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Potential of Cameroon-indigenous isolates of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* as microbial control agents of the flea beetle *Nisotra uniformis*

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ABSTRACT

The flea beetle *Nisotra uniformis* Jacoby is a widespread pest of malvaceous crops for which synthetic chemical insecticides remain the principal control tool, particularly in sub-Saharan Africa. With the ultimate aim of developing a microbial alternative for *N. uniformis* control, we conducted laboratory experiments (25 ± 1°C; 70 - 80% RH) to evaluate the biological attributes of three Cameroonian isolates of the entomopathogenic fungi *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin. Spore germination rates of the isolates varied from 0.5% after 4 hrs to 100% after 24 hrs on PDA medium, with MIITAC11.3.4 spores germinating fastest. Pathogenicity tests consisted of dipping adult beetles in conidial suspensions. All isolates were pathogenic to *N. uniformis*, with corrected mortalities varying between 23.3 - 86.7%. BIITAC-O-2 had the lowest LC_{50} at 5.17×10^6 conidia/ml, while MIITAC11.3.4 and BIITAC6.2.2 had LC_{50} at 4.11×10^7 conidia/ml, and 2.00×10^8 conidia/ml, respectively. BIITAC6.2.2 produced the shortest time to the highest mortality rate (1.17 days) and the shortest LT_{50} of 1.64 days but only at the highest concentration, followed by the two highest concentrations of MIITAC11.3.4 (LT_{50} at 3.43 and 5.70 days). The *c* parameter in the Weibull model showed that BIITAC-O-2 caused the highest rate of increase in mortality (2.42) at the highest concentration, followed by MIITAC11.3.4 (1.09) and BIITAC6.2.2 (0.50). The results of our study provide the basis for including *N. uniformis* in the ongoing development of BIITAC6.2.2 and MIITAC11.3.4 into biopesticide while continuing with further laboratory studies on BIITAC-O-2.

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Introduction

Nisotra uniformis (Coleoptera: Chrysomalidae) is considered one of the most destructive insect pests of several crop plant species, including *Abelmoschus esculentus* (L.) Moench (okra), *Corchorus olitorius* L. (jute mallow), *Gossypium* sp. L. (cotton), *Hibiscus cannabinus* L. (kenaf), *H. sabdariffa* L., *H. rosa-sinensis* L., and *Sesamum indicum* L. (sesame) (Donnelly, 1966; Jolivet, 1972; Sintim et al., 2014). *Nisotra uniformis* larvae live in the soil (up to 50 cm depth) where they feed on host plant roots; however, their underground damage has been considered negligible (Jolivet, 1972). Adult *N. uniformis* are light brown and feed on leaves and fruit structures, with leaf damage appearing characteristically as small round holes. Leaf damage by *N. uniformis* reduces host plant photosynthetic potential, leading to reduced vegetative growth, lower fruit yield, and marketable leaves (Odebiyi, 1980). Severe damage on young okra plants can also promote the development of various diseases by providing entry to pathogenic organisms (fungi, bacteria, viruses) which can lead to plant death (Vanlommel et al., 1996). Some *Nisotra* related species have been implicated in the transmission of okra mosaic virus (OKMV), one of the most important diseases of okra in Africa (Fajinmi & Fajinmi, 2006; Ndunguru & Rajabu, 2004). In addition, adult feeding on flowers and fruits may lead to flower abortion or loss of fruit marketability (George, 2003). Combined *N. uniformis* feeding on okra leaves, flowers and fruits can be responsible for up to 70% losses in okra fruit yield and quality (Programme Initiative Pesticides, 2008).

Control measures for the management of flea beetle populations are diverse, but applications of synthetic chemicals remain the principal control method used by farmers, especially in Africa (Abang et al., 2013; Mobolade et al., 2014). Most of the synthetic insecticides used by farmers have broad-spectrum activities that kill both beneficial and targeted insects, and they have been implicated in negatively affecting the health of users and consumers, as well as environmental pollution, insecticide resistance, and pest resurgence phenomenon (Devi et al., 1986; de Bon et al., 2014; Fan & Huang, 1991; Hassan et al., 2007). Alternative methods for flea beetle control in sub-Saharan Africa are rare (Abang et al., 2013; de Bon et al., 2014).

Biopesticides based on entomopathogenic fungi (EPF) have been shown repeatedly to be viable alternatives to synthetic chemical insecticides in insect and mite pest management (Srinivasan et al., 2019). More than 700 species of fungal pathogens of insects have been identified (Zimmermann, 2008) with numerous species or strains commonly used as biological control agents (Demir et al., 2013). The mode of action of EPF is complex enough to minimise the development of resistance. The host can be infected both by contact or ingestion, and the fungus can be dispersed within the target host population through horizontal transmission from infected to non-infected individuals (Kreutz et al., 2004). Another important aspect of the use of EPF is the ease of mass production of several well-known species (Shahid et al., 2012).

Numerous strains of two EPF species, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin, are the most used against a wide range of arthropod pests worldwide due to their demonstrated efficacy and ease of mass production (Chandler et al., 2011). The two EPF species have widely dominated the market of entomopathogenic fungi-based biopesticides in sub-Saharan Africa with more than 10 registered products (Hatting et al., 2019; Srinivasan et al., 2019).

In Cameroon, the International Institute of Tropical Agriculture (IITA) collected 40 local isolates of *B. bassiana* and *M. anisopliae* with the aim of establishing a biopesticide programme for multiple key crop pests in Central Africa (Mahot et al., 2019; Membang et al., 2020). Two of the collected isolates (MIITAC11.3.4 and BIITAC6.2.2) showed potential in the microbial control of the banana root borer *Cosmopolites sordidus* (Germar) and the cocoa mirid *Sahlbergella singularis* Haglund (Mahot et al., 2019; Membang et al., 2020). Considering their performance against the two pests, these two isolates were selected for pathogenicity tests against the flea beetle *N. uniformis,* in addition to a third isolate of *B. bassiana* (BIITA-C–O-2) isolated from *N. uniformis* in Cameroon (Bapfubusa, 2018). To our knowledge, no study related to the biological control of *N. uniformis* with fungal pathogens has been conducted, although recent field trials demonstrated the effectiveness of BotaniGard®, a *B. bassiana*-based biopesticide, against okra flea beetles of the genus *Podagrica* in Nigeria (Kudemepo et al., 2018). A biopesticide, particularly one based on local EPF isolate, would therefore provide an excellent alternative to the broad-spectrum synthetic pesticides that are widely used for the control of *N. uniformis* in Cameroon and elsewhere in sub-Saharan Africa.

The principal objective of this study was to evaluate the potential of three local isolates of *B. bassiana* and *M. anisopliae* against *N. uniformis* under laboratory conditions with the aim of selecting the most promising isolate(s) for the development of an effective biopesticide for this pest in vegetable production.

Materials and methods

Fungal cultures

Three isolates – two of *B. bassiana* (BIITAC6.2.2 and BIITAC-O-2) and one of *M. anisopliae* (MIITAC11.3.4) – were used in this study. BIITAC6.2.2 and MIITAC11.3.4 were isolated from soils collected in the Central region of Cameroon (N04°53′35.7″; E011°25′14.2″ and N04°35′68.0″; E011°15′81.3″ respectively) (Membang et al., 2020), while BIITAC-O-2 was isolated from a cadaver of an adult *N. uniformis* found on okra leaves at the IITA-Cameroon experimental farm (N03° 51′50.7″; E011°27′45″). Before the bioassays, BIITAC-O-2, BIITAC6.2.2, and MIITAC11.3.4 were re-isolated from adult *N. uniformis* infected in the laboratory. DNA barcoding correctly identified the two *B. bassiana* isolates; however, additional biological and morphological data were needed to support the identification of MIITAC11.3.4 as *M. anisopliae*, as two attempts at DNA barcoding of this isolate failed to conclusively place it in the phylogenetic tree of the isolates. All three isolates are maintained in the microbial collection of IITA-Cameroon in Yaoundé.

All the isolates were grown on Potato Dextrose Agar (PDA) in 9-cm plastic Petri dishes at 25 ± 1°C and 70-80% relative humidity. Fourteen-day-old conidia were harvested from the culture media by scraping the surface of fungal colonies with a sterile spatula and suspending the harvested fungal material in 10 ml of 0.1% sterile Tween 80 (1 ml/1 l). The suspension was vortexed (Vortex Genie 2TM; Bender & Hobein AG, Zurich, Switzerland) for 3 min to produce homogeneous conidial suspensions and filtered through sterile cheesecloth to remove mycelia and debris. Conidia concentrations

were determined using a haemocytometer (Malassez Cell) under the microscope (Leica) (40X). Five concentrations (3.2×10^5, 3.2×10^6, 3.2×10^7, 3.2×10^8, and 3.2×10^9 conidia/ml) were obtained through serial dilutions.

Assessment of spore germination overtime

To improve our isolate selection criteria, we modelled the germination response of all fungal isolates over time. Sixteen PDA plates (9 cm in diameter) per isolate were inoculated with 50 µl of conidial suspension at 3.2 × 106 conidia/ml and randomly assigned to four treatments. Each treatment represented a different period of post-inoculation inspection. Inoculated plates were sealed with Parafilm and stored at 25 ± 1°C, 12L:12D photoperiod, and relative humidity of 70-80%. The PDA plates were inspected for conidia germination at 4, 10, 14, and 24 hrs post-inoculation. Conidia with a germ tube were considered to have germinated (Milner et al., 1991). The germination process was stopped by spreading 40 µl of 0.1% formaldehyde (1 ml/l) on each plate to assess the exact number of germinated spores. One subsample of 100 conidia was observed at 40X magnification under the microscope in each of 4 quarter-fields of each replicate PDA plate.

Beetle collection

Adult *N. uniformis* were collected with a mouth aspirator from okra plants (var. Kirikou) grown in a field on the experimental farm of the IITA-Cameroon Yaoundé campus (03° 51′50N, 011°27′45E). The beetles were kept on 4-week-old potted okra plants (Kirikou variety) in cages (32.5 × 32.5 × 32.5 cm, W × D × H) and maintained under laboratory conditions (25 ± 1°C; 12L:12D; 70-80% RH). Prior to pathogenicity tests, a laboratory colony of *N. uniformis* was checked daily for 7 days. Dead individuals were removed from the colony and aseptically incubated in total darkness at 25 ± 1°C and 70-80% RH. Since mycosis did not develop on dead adult *N. uniformis*, the remaining living insects were used for pathogenicity tests.

Pathogenicity tests

Inoculum from the three isolates was used to infect *N. uniformis* in bioassays conducted under laboratory conditions (25 ± 1°C; 12L:12D photoperiod; 70-80% RH). Adult beetles were chilled for 90 sec at 4°C to immobilise them before their use in the bioassays. For each *M. anisopliae* and *B. bassiana* isolates, beetles were dipped individually for about 2 sec in 500 µl of conidial suspension (3.2×10^5, 3.2×10^6, 3.2×10^7, 3.2×10^8, and 3.2×10^9 conidia/ml); individuals in the control group were individually dipped in 0.1% Tween 80 (1 ml/l). The treated insects of each group were maintained in 9-cm plastic Petri dishes with muslin top covers. Each treatment was replicated three times in a complete randomised design. Ten insects were used per replicate. Each Petri dish contained a fresh okra leaf as food previously washed with 0.1% sodium hypochlorite (1 ml/l) and rinsed three times with distilled water. Leaves were replaced with fresh, similarly treated leaves at 2-day intervals. Beetle mortality was recorded daily for 14 days at the same period of the day. Dead insects were transferred aseptically to Petri dishes lined with

sterile cotton and moist filter paper and kept in a dark chamber at 25 ± 1°C and relative humidity of 70-80% for 7 days to encourage fungal outgrowth. Mycosis was used to confirm the pathogenic effect of the fungi. In our study, mycosis was characterised by fungal sporulation.

Statistical analysis

The effect of isolate and incubation time on germination rate (% germinated conidia) and the pathogenicity of these isolates to *N. uniformis* were averaged from all replicates. Germination rates did not fit a Gaussian distribution, and were therefore used in a Generalised Linear Model (GLM) assuming a quasi-binomial distribution and a logistic regression. Main factor effects were compared with Tukey HSD at $\alpha = 0.05$. When the interaction term was significant ($P < 0.05$), contrasts were used to compare the simple means of percent spore germination of the three isolates at each of the four incubation times based on a Tukey adjustment. Adult beetle mortality in pathogenicity tests was corrected with Abbott's formula (Abbott, 1925). Corrected mortality and mycosis data fit a Gaussian distribution with homogeneous variances and were used in 2-factor ANOVA with isolate and concentration as explanatory variables. Within factors, means were compared using the Tukey-HSD test. Mean corrected mortality for each concentration was used to estimate lethal concentrations at 50% (LC_{50}) of each isolate through Probit analysis, and a ratio test was conducted to compare the generated LC_{50} (Wheeler et al., 2006). The software Rstudio version 1.1.383 (RStudio Team, 2015) was used for the ANOVAs and Probit analyses.

Cumulative corrected mortality over the 14 days of the experiments was modelled using a modified Weibull growth model (Pinder et al., 1978; Meeker & Escobar, 1998):

$$F(T) = Mmax\left[1 - e^{-\left(\frac{T}{b}\right)^{c}}\right]$$

where $F(T)$ is the percent corrected mortality at a corresponding day (T) of observation and $Mmax$ is the maximum calculated average mortality at the end of the experiment, which replaces the asymptote parameter (a) in the Weibull growth model. The b coefficient represents the scale parameter and the time when mortality reaches its maximum rate of increase or the inflection point in the curve, while the c coefficient represents the rate of increase (or steepness) in mortality or the shape of the mortality curve. The model was fitted with the non-linear option in JMP Pro 14.3 (SAS Institute, 2019), which provided standard errors and confidence intervals of the two coefficients as well as time to 50% mortality (LT_{50}) through inverse predictions and estimation of confidence intervals. The model output also allowed comparisons of the model coefficients through the confidence intervals of their estimates, which is an important advantage not available with other models fitting mortality and mycosis over time (Pinder et al., 1978). All probabilities were considered at the 5% threshold.

Results

Spore germination over time

Spore germination rates (mean % ± SE) for all three isolates at four intervals over a 24-hr period are presented in Table 1. Spore germination differed among isolates and incubation period with a strong interaction between the two factors (Isolate: F = 169.9; df = 2, 180; $P < 0.001$; incubation period: F = 1230.4; df = 3, 180; $P < 0.001$; interaction: F = 85.4; df = 6, 180; $P < 0.001$). A total of 12 contrasts were generated, and significant differences were considered at $\alpha = 0.05$. At 4 hrs post-incubation, BIITAC-O-2 attained a higher germination rate than MIITAC11.3.4 ($P < 0.001$) and BIITAC6.2.2 ($P < 0.001$), which were both statistically similar ($P = 0.997$). Six hrs later, all isolates passed 50% germination except BIITAC6.2.2. Germination rates of the three isolates were different when incubated for 10 hrs ($P < 0.001$ for the three contrasts) with high rate for MIITAC11.3.4 (99.4% germination) compared with BIITAC-O-2 and BIITAC6.2.2. At 14 hrs post-incubation, the highest germination rate was attained by MIITAC11.3.4, followed by BIITAC-O-2 and BIITAC6.2.2 in decreasing order ($P < 0.001$ for the three contrasts). At 24 hrs post-incubation, all isolates exceeded 95% germination (100% for MIITAC11.3.4, 100% for BIITAC-O-2, and 96% BIITAC6.2.2), and they were all statistically similar ($P > 0.05$ for the three contrasts).

Pathogenicity

In this study, mean natural mortality and mean mycosis in the control group were respectively 16.7% and 0% at 14 days post-treatment. Overall, all isolates were pathogenic to *N. uniformis* but corrected mortality and mycosis of treated insects did not differ among isolates at all tested concentrations (corrected mortality: F = 3.03; df = 2, 30; $P = 0.063$; mycosis: F = 0.35; df = 2, 30; $P = 0.707$). All concentrations of each isolate were pathogenic, and corrected mortality and mycosis increased with concentration for all isolates and varied significantly by concentrations within each isolate (corrected mortality: F = 8.41; df = 4, 30; $P < 0.001$; mycosis: F = 10.7; df = 4, 30; $P < 0.001$). There was no interaction between isolate and concentration for both mortality (F = 0.48; df = 8, 30; $P = 0.86$) and mycosis (F = 0.46; df = 8,30; $P = 0.88$), indicating that mortality and mycosis increased similarly for all isolates. The Tukey test showed that the three highest concentrations of 3.2×10^7, 3.2×10^8, and 3.2×10^9 conidia/ml were statistically similar in terms of mortality and mycosis for all isolates, but only the conidial concentrations of 3.2×10^8 and 3.2×10^9 conidia/ml were significantly different from the lowest concentration of

Table 1. Conidia germination [mean (%) ± SE] at 4-time intervals during a 24-hr period for two *Beauveria bassiana* and one *Metarhizium anisopliae* isolates.

	Incubation time			
Isolate	4h	10h	14h	24h
BIITAC6.2.2	0.5 ± 0.18a	9.13 ± 0.94a	60.6 ± 2.07a	95.6 ± 0.89a
BIITAC-O-2	22.3 ± 2.73b	61.6 ± 3.50b	79.1 ± 2.78b	100 ± 0a
MIITAC11.3.4	2.13 ± 0.59a	99.4 ± 0.24c	98.8 ± 0.44c	100 ± 0a

For each isolate, means followed by the same lowercase letter in the same column (time of incubation) are not significantly different (ANOVA, Contrasts with corrected $\alpha = 0.05$).

3.2×10^5 conidia/ml in terms of mortality, whereas the concentration of 3.2×10^7 conidia/ml also differed from 3.2×10^5 conidia/ml in terms of mycosis (Table 2).

The results of the Probit analysis using corrected mortality of each isolate are presented in Table 3. Corrected mortality of all isolates at 14 days post-treatment fit the Probit model and allowed the estimation of median lethal concentrations (LC_{50}). BIITAC-O-2 had significantly the lowest LC_{50} (5.17×10^6 conidia/ml), followed by MIITAC11.3.4 (4.11×10^7 conidia/ml), and BIITAC6.2.2 (2.00×10^8 conidia/ml) (Table 3).

The Weibull growth model was applied to the isolates and concentrations that caused at least 50% beetle mortality at 14 days post-treatment since one of the objectives of using the Weibull model is to provide a prediction of the median lethal time (LT_{50}). Model parameter estimates and LT_{50} predictions (including 95% confidence limits) are presented in Table 4, and the graphical representations of model fitting are presented in Figures 1A-C. Of particular interest in using the modified Weibull model are (1) the rate at which mortality occurs (parameter *c*); (2) the time at which mortality reaches its greatest rate (parameter *b*); and (3) the advantage of using the model to provide an estimate of LT_{50} and associated confidence interval through inverse prediction.

The *b* coefficient of the Weibull model estimated the post-treatment time at which the mortality rate reached its maximum. The shortest time for this maximum mortality rate was reached in 1.17 days by BIITAC6.2.2 at 3.2×10^9 conidia/ml, followed by MIITAC11.3.4 at 3.2×10^8 conidia/ml and 3.2×10^9 conidia/ml in 4.63 and 2.89 days respectively (Table 4). On the other hand, the longest times to reach maximum mortality rate were those of BIITAC-O-2 at 3.2×10^6 conidia/ml in 9.01 days, followed by MIITAC11.3.4 at 3.2×10^7 conidia/ml in 8.69 days and BIITAC-O-2 at 3.2×10^8 conidia/ml in 7.80 days, which were all statistically similar based on the overlap in the confidence limits (CL) of the *b* values. Similarly, BIITAC-O-2 at 3.2×10^7 conidia/ml (7.51 days) and 3.2×10^8 conidia/ml (7.80 days) were statistically similar for the time at which maximum mortality was reached; however *b* values decreased with a higher concentration of this isolate (Table 4). In contrast, MIITAC11.3.4 recorded its lowest value of *b* at the second highest concentration of 3.2×10^8 conidia/ml (2.89). All *b* values for this isolate were statistically different based on comparisons of the 95% confidence limits.

The overall speed of mortality, as expressed in the *c* parameter, varied from the lowest overall rate of $c = 0.5$ for BIITAC6.2.2 at 3.2×10^9 to the highest of 2.58 for BIITAC-O-2

Table 2. Corrected cumulative mortality and mycosis rates (mean % ± SE) of *Nisotra uniformis* at 14 days when exposed to five concentrations of *Beauveria bassiana* and *Metarhizium anisopliae* isolates.

	Concentration (conidia/ml)				
Isolate	3.2×10^5	3.2×10^6	3.2×10^7	3.2×10^8	3.2×10^9
Mortality					
BIITAC6.2.2	23.3 ± 3.33a	33.3 ± 18.6ab	33.3 ± 3.33abc	43.3 ± 8.82bc	73.3 ± 14.5c
BIITAC-O-2	33.3 ± 12.0a	50.0 ± 17.3ab	56.7 ± 6.67abc	73.3 ± 17.6bc	86.7 ± 3.33c
MIITAC11.3.4	20.0 ± 5.77a	26.7 ± 12.0ab	56.7 ± 8.82abc	70.0 ± 15.3bc	70.0 ± 15.3c
Mycosis					
BIITAC6.2.2	20.0 ± 0a	50 ± 25.2ab	60.0 ± 17.32bc	60.0 ± 5.77c	80.0 ± 15.3c
BIITAC-O-2	23.3 ± 8.82a	46.7 ± 3.33ab	60.0 ± 5.77bc	83.3 ± 12.0c	90 ± 0c
MIITAC11.3.4	30.0 ± 15.3a	30.0 ± 10ab	53.3 ± 12.0bc	83.3 ± 16.7c	83.3 ± 16.7c

Row means followed by the same lowercase letter in the same row are not significantly different (ANOVA, Tukey HSD, α = 0.05)

Table 3. Median lethal concentrations (LC_{50}) and their 95% confidence limits (CL) – estimated through Probit analysis using mean corrected mortality at 14 days post-treatment of each of 5 concentrations of two *Beauveria bassiana* and one *Metarhizium anisopliae* isolates infecting *Nisotra uniformis*.

Isolate	n‡	Slope ± SE	LC_{50} (conidia /ml)	95% CL	$\chi^{2‡‡}$
BIITAC6.2.2	5	0.30 ± 0.13	2.00×10^{8c}	8.85×10^{6}-5.84×10^{14}	1.50
BIITAC-O-2	5	0.37 ± 0.14	5.17×10^{6a}	1.14×10^{4}-5.89×10^{7}	0.17
MIITAC11.3.4	5	0.39 ± 0.14	4.11×10^{7b}	2.39×10^{6}-5.39×10^{9}	0.93

‡number of concentrations; ‡‡Pearson chi-square goodness of fit test for the Probit model (α = 0.05).

at 3.2×10^{8} (Table 3). Differences observed among mortality parameters lead to a different form of the mortality function produced by each fungal concentration (Figure 1A-C). Generally, fungal concentrations with low values of *c* showed mortality curves characterised by rapid initial mortality, which then increased more slowly (e.g. BIITAC6.2.2 with a *c* of 0.5, Figure 1A), while those with higher values of the *c* parameter showed an initial delay before a rapid acceleration of mortality (e.g. BIITAC-O-2 with a *c* of 2.58) (Figure 1B). The *c* parameter decreased with higher concentrations in BIITAC-O-2 but not in MIITAC11.3.4 (Table 4).

Inverse prediction allowed us to generate LT_{50} values for fungal concentrations that killed at least 50% of the beetles (Table 4). All isolates produced their shortest LT_{50} at their respective highest concentration, except MIITAC11.3.4 at 3.2×10^{9} conidia/ml, which reached its LT_{50} at 5.7 days, while MIITAC11.3.4 at 3.2×10^{8} conidia/ml reached its LT_{50} 2.27 days earlier. Among the three isolates, BIITAC6.2.2 at 3.2×10^{9} conidia/ml was the most virulent as it reached the smallest LT_{50} in 1.81 days, while MIITAC11.3.4 at 3.2×10^{7} conidia/ml, BIITAC-O-2 at 3.2×10^{7} conidia/ml, and BIITAC-O-2 at 3.2×10^{8} conidia/ml were the least virulent, reaching 50% mortality in 13.9, 10.4 and 8.22 days respectively.

Discussion

In this study, we determined that the three EPF isolates, MIITAC11.3.4, BIITAC-O-2, and BIITAC6.2.2, were all pathogenic to *N. uniformis*. Nonetheless they were substantially different in terms of spore germination rates over time and their virulence to *N. uniformis* (LC_{50}, LT_{50}, and other parameters associated to the Weibull model).

Table 4. Predicted parameters and median lethal times LT_{50} for two *Beauveria bassiana* and one *Metarhizium anisopliae* isolates infecting *Nisotra uniformis*.

	Model parameters and their confidence limits				
Concentration(conidia/ml)*	*b*	95% CL	*c*	95% CL	Predicted LT_{50} (95% CL) days
3.2×10^{9}	1.17	0.72-1.62	0.50	0.31, 0.51	1.64 (1.12-2.16)
3.2×10^{6}	9.01	8.31-9.78	1.60	1.29-1.97	NA**
3.2×10^{7}	7.51	7.06-7.97	2.31	1.89-2.87	10.4 (9.47-11.4)
3.2×10^{8}	7.80	7.04-8.23	2.58	1.81-4.17	8.22 (7.41-9.04)
3.2×10^{9}	6.57	6.40-6.75	2.42	2.21-2.68	6.18 (6.01- 6.35)
3.2×10^{7}	8.69	7.76-9.71	1.61	1.14-2.42	13.9 (11.3-16.5)
3.2×10^{8}	2.89	2.38-3.45	1.30	0.94-1.90	3.43 (2.81-4.06)
3.2×10^{9}	4.63	4.11-5.17	1.09	0.90-1.31	5.70 (5.07-6.34)

* The Weibull model was fitted only for isolates that produced at least 50% mortality at day 14 post-treatment (see Table 2 for M*max* values used in the model)

** Inverse prediction of the LT_{50} was not generated for this treatment

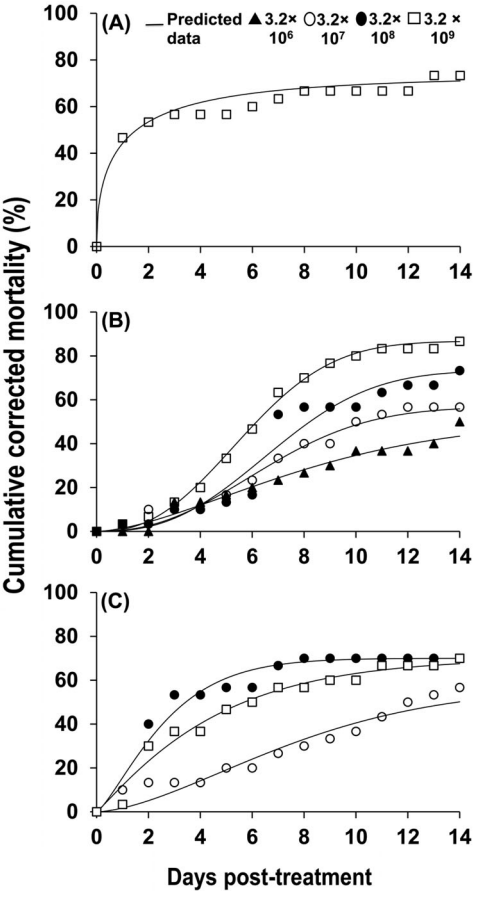

Figure 1. A-C. Actual and predicted cumulative corrected mortality of *Nisotra uniformis* after application at different concentration of two isolates of *Beauveria bassiana* (1A – BIITAC6.2.2 and 1B – BIITAC-O-2) and one isolate of *Metarhizium anisopliae* (1C – MIITAC11.3.4). For brevity and clarity, only data of isolates that caused higher than 50% mortality at 14 days post-treatment were included in Figures 1 A-C; see Table 2.

All evaluated EPF isolates displayed different germination rates, as expressed in the speed of spore germination and the total number of spores that germinated within 24 hrs. About 20% of spores of the *B. bassiana* isolate BIITAC-O-2 had already germinated at 4 hrs post-incubation, while MIITAC11.3.4 was the first to pass 90% germination before 10 hrs. Several studies have shown that rapid spore germination positively correlates with high virulence (Altre et al., 1999; Charnley, 1989). Therefore, considering the spore germination potential alone, MIITAC11.3.4 could be ranked highest in terms of promising potential for microbial control. However, other pathogenicity determinants related to spore biology must be considered (e.g. spore length, growth rate, and enzyme production; Samuels et al., 1989; Liu et al., 2015; Cortez-Madrigal et al., 2014).

Based on our results, all isolates were pathogenic to *N. uniformis*, and pathogenicity was significantly influenced by concentration. Mycosis was characterised by fungal sporulation and provided evidence of the pathogenic effect of the fungi. Indeed,

fungal sporulation considerably promotes the horizontal transmission of the disease within a host population, thus increasing the efficacy of the fungus (Toledo et al., 2007). Fungal sporulation can be influenced, however, by many factors, such as co-infection by other pathogenic organisms (Thomas et al., 2003), the presence of saprophytic microorganisms on the insect cuticle (Chouvenc et al., 2012), and the inoculated dose of spore (Luz et al., 1999).

During the 14 days of the pathogenicity experiments, *N. uniformis* mortality increased with time for all isolates at all concentrations. BIITAC6.2.2 resulted in the shortest LT_{50} although MIITAC11.3.4 was the fastest isolate to germinate and was expected to produce the shortest lethal time, according to Jackson et al. (1985). This result is similar to that reported by Safavi et al. (2007), who demonstrated that spore germination alone could not predict the virulence of a fungal isolate. Furthermore, it has been established that LT_{50} values decrease when test concentrations increase (Liu et al., 2002), but we observed that the shortest LT_{50} was not produced at the highest conidial concentration for the isolate MIITAC11.3.4. Since adult beetles were harvested from field-grown okra plants at an undetermined age, such observations could be due to differences in cuticle thickness (Anbutsu et al., 2017). Indeed, the infection success of a fungal spore depends highly on the texture and structure of the insect cuticle (Hajek & St Leger, 1994). We used the immersion method for our bioassay, where the conidia's position (and number) on the insect cuticle after treatment was not defined. Butt et al. (1992) observed significantly different LT_{50}s in bioassays when spore inoculation was done under the elytra of flea beetles versus when spores were applied on the dorsal and ventral thorax of beetles. Suitable biopesticide formulation and adequate application methods can increase spore hydrophobicity, allow spores to reach vulnerable sites of the insect, and result in better performances of the fungus (Ansari & Butt, 2012).

Of the three isolates, BIITAC-O-2 induced high mortality in *N. uniformis* populations at a relatively low concentration ($LC_{50} = 1.14 \times 10^4$ conidia/ml), which presumes its potential for biopesticide development over the two other isolates. Indeed, an EPF isolate that produces a low LC_{50} value is considered advantageous for field application purposes as less conidial material will be needed in the biopesticide composition (Ángel-Sahagún et al., 2010).

The change in beetle mortality over time was modelled with the Weibull growth model, which has been recommended for survival bioassays because of its flexibility (Carroll, 2003). The Weibull model generated estimates of two parameters, *b*, and *c*, that provided valuable insights into the performance of each fungus isolate at a specific concentration over time. The *b* parameter, the inflection point on the mortality curve, estimates how long it takes for an isolate to reach its maximum speed of host kill rate; as such, it distinguishes fast-acting isolates from those that are slower in killing their hosts. Based on the *b* parameter, the highest concentration of BIITAC6.2.2 is extremely fast-acting as it reached the highest mortality rate at 1.17 days post-treatment, while the time of maximum mortality rate ranged between 2.89 and 9.01 days for the two other isolates. Such rapid mortality was observed repeatedly with two coleopteran species in a bioassay with *B. bassiana*, and *M. anisopliae* isolates at a wide range of temperatures (Ak, 2019). Moreover, there is a very strong correlation between *b* and LT_{50} ($r = 0.920$, $P = 0.003$), as fast-acting isolates reach their 50% kill time (LT_{50}) in a shorter time than slower-acting isolates.

The *c* coefficient of the Weibull model expresses the overall rate of increase in mortality and provides an overview of the shape of the mortality curve. Hancock et al. (2009) demonstrated that preferred fungal agents for mosquito control are those that show strong virulence and higher rates of increase in mortality. Interestingly, BIITAC6.2.2 at 3.2×10^9 conidia/ml produced the lowest LT_{50} and the lowest rate of increase but the fastest initial kill rate at the highest concentration only, while all four lower concentrations produced less than 50% mortality at 14 days post-treatment (Table 2). Pinder et al. (1978) observed that a value of $c = 1$ indicates constant mortality. In our study, only MIITAC11.3.4 at 3.2×10^8 and 3.2×10^9 conidia/ml showed constant mortality as indicated in their *c* parameter values of 1.3 and 1.09 respectively, which were not significantly different from 1 (Table 4). These two highest concentrations of MIITAC11.3.4 caused high mortality rates in relatively short times (2.89 and 4.63 days post-treatment, respectively) and achieved median mortality at 3.43 and 5.7 days post-treatment, respectively. The aforementioned results suggest that MIITAC11.3.4 is a highly virulent strain. On the same basis, BIITAC-O-2 can be considered of intermediate virulence with its intermediate times to reach maximum kill speed and its median time to kill half of the adult beetles. However, BIITAC-O-2 showed the highest rates of increase (*c*) at three concentrations, and beetle mortality was concentrated around the LT_{50}. Indeed, BIITAC-O-2 induced a proportion of infected individuals higher than in the enzootic condition, i.e. a situation where the disease is constantly present in the host population ($c = 1$), demonstrating its epizootic potential. Isolates with lower *c* values caused earlier mortality, but then mortality got scattered in time.

According to several studies, fungal isolates with good germination capacities, low $LC5_0$, and LT_{50} values, and the potential to cause mycosis, have been demonstrated to be good biocontrol agents under field conditions (Ángel-Sahagún et al., 2010). Added to the previous parameters, we considered the mortality rate of increase (*c*) as an additional tool in the isolate screening process. Regarding germination capacities, the three isolates, MIITAC11.3.4, BIITAC-O-2, and BIITAC6.2.2, showed good performances, but MIITAC11.3.4 stood out by its rapid spore germination rate. Pathogenicity tests showed that BIITAC-O-2 was the isolate with the lowest LC_{50} value and the highest mortality growth rates, which underscore the epizootic potential of BIITAC-O-2; however, this isolate needed 6 days to reach 50% beetle mortality at the highest concentration (3.2×10^9 conidia/ml). Considering the germination rate, the LC_{50}, the LT_{50}, and the *c* parameter in the Weibull model, MIITAC11.3.4 stands out as the most balanced of the three isolates. Moreover, MIITAC11.3.4 possesses other desirable properties in terms of thermal tolerance and capacity for horizontal transmission (Mahot et al., 2019; Membang et al., 2021), which are yet to be examined for BIITA-C–O-2.

Although this is the first study of the use of Cameroon EPF against *N. uniformis*, it builds on several others that had selected the two isolates MIITAC11.3.4 and BIITAC6.2.2 for the development of biopesticides for *S. singularis* in cocoa plantations and *C. sordidus* in banana and plantain plantations (Mahot et al., 2019; Membang et al., 2020). The results of our study support the addition of *N. uniformis* to the targets of a potential biopesticide based on MIITAC11.3.4 and BIITAC6.2.2.

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