Efficacy of Chitosan and Salicylic acid for Controlling Gray mold Caused by *Botrytis cinerea* on Greenhouse Tomato

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Abstract

Application of Chitosan (CH) and salicylic acid (SA) in controlling the fungus *Botrytis cinerea* that causes gray mold on tomato fruits is a successful and safe alternative approach to chemical pesticides. Chitosan and salicylic acid were applied as Pre-harvest treatment at the concentration of 2000 mg/l and 250 mg/L under field the conditions on three different ripening stages of tomato fruits Turning, Pink, Light Red stages and to determine the best stage for application. The best concentration was determined in enhancing plant defenses against the pathogen and studying the effect of factors on the levels of some enzymes related to induction of resistance, such as peroxidase enzyme POD and PAL enzyme. The application of SA and CH had a significant effect in increasing the activity level of both enzymes, and treatment with salicylic revealed to an increase in the activity of POD enzyme in the treated plants and for the three fruit maturity stages (Turning, Pink, Light red), where the enzyme level was 17.33, 16.72 and 15.5 min /gm. The three stages of fruit ripening had an effect on increasing the activity of the POD enzyme, as it recorded 12.27, 12.12, 12.1 min/gm fresh weight, and the ripening stage affected the PAL enzyme, which was recorded at a level of 32.27, 31.53, 31.87 mg/gm fresh weight, for the three stages, respectively. Chitosan and salicylic did not differ in extending the shelf life of fruits and reducing losses depending on the rate of healthy fruits for the three stages at the end of the storage period at 25°C. Chitosan at a concentration of 2000 mg^{-1}/L differed significantly from other concentrations in inhibiting the pathogenic fungus, as well as salicyac at a concentration of 250 mg^{-1}/L was superior in its effectiveness compared with other concentrations. However, chitosan had a significant effect on the biofilm formation of *B. cinerea*, which displayed an average absorption degree of 0.53 (OD), while salicylic did not affect fungal biofilm formation, as the average absorption degree was 1.2 (OD) which did not differ from the control treatment.

Keywords: gray mold, tomato, chitosan, salicylic acid.

Introduction

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable or fruit crop after potato (13). Due to the expansion that occurred in recent years in the production of vegetables, including tomatoes, devastating diseases of economic importance fruit and vegetables including tomato, such as gray mold caused by the fungus *Botrytis cinerea* have appeared with appropriate conditions (28).

The fungus *B. cinerea* is a phytopathogenic, infecting more than 500 plant families with gray mold and causing losses of economically important fruits .The pathogen ranked second in the list of the ten most important fungal plant pathogens in the worldwide(10 and 38). The fungus causes losses of up to 40% in protected cultivation or cultivation in the fields if the control is not carried out in a timely manner, especially when the conditions are suitable for the spread and growth of the fungus (36)

The economic damage due to the fungus is caused by the invasion of flowers, florals and stems, and symptoms are characterized by yellowing and wilting of leaves and flowers (39). The virulence of the fungus isolates varies in the severity of infection due to the difference in the enzymes secreted to destroy the plant cell wall (16). Moreover, the ability of the fungus to form biofilm is an important factor in the virulence of the fungus in laboratory or field conditions on tomato stems (18). Therefore, the control of biofilms is important in controlling plant pathogens (22). In general, most of the losses caused by gray mold are during the production stages and before or through harvesting, storage, and marketing to the consumer disease control is very important during storage due to the rapid development of the disease at low temperatures (0 to 4 °C) (14). In the last century, farmers relied largely on chemical pesticides and fertilizers to increase crop production and reduce losses. Excessive and unregulated use of chemicals are resulting in pollution of the environment and affect human health. This prompted researchers to find alternatives to manage diseases and pests and reduce the use of chemical pesticides (9).Chitosan is a naturally occurring polysaccharide derived from Chitin that has been shown to control many pathogens and extend the shelf life of stored fruits and vegetables (24). Several reports indicated the possibility of using Chitosan as an antimicrobial, affecting spore germination and fungal growth, including *Botrytis cinerea* (20 and 21). Salicylic acid plays an important role in strengthening the plant's defenses. SA is found in different parts of vascular plants as it enhances the plant's ability to rapidly adapt when exposed to biotic or abiotic stress. The SA pathways within the plant lead to the stimulation of a number of genes and metabolic rate, and alter the plant's physiology by making it more resistant to various stress conditions including infection with various pathogens (15).

It is important to find an effective way to control gray mold on tomato crops using substitute factors that are safe for the environment and human health, few side effects, and, cost- effective. Therefore, the objective of this study is the evaluation of the efficiency of applying chitosan and

salicylic acid in reducing the losses during pre and post-harvest duration.

Materials and Methods

Samples of tomato fruits were collected from some tomato wholesaler locations and tomato fields in Al- Najaf province based on the common symptoms of the disease represented by gray to brown discoloration of the affected parts with the appearance of gray or brown thick growth, the pathogen was isolated (1). 10 different isolates of *B. cinerea* were obtained that were phenotypically diagnosed based on the taxonomic key (37). Then the most virulent isolate that was used in all subsequent experiments was selected and the molecular diagnosis was confirmed.

Pathogenicity test

Pathological testing of the fungus isolates was carried out on 4-week-old tomato Aigen plants grown in pots. The plants were treated by spraying with fungal spores at a concentration of 10^5 (which was calculated in advance by hemocytometer) and covered with plastic bags for 24 hours, after which the plants were ventilated and returned to the plastic house for 14 days. The severity of the disease was calculated using the injury severity scale (0-5) (1) .The results showed the superiority of the three isolates B.C1, B.C2 and B.C3 in terms of disease severity over tomato plants, and the three isolates were selected to conduct the rest of the pathogenicity experiments. The pathogenesis of B.C1, B.C2 and B.C3 isolates were tested on the leaves and fruit of tomato cultivar Aigen.

Efficacy of CHI and SA in inhibiting the fungus *Botrytis cinerea*

The effectiveness of chitosan was tested at concentrations (250, 500, 750, 1000, 1500 and 2000 mg^{-1}/L) in inhibiting pathogenic fungi in Petri dishes on a P.D.A. medium. Chitosan was dissolved according to the method used by Paul *et al. (*28) with some modifications where oxalic acid was used as a substitute for lactic acid in the same proportions used in the solution, while the effectiveness of salicylic acid in inhibiting the pathogenic fungus was tested at concentrations of 50, 75, 100, 150,200,250,300,400 mg^{-1}.L in Petri dishes on P.D.A medium (6).

The efficiency of chitosan and salicylic acid in inhibiting pathogenic fungus biofilm

The ability of Chitosan 0.002 $mg^{-1}.ml^{-1}$ and Salicylic acid 0.0025 mg^{-1}/ml to inhibit the biofilm formation of *Botrytis cinerea* was also tested in vitro according to the method described before (17). All wells were examined using a light microscope with a power of 40X magnification connected with a camera. The dry layer was dissolved in each hole by adding 200 µl of ethanol at a concentration of 80%. Absorbance rate was calculated for all treatments and comparison in the spectrophotometer after reading the ethanol and dye as a standard before measuring the treatments at a wavelength of 595 nm.

Efficacy chitosan and salicylic acid in inducing resistance enzymes Peroxidase (POD) and Phenylalanine ammonia-lyase (PAL) after greenhouse spraying

The treatment was done with fungus spores on the fruits and for three different stages of crop maturity (Turning, Pink, and Light Red) (31), each treatment was

repeated three times. The treatment was carried out with fungal spores according to the method used by Romanazzi *et al*. (30). The treatments were applied using a 2-liter manual sprayer. The treatments were with chitosan and salicylic acid. After 14 days of treatment. The estimation of the peroxidase enzyme activity was measured using a spectrophotometer to calculate the effect of the applied treatments on the peroxidase enzyme activity in tomato plants according to the (2).The activity of the enzyme PAL was also estimated based on the rate of conversion of phenylalanine to cinnamic acid at the wavelength of 290 nm by following the method (32).

Effect of applying chitosan and salicylic acid on tomato fruit shelf life

After applying the treatments on the ripening stages of tomatoes (Turning, Pink, Light Red), the fruits were harvested after reaching the stage of maturity and stored at room temperature for 10 days in plastic boxes 15 fruits for each replicate and three replicates for each stage (2). The efficiency of the bacteria was evaluated based on the number of non-infected fruits after the end of the storage period.

Experimental design and statistical analysis

All laboratory experiments were carried out according to the Complete Randomize Design (CRD). The field experiment was designed using Block Complete Randomize Design (RCBD) with three replications. Data were statically analyzed using Genstate 2009 computing program. Means were compared among treatments using Duncan's multiple range tests ($P \leq 0.05$)

Results

Isolation and identification

The results of the isolation showed that 10 different isolates of the pathogenic fungus *B. cinerea* were obtained out of 75 samples collected from different areas (Al-Haidriyah, Markaz district as well as local markets) of tomato fields in Najaf Governorate. The fungal isolates were purified and identified in the Plant Pathology Laboratory at the College of Agriculture / University of Kufa using a taxonomic key (38). The pathogenic isolate was confirmed molecularly in previous work using polymer chair reaction with ITS3- ITS4 primer (33)

Pathogenicity test

The results showed that *Botrytis cinerea* isolate B.C3 was the most virulent among the tested fungal isolated and was diagnosed phenotypically and molecularly. The B.C3 isolate showed high pathogenicity and severity on tomato plants, leaves and fruits.

Efficacy of chitosan in inhibiting the pathogen *Botrytis cinerea*

The results showed the effect of chitosan at several concentrations on the growth of the pathogenic fungus *B. cinerea* that ketosis at a concentration of 2000 mg/L showed to the highest inhibition rate of 94.4%, with a significant difference of 0.05 compared to the two control treatments (P.D.A + Oxalic acid), which allowed the fungus to grow completely (Figure1). In relation to the rest of the other concentrations, the concentration of 1500 mg/L exhibited the inhibition rate of 68.87% that was higher than that of 1000, 750, 500 and 250 mg/L inhibition of the

pathogenic fungus 66.97%, 66.97%, 66.03% and 66.67%, respectively, which did not differ among each other (Figure1).

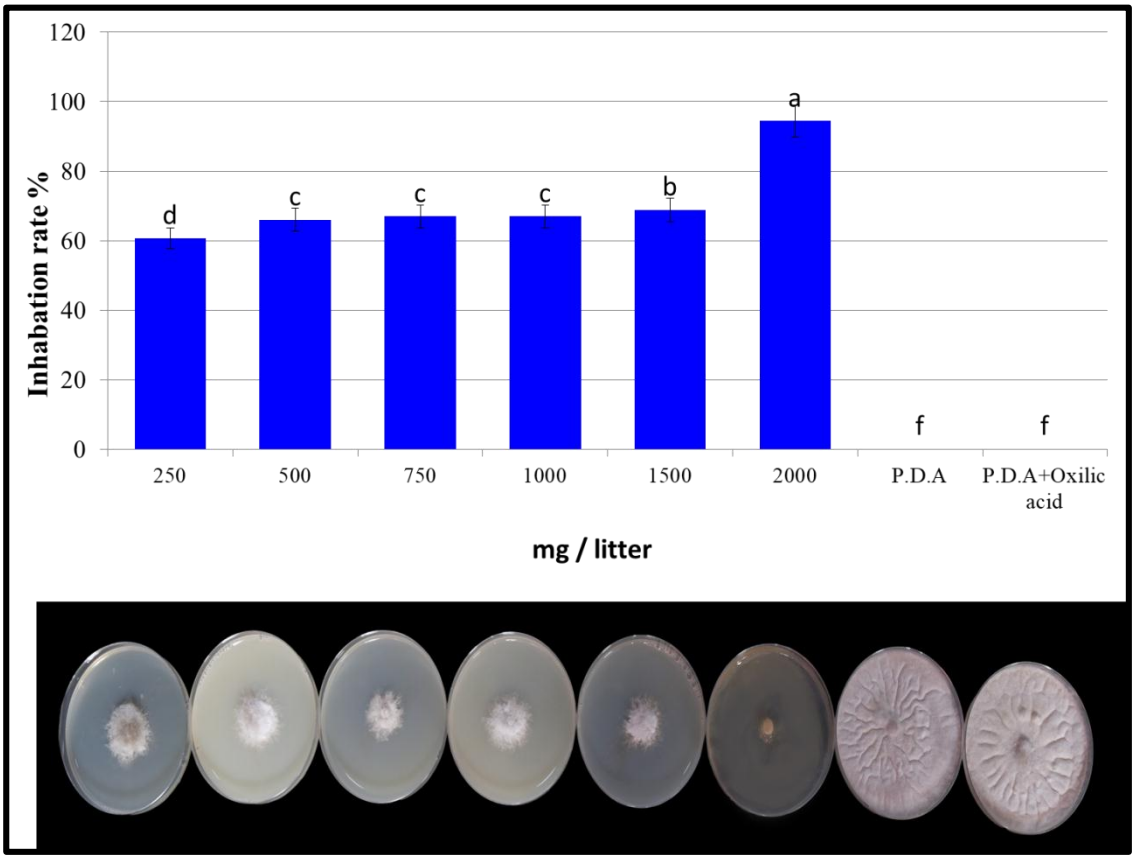

Figure1. effect of chitosan at different concentrations (column and plate) on growth (inhibition) of the pathogenic fungus *B. cinerea* on PDA or PDA + oxalic acid medium incubated at 25±2°C. Columns that have same letter are not significantly different according to Duncan's multiple range test (*P*≤0.05).

Effectiveness of salicylic in inhibiting the pathogenic fungus *Botrytis cinerea*

The results showed that salicylic acid (SA) at concentrations 250, 300 and 400 mg^{-1}/L inhibited the pathogenic fungus *B. cinerea* with 94.4% at 400 mg^{-1}/L which is a significant difference (P≤0.05) compared to the control treatment and the rest of the concentrations used, which did not differ among each (Figure2). In general, salicylic acid at concentrations of 200, 150 and 100 mg^{-1}/L had different inhibition rates of 91.2%, 91.1% and 90.7%, respectively. The lowest percentage of pathogenic fungi inhibition was at the concentrations 50 and 25 mg^{-1}/L, which showed the inhibition of pathogenic fungi by 36.97% and 14.77%, respectively (Figure2).

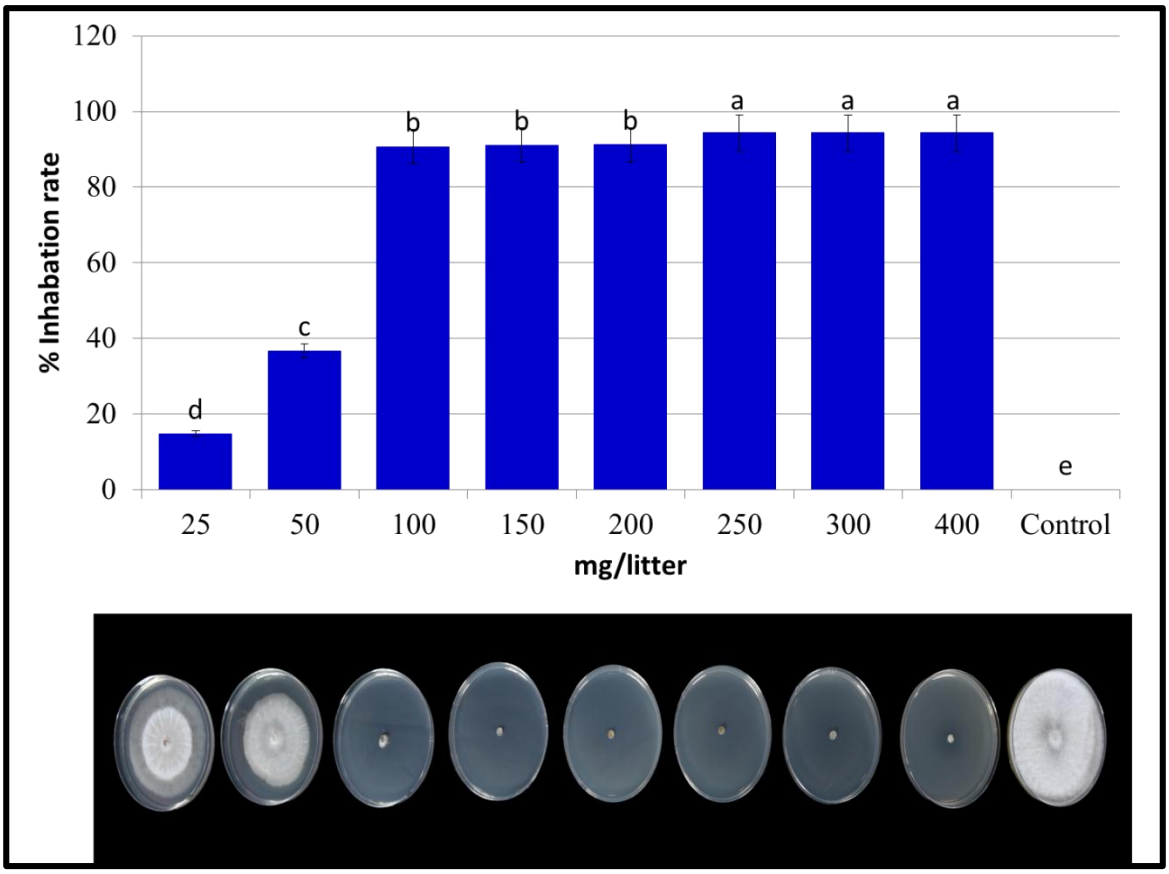

Figure2. Effect of Salicylic acid at different concentrations (Column and plate that correspond to) on growth (inhibition) of the pathogenic fungus *B. cinerea* on PDA medium incubated at 25±2°C. Columns that have same letter are not significantly different according to Duncan's multiple range test (*P*≤0.05).

The ability of CH and SA to inhibit the *Botrytis cinerea* biofilm formation

The results showed that chitosan a clear ability to inhibit fungal (Biofilm) formation with a significant difference from salicylic acid treatment (P≤0.05). It was found that the lowest rate of absorbance was 0.53 nm at the concentration of 0.002 mg/ml with a significant difference from salicylic at a concentration of 0.0025 mg/ml, and the control, (Figure3).

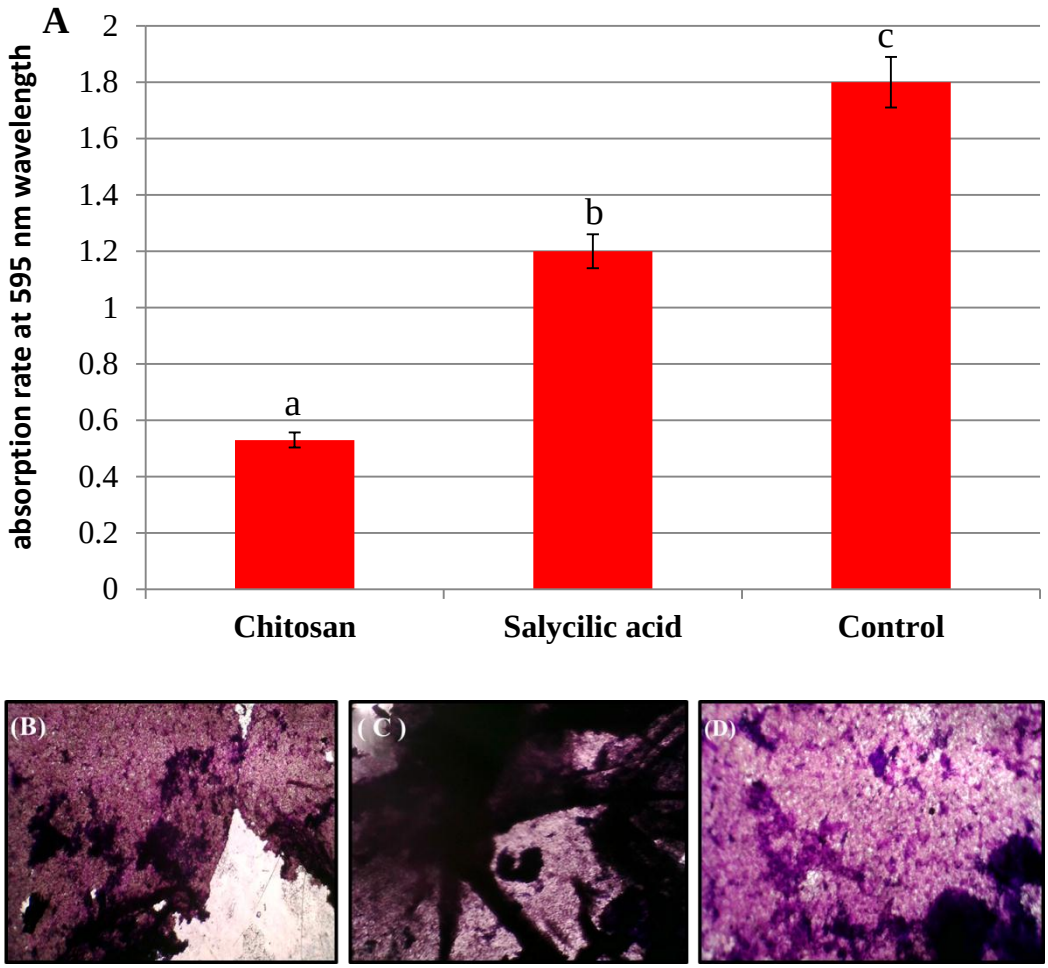

Figure 3. A) Effect of Chitosan and Salicylic acid on absorption rate at 595 nm wavelength. Bars are for mean and standard error (SE±). The different letters indicate the differences in the rate of absorption (OD) according to Duncan's test (P≤0.05) (B and C) Anti-biofilm activity of CHI and SA respectively using light microscopy at magnification (40X)D) Fungal Biofilm (control)

Effect of chitosan and salicylic on the activity of peroxidase enzyme POD and PAL enzyme in tomato plant

The results showed that the treatment of tomato plants with salicylic displayed an increase in the level of peroxidase (16.52 min/gm fresh weight) with a significant difference (P≤0.05) compared to the chitosan and control treatments, in which the enzyme level was 12.16, 6.95 min/gm fresh weight (Table 1). The Turning phase had the highest response to the effect of treatments on the level of peroxidase 12.58 min/gm fresh weight with a significant difference compared to the Pink and Light Red phases, in which the enzyme level was 11.75, 11.3 min/gm fresh weight. In general, the highest level of POD was in the treatment of salicylic in the turning stage, and Pink, which showed increasing in the enzyme to 17.33, 16.72 min/gm of fresh weight. Salicylic also had the same effect in the Light Red stage of the crop ripening in the Pink stage.

Table1. Effect of CHI and SA on the level of POD enzyme in tomato leaves after 14 days of treatment during the maturity stages (Turning, Pink, and Light Red)

Treatment	POD			Average
	Turning	Pink	Light Red	
Salicylic acid	17.33^{a}	16.72^{ab}	15.5^{b}	16.52^{a}
Chitosan	12.27^{c}	12.12^{c}	12.1^{c}	12.16^{b}
Negitive control	8.13^{d}	6.42^{e}	6.3^{e}	6.95^{c}
Average	12.58^{a}	11.75^{b}	11.3^{b}	

The values means for four replicates, means followed by the same letter(s) are not significantly different according to Duncan's multiple range test (P≤0.05).

The treatment of tomato plants with salicylic and chitosan did not differ in increasing the level of PAL enzyme with 32.09 and 32.08 mg/L fresh weight, respectively, but it differed significantly (P≤0.05) from the control treatment 21.36 mg/L of fresh weight Table (2). The Turning stage showed a greater response to the treatments and an effect on the level of PAL enzyme 29.03 mg/L of fresh weight, with a significant difference from the Pink and Light Red stages, which recorded 28.26 and 28.23 mg/g fresh weight, respectively.

In general, the highest level of PAL enzyme was recorded in the treatment of salicylic and chitosan in the Turning phase and in the treatment of salicylic in the Pink phase with significant difference from the other treatments. Treatment with salicylic and chitosan in the Light Red stage led to an increase in the level of the enzyme and did not differ from that of the chitosan treatment in the Pink stage. Salicylic and chitosan treatments in the Light Red phase had the same effect on the level of PAL enzyme (Table 2).

Table2. Effect of chitosan and salicylic acid on the level of PAL enzyme in tomato leaves after 14 days of treatment during the maturity stages (Turning, Pink, and Light Red)

Treatment	PAL			Average
	Turning	Pink	Light Red	
Salicylic acid	32.29^{a}	32.1^{a}	31.93^{bc}	32.09^{a}
Chitosan	32.27^{a}	31.53^{bc}	31.87^{c}	32.08^{a}
Negitive control	22.03^{d}	21.07^{e}	20.97^{e}	21.36^{b}
Average	28.86^{a}	28.23^{b}	28.25^{b}	

The values means for four replicates, means followed by the same letter(s) are not significantly different according to Duncan's multiple range test (P≤0.05).

The effect of CHI and SA on extending storage life and crop quality

The results showed that treatment with the study factors led to a prolongation of the storage life of tomato fruits. The Turning stage did not differ from Pink in the number of healthy fruits, but they differed significantly from the healthy fruits in the stored control treatment for both stages.

The application of salicylic and chitosan in the Light Red stage had the same effect on the average number of healthy fruits (11 healthy fruits) for both treatments compared with the control (Figure4).

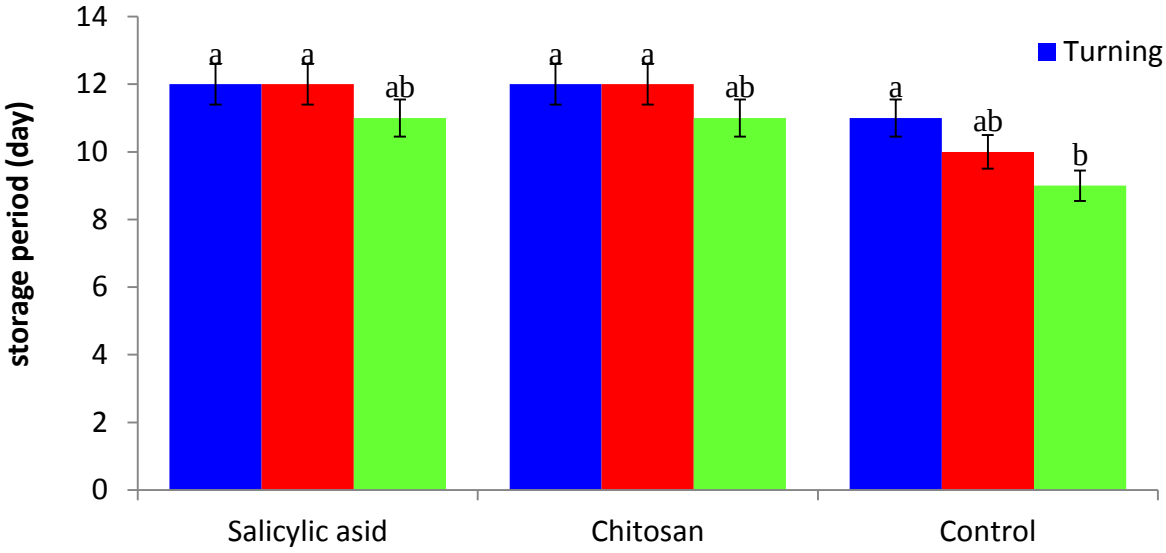

Figure4. Effect of applying SA and CHI on the average number of healthy fruits of ripening stages (Turning, Pink, Light Red) after 10 days of storage at a temperature of 25 °C, where the vertical bars show the averages and standard error (SE±). Bars that have different letters indicate significant differences according to Duncan's multiple range test (*P*≤0.05).

Discussion

Botrytis cinerea occupies the second place, in terms of economic and scientific importance, among the ten most important causes of fungal plant diseases globally, affecting many plant families of fruits and vegetables (39). It is considered one of the most important pathogens after harvest when favorable conditions exist in the post-harvest circulation chain that includes wounds, high humidity, tissue aging, high sugar content in fruits and vegetables (30).

The results showed the effectiveness of chitosan in inhibiting the growth of pathogenic fungus *B. cinerea*, as the percentage of inhibition increased with increasing concentrations.

In another study (3) indicated that the percentage of inhibition of *B. cinerea* increased by increasing the concentration of chitosan, which led to an inhibition of 87.84% at a concentration of 125 mg^{-1}/L

Chitosan affects the growth of the fungus and reduces the spore formation of *B. cinerea*. The inhibition of the fungus increases with the increase in the concentrations used (28) . The effectiveness of chitosan is attributed to the energetic effects the fungal cell and the plasma membrane that lead to the exudation of the cell contents. Subsequent studies indicated that chitosan, as a multi-

linked compound with molecular weight, directly affects cell walls, changes the permeability of cell membranes, and preventing DNA replication, and thus cell death (11)

The use of chitosan as an antibiotic is affected by a number of factors such as the type of pathogen, pH, structural properties, source of chitosan, and its concentration (19). Chitosan increases the level of peroxidase and makes the plant show higher defense reactions (7). It may lead to an increase in the activity of NADPH oxidase, causing an increase in peroxidase and activation of ROS (36). The foliar spray with chitosan on eggplant led to an increase in POD level after 7 days, the highest level was after 14 days, which decreased after 21 days (3). The increased levels of PAL may be attributed to the effect of chitosan on plant cell receptors leading to a molecular response and activation of reactive oxygen groups and an increase in the activity of pathogenicity-related genes (PRP) (7). The results were similar to (3) about the effectiveness of chitosan in increasing the level of PAL enzyme in the foliar-sprayed eggplant.

The results of the study agreed with the previous results about the efficiency of SA in inhibiting pathogenic fungi, where generally the percentage of inhibition increased with the increase in the concentration used (3). In culture media, SA has a direct effect on the biological processes of growth and development of the fungus, and leads to slow growth and inhibition and affecting the production and germination of spores, especially at high concentrations of SA (20).

The effect of salicylic acid in increasing the level of peroxidase enzyme is due to its regulatory role in the levels of reactive oxygen species (ROS), which have a role in controlling the activity of some enzymes, including peroxidase enzyme, thus improving growth processes and reducing infection with pathogens (14).

The effect of chitosan in extending storage life is attributed to the interaction of chitosan with plant tissues (30). The interaction by increasing secondary metabolites inside the fruits, which have a role in enhancing defenses and the formation of lignin, callus, phytoalexins, PAL enzyme and peroxidase (23) . Chitosan has an important role in inhibiting post-harvest fungal pathogens and reducing their losses, activating anti-enzymes such as PAL enzyme and Chatinase enzyme ß-1,3glucanase, on the other hand, salicylic acid leads to an enhancement of plant resistance before harvest, an increase in the content of phenols in fruits, including peroxidase and PAL enzyme, and the activity of antioxidant compounds after harvest, and an increase in the storage period of the yield (21).

Conclusion

The study showed that chitosan and salicylic acid had an inhibitory effect on the growth of pathogenic fungus *B. cinerea*. Chitosan showed a significant effect on inhibiting the biofilm formation of the fungus in vitro. The application of the two study factors together in the field on the stages of (Turning, Pink, Light Red) ripening of the tomato crop before harvest had a significant effect in increasing the level of the enzymes POD and PAL. This means enhancing plant

resistance, increasing storage life and reducing post-harvest losses by affecting the rate of the number of not-infected fruits.

Conflict of interest

The authors have no conflict of interest.

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