

ORIGINAL ARTICLES

Role of Some Additives in Enhancing the Formulation of Bacteria *Bacillus thuringiensis* against *Phthorimaea operculella* and *Helicoverpa armigera*.

1- Impact of Tween-80, Arabic gum, Molasses, cellulose, starch and talc powder

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ABSTRACT

Some additives have been adopted to improve the potency of the formulation of *Bacillus thuringiensis* var. *Kurstaki* (HD-234), and to increase its persistence. Results showed that: In case of 1st instar larvae of potato tuber moth, *Phthorimaea operculella*, the persistence of the modified *B. thuringiensis* var. *kurstaki* (HD-234) formulation suspension was 5.3, 6.9, 5.6 and **4.0** folds more stable than those of the commercial preparations of Dipel@2X, Agerin@, HD-1-S-1980 and *B. thuringiensis* var. *kurstaki* (HD-234). The mortality of *P.operculella* scord 60% when tween-80 was added at 0.1% to *B.t.* While in case of tomato fruit worm (American cotton boll worm), *Helicoverpa armigera*, the corresponding figure was 5.0, 7.1, 6.2 and 4.0 folds, and the mortality significantly increase to 65% as compared to 6% when the Tween 0.1% used alone, respectively, for the same preparations. The Arabic gum also increase the efficacy of the *B.t.* and the mean of the corrected mortality were **55** and 60% for *P. operculella* and *H. armigera*, respectively. When molasses was added as feeding stimulants the pests mortality were significantly increased to 76 and 77% for the corresponding two pests. After the additions of the carriers cellulose, starch and talc, the mortality of the *P. operculella* recorded 58, 58 and 31%, respectively.

Key words: Lepidopterous Insect Pests, Bioinsecticides, *Bt Kurstaki*, formulations

Introduction

The potato tuber moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) and tomato fruit worm (American cotton boll worm), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) are of the most important pests of vegetable plants belonging to family Solanaceae, mainly potato, tomato, eggplant and pepper (Sarhan, 2004; Soliman *et al.*, 2008). They are considered important economic pests because their larvae cause severe damage to solanaceous crops (Abul-Nasr *et al.*, 1971; Salama *et al.*, 1972; Sabbour, 2002).

Bacillus thuringiensis is a gram-positive endosporeforming bacterium, which produces protein toxins during sporulation and is toxic to various lepidopteran insect pests (Dulmage and Rhodes, 1971).

Effectiveness of *B. thuringiensis* var. *kurstaki* against larvae in the laboratory has been enhanced by the addition of toxic and non toxic compounds to the bacterial suspensions. The data of (Salama *et al.*, 1984) strongly suggested that the potency of *B. thuringiensis* var. *entomocidus* and var. *aizawai* against *Spodoptera littoralis* could be increased by modifying the conditions prevailing in the insect midgut through the incorporation of alkaline compounds, proteolytic activators, and some mildly toxic organic and inorganic compounds into the insect diet. In field trials against *S. littoralis* on soybean and *Agrotis ypsilon* on vegetable crops, the procedures carried out were based on the incorporation of some selected essentially non toxic and low cost compounds with different modes of action with the endotoxin to increase its activity. Among the compounds tested were wetting agents, stickers, food stimulants, carriers.

The present study, aims to evaluate Wetting agent, Stickers, Feeding stimulant or Carriers compounds that represent different groups that were used as additives to determine which of these additive compounds can potentiate the efficiency of *B. thuringiensis* var. *kurstaki* (HD-234) against 1st instar larvae of *Phthorimaea operculella* and *Helicoverpa armigera* to explore the possible development of more effective formulations of *B. thuringiensis* for field application.

Materials And Methods

Pathogen:

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B. thuringiensis var. *kurstaki* (HD-234) was used during this study. For production of endotoxin preparation, growing cultures were used to inoculate 500 ml-conical flasks each containing 50 ml of fermentation broth media (M_2 medium). To prepare 1 liter from this medium, Proflo (10g), Peptone (2g), Dextrose (15g), Yeast extract (2g), $MgSO_4 \cdot 7H_2O$ (0.3g), $FeSO_4 \cdot 7H_2O$ (0.02g), $ZnSO_4 \cdot 7H_2O$ (0.02g), $CaCO_3$ (1.0g) and $MnSO_4$ (0.02g) were dissolved in 1 liter distilled water and pH adjusted to 7.0. The flasks were incubated for 48-72 hrs in a controlled environment incubator shaker, operated at 300 rpm and 30°C, depending on time necessary to complete lysis. At the end of incubation period, the culture was centrifuged and the spore-endotoxin complex was precipitated using lactose-acetone procedure as described by Dulmage (1970).

Tested Insects:

Standard laboratory colony of the potato tuber moth *P. operculella* was reared on potato tubers *Solanum tuberosum* as a natural host plant under controlled conditions ($26 \pm 2^\circ C$ and $70 \pm 5\%$ R.H). Eggs were obtained from the stock culture and kept in Petri-dishes till larval hatch. The rearing technique by EL-Sherif (1966) was adopted. Pupae were individually kept in specimen tubes (1×3cm) till adult emergence. Adult moth were kept in oviposition cages that consist of chimney glass (8cm in diameter and 16cm height), the lower rim of which rested on the bottom of a Petri-dish lined with a disk of filter paper (Watman) and the upper rim covered with muslin. Each cage was provided with a small piece of cotton soaked in 5% honey solution as food supply. The deposited eggs were collected and kept in Petri-dishes till larval hatching. Groups of newly hatched larvae were transferred into Petri-dishes containing fresh pieces of potato. Larval development was allowed to continue until the adult emergence.

Multiplying of the stock culture of *H. armigera* reared in the laboratory under controlled conditions ($24 \pm 2^\circ C$ and $80 \pm 5\%$ R.H.) on semi-artificial diet described by Shorey and Hale (1965).

Additives:

The used additives were getting from Sigma[®] Company or local-made and they were grouped as the following:

1. Wetting agent: Tween-80 was tested as a wetting agent to ensure a good dispersal of the preparation.
2. Sticker: Arabic gum was tested as a sticker for binding the particulates in granules together.
3. Feeding stimulant: Molasses was tested as a feeding stimulant and sticker.
4. Carriers: Cellulose, Corn starch and Talc powder were tested as carriers.

Bioassay of *B. thuringiensis* var. *kurstaki* (HD-234):

To deduce the LC_{50} of *B. thuringiensis* (HD-234) against 1st instar larvae of *P. operculella*, six serial dilutions of *B. thuringiensis* (500, 250, 125, 62.5, 31.25, 15.625 $\mu g/ml$) were prepared. Then they were tested against 1st instar larvae of *P. operculella* by dipping potato slices for 1 minute in 1.5% ripening agar containing *B. thuringiensis* (HD-234) suspensions at one of the different concentrations mentioned above. The slices were picked up and left to dry at room temperature ($26 \pm 2^\circ C$), then placed in a small plastic cup (4cm height and 4.5cm diameter), each to confine 10 first instar larvae of *P. operculella*. Five replicates were made for each concentration. The same number was used as a control where potato slices were dipped in 1.5% ripening agar suspension without *B. thuringiensis* (HD-234) for 1 minute. The slice-agar coverage technique was used to ensure that the pathogen was well reached to the potato tuber moth larvae.

For *H. armigera*, bioassays were conducted by the surface contamination and by the diet incorporation methods, using 24-well tissue culture plates (Falcon 3047, sigma[®] company) and the six serial dilutions of *B. thuringiensis* plus a water-only for the control group. The surface contamination method was essentially that described by Beegle (1990) and Ferre *et al.* (1991) with 50 μl of each aliquots dilution or control being applied evenly to the surface of the artificial diet and allowed to dry before addition of the insects. The diet incorporation assay was based on the methods of Dulmage *et al.* (1971) and Beegle (1990). The water content of the diet was reduced by 10% and toxin solution stirred into the semi-artificial diet at $55-60^\circ C$ at a ratio of 1:9. A batch of control diet was prepared with water only. About 1 ml diet was poured into each well of a 24-well tissue culture tray. One neonate larva was added to each well. Forty-eight neonates were tested for each dilution.

For both tested insects, records on the number of surviving and dead individuals were taken after 7 days. The percentage of observed larval mortality reported and corrected according to Abbott's formula (Abbott, 1925). The median lethal concentration LC_{50} of *B. thuringiensis* was calculated according to Finney (1971).

Effect of additives on the efficiency of B. thuringiensis var. kurstaki (HD-234):

Additives are compounds that can enhance the formulation action (Rodham *et al.*, 1999) and reduce the effective biopesticide dose required (Behle *et al.*, 1999).

Selected groups of chemical compounds were tested in the present work with respect to their possible synergistic interactions with *B. thuringiensis* (HD-234) using the 1st instar larvae of *P. operculella* and *H. armigera* as the target insect pests. In selecting these compounds the following criteria were considered, the compound must be essentially nontoxic to man or animal, possesses no harm effect on plants at the tested concentration, biodegradable and commonly available at low price (Salama *et al.*, 1985 a and El-Moursy *et al.*, 1993) to assure the prospective of application in future for these compounds that would exhibit promising synergistic interaction with *B. thuringiensis*. Preliminary experiments using additives alone (without combination with *B. thuringiensis*) were tested firstly at 0.1, 0.05 and 0.01% concentrations to evaluate the effect of them against the target insect pests. The additive which causes larval mortality within normal limits (up to 20%) was selected for testing (at the three concentrations 0.1, 0.05 and 0.01%) in combination with *B. thuringiensis* (HD-234) at LC₅₀ (after El-Moursy *et al.*, 1993), and then bioassayed according to the previous mentioned technique for the target insect pests. Mortality was recorded after 7 days and corrected by Abbott's formula (Abbott, 1925). The collected data were statistically analyzed through comparing the treatment means by applying analysis of t-Test, ($P < 0.05$), using the software "SPSS for Windows (Version 7.5.1 by SPSS Inc. Chicago)".

Formulation of B. thuringiensis:

Water Dispersible Powder (WDP) formulation of *B. thuringiensis* (DH-234) was prepared using encapsulation technique. It consists of the biomass of *B. thuringiensis* (HD-234) as an active ingredient at 6.3% (w/w) and the following commercially available materials were used in the encapsulation procedure:

- a. Cellulose (78.9% w/w) as an inert carrier that aid in delivery of formulation to target pests (after Lisanky *et al.*, 1993).
- b. Arabic gum (5% w/w) as a sticker for binding the particulates in granules together (after Srivastava and Prasad 2000).
- c. Molasses (5% v/w) as feeding stimulant and sticker that stimulates feeding of formulation by pests and also as sunscreen.
- d. Tween-80 (3% w/w) as a wetting agent to ensure a good dispersal of the preparation and keep the formulation in suspension (after Foda *et al.*, 1993).

Results And Discussion*Additives effect on the efficiency of B. thuringiensis against the 1st instar larvae of P. operculella:**1-Wetting agents:*

Wetting agents are used for 3 reasons: to improve spray coverage on hydrophobic leaf surfaces, facilitate mixing into water of hydrophobic spores and toxin crystals, and to form emulsions between oil and water by reducing interfacial tension (Burgess, 1998). Tween-80 had been widely and successfully used in sprays as a wetting agent (Burgess, 1998).

Our results showed that when Tween-80 was ineffective as enhancer; this result agrees with that reported by Morris *et al.* (1995). On contrast, Salama *et al.* (1985 a) reported 3- and 5-fold increases in toxicity in *S. littoralis* larvae fed a diet supplemented with *B. thuringiensis* var. *kurstaki* with Tween-60 and 80 at 0.5%, respectively.

Results obtained in (Table 1) show that, larval mortality of *P. operculella* and *H. armigera* from Tween-80 alone ranged between (0.0 and 6.0%). There was no significance between *B. thuringiensis* (HD-234) when bioassayed alone at LC₅₀ and when combined with 0.1, 0.05 or 0.01% of Tween-80 against *P. operculella*, but there was a significance against *H. armigera* after the addition of Tween-80 at 0.1%; the larval mortality was slightly increased from 48 and 51% in the control to 60 and 65% after the addition of Tween-80 at 0.1% concentration, against 1st instar larvae of *P. operculella* and *H. armigera*, respectively, causing 1.25- and 1.27-fold increase in the potency, respectively. Our results for using Tweens as wetting agent was almost agree with many authors: Angus *et al.* (1961) reported that, there was no loss of toxicity, germination and growth on agar not inhibited, sprayed foliage readily eaten by bud worm larvae when Tween-80 was used as a wetting agent. Angus and Luthy (1971) said that, Tween-20 gave good results and no indication that additive reduced effectiveness of *B. thuringiensis*. Lisanky *et al.* (1993) mentioned that, Tween-80 was used as a wetting agent at 3% for water-based and 18% for oil-based *B. thuringiensis* flowable formulations to give adequate dispersion

into the spray and good cover of foliage. Also, Srivastava and Prasad (2000) mentioned that, Tween-80 was used as a spreader during the preparation of *B. thuringiensis* var. *kurstaki* formulation named 'Pusa B.t.' which gave good results against *S. litura*.

2. Stickers:

Arabic gum (Acacia Gum) by Sigma[®] Company, was tested as a sticker at 2.0 and 1.0% concentrations for binding the particles in granules together. It was ineffective as enhancer. Data in Table (2) show that, no significance difference was detected when Arabic gum incorporated at the tested concentrations with *B. thuringiensis* (HD-234) that larval mortality was slightly increased from 47 and 51% in the control to 55 and 60% after the addition of Arabic gum at 2.0% concentration, against 1st instar larvae of *P. operculella* and *H. armigera*, respectively, causing 1.25- and 1.17-fold increase in the potency, respectively. This result was in accordance with those reported by Lisanky *et al.* (1993) when used Arabic gum at 1% as drying protectant during the production of *B. thuringiensis*. Also, matched with Srivastava and Prasad (2000) who used Acacia Gum as a sticker during the preparation of *B. thuringiensis* var. *kurstaki* formulation named 'Pusa B.t.' which gave good results against *S. litura*.

3. Feeding stimulants:

In the field, feeding stimulation is a combination of attracting an insect to an area bearing the stimulant and encouraging it to eat more once it is there (Burges, 1998).

With *B. thuringiensis*, food intake should be increase by insect species to get the highest mortality, possibly a result of stimulants speeding up ingestion of a lethal dose before the antifeeding action of the crystal toxin stopped further feeding (Farrar and Ridgway, 1995; Andrews *et al.*, 1975; Burges, 1998).

Molasses was tested as a feeding stimulant at concentrations of 5.0, 2.0 and 1.0% to encourage pests to eat a maximum amount of pathogen. From our results in Table (3), molasses was a good enhancer. There was a highly significance between *B. thuringiensis* (HD-234) when bioassayed alone at LC₅₀ and when combined with 5.0% of molasses. Larval mortality of *P. operculella* and *H. armigera* increased from 49 and 52% for *B. thuringiensis* alone to 76 and 77% after incorporation of 5.0% molasses with *B. thuringiensis*, respectively. The efficiency was increased by factors of 1.55 and 1.48, respectively. Larval mortality from molasses alone ranged from an average 0.0 to 4.0% at the tested concentrations (Table 3).

Our results in accordance with Farrar *et al.* (1995) who found that molasses (12.5%) increased the larval gypsy moth feeding by 1.7 to 2.3-fold on lettuce leaf discs. Also, with the finding of McGuire *et al.* (1991& 1996), who mentioned that molasses increased the grasshopper mortality by 1.1- to 1.2-fold in assays with rye seedlings when added at 16% to corn starch granules with entomopox virus. Finally, our result was matched with those reported by Salama *et al.* (1985 b), who reported that molasses proved to be active feeding stimulant to *S. littoralis* and potentiated the effect of *B. thuringiensis* var. *entomocidus* HD-635 when combined with it.

4. Carriers:

Data in Table (4) showed that, Cellulose and starch have no inhibition or induction effects on the action of *B. thuringiensis* (HD-234) when incorporated at the tested concentrations against 1st instar larvae of *P. operculella* and/or *H. armigera* as there was no significance among *B. thuringiensis* (HD-234) when bioassayed alone at LC₅₀ and when combined with cellulose and starch.

Talc powder has inhibition effect on the action of *B. thuringiensis* when combined with it. The larval mortality was relatively high when talc powder tested alone; they were 21 and 23% at concentration of 2.0%, against 1st instar larvae of *P. operculella* and *H. armigera*, respectively, as compared to 4 and 6% larval mortality in the untreated control larvae, respectively.

These results were matched with those reported by Pramanik *et al.* (2000), who reported that the persistence toxicity of *B. thuringiensis* var. *kurstaki* increased in combination with some additives such as starch gave persistent toxicity value of 366.66 as compared to *B. thuringiensis* var. *kurstaki* alone (338.89) against fourth instar larvae of *Bombyx mori*, as the test insect, on mulberry leaves. Also, the finding of Ignoffo and Garcia (1996) who reported that starch-encapsulation of *B. thuringiensis* spores and endotoxin with UV screens such as Congo red to prolong the biological activity was accordance with our findings. However, one of the problems with this technique is that starch is the main component of formulation and is easily attacked by fungi and other saprophytic microorganisms if it remains wet for long periods as explained by Patel *et al.* (1996).

Finally, the findings of Ignoffo and Garcia (1996) and Lisanky *et al.* (1993) were matched with our results when used Talc powder as a carrier during the production of a *B. thuringiensis* dust.

Table 1: Effect of Tween 80 on the efficiency of *B. thuringiensis* var. *kurstaki* (HD-234) against 1st instar larvae of *P. operculella* and *H. armigera* after 7 days of treatment

insects Treatments	Tested	Mean% corrected mortality \pm SE		Mix	Mean% corrected mortality \pm SE		t-value	
		<i>P. operculella</i>	<i>H. armigera</i>		<i>P. operculella</i>	<i>H. armigera</i>	<i>P. operculella</i>	<i>H. armigera</i>
<i>B. thuringiensis</i> LC ₅₀	alone	48 \pm 3.54	51 \pm 4.25	<i>B.t.</i> + 0.1 % Tween 80	60 \pm 4.05	65 \pm 3.69	2.232 Ns	2.309*
				<i>B.t.</i> + 0.05% Tween 80	56 \pm 4.25	61 \pm 3.86	1.446 Ns	1.567 ^{NS}
				<i>B.t.</i> + 0.01% Tween 80	52 \pm 3.86	55 \pm 3.54	0.764 Ns	0.542 ^{NS}
0.1 % Tween 80 alone		4.0 \pm 1.15a	6.0 \pm 1.15a	-	-	-	-	-
0.05% Tween 80 alone		2.0 \pm 0.58a	4.0 \pm 0.58a	-	-	-	-	-
0.01% Tween 80 alone		0.0 \pm 0.0a	4.0 \pm 1.15a	-	-	-	-	-

NS = Not significant. * = Significant.

Table 2: Effect of Arabic gum on the efficiency of *B. thuringiensis* var. *kurstaki* (HD-234) against 1st instar larvae of *P. operculella* and *H. armigera* after 7 days of treatment

insects Treatments	Tested	Mean% corrected mortality \pm SE		Mix	Mean% corrected mortality \pm SE		t-value	
		<i>P. operculella</i>	<i>H. armigera</i>		<i>P. operculella</i>	<i>H. armigera</i>	<i>P. operculella</i>	<i>H. armigera</i>
<i>B. thuringiensis</i> LC ₅₀	alone	47 \pm 3.41	51 \pm 3.16	<i>B.t.</i> + 2.0 % Arabic gum	55 \pm 4.05	60 \pm 3.61	1.512 ^{NS}	1.877 ^{NS}
				<i>B.t.</i> + 1.0 % Arabic gum	51 \pm 4.25	53 \pm 2.75	0.734 ^{NS}	0.287 ^{NS}
2.0 % Arabic gum alone		4.0 \pm 1.15a	4.0 \pm 1.15a	-	-	-	-	-
1.0 % Arabic gum alone		2.0 \pm 0.58a	2.0 \pm 0.58a	-	-	-	-	-

NS = Not significant

Table 3: Effect of molasses as a feeding stimulant on the efficiency of *B. thuringiensis* var. *kurstaki* HD-234 against 1st instar larvae of *P. operculella* and *H. armigera* after 7 days of treatment

insects Treatments	Tested	Mean% corrected mortality \pm SE		Mix	Mean% corrected mortality \pm SE		t-value	
		<i>P. operculella</i>	<i>H. armigera</i>		<i>P. operculella</i>	<i>H. armigera</i>	<i>P. operculella</i>	<i>H. armigera</i>
<i>B. thuringiensis</i> LC ₅₀	alone	49 \pm 4.49	52 \pm 3.54	<i>B.t.</i> + 5.0 % Molasses	76 \pm 3.36	77 \pm 3.58	4.811**	4.970**
				<i>B.t.</i> + 2.0 % Molasses	67 \pm 3.42	71 \pm 3.10	3.187*	3.829**
				<i>B.t.</i> + 1.0 % Molasses	59 \pm 3.54	63 \pm 2.55	1.749 ^{NS}	2.524*
5.0 % Molasses alone		2.0 \pm 0.58a	4 \pm 1.15a	-	-	-	-	-
2.0 % Molasses alone		0.0 \pm 0.0 a	2 \pm 0.58a	-	-	-	-	-
1.0 % Molasses alone		0.0 \pm 0.0 a	0.0 \pm 0.0a	-	-	-	-	-

** = Highly significant. * = Significant. Ns = Not significant

Table 4: Effect of some carriers on the efficiency of *B. thuringiensis* var. *kurstaki* HD-234 against 1st instar larvae of *P. operculella* and *H. armigera* after 7 days of treatment

Insects Treatments	Tested	Mean% corrected mortality \pm SE		Mix	Mean% corrected mortality \pm SE		t-value	
		<i>P. operculella</i>	<i>H. armigera</i>		<i>P. operculella</i>	<i>H. armigera</i>	<i>P. operculella</i>	<i>H. armigera</i>
<i>B. thuringiensis</i> alone at LC ₅₀				<i>B.t.</i> + 2.0 % Cellulose	58 \pm 3.86	57 \pm 3.36	1.840 ^{NS}	1.017 ^{NS}
				<i>B.t.</i> + 1.0 % Cellulose	54 \pm 2.83	53 \pm 2.12	1.155 ^{NS}	0.063 ^{NS}
		50 \pm 3.54	51 \pm 2.40	<i>B.t.</i> + 2.0 % Starch	58 \pm 3.11	64 \pm 3.61	1.698 ^{NS}	2.256 ^{NS}
				<i>B.t.</i> + 1.0 % Starch	52 \pm 3.36	60 \pm 3.54	0.410 ^{NS}	1.574 ^{NS}
				<i>B.t.</i> + 2.0 % Talc	31 \pm 2.12	34 \pm 2.98	5.573**	4.847**
				<i>B.t.</i> + 1.0 % Talc	38 \pm 3.22	43 \pm 2.92	3.123*	2.319*
2.0 % Cellulose alone	2.0 \pm 0.58a	4 \pm 1.15a	-					
1.0 % Cellulose alone	0.0 \pm 0.0a	2 \pm 0.58a	-					
2.0 % Starch alone	2.0 \pm 1.73a	9 \pm 2.31a	-					
1.0 % Starch alone	0.0 \pm 0.0a	6 \pm 1.73a						
2.0 % Talc alone	21 \pm 4.04a	23 \pm 5.20a						
1.0 % Talc alone	15 \pm 2.89a	19 \pm 4.04a						

NS = Not significant. ** = Highly significant. * = Significant

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