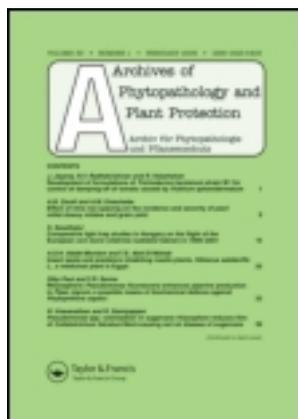


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In vitro* and *in vivo* antifungal activities of the essential oils of various plants against strawberry grey mould disease agent *Botrytis cinerea

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In order to investigate the effects of antifungal essential oils on postharvest decay and some quality factors of strawberry fruit, experiments were conducted under *in vitro* and *in vivo* conditions. The antifungal activities of essential oils obtained from fennel, anis, peppermint and cinnamon at concentrations 0, 200, 400, 600 and 800 $\mu\text{L L}^{-1}$ were investigated against *Botrytis cinerea* with four replications. *In vitro* results showed that the growth of *B. cinerea* was completely inhibited by fennel, cinnamon and anis essential oils at relatively low concentrations (400–800 $\mu\text{L L}^{-1}$). *In vivo* results showed that all the used essential oils at all applied concentrations caused an increase in the shelf life and inhibited *B. cinerea* growth on strawberry fruits completely in comparison to the controls. The results of this study confirmed the antifungal effect of four essential oils in both *in vitro* and on fruit postharvest.

Keywords: antifungal activities; essential oils; grey mould; strawberry

Introduction

Strawberry (*Fragaria ananassa* Duch.) is a non-climacteric fruit characterised by short postharvest life, often estimated less than a week even under ideal conditions at 8°C (Wills 1998). Loss of quality is frequently due to the onset of rots, which is caused by *Botrytis cinerea* (Dris et al. 2001; Elad et al. 2004; Williamson et al. 2007; Zhang et al. 2007). *Botrytis cinerea* Pers: Fr (grey mould rot) is a ubiquitous pathogen, which causes severe damage in many fruits, vegetables and ornamental crops in pre- and postharvest. The grey mould disease is very destructive on strawberry fruits in postharvest (Elad et al. 2004). Chemical control remains the main measure to reduce the incidence of postharvest disease in various fruits and vegetables. Antimicrobial chemicals belonging to the groups of benzimidazoles, aromatic hydrocarbon and sterol biosynthesis inhibitor are often used as postharvest treatment (Elad et al. 2004). A serious problem against the effective use of these chemicals is the development of resistance by the fungi (Bhaskara et al. 1998). The application of higher concentrations of chemicals in an attempt to

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overcome this problem increases the risk of high level toxic residues in the products, which is particularly serious because fruits and vegetables are consumed in a relatively short time after harvest (Elad et al. 2004). 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). Essential oils are naturally occurring terpenic mixtures, whose insecticidal action against specific pests and fungicidal action against some important plant pathogens have been recently reviewed (Isman 2000). It is believed that it is difficult for the pathogens to develop resistance to such a mixture of oil components with, apparently, different mechanisms of antimicrobial activity. In recent years, numerous studies have documented the antifungal effects of plant essential oils to control food spoilage fungi *in vitro* and *in vivo* (Feng and Zheng 2007; Omidbeygi et al. 2007; Amiri et al. 2008; Tian et al. 2011). Takayuki et al. (2007) measured the antifungal effects of 52 dried samples of spices and herbs against the soil-borne phytopathogenic fungus, *Fusarium oxysporum*. Black zira (*Bunium persicum*) showed the strongest effect, followed by cumin (*Cuminum cyminum* L.) and cardamom (*Elettaria cardamomum* Maton) (Takayuki et al. 2007). In this study, we used the essential oils of fennel, anis, peppermint and cinnamon. The aim of this study was to investigate the fungicidal potential of various essential oils to reduce *B. cinerea* *in vitro* conditions and to evaluate their effect on strawberry fruit quality after ambient storage *in vivo* conditions.

Materials and methods

Plant materials and extraction of essential oils

Air-dried seeds of fennel and anis, aerial parts peppermint and bark of tree cinnamon were supplied from agricultural research fields of Ferdowsi University of Mashhad, Iran. After the plant seeds and aerial parts had been authenticated, a 100 g portion of each was subjected to hydrodistillation 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. The yields from fennel, cinnamon, anis and peppermint extraction were 3.5, 1.0, 3.0 and 1.5% (w/w), respectively.

In vitro experiment

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

Antifungal activity was studied using a contact assay (*in vitro*) that produced hyphal growth inhibition. The assay was previously used for essential oil treatment on potato dextrose agar (PDA) medium by the “solution method” (SM) (Ozden and Bayindirli 2002). Briefly, the essential oils were dissolved in 50 mL L^{-1} Tween 80/water solution and the required amounts of these solutions were added to individual Petri dishes containing 20 mL of PDA medium at 45°C. Then, a 0.5 mm disc of mycelium was placed on the PDA medium in each dish. The treated media were incubated at 24°C and mycelia growth was measured daily. The inhibitory percentage (IP) was determined from the formula $\text{IP} = [(dc - dt)/dc] \times 100$, where dc is the mycelium diameter in the control Petri dish and dt is the mycelium diameter in the essential oil-treated Petri dish.

Spore germination assay

The effects of the essential oils on spore germination in PDA were tested. The oils were added to 10 mL glass tubes each containing 5 mL of PDA to obtain final concentrations of 0, 200, 400, 600 and 800 $\mu\text{L L}^{-1}$. A spore suspension (10^5 spores mL^{-1}) of *B. cinerea* was prepared from an actively growing culture (7–8 days old) in distilled sterile water. A 1 mL aliquot of this spore suspension was added to each tube. After 20 h of incubation at 28°C on a rotary shaker (at 200 rpm), at least 100 spores per replicate were observed microscopically (Olympus, Tokyo, Japan) to determine their germination rate (Xu et al. 2007).

In vivo experiment*Effect of these essential oils on postharvest decay and some qualities factors of B. cinerea inoculation on strawberry fruits*

Infected strawberry 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 use. Spore suspensions were prepared by removing spores from the sporulation edges of a 7- to 8-day-old culture with a bacteriological loop and suspending them in sterile distilled water. Spore concentration was determined with a haemocytometer and adjusted as required with sterile distilled water (10^5 spores mL^{-1}). Before infection, fruits were treated with sodium hypochlorite (100 $\mu\text{L L}^{-1}$). They were then sprayed in the prepared suspension and stored at room temperature for 2 h in order to fix the fungal inoculation (Asghari et al. 2009). In this phase, the SM was used as in the *in vitro* experiment. Fruits were treated with the different concentrations of cinnamon, anis, fennel and peppermint essential oils and stored in separate packages in order to prevent loss of essential oils. Treated and untreated (control) fruits were placed in cold storage (4°C) for 15 days.

Fruit storage life

The degree of infection on fruits (decay score) was rated using a scale of 0–8, where 0 = 0% infection, 1 \leq 10% infection, 2 = 10–20% infection, 3 = 20–30% infection, 4 = 30–40% infection, 5 = 40–50% infection, 6 = 50–65% infection, 7 = 65–80% infection and 8 \geq 80% infection.

pH, titrable acidity and total soluble solids

The pH of fruit juices was measured at 20°C using a pH meter (Jenway 3320; Bibby Scientific, Staffordshire, UK). Titratable acidity (TA) was determined by titration with 0.05 mol L^{-1} NaOH to pH 8.1 and reported as g malic acid per 100 g fresh weight (AOAC 2000). Total soluble solids (TSS) were determined at 20°C using a refractometer (RFM340; Bellingham and Stanley, Bellingham, UK) and reported as °Brix.

Weight loss percentage

In order to determine any weight loss during fruit storage, both the treated and untreated fruits were weighed at the beginning and end of the storage period.

Ascorbic acid content

Ascorbic acid contents was measured by classical titration method using 2,6-dichlorophenol indophenol solution and expressed as mg per 100 g (AOAC 2000).

Anthocyanin content

Total anthocyanin contents were determined by the pH-differential method (Rapisarda et al. 2000). A 1.0 ml aliquot strawberry fruit extract was diluted to 10 ml with a pH 1.0 solution (125 ml 0.2 M KCl and 375 ml 0.2 M HCl). A second 1.0 ml aliquot was diluted to 10 ml with a pH 4.5-buffered solution (400 ml of 1 M CH₃CO₂Na, (acetate Sodium) 240 ml 1 M HCl, and 360 ml H₂O). The absorbance of the solution was measured at 510 nm by using a UV spectrophotometer (CE2502, Bio Quest., and Cambridge, UK).

Statistical analysis

The experiment was conducted in a completely randomised factorial design with four replications consisting of 12 fruits each. Data were analysed using SAS version 9.1. (SAS Institute, Cary, NC, USA), and means were compared by Duncan's multiple range test at 5% level of confidence.

Results

Effect on radial growth fungus of *B. cinerea*

The effects of different concentrations of these essential oils on the radial growth of *B. cinerea* are shown in Figure 1. All four essential oils were found to inhibit the growth of *B. cinerea* in a dose-dependent manner. The results indicated that the highest radial growth was observed in the control (without essential oil application), while the lowest were obtained with 600 and 800 $\mu\text{L L}^{-1}$ of cinnamon, anis and fennel essential oils, respectively. When growth inhibition was calculated as the percentage of inhibition of *B. cinerea* radial growth relative to the control, cinnamon oil had the highest value 73.18%.

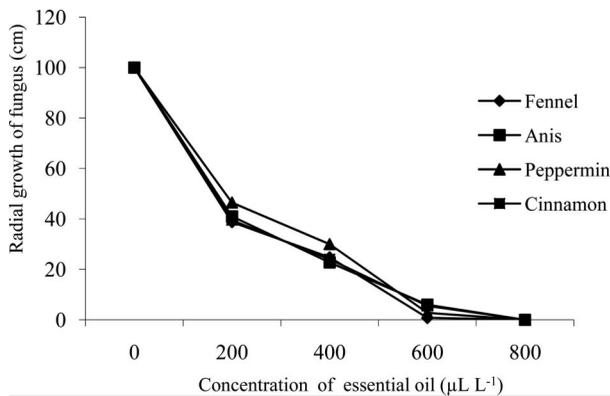


Figure 1. Effect of different concentrations of four essential oils on radial growth (cm) of *B. cinerea*.

Effect of essential oils on spore germination of *B. cinerea*

Spore germination of *B. cinerea* was inhibited by cinnamon, anis, fennel and peppermint oils at all concentrations (Figure 2). All four essential oils at 800 $\mu\text{L L}^{-1}$ inhibited spore germination completely. However, peppermint oil was generally slightly less effective than fennel, anis and cinnamon oils at inhibiting spore germination.

Effect of essential oils on postharvest quality factors of strawberry*Fruit storage life (decay)*

Essential oil-treated fruits were better maintained and had lower decay scores than control fruits, which showed maximum deterioration (Figure 3). Fruits treated with cinnamon oil at 600 $\mu\text{L L}^{-1}$ had the lowest decay score.

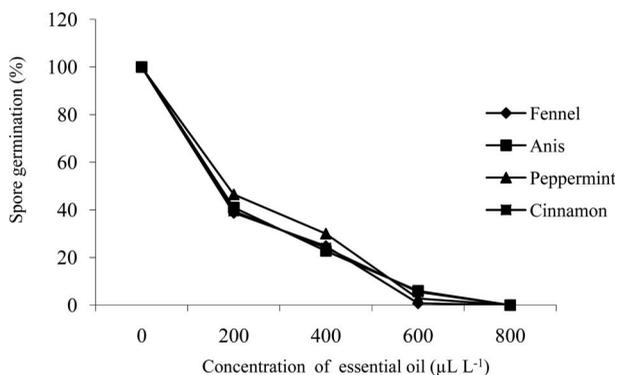


Figure 2. Effect of different concentrations of four essential oils on spore germination (%) of *B. cinerea*.

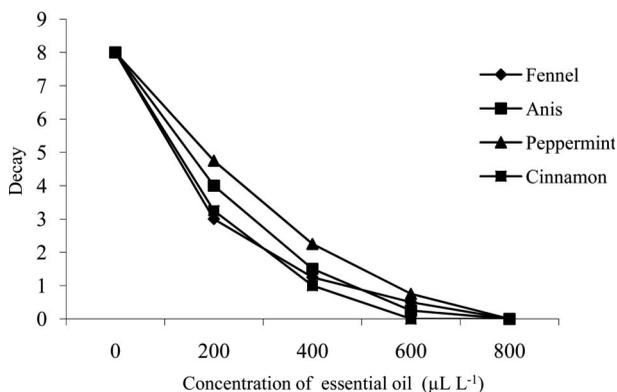


Figure 3. Effect of different concentrations of four essential oils on decay of strawberry fruits cv. Selva during storage.

Total soluble solids

The effect of the three essential oils on TSS is shown in Figure 4. Significant differences in TSS were observed between treated and control fruits. Fruits treated with fennel and anis oils at $800 \mu\text{L L}^{-1}$ had the highest TSS (11.75°Brix).

Titration acidity and taste index

There were significant differences in TA between treated and control fruits (Figure 5). The best essential oil was cinnamon oil with 0.935 (g per 100 g fresh weight) TA content. Cinnamon and anis oils at $800 \mu\text{L L}^{-1}$ proved to be the best treatment. There were no significant differences in taste index (TSS/TA) among treatments (data not shown).

pH

There were significant differences in pH between treated and control fruits (Figure 6). Fruits treated with fennel and cinnamon oils at $800 \mu\text{L L}^{-1}$ had the lowest pH (3.07 and 3.08).

Weight loss percentage

The weight loss percentage of essential oil-treated fruits was significantly lower than that of control fruits ($p < 0.01$). Fruits treated with anis oil at $600 \mu\text{L L}^{-1}$ showed the lowest weight loss percentage (6.57%), while control fruits followed by $800 \mu\text{L L}^{-1}$ essential oil-treated fruits showed the highest weight loss percentages (Figure 7).

Ascorbic acid content

The ascorbic acid content of fruits differed significantly among treatments when compared with the control (Figure 8). The best essential oil was cinnamon oil with 28.04 mg per 100 g ascorbic acid content. The results showed that cinnamon and anis oils at $800 \mu\text{L L}^{-1}$ were the lowest change in ascorbic acid content than the control with 32.53 and 32.12 mg per 100 g.

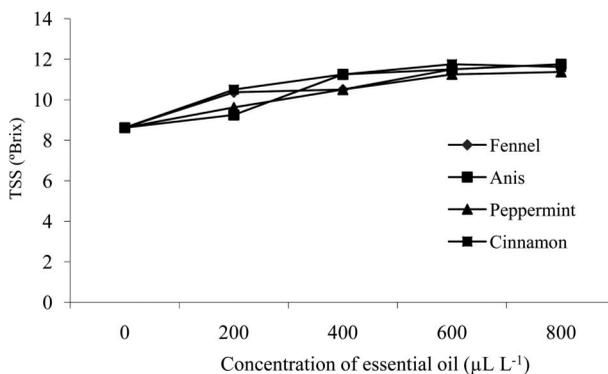


Figure 4. Effect of different concentrations of four essential oils on TSS (Brix) of strawberry fruits cv. Selva during storage.

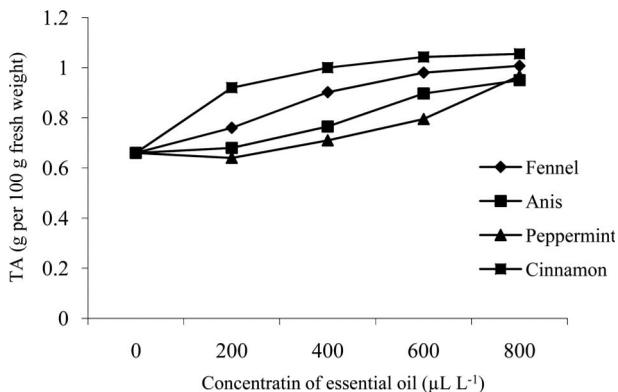


Figure 5. Effect of different concentrations of four essential oils on TA (g per 100 g fresh weight) of strawberry fruits cv. Selva during storage.

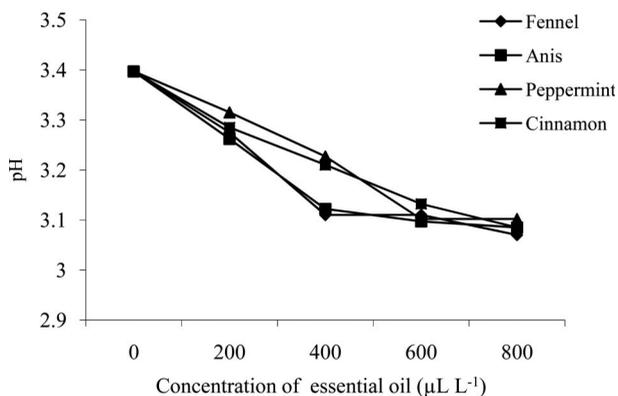


Figure 6. Effect of different concentrations of four essential oils on pH of strawberry fruits cv. Selva during storage.

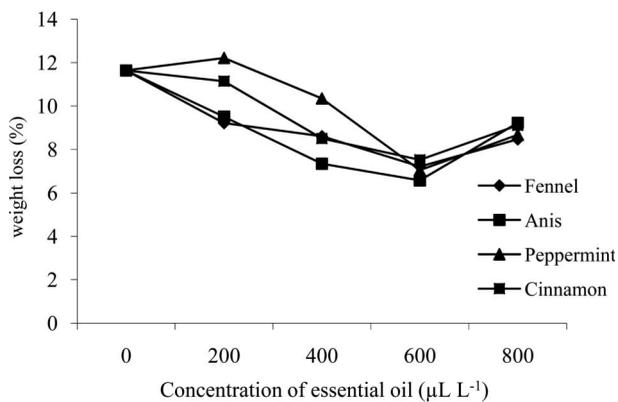


Figure 7. Effect of different concentrations of four essential oils on weight loss (%) of strawberry fruits cv. Selva during storage.

Anthocyanin content

The anthocyanin content of fruits differed significantly among treatments (Figure 9). The highest and lowest anthocyanin levels were found in $600 \mu\text{L L}^{-1}$ fennel oil-treated fruits (36.33 mg per 100 g) and control fruits (20.50 mg per 100 g), respectively.

Discussion

This study clearly showed that *Cinnamomum zeilanicom*, *Foeniculum vulgare*, *Pimpinella anisum* and *Mentha piperita* essential oils significantly reduced damage extent of the one important strawberry postharvest pathogens namely *B. cinerea*. This study indicated that fennel, anis and cinnamon oils had fungicide effective in concentrations of $400\text{--}800 \mu\text{L L}^{-1}$ *in vitro* conditions, respectively. Similarly, the

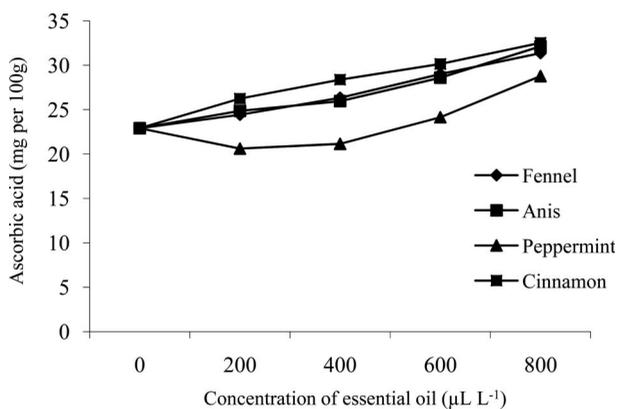


Figure 8. Effect of different concentrations of four essential oils on ascorbic acid (mg per 100 g) of strawberry fruits cv. Selva during storage.

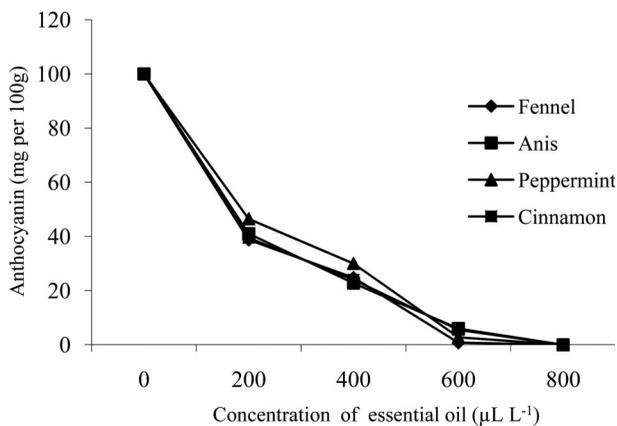


Figure 9. Effect of different concentrations of four essential oils on anthocyanin (mg per 100 g) of strawberry fruits cv. Selva during storage.

growth of *B. cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis* fungus was completely inhibited by oregano, thyme, dictamnus and marjoram essential oils (Bhaskara et al. 1998). Moreover, Chebli et al. (2003) indicated that essential oils of *Origanum compactum* and *Thymus glandulosus* inhibited the growth of the mycelium of *B. cinerea*. Spore germination and germ tube elongation were also inhibited by the essential oils which were tested, and among them, the essential oil of cinnamon had the highest inhibition on spore germination. The effect of essential oils on microbial growth has been reported by Fung et al. (1977), who thought it may be the result of phenolic compounds of essential oils that cause the altering of microbial cell permeability by interaction with membrane proteins. This would cause a deformation in cell structure and functionality and permit the loss of macromolecules from their interior (Pramila et al. 2012). Moreover, each of the essential oil components has its own contribution to biological activity of the oil.

The present results showed that the tested essential oils had a positive effect on storage life and reduced strawberry fruit decay, with 800 $\mu\text{L L}^{-1}$ cinnamon, anis and fennel oils treatment giving the longest storage life. The previous reports indicated that fruit decay reduced during postharvest treatments with volatile compounds for several products including raspberry and kiwifruit (Wang 2003; Williamson et al. 2007). Essential oils mainly conjugate to phenolic compounds, which accumulate in some plant cells, and have shown useful effects on these compounds for pathogen control (Plotto et al. 2003). It is known that oxidation products of phlorisidzin (an o-dihydroxyphenolic compound) inhibit fungal growth, and they are thought to inhibit the apple scab fungus *Venturia inaequalis* (Asghari et al. 2009). Fungal pectinases hydrolyse pectin, a cell wall compound that is abundant in the middle lamella and plays a role in cell adhesion. Thus, by inhibiting pectinases, the ability of fungi to hydrolyse and invade plant cell walls would be compromised (Vermeriss and Nicholson 2006). It seems that a similar role is played by phenolic compounds of essential oils. Thus, these findings reveal that exogenous essential oils may have a positive influence on the storage life and reduce the decay of strawberry fruits. Our study showed that the tested essential oils were effective in maintaining fruit quality. Essential oil-treated fruits had higher TSS, TA, ascorbic acid and anthocyanin content than control fruits, and fennel and anis oils at 800 $\mu\text{L L}^{-1}$ were the best treatment for TSS (11.75°Brix) and anthocyanin content (36.35 mg per 100 g), cinnamon oil had the highest TA content (1.05 g per 100 g fresh weight) and ascorbic acid content (32.53 mg per 100 g). The results were in agreement with those of Asghari et al. (2009) who reported that TSS and TA of strawberry infected with *B. cinerea* increased with the application of cumin oil. Our results indicated that essential oil application significantly decreased weight loss percentage, and fruits treated with anis oil at 600 $\mu\text{L L}^{-1}$ showed the lowest weight loss percentage (6.5%). Previous experiments using natural antifungal compounds (eugenol, thymol and menthol vapours) revealed benefits due to reduced weight loss percentage in cherry and grape (Serrano et al. 2005; Rattanapitigorn et al. 2006). Similar weight loss results were obtained when eucalyptus and cinnamon oils were applied to strawberry and tomato (Tian et al. 2011). In fact, there was a linear correlation between ethylene and damage, and thus, the fungus was responsible for the majority of ethylene production, a part of the basal level typical of non-climacteric fruits (Cristescu et al. 2002). Accordingly, it was reported that *B. cinerea* produced greater amounts of ethylene as the concentration of conidia inoculated *in vitro* or in the climacteric tomato fruit increased. The respiration rate was clearly affected by the essential oil

concentration and the degree of infection (Cristescu et al. 2002). Similarly, in our experiment, it could be concluded that the tested essential oils, by reducing the respiration rate, had a positive influence on the weight loss percentage of strawberry fruits.

Conclusion

Considering the reduction in mycelial growth and germination of *B. cinerea* *in vitro*, the reduced incidence of disease symptoms on essential oil-treated strawberry fruits and their increased storage life, we can conclude that cinnamon, fennel and anis essential oils could be used as possible biofungicides, as an alternative to synthetic fungicides, against phytopathogenic fungi on strawberry fruits. 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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