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EFFECT OF A NEW CHEMICAL FORMULA ON POSTHARVEST DECAY INCIDENCE IN CITRUS FRUIT

Nehal Samy El-Mougy, Mokhtar Mohamed Abdel-Kader, Mohamed Hasanen Aly*

Plant Pathology Department, National Research Centre, El-Behose St. Dokki, 12622, Giza, Egypt

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Abstract: Postharvest diseases caused by *Geotricum candidum* (sour rot), *Penicillium digitatum* (green mould), and *P. italicum* (blue mould) are the most important negative factors affecting handling and marketing of citrus fruits in Egypt. A new formula containing stevia leaf powder and a mixture of the three commercial chemical active ingredients: ketoconazole, fluconazole, and itraconazole has been successfully applied. Either chitosan or water wax were used as carriers, against fruit mould pathogenic fungi under laboratory and storage conditions. Results of the in vitro test showed that a complete reduction in linear fugal growth was observed when the ingredients of the new formula were used individually at a concentration of 400 µg/ml each, while a mixture of all the tested chemicals had a superior effect with all fungal growth completely inhibited with the use of the mixture at a concentration of 100 µg/ml. Similar results were recorded on citrus fruits which were coated with the suggested formula of chitosan or wax containing chemical compounds as a semi applicable technique using navel orange peel discs. Furthermore, the obtained results were confirmed using in vivo testing on navel orange and lime fruits, under artificial inoculation conditions of the pathogenic fungi within a storage period extended for four weeks. The proposed approach provides the treated agricultural products with long acting protection against microbial invasion and even association. This formula could be used as a fungicide alternative for protecting the agricultural products which have high moisture contents. The formula can be used against mould pathogens to prolong the healthy shelf life of the agricultural products. Such a treatment is safe, cheap, easily applied, and without residues which are harmful to people and the environment.

Key words: citrus fruit decay, ketoconazole, fluconazole, itraconazole, stevia

INTRODUCTION

Citrus are the most important exportation fruits in Egypt. Green and blue moulds as well as sour rot are the major postharvest diseases of citrus fruits caused by the pathogens *Penicillium digitatum*; *P. italicum* and *Geotricum candidum*, respectively (El-Mougy *et al*. 2008). They cause serious problems to the harvested citrus fruits during handling, transportation, exportation and the storage process. Although the use of chemical fungicides gave satisfactory control against mould infection, the fungicide residual can have a harmful effect on people and the environment (Eckert and Ogawa 1988). Moreover, successive use of fungicides could lead to some fungal isolates developing a significant resistance to the applied fungicides. Therefore, alternative fungicide treatments are needed for the management of postharvest diseases of citrus fruits. Carrying out good hygienic practice during production, transport and processing will certainly minimize the contamination of fruits and vegetables and reduce mould infection. These treatments are partially effective in removing disease-causing organisms from the surface of fruits and vegetables or from contact surfaces during handling. Under these conditions, development of nontoxic nonvolatile fungicides that could be surface-applied and add a level of protection to citrus fruits from mould infestations is one strategy to address this problem. Azoles constitute a major category of antifungal agents in clinical use (Pfaller *et al*. 1998; Uchida *et al*. 2000; Palou *et al*. 2001; Nakai *et al*. 2003; Pfaller *et al*. 2003). In general, they target inhibition of ergosterol synthesis. Like mammalian cells, fungi are eukaryotes, so agents that affect protein or nucleic acid biosynthesis are likely to display general eukaryotic toxicity (Gupte *et al*. 2002). Ergosterol, the predominant component of fungal cell membranes, is therefore an obvious and specific target for fungal inhibition. The original azoles approved for clinical use of miconazole, econazole, and ketoconazole have a complicated mode of action that involves inhibition of several membrane-bound enzymes as well as membrane lipid biosynthesis (Ghannoum and Rice 1999). The newer azole class of antifungal agents, *i.e.* fluconazole, itraconazole, and ketoconazole has also proven to be effective in treating invasive mycoses. Elewski and Hay (1996) reported that fluconazole has demonstrated good activity against dermatophyte fungi and many *Candida* spp. The broad-spectrum, synthetic antifungal triazole itraconazole was approved in 1995 for the treatment of onychomycosis and is available in capsule form and as an oral use (Elewski 1989). Moreover, many investigators reported the use of chitosan as a protective safe material against many

*Corresponding address:
mokh_nrc@yahoo.com

pathogens. Coating fruits with chitosan decreased postharvest diseases of apple, tomato, strawberry and lime fruits (El-Gaouth *et al*. 1991; Du and Sun 1994; Du *et al*. 1997; El-Mougy *et al*. 2002). On the other hand, Komissarenko *et al*. (1994), Tadhani and Subhash (2006), Anonymous (2007) recorded that stevia plant leaves also have an antifungal effect. Therefore, we used stevia plant leaf powder in our work, as a natural product for enhancing the inhibition efficacy of the chemical used. The objective of the present study was to evaluate a new formula that contains the azoles derivatives fluconazole, itraconazole, and ketoconazole compounds in combination to act as a fungicide that effectively controls the three mould fungi that invade citrus fruits. Chitosan or Carnova wax were used in the present study as carriers for the ingredients of azoles active derivatives.

MATERIALS AND METHODS

Tested materials

Fungi

Pathogenic isolates of citrus decay fungi, *i.e. P. digitatum, P. italicum* and *G. candidum* obtained from the Culture Collection Unit of the Department of Plant Pathology, National Research Centre, Egypt were maintained on 2% malt agar (Difco, Detroit, Michigan, USA). These fungal isolates proved their high virulence to cause decay to citrus fruits in previous work at the same Department.

Citrus fruits

Healthy citrus fruits, *i.e.* navel orange (*Citrus sinensis* L.) and lime (*Citrus aurantifolia* F. Muell) harvested freshly from citrus orchards were used in the present study.

Chemicals

Pure active ingredients of Ketoconazol (RAMADA Co., Egypt); Fluconazol (SEDICO Pharmaceutical Co., Egypt) and Itraconazol (MULTI-APEX Parma SAE, Egypt) were used. These chemicals rarely dissolve in water, therefore they were dissolved in polyethylene glycol (PEG) then diluted in distilled water (DI). PEG is used as an excipient in pharmaceutical products and the lower-molecular-weight variants are used as solvents (Smolinske 1992). The prepared solutions were kept in a refrigerator at 5°C till use.

Natural product

The plant material used in the present study was stevia, which is used traditionally for the source of natural sweetener. Stevia (*Stevia rebaudiana* Cav.) is the natural product obtained from the stevia plant leaves ground into a powder form and not chemically synthesized. It is sold commercially as a natural sweetener (SEKEM Co., Egypt).

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.

Water wax

Water wax (Carnauba wax) is used commercially for fruit-polishing in packing and exporting fruit stations, in order to retard yellowing. This wax extends the marketing life and helps the fruits avoid injury from brushing and bruising as well. Water wax was purchased from the packing and exporting fruit station at the Kalyoubia governorate, Egypt.

Laboratory tests

In vitro tests

The inhibitory effect of the tested chemical compounds and the carrier solutions chitosan and wax against the linear growth of mould incident fungi was evaluated under in vitro conditions.

Effect on linear growth

Chemical compounds individually or in combination at concentrations of 50, 100, 200 and 400 µg/ml, chitosan (2%) as well as water wax solutions at concentrations of 10, 20 and 40% were tested. The tested chemicals were added to sterilized malt agar 2% medium, before solidifying, to obtain the proposed concentrations. Then, gentle rotation was done for five min. to ensure the equal distribution of the added chemical(s). After this, the concentrations were dispensed in sterilized Petri plates (9-cm-diameter). Malt agar medium free of chemicals, was used for the check treatment. All plates were inoculated at the center with discs (5-mm-diameter) of 10-day-old culture of tested fungi. Five replicates were used for each particular treatment. Inoculated plates were incubated at 20±2°C. The average linear growth was measured after 5–10 days when the fungi reached full growth in the check treatment. Then, reduction (%) in mycelial growth was calculated in all treatments relative to the fungal growth (90-mm-diameter) in the control one.

The efficacy of the tested chemical compounds individually or in combination, diluted in chitosan or water wax, on either the growth inhibition of pathogenic fungi or their ability to induce mould infection of citrus fruits was evaluated under laboratory conditions using peel discs of navel orange fruits as a model to achieve this purpose.

Ethyl alcohol (70%) disinfested peel discs (10-mm-diameter) were dipped individually, for 5 min, in a volume of 100 ml of the tested material solutions, dissolved in either chitosan or wax, and air dried into laminar flow for 2 h. The discs were then transferred to the centre of Petri plates containing malt agar medium seeded with 3 ml of spore suspension (having a mean of 10^6 spore/ml) of either *P. digitatum, P. italicum* or *G. candidum.* The check treatment was peel discs plated on inoculated medium with tested fungi which were free of chemical compounds. Ten replicates were used for each treatment. All plates were incubated at 20±2°C for 7 days. The average diameter of inhibited fungal growth zone (mm) around the treated peel discs was measured.

Effect on mould incidence

To evaluate mould incidence in response to chemical compounds, peel discs were dipped, for 5 min, in the tested material solutions, in either chitosan or wax, then air dried. Treated discs were placed on moistened filter paper in Petri-plates, at a 5 disc/plate rate. The discs were then sprayed with spore suspension (having a mean of 10^6 spores/ml) of either *P. digitatum*, *P. italicum* or *G. candidum* at the rate of 3 ml/plate. Next, the sprayed discs were incubated at 20±2°C. Ten replicates were used for each particular treatment. The check treatment was peel discs free of both chemical compounds and pathogenic fungi. Mould incidence was recorded after 7 days of incubation. The average percentage of infected peel discs was calculated relative to the whole number of treated peel discs.

In vivo test

The citrus fruit used in this test were navel orange (*C. sinensis*.) and lime (*C. aurantifolia*). The citrus fruits were coated with either chitosan or water wax solutions containing a mixture of ketoconazole, fluconazole, itraconazole and stevia at equal concentrations of 100 µg/ml to reach a sum of 400 µg/ml of the mixture. The efficacy of coated citrus fruits against mould incidence under stress of artificial infestation, was evaluated during storage conditions. Fruits were surface sterilized by dipping them into 1% (v:v) sodium hypochlorite for 3 min. Then, fruits were rinsed 3 times with sterile distilled water and blotted dry on sterile filter paper. The fruits were wounded by a sterilized needle at one marked point and dipped individually into either chitosan or water wax solution made up of the proposed concentration of tested chemicals. Then, the treated fruits were artificially inoculated by spraying with tested fungi (having a mean of 10^6 spores/ml). Thereafter, all treated fruits were air dried, placed into carton boxes (46x23x30 cm) with a capacity of 20 fruits/box, covered with plastic sheets to maintain a relative humidity – RH (90–95%), and stored in a fruit store at 20±2°C for four weeks. Five boxes as replicates were used for each treatment as well as the control. Decayed fruits were counted periodically every week. The percentage of total disease incidence was calculated at the end of the storage period.

Statistical Analysis

All *in vitro* and *in vivo* experiments were set up in a completely randomized design. One way analysis of variance (ANOVA) was used to analyze the obtained data. General Linear Model option of the Analysis System SAS (SAS 1988) was used to perform the analysis of variance. Duncan's Multiple Range Test was used for the means separation (Winer 1971).

RESULTS AND DISCUSSION

***In vitro* tests**

The inhibitory effect of the tested chemical compounds as well as carrier solutions, *i.e.* chitosan and wax against the linear growth of mould incident fungi was evaluated under *in vitro* conditions. Results expressed as reduction in fungal linear growth or clear zone of growth inhibition are presented in tables 1 and 2. The results obtained show that the chemical compounds individually or in combination have an inhibitory effect against the tested fungal growth. The inhibitor effect increases in ascending order in parallel with the graduate increase of used concentrations of 50, 100 and 200 µg/ml of chemical compounds individually or in combination. This observation was also true with the Chitosan solution at 10, 20 and 40% concentrations. Complete reduction in linear fugal growth was observed at a concentration of 400 µg/ml, while the mixture of all the tested chemicals had the superior effect that fungal growth was completely inhibited at a concentration of 100 µg/ml. The fungi tested showed different responses against the chemicals used, especially *G. candidum* which showed a light tolerance against all the chemicals used. Stevia powder showed a similar trend for inhibiting fungal growth as the chemicals which were used, with no significant differences (Table 1). Although, stevia powder which had been dissolved in chitosan, showed a superior significant inhibitor effect over chitosan dissolved in wax (Table 2).

Data also show that water wax had no inhibitor effect against the tested fungi, at all used concentrations. Furthermore, the clear zone of fungal growth inhibition in response to chemicals used at different concentrations followed the same trend in respect to each diffusible rate. Evaluation of the excepted reaction of coating citrus fruits with the suggested formula of chitosan or wax which contained the chemical compounds, was carried out as a semi applicable technique using navel orange peel discs.

Data in table 3 showed that chemical compounds have a more protective effect against mould incidence of navel orange peel discs when dissolved in chitosan than in wax solutions. This observation was clearly true in the case of a combination of the tested three chemicals and stevia powder at concentrations of 25 ppm. Also, a highly significant reduction in mould incidence was observed in the peel discs treated with chemical compounds compared to the untreated control peels.

Complete reduction in mould incidence was observed at concentrations of 100 µg/ml of individual chemical compounds, while the mixture of all chemical compounds and stevia powder has superior efficacy and caused a similar effect at 50 µg/ml. These observations were true with chitosan or wax solutions. The use of chemical fungicides leads to satisfactory control, however, such use imposes selective pressure upon the pathogen population and the fungacides have a residual which can have a harmful effect on humans and can cause serious diseases. Moreover, some fungal isolates developed significant resistance to the used fungicides (Eckert and Ogawa 1988). These authors added, that fungicides utilized–borax, sodium carbonate and sodium O-henylphenate (SOPP) which had a broad antifungal spectrum and did not penetrate the fruit surface except at injured sites. Attempts to find alternatives to chemical control have been ongoing for some time, and indeed, many fungi have become resistant to commonly used fungicides. Therefore, looking for safe and cheap alternative approaches for controlling postharvest diseases of citrus fruits was of great interest to

Table 1. Reduction in fungal growth in response to different concentrations of some chemical compounds and chitosan *in vitro* conditions

Treatment	Concentration	Reduction in linear growth of fungi tested [%][1]		
		P. digitatum	*P. italicum*	*G. candidum*
Itraconazole (A)	50 ppm	22.2 e	23.3 e	18.9 ef
	100 ppm	45.4 d	46.7 d	22.2 e
	200 ppm	100 a	64.4 c	52.2 cd
	400 ppm	100 a	100 a	100 a
Ketoconazole (B)	50 ppm	24.4 e	35.6 de	15.6 ef
	100 ppm	41.1 d	56.7 cd	18.9 ef
	200 ppm	61.1 c	100 a	42.2 d
	400 ppm	100 a	100 a	100 a
Fluconazole (C)	50 ppm	31.1 de	38.9 de	17.8 ef
	100 ppm	47.8 d	55.6 cd	22.2 e
	200 ppm	100 a	68.9 c	43.3 d
	400 ppm	100 a	100 a	100 a
Stevia powder (D)	50 ppm	26.7 e	25.6 e	16.7 ef
	100 ppm	44.4 d	54.4 cd	23.3 e
	200 ppm	68.7 c	66.4 c	61.1 c
	400 ppm	100 a	100 a	100 a
A+B+C+D[2]	50 ppm	78.2 b	88.8 a	35.6 de
	100 ppm	100 a	100 a	100 a
	200 ppm	100 a	100 a	100 a
	400 ppm	100 a	100 a	100 a
Chitosan	10%	9.4 f	8.6 f	2.8 f
	20%	22.7 e	25.2 e	19.5 ef
	40%	75.3 b	76.6 b	64.2 c
Wax (Carnova wax)	10%	0.0 g	0.0 g	0.0 g
	20%	0.0 g	0.0 g	0.0 g
	40%	0.0 g	0.0 g	0.0 g

Mean values within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

[1] reduction [%] in mycelial growth was calculated according to chemical concentrations relative to the fungal growth (90-mm-diameter) in the control treatment

[2] concentration used is the sum of the equal concentration of each chemical used

Table 2. Average zone [mm] of growth inhibition of mould fungi in response to different concentrations of some chemical compounds in chitosan or wax under *in vitro* conditions

Treatment		Concentration [ppm]	Zone of inhibition growth [mm] of tested fungi		
carrier	chemical		*P. digitatum*	*P. italicum*	*G. candidum*
Chitosan	itraconazole (A)	25	9.2 c	8.8 c	6.2 d
		50	10.3 bc	9.8 c	7.3 d
		100	11.4 bc	11.1 bc	9.7 c
	ketoconazole (B)	25	9.7 c	9.2 8 c	7.4 d
		50	10.6 bc	9.8 c	8.8 c
		100	11.8 bc	11.2 bc	10.6 bc
	fluconazole (C)	25	9.6 c	8.9 c	7.6 d
		50	10.2 bc	9.7 c	8.2 c
		100	11.4 bc	10.8 bc	9.1 c
	stevia powder (D)	25	6.4 d	6.2 d	5.3 e
		50	7.8 d	6.8 d	5.8 e
		100	8.4 c	7.6 d	6.4 d
	(A+B+C+D)[1]	25	12.4 b	11.7 bc	9.4 c
		50	14.3 b	13.8 b	12.2 b
		100	16.8 a	15.4 a	13.4 b
Wax	itraconazole (A)	25	7.2 d	7.4 d	5.7 e
		50	8.3 c	8.7 c	6.8 d
		100	11.5 bc	12.2 b	8.6 c
	ketoconazole (B)	25	8.2 c	8.6 c	5.8 e
		50	8.8 c	6.2 d	7.4 d
		100	12.4 b	11.8 bc	9.1 c
	fluconazole (C)	25	9.4 c	9.6 c	6.4 d
		50	10.8 bc	11.2 bc	8.2 c
		100	12.3 b	12.8 b	9.5 c
	stevia powder (D)	25	9.1 c	9.3 c	5.9 e
		50	10.4 bc	10.3 bc	8.8 c
		100	11.6 bc	11.4 bc	10.7 bc
	(A+B+C+D)	25	12.4 b	12.3 b	9.2 c
		50	14.2 b	13.8 b	10.4 bc
		100	15.8 a	14.6 b	12.7 b
Control			0.0 f	0.0 f	0.0 f

Mean values within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

[1] concentration used is the sum of the equal concentration of each chemical used

Table 3. Mould incidence [%] on navel orange peel discs in response to different concentrations of some chemical compounds in chitosan or wax solutions *in vitro* conditions

Treatment		Concentration [ppm]	Mould incidence [%] on peel discs		
carrier	chemical		*P. digitatum*	*P. italicum*	*G. candidum*
Chitosan	itraconazole (A)	25	6.2 e	5.4 e	7.2 de
		50	2.2 f	1.9 f	2.4 f
		100	0.0 g	0.0 g	0.0 g
	ketoconazole (B)	25	8.3 d	8.6 d	10.4 c
		50	2.2 f	2.8 f	3.6 f
		100	0.0 g	0.0 g	0.0 g
	fluconazole (C)	25	5.8 e	4.8 ef	6.8 e
		50	1.8 f	1.6 f	2.6 f
		100	0.0 g	0.0 g	0.0 g
	stevia powder (D)	25	16.8 b	17.6 b	20.3 ab
		50	10.2 c	9.8 d	16.4 b
		100	0.0 g	0.0 g	0.0 g
	(A+B+C+D)[1]	25	4.3 f	4.6 ef	5.1 e
		50	0.0 g	0.0 g	0.0 g
		100	0.0 g	0.0 g	0.0 g
Wax	itraconazole (A)	25	7.3 de	6.2 de	8.4 d
		50	3.3 f	2.2 f	3.1 f
		100	0.0 g	0.0 g	0.0 g
	ketoconazole (B)	25	9.3 d	9.2 d	11.3 c
		50	2.8 f	3.1 f	4.3 ef
		100	0.0 g	0.0 g	0.0 g
	fluconazole (C)	25	6.6 de	5.2 e	7.2 de
		50	2.2 f	1.8 f	3.1 f
		100	0.0 g	0.0 g	0.0 g
	stevia powder (D)	25	18.2 b	19.5 b	22.2 ab
		50	11.3 c	10.6 c	18.3 b
		100	0.0 g	0.0 g	0.0 g
	(A+B+C+D)	25	5.1 e	5.3 e	6.3 de
		50	0.0 g	0.0	0.0 g
		100	0.0 g	0.0 g	0.0 g
Control			100 a	100 a	100 a

Mean values within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

[1] concentration used is the sum of the equal concentration of each chemical used

phytopathologists. There have been many attempts to achieve this target. Laboratory tests, in the present study, revealed that (Table 1) complete reduction in linear fugal growth was observed at a concentration of 400 µg/ml of these individual chemical compounds: itraconazole, ketoconazole, fluconazole, stevia. The mixture of all the tested chemicals had a superior effect at 100 µg/ml. Also, these chemicals could affect the growth of mould incidents expressed as clear zone of growth inhibition (Table 2) as well as decay incidence on treated citrus peel discs (Table 3). In this regard, the efficacy of these compounds, which are listed as azole derivatives, were reported to have an antifungal effect against human infectious diseases. In this regard, the newer azole class of antifungal agents (fluconazole, itraconazole, ketoconazole and stevia) has also proven to be effective in treating invasive mycoses (Zervos and Meunier 1993; Espinel-Ingroff *et al*. 2001; Rambali *et al*. 2001; Diekema *et al*. 2003; Clausen and Yang 2005; Tadhani and Subhash 2006). Hitchcock (1991) reported that the azole antifungal agents work by inhibiting cytochrome P-450-dependent 14 ά-sterol demethylase of ergosterol biosynthesis (P-450DM). Azole treated fungi are depleted of ergosterol, and accumulate 14 ά-methylated sterols which inhibit fungal growth. All of the azoles are fungistatic, as opposed to fungicidal, against *Candida* spp., underlining the importance of the host's immune system for eradicating the infecting organism and achieving a clinical cure (Rex *et al*. 1995). Resistance to fluconazole has been observed in a number of clinical fungal isolates, including *C. albicans*. Future work should be directed toward determining the prevalence of this problem and developing strategies for its prevention. The possibility of the resistance of *G. candidum*, *P. digitatum*, and *P. italicum* to ketoconazole, fluconazole, and itraconazole should be taken into consideration. Clausen and Yang (2005) reported that various concentrations of eight azoles were evaluated for their ability against pine wood mould infestation when challenged with *Aspergillus niger, Penicillium chrysogenum,* and *Trichoderma viride* spores. They also found that inhibiting the growth of mould fungi on cellulose-based building materials may be achievable through the use of azole-based antimycotics. Therefore, they concluded that azoles were variably effective against mould fungi that are frequently found on wood and wood products. Furthermore, the results of our study indicate that the stevia leaf powder has inhibitory activities against citrus fruits mould pathogens. This observation indicates that stevia leaf powder may be an ideal candidate to use with food preservation as well as pharmaceutical and natural plant-based products. The present in vitro and in vivo tests revealed that stevia powder had a synergistic effect when combined with the three azole compounds. The results showed a high inhibitor effect against mould fungi, although low concentrations of the combined formula were used (Tables 1, 2, 3). This result suggests that stevia leaves may be used as antimycotic ointment for various fungal skin diseases as well as bacterial diseases (Ghosh *et al*. 2008). This is in agreement with previous findings which concluded that *Stevia rebaudiana* Bertoni is a medicinal plant with a wide range of biological activity, which can be used for the preparation of various formulations for treatment of inflammation, wound and microbial infections (Komissarenko *et al*. 1994; Tadhani and Subhash 2006).

***In vivo* tests**

Chitosan or water wax containing four compounds, *e.g.* ketoconazole, fluconazole, itraconazole and stevia were tested for their protective effect against citrus fruit moulds. Results indicate that all treated fruits showed complete reduction in mould incidence. It is clear (Table 4) that efficacy of the two applied mixtures as fruit coating in chitosan or wax were able to highly protect both navel orange or lime fruits against mould incidence under stress of artificial inoculation with mould pathogens for over one month; the period of experimental test. In the untreated control fruits there were 100% green moulds, blue moulds, and sour rot infection. The new proposed formula is generally applied in combination with coating polymer such as chitosan or water wax. Coating fruits using a natural material such as chitosan for controlling postharvest diseases of apple, lime and valencia orange had satisfactory results (El-Gammal and El-Mougy 2002). The present investigation showed that applying the new formula as fruit coating obviously gave complete protection for citrus fruits against mould infection, even under

Table 4. Mould incidence [%] of citrus fruits coated with a chitosan or water wax solution containing a mixture[1] of ketoconazole, fluconazole, itraconazole and stevia, in which the fruits are under stress of artificial infestation during storage conditions

Mixture of chemical	Fruit decay incidence [%]					
	navel orange			lime		
	green mould	blue mould	sour rot	green mould	blue mould	sour rot
Chitosan solution	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
Wax solution	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
Untreated control	100 a	100 a	100 a	100 a	100 a	100 a

Mean values within columns followed by the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test

[1] the mixture used at a concentration of 400 ppm expressed as the sum of equal concentration of 100 ppm of each chemical used

artificial infestation with the pathogenic fungi under storage conditions for four weeks (Table 4). The new formula contains double-antifungal agents when using chitosan as a carrier for the chemical compounds. In addition to the antifungal activity of chemical compounds, chitosan is also reported to have an antifungal effect and had been widely used in medicine, agricultural production and the food industry (Hirano and Nagao 1989; Du and Sun 1994).

Coating fruits with chitosan decreased postharvest decay caused by fungal infection of citrus, tomato and strawberries (El-Gaouth *et al.* 1992 a, b; Benhamou 2004; Chien and Chou 2006; Chien *et al*. 2007). Chitosan, a by-product from the seafood industry, is a safe material as specified by toxicological studies (Hirano *et al*. 1990). The mode of action of chitosan was explained (Du *et al*. 1997) as a coating of fruit which significantly reduced the respiration rate, ethylene production, and interval O_2 level of peach, pear and kiwi fruits. The present findings demonstrated that the new formula containing a mixture of chemical compounds which are used in either chitosan or water wax as carriers, have the potential to be environmentally compatible, nontoxic postharvest fungicides to be used against citrus mould incidence of stored navel orange and lime fruits. The new formula could be for commercial use in packinghouses since this formula is considered to be safe for wide consumption.

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