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Case Studies of *Bacillus thuringiensis* Production and Biocontrol Applications

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17.1 Introduction

Despite a more than 10-fold increase in insecticide use since 1940 [1] 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 [1-3]. This fact 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 [4]. 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 increasingly becoming causes for concern,

underlining the need for development of alternative pest control methods [3]. 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 [4]. 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 [5-10].

The impact of biocontrol agents on beneficial organisms has been increasingly considered and evaluated worldwide because of the increased interest on conservation of biodiversity. Very little attention had been given in Brazil to this type of evaluation. Only recently a specific legislation for the registration of biopesticides and the pertinent generalized methodologies were proposed in Brazil [11].

17.2 Case Study I - *Bacillus thuringiensis kurstaki* application on *Podisus nigrispinus* Dallas (Heteroptera: Pentatomidae: Asopinae)

This study refers to an evaluation of the effect of a comercial formulation of *Bacillus thuringiensis* var. *kurstaki* (Btk) on the predator *Podisus nigrispinus* Dallas under laboratory conditions. Btk is a biopesticide widely used in Brasil for the control of lepidoptera pests in reforestation areas [12] and *P. nigrispinus* is common in such environments [13]. In addition, this predator has been mass produced in the laboratory for inundative releases in *Eucalyptus* plantations for the control of the lepidoptera *Thyrinteina arnobia* Stoll in parts of southeastern Brasil.

Tests were carried out under laboratory conditions to evaluate the effect of *B. thuringiensis* var. *kurstaki* on the predator *Podisus nigrispinus* Dallas through comparative analysis of fertility life tables. During two consecutives generations groups of 50 predators were fed larvae of *Bombyx mori* (L.) healthy or infected by Bt. Significant statistical differences were observed in both generations for all parameters under study except for the average generation time (T). Spores of Btk were not detected in the hemolymph of the predator. The results suggested that

the observed adverse effects of the Btk formulation were not related to a pathogenic effect of the product.

17.2.1 Materials and Methods

The tests were conduced in the laboratories of Embrapa Environment (CNPMA), at Jaguariúna, State of São Paulo, between October 1995 and July 1996, at 25? 2?C and 70? 10% R.H. (relative humidity) and 12 hours photophase. Predators used in the experiments, *P. nigrispinus,* were collected from a stock colony initiated with individuals from a mass rearing maintained by Industria Champion Papel e Celulose Ltda, from Mogi Guaçu State of São Paulo. Five couples of *P. nigrispinus* were hold individually in plastic cages (15 X 10 X 10 cm) for oviposition. The been of the cage was covered with a piece of filter paper on top of which these was a small cup with a piece of cotton seaked in distilled water. Twenty eggs were randomly taken from each cage to initiate live table studies. Eggs and the emmerged first stage nynphs were maintained together in Petri dishes (9.0 cm diameter) whose ban was covered with a piece of filter paper, on top of which these was a piece of cotton seaked in a 20% honey solution. All other stages, including adults, were with a maintained individually in plastic cups (10 cm diameter X 9 cm high) whose top was closed screen to allow air circulation; they were daily fed with a second on third instar larve of *B. mori* reared on cleam mulberry leaves (t1) or on mulberry leaves that had been submerged for 30 seconds in the Btk suspension (t2). In the latter case, caterpillars were allowed to feed for 16 min. time before they were offered to the predators. For both treatments, remaining prey were daily removed from the cups and new prey were added.

Eggs used in each treatment in the second generation were obtained from the corresponding treatments in the first generation. For each treatment, fertility life tables were constructed as suggested by Southwood [14], stations with 50 eggs for treatments. Calculated biological parameters were compared using t-test, after using the 'jackknife' method [15] to estimate variances. Daily oviposition rates were statiscally compared by F tests.

Microbiological observations of midgut of twenty adults of *P. nigrispinus* treated as described for t2 treatment, for 4 weeks, were externally cleaned (sodium hipoclorite 10%) for extration of the midgut. It was individually macerated, homogeneized (30 seconds) in sterilized water, diluted, unheated or heated (80^0C, 10 minutes) and inoculated into nutrient agar plates in order to obtain total and spore contents after an overnight incubation at 30^0C [16] hemolymph thirty adults of *P. nigrispinus,* alive, were fixed and had their legs removed.

The hemolinph drained from this intersection was diluted in sterilized water, homogeneized and inoculated into nutrient agar plates to obtain total and spore counts of Bt as described before. Part of the suspension was submitted to direct observation of characteristic Bt vegetative cells (at microscope) and spores or crystals with specific staining [17].

Feces were collected from the rearing flasks containing the insects treated for midgut examination. The material collected was diluted conveniently and inoculated into nutrient agar plates for Bt total and spore counts as described

above.

17.2.2 Results and discussion

In both generation, the intrinsic rate of increase (r_m), the liquid rate population increase (Ro) and finite rate of increase (?) were significantly lower (Table17.1), and period for population doubling was significantly higher for *P. nigrispinus* fed *B. mori* infected by Btk (Table17.2). The first three parameters were slightly lower and the latter parameters slightly big in the second generation. The duration of generation (T) was significantly longer for *P. nigrispinus* fed infected *B. mori* in the first but not in second generation. Total oviposition per female was slightly lower in the second generations for both treatments (Figure17.3, Figure17.4). In both generations total oviposition was significantly higher in the control treatment.

Also, in both generations oviposition started in the first week when *P. nigrispinus* was fed healthy *B. mori* and in the second week when it was fed infected *B. mori* (Figure17.1, Figure17.2). Peak oviposition rate was always reached a week after it started. When the predator was fed healthy *B. mori*, the oviposition period was longer. The oviposition period was also longer in the second generation, especially for predators fed healthy prey (Figure17.3, Figure17.4). Spores of Btk were not detected in the hemolymph of the predator.

The results of this study indicated an adverse effect of the biopesticide on the biology of *P. nigrispinus*, affecting negatively its development and reproduction. The absence of the pathogen in the hemolynph of the predator suggests that effect is not related to actual pathogenic effect of Btk on the predator. The actual cause for such effect could not be demonstrated by this experiment. It could be related to inert components of the formulation that may not affect the target organisms but may affect their non-target predators. Effect of inert components of a Btk formulation has been suggested [18] to explain the effect of that biopesticide on *Hippodamia convergens* Guérin-Méneville and *Chrysopa carnea* Stephens. Alternatively, the observed adverse effect could be related to conceivable toxin produced by Btk as observed [4] in louse.

The observed adverse effect could still be related to a quick deterioration of the food available to the predator because of eventual colonization of the moribund on dead prey by different microorganisms. This could conccilably occur despite of the daily replacement of the prey offered to the predators, and the fact that the prey usually were alive for about 2 hours after they were offered to the predators. To elucidate those different possibilities, future studies should include treatment corresponding to *B. mori* fed mulberry leaves treated with inactivated Btk.

The results of this study may not reflect the actual impact of the applications of Btk on the population of *P. nigrispinus* in the field. The work reported here correspond to the first phase of the evaluation of the impact of a biopesticide on a non-target organisms in accordance with protocols utilized for the purpose of registration of such products for commercial use [11,19]. In this phase, non-target organisms are exposed to high dosages of the biopesticides under conditions most favourable for deleterious effects to be shown. In case significant

Table 17.1 *The biological parameters of table of life associated of* P. nigrispinus *in two consecutive generations.*

Parameters	*Generation 1*		*Generation 2*	
	(t1)	(t2)	(t1)	(t2)
Ro	243.90	94.60	223.9	53.3
r_m	0.17	0.12	0.14	0.10
?	1.19	1.12	1.15	1.10
T	32.30	39.40	39.2	39.3
Td	4.00	5.00	6.0	7.0

Table 17.2 *Microbiological observations of midgut, hemolymph and faeces of* P. nigrispinus.

Observations	*Midgut (UFC)*	*Hemolymph*	*Faeces*
Spores	$0.8 \times 10^2 - 1.0 \times 10^4$	0	+
Total counts	$0.1 \times 10^4 - 2.0 \times 10^{5*}$	0	+
Microscope obervations	+	-	+

*Presence of others bacillus not identificable; + presence of Bt; - absence of Bt

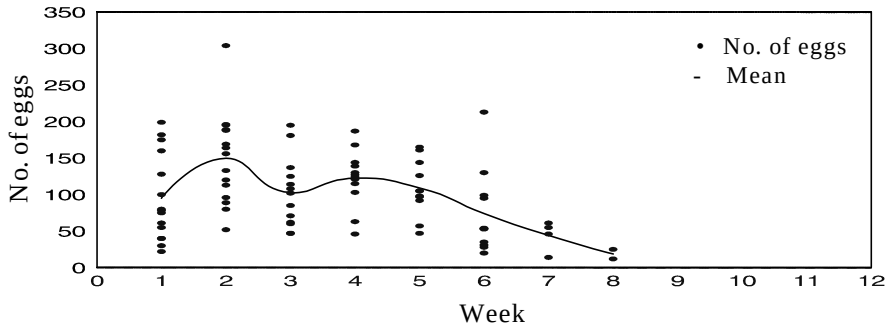

Figure 17.1 *Oviposition of* P. nigrispinus *fed with larve of* B. mori *healthy, generation I, 25?2°C, 70?10% RH.*

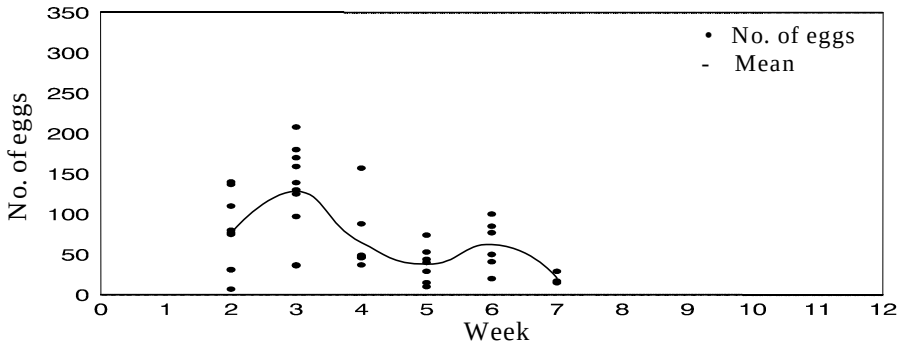

Figure 17.2 *Oviposition of* P. nigrispinus *fed with larvae of* B. mori *infected, generation I, 25?2?C, 70?10% RH.*

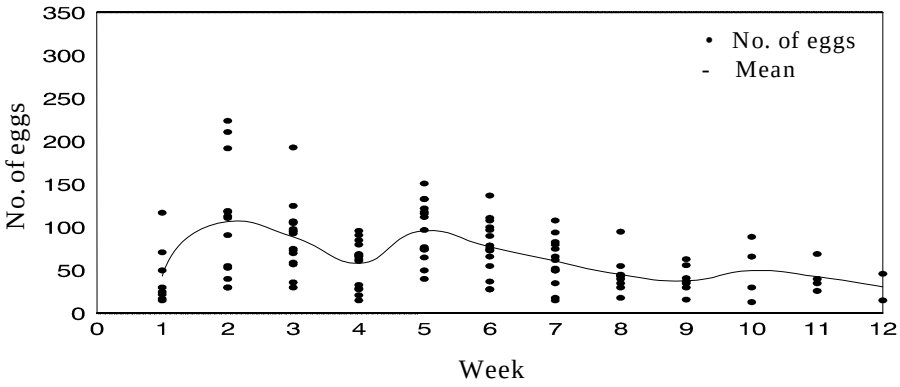

Figure 17.3 *Oviposition of* P. nigrispinus *fed with larvae of* B. mori *healthy generation II, 25?2?C, 70?10% RH*

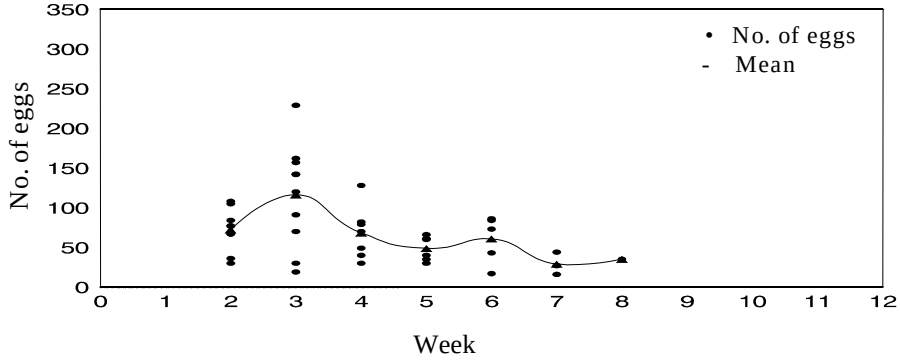

Figure 17.4 *Oviposition of* P. nigrispinus *fed with larve of* B. mori *infected, generation II, 25?2?C, 70?10% RH.*

effects are observed, the protocols more elaborated indicate the need for new, studies to be conducted, under conditions more similar to those prevailing in the field.

Under field conditions, *P. nigrispinus* would have available an array of alternative food items, which could allow it to escape the effects of a full dependence on prey infected by Btk, especially if it can distinguish between infected and non-infected prey. The results suggested that the observed adverse effects of the Btk formulation were not related to a pathogenic effect of the product.

17.3 Case Study 2 - *Bacillus thuringiensis tolworthi* production and application against *Spodoptera frugiperda*

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. 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.

17.3.1 Materials and Methods

17.3.1.1 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 [16]. 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 28°C. 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.

17.3.1.2 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 [20]. 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 [16] 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.

17.3.1.3 Fermentation parameters

The substrate moisture content corresponding to a water activity (a_w) of 0.92 was previously established [20] based on the adsorption isotherm method [21]. 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 [16] and expressed as Colony Forming Unit (CFU) / mL.

17.3.1.4 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 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.

17.3.2 Results and Discussion

17.3.2.1 Adsorption isotherm

The adsorption isotherm obtained for the rice utilized as substrate in this study is presented in Figure17.5. 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).

17.3.2.2 Solid-state fermentation parameters

The parameters studied during the fermentation process, namely substrate moisture and Btt sporulation, are presented in Figure17.6. 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 [5,8,20].

17.3.2.3 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 [22] 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).

With a concentration of 2 x 10^{11} CFU / g, as it 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 this case study was lower than that of Waquil [22] (2 x 10^6 compared to approximately 1.4 x 10^8 CFU / g). In their study, found no efficacy of Bt *kurstaki* against *S. frugiperda.*

17.3.2.4 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 70th 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

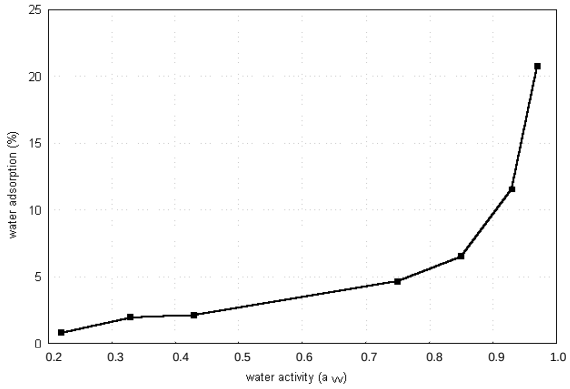

Figure 17.5 *Adsorption isotherm of the rice used as solid state substrate for Btt sporulation.*

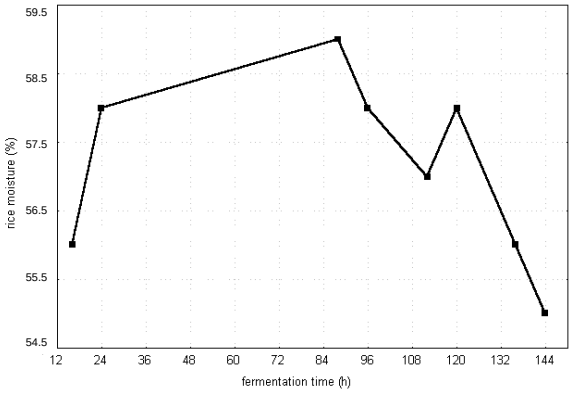

Figure 17.6 *Substrate moisture variation during the solid state fermentation process.*

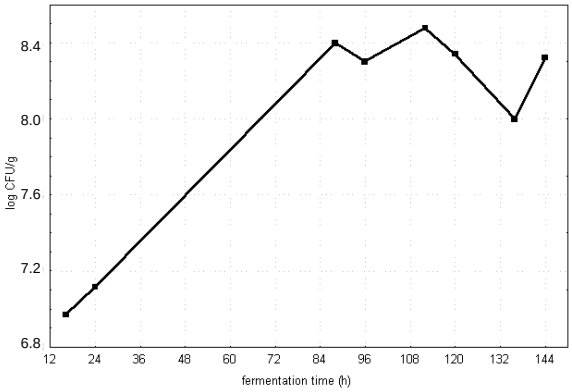

Figure 17.7 *Spore count during Btt growth by solid state fermentation on rice.*

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.

17.4 Conclusion

The results of the first case presented indicated an adverse effect of the biopesticide *Bacillus thuringiensis* on the biology of *P. nigrispinus*, affecting negatively its development and reproduction. It showed that under field conditions, *P. nigrispinus* would have available an array of alternative food items, which could allow it to escape the effects of a full dependence on prey, *Bombix mori*, infected by Btk, especially if it can distinguish between infected and non-infected prey.

The second case presented a successful way to achieve solid-state fermentation of active *Bacillus thuringiensis* 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) had 2 x 10^6 spores / mL and the final suspension was sprayed via tractor on corn fields. The objective of this study was acchieved, because it was produced 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.

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. Twenty four hours after the second application of Btt, mortality was 100% and no more Btt applications were necessary up to the 70th 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.

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