The impact of Pressurized CO_2 and N_2 Gases on the Various Developmental Stages of *Ephestia cautella* and *Oryzaephilus surinamensis* Store Insects

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Abstract

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The purpose of this study was to assess the impact of pressurized CO_2 and N_2 on the various stages of two well-known insect species found in storage, namely, *Ephestia cautella* (Walker) and *Oryzaephilus surinamensis* (L.).The insects were subjected to three different levels of pressurized gas (2, 4 and 6 Kg/cm^2) in a metal gas cylinder over a period of two days at room temperature of around 30°C. The results obtained suggested that CO_2 was significantly more effective than N_2 gas. The egg stage of both insect species exhibited the greatest tolerance, whereas the adult stage displayed the highest sensitivity to both gases, regardless of pressure level. It was observed that insect mortality increased as the pressure level of gas increased, with the highest mortality rates recorded at the highest gas pressure (6 kg/cm^2). Additionally, certain biochemical parameters were analyzed in adults of *O. surinamensis* that were exposed to 6 kg/cm^2 of CO_2 pressure for 2 days.

Keywords: *Ephestia cautella, Oryzaephilus surinamensis,* pressurized gases, CO_2, N_2, biochemical parameters.

Introduction

Conventional control methods, such as the use of methyl bromide and phosphine gases, have been widely utilized to effectively and affordably control stored products pests. However, excessive use of these chemicals has raised environmental concerns. Methyl bromide, in particular, has been recognized as a chemical that depletes ozone, leading to its phasing out of production and use by 2015 (UNEPA, 1995). In recent decades, extensive research has been conducted to explore the efficacy of modified atmosphere with different gases to control various stored pests. Depending on the concentrations of gas and temperature, treatments utilizing modified atmospheres at atmospheric pressure may take anywhere from a few days to several weeks to be successful in controlling all the developmental stages of pests depending on gas concentrations and temperature (Banks & Annis, 1990; White *et al*., 1995). To enhance effectiveness, researchers proposed the use of high pressure rather than atmospheric pressure (Calderon & Barkai-Golan, 1990; Navarro, 2006). The effectiveness of using high-pressure CO_2 for pest control depends on several factors such as the type of pest, the insect's life stage, the pressure level, and the duration of exposure (Khalil *et al*., 2020; Locatelli *et al*., 1990; Reza *et al*., 2021). These methods provide a means to eradicate insects from stored commodities without causing pollution to the environment and are considered safer than traditional fumigants. Additionally, there are no harmful residues left behind after treating the commodity with either nitrogen (N_2) or oxygen (O_2). The use of nitrogen is environmentally friendly, free from residues and can be employed in food storage and processing without requiring registration, according to current regulations (Sakka *et al*., 2020; Timlick *et al*., 2002). Nitrogen application has long been recognized as a promising method for the elimination of pests in stored products (Adler *et al*., 2000; Athanassiou *et al*., 2017; Navarro, 2006). This study aimed to determine the effectiveness of pressurized CO_2 and N_2 compared with pressurized air, at 3 different pressure levels (2, 4 and 6 kg/cm^2) to control the various life stages of *Ephestia cautella* (Walker) and *Oryzaephilus surinamensis* (L.). Additionally, biochemical analyses were conducted on *O. surinamensis* adults exposed to pressurized CO_2 and air.

Materials and Methods

Rearing technique of *Ephestia cautella*

Adults of *E. cautella* insects were collected and 50 pairs were put into individual plastic containers, 77 mm in diameter and 146 mm high. Each container housed one pair and contained cotton swabs soaked with a solution of 10% sugar, as described earlier (Shoukry *et al*., 1978). The mouth of the container was wrapped with a plastic mesh (Bell & Bowleyc, 1980) using a rubber band and inverted on a loosely affixed lid. The following items were observed: Muslin was used to cover each glass cage both from the upper and lower sides, in order to allow the eggs to easily fall into a Petri dish, muslin with a selected diameter was employed to cover the bottom side of the cage, the eggs were gathered on a daily basis and placed in 500 ml glass jars for incubation, for larval feeding, each jar containing 25 mg of eggs was provided with 150 g of artificial diet, the artificial diet was prepared using 500 g of yeast, 250 ml of natural honey, and 250 g of milk powder. Mixed together and placed in a glass jar covered with tulle fabric using a rubber band, and placed in a dark place. The jars were covered with muslin and incubated at the optimal rearing conditions of 27±1°C and 65±5% RH in

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complete darkness until cocoon formation (Hashem *et al*., 2016). Target insects were reared for several generations before using them in the experiments.

Preparing the insect stages for exposure to pressurized gases

Eggs stage- The experiment involved placing batches of 30 of *E. cautella* eggs, each being 0-24 hours old, into small cloth bags that were filled with approximately 25 g of medium and sealed using rubber bands.

Larval stage- The fourth larval developmental stage, 1-2 days old, were obtained from the main breeding population. A delicate brush was utilized to collect them at a rate of 10 larvae per vial, and then provided with 25 g of synthetic food, the vials containing the larvae were covered and subsequently exposed to gas as shown in Figure 1.

Pupal stage- 1-2 days old pupae were obtained from the original culture using a delicate brush, and 10 pupae were placed per vial. To prepare for gas exposure, the cages were covered with a small muslin fabric.

Adult stage- Newly hatched adults, 0-1 day old, were collected from the breeding colonies and placed inside vacant wire mesh cages in preparation for gas exposure.

Rearing technique of *Oryzaephilus surinaminsis*

Stock culture- The insect cultures were bred using a diet as described by (Miller *et al*., 1969). The cultures were made by mixing 930 g of white flour, 930 g of white corn meal, and 140 g of brewer's yeast. The white flour cornmeal was individually sifted through a sieve with 20 mesh per inch to eliminate any insect stages, and then stored at -13ºC for at least one week to eliminate any remaining insects and mites. The components of the medium were mixed together and placed in sealed plastic bags. These bags were stored in the deep freezer until needed. One-kg glass jars were used to house the cultures. Approximately 300 grams of the aforementioned medium was added to each jar, filling them up to about one-third full. Corrugated paper strips were placed on the surface of the medium. Each rearing jar had 200 adult insects collected and placed in it. One week after egg-laying, the adult insects were removed and transferred to a fresh rearing jar for another week before being disposed of. The jars were covered with muslin cloth, secured with rubber bands and kept in an incubator at 27±2ºC and 65±5% relative humidity. Once the adults of the new generation started to appear, they were collected from the medium using a number 20 sieve and prepared in the same manner as previously mentioned.

Rearing of *O. surinamiensis*

Egg stage- For egg laying, white flour was used as a substrate and carefully sifted through a 52-mesh/inch sieve to obtain a finely ground consistency. Any leftover residues were separated. Under a stereo microscope (KOPPACE 3.5X-90X) (KOPPACE Technology "SHENZHEN" CO., LTD.), the eggs laid on the artificial diet were easily visible. Approximately 200 g of sifted white flour was portioned and placed in glass jars weighing 500 g each. Additionally, strips of corrugated paper were placed on the surface of the flour in each jar. Separate prepared jars received around 200 ten-day-old adult insects. After one day, the adults and their laid eggs were sifted out from the white flour using a no.52 sieve. The eggs were collected, counted and subsequently examined, while the adults were moved to new jars. Each batch of 30 eggs was gently transferred to gauze cages measuring 3×8 cm. These cages contained 25 g of thoroughly mixed rearing medium. These cages were utilized for subsequent experiments.

Larval and pupal stages- Approximately 1000 eggs were divided into two groups. Each group was placed in a 1 kg glass jar with around 500 g of thoroughly mixed rearing medium. The jars were then placed in an incubator set at a temperature of 27±2ºC and 65±5% RH. 20 days later, the hatched eggs in both jars were developed into larvae (15 days old). The larvae from one group were used for testing, while the larvae from the other group were kept until the 25th day when they developed into pupae (Finkelman *et al*., 2003). The pupae, which were between 0-1 day old pupae were used for testing. To conduct the experiment, 10 insects were prepared as replicates for both the larvae and pupae, following the same procedure as described for the eggs.

Adult stage- Recently hatched adult flies, around one week old, were divided into groups of 20 and moved away from the cultured population. They were then placed in designated vials containing an adequate amount of food.

Figure 1. Wire gauze cages used for insect exposure to gases.

Insect exposure to gases

A cylindrical metal chamber with a capacity of 37 liters (Figure 2) was utilized to expose insects to gases. The chamber had a large opening at the top, which was equipped with a metal disc (cover), a rubber gas kit and six bolts. In addition, there were two taps on both sides of the chamber, with one tap connected to the upper part of one side and the other tap connected to the lower part of the opposite side. The vials containing the insects (Figure 3) were placed inside the exposure chamber and the chamber was sealed securely. To conduct the experiment, three replicates of each tested stage of both insects were used. The upper part of the chamber was connected to the source of the tested gas (gas cylinder or air compressor) using a suitable hose and secured tightly. A manometer was installed at the top of the chamber to measure the pressure. Initially, the lower tap was left open

to remove air from inside the chamber and then it was closed while the gas flow continued inside until the desired pressure level was reached. Three pressure levels (2, 4 and 6 kg/cm^2) were examined in the experiment. The exposure period for all stages of both insect species was two days. Once the exposure period was completed, the chamber was opened carefully and gradually. After each treatment, eggs, larvae, pupae and adults were transferred individually to 250 ml plastic jars that contained standard diets covered with muslin. These jars were kept at 27±1ºC and 65±5% relative humidity until they were examined for mortality. Adult mortality was recorded 4-5 days after exposure. Larval mortality was observed for those larvae that had not metamorphosed into pupae 9 days after exposure, pupal mortality was counted for those pupae that had not emerged as adults after 9 days and egg hatch was recorded 7 days after treatment. The mortality data obtained from the treatments were adjusted using Abbott's formula (Abbott, 1925). Statistical analysis of the data obtained was conducted using the MSTAT statistical analysis package (MSTAT-C, 1991), following the methods described earlier (Gomez & Gomez, 1984). To test the differences between treatment means at a 5% level of probability, the least significant differences (LSD) method was applied, (Steel *et al*., 1997).

Figure 2. Metal chamber connected with gas cylinder.

Figure 3. Metal chamber with air compressor.

Biochemical analysis

The biochemical analysis of adults of *O. surrinamensis* that were exposed to a pressure of 6 kg/cm^2 were made. The insects were prepared as described earlier (Amin, 1998). They were homogenized in distilled water (50 mg/1 ml). Homogenates were centrifuged at 8000 rpm for 15 min at 2°C in a refrigerated centrifuge. The pellets were discarded and the supernatant was to as the enzyme extract, and were kept in a deep freezer at -20°C until use. Double beam ultraviolet/visible spectrophotometer (spectronic 1201, Milton Roy Co., USA) was used to measure absorbance of metabolic compounds. Carboxylesterase activity was measured according to the method described by (Simpson *et al*., 1964), using methyl n-butyrate (MeB) as substrate. Acid and alkaline phosphatases were determined according to the method described by (Powell & Smith, 1954). Alpha esterases (α- esterases) were determined according to (van Asperen, 1962) using α-naphthyl acetate as substrates. Phenoloxidase activity was determined according to a modification of (Ishaaya, 1971). Total proteins were determined using the method of (Bradford, 1976). Total carbohydrates were estimated in acid extract of a sample by the phenol-sulphuric acid reaction of (Dubios *et al*., 1956). Total carbohydrates were extracted and prepared for assay according to (Crompton & Birt, 1967).

Statistical analysis

Each experiment included 3-4 duplicates of insect homo-gen, and the outcomes of biochemical tests were obtained from three separate evaluations. The gathered data were examined through one-way analysis of variance (ANOVA) using statistical software (cohort software Berkeley). If the ANOVA statistics were deemed significant ($P < 0.01$), the means were compared using Duncan's multiple range test.

Results and Discussion

The mortality rates of eggs, larvae, pupae, and adult from both insect (*E. cautella* and *O. surinamensis*) species were tested by exposing them to three different levels of pressurized air, CO_2, and N_2 for two days at room temperature (averaged at 30°C). The results obtained Table 1 indicated that as the pressure level increased, the mortality of *E. cautella* was clearly increased. Among the four stages of the insect, the adult stage exhibited the highest mortality rate compared to the other stages. Both the egg and pupal stages of *E. cautella* showed considerable tolerance to all pressurized gases, with no significant difference between them. When compared to the other stages, the larval stage of *E. cautella* displayed moderate sensitivity to CO_2 and N_2. The average mortality rates of the insect stages, regardless of the gas type and pressure level, were 39.50, 48.85, 42.93, and 59.59% for eggs, larvae, pupae, and adults, respectively. Results also indicate that N_2 was less effective against all stages of *E. cautella* as compared to CO_2. The overall mortality means caused by both gases and the control were 71.25, 66.01, and 5.90% for CO_2, N_2, and the control, respectively. Additionally, the results obtained revealed that the increase in pressure level significantly impacted the mortality rate of all stages of the tested insect. The mortality rates of the insect stages progressively and significantly

increased with higher pressure levels, reaching 40.88, 46.75, and 63.04% for pressure levels of 2, 4, and 6 kg/cm^2, respectively. The Lp_{50} of pressurized CO_2 for the adult stage of *E. cautella* was the lowest at 1.4 Kg/cm^2 compared to other insect stages (Figure 4-A). In contrast, the Lp_{50} for eggs was the highest at 2.7 kg/cm^2. Additionally, the Lp_{50} values of pressurized N_2 for the different stages of *E. cautella* (Figure 4-B) corresponded with the results presented in Table1, indicating that the egg stage was the most tolerant to pressurized N_2 at 3.1 kg/cm^2. Conversely, the adult stage was the most sensitive to pressurized N_2, with an Lp_{50} of only 1.5 kg/cm^2.

O. surinamensis generally showed less sensitivity to the tested pressurized gases to *E. cautella*.The mortality rate of *O. surinamensis* were lower, with 69.9% for CO_2 and 60.25% for N_2 Table 1. The sensitivity of eggs and larvae of *O. surinamensis* to the tested gases was relatively similar to that of *E. cautella*. However, the pupal and adult stages of *O. surinamensis* were less sensitive to the tested gases compared to *E. cautella* Tables 1 and 2. It was observed that the mortality rate of all stages of the insect followed the same trend as mentioned above for *E. cautella* As the pressure increased, the mortality rate increased, reaching the highest value for the highest pressure level. In most cases, the adult stages had the highest mortality rate for both gases at any pressure level (Table 1). According to the Lp_{50} values of pressurized gases applied to different stages of *o. surrinamensis* (Figure 5). It was evident that the egg stage exhibited the highest tolerance with the highest Lp_{50} value of 3.0 kg/cm^2 for CO_2 compared to other stages. The egg stage also showed a relatively high tolerance level of 3.0 kg/cm^2 to pressurized N_2, and higher than that of the larval (2.1 kg/cm^2) and pupal (2.1 kg/cm^2) stages. In contrast, the adult stage exhibited the lowest tolerance to pressurized N_2 (2.0 kg/cm^2), and the Lp_{50} value did not fall on the regression line.

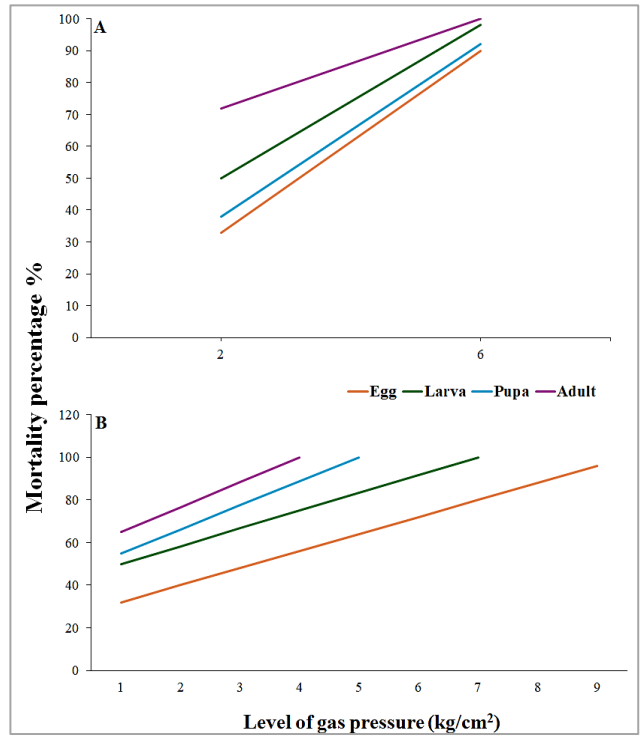

Figure 4. Toxicity lines for pressurized CO_2 (A) and N_2 (B) level-mortality Regression lines tested against the different stages of *E. cautella* for two days under room temperature.

Table 1. The average mortality percentages of *Ephestia cautella* (EC) and *Oryzaephilus surinamensis* (OS) under three different gas conditions over a period of two days.

Insect stages	Gases	Level of gas pressure (kg/cm^2) 2 EC	2 OS	4 EC	4 OS	6 EC	6 OS	Mortality rate means EC	Mortality rate means OS	General mortality means of stages EC	General mortality means of stages OS
Eggs	Control	1.11	1.11	2.20	2.20	3.30	3.30	2.20	2.20	39.50	38.13
	CO_2	39.99	29.99	53.44	52.22	93.33	93.33	62.25	58.51		
	N_2	36.66	34.44	52.22	49.99	73.33	76.66	54.07	53.69		
Larvae	Control	6.60	3.30	10.00	10.00	13.33	13.33	9.90	8.87	48.85	46.69
	CO_2	50.00	46.66	66.66	80.00	96.66	90.00	71.10	72.22		
	N_2	46.66	43.33	63.33	57.00	86.66	76.66	65.55	58.99		
Pupae	Control	3.30	3.30	6.60	6.60	10.00	10.00	6.60	6.63	42.93	30.72
	CO_2	40.00	40.00	56.66	70.00	93.33	93.33	63.33	67.77		
	N_2	43.33	36.66	53.33	50.00	80.00	73.33	58.88	17.77		
Adult	Control	3.30	1.60	5.00	3.30	6.60	5.00	4.90	3.32	59.59	53.14
	CO_2	68.33	60.00	96.66	83.33	100.00	100.00	88.33	81.11		
	N_2	61.66	51.66	95.00	80.00	100.00	93.33	85.55	74.99		
General mortality means of gas pressure		40.88	29.33	46.75	45.38	63.04	60.68				

$LSD_{0.05}$ for *Ephestia cautella* (*EC*) insect stages = 4.14%, for pressure =3.58%, for stages × gases= 6%, for stages pressure = 6%, for gases × pressure = 5.18%, and for stages × gases×pressure = 6%.

$LSD_{0.05}$ for *Oryzaephilus surinamensis* (*OS*) insect stages =4.38%, for pressure =3.79%, for gases = 3.79%, for insect stages × gases=6.33%, for insect stages × pressure =6.33%, for gases × pressure =5.48%, and for stages × gases× pressure =6%.

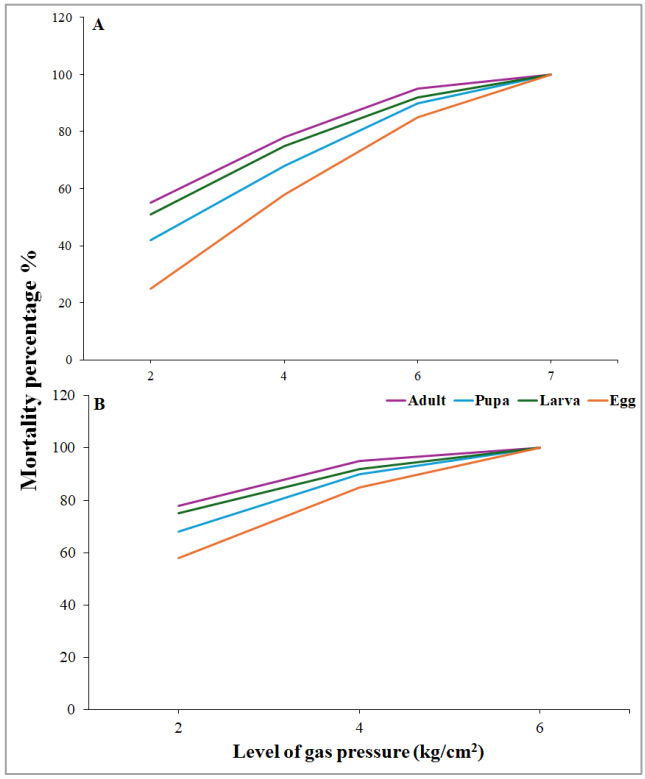

Figure 5. Toxicity lines for pressurized CO_2 (A) and N_2 (B) level-mortality Regression lines tested against the different stages of *O. surinamensis* for two days under room temperature.

Pressurized CO_2 produces an extraordinary impact by combining three effects: dissolving carbon dioxide in insects' body fluids, causing excessive acidity in both cell fluid and hemoglobin. Another effect is the deprivation of oxygen, and a crucial factor is the pressure effect. The combination of CO_2 and pressure can effectively yield results within a brief exposure time. For example, in the case of the dried fruit moth, the duration of treatment time was observed to increase significantly (Dumas *et al*., 1969). Numerous studies have demonstrated that vacuum fumigation combined with CO_2 can enhance the toxicity of fumigants, particularly substances like MB and phosphine (Calderon & Leesch,1983; Donahaye & Navarro, 1989; Dumas *et al*., 1969; Monro *et al*., 1966; Williams,1985). The different effects of CO_2 and N_2 treatment on insects can be explained by the varying solubility of these gases in insect haemolymph. Additionally, CO_2 is known to impede respiration, possibly due to reduced oxygen levels and increased CO_2 levels. The lethal impact of CO_2 is attributed to its high solubility in insect body fluids under high pressure, leading to a decrease in ph. It has also been observed that the rapid uptake of CO_2 under high pressure causes expansion, and causes evaporation when the pressure is lowered, resulting in damage to the insect cell membrane (Oliver & Herbert, 2018). Treated insects experienced severe damage to their integument due to the internal expansion of dissolved CO_2 when the gas pressure quickly returned to atmospheric pressure. According to a previous study (Riudavets *et al*., 2010), the exposure time needed to achieve 100% control was significantly shorter at higher CO_2 pressure, compared to atmospheric pressure, as the effectiveness of CO_2 depends on how quickly it dissolves under pressure of CO_2 in the atmosphere.

Biochemical studies

Results presented in Table 2 show the effect of exposure to 6 kg/cm^2 of CO_2 gas for 2 days on the biochemical parameters of *O. surinamensis* adults. The results indicated that the level of alkaline phosphatase and carboxyl esterases were significantly higher in the treated insects compared to the control group. There was a slight influence on total carbohydrates and peroxidase, but no significant differences were observed. The treated insects had lower levels of alpha esterases and phenol oxidase compared to the control group, with significant differences between them. Total proteins were also lower in the treated insects, with a significant difference from the control group Table 3. Findings are consistent with previous studies (Crompton & Birt, 1967; Ghoneim *et al*., 2014; Qari *et al*., 2017; Mohamed *et al*., 2018). Additionally, proteins may act as antioxidants and protect against damage caused by aerobic metabolism and dietary pro-oxidants, which can harm proteins, lipids, and other crucial metabolites. The exact way in which carbon dioxide becomes toxic under high pressure is not yet completely understood, however, it could be related to an increase in respiration and its dissolution in the fluids of the intestines (Stahl *et al.,* 1985), as well as causing damage to cell membranes during sudden decompression. **(**Wei *et al*., 1998) provided two main reasons behind the killing effect of pressurized CO_2 on insects. Firstly, it causes significant damage to the insects' body walls, resulting in their death in large numbers. Secondly, the combination of high pressure and amount of CO_2 enables a swift penetration, leading to toxic effects and rapid death.

Table 2. Changes in some biochemical content in *O. surrinamensis* adults treated for 2 days under 6 kg/cm^2 of pressurized CO_2.

	Biochemicals content						
Parameters	**Alkaline phosphatase (mg/g)**	**Alpha esterases (mg/g)**	**Carboxyl esterases (mg/g)**	**Total proteins (mg/g)**	**Total carbohydrates (mg/g)**	**Phenol oxidase (Δ O.D.units /min/g)**	**Peroxidase (Δ O.D.units /min/g)**
Control	258.33	1286.00	45.17	34.73	47.80	12.73	15.97
Treated	486.33	1004.33	69.40	31.43	45.77	7.67	16.17
$LSD_{0.01}$	57.76	126.97	6.34	1.58	-	1.38	-

الملخص

أمين، منار يوسف، إيمان لطفي عياد ومحمد محمد أبراهيم عامر. 2025. تأثير غازي ثاني أكسيد الكربون والنيتروجين المضغوطين على الأطوار المختلفة لحشرتي فراشة المخازن الاستوائية (دودة البلح العامري) وخنفساء السورينام. مجلة وقاية النبات العربية، 43(4):480–487.

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هدفت هذه الدراسة إلى تقدير تأثير غازي ثاني أكسيد الكربون والنيتروجين المضغوطين على الأطوار المختلفة لنوعين معروفين من حشرات المواد المخزونة، وهما: فراشة المخازن الاستوائية (دودة البلح العامري) (*Ephestia cautella*) وخنفساء السورينام (*Oryzaephilus surinamensis*). تمّ تعريض الأطوار المختلفة لنوعي الحشرات المدروسة للغازين المضغوطين عند ثلاثة مستويات من الضغط (2، 4 و 6 كغ/سم^2) وذلك في أسطوانة معدنية لمدة يومين متتالين عند حرارة الغرفة (متوسط 30°س). أوضحت النتائج أن غاز ثاني أكسيد الكربون المضغوط كان أكثر فعالية من غاز النيتروجين. كان طور البيضة لنوعي الحشرات كلتيهما أكثر تحملاً من باقي الأطوار، بينما كان طور الحشرة الكاملة لكليهما أيضاً أكثر الأطوار حساسية لكلا الغازين، بصرف النظر عن مستوى الضغط. لوحظ أيضاً أنه كلما ازداد مستوى الضغط كلما زادت نسبة القتل لكلا النوعين الحشريين. بالإضافة إلى ما سبق، تمّت دراسة بعض التأثيرات البيوكيميائية لغاز ثاني أكسيد الكربون المضغوط عند أعلى مستوى من الضغط (6 كغ/سم^2) على الحشرات الكاملة لخنفساء السورينام.

كلمات مفتاحية: فراشة المخازن الاستوائية، خنفساء السورينام، غازات مضغوطة، ثاني أكسيد الكربون، النيتروجين، تحليلات بيوكيميائية.

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