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Evaluation of the Entomopathogenic Potential of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* for Management of *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae)

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Abstract: The banana weevil (BW), *Cosmopolites sordidus*, is the main coleopteran pest of banana, causing up to 100% yield loss. In this study, we screened 20 isolates of entomopathogenic fungi (EPF) for the management of BW. In the lab, eight *Beauveria bassiana* isolates caused >50% mortality of the adult BW, whereas *Metarhizium anisopliae* and *Isaria fumosorosea* isolates were less pathogenic. *B. bassiana* isolates ICIPE 648, ICIPE 660 and ICIPE 273 were the most pathogenic, killing ≥80% of adult BW. *B. bassiana* isolate ICIPE 622 yielded the highest spores per BW cadaver (1.84×10^8 spores), followed by ICIPE 660, ICIPE 273 and ICIPE 648—1.17×10^8, 3.8×10^7 and 3.6×10^7 spores, respectively. ICIPE 273 had the shortest LT_{50} (5.3 days) followed by ICIPE 648 (9.8 days) and 660 (11.1 days). Similarly, the LC_{50} values for the three isolates were 5.18×10^7, 5.49×10^7 and 5.2×10^7 spores mL^{-1}, respectively. In the field, ICIPE 273 and ICIPE 648 had the highest (31.3%) and lowest (20.8%) pathogenicity, respectively. This study indicates that the *B. bassiana* isolates ICIPE 273, ICIPE 648 and ICIPE 660 are potential candidates for the environmentally sustainable management of BW.

Keywords: banana weevil; spore production; pathogenicity; biological control; banana

1. Introduction

Banana (*Musa* spp.) is an important food and cash crop feeding more than 400 million people, with an annual global production estimated at approx. 167 million metric tons [1]. The estimated international trade value for banana in 2016 was US$ 11.5 billion [2]. In 2018, approximately 10% of the global banana production was produced in the East African region [1] with most of the production coming from small plots and backyard gardens. For some countries in East Africa, the annual per capita consumption exceeds 200 kg of banana. Nonetheless, banana production in this region has declined over the years. Biotic and abiotic constraints have been reported as major impediments to banana production in the region [3,4].

The banana weevil (BW), *Cosmopolites sordidus* Germar (Coleoptera: Curculionidae), is the most important insect pest hindering banana production in the major banana growing areas [5,6]. The adult BW are cryptic and nocturnal, sheltering in or around the banana corms and between leaf sheaths. The female adult lays between one to four eggs per week, which it deposits in cavities that it makes with its rostrum in the banana corm and

lower pseudostem. The newly hatched larvae tunnel their way into the corm as they feed and grow, making it the most destructive stage of the insect. The BW is disseminated primarily as eggs or larvae in infested planting materials. Banana weevil infestation can deter crop establishment or shorten plantation life [5,7–10]. In the 1980s, BW infestation was implicated as a primary factor that contributed to the shift in cultivation of East African highland banana (*Musa* spp., genome group AAA-EA, EAHB) from its traditional central region of Uganda to the South-Western part of the country [11]. In on-station trials, yield losses of more than 40% have been attributed to BW infestation [5,6], while up to 100% yield loss has been observed in severely infested farmer's fields [12]. Moreover, recent studies have demonstrated the potential of BW as a vector for several banana diseases, including *Fusarium oxysporum* f. sp. *cubense* tropical race 4 [13,14]. The design and development of new and sustainable management options for this pest is, therefore, of vital importance.

Control and management of BW in smallholder farming systems has relied on the use of pseudostem traps and maintenance of a clean, healthy banana plantation [7,10,15], with other cultural, chemical and biological strategies practiced to a lesser extent [16–18]. Most farmers in the East African region grow banana without practicing any control measures against BW infestation. Currently, breeding for resistance to BW is one of the main targets of banana breeding activities in the region [17,19–21]. Due to the cryptic nature of the adults and given that other life stages grow within and are protected by the banana corm, the use of systemic synthetic pesticides would be recommended. However, the increase in the global ban on most of the synthetic pesticides coupled with the high cost of the pesticides leaves banana farmers with few alternative management options. The possibility of using entomopathogenic fungi (EPF) in the management of BW has been studied in vitro [22–25] and in the field [26,27].

The possibility to identify and develop new potential fungal isolates against BW is dependent on continued bioprospecting and screening of EPFs. Using local and indigenous isolates provides a further advantage, as these are not subjected to phytosanitary restrictions and require no permit for introduction into the country; in addition, they are adapted to local environmental conditions. The Arthropod Pathology Unit (APU) of the International Centre of Insect Physiology and Ecology (*icipe*) has a collection of more than 300 EPFs classified into *Beauveria*, *Metarhizium* and *Isaria,* among others [28]. Efficacy of these EPFs has been tested against a number of plant pests including leaf miners: *Liriomyza sativae* (Blanchard) and *L. trifolii* (Burgess) [29,30], *L. huidobrensis* (Blanchard) [31], *Spoladea recurvalis* (Fabricius) [32], stem borers: *Chilo partellus* (Swinhoe) and *Busseola fusca* (Fuller) [29,33,34], pod borers: *Maruca vitrata* (Fabricius) [35], sap-sucking: *Megalurothrips sjostedti* (Trybom) [36], *Aphis craccivora* (Koch) [37] and fruit flies: *Ceratitis capitata (*Weidemann), *C. rosa* (Karsch), *C. cosyra* (Walker) [38] and *Zeugodacus cucurbitae* (Coquillett) [39], among others. Similarly, Real IPM Ltd., Thika, Kenya, has a collection of several potential fungal isolates isolated from various locations within Kenya. However, the potential of Kenyan isolates of EPF for the management of BW is not well determined.

This study was, therefore, conducted to assess the efficacy of selected *Beauveria*, *Metarhizium* and *Isaria* EPF isolates from the *icipe* and Real IPM fungal repositories against adults of the BW in the laboratory, and to identify the most effective isolate(s) based on mortality rate, lowest effective dosage and highest spore production on BW cadavers. The selected effective isolate(s) from the pathogenicity screening in the laboratory were further tested for efficacy under the field and these would be recommended to be developed into mycoinsecticides.

2. Materials and Methods

2.1. Trapping and Maintenance of the Adult Banana Weevil

Adult BW of indeterminate age were obtained from a naturally infested EAHB field at the Industrial Crops Research Institute (ICRI) of the Kenya Agricultural and Livestock Research Organization (KALRO), Mwea, Kenya. The BW were trapped using the split banana pseudostem trap [40]. Pieces of banana pseudostems (~30 cm long) were split and

placed around banana mats in the field. The BW were collected from the sliced part of the pseudostems facing the ground after 1–2 days. Identity of the collected BW was made based on the description provided by Treverrow [10] and Viljoen et al. [40]. Collected BW were maintained in 10 L buckets with screened lids. A banana corm piece (~400 g) from a susceptible EAHB cultivar (Ngombe) was provided for BW to feed on and was changed weekly; the BW were maintained in the laboratory (25 ± 2 °C) for at least two weeks before they were used in bioassays.

2.2. Fungal Isolates

A total of 20 fungal isolates, 15 *B. bassiana*, 4 *M. anisopliae* and 1 *I. fumosorosea* were screened for their entomopathogenic potential against the adult BW. Fungal cultures were obtained from the *icipe* and Real IPM fungal germplasm collections. Origin, year of isolation and primary host are presented in Table 1. The identities of these fungal isolates were previously established based on morphological features as described by Goettel et al. [41] and Humber [42], and molecular tools based on the conserved internal transcribed spacer (ITS) regions of the respective EPF isolate DNA. *Beauveria bassiana* and *I. fumosorosea* isolates were sub-cultured on potato dextrose agar (PDA) medium (39 g/L distilled water), while *M. anisopliae* isolates were sub-cultured on Sabouraud dextrose agar (SDA) medium (65 g/L distilled water). All isolates were cultured in sterile 90 mm plastic Petri dishes and maintained in the incubator (25 ± 2 °C) in the dark for 3–4 weeks until sporulation.

Table 1. Identity of selected entomopathogenic fungal isolates and germination of their respective spores on Sabouraud dextrose agar (SDA) media after 18 h incubation at 25 ± 2 °C.

Fungal Species	Isolate	Year of Isolation	Isolation Host/Source	Locality (Country) of Isolation	Repository	% Viability (Mean ± SE)
Beauveria bassiana	ICIPE 273	2006	Soil	Mbita (Kenya)	ICIPE, Kenya	94.2 ± 1.36 ab
	ICIPE 279	2005	Coleopteran larvae	Kericho (Kenya)	ICIPE, Kenya	92.2 ± 1.22 b
	ICIPE 281	2005	Soil	Mauritius	ICIPE, Kenya	94.1 ± 0.10 ab
	ICIPE 284	2005	Soil	Mauritius	ICIPE, Kenya	94.5 ± 1.07 ab
	ICIPE 603	2007	Hymenoptera	Taita hills (Kenya)	ICIPE, Kenya	92.6 ± 1.15 ab
	ICIPE 609	2008	Soil	Meru (Kenya)	ICIPE, Kenya	93.9 ± 0.92 ab
	ICIPE 621	2008	Soil	Kericho (Kenya)	ICIPE, Kenya	93.5 ± 1.07 ab
	ICIPE 622	2008	Soil	Kericho (Kenya)	ICIPE, Kenya	94.0 ± 1.79 ab
	ICIPE 644	2007	Soil	Mauritius	ICIPE, Kenya	96.1 ± 1.44 ab
	ICIPE 647	2005	Soil	Mauritius	ICIPE, Kenya	94.4 ± 0.02 ab
	ICIPE 648	2007	Soil	Kericho (Kenya)	ICIPE, Kenya	95.5 ± 1.00 ab
	ICIPE 660	2008	Soil	Kemokock (Kenya)	ICIPE, Kenya	93.0 ± 1.36 ab
	ICIPE 662	2008	Soil	Mariakani (Kenya)	ICIPE, Kenya	94.9 ± 0.68 ab
	SD 229-Bb01	2008	Soil	Thika, Kenya	Real IPM, Kenya	96.3 ± 1.62 ab
	SD 277-Bb02	2018	White scale insect	Thika, Kenya	Real IPM, Kenya	93.8 ± 0.48 ab
Metarhizium anisopliae	ICIPE 18	1989	Soil	Mbita (Kenya)	ICIPE, Kenya	96.9 ± 0.48 ab
	ICIPE 62	1990	Soil	Matete (DRC)	ICIPE, Kenya	95.8 ± 0.74 ab
	ICIPE 69	1990	Soil	Matete (DRC)	ICIPE, Kenya	97.9 ± 0.37 a
	ICIPE 78	1990	*Temnoschoita nigroplagiata*	Ungoe (Kenya)	ICIPE, Kenya	98.0 ± 0.59 a
Isaria fumosorosea	ICIPE 682	2015	Soil	Masai Mara (Kenya)	ICIPE, Kenya	92.6 ± 0.32 ab

Means within same column followed by same letter(s) are not significantly different by Student-Newman-Keuls (SNK) test at $p < 0.05$.

For each individual fungal isolate, a spore suspension was prepared by washing the plates containing the spores with 10 mL of a sterile aqueous solution of 0.01% (*v/v*) Triton X-100 and spores scraped off using a sterile metal spatula. Individual suspensions were each collected into a sterile 30 mL universal bottle containing 3 mm diameter glass beads; this formed the stock suspensions.

2.3. Spore Viability

For each isolate, the fungal spores were quantified using a Neubauer hemocytometer under a light microscope (LEICA DMLS, Leica Microsystems GmbH, Wetzlar, Germany) at 400× magnification; the spore concentrations for each isolate were adjusted to 3.0×10^6 spores mL^{-1}. For each isolate a 100 µL spore suspension was spread plated onto SDA media in individual 90 mm Petri dishes using a drigalski spatula. Four replicate plates were used for each isolate. The plates were sealed with parafilm and maintained in an incubator (25 ± 1 °C) for 16–18 h. Spore germination was halted by spreading ~1 mL lactophenol cotton blue solution on the agar surface in each Petri dish. Four sterile glass cover slips were then placed on the agar surface of each Petri dish. The percentage spore viability was determined by selecting ~100 spores under each coverslip and both the germinated and non-germinated spores counted using a light microscope (400× magnification). Spores were considered as germinated if their germ tubes were two times longer than the propagule diameter [41].

2.4. Bioassays

2.4.1. Pathogenicity against Adult *Cosmopolites sordidus* in the Laboratory

Prior to use in any bioassay, the adult BW were placed on a kitchen sieve, washed with sterile distilled water and then blotted dry with a paper towel. The BW were further subjected to a 'fitness test' in which they were placed on one side of a rectangular plastic container (20 × 12 × 5 cm, L × W × H) and a piece of banana corm (~100 g) placed on the opposite side and then left to stand for 1 h. Only BW that moved towards the banana corm were considered as fit to be used in a bioassay and were selected for further use.

To screen out the most effective isolates among the 20 under study, 10 fit adult BW were placed on a plastic 90 mm Petri dish lined with sterile filter paper and sprayed (treated) with a 10 mL suspension of 1.0×10^8 spores mL^{-1} of respective fungal isolates [25,32,37]. Ten untreated control BW were sprayed with 10 mL of sterile 0.01% (*v/v*) Triton X-100. The spraying was performed using a potter precision laboratory spray tower (Burkard Scientific, London, UK) [43]. Rotation of the spray tower from the base ensured a homogeneous distribution of the designated spore suspension onto the plate containing the test BW. The treated BW remained in the Petri dish for 24 h, before transferring into plastic containers (20 × 12 × 5 cm, L × W × H) with screened lids (~1 mm mesh) using sterile forceps [44], along with ~150 g banana corm as food, which was changed every three days. All the treatments were maintained in a dark room at 25 ± 2 °C. Each treatment was replicated four times and the treatments were arranged in a completely randomized design; the experiment was repeated once in time.

Observation of dead BW was conducted at three-day intervals for 35 days [22,23]. Since disturbed adult BW display thanatosis (feigning death) [45], a 'confirmation of death' test was performed before dead BW were removed from the containers. The BW were separated from the banana corm, placed on one side of the container and fresh banana corm placed on the opposite side. The containers were left to stand in darkness for 1 h. Only those BW that did not move towards the banana corm after 1 h were considered as dead. Dead BW were disinfected with 1% sodium hypochlorite and 70% ethanol, followed by three rinses in sterile distilled water for ~1 min. The disinfected BW were placed in a plastic sterile Petri dish lined with moist sterile filter paper; Petri dishes were sealed with parafilm and incubated (25 ± 2 °C) for 14 days to monitor for mycosis [46].

2.4.2. Assessment of Spore Production on Banana Weevil Cadavers

Eight fungal isolates that displayed >50% BW mortality were selected for determination of spore production levels on BW cadavers. Treatment and spraying of BW with the fungal isolates was performed as described for the pathogenicity assay above. For each isolate, the setup was maintained until three cadavers were picked from each replicate, thus, a total of 12 cadavers were assessed for each isolate. The cadavers were incubated in the dark (25 ± 2 °C) for 14 days in sterile plastic Petri dishes lined with sterile moist

filter paper. Each mycosed BW cadaver was individually vortexed in 5 mL of 0.01% sterile Triton X-100 contained in 30 mL universal bottles. Spore production was quantified using a Neubauer counting chamber [46] under a light microscope at 400× magnification.

2.4.3. Dose-Mortality Bioassay

Three fungal isolates were selected for dose-mortality bioassays based on: (1) High mortality potential, (2) low LT_{50} and (3) high spore production on BW cadavers. Based on these criteria, three *B. bassiana* isolates: ICIPE 273, ICIPE 648 and ICIPE 660, were selected for dose-mortality assessments. Ten fit adult BW were sprayed with 10 mL of each test isolate at five levels of concentrations: 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^8 spores mL^{-1}. Untreated control BW were sprayed with 10 mL

Sterile 0.01% Triton X-100. Each treatment was replicated four times and the treatments were arranged in a completely randomized design; the experiment was repeated once in time. As described in the pathogenicity assay above, a 'confirmation of death' test was performed prior to recording of weevil mortality. Mortality data was recorded every two days for 34 days. Dead BW were disinfected in a 1% sodium hypochlorite and 70% ethanol solution, followed by three rinses in sterile distilled water. The BW cadavers were placed in sterile plastic Petri dishes lined with moist sterile filter paper and incubated (25 ± 2 °C) in the dark for mycosis.

2.4.4. Efficacy of EPFs under Field Conditions

The field trial was conducted at the *icipe* campus, Nairobi, Kenya. The banana field contained 20 mats spaced at 2.5 × 2.5 m, and previously infested with BW.

Three fungal isolates (ICIPE 273, ICIPE 660 and ICIPE 648) were selected to test for pathogenicity against BW in the field. Fungal spores for each EPF isolate were produced on PDA as described in Section 2.2 above and a spore suspension of 1.0×10^8 spore mL^{-1} was prepared for each isolate. Banana pseudostem traps were prepared from freshly harvested pseudostems of EAHB plant as described by Viljoen et al. [40]. The banana pseudostems were cut into ~30 cm pieces and longitudinally split into two halves. Prior to setting up the weevil traps, each trap was dosed with a 10 mL suspension of 1.0×10^8 spore mL^{-1} of the respective EPF isolate. The EPF spore suspension was applied on the sliced part of the trap and the traps were left to stand for 15–20 min to soak the spore suspension. Control traps were applied with 10 mL of 0.01% (*v/v*) Triton X-100. Four weevil traps were used for each fungal isolate.

The traps were randomly placed on individual banana mats close to the base of the plant with the sliced part facing the ground. Traps were set up in the evening and the trapped weevils were collected from each trap daily (between 8–9 am) for seven consecutive days. For each day, weevils collected from each trap were separately placed in a rectangular plastic container (20 × 12 × 5 cm, L × W × H) and supplied with ~100 g piece of banana corm as food. The weevils were maintained in the laboratory at ambient temperature (25 ± 2 °C) for 21 days. Weevil mortality was checked at 3-day intervals for 21 days.

2.4.5. Viability of Fungal Spores after Exposure to Field Conditions

To assess viability of fungal spores in the field, ~1 g of plant tissue was scraped from each pseudostem trap. The plant tissue samples were collected at 3-day intervals for 9 days. The plant tissue was scraped from the part of the pseudostem where the spore suspension was applied. Plant tissue collected from each trap was placed in a separate sterile 15 mL Falcon tube and ~2 mL of sterile 0.01% Triton X-100 was added. The contents of the Falcon tube were vortexed to separate the fungal spores from the plant tissue. The fungal spores were quantified using a Neubauer hemocytometer and the spore concentration for each isolate was adjusted to 3.0×10^6 spores mL^{-1}. Spore viability was determined as described before. However, the SDA media used was supplemented with the antibiotics: streptomycin sulphate (0.2 g L^{-1}), penicillin G (0.1 g L^{-1}) and chlortetracycline (0.05 g L^{-1}).

2.5. Statistical Analysis

Percentage spore viability and percent mortality of the adult BW following exposure to the EPF were subjected to analysis of variance (ANOVA) and the means separated using the Student-Newman-Keuls (SNK) test from the package 'agricolae' [47]. Prior to analysis, percent mortality data were first corrected for natural mortality [48] then angular transformed to conform to the requirement of normality of variances [49]. Time- and concentration to mortality data were analyzed using the generalized linear model (GLM) with binomial distribution to generate the slope and intercept of the regression curves. Lethal time and lethal concentration to 50% mortality (LT50 and LC50) values were estimated using the 'dose.p' function of the package 'MASS' [50]. LT50 was evaluated only for isolates that yielded >50% mortality of adult BW. GLM analysis with binomial distribution was run for each replication to determine the lethal concentration to 50% mortality. For each EPF test isolate, insect mortality between doses was analyzed using the Kaplan–Meier survival analysis (log-rank method) using the R package 'survival' [51]. Data on production of spores on BW cadavers were checked for normality [49], then fitted to GLM analysis using negative binomial regression analysis of the package 'MASS' [50] and group means were separated using SNK test from the package 'agricolae' [47] at $p = 0.05$. Analysis of variance was performed on the number of captured weevils from the traps fitted with a linear mixed effects model using the 'lmer' function of the package 'lme4' [52]. EPF treatment and number of days to weevil capture were specified as the fixed variables, while trap was specified as the random variable. Computation of least square means was performed using the 'lsmeans' function of the package 'emmeans' [53] and group means were separated using the adjusted Tukey's method executed using the 'cld' function from the package 'multicomp' [54]. All data analyses were performed using R (Version 4.0.2) statistical software [55].

3. Results

3.1. Spore Viability and Pathogenicity of Fungal Isolates to Adults of Cosmopolites sordidus in the Laboratory

Spore viability differed significantly among the 20 EPF isolates ($F = 2.469$, $df = 19$, $p = 0.008$). Generally, spores of all the isolates were viable. However, viability of ICIPE 69 and 79 was significantly higher than that of ICIPE 279 (Table 1).

There was no significant effect between the repeat experiments on pathogenicity of the fungal isolates ($F = 1.67$, $df = 1$, $p = 0.76$), thus data from both experiments were pooled for analysis. Among the 20 isolates tested, 18 were pathogenic to adults of BW at 35 days post exposure (DPE). BW mortality differed significantly among the EPF isolates. The *B. bassiana* isolates ICIPE 660, 648, 273, 284, 622, 644, SD-229-Bb01 and SD-277-Bb02 caused significantly higher BW mortality in comparison to other tested isolates ($F = 12.53$, $df = 19$, $p < 0.001$). Among the eight highly pathogenic *B. bassiana* isolates, ICIPE 660, 648 and 273 resulted in the highest BW mortality ($\geq$ 80%). EPF isolates ICIPE 69 and 281 were non-pathogenic to adults of BW (0%) (Table 2). All four *Metarhizium* isolates: ICIPE 18, 62, 69 and 78 caused low mortality from 0% to 7.5%. The *Isaria fumosorosea* isolate ICIPE 682, also caused low BW adult mortality (2.5%). Only the *Beauveria* isolates caused >50% mortality of BW. The lethal time to 50% mortality (LT_{50}) was calculated for the eight *Beauveria* isolates that yielded >50% mortality 35 DPE. Among the eight isolates, ICIPE 273 exhibited the highest mortality rate, yielding the least LT_{50} value (5.3 days), while the Real IPM isolate SD-277-Bb02 exhibited the lowest rate of mortality, consequently yielding the highest LT_{50} value (26.9 days) (Table 2).

Table 2. Pathogenicity of entomopathogenic fungal isolates against adults of *Cosmopolites sordidus*.

Fungal Species	Isolate	% Weevil Mortality (Mean ± SE)	LT_{50} (Days) (95% FL)	Regression Line	
				Slope (± SE)	Intercept
Beauveria bassiana	ICIPE 273	80 ± 14.1 a	5.3 (1.9–8.8)	0.04 ± 0.008	−0.21
	ICIPE 279	7.8 ± 2.6 c	na	na	na
	ICIPE 281	0 ± 0 c	na	na	na
	ICIPE 284	62.5 ± 16.5 ab	18.3 (16.2–20.4)	0.037 ± 0.008	−0.68
	ICIPE 603	2.5 ± 2.5 c	na	na	na
	ICIPE 609	30 ± 17.8 bc	na	na	na
	ICIPE 621	2.5 ± 2.5 c	na	na	na
	ICIPE 622	53.6 ± 8.5 ab	24.2 (21.7–26.7)	0.033 ± 0.008	−0.79
	ICIPE 644	62.5 ± 9.5 ab	17.4 (15.2–19.7)	0.035 ± 0.008	−0.61
	ICIPE 647	20 ± 4.1 bc	na	na	na
	ICIPE 648	82.5 ± 8.5 a	9.8 (7.9–11.7)	0.057 ± 0.009	−0.56
	ICIPE 660	82.5 ± 6.3 a	11.1 (9.4–12.8)	0.061 ± 0.009	−0.68
	ICIPE 662	27.5 ± 2.5 bc	na	na	na
	SD-229-Bb01	62.5 ± 4.8 ab	14.7 (11.5–18)	0.026 ± 0.008	−0.39
	SD-277-Bb02	50.8 ± 10.3 ab	26.9 (24.3–29.6)	0.035 ± 0.008	−0.95
Metarhizium anisopliae	ICIPE 18	6.7 ± 3.5 c	na	na	na
	ICIPE 62	7.5 ± 7.5 c	na	na	na
	ICIPE 78	7.5 ± 4.8 c	na	na	na
	ICIPE 69	0 ± 0 c	na	na	na
Isaria fumosorosea	ICIPE 682	2.5 ± 2.5 c	na	na	na

Means within same column followed by same letter(s) are not significantly different by Student-Newman-Keuls (SNK) test at $p < 0.05$. [na] values not estimated (<50% mortality achieved). FL: 95% fiducial limits.

3.2. Spore Production per Cosmopolites Sordidus Cadaver

After 14 days of incubation, production of spores on the BW cadavers varied significantly between the eight selected *Beauveria* EPF isolates ($\chi^2 = 116.21$, $df = 7$, $p < 0.001$). The isolates ICIPE 622, ICIPE 660, ICIPE 273 and ICIPE 648 produced 1.8×10^8, 1.2×10^8, 3.8×10^7 and 3.3×10^7 spores per cadaver, respectively, which was significantly higher than spores produced by isolates SD-277-Bb02 (4.4×10^6) and ICIPE 644 (2.8×10^6), respectively (Figure 1).

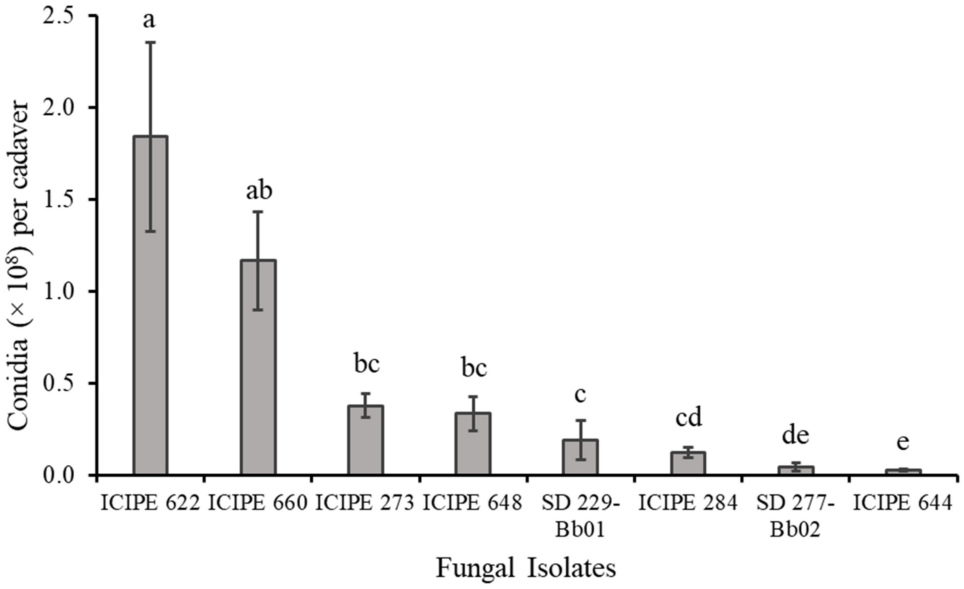

Figure 1. Spore production on individual *Cosmopolites sordidus* cadavers after 14 days of incubation at 25 ± 2 °C. Bars denote mean ± SE, bars with same lower-case letter(s) are not significantly different (Student-Newman-Keuls (SNK) test) at $p < 0.05$.

3.3. Dose Response

At 34 days post treatment, the lethal concentration to 50% mortality (LC_{50}) did not vary between the three EPF isolates ($p > 0.05$). The isolates ICIPE 273, ICIPE 660 and ICIPE 648 exhibited LC_{50} of 5.18×10^7, 5.2×10^7 and 5.49×10^7 spores mL^{-1}, respectively.

Survival analysis indicated that an increase in spore concentration significantly reduced the survival rate of the BW following exposure to ICIPE 273 (log-rank test, $\chi^2 = 69.2$; $df = 4$; $p < 0.0001$), ICIPE 648 (long-rank test, $\chi^2 = 92$; $df = 4$; $p < 0.0001$) and ICIPE 660 (log-rank test, $\chi^2 = 69.7$; $df = 4$; $p < 0.0001$). The isolates ICIPE 273, ICIPE 660 and ICIPE 648 attained a cumulative BW mortality of 76%, 80% and 80% at spore concentration 1.0×10^8 spore mL^{-1}, respectively (Figure 2).

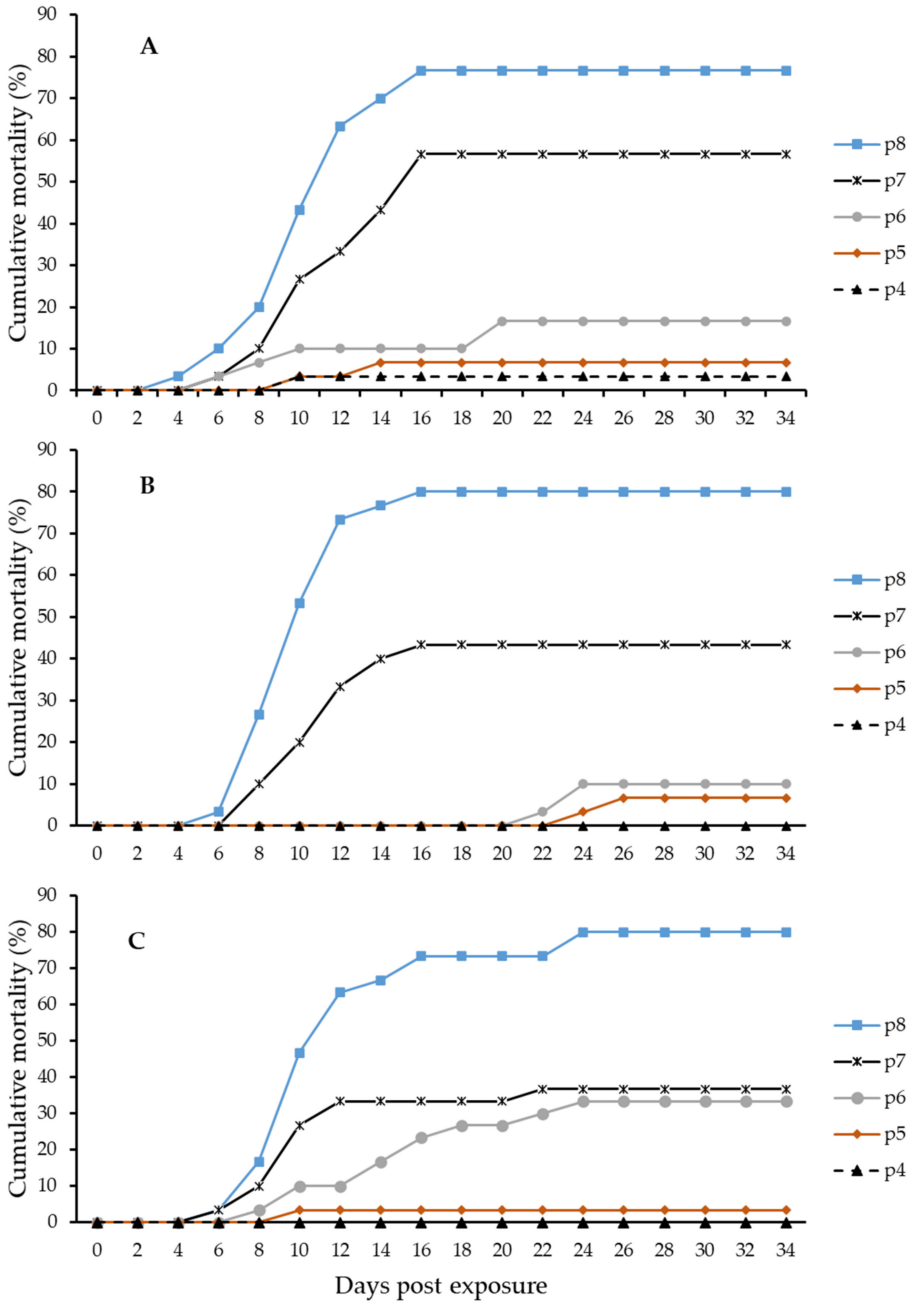

Figure 2. Dose-dependent cumulative mortality of adult *Cosmopolites sordidus* weevils induced by exposure to ICIPE 273 (**A**), ICIPE 648 (**B**) and ICIPE 660 (**C**) at spore concentrations of 1.0×10^4 (p4), 1.0×10^5 (p5), 1.0×10^6 (p6), 1.0×10^7 (p7) and 1.0×10^8 (p8) spores mL^{-1}.

3.4. Number of Captured Weevils and Pathogenicity of EPFs in the Field

There was no effect of EPF treatment on the number of weevils captured per trap per day during the assessment period (F = 0.86, df = 3, p = 0.46). However, there was a significant effect of time of capture on number of captured weevils (F = 13.91, df = 6, $p < 0.001$), and there was no interaction effect between EPF treatment and time of capture (F = 0.45, df = 18, p = 0.97). Overall, the number of weevils captured per trap during the seven-day period gradually reduced from >three weevils per trap on day one to <one weevil per trap on day seven (Table 3).

Table 3. Number of weevils captured per trap during a seven-day period following set up of the traps in the field.

Treatment	Days Post Trap Set Up in the Field						
	1	2	3	4	5	6	7
Control	3.8 ± 0.9 aA	2.8 ± 1.5 abA	1.8 ± 0.5 abA	2.0 ± 0.0 abA	1.0 ± 0.4 bA	1 ± 0.4 bA	0.3 ± 0.3 bA
ICIPE 273	3.3 ± 0.3 abA	3.8 ± 0.6 aA	2.3 ± 0.9 abcA	1.3 ± 0.6 abcA	0.8 ± 0.3 bcA	0.8 ± 0.3 bcA	0.3 ± 0.3 cA
ICIPE 648	4.3 ± 0.6 aA	3.0 ± 1.2 abA	2.0 ± 0.7 abA	1.8 ± 0.6 abA	1.8 ± 0.6 abA	1.3 ± 0.6 bA	1.0 ± 0.4 bA
ICPE 660	3.3 ± 0.3 abA	2.0 ± 0.4 abA	2.0 ± 0.4 abA	1.8 ± 0.5 abA	1.3 ± 0.6 abA	1.0 ± 0.0 abA	0.5 ± 0.3 aA

Values represent means ± standard error. At each day, means followed by the same upper-case letter indicate no treatment difference. While for each treatment, means with the same lower-case letter(s) indicate no difference between days (Tukey HSD test) at $p < 0.05$.

Mortality of the captured weevils varied significantly between EPF treatment (χ^2 = 12.61, df = 3, $p < 0.006$) and time of capture (χ^2 = 19.57, df = 6, p = 0.003); however, there was no interaction effect (χ^2 = 2.89, df = 18, p =1). While none of the captured weevils from the control treatment died during the 21 days of incubation in the laboratory, mortality of weevils captured from traps sprayed with fungal isolates ICIPE 273 and ICIPE 684 was significantly higher than weevils captured from the control treatment on day one. There was no mortality amongst the weevils captured from the traps after three days following set up of the weevil traps (Table 4).

Table 4. Weevil mortality (%) at three weeks of incubation in the laboratory after capture from the EPF-treated traps in the field.

Treatment	Days post EPF Application and Trap Set Up in the Field			
	1	2	3	4 *
Control	0 ± 0 aB	0 ± 0 aA	0 ± 0 aA	0 ± 0 aA
ICIPE 273	31.3 ± 2.1 aA	17.5 ± 6.0 aA	8.3 ± 8.3 abA	0 ± 0 bA
ICIPE 648	20.8 ± 7.2 aA	8.3 ± 8.3 abA	0 ± 0 bA	0 ± 0 bA
ICIPE 660	22.9 ± 7.9 aAB	8.3 ± 8.3 abA	8.3 ± 8.3 abA	0 ± 0 bA

Values represent means ± standard error. At each day, means followed by the same upper-case letter(s) indicate no treatment difference. While for each treatment, means with the same lower-case letter(s) indicate no difference between days (Tukey HSD test) at $p < 0.05$. * No mortality for weevils captured on days 4, 5, 6 and 7 post EPF application.

3.5. Spore Viability after Exposure to Field Conditions

Viability of fungal spores recovered from the weevil traps was significantly different between EPF isolates (χ^2 = 51.63, df = 2, $p < 0.001$), time of exposure (χ^2 = 2228, df = 3, $p < 0.001$), and there was a significant interaction (χ^2 = 16.96, df = 6, p =0.009). Prolonged exposure of fungal spores to field conditions significantly reduced spore viability of all the three EPF isolates. Viability of fungal spores at day zero (90.3–92.3%) did not vary significantly between the three EPF isolates. However, at day three, viability of spores of ICIPE 273 (40.9%) remained significantly higher compared to ICIPE 648 (21.6%) and ICIPE 660 (16.2%). Conversely, spore viability did not differ significantly between the three EPF isolates at days six and nine, and this had considerably reduced to <10% by day nine post field application (Figure 3).

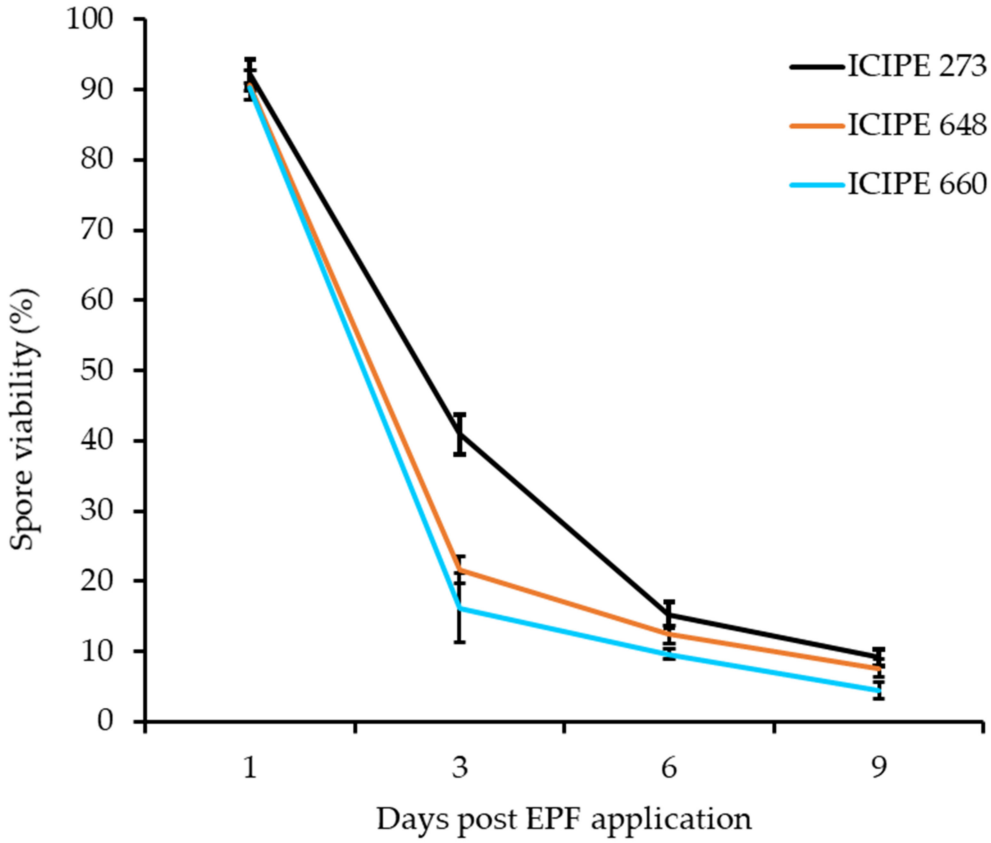

Figure 3. Viability of fungal spores collected from the pseudostem traps at zero, three, six and nine days post entomopathogenic fungal isolate application in the field.

4. Discussion

Spore viability is a vital component to determine prior to screening of any EPF isolate for use as a biological control agent, as spore viability can influence the pathogenicity of EPF isolates [56]. The viability of the EPF isolates used in our study varied between 92.5% and 98%, ranges that were considered sufficiently high for all isolates to be deemed fit for further studies. Whereas spore viability can be influenced by various factors, including temperature, humidity, culture media, age of the culture [57,58], among others, these were all uniform during the in vitro culture of the isolates used in this study, which may explain the minimal variation in spore viability values of the test isolates prior to exposure to environmental conditions.

However, exposure of the spores of the fungal isolates ICIPE 273, ICIPE 648 and ICIPE 660 to environmental conditions in the field led to a substantial reduction in spore viability for all the three EPF isolates tested. While the three selected EPF isolates were originally isolated from soil (Table 1), and it would be assumed that they would withstand environmental conditions and naturally survive in soil, their continued maintenance on artificial culture media prior to use in our study could have negatively affected their viability upon re-exposure to environmental conditions. In fact, while Wang et al. [59] demonstrated that fungal conidia synthesize and store messenger RNA (mRNA) transcripts for future use in the production of enzymes and metabolites needed for conidial germination and virulence, they also established that the type of mRNA transcripts generated is dependent on the state of the environment in which the conidia are exposed to at the time. Consequently, while the spore conidia were formed on synthetic PDA media, exposure of the spores to the soil and pseudostem trap conditions in the field could have rendered the transcripts less effective in modulating spore viability, hence the observed considerable reduction in spore viability from >90% on day zero to ≤41% on day three with prolonged time of exposure. Furthermore, corms and pseudostems of freshly cut banana plants have been

shown to emit volatile compounds with an attractive effect on the BW [60–62]. Whether these biomolecules and compounds have any effect on fungal spore viability, or how they affect the viability is not fully understood.

Our study in the laboratory revealed variable pathogenicity between the EPF isolates, as depicted by the mean mortality (20–82.5%) and LT_{50} values (5.3 days–∞). Of the 15 *B. bassiana* isolates tested in the lab, eight were highly pathogenic to adults of BW (mortality >50%), while seven were less pathogenic (mortality <50%). Pathogenicity of *B. bassiana* isolates against the adult BW has been studied previously in the lab, with Lopes et al. [44] reporting mortality levels of 6–96%, while Omukoko et al. [25] reported 4–51%, Membang et al. [24] 34.8–96.3% and Kaaya et al. [22] 63–97%. Our range of values for adult BW mortality following exposure to *B. bassiana* reflect the findings of these studies. Similar to our methods, Omukoko et al. [25] and Membang et al. [24] used spore suspensions, while Kaaya et al. [22] used dry spores. This indicates that *B. bassiana* isolates can be developed as either a liquid or powder formulation and still be able to achieve the same effect. On the other hand, all isolates of *M. anisopliae* and *I. fumosorosea* used in our study were less pathogenic to adult BW (mortality $\leq$ 7.5%). Kaaya et al. [22] also reported low mortality levels (11.7%) of the adult BW following exposure of BW to spores of *M. anisopliae*. In contrast, Lopes et al. [63] and Membang et al. [24] reported mortality rates of 24–79.5% and 7.4–92.6% following exposure of the adult BW to isolates of *M. anisopliae*, respectively. The low pathogenicity of *M. anisopliae* isolates exhibited in our study could be attributed to inter-species variation in virulence of the fungus.

Efficacy of an EPF depends on its ability to attach onto the insect host body (cuticle), germinate, invade and proliferate in its hemocoel. Host death results from nutrient depletion, physical obstruction and/or toxinosis [41]. The insect cuticle is the primary physical barrier that EPFs encounter prior to establishment of an epizootic in the host insect population. Studies have shown EPFs to produce proteases, lipases, chitinases and other bioactive metabolites [64,65]. These enzymes play an important role in the pathogenesis and physiological processes, and form part of the initial host infection process. While testing the efficacy of three *Cordyceps fumosoroseus* (Wize) isolates against nymphs of the whitefly *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae), Castellanos-Moguel et al. [66] established that efficacy was proportional to the amount of protease enzyme produced by the respective isolates. Similarly, while testing the efficacy of six *B. bassiana* isolates against larvae of the cabbage pest *Pieris brassicae* (L.), Dhawan and Joshi [67], established that there was variation in the amount and type of enzyme (chitinase, protease and lipases) produced by *B. bassiana* isolates. Furthermore, efficacy of each isolate was proportional to the total amount of enzymes produced by the respective *B. bassiana* isolate [67]. Consequently, EPF isolates belonging to the same (or different) species express variations in the level (and type) of bioactive metabolite(s). Thus, the observed inter- and intra-species mortality variations in our study could be attributed to variations in the type and level of bioactive metabolites produced by the different EPF isolates. However, the type and levels of metabolites produced by each isolate used in our study are yet to be established.

Short kill time and low effective dose are critical factors to consider in the selection and development of an effective mycoinsecticide, which positively impacts on the subsequent selection for commercial production [68]. One of the major reasons why farmers continue to use synthetic chemicals is due to their rapid knock-down effect, while microbial alternatives tend to have a slow and more prolonged killing time. Thus, selecting EPF isolates with a shorter kill time can help facilitate adoption and positively impact the acceptance of these mycoinsecticides by the banana farmers as alternatives to synthetic chemical pesticides. In our in vitro pathogenicity study, the three *B. bassiana* isolates—ICIPE 273, ICIPE 648 and ICIPE 660—exhibited the shortest kill time ($LT_{50} \leq 11.1$ days) and lowest effective dose ($LC_{50} \leq 5.3 \times 10^7$ spores mL^{-1}), thus presenting them as promising isolates for development into mycoinsecticides. Furthermore, the commercial production of microbial insecticides is largely dependent on cost-effective production systems that can produce

high yields of highly infective fungal spores [69]. Consequently, achieving high efficacy at a low spore dose and high degree of sporulation are key aspects in the selection and potential commercialization of a given microbial insecticide.

Pathogenicity of ICIPE 273 has been studied previously; among the nine *B. bassiana* isolates screened, Omukoko et al. [25] established that ICIPE 273 was the most pathogenic causing 51% mortality of adult BW, which is a relatively low mortality level compared to the 80% observed in our study. The differences observed between the two studies in BW mortality following exposure to ICIPE 273 could be attributed to the difference in method of application used. In our study, the adult BW were sprayed with the fungal suspension and left in the Petri dish lined with sterile filter paper for 24 h, while Omukoko et al. [25], dipped the BW in a spore suspension for 11 s followed by draining off the excess suspension. Spore viability is humidity depended, the moist, EPF-soaked filter papers used in our study could not only have provided a prolonged conducive environment for spore germination, but also helped maintain a relatively moist BW exoskeleton thus facilitating the infection process. On the other hand, the immediate draining off of the excess EPF suspension from the BW body by Omukoko et al. [25] meant a gradual loss of moisture, hence could have rendered the environment suboptimal for EPF spore germination on the BW body, and hence the observed difference in the infection levels of ICIPE 273 between the two studies.

Efficacy of the selected three *B. bassiana* isolates (ICIPE 273, ICIPE 648 and ICIPE 660) in the field reduced to $\leq$31% BW mortality on day one, with no weevil mortality reported after three days post field application. The observed variation in the laboratory versus field BW mortality levels across the three EPF isolates could be attributed to the sudden reduction in spore viability upon application in the field. Efficacy of an EPF can be improved by manipulating the formulation or mode of delivery of the fungal spores [28]. Generally, oil-based formulations of the mycoinsecticides are reported to be more effective than aqueous, granular or dry spore formulations, as the oil improves the shelf life of the mycoinsecticide, improves the thermotolerance, protects the propagules against the UV radiation, enhances attachment of the conidia onto the insects body and maintains spore viability for a longer time [70–74].

The potential of a mycoinsecticide to produce spores on insect cadavers is beneficial as this acts as a source of inoculum for secondary infections and secondary control, thus increasing the probability of spreading the epizooty among the pest population [75]. Furthermore, the potential of the candidate EPF isolates to sporulate on BW cadavers results in a reduction in the number of applications needed in the field, as initial culture can be maintained through self-proliferation on cadavers, requiring only a small booster dose over time. This would ultimately result in a reduction in cost to the farmer, due to the reduction in frequency of application. In our study, *B. bassiana* isolates: ICIPE 273, ICIPE 648 and ICIPE 660 were able to produce high spore concentrations ($> 3.3 \times 10^7$ spores) in BW cadavers. In addition to host behavior, secondary infection can be aided by environmental conditions. Rain has been reported to aid dispersal of fungal spores over greater distances through run off water, the splashing droplets and vibration made by the impact of rain droplets [75]. In addition, wind can support both short- and long-distance dispersal of fungal spores. While BW numbers are reported to surge during the wet and humid season [76], fungal spore production and efficacy are also aided by high humidity [77]. Thus, field application of fungal-based microbial insecticides during the rainy season has the potential of counteracting the surge in BW numbers. This can further be aided by the spreading of the cadaver-generated fungal spores by the splashing of droplets and by runoff water, in addition to the humid environment being favorable for fungal sporulation, spore germination and epizooty [77].

5. Conclusions

The *B. bassiana* isolates ICIPE 273, ICIPE 648 and ICIPE 660 exhibited high mortality levels and low median lethal time against the BW in the lab. In addition, the isolates were able to sporulate efficiently on BW cadavers. Furthermore, the three EPF caused BW

mortality when applied in the field. Thus, these three isolates can be regarded as potential candidates for the management and control of BW. However, different formulations of these isolates need to be assessed across a range of environmental conditions. In addition, due to the genetic variability in BW from the different banana growing regions, it is crucial that the candidate isolates be tested against BW from different geographical locations. Furthermore, testing compatibility and performance of these candidate isolates with already existing environmentally friendly management strategies will help develop a strong IPM strategy against the BW. Since *B. bassiana* isolates are known to have a wide host range, including non-target species, testing of the selected pathogenic isolates against non-target organisms is recommended. This will help establish their direct effect on the environment and thus guide decisions on developing them into environmentally friendly biopesticides. In addition, the endophytic potential of the fungal isolates for the management of BW in banana would be useful to study. This will ultimately help to reduce the reliance on synthetic agrochemical-chemical pesticides, towards better protection of ecological services, the environment and human life. In turn, this will boost the use of climate and environment smart management strategies against the BW.

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Data Availability Statement: The raw data supporting the conclusions of this study will be made available by the authors, without undue reservation.

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