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Research Article

Biocontrol of *Penicillium digitatum* on Postharvest Citrus Fruits by *Pseudomonas fluorescens*

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The effectiveness of the bacteria antagonist *Pseudomonas fluorescens* to control green mold caused by *Penicillium digitatum* on oranges (*Citrus sinensis Osbeck*, cv. Jincheng) and the possible modes of action were evaluated. Whether *in vitro* or *in vivo*, treatments with cell-free autoclaved cultures or culture filtrate had limited capacity to suppress *P. digitatum*, while *P. digitatum* was significantly inhibited by bacterial fluid (*P. fluorescens* in the nutrient broth liquid medium) and bacterial suspension (*P. fluorescens* in sterile distilled water) with living cells. There was a positive relationship between the concentration of *P. fluorescens* in bacterial suspension and its biological efficacy. In addition, *P. fluorescens* was effective when applied preventatively but not when applied curatively. In the inoculated wounds, the population of *P. fluorescens* was an approximately 28- and 34-fold increase after being incubated at 20°C for 8 d and at 4°C for 16 d, respectively, and *P. digitatum* could effectively stimulate the growth and reproduction of *P. fluorescens*. Moreover, *P. fluorescens* was able to inhibit spore germination and germ tube elongation of *P. digitatum* as well as induce resistance on citrus peel by increasing the chitinase (CHI) activity and advancing the activities peaks of β-1,3-glucanase (GLU), peroxidase (POD), and phenylalanine ammonia lyase (PAL). All of these results support the potential application of *P. fluorescens* against green mold on postharvest citrus.

1. Introduction

Citrus fruits are important commercial fruits and widely distributed in the world. It is estimated that the global citrus production in 2017 was up to around 50 million metric tons [1]. Besides good sensorial characteristics, citrus contain high levels of antioxidant compounds, including vitamin C, flavanones, and anthocyanins [2, 3]. However, citrus fruits are exposed to many postharvest diseases during transportation and storage, among which green mold, caused by *Penicillium digitatum*, is one of the most devastating diseases, causing significant economic and resource losses in the world [4–6]. In addition, *P. digitatum* can cause an allergic response by producing countless air-borne spores [3, 7]. Traditionally, application of synthetic fungicides such as thiabendazole and imazalil was the main method to control green mold [8, 9], while resulted in pathogen resistance [10]. Public pressure to reduce fungicide use and to obtain healthy and safe fruits has driven research for development of no-chemical approaches to control postharvest diseases [3, 6, 11]. Among the different means, the use of antagonistic microorganisms for biological control of fruits decay appears to be an excellent option [12–14].

The biological control of major postharvest pathogens for citrus was reported by all kinds of microbial antagonists such as *Bacillus subtilis* [15, 16], *Pseudomonas* spp. [17], *Debaryomyces hansenii* [18], *Kloeckera apiculate* [13], *Candida membranifaciens* [6], and so forth. *Pseudomonas* *fluorescens*, a Gram-negative bacterium that is a common and abundant inhabitant in the soil and plant surfaces [19], has the capacity to inhibit or suppress a variety of pathogenic fungi [20, 21]. As an effective biocontrol agent, *P. fluorescens* has been studied extensively for plant disease in the rhizosphere for producing antibiotics such as phenazine-1-carboxylicacid (PCA) and 2,4-diacetylphloroglucinol (DAPG) [22, 23], producing volatile compounds [24, 25], excreting siderophore to compete with iron [26], competing for nutrients and space sites [27] and inducing systemic resistance [28, 29]. However, there are few researches or reports on its potential as a biocontrol agent in postharvest disease of fruits, especially for citrus fruits.

Therefore, the main objective of this investigation was to evaluate the effectiveness of *P. fluorescens* in the control of citrus green mold caused by *P. digitatum in vitro* via measuring the pathogen colony diameter on agar plates and counting spore germination rate and *in vivo* via calculating the disease incidence, investigating the population dynamics of *P. fluorescens* in wounds sites and its influences on the activities of defensive ferments chitinase (CHI), β-1,3-glucanase (GLU), peroxidase (POD), and phenylalanine ammonia lyase (PAL).

2. Materials and Methods

2.1. Fruit Material. Orange (*Citrus sinensis Osbeck*) fruits of cv. Jincheng were handharvested at commercial maturity from adult trees grown in an orchard where standard culture practices were employed, in Beibei, Chongqing, China, and oranges were transported to our laboratory within 4 h for this study. The fruits were selected for their uniform size, color, and absence of physical injuries or pests and pathogens infection. Four wounds (5 mm wide × 4 mm deep) were made using a sterile needle at the equatorial side. Then, the fruits were placed on a bench and divided into groups in a complete randomized block design (CRBD).

2.2. Pathogens. Penicillium digitatum was kindly provided by Dr. Wen from College of plant protection, Southwest University, Chongqing, China, and maintained on the potato dextrose agar medium (PDA: liquid extract from 200 g fresh potato, 20 g dextrose, 20 g agar, and water with total volume of 1000 mL) at 4°C. After culturing the pathogens on PDA at 25°C for one week, the cultures were scraped using a sterile loop and washed with sterile distilled water (SDW) containing 0.05% (v/v) Tween-80 to prepare the conidial suspension. Spore concentration was determined and adjusted to desired concentration by using a hemocytometer (Qiujing Biochemical reagent Instruments Co., Ltd., Shanghai, China).

2.3. Antagonist. Pseudomonas fluorescens was obtained from Dr. Zsolt Zalán, National Agricultural Research and Innovation Centre Food Science Research Institute, Budapest, Hungary, and was maintained at 4°C on the nutrient broth agar medium (NA: 18 g nutrient broth (NB) and 20 g agar in 1000 mL deionized water). Liquid cultures were inoculated with a loop of original culture in 50 mL of NB in 250 mL Erlenmeyer flasks for 16 h on a rotary shaker at 200 rpm/min. After this, the bacterial concentration was around 1.5×10^{10} CFU/mL. Different preparations of antagonist were prepared based on this bacterial fluid. Cell culture was centrifuged at 4000 × g for 10 min, and bacteria were precipitated in the bottom of the tube, while the supernatant contained only a few of bacteria. Then, (a) *P. fluorescens*-free medium (culture filtrate) was prepared by using a 0.22 μm polycarbonate membrane filter (Hefei Biosharp Co., Ltd., China) to filter the supernatants which allowed investigating the independent effect of bacteria metabolites secreted by *P. fluorescens*; (b) dilutions of bacterial fluid. Bacterial fluid (about 1.5×10^{10} CFU/mL) was diluted into 1×10^8 CFU/mL by adding the bacteria-free medium obtained in (a); (c) autoclaved *P. fluorescens* cultures, which were prepared by autoclaving 1×10^8 CFU/mL bacterial fluid that was obtained in (b) at 121°C for 20 min; and (d) bacterial suspension was prepared by using SDW to wash the bacterial precipitate twice to remove the residual culture medium and adjusted to 1×10^8 CFU/mL with the addition of SDW. SDW was used as the control in our investigation. The concentration was adjusted as desired by nephelometry (WZT-1M, Jinjia Scientific Instruments Co., Ltd., Shanghai, China).

2.4. The Effect of P. fluorescens on the Mycelium Growth of P. digitatum. The assay was performed according to [3, 18] with minor modifications. A hole (6 mm in length × 2 mm in depth) was made by using a hole puncher in centre of 1/2 PDA/NA (500 mL PDB, 9 g NB, 20 g agar, and water with total volume of 1000 mL), and 20 μL 1×10^6 spores/mL spore suspension were injected. Concomitantly, various processing fluids of antagonist were (1) injected into the same hole with the same volume of spore suspension, and (2) independently, various processing fluids were inoculated by a sterile loop to draw two lines (about 30 mm) symmetrically above and below (25 mm off the center) the spore hole. The plates were incubated at 25°C for 7 d, and the efficacy of *P. fluorescens* was determined by measuring the horizontal and vertical diameters of each mold plaque with the help of a Vernier caliper (Feng Liang International Group Co., Ltd., Hong Kong, China).

2.5. The Effect of P. fluorescens on Spore Germination and Germ Tube Elongation of P. digitatum. The assay was carried out as described by Wang et al. [30] with slight modification. Four mL of a 5×10^6 spores/mL suspension and 2 mL of bacteria-free medium (Section 2.3 (a)), bacterial fluid (Section 2.3 (b)), autoclaved cultures (Section 2.3 (c)), and bacterial suspension (Section 2.3 (d)) with different concentrations and SDW were added into 50 mL Erlenmeyer flasks containing 14 mL PDB, respectively. At least 100 *P. digitatum* spores per replicate were checked microscopically for germination percent and germ tube length after 12 h of incubation at 28°C on a rotary shaker at 150 rpm/min. When the size of the germ tube was equal to or greater than spore length, the conidia were considered germinated [30, 31].

2.6. The Effect of P. fluorescens on Citrus for the Control of P. digitatum. Citrus was wounded as described above (Section

2.1). The wound was then treated with 20 μL of one of the five mentioned bacterial preparations and allowed to dry for 2 h. Then, the same volume of 1×10^4 spores/mL conidial suspension of *P. digitatum* was inoculated into each wound site with a micropipette. When the surfaces of the fruits were dry, the fruits were put into fresh-keeping bags, and each orange was put in a fresh-keeping open bag in order to avoid mutual interference. All treated fruits were placed in a constant temperature and humidity incubator (LHS-150CLY, Qixin Scientific Instruments Co., Ltd. Shanghai, China) at 20°C under 90% relative humidity (RH). The lesion diameters were determined by taking the mean of the horizontal and vertical diameters of each lesion, and the disease incidence was calculated by the number of infected wounds. Any fruit wound with visible mold growth was considered to be infected.

2.7. The Effect of P. fluorescens Concentration on Biocontrol Efficacy. The wounded fruits previously prepared were inoculated with 20 μL of bacterial suspension at concentrations of 10^6, 10^7, 10^8, and 10^9 CFU/mL. Two hours later, wounded fruits were treated by the same volume of 1×10^4 spores/mL conidial suspension of *P. digitatum*. Wounds treated with 20 μL SDW before pathogen inoculation served as a control. All treated fruits were packed and placed in a constant temperature and humidity incubator at 20°C under 90% RH for 8 d; afterwards, the lesion diameters and disease incidence were determined as described earlier.

2.8. Preventative Action and Curative Action of P. fluorescens Antagonistic to P. digitatum. This part of experiment was divided into two tests. (1) The wounded fruits were treated with 20 μL of 1×10^8 CFU/mL bacterial suspension; after the wound site had dried for 0 h, 6 h, 12 h, and 24 h, each wound was inoculated with 20 μL of 1×10^4 spores/mL conidial suspension of *P. digitatum*. (2) The wounded fruits were treated with 20 μL of 1×10^4 spores/ml conidial suspension of *P. digitatum*; after the wound site had dried for 6 h, 12 h, 24 h, and 48 h, each wound was inoculated with 20 μL of 1×10^8 CFU/mL bacterial suspension. Then, all treated fruits were packed and placed in a constant temperature and humidity incubator at 20°C and 90% RH for 8 d; afterwards, the lesion diameters and disease incidence were determined as described earlier.

2.9. Population Dynamics of P. fluorescens in Fruit Wound. Aliquots (20 μL) of 1×10^8 CFU/mL bacterial suspension were applied to each wound site; 2 h later, the same volume of SDW or 1×10^4 spores/mL conidial suspension of *P. digitatum* were treated into the wounds, respectively. Then, the treated fruits were incubated at 20°C or 4°C, respectively. The population of *P. fluorescens* was enumerated at various time intervals (0, 2, 4, 6, and 8 d at 20°C and 4, 8, 12, and 16 d at 4°C) during incubation. The wounded areas from 5 fruits were gouged out with a sterile hole puncher (10 mm in diameter) and ground in a sterile mortar in 25 mL of SDW, grinding repeatedly. After that, serial 10-fold dilutions were prepared, and an aliquot of 100 μL of each dilution was plated on the NA medium. The plates were incubated at 28°C for 2 d, and the population density (expressed as log10 CFU/wound) was determined by counting the colonies.

2.10. Effects of P. fluorescens on the Defense Enzymes of Fruit. Citrus were wounded as described above (Section 2.1). The wounds were treated with 20 μL of 1×10^8 CFU/mL bacterial suspension and allowed to dry for 2 h, and the same volume of 1×10^4 spores/ml conidial suspension of *P. digitatum* was inoculated into each wound site with a micropipette. Wounds treated with SDW served as a control. After that, all treated fruits were packed and placed in a constant temperature and humidity incubator at 20°C under 90% RH. At various time intervals (0, 2, 4, 6, and 8 d), samples were taken from 10 independent fruits to analyze defense enzyme activities and protein content. Activities of chitinase (CHI) and β-1,3-glucanase (GLU) were determined as previously described in [32]. One unit of CHI was defined as the amount of enzyme required to catalyze the production of 1 μg *N*-acetylglucosamine per minute at 37°C. One unit of GLU was defined as the amount of enzyme required to catalyze the production of 1 μg glucose equivalents per minute at 37°C. Enzyme extraction and enzymatic assays for peroxidase (POD) activity and phenylalanine ammonia lyase (PAL) activity were measured according to the method of [33] with minor modifications. POD activity was expressed as one increase in absorbance at 470 nm per minute by using a spectrophotometer (T6, Puxi General Instrument Co., Ltd., Beijing, China). PAL activity was expressed as one increase in absorbance at 290 nm per minute.

2.11. Statistical Analysis. All the experiments were conducted twice using CRBD, and each treatment was replicated three times. Statistical analysis was performed with one-way analysis of variance (ANOVA) test using SPSS Version 19.0 software. All experimental data were expressed as mean ± standard deviation (X ± SD). Differences were considered to be statistically significant when $P < 0.05$ according to Dunnett's test.

3. Results

3.1. In Vitro Antifungal Assay. Antagonism of *P. fluorescens* against *P. digitatum in vitro* was determined with two different treatments in 1/2 PDA/NA (Table 1). *P. digitatum* was significantly ($P < 0.05$) inhibited by various processing fluids of *P. fluorescens*. No statistically significant differences were found between autoclaved cultures and culture filtrate, as well as between bacterial suspension and bacterial fluid. However, the antagonistic effectiveness of bacteria suspension and bacteria liquid was much higher than that of autoclaved cultures and culture filtrate. When bacterial suspension or bacterial liquid was cultured with spore suspension of *P. digitatum* together in the plate centre, the growth and reproduction of *P. digitatum* was completely inhibited. The inhibition obtained was around 40% when cultured with *P. digitatum*.

Table 1: The effect of *P. fluorescens* on the mycelial growth of *P. digitatum*.

Treatments	Plate central mixed culture		Plate confrontation culture	
	Mycelium growth (cm)	Inhibition rate (%)	Mycelium growth (cm)	Inhibition rate (%)
SDW	6.20 ± 0.17a	0.00 ± 0.00c	6.28 ± 0.07a	0.00 ± 0.00c
Autoclaved cultures	4.88 ± 0.08b	21.23 ± 1.61b	5.18 ± 0.09b	17.52 ± 1.41b
Culture filtrate	4.62 ± 0.11b	25.42 ± 2.26b	5.06 ± 0.12b	19.51 ± 1.90b
Bacterial suspension	0.00 ± 0.00c	100.00 ± 0.00c	3.70 ± 0.08c	41.04 ± 1.30a
Bacterial fluid	0.00 ± 0.00c	100.00 ± 0.00c	3.67 ± 0.0.13c	41.56 ± 2.13a

SDW, sterile distilled water, was prepared by autoclaving deionized water at 121°C for 20 min. Autoclaved cultures were prepared by autoclaving 1×10^8 CFU/ml bacterial fluid at 121°C for 20 min. Culture filtrate was prepared by using a 0.22 μm polycarbonate membrane filter to filtrate the supernatants. Bacterial suspension (1×10^8 CFU/mL) was prepared by using SDW to wash the bacterial precipitation twice to remove the residual culture medium. Bacterial fluid (1×10^8 CFU/mL) was prepared by diluting the culture (about 1.5×10^{10} CFU/mL) with culture filtrates. Values in a column followed by a different letter are significantly different according to Duncan's multiple range test at $P < 0.05$ level.

Table 2: The effect of *P. fluorescens* on spore germination and germ tube elongation of *P. digitatum*.

Treatments	Spore germination (%)	Germ tube length (μm)
Control	87.32 ± 1.14a	49.56 ± 7.29a
Culture filtrate	30.03 ± 0.28b	29.90 ± 3.76c
Autoclaved cultures	29.67 ± 0.69b	39.42 ± 3.56b
1×10^6 CFU/mL bacterial suspension	19.80 ± 0.59c	13.27 ± 3.85d
1×10^7 CFU/mL bacterial suspension	5.66 ± 0.43d	7.86 ± 1.60e
Bacterial fluid (1×10^8 CFU/mL)	1.00 ± 0.82e	4.17 ± 1.18e
1×10^8 CFU/mL bacterial suspension	0.66 ± 0.47e	3.75 ± 1.25e

Autoclaved cultures were prepared by autoclaving 1×10^8 CFU/ml bacterial fluid at 121°C for 20 min. Culture filtrate was prepared by using a 0.22 μm polycarbonate membrane filter to filtrate the supernatants. Bacterial suspension was prepared by using SDW to wash the bacterial precipitation twice to remove the residual culture medium and adjusted to designed one with the addition of sterile distilled water. Bacterial fluid (1×10^8 CFU/mL) was prepared by diluting the culture (about 1.5×10^{10} CFU/mL) with culture filtrates. Values in a column followed by a different letter are significantly different according to Duncan's multiple range test at $P < 0.05$ level.

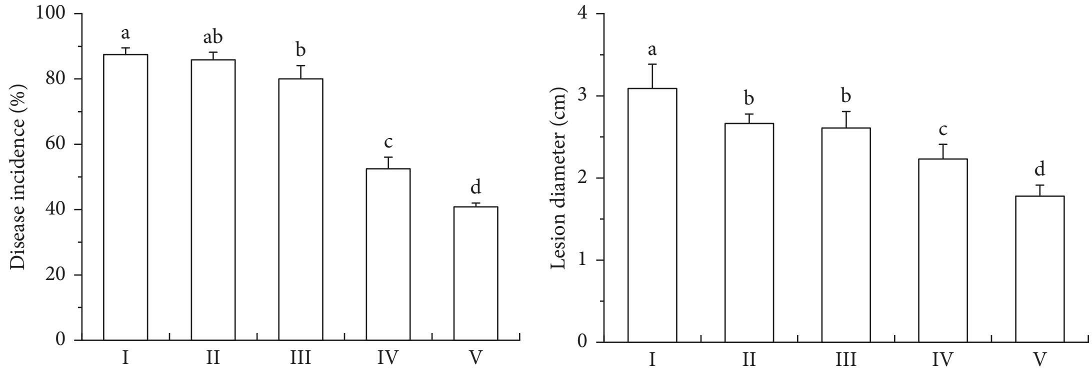
As shown in Table 2, various processing fluids of *P. fluorescens* significantly ($P < 0.05$) inhibited spore germination, and germ tube elongation of *P. digitatum*, among which bacterial suspension and bacterial fluid, had the greatest antagonistic capacity. The concentration markedly influenced the effectiveness of bacterial suspension, the higher concentration, the lower spore germination rate, and the smaller germ tube length. The spore germination rate was only 0.66%, and the germ tube length was only 3.75 μm, when the concentration of bacterial suspension was 1×10^8 CFU/mL.

3.2. The Effect of P. fluorescens on Citrus for the Control of P. Digitatum. The effects of *P. fluorescens* on citrus for the control of *P. digitatum in vivo* are presented in Figures 1 and 2. There were no significant differences in the disease incidence or lesion diameter between autoclaved cultures and culture filtrate, which had limited protection against pathogen infection. The bacterial fluid remarkably inhibited *P. digitatum*, but its effectiveness was significantly less ($P < 0.05$) than that of bacterial suspension. The highest level of the antagonistic effect of *P. fluorescens* to inhibit green mold decay, as reflected by the lowest disease incidence and the smallest lesion diameter, was observed with the treatment of bacterial suspension (Figure 1). At the same time, the statistical analysis revealed a significant ($P < 0.05$) effect of concentration of bacterial suspension on disease incidence and lesion diameter. The protection offered by bacterial suspension was higher with increasing concentrations of antagonist. When the bacterial suspension was applied at 1.0×10^9 CFU/mL, the disease incidence was reduced from 87.50% to 30.00%, and the lesion diameter was reduced from 3.09 cm to 1.27 cm, respectively, compared with the control treated with SDW (Figure 2).

3.3. Preventative Action and Curative Action of P. fluorescens Antagonistic to P. digitatum. As shown in Figure 3, significant differences ($P < 0.05$) were observed on disease incidence and lesion diameter corresponding to different periods separating the *P. fluorescens* and the *P. digitatum* inoculation. When bacterial suspension was inoculated later than the pathogen or applied simultaneously to the wound, the incidence of green mold decay ranged from 46.67% to 81.67%, and the lesion diameter ranged between 1.88 cm and 2.72 cm. While *P. fluorescens* was inoculated before the pathogen, the disease incidence was below 35%, and the lesion diameter did not exceed 1.7 cm.

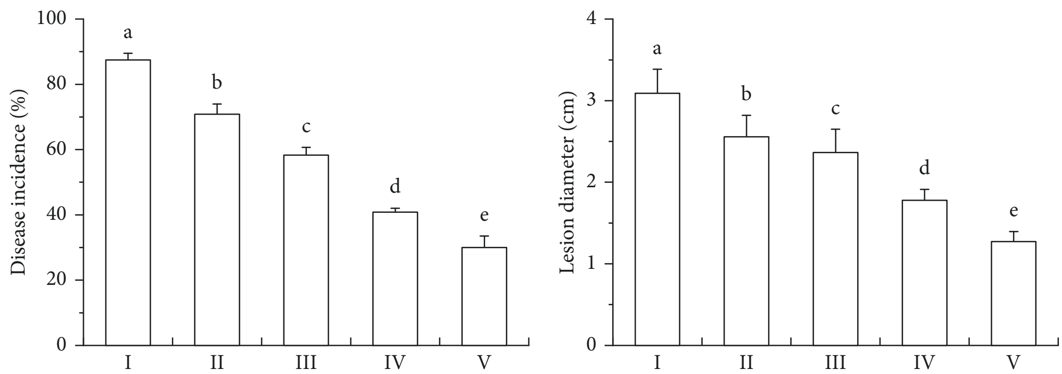
3.4. Population Dynamics of P. fluorescens in Fruit Wound. As shown in Figure 4, the population of *P. fluorescens* increased quickly in wounded fruit at 20°C, from an initial level of 1.44×10^5 CFU/wound to 4.05×10^6 CFU/wound after 8 d. Obviously, low temperature (4°C) inhibited the growth of *P. fluorescens* with the population being up to 4.84×10^6 CFU/wound after 16 d. On the contrary, *P. digitatum* could effectively stimulate the growth and reproduction of *P. fluorescens* both at room temperature and low temperature. The relationship between $\log_{10}$ CFU/wound (y) and incubation time (x) is described by the regression equations shown inside Figure 4.

3.5. Effect of P. fluorescens on CHI and GLU Activities. The CHI activity of each treatment group increased in the initial period of storage and reached the peak on the forth



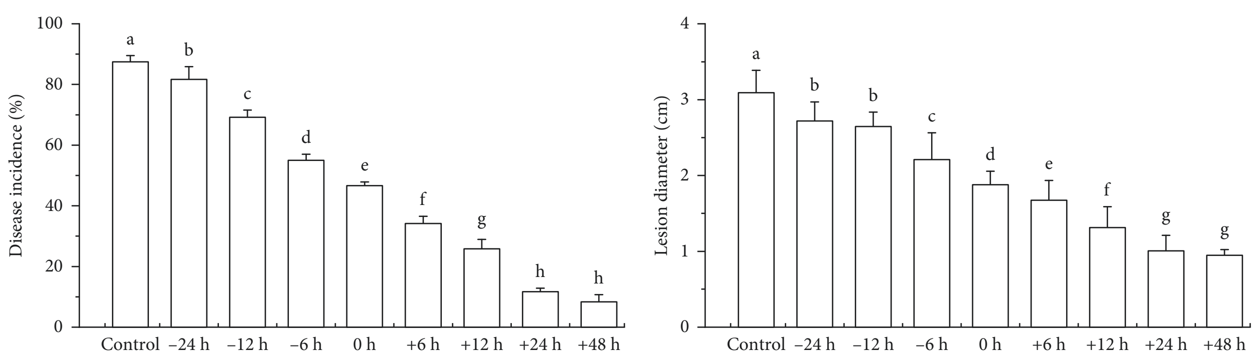
(a) (b)

Figure 1: The effect of *P. fluorescens* on citrus for the control of *P. digitatum*: I, SDW; II, autoclaved cultures; III, culture filtrate; IV, 1×10^{8} CFU/mL bacterial fluid; V, 1×10^{8} CFU/mL bacterial suspension. Columns with different lowercase letters within the same panel are significantly different at the $P<0.05$ level by Duncan's multiple range test.



(a) (b)

Figure 2: The effect of *P. fluorescens* concentration on biocontrol efficacy: I, SDW; II, 1×10^{6} CFU/mL; III, 1×10^{7} CFU/mL; IV, 1×10^{8} CFU/mL; V, 1×10^{9} CFU/mL. Columns with different lowercase letters within the same panel are significantly different at the $P<0.05$ level by Duncan's multiple range test.



(a) (b)

Figure 3: Preventative action and curative action of *P. fluorescens* against to *P. digitatum*.−, citrus were inoculated *P. digitatum* prior to *P. fluorescens*; +, citrus were inoculated *P. fluorescens* prior to *P. digitatum*. Columns with different lowercase letters within the same panel are significantly different at the $P<0.05$ level by Duncan's multiple range test.

day except in the control (SDW), with highest CHI activity occurring on the second day (Figure 5(a)). Both treatments of *P. fluorescens* and *P. fluorescens* + *P. digitatum* induced significantly ($P<0.05$) higher activity of CHI during the whole incubations, compared with the control. The changes of GLU activity in all treatments were similar to that of CHI with the tendency to rise first and decline latter (Figure 5(b)). The GLU activity of citrus treated with *P. fluorescens* was also

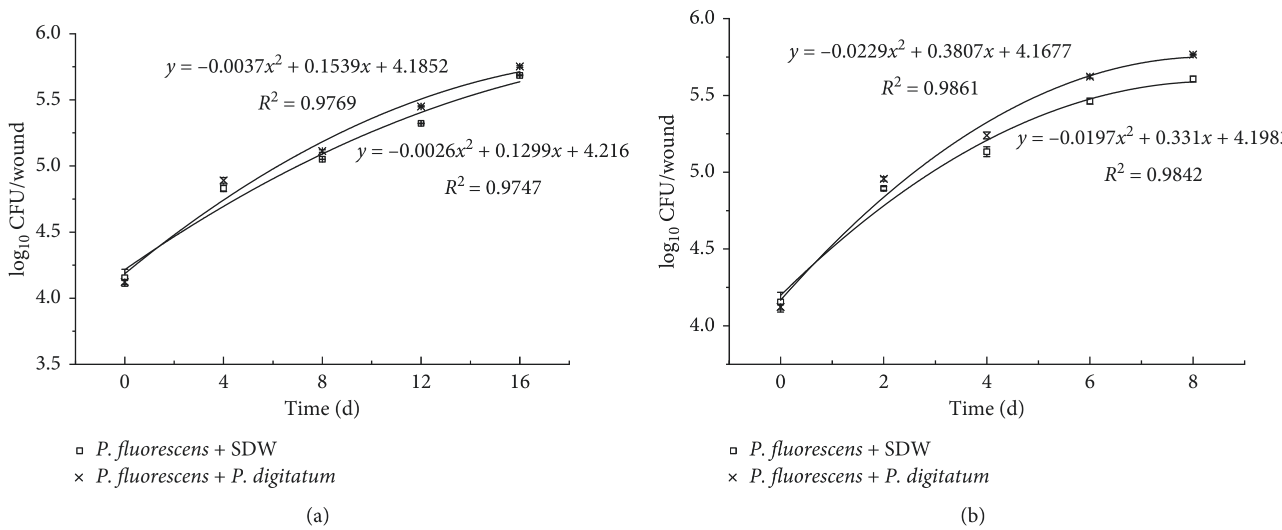


Figure 4: Population dynamic of *P. fluorescens* in citrus wounds at 4°C for 16 days (a) and 20°C for 8 days (b).

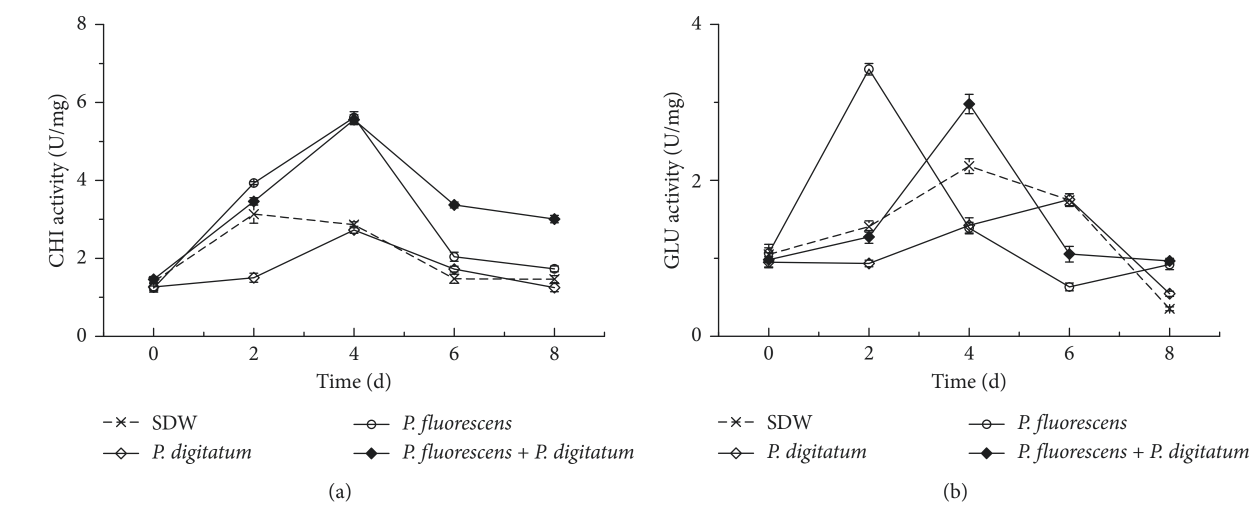


Figure 5: The effect of *P. fluorescens* on activities of CHI (a) and GLU (b). Each value is the mean of three experiments. Bars represent standard errors.

induced, and the induction lasted for 4 days. In addition, treatment with *P. digitatum* reduced the activities of both CHI and GLU (Figures 5(a) and 5(b)).

3.6. Effect of P. fluorescens on POD and PAL Activities. The activity changes of POD and PAL in citrus for all treatments are presented in Figure 6. The control POD activity increased gradually and reached the peak on the sixth day. The POD activity of treatment with *P. fluorescens* + *P. digitatum* increased sharply in the initial 2 days and then decreased gradually and was lower than the control after the forth day. The POD activity of citrus treated with *P. fluorescens* was 21.2% higher than the control while reaching the peak at the forth day with the level of 4.35 U/mg. Except for the treatment with *P. fluorescens* + *P. digitatum*, the PAL activity of the other treatment groups reached the peak at the second day, among which the citrus inoculated with *P. fluorescens*, had the highest activity level.

4. Discussion

Compared with chemical pesticides, biological control is a safer and more environmentally friendly approach to manage postharvest decay of fruits and vegetables [3, 16]. More and more investigators have focused their research efforts on the use of biological control agents to take the place of chemical fungicides over the several decades [21]. *P. fluorescens* is widely used as a biocontrol agent in agricultural practices. To date, however, little is known about its biocontrol efficacy in postharvest diseases of fruits. Therefore, we carried out the research to evaluate the effect of *P. fluorescens* on the disease control of citrus fruits in order to provide an experimental basis for its further application.

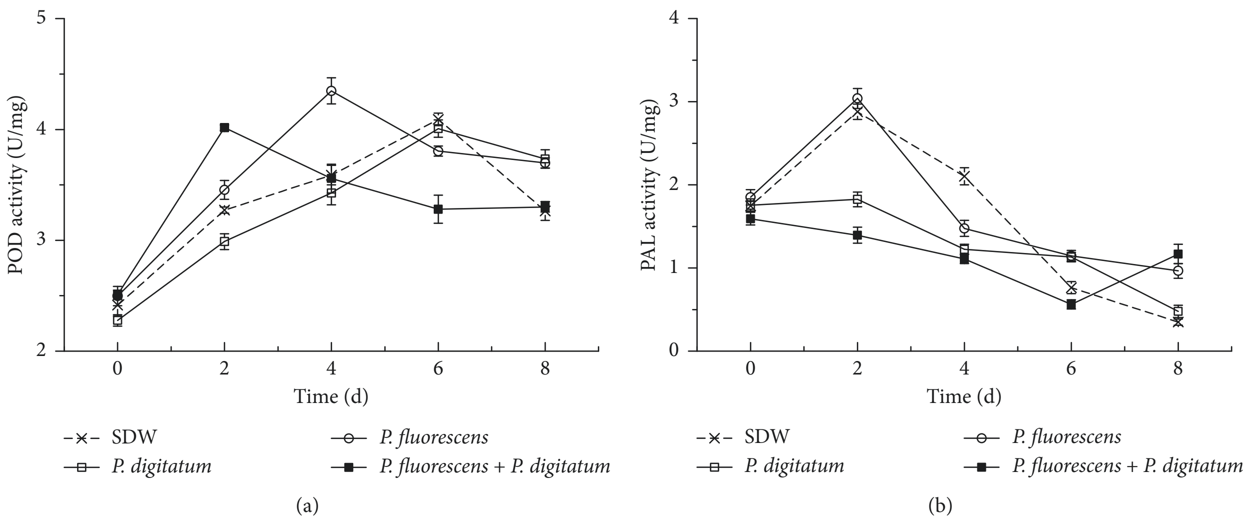


Figure 6: The effect of *P. fluorescens* on activities of POD (a) and PAL (b). Each value is the mean of three experiments. Bars represent standard errors.

In this study, whether *in vitro* or *in vivo*, autoclaved cultures and culture filtrate could inhibit *P. digitatum*, but the inhibitory effect was very limited. This result indicated that this *P. fluorescens* strain may produce few antibiotic substances, and this was not the main way to inhibit *P. digitatum*. There have been a lot of reports finding that the production of DAPG, PCA, pyrrolnitrin (Prn), pyoluteorin (Plt), and hydrogen cyanide (HCN) by *P. fluorescens* is very important to control plant diseases [22, 34]. For example, DAPG, Prn, and Plt produced by *P. fluorescens* Pf-5 play significant roles in controlling *Pythium ultimum* [23]. Maurhofer et al. [34] reported that the primary mechanism of action of *P. fluorescens* CHAO to inhibit *Pythium ultimum* and *Fusarium oxysporum* was attributed to the production of DAPG, Plt, and HCN. Most *P. fluorescens* strains have the ability to compete for iron with the pathogen by producing siderophores [26, 35], as the content of iron in fruits is limited, though fruit wounds are nutrient rich [21]. Besides, gas, cellulose, glucanase, and protease can also be produced by *P. fluorescens* [21, 36]. Our isolate of *P. fluorescens* may not have the ability to produce antibiotics since the antibiotics can extremely inhibit the growth of pathogen even at very low concentrations. Also, we screened for the presence of biosynthesis genes encoding the production of antibiotics commonly associated with pseudomonad biocontrol agents. However, no molecular evidence for genes coding for the antibiotics DAPG, PCA, Prn, Plt, or HCN was obtained by the polymerase chain reaction (PCR) (Supplementary Materials (available here)). It is not clear whether other antifungal substances are produced by our *P. fluorescens* strain.

P. digitatum was significantly inhibited by bacterial fluid and bacterial suspension both *in intro* and *in vivo*, and bacterial suspension showed increased biocontrol efficacy compared with bacterial fluid against green mold on postharvest citrus, implying that competition for nutrients may be one of the main modes of action of *P. fluorescens*. The result is in agreement with O′Sullivan et al. [27], who found that *P. fluorescens* M14 could make full use of a large amount of different carbon sources. The commercially available biocontrol agent Bio-Save@ (*P. seudomonas syringae*) can inhibit various kinds of postharvest diseases mainly through competing for nutrients and space sites [21]. In the dosage trial, increments in bacterial suspension concentration led to higher biocontrol efficacy. The result was consistent with previous studies by Zamani et al. [17] and Nunes et al. [37], who observed that there was a positive relationship between the population density of an antagonist and its biological efficacy. In addition, inoculation order and inoculation time of antagonist and pathogen also significantly affected the biocontrol efficacy. In general, *P. fluorescens* gave a significant reduction of disease incidence when applied before inoculating *P. digitatum*, and the earlier the *P. fluorescens* inoculation, the lower the disease incidence, and the smaller the lesion diameter. This result was in agreement with other studies that *Candida saitoana* could inhibit *Penicillium expansum* more effectively when applied to the apple fruit before pathogen inoculation than after pathogen inoculation [38]. More recently, Abraham et al. [39] showed that antagonists of *Bacillus* and yeast were effective when applied preventatively but not when intending to cure. Mercier and Smilanick [40] suggested that the pathogen penetration into the fruit tissues and lack of access for the antagonist leads to the failure of curative control of antagonist. It is generally accepted that the capacity for rapid colonization by an antagonist in fruit wounds is critical to biocontrol activity [30, 41]. In our study, the population of *P. fluorescens* increased 28- and 34-fold more, being incubated at 20°C for 8 d and 4°C for 16 d, respectively. One interesting phenomenon was also observed that *P. digitatum* could effectively stimulate the growth and reproduction of *P. fluorescens* both at room temperature and low temperature (Figure 4), which was similar to the results reported by another author [42]. The results suggested that *P. fluorescens* could grow and utilize most of the nutrients released from wounds faster than *P. digitatum*; therefore, there were not enough nutrients and space sites left to *P. digitatum* spores for colonization. The results of these trials further indicated that competition for nutrient and space sites played an important role in the biocontrol capability of *P. fluorescens* against *P. digitatum*.

Most fungal pathogens infect fruit from wounds, stomata, and lenticels through spore germination to form germ tubes, causing postharvest diseases [8, 21]. Therefore, it is logical to investigate the inhibitory effect of antagonist on the germination of pathogenic fungi. Our study showed that there were significant effects ($P < 0.05$) on inhibiting spore germination and germ tube elongation of *P. digitatum* by *P. fluorescens* with living cells, even when present in PDB where nutrition and space sites were abundant. Our findings were similar to Wallace et al. [21] who found that the isolates of *P. fluorescens* 1–112, 2–28, and 4–6 inhibited conidial germination of *P. expansum* by over 90% compared with the control. In addition, these results implied that there may be other modes of action for *P. fluorescens* against fruit disease besides competition for nutrient substance and space sites.

The induction of defense response in fruit has been considered as another major mechanism of antagonists to suppress infection with pathogens, and growing evidences have supported this point of view [13, 43, 44]. It is commonly believed that induced resistance has been associated with induction of the pathogenesis-related (PR) proteins and a series of defensive enzymes [4, 8, 45, 46]. Among PR proteins, CHI and GLU, which can degrade the cell walls of pathogens separately or synergistically, are the most important detected PR proteins and can be used as markers for the establishment of plant disease resistance after induced treatments [8, 45, 47]. POD is one of the key enzymes of reactive oxygen metabolism and can participate in the synthesis and metabolism of secondary metabolites [8, 33, 48]. PAL is the first gateway enzyme in the phenylpropanoid pathway for the biosynthesis of many plant secondary metabolites, such as flavonoids, phenols, lignin, salicylic acid, and so on, related closely to plant disease resistance closely [4, 33, 49]. Many researchers have reported that antagonist treatments can induce systemic resistance in harvested orange [6, 13, 50], apple [51, 52], and grapefruit [53]. In this study, we found that *P. fluorescens* was able to induce resistance on citrus peel, increasing the CHI activity during the storage period and advancing the activity peaks of GLU, POD, and PAL.

P. fluorescens is ubiquitter in natural water, soil, leaf, and fruit surfaces, suggesting that it is not likely to pose additional risk to human health. However, it is necessary that rigorous and further toxicity studies should be designed and conducted before using the strain as a biocontrol agent.

5. Conclusions

In conclusion, the result of this study showed that the application of *P. fluorescens* was observed to be effective in controlling green mold caused by *P. digitatum*. The possible modes of action may include inhibiting spore germination and mycelium growth, competition for nutrient substance and space sites, and inducing disease resistance. Therefore, we suggested that *P. fluorescens* can potentially be used as a biocontrol agent against *P. digitatum* in postharvest citrus.

Data Availability

All the authors agree to make freely available any materials and information described in the manuscript that may be reasonably requested.

Additional Points

(i) *P. digitatum* may effectively stimulate the growth of *P. fluorescens* in fruit wounds. (ii) *P. fluorescens* was effective when applied preventatively but not when applied curatively. (iii) *P. fluorescens* could potentially be used as a biocontrol agent against green mold on postharvest citrus.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

The presence of genes for the biosynthesis of 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyrrolnitrin (Prn), pyoluteorin (Plt), and hydrogen cyanide (HCN) was determined by the polymerase chain reaction (PCR) using the primer sets described in Supplementary Table 1. The PCR reactions were performed according to [21, 54–56]. Our *P. fluorescens* strain was negative for the genes encoding the production of DAPG, PCA, Prn, Plt, and HCN (Supplementary Figure 1). (*Supplementary Materials*)

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