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Evaluation of the effectiveness of *Metarhizium anisopliae* on *Callosobruchus maculatus* Fab. (Coleoptera: Bruchidae) and *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) in laboratory

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Abstract

Callosobruchus maculatus and *Sitophilus zeamais* are major storage insect pests of cowpea, Kersting's groundnut and maize in tropical Africa. These pests can cause post-harvest losses of up to 100% in a few months. The most common suppression method is the use of pesticides, with unfortunately important hazards and side effects. This study, evaluated the effectiveness of four isolates of *Metarhizium anisopliae* (ma351, ma356, ma357 and ma358) on *C. maculatus* and *S. Zeamais* in stored grains of cowpea, Kersting's groundnut and Maize, respectively. The pathogenicity of the four isolates was assessed and two of them (ma356, ma357) were the most effective. To study the virulence of these two isolates, four doses were used 0, 10^8, 10^9, 10^{10} conidia/100 grains. Results from the pathogenicity test indicated that all studied isolates were pathogenic to insects at a dose of 10^9 conidies/100 grains equivalent to 0.002 g/100 grains powder form. In the presence of isolates, the lifespan of treated pests was reduced compared to the control insect pests. The effectiveness of the isolates was significantly dose-dependent for all parameters evaluated except for imagos hatching rate of *C. maculatus* on cowpeas and seed weight loss in maize. The dose of 10^{10} conidia/100grains significantly reduced longevity of *S. zeamais* whereas with the dose of 10^9 conidia/100 grains reduction was already significant on *C. maculatus* for all parameters. This study built from experiments and speculated on how the production of entomophagenic fungi at large scale would be an alternative to the use of chemical pesticides in controlling population of *C. maculatus* and *S. zemaïs* in stored products and therein significantly reduce the overall post-harvest losses.

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Introduction

Legumes and cereals are the main groups of food crops in Benin. Maize is the most widely grown cereal worldwide representing 38% of the world production, followed by wheat (29%) and rice (21%) (USDA, 2013). In Benin, cowpea (*Vigna unguiculata* L.) is one of the major pulses produced and consumed (Pronaf, 2002) and Maize (*Zea mays* L.) represents the main cereal food crop (Yallou, 1994). Kersting's groundnut (*Macrotyloma geocarpum* Harms) is a crop with an increasing demand for consumption although it categorization as neglected crop (Béhanzin, 1986). For all these speculations, productions are far below the demands for consumption (Gueye *et al.*, 2011) to the extent that they are affected by climate hazards, and losses caused by pre-harvest and post-harvest insects pests. Despite the efforts to increase production, food insecurity is still being accentuated by significant post-harvest losses. In Benin, farmers annually register 20 to 50% of post-harvest losses after six months of storage (Adegbola *et al.*, 2011). According Gansou *et al.* (2000) and PADSA (2000) up to 75% losses can be incurred in absence of proper phytosanitary treatment. During storage, the insects pests especially some species of Coleoptera (Bruchidae and Curculionidae) including *Callosobruchus maculatus* (Fab.) (Coleoptera: Bruchidae) and *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) attack the grain legumes and cereals. *C. maculatus* mainly attacks Kersting's groundnut and Cowpea grains and *S. zeamais* is primarily found in Maize grains. These insects may cause a complete loss of the stocked products if effective protection measures are not taken in seven months storage time (Ngamo and Hance, 2007). Faced with the threat of storage insect pests, the use of synthetic pesticides stands as the major control measures. Under optimal conditions, they can effectively control the storage pests (Salim, 2011). However, they have many drawbacks, including the emergence of resistant strains (Benhalima *et al.,* 2004), poisoning, environmental pollution and ecological disorders (Regnault-Roger, 2002).

Given the adverse effects associated with the use of chemical pesticides, it is urgent to develop alternative control methods of these pests. These include the use of entomopathogenic fungi which inhibit the development of bruchids and maize weevils. Among these fungi, *Metarhizium anisopliae* (Metschnikoff) Sorokin proved to be effective for pest management (Inglis *et al.,* 2001). This study aims to assess the effectiveness of *M. anisopliae* on *C. maculatus* in stocked cowpea and Kersting's groundnut and on *S. zeamais* in Maize as well.

Material and methods

Study area

The study was conducted in the Phytopathology Unit of the Laboratoire de Diagnostic et de Soutien à la Protection des Végétaux, Direction of Crop Production in Porto-Novo, Benin. The ambient temperature within the laboratory at the time of the experiments was 26 ± 1°C.

Biological material

The biological material used consists of various storage pests, including *C. maculatus* and *S. zeamais* as well as *M. anisopliae* isolates (ma351, ma356, ma357, ma358) (Table 1). These isolates were obtained from the collection unit of the International Institute of Tropical Agriculture (IITA).

Plant Material

The plant material consisted of white grains of *V. unguiculata*, grains of white variety of *M. geocarpum* and white grains of *Z. mays*. The choice of these varieties is guided by consumer preference. The grains of various crops were bought at Ouando market in Porto-Novo, Benin. These seeds were cleared from all insects (*C. maculatus, S. zeamais*) through their sterilization at a temperature of 121 ° C and 15 psi of pressure after they had been frozen at -18 ° C for two weeks.

Mass rearing of the insects

The strain of *C. maculatus* used, was from infested seeds of cowpea and kersting's groundnut obtained from the Post-harvest Unit of the Directorate of Plant Protection.

These seeds were sieved out using a Retsch brand sifter made of a series of five sieves having different mesh sizes and the collected adults *C. maculatus* (of undefined age) were introduced into 225 ml jars containing 250 g of healthy grains of cowpea and kersting's groundnut. The jars were sealed with wire mesh lids. Seventy-two hours later, these adult insects were removed and the infested seeds were incubated until the emergence of F1 insects. Thereafter, the jars were sieved in order to obtain adult insects of the same age (24 h). These are the adult insects used to initiate the experimentation. The rearing process is the same for *S. zeamais* with the only difference that maize grains were used as food diet.

Experimental protocol

Two experiments were conducted. In the first experiment the pathogenicity of isolates was tested while the second experiment allowed determination of the optimal dose.

Pathogenicity test of the isolates

Five 24 hours old couples of insects were introduced into 36 jars of 225 ml each containing 100 healthy maize grains (about 21 g) for *S. zeamais*, and cowpea (about 18 g) and kersting's groundnut (about 27 g) for *C. maculatus*. The grains were introduced into 225 ml sterilized jars and 0.02 g powder (or 109 conidia) of each isolate of *Metarhizium* was added to assess the pathogenicity of different isolates on the studied storage pests under proper laboratory conditions. The ambient temperature in the laboratory was 26 ± 1 ° C. Dead insects were removed and counted daily for a period of 5 days for *C. maculatus* and 21 days for *S. zeamais*. Each treatment was replicated three times, as well as the control which contains grains and insects without conidia of the isolates. The most effective isolates were selected for virulence testing.

Determination of the optimal dose

To determine the appropriate and optimal dose to use, five couples of 24 hours old insects were also introduced in 225 ml jars, each containing 100 healthy grains (maize for *S. zeamais*, cowpea and kersting's groundnut for *C. maculatus*), to which were added in powder form, different doses (0 g, 0.0002 g, 0.002 g, 0.02 g per jars representing 0, 10^8, 10^9, 10^{10}conidies/100 grains respectively) of each of the two isolates (ma356 and ma357) identified as virulent during the pathogenicity test. Dead insects were daily removed for 5 days and 21 days for *C. maculatus* and *S. zeamais* respectively; and incubated for evaluation of sporulation. Each dose was replicated three times for each commodity as well as the control. The mortality speed and rate of the insects associated to each dose, were used to determine thqe appropriate dose for management of the studied pests. The following parameters were evaluated:

Insect Mortality: after infestation by the isolates, the dead individuals were daily counted from the beginning until the fifth day for *C. maculatus* and the twenty-first day for *S. zeamais*.

Sporulation: sporulation rate was assessed by incubating the dead insects in petri dishes containing slightly wet filter papers.

Eggs-hatching: 15 days after infestation, the number of eggs (hatched and unhatched) laid by surviving females of *C. maculatus* was counted under a binocular magnification 40.

Adults' emergence: from the 21st day until the 40th day, imagos were removed and counted as soon as they emerge.

Seed weight loss: 40 days after infestation, seeds used in the experiments were weighed to determine the loss in weight of the grains.

Statistical analysis

Data in percentage were arcsine-transformed and then subjected to analysis of variance using SAS software version 9.1 (2003). Means were separated using the SNK (Student Newmans Keuls) test at 5% level of confidence. Average Survival Time (AST) were calculated using Kaplan-Meier survival analysis. The log-rank test was used for pairwise comparison of the ASTs.

The hatching rate was determined as the ratio in percentage of the number of hatched eggs and the total number of eggs laid. The emergence rate was determined as the ratio in percentage of the total number of adults insects emerged and the total number of eggs laid. Seed weight Loss was determined as the difference in weight of the grains before and after infestation.

Lethal Dose (LD50) *estimation*

Analysis and modeling of time-dose-mortality data were performed, using the model "Cox regression" (SPSS, 1989-2007). Cox regression models use the hazard function for estimating the relative risk of failure. The hazard function, h (t) is an assessment of the potential death of an individual per unit time at a given time, as the individual has survived until now. Cox regression models were expressed in terms of hazard function as follows:

$$h(t) = [h_0(t)]\, e^{(BX)} \quad (1)$$

Where X represents log (dose), B the regression coefficient is the relative risk (here instantaneous risk of death) associated to a treatment compared to another treatment, *e* is the base of the natural logarithm and h_0 (t) is the hazard function when X is equal to 0.

Cumulative hazard function, H (t), is related to the survival function and may be derived from the survival function as follows:

$$H(t) = -\ln S(t) \quad (2)$$

The hazard function and survival function are closely linked and both have been calculated using the method of Cox regression (SPSS, 1989-2007). LD50 can be derived from equations (1) and (2) as follows:

$$X = 10\text{^}\ln(\ln(0.5) - \ln(h_0(t)))/B \quad (3)$$

The confidence intervals for the LD50 were calculated on the basis of the same equations, using the following information: standard deviation (SD) of B and standard deviation (SD) of h_0 (t).

Results

Pathogenicity of M. anisopliae isolates on C. maculatus in kersting's groundnut and cowpeas

Results indicated significant difference between treated and untreated insects in kersting's groundnut (P = 0.0057) as well as cowpea (P = 0.0050). However, there were no significant differences between the isolates on both diets, although ma356 was the isolate that caused the highest mortality rate in the kersting's groundnut and cowpea.

Table 1. Average survival Times (Days) of *C. maculatus* and *S. zeamais* after infestation with *M. anisopliae* isolates.

Insects	Crops	Control	ma351	ma356	ma357	ma358
C. maculatus	Cowpea	5.60 ± 0.13 a*	3.50 ± 0.24 bc	3.43 ± 0.25 c	3.60 ± 0.29 bc	4.33 ± 0.31 b
	Kersting's groundnut	5.47 ± 0.23 a	3.53 ± 0.27 bc	3.27 ± 0.22 c	4.00 ± 0.32 b	3.63 ± 0.12 bc
S. zeamais	Maize	21.03 ± 0.39 a	18.93 ± 0.79 b	17.77 ± 1.07 b	14.80 ± 1.19 c	17.80 ± 0.89 b

*Means with a row followed by same letter are not significantly different (t-student test, with $p<0.05$).

Table 2. Estimates of B value resulting from the Cox regression for *Metarhizium anisopliae* isolates (ma356 and ma357) on the tested storage pests, including Wald coefficients.

Insects	Crops	B	SE	Wald	Df
C. maculatus	Kersting's groundnut	0.406	0.062	43.241	1
	Cowpea	0.308	0.046	44.296	1
S. zeamais	Maize	0.613	0.094	42.802	1

These rates were 93.3 ± 3.3 % and 100 ± 0.0% in the Kersting's groundnut and cowpea respectively (Fig. 1 & 2). Overall, the average survival time of treated insects was significantly lower than those of untreated individuals.

The analysis of variance showed that in both cases the individuals treated with the ma356 isolate had the lowest average survival times (Table 2). In addition, sporulation rates of dead individuals of *C. maculatus* were the highest in both diets and with the same isolate (Fig. 4).

Pathogenicity of M. anisopliae isolates on S. zeamais in Maize

Fig. 3 shows the evolution of mortality of *S. zeamais* over time, after inoculation of maize kernels with *M. anisopliae* isolates. The highest mortality rates were obtained in individuals treated with the isolates. The highest mortality rate was obtained with the ma357 isolate; however, no significant differences were recorded between the isolates used. Whether in cowpea or kersting's groundnut, the *M. anisopliae* isolates seem to be less effective on *S. zeamais* in maize with a higher average survival time compared to those obtained on *C. maculatus*.

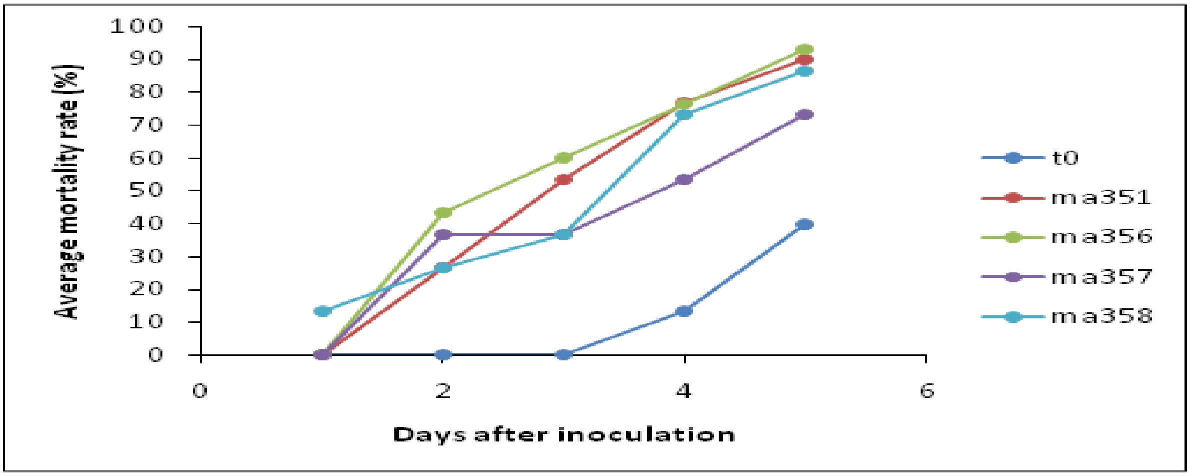


Fig. 1. Evolution over time of the mortality of *C. maculatus* after inoculation with isolates of *M. anisopliae* in the Kersting's groundnut.

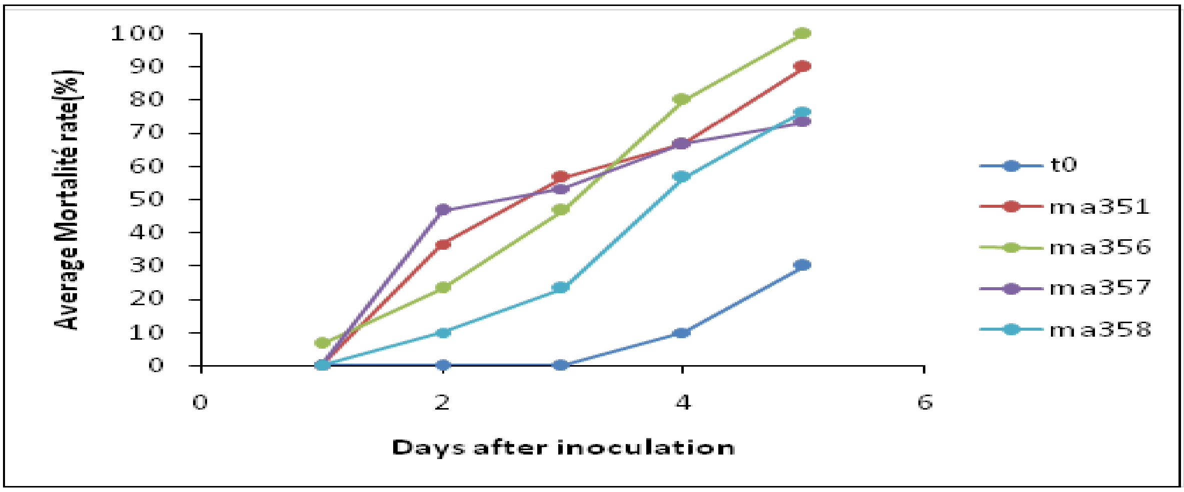


Fig. 2. Evolution over time of the mortality of *C. maculatus* after inoculation with *M. anisopliae* isolates in cowpeas.

The lowest average survival time was obtained on insects treated with ma357. This isolate was the most effective in controlling *S. zeamais* (Table 2).

Determination of the optimal dose of M. anisopliae on C. maculatus and S. zeamais

Determination of the optimal dose of ma356 on C. maculatus in Kersting's groundnut *and cowpeas*

Evolution of mortality rates of *C. maculatus* individuals subjected to the isolate Ma356 in cowpea followed a similar variation as that of the mortality rates in kersting's groundnut (Fig. 5 & 6). The dead insects' sporulation rates were significantly different among doses (P = 0.001). The sporulation rate varied according to the conidia dose used. The higher the dose, the more was the dead insects' sporulation.

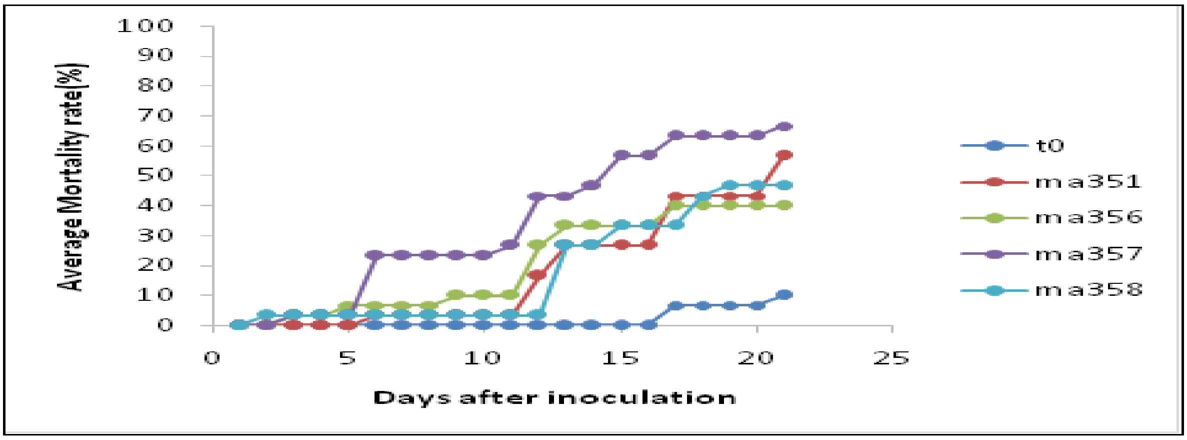


Fig. 3. Evolution over time of mortality of *S. zeamais* after inoculation with isolates of *M. anisopliae* in Maize.

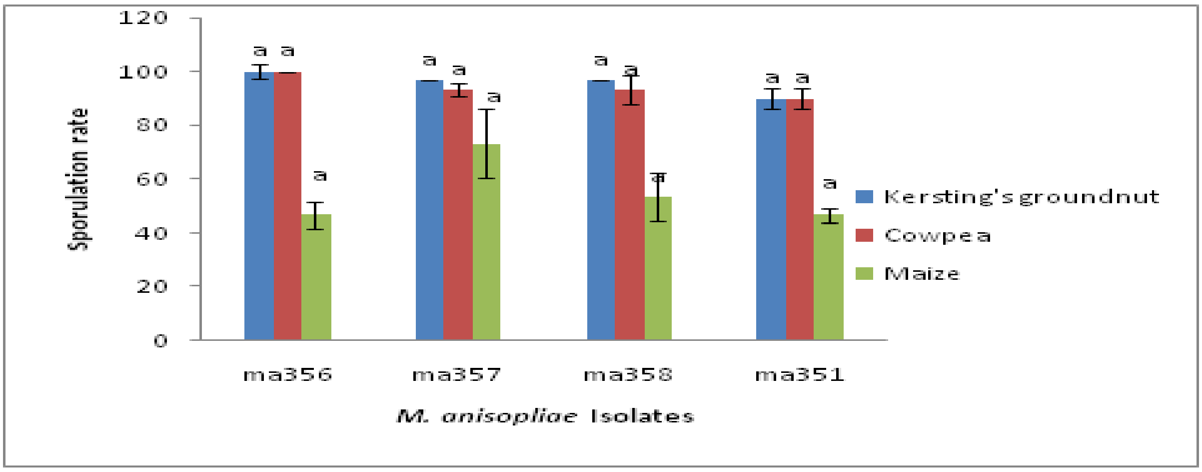


Fig. 4. Average sporulation rates of dead insects from treatments with *M. anisopliae* isolates.

Determination of the optimal dose of ma357 on S. zeamais in Maize

Contrary to the trend observed in the experiment on *C. maculatus*, ma357 had eliminated individuals of *S. zeamais* from the 15th day after inoculation and this at all doses. The evolution of the mortality rate over time was almost the same at 10^{10} and 10^{9} doses of conidia/100grains (Fig. 7). The sporulation rates of dead individuals of *S. zeamais* were dose dependent. The higher the dose, the greater is the sporulation rate. The analysis revealed a significant difference between the sporulation rates of the different doses (P = 0.015).

Determination of the LD50

LD50 after treatment with ma356 on C. maculatus

Adjustment of the data by the Cox regression model to the curves of LD 50 was relatively good, and was based on the value of B (0.308 in cowpeas and 0.406 in kersting's groundnut).

The higher the B value, the narrower were the confidence intervals. LD50 values were similar for *C. maculatus* in various diets. Thus, a dose of 10^{4} conidia (2.00 x10^{-7} g) in 100 cowpea seeds was necessary to kill 50% of individuals of *C. maculatus* in four days or a dose of $10^{4.1}$ (2.52 x10^{-7} g) conidia in 100 grains of kersting's groundnut to kill 50% of individuals of *C. maculatus* in four days as well (Fig. 8 & 9).

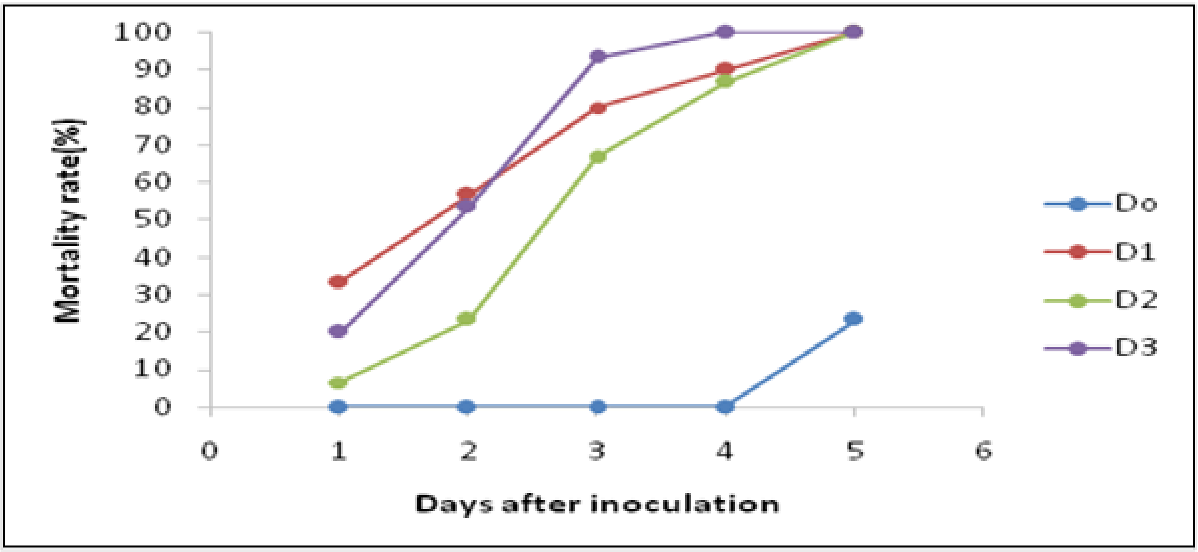
LD50 after treatment with ma356 on S. zeamais

The Cox regression model of the LD 50 curve for ma356 had well-adjusted the data, based on the high value of B (B = 0.613). This is also evident by the size of the confidence intervals. The relationship dose-response was a very strong for ma356. The effect dose/response was higher for this insect, but the LD50 was the highest with $10^{8.9}$ conidia (1.59 x 10^{-2} g) in 100 maize kernels, to kill 50% of individuals *S. zeamais* in four days (Fig. 10).

Effectiveness at different doses of the isolate ma356 on egg-laying of females, hatching rate and emergence rate of C. maculatus individuals

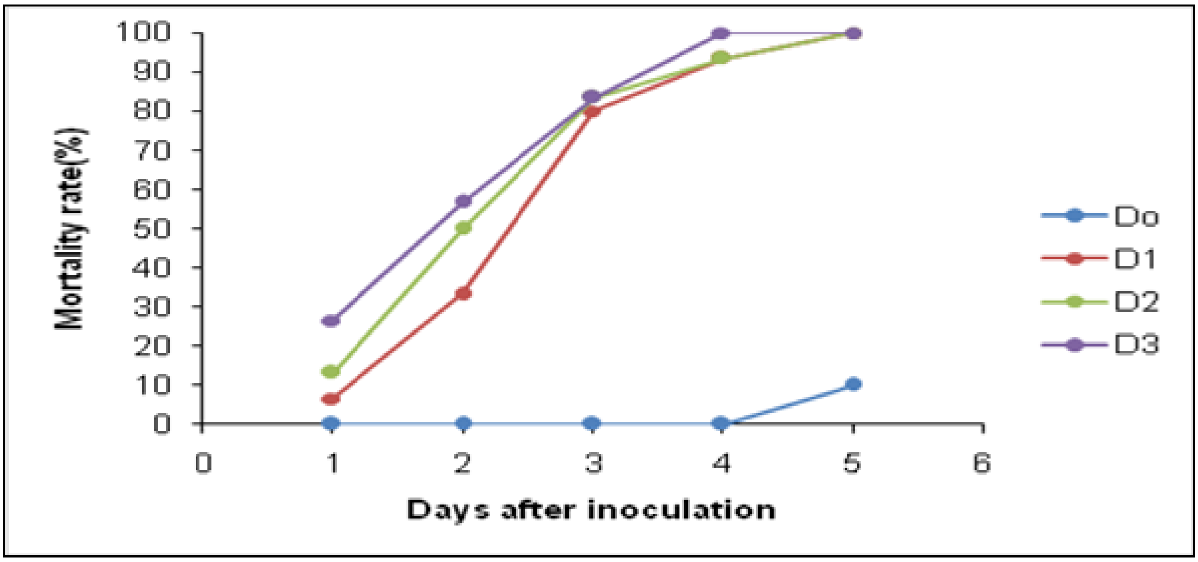
Results showed that ma356 influences egg-laying of the females of *C. maculatus* and this in both food diets used. There were no significant differences for the eggs hatching rates in kersting's groundnut (P = 0.89). Controversially, a significant difference was observed when cowpea was used as diet (P = 0.001) (Fig. 11 & 12).

As concerning the imagos emergence rate, there was no significant difference between the fungi doses used. At the dose of 10^{10} conidia in 100 grains all hatched eggs have emerged (Fig. 13). However, a very low egg-laying rate was recorded with both food diets.



Do= 0g /100 grains; D1= 0.0002g /100grains; D2= 0.002g /100grains;
D3= 0.02g /100grains

Fig. 5. Evolution over time of mortality of *C. maculatus* after inoculation with different doses of ma356 in Kersting's groundnut.

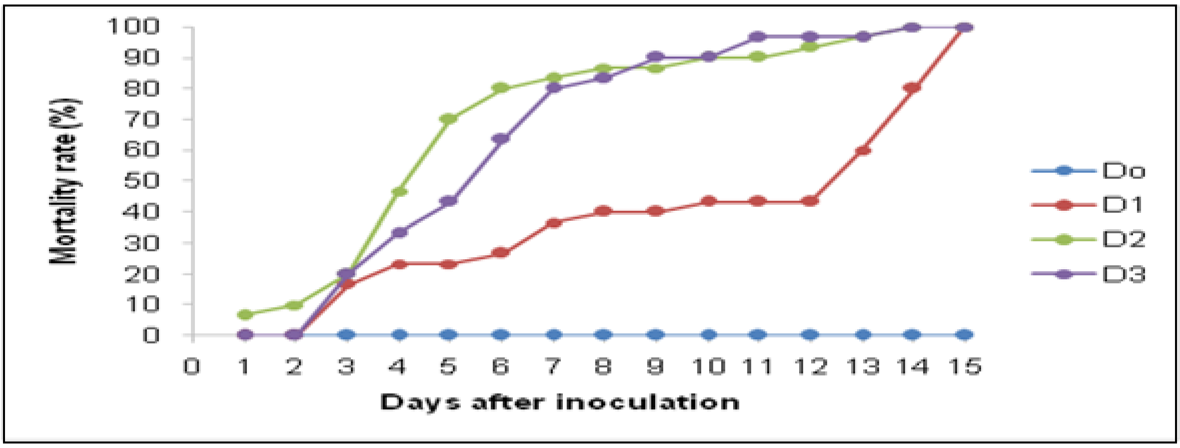


Do= 0g /100 grains; D1= 0.0002g /100grains; D2= 0.002g /100grains;
D3= 0.02g /100grains

Fig. 1. Evolution over time of mortality of *C. maculatus* after inoculation with different doses of ma356 in Cowpea.

Seed weight loss after treatments

The seed weight loss varied between 0.21 ± 0.09 g (D3, cowpea) and 2.06 ± 0.43 g (Do, cowpea). There was no significant difference (P = 0.088) was recorded in the maize kernels from the treatments though the seed weight loss of the dose Do was significantly higher than those recorded in other treatments. Similar results were observed in kersting's groundnut (P = 0.293). On the other hand, the difference was significant on cowpea (P = 0.032) (Fig. 14).



Do= 0g /100 grains; D1= 0.0002g /100grains; D2= 0.002g /100grains;
D3= 0.02g /100grains

Fig. 7. Evolution over time of mortality of *S. zeamais* after inoculation with different doses of ma357 in maize.

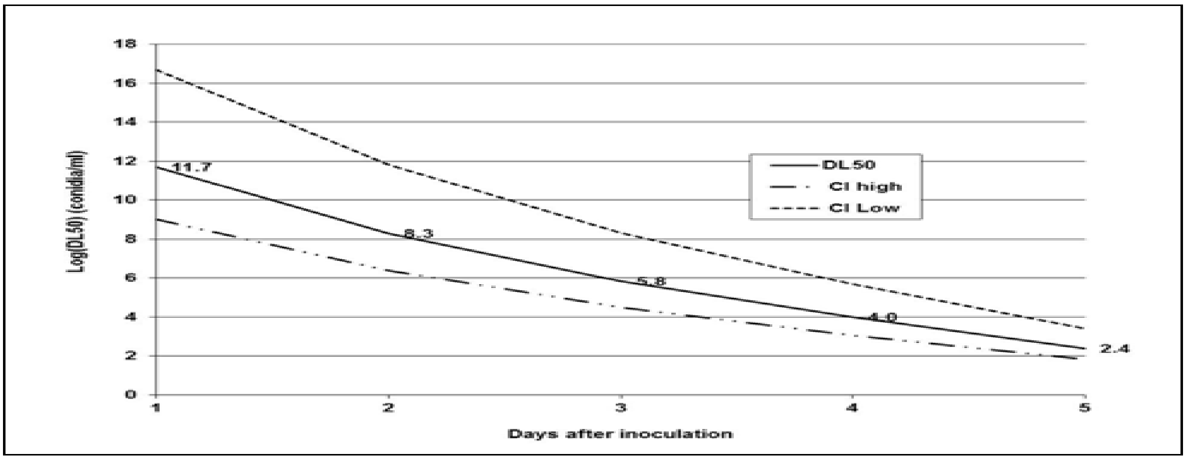


Fig. 8. LD50 Values after treatment of individuals of *C. maculatus* with different doses of ma356 in cowpea.

Discussion

Pathogenicity of M. anisopliae isolates on C. maculatus and S. zeamais

The assessment of pathogenicity *of M. anisopliae* isolates on the studied storage pests, including *C. maculatus* and *S. zeamais* showed they have a lethal effect on these insects. Generally, in the presence of *M. anisopliae* isolates, the lifespan of the treated insects was significantly lower than those of untreated insects.

On *C. maculatus*, the analysis revealed a significant difference in mortality rates between treated and untreated individuals for both food diets (cowpea and Kersting's groundnut). However, no significant differences were observed between the different isolates while the highest mortality rate of pests (80.9% cowpea and 74.7% Kersting's groundnut) was obtained with the isolate ma356. The lowest average survival times of the pests were obtained with this same isolate (3.43 ± 0.25 days and 3.27 cowpea ± 0.22 days in Kersting's groundnut).

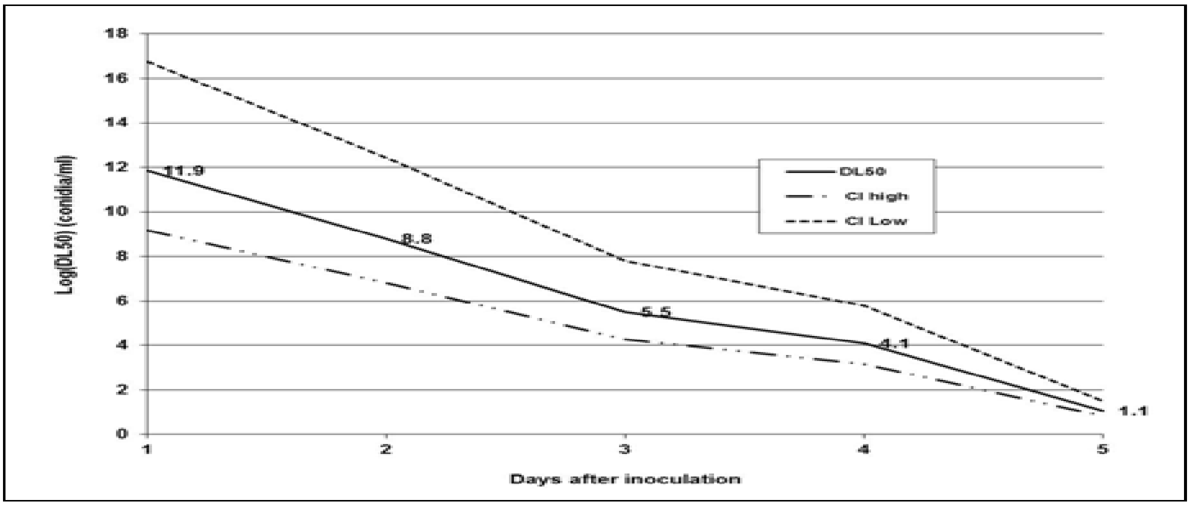


Fig. 9. LD50 Values after treatment of individuals of *C. maculates* with different doses of ma356 in Kersting's groundnut.

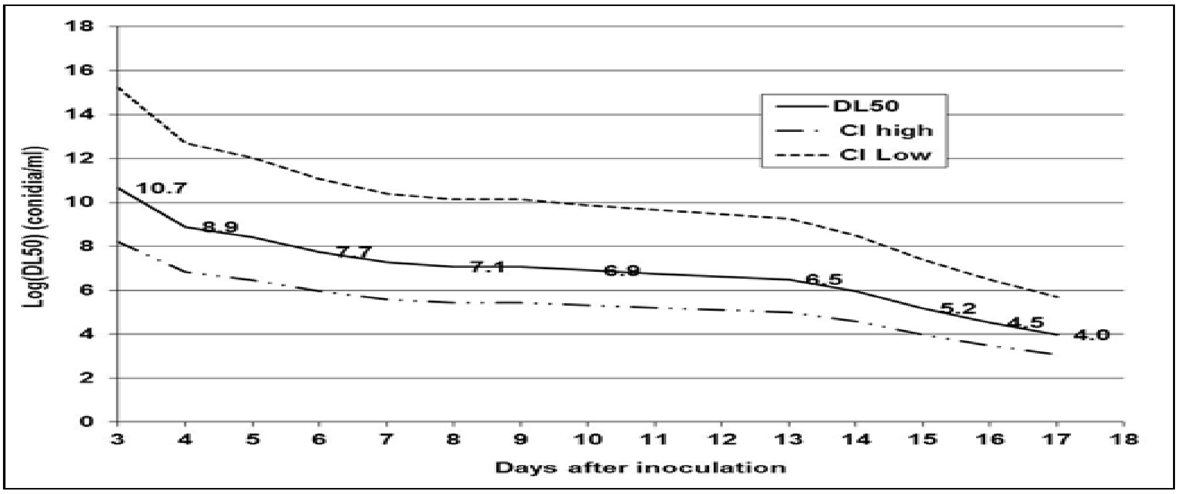


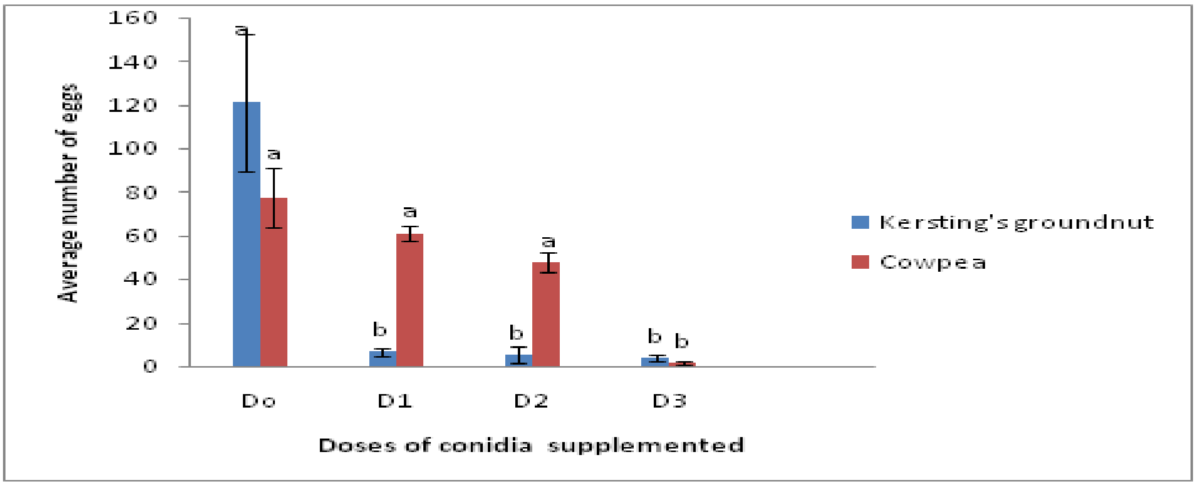
Fig. 10. LD50 values after treatment with individuals of *S. zeamais* at different doses of ma356 in maize.

In addition, the highest sporulation rates (100%) were obtained on the dead insects treated with this isolate.

On *S. zeamais*, the means comparison test showed that ma357 is the most effective of all in that it generated the highest mortality rate (56.97%), sporulation rate (73%) and the lowest average survival time (14.80 ± 1.19 days). Therefore, the use of pathogenic fungi especially *M. anisopliae* in post-harvest could be an effective control method to promote. Indeed, studies showed that the use in post-harvest of entomopathogenic fungi especially *M. anisopliae* and *B. bassiana* may be effective for controlling pests (Hidalgo *et al.*, 1998; Kassa *et al.*, 2002; Batta, 2005).

The low rates of mortality and higher average survival time observed in individuals of *S. zeamais* could be due to the fact that this pest is more resistant than *C. maculatus*. This could be explained by the fact that the immune system of *S. zeamais* did not favor the germination of conidia, which reduced their percentage of penetration.

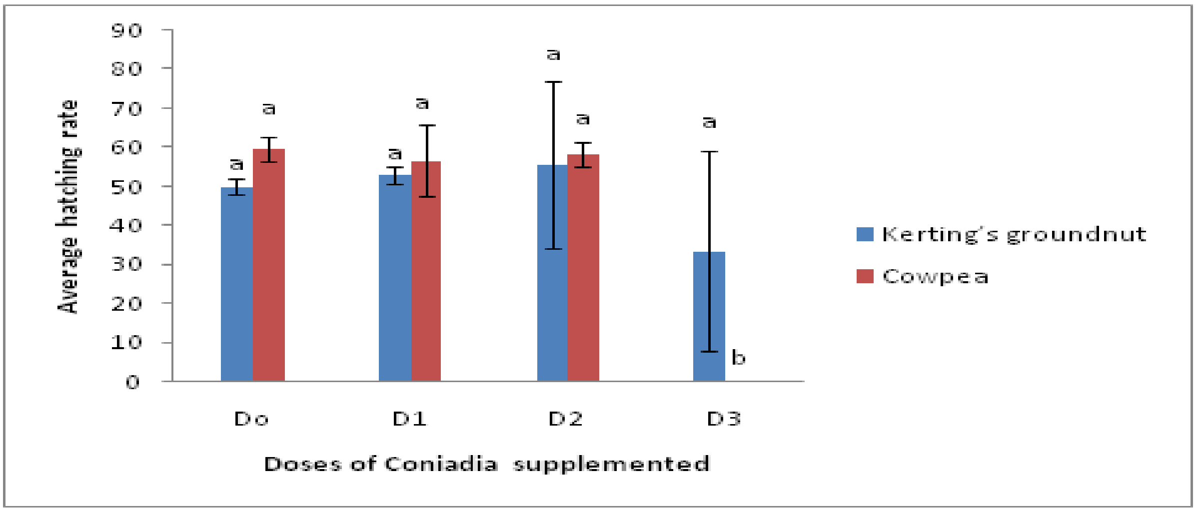
This confirms the results of Andersen (1979) who showed that the immune system of insects can strongly influence the pathogenicity of entomopathogenic. Thus, insect sensitivity to *M. anisopliae* toxin differs from one pest to another. This supports the assertions of Kershaw *et al.* (1999) who asserted that insects are differently sensitive to the toxins produced by entomopathogenic fungi.



Do= 0g /100 grains; D1= 0.0002g /100grains; D2= 0.002g /100grains;

D3= 0.02g /100grains

Fig. 11. Average number of eggs laid by females' individuals of *C. maculatus* after inoculation with *M. anisopliae* isolate ma356 on Kerting's groundnut and cowpea diets.



Do= 0g /100 grains; D1= 0.0002g /100grains; D2= 0.002g /100grains;

D3= 0.02g /100grains

Fig. 12. Average hatching rate of eggs of *C. malaculatus* after inoculation with ma 356.

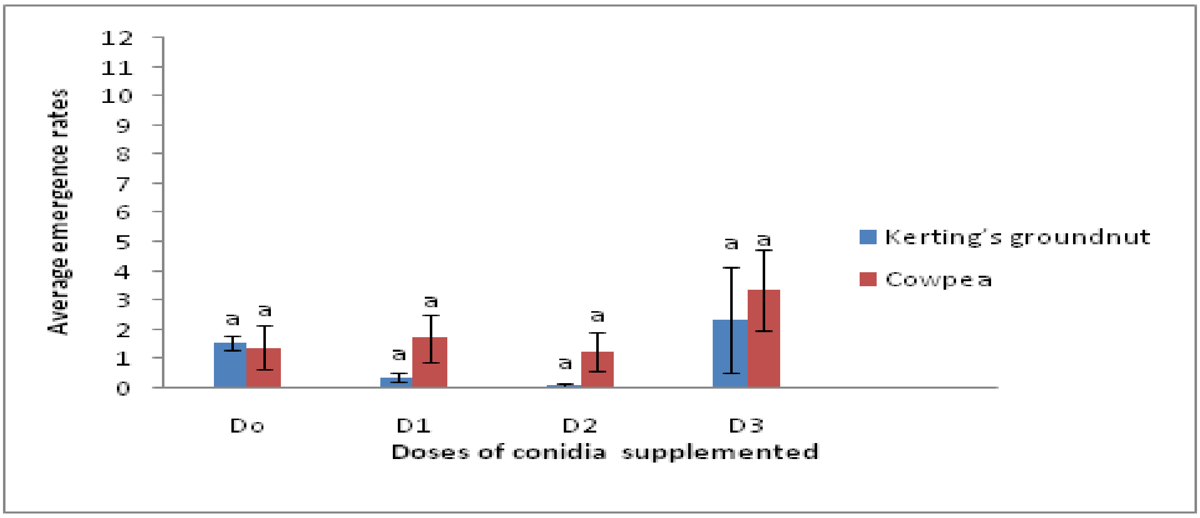
Lethal dose and effects *of M. anisopliae isolates on growth parameters, lifespan and survival of S. zeamais and C. maculatus in stored products (Maize, Cowpea and Kerting's groundnut respectively)*

Assessment of virulence of the isolates which proved to be effective for the control of *C. maculatus* (ma356) and *S. zeamais* (ma357) at different conidia doses (0, 10^8, 10^9 and 10^{10}conidies/100 grain) showed that mortality of the pests and sporulation of dead individuals vary depending on the doses. The higher the dose, the higher the mortality rate increases and the sporulation rate as well.

Our results corroborate those of Douro Kpindou *et al.* (2012) who demonstrated that the number of L3 larva of *Helicoverpa armigera* died after inoculation of *Metarhizium* is dose dependent. Khashaveh (2011) showed that the use of Boverosil (*B. bassiana* formulation containing 5.92 x 10^9 conidia/g) caused 90% mortality in adult *S. granarius* at a dose of 5.92 x 10^8 conidia/ml. In addition, the ma356 isolate used for the control of *C. maculatus* had significantly reduced the egg-laying percentages, hatching and emergence of the imagos. However, the analysis of variance revealed no significant difference in the egg hatching rate.

The isolate ma356 has thus no effect on fertility of *C. maculatus* female and is not an ovocide type isolate but a larval type since it reduced adult emergence.

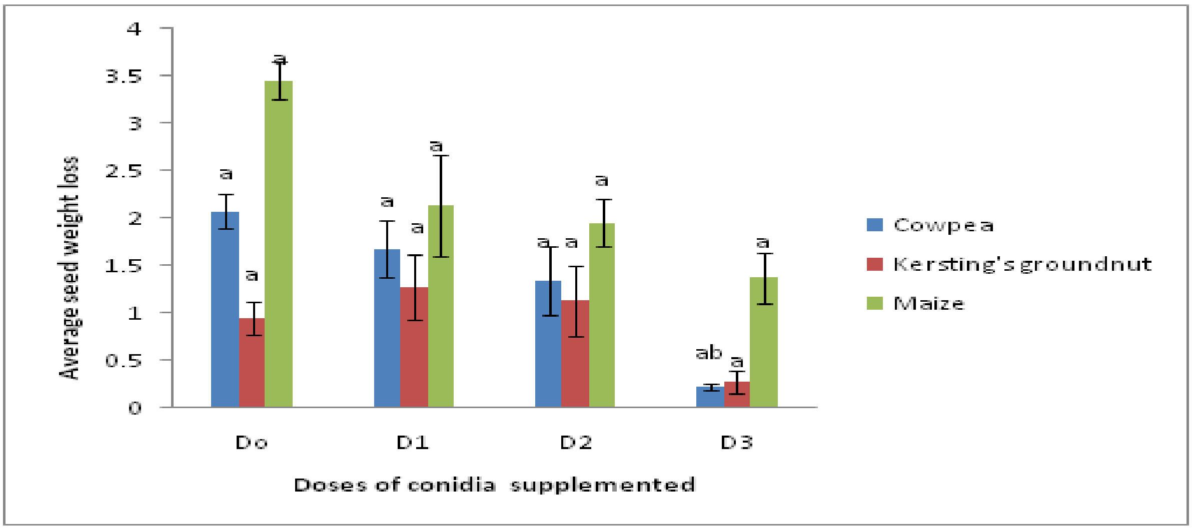
There was a reduction in the rate of emergence of the females in presence of ma356 isolate in both diets. The same toxic action of this fungus was recorded by treating different stages of *Lymantria dispar* (Ouakid *et al*., 2005).



Do= 0g /100 grains; D1= 0.0002g /100grains; D2= 0.002g /100grains;

D3= 0.02g /100grains

Fig. 13. Average emergence rates of imagos of *C. malaculatus* after inoculation with ma356.



Do= 0g /100 grains; D1= 0.0002g /100grains; D2= 0.002g /100grains;

D3= 0.02g /100grains

Fig. 14. Average seed weight loss per treatment.

The action of individuals of *C. maculatus* is more limited on the Kersting's groundnut grains than on cowpea grains. This is justified by the fact that low egg hatching, emergence and seed weight loss were recorded on the Kersting's groundnut grains. This underlines that cowpea is the preferred host plant of *C. maculatus*.

This confirms the results obtained by Sankara *et al.* (2012) that demonstrated that the transfer of *C. maculatus* on other legumes and its incubation on these hosts for a relatively long time; influences its preference for this host, but does not change its ovipositional preference for the reference host plant cowpea.

Conclusion

Overall, this study showed that *Metarhizium anisopliae* isolates used are pathogenic to the targeted storage pests but their effectiveness varies from one insect to another. This effectiveness is function of the dose of conidia used and the pest. From all isolates, ma356 and ma357 proved to be the most virulent on *C. maculatus* and *S. zeamais* respectively. Thus the dose of 10^{10} ma357 is the optimal dose to use for better management of *S. zeamais* while a dose of 10^9 conidia of ma356 could be effective in managing *C. maculatus*. LD50 values obtained on *C. maculatus* are similar for both diets (cowpea and kersting's groundnut) while the value obtained on *S. zeamais* is much higher and this in four days. Nevertheless, *C. maculatus* caused more damage on cowpea as compared to kersting's groundnut.

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