ENTOMOLOGY

Synergistic effect of certain insecticides combined with *Bacillus thuringiensis* on mosquito larvae

C.P. Narkhede,[1] C.D. Patil,[1] R.K. Suryawanshi,[1] S.H. Koli,[1] B.V. Mohite,[1] S.V. Patil[1,2]

[1]School of Life Sciences, and [2]North Maharashtra Microbial Culture Collection Centre, North Maharashtra University, Maharashtra, India

Abstract

For effective vector control it is essential to formulate new preparations having multiple action against the vector pest. Developing combined formulation of biopesticide and chemical pesticide is one of the novel concept to fight against the vectors with new weapons; however, compatibility of biopesticide *i.e. Bacillus thuringiensis* (Bt) and chemical pesticide is a real hurdle. In this investigation, local isolate *Bacillus thuringiensis* SV2 (BtSV2) was tested for its compatibility with various available mosquito larvicides. Temephos was most compatible with BtSV2 than with other tested pesticides. These two compatible agents were tested for larvicidal potential. Our study revealed that the synergistic effect of both agents reduces LC_{50} value by 30.68 and 22.36% against the *Ae. aegypti* and *An. stephensi,* respectively. The larvicidal potential increased when compared to individual pesticides. It was also observed a biochemical change in larvae after the TBT (Temephos + Bacillus thuringiensis) combination treatment; it involves decreased level of alpha esterase, acetylcholine esterase and protein while level of beta esterase and acid phosphatase was unchanged and alkaline phosphatase activity was increased. Increased potential of combined formulation may be due to altered physiological condition.

Correspondence: Satish V. Patil, School of Life Sciences, North Maharashtra University, Post Box 80, Jalgaon 425001, Maharashtra, India
E-mail: satish.patil7@gmail.com

Key words: Combination; *Bacillus thuringiensis* (Bt); Temephos; Esterase; Protease; Larvicidal.

Acknowledgements: Authors are indebted to University grant commission and Department of Science and technology, India for making the research facilities available under the UGC-SAP and UGC-FIST programme sanctioned to the School of Life Sciences. Mr. Chandrakant P Narkhede also thankful to UGC-BSR for providing fellowship (File No.-NMU/SLS/491/2015 UGC-BSR dated 11 August 2015)

Contributions: the authors contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Received for publication: 2 September 2016.
Revision received: 29 January 2017.
Accepted for publication: 30 January 2017.

Journal of Entomological and Acarological Research 2017; 49:6265
doi:10.4081/jear.2017.6265

Introduction

Vector-borne diseases affect two third of the world population as reported to kill millions of people every year; these diseases are directly associated with economic growth and costs limit of several undeveloped and developing countries.

Mosquitoes are still being the most important vectors spreading life-threatening human diseases. It is well documented that the synthetic insecticides dramatically reduce the risk of vector born diseases. However, the indiscriminate use of chemical pesticides like organophosphates and pyrethroids has led to the development of resistance in mosquito populations (McGaughey, 1985; Huang & Ottea, 2004).

Similarly, the eco-friendly and safe bioinsecticide *Bacillus thuringiensis* is being used worldwide as an effective mosquito control agent. The intensive use of *Bacillus thuringiensis* has led to the development of resistance in natural mosquito population (Boyer *et al*., 2012). However, some reports suggest a cross development against Bt, due to the increase in detoxifying activity of enzyme in xenobiotic reaction, decreasing activity of mid gut protease and a modification of specific receptor of mid gut (Jurat-Fuentes *et al*., 2004; Boyer *et al.,* 2007; Saengwiman *et al.,* 2011).

Despite that, majority of health and agriculture pest, which were brought under control by various pesticides, are now on rebound. The major vector pests-mosquitoes are well known for transferring dengue and malaria and are becoming resistant to most of the available pesticides.

To fight against the resistance problem, various methods were advocated like the use of different pesticides especially in combination with different chemical class/family that has a different mode of actions against the pest. This strategy definitely leads to prevent or to delay the development of resistance against pesticides.

The combined use of biological and chemical pesticides, having different mode of action, is one of the promising economic and eco-friendly strategies to fight the resistance problem.

However, the applicability and compatibility of this perspective is a real challenge. The incompatibility of pesticides of bio and synthetic origin may lead to a loss of efficacy of pest control, as well as economic loss and environmental hazards.

Compatibility is the capacity to mix different pesticides without physical or chemical interactions or changes, leading to the enhancement of their biological efficiency. The majority of researchers extensively studied the mode of action and resistance development mechanisms of biological and chemical pesticide in the past. This study aims to test the combination between Bt and chemical pesticides and its synergistic impact on larvicidal efficiency and mosquito larvae metabolism.

Materials and Methods

Chemicals and reagents

Nutrient broth, α-napthyl acetate, β-napthyl acetate, α-naphthol, β-naphthol, ethyl acetate were procured from Hi-Media (Mumbai, India) and temephos (EC 50%), chloropyrifos (EC 20%), cypermethrin (EC 25%), cyhalothrin (EC 5%), malathion (EC 50%), spinosad (EC 45%), azadirachtin (3000 ppm) was obtained from M/S Gharda chemicals limited (India).

Mosquito rearing

For the laboratory trial, early fourth instars larvae of *Ae. aegypti* and *An. stephensi* were maintained as Patil *et al*., 2012 In brief, the larvae were maintained in plastic enamel trays containing dechlorinated tap water and all the experiments were carried out at 28±2°C and 75-85% relative humidity under 14:10 light and dark cycles. Larvae were fed with a diet of finely ground brewer's yeast and dog biscuits (3:1).

Pesticide tolerance tests for *B. thuringiensis* SV2

The pesticide tolerance test was performed with previously isolated *B. thuringiensis* SV2 (BtSV2) strain (HM854748) (Patil *et al.,* 2012) using solid and liquid media. The compatibility and tolerance of BtSV2 isolate was tested at 5 ppm concentration of temephos, chloropyrifos, cypermethrin, cyhalothrin, malathion, spinosad and azadirachtin in nutrient agar and nutrient broth respectively at 28±2°C for 24 hrs.

The pesticide accumulation was studied by inoculating BtSV2 spore suspension (2×10^5/mL) in nutrient broth containing various pesticides at their highest concentrations as per WHO recommendations *i.e*. temephos (1 ppm), chloropyrifos (2.5 ppm), cypermethrin (20 ppm), cyhalothrin (25 ppm), malathion (15 ppm), spinosad (0.5 ppm) and azadirachtin (50 ppm) and incubated at 28±2°C for 48 hrs. Growth pattern of BtSV2 was observed by measuring OD spectrophotometrically at 600 nm in comparison to control (Harley & Prescott., 1996; Li *et al*., 2012)

Sample preparation for bioassay

The pesticides grown lyophilized culture of BtSV2 was dissolved in distilled water to prepare test concentrations and used to make different combinations with temephos. The lyophilized BtSV2 biomass without pesticide was dissolved in distilled water, which served as a control. The solutions were homogenized by stirring on shaker for 30 min at 120 rpm and used for mosquito larvicidal assay.

Larvicidal assay

The larvicidal assay was performed by individual lyophilized BtSV2 isolate, temephos and combined formulation of BtSV2 (TBT) and temephos at sub lethal concentrations of both temephos and BtSV2 *i.e.* A (1:1), B (1:2), C (1: 3) and D (1:4).

The twenty numbers of fourth instars larvae were introduced in 100 mL of the test medium containing a particular concentration of pesticide against the tap water control. All containers were maintained at room temperature with naturally prevailing photoperiod in the laboratory. Larval mortality was checked after 24 hrs of incubation. Each treatment was performed in triplicates. In all assays, the mortality of larvae was recorded and calculated by Abbott formula (Abbott, 1925). The larvicidal activity of BtSV2, temephos and TBT at various concentrations was subjected to probate regression analysis. The lethal concentrations in ppm (LC_{50}, LC_{90}) were calculated and recorded.

Preparation of whole body homogenates

Physiological and biochemical changes in larvae were analysed by biochemical experiments using the procedures outlined by WHO (WHO, 1998).

Preparation of larval extracts

For each replicate, twenty early fourth instars larvae treated with LC_{30} after 12 hrs were washed with distilled water and the adhering water was completely removed from the body surface by blotting with tissue paper and chilled in ice. The larvae then pooled and homogenized in eppendorf tubes (held in ice) using a Teflon hand homogenizer, homogenized using a vortex for 1 minute and centrifuged at 15,000 *g* for 10 min at 4°C. The clear supernatants from larvae of the same biological replicate were kept at 4°C, and further used for biochemical assays like protein, phosphatase, esterase, protease etc.

Total protein estimation

The proteins in the larval homogenates of TBT(A) treated larvae were first precipitated by 80% ethanol (Subhashini & Ravindranath, 1980) and the protein concentration (Lowry *et al.,* 1951) was estimated and used for further enzyme profile analysis.

Carboxyl esterase assay

Carboxyl esterase activity in the treated larval homogenates was measured with appropriate modifications (Van Asperen, 1962). Larval homogenate (200 µL) was mixed with 2 mL of the alpha and beta naphthyl acetate solution, reaction was allowed for 30 min at room temperature. After incubation, 500 µL of the fast blue SDS reagent was added (22.5 mg fast blue salt in 2.25 mL distilled water and 5% SDS in 0.2 M phosphate buffer (pH 7.2). Colour was allowed to develop for 15 min at RT. The optical density of the sample was read at 588 nm in the spectrophotometer against the respective reagent blank.

Phosphatase assay

Phosphatases in the treated larval homogenate were determined by the method of Powell & Smith, 1954. The reaction mixture consists of 1 mL carbonate buffer (pH 10.4) in 1 mL of 0.01 M disodium phenyl phosphate (substrate), and 100 µL of larval homogenate then incubated for 30 min at 37°C. At the end of incubation period, 0.8 mL of 0.5 N NaOH was added in 1.2 mL of 0.5 N $NaHCO_3$, followed by 1 mL of 4-aminoantipyrine solution and 1 mL of potassium ferricyanide. The produced colour was measured immediately by spectrophotometer at 510 nm. The enzymatic activity was expressed as µM/mg protein/min.

General proteases assay

Proteolytic activity was assayed by using azocasein (Sigma System, Marlborough USA) as a substrate. Briefly, 120 µL of enzyme solution was added to 480 µL of azocasein (1%, w/v) in reaction buffer (Tris buffer containing 5 mM $MgCl_2$) and the mixture was incubated at 30°C for 30 min. The reaction was terminated by adding 600 µL of 10% (w/v) trichloroacetic acid and incubated for 30 min at 4°C. Then reaction mixture was centrifuged at 15,000 *g*, at 4°C for 10 min. The 800 µL of resulting supernatant was removed and neutralized by adding 200 µL of 1.8 N NaOH, and the absorbance was measured at 420 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). One unit of enzyme activity was calculated as the amount product produced which leads to an increase in absorbance at 420 nm of 0.01 in 30 min at 30°C (Oppenoorth & Van Asperen, 1960; Elpidina *et al.,* 2001).

Synergistic effect

Synergistic effect of different combination was determined by the methods of Sun & Johnson (1960) and Clausing & Bieleke (1980). According to the formula reported by these authors, if CTC value >100, the effect is synergistic, V<0.8 corresponds to antagonism action, V from 0.8 to 1.5 corresponds to additive action and V>1.5 means potentiation.

Statistical analysis

Each experiment was performed with five determinations using samples from different preparations. The difference in the levels of various biochemical parameters between control and experimental larvae were tested for statistical significance using mean difference Student's t-test (MINITAB Software package). The acceptance level of statistical significance was $P \leq 0.05$ in all instances.

Results and discussion

On nutrient agar, *B. thuringiensis* SV2 grew as typical off-white coloured colonies with slightly raised elevation and regular margins in presence of malathion, temephos, cyhalothrin, spinosad, azadirachtin and cypermethrin supplemented with respective pesticide each at 5 ppm with zones of clearance were observed on solid medium after 8 days of incubation.

However, in nutrient broth it was found that, in presence of malathion and temephos, BtSV2 grows well without degrading pesticide but it degrades cyhalothrin, spinosad, azadirachtin, cypermethrin.

These results confirm that temephos and malathion have no inhibitory effect on growth of BtSV2 in broth culture (Figure 1). Hence temephos and malathion are the most suitable pesticides for combination. As per the WHO recommendations, temephos is used as larvicide while malathion as adulticide; so, temephos and BtSV2 combination was evaluated and recorded as a potent larvicidal formulation.

Mosquito larvicidal potential of individual Bt, temephos and their combinations were studied against the fourth instars larvae of *Ae. aegypti* and *An. stephensi*. It was observed that in combination, the larvicidal potential of TBT (A) was increased significantly as compared to individual applications of both pesticides.

In TBT the LC_{50} value against the *Ae. aegypti* and *An. stephensi* decrease by 30.68 and 22.36%. These results indicate that the reduction in LC_{50} values is due to synergistic action of both. For further combination studies, major three preparations were designed *i.e.* temephos plus Bt A (1:1), B (1:2), C (1:3) and D (1:4) to find out type of interaction taking place during the combined effect of temephos and BtSV2 at different ratio against *Ae. aegypti* and *An. stephensi*. Similarly co-toxicity indexes (CTC) for each combination were analysed. In all combinations CTC value was always above 100, indicating synergistic action of combined pesticide. In case of *An. stephensi* TBT(A) preparation (1:1), showed highest CTC value *i.e.* 1021.5 (Table 1) while in case of *Ae. aegypti,* TBT(C) preparation (1:3) with highest CTC value *i.e.* 1434.75 (Table 2).

The biochemical changes in treated larvae were monitored for BtSV2 and its combinations. It was observed that in both larval treatment TBT caused reduction in protein content as compared to control; on the contrary, in BtSV2 treatment the protein content

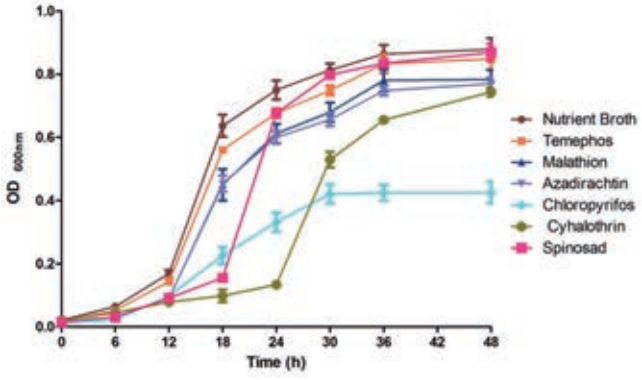

Figure 1. Effect of different pesticides on growth of *Bacillus thuringiensis* SV2.

Table 1. Effect of different combinations of temephos and BtSV2 on *Anopheles stephensi* fourth instar larvae.

Treatment	*Anopheles stephensi*			
	LC_{50} (ppm)	CTC	V index	Effect
Temephos	0.0088±0.00030	NA	NA	NA
BtSV2	3.154±0.185	NA	NA	NA
TBT (1:1)	0.006158±0.00023	1021.5	258.85	Synergistic
TBT (1:2)	0.0052±0.00023	996.59	304.11	Synergistic
TBT (1:3)	0.0043±0.00025	977.59	366.13	Synergistic
TBT (1:4)	0.0041±0.00032	895.38	384	Synergistic

CTC, co-toxicity coefficient. CTC<100, antagonism; CTC>100, synergistic effect. V-index (Clausing & Bieleke, 1980) >1.5 effect is synergistic.

Table 2. Effect of different combinations of temephos and BtSV2 on *Aedes aegypti* fourth instar larvae.

Treatment	*Aedes aegypti*			
	LC_{50} (ppm)	CTC	V index	Effect
Temephos	0.007462±0.00021	NA	NA	NA
BtSV2	3.601±0.231	NA	NA	NA
TBT (1:1)	0.0059±0.00023	1233.57	305.80	Synergistic
TBT (1:2)	0.0052±0.00029	1157.28	346.96	Synergistic
TBT (1:3)	0.0034±0.00034	1434.75	530.64	Synergistic
TBT (1:4)	0.0035±0.00031	1306.86	515.48	Synergistic

CTC, co-toxicity coefficient. CTC<100, antagonism; CTC>100, synergistic effect. V-index (Clausing & Bieleke, 1980) >1.5 effect is synergistic.

was slightly increased as compared to control. In an enzymatic study with TBT treatment, the activity of some enzymes were found to be decreased significantly *i.e.* α-esterase, acetylcholine esterase, while β-esterase and acid phosphatase were found to remain unchanged in any treatment and alkaline phosphatase level was found to be increased. The altered physiological condition in TBT treatment may have contributed to larval toxicity. Total protease level remained unchanged in TBT treatment as compared to untreated control while in BtSV2 treatment it was found to be slightly increased (Figure 2).

Gut proteases, modified receptor and other detoxifying enzymes like esterases, monooxygenase etc. have previously been reported for their major role in mechanism for insect resistance to Bt cry proteins (Rahman *et al.,* 2004; Bravo *et al.*, 2007; 2011). It was observed that in combination study, level of α carboxyl esterase and acetylcholine esterase was decreased than control, and enzymes like total proteases level remain unchanged. So with the use of combined formulation we may expect to have decreased chances of resistance generation against Bt.

In a previous report, the expression of several proteases has

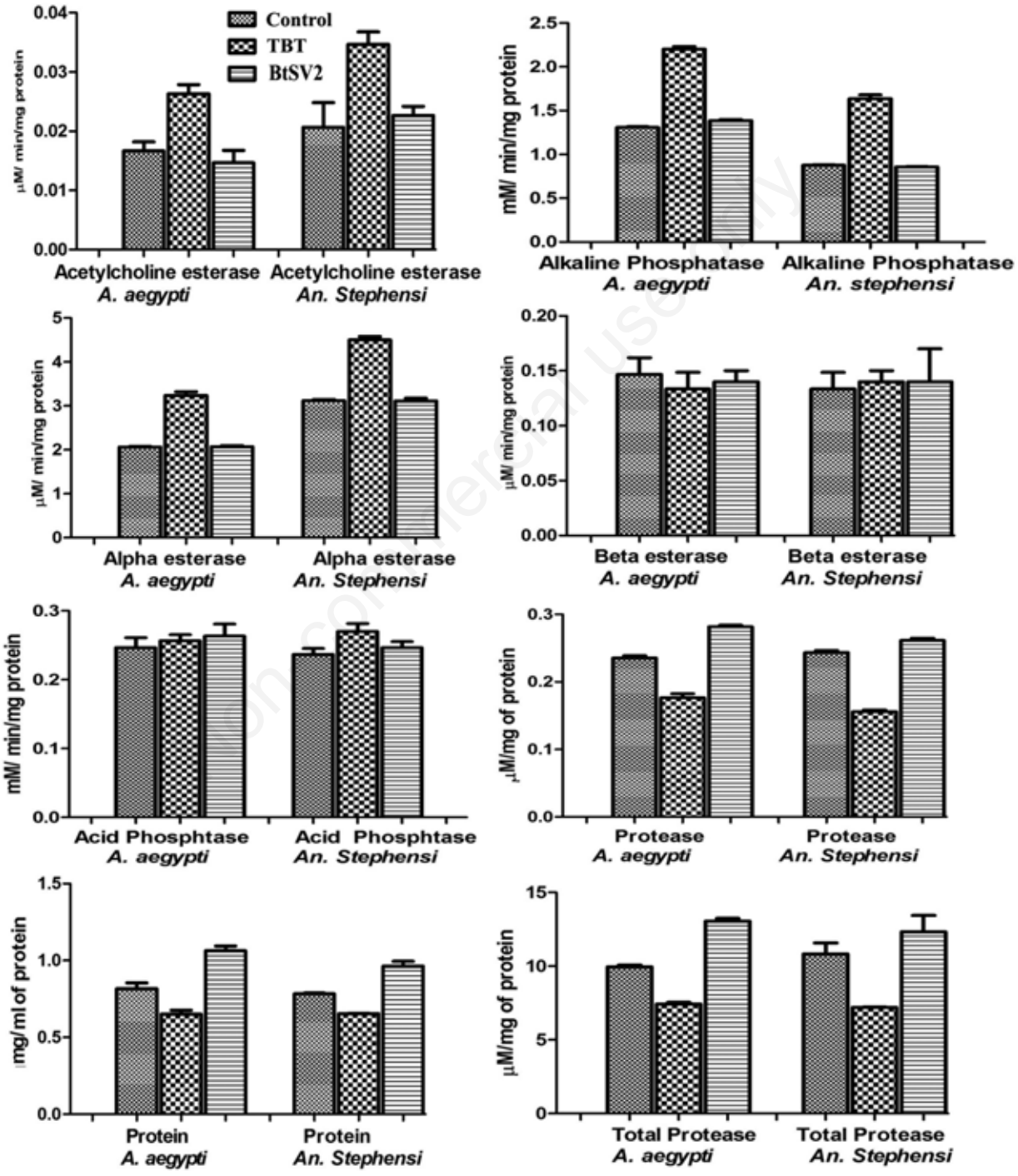

Figure 2. Effect of normal BtSV2 and TBT treatment on biochemical changes in mosquito larvae.

been reported in Bt resistant *Ae. aegypti* (Silva-Filha *et al*., 2014), but in our observation during TBT treatment protease neither increased or decreased. The level of proteases might have been maintained fixed, due to the contradicting effect of the individual components of the formulation. In organism on one side, Bt toxin might induce activity of proteases as a part its natural protection mechanism while on the other side temephos may be working to decrease activity of proteases. Therefore in the treated larvae finally the activity of proteases seems to remain unaltered. This is also supported by the increased protease activity in BtSV2 treatment.

In mixture treatment the level of acetylcholine was found to be decreased, similar reduction in acetylcholine esterase was previously reported by Koodalingam *et al*. (2012).

Although the mixture contains chemical pesticide *i.e.* temephos as well as BtSV2, it was observed that in combination treatment acetylcholine esterase and α-esterase levels were decreased. Previous report advocates rise of esterase level in chemical pesticide exposed insect pest or higher levels of esterase mainly concern with chemical pesticide resistance in mosquitoes and other insects *e.g. Culex quinquefasciatus* (Sahgal *et al*., 1994), *Blattella germanica* (Anspaugh *et al*., 1994; Polson *et al.,* 2011). Similarly the major role of carboxyl esterase has been reported for its detoxification of organophosphates in resistant pest (Rossiter *et al.*, 2001; Yang *et al*., 2004).

Besides these, level of alkaline phosphatase (ALP) was found to be increased in mixture treatment than untreated and BtSV2 exposed larvae. The justification for this may lie in fact that, ALP plays vital role in cry toxicity, which is already evaluated in *Ae. aegypti.* It is well known that ALP found in regions of microvilli where cry toxin binds to microvilli of gastric caeca. Similarly it was also reported that the ALP expression was significantly reduced in Cry1 resistance in insects (Jurat-Fuentes & Adang, 2004; Jurat-Fuentes *et al*., 2011; Flores-Escobar *et al*, 2013). In addition, ALPs have also been observed to bind a number of Cry toxins (Jiménez *et al*., 2012), as in *Manduca sexta*, *Heliothis virescens* and *Spodoptera frugiperda* (McNall & Adang, 2003; Jurat-Fuentes & Adang, 2004; Flores-Escobar *et al*., 2013).

Although during treatment (mixture treatment, untreated and BtSV2) there was no alteration of acid phosphatase activity in treated larvae. Decreased level of esterases, constant level of gut proteases and increased level of ALP is the ideal physiology reported in susceptible or sensitive pest, such ideal physiology was found to be maintained by combined formulation of temephos and BtSV2.

Conclusions

The combined preparation of Bt and temephos leads to biochemical changes in mosquito larvae, which eventually contributes to increase their toxicity. The formulation may have its implications on the use of reduced quantity of chemical pesticides, which is a major cause of resistance development and ecological pollution. Similarly, due to the differential mode of action, this combination may also contribute to prolong the generation of resistance in pest. Significantly, the cumulative strategy may improve performance of integrated pest management programs.

References

ABBOTT W.S., 1925 - A method of computing the effectiveness of an insecticide. - J. Eco. Entomol. 18: 265-266.

ANSPAUGH D.D., ROSE R.L., KOEHLER P.G., HODGSON E., ROE R.M., 1994 - Multiple mechanisms of pyrethroid resistance in the German cockroach, *Blattella germanica* (L.) - Pesti. Biochem. Phys. 50: 138-148.

BOYER S., PARIS M., JEGO S., LEMPERIERE G., RAVANEL P., 2012 - Influence of insecticide *Bacillus thuringiensis* subsp. israelensis treatments on resistance and enzyme activities in *Aedes rusticus* larvae (Diptera: Culicidae). - Biol. Control 62: 75-81.

BOYER S., TILQUIN M., RAVANEL P., 2007- Differential sensitivity to *Bacillus thuringiensis* var. *israelensis* and temephos in field mosquito populations *of Ochlerotatus cataphylla* (Diptera: Culicidae): toward resistance. - Environ. Toxicol. Chem. 26: 157-162.

BRAVO A., GILL S. S., SOBERON M., 2007 - Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. - Toxicon 49: 423-435.

BRAVO A., LIKITVIVATANAVONG S., GILL S.S., SOBERÓN M., 2011 - *Bacillus thuringiensis*: a story of a successful bioinsecticide. - Ins. Biochem. Mol. Biol. 41: 423-431.

CLAUSING P., BIELEKE R., 1980 - Aspects of methodology employed in the investigation of combined chemical effects on acute oral toxicity. - Arch. Toxicol. Suppl. 4: 394-395.

ELPIDINA E.N., VINOKUROV K.S., GROMENKO V.A., RUDENSKAYA Y.A., DUNAEVSKY Y.E., ZHUZHIKOV D.P., 2001 - Compartmentalization of proteinases and amylases in *Nauphoeta cinerea* midgut. - Arch. Insect. Biochem. Physiol. 48: 206-216.

FLORES-ESCOBAR B., RODRÍGUEZ-MAGADAN H., BRAVO A., SOBERÓN M., GÓMEZ I., 2013 - Differential role of *Manduca sexta* aminopeptidase-N and alkaline phosphatase in the mode of action of Cry1Aa, Cry1Ab, and Cry1Ac toxins from *Bacillus thuringiensis*. - Appl. Environ. Microbiol. 79: 4543-4550.

HARLEY J.P., PRESCOTT L.M., 1996 - Laboratory exercises in microbiology. 3rd ed. McGraw-Hill education, Columbus: 156-159 pp.

HUANG H., OTTEA J.A., 2004 - Development of pyrethroid substrates for esterase associated with pyrethroid resistance in the tobacco budworm, *Heliothis virescens* (F.). - J. Agric. Food Chem. 52: 6539-6545.

JIMÉNEZ A.I., REYES E.Z., CANCINO-RODEZNO A., BEDOYA-PÉREZ L.P., CABALLERO-FLORES G.G., MURIEL-MILLAN L.F., LIKITVIVATANAVONG S., GILL S.S., BRAVO A., SOBERÓN M., 2012 - *Aedes aegypti* alkaline phosphatase ALP1 is a functional receptor of *Bacillus thuringiensis* Cry4Ba and Cry11Aa toxins. - Insect. Biochem. Mol. Biol. 42: 683-689.

JURAT-FUENTES J.L., ADANG M.J., 2004 - Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. - Eur. J. Biochem. 271: 3127-3135.

JURAT-FUENTES J., GAHAN L., GOULD F., HECKEL D., ADANG M., 2004 - The HevCaLP protein mediates binding specificity of the Cry1A class of *Bacillus thuringiensis* toxins in *Heliothis virescens*. - Biochem. 43: 14299-14305.

JURAT-FUENTES J.L., KARUMBAIAH L., JAKKA S.R., NING C., LIU C., WU K., JACKSON J., GOULD F., BLANCO C., PORTILLA M., PERERA O., ADANG M., 2011 - Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from *Bacillus thuringiensis*.- PloS. One 1: e17606.

KOODALINGAM A., MULLAINADHAN P., RAJALAKSHMI A., DEEPALAKSHMI R., AMMU M., 2012 - Effect of a Bt-

based product (Vectobar) on esterases and phosphatases from larvae of the mosquito *Aedes aegypti*. - Pest. Biochem. Physiol. 104: 267-272.

LI X., DING X., XIA L., SUN Y., YUAN C., YIN J., 2012 - Proteomic analysis of *Bacillus thuringiensis* strain 4.0718 at different growth phases. - Sci. World. J. 2012: 1-10.

LOWRY O.H., ROSENBROUGH N.J., FARR A.L., RANDALL R.J., 1951 - Protein measurement with the Folin-phenol reagent. - J. Biol. Chem. 193: 265-275.

MCGAUGHEY W.H., 1985 - Insect resistance to the biological insecticide *Bacillus thuringiensis*. - Science 229: 193-195.

MCNALL R.J., ADANG M.J., 2003 - Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. - Insect Biochem. Mol. Biol. 33: 999-1010.

OPPENOORTH F.J., VAN ASPEREN K., 1960 - Allelic genes in the housefly producing modified enzymes that cause organophosphate resistance. - Science 13: 2298-2299.

PATIL C.D., PATIL S.V., SALUNKE B.K., SALUNKHE R.B., 2012 - Insecticidal potency of bacterial species *Bacillus thuringiensis* SV2 and *Serratia nematodiphila* SV6 against larvae of mosquito species *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. - Parasitol. Res. 110: 1841-1847.

POLSON K.A., BROGDON W.G., RAWLINS S.C., CHADEE D.D., 2011 - Characterization of insecticide resistance in *Trinidadian* strains of *Aedes aegypti* mosquitoes. - Acta Trop. 117: 31-38.

POWELL M.E.A., SMITH M.J.H., 1954 - The determination of serum acid and alkaline phosphatase activity with 4-aminoantipyrine. - J. Clin. Pathol. 7: 245-248.

RAHMAN M.M., ROBERTS H L., SARJAN M., ASGARI S., SCHMIDT O., 2004 - Induction and transmission of *Bacillus thuringiensis* tolerance in the flour moth *Ephestia kuehniella*. - Proc. Natl. Acad. Sci. USA 101: 2696-2699.

ROSSITER L.C., GUNNING R.V., ROSE H.A., 2001 - The use of polyacrylamide gel electrophoresis for the investigation and detection of fenitrothion and chlorpyrifos-methyl resistance in *Oryzaephilus surinamensis* (Coleoptera: Silvanidae). - Pest. Biochem. Physiol. 69: 27-34.

SAENGWIMAN S., AROONKESORN A., DEDVISITSAKUL P., SAKDEE S., LEETACHEWA S., ANGSUTHANASOMBAT C., 2011 - In vivo identification of *Bacillus thuringiensis* Cry4Ba toxin receptors by RNA interference knockdown of glycosylphosphatidylinositol-linked aminopeptidase N transcripts in *Aedes aegypti* larvae. - Biochem. Biophys. Res. Commun. 4: 708-713.

SAHGAL A., KUMAR S., PILLAI M.K.K., 1994 - Microplate assay of elevated esterase activity in individual pyrethroid-resistant mosquitoes. - J. Biosci. 19: 193-199.

SILVA-FILHA M.H.N.L., BERRY C., REGIS L., 2014 - *Lysinibacillus sphaericus*: Toxins and mode of action, applications for mosquito control and resistance management. In: Advances in insect physiology: insect midgut and insecticidal proteins. Dhadialla T.S., Gill S.S., Eds. Elsevier, Oxford: pp 89-176.

SUBHASHINI M.H., RAVINDRANATH M.H., 1980 - Haemolymph protein concentration of *Scylla serrate*: assessment of quantitative methods and intra-individual variability. - Arch. Int. Physiol. Biochem. 88: 47-51.

SUN Y.P., JOHNSON E.R., 1960 - Analysis of joint action of insecticides against house flies. - J. Ecol. Entomol. 53: 887-892.

VAN ASPEREN R., 1962 - A study of housefly esterase by means of sensitive colorimetric method. - J. Insect. Physiol. 8: 401-416.

WHO, 1998 - Techniques to detect insecticide resistance mechanisms (field and laboratory manual). - WHO, Geneva.

YANG Y., WU Y., CHEN S., DEVINE G.J., DENHOLM I., JEWESS P., MOORES G.D., 2004 - The involvement of microsomal oxidases in pyrethroid resistance in *Helicoverpa armigera* from Asia. - Insect. Biochem. Mol. Biol. 34: 763-773.