14

Biological Activity of Insecticidal Toxins: Structural Basis, Site-Directed Mutagenesis and Perspectives

Silvio Alejandro López-Pazos[1,3] and Jairo Cerón[2]
[1]*Facultad de Ciencias de la Salud, Universidad Colegio Mayor de Cundinamarca,*
[2]*Instituto de Biotecnología, Universidad Nacional de Colombia, Santafé de Bogotá DC,*
[3]*Biology & Rural Ecology research group, Corporación Ramsar Guamuez,*
El Encano (Pasto-Nariño)
[1,2,3]*Colombia*

1. Introduction

Insect pests destroy about 18% of crop production each year and transmit disease agents (Oerke & Dehn, 2004). Beetles (order Coleoptera) are the largest and most diverse group of eukaryotes. They contain species of harvest pests that produce major losses around the world (Wang et al., 2007). Some examples of coleopteran pests follow: *Dectes texanus* [Coleoptera (order): Cerambycidae (family)], attacks soybeans; *Tribolium castaneum* (Coleoptera: Tenebrionidae), a biological problem of stored products; *Hypothenemus hampei* (Coleoptera: Scolytidae), an entomological problem of coffee crops; and *Premnotrypes vorax* (Coleoptera: Curculionidae), a potato pest in South America (Abdelghany et al., 2010; Tindall et al., 2010; López-Pazos et al., 2009b; Pai & Bernasconi, 2008; Damon, 2000). Lepidopteran species constitute an important group of harmful harvest pests that affect commercial agriculture. Among them are the following: the cotton bollworms, *Helicoverpa armigera* and *H. zea* (both Lepidoptera: Noctuidae); *Tecia solanivora* (Lepidoptera: Gelechiidae), a pest in potato crops of the Americas; *Plutella xylostella* (Lepidoptera: Plutellidae), of great importance in cruciferous crops; and the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), which causes losses in corn, cotton and rice (Keszthelyi et al., 2011; Du et al., 2011; Chagas et al., 2010; Suckling & Brockerhoff, 2010; Bosa et al., 2006; Monnerat et al., 2006).

The biological control of insect pests is an important alternative to the management of insects (or Integrated Pest Management-IPM). Unfortunately insect pests have been attacked primarily with chemical products, which cause huge environmental losses and adverse effects on human health. However, biological control and IPM-compatible chemicals can be used together [as outlined in a recent review by Gentz et al., (2010). Extensive research has centred on the search for an appropriate insecticidal peptide or polypeptide with toxicity to pest organisms, but not to flora and fauna. Researchers also hope to establish the most appropriate means of delivering the biological molecule to its site of action (De Lima et al., 2007). Recombinant DNA technology allows the exploitation of the insecticidal properties of

entomopathogenic organisms. It offers environmentally friendly options for the cost-effective control of insect pests (St Leger & Wang, 2010). Bioinsecticides include microbial agents, natural enemies, plant defences, metabolites, pheromones and genes that transcribe toxic peptides or proteins. The number and variety of toxins is extensive. For example, there are at least 0.5 million insecticidal toxins from arachnids, and evidence suggests that the use of novel toxic factors is likely to be extensive (Whetstone & Hammock 2007).

2. Typical anti-insect toxins

There are two classes of insecticidal toxins: (1) peptide-like toxins (3-10 kDa) from some scorpion and spider venoms and (2) the high molecular mass toxins (*i.e.*, about 1000 residues), such as the latrotoxins from the venom of the spider *Latrodectus* or the crystal proteins of *Bacillus thuringiensis* (De Lima et al., 2007; Schnepf et al., 1998). The toxins of the first group consist of one chain that contains many cysteine residues and intramolecular disulfide bridges. These peptides interact with ion channels (*i.e.*, those for Na^+, K^+, $Ca2^+$ and Cl^-) on cellular membranes (De Lima et al., 2007). Recently a peptide-like toxin nomenclature has been proposed that takes into account the basis of activity, the biological source and the relationship with other toxins (King et al., 2008). The primary sources of entomopathogenic proteins in the second group of toxins are several organisms, including spiders, snakes, scorpions, anemones, snails, lacewings, insects, fungi and bacteria (De Lima et al., 2007; Schnepf et al., 1998).

Toxins from arthropod venoms consist of combinations of biologically active compounds (peptides, proteins, nucleotides, lipids and other molecules). They are used for paralysing insects and for defence against natural enemies. They interact with ion channels and/or receptors from neurological systems in the target organism (De Lima et al., 2007).Venom-derived peptide toxins target voltage-gated Na^+, K^+, Ca^{2+}, or Cl^- channels. Proteins, such as neuropeptides and hormones, are analogous. Their effects depend upon their specific activities (Whetstone & Hammock, 2007). Antagonists disrupt and interfere with development and behaviour. Spiders and scorpions maybe the most important arthropods having insecticidal toxins. Many spider venoms contain a complex mixture of both neurotoxic and cytolytic toxins (see: www.arachnoserver.org). Virtually all insecticidal spider toxins contain a cystine-knot motif that provides them with chemical and biological stability (King et al., 2002; Tedford et al., 2004). These types of venoms contain acylpolyamines (from the Araneidae family), cytolytic toxins (from the Zodariidae family) and neurotoxic peptides (J-atratoxins), and neurotoxins (>10 kDa) and enzymes (~35 kDa) in the Sicariidae and Theridiidae families respectively (Vassilevski et al., 2009; Gunning et al., 2008). *Agelenopsis aperta* employs venom that is very active against insects. It is composed of toxins (agatoxins) that attack transmitter-activated cation channels, voltage-activated sodium channels and voltage-activated calcium channels. The α-agatoxins, μ-agatoxins and ω-agatoxins alter insect ion channels (Adams, 2004). Australian funnel-web spiders [Mygalomorphae (order): Hexathelidae (family): Atracinae (subfamily)] have ω-atracotoxins (36–37 residues with six cysteines in a disulfide pattern), which slow insect cation voltage-dependent channels (Chong et al., 2007).

Scorpions are a special group of organisms that have interesting toxins. These toxins have 23-78 residues. Generally the conformation has an α-helix packed against a three-stranded β-sheet stabilized by four disulfide bonds. Scorpion toxins recognize the face of voltage-

dependent sodium channels and alter their gating. They are defined as α-or β-toxins, based on their mechanism of action (Rodríguez de la Vega et al., 2010; Gurevitz et al., 2007; Karbat et al., 2004). Anti-insect α-toxins bind to voltage-dependent sodium channels with high affinity (Gordon et al., 2007). Scorpion β-toxins change the voltage dependence of channel activation. The first class of entomopathogenic scorpion β-toxins is comprised of excitatory toxins. They are composed of 70-76 amino acids. These toxins may induce spastic paralysis by the activation of sodium flux at negative membrane potential. A second group consists of depress ant toxins, which induce flaccid paralysis by depolarization of the axonal membrane. A third set is composed of active toxins, which act on both insect and mammalian sodium channels, with typical depressant effects on insects (Gurevitz et al., 2007).

Surprisingly, some insects (such as the tobacco hornworm *Manduca sexta*) produce insecticidal peptides (each peptide has 23 amino acids) from haemolymph. These molecules can cause paralysis in the larvae of many insects (Skinner et al., 1991). For example, a dose of 105 plaque-forming units of baculovirus containing a poneratoxin DNA sequence from the ant, *Paraponera clavata*, was adequate for controlling lepidopteran individuals (*S. frugiperda*) (Szolajska et al., 2004).

Microorganisms possess toxins for the biological control of insects. Fungus is an entomopathogenic option. *Beauveria bassiana* has a long history in relation to the control of lepidopteran, coleopteran and dipteran species (Howard et al., 2010; Qin et al., 2010; Cruz et al., 2006; Shah & Pell, 2003). *Metarhizium anisopliae* has been used against ticks and insects, this fungus has a wide set of virulent factors, such as lipolytic enzymes, proteases, chitinases and toxins (destruxins) (Schrank & Vainstein, 2010; Pava-Ripoll et al., 2008). Ascomycota (genera *Cordyceps*, *Hypocrella* and *Torrubiella*), Zygomycota (genera *Conidiobolus* and *Entomophaga*), Deuteromycota (genus *Aschersonia*), Zygomycetes (genus *Entomophthora*) and Hyphomycetes (genus *Hirsutella*), which have activity against lepidopterans and coleopterans (Shah & Pell, 2003). Many bacteria, such as *Serratia marcescens*, *Photorhabdus luminescens, B. thuringiensis* and *Xenorhabdus nematophilus*, can produce entomopathogenic toxins (Roh et al., 2010; Whetstone & Hammock, 2007). Baculoviruses have been used as safe and effective biopesticides for the protection of crops and forests in the Americas, Europe and Asia. The *oryctes* virus has also demonstrated insecticidal activity against the rhinoceros beetle. The entomopathogenic parvoviruses are an insecticidal option. The *H. armigera* stunt virus (a tetravirus) has been isolated from pests and may be useful for the development of genetically modified plants (Whetstone & Hammock, 2007).

Plants produce a great variety of toxic compounds that are responsible for insect self-defense mechanisms. Plant cyclotides contain 30 amino acids with acyclic peptide backbone and a knotted alignment of three conserved disulphide bonds connected in a "cystine knot" motif. Members of Lepidoptera and Coleoptera are susceptible to plant cyclotides from the Violaceae, Rubiaceae and Cucurbitaceae families (Gruber et al., 2007). Plant cysteine proteases are accumulated after lepidopteran infestation affecting insect growth (Pechan et al., 2002). Plant defensins are antimicrobial proteins with eight conserved cysteines and four disulfide bridges. Defensins attack lepidopteran α-amylases, causing feeding inhibition (Kanchiswamy et al., 2010; Rayapuram & Baldwin, 2008). Plant glucanases, chitinases, lectins and dehydrins are induced after attack by lepidopteran and coleopteran pests (Ralph et al., 2006).

3. The phylogenetic relationship of insecticidal toxins and their comparison with lepidopteran- and coleopteran-specific molecules

Twenty-seven amino acid sequences from the RCSB Protein Data Bank (PDB) (http://www.pdb.org/pdb/home/home.do) were selected by a bibliographical revision, using the criteria of established insect-specific toxicity. Next a phylogenetic analysis of insect-specific toxins was performed (Figure 1) by means of Phylogeny.fr platform (http://www.phylogeny.fr/) (Dereeeper et al., 2008). The available data from a bibliographical search,show insecticidal protein sequences from a large variety of organisms with toxicity against several orders of targets, including 11 anti-lepidopteran toxins and five coleopteran-specific toxins (Table 1).

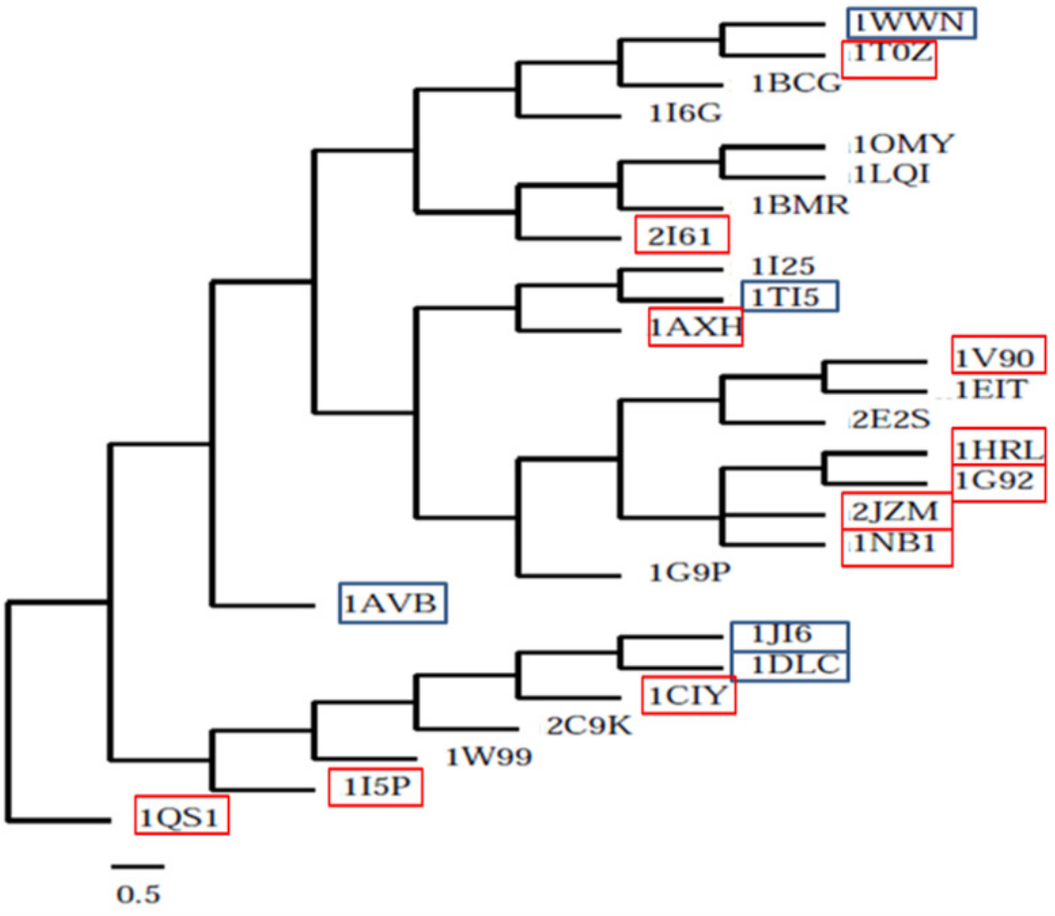

Fig. 1. Phylogenetic tree for insecticidal toxins. The blue squares indicate the coleopteran-specific amino acid sequences and the red squares show antilepidopteran toxins. The analysis of the toxins was done by the parsimony method with the TNT 1.1 program, using the alignment previously obtained with MUSCLE 3.7. The analysis was carried out 1000 times in order to obtain a strict consensus tree by using the bootstrapping tool. The consensus phylogenetic tree was computed by the TreeDyn 198.3. See the text for an analysis.

ID PDB	TOXIN	SOURCE	ORDER TARGET	REFERENCES
1AVB	Arcelin 1	*Phaseolus vulgaris*	Coleoptera	Fabre et al., 1998; Mourey et al., 1998
1AXH	ω-ACTX-HV1	*Hadronyche versuta*	Lepidoptera, Diptera, Ixodida	Chong et al., 2007; Fletcher et al., 1997
1BCG	Bjxtr-IT	*Buthotus judaicus*	Blattaria	Possani et al., 1999; Oren et al., 1998
1BMR	Lqh III	*Leiurus quinquestriatushebraeus*	Blattaria	Krimm et al., 1999
1CIY	Cry1Aa	*Bacillus thuringiensis*	Lepidoptera	Grochulski et al., 1995; López-Pazos & Cerón, 2007
1DLC	Cry3A	*Bacillus thuringiensis*	Coleoptera	Li et al., 1991; López-Pazos & Cerón, 2007
1EIT	μ-agatoxin	*Agelenopsis aperta*	Diptera	Adams, 2004; Omecinsky et al., 1996
1G92	Poneratoxin	*Paraponera clavata*	Lepidoptera	Szolajska et al., 2004
1G9P	ω-Atracotoxin-HV2A	*Hadronyche versuta*	Orthoptera	Chong et al., 2007; Wang et al., 2001
1HRL	PP1	*Manduca sexta*	Lepidoptera	Yu et al., 1999; Skinner et al., 1991
1I5P	Cry2Aa	*Bacillus thuringiensis*	Lepidoptera, Diptera	Morse et al., 2001; López-Pazos & Cerón, 2007
1I6G	CsE-v5	*Centruroides sculpturatus Ewing*	Blattaria	Jablonsky et al., 2001; Possani et al., 1999; Lee et al., 1994
1JI6	Cry3Bb1	*Bacillus thuringiensis*	Coleoptera	Galitsky et al., 2001; López-Pazos & Cerón, 2007
1LQI	Lqh(α)IT	*Leiurus quinquestriatus hebraeus*	Diptera	Tugarinov et al., 1997; Zilberberg et al., 1997
1I25	Huwentoxin-II	*Selenocosmia huwena*	Blattaria	Liang., 2004; Shu et al., 2002
1NB1	Kalata B1	*Oldenlandia affinis*	Lepidoptera	Rosengren et al., 2003; Gruber et al., 2007
1OMY	BmKaIT1	*Buthus martensii* Karsch	Diptera, Orthoptera	Ji et al., 1996

ID PDB	TOXIN	SOURCE	ORDER TARGET	REFERENCES
1QS1	VIP2	*Bacillus thuringiensis*	Lepidoptera	Han et al., 1999
1TI5	VrD1	*Vigna radiata*	Coleoptera	Liu et al., 2006
1T0Z	BmK IT-AP	*Buthus martensii* Karsch	Lepidoptera	Li et al., 2005; Hao et al., 2005
1V90	δ-palutoxin IT1	*Paracoelotes luctuosus*	Lepidoptera	De Lima et al., 2007; Ferrat et al., 2005
1WWN	BmK-βIT	*Buthus martensii* Karsch	It displays toxicity against Diptera and is related with AaIT from *Androctonus australis* Hector with activity against Blattaria, Orthoptera, Diptera and Coleoptera	Pava-Ripoll et al., 2008; Zlotkin et al., 2000
1W99	Cry4Ba	*Bacillus thuringiensis*	Diptera	Boonserm et al., 2005; López-Pazos & Cerón, 2007
2C9K	Cry4Aa	*Bacillus thuringiensis*	Diptera	van Frankenhuyzen, 2009; Boonserm et al., 2006
2E2S	Agelenin	*Agelena opulenta*	Orthoptera	Yamaji et al., 2007
2I61	LqhIT2	*Leiurus quinquestriatushebraeus*	Lepidoptera, Diptera	Karbat et al., 2007; De Lima et al., 2007
2JZM	Chymotrypsin inhibitor C1	*Nicotiana alata*	Lepidoptera	Schirra et al., 2008; Schirra et al., 2001; Miller et al., 2000

Table 1. Some toxins from several sources for which **experimentally determined structures** are available in the Protein Data Bank (PDB).

The observed toxin phylogenies - specifically active against lepidopteran species - have several relationships among them and are distributed along all of the branches (Figure 1). *B. thuringiensis* proteins (Cry and vegetative insecticidal protein (VIP)) are closely related in a separated branch, containing three lepidopteran-specific proteins (Cry1Aa, Cry2Aa and VIP2). BmK IT-AP is related with BmK-βIT, Bjxtr-IT and CsE-v5. The antilepidopteran structure 2I61 is in the same group as 1BMR, 1LQI and 1OMY. The Hadronyche versuta toxin (ω-ACTX-Hv1a) has proximity with Huwentoxin-II (*Ornithoctonus huwena*) and the coleopteran-specific VrD1 from the wild mung bean. 1V90 (a lepidopteran-specific toxin), 1EIT and 2E2S are close. The antilepidopteran toxic factors PP1, Poneratoxin, Kalata B1 and

chymotrypsin inhibitor C1, have proximity with ω-Atracotoxin-Hv2A from *H. versuta*. Only arcelin1 is in a different site. One might ask whether the amino acid sequences associated with antilepidopteran toxins could have the same biological role, such as 1G9P, 2E2S, 1EIT, 1I25 or 1WWN. Moreover, the phylogenetic tree showed no relationship among Coleopteran-specific sequences, except for 1DLC and 1JI6, which belong to the family of *B. thuringiensis* Cry toxins (Figure 1, Table 1). However, this analysis indicates that 1T0Z (from the Asian scorpion *Buthus martensi* Karsch) and 1I25 (from the Chinese bird spider *O. huwena*) may have anti-coleopteran properties due to the fact that they are in the same branch as 1WWN and 1TI5, respectively (Figure 1). Studies have shown that insecticidal toxins purified from arthropod venoms exert their effects via specific interactions with ion channels and receptors in the central or peripheral nervous system (De Lima et al., 2007; Bloomquist, 2003; Johnson et al., 1998; Fletcher et al., 1997). *B. martensi* Karsch venom has four peptides related to the excitatory insect toxin family and 10 related to the depressant insect toxin (Goudet et al., 2002). Huwentoxin-II (from the spider *O. huwena*) can paralyse cockroaches for hours (ED50 of 29 ± 12 nmol/g) and increase the activity of Huwentoxin-I (a toxin targeting ion channels) (Liang, 2004).

4. Insecticidal toxins and site-directed mutagenesis: case reports

Site-directed mutagenesis is a powerful methodology for studying function and protein structure through manipulation at the level of the DNA molecule. Advances in site-directed mutagenesis have allowed the transfer of new or improved gene roles between organisms, such as bacteria, plants and animals (Adair & Wallace, 1998; James & Dickinson, 1998). In this section, we describe several experiences of the application of site-directed mutagenesis on insecticidal toxin sequences.

4.1 Mutagenesis exposes essential residues in the anti-insect toxin Av2 from *Anemonia viridis*

Sea anemones (Metazoa, Cnidaria, Anthozoa, and Hexacorallia) are sessile predators that are highly dependent on their venom for prospering in a wide range of ecological environments. Venom analysis shows a significant collection of low molecular weight toxins: ~20 kDa pore-forming toxins, 3.5–6.5 kDa voltage-gated potassium channel-active toxins and 3–5 kDa polypeptide toxins active on voltage-gated sodium channels (Navs) (Moran et al., 2009). [A Nav has a central role in the excitability of animals. It functions in the initiation and propagation of action potentials (Goldin, 2002).]

The *Anemonia viridis* toxin 2 (Av2) is a lethal neurotoxin. Av2 has shown a clear preference for insect Nav from the assessment of toxin effects on the *Drosophila melanogaster* sodium channel (DmNav1) expressed in *Xenopus laevis* oocytes (Moran et al., 2009; Warmke et al., 1997). Hence, mutagenesis offers a means of examining residues thought to be important for Av2 activity on insect Navs. A synthetic gene coding for Av2 was designed. It was cloned into the expression vector pET-14b and used to transform appropriate *Escherichia coli* cells (strain BL21). Av2 point mutations (Note: amino acid abbreviations and single-letter designations are given in Table 1 of the chapter by Figurski et al.) [V2A (*i.e.*, residue 2 changed from V to A), P3A, L5A, D7A, S8A, D9A, G10A, G10P, S12A, V13A, R14A, G15A, G15P, N16A, T17A, L18A, G20P, I21A, P28A, S29A, W31A, H32A, N33A, K35A, K36A,

H37A, P39A, T40A, I41A, W43A and Q47A] were established by means of PCR (Polymerase Chain Reaction) using the appropriate primers and the synthetic Av2 gene as the DNA template. The mutant proteins were purified by reverse-phase high performance liquid chromatography. Toxicity assays were done on *Sarcophaga falculata* blowfly larvae. (They were scrutinized for immobilization and contraction). Competition binding assays were done with the neuronal membranes of adult cockroaches (*Periplaneta americana*). The toxicity correlated well with the results of the binding assays. This study indicated that N-terminal aliphatic residues (V2 and L5) play a role in such activity. The central region of the toxin is not involved in the toxic activity. W23 and L24 are important residues in toxin structure. At the C-terminus, it is noteworthy that residue I41 is involved in the bioactive surface of Av2. Residues V2, L5, D9, N16, L18 and I41 are pivotal amino acids for toxicity to blowfly larvae and for binding to cockroach neuronal membranes. The information from these mutants may be applicable to other insect orders (Moran et al., 2006).

4.2 Mutagenesis demonstrates that N183 is a key residue for the mode of action of the Cry4Ba protein

B. thuringiensis is a biopesticide bacterium. Its insecticidal properties are attributed (predominantly) to Cry toxins (a protein family), which are synthesized during the sporulation phase of the organism (Roh et al., 2007). The Cry protein is ingested by the susceptible insect, solubilized in the gut lumen, and cleaved by proteases to yield the activated 60 kDa toxin. Next Cry toxins are recognized by cadherin-like receptors (CADR) to assemble oligomeric forms of the toxin. The toxin oligomers have binding affinities to the secondary receptors: aminopeptidase N (APN), alkaline phosphatase (ALP), ADAM metalloprotease or glycosylphosphatidyl-inositol (GPI)-anchored proteins. The oligomers insert into the apical membrane of midgut-generating pores to cause osmotic lysis and insect death (Ochoa-Campuzano et al. 2007; Pigott & Ellar, 2007). Cry toxin is composed of three functional domains. Domain I comprises seven hydrophobic and amphipathic α-helices and is capable of forming pores in the apical membrane of the insect midgut. Domain II is made of three variable anti-parallel β-sheets, which are responsible for receptor recognition. Domain III has two anti-parallel β-strands involved in structural stability and receptor binding (Schnepf et al., 1998). Site-directed mutagenesis on Cry proteins revealed the function of each domain in the toxicity to the target insect. This fact provides a perspective on the generation of toxins with enhanced toxicity or new specificities.

A collection of Cry4Ba mutants (Figure 2), which are modified in polar uncharged residues (Y178, Q180, N183, N185, and N195) within α-helix 5, were developed to observe their effects on biological activity. All mutant toxins were generated using PCR-based site-directed mutagenesis, and each mutant was expressed from the *lac* promoter in *E. coli* upon IPTG (isopropyl β-D-thiogalactopyranoside) induction. The Cry4Ba-N183A mutant does not display lethality, while alanine substitutions for other residues (Y178, Q180, N185, and N195) still maintained more than 70% of the insect toxicity of the Cry4Ba standard (Figure 2). This result indicated that N183 plays an important role in the functionality of the Cry4Ba toxin (Likitvivatanavong et al., 2006).

Other studies indicated that N183 plays a crucial role in both toxic and structural properties. Mutants N183Q and N183K were made so as to be insoluble at alkaline pH. Mutations at N183 using several residues (with different structural characteristics) revealed that

substitutions with a polar amino acid still retained lethal activity similar to the Cry4Ba standard. Nevertheless, changes to charged or nonpolar residues suppressed biological activity (Figure 2). In conclusion, N183 polarity and α-helix 5 localization (in the middle of domain I) are very important to the toxicity of the Cry4Ba protein (Likitvivatanavong et al., 2006).

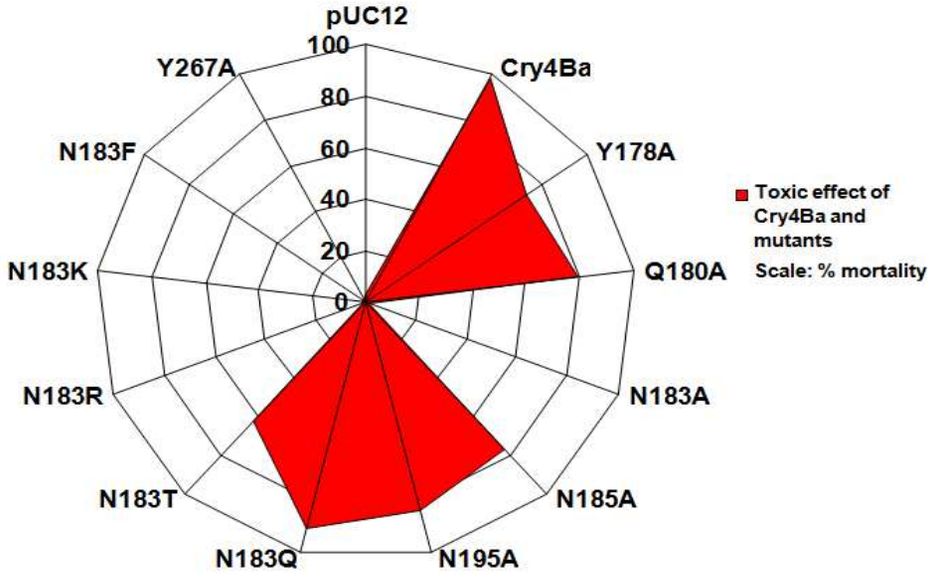

Fig. 2. Biological activity of Cry4Ba and mutants. The red colour indicates lethality and level. Bioassays for mosquito-larvicidal activity were performed using 2-day-old *Stegomyia* (*Aedes*) *aegypti* (mosquito) larvae. The altered residues in the mutant proteins are given on the outside of the graph. The gene for the mutant protein was inserted into the plasmid expression vector pUC12 and induced from the *lac* promoter. pUC12 on the graph depicts the toxicity of the vector alone.

4.3 A Juvenile hormone esterase with a mutated α helix shows improved insecticidal effects

Juvenile hormone (JH) regulates several physiological events in insects (development, metamorphosis, reproduction, diapause, migration, polyphenism and metabolism). JH esterase (JHE) is a hydrolytic enzyme from the α/β-hydrolase fold family, which metabolizes JH (Kamita et al., 2003). When JHE is injected into lepidopteran larval states, it causes a darkening and a decrease in feeding (Hammock et al., 1990; Philpott & Hammock, 1990). JHE is rapidly cleared from the haemolymph following inoculation, suggesting a discriminatory system for its elimination (El-Sayed et al., 2011). In testing, it was revealed that the double histidine mutated JHE [JHE K204H and R208H (in an amphipathic α helix)] is capable of blocking clearance from the haemolymph by reducing its binding to the JHE receptor. These experiments used *Autographa californica* NPV (AcMNPV, a baculovirus with pathogenic activity towards insect pests) as an expression vehicle. JHE shows enhanced insecticidal activity against the lepidopteran larvae of *M. sexta* (tobacco hornworm), *Heliothis virescens* (tobacco budworm) and *Agrotis ipsilon* (black cutworm) (El-Sayed et al., 2011).

Mutant and wild-type JHEs were produced and purified from insect cells, and their activities were found in the culture supernatants of insect cells. The specific activity of

mutant JHE was 6.5 nmol of JH III acid (a metabolism product of JH by JHE) formed min^{-1} mg^{-1}. The specific activity of wild-type JHE was 61.3 nmol of JH III acid formed min^{-1} mg^{-1}. The K204H and/or R208H alterations, although far-removed from the catalytic site of the protein, induced allosteric properties that led to a decrease in activity. No statistically significant differences were seen in the clearance of JH hydrolysis activity in the fourth instars of *H. virescens*, *A. ipsilon* and *M. sexta*. Bioassays (using the first instars of *H. virescens* and *A. ipsilon*) were done to establish the lethal concentration and the lethal time and to determine the result of the expression of mutant JHE on the insecticidal lethality of the baculovirus. The results showed that the median lethal concentration of mutant JHE was 3.2-fold lower in *H. virescens*, in contrast to the effect of AcMNPV. There is no effect on *A. ipsilon*, as observed by the bioassay (Table 2). The most notable difference between the esterases was the higher median lethal concentration (1.9-fold) of mutant JHE compared to a non-mutant JHE against *A. ipsilon* (Table 2). The median lethal concentration of mutant JHE in *H. virescens* was 3.5-fold lower than mutant JHE in *A. ipsilon*. The median lethal time of *H. virescens* and *A. ipsilon* treated with mutant JHE was about 4.8 and 5.3 days, respectively. It was about the same for non-mutant JHE. In addition, feeding assays were carried out using the first instars of *M. sexta* (for 4 days on an artificial diet or on a tomato leaf). The results showed 41–90% lower mass for the mutant than for the JHE wild type (non-mutant) at the end of the experiment. The study showed that point mutations of the amphipathic α-helix were sufficient for improving insecticidal activity (El-Sayed et al., 2011).

Insect	Esterase	Median lethal concentration ($x10^5$) (95% Confidence Limits)
H. virescens	Mutant JHE	1.8 (1.0-2.6)
	Wild type JHE	**2.7** (1.8-3.8)
A. ipsilon	Mutant JHE	6.3 (3.6-13)
	Wild type JHE	3.3 (2.3-4.6)

Table 2. Lethal concentrations of mutant and wild-type versions of JHE in the first instar larvae of *H. virescens* and *A. ipsilon*. Insects were inoculated with recombinant JHEs in a polyhedral virus vehicle. The median lethal concentration is expressed as polyhedra per ml (modified of El-Sayed et al., 2011).

4.4 Predicting important residues responsible for the capacity of scorpion α-toxins to discriminate between insect and mammalian voltage-gated sodium channels

Scorpion toxins are poison molecules (61–67 amino acids). Scorpion α-toxins recognize voltage-gated sodium channels (NaCh). NaChs mediate the temporary increase in sodium ion permeability thereby generating action potentials. The toxin expands the action potential by delaying the inactivation stage (Gordon et al., 2007). LqhαIT, from the scorpion *Leiurus quinquestriatus hebraeus*, is an α-toxin that is highly active on insect NaChs. A mutagenic analysis of LqhαIT was performed, revealing that the residues important for function are grouped into two different domains. A new toxin made by putting the efficient region of LqhαIT onto Aah2 (an anti-mammalian α-toxin from the scorpion *Androctonus australis* Hector) proved to be anti-insect (Karbat et al., 2004).

Mutations in the cDNAs of *L. quinquestriatus hebraeus* encoding LqhαIT were generated by PCR (Gurevitz et al., 1991). A CD (Circular Dichroism) Spectroscopy analysis was recorded at 25°C (Karbat et al., 2004). Some residues (Y14, E15, D19, Y21, E24, L25, K28, A39, N54 and P56) had no effect on the biological action or alteration of the CD spectrum. N44 and mutants F17G/A, R18A, W38A had decreased lethality and an unchanged CD spectrum. The F17W and W38Y mutants had activities similar to wild-type LqhαIT, so aromatic side chains affect toxin function. The substitutions I57A/T, R58K, V59A/G, R58K/V59A, K62A/L/R and R64N in the C-terminal region reduced biological activity. The substitution R58N had a marked negative effect on biological activity. This result implies that both charged amine groups and the aliphatic moiety in R58 are principal determinants in functionality. Biologically important residues appear in two domains. The first domain (core-domain) consists of F17, R18, W38 and N44. The second domain (NC-domain) is formed by residues K8, Y10, P56, I57, R58, V59, K62 and R64 (Karbat et al., 2004). LqhαIT and Aah2 have an overall similarity of 70%, although the similarity varies in the NC-domain. The core-domain and the NC-domain of Aah2 were replaced by the LqhαIT counterparts to generate four hybrids (Table 3). The constructs were evaluated with biological assays using *S. falculata* blowfly larvae. Immobilization and contraction were measured, and an effective dose of 50% (ED50) was calculated (Table 3) (Karbat et al., 2004).

Toxin	**ED50/100 mg of *S. falculata* body weight**
Parental	
LqhαIT	13 ng
Aah2	> 10 μg
Mutant toxin	
$Aah2^{LqhαIT(8–10)}$	> 10 μg
$Aah2^{LqhαIT(56–64)}$	> 10 μg
$Aah2^{LqhαIT(8–10,\ 56–64)}$	64 ng
$Aah2^{LqhαIT(8–10,\ G17F,\ 56–64)}$	37 ng

Table 3. Toxicity assays of Aah2 and its counterpart mutants (Karbat et al., 2004).

The similar activities of $Aah2^{LqhαIT(8–10,\ G17F,\ 56–64)}$ and LqhαIT indicate that their functional NC-domains are equally oriented. This indicates that the increase of insecticidal activity is related to the arrangement of the NC-domain in a structure that projects into the solvent. Remarkably this conformation is universal to all scorpion α-toxins with lethality on insects, in contrast with the flat face in α-toxins that are toxic to mammals (Karbat et al., 2004).

5. Final remarks

5.1 Novel sources?

Whole-genome sequencing projects are a resource of biological functions and their annotation allows for the detection of proteins through orthologous sequences (common ancestry), searches and primary and tertiary structure correlation - a process named "comparative genomics" (Lee et al. 2007; Ellegren, 2008). This theoretical approach makes it possible to find candidate toxins in sequenced genomes. An appropriate criterion for the identification of novel lepidopteran and coleopteran candidate toxins can be understood in

terms of the "guilt by association" principle (Gabaldon &Huynen, 2004; Aravind, 2000). For this reason, we applied a very basic protocol (Figure 3). BLAST (tblastn) searches from the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov /Blast.cgi). Searches were done using each toxin (from Table 1) as a query. The iterative searches were done for proteins larger than 100 aminoacids with an inclusion threshold of 0.01 (the statistical significance limit for inclusion of a sequence in the process) and for proteins smaller than 100 aminoacids with an inclusion threshold of 0.1. The searches used the 881 completely sequenced bacterial and archaeal genomes available on the NCBI Microbial Genomes website at the time of this analysis (January 2011) and the entire NCBI environmental samples database (1.66 million Whole Genome Shotgun reads) (see http://www.ncbi.nlm.nih.gov/). The searches were done until either convergence was achieved or until the last iteration before the first known false positives appeared. Significant hits to proteins encoded in these genomes were further classified as possible insect-specific toxins. The BLAST analysis showed fourteen microbial sequences with a high similarity to insecticidal queries (Table 4). There is a version of Arcelin 1 encoded in the genome of the cyanobacterium *Acaryochloris marina* (Tables 1 and 4). Cry proteins from *B. thuringiensis* have a degree of correspondence to sequences in the genomes of four bacteria and one archaeon (Table 4). The VIP2 toxin from *B. thuringiensis* appears to be very diverse in nature. We found VIP-like toxins encoded by eleven bacterial genomes (Table 4). The identified lepidopteran-active toxins are associated with Cry1Aa, Cry2Aa and VIP2. Anti-coleopteran-like toxins were identified, and they are related to Arcelin 1 and Cry3A (Table 4). The search in the Environmental Sample Database showed seven most probable insecticidal sequences related with a Blattaria-active toxin, a coleopteran-specific toxin, four lepidopteran-active toxins and an anti-dipteran toxin (Table 4).

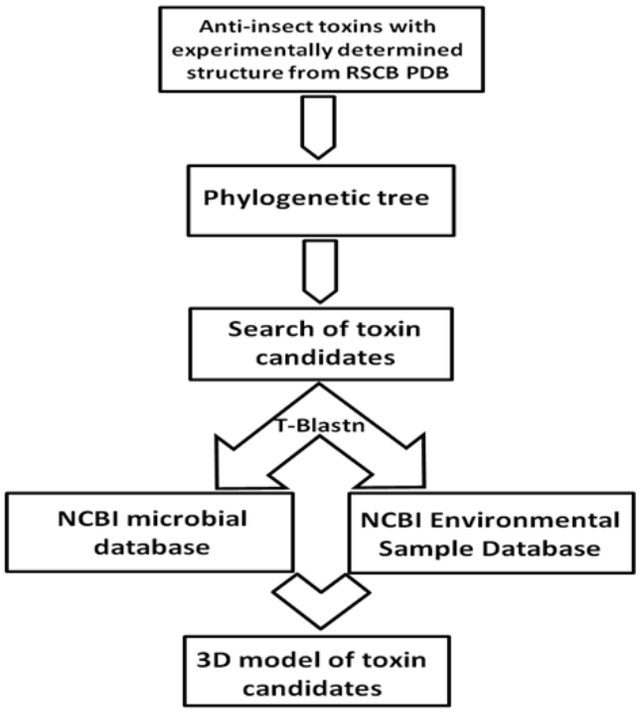

Fig. 3. Diagram of the work.The search for lepidopteran- and coleopteran-specific toxins was done through a basic strategy with the BLAST program on microbial and environmental genomes.

For our trial, the most important organisms harbouring lepidopteran- and coleopteran-active toxins are *A. marina*, *B. weihenstephanensis* and *Clostridium difficile*. First, *A. marina* is a unicellular cyanobacterium containing chlorophyll d as a major pigment (Ohashi et al., 2008). Second, *B. weihenstephanensis* is a Gram-positive, facultatively anaerobic, spore-forming bacterium. This organism has food poisoning potential and is able to grow aerobically at 7ºC. *B. weihenstephanensis* has a 16s rDNA signature sequence ^{1003}TCTAGAGATAGA and the signature sequence ^{4}ACAGTT of the gene for CspA (a major cold shock protein) (Lechner et al., 1998). Third, *C. difficile* is a Gram-positive spore-forming anaerobic bacterium thought to be involved in diarrhoea and colitis. *C. difficile* codes for two potent toxins (A and B), which attach to specific receptors in the lumen of human colonic epithelium (Vaisnavi, 2010). It is interesting to note that "particular" organisms have versions of these kinds of toxins, such as *Methanosarcina acetivorans* (an acetate-using methanogen archaeon), *Dyadobacter fermentans* (a Gram-negative bacterium isolated from maize and related to *Runella slithyformis*), the marine bacterium *Microscilla furvescens*, and *Cupriavidus necator* - previously known as *Ralstonia eutropha*, a microorganism that can be isolated from several environmental sources, such as soil and water, and which is important in polyhydroxyalkanoate production and bioremediation by the degradation of chlorinated aromatic pollutants (Galagan et al., 2002;Chelius & Triplett, 2002; Lykidis et al., 2010). In addition, we detected other *Clostridium* and *Bacillus* species. The NCBI environmental samples database, a metagenome of the Sargasso Sea genetic diversity from the Venter et al. (2004) project, shows environmental sequences with anti-lepidopteran and anti-coleopteran potential (Table 4).

We built tertiary (3D) structures of some of the predicted toxins: a lepidopteran-active toxin, a coleopteran-specific toxin and a toxin from a metagenome sequence. Approximately 30% sequence identity in the primary sequence is required for the generation of useful structures (Forster, 2002; Paramasivan et al., 2006). Tertiary models of candidate insecticidal sequences were constructed by homology modelling using the crystal structure of homologous protein from the RCSB PDB database (http://www.pdb.org/pdb/home/home.do). We used SWISS-MODEL (http://swissmodel.expasy.org/) (Arnold et al., 2006) for the identification of templates (Table 4 footnotes). The structural alignments were generated with DeepView Swiss-PdbViewer 4.0 software (http://spdbv.vital-it.ch/) (Guex & Peitsch, 1997).

The final models (Figure 4) have a range of 33% to 37% identity with the templates. The toxins in Figure 4 correspond to the following (A) NCBI ID NC_009925.1 from the *A. marina* MBIC11017 genome (33% identity), (B) NCBI ID NC_010180 from the *B. weihenstephanensis* KBAB4 plasmid pBWB401 (37% identity) and (C) the hypothetical protein GOS_5670768 from the marine metagenome (33% identity) (Table 4). The most striking feature of the predicted structure of the candidate insect toxin from the *A. marina* genome consists of two large β-pleated sheets that form a scaffold on which is a possible a carbohydrate-binding region (Figure 4). These architectures and topologies are found in a wide variety of carbohydrate recognizing proteins, such as plant lectins, galactins and serum amyloid proteins (Loris et al., 1998). The model is structurally related to the jelly-roll topology, which facilitates viral entry into bacterial cells. Entry is mediated by interactions with sugar-modified proteins on the cell surface (Petrey & Honig, 2009). It has been postulated that the binding of the lectin to the sugar moiety of any of the glycosylated digestive enzymes is a potential factor of insecticidal activity (Peumans & Van Damme, 1995a, b). Based on the structural alignment of the aminoacid sequences of the toxin from *B. weihenstephanensis* with

INSECTICIDAL TOXIN (ID PDB)	ORGANISM TARGET GENOME/ENVIRONMENTAL SOURCE	ID NCBI	E-VALUE	REGION
Microbial database				
1AVB[A]	*Acaryochloris marina* MBIC11017	NC_009925.1	3e-10	1669294-1669911
1CIY, 1DLC[B], 1I5P, 1JI6*, 1W99 and 2C9K**	*Bacillus weihenstephanensis* KBAB4 plasmid pBWB401	NC_010180	8e-97-2e-10	139296-138751
	Methanosarcina acetivorans C2A	NC_003552.1	4e-19-1e-04	3249335-3249832
	Dyadobacter fermentans DSM 18053	NC_013037.1	1e-15-4e-06	2869719-2870441
	Bacillus brevis NBRC 100599	NC_012491.1	5e-16-0.026[1]	4962833-4963585
	Ralstonia eutropha JMP134 Chromosome 1	NC_007347.1	1e-08-3.3[2]	411729-411409
1QS1	*Clostridium difficile*	ABHF02000033.1	2e-41	223624-224649
	Clostridium perfringens, E str. JGS1987	NZ_ABDW01000012.1	3e-39	66996-65971
	Clostridium botulinum, D str. 1873 plasmid pCLG1	NC_012946.1	1e-33	103322-104389
	Clostridium acetobutylicum ATCC 824	NC_003030.1	5e-17	398379-398876
	Bacillus cereus Rock4-18	NZ_ACMN01000162.1	1e-21	17703-17065
	Bacillus halodurans C-125	NC_002570.2	4e-15	3637460-3636978
	Streptomyces avermitilis MA-4680	NC_003155.4	5e-12	6590878-6591372
	Listeria monocytogenes FSL R2-561	AARS01000007.1	8e-12	72280-71786
	Lactobacillus brevis subsp. *gravesensis*	NZ_ACGG01000118.1	3e-11	220449-220006
	Aeromonas hydrophila subsp. *hydrophila*	NC_008570.1	2e-05	1214897-1215424
	Enterococcus faecalis V583	NC_004668.1	2e-05	311391-311870
Environmental database				
1BMR	hypothetical protein GOS_4202115 marine	gb|ECA60195.1	0.057	88-243

INSECTICIDAL TOXIN (ID PDB)	ORGANISM TARGET GENOME/ENVIRONMENTAL SOURCE	ID NCBI	E-VALUE	REGION
	metagenome			
1DLC[C]	hypothetical protein GOS_5670768 marine metagenome	gb|ECH33518.1	0.014	12-142
1QS1	hypothetical protein GOS_355881 marine metagenome	gb|EBA70908.1	6e-04	102-270
	hypothetical protein GOS_1734861 marine metagenome	gb|EDJ21677.1	8e-04	416-584
	hypothetical protein GOS_9568803 marine metagenome	gb|EBF61568.1	0.003	5-173
	hypothetical protein GOS_7854205 marine metagenome	gb|EBP79016.1	0.004	78-232
1W99	hypothetical protein GOS_6575573 marine metagenome	gb|EBX51304.1	0.010	29-95

Table 4. Results of the BLAST search in a microbial database (Blosum 62, E threshold 0.01) and Environmental Sample Database (Blosum 62, E threshold 0.01) (underlined by modelled sequences). * It is not compatible with *B .weihenstephanensis*. ** Only compatible with *B. weihenstephanensis* and *M. acetivorans*. [A] PDB template: 1G7Y chain C (lectin from the legume *Dolichos biflorus*). Model residues: 72-289.[B] PDB template: 3EB7 (Cry8Ea1). Model residues: 64-648.[C]PDB template: 2E58 (MnmC2 from *Aquifex aeolicus*). Model residues: 38-136. The ID PDB refers to code in Protein Data Bank; the ID NCBI refers to accession number in National Center for Biotechnology Information. The region column refers to the specific segment inside the DNA sequence from the ID NCBI column.

the Cry8Ea1 protein, a model of the toxin was obtained; and it corresponds to the general model for a Cry protein (Figure 4). The last structure corresponds to a sequence from the marine metagenome. It was built by homology to a possible transferase of *Aquifex aeolicus*, a hyperthermophilic microorganism that grows at 85-100°C. It has been suggested that this organism may be the earliest diverging eubacterium (Deckert et al., 1998). The model is composed of three α-helices and a large β-sheet, in which the first and second β-strands are arranged in parallel; and the third and fourth are anti-parallel. Interestingly, the model is somewhat similar to that of the aminoacyl-tRNA synthetase editing domain (Ribas de Pouplana & Schimmel, 2000; Naganuma et al., 2009). The phylogenetic relationships amongst these enzymes are clustered around substrate specificity (Guo et al., 2009). That the amino acid sequence from an ancient bacterium has identity with the Cry protein of *B. thuringiensis*, and that the toxin structure is similar to that of an aminoacyl-tRNA synthetase

editing domain and that it has a helix-sheet formation, hints at the origin of these toxins and their specificities.

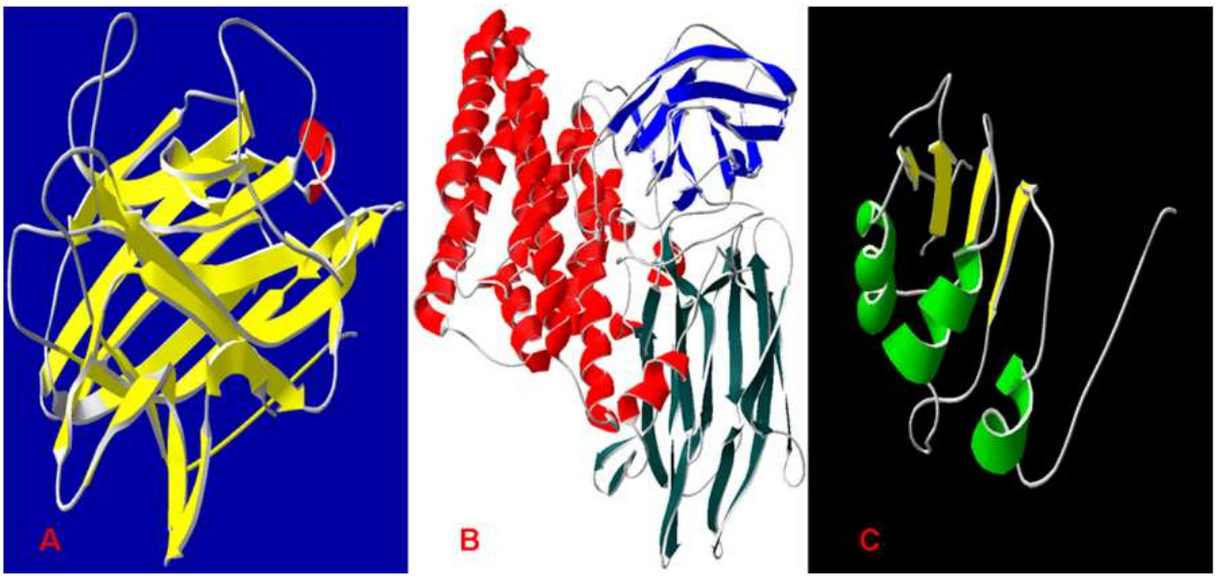

Fig. 4. Models of candidate toxins. (A) Insect toxin the from the *A. marina* genome (β-pleated sheets are in yellow); (B) Structure of the toxin from the *B. weihenstephanensis* genome (domain I is red; blue represents domain II; and domain III is green); and (C) model of the toxin from the marine metagenome (the helices are green, and the β-sheet is yellow). Also see the text.

5.2 *B. thuringiensis* vs. lepidopteran and coleopteran pests

The entomopathogenic bacterium *B. thuringiensis* has been used to help thwart the development of insect and plant resistance by using *cry* genes to construct lethal toxins against pest larvae. Some Cry proteins display biological activity against lepidopteran (Cry1, Cry2, Cry7, Cry8, Cry9, Cry15, Cry22, Cry32 and Cry51) and coleopteran (Cry1B, Cry1I, Cry3, Cry7, Cry8, Cry9, Cry14, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43 and Cry55) organisms (van Frankenhuyzen, 2009). Over the past fifteen years, research in our laboratory has focused on the study of the Cry proteins of the entomocidal bacterium *B. thuringiensis* for the biological control of insect pests in Colombia. This country is severely affected by lepidopteran and coleopteran pests, such as larvae of the potato tuber moth, *T. solanivora*; the armyworm, *S. frugiperda*; the Andean weevil, *Premnotrypes vorax* and the coffee berry borer (CBB), *Hypothenemus hampei.*

5.3 Our experience with lepidopterans

We worked with the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), an important pest in the Americas. This insect is susceptible to the Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ae, Cry1B, Cry1F, Cry1I, Cry1J, Cry2, Cry8 and Cry9 toxins. Cry1Ac is the most active toxin against this pest (van Frankenhuyzen, 2009). In collaborative work, we tested chimeric Cry1 proteins (Cry1Ba, Cry1Ca, Cry1Da, Cry1Ea, and Cry1Fb) containing domain III of Cry1Ac, which shows higher toxicity in the Cry1Ba, Cry1Ca and Cry1Fb proteins. In addition, we considered an analysis for toxicity against *H. virescens* with the Cry1Ac domain

III triple-mutant toxin, named Tmut (N506D, Q509E, Y513A), supplied by Dr. Ellar (Burton et al., 1999). The test was done by means of a competition-binding assay using an immunoblotting method on nitrocellulose paper. Brush border membrane vesicles (BBMVs) from the *H. virescens* midgut were incubated with biotin-labelled toxin and with increasing concentrations of homologous (identical) or heterologous (mutant) toxin (Figure 5). The Tmut toxin was not able to compete with the Cry1Ac protein for binding to BBMVs (Figure 5). Also the mutant toxicity was 7-fold lower than the toxicity of the reference Cry1Ac. It indicates that at least one of the three residues (N506, Q509 and Y513) has an important role in the biological activity of the toxin (Karlova et al., 2005).

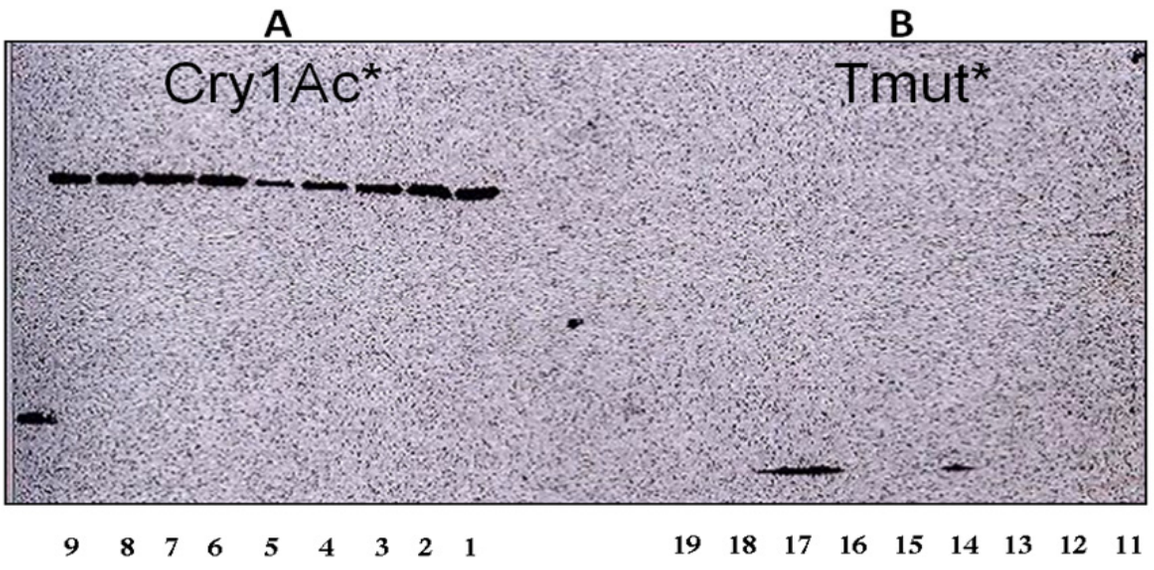

Fig. 5. The Cry1Ac binding reaction on *H. virescens* BBMVs. A. Lane 1, control with nothing added; lanes 2-5, homologous competition between parental Cry1Ac (10, 30, 90, 270 ng of the protein for each lane, respectively) and Cry1Ac labelled with biotin (10 ng); lanes 6-9, heterologous competition between the Cry1Ac domain III triple-mutant (named Tmut, which has the point mutations N506D, Q509E, and Y513A) toxin (10, 30, 90, 270 ng of the protein for each lane, respectively) and Cry1Ac labelled with biotin (10 ng). The first experiment (lanes 2-5) shows that the Cry1Ac wild-type protein (both the labelled and unlabelled proteins) binds to BBMVs (*i.e.*, competition was observed); the second experiment (lanes 6-9) indicates that the Cry1Ac domain III triple-mutant (Tmut) toxin was not able to bind to BBMVs and compete with the bound Cry1Ac wild type (labelled) protein (*i.e.*, competition was not visible). B was set up as follows: lane 11, a no-competitor control; lanes 12-15, heterologous competition between parental Cry1Ac (10, 30, 90, 270 ng of the protein for each lane, respectively) and the Cry1Ac domain III triple-mutant (Tmut) toxin labelled with biotin (10 ng); lanes 16-19, homologous competition between the Cry1Ac domain III triple-mutant (Tmut) toxin (10, 30, 90, 270 ng of protein for each line, respectively) and the Cry1Ac domain III triple-mutant (Tmut) toxin labelled with biotin (10 ng). However, the absence of bands in B confirmed that Tmut is unable to bind to BBMVs. The asterisk indicates the toxin labelled with biotin. Also see the text.

We collaborated in the genetic characterization of *S. frugiperda* (fall armyworm) strains from Brazil, Colombia and Mexico, all of which were correlated with vulnerability to the Latin American *B. thuringiensis* isolates and recombinant toxins (Monnerat et al., 2006). The recognition of genetic variability among insect strains is a decisive analysis for the development of improved pest control strategies, since the biological behaviour of Cry proteins on insect populations is dependent on the specific alleles (specially receptor related), the gene flow and fitness performance. Genetic analysis [molecular analysis for genetic variability was done with Random Amplification of polymorphic DNA (RAPD)] showed that these *S. frugiperda* populations had different levels of similarity among them (between 22% and 37%). *B. thuringiensis* isolates were found to have genes for Cry1 (Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1D, Cry1E, Cry1G and Cry1I) and Cry2. The fall armyworm (*S. frugiperda*) groups differ in their susceptibilities to *B. thuringiensis*. The most toxic *B. thuringiensis* isolates for *S. frugiperda* had a mixture of genes for Cry1Aa, Cry1B and Cry1D. The Colombian population of this insect was the most susceptible to Latin American *B. thuringiensis* strains. The Mexican *S. frugiperda* was sensitive to recombinant Cry1Ca and Cry1Da. *S. frugiperda* from Brazil was highly susceptible to recombinant Cry1Ca, while the Colombian insects were susceptible to recombinant Cry1B, Cry1C and Cry1D proteins (Monnerat et al., 2006).

Recently we contributed to the determination of Cry1 toxicity against the first instar larvae of *T. solanivora*. We evaluated the products of the *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ca*, *cry1Da*, *cry1Ba*, *cry1Ea*, *cry1Fa* and *cry1Ia* genes and the gene for the hybrid protein SN1917 (encoding Cry1Ba and Cry1Ia in domain II) against the first instar larvae of this pest. We identified toxins with high activity relative to the Cry1Ba, Cry1Ac and SN1917 toxins (Martinez et al., 2003; López-Pazos et al., 2010).

5.4 Our experience with coleopterans

We researched the relationship between ecological niches of the Andean weevil, *P. vorax*, and the bacterium *B. thuringiensis*. We isolated and molecularly characterized *B. thuringiensis* native strains from potato areas (soil, store products and dead *P. vorax*). Bioassays were done using neonate larvae. In addition, the Cry3Aa recombinant toxin and its mutants (mutant 1: D354E; mutant 2: R345A, ΔY350, ΔY351; and mutant 3: Q482A, S484A, R485A) were constructed; and biological assays were performed. We found 300 strains (Bt index was 0.43, calculated as *B. thuringiensis* strains divided by the total amount of *Bacillus* strains) with 21 *cry* gene profiles. Unfortunately neither the isolates nor the recombinant Cry3Aa toxin were toxic against this coleopteran. However, a Cry3A triple mutant [R345A, ΔY350 (deletion), ΔY351 (deletion)] had a minor level of biological activity (mortality 21.87%), in contrast to wild-type Cry3Aa (<6%). This was probably due to site-directed modifications (López-Pazos et al., 2009b).

Coffee crops are severely affected by the CBB (coffee berry borer, *H. hampei*). Female insects drill fissures into the berry and lay their eggs, causing severe losses in production and quality. The entire metamorphosis takes place in the fruit (Damon 2000). This pest is currently present in more than 90% of the planted area (Bustillo 2006; Ramírez 2009). Recently, our research has been centred on the study of Cry toxins for the biological control of CBB, using recombinant proteins of Cry1B, Cry1I, Cry3A, Cry4, Cry9 and SN1917. Although the Cry1B and Cry3A proteins showed minor activity against the pest, the results

support the hypothesis that toxicity could be indirect and due to physiological factors of the insect rather than directly from the toxicity of dedicated toxin molecules. Unfortunately the Cry1I, Cry4, Cry9 and SN1917 hybrids were not toxic to CBB (López-Pazos et al. 2010, 2009a). We wanted to learn about the possible interaction between Cry toxins and the receptors in midgut CBB. Brush border membrane vesicles (BBMVs) from the midgut of *H. hampei* were prepared according to Wolfersberger et al. (1987). We used the Cry1B, Cry1I, Cry3A (López-Pazos et al. 2009a; López-Pazos et al. 2010), Cry4 and Cry9 proteins (Figure 6). BBMVs divided by protein electrophoresis showed bands between 20–220 kDa (Figure 6). A blotting test was prepared to determine the weight of Cry-binding proteins in CBB-BBMVs. Cry1B recognized proteins of ~190, 140, 80, 75, 60, 50 and 40 kDa (Figure 6). A signal for Cry1I was also visible at 140 kDa (Figure 6). Cry3A binding proteins were detected at ~140 kDa, 120 kDa and 70 kDa (Figure 6). Cry4 and Cry9 were not detected by any protein on BBMVs (Figure 6). There appeared to be several Cry1B and Cry3A toxin binding sites and/or receptors in the midgut epithelia of CBB.

5.4.1 The modes of action of Cry toxins in coleopterans: the case of CBB

The specific conditions in CBB gut physiology (acidic pH, types of proteases or high proportions of insecticide resistance alleles) are not favourable to the modes of action of the Cry proteins (López-Pazos et al. 2009a). The presence of candidate receptors for Cry proteins in CBB offers evidence for the potential of Cry protein use for the control of this pest. Cadherin-like receptors (CADR) have been studied in lepidopteran and dipteran insects. CADRs were isolated from the coleopterans *Diabrotica virgifera virgifera* (191 kDa) and *Tenebrio molitor* (179 kDa) (Sayed 2007; Fabrick et al. 2009). The CADR receptors are highly variable, with molecular weights ranging from 175 to 210 kDa. An important Cry protein binding site was found to be contained in CADR repeat number 12 (Pigott & Ellar 2007; Hua et al. 2004). It was possible improve the toxicity of Cry3 proteins against coleopterans by adding a CADR fragment containing Cry protein binding site (Park et al. 2009).

Aminopeptidase N (APN) is an N-acetyl-D-galactosamine (GalNAc)-bearing glycoprotein. APN is a receptor for Cry toxins. Different APNs have molecular weights of 90-170-kDa. It was proposed that the Cry-APN interaction has two steps: carbohydrate recognition and irreversible protein-protein interaction (Pigott & Ellar 2007). More than 60 different APNs have been registered in databases. They are from 26% to 65% similar (Herrero et al. 2005, Nakanishi et al. 1999). The 140 kDa protein (from BBMV analysis) is consistent with its being an APN. We do not know if the multiple Cry-binding polypeptides detected in CBB are different proteins or if they are one APN glycosylated differently.

It is also known that CADRs are susceptible to proteolytic digestion and for producing a ~120 kDa fraction. For this reason, CADRs can be confused with APNs in protein-protein interaction blots (Martínez-Rámirez et al. 1994). Cry proteins have multiple binding determinants, possibly specified independently by domains II and III. Moreover, Cry toxins interact with other classes of proteins in the Coleoptera order, such as ALP (molecular weight ~65 kDa), V-ATPase and the Heat-Shock Cognate protein (~ 80 kDa) and the ADAM metalloprotease (~30 kDa) (Hua et al. 2001; Ochoa-Campuzano et al. 2007; Martins et al. 2010; Nakasu et al. 2010). Any signals in the ligand blot for Cry1B and Cry3A would be related with these proteic groups. However, we identified the minor biological activity of Cry1B and Cry3A proteins on CBB larvae (López- Pazos et al. 2009a); and none was seen

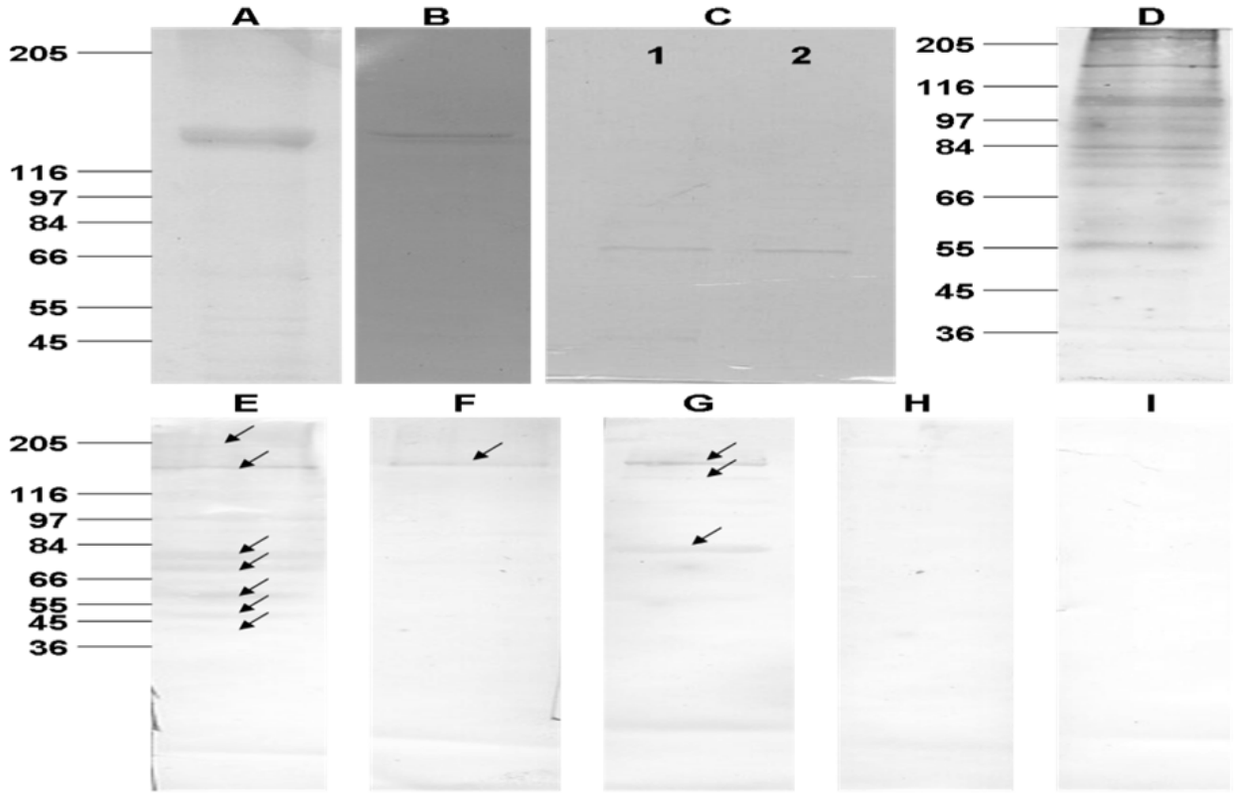

Fig. 6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant toxins (A, B, and C) and ligand blots of Cry proteins on membrane vesicles from the midgut of the coffee berry borer (CBB-BBMVs) (E, F, G, H, and I). D shows SDS-PAGE of CBB-BBMV proteins. (A) Cry4 protoxin, (B) Cry9 protoxin, and (C) Cry4 (1) and Cry9 (2) protease-treated toxins; (D) brush-border-membrane-vesicle (BBMV) proteins from CBB. Cry-binding proteins (E-I) are indicated by the arrows. The biotin-labelled ligands (see below) are the following: (E) Cry1B, (F) Cry1I, (G) Cry3, (H) Cry4, and (I) Cry9. The numbers are molecular masses (kDa). Specifically, Cry4 and Cry9 were prepared for cloning by PCR amplification using the primers Cry4F (5′-ATGGGATCCTATCAAAATAAAAATGAATAT-3′) with Cry4R (5′-TCACTCGTTCATGCCTGCAGATTCAAT GCT-3′) and Cry9F (5′-ATGGGTACCAATAAACACGGAATTATTGGC-3′) with Cry9R (5′-TTACTGCAGTGTTTCAACGAA TTCAATACT-3′), respectively. *Bam*HI and *Kpn*I restriction sites were added to the sequences of the Cry4 and Cry9 forward primers (underlined), respectively. *Pst*I restriction sites were added to both the Cry4 and Cry9 reverse primers (underlined). The restriction sites were added to clone the amplified DNA fragment. The brush border membrane protein resolved on SDS-PAGE was transferred onto an Immobilon-P polyvinylidene difluoride (PVDF) membrane for blotting. The PVDF membrane was incubated with a biotin-labelled activated Cry toxin for binding, followed by washing with PBS/Tween (phosphate-buffered saline, pH7.4, containing 0.05% Tween-20) and incubation with streptavidin conjugated to peroxidise. The bands were visualized by peroxidase reacting with diaminobenzidine.

with Cry1I, Cry4, Cry9 and SN1917 hybrids. In this sense, there is a correlation between our data and ligand blot observations.

6. Conclusion

Insecticidal toxins are an important option for the biological control of lepidopteran and coleopteran insects. Their use in the genetic engineering of plants could provide a new generation of resistant crops. Such recombinant plants, thanks to their significant environmental and economic benefits, could help agricultural families in poor countries.

7. Acknowledgments

The authors are grateful to the Instituto de Biotecnología de la Universidad Nacional de Colombia. López-Pazos S.A. is grateful to Colciencias for a doctoral fellowship.

8. References

Abdelghany A. Y., Awadalla S. S., Abdel-Baky N. F., El-Syrafi H. A., Fields P. G. (2010). Effect of high and low temperatures on the drugstore beetle (Coleoptera: Anobiidae). *J. Econ. Entomol*. 103:1909-1914.

Adair J. R., Wallace T. P. (1998). Site-Directed Mutagenesis. *Molecular Biomethods Handbook*, Pages 347-360.

Adams M. E. (2004). Agatoxins: ion channel specific toxins from the American funnel web spider, *Agelenopsis aperta*. *Toxicon*. 43: 509–525.

Aravind L. (2000). Guilt by association: contextual information in genome analysis. *Genome Res*. 10: 1074–1077.

Arnold K., Bordoli L., Kopp J., Schwede T. (2006). The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. *Bioinformatics*. 22:195-201.

Bloomquist J.R. (2003). Mode of action of atracotoxin at central and peripheral synapses of insects. *Invertebr. Neurosci*. 5:45-50.

Boonserm P., Davis P., Ellar D. J., Li J. (2005). Crystal structure of the mosquito larvicidal Toxin Cry4Ba and its biological implications. *J. Mol. Biol*. 348: 363-382.

Boonserm P., Mo M., Angsuthanasombat C., Lescar J. (2006). Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution.*J. Bacteriol*.188: 3391-401.

Bosa C. F., Cotes A. M., Osorio P., Fukumoto T., Bengtsson M., Witzgall P. (2006). Disruption of pheromone communication in *Tecia solanivora* (Lepidoptera: Gelechiidae): flight tunnel and field studies. *J. Econ. Entomol*. 99:1245-1250.

Burton S.L., Ellar D.J., Li J., Derbyshire, D. J. (1999). N-Acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *J. Mol. Biol*. 287:1011–1022.

Bustillo A. E. (2006). Una revisión sobre la broca del café, *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae), en Colombia. *Rev. Colomb. Entomol*.32: 101-116.

Chagas Filho N. R., Boiça A. L. Jr., Alonso T. F. (2010). Biology of *Plutella xylostella* L. (Lepidoptera: Plutellidae) reared on cauliflower genotypes. *Neotrop. Entomol*. 39:253-259.

Chelius M. K., Triplett E. W. (2000). *Dyadobacter fermentans* gen. nov., sp. nov., a novel gram-negative bacterium isolated from surface-sterilized Zea mays stems. *Int. J. Syst. Evol. Microbiol*. 50 Pt 2: 751-758.

Chong Y., Hayes J. L., Sollod B., Wen S., Wilson D. T., Hains P. G., Hodgson W. C., Broady K. W., King G. F., Nicholson G. M. (2007). The ω-atracotoxins: selective blockers of insect M-LVA and HVA calcium channels. *Biochem. Pharmacol*. 74:623-638.

Cruz L. P., Gaitan A. L., Gongora C. E. (2006). Exploiting the genetic diversity of *Beauveria bassiana* for improving the biological control of the coffee berry borer through the use of strain mixtures. *Appl. Microbiol. Biotechnol*. 71: 918-926.

Damon A. (2000). A review of the biology and control of the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae). *Bull. Entomol.Res*. 90:453-465.

Deckert G., Warren P. V., Gaasterland T., Young W. G., Lenox A. L., Graham D. E., Overbeek R., Snead M. A., Keller M., Aujay M., Huber R., Feldman R. A., Short J. M., Olsen G. J., Swanson R. V. (1998). The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature*.392: 353-358.

De Lima M. E., Figueiredo S. G., Pimenta AM. C., Santos D. M., Borges M. H., Cordeiro M. N., Richardson M., Oliveira L. C., Stankiewicz M., Pelhate M. (2007). Peptides of arachnid venoms with insecticidal activity targeting sodium channels. Comp.*Biochem.Physiol*. Part C. 146: 264–279.

Dereeper A., Guignon V., Blanc G., Audic S., Buffet S., Chevenet F., Dufayard J. F., Guindon S., Lefort V., Lescot M., Claverie J. M., Gascuel O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic. Acids. Res*. 36 (Web Server issue):W465-W469.

Du E., Ni X., Zhao H., Li X. (2011). Natural history and intragenomic dynamics of the Transib transposon Hztransib in the cotton bollworm *Helicoverpa zea.Insect. Mol. Biol*.20:291-301.

Ellegren H. (2008).Comparative genomics and the study of evolution by natural selection. Mol. Ecol. 17:4586-4596.

El-Sayed A., El-Sheikh Shizuo G. Kamita, Kiem Vu , Bruce D. Hammock. (2011). Improved insecticidal efficacy of a recombinant baculovirus expressing mutated JH esterase from *Manduca sexta*. *Biological control.* 58: 354-361.

Fabre C., Causse H., Mourey L., Koninkx J., Rivière M., Hendriks H., Puzo G., Samama J. P., Rougé P. (1998). Characterization and sugar-binding properties of arcelin-1, an insecticidal lectin-like protein isolated from kidney bean (*Phaseolus vulgaris* L. cv. RAZ-2) seeds. *Biochem. J*. 329: 551-560.

Fabrick J., Oppert C., Lorenzen M. D., Morris K., Oppert B., Jurat-Fuentes J. L. (2009). A novel *Tenebrio molitor* cadherin is a functional receptor for *Bacillus thuringiensis* Cry3Aa toxin. *J. Biol. Chem*. 284: 18401-18410.

Ferrat G., Bosmans F., Tytgat J., Pimentel C., Chagot B., Gilles N., Nakajima T., Darbon H., Corzo G. (2005). Solution structure of two insect-specific spider toxins and their pharmacological interaction with the insect voltage-gated Na+ channel. *Proteins* 59:368-379.

Fletcher J. I., Smith R., O'Donoghue S. I., Nilges M., Connor M., Howden M. E., Christie M. J., King G. F. (1997). The structure of a novel insecticidal neurotoxin, ω-atracotoxin-HV1, from the venom of an Australian funnel web spider. *Nat.Struct.Biol*. 4: 559-566.

Forster M. J. (2002).Molecular modeling in structural biology.*Micron*.33: 365–384.

Gabaldon T., Huynen M. A. (2004).Prediction of protein function and pathways in the genomeera.*Cell. Mol. Life Sci*. 61: 930–944.

Galagan J. E., Nusbaum C., Roy A., Endrizzi M. G., Macdonald P., FitzHugh W., Calvo S., Engels R., Smirnov S., Atnoor D., Brown A., Allen N., Naylor J., Stange-Thomann N., DeArellano K., Johnson R., Linton L., McEwan P., McKernan K., Talamas J., Tirrell A., Ye W., Zimmer A., Barber R. D., Cann I., Graham D. E, Grahame D. A., Guss A. M., Hedderich R., Ingram-Smith C., Kuettner H. C., Krzycki J. A., Leigh J. A., Li W., Liu J., Mukhopadhyay B., Reeve J. N., Smith K., Springer T. A., Umayam L. A., White O., White R. H., Conway de Macario E., Ferry J. G., Jarrell K. F., Jing H., Macario A. J., Paulsen I., Pritchett M., Sowers K. R., Swanson R. V., Zinder S. H., Lander E., Metcalf W. W., Birren B. (2002). The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. *Genome Res*. 12: 532-542.

Galitsky N., Cody V., Wojtczak A., Ghosh D., Luft J. R. (2001). Structure of the insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. *Act Crystallogr* 2001; D57:1101-1109.

Gentz M. C., Murdoch G., King G. F. (2010). Tandem use of selective insecticides and natural enemies for effective, reduced -risk pest management. *Biol. Control*. 52: 208-215.

Goldin, A.L. (2002). Evolution of voltage-gated Naþ channels.*J. Exp. Biol*. 205: 575-584.

Gordon D., Karbat I., Ilan N., Cohen L., Kahn R., Gilles N., Dong K., Stühmerd W., Tytgat J., Gurevitz M..(2007). The differential preference of scorpion α-toxins for insect or mammalian sodium channels: Implications for improved insect control. *Toxicon*. 49: 452–472.

Goudet C., Chi C.-W., Tytgat J. (2002). An overview of toxins and genes from the venom of the Asian scorpion *Buthus martensi* Karsch. *Toxicon*. 40: 1239–1258.

Gunning S. J, Maggio F., Windley M. J., Valenzuela S. M., King G. F., Nicholson G. M. (2008). The Janusfaced atracotoxins are specific blockers of invertebrate KCa channels. *FEBS J*. 275: 4045-4059.

Grochulski P., Masson L., Borisova S., Pusztai-Carey M., Schwartz J. L., Brousseau R., Cygler M. (1995). *Bacillus thuringiensis* CryIA(a) Insecticidal Toxin: Crystal Structure and Channel Formation. *J. Mol. Biol*. 254: 447-464.

Gruber C. W., Cemazar M., Anderson M. A., Craik D. J. (2007). Insecticidal plant cyclotides and related cystine knot toxins. *Toxicon*. 49: 561-575.

Guex, N., Peitsch, M. C. (1997). Swiss-model and the Swiss-PdbViewer: An environment for comparative protein modelling. *Electrophoresis*. 18: 2714-2723.

Guo M., Chong Y. E., Beebe K., Shapiro R., Yang X. L., Schimmel P. (2009). The C-Ala domain brings together editing and aminoacylation functions on one tRNA. *Science*. 325:744-747.

Gurevitz M., Karbat I., Cohen L., Ilan N., Kahn R., Turkov M., Stankiewicz M., Stuhmerc W., Dong K., Gordon D. (2007). The insecticidal potential of scorpion β-toxins. *Toxicon*. 49: 473-489.

Gurevitz M., Urbach D., Zlotkin E., Zilberberg N. (1991) Nucleotide sequence and structure analysis of a cDNA encoding anα-insect toxin from the scorpion *Leiurus quinquestriatus hebraeus*. *Toxicon* 29:1270-1272.

Hammock B.D., Bonning B.C., Possee R.D., Hanzlik T.N., Maeda S. (1990). Expression and effects of the juvenile hormone esterase in a baculovirus vector. *Nature*.344: 458–461.

Han S., Craig J. A., Putnam C. D., Carozzi N. B., Tainer J. A. (1999) Evolution and mechanism from structures of an ADP ribosylating toxin and NAD complex. *Nat. Struct.Biol*. 6:932-936.

Hao C. J., Xu C. G., Wang W., Chai B. F., Liang A. H. (2005). Expression of an insect excitatory toxin, BmK IT, from the scorpion, *Buthus martensii* Karsch, and its biological activity. *Biotechnol. Lett*.27:1929-1934.

Herrero S., Gechev T., Bakker P. L., Moar W. J., de Maagd R. A. (2005).*Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four aminopeptidase N genes. *BMC Genomics*.6: 96.

Howard A. F., N'guessan R., Koenraadt C. J., Asidi A., Farenhorst M., Akogbéto M., Thomas M. B., Knols B. G., Takken W. (2010). The entomopathogenic fungus *Beauveria bassiana* reduces instantaneous blood feeding in wild multi-insecticide-resistant *Culex quinquefasciatus* mosquitoes in Benin, West Africa. *Parasit. Vectors*. 15: 87.

Hua G., Jurat-Fuentes J. L., Adang M. J. (2004). Bt-R1a extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and toxicity. *J. Biol. Chem*. 279: 28051–28056.

Hua G., Masson L., Jurat-Fuentes J. L., Schwab G., Adang M. J. (2001). Binding analyses of *Bacillus thuringiensis* Cry δ-endotoxins using brush border membrane vesicles of *Ostrinia nubilalis*. *Appl. Environ. Microbiol*.67: 872-879.

James R. M., Dickinson P. (1998). Site-Directed Mutagenesis. *Molecular Biomethods Handbook*. Pages 361-381.

Ji Y. H., Mansuelle P., Terakawa S., Kopeyan C., Yanaihara N., Hsu K., Rochat H.(1996). Two neurotoxins (Bmk I and Bmk II) from the venom of the scorpion *Buthus martensi* Karsch: purification, amino acid sequences and assessment of specific activity. *Toxicon*.34: 987-1001.

Jablonsky M. J., Jackson P. L., Krishna N. R. (2001). Solution structure of an insect-specific neurotoxin from the new world scorpion*Centruroides sculpturatus* Ewing. *Biochemistry*. 40: 8273-8282.

Johnson J. H., Bloomquist J. R., Krapcho K. J., Kral R. M. Jr, Trovato R., Eppler K. G., Morgan T. K., DelMar E. G. (1998). Novel insecticidal peptides from *Tegenaria agrestis* spider venom may have a direct effect on the insect central nervous system. *Arch Insect Biochem Physiol*. 38: 19-31.

Kamita S. G., Hinton A. C., Wheelock C. G., Wogulis M. D., Wilson D. K., Wolf N. M., Stok J. E., Hock B., Hammock B. D. (2003). Juvenile hormone (JH) esterase: why are you so JH specific? *Insect Biochemistry and Molecular Biology*. 33: 1261–1273.

Kanchiswamy C. N., Takahashi H., Quadro S., Maffei M. E., Bossi S., Bertea C., Zebelo S. A., Muroi A., Ishihama N., Yoshioka H., Boland W., Takabayashi J., Endo Y., Sawasaki T., Arimura G. (2010). Regulation of *Arabidopsis* defense responses against Spodoptera littoralis by CPK-mediated calcium signaling.*BMC Plant.Biol*. 10: 97.

Karbat I., Frolow F., Froy O., Gilles N., Cohen L., Turkov M., Gordon D., Gurevitz M. (2004). Molecular Basis of the High Insecticidal Potency of Scorpion α toxins. *The Journal of Biological Chemistry*.279: 31679–31686.

Karbat I., Turkov M., Cohen L., Kahn R., Gordon D., Gurevitz M., Frolow F. (2007). X-ray structure and mutagenesis of the scorpion depressant toxin LqhIT2 reveals key determinants crucial for activity and anti-insect selectivity. *J. Mol. Biol*. 366: 586-601.

Karlova R., Weemen-Hendriks M., Naimov S., Ceron J., Dukiandjiev S., de Maagd R. (2005).*Bacillus thuringiensis*δ-endotoxin Cry1Ac domain III enhances activity against *Heliothis virescens* in some, but not all Cry1-Cry1Ac hybrids. *J. Invertebr. Pathol*. 88: 169–172.

Keszthelyi S., Pál-Fám F., Kerepesi I. (2011). Effect of cotton bollworm (*Helicoverpa armigera* Hübner) caused injury on maize grain content, especially regarding to the protein alteration. *Acta Biol. Hung*. 62: 57-64.

King G. F., Gentz M. C., Escoubas P., Nicholson G. M. (2008). A rational nomenclature for naming peptide toxins from spiders and other venomous animals. *Toxicon*. 52: 264-276.

King G. F., Tedford H. W., Maggio F. (2002). Structure and function of insecticidal neurotoxins from Australian funnel web spiders. *Toxin Reviews*. 21: 361-389.

Krimm I., Gilles N., Sautière P., Stankiewicz M., Pelhate M., Gordon D., Lancelin J. M. (1999). NMR structures and activity of a novel α-like toxin from the scorpion *Leiurus quinquestriatus* hebraeus. *J. Mol. Biol.* 285:1749-1763.

Lechner S., Mayr R., Francis K. P., Prüss BM, Kaplan T., Wiessner-Gunkel E., Stewart G. S., Scherer S. (1998).*Bacillus weihenstephanensis* sp. *nov*. is a new psychrotolerant species of the Bacillus cereus group. *Int. J. Syst. Bacteriol*.48: 1373-1 382.

Lee W., Moore C. H., Watt D. D., Krishna N. R. (1994). Solution structure of the variant-3 neurotoxin from *Centruroides sculpturatus* Ewing. *Eur. J. Biochem*.218: 89-95.

Lee D., Redfern O., Orengo C. (2007). Predicting protein function from sequence and structure. *Nat. Rev. Mol. Cell. Biol*. 8: 995-1005.

Li C., Guan R. J., Xiang Y., Zhang Y., Wang D. C. (2005). Structure of an excitatory insect-specific toxin with an analgesic effect on mammals from the scorpion *Buthus martensii* Karsch. *Acta Crystallogr. D Biol. Crystallogr*. 61(Pt 1):14-21.

Li J., Carroll J., Ellar D. J. (1991).Crystal Structure of Insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å Resolution.*Nature*.353:815-821.

Liang S. (2004). An overview of peptide toxins from the venom of the Chinese bird spider *Selenocosmia huwena* Wang [=*Ornithoctonus huwena* (Wang)] *Toxicon*.43: 575-585.

Likitvivatanavong S., Katzenmeier G., Angsuthanasombat C. (2006). Asn183 in α5 is essential for oligomerisation and toxicity of the *Bacillus thuringiensis* Cry4Ba toxin. *Archives of Biochemistry and Biophysics*.445: 46–55.

Liu Y. J., Cheng C. S., Lai S. M., Hsu M. P., Chen C. S., Lyu P. C. (2006). Solution structure of the plant defensin VrD1 from mung bean and its possible role in insecticidal activity against bruchids. *Proteins*.63:777-786.

López-Pazos S. A., Cerón J. A. (2007). Three-dimensional structure of *Bacillus thuringiensis* toxins: a review *Acta. Biol. Colomb*.12: 19-32.

López-Pazos S. A., Cortázar J. E., Cerón J. A. (2009a). Cry1B and Cry3A are active against *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae*). J. Invertebr. Pathol*.101: 242-245.

López-Pazos S. A., Martínez J. W., Castillo A. X., Cerón Salamanca J. A.(2009b). Presence and significance of *Bacillus thuringiensis* Cry proteins associated with the Andean

weevil *Premnotrypes vorax* (Coleoptera: Curculionidae). *Rev. Biol. Trop*. 57: 1235-1243.

López-Pazos S. A., Rojas Arias A. C., Ospina S. A., Cerón J. (2010). Activity of *Bacillus thuringiensis* hybrid protein against a lepidopteran and a coleopteran pest. *FEMS Microbiol. Lett*. 302: 93-98.

Loris R., Hamelryck T., Bouckaert J., Wyns L. (1998). Legume lectin structure.*Biochim. Biophys*. Acta.1383: 9–36.

Lykidis A., Pérez-Pantoja D., Ledger T., Mavromatis K., Anderson I. J., Ivanova N. N., Hooper S. D., Lapidus A., Lucas S., González B., Kyrpides N. C. (2010). The complete multipartite genome sequence of *Cupriavidus necator* JMP134, a versatile pollutant degrader. *PLoS One*. 5: e9729.

Martínez-Ramírez A. C., González-Nebauer S., Escriche B., Real M. D. (1994). Ligand blot identification of a *Manduca sexta* midgut binding protein specific to three *Bacillus thuringiensis* CryIA-type ICPs. *Biochem. Biophys. Res. Commun*.201: 782–787.

Martínez W., Uribe D., Cerón J. (2003). Efecto tóxico de proteínas Cry1 de *Bacillus thuringiensis* sobre larvas de *Tecia solanivora* (Lepidoptera: Gelechiidae). *Rev. Colomb. Entomol*.29: 89–93.

Martins E. S., Monnerat R. G., Queiroz P. R., Dumas V. F., Braz S. V., de Souza Aguiar R. W., Gomes A. C., Sánchez J., Bravo A., Ribeiro B. M. (2010). Midgut GPI-anchored proteins with alkaline phosphatase activity from the cotton boll weevil (Anthonomus grandis) are putative receptors for the Cry1B protein of *Bacillus thuringiensis*. *Insect. Biochem. Mol. Biol*. 40: 138-145.

Miller E. A., Lee M. C. S., Atkinson A. H. O., Anderson M. A. (2000). Identification of a novel four-domain member of the proteinase inhibitor II family from the stigmas of *Nicotiana alata*. *Plant. Mol. Biol*. 42: 329-333.

Monnerat R., Martins E., Queiroz P., Ordúz S., Jaramillo G., Benintende G., Cozzi J., Real M. D., Martinez-Ramirez A., Rausell C., Cerón J., Ibarra J. E., Del Rincon-Castro M. C., Espinoza A. M., Meza-Basso L., Cabrera L., Sánchez J., Soberon M., Bravo A. (2006). Genetic variability of *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) populations from Latin America is associated with variations in susceptibility to *Bacillus thuringiensis* Cry toxins. *Appl. Environ. Microbiol*. 72:7029-7035.

Moran Y., Cohen L., Kahn R., Karbat I., Gordon D., Gurevitz M. (2006). Expression and Mutagenesis of the Sea Anemone Toxin Av2 Reveals Key Amino Acid Residues Important for Activity on Voltage-Gated Sodium Channels.*Biochemistry*. 45: 8864-8873.

Moran Y., Gordon D., Gurevitz M. (2009). Sea anemone toxins affecting voltage-gated sodium channels-molecular and evolutionary features. *Toxicon*. 54: 1089–1101.

Morse R. J., Yamamoto T., Stroud R. M. (2001). Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure*.9:409-417.

Mourey L., Pédelacq J. D., Birck C., Fabre C., Rougé P., Samama J. P. (1998). Crystal structure of the arcelin-1 dimer from *Phaseolus vulgaris* at 1.9-A resolution.*J. Biol. Chem*. 273:12914-12922.

Naganuma M., Sekine S., Fukunaga R., Yokoyama S. (2009). Unique protein architecture of alanyl-tRNA synthetase for aminoacylation, editing, and dimerization. *Proc. Natl. Acad. Sci*. USA.106: 8489-8494.

Nakanishi K., Yaoi K., Shimada N., Kadotani T., Sato R. (1999).*Bacillus thuringiensis* insecticidal Cry1Aa toxin binds to a highly conserved region of aminopeptidase N in the host insect leading to its evolutionary success. *Biochim. Biophy. Acta*.1432: 57-63.

Nakasu E. Y., Firmino A. A., Campos Dias S., Lima Rocha T., Batista Ramos H., Ramos de Oliveira G., Lucena W., Ribeiro da Silva Carlini C. R., Grossi de Sá M. F. (2010). Analysis of Cry8Ka5-binding proteins from *Anthonomus grandis* (Coleoptera: Curculionidae) midgut.*J Invertebr. Pathol*. 104: 227-230.

Ochoa-Campuzano C., Real M. D., Martinez-Ramirez A. C., Bravo A., Rausell C. (2007). An ADAM metalloprotease is a Cry3Aa *Bacillus thuringiensis* toxin receptor.*Biochem. Biophys. Res. Comm*. 362: 437-442.

Oerke E.-C., Dehne H.-W. (2004). Safeguarding production-losses in major crops and the role of crop protection. Crop Protection. 23: 275-285.

Ohashi S., Miyashita H., Okada N., Lemura T., Watanabe T., Kobayashi M. (2008). Unique photosystems in *Acaryochloris marina*. Photosynth.Res. 98:141-149.

Omecinsky D. O, Holub K. E., Adams M. E., Reily M. D. (1996). Three-dimensional structure analysis of μ-agatoxins: further evidence for common motifs among neurotoxins with diverse ion channel specificities. *Biochemistry*.35: 2836-2844.

Oren D. A., Froy O., Amit E., Kleinberger-Doron N., Gurevitz M., Shaanan B. (1998). An excitatory scorpion toxin with a distinctive feature: an additional α helix at the C terminus and its implications for interaction with insect sodium channels. *Structure*. 6:1095-1103.

Pai A., Bernasconi G. (2008). Polyandry and female control: the red flour beetle *Tribolium castaneum* as a case study. *J. Exp. Zool. B. Mol. Dev. Evol*. 310:148-159.

Paramasivan R., Sivaperumal R., Dhananjeyan K. J., Thenmozhi V., Tyagi B. K.(2006). Prediction of 3-dimensional structure of salivary odorant-binding protein-2 of the mosquito Culex quinquefasciatus, the vector of human lymphatic filariasis. *In Silico Biol*. 7: 1-6.

Park Y., Abdullah M. A., Taylor M. D., Rahman K., Adang M. J. (2009). Enhancement of *Bacillus thuringiensis* Cry3Aa and Cry3Bb Toxicities to Coleopteran Larvae by a Toxin-Binding Fragment of an Insect Cadherin.*Appl. Environ. Microbiol*.75: 3086-3092.

Pava-Ripoll M., Posada F. J., Momen B., Wang C., St Leger R. (2008). Increased pathogenicity against coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) by *Metarhizium anisopliae* expressing the scorpion toxin (AaIT) gene.*J. Invertebr. Pathol*.99: 220–226.

Pechan T., Cohen A., Williams W. P., Luthe D. S. (2002). Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. *Proc. Natl. Acad. Sci. U S A*. 99: 13319-13323.

Petrey D., Honig B. (2009). Is protein classification necessary? Toward alternative approaches to function annotation.*Curr Opin Struct Biol* 19:363–368.

Peumans W. J., Van Damme E. J. (1995a). Lectins as plant defence proteins. *Plant.Physiol*. 109: 347–352.

Peumans W. J., Van Damme E. J. (1995b).Role of lectins in plant defense.*Histochem.J.* 27: 253–271.

Philpott, M.L., Hammock, B.D. (1990). Juvenile hormone esterase is a biochemical anti-juvenile hormone agent. *Insect Biochemistry* 20: 451–459.

Pigott C.R., Ellar D.J. (2007). Role of Receptors in *Bacillus thuringiensis* Crystal Toxin Activity.*Microbiol. Mol. Biol. Rev*. 71: 255–281.

Possani L. D., Becerril B., Delepierre M., Tytgat J. (1999).Scorpion toxins specific for Na+-channels.*Eur. J. Biochem*. 264: 287-300.

Qin Y., Ying S. H., Chen Y., Shen Z. C., Feng M. G. (2010). Integration of insecticidal protein Vip3Aa1 into *Beauveria bassiana* enhances fungal virulence to *Spodoptera litura* larvae by cuticle and per Os infection. *Appl. Environ. Microbiol*.76:4611-4618.

Ralph S. G., Yueh H., Friedmann M., Aeschliman D., Zeznik J. A., Nelson C. C., Butterfield Y. S., Kirkpatrick R., Liu J., Jones S. J., Marra M. A., Douglas C. J., Ritland K., Bohlmann J. (2006). Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant. Cell. Environ*. 29: 1545-1570.

Ramírez R. (2009). La broca del café en Líbano. Impacto socioproductivo y cultural en los años 90.*Revista de Estudios Sociales*.32: 158-171.

Rayapuram C., Baldwin I. T. (2008). Host-plant-mediated effects of Nadefensin on herbivore and pathogen resistance in *Nicotiana attenuata. BMC Plant.Biol.* 8: 109.

Ribas de Pouplana L., Schimmel P. (2000). A view into the origin of life: aminoacyl-tRNA synthetases *Cell. Mol. Life Sci*. 57: 865–870.

Rodríguez de la Vega R. C., Schwartz E. F., Possani L. D. (2010). Mining on scorpion venom biodiversity. *Toxicon*. 56: 1155–1161.

Roh J. Y., Choi J. Y., Li M. S., Jin B. R., Je Y. H.(2007). *Bacillus thuringiensis* as a Specific, Safe, and Effective Tool for Insect Pest Control. *J. Microbiol. Biotechnol*. 17: 547–559.

Rosengren K. J., Daly N. L., Plan M. R., Waine C., Craik D. J. (2003). Twists, knots, and rings in proteins. Structural definition of the cyclotide framework. *J. Biol. Chem*. 278:8606-8616.

Sayed A., Nekl E. R., Siqueira H. A., Wang H. C., Ffrench-Constant R. H., Bagley M., Siegfried B. D. (2007). A novel cadherin-like gene from western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), larval midgut tissue. *Insect.Mol. Biol*. 16: 591–600.

Schirra H. J., Anderson M. A., Craik D. J. (2008). Structural refinement of insecticidal plant proteinase inhibitors from *Nicotiana alata. Protein. Pept. Lett*.15:903-909.

Schirra H. J., Scanlon M. J., Lee M. C., Anderson M. A., Craik D. J. (2001). The solution structure of C1-T1, a two-domain proteinase inhibitor derived from a circular precursor protein from *Nicotiana alata. J. Mol. Biol*. 306:69-79.

Schrank A., Vainstein M. H. (2010). *Metarhizium anisopliae* enzymes and toxins.*Toxicon*. 56: 1267-1274.

Schnepf E., Crickmore N., Van Rie J., Lereclus D., Baum J., Feitelson J., Zeigler D.R., Dean D.H. (1998). *Bacillus thuringiensis* and Its Pesticidal Crystal Proteins. *Microbiol. Mol. Biol. Rev*. 62: 775-806.

Shah P. A., Pell J. K. (2003). Entomopathogenic fungi as biological control agents.*Appl. Microbiol. Biotechnol*. 61: 413-423.

Shu Q., Lu S. Y., Gu X. C., Liang S. P. (2002). The structure of spider toxin huwentoxin-II with unique disulfide linkage: evidence for structural evolution. *Protein Sci*. 11:245-252.

Skinner W. S., Dennis P. A., Li J.P., Summerfelt R.M., Carney R. L., Quistad G. B. (1991). Isolation and Identification of Paralytic Peptides from Hemolymph of the Lepidopteran Insects Manduca sexta, Spodoptera exigua, and *Heliothis virescens*. *J. Biol. Chem*. 266: 12873-12877.

St. Leger R. J., Wang C. (2010). Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests.*Appl. Microbiol. Biotechnol*. 85:901–907.

Suckling D. M., Brockerhoff E. G. (2010). Invasion biology, ecology, and management of the light brown apple moth (Tortricidae). *Annu. Rev. Entomol*. 55:285-306.

Szolajska E., Poznanski J., López M., Michalik J., Gout E., Fender P., Bailly I., Dublet B., Chroboczek J. (2004). Poneratoxin, a neurotoxin from ant venom. Structure and expression in insect cells and construction of a bio-insecticide.*Eur. J. Biochem*.271: 2127–2136.

Tedford H. W., Sollod B. L., Maggio F., King G. F.(2004). Australian funnel-web spiders: master insecticide chemists. *Toxicon*. 43:601-618.

Tindall K. V., Stewart S., Musser F., Lorenz G., Bailey W., House J., Henry R., Hastings D., Wallace M., Fothergill K. (2010). Distribution of the long-horned beetle, *Dectes texanus*, in soybeans of Missouri, Western Tennessee, Mississippi, and Arkansas.*J. Insect.Sci*. 10:178.

Tugarinov V., Kustanovich I., Zilberberg N., Gurevitz M., Anglister J. (1997). Solution structures of a highly insecticidal recombinant scorpion α-toxin and a mutant with increased activity. *Biochemistry*.36: 2414-2424.

van Frankenhuyzen K. (2009).Insecticidal activity of *Bacillus thuringiensis* crystal proteins. *J. Invertebr. Pathol*.101: 1–16.

Venter, J.C., et.al.(2004). Environmental Genome Shotgun Sequencing of the Sargasso Sea.*Science*.304: 66-74.

Vaishnavi C. (2010). Clinical spectrum & pathogenesis of *Clostridium difficile* associated diseases. *Indian. J. Med. Res*. 131: 487-499.

Vassilevski A., Kozlov S. A., Grishin E. V. (2009). Molecular Diversity of Spider Venom. *Biochemistry (Moscow)*.74: 1505-1534.

Wang X. H., Connor M., Wilson D., Wilson H. I., Nicholson G. M., Smith R., Shaw D., Mackay J. P., Alewood P. F., Christie M. J., King G. F. (2001).Discovery and structure of a potent and highly specific blocker of insect calcium channels.*J. Biol. Chem*. 276: 40306-40312.

Wang L., Wang S., Li Y., Paradesi M. S. R., Brown S. J. (2007). BeetleBase: the model organism database for *Tribolium castaneum*. Nucleic. Acids. Res. 35: D476–D479.

Warmke, J. W., Reenan, A. G. R., Wang, P., Qian, S., Arena, J. P., Wang, J., Wunderler, D., Liu, K., Kaczorowski, G. J., Van der Ploeg, L. H. T., Ganetzky, B., and Cohen, C. J. (1997). Functional expression of Drosophila para sodium channels.Modulation by membrane protein TipE and toxin pharmacology.*J. Gen. Physiol*. 110: 119-133.

Whetstone P. A., Hammock B. D. (2007).Delivery methods for peptide and protein toxins in insect control.*Toxicon*. 49: 576–596.

Wolfersberger M. G., Luethy P., Maurer A., Parenti P., Sacchi F. V., Giordana B., Hanozet G. M. (1987). Preparation and partial characterization of amino acid transporting

brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). *Comp. Biochem.Physiol*. 86:301–308.

Yamaji N., Sugase K., Nakajima T., Miki T., Wakamori M., Mori Y., Iwashita T. (2007). Solution structure of agelenin, an insecticidal peptide isolated from the spider *Agelena opulenta*, and its structural similarities to insect-specific calcium channel inhibitors. *FEBS Lett*.581: 3789-3794.

Yu X. Q., Prakash O., Kanost M. R. (1999).Structure of a paralytic peptide from an insect, *Manduca sexta*.*J. Pept. Res*. 54:256-261.

Zilberberg N., Froy O., Loret E., Cestele S., Arad D., Gordon D., Gurevitz M. (1997). Identification of structural elements of a scorpion α-neurotoxin important for receptor site recognition.*J. Biol. Chem*. 272: 14810–14816.

Zlotkin E., Fishman Y., Elazar M. (2000).AaIT: from neurotoxin to insecticide.*Biochimie*.82: 869-881.