Mass production of *Bacillus subtilis* and *Trichoderma viride* for the control of *Phyllosticta citricarpa* (teleomorph: *Guignardia citricarpa)*

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RESUMO

Bettiol, W.; Kupper, K.C.; Goes, A. de; Moretto, C.; Correa, E.B. Produção massal de *Bacillus subtilis* e *Trichoderma viride* para o controle de *Phyllosticta citricarpa* (teleomorfo: *Guignardia citricarpa). **Summa Phytopathologica,*** v.31, p.276-278, 2005.

O objetivo foi estudar a produção de células e de metabólitos de *Bacillus subtilis* (ACB-69) e de conídios de *Trichoderma viride* (ACB-14) em diferentes substratos, pois apresentam potencial para o controle de *Phyllosticta citricarpa*. O meio constituído de farelo de algodão acrescido de proteína hidrolisada foi o que proporcionou maior produção de células de *B. subtilis* (2,4 x 10^9 células/mL), após três dias de incubação da cultura. Esse substrato líquido também propiciou condições para que a bactéria produzisse metabólitos termoestáveis e, em quantidades suficientes para inibir o crescimento micelial do fitopatógeno. A produção de *B. subtilis* pelo sistema de fermentação sólida foi melhor no substrato quirera de arroz sendo que o número de células da bactéria diminuiu à medida que aumentou a concentração do substrato. De um modo geral, o meio líquido foi superior ao sólido para a produção de *B. subtilis*. Com relação à produção de *T. viride*, verificou-se que os substratos testados apresentaram baixa produção de esporos, sendo que o melhor substrato testado (sabugo de milho + proteína hidrolisada) produziu apenas 2,2 x 10^6 conídios/mL.

Palavras-chave adicionais: Controle biológico, meios de cultura, antagonistas.

ABSTRACT

Bettiol, W.; Kupper, K.C.; Goes, A. de; Moretto, C.; Correa, E.B. Mass production of *Bacillus subtilis* and *Trichoderma viride* for the control of *Phyllosticta citricarpa* (teleomorph: *Guignardia citricarpa). **Summa Phytopathologica,*** v.31, p.276-278, 2005.

The work was aimed at studying the production of cells and metabolites of *Bacillus subtilis* (ACB-69) and of *Trichoderma viride* conidia (ACB-14) on different substrates, since they can potentially control *Phyllosticta citricarpa*. Our results showed that the medium consisting of cotton meal added of hydrolized protein provided the highest yield of *B. subtilis* cells (2.44 × 10^9 cells/mL), after the culture had been incubated for three days. This liquid substrate also provided conditions for the bacterium to produce thermostable metabolites, in sufficient amounts to inhibit the plant pathogen's micelial growth. The production of *B. subtilis* under the solid fermentation system performed better on the brewers rice substrate; the number of bacterial cells decreased as the substrate concentration increased. In general, the liquid medium yielded a higher amount of *B. subtilis* than the solid medium. With regard to the large scale production of *T. viride*, it was verified that the substrates tested had a low spore production; the best substrate among those tested (corn cob + hydrolized protein) only yielded 2.17 × 10^6 conidia/mL.

Additional keywords: Biological Control, citrus black spot, substrates, antagonistics.

Interest in the biological control of plant diseases has increased in recent years, since society has demanded foods that are free from agrochemical residues and are produced with respect for the environment. *Trichoderma* and *Bacillus subtilis* are among the most studied biocontrol agents. The efficacy of these antagonists has been demonstrated by several authors (1, 2, 4, 5, 7). There are several commercial products based on these antagonists; the product Kodiak® (made from *B. subtilis*) has been used to control citrus postbloom fruit drop (PBFD), caused by *Colletotrichum acutatum*, in 'Natal' orange orchards in Florida (7). Moretto (6) verified the potential of *B. subtilis* and *Trichoderma* in the control of *C. acutatum B. subtilis* and *Trichoderma* isolates also have been studied for the control of citrus black spot, caused by *Guignardia citricarpa*, with promising results (data not published). In these studies, the main difficulty was the mass production of the antagonists for field applications, which require large quantities of the product at low cost. The purpose of this work was to study the production

of *B. subtilis* (isolate ACB-69) and *Trichoderma viride* (ACB-14) inoculum, selected by Moretto (6), on low-cost substrates.

Culture media for *B. subtilis* multiplication

The medium consisting of residue of the glutamic fermentation of molasses (RGFM), a commercial product known as Aminofértil, was prepared by dilution in water at 5% + 2 g dextrose for each 100 mL. The media containing cotton meal, corn bran, soybean bran, and wheat bran were prepared by putting 60 g of each of these into 400 mL water, maintaining them under room conditions for 24 h and then straining. Two g of dextrose/100 mL were added to the broth. Later, 100 mL of the media were transferred into a 250 mL Erlenmeyer flask and autoclaved at 120°C for 20 min., at 1 atm. The media were inoculated with 2 mL of a bacterial suspension and maintained for 48 h under constant agitation in the dark. A completely randomized experimental design with four replicates was adopted, and Potato-Dextrose (PD) medium was considered a control. Cells were counted in a hemocytometer. The best media were those consisting of cotton meal and wheat bran, producing 1.4×10^8 cells/mL, being different from the PD medium, which produced 6×10^7 cells/mL, and from the media made from corn and soybean bran and RGFM, which produced 8.8; 6.7; and 7.1×10^7 cells/mL, respectively.

Determination of *B. subtilis* incubation period for metabolites production

The liquid media tested were made from rice bran, wheat bran, cotton meal, brewers rice, and potato-dextrose. In order to prepare the media, 200 g of each product were boiled in 1 L water for 20 min. After boiling, the broth was filtered, added of 20 g dextrose, and the volume completed to 1 L. One hundred milliliters of each medium were distributed into 250 mL Erlenmeyer flasks and then sterilized. *B. subtilis* colonies were transferred to each corresponding medium with a loop, and incubation occurred under laboratory room conditions for 120, 96, 72, 48, and 24 h, under constant agitation, in the dark. Each treatment consisted of five replicates. In order to evaluate the inhibition potential of *Phyllosticta citricarpa*, 10 mL samples of each broth were transferred into Erlenmeyer flasks with 250 mL capacity, containing 90 mL PDA, and then sterilized and poured into Petri dishes (20 mL/dish). One culture medium disc, 0.5 cm in diameter, containing a 10-day-old *P. citricarpa* colony, was transferred to the center of each plate. The control treatment consisted of Petri dishes containing PDA without the metabolites. The cultures were incubated under laboratory room conditions for eight days, and the diameter of the fungus colonies was determined. A completely randomized design was used, with five replicates. *B. subtilis* grown in liquid medium prepared with wheat bran or cotton meal released thermostable metabolites at concentrations high enough to inhibit the mycelial growth of *Phyllosticta*, regardless of incubation period (Table 1). These two culture media were more efficient than rice bran or brewers rice.

***B. subtilis* production in cotton-meal liquid substrate, added of carbon and nitrogen sources**

Treatments consisted of: 1) Cotton meal (CM) + sucrose, obtained from white granulated sugar; 2) CM + hydrolyzed protein; 3) CM +dextrose; 4) CM + sucrose, obtained from brown sugar; 5) CM + glucose; 6) CM + maltodextrin, and 7) CM + sugarcane molasses. In order to prepare each media, the CM was mixed into 1 L water and boiled for 20 min. After boiling, the broth was filtered and added of 20 g of the supplements corresponding to the treatments, and the volume completed to 1 L. One hundred milliliters of each medium were poured into 250 mL Erlenmeyer flasks and then sterilized in an autoclave for 20 min. at 120°C and 1 atm. After inoculation, the cultures were incubated for 72 h under constant agitation, in the dark. The experimental design was completely randomized, with four replicates (flasks). Cells were counted in a hemocytometer. The CM substrate supplemented with hydrolyzed protein favored the production of *B. subtilis* cells (2.4×10^9 cells/mL), although it was not statistically different from the treatment supplemented with glucose (1.9×10^9 cells/mL). Both media were different from the other treatments, which yielded 0.59; 0.71; 0.90; 0.91; and 1.03×10^9 cells/mL, for media containing CM added of dextrose, white granulated sugar, brown sugar, molasses, and maltodextrin, respectively.

Solid culture media for *B. subtilis* production

Quantities of 100, 150, and 200 g of cotton meal (CM) and wheat bran (WB), or brewers rice (BR) and citrus pulp (CP), were weighed in autoclavable polypropylene bags. Thirty-five, 50, and 70 mL of distilled water were added to the BR media, respectively; for CM and CP, 25, 35, and 50 mL were added, respectively, and for WB, 50, 75, and 100 mL, were added, respectively. These media were autoclaved at 120°C and 1 atm, for 1 h. Two substrate inoculation methods were used. In the first, two-day-old *B. subtilis* colonies were removed from cultures grown in PDA medium and transferred into Erlenmeyer flasks containing 100 mL potato-dextrose liquid medium, and incubated at laboratory room conditions for 48 h, under

Table 1. Mycelial growth inhibition of *Phyllosticta citricarpa* by *Bacillus subtilis* thermostable metabolites produced in several substrates and different incubation times.

Substrate	Incubation time (hour)				
	24	48	72	96	120
Rice bran	5.03 Aa[1,2]	3.03 Ba	2.80 Ba	2.85 Bab	2.59 Ba
Potato dextrose	1.64 BCc	1.93 ABCb	2.65 Aa	2.40 ABb	1.35 Cb
Cotton meal	1.14 Ac	0.96 Ac	0.91 Ab	1.10 Ac	1.40 Ab
Wheat bran	1.45 Ac	1.50 Abc	1.45 Ab	1.05 Ac	1.18 Ab
Brewers rice	2.90 Ab	2.96 Aa	3.41 Aa	3.40 Aa	2.93 Aa

[1]Mean diameter (cm) of the *P. citricarpa* colony; [2]Means followed by a common upper case letter in rows and lower case letter in columns are not statistically different (Tukey, P≥0.05).

constant agitation, in the dark. Next, 10 mL samples of the fermented broth were transferred into each polypropylene bag. In the second, the *B. subtilis* colonies developed in PDA for two days were suspended with 100 mL sterilized distilled water per plate. Samples containing 10 mL of the bacterial suspension were transferred into each polypropylene bag. The cultures were incubated for seven days at a laboratory environment, in the dark. The bags were agitated daily. Three replicates were adopted per treatment. After the incubation period, 1 g of each substrate was added to 10 mL sterilized distilled water and the number of bacterial cells was determined with a hemocytometer. The best substrate was BR at the three concentrations studied. The solid medium consisting of BR was statistically different from the CM, WB, and CP substrates. At the highest concentration, however, the media prepared from BR and WB did not show significant differences (Table 2). The medium concentrations showed statistical differences among themselves for the BR substrate, and the number of cells decreased as substrate concentration increased. There were no statistical differences between inoculation methods and substrate used (Table 2). The solid media were inferior in relation to liquid media for the production of *B. subtilis*, with yields of 1.4×10^7 cells in the solid medium and 2.4×10^9 cells/mL in the liquid medium. These results indicate that liquid media should be preferred, because, in addition to superior cell yields, they present advantages from an industrial point of view.

Media for *Trichoderma viride* production

The following culture media were studied: 1- Brewers Rice (BR) + Hydrolyzed Protein (HP); 2- BR + HP + Dextrose (DE); 3- BR + HP + Glucose (GLU); 4- Ground Corn Cob (CC) + HP; 5- CC + HP + DE; 6- CC + HP + GLU; 7- Wheat Bran (WB) + HP; 8- WB + HP + DE; 9- WB + HP + GLU. One hundred g of each substrate were weighed in polypropylene bags; 35 mL distilled water were added for the BR medium and 70 mL were added for WB and CC; 2 g of the supplements were also added. The media were autoclaved at 120°C and 1 atm, for 1 h. Ten-mL aliquots of a suspension containing *T. viride* conidia were transferred into each polypropylene bag. Incubation was carried out for 10 days in a laboratory environment, in the dark. The media were agitated daily. Three replicates were adopted per treatment. After incubation, the number of spores was counted in a hemocytometer. The best substrate was CC + HP (2.2×10^6 conidia/mL), which was not statistically different from the CC + HP + DE (1.4×10^6 conidia/mL) media and from the BR + HP + DE media (1.5×10^6 conidia/mL), but was different from the other treatments, which produced 1.3; 1.1; 1.0; 0.90; 0.88; and 0.85×10^6 conidia/mL in the BR + HP + GLU; BR + HP; CC + HP + GLU; WB + HP; WB + HP + DE; and WB + HP + GLU media, respectively. Spore yields in the CC, BR, and WB media were low (around 10^6 conidia/mL). This value is lower than those obtained with several substrates, such as rice grain, and even corn cob, which yield around 10^8 conidia/mL. Concentration is even lower than in products available in the Brazilian market, such as Trichovab®, produced by CEPLAC, which has up to 10^{10} cfu/mL (3).

Table 2. Effect of solid substrate concentrations and inoculation methodology on the production of *Bacillus subtilis* colony-forming units.

Substrates	Substrate concentration		
	100 g	150 g	200 g
Brewers rice	14.30 aA[1, 2]	9.63 aB	4.70 aC
Cotton meal	0.51 bA	0.36 bA	0.30 cA
Citrus pulp	0.35 bA	0.57 bA	0.40 bA
Wheat bran	3.15 bA	5.05 cA	3.54 abA
Inoculation methodology			
Bacterial cells + metabolites	4.01 aA	4.11 aA	2.95 aA
Bacterial cells only	5.15 aA	3.69 aA	1.52 aB

[1]Mean number of bacterial cells × 10^6, obtained with both bacterium inoculation methods in the substrates; [2]Means followed by a common upper case letter in the row, and lower case letter in the column, are not different by Tukey test at 5% probability.

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