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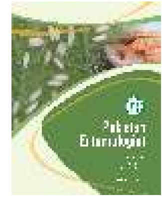

Hvt SPIDER TOXIN CONFERRED INSECTICIDAL ACTIVITY AGAINST *APHIS GOSSYPII* AND *MYZUS PERSICAE* SULZER

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ABSTRACT

Plant sucking insects being brutal competitors are active during all developmental phases of economic crop plants and cause massive yield and quality losses of agricultural produce. Potato Virus X (PVX) based transient expression was used to achieve high throughput of Hvt peptide toxin from *H. versuta* spider in tobacco plants were bioassayed in aim to test the insecticidal activity of Hvt again aphids of species *Aphis gossypii* and *Myzus persicae*. The insecticidal and antagonistic effects of Hvt were evaluated in *A. gossypii* and *M. persicae* in terms of mortality, population survival and nymph production by feeding adult aphids. The characteristic symptoms of Hvt toxicity were characterized and results strongly revealed significant insecticidal activity of Hvt in tested insect species at $p<0.01\%$. In a positive control bioassay, *Helicoverpa armigera* and *Spodoptera exigua* fed on transiently expressed Hvt/PVX plants showed significant insecticidal and entomo-toxic effects of Hvt in terms of larval mortality and lower dry weight of survived larvae. The results demonstrated in this study are important to evaluate the substantial insecticidal activity of Hvt for the purpose of its potential application in developing insect resistant transgenic plants technology.

Keywords: Hvt, PVX, transient expression, Agrobacterium infiltration, RT-PCR,

INTRODUCTION

Sucking insects are the most destructive pests of economic crop plants as they directly feed on plant nutrients through extracting food assimilates from phloem cells, deprive of plants from essential nutrients, vie with plant vigor and serve as plant disease vectors (Chakraborti *et al*., 2009; Dhaliwal *et al*., 2010., Bharathi *et al.,* 2011). No doubt, transgenic plant technology based on *δ*-endotoxins of *Bacillus thuringiensis* (Bt), a gram-positive, spore forming soil bacterium has transfigured global crop production through development of insect resistant transgenic plants (Gatehouse *et al.,* 2011; Kos *et al.,* 2009). Although, Bt transgenic technology is effectively contributing to control herbivorous insect species but commercialized *δ*-endotoxins are not effective against plant sucking insects (Malone *et al*., 2008; Chougule and Bonning, 2012). Hence, there is a solemn need of evaluation of other non-Bt insecticidal proteins against economically important plant sucking insects (Gatehouse, 2008).

The seminal advancements in plant biotechnology have driven the development of new strategies for plant protection and search for novel entomo-toxic molecules with substantial insecticidal activity is of challenging interest to develop insect resistant crop plants (Gatehouse et. al., 2011). Plant viral vectors offer a useful tool in molecular biology for high throughput transient expression of proteins in plants (Donini et al., 2005; Gleba et al., 2007). However, the preliminary screening and evaluation of candidate insecticidal gene constructs is an expensive, laborious and time-consuming task compared to stable transformation (Lawrence and Novak, 2001; Lawrence and Novak, 2006; Leckie and Neal Stewart, 2011). Previously, Potato Virus X (PVX) vector based transient expression system has been extensively utilized for high throughput expression of wider array of valuable proteins and functional gene assays in plants (Donini et al., 2005; Lacomme and Chapman, 2005; Maimbo et al., 2007).

Two cosmopolitan aphid species *Aphis gossypii* and *Myzus persicae* are major pests of wide range of economic crops like cotton, maize, tobacco, potato and other vegetables (Ahmad

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and Iqbal, 2008). The incredible diversity of peptide toxins from various spiders is a fanatical interest area for development of bio-pesticides to protect crop plants (Tedford *et al*., 2004t; Maggio *et al*., 2005; Windley *et al*., 2012). The ω-HV1a (Hvt) is a fractionated peptide toxin from *Hydronyche versuta* (funnel web spider) renowned for its selectivity, specificity and antagonistic effect over insect specific voltage gated calcium channels (Atkinson *et al*., 1996; Fletcher *et al*., 1997; Bloomquist, 2003; Chong *et al*., 2007). Previously, the insecticidal activity of Hvt through transgenic plants was tested against herbivorous insect species (Khan *et al*., 2006; Shah *et al*., 2011; Javaid *et al.,* 2016). Potato Virus X (PVX) based transient expression system is inexpensive, swift, robust and high throughput system as compared to constitutive transgene expression (Wagner *et al*., 2004; Donini *et al*., 2005). Previously, PVX based transient expression established for swift, inexpensive and quick assessment of candidate insecticidal proteins against herbivorous insect species (Lawrence and Novak, 2001; 2006; Lawrence and Koundal, 2002). Because PVX replicate and move systemically in phloem tissue of host plants (Cruz et al., 1998), therefore, PVX has been employed to specifically target aphids of species *Aphis gossypii* and *Myzus persicae*. We have employed PVX vector based high throughput transient expression of insecticidal proteins Hvt (ω-HV1A) from Australian funnel-web spider (*Hydronyche versuta*). This objective of this study was the identification and evaluation of insecticidal properties of Hvt in *A. gossypii* and *M. persicae*. PVX based transient expression is saving time and cost effective methodology in contrast to conventional transgenic expression and this study will boost the efforts of biologist to screen potential candidate proteins against sucking insect species to complement insect resistant transgenic plant technology.

MATERIALS AND METHODS

Gene sequence and primers designing

Hvt, a 37 amino acid long synthetic peptide toxin was deduced from published nucleotide sequence (Fletcher *et al*., 1997). The codon optimization was performed for its proper expression in plants (Mukhtar *et al*., 2004). Primers were designed using (NCBI, BLAST, DNASTAR) bioinformatics tools for further analysis.

Cloning of PVX based expression construct

A PVX based binary vector pgR107 (Gene Bank Acc# AY297842.1) was used to express the Hvt protein in *Nicotiana tabacum* L. plants. The 117nucleotide gene sequence of Hvt was cloned in pgR107 vector using MCS restriction sites *Cla*I and *Sal*I. For the amplification of Hvt by PCR, a reaction mixture of 25µl containing 1µg DNA template, 5µl 10X *Taq* polymerase buffer, 5µl 2mM dNTPs, 1.5mM $MgCl_2$, 0.5µM of each primer and 1 unit of *Taq* DNA polymerase enzyme (Fermentas, Life Sciences) in thermal cycler. The PCR profile 94^{0}C 5min, 94^{0}C 3min, 48^{0}C 30sec, 72^{0}C 45sec, 72^{0}C 10min followed 4^{0}C 10min using gene specific primers 5′<u>*ATCGAT*</u>ATGTCACCAACTTGCATACCG and 3′<u>*GTCGAC*</u>TTAATCGCATCTTTTTACGGTAT from commercially synthesized 117bp nucleotide DNA from Medigenomix, Germany (Khan *et al*., 2006). DNA products were resolved on 1.7% agrose gel stained with ethidium bromide (0.5µg/ ml) in 0.5X TAE buffer (20mM Tris acetate and 0.5mM EDTA [pH 8.0] co-electrophoresis a100bp DNA ladder. The DNA/RNA were analyzed in UV transilluminator (Eagle Eye, Strategene). The DNA from agrose gel was isolated and purified by Wizard SV Gel and PCR Clean-Up System kit (Promega). The 500ng Hvt DNA from PCR amplification and circular cloning vector pgR107 were digested separately to expose sticky ends (10.0µl volume) using 1.5µl of 10X Tango Buffer (Final conc. 1X Tango), 5 units of *Cla*1 (0.5µl)*,* 0.5µl of RNAase 2.5µl sterile ddH_2O (final volume of reaction 15µl) and incubated for 3-4H. Following 2nd enzyme *Sal*1 (0.5µl), 0.5µl of 10X Tango Buffer (Final conc. 2X Tango)*,* sterile ddH_2O of 4.0µl was added (final volume 20µl of reaction) and incubated at 37^{0}C for 3-4H. The restricted vector and DNA products were purified from proteins by phenol-chloroform (24:1) Treatment. To ligate both PCR product and the vector, and 500ng of restricted DNA, 3µl restricted pgR107 vector, 6µl 5X ligation buffer and 5 units (0.5µl) of T4 DNA Ligase enzyme (Fermentas, Life Sciences) were added to obtain a final volume of 30µl and incubated at 16^{0}C overnight. The Recombinant PVX vector was confirmed by respective restriction endonucleases *Cla*1 and *Sal*1 (Fermentas, Life Sciences) after miniprep plasmid isolation using (GeneJET^{TM} Plasmid Miniprep Kit, Thermoscientific). The positive clones of Hvt/PVX were confirmed by sequencing from (Macrogen, Korea).

Transformation in Escherichia coli cells

The recombinant PVX vector was transformed into *E. coli* Top10 cells by heat shock method at 42^{0}for 30sec and cultured in sterilized solid Luria Bertani (LB) agar medium (1% Tryptone, 0.5% yeast extract and 1% NaCl) supplemented with Kanamycin (25µg/ml) and incubated at 37°C 220-rpm overnight.

Agrobacterium transformation and infiltration

For transient expression of Hvt in tobacco, recombinant PVX was co-transformed with pSoup binary vector into *Agrobacterium tumefaciens* strain (GV3101) by electroporation at 1.44kV (2.5kv) using (ECM 600, BTX, U.S.A) apparatus. Cultures of *Agrobacterium* cells were grown at 28^{0}C for 48H and 125-rpm with Kanamycin(25µg/ml), Rifamycin (25µg/ml) and Tetracycline (12.5µg/ml) antibiotics in LB liquid medium and inoculum was prepared by pelleting and suspending agrobacterium cells in 30ml of 10mM $MgCl_2$ and 30µl of 100µM acetosyringone added and incubated at 4^{0}C overnight. The tobacco plants of 4-6 weeks old were agrobacterium-infiltrated with 1ml surgical syringe (B.D Syringe) following a method illustrated by (Leckie and Stewart 2011).

Reverse transcriptase (RT) PCR of tobacco plants

In order to confirm the expression of Hvt in tobacco, total RNA was extracted from tobacco plants by TRIREAGENT (Invitrogen). The 1µg quantity of RNA quantified on Spectrometer (Smart spec, BioRad) was used to synthesize first strand complementary DNA (cDNA) by the RevertAid® H Minus first strand cDNA synthesis kit (Fermentas, Life

Sciences), oilgo $_{(dT)18}$ primers in PCR 94^{0}for 30sec, 50^{0}for 30sec, 72^{0}for 30sec and gene specific primers, 500bp of Coat Protein Promoter (CPP) of PVX as (+ive internal control) from Hvt/PVX, PVX empty vector and Healthy plants control plants (Fig.1).

Plant-insect bioassays

Tobacco plants of *N. tabacum* L. were raised in double containment at 22-25^{0}C and 65-70% RH. The bollworms of *Helicoverpa armigera* and *Spodoptera exigua* eggs and neonate larvae were acquired from Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Boyace Thompson Research Institute (BTI) Cornell University Ithaca, USA. Aphid species were provided by insect rearing facility centre at National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad and Aphid Rearing Laboratory in Boyace Thompson Institute (BTI) Ithaca, USA. The cotton aphid, *A. gossypii* were reared in glass house at 25-30^{0}C and 60-70% RH on cotton seedlings and *N. tabacum* while *M. persicae* aphids were reared at 20-25^{0}C and 70-75% RH on cabbage and *N. tabacum* plants respectively. After two rounds of aphid generation, adult aphids were collected from host plants and bioassayed. The tobacco plants of 10-12 day post-infiltration (dpi) after arrival of mild PVX symptoms on systemic leave were used. Bioassays were designed in Complete Randomized Block Design (CRD) with ten replications. Hvt was tested on neonate larvae of bollworms as positive control. Data was scored for mortality and dry weights gain of surviving larvae. The surviving larvae were collected in 1.5ml eppendorf tubes, lyophilized using dry hot oven at 50^{0}C for 48H and weighed on digital weigh balance (Sturtious®) of 0.001mg accuracy. The data were scored for mortality, number of nymphs produced by adult aphids that survived on Hvt/PVX plants were compared to PVX empty vector and healthy as control plants. The 25 adult aphids of 12-14 days old (*M. persicae*) and 8-10 days old (*A. gossypii*), while 5 neonate larvae of bollworm species were introduced on each symptomatic leaf of treatment plants placed on moist filter paper, caged in petriplate wrapped with cling film. The statistical data was analyzed by computing means and variances Analysis of Variance (ANOVA) using MSTAT-C software and Fisher's F-test and Student's t-test were applied for estimation of significance as p-value against p<0.01% and p<0.05% probability. The bioassay pictures were captured by using Sony Cybershot® 16mpx with 10X Zoom and data scored were graphically plotted using Means, Standard Deviation (S.D) and Standard Error (S.E) in Microsoft Office Excel 2010.

RESULTS

Insecticidal activity of Hvt in bollworms, *S. exigua* and *H. armigera*

Hvt is known for strong insecticidal activity in herbivorous bollworm species therefor, PVX based expression of Hvt was tested in *S. exigua* and *H. armigera* as positive control bioassay. The 5 numbers of neonate larvae of *S. exigua* were released on each symptomatic leaf in petriplates with five replications for each treatment. The results reveled 83% scored mortality in *S. exigua* larvae after feeding Hvt/PVX plants for 5 days feeding duration (Fig. 2). The results of positive control bioassay also revealed that Hvt exerted strong insecticidal effects in bollworm that caused significant lethality with obvious symptoms of Hvt toxicity in larvae of tested insect species. The results of positive control bioassay confirmed that PVX based transient insecticidal protein expression system is potentially working and ready to bioassay other insect species.

Insecticidal activity of Hvt in cotton aphid, *A. gossypii*

The 25 adult aphids were released on each treatment plants of Hvt/PVX, PVX and Healthy as control plants with 15 replications for 5 days feeding duration. The Hvt exerted significant insecticidal effects on aphids and characteristic symptoms of Hvt appeared like melanization of cuticle, disturbed locomotion, disoriented coordination with continuous antennal and cerci movements, resting body posture with reduced fecundity of adult aphids fed on Hvt/PVX plants compared to control plants (Fig. 3). The Hvt exerted significant insecticidal effects on aphids and characteristic symptoms of Hvt appeared like melanization of cuticle, disturbed locomotion, disoriented coordination with continuous antennal and cerci movements, resting body posture with reduced fecundity of adult aphids fed on Hvt/PVX plants compared to control plants (Fig. 3). The mean number of survivors of adult aphids of *A. gossypii* on (Hvt/PVX 4.3, PVX 21.3, healthy 21.4 ±1.513 S.E) and mean number of nymphs produced that survived on (Hvt/PVX 38.30, PVX 57.1, healthy 61.66 ±0.281 S.E) were found significant at p<0.01% (Table 1). The insecticidal effects of Hvt on adult aphid, *A. gossypii* in terms of adult aphid mortality and nymphs produced that survived on Hvt/PVX were compared to control plants and plotted for 5 days feeding duration as illustrated in (Fig. 4).

Insecticidal activity of Hvt in potato peach aphid, *Myzus persicae*

The 25 adult aphids were released on ACA Lectin/PVX plants, PVX and Healthy as control plants with 15 replications bioassayed for 5 days feeding duration. The Hvt exerted significant insecticidal effects on aphids and characteristic symptoms of Hvt appeared like melanization of cuticle, disturbed locomotion, disoriented coordination with continuous antennal and cerci movements, resting body posture with reduced fecundity of adult aphids fed on Hvt/PVX plants compared to control plants (Fig. 5). Hvt spider toxins exerted sever entomotoxic effect on adult aphid population and reproduction that significantly declined as less number of adult aphids were survived to reproduce normally Secondly, less number of nymph were scored because newborn aphid babies could not survive soon after started feeding on Hvt/PVX plants. The mean number survivors of *M. Persicae* were found significant on (Hvt/PVX 2.400 PVX 21.9, healthy 21.4)±1.704 S.E and mean numbers of nymphs produced that survived on (Hvt/PVX 23.80, PVX 59.30, healthy 62.1 ±3.384 S.E) after 5 days feeding duration at p<0.01% (Table 1). The insecticidal effects of Hvt on adult aphids *M. persicae* in terms of population survival and nymphs produced on Hvt/PVX compared to control plants as illustrated in (Fig. 6).

Table1

ANOVA of Hvt for adult aphid mortality and nymph production and survival.

Insect species	Mean		SOV	*df*	MS	*p*-value	S.D	S.E
S. litturalis	Hvt/PVX	1.0	Rep.	9	0.578^{NS}	0.1131		
(Larval mortality)	PVX	4.7	Treat.	2	43.30^{**}	0.0000	1.831	0.334
	Healthy	4.5	Error	18	0.300	-		
S. litturalis	Hvt/PVX	0.054	Rep.	14	0.005	-		
(Dry Weight)	PVX	0.806	Treat.	2	1.836^{**}	0.000	0.037	0.06
	Healthy	0.786	Error	28	0.015	-		
H. virescens	Hvt/PVX	0.8	Rep.	9	0.389^{NS}	0.1703		
(Larval mortality)	PVX	4.7	Treat.	2	54.90^{**}	0.0000	2.013	0.368
	Healthy	5.0	Error	18	0.233	-		
H. virescens	Hvt/PVX	0.143	Rep.	14	0.045^{NS}	0.2543		
(Dry weight)	PVX	0.806	Treat.	2	1.466^{**}	0.0000	0.37	0.067
	Healthy	0.872	Error	28	0.032	-		
A. gossypii	Hvt/PVX	4.30	Rep.	9	1.407	-		
(Adult)	PVX	21.3	Treat.	2	969.033^{**}	0.0000	8.285	1.513
	Healthy	21.0	Error	18	2.219	-		
A. gossypii	Hvt/PVX	23.8	Rep.	9	34.281^{NS}	0.3799		
(Nymphs)	PVX	59.3	Treat.	2	4558.30	0.0000	18.533	3.384
	Healthy	62.1	Error	18	29.781	-		
M. persicae	Hvt/PVX	2.4	Rep.	9	2.300	0.3622		
(Adult)	PVX	21.9	Treat.	2	1235.83	0.0000	9.335	1.704
	Healthy	21.4	Error	18	1.944	-		
M. persicae (Nymphs)	Hvt/PVX	38.3	Rep.	9	89.852	-		
	PVX	57.1	Treat.	2	1527.63^{**}	0.0088	1.513	0.281
	Healthy	61.6	Error	18	245.152	-		

SOV=Source of variation, *df*=degree of freedom, MS= Mean squares, Treat= Treatments, Rep= Replications, Err= Error, p-value= estimated at corresponding degree of freedom, *df*= degree of freedom, S.D=Standard deviation, S.E=Standard Error., NS= non significance, ** significant at $p \leq 0.01\%$

DISCUSSION

PVX vector is a promising and attractive tool for screening and evaluation of gene assays accompanied with rapid high replication, strong gene expression, robust results analysis and inexpensive methodology of easy manipulation, inoculation and good range of solanaceae host plants (Lawrence and Novak, 2001). Previously, PVX has been evaluated for high throughput expression, screening and evaluation of candidate insecticidal proteins against chewing insects (Lawrence and Koundal, 2002; Lawrence and Novak, 2001; Lawrence and Novak, 2006). Besides, PVX was demonstrated as simple cheap, robust and time saving screening tool for insecticidal protein expression and evaluation of candidate proteins under attestation against sucking insect pest species. Moreover, the statistical results and commonality in phenotypes are evident of oral insecticidal toxicity of the Hvt in aphids and bollworms (Khan *et al*., 2006; Shah *et al*., 2011, Javaid *et al.*, 2016). The paralysis, convulsions, disoriented/irregular movement and resting appendages are the common characteristics of the venom toxication in insects as previously characterized by (Manzoli-Palma *et al*., 2003). The toxicity symptoms of Hvt were characterized as feeding cessation; legs protruding out and disoriented locomotion of the insects might be due to paralysis of mouthparts of insects and motor muscles (Vonorax et al., 2006). The blockade and inactivation of moderate MLVA and insect HLVA with high affinity might be responsible for weakness and paralysis in test insects. The continual and disoriented antennal and cerci movements and loss of rightening reflex may be due to perturbing effect due to slower intoxication and blocking of calcium channel in CNS of the insect (Pringos et al., 2011). Mortality induced in insects by Hvt toxin (ω -Hv1a) is due to inactivation of voltage-gated calcium channels (VGCCs) in the central nervous system because P/Q-type Calcium channel widely expressed in the CNS are the primary targets of ω-Hv1a peptide toxin (King et al., 2008). The complementary results of this study indicated the substantial insecticidal activity of Hvt spider toxin against plant aphids and this study will boost the efforts to test this candidate protein against other important sucking insect species in perspective to develop insect resistant transgenic plants.

The use of agrochemicals with few target receptors has promoted the evolution of resistance to a large number of insecticides that indicated a solemn need to identify and evaluate some novel candidate insecticidal compounds/proteins with novel insecticidal targets (Windley et al., 2011). Hvt is a peptide toxin from *H. versuta* funnel web-spider has high affinity and specificity for insect CaVα-1 channels as primary target and moderately potent blocker of putative CaV2 channels with no effect in higher vertebrates (Pringos et al., 2011; Yamaji et al., 2007). The insecticidal activity Hvt toxin is based on inhibitory cysteine knot (ICK)

of the three key amino acids Pro10, Asn27 and Arg35 of ω-Hv1a are considered to form a tight and stable toxin-target complex critical for receptor binding, inhibition of insect calcium channel, selectivity and effective insecticidal activity (Pringos et al., 2011). These residues have been proposed to bind the calcium channel pore forming ion conducting unit through hydrophobic and electrostatic interaction that perturb the Ca^{2+} ion conductance of the channel and cause inactivation of Ca^{2+} mediated gene transcription, gene regulation cascades and synapses transmission in P/Q type neuron cells in CNS (Pringos et al., 2011). Hvt selectively block insect P/Q type calcium channel by depolarization of the resting membrane potential and reduction in spontaneous firing to induce a depressant phenotype, a characteristic toxic symptom of ω-Hv1a in insects (Bloomquist, 2003; King, 2007). Hvt is a potential bioinsecticide combined with the desirable attributes of novel target selectivity, high potency of structural stability, target sites activity and nanomolar concentrations required for insecticidal activity than conventional agrochemicals (Windley et al., 2011). Hvt being a potential insecticidal protein with substantial insecticidal activity and insect selectivity implicated to play an eminent and competitive role in crop protection after assessments of low toxicity in non-target organisms, less persistence in environment and cost of technology transfer to farmers.

CONCLUSIONSPVX vector was established as potential and valuable screening tool for the assessment of general efficacy of candidate insecticidal proteins against economically important phloem feeding insects in model plants. Moreover, the significant results of feeding bioassays of Hvt against cotton aphid and potato peach aphid strongly recommend the potential utilization of insecticidal characteristics of Hvt to develop insect resistant transgenic crop plants after assessment of all biosafety considerations.

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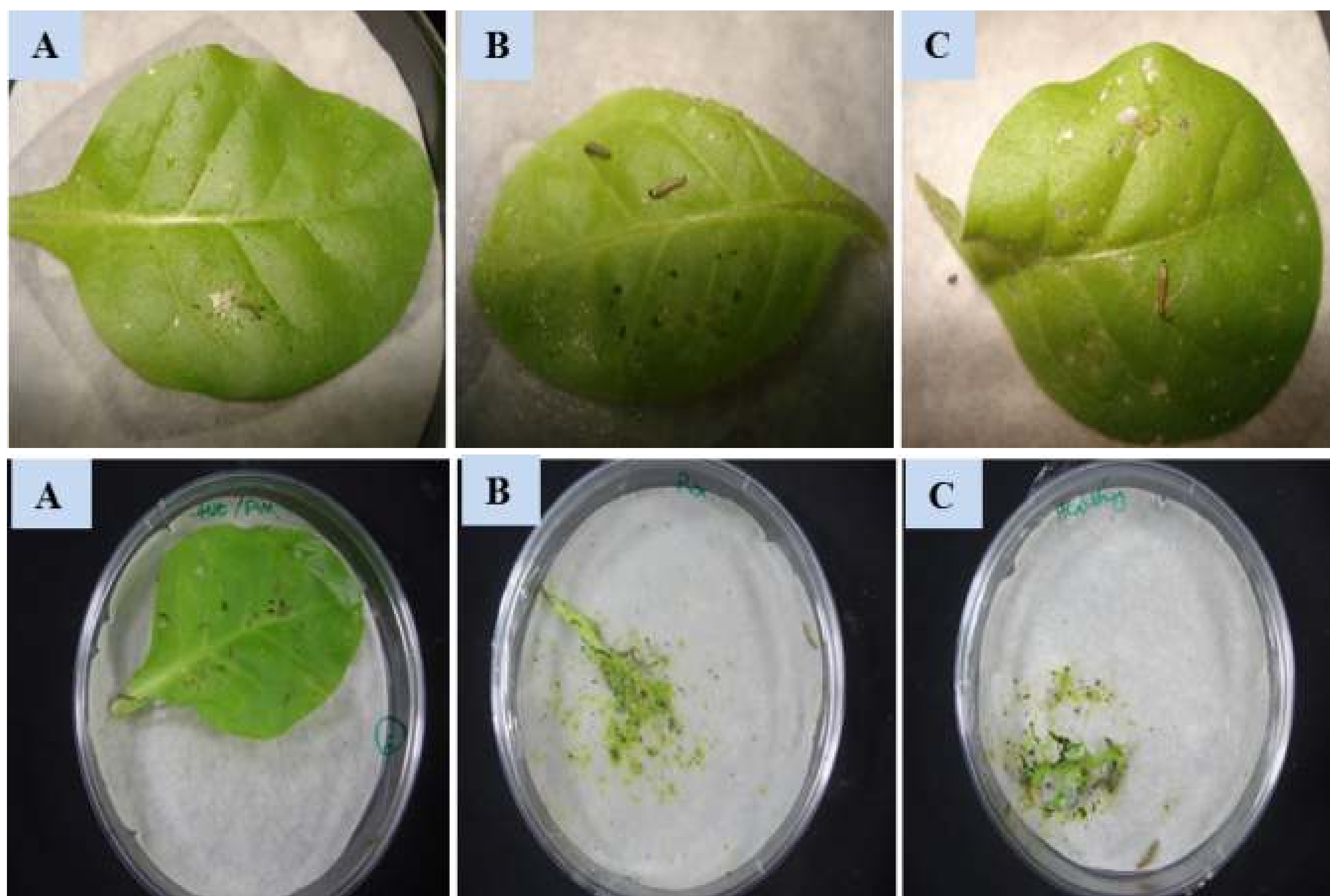

Fig.1

Transient expression of Hvt through PVX in *N. tabacum* L. plants showing insecticidal activity of Hvt spider toxin in *S. exigua* and *H. armigera* for 5 days feeding duration compared to control plants.
(A)=Hvt/PVX plants, (B)=PVX empty vector and (C)=Healthy as control plants.

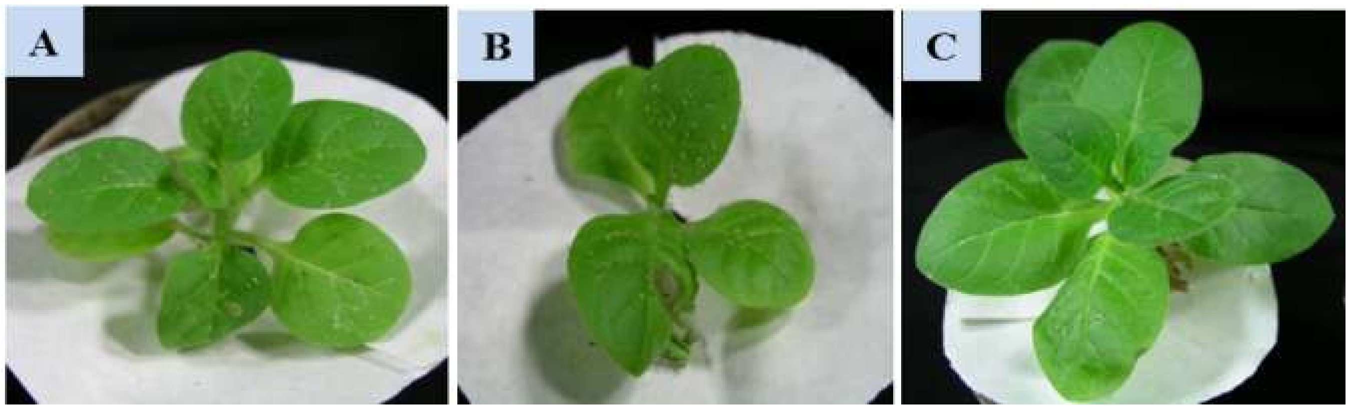

Fig. 2

Transient expression of Hvt through PVX in *N. tabacum* L. plants showing insecticidal activity of Hvt spider toxin in *A. gossypii* Larvae for 5 days feeding duration compared to control plants. Where, (A)=Hvt/PVX plants, (B)=PVX empty vector and (C)=Healthy as control plants

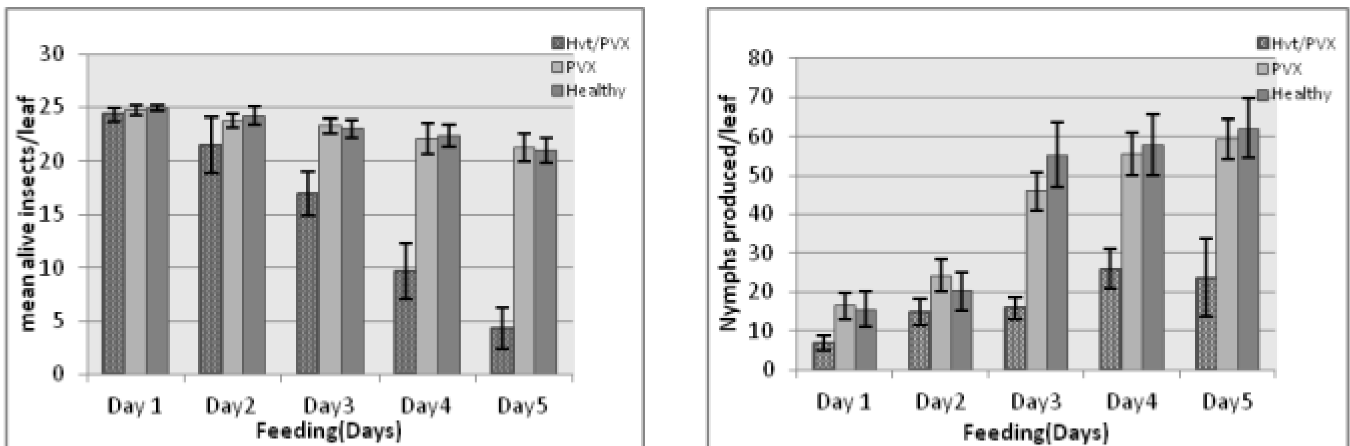

Fig. 3

Insecticidal effects of Hvt spider toxin on population survival of A. gossypii Larvae for 5 days feeding duration compared to control plants. Where, (A)=Adult aphid population, (B)=Number of nymphs produced and survived.

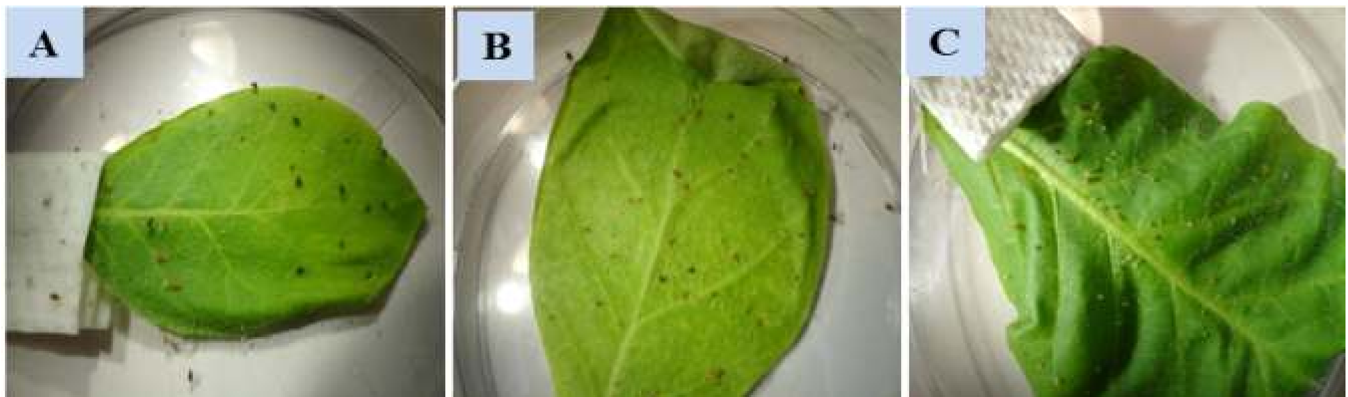

Fig. 4

Transient expression of Hvt through PVX in *N. tabacum* L. plants showing insecticidal activity of Hvt spider toxin in *M. persicae* Sulzer for 5 days feeding duration compared to control plants. Where, (A)=Hvt/PVX plants, (B)=PVX empty vector and (C)=Healthy as control plant.

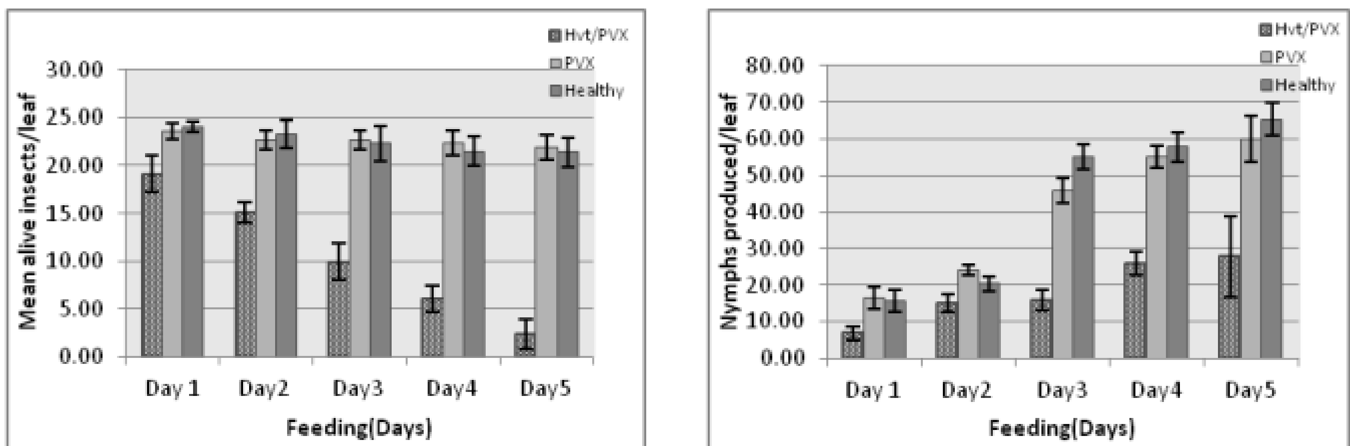

Fig. 5

Insecticidal effects of Hvt spider toxin on population survival of *M. persicae* Sulzer for 5 days feeding duration compared to control plants. Where, (A)=Adult aphid Population, (B)=Number of nymphs produced and survived

REFERENCES

Ahmad, M. and A.M. Iqbal, 2008. Susceptibility of Pakistani populations of cotton aphid *Aphis gossypii* (Homoptera: Aphididae) to endosulfan, organophosphorus and carbamate insecticides. Crop Prot., 27: 523-531.

Atkinson, R.K., E.J. Vonarx and M.E.H. Howden, 1996. Effects of whole venom and venom fractions from several Australian spiders, including Atrax (Hadronyche) species, when injected into insects. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. Endocrinol., 114: 113-117.

Bharathi, Y., S. Vijaya Kumar, I.C. Pasalu, S.M. Balachandran, V.D. Reddy and K.V. Rao, 2011. Pyramided rice lines harbouring *Allium sativum* (asal) and *Galanthus nivalis* (gna) lectin genes impart enhanced resistance against major sap-sucking pests. J. Biotechnol., 152: 63-71.

Bloomquist, J., 2003. Mode of action of atracotoxin at central and peripheral synapses of insects. Invert. Neurosci., 5: 45-50.

Chakraborti, D., A. Sarkar, H.A. Mondal and S. Das, 2009. Tissue specific expression of potent insecticidal, *Allium sativum* leaf agglutinin (ASAL) in important pulse crop, chickpea (*Cicer arietinum* L.) to resist the phloem feeding *Aphis craccivora*. Transgenic Res., 18: 529-544.

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

Chougule, N.P. and B.C. Bonning, 2012. Toxins for transgenic resistance to hemipteran pests. Toxins., 4: 405-429.

Cruz, S.S., A.G. Roberts, D.A.M. Prior, S. Chapman, and K.J. Oparka, 1998. Cell-to-Cell and phloem-mediated transport of Potato Virus X: The role of virions. The Plant Cell Online., 10: 495-510.

Dhaliwal, G.S., V. Jindal and A.K. Dhawan, 2010. Insect pest problems and crop losses: changing trends. Indian J. Ecol., 37: 1-7.

Donini, M., C. Lico, S. Baschieri, S. Conti, W. Magliani, L. Polonelli and E. Benvenuto, 2005. Production of an engineered killer peptide in Nicotiana benthamiana using a Potato virus X expression system. App. Environ. Microbiol., 71: 6360-6367.

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

Gatehouse, J.A., 2008. Biotechnological prospects for engineering insect-resistant plants. Plant Physiol., 146: 881-887.

Gatehouse, A.M.R., N. Ferry, M.G. Edwards and H.A. Bell, 2011. Insect-resistant biotech crops and their impacts on beneficial arthropods. Phil. Trans. R. Soc. B., 366: 1438-1452.

Gleba, Y., V. Klimyuk and S. Marillonnet, 2007. Viral vectors for the expression of proteins in plants. Curr. Opinion Biotechnol., 23: 134-141.

Javaid, S., I. Amin, G. Jander, Z. Mukhtar, N.A. Saeed, and S. Mansoor, 2016. A transgenic approach to control hemipteran insects by expressing insecticidal genes under phloem specific promoters. Sci. Rep., 6: e34706,

Khan, S., Y. Zafar, R. Briddon, K. Malikand Z. Mukhtar, 2006. Spider venom toxin protects plants from insect attack. Transgenic Res., 15: 349-357.

King, G.F., P. Escoubas and G.M. Nicholson, 2008. Peptide toxins that selectively target insect NaV and CaV channels. Channels., 2: 100-116.

Kos, M., J.J.A. van Loon, M. Dicke and L.E.M. Vet, 2009. Transgenic plants as vital components of integrated pest management. Trends Biotechnol., 27: 621-627.

Lacomme, C. and S. Chapman, 2005. Use of Potato Virus X (PVX) Based Vectors for Gene Expression and Virus-Induced Gene Silencing (VIGS), Current Protocols in Microbiology. John Wiley & Sons, Inc.

Lawrence, S.D. and N.G. Novak, 2001. A rapid method for the production and characterization of recombinant insecticidal proteins in plants. Mol. Breed., 8: 139-146.

Lawrence, S.D. and N.G. Novak, 2006. Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. Biotechnol. Lett., 28: 593-599.

Leckie, B. and C.N. Stewart, 2011. Agroinfiltration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants. Plant Cell Rep., 30: 325-334.

Maimbo, M., K. Ohnishi, Y. Hikichi, H. Yoshioka and A. Kiba, 2007. Induction of a small heat shock protein and its functional roles in nicotiana plants in the defense response against *Ralstonia solanacearum*. Plant Physiol., 145: 1588-1599.

MacIntosh, S.C., 2009. Managing the risk of insect resistance to transgenic insect control traits: practical approaches in local environments. Pest Manag., Sci. 66: 1-15.

Maggio, F., B.L. Sollod, H.W. Tedford, G.F. King, I.G. Lawrence, I. Kostas and S.G. Sarjeet, 2005. Spider toxins and their potential for insect control In: Comprehensive Molecular Insect Science, Elsevier, The Amsterdam, p. 221-238.

Malone, L.A., A.M.R. Gatehouse, B.I.P. Barratt, J.R. Romeis, A.M. Shelton and G.G. Kennedy, 2008. Byond Bt: alternative strategies for insect-resistant genetically modified crops., Integration of Insect-Resistant Genetically Modified Crops within IPM Programs, Springer Netherlands, pp. 357-417.

Manzoli-Palma, M.F., N. Gobbi and M.S. Palma, 2003. Insects as biological models to assay spider and scorpion venom toxicity. J. Venom. Anim. Toxins incl. Trop. Dis., 9:174-185.

Mukherjee, A.K., B.L. Sollod, S.K. Wikel and G.F. King, 2006. Orally active acaricidal peptide toxins from spider venom. Toxicon., 47: 182-187.

Mukhtar, Z., S.A. Khan, Y. Zafar and K.A. Malik, 2004. Synthetic omega-atracotoxin Hvla gene of funnel web spider (*Hadronyche versuta*) for development of insect resistant plants. Patent No. 139032, IPO, Pakistan.

Pringos, E., M. Vignes, J. Martinez, and V. Rolland, 2011. Peptide Neurotoxins That Affect Voltage-Gated Calcium Channels: A Close-Up on omega-Agatoxins. Toxins., 3: 17-42.

Shah, A., M. Ahmed, Z. Mukhtar, S. Khan, I. Habib, Z. Malik, S. Mansoor and N. Saeed, 2011. Spider toxin gene cloned under phloem specific promoters provides resistance against american bollworm. Biotechnol. Lett., 33:1457-1463.

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

Vonorax, E.J., M.I. Tyler, R.K. Atkinson and M.E.H. Howden, 2006. Characterization of insecticidal peptides from venom Australian funnel-web spiders. J. Venom. Anim. Toxins incl. Trop. Dis., 12: 215-233.

Wagner, B., H. Fuchs, F. Adhami, Y. Ma, O. Scheiner and H. Breiteneder, 2004. Plant virus expression systems for transient production of recombinant allergens in *Nicotiana benthamiana*. Methods., 32: 227-234.

Windley, M.J., P. Escoubas, S.M. Valenzuela, and G.M. Nicholson, 2011. A novel family of insect-selective peptide neurotoxins targeting insect large-conductance calcium-activated K+ channels isolated from the venom of the theraphosid Spider *Eucratoscelus constrictus*. Mol. Pharmacol., 80: 1-13.

Windley, M.J., V. Herzig, S.A. Dziemborowicz, M.C. Hardy, G.F. King and G.M. Nicholson, 2012. Spider-venom peptides as bioinsecticides. Toxins., 4: 191-227.

Yamaji, N., K. Sugase, T. Nakajima, T. Miki, M. Wakamori, Y. Mori, and T. Iwashita, 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: 3789-94.