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Original Research Article

Effects of Essential Oils to Control *Rhizopus stolonifer in vitro* and *in vivo* on Strawberry

Samane Mohammadi[1], Hossein Aroiee[1], Mohammad Hossein Aminifard[2*], Ali Tehranifar[1] and Vahid Jahanbakhsh[3]

[1] Department of Horticultural Science, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, IRAN
[2] Department of Horticultural Science, College of Agriculture, Birjand University, Birjand, IRAN
[3] Department of Plant Protections, College of Agriculture, Ferdowsi University of Mashhad, IRAN

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ABSTRACT

The aim of this study was to evaluate the antifungal effects of essential oils (fennel, black caraway and anis) at different concentrations against the fungal pathogen, *Rhizopus stolonifer*, the causal agent of smooth rot disease of strawberry (*Fragaria ananassa* Duch.) under in vitro and in vivo conditions.The results of in vitro showed the growth of *R. stolonifer* was completely inhibited by the application of fennel, anis and black caraway oils at concentrations 600 and 800 ML.L^{-1}, respectively. The results of in vivo indicated that fennel, anis and black caraway oils at all applied concentrations inhibited *R. stolonifer* growth on strawberry fruits compared with control. Also the mentioned oils at upper concentrations showed positive effects on fruit quality characteristics like titrable acidity, total soluble solids, anthocianin content, ascorbic acid and weight loss percentage. These essential oils inhibited to infection fruits by *R. stolonifer* and increased life storage fruits. The present research confirms antifungal effects of these essential oils of both in vitro and in vivo on fruit postharvest.

Key Words: Antifungal activity, Essential oil, *Rhizopus stolonifer*, Strawberry

INTRODUCTION

The control of fungal decay of fruit is closely linked to the use of pesticides. However, throughout the world, the use of synthetic agricultural fungicides is increasingly being restricted (Dayan *et al*., 2009) to promote safer and biodegradable alternatives (Wisniewski *et al*., 2001). Fungicides including prochloraz, guazatine, imazalil, thiabendazole and pyrimethanil (Smilanick *et al*., 2008), serious environmental problems can result from irresponsible disposal of this hazardous waste. Fungicides can be retained by agricultural soils (Moller *et al*., 1999), before penetrating into ground water (Taube *et al*., 2002). Efforts to find alternative antimicrobial agents are focused on chemical classes that function by novel modes of action (Wilson *et al*., 1997), thereby preventing the development of pathogen resistance against protective products (Eckert *et al*., 1994). 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), and indeed, essential oils have been shown to possess antimicrobial and antifungal properties (Ahmet *et al*., 2005, Karmen *et al*., 2003). Takayuki *et al*. (2007) applied to measure the antifungal effects of 52 dried samples of spice and herbs against a soil-borne phytopathogenic fungus, *Fusarium oxysporum*. Soylu *et al.* (2010) investigated antifungal activities of essential oils obtained from aerial parts of aromatic plants, such as origanum (*Origanum syriacum* L. var. bevanii), lavender (*Lavandula stoechas* L. var. stoechas) and rosemary (*Rosmarinus officinalis* L.), against *Botrytis cinerea*. They showed that complete growth of pathogen was inhibited by the essential oil of lavender and rosemary. The main objectives of the present study were to assess and compare the inhibitory effects of fennel (*Foeniculum vulgare*), black caraway (*Carum carvi*) and anis (*Pimpinella anisum*) essential oils against *Rhizopus stolonifer* and evaluate the potential application of essential oils to control postharvest spoilage on stored strawberry (*Fragaria ananassa* Duch. cv. Selva).

MATERIALS AND METHODS

Plant materials and extraction of essential oils

Air-dried seeds of fennel, black caraway and anis were supplied from agricultural research fields of Ferdowsi University of Mashhad, Iran. After the plant seeds were authenticated, then 100 g of these medicinal plants were subjected to hydro distillation for 3 hours using a Clevenger type apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4 ºC for future analysis. The extracted yields from fennel, black caraway and anis extraction were 3.5%, 4% and 3%, respectively.

* Corresponding author: aminifard_mh55@yahoo.com

Gas chromatography (GC)
The essential oils were analyzed by using a Shimadzu GC-9 a gas chromatograph equipped with a DB-5 fused silica column (J and W Scientific Corporation) (30 m × 0.25 mm i.d., film thickness 0.25 µm). Helium was used as the carrier gas with a linear velocity of 32 cm s^{-1}, then the percentages of compounds were calculated by the area normalization method without considering response factors.

Gas chromatography–mass spectroscopy (GC-MS)
GC–MS analyses were carried out in a Varian 3400 GC–MS system equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm); oven temperature was 50–240 °C at a rate of 4 °C/min, transfer line temperature 260 °C, carrier gas, helium, with a linear velocity of 31.5 cm/s, split ratio 1:60, ionization energy 70 eV, scan time 1 s, and finally mass range was 40–300 amu.

Component identification
The components of the oils were identified by comparison of their mass spectra with those of a computer library or with authentic compounds and confirmed by comparison of their retention indices, either with those of authentic compounds or with data published in the literature. The retention indices were calculated for all volatile constituents using a homologous series of alkanes (Davies 1990).

Design of experiments and treatments
All the experiments (in vitro and in vivo) were carried-out in randomized factorial design with two factors; including three essential oils (fennel, black caraway and anis) and five concentrations (0, 200, 400, 600 and 800 $\mu L.L^{-1}$)with four replications.

***Antifungal effects of essential oils on mycelial radial growth* in vitro**
Antifungal activity was studied by using a contact assay (in vitro), which produces hyphal growth inhibition. The method was used for essential oils treatment on potato dextrose agar (PDA) medium "Solution Method"(SM) (Ozden *et al.* 2002). In this method the essential oils were dissolved in tween 80-water solution (5% v/v) and required amounts of the solutions were added to each of the PDA plates containing 20 ml of agar at 45 °C in petri dish. A 0.5 mm disk of mycelium of 3 days old culture *R. stolonifer* was located on PDA medium. The treated medium was incubated in 24°C and mycelium growth was determined daily. Inhibitory percentage was determined according to the following formula. IP= dc-dt×100/dc. IP= Inhibitory percent, dc= mycelium growth diameter in control and dt= mycelium growth diameter in essential oil treated petri dish. Four replications used for each treatment.

Spore germination assay
The effect of fennel, black caraway and anis oils on spore germination were tested in potato dextrose Agar (PDA). The used oils were added to a 10 ml glass tube containing 5 ml PDA to obtain final concentrations 0, 200, 400, 600 and 800 $\mu L.L^{-1}$. Spore suspension (10^5spores ml^{-1}) of *R. stolonifer* was prepared from actively growing culture (3 days old) in distilled sterile water. At the same time, aliquots (1 ml) of spore suspensions of *R. stolonifer* were added to each tube. After 18 h of incubation at 28 °C on a rotary shaker (200 rpm), at least 100 spores per replicate were observed microscopically to determine germination rate (Xu *et al.,* 2007).

Effect of essential oils on postharvest decay and some qualities factors of* R. stolonifer *inoculation on strawberry fruits
R. stolonifer was isolated from infected strawberry fruit. Spore suspensions were prepared by removing the spores from the sporulation edges of a 3 days old culture with a bacteriological loop, and suspending them in sterile distilled water. Spore concentration was determined with a hemocytometer (1×10^5), and adjusted as required with sterile distilled water. At first, fruit were treated by sodium hypochlorite (100 $\mu L.L^{-1}$). Then fruits were dipped in prepared suspension and located in room temperature for 2 h in order to fixed fungal inoculation (Asghari *et al.,* 2009). According to the in vitro experiment, SM method (solution method) was used. Fruits were treated by required essential oils, treated the fruit, by dipping at concentrations of 0, 200, 400, 600 and 800 $\mu L.L^{-1}$ for 30 second and located in the packages separately (disinfection of plastic containers that full were closed). Essential oil-treated and untreated fruits together with control were transferred into packages and were steeked in order preventing loss of oils and then put into the cold storage (4°C).

Decay incidence in the cold storage conditions
The degree of infection on fruit was rated using a scale of 0 to 9, where 1= no infection; 2= trace infection low than 10%, 3= infection between 10- 20%, 4= infection between 20- 30%, 5= infection between 30- 40%, 6= infection between 40- 50%, 7= infection between 50- 65%, 8= infection between 65- 80% and 9 = infection more than 80% (Asghari *et al*, 2009).

Quality parameters

Titrable acidity (TA), Total soluble solids (TSS) and pH
The pH value of fruit was measured with a pH meter (model Jenway 3320) at 20°C. Titratable acidity (TA) was determined by titration with 0.1 N NaOH (Horwitz 1975). Total soluble solids (TSS) was determined at 20°Cwith a digital Refractometer (model RFM340, UK)

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 (Horwitz 1975).

Weight loss percentage
In order to determine any weight loss during the storage, both treated and untreated fruit were weighted at the previous and end of the storage day.

Anthocyanin
Total anthocyanin content was determined by the pH differential method (Rapisarda 2000).

Statistical analysis
Data were analyzed using SASS 9.1 statistical software and means were compared by Duncan's multiple range test (DMRT) at 5% level of confidence.

RESULTS

Essential oils composition
The chemical compositions of the essential oils were determined by the GC-MS analysis. The identification of the unknown compounds was based on their relative retention time. The results are presented in Table 1. Components were mainly composed of monoterpenes and terpenes. The oil of black caraway was found to contain mainly cumin aldehyde [50% (v/v)], followed by perill aldehyde [21.8 % (v/v)]. The sample of fennel oil contains anethole as a major component [75.2 % (v/v)] followed by fenchone [9.8 % (v/v)]. The major constituents of anis oil were trans-anethole [90.7 % (v/v)] and estragole [4.3% (v/v)].

Table 1. Composition of the fennel, black caraway and anis essential oils of experiment.

Essential oils	RI*	Compounds	Percent
Fennel	935	α-thujone	0.73
(*Foeniculum vulgare*)	981	Myrcene	0.43
	1,000	α- Phelandrene	2.74
	1,071	Fenchone	9.37
	1,016	p-cymene	0.54
	1,221	Estragole	3.51
	975	β-Pinene	2.23
	1,279	Anethole	75.15
Black caraway	975	β- Pinene	0.7
(*Carum carvi*)	1,000	α- Phelandrene (4.5%)	4.5
	1,016	Para- Cymene	4.3
	1,060	Gama- Terpinene	10
	1,266	Cumin aldehyde	50
	1,181	Perill aldehyde	21.8
	939	α- Pinene	0.19
Anis	1,163	Estragole	4.3
(*Pimpinella anisum*)	1,200	Anisaldehyde	2.5
	1,254	trans-Anethole	90.7
	1,778	trans-Psedoisoeugeuyl 2-methylbutyrate	0.3

[+]Retention index

Effect of essential oils on radial growth of* R. stolonifer *and spore germination

The effects of different concentrations of these essential oils on the radial growth of *R. stolonifer* are shown in Fig 1. All used essential oils were found to inhibit the growth of *R. stolonifer* in a dose-dependent manner. The result indicated that the highest radial growth was observed in control (without essential oil application), while the lowest radial growth were in 600 and 800 $\mu L.L^{-1}$ of fennel and anis essential oils. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control, that the fennel oil had the highest percentage of inhibition with 76.29 %. Spores germination of *R. stolonifer* was inhibited by fennel, anis and black caraway essential oils (Fig 2). All of these essential oils at 800 $\mu L.L^{-1}$ had highest inhibitory effect of germination of spores of *R. stolonifer*. There were no significant differences between fennel and anis essential oils on this variable.

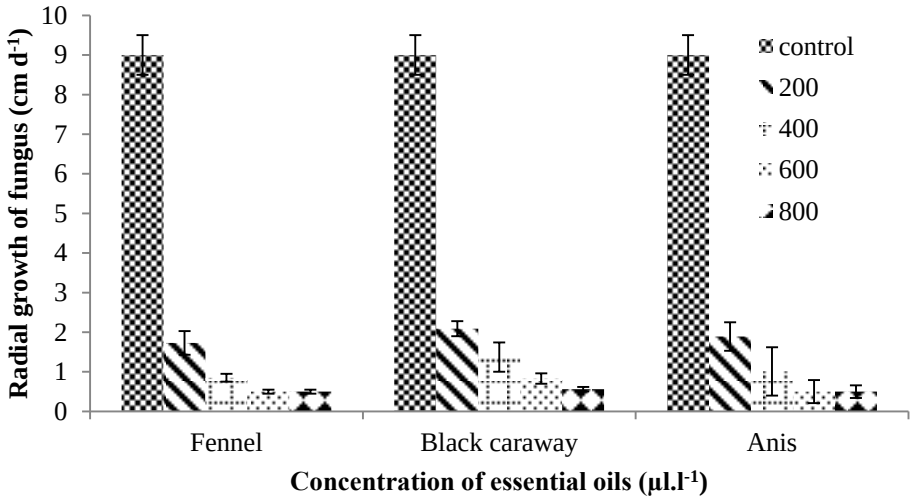

Figure 1. Effect of different concentrations of each of three essential oils on the radial growth (cm d^{-1}) of *Rhizopus stolonifer*.

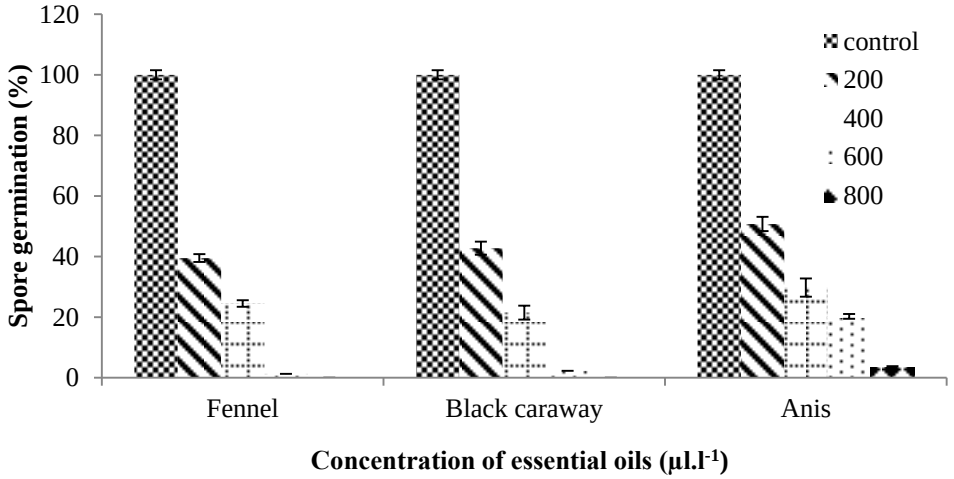

Figure 2. Effect of different concentrations of each of three essential oils on spore germination (%) of *Rhizopus stolonifer*.

Effect of essential oils on postharvest quality factors of strawberry

Essential oils-treated fruit could be maintained better and had low severity of decay scores, whereas non-treated fruit showed increased fruit deterioration (Fig 3). Among essential oils, treated fruits with fennel and anis essential oils with 600 $\mu L.L^{-1}$ had the lowest decay scores, while control had the highest decay score. The effect of these essential oils on TSS is shown in Table 2. The result indicated that significant differences were observed in TSS among treated fruits and control. Data showed that treatment with fennel essential oil at 800 $\mu L.L^{-1}$ had the highest TSS (12.13 ^{o}Brix). There was a significant difference in TA between treated fruits and control (Table 2), but there was no significant difference between fennel and anis oils applications. Among essential oils, fennel oil at 800 $\mu L.L^{-1}$ was the best treatment (1.09 g per 100 g FW). There was no significant difference in TSS/TA content among treatments (data not shown). The ascorbic acid content of fruits differed significantly among treatments when compared with the control (Table 2). The best essential oil was black caraway oil with 27.64 mg per 100 g ascorbic acid content. The results showed that fennel and black caraway oils at 800 $\mu L.L^{-1}$ were the lowest change in ascorbic acid content than the control with 33.06 and 32.56 mg per 100 g. The result showed that there were significant differences in pH value, between treatments and control (Table 2). In comparison with control, the fennel essential oil in the highest concentration tested had the lowest pH value (3.09). The weight loss percentage was very low for treated fruits with these essential oils and it showed a significant difference compared with control ($p< 0.01$). Among samples, treated fruits with fennel essential oil at 600 $\mu L.L^{-1}$ had the lowest percentage of weight loss (5.53 %), while control, followed by 800 $\mu L.L^{-1}$ concentration all of essential oils had the highest percentage of weight loss (Table 2). The anthocyanin content of treated fruits showed significant differences among treatments (Table 2). The highest and lowest anthocyanin levels were obtained by applying fennel oil at 800 $\mu L.L^{-11}$ (34.03 mg $100g^{-1}$ FW) and control (19.18 mg $100g^{-1}$ FW), respectively.

Table 2. Effect of three essential oils on postharvest quality factors in strawberry fruit

Treatment	TSS (°Brix)	TA ($g100g^{-1}$)	Ascorbic acid (mg 100 g^{-1})	pH	Weight loss (%)	Anthocyanin (mg 100 g^{-1} FW)
0 (control)	9.12^{g}	0.56^{h}	19.17^{f}	3.50^{a}	12.20^{a}	19.18^{f}
F_{200}	10.88^{c-e}	0.82^{e}	22.95^{e}	3.28^{bc}	$8.01d^{e}$	23.01^{de}
F_{400}	11.25^{bc}	0.91^{d}	26.28^{d}	3.17^{de}	7.70^{ef}	28.72^{bc}
F_{600}	12.00^{ab}	0.97^{cd}	29.27^{c}	3.13^{d}	5.53^{g}	33.13^{a}
F_{800}	12.50^{a}	1.09^{a}	33.06^{a}	3.09^{g}	8.03^{de}	23.03^{de}
B_{200}	9.78^{fg}	0.73^{f}	26.18^{d}	3.40^{b}	10.00^{b}	23.12^{de}
B_{400}	10.38^{de}	0.80^{e}	29.04^{c}	3.30^{b}	9.09^{cd}	23.63^{de}
B_{600}	11.05^{bc}	0.94^{cd}	31.24^{ab}	3.18^{de}	7.70^{ef}	29.32^{bc}
B_{800}	11.56^{ab}	1.04^{ab}	32.56^{ab}	3.15^{de}	9.89^{c}	29.07^{a-c}
A_{200}	10.04^{e-g}	0.66^{g}	24.19^{e}	3.29^{bc}	8.24^{de}	21.17^{ef}
A_{400}	11.38^{cd}	0.88^{e}	26.98^{d}	3.15^{de}	7.09^{ef}	26.78^{c}
A_{600}	11.65^{ab}	0.99^{bc}	28.88^{c}	3.17^{de}	6.87^{g}	30.18^{b}
A_{800}	11.78^{ab}	1.07^{a}	31.08^{b}	3.10^{e}	9.01^{de}	30.73^{a}

F: fennel seed essential oil; B: black caraway seed essential oil; A: anis seed essential oil. 200, 400, 600 and 800 $\mu L.L^{-1}$ concentrations of essential oils. Within each column, same letter indicates no significant difference between treatments at 5% levels.

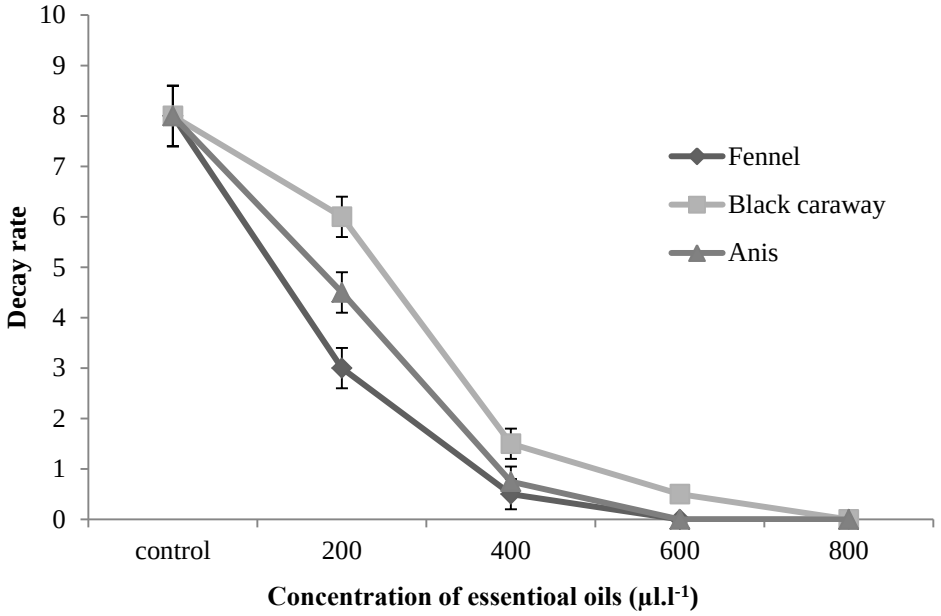

Figure 3. Effect of different concentrations of each of three essential oils on decay rate of strawberry fruit.

DISCUSSION

Natural antimicrobial agents such as essential oils can be used in food industry only if the compounds they release over time and their effects on target plant and pathogen are well-known and understood (Isman 2000). In vitro data presented in this study indicated that tested essential oils had fungicide effective in upper concentrations especially fennel essential oil. Similarly, the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis* fungus were completely inhibited by oregano, thyme, dictamnus and marjoram essential oils (Dimitra *et al*., 2003). Also Chebli *et al.* (2003) indicated that essential oils of *Origanum compactum* and *Thymus glandulosus* inhibited the growth of the mycelium of *Botrytis cinerea*. Spore germination and germ tube elongation were also inhibited by the essential oils which were tested, and among them thyme oil had the lowest 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. For example, the oil of fennel was found to contain ainly anethole as a major component [75.2 % (v/v)] followed by fenchone [9.8 % (v/v)]. The sample of black caraway oil contains cumin aldehyde [50 % (v/v)], followed by perill aldehyde [21.8 % (v/v)]. The major constituent of anis oil is trans-anethole [90.7% (v/v)] and estragole [4.3% (v/v)] and the previous reports showed these compounds have more fungicide effect (Takayuki *et al*., 2007). The results showed that tested essential oils had the positive effect on shelf life and reduced decay content, and fennel essential oil with-concentration of 800 $\mu L.L^{-1}$ had the most shelf 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 conjugated to phenolic compounds that accumulate in some plants cells and show useful effect for pathogen control (Plotto *et al.,* 2003). It is known that those oxidation products of phlorsidzin (an orhto-dihidroxyphenolic compound) inhibit fungal and are thought to inhibit the apple scab fungus venturia inaequalis (Asghari *et al*., 2009). Fungal pectinases hydrolyze 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 the fungus to hydrolyze and invade the plant cell wall would be compromised (Vermeriss and Nicholoson, 2006). It seems that similar role was played by phenolic compound of essential oils. Thus, these findings reveal that exogenous essential oils may have a positive influence on shelf life and the reduce decay of strawberry fruits. This study showed that essential oils were effective to maintain fruit quality. Treated fruits with essential oils had more total soluble solids, TA, ascorbic acid and anthocyanin content in comparison to control, and fennel essential oil in concentration 800 $\mu L.L^{-1}$ was the best treatment for TSS (11.08 $^{\circ}$Brix), TA (0.87 g 100 g^{-1} FW) and anthocyanin content (26.09 mg 100 g^{-1} FW). The obtained results were in agreement with Asghari *et al.* (2009), who reported that TSS and titrable acidity of strawberry infected *B. cinerea* increased with the applications of cumin essential oil. These results indicate that essential oils application significantly decreased weight loss percentage, and fruits treated with fennel essential oil at 600 $\mu L.L^{-1}$ had the lowest the percentage of weight loss (5.53 %). The previous experiments using natural antifungal compounds (eugenol, thymol and menthol vapors) revealed benefits due to reduced weight loss percentage in cherries and grapes (Rattanapitigorn *et al*., 2006, Serrano *et al*., 2005). Similar results were found with eucalyptus and cinnamon oil in strawberry and tomato on reducing weight loss percentage (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 has been 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 concentrations of these essential oils and dimension of infection (Cristescu *et al*., 2002). Similarly, (in our experiment), it could be concluded that essential oils by reducing respiration rate of fruit had a positive influence on weight loss percentage of strawberry fruit.

CONCLUSIONS

Considering the reduction in the mycelial growth and germination of *Rhizopus stolonifer* in vitro and incidence of disease symptoms on essential oil treated strawberry fruits and increasing shelf life, we can conclude that fennel, anis and black caraway essential oils could be used as possible bio fungicides alternative to synthetic fungicides against phytopathogenic fungi on strawberry fruits. However, more studies are required to recommend these essential oils as a commercial and natural antifungal to increase postharvest and shelf life on other horticultural crops.

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