J. Entomol. Res. Soc., 19(2): 83-93, 2017 *ISSN:1302-0250*

Effects of Indole-3-Acetic Acid on Hemocytes of *Achoria grisella* Fabr. (Lepidoptera: Pyralidae)

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ABSTRACT

In order to provide a more complete understanding of physiological impacts of IAA on insects, we investigated the cytotoxic effects of variable doses of IAA on hemocytes of *Achoria grisella* Fabr. (Lepidoptera: Pyralidae) larvae. The results revealed that addition of IAA (2 to 1,000 ppm) in diet of *A. grisella larvae* resulted in an increase in the total hemocyte counts at all doses tested. The percentage of plasmatocytes decreased but granulocytes increased at 2, 5, 100, 200 and 1,000 ppm. Nevertheless, the application of IAA did not alter mitotic indices, the percentage of spherulocytes, prohemocytes and oenocytoids. The percentage of living cells decreased at all treated doses compared to control interrelated with the elevated ratio of early apoptotic hemocytes at 5, 10, 50, 100 and 200 ppm. Significant reductions were observed at 2, 10, 50 and 100 ppm in the ratio of necrotic hemocytes, and at 10,100 and 200 ppm in the late apoptotic cells in IAA-treated *A. grisella* larvae. Our findings demonstrate that IAA exhibits detrimental effects on the hemocytes of *A. grisella* larvae that are the main components of insect immunity.

Key words: Achoria grisella, apoptosis, cytotoxic, hemocyte, indole-3-acetic acid, mitosis.

INTRODUCTION

In the last fifty years, the world population multiplied more rapidly than even before. At the same rate, the requirement of agricultural production has increased and the application of plant growth regulators (PGRs), which promote agricultural production and prevent damage of pests, has become widespread (McDonald *et al.*, 1988; Silva *et al.*, 2003; Paulson *et al.*, 2005). Auxins are a class of PGRs which are involved in many developmental processes like cell enlargement, cell division, vascular tissue differentiation and root initiation (Davies, 1995). Indole-3-acetic acid (IAA) is one of the most important natural auxins (Davies, 1995) which affects plant growth and development. However, it has also adverse impacts on non-target organisms such as insects by altering developmental time, longevity, reproductive potential and hemolymph metabolites (Rup *et al.*, 2000, 2002; Kaur and Rup, 2003; Uçkan *et al.*, 2011; Uçkan *et al.*, 2014; Uçkan *et al.*, 2015).

The known harmful effects of insecticides have led to search for ecologically innocent compounds, which can repress pest insects. Authors have suggested that PGRs can be used instead of pesticides in Integrated Pest Management (IPM)

programs (Posnava, 1974; Kaur and Rup, 2002). However, the effects of these agents on different organisms still need to be clarified. The toxicological studies on the effects of PGRs including IAA have been conducted on different animals. De Melo *et al*., (1997, 2004) have suggested that IAA is cytotoxic for leukocytes by inducing necrosis and apoptosis. The adverse effects of PGRs on insects' physiology have also been reported by using *Bactrocera cucurbitae* (Kaur and Rup, 2002), *Spilarctia obliqua* (Gupta *et al*., 2009), *Galleria mellonella* (Altuntaş *et al*., 2012; Er and Keskin, 2015), *Apanteles galleria* (Uçkan *et al*., 2014).

The innate immune system of insects involves cellular and humoral defence (Strand, 2008b). Cellular defence system includes phagocytosis, encapsulation, nodulation and clotting that are directly mediated by immune cells called hemocytes (Lackie, 1988; Strand and Pech, 1995; Irving *et al*., 2005; Strand, 2008b). Hemocytes also play a role in humoral defence by producing soluble effector molecules (Imler and Bulet, 2005; Kanost and Gorman, 2008; Strand, 2008b).

Another immune defence in insects is apoptosis, which occurs generally against viral infections (Clem, 2005) and can be also induced by several environmentally stimuli such as UV-irradiation or chemicals (Sun *et al*., 1999, Kim *et al*., 2001). Furthermore, cell death caused by toxic agents has a different morphology and is called necrosis (Wyllie, 1981). Because hemocytes are so sensitive against environmental impacts, the total count of hemocytes and apoptotic indices can be used as indicators for detecting cytotoxic effects of chemicals such as PGRs also stated by Altuntaş *et al*., (2012).

The smaller wax moth, *Achoria grisella* Fabr. (Lepidoptera: Pyralidae) is ubiquitous pest of honey bee colonies. Only *A. grisella* at its larval stage feeds on honey combs and causes damage in apiculture (Chariere and Imdorf, 1997; Ellis *et al*., 2013). *A. grisella* larvae can also be used as model organisms model organisms in toxicological and immunological studies depending on its properties such as short life cycle, large hemolymph amount, easy and relatively cheap rearing conditions.

Therefore, *A. grisella* larvae were selected as a model organisms to investigate the cytotoxic impacts of IAA. To our knowledge this is the first report that demonstrates total and differential hemocyte counts, and also mitotic and apoptotic indices of hemocytes in *A. grisella* larvae with and without IAA application. Our study will provide a more complete understanding of effects of IAA on insect hemocyte physiology and chemically- induced apoptosis.

MATERIALS AND METHODS

Insect rearing

Stock culture of the *A. grisella* was reared with the temperature of 25± 2ºC, 60± 5% RH and 12:12 (L:D) photoperiod in our laboratory. The stock and successive cultures of *A. grisella* were established and maintained according to Uçkan and Gülel (2000) and Uçkan *et al*., (2011).

Bioassays

Newly hatched *A. grisella* larvae were reared on IAA-treated artificial diet. Various doses (2, 5, 10, 50, 100, 200, 500 and 1,000 ppm) of IAA (Merck 10 g, Darmstadt, Germany) were added to distilled water and homogenized with artificial diet (Sak *et al*., 2006). The last instar larvae of *A. grisella* (45-50 mg) were used in all experiments.

Total and differential hemocyte counts

In order to detect the impacts of various doses of IAA (2- 1,000 ppm) on the total hemocyte count (THC), last instars were washed in 70% ethyl alcohol and distilled water, and then air dried. After sterilization hemolymph was collected in anticoagulant buffer as described by Er *et al*., (2010). Ten microliters of these hemolymph suspension was used to count the total hemocyte number with a Neubauer Hemocytometer. To investigate the effects of various doses of IAA on differential hemocyte count (DHC), 5 µl of pure hemolymph was spread directly on sterile microscope slides and prepared for analysis according to Er *et al*., (2010). THC and DHC were assayed under an Olymphus BX51 (Olymphus Corp., Tokyo, Japan). All experiments were replicated five times with 3 larvae in each. Four hundred cells per larva were analysed for DHC.

Analysis of cell viability and mitotic indices

The rate of dead and alive hemocytes, and mitotic indices of IAA-treated and untreated *A. grisella* larvae were investigated using acridine orange/ethidium bromide (Sigma Chemical Co.) double staining according to Er *et al*., (2010) and Altuntaş *et al*., (2012). Five microliters of hemolymph per larva and 10 microliters of dye cocktail were spread on slides, respectively and instantly examined under an Olymphus BX51 (Er *et al*., 2010, Altuntaş *et al*., 2012). Using double staining method, cell stages were identified as living cells, early apoptosis, late apoptosis and necrosis according to Cendoroglo *et al*., (1999) and (Kosmider *et al*., 2004). Three larvae were examined for each experimental group and replicated 5 times. Three hundred cells per larva were investigated for apoptotic, and mitotic indices.

Statistical analysis

IAA-releated differences in the means were analysed using One-way Analysis of Variance (ANOVA) and comparison was done by Tukey's Honestly Significant Difference (HSD) test when variances were homogenous; otherwise Tamhane T2 test. Percentage data were normalized by arcsine transformation prior to analysis. All data were analysed by the SPSS software (SPSS 18.0 for windows). Differences were considered statistically significant when $P < 0.05$.

RESULTS

Addition of IAA (2- 1,000 ppm) to diet resulted in an increase in the total hemocyte number of *A. grisella* at all doses but the differences were significant at only 2 and 100 ppm when compared to the control ($F=5.404$; $df=8, 126$; $P=0.000$) (Table 1). The mean percentage of granulocyte of IAA- treated *A. grisella* increased at 2, 5, 100,

200, and 1,000 ppm, but changes were not significant (F=1.758; df=8, 126; P= 0.092) (Table 2). On the other side, the percentage of plasmatocyte significantly decreased at 5 ppm when compared to the control, 10, 50, and 500 ppm (F= 2.240; df=8, 126; P= 0.029) (Table 2). In contrast, the application of IAA did not alter the percentage of spherulocytes (F=1.010; df=8, 126; P= 0.432), prohemocytes (F= 1.626; df=8, 126; P= 0.124), oenocytoids (F= 0.921; df=8, 126; P= 0.502) (Table 2) and also mitotic indices (F= 0.528; df=8, 126; P=0.833) (Table 1) significantly when compared to control.

The mean percentage of living hemocytes of IAA treated *A. grisella* larvae declined at all tested doses, but the changes were not significant compared to the control and other doses (F=0.775; df=8, 126; P= 0.625) (Table 3). The mean early apoptotic hemocytes of IAA treated *A. grisella* significantly increased at 5, 10, 50, 100, and 200 ppm compared to control (F= 6.629; df=8, 126; P=0.000) (Table 3). However, IAA addition in *A. grisella* diet caused a decrease in the percentage of late apoptosis at 2, 10, 50, and 100 ppm (F= 3.599; df=8, 126; P=0.001) (Table 3) and necrosis at 5, 10, 50, and 100 ppm (F= 2.721; df=8, 126; P= 0.008) (Table 3).

Table 1. IAA-related changes in total hemocyte counts and mitotic indices of *A. grisella* larvae.

IAA (ppm)	Total hemocyte count[a] (x10^6 cell/ml) (Mean± SE)[b]	Mitotic indices [a] (cells/300) (% ± SE)[b]
0	6.38**±0.38a**	0.88±0.13a
2	9.55**±0.56c**	0.72±0.14a
5	6.91**±0.42ab**	0.92±0.20a
10	6.72**±0.32ab**	0.95±0.16a
50	7.19**±0.31ab**	0.75±0.15a
100	8.38**±0.44bc**	0.97±0.21a
200	8.08**±0.45abc**	0.80±0.18a
500	7.47**±0.44ab**	0.88±0.16a
1,000	7.26**±0.39ab**	0.78±0.16a

[a]The mean of 15 last instars per treatment.

[b]Columns followed by the same letter (a-c) are not significantly different (P>0.05; Tukey's HSD)

The mean percentage of living hemocytes of IAA treated *A. grisella* larvae declined at all tested doses, but the changes were not significant compared to the control and other doses (F=0.775; df=8, 126; P= 0.625) (Table 3). The mean early apoptotic hemocytes of IAA treated *A. grisella* significantly increased at 5, 10, 50, 100, and 200 ppm compared to the control (F= 6.629; df=8, 126; P=0.000) (Table 3). However, IAA addition in *A. grisella* diet caused a decrease in the percentage of late apoptosis at 2, 10, 50, and 100 ppm (F= 3.599; df=8, 126; P=0.001) (Table 3) and necrosis at 5, 10, 50, and 100 ppm (F= 2.721; df=8, 126; P= 0.008) (Table 3).

Table 2. IAA-related changes in percentage of differential hemocyte counts A. grisella larvae.

IAA (ppm)	Differential hemocyte count (cells/400) (% ± SE)[a]				
	Hemocyte type[b]				
	PLs	GRs	SPs	PRs	OEs
0	75.17 ± 1.67ac	20.22±1.95a	0.60±0.18a	2.88±0.48a	1.13±0.20a
2	71.35 ± 1.98abc	24.42±1.87a	0.78±0.16a	2.38±0.33a	1.07±0.28a
5	69.20 ± 1.87b	26.82±1.86a	0.90±0.22a	2.18±0.52a	0.90±0.22a
10	76.32 ± 1.34a	20.00±1.47a	0.52±0.08a	1.82±0.38a	1.35±0.19a
50	75.98 ± 1.76a	20.47±1.72a	0.45±0.13a	2.12±0.34a	0.98±0.24a
100	70.92 ± 1.83bc	25.07±1.87a	0.53±0.15a	2.45±0.45a	1.03±0.17a
200	71.22 ± 2.28ab	23.85±2.39a	0.68±0.19a	3.12±0.49a	1.13±0.20a
500	76.05 ± 1.90a	21.30±1.82a	0.48±0.13a	1.30±0.24a	0.87±0.16a
1,000	71.88 ± 1.73ab	24.23±1.65a	0.68±0.23a	2.52±0.40a	0.68±0.15a

[a]The mean of 15 last instars per treatment.

[b]Columns followed by the same letter (a-c) are not significantly different (P>0.05; Tukey's HSD; PLs, Plasmatocytes; GRs, Granulocytes; SPs, Spherulocytes; PRs, Prohemocytes; OEs,Oenocytoids)

Table 3. IAA-related changes in percentage of viable, early apoptosis, late apoptosis, necrosis of *A. grisella* larval hemocytes.

IAA (ppm)	Apoptotic indices[a] (cells/300) (%± SE)[b]			
	Viable	Early Apoptosis	Late Apoptosis	Necrosis
0	55.22±4.19a	34.80±3.86a	7.00±1.44a	2.98±0.77a
2	53.87±3.46a	37.67±3.13a	6.91±1.54a	1.56±0.40bc
5	47.84±3.94a	45.87±3.92b	4.09±1.51ab	2.20±0.64acd
10	49.96±3.61a	46.22±3.55 b	2.98±0.80 b	0.84±0.17b
50	45.04±4.48a	49.36±3.81 b	4.67±1.41ab	0.93±0.23bd
100	47.09±3.34a	48.02±3.17 b	3.49±0.56 b	1.40±0.29bd
200	49.47±3.45a	45.56±3.68 bc	3.18±0.55 b	1.80±0.34ab
500	50.40±3.53a	41.00±2.65a	5.96±1.31a	2.65±0.50ac
1,000	47.98±2.86a	43.44±2.33ac	6.84±1.62a	1.73±0.39ab

[a]The mean of 15 last instars per treatment.

[b]Columns followed by the same letter (a-d) are not significantly different (P> 0.05; Necrosis: Tamhane test, Other data sets: Tukey's HSD).

DISCUSSION

We had previously determined the impacts of IAA on the developmental characteristics of *Apanteles galleria, G. mellonella and Pimpla turionellae* (Uçkan *et*

al., 2011; 2015). In order to characterise further effects of IAA on insect immunity, IAA induced alterations in THCs, DHCs, mitotic and apoptotic indices of *A. grisella* hemocytes were investigated.

Five distinct classes of hemocytes have already been identified in different Lepidopteran species, such as *Manduca sexta* (Horohov and Dunn, 1982), *Poekilocerus bufonius* (Al-Robai *et al*., 2002), *Bomby xmori* (Ganie *et al*., 2015), *G. mellonella* (Altuntaş *et al*., 2012), *Spodoptera litura* (Sharma *et al*., 2003), *Ostrinia furnacalis* (Jian *et al*., 2003), and *Spodoptera littoralis* (Ghoneim *et al*., 2015). In the present study the former five main hemocyte types were also identified in the hemolymph of last instars of *A. grisella*: prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids. All of the treated IAA doses caused an increase in THC of *A. grisella.* Several reports have suggested that stress, wounding or infection can cause an increase in the number of hemocytes in circulation (Ratcliffe *et al*., 1985; Lackie, 1988; Strand 2008b). Similarly, Altuntaş *et al*., (2012) reported that, GA_3 caused a rise in THC in *G. mellonella* larvae. George and Ambrose announced (2004) that exposing to an organophosphate led to an increase in total hemocyte count and granulocyte numbers but a decrease in prohemocyte and plasmatocyte numbers of *Rhynocoris kumarii.* Similarly, we found that treatment with IAA in diet of *A. grisella* caused a decrease in the percentage of plasmatocytes whilst an increase in that of granulocytes. None of the applied doses led to significant changes in the percentage of spherulocyte, prohemocyte, and oenocytoids when compared to the control. Likewise, a various botanical originated insecticides such as azadirachtin (Azambuja *et al*., 1991; Er *et al*., 2017) and extract of *Artemisia annua* (Zibaee and Bandani) also alter hemocyte number of insects (James and Xu, 2012). Several reports in Lepidopteran demonstrated that the origins of hemocyte population in circulation during larval stage are both hemocytes, which can proliferate and differentiate, and hematopoietic organs (Strand 2008b). Our results revealed insignificant fluctuation in mitotic indices of hemocytes of IAA-treated *A. grisella.* Furthermore, the mitotic index fluctuations were not synergistic with dose depended increases in THC. Similarly, Altuntaş *et al*., (2012) demonstrated that GA_3 treatment in *G. mellonella* larvae caused an increase in mitotic indices, but there was no correlation between induced mitotic indices and THC. As a typical Lepidopteran insect, *Bombyx mori* has four hematopoietic organs, which produce hemocytes and continuously release into the hemolymph during larval stages (Strand, 2008a, 2008b). It seems likely that, the source of incremental THC of IAA-treated *A. grisella* may be hematopoietic organs instead of mitosis of hemocytes already in circulation. Some evidences have suggested that there is a bidirectional connection between the immune system and nervous system (CNS) in insects (Beckage, 2008). Moreover, CNS regulates directly or indirectly insect hormones, which affect immune responses (Niijhout, 1994; Adamo, 2006; Beckage, 2008). Ecdysteroids and juvenile hormones (JHs) regulate many developmental and physiological processes including immune responses (Gade *et al*., 1997; Rantala *et al*., 2003; Franssens *et al*., 2006; Flatt *et al*., 2008). Several reports have suggested that the endocrine organs regulate hemocyte populations and differentiation (Riziki,

1957, 1962; Hoffman, 1970; Judy and Marks, 1971; PrasadaRao *et al*., 1984; Ahmad and Khan, 1988). Previously, we have demonstrated that IAA treatment led to a delay in larval developmental time of *G. mellonella* and immature developmental time of parasitoid *P. turionellae,* which are regulated by ecdysteroids and JHs. The observations presented here and previously described have led us to conclude that IAA may alter hormone balance between ecdysteroids and JHs and cause alternations in THC and DHC in *A. grisella.*

As also stated above, the environmentally and internally regulatory stimuli (Kim *et al*., 2001) such as DNA damage, growth factor deprivation, death receptor signaling, radiation, chemicals or injury may cause apoptosis or necrosis (Dragovich *et al*., 1998; Sun *et al*., 1999; Bezabeh *et al*., 2001; Opferman and Krosmeyer, 2003; Nikoletopoulou *et al*., 2013). Our results indicated that whereas early apoptotic cells increased at low doses of IAA, the percentage of late apoptosis and necrosis decreased when compared to untreated larvae. Furthermore, there was a significant reduction in the percentage of living cells at all treated doses. Apoptosis inductive effect of IAA was also observed by Frukawa *et al*., (2004) in different tissue cells of mice. Altuntaş *et al*., (2012) also reported that GA_3 treatment on *G. mellonella* larvae induced apoptotic and necrotic cell death and reduced cell viability when compared to untreated larvae. Microbial infections or pollutants/toxins such as UV radiations, pesticides, and ozone trigger production of reactive oxygen species (ROS) (Kohen and Nyska, 2002; Poljsak *et al*,. 2013). When ROS is not sufficiently reduced by antioxidant enzymes (Turrens, 2003 ; James and Xu, 2012), they may affect adversely on the organism by reacting with macromolecules of biological importance (James and Xu, 2012). Increased oxidative stress causes death of cells either by necrosis or by apoptosis (Zamzami *et al*., 1995, 1996; Tan *et al*., 1998; Kannan and Jain, 2000; James and Xu, 2012). A wide variety of synthetic insecticides are known to supress the activity of antioxidant enzymes (James and Xu, 2012). Olivera *et al*., (2007) reported that, IAA treatment on rat liver caused decrease in the activity of catalase and glutathione S- transferase. Likely, Çelik and Tuluçe (2006) reported that, IAA and kinetin increased the production of lipid peroxidases, and inhibited antioxidant defence in various rat tissues. Therefore, IAA treatment on *A. grisella* may cause a decrease in antioxidant enzyme activity and induce apoptosis.

The increased interest to use eco-friendly products to promote agricultural production and to prevent damage of pests led us to investigate effects of ecologically innocent chemicals such as IAA in the model *A. grisella* larvae. Our data demonstrated that IAA affected THC and DHC, but has not effect on mitotic indices. In addition, IAA treatment caused a decrease in the percentage of living cells, late apoptosis and necrosis, but an increase in early apoptosis of *A. grisella* larval hemocytes. IAA showed similar effect with synthetic insecticides, which is harmful for target and non-target organism.

Effects of IAA on other physiological parameters of insects such as hemolytic and phenoloxidase activity and antioxidant enzyme activity should also be explored before suggesting this plant derived compound as a good candidate for pest control in IPM programs.

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Received: April 14, 2016 *Accepted: June 23, 2017*