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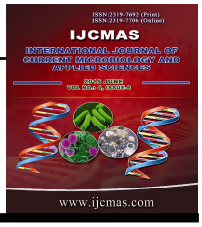

Original Research Article

Application of Chitosan and essential oils as alternatives fungicides to control green and blue moulds of citrus fruits

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Keywords

Essential oil, Chitosan, Green mould, Blue mould, Citrus fruits

A B S T R A C T

The objective in the present study was to investigate the application of chitosan - essential oils amended coatings of citrus fruits to control postharvest diseases as fungicides alternatives. Different concentrations of chitosan and essential oils EOs such Lemongrass and citral were applied as individually or in combination treatments against *Penicillium digitatum* and *P. italicum* the main pathogens of rotting citrus fruit. *In vitro*, chitosan, lemongrass EO, citral EO and chitosan- EOs mixtures significantly reduced the linear growth and spore germination of *P. digitatum* and *P.italicum*. Lemongrass and citral EO s at 6 ml / L as well as chitosan + citral or lemongrass EOs mixtures at 3g/L+ 3 ml / L or 4 g/L+ 4 mL / L caused complete growth reduction of *P.digitatum* and *P.italicum*. Moreover these treatment cause 100 % protection of peel dices of orange and lime fruits artificially infected with *P.digitatum and P.italicum.* In storage trials, coated orange and lime fruits with combined treatments of chitosan + citral or lemongrass at concentrations 4.0 g / L+ 4.0 ml / L or 3g/L+3 ml/L caused significantly protective effect against green and blue mold's diseases and prevent the development of fruit decay due the mould's incidence during 40 days of storage at 20°C. Therefore, combination between chitosan and essential oils as fruit coating could be applicable safely for controlling green and blue moulds decay of citrus fruits instead of fungicidal treatments.

Introduction

Post-harvest decay of citrus fruit caused by *Penicillium digitatum* Pers. Sac (green mould), *Penicillium italicum* Whemer (Blue mould) and *Geotrichum candidum* Link. (Sour rot) have been reported all over the world and represents major losses in production, during harvest, storage and exportation (Morsy and Abd El-Kader, 1994; Joseph and Korsten, 2003). Postharvest chemical treatments are usually applied to protect fruit and vegetables during transit, storage and marketing (Eckert and Ogawa, 1998).In Egypt, the citrus industry depends on synthetic fungicides as standard practice for the control of post-harvest citrus fruit rots. However, loss of efficacy of some

fungicides, increasing public concern over food safety and constant reviewing of residue limits, are challenges for the industry. There is a growing need to develop alternative approaches for controlling post harvest decay pathogens.

Application of essential oil is a very attractive method for controlling postharvest diseases. Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey,*et al*., 2001; Tripathi,*et al*., 2008). In this regard, lemongrass (*Cymbopogon citratus* L.) oil was reported to be antifungal activity against several plant pathogens. Fungal spore production, spore germination and germ tube length of *C. coccodes, B. cinerea, C. herbarium* and *R. stolonifer* was inhibited with lemongrass oil treatments (Tzortzakis and Economakis 2007). Moreover, using lemongrass essential oils by spraying or dipping fruits for controlling postharvest diseases of several fruits has been reported (Tzortzakis and Economakis, 2007). Essential oils or their constituents have been shown to have fungicidal activities against several post harvest pathogens of citrus fruits (Caccioni *et al*., 1998; Rio del *et al*., 1998 ; Fiori, *et al*., 2000). Faten (2010) indicate that combined treatments between citral at 4.0 or 5.0 ml / l and chitosan at 6.0 or 8.0 g / l reduced sour rot of lime fruit and rotted part tissue more than 89.5 and 93.5% respectively. Many authors reported using citral for controlling postharvest diseases of citrus fruits (Abd-El-Kareem and Abd-Alla, 2002; Abd-El-Kareem,*et al*., 2002, Chen *et al*., 2007, Tripathi, *et al*., 2008).

Chitosan, a natural biopolymer with antifungal and eliciting properties, also, a common food additive with antifungal properties, able to reduce postharvest decay of fruits and vegetables was reported by many investigators (Xianghong and Shiping,2009 ; Abd-El-Kareem, *et al*.,2002). Chitosan has become a prospective alternative treatment for fruit and vegetables due to its natural character, antimicrobial activity, and elicitation of defense responses in plant tissue (Cheah, *et al*., 1997).Chitosan has been used to control postharvest diseases of many fruits such as pear (Meng,*et al*., 2010), strawberry (El-Gaouth,*et al*., 1992, Morsey, *et al*., 1999) ;table grape (Granfrance *et al*., 2007, Ait Barka,*et al*., 2000), tomato (Liu *et al*., 2007; Ben-shalom, *et al*., 2003; Bdawaya and Rabeab,2009), citrus (Abd-El-Kareem,*et al*.,2002,Chen, *et al*.,2007; El-Mohamedy,2008), and longan (Jang and Liu,2001).cucumber (Ben-Shalom,*et al*., 2007; Bhaskara,*et al*., 2000 ;)

The antifungal activity of chitosan were examined against fungi including *Penicillium digitatum, Penicillium italicum, Botrydiplodia lecanidion* and *Botrytis cinerea*. Hongyin, *et al*., (2011) indicated that chitosan significantly inhibited the decay of citrus fruit caused by *Penicillium digitatum, Penicillium italicum, Botrydiplodia lecanidion*, and *Botrytis cinerea* after 14 days storage at 25 °C, and is more effective than TBZ(fungicide).Yu,*et al*. (2007) found that chitosan applied alone or with *Cryptococcus laurentii* could effectively inhibit the blue mold rot caused by *Penicillium expansum* in apple fruit after seven days of incubation at 20°C.

The objective of this study was to evaluate the effectiveness of chitosan and two essential oils EOs (Lemongrass and citral oils) alone or in combination on the mycelial growth and spore germination of *Penicilium digitatum* and *P. italicum*, their efficacy in the control of citrus green and blue molds under storage conditions.

Materials and Methods

Source of citrus fruit and fungal cultures

Commercially harvested Navel oranges (*Citrus sinensis* L. Osbeck) and lime (*Citrus aurantifolia* F. Muell), with healthy appearance from citrus orchards were used in this investigation.A highly aggressive isolates of *P. digitatum* and *P. italicum* originally isolated from rotted citrus fruit in previous studies(El-Mohamedy,2008),were used in this investigation. Isolates were grown on potato dextrose agar (PDA) at 25°C for 7 days. Spore suspension was obtained by flooding 7-day-old PDA cultures of pathogen with sterile distilled water containing 0.01% (v⁄ v) Tween 80. Spore concentrations of the pathogen was determined by a haemocytometer and adjusted with sterile distilled water to 106 spores / ml (Zhang *et al.* 2003).

Essential oils

Essential oils of lemongrass oil (*Cymbopogon citrates*) and citral oil (constituent of citrus fruit essential oil or lemongrass plant) were purchased from International Flavors and Plant oils Inc., Giza, and Egypt. These essential oils were stored in dark bottles at 4ºC for further studies. Oil suspensions were prepared by adding commercial oils to sterile distilled water containing 0.01 Tween 80 (Letessier, *et al.*, 2001) to obtain concentration of 2, 3, 4, 6, and 8ml/L. The emulsifier Tween was added to enhance the solubility of oils (Fiori, *et al*., 2000).

Chitosan

Chitosan, a non-toxic polymer of ß-1, 4-glucosamine, was obtained from the chitin of crustacean shell wastes manufactured commercially by Sigma chemical Co., St.Louis, Mo, USA.

Antifungal activity of Chitosan and essential oils *in vitro*

Antifungal activity of lemon grass, citral essential oils EOs and chitosan was studied by determined their inhibitory effect on linear growth and spore germination *of Penicilium digitatum* and *P. italicum* on PDA medium.

Effect on linear growth

Different concentrations of chitosan at 2, 4, 6 and 8 g/L (prepared according the method stated by El -Ghaouh *et al*.(1991) ; Essential oils of lemongrass and/or citral at 2, 4, 6 and 8 ml/L,as well as chitosan –oil mixtures, chitosan + citral and chitosan + lemongrass with different concentrations 2g/L+ 2ml/L, 3g/L+ 3ml/L and 4g/L+ 4 ml/L.All tested compounds were dissolved individually in Tween 80 (0.1%) then added to PDA medium before solidifying and gently agitated to ensure even distribution of the oils in the medium, then dispensed in Petri - plates (9 cm-diameter).Discs (5.mm diam) of the of *Penicillium digitatum* and *Penicillium italicum* was aseptically transferred to the center of five plates of each treatment as a replicates. Five not amended plates containing PDA medium and Tween 80 (0.1 %) were used as a control. All plates were incubated at 20C. When the colonies on the untreated plates were about (90 mm), records were taken for the other treatments as measurement of 2 perpendicular diameters, and the average linear growth in mm and then, reduction (%) in mycelial growth was calculated in all treatments relative to the fungal growth (90-mm-diameter) in the control one.

Effect on Spore Germination

Spores of 10-days-old cultures of *P. digitatum* and *P. italicum* were harvested in sterilized water (containing 0.01% Tween

80) then adjusted to reach concentration of 10^6 spores / ml. One ml of spore suspension was placed in Petri plates. PDA media supplemented with previous concentrations of chitosan and essential oil EOs treatments, then poured into the previous inoculated plates and rotated gently to ensure even distribution of fungal spores. Inoculated plates were incubated at 20°C for 24 h. Germinated spores were counted and percentage of spore germination was calculated.

Protective effect of chitosan, essential oils and chitosan + oils mixtures on Orange and lime peel dices against fungal infection

Discs of orange or lime peel were taken from healthy fruits by use of a sterile cork borer. The discs were dipped in 95 % ethanol for about 10s, then washed with sterile distilled water three times. The peel discs were then dipped for 10 min. in solutions of chitosan at 2, 4, 6 and 8 g / L; lemongrass and citral at 2, 4, 6 and 8 ml / L; chitosan + citral and/or lemongrass EOs at 2g/L+ 2ml/L, 3g/L+ 3ml/L and 4g/L+ 4 ml/L treatments, then the discs of orange and lime peels sprayed individually with spore suspension of *P. digitatum* and *P. italicum* (10^6 /ml). The peel discs were placed in Petri dishes (10 peel discs/dish) that had been moistened with moistened filter paper and incubated at 25^{o}C for 72 hours in the dark, ten petri dishes were used for each treatment . Percentage of infected peel discs was determined and reduction of infection was calculated.

Management of green and blue moulds diseases of orange and lime fruits during storage

Different concentrations of chitosan, Citral, lemongrass, chitosan + citral and chitosan+ lemongrass treatments were tested to study their efficiency on management of green and blue moulds incidence on orange and lime fruits during different periods of storage.The promising concentrations of chitosan, lemongrass and citral were applied as single or in combination treatments as follow :-

Chitosan at 4 and 6g/L - Citral at 4 and 6 ml /L. Lemongrass at 6 and 8 ml /L - Chitosan + Citral at 2g/L+ 2ml/L, 3g/L+ 3ml/L and 4g/L+ 4 ml/L- Chitosan +Lemongrass at 2g/L+ 2ml/L, 3g/L+ 3ml/L and 4g/L+ 4 ml/L - Control (untreated inoculated fruits).

Fresh orange and lime fruits apparently free from physical damage and diseases were artificially wounded using sterilized scalpel. Inoculation of wounded fruits was carried out by spraying fruits with spore suspension (10^6 spores/ml) of *P. digitatum* and / or *P. italicum* then air dried at room temperature 23-25°C.

Inoculated fruits were dipped for 4 min in solutions of each chitosan at concentrations of 4.0 and 6.0 g / L; citral at 4.0 and 6.0 ml / L; lemongrass oil at 6.0 and 8.0 ml / L and chitosan + citral and /or chitosan + lemongrass at concentrations of 2g/L+ 2ml/L, 3g/L+ 3ml/L and 4g/L+ 4 ml/. All treated or un-treated (control) orange and lime after air-dried, placed into carton boxes at the rate of 25 or 40 fruit / box of orang and/or lime fruits respectively.

Each particular treatment as well as control treatment was represented by five carton boxes. All boxes were stored at 20±2°C for 10, 20, 40 days. Number of infected orange and lime fruits (disease incidence) was recorded and percentages of blue and green moulds reduction were calculated. Percentages of rotted parts of orange and lime fruits were recorded and calculated.

Statistical analysis

Statistical analyses were performed with descriptive statistics (mean) and inferential tests (ANOVA followed by Tukey test) to determine statistically significant differences ($P < 0.05$) between treatments with Sigma Stat software 2.03. (Neter et al., 1985).

Results and Discussion

Effect of chitosan and essential oils on linear growth and spore germination

Lemongrass, citral EOs and Chitosan at different concentrations *i.e*. 2,4,6 and 8 g/L or ml/L as well as chitosan + essential oils EOs mixture three at three concentrations *i.e*. 2g/L+2 ml/L, 3g/L+3 ml/L and 4 g/L+ 4 ml/L of Chitosan + Citral or Lemongrass EOs were tested to study their inhibitory effect on linear growth and spore germination of *Penicillium digitatum* and *P.italicum*.

Results in Table (1) indicate that all treatments of chitosan and essential oils reduce the linear growth and spore germination of *P. digitatum* and *P. italicum*. Chitosan-essential oils EOs treatments were most effective against two pathogens compared with chitosan or essential oils alone treatments. Chitosan at 6 g/L and 8 g/L ; citral and lemonrass EOs at 6 ml/L and 8 ml/L, as well as chitosan - citral or lemongrass EOs mixtures at 4 g/L+ 4 ml/L treatments caused complete growth reduction of *P. digitatum* and *P. italicum*. Chitosan and essential oils at 4 g/L or ml/L cause condesdrable reduction on growth of the two pathogens. Citral at low concentration (2/ml/L cause considerable reduction more than 50% of growth and spore germination of the two pathogens, meanwhile, chitosan and lemongrass at 2g/L cause less effect as the reduction was less 50% of same pathogens.

Table.1 Reduction in the linear growth and spore germination (%) of *Penicillium digitatum* and *P.italicum* as affected by different concentrations of chitosan, lemongrass and citral EOs on PDA medium

Treatment	Concentration	*P. digitatum*		*P. italicum*	
		linear growth	Spore germination	linear growth	Spore germination
Chitosan	2 g/L	48.9	60.6	44.4	56.5
	4 g/L	68.8	72.3	63.3	69.5
	6 g/L	100	100	100	100
	8 g/L	100	100	100	100
Lemongrass	2 ml/L	42.2	55.0	42.2	58.8
	4 ml/L	66.7	70.0	63.3	74.0
	6 ml/L	100	100	100	100
	8 ml/L	100	100	100	100
Citral	2 ml/L	53.3	68.0	50.0	77.0
	4 ml/L	71.1	90.0	66.7	92.0
	6 ml/L	100	100	100	100
	8 ml/L	100	100	100	100
Chitosan + Citral	2g/L+2 ml/L	58.9	77.0	51.1	64.0
	3g/L+3 ml/L	88.9	95.0	83.3	90.0
	4 g/L+ 4 ml/L	100	100	100	100
Chitosan +Lemongrass	2g/L+2 ml/L	53.3	70.0	50.0	66.0
	3g/L+3 ml/L	84.4	90.0	80.0	88.0
	4 g/L+4 ml/L	100	100	100	100
Control		0.0	0.0	0.0	0.0

The highest reduction on linear growth of *P. digitatum* and *P. italicum* were 68.8 and 63.3 % at 4 g/L of chitosan; 66.7 and 63.3% at 4 ml/L of lemongrass; 71.1 and 66.7% at 4 ml/L of citral; 88.9 and 83.3% at 3g/L+3 ml/L of chitosan + citral ; 84.4 and 80.0 % at 3g/L+3 ml/L of chitosan + lemongrass oil . Chitosan and essential oils individually or in combination at high concentrations 6 and 8g/L or 4 g/L + 4ml/L completely reduce spore germination of both pathogens .At half concentrations of such treatments,spore germination reduced by up to 92% of both pathogens ,but at the least concentration considerable effects were recoded .Spore germination was more sensitive to all concentrations of tested compounds than linear growth.

Protective effect of chitosan, essential oils and chitosan-EOs mixtures on peel dices in *vitro*

Three concentrations of chitosan, citral and lemongrass single or in combined treatments with highest effect on linear growth and sporulation of *P.digitatum* and *P.italicum* were tested for their ability to prevent the infection and colonization of orange and lime peel discs by of *P.digitatum* and *P.italicum.*

The protection effect of different concentrations of chitosan and chitosan-oil mixtures was presented in Table (2).All chitosan and essential oils treatments cause highly protective effects of orange and lime peel dices against *P.digitatum and P.italicum* compared with other treatments. Combined treatments of chitosan +essential oils were the most effective in decreasing decay infection by the two pathogens compared with single treatment and control. Results indicate that the protection effect was increased by increasing the concentrations of chitosan and chitosan-oil mixtures. Chitosan at 8 g/L and citral or lemon grass at 8ml/L and chitosan + citral or lemon grass at 4g/l+4ml/L cause 100 % protection against *P.digitatum* and *P.italicum* infection, as there is no infection of peel dicks of lime and orange were recorded.

Table.2 Reduction (%) in Peel disks of orange and lime fruits artificially infected with *P. digitatum* and *P. italicum* in response to long acting protection effect of different chitosan-oil treatments *in vitro*

Treatment	Concentration	Peel disks infection %			
		P. digitatum		*P. italicum*	
		Orange	Lime	Orange	Lime
Chitosan	4 g/L	74	73	72	70
	6 g/L	88	83	85	81
	8 g/L	100	100	100	100
Citral	4 ml/L	71	70	74	71
	6 ml/L	84	80	85	81
	8 ml/L	100	100	100	100
Lemongrass	4 ml/L	68	66	65	62
	6 ml/L	88	85	82	80
	8 ml/L	100	100	100	100
Chitosan + Citral	2g/L+2 ml/L	73	71	72	70
	3 g/L+3 ml/L	95	92	92	90
	4 g/L+ 4 ml/L	100	100	100	100
Chitosan +Lemongrass	2g/L+2 ml/L	72	70	72	70
	3g/L+3 ml/L	92	88	88	85
	4 g/L+4 ml/L	100	100	100	100
Control		0.0	0.0	0.0	0.0

Chitosan-essential EOs treatments reduced colonization of orange and lime peel discs with *P. digitatum* and *P.italicum* by 100 % at 4g/l+4ml/L and by more than 92 % and 74 % at 3g/l+3ml/L and 2g/l+2ml/L respectively. Meanwhile, chitosan +citral or lemongrass EOs at 3g/l+3ml/L , chitosan 6g/L, citral 6 ml/L and lemongrass at 6 ml/L cause reduction of infection by the two pathogens *P. digitatum* and *P. italicum* reach to 90-95%,85-92% 81-88%,70-76% and 62-68% respectively. The least protection effect (less than 75 % protection) was noticed at the low tested concentrations of such treatments.

Effect of chitosan - essential oils mixtures on management of green and blue moulds diseases:

A- Effect on green and blue diseases incidence:

Promising treatments of chitosan, citral and lemon grass EOs at 6 and 8 ml/L and chitosan + citral or lemongrass EOs at 2g/L+2 ml/L, 3g/L+3 ml/L and 4g/L+4 ml/L were applied to control green and blue moulds diseases of orange and lime fruits during 10, 20 and 40 days of storage at 20°C.

Results in Tables (3 and 4) indicate that chitosan and chitosan- EOs mixtures significantly prevent blue and green moulds incidence on orange and lime fruit up to 40 days of storage at 20°C, as the least number of infected fruits were recorded compared with uncoated fruit (control).The percentages of reduction in both mould diseases on orange and lime fruits were increasing by increasing concentration of chitosan and essential oils. Results in Table (3) show that the most effective treatments in protective effect of orange fruits against blue and green moulds during 40 days of storage at 20°C were combined treatments between chitosan + citral or lemon grass at concentrations 4.0 g / L+ 4.0 ml / L and followed by chitosan + citral at 3g/L+3 ml/L, these treatments cause reduction of blue and green moulds incidence on orange fruits reach to 96 – 92 %, 92 – 84 % and 92 – 80 % of blue moulds ; 96 -88 % , 92-80 % and 88-80 % of green mould after 10-40 days of storage at 20°C, respectively.

Single treatments of Chitosan at 8g/L, citral and lemongrass EOs at 8 ml/L cause protective effect against the two tested pathogens, as reduction of blue and green moulds on orange fruits reach to 88 – 76 %, 88 – 72 % and 80 – 72 % of blue mould ; 88 -72 % , 88-64 % and 84-60 % of green mould after 10 - 40 days of storage at 20C respectively. Meanwhile. chitosan + citral or lemongrass Eos at low concentration 2g/L+2 ml/L, chitosan 6g/L, citral and lemongrass EOs at 6.0 ml / L cause less protective effect against two mould diseases epically after 40 days of storage.

As for protective effect of chitosan and essential oils against blue and green moulds of lime fruits (Table 4), the same trend of results followed by the same treatments. The highest reduction in blue and green moulds incidence on lime fruits were obtained with chitosan + citral or lemon grass at concentrations 4.0 g / L+ 4.0 ml / L followed by chitosan + citral at 3g/L+3 ml/L treatments, they cause reduction reach to 94 – 92 %, 90 – 88 % and 88 – 80 % of blue moulds ; 92 -90 % , 90-88 % and 86-78 % of green mould after 10-40 days of storage at 20C respectively.

Chitosan at 8g/L, citral and lemongrass EOs at 8 ml/L cause considerable protective effect against the two tested pathogens, as reduction of blue and green moulds on lime fruits reach to 80 – 78 %, 84 – 74 % and 80 – 74 % of blue mould ; 86 -76 % , 84-72 % and 80-70 % of green mould after 10 - 40

days of storage at 20C respectively. Meanwhile. chitosan + citral or lemongrass Eos at low concentration 2g/L+2 ml/L, chitosan 6g/L, citral and lemongrass EOs at 6.0 ml / L cause less protective effect against two mould diseases epically after 40 days of storage.

Table.3 Reduction (%) in green and blue moulds on orange fruits in response to long acting protection effect of different chitosan-oils treatments during storage at 20°C

Treatment	Concentration	% Disease incidence(DI) and reduction (R)											
		Blue mould						Green mould					
		10 day		20 day		40 day		10 day		20day		40 day	
		DI	R%	DI	R%	DI	R%	DI	R%	DI	R%	DI	R%
Chitosan	6 g/L	12d	88	20c	80	20d	68	20b	80	28b	72	32c	68
	8 g/L	12d	88	12d	88	20d	76	12d	88	16d	84	28d	72
Citral	6 ml/L	20c	80	24c	76	24c	76	16c	84	20c	80	32c	68
	8 ml/L	12d	88	20c	80	28c	72	12d	88	16	84	36c	64
Lemongrass	6 ml/L	28b	72	40b	60	40b	60	24b	76	28b	72	48b	52
	8 ml/L	20c	80	24c	76	32b	68	16c	84	20c	80	40b	60
Chitosan + Citral	2g/L+2 ml/L	24b	76	28c	72	20d	72	16c	84	20c	80	24d	76
	3g/L+3 ml/L	8e	92	12d	88	20d	80	12d	88	20c	80	16e	80
	4g/L+ 4 ml/L	4e	96	8d	92	8e	92	4e	96	8e	92	12e	88
Chitosan +Lemongrass	2g/L+2 ml/L	24b	76	24c	76	24c	72	20b	80	24b	76	32c	68
	3g/L+3 ml/L	12e	88	24c	76	20e	76	12d	88	8e	82	24d	76
	4 g/L+4 ml/L	8e	92	12d	88	12e	84	8e	92	12d	88	20de	80
Control		100a	0.0	100a	0.0	100a	0.0	100a	0.0	100a	0.0	100a	0.0

Data in each column with the same letter are not significantly difference (*P*=0.05)according to Tukey test(Neter et al.,1985).

Table.4 Reduction (%) in green and blue molds on lime fruits in response to long acting protection effect of different chitosan-oils treatments during storage at 20°C

Treatment	Concentration	% Disease incidence(DI) and reduction (R)											
		Blue mould						Green mould					
		10 day		20 day		40 day		10 day		20 day		40 day	
		DI	R%	DI	R%	DI	R%	DI	R%	DI	R%	DI	R%
Chitosan	6 g/L	22c	78	30b	70	30c	70	24c	76	28c	72	32b	68
	8 g/L	20c	80	20c	80	32b	78	14e	86	20d	80	24c	76
Citral	6 ml/L	20c	80	25c	75	30c	70	28c	72	34b	66	28c	62
	8 ml/L	16cd	84	20c	80	26c	74	16e	84	20d	80	28c	72
Lemongrass	6 ml/L	30b	70	38b	62	40b	60	30b	70	36b	64	40b	60
	8 ml/L	20bc	80	20c	80	26c	74	20d	80	23c	77	30c	70
Chitosan + Citral	2g/L+2 ml/L	18cd	82	24b	78	25c	75	20d	80	24d	76	30c	70
	3g/L+3 ml/L	12cd	88	16d	84	20d	80	14e	86	18e	82	22c	78
	4 g/L+ 4 ml/L	6e	94	8e	92	8e	92	8f	92	10f	90	10d	90
Chitosan +Lemongrass	2g/L+2 ml/L	22c	78	18d	72	32b	68	20d	80	28c	72	36b	64
	3g/L+3 ml/L	18cd	82	20c	80	20d	80	12e	88	18e	82	22c	78
	4 g/L+4 ml/L	10e	90	10e	90	12e	88	10f	90	10f	90	12d	88
Control		100a	0.0	100a	0.0	100a	0.0	100a	0.0	100a	0.0	100a	0.0

Data in each column with the same letter are not significantly difference (*P*=0.05)according to Tukey test(Neter et al.,1985).

B- Effect on rotted parts tissue of orange and lime fruits

Results in Table (5and 6) indicate that all chitosan and essential oils treatments prevent the development of blue and green moulds incidence and significantly reduced rotted part tissue of orange and lime fruits. Results in Table (5) indicate that chitosan + citral or lemongrass EO at 4 g/L+ 4 ml/L and chitosan + lemongrass EO 3 g/L+ 3 ml/L treatments are the most effective in protective of orange and lime against development of both decay moulds, which reduced the rotted tissue part by 92.7%, 91.7% and 87.2%of blue mould ; 92.4,92.0and 78.7% of green mould of blue and green mould on orange fruits after 40 days of storage at 20C. While single treatments of chitosan 8 g/L, citral and lemon grass EO at 8 ml/L reduced the rotted tissue part by 90.5%, 90.5% and 88.2%of blue mould ; 89.7, 89.5 and 89.1% of green mould.

Table.5 Rotted tissue part (%) of orange fruits caused by blue and green moulds diseases in response to long acting protection effect of different chitosan-oil treatments

Treatment	Concentration	% Rotted tissue part (RTP) and Reduction (R)											
		Blue mould						Green mould					
		10 days		20 days		40 days		10 days		20 days		40 days	
		RTP	R	RTP	R	RTP	R	RTP	R	RTP	R	RTP	R
Chitosan	6 g/L	11.0c	29.2	12.5c	65.8	12.8c	84.9	11.6c	66.8	13.0b	83.2	14.0b	84.4
	8 g/L	5.8e	78.5	7.4e	88.2	8.0e	90.5	7.0d	80.0	7.8d	89.9	9.2c	89.7
Citral	6 ml/L	12.4b	54.1	12.6c	79.9	13.2b	84.4	12.0b	65.7	14.0b	81.9	14.4b	84.0
	8 ml/L	6.0e	77.8	8.2e	86.9	8.0e	90.5	7.2d	79.4	8.0d	89.7	9.4c	89.5
Lemongrass	6 ml/L	13.2b	51.1	14.4b	77.1	14.8b	82.5	13.4b	61.7	14.4b	81.4	15.0	83.3
	8 ml/L	6.0e	77.8	8.4e	86.6	10.0d	88.2	7.4d	78.8	9.0c	88.4	9.8c	89.1
Chitosan + Citral	2g/L+2 ml/L	12.4b	54.1	12.0c	80.8	13.2b	84.4	11.0c	68.6	13.4b	82.7	14.0b	84.4
	3g/L+3 ml/L	8.0d	70.4	10.0e	84.1	10.8d	87.2	8.4d	76.0	9.0c	88.4	11.0c	87.7
	4 g/L+ 4 ml/L	4.8e	82.2	5.0f	92.0	6.2e	92.7	6.2e	82.2	6.0d	92.2	6.8d	92.4
Chitosan +Lemongrass	2g/L+2 ml/L	10.0c	62.9	12.0c	80.9	12.8c	84.9	11.0c	68.6	12.8b	83.5	13.2b	85.3
	3g/L+3 ml/L	9.2cd	65.9	10.4d	83.4	11.4c	86.5	10.8c	69.1	12.0b	84.5	12.8b	85.7
	4 g/L+4 ml/L	5.0e	81.4	5.4f	91.4	7.0e	91.7	6.8e	80.6	6.0d	92.2	7.2d	92.0
Control		27.0a	0.0	62.8a	0.0	84.8a	0.0	35.0a	0.0	77.7a	0.0	90.0a	0.0

Data in each column with the same letter are not significantly difference (*P*=0.05)according to Tukey test(Neter et al.,1985).

The same trend followed by the treatment in Table (6). All concentrations chitosan and essential oil in single or combined treatments significantly decrease the decayed tissue parts of orange and lime fruits during up to 40 days of storage at 20°C. As, the highest records of rotted tissue parts of lime fruits were obtained with high concentrations of chitosan + citral or lemongrass EO at 4 g/L+ 4 ml/L and chitosan + citral 3 g/L+ 3 ml/L treatments, as the reduction of lime rotted tissue parts reach to 92.8%, 92.1% and 89.1%of blue mould ; 92.0,90.5and 86.5% of green mould after 40 days of storage at 20C. While single treatments of chitosan 8 g/L, citral and lemon grass EO at 8 ml/L reduced the rotted tissue part by 91.6%, 81.9% and 89.6%of blue mould ; 90.3, 88.4 and 88.0% of green mould.

Table.6 Rotted tissue part (percentage) of lime fruits caused by blue and green molds in response to long acting protection effect of different chitosan-oil treatments

Treatment	Concentration	% Rotted tissue part (RTP) and Reduction (R)											
		Blue mold						Green mold					
		10 days		20 days		40 days		10 days		20 days		40 days	
		RTP	R	RTP	R	RTP	R	RTP	R	RTP	R	RTP	R
Chitosan	6 g/L	10.2b	53.6	13.0b	76.5	13.4b	83.4	12.4b	58.6	13.8b	76.3	14.4b	83.3
	8 g/L	5.8d	73.6	6.0d	89.1	6.8d	91.6	6.8d	77.3	7.5d	87.1	8.2d	90.3
Citral	6 ml/L	11.0b	50.0	13.4b	75.8	13.8b	82.9	13.2b	56.0	13.0b	77.7	14.8b	82.5
	8 ml/L	6.4d	70.9	7.2d	87.0	7.8c	90.3	7.4cd	75.3	8.0d	86.3	9.8c	88.4
Lemongrass	6 ml/L	11.0b	50.0	14.0b	74.7	14.6b	81.9	13.8b	54.0	14.4b	75.3	15.2b	82.1
	8 ml/L	7.0c	68.1	7.5d	86.4	8.4c	89.6	8.4c	72.0	10.2	82.8	10.2c	88.0
Chitosan + Citral	2g/L+2 ml/L	7.5c	65.9	8.8d	84.1	10.4	87.1	10.6b	64.6	12.0b	79.4	13.0b	84.7
	3g/L+3 ml/L	7.2c	67.2	7.6d	86.2	8.8c	89.1	9.2bc	69.3	10.8c	81.5	11.4c	86.5
	4 g/L+ 4 ml/L	4.4d	80.0	5.0e	90.9	5.8d	92.8	6.0d	80.0	6.5d	88.8	6.8d	92.0
Chitosan +Lemongrass	2g/L+2 ml/L	10.0b	54.5	12.0c	78.3	13.2b	83.7	11.4b	62.0	13.4b	77.0	14.4b	83.0
	3g/L+3 ml/L	8.0c	63.6	11.8c	78.7	12.0b	85.1	11.0b	63.3	13.0b	77.7	13.6b	84.0
	4 g/L+4 ml/L	5.0d	77.2	5.4e	90.2	6.4d	92.1	6.2d	79.3	7.0d	88.0	8.0d	90.5
Control		22.0a	0.0	55.4a	0.0	81.0a	0.0	30.0a	0.0	58.4a	0.0	85.0a	0.0

Data in each column with the same letter are not significantly difference (P=0.05)according to Tukey test(Neter et al.,1985).

A new approach to the control of postharvest pathogens, while maintaining fruit quality, has been implemented by the application of chitosan - essential oils amended coatings to citrus fruits. This approach eliminates the need for synthetic fungicides and reducing environmental pollution (Mari *et al.*, 2007).Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey *et al*., 2001, El-Mohamedy 2002 and 2008, El-Mohamedy *et al*., 2003). Chitosan, a natural biopolymer with antifungal and eliciting properties, also, a common food additive with antifungal properties, able to reduce postharvest decay of table grapes was reported by Gianfranco *et al*., (2007).

In this research the results show that chitosan, lemongrass and citral essential oils as single or in combination treatments were significantly reduce the linear growth and spore germination of *P. digitatum* and *P. italicum* the causal agents of green and blue moulds of orange and lime fruits and decreased the potential of pathogenic capillary to infect and colonized peel discs of orange and lime fruits under artificially infection in *vitro* trials.As, the treatments of chitosan at 6 g/L and 8 g/L ; citral and lemonrass EOs at 6 ml/L and 8 ml/L, as well as chitosan - citral or lemongrass EOs mixtures at 4 g/L+ 4 ml/L caused complete growth reduction of *P. digitatum* and *P. italicum*. Meanwhile, chitosan, citral and lemongrass EOs at low concentration cause less effect as the reduction of same pathogens was less 50%. These results are agreement with those reported by many researches (El-Mohamedy,2002 and2008,El-Mohamedy,*et al*.,2003).Tzortzakis and Economakis, (2007) stated fungal spore production, spore germination and germ tube length of *C. coccodes, B. cinerea, C. herbarium* and *R. stolonifer* was inhibited with lemongrass oil treatments. Citral is the most effective constituent of citrus essential oil are shown to have fungicidal activities against postharvest pathogens of citrus (Ben-Shlom *et al.*, 2003 ; Cacioni *et al*., 1998 ;Cheah *et al*., 1997). Rodov *et al*., (

1995) reported that resistance of young lemon fruit to decay development is related to citral level in lemon flavedo. The inhibitory effect of citral on postharvest pathogens was reported by (Abd-El-Kareem et al., 2002; Chein et al., 2007, El-Mohamedy, 2002 and 2008; Faten, 2010).

Many researchers investigated the mode of action of essential oils on/in the fungal cell in order to promote fungistatic or fungicide effect.In general, inhibitory action of natural products on moulds involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intercellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition (Campo *et al.* 2003). Also, it is reported that plant lytic enzymes act in the fungal cell wall causing breakage of b-1,3 glycan, b-1,6 glycan and chitin polymers (Brull and Coote 1999). The mode by which microorganisms are inhibited by essential oils and their chemical compounds seem to involve different mechanisms. It has been hypothesized that the inhibition involves phenolic compounds, because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing increased permeability and unavailability of vital intracellular constituents (Juven *et al*., 1994).

Chitosan is seafood industry was reported by many investigators as a protective safe material against many pathogens (Chien *et al*., 2007; Badawya and Rabeab,2009).The antifungal activity of chitosan were examined at various concentrations against fungi including *Penicillium digitatum, Penicillium italicum, Botrydiplodia lecanidion* and *Botrytis cinerea.* (El-Mohamedy, 2002, 2003 and 2008; El-Mohamedy *et al*., 2002; Hongyin *et al*., 2011).The mechanism by which chitosan affects the growth of several phytopathogenic fungi has been postulated in several hypotheses, first: its polycationic nature, it is believed that chitosan interferes with negatively charged residues of macromolecules exposed on the fungal cell surface. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents (Papineau *et al*., 1991). Second: the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of mRNA and protein synthesis, third : the interaction of chitosan with fungal DNA and RNA. fourth: malformation of fungal mycelial i.e., excessive mycelial branching, abnormal shapes, swelling, and hyphae size reduction (Cheah *et al.*,1997). Numerous previous studies have shown that chitosan could directly inhibit spore germination, germ tube elongation and mycelial growth of many phytopathogens, such as *Botrytis cinerea* (El-Gaouth *et al*., 1992; Liu *et al*., 2007; Chien and Chon, 2006), *Fusarium solani* (Eweis *et al*., 2007), *Rhizopus stolonifer* (El-Gaouth *et al*., 1992), *Penicillium* (Chien and Chon, 2006), and *Sclerotium rolfsii* (Eweis *et al*., 2007).Liu *et al*.(2007) reported that chitosan completely inhibited spore germination of *P. expansum* and *B. cinerea, significantly* inhibited germ tube elongation of both pathogens and the plasma membranes of spores of both pathogens were damaged.

Our results indicate that the most effective treatments in protective effect of orange fruits against blue and green moulds during 40 days of storage at 20°C were combined treatments between chitosan + citral or lemon grass at concentrations 4.0 g / L+ 4.0 ml / L and followed by chitosan + citral at 3g/L+3 ml/Meanwhile,least protective effects against two pathogens was achieved at low concentration of chitosan or essential

oils single treatments.In this respect,Using of citral for controlling postharvest diseases of navel orange was reported by many researchers (Abd-El-Kareem and Abd-Alla, 2002;Abd-Alla,*et al*., 2011; El-Mohamedy, 2008).

Chitosan has become a prospective alternative treatment for fruit and vegetables due to its natural character, antimicrobial activity, and elicitation of defense responses in plant tissue (Hongyin *et al*., 2011;Chen *et al*., 2005).Gianfranco *et al*., (2007) noted that coating fruits with chitosan decreased postharvest diseases of tomato, strawberry and lime fruits.The application of chitosan coating reduced respiration rate and weight loss, delayed the increase in PPO activity and the changes in colour, and eating quality, and partially inhibited decay of fruit during storage (Meng *et al*., 2008 and 2010). Liu *et al*. (2007) found that treatment of chitosan induced the activities of PPO and POD, and increased the content of phenolic compounds in tomato fruit stored at 25 and 2 °C, these results possibly being related to the effective control of chitosan on gray mold rot and blue mold rot of tomato fruit. Many invitagatoers found that coating fruits with chitosan reduced the respiration rate, ethylene production, interval O2 levels and increased the interval CO2 of peach and pear fruits. They added that coated fruits were markedly firmer and less mature of the end of storage. Moreover, chitosan is able to extend storage life and to control decay of strawberries, apples, peaches, pears, kiwfruit, cucumbers sweet cherries, and citrus fruit (Abd-El-Kareem *et al*., 2002, Chen et al., 2007; Liu *et al*., 2007; Ben-shalom *et al*., 2003; Ewies *et al*., 2006 ; Meng *et al*., 2008 and 2010).The present results may lead to the conclusion that application of essential oils is applicable, safe and cost-effective method for controlling postharvest diseases. Also, the use of chitosan and essential oils in agriculture could be a suitable alternative for inclusion in disease control systems and could act sometimes as main or adjuvant antifungal compounds and do not leave a toxic residue in the product.

In conclusion, the improved formulation of chitosan – essential oils mixtures provided an effective control for orange and lime fruit against postharvest pathogen infections and artificial infections of *Penicillium digitatum* and *P. italicum* with values of decay reduction higher than 90 %. These results demonstrated that the commercialization of some of chitosan – essential oils mixtures to control post-harvest decay of fruits and vegetables appears to be feasible and may present an alternative to synthetic pesticides. However, the potential use of chitosan and essential oils to control post harvest diseases requires a detailed examination of their biological activity and dispersion in fruit tissues and the development of a formulation, which inhibits the growth of pathogens at non-phytotoxic concentrations.

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