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ORIGINAL PAPER

Control of Green and Blue Molds of Citrus Fruits Using some Biocontrol Agents under Egyptian Conditions

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ABSTRACT

Green mold (*Penicillium digitatum* (Pers.: Fr.) Sacc.) and blue mold (*Penicillium italicum* Wehmer.) are the main postharvest diseases attacking citrus fruits and responsible for huge losses. The efficacy of three bio-commercial products namely Biocontrol T34 (*Trichoderma asperellum*), BAS (*Bacillus amyloliquefaciens*) and SerenadaASO (*Bacillus subtilis)*, were tested *in vivo* to control Citrus green and blue molds on orange fruits of cv. Valencia late (*Citrus sinensis* L. Osb.). Tecto (Thiabendazole) was used to compare the effectiveness of the three bio-commercial products. Concerning decay incidence, Tecto and Biocontrol T34 were the best treatments, completely inhibited green mold as compared to water control. Serenada ASO and BAS reduced the percentage of infection by 92.8 and 85.7%, respectively as compared to water control. In addition, regarding disease severity, also Tecto and Biocontrol T34 were the best treatments, completely inhibited green mold lesion development. Serenada ASO and BAS reduced the percentage of lesion diameter by 95.7 and 97.8%, respectively compared to untreated control. Biological control agents are promising alternatives control methods to manage citrus postharvest decays.

Keywords: Citrus, *Citrus sinensis*, *Penicillium digitatum*, green mold. *Penicillium italicum*, blue mold, Biological Control.

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INTRODUCTION

Citrus is commercially produced in more than 137 countries worldwide, meanwhile climate is suitable for citrus production in Egypt. (Ismail and Zhang 2004). The total cultivated area of citrus fruits in Egypt in 2021 reached 493,925 Feddan, while the fruitful area is 440,210 Feddan, and the amount of production is around 4503,226 tons. The total cultivated area of oranges reaches 329,729 Feddan, and the fruitful area is 302,064 Feddan. The average production of an acre is 10.5 tons per Feddan, and the amount of total production is 3,173,430 tons. (Anon 2021). The genus Penicillium includes 150 species, but only a few species are economically important plant pathogens (Pitt, 1987). Green and blue rots, caused by *Penicillium digitatum* (Pers. Fr.) Sacc. and *P. italicum* Wehmer, respectively, are the most important postharvest diseases of citrus fruit grown under Mediterranean climate conditions. Both pathogens are necrotrophs that require wounds through the flavedo to enter the fruit (Ballester *et al*., 2010). Blue mold is generally of lesser overall importance but may become a major problem under prolonged storage conditions, which suppress development of green mold (Eckert and Eaks, 1989). At room temperature, green mold invades fruit much more rapidly than blue mould and predominates in mixed infections (Brown *et al*., 1995). Meanwhile, other pathogens are generally low incidence and affecting the decay to stored citrus fruit (Snowdon, 1990 a, b and Youssef *et al*., 2010). Synthetic fungicides application in packinghouses, before storage of citrus fruit is the most commonly used worldwide for management of citrus postharvest diseases. However, due to pathogen resistance regarding the fungicides and the hazards to health and environment because of the side effect of the use of fungicides there has been considerable interest in alternative control methods (Youssef *et al*., 2012).

Biological control has emerged as one of the most promising alternatives to the use of chemicals and fits in well with the concept of sustainable agriculture because it mostly exploits natural cycles with reduced environmental impact. During the last twenty years, several biological control agents have been widely investigated for use against different pathogens on fruit and vegetable crops. Many biocontrol mechanisms have been suggested for use on fruit including antibiosis, parasitism, competition, and induced resistance in the host tissue. Several antagonistic microorganisms were found capable of reducing the incidence of postharvest diseases of various fruits, both in small scale and semi-commercial conditions, such as *Pseudomonas* spp. (Smilanick and Denis-Arrue, 1992, Huang *et al*., 1995), *Bacillus* spp. (Arras and D'hallewin, 1994), *Debaryomyces hansenii* (Chalutz and Wilson, 1990), *Trichoderma viride* (Ubalua and Oti, 2007) and *Pichia guilliermondii* (Droby *et al*., 1997).

The main objective of this investigation was to evaluate the effectiveness of three bio-commercial products namely Biocontrol T34 (*Trichoderma asperellum*), BAS (*Bacillus amyloliquefaciens*) and Serenada ASO (*Bacillus subtilis*) were tested *in vivo* to control green and blue molds on orange fruits of cv. Valencia late (*Citrus sinensis* L. Osb.).

MATERIALS AND METHODS

Isolation and identification of the associated fungi:

The used *Penicillium digitatum* and *P. italicum* isolates in this study were isolated from infected fruits of *Citrus sinensis* L. Obseck cultivar Valencia late showing typical green and/or blue mold symptoms, respectively. According to the morphological and cultural characteristics both the two isolated fungi were identified, and the identification was confirmed at Mycology Research and Plant Disease Survey Department, Plant Pathology Research Institute, A.R.C., Egypt.

Preparation of the pathogens inocula:

P. digitatum and *P. italicum* inocula were prepared by growing each fungus on potato dextrose agar (PDA) in Petri plates incubated in the dark at 24°C. From cultures one week-old, conidia were collected using sterile spatula scraping, and then in distilled water containing 0.05% Tween suspended in sterile 80 (v/v). Through sterile gauze two layers the resulting suspension was filtered. Then using a Thom counting chamber the spore counts were made (Tiefe 0.100 mm, 1/400 qmm, HGB Henneberg-Sander GmbH, Lutzellinden, Germany) and to obtain a final concentration of 10^5 conidia/mL, dilute the suspension with sterile distilled water.

Pathogenicity tests:

Uniform in size fruits cv. Valencia late oranges were selected (diameter 9±0.5cm) appearance No sores or mechanical injuries or plague, no disease or disorders of any considerate. Orange fruits were surface sterilized for about 3-5 min, with a sodium hypochlorite 2% solution and washed with tap water and air dried at room temperature. Fruits were inoculated with 10 μL of a conidial suspension of each of *P. digitatum* and *P. italicum* adjusted to 10^5 conidia/mL after wounded at four places around the equatorial axis and as well as inoculated fruits were incubated for two weeks in high relative humidity at 24°C. Three replicates of five fruits for each treatment. The incidence of decay (infected wounds, %) was recorded. Orange fruits treated with sterile distilled water served as control.

Source of bioagents:

Two naturally existing potential bacterial bioagents, *Bacillus subtilis* and *Bacillus pumilus* isolates against fungal pathogens were isolated from the phyllospheric bacteria of guava leaves (Kamhawy, 2012) were used in this study.

Efficacy of *Bacillus subtilis* and *Bacillus pumilus* isolates on linear growth of *Penicillium italicum and Penicillium digitatum in vitro:*

An *in vitro* experiment was conducted to study the antagonistic effect of two bacterial isolates against *P. italicum and P. digitatum* on PDA by dual culture technique using Noval ring method (Adetuyi and Cartwright 1985). A mycelial plug of *Penicillium* was cut from colony margin by a 0.8 cm in diameter cork borer and placed in the center of a Petri dish containing PDA medium. A circular line was made with the edge of sterile 50 mm diameter glass tube dipped in suspension of the tested bacteria (10^8 cfu/mL) and stamped on the medium surrounding the fungal inoculum. The bacterial suspension was made according to the methods described by Yenjit *et al*. (2004). Plates were incubated for 7 days at 28°C. The colony diameter of the desired *Penicillium* sp. was measured and compared to a control experiment where the bacteria were replaced by sterile distilled water. Four replicates were used for each bacteria isolate. The results expressed as mean the percentage inhibition of the growth of the fungus using the following formula: fungal growth in control (mm) – fungal growth in treatment (mm) / fungal growth in control (mm) × 100.

Efficacy of biocontrol agents on Penicillium rot of citrus fruits *in vivo:*

Citrus sinensis (L. Obseck) Valencia late fruits were collected from a local market located at Giza governorate in Egypt. Oranges were selected by uniformity of size and health of any diseases or disorders, and directly processed. The oranges were surface sterilized with sodium hypochlorite 2% for about 3-5 min, rinsed with tap water and dried at room temperature.

Citrus fruits in post-harvest stage were used in this experiment. The fruits were disinfected with 70% alcohol and flamed before getting pierced with a sterilized needle to open 10 wounds on each fruit. Two experiments were separately performed. In the first experiment, 0.1 mL spore suspensions of *P. italicum* and *P. digitatum*, (4×10^5 spores /mL) was inoculated at the wounded areas of the citrus fruits, then incubated at room temperature for 24 h prior to the spray of cell suspension of bacterial antagonist (10^8 cfu/mL). For the second experiment, citrus fruits were sprayed with the bacterial antagonist and kept for 24 h at room temperature before they were inoculated with *P. italicum or P. digitatum*, the fruits from two experiments were incubated at room temperature (27+2ºC) before symptoms were observed and disease severity was recorded. There were five replicates in each treatment. The disease severity was recorded after 10 days incubation by measuring rotted areas occurring on the fruit surface were evaluated according to Ippolito *et al*. (2005).

Effect of different products against molds *in vivo*:

Product solutions:

For experimental use, the required concentration of any product was prepared in 100 mL distilled water as shown in Table 1.

Table (1): Product solutions, their active ingredient and recommended dose used.

Product	Active ingredient	Dose
Tecto 50% SC	Thiabendazole	1.5 mL/L
Biocontrol T34 12% WP	*Trichoderma asperellum*	0.85 g/L
BAS	*Bacillus amyloliquefaciens*	0.3 g/L
Serenade ASO	*Bacillus subtilis* QST 713	3.3 mL/L

Effect of different commercial products against molds *in vivo*:

Citrus sinensis (L. Obseck) Valencia late fruits were collected from a local market located at Giza governorate in Egypt. Oranges were selected by uniformity of size and health of any diseases or disorders, and directly processed. The oranges were surface sterilized with sodium hypochlorite 2% for about 3-5 min, rinsed with tap water and dried at room temperature. The collected fruits using sterile nail-head were wounded in an equidistant point in the equatorial zone (5 mm × 3 mm, depth: wide). Representing each treatment 30 µl of the desired product solution were applied in each wound. After 2 hours, 10 µl of a 10^5 CFU suspension of *P. digitatum* or *P. italicum* in the same wound were inoculated each alone. The control was treated with distilled water and then inoculated with the same pathogen concentration. Three replicates were used for each treatment and each replicate consisted of 5 oranges. Treated fruits were kept in plastic bags and incubated for two weeks in high relative humidity at 20±1°C. The decay incidence (% infected wounds) and severity of disease (lesions diameters, mm) were determined and recorded according to Ippolito *et al*. (2005)

Statistical analysis:

The infected wounds percentage (disease incidence) and diameters of lesion (disease severity) data, taken from each wound, were elaborated, and analyzed by one-way analyses of variance using the software Statistica 6. Using Duncan's Multiple Range Test at $P\leq0.05$ level mean values were compared (Gomez and Gomez, 1984).

RESULTS

Identification of the isolated fungi:

The isolated fungi from naturally decayed citrus fruit were purified and identified as: *Penicillium digitatum* (Pers.: Fr) Sacc. and *Penicillium italicum* Wehmer. according to its morphological and cultural characteristics (Visagie *et al*., 2014). A pure culture of each fungus was maintained on potato dextrose agar (PDA) and stored at 4°C, the pathogen inoculum was obtained from 7-day-old culture plates incubated at 25°C. The spore concentration was determined with the aid of a haemocytometer and adjusted to 10^6 spores per ml with sterile distilled water.

Pathogenicity tests:

Pathogenicity test proved that all orange fruits artificially inoculated with the pathogens were infected and the incidence of decay (infected wounds, %) was 100%. Also, untreated fruits (control) remained healthy without any infections.

Efficacy of *Bacillus subtilis* and *Bacillus pumilus* isolates on linear growth of *Penicillium italicum* and *Penicillium digitatum in vitro:*

The two-biocontrol agents, *B. subtilis* and *B. pumilus* were tested for their antagonistic effect against *P. italicum* and *P. digitatum*_in dual culture technique. The growth inhibition of values *P. italicum* and *P. digitatum* by the two-biocontrol agents were determined. As shown in Figure (1), the two isolates of the biocontrol agents gave almost a total reduction of the growth of the two isolates of Penicillium in the laboratory after incubation for a week.

Efficacy of biocontrol agent on Penicillium rot of citrus fruits *in vivo:*

Data presented in Table 2 and Fig. 2 clearly show that all promising isolates of bacteria are able to reduce disease severity on citrus fruits. It was noticed that when bacterial antagonists were applied as spray application at 24 h before inoculation by*P. italicum* or *P. digitatum* was more efficient than after inoculation. *B. pumilus* provided the highest disease reduction percentage for both *P. italicum* and *P. digitatum* being 79.60 and 67.88 %, respectively of in comparison with control. The antagonistic activities of *Bacillus* spp. could be attributed to the production of endospores so, it more adapted to environmental extremes than the pathogen.

Effect of different biological products against molds *in vivo*:

Decay incidence (%) and severity of disease (lesion diameter (mm)) caused by *P. digitatum* on "Valencia late" oranges treated or untreated with some biological products are reported in Table (3) and Figs. (3 and 4) and for *P. italicum* are reported in Figs. (5 and 6) respectively.

Regarding green mold, concerning decay incidence, Tecto and Biocontrol T34 were the best treatments, completely inhibited (100%) green mold. Serenada ASO and BAS reduced the percentage of infection by 92.9 and 85.7%, respectively, as compared to water control (Fig. 3).

In addition, concerning disease severity, also Tecto and Biocontrol T34 were the best treatments, completely inhibited (100%) green mold lesion development. Serenada ASO and BAS reduced the percentage of lesion diameter by 95.7 and 97.8%, respectively, as compared to un-treated control (Fig. 4).

Overall, no differences were found between the chemical fungicide Tecto and the biological product Biocontrol T34 (*Trichoderma asperellum*) in terms of disease incidence and disease severity.

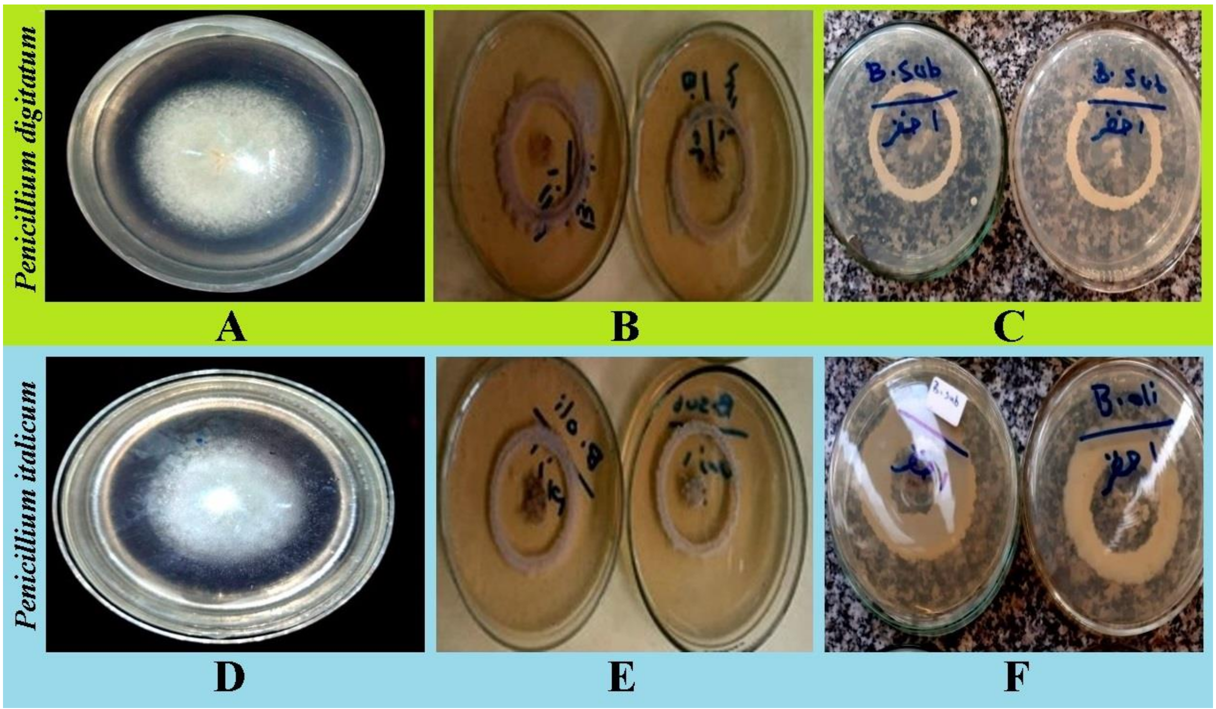

Fig. (1). Novel ring bioassay showing the highest percentage inhibition of B. subtilis & B. pumilus isolates on *P. italicum* or *P. digitatum* After 10 days incubation. A&D: Control, B&E *Bacillus subtilis*., C, and F *Bacillus pumilus* isolates.

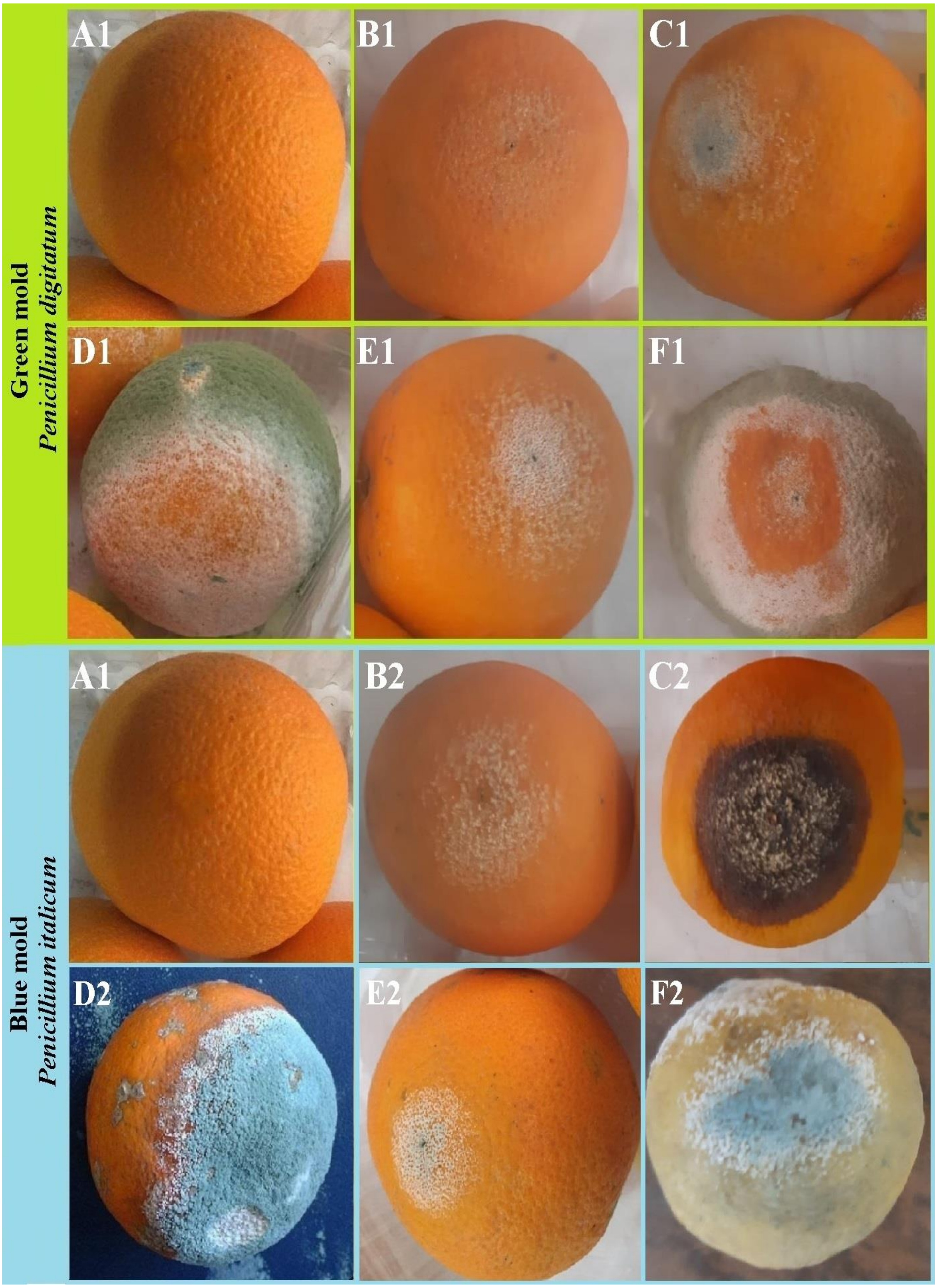

Fig. (2). Efficacy of bacterial antagonists, *B. subtilis* or *B. pumilus* isolates for reducing the amount of disease caused by *P. digitatum* or *P. italicum* disease on citrus fruits when sprayed before and after inoculation each pathogen by 24h (A1) control treatment with water (D1 and D2) infected control with *P. digitatum* or *P. italicum*, (B1 and B2) *B. pumilus* treatment before inoculation by *P. digitatum* or *P. italicum*. (C1 and C2) *B. pumilus* treatment after inoculation by *P. digitatum* or *P. italicum* (E1 and E2) *B. subtilis* treatment before inoculation by *P. digitatum* or *P. italicum*. (F1 and F2) *B. subtilis* treatment after inoculation by *P. digitatum* or *P. italicum*.

Table 2. Efficacy of bacterial antagonists for reducing the amount of disease caused by *P. italicum or P. digitatum* on citrus plants when sprayed before or after treatment by *B. subtilis* and *B. pumilus* for 24h.

Treatments	*P. digitatum*		*P. italicum*		Mean	
	% Disease severity	% Disease reduction*	% Disease severity	% Disease reduction*	% Disease severity	% Disease reduction*
	Application treatment 24hr.before inoculation					
B. pumilus	10.20	79.60	22.00	67.88	16.10	73.74
B. subtilis	30.00	40.00	32.00	53.28	31.00	46.64
** Control	50.00	-	68.50	-	59.25	
Means	-	59.80	-	60.58		
	L.S.D. at %5 for Treatments (T) = 2.1; Spray (S)= 1.9; T × S = 3.1					
	Application treatment 24hr.after inoculation by					
B. pumilus	35.00	30.00	55.00	19.71	45.00	24.85
B. subtilis	42.00	16.00	57.00	16.79	49.50	16.39
** Control	50.00	-	68.50	-	59.25	
Means	-	23.00	-	18.25		
	L.S.D. at %5 for Treatments (T) = 2.7; Spray(S)= 2.2; T × S = 3.6					

* Disease reduction (%) recorded 10 days after inoculation. ** Control = Inoculated fruits, sprayed with water

Table (3): Disease incidence (%) and Lesion diameter (mm) due to artificial inoculation by *P. digitatum* and *P. italicum* on oranges "Valencia late" treated with different products.

Product	*P. digitatum*				*P. italicum*			
	*DI (%)	**R %	***L (mm)	**R %	*DI (%)	**R %	***L (mm)	**R %
Tecto 50% SC	0.0	100.0	0.0 b	100.0	0.0	100.0	0.0 b	100.0
Biocontrol T34 12% WP	0.0	100.0	0.0 b	100.0	13.3	86.7	4.0 b	93.3
BAS	13.3	85.7	2.3 b	95.7	20.0	80.0	4.7 b	92.2
Serenade ASO	6.7	92.9	1.2 b	97.8	26.7	73.3	7.3 b	87.8
Water (control)	93.3		54.7 a		100.0		60.0 a	

* DI = disease incidence; **R = reduction; ***LD = Lesion diameter (mm).

Regarding blue mold, concerning decay incidence, only the chemical fungicide Tecto was able to completely inhibit (100%) blue mold caused by *P. italicum*. However, Biocontrol T34, BAS and Serenada ASO reduced the percentage of infection by 86.7, 80 and 73.3%, respectively compared with un-treated control (Fig. 5).

In addition, concerning disease severity (lesion diameter), the products also showed the same pattern as disease incidence. In particular, Biocontrol T34, BAS and Serenada ASO reduced the percentage of lesion diameter by 93.3, 92.2 and 87.8%, respectively compared with un-treated control (Fig. 6).

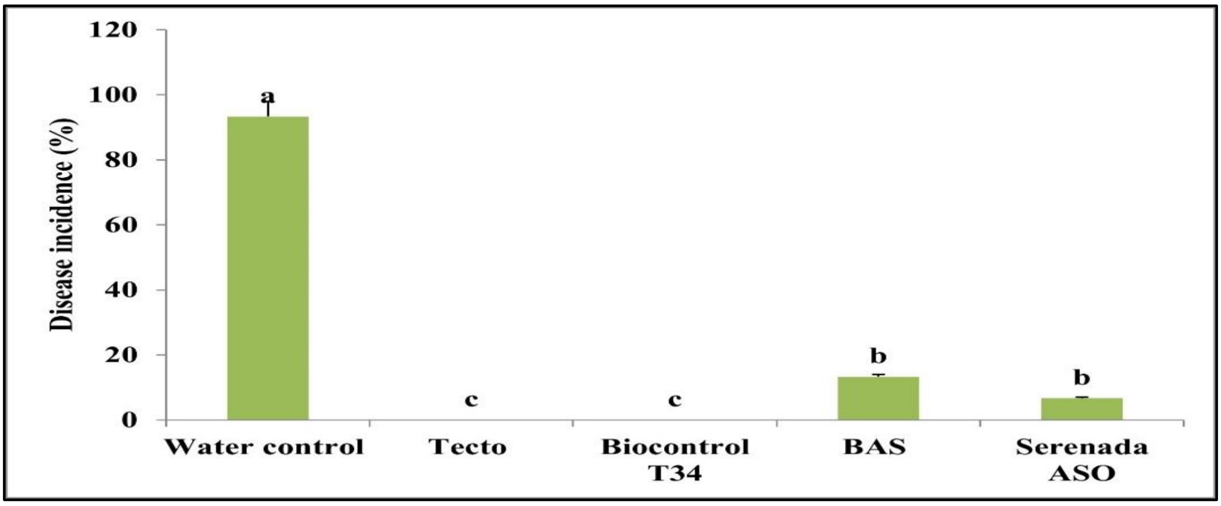

Fig. (3): Disease incidence (%) due to artificial inoculation by *P. digitatum* on oranges "Valencia late" treated with different products. Treatments with different letters significantly difference at $P \leq 0.05$.

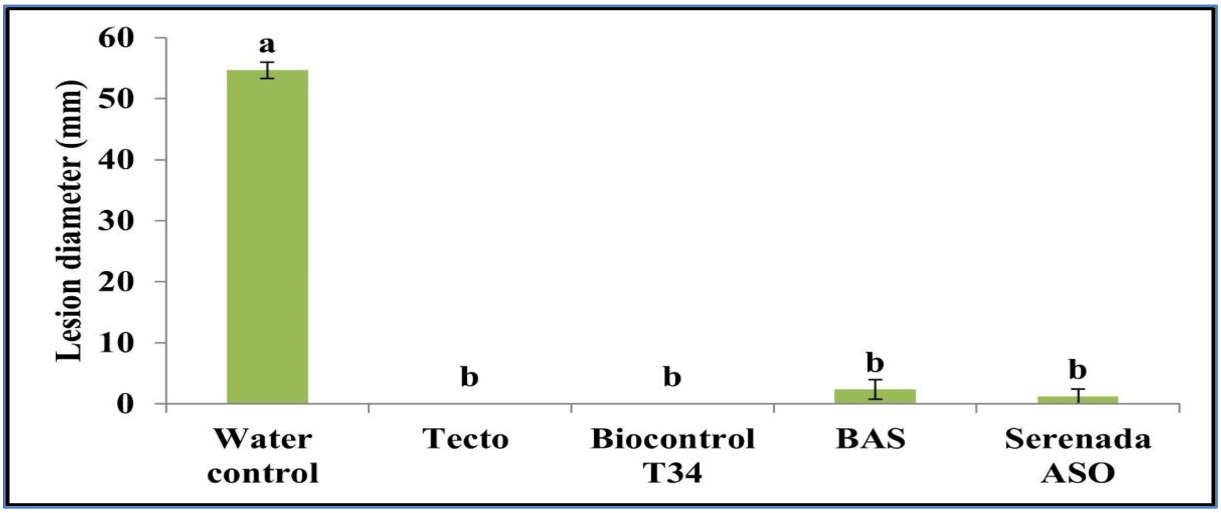

Fig. (4): Lesion diameter (mm) due to artificial inoculation by *P. digitatum* on oranges "Valencia late" treated with different products. Treatments with different letters significantly difference at P ≤ 0.05.

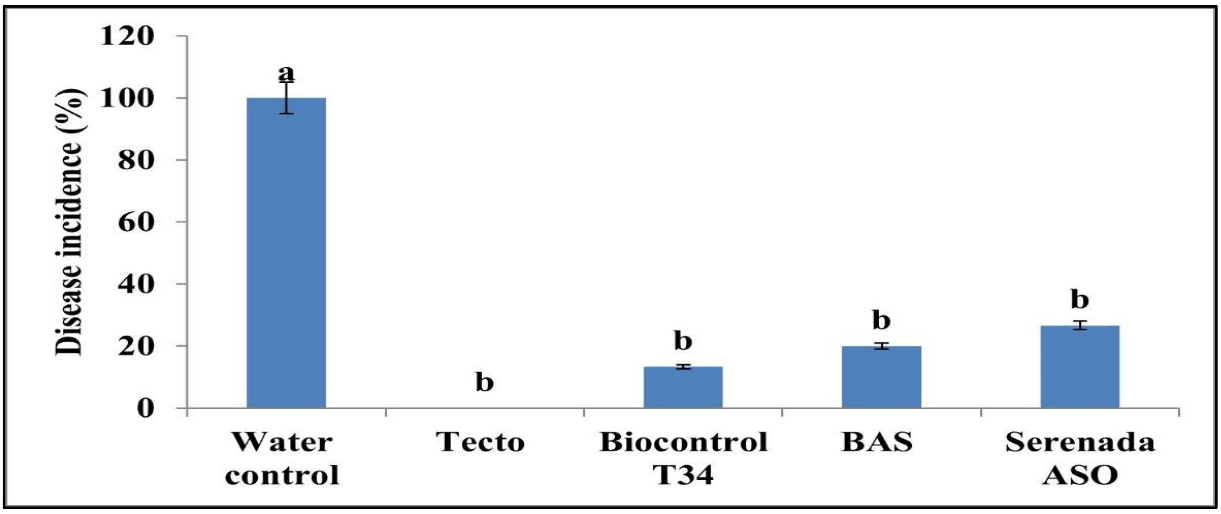

Fig. (5): Disease incidence (%) due to artificial inoculation by *P. italicum* on oranges "Valencia late" treated with different commercial products. Treatments with different letters significantly difference at *P* ≤ 0.05.

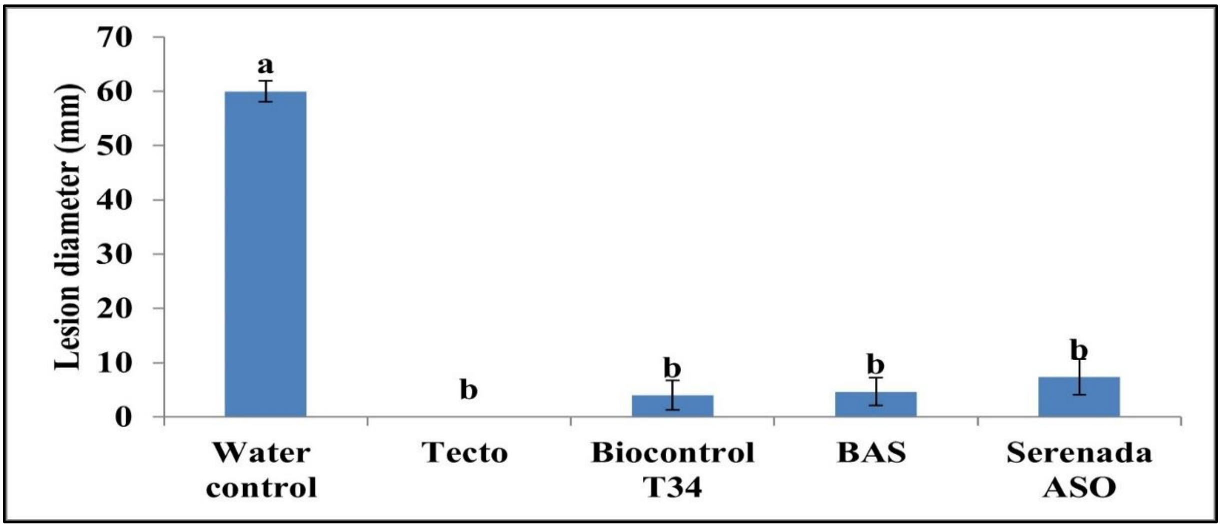

Fig. (6): Lesion diameter (mm) due to artificial inoculation by *P. italicum* on oranges "Valencia late" treated with different products. Treatments with different letters significantly difference at *P* ≤ 0.05.

DISCUSSION

Green and blue molds are the major diseases that cause the greatest loss of postharvest citrus fruits (Zhang *et al* 2009). Researchers are trying to find alternative means to control plant pathogenic fungi in general and especially those causing postharvest diseases to reduce and/or eliminate the use of fungicides. This study aims to investigate the effect of some bioproducts against blue and green molds in vivo on "Valencia late" oranges by using Biocontrol T34 (*Trichoderma asperellum*), BAS (*Bacillus amyloliquefaciens*) and SerenadaASO (*Bacillus subtilis*), against *P. italicum* and *P. digitatum* under artificial inoculation. Regarding green mold, concerning decay incidence, Tecto and Biocontrol T34 were the best treatments, completely inhibited green mold. Perhaps this is due to the ability of *Trichoderma asperellum* to parasitize the fungus that causes the disease. Serenada ASO and BAS reduced the percentage of infection by 92.9 and 85.7%, respectively as compared to water control. This efficacy may be due to the ability of *Bacillus amyloliquefaciens* and *Bacillus subtilis* to secrete antibiotics that have the ability to inhibit pathogenic fungi that cause blue and green rots of citrus fruits. In addition, concerning disease severity, also Tecto and Biocontrol T34 were the best treatments that completely inhibited (100%) green mold lesion development. Serenada ASO and BAS reduced the percentage of lesion diameter by 95.7 and 97.8%, respectively as compared to water control. *B. amyloliquefaciens* and *B. subtilis* could inhibit the spore germination and fungal growth of *P. italicum* and *P. digitatum. B. amyloliquefaciens* and *B. subtilis* may be responsible for some of the exopolysaccharide (slime or gum) production seen in sugarcane processing plants. Amylolysis is usually not associated with dextran production. However, these biologics can produce LeVan (a fructose-based exopolysaccharide) from sucrose (Marvasi *et al*. 2010 and Tian *et al*. 2011). As mentioned before, decay pathogens including *P. digitatum* and *P. italicum* enter fruit through wounds sustained during harvesting and handling. This implies that the pathogen is already in the fruit before treatment is applied. Any antagonist to be beneficial to growers must be able to stop further development of a pathogen that is already in the fruit.

Mechanism of biological control of plant pathogens is generally involve competition for nutrients, production of bacterial metabolites such as iron chelating siderophores, hydrogen cyanide (HCN), antibiotics, extracellular lytic enzymes induced systemic resistance (O'Sullivan and O'Gara, 1992; Van Loon *et al*., 1998). O'Sullivan and O'Gara,1992 reported that, successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal. Huang (2008) reported, with Bacillus showing strong antagonistic activity against *Penicillium digitatum*. B. (1.6×10^{10} to 1.6×10^{12} CFU ml^{-1}) gave significant control of *P. digitatum* infection in Valencia orange, which was as effective as imazalil (500 µg ml^{-1}) and was significantly better than benomyl treatment (500 µg ml-^{1}). When lower concentrations of the bioagents (1.9×10^{7} to 1.9×10^{9} CFU ml^{-1}) were tested (Yenjit *et al*. 2004) on Washington Navel orange and Lisbon lemon fruit, the antagonist caused significant control of *P. digitatum* infection at two inoculum levels (6.5×10^{4} and 6.5×10^{5} spores ml-5) (Youssef *et al*., 2010). Concentrations of both the pathogen and the antagonist affected the biocontrol effect. Hammami et al. (2022) reported that overall, 180 yeasts and bacteria isolated from the peel of citrus fruits were screened for their in vitro antagonistic activity against *Penicillium digitatum* and *P. italicum*, causative agents of green and blue mold of citrus fruits, respectively. Three bacterial isolates were selected for their inhibitory activity on mycelium growth. The bacterial isolates were identified as *Bacillus amyloliquefaciens*, *B. pumauis* and *B. subtilis* isolates significantly reduced the incidence of decay incited by *P. digitatum* and *P. italicum* on 'Valencia' orange and 'Eureka' lemon fruits. Moreover, they were effective in preventing natural infections of green and blue mold of fruits stored at 4 ◦C. The antagonistic efficacy of the three isolates depended on multiple modes of action, including the ability to form biofilms and produce antifungal lipopeptides, lytic enzymes and volatile compounds.

Huang *et al*. (2008) reported a new antagonist/pathogen combination, with *Bacillus pumilus* showing strong antagonistic activity against *P. digitatum*. *B. pumilus* gave significant control of *P. digitatum* infection in "Valencia" orange, which was as effective as imazalil and was significantly better than benomyl treatment. In addition, Hernández-Montiel *et al*. (2010) tested the performance of twelve native isolates of *Debaryomyces hansenii* obtained from the marine environment and the pericarp of Mexican lime. Native pericarp isolates were more effective both *in vitro* and in simulated industrial packinghouse conditions for the postharvest control of blue mold on Mexican lime. The performance of the yeast was partially linked to a rapid consumption of available sugars in the medium, and *D. hansenii* isolates DhhBCS06, LL1, and LL2 were able to reduce incidence of the disease by up to 80% after two weeks of storage.

CONCLUSION

Biocontrol agents have emerged as one of the most promising alternatives to the use of chemicals and fits in well with the concept of sustainable agriculture because it mostly exploits natural cycles with reduced environmental impact. This study suggested that biocontrol agents can be easily included among the integrated pest management strategies to manage postharvest decays of citrus fruits.

CONFLICTS OF INTEREST

The author(s) declare no conflict of interest.

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