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SHORT COMMUNICATION

Solid-state fermentation of *Bacillus thuringiensis tolworthi* to control fall armyworm in maize

Deise Maria Fontana Capalbo*
Embrapa Meio Ambiente
C.P. 69, CEP: 13820 000
Jaguariúna, São Paulo, Brasil
Tel: (19) 323333764
Fax: (19) 38678740
E-mail: deise@cnpma.embrapa.br

Fernando Hercule Valicente
Embrapa Milho e Sorgo
C.P. 151, CEP: 35701 970
Sete Lagoas, Minas Gerais, Brasil
Tel: (31) 7791000
Fax: (31)7791088
E-mail: valicenti@cnpms.embrapa.br

Iracema de Oliveira Moraes
Universidade Guarulhos
Praça Tereza Cristina, 01, CEP: 07023-070
Guarulhos, São Paulo, Brasil
Tel: (19) 32582001
Fax: (19) 32582001
E-mail: iomoraes@hotmail.com

Lúcia Helena Pelizer
Fundação de Amparo à Pesquisa do Estado de São Paulo Fellow
Tel: (16) 91211736
Fax: (16) 91211736
E-mail: lucia.pelizer@uol.com.br

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The well-known entomopathogenic bacterium *Bacillus thuringiensis* (Bt) produces a spore-crystal complex which is responsible for its biocide characteristic, and the bacterium can be obtained by fermentation, either in liquid or semi-solid substrates. This paper presents a successful way to achieve solid-state fermentation of active Bt var. *tolworthi* (Btt) against *Spodoptera frugiperda* (fall armyworm) in corn. More than 10^9 CFU/g were obtained using humidified rice as substrate maintained in polypropylene bags. This active complex (substrate plus spore-crystal of Bt) was prepared in order to obtain 2 x 10^6 spores/mL; the final suspension then sprayed via tractor on corn fields. On the treated plants, mortality of neonate larvae was 100% within two days of spraying, and all larvae were found dead on leaves. During one maize crop cycle, two applications were made, and up until 70 days after emergence it was not necessary to apply any other insecticide for fall armyworm control.

Despite a more than 10 - fold increase in insecticide use since 1940 (Lysansky and Coombs, 1994), crop losses due to insects have nearly doubled in the same period. This situation accelerates the movement towards better control methods among which microbial control is one of the most efficient. The most promising biological control agent to date is the bacterium *Bacillus thuringiensis* (Bt), the leading organism used in commercial microbial pesticides (Lambert and Pferoen, 1992; Meadows, 1993; Lysansky and Coombs, 1994). It has attracted the attention of both microbiologists and entomologists for many years because of its unique capacity to synthesize insecticidal protein crystals. This protein has allowed use of Bt as a natural biological control agent in agriculture and forestry for elimination of pests, and in human health for the elimination of disease vectors.

The microbial control of insect pests is of crucial importance to developing countries (Dulmage, 1993). The overuse or misuse of chemical pesticides and their negative impacts on soil and water quality, human health, wildlife and the ecological balance within agro-ecosystems are

*Corresponding author

increasingly becoming causes for concern, underlining the need for development of alternative pest control methods (Meadows, 1993). Although Bt has proved to be a highly successful weapon for fighting some agricultural pests and some vectors of diseases, its use is still limited in developing countries except in China, where it is widely produced and used (Dulmage, 1993).

Constraints to greater use of Bt in developing countries are: (1) scientific and technical: the difficulty in increasing effectiveness of products against specific pests and under specific agro-ecological conditions of individual countries; (2) micro and macro-economics: efforts to reduce costs of production lead developing countries to make Bt useful only for small scale application and this has limited its large-scale commercialization; (3) farmer acceptability: the longer period necessary to obtain high levels of mortality of pest larvae with Bt compared to chemical pesticides may be a problem from the point of view of the farmer, restricting the adoption of Bt.

There are two main advantages in promoting development of local production facilities for microbial insecticides in developing countries: (1) stability: locally produced microbial insecticides avoid lengthy shipping periods and long storage at variable temperatures before the product reaches the consumer; (2) formulations: local production provides material for appropriate field studies and for formulations suitable for local environmental conditions (Moraes et al. 1990; Moraes et al. 1994; Capalbo, 1995; Arruda, 1999; Moraes, 1999; Rizzatto, 1999).

The objective of this study was to produce Bt var. *tolworthi* (Btt), a strain active against *Spodoptera frugiperda* (fall armyworm), by a simple and effective process (solid-state fermentation) in order to obtain an active, low-cost and locally-produced biological control agent.

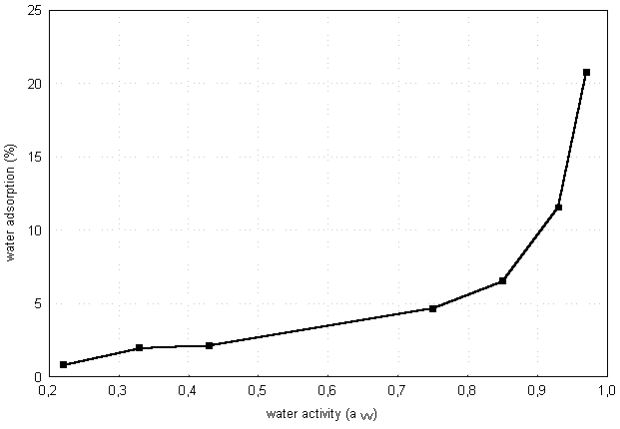

Figure 1. Adsorption isotherm of the rice used as solid-substrate for Btt sporulation.

Materials and Methods

Growth of bacterial strain for previous laboratory evaluation

Institute Pasteur, Paris, France, supplied Btt (strain T09). Btt was grown in nutrient broth supplemented with salts ($MgSO_4$, $FeSO_4$, $ZnSO_4$ and $MnSO_4$, final pH adjusted to 7,5) in a shaker at 30°C for 4 days, until a spore concentration of 10^9 CFU/mL (CFU stands for "colony-forming units", as described in Thompson and Stevenson, 1984). After centrifuging at 3,000 rpm in a bench top Tecnal centrifuge, followed by suspending the pellet with sterile water and centrifuging again at the same speed, a sample of the pellet was collected and the pellet was frozen. The spore content was determined in the sample, in order to have a relation weight x spore content, making it easier to be used in the field experiments. Whenever needed, part of the pellet was thawed, weighed and added to an aqueous solution of 0.1% (v/v) of Tween 80® (poly-oxy-ethylene sorbitan monooleate, Atlas Chemie), and each suspension was offered, on maize leaf discs, to two-day-old *S. frugiperda* larvae, maintained individually, in glass plates, in a chamber at 28ºC. Mortality was evaluated daily and standard laboratory procedures were used for lethal concentration (LC_{50}) and lethal time (LT_{50}) determinations, followed by statistic analysis by the Mstat computer model. With these values, it was possible to define the concentration of spores to be used in field experiments.

Solid-state fermentation

For field evaluations, a small amount of a Btt colony grown on a nutrient agar slant was inoculated into erlenmeyers with nutrient broth and incubated overnight (30ºC, 15 h, 150 rpm). This activated Btt was then inoculated into sterile polypropylene bags containing sterile moist rice: The desired moisture content was determined from an adsorption isotherm, the proportion of Btt inoculum and water was established in previous experiments (Pelizer, 1997). The bags were sealed and incubated at 30ºC for at least 4 days, until sporulation had occurred. The spore concentration in the final biomass (culture medium + microorganism) was determined as described by Thompson and Stevenson (1984) and then it was frozen for later use. This process, hereafter named Solid-state fermentation, was evaluated as mentioned in Fermentation parameters before using its product for field evaluation purposes. For field application it was thawed and the necessary weight of biomass was taken (determined as described in Growth of bacterial strain for previous laboratory evaluation).

Fermentation parameters

The substrate moisture content corresponding to a water activity (a_w) of 0.92 was previously established (Pelizer, 1997) based on the adsorption isotherm method of Rockland (1960). The moisture content of the medium was

determined in triplicate samples that were taken at different fermentation times and dried at 100°C until constant weight. Spores were counted (triplicate determinations) by the pour-plate counting technique after heat shock (Thompson and Stevenson, 1984) and expressed as Colony Forming Unit (CFU)/mL.

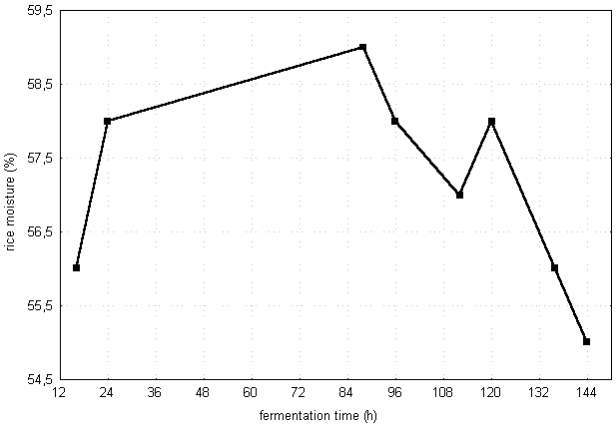

Figure 2. Substrate moisture variation during the solid-state fermentation process.

Field experiments

Spore suspension obtained by solid state fermentation was mixed with a dispersant and sprayed in a maize field with a tractor, at a rate of 300 L/ha. The nozzles used were 6504, as recommended by researchers from the Brazilian Agricultural Research Corporation (Embrapa) at the Embrapa Corn and Sorghum Research Center. About three hectares of fifteen-day-old maize crop showing symptoms of the presence of fall armyworm on the leaves (scratched leaves) was chosen. About 20-25 maize plants having fall armyworm eggs on the leaves, chosen at random in the area were marked with a red ribbon. A non-sprayed field was established to be used as "control". The tractor sprayed Btt at 4 pm. The crop was monitored for presence of *S. frugiperda* before the field application, and daily after the first Btt spray. If initial infestation level was reached, new Btt spraying should be applied following the same previously described procedures.

Results and Discussion

Adsorption isotherm

The adsorption isotherm obtained for the rice utilized as substrate in this study is presented in Figure 1. This relationship between the water adsorption and the water activity (a_w) allowed the establishment of the initial moisture content of the rice-culture-medium that corresponded to approximately 0.92 (considered a good a_w level for Btt and other bacteria).

Solid-state fermentation parameters

The parameters studied during the fermentation process, namely substrate moisture and Btt sporulation, are presented in Figure 2 and Figure 3 respectively.

Although there was a variation in moisture content of the medium during the fermentation process, it was not a restraining factor for Btt growth and sporulation in the experiments because the final moisture (55%) still represents a good a_w for Btt.

The level of spores attained in the solid-state fermentation is comparable to that obtained in other solid state fermentation processes with various different substrates and Bt subspecies (Moraes et al. 1990; Pelizer, 1997; Arruda, 1999).

Laboratory experiments

The LC_{50} was 0.37 mg of biomass pellet/mL of water. Lethal time (LT_{50}) was 2.8 days for 0.16 mg of pellet/mL. Mortality rose to 98% in 24 hours when 2 mg of pellet/mL was used.

With these results, a concentration of approximately 2 x 10^6 CFU/mL (final application volume in the field) was selected for the field experiments. Waquil et al. (1982) used Dipel M (Bt var. *kurstaki*) at the recommended dosage (0.72 kg/ha), in similar conditions of field experiments (experimental area of Embrapa Corn and Sorghum in Brazil, Brazilian variety of maize, tractor pulverization). With a concentration of 2 x 10^{11} CFU/g, as is usual nowadays with commercialized Dipel products in Brazil, and a 3% concentration of the active ingredient (spores of Bt) in the formulation, the concentration of spores used in our study is lower than that of Waquil (2 x 10^6 compared to approximately 1.4 x 10^8 CFU/g). In their study, Waquil et al. (1982) found no efficacy of Bt *kurstaki* against *S. frugiperda.*

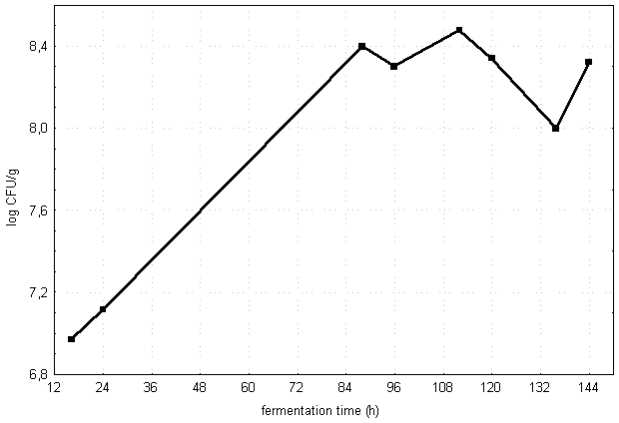

Figure 3. Spore-count during Btt growth by solid-substrate fermentation on rice.

Field experiments

At 24 hours after first Btt application, eggs had hatched and small larvae were feeding on the leaves of the marked plants. At the second evaluation, 48 hours after spraying, only dead, black larvae were found on the marked plants. The "control" field showed normal healthy larvae for the same observation dates.

Twenty days after the first spraying, the same level of initial larvae infestation was detected in the area, so Btt was sprayed again following the same procedures as stated before. This larvae appearance is not abnormal in field treated with Bt because sunlight and other environmental factors inactivate its spores and toxins.

Twenty four hours after the second application of Btt, mortality was 100% and no more Btt applications were necessary up to the 70^{th} day after emergence of the plants; two applications of Btt provided protection through the cycle of the crop.

Btt produced by the proposed solid-state fermentation generated spores active against *S. frugiperda* under laboratory and field conditions. The fermentation process proposed was easy and simple to run, and generated active product easy to apply with conventional machinery. So, this simple fermentation process, combined with usual application procedures, resulted in a good biological control product, indicating that the whole process could be used for local small-scale production and application.

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