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Postharvest Control of Orange Green Mold caused by *Penicillium digitatum* using Chitosan and Salicylic acid

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A B S T R A C T

In this study, *Penicillium digitatum* was isolated and identified as the causative agent of green mold disease in orange fruits based on its growth and morphological characteristics. Inhibition results showed that chitosan and salicylic acid exerted an inhibitory effect on the growth of *P.digitatum*. Chitosan achieved complete inhibition at 1000 mg.L^{-1}, while salicylic acid achieved 55% inhibition at a concentration of 500 mg.L^{-1}. Treatments with chitosan and salicylic acid also reduced the incidence, disease severity, and mold area of stored fruit compared to untreated fruit. Furthermore, these treatments contributed to improved fruit quality during storage, reducing weight loss and increasing vitamin C and total soluble solids content TSS . After 96 hours, peroxidase activity increased to 95.83 and 1.46 units in chitosan treatments and to 96.59 and 1.69 units, respectively, in salicylic acid treatments.It also increased the accumulation of total phenols to 16.4 and 16.3 mg.g.fw^{-1} in chitosan and salicylic acid treatments, respectively. These results indicate the effectiveness of chitosan and salicylic acid as antifungal agents and improve the resistance of orange fruits during storage, enhancing their potential as safe and environmentally friendly alternatives for controlling postharvest diseases.

Introduction

Citrus fruits represent one of the most extensively cultivated and economically significant fruits worldwide, owing to their rich nutritional value and diverse uses. Industry [1]. Citrus fruits are particularly appreciated for their rich content of health-promoting compounds including vitamin C, ascorbic acid, flavonoids, Carotenoids essential oils, antioxidants, essential nutrients and minerals. [2]. Postharvest diseases, pose a major threat to citrus production with green mold caused by *Penicillium digitatum*, being one of the most destructive, particularly in oranges and lemons [3]. The pathogen invades fruit through wounds leading to rapid decay and substantial economic losses during storage and transportation**.** *P. digitatum* is characterized by the formation of velvety green colonies accomplished by a strong, unpleasant odor. Infection begins when pathogen spores adhere to the fruit surface, germinate, and penetrate host tissues [4]. The pathogen then secretes cell wall-degrading enzymes such as pectinase and cellulase, which break down plant cell wall components, resulting in tissue maceration and progressive fruit mold.[5]. Infection occurs when pathogen spores adhere to the fruit surface, then germinate, penetrate the host tissues, The pathogen then secrete cell wall degradative enzymes such as pectinase and cellulase, resulting in tissue maceration and progressive fruit mold . [6]. Effective control of green mold requires an integrated approach that includes proper agricultural practices, including careful handling of fruit during harvest and maintaining optimal storage conditions in terms of hygiene, temperature, and humidity. Although Chemical fungicide such as Imazalil and Thiabendazole are commonly employed in postharvest treatments, their excessive use has contributed to development of fungicide resistance strains of *P. digitatum,* raising significant concerns regarding human health and environmental safety. [7]. As a result, Promising alternatives have emerged, such as biological control methods and the application of natural resistance-inducing compounds. Among these, Chitosan, a naturally occurring, positively charged polymer derived from chitin has exhibits antimicrobial activity through electrostatic interactions that disrupt the integrity of cell membranes.[8].It also stimulates plant defenses such as defensive enzymes and phenolic compounds. Chitosan has promising efficacy in reducing the development of green mold disease, reducing disease incidence and severity in grapefruit and mandarin. [9].

The efficacy of chitosan is related to its concentration. Concentrations exceeding 0.1% were sufficient to inhibit pathogen growth, while a 0.5% concentration significantly reduced disease development in Washington Navel, Valencia, Femminello, and Marsh Seedless orange varieties. [10, 11]. Chitosan treatments also enhanced plant defense mechanisms by increasing the activity of chitinase and β-1,3-glucanase, and raising levels of antioxidants SOD, POD, H_2O_2, and GSH, while inhibiting catalase activity and maintaining ascorbic acid content. In navel oranges, immersing the fruit in a 2% chitosan solution in 0.5% acetic acid reduced green and blue mold infection and stimulated the fruit's defenses. Chitosan films supplemented with lemon peel extracts (AntiFun-LM) showed high inhibition capacity against pathogen.[12,13]. Salicylic acid is a phenolic compound characterized by a benzene ring bearing both hydroxyl and carboxyl functional group, these structure features confer acidic and lipophilic properties that enable it to penetrate plant tissues and interact with their cellular components.SA exists in plants either as freeform or as conjugated compounds, stored and released as needed to perform vital physiological functions, including defense responses and growth regulation. SA plays a central role in activating systemic acquired resistance (SAR), which increases the plant's overall defense against pathogens, particularly *P. digitatum* and *P. italicum*, which cause green and blue mold in citrus.[14, 15,16]. This resistance occurs by stimulating defense enzymes such as chitinase and Glucanases. Treating fruit with salicylic acid at concentrations ranging from 0.5 to 16 mM effectively inhibits *P. digitatum* growth. It causes structural changes in the fungus, including cell wall rupture, cytoplasmic shrinkage, and leakage of cytoplasmic components. This reduces green mold and preserves fruit quality. [17]

Materials and Methods

Isolation of pathogenic fungi

The fungus was isolated from orange fruits infected with green mold. The infected tissue was cut and sterilized with 5% sodium hypochlorite for 2 minutes, then washed with sterile distilled water. The pieces were placed on filter paper and transferred to Petri dishes containing PDA medium with 200 mg/L chloramphenicol, five pieces per dish, and incubated at 25°C for 7 days. Colonies were purified into fresh medium and identified according to [18] .

The effect of salicylic acid on the mycelial growth of *P.digitatum* .

The following concentrations of the salicylic acid were used:0, 5, 10, 50, 500, and 1000 mg/L, incorporated into PDA supplemented with 200 mg/L Chloramphenicol. After the medium

solidified, agar plugs (5 mm in diameter) taken from 7-day-old *P. digitatum* culture, were placed at the center of the plates. The plates were then incubated at 27°C for 7 days. After incubation the colony areas of *P. digdigitatum* were measured, and the percentage of growth inhibition was calculated.:

The *P. digitatum* growth inhibition rate (IR) was calculated as follows:

IR = [C2 - C1 / C2] × 100, where C2 is the area of the control colony and C1 is the area of the *P. digitatum* colony in the presence of salicylic acid .The experiment included four replicates. [19]

The effect of chitosan on the mycelial growth of *P. digitatum*.

The effect of chitosan on the mycelial growth of *P. digitatum* was evaluated using concentrations of 0, 5, 10, 500, and 1000 mg. L^{-1}, following the same procedure applied above

Storage Experiment

Sound orange fruits free of wounds and physical damage were selected. fruits were first washed with running water, then treated with an 8% sodium carbonate solution, followed by a water rinsing tap water. Surface sterilized was carried out by immersion in 70% ethyl alcohol for two minutes, then placed on sterile filter paper and allowed to dry at room temperature.

Each fruit was wounded at four equidistant points using a sterile inoculating needle to a depth of 3 mm. The experiment included four replicates, each consisting of four fruits. The following treatments were applied:

The following treatments were applied:

1. Negative control treatment: The fruits were immersed in sterile distilled water for 15 minutes.
2. Positive control treatment: The fruits were inoculated only with *P. digitatum* spore suspension.
3. Chitosan treatment: The fruits were immersed in a 500 mg/L chitosan solution for 15 minutes. according to [19]
4. Salicylic acid treatment: The fruits were immersed in a salicylic acid solution (100 mg/L) for 15 minutes.

After the treatments, fruits in the inoculated groups were subjected to fungal inoculation by applying 30 µL of P. digitatum spore suspension at a concentration of 2×1052 x 10^5 spores/ml. The fruits were stored at 5°C for 10 days.

The following parameters were calculated: disease incident and severity according to [19], fruit weight loss, and total soluble solids (TSS) content, which was measured in fruit juice using a refractometer according to [20] . Ascorbic acid content was determined according to [21],peroxidase activity and polyphenol oxidation and total phenolics content were measured using the method described in [22, 23]

Statistical analysis

All experiments were conducted according to a completely randomized design (CRD), The obtained data were statistically analyzed using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Treatment means were compared using the least significant difference (LSD) test at a significance level of 0.05.

Results and discussion

Isolation and identification

Isolation and identification results from orange fruits showing symptoms of green mold revealed the presence of the fungus Penicillium digitatum. Colonies were gray green to olive-brown in color, with white mycelium in the early stages, and produced conidia at medium to high densities (Fig. 1a). Colony diameters averaged 3-4 cm after 7 days of incubation at 25°C. The conidiophores were columnar-shaped, thin-walled, and smooth, bearing brush-like structures (penicilli) at their ends, with pointed, cylindrical phials terminating in oval to cylindrical, smooth-walled, white-to-light-green spores arranged in irregular chains (Fig. 1b). These characters are consistent with the taxonomic key of [18]

Figure 1. A: Colony of *P. digitatum* B: Conidiophores of P.digitatum

The effect of chitosan and salicylic acid on the mycelial growth of P. digitatum

The results in Figure (**2**) show the inhibitory effect of varies concentrations of chitosan and salicylic acid on P. digitatum. At the lowest concentration of 5 mg L-1, chitosan and salicylic acid showed inhibition rates of 4.63% and 2.27%, respectively. As the concentrations increased to 10 mg L^{-1}, the inhibition rate increased to 7.37% for chitosan and 4.1% for salicylic acid. A significant increase was observed at 100 mg L-1, where chitosan achieved an inhibition rate of 27.5% and salicylic acid 20.6%. At 500 mg L^{-1}, the inhibition rate of chitosan increased significantly to 80.13%, while that of salicylic acid increased to 55.23%. At a concentration of 1000 mg L-1, chitosan achieved 100% complete inhibition.

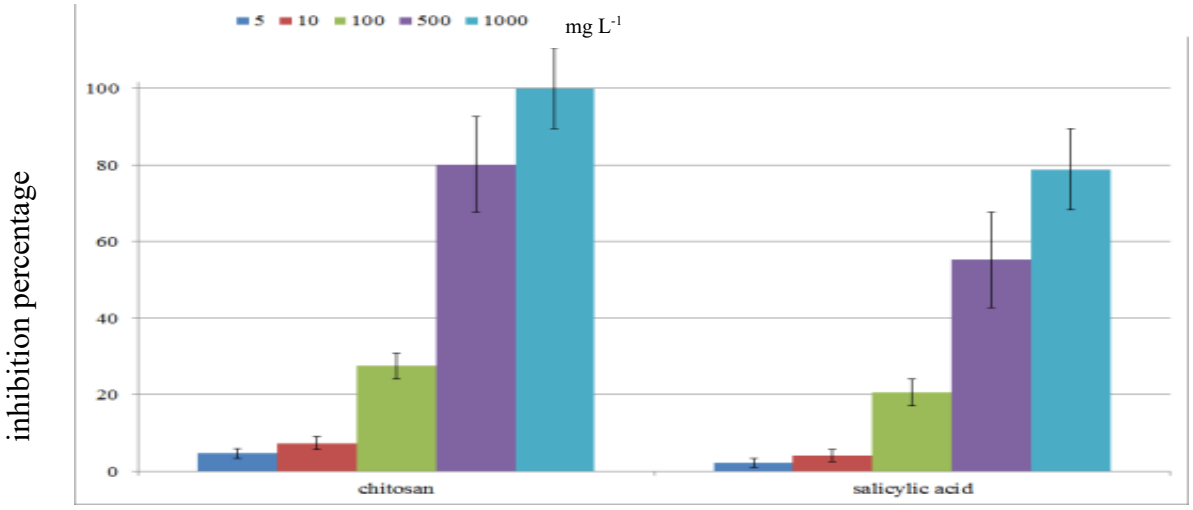

Figure 2. Effect of chitosan and salicylic acid on the mycelial growth of *P. digitatum*

The effect of chitosan and salicylic acid on the incidence and severity of green mold disease in orange fruits

The results in figure (3) show that both salicylic acid and chitosan significantly reduced the incidence and severity of green mold disease in orange fruits during storage compared to untreated fruits, fruit in control treatment showed 100% disease incidence, with severity of 0.9%and lesion diameter 4.88 cm. Treatment with chitosan reduced disease incidence to 68.75%, severity to0.35%, and lesion diameter to 1.50 cm .salicylic acid was even more effective ,lowering incidence to 56.25%, severity to 0.25%,and lesion diameter to 1.28 cm

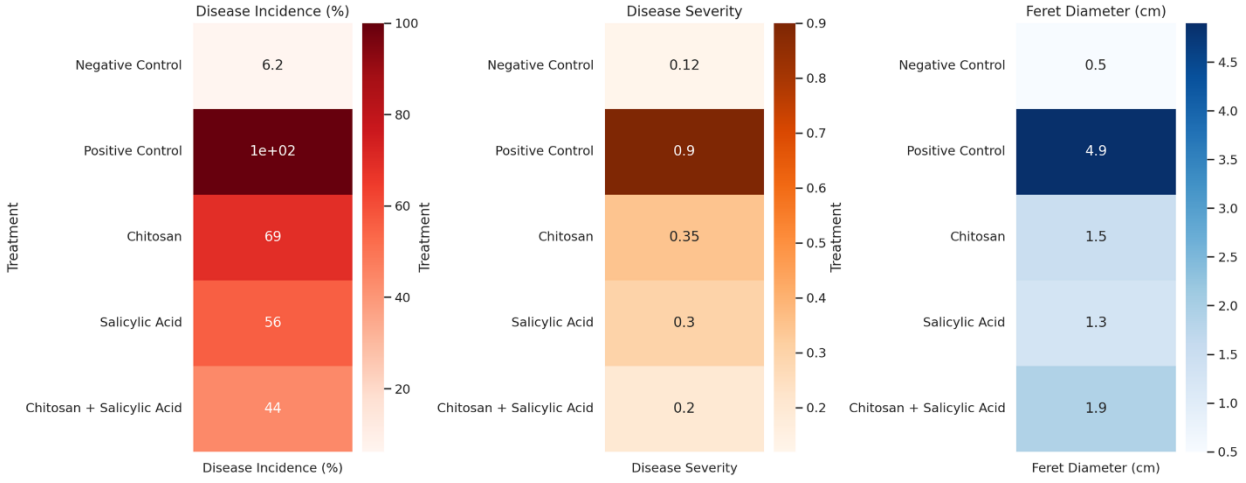

Figure 3. Heat map comparing the effect of salicylic acid and chitosan on the pathological parameters of green mold disease in orange fruits under storage conditions.

The Effect of Salicylic Acid and Chitosan on Orange Fruit Quality infected with *P.digitatum* under Storage Conditions

The results in Table (1) show the effect of different treatments on the quality characteristics of orange fruits infected with green mold during storage. Weight Loss: Untreated fruit exhibited the highest weight loss at 4.87%, chitosan treatment showed the lowest weight loss at 2.12%. Salicylic acid showed a weight loss of 3.15%, less than the positive control but higher than chitosan. Total Soluble Solids (TSS): TSS levels showed minor variation across treatments, fruit treated with salicylic acid had a TSS 12.49% closely followed by chitosan treated fruits at 12.35% both values were slightly higher than control treatment 11.98% . Ascorbic Acid: the ascorbic acid content Chitosan-treated fruits was 125.48 mg/100 g fresh weight. While Salicylic acid content was 123.72 mg.100 g^{-1}, compared to 119.23 mg.100 g^{-1} in the positive control .

Table 1. Effect of Salicylic Acid and Chitosan on Orange Fruit Quality infected with *P.digitatum* under Storage Conditions

Tr,	W.Loss %	TSS%	As mg.100g^{-1}
N.C.	1.72 ±0.043	12.45±0.72	125.45±1.63
P.C.	4.87 ±0.025	11.98±0.74	119.23±1.41
Ch.	2.12±0.029	12.35±0.78	125.48±1.67
SA	3.15±0.037	12.49±0.69	123.72±1.58
L.S.D.	0.89	1.45	3.27

Tr. Tretments , N.C. Negative control , P.C. Positive control, Ch. Chitosan , SA Salicylic acid

The effect of chitosan and salicylic acid on the activity of the peroxidase enzyme in orange fruits infected with *P. digitatum* under Storage Conditions

The results in Table (2) showed that chitosan and salicylic acid significantly enhanced peroxidase (POD) activity in orange fruits infected with green mold during storage. In uninfected fruits (negative control), enzyme activity remained low and stable 71.51–75.88 units , while untreated infected fruits (positive control) showed moderate stimulation 81.49–88.08 units . Chitosan treatment significantly increased POD activity, particularly after 48 , 72 and 96 hours 86.71,89.95,95.83 units , with a slight decrease at 96 hours, although still higher than in the positive control. Salicylic acid demonstrated a strong and sustained stimulatory effect, increasing activity from 71.06 units at 24 hours to 96.59 units at 96 hours, indicating sustained activation of fruit defense mechanisms.

The effect of chitosan and salicylic acid on the activity of the polyphenol oxidase enzyme in orange fruits infected with *P.digitatum* under Storage Conditions

The results in Table (3) indicate that all treatments affected the activity of polyphenol oxidase (PPO) in orange fruits infected with green mold during different storage periods 24, 48, 72, and 96 hours . The negative treatment (healthy, uninfected fruits) maintained a low and stable enzyme activity ranging between 1.38 and 1.49 units, which reflects the normal level in unaffected fruits. In contrast, infected fruits (positive control) recorded a temporary increase in enzyme activity, peaking at 72 hours 1.71 units , as a natural defense response. The chitosan treatment showed a moderate increase in PPO activity, reaching 1.64 units after 96 hours, while the use of salicylic acid further enhanced the enzyme activity, reaching 1.68 units during the same period, indicating the role of both compounds in stimulating the defense systems and antioxidant enzymes in fruits during storage.

Table 2. Effect of chitosan and salicylic acid on the activity of the peroxidase enzyme in orange infected with *P.digitatum* under Storage Conditions.

Tr.	peroxidase activity (uint.min .g.fw-1)			
	24 h	48h	72h	96h
N.C.	71.51±2.2	75.88±1.82	75.58±2.4	74.99±1.8
P.C.	72.53±2.3	81.49±1.87	88.08±2.4	85.59±1.9
Ch.	75.83±2.2	86.71±1.92	89.95±2.4	95.83±1.9
SA	71.06±2.1	86.00±1.94	84.39±2.3	96.59±1.8
LSD	4.52	3.78	4.91	3.73

Tr. Tretments , N.C. Negative control , P.C. Positive control, Ch. Chitosan , SA Salicylic acid

Table 3. Effect of chitosan and salicylic acid on the activity of the polyphenol oxidase activity in orange fruits infected with *P.digitatum* under storage conditions.

Tr.	polyphenol oxidase activity (uint.min .g.fw^{-1})			
	24 h	48h	72h	96h
N.C.	1.45±0.06	1.49±0.11	1.38±0.06	1.45±0.07
P.C.	1.45±0.06	1.45±0.09	1.71±0.06	1.45±0.07
Ch.	1.42±0.05	1.53±0.07	1.63±0.05	1.64±0.07
SA	1.49±0.69	1.59±0.10	1.61±0.05	1.68±0.06
LSD	1.32	0.28	0.12	0.38

Tr. Tretments , N.C. Negative control , P.C. Positive control, Ch. Chitosan , SA Salicylic acid

The effect of chitosan and salicylic acid on the activity of the total phenol content in orange fruits infected with *P.digitatum* under Storage Conditions.

The results in Table (4) indicate that all treatments significantly affected the total phenol content (mg/g fresh weight) in orange fruits infected with green mold during storage 24, 48, 72, and 96 hours . The negative treatment maintained low and stable levels 11.4–11.9 mg.g.fw^{-1} , while the positive treatment showed a temporary increase of 13.5 mg/g after 48 hours, which subsequently declined. Chitosan gradually stimulated the accumulation of phenolics, reaching 16.4 mg.g.fw^{-1} after 96 hours, with its effect clearly evident between 48 and 72 hours. Salicylic acid, on the other hand, gradually and continuously enhanced this accumulation, reaching 16.3 mg/g after 96 hours, reflecting its role in activating systemic acquired resistance and enhancing phenol-related defense pathways.

Dissection

The results of the study demonstrated that the two natural compounds, chitosan and salicylic acid had clear and complementary effects in reducing green mold disease in citrus fruits caused by the fungus *P.digitatum*. These effects occurred through two main pathways: the first was a direct effect on the pathogenic fungus, and the second was stimulating the fruit's biological defenses and reducing the severity of infection, in addition to improving the storage and physiological properties of the fruit during storage.Chitosan treatment demonstrated significant effectiveness in inhibiting the fungal growth of P. digitatum in laboratory conditions. Chitosan concentrations ≥0.1% caused significant inhibition of mycelium growth, while a

concentration of 0.5% resulted in almost complete inhibition. This effect is attributed to chitosan's interaction with the fungal cell wall and plasma membrane, where it binds to the fungi's negatively charged groups, increasing membrane permeability and, consequently, leakage of vital cellular components such as poldines and cations, ultimately leading to fungal cell death.[5, 24].

Table 4. The effect of chitosan and salicylic acid on the activity of the Total phenol contain in orange fruits infected with *P.digitatum* under Storage Conditions

Tr.	Total phenol(mg .g.fw^{-1})			
	24 h	48h	72h	96h
N.C.	11.9±0.14	11.4±0.13	11.69±0.08	11.8±0.13
P.C.	10.7±0.18	13.5±0.14	12.6±0.11	13.2±0.18
Ch.	11.6±0.14	14.2±0.11	15.7±0.09	16.4±0.17
SA	11.9±0.15	15.1±0.09	15.4±0.12	16.3±0.15
LSD	0.24	0.93	0.47	0.54

Tr. Tretments , N.C. Negative control , P.C. Positive control, Ch. Chitosan , SA Salicylic acid

In addition, chitosan affects protein synthesis within the fungal cell , prevents the absorption of nutrients from the surrounding environment, and chelates trace elements essential for fungal enzymes, contributing to the inhibition of fungal vital processes .[25]. Salicylic acid has also been shown to inhibit fungal growth and spore germination, particularly when used at concentrations ranging from 6 to 16 mM. Studies have shown that these concentrations disrupt the integrity of the spore plasma membrane, increased permeability, leakage of cellular contents and the accumulation of reactive oxygen species (ROS), which are indicators of oxidative stress in fungal cells, leading to their death .[26,27].. The effect of salicylic acid also extends to influencing vital processes by inhibiting certain fungal enzymes associated with growth and penetration. This direct effect on the fungus was clearly reflected in the reduction in the incidence and severity of green mold infection on orange fruits. It was observed that treatment with chitosan and salicylic acid, either alone or in combination, significantly reduced the development of disease symptoms compared to the control. The combined treatment with chitosan (0.5%) and salicylic acid (8 mM) demonstrated a synergistic effect, recording the lowest infection rate and disease severity during the storage period, indicating the effectiveness of this treatment in protecting the fruit from fungal infection .[24,28]. This reduction is attributed to limiting fungal penetration into the pericarp tissue, in addition to stimulating fruit defenses. The studied treatments demonstrated an important role in improving fruit storage properties during cold storage. Chitosan contributed to reducing weight loss by forming a semi-permeable layer on the fruit surface, which reduces evaporation and moisture loss, and limits the entry of oxygen and the exit of carbon dioxide, thus reducing respiration rates and delaying ripening .[5]. On the other hand, salicylic acid helped delay physiological changes associated with storage, such as the reduction in ascorbic acid (vitamin C) and total soluble solids (TSS) content,by reducing oxidative stress and maintaining the stability of juice components. .[29]. Bioassay results showed that treatment with chitosan and salicylic acid significantly stimulated the activity of defense enzymes in the fruit, particularly peroxidase (POD) and polyphenol oxidase (PPO), two key enzymes involved in the formation of defense barriers against pathogens. POD and PPO activity gradually increased in treated fruits compared to untreated fruits, with the highest activity recorded in fruits that received the dual treatment, indicating an enhanced defense response .[30,31]. This increase in enzyme activity was accompanied by an increase in the total content of phenolic compounds, which are secondary defense compounds that contribute to fungal resistance through their antioxidant and pathogen-inhibiting properties. The phenolic pathway is believed to be stimulated by the activation of Phenylalanine Ammonia-Lyase (PAL), the key enzyme in the synthesis of phenolic compounds in response to biotic stress .[31]. This enhances the fruit's ability to repel the fungus and prevent its progression within plant tissues, which explains the reduced severity of infection and improved quality of treated fruits.

Conclusions

Pnicillium digitatum was isolated and identified as the causative agent of green mold disease in orange fruits. Treatments with chitosan and salicylic acid demonstrated effective inhibition of fungal growth and reduced the incidence and severity of infection during storage. These treatments also improved fruit quality by reducing weight loss and maintaining high levels of total soluble solids and vitamin C. Furthermore, the treatments increased the activity of defense enzymes (POD and PPO) and phenolic content, enhancing fruit resistance to the disease. The results indicate that chitosan and salicylic acid represent environmentally friendly alternatives for postharvest control of green mold disease

Conflicts of Interest

The authors declare no conflict of interest.

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