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RESEARCH PAPER

Effect of azadirachtin on digestion, cytoskeletal structure and fecundity of *Bemisia tabaci*

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ABSTRACT

Three digestive enzymes namely trypsin, alkaline phosphatase, lipase and two genes knottin 3 and vitellogenin were evaluated against whitefly after feeding with 5 ppm azadirachtin for 12 hours. Down regulated activity of all the three enzymes were observed. The transcript level of the two genes, related to reproductive system and cytoskeleton respectively were observed feeding with azadirachtin at similar concentration and exposure period as enzyme activity. It was observed that azadirachtin significantly inhibited the expression of both of the genes vitellogenin and knottin 3.

Key words: Azadirachtin, *Bemisia tabaci*, digestive enzyme activity, gene expression.

Introduction

Azadirachtin, a biodegradable compound under limnoid group having different biological activities such as insect growth and development (Schmutterer, 1990; Ascher, 1993; Mordue (Luntz) and Blackwell, 1993; Morgan, 2009). Pesticidal properties of this compound found to be effective against nearly 550 insect pest species including other arthropods, nematodes, annelids and fungi (Anuradha and Annadurai, 2008). It is a strong anti-feedent, repellent and growth regulating compound for a wide variety of phytophagous insects. Inhibition of ecdysteroids synthesis results in insect growth inhibition while reduced fecundity and fertility due to affecting development of reproductive organ and vitellogenesis. Presence of azadirachtin in insect body making hormonal imbalances resulting failure of molt or heavily deformed in immatures. In addition, azadirachtin has medium to strong cytotoxicity, antiproliferative and antimitotic effects in insect cell lines (Salehzadeh et al., 2003; Anuradha et al., 2007; Kumar et al., 2007). Proliferation inhibition, cell cycle arrest and apostosis reported from *Spodoptera litura* SI-1 cell line (Zhong et al., 2008; Huang et al., 2011). Azadirachtin inhibited the expression of defense related ferritin and thiredodoxin peroxidase genes (Asaduzzaman et. al., 2016). Furthermore, it has several advantages such as, low human toxixity (Raizada et al., 2001), rapid degradation, low risk of develops resistance and specific properties to non-target organisms (Feng and Isman, 1995; Immaraju, 1998; Walter, 1999).

Globally distributed pest, *B. tabaci* ranked one of the top 100 most damaging pests (Touhidul and Shunxiang, 2007; Abdel-Baki and Al-Deghairi, 2008) in this world due its multidimensional damage over 600 host plants (Mound and Halsey, 1978; Greathead, 1986; Secker et al., 1998) like direct feeding from phloem, transmitting over 100 plant viruses (Jones et al., 2003; Hogenhout et al., 2008) and provide suitable environment for growth and development of sooty mold fungus that reduce the quality and quantity of the harvested products (Nomikou et al., 2001; Naranjo et al., 2002), it is the major considerable economic pest in the field of agriculture. In addition to difficulties of control because of abaxial surface habitat, inter crop movement, continual development of resistance; scientists around the globe are trying to develop eco-friendly control approaches.

Here, the efficacy of azadirachtin in changes the activity of digestive enzymes upon feeding and the transcription level of 2 genes related to cytoskeleton and reproduction were evaluated against the most important agronomic and horticultural pest, *B. tabaci.*

Materials and methods

Insect rearing

The colony was maintained in an insect proof case (60"×60"×45") providing tomato plants in an air conditioned room at 25±1 ℃, 62±2 RH and 16:8 (hours L:D).

Preparation of azadirachtin formulation

Azadirachtin (>95% purity; Sigma-Aldrich, St Louis, MO) was dissolved in isopropanol to prepared 100 ppm stock and kept in -20 ℃ until use, further diluted with artificial diet 20% sucrose solution to obtain expected concentration for testing mortality of *B. tabaci.*

Collection of day 0 whitefly

About 30 leaves with mature nymphs that were ready to

hatch collected from rearing case and kept in petridishes. Hatching of eggs was monitored each day and collected day 0 whitefly for using the experiments.

Enzyme Assays

Protein extraction for enzyme assay

400 Day 0 whitefly collected by previously described method and allowed to feed 200 each 0 ppm (only sucrose 20%) and 5 ppm 300 µl azadirachtin solution using parafilm sandwich in separate glass chamber for 12 hours followed by homogenized with protein digestion buffer and extracted protein through centrifuging at 12000 rpm 15 min at 4 ℃. Protein concentration was measured by the Bradford dye essay (Bradford, 1976) using immunogolobulin as a standard like bovine serum albumin fraction (BSA).

Chemicals

Nα-Benzoyl-D,L-arginine b-naphthylamide hydrochlori de (L-BApNA), p-Nitrophenyl phosphate hexahydrate (pNPP) were purchased from Sigma (www.sigmaal drich.com). 2, 3-Dimercapto-1-propanol tributyrate for lipase activity from BioAssay systems were also purchased (BioAssay systems, Hayward, CA, USA).

Proteneous digestive enzyme

Synthetic substrates provide assay of enzymatic activities. Use of chromogenic substrates allows identification, localization and quantitation of specific enzymes in insect tissue samples for suitable spectrophotometric assays (Christeller et al., 1992; Johnston et al., 1995). Assays of enzyme activities are greatly simplified by using synthetic substrates. Activities for different enzymes were determined using substrates L-BApNA for trypsin, pNPP for alkaline phosphatase. Substrates for trypsin, and alkaline phosphatase were prepared at a final concentration of 2 mM in 50 mM Tris-HCl at pH 8.6 (Erlanger et al., 1961). The assays were carried out by adding 5 µl of enzyme solution to 195 µl of substrates. Enzyme activities were monitored in the linear portion of the assay, which is referred to as the initial velocity, by measuring continuous hydrolysis of substrates at 30 ℃ for 10 min at 405 nm using a Tecan Sunrise microplate reader (Tecan, San Jose, CA). One unit (U) of enzyme activity was defined as the hydrolysis of 1 µmol of substrate per min under the assay conditions. Specific activity was expressed as mU/mg.

Lipase assay

Lipase activity was determined by measuring the absorbance at 405 nm of mixtures that including 10 µL enzyme extract and 140 µL of 0.1% 2,3-dimercapto-1-propanol tributyrate (w/v) as lipase substrate. All mixtures were incubated for 10 to 20min before to absorbance reading. Lipase activity was determined as follows: Activity (U/L)= $(OD_{20\ min}-OD_{10\ min})/(OD_{Cal}-OD_{Water})\times 735$, where $OD_{20\ min}$ and $OD_{10\ min}$ are the OD_{412} nm values of the sample at 20 min and 10 min, OD_{CAL} and OD_{H20} are the $OD_{412\ nm}$ values of the calibrator and water at 20 min, n is the dilution factor, and the number "735" is the equivalent activity of the calibrator under the assay conditions.

RNA extraction and cDNA preparation

Day 0 whiteflies 100 each of the chamber were feed 12 h at 0 ppm and 5 ppm azadirachtin through parafilm method followed by total RNA was isolated from whole bodies of adults using RNeasy mini kit (Qiagen, USA). All extract were treated with DNase (RNase free) and quantified using an IMPLEN Nano Photometer (Implen GmbH, Munich, Germany). The cDNA synthesis reactions for each total RNA (2 µg) were prepared using a Reverse Transcriptase System Kit (Applied Biosystems, USA) and done in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA).

Quantitative real-time RT-PCR (qRT-PCR) analysis

Using nucleotide sequences from the NCBI database, Gene specific primers were designed for qRT-PCR a according to Mahadav et al. (2009) (Table 1). The cDNA samples (0.2 µl) in triplicate were run in a 7300 Sequence Detection System (Applied Biosystems, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) for 1 cycle (95 ℃ for 10 min) followed by 40 cycles (95 ℃ for 15 sec; 60 ℃ for 20 sec; 72 ℃ for 35 sec) followed by 1 cycle for the dissociation stage (95 ℃ for 15 sec; 60 ℃ for 30 sec; 95 ℃ for 15 sec). The expression level of each gene was determined by measuring the relative quantities of the cDNAs from their respective mRNA. The Ct (Threshold cycles) values were used to calculate the mRNA levels. The data were analyzed using the formula, $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ treatment-\Delta Ct\ control]}$ (Livak and Schmittgen, 2001). The partial nucleotide sequence of *actin* gene from *B. tabaci* (AF071908) was identified from the *Bemisia* EST database in the NCBI Genbank. Actin level was used as a reference to normalize the expression levels of the other genes.

Table 1. Gene specific nucleotide primer sequences for real-time RT-PCR.

Target genes	Sequences (5′→3′)	Product lengths (bp)	GenBank accession numbers
knottin 3	CAT TCC AAT CCC TCC GAA AA CGA CCC TAG GCA AGT GTG AAC	-	EE597369
Vitellogenin	GAC AAA ATA GCA ACG GCC AAA A GGC TGG TTG CAT GAA GAT TTCT	71	EE597946

Statistical analysis

The mRNA levels were reported as the mean ± standard error. Analysis of variance (ANOVA) was done to analyze the means using PROC General Linear Model

(GLM) by the Statistical Analysis System program (SAS, 2003) version 9.1 to identify significant effects of the dose specific treatments. The separation of treatment means was done using Duncan's multiple range tests at a 95% confidence level. Data were analyzed by completely randomized design with three replications.

Results and discussions

To evaluate the digestive enzyme activity, 200 adult 3-4 days old *B. tabaci* were feed to 5 ppm for 12 hours, after protein extraction it was measured by Bradford assays followed by absorbance difference per minute, total activity, specific activity were measured of 3 enzymes, trypsin, alkaline phosphatase, and lipase using their substrate activities. The specific activity all enzymes were down-regulated but their effect were not significant statistically (fig. 1)

Two tested proteineous enzymes were down regulated though their effects were not significant. Azadirachtin affects many enzymatic activities in different tissues where cells are producing enzymes. Reduced activities of gut enzymes have been documented using azadirachtin in *S. litura* (Senthil et al., 2005a), *Cnaphalocrocis medinalis* (Senthil et al., 2005b), and *P. interpunctella* (Rharrabe et al., 2008), which may be caused by either inhibition of protein synthesis or toxicity of azadirachtin. Four major groups of proteinases enzymes, (1) serine proteinases; (2) cysteine proteinases; (3) aspartic proteinases, and (4) metalloproteinases (Bode and Huber, 1992) are involved in digestive processes, proenzyme activation, discharge of physiologically active peptides, complement activation, and inflammation processes amongst others (Neurath, 1984). Detailed studies from Murdock *et al.* (1987) and Srinivasan *et al.* (2006) about the midgut enzymes suggest that Serine proteases such as trypsin and chymotrypsin are dominate the larval gut environment and add to about 95 % of the total digestive activity in Lepidoptera, whereas the range is wider in Coleopteran species. Inhibits secretion of trypsin feeding with azadirachtin in the midgut of *M. sexta* was observed (Timmins and Reynolds, 1992).

Alkaline phosphatase is the hydrolytic enzymes, which hydrolyze phosphomonoesters under alkaline condition. It is elementarily found in the intestinal epithelium of animals and provide phosphate ions from mononucleotide and ribonucleoproteins for a variety of metabolic processes. ALP is involved in the transphosphorylation reaction and the midgut has the utmost ALP activity as compared to other tissues (Sakharov *et al.* 1989). Senthil Nathan & Kalaivani (2005) reported that feeding with azadirachtin decreased the amount of alkaline phophatase enzyme in the midgut *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) on *Ricinus communis* L.

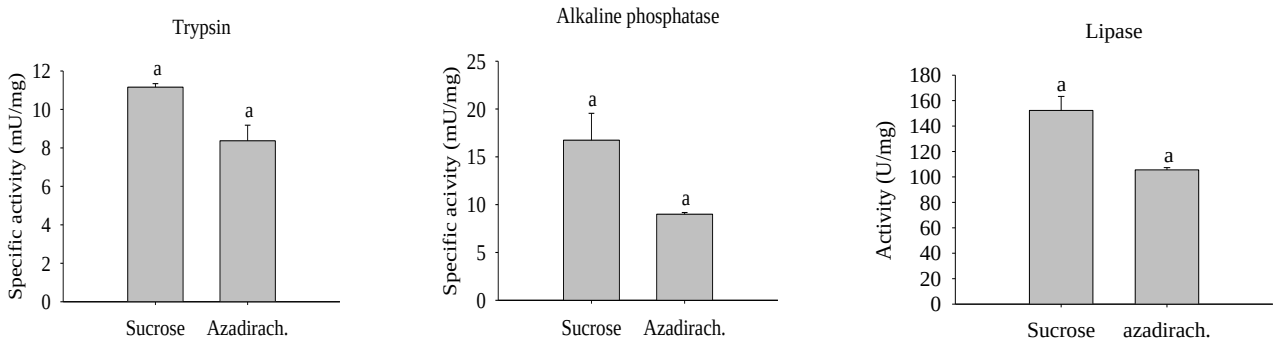

Figure1. Effect of azadirachtin on the digestive enzymes activity of *B. tabaci.* Whiteflies (n=200) were allowed to feed for 12 hours with 5 ppm azadirachtin. Protein was extracted and measured by Bradford assay. Enzyme assays were determined using substrates activity by Tecan sunrise microplate reader. Bars bearing the same letter did not differ significantly at 95% confidence level

Reduced activity of lipase was observed in this study. The activity of enzyme lipases significantly changes due to using botanical insecticides. Senthil Nathan et al. (2006) reported sharply decreased the activity level of lipase in the midgut of *Cnaphalocrocis medinalis* (Guenee) (Lepidoptera: Pyralidae), the rice leaffolder, treating with Btk, NSKE and VNLE (azadirachtin and neem components) and reduced **α**-amylase activity was monitored after feeding with azadirachtin in *P. interpunctella*. (Rharrabe et al., 2008).

This reduction can be due to an inhibition of enzyme activity or to the cytotoxic effect of azadirachtin on midgut epithelial cells which synthesize this enzyme. In *R. prolixus* (Nogueira et al., 1997) and *L. migratoria* (Nasiruddin and Mordue, 1993) azadirachtin caused severe cytotoxicity to midgut epithelial cells.

Azadirachtin may induce its effect by modifying the protein synthesis capacity of the fat body as reported in *L. migratoria* (Rembold et al., 1987), *R. prolixus* (Feder et al., 1988), *L. riparia* (Sayah et al., 1996) and *S. litura* (Huang et al., 2004).

Gene expression of two genes was observed. To determine gene expression at molecular level, the mRNA level of each gene was compared after feeding with artificial diet (sucrose 20%) and sucrose with 5 ppm concentration of azadirachtin. Feeding the whiteflies

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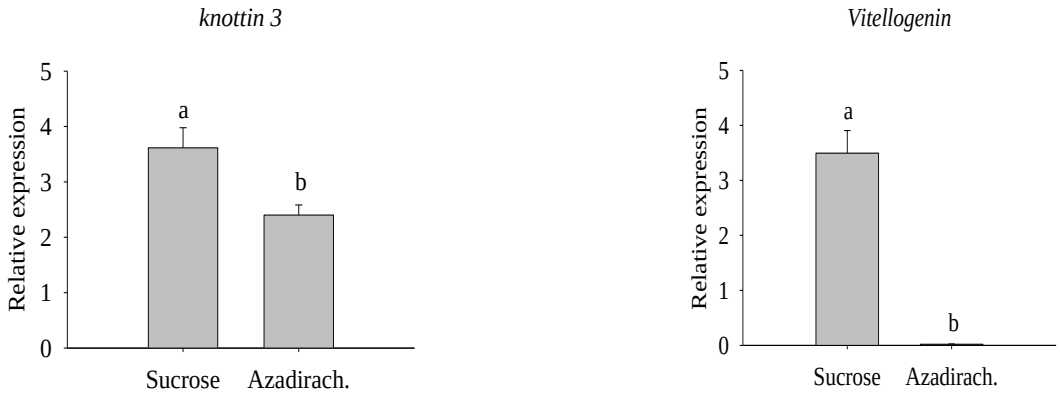

Figure 2. Effect of azadirachtin on gene expression of *B. tabaci*. Whiteflies (n=100) were feed to 5 ppm azadirachtin for 12 hours. Each point of bars represents mean±SE of relative expression in response to azadirachtin treatment of three replications. Same letters on the bar did not differ significantly at 95% confidence level by DMRT.

with 5 ppm azadirachtin for 12 hours total RNA was extracted followed by RT-PCR was done using gene specific primers. Transcription level of two genes was observed and they were down regulated (fig. 2).

Different organisms having Knottins that are miniprotein shared different biological functions (Chiche et al., 2004) and interaction of this may contribute to resistance against different stresses, with antimicrobial activity. They have the ability to bind with proteins, carbohydrates, and lipids (Smith et al., 1998). The genes of UGT isozymes, induced following heat stress, catalyze the detoxification of numerous chemical toxins by conjugation to glucuronic acid. In addition knottin expression is modulated by hormones, drugs and other foreign chemicals through the action of proteins that bind and/or sense the presence of these chemicals. These proteins include transcription factors that respond to stress and the response of UGT to higher temperatures may therefore be related to the accumulation of toxic materials in response to those temperatures (Mackenzie et al., 2003).

Vitellogenin is a storage protein synthesized from the fat body and transported into the ovary for the development of eggs. Singnificantly down regulated exression of vitellogenin feeding with azadirachtin suggested reduced oviposition. Azadirachtin interfere with vitellogenesis with the reduction of ovarian content such as proteins, lipids and carbohydrates (Tine et al., 2011).

References

Abdel-Baky, N.F., Al-Deghairi, M.A., 2008. Role of host plants on the biological aspects and parasitism levels of *Eretmocerus mundus* Mercet (Hymenoptera: Apheinidae) a parasitoid of Bemisia tabaci Gennadius (Aleyrodidae: Hemiptera). J. Entomol., 6, 356-368.

Anuradha A., Annadurai R.S., 2008. Biochemical and molecular evidence of azadirachtin binding to insect actins, *Curr. Sci.*, 95

Anuradha, A.. Annadurai, R.S., Shashidhara, L.S., 2007. Actin cytoskeleton as a putative target of the neem limonoid Azadirachtin A, Insect Biochem. Mol. Biol. 37, 627–634.

Asaduzzaman, M., Shim, J.K., Lee, S., Lee, K.Y., 2016. Azadirachtin ingestion is lethal and inhibits expression of ferritin and thioredoxin peroxidase genes of sweetpotato whitefly *Bemisia tabaci*. J. Asia-Pacific Entom. 19, 1-4.

Ascher, K.R.S., 1993. Nonconventional insecticidal effects of pesticides available from the neem tree, Azadirachta indica. Arch. Insect Biochem. Physiol. 22, 433–449.

Bode, W., Huber, R., 1992. Natural protein proteinase inhibitors and their interaction with proteinases. Eur. J. Biochem. 204, 433-451.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analyt. Biochem. 72, 248-254.

Chiche, L. Heitz, A. Gelly, J.C. Gracy, J. Chau, P.T. Ha, P.T. Hernandez, J.F. Le-Nguyen D. 2004. Squash inhibitors: from structural motifs to macrocyclic knottins Curr. Protein Pept. Sci., 5, 341–349.

Christeller, J.T., Laing W.A., Markwick N.P., Burgess, E.P.J., 1992. Midgut protease activities in 12 phytophagous lepidopteran larvae: Dietary and protease inhibitor interactions. Insect Biochem. Mol. Biol. 22, 735–746.

Erlanger, B.F., Kokowsky, M., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates for trypsin. Arch. Biochem. Biophys. 95, 271-278.

Feder, D., Valle, D., Rembold, H., Garcia, E.S., 1988. Azadirachtin-induced sterilization in mature females of *Rhodnius prolixus*. Zeitschrift Fu¨ r Naturforschung C-A. J. Biosci. 43c, 908–913.

Feng, R., Isman, M. B., 1995. Selection for resistance to azadirachtin in the green peach aphid, *Myzus persicae*. Experientia 51, 831–833.

Greathead, A.H., 1986. Host plants. In: Cock, M.J.W. (Ed.), *Bemisia tabaci* FA Literature Survey on the Cotton Whitefly with an Annotated Bibliography. CAB International Institutes, Biological Control. Silwood Park, UK, 17–26.

Hogenhout, S.A., Ammar, E.D., Whitfield, A.E., Redinbaugh, M.G. 2008. Insect vector interactions with persistently transmitted viruses. Annu. Rev. Phytopathol. 46, 327–359.

Huang, J.F., Shui, K.J., Li, H.Y., Hu, M.-Y., Zhong, G.-H., 2011. Antiproliferative effect of azadirachtin A on *Spodoptera litura* Sl-1 cell line through cell cycle arrest and apoptosis induced by up-regulation of p53. Pestic. Biochem. Physiol. 99, 16–24.

Huang, Z., Shi, P., Dai, J., Du, J., 2004. Protein metabolism in *Spodoptera litura* (F.) is influenced by the botanical insecticide azadirachtin. Pestic. Biochem. Physiol. 80, 85–93.

Immaraju, J. A., 1998. The commercial use of azadirachtin and its integration into viable pest control programmes. Pesticide Sci. 54, 285–289.

Johnston K.A., Lee, M.J., Brough, C., Hilder, V.A., Gatehouse, A.M.R., Gatehouse J.A., 1995. Protease activities in the larval midgut of *Heliothis virescens*: Evidence for trypsin and chymotrypsin-like enzymes. Insect Biochem. Mol. Biol. 25, 375–383.

Jones, D.R. 2003. Plant viruses transmitted by whiteflies. Eur. J. Plant Pathol. 109, 195–219.

Kumar, P., Poehling, H.M., 2007. Effects of azadirachtin, abamectin, and spinosad on sweetpotato whitefly (Homoptera: Aleyrodidae) on tomato plants under laboratory and greenhouse conditions in the humid tropics. J. Econ. Entomol. 100, 411-20.

Livak, K.J., Schmittggen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402– 408.

Mackenzie, P.I., Gregory, P.A., Gardner-Stephen, D.A., Lewinsky, R.H., Jorgensen, B.R., Nishiyama, T., Xie, W., Radominska-Pandya, A., 2003. Regulation of UDP glucuronosyltransferase genes. Curr. Drug Metab. 4, 249–257.

Mahadav, M., Kontsedalov, S., Czosnek, H., Ghanim, M., 2009. Thermotolerance and gene expression following heat stress in the whitefly *Bemisia tabaci* B and Q biotypes. Insect Biochem. Mol. Biol. 39, 668-676.

Mordue (Luntz), A.J., Morgan, E.D., Nisbet, A.J., 2005. Azadirachtin, a natural product in insect control. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Science, vol. 6. Elsevier, Oxford, pp. 117–135.

Morgan, E.D., 2009. Azadirachtin, a scientific goldmine. Bioorg. Med. Chem. 17, 4096–4105.

Mound, L.A., Halsey, S.H., 1978. Whitefly of the World: a Systematic Catalogue of the Aleyrodidae (Homoptera) with Host Plant and Natural Enemy Data. Wiley, New York, 340pp.

Murdock, L.L., Brookhart, G., Dunn, P.E., Foard D.E., Kelley, S., 1987. Cysteine digestive proteinases in Coleoptera. Comp. Biochem. Physiol. 87B, 783-787.

Naranjo, S. E., Ellsworth, P. C. Chu, C. C. Henneberry, T. J., 2002. Conservation of predatory arthropods in cotton: Role of action thresholds for *Bemisia tabaci* (Homoptera: Aleyrodidae). J. Econ. Entomol., 95, 682-691.

Nasiruddin, M., Mordue, A.J.L., 1993. The effects of azadirachtin on the midgut histology of the locusts *Schistocerca gregaria* and *Locusta migratoria*. Tissue and Cell 25, 875–884.

Neurath, H., 1984. Evolution of proteolytic enzymes. Sci. 224, 350-357.

Nogueira, N.F.S., Gonzalez, M.S., Garcia, E.S., De Souza, W., 1997. Effect of azadirachtin A on the fine structure of the midgut of *Rhodnius prolixus*. J. Invert. Pathol. 69, 58–63.

Nomikou, M. Janssen, A. Schraag, R., Sabelis, M. W., 2001. Phytoseiid predators as potential biological control agents for *Bemisia tabaci*. Exp. Appl. Acarol. 25, 271-291.

Raizada, R. B., Srivastava, M. K., Kaushal, R. A., Singh, R. P., 2001. Azadirachtin, a neem biopesticide: subchronic toxicity assessment in rats. Food Chem. Toxicol. 39, 477– 483.

Rembold, H. Annadurai, R.S. 1993. Azadirachtin inhibits proliferation of Sf9 cells in monolayer-culture, J. Biosci. 48, 495–499.

Rharrabe, K., Amri, H., Bouayad, N., Sayah, F., 2008. Effects of azadirachtin on post-embryonic development, energy reserves and a-amylase activity of Plodia interpunctella Hu¨ bner (Lepidoptera: Pyralidae) J. Stored Prod. Res. 44, 290–294.

Sakharov I. Y., Makarova, I. E. Ermolin' G.A., 1989. Chemical modification and composition of tetrameric isozyme K of alkaline phosphatase from harp seal intestinal mucosa Comparative Biochemistry and Physiology. Part B: Comparative Biochemistry. 92, 119–122.

Salehzadeh, A. Akhkha, A., Cushley, W. Adams, R.L., Kusel, J.R., Strang, R.H. 2003. The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells, Insect Biochem. Mol. Biol. 33, 681–689.

SAS Institute, 2003. SAS Version 9.1, SAS Institute Inc., Cary. NC, USA.

Sayah, F., Fayet, C., Idaomar, M., Karlinsky, A., 1996. Effect of azadirachtin on vitellogenesis of *Labidura riparia* (Insecta, Dermaptera). Tissue and Cell 28, 741–749.

Schmutterer, H., 1990. Properties and potential of natural pesticides from the neem tree, Azadirachta indica. Annu. Rev. Entomol. 35, 271–297.

Secker, A.E., Bedford, I.A., Markham, P.G., William, M.E.C., 1998. Squash, a reliable field indicator for the presence of B biotype of tobacco whitefly, *Bemisia tabaci*. In: Brighton Crop Protection Conference-Pests and Diseases. British Crop Protection Council, Farnham, UK, pp. 837–842.

Senthil Nathan, S., Kalaivani, K., 2005. Efficacy of nucleopolyhedrovirus (NPV) and azadirachtin on Spodoptera litura Fabricius (Lepidoptera: Noctuidae). Biol. Control 34, 93–98.

Senthil Nathan, S., Kalaivani, K., Chung, P.G., 2005a. The effects of azadirachtin and nucleopolyhedrovirus on midgut enzymatic profile of *Spodoptera litura* Fab. (Lepidoptera: Noctuidae). Pestic. Biochem. Physiol. 83, 46–57.

Senthil Nathan, S., Kalaivani, K., Murugan, K., Chung, P.G., 2005b. The toxicity and physiological effect of neem limonoids on *Cnaphalocrocis medinalis* (Guene´ e) the rice leaffolder. Pestic. Biochem. Physiol. 81, 113–122.

Senthil-Nathan S. 2006. Effect of *Melia azedarach* on nutritional physiology andenzyme activities of the rice leaffolder *Cnaphalocrocis medinalis* (Guenee) (Lepidoptera: Pyralidae). Pestic. Biochem. Physiol. 84, 98-108.

Smith, G.P. Patel, S.U. Windass, J.D. Thornton, J.M. Winter, G. Griffiths A.D.,1998. Small binding proteins selected from a combinatorial repertoire of knottins displayed on phage J. Mol. Biol., 277, 317–332.

Srinivasan, A., Giri, A.P., Gupta, V.S., 2006. Structural and functional diversities in lepidopteran serine proteases. Cell. Mol. Biol. Lett. 11, 132-154.

Timmins, W.A., Reynolds, S.E., 1992. Azadirachtin inhibits secretion of trypsin in midgut of Manduca sexta caterpillars: reduced growth due to impaired protein digestion. Entomologia Experimentalis et Applicata 63, 47–54.

Tine, S., Aribi, N., Soltani. N., 2011. Laboratory evaluation of azadirachtin against the oriental cockroach, *Blatta orientalis* L. (Dictyoptera, Blattellidae): Insecticidal activity and reproductive Effects African J. Biotechnol. 10(85), 19816-19824.

Touhidul, M., R. Shunxiang, 2007. Development and reproduction of *Bemisia tabaci* on three tomato varieties. J. Entomol. 4, 231-236.

Walter, J. F., 1999. Commercial experience with neem products. In: Methods in biotechnology. Vol. 5: Biopesticides. Ed. by Hall, F. R.; Menn, J.J. Totowa, N.J: Humana Press, 155–170.

Zhong, G.H. Shui, K.J. Lu, Ch.J. Jia, J.W. Ren, T.J. Hu, M.Y., 2008. Induction of apoptosis by azadirachtin, a botanical insecticidal component, in *Spodoptera litura* Sl-1 cultured cell line, Acta Entomol. Sin. 51, 618–627.