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RESEARCH ARTICLE

Mortality of *Maruca vitrata* (Lepidoptera: Crambidae) larval stages induced by different doses of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*

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Abstract

The dose-dependent mortality of second (3-4 days old), third (5-6 days old) and fourth (7-9 days old) larval instars of *Maruca vitrata* as induced by the entomopathogenic fungal isolates, *Metarhizium anisopliae* Ma 29 and *Beauveria bassiana* Bb 115 was determined in a laboratory experiment. For each fungal isolate, five doses (0, $2x10^4$, $2x10^5$, $2x10^6$ and $2x10^7$ conidia/larva, respectively) were applied topically to one individual larva at the rate of 2 µl of an oil formulation. Observations were focused on mortality and sporulation of treated larvae. Cox regression was used to determine the lethal dose 50 (LD50). Results revealed high mortality of the third larval stage (L3) at 2×10^6 conidia for Ma 29 and Bb 115 with 49% and 96% mortality, respectively. No significant difference in mortality was observed between larval stages exposed to $2x10^7$ conidia/ larva. The sporulation rate did not differ significantly between larval stages. Regardless of application doses, the Mean Survival Times (MSTs) of larvae were significantly different between doses for both fungal species. The lowest MSTs were recorded for the younger larvae (L2, L3) at higher doses ($2x10^6$ and $2x10^7$ conidia/larva). Larval mortality increased with increasing dose, regardless of fungal species and larval stages, suggesting a dose-dependent response of *M. vitrata* larvae

Introduction

Cowpea, *Vigna unguiculata* Walp. (Fabaceae) is a major grain legume produced and consumed in Africa. As a good source of protein, it plays a significant role in human nutrition and thereby in food security and poverty reduction (Singh et al., 1990). However, its production is limited by several constraints, mainly biotic pressure from insect pests and diseases. Several insect species have been reported damaging cowpea at different growth stages: among them, the legume pod borer (LPB) *Maruca vitrata* Fabricius, (Lepidoptera: Crambidae) is the most serious worldwide (Liao and Lin, 2000). Larvae of this insect attack flower buds, flowers, and young pod (Singh and Jackai, 1985; Okech and Saxena, 1990), inducing over 80% yield losses (Okeyo-Owuor et al., 1983; Singh et al., 1990; Tamò et al., 2003).

To control *M. vitrata*, synthetic pesticides can give a quick relief and are commonly applied in Africa. However, the overreliance on synthetic pesticides leads to some unwanted side-effects such as environmental pollution, pest resistance and toxicity to other non-target organisms (Ekesi et al., 2002). The limited success of these control methods calls for the development of alternatives that are more effective and respectful of the environment and human health, and more economically profitable.

Such alternative methods include the use of natural enemies of insects such as bacteria, viruses and fungi. Srinivasan et al., 2009 have reported biological control as a key component of integrated pest management of cowpea, and this includes the use of micro-organisms such as entomopathogenic fungi. *Metarhizium anisopliae* (Metchinikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales:

Ophiocordycipitaceae) have already been the object of a previous study evaluating the effects of different concentrations of 11 isolates of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* on the five larval stages of *M. vitrata* in the laboratory (Toffa et al., 2014). This study has permitted to identify the most promising isolate each from both fungal species, which are now selected for more in-depth studies in the present work.

Douro Kpindou et al., (2012b) performed lab tests with isolates of *M. anisopliae* and *B. bassiana* on larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and reported mortality rates ranging from 58% to 74%. The same study indicated that, for all isolates tested and irrespective of the larval stage of *H. armigera*, the dose/effect responses were always significant. Although Soundararajan and Chitra (2011) showed that *B. bassiana* significantly reduced the damage caused by *M. vitrata* in the field in India, very little work has been carried out so far on the use of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* in controlling the larval populations of this pod borer on cowpea in Africa.

More specifically, this investigation was conducted to assess the efficacy at various doses of *M. anisopliae* and *B. bassiana* on larval stages of *M. vitrata*, also in view to determine the lethal dose (LD50) for both fungal species.

Materials and methods

Study site

All experiments were conducted at the International Institute of Tropical Agriculture(IITA),Benin-Station in Cotonou at a temperature of 26 ± 0.50 ºC and 65.5 ± 5% RH.

Mass rearing of *M. vitrata* larvae

Pupae of *M. vitrata* obtained from a stock culture at IITA-Benin were placed in open Petri dishes. They were incubated in wooden cages (44 x 45 x 58 cm) with sleeves, having sides of fine mesh and a glass top, at 27.0 ± 0.6 °C and 60.9 ± 4.6 % relative humidity. Emerging adults were fed using cotton fibres moistened with 10% glucose solution. Four-day-old female moths were transferred in groups of 4 or 5 individuals to transparent cylindrical plastic cups (3 cm diameter x 3.5 cm height) and kept for 24 h to allow for oviposition, which occurred on the inner surface of the cups. Ovipositing females were fed using small pieces of filter paper moistened with 10% glucose solution, which were replaced every 24 h. Cups carrying eggs were kept at the same experimental conditions until the larvae hatched. Larvae were transferred to large cylindrical plastic containers (9 cm diameter x 12 cm height) provided with artificial diet prepared according to Jackai and Raulston (1988), and reared until pupation. Pupae were collected and placed in cages until adult emergence. Eggs used in the experiments were obtained from this mass production.

Origin of the entomopathogenic fungi

The isolates of *M. anisopliae* and *B. bassiana* used in this study were obtained from the fungal collection of IITA-Benin and selected during a previous study for their pathogenicity to *M. vitrata* (Toffa et al., 2014). *Beauveria bassiana* was produced using Potato Dextrose Agar (PDA) (Becton, Dickinson and Company; Sparks, MD 21152 USA) while *M. anisopliae* was produced on Sabouraud Dextrose Agar (SDA) (Topley House, 52 Wash Bury, Lancashire, BL96AS, UK.).The isolate of *M. anisopliae* was 'Ma 29', whereas the isolate of *B. bassiana* was 'Bb 115'. For the two fungi, germination rate after 24h of incubation at 26°C ranged between 74.5 ±0.05% and 96.06±0.03%.

Experimental design

Different doses of the entomopathogenic fungi were used to inoculate second (3-4 days old, henceforth called L2), third (5-6 days old, L3) and fourth (7-9 days old, L4) larval instars of *M. vitrata*. For each fungus, six treatments were performed including two controls (i.e. without fungal conidia) and four doses of conidia at the application rates of $2x10^4$, $2x10^5$, $2x10^6$ and $2x10^7$ conidia per individual insect, mixed with peanut oil. In the first control *M. vitrata* larvae were inoculated with a peanut oil solution without any conidia (i.e. a white formulation), while in the second control *M. vitrata* larvae were not treated at all. Treatments were replicated three times in a randomized complete block design.

The fungi were inoculated by topical application (Bateman et al., 1996) of 2 µl of the oil formulation on the pronotum of the larva. For each treatment and each larval instar of *M. vitrata*, 40 larvae were used per replicate. Each larva was placed individually in a plastic cup (3.8 cm top diameter, 2.9 cm bottom diameter 4.0 cm height) with perforated cover for ventilation. The cups contained a piece of artificial diet and were kept on a bench in the laboratory at 20.7-24.2˚C and 80-97.5 relative humidity %. Holes (2 cm) punched in the lid of the cups were covered with fine mesh for aeration. Larval survival was checked daily. Cadavers were collected daily and put in Petri dishes

(diameter 9 cm) for 24 hours to dry out. They were subsequently incubated in Petri dishes containing wet filter paper and the presence or absence of sporulation was noted after 10 days.

Data analysis

Median Survival Times (MSTs) were calculated using Kaplan Meier Survivorship analysis(SPSS, 1989-2003). Mortality and sporulation rates MSTs were subjected Analysis of Variance (ANOVA), using the general linear model (GLM) procedure of SAS software version 9.1(2003). In case of significant F values, means were compared using SNK (Student-Newman-Keuls) at the probability level of 5%. Percentages were arcsine transformed and the MSTs were log transformed, before being subjected to ANOVA. The 15 days cumulative mortalities were used to draw graphs and to compare treatments.

Estimate of the LD50 (Lethal Dose 50)

Time-dose-mortality modeling was performed using the "Cox regression" model (SPSS, 1989-2003), which uses the Cox hazard function to estimate the relative risk of failure (Maddox, 1982). The hazard function, h (t) is an assessment of the potential death of an individual per unit of time at a defined time, given that the individual has survived until now. Cox regression models are expressed in terms of the hazard function as follows:

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

where X is log (dose), the regression coefficient B is the relative risk (here instantaneous risk of death) associated with one treatment versus another treatment, and h0 (t) is the function risk when X is 0. The cumulative risk, 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 the survival function are closely related, and both were calculated using the method of Cox regression (SPSS, 1989-2003).

The LD50 is defined as the dose of a product (chemical or biological) needed to induce the death of half of the tested organisms at a given time after application (Maddox, 1982). The LD50 can be derived from equations (1) and (2) as follows:

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

Confidence intervals for the LD50 were calculated based on the same equations.

Results

Susceptibility of M. vitrata larvaeto B. bassiana (Bb 115) and M. anisopliae (Ma 29)

Mortality

Bb 115 was pathogenic to all tested larval instars, but caused less mortality on L4than in younger stages. The doses of $2x10^4$ and $2x10^5$ conidia induced lower mortality in L2 with45.0 ± 0.8% and 59 ± 1.7% mortality, respectively ($F_{4,28}$ = 26.40, P <0.0001). On the other hand, significantly higher mortality rates across larval instars were obtained when $2x10^6$, $2x10^7$ conidia were applied. A statistically significant decrease in mortality rates was observed with increasing host age (F= 29.22, P <0, 0001) (Figure 1a).

The isolate Ma 29 produced no significantly different mortality rates between the different doses, regardless of larval age even if slight increases in mortality were observed at higher doses. However, mortality rates were significantly higher in fungal treatment compared to untreated controls (Figure 1b). High mortality rates were observed in L2, L3 and L4 when $2x10^6$, $2x10^7$ conidia were applied.

Table1:Mean survival time (MST) (days ± SE) and 95% CI [in square brackets]of L2, L3 and L4 instars of *M. vitrata* inoculated with fungal isolate Bb 115.

Treatments	Stades larvaires		
	L2	L3	L4
Témoin 1	13.5 ± 0.2 a	14.4 ± 0.2 a	14.7 ± 0.3 a
	[13.8 – 15.3]	[14.1 – 14.8]	[14.4 – 15.0]
Témoin 2	13.8 ± 0.4 a	14.2 ± 0.2 a	14.3 ± 0.4 a
	[13.8 – 15.9]	[13.2 – 15.2]	[13.7 – 15.0]
$2x10^4$ conidia/insect	7.2 ± 0.6 ab	10.4 ± 0.5 ab	11.3 ± 0.4 ab
	[6.2 – 8.2]	[9.5 – 11.3]	[10.0 –12.7]
$2x10^5$ conidia/insect	6.8 ± 0.5 ab	8.1 ± 0.6 b	10.4 ± 0.3 ab

	[5.9 - 7.8]	[7.2 - 9.0]	[9.0 – 11.8]
$2x10^6$ conidia/insect	4.3 ± 0.5 b	5.6 ± 0.5 c	7.8 ± 0.4 b
	[3.4 – 5.3]	[4.7 – 6.6]	[6.9 – 8.8]
$2x10^7$ conidia/insect	3.2 ± 0.7 b	4.6 ± 0.6 c	4.8 ± 0.4 c
	[2.3 – 4.1]	[3.7 – 5.6]	[3.9 – 5.8]
$F_{5.30}$	102.0	70.12	226.84
P	< 0.0001	< 0.0001	< 0.0001

In the same column, means followed by the same letter are not significantly different (ANOVA followed by SNK test at 5%

Table 2: Mean survival time (MST) (days ± SE) and 95% CI [in square brackets]of larvae L2, L3 and L4 of *M. vitrata* inoculated with fungal isolate Ma29.

Treatments	Larval Stages		
	L2	L3	L4
Control 1	13.4 ± 0.2 a	13.6 ± 0.2 a	14.8 ± 0.1 a
	[12.1 – 14.8]	[12.3 – 14.9]	[14.6 – 15.0]
Control 2	13.6 ± 0.1 a	14.5 ± 0.2 a	14.9 ± 0.1 a
	[12.4 – 14.9]	[14.1 – 14.9]	[14.7 – 15.0]
$2x10^4$ conidia/insect	9.7 ± 0.4 b	10.0 ± 0.3 ab	12.2 ± 0.4 ab
	[8.0 – 10.5]	[9.4 – 11.6]	[11.4 –13.0]
$2x10^5$ conidia/insect	10.0 ± 0.3 ab	10.6 ± 0.4 ab	10.8 ± 0.3 b
	[9.4 - 11.6]	[9.9 - 11.4]	[9.2 – 11.4]
$2x10^6$ conidia/insect	8.6 ± 0.6 b	8.1 ± 0.7 b	1 0.4 ± 0.3 b
	[7.4 – 9.7]	[7.2 – 9.9]	[9.8 – 11.9]
$2x10^7$ conidia/insect	7.3 ± 0.6 b	7.2 ± 0.5 b	8.6 ± 0.6 b
	[6.1 – 8.6]	[6.0 – 8.5]	[7.5 – 9.8]
$F_{5.30}$	16.74	9.70	3.87
P	< 0.0001	< 0.0001	< 0.0482

In the same column, means followed by the same letter are not significantly different (ANOVA followed by SNK test at 5%)

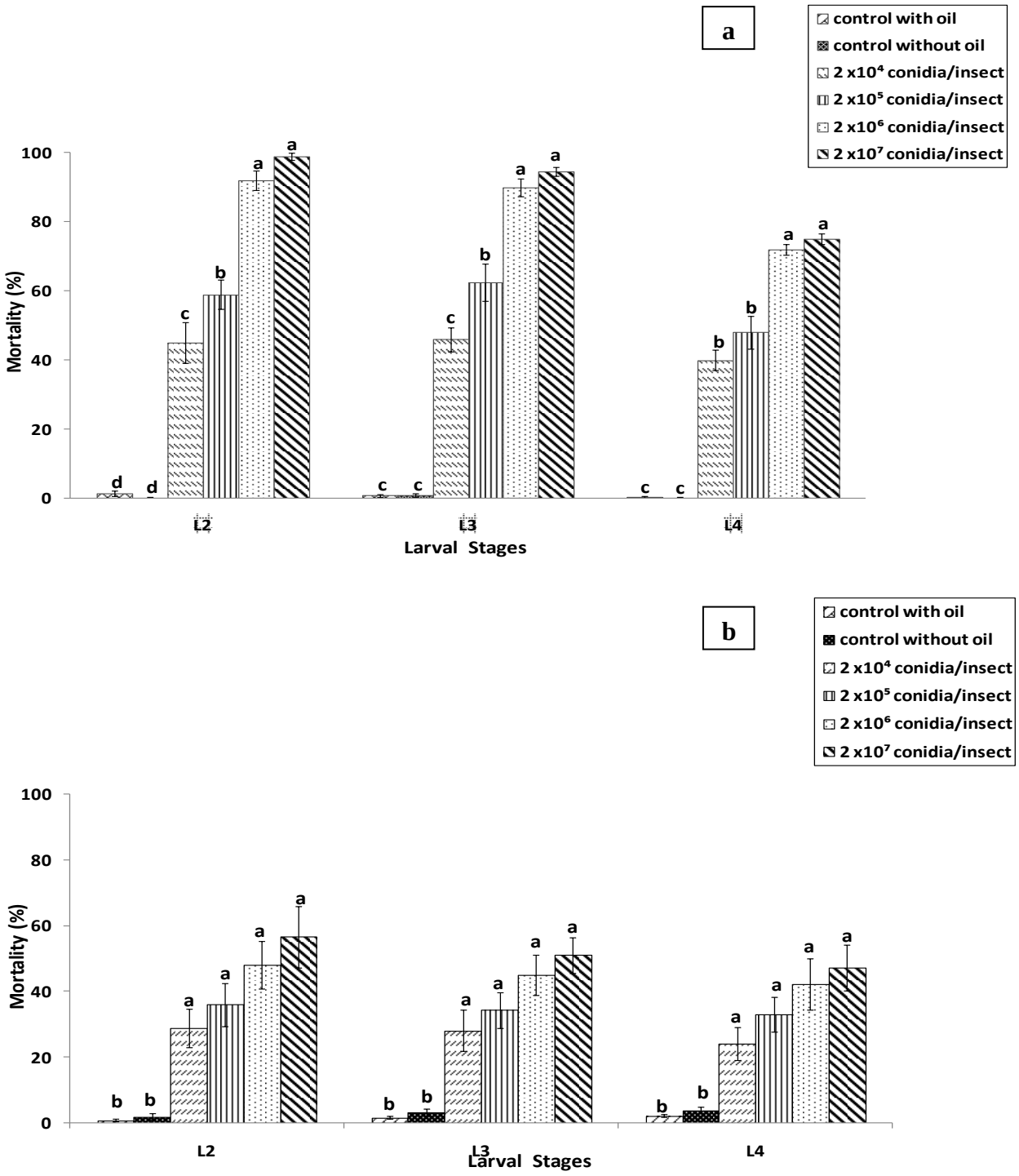

Fig. 1: Mortality of *Maruca vitrata* larvae treated with different doses of Bb115 (a) and Ma 29 (b) Treatments followed by the same letters are not significantly different at the 5% level (ANOVAfollowed by SNK test).

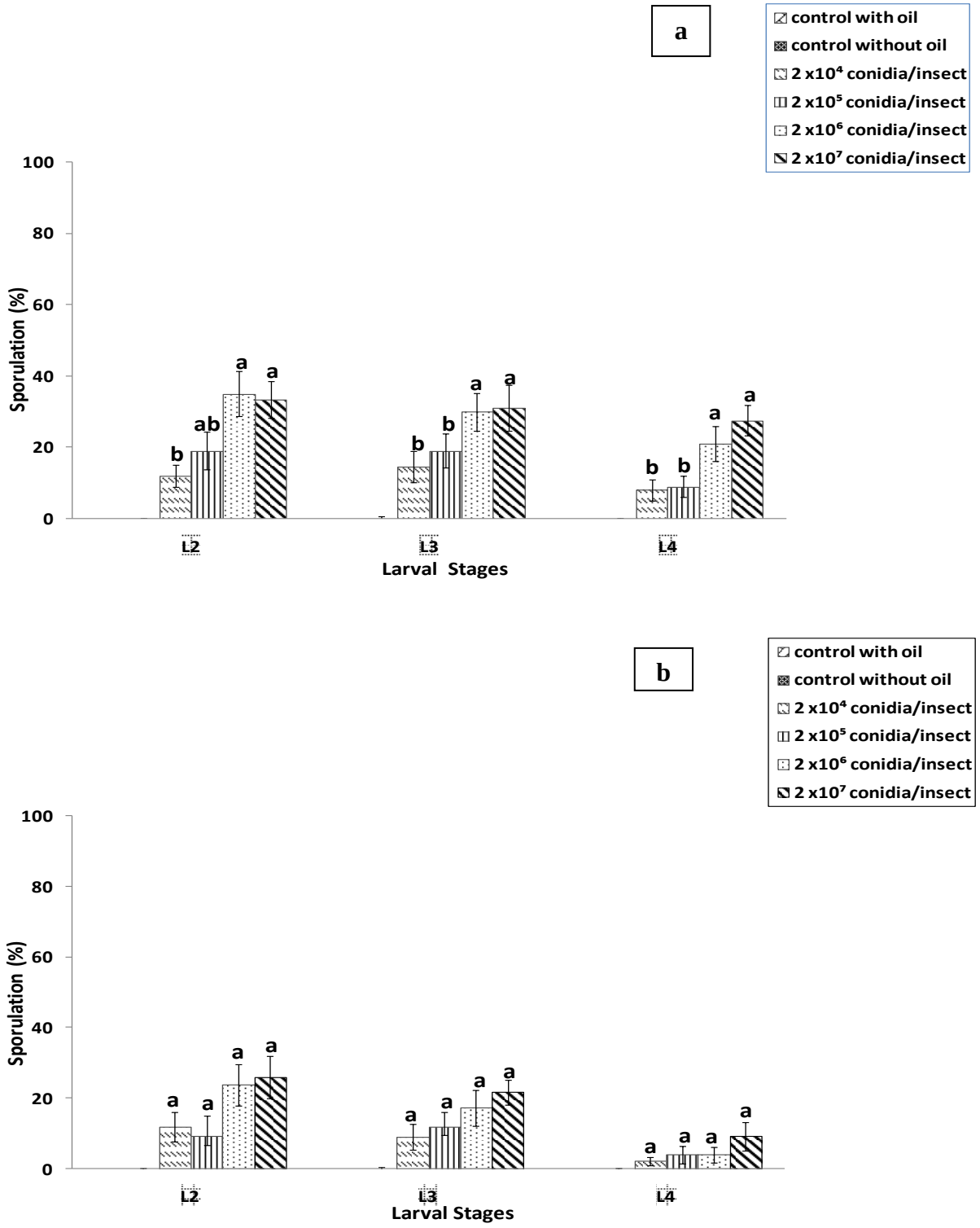

Fig. 2: Sporulation rate of *Maruca vitrata* larvae treated with different doses of Bb115 (a) and Ma 29 (b) Treatments followed by the same letters are not significantly different at the 5% level (ANOVA, followed by SNK test).

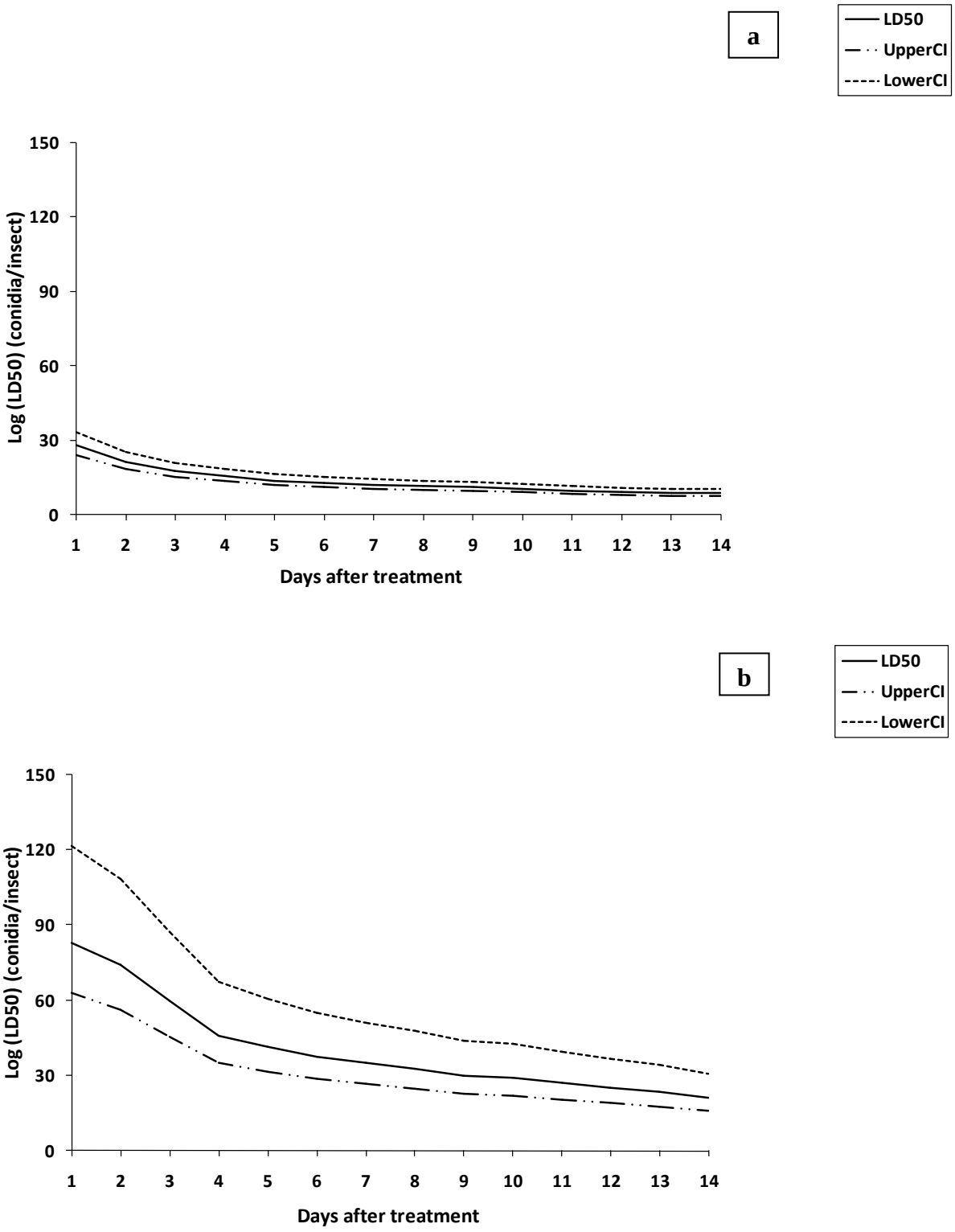

Fig. 3: LD50 values after treatment of the second stage of *Maruca vitrata* to various doses of Bb 115 (a) and Ma 29 (b).

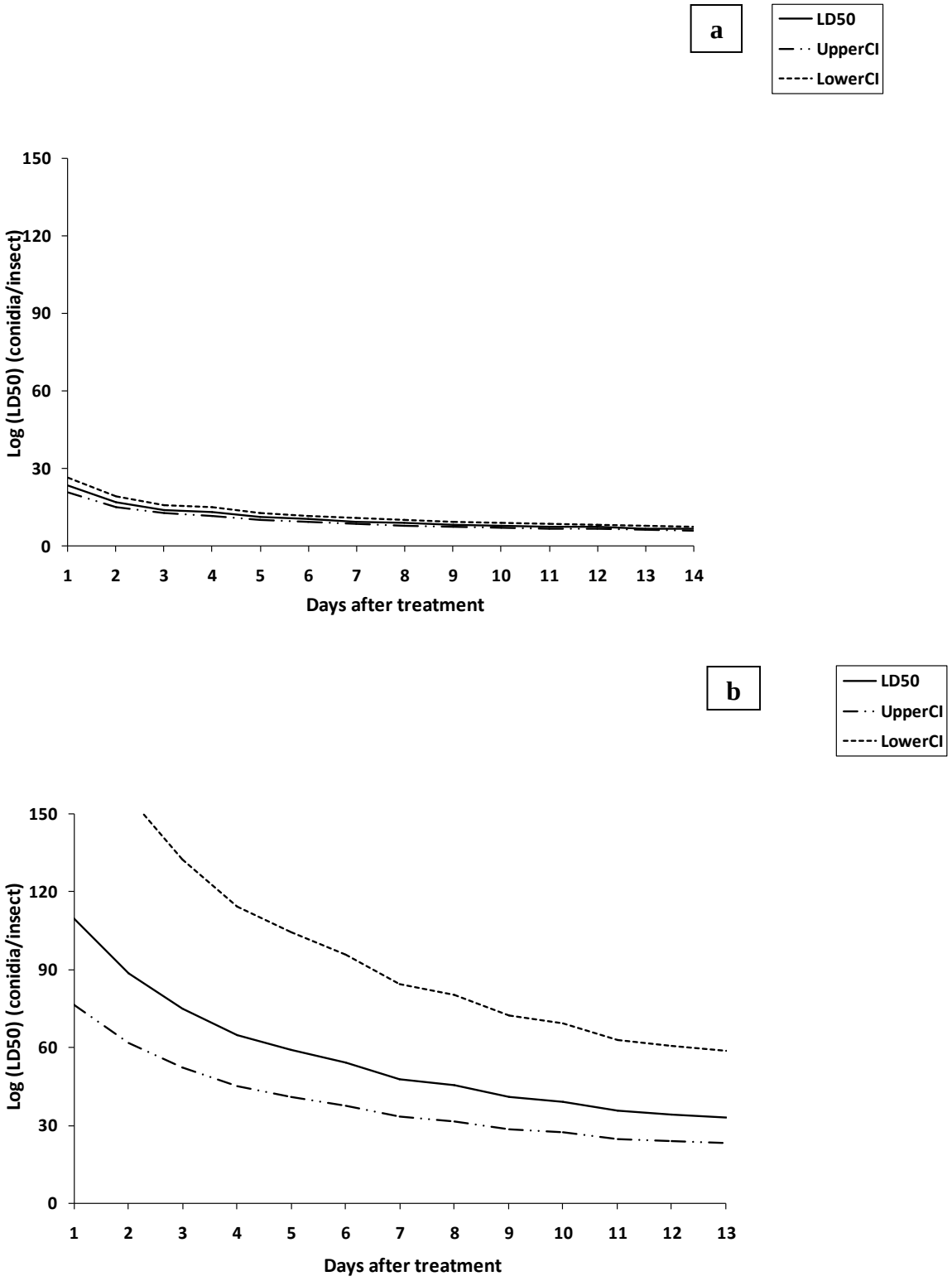

Fig. 4: LD50 values after treatment of the third stage of *Maruca vitrata* to various doses of Bb 115 (a) and Ma 29 (b).

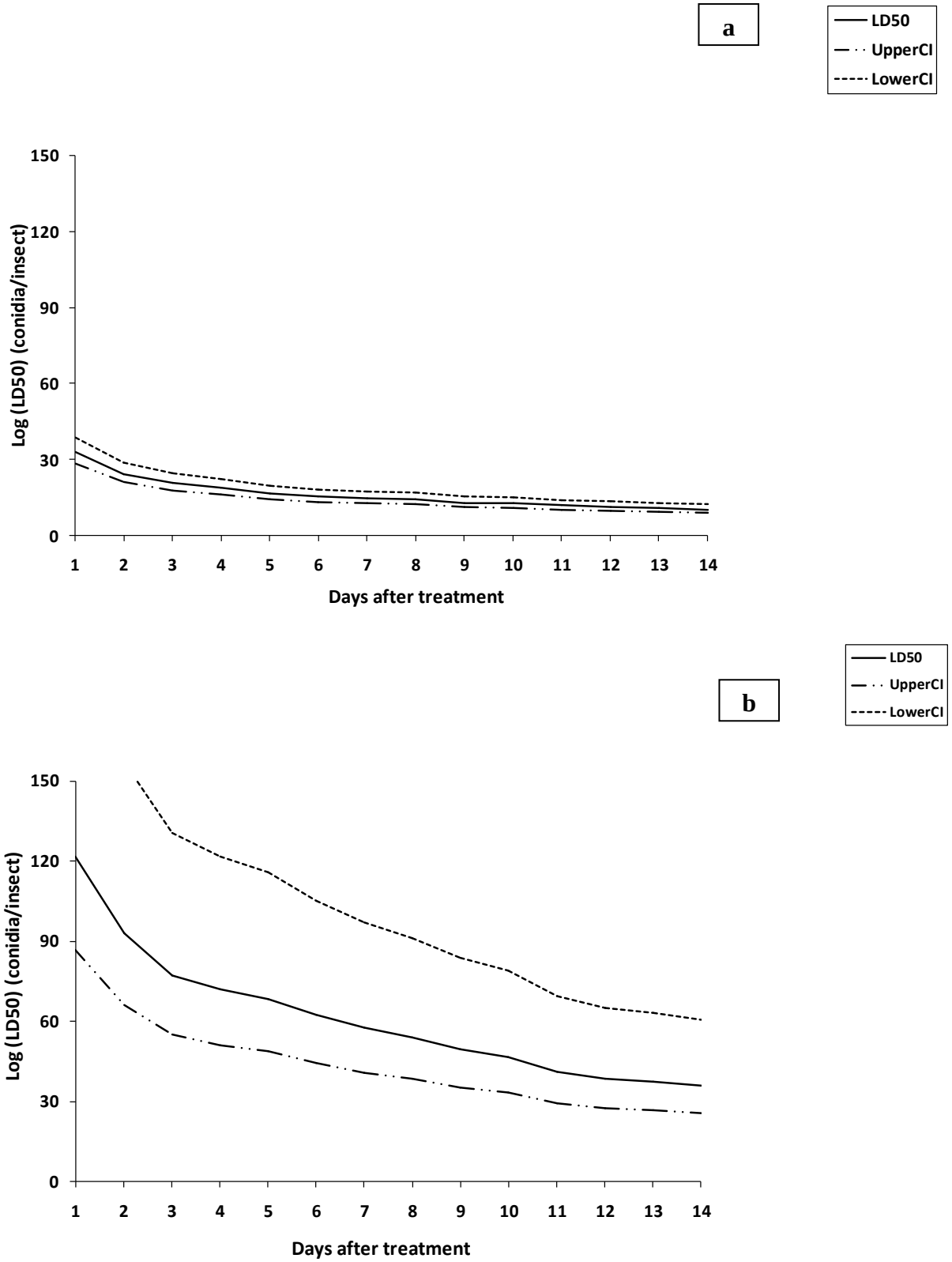

Fig. 5: LD50 values after treatment of the fourth stage of *Maruca vitrata* to various doses of Bb 115 (a) and Ma 29 (b).

Sporulation

Sporulation rates of larval cadavers (pooled from all concentrations) infected with Bb 115 where quite consistent and decreased only slightly with larval age. However, these sporulation rates induced by applying the different doses varied from 22.0 ± 3.2% (fourth stage) to 31.5 ± 4.9% (second stage) and from 6.0 ± 3.0% (fourth stage) to 15.2 ± 4.8% (second stage) when $2x10^7$ conidia per insect and $2x10^4$ conidia per insect were applied, respectively (Figure 2a).

Doses $2x10^4$ and $2x10^5$conidia/insect caused the lowest sporulation regardless of larval stage while $2x10^6$ and $2x10^7$conidia/insect induced the highest sporulation rate to all tested larval stages. These sporulation rates were significantly higher than those caused by each of the other doses ($F = 16.34$, $P<0.0001$; $F = 14.43$; $P <0.0001$; $F= 8.67$; $P = 0.0003$ for second, third and fourth, respectively). In contrast to mortality, the interaction, larval age-dose was not significant for all doses ($F= 0.449$, $P= 0.9668$).

Moreover, dead larvae due to *M. anisopliae* application sporulated regardless of larval stages or fungal doses. The values of sporulation are summarized in Figure 2b. Contrary to mortality, isolate Ma 29 x larval age interactions were not significantly determinant for all doses ($F=0.81$;$P< 0.7562$). No sporulation was observed on dead insects in the controls.

Larval Survival

Tables 1 and 2 summarize the survival time (MST) of *M. vitrata* after application of Bb 115 and Ma 29, respectively.

Compared with the two controls, survival of L2, L3 and L4 were significantly affected by the isolate Bb 115 ($F_{5, 30}$ = 102.0, $P <0.0001$; $F_{5, 30} = 70.12$, $P <0.0001$ $F_{5, 30} = 226.84$; $P <0.0001$, respectively, for L2, L3, L4). In addition, a decreasing trend in MST was observed within doses in L2 from the controls (13,5±0,2 days and 13,8±0,4 days for control 1 and 2, respectively) to higher doses (3.2 ± 0.7 days for $2x10^7$ conidia per insect) and this was 10 days less compared to controls. The highest MST was observed in the larval stage L4 (Table 1).

However, the survival of the second larval stage treated with Ma29 ranged from 7.3 ± 0.6 days ($2x10^7$ conidia per insect) to 10.0 ± 0.3 days ($2x10^5$ conidia per insect); whereas it ranged from 13.4 ± 0.2 to 13.6 ± 0.1days with control 1 and control 2 respectively. Also, from the third larval stage, no significant difference was observed between the doses. The values of mean survival times (MST) are summarized in Table 2. Irrespectively to the larval stage, MST decreased from control to the treated. Indeed, the survival of the fourth larval stage treated was about 8.6 ± 0.6 with dose $2x10^7$ conidia per insect.

Determination of LD50

The Cox regression analysis demonstrates that the different fungal doses used were significant indicators of *M. vitrata* larval survival ($P <0.05$). The values of B showed the existence of a dose – mortality response. This relationship was stronger for isolate Bb 115 with higher B value ($B = 0.382, 0.544, 0.451$ for L2, L3 and L4, respectively).

Evolution of the LD50 after applying Bb115 and Ma29

The fungal concentration required to kill 50% of L2 larvae (LD 50) in 5, 8 and 10 days was 10^{10}, 10^8 and 10^5 conidia/insect, respectively, for Bb 115 (Figure 3a) and 10^{41}, 10^{32} and 10^{25} conidia/insect, respectively, for Ma 29 (Figure 3b).

As illustrated in Figure 4a, 10^{12}, 10^{10} and 10^7 conidia/insect of Bb 115, and 10^{58}, $10^{45}$and 10^{34} conidia/insect of Ma 29 (Figure 4b) were needed to kill 50% of L3 in 5, 8 and 11 days, respectively.

For Bb 115, 10^{14}, 10^{12} and 10^9 conidia/insect were needed to kill 50% of L4 in 5, 8 and 12 days, respectively (Figure 5a), while 10^{68}, 10^{54} and 10^{38} conidia/insect were required for Ma 29 to achieve the same LD50 (Figure 5b).

Discussion

In general, both entomopathogenic fungi *M. anisopliae* and *B. bassiana* induced mortality and sporulation on *M. vitrata* larvae, thus demonstrating their pathogenicity against this insect, and corroborating similar results with other pest organisms (Toffa, 2004; Gundannavar et al., 2006). The relatively high larval mortality (30-100%) observed shortly after inoculation (2-10 days) in our study evidenced the virulence of the two isolates, which varied depending on fungal species, larval stage and applied doses. These results are consistent with those obtained by Valda et al., (2003), where 70-96% mortality were induced while applying *B. bassiana* on larvae of the diamond

back moth, *Plutella xylostella*. Like in our study, the mortality of *P. xylostella* larvae treated with *M. anisopliae* was lower than when exposed to *B. bassiana*.

Indeed, the higher virulence of Bb115 isolate could be explained by the fact that this isolate was isolated from an insect of the family Pyralidae in Central Benin. These results corroborate several earlier studies, such as the ones by Mc Coy et al., (1988) who reported that an isolate is usually more virulent to the host family, from which it was isolated. However, the virulence of a isolate reveals the complexity of the factors involved in the specificity of entomopathogen (climate of the region of collection, the storage conditions of the inoculum, the species of insect host on which the fungus was originally isolated, number of re-inoculation of artificial media, etc.).

Lower mortality rates were obtained when L3 and L4 larval stages were treated with *B. bassiana* Bb 115 at $2x10^4$ and $2x10^5$ conidia/insect, the dose/effect response becoming more significant beyond $2x10^6$ conidia/insect on L3 and L4 than the other larval stages. Sporulation rates after *B. bassiana* treatment followed the same trend as mortalities and were dose-dependent. As expected, younger larvae (L2 and L3) sporulated better than older ones, owing to their relative higher ratio of body weight per conidia applied. These results corroborate those of Kulkarnia et al., (2008) and Douro Kpindou et al., (2012b) who observed that after application of different doses (10^4, 10^5, 10^6, 10^7 conidia per insect) of *B. bassiana* on larvae of *H. armigera*, younger larvae were more susceptible and sporulated more frequently than older instars. However, with *M. anisopliae* Ma 29 isolate, no significant difference was observed in the different treatments suggesting fungus-specific activity.

Cox regression analysis revealed that the mean survival time (MST) of fungus-inoculated larvae was higher (2-10 days) compared to control. The two isolates Bb 115 and Ma 29 were able to kill within few days. Similar observations were reported by Tanada and Kaya (1993), while Lozano and Espaňa-Luna, (2008) claimed that insects subjected to virulent isolates generally die within 3 to 10 days. In addition, the Cox regression model allowed for a more appropriate analysis of biopesticide bioassays. As put forward by Preisler and Robertson, (1989), the conventional way to separate time effect from dose effect in bioassays was found unsuitable, arguing that models such as the Cox regression would be more appropriate for evaluating the effectiveness of a pathogen or pesticide on the target host. All Cox regressions curves revealed significant dose-mediated responses as indicated by the risk coefficients B and adjusted B values. Our results were consistent with those reported by Abebe, (2002) and Douro Kpindou et al., (2012a) for *M. anisopliae* and *B. bassiana* applied on *Macrotermes subhyalinus* and on *H. armigera* larvae, respectively.

In this study, dose, fungal species and the larval stage had an influence on the LD50. The highest LD50 values were obtained from older larvae (L4, L5): e.g. for Bb 115, 10^{13} conidia/insect were needed to kill 50% of L3 in 5 days, against 10^{16} conidia for L4.

From the results obtained it appears that the isolate Bb 115 gave the highest B value (0.544; section 3.3), narrower confidence intervals and lowest LD50, thus confirming dose-response pattern of the young larvae (L2, L3) of *M. vitrata* as revealed by Cox regression. In contrast, with the isolate Ma 29, LD50 values were significantly much higher. Parameters such as the high mortality rate of larvae, the sporulation rate and value of the lethal dose obtained make the Bb115 isolate, an excellent candidate for the development of microbial insecticides.

Conclusion

Our results showed that the dose-response was significant for all larval stages. At a given time, the effect of dose applied depends on the larval stage, the young larvae L2, L3 being more susceptible than the older ones. The fungal isolate Bb 115 (*B. bassiana*) has revealed to be of interest for the control of *M. vitrata* and therefore warrants further testing under field conditions.

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