Tropical Plant Pathology, vol. 38(6):505-512, 2013

www.sbfito.com.br

RESEARCH ARTICLE / *ARTIGO*

Application of microorganisms, alone or in combination, to control postbloom fruit drop in citrus

Mariana N. Klein[1], Aline C. Silva[1], Marcos R. Lopes[1] & Katia C. Kupper[2]

[1]Universidade Federal de São Carlos, 13600-970, Araras, SP, Brazil; [2]Centro de Citricultura Sylvio Moreira, Instituto Agronômico de Campinas, 13490-970, Cordeirópolis, SP, Brazil

Author for correspondence: Katia Cristina Kupper, e-mail: katia@centrodecitricultura.br

ABSTRACT

Isolates of *Trichoderma* spp. (ACB-14, ACB-33, ACB-37, and ACB-40) and *Bacillus subtilis* (ACB-66, ACB-69, ACB-77, and ACB-83) were tested separately or in mixtures for suppression of postbloom fruit drop in citrus, caused by *Colletotrichum acutatum*. This work aimed at: (i) determining the incubation time and temperature for production of cells of biocontrol agents; (ii) determining the effect of the isolates, separately or in mixture on the germination conidia of *C. acutatum*; (iii) evaluating the efficiency of antagonistic isolates on detached citrus flowers and under field conditions. The results of the interactions *in vitro* showed that there was little differentiation in cell production among the species, and the optimum temperature was 27°C. The best time for multiplication of bacterial cells was 36 hours, whereas for *Trichoderma*, the production of conidia continued to increase up to 120 hours of incubation. The mixtures of the ACB-77 plus ACB-66, ACB-33, or ACB-37 inhibited pathogen germination from 84% to 89%. Studies with detached citrus flowers showed that ACB-69 alone gave 99% control. The use of mixture ACB-69 plus ACB-37 proved to be viable in the control of disease under field conditions, but the efficiency of the control was lowest than the obtained by applications of ACB-69 alone.
Key words: *Bacillus subtilis*, *Colletotrichum acutatum*, *Trichoderma* spp., biological control.

INTRODUCTION

Brazil is the world's largest producer of oranges, responsible for about 30% of fruit and 50% of juice production, with 85% of the juice marketed internationally. The crop occupies an area of approximately 839000 ha, of which 77% are located in the southeastern region of Brazil. Around 80% of the orange production in São Paulo State is destined to processing, and the juice produced is exported to countries such as Russia, Belgium, Netherlands, United States, and Japan. The remaining 22% is sent to the fresh fruit market, both for internal market and for export (Agrianual, 2011).

Though the citrus sector is of great importance, this crop faces serious phytosanitary problems. Postbloom fruit drop (PFD) caused by the fungus *Colletotrichum acutatum* is one of the most severe diseases of citrus. All sweet orange varieties cultivated in São Paulo State are susceptible to this disease, which is controlled with protectant and systemic fungicides sprays (Goes et al., 2008). One of the difficulties in efficiently controlling the disease by spraying chemicals is that it is more severe when long periods of rain or high humidity occur during peak flowering periods (Denham & Waller, 1981). The occurrence of several bloom cycles also requires higher number of sprays, strongly increasing the production costs, and causing undesirable environmental consequences. Biological control is one of the alternatives to control the disease. Among the most studied antagonists are the bacterium *Bacillus subtilis* and the fungus *Trichoderma* spp. (Pusey et al., 1986; Sonoda & Guo, 1996; Kupper & Gimenes-Fernandes, 2002; Kupper et al., 2011, and 2012; Tronsmo & Dennis, 1977; Gullino & Garibaldi, 1983; Moretto et al., 2001).

According to Guetsky et al. (2001), the introduction of two or more biocontrol agents on the phylloplane may facilitate disease control, assuming that each one has different ecological needs and diverse modes of action. It may even result in an increasing control consistency. The application of antagonist mixtures has reduced the variability and enhanced the efficiency of biocontrol agents in multiple systems (Janisiewicz & Korsten, 2002).

This work aimed to study the use of biological control agents alone, or in mixtures, for the control of postbloom fruit drop of citrus.

MATERIALS AND METHODS

Microorganisms

This study used four isolates of *Bacillus subtilis* (ACB-66, ACB-69, ACB-77, and ACB-83) from citrus flowers and leaves (Kupper & Gimenes-Fernandes, 2002), four isolates of *Trichoderma* spp., ACB-14 and ACB-40 (*Trichoderma* sp.), ACB-33 (*T. aureoviride*), and ACB-37 (*T. pseudokoningii*) from citrus soil (Moretto et al., 2001). All the microorganisms studied are deposited in the collection of APTA Center Citros Sylvio Moreira/ IAC, Cordeirópolis, São Paulo, Brazil.

Growth of biological control agents

Colonies of each biological control agent (BCA) cultured on potato dextrose agar (PDA) were transferred to glass tubes containing 10 mL of sterile distilled water and concentrations were determined and adjusted to 1 x 10^4 cells mL^{-1}, using a hemocytometer. An aliquot of 0.2 mL of each suspension of biocontrol agent was plated in Petri dishes with PDA. The cultures of bacteria and the fungi were incubated, in the dark, photoperiod of 12/12h, at 15ºC, 20ºC, 27ºC, and 35ºC. Cultures from different plates were harvested at different times (6, 12, 18, 24, 36, 48, 72, and 120 h) by scraping the surface of microorganism with a sterile microscope slide. The experiment was carried out in a completely randomized design with a factorial arrangement of the treatments. Cell production of the microorganisms was determined with a hemocytometer. There were three replicates (Petri dishes) for each combination of biocontrol agent x temperature x time. The data were subjected to analysis of variance (ANOVA) using the SAS statistical software (SAS Institute) and the response surface graphs were generated using the software Statistica for Windows V7.0 (Statsoft Inc.).

Effect of biocontrol agents on *Colletotrichum acutatum* conidial germination

The effect of BCAs alone or in mixtures on the conidial germination of the plant pathogen was assessed by the technique of water-agar placed on glass slides. Ten µL aliquots from each suspension of BCA (1 x 10^6 cells mL^{-1}) and conidial suspension of *C. acutatum* (1 x 10^4 conidia mL^{-1}) were placed simultaneously in demarcated areas of glass slides, previously prepared with water-agar. Controls were placed in aliquots of sterile distilled water instead of BCA. After preparing the glass slides, they were placed in tightly sealed plastic containers containing a cotton swab soaked in sterile distilled water to maintain humidity. The cultures were incubated at 25ºC for 14 h, in the dark. After this period, 100 conidia selected arbitrarily were observed under an optical microscope and the percentage of germinated conidia was calculated. Conidia of *C. acutatum* were considered germinated if the length of the germ tube was equal or greater or similar to the width of the conidium. Before counting, 10 µL of lactophenol cotton blue was added on the conidial suspension to prevent the development of pathogen. The experiment was arranged in a completely randomized design, with each treatment repeated eight times. The data were analyzed by ANOVA using the statistical program ASSISTAT 7.6 and the means were compared by Scott-Knot's test at 5% probability.

***In vivo* assay on detached flowers**

Detached Lima sweet orange (*Citrus sinensis*) flowers were placed in plastic boxes (Germbox), with the stems inserted in holes made into 0.5-cm thick synthetic foam over filter paper that was soaked with sterile distilled water. The boxes were placed 70 cm below two germicidal lamps (Sankyo Denki G30T8, 30 watts) for 20 min before antagonist application and pathogen inoculation (Moretto et al., 2001). Biocontrol agents were grown on PDA and 10 µL of suspensions containing 1 x 10^6 cells mL^{-1} were applied alone or in combinations on each petal. The flowers were treated with BCAs 24 h before inoculation with the pathogen (1 x 10^4 conidia mL^{-1}). Flowers sprayed with *C. acutatum* were used as controls. Each treatment was replicated three times, and each replication consisted of one Germbox with 10 flowers. The boxes were maintained in a growth chamber at 22ºC and a 12-h photoperiod. The evaluation was done 72 h after inoculation, determining the percentage of healthy flowers. The data were analyzed by ANOVA using statistical program ASSISTAT 7.6, and the means were compared by Scott-Knott test at 5% probability.

Field experiment

Based on the results obtained *in vitro* and on detached citrus flowers, the best mixtures of biocontrol agents were chosen to be tested under field conditions. The treatments were: ACB-69+ACB-37; ACB-69+ACB-77; ACB-66+ACB-37; ACB-66+ACB-77, and ACB-77+ACB-37. These were compared with ACB-66, ACB-69, ACB-77, ACB-37, and with the chemical carbendazin in the growing season 2009/2010 and thiophanate-methyl in the growing season 2011/2012 following the standard strategies already used by the private property and the control without treatment. Two trials were done to test the effect of *B. subtilis* and *Trichoderma* alone, or in mixture, in preventing infection by *C. acutatum*.

Field Experiment 1 was conducted during the growing season 2009/2010 in 12-year-old orchards of 'Lima' sweet orange (*C. sinensis*) grafted on Rangpur lime (*Citrus limonia)* located in the municipality of Engenheiro Coelho, SP. Field experiment 2 was conducted during the growing season 2011/2012 in 20-year-old orchards of 'Lima' sweet orange (*C. sinensis*) grafted on Rangpur lime, located in the municipality of Estiva-Gerbi, SP. Inoculum of *B. subtilis* was produced on glass vessels containing 15 L of medium comprised of the foliar fertilizer base Ajifol at 5% (v/v), autoclaved at 120ºC for 60 min, at 1 atm. This medium contains carbon, nitrogen, and mineral sources; it is inexpensive and widely used in several citrus orchards as a foliar fertilizer. The medium was seeded with a bacterial suspension and incubated at room temperature (22ºC ± 2ºC), for 72 h under constant agitation (Kupper & Gimenes-Fernandes, 2002). The multiplication of *Trichoderma* spp. was carried out in a culture medium based on potato (200 g/L) and dextrose (20 g/L), in a 15-L Microferm Fermentor under controlled incubation conditions (22ºC ± 2ºC) for 120 h, under constant agitation. The treatments were applied using a tractor-powered sprayer, with two guns calibrated to provide an application volume of 2.000 L/ha, using 6 L/plant. The working pressure used was 248 kpascals, with motor rotation of 540 rpm. The treatments were applied weekly in a total of four applications during the blossom

period according to Agustí et al. (2002): (1) closed visible flowers, or green bud, (FGB); (2) elongating flower petals; sepal covering corolla or white bud (FWB); (3) most flowers with petals forming a hollow ball (HB); and (4) open flowers (FO).

In field Experiment 2, the treatments were applied using an air-assisted sprayer calibrated to provide an application volume of 4.750 L/ha, using 10 L/plant. The working pressure used was 690 kpascals, with motor rotation of 540 rpm. Three applications were performed during flower growth stages (white bud, hollow ball, and open flowers). In both field experiments a final concentration of 10^7 cells/mL of each microorganism was used. A randomized block design was used with 11 treatments and four repetitions. Each experimental plot was composed of four plants, and the evaluations were conducted on a sample of four branches per plant from the two central plants in the plot. The evaluations were based on counting the number of diseased and healthy flowers in a sample of 12 branches per plant in Experiment 1 and three branches per plant in Experiment 2. Samples were taken from plants located at the centre of each plot. The percentage of diseased flowers followed the method adapted from Timmer & Zitko (1996), where up to 100 flowers that had recently opened or were about to open were observed on each branch. A second evaluation was performed 90 days after the first evaluation, counting the number of fruit set and calyces retained and/or of the yellow caused by the disease to obtain the average number of effective fruits (ANEF), using the following equation: ANEF= [A/ (A+B)] x 100, where: A = number of fruit sets and B = number of persistent calyces and/or number of yellow fruits due to the disease, according to Goes (1995). The data were analyzed by ANOVA using statistical program ASSISTAT 7.6, and the means were compared by Tukey's test at 5% probability.

RESULTS

Growth of biological control agents

There were differences among the isolates, particularly between ACB-83 and ACB-77 that produced significantly more bacterial cells, with 2.24 and 1.91 x 10^9 cells mL^{-1}, respectively, than isolates ACB-69 (1.15 x 10^8 cells mL^{-1}) and ACB-66 (9.43 x 10^7 cells mL^{-1}). The most favorable temperature for bacterial growth was 27ºC with an average production of 2.55 x 10^9 cells mL^{-1}, whereas at 15ºC the yield was 3.69 x 10^7 cells mL^{-1}. The yields were 9.78 x 10^8 cells mL^{-1} at 20ºC and 8.02 x 10^8 cells mL^{-1} at 35ºC. There was an increase in the production of bacterial cells up to 36 h (5.24 x 10^8 cells mL^{-1}), a drop after 48 h of incubation (2.44 x 10^8 cells mL^{-1}), the bacterial concentration increased again at 72 h (1.48 x 10^9 cells mL^{-1}) up to 120 h (9.61 x 10^8 cells mL^{-1}). The isolate ACB-14 (*Trichoderma* sp.) had the lowest number of conidia produced (1.8 x 10^9 conidia mL^{-1}), differing significantly from the other isolates: ACB-40 (2.98 x 10^9 conidia mL^1), ACB-37 (2.26 x 10^9 conidia mL^{-1}), and ACB-33 (2 x 10^9 conidia mL^{-1}), which did not differ among themselves. The best temperature for fungal multiplication was 27ºC (9.02 x 10^9 conidia mL^{-1}) whereas 15ºC permitted less development of *Trichoderma* isolates (4.2 x 10^6 conidia mL^{-1}). The largest number of conidia was obtained after an incubation period of 120 h (1.76 x 10^{10} conidia mL^{-1}). From 6 to 18 h fungal sporulation almost did not occur, while at 24 h spore concentration was approximately 1 x 10^5 conidia mL^{-1}. The concentration of conidia gradually increased after 36 h (2.70 x 10^6 conidia mL^{-1}), 48 h (1.84 x 10^7 conidia mL^{-1}), and 72 h (5.08 x 10^8 conidia mL^{-1}). Figure 1 shows some selected biocontrol agents used in this study that presented different growth and sporulation patterns.

Effect of biocontrol agents on the germination of *Colletotrichum acutatum*

All treatments inhibited germination of the phytopathogen, with values that ranged from 29% to 98% inhibition. Isolates of *B. subtilis* ACB-69, ACB-83, ACB-66, and ACB-77 inhibited 98%, 95%, 92%, and 88% of *C. acutatum* germination, respectively. Isolates ACB-40, ACB-14, ACB-37, and ACB-33 of *Trichoderma* spp. inhibited 91%, 86%, 85%, and 61%, respectively. The mixtures of the ACB-77 plus ACB-66, ACB-33, or ACB-37 inhibited pathogen germination from 84% to 89% (Table 1).

In vivo assay on detached flowers

Most isolates of *B. subtilis* and *Trichoderma* spp. (except ACB-33), when used alone, resulted in more than 86% of symptomless flowers. The bacterial isolates ACB-66, ACB-83, and ACB-69 showed 95%, 96%, and 99% healthy flowers, respectively. The best treatments for controlling disease in detached flowers, using the combination of biological control agents were ACB-77 with ACB-37 or with ACB-66, with mixtures showing around 88% healthy flowers (Table 2).

Control of postbloom fruit drop in the field

In the 2009/2010 growth season (Table 3), only treatment with *T. pseudokoningii* (ACB-37) did not differ from the control (without disease control). The combined treatment ACB-37 and ACB-69 (*B. subtilis*) provided 73% of healthy flowers, which did not differ from the chemical control, from ACB-66 and ACB-69; these differed from each other and had 90%, 86%, and 81% of flowers without pathogen infection, respectively. Other treatments had intermediate results, showing percentages of healthy flowers that ranged from 33% to 65%.

The average number of remaining fruits showed that although the disease had occurred with great intensity, it was possible to observe the effect of treatments on disease incidence. Plants treated with isolate ACB-69 (*B. subtilis*) alone or combined with ACB-37 (*Trichoderma pseudokoningii*) did not differ significantly, either among themselves or from the standard fungicide used for the disease control, showing an average amount of healthy

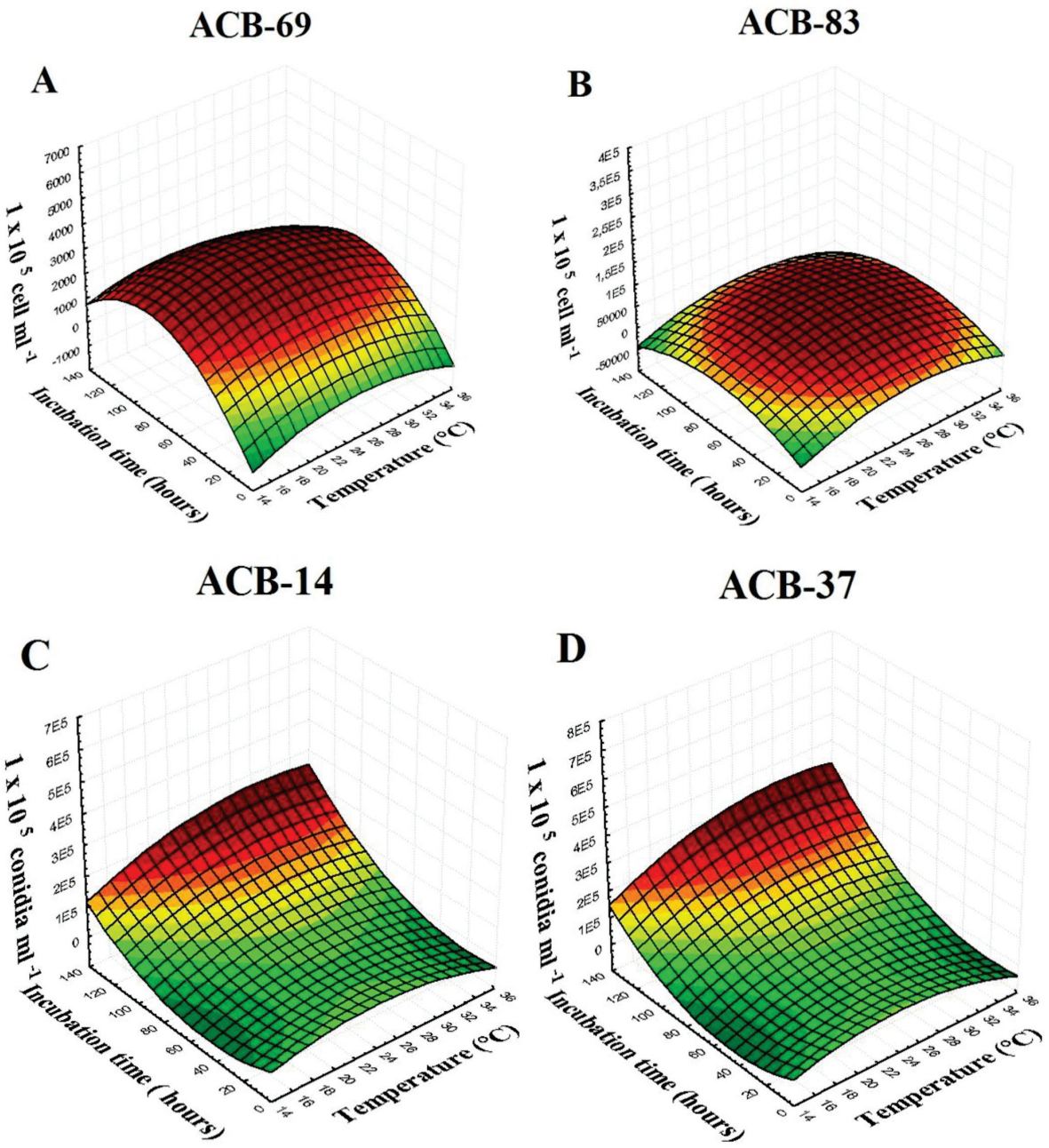

FIGURE 1 - Response surface graphs of incubation time (6, 12, 18, 24, 36, 48, 72, and 120 h) and temperature (15, 20, 27, and 35ºC) on production of cells of selected isolates of *Bacillus subtilis*: ACB-69 (**A**), ACB-83 (**B**), and *Trichoderma* sp.: ACB-14 (**C**) and ACB-37 (**D**). These isolates are shown for presenting different patterns of growth and sporulation. The experiment was done with 4 isolates of *Trichoderma* spp. (ACB-14, ACB-33, ACB-37 and ACB-40) and 4 isolates of *Bacillus subtilis* (ACB-66, ACB-69, ACB-77, and ACB-83). Isolates were incubated on different growth conditions and production of *B. subtilis* cells and *Trichoderma* spp. spores were determined in each period of incubation.

fruit of 41%, 45%, and 40%, respectively. Isolate ACB-66, that presented 81% of healthy flowers, showed an intermediate behavior, with 36% of an average number of effective fruits, not differing from the fungicide and the treatment with ACB-69, but differed from the mixture ACB-69 +ACB-37.

The percentage of healthy flowers in the second season (Table 3) showed that, except for the treatment with ACB-37 alone, the others differed from the control. The best treatments, the chemical treatment, ACB-69 alone and the mixture of ACB-69+ACB-37 did not differ significantly from each other, with percentage of healthy flowers of

TABLE 1 - Effect of the biological control agents, *Bacillus subtilis* (ACB-66, ACB-69, ACB-77 and ACB-83), and *Trichoderma* spp. (*Trichoderma* sp. - ACB-14 and ACB-40, *T. aureoviride* - ACB-33, and *T. pseudokoningii* - ACB-37), in combination or not, on the inhibition of *Colletotrichum acutatum* spore germination

Treatments	% Inhibition[a]	Treatments	% Inhibition[a]
ACB-69	98.38 a[b]	ACB-69+33	58.12 j[b]
ACB-83	95.25 b	ACB-77+14	57.62 j
ACB-66	92.25 b	ACB-37+40	56.00 j
ACB-40	90.62 c	ACB-33+37	55.50 j
ACB-66+77	89.12 c	ACB-69+66	55.25 j
ACB-77+37	88.62 c	ACB-33+40	51.00 l
ACB-77	87.87 c	ACB-83+40	50.12 l
ACB-14	85.62 d	ACB-66+40	48.37 m
ACB-37	85.37 d	ACB-83+33	41.37 n
ACB-77+33	84.25 d	ACB-69+40	40.37 n
ACB-69+77	76.00 e	ACB-83+37	39.50 n
7ACB-66+37	74.25 f	ACB-66+33	38.25 n
ACB-69+37	72.00 g	ACB-14+37	34.75 o
ACB-77+40	71.62 g	ACB-14+40	33.37 o
ACB-83+77	69.75 h	ACB-14+33	31.87 o
ACB-66+14	69.37 h	ACB-69+14	29.75 o
ACB-66+83	63,00 i	ACB-69+83	28.87 o
ACB-33	60.75 i	Control	0.00 p
ACB-83+14	58.62 j		

[a]100 conidia selected arbitrarily were observed under an optical microscope and the percentage of germinated conidia was calculated. [b] Means followed by the same letter are not significantly different according to Scott-Knot's test ($P \leq 0.05$).

TABLE 2 - Percentage of flowers without symptoms of *Colletotrichum acutatum*, in detached citrus flowers, treated with *Bacillus subtilis* (ACB-66, ACB-69, ACB-77 and ACB-83), and with *Trichoderma* spp. (*Trichoderma* sp. ACB-14 and ACB-40), *T. aureoviride* ACB-33, and *T. pseudokoningii* ACB-37), in mixture or not, 24 h before inoculation with *Colletotrichum acutatum*

Treatments	% Healthy flowers[a]	Treatments	% Healthy flowers[a]
ACB-69	98.58 a[b]	ACB-33+37	58.95 c[b]
Control	96.90 a	ACB-83+14	57.67 c
ACB-83	95.67 a	ACB-77+14	57.51 c
ACB-40	94.72 a	ACB-69+33	56.40 c
ACB-66	92.05 a	ACB-40+37	55.35 c
ACB-77+37	88.58 a	ACB-66+69	52.47 c
ACB-77+66	88.01 a	ACB-40+33	51.95 c
ACB-37	87.11 a	ACB-66+40	50.24 c
ACB-77	86.95 a	ACB-83+40	49.24 c
ACB-14	85.74 a	ACB-69+40	41.64 d
ACB-77+69	76.78 b	ACB-66+33	41.60 d
ACB-66+37	74.99 b	ACB-83+33	41.60 d
ACB-77+40	72.81 b	ACB-40+14	41.38 d
ACB-69+37	72.14 b	ACB-83+37	39.97 d
ACB-66+14	70.13 b	ACB-14+37	35.04 d
ACB-83+77	69.90 b	ACB-14+33	32.10 d
ACB-77+33	65.06 c	ACB-69+14	31.57 d
ACB-83+66	62.61 c	Inoc. Control	31.07 d
ACB-33	60.24 c	ACB-83+69	30.37 d

[a]Detached flowers were placed in plastic boxes (Germbox), and the biocontrol agents were inoculated, alone or in mixture, on each petal. The evaluation was done 72 h after inoculation with *C. acutatum*, determining the percentage of healthy flowers. [b]Means followed by the same letter are not significantly different according to Scott-Knot's test ($P \leq 0.05$).

TABLE 3 - Effect of the mixture of biological control agents in the percentage of flowers without infections by *Colletotrichum acutatum,* and the average number of effective fruits (ANEF) in 'Lima 'orange plants under field conditions in Engenheiro Coelho-SP, Brazil, during the 2009/2010 season and in Estiva Gerbi-SP, Brazil, during the 2011/2012 season

Treatments	% Healthy flowers[a]	ANEF[b]	% Healthy flowers[a]	ANEF[b]
	2009/2010 season		2011/2012 season	
Chemical control	90.25 a [c]	40.20 ab [c,d]	89.32 a [c]	68.85 a[c,d]
ACB-69	86.12 a	40.55 ab	85.15 a	58.16 a
ACB-66	80.87 ab	35.85 b	74.53 b	45.10 b
ACB-69+37	72.50 abc	44.58 a	84.15 ab	64.64 a
ACB-69+77	65.00 bcd	9.62 fg	50.98 c	23.23 c
ACB-77+66	56.62 cd	28.38 c	37.73 d	40.78 b
ACB-77	55.12 cd	25.69 cd	50.35 c	27.77 c
ACB-77+37	47.62 de	19.30 de	9.28 d	24.22 c
ACB-66+37	32.50 ef	26.25 cd	43.15 cd	29.13 c
ACB-37	17.62 fg	16.34 ef	18.53 e	9.40 d
Control	8.50 g	8.92 g	16.98 e	4.22 d

[a]The evaluations were based on counting the number of healthy flowers in a sample of branches per plant. [b]ANEF = (A/(A + B)) x 100 (where A = number of fruit sets and B = number of persistent calyces and/or number of yellow fruits due to the disease). [c]Means followed by the same letter are not significantly different to according to the Tukey's test. ($P \leq 0.05$). [d]Data transformed to arc sen sqrt (x+0.5).

89%, 85%, and 84%, respectively. Other treatments showed intermediate control with the percentage controls ranging from 74.5% (ACB-66) and 38% (ACB-77+ACB-66). In evaluating the average number of effective fruits produced, except for treatment with the ACB-37, the other treatments differed from the control, with percentages of average number of effective fruits ranging from 69% to 23%. The most effective treatments were the chemical control, the mixture (ACB-69+ACB-37) and ACB-69, with average numbers of effective fruits at 69%, 65%, and 58%, respectively.

DISCUSSION

In this study we investigated the effects of mixtures between species of *Bacillus* and *Trichoderma* to control postbloom fruit drop of citrus and observed that when these microorganisms were applied in combination disease control was reduced. The levels of control of postbloom fruit drop by isolate ACB-69 in the experiments reported here were similar to what was obtained in other trials with the same isolate (Kupper et al., 2003, 2009, 2012). However, this was the first attempt of mixing this isolate with other biocontrol agents for control of postbloom fruit drop. We believe that isolate ACB-37 diminishes the biological control activity of ACB-69 thereby reducing the efficacy of the mixtures. This study demonstrates that certain biological control agents are incompatible. One of the isolates interferes with the mechanism by which a second isolate suppresses plant disease.

As in our study, other authors found more efficient results when the microorganisms were used individually. In studies with *Trichoderma* isolates for controlling root-rot of strawberry caused by *Armillaria mellea*, Raziq & Fox (2005) observed that some isolates of *T. harzianum* and *T. hamatum* were more efficient when applied individually than when mixed. Georgakopoulos et al. (2002) studied *B. subtilis*, *Pseudomonas fluorescens*, *P. corrugata*, *T. viride* and *Gliocladium virens* applied alone or in combination for controlling *Pythium ultimum*, and found that mixtures were less effective than individual antagonist treatments. On the other hand, Roberts et al. (2005) studied different isolates of *Trichoderma* and bacteria such as *Serratia marcescens, Burkholderia ambifaria*, and *Burkholderia cepacia* for biocontrol of different fungal diseases caused by *Rhizoctonia solani* and *Pythium ultimum* in cucumber and found that the combinations of biocontrol agents were more effective when compared to applications of the agents alone. The same result was found by Maketon et al. (2008), which showed that neither *B. subtilis* nor *T. harzianum* alone could control *Ralstonia solanacearum*, causal agent of bacterial wilt in tobacco, but when combined, they provided control levels similar to the chemical treatment.

Considering that microorganisms involved in mixtures may have different mechanisms of action, it is believed that the control could be optimized by reducing the variability during the suppression of diseases (Punja, 1997; Guetsky et al., 2002; Jetiyanon & Kloepper, 2002). Biocontrol by mixing antagonists is maximized when knowledge on the biology and ecological needs of each microorganism involved is available. Compatible mixtures of biocontrol agents with different mechanisms of action may reduce the chance of resistance development by phytopathogens and may be effective over a wider range of environmental conditions. In particular, mixtures of fungi and bacteria may provide protection at different times due to their differential physiological requirements

and mechanisms of action, which may help overcome inconsistencies in the performance of individual isolates.

According to Kupper et al. (2003) antibiosis seems to be a major mechanism involved in the interaction between *B. subtilis*, especially the ACB-69 isolate and *C. acutatum*. On the other hand, in species of *Trichoderma,* production of cell-wall-degrading enzymes (Kubicek et al., 2001) and mycoparasitism (Moretto et al., 2001) have been shown to be key factors in other systems. Further studies need to be performed to reveal the mechanisms used by *Trichoderma* to antagonize *C. acutatum* in citrus and improve the current understanding about its incompatibility with the *Bacillus* isolates used in our experiments.

The data obtained under field conditions in our study showed that when spraying biological products, the association of postbloom drop and high humidity should be considered. Prolonged periods of rain, as occurred during installation and evaluation of the experiment in August, September, and October (2009/2010 growth season) favored the development of an epidemic that diminished the effects of the biological treatments. Examples reported in the literature show that environmental conditions affect the stability, survival, and antagonistic activity of biological control agents (Elad & Zimand, 1993; Elad et al., 1994; Dik & Elad, 1999). This might explain why mixtures of ACB-77 plus ACB-66 or ACB-37 showed efficiency only *in vitro* and on detached citrus flowers, where environmental conditions were controlled.

Another aspect that should be considered about mixtures of microorganisms is the formulation of the bioproduct and the concentration of every agent. In general the cost of registration of biocontrol agents is high. If a combination of biocontrol agents is attempted, each agent needs to be registered independently. This requirement will certainly increase the cost of developing a biological product as well as the cost of its use. Considering the results of our work, the application of isolate ACB-69 presents advantages to postbloom fruit drop control in terms of cost and efficacy.

ACKNOWLEDGMENTS

The financial support of Fundação de Apoio à Pesquisa do Estado de São Paulo - FAPESP is gratefully acknowledged (processes no. 2008/56390-3 to K.C. Kupper and no. 2008/09821-9 - scholarship to M.N. Klein).

REFERENCES

Agrianual (2011) Yearbook of Brazilian Agriculture. São Paulo SP, Brazil. FNP.

Agustí M, Zaragoza S, Bleiholder H, Buhr L, Hack H, Klose R, Staub R (2002) The BBCH scale for describing phenological stages in citrus development. Proceedings of the Internacional Society of Citriculture 9:445-446.

Denham TG, Waller JW (1981) Some epidemiological aspects of post-bloom fruit drop disease (*Colletotrichum gloeosporioides)* in citrus. Annals of Applied Biology 98:65-67.

Dik AJ, Elad Y (1999) Comparison of antagonists of *Botrytis cinerea* in greenhouse-grown cucumber and tomato under different climatic conditions. European Journal of Plant Pathology 105:123-137.

Elad Y, Köhl J, Fokkema NJ (1994) Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic bacteria and fungi. European Journal of Plant Pathology 100:315-336.

Elad Y, Zimand G (1993) Use of *Trichoderma harzianum* in combination or alternation with fungicides to control cucumber grey mould (*Botrytis cinerea*) under commercial greenhouse conditions. Plant Pathology 42:324-332.

Georgakopoulos, DG, Fiddaman, P, Leifert C, Malathrakis, NE (2002) Biological control of cucumber and sugar beet damping-off caused by *Pythium ultimum* with bacterial and fungal antagonists. Journal of Applied Microbiology 92:1078-1086.

Goes A (2005) Podridão floral de frutos cítricos: Caracterização do agente causal, *Colletotricum gloeosporioides* Penz. (Sensu Arx, 1957), e controle da doença. PhD Thesis, Universidade de São Paulo. Piracicaba SP, Brazil.

Goes A, Garrido RBO, Reis RF, Baldassari RB, Soares MA (2008) Evaluation of fungicide applications to sweet orange at different flowering stages for control of postbloom fruit drop caused by *Colletotrichum acutatum*. Crop Protection 27:71-76.

Guetsky R, Shtienberg D, Elad Y, Dinoor A (2002) Establishment, survival and activity of biocontrol agents applied as a mixture in strawberry crops. Biocontrol Science and Technology 12:705-714.

Guetsky R, Shtienberg D, Elad Y, Dinoor A (2001) Combining biocontrol agents to reduce the variability of biological control. Phytopathology 91:621-627.

Gullino ML, Garibaldi A (1983) Situation actuelle et perspectives d'avenir de la lutte biologique et integree contre la pourrite grise de la vigne en italie. Les Colloques de L'INRA Bordeaux 18:91-97.

Janisiewicz WJ, Korsten L (2002) Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40:411-441.

Jetiyanona K, Kloepper JW (2002) Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. Biological Control 24:285-291.

Kubicek CP, Mach RL, Peterbauer CK, Lorito M (2001) *Trichoderma*: from genes to biocontrol. Journal of Plant Pathology 83:11-23.

Kupper KC, Bellotte JA, Goes A (2009) Controle alternativo de *Colletotrichum acutatum*, agente causal da queda prematura dos frutos cítricos. Revista Brasileira de Fruticultura 31:1004-1015.

Kupper KC, Corrêa EF, Azevedo FA, Silva AC (2012) *Bacillus subtilis* to biological control of postbloom fruit drop caused by *Colletotrichum acutatum* under field conditions. Scientia Horticulturae 134:139-143.

Kupper KC, Corrêa EB, Moretto C, Bettiol W, Goes A (2011) Control of *Guignardia citricarpa* by *Bacillus subtilis* and *Trichoderma* spp. Revista Brasileira de Fruticultura 33:1111-1118.

Kupper KC, Gimenes-Fernandes N, Goes A (2003) Controle biológico de *Colletotrichum acutatum,* agente causal da queda prematura dos frutos cítricos. Fitopatologia Brasileira 28:251-257.

Kupper KC, Gimenes-Fernandes N (2002) Isolation and selection of *Bacillus* spp. for biocontrol of *Colletotrichum acutatum* on 'Tahiti' lime detached flowers. Summa Phytopathologica 28:292-295.

Maketon M, Apisitsantikul J, Siriraweekul C (2008) Greenhouse evaluation of *Bacillus subtilis* AP-01 and *Trichoderma harzianum* AP-001 in controlling tobacco diseases. Brazilian Journal of Microbiology 36:296-300.

Moretto KCK, Gimenes-Fernandes N, Santos JM (2001) Influence of *Trichoderma* spp. on *Colletotrichum acutatum* mycelial growth and morphology and on infection of 'Tahiti' lime detached flowers. Summa Phytopathologica 27:357-364.

Punja ZK (1997) Comparative efficacy of bacteria, fungi, and yeasts as biological control agents for diseases of vegetable crops. Canadian Journal of Plant Pathology 19:301-306.

Pusey PL, Wilson CL, Hotchkiss MW, Franklin JD (1986) Compatibility of *Bacillus subtilis* for postharvest control of peach brown rot with commercial fruit waxes, dicloran, and cold-storage conditions. Plant Disease 70:587-590.

Raziq F, Fox RTV (2005) Combinations of fungal antagonists for biological control of *Armillaria* root rot of strawberry plants. Biological Agriculture and Horticulture 23:45-57.

Roberts DP, Lohrke SM, Meyerb SLF, Buyer JS, Bowers JH, Bakerd CJ, Lie W, Souza JT, Lewis JA, Chung S (2005) Biocontrol agents applied individually and in combination for suppression of soilborne diseases of cucumber. Crop Protection 24:141-155.

Sonoda RM, Guo Z (1996) Effect of spray applications of *Bacillus subtilis* on postbloom drop of citrus. Phytopathology 86:52-60.

Timmer LW, Zitko SE (1996) Evaluation of a model for prediction of postbloom fruit drop of citrus. Plant Disease 80:380-383.

Tronsmo A, Dennis C (1977) The use of *Trichoderma* species to control strawberry fruit rots. Netherland Journal of Plant Pathology 83:449-455.

TPP 2013-0017 - Received 31 January 2013 - Accepted 23 September 2013
Section Editor: Jorge Teodoro de Souza