Inhibition of grey mould *in vitro and in vivo* with essential oil of fennel (*Foeniculum vulgare* L.)

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Abstact

The aim of the study was to determine the antifungal effects of the fennel essential oil against fungal pathogen *Botrytis cinerea* the causal agent of grey mould disease of tomato fruit under *in vitro* and *in vivo* conditions. Treatments consisted of five concentrations (0, 200, 400, 600 and 800 μlL^{-1}).The fennel oil had a remarkable effect on spore germination of grey mould. The growth of grey mould was completely inhibited by fennel oil at 600 and 800 μlL^{-1}.The results *in vivo* showed that fennel oil increased the shelf life and decreased decay rate of tomato fruits. Also, fennel essential oil positively affected on postharvest quality factors. Treated fruits with fennel oil had significantly higher titrable acidity, total soluble solids, ascorbic acid, and lycopene and ß-carotene content comparison to control. Thus, these results showed that fennel essential oil has impact on postharvest decay and fruit quality of tomato.

Keywords: antifungal activity, essential oil, fennel, tomato

Introduction

The pre- and postharvest losses in world crops due to fungal disease may amount to more than 12% in developing countries (Agrios 1997).Grey mould (*Botrytis cinerea*) reduces the shelf life and market values of food commodities and renders them unfit for human consumption and cause undesirable effect on human health (Sharif, et al., 2010, Williams, et al., 2004). For many years, a variety of different synthetic chemicals (benzimidazoles) have been used as antifungal agents to inhibit the growth of plant pathogenic fungi (Brent and Hollomon,1998).However, there is a series of problems against the effective use of these chemicals in areas where the fungi have developed resistance (Brent and Hollomon, 1998).Some synthetic fungicides can also cause environmental pollution owing to their slow biodegradation in the environment(Barnard, et al., 1997).This has also increased the need for the development of new safe and biodegradable alternatives as natural fungicides. There is a growing interest on the research of the possible use of natural products such as plant-based essential oils, which may be less damaging for pest and disease control (Costa, et al., 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 (Tian, et al., 2011, Tzortzakis 2007).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*. Essential oils of seven Moroccan Labiatae were chemically analysed and evaluated for their *in vitro* antifungal activity against *Botrytis cinerea* (Chebli, et al., 2003). Among them, *Origanum compactum* and *Thymus glandulosus* greatly inhibited the growth of the mycelium and the inhibition of *Botrytis cinerea* was 100% for both oils at 100 ppm. 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 inhibition of pathogen by essential oil of lavender and rosemary was.Tomato is susceptible to postharvest decay caused by several pathogenic fungi such as by *Botrytis cinerea*, is a major disease of tomato. Therefore, we have undertaken to investigate the antifungal activity of the fennel essential oil against grey mould (*Botrytis cinerea)* and evaluate the potential application of fennel oil to control postharvest spoilage on stored tomato fruits.

Materials and methods

Plant materials and Extraction of essential oils

Air-dried seeds of fennel were supplied from agricultural research fields of Ferdowsi University of Mashhad, Iran. After the plant seeds had been authenticated, a 100g portion of each was subjected to hydro distillation for 3h 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 yield from fennel extraction was 3.5% (w/w).

Gas chromatography

The essential oil was analyzed using a Shimadzu GC-9 (Kyoto, Japan) gas chromatograph equipped with a DB-5 fused silica column (30 m× 0.25 mm i.d., film thickness 0.25m; J&W Scientific, California, USA).Helium was used as carrier gas at a linear velocity of 32 cm s^{-1}.The percentages of different compounds present in the oils were calculated by the area normalization method without considering response factors.

Gas chromatography–mass spectroscopy

Gas chromatography/mass spectrometry (GC/MS) analysis was carried out in a Varian 3400 GC/MS (California, USA) system equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific).The oven temperature was raised from 50 to 240 ˚C at a rate of 4 ˚C min^{-1}, the transfer line temperature was 260 ˚C, the carrier gas was helium at a linear velocity of 31.5 cm s^{-1}, the split ratio was 1:60, the ionization energy was 70 eV, the scan time was 1s and the mass range was 40–300 amu.

Identification of components

The components of the oil was 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).

The First experiment (In vitro experiment)

Antifungal effects of fennel essential oil on mycelial radial growth in vitro

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) (Özden and Bayindirli, 2002).Briefly, the essential oil was 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 IP = [(*d*c − *d*t)/*d*c] × 100, where *d*c is the mycelium diameter in the control Petri dish and *d*t is the mycelium diameter in the essential oil-treated Petri dish.

Spore germination assay

The effects of the essential oil on spore germination in PDA were tested. The oil was added to 10 mL glass tubes each containing 5 mL of PDA to obtain final concentrations of 0, 200, 400, 600 and 800 µlL^{-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 5 days of incubation at 28 ˚C a thin layer of mycelium was aseptically removed, placed in a drop of 1 g L^{-1} lactophenol/cotton blue on a microscope glass slide, stained for 2h and observed under a microscope (Olympus, Tokyo, Japan).For each treatment, four replicate tubes were used (Xu, et al., 2007).

The second experiment (In vivo experiment)

Infected tomato 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 7-8-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 µlL^{-1}).They were then dipped in the prepared suspension and stored at room temperature for 2 h in order to fix the fungal inoculation (Asghari Marjanlo, et al., 2009).In this phase the SM was used as in the *in vitro* experiment. Fruits were treated with the different concentrations of fennel essential oil and stored in separate packages in order to prevent loss of essential oil.Treated and untreated (control) fruits were placed in cold storage (5 ˚C) for 60 days.

Life storage (Decay rate)

Decay rate was calculated by number of fruits with visible fungal infection for each package.

Weight loss percentage

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

Carotenoids determination

Sixteen milliliters of acetone- hexane (4:6) solvent were added to 1.0 g of tomato homogenate and mixed in a test- tube. Automatically, two phases separated, and an aliquot was taken from the upper solution for measurement of optical density at 663, 645, 505 and 453nm in a spectrophotometer. Lycopene and β-carotene contents were calculated according to the Nagata and Yamashita (Nagata and Yamashita, 1992) equations: Lycopene (mg 100 ml^{-1} of extract) = -0.0458 *A_{663} + 0.204* A_{645} + 0.372* A_{505} - 0.0806 * A_{453}. β -Carotene (mg 100 ml^{-1} of extract) = 0.216*A_{663} - 1.22*A_{645} - 0.304*A_{505} + 0.452*A_{453}.

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 citric acid per 100 g fresh weight (Horwitz 1975).Total soluble solids(TSS) were determined at 20 ˚C using a refracto meter (RFM340, Bellingham and Stanley, Bellingham, UK) and reported as ˚Brix.

Ascorbic acid

Ascorbic acid contents was measured by classical titration method using 2, 6-dichlorophenol indophenols solution, and expressed as g 100 mg^{-1} (Miller 1998).

Statistical analysis

The analysis of variance was carried out according to the General Linear Models (GLM) procedure developed by SAS (SAS Institute, v 9.1).

Results

The identified components of fennel essential oil are listed in Table 1. A total of 8 compounds were identified from all the samples, accounting for 94.7 % of the total compositions of individual samples.

Table 1: Composition of fennel essential oil of experiment

Essential oil	RI*	Compound	Percent
Fennel (*Foeniculum vulgare*)	935	α-thujone	0.73
	981	Myrcene	0.43
	1000	α- Phelandrene	2.74
	1071	Fenchone	9.37
	1016	p-cymene	0.54
	1221	Estragole	3.51
	975	β-Pinene	2.23
	1279	Anethole	75.15

*Retention Index

Effect fennel oil on radial growth of grey mould and spore germination

The effects of different concentrations of fennel oil on the radial growth of grey mould were shown in Figure 1. Result indicated that the highest radial growth was observed in control (without essential oil application), while in concentration 600 and 800 μlL^{-1} of oil were no radial growth of fungus. Control of germination spores of grey mould was totally inhibited by fennel essential oil at upper concentrations and it had significant effect on inhibition of germination spores of grey mould. Data showed in concentration 800 μlL^{-1} oil was without germination spores of grey mould (Figure 2).

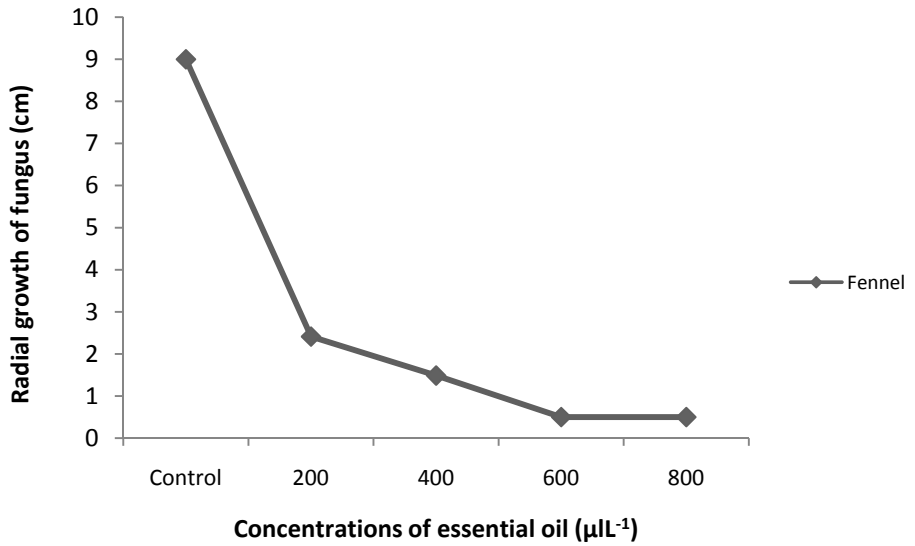

Figure 1. Effect of fennel essential oil on radial growth (cm) of grey mould

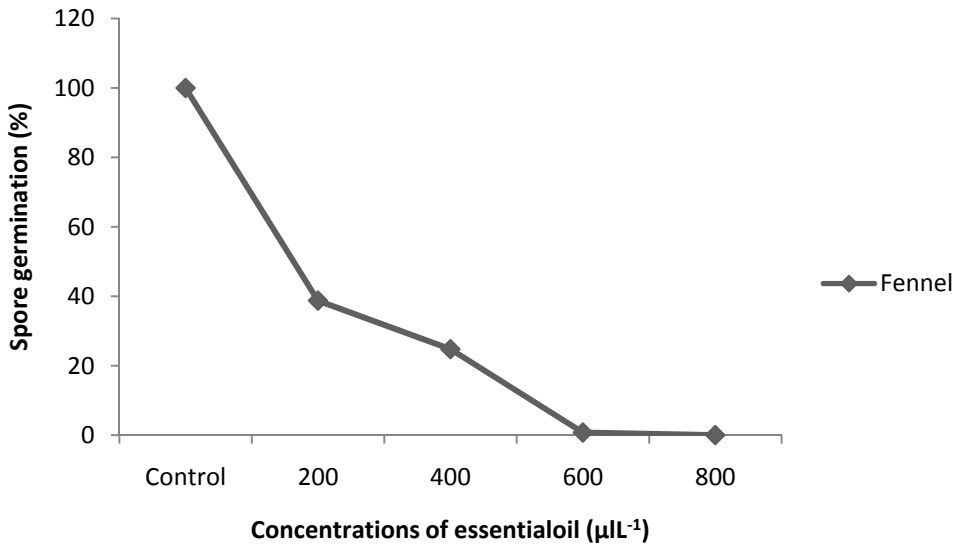

Figure 2. Effect of fennel essential oil on spore germination of grey mould

Effect of essential oils on postharvest quality factors of tomato

Life storage fruit (Decay rate)

It was found that treated fruits better maintained and had low severity of decay scores; whereas non-treated fruit showed increased fruit deterioration (Figure 3).The lowest decay scores were observed in concentrations 800 and 600 μlL^{-1}.

Weight loss percentage

The percentage of weight loss was very low for fruit treated by fennel oil and was significant comparison to control (Figure 4).Treated fruits by the fennel oil in concentrations 600 and 800 μlL^{-1} had the lowest weight loss percentage, while control had the highest weight loss percentage.

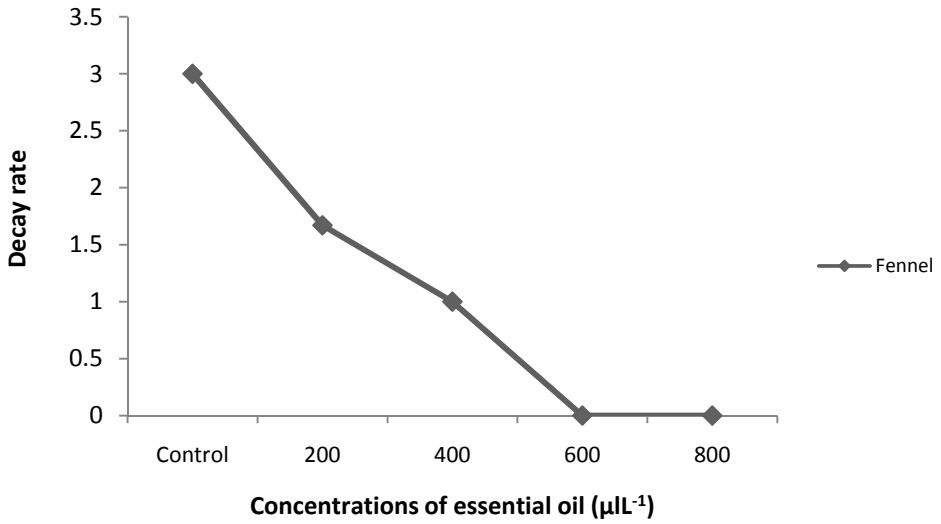

Figure 3. Effect of fennel essential oil on decay rate of tomato during storage

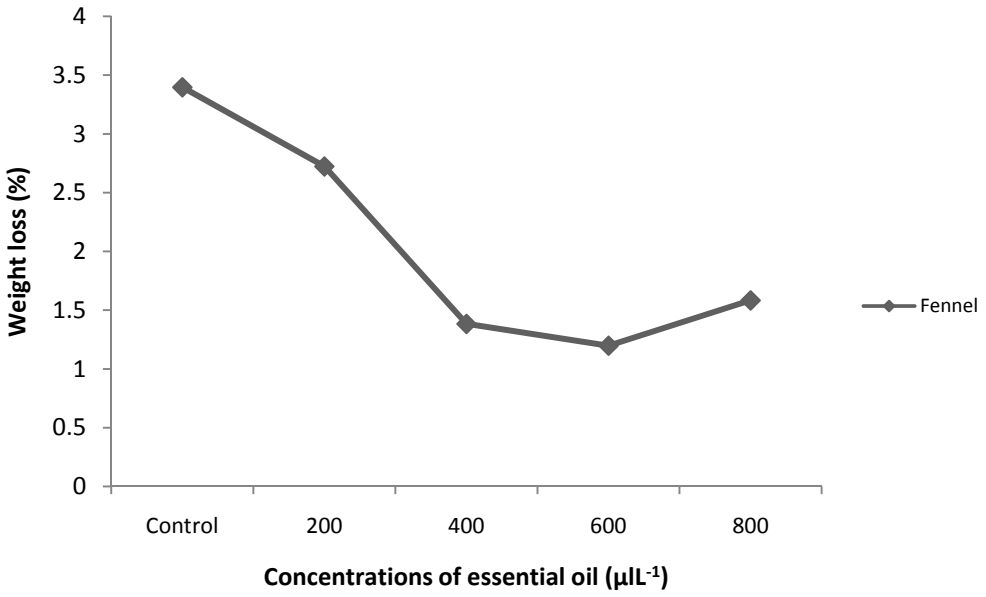

Figure 4. Effect of fennel essential oil on weight loss of tomato during storage

Lycopene and ß-carotene

It was found that lycopene and ß-carotene of treated fruits significantly improved by fennel oil in compared to the control. (Figure 5 and Figure 6).The maximum values of lycopene and β –carotene were obtained at 800 μlL^{-1} with 2.01 and 3.1 mg 100 ml^{-1}, respectively, while the minimum values were recorded in the control.

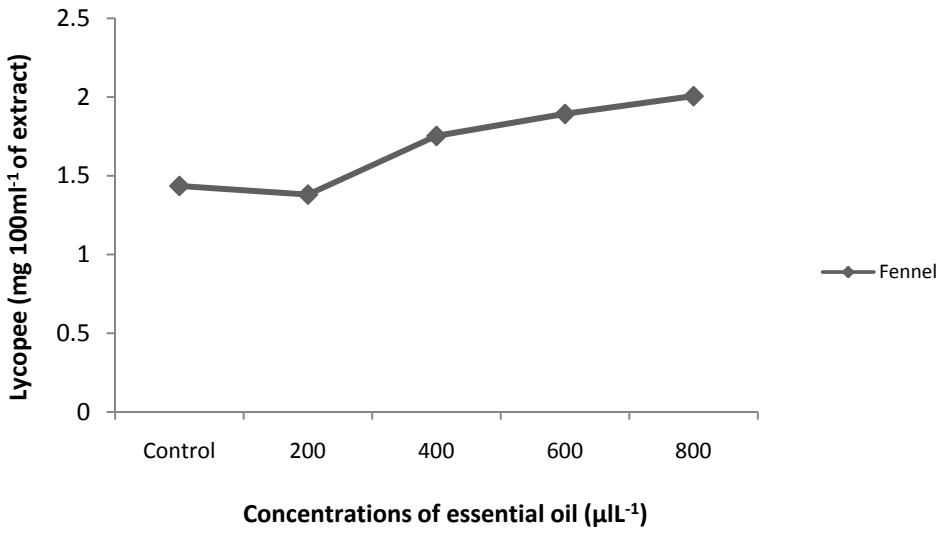

Figure 5. Effect of fennel essential oil on lycopene content of tomato during storage

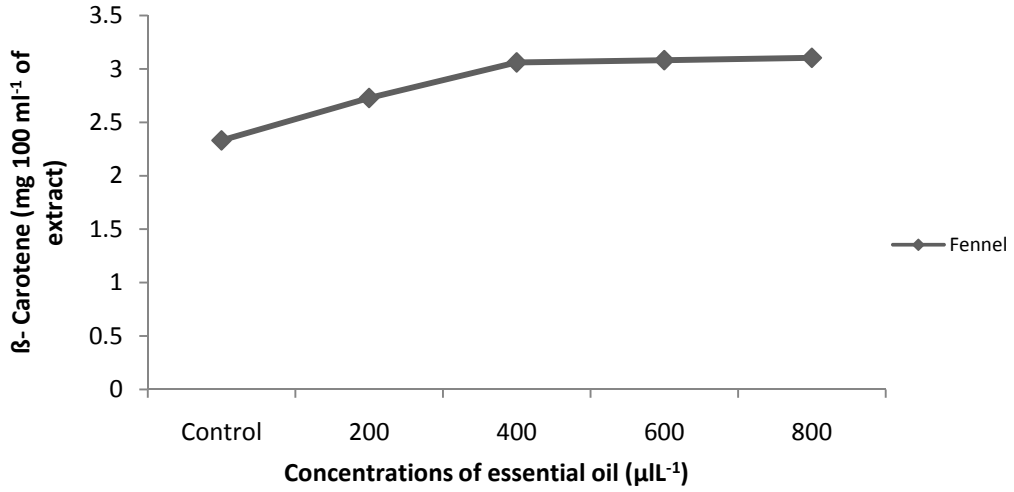

Figure 6**.** Effect of fennel essential oil on ß-carotene content of tomato during storage

Total soluble solids (TSS)

The result showed that a significant total soluble solid was observed in treated fruits compared to the control (Table 2).The highest TSS was recorded in concentration 800 μlL^{-1} (4.17 $^{\circ}$Brix), while the lowest value was in control (3 $^{\circ}$Brix).

Titrable acidity

There was significant difference in TA content of treated fruits with control fruits (Table 2).The fennel oil with concentration 800 μlL^{-1} had the highest TA content with 0.93 g 100 g^{-1}, while control had the lowest value with 0.58 g 100 g^{-1}.

Ascorbic acid

The effects of different concentrations of fennel oil on the ascorbic acid were shown in Table 2. The highest and the lowest ascorbic acid were obtained at 800 μlL^{-1} with 26.61 mg100 g^{-1} and control with 20.83 mg100 g^{-1}, respectively.

pH

The pH value of the treated tomato juice was significantly different from among concentrations of fennel oil and control (Table 2).Treated fruits in concentration 800 μlL^{-1} had the lowest pH value with 4.01, while control fruits had the highest pH value concentrations (4.34).

Table 2: Effect of fennel essential oil on postharvest quality factors

Treatment	TSS ($^{\circ}$Brix)	Titrable acidity (g 100 g^{-1})	Ascorbic acid (mg100 g^{-1})	pH
Control	3.0cd	0.58dc	20.83e	4.34a
F_{200}	3.17cd	0.65cd	22.59d	4.12b
F_{400}	3.5c	0.72c	24.05c	4.08bc
F_{600}	4.0ab	0.87ab	25.97ab	4.02bc
F_{800}	4.17a	0.93a	26.61a	4.01bc

F: Fennel oil, 200, 400, 600 and 800: concentrations of the essential oil (μlL^{-1}), Within each column, same letter indicates no significant difference between treatments at 5% levels.

Discussion

The *in vitro* data presented here indicated that fennel essential oil tested had a fungicidal effect at high concentrations. Similarly, growth of *B. cinerea, Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis* were completely inhibited by oregano, thyme, dictamnus and marjoram essential oils (Bhaskara Reddy, et al., 1998).Chebli, et al. (2003) also reported that the essential oils of *Origanum compactum* and *Thymus glandulosus* inhibited the mycelial growth of *B. cinerea*. Spore germination and fungal germ tube elongation were also inhibited by the essential oils tested here. The effects of essential oils on microbial growth were reported by Fung, et al. (1977), who thought that phenolic compounds in the essential oils altered microbial cell permeability by interacting with membrane proteins. This would then cause the deformation of cell structure, disrupt functionality, and permit the loss of macromolecules (Pramila, et al., 2012).Moreover, each component of an essential oil makes its own contribution to the overall biological

component (75.15%) followed by fenchone (9.37%), and previous research has shown these compounds have a strong fungicidal effect (Takayuki, et al., 2007).The present study showed that fennel essential oil tested had positive effects on the storage-life of tomato and reduced fruit decay, with 800 μlL^{-1} fennel oil giving the longest storage-life. Previous reports have indicated that fruit decay could be reduced by post-harvest treatment with volatile compounds from several plants, including raspberry and kiwifruit (Wang, et al., 2003; Williamson, et al., 2007). Essential oils are mainly conjugated to phenolic compounds which accumulate in some plant cells, and have positive effects on pathogen control (Plotto, et al., 2003). It is known that oxidation products of phlorsidzin (an *o*-dihydroxyphenolic compound) inhibit fungal growth, and are thought to inhibit growth of the apple scab fungus, *Venturia inaequalis* (Asghari Marjanlo, et al., 2009). Fungal pectinases hydrolyse pectin, a plant cell wall compound that is abundant in the middle lamella and has a key role in cell adhesion.Thus, by inhibiting its pectinases, the ability of fungus to hydrolyse and invade plant cell walls would be compromised (Vermeriss and Nicholoson, 2006). A similar inhibitory role appears to be played by the phenolic compounds in essential oils.Thus, our findings revealed that exogenous essential oils may have a positive influence on the storage-life of tomato fruit and reduce decay. Our study also showed that the essential oil tested was effective at maintaining fruit quality. Essential oil-treated fruit had higher TSS, TA, and lycopene and ß-carotene contents than control fruits. Our results agreed with those of Asghari Marjanlo, et al. (2009), who reported that, the TSS content and TA values of strawberry fruit infected with *B. cinerea* increased following the application of cumin (*C. cyminum*) oil. Our results also indicated that the application of essential oil significantly decreased weight loss percentage values. Previous experiments using natural anti-fungal compounds such as eugenol, thymol and menthol vapours, also resulted in reduced weight loss percentage in cherry and grape (Rattanapitigorn, et al., 2006; Serrano, et al., 2005). Similar weight loss results were obtained when eucalyptus or cinnamon oils were applied to strawberry and tomato fruit (Tian, et al., 2011). In fact, there was a linear correlation between ethylene levels and fruit damage as thus the fungus was responsible for the majority of ethylene production in non-climacteric fruit (Cristescu, et al., 2002).Thus, it has been reported that *B. cinerea* produced higher amounts of ethylene as the concentration of conidia inoculated *in vitro* increased in climacteric tomato fruit.The rate of respiration was also affected by the concentration of essential oil applied and the degree of fungus infection (Cristescu, et al., 2002). Similarly, in our experiments, it could be concluded that, by reducing the rate of respiration, the essential oil had a positive influence on the weight loss percentages in tomato fruit.

Conclusion

Considering the reduction in mycelial growth and germination of *B. cinerea in vitro*, the reduced incidence of disease symptoms on essential oil-treated tomato fruits and their increased storage life, we can conclude that fennel essential oil could be used as possible biofungicides, as an alternative to synthetic fungicides, against phytopathogenic fungi on tomato fruits. However, more studies are required before these essential oil can be recommended as commercial and natural antifungal agents to increase the postharvest storage life of other horticultural crops.

References

Agrios, G.N., (1997) Significance of plant diseases, in: Plant Pathology, fourth ed., Academic Press, San Diego, 25–37.

Asghari Marjanlo, A., Mostofi, Y., Shoeibi, Sh., Fattahi, M., (2009) Effect of Cumin Essential Oil on Postharvest Decay and Some Quality Factors of Strawberry. Journal of Medicinal Plants, 8(31), 25- 43.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., (2008) Biological effects of essential oils – A review. Food and Chemistry Toxicology, 46, 446 – 475.

Barnard, M., Padgitt, M., Uri, N.D., (1997) Pesticides use and its measurement. International Journal Pest Control, 39, 161–164.

Brent, K.J., Hollomon, D.W., (1998) Fungicide Resistance: The Assessment of Risk, FRAC, Global Crop Protection Federation, Brussels, 1–48.

Chebli, B., Mohamed, A., Idrissi, H., Mohamed, H., (2003) Chemical composition and antifungal activity of essential oils of seven Moroccan Labiatae against *Botrytis cinerea*. Journal of Ethnopharmacology, 89,165–169.

Costa, T.R., Orionalda, F.L.F., Santos, S.C., Cecília, M.A.O., Luciano, M.L., Pedro, H.F., Paula, J.R., Ferreira, H.D., Beatriz, H.N.S., Maria do, R.R.S., (2000) Antifungal activity of volatile constituents of Eugenia dysenterica leaf oil. Journal Ethnopharmacol, 72,111–117.

Cristescu, S.M., De Martinis, D., Hekkert, S.L., Parker, D.H., Harren, F.J.M., (2002) Ethylene production by *Botrytis cinerea* in vitro and in tomatoes. Applied Environmental Microbiology, 68, 5342–5350.

Davies, NW., (1990) Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and carbowax 20 M phases. Journal of Chromatography, 503, 1- 24.

Dimitra, J., Daferera, N., Ziogas, M., Polissiou, G., (2003) The effectiveness of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium* sp. and *Clavibacter michiganensis* subsp. Michiganensis. Journal Crop Protection, 22, 39–44.

Fung, DYC., Taylor, S., Kahan, J.,(1977) Effects of butylated hydroxyanisole (BHA) and butylated hydroxitoluene (BHT) on growth and aflatoxin production of Aspergillus flavus. Journal of Food Safety, 1, 39–51.

Horwitz, W.,(1975) Official methods of analysis of the Association of Official Analytical Chemist (AOAC). Washington, USA.

Miller, D., (1998) Food chemistry: a laboratory manual. John Wiley and Sons Press.

Nagata, M., Yamashita, I., (1992) Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. Journal of Japanese Society of Food Science and Technology, 39, 925- 928.

Özden, C., Bayindirli, L., (2002) Effects of combinational use of controlled atmosphere, cold storage and edible coating applications on shelf life and quality attributes of green peppers. Food Research Technology, 21, 320 - 326.

Plotto, A., Roberts, RG., Roberts, DD., (2003) Evaluation of plant essential oils as natural postharvest disease control of tomato (*Lycopersicon esculentum*). Acta Horticulture, 628,737- 745.

Rasooli, I., Rezaei, M.B., Allameh, A., (2006) Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlock*. Food Control, 17,359–364.

Rattanapitigorn, P., Arakawa, M., Tsuro, M., (2006) Vanillin enhances the antifungal effect of plant essential oils against Botrytis cinerea. The International Journal of Aromatherapy, 16,193–198.

Serrano, M., Martinez Romero, D., Castillo, S., Guillen, F., Valero, D., (2005) The use of the natural antifungal compounds improves the beneficial effect of MAP in sweet cherry storage. Innovative Food Science and Emerging Technology, 6,115- 121.

Sharif, M., Atiqur, R., Yunus, A., Sun Chul, K., (2010) Inhibition of plant pathogens in vitro and in vivo with essential oil and organic extracts of *Cestrum nocturnum* L. Journal Pesticide Biochemistry and Physiology, 96,86–92.

Soylu, M., Kurt, S., Soylu, S., (2010) In vitro and in vivo antifungal activities of the essential oils of various plants against tomato grey mould disease agent *Botrytis cinerea*. International Journal of Food Microbiology, 143, 183–189.

Takayuki, S., Mami, S., Azizi, M,, Yoshiharu, F., (2007) Antifungal effects of volatile compounds from black zira (*Bunium persicum*) and other spices and herbs. Journal Chemistry Ecology, 33, 2123- 2132.

Tian, J., Ban, X.Q., Zeng, H., He, J.S., Huang, B., Wang, Y.W., (2011) Chemical composition and antifungal activity of essential oil from *Cicuta virosa* L. var. latisecta Celak. International Journal of Food Microbiology, 145, 464-470.

Tzortzakis, N.G., (2007) Maintaining postharvest quality of fresh produce with volatile compounds. Innovative Food Science and Emerging Technologies, 8,111- 116.

Vermeriss, W., Nicholson, R., (2006) Phenolic Compound Biochemistry. Springer, New York, NY, pp. 273–274.

Wang, C.Y., (2003) Maintaining postharvest quality of raspberries with natural volatile compounds. Postharvest Biology and Technology, 28,181–186.

Williams, H.J., Philliips, T.D., Jolly, E.P., Stiles, K.J., Jolly, M.C., Aggrawal, D., (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, Potential health consequences, and interventions. The American Journal of Clinical Nutrition. 80, 1106–1120.

Williamson, B., Tudzynski, B., Tudzynski, P., Vankan, JAL., (2007) *Botrytis cinerea*: the cause of gray mould disease. Journal of Molecular plants Pathology, 8,561-580.

Xu, W., Huang, K., Guo, F., Qu, W., Yang, J., Liang, Z., Luo, Y., (2007) Postharvest grapefruit seed extract and chitosan treatments of table grapes to control *Botrytis cinerea*. Postharvest Biology and Technology, 46, 86–94.