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Antifungal activity of a chitosan and polyvinyl alcohol (PVA) mixture on damage-causing strains isolated from postharvest oranges grown in Vietnam

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Abstract

The identification and development of a biofilm that is highly efficacious against disease-causing microorganisms in postharvest fruits are pivotal in ensuring the stability and security of agricultural supply chains. The objectives of the present work were to assess the antifungal ability of a chitosan and polyvinyl alcohol (PVA) mixture on postharvest oranges using the agar-well diffusion method, and to determine the minimum inhibitory concentration of the mixture. Four fungal species responsible for causing damage and rot in oranges were isolated from oranges namely *Penicillium* sp., *Aspergillus niger*, *Rhizopus delemar*, and *Colletotrichum* sp. Overall, the chitosan and PVA combination showed antifungal activity against the four strains, but the composition at which inhibition was maximised depended on the fungal species tested. The minimum inhibitory concentration of the chitosan/PVA mixture against the four isolated fungal strains was 1.15% chitosan + 0.39% PVA, 0.83% chitosan + 0.56% PVA, 1.1% chitosan + 0.37% PVA, and 0.41% chitosan + 0.41% PVA for *Penicillium* sp., *A. niger, R. delemar*, and *Colletotrichum* sp., respectively. These results are expected to aid in further developments on the management of postharvest spoilage during storage of fruits and vegetables.

Introduction

Orange is a tropical / subtropical fruit belongs to the Rutaceae family. Orange is non-climacteric, thus having reduced respiration and ethylene production during its ripening period. Orange is also susceptible to physiological disorders and weight loss under unsuitable storage conditions, both of which have been shown to be linked to the dehydration of the fruit (Grierson and Miller, 2006). In addition, the quality and shelf life of postharvest oranges can be adversely affected by a number of pathogens including *Lasiodiplodia theobromae, Phomopsis citri, Alternaria citri, Botrytis cinerea, Colletotrichum musae*, and *Phytophthora citrophthora* (Eckert and Eaks, 1989; Snowdon, 1990). Most notably, *Penicillium digitatum* and *P. italicum* are fungal species that are capable of causing orange to rot through wound infection after 7 - 10 d of storage at 20 - 25°C (Palou, 2014). Thus, prevention of fungal spoilage in oranges has been extensively studied and approached with different strategies.

Chitosan is a biological polymer that possesses a wide range of beneficial properties including ease of dissolution, safety, and eco-friendliness; and has been shown to exert antibacterial and antifungal effects (Rabea *et al.,* 2003; Cagri *et al.,* 2004). Chitosan has been widely applied in the manufacturing industry to prolong the storage time of food products (Vasilatos and Savvaidis, 2013), and can be easily combined with other polymers, thus resulting in biofilms with improved mechanical properties and bioactivity (Bano *et al.,* 2014). Previous studies have attempted to combine chitosan with other materials such as polyvinyl alcohol (PVA; Kanatt *et al.,* 2012), polyethylene terephthalate (PET; Torres-Huerta *et al.,* 2014), starch (Liu *et al.,* 2009; Kowalczyk *et al.,* 2015), and wheat gluten (Chen *et al.,* 2014) to produce composite films that can prevent bacterial and fungal growth (El-Salmawi, 2007; Sung *et al.,* 2010). Among them, PVA is a synthetic polymer which has good water solubility and mechanical properties, high biocompatibility, low toxicity, and ease of preparation (Park *et al.,* 2001; Silva *et al.,* 2013).

The antifungal and antibacterial properties of chitosan combinational biofilms such as poly (acid

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lactic)/starch/chitosan (Bie *et al.,* 2013) and chitosan/poly (vinyl alcohol)/pectin (Tripathi *et al.,* 2010) have been evaluated against foodborne pathogens. In addition, many other biofilms such as LDPE/chitosan have also been investigated for their capability to prolong storage time and to improve red meat sliceability and antibacterial properties against *L. monocytogenes, Escherichia coli*, and *Salmonella* Enteritidis (Park *et al.,* 2010). Davidovich-Pinhas *et al.* (2014) showed that chitosan, when combined in a biofilm with either low-density polyethylene (PE) or polyethylene glycol (PEG) can efficiently inhibit the growth of *Bacillus subtilis* and *E. coli*. In another study, the combination of plasma-treated polyethylene terephthalate/polypropylene (PET/PP) and chitosan yielded a biofilm that completely inhibited the growth of *B. subtilis* and *E. coli*, and killed most of *Staphylococcus aureus* at the ratio of 85% (Lei *et al.,* 2014). Despite the advantages of applying chitosan as food preservative agent, little information about the antifungal efficiency of chitosan in combination with PVA has been reported so far. In the present work, we carried out a survey and assessment of the antifungal efficiency of a chitosan and PVA mixture on postharvest oranges during storage. From the results, we proposed a measure for controlling fungus-derived damage of oranges during postharvest which greatly contributes to further developments on extending the shelf life of postharvest agricultural produce.

Materials and methods

Isolation of fungi causing damage on postharvest oranges

Oranges (*Citrus sinensis* L. Osbeck cultivar) were harvested at a mature green stage from a garden in Dong Thap Province, Vietnam, packaged in plastic bags, and stored at 30 ± 2°C at 80 ± 5% RH. After disease symptoms were clearly visible, the infected part was cut out and immersed in sodium hypochloride solution for 2 min. The samples were then washed with distilled water, and immersed in 75% ethanol solution for 30 s. Following drying for 15 min, the samples were cut by the lamella, and placed in Petri plates containing PDA growth medium. The plates were incubated for 3 w to allow disease development. The morphology and spore formation of the pure fungal cultures in PDA growth medium were observed using a light microscope.

Assessment of in vitro antifungal properties of chitosan/PVA mixture on postharvest oranges

The experiment was performed by using a Response Surface Method (RSM) utilising the Central Composite Design (CCD) according to Kanatt *et al.* (2012), with chitosan, PVA, and chitosan/PVA ratios in the ranges 0.5 - 1.5, 1 - 5, and 1 - 2%, respectively. The experimental treatments are shown in Table 1.

Chitosan at concentrations of 0.5, 1, and 1.5% (w/v) was prepared by dissolving it in 1% acetic acid (v/v) at room temperature under stirring at 500 rpm.

Table 1. Experimental treatments.

Treatment	Pattern	Chitosan (%, w/v)	PVA (%, w/v)	Chitosan/PVA ratio
1	0A0	1	5	1.5
2	000	1	3	1.5
3	++−	1.5	5	1
4	+−+	1.5	1	2
5	a00	0.5	3	1.5
6	+−−	1.5	1	1
7	000	1	3	1.5
8	+−+	1.5	1	2
9	00a	1	3	1
10	−−−	0.5	1	1
11	A00	1.5	3	1.5
12	−−+	0.5	1	2
13	00A	1	3	2
14	−+−	0.5	5	1
15	−++	0.5	5	2
16	0a0	1	1	1.5
17	Control	0	0	-

PVA (1, 3, and 5%, w/v) was prepared by dissolving it in boiling water at 90°C, also under stirring at 500 rpm. Chitosan and PVA were mixed at different ratios (1 : 1, 1 : 1.5, and 1 : 2), and 0.1% glycerol (w/v) was added to the mixtures as a pliable substance for films (adjusted pH = 5.5), and stirred (200 rpm) at room temperature for 15 min to remove gas / bubble in the mixture (Bonilla *et al.,* 2014). The mixtures of chitosan and PVA were added to PDA growth medium to evaluate the antifungal efficiency by using a perforated agar method. The fungal cultures were placed in the middle of the Petri plate, followed by incubation at 32°C, and observation of the fungal growth after 5 - 7 d. Distilled water was used as the negative control. The diameter of the fungi formed was determined using the formula: $\Delta A = A - a$, where A was the diameter of the fungi formed (mm), and a was the diameter of the perforated agar (mm). Finally, antifungal efficiency (H%) was calculated by the agar-well diffusion method (Chand, 2013) using the formula: H (%) = 100 × [(diameter of fungi formed on the control plate – diameter of fungi formed on the tested plate)] / diameter of fungi formed on the control plate (Zygadlo *et al.,* 1994; Elshafie *et al.,* 2018). The experiments were performed in triplicate (15 plates for each fungal species).

Determination of minimum inhibitory concentration (MIC 90%) of chitosan/PVA mixture in vitro

After determining the chitosan and PVA mixture that exhibited the highest antifungal efficiency, the MIC assay was performed for each fungal strain. This mixture of chitosan and PVA was previously prepared, and then increased by up to 10% for the MIC assay. The experiment was also carried out by using the perforated agar method, followed by 5-d or 7-d incubation (depending on fungal species) at 32°C. MIC values were defined as the lowest sample concentration that could inhibit fungal growth. The experiments were performed in triplicate (15 plates for each fungal species) in a completely randomised factorial design (CRD).

Statistical analysis

All data were analysed using JMP 10.0 software (SAS Institute Inc., Cary, NC, USA). Significant differences between treatments were shown through the Duncan test at 95% confidence level ($p < 0.05$).

Results and discussion

Isolation of fungi that caused damage to postharvest oranges

In the present work, four fungal species were isolated from the decayed oranges namely *Penicillium* sp., *Aspergillus niger, Rhizopus delemar*, and *Colletotrichum* sp. The characteristics of the isolated fungal species were then observed on PDA growth media. *R. delemar* is a fibrillar fungus that exhibited flaked form, rapid growth, and eventually turned from white to grey, forming small grey-black punctiform

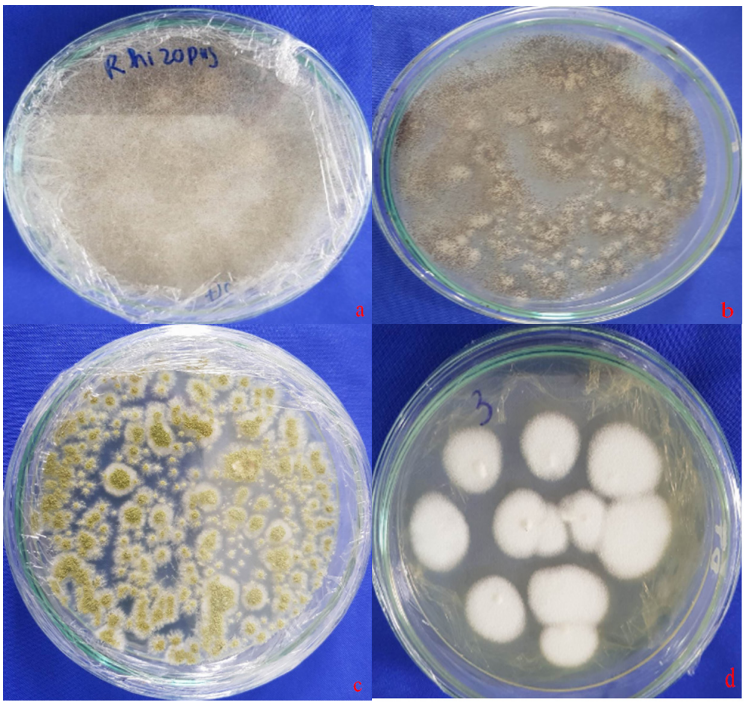

Figure 1. Fungal species isolated from processing-damaged oranges in Potato Dextrose Agar growth medium; (a) *Rhizopus delemar*, (b) *Aspergillus niger*, (c) *Penicillium* sp., and (d) *Colletotrichum* sp.

colonies (Figure 1a). *A. niger* is a hyphate fungus that was white initially, and then turned black, and eventually formed black spots (Figure 1b). *Penicillium* sp. is a hyphate fungus with a distinctive green colour (Figure 1c). *Colletotrichum* sp. is a white fungus with short hyphae (Figure 1d).

In vitro assessment of antifungal efficiency of chitosan/PVA mixture against damage-causing fungi in postharvest oranges

Chitosan and PVA mixtures were evaluated for their *in vitro* antifungal activity in PDA growth medium. Overall, all mixtures tested exhibited significant inhibitory action against *R. delemar* as compared to the control (Table 2). The highest antifungal efficiency (88.41%) with an inhibitory zone diameter of 9.27 ± 0.09 mm was obtained in Treatment 4 (1.5% chitosan and 1% PVA at a ratio of 2 : 1). This result was followed by those for Treatments 3, 8, and 9 with antifungal efficiency of 86.64, 88.33, and 88.40%, respectively.

Against *A. niger*, Treatments 4, 8, 11, and 13 resulted in 100% inhibition, while Treatment 17 (control) was unable to inhibit the fungus. In the case of *Penicillium* sp., no treatment was found to completely inhibit the fungal growth, and only Treatments 4 and 8 showed high and statistically similar antifungal efficiencies with diameters of 7.61 ± 0.09 and 7.51 ± 0.09 mm, respectively. This was contrasted by the control with having no activity and the largest diameter of 67.13 ± 1.43 mm. On the other hand, *Colletotrichum* sp. seemed to be most susceptible to many of the formulated treatments. To be specific, similar to the results for *Penicillium* sp., Treatments 4 and 8 also completely inhibited *Colletotrichum* sp. in addition to Treatments 6, 2, 7, 11, 13, and 16. The inhibition zone of the control against *Colletotrichum* sp. was 21.69 ± 0.38 mm in diameter, which was indicative of no inhibitory action against the control.

According to El-Salmawi (2007) and Sung *et al.* (2010), the combination of PVA and chitosan could deter the growth of microorganisms. However, it is chitosan that confers the mixture its potent antimicrobial ability, and a high concentration of PVA was found to play the role of improving the mixture's

Table 2. Antifungal efficiency of chitosan/PVA mixture against fungal strains that cause damage in postharvest oranges.

Treatments	*R. delemar*		*A. niger*		*Penicillium* sp.		*Colletotrichum* sp.	
	Diameter of fungal growth (mm)	Antifungal efficiency (%)	Diameter of fungal growth (mm)	Antifungal efficiency (%)	Diameter of fungal growth (mm)	Antifungal efficiency (%)	Diameter of fungal growth (mm)	Antifungal efficiency (%)
1	17.85 ± 0.18^{ef}	77.69	8.62 ± 0.35^{e}	89.22	13.68 ± 0.57^{e}	79.62	6.55 ± 0.10^{c}	69.80
2	16.32 ± 0.31^{e}	79.60	7.40 ± 0.11^{d}	90.75	10.86 ± 0.56^{cd}	83.82	++	100
3	10.69 ± 0.28^{ab}	86.64	6.36 ± 0.45^{c}	92.05	11.81 ± 0.36^{d}	82.41	7.89 ± 0.34^{d}	63.62
4	9.27 ± 0.09^{a}	88.41	++	100	7.61 ± 0.09^{a}	88.66	++	100
5	27.29 ± 0.32^{gh}	65.89	15.75 ± 0.46^{h}	80.31	27.54 ± 0.52^{i}	58.98	10.29 ± 0.22^{e}	52.56
6	11.75 ± 0.34^{bc}	85.31	4.56 ± 0.61^{b}	94.30	9.71 ± 0.18^{bc}	85.54	++	100
7	16.44 ± 0.47^{ef}	79.45	5.52 ± 0.49^{bc}	93.10	11.06 ± 0.20^{cd}	83.52	++	100
8	9.34 ± 0.18^{a}	88.33	++	100	7.51 ± 0.09^{a}	88.81	++	100
9	18.57 ± 0.09^{f}	76.79	8.66 ± 0.47^{e}	89.18	13.48 ± 0.65^{e}	79.92	8.33 ± 0.23^{d}	61.60
10	30.53 ± 0.40^{i}	61.84	13.55 ± 0.41^{g}	83.06	18.31 ± 0.32^{g}	72.72	12.39 ± 0.37^{f}	42.88
11	9.28 ± 0.31^{a}	88.40	++	100	8.34 ± 0.46^{ab}	87.58	++	100
12	26.36 ± 0.37^{g}	67.05	6.52 ± 0.20^{cd}	91.85	15.47 ± 0.22^{f}	76.96	7.66 ± 0.34^{d}	64.68
13	13.82 ± 0.27^{cd}	82.73	++	100	9.46 ± 0.22^{bc}	85.91	++	100
14	29.48 ± 0.53^{hi}	63.15	18.49 ± 0.39^{i}	76.89	29.73 ± 0.45^{j}	55.71	15.58 ± 0.59^{g}	28.17
15	26.23 ± 2.74^{g}	67.21	10.35 ± 0.21^{f}	87.06	20.77 ± 0.30^{h}	69.06	12.87 ± 0.29^{f}	40.66
16	15.65 ± 0.40^{de}	80.44	4.73 ± 0.37^{b}	94.09	9.87 ± 0.79^{bc}	85.30	++	100
17	80.00 ± 0.00^{j}	0	80.00 ± 0.00^{j}	0	67.13 ± 1.43^{k}	0	21.69 ± 0.38^{h}	0

Means followed by similar superscript letter within a column are not significantly different at confidence interval 95%; ++ = complete inhibition.

mechanical properties and gelation. The current results are also in line with a previous study where chitosan was found to inhibit *Penicillium* sp., *Aspergillus* sp., *Rhizopus* sp., and *Colletotrichum* sp. (Cota-Arriola *et al.,* 2011).

A higher chitosan concentration and mixing ratio seemed to be associated with improved antifungal results possibly due to the known antifungal ability of chitosan, and the encapsulation effect of PVA (El-Salmawi, 2007). To be specific, when increasing the PVA concentration, chitosan molecules can be covered by excess PVA, thus deterring the contact of chitosan with microorganisms, and reducing the antifungal efficiency of chitosan and PVA compounds.

Determination of MIC of chitosan and PVA compounds in vitro

In this assay, we determined the MIC against each fungal strain by selecting one treatment that showed the greatest inhibition but lower than 100% against that strain. We selected Treatment 4 (1% chitosan and 0.33% PVA) to determine the MIC of the mixture of chitosan and PVA against *R. delemar.* Increasing the concentration of the two compounds by 10% (1.1% chitosan and 0.37% PVA) resulted in complete inhibition of fungal growth. Hence, the MIC against *R. delemar* was 1.1% chitosan and 0.37% PVA (Figures 2a and 2b).

For *A. niger*, Treatment 6 consisting of 0.75% chitosan and 0.5% PVA was selected to study the MIC. Zero growth of *A. niger* was observed on the application of the mixture that contained 0.83% chitosan and 0.56% PVA, which was 10% higher than used in Treatment 6. Therefore, the MIC against *A. niger* was 0.83% chitosan and 0.56% PVA (Figures 2c and 2d).

Against *Penicillium* sp., Treatment 8 containing 1% chitosan and 0.33% PVA was selected for the *in vitro* MIC assay. Complete fungal inhibition was observed for the mixture consisting of 1.15% chitosan and 0.39% PVA. This suggests that the MIC against *Penicillium* sp. was 1.15% chitosan and 0.39% PVA, which was 15% higher than the concentrations used in Treatment 8 (Figures 3a and 3b).

Against *Colletotrichum* sp., Treatment 12 containing 0.33% chitosan and 0.33% PVA was used to determine the MIC of chitosan and PVA compounds. It was found that increasing the chitosan and PVA concentrations of Treatment 12 by 20% yielded a mixture that was capable of completely inhibiting *Colletotrichum* sp. growth. Therefore, the MIC against this strain was 0.41% chitosan and 0.41% PVA (Figures 3c and 3d).

According to Ahmed *et al.* (2017), the MIC of chitosan against *A. niger*, *Penicillium* sp., and *Rhizopus* sp. was 0.35%. In the present work, chitosan was used in combination with PVA, hence a higher

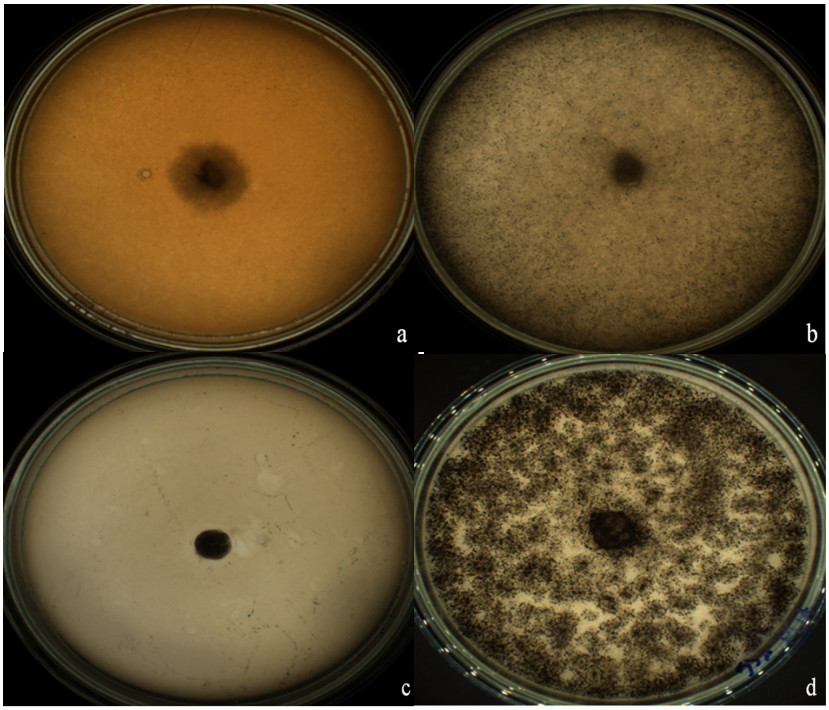

Figure 2. Antifungal ability of chitosan/PVA mixture against *Rhizopus delemar* with (a) Treatment 4, and (b) the control; *Aspergillus niger* with (c) Treatment 6, and (d) the control, after 5 d at 32°C.

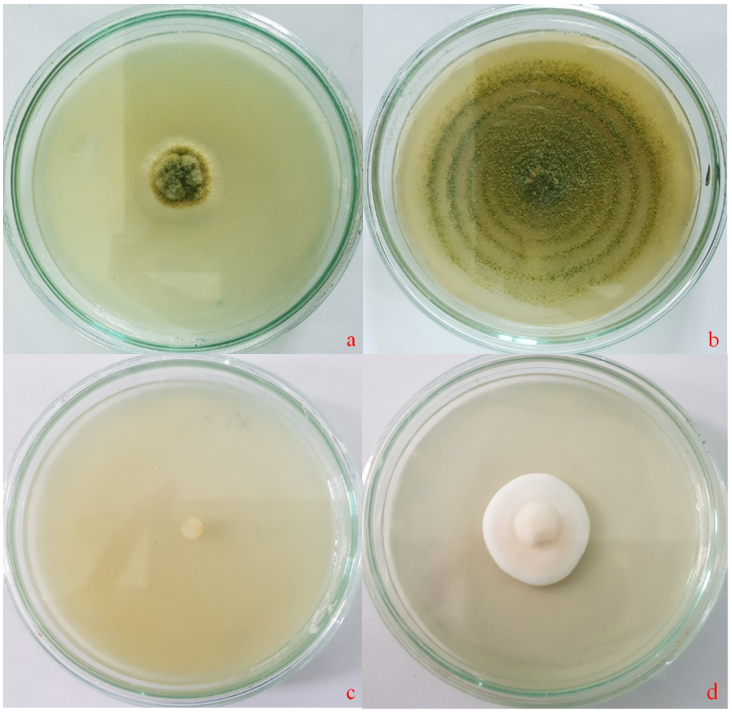

Figure 3. Antifungal ability of chitosan/PVA mixture against *Penicillium* sp. with (a) Treatment 8, and (b) the control; *Colletotrichum* sp. with (c) Treatment 12, and (d) the control, after 7 d at 32°C.

concentration of chitosan was required to inhibit the fungal strains. In addition, the presence of PVA might encapsulate chitosan molecules, thus reducing the contact between chitosan and the microorganism, which in turn reduces the antifungal efficiency of the mixture. The higher MIC could also be attributable to differences in the molecular weight of chitosan, which has been shown to affect its antimicrobial ability. Another explanation is that fungal strains isolated from different sources also have different levels of sensitivity to chitosan (Junior, 2016).

Conclusions

In the present work, we determined and isolated four fungal strains that cause diseases in postharvest oranges during storage namely *Penicillium* sp., *A. niger, R. delemar*, and *Colletotrichum* sp. Chitosan/PVA mixtures were studied as potential biofilms to inhibit the growth of the four strains. The MICs of chitosan/PVA mixtures against four isolated fungal species were also determined. Against *Penicillium* sp., *A. niger, R. delemar*, and *Colletotrichum* sp., the MICs of chitosan and PVA in the mixture were 1.15% chitosan + 0.39% PVA, 0.83% chitosan + 0.56% PVA, 1.1% chitosan + 0.37% PVA, and 0.41% chitosan + 0.41% PVA, respectively. The results substantiate the use of chitosan/PVA mixtures for extending the shelf life of postharvest oranges and provide insight for further developments of preventive measures against rot diseases in fruits.

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