Effects of Gibberellic Acid on Hemocytes of *Galleria mellonella* L. (Lepidoptera: Pyralidae)

H. ALTUNTAŞ,[1,2] A. Y. KILIÇ,[1] F. UÇKAN,[3] AND E. ERGIN[4]

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ABSTRACT The impacts of different doses of the plant growth regulator gibberellic acid (GA_3) in diet on the number of total and differential hemocytes, frequency of apoptotic, and necrotic hemocytes, mitotic indices, encapsulation, and melanization responses were investigated using the greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae. Total hemocyte counts increased in *G. mellonella* larvae at all treatment doses whereas GA_3 application had no effect on the number of different hemocyte types. The occurrence of apoptosis, necrosis and mitotic indices in GA_3 treated and untreated last instars were detected by acridine orange or ethidium bromide double staining by fluorescence microscopy. While the ratio of necrotic hemocytes increased at all GA_3 treatments, that of late apoptotic cells was only higher at doses >200 ppm when compared with untreated larvae. The percentage of mitotic index also increased at 5,000 ppm. Positively charged DEAE Sephadex A-25 beads were used for analysis of the levels of encapsulation and melanization in GA_3 treated *G. mellonella* larvae. At four and 24 h posttreatments with Sephadex A-25 bead injection, insects were dissected under a stereomicroscope. Encapsulation rates of larval hemocytes were dependent on the extent of encapsulation and time but not treatment groups. While the extent of melanization of hemocytes showed differences related to time, in general, a decrease was observed at all doses of GA_3 treated larvae at 24 h. We suggest that GA_3 treatment negatively affects hemocyte physiology and cell immune responses inducing cells to die by necrosis and apoptosis in *G. mellonella* larvae.

KEY WORDS gibberellic acid, hemocyte, apoptosis, encapsulation, *Galleria mellonella*

Plant growth regulators (PGRs), inhibiting or inducing plant growth, are widely used for two aims recently; increasing agricultural production and resistance of plants to pests (McDonald et al. 1988, Silva et al. 2003, Paulson et al. 2005). Gibberellic acid (GA_3) is a type of gibberellins and a large family of tetracyclic diterpenoid PGRs that have been identified in different plants. GA_3 is used extensively in agriculture to stimulate both cell division and cell elongation that affect leaves as well as stems (Carslon and Crovetti 1988, Salisbury and Ross 1992). However, GA_3 treatment is not only effective on plant growth, but also it affects the development, survival, longevity, and reproductive potential of insects and other animals as reported in several studies (Visscher 1980; Harikesh and Bhattacharya 2003; Kaur and Rup 2000, 2003a,b; Uçkan et al. 2008, 2011a). Moreover, Posnova (1974) stated that PGRs might be used in integrated pest management (IPM) programs with the aim of suppressing insects.

Studies on the toxicological properties of various PGRs including GA_3 were mostly conducted on mammals. It has been observed that abscisic acid and GA_3 cause lipid peroxidation in some tissues of rats and they change the activities of the enzymes in antioxidant defense system (Çelik et al. 2002, Furukawa et al. 2004, Tuluce and Çelik 2006). Exposure of the toads *Bufo regularis* Reuss (1833) to a diet including GA_3 induced hepatocellular carcinomas in 16% of the animals. Moreover, El-Mofty et al. (1994) showed that GA_3 induced breast and lung adenocarcinomas in mice. Gibberellic acid was found to induce chromosomal aberrations in human lymphocytes and mice (Zalinian et al. 1990, El-Mofty et al. 1994, Bakr et al. 1999). The World Health Organization also listed GA_3 as a plant growth regulator related to pesticides (WHO 1990).

Insects have developed an innate immune system of defense against invading organisms in the hemolymph. Generally, the innate immune response of invertebrates, including insects consists of phagocytosis, nodulation, and encapsulation. These reactions are the product of cellular and humoral responses (Carton and Nappi 1997, Schmidt et al. 2001, Lavine and Strand 2002, Jiravanichpaisal et al. 2006). The free hemocytes in the hemolymph of insects have important functions in cellular defense (Lavine and Strand 2002, Jiravanichpaisal et al. 2006). Hemocytes play role in many

[1] Department of Biology, Faculty of Science, Anadolu University, Eskişehir, Turkey, 26470.
[2] Corresponding author, e-mail: hyalcitas@anadolu.edu.tr.
[3] Department of Biology, Faculty of Science-Literature, Kocaeli University, İzmit–Kocaeli, Turkey, 41300.
[4] Nursing School, Gülhane Military Medical Academy, Ankara, Turkey, 06018.

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functions such as phagocytosis, encapsulation, nodule formation, cell agglutination, detoxification, as well as synthesis and secretion of the humoral immunity factors of the immune system of insects. These functions of hemocytes make them more sensitive than any other cells against environmental factors such as PGRs and other xenobiotics in bringing and accelerating the emergence of cellular oxidative stress (Kowalska 1991, Yılmaz and Yuksel 2005, Pugine et al. 2007). In this case, irreversible damage can occur in cells and they are quickly overcome by apoptotic or necrotic mechanisms (Pech and Strand 2000; Sung et al. 2003a,b). These changes caused by various environmental pollutants such as PGRs can be observed morphologically. They include increases in cell volume (hypertrophy) and the number of cells (hyperplasia), decreases in cell size (atrophy), cell proliferation arrest (aplasia), cell number (hypoplasia), and abnormal cell growth (Brown 2002). For these reasons, hemocytes are frequently used to ascertain the cytogenetic damage of toxic chemicals (Begum and Gohain 1996, Gayfullina et al. 2006).

Previous studies of insect hemocytes have focused on counts in the circulating hemolymph, the mitotic index, the rates of apoptosis, and encapsulation in response to starvation, injury, infection, and insecticide treatment (Jones and Liu 1968, Lastra and et al. 2001, Pech and Strand 2000, Sewify and Hashem 2001, Teramoto and Tanaka 2004, Suzuki and Tanaka 2006). Of those studies dealing with environmental pollutants, there is little clear information on the effects that PGRs, such as GA_3, have on the immune system. To further enhance our understanding of how the immune physiology can be influenced by environmental factors, we studied the exposure response to different doses of GA_3 in *Galleria mellonella* L. (Lepidoptera: Pyralidae). This is one of a number of important phytophagus pest species that take in a variety of plant secondary compounds and environmental pollutants (Sak et al. 2006, Ergin et al. 2007). We observed changes to the hemocytes and cell immune response that suggest GA_3 negatively impacts these insects.

Materials and Methods

Insect Rearing. *G. mellonella* original stock came from Kocaeli University, Kocaeli, Turkey. Laboratory colonies were kept in our laboratory in Anadolu University, Eskişehir, Turkey. *G. mellonella* were maintained by feeding the insects on a diet described by Bronskill (1961) and modified by Sak et al. (2006). Insects were reared at 25 ± 1°C, 60 ± 5% RH, and a photoperiod of 12:12 (L:D) h.

Bioassays. An individual mating pair of the host, 1- to 2-d-old *G. mellonella* was placed in 1-liter jars containing 1 g honeycomb to provide a mating and oviposition substrate. The adults were removed from the jars on the fifth day. Early instars were exposed to 10 g of host diet (Sak et al. 2006) treated with 50, 100, 200, 500, 1,000, and 5,000 ppm GA_3 (Merck 5 g, Darmstadt, Germany) homogenized with doses in separate jars. Larvae reared on GA_3-free diet were controls. Both experimental and control group diets were replenished at every day. To determine the impact of GA_3-related doses on the number, apoptotic and mitotic indices, encapsulation, and melanization responses of the hemocytes, last instars of *G. mellonella* (0.18 ± 0.02 g) were randomly selected from different populations at different times. Assays with GA_3 and controls were also carried out in the same laboratory condition with the stock culture.

Total and Differential Hemocyte Counts. To investigate the effects of different doses of GA_3 (50–5,000 ppm) on the number of circulating hemocytes, last instars were bled on the first hind leg with a sterile 19-gauge needle. Four microliters of hemolymph from each larva were collected with a glass microcapillary tube (Sigma, St. Louis MO). To avoid hemocyte aggregation, hemolymph collected from larvae was immediately mixed to 36 μl ice cold anticoagulant buffer (0.098 M NaOH, 0.186 M NaCl, 0.017 M Na_2EDTA, and 0.041 M Citric acid, pH 4.5) in sterile eppendorf tubes. Ten microliters of hemolymph suspension diluted in anticoagulant solution were applied to an improved Neubauer Hemocytometer (Superior, Bad Mergentheim, Germany). To determine the effects of all treatment doses of GA_3 on the number of different hemocyte types, last instars were also assayed by piercing the larvae at the same site and immediately spreading five microliters of hemolymph on sterile microscopic slides. The slides were allowed to air dry for 20–30 min to facilitate the adhesion of hemocytes to the glass. Cells were then fixed in methanol: acetic acid (3:1 vol:vol) for 5 min. Once the fixative had evaporated, hemocytes were stained with Giemsa (3 ml of Merck Giemsa's azure eosine methylene blue solution in 57 ml of PBS, pH 7.4) for 10 min, and slides were rapidly washed with distilled water and PBS and then air dried. Hemocyte types were classified by morphological characteristics according to study of Er et al. (2010). Total (THC) and differential hemocyte counts (DHC) were assayed under a Leica DM6000 B microscope (Leica Microsystems, Istanbul, Turkey) with bright field and phase contrast optics. Three larvae were evaluated for each experimental and control assays in five replicates for total and differential hemocyte counts. A thousand cells from an individual larva were counted on each slide for DHC.

Analysis of Cell Viability and Mitotic Indices. The impacts of different doses of GA_3 (50–5,000 ppm) in diet on the frequency of apoptotic and necrotic hemocytes, hemocyte viability, and mitotic indices were investigated using last instars (0.18 ± 0.02 g). The occurrence of apoptosis in GA_3 treated and untreated last instars was detected by acridine orange or ethidium bromide double staining by fluorescence microscopy (Er et al. 2011). Five microliters of hemolymph from each larva were collected with a glass microcapillary tube and immediately poured on a sterile microscope slide. The slides were allowed to stand at room temperature and 10 μl of dye suspension containing equal volumes of ethidium bromide (100 μg/ml in PBS, Sigma) and acridine orange (100 μg/ml in PBS, Sigma) was spread on the glass slide, covered

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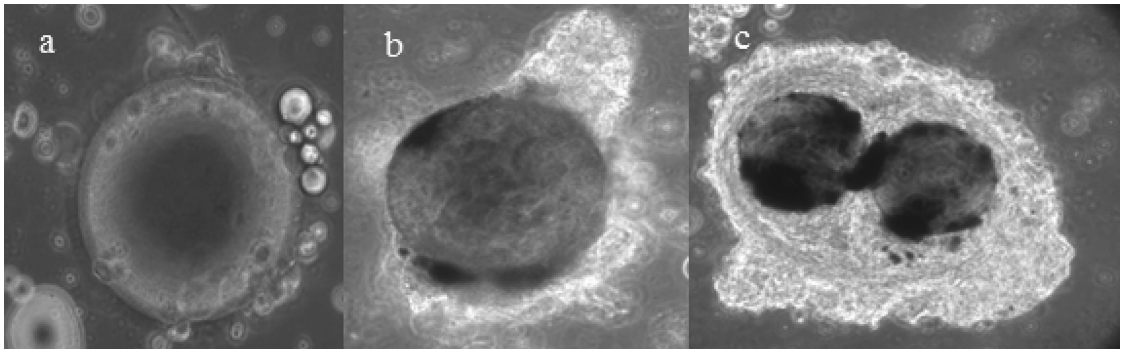


Fig. 1. Encapsulation of Sephadex A-25 beads in *G. mellonella* larvae. (a) Negative (no or only a few beads attached to the bead), (b) weak (2–10 layers of hemocytes around the bead), (c) strong (more than 10 layers of hemocytes around the bead).

with a cover slip and immediately examined using a fluorescence microscope at blue filter (Leica DM6000 B). Cells were identified as viable (green nucleus with red-orange cytoplasm with an intact membrane), early apoptotic (cell membrane still continuous but chromatin condensation and an irregular green nucleus are visible), late apoptotic (ethidium bromide penetrates through altered cell membrane and stains the nuclei orange, while fragmentation or condensation of chromatin is still observed), and necrotic (orange nucleus with intact structure) (Kosmider et al. 2004, Er et al. 2011). The percentages of hemocytes identified as viable, early apoptotic, late apoptotic, and necrotic were determined with respect to GA_3 doses. Five larvae were evaluated for each experimental and control assay and 500 cells from an individual larva were counted in each of four replicates for hemocyte viability and mitotic indices.

Encapsulation and Melanization. The positively charged DEAE Sephadex A-25 beads (40–120 μm in diameter; Sigma), evoking a strong encapsulation response (Uçkan et al. 2010), were used for analysis of the levels of encapsulation and melanization in GA_3 treated and untreated *G. mellonella* last instars. These chromatography beads were injected into hemocoel of the last instars. Before injection, these beads were stained with 0.1% coomassie blue in PBS for 1 h. After staining, the supernatant was removed and washed three times in fresh PBS (Uçkan et al. 2010). The beads were resuspended in sterile PBS at a concentration of $\approx$15–20 beads/10 μl. Finally, 10 μl of PBS containing 15–20 Sephadex A-25 beads were injected on the first hind leg of last instar larvae for all doses of GA_3 treated and untreated larvae by using a 50 μl Hamilton microsyringe with 22-gauge needle (Hamilton Co., Reno, NV). After bead injection, all experimental and control larvae were maintained at room temperature for 4 and 24 h. At 4 and 24 h posttreatments with Sephadex A-25 bead injection, larvae were dissected under a stereomicroscope. All beads that could be found were removed, added to a drop of PBS on a microscopy slide, and overlaid with a cover slip. Beads were observed by phase contrast microscope (Leica DM6000 B). The beads were scored for encapsulation and melanization. The encapsulation response was defined as negative (no, or only a few hemocytes attached to beads), weak (2–10 layers of hemocytes around beads) and strong (>10 layers of hemocytes around beads) (Fig. 1) (Uçkan et al. 2010). At the same time, the extent of the melanization of each capsule was determined by observing the blackening of the capsule and the beads were defined as melanized if melanized area covered more than the quarter of the capsule. Five larvae were evaluated for each experimental and control assays in three replicates at two time points in encapsulation and melanization responses of hemocytes.

Statistical Analysis. Means were compared using one way analysis of variance (ANOVA). Subsequently, means were subjected to Tukey's honestly significant difference (HSD) post hoc tests when variances (tested in SPSS by Levene statistics for homogeneity of variances) are homogenous, but Tamhane tests otherwise to assess the significance of the effects of GA_3 doses. Percentage data were normalized by arcsine transformation before analyses. Encapsulation means were also compared using three-way ANOVAs to determine the effects of different treatments (50–5000 ppm), time, encapsulation levels, and their interactions on the encapsulation response. *t*-tests were also inferred to determine the significance of the effects of GA_3 doses on the melanization response of hemocytes with respect to control group. A statistical software program (SPSS, version 15.0 for Windows; SPSS Science, Chicago, IL) was used for data analysis. Results were considered statistically significant when $P < 0.05$.

Results

Effects of GA_3 Doses on THC and DHC. Total hemocyte number of untreated last instars of *G. mellonella* were determined as $21.23 \pm 1.57 \times 10^6$ cell/ml. Exposure of *G. mellonella* to a diet containing GA_3 resulted in a remarkable increase in the number of circulating hemocytes at doses 100, 200, and 500 ppm with respect to untreated larvae. The maximum count of $32.63 \pm 0.98 \times 10^6$ cell/ml was observed at 500 ppm when compared with other doses and untreated lar-

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Table 1. GA_3-related changes in total hemocyte counts ($\times 10^6$ cell/ml) of *G. mellonella* larvae

GA_3 (ppm)	Total hemocyte count[a] ($\times 10^6$ cell/ml) (mean ± SEM)[b]
Untreated	21.23 ± 1.57abc
50	26.55 ± 1.29cd
100	28.89 ± 0.71de
200	30.75 ± 1.45de
500	32.63 ± 0.98e
1,000	18.84 ± 1.08ab
2,000	23.01 ± 0.70bc
5,000	16.72 ± 0.81a

[a] Each represents the mean of hemocyte counts from 15 larvae.
[b] Means within a column (a–e) followed by the same letter are not significantly different ($P > 0.05$; Tamhane test).

Table 2. GA_3-related changes in differential hemocyte counts (cells/1,000) of *G. mellonella* larvae

GA_3 (ppm)	Differential hemocyte count (cells/1,000) (mean ± SEM)[a]		
	Hemocyte type[b]		
	PL	GR	OT
Untreated	464.09 ± 19.09a	466.00 ± 19.78a	69.91 ± 4.32a
50 ppm	439.58 ± 16.70a	479.48 ± 16.02a	80.94 ± 7.48a
100 ppm	482.84 ± 21.87a	455.14 ± 22.71a	62.02 ± 4.79a
200 ppm	500.23 ± 33.93a	412.56 ± 38.14a	87.21 ± 12.62a
500 ppm	522.90 ± 22.93a	411.74 ± 22.60a	65.37 ± 7.32a
1,000 ppm	530.33 ± 27.35a	414.97 ± 27.99a	57.70 ± 5.19a
2,000 ppm	451.47 ± 15.15a	472.87 ± 16.12a	75.66 ± 7.68a
5,000 ppm	489.74 ± 21.48a	436.93 ± 23.73a	73.33 ± 7.85a

[a] Each represents the mean of hemocyte counts from 15 larvae.
[b] Means within a column (a) followed by the same letter are not significantly different ($P > 0.05$; PL: Tukey HSD, GR and OT: Tamhane test). PL, plasmatocytes; GR, granulocytes; OT, others.

vae. In addition, total hemocyte number decreased at doses 1,000 and 5,000 ppm with respect to other doses of GA_3 (Table 1; $F = 26.495$, df = 7, 112, $P < 0.05$). Different hemocyte types of last instars were classified into granular cells (GR), plasmatocytes (PL), spherulocytes, onositoid, and prohemocytes (Er et al. 2010; Fig. 2). Spherulocytes, onositoids, and prohemocytes were collectively named as others (OT). GR and PL, as the most common type of hemocytes, were detected in the total hemocyte population of untreated larvae of *G. mellonella* (Table 2; Fig. 2). Exposure of *G. mellonella* to a diet containing different doses of GA_3 had no effect on the number of different hemocyte types (GR: $F = 1.357$; df = 7, 112; $P > 0.05$, PL: $F = 1.989$; df = 7, 112; $P > 0.05$, OT: $F = 1.916$; df = 7, 112; $P > 0.05$, Table 2).

Effects of GA_3 Doses on Cell Viability and Mitotic Indices. Hemocytes of *G. mellonella* last instars were identified as viable, early apoptotic, late apoptotic, and necrotic as showed in Fig. 3. Acridine orange or ethidium bromide double staining indicated that the ratio of apoptotic and necrotichemocytes circulating in larval hemolymph was influenced by GA_3 treatment in diet depending on the treatment doses. A decrease in viable hemocyte indices was observed at the highest dose of 5,000 ppm ($F = 4.674$, df = 7, 152, $P < 0.05$; Table 3) but no significant difference was observed among all other doses and control. While the ratio of necrotic hemocytes increased at all GA_3 treatments ($F = 11.978$; df = 7, 152; $P < 0.05$), that of late apoptotic cells only raised at doses >200 ppm when compared with untreated larvae ($F = 6.038$; df = 7, 152; $P < 0.05$). In contrast, no difference was observed in early apoptotic indices of larvae at all treatments ($F = 0.787$, df = 7, 152, $P < 0.05$; Table 3). The percentage of mitotic index also increased at 5,000 ppm ($F = 3.894$; df = 7, 112; $P < 0.05$). However, mitotic frequency did not significantly change among all other doses of GA_3 (Table 4).

Effects of GA_3 Doses on Encapsulation and Melanization Levels. In the hemolymph of last instars of *G. mellonella*, Sephadex A-25 beads induced a strong encapsulation reaction. The thicknesses of the capsules changed at two time points checked (4 and 24 h) following bead injection (Fig. 1). After injection of the beads into the hemocoel of untreated *G. mellonella* larvae 15.72 and 87.65% of the covered beads were surrounded by multiple layers of hemocytes at 4 and 24 h, respectively, so the level of strong encapsulation level was higher at 24 h compared with that of at 4 h (Table 5). Similar results observed in the larvae of *G. mellonella* treated with different doses of GA_3.

The percentage of nonencapsulated beads increased at doses >500 ppm ($F = 8.657$; df = 7, 112; $P < 0.05$) with respect to untreated larvae at 4 h after injection of the beads into larvae. Exposure of *G. mellonella* to a diet containing different doses of GA_3 resulted in a remarkable decrease in the percentage of weak encapsulated beads at all treatments at 4 h when compared with untreated larvae. Among doses, the levels of weak encapsulated beads significantly differed at 50 ppm from other doses of GA_3. In general, the number of weak encapsulated beads indicated a considerable decline depending on dose increases of GA_3 at 4 h ($F = 24.150$; df = 7, 112; $P < 0.05$). By contrast, the percentage of strongly encapsulated beads increased at all treatments and especially the increase was significant at doses

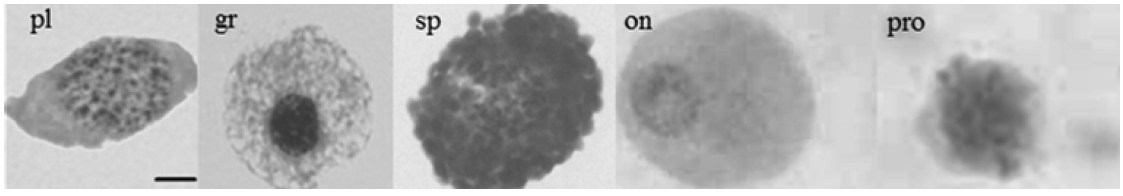


Fig. 2. Plasmatocyte (pr), granulocyte (gr), spherulocytes (sp), onositoids (on), and prohemocytes (pro) from last instars of *G. mellonella*. Photographs were made using a 1,000× objective. Bars = 10 μm.

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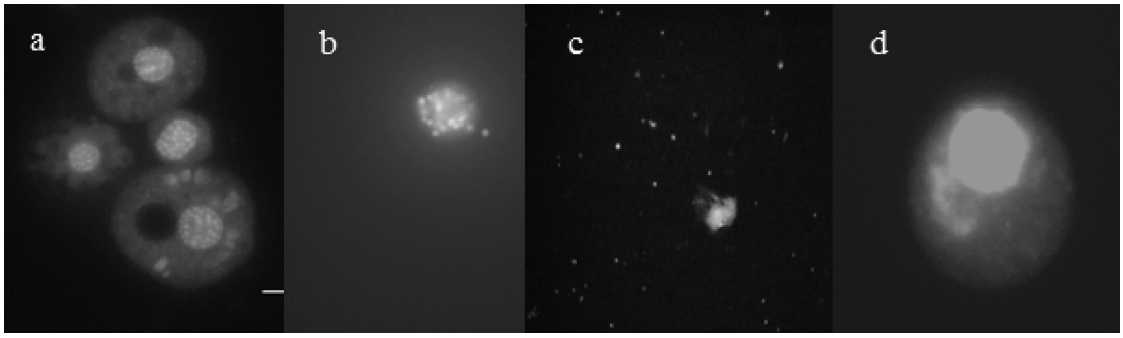


Fig. 3. Acridine orange or ethidium bromide double staining of *G. mellonella* hemocytes with symptoms of apoptosis. (a) Normal hemocytes, (b) early apoptosis, (c) late apoptosis, and (d) necrosis. Scale bar = 10 μm.

≥100 ppm when compared with untreated larvae (F = 22.260; df = 7, 112; $P < 0.05$).

At 24 h upon injection of the beads into the hemocoel of last instars, the percentage of nonencapsulated beads displayed no significant differences at all treatments with regard to untreated larvae except for 5,000 ppm that represents a threefold decrease in the number of none encapsulated beads when compared with that of at 50 ppm (F = 3.116; df = 7, 112; $P < 0.05$). Unlike 4 h after injection, the percentage of weak encapsulated beads increased at all doses except for 500 ppm with regard to untreated larvae at 24 h. Meanwhile, the highest percentage of weak encapsulation response was observed at 200 ppm (F = 26.905; df = 7, 112; $P < 0.05$). By 24 h after injection, the ratio of strongly encapsulated beads significantly decreased at 50, 100, and 200 ppm doses of GA_3 whereas it did not differ at 500 ppm with respect to untreated larvae. However, the ratio of strongly encapsulated beads decreased again at doses >500 ppm (F = 28.998; df = 7, 112; $P < 0.05$).

Three way ANOVAs indicated that the effects of all treatment doses of GA_3 on the encapsulation rates of larval hemocytes were dependent on the extent of encapsulation (F = 2883.328; df = 2; $P < 0.05$) and time (F = 26.798; df = 1; $P < 0.05$) but not treatment groups (F = 0.828; df = 7; $P > 0.05$). While experimental groups and time interactions were not significant for encapsulation responses of larvae (F = 0.847; df = 7; $P > 0.05$), the extent of encapsulation was significantly affected by all treatments and time point (F = 33.149, df = 14, $P < 0.05$; Table 6).

After injection of the beads into the hemocoel of untreated *G. mellonella* larvae, a greater percentage of capsules were melanized and the ratio of melanized hemocytes were determined as 54.29 and 77.87% at 4 and 24 h, respectively (Table 7; $P < 0.05$, t-test). The percentage of capsules that were melanized decreased at doses <1,000 ppm at 24 h when compared with that of at 4 h ($P < 0.05$), but there were not significant differences between the two time points at higher doses of GA_3 treated larvae ($P > 0.05$). The extent of melanization of hemocytes showed differences in relation with time in doses of GA_3 treated larvae. By 4 h after injections, the ratio of melanization increased at doses <1,000 ppm and decreased significantly at 5,000 ppm with respect to untreated larvae (F = 24.166; df = 7, 112; $P < 0.05$; Tukey's HSD post hoc tests). A significant decrease was observed at all doses of GA_3 treated larvae except for 500 ppm when compared with untreated larvae at 24 h (F = 16.435; df = 7, 112; $P < 0.05$; Tukey's HSD post hoc tests).

Discussion

Effects of various chemicals that cause environmental pollution on the hemocytes and other tissue cells of invertebrate organisms were investigated in several studies (Jones and Liu 1968, Pech and Strand 2000, Lastra et al. 2001, Sewify and Hashem 2001, Teramoto and Tanaka 2004, Suzuki and Tanaka 2006). Here, experiments were conducted to determine whether GA_3 affected the hemocytes of *G. mellonella* that is a test

Table 3. GA_3-related changes in apoptosis, necrosis, and viability of *G. mellonella* larval hemocytes

GA_3 (ppm)	Apoptotic indice[a] (cells/500) (% ± SE)[b]			
	Early apoptosis	Late apoptosis	Necrosis	Viable
Untreated	6.36 ± 0.81a	2.18 ± 0.26a	0.04 ± 0.04a	91.42 ± 0.94a
50	6.67 ± 0.65a	3.39 ± 0.41ab	1.04 ± 0.21b	88.91 ± 0.91ab
100	5.74 ± 0.48a	3.79 ± 0.41ab	1.16 ± 0.21b	89.31 ± 0.69a
200	5.55 ± 0.47a	2.74 ± 0.48ab	0.96 ± 0.24b	90.75 ± 0.83a
500	5.87 ± 0.75a	3.93 ± 0.44bc	1.53 ± 0.18b	88.67 ± 1.07ab
1,000	5.47 ± 0.40a	3.45 ± 0.31bc	1.12 ± 0.20b	89.96 ± 0.63a
2,000	6.36 ± 0.44a	3.96 ± 0.56bc	1.32 ± 0.18b	88.36 ± 0.96ab
5,000	6.75 ± 0.46a	5.95 ± 0.48c	2.14 ± 0.24b	85.16 ± 0.89b

[a] Each represents the mean of apoptotic indices (%) from 20 last instars.
[b] Columns followed by the same letter (a–c) are not significantly different ($P > 0.05$; Viable: Tukey HSD, Other data sets: Tamhane test).

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Table 4. GA_3-related changes in frequency of mitosis (%) in hemocytes of *G. mellonella* larvae

GA_3 (ppm)	Frequency of mitosis[a] (cells/1,000) (% ± SE)[b]
Untreated	1.146 ± 0.14a
50	1.321 ± 0.16ab
100	1.649 ± 0.12ab
200	1.485 ± 0.25ab
500	1.263 ± 0.18ab
1,000	1.177 ± 0.16ab
2,000	1.329 ± 0.06ab
5,000	2.335 ± 0.31b

[a] Each represents the mean of mitotic indices (%) from 15 last instars.
[b] Columns followed by the same letter (a, b) are not significantly different ($P > 0.05$; Tamhane test).

organism for physiological studies. First, we determined that hemocyte types of last instars of *G. mellonella* include granulocytes, plasmatocytes, spherulocytes, onositoids, and prohemocytes. Similar results also have been reported for larvae and pupae of *G. mellonella* by Er et al. (2010).

Our results indicated that the number of circulating hemocytes in last instars increased markedly at lower doses of GA_3 (50–500 ppm), but at high doses of GA_3 (1,000 and 5,000 ppm) no difference was observed when compared with untreated larvae. In several studies on lepidoptera, it is reported that the amount of circulating hemocyte in the hemolymph may change similarly in response to stress conditions, wounding or bacterial, fungal, and viral infections (Norayanan 2004, Mowlds et al. 2008, Phukan et al. 2008, Abd El-Aziz and Awad 2010, Kim and Kim 2010). We suggest that lower doses of GA_3 may induce a hormesis effect that is characterized as low dose stimulation of potentially toxic substances at sublethal concentrations that is not elicited by a higher dosage (Calabrase and Baldwin 2002, Kefford et al. 2008). The phenomenon of hormesis has been widely reported lately in arthropod pest species exposed to pesticides and other toxicants. These previous studies indicated that sublethal doses of various insecticides, heavy metals, and insect growth inhibitors have hormetic influence on the juvenile hormone (JH) levels, esterases, fecundity, and other biological parameters of some insect pest species (Mukherjee 1993, Nair et al. 2002, Nascarella et al. 2003, Zanuncio et al. 2003, Pereira et al. 2009, Yu et al. 2010). Low doses may exert positive effect on the immune system of pests. That is, a substance expected to elicite negative effect may trigger immune responses at low doses. However, these responses are likely to be eliminated at high doses. Furthermore, GA_3, a terpenoid compound like JH may affect hormetic response like JH analogues or mimics (Nair et al. 2002).

In lepidoptera and other insect orders, fluctuations in hemocyte number are influenced by mitosis of the circulating hemocytes itself, as well as the release of hemocytes from the hemopoietic organ (Gardiner 2000, Huang et al. 2009). Our results confirm those of others reporting that normally 1–8% of the population of circulating hemocytes in untreated larvae (1.15%) are in the mitotic phase in *G. mellonella* (Arnold and Hinks 1976, Er at al. 2010). Exposure of *G. mellonella* to a diet containing 5,000 ppm doses of GA_3 resulted in a twofold increase in mitotic indices of the circulating hemocytes. Yılmaz and Yüksel (2005) also determined that IAA, a stimulatory PGR like GA_3, increased the mitotic index of the bone marrow cells of the third generation of mice. Kowalska (1991) also showed that IAA increased the amount of DNA and RNA of human fibroblasts in cell culture. The authors suggested that induction of mitotic division could be related with the enhancing of the amount of DNA and RNA depending on IAA treatment (Kowalska 1991, Yılmaz and Yuksel 2005). These results indicate that PGRs stimulating cell division in plants may also stimulate molecules that control cell cycle regulation in larvae.

However, we determined that the increase in mitotic indices did not have any effect on the number of total and different types of hemocytes. Larval development time depends on the interval between spikes of JH and ecdysteroids, especially insect molting hormone ecdysone causes alterations on hemocyte division and mitotic rate (İzzetoğlu and Karaçalı 2003, Kiuchi et al. 2008). İzzetoğlu and Karaçalı (2003) also determined a dose-wise decrease in the number of hemocytes in the hemolymph of *G. mellonella* larvae after 20-hydroxyecdysone treatment. Therefore, GA3

Table 5. GA_3-related changes in encapsulation of Sephadex DEAE A-25 beads in *G. mellonella* larvae

GA_3[a] (ppm)	Total beads assessed		Extent of encapsulation (% ± SE and ≠ of beads encapsulated)[b]					
			None		Weak		Strong	
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
Untreated	223	226	6.26 ± 0.79a 14	2.64 ± 0.86ab 6	78.02 ± 1.23a 174	9.71 ± 0.83a 22	15.72 ± 0.87a 38	87.65 ± 1.03a 199
50	227	226	9.26 ± 0.87ab 21	5.78 ± 0.61a 13	71.27 ± 1.21b 162	24.26 ± 1.43b 55	19.47 ± 0.92ab 44	69.96 ± 1.48bc 158
100	226	225	12.37 ± 0.85bc 28	2.62 ± 0.86ab 6	65.98 ± 1.83bc 149	15.55 ± 1.01cd 35	21.65 ± 1.33bc 49	81.83 ± 1.11d 184
200	232	225	6.06 ± 0.44a 14	4.37 ± 0.83ab 10	53.42 ± 2.31d 124	31.92 ± 1.61e 72	40.53 ± 2.47d 94	63.71 ± 1.91c 143
500	221	223	9.98 ± 1.28ab 22	3.62 ± 0.91ab 8	57.94 ± 2.19d 127	10.87 ± 1.76ac 24	32.08 ± 2.74cde 72	85.51 ± 2.21ad 191
1,000	224	218	11.55 ± 0.96bc 26	6.00 ± 1.14ab 13	59.47 ± 1.22cd 133	30.26 ± 1.41be 66	28.98 ± 0.64ef 65	63.74 ± 1.71c 139
2,000	223	223	11.73 ± 1.29bc 25	4.93 ± 0.80ab 11	58.05 ± 1.84d 134	15.64 ± 1.02cd 35	30.22 ± 2.29cdf 64	79.43 ± 0.63d 177
5,000	224	220	15.63 ± 1.27c 35	1.75 ± 0.78b 4	58.96 ± 1.46cd 132	22.78 ± 2.85bde 50	25.40 ± 0.84cf 57	75.47 ± 2.80bd 166

[a] Larvae untreated and treated with different doses of GA_3, injected 10 μl PBS containing 15–17 Sephadex DEAE A-25 beads. Beads were dissected out and assessed at 4 h and 24 h posttreatment.
[b] Numbers in columns (a–f) followed by the same letter are not significantly different ($P > 0.05$; None 4 h and Weak 4 h: Tukey HSD, Other data sets: Tamhane test).

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Table 6. ANOVAs effects of different treatments (50–5,000 ppm), time, encapsulation levels, and their interactions on the encapsulation response by *G. mellonella* larvae

Source	df	MS	*F*	*P*	R^2
Treatment	7	0.06214	0.828	0.564	0.94
Time	1	0.201	26.798	0.000	
Extent of encapsulation	2	21.637	2,883.328	0.000	
Treatment × time	7	0.06355	0.847	0.549	
Time × extent of encapsulation	2	15.704	2,092.666	0.000	
Extent of encapsulation × treatment	14	0.100	13.884	0.000	
Treatment × time × extent of encapsulation	14	0.249	33.149	0.000	
Error	672	0.07504			

reducing larval development time of *G. mellonella* (Uçkan et al. 2011b) may also hold down the level of JH and stimulation of the ecdysteroids in hemolymph. Together with the data obtained from this point of view, these effects might also release important results on the hemocyte division.

Hemocytes become necrotic or undergo apoptosis (programmed cell death) as immune defense responses to foreign organisms and toxic compounds (Pech and Strand 2000; Sung et al. 2003a,b). The present work demonstrated that while late apoptotic cells increased at high doses of GA_3, the ratio of necrotic hemocytes increased at all GA_3 treatments when compared with untreated larvae, and a decrease in viable hemocyte indices was determined at the highest dose of 5,000 ppm. These results are similar to those of others reporting that necrosis has been induced by the organophosphorous insecticides in insects (Saleem and Shakoori 1993, İçen et al. 2005, Uçkan and Sak 2010). These earlier studies determined that organophosphorous insecticides caused stimulation of lipid peroxidation, increase in free radical formation and secretion of the tumor necrosis factors, various stress proteins, and cellular enzymes (Saleem and Shakoori 1993, İçen et al. 2005, Uçkan and Sak 2010). Few studies with PGRs demonstrated that IAA increased the amount of the DNA and RNA in the culture of human fibroblasts (Kowalska 1991) and the apoptosis of different tissue cells of mice (Furukawa 2004) and GA_3 induced the formation of tumor as well as stimulate the activity of mast cells by increasing oxidative stress in rats (Erin et al. 2008). These early studies support the reliability of our results; therefore, it is likely that, in insects, GA_3 might cause similar effects on the metabolic pathway of cell death mechanisms like organophosphorous insecticides.

The present work aimed to analyze whether different doses (50–5,000 ppm) of GA_3 affects the rate of the encapsulation and melanization response of *G. mellonella* in larval stage. Nonencapsulated beads increased at 24 h after injections whereas strongly encapsulated and melanized beads decreased at all doses of GA_3 treated larvae at the same time. Because the melanization response is hemocyte-mediated (Schmidt et al. 2001, Lavine and Strand 2002), it is likely that the negative effects of GA_3 on cell immune defense was likely because of the observed increases in hemocyte necrosis and apoptosis (Suzuki and Tanaka 2006). Nappi et al. (2000) also suggested that melanization reactions were associated with phenoloxidase, the terminal enzyme of melanization cascade, which is released from hemocytes. Therefore, the decreasing melanization may be related with the decreasing activity of phenoloxidase in hemolymph after GA_3 treatment. In a recent study, a decrease in phenoloxidase and an increase in lysosomal activities were also reported for *G. mellonella* larvae exposed to fungal infection (Bogus et al. 2007).

Collectively, our results indicate that GA3 ingestion by *G. mellonella* larvae has substantial negative effects on the rates of encapsulation and melanization, important functions of the immune system. Combined with our previously identified effect of GA_3 on larval development times (Uçkan et al. 2011b), our current results support the use of GA_3 as a substitute for traditional synthetic organopesticides in IPM programs.

Table 7. Effects of different GA_3 doses on the melanization (%) of Sephadex-A25 beads in *G. mellonella* larvae

GA_3 (ppm)[a]	Melanization (%)[b] time after treatment	
	4 h	24 h
Untreated	54.29 ± 2.96ax	77.87 ± 1.62ay
50	75.50 ± 2.91bcx	55.36 ± 2.61bcdy
100	78.54 ± 3.09bcx	49.39 ± 2.38dy
200	73.78 ± 2.43bcx	57.05 ± 1.29bcdy
500	74.99 ± 1.68cx	68.66 ± 4.20acey
1,000	55.04 ± 2.63ax	49.54 ± 3.19bdx
2,000	64.16 ± 1.90abx	69.55 ± 1.35ex
5,000	40.71 ± 1.00dx	56.28 ± 2.18bcdx

[a] Each represents the mean of melanized beads from 15 larvae.
[b] Numbers in columns (a–d) and rows (x–y) followed by the same letter (a–b) are not significantly different ($P > 0.05$; Tamhane tests and *t*-test).

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