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Postharvest Decay Control of Strawberry Gray Mold by Application of SodiumBicarbonate and Vinegar Combination

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Abstract
Gray mold caused by *Botrytis cinerea* is one of the most prevalent fungal diseases present in fruits and vegetables worldwide. Four *Botrytis* isolates collected from different sites in Al-Najaf province in Iraq were identified morphologically and confirmed by molecular analysis using ITS3- ITS4 region. Pathogenicity test was performed on detached leaves and strawberry fruit to determine the most pathogenic *Botrytis* isolates. Lesion diameter was the highest when detached leaves and fruit were inoculated with Bc1. However,the development of resistant isolates and people concern for food safety leads to explore new strategies for controlling postharvest disease. In current study, the effect of Sodium bicarbonate (SB) with Vinegar on *B.cinerea* using Bc1 isolate as a model of high virulence was evaluated. Complete inhibition of fungal mycelium and spore germination were achievedby SB-vinegar mixture at 50 mM. Fungicidal activity was validated by quantification of soluble proteins released from treated mycelium with different concentrations of SB-Vinegar mixture. Gray mold severity on inoculated strawberry fruit was significantly reduced by dipping diseased fruit in SB-Vinegar mixture at 50 mM for 10 min. SB-Vinegar mixture is safe compounds for human health and promising alternative approach to other fungicides for controlling postharvest-gray mold disease of strawberry caused by *B.cinerea*.
***Key words*:** Postharvest disease, *Botrytis cinerea*, Strawberry, Sodium bicarbonate, Vinegar

Introduction

Strawberry (*Fragaria x ananassa* Duch) is one of the most diet rich fruit worldwide. Strawberry possesses several nutrition compositions including vitamin C, Minerals, folates and antioxidant compounds that are crucial for human health (Giampieri et al., 2014). Strawberry is highly perishable and susceptible host for postharvest decay. *Botrytis cinerea* can significantly cause quantitative losses on strawberry and considered to be one of the most a predominant and aggressive disease in both pre- and postharvest period. Fungal infection can be initiated in the field, storage and during transit (Mertely *et al*., 2000). *B. cinerea* is ubiquities filamentous fungus, which rated the second economic importance fungal pathogen .Gray mold disease, is complicated to control if the environmental conditions are appropriate for it (Dean *et al*., 2012). Humidity of 85% and temperature between 15-23˚C are preference in development this pathogen on strawberry. *Botrytis cinerea* shows apparent disease symptoms on any parts of infected plant or remain as latent infection until reach a costumer (Elad *et al*, 2007). The sexual reproduction of *B.cinerea* is seclortia, which formed for survival throughout inappropriate and sever conditions. However, Conidia, asexual stage of the pathogen format in enormous numbers are able to be dispersion by wind and water to other plants. To date, synthetic fungicides are the core approach for the control of pre- post harvest diseases. Application of fungicides is not adequate to control gray mold because of

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the ability of *B.cinerea* to change its genome functionality in various environments and habitats. Public concern of food safety and using of pesticides, development of resistant by *B.cinerea* to most used fungicides, few efficient methods to decontaminated infected strawberry fruit by *B.cinerea* leads to investigate alternative and safe strategies to control the gray mold.

Recently, studies have revealed the efficiency of diverse organic and inorganic salts including potassium tetraboarte tetrahydrate, potassium carbonate, sodium benzoate to control postharvest disease such as gray mold on grape (Qin *et al*, 2010) soft rot of tomato (Ahmed *et al*., 2017), anthracnose decay on mango (Shi *et al*., 2012). In addition, Ahmed *et al*., 2018 showed that Potassium tetraboarte tetrahydrate was successful inorganic salt to reduce gray mold of tomato when applied as pre-harvest treatment in the greenhouse. Sodium bicarbonate SB ($NaHCO_3$) is recognized as baking powder and it one of the most widespread, safe, and available salts used as an additive in food, texture modification, dough leavening, mineral water and medicinal treatments worldwide (Medeswaran *et al*., 2018, Mirrakhimov *et al*., 2017). SB has been used in industry in considerable amount as safe compound for making all dough products. The US Food and Drug Administration approved that sodium bicarbonate as nontoxic compound on all Agriculture products. Several studies have been documented that sodium bicarbonate possess low fungistatic action (Fallir *et al*., 1997; Zamani *et al*., 2007). Vinegar is acetic acid compound resulted from the fermentation of macerated apple in distilled water for certain time. The reaction of SB and vinegar mixture generates dioxide carbon which can be highly effective agent to inhibit and kill the fungus. The combination of SB and Vinegar would work as fungicide effect. However, there is not documentation of use sodium bicarbonate with vinegar to control postharvest diseases caused by *B.cinerea.* The aim of this study was to examine the effectiveness of sodium bicarbonate with vinegar combination for decreasing gray mold of strawberry.

Material and Methods

Fungal isolate and pathogenicity tests

Four fungal isolate were isolated from diseased strawberry fruit collected from 12 stores located throughout Al-Najaf province, Iraq. Small pieces were cut from the edge of lesion and cultured on water agar. Single spore or hyphal tip were transferred to V8 medium and incubated for 10 d at 23˚C. The isolates were stored at 4C˚ on sterile toothpicks for further experiments. Pathogenicity tests were conducted to verify the most pathogenic isolates of *B.cinerea* (Bc1, Bc2, Bc3, and Bc4) on detached leaves and strawberry fruit. Three weeks-old strawberry leaves were surface sterilized with 70% ethanol, sterilized leaves positioned in Petri dishes including wet filter paper. Fungal plug were placed on the leaves surface and plates held for 72 h at 23˚C. PDA plug with no fungus saved as control. On strawberry fruit, spore suspension of $1x10^5$ spores/ml was prepared from 10-day-old culture of each isolate of *B.cinerea.* Healthy fruits inoculated with 10 µl of spore suspension using pipette tips. Fruits inoculated with sterilized water saved as control treatment. Lesion diameter of inoculated fruit and detached leaves were measured (Ahmed *et al*., 2016)..

DNA isolation and Endpoint PCR

The microbial DNA isolates kit (Promega, Madison, WI) was used to extract the DNA from all fungal isolates following the manufactures' instructions. The ITS gene region of fungal

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isolates was amplified using the primer set ITS3 (Evan *et al*., 2014). Each PCR reaction included 10 µl of Red Taq (Promega), 1µl of each primer, 4 µl templates DNA, and 4µl Pure-distilled water. PCR was performed using a PCR system (BIO-RAD) with initial denaturation for 3 min at 95°C, succeeding by 36 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C, and extension for 1 min at 72°C. A final extension for 5 min at 72°C was performed. Each PCR reaction was run with a negative control (no DNA). Agarose gels 1.5% was used to separate all PCR amplicons, stained with 0.4 µg/ml ethidium bromide, and bands visualized with a UV illuminator. DNA concentration estimates were considered by comparing the fluorescent intensities of the product with DNA standards on a 2-log ladder (iNtRON Biotechnology, Inc., Korea). Amplified PCR product treated with ExoSAP-1T (iNtRON Biotechnology, Inc., Korea). A mix of 5 µl of post-PCR reaction and 2 µl ExoSAP-IT reagents were combined. The mix was incubated at 37°C for 15 min subsequent by incubation at 80°C for 15 min. Each purified template was sequenced on both strands using two contiguous primers (ITS3- ITS4). The sequences of ITS 3 and 4 regions of the tested isolates were edited in order to generate arranged sequences from forward and reverse sequences in the amplicons using sequence assembly software (DNA BASER). A consensus sequence was analyzed by NCBI BLAST database seeking fungal identities.

The Antifungal Activity of Sodium Bicarbonate-Vinegar Mixture on Mycelial Growth

The pathogenic isolates of *B. cinerea* (Bc1) were tested for susceptibility on sodium bicarbonate and vinegar mixture. PDA medium was autoclaved and allowed to cool at 45˚C and supplemented with sterilized SB and vinegar using filter Millipore 0.22 mm at final concentration of 0, 10, 25, 50, 100 mM. Fungal plugs 6 mm in diameter were acquired from 10 days old culture and placed on 90 mm plate containing SB-Vinegar mixture. All plates and control (PDA alone) were incubated at 23˚C. The measurement of mycelial growth was conducted when the growth in control Petri dishes contact the edge of the plate.

Inhibitory Effect of SB – Vinegar mixture on Spore Germination

A test of inhibitory effect of sodium bicarbonate-Vinegar mixture on the spore germination of *B. cinerea* (Bc1) was examined following (Qin *et al*., 2003) with slight modification. Potato dextrose broth (PDB) was autoclaved and allowed to cool at 45˚C. Sterilized PDB supplemented with and without 0, 10, 25, 50, and 100 SB-Vinegar mixture mM/mM and transferred into 96 wells micro plate. Spores suspension ($1x10^5$ spore/mL) of *B.cinerea* were prepared and 50 µl of aliquots were inoculated into the wells. The micro plates were incubated for 8 h at 23 ˚C. Ten µl of incubated broth placed on slide and visualized under a light microscope. At least 200 spores were calculated in the microscope field for germination average and germinated spores were presented as percentage of the total number of calculated spores. There were four replicates for each treatment.

Determination of soluble proteins leakage

The assessment of soluble protein leakage from mycelium of *B.cinerea* was performed following (Lewis et al., 1987) with some modifications. Mycelium of *B.cinerea* (Bc1) was grown on PDB in (500 ml flask for 4 days at 23˚ C with shaking at 100 rpm. The mycelia were collected and washed twice with sterile dH_2O, then washed mycelia resuspended in sterile dH_2O containing sodium bicarbonate-vinegar mixture (0, 10, 25, 50, and 100 mM/mM) and shacked on a rotary at 23˚C for 4 and 8 h. The dry weight of mycelia was measured after filtered from the solutions that were used to determine the soluble proteins.

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Released proteins were quantified following Bradford test (Bradford 1976). Values of soluble proteins were measured as milligrams per gram of dry weight of mycelium.

Efficacy of SB –Vinegar Mixture on Gray Mold disease Symptoms on Strawberry Fruit

A test of protection strawberry fruits from gray mold caused by *B.cinerea* was conducted using sodium carbonate- Vinegar mixture following (Ahmed *et al*., 2017) with some modifications. Strawberry fruits were selected as uniform in size, free from wounds and decay. The fruit were washed two times with tap water and surface sterilized in 1% NaOH for 5 min, and then fruits were rinsed in sterilized water twice and dried at room temperature in sterilized conditions. Fruits were wounded (1 mm diameter) using a pipette tip and immersed in the different SB-Vinegar concentrations (0, 10. 25, 50, and 100 mM) for 10 min, then dried at room temperature. Wounds were inoculated with a 20 µl aliquot including $1x10^5$ spore/ml of *B.cinerea*. Fruit were transferred into plastic container containing wet paper towel and incubated for 3 days at 23˚C. The assessment of disease severity was conducted by measuring the lesion diameter on each fruit. The lesion diameter was the average of eight fruits in each treatment.

Data Analysis

The experiments were arranged as complete randomized design (CRD) with five replications. All data analyses were accomplished using (SAS version 9.2), and means dissimilarity were compared by Duncan's multiple range test. Different at ($P < 0.05$) were considered significant. All experiments were repeated twice.

Results and discussion

Isolates pathogenicity

The pathogenicity test showed that *B.cinerea* (Bc1) was the most pathogenic and virulent isolates on deatached leaves and strwberry fruit moung other three fungal isolates (Bc2, Bc3, and Bc4) **(Table 1 and Fig. 1)**. The heighest lesion diameter on detached leaves and fruit was in *B.cinerea* (Bc1) with 45, 69 mm respectively. The surface of inoculated fruit and deatached leaves with *B.cinerea* (Bc1) was mostly covered with fungal mycelim. Several studies rported that fungi grow saprophytically on strawberry and tomato (Ellis, 2008; Agrios, 2005; Elad *et al*., 2007; Ahmed *et al*., 2016; Ahmed *et al*., 2017). Deviation of pathogenic fungi in secretion of pathogenecity factors including cell wall degardin enz

Table 1. Lesion diameter (mm) of *Botrytis cinerea* isolates on detached leaves and strwberry fruit 72h after incubation at 23˚C

Fungal isolates	Lesion diameter (mm)	
	Detached leaves	Fruit
Bc1	45.00^a	69.00^a
Bc2	3.00^b	5.00^b
Bc3	5.00^b	6.00^b
Bc4	3.00^b	4.00^b

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Control (Distilled water)	0.00^{c}	0.00^{c}

*Mean values followed by different letters within a column are different according to Duncan's multiple range tests ($p \leq 0.05$)

and oxalic acid are regularly as a consequence of variable virulences among pathogenes (Ten Have *et al*., 2002; Bellincampi *et al*., 2014). Other studies documented that the pathogenicity of *B.cinerea* is relied on its ability for production of several pathogenic factors such as toxins, reactive oxygen species and cell degarding enzymes (Choquer *et al*., 2007; Dalmais *et al*., 2011).

Confirmation of Molcular Idntification

All fungal isolates were confirmed by successful amplification of the ITS3-ITS4 region **(Fig.2)**. Obtained sequences of identified fungi were < 98% similarity compared with NCBI BLAST (https://www.ncbi.nlm.nih.gov/). *B. cinerea* was counterpart at 100 % similarity.

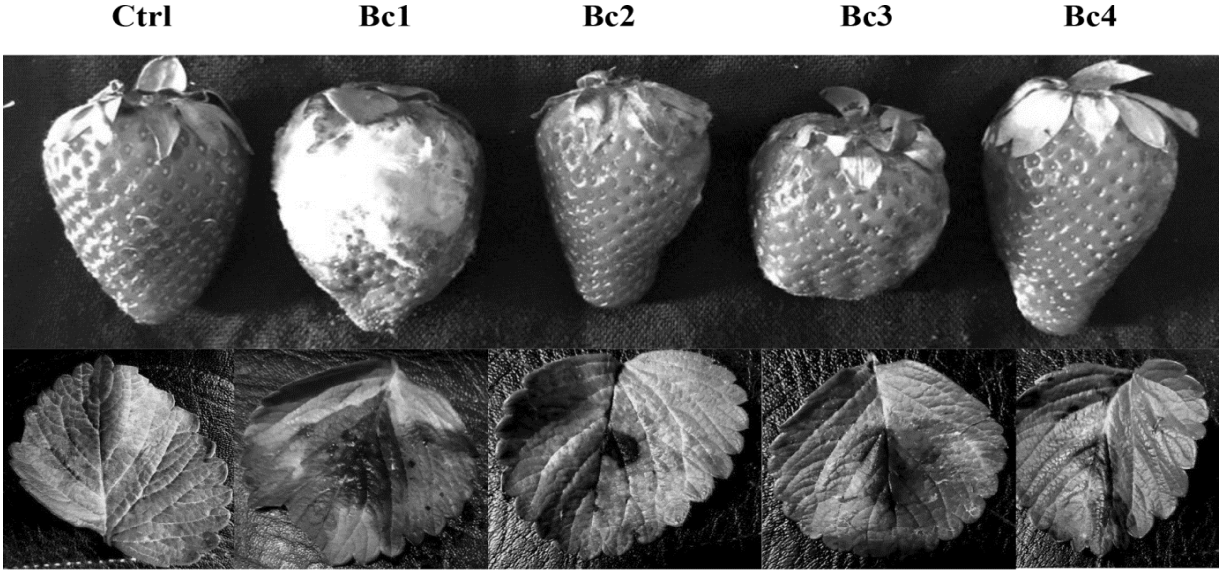

Fig. 1 Pathogeicity test of *Botrytis cinerea* isolates on detached leaves and strawberry fruit 72 h after incubation with spore suspension of *Botrytis cinerea* (Bc1) at 23˚C.

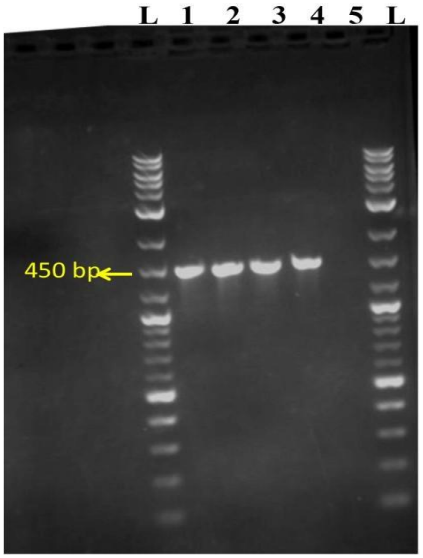

Fig. 2 The gel electrophoresis of PCR products using DNA from isolated fungi. L= DNA ladder (0.1- 10.0 kb); 1-4= *Botrytis cinerea* (Bc1, Bc2, Bc3, Bc4); 5= NC (No DNA).

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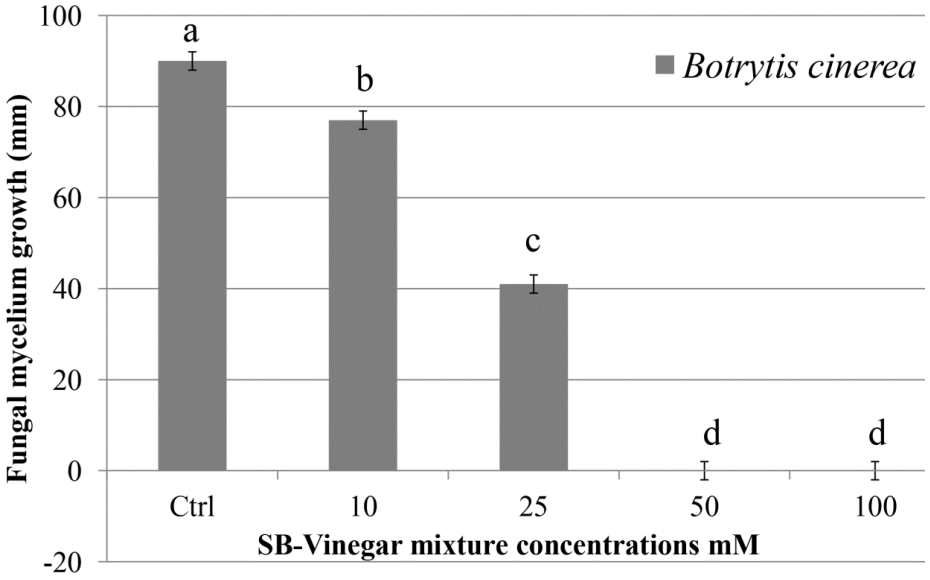
Inhibitory Effect of Sodium Bicarbonate-Vinegar Mixture on Mycelial Growth and spore germination

The sodium bicarbonate-vinegar mixture was efficient in inhibition the mycelium growth of *B.cinerea* on PDA **(Fig. 3)**. The most effective concentration of SB-vinegar concentration

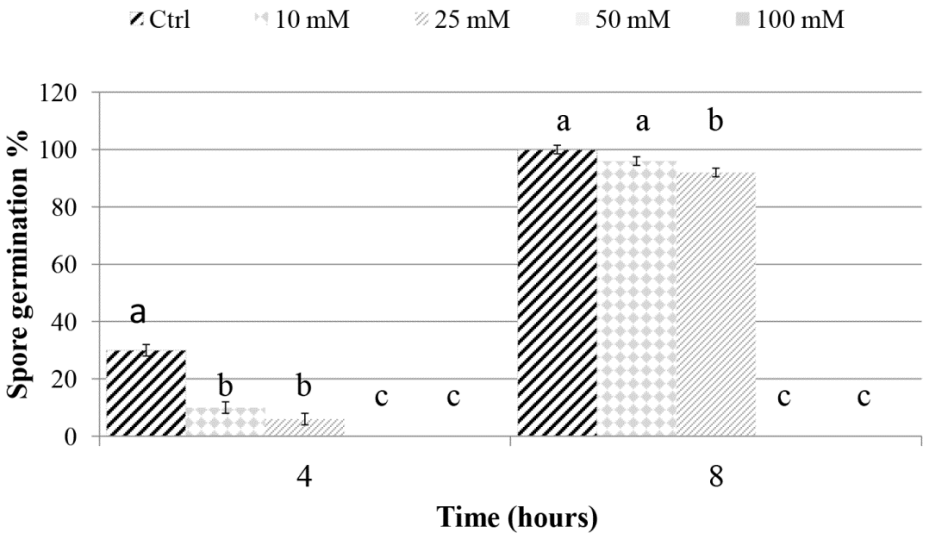


that completely inhibited the mycelial growth was 50 mm and there was no significant

Fig. 3 Efficacy of sodium bicarbonate-vinegar mixture at different concentrations on mycelial growth of *Botrytis cinerea* (Bc1) on PDA medium at 23˚C. Treatments showing different letters indicating statistically different by Duncan's multiple range test ($P < 0.05$)

different with 100 mm of the mixture. In addition, the results showed that 50 mm of mixture was highly significant different compared with control ($P < 0.05$). The antifungal activity of sodium bicarbonate- vinegar mixture on spore germination of *B.cinerea* on PDB was revealed after incubation for 4 and 8 h **(Fig. 4)**.

Fig. 4 Efficacy of sodium bicarbonate-vinegar mixture at different concentrations on spore germination of *Botrytis cinerea* (Bc1) on PDB at 23˚C for 4 and 8 h. Treatments showing



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different letters indicating statistically different by Duncan's multiple range test ($P < 0.05$).
The SB-vinegar mixture 50 mM completely inhibited the spore germination of *B.cinerea* and the

results were highly significant difference compared with other concentration and the control treatment. Several studies found that SB poses direct action for inhibition the mycelial growth and sporulation of *Penecillium* and reduces the incidence of postharvest green and blue mold on citrus (Smilanick *et al*., 2008; Droby *et al*., 2003). In addition, (Turkkan *et al*., 2017) observed that SB at 0.8% w/v totally inhibited the mycelial growth and spore germination of *B.cinerea* on Kiwifruit.

Proteins Leakage

To evaluate the possible mechanisms of SB-vinegar mixture on *B.cinerea*, released cytoplasmic content including soluble proteins were measured from treated the fungal mycelium. The results showed that simulated the soluble protein significantly and the leakage started at 50 mM after 4 and 8 h **(Fig. 5)**. The highest weight of leakage of soluble proteinwas at 100 mM indicating there was linear relationship between the concentrations of SB- Vinegar mixture with the quantity of released protein from the mycelium of *B.cinerea.* This experiment indicates that the cell membrane of fungal pathogen was degraded, damaged and exudation of cytoplasm including proteins were released. These results showed the fungicide effect of SB- Vinegar mixture on treated *B.cinerea.* The fungicidal activity of SB- vinegar mixture for releasing soluble protein was consistent with results of inhibitory effect of mixture at 50 mM on the mycelial growth and spore germination. These data suggested that the possible mechanisms of fungicidal activity of the mixture are disruption of cell membrane, releasing the cytoplasmic constituents and eventually cell killed. Some studies found that the possible action of boron salts activity against *B. cinerea* on table grape was damage the cell membrane and releasing the soluble protein and carbohydrate (Qin *et al*., 2003). There was no report showing the activity of SB as fungicide. In our study we approvedthat SB-vinegar mixture pose fungicide activity against *B.cinerea*.

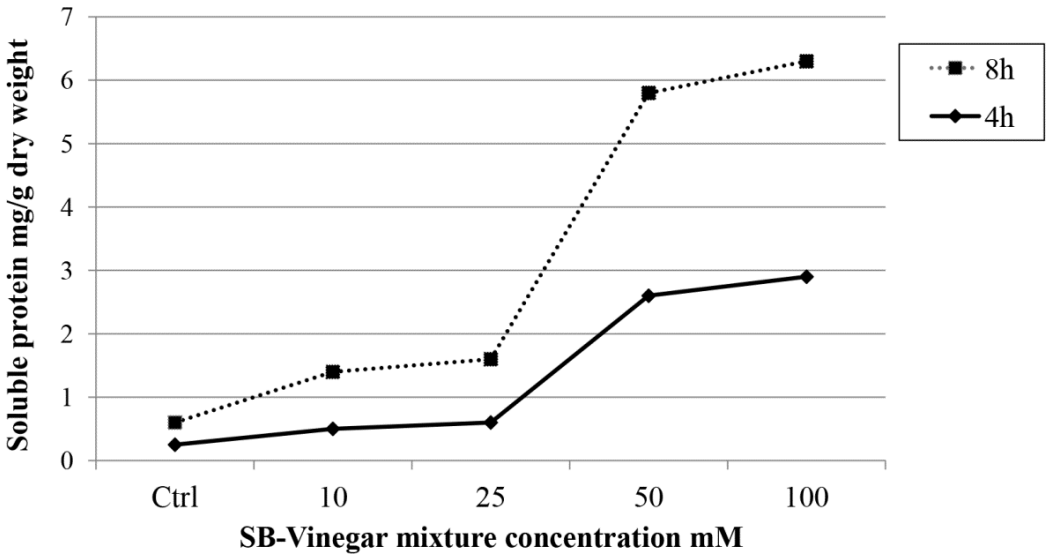

Fig. 5 Soluble protein released from mycelium of *Botrytis cinerea* supplemented with different concentrations of sodium bicarbonate and vinegar mixture and incubated at 23˚C for 4 and 8 h. Treatments showing different letters indicating statistically different by Duncan's

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multiple range test ($P < 0.05$).

Effect of SB- Vinegar Mixture on Gray Mold Disease of Strawberry Fruit

Sodium bicarbonate- vinegar mixture application at 50 and 100 Mm completely inhibited the lesion enlargement and development. Strawberry fruit immersed into SB-Vinegar mixture at 50 and 100 mM for 10 min before spore inoculation completely reduced the gray mold disease compared with control and other treatments **(Fig. 6)**. These results were compatible with data from mycelial growth, spore germination, and the leakage of soluble proteins.

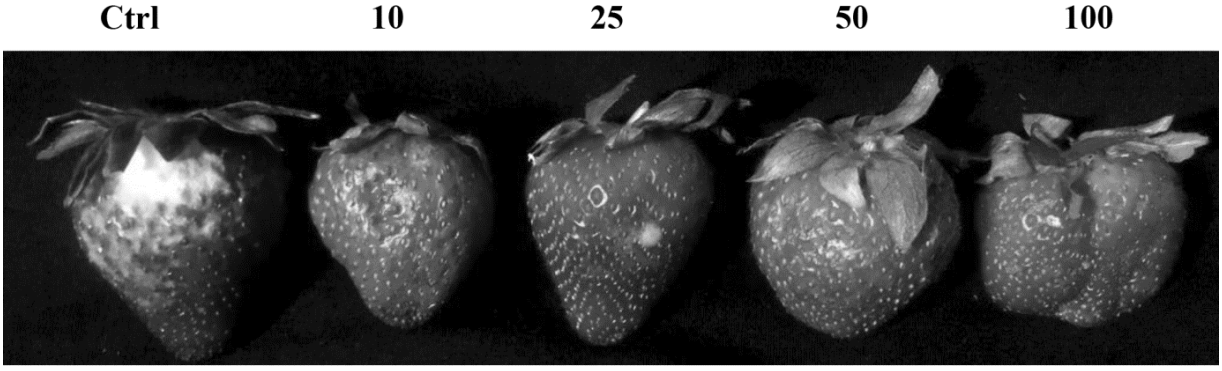

Fig. 6 Effect of different sodium bicarbonate (SB)- vinegar mixture concentrations at 10 minutes immersion times on disease severity in strawberry fruit following inoculation with *B. cinerea*. Treated fruit were stored at 25˚C for 7 days.

Conclusion

SB-Vinegar mixture was obviously effective against *B.cinerea* and its fungicidal activity was confirmed by leakage of soluble proteins. Dip application of sodium bicarbonate- Vinegar mixture at 50 mM for 10 min completely decreased lesion diameters in strawberry fruit inoculated with *B.cinerea.* SB-Vinegar can be safe and cost effective approaches for preventing gray mold disease in post-harvest strawberry fruit.

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