Egy. J. Pure & Appl. Sci. 2023; 61 (2):1-18

Egyptian Journal of Pure and Applied Science

Citrus Green Mold Biocontrol by Novel Epiphytic Antagonist Combined with Safe Compounds Formulations

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ARTICLE INFO

Received 24 February 2023
Accepted 6 April 2023

Keywords
Biological control agents ,
Bioformulations ,
Penicillium digitatum ,
Enterobacter ,
cinnamic acid.

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ABSTRACT

We obtained 15 bacterial isolates, that were isolated from the peels of orange fruits. They were screened for their bioactivity against *Penicillium digitatum*, the causative agent of green mold disease. Only one isolate showed the highest antifungal activity, where the inhibition percentage reached 67% in the dual culture assay. Furthermore, this isolate was identified by the molecular technique of 16S rRNA. It is *Enterobacter* sp. (SH-87) strain with the accession number (GenBank accession number OP481922). The biological activity of *Enterobacter* sp. (SH-87) was investigated on *in-vivo* and *in-vitro*. The *in-vitro* results demonstrated that the metabolites in the filtrate, are effective in controlling the mycelial growth, also, it decreased the spore germination. On the other hand, the *in-vivo* findings clarified that the fermentation liquid (FL) was effective in reducing both disease incidence of artificially infected oranges. So, it has been formulated with different safe compounds including chitosan CHS, salicylic acid SA, and cinnamic acid CA. The selected compounds showed an activity when processed with *Enterobacter* sp. into edible coatings (ECs) formulations, to be applied against citrus green mold decay. All additives were effective in controlling *P. digitatum* on plates and in planta. ECs displayed an efficacy when used to control green mold on orange fruits. Disease incidence declined significantly in response to preventive treatment by coating orange fruits with film solutions enriched with *Enterobacter* sp. This is the first study highlights that *Enterobacter* epiphytic antagonistic bacterium is a novel safe biocontrol agent that could be formulated to control citrus green mold disease.

1. Introduction

Use of chemical fungicides has posed obstacles in the agricultural domain; these chemicals have led to prodigious environmental issues, consequently, they have raised the global concern to search for more green and ecofriendly strategies for disease management. Biological control, based on employing antagonistic microorganisms, is an alternative sustainable approach to control vast of postharvest pathogens. Furthermore, employing some safe compounds that belong to generally recognized as safe (GRAS) has emphasized its efficacy in controlling various plant pathogens.

Impaired quality of fruit during storage was caused by infection with various fungal diseases has led to a marked reduction of their yield, shelf life, quality, and massive economic losses. Citrus is one of the highly consumed kinds of fruit globally, they are popular by their pleasant taste and flavor **(Mditshwa *et al.,* 2017)**. They provide humans with essential nutritional compounds, such as vitamin C, carotenoids, and flavonoids. These are renowned for their bioactivity; they are characterized by their antioxidant, anti-cancer, and anti-inflammatory characteristics. Citrus family encompasses various species, such as oranges, lemons, limes, grapefruits, mandarins, and tangerines, in which orange accounts for half of the global production followed by mandarins and tangerines.

It is cultivated in many countries including, tropical and subtropical ones, assigning the top countries including China, Brazil, the European Union, and the United States **(Liu *et al.,* 2012)** Egypt is one of the leading exporters of citrus; where the number of importing countries has reached 98 countries. These countries include, Bangladesh, China, Ukraine, India, Malaysia, United Kingdom, United Arab Emirates, Saudi Arabia, The Netherlands, and Russia **(Wally and Akingbe 2019)**.

Citrus is a non-climacteric fruit; therefore, they have to be picked at their full maturation. However, ripe orange fruits are highly perishable to a wide spectrum of phytopathogens including bacteria, viruses, and fungi that cause more than 20 diseases resulting in fruit spoilage and deterioration on both quality and yield levels **(Chen *et al.,* 2020)**. The key pathogenic fungi invading citrus are *Penicillium digitatum, Penicillium italicum, Geotrichum citri-aurantii, Aspergillus niger,* and *Aspergillus flavus* **(Bazioli *et al.,* 2019)**. The most destructive fungal pathogens of citrus belong to *Penicillium* sp; *Penicillium italicum* and *Penicillium digitatum* are the causative agents of blue and green molds, respectively **(Droby *et al.,* 2002)**.

Chemical control based on using synthetic fungicides is considered the key strategy for disease management, such as imazalil, thiabendazole, prochloraz, and pyrimethanil are reported to effectively control *Penicillium* pathogens **(Bazioli *et al.,* 2019; Yu *et al.,* 2020)**. Although these chemicals have been utilized on a large scale, they have a deleterious impact on the environment. They have caused emerging of resistant strains of these pathogens, and consequently, they become less efficient in combating the disease. Moreover, they have afflicted human health due to the persistence of the residues in the fruits after harvest. Hence, public and consumer concerns have been raised forcing the researchers and people working in the agricultural sector to seek for alternative methods that are safer to tackle the repercussions resulting from the residual contamination of fungicides.

Biological control based on the exploitation of microbial antagonists has been employed as an alternative strategy replacing synthetic fungicides. Antagonistic microorganisms including bacteria, yeasts, and fungi inhabit various ecological niches, and could be found on vegetables and fruit surfaces named as epiphytes. Biological control agents (BCAs) are good candidates in agricultural practices; they are efficient, sustainable, green, and eco-friendly approaches for protecting different postharvest commodities **(Dukare *et al.,* 2020).**

Numerous (BCAs) have been employed as microbial biocides against a wide spectrum of postharvest pathogenic fungi, including *Monilinia* spp., *Penicillium expansum*, and *Botrytis cinerea*, **(Bazioli *et al.,* 2019)** For example, *Aureobasidium pullulans* **(Galli *et al.,* 2021)**, *Bacillus amyloliquefaciens* **(Ye *et al.,* 2021)**, and *Enterobacter* spp **(Pereira *et al.,* 2011)**. They are able to reduce disease incidence by affecting the growth of fungal pathogens since they display varied modes of action including parasitism, competition for nutrients and space, induced of host resistance, production of antimicrobial secondary metabolites, production of cell wall degrading enzymes, and formation of biofilm **(Saraf *et al.,* 2014)**.

Along with the biological control agents, generally recognized as safe (GRAS) are known as low-toxic compounds with the least effects on the environment. They encompass various genres of compounds, such as essential oils, food additives, peptides, plant extracts, and organic acids, in addition to these materials, inorganic salts including carbonates, sorbates, benzoates, and acetates **(Palou and Pérez-Gago, 2021)**.

The combination of these GRAS with BCAs has been applied as a control measure strategy against different plant diseases, and citrus diseases, in particular. This approach points at the significance of such integration in order to reinforce the overall efficacy of the biological agent, because they behave synergistically to control fruit decay. Therefore, the present work has been conducted, firstly: to isolate new bacterial antagonists from peels of citrus fruit, secondly: to evaluate the biological activity of the obtained isolates against *Penicillium digitatum in vitro* and *in vivo*, thirdly: to elucidate the various mechanisms exerted by the isolated antagonistic bacteria, and ultimately: to prepare liquid bio formulations based on the isolated bioagent and some GRAS compounds for coating citrus fruit. That would be very helpful to increase shelf life and maintain the quality and quantity of the fruit under investigation.

2. Materials and methods

2.1. Isolation of bacterial strains

For isolation of bacteria from the surface of citrus fruit, they were selected to be healthy and mature and then placed in a sterile beaker (1000 ml) containing 300 mL of saline solution (0.85% wt./ vol.) supplemented with 0.5% Tween 80. After that, they were shaken on an orbital shaker at 180 rpm for an hour. The resultant solution was serially diluted using sterile distilled water (SDW). Hundred microliter was plated on nutrient agar (NA) medium (beef extract 1 g/ L, peptone 5 g/L, yeast extract 2 g/L, NaCl 5 g/L, Agar 20 g/ L) **(Jacobs and Gerstein, 1960)** the plates were incubated for 48 hours at 30 ℃. Distinctive colonies were monitored, and they were transferred to be inoculated on pure NA dishes for obtaining axenic cultures that were preserved in 20% glycerol at -80 ℃ for long-term storage.

2.2. Pathogen

Penicillium digitatum, the causal agent of green mold disease, was isolated from naturally infected sweet orange that has shown the green mold decay. A single spore colony was subjected for morphological identification, based on the morphology of the fungal spores and bodies. After that, the fungus maintained on PDA medium (potato: 200 g, dextrose: 20 g, agar: 20 g / L) **(Zhao *et al.,* 2009)**, the plates were incubated at 28 °C for 7 days, then stored at 4 °C for further experiments.

2.3. Citrus fruit preparation

Fresh mature healthy sweet orange fruit (Citrus sinensis) were divided into various groups (10 fruits per group). Fruits were washed under running tap water and then disinfected by immersion in 70% ethyl alcohol for 1 min, followed by rinsing with sterile distilled water (SDW) to remove any alcohol residues, left in the laminar hood flow to dry at room temperature. For artificially infected orange, a single wound was made (5 mm wide and 4 mm deep) at the equator for each fruit.

2.4. Screening of obtained isolates for antagonistic activity

To investigate the antagonistic activity of the different epiphytic isolates, a dual culture assay was applied. For making a seed culture, a single colony was taken from each isolate and revived in 25 ml Luria Bertaine (LB) broth medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) (Sezenove et al., 2007) and incubated under shaking at 180 rpm overnight. Take 20 µl to be streaked in the middle of PDA plates, incubate the plates at 30 °C for 24 hours. After that, a mycelial fungal plug from P. digitatum excised from the margin of 7-day-old cultures to be placed on both sides of the plate away from the microbial antagonist by 3 cm.

The dishes were wrapped with parafilm and incubated at 28 °C for 7 days, the plates without the antagonist inoculum used as control. The plates were observed after 7 days to measure the radial growth (RG) of the fungal colonies, and then calculate the inhibition percentage (IP) according to the following formula: IP (%) = RG_C- RG_T/ RG_C × 100, where $RG_{C:}$ radial growth of the control, $RG_{T:}$ radial growth of the treatment.

2.5. Molecular identification of the selected bacterial isolate

The most effective bacterial isolate was subjected to molecular identification using 16S rRNA gene sequencing. DNA extraction from pure bacterial colonies was performed using the cetyl trimethyl ammonium bromide (CTAB) method (Wilson, 1987). The 16S rRNA primer that was used for the PCR: forward 16S rRNA sequence (5'CCAGCAGCCGCGGTAATACG3) and reverse 16S rRNA sequence (5'ATCCGCTACCTTGTTACGACTTC3'), in which sanger sequencing was used. The purified PCR product was sequenced, and after that, the procured sequences were subjected to Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. For the construction of the phylogenetic tree, the PCR product were analyzed with the help of MegAlign (DNA Star) software version 5.05., the obtained sequence from the study had been submitted to the GenBank, NCBI database.

2.6. Modes of action exerted by biocontrol bacterium (SH-87)

2.6.1. Effect of cell-free supernatant (CFS) on mycelium growth

Inoculate 100 ml fresh LB broth media contained in 250 ml Erlenmeyer flask with (3% v/v) cell suspension from the bacterial antagonist SH-87 incubated at 30 °C with shaking at 200 rpm for 48 hours. To obtain the CFS, centrifuge the fermentation liquid (FL) at 10000 rpm for 10 min at 20 °C, then pass the supernatant through a 0.45µm syringe membrane filter to remove any cells and obtain pure CFS.

Molten PDA media was amended with CFS to obtain a series of concentrations (10, 20, 30, 40, and 50%), mix well, and then poured into Petri plates. After that, inoculate the plates with a mycelial plug taken from the margin of the fungal cultures (7-day old culture), placed at the center of the plate, and incubated at 28 °C with a daily observation of the radial growth which was measured after 3, 5, and 7 days from incubation.

2.6.2. Effect of cell-free supernatant (CFS) of biocontrol bacterium (SH-87) on spore germination of Penicillium digitatum

In a 100 ml conical flask containing 20 ml PDB inoculate them with 1 ml CFS obtained as mentioned in section (2.6.1.), with 1 ml of *Penicillium digitatum* spore suspension of concentration ($4X10^5$spore/ml) which was counted using the hemocytometer, uninoculated PDB kept as control. Incubate at 120 rpm for 12 hr., at the end of the incubation period count at least 100 spores that appeared germinated, the spore was considered to be germinated when the length of the germ tube is equal or greater than the diameter of spore. Calculate the inhibition percentage of spore germination as following: the number of germinated spores control treatment/numberer of un-germinated spores X100.

2.7. In vivo application of biocontrol bacterium (SH-87) on citrus fruits

Fruits were divided into three different groups: 1. Fruit treated with dipping in cell suspension (CS) at a concentration of (1 X 10^8 CFU/ ml), 2: fruit dipped in the fermentation broth (FB) where the concentration was adjusted at (1 X 10^8 CFU/ ml), and 3: fruit treated with the cell-free supernatant (CFS), fruit that did not receive any kind of these treatments were used as control. All treated fruits were left to dry in the lamina hood flow for 2 hours. Afterward, the fruits were sprayed with a spore suspension made from a 7-day-old culture of *Penicillium digitatum* at concentration of (5×10^4 spore/ ml) and left to dry.

They were placed in plastic boxes lined with moistened paper towels to maintain the humid conditions and wrapped with plastic film, then incubated at 25 ºC with RH (70-80%). Fruits were daily monitored for up to 7 days till the appearance of the decay on the control fruits. Each group had 5 fruits with duplicates, and the experiment was repeated twice. Disease parameters were recorded by measuring lesion diameter (LD) using a graded caliper, disease incidence percentage (DI%) which was calculated as (number of infected fruits /total number of fruits) X 100.

2.8. Investigation of some safe compounds

2.8.1. In-vitro investigation of chitosan (CHS), salicylic acid (SA), and cinnamic acid (CA) against P. digitatum

Chitosan (CHS) of high molecular weight 420 KDa, degree of deacetylation (DD) ≤ 85%) was used, in addition, salicylic acid SA and cinnamic acid CA were selected.

To test the inhibitory effect of these compounds *in-vitro*, a poisoned food technique was applied where molten PDA media were amended with CHS, SA, and CA solutions, to obtain the required concentrations of (0.5, 1, 1.5, and 2% w./v.), (2, 4, 6, and 8 mM), (0.5, 1, 1.5, and 2 mM), respectively. After solidification. the plates were inoculated with a mycelial fungal plug that was taken from *P. digitatum* culture to be placed in the center of the plate. The plates were incubated at 28 ℃ and the radial growth was observed and compared with the control PDA plates which were not supplied with any of tested compounds, the percentage of mycelium inhibition was calculated, there were three replicates for each concentration, and the entire experiment was repeated twice.

2.8.2. In vivo testing of the activity of CHS, SA, and CA against Penicillium digitatum on artificially infected orange fruits

Fruits were classified into 4 groups; first: fruits dipped in CHS (0.5, 1, 1.5, & 2% (w./v.), second: fruits dipped in SA (2, 4, 6, & 8 mM), the third group where fruits were immersed in CA (0.5, 1, 1.5 & 2 mM), and fourth group fruits dipped into sterile distilled water and used as control. After being dipped in the previous solutions, they were left to dry for 2 hours, and then, they were sprayed with a spore suspension of green mold (5×10^4 spores/ml), and dried in the laminar airflow for 2 hr. Each group has contained 5 fruits with duplicates, and the experiment was carried out two times. The fruits were incubated at 28 °C, RH (70-80%), and they were daily observed for decay occurrence for up to 7 days. Calculate the disease parameters including lesion diameter (LD) and disease incidence percentage as mentioned in section (2.7).

2.9. Investigating the biological activity of the antagonistic bacterium (SH-87) integrated with GRAS compounds in a liquid bioformulations against Penicillium digitatum on artificially infected citrus fruits

Fruits were divided into 4 groups; G1: fruits treated with fermentation broth (FB) taken from the biocontrol bacterium SH-87 + (0.5, 1 1.5, 2%) CHS, G2: fruits that were treated with FB + (2, 4, 6, 8 mM) SA, G3: treated fruits with the combination of FB +(0.5, 1, 1.5, 2 mM) CA, and G4: untreated control fruit. All fruits were treated by dipping in the aforementioned liquid formulations for 10 min. and then left to dry on a clean bench for 2 hours, then they were sprayed with a spore suspension of green mold (5×10^4 spores/ml). After that, they were dried at room temperature. Each group has contained 5 fruits with duplicates, incubated at 28°C, RH (70-80 %), and daily observed for decay occurrence up to 7 days. Calculate the disease parameters including lesion diameter (LD) and disease incidence percentage (DI), the experiment repeated twice.

2.10. Preparation of film-forming solutions (FFS) made by GRAS integrated with biocontrol bacterium (SH-87) for coating

2.10.1. Preparation of chitosan CHS, CHSSA, and CHSCA film solution

Edible films were prepared based on the biopolymer chitosan integrated with SA and CA. Chitosan (2% wt. /vol.) dissolved in (1% vol./ vol.) glacial acetic acid at room temperature under stirring for 12 hr., then add (1% wt./ vol) glycerol as a plasticizer and (0.5% wt./ vol.) Tween 80 as a surfactant, let the FFS under stirring till complete solubility for 24 hr. After complete solubility, adjust the pH at 5.8-6.For the preparation of the chitosan/ SA blend and chitosan / CA blend, chitosan (2% wt./ vol.) was dispersed in (1% vol./ vol.) glacial acetic acid with a certain ratio of SA and CA to obtain concentrations of (4 and 6 mM) and (1.5 and 2 mM), respectively, and then add the same adjuvants (glycerol and Tween 80) as mentioned above.

2.10.2. Enriching the FFS with biocontrol bacterium (SH-87) fermentation liquid (FL)

Fermentation liquid of the antagonistic bacterium SH-87 was incorporated into the prepared film-forming solutions by adding 3% v./ v. at concentration of ($1X10^8$ CFU/ml), and mix well on a shaker, the FFS was ready for use and application.

2.11. Effect of coating with the different prepared FFS on artificially inoculated citrus fruit with P. digitatum

Orange fruits were coated with different FFS and then they were challenged with the pathogen spore suspension. Treatment groups were: G1: CHS+SA (4mM), G2: CHS+SA (6 mM), G3: CHS+CA (1.5 mM), G4: CHS+ CA (2mM), all mixed with biocontrol bacterium SH-87 fermentation liquid ($1X10^8$ CFU/ml). After that, fruits were dried for 2 hr., and then they were sprayed with fungal spore suspension ($5x10^4$ spores/ml) obtained from 7-day *P. digitatum* fungal cultures, the untreated fruit was used as a control group, place the fruit in plastic boxes lined with moistened paper towels to maintain the relative humidity between (80-90%), incubate at room temperature and follow the disease incidence after 7 days.

2.12. Statistical analysis

All data were analyzed using analysis of variance (ANOVA), and the means were subjected to Tukey's test ($P < 0.05$ was considered significant) using Minitab16 software.

3. Results

3.1. Isolation and identification

Fifteen bacterial isolates were procured from the peels of healthy orange fruits, they were investigated for screening their antagonistic activity against *Penicillium digitatum* (unpublished data). One isolate (SH-87) was selected as it showed the maximum antifungal activity.

It was identified morphologically and biochemically. The bacterium was appeared under microscope as Gram -ve short rods. The strain was positive for catalase reaction, and negative for oxidase reaction. For more confirmation, the strain was identified using molecular techniques. Results showed that the sequence was 100% similar to the counterpart sequence of *Enterobacter* sp. strain M5 (GenBank accession number MT641242.1). The PCR product (854 bp) was analyzed for the construction of the phylogenetic tree shown in Fig. 1, and it was submitted to the NCBI GenBank and acquired the accession number (OP481922).

3.2. Dual culture assay of Enterobacter sp. (SH-87)

Fig. 2 demonstrated the highest antagonistic activity of *Enterobacter* sp. (SH-87) on the radial growth of *Penicillium digitatum* in a dual culture test based on the agar diffusion method. The results indicated the presence of a significant difference ($P < 0.05$) between the treated and the control plates since the colony diameter decreased by 47 mm giving an inhibition percentage of 67%.

3.3. Antifungal activity of CFS on radial growth of P. digitatum

The effect of cell-free supernatant obtained from *Enterobacter* fermentation liquid after centrifugation is presented in Table 1, Fig. 3. Data emphasized the efficacy of CFS since it exerts an antifungal activity on the radial growth of fungal pathogens. Results showed that the inhibitory effect of the CFS is dose-dependent as the inhibition percentage decreased gradually with increasing CFS concentrations, till reaching complete inhibition at the maximum concentration of 50%. Regarding the fungal susceptibility to the suppressing effect of the CFS, results revealed that *P. digitatum* is susceptible to the inhibitory effect of CFS.

3.4. Effect of CFS on spore germination of P. digitatum

As shown in Fig. 4, the figure depicted the spore germination after being exposed to the effect of CFS obtained from *Enterobacter* sp. after 48 hours. CFS adversely affected the ability of the fungus to sporulate, since the spore germination dropped to zero, no germinated spores detected in the CFS treatments.

3.5. In vivo evaluation of different fractions obtained from Enterobacter sp. (SH-87) on artificially infected citrus fruit

Based on the *in vitro* assessment of the antagonistic activity of *Enterobacter* cells against the green mold pathogen, it is harnessed to be applied for the treatment of artificially infected citrus fruit. Different fractions (CS, FB, and CFS) were used as the infected fruits were dipped for 10 min. As shown in Table 2 and Fig. 5 all fractions had a potential activity in reducing both the lesion diameter and disease incidence percentages showing a significant difference among them. It was observed that FB was the most effective treatment causing a sharp decrease in the lesion diameter from 70.7 mm for control untreated fruits to 32.3 mm and infection incidence is only 20% as compared with control fruits, followed by the CFS with a recorded disease of incidence of 44%.

3.6. In vitro evaluation of some GRAS compounds against P. digitatum

Chitosan (CHS), salicylic acid (SA), and cinnamic acid (CA) are reported as GRAS. Results of the inhibitory effect of CHS, SA, and CA on the radial growth of *Penicillium digitatum* are presented in Table 3. Data demonstrated the significant inhibitory effect of all tested GRAS on green mold pathogen, it is noticed that the inhibitory effect is directly proportional to the used concentrations. Complete fungal inhibition was achieved at 1, 1.5, and 2% chitosan concentrations, while it was obtained after SA treatments at 6 and 8 mM. For cinnamic acid, the inhibition percentage was 100% at 2mM concentration.

3.7. In vivo evaluation of CHS, SA, and CA on artificially infected citrus fruit with P. digitatum

In-vivo investigation of the activity of CHS, SA, and CA was conducted on artificially infected orange fruits with green mold pathogen. Table 4 showed the lesion diameter (LD) and disease incidence (DI) of citrus fruit treated by dipping in CHS, SA, and CA solutions. Notably, chitosan has a potent antifungal action on *P. digitatum* which relies on the used concentration since the lesion diameter decreased dramatically with increasing the applied concentrations. LD recorded 79.6 mm for untreated control oranges while for the treated ones the diameters of the decayed area were 27, 14.2, and 11 mm for treatments of 0.5, 1, and 1.5% of chitosan concentrations, respectively. Moreover, 2% CHS completely inhibited the fungal growth.

Similarly, increasing the dose of SA from 2 up to 8 mM affected the lesion diameter positively, lesion diameter was affected significantly post the SA treatment; where the LD recorded 13.5, 3.7, 5.1, and 4.6 mm for the tested concentrations of 2, 4, 6, and 8 mM respectively. Regarding the effect of CA, LD showed a significant difference between the control fruits and the treated ones ($P < 0.05$), the measured LD after 7 days were 27.2, 25.5, 23, and 22.4 mm for the treatments of 0.5, 1, 1.5, and 2mM, respectively. Conversely, CA did not affect the DI percentage, as data illustrated no significant difference among all treatments indicating that CA has a weak inhibitory action on *P. digitatum* in the presence of the host.

3.8. In-vivo evaluation of CHS, SA, and CA combined with Enterobacter antagonist on citrus fruit artificially inoculated with P. digitatum

A combination of *Enterobacter* bioagent with the studied GRAS compounds has been investigated. Results of the overall treatments were positive and significant. *Enterobacter* fermentation broth suspended in different CHS, SA, and CA concentrations reduced both LD and DI effectively. They could inhibit fungal growth on the artificially inoculated orange fruits. Data presented in Table 5, Fig. 6 demonstrated the *in vivo* effect of CHS, SA, and CA combined with *Enterobacter* sp. (SH-87) fermentation broth. Results underlined the efficacy of the combined mode treatments against green mold disease. CHS treatments as combined with *Enterobacter* fermentation broth conferred a significant difference for all treatments in comparison with the untreated fruits, disease incidence percentage was 100% for control fruits while it abated for all tested CHS concentrations combined with a bacterial antagonist to reach 33, 25, 17, and 17% for 0.5, 1, 1.5, and 2% CHS concentrations, respectively. Furthermore, the lesion diameter reduced significantly with increasing CHS concentrations and it reached 4.6 mm at 2% CHS while it was 76.6 mm for control fruits.

Regarding the SA impact, it highlighted that SA could effectively curb green mold incidence when combined with the bacterial antagonist *Enterobacter* fermentation broth. A significant difference was recorded for all used concentrations. Disease incidence decreased from 100% for untreated fruit to 17% at 2 mM SA, while for both 4 & 6 mM treatments disease was completely inhibited and DI reached 0. On the other hand, CA did not affect disease parameters when used solely, however, it showed bioactivity when combined with *Enterobacter* fermentation liquid. Lesion diameter decreased in a dose-dependent way, also, disease incidence declined from 100% for control to 8% at 1.5 and 2 mM, respectively.

3.9. Effect of coating with different prepared film-forming solutions (FFS) on artificially inoculated citrus fruit with P. digitatum

Two film-forming solutions were prepared, CHS/SA (4, 6 mM) and CHS/CA (1.5, 2 mM) amended with 3% (v/. v) *Enterobacter* fermentation liquid. Oranges artificially contaminated with *P. digitatum* spores were coated with the prepared coating solutions for 10 min. The obtained results are shown in Table 6**.** After 7 days of incubation at 28 °C, the decay incidence was significantly controlled in coated fruits compared to the uncoated ones. Disease incidence ranged between 10-15% at all treatments, moreover, the lesion diameter of the decayed area recorded a significant reduction for coated orange fruits compared with the untreated fruits. It was 69 mm and reached 6 and 4.8 mm for SA based edible films at 4 and 6 mM concentrations, for CA edible films, LD was 8.9 and 5.3 mm at 1.5 and 2 mM, respectively.

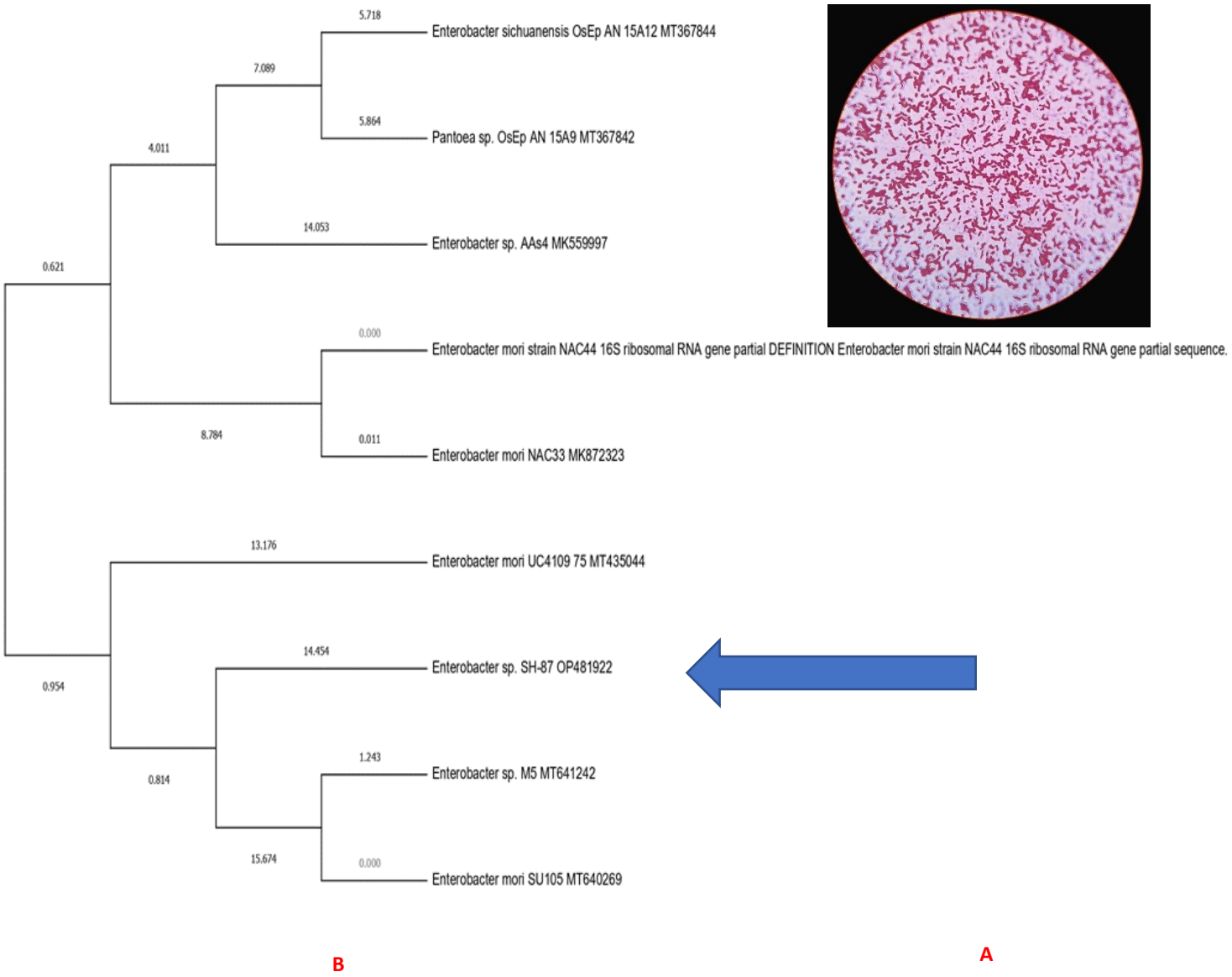

Fig. 1 A: colony morphology of *Enterobacter* sp. under microscope, **B:** Phylogenetic tree representing the relationships of 16S rRNA gene sequence analysis of the isolated epiphytic bacterial antagonist, *Enterobacter* sp. (SH-87*)*, the arrow showed the strain and the closely related strain of *Enterobacter* sp.

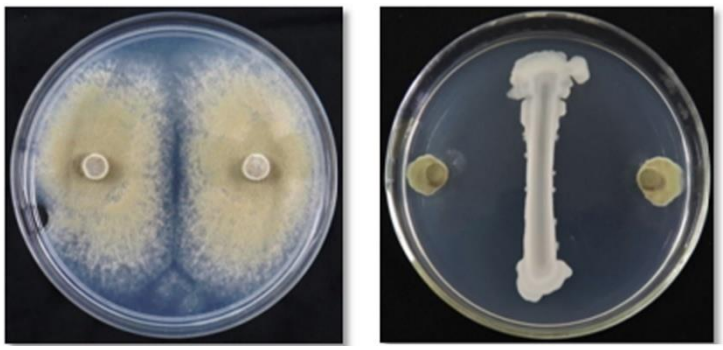

Fig. 2 Dual culture of *Enterobacter* sp. (SH-87) against *Penicillium digitatum,* A: control plates, B: plates inoculated with *Enterobacter* cells in the middle of the plate.

Table 1. Effect of different concentrations of cell-free supernatant (CFS) of *Enterobacter sp.* (SH-87) on mycelium growth of *Penicillium digitatum*.

Conc. %	Days after-inoculation (DPI)		
	3	5	7
0	3.2±0.17[a]	5.5±0.15[a]	7.4±0.1[a]
10	1.9±0.15[b]	3.2±0.42[b]	5±0.05[b]
20	1.2±0.05[c]	2.5±0.1[c]	3.9±0.25[c]
30	0.0	0.0	2.2±0.05[d]
40	0.0	0.0	1.3±0.14[e]
50	0.0	0.0	0.0

Data are the means of 3 replicates of two complete experiments, the mean colony diameters having the same letters are not significant ($P > 0.05$) according to Tukey's test. Radial growth was measured at intervals (3, 5, and 7 days).

Table 2. Effect of fermentation broth (FB), cell-free supernatant (CFS), and cell suspension (CS) of Enterobacter sp. (SH-87) on lesion diameter and disease incidence of citrus fruits inoculated with spores of Penicillium digitatum.

Treatments	Lesion diameter (mm)	Disease incidence (%)
Control	70.7±6.5[a]	100
CS	55.8±8.7[b]	33
CFS	44.2±5.3[d]	25
FB	32.3±6.8[c]	20.8

Data are the means of 10 replicates of two complete experiments, the mean diameters having the same letters are not significant ($P > 0.05$) according to Tukey's test.

Table 3. *In vitro* effect of chitosan (CHS), salicylic acid (SA), and cinnamic acid (CA) on radial growth of *Penicillium digitatum*

Treatments		Colony diameter (cm)	Inhibition percentage (%)
Control		8.2±0.5[a]	0.0
CHS	0.5%	1.1±0.08[b]	86.5
	1%	0.0[c]	10
	1.5%	0.0[c]	100
	2%	0.0[c]	100
SA	2mM	7.8±0.3[a]	4.9
	4mM	6±0.1[a]	26.8
	6mM	0.0[b]	100
	8mM	0.0[b]	100
CA	0.5mM	6.6±0.2[b]	19.5
	1mM	1.6±0.2[c]	80.5
	1.5mM	1.2±0.1[d]	85.4
	2mM	0.0[e]	100

Data are the means of 3 replicates of two complete experiments, the mean diameters having the same letters are not significant ($P > 0.05$) according to Tukey's test. CHS: chitosan, SA: salicylic acid, and CA: cinnamic acid.

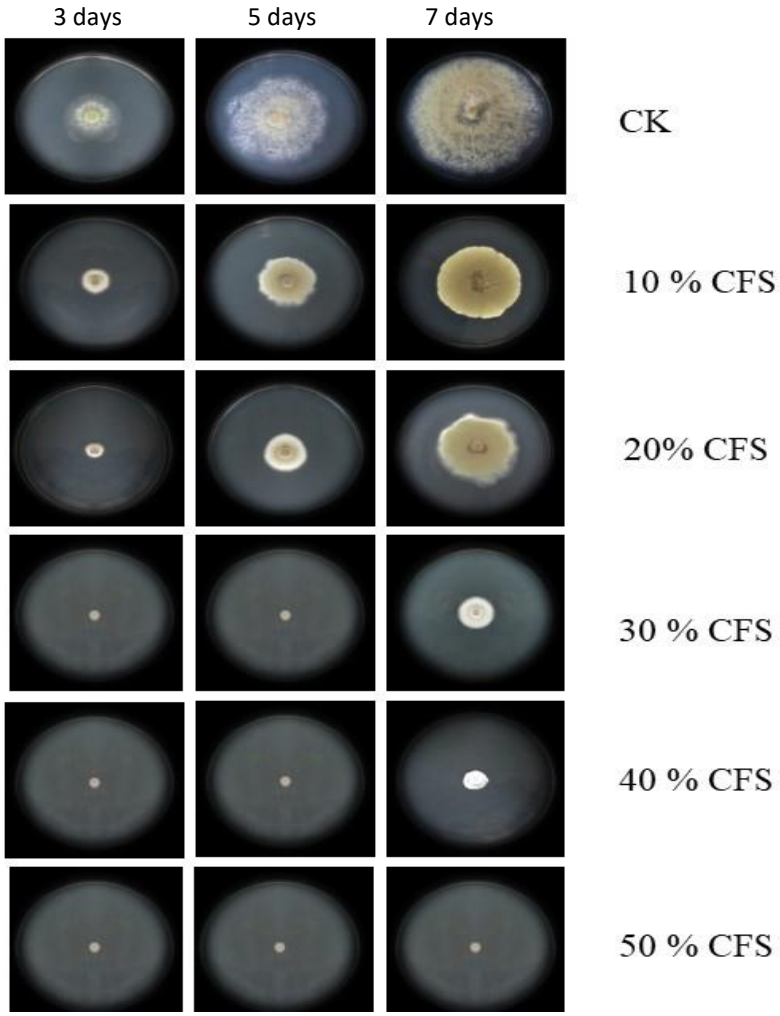

Fig. 3 Effect of cell free supernatant (CFS) on mycelium growth of *P. digitatum*.

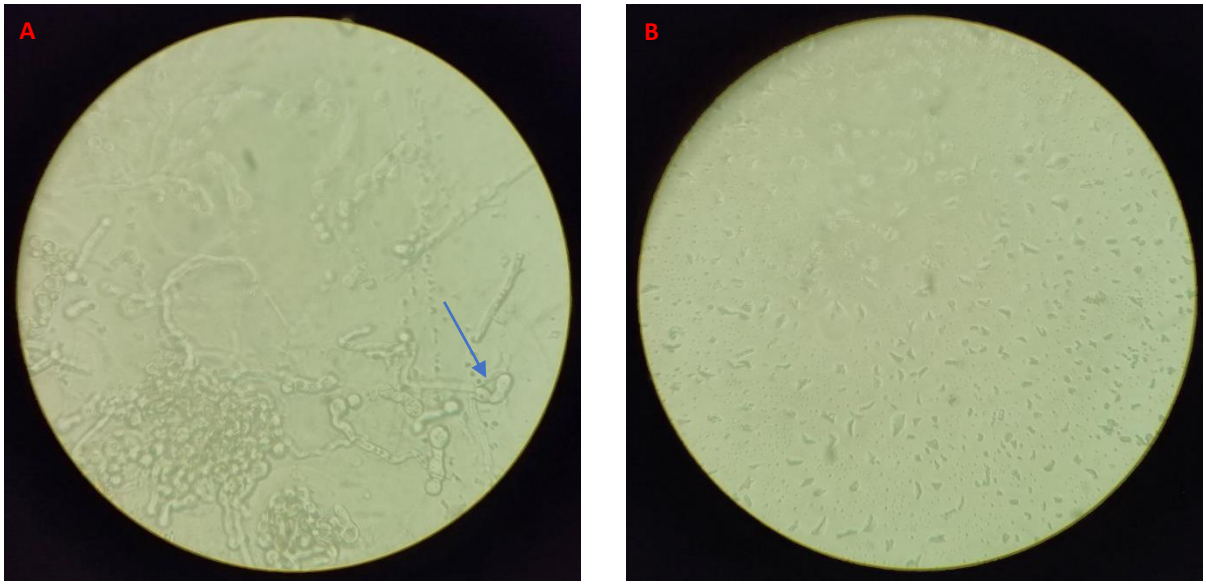

Fig. 4 Effect of CFS on spore germination of *P. digitatum*, A: control, B: treatment.

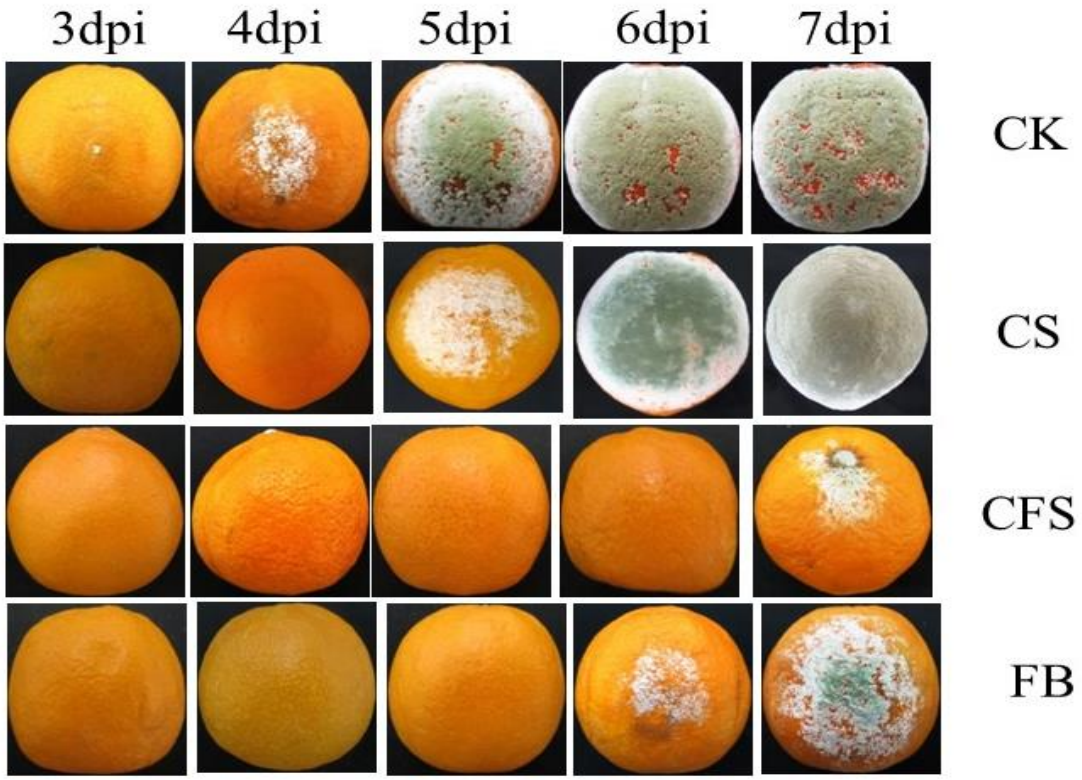

Fig. 5 *In-vivo* effect of *Enterobacter* sp. (SH-87) on artificially infected citrus fruit with *Penicillium digitatum*, CK: untreated citrus fruit, FB: fruits treated with fermentation broth of concentration (1X10^7 CFU/ ml) of *Enterobacter* cells grown for 24 hr., CFS: fruits treated with cell-free supernatant, and CS: fruits treated with cell suspension free from the filtrate of concentration (1X10^7 CFU/ ml), all fruits were sprayed with the green mold spore suspension of concentration (5X10^4 spores/ ml).

Table 4. Lesion diameter and disease incidence of oranges artificially inoculated with *P. digitatum* treated with different concentrations of CHS, SA, and CA

Treatments		Lesion diameter (mm)	Disease incidence (%)
Control		79.6±2.4^a	100
CHS	0.5%	27±4.9^b	45
	1%	14.2±2.4^c	25
	1.5%	11±3.7^{bc}	20
	2%	0.0^e	0.0
SA	2mM	13.5±2.6^b	41.7
	4mM	3.7±8.3^c	16
	6mM	5.1±10.3^c	25
	8mM	4.6±2.2^c	25
CA	0.5mM	27.2±1.9^b	90
	1mM	25.5±5.5^b	90
	1.5mM	23±3^c	90
	2mM	22.4±3.4^c	90

Data are the means of 3 replicates of two complete experiments, the mean diameters having the same letters are not significant ($P > 0.05$) according to Tukey's test. CHS: chitosan, SA: salicylic acid, and CA: cinnamic acid.

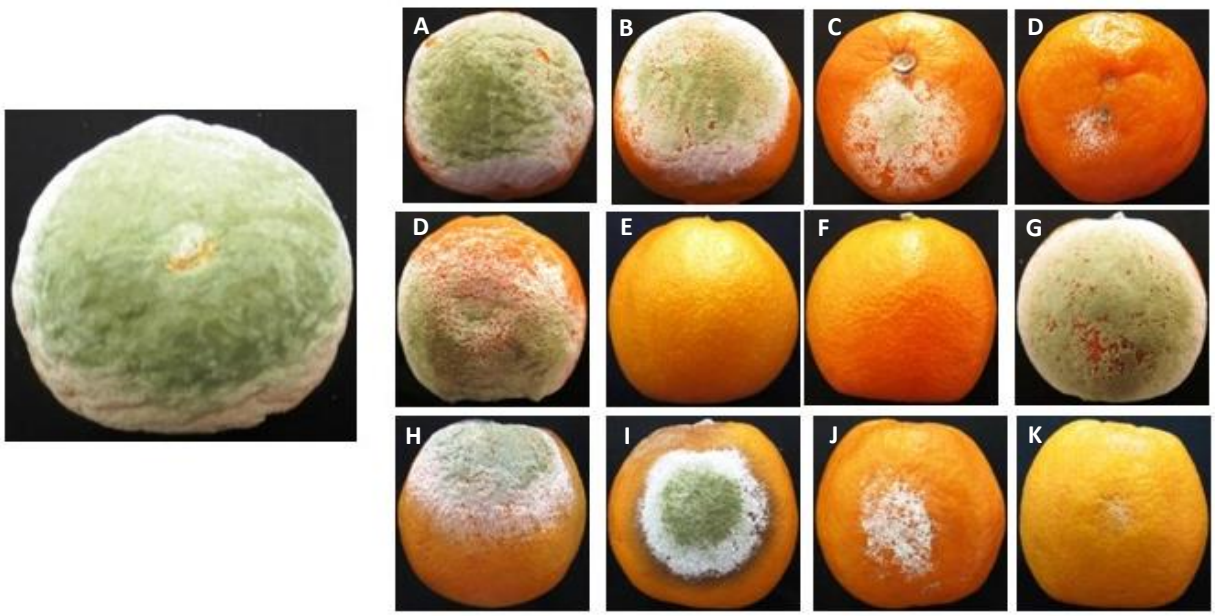

Fig. 6 Preventive treatment of oranges artificially infected with *P. digitatum*, oranges were dipped into liquid formulations made of 3% *Enterobacter* fermentation broth + (A-D) chitosan at (0.5, 1, 1.5, and 2%), (D-G): salicylic acid at (2, 4, 6, and 8mM), (H-K): cinnamic acid at (0.5, 1, 1.5, and 2 mM) incubated for 7 days at 28 ℃.

Table 5. Effect of CHS, SA, and CA combined with 3% *Enterobacter* sp. (SH-87) on lesion diameter and disease incidence (%) of orange fruits infected with green mold pathogen, incubated for 7 days at 28 ℃

	Treatments	Lesion diameter (mm)	Disease incidence (%)
	Control	76.6±0.68^{a}	100
	0.5% + SH87	11.4±5.3^{b}	33
	1% +SH87	8.2±4.5^{b}	25
	1.5% +SH87	5.5±3.8bc	17
	2% +SH87	4.6±3.2^{c}	17
SA	2mM +SH87	5.4±3.7^{a}	17
	4mM +SH87	0.0^{c}	0
	6mM +SH87	0.0^{c}	0
	8mM +SH87	4.8±3.3 ^{b}	17
	0.5mM +SH87	5.1±3.5^{b}	30
	1mM +SH87	4.5±3.2^{bc}	15
	1.5mM +SH87	1.7c^{d}	8
	2mM +SH87	1.4^{d}	8

Data are the means of 3 replicates of two complete experiments, the mean diameters having the same letters are not significant ($P > 0.05$) according to Tukey's test. CHS: chitosan, SA: salicylic acid, and CA: cinnamic acid.

Table 6. Biocontrol effectiveness of coating with film-forming formulations made of chitosan (2%) blended with (4, 6 mM) SA and (1.5, 2 mM) CA in combination with *Enterobacter* cells on infected oranges with *Penicillium digitatum*

	Treatments	Lesion diameter (LD) (mm)	Disease incidence (%)
	Control	69±6.8^{a}	100
CHS	2% CHS+4 mM SA+SH87	6±4.1^{b}	10
	2% CHS+6 mM SA+SH87	4.8±3.3^{c}	10
	2% CHS+1.5 mM CA+SH87	8.9±4.8^{ab}	15
	2% CHS+2 mM CA+SH87	5.3±3.7^{c}	10

Data are the means of 3 replicates of two complete experiments, the mean diameters having the same letters are not significant ($P > 0.05$) according to Tukey's test. CHS: chitosan, SA: salicylic acid, and CA: cinnamic acid

4. Discussion

In the present work, we aimed at controlling green mold disease by harnessing bacterial antagonists. An epiphytic *Enterobacter* sp., the strain was isolated from the surface of healthy orange fruit indicating that fruit surfaces have beneficial microflora that displays a role in protecting against various pathogens because they are highly adapted to the surrounding environmental conditions. The results are in agreement with (**Hammami *et al.,* 2022)**, as they got 5 active antagonistic yeasts and bacterial isolates from the surface of oranges and lemons. Bacterial bioagents including *Bacillus* sp., *Pseudomonas* sp., and *Enterobacter* sp. are reported to have a potent biological efficacy against a broad spectrum of bacterial and fungal phytopathogens; they exerted different modes of action to afflict microbial growth. In our study, we tracked the different mechanisms of activity that could be displayed.

The dual culture assay depicted the potential activity of *Enterobacter* against *P. digitatum*, it could positively inhibit the linear growth of the fungal pathogen**.** In line with our results, **Guo *et al.* (2020)** investigated the biocontrol activity of different endophytic *Enterobacter* species isolated from sugarcane roots against different fungal pathogens, such as *Fusarium moniliforme*, *Fusarium cubense*, *Botrytis cinerea*, *Ceratocystis paradoxa*, and *Sporisorium scitamineum*. They reported that the obtained *Enterobacter* isolates were powerful as they could suppress fungal pathogens' growth attributed to the production of potent secondary metabolites that diffuse through the agar.

We have also studied the activity of the water-soluble secondary metabolites being excreted in the growth medium and that through obtaining the cell-free supernatant (CFS) from *Enterobacter* cells. The results marked an evident activity of the CFS on the mycelial growth of green mold pathogen. The inhibitory action of CFS might be attributed to the presence of bioactive secondary metabolites that have been synthesized by bacterial cells during their growth. Likewise, **Tian *et al.* (2020)** demonstrated the antifungal action of CFS taken from *Bacillus* sp. w176 against *P. digitatum in vitro*.

They explicated their results to the presence of active peptide molecules in the CFS. Furthermore, CFS could impact the genetic expression of PdOs2 and PdAqu genes in green mold pathogen by their downregulation, which displays a crucial role in cell structures development in various phytopathogens **(An *et al.,* 2016).** Regarding the *in vivo* activity of *Enterobacter* sp. (SH-87) on citrus fruit being artificially contaminated with *P. digitatum,* all tested fractions got from the bacterial antagonist (CS, FB, and CFS) showed a significant reduction of both disease incidence and lesion diameter. It is noticeable that FB treatment was the most effective one among others, and this might be due to the combination of both vegetative cells and metabolites in this liquid, so boosting its overall activity. These results are congruent with **Chen *et al.* (2018)** who applied the supernatant and cell suspension of *Bacillus amyloliquefaciens* DH-4 for the treatment of citrus inoculated with *P. digitatum*.

They demonstrated that the supernatant was more powerful than cell suspension for eradication of green mold disease on citrus fruit. They interpreted that as the citrus peel has not conferred an appropriate environment for microbial cells to grow, conversely, they employed both the supernatant and the fermentation broth of same bacterial strain for storage, the results revealed that the whole fermentation broth was more effective for storage than CFS standing alone because it contains both cells and potent metabolites. Generally recognized as safe (GRAS) compounds have been approved by the food and drug administration (FDA) to be incorporated as safe constituents of edible films, food products, and food packaging. Chitosan (CHS), salicylic acid (SA), and cinnamic acid (CA) are reported as GRAS.

In vitro and *in vivo* evaluation of the suppressive effect of CHS, SA, and CA against *P. digitatum* was examined. The obtained data highlighted the bioactivity of all tested GRAS against the green mold pathogen, they could inhibit the mycelium growth on PDA plates, in addition, they abolished the fungal growth on artificially infected oranges by diminishing both disease incidence (DI) and lesion diameter (LD). Chitosan (CHS) effect on *P. digitatum* growth was tangible; it could inhibit mycelial growth on PDA plates at all tested concentrations.

Likewise, our data are in agreement with **Coutinho *et al.* (2020)**, who applied different forms of CHS against two *Penicillium* species. The results demonstrated the antifungal action of CHS against those fungal pathogens in a dose-directed behavior. Similarly, different CHS concentrations were assessed against *P. digitatum*, and the results highlighted the lethal action of CHS at higher concentrations on both *in vitro* and *in vivo* experiments **(Bagy *et al.,* 2020)**. Regarding the effect of salicylic acid (SA), the present results are consistent with those of **Allahverdi *et al.* (2021)** who studied the *in vivo* and *in vitro* impact of SA on the growth of *P. digitatum*. They observed that the radial growth of the pathogen was greatly reduced at the maximum SA concentration 16 mM. Moreover, the lesion diameter of treated lemon fruit was diminished at increased SA concentrations. Consequently, they stated that SA encouraged pathogen eradication in a dose-dependent way because of its fungitoxic activity on green mold pathogen. Similarly, increasing the dose of SA from 2 up to 8 mM affected the lesion diameter positively. Along with our findings, **Moosa *et al.* (2019)** studied the applicability of using SA at different concentrations as a preventive strategy against green and blue mold pathogens.

They demonstrated that SA could suppress fungal growth, disease incidence, and disease severity. The efficacy of the treatment positively increased by increasing the concentrations from 2 mM to 8 mM. They credited their results to the ability of SA to induce the defensive mechanisms of citrus fruit, hence, SA can be harnessed for reducing green and blue mold rots of citrus. The impact of CA on *P. digitatum* growth was studied, the results indicated that CA could inhibit the fungal radial growth on PDA plates in a concentration-dependent style reaching complete mortality at 2 mM. Similarly, **Li *et al.* (2019)** reported the vigorous antifungal action of CA on *P. italicum*, the causative agent of blue mold disease of citrus, where CA could completely inhibit mycelium growth on PDA medium at 3 mM and the effect was positively correlated to the increased concentrations. Conversely, our data depicted that the application of CA concentrations in planta could not significantly achieve the control measure of green mold disease; disease incidence was not reduced as a result of dipping fruits into CA solutions.

This might be interpreted that *P. digitatum* has responded aggressively to CA on oranges triggering certain metabolic pathways in the fungus which encourages pathogen resistance to the treatment. A combination of *Enterobacter* bioagent with the studied GRAS compounds has been investigated. In accordance to our knowledge, this is the first study to employ *Enterobacter* as biocide to eradicate citrus green mold disease, and to develop liquid formulations based on this bacterium with some active GRAS compounds. Results of the overall treatments were positive and significant. *Enterobacter* fermentation broth suspended in different CHS, SA, and CA concentrations reduced both LD and DI effectively. They could inhibit fungal growth on the artificially inoculated orange fruits. The obtained data are consistent with studies that elucidated the bioactivity of the application of biocontrol agents with different bioactive compounds. **Li *et al.* (2019)** showed the activity of the combined treatment of the yeast, *Cryptococcus laurentii* with CA, and reported that the efficacy of the combined treatment was greater than of the single treatment of either the yeast or the CA in reducing disease incidence of blue mold disease.

Notably, *Enterobacter* cells suspended in SA have conferred promising results in controlling green mold pathogen on infected oranges as compared to the untreated fruits. Applied treatments caused a significant reduction of both LD and DI on fruits, these results are in line with the findings of **Lyousfi *et al.* (2021)**, they evaluated the impact of SA at different concentrations combined with *Bacillus amyloliquefaciens* (SF14) and *Alcaligenes faecalis* (ACBC1) antagonistic bacteria to control *Monilinia fructigena*, the causative agent of brown rot disease. They indicated that the combined effect of SA at tested concentrations with both antagonistic bacteria significantly inhibited the mycelia growth *in vitro* and disease severity on nectarines, as well. Although the individual treatment of oranges with SA has impacted positively the disease parameters, such as LD and DI, our data illustrated the adverse effect of *Enterobacter* cells when introduced with higher concentration of 8 mM SA. Similarly, obtained results are in harmony with those of **Lyousfi *et al.* (2021)**, who emphasized that disease severity increased when fruits were treated with SA at higher concentration and bacterial bioagents.

It could be inferred that SA at higher amounts could not improve either the bioactivity of the antagonists or the resistance of the fruits against the disease. *Enterobacter* sp. (SH-87) was incorporated into preparation of edible films for coating purposes. Two film-forming solutions were prepared, CHS/SA (4, 6 mM) and CHS/CA (1.5, 2 mM) amended with 3% (v/. v) *Enterobacter* fermentation liquid. The obtained results were promising, as both the DI and LD were significantly reduced compared with the uncoated fruits. Recently, few studies shifted to employing edible films for the encapsulation of microbial antagonists. Chitosan-based coatings have been broadly applied for coating purposes because of their effectiveness in curbing fungal decay, in addition to delaying fruit ripening **(Aloui *et al.*, 2014)**.*Enterobacter* was incorporated into different bioformulations. **Shabbir *et al.* (2020)** employed *Enterobacter* sp. UPMSSB7 in a peat-based formulation associated with arbuscular mycorrhizal fungus *Glomus mosseae* in addition to silicon. They assessed the efficacy of this formulation against *Rigidoporus microporus* which causes the white root rot disease of rubber.

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Table. Volatile compounds extracted from *Enterobacter* sp. SH-87 using HS-SPME-GC/MS by two different fibers.

(A): Red fiber

Fiber type	Retention time RT (min)	Component name	Area Sum%
	3.15	Z,Z,Z-1,4,6,9-Nonadecatetraene	1.03
(A)	18.113	.2-Methylundecanoic acid	1.42
	20.024	Isovanillin	1.74
	21.414	Ethyl Vanillin	1.11
	24.522	Ethylene glycol - Adipate - Diethylene glycol	0.91
	25.111	Methyl myristate	3.04
	25.929	1-Pentylhexylamine	1.79
	25.975	Methyl hexadecanoate	20.7
	26.061	Methyl hexadecanoate	7.3
	26.181	Palmitic acid	0.97
	26.421	Margaric acid methyl ester	3.9
	26.478		1.09
	26.69	Oleic acid, methyl ester	28.88
	26.753	Methyl isostearate	14.64
	27.114	Methyl nonadecanoate	4.29
	27.497	Methyl eicosanoate	6.34

(B): Black fiber

RT (min)	Component name	Area Sum %
13.999	3,5-Di-O-benzoyl-2-deoxypentofuranose	1.5
16.116	Cuminaldehyde	3.39
16.826	α-Citral	2.71
17.243	Anethole	3.4
17.318	Thymol	5.12
17.592	p-Isopropenylphenol	2.58
18.228	Dodecamethylcyclohexasiloxane	0.93
18.914	2,4-Diisocyanato-1-methylbenzene	9.63
19.137	Neryl acetate	2.57
19.612	Geranyl acetate	1.84
19.692	Toluene-2,3-diamine	0.85
20.019	.6-Amino-1,3-dihydro-2H-indol-2-one	4
20.156	4-Pyrrolidinopyridine	3.34
20.236	2-Benzimidazolinone, 5-methyl	4.54
20.665	Caryophyllene	1.57
20.98	cis-α-Bergamotene	4.12
21.667	.(E)-Ethyl cinnamate	0.54
22.45	Tetradecamethylcycloheptasiloxane	5.2
22.553	cis-α-Bisabolene	0.57
22.708	β-Bisabolene	6.87
24.11	2,6,10-Trimethyltetradecane	0.74
24.522	Ethylene glycol - Adipate - Diethylene glycol	2.05
24.745	Hexadecamethyl-cyclooctasioxane	1.1
25.071	Stilbene	0.58
25.666	Cycloartenol acetate	1.35
25.867	1-Heptatriacontanol	0.99
26.073	1-Heptatriacontanol	1.19
26.118	Ethyl iso-allocholate	1.49
26.187	Palmitic acid	3.36
26.582	Dihydroxanthin	0.56
26.696	Lupeol	1.52
26.828	Oleic Acid	1.75
26.885	Stearic acid	0.86
27.068	Biphenol A	9.76
27.308	3-Hydroxyspirost-8-en-11-one	1.64
27.474	Brassicasterol acetate	5.78