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Using Hot Water and Chitosan for Controlling Green and Blue Moulds of Navel Orange Fruits

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Abstract : Evaluating of hot water and chitosan alone or in combination applied as curative or protective effect on green and blue moulds incidence of navel orange fruits was tested. *In vitro,* studies were carried out on agar disk and spore suspensions of both *P. digitatum* and *P. italicum.* The lethal temperatures to *P. digitatum* and *P. italicum* were 56.0 and 54 °C when were exposed to temperatures for 10 seconds as agar disks or growth suspension respectively. Data also showed that chitosan at different concentrations, *i.e.* 2 , 4 and 6 g / L were effective in reducing the growth of both pathogens. Complete inhibition of linear mycelial growth and spore germination of both pathogens was obtained with chitosan at 6.0 g / L. *In vivo* studies, single applications of hot water and chitosan were applied to artificially inoculated citrus fruits to test their efficacy in the control of *Penicillium digitatum* and *P. italicum*. Treated fruits were stored at 20±2°C° and 90-95% relative humidity for 15 days. Results revealed that hot water and chitosan showed high curative effects against both green and blue moulds. The highest reduction in the disease incidence was obtained with hot water at 60°C for 30 seconds, being 87.5 and 86.0 % reductions for green and blue moulds respectively. Dipping navel orange fruits in hot water plus coating with chitosan showed high protective effects against both green and blue moulds. A remarkable decrease in the incidence of both mould incidence and rotted part tissues was observed when navel orange fruits were dipped in hot water at 60°C for 30 seconds followed by chitosan coating at the concentration of 6.0 g / L , being 88.0 & 86.0 and 90.0 & 88.0 % reduction for both green and blue moulds respectively. Meanwhile, orange fruits dipping in hot water only showed less protective effect against both green and blue moulds.
Key words. Hot water, chitosan , green and blue moulds , navel orange fruits.

Introduction

Penicillium digitatum Pers. Sacc. and *P. italicum* Wehmer, the causal organisms of citrus green and blue mould respectively, are the most important postharvest pathogens and cause serious losses annually [1]. These diseases affecting harvested citrus fruits during handling, transportation, exportation and storage[2,3,4,5,6,7]. The pathogen penetrates the fruit through wounds that are formed during harvesting, improper handling and by insects [8]. The availability of nutrients and moisture in the wounds favour cause the germination of spores[9,10]. In addition, some physiologically induced injuries such as chilling injuries and stem-end rind breakdown can also provide entry for the pathogen into the fruit. Therefore, disease management strategies employed to control the occurrence of green and blue moulds encompass sanitation and application of synthetic fungicides[11,12,13].

There is, however, a growing concern globally, over the continuous use of synthetic chemicals on food crops because of their potential effects on human health and the environment[14,15,16]. Pathogen resistance is

another factor mitigating against the continuous use of synthetic fungicides[17]**.** These concerns have resulted in a renewed interest in the search for alternative control measures.

The use of heat is a method which has been studied for controlling postharvest diseases of several fruits species [18,19,20,21,22,23]**.** Hot water treatment on citrus fruits have been well studied for controlling postharvest green and blue moulds of citrus fruits[1,24].

Chitosan is a linear amino polysaccharide of glucosamine and N-acetylglucosamine units and is obtained by alkaline deacetylation of chitin extracted from the exoskeleton of crustaceans such as shrimps and crabs, as well from the cell walls of some fungi[25]**.** Chitosan is soluble in dilute organic acids, and could theoretically be used as a preservative for coating fruit. The coating is non-toxic and safe[26]**,** and exhibits antifungal activity against several fungi[27,28]**.** A chitosan coating is known to have the potential to prolong the storage life and control the decay of strawberries, tomatoes, peaches, pears, kiwifruit, litchi, apples, longan and citrus fruits, as examined by[7,29,30,31,32,33,34]**.** The aim of this study was to evaluate the efficacy of hot water dipping, either alone, or in combination with chitosan coating for the control of green and blue moulds on artificially inoculated navel orange fruits.

Materials and Methods

Navel orange fruits

The mature navel orange (*Citrus sinensis* L., osbeck) fruits used in the experiment were grown in National Research center NRC orchards, and brought to the laboratory immediately after harvest. They were selected for their uniformity, size, color and shape, and for being free of damage and fungal infection.

Fungal isolates

High virulent isolates of *Penicillium digitatum* and *P. italicum*, the cause of citrus green and blue mould, respectively were obtained from Plant Pathol. Dept., (NRC). The pathogenic isolates were isolated and selected based on their pathogenic ability to navel orange fruits in previous study**.** *Penicillium digitatum* and *P. italicum*, were maintained on potato dextrose agar PDA (potato 20 g ; dextrose 20 g ; Streptomycin 0.03 g and agar 20g in L liter of distilled water) at 25 ± 2°C for two weeks. A spore suspension was obtained by flooding 2-week-old cultures of *P. digitatum* and *P. italicum* with sterile distilled water that contained 0.1% (v/v) Tween 80. Spores were counted using a hemacytometer slid, and the spore concentrations from the pathogens were adjusted with sterile distilled water to obtain 1×10^6 spores per ml.

Hot water treatment

The digital water bath apparatus (Neslab GP-300 Series Constant Temperature Bath, Union City, CA) were used *in vitro* and *in vivo* experiments.

Chitosan treatment

Chitosan powder were purchased from Sigma chemical Co. *In vitro* tests, chitosan were prepared at the concentration of 0.0, 2.0, 4.0 or 6.0 g / L PDA and /or PDB medium. *In vivo* tests, navel orange fruits were coated with the prepared chitosan solutions at the concentration of 0.0, 2.0, 4.0 or 6.0 g / L water. Chitosan were dissolved by dispersed in dilute glacial acetic acid. The pH of the solution was adjusted to pH 5.0 with 0.1 M NaOH. Acid solution without chitosan, pH 5.0, was used as control. All treatments and controls contained 0.1% Tween 80 (Sigma chemical Co.) to improve wettability.

Controlling fungal growth with hot water in vitro

Agar disks (6- mm diameter) and spore suspension (1×10^6 / mL) obtained from two-week-old cultures of both *P. digitatum* and *P. italicum* was subjected to evalute the effect of hot water on the viability of both pathogens according to the method described by [35]**.** Agar disks and/or one mL of spore suspensions were transferred to Screw-cap glass vials, 20 cm long and 20 mm in diameter, containing 20.0 ml sterilized water. Then dipped in hot water of a wide range of temperatures, *i.e.* 25, 50, 52,54,56,58 and 60^{o}C from various exposure times, *i.e.* 10, 20, 30 and 40 seconds. Treated agar disks were dried using sterilized filter paper and

transferred into Petri-plates containing PDA medium. Five Screw-cap glass vials were used for each treatment as well as the control (un-treated agar disks and/or one mL of un-treated spore suspensions). Viability of the pathogens from both agar disks and spore suspensions that had been subjected to previous temperatures with different exposure times was assessed by planting on PDA medium and incubated at 25±2 ºC for 5 days. Plates showing growth (+) or non-growth (-) were recorded and compared with the control plates (un-treated agar disks and/or one mL of un-treated spore suspensions).

Controlling fungal growth with chitosan in vitro

The inhibition by chitosan toward the linear mycelial growth of *P. digitatum* and *P. italicum* were determined on potato dextrose agar (PDA) at 25 ± 2°C according to[36]. The prepared PDA medium was dispersed in 100 ml quantities into 250 ml Erlenmyer flasks and sterilized by autoclaving at 121°C for 15 min. Chitosan were prepared as described previously and then added to PDA medium before its solidification to obtain the final concentrations of 0, 2, 4 and 6 g/L (w/v) and mixed gently with 0.1% Tween 80 (Sigma) to enhance solubility. Each flask was then disbanded in sterilized Petri- plates (9- cm diameter) before its solidification. Plates were individually inoculated with equal disks (6- mm diameter) taken from 7-days old cultures of each *P. digitatum* and *P. italicum*, then incubated at 25 ± 2°C. Linear mycelial growth of fungus was measured, when the control plates reached full growth and the average growth diameter was calculated. Each treatment was represented by 5 plates as replicates.

Meanwhile, the inhibition by chitosan toward the spores germination of both *P. digitatum* and *P. italicum* were determined on potato dextrose broth (PDB) at 25 ± 2°C according to[36]. The prepared potato dextrose broth (PDB) was dispersed in 5 ml quantities into 10 ml test tube and sterilized by autoclaving at 121°C for 15 min. Chitosan were prepared as described previously and then added to PDB to obtain the concentrations of 0, 2, 4 and 6 g/L (w/v) and mixed gently with 0.1% Tween 80 (Sigma) to enhance solubility. Each tube was then inoculated with 1.0 ml of the spore suspension at a concentration of 10^3/ml. Inoculated test tubes were incubated at25 ± 2°C for 20 hours on rotary shaker (200 rpm). Germinated spores were examined microscopically to determine the germination rate[37]**.** Experiment was represented by one handred spores per replicate and five replicates per treatment were used.

Curative effect of hot water and chitosan against green and blue moulds *in vivo*

Navel orange fruits were surface-sterilized with 2% sodium hypochlorite for 2 min at room temperature, rinsed with tap water in order to remove the heavy dirt, pesticides and fungal spores that are covering the fresh harvested produce and allowed to dry at room temperature. Fruits 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* or *P. italicum*, individually then air dried. Inoculated fruits were dipped in hot water of wide range of temperatures, *i.e.* 25, 50, 52,54,56,58 and 60°C from various exposure times, *i.e.* 10, 20 , 30 and 40 seconds.

Meanwhile, other inoculated fruits were dipped in chitosan at the concentations of 2.0, 4.0 and 6.0 g/ L water individually and air dried. A set of inoculated fruits with *P. digitatum* or *P. italicum*, individually only were left as control. Each treatment as well as the control was performed in triplicate. All treated or un-treated (control) fruits were placed into carton boxes at the rate of 10 fruits/box and stored for 15 days at 20±2°C and 90-95% relative humidity for assessment. The fruits were examined regularly to detect mould and regarded as infected if a visible lesion was observed. Results were expressed as percentage of fruit infected.

Protective effect of hot water and chitosan alone or in combination against green and blue moulds *in vivo*

The treatments of dipping navel orange fruits in hot water at 60°C for 30 seconds and chitosan at 6.0 g / L water, were applied alone or in combination to study their protective effect against green and blue moulds. Navel orange fruits were surface-sterilized and wounded as mentioned before. Wounded fruits were subjected to the flowing treatments: (i) dipping in hot water at 60°C for 30 seconds, (ii) caoting with chitosan at the concentration of 6.0 g / L water, (iii) dipping in hot water at 60°C for 30 seconds followed by chitosan at the concentration of 6 g / L water, and (iv) control (untreated fruits). Inoculation of fruits was carried out by spraying fruits with spore suspension (10^6 spores/ml) of *P. digitatum* or *P. italicum*, individually then air dried. Each treatment as well as the control was performed in triplicate. All treated or un-treated (control) fruits were placed into carton boxes at the rate of 10 fruits/box and stored for 15 days at 20±2°C and 90-95% relative

humidity for assessment. The fruits were examined regularly to detect mould and regarded as infected if a visible lesion was observed.

Disease incidence (%) were expressed as percentage of fruit infected, while disease severity (%) were expressed as percentage of rotted part of fruit which was calculated from the following formula:

$$\text{Percentage of rotted part (\%)} = \frac{\text{Rotted part weight of fruit}}{\text{Fruit weight}} \text{ X } 100$$

Statistical analysis

Tukey test for multiple comparison among means was utilized [38].

Results

Effect of hot water treatment on *P. digitatum* and *P. italicum* growth *in vitro*

Effect of hot water on the viability of both *P. digitatum* and *P. italicum* are shown in Table 1. Results indicate that spore suspensions were more sensitive to hot water than agar disks. For agar disk method, at the high exposure time, *i.e.* 30 and 40 seconds, no growth was observed when agar disks of both pathogens was incubated at 52°C. At the medium exposure time, *i.e.* 20 seconds, no growth was observed when agar disks of both pathogens was incubated at 54°C, while at the low exposure time, *i.e.* 10 seconds, no growth was observed when agar disks of both pathogens was incubated at 56°C. For spore suspension method, at the high exposure time, *i.e.* 30 and 40 seconds and at the medium exposure time, *i.e.* 20 seconds, no culturable conidia was observed when conidial suspension of both pathogens was incubated at 52°C. While at the low exposure time, *i.e.* 10 seconds, no conidia was observed when conidial suspension of both pathogen was incubated at 54 and 56°C.

Table 1. Effect of hot water dipping temperatures and exposure times on the viability of agar disk and spore suspention of *P. digitatum* and *P. italicum*.

Hot water (C^o)	Viability of pathogenic fungi*							
	Exposure time (second)							
	P. digitatum				*P. italicum*			
	10	20	30	40	10	20	30	40
Agar disk method								
25	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+
52	+	+	—	—	+	+	—	—
54	+	—	—	—	+	—	—	—
56	—	—	—	—	—	—	—	—
Spore suspension method								
25	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+
52	+	—	—	—	+	—	—	—
54	—	—	—	—	—	—	—	—
56	—	—	—	—	—	—	—	—

* (+) = Indicate growth and (—) = Indicate no growth

Inhibitory effect of chitosan against *P. digitatum* and *P. italicum* growth *in vitro*

The *in vitro* antifungal activities of different concentrations of chitosan are shown in Table 2. Results indicate that all the tested concentrations of chitosan significantly reduced the linear mycelial growth of both *P. digitatum* and *P. italicum*. It is clear that the antifungal activity increased with the increase in concentrations of chitosan. The highest reduction was obtained with chitosan at 4.0 g / L, which reduced the linear mycelial growth by 73.3 and 63.3 for *P. digitatum* and *P. italicum*, respectively. Complete inhibition of linear mycelial growth was obtained with chitosan at 6.0 g / L, while chitosan at 4.0 g / L, showed the less one.

Meanwhile, the effect of PDB amended with different concentrations of chitosan on conidial germination (%) are shown in Table 2. Results indicate that all the tested concentrations of chitosan significantly reduced the germinated spores of both *P. digitatum* and *P. italicum*. With increase in concentration, the efficacy of chitosan was increased, while the complete inhibition of the conidial germination of both pathogens was obtained with chitosan at 6.0 g / L PDA medium.

Table 2. Inhibitory effect of chitosan against *P. digitatum* and *P. italicum* growth *in vitro*.

Chitosan treatment (g/L)	*P. digitatum*		*P. italicum*	
	Linear growth (mm)			
	Growth (mm)	Reduction (%)	Growth (mm)	Reduction (%)
0.0	90.0 a	-	90.0 a	-
2.0	55.4 b	38.4	60.0 b	33.3
4.0	24.0 c	73.3	33.0 c	63.3
6.0	0.0 d	100	0.0 d	100
Spore germination (%)				
	Germination (%)	Reduction (%)	Germination (%)	Reduction (%)
0.0	94.0 a	6.0	92.0 a	8.0
2.0	42.0 b	58.0	49.0 b	51.0
4.0	17.0 c	83.0	25.0 c	75.0
6.0	0.0 d	100	0.0 d	100

Means designated with the same letter in the same column are not significantly different at 0.05 level of probability.

Curative effect of hot water on postharvest decay of navel orange fruits

Curative effect of hot water at a wide range of temperatures *i.e.* 25, 50, 52,54,56,58 and 60^{o}C and various exposure times, *i.e.* 10, 20 , 30 and 40 seconds against green and blue moulds incidence on navel orange are shown in Table 3. Results indicate that all the tested temperatures and exposure times of hot water treatment significantly reduced the disease incidence. With increase in temperature and exposure times, the efficacy of hot water was increased. Complete reduction of green and blue moulds was obtained with hot water at 60^{o}C for 40 seconds. The highest reduction in the disease incidence was obtained with hot water at 60^{o}C for 30 seconds, being 87.5 and 86.0 % reductions for green and blue moulds respectively. Meanwhile, other treatments showed moderate effect. It was noticed that hot water at 60^{o}C for 40 seconds resulted in some damage on orange fruits.

Table 3. Curative effect of hot water temperatures and exposure times against green and blue moulds of navel orange fruits.

Hot water (C^{o})	Disease incidence (%)							
	Exposure time (second)							
	Green mould				Blue mould			
	10	20	30	40	10	20	30	40
25	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a
54	57.0 b	51.0 b	44.0 b	37.0 b	62.4 b	55.0 b	51.0 b	43.0 b
56	52.0 c	44.0 b	38.0 c	30.0 c	59.4 b	52.0 b	47.0 b	38.0 b
58	44.0 d	37.0 d	33.2 c	15.0 d	48.0 c	43.2 c	32.2 c	22.1 c
60	28.0 e	20.1 e	12.5 d	0.0 e	31.0 d	24.0 d	14.0 d	0.0 d

Means designated with the same letter in the same column are not significantly different at 0.05 level of probability.

Curative effect of chitosan on postharvest diseases of navel orange fruits

Table 4 shows the *in vivo* curative effect of different chitosan concentrations in controlling the postharvest decay of navel orange fruits caused by *P. digitatum* and *P. italicum* as disease incidence (%) after 15 d. of inoculation and treatment, when fruit were stored at 20±2^{o}C. Results indicate that all the tested chitosan concentrations reduced significantly postharvest decay of navel orange fruits. With increase in chitosan

concentrations, the efficacy was increased. After storage period, 100% of control fruit developed green and blue moulds in navel orange fruits. It can be noticed that chitosan inhibited fungal decays by 59.0 and 61.6 % for blue and green moulds, respectively, when used at the concentrations of 6.0 g/ L water.

Table 4. Curative effect of different chitosan concentrations against green and blue moulds of navel orange fruits.

Chitosan coating (g/L)	Postharvest diseases of navel orange fruits			
	Blue mould		Green mould	
	Disease incidence (%)	Reduction (%)	Disease incidence (%)	Reduction (%)
0.0	100 a	-	100 a	-
2.0	62.1 b	37.8	67.2 b	32.8
4.0	50.0 c	50.0	47.0 c	53.0
6.0	41.0 d	59.0	38.4 d	61.6

Means designated with the same letter in the same column are not significantly different at 0.05 level of probability.

Protective effect of hot water and chitosan alone or in combination on postharvest diseases of navel orange fruits

Table 5 shows the *in vivo* protective effect of hot water and chitosan alone or in combination in controlling the postharvest decay of navel orange fruits caused by *P. digitatum* and *P. italicum* as disease incidence (%) and disease severity (%) after 15 d. of treatment and inoculation, when fruit were stored at 20±2^{o}C. Results indicate that all treatments reduced significantly postharvest decay incidence (%) and severity (%) of navel orange fruits compared to the control. After storage period, 100% of control fruit developed green and blue moulds in navel orange fruits. It was noticed that, the highest reduction in postharvest decay of navel orange fruits was obtained with combined treatments of dipping in hot water at 60^{o}C for 30s followed by chitosan caoting at 6 g / L water, which reduced the disease incidence and disease severity by 88.0 and 86.0 and 90.0 & 88.0 % for green and blue moulds, respectively. Single treatments of chitosan coating reduced the disease incidence and disease severity by 72.0 & 69.6 % and 76.5 & 75.5 % for green and blue moulds, respectively. Meanwhile, single treatment of hot water was less protective effect.

Table 5. Protective effect of hot water and chitosan alone or in combination on postharvest diseases of navel orange fruits.

Treatment condition	Postharvest diseases of navel orange fruits*			
	Disease incidence (%)		Disease severity (%)	
	Blue mould	Green mould	Blue mould	Green mould
Control	100 a	100 a	100 a	100 a
Hot water at 60^{o}C for 30 s	70.0 b	78.3 b	66.0 b	72.0 b
Chitosan coating at 6.0g/L	28.0 c	30.4 c	23.5 c	24.5 c
Hot water at 60^{o}C for 30 s + chitosan at 6 g /L	12.0 d	14.0 d	10.0 d	12.0 d

Means designated with the same letter in the same column are not significantly different at 0.05 level of probability.

Discussion

Results obtained from the present study indicate that hot water and chitosan have a significant effect on the growth (*in vitro* and *in vivo*) of both *P. digitatum* and *P. italicum*. *In vitro* studies, the growth of agar disks of both pathogens were completely inhibited when exposed to hot water at 56.0^{o}C for 10 and/or seeded on PDA medium amended with chitosan at 6.0 g / L. Also, conidial suspensions of both pathogens exposed to the same conditions were not capable of further growth when plated on PDA, thus indicating that the treatments affected

both mycelial growth and spores germination and further pathogens development. *In vivo* studies, the present study revealed that hot water at 60°C for 30 seconds and/or chitosan at 6 g/ L, showed high curative effects against postharvest decay on navel orange, being 87.5 & 86.0 % and 59.0 & 61.6 %, reduction in the incidence of blue and green moulds, respectively. This finding agrees with earlier [36,39,40] and recent reports [7,22,41] on the suppressive and antifungal properties of hot water and chitosan. Heat treatment directly affect on hyphae and spores by retarding germ tube growth, reducing activity or completely killing germinating spore, thus reducing the rate of infection effective inoculums[24]. A longer dipping time of hot water were required to achieve a reduction in fungal growth in the fruit tissues than in agar. This suggests that the effective hot water temperature or dipping time to which the fungus is exposed is much lower than the dipping time applied to the fruit surface and high lights the penetration of pathogen in first few layer of living fruit peel tissues. The effect of hot water on blue and green moulds is basically due to reduction in the viability of fungi spores. Heat may also reduce pathogen growth by inducing resistance mechanisms in the outer layers of epicarp [40] This is due to the enhanced formation of lignin, which is a related compound that prevents invasion by mould spores. Hot water brushing HWB treatment at 62°C for 20 sec induced resistance against *P. digitatum* in 'Star Ruby' grapefruit. The main factor responsible for the induction of disease resistance by the HWB treatment was the exposure to the high temperature, since rinsing and brushing the fruit with tap water (approximately 20°C) or with hot water at 53°C did not affect the percentage of decay development nor the rot diameter in the infected wounds. HWB using heated water induced the accumulation of heat shock and pathogenesis-related proteins, which were not observed in HWB using unheated tap water. On the other hand, several hypotheses have been postulated by which chitosan affects the growth of pathogenic fungi [42] first: by 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. 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.

However, in the present study, protective effect of hot water were evaluated alone or in combination with chitosan coating. Protective effect of chitosan coating were more effective in inhibiting *P. italicum* and *P. digitatum* than hot water. A remarkable improvement in suppressive and antifungal activity was observed when hot water treatment were combined with chitosan coating. As a result, the treatment comprising of hot water at 60°C for 30 seconds followed by chitosan coating with the concentration of 6g/L water, was more effective in controlling both green and blue moulds than single treatment. The reason(s) for this observation is well understood. The killing of pathogenic fungal spores by heat treatment and the stronger fungicidal effect of chitosan coating [43]**.** These results are concurrence with the work of[44], they reported that combination of hot water and chitosan treatments is a valid strategy to improve the existing ones already used in controlling postharvest decay of sweet cherries. The advantage of hot water dipping is that it can control surface infections as well as infections that have penetrated the skin, without leaving no chemical residues on the fruits[45]**.** Postharvest heat treatment also can reduce chilling injury in many wounds of fruits during subsequent low temperature storage as well as reduce pathogens level and disease development. The beneficial effect of hot water may be enhanced by following with chemical and biological treatments (**Francesco and Mari, 2014).** Hot water may be supplied to fruits in many ways: by hot water dips, vapor heat, hot dry air or by hot water rinsing and brushing[40]. Treatment with hot water has become increasingly accepted commercially, and significant improvement has been made with the addition of brushing [39]**.**

Controlling of plant diseases depends mainly on fungicides application such chemicals are not always desirable due to potential hazards to human beings and the environment. Alternative approaches to fungicides are needed for controlling plant diseases [46,47,48,49,50,51,52,53, 54, 55, 56].

It could be suggested from the present study that combination treatment between hot water dipping and chitosan as fruit coating considered as one of applicable safely for controlling postharvest diseases of navel orange fruits.

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