# BIOLOGICAL AND MOLECULAR STUDIES ON THE TOXIC EFFECT OF VEGETATIVE INSECTICIDAL PROTEIN (VIPs) OF *Bacillus thuringiensis* EGYPTIAN ISOLATES AGAINST WHITEFLIES

**LAMIAA F. EL-GAIED, H. EL-SHESHTAWY AND W. EL-MENOFY**

*Departement of Microbial Molecular Biology, Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt*

The whitefly *Bemisia tabaci* (Gennadius) is a taxonomically diverse group, consisting of more than 20 cryptic members worldwide with enormous variation in ecological and biological traits. Whiteflies have been recognized as virus vectors since 1930. However, they have become very important pests during the last three decades. Whiteflies cause plant damage either directly, by the extraction of phloem nutrients which can result in 50% yield reduction, by feeding punctures and the honey dew extracted by these insects serve as medium for sooty mold fungi, or indirectly by acting as vectors of several economically important viral plant pathogens (Costa, 1979; Abdallah *et al*., 1993; Bedford *et al*., 1994; Inbar and Gerling, 2008). In Egypt, the most severe damage is associated with infections occurring in April till November.

Some factors that result in whiteflies to be so widespread and cause a lot of damage are that it has a wide host range, resistance to many insecticides, a high reproductive capacity, and capability of transmitting viruses (Mohammad, 2005; Schuster *et al*., 2008 & 2010).

Whitefly transmitted geminiviruses (WTGs) cause epidemics in vegetable and fiber crops with an increasing prevalence and distribution in tropical, sub tropical regions of the world and can infect more than 600 species of host plants (Bird and Maramorosch, 1978; Thiago *et al*., 2012; El-Gaied *et al*., 2014). It also considered as the major vector transmitting various types of geminiviruses as tomato yellow leaf curl virus (TYLCV) which cause great damage to tomatoes crop. Because of the increasing importance of WTGs, rapid and accurate methods are needed for controlling such insects (Gottlieb *et al.*, 2010).

*Bacillus thuringiensis* (*Bt*) is an important biological control agent, widely used in agriculture around the world due to its insecticidal activity. *B. thuringiensis* belongs to the *Bacillaceae* family, is a gram positive, rod –shaped, spore-forming bacterium produces parasporal bodies (inclusions) during the process of sporulation. These inclusions are pro-toxins which when ingested by susceptible insect larvae are activated into toxins (δ-endotoxins) into midgut leading to paraly-

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sis of mouth parts and gut and eventually death of the larvae (Balaraman, 2005). Due to its well-known insecticidal activity, many Bt-based commercial formulations are available on the market (Burges, 1982).

In addition to δ-endotoxins, *B. thuringiensis* produces a novel family of insecticidal proteins named vegetative insecticidal proteins (Vips) during its vegetative stage (Estruch *et al*., 1996; Yu *et al*., 2011). The type of Vip toxins have been classified as *vip1- vip4*, are ranked according to their homology (http://www.lifesci.sussex.ac. uk/home/ Neil_Crickmore/Bt/vip.html).

Vegetative insecticidal proteins (*Vips*) are secretable proteins from *B. thuringiensis* (Estruch *et al*., 1996; Warren, 1997) which do not share sequence homology with known toxic Cry proteins. It was reported that *B. thuringiensis* VIPs have display insecticidal activity against a wide variety of lepidopterans (Yu *et al*., 1997; Estruch and Yu, 1998), coleopterans (Warren, 1997) and some sap-sucking insect pests (Sattar and Maiti, 2011). In this study, we are specifically investigating the pathogencity of *B. thuringiensis* vegetative proteins as a new approach for controlling whiteflies population in Egypt. A better understanding of such interactions will, in turn, offer valuable insight into the long-term sustainable control of whiteflies.

## MATERIALS AND METHODS

### *Bacterial strains and culture media*

Bacterial strains used in this study are *Bacillus thuringiensis* Subsp aegypti, BtC-18 and BtDI-29, were isolated from soil samples from Egypt. *B. thuringiensis* strains C18 and DI29 were obtained from AGERI and grown on LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) overnight at 28-30°C for 16 hour to reach the vegetative phase of bacterium growth. BtC-18 was isolated, characterized and patented in USA under NO: 5986177.

### *Isolation of B. thuringiensis DNA*

*Bacillus thuringiensis* strain C18 and DI29 were grown in LB agar medium at 30°C for 16 h. The cells (about 100 mg) were pelleted and re-suspended in 100 µl SI (1 M sucrose, 25 mM Tris-HCl (pH 8.0), 25 mM EDTA, 20 mg/ml lysozyme). Then, the mixture was incubated at 37°C for 30 min. For cell lysis, 200 µl of SII (200 mM Tris-HCl, 10 mM EDTA, 1 M NaCl, 2% SDS, 1/10 volume phenol, pH 8.0) was added and mixed gently. After incubated at 37°C for 1 h, the mixture was mixed with 400 µl chloroform gently and centrifuged at 12,000 xg for 10 min. The genome DNA was precipitated by mixing the supernatant from the last step with an equal volume of isopropanol and the pellet washed twice by 70% ethanol. Finally, the DNA pellet was eluted in 100 µl sterilized water and used as template for PCR amplification of *vip* genes fragments.

***PCR amplification of vip genes***

Three sets of specific primers were designed and used to check the presence of *vip1*, *vip2* and *vip3* genes in BtC-18 isolate based on the published sequences local isolates in GeneBank under accession numbers; JN008908, JN035904 and JF811911 for *vip1, vip2* and *vip3*, respectively. The PCR reactions were performed in a total volume of 50 µl containing 1 µl of the appropriate upper-primer (10 pmol/µl), 1 µl of the appropriate lower-primer (10 pmol/µl), 1.5 µl $MgCl_2$ (50 mM), 1.5 µl of dNTPs mixture solution (10 mM), 2 µl of DNA template (0.1-0.5 µg), 5 µl of 10X PCR reaction buffer and 0.5 µl of Taq DNA polymerase (5 U/µl). The reaction volume was completed to 50 µl using autoclaved $ddH_2O$. For a typical PCR the following programme was used: an initial 95°C for 3 min; a total of 30 cycles of denaturation at 95°C for 1 min, primer annealing at 50/55°C for 1 min and primer extension at 72°C for 1 min, final cycle at 72°C for 7 min to allow the completion of primer extension. PCR-amplified DNA fragments were analyzed by electrophoresis on a 1% agarose gel prepared in 1X TAE buffer. Primers used in the reaction are illustrated in Table (1).

***SDS-PAGE and protein determination***

Total bacterial protein extracts were separated according to its molecular mass using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). For the experiments, the Bio-Rad mini-protein II dual slab gel was used with 10 ml of 15% separating gel containing 5 ml of 30%/0.8% (v/v) acrylamid/ bisacrylamid (Carl Roth GmbH, Germany), 2.3 ml $ddH_2O$, 2.5 ml of 1.5 M Tris-HCL (pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulfate (APS) and 0.004 ml of TEMED (N,N,tetramethylethylenediamine). The mixture was poured slowly between two glass plates and overlaid with 1-2 ml of $ddH_2O$ saturated Butanol. After complete polymerization of the gel (15-20 min), the overlay was removed and the surface was rinsed with 1X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). About 2 ml of 5% stacking gel containing 0.33 ml of 30%/0.8% acrylamid/ bisacrylamid, 1.4 ml $ddH_2O$, 0.25 ml of 1 M Tris-HCL (pH6.8), 0.02 ml of 10% SDS, 0.02 ml of 10% APS and 0.002 ml of TEMED was poured onto the separating gel and then the combs were placed. Electrophoresis was carried out in 1X SDS-PAGE running buffer. For preparation of the protein samples, about 0.25 volume of 4 X SDS protein sample buffer was added and the samples were boiled for 5 min. The samples were loaded and the gel was run at 75V until the dye reached the separating gel and then the gel was run at 150V for 60 min. After running, the gels were stained in coomassie brilliant blue protein stain solution with gentle shaking overnight and then destained in protein destaining solution for 30-45 min according to the standard protocol (Sambrook *et al*., 1989).

***Ammonium sulphate precipitation of VIPs***

*Bacillus thuringiensis* C18 subsp aegypti was grown for 16 h at 28°C on LB medium. Solid ammonium sulphate was added to the *B. thurenginsis* culture to 80% relative saturation. After storing at 4°C overnight, the precipitate was collected by centrifugation (12,000 g for 30 min). Precipitates were redissolved in distilled water, and dialyzed extensively against distilled water for 2 days to remove ammonium sulphate. The solution was centrifuged at 10,000 g for 20 min at 4°C to remove the insoluble components, before freeze-drying to yield crude proteins. The crude proteins were dissolved in a small volume of distilled water to prepare a crude protein solution to be applied for SDS-PAGE analysis.

***Insect rearing***

Whiteflies (*Bemisia tabaci*) collected from tomato plants were reared on cotton plants (*Gossypium hirsutum* L.*)* grown in insect wooden proof cage in controlled room temperature provided with 16 h illumination/day for one month.

***$LC_{50}$ and $LT_{50}$***

A group of 30 newly hatched whiteflies were collected from cotton plants and left starved for 4h in conical flask covered with a Parafilm membrane, then fed on 3% sucrose solution supplemented with different concentration of bacterial culture added over the Para film for insect feeding. Different concentration of bacterial culture were used as follow; 25, 50, 100, 200 ppm for each type of bacteria and kept for for 3, 6, 12 and 24 h for each concentration. Mortality of whiteflies was recorded compared with controlled insects feed on 3% sucrose solution. The experiment was repeated three times and the average were taken to determine $LC_{50}$ and $LT_{50}$ values**.** Further purified VIP protein of BtC-18 using 80% ammonium sulphate concentration precipitation were used to determine its $LC_{50}$ using serials of concentration as follow; 50, 250, 500 and 1000 ppm using previous conditions each test were repeated three times for confirmation and the average was taken to determine the $LC_{50}$ and $LT_{50}$ values by probit analysis according to Finny *et al.* (1971).

## RESULTS AND DISCUSSION

***Determination of $LC_{50}$ and $LT_{50}$ values***

In this study we investigating the insecticidal protein toxicity of VIPs produced during the vegetative stage of *B. thuringiensis* growth of isolates BtC-18 and BtDI-29 as a new approach for controlling of whiteflies population. For that, the biological activity of VIPs was evaluated against whiteflies population. The $LC_{50}$ value for BtC-18 and BtDI-29 were determined by feeding of whiteflies on 3% sucrose solution with different concentrations of the whole culture ranging from 25-1000 ppm for both isolates. Control treatments consisted of uninfected insects and insects fed on 3% sucrose solution. The results of the bioassay revealed that the $LC_{50}$ value for BtC-18 was 90 ppm,

compared to the $LC_{50}$ value for BtDI-29 which was 160 ppm. Regression analysis of the whiteflies response to BtC-18 and BtDI-29 are illustrated in (Fig. 1). The $LC_{50}$ was determined by probit analysis plot (Finny, 1971). The result of the bioassay revealed that the BtC-18 is about two-fold more effective than BtDI-29. On the other hand, the calculated $LT_{50}$ was 10 h for BtC-18, where it was 11 h for BtDI-29. The $LT_{50}$ value for BtC-18 and BtDI-29 was similar if not identical and thus was not statistically different. From the $LC_{50}$ and $LT_{50}$ analysis it was concluded that insects fed on sucrose solution contains BtC-18 vegetative bacterial cells gave relatively higher level of mortality compared to insects fed on sucrose solution contains BtDI-29 bacterial cells based on $LC_{50}$ and $LT_{50}$ analysis. These results led to further characterization of VIP proteins of BtC-18 isolate as a promising biopesticides against whiteflies. For that, BtC-18 will be subjected to further protein purification and PCR analysis.

***PCR amplification of vip genes***

In order to check the presence of *vip* genes into the genome of *B. thurengiensis* BtC-18, three sets of specific primers were designed. As shown in Fig. (2), the predicted molecular size of the amplified gene fragments was successfully detected as: 420 bp, 790 bp and 530 bp for *vip1, vip2 and vip3* genes, respectively. The nonspecific bands that shown with vip1 PCR fragment could be due to the unspecific annealing temperature that was used. Optimization of the vip1 PCR amplification program are still needed. Detection of *vip* genes expected molecular size suggested that *B. thuringiensis* strain BtC-18 harboring the three *vip* gens on its genome. These in accordance of the results obtained by Osman *et al*. (2013), who confirmed the presence of Vip3A into BtC-18 genome through the detection of the expressed VIP3 protein using Vip3A antibody by western blotting and amino acid sequencing.

***Purification and insecticidal activity of VIPs against whitefly***

To further examine the insecticidal activity of vegetative proteins fractions against whiteflies population, *B. thuringiensis* bacterial culture was purified using ammonium sulphate precipitation. The 80% ammonium sulfate saturation was used to precipitate the high molecular weight of vegetative proteins. For that, 100 ml overnight culture was grown on LB medium at 28-30°C for 16 hour to reach the vegetative phase of bacterium growth. Total protein was extracted from bacterial pellets and applied for protein purification using 80% ammonium sulphate precipitation method. The purified protein was electrophoretically separated (Fig. 3, A and B). After storing at 4°C overnight, the precipitate was collected by centrifugation (12,000 g for 30 min) and the lystae were subjected to SDS-PAGE to check the degree of purity and to estimate the molecular weights of the protein components.

The results showed that a band at ~88 kDa was clearly detected (Figure 3B)

corresponding to the molecular mass of VIP3. This result is in accordance with Sellami *et al*. (2001), Shi *et al*., (2006), Sattar *et al*. (2011) and Osman *et al.* (2013). They investigated the presence of VIP3 at ~88 kDa isolated from *B. thuringiensis* species and its efficacy as insecticide against different insect species. The ~88 kDa protein fraction was bioassayed against whiteflies' population and $LC_{50}$ was calculated using USEPA Probit Analysis Program v1.5. As shown in Table (2), the calculated $LC_{50}$ for BtC-18 precipitated ~88 KDa protein was 898 ppm. Control insects were fed on 3% sucrose solution.

The results showed that the ~88 kDa purified protein was active against white fly with an $LC_{50}$ of 898 ppm. Whereas total vegetative protein extracts killed 50% of white flies adults with $LC_{50}$ of 90 ppm. These results demonstrated that the activity of the whole bacterial culture was about 9-fold more potent than the VIP3 as a separate protein fraction against whitefly insects. Recently, the insecticidal activity of purified proteins from BtC-18 strain isolated during vegetative states has been examined against different insect species. A protein banding pattern showed a protein band at ~88 kDa that showed insecticidal activity against Black Cutworm (BCW) first instar larvae with an estimated $LC_{50}$ of 26 ng cm $^{-2}$. The results of western blot analysis showed that Vip3A antibody cross-reacted with the protein band at the size of ~88 kDa suggested that the detected protein band at 88 KDa is corresponding to the Vip3A protein (Osman *et al.*, 2013). By further alignment of amino acids sequence of the purified vegetative protein fraction of 88 kDa using BtC-18 vegetative culture with the published protein database, it was showed that it matched with a similar stretch of N-terminal amino acids of the Vip3Aa toxin protein with 92% amino acid sequence identity. While the analysis showed 92% identity with Cry35Aa for a 44 kDa fraction obtained after FPLC vegetative proteins fractionation Osman *et al*. (2013). This also clearly agrees with the results obtained by Sellami *et al*. (2001). They had examined the maximal production of the secreted VIP3LB (also called Vip3Aa16 with ~88.5 KDa) during the growth of the wild-type strain *B. thuringiensis* BUPM 95) against *S. littoralis* second instart larvae. It was concluded that *B. thuringiensis* Vip3LB protein affects clearly the growth of *S. littoralis* second-instars larvae. Recently, several isolates of *B. thuringiensis* were found to have also some activity on Dipteran insects such as mosquitoes. However, the significant concentrations were too high for offering any operational promise (Balaraman, 2005).

In a PCR screening of *vip* genes of *B. thurengiensis* isolates, it was found that the occurrence of *vip3* genes was more common than the occurrence of *vip1* and *vip2* genes. Whereas only around 10% of the isolates showed *vip1* or *vip2* gene amplification, almost half of them contained genes belonging to the vip3 family (Hernandez-Rodryguez *et al.*, 2009). This frequency of vip3 genes was similar to

that observed by Espinasse *et al.* (2003). This finding can explain the only using of VIP3 in this study for further purification and inscectisidal activity determination against whitefly.

It is a well-established fact that the presence of both the components of the binary toxinVIP1-VIP2 is essential for their activities against some coleopteran insects (Carozzi and Koziel, 1997). These may explain the high potency of the *B. thurengiensis* whole culture against whitefly compared to VIP3 separate protein fraction that was observed in this study.

Further studies on the individual proteins toxicity component of VIP1 and VIP2 of *B. thuringiensis* BtC-18 are still needed in order to investigate the rule of VIP proteins and *vip* gene family for the control of whitefly population.

## SUMMARY

Whitefly *Bemisia tabaci* (Gennadius) transmitted geminiviruses cause epidemics in vegetable and fiber crops. It can infect more than 600 species of host plants. It also considered as the major vector transmitting various types of geminiviruses such as tomato yellow leaf curl virus (TYLCV) which cause great damage to tomatoes crop. Previous studies have reported that the vegetative insecticidal proteins (VIPs) of *Bacillus thuringiensis* (*Bt*) revealed insecticidal activity against different insect species. In this study, two local isolates of *B. thuringiensis* named BtC-18 and BtDI-29 were screened for the insecticidal activity of VIPs against whitefly population. Analysis of median lethal concentration ($LC_{50}$) revealed that *B. thuringiensis* strain BtC-18 is more potent and toxic than BtDI-29 strain against whiteflies with an estimated $LC_{50}$ of 90 ppm and 160 ppm for BtC-18 and BtDI-29, respectively. However, the median lethal time ($LT_{50}$) value did not show significant difference between both isolates. PCR analysis of *vip* genes confirmed the presence of *vip1, vip2* and *vip3* genes on BtC-18 genome. Proteins extract from the BtC-18 culture pellet were further purified by 80% saturation of ammonium sulfate precipitation. The purified protein showed a clear band at ~88 KDa corresponding to Vip3A protein as previously demonstrated. The $LC_{50}$ of the purified band at 88KDa showed insecticidal activity against white fly with an estimated $LC_{50}$ of 898 ppm.

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Table (1): Nucleotide sequences of the primers used to amplify *vip1*, *vip2* and *vip3* gene fargments.

| Primers name | Primer sequence (5'….3') | Product size (Bp) |
|---|---|---|
| Vip1int-F | AGGCCGCAAGGGATTTAGAT | 420 |
| Vip1int-R | CGTTGCGATGGTATTGTTATT | |
| Vip2int-F | AATTACTTTTTCTATGGCAGGTTC | 790 |
| Vip2int-R | TAAGGCAGGTAAAGGCTCACT | |
| Vip3int-F | GATCGTCCCGCCAAGTG | 530 |
| Vip3int-R | TCCCTCGTCCTCCCTGATA | |

Table (2): $LC_{50}$ calculation of purified VIP3 protein fraction against whiteflies.

| Toxin | Nr. larvae | $LC_{50}$ (Cl) | Slope (SE) | $\chi^2$ |
|---|---|---|---|---|
| VIP3 | 30 | 898[a] (609 - 2489) | 2.10 (0.75) | 5.991 |

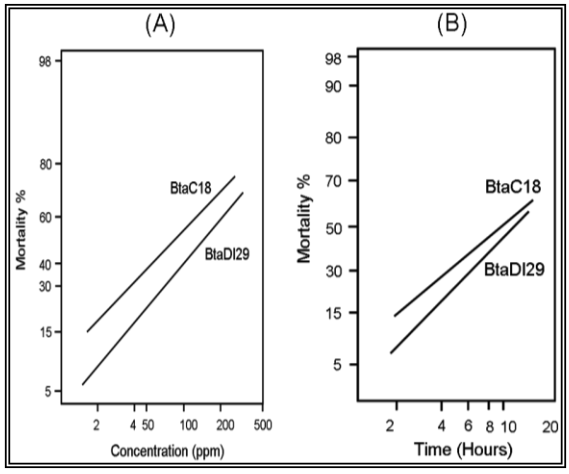


Fig. (1): $LC_{50}$ and $LT_{50}$ regression line of whiteflies response to different vegetative culture of *B. thurengiensis* BtC-18 and BtDI-29. (A) Median lethal concentration ($LC_{50}$) of BtC-18 and BtDI-29 culture. (B) Median lethal time ($LT_{50}$) of BtC-18 and BtDI-29 culture.

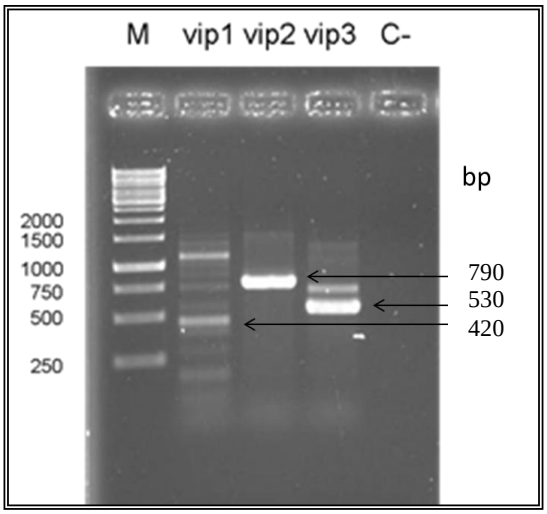


Fig. (2): PCR amplification of *vip* genes using BtC-18 total DNA. The PCR amplifications shows the expected molecular weight of each gene fragnemnts at 420 bp, 790 bp and 530 bp for *vip1*, *vip2* and *vip3* genes, respectivily. M: I kb DNA ladder

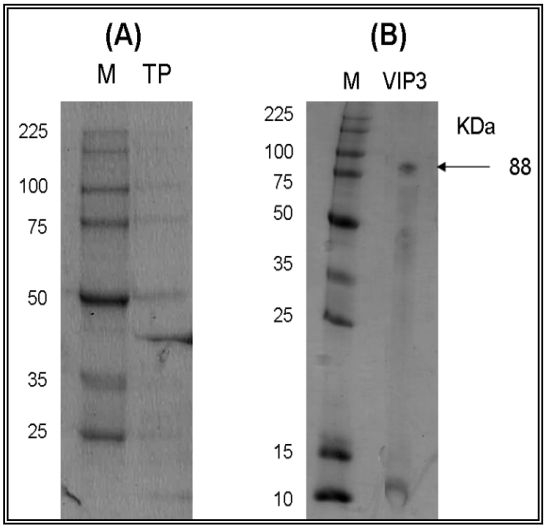


Fig. (3): SDS-PAGE analysis of purified VIP proteins of *B. thurenginsis* BtC-18 using ammonium sulfate precipitation. (A) Total protein extracts of BtC-18 overnight culture. (B) Purified VIP protein using ammonium sulphate precipitation at ~88 KDa corresponding to VIP3A. M: Broad range protein ladder.