Toxic and Synergistic Action of α-Amyrin Acetate with *Bacillus thurigiensis* Berliner Aganist the Diamonback Moth, *Plutella xylostella* Linn. (Lepidoptera: Plutellidae)

Kuppusamy CHENNIAPPAN*   Kadarkarai MURUGAN

Division of Entomology, Department of Zoology, Bharathiar University, Coimbatore-641046. Tamil Nadu, INDIA. *Corresponding author: C. Kuppusamy, Phone: +91-422-2422 222, Mobile: 91 + 09944713751, e-mail: lionkupps@yahoo.com

ABSTRACT

The diamondback moth, *Plutella xylostella* L. is the most destructive insect pest attacking crucifers. Toxic and synergistic activity of α-amyrin acetate with *Bacillus thuringiensis* Berliner (Bt) were evaluated by adopting drench and leaf dip bioassays for 24, 48 and 72 hours. Bt treatments showed most of the larval mortalities occurred after 24 hours. The LC_{50} values were 5.77µg/ml, 6.63µg/ml,6.80 µg/ml, 7.22 µg/ml and 122.35 µg/ml, 124.70 µg/ml, 134.28 µg/ml, 145.97 µg/ml for 1st, 2nd, 3rd, 4th larval instars at 72hrs of α-amyrin acetate and Bt treatment respectively. α-amyrin acetate was toxic to all the larval instars. Higher concentration of α-amyrin acetate caused high mortality of larvae even though only small portions of the leaf disc was consumed and larval death was due to a combination of poor feeding and midgut membrane disruption of α-amyrin acetate. Susceptibilty was observed to decrease with increasing larval age. The α-amyrin acetate had an enhancing synergistic influence on the Bt with synergistic factors ranging from 104.81(1:1), 129.47(1:2) to 142.41 (1:4) for first instar larvae; 89.36(1:1), 118.35 (1:2) to 146.83 (1:4) for second instar larvae; 76.96 (1:1), 112.57 (1:2) to 175.30 (1:4) for third instar larvae and 65.31 (1:1),117.01(1:2) to194.73(1:4) for fourth instar larvae at 72 hrs. The combination treatments being more efficient than the individual α-amyrin acetate or Bt treatments alone. Such integrated approach has important implication in the environmentally safe control of pests.

Key words: *Plutella xylostella, Bacillus thuringiensis*, α-amyrin acetate, *Catharanthus roseus*, Synergism.

INTRODUCTION

In India, Crucifers are attacked by several insect pests, among these insect pests, diamondback moth *Plutella xylostella* L. is the most destructive (Hassanein *et al*., 1958).It occurs through out the world on cruciferous plants. The level of diamond back

moth infestation varies according to locality, type of cabbage plants, outlining plants and the level of natural enemies. If no control measures are undertaken, feeding injury caused by this caterpillar may reduce production to zero. Its exceptional pest status is due to the diversity and abundance of host plants, lack of disruption of its natural enemies, its reproductive potential (over 20 generations per year in the tropics) and its genetic elasticity facilitating rapid development of resistance to chemical insecticides (Mohan and Gujar, 2003; Shelton, 2004;Vickers *et al*., 2004). Its control on cruciferous crops worldwide costs about $1 billion annually (Talekar *et al.*, 1998), in addition to the crop losses it causes. Diamond back moth was reported as the first crop pest in the world to develop resistance to DDT in Java, Indonesia (Ankersmith, 1953; Johnson, 1953) and now has become resistant to most synthetic insecticides used against it in the field in many countries (Talekar and Shelton, 1993). Diamond back moths also have the distinction of being the first insect to have developed resistance in the field to the bacterial insecticide *Bacillus thuringiensis* Berliner (Bt) (Kirsch and Schmutterer, 1988; Tabashnik *et al*., 1990; Shelton and Wyman, 1992). The situation regarding farmer's perception and use of pesticides in India fully justifies the hesitancy exhibited so far with respect to recommendation of *Bacillus thuringiensis* Berliner (Bt) as the sole control measures, Use of Bt can be and should be, advocated within the integrated programme of pest management. Integrated pest control is an effective and essential part of any successful pest control program. Over the past decades, phytochemicals have received progressively more attention as insecticide alternatives and selected phytochemicals have been shown to be potentially effective as part of biocontrol programs. So far only azadirachtin-an Insect Growth Regulator (IGR) derived from Indian neem (*Azadirachta indica*) seed kernel is being commercialized and marketed for managing agricultural insect pests. However substitute(s) of such naturally occurring prototypes are in great demand at a global level with a view to manage the insect resistance and safety to environment. The n-hexane fraction of acetone extract from *Catharanthus roseus* leaf contains α-amyrin acetate and oleanolic acid as natural source of pesticides against the gram pod borer *Helicoverpa armigera* Hub., tobacco caterpillar *Spodoptera littura* F. The purified α-amyrin acetate had given 35.71% IGR activity against *Helicoverpa armigera* (Dwijendra Singh *et al*., 2003). The current study evaluates the combined influence of Bt with α-amyrin acetate, a botanical pesticide from *Catharanthus roseus* for the control of diamondback moth *P. xylostella* L. This combined approach would, on one hand, reduce the amount of the Bt formulation needed (thereby reducing costs, risk to non-target organisms and the risks of resistance), and on the other, significantly decrease farmer dependence on synthetic pesticides. In the larger context, this would translate into reduced pesticide contamination of the environment.

MATERIALS AND METHODS

Insects

Diamondback moth *P. xylostella* larvae used in this study were obtained from a laboratory colony reared on cabbage plants (*Brassica oleracea* var *capitata* "stone

head") and maintained at room temperature (20-23 ^{0}C) and a 16: 8 LD photo period. The colony had been maintained continuously for one year in our laboratory.

Test materials

Bacillus thurnigiensis: *Bacillus thuringiensis subsp kurstaki* Berliner was used as commercial formulation (DiPel® 2X, 32,000 International Units per mg of product, Abbot, North Chicago, IL).

α-amyrin acetate: Extraction, isolation and characterization

The leaves of *C. roseus* were collected locally from the foothills of the Western Ghats area adjacent to Bharathiar University, Coimbatore, Tamil Nadu, India. The leaves were washed with double distilled water and were shade dried at room temperature. The dried parts were chopped into small pieces of approximately 1 cm size by a falcon stem cutter (Biocraft Scientific India, Uttar Pradesh, India) and powdered with the help of an electric blender. The dried powder was subjected to acetone in a Soxhlet apparatus (Borasil, Mumbai, India) for 72 h (Saxena *et al.*1994). The solvent was then filtered and evaporated under reduced pressure in a rotary vacuum evaporator to afford crude extract (41.28gm), as greenish orange mass. The compound α-amyrin acetate was isolated from the acetone extract by column chromatography (Backett and Stenlake 1986) on silica gel of 60-120 mesh size. The column was first eluted with n-hexane: ethyl acetate (1:5) and then polarity of the solvent system was gradually increased and 50 ml were collected in each fraction. The fractions 3 to 6 showed almost identical spots on TLC. These fractions were combined and subjected to preparative Thin Layer Chromatography (Egon and Stahi 1969) using solvent system chloroform: methanol (7:1) to afford the compound α-amyrin acetate as yellowish syrup. The compound α-amyrin acetate was characterized on the basis of its ^{1}H-NMR, ^{1}H-^{1}HCOSY 90^0 spectrum, ^{13}C complete decoupling, ^{13}C DEPT 135^0 spectra and long range correlation spectra.

The volume of stock solution of 1% obtained by weighing 200mg of the material and adding 20ml of acetone to it and kept in a screw cap vial with aluminum foil over the mouth of the vial. Homogenous solutions were obtained by gentle shaking or stirring. The test concentrations are then obtained by adding appropriate dilution to distilled water. A wetting agent (Tween 20) was used at the rate of 0.63ml/L of spray with both bacterial and α-amyrin acetate treatments.

Drench Bioassay

Neonate and second instar larvae of diamondback moth, *Plutella xylostella* from the laboratory colony were used in drench bioassays .Ten larvae were placed in a 60 ml plastic cup (B200 Solo cup Company, Urbana, Illinois) lined with a 42.5mm diameter Whatman No.1filter paper. Larvae were drenched with 223 µl of α-amyrin acetate or Bt or control solutions (Sparks *et al.*, 1998) with a different rate of concentrations. Larvae were provided with two cabbage-leaf discs (*Brassica oleracea* var. capitata "stone head") 1cm diameter, 1 h after the treatment. Acetone or Water plus powdered laundry detergent (0.05 %w/v) was used as a control. There were six replicates for each treatment. The cups were placed in a plastic box lined with moistened towel

paper in a growth chamber at 26 ^{0}C and a photoperiod of 16:8 LD. Mortality was recorded after 24, 48 and 72 hours. Each experiment was conducted four times and the data combined for analysis.

Leaf dip Bioassay

The 3rd and 4th instar larvae of the diamondback moth obtained from the laboratory colony were used in leaf-dip bioassays. Although the drench bioassay could have been used for the larger larvae as well, we believe that the leaf dip bioassay more closely approximates field exposures. The following method was used. Cabbage leaves (*Brassica oleracea* var capitata "stonehead") were washed with distilled water and allowed to air dry, leaf discs (2 cm diameter),cut from these leaves with a cork borer, were dipped in one of five concentrations for 5 s and allowed to air-dry. One disc was placed in a 5cm diameter Petri dish lined with moistened Whatman (No.1) filter paper and a larvae was placed individually in the Petri dish. Petri dishes were placed in a plastic box lined with moistened towel paper in a growth chamber at 26 ^{0} C and a photoperiod of 16 : 8 LD. Mortality was recorded after 24, 48 and 72 hours. Each of the five concentrations was tested on 20 larvae and distilled water plus powdered laundry detergent (0.05 %w/v) was used as control. The experiment was repeated four times and the data combined for analysis.

Combined treatment

These experiments were aimed to check for synergism or antagonism between α-amyrin acetate and *Bacillus thuringiensis* Berliner in combined treatment. α-amyrin acetate was suspended in distilled water at desired concentrations, as well as *Bacillus thuringiensis* and Tween 20 was added as a wetting agent. Test concentrations for each of the mixed formulation ratios were prepared by adding together above preparations at desired concentrations as 1:1 ratio (one part correspond to α-amyrin acetate and one parts correspond to Bt), for 1:2 ratio one part correspond to α-amyrin acetate and two parts of Bt and for 1:4 ratio (one part correspond to α-amyrin acetate and four parts to Bt). The drench and leaf dip bioassays (described above) was carried out. Mortality counts were recorded for 24, 48 and 72 hours after treatment.

Data analysis

Mortality data produced for α-amyrin acetate and *Bacillus thuringiensis* Berliner and for combined treatment were analyzed by probit analysis (Finney, 1971). The corrected percent mortality was calculated by Abbot's formula (Abbot 1925). Regression equations were obtained, along with LC_{50} and its 95% confidence level. A co-toxicity co-efficient (Sarup *et al*., 1980) and a synergistic factor (Kalyanasundaram *et al*., 1985) for combined experiments were calculated using $LC_{50.}$

Synergistic factor (SF) = toxicity of insecticide (alone)/toxicity of insecticide with α- amyrin acetate.

A value of SF >1 indicates synergism and SF<1 indicates antagonism.

RESULTS

The results after the treatment with α-amyrin acetate, mortality were concentration dependent (Figs 1, 2). The lower concentrations (2-4 µg/ml) showed mortality percentages ranging from 19.0, 10.0, 9.0 and 7.0 within 24hrs, increased to 35.0, 29.0, 28.0, and 26.0 % after 72 hours for 1st, 2nd , 3rd , and 4th larval instars respectively. On the other hand the higher concentrations (8-10 µg/ml) showed mortality percentage ranging from 69.0, 60.0, 58.0 and 56.0 with 24 hours. After 72 hours, the mortality increased to reach 85.0, 82.0, 80.0 and 77.0 for 1st, 2nd, 3rd, and 4th larval instars respectively. LC_{50} values were 7.42, 6.86, 5.78; 9.86, 7.75, 6.63 µg/ml for first and second instars larvae 9.31, 7.98, 6.81; 9.43, 8.23, 7.96 µg/ml for 3rd and 4th instars larvae respectively at 24, 48, and 72 hours (Table 1)
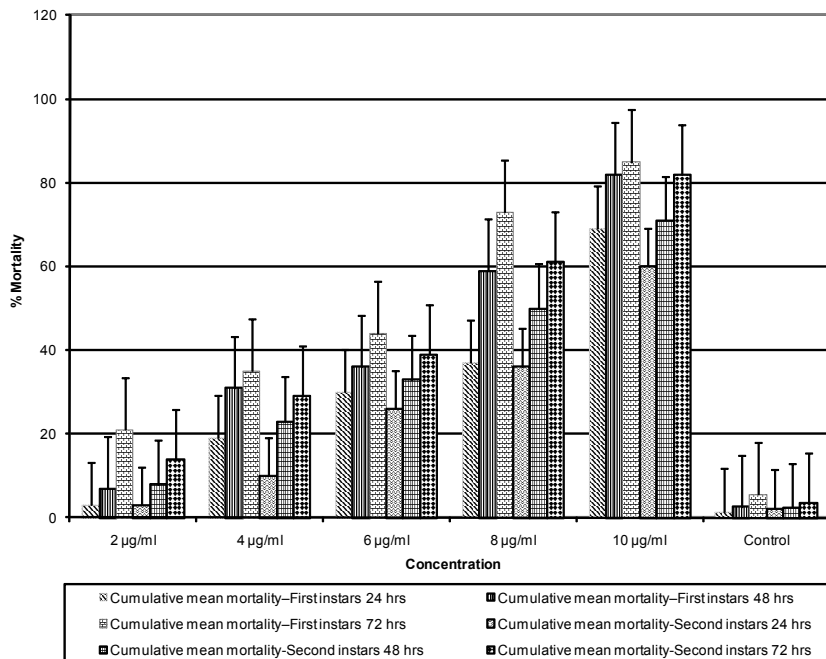

Fig. 1. Efficacy of α-amyrin acetate against the 1st and 2nd larval instars of *Plutella xylostella* L .in drench bioassay treated with 24, 48 and 72 hours.

Mortality with Bt treatment was concentration dependent for all larval instars (Figs 3, 4). The lower concentrations (54-81 µg/ml) showed mortality percentages ranging from 14.0, 11.0, 8.0, and 6.0 within 24 hours, increased to 33.0, 31.0, 27.0 and 25.0 % after 72 hours for 1st, 2nd, 3rd and 4th instars larvae respectively. On the other hand the higher concentrations (135-189µg/ml) showed mortality percentage ranging from 75.0, 60.0, 58.0 and 54.0 within 24 hours. After 48 hours, the mortality increased to reach 79.0, 78.0, 71.0, and 65.0 % for 1st, 2nd, 3rd, and 4th larval instars respectively. LC_{50} values were 151.06, 135.59, 122.35; 167.43, 142.62 and 124.71 µg/ml for1st, 2nd larval instars and 172.16, 150.76, 134.28; 178.85, 158.16, 145.98 µg/ml for 3rd and 4th larval instars at 24, 48 and 72 hours respectively (Table 2).

Mortality percentages among 1st instars larvae of *Plutella xylostella* were high due to the combined application of α-amyrin acetate with Bt, being 54.0, 49.6, 45.6; 41.6 at 24 hrs for 1st, 2nd, 3rd and 4th instars larvae at 1:1 ratios (Table 3). After 72 hours the mortality increased to reach 66.0, 64.0, 59.6 and 54.2 at 72 hours for the same concentrations. On the other hand the 1:2 ratio combination caused mortality percentage ranging from 56.4, 54.6, 52.6, and 50.6 within 24 hours, the mortality increased to reach 71.2, 69.2, 67.0 and 64.2 at 72 hours for 1st, 2nd, 3rd and 4th larval instars respectively. The similar trend was also observed for 1:4 ratio combinations indicating a considerable improvement in the efficacy of α-amyrin acetate with Bt applied in combination against *Plutella xylostella* and proved this combination acted synergistically. The ratio 1:1 had LC$_{50}$ of 2.28, 1.77, 1.18; 2.69, 2.83, and 1.39 for 1st and 2nd instars and 3.09, 2.42, 1.74; 3.58, 2.80, and 2.23 for 3r and 4th instars at 24, 48 and 72 hours respectively. The synergistic factors at 1:1 ratios were 66.12, 76.54, 104.81 for first instars 62.12, 90.12, 89.36 for 2nd instars, 55.64, 62.28, 76.96 for 3rd instars and 49.90 and 49.90, 55.19, 65.31 for fourth instars at 24, 48 and 72 hours respectively (Table 4). The ratio 1:2 had Synergistic values of 72.74, 89.48, 129.0 for first instars, 75.59, 86.04,118.35 for 2nd instars, 77.75, 82.51,112.57 for 3rd instars 62.09, 76.07, 117.01 for 4th instars at 24,48 and 72 hours respectively (Table 5). The ratio 1:4 had Synergistic values of 78.56, 92.03, 142.41 for 1st instars, 86.49, 102.31, 146.83 for 2nd instars, 82.50, 97.88, 175.30 for 3rd instars, 81.15, 98.50, 194.73 for 4th instars at 24,48 and 72 hours respectively (Table 6).
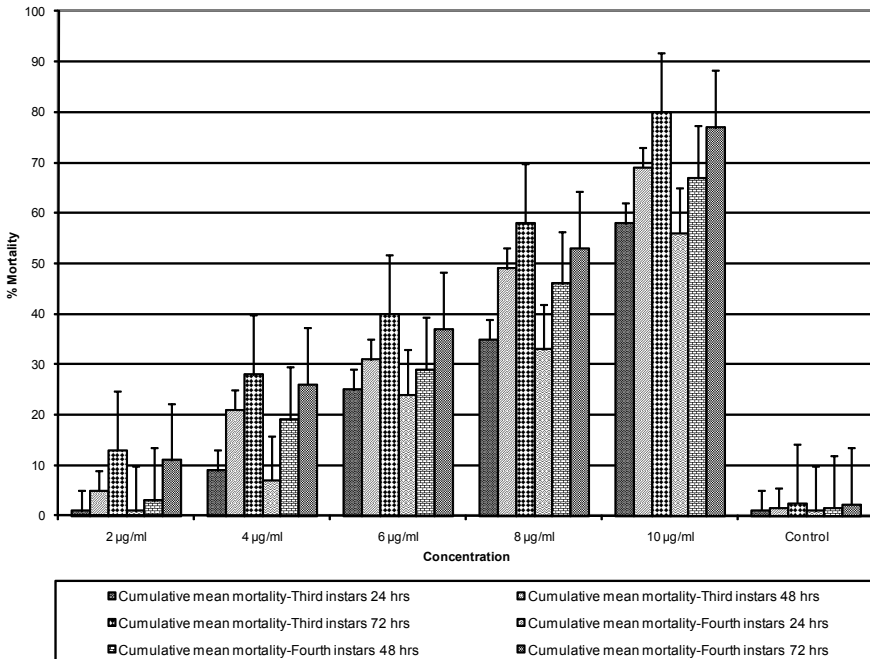

Fig. 2. Efficacy of α-amyrin acetate against the 3rd and 4th larval instars of *Plutella xylostella* L. in leaf dip bioassay treated with 24, 48 and 72 hours.
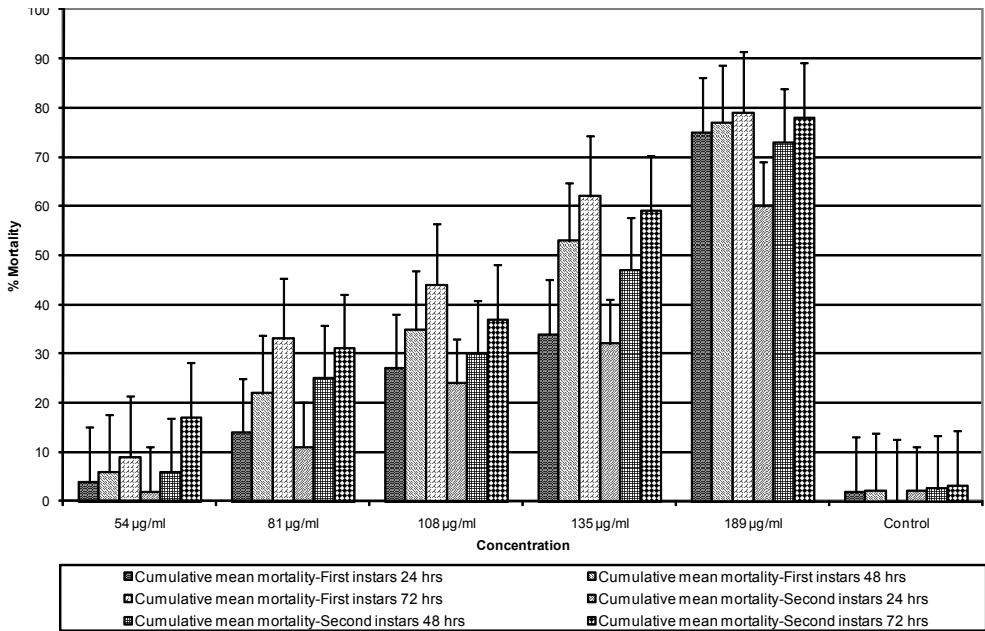
Fig. 3. Efficacy of *Bacillus thuringiensis* Berliner against the and 1st and 2nd larval instars of *Plutella xylostella* L. in drench bioassay treated with 24, 48 and 72 hours.
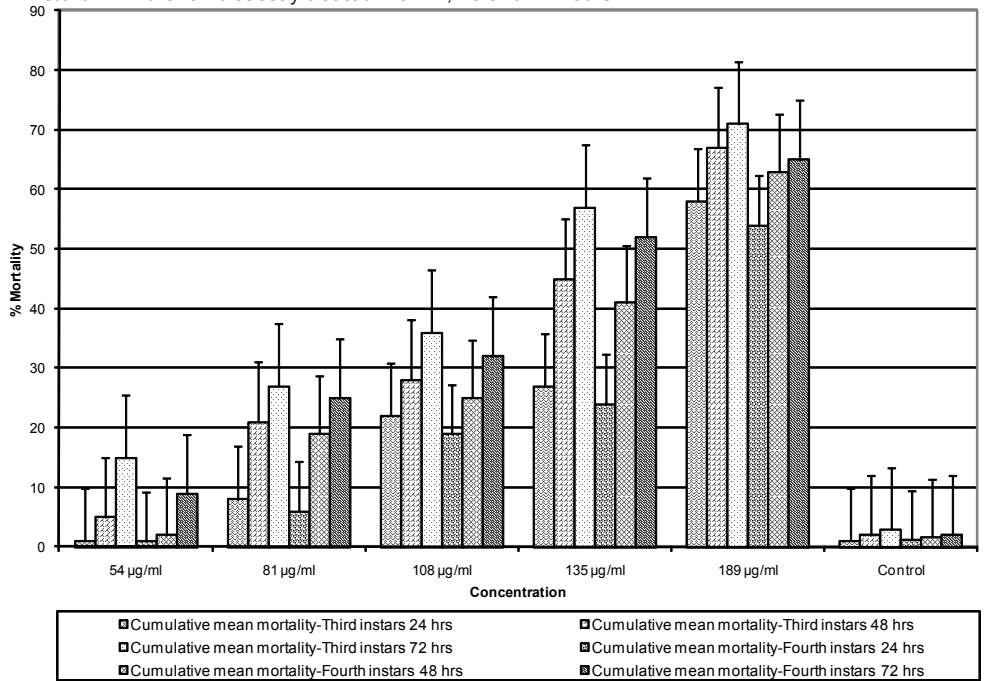

Fig. 4. Efficacy of *Bacillus thuringiensis* Berliner against the and 3rd and 4th larval instars of *Plutella xylostella* L .in leaf dip bioassay treated with 24, 48 and 72 hours.

Table 1. Lethal concentration values of α-amyrin acetate against the different larval instars of *Plutella xylostella* L. after 24, 48 and 72 hours.

Larval instars	Bioassay	Exposure hours	Regression co efficient	LC_{50} (μg/ml)	Lower fiducial limits (μg/ml)	Upper fiducial limits (μg/ml)	X^2	P value
1st instars	Drench Bioassay	24	Y=0.2465 + -2.0754X	8.42	7.0823	10.97	7.741	0.455
		48	Y=0.2643+-1.8104X	6.85	5.69	8.27	6.844	0.347
		72	Y=0.2339+-1.3511X	5.78	5.25	6.29	3.546	0.052
2nd instars		24	Y=0.2519+-2.1812X	9.05	8.46	9.83	1.596	0.801
		48	Y=0.2292+-1.7780X	7.75	5.29	8.41	1.382	0.598
		72	Y=0.2402+-1.5940X	6.63	4.14	7.16	2.071	0.203
3rd instars	Leaf dip Bioassay	24	Y=0.2768+-2.5284X	9.43	8.58	9.85	5.183	0.234
		48	Y=0.2432+-1.9382X	7.97	7.44	8.60	2.199	0.431
		72	Y=0.2346+-1.5972X	6.81	6.30	7.36	1.377	0.295
4th instars		24	Y=0.28063+-2.6139X	9.31	8.75	10.05	5.268	0.060
		48	Y=0.2518+-2.0729X	8.23	7.70	8.87	3.353	0.132
		72	Y=0.2306+-1.6654X	7.22	6.70	7.81	1.912	0.214

Table 2. Lethal concentration values of *Bacillus thuringiensis* Berliner against the different larval instars of *Plutella xylostella* L. after 24, 48 and 72 hours.

Larval instars	Bioassay	Exposure hours	Regression co efficient	LC_{50} (μg/ml)	Lower fiducial limits (μg/ml)	Upper fiducial limits (μg/ml)	X^2	P Value
1st instars	Drench Bioassay	24	Y=0.0166+-2.5189X	151.05	142.65	161.18	2.613	0.052
		48	Y=0.0157+-2.1412X	135.59	127.66	162.75	3.303	0.077
		72	Y=0.0145+-1.7784X	122.35	99.78	150.15	7.739	0.312
2nd instars		24	Y=0.01462+-2.4480X	167.43	156.79	181.41	3.519	0.660
		48	Y=0.0142+-2.0358X	142.62	133.66	153.37	4.635	0.710
		72	Y=0.0130+-1.6245X	124.71	115.67	134.68	1.753	0.358
3rd instars	Leaf dip Bioassay	24	Y=0.0157+-2.7172X	172.15	149.62	218.87	6.694	0.491
		48	Y=0.00137+-2.0696X	150.76	141.01	162.96	4.263	0.532
		72	Y=0.01190+-1.5982X	134.28	124.28	146.11	2.754	0.711
4th instars		24	Y=0.0157+-2.8186X	178.85	150.60	224.60	5.685	0.336
		48	Y=0.0141+-2.2154X	158.16	134.68	206.17	7.418	0.340
		72	Y=0.0119+-7457X	145.98	123.98	184.65	5.503	0.591

Table 3. Efficacy of *Bacillus thuringiensis* Berliner in combination with α-amyrin acetate in different ratios against different larval instars of *Plutella xylostella* L. after 24, 48 and 72 hours in drench and leaf dip bioassays.

Ratios	Cumulative mean mortality-First instars			Cumulative mean mortality-Second instars		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
1:1	54.0 ± 1.3[c]	60.0 ±0.5[d]	66.0 ±3.4[bc]	49.6 ± 4.7[c]	57.0 ± 5.2[dc]	64.0 ± 6.5[d]
1:2	56.4 ± 1.9[b]	63.6 ± 0.3[bc]	71.2 ± 2.7[d]	54.6 ± 7.2[b]	61.6 ± 4.3[bc]	69.2 ± 9.2[c]
1:4	58.2 ± 1.5[a]	64.4 ± 0.6[ab]	73.0 ± 1.9[ab]	58.2 ± 3.1[a]	65.4 ± 4.6[ab]	73.0 ± 4.0[bc]
Control	2.0 ± 0.1[f]	2.8 ± 0.1[f]	4.1 ± 0.1[f]	1.9 ± 0.1[f]	2.4 ± 0.6[f]	3.0 ± 0.05[f]
Ratios	Cumulative mean mortality-Third instars			Cumulative mean mortality-Fourth instars		
1:1	45.6 ± 5.2[d]	52.4 ± 5.3[c]	59.6 ± 4.0[de]	41.6 ± 3.9[cd]	47.6 ± 3.4[c]	54.2 ± 0.5[d]
1:2	52.6 ± 4.5[bc]	58.6 ± 6.7[b]	67.0 ± 5.1[cd]	50.6 ± 5.0[c]	56.4 ± 6.1[b]	64.2 ± 6.7[c]
1:4	56.4 ± 7.2[ac]	63.2 ± 4.1[a]	70.6 ± 6.2[ab]	55.0 ± 6.1[b]	61.0 ± 3.9[a]	66.0 ± 8.1[bc]
Control	1.7 ± 0.2[f]	1.9 ± 0.1[f]	2.7 ± 0.1[f]	1.2 ± 0.1[f]	1.6 ± 0.2[f]	1.6 ± 0.1[f]

The mean values have been obtained averaging the drench and leaf dip bioassays, with all the concentrations tested.

± SE: Standard error. Means (± S E) followed by the same letters (a-f) within columns indicate no significant difference in a Tukey test.

Table 4. Lethal concentration and synergistic values of 1:1 combinations of α-amyrin acetate and *Bacillus thuringiensis* Berliner against the different larval instars of *Plutella xylostella* L.

Larval instars	Bioassay	Exposure hours	Regression equation	X²	LC$_{50}$ (Fidicial Limits) (µg/ml)	Co toxicity factor	Synergistic factor	P Values
1st instars	Drench bioassay	24	Y=0.3655+-0.8350X	21.06	2.28 (0.16-4.33)	6612.16	66.1218	0.001
		48	Y=0.4117+-0.7294X	21.54	1.77 (0.00-3.41)	7654.41	76.5441	0.001
		72	Y=0.4608+-0.5380X	13.574	1.17 (0.87-2.17)	10481.55	104.8155	0.004
2nd instars		24	Y=0.3226+-0.8696X	23.575	2.69 (0.38-6.29)	6212.42	62.1242	0.001
		48	Y=0.3945+-0.8024X	23.44	2.03 (0.81-4.00)	7012.48	70.1248	0.001
		72	Y=0.3832+-0.5347X	17.91	1.39 (0.98-2.80)	8936.95	89.3695	0.000
3rd instars	Leaf dip bioassay	24	Y=0.3095+-0.9576X	24.48	3.10 (0.23-9.09)	5564.16	55.6416	0.001
		48	Y=0.3687+-0.6925X	19.16	2.42 (0.41-4.34)	6228.55	62.2855	0.001
		72	Y=0.3474+-0.6061X	15.330	1.74 (0.77-3.18)	7696.50	76.9650	0.002
4th instars		24	Y=0.2866+-1.6273X	24.184	3.58 (1.00-19.96)	4990.19	49.9019	0.001
		48	Y=0.3448+-0.9881X	23.54	2.86 (0.49-6.17)	5519.60	55.1960	0.001
		72	Y=0.2984+-0.6671X	15.665	2.23 (0.45-4.11)	6531.37	65.3137	0.001

Table 5. Lethal concentration and synergistic values of 1:2 combinations of α-amyrin acetate and *Bacillus thuringiensis* Berliner.

Larval instars	Bioassay	Exposure hours	Regression equation	X²	LC$_{50}$ (Fidicial Limits) (µg/ml)	Co toxicity factor	Synergistic factor	P Values
1st instars	Drench bioassay	24	Y=0.3679+-0.7639X	23.966	2.08 (1.34-4.28)	7274.86	72.74	0.001
		48	Y=0.4428+-0.6711X	24.421	1.51 (0.32-3.18)	8948.08	89.48	0.002
		72	Y=0.4893+-0.4624X	18.625	0.94 (0.31-2.11)	12947.21	129.47	0.005
2nd instars		24	Y=0.3499+-0.7751X	27.872	2.21 (1.09-5.42)	7559.64	75.59	0.001
		48	Y=0.4127+-0.6842X	24.096	1.66 (0.09-3.89)	8604.00	86.04	0.002
		72	Y=0.4429+-0.4667X	22.197	1.05 (0.87-2.47)	11835.08	118.35	0.002
3rd instars	Leaf dip bioassay	24	Y=0.3499+-0.7751X	27.872	2.21 (1.09-5.42)	7775.75	77.75	0.001
		48	Y=0.3805+-0.7126X	24.027	1.87 (0.89-3.88)	8251.31	82.51	0.001
		72	Y=0.4072+-0.4858X	24.548	1.19 (0.77-2.84)	11257.62	112.57	0.004
4th instars		24	Y=0.3314+-0.8552X	27.00	2.88 (1.62-6.90)	6209.80	62.09	0.001
		48	Y=0.3733+-0.7763X	26.597	2.07 (1.05-4.58)	7607.02	76.07	0.001
		72	Y=0.3462+-0.4319X	25.118	1.25 (0.39-3.19)	11701.49	117.01	0.002

Table 6. Lethal concentration and synergistic values of 1:4 combinations of α-amyrin acetate and *Bacillus thuringiensis* Berliner against the different larval instars of *Plutella xylostella* L.

Larval instars	Bioassay	Exposure hours	Regression equation	X²	LC$_{50}$ (Fidicial Limits) (µg/ml)	Co toxicity factor	Synergistic factor	P Values
1st instars	Drench bioassay	24	Y=0.3770+-0.7248X	22.335	1.92 (0.26-3.81)	7856.82	78.56	0.000
		48	Y=0.4531+-0.6602X	23.197	1.45 (0.07-3.00)	9303.33	93.03	0.000
		72	Y=0.5436+-0.4671X	15.60	0.85 (0.01-1.82)	14241.78	142.41	0.001
2nd instars		24	Y=0.3989+-0.7722X	24.93	1.93 (0.37-3.95)	8649.19	86.49	0.000
		48	Y=0.4700+-0.8552X	29.142	1.39 (0.08-3.31)	10231.72	102.31	0.001
		72	Y=0.5298+-0.4500X	26.228	0.85 (0.06-2.30)	14683.42	146.83	0.001
3rd instars	Leaf dip bioassay	24	Y=0.3952+-0.8247X	25.525	2.08 (1.04-4.27)	8250.90	82.50	0.002
		48	Y=0.4281+-0.6595X	31.015	1.54 (0.07-3.83)	9788.5	97.88	0.001
		72	Y=0.5321+-0.4520X	24.015	0.83 (0.05-3.23)	17530.46	175.30	0.002
4th instars		24	Y=0.3813+-0.8405X	26.27	2.20 (1.11-4.67)	8115.45	81.15	0.000
		48	Y=0.3723+-0.5970X	29.27	1.60 (0.55-4.09)	9850.82	98.50	0.001
		72	Y=0.3381+-0.2535 X	20.452	0.75 (0.02-2.35)	19473.07	194.73	0.004

In general, the lethal concentration values decreased with time exposure but increased with larval instar stage. The lethal concentration values, for example, decreased from 2.28µg/ml at 24 hrs to 1.17 µg/ml at 72 hrs for 4th instar larvae at 1:1 but increased with larval instar stage from 1.17 µg/ml at 72hrs for 1st instars larvae to 2.23 µg/ml at 72 hrs for 4th instar larvae at 1:1 ratio. Further, the increment of Bt proportion produced a general lowering of LC_{50} for example, the lethal concentration values were 2.23 µg/ml at 72hrs of 4th instar larvae for 1:1 ratio (one part corresponds to α-amyrin acetate and one parts to Bt) is decreased to 1.24 µg/ml at 72 hrs of 4th instar larvae at increment of Bt proportion in 1:2 ratio (one parts corresponds to α-amyrin acetate and two pars correspond to Bt) and further decreased to 0.74 µg/ml at 72hrs of 4th instar larvae at 1:4 ratio (one part of α-amyrin acetate and four parts to Bt).

DISCUSSION

α-amyrin acetate treatment

The results revealed that α-amyrin acetate is toxic to all the larval instars of *Plutella xylostella*. In the leaf dip bioassays, high concentrations of α-amyrin acetate caused high mortality of larvae even though only very small portions of the leaf consumed. This suggests that insect death was due to a combination of starvation, contact and gut toxicity of α-amyrin acetate. Susceptibility was observed to decrease with increasing larval age possibly due to increased detoxification metabolism in older larvae. The first and second instars of the *Plutella xylostella* were more susceptible stages to α-amyrin acetate than the older age larvae; therefore control measures should be targeted at the earlier larval stages of this pest. Kohyama (1986) and Abdul-Kadir *et al*., (1999) reported similar results with an insect growth regulator (Teflubenzuron) and three baculoviruses tested against *Plutella xylostella*.

The LC_{50} values indicated that the young instars was more susceptible than the older one, which can be comparable to the results of Audrey Leatemia and Murray, (2004) reported that crude aqueous seed extracts and ethanol seed extracts of *Annona squamosa* caused significant mortality at 0.2 %w/v, 0.6 % w/v at leaf dip bioassays against *P. xylostella.* The results obtained herein were similar to those reported by Moeschler *et al*., (1987) in *Plutella xylostella* the 100 % mortality was observed after treatment with 40 ppm of pure annonin an active compound extracted from *Annona squamosa*.

Furthermore the study was favorably supported by the results of Dwijendra Singh *et al*., (2003) reported that α-amyrin acetate and acetone extracts from C. roseus showed 35.71% and 82.2% toxic and IGR activity against *Helicoverpa armigera* and *Spodoptera litura* respectively and confirmed its toxic effects against serious field pests.

Bacillus thuringiensis treatment

The drench and leaf dip bioassays showed that most of larval mortalities of *Plutella xylostella* L.occurred after 24 hours, then increased slightly and gradually till the 72 hours. Mortality readings; however remained almost constant from 24 to 74 hours at high concentrations. The lethal concentrations, there were not great difference between the instars of the caterpillar, indicated that young larvae were as a resistant as the older

to Bt. In general in both bioassays larval mortalities occurred after 24 hours. Mortalities then increased slightly and gradually. In some cases, however mortality readings remained almost unchanged. This indicates that young as well as older larvae which escaped from the rapid death by endotoxins, later on suffered from starvation due to possible paralysis of the gut muscles, one of the effects known for Bt (Baur *et al.*, 1998). Further more, surviving larvae that fed on treated food appeared dwarfed and much smaller in size than those of the untreated control. Therefore, longer exposure and higher amount of ingested contaminated food are needed to show more effect.

Felke and Langerbruch (2001) recorded that larvae of *Plutella xylostella* L. showed significant mortality, fed less and grew more slowly than larvae of untreated control group.

Combined treatment

The results revealed that an apparent increase in efficacy of the combination between Bt and α-amyrin acetate in comparison with Bt and α-amyrin acetate when each was used alone. The combination of Bt with α-amyrin acetate was greatly enhanced at different ratios. However, the activity is minimal when the mixed formulations contained an equal amount of both constituents (i.e. the 1:1 ratio). The synergistic factors for ratio 1:4 were higher than for the other ratios of the mixed formulations tested. Among different combination ratios, the 1:4 ratio, the mortality percentage and synergism was high, moreover the synergistic activity proportional to the increasing concentration of α-amyrin acetate. A ratio of 1:4 of Bt and α-amyrin acetate was 142.41 fold more toxic at LC_{50} for 1st instars; 146.8 fold for 2nd instars; 175.3 fold for 3rd instars and 194.7 fold for 4th instars at 72 hours respectively, than Bt was used alone and this high level of activity resulted from synergism between the Bt and α-amyrin acetate. In all the ratios the synergistic value increases from 24 hour to 72 hours steadily at one hand. On the other hand the mortality values increases and LC_{50} values decreased. The results indicating that in all the combinations α-amyrin acetate acted synergistically. A possible explanations may be that the Bt crystal proteins paralyses the gut, and prevents further feeding and the neuro-toxic effect of α-amyrin acetate increases the toxicity combined with Bt crystals and caused higher mortality in treated larvae indicated that increased toxic effect of combined treatments on the neuromuscular system. (Dwijendra Singh *et al.*, 2003) Ludlum *et al.*, (1991) have reported that aromatic compounds and plant allelochemicals increase Bt activity and caused swelling of the gut epithelial cells. The present work is favorably supported by the finding of El-Bishry (1994) who reported that increased mortality rates against larvae of *Agrotis ipsilon* resulted due to mixing infective juveniles of the nematode *Steinernema carpocapsae* with Bt Dipel 2x. The LC_{50} values was reduced from 56.26 Ijs incase of nematode treatment alone to 10.79 Ijs in combination with Bt. Baur *et al.*, (1998) reported that in the laboratory and in the field experiments, combining nematode and Bt yielded an increase in mortality effect was detected between Bt *aizawai* and *Xenorhbdus nematophilus* or *Photorhabdus luminescence*, the symbiotic bacteria associated with *S.carpocapsae* and *H.bacteriophora* respetively. Chiu Shin-Foon (1990) demonstrated the efficacy of Bt with ethanol extracts of Derris roots on *Crocidolomia binotalis*, while Ramaprasad *et al.*, (1982) recorded the efficiency of Bt with aqueous extracts of *Pongamia galoabra* on *Spodoptera litura*. While

neem enhanced the action of Bt against *Spodoptera frugiperda* (Hellpap and Zebitz, 1986). The synergistic effect depend not only on the nature of the micro organisms participating in the process but also on other parameters such as their virulence, the relative dose applied and the sequence time of infection. α-amyrin acetate and Bt can together constitute an effective and environmentally safe IPM programme. Coupled with farmer's education and strong extension activities, such a combination approach can definitely help to reduce pesticide use.

ACKNOWLEDGEMENTS

REFERENCES

Abbot, W. S., 1925, A method of computing the effectiveness of an insecticide *Journal of Economic Entomology*, 18: 265-267.

Ankersmith, G. W., 1953, DDT resistance in *Plutella maculipennis* (Curt.) (Lepidoptera) in Java .*Bulletin of Entomological Research*, 44: 421-425.

Audrey Leatemia, J., Murray B. Isman., 2004, Toxicity and antifeedant activity of crude seed extracts of *Annona squamosa* (Annonaceae) against lepidopteron pests and natural enemies. *International Journal of Tropical Insect science*, 24: 150-158.

Baur, M. E., Kaya, H. K., Tabashnik, B. E., Chilcutt, C., 1998, Suppression of diamond back moth (Lepidoptera: Plutellidae) with an entomopathogenic nematode (Rhabditidae: Stinernematidae) and *Bacillus thuringiensis Berliner. Journal of Economic Entomology*, 91: 1089-1095.

Beckett, A. H., Stenlake, J. B., 1986, Chromatography. In: Practical Pharmaceutical chemistry, Vol.23rd edition, Delhi, India, 75-76.

Chiu Shin-Foon., 1990, Investigations on botanical insecticides in South China-an update. In: Proc.Symp. Bot.Pesticides in IPM, Rajahmundry, 134-137

Claude B-Bernard., Bernard J. R. Philogene., 1993, Insecticide synergists: role, importance and perspectives. *Journal of toxicology and Environmental biology*, 38:199-223.

Dwijendra Singh., Sucheta S. Metha., Nirmal K. Neoliya., Yogendra N. Shukla., Mamta Mishra., 2003, New possible insect growth regulators from *Catharanthus roseus*, *Current Science*, 84: 1184-1186.

Egon Stahl., 1969, A laboratory hand book, 2nd ed. Springer Verlag, New York, USA.

EI-Bishry, M.H., 1994, Interaction between entomopathogenic nematodes and *Bacilus thuringiensis*: a new approach for biological control of insects. Egyptian. *Journal of Biology and Pest control*, 4:105-111.

EI-Hussini, M. M., Afifi AI., 1981. Effect of Entobacterin -3 on the spiny boll worm *Earias insulana* (Boisd), *Bulletin of Entomological Research* 75: 596-598.

Felke, M., Langerbruch, G. A., 2001, Does Bt pollen harm butterflies? Gesunde-Pflanzen, 53: 24-28.

Finney, D. J., 1971. Probit Analysis, 3rd edn.Cambridge University Press, Cambridge.

Hassanein, M. H., 1958, Biological studies on diamond back moth, *Plutella xylostella* (Curtis) (Lepidoptera: Plutellidae).*Bulletin of Entomology Society Egypt*, 42: 325-337.

Hellpap, C., Zebitz, C. P. W., 1986, Combined application of neem seed kernel extracts with *Bacillus thuringiensis* products for the control of *Spodoptera frugiperda* and *Aedes togoi*. *Journal of Applied Entomology*, 101: 515-524.

Hussan Bin Abdul Kadir., Christopher C. Payne., Norman E. Crook., John S. Fenlon., Doreen Winstanley., 1999,The comparative susceptibility of the diamond back moth *Plutella xylostella* and some other

major lepidopteran pests of brassica crops to a range of Baculoviruses. *Biocontrol science and technology*, 9: 421-433.

Javir, E. Q., 1992, *Foreward*. *In* : Talker, (Ed.) Diamondback moth and other crucifer pests, Proceeding of the Second International Workshop,10-14 December 1990, Asian Vegetable Research and Development Centre,Tainan,Taiwan, 11.

Johnson, D. R., 1953, *Plutella maculapennis* resistance to DDT in Java. *Journal of Economic Entomology*, 46: 176.

Kalyanasundaram, M., Das, P. K., 1985, Larvicidal and synergistic activity of plant extracts for mosquito control. *Indian Journal of Medical Research*, 82: 12-19.

Kirsch, K., Schmutterer, H., 1988, Low efficacy of a *Bacillus thuringiensis* (Berl.) formulation in controlling the diamondback moth *Plutella xylostella* (L.) in the Philippines. *Journal of Applied Entomology*, 105: 249-255.

Kohyama, Y., 1986, *Insecticidal activity of MK-139 (CME 134) against diamond back moth*. *In*: Diamondback moth management (Edited by N. S.Talekar and T. D.Griggs). Proceedings of the first International workshop, Taiwan, 265-269.

Ludlum, C. T., Felton, G. W., Duffey, S. S., 1991, Plant defense: Chlorogenic acid and poly phenol oxidase enhances toxicity of *Bacillus thuringiensis var kurstaki* to *Heliothis zea*. *Journal of Chemical Ecology*, 17: 217-237.

Moeschler, H. F., Pfuger W., Wendlisch, D., 1987, Pure annonin and a process for the preparation thereof, US Patent No.4, 689,323.

Mohan, M., Gujar, G. T., 2003, Characterization and comparison of midget proteases of *Bacillus thuringiensis* susceptible and resistant diamond back moth (Lepidoptera: Plutellidae).*Journal of Invertebrate Pathology*, 82: 1-11.

Molyneux, A. M., Bedding, R. A., Akhurst, R. J., 1983, Susceptibility of larvae of the sheep blowfly *Lucilia cuprina* to various Heterorhabditis spp., Neoaplectana spp .and undescribed Steinernematid nematode. *Journal of Invertebrate Pathology*, 42: 1-7.

Sarup, P., Dhingra, S., Agarwal, K. N., 1989, Newer dimensions for evaluating the synergistic effect of non-toxic chemicals in the mixed formulations against the adults of *Cylas formicarius* Fabricius. *Journal of Entomological Research*, 4: 1-14.

Saxena, R. C., Jayashree, S., Padma, S., Dixit, O. P., 1994, Evaluasion of growth disrupting activity of *Ageratum conyzoides* crude extract on *Culex quinquefasciatus* (Diptera: Culicidae). *Journal of Environmental Biology,* 15: 67-74.

Shelton, A. M., 2004, Management of the diamond back moth: Déjà vu all over again? In: Endersby, N. M., Ridland, P. M., (Eds).The management of diamondback moth and other crucifer pests proceeding of the Fourth International Workshop, 26-29 November 2001.Development of Natural Resources and Environment, Melbourne, Australia.

Shelton, A. M., Wyman, J. A., 1992, Insecticide resistance of diamond back moth in North America. In: Diamondback moth and other Crucifer Pests (edited by N.S.Talekar). Proceedings of the Second internationals Workshop, Taiwan, 447-454.

Tabashnik, B. E., Cushing, N. L., Finson, N., Johnson, M. W., 1990. Field development of resistance to *Bacillus thuringiensis* in diamond back moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology*, 83: 1671-1676.

Talekar, N. S., Shelton, A. M., 1993, Biology, ecology and management of the diamondback moth .*Annual Review of Entmology*, 38: 275-301.

Vickers, R. A., Furlong, M. J., White, A., Pell, J.K., 2004, Initiation of fungal epizootics in diamondback moth populations within a large field cage: proof of auto-dissemination. *Entomologica Experimentalis et Applicata*, 111: 7-17.