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Toxicity of ozone fumigation on *Galleria mellonella* larvae and latent effects on pupation and adult emergence

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Summary The greater wax moth *Galleria mellonella* L. is a serious pest of honey bee combs in hives. Ozone fumigation was applied on fourth instar larvae at three concentrations, 0.5, 1.0 and 2.0 g/m^3 for 30, 60, 90 and 120 min. Our findings showed that the two concentrations of 0.5 and 1.0 g/m^3 were less effective than that of 2.0 g/m^3 on larval mortality with 40 and 60%, respectively, at 120 min of exposure time after 7 days. Full larval mortality (100%) was obtained by 2.0 g/m^3 of ozone at 90 min exposure time in 3 and 7 days post treatment. Our findings demonstrated that ozone had latent effects on pupation as well as adult emergence from treated larvae of *G. mellonella*. Exposure to 0.5 g/m^3 for 120 min, 1.0 g/m^3 for 120 min and 2.0 g/m^3 for 60 min resulted in the lowest pupation percentage (60, 40 and 0%, respectively) and strong inhibition of adult emergence (16.7, 0 and 0% adult emergence, respectively. Our research indicates that ozone fumigation is a useful strategy for the control of the greater wax moth.

Additional keywords: adult emergence, fumigation, greater wax moth, larval mortality, ozone, pupation

Introduction

The wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), is a major insect pest of honey bee combs in hives (Hanley *et al.,* 2003). Colony losses associated with the pest are considerable in a lot of places; the effect is stronger in weaker colonies and stored combs (Graham 1992; Ellis *et al.,* 2013; Sohail *et al.,* 2017). The larvae consume pollen, honey, wax combs, and the skins of cast honey bee larvae. The presence of larval wax moth in combs is indicated by their production of silken threads, which are frequently tunnel-like inside the combs.

Management of *G. mellonella* is applied by synthetic fumigants which question the potential risks on honey bee and human health, and the environment (Desneux *et al.,* 2007). In this respect, safe and effective control methods such as ozone fumigation and freezing have been tested for the control of *G. mellonella* (Zhu *et al.,* 2016; Nofal *et al.,* 2024). Ozone (O_3) is an unstable gas created when oxygen is exposed to a high voltage electric discharge (Kim *et al.,* 1999). It has been suggested as an excellent alternative for conventional fumigants such as phosphine gases since it has a short half-life (20–50 min) due to its rapid breakdown into oxygen, and therefore does not leave any residues on treated stored products, significantly deplete nutrients in commodities or cause insect resistance. (Mendez *et al.,* 2003).

Several studies illustrate how ozone applied to stored goods effectively combated a range of insect pests (Isikber *et al.,* 2007; Niakousari *et al.,* 2010; James, 2011; Jemni *et al.,* 2015; Ayad, 2019; Gad *et al.,* 2021a, b; Ingegno and Tavella, 2022; Sitoe *et al.,* 2024; Metwaly *et al.,* 2024). The mortality of *G. mellonella* caused by ozone gas has been reported by other studies (James, 2011; Nofal *et al.*, 2024). However, the latent effect of ozone on biological aspects such as pupation and adult emergence from treated larvae is limited. As a result, the current study focuses on studying the susceptibility of fourth instar larvae to ozone and their latent effects on pupation and adult emergence.

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Materials and Methods

Greater wax moth rearing

Galleria mellonella was mass cultured in the laboratory using larvae that were collected from infested beehives. Fourth instar larvae which were raised on bee wax for many generations at 26°C and 65% R.H. were used in the tests.

Ozone production

The ozone was generated using techniques reported by Gad *et al.* (2021b) at the Centre of Plasma Technology, Al-Azhar University, Nasr City, Cairo, Egypt.

Exposure of larvae of greater wax moth to ozone fumigation

To determine the effect of ozone on fourth instar larvae of the greater wax moth, 20 larvae were introduced inside small plastic tubes (5 cm high and 3 cm diameter) which were then placed into glass fumigation jars (35 cm high and 20 cm diameter). A metal lid with an inlet and an exit was used to cover the fumigation jar. The ozone generator was connected to the fumigation jars through a plastic tube extending from the entrance to the bottom of the fumigation jar. Both openings of the lid were secured with a valve. The larvae were exposed to the desired gas concentrations (0.5, 1.0 and 2.0 g/m^3) for 30, 60, 90 and 120 min and then the treated larvae were moved to 500 ml glass jars. For the control group, the larvae were exposed to air without ozone. For each ozone concentration and the control, four replicates (jars of 20 larvae) were set up. To determine the percentage of mortality, the mortality of larvae was recorded 3 and 7 days post exposure. After that, treated larvae were transferred into glass jars (3 cm diameter and 5 cm depth) with muslin cloth coverings for incubation under the same conditions mentioned above. They were checked every day until pupation and adult emergence (Al-Ayat *et al.,* 2025). Percent pupation, adult emergence and survival were calculated using the following formulas:

Pupation (%) = (Number of pupae/Total number of larvae) × 100

Adult emergence (%) = (Number of moths/Total number of pupae) × 100

Survival (%) = (Number of moths/Total number of larvae) × 100

Data analysis

The data of mortality, pupation adult emergence were transformed Arcsine. ANOVA was performed and the means were compared using Tukey's test ($P < 0.05$) in SPSS version 21 (Haynes, 2013).

Results

Larval mortality of *G. mellonella*

The mortality percentage of treated larvae of *G. mellonella* increased gradually with an increase in either concentration of ozone or exposure time. The two concentrations of 0.5 and 1.0 g/m^3 were less effective than that of 2.0 g/m^3 on larval mortality with 30 and 40% ($P < 0.01$; $F = 2.5$; df = 3, 12), respectively (Table 1) at 120 min of exposure time and 3 days post treatment. After 7 days of treatment, the two concentrations of 0.5 and 1.0 g/m^3 induced larval mortalities (40 and 60%) ($P < 0.01$; $F = 3.1$; df = 3, 12), respectively at 120 min of exposure time (Table 1). The highest concentration (2.0 g/m^3) induced the maximum larval mortalities (80 and 90%) at 60 min of exposure, 3 days ($P < 0.01$; $F = 763.7$; df = 3, 12) and 7 days after treatment ($P < 0.01$; $F = 907.6$; df = 3, 12), respectively. Full larval mortality (100%) was obtained by 2.0 g/m^3 of ozone at 90 min exposure time, 3 and 7 days post treatment.

Latent effects of ozone fumigation on *G. mellonella*

The delayed effect of ozone fumigation on pupation, adult emergence and survival percentage of *G. mellonella* is shown in Table 2. The lowest pupation percentage (60, 40 and 0%) was obtained from treated larvae by 0.5, 1.0 and 2.0 g/m^3 at 120, 120 and 60 min exposure time, respectively ($P < 0.01$; $F = 209.0$; df = 4, 15), ($P < 0.01$; $F = 38.9$; df = 4, 15) and ($P < 0.01$; $F = 600.0$; df = 4, 15).

Table 1. Percentage larval mortality of *Galleria mellonella,* 3 and 7 days after fumigation with different ozone concentrations for different exposure times.

Concentration (g/m^3)	% Larval mortality (mean ± SE) after 3 days				% Larval mortality (mean ± SE) after 7 days			
	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min
Control	0.0±0.0c	0.0±0.0d	0.0±0.0d	0.0±0.0d	0.0±0.0b	0.0±0.0d	0.0±0.0d	0.0±0.0d
0.5	5.0±2.9bc	17.5±2.5c	25.0±1.2b	30.0±1.2c	10.0±1.4b	27.5±2.5c	30.0±1.6c	40.0±4.1c
1.0	10.0±2.0b	30.0±2.6b	40.0±1.0b	40.0±2.9b	30.0±2.0a	40.0±2.6b	50.0±1.0b	60.0±4.1b
2.0	20.0±2.0a	80.0±2.2a	100.0±0.0a	100.0±0.0a	40.0±4.0a	90.0±2.0a	100.0±0.0a	100.0±0.0a
F	15.0	763.7	883.0	2.5	40.0	907.6	8.6	3.1
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

There is no significant difference between the mean values in each column that are followed by the same letter (df = 3, 12, P < 0.05).

Table 2. Latent effects of ozone fumigation at different concentrations and different exposure times, on pupation, adult emergence and survival of *Galleria mellonella* treated as fourth instar larvae.

Exposure times (min)	Ozone concentration of 0.5 g/m^3			Ozone concentration of 1.0 g/m^3			Ozone concentration of 2.0 g/m^3		
	Pupation (%)	Adult emergence (%)	Survival (%)	Pupation (%)	Adult emergence (%)	Survival (%)	Pupation (%)	Adult emergence (%)	Survival (%)
0	100±0.0a	100±0.0a	100±0.0a	100±0.0a	100±0.0a	100±0.0a	100±0.0a	100±0.0	100±0.0
30	90.0±0.8b	88.9±3.4b	80.0±1.9b	70.0±4.0b	28.9±1.8b	20.0±4.1b	50.0±3.5b	0.0±0.0	0.0±0.0
60	72.5±2.5c	55.4±1.8c	40.0±2.2c	60.0±1.2bc	16.9±1.0c	10.0±2.0c	0.0±0.0c	0.0±0.0	0.0±0.0
90	70.0±1.2c	25.0±3.6d	17.5±2.5d	50.0±4.0cd	0.0±0.0d	0.0±0.0d	0.0±0.0c	0.0±0.0	0.0±0.0
120	60.0±4.1d	16.7±1.0e	10.0±1.7e	40.0±4.1d	0.0±0.0d	0.0±0.0d	0.0±0.0c	0.0±0.0	0.0±0.0
F	209.0	433.0	1231.0	39.8	1997.0	9.4	600.0		
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		

There is no significant difference between the mean values in each column that are followed by the same letter (df = 4, 15, P < 0.05).

The lower concentration (0.5 g/m^3) caused strong suppression of adult emergence and survival (16.7 and 10.0%), ($P < 0.01$; $F = 433.0$; df = 4, 15) and ($P < 0.01$; $F = 1231.0$; df = 4, 15), respectively. The two concentrations (1.0 and 2.0 g/m^3) induced full suppression (0%) of adult emergence and survival ($P < 0.01$; $F = 1997.0$; df = 4, 15) and ($P < 0.01$; $F = 9.4$; df = 4, 15), respectively.

Discussion

Since methyl bromide was added to the list of chemicals that cause ozone depletion, many fumigants have been proposed as an alternative to methyl bromide (UNEP, 1995). Ozone is one of these fumigants, which has been demonstrated to have some potential for controlling postharvest insects (Mahroof *et al.,* 2018). Our investigation demonstrated that the larvae of *G. mellonella* were highly sensitive to ozone fumigation. The larval mortality increased with increasing ozone concentration and exposure time. The ozone concentration of 2.0 g/m^3 was effective to reach complete larval mortality in two exposure times, 90 and 120 min. Our findings are in line with several reports on other insects indicating that ozone fumigation was effective on larvae of postharvest insects. Kells *et al.* (2001) showed that ozone induced higher larval mortality of *Plodia interpunctella* (Hübner) by 50 ppm after three days of treatment. Isikber *et al.* (2007) reported that 300 ppm of ozone for 2 h of exposure time caused complete larval mortality of *Ephestia kuehniella* (Zeller). Osman (2009) found that 1 g/m^3 of ozone caused complete mortality of *E. kuehniella* larvae after 6 days of exposure. Hussain (2014) stated that larvae of *Ephestia cautella* (Walker) treated with 80 ppm caused 100% mortality after 5 h of exposure. Ibrahim and Al-Ahmadi (2014) observed that 80 ppm for 1 h caused the larval mortality of *Phthorimaea operculella* (Zeller) (80.0%). Keivanloo *et al.* (2014) demonstrated that the larval mortality of *P. interpunctella* (85.0%) was achieved by ozone treatment (5 ppm) for 2 h. Husain *et al.* (2015) ozone fumigation caused complete larval mortality of *E. cautella* by 22 ppm for 24 h of exposure time. Zinhoum and El-Shafei (2019) showed that complete larval mortality of *P. interpunctella* was obtained after 90 min of exposure to 1.0 g/m^3 of ozone. Mahmoud *et al.* (2023) revealed that the LC_{50} values were 275.3 and 446.8 ppm for the second and fifth larval instars of the khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermetstidae), after 2 h of exposure time.

The high toxicity of ozone against larvae of *G. mellonella* found in the present investigation may be due to the strong oxidizing properties of the gas. According to Hermes-Lima (2004), when ozone disintegrates into dioxygen, free radicals of reactive oxygen species (ROS) are produced which can significantly change DNA and protein molecules as well as oxidize polyunsaturated fatty acids. As a result, according to Holmstrup *et al.* (2011), these effects may damage cells and kill insects.

Moreover, our findings demonstrated full suppression of pupation, adult emergence and survival, by ozone fumigation at the concentration of 2.0 g/m^3. Similarly, Bonjour *et al.* (2011) demonstrated that ozone induced full inhibition of *Tribolium castaneum* (Herbest) progeny by 70 ppm treatment for four days. Abd El-Ghaffar *et al.* (2016) mentioned the progeny of *Sitotroga cerealella* (Olivier) was completely suppressed by treatment of the larval stage with 5 g/m^3 for 5 h. Abd El-Ghaffar *et al.* (2017) showed that the complete suppression of pupation of red flour beetle from treated larvae with ozone was achieved after 5 h exposure of 5 g/m^3. Subramanyam *et al.* (2017) indicated that the ozone fumigation was effective in progeny suppression of *Rhyzopertha dominica* (F.) at lower concentrations (0.42 and 0.84 g/m^3). The same findings were obtained by Xinyi *et al.* (2019).

Finally, the present study provided valuable and promising data on the application of ozone fumigation against the greater wax moth under laboratory conditions. However, there are some limitations to the application of ozone gas as a fumigant to control this insect pest in stored hives such as the difference between fumigation chambers used in this study and places of stored hives, which are far more complex, with irregular internal structures, variable airflow, and potentially porous materials like wax, wood, and propolis that could absorb or react with ozone. These factors may lead to uneven ozone distribution, reducing its efficacy against pests in some parts of the hive. Also, field trials are needed to determine whether ozone-treated comb is acceptable to bees, and if adequate fumigation conditions can be achieved on a large field-use scale, where the woodenware will slow the accumulation of ozone within a chamber (James, 2011). Also, the cost of ozone generators is still high for beekeepers. However, the sustainable application for ozone fumigation will reduce the cost and might become cheaper if the application of this technology increases under field conditions.

Conclusion

Based on our findings, the ozone fumigation can cause full larval mortality of *G. mellonella* after exposure for 90 min to 2.0 g/m^3 while lower time exposure of 30 min at this concentration can cause full inhibition of adult emergence similarly with 90 min exposure to 1.0 g/m^3, supporting the potential use of ozone in IPM strategies against the pest. Further studies are needed on the efficacy of ozone against the pest under field conditions and its impact on treated stored hives in terms of delay in the development of insect resistance as well as to reduce the risk to honeybees, humans and the environment.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Τοξικότητα του υποκαπνισμού με όζον στις προνύμφες του κηρόσκωρου, *Galleria mellonella*, και λανθάνουσες επιδράσεις στη νύμφωση και την έξοδο των ενηλίκων

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Περίληψη Ο κηρόσκωρος, *Galleria mellonella* L., είναι ένας σοβαρός εχθρός των κηρηθρών των μελισσών στις κυψέλες. Ο υποκαπνισμός με όζον εφαρμόστηκε σε προνύμφες τέταρτης ηλικίας σε τρεις συγκεντρώσεις, 0,5, 1,0 και 2,0 g/m^3 για 30, 60, 90 και 120 λεπτά. Τα αποτελέσματα έδειξαν ότι οι συγκεντρώσεις των 0,5 και 1,0 g/m^3 ήταν λιγότερο αποτελεσματικές από αυτή των 2,0 g/m^3, με 40 και 60% θνησιμότητα των προνυμφών, αντίστοιχα, σε χρόνο έκθεσης 120 λεπτών, 7 ημέρες μετά την εφαρμογή. Καθολική θνησιμότητα (100%) των προνυμφών επιτεύχθηκε με 2,0 g/m^3 όζοντος σε χρόνο έκθεσης 90 λεπτών, 3 και 7 ημέρες μετά την εφαρμογή. Τα αποτελέσματα έδειξαν ότι το όζον είχε λανθάνουσες επιδράσεις στη νύμφωση καθώς και στην έξοδο των ενηλίκων του *G. mellonella*. Τα χαμηλότερα ποσοστά νύμφωσης και εξόδου ενηλίκων καταγράφηκαν μετά από έκθεση σε 0,5 g/m^3 για 120 λεπτά, 1,0 g/m^3 για 120 λεπτά και 2,0 g/m^3 για 60 λεπτά και ήταν 60, 40 και 0% για τη νύμφωση, αντίστοιχα, και 16,7, 0 και 0% για την έξοδο των ενηλίκων, αντίστοιχα. Συμπερασματικά, φαίνεται ότι ο υποκαπνισμός με όζον είναι μια χρήσιμη πρακτική για την αντιμετώπιση του κηρόσκωρου.

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