J Food Sci Technol (November 2015) 52(11):7441–7448
DOI 10.1007/s13197-015-1871-7

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ORIGINAL ARTICLE

The control of Botrytis fruit rot in strawberry using combined treatments of Chitosan with *Zataria multiflora* or *Cinnamomum zeylanicum* essential oil

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Revised: 31 March 2015 / Accepted: 4 May 2015 / Published online: 22 May 2015
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Abstract This study evaluated the efficacy of the combined application of chitosan (CS) and *Zataria multiflora* (ZEO) or *Cinnamomum zeylanicum* essential oils (CEO) on the inhibition of *Botrytis cinerea*, the causal agent of strawberry gray mold on laboratory media and fruits during storage (7 days at 4 °C followed by 3 days at 20 °C). The application of different CS, CEO and ZEO concentrations inhibited the mycelial growth of the assayed fungus. The mixtures of CS and CEO (CC) or ZEO (CZ) inhibited the mycelia growth in potato dextrose agar, as well as the growth of *B. cinerea* in artificially infected strawberries stored at both room and cold temperature. Moreover, combined treatments showed more significant mycelial inhibition results and reduction of the IC_{50}, MIC and MFC values compared to pure EOs or CS ($p<0.05$). In fruit decay assays, combined treatments (CC or CZ) were able to reduce fungal decay in the range of 60–85 % at 9th day of storage. These results demonstrate the potential of the combined application of CS and CEO or ZEO at sub-inhibitory concentrations to control post-harvest pathogenic fungi in fruits, in particular, *B. cinerea* in strawberries.

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Keywords *Zataria multiflora* · Essential oil · *Cinnamomum zeylanicum* · Chitosan · Strawberry · *Botrytis cinerea*

Introduction

Strawberry (*Fragaria x ananassa*) is a highly perishable fruit crop of great importance throughout the world. It is considered as a 'soft fruit' due to delicate texture, coated by a very thin cuticle and presenting high susceptibility to physical damage and fungal decay (Amil-Ruiz et al. 2011). Botrytis fruit rot, also known as gray mold, is the most economically significant postharvest pathogen of strawberry fruits that caused by the fungus *Botrytis cinerea* (Pers.) (Bouchra et al. 2003). The disease affects fruit in the field, resulting in severe preharvest losses as well as postharvest, since *B. cinerea* fungus remains latent underneath the sepals until fruit ripening, then close to or after harvest it can turn from a saprophyte into a parasite (Powelson 1960).

Chemical fungicides provide the primary means for controlling postharvest fungal decay of fruit and vegetables. However, continuous and widespread use of fungicides has significant drawbacks including cost, handling hazards, contamination of fruits and vegetables with fungicide residues, and threats to human health and the environment (Paster and Bullerman 1988). Therefore, it has increased interest in finding safe and biodegradable alternatives as natural products to replace synthetic fungicides (Tripathi and Dubey 2004). In this context, environmentally friendly plant extract agents, such as essential oils (EOs), has shown great potential as alternatives to synthetic fungicides in disease control and quality maintenance in avocados (Sellamuthu et al. 2013), strawberries (Shao et al. 2013), and tomatoes (Soylu et al. 2010).

Extracts and EOs derived from Zataria and cinnamon are among some of the plant extracts that have been investigated

for their antimicrobial effects against plant pathogens. Zataria oil (ZEO), extracted from the dried *Zataria multiflora* Boiss. (*Lamiaceae*), is widely used and well known for its antimicrobial, antioxidant and antifungal activity (Sajed et al. 2013). The main compounds of ZEO are phenylpropanoids and terpenes such as thymol (46.61 %), carvacrol (17.26 %), *p*-cymene (11.51 %), γ-terpinene (4.01 %) and β-caryophyllene (2.91 %) (Saei-Dehkordi et al. 2010). ZEO has been proven to be very effective against *B. cinerea* (Ghorbani et al. 2014). In addition, cinnamon oil (CEO), extracted from the *Cinnammum zeylanicum* Blume (*Lauraceae*), is widely used as a spice and flavoring agent in foods for thousands of years and has many applications in the perfume, food and pharmaceutical industries (Bakkali et al. 2008). Modern studies showed that CEO also possesses anti-fungal (Carmo et al. 2008) and antibacterial activities (Matan et al. 2006).

The use of EOs in food preservation has been often limited because of their application costs and other drawbacks, such as their intense aroma and potential toxicity. An interesting approach to reduce the doses of EOs while maintaining their effectiveness could be to incorporate these compounds into the formulation of edible coatings (Perdones et al. 2012). In this sense, some researchers have reported the effects of EOs combined with hydroxypropylmethylcellulose, wax or chitosan (CS) to control the decay development and extend storage life of different fruits (Perdones et al. 2012; Sánchez-González et al. 2011).

Among these polysaccharides, CS [poly-(β-1/4)-2-amino-2-deoxy-D-glucopyranose] is the most interesting edible coating combined with EOs. It is a natural cationic polysaccharide that traditionally obtained from crustacean shells (crabs, shrimps and crayfish) using either chemical or microbiological procedures (Tikhonov et al. 2006). CS is generally recognized as safe (GRAS) by the FDA showing a broad-spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria and also fungi that could effectively control the fruit decay (Aider 2010).

CS-EOs edible coatings have proven to be effective at extending the shelf-life of some fruit and vegetables, such as table grapes (Sánchez-González et al. 2011), sweet pepper (Xing et al. 2011) and orange fruit (Cháfer et al. 2012). Shao et al. (2015) reported higher antifungal ability of CS– clove oil coatings compared to individual treatments, both in in vitro tests and in *Penicillium digitatum*- inoculated citrus fruits (Shao et al. 2015). Similarly, Perdones et al. (2012) stated that a CS– lemon oil coating might be a promising candidate to maintain the quality of strawberries (Perdones et al. 2012). However, to the best of our knowledge, no data are available regarding CS coatings with ZEO or CEO and their role against *B. cinerea,* the causal agent of gray mold, in in vitro tests and in strawberry fruit. Therefore, the combining effects of using CS with ZEO and CEO against this fungus under both in vitro and in vivo (artificially inoculated fruits) conditions were investigated in this study.

Materials and methods

Materials

C. Zeylanicum and *Z. Multiflora* essential oils (100 % pure) used in this study were purchased from Magnolia Co, IRAN. CEO and ZEO quality parameters such as odor, color, appearance, purity, solubility and also chemical properties including pH, acidity and brix were described in an accompanying technical report. The chemical compositions of applied essential oils were cinnamaldehyde (68.95 %), benzaldehyde (9.94 %), (E)-cinnamyl acetate (7.44 %), limonene (4.42 %) and eugenol (2.77 %) for CEO and thymol (51.2 %), p-cymene (13.8 %), carvacrol (11.26 %), linalool (6.83 %) and γ-Terpinene (4.46 %) together with other minor compounds for ZEO.

Growth media, Tween-80 and CS of low molecular weight (CZS 9012-76-4, Brookfield viscosity 20–300 cps and degree of deacetylation (DDA)>75.0 %) was obtained from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Each EOs was dissolved in distilled water with 0.05 % (*v/v*) Tween-80 as a surfactant to make 1 % (*v/v*) stock solution. For preparation of 1 % (*w/v*) CS stock solution, 5 g of CS were dissolved in 500 ml of 1 % (*v/v*) acetic acid and mixed by stirring at 40 °C for 2 h. Furthermore, each EOs was added separately to CS (1 %, *w/v*) at 1:1 ratio to make the combination of 1 % CS with 1 % CEO (CC) or ZEO (CZ). Finally, all stock solutions were filter-sterilized through a 0.2 μm microporous membrane and stored at 4 °C until testing time.

Microbial strain and culture media

Botrytis cinerea, IRAN 1304C was supplied from the Iranian Research Institute of Plant Protection (IRIPP). The fungi cultures were maintained and grown on Potato Dextrose Agar (PDA) slants at 25 ° C for 5 days. Cultures were stored at 4 °C and subcultured once a month.

Effects of CS, ZEO, CEO and their combinations on mycelial growth

Antifungal assays of CS, ZEO, CEO and their combinations were performed with the pour plate method as described by Askarne et al. (2012). In this method, solutions of serial concentrations of each treatment were mixed with sterilized potato dextrose agar (PDA) in the petri dish (9 cm dia.) containing 15 ml agar to obtain final concentrations from 0.01 to 0.15 %. Tween-80, 0.05 % (*v/v*) was added for enhancing the oil solubility. After inoculating the mycelia of fungus onto the center

of agar, the dishes were sealed with parafilm and incubated at 25 °C for 5–7 days, until the growth in the control plates (without the EOs and CS) reaches the edge of the plates. Then, the antifungal index (AI) of treatment was calculated as follows:

$$\text{Antifungal index}(\%) = [(C - T)/C] \times 100$$

Where C and T are the radial growth (mm) of fungus in the control and treated plates, respectively. The IC_{50} values (the concentration inhibited 50 % of the mycelium growth) were calculated by probit analysis. Minimal inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) were also examined using the methods reported by Yen and Chang (2008). When the mycelium of fungi reached the edges of the control dishes, the lowest concentration with no sign of growth was defined as MIC. After the MIC was determined, a small piece of agar ($2 \times 2 \times 2\ mm^3$) was taken from the colony of the MIC plate, and was inoculated on a drug-free PDA medium. After 5 days, MFCs were determined by the lowest concentration of the test compounds in which no recovery of microorganism was observed (Yen and Chang 2008).

Effects of CS, ZEO, CEO and their combinations on *B. cinerea* artificially infected strawberries

The in vivo study conducted on strawberry that obtained from the commercial market. The fruits were selected for the absence of defects, uniformity in size, and degree of ripening (2/3 red on the surface), and were disinfected with sodium hypochlorite solution (2.5 %) for 2 min. After abundant washing with distilled water (×3) the fruits were immersed separately for 1 min in the respective treatments (CS, CEO, ZEO, CC and CZ) at a concentration of 0.15 %. Strawberries immersed in distilled water were used as a control. Conidia of *B. cinerea* were recovered from 2-week old cultures by adding 10 ml of sterile water to each plate. The concentration of the conidial suspension was adjusted to 10^5 conidia per ml and a drop of Tween 80 was added to the suspension. Strawberries were inoculated and dried in air for 1 h, placed in covered plastic boxes and stored for 7 days at 4 °C, 95–98 % RH, and next exposed to 3 days shelf-life at 20 °C, 95–98 % RH. Five replicates of 30 strawberries were used for each of the treatments.

During the storage, the percentage of decayed strawberries was recorded. Disease severity was also recorded according to an empirical scale with six degrees: 0, healthy fruit; 1, 1–20 % fruit surface infected; 2, 21–40 % fruit surface infected; 3, 41–60 % fruit surface infected; 4, 61–80 % fruit surface infected; 5, more than 81 % of the strawberry surface infected and showing sporulation (Romanazzi et al. 2000).

Statistical analysis

Data on effects of the tested extracts on the growth of pathogens was analyzed by one-way analysis of variance and comparison of means using the Tukey's test at the level $P<0.05$. The statistical analysis was performed using GraphPad Prism, version 5.02 (GraphPad Software, Inc., San Diego, CA, USA) statistic software.

Results

Antifungal activity of individual CS, ZEO and CEO

The antifungal activities of tested compounds were first examined at various concentrations (Table 1). All tested CS, ZEO and CEO concentrations had different degrees of antifungal activity against *B. cinerea*. The results indicated that the test treatments reduced the fungal growth in a concentration-dependent manner, i.e., as the concentration increases the radial growth decreases.

Table 1 Antifungal activity of *Z. multiflora*, *C. zeylanicum* essential oil and chitosan against *B. cinerea*

Compounds	Concentration (%)	RG[a] (mm)	AI[b] (%)
Controls	–	55.00±0.00 a	0.00 a
Cinnamomum zeylanicum Blum (CEO)	0.01	50.00±1.50 b	9.09 b
	0.02	47.50±1.75 c	13.64 c
	0.04	39.00±1.33 d	29.09 d
	0.08	30.00±0.60 e	45.45 e
	0.1	20.66±1.60 f	62.44 f
	0.15	12.85±2.33 g	76.64 g
Zataria multiflora (ZEO)	0.01	55.00±1.50 a	0 a
	0.02	51.50±1.50 h	6.36 h
	0.04	43.50±2.20 i	20.91i
	0.08	32.50±1.33 j	40.91 j
	0.1	24.66±0.44 k	55.16 k
	0.15	14.85±0.60 l	73.45 l
Chitosan (CS)	0.01	50.33±2.45 m	8.49 m
	0.02	47.33±1.45 c	13.94 c
	0.04	30.67±4.33 n	44.24 n
	0.08	19.33±0.67 q	64.85 q
	0.1	16.83±0.44 r	69.61 r
	0.15	14.33±0.33 l	73.94 l

[a] Radial Growth of fungus pathogen

[b] Antifungal Index (%)

The results are means±standard errors of three replications. Means within a column indicated by the same letter were not significantly different according to Tukey's test at the level $P<0.05$

For ZEO and CEO, all concentrations showed low to moderate effect on the radial growth of *B. cinerea* with antifungal index <80 %. Moreover, no difference in radial growth from the non-amended control was noted in 0.01 and 0.02 % (*v*/*v*) ZEO and CEO. The highest antifungal activity was recorded at a concentration of 0.15 % ZEO and CEO with antifungal indices of 73.45 % and 76.64 %, respectively ($p<0.05$). While other concentrations inhibited between 0 and 60 % radial growths of *B. cinerea* (Table 1).

For CS, ANOVA results demonstrated that three tested concentrations out of six reduced mycelial growth of *B. cinerea* more than 50 % ($p<0.05$). The antifungal index of 0.15 %, 0.1 % and 0.08 % (*w*/*v*) of CS was recorded at 73.94, 69.61 and 64.85 %, respectively, while other concentrations (0.01–0.04 %) inhibited only 44.24, 13.94 and 8.49 % radial growth of *B. cinerea*, respectively (Table 1).

Combined effects of CS with ZEO or CEO on in vitro pathogen growth

The combination of CS with CEO or ZEO was prepared in 1:1 ratio (described previously) with serial concentrations (0.01–0.15 %, *v*/*v*) for assaying, and the antifungal index, IC_{50}, MIC and MFC values were given in Tables 2 and 3.

ANOVA results demonstrated that all combined treatments significantly reduced the growth area of *B. cinerea* compared with untreated control, resulting in 30–100 % growth inhibition ($p<0.05$). Among all tested combinations, 10 concentrations out of 12 reduced mycelial growth of *B. cinerea* more than 50 % ($p<0.05$). Moreover, the strongest antifungal effect was discovered in CS combined with either CEO (CC) at the concentration of ≥0.1 % or ZEO (CZ) at the concentration of ≥0.15 %, which resulted in complete growth inhibition, compared with the control ($p<0.05$).

According to the results from the in vitro inhibition of mycelial growth assay, only the treatments with 0.15 % (*v*/*v*) of CC and CZ which reduced mycelial growth of *B. cinerea* by more than 70 %, were screened for the in vivo assay in mold-inoculated strawberry fruit.

Table 2 Antifungal activity of the combined treatments of chitosan and cinnamon (CC) or *Z. multiflora* (CZ) essential oil against *B. cinerea*

Compounds	Concentration (%)	RG[a] (mm)	AI[b] (%)
Controls	–	55.00±0.00 a	0.00 a
CC (CS+CEO)	0.01	37.67±2.26 b	31.51 b
	0.02	21.83±3.33 c	60.31 c
	0.04	12.65±1.66 d	77.00 d
	0.08	5.5±0.33 e	90.00 e
	0.1	0.00±0.00 f	100.00 f
	0.15	0.00±0.00 f	100.00 f
CZ (CS+ZEO)	0.01	40.55±1.75 g	26.36 g
	0.02	23.83±2.66 h	56.67 h
	0.04	15.25±0.80 i	72.27 i
	0.08	8.33±1.33 j	84.85 j
	0.1	2.63±0.66 k	95.22 k
	0.15	0.00±0.00 f	100.00 f

[a] Radial growth of fungus pathogen

[b] Antifungal index (%)

The results are means±standard errors of three replications. Means within a column indicated by the same letter were not significantly different according to Tukey's test at the level $P<0.05$

Table 3 MIC, MFC and IC_{50} values of test compounds against *B. cinerea*

Compounds	IC_{50} (%)	MIC (%)	MFC (%)
Cinnamomum zeylanicum Blum (CEO)	0.087 a	–	–
Zataria multiflora (ZEO)	0.098 b	–	–
Chitosan (CS)	0.069 c	–	–
CEO+CS	0.019 d	0.1 a	0.15
ZEO+CS	0.023 e	0.15 b	–

MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; $IC_{50,}$ median inhibition concentration

Within the column, mean values followed by the same letter are not significantly different according to Tukey's test at the level $P<0.05$

In vivo antifungal effects of CC and CZ on strawberry fruit

The efficacy of the different coatings in reducing disease severity and incidence of gray mold on infected strawberry (%) is shown in Figs. 1, 2 and 3. The statistical analysis revealed that the lowest percentage of infected strawberries was recorded for 0.15 % (*v*/*v*) CC (16.67 %), followed by 0.15 % CZ (41.67 %), compared with the control ($p<0.05$) at day 9 (Fig. 1). Meaning, the most effective treatments to reduce the disease incidence were those based on the combination of CS with CEO or ZEO.

The combined treatments of CS-CEO or CS-ZEO were not only effective in controlling strawberry decay, but also in delaying the onset of disease symptoms and slowing down *B. cinerea* growth during the storage period ($p<0.05$). Uncoated strawberries showed signs of fungal decay after 4 days of storage at 4 °C (Fig. 1). However, only 15 % of uncoated fruit were infected by mold while no sign of fungal decay could be detected by visual inspection of strawberries coated with CS-CEO or CS-ZEO treatments after 6 days of storage. Of the fruit coated with the combined treatments, 41.67 and 16.67 % was observed to be infected on the ninth day of storage (Fig. 1). Treatments based on 0.15 % of CS, CEO or ZEO showed lower antifungal activity compared to treatments with CS in combination with CEO or ZEO ($p<0.05$), resulting in a fungal decay reduction of only 33.33 %, 34.75 % and 21/67 %, at day 9, respectively.

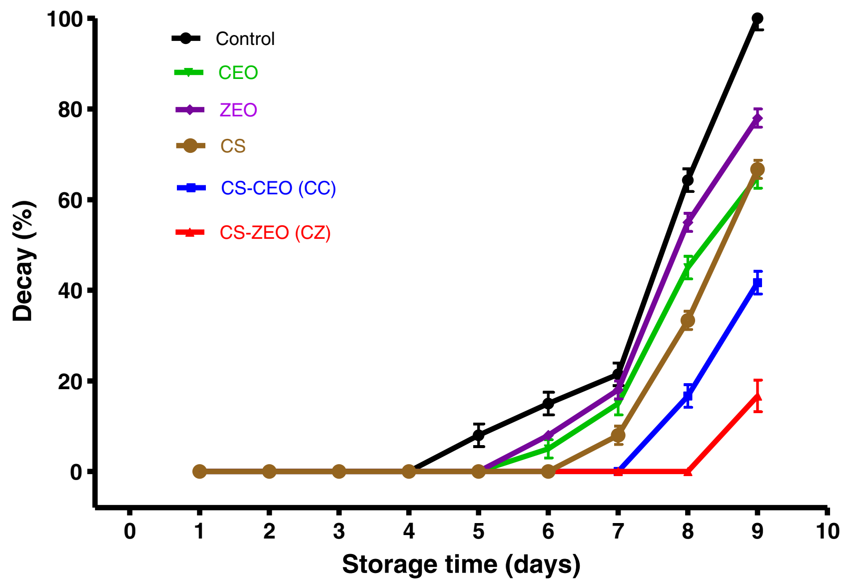

Fig. 1 Effect of *Zataria multiflora* (ZEO) and *Cinnamomum zeylanicum* essential oils (CEO) and chitosan (CS), alone and in combination on disease incidence of artificially infected strawberries during 7 days storage at 4 °C followed by 2 days at 20 °C. In each treatment, vertical bars represent standard deviations of three replications. Statistical significance determined at $p \leq 0.05$ according to the Tukey's range test

Similarly, strawberries immersed in CEO, ZEO, CS, CS-CEO and CS-ZEO, showed significant reduction of gray mold disease severity, when compared to the control fruit after 2 days of storage at 20±1 °C (Fig. 2). They were able to reduce disease severity to less than 1.5 and 2.4 in CS-ZEO and CS-CEO treated fruit, compared to 4.86 units (based on a 0–6 empirical scale) in the control strawberry. By the end of the 7-day storage period, disease severity values ranged between 0 (for both combined treatments) and 1 (for 0.15 % CS, ZEO or CEO); by the end of the 9-day storage period, the severity of disease varied from 1.5 (for CS-ZEO treatment) to 3.5 (for ZEO). However, strawberries treated with CEO or ZEO exhibited high mold development at both storage periods (Fig. 2). Furthermore, the combined treatments did not cause any apparent phytotoxicity on strawberries. However, fruit treated with pure ZEO and CEO exhibited phytotoxicity symptoms, including drying and browning, compared to the non-treated fruit.

Discussion

In current study, the in vitro antifungal activity of CEO, ZEO and CS, alone and their combinations and their in vivo efficacy on strawberry fruit were evaluated. The results showed that the tested compounds had a moderate effect on mycelia

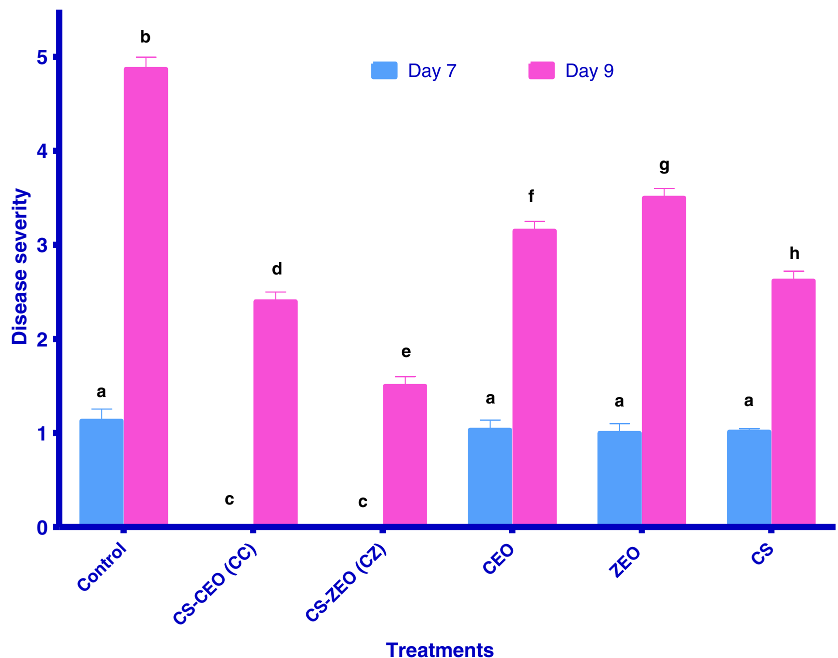

Fig. 2 Effect of *Zataria multiflora* (ZEO) and *Cinnamomum zeylanicum* essential oils (CEO) and chitosan (CS), alone and in combination on disease severity of artificially infected strawberries during 7 days storage at 4 °C followed by 2 days at 20 °C. In each treatment, vertical bars represent standard deviations of three replications. Statistical significance determined at $p \leq 0.05$ according to the Tukey's range test

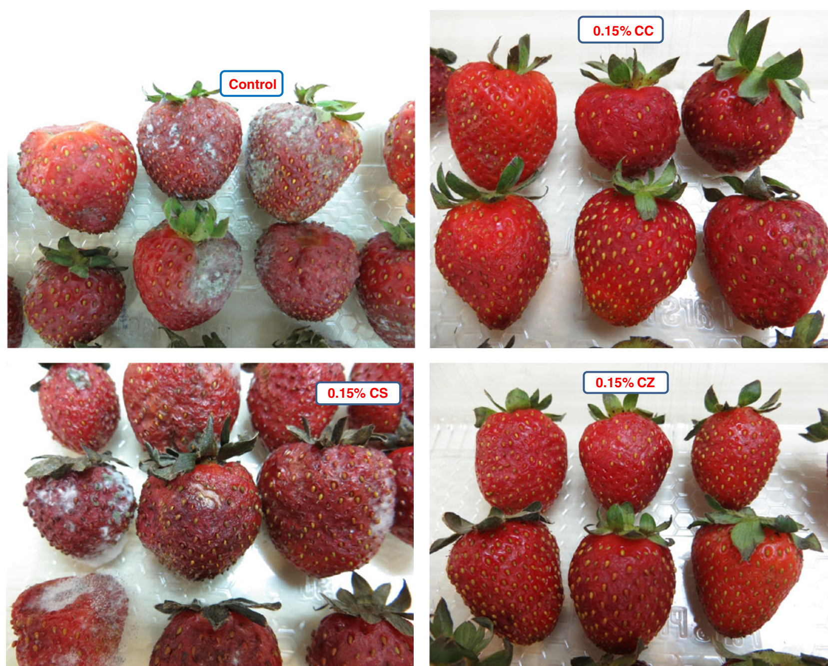

Fig. 3 Appearance of strawberries coated with CS alone and in combination with ZEO (CZ) or CEO (CC) a concentration of 0.15 % (*v*/*v*) during storage. Fruits were treated, inoculated with fungus and stored for 7 days at 4 °C followed by 2 days at 20 °C

growth of *B. cinerea*, with different degrees depending on the type of treatments used. Previous studies indicate that the effect of plant extracts on fungal pathogen may be attributed to the content of secondary metabolites (e.g., alkaloids, phenolic, flavonoids and terpenoids compounds) with known antifungal activity (Mohamed and El-Hadidy 2008). Phenolic compounds (such as carvacrol), which are found in the hydrophobic fractions of EOs, may be dissolved in the hydrophobic domain of the cytoplasmic membrane, resulting in cell death (dos Santos et al. 2012). It has been accepted that the antifungal activity of the CEO is related to its large amounts of eugenol (Burt 2004) and cinnamaldehyde (Bassolé and Juliani 2012), while the good activity of ZEO would seem to be related mainly to the high percentage of thymol and carvacrol which are well-known as antifungal agents (Saei-Dehkordi et al. 2010).

Numerous studies have reported the efficacy of CS in the inhibition of the mycelial growth of postharvest pathogenic fungi (Dutta et al. 2012; Jianglian and Shaoying 2013). The CS has been reported previously as a natural fungicide that could be considered as a suitable alternative to synthetic antifungal agents for managing gray mold caused by *B. cinerea* in fruit and vegetables at harvest and during storage time (Badawy and Rabea 2009). Perdones et al. (2012) reported that CS coatings reduced the percentage of infected strawberries as compared to non-coated ones after 3 days of storage (Perdones et al. 2012). It does an antifungal activity with different mechanisms. For example, this feature in fungi is done by suppressing sporulation and spore germination (Hernández-Lauzardo et al. 2008) and penetrating into fungal cells. In general, it is known that CS through the outer (plasma membrane) and intracellular could have fungi toxic effects on phytopathogen fungi (Guo et al. 2008).

In our study, CS combined with CEO or ZEO also was studied to determine whether the combination of two compounds has synergistic, additive or antagonistic effects against *B. cinerea*. The interaction was evaluated by comparing the isoeffective concentration (IC_{50}), MIC and MFC values of test compounds and designated combinations. It is considered synergy when the isoeffective concentration of combination was significantly lower than those of compounds acting alone (Yen and Chang 2008).

The obtained results indicate that the combined application of CS and ZEO or CEO showed more significant mycelial inhibition results compared with pure EOs or CS ($p<0.05$). The strongest antifungal effect was observed with the combination of CS and CEO (CC) ($p<0.05$). The results, as seen in Table 3, revealed that the growth inhibitory action of CEO and CS at the concentration of 0.1 % has been enhanced from 62.44 and 69.61 % respectively, to 100 % (CC), indicating portent of synergistic effect. This combination effect was further confirmed by comparing their isoeffective concentrations. The values of IC_{50}, MIC and MFC for CC were 0.019, 0.1 and 0.15 %, respectively, which were significantly lower than those of using either CEO or CS alone ($p<0.05$). Moreover, the synergistic effect was observed for the combination of CS and ZEO with significantly lower values of IC_{50} (0.023 %) and MIC (0.15 %) than that of CS or ZEO alone. The AI% of ZEO at the concentration of 0.15 % was 73.45 %, and that of CS at the same concentration was 73.94 %, while the AI of their combination (CZ) against *B. cinerea* dramatically increased to 100 %. It seems to be consistent with the findings

of Moradi et al. (2011) who reported the synergistic interaction of ZEO with CS by enhancing the antibacterial activity of the ZEO and CS against *Listeria monocytogenes* on agar culture media (Moradi et al. 2011). In another study, Hu et al. (2003) showed that thymol (the major phenolic components of ZEO) in combination with CS significantly improved the antimicrobial activity (Hu et al. 2003). Our results also support Wang et al. (2011) study that reported the synergistic interaction of cinnamon oil with CS by enhancing the antimicrobial activity of the CEO. Similarly, Avila-Sosa et al. (2012) showed that CS edible films incorporating CEO could improve the quality of foods by the action of the volatile compounds on surface growth of molds such as *Aspergillus niger* and *Penicillium digitatum*.

The mechanism of how these EOs and CS synergistically enhance the antifungal properties of each other remains obscure. Results of Bennis et al. (2004) showed that antifungal activity of eugenol and thymol involve alternations of both membrane and cell wall of *Saccharomyces cerevisiae* that resulting in increase of permeability to allow foreign particles entering fungal cell causing yeast death (Bennis et al. 2004). The antifungal mechanisms of CS in combination with CEO or ZEO in *B. cinerea* are very possible performing in the same way as they do in the yeast; eugenol or thymol alter the surface and structure of fungal cell wall, and CS acts as a potentiator by reducing the cell wall synthesis and facilitating the death in an energy-dependent manner.

Although in vitro tests of plant extracts is an important first step in selecting plants with antifungal potential against plant pathogens, in vivo tests are needed to check whether the positive results of the in vitro tests can be obtained too (Askarne et al. 2012). Results obtained in this study indicated that CS combination with ZEO or CEO significantly decreased both disease severity and incidence of infected strawberries by *B. cinerea*, after 1 or 2 days shelf-life at 20 °C following removal from storage at 4 °C. Indeed, the in vivo trials confirmed the strong efficacy shown in vitro by combined CS-ZEO treatment. Nonetheless, the efficacy of extracts on fruit was not always in accordance with the antifungal activity shown in in vitro trials as in case of combined CS-CEO, which was effective in vitro against *B. cinerea* but not very effective on strawberry. No clear explanation for this fact was found, although in the complex host/antimicrobial compound/pathogen system, numerous factors can lead to divergent behavior with respect to that observed in the in vitro studies. It has been reported that these differences could be attributed to the alternation of site action (Abdolahi et al. 2010) or structural changes (degradation, hydrolysis, polymerization, etc.) of treatments under in vivo condition (Gatto et al. 2011). In this sense, Vu et al. (2011) reported a higher antifungal effect of limonene than peppermint essential oil, encapsulated in modified CS, when applied to cold-stored strawberries, although the in vitro antifungal effect of the free (non-encapsulated) oils showed the opposite trend (Vu et al. 2011).

In agreement with the results obtained in this study, other studies have reported that the application of CS and EOs alone or in combination as a coating material preserved or improved the physical and physicochemical quality of fruits (Hassani et al. 2012; Perdones et al. 2012). For example, Xing et al. (2011) showed that the use of coatings composed of CS (10, 000 ppm) and CEO (2500 ppm) was effective in reducing the microbial deterioration of sweet peppers throughout 35 days of storage at room temperature.

Conclusions

The results obtained from both in vitro and in vivo trials indicated that the combination of CS with ZEO or CEO was able to potentiate antimicrobial activity against *B. cinerea* and at the same time, allow their use at lower concentrations. Moreover, it demonstrated the potential use of these combined treatments as a preservative agent to control postharvest gray mold of strawberries stored at room and cold temperatures. These findings reveal the potential of the combined application of CS-CEO or CS-ZEO at sub-inhibitory concentrations to control the growth and survival of pathogenic fungi in fruits, particularly *B. cinerea* in strawberries, which may be an alternative to synthetic antifungal agents that currently applied to reduce post-harvest losses from fruits and vegetables. However, further studies are needed to better understand the mechanisms of action of these combined treatments.

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