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Susceptibility of immature stages of the Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) to ozonated water

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Abstract: Ozone is a powerful oxidant capable of killing insects and microorganisms and has been used in the food processing industry in the gaseous and aqueous states. In a laboratory study, the susceptibility of immature stages of an important stored-product pest, the Indian meal moth, *Plodia interpunctella* Hubner to ozonated water was investigated. Ozone was applied in aqueous form at four concentrations (0, 2, 3 and 5 ppm) for four different periods (30, 60, 90 and 120 min) on eggs, larvae and pupae of, *P. interpunctella*. The results indicated that in all tested stages, the rate of mortality increased with increasing of concentration and exposure time. This study showed that 5-day old larvae were more susceptible than other stages (12-, 17-day old larvae, pupae and eggs) when exposed to 5 ppm ozone for 120 min. Following 5-day old larvae, 12-day old larvae, 17-day old larvae and pupae had the highest sensitivity to ozonation. At the highest concentration of ozone for the longest time, the least mortality rate was recorded for one day old eggs. According to these preliminary results, ozonated water has potential of reducing population density of *P. interpunctella*, one of the most important pests of dried fruits such as date, almond and pistachio, in storage.

Keywords: Post harvest pests, Ozonated water, *Plodia interpunctella*.

Introduction

Ozone is a triatomic form of oxygen (O_3) and is referred to as activated oxygen, or allotropic oxygen. It is an unstable gas with a half life of about 20 minute, depending on the temperature (Mullen and Arbogast, 1979). It has a longer half-life in the gaseous state than in aqueous solution (Rice, 1986). Ozone has been used as a water treatment to disinfect, eliminate odors, taste, color, and to remove pesticides, inorganic and organic compounds (Legeron, 1984; Kim *et al*., 1999; EPA, 1999). Ozone has many advantages as a sanitizer. Some of these advantages are: gaseous ozone and ozonated water can be generated on site, eliminating the need to store or dispose of chemical containers, no residue on treated product, and chemical reaction of ozone with organic material occurs at very rapid rates and short reaction times, which effectively prevents microorganisms from developing tolerance to ozone (Mendez *et al*., 2003). Also, ozone is attractive because its degradation product is oxygen, thus leaving no undesirable residue. Such advantages make ozone attractive to the food industry and consequently it has been affirmed as Generally Recognized as Safe (GRAS) for use in food processing (Graham, 1997).

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Ozone is mainly used in agriculture against stored product pests (Erdman, 1980; Mason *et al*., 1997; Kells *et al*., 2001; Sousa *et al*., 2008, Bonjour *et al*., 2011; Hansen *et al*., 2012), for inactivation of bacterial growth (Achen and Yousef, 2001; Sharma *et al*., 2002; Habibi Najafi and Haddad Khodaparast, 2009), prevention of fungal decay (Palou *et al*., 2002), destruction of pesticides and chemical residues (Hwang *et al*., 2001), and degradation of aflatoxin from dried fruits (Zorlugenc *et al*., 2008).

Iran is the largest pistachio nut producer in the world (FAO, 2011; Cheraghali and Yazdanpanah, 2010). Stored nuts suffer severe qualitative and quantitative losses due to the *Plodia interpunctella* infestation (Shojaaddini *et al*., 2008). This moth is well adapted to storage conditions and presents an immediate threat to the nuts. The larvae are able to penetrate and infest a wide range of packaged foods (Cline, 1978), and can have a great economic impact due to direct product loss and indirect factors such as, the cost of pest control and loss of sales from consumer complaints (Sauer and Shelton, 2002).

Currently, phosphine is a common fumigant used for stored-pistachio protection. However, due to development of pest resistance, health hazards and risk of environmental contamination, this fumigant is to be restricted (Shadia, 2011). Therefore, several non-chemical control methods for *P. interpunctella* infestations have been suggested as alternatives for fumigants (Shojaaddini *et al*., 2008). Recently, there has been a growing interest in research concerning the possible use of ozone gas as an alternative to chemical control of storage pests (Kells *et al*., 2001; Sousa *et al*., 2008, Bonjour *et al*., 2011; Hansen *et al*., 2012). However, limited research has been performed on the effect of ozonated water on stored product pests.

The overall aim of the present study was to determine the influence of ozone treatment in gaseous and aqueous forms on immature life stages (egg, larvae & pupae) of Indian meal moth, *P. interpunctella*. By simulation of a floating tank in pistachio processing, this work presents only the results of ozonated water on the studied insect.

Materials and Methods

Test insect

P. interpunctella were collected from an infested food warehouse in Mashhad (Razavi Khorasan province, NE of Iran) in 2011. The larvae were reared in the laboratory on an artificial diet consisting of 160 g yeast, 200 ml glycerol, 200 ml honey and 800 g wheat bran (Sait *et al*., 1997) in transparent plastic containers of 25 cm in diameter and 30 cm in height. The top of containers were covered with muslin cloth to avoid the escape of the moths. For each experiment, a group of twenty adults were transferred to oviposition funnel, 22 cm in diameter and 20 cm in height, covered with a 20 mesh cloth net and reversed on a black plastic sheet. After 24 h, the adults were removed and the eggs laid on plastic sheets were collected and used in experiments or incubated at the rearing conditions for rearing other stages needed in further trails. All cultures were maintained at 28 ± 1 ºC, 65 ± 5% r. h. and a photoperiod of 13:11 h (L:D) in an incubator (Razi Company Inc., Mashhad-Iran).

Experimental setup

The experimental setup for ozone application consisted of an ozone generator (a laboratory corona discharge ozone generator (AS-1200 M, Ozoneab Company Inc., Iran), monitor-controller and ozone detector. Aqueous ozone was produced by forcing ozone into distilled water with pH 7 and room temperature. Ozone injection was administered by a venturi system into a 75 liter tank filled with water. The ozone concentration in water was monitored using an ozone analyzer (Micro 1000M2) in the range between 0 and 20 ppm with the accuracy of 0.01. To do this, a sample of ozonated water was placed in small vial of microprocessor-based instrument designed to measure the amount of dissolved ozone in water. Before placing the water vial into a horizontal analyzer, a pill of DPD No4, was added to the sample. When water vial

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was fitted tightly, the analyzer was turned on and the concentration of ozone in water on the screen of the analyzer was read. The amount of ozone was measured at the contact tank.

Bioassays

For each growth stage, 16 combinations of ozone concentration and exposure time were tested. Eggs attached to a piece of plastic sheet, pupae, and larvae (5, 12 and 17 day-old) were placed in 130-mL jars with a hole at the center of the lids covered with a 40 mesh cloth net. Pupae, larvae (5, 12 and 17 day-old) were then exposed to ozone concentrations of 0, 2, 3 and 5 ppm for 30, 60, 90 and 120 min. For each stage of the studied insect, the experiment was replicated six times by using at least 10 specimens of the same age per replicate. After treatment, eggs, larvae and pupae were held at28 ± 2 °C and 65 % ± 5% R.H, on the same diet until scoring of the mortality. Following Isikber and Oztekin (2009), mortality counts for eggs were done 7 days after treatment and in cases of larvae and pupae 9 days after treatment. In all cases, individuals that failed to enter to next life stage were considered dead.

Data analysis

Statistical analyses were performed using the GLM procedure in SAS, version 9.1 (SAS Institute, 2002, Cary NC, USA). Data were first transformed using the arcsine square-root transformations to stabilize variances and then analyzed separately for each life stage using one-way analysis of variance (ANOVA). Tukey's Studentized Range (HSD) Test (at $\alpha = 0.05$) was used to compare means among treatments using. A multiple linear regression was performed using the REG procedure in SAS software to analyze the relationship between mortality percent in different ozone concentration and exposure time combinations.

Results

Eggs

Mortality of immature stages of *P. interpunctella* at 4 ozone concentrations and 4 exposure times is summarized in Table 1. Egg mortality increased from zero (control) to a maximum of 56.66% at 5 ppm for exposure time of 120 min. The lowest egg mortality (25.55 %) was observed at ozone concentration of 2 ppm for 30 min. Statistical analyses showed that mortality rate of eggs was significant in all ozonated water treatments ($F = 3.87$; $df = 9,95$; $P = 0.0004$). This study showed that eggs were more tolerant to ozone than the other stages. Figure 1 represents the results obtained for treatment of eggs by ozonated water with ozone concentration of zero to 5 ppm for 30, 60, 90 and 120 min.

Larvae

Larvae showed to be more susceptible to ozone than other immature stages. However, there was a variation in susceptibility of larvae of different ages to ozone. 5-day old larvae were more susceptible to ozone than 12-d and 17-d old larvae. Mortality ratios of 5-d old larvae increased from 0.00 at control treatment to 88.3 after 2 hrs of exposure to ozone at 5 ppm concentration. ANOVA results for 5-day old larvae was ($F = 5.67$; $df = 9, 95$; $P < 0.0001$). In cases of 12-d- and 17-d old larvae which the interactions between ozone concentration and exposure period were not significant, data were analyzed separately for each larval stage (Table 2). The highest mortality (88.33%) was achieved for 5-day old larvae when exposed to 5 ppm ozone for 2 hrs (Fig. 2). In case of 12-d and17-d old larvae with increasing ozone concentration from zero to 5 ppm the mortality ratios increased from 0-62.4 and 0-70.4 respectively. Also, with increasing exposure time from 30 min to 120 min mortality ratios of 12-d old larvae increased from 33.7 to 56.6, and of 17-d old larvae increased from 36.2 to 59.5 (Table 2).

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Table 1 Effects of ozone concentration and exposure time on mortality (mean ± SE) of *Plodia interpunctella* in different life stages.

Life stage	Exposure periods (min)	Ozone concentration (ppm)			
1 day old Eggs	30	0 ^{f}	25.55 ± 2.48 ^{e}	31.66 ± 4.77 ^{de}	31.66 ± 3.07 ^{de}
	60	0 ^{f}	36.66 ± 3.33 ^{cde}	41.66 ± 4.77 ^{abcde}	45.00 ± 2.23 ^{abcd}
	90	0 ^{f}	36.66 ± 2.10 ^{cde}	40.00 ± 3.65 ^{bcde}	51.66 ± 3.07 ^{abc}
	120	0 ^{f}	51.66 ± 4.77 ^{abc}	55.00 ± 5.62 ^{ab}	56.66 ± 4.21 ^{a}
5 day old Larvae	30	0^{e}	30.00 ± 2.58 d	31.66 ± 3.07 ^{cd}	40.00 ± 8.56 ^{cd}
	60	0 ^{e}	40.00 ± 3.65 ^{cd}	41.66 ± 3.07 ^{cd}	45.00 ± 7.63 ^{bcd}
	90	0^{e}	51.66 ± 5.42 ^{bcd}	55.00 ± 5.62 ^{bc}	66.66 ± 6.14 ^{ab}
	120	0 ^{e}	83.33 ± 6.66 ^{a}	86.66 ± 6.14 ^{a}	88.33 ± 4.77 ^{a}
Pupae	30	0 ^{f}	32.96 ± 5.30 ^{e}	45.00 ± 4.28 ^{cde}	46.66 ± 7.14 ^{cde}
	60	0 ^{f}	36.66 ± 3.33 ^{ed}	43.33 ± 4.21 ^{cde}	65.00 ± 5.62 ^{abc}
	90	0 ^{f}	35.00 ± 5.62 ^{e}	45.00 ± 6.19 ^{cde}	55.00 ± 4.28 ^{bcde}
	120	0 ^{f}	60.00 ± 2.58 ^{abcd}	78.33 ± 7.49 ^{ab}	81.66 ± 7.03 ^{a}

For each life stage separately, means followed by different superscript letters are significantly different ($P < 0.05$) by Tukey's HSD test. ANOVA results for eggs, 5d larvae and pupae were *($F = 3.87$; df = 9,95; $P < 0.0004$), ($F = 5.67$; df = 9,95; $P = <.0001$), ($F = 6.55$; df = 9,95; $P <.0001$),* respectively.

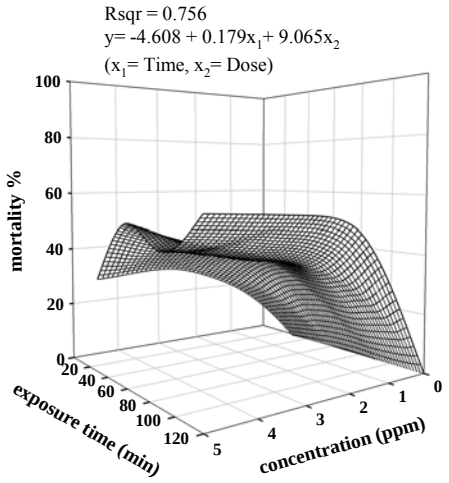

Figure 1 Effect of ozone concentration and exposure time on mortality (%) *P. interpunctella* in egg stage.

Pupae

Similar to larval stage, pupal mortality was increased with increasing of exposure time and ozone concentration (Fig. 3). Results showed that differences in mortality rates of pupae between treatments were significant (F = 6.55; df = 9, 95; P = <.0001).

A period of 120 min exposure of pupae to 5 ppm of ozone reduced adult emergence from 100 % (control) to 18.34 %.

Table 2 Effects of ozone concentration and exposure time on mortality (mean ± SE) of *P. interpunctella* in 12-and 17-day old larvae.

Ozone concentration (ppm)	12-day old larvae	17-day old larvae
0	0 ^{b}	0^{b}
2	54.58 ± 3.98^{a}	58.06 ± 4.06^{a}
3	57.07 ± 3.5^{a}	67.21 ± 4.19^{a}
Exposure period (min)		
30	33.75 ± 4.65^{b}	36.24 ± 5.47^{b}
60	38.33 ± 5.23^{ab}	46.25 ± 6.42^{ab}
90	45.41 ± 6.13 ^{ab}	53.68 ± 7.62^{a}
120	56.66 ±7.62^{a}	59.52 ± 7.60^{a}

For ozone concentration and exposure period separately, means followed by different superscript letters are significantly different (P < 0.05) by Tukey's HSD test.

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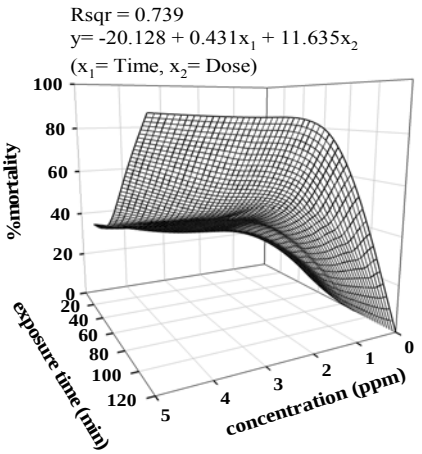

Figure 2 Effects of ozone concentration and exposure time on mortality (%) of *P. interpunctella* 5 days old larvae.

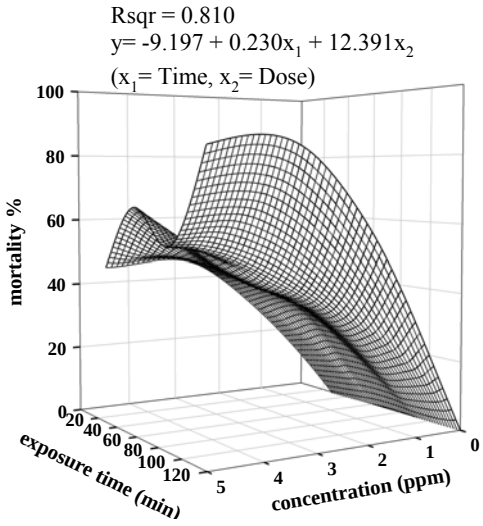

Figure 3 Effect of ozone concentration and exposure time on mortality (%) of *P. interpunctella* pupae.

Discussion

Ozonated water effectively killed all immature life stages of *P. interpunctella*, a serious pest of stored pistachio nuts and many other dried fruits. However, the effectiveness of aqueous ozone on mortality of this insect varied according to the life stage tested. Not surprisingly, ozone had less effect on *P. interpunctella* egg mortality. Results presented by others are in agreement with our findings. For example, Leesch (2003) who tested the toxicity of gaseous ozone to different stages of *P. interpunctella* and adults of *T. confusum*, reported that eggs of *P. interpunctella* were not killed 100% at high concentrations of ozone (10,000 ppm). Wood (2008) found eggs of the greater wax moth, *Galleria mellonella*, were more tolerant to ozone compared with larvae and adults. Also, Niakousari *et al*., (2010) reported that a concentration of 4,000 ppm of ozone for 2 hrs resulted only in 80% mortality of *P. interpunctella* eggs on date fruits. Similarly, Bonjour *et al*., (2011) found that ozone at a concentration of 70 ppm for a period of 4 days didn't have significant effect on eggs of *P. interpunctella*. Overall, these results show that insect eggs are more resistant to ozone than other immature stages and this can be explained by the fact that ozone has an initial problem in being able to penetrate through the egg shield.

Our data indicated that pupae are more tolerant than larvae. The present results support those of Kells *et al*., (2001) who found a higher mortality rate for larval stage compared with other stages of *P. interpunctella* exposed to 50 ppm ozone for 3 days, Leesch (2003) reported that, all life stages of *P. interpunctella* except the egg stage were more or less susceptible to laboratory treatment with ozone at 300 ppm for a 4-h exposure period. Also Isikber and Oztekin (2009) reported that pupae of *T. confusum* were more resistant to ozone than larvae. Similarly, James (2011) reported that pupae of *Galleria mellonella* were more resistant to ozone compared with larvae. In contrast to the present results, Bonjour *et al*., (2011) evaluating the efficacy of ozone fumigation against the major grain pests in stored wheat reported that pupae of *P. interpunctella* were more susceptible than eggs and larvae. This controversy may be due to the differences in experimental conditions.

Based on the present results and those of others, it seems that ozone toxicity for insects varies depending on the insect species, application method, its growth stage in the life cycle and method of ozone application. For example, larval and pupal stages of *T. castaneum* are ozone sensitive with sensitivity

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decreasing with age (Erdman, 1980). Also, Bonjour *et al*., (2011) showed that ozone treatment on pupae were more effective than on eggs and larvae of *P. interpunctella*. Isikber and Oztekin (2009) studied the mortality rate of *Ephestia kuehniella* and *T. confusum* and observed that insect mortality during ozonation was not only dependent upon the life stages for both of the species but also was insect specific. They observed a higher susceptibility and higher mortality for larvae, pupae and adult stages of *E. kuehniella* (90-100%) compared to *T. confusum* (1.3-22.7%) under similar experimental conditions. Also, Leesch (2003) reported a higher susceptibility rate for *P. interpunctella* compared to *T. confusum*.

Lethal concentrations and exposure times for ozone gas have been reported to range between 5 ppm for 5 days to 300 ppm for 18 hrs for insects living within commodities. The present study showed that much less concentration and time period (5 ppm for 120 min) can be effective for larval stage and pupae of *P. interpunctella*. The variation in effective level of ozone among different studies is considered to be acceptable considering the fact that majority of studies, evaluating the efficacy of ozone gas to control stored product insects, have used specimens of insects placed inside the grain masses or inside the kernels of grain or other products. In such cases, it is obvious that insects are, to some extent, protected from being exposed to ozone. So, much higher concentration and longer exposure time is needed to obtain a full control of treated insects. Moreover, the efficacy of ozone to control insect pests in deeper layers of commodities is less than that for insects that are on the surface of commodity. As shown by Hansen *et al*., (2012) full mortality of those stored product pests feeding within kernels needed 135 ppm of ozone for 8 days compared with 35 ppm for 6 days for freely exposed stages of main stored pests. Also, some differences in mortality rates of different immature stages of *P. interpunctella* might be due to the fact that physically larger larvae, pupae and eggs are more robust than younger larvae.

Overall, the present study showed that sensitiveness to ozonation depended on ozone concentration, exposure time, ozone application method and the life stage of the study insect. The results show that ozone, particularly at low concentrations, required much longer exposure times to be effective. This suggests the recommended concentration of ozone may need to be increased if 100% mortality is desired. This could be related to the unstable nature of ozone, its short half-life, its high reactivity, or its conversion to O_2 during the ozonation process.

These preliminary experiments showed that aqueous ozone has the potential to reduce the population of immature stages of *P. interpunctella*, and could be applied as a management tool during pistachio processing. However, before any recommendations can be made, the scale of the experiments should be expanded and taken from the laboratory to the field.

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ارزیابی حساسیت مراحل نابالغ شب‌پره هندی (*Plodia interpunctella* Hubner) به ازن محلول در آب

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چکیده: ازن یک اکسیدکننده قوی است که هم به‌صورت ازن گازی و هم به‌صورت ازن محلول در آب در صنایع غذایی مورد استفاده قرار می‌گیرد. در این مطالعه، تأثیر ازن به‌صورت ازن محلول در آب در ۴ غلظت (۰، ۲، ۳ و ۵ پی‌پی‌ام) و چهار زمان (۳۰، ۶۰، ۹۰ و ۱۲۰ دقیقه) بر مراحل نابالغ شب‌پره هندی، *Plodia interpunctella* Hubner آزمایش شد. نتایج نشان داد در همه‌ی مراحل زیستی مورد آزمایش با افزایش غلظت و زمان، درصد مرگ‌ومیر افزایش یافت. این مطالعه نشان داد، زمانی‌که نمونه‌ها در معرض غلظت ۵ پی‌پی‌ام و زمان ۱۲۰ دقیقه قرار گرفتند، لارو ۵ روزه نسبت به سایر مراحل زیستی مورد آزمایش حساس‌تر بود. به‌ترتیب لارو ۱۲ و ۱۷ روزه، شفیره و تخم، بعد از لارو ۵ روزه به ازن‌دهی حساس بودند. در بیشترین غلظت و طولانی‌ترین زمان مورد استفاده، تخم کمترین درصد مرگ‌ومیر را نشان داد. براساس نتایج، ازن محلول در آب پتانسیل کاهش جمعیت شب‌پره هندی یکی از مهم‌ترین آفات خشکبارهایی نظیر خرما، بادام و پسته را در انبار دارد.

واژگان کلیدی: آفات پس از برداشت، ازن محلول در آب، شب‌پره هندی

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