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Bio-Residual Effects of Certain Compounds on the Cowpea Beetle under Store Conditions

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Abstract

The cowpea beetles cause severe damage to legume seeds in storage. One of the most common control methods is mixing seeds with synthetic or natural agents to reduce heavy losses. This study was conducted to assess the residual toxicity of the evaluated compounds over a three-month storage period and to investigate the enzymatic activities of certain chemical agents in comparison with the organophosphorus insecticide malathion 1% dust against *Callosobruchus maculatus*. The results show that the mortality percentages decreased with increasing periods of exposure. Additionally,*C. maculatus* was more sensitive to kaolin and malathion than the other tested compounds. Based on the DT_{50} values, the tested compounds could be ranked as follows: Talc powder > thiamethoxam > lemongrass > *B. bassiana* > *M. anisopliae*. The highest persistent compound after kaolin and malathion was thiamethoxam, and the highest degradable compound was *M. anisopliae* after hexaflumuron and indoxacarb. The seed damage (%) recorded in all seed treatments was lower than that of the control during storage. Lemongrass EO achieved the highest protection after 3 months of storage and tested insecticides (thiamethoxam, malathion, hexaflumuron and indoxacarb). None of the treatments, including the control, had a significant effect on the germination percentages of treated cowpeas, except for lemongrass essential oil (EO), which significantly reduced germination. According to the findings, lemongrass EO significantly increased carboxylesterase (CarEs) and acetylcholinesterase (AChE) activity compared to other treatments. Lemongrass oil shows promise as a seed protectant; however, its dosage must be carefully controlled, as high doses may negatively affect seed germination and enzyme activity.

Keywords: Bio-Residual, Cowpea Beetle, Store Conditions.

INTRODUCTION

Infestation of stored legumes by bruchid insects leads to significant damage, including loss of seed weight, deterioration in seed quality, and reduced germination rates (Aly *et al*., 2005). Although synthetic insecticides and fumigants are commonly used to manage bruchid populations, their application has contributed to developing insect resistance, environmental contamination, and negative impacts on mammals and beneficial insects (Phillips and Throne, 2010). Consequently, alternative strategies have been developed and successfully employed, including the use of natural products and their derivatives, modified atmosphere storage, and bioinsecticides (Tiroesele *et al.,* 2014; Abdelgaleil *et al*., 2021; Gad *et al*., 2022, 2023). Successful crop production relies on the availability of high-quality seeds. However, seed quality may decline during storage due to various factors, among which infestation by storage pests is particularly significant. The larvae of the cowpea beetle (*Callosobruchus maculatus*) feed on legume seeds, often causing up to 100% loss of stored cowpea. To mitigate such substantial storage losses, a range of control strategies has been developed, with ongoing efforts to improve and expand these methods. The application of plant-derived products and certain synthetic insecticides has been recognized as an environmentally friendly and safer approach (Saber *et al*., 2017). The percentage of seed germination and esterase activities of the tested insects are bio-indicators of insecticide residues. Certain seed treatment residues, as protectant agents, affect the quality of seed, such as germination (Adetumbi *et al.,* 2011). Moreover, these residues may change the enzyme activity of tested insects (El-Sayed *et al.,* 2015). This study aimed to examine the effect of long-term storage on seed germination and seed weight loss for 3 months, plus the enzyme activity of the insect exposed to sublethal concentrations of the tested compounds under storage conditions.

MATERIALS AND METHODS

1- Insect culture

The cowpea beetle, *C. maculatus* was reared according to Suleiman *et al.* (2014)

2- Residual activity of tested materials at tested storage periods

For the tested pesticides (thiamethoxam, indoxacarb and hexaflumuron), LC_{50} values were determined. Subsequently, 500 gm of cowpea seeds were dipped in the LC_{50} dilution and placed in a 2 kg glass jar under storage conditions. Five grams of the tested compound powder (*B. bassiana, M. anisopliae,* talc powder, kaolin and malathion dust) were mixed with 500 grams of cowpea seeds and stored in a glass jar. According to Ilesanmi and Gungula, (2010), 3.75 ml of lemongrass oil was mixed with 500 grams of cowpea seeds. Three seed samples (10 grams each) were collected at different intervals ranging from 1 week to 12 weeks. Subsequently, ten unsexed adults of *C. maculatus* were introduced to the treated seeds placed in Petri dishes and exposed for three days. The treated seeds were then stored for three months (Saber *et al*., 2017). Mortality percentages were recorded from the first week until the 12th week of exposure to cowpea treated with different concentrations of pesticides.

3- Seed germination

Twenty-five treated cowpea seeds from each previously mentioned treatment were placed in Petri dishes. Each dish was lined with two layers: a cotton layer followed by filter paper, both moistened with water. The seeds were then arranged on the filter paper. After three days, the number of germinated seeds was recorded. Similarly, three replicates of untreated cowpea seeds were used as controls. The germination percentage and the percentage reduction in germination were calculated. All treatments, including untreated control, were repeated three times at monthly intervals (Saber *et al*., 2017). The Germination Index (GI) was calculated based on germination data following the method described by Olisa *et al*. (2010) as follows:

$$\%G = \frac{\text{No of emerged seedlings at the final count}}{\text{Total number of seeds planted}} \times 100$$

4- Adult emergence % and weight loss

This experiment was carried out over a three-month storage period, during which seed weight loss was recorded monthly. The procedure involved introducing ten pairs of *Callosobruchus maculatus* (0–48 hours old) into glass jars containing 300 grams of healthy cowpea seeds. Each treatment was replicated three times. The adult females were allowed to remain in the jars until natural death to ensure complete oviposition. Following egg-laying, the dead adults were removed. The hatched eggs were allowed to develop until adult emergence, which was recorded. The percentage reduction in adult emergence was then calculated according to Abbott's formula (Abbott, 1925).

$$\text{Reduction \%} = \frac{\text{Number of adults in control - Number of adults in treated}}{\text{Number of adults in control}} \times 100$$

Seed weight loss was assessed from the time of insect introduction until the emergence of adults. The calculation of seed weight loss was performed using the equation described by Mebarkia *et al.* (2010).

$$\text{\% weight loss} = \frac{\text{Initial weight of grain - Final weight of grains}}{\text{Initial weight of grains}} \times 100$$

5- Measurement of esterase activity

Enzyme preparation: Ten cowpea beetle adults from each treatment were homogenized in sodium phosphate buffer (0.1 M, pH. 7.4) using a glass homogenizer at a concentration of 0.02 mg homogenized cowpea beetle /1ml extract solution. Homogenates were centrifuged at 5000 rpm for 20 minutes at room temperature. The supernatant was used for a general esterase assay and for determining its protein content.

α - naphthyl acetate (α - NA)

Esterase activity: The activity of esterase towards α-naphthyl acetate was determined calorimetrically according to the method of Bracha and Bonard (1966) Absorbance at 500 nm was recorded exactly 20 min after the addition of potassium ferricyanide using JINGHUA Model 752 UV-Vis Spectrophotometer. A control tube containing everything except the enzyme solution was used to correct for the non-enzymatic hydrolysis of the substrate. In all assays, the activity was the mean of 3 replicates.

P - Nitrophenyl Acetate (P-NPA)

Esterase activity: The activity of esterase toward 4- nitrophenyl acetate as substrate (2×10^{-4} M p - NPA) was determined calorimetrically using JINGHUA Model 752 UV-Vis spectrophotometer as follows: To the colorimeter tube, 3.9 ml sodium phosphate buffer (0.1 M, pH 7.4) followed by 1 ml of enzyme solution were added and the tube was kept in the water bath at 30°C. After 5 min for equilibrium, 0.1 ml of 4-NPA dissolved in acetone was added and the reaction was incubated for 20 min at the same temperature. The p-nitrophenol released at the end of the incubation period was measured calorimetrically at 405 nm. A control tube containing everything except the enzyme solution was used to correct the non-enzymatic hydrolysis of the substrate. In all assays, the enzyme activities were taken as the average of 3 replicates.

Acetylcholinesterase (AChE) assay

AChE activity: The enzymes were prepared as mentioned above, and enzyme activity was measured spectrophotometrically according to the method of Ellman *et al*. (1961) using a JINGHUA Model 752 UV-Vis spectrophotometer by monitoring changes in absorbance at 412 nm. In a test tube, 3.8 ml of sodium phosphate buffer (0.1 M, pH 7.4), 1 ml of enzyme solution and 0.1 ml of DTNB in distilled water were added. After reaching equilibrium, 0.1 ml of acetylthiocholine iodide (2×10^{-4} M of ATChI) as a substrate was prepared in distilled water. The reaction mixture was allowed to react at 30°C for 20 minutes. A complete assay mixture without enzyme served as a control. In all essays, enzyme activities were taken as the means of 3 replicates.

Determination of protein

The diluted homogenate was used to determine the protein content using a biuret solution according to the method of Gornall *et al*. (1949). The biuret solution was prepared by dissolving 1.5 g of copper sulfate and 6.0 mg of sodium potassium tartrate in 500 ml of distilled

water. To this solution, 300 ml of 10 % sodium hydroxide was slowly added, and then the solution was diluted to 1 liter.

Protein measurement

To 1 ml of the homogenate solution, 4 ml of the biuret reagent was added, and the resulting solution was kept for 24 hours at room temperature. Then the absorbance at 540 nm was read against a blank containing 4 ml of biuret solution and 1 ml of distilled water. Three replicates were compared against a standard solution of bovine serum albumin (BSA) as a standard protein.

RESULTS AND DISCUSSION

1. Residual activity of the tested compounds against the cowpea beetles

The bio-residual activity of thiamethoxam, indoxacarb, and hexaflumuron on germination and insect enzymes was studied under laboratory conditions. Data in Table 1 show the efficacy of the tested compounds on the cowpea beetles for 12 weeks post-treatment. Kaolin, as a natural agent and malathion, an OP synthetic compound caused 100 % mortality over the tested period. Otherwise, indoxacarb and hexaflumuron had the lowest mortality effects from 0.0 to 10%. The remaining tested compounds caused high mortality during the initial weeks of storage, after which mortality rates gradually declined. Based on the DT_{50} values, the persistence of the tested compounds ranked in descending order as follows: talc powder > thiamethoxam > lemongrass > *Beauveria bassiana* > *Metarhizium anisopliae*. Following kaolin and malathion, thiamethoxam was the most persistent compound, while *M. anisopliae* was the most rapidly degradable compound after hexaflumuron and indoxacarb (Table 2). From the present results, kaolin could be promising as a candidate for malathion as a seed protectant after 3 days. These findings are consistent with those reported in previous studies. Traditionally, powders such as clay, sand, soil, and wood ash have been employed as control measures by applying a thick layer of dust over the surface of stored grain and mixing it thoroughly with the bulk. These materials fill the intergranular spaces, thereby restricting the movement and spread of insects within the stored grain mass (Inge, 2004).

Table 1. Mortality percentage (Mean ± SE) of cowpea beetles, C. maculatus adults exposed to treated cowpea seeds at storage periods (weeks) of the tested compounds.

Time / Compounds	%Mortality (Mean ± SE)											
	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	12 weeks
Thiamethoxam	73.33± 8.82	53.33± 3.33	53.33± 3.33	53.33± 3.33	53.33± 3.33	53.33± 3.33	46.67± 6.67	43.33± 3.33	43.33± 3.33	43.33± 3.33	40±0.0 0	36.67± 3.33
Indoxacarb	6.67±3 .33	6.67±3. 33	6.67±3 .33	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00
Hexaflumuron	0.00±0 .00	0.00±0. 00	0.00±0 .00	0.00±0 .00	0.00±0 .00	0.00±0 .00	0.00±0 .00	0.00±0 .00	0.00±0 .00	0.00±0 .00	0.00±0 .00	0.00±0 .00
B. bassiana	53.33± 3.33	53.33± 3.33	53.33± 3.33	53.33± 3.33	53.33± 3.33	53.33± 3.33	33.33± 3.33	30.00± 0.00	30.00± 0.00	30.00± 0.00	30.00± 0.00	23.33± 3.33
M. anisopliae	46.67± 6.67	40.00± 5.77	40.00± 5.77	40.00± 5.77	40.00± 5.77	40.00± 5.77	20.00± 0.00	16.67 ± 3.33	16.67 ± 3.33	16.67 ± 3.33	16.67 ±3.33	10.00 ±0.00
Talc powder	86.67± 3.33	80.00± 5.77	80.00± 5.77	80.00± 5.77	80.00± 5.77	80.00± 5.77	50.00± 0.00	40.00± 0.00	40.00± 0.00	36.67± 3.33	33.33± 3.33	30.00± 0.00
Kaolin	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	90.00± 10.00	90.00± 10.00
Malathion dust	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00
Lemongrass oil	90.00± 0.00	70.00± 0.00	63.33± 3.33	63.33± 3.33	63.33± 3.33	63.33± 3.33	36.67± 3.33	26.67± 3.33	26.67± 3.33	26.67± 3.33	23.33± 3.33	20.00± 0.00

Table 2. DT_{50} Values (Mean ± SE) of the tested compounds on cowpea beetles, *C. maculatus* adults at storage

Toxicity / Compounds	DT_{50} (week) (C. Ls 95%)	Slope ± SE	x^2	P-Value
B. bassiana	2.77 (1.08-4.19)	0.79±0.12	27.7	0.002
M. anisopliae	1.37 (0.39-2.28)	1.01±0.12	31.35	0.001
Talc powder	7.76 (5.91-11.48)	1.80±0.14	61.09	0.000
Thiamethoxam	5.07 (3.93-6.47)	0.7±0.12	5.96	0.818
Lemongrass oil	5.05 (4.11-6.09)	1.922±0.14	32.83	0.000

2- Effects of the tested compounds on the seed germination, weight loss of treated cowpea seeds and reduction of adult emergence of cowpea beetles

In the second experiment, the effects of treated seeds on the germination percentage, seed weight loss, and percentage reduction of adult emergence for 3 months of storage, the insect damage (%) recorded in all seed treatments was significantly lower than the control, except for lemongrass during storage. Regarding seed loss after three months of storage, lemongrass provided complete protection. For the remaining tested compounds, seed weight losses varied depending on the compound and the storage duration. According to Inge (2004), oil may also be effective in killing insect eggs. When eggs are present on the seed surface or within the seed, the oil coating can inhibit gas exchange. As a result, larvae inside the eggs or seeds may die due to oxygen deprivation. Lemongrass, thiamethoxam, indoxacarb, and hexaflumuron could equal malathion in reduction % of cowpea adult emergence through 3 months. Pyriproxyfen is an insect growth regulator that functions as a juvenile hormone (JH) analog. These JH analogues interfere with the development and metamorphosis of immature insects. Larvae at the late developmental stages are particularly susceptible to the effects of JH analogues (Alejandro *et al*., 2020).

Table 3. Germination percentage and weight loss of treated cowpea seeds and reduction of adult emergence (Mean ± SE) at different storage periods.

Time / Compounds	% Germination (Mean ± SE)			% Weight loss (Mean ± SE)			%Reduction of adult emergence (Mean ± SE)		
	1 month	2 month	3 month	1 month	2 month	3 month	1 month	2 month	3 month
Control	96±2.31	100±0	100±0	7.52	34.32	51.13	-	-	-
Thiamethoxam	88±2.31	98.67±1.33	100±0	0	0.1	0.67	100	99.64	99.06
Indoxacarb	93.33±1.33	96±2.31	98.67±1.33	0.33	2	3	96.01	96.96	96.56
Hexaflumuron	92±2.31	96±2.31	97.33±1.33	1.67	2	3.33	89.36	96.79	95.63
B. bassiana	96±4	97.33±2.67	98.67±1.33	4.77	26.33	29.77	28.86	46.43	40.63
M. anisopliae	93.33±4.81	96±2.31	97.33±2.67	0	33.59	39.85	53.06	10.71	12.5
Talc powder	97.33±1.33	98.67±1.33	100±0	1.67	26.44	30.32	60.11	40.71	42.19
Kaolin	93.33±1.33	96±0	98.67±1.33	0	11.23	16.43	77.39	67.86	62.5
Malathion dust	82.67±3.53	96±2.31	97.33±1.33	3.82	4.84	6.82	96.41	92.86	89.69

Lemongrass oil	16±2.31*	21.33±3.53 *	41.33±3.53 *	0	0	0	100	99.89	99.69

Acetylcholinesterase (AChE) is a key component of cell-to-cell signaling in the nervous system, where it breaks down the neurotransmitter acetylcholine to terminate nerve impulses. Inhibition of this enzyme disrupts the normal signaling process. Although not yet definitively proven, octopaminergic receptors, γ-aminobutyric acid (GABA) receptors, and AChE have been proposed as potential target sites for certain essential oil constituents (Kostyukovsky *et al.,* 2002; Priestley *et al*., 2003; Yeom *et al.,* 2012). Data in Table 4 show the general esterases (CaEs) and AChE activity in treated cowpea adults after 11 weeks of storage. In most treatments, high enzyme activity was observed, with no significant differences between carboxylesterase (CaEs) and acetylcholinesterase (AChE) activities, except in the lemongrass essential oil (EO) treatment compared to the control. Insects treated with lemongrass EO exhibited elevated CaEs activity when using α-naphthyl acetate (α-NA) and p-nitrophenyl octanoate (p-NO_2) as substrates, recording 46.05 and 29.01 nmoles of substrate hydrolyzed/mg protein/min, respectively, compared to 12.41 and 12.76 nmoles in the control. A similar significant increase was observed in AChE activity in insects treated with oil (26.2) compared to untreated insects (13.07). Lemongrass essential oil (EO) significantly influenced the activities of acetylcholinesterase (AChE), glycogen, and lactate dehydrogenase. The variation in AChE activity was predominantly attributed to the duration of exposure. Notably, the activities of AChE and aspartate aminotransferase increased significantly after 18 hours of inhalation. The observed inhibition of AChE may result from the formation of a reversible complex at the enzyme's active site, as evidenced by the significant recovery of AChE activity following 18 hours of exposure to the EO (Omotoso *et al.,* 2020). Furthermore, lemongrass oil demonstrated effective insecticidal activity against Callosobruchus maculatus, suggesting its potential as an alternative agent for the protection of cowpea seeds during storage, particularly for rural farmers in tropical and subtropical regions (Omotoso *et al.,* 2020). Essential oils contain various selective bioactive compounds that exert minimal or no adverse effects on non-target organisms and the environment (Vinayaka *et al.,* 2010). The use of plant-based oils is both cost-effective and sustainable, especially considering the ease of cultivating lemongrass. Additionally, the safety of lemongrass for users is supported by its widespread use as a culinary spice and herb (Omotoso *et al*., 2020). The insensitivity of AChE to organophosphate (OP) and carbamate insecticides has been identified as a major resistance mechanism in numerous arthropod species. The occurrence of mutations in the AChE genes especially in or close to the catalytic site was linked to resistance to OP and has been confirmed using functional expression (Lee and Barron 2016; Mwila *et al.,* 2013; Scaps *et al.,* 1997). There is a necessity to explore the role of synergists such as S, S , S-tri-n-butyl phosphorotrithioate, which acts as a specific inhibitor of esterases and glutathione S-transferases (GST), for resistance management (Roditakis *et al*., 2006; Carletto *et al*., 2009). Findings by Houghton *et al.* (2006) demonstrated that monoterpenoid compounds influence insect mortality, potentially through the modulation of certain enzymatic activities. In the present study, there was no significant difference in the activity of CaEs in insects with neurotoxic insecticides (thiamethoxam) when using α-NA and P-NO2, with values of 21.61 and 15.29, respectively, compared to 12.41 and 12.76 nmoles of substrate per mg of protein per minute in the control group. Similarly, there was a similar trend with values of 14.39 compared to 13.07 in untreated insects. Conversely, significant inhibition of acetylcholinesterase (AChE) was observed in *Callosobruchus maculatus* adults treated with malathion and cassia oil, whereas other tested essential oils did not produce such effects (El-Sayed *et al*., 2015). Lee *et al*. (2000) also identified 1,8-cineole as the most potent inhibitor of eel AChE among the monoterpenes examined. This inhibition may represent a mode of action underlying the toxicity of essential oils and monoterpene fumigation against stored-grain

insect pests. Additionally, alternative mechanisms have been proposed; for instance, the insecticidal effect of compounds found in spearmint is largely attributed to their fumigant action, with toxicity resulting from penetration of the insect body via the respiratory system (Shaaya *et al.,* 1997; Park *et al*., 2003).

Table 4. α- naphthyl acetate (α- NA) and P- nitrophenyl acetate (P-NO2) hydrolyzing activities of Carboxylesterase (s) and acetylthiocholine iodide (ATchI) hydrolyzing activities of acetylcholine esterase (s) (AChE) from *C. maculatus* treated with half lethal concentration of tested pesticide after 11 weeks from treatment.

Substrate / Compounds	CaEs activity (V)* ±SE		AChE activity (V)* ±SE
	α- NA	P-NO2 phenyl acetate	ATchI
Control	12.41±0.72 a	12.76±0.37 ab	13.07±0.62 a
Talc powder	20.08±2.22 ab	20.20±3.89 abc	15.81±2.08 a
Lemongrass oil	46.03±2.83 c	29.61±5.08 c	26.20±4.22 b
B. bassiana	22.81±3.08 ab	6.24±1.18 a	19.73±1.74 ab
M. anisopliae	12.87±2.40 a	22.95±0.83 bc	19.87±0.74 ab
Indoxacarb	27.98±1.57 b	25.56±4.39 bc	12.44±0.99 a
Thiamethoxam	21.61±1.41 ab	15.29±1.63 abc	14.39±0.76 a
Hexaflumuron	19.80±2.24 ab	7.21±2.32 a	13.25±0.94 a

***** V (The velocity): residual free enzyme concentration

CONCLUSION

The findings of this study demonstrate that several synthetic and natural compounds can effectively reduce Callosobruchus maculatus infestation and seed damage during cowpea storage. Among the tested treatments, kaolin and malathion exhibited the highest residual toxicity, while thiamethoxam showed notable persistence over time. Lemongrass essential oil (EO) provided strong protection against seed damage and enhanced enzymatic activity (CarEs and AChE), indicating a potential mode of action. However, its negative impact on seed germination highlights the need for careful dose optimization. Overall, lemongrass EO emerges as a promising natural alternative to conventional insecticides for seed protection, but further studies are recommended to refine its application rate and evaluate its long-term safety and efficacy.

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