Microbial Pathogenesis 114 (2018) 139–146

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Biological control of the Pulse beetle, *Callosobruchus maculatus* in stored grains using the entomopathogenic bacteria, *Bacillus thuringiensis*

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ARTICLE INFO

Keywords:
Entomopathogen
Biological control
Pulse beetle
Essential commodity
Digestive enzymes

ABSTRACT

In the present study, we reported the biological control of stored product insect pest, *Callosobruchus maculatus* using the entomopathogenic bacteria, *Bacillus thuringiensis*. A significant delay in the larval, pupal and total development period of *C. maculatus* was observed after treatment with *B. thuringiensis* at 4×10^8 cells/mL. Furthermore, *B. thuringiensis* are highly effective in the control of *C. maculatus* and produced 100% mortality at 4×10^8 cells/mL. The LC_{50} value was estimated to be 3×10^7 cells/mL. In addition, a significant decrease in the activity of mid-gut α-amylase, cysteine protease, α & β-glucosidases, lipase, glutathione S-transferase (GST) and lactate dehydrogenase (LDH) was observed after treatment with *B. thuringiensis* at 4×10^8 cells/mL. This study concludes that *B. thuringiensis* are more effective against *C. maculatus* and could be used as a potential biological control agent in the management of stored product insect pests in the future.

1. Introduction

Coleopteran insects infest stored food products and cause post harvest losses of grains. *Callosobruchus maculatus* is the destructive pests of stored commodities worldwide that cause quantitative and qualitative damage to grains [1]. Quantitative damage is caused by insect feeding resulting in weight loss. Qualitative damage occurs due to the loss of nutritional and aesthetic value [2]. Therefore, it is essential to prevent such losses by controlling the pest on stored grains. The control of coleopteran pests is still depended on the application of chemical insecticide. In recent years, pest resistance and environmental concerns have forced us to search for alternative methods that are effective and eco-friendly [3]. Biological control methods including microbial pesticides is a kind of safety ecological measures and are included in integrated pest management (IPM) programmes. Biopesticides or biological pesticides based on entomopathogenic microorganisms provides an ecologically sound and effective strategies to pest problems. The commonly used biopesticides are *B. thuringiensis*, *Trichoderma* and *Phytopthora*. The potential benefits of biopesticides to agriculture are considerable. The advantages of biopesticides are: (i) inherently less harmful to environment, (ii) affect only the target organisms, (iii) very effective in minute quantities and decompose quickly and (iv) it can contribute significantly in the pest management programs, and minimize the use of chemical pesticides to a greater extent.

B. thuringiensis is a crystalliferous spore forming aerobic, gram-positive bacteria of the family *Bacillaece* [4]. It produces parasporal crystals containing *Cry* proteins that may be toxic for different insect pests damaging agricultural plants and products. The *Cry* proteins are encoded by *Cry* genes and approximately 300 *Cry* genes have been identified based on the similarity in amino acid sequence [5]. Because crystal proteins are highly specific and environmentally safe [6], they have been successful as bioinsecticides against the larvae of Lepidoptera, Diptera and Coleoptera [7,8]. *B. thuringiensis* (Bt) preparations were directly sprayed on plants for several years to protect them against insect pests of different orders. In 1981, transgenic plants were developed by successfully transferring the genes encoding the pesticide proteins. Transgenic plants containing Bt gene such as maize and potato were raised for providing protection against pests without the need for spraying [9]. Very little information is available on the use of microbial pesticides in the insect pest control of stored grains, especially *C. maculatus*. Therefore, the present study was aimed to find and recommend possibly the effective control measures against *C. maculatus* in the stored grains. In this study, the insecticidal properties of *B. thuringiensis* against the cowpea weevil, *C. maculatus* was tested. Further, the inhibitory effects of *B. thuringiensis* on the mid-gut digestive enzymes of *C. maculatus* were determined.

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https://doi.org/10.1016/j.micpath.2017.11.046
Received 11 September 2017; Received in revised form 24 November 2017; Accepted 24 November 2017

2. Materials and methods

2.1. Bacterial culture conditions

Gram positive *B. thuringiensis* P1 (KC465903) was procured from Microbial type culture collection (MTCC), Chandigarh. Colonies were inoculated in nutrient broth containing penicillin G and placed in an orbital shaking incubator (REMI CIS-24 Plus) set at 28 °C and 180 rpm for about 48 h. The colonies that grew were observed under a phase contrast microscope (1000× magnification), to determine the presence of parasporal body morphology. The spore count of *B. thuringiensis* was made by Neubauer's haemocytometer as per the procedure described by Gahukar [10].

2.1.1. Characterization of crystal protein

The purification of crystal proteins was performed following the method by Bel et al. [11]. *B. thuringiensis* were cultured and allowed to sporulate on nutrient agar plates. Then, two loops of the colony were removed from the plate and transferred to a sterile eppendorf tubes containing 1 mL of ice-cold sodium hydroxide. They were centrifuged for 5 min at 12000*g* and the supernatants were discarded. The pellets were resuspended in 150 μL of SDS (1%) and β-mercaptoethanol (0.01%), and boiled for 10 min to dissolve the crystals. The samples were then centrifuged at 12000*g* for 10 min and the supernatants were removed. For protein precipitation, 150 μL of 20% TCA (thricholoroaceticacid) was added onto the protein pellet and incubated for 10 min on ice. Thereafter, the resuspended pellets were centrifuged at 10000*g* for 15 min and the supernatants were removed. An equal volume of ice-cold acetone was added and centrifuged for 5 min at 12000*g*. Finally, the supernatants were removed and the pellets were resuspended in SDS-PAGE loading buffer (0.15 M Tris/Cl pH 8.8, 3.75 mM EDTA, 0.75 M sucrose, 0.075% bromophenol blue, 2.5% SDS, and 7.4 mM dithiothreitol) in equal volumes. The samples were then boiled for 10 min. Electrophoresis was carried out in 10% polyacrylamide gel, at 150 V. After electrophoresis, the gel was stained using coomassie brilliant blue R250 (1.25 g of dye in 450 mL of methanol: H_2O (1:1 v/v) and 50 mL of glacial acetic acid). Finally, the gel was destained in methanol/acetic acid solution (90 mL of methanol: H_2O (1:1 v/v) and 10 mL of acetic acid) kept on an orbital shaker for 4 h. After destaining, the gel was dried in gelatin.

2.1.2. Scanning electron microscopy

Briefly, thin film of *B. thuringiensis* crystal protein was prepared on the carbon coated copper grid and the film on SEM grid was then allowed to dry under a mercury lamp for 5min and observed under a scanning electron microscope (Hitachi S-4500, Krefeld, Germany).

2.2. Insecticidal activity of B. thuringiensis

2.2.1. Culture maintenance of C. maculatus

Adult *C. maculatus* was obtained from the Department of Entomology, Agricultural College and Research Institute, Madurai, Tamil Nadu. The culture was reared on healthy, sterilized seeds of green gram, *Vigna radiata* and maintained at 30 ± 1 °C, 60 ± 5% relative humidity (RH), 2 h light: 12 h dark period for three generations before experimentation to ensure that they were genetically and phenotypically alike. The beetles were grown under moderately crowded conditions to ensure proper development with equal size of the resultant adults and used as a source for bioassay [1].

2.2.2. Insect bioassay

The efficacy of *B. thuringiensis* against the fourth instar larvae of *C. maculatus* was tested at five different spore concentrations (3×10^5, 3×10^8, 3×10^9, 4×10^5 and 4×10^8 cells/mL). Briefly, bean-shaped artificial diets for feeding tests were formulated from cowpea seeds as per the composition shown in Table 1. The mixture was autoclaved for 15 min at 121 °C and 15 psi pressure, allowed to cool and approximately 1 g cakes were casted in pet jars. Then, the artificial diets were treated with different spore concentrations of *B. thuringiensis*, air dried and kept in a plastic jar. Sterilized untreated seeds were used as control. Ten newly emerged males and females (1:1) of *C. maculatus* were introduced in pairs into the plastic jar and covered with muslin cloth. Introduced pairs were allowed to oviposit and develop on the treated artificial seeds. Five replicates were performed for each concentration of *B. thuringiensis*. Bioassay was maintained at a controlled conditions of 30 ± 1 °C and 60 ± 5% RH. The grub (larval) period, pupal period, total development period and mortality of *C. maculatus* were determined for each concentration of *B. thuringiensis* as described by Thiravia Raj and Malaikozhundan [12].

Table 1
Composition of artificial diet for insect bioassay.

Ingredient	Quantity (g)
Cowpea flour	80
Sorbic acid	1.5
Sucrose	40
Casein	40
Yeast	20
Ascorbic acid	4
Vitamin	1
Antimold solution agar	24
Distilled water	1000 mL

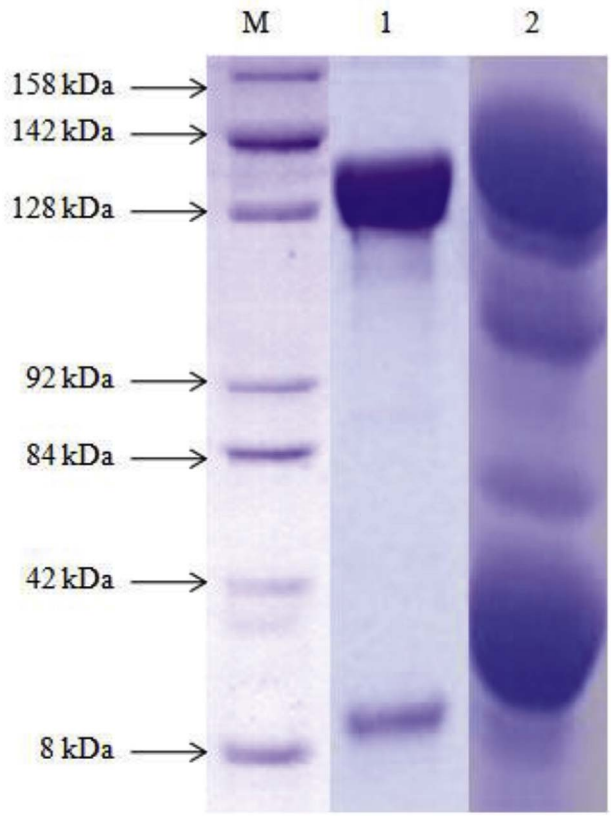

Fig. 1. SDS-PAGE of spore-crystal protein from *B. thuringiensis*. Lane M: Molecular marker; Lane 1: crystal protein from *B. thuringiensis*; Lane 2: crystal protein from reference strain (*B. thuringiensis var kurstaki*).

2.2.3. Development of C. maculatus

The duration of larval and pupal stages was recorded from the time of egg hatching until the emergence of adults. The total developmental period of *C. maculatus* was determined by counting the days between