CONTROL OF *Guignardia citricarpa* BY *Bacillus subtilis* AND *Trichoderma* spp.[1]

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ABSTRACT – The ability of isolates of *Bacillus subtilis* and *Trichoderma* spp. to control citrus black spot (CBS) was investigated in ´Natal´ sweet orange orchards. The first experiment was conducted during the 2001/2002 season and four isolates of *B. subtilis* (ACB-AP3, ACB-69, ACB-72 and ACB-77), applied every 28 days, alone or in combination were tested and compared with fungicide treatments. Two other experiments were carried out during the 2002/2003 season, where the same isolates of *Bacillus* and two isolates of *Trichoderma* (ACB-14 and ACB-40) were tested being applied every 28 days in the second experiment, and every 15 days in the third experiment. In the first experiment, the treatment with ACB-69 differed statistically from the control, but did not differ from other biological control agents or mixture of *Bacillus* isolates. In the second experiment, the treatments with ACB-69 and ACB-AP3 resulted in smaller disease index compared with the control treatment. However, this result was not repeated in the third experiment, where the isolates were applied every 15 days. Disease severity was high in both evaluated seasons and the fungicide treatment was the most effective for disease control.
Index terms: *Citrus sinensis*, *Phyllosticta citricarpa,* citrus black spot, biological control.

CONTROLE DE *Guignardia citricarpa* POR *Bacillus subtilis* E *Trichoderma* spp.

RESUMO – A habilidade de isolados de *Bacillus subtilis* e *Trichoderma* spp. em controlar a mancha preta dos frutos cítricos (MPC) foi avaliada em pomares de laranjeira 'Natal'. O primeiro experimento foi conduzido durante a safra de 2001/2002, no qual foram testados quatro isolados de *B. subtilis* (ACB-AP3, ACB-69, ACB-72 e ACB-77) aplicados de forma isolada ou em mistura, em intervalos de 28 dias, comparando-se com o tratamento fungicida. Dois outros ensaios foram conduzidos durante a safra de 2002/2003, em que os mesmos quatro isolados de *B. subtilis* e dois isolados de *Trichoderma* spp. (ACB-14 e ACB-40) foram testados, sendo os isolados aplicados em intervalos de 28 dias no segundo experimento e em intervalos de 15 dias no terceiro experimento. No primeiro experimento, o tratamento com ACB-69 diferiu estatisticamente do tratamento-testemunha, porém não diferiu dos outros agentes de controle biológico ou da mistura dos isolados de *Bacillus*. No segundo experimento, os tratamentos com ACB-69 e ACB-AP3 resultaram em menores índices de doença em comparação com o tratamento-testemunha. Entretanto, este resultado não se repetiu no terceiro experimento, em que os isolados foram aplicados em intervalos de 15 dias. A severidade de doença foi alta em ambas as safras avaliadas, e o tratamento com fungicida foi o mais efetivo para o controle da doença.
Termos para indexação: *Citrus sinensis, Phyllosticta citricarpa,* mancha preta dos frutos cítricos, controle biológico.

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INTRODUCTION

Control of citrus black spot (CBS) is mainly carried out by the use of protective or systemic fungicides, either alone or in combination with or without mineral or vegetable oil (FUNDECITRUS, 1998; GOES et al., 1990). Good results have been obtained with systemic and protectant fungicides applied at 50 to 55-day intervals (GOES, 1998), or 28-day intervals for the protectant ones (SCHUTTE et al., 1997). Nevertheless, the continuous use of fungicides can lead to the appearance of resistant strains. Martins et al. (1998) published the first case of *G. citricarpa* resistance to benomyl in citrus plants in Brazil. According to Rodrigues et al. (2007) isolates of *G. citricarpa* from lesions on *Citrus sinensis* fruits exhibited resistance to different concentrations of carbendazim, also a fungicide of the benzimidazole group. In addition to that, the fungicides can have deleterious effects on the environment. Akinnifesi et al. (2006) concluded that the copper-based fungicide residues predispose the soils to nitrogen, phosphorus and potassium deficiencies and perhaps copper toxicity if not properly managed. Therefore, it is essential to search for new alternatives to control this disease, reducing the costs, the dependency of agrochemicals, and using more adequate pathways.

Species belonging to the genus *Trichoderma* (DUBOS et al., 1982; MELO, 1998) and *Bacillus subtilis* (BETTIOL et al., 1994; KALITA et al., 1996; SONODA; GUO, 1996; KUPPER; GIMENES-FERNANDES, 2002; KUPPER et al., 2003; KUPPER et al., 2009) are among the most studied antagonists in biological control. Kupper et al. (2003) reported that the treatment with the isolate of *B. subtilis* ACB-69, at 10% of concentration, during the blossom period differed from the non-controlled plots and was equal to the benomyl treatment on the control of *Colletotrichum acutatum,* causing a lower percentage of symptomatic flowers and a greater number of effective fruits. In this sense, biological control emerges as a possible alternative to solve several of these problems, besides allowing the production of healthier food with the increase in organic agriculture.

The aim of the present study was to evaluate the potential of *B. subtilis* and *Trichoderma* spp., isolated from citrus plants and citrus orchard soil, respectively, for control of CBS in the field.

MATERIAL AND METHODS

The antagonists used in the biological control tests were obtained from samples of soil (*Trichoderma* spp.), leaves (*Bacillus subtilis*), and flowers of citrus trees (*Bacillus subtilis*) collected in commercial citrus orchards in the State of São Paulo (MORETTO et al., 2001; KUPPER; GIMENES-FERNANDES, 2002).

Fifteen isolates of *Trichoderma* spp. and four isolates of *B. subtilis* were screened individually against *G. citricarpa* (=*Phyllosticta citricarpa*) in the Fitossanity Department of the University of the state of São Paulo (UNESP, Jaboticabal) by using dual culture techniques in 90mm Petri dishes containing Potato-Dextrose-Agar (PDA) (DENNIS; WEBSTER, 1971). The microorganisms (pathogen and antagonist) were transferred to Petri dishes at the same time using disks (5mm in diameter), 3 cm apart. Controls were prepared without the antagonists. The diameters of *P. citricarpa* colonies were measured after incubation at 25 ± 2ºC for five days. The experimental design was completely randomized with five replicates with each dish as an experimental unit. Means were compared by Tukey's test at 5% probability.

Field experiment 1 was conducted during the growing season (2001/2002) in 12-year-old orchards of 'Natal' sweet orange [*Citrus sinensis* (L.) Osbeck] grafted on Rampur lime (*Citrus limonia* Osbeck), with spacing of 7 m x 3.5 m, located in the municipality of Rincão (State of São Paulo). Isolates of *B. subtilis* (ACB-69, ACB-72, ACB-77 and ACB-AP3) were grown on a medium based on the residue of Aminofertil® (glutamic fermentation of molasses). The growth medium was prepared by diluting the Aminofertil® residue in water at 5% with the addition of dextrose (20g/L), in a 15-liter Microferm Fermentor under controlled incubation conditions (25ºC ± 2ºC) for 72 hours, under constant agitation, in the dark. The residue was used for multiplication of the antagonists because of its high content of carbon, nitrogen and salts in addition to its low cost and being utilized as foliar fertilizer for citrus (BETTIOL; ASTIARRAGA, 1998).

Field experiments 2 and 3 were carried out in two different 8-year-old orchards of 'Natal' sweet orange on Rampur lime, located in the municipality of Santa Rita do Passa Quatro (State of São Paulo) during the 2002/2003 season. Four isolates of *B. subtilis* (ACB-AP3, ACB-69, ACB-72 and ACB-77), and two isolates of *Trichoderma* [*T. viride* Pers. Ex S. F. Gray (ACB-14) and *Trichoderma* sp. (ACB-40)], which showed potential for control of

P. citricarpa in dual culture technique evaluations, were tested. The multiplication of the isolates of *B. subtilis* was done in culture medium based on cotton meal (200gL^{-1}) with dextrose (20g.L^{-1}) (in the first application), or hydrolyzed protein (20g.L^{-1}) (in the second application). The cotton meal substrate was chosen because of its high efficiency in the production of bacterial cells and metabolites that inhibited the development of the plant pathogen (BETTIOL et al., 2005), besides its low cost. The multiplication of *Trichoderma* spp. was carried out in a culture medium based on potato (200g.L^{-1}) and dextrose (20g.L^{-1}), in a 15-liter Microferm Fermentor under controlled incubation conditions (25^{o}C ± 2^{o}C) for 120 hours, under constant agitation, in the dark.

For all experiments, the fermented broth of the isolates of *B. subtilis* or *Trichoderma* spp. was diluted to 10% and compared to: (i) standard fungicide treatment (copper oxychloride at 100 g of metallic copper in 100 L of water and carbendazim + mancozeb containing 25g of a.i + 160g of a.i/100 L of water, respectively, with the addition of mineral oil at 0.5%) (FUNDECITRUS, 1998) and (ii) control (water).

In the experiment 1, two additional treatments were included, a combination of *B. subtilis* isolates (ACB-69+ACB-72+ACB-77+ACB-AP3) and a biofertilizer produced under anaerobic conditions (at a concentration of 10%) (KUPPER et al., 2006). The first spray in all treatments was done on October 15, 2001 (at ¾ petal fall) and repeated at 28, 56, 84, 112, and 140 days after the first. In the standard fungicide treatment, two sprays were done with copper oxycloride, on the same day as the first spray with the biological agents and 28 days after that; and two others with the mixture of carbendazim + mancozeb + mineral oil at 0.5% at 84 and 140 days after the first spray. The combination of *B. subtilis* isolates was prepared using equal parts of the four isolates.

For the 2002/2003 season, in the experiment 2, microbial antagonists and fungicides were applied according to the scheme described for the 2001/2002 season (at 28-day intervals). The first spray of all treatments was applied on October 8, 2002 and the last on 140 days after the first application. In the experiment 3, the fungicides were applied at 28-day intervals (6 applications) and the sprays with microbial antagonists were applied at 15-day intervals (9 applications), the first spray of all treatments was applied on October 23, 2002.

Fungicides and antagonists were sprayed using two tractor-powered sprayers, one for application of the biological control agents and another one for application of the fungicides, with two guns and a 150L tank. Each treatment received 100L of liquid spray (8.33L per tree, in the 2001/2002 season and 6.66L per tree, in the 2002/2003 season). The concentration of bacterial cells applied was 10^7 cfu/mL per isolate, where the spray with combination of antagonists consisted of 2.5L of fermented broth of each *Bacillus* isolate, in the same concentration of cells (2001/2002 season) and 10^8 cfu/mL and 10^6 conidia/mL for *B. subtilis* and *Trichoderma* spp., respectively (2002/2003 season).

The evaluations were performed during harvest on 50 arbitrarily selected fruits from the center of the tree in each experimental unit. A severity rating scale for CBS adapted from Fagan (1999): 0-absence of symptoms; 1-0.9%; 2-1.4%; 3-2.8%; 4-5.6%; 5-9.2%; and 6-17.3% of the area covered with symptoms, was used. Later, the disease index (DI) was determined using the formula according to Wheeler (1969), with modifications: $DI = \Sigma ((fv) \times 100)/n)$, where; f = number of fruits with a certain degree of infection; v = degree of infection and n = total number of sampled fruits.

The experimental design was in randomized blocks with four and five replicates in the assays of the Experiment 1 (2001/2002) and Experiments 2 and 3 (2002/2003), respectively. Each experimental plot consisted of three trees. Analysis of variance and comparison of averages were done by the Duncan's test (0.05).

RESULTS AND DISCUSSION

Bacillus subtilis and *Trichoderma* spp. significantly inhibited the mycelial growth of *P. citricarpa* (Table 1). *Bacillus subtilis* isolates inhibited the mycelial growth of *P. citricarpa* around 15 to 23%, and the isolates of *Trichoderma,* such as ACB-14 and ACB-40, inhibited the development of the pathogen by 50 and 57%, respectively, when compared to the control. The isolates of *Trichoderma* spp., when paired with *P. citricarpa*, completely covered the culture surface in the Petri dishes six days after the beginning of the incubation. This was not observed by Moretto et al. (2001), who verified that these same isolates when in paired culture with *Colletotrichum acutatum* had faster mycelial growth in PDA and after two days of incubation, the antagonists completely covered the culture surface, hindering the development of the plant pathogen, when compared with the control. Possibly the temporal change in the growth pattern of isolates of *Trichoderma* spp., as observed in this work has probably resulted from the production of

metabolites by *P. citricarpa* that offered resistance to the growth of the antagonist, especially if considered the slow growth of the plant pathogen in the culture medium. Moreover, the inhibitions on the development of the pathogen may have occurred not only by direct action of the antagonist, but also by the lack of nutrients in the medium colonized by the antagonists. This was deduced due the slow growth of *P. citricarpa* in culture media without the presence of the antagonists. Given the above, it is believed that for future work belonging *P. citricarpa* and antagonists with faster growth, the pathogen should be transferred hours or days before of the antagonists when evaluate in dual culture.

During the 2001/2002 season (Experiment 1), the severity of black spot in citrus fruits was high. The high levels of disease symptoms observed are due to the susceptibility of the variety, levels of inoculum of *G. citricarpa* and suitable climatic conditions. In the period corresponding to the critical stage of the disease, between October 2001 and March 2002, the average temperature for Rincão ranged from 18.8 to 28.7°C with rainfall index at 217.3 mm. The control of the disease in the biofertilizer treatment was effective (DI=2.48), being statistically different from the control (DI=3.43), however, it was not as efficient as the chemical control. Kupper et al. (2006) reported the potential use of biofertilizer in the control of CBS. In this study, the authors verified the possibility of using it as a protecting biofungicide, replacing copper oxychloride. Among the isolates of *B. subtilis,* only the isolate ACB-69, with DI=2.42, differed from the control (DI=3.43), but no difference was observed compared to the other isolates and the treatment with the combination of isolates (DI=3.05).

In the assays conducted in the 2002/2003 season (Experiments 2 and 3) the disease also occurred in high intensity, for the case of experiments conducted at Santa Rita do Passa Quatro, disease levels were high as well as the climate conditions were favorable for the pathogen. The temperature ranged from 19 to 29,5°C, with rainfall index at 226 mm in the similar period to that of Rincão. Although these conditions provide the development of pathogen, they also promote the development of the biological control agents. Regarding to the control of CBS, the chemical one was the most effective in the experiments 2 and 3 (Table 3). In the experiment 2, the isolates ACB-69 and ACB-AP3 of *B. subtilis*, showed the lowest disease index (DI=1.70 and 1.72, respectively), when compared with the other biological control agents, statistically differing from the control (DI=2.12), when sprayed in intervals of 28 days (Table 3). Differences were not demonstrated between biological treatments and the control when the biological control agents were applied every 15 days (Table 3). According to Korsten and Jeffries (2000), biological control agents need to establish themselves to a certain critical level inside the canopy before the control can occur. Consequently, the data observed for 2002/2003 season provided an opportunity to reevaluate the use of biocontrol agents at shorter spray intervals. It is believed that higher cell concentrations of the antagonist after the 15-day interval sprays of biocontrol agents could have resulted in competition for space and/or nutrients, or even greater production of toxic metabolites that could be self-harmful. Consequently such unfavorable conditions in the field could be cited as a reason for failure or inconsistent performance of biological treatments, when the results from experiment 2 and 3 are compared. Another reason for the possible inefficacy of the biological control agents is the possible unfavorable weather conditions and deleterious effect to the antagonist populations caused by the use of acaricides and insecticides in the experimental areas. Studies about sensitivity of *B. subtilis* (ACB-69) to the chemicals products used to control pests and diseases in citrus showed that concentrations of 100 to 1000 $\mu g.mL^{-1}$ of dicofol and propargite acaricides affected the development of the bacteria (BEZERRA et al., 2006). Since the integration of the different forms of control is essential for integrated pest management, it is necessary to know the sensitivity of the biocontrol agents to the applied products. Korsten et al. (1997) demonstrated that pre-harvesting spraying of *B. subtilis,* alone or together with fungicides, especially copper oxychloride, efficiently reduced natural infections of *Pseudocercospora purpura* in avocado under field conditions. The authors discussed the importance of a combination of biological products and conventional procedures used in crop protection that can increase the acceptance by the growers.

Regarding to the results obtained on the behavior of *Trichoderma* spp., under natural conditions, it was found that the isolates did not control the disease, although they have shown a greater ability to inhibit the development of colonies of *P. citricarpa* when in controlled laboratory conditions. Differences in the efficacy of biological control between controlled conditions and commercial situations may have several causes. The occurrence of diseases as well as the effective suppression of plant diseases by biocontrol agents is largely affected by environmental conditions. The environment affects the establishment, survival, and activity of the biocontrol (ELAD;

ZIMAND, 1993; ELAD et al., 1994; DIK; ELAD, 1999). For example, high temperature during the day and high vapor pressure deficit during the night were associated with reduced efficacy of *Botrytis cinerea* suppression in cucumber and tomato by *Trichoderma harzianum* T39, *Aureobasidium pullulans*, and *Candidus albidus* (DIK; ELAD, 1999; DIK et al., 1999). Similarly, Hannusch and Boland (1996) indicated that most of the biocontrol agents they studied were highly dependent on the environment for efficacy. Species of *Trichoderma* used in this study were isolated from citrus orchard soil, and it can be the possible cause of its inefficacy in controlling CBS in citrus plant canopy, due the lack of capacity of adaptation and colonization in fruit of citrus for controlling the disease.

Kupper et al. (2003) reported that, under natural conditions, the *B. subtilis* isolate ACB-69 was as effective as benomyl in the reducing of the percentage of symptomatic flowers of post bloom citrus fruit drop caused by *Colletotrichum acutatum,* allowing an increase in the average number of effective fruits. Therefore, these results along with the present study demonstrate the potential of this isolate in the control of diseases that affect young flowers and fruit of citrus, especially when there is a compatibility of the control periods of such diseases, as related in literature (FUNDECITRUS, 1998).

Because of the importance of citrus diseases, the prices in the national and international markets and the demand for fruits free of chemical residues, control measures that cause the lowest environmental and social impact are gradually being accepted as the most adequate. For this reason, this study shows that the biological control can be an additional alternative to CBS control even though the antagonists tested did not attain the control level of the chemicals used in the experiment. The disease control level can be inadequate for the conventional fresh fruits as discussed by Reis et al. (2003), but it could be sufficient for the Brazilian organic market. Even so it is necessary to continue the tests to establish the most adequate spray timing, the best concentration and formulation of the antagonists, and the role of the weather conditions in the sprays of biological control agents. These studies must be done in the field since a good correlation between data obtained *in vitro* and *in vivo* is not always observed (LOPES; STALL, 1990).

TABLE 1- Effect of *Trichoderma* spp. and *Bacillus subtilis* isolates on the linear growth of *Phyllosticta citricarpa***,** evaluated through the dual culture technique on PDA medium after five days of incubation at 25 ± 2°C.

Treatments	Species	Diameter of the colony (cm) of *P. citricarpa*	Percentage of colony inhibition
Control	-	2.06 a [(1)]	-
ACB-72	*Bacillus subtilis*	1.75 b	15.05
ACB-77	*Bacillus subtilis*	1.70 bc	17.48
ACB-AP3	*Bacillus subtilis*	1.66 bc	19.42
ACB-69	*Bacillus subtilis*	1.59 bcd	22.82
ACB-37	*Trichoderma pseudokoningii*	1.53 bcde	25.73
ACB-39	*Trichoderma aureoviride*	1.47 cdef	28.64
ACB-30	*Trichoderma harzianum*	1.42 defg	31.07
ACB-35	*Trichoderma koningii*	1.40 defgh	32.04
ACB-05	*Trichoderma koningii*	1.35 efgh	34.47
ACB-38	*Trichoderma koningii*	1.31 efghi	36.41
ACB-32	*Trichoderma virens*	1.28 fghi	37.86
ACB-33	*Trichoderma aureoviride*	1.26 fghi	38.83
ACB-31	*Trichoderma harzianum*	1.26 fghi	38.83
ACB-03	*Trichoderma aureoviride*	1.24 ghij	39.81
ACB-36	*Trichoderma aureoviride*	1.22 ghij	40.78
ACB-04	*Trichoderma harzianum*	1.19 hij	42.23
ACB-34	*Trichoderma aureoviride*	1.11 ijk	46.12
ACB-14	*Trichoderma viride*	1.03 jk	50.00
ACB-40	*Trichoderma* sp.	0.89 k	56.80

[(1)]Averages followed by the same letter did not show statistical difference (Tukey's test, 0.05). Analysis done with data transformed by $(x+0.5)^{1/2}$.

TABLE 2 – Disease index of citrus black spot, caused by *Guignardia citricarpa*, on 'Natal' sweet orange sprayed with different treatments (*Bacillus subtilis* ACB-AP3, ACB-77, ACB-72, and ACB-69; combination of all *B. subtilis* isolates; chemical control; and control) during the 2001/2001 season (Experiment 1), in the municipality of Rincão, State of São Paulo.

Treatments	Average
Control	3.43 a [1]
ACB-AP3 (*Bacillus subtilis*)	3.14 ab
ACB-77 (*Bacillus subtilis*)	3.09 ab
Combination (ACBs AP3, 77, 72 and 69)	3.05 ab
ACB-72 (*Bacillus subtilis*)	2.94 ab
Biofertilizer [2]	2.48 b
ACB-69 (*Bacillus subtilis*)	2.42 b
Chemical control [3]	1.19 c

[1] Averages followed by the same letter did not show statistical difference (Duncan's test, 0.05). [2]Biofertilizer produced under anaerobic conditions (at a concentration of 10% v/v) (Kupper et al., 2006) [3] Copper oxychloride at 1g of metallic copper in 1 L of water and carbendazim+mancozeb containing 0.25g of a.i+1.6g of a.i./L of water, respectively, with the addition of mineral oil at 0.50% v/v. [4] Treatments were applied at 28 days of interval.

TABLE 3- Disease index of citrus black spot, caused by *Guignardia citricarpa*, on 'Natal' sweet orange sprayed with different treatments (*Bacillus subtilis* ACB-AP3, ACB-77, ACB-72, and ACB-69; *Trichoderma viride* ACB-14; *Trichoderma* sp. ACB-40; chemical control; and control) during 2002/2003 season, in two experiments (Experiments 2 and 3) at Santa Rita do Passa Quatro, State of São Paulo. For experiment 2 the treatments were applied every 28 days, and for experiment 3, every 15 days.

Treatment	Experiment 2	Experiment 3
ACB-77 (*Bacillus subtilis*)	2.19 a [1]	1.83 a
Control	2.12 ab	1.90 a
ACB–14 (*Trichoderma viride*)	1.94 abc	1.82 a
ACB–40 (*Trichoderma* sp.)	1.91 abc	1.65 a
ACB-72 (*Bacillus subtilis*)	1.78 bc	1.80 a
ACB-AP3 (*Bacillus subtilis*)	1.72 c	1.92 a
ACB-69 (*Bacillus subtilis*)	1.70 c	1.68 a
Chemical control [2]	1.08 d	0.29 b

[1]Averages followed by the same letter within a column did not show statistical difference (Duncan's test, 0.05). [2]Copper oxychloride at 1g of metallic copper in 1 L of water and carbendazim+mancozeb containing 0.25g of a.i+1.6g of a.i./L of water, respectively, with the addition of mineral oil at 0.5% v/v.

CONCLUSIONS

1-The good results in the laboratory conditions of antagonistic action of *Trichoderma* spp. is not always reflected in the control of CBS in field conditions;

2-Bacillus subtilis shows potential to control the disease, but the efficiency of disease control, under field conditions, proved to be unstable, requiring further studies with a view to selecting the most efficient isolates and the best period of application.

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