Effectiveness of a Biological Insecticide Derived from Scorpion Fish Offal against the Chickpea Weevil

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Abstract
The aim of this study is to evaluate the biological activity of a crude chitinase extracted from fish offal (scorpion) on the chickpea weevil Callosobruchus maculatus (Coleoptera: Bruchidae) at various concentrations: 3%, 6%, 9%, 12%, 15%, 18%, 21% and 24% (V/V). The biological tests were performed under the typical experimental conditions: temperature and relative humidity at 28°C and 75%, respectively. The results have shown that the concentration of 24% of crude chitinase extracted has 100% of insecticidal activity after a contact time of 1 h. The lethal dose (LD50) is estimated for every sample and for a range time from 1 to 72 h. The lower percentage of mortality (10%) is obtained for low values of concentrations and contact time (dose of 3% of crude chitinase and 1 h of contact time). The maximum mortality rate is observed for the high values of two parameters (dose of 24% of crude chitinase and 72 h contact time). However, it is observed that for a higher dose (24%) and for a lower contact time (1 h), the mortality rate increased rapidly. The novelty of this work consists in the use of a new bioinsecticide obtained from offal scorpion fish.

Keywords: Crude chitinase; Scorpion fish; Extraction; Bio-insecticide; Callosobruchus maculatus

Introduction
Around the world, stored products are attacked by several species of pests. Chemical control against the various pests, using essentially conventional pesticides, continues to be the major means of protecting foodstuffs. Secondary effects of insecticides and environmental imperatives have encouraged the search for new approaches to protect these pests. To fight against the food insecurity and to protect the environment, we should increase agricultural production through the judicious use of scientific and biotechnological tools. Leguminous seeds represent the main source of protein in many developing countries [1]. Unfortunately, they suffered considerable losses during storage [2]. The stored products can be destroyed by insects, fungi and rodents. Losses caused by insects are considerable in countries where modern techniques of storage are not yet introduced. Larvae of cleopter Bruchidae consume and grow only in the seeds of leguminous [3,4].

Also, the chickpea weevil Callosobruchus maculatus may infest the plant Cicer arietinum in fields and stocks, in addition it infects other leguminous non-host plants which have an economic importance in developing countries as cowpea (Vigna unguiculata) and sap (Vicia faba) [5].

Chemical control using synthetic insecticides has dominated control tactics against insect pests of stored products [6-8]. In this study a new bio-insecticide obtained from offal scorpion fish was utilized. For this purpose, chitinase was extracted from scorpion’s stomach and tested as a protective film of chickpea weevil.

Experimental design and statistical analysis were utilized to study the effects of two independent variables, namely concentrations of crude chitinases (X1) and contact time (X2) on mortality rate of Callosobruchus maculatus. The planning of the experiments was carried out in order to evaluate the effects of the parameters and their interactions using the two levels factorial planning of the two studied variables 22. A total of 8 experiments were formulated including 22 full factorial design (+1) with four replicates at central point (coded level 0). The factors and the experimental domain of variation of factors (coded and uncoded levels) are given in Table1.

The functional relationship between the two independents variables (X1, X2) and the response (Y) is expressed by a first order polynomial function (Eq.1):

\[ Y = a_0 + a_1 X_1 + a_2 X_2 + a_{1.2} X_1 X_2 \]  

Y represents the mortality rate of Callosobruchus maculatus, X1, X2 are independent variables representing the coded values of the factors according to Table 1, a0 (center point of system) is the average of the results of the replicated central point, a1, a2 are coefficients of linear effects and a1.2 is coefficient of interactive effect.

In the present work, the influence of the concentration of the crude chitinase and the contact time on the mortality rate of insects has been estimated.

Material and Methods
The insect cuticle is composed of chitin. Chitin is a polymer of N-acetyl glucosamine and the second abundant component on the surface of the cuticle is chitinase.

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Table 1: Domain of variation of the parameters for the factorial planning 22.

| Parameter | Lower level | Higher level | Centre Co-ordinates |
|-----------|-------------|--------------|---------------------|
| Doses of crude chitinases (%) | 3 | 24 | 14 |
| Contact time (h) | 1 | 72 | 37 |

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surface of the earth after cellulose [9]. It is present in bacterial, yeasts and fungi cell walls [10]. In animal reign, chitin is essentially present in exoskeleton of arthropods, and cuticle, trachea, diaphragm, peritrophic membrane and exchorion of insect [11]. However, chitinases may be used as antagonist to pests. Chitinases (EC3.2.1.14) are enzymes that are widely distributed in nature and play an important part in degradation of chitin. They occur in many fishes where they are produced by the digestive glands for the digestion of chitinous foods [12].

Choice of biological material and insects breeding

The choice of chitinases from offal scrap fish is due to their high enzymatic activity (40%) compared to others fishes as Red mullet (24%), burbot (14%), bougue (13%) and silt (5%). Among the insects, Callosobruchus maculatus (chickpea weevil) has been chosen for several reasons: the species is cosmopolitan and infests stored products of economic importance in Algeria, and may be easily cultivated in laboratory for testing the efficiency of crude chitinase on a large number of individuals.

The insects were mass reared on chickpea in jars (18 cm in height and 11 cm in diameter). The jars are kept at a controlled temperature T=28 ± 2°C, in obscurity and at a relative humidity of about 75 ± 2%.

Extraction of crude extracts chitinase

The samples were prepared at 1/6 ratio [13] in weight of offal per volume of buffer solution prepared from citric acid (0.15 M) and disodium phosphate (0.3 M) at pH 5. The reaction mixture was incubated at 37°C during 3 h at 120 rpm.

At the end of the incubation, the macerated sample was filtered through a gauze fabric. The obtained filtrate was centrifuged at 6000 g during 30 min. The recovered supernatant is the crude enzyme extract.

Dosage of N-Acetyl-Glucosamine (NAG)

The dosage of NAG was performed using Reissig method [14] modified by Gustowska et al. [15]. This technique requires a reagent A, which is composed of potassium tetraborate solution (0.8 M) adjusted to pH 9.1 with 1 N KOH, and a reagent B involving 1.5 g of dimethylaminobenzaldehyde (DMAB) dissolved in 100 ml of pure glacial acetic acid containing 1.25% of HCl (12 N). The concentration of NAG was determined by adding 0.5 ml of supernatant and a blank (0.5 ml of distilled water) to 0.1 ml of reagent A.

The mixture was heated in a water bath at 100°C for about 3 min and then cooled in a cold water bath, followed by addition of 3 ml of reagent B and homogenization of the solution. The samples were immediately incubated in a thermostatic bath at 37°C during 20 min. The NAG concentration was determined using spectrophotometer at various concentrations (3%, 6%, 9%, 12%, 15%, 18%, 21% and 24%) were homogeneously dispersed in 100 g of seeds. The experiments were repeated three times. All batches of treated seeds were infested with 10 pairs of insects aged between 0 and 24 h. The counting of dead insects is carried out after 1, 24, 48 and 72 h. The death recorded in batches of treated seeds was calculated and corrected for mortality in accordance with Abbott’s formula [18] taking into account the natural mortality in samples.

Bio essay of crude chitinase on larval feeding

The method described by Xie [19] to measure chitinases effect on the insect feeding was utilized in our present work. A mix of 50 g of chickpea flour and 14 ml of the lethal dose (24%) was used for making meatballs the size of a chickpea. The seeds used for the control were made with chickpea flour and citrate buffer. The reconstituted seeds were dried for 24 h at a temperature of 30°C (to avoid distortion of chitinases and protein fractions) and at humidity of 75%. Chickpeas well prepared were placed in Petri dishes of 14 cm diameter with 10 insects couples to obtain the eggs. The eggs laid were counted daily until the death of the females and placed in an oven to assess fertility. The experiment was repeated four times for each dose and for the control.

The number of died larvae found in treated population with those concentrations previously cited were counted. The percentage of mortality was calculated and corrected with the control mortality [18],

\[
\text{MC} = \frac{(M-\text{Mt})}{(100-\text{Mt})} \times 100
\]

MC is the percentage of the mortality corrected, M is the percentage of the mortality observed in the treated population and Mt is the percentage of the mortality in the control population. One way to test the effectiveness of a product is the calculation of Lethal Dose 50 (LD50) which corresponds to the amount killing 50% of individuals of the same batch. It is deduced by drawing the regression line. The percentages of mortality were transformed into corrected probit were plotted as a function of natural logarithm of the LD50 which was determined from the Eq.2 of a straight line obtained theoretically [20].

The experimental design and the results evaluation were performed using Statistica Software (Stat Soft France Version 9.0).

Results and Discussion

Evaluation of the effect of crude chitinase contact

The obtained results show that crude chitinases manifests insecticidal activity by contact. The percentages of mortality obtained ranged from 10 to 100% after only 1 h of contact. The control gave a percentage of mortality close to 0% (Figure 1). Other studies [21] using essential oils from aromatic plants were carried out on the same pest. Melaleuca quinquenervia, Citrus aurantifolia and rosemarinus officinalis revealed mortality rate of 60%, 48% (After a 24 h treatment) and 25% (after 96 h of treatment) respectively. The biopesticide produced in this study appeared more efficient than the essential oils reported in literature.

The results were confirmed by observation of scanning electron microscope SEM (JEOL 6360). Indeed, the deterioration of the insect’s
The cuticule by the crude chitinases compared to control (Figure 2) was observed.

The LD50 of crude chitinases obtained against the contact time ranging from 1 to 72 h were close to 14%. Indeed, LD50 of 5% (1 h of contact), 15% (24 h of contact), 14% (48 h of contact) and 13% (72 h of contact) were obtained (Figure 3). However, for Callosobrochus maculatus, in all studies undertaken the essential oils have been used.

Contact test on the treated seeds and larval feeding

The results obtained after contact tests on the treated seeds showed similar mortality percentages compared to the previous experiments. Nevertheless, chitinases extracted raw from fish offal was very toxic against Callosobrochus maculatus with 100% of mortality rate at a dose of 24% after 24 h (Figure 4). Low doses (3%, 6%, 9% and 12%) showed a less active insecticidal effect.

To evaluate the effect of chitinase on larval feeding, five days treatment, all eggs laid entered reconstructed seeds. The results obtained show that after 10 days, all the larvae died after leaving the seeds. In the control, the rate of emergence is about 95% was obtained.

Experiments planning study

The matrix of the experimental design and the experimental results are shown in Table 2. By comparing the different obtained mortality rates, the mortality is mostly affected by the dose's variation of crude chitinases (%) and the contact time (h).

The lower percentage of mortality (10%) is obtained for low values of concentrations and contact time (dose of 3% of crude chitinase and 1 h of contact time). The maximum mortality rate is observed for the high values of two parameters (dose of 24% of crude chitinase and 72 h contact time). However, it is observed that for a higher dose (24%) and for a lower contact time (1 h), the mortality rate increased rapidly.

Table 3 presents the statistical significance of each effect of model's effect. According to the regression analysis, effects lower than 0.05 in the P-value column (95% confidence level) is significant (P < 0.05). In this case, it can be seen that the dose of crude chitinases was the most important influence on the mortality rate of Callosobrochus maculatus.
The analysis of variance was used for testing the significance of the chosen parameters.

The contact time and the interaction between the crude chitinase doses and the time were not statistically significant on the results obtained from the model is represented by the following Eq.3:

\[ Y = 46.5787 + 44.1750 X_1 + 0.0891 X_2 - 0.0891 X_1X_2 \]  

where Y is the percentage of mortality, and X_1 and X_2 represent the doses and contact time, respectively.

The value of adjusted R^2 (0.860480) suggests that the total mortality rate variation of 86% is attributed to the independent variables, and only about 14% of the total variation cannot be explained by the model.

Table 3: Effects of the coefficient of the model.

| Coefficient | Statistic (t) | Limit confianding (-95%) | Limit confianding (+95%) |
|-------------|--------------|--------------------------|--------------------------|
| Constant    | 46.5787      | 10.0754                  | 37.7343                  |
| X_1         | 44.1765      | 6.7938                   | 28.2654                  |
| X_2         | 0.0891       | 0.0091                   | -0.0091                  |
| X_1X_2      | -0.0891      | -0.0891                  | -0.0891                  |

R^2 = 0.920274, Adjusted R^2 = 0.860480

Table 4: Analysis of variance.

| Sum square | Freedom degree | Mean square | F     | P   |
|------------|----------------|-------------|-------|-----|
| Regression | 7894.372       | 3           | 2631.457 | 15.3 | 0.001596 |
| Résidu    | 683.9076       | 4           | 170.9769 | 10.075 | 0.001596 |
| Total     | 8578.2796      | 7           | 125.9248 | 15.3 | 0.001596 |

The response surface plot which gives the mortality rate (%) as a function of dose of crude chitinase and the contact time, shows that the mortality rate increases with augmentation of crude chitinase dose and contact time.

Conclusion

The insects were sensitive to biological tests. The crude chitinase extract was used as a biological insecticide and its effect is related to the dose and to the contact time. Thus, the approaches were designed to determine the effectiveness of crude chitinases contact. Interesting results are obtained for 100% of insecticide activity after 1 h contact at a dose of 24%.

The difference noted in the mortality rate of the insects at different doses attracts us to investigate many other aspects in this field. It would be appropriate to extend these trials to all other insects attacking leguminous seeds stock and other grains such as Sitophilus oryzae, to effectively assess their actual action in stored-product protection.

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