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Research Article

BIOASSAY OF EFFICACY OF FIVE ISOLATES OF *BACILLUS THURINGIENSIS* SSP. *KURSTAKI* AGAINST LARVAE OF THE SPOTTED BOLLWORM (*ERIAS VITTELLA*) ON OKRA [*ABELMOSCHUS ESCULENTUS* L. (MOENCH)]

Mona E. Elyass [1,3], Ahmed A. Mahdi [2,3]* and Idress H. Atttalla [3]
[1]Commission for Biotechnology and Genetic Engineering, National Center for Research, Khartoum, Sudan
[2]Department of Agric. Biotechnology, Fac. of Agriculture, Univ. Khartoum, Sudan
[3]Department of Microbiology, Fac. Science, Omar Al-Mukhtar University, Beida, Libya
*Corresponding Author Email: monadaliaya@gmail.com; idress174@gmail.com

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ABSTRACT

In a bioassay of the toxicity of four local isolates and a reference (HD-1) strain of *Bacillus thuringiensis* subsp. *kurstaki* (Bt *kurstaki*) to larvae of the spotted bollworm (*Erias vittella*), different concentrations of spore-crystal mixture were offered to third-instar larvae of the spotted bollworm (*Erias vitella*) utilizing okra seeds as diet. The number of dead larvae and the time of death were recorded, and corrected mortality was computed using Sun-Shepard formula. Most isolates exhibited LT_{50} values far lower than the reference strain after 24 and 48 h of incubation. One such isolate (BO5) required just 22.54 h to kill 50% of *E. vittella* larvae when used at $2.4x10^9$ spore-crystals/mL. Similarly, most isolates exhibited LC_{50} values lower than the reference strain at 24 and 48 h. However, despite the unsatisfactory performance by the reference strain at 24 and 48 h, it showed much improved performance at 72h, where only one isolate (K21) showed relative potency (RP) value higher than the reference strain, while another (KO9) was almost as potent as the reference strain. It is concluded that continued search for and evaluation of Bt isolates obtained from different environments could uncover great capabilities in these isolates.

Keywords: *Bacillus thuringiensis kurstaki*, *Erias vittella*, biopesticide, okra.

INTRODUCTION

In the Sudan, the spotted bollworm (*Earias vittella* Fab.) (Lepidoptera: Noctuidae) attacks okra [*Abelmoschus esculentus* L. (Moench)] as the major host, followed by cotton, and very rarely *Abutilon* weeds. The first report on the incidence of this species in East Africa was published in 1986[1]. An incidence of >85% infestation was recorded by *E. vittella* on autumn okra in Khartoum area[2,3], reflecting how deleterious effect this pest imposed on the production of this vital and staple vegetable crop[4]. Larvae of the spotted bollworm bore into terminal shoots of young plants, flower buds and young bolls, leading to death of shoots and shedding of flower buds and bolls[5]. *E. vittella* infestation on okra starts as soon as the fruits set and attains a maximum (69.91%) 3-4 weeks later[6].

Okra is one of the most popular vegetable crops in the Sudan, and is thought to be native to an area extending from Ethiopia to the Sudan[7]. Like other vegetables, okra is grown mostly as intensive crops with considerable input of fertilizers, irrigation water, insecticides, etc, and is often cultivated continuously in a limited area with narrow crop rotations which provides optimum conditions for development of high pest populations. Because of the high susceptibility of okra to insects, the high profitability and lack of knowledge, farmers rely heavily on insecticides. In crops, such as okra, the short interval between spraying and picking of fruits poses serious residue hazards to the consumers, as well as serious resistance problems. In order to support sustainable vegetable production, it is important to develop alternative methods of pest control. Biological control represents a desirable technique for controlling insects, due to its minimal environmental impact and its avoidance of problems of resistance. Among biocontrol agents, *Bacillus thuringiensis* Berliner (Bt) plays a very important role. This bacterium is currently being used worldwide, mainly for the management of lepidopterous, coleopterous and dipterous pests[8]. More than 360 products manufactured from *B. thuringiensis* spores and toxins or toxins alone are registered for use in the United States alone[9], and today several crop plants have been genetically modified by incorporation of *B. thuringiensis* toxin genes into their genomes as a means of pest control[10,11,12].

Bacillus thuringiensis is a gram-positive spore-forming bacterium acclaimed as the most widely used biopesticide. The application of this entomopathogen is estimated at 13,000 tons of commercial formulated in the world, mainly applied against Lepidoptera[13]. During sporulation, Bt synthesises two types of proteinaceous toxins (δ-endotoxin): Crystal (Cry) and Cytolytic (Cyt) parasporal toxins[14]. Cry toxin is toxic to a wide range of insect orders, and some of the Cry toxins have an insecticidal spectrum covering two or three orders[15,16]. This toxin is usually exhibited as a parasporal crystal[17]. The Cyt toxin, on the other hand, is toxic to dipteran larva *in vivo*, but exhibits a broad range of cytotoxicity against dipteran and mammalian cells *in vitro*[18]. Cyt toxin is also exhibited as a parasporal inclusion (Crystal) protein. Certain Bt strains also produce non-insecticidal parasporin toxins with strong cytocidal activity against human cancer cells[19,20].

The δ-endotoxin in these spore crystals is solubilized in the midgut of susceptible insect larvae after ingestion of the crystals. The δ-endotoxins are protoxins which are then

proteolytically cleaved by midgut proteases[21] into active toxic peptides that bind to certain receptors in the midgut cells leading to the formation of pores that lead to osmotic lysis of the cells lining the midgut[22,23] usually causing death within 2 days[24].

Various criteria have been used to classify Bt subspecies, including at least 71 H serotypes by their flagellar immunological reactions[25,26] and more than 300 holotypes based on the homology of amino acid sequence of the Bt toxins which have been categorized into 73 *cry* and 3 *cyt* families[17,27]. There is a good correlation between these subclasses and insect host range. However, Bt strains can synthesize more than one toxin, resulting in complex and overlapping host profiles[28]. Most Bt *kurstaki* strains are specific for lepidopteran insects (butterflies and moths), whereas other strains specialize in other insects. The present study aimed at conducting comparative bioassay for toxicity of four *Bt kurstaki* isolates obtained from different habitats in Sudan, and a reference *Bt kurstaki* strain against larvae of the spotted bollworm (*E. vittella*).

MATERIALS AND METHODS

Bacterial strains

The *Bacillus thuringiensis* strains used in this study (Table 1) were isolated from various habitats in different locations in Sudan[29] and were characterized through biochemical, serological and entomopathogenic methods. The isolates were continually refreshed by sub-culturing in Nutrient Agar. A reference strain (*Bacillus thuringiensis* ssp. *kurstaki*, strain HD-1, serotype H3a 3b) isolated from the plant protection product Dipel ES (obtained from Bonide Products Inc., Yorkville, N.Y.) was also included in the study.

Preparation of the spore-crystal mixtures

Each isolate and the reference strain were grown in 100 ml of Luria-Bertani (LB) broth medium in Erlenmeyer flasks of 250 mL capacity, the flasks were continuously shaken at 200 rpm using a Junior orbital shaker (Lab-line instrument inc., Melrase Park, 111, USA.) at room temperature for eight days. Cell autolysis was checked microscopically. Cultures were centrifuged at 4000 rpm in a Labofuge 1 bench –top centrifuge (Herraeus-Christ GMBH, Osterol, Germany) after which the pellets were suspended in sterile water and centrifuged again at the same speed. The final pellet was then suspended in 50 mL of sterile distilled water to give a thick suspension, the spore-crystal count in which was determined by a haemocytometer. The spore-crystal suspensions were adjusted to give $1.3x10^7$, $4.5x10^8$ and $2.4x10^9$ spore-crystals/mL.

Bioassays for insect toxicity

The above three concentrations of spore-crystals were offered to spotted bollworm larvae in mashed okra [*Abelmoschus esculentus* (L) Moench] seeds. For each Bt isolate, four sets of petri-dishes fitted with Whatman No. 1 filter papers were used for assay of larval mortality. Three sets served for the three spore-crystal concentrations while the fourth served as control. Each set was replicated three times. Okra seeds were mashed, thoroughly mixed with 4.0 mL of each of the four concentrations of the spore-crystal suspensions of each isolate and the reference strain, and were put in petri-dishes. Controls received 4.0 mL distilled water. Batches of ten third-instar larvae of spotted bollworm were added to the dishes and were incubated at room temperature for 72 h.

The dishes were observed regularly, and the number of dead larvae was recorded after 24, 48 and 72 h. The corrected percentages of larval mortality were computed according to the Sun-Shepard formula for estimating pesticide efficiency[30] in which % larval death was calculated as =[(Pt ± Pck) ÷ (100 ± Pck)]× 100

Where:
Pt = [(Tb – Ta) ÷ Tb] × 100
Pck = [(Ca – Cb) ÷ Cb] × 100 where:
Tb = the number of larvae in the treated dishes before the start of the treatment,
Ta = the number of larvae remaining in the treated dishes after 24, 48 or 72 hours after the start of the treatment,
Cb = the number of larvae in the control dishes before the start of the treatment, and
Ca = the number of larvae remaining in the control dishes after 24, 48 or 72 hours after the start of treatment.

The lethal concentration that causes death of 50% of the treated larvae (LC_{50}) and the time required for death of 50% of the larvae (LT_{50}) were calculated from linear regression equations of the dose-response relationship curves. The relative toxicity of each strain was expressed as the potency ratio (RP) which was calculated by dividing the LD_{50} of the reference strain by that of the test strain[31]. RP values were calculated at 72 h only because of the wide confidence intervals obtained at 24 and 48 h.

RESULTS AND DISCUSSION

On application of $2.4x10^9$ spore-crystals/mL, isolate BO5 achieved high rates of larval mortality than all other test isolates, including the reference strain, at 24 and 48 h. However, by 72 h of incubation, it was overtaken by both the reference strain and isolate K21, and was level with isolate KO9. Isolate SO6 was the least effective of all five Bt isolates (Figure 1).

When the spore-crystal concentration was lowered to 4.5×10^8, isolate BO5 was again the better strain at 24 and 48 h, but was on the same level with isolates KO9, K21 and the reference strain at 72h from start of incubation. It is to be noted here that although isolate SO6 was better than the other three isolates at 24 h, it was again the least effective at 72 h. Moreover, although the reference strain exhibited low mortality rates at 24 and 48 h, it picked up to achieve 87.3% mortality by 72 h (Figure 2).

On lowering the spore-crystal concentration to $1.3x10^7$/mL, again a better mortality performance was shown by isolate BO5 at 24 and 48 h, but was overtaken by isolates KO9, K21 and the reference strain at 72 h. Interestingly, although isolate K21 did not achieve any mortality at 24 h, it was the better strain at 72 h, with 85.7% mortality. As in the other two concentrations, isolate SO6 was the least effective strain (Figure 3).

The times required to kill 50% of the spotted worm larvae (LT_{50}) using the three spore-crystal concentrations were calculated from linear trend lines fitted to the response curves for each concentration. On application of 1.3×10^7 spore-crystals/mL, isolate BO5 required 40.60 h to kill 50% of the larvae as compared to 54.62 h by the reference strain, and as much as 96.11 h by isolate SO6. However, when the spore-crystal concentration was raised to 4.5×10^8, the LT_{50} was reduced to 26.76, 44.51 and 55.37 h for isolate BO5, the reference strain and isolate SO6, respectively. A further increase in the spore-crystal concentration to 2.4×10^9 resulted in LT_{50} values of 22.54, 39.16 and 43.52 h for isolate BO5, the reference strain and isolate SO6, respectively (Table 2).

Table 1: Habitats and locations where *Bacillus thuringiensis* isolates were obtained

Isolate code	Habitat of isolation	Location
B05	Stagnant rain water	Elobeid, western Sudan
S06	Dry clay soil	Kenana sugar cane farm
K09	Dry clay soil	Kosti, central Sudan
K21	Moist mud	Kosti

Table 2: The time (h) required for death of 50% of *E. vittella* larvae (LT_{50}) by 5 Bt *kurstaki* isolates

Bt *kurstaki* Isolate	Spore-crystal mixture/ mL		
	$1.3x10^7$	$4.5X10^8$	$2.4x10^9$
BO5	40.60	26.76	22.54
SO6	96.11	55.37	43.52
KO9	52.21	44.85	39.56
K21	50.28	44.17	37.78
Reference strain	54.62	49.51	39.16

Table 3: LC50 (Spore-crystal mixture/mL) and potency ratios (bracketed) of the test strains

Bt *kurstaki* Isolate	24h	48h	72h	RP ratio (72 h)
BO5	$1.92x10^6$	$1.37x10^5$	$1.37x10^5$	0.53
SO6	$3.28x10^{14}$	$2.19x10^9$	$6.25x10^6$	0.01
KO9	$9.60x10^{25}$	$4.31x10^7$	$7.50x10^4$	0.96
K21	$9.57x10^7$	$9.57x10^7$	$3.83x10^4$	1.89
Reference strain	$1.18x10^{18}$	$4.15x10^{10}$	$7.23x10^4$	

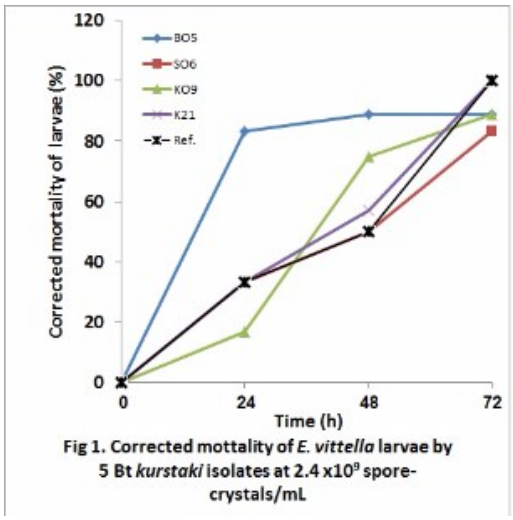

Fig 1. Corrected mottality of *E. vittella* larvae by 5 Bt *kurstaki* isolates at 2.4 $x10^9$ spore-crystals/mL

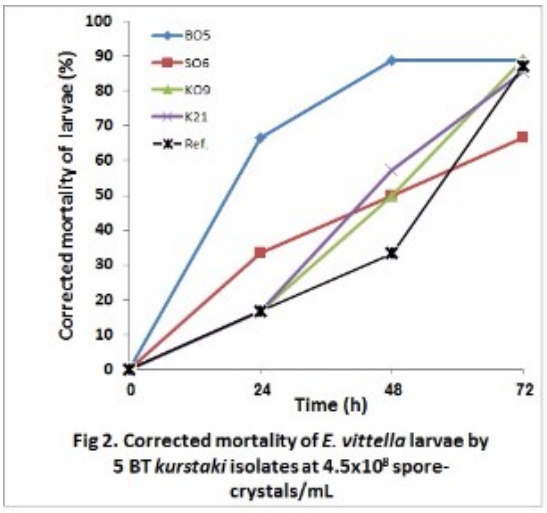

Fig 2. Corrected mortality of *E. vittella* larvae by 5 BT *kurstaki* isolates at $4.5x10^8$ spore-crystals/mL

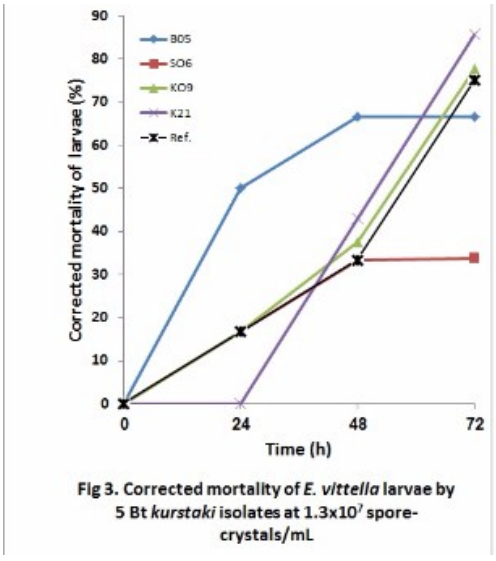

Fig 3. Corrected mortality of *E. vittella* larvae by 5 Bt *kurstaki* isolates at $1.3x10^7$ spore-crystals/mL

Table 3 shows the spore-crystal concentrations required to kill 50% of *E. vittella* larvae (LT_{50}) after 24, 48 and 72 h of incubation. As expected, isolate BO5 was by far the better strain at both 24 and 48 h. However, at 72 h it required $1.37x10^5$ spore-crystals/mL as compared to $3.83x10^4$, $7.23x10^4$ and $7.50x10^4$ spore-crystals/mL for isolate K21, the reference strain and isolate KO9, respectively. As usual, isolate SO6 lagged, with an LC_{50} of $6.25x10^6$ spore-crystals/mL.

It has been shown that some Bt *kurstaki* strains were more effective than the commercially developed HD-1 strain against many lepidopteran larvae[32]. The spectrum of activity of a particular strain against different insect species will vary such that a strain particularly potent against one insect species is not necessarily equally potent against another. The differences are more likely associated with biological and ecological differences in the areas where Bt strains are isolated[33]. For that reason each strain has to be evaluated against each insect species. The differences in potency have been attributed to quantitative and qualitative differences in the Delta-endotoxins produced by different strains. Great differences in quantities of spore-crystals and mortalities produced by Bt *kurstaki* isolates obtained from this same place have previously been recorded[34].

In the present study, the reference strain exhibited comparatively low potency at 24 and 48 h when compared to the four test strains. However, it succeeded in achieving larval mortalities of 75%, 87.3% and 100% at the spore-crystal concentrations of $1.3x10^7$, $4.5x10^8$ and $2.4x10^9$, respectively by 72 h. Although very wide RP ratios were obtained when comparing the LD_{50} values of the four test strains to those of the reference strain at 24 and 48 h (indicating low potency of the reference strain), these values were rejected due to the wide confidence intervals. The RP values obtained at 72h were more realistic, showing relative potencies of 1.89, 0.96, 0.53 and 0.01 for the isolates K21, K09, BO5 and SO6, respectively. Interestingly, although isolate BO5 was consistently the best of all five strains at 24 and 48 h, its final record, as judged by the RP ratio at 72 h, was not particularly impressive.

CONCLUSION

The present results have demonstrated that biocontrol of *E. vittella* using biopesticides such as Bt *kurstaki*, alone or in conjunction with other treatments in integrated biocontrol, is a viable alternative to chemical pesticides. This is of prime importance in vegetable crops such as okra the pods of which are picked at very short intervals for human consumption. Some of the present isolates have exhibited larval toxicities as good as the reference strain (HD-1), and some have shown even better performance, particularly at early stages of incubation. Due to the limited number of formulated Bt products in the market, resistance in insect populations may emerge quickly to these products[35]. This resistance has been particularly demonstrated in lepidopteran populations exposed to Cry toxins[36]. To offset this, indigenous Bt isolates which exhibit good potential can be utilized in the development of new commercial products and formulations that widen the possibilities of avoiding such setbacks in the management of lepidopteran pests such as *E. vittella*.

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