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EFFECTS OF AZADIRACHTIN ON DEVELOPMENT OF MODEL INSECT *Galleria mellonella* L. (LEPIDOPTERA: PYRALIDAE)

Beyza DERE [1], Z. Ulya NURULLAHOĞLU [1], Hülya ALTUNTAŞ [2,*]

[1] Department of Biology, Faculty of Science, Marmara University, İstanbul, Turkey
[2] Department of Biology, Faculty of Science, Eskişehir Technical University, Eskişehir, Turkey

ABSTRACT

Azadirachtin (AZA), a plant allelochemical, is used against pest organisms and Lepidopteran species are quite sensitive to this substance. In this study, the developmental effects of AZA on model insect and greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae via force feeding method were investigated. To investigate the effects of different doses of pure AZA (0.2 - 5 µg/larva), seventh instar larvae were fed by a force feeding method. The results showed that all doses of AZA caused lethal and sublethal effects on the biological parameters of *G. mellonella*. Larval developmental time increased while that of pupa did not change depending on the increasing doses of AZA. In addition, adult longevity, and weights of pupae and adults decreased at all AZA doses in a dose-dependent manner. In conclusion, this study, using *G. mellonella* as a model organism, showed that AZA has insecticidal activity and causes adverse effects on the life history traits of Lepidopteran pest insects.

Keywords: Azadirachtin, *Galleria mellonella*, Model insect, Developmental time, Integrated pest management

ÖZET

Bitkisel kökenli bir allelokimyasal olan Azadirachtin (AZA), zararlı organizmalara karşı kullanılır ve Lepidopter türleri bu maddeye karşı oldukça duyarlıdır. Bu çalışmada model böcek ve büyük balmumu güvesi olarak bilinen *Galleria mellonella* L. (Lepidoptera: Pyralidae)'nın larvaları üzerinde zorla besleme yöntemi ile uygulanan AZA'nın, gelişimsel etkileri araştırılmıştır. Farklı dozlardaki saf AZA (0,2 - 5 µg/larva)'nın etkilerinin araştırılması için, 7. instar larvalara zorla besleme yöntemi ile besleme yapıldı. Sonuçlar, AZA'nın farklı dozlarının *G. mellonella*'nın biyolojik parametreleri üzerinde letal ve subletal etkilerinin olduğunu gösterdi. Artan AZA dozlarına bağlı olarak larval yaşam süresi artarken, pupaların yaşam süresi değişmemiştir. Ayrıca, ergin yaşam uzunluğu, pupal ve ergin ağırlıkları, doz bağımlı bir şekilde tüm AZA dozlarında azalmıştır. Sonuç olarak, bu çalışmada AZA'nın insektisidal aktiviteye sahip olduğu ve Lepidopter zararlı böceklerin yaşam süreleri üzerinde olumsuz etkilere neden olduğu model böcek *G. mellonella* kullanılarak gösterilmiştir.

Anahtar kelimeler: Azadirachtin, *Galleria mellonella*, Model böcek, Gelişim süresi, Entegre mücadele

1. INTRODUCTION

Integrated Pest Management (IPM), which allows the use of a combination of many methods to suppress pest populations, has an increasing importance. In particular, usage of eco-friendly biopesticides in this process is at least as important as biological control agents [1]. Bioinsecticides are safer than chemical pesticides since they do not leave any residue and exhibit rapid degradation from at the environment. Senthil-Nathan [2] also stated that bioinsecticides show low toxicity by using at higher doses against vertebrates. Azadirachtin (AZA) which is a molecule belonging to the tetranortriterpenoid (limonoid) family is obtained from *Azadirachta indica* (Neem). AZA has been assessed in significant biopesticides and has been included in IPM programs [3,4]. *A. indica* is an evergreen tree in all seasons and it belongs to the Meliaceae (Mahogany) family. *A. indica* contains approximately 99 active compounds that are mainly known as Azadirachtin, Nimbin, Nimbidin and

*Corresponding Author: hyalcitas@eskisehir.edu.tr
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Nimbolides molecules [5, 6]. The species belonging Meliaceae family have also been investigated due to the fact that they; (1) have significant chemical groups that have insecticidal effect on the insects; (2) are grown in several tropical areas; (3) show biological activity against insects [7]. In the open literature, there are various studies related to AZA and these show that AZA has biological effects such as antifeedant, sterilizing, reducing egg production, and inhibiting growth and development in insects [5,8-10]. Additionally, AZA has been used as a medical product for treating various infections in some countries such as India and Pakistan [11-13]. Several earlier studies revealed that even very low doses (<1 to 50 ppm) of AZA were highly toxic on insects [14-16] and had antifeedant effects or caused developmental anomaly on the various Lepidopteran species [17]. The inhibitory effects of AZA on the growth and development of insects were to be due to the inhibition of ecdysteroid hormone secretion from prothoracic glands [18, 19].

We selected the greater wax moth, *G. mellonella* larvae because of two reasons to investigate the biological effects of AZA on the storage pest insects. First, this species causes significant economic loss in the beekeeping industry by feeding and laying eggs on the honeycombs of *Apis mellifera* L. (Hymenoptera: Apidae) during storage [20]. Several fumigant insecticides such as sulphur dioxide, acetic acid, formic acid, para dichloro benzene (PDCB) or phosphine have been used to control the infestation of the wax moth on beeswax combs during storage, however, use of these chemicals is harmful to bee populations [21, 22]. For this reason, we think that eco-friendly chemicals such as AZA can be used as alternative botanical insecticide. Secondly, *G. mellonella* is an excellent model insect used for applications in vivo toxicity and physiological investigations of various environmental chemicals, and also quite easy to rear in laboratory conditions [16, 22-25]. Furthermore, we determined in a previous study that LD_{50} value for larvae force fed with AZA was 2.1 µg/larva and it caused oxidative stress in a dose-dependent manner on *G. mellonella* larvae [16]. Therefore, the aim of the present study was to determine the adverse effects of AZA applied by force feeding assay on the development of *G. mellonella*.

2. MATERIALS AND METHODS

2.1 Insect Rearing

G. mellonella colony was cultured by feeding the insects with a modified Bronskill's [26] artificial diet including 340 g of bran, 20 g of pollen, 75 ml of filtered honey, 150 ml of glycerol, 100 g of ground old dark honeycomb and 75 ml of distilled water. All stock and experimental laboratory cultures were maintained at Marmara University, İstanbul, Turkey at 27 ± 1° C, 60 ± 5 % RH in constant darkness.

2.2 Bioassays

Technical AZA (Sigma, St. Louis, MO) was used for experimental analysis. The stock AZA solution was prepared as 2 mg/ml (w:v) and dissolved in 10 % ethanol. To investigate the effects of different doses of AZA (0.2, 0.5, 1, 1.5, 2, 3, and 5µg/larva) on the development of *G. mellonella*, larvae were fed by insect force feeding method [16]. Selected larvae weighing 0.16 ± 0.01 g were starved for 3 h before force feeding treatment. To the force feeding, the Hamilton injector (22 gauges) was fixed to the injector manipulator and starved larvae were fed with 5 µl of AZA solution by entering the fixed needle through the mouth into the esophagus (Figure 1). After force feeding, each larva was maintained in a sterile plastic box (50 ml) containing 2 g diet at 27 ± 1°C, 60 ± 5 % RH. Larvae force fed with 10 % ethanol solution and distilled water were served as positive and negative control, respectively. All larvae were observed daily to determine the various life history traits daily. Hence, larval and pupal developmental time (day), adult life span, pupal and adult weights were determined in all experimental groups. Assays for each experimental and control groups were replicated four times with 20 larvae completely randomized from different populations at different times.

Figure 1. Force feeding method; *G. mellonella* larvae were force-fed for controls and experimental groups: the needle enters the mouth of the larva.

2.3 Statistical Analysis

Firstly, all data obtained from biological analysis were checked for normality of data distribution. Data obtained from larval and pupal developmental time were not normally distributed, so the nonparametric statistical test: Kruskall Wallis (K-independent samples test) was performed and subsequently significant differences were determined using Mann-Whitney U tests in SPSS program. However, data from adult longevity, pupal and adult weights were normally distributed, so the parametric statistical test; One-way analysis of variance (ANOVA) was used and subsequently significant differences were determined using Tamhane T2 test in SPSS program. Significances were ascribed at $P < 0.05$.

3. RESULTS

Larval developmental time increased due to increase of AZA doses (x^2=303.215; df= 8; P=0.000, Table 1). It was observed that all larvae died after 27.25±0.97 days at the highest doses of AZA (5 µg/larva) (Table 1). However there was no significant difference in in pupal developmental time at all AZA doses except for 3 µg/larva dose when compared with control groups (x^2=13.836, df=7, P=0.054, Table 1). It was found that pupal development did not complete at 3 µg/larva dose. Therefore, data for pupal developmental time and adult longevity were not obtained at the highest doses of AZA. Adult longevity was considerably affected by AZA treatment and decreased at all doses of AZA when compared with those of controls. In particular, adult longevity time shortened significantly at 0.5 (9.76±0.69 day), 1.5 (9.10±0.95 day) and 2 µg/larva (9.00±0.91 day) AZA doses in comparison to the control (F=32.788; df= 6, 263; P=0.000, Table 1). In general pupal and adult weight of AZA treated groups decreased significantly when compared with those of controls (Table 2). Furthermore a significant decrease was observed in pupal and adult weight at 1.5 and 2 µg/larva AZA doses, but this trend in decrease was not dose-dependent at all doses of AZA (F=20.797; df =6, 263; P=0.000 and F=10.477; df=6, 263; P=0.000).

Table 1. Developmental effects of AZA on force fed larvae of *G. mellonella*

AZA Doses (µg/larva)	Mean ± SE*		
	Larval Developmental Time (day)**	**Pupal Developmental Time (day)****	**Adult Longevity (Day)****
dH_2O	6.43 ± 0.17 a	10.05 ± 0.17 a	17.75 ± 0.48 a
%10 Ethanol	6.05 ± 0.18 a	9.27 ± 0.37 a	19.52 ± 0.57 a
0.2	5.40 ± 0.13 b	9.51 ± 0.40 a	12.47 ± 0.81 b
0.5	5.78 ± 0.20 b	11.73 ± 1.02 a	9.76 ± 0.69 b
1	11.61 ± 1.02 c	9.49 ± 0.44 a	11.88 ± 1.29 b
1.5	13.75 ± 1.10 c	10.04 ± 0.57 a	9.10 ± 0.95 b
2	18.58 ± 1.17 d	9.39 ± 0.68 a	9.00 ±0.91 b
3	22.70 ± 1.05 e	-	-
5	27.25 ± 0.97 f		-

* All data represent mean ± standard error of 4 replicates with 20 larvae in each replicate
**Significant differences are indicated by a-f ($P<0.05$) among doses in the same dose column.

Table 2. Effects of AZA on pupal and adult weight of *G. mellonella* force fed larvae.

Doses (µg/larva)	Mean ± SE*	
	Pupal Weight (g)**	**Adult Weight (g)****
dH_2O	0.13 ± 0.003 a	0.09 ± 0.002 a
%10 Ethanol	0.13 ±0.003 a	0.08 ±0.001 a
0.2	0.12 ± 0.002 b	0.07 ±0.001 b
0.5	0.12 ± 0.002 b	0.07 ±0.002 b
1	0.11 ± 0.004 b	0.07 ±0.003 b
1.5	0.10 ± 0.003 c	0.06 ± 0.002 c
2	0.09 ± 0.007 c	0.05 ±0.004 c
3	-	-
5	-	-

* All data represent mean ± standard error of 4 replicates with 20 larvae in each replicate.
**Significant differences are indicated by a-c ($P<0.05$) among doses in the same dose column.

4. DISCUSSION

All data obtained in our study are similar with earlier studies and also add new information to the literature. Previous study revealed that Neem EC (containing 100 ppm AZA) applied to third instar larvae of *Mamestra brassicae* L. (Lepidoptera, Noctuidae) caused deaths in the larval, prepupal and

pupal stages [27]. There are also numerous studies about the insecticidal and biological effects of technical or commercial AZA on various insects in literature [5,8-10,16,17,28,29]. The above mentioned results showed that larval and pupal mortality increased and could not molt to the next stage as AZA concentration increased. Similarly, our results showed that technical AZA caused a significant dose-dependent prolongation of larval developmental time of *G. mellonella*. In addition, a high larval and pupal mortality was observed due to increase of AZA. The present findings are also in agreement with Simmonds et al. [30] who found that neem tree extracts applied topically caused mortality in a dose dependent manner on some Lepidopteran species after 72 hour. Another study conducted with AZA also concluded that exposure the AZA by force feeding assay caused formational anomaly and had adverse effect on the detoxification responses (SOD, CAT, and GST activities) of *G. mellonella* larvae [16]. Therefore, we infer that AZA might have inhibitory effect on the mechanism of the juvenile hormone secretion of *G. mellonella* larvae [18].

It is known that AZA even at low doses is highly toxic on insects. Low concentrations of AZA have various toxic effects such as antifeedant and sterilizing agent, reducing egg production, inhibiting growth and development of insects [8, 9, 17, 29]. Additionally, researchers demonstrated that *A. indica* extract caused cell division and inhibition of protein synthesis during development of several lepidopteran species [17]. Therefore, these toxic effects of AZA could be the reason of the decrease in adult longevity in AZA treated larvae. A previous study showed that pupal weight of the *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) reduced by only the 5% and 10% concentrations of the neem seed extract [31]. Jogar et al. [32] reported that different commercial preparations of AZA significantly caused weight loss in *Pieris brassicae* L. (Lepidoptera: Pieridae) and *G. mellonella* pupae. In a study with neem oil applied to the third instar larvae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae), it was reported that pupal weight decreased and the histopathology occurred [33]. In our study, similar to these previous studies, significant decreases in pupal and adult weight were detected in larvae with AZA doses in particular the higher doses. The reduction in the pupal and adult weight indicate that AZA negatively affects both larval feeding and egg production in female adult. In other words, present results obtained from *G. mellonella* support the previous studies about antifeedant effects of AZA on insects.

In conclusion, our study using model insect *G. mellonella* showed that pure AZA has negative effects on life history traits of Lepidopteran pests even at low concentrations. Therefore, we think that the present results will be useful for the production of novel biopesticide formulations containing pure AZA.

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