Alternaria Spots in Tomato Leaves Differently Delayed by Four Plant Essential Oil Vapours

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Alternaria leaf spot disease has been a concern during a tomato production in greenhouse. In vitro antifungal activities of vapours of four plant essential oils, cinnamon oil, fennel oil, origanum oil and thyme oil, were investigated during in vitro conidial germination and mycelial growth of Alternaria alternata causing the tomato leaf spots to find eco-friendly alternatives for chemical fungicides. The four plant essential oils showed different antifungal activities against in vitro conidial germination of A. alternata in dose-dependent manners, and cinnamon oil vapour was most effective to suppress the conidial germination. The four plant essential oils showed similar antifungal activities against the in vitro mycelial growth of A. alternata in dose-dependent manners, but low doses of thyme oil vapour slightly increased in vitro mycelial growth of A. alternata. Necrotic lesions on the A. alternata-inoculated tomato leaves were reduced differently depending on kinds and concentrations of plant essential oils. Delayed conidial germination and germ-tube elongation of A. alternata were found on the tomato leaves treated with cinnamon oil and origanum oil vapours at 6 hpi. These results suggest that volatiles from cinnamon oil and origanum oil can be provided as alternatives to manage Alternaria leaf spot during the tomato production eco-friendly.

Keywords: Alternaria alternata, Disease management, Eco-Friendly, Plant essential oil vapour, Tomato

Introduction

Tomato plants have been infected by several Alternaria species including A. alternata, A. solani and A. tenuissima causing early blight and leaf spot symptoms (Agamy et al., 2013; Chaerani and Voorrips, 2006; Egusa et al., 2009; Jambhulkar et al., 2016; Prasad and Upadhyay, 2010). A. alternata invaded many organs of the tomato plants, and caused leaf blight, stem canker and fruit rot of tomato plants (Gherbawy et al., 2018; Grogan et al., 1975; Spalding, 1980). A. alternata-produced toxic metabolites such as AAL toxin, alternariol, alternariol monomethyl ether and tenuazonic acid might be involved in the fungal pathogenicity to tomato plants (Egusa et al., 2009; Meena et al., 2016). A variety of chemical and biological controls of tomato early blight caused by Alternaria solani infection have been suggested (Abu-El Samen et al., 2016; Brame and Flood, 1983; Song et al., 2011; Spletzer and Enyedi, 1999). Several chemical fungicides were suggested to control diseases by A. alternata in tomato plants and fruits (Abdel-Mallek et al., 1995; Malathrakis, 1983; Spalding, 1980). However, recent occurrence of fungicide-resistant isolates of A. alternata and A. solani in tomato fields has warned of fre-
quent uses of the chemical fungicides (Chavan et al., 2017). Only limited information has been available for eco-friendly management of *A. alternata*-mediated tomato diseases using antagonistic bacteria and plant defence-inducing chemical agents. Different bacterial strains of *Pseudomonas* spp. such as *P. fluorescens*, *P. putida* and *P. aeruginosa* inhibited *in vitro* mycelial growth of *A. alternata* causing a damping-off of tomato seedlings (Hammami et al., 2013). Application of whey compost-tea and rhizobacteria such as *Serratia liquefaciens* and *Pseudomonas putida* in soils decreased disease severity of tomato leaves inoculated with *A. alternata* (Pane et al., 2012; Schuhegger et al., 2006). Pretreatment with salicylic acid mediated induced disease resistance in tomato leaves against challenging *A. alternata* inoculation, and both L-glutamate and γ-aminobutyric acid induced resistance and reduced tomato fruit rot caused by *A. alternata* (Esmailzadeh et al., 2008; Yang et al., 2017a, 2017b).

Application of plant essential oils has been effective to reduce bacterial and fungal diseases in various horticultural crops and suggested as one of the eco-friendly alternatives for disease management by chemical pesticides. In our previous studies, soil-drenching clove oil solution significantly reduced tomato bacterial wilt by *Ralstonia pseudosolanacearum* (Lee et al., 2012). Distinctly suppressed *in vitro* conidial germination and mycelial growth of *Colletotrichum gloeosporioides* were demonstrated by treatment with cinnamon oil and clove oil vapours, and followed by reduced anthracnose lesions on pepper fruits treated with the two vapours (Hong et al., 2015). Recently, different *in vitro* antifungal activities of four plant essential oils, cinnamon oil, fennel oil, origanum oil and thyme oil, were investigated on *Fusarium oxysporum* f. sp. *fragariae* that causes Fusarium wilt in strawberry plants, although *in planta* crop protection efficacies were not evaluated yet, because *in vitro* and/or *in planta* antifungal activities of these four plant essential oils were prevalently demonstrated against diverse plant pathogenic fungal species (Park et al., 2017).

Diverse plant essential oils inhibiting *Alternaria* spp. including *A. alternata* have been shown to control diseases in many crops such as citrus, potato and tomato and summarised in Table 1. Essential oils from Cretan oregano, thyme, fennel, bay laurel and French lavender showed different *in vitro* antifungal activities during conidial germination, germ tube elongation and mycelial growth of *A. alternata* causing black rot in Minneola tangelo fruits (Soylu and Kose, 2015). However, control efficacies of the citrus black rot disease by the plant essential oils were not evaluated. Essential oils from carnation, caraway and thyme plants have shown *in vitro* antifungal activities against *A. solani* as well as reducing effects of early blight of potato plants in fields leading to increased potato tube yields (El-Mougy, 2009). Early blight in tomato seedlings and black rot in tomato fruits by *A. alternata* infection could be controlled by origanum oil and citronella oil treatments, respectively (Chen et al., 2014; Pérez-González et al., 2016). Thyme oil exerted both antifungal activity and disease control effect on *in vitro* growth of *A. alternata* and *A. alternata*-infected tomato fruits via fumigation and contact treatments (Feng et al., 2011).

In this study, we applied the four plant essential oils to *A. alternata* to investigate their antifungal activities to suppress *in vitro* conidial germination and mycelial growth of *A. alternata*, as well as to control Alternaria leaf spot disease in tomato leaves. Fungal developments regulated by the plant essential oils on the inoculated leaves were also observed microscopically.

### Table 1. *in vitro* antifungal activities against *Alternaria alternata* and disease reduction efficacies of plant essential oils

| Plant essential oils | *in vitro* activity | Disease reduction | Reference |
|---------------------|---------------------|-------------------|-----------|
| Cinnamon oil        | M                   | –                 | Sukatta et al., 2008 |
| Citronella oil      | C, M                | Tomato fruits     | Chen et al., 2014 |
| Clove oil           | M                   | –                 | Sukatta et al., 2008 |
| Fennel oil          | M                   | –                 | Soylu and Kose, 2015 |
| Origanum oil        | C, GT, M            | –                 | Soylu and Kose, 2015 |
| Peppermint oil      | M                   | Tomato fruits     | Abd-Alla et al., 2009 |
| Thyme oil           | C, M                | Ponkan tangerine fruits | Perina et al., 2015 |

C, conidial germination; GT, germ tube elongation; M, mycelial growth.
Materials and Methods

Fungal culture. *Alternaria alternata* KACC 40019 was originated from diseased sesame plant showing leaf blight symptom, and maintained on 1/2-strength potato dextrose agar (PDA) at 25°C under dark condition. The conidial suspension was obtained from 6 day old-cultured colonies on the PDA using pouring quarterly diluted (1/4) potato dextrose broth. Concentration of the conidial suspension was adjusted using a haemacytometer under a light microscope. To obtain mycelial agar plugs (5 mm in diameter) from the growing colony edge, *A. alternata* was inoculated on the center of PDA media and cultured for 6 days at 25°C under dark condition.

Plant growth. Tomato plants (cv. Cupirang) were grown in a commercial soil mixture ‘Toshil’ in pots (8 cm in diameter, 7.5 cm in height) in a walk-in growth at 26–28°C in the daytime and at 22–24°C in the night under a 12-h fluorescent light illumination as slightly modified method described in our previous studies (Hong et al., 2016). Second true leaves from the bottom were detached from the 5-leaf stages of tomato plants and used for the fungal inoculation.

Preparation of paper discs harbouring plant essential oils. Four plant essential oils cinnamon oil, fennel oil, origanum oil and thyme oil were purchased and prepared in paper discs as described in our previous studies (Hong et al., 2015; Park et al., 2017). Each plant essential oil was dropped onto sterile paper discs (8 mm in diameter) to contain 0, 0.1, 0.2, 0.5, 1, 2 and 5 µl/paper disc, and the paper discs were attached inside the covers of circular Petri dishes, square dishes and plastic boxes for *in vitro* conidial germination, *in vitro* mycelial growth inhibition and *in planta* protection assay, respectively, to exert their volatile activities. Final vapour concentrations of each plant essential oil for *in vitro* conidial germination and mycelial growth inhibition assays as well as for *in planta* protection assay were calculated and summarised in Table 2.

Table 2. Concentration of plant essential oil volatiles s used in this study for *in vitro* antifungal activities and *in planta* crop protection.

| Plant essential oils (µl/paper disc) | *in vitro* conidial germination (µl/cm³ air) | *in vitro* mycelial growth (µl/cm³ air) | *in planta* protection (µl/cm³ air) |
|-------------------------------------|---------------------------------------------|----------------------------------------|-----------------------------------|
| 0.1                                 | 0.00077                                      | 0.0025                                 | 0.00019                           |
| 0.2                                 | 0.00154                                      | 0.0050                                 | 0.00038                           |
| 0.5                                 | 0.00385                                      | 0.0125                                 | 0.00095                           |
| 1                                   | 0.00772                                      | 0.0250                                 | 0.00190                           |
| 2                                   | 0.01538                                      | 0.0500                                 | 0.00380                           |
| 5                                   | 0.03846                                      | 0.1000                                 | 0.00950                           |

Inhibition of *in vitro* conidial germination and mycelial growth of *A. alternata*. Antifungal activities of vapours of the four plant essential oils against *in vitro* conidial germination and mycelial growth of *A. alternata* were evaluated as experimental procedures described in our previous studies (Hong et al., 2015; Park et al., 2017). Conidial suspensions treated with different concentrations of the plant essential oil vapours on glass slides were incubated in plastic square dishes for 3 h at 25°C under dark and humid conditions, and numbers of germinated conidia and total conidia were measured under a light microscope. Conidial germination (%) of the vapour-treated samples was compared to the conidial germination on the mock-treated control. Mycelial growth inhibition assay, fungal colony diameters formed on the PDA media were measured at 6 days after culture at 25°C under dark condition. Relative mycelial growth was calculated compared to the colony diameter on the mock-treated control. Independent experiments were conducted repeatedly four times for means and standard errors, and four replicates were prepared for each experiment.

Fungal inoculation and disease evaluation. One drop (10 µl) of the conidial suspension (2×10⁶ conidia/ml) of the *A. alternata* was inoculated onto centre of adaxial surface of the detached tomato leaves, and the inoculated leaves were placed on sterile water-saturated gauzes laid in plastic boxes at the bottom at 25°C to allow the necrotic spot disease symptom development under humid and dark conditions. Lesion diameters were measured at 5 days post-inoculation (dpi). Independent experiments were conducted repeatedly four times for means and standard errors, and four replicates were prepared for each experiment.

Fungal development including conidial germination and germ-tube elongation on the inoculated tomato leaves were observed at 6 hours post-inoculation (hpi) under a light microscopy after fungal staining with lactophenol-trypan blue solution after clearing chlorophylls of the leaf tissues with
boiling ethanol (Park et al., 2017). Independent experiments were conducted repeatedly three times for means and standard errors of conidial germination and germ-tube length of *A. alternata* on the inoculated leaves, and eight photos were prepared for each experiment.

**Statistical analyses.** Data were subjected to analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) to determine effects of plant essential oil treatments on *in vitro* growth of *A. alternata*, disease suppression and the fungal development on the leaves. Means were compared by least significant difference (LSD) test at *P* < 0.05. Graphs were prepared using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

**Results**

**Antifungal activities of the four plant essential oils against *in vitro* conidial germination of *A. alternata.* In *vitro* conidial germination of *A. alternata* was differently inhibited by treatments with vapours of the four different plant essential oils (Fig. 1). The lowest dose of cinnamon oil (0.1 µl/disc) resulted in drastic suppression of the conidial germination by ca. 35.1%, and doubling dose to 0.2 µl/disc more decreased the conidial germination by ca. 19.4%. Increasing doses to 0.5–5 µl/disc only led slightly augmented antifungal activities against the conidial germination. Antifungal activity of fennel oil against the conidial germination was demonstrated but relatively higher doses were necessary for reducing the germination below the 50% level. The conidial germination was first inhibited by 0.5 µl/disc of fennel oil to ca. 87% and then increasing doses of the fennel oil to 0.5, 1, 2 and 5 µl/disc gradually decreased the conidial germination to ca. 80.4, 72.8, 38.2 and 25.9%, respectively. Treatment with origanum oil vapour showed dose-dependent antifungal activity against the conidial germination. Increasing dose of origanum oil to 0.1, 0.2, 0.5 and 1 µl/disc resulted in decreases in the conidial germination by ca. 71.6, 42.1, 21.6 and 16.3%, respectively. However, more than 1 µl/disc of origanum oil could not significantly increase the suppression efficacies against the conidial germination. Thyme oil also showed dose-dependent antifungal activity against the conidial ger-

![Fig. 1. Conidial germination of *Alternaria alternata* inhibited by volatiles of four different plant essential oils, cinnamon oil, fennel oil, origanum oil and thyme oil. Germinated conidia of *A. alternata* on the glass slides by treatment with different doses (0, 0.1, 0.2, 0.5, 1, 2 and 5 µl/disc) of each volatile essential oil were observed under a light microscope. Relative conidial germination (%) affected by different doses of each essential oil was shown as percentage of the conidial germination. Error bars represent the standard errors of the mean conidial germination of four independent experimental replications. Means followed by the same letter are not significantly different at 5% level by least significant difference test. The same letter above bars represented no significant difference between treatments.](image-url)
mination similar to those of origanum oil. Increasing dose of thyme oil to 0.1, 0.2, 0.5 and 1 µl/disc resulted in decreases in the conidial germination by ca. 87.8, 86.8, 31.8 and 17.9%, respectively. However, more than 1 µl/disc of origanum oil could not significantly increase the suppression efficacies against the conidial germination.

**Different antifungal activities of the four plant essential oils against in vitro mycelial growth of A. alternata.**

In vitro mycelial growth of A. alternata was also differently inhibited by treatments with vapours of the four different plant essential oils (Fig. 2). At least 1 µl/disc of cinnamon oil was necessary to reduce the mycelial growth with ca. 82.7% of the untreated control. Increasing dose to 2 µl/disc of cinnamon oil resulted in ca. 46.2% of the mycelial growth and 5 µl/disc of cinnamon oil completely inhibited the mycelial growth. Fennel oil has shown relatively lower antifungal activity against the mycelial growth compared to those of cinnamon oil. Most of the mycelia ca. 87.9% was still grown by 2 µl/disc of fennel oil, and even increasing dose to 5 µl/
disc moderately inhibited the mycelial growth by ca. 67.6%. Increasing origanum oils also arrested the mycelial growth in dose-dependent manner. At least 0.5 µl/disc of origanum oil reduced the mycelial growth by ca. 90.0%, and 1 µl/disc reduced the mycelial growth by ca. 60.5%. More than 2 µl/disc showed strong antifungal activity against the mycelial growth that was completely arrested. Thyme oil showed different effects on the mycelial growth dependent on doses applied. Low doses 0.1 and 0.2 µl/disc of thyme oil rather enhanced the mycelial growth but 0.5 µl/disc of thyme oil did not alter the mycelial growth. One µl/disc slightly decreased the mycelial growth by ca. 84.3% and increasing to 2 µl/disc led to more decrease in the mycelial growth showing ca. 39.1% compared to that of untreated control.

**Plant protection efficacies of the four plants essential oil vapours against Alternaria leaf spot.** Treatment with vapours of the four plant essential oils resulted in different protection efficacies against tomato Alternaria leaf spot disease (Fig. 3). Treatments with cinnamon oil (1 µl/disc), fennel oil (1 µl/disc) and origanum oil (1 µl/disc) resulted in reduced diameters of necrotic lesion on the A. alternata-inoculated tomato leaves at 5 dpi, and there were no difference in protection efficacies. However, other treatments with cinnamon oil (0.5 µl/disc), fennel oil (1 µl/disc), origanum oil (0.5 µl/disc) and thyme oil (0.5 and 1 µl/disc) did not change the lesion size compared to that of mock-treated control.

**Fungal development of A. alternata on the tomato leaves.** Growing behaviour of A. alternata on the detached tomato leaves with or without the plant essential oil vapours was observed under a light microscope (Fig. 4A). Many conidia germinated and germ-tube elongated in mock-, fennel oil- and thyme oil vapour-treated leaves, but distinct conidial germination and germ-tube elongation of A. alternata was not observed on cinnamon oil and origanum oil vapour-treated tomato leaves. Early conidial germination and germ-tube elongation on the tomato leaves were quantified at 6 hpi (Fig. 4B). On the mock-treated tomato leaf surface, ca. 46.2% of A. alternata conidia germinated and germ-tubes grew well with average length 8.47 µm. Treatments with vapours of the four plant essential oils (1 µl/disc each) resulted in different conidial germination and germ-tube elongation of A. alternata on the leaves. Treatments with cinnamon oil and origanum oil vapours resulted in suppressed conidial germination of A. alternata shown by ca. 36.4% and ca. 36.1% germination rates, respectively. No significant reduction in the germination was found in the conidia treated with fennel oil and thyme oil vapours. More distinct suppression by cinnamon oil and origanum oil vapours was demonstrated by reduced germ-tube lengths of ca. 2.44 µm and ca. 3.35 µm, respectively. Thyme oil vapour slightly reduced the germ-tube lengths with ca. 7.16 µm.

**Fig. 3.** Disease control of tomato Alternaria leaf spot by treatments with the four different plant essential oils. (A) Symptom development on the Alternaria alternata-inoculated tomato leaves at 5 dpi. (B) Lesion diameters (mm) of the inoculated tomato leaves at 5 dpi after treatment with plant essential oils. Error bars represent the standard errors of the mean lesion diameter of four independent experimental replications. Means followed by the same letter are not significantly different at 5% level by least significant difference test. The same letter above bars represented no significant difference between treatments.
Discussion

Treatment with plant essential oils has been suggested as one of eco-friendly disease management during plant production and food preservation (Sakkas and Papadopoulou, 2017; Sivakumar and Bautista-Baños, 2014). Their volatile activities contribute to effective control efficacies of plant diseases, and various constituents originated from many antifungal plant essential oils have been identified (Lee et al., 2007; Neri et al., 2007; Oliva et al., 2015; Soylu et al., 2006; Wei and Shibamoto, 2010). In this study, vapours of the four plant essential oils, cinnamon oil, fennel oil, origanum oil and thyme oil, were evaluated to investigate whether they inhibit in vitro fungal development of A. alternata and protect...
tomato leaves against A. alternata infection in planta. These four plant essential oils have shown their in vitro antifungal activities against a variety of phytopathogenic fungi or in planta protection efficacies to broad ranges of fungal diseases in grape, pepper and tomato plants (Hong et al., 2015; Soylu et al., 2007, 2010; Walter et al., 2001). Cinnamon oil as a vapour significantly suppressed in vitro conidial termination and mycelial growth of C. gloeosporioides, and reduced anthracnose lesion development on pepper fruits (Hong et al., 2015). The four plant essential oils also exerted in vitro volatile antifungal activities dose-dependently against F. oxysporum f. sp. fragariae causing Fusarium wilt in strawberry plants in our previous study (Park et al., 2017). In the present study, vapour treatments of the four plant essential oils have showed differential antifungal activities during in vitro conidial germination and mycelial growth of A. alternata in dose-dependent manners. Generally, increasing doses of the plant essential oils highly limited in vitro growth of A. alternata. A variety of chemical constituents were indentified in cinnamon oil and origanum oil (Oliva et al., 2015; Soylu et al., 2006; Zamani-Zadeh et al., 2013). It remains elucidated which volatile constituents are related to antifungal activities of cinnamon oil and origanum oil against A. alternata. Recently, synergistic antifungal activities of origanum oil and thyme oil were demonstrated against mycelial growth of F. oxysporum f. sp. fragariae in our study (Park et al., 2017). Combined treatments with more than 2 plant essential oils may lead to enhanced antifungal activities against A. alternata as well, which remains investigated for the better disease control efficiencies.

In vitro antifungal activities of the three plant essential oils, cinnamon oil, fennel oil and origanum oil, were closely associated with in planta protection against A. alternata infection in tomato leaves without any phytotoxic effect. Arrested conidial germination and germ-tube elongation during the early infection stages of A. alternata observed under a light microscope were related to the retarded lesion development on the inoculated tomato leaves treated with cinnamon oil and origanum oil vapours. More investigation of morphological and subcellular changes of A. alternata by cinnamon oil and origanum oil vapours will provide better information on the antifungal activities of the two plant essential oils. Reducing A. alternata inoculum density in the tomato leaves by the plant essential oils can mediate in decrease of fruit rot during harvest and storage periods, because A. alternata also caused black rot mould in ripe tomato fruits (Abdel-Mallek et al., 1995; Bessadat et al., 2017; Gherbawy et al., 2018; Malatharakis, 1983; Xie et al., 2012). These three plant essential oils can be suggested to control other foliar diseases in tomato plants by bacterial and fungal infections simultaneously.

By contrast, thyme oil treatment did not mediate disease protection in planta, which may be related to increase in mycelial growth by low doses (0.1 and 0.2 µl/paper disc) of thyme oil vapours in Fig. 2. Major components of thyme oil were identified as p-cymene, carvacrol and χ-terpinene, and they have shown antimicrobial activities, and none of them was reported to enhance fungal growth. It is necessary to answer why thyme oil vapour enhanced fungal growth of A. alternata. Disease controls by thyme oil treatment have been prevalently demonstrated in tomato fruits infected by A. alternata, Ponkan tangerine fruits infected by A. alternata and potato plants infected by A. solani (El-Mougy, 2009; Feng et al., 2011; Perina et al., 2015). We cannot exclude that increasing thyme oil dose or changing treatment method might be effective to control Alternaria leaf spot in tomato plants infected by A. alternata.

In conclusion, we applied vapours of the four different essential oils, cinnamon oil, fennel oil, origanum oil and thyme oil, to A. alternata causing leaf blight, stem canker and fruit rot in tomato plants. Cinnamon oil and origanum oil volatiles played roles in disease suppression of Alternaria leaf spot by the current detached leaf assay. Further evaluation for the tomato protection efficacies of the cinnamon oil and origanum oil volatiles under greenhouse conditions will be more valuable for integrated disease management eco-friendly.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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