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 limno 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 antimiotic effects in insect cell lines (Salehzadeh et al., 2003; Anuradha et al., 2007; Kumar et al., 2007). Proliferation inhibition, cell cycle arrest and apoptosis 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; Imamarius, 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-Deghairy, 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; Hohenhout 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 °C, 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 °C 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
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 20 min before to absorbance reading. Lipase activity was determined as follows: Activity (U/L) = \( \frac{(OD_{405\,nm} - OD_{water}) \times 735}{OD_{cal} - OD_{water}} \), 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_{water} 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 RNaseasy 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 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) and done in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA).

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

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Sequences (5’→3’)</th>
<th>Product lengths (bp)</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>knottin 3</td>
<td>CAT TCC AAT CCC TCC GAA AA</td>
<td>71</td>
<td>EE597369</td>
</tr>
<tr>
<td></td>
<td>CGA CCC TAG GCA AGT GTG AAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitellogenin</td>
<td>GAC AAA ATA GCA ACG GCC AAA A</td>
<td></td>
<td>EE597946</td>
</tr>
<tr>
<td></td>
<td>GGC TGG TTG CAT GAA GAT TTCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

database in the NCBI Genbank. Actin level was used as a reference to normalize the expression levels of the other genes.

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
Results and discussions

To evaluate the digestive enzyme activity, 200 adult 3–4 days old B. tabaci were fed 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 proteaceous 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) metallocproteinases (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 phosphatase enzyme in the midgut Spodoptera litura Fabricius (Lepidoptera: Noctuidae) on Ricinus communis L.

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 Bk, 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
**Figure 2.** Effect of azadirachtin on gene expression of *B. tabaci*. Whiteflies (n=100) were fed 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. Significantly down regulated expression 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**


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