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EVALUATION OF *BACILLUS SUBTILIS* AS POTENTIAL BIOCONTROL AGENT FOR POSTHARVEST GREEN MOLD CONTROL ON 'VALENCIA' ORANGE

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Abstract. **Effective biocontrol of postharvest decay could provide an alternative approach for chemical control and for shipping chem-free citrus. Developing effective biocontrol agents, formulations and application methods for postharvest decay control of citrus is obviously needed. With the effort to look for effective biocontrol agents, two *Bacillus subtilis* strains GB03 and GB07, were tested for their potential biocontrol activity of green mold caused by *Penicillium digitatum* on 'Valencia' orange. *B. subtilis* strain GB07 achieved green mold control of 72.2 to 100% in four separate experiments when a preventive test method was used. GB03 only showed green mold control of 11.1 to 55.6% under the same test conditions. GB07 also reduced green mold incidence by 83% when it was applied to naturally infected fruit using a simulated commercial application system through a packingline. The performance of GB07 for green mold control was similar to that of standard chemical thiabendazole (TBZ) at 1,000 ppm level. To achieve good biocontrol activity, GB07 should be present in injury sites prior to *P. digitatum* infections. A minimum required concentration of GB07 at a 3×3 mm injury has been calculated as 2×10^6 cells. Under temperatures from 10, 15, 20, 25, and 30 °C, the best effect of GB07 on decay reduction was obtained at 30° C, followed by 25 °C. GB07 has also been demonstrated to produce antifungal compounds which actively suppressed the growth of *P. digitatum in vitro*. *B. subtilis* GB07 may have good potential to be developed as a new biocontrol agent of green mold and other decays on Florida citrus fruit.**

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Postharvest decay is one of the major factors affecting fresh citrus fruit quality, shelf life and economic returns in Florida. Postharvest disease control in Florida is conducted by an integrated procedure using synthetic fungicides as the core component. There are three registered fungicides, thiabendazole (TBZ), imazalil and sodium o-phenylphenol (SOPP), for postharvest treatments of citrus fruit. However, there is a great potential risk that one or more of these fungicides will be removed from markets due to increasingly restrictive federal regulations, pathogen resistance or other factors. TBZ and imazalil are currently under the EPA review for re-registration, and SOPP will be reviewed later. Currently there are no alternatives to these postharvest chemicals in citrus industry. Biological control of postharvest decays of fruit and vegetables has been an active research area and may provide a good alternative to chemicals (Droby and Chalutz, 1999; Wilson et al., 1991). Biocontrol may also provide a good tool to solve the problems of chemical residues in fruit and juice and pathogen resistance development. Compared with root or foliar diseases, postharvest disease is more suitable for biocontrol since the environmental factors are more stable and can be controlled. BioSave® 10 LP and Aspire are two commercial biocontrol products registered for the control of decays caused by injury-mediated pathogens on citrus fruit, but their use is very limited in Florida due to the packinghouse operation conditions (Brown and Chambers, 1996). There is a need to look for more practical and effective biocontrol agents for the control of citrus postharvest diseases in Florida. The objective of this study was to evaluate two *Bacillus subtilis* strains as the potential biocontrol agents for citrus postharvest green mold control.

Materials and Methods

Fungal pathogen. A *Penicillium digitatum* strain (PD-9) was originally isolated from decayed citrus fruit. The pathogen was maintained in a soil tube and stored at 22 °C. The fungus was recovered from the soil tube and grown on potato dextrose agar (PDA) when it was needed prior to each experiment.

Bacillus subtilis strains. Two *B. subtilis* strains, GB03 and GB07, were obtained from Gustafson, Inc., Plano, TX. GB03 and GB07 are active ingredients of commercial biocontrol products, Kodiak and Epic, respectively. These two biocontrol products were registered and used for control of root diseases of cotton and other crops. These two strains have been maintained in original product materials and kept in a refrigerator for about 10 yr.

Effects of B. subtilis strains on green mold on 'Valencia' orange. Prior to fruit inoculations, *P. digitatum* PD-9 was grown on PDA for 4 d, and *B. subtilis* GB03 and GB07 were grown for 7 d. The cell suspensions of *P. digitatum* PD-9 and *B. subtilis* GB03 and GB07 were prepared from the PDA cultures. The cell concentration of PD-9, GB03 and GB07 in suspensions was 10^6, 10^8 and 10^8 cells/mL, respectively, based on a hemacytometer determination. Two test methods were used in this study: (1) Artificial application of biocontrol agents: 'Valencia' orange were obtained from a local commercial packinghouse in Polk County, Fla. Fruit were not treated with any chemicals before they were brought to the facility. Fruit were washed, randomized, and dried through a simulated commercial packingline. Fruit were wounded 3 mm deep and 3 mm diameter at a fruit equator site. One wound was made on each fruit. Thirty fruit were used for each treatment. Twenty µL of cell suspension (10^8 cells/mL) of GB03 or GB07 were placed into each wound. The fruit treated with water were used as control. Biocontrol treated fruit then were incubated in a dish pan with moist paper at 25 °C. After 24 h the same fruit were inoculated with PD-9 by placing 20 µL of the spore suspension (10^6 spores/mL) at each of the same wounds. Inoculated fruit were incubated at 25 °C in the same dish pans. Decay was counted every 2 d. (2) Simulated commercial application of biocontrol agent: The fruit were not inoculated with the pathogen PD-9. The fruit were dripped with GB07 suspension (10^8 cells/mL) or a postharvest chemical thiabendazole (TBZ) at 1,000 ppm when the fruit were run through a packingline. The fruit were treated with GB07 or TBZ on brush bed of the packingline for approximately 15 seconds. Three replicates were used for each treatment. Each replicate had about 40 to 50 fruit. Treated fruit were dried at 50-60 °C for 1-2 min and then incubated at 21 °C and 90 to 100% relative humidity. Decay incidence was recorded weekly up to 4 weeks.

Effects of B. subtilis GB07 concentrations on decay control. GB07 cell concentration was made at 0, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 cells/mL in the suspensions. These suspensions and PD-9 spore suspension (10^6 spores/mL) were used to treat fruit wounds following the procedure of the artificial application of biocontrol agents described as above. Treated fruit were incubated at 25 °C, and decay was recorded at 4 d after fruit inoculation with *P. digitatum* PD-9.

Effects of temperatures on the performance of GB07 on decay control. Fruit were treated with GB07 (10^8 cells/mL) and *P. digitatum* PD-9 (10^6 spores/mL) following the procedure of the artificial application of biocontrol agents and pathogen described previously. Treated fruit were incubated at a temperature of 10, 15, 20, 25, and 30 °C. Decay was recorded every day up to 8 d.

Effects of time periods of fruit injury colonization by GB07 on green mold control. Fruit were wounded as described previously, and 20 µL of GB07 (10^8 cells/mL) were placed into each wound at 0, 4, 8, 16 and 24 h before inoculating the same wound site with *P. digitatum* PD-9 (20 µL of 10^6 spores/mL). Treated fruit were incubated at 25 °C, and decay was recorded at 4 d after fruit inoculation with PD-9.

Antibiotic activity of GB07 against P. digitatum in vitro. B. subtilis GB07 was cultured on PDA by making a single streak across the center of the plate. After 2 d, 10 µL of PD-9 suspension (10^6 spores/mL) were placed on either side of the GB07 streak at a distance of 2 cm. Control plates were streaked with autoclaved water and inoculated with PD-9 spore suspension. Five plates were used for each treatment. The inhibitory activity of GB07 to PD-9 was recorded after the plates were incubated at 25 °C for 4 d.

Data analysis. Analysis of variance and regression was performed using the general linear model procedure of SAS. Treatment means were compared using the Ryan-Einot-Gabriel-Welsch multiple range test ($P \leq 0.05$).

Results

Effects of B. subtilis strains on green mold control on 'Valencia' orange. When *P. digitatum* PD-9 was applied into artificial injury sites 24 h after *B. subtilis* GB03 and GB07 were applied, GB03 and GB07 showed green mold control of 11.1 to 55.6% and 72 to 100%, respectively, in four separate experiments (Table 1). GB07 performed much better than GB03 for green

mold control under the same test conditions. When GB07 was applied to naturally infected fruit using a simulated commercial dripping system through a packingline, GB07 reduced decay by 83%, and TBZ at 1,000 ppm reduced decay by 73.6% compared to non-treated control (Fig. 1). Statistical analysis indicated that GB07 reduced green mold similar to commercial standard chemical TBZ under the same test conditions.

Effects of B. subtilis GB07 concentrations on green mold control. Green mold incidence increased as GB07 concentration at fruit injury sites decreased (Fig. 2). There was a significant negative correlation between green mold incidence and GB07 concentration (r = 0.8838, P = 0.0036). The GB07 at 10^8 and 10^9 cells/mL achieved 78.9% and 82.4% of green mold control, respectively.

Effects of temperatures on the performance of GB07 on green mold control. When the biocontrol tests were performed under temperatures of 10, 15, 20, 25, and 30 °C, the best effect of GB07 on green mold control was obtained at 30 °C, followed by 25 °C (Fig. 3). Under 10 °C, green mold developed rapidly 7 d after fruit inoculation with *P. digitatum* PD-9. At 15 °C the green mold increased significantly 5 d after inoculation.

Effects of time periods of fruit injury colonization by GB07 on green mold control. The percentage of green mold control increased as the colonization time of GB07 at injury sites increased (Fig. 4). A significant positive correlation (r = 0.9938, P = 0.00059) was obtained between them. When GB07 and PD-9 were presented at fruit injury sites at the same time, the percentage of green mold control by GB07 was only 33.3%, while a 24-h of colonization of injury sites by GB07 prior to the PD-9 inoculation showed a 100% green mold control.

Antibiotic activity of GB07 against P. digitatum in vitro. When *P. digitatum* PD-9 was cultured on the PDA with GB07 streaked culture, PD-9 had a little or no visual growth compared with that in control plates (Fig. 5). The antibiotics produced by GB07 strongly suppressed the growth of PD-9.

Discussion

Biocontrol of postharvest diseases has been an active research area for many years (Droby and Chalutz, 1999). Two biocontrol products, Aspire (*Candida oleophila*) and BioSave® 10 LP (*Pseudomonas syringae*), have been registered for commercial postharvest treatment of citrus fruit for decay control (Brown and Chamber, 1996). Since the decay control efficacy and consistency of these biologicals are usually less compared to the fungicides TBZ and imazalil, they have not been used in Florida packinghouses (Brown and Chamber, 1996). In this study, two *B. subtilis* strains were subjected to test their potential for postharvest citrus green mold control. The data of this study indicated that *B. subtilis* GB07 significantly reduced green mold caused by *P. digitatum* if GB07 presented at injuries prior

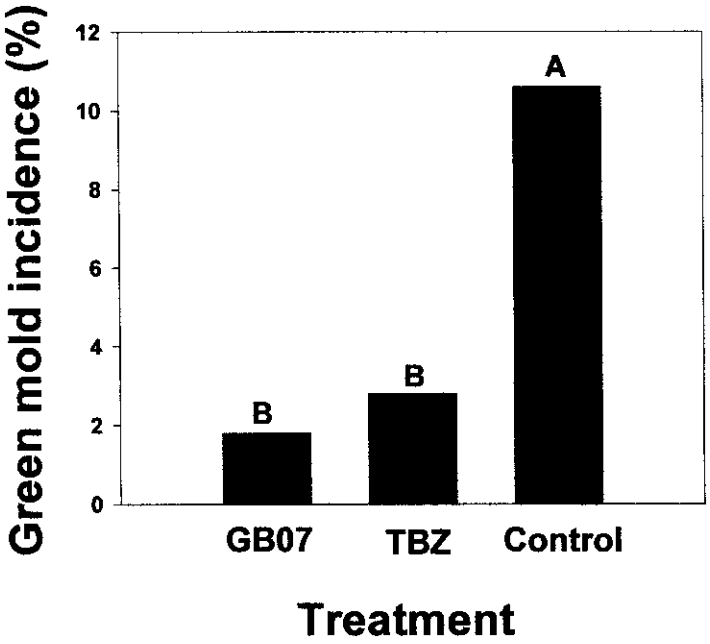

Fig. 1. Effect of *Bacillus subtilis* GB07 on green mold control of 'Valencia' orange using a simulated commercial application procedure. Means with the same letter on each bar are not significantly different based on the Ryan-Einot-Gabriel-Welsch multiple range test ($P \leq 0.05$).

Table 1. Effects of *Bacillus subtilis* strains on green mold on 'Valencia' orange inoculated with *Penicillium digitatum*.

Experiment	Bacterial strain	% Fruit decay[y]	% Decay control
Expt. I	Control	75.0 A[z]	
	B. subtilis GB03	33.3 B	55.6
	B. subtilis GB07	0.0 C	100.0
Expt. II	Control	90.0 A	
	B. subtilis GB03	75.0 A	16.7
	B. subtilis GB07	15.0 B	83.3
Expt. III	Control	90.0 A	
	B. subtilis GB03	80.0 A	11.1
	B. subtilis GB07	25.0 B	72.2
Expt. IV	Control	83.3 A	
	B. subtilis GB03	50.0 B	40.0
	B. subtilis GB07	10.0 C	88.0

[y]Decay incidence was obtained after fruit inoculation with *P. digitatum* PD-9.
[z]Means with the same letter within the same column are not significantly different (P ≤ 0.05) based on the Ryan-Einot-Gabriel-Welsch multiple range test.

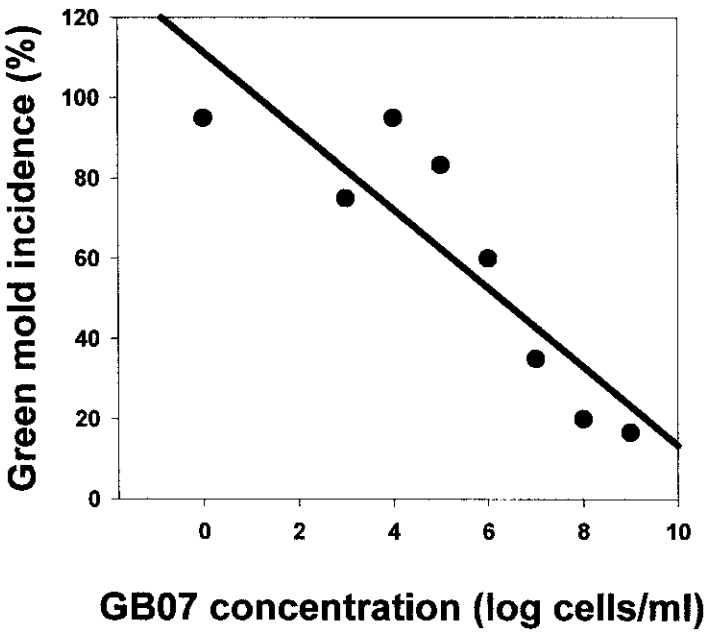

Fig. 2. Correlation between green mold incidence and *Bacillus subtilis* GB07 concentrations at 'Valencia' orange fruit injury sites.

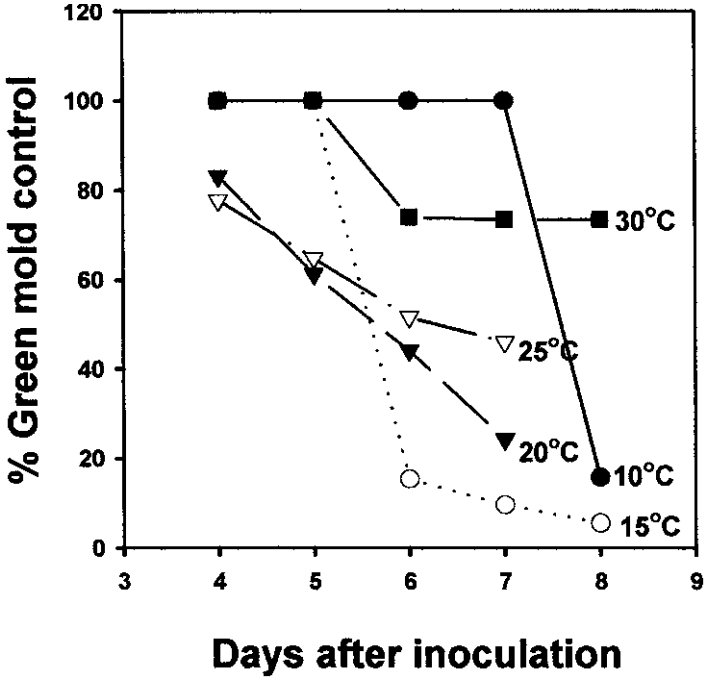

Fig. 3. Effect of *Bacillus subtilis* GB07 on green mold incidence on 'Valancia' orange under different temperatures.

to *P. digitatum* infections. This indicated that *B. subtilis* strains may have good potential to be developed as new biocontrol agents for postharvest decay control of citrus fruit. Similar information about *B. subtilis* strains as potential biocontrol agents has also been reported by others on citrus and other fruits (Pusey and Wilson, 1984; Pusey et al., 1988; Singh and Deverall, 1984). Since GB07 performed significantly better than GB03 on green mold control, it is possible that a good strain of *B. subtilis* might be discovered or selected for green mold control from screening a large number of *B. subtilis* isolates.

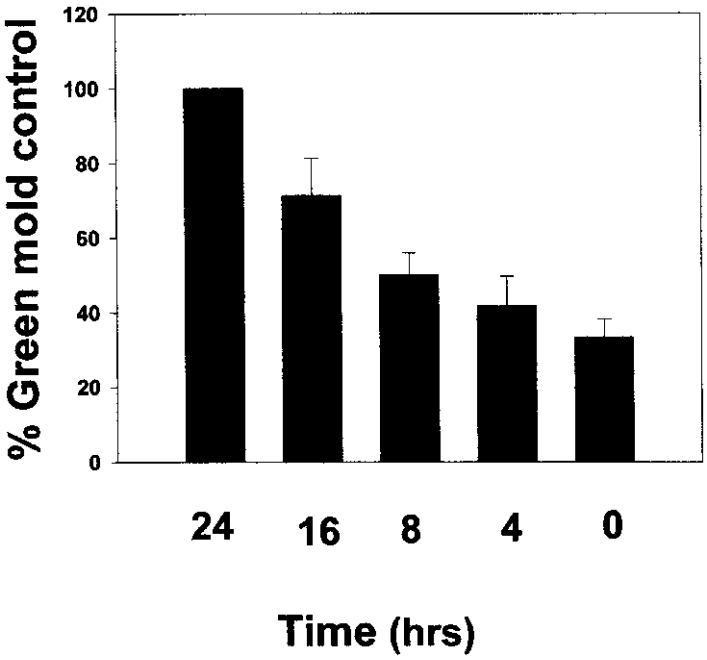

Fig. 4. Effect of pre-colonization time of *Bacillus subtilis* GB07 at fruit injury sites on green mold incidence. Standard errors were calculated based on three replicates.

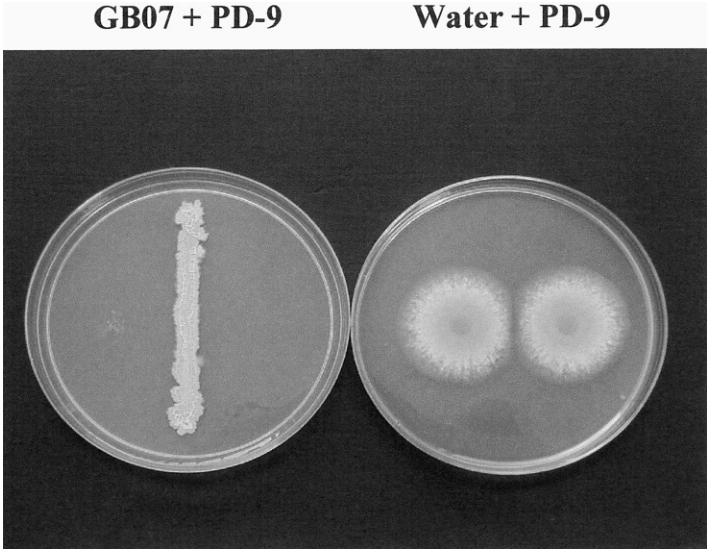

Fig. 5. Antibiotic activity of *Bacillus subtilis* GB07 against *Penicillium digitatum* PD-9.

Generally, most biocontrol agents lack eradicant activity compared with chemicals, and usually exhibit a preventive activity by competing for nutrients or spaces with pathogens in infection courts. Our study also demonstrated that GB07 primarily exhibited a preventative activity. This model of action is similar to that of the commercial biocontrol products, Aspire and BioSave® 10 LP. Our study of GB07 on the relationship between the pre-colonization time and decay control efficacy showed that GB07 had to be presented at injuries at least 16 h prior to *P. digitatum* infection in order to achieve a more than 80% decay control. This can be a limitation for using GB07 to control green mold after pathogen infections. However, in one of our tests, when naturally infected fruit were treated with GB07 through a simulated commercial dripping system through a packingline, GB07 significantly reduced green mold incidence. Since this was a simulated commercial operation, the mechanisms of decay control by GB07 in this case might be due to a complex interactions among fruit injury occurring time, injury healing degree, fruit tissue resistance, GB07 colonization and *P. digitatum* infection. Therefore, further studies of GB07 for decay control are needed at commercial application procedure to fully demonstrate its potential for postharvest decay control.

To achieve a successful decay control, the biocontrol organisms have to colonize injury sites with a concentration at a certain level. Under our test conditions, a minimum GB07 concentration of 2×10^6 cells at a 3×3 mm injury site was needed to achieve about 80% decay control. In addition, as a successful biocontrol agent, it should survive at the injury site, proliferate and compete with pathogen for nutrients and space, or produce antibiotics against the pathogens. It is not clear how well GB07 can adapt to the citrus fruit injuries. Biological control agents also can be greatly affected by environmental factors such as temperature and humidity. These factors not only affect the performance of biocontrol agents, but also affect host fruit physiology, pathogen infection and pathogenesis. The results in the current study indicated that GB07 performed the best at 30 °C, and followed by 25 °C. The best efficacy of GB07

at 30 °C might result from a combination effect of fruit injury healing and suppression of pathogen growth by high temperature. At low temperature, 10 and 15 °C, green mold was less in the initial several days after fruit inoculation with the pathogen. This might be due to the slow proliferation and infection of pathogen at the low temperatures. The best biocontrol of green mold may be achieved under particular environmental conditions which promote biocontrol agent's activities and suppress the pathogen's activities.

In this study, the antibiotic activity of *B. subtilis* GB07 against *P. digitatum in vitro* has been demonstrated. The antibiotic production by *B. subtilis* has also been reported by many other researchers (Burachik et al., 1964; McKeen et al., 1986). Most of the known antibiotics produced by *B. subtilis* are polypeptides (Burachik et al., 1964; Katz and Demain, 1977; McKeen et al., 1986). However, it is still not known if GB07 produces the antibiotics at citrus fruit injuries. If so, what role that the antibiotics may play for decay control in citrus fruit needs further study.

Compared to the commercial biocontrol agents BioSave® 10 LP and Aspire, the advantages of *B. subtilis* strains as biocontrol agents are their strong resistance to adverse environmental factors since *B. subtilis* can produce endospores. The endospores are dormant resting cells with distinctive properties of great resistance to external environmental factors such as heat, UV irradiation and toxic chemicals. Because of this characteristic of *B. subtilis*, the cell viability maintenance of GB07 should be much easier and effective during storage and delivery compared to other biocontrol agents. This study mainly focused on the potential of GB07 for green mold control. It is likely that GB07 may also have potential for the control of blue mold caused by *P. italicum* and sour rot caused by *Geotrichum candidum* since these decays are also caused by injury-mediated fungal pathogens. Probably GB07 has no effect on the control of citrus stem-end rot caused by *Diplodia natalensis* and *Phomopsis citri* since these pathogens are latent infection fungi and colonize fruit bottom tissue and develop when natural openings occur during abscission (Brown and Wilson, 1968). Further studies are needed before GB07 can be recommended and developed as a new effective biocontrol agent for the integrated postharvest disease control of citrus in Florida.

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