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Essential oils to control *Botrytis cinerea* in vitro and in vivo on grape fruits

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ABSTRACT

Purpose: The effect of abusing chemical biocides in controlling pests and diseases has drawn the attention of policymakers to the development of methods potentially available in nature for this purpose. **Research method:** In the present study, the inhibitory effects of four different essential oils against *Botrytis cinerea* were tested at various concentrations (0, 200, 400, 600, and 800 µL L^{-1}) in *in vitro* and *in vivo*. **Main findings:** The *in vitro* results showed that the growth of *B. cinerea* was completely inhibited by the application of anise oil at concentrations of 800 µL L^{-1}. The *in vivo* results indicated that treated fruits with marjoram oil had more total soluble solids, and anthocyanin content in comparison to anise, chamomile, and black caraway oil. Furthermore, among essential oils, treated fruits with black caraway essential oils had the lowest pH, while anise, chamomile, and marjoram oil had the highest pH. The highest anthocyanin content and pH were obtained at 200 and 400 µL L^{-1} concentration and lowest values were found at a control treatment (respectively). The most total soluble solids were observed at control treatment and the lowest values were recorded at 600 µL L^{-1}. The application of each essential oil decreased the percentage loss in fresh weight significantly and increased the storage-life of the fruit. **Limitations:** Higher cost of application was a limitation. **Originality/Value:** This research confirms the antifungal effects of anise, fennel, chamomile, and marjoram essential oils both *in vitro* and *in vivo* on grape fruits postharvest. Therefore, these essential oils could be an alternative to chemicals to control postharvest phytopathogenic fungi on grape fruits.

INTRODUCTION

Grape (*Vitis vinifera* L.) is a plant from the family Vitaceae. In this family, there are about 10 genus and more than 600 species. The most important genus of this family is the economically and nutritionally Vitis (Jalili Marandi, 2007). The total grape production in the world in 2016 was reported to be about 77.4 million tons, and Iran was ranked ninth in the world with about 2.45 million tons (FAO, 2016).

Botrytis cinerea, which causes severe damage to many fruits, (gray mold) is a ubiquitous pathogen vegetable, and ornamental crops, both pre and postharvest (Elad, 1997). *B. cinerea* is one of the commonly present fungi causing severe postharvest losses to fruits and vegetables. In recent years and according to its scientific and economic extent, this, the fungus was placed in the second rank in the world top ten pathogens listed (Feliziani & Romanazzi, 2016). It infects the plant both during the growing season and during storage and causes up to 55% loss during post-harvest storage (Martínez-Romero et al., 2007).

Essential oils or the so-called volatile or ethereal oils are aromatic oily liquids obtained from various plant organs which include: flower, bud, seed, leaf, twig, bark, herb, wood, fruit and root (Serrano et al., 2005). The application of essential oil is a very attractive method to control postharvest diseases. The production of essential oils by plants is believed to be predominantly a defense mechanism against pathogens and pests (Oxenham, 2003). Essential oils are volatile, natural, complex compounds, characterized by having a strong odor, and are formed as secondary metabolites by aromatic plants. In nature, essential oils play important roles as anti-bacterial, antiviral, anti-fungal, and insecticidal protectants in plants. They also act against herbivores by reducing the dietary appeal of such plants (Bakkali et al., 2008). Considerable effort has been focused on such plants as sources of potentially useful natural products for use as commercial fungicides (Tian et al., 2011; Tzortzakis, 2009). Generally, the use of plants essential oils has been broadly used as postharvest treatments that are applied to fruits (Maqbool et al., 2011). The exploitation of natural substances such as the essential oils is safer to consumers and the environment for the control of postharvest disease (Isman, 2000). In recent years, numerous studies have documented the antifungal effects of plant essential oils to control food spoilage fungi *in vitro* and *in vivo* (Amiri et al., 2008; Feng & Zheng, 2007; Omidbeygi et al., 2007; Tian et al., 2011). The quality of essential oils depends on several physical parameters such as specific gravity, optical rotation, refractive index, and solubility in different organic solvents, acid number, saponification value, ester value, and phenolic contents (Chowdhury & Kapoor, 2000). However, their data indicates many variations between the same essences.

Antifungal activity of various essential oils from aromatic plants against *B. cinerea* has been documented (Bishop & Reagan, 1998; Chebli et al., 2003; Daferera et al., 2003; Reddy et al., 1998; Wilson et al., 1997) . According to results Mohammadi et al. (2011) application of fennel, anise, peppermint, and cinnamon oils showed that all these essential oils caused an increase in the shelf life of fruits and inhibited *Botrytis cinerea* growth as compared to the controls. Based on the report of Ozcan (2003), antagonistic properties of some essential oils such as salvia, laurel, dill, cumin, fennel, and thyme were found to be in control of *B. cinerea*. Antifungal effects of plant essential oils to control food spoilage fungi *in vitro* and *in vivo* were studied in apple (Amiri et al., 2008), mango (Dubey et al., 2008; Regnier et al., 2008), citrus (Du Plooy et al., 2009), tomato (Omidbeygi et al., 2007), *Malus pumilo* (Shahi et al., 2003), avocado (Sellamuthu et al., 2013) and plum (Aminifard & Mohammadi, 2013). Among post-harvest diseases, *B. cinerea* is an important pathogenic pathogen in a 12-year-old study in the United States that the causal agent of *B. cinerea* was more than 32.5% of the damage to the fresh grapes (Kulakiotu et al., 2004).

The objectives of this study were to test and to compare the inhibitory effects of the essential oils of anise (*Pimpinella anisum*), fennel (*Foeniculum vulgare*), chamomile (*Matricaria chamomilla*), black caraway (*Carum carvi*) and marjoram (*Origanum majorana*), at different concentrations, in post-harvest control of *B. cinerea* on grape fruits.

MATERIALS AND METHODS

Plant materials and extraction of essential oils

In this study, essential oils of fennel and black caraway were obtained from Mashhad Golfa Shafa Company and Chamomile and marjoram oil from Gorgan Essential Oil Company. Air-dried seeds of anise were supplied from agricultural research fields of University of Birjand, Iran. After the plant seeds parts had been authenticated, a 100 g portion of each was subjected to hydro distillation for 3 h in a Clevenger-type apparatus. The resulting oils were dried over anhydrous Na_2SO_4 and preserved in sealed vials at 4 °C for future analysis (Fatemi et al., 2013).

Experiment I (*In vitro* experiment)

Design of experiments and treatments

In vitro experiments were carried out in a randomized factorial design with two factors; including four essential oils (anise, fennel, chamomile, and marjoram) and five concentrations (0, 200, 400, 600, and 800 μL L^{-1}) with three replications.

Antifungal effects of the essential oils on mycelia radial growth in *in vitro* conditions

Antifungal acting was studied using a contact assay (*in vitro*) that produced hyphal growth inhibition. The test was previously used for essential oil treatment on potato dextrose agar (PDA) medium by the “solution method” (SM) (Özden & Bayindirli, 2002). In this method, each essential oil was dissolved in 5% (v/v) Tween-80 and the required amount was added to each 9 cm Petri plate containing 20 ml PDA-agar at 45ºC. A 0.5 mm disc of *B. cinerea* mycelium was placed on the treated PDA medium and the plate was incubated at 24ºC. Radial mycelia growth was determined each day (up to ten days). The inhibitory percentage (IP) was determined using the formula (1):

$$IP = [(dc \times dt) / dc] \times 100 \quad (1)$$

Where dc was the mycelium diameter in a control Petri dish, and dt was the mycelium diameter in the essential oil-treated Petri dish measured daily (Aminifard & Mohammadi, 2013).

Experiment II (*In vivo* experiment)

Design of experiments and treatments

In vivo experiments were carried out in a randomized factorial design with two factors; including four essential oils (anise, black caraway, chamomile, and marjoram) and five concentrations (0, 200, 400, 600, and 800 μL L^{-1}) with three replications.

Application of essential oils and *B. cinerea* inoculation on grape fruits

The experiment was carried out using grape cultivar Rish Baba that prepared from Birjand local Market. First, remove the grapes from the cluster with scissors with a 1 cm of tail, then rinse the surface and spread on sterile paper to dry. Infected grape fruits were selected and collected from storage to isolate *B. cinerea*. The culture was maintained on PDA at 4 °C. Fresh cultures were grown on PDA plates before usage. Spore suspensions were collected by removing spores from the sporulation edges of a 7–8-day-old culture with a bacteriological loop and suspending them in sterile distilled water. Spore concentration was determined with a hemocytometer and adjusted as required with sterile distilled water (10^5 spore's mL^{-1}). Before infection, fruits were treated with sodium hypochlorite (100 µL L^{-1}) for 5 minutes. They were then sprayed in the prepared suspension and stored at room temperature for 2 h to fix the fungal inoculation (Asghari Marjanlo et al., 2009). In this experiment, three replicates were used for each treatment and 20 experimental units (fruit) for each replicate. We then put the grapes in a zipper pack and sprayed the essential oils on various concentrations (0, 200, 400, 600, and 800). We prepared the essential oil solution from the essential oil mixture with acetone and twin 80 (0.05%) for better solubility and uptake by the fruit. Of course, the solvents were selected according to the experiments performed with acetone, since it did not affect the growth of the fungus. Samples were placed in disposable containers and refrigerated and stored at 4 °C for 10 days.

Anthocyanin contents

Total anthocyanin contents were determined by the differential pH method (Rapisarda et al., 2000). A 1.0 ml aliquot of each grape fruit extract was diluted to 10 ml with a pH 1.0 solution made from 125 ml of 0.2 M KCl plus 375 ml of 0.2 M HCl. A second 1.0 ml aliquot of fruit extract was diluted to 10 ml with a pH 4.5 solution made from 400 ml of 1 M sodium acetate, 240 ml 1 M HCl, and 360 ml H_2O. The absorbance of each solution was measured at 510 nm using a UV spectrophotometer (BioQuest CE 2502; Cecil Instruments Ltd., Cambridge, UK) and the concentration of anthocyanins was calculated using the equation (2):

$$C_{mg\ 100\ g^{-1}} = [(A_{pH1.0} - A_{pH4.5}) \times 484.82 \times 1{,}000/24{,}825] \times DF \quad (2)$$

Where the period in parentheses was the difference in absorbance at 510 nm among the pH 1.0 and pH 4.5 solutions, 484.82 was the molecular mass of cyanidin-3- glucoside chloride, 24,825 was its molar absorption at 150 nm in the pH 1.0 solution, and DF was the dilution factor (Aminifard & Mohammadi, 2013).

Total soluble solids

Total soluble solids (TSS) were determined at 20 °C using a refractometer (RF 10, 0-32° Brix, Extech Co., USA) and reported as °Brix.

pH

The pH of fruit juices was measured at 20 °C using a pH meter (Metro model, manufactured by the Swiss Metro Company).

Weight loss percentage

Weight loss was determined by weighting the whole grape before and after the storage period. Weight loss was expressed as the percentage of loss of weight with respect to the initial weight in the formula (3) (Hosseini & Moradinezhad, 2018).

$$WL = (\text{Initial weight} - \text{Secondary weight}) / (\text{Initial weight}) \times 100 \quad (3)$$

Statistical analysis
The experiment was conducted in a completely randomized factorial design with three replications consisting of twenty fruits each. Data were analyzed using SAS Version 9.1. (SAS Institute, Cary, NC, USA) and means were compared by Duncan's multiple range test at 1 and 5% level of confidence.

RESULTS

In vitro experiment
Effect of essential oils on radial growth of *B. cinerea* in *in vitro* conditions
The results of the analysis of variance on the tenth day showed that the effect of type and concentration of essential oil and their interactions on the growth rate of *B. cinerea* fungi were significant (at 5% level) (Table 1). The highest fungal growth rate was observed in essential oil of the chamomile with a growth rate of 25.70 mm, while the least fungal growth rate was recorded at anise oil (11.06 mm). Marjoram and fennel oil after anise oil showed the greatest inhibitory effect (14.3 and 15.3 mm, respectively) (Fig. 1). The results of the mean comparison of essential oil concentration on the tenth day indicated that the control had the highest growth rate with the growth rate of the fungus 40.6 mm, and the concentration of 800 μL L^{-1} with 5.5 mm had the least growth, and the rest concentrations showed significant differences (Fig. 2).

On the tenth day, the growth rate of *B. cinerea* was significantly different in control treatment and all anise oil treatments (Fig. 3). Concentrations of 200, 400 and 600 μL L^{-1} of fennel oil did not differ significantly on 10 days, but all of them had a significant difference with the control of *B. cinerea* fungus and 800 μL L^{-1} (Fig. 3). The growth rate of *B. cinerea* fungi on day 10 was not significantly different in 400 and 600 μL L^{-1} of chamomile oil, but showed a significant difference with other treatments, there was a significant difference between control treatment and 200 and 800 μL L^{-1} concentration. On the tenth day, the growth rate of *B. cinerea* fungus in the control treatment and all treatments of marjoram oils were statistically significant. Generally, the highest growth rate of fungi was observed in control treatment and the lowest values in 800 μL L^{-1} concentration anise, fennel, chamomile and marjoram oils (Fig. 3).

In vivo experiment
Anthocyanin contents
There was a statistically significant difference in the amount of anthocyanin in treated grapes with *B. cinerea* fungus (at 1% level) (Table 2). The highest amount of anthocyanin in grapes treated with marjoram oil (2216.6 mg $100g^{-1}$) and the lowest amount of it was observed in grapes treated with chamomile oil (936.25 mg $100g^{-1}$). The results of mean comparison of grapes incubated with *B. cinerea* fungus between different concentrations on the amount of grape anthocyanin showed a significant difference. The highest amount of anthocyanin was observed at 200 μL L^{-1} concentration (2084.24 mg $100g^{-1}$) and the lowest amount were recorded at control treatment (1157.7 mg $100g^{-1}$).

Total soluble solids
There was a significant difference between the results of mean comparison of grapes treated with *B. cinerea* fungus between different essential oils on the amount of grape total solids content (at 1% level). The highest amount of total soluble solids in the grapes treated with marjoram oil (32.2 °Brix) and the lowest value in the grapes treated with chamomile oil (22.04 °Brix). The results of mean comparison of grapes treated with *B. cinerea* fungus

between different concentrations showed a significant difference in the amount of total solids content of grape (at 1% level). The highest amount of total soluble solids was observed at control treatment (25 °Brix) and the lowest values are at 600 µL L^{-1} (22.32 °Brix) (Table 2).

pH

The results of mean comparison of grapes treated with *B. cinerea* fungus showed significant differences between varieties of essential oils on grape pH. There was no statistically significant difference between the anise, chamomile, and marjoram oils, but all three showed significant differences with black caraway oil. The lowest amount of pH in grapes treated with black caraway oil (4.44). The results of mean comparison of grapes treated with *B. cinerea* fungi between different concentrations showed a significant difference in grape pH. The lowest pH was found at a control treatment (4.43) and the highest values were recorded of 400 µL L^{-1} (4.53) (Table 2).

Table 1. Analysis variance of for the effect of type and concentration of essential oil on radial growth for *B. cinerea* fungi treatments in *in vitro* conditions

Source of variation	df	Radial growth of fungus (tenth day) (mm)
Type essential oils	3	603.8*
Concentrations of essential oils	4	2347.2*
Type essential oils × Concentrations of essential oil	12	51.2*
Error	40	56.6

*, ** Significant at 5%, and 1% probability level, respectively.

Table 2. Comparison of means of the effect of type and concentration of essential oils on quality factors for *B. cinerea* fungi treatments in *in vivo* conditions

Treatment		Anthocyanin content (mg 100 g^{-1})	TSS (°Brix)	pH	Weight loss percentage (%)
Essential oils	Anise	1227.4^{c}	22.86^{c}	4.50^{a}	3.17^{a}
	Chamomile	936.25^{d}	22.04^{d}	4.50^{a}	0.83^{c}
	Black caraway	2077.7^{b}	23.52^{b}	4.44^{b}	0.81^{d}
	Marjoram	2216.6^{a}	32.2^{a}	4.50^{a}	1.45^{b}
Concentrations of essential oils	Control	1157.7^{e}	25.00^{a}	4.43^{e}	2.53^{a}
	200	2080.24^{a}	22.37^{d}	4.49^{c}	1.55^{b}
	400	1596.37^{c}	23.22^{c}	4.53^{a}	1.29^{d}
	600	1658.4^{b}	22.32^{d}	4.51^{b}	1.34^{c}
	800	1579.8^{d}	24.10^{b}	4.47^{d}	1.25^{e}

Within each column, the same letter indicates no significant difference between treatments at 5% levels.

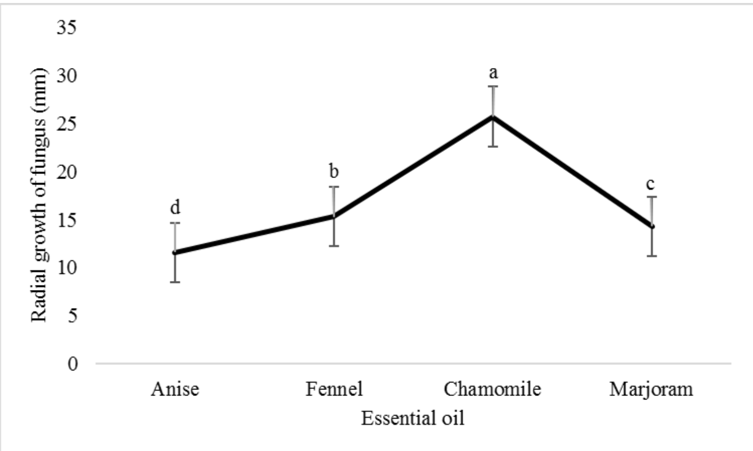

Fig. 1. Effect of four essential oils on radial growth (mm) of *B. cinerea* in *in vitro* conditions

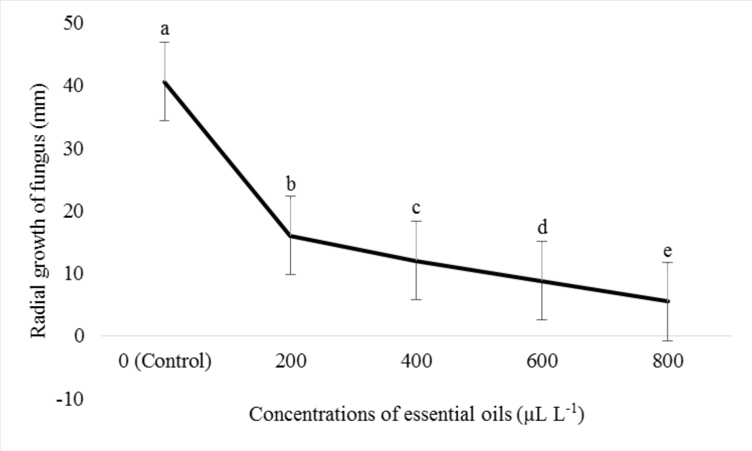

Fig. 2. Effect of different concentrations of essential oils on radial growth (mm) of *B. cinerea* in *in vitro* conditions

Weight loss percentage

Fruit treated with black caraway oil had the lowest weight loss percentage (0.81%), while fruit treated with anise oil had the highest weight loss percentages (Table 2). Furthermore, the weight loss percentage of essential oil-treated fruits was significantly lower than that of control fruits ($p < 0.01$). Fruits treated at 800 µL. L^{-1} of oils showed the lowest weight loss percentage (1.25%), while control fruits showed the highest weight loss percentages (Table 2).

DISCUSSION

The antifungal property of several essential oils on postharvest pathogens of fruits and vegetables under *in vitro* and *in vivo* conditions have been investigated previously (Feng & Zheng, 2007).

The *in vitro* data presented in this study showed that the examined essential oils had a fungicidal effect at higher concentrations, particularly anise essential oil. The results indicated that the control treatment had the highest growth rate of *B. cinerea* and the concentration of 800 µL. L^{-1} had the least growth of *B. cinerea*. Fatemi et al. (2013) reported that the examined black caraway and anise essential oils at 800 µL L^{-1} had a fungicidal effect at higher concentrations, especially anise essential oil. Huang et al. (2010) reported that the antifungal activity of the star anise was related to Trans Antoul. Ćosić et al. (2010) in the study of antifungal activity, several essential oils such as cloves, peppermint, salvia, thyme, cinnamon, anise, black caraway, orange, rosemary, lavender, and pine against some fungal phytopathogens including *Colletotrichum coccodes*, etc., the most antifungal activity was observed in the essential oils of black caraway, thyme, cloves, peppermint, cinnamon, and anise. Similarly, the growth of *B. cinerea* was inhibited by thyme, oregano, dictamnus, and marjoram essential oils (Daferera et al., 2003). Additionally, the mycelia growth of *B. cinerea* was reported by Bouchra et al. (2003) to be inhibited by *Origanum compactum* and *Thymus glandulosus* essential oils. Also, Chebli et al. (2003) reported that the essential oils of *Thymus glandulosus* and *Origanum compactum* inhibited the mycelia growth of *B. cinerea*. The antimicrobial attributes of essential oil and major constituents of fennel can suppress several plant pathogenic fungi (Soylu et al., 2007).

This difference in the antifungal activity may be due to their chemical composition, the structural configuration of these constituents, the activity of their functional groups and possible synergistic interactions between these constituents (Bajpai et al., 2013). Daferera et al. (2003) investigated the antifungal effects of some essential oils against *B. cinerea* and *Fusarium* Sp. on an artificial culture medium, they reported that marjoram essential oil in low concentrations showed a completely inhibitory effect. Sekine et al. (2007) investigated an antifungal effect of black caraway compounds and 52 herbaceous plants in the study of black caraway and followed by cumin and cardamom strong inhibitory effects against *oxysporum Fusarium*, which is consistent with the results of this study. They also stated that this antifungal activity is related to the composition of the cumin aldehyde in the black caraway.

Treated fruits with marjoram oil had more total soluble solids, and anthocyanin content in comparison to anise, chamomile, and black caraway oil. Furthermore, fruits treated with black caraway oil showed the lowest pH, while treated fruits with anise, chamomile, and marjoram essential oils showed the highest pH. The highest anthocyanin content and pH were obtained at 200 and 400 μL L^{-1} concentrations respectively, and lowest values were found at a control treatment. The highest amount of total soluble solids was observed at control treatment and the lowest values were recorded at 600 μL L^{-1}.

The results weren't in agreement with those of Asghari Marjanlo et al. (2009) who reported that TSS of strawberry infected with *B. cinerea* increased with the application of cumin oil. Mahmoud and Abd El- Salam (2014) reported that essential oils of celery, cinnamon, and coriander, positively, affected postharvest total soluble solids compared with control. Moreover, Abd El Wahab (2015) worked on 'Florda 7.2' nectarine to tested some essential oils to maintain postharvest fruit quality and reported that TSS increased with increasing storage and marketing periods, moreover, Coriander oil during cold storage and market life periods delayed the changes in total soluble solids compared with control. The results disagree with Rattanapitigorn et al. (2006) and Serrano et al. (2005) previous experiments using natural antifungal compounds (eugenol, thymol and menthol vapors) revealed benefits due to reduced weight loss percentage in cherry and grape. Similar weight loss results were obtained when eucalyptus and cinnamon oils were applied to strawberry and tomato (Tian et al., 2011).

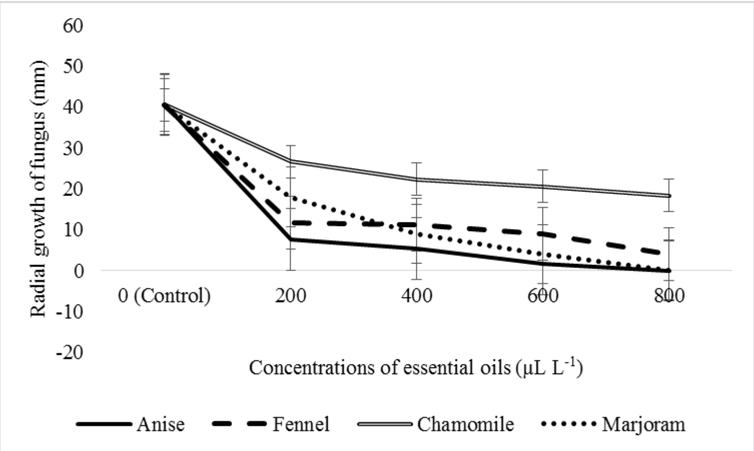

Fig. 3. Interaction effect of different concentrations of four essential oils on radial growth (mm) of *B. cinerea* in *in vitro* conditions

CONCLUSION

Considering the reduction in mycelia growth of *B. cinerea* in *in vitro*, we can conclude that anise oil could be used as possible bio fungicides, as an alternative to synthetic fungicides, against *B. cinerea*. However, more studies are required before these essential oils can be recommended as commercial and natural antifungal agents to increase the postharvest storage life of other horticultural crops.

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Conflict of interest

The authors declare that they have no conflict of interest.

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