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THE USE OF FUNGICIDE ALTERNATIVES FOR CONTROLLING POSTHARVEST DECAY OF STRAWBERRY AND ORANGE FRUITS

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Abstract: Control measures of postharvest diseases of strawberry and navel orange fruits using hydrogen peroxide, calcium chloride and chitosan were evaluated under *in vitro* and *in vivo* conditions. All tested concentrations of chemicals used were able to reduce the linear growth and spore germination of *B. cinerea; R. stolonifer; P. digitatum* and *P. italicum.* Complete inhibition of linear growth and spore germination was obtained with concentrations of 1.5 and 2.0% of all treatments. Under storage conditions, significant reduction in descending order of mould incidence was observed in strawberry and orange fruits treated with ascending concentrations of calcium chloride, hydrogen peroxide and chitosan. Obtained data revealed significant reduction in mould incidence in fruits when treated by calcium chloride and chitosan 12h before artificial inoculation with the mould pathogens, while hydrogen peroxide showed the opposite result. The present study demonstrated that the application of hydrogen peroxide is superior to treatment with calcium chloride or chitosan enhanced the control activity against mould pathogens which as it expressed was as either percentage of diseased fruits or decay development as rotted tissue weight of strawberry and navel orange. The applied tested chemical might act as contact and systemic fungicides which have a protective or therapeutic effect.

Key words: calcium chloride, chitosan, hydrogen peroxide, moulds, orange, postharvest decay, strawberry, storage

INTRODUCTION

The fungal decay of fruits and vegetables in postharvest storage greatly limits their economic value. Grey mould and soft rot of strawberries (*Fragaria ananassa*

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Duchesne) are postharvest diseases caused by *Botrytis cinerea* Pers.ex Fr. and *Rhizopus stolonifer* Ehrenb. Fr. Vill. (Ceponis *et al*. 1987). They were reported to cause severe problems during storage period and shelf life (El-Kazzaz *et al.* 1983; Li and Kader 1989). Moreover, *Penicillium digitatum* (Pers. Sacc.) (Green mould) and *P. italicum* When, (blue mould) are the most important factors affecting harvested orange fruits during handling, transportation, exportation and storage (Morris 1982; Eckert and Brown 1986). All these pathogens infect fruits through epicarpic wounds caused during harvesting and handling, in the backing–house processing lines, so an entry site is required for setting, infection, and decay development (Spotts and Cervantes 1986). Although fungicide treatments have been the main methods for controlling postharvest diseases, public concern about fungicide residues in food and the development of fungicide resistance by pathogens has increased the search for alternative means of controlling the disease. Certain strategies, such as pre- or postharvest application of calcium salts, hydrogen peroxide and chitosan against fruit decay are proposed (Conway *et al.* 1994; Sapers and Simmons 1998; El-Gaouth *et al*. 1992). Pre- and postharvest calcium applications have been used to delay ageing or ripening to reduce postharvest decay and control of many diseases in fruits and vegetable (Poovaiah 1986). Saftner *et al.* (1997) reported that postharvest calcium treatment of apples provided broad-spectrum protection against the postharvest pathogens of *P. expansum* and *B. cinerea.* Also, the use of hydrogen peroxide on whole and fresh-cut produce has been investigated in recent years (Ralph 2003). In this regard, Juven and Pierson (1996) reviewed research reports on the antimicrobial activity of H_2O_2 and its use in the food industry. Microbial populations on whole cantaloupes, grapes, prunes, raisins, walnuts, and pistachios were significantly reduced upon treatment with hydrogen peroxide (Sapers and Simmons 1998). In addition many investigators reported the use of chitosan as a protective safe material against many pathogens. Coating fruits with chitosan decreased postharvest diseases of apple, tomato, strawberry and lime fruits (El-Gaouth *et al.* 1991; Du *et al.* 1997; El-Mougy *et al.* 2002). The purpose of the present study was to evaluate the effect of calcium chloride, hydrogen peroxide and chitosan on the *in vitro* growth and spore germination of the decay fungi of strawberry and orange fruits. Moreover, their effects against the incidence and disease development of gray mould, soft rot of strawberry and blue green mould and of orange were also tested under *in vivo* conditions.

MATERIALS AND METHODS

Pathogen inocula

An aggressive isolate of each *B. cinerea*, *R. stolonifer*, *P. digitatum* and *P. italicum* obtained from Plant Pathology Department, National Research Centre, Egypt were used in the present work. These isolates developed well and were maintained on Potato-Dextrose-Agar (PDA) media, with periodic recultured as test as needed.

Strawberry and orange fruits

Recent fresh harvested land apparently healthy fruits of strawberry and navel orange were collected from El-Ebour commercial principal market at Cairo, Egypt. Collected fruitswere transported to the laboratory and kept in refrigerat or at 5°C until needed.

***In vitro* growth and spore germination of the decay fungi**

Calcium chloride, hydrogen peroxide and chitosan at five concentrations each *i.e*. 0.0, 0.5, 1.0, 1.5, and 2.0% (w:v) were tested for their inhibitory effect on linear growth and spore germination of *B. cinerea*, *R. stolonifer, P. digitatum* and *P. italicum.* Tested chemicals were added to conical flasks containing sterilized PDA to obtain the proposed concentrations, then rotated gently and dispensed in sterilized Petri plates (9 cm–diameter). PDA medium free of chemicals was used for check treatment. All plates were inoculated at the centre with disks (5-mm diameter) of 10-days old culture of tested fungi. Five plates were used as replicates for each particular treatment. Inoculated plates were incubated at 20±2°C. The average linear growth of tested fungi was calculated after 5–10 days when they reached full growth in check treatment.

The effect of Calcium chloride, hydrogen peroxide and chitosan at the same previous concentrations on conidia germination of *B. cinerea*, *R. stolonifer, P. digitatum* and *P. italicum* was assessed in potato dextrose broth (PDB) using a modified method of Piano *et al*. (1997). Spore suspensions were prepared from 2-week old cultures grown on PDA by removing spores from the surface of the cultures with a sterile bacteriological loop in 5 ml of sterile distilled water. Suspensions were filtered through four layers of cheesecloth to remove fungal mycelia. Spore concentration was determined with a hemacytometer, and adjusted to 6×10^6 spores/ml. Aliquots of 100 μL of the pathogen suspension were transferred to glass tubes (180 × 16 mm) containing 5 mL PDB, then the tested chemicals were added to each tube to achieve the proposed concentration. All tubes were put on a rotary shaker at 50 rpm at 25°C for 20 h. Percent of spore germination was determined in three different microscopic fields. A total of 100 spores per replicate were observed. All treatments consisted of three replicates, and experiments were repeated three times. The efficacy of each treatment on either fungal growth or spore germination was calculated according to the following formula:

$$R(\%) = \frac{X - Xa}{X} \text{ x } 100$$

Where: R: Reduction (%), X: Estimation of germination or growth in control medium and Xa: Estimation of germination growth in a medium with tested chemicals.

***In vivo* incidence of postharvest decay of strawberry and navel orange fruits**

Calcium chloride, hydrogen peroxide and chitosan at the above concentrations were applied to strawberry and navel orange fruits for evaluating their effect against decay incidence during storage period. Fruits were surface sterilized by dipping them into 1% (w:v) sodium hypochlorite for 1 min, rinsed 3 times with sterile distilled water and blotted dry on sterile filter paper. The orange fruits were wounded by a 1 mm diameter needle at one marked point and dipped individually into the solution of proposed concentrations of tested chemicals. After 12 h, the treated fruits were artificially inoculated by spraying with tested fungal ($1\text{x}10^6$/ml) spore suspension. Inoculation of fruits was carried out one day before or after chemical treatments. Thereafter, all treated fruits were air dried, placed into carton boxes (20 fruits per each), covered with plastic sheet to maintain a relative humidity at 100% and stored in cold room at 20 ± 2°C for three weeks. Five boxes as replicates were used for each particular treatment as well as the control. Decayed fruits were counted and then the percentage of disease incidence calculated in relative to control treatment.

In vivo integrated treatments with hydrogen peroxide and chitosan or calcium chloride to control of strawberry and navel orange fruits

Hydrogen peroxide, as fruits surface disinfectant, in combination with chitosan or calcium chloride were tested against postharvest diseases of strawberry and navel orange fruits. Fruits were dipped in hydrogen peroxide at concentrations 2.0% for 5 min then air dried. Thereafter, disinfected fruits were wounded as stated before, then dipped in chitosan or calcium chloride at concentrations of 1.5 or 2.0 % and left to air drying before artificial inoculation. Inoculation of fruits and storage process were carried out as mentioned before. All fruits were stored at 20±2C° for 21 days. Percentage of disease incidence was calculated as stated before. The disease development was expressed as weight of the rotted tissue relatively to the whole weight of infected fruits.

Statistical Analysis

All data were analyzed according to standard procedures for analyses of variance (Steel and Torrie 1980).

RESULTS AND DISCUSSION

In vitro growth and spore germination of decay fungi

All tested concentrations of used chemicals were able to reduce the linear growth and spore germination of *B. cinerea; R. stolonifer; P. digitatum* and *P. italicum* (Tables 1, 2). Complete inhibition of linear growth and spore germination was obtained with concentrations of 1.5 and 2.0% of all chemicals. *R. stolonifer* showed a slight tolerance against calcium chloride and chitosan at 1.5% whereas its growth and spore germination amounted to (80.0, 64.2%) and (61.1, 74.4%), respectively. Moderate effect was obtained with the other concentrations of chemicals used. In this regard, application of these chemicals had similar results which were recorded by several investigators. Tian *et al* (2002) recorded that calcium chloride at 2% inhibited the growth and spore germination of *R. stolonifer*, although $CaCl_2$ was tolerated by *Alternaria alternata* and *P. expansum in vitro* where their growth was highly affected at 6% concentration (Maouni *et al.* 2007), while pronounced inhibition of spore germination of *P. digitatum* occurred at a concentration of 272 mM (4% wt/vol) of $CaCl_2$ (Droby *et al.* 1997). On the other hand, hydrogen dioxide is purported to control plant diseases by killing bacteria or fungi on contact, including those that have invaded the tissue (Miller 2006). Hydrogen peroxide is a strong oxidizer used for high-level disinfection and sterilization. It produces reactive hydroxyl free radicals and ions that can attack membrane lipids, DNA and other essential cell components (Ralph 2003). The present data also indicate that chitosan treatment reduced the linear growth and spore germination of tested fungi. The inhibitory effect on pathogenic fungal growth by chitosan was also reported by Leuba and Stossel (1986) and El-Mougy *et al*. (2002, 2006).

In vivo incidence of postharvest decay of strawberry and navel orange fruits

Under storage conditions, significant reduction in descending order of mould incidence was observed in strawberry and orange fruits treated with ascending concentrations of calcium chloride, hydrogen peroxide and chitosan (Table 3). The tested fruits were subjected to chemical application 12h before or after artificial inoculation in order to allow the fungal spore germination, invasion and infection fruit tissues to take place (Agrios 1988). Presented data revealed a significant reduction in mould incidence in fruits treated by calcium chloride and chitosan 12h before artificial inoculations with the mould pathogens, while hydrogen peroxide showed the opposite results. Regarding the mode of action of tested chemicals, hydrogen peroxide is reported to have highly reactive and short-lived effect due to instability of the peroxide bond, which leads to rapid degradation and low residues of hydrogen peroxide expected after application (Ralph 2003). Therefore, the application of hydrogen peroxide in the presence of fungal inoculum (after fruit inoculation) resulted in a higher effect than earlier application due to the direct effect of the released oxygen atom. Hydrogen peroxide is an unstable molecule, when it breaks down a single oxygen atom and a molecule of water is released. Many spores of organisms causing diseases are killed by oxygen, the free oxygen atom release a from H_2O_2 which is extremely effective. Hydrogen peroxide will help to eliminate existing infections and will help prevent the future ones (Fredrickson 2005).

Table 1. Reduction (%) in the linear growth of pathogenic fungi causing strawberry and navel orange fruit decay in response to different concentrations of calcium chloride, hydrogen peroxide and chitosan

Chemicals	Concentrations [%] (w/v)	Reduction in linear fungal growth [%]*			
		strawberry pathogens		navel orange pathogens	
		B. cinerea	*R. stolonifer*	*P. digitatum*	*P. italicum*
Calcium chloride	0.5	38.3*	46.4	68.3	66.4
	1.0	77.8	61.1	80.9	78.3
	1.5	100	80.0	100	100
	2.0	100	100	100	100
Hydrogen peroxide	0.5	65.6	63.3	55.6	52.2
	1.0	80.0	76.6	79.4	75.6
	1.5	100	100	100	100
	2.0	100	100	100	100
Chitosan	0.5	72.2	31.1	73.1	79.4
	1.0	92.8	41.9	100	100
	1.5	100	64.2	100	100
	2.0	100	100	100	100

*reduction in fungal growth was calculated relatively to the growth in check treatment (90 mm)

Recently there are many commercial products on base of hydrogen peroxide. Storox, a relatively new broad spectrum sanitizer, containing a mixture of hydrogen peroxide and peroxyacetic acid, reduced mould contamination For example the Storox treatment was also tested on wooden bins and reduced *P. expansum* populations by 97.9% when used at 2700 ppm (Sholberg 2004). The highest concentration of hydrogen peroxide (2%) in the present study was able to reduce infection of strawberry fruits with grey mould and soft rot down to 7 and 10% when applied after fungal inoculation, while the recorded disease incidence was 70 and 90% in case of later inoculation. Also, green and blue mould incidence of navel orange fruits showed the same phenomena: were 10 and 14% and 70 and 80%, of infected fruits in respective order to moulds and application time. The opposite feature of fungal fruit infectivity was observed with calcium chloride and chitosan applications.

Table 2. Reduction [%] in spore germination of pathogenic fungi causing strawberry and navel orange fruit decay in response to different concentrations of calcium chloride, hydrogen peroxide and chitosan

Chemicals	Concentrations % (w/v)	Reduction in spore germination [%]*			
		strawberry pathogens		navel orange pathogens	
		B. cinerea	*R. stolonifer*	*P. digitatum*	*P. italicum*
Calcium chloride	0.5	22.0*	17.2	29.0	32.0
	1.0	43.0	48.3	71.6	61.0
	1.5	100	61.1	100	100
	2.0	100	100	100	100
Hydrogen peroxide	0.5	46.2	50.6	55.6	64.4
	1.0	73.7	72.8	83.4	86.8
	1.5	100	100	100	100
	2.0	100	100	100	100
Chitosan	0.5	54.8	20.0	46.4	58.2
	1.0	72.6	59.6	100	100
	1.5	100	74.4	100	100
	2.0	100	100	100	100

*reduction in spore germination was calculated relatively to the average of three replicates each 100 spores of check treatment

Data in table 3 show that the highest concentration of calcium chloride (2%) could reduce grey and soft rot incidence of strawberry fruits down to 10 and 12% when applied after fungal inoculation, while in case of its application before inoculation more reduction was recorded. Similar records were also observed in navel orange infection with green and blue moulds: 30.2 and 34.2% and 20 & 22% in respective conditions. Similar results were reported concerning the efficacy of calcium chloride against fruit decay incidence. Postharvest calcium treatment significantly limited decay in peaches by *Monilinia fructicola* (Conway *et al.* 1987) and in apples by *Botrytis cinerea* (Lein *et al.* 1997). The precise mechanism by which calcium chloride reduces fungal infection is not yet understood, but the role of calcium in resistance may be one to interfere with the activity of pectolytic enzymes (Conway *et al.* 1992) and may be partially attributable to the decrease in maceration of cell walls by polygalacturonase (PG) due to the improved structural integrity caused by the increase in calcium chloride content (Conway *et al.* 1988). Also, previous studies indicated that the increase in the calcium content of the cell walls of apples reduces the activity of polygalacturonase extracted from *P. expansum* culture (Conway *et al.* 1988; Saftner *et al.* 1997). The Ca^{++} ions might bind with intercellular pectic acids and constitute pectate chloride; which is resistant to the fungal pectolytic enzyme polygalactouronase (Bateman 1964; Conway and Sams 1984). On the other hand, data in table 3 showed that coating fruits with chitosan either before or after fungal inoculation decreased strawberry and navel orange fruit decay. These results are in agreement with those reported by El-Ghaouth *et al.* (1992) and Abd El-Kareem *et al*. (2001) who recorded that coating fruits with chitosan decreased postharvest diseases of tomato, strawberry and lime fruits. In this respect, Du *et al*. (1997) reported that coating fruits with chitosan reduced the respiration rate, ethylene production, internal O_2 levels and increased the internal CO_2 of peach and pear fruits. They added that coated fruits were markedly firmer and less mature at the end of storage.

Table 3. Effect of different concentrations of calcium chloride, hydrogen peroxide and chitosan applied before or after artificial inoculation with pathogenic fungi on the incidence (%) of strawberry and navel orange fruits decay during storage

Chemicals	Concentrations % (w/v)	Inoculation time*	Decay incidence [%]			
			strawberry		navel orange	
			grey mould	soft rot	green mould	blue mould
Calcium chloride	0.5	A	28.0 c	35.0 c	61.0 cd	65.0 c
		B	23.0 cd	28.0 c	45.8 d	45.0 d
	1.0	A	17.0 e	24.0 d	49.5 d	55.0 d
		B	14.0 ef	18.5 e	35.8 d	33.5 ed
	1.5	A	10.2 f	17.0 e	41.0 d	45.0 d
		B	5.0 gh	13.0 f	28.5 e	30.2 e
	2.0	A	10.0 f	12.0 f	30.2 e	34.2 ed
		B	5.0 gh	9.5 h	22.5 f	24.0 e
Hydrogen peroxide	0.5	A	27.0 c	32.0 c	20.0 f	22.0 e
		B	90.0 ab	100.0 a	100.0 a	100.0 a
	1.0	A	17.5 e	25.0 d	14.5 gh	16.2 g
		B	90.0 ab	100.0 a	95.0 ab	90.0 ab
	1.5	A	10.2 f	14.5 f	10.0 hi	14.0 g
		B	80.0 b	90.0 ab	85.5 b	85.0 b
	2.0	A	7.0 g	10.0 gh	10.0 hi	14.0 g
		B	70.0 cd	90.0 ab	70.0 c	80.0 b
Chitosan	0.5	A	30.2 c	41.0 c	22.5 f	28.0 e
		B	18.0 e	33.2 c	22.5 f	23.0 e
	1.0	A	17.0 e	22.0 d	17.0 g	21.0 e
		B	10.0 f	24.5 d	17.0 g	19.5 f
	1.5	A	8.5 g	14.5 f	12.0 h	15.4 g
		B	6.5 g	18.4 e	12.0 h	12.0 h
	2.0	A	7.0 g	14.0 f	11.2 h	14.0 g
		B	4.0 gh	12.4 g	11.2 h	12.5 h
Control			100.0 a	100.0 a	100.0 a	100.0 a

*A: Fruits were inoculated 24 h after chemical treatment, while B: Fruits were inoculated 24 h before chemical treatment

Figures with the same letter are not significantly different (p = 0.05)

Table 4. Effect of calcium chloride or chitosan in combination with hydrogen peroxide on the incidence (%) of strawberry and navel orange fruits decay during storage

Chemicals*	Concentrations % (w/v)	Decay incidence (%)			
		strawberry		navel orange	
		grey mould	soft rot	green mould	blue mould
Calcium chloride	1.5	20.0 b	17.5 b	18.0 b	22.0 b
	2.0	10.0 c	8.0 d	12.5 c	12.0 c
Chitosan	1.5	4.0 d	14.0 c	6.5 d	8.0 d
	2.0	0.0 e	8.0 d	0.0 e	0.0 e
Control	0.0	100.0 a	100.0 a	100.0 a	100.0 a

Figures with the same letter are not significantly different ($p = 0.05$)

*hydrogen peroxide at 2% was applied to all fruit before treatments with either calcium chloride or chitosan

The present study demonstrated that the application of hydrogen peroxide is a superior combination to calcium chloride or chitosan enhanced the control which activity against mould pathogens, this was expressed as either percentage of diseased fruits or decay development as rotted tissue weight of strawberry and navel orange tables 4, 5. The beneficial effect of combining calcium with chitosan may be a result of several different interactions taking place between disinfectant action of hydrogen peroxide, calcium ions, chitosan and the pathogen. The application of 2% chitosan approximately caused a complete reduction of decay infection of strawberry and navel orange, while no more than 12.5% of decayed fruit treated with calcium chloride at the same concentration was observed. The mechanism(s) by which calcium or chitosan reduce infection of citrus wounds is not yet fully understood.

Table 5. Effect of calcium chloride or chitosan in combination with hydrogen peroxide on the decay development [%] of strawberry and navel orange fruits during storage

Chemicals*	Concentrations % (w/v)	Percentage of decay development (rotted tissue part)*			
		strawberry		navel orange	
		grey mould	soft rot	green mould	blue mould
Calcium chloride	1.5	14.0 b	12.5 b	13.5 b	17.2 b
	2.0	8.2 c	6.5 d	9.0 c	7.5 c
Chitosan	1.5	3.0 d	10.5 c	3.0 d	2.0 d
	2.0	0.0 e	4.0 e	0.0 e	0.0 e
Control	0.0	100.0 a	100.0 a	100.0 a	100.0 a

Figures with the same letter are not significantly different ($p = 0.05$)

*hydrogen peroxide at (2% was applied to all fruit before treatments with either calcium chloride or chitosan

The models which have been proposed to explain the anti-fungal activity of chitosan revealed that the activity of chitosan is related to its ability to interfere with the plasma membrane function (Leuba and Stossel 1986) and the interaction with fungal

DNA and RNA (Hadwiger and Loschke 1981). In this respect, Du *et al*. (1997) reported that coating fruits with chitosan reduced the respiration rate, ethylene production, interval O_2 levels and increased the internal CO_2 of peach and pear fruits. They added that coated fruits were markedly firmer and less mature at the end of storage. In an attempt to study the direct effect of calcium on citrus fruit tissue, it was shown that calcium stimulates ethylene production in grapefruit peel. Such an increase in ethylene production in grapefruit peel may signal the activation of defense reactions against pathogens. The involvement of ethylene in the induction of physiological and compositional changes in citrus fruit tissue and increased resistance of fruit to infection has been reported previously (Brown and Barmore 1983; El-Kazzaz *et al.* 1983). The applied tested chemicals might act as contact and systemic fungicides which have protective or therapeutic effect. These chemicals seem to have a broad spectrum of disease control activity. They directly inhibit the fungi on the fruit surface, usually spore germination, so the fungus will not be able to infect the fruit tissues. Moreover, they could interfere with the plant cell induced defense and inhibit the penetrating or invading fungi and disease development as well. In the light of the present findings, it could be suggested that integrated treatment withhydrogen dioxide followed by Chitosan or calcium chloride might be considered as safe commercially method for controlling such postharvest diseases of strawberry and navel orange fruits.

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POLISH SUMMARY

WYKORZYSTANIE ALTERNATYWNYCH ŚRODKÓW (FUNGICYDÓW) ZAPOBIEGAJĄCYCH ZGNILIZNOM OWOCÓW TRUSKAWKI I POMARAŃCZY PO ZBIORACH

Działanie nadtlenku wodoru, chlorku wapnia i chitosanu w zapobieganiu gniciu truskawek i pomarańczy po zbiorze oceniono w warunkach *in vitro* i *in vivo.* Testowane preparaty we wszystkich badanych stężeniach zredukowały wzrost liniowy kultur i kiełkowanie zarodników grzybów: *B. cinerea, R. stolonifer, P. digitatum* i *P. italicum.* Całkowite zahamowanie nastąpiło przy stężeniach 1,5 i 2,0%. Podczas przechowywania obserwowano istotną, zwiększająca się wraz ze wzrostem stężenia testowanych środków, redukcję liczby gnijących truskawek i pomarańczy. Chlorek wapnia i chitosan zastosowane 12 godzin przed inokulacją, istotnie ograniczyły gnicie owoców, natomiast nie powodował tego nadtlenek wodoru. Uzyskane wyniki badań wskazują, że nadtlenek wodoru użyty po zastosowaniu chlorku wapnia lub chitozanu wyraźnie zwiększył aktywność preparatów ograniczających gnicie zarówno truskawek jak i pomarańczy. Zastosowane związki mogą dawać podobny efekt do kontaktowych i systemicznych fungicydów, które działają zapobiegawczo lub leczniczo.