Journal of Toxicological Studies 2024, 2(2), 1393.
https://doi.org/10.59400/jts.v2i2.1393

Article

Evaluation of the crude chitinases toxicity on the reproductive system of *Callosobruchus maculatus* (Coleoptera: Bruchidae)

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CITATION

Hassiba LH, Zoheir A, Nouara B, et al. Evaluation of the crude chitinases toxicity on the reproductive system of *Callosobruchus maculatus* (Coleoptera: Bruchidae). Journal of Toxicological Studies. 2024; 2(2): 1393.
https://doi.org/10.59400/jts.v2i2.1393

ARTICLE INFO

Received: 20 May 2024
Accepted: 22 July 2024
Available online: 3 August 2024

Abstract: Insecticides represent the most used control method against the insect pests of stored food products. But there are strains of insects that are resistant to these insecticides; this is the case of the beetle of the chickpea, *Callosobruchus maculatus*. The present study has for objective the evaluation of the biological activity of chitinolytic enzymes extracted from the fish offals *Scorpaena scrofa* (scorpion fish) on this beetle at different doses (3%, 6%, 9%, 12%, 15%, and 21%) prepared with the buffer solution. The results obtained from three treatments (T_1, T_2, and T_3) realized according to the dose of the crude enzyme and the time of exposure were compared with those of the controls and have shown a very significant efficiency of our crude enzyme on the reduction of the fertility rate of 100% by treating the couple (T_1) at the same time as to treat the male (T_3) and the female (T_2) separately with the strongest dose (21%) for 48 h of exposure. It would be thus desirable to demonstrate the activity of these enzymes in the real conditions of storage.

Keywords: chitinase; *Scorpaena scrofa*; bioinsecticide; stored foods; *Callosobruchus maculatus*

1. Introduction

The leguminous plant seeds represent the main source of interesting proteins as a supplement to cereal for human food [1,2]. The man always thought of protecting stored foodstuffs, and this protection has known enormous progress during the last century. The use of synthetic chemical insecticides represents the most widely used pest control method [3,4], but the development of resistance to the latter makes them more and more insensible to pesticides [5–8]. Compounds are often found in the form of residues in food and present risks for human health, animal health, and the environment [9,10]. The animal and bacterial reigns can present many possibilities: the use of enzymes with insecticidal properties in certain developing countries is told by plentiful literature [11,12]. In a concern of environmental respect and within the framework of sustainable development, it is advisable to reduce considerably the quantities of synthetic inputs of synthesis.

The efforts of researchers must therefore be directed towards the elaboration of

alternative control strategies. These new approaches must be based on the joint use of biomolecules with insecticidal properties. The bacterial chitinases of certain insects have already been the object of some works showing their insecticidal potential [13–21]. *Chitinases* (E.C. 3.2.2.14) are glycosyl hydrolases that hydrolyze the chitin [10,22–24], which is a polymer of N-acetylglucosamine, the second compound of the living organism the most represented on the surface of the earth after cellulose [25]. Chitin is present in the shells of crustaceans, the cuticles of insects [26], in the walls of mushrooms (*Aspergillus nigers*, *Penicillium notatum*), and in certain microorganisms such as yeasts (*Sacharomyces cerevisiae*, *Candida albicans*) fungi [27–29].

Chitin is also found in a more minor way in squid feathers [30–32]. It constitutes the sclerified exoskeleton, or cuticle [10,33], and is also present in the trachea of the peritrophic membrane of the exochorion [9,21]. Chitin is a potential target for crop pest control. The use of chitinase extracted from the fish offal (scorpion fish) allows to confirm its potential effect against the populations of the chickpea beetle *Callosobruchus maculatus.* A study carried out by Laribi-Habchi et al. [21] has shown the efficiency of the chitinase on the mortality rate of the insect and its impact on the digestive tract. In this context, we were interested in the toxic effect of the enzyme on the male and female reproductive systems of the beetle.

2. Material and methods

2.1. Biological materiel

2.1.1. Marine biomass

The marine biomass used for our study is a fish (scorpion fish), from the family Scopaenidae and the species *Scorpaena scrofa*. The criteria of choice were based on the availability of this fish on the Algerian coast and on the study made preliminary by Laribi-Habchi et al. [10]. The offals were got back and transported in a cooler to the laboratory for the cleaning, grinding, and storage at −20 °C for the extraction of the chitinase enzyme.

2.1.2. Insect

The target insect in our experiment is the chickpea beetle *Callosobruchus maculatus* [34] from the family Bruchidae and the order Coleoptera. This species is cosmopolitan, with fast reproduction and infests stored economically important commodities (chickpea). The strain of *C. maculatus* was supplied by the laboratory of Agricultural and Forest Zoology of the National School of Agronomy of El Harrach—Algeria. Mass breeding of insects was carried out in the laboratory in 100 cm^3 glass jars closed by a mosquito net to ensure oxygenation conditions. These jars were kept in the dark in an oven at a temperature of 28 °C ± 2 °C and a relative humidity of 75% ± 5%. During our experimentation, we have worked on adult's males and females aged 0–15 min recovered by sieving.

2.1.3. Chickpeas

The used variety of chickpea is *Cicer arietinum*, not treated with conventional insecticide, which was provided by the Technical Institute of Great Cultivation of Oued Smar El Harrach—Algeria. The choice of this species depends on its

availability in the Algerian market. 500 g of chickpeas were put in every jar in which were added the adults of opposite sex.

2.2. Preparation of the crude extract

The method of extraction of the crude chitinases was inspired by the experimental protocol of Laribi-Habchi et al. [26]. The samples were prepared by respecting the proportion 1/6 in weight of offal by volume of citrate (w/v) buffer solution, pH 5 (citric acid at 0.15 M and dissodic phosphate at 0.3 M). The reactive mixture was then centrifuged at 37 °C for 3 h at 120 rpm. At the end of the incubation, the macerated sample was filtered through a gauze cloth. The filtrate thus obtained was centrifuged at 6000 rpm for 30 min. The recovered supernatant constitutes the crude enzymatic extract.

2.3. Preparation of the natural substrate (colloidal chitin)

The colloidal chitin was prepared from the commercial chitin (Sigma) by the method of Robert and SelitreniKoff, modified by Lee et al. [35]. Dissolve 5 g of chitin powder in 60 mL of concentrated HCl at 4 °C. The mixture was stirred and then added to two liters of ethanol (95%), with a rapid stirring overnight at −20 °C. The precipitate recovered by centrifugation at 5000 rpm for 20 min at 4 °C was washed with sterile distilled water until neutrality (pH 7). The colloidal solution was thus prepared and stored at 4 °C for other applications (in this case the colloidal chitin has been lyophilized).

2.4. Chitinase activity test

The analytical method used has served to study the chitinase activity in the various doses of the crude extract prepared with the citrate buffer solution (3%, 6%, 9%, 12%, 15%, and 21%) (v/v). The reaction mixture consists of 0.5 mL of the mixed enzyme solution with 0.5 mL of 50 Mm buffer of 2-(N-morpholino) ethane sulfonic acid (buffer A) and supplemented with 2 Mm $CoSO_4$ at pH 5 containing 10 mg/mL chitin colloidal, incubated for 1 h at 37 °C. The mixture is boiled for 10 min, then cooled and centrifuged (30 min at 13,000 g) to eliminate non-degraded chitin. The total production of N-Acetyl-Glucosamine (NAG) was determined by the specific method for amino sugar [36]. Amino sugars are characterized by the Elson and Morgan reaction. When hot and in an alkaline environment, they cyclize in furan form and, by elimination of a water molecule, acquire a double bond. The product formed complexes in an acidic medium with paradimethyl aminobenzaldehyde to give a purplish color. Reading is compared with a standard curve prepared with a dilution series of NAG (0 to 10 mg/m).

2.5. Toxicity test

In order to estimate the toxicity of the enzyme studied, we calculated the fertility rate of the number of eggs laid and the viability rate of submerged adults, taking into account three factors: sex, the dose of the enzyme tested, and the duration of treatment. We impregnated the internal surface of the Petri dishes (including the cover) with 1 mL of each dose of crude chitinase extract (3%, 6%, 9%, 12%, 15%,

and 21%) and 1 mL of the buffer solution (control). After drying the 14.5 cm Petri dishes, five pairs of *C. maculatus* insects aged 0 to 15 min were placed separately. The Petri dishes were incubated for 24 and 48 h at 27 °C. Several test cases were performed on the male and female reproductive systems (**Table 1**). For each dose, the tests were repeated five times. Adult bruchids that resisted the treatment were maintained on chickpea seeds until their death. After reproduction, the eggs laid were counted using a binocular microscope from the 5th day. The chickpeas were then placed in the rearing incubator until the adults emerged. Insects were removed from the petri dishes as they emerged from the chickpea seeds.

Table 1. Toxicity test of the crude chitinases on the reproductive system of the couple.

Treatment	Female	Male
T_1	T	T
T_2	T	NT
T_3	NT	T

NT: Untreated T: Treated.

Calculation methods:

- Preparation of dilutions: The tested doses were obtained by diluting a volume of the crude chitinase extract in 100 mL of the citrate buffer solution at 0.05 M.
- Fertility rate: The fertility rate was calculated by the following formula [37].

$$Tf = (\text{Number of laid eggs}/\text{Total number of laid eggs in the control}) \times 100 \quad (1)$$

- Reduction rate of the laying: The reduction rate of the laying is given by the following formula [37]:

$$R_r\ (\%) = (N_c - N_t)/N_c \times 100 \quad (2)$$

R_r: Reduction rate compared to the control (%).

N_c: Number of eggs in the control.

N_t: Number of eggs in the test.

- Viability rate: The adults which begin to emerge from the 30th day were regularly counted and removed from the boxes as they emerged from the seeds. The viability rate was calculated by the formula:

$$\text{Viability rate}\ (\%) = (\text{Number of emerged adults}/\text{Number of laid eggs}) \times 100 \quad (3)$$

2.6. Sampling of the female genital tract

The dissection was carried out under a binocular magnifying glass (magnification isotonic). We then make a tear incision using forceps on either side of the lateral part of the abdomen at the level of the sixth sternite, and then with the forceps we grasp the posterior part of the abdomen. Abdomen, and by slow, firm, and progressive traction, we extract the genital tract.

2.7. Statistic study

The results were analyzed by the analysis of variance (ANOVA) in order to determine the effect of three criteria (dose of the crude enzyme, time, and sex) on the fertility rate and the viability of the insect C. This test was realized by the software

Statistica version 7.

3. Results and discussion

3.1. Effect of toxicity on the reproductive system

3.1.1. Fertility rate

Figure 1 shows the fertility rate of three treatments realized according to the dose of the crude enzyme (chitinase) and exposure time. We noticed that the fertility rate decreases clearly and in a progressive way for T_1 with regard to T_2 and T_3, and this with an increase in the doses and as a function of time. The fertility rates for T_1 (49 and 31%), T_2 (62 and 42%), and T_3 (73 and 68%) were recorded with the lowest dose (3%) during 24 h and 48 h, respectively (**Figures 1a** and **1b**). Contrary to the strong dose (21%), the fertility rates for T_1 (3 and 0%), T_2 (3 and 1%) and T_3 (9 and 5%) were noted after 24 h and 48 h, respectively. According to these results, we noticed that the efficiency of the chitinase enzyme on the reduction of the fertility rate is 100% for a high dose during 48 h by treating the couple (T_1) at the same time as to treat the male (T_3) and the female (T_2) separately.

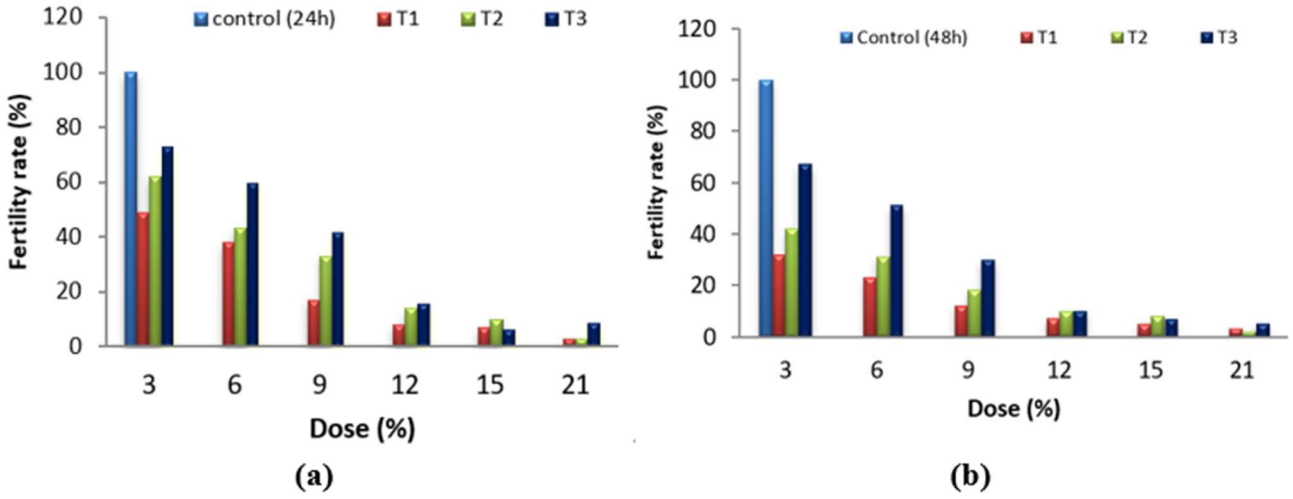

Figure 1. Fertility rate of three treatments according to the dose of the enzyme tested during **(a)** 24 h; and **(b)** 48 h.

3.1.2. Reduction rate

Figure 2 shows the reduction rate of egg-laying of the treated couple at a maximum dose (21%) as a function of the exposure time. The results showed that the reduction rate is important (100%) with T_1 with regard to T_2 (97.47%) and T_3 (93.27%) for a treatment time of 48 h. The effect of the crude enzyme has shown its toxicity effect on the reproductive system when the female and the male were treated at the same time.

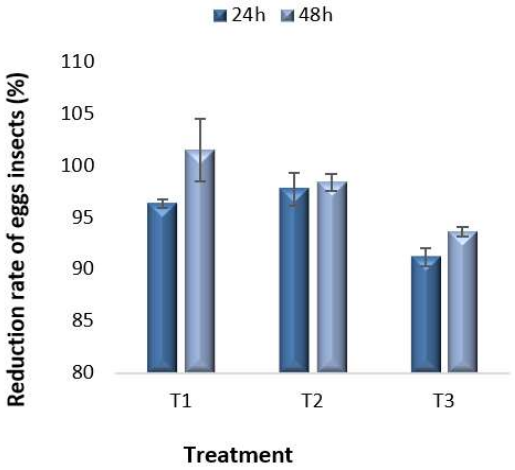

Figure 2. Reduction rate of the eggs-laying according to the maximal dose (21%) and time of treatment.

3.1.3. Viability rate

Figure 3 shows that the viability rate of the *C. maculatus* insect progressively decreases with an increase in dose and as a function of time. We have registered the same curve as the fertility rate. On the other hand, it is not the totality of the laid eggs that are emerged to give adults. The viability rates for T_1 (2 and 0%), T_2 (3 and 0%), and T_3 (5 and 5%) were recorded with the highest dose (21%) for 24 h and 48 h, respectively (**Figures 3a** and **3b**). According to these results, we noticed that the toxicity test has a significant impact on the female and male reproductive systems.

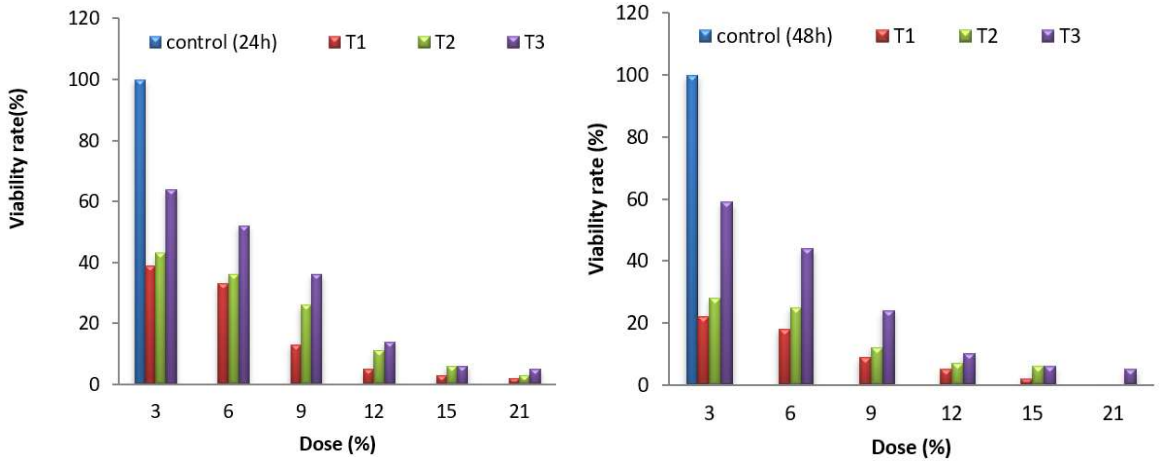

Figure 3. Viability rate of three treatments according to the dose of the enzyme tested during **(a)** 24 h; and **(b)** 48 h.

This test seems interesting to us due to the fact that it has not been used in this area by other authors. The obtained results concerning the toxicity of the crude chitinases of the scorpion fish showed an effect on the reproductive system of the adult female insects according to the dose used and the time of exposure. This toxicity was probably due to a disturbance of the ovogenesis of the female insect by the high chitinase activity, which is 0.04 U/mg at the dose of 21%. The later caused the hydrolyze of the chitine of the ovarian membrane of the female genital system [38].

The toxicity was observed by Wiwat et al. [39] on the insect pest eggs of cabbage *Plutella xylostzlla* treated with crude chitinases extracted from the bacterial strain *Bacillus thuringiensis* ssp HD1 after 6 h of treatment. The toxicity was also observed by Laribi-Habchi et al. [40] on the insect pest *Callosobruchus maculates* with pure chitinase extracted from fish *Scorpeana scrofa* after 1 h of treatment. The works of Rishad et al. [41] have also shown that the chitinases extracted from the bacterial strain *Bacillus pumilus* MCB-7 have an insecticidal effect on the eggs of the insect pest of rice, *Scirpophagea incertulas*, with an inhibition rate of 68% after 12 days of treatment.

3.2. Microscopic observation of female reproductive system

Observation of the genital tract of females treated with the crude enzyme showed a physiological difference compared to the control (**Figure 4A**): deterioration of the genital tract with atrophy and an atretic appearance of the oocytes, visible through transparency (**Figure 4B**). We noticed that the oocytes, detectable by their yellowish-yellow charge, are no longer visible in the ovarioles. Indeed, the ovarian structure includes a protective cuticle based on sclerotized chitin, thus hardened by new bonds of chitino-protein fibers [38]. Therefore, the presence of chitin at the level of the ovarian membrane led to its degradation in the presence of chitinases at high doses, which overall led to the total deformation of the reproductive system (which confirms our results on fecundity and fertility, which are marked by a reduction in egg laying).

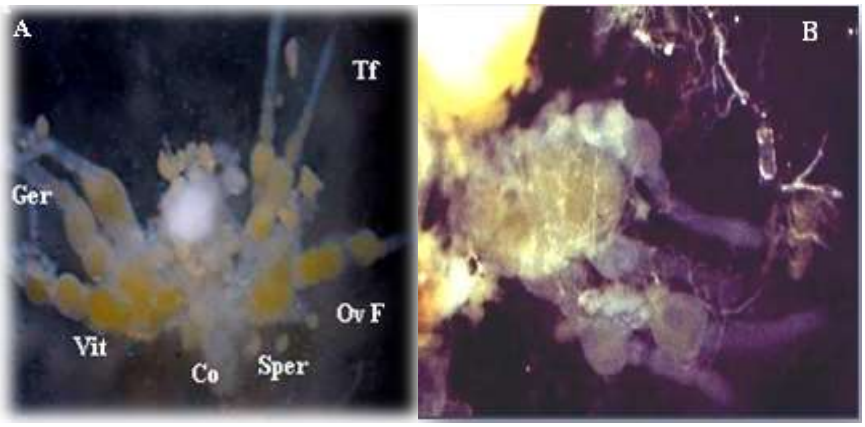

Figure 4. Optical microscopic observation G X 40 of the reproductive system of the female insect treated with the crude enzyme (21%) for **(B)** 48 h; and **(A)** control.
(OvF: ovarian follicles, Tf: terminal filament, Ger: germarium, Co: common oviduct, Sper: spermatheca, Vit: vitellarium).

3.3. Statistical study

Depending on the doses of the crude enzyme chitinase, the ANOVA showed a significant difference with $F = 6.66$ for $P = 0.00$. Depending on the duration of exposure, there is also a significant variation with $F = 9.38$ for $P = 0.00$.

Figure 5 shows a very important positive correlation between egg viability and the number of emerging insects for all treatments performed and dose-related to time. The dose factor is an important parameter which has a high impact on viability and production.

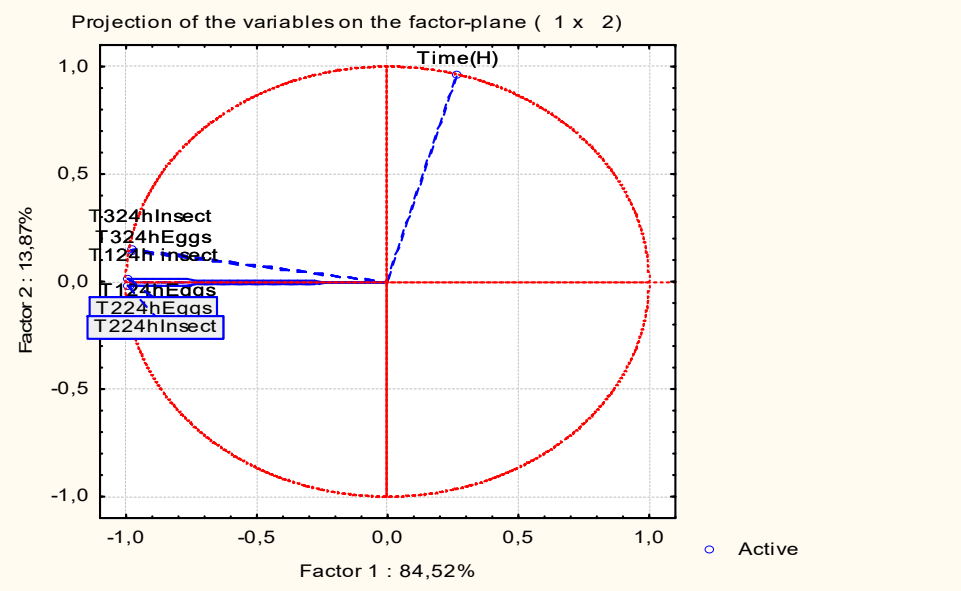

Figure 5. Principal component analysis (PCA) showing the correlation between biological variables (number of eggs and number of emerging insects) as a function of dose and treatment time.

P: Probability, F: Factor of fisher.

4. Conclusion

The use of chitinases capable of fighting harmful insects could constitute an alternative approach complementary to traditional insecticide treatment. The effectiveness of crude chitinases extracted from the offal of scorpion *scorpeana scrofa* has been demonstrated. In fact, they influence the population of harmful insects through a double action: toxicity acting on the reduction to the cancellation of female fertility as well as that of viability. Despite certainly encouraging results, the effectiveness of these enzymes still remains to be demonstrated in a real situation. Additional experiments are necessary, such as repeating these tests on other harmful insects, in order to confirm the effectiveness of these enzymes on a wide range of insects and also to understand and specify the mechanism of action of these enzymes responsible for this activity.

Author contributions: Conceptualization, LHH; methodology, LHH; software, BN, AAD, AH and MO; validation, BN and LHH; formal analysis, LHH and BN; investigation, LHH; resources, LHH; data curation, LHH and BN; writing—original draft preparation, LHH, BN, MMF and AZ; writing—review and editing, LHH; visualization, MMF; supervision, LHH; project administration, LHH; funding acquisition, LHH. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: The authors declare no conflict of interest.

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