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# Isolation of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) from the soils of coffee fields and insecticide activity against *Hypothenemus hampei* (Coleoptera: Scolytidae)

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**ABSTRACT**

Coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) is one of the most important pests of coffee marketed in Cameroon and the world. This insect lives an endophytic life in the berries and remains very difficult to fight with the spraying of synthetic pesticides. This study aims at investigating the potential impact of two native strains of *Beauveria bassiana* (Balsamo) Vuillemen (Deuteromycotina: Hyphomycetes) from Cameroon against the CBB. *B. bassiana* strains were isolated from rhizosphere strains of coffee farms. The efficiency tests were conducted on adult *H. hampei*. Five treatments of *B. bassiana* were used compared with Pyriforce (Chlorpyriphos-ethyl). Results indicate that, the *B. bassiana* strains were different in terms of their macromorphological characteristics. They were virulent against the CBB irrespective of the mode of application of spores directly to the borer as well as to the berries. These results clearly showed that the two isolates of *B. bassiana*, native to coffee growing areas in Cameroon, can be used as potential biological control agents against *H. hampei* and as alternatives to chemical insecticides in integrated pest management programmes for coffee.

**Keywords:** Biological control, Insecticide activity, *Coffea* sp., *Hypothenemus hampei*, *Beauveria bassiana*, Cameroon.

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## INTRODUCTION

The two main commercial species of coffee trees (*Coffea* L.) are produced in Cameroon. Coffee is an agricultural export product contributing to the improvement of producers' incomes and the gross domestic product (GDP) of this country (Mahob *et al.*, 2006). However, their production is severely disrupted by attacks from pests and diseases. One of the most important damages on coffee is caused by the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) (Amang-Mbang *et al.*, 2012).

Native to Africa (Perez-Lachaud *et al.*, 2002), the coffee berry borer, particularly adult females, cause damage by piercing the lower base of the berries and creating galleries inside where they lay their eggs. These eggs then consume the endosperm of the berries (Damon, 2000; Vega *et al.*, 2009). Through this mechanism, the coffee berry borer becomes the most devastating pathogen of coffee berries in the world (Infante *et al.*, 2014; Vega *et al.*, 2015), reaching attack rates of 10-100% in Cameroon (Mahob *et al.*, 2006).

Attempts at chemical control of the borer with the use of Chlorpyriphos-ethyl 600 g/l and endosulfan (Aristizabal *et al.*, 2012), cultural control with sanitary harvesting (Dufour and Frérot, 2008) or biological control with pesticidal plant extracts

(Nguema Ndoutoumou *et al.,* 2015; Manga Essouma *et al*., 2021) have had interesting effects. However, this remain limited because of the insect's endophytic lifestyle at all stages of development (Cochereau and Potiaroa, 1994; Dufour, 2013), theirs impacts on natural strain of Beauveria (Ribeiro *et al.,* 2012) and, in addition the costs in time, energy, and the resistance developed by the berry borers (Nguyen Ban, 1977). Biological control with the use of parasitoids, nematodes and entomopathogens remains promising (Rodríguez *et al.,* 2017). Moreover, there is growing demand today for better quality coffee with less or almost no chemical residues and above all for the protection and conservation of biodiversity and the environment (Bourguet and Guillemaud, 2016).

Chemical pesticides must therefore be reduced in coffee production today. This can be done by substituting chemical methods or combining them with organic methods. The use of entomopathogenic fungi (EPFs), natural enemies against plant pathogens (Medeiros and Silva, 2019) is in line with this need for sustainable production. EMFs are suitable for obtaining healthy products and are related to insects because they use chitin (the main component of the insect exoskeleton) as a source of carbon. The best known and studied worldwide are the genera *Beauveria* and *Metarhizium.* These, are frequently observed in nature and have a large number of hosts (Vázquez, 2019; Barra-Bucarei *et al.,* 2019).

*Beauveria bassiana* (Balsamo) Vuillemen (Deuteromycotina: Hyphomycetes) is commonly found parasitizing insects and has specificity for more than 200-700 insect species such as whiteflies, termites, aphids, beetles, lepidoptera and even several phytopathogenic fungi such as *Colletotrichum* (Sanivada and Challa, 2014; Serrato-Diaz *et al.,* 2020). It causes a disease called "white muscardine" once in contact with insects (Van Lenteren *et al.,* 2018; Paiva-Guimarães *et al.,* 2019). In several studies, it has been shown to naturally and effectively control *H. hampei* in several coffee producing countries (Wraight *et al.,* 2018; Serrato-Diaz *et al.,* 2020).

The infection rate of the borer varies from 1% in Brazil (Costa *et al.,* 2002), 44% in Nicaragua (Monzón *et al.,* 2008) and up to 70.6% in Cameroon (Amang-Mbang *et al.,* 2012).

In addition, Escobar-Ramíreza *et al.,* 2019 showed that the coffee berry borer population is significantly reduced by conventional biological control rather than natural control.

In view of these findings, this study will evaluate in vitro the insecticidal activity of *B. bassiana* strains native to coffee producing areas in Cameroon on *H. hampei*. In addition, the presence of *B. bassiana* in the soils of coffee plantations in Cameroon will be proven and their insecticidal effect on the coffee berry borer will be tested. This type of control was chosen because in biological control, it is recommended to use products developed from native or local species (Gutiérrez and Maldonado, 2010).

## MATERIALS AND METHODS

### Study sites

Soil samples, berries and berry borers were collected in the coffee collection and production fields of the stations of the "Agricultural Research Institute for Development" (IRAD). These are located in the sites of Foumbot (Latitude 05°29'034" and Longitude 010°33'33. 0"; altitude 1,240 m to 2,740 m; annual average temperatures 19°C) and Nkoémvone (2°90 of latitude North to 12°2 of longitude East; altitude 500 and 1,000 m; annual average temperatures 25°C). The tests were carried out in the Phytopathology and Entomology Laboratories of the "IRAD", located in Yaoundé, Cameroon.

### Isolation and characterization of isolates

The *Beauveria bassiana* strains used in this study were isolated from composite soil samples and using PDA media (Meyling and Eilenberg, 2007). Healthy late instar larvae of *Rhynchophorus* spp. were used to bait the fungus. Colonies developed around the insects were identified on the basis of macromorphological criteria and successive transplantations were carried out until pure isolates were obtained (De kouassi, 2001).

Isolates were characterised by electron microscopic observation at ×40 magnification of the 21-day pure cultures. Spore production and germination, mycelial growth, virulence and pathogenicity of the isolates were measured (Hussein *et al.*, 2010). Finally, two isolates were obtained: *B. bassiana* from Foumbot (Bb-IRAD.Fbt) and *B. bassiana* from Nkoemvone (Bb-IRAD.Nkoe).

**Spore production, germination and radial growth**

Spore production and germination were assessed according to the methods described by Mboussi *et al.* (2016) and Messi *et al.* (2018). To assess radial growth, a mycelium disc (0.6 cm of diameter) of 21-day pure culture of each isolate was taken and placed in the centre of 90 mm Petri dish containing pre-prepared PDA medium. The plates were incubated under high humidity conditions and each diameter or straight line was measured daily for 21 days using the perpendicular line method. Radial growth was evaluated using the formula of Singh *et al.* (1993).

**Pathogenicity and virulence of isolates after direct infection of berry borers**

Pathogenicity here is expressed by the ability of the isolates to make white muscardine appear on berry borers and virulence is their ability to kill insects. As before, 10 mL stock solutions were prepared. Concentrations of $2 \times 10^2$ and $3 \times 10^2$ spores/ml were calibrated. About 40 non-sexed adult berry borers were disinfected with hypochlorite (2%), rinsed 3 times in sterile distilled water and soaked in spore solutions of each isolate during 10 seconds (Butt and Goettel, 2000; Manga Essouma *et al.*, 2021). The insects (05) inoculated with *Beauveria bassiana* isolates and mature coffee berries (05), were incubated in cups (10 cl) with bottoms filled with cotton and blotting paper soaked in sterile distilled water. The number of dead beetles with or without a sporulation signature was assessed.

**Virulence of *B. bassiana***

The millilitres spore solutions were prepared and five (05) concentrations of each *B. bassiana* isolate were calibrated according to the formula of Gata-Gonçalves (2003). Batches of adult, non-sexed, first-generation insects were prepared and soaked in the different treatments. Each box prepared as before contained ten treated insects and ten untreated mature berries. The boxes were incubated in the incubation chamber and inspection of the boxes was carried out daily for 10 days (Benavides, 2012). Dead insects with or without sporulation signatures were counted. Observed or gross mortality and corrected cumulative mortality rates were calculated according to Abbott (1925) formulae.

Five treatments were used : the two isolates of *B. bassiana* at five concentrations including $C_1$ ($3\times10^2$ spores/mL), $C_2$ ($3\times10^4$ spores/mL), $C_3$ ($3\times10^6$ spores/mL), $C_4$ ($3\times10^8$ spores/mL) and $C_5$ ($3\times10^{10}$ spores/mL); Sterile distilled water which was the negative control ($C_{0-}$) and; pyriforce (chlorpyriphos-ethyl 600 g/L; EC) at the recommended dose of 1 l/ha or 4.6 µL/mL which was the positive control ($C_{0+}$). Each treatment was repeated five times and each repetition corresponding to the box in which the bark beetles were incubated. The whole experiment was repeated twice.

**Pathogenicity of *B. bassiana***

Dead insects with sporulation signatures were counted. To confirm the actual cause of death, insects without sporulation signatures were disinfected and incubated in 90mm Petri dishes lined with cotton wool lined with blotting paper moistened with sterile distilled water. These insects were observed for seven days and those with sporulation signatures were counted. The sporulation rate was calculated in relation to the number of dead insects directly showing the sporulation signature according to Lopes *et al.* (2011) formula.

**Virulence of *B. bassiana* after direct infection of berry borers**

The virulence of *B. bassiana* on *H. hampei* by infection of the berries made it possible to apprehend the capacity to indirectly infest berry borers. The insects were primed and placed in tins with coffee berries soaked in a solution of $3 \times 10^8$

spores/ml, the only concentration used for this test. The number of dead berry borers in each can was counted daily and mortality rates calculated as in the evaluation of virulence by insect infection.

**Statistical analysis of the collected data**

The tests in this study are tri-factorial with: a first factor which is the treatment (inoculum, water and chemical insecticide), a second factor which is the concentration of the treatments and a third factor which is the incubation time. The collected data have been analysed with the XLSTAT 2014 software. Observed, cumulative and corrected mortality rates and sporulation rates were normality tested by Shapiro-Wilk and Jarque-Bera (1965). Angular transformations (ArcSin) were performed (Fisher and Yates, 1963; Jayarama, 1999). The transformed data underwent an analysis of variance (ANOVA) followed by a multiple mean comparison test. The mean values obtained were presented with their standard deviation.

Subsequently, these mean values were transformed into probits, and the concentrations and observation time were transformed into a decimal logarithm (Finney, 1971). From these transformations, "probit-logarithm" regression lines were generated of the type $y = ax + b$ ($y$ = probit of the mortality rate; $x$ = logarithm of the concentration or time; $a$ = slope of the regression line). From these regression equations, lethal concentrations (LC) and lethal times (LT) of 50 and 90% mortality were determined by the maximum likelihood method, or working probit method (Finney, 1952; Lazar, 1968). Conclusions were drawn at the transformed scale, but the results presented (LC and LT 50 and 90, means, standard deviations, concentration and observation time) were converted back to the original units (Jayarama, 1999).

## RESULTS

**Characterisation of *B. bassiana* isolates**

The pure isolates of *B. bassiana* were isolated from the soil samples collected in the coffee fields. The number of spores produced was significantly identical between the two isolates. It reaches an average total of $8.5 \times 10^5$ spores/mL after 24 hrs incubation ($Pr = 0.074 > \alpha = 0.05$; Fisher's test). Evaluation of spore germination showed that there was a significant difference between the different isolates ($Pr = 0.007 < \alpha = 0.05$; Fisher's test). It was significantly higher in Bb-IRAD.Nkoe (60.3% germinated spores) than in Bb-IRAD.Fbt (56.3%). However, a large variation in germination rate was observed between replicates of Bb-IRAD.Fbt (Standard deviation = 1.2). Mycelial growth was significantly different between the two isolates ($Pr < 0.0001 < \alpha = 0.05$; Fisher's test) and significantly higher (2.09 cm) for Bb-IRAD.Nkoe (Table 1). The Nkoemvone isolate showed a volatile character with jumps in the petri dish.

Pathogenicity and virulence testing confirmed that the isolates belonged to the genus *Beauveria* sp. and their efficacy/virulence against the resulting berry borer's population. After 7 days of incubation, the concentration $2 \times 10^2$ spores/ml caused 42.3% and 50.4% mortality of *H. hampei* for Bb-IRAD.Fbt and Bb-IRAD.Nkoe, respectively, while the concentration $3 \times 10^2$ spores/ml caused 82.7% and 100% mortality for Bb-IRAD.Fbt and Bb-IRAD.Nkoe, respectively (Table 1). All deaths were covered with *B. bassiana* mycelium.

**Adults *Hypothenemus hampei***

**Virulence of *B. bassiana***

The spores of Bb-IRAD.Fbt and Bb-IRAD.Nkoe caused mortality of the berry borers after direct contact between the two living organisms. The results in (Table 2) showed that these isolates had a highly significant and concentration-dependent insecticidal effect ($Pr < 0.0001 < \alpha = 0.05$; Fisher's test). Pyriforce (Chlorpyriphos-ethyl 600 g/L) ($C_{0+}$) was almost totally effective (97.6% dead insects on average after 10 days observation) while with sterile distilled water ($C_{0-}$) no mortality was observed. The average corrected cumulative mortality rate is between 35.5% ($C_1$) and 87.8% ($C_5$) for Bb-IRAD.Fbt. For Bb-IRAD.Nkoe, mortality is between 41% ($C_1$) and 90.7% ($C_5$) after ten days of observation (Table 2).

Figure 1 showed that the average percentage of corrected cumulative mortality increased with *B.*

*bassiana* concentrations and with time. In general, it appears that Bb-IRAD.Nkoe was more effective than Bb-IRAD.Fbt. More than 50% mortality was observed with the highest concentration ($C_5$) in both isolates on day three, day four with $C_4$, day five with $C_3$, day six with $C_2$ and day seven with $C_1$. With Bb-IRAD.Fbt, concentrations 1, 2 and 3 did not cause 100% mortality after 10 days of observation. Similarly, concentrations 1 and 2 did not cause 90% mortality after 10-days of observation. With Bb-IRAD.Nkoe, only concentrations 1 and 2 did not cause 100% mortality after 10 days, although $C_2$ caused more than 90% mortality of *H. hampei* (Fig. 1).

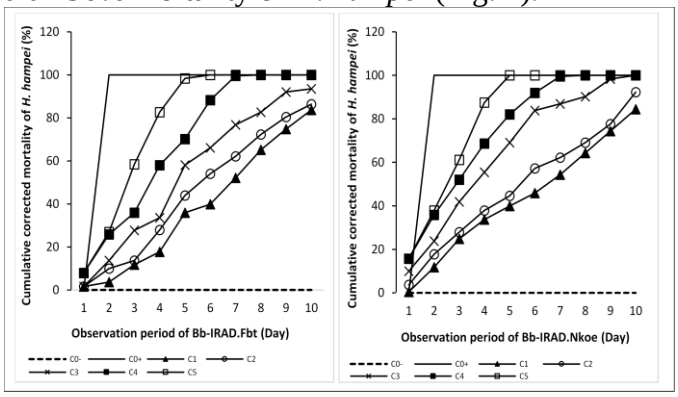


**Fig. 1.** Cumulative mean corrected mortality of berry borers as a function of time and concentrations of *B. bassiana* Foumbot and Nkoemvone, water and pyriforce. $C_{0+}$= pyriforce (Chlorpyriphos-ethyl 600 g/l); $C_{0-}$= water +tween 80; $C_1 = 3 \times 10^2$ spores/mL; $C_2 = 3 \times 10^4$ spores/mL $C_3 = 3 \times 10^6$ spores/mL; $C_4 = 3 \times 10^8$ spores/mL; $C_5 = 3 \times 10^{10}$ spores/mL.

The highest observed daily mortality was 100% with pyriforce on the second day of observation while it was 29% on the third day at $C_5$ of Bb-IRAD.Fbt. The insecticidal effect of *B. bassiana* was significant between the second and sixth day with Bb-IRAD.Fbt and between the second and fifth day with Bb-IRAD.Nkoe. During these same periods, daily mortalities between 7-25% were observed in all concentrations of both isolates. On the tenth day, mortalities of 4% for Bb-IRAD.Fbt and 10% for Bb-IRAD.Nkoe were still observed.

**Determination of $LC_{50}$ and $LC_{90}$**

Adjusted cumulative mortalities from day four were used to remain within the ranges of the applied concentrations. A strong positive relationship ($R^2$=0.94 and 0.95) was observed between the probits of the mortalities and the logarithms of the applied concentrations (Fig. 3). lowest concentrations ($C_1$ and $C_2$) (Fig. 2).

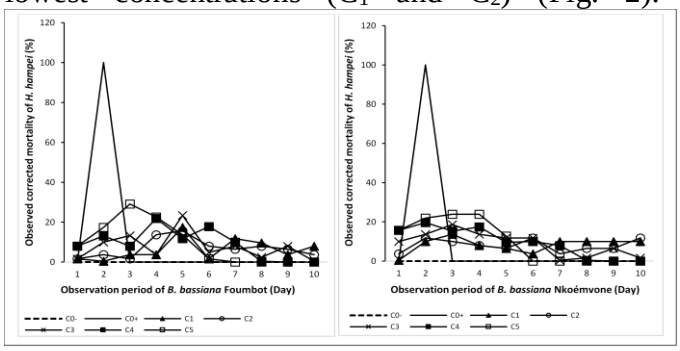


**Fig. 2.** Observed mean corrected mortality of berry borers as a function of time and doses of *B. bassiana* Foumbot and Nkoemvone, water and pyriforce. $C_{0+}$ = pyriforce (Chlorpyriphos-ethyl 600 g/l); $C_{0-}$ = water +tween 80; $C_1 = 3 \times 10^2$ spores/mL; $C_2 = 3 \times 10^4$ spores/mL $C_3 = 3 \times 10^6$ spores/mL; $C_4 = 3 \times 10^8$ spores/mL; $C_5 = 3 \times 10^{10}$ spores/mL.

The inverse logarithm of the linear regression equation revealed that the $LC_{50}$ of *H. hampei* are $1.51 \times 10^7$ and $2.63 \times 10^4$ spores/mL for Bb-IRAD. Fbt and Bb-IRAD.Nkoe respectively. Lethal concentrations causing 90% mortality of berry borers are $7.18 \times 10^{12}$ and $8.49 \times 10^{11}$ spores/ml for Bb-IRAD.Fbt and Bb-IRAD.Nkoe respectively. These results showed that it takes about 574 times fewer spores/ml of Bb-IRAD.Nkoe to kill 50% of berry borers and about 8 times fewer spores to kill 90% of berry borers compared to Bb-IRAD.Fbt (Fig. 3).

**Determination of $LT_{50}$ and $LT_{90}$**

The regression lines obtained revealed strong positive correlations between these two variables. They varied in the same direction and all slopes were positive and greater than two (2). By observing Bb-IRAD.Fbt, we can see that the $TL_{50}$ decreased with increasing concentration from 11.98 days for the concentration $3 \times 10^2$ spores/mL to 2.52 days for the concentration $3 \times 10^{10}$ spores/mL. Similar observations were made by observing the $TL_{90}$ where 90% mortality was obtained after 50.3 days for $3 \times 10^2$ spores/mL and after 5.5 days for $3 \times 10^{10}$ spores/mL. The lethal times of Bb-IRAD.Nkoe were lower. There was a difference of 7.3 days ($TL_{50}$) between the two isolates for the $3 \times 10^2$ spores/mL concentration

**Table 1.** Production, spore germination and mycelial growth after 21 days.

| Characterisation parameters | | Isolates of *Beauveria bassiana* | | |
|---|---|---|---|---|
| | | Foumbot | Nkoémvone | Total |
| Average spore production ($1 \times 10^5$ spores/ml) | | $8.1 \pm 0.5^a$ | $9.0 \pm 0.5^a$ | $8.5 \pm 0.7$ |
| Average spore germination rate (%) | | $56.3 \pm 1.2^a$ | $60.3 \pm 0.6^b$ | $58.3 \pm 2.4$ |
| Average mycelial growth (cm) | | $1.95 \pm 1.2^a$ | $2.1 \pm 1.1^b$ | $2.0 \pm 1.2$ |
| Mortality rate (%) | $2 \times 10^2$ spores/ml | 42,3 | 50,4 | 46,4 |
| | $3 \times 10^2$ spores/ml | 82,7 | 100 | 91,4 |

*The values of the same line with different letters are significantly different at $p < 0.05$ according to the Fisher test.*

**Table 2.** Percentages of cumulative mortality corrected for Bb-IRAD.Fbt and Bb-IRAD.Nkoe.

| Treatments applied | Cumulative mortality corrected for each isolate of *Beauveria bassiana* (%) | |
|---|---|---|
| | Bb-IRAD.Fbt | Bb-IRAD.Nkoe |
| $C_{0-}$ (Water + tween 80) | $0 \pm 0^a$ | $0 \pm 0^a$ |
| $C_{0+}$ (Chlorpyriphos-ethyl 600 g/l) | $97.6 \pm 21^g$ | $97.6 \pm 21^g$ |
| $C_1$ ($3 \times 10^2$ spores/ml) | $35.5 \pm 13{,}3^b$ | $41 \pm 10.1^b$ |
| $C_2$ ($3 \times 10^4$ spores/ml) | $43.1 \pm 12{,}01^c$ | $48.4 \pm 10.1^c$ |
| $C_3$ ($3 \times 10^6$ spores/ml) | $54.5 \pm 15^d$ | $70.6 \pm 15.8^d$ |
| $C_4$ ($3 \times 10^8$ spores/ml) | $77.5 \pm 20{,}4^e$ | $83 \pm 16.4^e$ |
| $C_5$ ($3 \times 10^{10}$ spores/ml) | $87.8 \pm 21{,}0^f$ | $90.7 \pm 17.8^f$ |
| Analysis of variance | $R^2 = 0{,}978$<br>$F = 182{,}597$<br>$Pr < 0{,}0001$ | $R^2 = 0{,}980$<br>$F = 201{,}094$<br>$Pr < 0{,}0001$ |

*abc : Values with different letters in the same column are significantly different at the critical threshold of $\alpha = 0.05$ according to the Fisher test.*

**Table 3.** Linear probit regression of percentages of cumulative mortality corrected for decimal logarithms of observation time for each concentration of *Beauveria bassiana* up to the fourth day of observation.

| ***Beauveria bassiana* concentrations (spores/ml)** | Linear regression parameters | | | |
|---|---|---|---|---|
| | **Bb-IRAD.Fbt** | | **Bb-IRAD.Nkoe** | |
| | Regression equation | $LT_{50}$ & $LT_{90}$ (Day) | Regression equation | $LT_{50}$ & $LT_{90}$ (Day) |
| $3 \times 10^2$ | $y = 2{,}0563x + 2{,}7824$<br>$R^2 = 0{,}9463$ | 11,98 & 50, 31 | $y = 3{,}7423x + 2{,}4763$<br>$R^2 = 0{,}9715$ | 4,72 & 10,39 |
| $3 \times 10^4$ | $y = 2{,}4304x + 2{,}8873$<br>$R^2 = 0{,}972$ | 7,40 & 24, 92 | $y = 2{,}4507x + 3{,}2524$<br>$R^2 = 0{,}9915$ | 5,17 & 17,22 |
| $3 \times 10^6$ | $y = 2{,}9213x + 2{,}9312$<br>$R^2 = 0{,}9818$ | 5,11 & 14, 02 | $y = 2{,}3542x + 3{,}6722$<br>$R^2 = 0{,}9888$ | 3,66 & 12,83 |
| $3 \times 10^8$ | $y = 2{,}5379x + 3{,}5705$<br>$R^2 = 0{,}9765$ | 3,66 & 11, 70 | $y = 2{,}4219x + 3{,}9554$<br>$R^2 = 0{,}9898$ | 2,7 & 9,13 |
| $3 \times 10^{10}$ | $y = 3{,}8264x + 3{,}4619$<br>$R^2 = 0{,}9663$ | 2,52 & 5, 46 | $y = 3{,}3973x + 3{,}8568$<br>$R^2 = 0{,}9388$ | 2,17 & 5,17 |

*LT 50 or 90 = Lethal Time required to achieve 50 or 90% coffee berry borer mortality; Probit-logarithm regression equation of type $y = ax + b$ where y= probit of the percentage of mortality; x= logarithm of time or day; a= slope of the line; $R^2$= the coefficient of determination.*

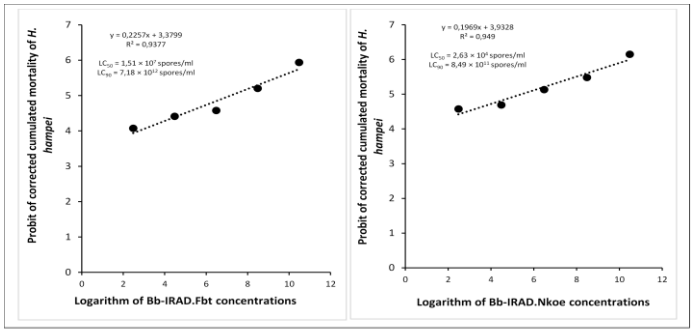


**Fig. 3.** Linear probit regression of the corrected cumulative mortality of *Hypothenemus hampei* as a function of the decimal logarithms of the doses of *Beauveria bassiana* after 4 days of incubation. LC 50 or 90 = Lethal Concentration causing 50 or 90% coffee berry borer mortality; Probit regression equation - logarithm of type y = ax + b where y= probit of the percentage of the corrected cumulative mortality of *H. hampei*; x= logarithm of the *B. bassiana* dose; a= slope of the line; $R^2$= the coefficient of determination.

and 2.6 days difference between the $3 \times 10^2$ and $3 \times 10^{10}$ spores/mL concentrations. This lethal time was about four times less than that observed with Bb-IRAD.Fbt for the same concentration range (Table 3). These results showed that Bb-IRAD.Nkoe was more virulent than Bb-IRAD.Fbt because the former infects and kills 50 and 90% of berry borers in less time than the latter.

**Pathogenicity of *B. bassiana***

The analysis of cumulative sporulation rates obtained from dead insects with sporulation signature at the time of observation, showed a positive but average relationship with the concentrations of Bb-IRAD.Fbt ($R^2$= 0.657; F= 7.772; Pr< 0.0001< α= 0.05; Fisher's test) and Bb-IRAD.Nkoe ($R^2$= 0.512; F= 4.254; Pr< 0.0001< α= 0.05; Fisher's test).

Between the concentrations $3 \times 10^2$ spores/mL and $3 \times 10^8$ spores/mL (16.4%) of Bb-IRAD.Fbt, one could say that the sporulation rate increased with concentration, but decreased at the concentration $3 \times 10^{10}$ spores/mL (12%). The same observations were made with Bb-IRAD.Nkoe, but with higher sporulation rates compared to Bb-IRAD.Fbt for the same concentrations (Fig. 4). There were no significant differences observed between the averages of the corrected cumulative sporulation rates of Bb-IRAD.Nkoe at $3 \times 10^4$, $3 \times 10^6$ and $3 \times 10^8$ spores/mL as was the case with Bb-IRAD.Fbt at the same concentrations. In addition, all berry borers cadavers incubated after observation showed a sporulation signature 2-3 days after incubation (Fig. 4).

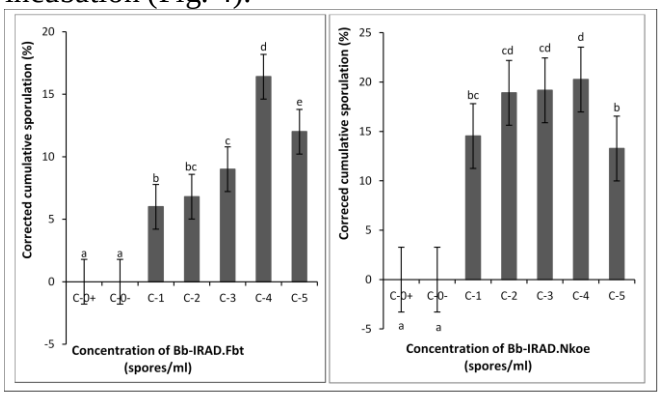


**Fig. 4.** Mean corrected cumulative sporulation of berry borers as a function of *Beauveria bassiana* Foumbot, water and pyriforce concentrations. $C_{0+}$ = pyriforce (Chlorpyriphos-ethyl 600 g/l); $C_{0-}$ = water + tween 80; $C_1 = 3 \times 10^2$ spores/mL; $C_2 = 3 \times 10^4$ spores/mL $C_3 = 3 \times 10^6$ spores/mL; $C_4 = 3 \times 10^8$ spores/mL; $C_5 = 3 \times 10^{10}$ spores/mL. **Values with different letters are significantly different at the threshold α= 0.05 according to the Fisher test.*

Daily analysis of the corrected observed sporulation rates showed that sporulation was greatest between the fourth day of incubation for the $3 \times 10^4$ and $3 \times 10^8$ spores/mL concentration, the fifth day for the $3 \times 10^6$ spores/mL concentration (8%) and the sixth day of incubation for the $3 \times 10^8$ spores/mL concentration (10%) of Bb-IRAD.Fbt. With Bb-IRAD.Nkoe, sporulation was higher for the concentrations $3 \times 10^4$, $3 \times 10^6$, $3 \times 10^8$ and $3 \times 10^{10}$ spores/mL between the second and fifth day. The $3 \times 10^2$ spores/mL concentration showed the highest sporulation from fifth day onwards and remained constant from day eighth to tenth day at 2% berry borers (Fig. 5).

Daily analysis of the corrected cumulative sporulation rates showed that these increase over time. With a concentration of $3 \times 10^6$ spores/ml of Bb-IRAD.Fbt, the sporulation rate on the sixth day reached an average of 26% of dead berry borers with a sporulation signature. The lowest rates (12% of berry borers on average) were observed from day eight with the lowest concentrations of $3 \times 10^2$ and $3 \times 10^4$ spores/mL. Sporulation rates were not correlated with Bb-IRAD.Nkoe

concentrations. The lowest rate (16% of dead berry borers with sporulation signature) was observed with the highest concentration $3 \times 10^{10}$ spores/mL. The concentrations $3 \times 10^{6}$ and $3 \times 10^{4}$ spores/mL showed the highest sporulation rates of 28.4% and 27.8%, respectively, on the tenth day (Fig. 6).

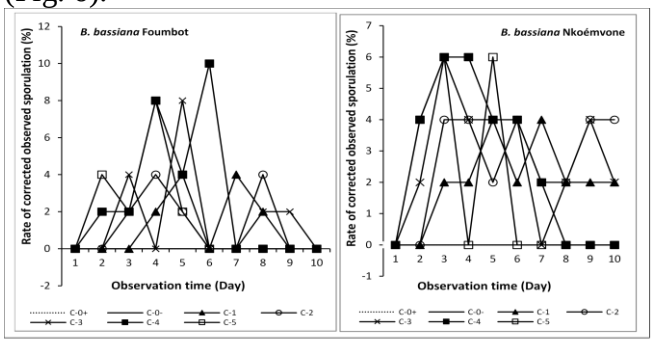


**Fig. 5.** Mean corrected observed sporulation of berry borers as a function of observation time. $C_{0+}$= pyriforce (Chlorpyriphos-ethyl 600 g/l); $C_{0-}$= water+tween 80; $C_1 = 3 \times 10^2$ spores/mL; $C_2 = 3 \times 10^4$ spores/mL $C_3 = 3 \times 10^6$ spores/mL; $C_4 = 3 \times 10^8$ spores/mL; $C_5 = 3 \times 10^{10}$ spores/mL.

### Virulence of *B. bassiana*

Table 4 shows that mortalities of more than 80% of berry borers were obtained by direct infestation of coffee berries with the $3 \times 10^8$ spores/ml *B. bassiana* concentration of each isolate. However, these rates were not significantly different between the two isolates. Sporulation rates reached 10% dead berry borers with a sporulation signature with Bb-IRAD.Fbt and 7% with Bb-IRAD.Nkoe. Analysis of variance shows that mortality was explained by the type of isolate and its concentration at 93% while sporulation was explained at only 48%. These results showed that the link between mortality, sporulation and type of isolate was very weak. Infestation of coffee berries at a concentration of $3 \times 10^8$ spores/mL of *B. bassiana*, caused 100% mortality to *H. hampei* on the seventh and eighth day with Bb-IRAD.Nkoe and Bb-IRAD.Fbt. respectively. The daily evolution of this mortality was increasing and almost identical with the two isolates. The sporulation rate reached 21.53% on the eighth day with Bb-IRAD.Fbt and 17.38% on the seventh day with Bb-IRAD.Nkoe at a concentration of $3 \times 10^8$ spores/mL. The number of berry borers that died with a sporulation signature was low compared to the number of berry borers that died in total with or without a signature (Fig. 7).

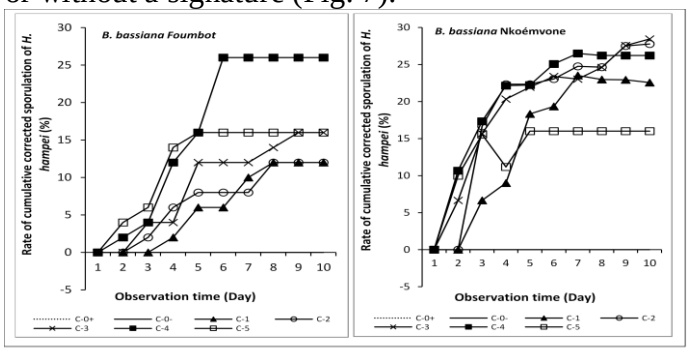


**Fig. 6.** Cumulative corrected berry borers sporulation caused by concentrations of *Beauveria bassiana* Foumbot and Nkoemvone as a function of observation time. $C_{0+}$= pyriforce (Chlorpyriphos-ethyl 600 g/l); $C_{0-}$= water + tween 80; $C_1 = 3 \times 10^2$ spores/mL; $C_2 = 3 \times 10^4$ spores/mL $C_3 = 3 \times 10^6$ spores/mL; $C_4 = 3 \times 10^8$ spores/mL; $C_5 = 3 \times 10^{10}$ spores/mL.

## DISCUSSION

*B. bassiana* could be isolated from soil samples (Elmholt and Kjøller, 1989) from coffee fields in Cameroon confirming its presence in coffee producing areas such as Amang-Mbang *et al.*, 2012. Significant differences were observed between the two isolates in spore production and radial growth. This showed that the two isolates were different and that this difference could be related either to their locality of origin, genetic recombination's or their cosmopolitan nature. Bb-IRAD.Fbt came from a locality marked by a milder and more humid climate, high altitude and Arabica production fields, while Bb-IRAD.Nkoe came from a locality marked by a milder and less humid climate, low altitude and Robusta production fields

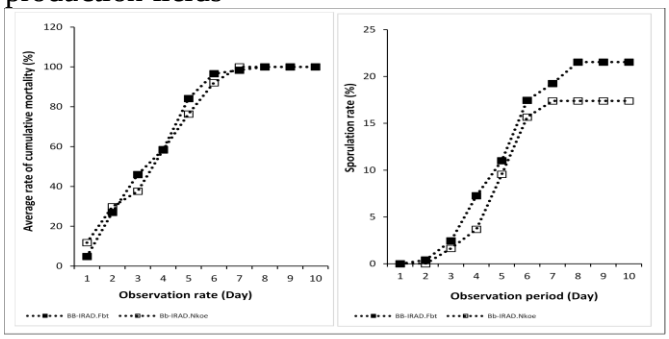


**Fig. 7.** Cumulative mortality of *Hypothenemus hampei* and sporulation rate of *Beauveria bassiana*.

**Table 4.** Cumulative mortality of *Hypothenemus hampei* and sporulation rate of *Beauveria bassiana* at a concentration of $3 \times 10^8$ spores/ml.

| Isolate | Cumulative mortality (%) | Cumulative sporulation rate (%) |
|---|---|---|
| Bb-IRAD.Fbt | 80.1 ± 22.2[a] | 9.7 ± 8.3[a] |
| Bb-IRAD.Nkoe | 80.1 ± 19.8[a] | 7,44 ± 4.2[a] |
| Analysis of variance | $R^2$ = 0,927<br>F = 53,282<br>Pr< 0,0001 | $R^2$ = 0,481<br>F = 3,903<br>Pr< 0,0001 |

**Values with different letters in the same column are significantly different at the threshold α= 0.05 according to the Fisher test.*

*Estrada et al*. (2007) and Wang *et al.* (2013) characterised strains of *B. bassiana* from several countries and found a great diversity related to the geographical origin of these strains. However, Toledo *et al.* (2018) found that the high genetic diversity among 36 isolates of *B. bassiana* in Argentina was not related either to the geographical origin of the strains or to their insect hosts.

After 21 days of incubation at 25±2 °C, the isolates were able to grow to mean radial growths of 1.9 and 2.0 cm for Bb-IRAD.Fbt and Bb-IRAD.Nkoe, respectively. This growth was relatively low and differs between isolates, which could be due either to the genetic characteristics of the isolates or to the PDA medium used, as temperatures of 20, 25 and 30 °C and the Agar SDA and PDA media were favourable for its development according to Heviefo *et al.* (2018). The same authors showed that the temperature of 25 ± 1 °C and SDA Agar were more suitable for mycelial development and spore production of *B. bassiana* while PDA was good for the isolation of *B. bassiana* from its host.

Mortality of berry borers reached peak rates of 88 and 91% with the two isolates of *B. bassiana*. These rates were almost identical to that (98%) caused by the chemical insecticide (Chlorpyriphos-ethyl 600 g/L) but at different times. These results confirmed that our two isolates native to Cameroon were effective against the coffee berry borer. The efficacy of these isolates could be better because the substrate in which the conidia were produced influences their effectiveness. Ludmilla *et al.* (2017) found that conidia of *B. bassiana* produced on rice and potatoes totally controlled eggs of *Tuta absoluta*.

Differences in time between the effect of the fungi and that of the chemical insecticide revealed the difference in the attack mechanism of Chlorpyriphos-ethyl and *Beauveria bassiana*. The chemical insecticide attacks directly (acting as a neurotoxin that inhibits acetylcholine esterase) with a rapid and total action from the second day, while the fungus attacks for several days in phases (adhesion, penetration, establishment or sporulation and dissemination) corresponding to its life cycle, which is also synchronous with the insect's development stages and environmental conditions (Shah and Pell, 2003; Manga Essouma *et al.,* 2021). This assertion agrees with that of Längle who stated in 2006 that biological control agents such as *B. bassiana* are significantly different from chemical pesticides in their properties and this should be taken into account when integrating and reviewing efficacy studies. Nevertheless, *B. bassiana* to kill berry borers during the important penetration phase corresponding to the second day after incubation (Barra-Bucarei *et al.*, 2019) or within 5 days (Cochereau and Potiaroa, 1994).

The efficiency of our isolates increased with the spores concentration and time. Likewise, this efficiency depended on the isolates in view of the concentrations and lethal times necessary to kill 50 and 90% of berry borers. The best effect was observed with Bb-IRAD.Nkoe in view of its $LC_{50}$, $LC_{90}$, $LT_{50}$, $LT_{90}$ and life cycle characteristics which were significantly better than those of Bb-IRAD.Fbt. The low altitude of this locality would act favourably on the virulence gene of this isolate. Amang-Mbang *et al.* (2012) showed that the presence and mortality rates of the berry borers due to *B. bassiana* were higher in the locality of Nkoémvone after berry harvest. Rates of dead berry borers with a sporulation signature were lower than those without a sporulation signature. From these results, we observed that our isolates acted inside the berry borers before showing signs

outside the infected host and this effect increased with concentration and time.

Direct infestation of coffee berries always results in a high mortality of berry borers, reaching 80%. *B. bassiana* spores are therefore able to infest berry borers even indirectly when they come into contact with pre-treated coffee berries (Mota *et al.*, 2017). Observation of sporulation rates showed that the majority of insects died before entering the berry by two cumulative effects, including the development of spores of the entomopathogenic fungus in their tissues, hunger and fatigue due to the efforts made to enter the berry. This ability to infest and kill berry borers is important in the natural environment to cope with berry attacks. Daily observation of this phenomenon showed that *B. bassiana* always acted with a latency time of between five and six days for a lethal effect on the berry borers.

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