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RESEARCH PAPER

Effect of azadirachtin on digestive enzyme activity and gene expression of sweetpotato whitefly, *Bemisia tabaci*

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ABSTRACT

Pure compound of azadirachtin tested against adult *B. tabaci* using artificial diet. Digestive enzymes activity was observed after feeding with 5 ppm azadirachtin for 12 hours. The specific activity of the tested 3 protease enzymes- trypsin, chymotrypsin and aminopeptidase were down regulated. The transcript level of eight genes, two stresses, and six cytoskeleton genes were observed feeding with azadirachtin 5 ppm for 12 hours. Among the tested genes, the expression level of all genes, hsp70, hsp90, p8 protein, myosin, profiling, myofilin, paramryosin, tropomyosin were down regulated.

Key words: Azadirachtin, digestive enzyme activity, gene expression, whitefly

Introduction

Azadirachtin is a strong anti-feedent, repellent and growth regulating biodegradable compound for a wide range of phytophagous insects originated from neem seeds. Effectiveness of this compound has been proven against nearly 550 insect pest species including other arthropods, nematodes, annelids and fungi (Anuradha and Annadurai, 2008). 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). 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.

Insect physiology can be regarded as key to successful control of insect pests. Effectiveness of azadirachtin have been documented in the alkaline phosphatase and amylase digestive enzymes activity and also the expression of defense related genes such as ferritin and theoredoxin in sweetpotato whitefly (Asaduzzaman et al., 2016 ab). Here the efficacy of azadirachtin evaluated against the activity of three protease digestive enzymes and the transcription level of 8 genes related to stress and cytoskeleton.

Materials and methods

Insect rearing

The colony was maintained in a 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 hydrochloride (L-BApNA), N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SA2PFpNA), 4-Nitrophenyl acetate (pNPAC), p-Nitrophenyl phosphate hexahydrate (pNPP), L-Leucine p-Nitroanilide (LPNA) and 5,5'-Dithio-bis (2-Nitrobenzoic Acid) (DTNB) with Acetylecholine iodide were purchased from Sigma (www.sigmaaldrich.com). 2, 3-Dimercapto-1-propanol tributyrate for lipase and Amylose azure with potassium phosphate and sodium chloride for amylase activity from BioAssay systems were also purchased (BioAssay systems, Hayward, CA, USA).

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

Target genes	Sequences (5′→3′)	Product lengths (bp)	GenBank accession numbers
hsp70	TCC CTC GAG TCC TAC TGC TTT AA	71	DQ093377
	TCG CTG ATC TTG TCC TTC AGT TT		
hsp90	GCT CCG AGA CTC TTC GAC AAT G	71	DQ093381
	CAG GGT GGT CAG GGT TGA TTT		
P8 protein	CCA CCA GAC CGA AAC CTG TT		EE600305
	CGG TTC AGG AGG CAG CTT ATT		
paramyosin	GCA ACC TGTCCTTAATCCGC	-	EE597453
	TGT TGG TGG TGA CGA AGG TG		
tropomyosin	GAA CAG TTC ATG CAA GTC TCC G		EE601593
	GAG CGC CTTG TCC TTC TCC T		
Myosin H chain	ATT CCG CAA GGC ACA ACA AG	-	EE599189
	AAG TCG GCT CGT TCT TCA GC		
myofilin	AGC AACCTCATCATCCGCAC	-	EE674613
	CGC GTG ATT TGA TTG TCG AG		
Profilin	ACG GGC TAA AC TTG GCA AAG	-	EE597329
	GCT TGG TGT GTT TTC ATG CAG T		
actin	GAC GGA CAG GTC ATC ATA ATC G	78	AF071908
	CAT ACC CAA GAA GGA TGG CTG		

Proteineous 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, SA2PFpNA for chymotrypsin, SA2 PLpNA for chymotrypsin, LpNA for aminopeptidase, o-NPAc for esterase pNPP for alkaline phosphatase and DTNB for acetylcholine esterase. Substrates for trypsin, chymotrypsin, esterase and aminopeptidase were prepared at a final concentration of 2 mM in 50 mM Tris-HCl at pH 8.6 (Erlanger et al., 1961). Acetylcholine esterase reaction mixture contained 100 mM Tris-HCl (pH 7.8), 0.4 mM 5,5-dithio-bis 2-nitrobenzoic acid (DTNB), 5 mM acetylcholine iodide. 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.

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 quantitative real-time PCR (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, $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ \text{treatment}-\Delta Ct\ \text{control}]}$ (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.

Statistical analysis

Mortality rates and 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

To evaluate the digestive enzyme activity, 200 day-0 adult *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, chymotrypsin, and aminopeptidase using their substrate activities. The specific activity almost all enzymes were down-regulated though their effects were not significant statistically (fig. 1)

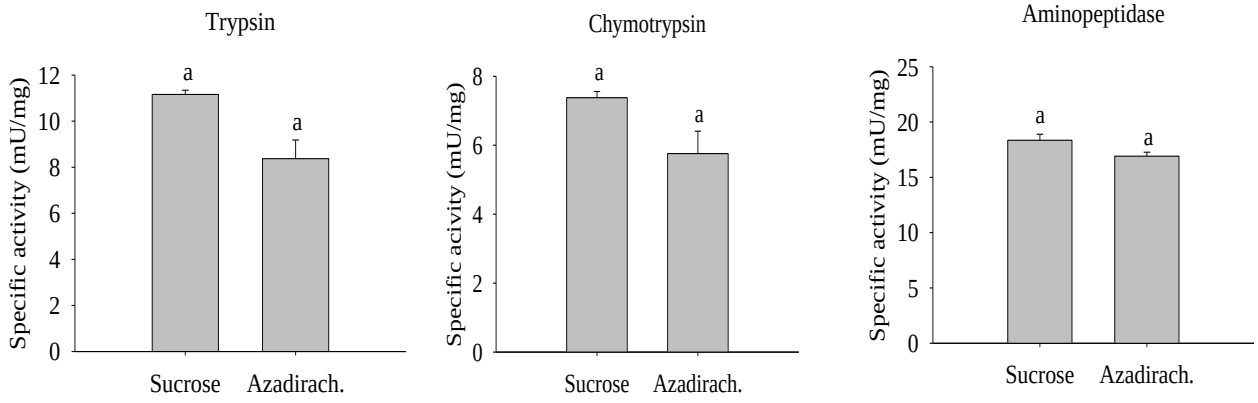

Fig.1. Effect of azadirachtin on the digestive enzymes activity of *B. tabaci.* Whiteflies (n=200) were allowed to feed for 12 hours with 5 ppm azadirachtin. Protein was extracted and measured by Bradford assay. Enzyme assays were determined using substrates activity by Tecan sunrise microplate reader. Bars bearing the same letter did not differ significantly at 95% confidence level.

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 with 5 ppm azadirachtin for 12 hours total RNA was extracted followed by RT-PCR was done using gene specific primers. Tansciption level of Eight genes, two stresses and six cytoskeleton genes were observed feeding with azadirachtin 5 ppm for 12 hours. The expression levels of all the tested genes were down regulated (fig. 2).

Discussion

Here tested three proteineous enzymes all were down regulated though their effects were not significant. Azadirachtin affects many enzymatic activities in different tissues where cells are producing

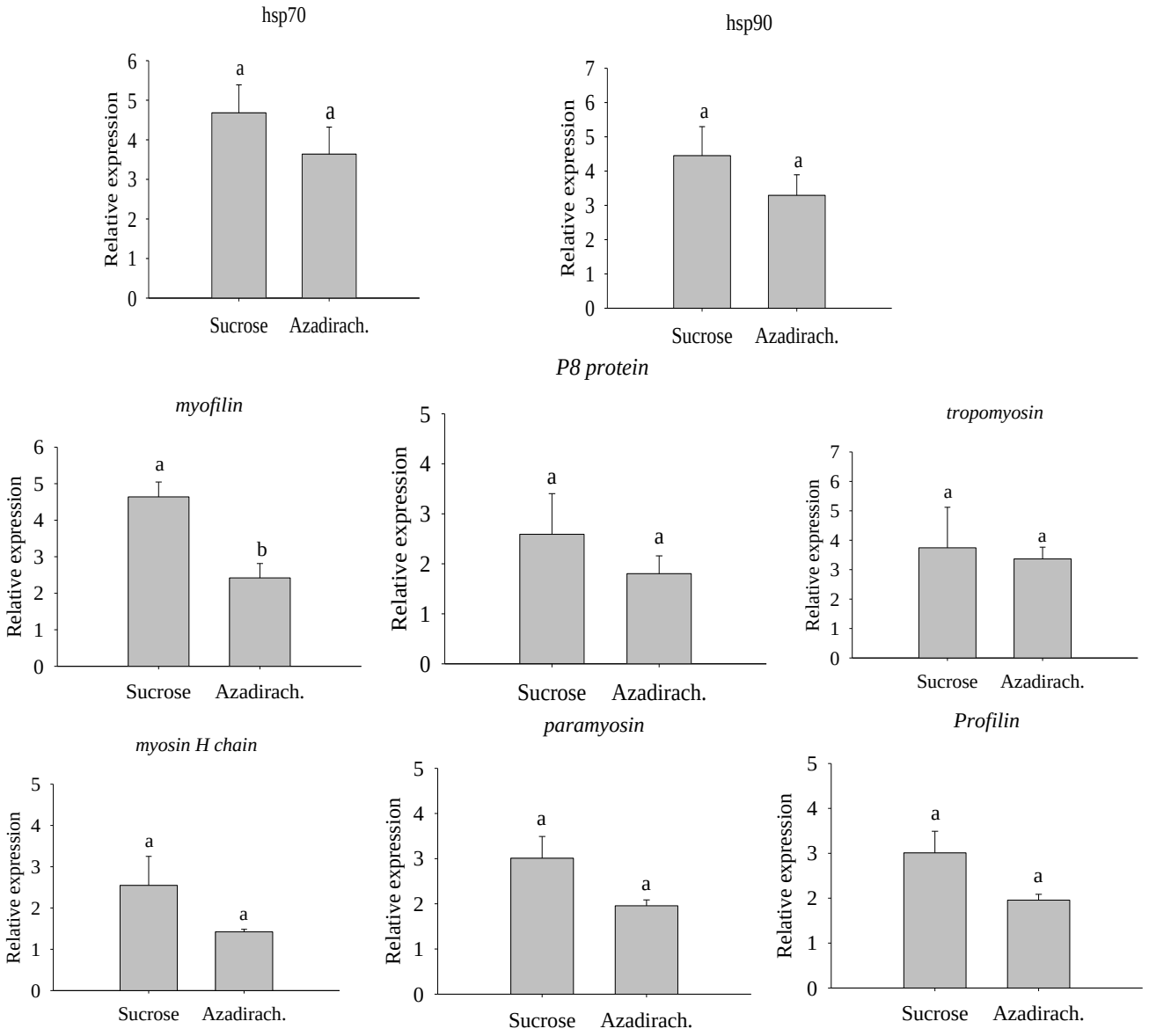

Fig. 2. Effect of azadirachtin on gene expression of *B. tabaci*. Whiteflies (n=100) were feed to 5 ppm azadirachtin for 12 hours. Each point of bars represents mean±SE of relative expression in response to azadirachtin treatment of three replications. Same letters on the bar did not differ significantly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

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).

Three heat shocks proteins genes (hsp70 and hsp90) tested in this study expressed down regulated. Heat shock proteins are large group of cellular protein act primarily as molecular chaperones, influencing exact refolding and preventing accumulation of denatured proteins (Johnston et al, 1998) in response to different stress like extremity of temperature, toxic substances, pathogens (Dellinger 2001, Sorensen et al., 2003). Down regulated expression of stress genes suggested that azadirachtin feeding adult *B. tabaci* might be susceptible to heat stress.

In this study it was observed that all cytoskeleton genes were down regulated, among them myofilin expression was significantly reduced which indicated azadirachtin might have effects on insect cytoskeleton. It is the sensitive and target site of stress, both thermal and oxidative stress can disrupt cell integrity (Dalle-Donne et al., 2001). The cytoskeleton provides to sculpting cell morphology, exo and endocytosis, cell division, cell polarity, intracellular migration, intercellular adhesion, signal transduction, and regulation of ion channel populations and activity (Molitoris, 1997).

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