

Biological Control of Pink Bollworm *Pectinophora gossypiella* (Saunders) by *Bacillus cereus* MA7

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Abstract

The present study describes the insecticidal potentiality of spore-crystal complex and supernatant of *Bacillus cereus* strain isolated from dead or moribund pink bollworm. Three isolates of actinomycetes, two gram-negative and two spore-forming bacteria were isolated from the larvae and complete identification was performed for the most effective one. The toxicity of the two preparations was evaluated against 1st and 4th instars larvae of *P. gossypiella*. The LC_{50s} of *B. cereus* spore-crystals for the tested insect were; 88.5, 200µg/g, respectively. While, LC_{50s} of the supernatant were; 284.8 and 277.5µl/g, respectively. The retarding effects of the two preparations were slightly extended to the pupa and adult stages.

Keywords: Biological control; Pink bollworm; *Bacillus cereus*; Spore-crystal complex; Supernatant; Mortality

Introduction

The society is faced with the problem of increasing the use of pesticides to control pests in the absence of their predators or bio-agents. On the other hand, there is an ever-increasing need for food and especially for improved crop production in the developing countries. Therefore, some of the methods currently used to achieve higher yields, especially by pest and disease control, are environmentally undesirable. Also, manufacturing and application of conventional chemical pesticides has direct and indirect risks to man [1]. Besides, many insects have developed marked or complete resistance to many chemical insecticides [2,3]. During the last few years, biologists have turned their attention to the possibility of using other organisms as biological control agents, and the microbiologists contributing in the development of the efficacy of microbial substances (bacteria, fungi, virus and protozoa) for the control of many insect pests. Although a 100 or so bacteria cause diseases of insects [4], only few are used commercially as control agents. Some bacteria have been isolated from soil, insect habitats [5,6], insect larvae [7,8] or stored products [9]. In their work on the application of *Bacillus* species in control of *Meloidogyne javanica* (Treb) chitwood on Cowpea and Mash bean, Dawar et al. [10] reported that the application of *B. cereus* cell suspension reduced the hatching of the eggs and caused a mortality reaching more than 50%. When *B. cereus* strains; Ae10 and Cx5 were used for the bio-control of mosquitoes, Luxananil et al. [11] concluded that the results raised the possibility that *B. cereus* strains may be applicable as new mosquito larvicide host cells with long persistence. They also added that oral feeding to mice did not lead to abnormal symptoms, and negative results were obtained in a rabbit skin irritation assay.

Diverse population of aerobic endospore-forming bacteria occurs in agricultural fields and may directly or indirectly contribute to crop productivity. *B. thuringiensis* is very well-known as a bio-control agent against many insects especially its crystal protein [12,13]. Despite most species of *Bacillus* are harmless saprophytes, two species viz., *B. thuringiensis* and *B. cereus* are considered medically and environmentally important especially in field of controlling some plant insects [14]. Currently, pathogenicity of *B. cereus*, for example, mainly relies on the secretion of a wide array of toxins and enzymes [15]. Although *B. cereus* UW85 was identified for its ability to suppress alfalfa damping-off caused by *Phytophthora medicaginis* [16], it also

suppresses other diseases of cucumber, peanuts, tobacco and tomato which some of them are caused by insects [17].

The present study was initiated to determine the pathogenic bacteria associated with the pink bollworm *P. gossypiella* (Saunders) which is the most important insect pest of cotton in Egypt. This insect causes considerable damage to both quality and quantity of cotton staple in spite of the wide spread use of insecticides to control it.

Materials and Methods

Collecting larvae

Green cotton bolls were collected from fields at Fayoum Governorate, Egypt during September and October, 2009. The bolls were dissected and inspected thoroughly for infestation with *P. gossypiella* larvae. Active and healthy larvae were collected and reared at 27±2°C and 65±3% relative humidity (RH), on a formulated diet previously described by AbdEl-Hafez et al. [18]. Emerging moths were kept under the above mentioned laboratory conditions and provided with 10% sucrose solution. After mating, female moths were allowed to lay their eggs in glass chimney cages covered with muslin. The dead or moribund larvae were stored solely in sterilized tightly closed vials at 4°C in refrigerator until needed.

Bacterial isolation techniques

In order to reveal any microorganisms associated with the subjected insects, each of the refrigerated individuals was examined through 24-72 hrs from the time of storage under aseptic conditions. The larvae were surface sterilized by dipping in 2% sodium hypochlorite for 3-5 minutes, then passed through five separate washing with sterile distilled water [19]. For insuring the appropriate surface sterilization,

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checks were made by spreading the last washing solution on nutrient agar. Sterilized larvae were dried up between two sterilized filter papers (Whatman No1). After that, these larvae were transferred aseptically into a sterile mortar and macerated with a sterile pestle, then diluted and plated on nutrient agar for growth incubating them at 27°C for 24-48 hrs. Some dishes were incubated for extra-time in order to check the presence of slow growing microorganisms. Incubated plates were inspected daily to observe the bacterial colonies' growth that were then purified, used by following Koch's postulates and stored on slants of the desired artificial media at 4°C. The isolates were cultured periodically until they had been used in the subsequent experiments. Healthy larvae were subjected to the same procedures of isolation for obtaining the expected dormant pathogens.

Identification of the bacterial isolates

The isolates were taken and sub-cultured till full purification. Gram staining was used to differentiate between the isolates. For spore-forming bacteria, the diluted tubes were pasteurized at 63°C. All bacterial isolates were tested for their toxicity on larvae and the most effective one was subjected to further identification to the species level according to [20] Krieg and the use of API50-CHB (France Biomerica).

Preparation of bacterial cell-free supernatant

The bacterial species were grown on nutrient agar at 27°C. Spores and crystals were obtained by washing the old slant of each species [21, 22] then, inoculating a 100 ml of nutrient broth in a 250ml Erlenmeyer flask with each suspension. The inoculated broth was incubated in a shaking-incubator at 30°C and 200 rpm for 24 hrs, after which the entire culture was used as an inoculum for 1 liter of nutrient broth in a 2800 ml flask. The broth was incubated at 30°C and 200 rpm until the bacteria sporulated and lysed releasing crystals and spores into the medium.

Cells, spores and crystals were removed by centrifugation in a cooling centrifuge at approximately 20,000g for 30 min at 4°C. The supernatant which contained the exotoxin was then examined for its thermal stability and stored at 4°C until needed. The pelleted spores and crystals were collected and centrifuged again then freeze-dried [23].

Toxicity test and bioassays

The stock solutions of spore-crystals were prepared by suspending 15 mg of spore-crystal powder in 25 ml saline buffer containing 1% Tween 80. Concentrations of 50, 100, 200, 400 and 800 µg/g diet were prepared and tested for their toxicity to 1st & 4th instars larvae of pink bollworm *P. gossypiella* (Saunders). An aliquot of each suspension was added to a plastic cup containing 300g of formulated diet and mixed at high speed for 2 min with a malt mixer. A diet treated with each concentration was dispensed into fifty (30ml) another plastic cups. Another fifty cups containing the formulated diet to which buffered saline solution was added were used as control. The diet was stored at room temperature overnight to solidify, two pink bollworm neonate larvae were placed in each cup and the cups were incubated at 27°C and 65% RH. Larval mortality was recorded after 48 hrs of incubation. Mortality was calculated according to Abbott's formula [24]:

$$\text{Corrected mortality (\%)} = \frac{P - P_0}{100 - P_0} \times 100$$

Where P is the percent mortality of treated larvae, P₀ is the percent mortality of the untreated control.

The toxicity of the supernatant containing the thermostable exotoxin (TET) for the pink bollworm was tested by adding supernatant of bacterial species, heated at 121°C, to 300g of formulated diet to

obtain serial concentrations of supernatant; 50, 100, 200, 400 and 800 µl of the supernatant, respectively. The diet and supernatant were mixed thoroughly for 2 min with a malt mixer and dispensed into fifty (30ml) plastic cups. Pink bollworm neonate larvae were placed in each cup (2 larvae/cup) and incubated at 27°C and 65% RH. Larval mortality was recorded after 48 hrs of incubation. The test was replicated four times and the percentages of pupation and adult emergence were recorded.

Results and Discussion

Identification of the isolated entomogenous microorganisms

A total of 8 internal bacteria, isolated from naturally infested larvae of pink bollworm were; 3 actinomycetes, 2 gram-negative short rods bacteria and 3 spore-forming. The three spore-forming isolates related to the genus *Bacillus* and the most effective one, used in this study, was characterized as *Bacillus cereus* MA7. In the preliminary bioassays, the three bacterial isolates were assayed for pathogenesis against neonate larvae of *P. gossypiella*. Only one isolate of bacteria increase mortality among neonate larvae when all treatments were compared with the control using Abbott's formula. Mortality in these treatments ranged between 20 to 100% and the bacterial strain which was readily re-isolated from dead larvae was confirmed to be *B. cereus* MA7.

Toxicity of *B. cereus* MA7 spore-crystal complex

In the present study, the two insect larvae were markedly affected with *B. cereus* spore-crystal. The LC_{50s} were; 88.5 (1st instars larvae) and 200 (4th instars larvae) Table (2). The toxic action of *B. cereus* spore-crystal complex increased with the increase of concentration level to reach 100% for the 1st instars and 77.5 for the 4th instars at 800µg/g, respectively with a gradual increase for both instars. (Tables 1 and 2). Also, the relative susceptibility of the 4th instars larvae to the 1st instars of the pink bollworm reached 2.25 times only. These results are in agreement with the findings of Mohd-Salleh and Lewis [22] who reported the similar effect of spore-crystal complex of *B. thuringiensis* against corn insects.

Toxicity of *B. cereus* MA7 supernatant containing TET

Sterile supernatant of *B. cereus* showed toxic activity against the pink

Treated instars larvae	Concentration (µg/g)	% Larval mortality				
		Mortality of tested larvae	Total larval mortality	% pupation	Pupal mortality	% adult emergence
1 st instars larvae	50	37.0	49.8	50.2	6.7	45.5
	100	61.5	76.2	23.8	6.5	17.3
	200	85.0	91.5	8.5	3.0	5.5
	400	93.5	98.6	1.4	1.4	0.0
4 th instars larvae	50	22.6	29.5	70.5	5.3	65.2
	100	41.4	46.0	54.0	6.0	48.0
	200	50.0	53.8	46.5	6.5	39.7
	400	62.8	66.5	33.5	6.8	27.5
	800	77.5	80.4	19.6	4.6	15.0

N.B. Percentages of larval mortality were corrected using Abbot's formula [24]

Table 1: Effect of spore-crystal complex of *B. cereus* MA7 as tested against 1st and 4th instars larvae of the pink bollworm *P. gossypiella* (Saunders).

Treated instars larvae	LC ₅₀ µg/g of diet	Slope	95% confidence interval /µg/g	Relative susceptibility
1 st instars larvae	88.5	5.84 ± 0.347	75.5-98.6	2.25
4 th instars larvae	200	3.45 ± 0.38	189.4-213	1.30

Table 2: Potencies of spore-crystal complex of *B. cereus* MA7 as tested against neonate larvae of the pink bollworm *P. gossypiella* (Saunders).

Treated instars larvae	Concentration (µl/g)	% Larval mortality				
		Mortality of tested larvae	Total larval mortality	% pupation	Pupal mortality	% adult emergence
1 st instars larvae	50	82.5	100	0.0	0.0	0.0
	100	75.5	80.4	19.6	6.6	13.0
	200	63.8	68.6	31.4	5.5	26.0
	400	44.6	50.0	50.0	6.0	44.0
	800	31.7	36.5	63.5	4.4	59.5
4 th instars larvae	50	80.9	83.5	16.5	4.8	11.7
	100	66.5	70.0	30.0	4.6	25.4
	200	54.6	56.8	43.2	5.3	38.0
	400	40.4	42.4	57.6	3.6	54.0
	800	29.8	31.5	68.5	4.0	64.5

N.B. Percentages of larval mortality were corrected using Abbot's formula [24].

Table 3: Effect of different concentrations of *B. cereus* MA7 supernatant as against the neonate larvae of the pink bollworm *P. gossypiella* (Saunders).

Treated instars larvae	LC ₅₀ µl/g of diet	Slope	95% confidence interval /µg/g	Relative susceptibility
1 st instars larvae	284.8	4.92	275.6-292.4	1.050
4 th instars larvae	277.5	4.56	283.7-295.6	1.026

Table 4: Potencies of *B. cereus* MA7 supernatant as tested against neonate larvae of the pink bollworm *P. gossypiella* (Saunders).

bollworm (Table 3). The presence or absence of TET in this supernatant was confirmed in these tests where the mortality percentages of the tested insect were correlated negatively with the increase of concentration level of bacterial supernatant. The mortality percentage was higher at lower concentration; 82.5 for 1st instars and 80.9 for the 4th instars. While, the lower percentages were recorded at higher concentrations where it was 31.7 and 29.8 for 1st and 4th instars, respectively. This phenomenon has been substantiated with other lepidopterous insects and could be attributed to feeding deterrence associated with TETs [22]. *P. gossypiella* was found to have low sensitivity with regard to LC₅₀ where it showed 284.8 and 277.5 for the 1st and 4th instars, respectively (Table 4). It's worth mentioning that the spore-crystal complex was more effective than the supernatant containing TETs. The general phenomenon is in an agreement with the findings of Feng et al. [25] and Abou El-Ela [8]. The present investigation also showed that the supernatant of *B. cereus* containing TET killed more than 80% of first instar larvae for the tested insect and these results disagree with what was reported by Raun et al. [26], who showed that TET had only a limited effect on the tested corn borer insect.

The retarding effect

The retarding effect of spore-crystal complex or supernatant has been kept in the survivors from the treatments. Thus, the percentages of pupation and adult emergence were negatively correlated with the increase of spore-crystal concentration and positively with the increase in the concentration of the supernatant. The obtained results are in parallel with the findings of Azab [27].

Finally, it appears that *B. cereus* supernatant or spore-crystal complex may be used as a biological control agent for the tested insect although further study is still needed regarding the chemical identification of the metabolites as well as other host insects to be tested.

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