International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(1): 2544-2552

Received: 24-11-2018
Accepted: 28-12-2018

Shahanaz
Department of Entomology, College of Horticulture, Mojerla, Sri Konda Laxman Telangana State Horticultural University, Wanaparthy, Telangana, India

Correspondence
Shahanaz
Department of Entomology, College of Horticulture, Mojerla, Sri Konda Laxman Telangana State Horticultural University, Wanaparthy, Telangana, India

Spider venom peptides: A potential insecticide

Shahanaz

Abstract
This review provides an overview of the development of spider venom research, focusing on the structure and function of venom components and analysis techniques. The major groups of venom components are low molecular weight compounds, antimicrobial (Also called cytolytic or cationic) peptides (only in some spider families), cysteine-rich peptides (Neurotoxic), and enzymes and proteins. Cysteine-rich peptides are reviewed with respect to various structural motifs, their targets (ion channels, membrane receptors), nomenclature, and molecular binding. We further describe the latest findings regarding the maturation of antimicrobial and cysteine-rich peptides, which in most known cases are expressed as propeptide-containing precursors.

Keywords: Spider, venom, peptides, bioinsecticide

Introduction
Over 10,000 arthropod species are currently considered to be pest organisms. They are estimated to contribute to the destruction of ~14% of the world's annual crop production and transmit many pathogens. Presently, arthropod pests of agricultural and health significance are controlled predominantly through the use of chemical insecticides. Unfortunately, the widespread use of these agrochemicals has resulted in genetic selection pressure that has led to the development of insecticide-resistant arthropods, as well as concerns over human health and the environment. Bio insecticides represent a new generation of insecticides that utilise organisms or their derivatives (transgenic plants, recombinant baculoviruses, toxin-fusion proteins and peptidomimetics) and show promise as environmentally-friendly alternatives to conventional agrochemicals. Spider-venom peptides are now being investigated as potential sources of bio insecticides. With an estimated 100,000 species, spiders are one of the most successful arthropod predators. Their venom has proven to be a rich source of hyper stable insecticidal mini-proteins that cause insect paralysis or lethality through the modulation of ion channels, receptors and enzymes. Many newly characterized insecticidal spider toxins target novel sites in insects. The structure and pharmacology of these toxins and discuss the potential of this vast peptide library for the discovery of novel bioinsecticides (Windley *et al*., 2012) [19].
Spider venoms are an incredibly rich source of disulfide-rich insecticidal peptides that have been tuned over millions of years to target a wide range of receptors and ion channels in the insect nervous system (King and Hardy, 2013) [6]. These peptides can act individually, or as part of larger toxin cabals, to rapidly immobilize envenomated prey owing to their debilitating effects on nervous system function. Most of these peptides contain a unique arrangement of disulfide bonds that provides them with extreme resistance to proteases. As a result, these peptides are highly stable in the insect gut and hemolymph and many of them are orally active. Thus, spider-venom peptides can be used as stand-alone bioinsecticides, or transgenes encoding these peptides can be used to engineer insect-resistant crops or enhanced entomopathogens. The potential of spider-venom peptides to control insect pests and highlight their advantages and disadvantages compared with conventional chemical insecticides (Khan *et al*., 2006) [5].
In contrast, the global biopesticide sector has grown more strongly, with a CAGR of 15.6% and an increase in value from USD1.6 billion in 2009 to an estimated USD3.3 billion in 2014. In 2006, orchard crops had the largest share of biopesticide use at 55%, and in the same year biopesticides represented roughly 2.5% of the global pesticide market. However, synthetic pesticides still retain the highest market share, with a CAGR of 3% leading to an estimated value of USD48 billion in 2014. Nevertheless, the 5-fold higher CAGR for biopesticides has resulted in increased interest in this sector of the market.

The potential sources of biopesticides include microbes (viral, fungal, bacterial), entomophagous nematodes, plant-derived products, insect pheromones and insect resistance genesexpressed in crops. In particular, insecticidal toxins derived from insect predators and parasitoids are of growing interest in the development of bioinsecticides, and these include peptide neurotoxins derived from the venoms of scorpions. Parasitic wasps the straw itch mite and spiders. Currently, there is a great deal of interest in spider venoms as they comprise an extensive library of potent insecticidal, neurotoxic peptides.

Spider venoms sources of novel bio insecticides

Spiders are ancient creatures that evolved from an arachnid ancestor around 300 million years ago during the Carboniferous period. This highlights the long evolutionary timescale over which spiders have evolved their complex venom. Spiders are the most speciose venomous animal and along with predatory beetles are the most successful terrestrial predators, with over 42,000 extant species described to date. This may be an under-representation of their true speciation, with about four times as many species predicted to exist, but not yet characterised (Nachman and Pietrantonio, 2010) [10].

One of the major features contributing to the overall success of spiders is the production of a highly toxic venom from their venom glands that they employ to subdue prey and deter predators. Since they rely completely on predation as a trophic strategy, spiders have evolved a complex pre-optimized combinatorial library of enzymes, neurotoxins and cytolytic compounds in their venom glands. These venom components fall into three classes delineated by their molecular mass (i) low molecular mass acylpolyamines and other non peptidic molecules (<1 kDa), (ii) disulfide-rich neurotoxins and linearcytolytic peptides (1–10 kDa), and (iii) high molecular mass proteins (>30 kDa) comprising mainly enzymes and neurotoxins.

Most spider venoms are dominated by small disulfide-rich peptide neurotoxins (Figure 1B), and these are the largest and most extensively studied group of spider toxins. To date, around 800 peptide toxins from 78 spider species have been described in ArachnoServer 2.0 (www.arachnoserver.org), a curated database containing available information on spider-venompeptides and proteins. These toxins were isolated from the venom of 20 of the 110 extant spider families, including representatives from the two major infraorders Araneomorphae (modern spiders) and Mygalomorphae (primitive spiders) (Figure 1A). Araneomorphs represent >90% of all known spider species. However, mygalomorphs are a more sustainable and convenient source of venom due to their large venom glands and their longevity (they can live for over 25 years).

Spiders master insect predators

The ecological advantages conferred by the possession of a venom system are evident from the extraordinarily diverse phyla that have evolved venoms for predation, defence, and competitor deterrence. The extant suite of venomous taxa includes cnidarians, echinoderms, molluscs, vertebrates, and arthropods (e.g., ants, bees, centipedes, scorpions, spiders, and wasps) (King, 2011) [7]. Spiders are the most successful venomous animals and, with the possible exception of predatory beetles, they are the most abundant terrestrial predators (Windley *et al*., 2012) [19]. The number of extant spider species, which is predicted to be greater than 150,000 (Coddington and Levi, 1991) [3], is likely to be larger than the total number of venomous predators in all other terrestrial phyla. The remarkable evolutionary success of spiders is due in large part to their ingenious exploitation of silk and the evolution of a pharmacologically complex venom that ensures rapid subjugation of prey. All spiders, with the exception of the hackled orb weavers (Uloboridae) and possibly certain species of primitive mesothelids, produce venom in paired glands that reside either in the basal segment of the chelicerae in primitive mygalomorph spiders or in the anterior of the prosoma in modern araneomorph spiders. A duct from each venom gland leads to a small opening near the tip of the corresponding fang. Compression of the muscles encircling each venom gland forces venom along the duct and out through the opening in the fang tip. Thus, the envenomation apparatus of spiders acts like a pressurized hypodermic needle that is capable of delivering controlled microliter doses of venom. His primary purpose of spider venom is to rapidly subdue prey.

Spiders often tackle large prey that can be physically dangerous or even venomous; for example, crab spiders of the family Thomisidae prey primarily on venomous hymenopterans, whereas the so-called assassin spiders in the family Archaeidae are obligate predators of other spiders.

Chemistry and pharmacology of spider venoms

The venoms of spiders are less well studied than those from other venomous taxa such as marine cone snails, scorpions, and snakes. Venom components from only 174 (~0.4%) of the 43,244 extant species cataloged to date have been described. However, the taxonomic coverage is better than these numbers suggest, as these 174 spiders belong to 32 (30%) of the 109 extant families of venomous spiders. The chemical complexity of spider venoms is extraordinary, ranging from salts and small organic compounds to large presynaptic neurotoxins found in the venom of widow spiders (*Latrodectus* spp.) (Vassilevski *et al*., 2009) [18]. These venom compounds can be broadly grouped into five classes on the basis of their chemical structure and mechanism of action. We examine each of these toxin classes in the context of insecticide development.

a) Salts and small organic compounds

The limited data available on the ionic composition of spider venoms indicate that they havea very low concentration of Na^+ (~10 mM) and a high concentration of K^+ (70–200 mM) (Vassilevski *et al*., 2009) [18], which is the inverse of the Na^+/K^+ ratio in the hemolymph of most insects. The high K^+ concentration may contribute to venom toxicity by causing depolarization of axonal fibers in the vicinity of the venom injection site, as proposed for the K^+-rich prevenom in scorpions. A wide range of small organic compounds (<1 kDa) have been found in spider venoms, including amino acids (e.g., GABA, glutamate, and taurine), acylpolyamines, biogenic amines (histamine and octopamine), neurotransmitters (acetylcholine), nucleosides (adenosine), and nucleotides (ATP) (Vassilevski *et al*., 2009) [18]. However, because the molecular components that make up the neuronal circuitry of insects and vertebrates are highly conserved, most of these small molecules have limited phylum selectivity, being active in both arthropods and humans. Consequently, none of these compounds have been seriously pursued as insecticide leads.

b) Linear cytolytic peptides

Peptides (defined as proteins less than 10 kDa) are the dominant components of most spider venoms and the primary source of their pharmacological diversity (Figure 1). Proteomic analyses have revealed that some spider venoms contain more than 1,000 unique peptides. Thus, as a group, spider venoms might contain as many as 20 million bioactive peptides based on a conservative estimate of 200 peptides per venom and 100,000 extant species. This incredible chemical diversity is one reason why spider venoms are widely used as natural sources for drug and insecticide discovery programs.

The majority of spider-venom peptides have a mass of 3.0 to 4.5 kDa (composed of 25 to40 amino acid residues), but there is a significant fraction with a mass of 6.5 to 8.5 kDa (composed of 58 to 76 residues). As in scorpion-venom peptides, posttranslational modifications (PTMs) are rare in spider-venom peptides, with the exception of disulfide bonds and C-terminal a midation (Windley *et al*., 2012) [19]. This situation contrasts with the very high frequency of PTMs other than disulfide bonds in venom peptides from marine cone snails.

No cytolytic peptides have been reported from the venoms of mygalomorph spiders (Saez *et al*., 2010) [15]. At this stage it is unclear whether this absence is simply due to the limited taxonomic sampling of spider venoms or whether the cytolytic peptides are an araneomorph-specific innovation. Like most cytolytic peptides found in scorpion and hymenopteran venoms the cytolytic peptides from spider venoms have only weak insecticidal activity compared with their disulphide reticulated counter parts. It has been proposed that their primary role is to facilitate the act ion of the disulfide-rich neurotoxins by breaking down anatomical barriers, dissipating transmembrane ion gradients, and/or perturbing the membrane potential across excitable cells. These peptides have not been used as insecticide leads owing to their intrinsically weak insecticidal activity and their lack of selectivity; most of them are hemolytic and broadly cytolytic in both vertebrates and invertebrates, with many having antimicrobial activity (Vassilevski *et al*., 2009) [18].

c) Disulfide-Rich Peptide Neurotoxins

Disulfide-rich (SS-rich) peptides are the dominant compounds in most spider venoms and theyare the major contributors to the venom's insecticidal activity. Only a few atypical spider venomsdefy this archetype, such as those from widow spiders (*Latrodectus* spp.), which contain a large proportion of large presynaptic neurotoxins (Rohou *et al*., 2007) [14], and the hobo spider *Tegenaria agrestis*, in which sulfated nucleosides constitute approximately 50% of the venom dry weight. However, even the venoms of sicariid spiders, which are better known for containing sphingomyelinase A (SMaseA) the cause of dermo necrotic lesions in humans are richly populated with SS-rich peptides (Chaim *et al*., 2011) [2].

In terms of insecticidal activity, the SS-rich peptide neurotoxins are typically at least 10-foldmorepotent than most cytolytic venom peptides (Windley *et al*., 2012) [18]. Most SS-rich venom peptides target presynaptic ion channels or postsynaptic receptors either at peripheral neuromuscular junctions (NMJs) or at synapses in the insect central nervous system (CNS) (Figure 3). Individually, or in combination, these peptides can either deaden the insect nervous system and cause flaccid paralysis or over activate the nervous system and cause convulsive paralysis. In either case, the end effect is to rapidly incapacitate envenomated prey.

However, the overall effect induced by the SS-rich peptide neurotoxins is complex and involves groups of toxins that act at different times and at different anatomical sites following envenomation. The range of pharmacologies exhibited by the SS-rich neurotoxins is extraordinary and includes lectins, protease inhibitors, and modulators of transient receptor potential (TRP) channels, mechano-sensitive channels, acid-sensing ion channels, ionotropic glutamate receptors, glutamate transporters, calcium-activated potassium (KCa) channels, voltage-gated calcium (CaV) channels, voltage-gated sodium (NaV) channels, and voltage-gated potassium (KV) channels (Figure 1*b*). It is evident that spiders developed molecules that modulate the activity of key insecticide targets, such as NaV channels, hundreds of millions of years ago.

The pharmacology of some venom peptides suggests that they are involved in predator deterrence rather than prey capture. Thus, it seems likely that some SS-rich spider-venom peptides, along with certain venom enzymes have evolved specifically to ward off vertebrate predators rather than aid in capture of insect prey.

d) Enzymes

A number of enzymes, including collagenase, hyaluronidase, phospholipase A2, SMase A, andvarious proteases, are present in spider venoms. With the exception of SMase A, only limitedsequence information and functional data have been obtained for these enzymes; hence their evolutionary history and true function remain unclear. It has been proposed that their primary role is to degrade extracellular matrix (collagenase, hyaluronidase, proteases) and the underlying cell membrane (SMase A, phospholipaseA2) in envenomated prey to facilitate the spread of peptideneurotoxins.

e) Large pre synaptic neurotoxins

With the exception of enzymes, most spider-venom proteins are smaller than 12 kDa. A notable exception occurs in the venoms of widow spiders (*Latrodectus* spp.), which contain a family of high molecular-weight proteins known as latrotoxins. These 110- to 140-kDa proteins have a similar domain architecture that consists of a unique N-terminal region and a C-terminal region composed of 13 to 22 an kyrin repeats (Rohou *et al*., 2007). However, they have remarkably different phylum selectivity. For example, the venom of the European black widow spider, *Latrodectus tredecimguttatus*, contains avertebrate-specific α-latrotoxin, a crustacean-specific α-latrocrustatoxin, and insect-specific α-,β-, γ-, δ-, and ε-latroinsectotoxins (Rohou *et al*., 2007;Vassilevski *et al*., 2009;Windley *et al*., 2012) [14, 18, 19].

α-Latrotoxin is widely used as a pharmacological tool because of its ability to induce massive neurotransmitter release from vertebrate nerve terminals.

It is thought to be the major component responsible for the potentially fatal effects of *Latrodectus* envenomation α-Latrotoxinbinds to specific receptors on presynaptic nerve terminals, which enables it to subsequently insert into the nerve terminal membrane to form a non-selective cation channel. It then causes massive exocytosis of synaptic vesicles by a complex set of calcium-dependent and calcium-independent pathways that remain to be fully elucidated. Specific receptors for the latroinsectotoxins have yet to be identified, but orthologs of all three classes of vertebrate α-latrotoxin receptors are present in insects. Moreover, analogous to the function of α-latrotoxin invertebrates, the

latroinsectotoxins induce exhaustive neurotransmitter release at insect NMJs (Rohou *et al*., 2007) [14].

Spider venoms contain enzymes that facilitate access of peptide and protein neurotoxins to their molecular targets by degrading the myelin sheath around axons as well as the extracellular matrix of the synaptic cleft. The α-latrotoxins cause massive neurotransmitter release by promoting synaptic vesicle exocytosis (King and Hardy, 2013) [6].

Latrotoxins are also found in the closely related the ridiid spider *Steatoda grossa* but they have not been found in venoms outside of the family. The ridiidae. The latroinsectotoxins are the most potent insecticidal toxins isolated from spider venoms, with extremely low 50% lethal dose (LD50) values of less than 1 pmol g−1 in both lepidopterans and dipterans (Windley *et al*., 2012) [19]. However, they have not been pursued as bioinsecticide leads because of their large size, complex mode of action, and the difficulty of producing those using synthetic or recombinant methods.

Toxin cabals making venom more potent than the sum of its parts

The concept of toxin cabals was first introduced to explain how groups of venom peptides from marine cone snails could act synergistically to enhance venom potency. Spider venoms also contain toxin cabals that differ in their time and site of action. For example, both δ- and κ-toxins are present in the venom of the Chinese earth tiger tarantula, *Chilobrachys guangxiensis*, indicating that at least some spider venoms might contain lightning-strike cabals. (King and Hardy, 2013) [6].

Small molecules in spider venoms can also form toxin cabals. For example, free glutamate present in spider venoms activates ion tropic glutamate receptors at prey NMJs immediately after venom injection, which allows acylpolyamines present in the venom to access these channels where they act as open-channel blockers (Vassilevski *et al*., 2009) [18].

However, in contrast with cone snails, spiders seem to have developed toxin cabals that are designed specifically for long-term prey capture and storage. Unlike cone snails, which immediately consume envenomated prey, some spiders store prey for hours to days prior to consumption. Thus, it is important that prey is immediately immobilized by the spider's venom to aid capture, but it is equally important that venom causes irreversible immobilization (which is not the case for the lightning-strike cabal) so that prey can be stored if required. For example, Australian funnel web spiders (*Atrax* and *Hadronyche*) contain two different peptidic blockers of insect CaV channels, which ostensibly appear redundant. However, they have different sites and time courses of action (Oerk and Dehne, 2004) [11].

One of these toxins causes almost instantaneous paralysis, presumably by blocking CaV channels at peripheral NMJs, but its effects are reversible within a few hours. In contrast, the other peptide has no effect on insect NMJs, but it causes a flaccid paralysis by blocking CaV channels in the CNS; it takes 20 to 30 min for the effects of this toxin to become apparent owing to the time it takes for the peptide to traverse the insect blood-brain barrier, but its effects are irreversible. When these two SS-rich neurotoxins are combined, the prey is rapidly paralyzed and remains so until the spider is ready to consume it. Thus, although at first glance it seems difficult to rationalize the remarkable chemical and pharmacological complexity of spider venoms, detailed examination reveals that the venom is an extremely fine-tuned system of different components, sometimes with seemingly contradictory modes of action, acting together in a synergistic, time-dependent manner to maximize the overall effect of the venom on prey.

Structure of spider-venom peptides

Knots and Helices

An attempt has been made to classify spider-venom peptides on the basis of their disulfide frame work and primary structure. But in reality we know little about the structure of spider-venom peptides. Three-dimensional structures have been determined for only 44 spider-venom peptides, and 39 of these conform to a single structural class, known as the inhibitor cystine knot (ICK) motif of the remaining five structures, three are helical cytolytic peptides, onehas a Kunitz domain fold, and one is a novel scaffold. However, on the basis of sequence homology with peptides from other venoms, there are other three-dimensional folds present in spider venoms, including cysteine-rich secretory protein (CRISP) domains and prokinetic in scaffolds. (Saez *et al*., 2010) [15].

The ICK motif is defined as an antiparallel β sheet stabilized by a cystine knot In spider toxins, the β sheet typically comprises only two β strands (structure 2A2V in Figure1*a*), although a third N-terminal strand is sometimes present (King *et al*., 2002; Saez *et al*., 2010) [20, 15]. The cystine knot comprises a ring formed by two disulfide bridges and the intervening sections of peptide backbone, with a third disulfide bond piercing the ring to create a pseudoknot. The two central disulfide bridges that emanate from the two β strands are closely packed against oneanother and form the compact hydrophobic core of these hyperstable mini-proteins.

The ICK motif provides these small peptides with exceptional chemical and thermal stability; they are resistant to extremes of pH, organic solvents, and high temperatures (Saez *et al*., 2010) [15]. However, from a biological perspective, their most important property is resistance to proteases. ICK peptides are typically stable in insect hemolymph for several days and have half-lives of longer than 12 h in gastric fluid (Saez *et al*., 2010) [15]. The marked insensitivity of the ICK scaffold to changes in intercystine residues has enabled spiders to develop diverse pharmacologies using this disulfide framework (Sollod *et al*., 2005) [17].

All insecticidal spider-venom toxins display a classical prepropeptide paradigm except LITs (e.g., α-LIT-Lt1a) from *Latrodectus* spp. ω-AGTX-Aa1a from *Agelenopsis aperta* is a heterodimer consisting of a 66-residue major chain that is linked via a disulfide bond to a 3-residue minor chain. Known PTMs in spider-venom peptides (dark grey bars) as well as probable PTMs (light grey bars) and those predicted from sequence homology (white bars).

Insecticidal spider-venom peptides

The natural preys of most spiders are invertebrates, mainly insects, although other arachnids such as mites, opilionids, and both conspecific and non-conspecific spiders often contribute to their diet. Because most spiders are polyphagous (i.e., they do not feed on a restricted prey type or taxon)., their venoms have evolved to contain an array of compounds that target a broad spectrum of insect prey. Moreover, although some large spiders consume small vertebrates, very few are toxic to humans. Hence, the primary rationale for investigating spider venoms as a potential source of bioinsecticides is that their venoms are expected to contain a wide range of insecticidal peptides that mostly have little or

no mammalian activity. The past two decades of research on spider venoms has largely validated this hypothesis. To date, more than 200 SS-rich insecticidal spider-venom peptides (ISVPs) have been sequenced (Windley *et al*., 2012) [19]. They range in size from 3.3 to 9.0 kDa and contain3 to 6 disulfide bonds. However, only several dozen of these peptides are sufficiently potent (i.e.,LD50 <1500 pmol g−1) to warrant serious consideration as bioinsecticides, and even fewer have been shown to be harmless to vertebrates.

The attributes of those ISVPs that appear to hold most promise as bioisecticides are summarized in Table 1. Notably, 10 of these 13 ISVPs cause no adverse effects when injected into rodents, indicating that they are highly selective for insects.

Many of these ISVPs have molecular targets that are distinct from those of extant chemical insecticides, including CaV channels, NMDA receptors, and glutamate transporters. Some of these, such as CaV channels, have been validated as insecticide targets by gene knockout and inducible expression of ISVP transgenes in *Drosophila melanogaster.* Thus, in addition to their potential as bioinsecticides, ISVPs have helped expand the range of validated insecticide targets. Because ISVPs are genetically encoded mini-proteins, they provide more options for insect control than conventional chemical insecticides. Aside from the traditional chemical approach of using them as foliar sprays or incorporating them into baits, ISVP transgenes can be incorporated into plants as an insect-resistance trait or used to enhance the efficacy of entomopathogens (Richards, 1997) [21].

Potential of Spider Venom Peptides for control of Insect Pests.

a) Spider-venom peptides as bio insecticides

In contrast with most chemical insecticides, ISVPs are unlikely to be topically active, because in order to access their sites of action in the insect nervous system, they would have to penetrate the insect exoskeleton, which comprises an outer lipophilic epicuticle and a heavily sclerotized exocuticle. In the only report that describes topical activity for a spider-venom peptide, a fusion of ω-HXTX-Hv1a to the C terminus of thioredoxin was topically active to second-instar *Helicoverpa armigera* and *Spodoptera littoralis* larvae (Khan *et al*., 2006). However, the fusion protein was applied in a solution containing a very high concentration of imidazole, a compound known to have contact insecticidal activity, so it remains uncertain whether ω-HXTX-Hv1a is indeed topically active. It is possible that clever peptide analoging, as has been used to confer both oral and topical activity on small insect kinin neuropeptides could be used to engineer topically active ISVPs.

However, this method would substantially increase the cost of ISVP manufacture and render the peptide non-natural, potentially increasing the time and cost of product registration. If we exclude the topical route, then ISVPs must be delivered via a vector such as an entomopathogen or, alternatively, ingested by the targeted insect pest (if they have oral activity) in order to be effective. Very few studies have explored the oral activity of ISVPs, but emerging evidence indicates that many of them, particularly ICK peptides with high levels of protease resistance, will have some level of oral activity. These hyper stable peptides are likely to have long residence times in the insect gut, and therefore even low rates of intestinal absorption will make them orally available. For example, the ICK-containing insecticidal peptide ω-HXTX-Hv1a was orally active against the lone star tick, *Amblyomma americanum*, and it's per os activity was only slightly lower than when the peptide was injected (Mukherjee *et al*., 2006) [9]. Consistent with this observation, the same peptide (or its ortholog ω-HXTX-Ar1a) was orally active against lepidopteran pests when expressed in cotton (*Gossypium* spp.), poplar (*Populus* spp.), and tobacco (*Nicotiana tabacum*) plants (Khan *et al*., 2006) [5].

In general, however, the LD50 for ISVPs fed to insects is ~90-fold higher than when they are injected. This higher LD50 presumably results from a slow rate of absorption in the insect gut, as observed previously for insect neuropeptides and SS-rich peptides from scorpion and snake venoms. For highly potent ISVPs, their lower but still substantial per os activity does not preclude their potential use as stand-alone insecticides. However, the commercial potential of ISVPs would clearly be enhanced by any method that significantly improved their oral activity. One option is to decorate ISVPs with covalently attached polyethylene glycol polymers, an approach that substantially improved the oral insecticidal activity of the trypsin-modulating ostatic factor. However, this approach suffers from the same disadvantages as chemically synthesized peptide analogs, including increased costs of manufacture and longer and costlier product registration.

A promising alternative approach is fusion of ISVPs to a carrier protein that facilitates transport across the insect gut into the hemolymph. The best-studied fusion protein for this purposeis *Galanthus nivalis* agglutinin (GNA), a mannose-specific lectin from the snowdrop plant. When ingested by insects, GNA binds to glycoproteins in the digestive tract and is subsequently transported across the gut epithelium into the hemolymph over a period of 24 hours, the protein accumulates in the insect gut, Malpighian tubules, and hemolymph. GNA itself is moderately insecticidal in some insects but inclusion of GNA at 2%of total dietary protein had no effect on survival or growth of larvae of the tomato moth, *Lacanobia oleracea* Thus, GNA is a relatively innocuous fusion protein that can be used to transport insecticidal peptides across the insect gut and thereby enhance their oral activity.

In the case of *L. oleracea* and *N. lugens*, the presence of intact fusion protein in insect hemolymph was confirmed by Western blot analysis. Other fusion proteins have been investigated for their ability to enhance the oral activity of SS-rich venom peptides. For example, fusion of AaIT, a 70-residue scorpion-venom peptide, to an N-terminal portion of the coat protein from barley yellow dwarf luteovirus substantially enhanced its oral activity against the aphids *M. persicae* and *Rhopalosiphum padi* the viral coat protein binds to epithelial receptors in the aphid hindgut and mediates virus uptake into the hemocoel.

This approach should be equally applicable to ISVPs, but because luteoviruses are vectored specifically by aphids, it is unclear whether the luteoviral coat protein will facilitate transport of ISVPs across the gut of insects other than aphids.

A significant advantage of the fusion protein approach over chemical modification approaches for improving the oral activity of ISVPs is that the fusion protein can still be produced cheaply by recombinant methods, and transgenes encoding the fusion protein can be engineered into entomopathogens and plants. However, covalent attachment to a fusion protein might alter ISVP selectivity not just with respect to targeted pests but also predators and parasitoids; for example, GNA has deleterious effects on some parasitoids, although only at very high doses Thus, it will be important,

especially in the context of integrated pest management programs, to establish whether covalent attachment to a fusion protein alters ISVP selectivity.

Many ISVPs, either alone or fused to carrier proteins, are likely to have sufficient oral activity, act with sufficient speed (i.e., death or irreversible paralysis within 24 h), and be cheap enough to produce to be competitive with chemical insecticides; for example, Vestaron Corporation recently reported that at least some ISVPs can be produced by yeast fermentation at a cost of less than 20cents per gram and that these ISVPs have activity comparable to chemical insecticides when used as a foliar spray. However, much work remains to be done to determine the eco- toxicological and environmental profile of ISVPs and whether their activity can be improved significantly by formulation as for chemical insecticides (Hardy *et al*., 2013) [4].

A potential advantage of some ISVPs is that they have novel modes of action compared with extant chemical insecticides; hence they might be particularly useful for control of arthropod pests that have developed resistance to multiple classes of chemical insecticides. ISVPs can be useful even in situations where they have the same molecular target as an insecticide to which an insect population has developed resistance. This seems counterintuitive, but it is possible because most arachnid toxins act at sites different from those targeted by chemical insecticides. Thus, target-site mutations that confer resistance to chemical insecticides can increase susceptibility to peptide neurotoxins that act on the same target. For example, even though the scorpion toxin AaIT and pyrethroids both target NaV channels, a pyrethroid-resistant strain of *Heliothis virescens*was more susceptible than non resistant strains to a recombinant baculovirus expressing AaIT.

Many insect pests have developed resistance to existing chemical insecticides and consequently there is much interest in the development of new insecticidal compounds with novel modes of action. Thus, it has been assumed that spider-venom peptides are not orally active and are therefore unlikely to be useful insecticides. Contrary to this dogma, they show that it is possible to isolate spider-venom peptides with high levels of oral insecticidal activity by directly screening forper os toxicity. Using this approach, isolated a 34-residue orally active insecticidal peptide (OAIP-1) from venom of the Australian tarantula *Selenotypus plumipes.* The oral LD50 for OAIP-1 in the agronomically important cotton bollworm *Helicoverpa armigera* was 104.260.6 pmol/g, which is the highest per os activity reported to date for an insecticidal venom peptide. OAIP-1 is equipotent with synthetic pyrethroids and it acts synergistically with neonicotinoid insecticides (Fig 7). The three-dimensional structure of OAIP-1 determined using NMR spectroscopy revealed that the three disulfide bonds form an inhibitor cystine knot motif; this structural motif provides the peptide with a high level of biological stability that probably contributes to its oral activity. OAIP-1 is likely to be synergized by the gut-lytic activity of the *Bacillus thuringiensis* Cry toxin (*Bt*) expressed in insect-resistant transgenic crops, and consequently it might be a good candidate for trait stacking with Bt (Hardy *et al*., 2013) [4].

Dose-response curve resulting from feeding sOAIP-1 to cotton bollworms (larval *H. armigera*) (Fig. B). Thecalculated LD50 values are shown. (Fig C) Mortality observed at 48 h after feeding 100 pmol imidacloprid, 100 pmol sOAIP-1, or a 50:50 mixture of these compounds into *H. armigera*. Each data point is the mean 6SEM of three replicates of 10 individuals.

The oral toxicity of OAIP-1 against *H. armigera* with that of several commercially available pyrethrod insecticides compared and found that on molar basis OAIP-1is more potentthan any of these chemical insecticides.

Enhancing entomopathogens using spider-toxin transgenes

Entomopathogenic Fungi

Insects can be infected by a wide range of bacterial, viral, protozoan, and fungal pathogens. Many of these have potential as bioinsecticides, but fungal entomopathogens such as *Metarhizium* have the distinct advantage that they are contact active and do not require ingestion; fungal spores (conidia) germinate on the surface of the host and penetrate through the insect cuticle before proliferating in the hemocoel. Wild-type strains of entomopathogenic fungi have been developed commercially for control of crop pests; for example, Green Guard TM, an oil-based formulation of *Metarhizium acridum* spores, is used for locust control in Australia. However, a major disadvantage of entomopathogenic fungi compared with chemical insecticides is their slow kill time (typically more than 7 days).It was recently demonstrated that the potency and speed of kill of *M. anisopliae* could be substantially improved by engineering it to express the scorpion-venom peptide AaIT.

The transgenic fungus caused 50% mortality of the tobacco hornworm, *Manduca sexta*, and the dengue vector *Aedes aegypti* using conidial doses 22-fold and 9-fold lower than required with wild-type fungus. When applied at the same dose, the engineered fungus greatly reduced the kill time compared with wild-type fungus; for example, at a dose of 107 conidia ml−1, 50% mortality of *Ae. Aegypti* was attained in 5 days with transgenic fungus compared to >10 days for wild-type fungus. Moreover, mosquitoes infected with transgenic fungus had a reduced tendency to blood feed.

This study suggests that engineering *Metarhizium* to express ISVP transgenes should be an efficient approach for delivering ISVPs to both crop pests and disease vectors because it mitigates two of the potential disadvantages of ISVPs. First, it obviates the need for the ISVP to be orally active as the peptide is produced in situ in the insect hemocoel, and second, the selectivity of the ISVP becomes relatively unimportant as the range of affected insects is largely determined by the host range of the fungus. Thus, off-target effects, particularly on predators and parasitoids, can be minimized by choosing *Metarhizium* species that have a restricted host range; for example, *M. acridum* exclusively infects grasshoppers in the suborder Caelifera.

Baculoviruses

Baculoviruses are double-stranded DNA viruses that have been used commercially for several decades to manage insect pests. They are used extensively in Brazil to protect soybean (*Glycinemax*) crops from the velvet bean caterpillar, *Anticarsia gemmatalis*, and in China to safeguard cotton (*Gossypium* spp.) from the cotton bollworm, *H. Armigera.* For most members of thenucleo polyhedron virus subgroup, infectivity is restricted to a few closely related Lepidopterans. (In most situations, wild-type nucleopolyhedro viruses are not competitive with chemical insecticides because they typically take 5 to 10 days to kill their host, during which time the insect continues to feed and cause crop damage.

This shortcoming has been addressed by engineering transgenic nucleopolyhedro viruses that express insecticidal venom peptides from sea an venoms, scorpions, or spiders in all cases, expression of the toxin transgene reduced the time between virus application and cessation of feeding or death. Notably, however, the most dramatic improvement in insecticidal activity resulted from incorporation of a transgene-encoding μ-agatoxin-Aa1d (μ-Aga IV) a 37-residue ISVP from the venom of the American funnel-web spider *Agelenopsis aperta*. Thus, engineering baculoviruses to express ISVP transgenes should be an efficient approach for delivering ISVPs to insect pests. As for the incorporation of ISVP transgenes into *Metarhizium*, this approach obviates the need for the ISVP to be orally active and the range of susceptible insects will be determined primarily by the host range of the virus (Hardy *et al*., 2013) [4].

Two genetically enhanced isolates of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) expressing insect-specific neurotoxin genes from the spiders *Diguetia canities* and *Tegenaria agrestis* were evaluated for their commercial potential. Since prevention of feeding damage is of primary importance in assessing agronomic efficacy, a method for estimating the median time to cessation of feeding (FT_{50}) was developed. Neonate droplet feeding assays with preoccluded virus samples were conducted to compare the FT_{50}s and median survival times (ST_{50}s) of larvae infected by the toxin-expressing recombinant viruses with those of larvae infected by wild-type AcMNPV and the appropriate polyhedrin-minus control viruses. Low dosages were used to minimize the effect of dosage on the response times, and the time to molting of noninfected larvae was used to audit variability among batches of larvae within and between tests. Appropriate statistics are discussed. To evaluate host spectrum, response times were compared in three lepidopteran insect pests, *Trichoplusia ni* Hubner, *Spodoptera exigua* (Hubner), and *Heliothis virescens* (Fabricius). The recombinant viruses expressing insect-specific toxin genes from *T. agrestis* and *D. canities*, designated vAcTalTX-1 and vAcDTX9.2, respectively, significantly reduced both FT_{50} and ST_{50} in all three lepidopteran pests. Reductions in feeding times compared to the wild-type virus ranged from 16 to 39% with vAcTalTX-1 and 30 to 40% with vAcDTX9.2 (Table 3) Reductions in survival time were lower, ranging from 18 to 33% with vAcTalTX-1 and 9 to 24% with vAcDTX9.2. While vAcTalTX-1 tended to kill faster than vAcDTX9.2, vAcDTX9.2 stopped feeding faster than vAcTalTX-1, suggesting that it would be more effective in reducing crop damage (Hughes *et al*., 1997**)** [22].

Spider-toxin transgenes for engineering insect-resistant crops

The introduction of genetically modified (GM) crops in themid-1990s revolutionized global crop production. In 2015, 179.7 million hectares of GM crops were planted in 28 countries, representing10% of all cropland. The introduction of insect-resistant GM crops, mainly corn and cotton, carrying an insecticidal protein (known as δ-endotoxin, Cry toxin, or simply *Bt*) from the bacterium *Bacillus thuringiensis* dramatically reduced insecticide use and improved crop yields.

Although here have been only a few reports of field resistance to *Bt* crops, constitutive expression of the toxin in transgenic plants will ultimately expedite resistance development. Resistancec can be delayed through the use of non-GM refuges and/or by engineering *Bt* plants to express insecticidal genes that act via different mechanisms, an approach known as pyramiding or trait stacking. ISVP transgenes appear to be good candidates for trait stacking with *Bt* because (*a*) They have completely different mechanisms of action (*b*) They are likely to be synergized by *Bt*, which causes lysis of midgut epithelial cells and therefore should facilitate movement of ISVPs into the hemocoel (*c*) whereas *Bt* toxins are specific for the insect orders Lepidoptera, Coleoptera, Hymenoptera, and Diptera,

ISVPs with complementary selectivity, particularly against sap-sucking hemipterans, can be selected for trait stacking.

Attempts to engineer plants expressing ISVPs began 16 years ago with the demonstration that transgenic tobacco expressing ω-HXTX-Ar1a from the Australian funnel-web spider *Atraxrobustus* had enhanced resistance to *H. armigera* transgenes encoding this ISVP (or its ortholog ω-HXTX-Hv1a) have subsequently been engineered into cotton, tobacco (as a fusion to thioredoxin). All ISVP-expressing transgenic plants developed thus far have shown significantly increased resistance to insect pests. For example, the mortality of second-instar *H. armigera* fed on transgenic tobacco expressing ω-HXTX-Hv1a was 75–100% after 72 h compared to 0% for larvae fed on untransformed plants, regardless of whether the peptide was expressed under the strong *35S* promoter from *Cauliflower mosaic virus* (Khan *et al*., 2006) or weaker phloem-specific promoters (Table 4). It has even been claimed that transgenic cotton expressingω-HXTX-Hv1a is as effective as Monsanto' spyramided Bollgard II R cotton in controlling major cotton pests. Thus, ISVP transgenes seem to hold great promise as a stand-alone insect-resistant plant trait or for trait stacking with *Bt* in order to minimize resistance development and expand the range of pests to which GM crops are resistant (Khan *et al*., 2006) [5].

Insect larvae were caged on detached tobacco leaves of transformed (a, c, e) and control, non-transformed (b, d, f) plants. First instar (a, b) and second instar (c, d) *H. armigera* larvae and second instar *S. littoralis* larvae (e, f) were released on the leaves and photographed after 24 h. Right side, Whole plant toxicity assay. Second instar *S. littorallis* larvae, 32 per plant, were released on (a) transformed (line T21) and (b) non-transformed tobacco plants. The photograph was taken 5 days after release of the insects.

Transgenic expression of Hvt in tobacco effectively protected the plants from *H. armigera* and *S. littoralis* larvae, with 100% mortality within 48 h.

They conclude that the Hvt is an attractive and effective molecule for the transgenic protection of plants from herbivorous insects which should be evaluated further for possible application in agriculture.

Experiments were carried out challenging detached leaves or whole plants with the herbivorous insect *Spodoptera frugiperda*. The bioassays indicated that the transgenic lines were significantly more resistant than the wild type plants (Campuzano *et al*., 2009) [23].

Experimental assays on tobacco leaves were conducted using *Spodoptera frugiperda* larvae from neonatal to 6 instar. Five groups of ten larvae from a single stage of development (neonatal and 2–3, 3–4, 4–5, and 5–6 instar) were placed in humid Petri dishes with detached leaves from 8-week-old transgenic tobacco plants from lines 56, 57 and 104, respectively. When neonatal larvae were used, a 100% mortality rate was observed on larvae fed on leaves from wild type plants, as well as on those fed on leaves from transgenic plants expressing Magi 6.

This is not surprising since it is well known that tobacco has insecticide properties per se, it is rich in alkaloids such as nicotine, and because neonatal larvae are not used to a diet based on this plant they die. In contrast, the larvae of the 3–4 and 4–5 instar fed on wild type plants demonstrated a mortality of 25% and 36%, respectively, whilst the same instars fed on transgenic plants demonstrated a mortality of 75% and 86%, respectively, representing a significant ($p< 0.05$) 2.5 fold difference when compared to the non-transformed control. This work demonstrated that the expression of Magi 6 peptide in transgenic plants conferred resistance to insect attack and opens the possibility of employing this peptide to improve the resistance of diverse plants.

The response of the Asian gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) to a fusion gene consisting of the spider, *Atrax robustus* Simon (Araneae: Hexanthelidae) ℵ-ACTXAr1 sequence coding for an □-atracotoxin and a sequence coding for the *Bt*-toxin C-peptide, expressed in transgenic poplar *Populus simonii* x *P. nigra* L. (Malphigiales: Salicaceae) was investigated. Individual performance, feeding selection, midgut proteinase activity and nutrition utilization were monitored. The growth and development of *L. dispar* were significantly affected by continually feeding on the transgenic poplar, with the larval instars displaying significantly shorter developmental times than those fed on nontransgenic poplar, but pupation was delayed. Mortality was higher in populations fed transgenic poplar leaves, than for larvae fed nontransgenic poplar leaves. The cumulative mortality during all stages of larvae fed transgenic leaves was 92% compared to 16.7% of larvae on nontransgenic leaves (Cao *et al*., 2010) [1].

The highest mortality observed was 71.7% in the last larval instar stage. A two-choice test showed that fifth-instar larvae preferred to feed on nontransgenic leaves at a ratio of 1:1.4. Feeding on transgenic leaves had highly significant negative effects on relative growth of larvae, and the efficiency of conversion of ingested and digested food. Activity of major midgut proteinases was measured using substrates TAME and BTEE showed significant increases in tryptase and chymotrypsin like activity (9.2- and 9.0-fold, respectively) in fifth-instar larvae fed on transgenic leaves over control. These results suggest transgenic poplar is resistant to *L. dispar*, and the mature *L. Dispar* may be weakened by the transgenic plants due to *Bt* protoxins activated by elevated major midgut proteinase activity. The new transgenic poplar expressing fusion protein genes of *Bt* and a new spider insecticidal peptide are good candidates for managing gypsy moth.

Conclusions

The pesticide market is a multibillion-dollar industry. Agrochemicals dominate the marketplace with >95% of the market share, but their spectrum of activity is often too broad with significant non-target toxicity. Additionally, the restricted range of targets limits their long-term viability in the face of growing insecticide resistance. Since resistance development should be anticipated for any insecticide the development of new insecticides with specificity and effectiveness against target species, together with minimal non-target toxicity and environmental persistence, will continue to be in demand. Spider-venom peptides are a rich source of potential bioinsecticides that can combine the desirable attributes of high potency, novel target activity, structural stability and phyletic selectivity. Moreover, pharmacological characterisation of spider toxins is revealing novel target sites not previous exploited by conventional agrochemicals, thereby validating new insecticide targets for future screening programs. These peptides can be delivered to insect pests via many different routes, including incorporation of transgenes encoding the peptides into entomopathogens or crop plants. For venom peptides to play a competitive role in the bioinsecticide market they must:

1. Have broad pest-species specificity
2. Have low toxicity in non-target organisms
3. Remain in the environment long enough to be effective, but not so long as to induce
4. resistance development within pest species
5. Be cheap to produce
6. Be easy to formulate and deliver
7. Be publicly perceived as innocuous and
8. (vii)Be readily accessible to both small farmers as well as large agribusinesses

Compared with existing agrochemicals, some of these latter goals have yet to be fully achieved with spider-venom peptides although significant technological improvements continue to emerge. Future research will undoubtedly continue to facilitate the realization of these objectives.

References

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