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CONTROL OF GREEN MOLD DISEASE IN ORANGES CAUSED BY *PENICILLIUM DIGITATUM* BY USING CHITOSAN AND SALICYLIC ACID

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ABSTRACT

This research delved into the impact of Chitosan and Salicylic Acid (SA) on disease incidence (DI) and the quality of citrus fruit, specifically concerning *Penicillium digitatum* infection. The study finds the use of the SA effect curbed the growth of green mold decay in citrus fruit due to *P. digitatum*. Meanwhile, Chitosan-treated fruit exhibited reduced (DI) and lesion diameters in comparison to the control, after 3 or 7-days. However, the combination of Chitosan and SA reduced (DI), plummeting to 36.67% from the control's 83.33% after 7 days. The combined treatment resulted in smaller lesion diameters. (PAL) activity exhibited a consistent increase in all treated fruit during storage. In conjunction with SA, Chitosan demonstrated the highest PAL activity after four weeks. In the case of (POD) activity, treated fruit showcased an increase compared to the control. (C) activity was notably elevated in treated fruit, with the combined treatment revealing the highest activity level. The total phenolic content throughout the treatments and the content of phenolic compounds saw a gradual ascent, culminating after four weeks. The combined treatment displayed elevated phenolic content in contrast to individual treatments and the control. The activities of chitinase and β-1,3-glucanase exhibited an upward trajectory in citrus peel over the storage period. Applying Chitosan and SA led to increased Total Soluble Solids (TSS) and ascorbic acid content after four weeks, with no significant differences in weight loss compared to the control.

INTRODUCTION

Fungi cause significant losses in crops before and after harvest (Strano *et al.*, 2022). Green mold is one of the major post-harvest diseases caused by *Penicillium digitatum* infects citrus fruits, and severe losses of up to 80% occur under suitable storage conditions (Ahmad Salah O. and Abdul Salam Shimmaa R. 2008). This leads to the production of green spores and fruit shrinkage over time (Gergis, R. K., & Mohamed, N. I. (2019; Li *et al.*, 2022). The control of post-harvest citrus diseases mainly relies on using synthetic fungicides through different protocols, such as dipping citrus fruits in soak tanks, spraying an aqueous solution, and using fumigants (Liu *et al.*, 2022). Due to the health and environmental hazards associated with these chemicals, plant pathologists have been seeking valuable alternatives. Moreover, the continuous and indiscriminate use of chemicals has resulted in the development of resistant strains of pathogens. (Da Costa Gonçalves *et al.*, 2022; Aljuboori *et al.*, 2022; Ahmed *et al.,* 2023). In recent times, natural products have emerged as a promising substitute for synthetic fungicides (Mohammadi, P. *et al*. 2017; Samada *et al.,* 2022). The induction of pathogen resistance IPR represents a good promising approach to control plant diseases, offering long-term systemic protection against a wide range of pathogens (Parker, 2020; Bhatta, U. K. 2022; Ismail *et al.,* 2023). Salicylic acid (SA), a phytohormone, plays crucial roles in various aspects of plant development and growth. (Arif *et al.,* 2020). SA acts as a signaling molecule that triggers the production of defense compounds, including pathogenesis-related proteins. (Saleem *et al.,* 2020). Numerous studies have demonstrated that the application of exogenous SA enhances pathogen resistance and reduces decay incidence in fruits. (Wang *et al.,* 2020). Recent research has specifically highlighted the notable effectiveness of Salicylic acid in combating green mold decay." when it is applied "to manage green mold in citrus fruits.". (Moosa *et al.,* 2019). Chitosan, derived from deacetylated chitin, is a polysaccharide with a substantial molecular weight, primarily involving D-glucosamine and N-acetyl-D-glucosamine connected through b-1,4-linkages. (Araújo *et al.,* 2020). Chitosan and its derivatives, such as oligochitosan, appear safe, biodegradable, and compatible with other microorganisms. It is a good and attractive option to study its effectiveness in preventing deterioration and prolonging the storage period of fruits. (Kumar *et al.,* 2020). Chitosan and its modified forms have exhibited the capacity to hinder the growth of many fungi and can trigger the plant's and fruit's defense against infections stemming from various pathogens (Lopez-Moya *et al.,* 2020). Prior research has utilized chitosan for preserving fruit quality and controlling post-harvest deterioration in fruit, specifically, green mold in citrus ". (Shi *et al.,* 2019). In addition to its direct antifungal effects, many researchers have explored its potential to induce a defensive response in citrus fruits. Many studies have provided evidence that chitosan and salicylic acid (SA) can trigger production of reactive oxygen species (ROS) and improve the functions of enzymes like phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (POD), chitinase, and β-1,3-glucanase, and stimulate the expression of defense-related genes and resistance to diseases and maintaining fruits quality (Wang *et al.,* 2020; Al-Mokadem *et al.,* 2022). This study aims to find the separate and combined effects of post-harvest chitosan and salicylic acid treatments on the regulation of green mold fungi caused by *Penicillium digitatum* in orange fruits. In addition, the study aims to evaluate how these applications affect important host defense enzymes and fruit quality during storage.

MATERIALS AND METHODS

Citrus fruits

Orang fruits were carefully selected based on comparable size, ripeness, and absence of external harm and then randomly divided into four groups. Initially then, the fruits were surface-disinfected by immersing them in 1% (v/v) sodium hypochlorite for 2 minutes. Afterward, they were washed with sterilized distilled water and left to air-dry at room temperature (Zhou *et al.,* 2014).

Pathogen inoculum

P. digitatum isolates were extracted from the rotted orange fruits and used in this experiment. The fungi colonies were identified based on the morphological characteristics of the colony (white mycelium and green conidia) on agar medium after incubation at 25 ±2 °C for 7 days. The isolates were cultured on potato dextrose agar (PDA) at 25 ±2 °C for a week. Spores were then harvested by flooding the media surface with sterile distilled water technique, and the plate was gently agitated to dislodge the spores. A hemocytometer was used for spores counting, and spore concentrations of pathogens were adjusted using sterile distilled water containing 0.05% (v/v) Tween 80 to obtain 105 spores/ml (Zhou *et al.,* 2014).

Treatments

The treatments applied were as follows:

1. Control treatment: Fruits were immersed in sterilized distilled water for a quarter of an hour.
2. Chitosan treatment: Fruits were immersed in ten grams. L−1 (w/v) chitosan solution for a quarter of an hour
3. Salicylic Acid treatment: Fruits were immersed in 2 mM Salicylic Acid solution for 15 minutes.
4. Salicylic Acid + Chitosan treatment: Fruits were first immersed in 2 mM Salicylic Acid solution for 15 minutes, then after air-drying for 2 hours, they were dipped again in ten grams L−1 (w/v) chitosan solution for another 15 minutes.

Air drying process was applied to all treated fruits. Then cartons were used to pack the fruits and kept at room temperature for future experiments. Each treatment group consisted of triplicate samples, with 10 fruits per replicate.

The impact of Chitosan and Salicylic Acid on the occurrence of diseases and the lesions diameter caused by P. digitatum in citrus fruit

To assess the effectiveness of Fruits immersed in Chitosan and Salicylic Acid solutions. treatments for managing *P. digitatum* green mold, each fruit was wounded at three equatorial points using a sterilized borer (2 mm deep × 5 mm wide). Following this, 30 µl of a 1×106 suspension of *P. digitatum* containing spores at a concentration of ml−1 was introduced into each wound. Subsequently, the fruits were air-dried and individually placed in plastic bags, followed by incubation at room temperature 27±2 for a period of 7 days. The assessment of disease incidence and measurement of lesion diameter were conducted daily, beginning from the day of inoculation. Each treatment included three replicates, each consisting of 10 fruits

The influence of Chitosan and Salicylic Acid on the overall content of phenolic compounds and the activities of defense-related enzymes in citrus fruit

Tissue samples of four grams were taken from areas located 3 to 6 mm beneath the fruit's skin toward its equator at 1, 2, 3, 4, and 5 weeks after the treatment. Samples were separately packaged, frozen, and stored at −20 °C until biochemical analysis. Peroxidase (POD) and polyphenol oxidase (PPO) activities were determined following the method outlined by Moosa et al. (2019), with POD activity quantified as the enzyme amount inducing a difference of 1 in absorbance per minute at a 470 nm. Similarly, PPO activity was quantified as the enzyme amount causing a minutely variation of 1 in absorbance at 420 nm. The method outlined by Zhou et al. (2014) was used to measure the activity of phenylalanine ammonia-lyase (PAL), which was then expressed in units (U) per gram of fresh weight (FW). The amount of enzyme that caused a 0.01 rise in absorbance at 290 nm per hour was designated as one unit of PAL activity. Based on the methods described by Peng et al. (2016), the activities of chitinase (CHI) and β-1,3-glucanase were measured and expressed in units (U) per gram of fresh weight (FW). One unit of CHI activity was defined as the enzyme amount required for catalyzing the production of 1 µg NAG per hour at 37° C. β-1,3-glucanase activity was measured by the amount of reducing sugar at 540 nm after reacting with 250 µl of 3,5-dinitrosalicylic reagent. The total phenolic compound content was determined spectrophotometrically using Folin-Ciocalteu reagent, following the method outlined by Tian *et al.,* (2021). The results were expressed as grams of gallic acid equivalent (GAE) per gram of fresh weight (FW).

Effects of Chitosan and SA on Post-harvest Quality Parameters in citrus fruit

Weight loss was assessed at the start and during storage for each treatment. The calculation was done as follows: $loss\ of\ weight = (Wi - Wf)\ /\ Wi \times 100\%$, where the initial sample weight is denoted by Wi, while the final specimen weight is denoted by Wf. The initial weight served as the basis for calculating the percentage of fresh weight loss. For each replicate, three fruits were peeled, and the total soluble solids (TSS) content was determined using a portable refractometer, with results expressed as percentages (g/100 g of FW). Ascorbic acid content was quantified using 2,6-dichloroindophenol titration, and the results were expressed as milligrams per 100 g of FW, following the method described by Shende et al. (2020).

RESULTS AND DISCUSSION

The Incidence of disease and lesions diameter caused by P. digitatum in citrus fruit

As depicted in Table (1), Salicylic Acid (SA) alone had a significant inhibitory effect on the development of green mold decay in citrus fruit when inoculated with *P. digitatum*. After 3 or 7 days of incubation, the fruits treated with chitosan exhibited reduced lesion diameters as well as a lower incidence of disease of *P. digitatum* compared to the control.

Table (1): Impact of chitosan and Salicylic Acid (SA) treatments on disease incidence and lesion diameter in citrus fruit infected by P. digitatum.

Treatments	Disease incidence %		lesion diameter mm	
	3 days	7 days	3 days	7 days
Control	56.67	83.33	18.63	35.40
Chitosan 2 g. $L^{-1.}$	40.00	56.67	10.20	21.18
SA 2 mM	26.67	46.67	11.77	22.36
SA + Chitosan	23.33	36.67	9.47	16.89
LSD	10.23	15.54	3.24	3.25

Nevertheless, with the increasing duration of storage, the antifungal efficacy in reducing incidence of disease. When Chitosan was used in combination with SA, the disease incidence caused by *P. digitatum* reduced to 36.67%, in contrast to 83.33% in the control after 7 days of storage. The lesion diameter for the combination was

16.89 mm, significantly smaller than those observed in fruits treated with either chitosan or just SA after incubation of 7 days.

The influence of both Salicylic Acid (SA) and Chitosan on PAL, POD, and PPO enzymes activities, as well as the overall phenolic concentration in citrus fruit.

As depicted in Table (2), activity of PAL enzyme in all treated citrus samples unceasingly increased throughout the entire storage period. PAL activity in the combined treatment peaked after four weeks, being 53.11, 83.62 and 108.48 (U g. fw^{-1}) For chitosan, SA and SA+Chitosan respectively, compared with the control fruit 35.03(U g. fw^{-1}). Regarding POD activity, there was no remarkable change in the control fruit. However, in the treated fruits, POD activity increased rapidly and peaked at two weeks, after which it subsequently decreased. The chitosan plus SA treatment significantly improved POD activity in comparison to the control. The activity with the combined application was 155.94 (U g. fw^{-1}) compared with control fruit at three weeks 36.16(U g. fw^{-1}). As shown in Table 2, chitosan and Salicylic Acid (SA) application caused noteworthy increase in PPO enzyme activity. When compared to the control group during storage. The activity with the combined application was 3.25 (U g. fw^{-1}) compared with control fruit at four weeks 1.65 (U g. fw^{-1}). In all treatments, as shown in Table (3), The content of total phenolic compounds exhibited a gradual increase, reaching its peak at four weeks. Notably, the combined sample showed a rapid and significant increase, reaching levels of 37.8 (mg. g. fw^{-1}), which were substantially higher than both the individual treatments and the control, which registered at 18.4 (mg. g. fw^{-1}).

Table (2): The impact of both Salicylic Acid (SA) and Chitosan on the activities of PAL and POD enzymes in citrus fruit.

Treatments	PAL (U g. fw^{-1})					POD (U g. fw^{-1})				
	1week	2week	3week	4week	5week	1week	2week	3week	4week	5week
Control	27.12	23.73	28.25	35.03	31.64	41.81	36.16	36.16	47.46	48.59
Chitosan	31.64	30.51	37.29	53.11	54.24	64.41	74.58	75.71	63.28	57.63
SA	44.07	38.42	35.03	83.62	80.23	98.31	102.83	128.82	81.36	70.06
SA+Chitosan	46.33	42.94	50.85	108.48	100.57	126.56	107.35	155.94	92.66	83.62
LSD	5.47	7.37	17.28	15.48	18.47	19.25	25.29	24.82	11.53	18.25

Table (3): Influence of both Salicylic Acid (SA) and Chitosan on PPO and Chitosan on PPO enzyme activities and the total phenolic content in citrus fruit.

Treatments	PPO (U g. fw^{-1})					Total phenol (mg. g. fw^{-1})				
	1week	2week	3week	4week	5week	1week	2week	3week	4week	5week
Control	1.54	1.65	1.89	1.65	2.21	12.4	13.8	12.8	18.4	16.7
Chitosan	1.82	2.45	2.65	1.89	2.34	13.2	18.4	18.3	27.4	23.9
SA	1.85	2.35	2.71	2.45	2.75	13.6	18.5	18.6	33.2	26.8
SA+Chitosan	2.45	3.45	2.98	3.25	3.17	13.7	25.4	27.4	37.8	32.4
LSD	5.47	7.37	17.28	15.48	18.47	19.25	24.82	25.29	11.53	18.25

The impact of both Salicylic Acid (SA) and Chitosan on Chitinase and the activities of β-1,3-glucanase in citrus fruit

As depicted in Table (4), the chitinase activity in citrus peel exhibited an increase over the storage period. At five weeks, fruits treated with chitosan, SA only, or the combination of both treatments exhibited chitinase activities of 64.26, 74.34, and 81.90 U g. fw^{-1}, respectively, which were significantly higher than the control at 60.48 U g. fw^{-1}. Similarly, the longer the storage time, the higher the activity of β-1,3-glucanase in the peel of citrus fruit. (Table 4). The SA and chitosan treatments, when applied individually, resulted in significantly higher enzyme activities after two and four weeks, respectively, in comparison to the control throughout the storage period. The combo of SA and chitosan induced that the enzyme activity of grapefruit fruit is significantly higher than that of SA or chitosan alone. After five weeks, the combined fruit exhibited a peak β-1,3-glucanase activity of 7.06 U g. fw^{-1}, significantly greater than the control's 5.29 U g. fw^{-1}.

Table (4): The impact of Chitosan and Salicylic Acid (SA) on the activities of Chitinase and -1,3-glucanase in citrus fruit.

Treatments	Chitinase (U g. fw^{-1})					β-1,3-glucanase (U g. fw^{-1})				
	1week	2week	3week	4week	5week	1week	2week	3week	4week	5week
Control	23.94	27.72	36.54	65.52	60.48	4.41	5.17	4.91	5.17	5.29
Chitosan.	30.24	44.10	49.14	79.38	64.26	4.03	5.42	5.29	5.42	5.80
SA	32.76	51.66	61.74	73.08	74.34	4.54	5.54	5.42	5.54	5.92
SA+Chitosan	35.28	65.52	79.38	89.46	81.90	4.16	5.80	5.54	6.55	7.06
LSD	4.24	9.25	11.24	12.68	12.89	0.21	0.12	0.29	0.23	0.45

The impact of Chitosan and Salicylic Acid (SA) on the quality of citrus fruit after harvest.

Table (5) presents data indicating that the use of chitosan and Salicylic Acid (SA), whether applied separately or in combination, led to a significant increase in the Total Soluble Solids (TSS) and ascorbic acid content compared to control fruit after four weeks of storage. Importantly, no significant differences in weight loss were observed among the treatments.

Table (5): The impact of Chitosan and Salicylic Acid (SA) on the post-harvest quality of citrus fruit.

Treatments	Weigh Loss (%)	Total Soluble Solid(%)	Ascorbic Acid (mg.100 g^{-1} FW)
Control	4.67	12.31	105.62
Chitosan.	3.96	13.53	120.030
SA	4.34	13.46	136.05
SA+Chitosan	4.04	13.79	135.31
LSD	1.87	0.32	10.28

In recent times, inducing resistance in fruits has emerged as a promising strategy to control post-harvest diseases (Yang, Q. *et al.* 2019). Chitosan, among compounds that are natural elicitors, stands out as a biodegradable substance with both eliciting and antimicrobial properties (Betchem *et al.,* 2019). Previous research demonstrated that immersing apple fruits in a 1% chitosan solution, the blue mold that was caused by *Penicillium expansum* during fruit storage was reduced. Similarly, we observed that chitosan treatment effectively inhibited green mold in citrus (Bhatta *et al.,* 2019).

On the other hand, Salicylic Acid (SA) is produced naturally in plants and acts as a signaling molecule that plays a vital role in triggering mechanisms to counter disease resistance (Ahmad *et al.,* 2019). Our study confirmed that the Fruit treated with SA had a significant decrease in the incidence of green mold than in control fruit, consistent with earlier findings. Additionally, Fruit inoculated with *P. digitatum* showed a significant reduction in lesion diameter and disease incidence with the combination of chitosan and SA in comparison to treatments that utilize chitosan or SA alone, indicating a better defense mechanism against green mold in citrus fruit was activated by using the combination (Shi *et al.,* 2019).

The mechanism behind SA enhancing the antifungal effect of chitosan is intricate and may result from the synaxis of chitosan and SA. This antibacterial synergy of SA and chitosan aligns with the findings made by Urban et al.2022).
Exogenous SA has been reported by studies to increase endogenous SA levels, which can stimulate resistance to Fusarium and Significantly decrease the severity of disease in chickpea and tomato. (Bawa *et al.,* 2019). Enhancing the defense mechanisms of plants and post-harvest fruit can be achieved in part by inducing disease resistance. Peroxidase and polyphenol oxidase are crucial defense enzymes associated with plant disease resistance. (Moosa *et al.,* 2019; Baker *et al.,* 2022). Poly phenol oxidase and peroxidase take part in the activation of oxygen metabolism in fruits and contributes to the transformation of phenolic compounds into harmful quinones. (Jiang, *et al.,* 2019).

The storage period after dipping revealed an increase in POD activity in citrus fruit treated individually or in combination, as shown in our experiment. POD activity in combined fruit was significantly higher than in the alone citrus fruit, indicating induced resistance. The defense reaction against pathogens involves PPO as a key enzyme, responsible for lignification of plant cells and catalyzing the formation of lignin and phenols, providing protection for plant cells to prevent pathogen infection. Moreover, it can also catalyze phenols to produce highly toxic quinone substances that are toxic to invading pathogens. (Kaur *et al.*, 2022).

In this study, the treatment with Salicylic Acid (SA) and chitosan significantly boosted the activity of Polyphenol Oxidase (PPO) in citrus fruit peel, which aligns with findings in apples and apricots (He *et al.,* 2022; Liu *et al.,* 2023). The results indicated that the enhancement of PPO activity was found to have a positive correlation with increased induced fruit resistance (Zhao *et al.,* 2019).
Phenylalanine Ammonia-Lyase (PAL) plays a key role in regulating the phenolic pathway by converting phenylalanine into trans-cinnamate. The resistance that is a result of abiotic elicitors could be attributed to their ability to stimulate PAL activity, leading to the accumulation of total phenolic compounds in fruits (Guarnizo *et al.,* 2022).

Our findings demonstrated Citrus fruit's PAL activity during storage was significantly enhanced by all treated groups. and there was a substantial increase in the content of total phenolic compounds. This outcome in our study is likely a result of abiotic elicitors, such as chitosan and SA, triggering an increase in PAL activity, which, in turn, promotes the production of phenolics and strengthens the defense resistance of citrus fruit.

The combination of chitosan with SA, as shown in our study, rapidly and robustly induced defense-related reactions in citrus fruit. This effect may be linked to the reduced the diameter of the lesion and the incidence of infection, potentially explained by the synergistic interaction amid chitosan and SA, suggesting a combined effect of both compounds in enhancing induced resistance. Furthermore, the combination of chitosan and SA treatment did not affect the quality of citrus fruits after harvest, while boosting disease resistance in the control of Citrus fruits with green mold.

CONCLUSIONS

These findings suggest that the combo of both Salicylic Acid (SA) and chitosan could serve as a promising strategy for commercial applications aimed at controlling post-harvest green mold in citrus.

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CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest with the publication of this work.

مكافحة مرض العفن الأخضر في البرتقال المتسبب عن الفطر *Penicillium digitatum* باستخدام الكايتوسان وحامض الساليسيليك

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الخلاصة

تناول البحث تأثير المعاملة بالكايتوسان وحامض السالسليك (SA) في نسبة الإصابة بالفطر المسبب للعفن الاخضر Penicillium digitatum وجودة ثمار البرتقال تحت ظروف الخزن. أظهرت نتائج الدراسة تأثير المعاملة بحامض السالسليك في كبح نمو الفطر وفي الوقت نفسه أظهرت الثمار المعاملة بالكايتوسان انخفاضًا في معدل الإصابة بالمرض فضلا عن صغر المساحة المصابة مقارنة بمعاملة السيطرة بعد 3 أو 7 أيام من المعاملة. كما أدى الجمع بين معاملتي الكايتوسان وحامض السالسليك إلى انخفاض ملحوظ في حدوث المرض، حيث انخفض إلى 36.67% مقارنة بمعاملة السيطرة 83.33% بعد 7 أيام من المعاملة إضافة لصغر المساحة المصابة. كما ظهرت زيادة ثابتة في نشاط انزيم فينايل النين امونيالاييز PAL في جميع الفاكهة المعاملة أثناء التخزين فقد كان للمعاملة المشتركة بالكايتوسان وحامض السالسليك أعلى نشاط لأنزيم PAL بعد

أربعة أسابيع من المعاملة. وسجلت النتائج أيضا نشاطا لأنزيم البيروكسيديز PODحيث أظهرت الفاكهة المعاملة زيادة كبيرة مقارنة بمعاملة السيطرة. سجل نشاطا ملحوظا لانزيم البوليفينول أوكسيديز (PPO) في الفاكهة المعاملة، حيث كشفت المعاملة المشتركة عن أعلى مستوى للنشاط الانزيمي. كما شهد محتوى المركبات الفينولية الكلي ارتفاعًا تدريجيًا، وبلغ ذروته بعد أربعة أسابيع. ومن اللافت للنظر أن المعاملة المشتركة أظهرت محتوى فينوليًا مرتفعًا على عكس من المعاملات الفردية فضلا عن معاملة السيطرة. وشهد نشاط انزيمي الكيتيناز و-β 1,3-جلوكاناز مسارًا تصاعديًا في قشرة الحمضيات خلال فترة التخزين. وأظهرت المعاملات المشتركة نشاطا انزيميا أكبر مما يدل على زيادة في آليات الدفاع. أعطت المعاملة بالكايتوسان وحامض السالسليك زيادة في محتوى المواد الصلبة الذائبة الكلية (TSS) وحمض الأسكوربيك بعد أربعة أسابيع، مع عدم وجود فروق معنوية في فقدان وزن الثمار مقارنة بمعاملة السيطرة.
الكلمات المفتاحية: PAL، PPO، الخزن، ثمار الحمضيات.

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