Efficacy of *Beauveria bassiana* (Balsamo) Vuillemin against the bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidea) under laboratory conditions.

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1 SUMMARY

Damage of cotton by the bollworm *Helicoverpa armigera* heavily compromises the cotton production system in West Africa, particularly in Benin. Control of this insect pest was mainly done by the application of chemicals leading to the build-up of resistant population and other side effects. To develop an environmentally friendly strategy against the pest, this study aimed to evaluate the efficacy of *Beauveria bassiana* isolates on *H. armigera* larvae. Thirteen isolates of the fungus were screened for their virulence to *H. armigera* third instar larvae using at 10^7 conidia.mL^{-1}. In a second trial the effects of five concentrations (10^5, 10^6, 10^7, 10^8, 10^9 conidia.mL^{-1}) of the two most virulent isolates were performed. Conidia suspension was applied on each larva topically. This study finding showed in the first trial, four isolates with high mortality, namely Bb71, Bb11, Bb3, and Bb339. In addition, in the second ones, mortality rates of caterpillars increased with fungal concentrations. The induced mortality varied from 20.00 ± 2.88% to 63.33 ± 6.66% and from 33.33 ± 7.26% to 71.66 ± 4.40% for Bb3 and Bb11 isolates, respectively. Different lethal concentrations (LC50) were estimated to 9.68 x 10^{14} conidia.mL^{-1}, 1.70 x 10^{30} conidia.mL^{-1}, 9 days after inoculation for Bb11 and Bb3, respectively. In conclusion, this alternative strategy using *B. bassiana* as a biopesticide to control *H. armigera* was promising. It could better manage the pest in a perspective of synergistic effect evaluations with other biocontrol agents.

2 INTRODUCTION

Cotton is heavily attacked by many pests in West Africa farming system. Of these, carpophagous and phyllophagous caterpillars were major constraint to cotton production in Benin (Dannon *et al.,* 2020). One of the most destructive carpophagous is cotton bollworm *Helicoverpa armigera* (L.) (Hübner) (Lepidoptera: Noctuidea). Caterpillars of this insect species are known to damage resulting in the reduction of cotton production potential by 40-90%. (Djihinto, 2006). In Benin, many other cultivated crops were identified as host plants for this insect pest namely cotton, tomato, chili, okra, corn, sorghum and sunflower (Katary, 2003; Djihinto, 2014). Resistance of *H. armigera* to synthetic pyrethroids become a worldwide issue. It has been reported by scientists in Australia, China, India, Spain, Ivory Coast and Benin (Martin *et al.,* 2000; Djihinto *et al.,* 2009; Brun-Barale *et al.,* 2010; Tossou *et al.,* 2019). The first case was recorded since 1998 when the pest started to become resistant to chemical insecticides use in West Africa (Martin *et al.,* 2005), particularly in cotton fields from Benin. For example, the pest resistance was proved under laboratory conditions, against the following compounds cypermethrin, deltamethrin, bifenthrin, and fenvalerate (Djihinto *et al.,* 2009). Chemical insecticides used in Benin, often consist of combining molecules when used single do not always provide the expected control. Beside the effect of resistance, environmental pollution remains another major issue to be targeted (Lawani *et al.,* 2017; Gouda *et al.,* 2018). Facing all these issues, alternative methods become attractive option with regard to the pest resistance and other side effects. In this such perspective, several natural enemies are able to attack *H. armigera* such as parasitoids (Diabatté, 2018) and microorganisms (Kulkarni *et al.,* 2008; Lawo *et al.,* 2008; Nahar *et al.,* 2008; Douro Kpindou *et al.,* 2012a). Thus, the fungus *Beauveria bassiana* (Bals.) Vuil. was reported to be a promising option as an entomothogenic fungal species for the control of *H. armigera* (Elham Kalvnadi, 2018). The fungus can infect all *H. armigera* larvae instars (Douro kpindou *et al.,* 2012b) and use several modes of action like infection by conidia and toxins (Mascarin and Jaronski, 2016; Dannon *et al.,* 2020). But only one isolate of the fungus was used by Douro Kpindou *et al.* (2012a) on *H. armigera* while there are more than 20 isolates available at the entomopathogen bank of International Institute of Tropical Agriculture (IITA), Benin Station (Toffa Mehinto, 2014). The current study was carried out to evaluate the efficacy of *B. bassiana* on *H. armigera* under laboratory conditions.

3 MATERIELAND METHODS

3.1 Insect collection: Two hundred eighty five (285) caterpillars of *H. armigera* were collected in October 2017 on cotton fields in communes of Benin namely Malanville, Savalou, glazoué, Savè and Bohicon. They were reared in insectarium at International Institute of Tropical Agriculture (IITA), Benin station under a temperature averaging 26 ± 1 °C, 70 ± 5 % relative humidity and a photoperiod of 12:12 (L: D) h. Third *H. armigera* instars larvae from the first generation were tested using *B. bassiana* isolates. Larvae were fed using the modified artificial diet from Teakle and Jensen (1985). The method consisted of mixing cowpea and maize flour, tap water, agar, ascorbic acid, Methyl p-hydroxy benzoate, sorbic acid and formaldehyde.

3.2 Fungal isolates :Isolates were obtained from the entomopathogen bank of IITA-Benin (**Table 1**).

Table 1: Summary on *B. bassiana* isolates tested on *H. armigera*

Range	Abbreviation of fungal isolates	Register N°	Host (Country of origin)	Auteur (Year of isolation)
1	Bb2	5644	*Eldana sacharina (Benin)*	-IITA-Benin (1997)
2	Bb3	5645	*Eldana sacharina (Benin)*	-IITA-Benin (1997)
3	Bb5	5647	*Acigona sp. (Nigeria)*	- IITA-Benin (1997)-
4	Bb6	5648	*Acigona sp. (Benin)*	- IITA-Benin (1997)-
5	Bb11	5653	*Sesamia calamistis (Benin)*	-IITA-Benin (1997)
6	Bb115	I93-841	*Locusta migratoria (Madagascar)*	-Madagascar (1993)
7	Bb69	I91-623	*Zonocerus variegatus (Benin)*	-IITA-Benin (1991)
8	Bb71	I91-592	*Zonocerus variegatus (Benin)*	-IITA-Benin (1991)
9	Bb84	I91-679	*Hieroglyphus (Benin)*	-IITA-Benin (1998)
10	Bb353	-	*Callosobruchus sp. (Benin)*	-IITA-Benin (2001)
11	Bb116	I93-842	*Locusta migratoria (Madagascar)*	-Madagascar (1993)
12	Bb338	2191 ARSEF	*Pentatomidae Oebalus (Brazil)*	- Cornell University (USA, 1986)
13	Bb339	3086 ARSEF	*Leptoglossus fulvicorni (USA)*	-Cornell University (USA, 1990)

3.3 Laboratory experiments

3.3.1 Test of germination and determination of concentrations: Serial dilution was performed using each isolate for the counting of conidia, germination test on PDA and titration of concentrations. Germination rates of conidia and concentrations to be tested on larvae were estimated by Douro Kpindou et al. (2012b) approach (line 1 and 2).

$$\%germination = \left[\frac{x}{x+y}\right] \text{x } 100 \qquad \text{(line.1)}$$

x=number of germinated conidia within 24 hours; y= number of non-germinated conidia.

$$C' = \frac{Co \text{ x } Vo}{Vo + V'} \qquad \text{(line.2)}$$

C' = concentration to be tested, Co = concentration of initial conidial suspension; Vo= volume needed and V'= volume to be added.

Fungal isolates were used in the first trial and the two most virulent ones were retained for the second bioassay.

3.3.2 Screening of B. bassiana isolates: Thirteen (13) *B. bassiana* isolates were applied at a concentration of 10^7 conidia. mL^{-1} (formulated with Tween 80 (0.05%)) on third *H. armigera* larvae. Larvae were individually put in rearing boxes (3.8 cm x 2.9 cm x 4.0 cm) with punched tiny holes for ventilation. Hundred-twenty larvae of *H. armigera* were treated using each of the 13 isolates. Larvae were inoculated by the method of Bateman et al. (1996) and Peveling et al. (1997). Each larva received topically 1µL of the fungal suspension on the pronotum. Germination rates were up to 85%, 20 hours after incubation. Laboratory temperatures averaged 26 ± 1°C with 70 ± 5% relative humidity (RH). Mortality of larvae was checked daily over 15 days per isolate. Cadavers were dried for 48-72 hours, and transferred in petri dishes (9 cm diameter) containing humidified Whatman filter paper, sealed with parafilm and incubated at 26 °C to check sporulation. The number of pupae dead and sporulated larvae and adults emerged were recorded.

3.3.3 Assessing of effect of different concentrations* of *B. bassiana* on the survival of *H. armigera larvae: Two isolates (Bb3, Bb11) of the fungus were used in the current bioassay. Treatments consisted of a control (Tween 80 à 0.05% without fungus) and five different concentrations (10^5, 10^6, 10^7, 10^8, 10^9 conidia.mL^{-1} corresponding to 10^2, 10^3, 10^4, 10^5, 10^6 conidia per insect). Third *H. armigera* instars larvae were treated with each concentration.

3.4 Data analysis: Mortality rate was estimated based on the OECD approach (OECD, 2010).

$$MR = \frac{Nd}{Nu}$$

MR= mortality rate, Nd= number of dead larvae, Nu= number of larvae used (normally 20).

Sporulation rate was assessed basing on the number of larvae dead in each treatment. Likewise, the emergence rate was estimated based on the number of pupae formed by treatment. Percent data (mortality, sporulation and emergence) were Arcsin (square (p)) transformed prior to the analysis of variance (ANOVA) (Steel *et al.,* 1997), using R version 4.0.2. Tukey (HSD) test was used to discriminate means in the case of significant differences. The lethal concentrations and lethal times were estimated using the model of Cox regression (SPSS, 1989- 2007) as described by Douro Kpindou *et al.* (2012a).

4 RESULTS

4.1 Screening test of isolates: The screening test revealed the pathogenicity of *B. bassiana* isolates on third larval instars of *H. armigera* (**Fig. 1**).

Fig. 1: *B. bassiana* growth in full sporulation on third larval instars of *Helicoverpa armigera.*

There were significant differences between the isolates (**Fig.2**) when considered the mortality of caterpillars (F=6.7801, P<0.0001). The cumulative mortality after fifteen days varied from 3.33 ± 1.66% to 53.33 ± 6.01% compared to the control (3.33 ± 1.66%). Four isolates showed high mortality, namely Bb71 (53.33 ± 6.01%), Bb11 (33.33 ± 6.66%), Bb3 (21.67 ± 1.66%) and Bb339 (18.33 ± 6.01%). Likewise, significant differences were observed between isolates (**Fig.3**) for the mycosis rate of caterpillars (F=2.7824, P<0.05). The recorded mycosis rates of cadavers after incubation were 25.00± 7.21%, 11.11± 11.1%, 11.11± 11.1% and 6.67± 6.6% for Bb11, Bb115, Bb339 and Bb3, respectively. No mycosis was observed for other isolates.

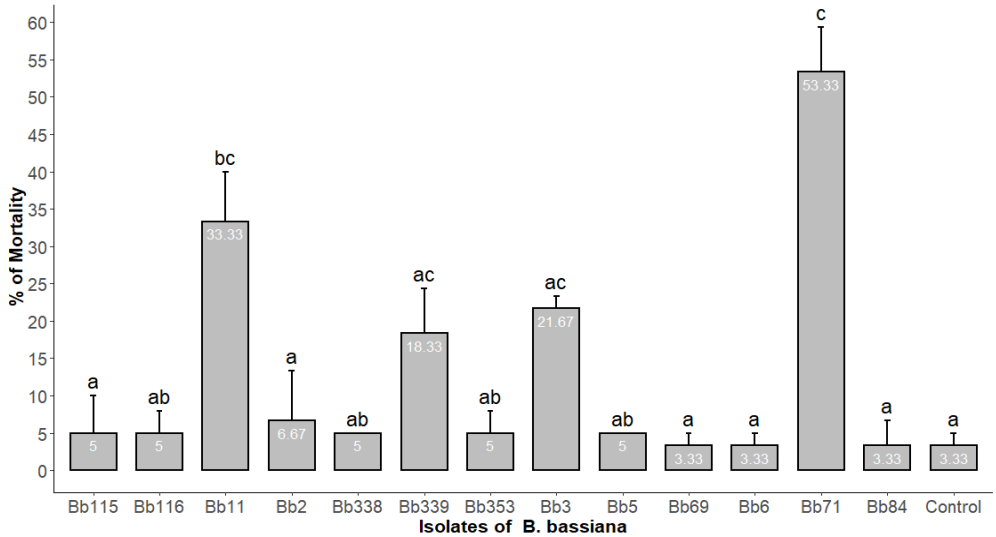

Fig. 2 Mean mortality rates induced by 13 *B. bassiana* isolates at 10^7 conidia.mL^{-1} on third larval instars (L3) of *H. armigera.*

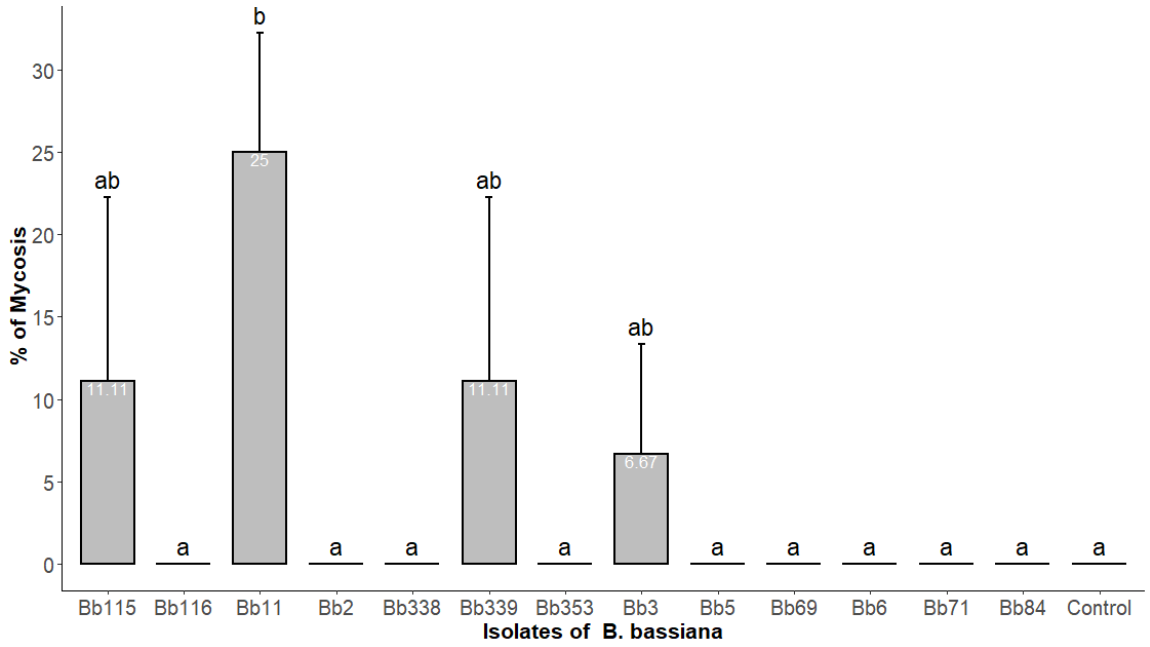

Fig. 3 Mean mycosis rates of 13 *B. bassiana* isolates at 10^7 conidia.mL^{-1} on third larval instars (L3) of *H. armigera.*

4.2 Assessing of effect of different B. bassiana concentrations* on the survival of *H. armigera caterpillars

4.2.1 Effect of B. bassiana on third larvae stage and adults' emergence: Results of this study showed the mortality-dose effect significant for all isolates (P<0.0001) at 5% on L3 larvae of *H. armigera* (Table 2). Mortality rates increased with fungal concentrations. The induced mortality varied from 20.00 ± 2.88% to 63.33 ± 6.66% and from 33.33 ± 7.26% to 71.66 ± 4.40% for Bb3 and Bb11 isolates, respectively. Isolate Bb3 activity varied strictly with fungal concentrations, while Bb11 isolate induced the same effects with the concentrations of 10^5 and 10^6 on the first hand, then at those of 10^8 and 10^9 on the other hand. An optimal concentration could already be indicated for Bb11 isolate at the concentration of 10^8 conidia.mL^{-1}. Moreover, when comparing isolates within each concentration, significant differences were observed with the concentrations of 10^7 and 10^8 conidia.mL^{-1} (P<0.05). For this purpose, the high mortality rates were 51.66 ± 6.01% (Bb11) against 25.00 ± 2.88% (Bb3) and 71.66 ± 1.66% (Bb11) against 41.66 ± 4.40% (Bb3) for 10^7 and 10^8 concentrations, respectively.

Table 2: Effects of *B. bassiana* on third larval instars of *Helicoverpa armigera*

Bb Concentration (Conidia/ml)	Mortality rate of larvae (%)		*T test*
	Bb3	Bb11	
10^5	20.00±2.88 abA	33.33±7.26 bA	*t=1.7097 P=0.1625*
10^6	28.33±4.40 bcA	41.66±4.40 bA	*t=2.1207 P=0.1013*
10^7	25.00±2.88 acA	51.66±6.01 bcB	*t=4.0474 P=0.01551*
10^8	41.66±4.40 cdA	71.66±1.66 cB	*t=6.3699 P=0.003115*
10^9	63.33±6.66 dA	71.66±4.40 cA	*t=1.0538 P=0.3514*
Control	8.33±1.66 a	5.00±2.88 a	
ANOVA	*F=19.9626 P=6.526e-05*	*F=25.6728 P=2.107e-05*	

*There is no significant difference between means followed by the same lowercase letters shown in the same column (ANOVA followed by Tukey test at 5%).
*There is no significant difference between means followed by the same capital letters shown in the same line (ANOVA followed by Tukey test at 5%).

Adult emergence of *H. armigera* was significantly affected by concentrations in each isolate ($P<0.05$) (**Table 3**). However, when comparing isolates for each concentration, a significant difference was occurred at 10^7 conidia.mL^{-1}.

Table 3: Effects of *B. bassiana* on emergence of *Helicoverpa armigera*

Bb Concentration (Conidia/ml)	Emergence rate of moths (%)		*T test*
	Bb3	Bb11	
10^5	75.47±9.22 aA	72.92±9.71 abA	*t= -0.22167 P= 0.8354*
10^6	78.61±4.71 abA	65.64±8.07 abA	*t= -1.4124 P= 0.2307*
10^7	88.84±2.30 abB	63.42±8.03 abA	*t= -3.2949 P= 0.03008*
10^8	77.86±6.75 abA	53.33±10.08 aA	*t= -2.0271 P= 0.1126*
10^9	58.88±4.84 aA	55.15±12.06 aA	*t=-0.2656 P=0.8036*
Control	98.14±1.85 b	94.62±3.21 bA	
ANOVA	*F= 5.9352 P= 0.008383*	*F= 4.0785 P= 0.02154*	

*There is no significant difference between means followed by the same lowercase letters shown in the same column (ANOVA followed by Tukey test at 5%).
*There is no significant difference between means followed by the same capital letters shown in the same line (ANOVA followed by Tukey test at 5%).

4.2 Lethal concentration (LC50) of *B. bassiana* on third larvae stage of *H. armigera*: Cox regression analysis revealed that the different concentrations of the fungus used were significant and affected larval survival (P <0.05) (table 4). Values of B showed the existence of a dose-response relationship. This relationship was strong for Bb11 with the highest value of B (0.097).

Table 4: Estimation of B values and Wald coefficients with Cox regression model for *B. bassiana* and third instars larvae of *H. armigera*, including.

B. bassiana Isolate	*Helicoverpa armigera* Stage	B	SE	Wald	df	Sig.
Bb3	L3	0.060	0.019	9.902	1	0.002
Bb11	L3	0.097	0.019	26.028	1	0.000

B: B value of the Cox regression; **SE:** standard error; Wald: Wald coefficient; **df:** degree of freedom; **Sig:** probability.

Isolates LC50 curves are represented in Figures (4 and 5). For all isolates, the dose-response effect was significant and depends on the value of B. Higher is B value, narrower are confidence intervals. It had to 6.59 x 10^{20}, 8.76 x 10^{16} and 9.68 x 10^{14} conidia.mL-1 of Bb11 isolate to kill 50% of the 3rd instar larvae *of H. armigera* respectively in 5, 7 and 9 days after inoculation. Likewise, 8.76 x 10^{38}, 5.96 x 10^{32} and 1.70 x 10^{30} conidia.mL^{-1} of Bb3 isolate are required to kill 50% respectively within 5, 7 and 9 days after inoculation.

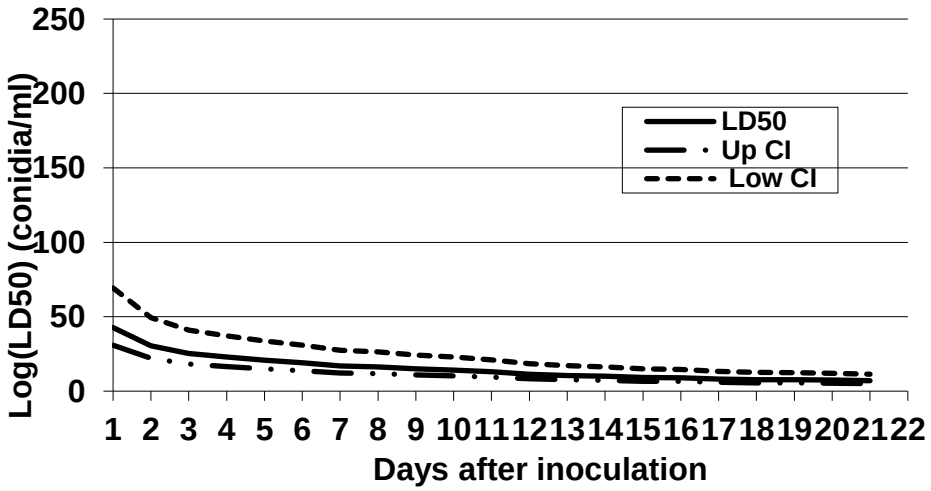

Fig. 4 LC50 values after treatment of the third stage of *H. armigera* to various concentrations of Bb11.

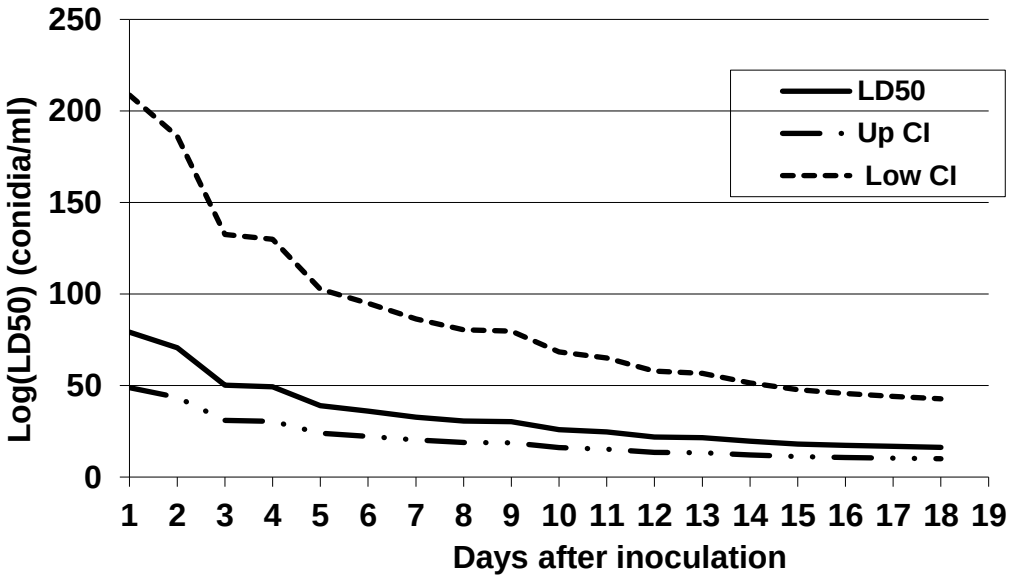

Fig. 5 LC50 values after treatment of the third stage of *H. armigera* to various concentrations of Bb3.

5 DISCUSSION

Screening of *B. bassiana* isolates for their virulence to *H. armigera* third instars larvae revealed that the insect was susceptible to the fungus. All isolates were pathogenic to third larval instars and their virulence was fairly varied. A few number of isolates (Bb71, Bb11, Bb3 and Bb339) induced a high fairly mortality. Among these, three namely Bb11, Bb339 and Bb3 proved the full sporulation activity on cadavers suggesting a variability within the tested isolates. In this perspective, Safavi (2010) described virulence of isolates as its ability to induce infection and disease in insects by recovering the fungus on dead insect than others. Some isolates induced a less virulence in our experiment. That could be explained by the insect host resistance to those isolates. Petlamul et al. (2019) reported that insects can resist microorganisms present in their environment by efficient immune systems. It may also be related to their innate immune systems of caterpillars based on humoral and cellular components (Lavine and Strand, 2002). The most promising isolates Bb3, Bb11 used for assessing the effect of different concentrations were selected based on their origin (Benin), and their virulence and sporulation rate. Mortality rates increased with different fungal concentrations and provided the effectiveness of *B. bassiana* on larvae and adults emergence. The increase in mortality rate could be explained by the quantity of conidia received by the host insect. The optimal concentration was 10^8 conidia mL^{-1} for Bb11 [1]. Our findings were in concordance with previous studies done by Douro Kpindou et al. (2012a) reporting the same optimum when testing some *B. bassiana* isolates on *H. armigera*. Furthermore, isolates Bb3 and Bb11 showed significant differences with the concentrations of 10^7 and 10^8 conidia.mL^{-1}. Indeed, Bb11 isolate was more virulent than Bb3 by the highest mortalities recorded on third larval instars. In addition, emergence rate of moths was significant and less with Bb11 compared to Bb3 at 10^7 conidia.mL^{-1}. These observations were in agreement with those done by Toffa Mehinto et al. (2014) who reported a variability of virulence between *B. bassiana* isolates on *Maruca vitrata* Fabricius (Lepidoptera; Crambidae). Such virulence has been reported by Ferron (1981), De Kouassi (2001) to be related to production of enzymes and toxic metabolites by isolates. Models that combine time and dose effects seem to be more appropriate for assessing the effectiveness of a

pathogen or pesticide on target insect species (Robertson and Preisler, 1992). Cox regression models fitted well in the current study. All B values given by Cox regression model were all significant ($P<0.05$). In consequent, our data showed a significant effect / dose response. Similarly, a good trend of the LC50 curve was observed with Bb11 isolate and the confidence interval was relatively narrower. Based on LC50, isolate Bb11 was found to be more virulent than Bb3. However, LC50 were a bit high suggesting a possibility of the complementary trials with other biocontrol agents in the way to evaluate the synergic effects.

6 CONCLUSION

The *H. armigera* bollworm was susceptible to all *B. bassiana* isolates tested in the current study. However, significant differences occurred between these isolates when considering larval mortality and mycosis rates. The isolate Bb11 was identify to be the most promising one for the management of *H. armigera*. Values of LC50 provides would be useful for the development and formulation of *B. bassiana*-based biopesticide for managing insect pests in cotton.

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