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Preserving Wheat Seed Quality: Low-Level Ozone Fumigation as a Strategy Against Khapra Beetle Damage

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ABSTRACT

This study evaluated the efficacy of ozone fumigation against different life stages of the khapra beetle, *Trogoderma granarium* (Coleoptera: Dermestidae), a critical stored-product pest, under both laboratory and simulated storage conditions. A laboratory colony was established from field-collected adults and reared on sterilized wheat at 30 ± 2°C, 65 ± 5% RH, and a 0:24 (L:D) h photoperiod. Synchronized third-instar larvae, fourth-instar larvae, and adults (<24 h old) were exposed to 50 ± 5 ppm ozone gas in a sealed 1000 L fumigation chamber at 38 ± 2°C and 49 ± 5% RH. A factorial completely randomized design (CRD) with life stage and exposure time (0, 6, 12, 18, 24, 48, 72 h) as factors was employed, including a positive control (aluminum phosphide at 3 g/tonne for 72 h). In laboratory bioassays, insects were directly exposed in gas-permeable mesh bags. For simulated storage trials, insect samples were embedded within 10 kg wheat bags inside the fumigation chamber. Mortality was assessed 14 days post-treatment. Results indicated a time-dependent mortality for all stages. Under direct exposure, adults were highly susceptible, reaching 100% mortality within 12 hours. Larval stages were more tolerant, requiring 36 hours to achieve 96.6% mortality. In simulated storage, the efficacy of ozone was attenuated due to reduced penetration, requiring 72 hours of exposure to achieve 100% mortality for both larvae and adults. At 72 hours, ozone efficacy was statistically equivalent to aluminum phosphide for adults and superior for larvae. Seed germination was not adversely affected by any treatment. However, biochemical analysis revealed that ozone treatment caused a significant reduction in seed fat content (from 0.36% to 0.10%) and an increase in soluble carbohydrates, attributable to oxidative processes. Protein content showed a relative increase, likely due to the reduction in fat mass. The study concludes that ozone fumigation at 50 ppm is a viable alternative to phosphine for controlling *T. granarium*, achieving complete mortality across life stages within 72 hours in a storage simulation without compromising seed viability. However, its potential to induce oxidative biochemical changes in grains necessitates further optimization for commercial application.

1- Introduction

Wheat (Triticum aestivum L.) is a staple crop that is critical for global food security, feeding more than one-third of the world's population [1]. However, maintaining the quality and quantity of wheat stocks during storage is faced with a serious challenge due to contamination by storage pests. Estimates indicate that between 10 and 40% of the total global cereal production is lost annually due to storage pest damage, posing a serious threat to the sustainability of the food chain [2]. Among storage pests, the Khapra beetle (*Trogoderma granarium* Everts) is known as one of the most destructive and resistant pests of stored grains, especially wheat [3]. This pest has become a global challenge and an important quarantine pest due to its unique characteristics, such as its ability to withstand starvation for years, hiding in crevices, and having a very hard larval shell that protects it from many insecticides [4].The damage caused by this pest is not limited to direct consumption of the grain; contamination with larvae shells and feces severely reduces the nutritional quality and marketability of the product and can in some cases cause sensitization [5].

For decades, the control of the Khapra beetle has relied mainly on the use of strong chemical fumigants such as methyl bromide and phosphine [6]. However, methyl bromide has been phased out worldwide under the Montreal Protocol due to its severe ozone depletion effects [7]. On the other hand, there have been alarming reports of resistance of Khapra beetle populations to phosphine worldwide, compromising the effectiveness of this last line of chemical defense [8]. In addition, there is growing concern about the residues of chemical pesticides in food and their negative effects on human health and the environment [9]. These challenges have highlighted the urgent need to develop and optimize efficient, safe, and environmentally friendly solutions for the management of this pest. In this regard, ozone (O_3) has attracted much attention as a non-chemical and "green" fumigant. Ozone is an unstable and powerful gaseous molecule that, due to its high oxidation potential, can directly affect the respiratory system of insects and cause their death [10]. After its action, the gas rapidly decomposes to ordinary oxygen (O_2) and leaves no toxic residue on the product, making it ideal for the food industry [11]. Previous studies have shown the effectiveness of ozone at high concentrations (often above 50 ppm) on a range of storage pests [12]. However, the use of high concentrations can be economically costly and, in some cases, there is a potential risk of oxidative stress and negative effects on the physiological quality of the seed, especially on its germination and vigor [13].

Therefore, there is a critical knowledge gap regarding the optimization of this technology: can effective control of the seed beetle be achieved within a reasonable time using low-level and more cost-effective ozone, while fully preserving the quality of the wheat seed? Most previous studies have focused on the maximum insecticidal efficacy of ozone and have not comprehensively investigated the simultaneous impact of low-dose methods on the seed beetle and vital properties of wheat seeds. The main objective of this study is to comprehensively evaluate the efficacy of fumigation with low ozone levels in controlling different life stages of the grain borer beetle and also to investigate its effect on germination and quality indices of wheat seeds. We hypothesize that treatment with low ozone concentrations, if the exposure time is extended, can achieve high mortality rates against the grain borer beetle without having a negative impact on germination and wheat seed health. This research will provide a practical and sustainable solution for integrated pest management (IPM) in grain storages.

2-Materials and Methods

2-1 Insect Rearing and Colony Establishment

A founding population of *T. granarium* (Coleoptera: Dermestidae) adults was collected from infested wheat stocks at the Mesopotamia General Company for Seed Production, Ministry of Agriculture, Iraq. Fifty male-female pairs were isolated and introduced into 1 L glass rearing jars. Each jar contained 200 g of sterilized soft white wheat (*T. granarium*), which had been frozen at -20°C for 72 hours to eliminate any contaminating fauna or flora. Jars were sealed with breathable cotton muslin cloth secured with rubber bands.

The insect colony was maintained in a controlled environment incubator (Model:507) at 30 ± 2°C and 65 ± 5% relative humidity, with a photoperiod of 0:24 (L:D) h. To maintain a continuous culture, new jars with fresh, sterilized wheat were inoculated with adults and larvae every two weeks. Species identification was confirmed based on morphological characteristics using established taxonomic keys [14], and a voucher specimen was deposited at the institutional collection.

2-2 Insect Stage Isolation and Sex Determination

To obtain synchronized age classes for bioassays, ten pairs of adults were introduced into plastic containers (500 g capacity) containing sterilized wheat. The containers were monitored daily for egg laying, after which adults were removed. The developing larvae were observed, and their instar was determined based on head capsule width and body size [15]. Third and fourth instar larvae, identified by their distinct size and setal characteristics, were collected for experiments.

For adult studies, pupae were isolated and held until eclosion. Newly emerged adults (< 24 h old) were briefly anesthetized by placement at -20°C for 60 seconds to facilitate handling. Sex differentiation was performed under a stereo-microscope based on established morphological dimorphism: females are typically larger (1.6-1.7 mm long) with 4-segmented antennae, while males are smaller (1.3-1.4 mm long) with 5-segmented antennae and generally darker, near-black coloration.

2-3 Experimental Design and Ozone Fumigation Setup

The study was conducted as a completely randomized design (CRD) with a factorial arrangement of treatments. The factors were insect life stage (adults, 3rd instar larvae, 4th instar larvae) and ozone exposure time (0, 6, 12, 18, 24, 72 h). A commercial phosphine fumigant (aluminum phosphide) treatment at the recommended dosage and a 72-hour exposure was included as a positive control. Each treatment combination was replicated three times .The experiment was conducted in a warehouse at the Mesopotamia General Company for Seeds, maintained at 38 ± 2°C and 49 ± 5% RH to simulate realistic storage conditions. The primary fumigation chamber consisted of a sealed, 1000 L polypropylene tank. Ozone was generated using an aluminum phosphide ozone generator and introduced into the chamber via chemical-resistant tubing. To ensure precise and reliable ozone concentration throughout the experiments, the ozone generator was calibrated prior to the study. A calibrated ozone analyzer (Model 49i, Thermo Scientific, Range: 0-100 ppm, Accuracy: ±2% of reading) was used for this purpose. The analyzer itself was calibrated according to the manufacturer's instructions using a certified zero-air source and a traceable ozone standard generator. During fumigation, the ozone concentration inside the chamber was continuously monitored and maintained at the target concentration of 50 ± 5 ppm by dynamically adjusting the output of the ozone generator based on real-time feedback from the analyzer.

2-4 Bioassay Procedure

Test insects (20 individuals per replicate) were placed inside small, gas-permeable mesh bags (9.5 x 6.5 cm) along with 5 g of uninfested, sterilized wheat seeds (cv. Aba 99). Three such bags—one for each life stage—were placed in the geometric center of a 10 kg polypropylene bag filled with wheat, mimicking the conditions within a stacked storage bag. Six of these 10 kg bags were then strategically positioned inside the 1000 L fumigation tank to represent a stacked configuration .Ozone fumigation was initiated, and bags were removed from the chamber at their respective exposure endpoints (6, 12, 18, 24, 48, 72 h). Control groups (0 h exposure) were handled identically but without ozone application. Following fumigation, the mesh bags were immediately transferred to clean plastic containers with fresh, untreated wheat and held in the incubator under standard rearing conditions. Mortality was assessed 14 days post-treatment. Insects were considered dead if they showed no movement upon prodding with a fine brush. For the positive control, the same procedure was followed using aluminum phosphide tablets at the manufacturer's recommended dosage (typically 3 g/tonne) in a separate, similarly sealed chamber for a 72-hour exposure.

2-5 Data Analysis

Mortality data were corrected for natural mortality in the control groups using Abbott's formula [17] where necessary. Data were subjected to a two-way analysis of variance (ANOVA) using SAS software (version 9.4, SAS Institute, 2012) to determine the main effects of life stage, exposure time, and their interaction. Where significant F-tests ($P \leq 0.05$) were found, means were separated using the Least Significant Difference (LSD) test. Results are presented as mean percentage mortality ± standard error.

3-Results and Discussion

3.1. Efficacy of Ozone Fumigation Under Laboratory Conditions

Direct exposure to 50 ppm ozone gas resulted in significant and time-dependent mortality in both larval and adult stages of *T.granarium* (Table 1). Adult insects exhibited remarkable susceptibility, achieving 100% mortality after just 12 hours of exposure. In contrast, larval stages demonstrated greater resilience, with mortality rates of 50.0%, 76.6%, and 96.6% recorded at 12, 24, and 36 hours, respectively. The control mortality remained low at 3.6%, confirming the observed effects were due to the ozone treatment.

Table 1. Effect of ozone at 50 ppm on the activity of larvae and adults of the Khapra beetle *T. granarium* under laboratory conditions

Exposure time hours	Larvae mortality %	Adult mortality%
12	50	100
24	76.6	100
36	96.6	100
CONTROL	3.6	3.6
L.S.D. ($P \leq 0.05$)	13.205	5.1635

The pronounced susceptibility of adults is likely attributable to their direct and unimpeded contact with the gaseous ozone, which acts as a powerful oxidizing agent. Ozone is known to degrade vital biomolecules, including unsaturated lipids in cell membranes and respiratory enzymes, leading to rapid physiological failure [17, 20]. The higher tolerance observed in larvae, particularly the 3rd and 4th instars used here, may be linked to their thicker cuticle and potentially different respiratory physiology, which could impede ozone penetration or mitigate oxidative damage [21]. These findings align with studies on other stored-product pests, such as Tribolium castaneum, where increased exposure times were necessary to achieve high mortality in larval stages [18].

3.2. Efficacy of Ozone Fumigation Under Simulated Storage Conditions

When evaluated in a more realistic scenario with insects embedded within 10 kg polypropylene bags of wheat, the efficacy of ozone was attenuated and required significantly longer exposure times, highlighting the impact of commodity penetration (Table 2). No significant larval mortality was observed within the first 18 hours. Mortality commenced at 24 hours (20.0%) and increased to 73.3% at 48 hours, reaching 100% only after a 72-hour exposure. A similar, though less pronounced, delay was observed for adults. Mortality began to manifest at 12 hours (10.0%), rising to 33.3% at 24 hours and 96.6% at 48 hours, before also reaching 100% at 72 hours.

Table2. Effect of Ozone on *T. granarium* larvae and adults in wheat bags under storage conditions

Exposure time (hours)	Larvae mortality %	Adult mortality%
O_3 6	0	0.5
12	0	10
18	0	10
24	20	33.3
48	73.3	96.6
72	100	100
CONTROL (72 h)	0.5	0.5
Aluminum phosphide (72 h)	86.6	96.6
L.S.D. ($P \leq 0.05$)	6.1196	6.1196

This time-lag effect is a critical consideration for practical application. The gradual increase in mortality underscores the time required for ozone to diffuse through the inter-kernel spaces and the grain mass to reach lethal concentrations at the center of the bag [22]. Despite this delay, ozone at 72 hours proved statistically equivalent to the conventional fumigant aluminum phosphide (96.6% mortality for adults) and was superior for larval control (100% vs. 86.6%). This demonstrates ozone's potential as an effective alternative, particularly against phosphine-resistant populations or in situations where residue-free treatment is paramount.

3.3. Impact of Fumigation on Seed Viability and Quality

A primary concern with any novel fumigation treatment is its impact on seed quality. As shown in Figure 1, neither ozone nor aluminum phosphide treatment significantly affected the germination capacity of wheat seeds. The germination percentage across all treatments, including the control, averaged 86.66%, with no statistically significant differences (L.S.D. = 11.535). This indicates that the applied dosages and exposure times are within a safe threshold for preserving seed viability.

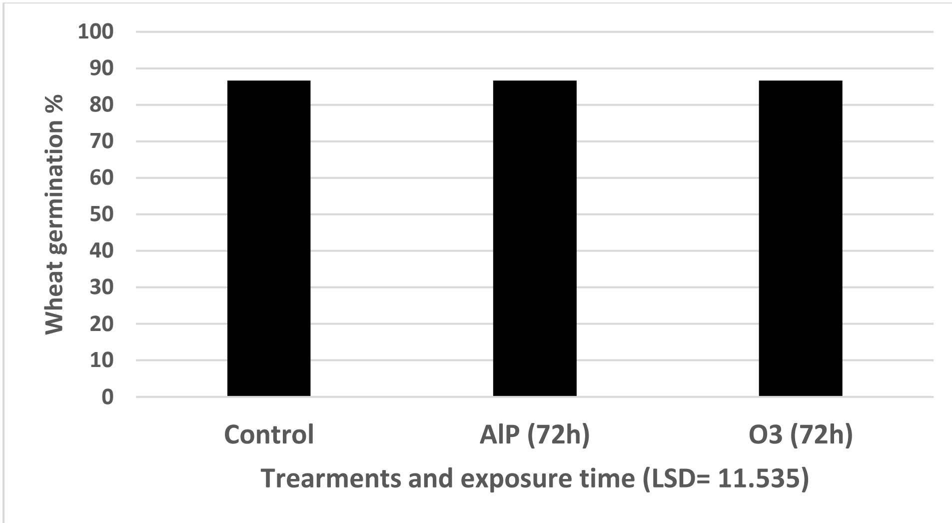

Figure1. Effect of the experimental treatments on wheat seed germination percentage

Effect of Treatments on Seed Nutrient Content: Carbohydrate and Protein Analysis

The soluble carbohydrate, protein, fat, and ash content in wheat seeds exposed to 50 ppm ozone for 72 hours, seeds exposed to the recommended concentration of aluminum phosphide for 72 hours, control seeds, and seeds from the previous season harvested in 2023 that had been treated multiple times with aluminum phosphide were analyzed to compare the results (Table3). The control, ozone, and phosphide treatments significantly outperformed the single-dose phosphide treatment in the ash analysis. In the fat analysis, the control treatment significantly outperformed the other treatments. Protein levels were significantly highest in the ozone treatment. Similarly, the soluble carbohydrate content of the seeds was highest in the ozone and phosphide treatments compared to the single-dose phosphide and control treatments. However, analysis of biochemical composition revealed more nuanced effects (Table 3). While ash content remained stable across treatments, a significant reduction in fat content was observed in ozone-treated seeds (0.10%) compared to the control (0.36%). This is a direct consequence of ozonation, a process known to oxidize unsaturated fatty acids, leading to rancidity [24]. Conversely, soluble carbohydrate content was significantly higher in ozone and multiple-phosphide treatments. This may result from the oxidative stress induced by the treatments, potentially stimulating the hydrolysis of complex starch molecules into simpler sugars [24].

Table3. Effect of treatments on seed nutrient content: Carbohydrate and Protein Analysis

Treatment	Ash %	Fat %	Protein%	Carbohydrates%
Control	2.06	0.36	14.38	9.187192
Aluminum phosphide	1.93	0.26	14.17	9.582049

O3	2.13	0.1	15.4	15.16755
Aluminum phosphide multiple	2.03	0.14	13.89	15.06645
L.S.D. ($P \leq 0.05$)	0.1438	0.0776	0.7867	2.2444

Interestingly, the protein content was significantly highest in the ozone-treated seeds (15.40%). This apparent increase is likely relative, caused by the reduction in fat content, thereby increasing the proportional percentage of other components like protein and carbohydrates on a dry-weight basis, rather than an actual synthesis of new protein. The biochemical changes, particularly lipid oxidation, highlight a trade-off. While germination is unaffected, prolonged ozonation could potentially impact the sensory and long-term storage quality of grain intended for human consumption due to rancidity. Therefore, optimizing the concentration-exposure time product to achieve pest control with minimal biochemical alteration is essential for future commercial application [19, 24].

4-Conclusion

This study confirms that low-level ozone fumigation (50 ppm) is a viable strategy for controlling the Khapra beetle, *T. granarium*, in stored wheat. Its efficacy is highly dependent on exposure time and the life stage of the insect, with adults being more susceptible than larvae. Under practical storage conditions, a 72-hour exposure was required to achieve complete mortality, performing comparably or superiorly to aluminum phosphide. Critically, this treatment protocol did not compromise seed germination. The main significant effect was on seed biochemistry, specifically the oxidation of fats. This underscores that ozone is a potent biocide with clear, measurable effects on biological systems beyond the target pest. Future research should focus on fine-tuning ozone application protocols (e.g., pulsed application) to minimize these biochemical changes while maintaining lethal efficacy, and to assess its impact on the functional properties of flour and end-products. Nonetheless, ozone fumigation presents a promising, residue-free alternative for integrated pest management in stored wheat, particularly in the face of increasing phosphine resistance.

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