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Research

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Biological and Microbial Control

Susceptibility of the Western Honey Bee *Apis mellifera* and the African Stingless Bee *Meliponula ferruginea* (Hymenoptera: Apidae) to the Entomopathogenic Fungi *Metarhizium anisopliae* and *Beauveria bassiana*

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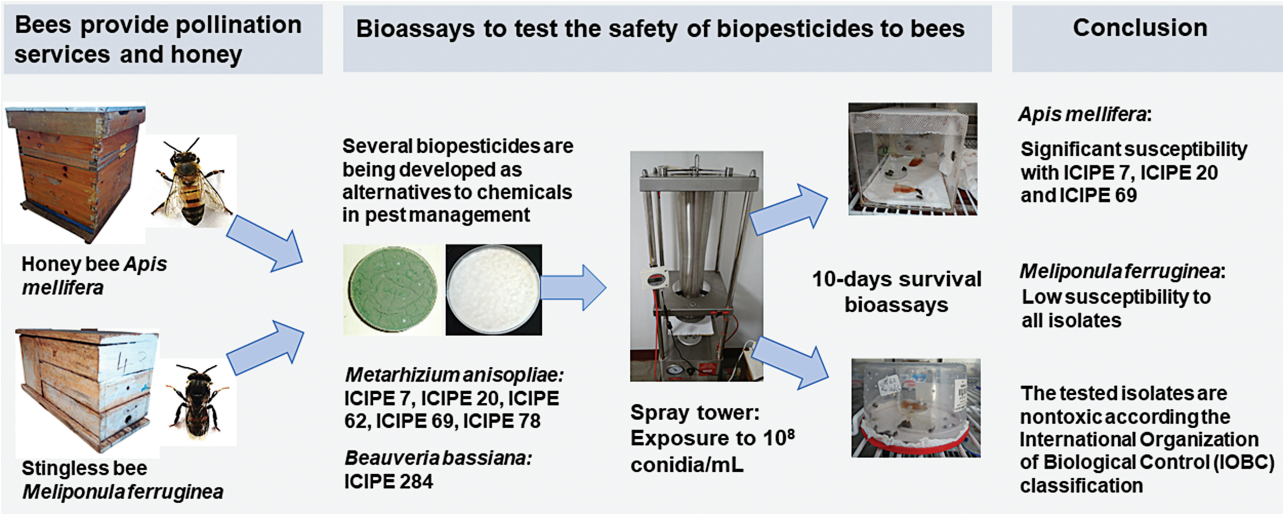
Abstract

This study assessed the nontarget effect of entomopathogenic fungi on the Western honey bee *Apis mellifera* L. and the African stingless bee *Meliponula ferruginea* Cockrell (Hymenoptera: Apidae). Pathogenicity of five *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, and ICIPE 78) (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) and one of *Beauveria bassiana* (ICIPE 284) (Balsamo) Vuillemin (Hypocreales: Cordicipitaceae) isolates were evaluated on bees at 10^8 conidia/ml. Conidial acquisition was evaluated immediately after exposure. *Apis mellifera* acquired more conidia (2.8×10^4–1.3×10^5 conidia per bee) compared to *M. ferruginea* (1.1×10^4–2.3×10^4 conidia per bee). In the bioassay with *A. mellifera*, ICIPE 7, ICIPE 20, and ICIPE 69 moderately reduced the survival by 16.9, 17.4, 15.3%, with lethal times LT_{10} = 7.4, 7.6, 8.1 d and LT_{25} = 8.7, 10.0, 9.9 d, respectively. The three isolates caused *A. mellifera* mycosis of 11.6–18.5%. None of the isolates had a significant effect on *M. ferruginea*. The tested isolates are nontoxic to bees according to the International Organization of Biological Control (IOBC) classification. However, the effect of ICIPE 7, ICIPE 20, and ICIPE 69 merits further studies on bee colonies, especially those of *A. mellifera*, under field conditions.

Graphical Abstract



Key words: biopesticide, conidia acquisition, nontarget effect, pollinator, survival

Bees, principally the Western honey bee *Apis mellifera* L. (Hymenoptera: Apidae: Apini) and stingless bees (Hymenoptera: Apidae: Meliponini), are among the most culturally and economically important insects worldwide, providing essential pollination services to a wide range of flowering plants, thereby contributing to ecological well-being, crop productivity and food security (IPBES 2016). Insects pollinate 75% of world crop species, accounting for 35% of food production (IPBES 2016, Klein et al. 2007) valued at $267–657 billion USD annually (Porto et al. 2020). Additionally, honey bees and stingless bees produce several hive products including honey, wax, cerumen, bee bread, royal jelly, bee venom, and propolis, commonly used in nutritious food, pharmacology, cosmetics, generating income to many beekeepers (Raina 2000, Pasupuleti et al. 2017). During the last decade, apiculture has been growing in Africa and accounts for 10% and 25% of the global production of honey and wax, respectively (Moinde 2016). Honey bees and stingless bees exist in several African biodiversity hotspots (Eardley et al. 2009, Anguilet et al. 2015), and increasing interest in meliponiculture of stingless bees is associated with their Afrotropical existence (Eardley and Kwapong 2013, Kiatoko et al. 2016, Yurrita et al. 2017), pollination services (Kajobe 2006, Slaa et al. 2006, Kiatoko et al. 2014), and production of high quality and medicinal honey (Eardley and Kwapong 2013, Souza et al. 2006).

Insect pollination services and beekeeping are at risk owing to several factors, including the heavy applications of broad-spectrum chemical pesticides in response to significant damages inflicted by pests and diseases (Brittain et al. 2010, Sponsler et al. 2019). Impacts of chemical pesticides on nontarget and beneficial insects are well documented (Brittain et al. 2010, Wiest et al. 2011, Ndakidemi et al. 2016), and they partly constitute key drivers to the unprecedented declines of bee pollinators across the world (IPBES 2016, Kumar et al. 2018).

The entomopathogenic fungi *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin are formulated and used worldwide as biopesticides, and these biopesticides are safer alternatives to chemical pest control based on their persistence in the field and environmental compatibility (Shah and Pell 2003, Maina et al. 2018). During the last two decades, *M. anisopliae* isolates researched at the International Centre of Insect Physiology and Ecology (*icipe*, Nairobi, Kenya) have been developed into biopesticides, and they are currently applied on 133,000 ha to manage several insect pests in sub-Saharan Africa (Akutse et al. 2020). Currently, *M. anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and *B. bassiana* ICIPE 284 isolates are in the pipeline or have been commercialized for the management of several pests (Table 1).

Conidia of entomopathogenic fungi applied on blooming crops may be acquired by forager bees and ultimately carried to their colonies. Laboratory studies on the effect of entomopathogenic fungi on bees have yielded variable results, which may be associated with the tested bee species (Toledo-Hernandez et al. 2016), isolates of entomopathogenic fungi (Espinosa-Ortiz et al. 2011), methods of exposure (Potrich et al. 2018, Colombo et al. 2020) or tested concentrations of entomopathogenic fungi (Conceição et al. 2014). For instance, exposure to some isolates of *M. anisopliae* caused 100% mortality to *A. mellifera* in 10-d bioassays (Bull et al. 2012), and 50.1–94.2% mortality and 40.0–53.0% mortality to the neotropical stingless bees *Tetragonisca angustula* Latreille and *Melipona beecheii* Bennett (Hymenoptera: Apidae) in 20-d bioassays, respectively (Toledo-Hernandez et al. 2016). Similarly, exposure to some isolates of *B. bassiana* caused high mortality in 10-d bioassays with the stingless bee *Melipona scutellaris* Latreille (30.9–79.6%) (Conceição et al. 2014). However, in these bioassays, certain isolates of *B. bassiana* and *M. anisopliae* caused low mortality (<40.0%) to the stingless bees *Scaptotrigona mexicana* Guérin–Méneville (Hymenoptera: Apidae) and *M. beecheii* (Toledo-Hernandez et al. 2016).

The process of registration of fungal-based biopesticides requires the provision of their ecotoxicological test results on vertebrates and nontarget invertebrates. Ecotoxicological dossier of *M. anisopliae* ICIPE 7, ICIPE 62, ICIPE 69, and ICIPE 78 registered in sub-Saharan Africa indicate that they are nontoxic to bees. However, these ecotoxicological results are obtained according to the guidelines of OECD (1998) by testing suboptimal doses (<10^7 conidia/ml) through oral exposure in short bioassays (<96 hr). In these studies, only *A. mellifera* is used as a model insect, yet the susceptibility of other bees such as stingless bees to these biopesticides may vary significantly and remains unexplored. Entomopathogenic fungi typically kill the target insect within 3–14 d after exposure (Maina et al. 2018) and their toxicity to *A. mellifera* is arguably higher through contact exposure than through oral exposure (Potrich et al. 2018, Colombo et al. 2020).

Therefore, the objective of the present study was to assess the nontarget effect of *M. anisopliae* and *B. bassiana* isolates in the pipeline and already commercialized on *A. mellifera* and *M. ferruginea* through contact exposure to 1 × 10^8 conidia/ml in 10-d bioassays under laboratory conditions.

Materials and Methods

Fungal Isolates

Five isolates of *M. anisopliae* and one isolate of *B. bassiana* were used in this study (Table 1). Isolates were obtained from *icipe* where they had been preserved as slant cultures in 10% glycerol at –80°C. Virulence of each isolate was revived by injecting 7th instar larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) with 5 µl water containing ≈5,000 conidia followed by 7 d incubation at 25 ± 2°C and 0:24 L:D (light:dark photoperiod). The conidia were harvested, streak-plated on media surfaces, and incubated for 21 d at 25 ± 2°C and 0:24 L:D. Sabouraud dextrose agar (SDA) (Oxoid, Hampshire, UK) and potato dextrose agar (PDA) (Oxoid) were used for *M. anisopliae* and *B. bassiana* isolates, respectively, after being autoclaved at 121°C for 15 min and 15 PSI in a 63 liter autoclave (AMA440, Astell Scientific, Kent, UK). A selective antibiotic agent (0.25 g/liter of streptomycin sulfate) was added to the media (cooled to 45°C) followed by dispensing in 95 mm (diameter) × 15 mm (height) plastic Petri dishes. Inoculated media in Petri dishes were incubated for 21 d at 25 ± 2°C and 0:24 L:D before bioassays.

Preparation of Fungal Suspensions

The viability of each isolate was assessed before bioassays as follows. Conidia were harvested from 21-d-old cultures, transferred into a 25 ml universal bottle containing 10 ml of sterile 0.05% Triton-X-100 (Triton, Darmstadt, Germany), and 4 sterile 1–2 mm (diameter) glass beads, and vortexed for 3 min at 700 rpm to ensure homogeneity. The suspensions were serially diluted (10^{-2}) in

Table 1. Overview of entomopathogenic fungal isolates that were assessed for pathogenicity to *Apis mellifera* and *Meliponula ferruginea*

Fungal isolate	Origin			Current target pests	Trade name	Future target pests
	Host/substrate	Year	Location			
Metarhizium anisopliae						
ICIPE 7	*Amblyomma variegatum*	1996	Rusinga Island (Kenya)	*Amblyomma* sp.,*Rhipicephalus*sp.,*Hyalomm a* sp.	Mazao TickOff	*Spodoptera frugiperda, Frankliniella occidentalis*
ICIPE 20	Soil	1989	Migori (Kenya)	–	–	*Ceratitis* sp., *Tuta absoluta, Liriomyza huidobrensis, S. frugiperda*
ICIPE 62	Soil	1989	Kinshasa (DR Congo)	*Aphis craccivora*	Mazao Supreme	*Bactrocera dorsalis*
ICIPE 69	Soil	1990	Kinshasa (DR Congo)	*Ceratitis* sp., *Rastrococcus invadens, F. occidentalis, T. absoluta, L. huidobrensis*	Mazao Campaign, Real Metarhizium 69, Real Metarhizium SC	*Thaumotoibia leucotreta, Maruca vitrata*
ICIPE 78	*Temnoschoita nigroplagiata*	1990	Ungoe (Kenya)	*Tetranynchus urticae*	Achieve, Mazao Achieve	*S. frugiperda*
Beauveria bassiana						
ICIPE 284	Soil	2005	Unknown (Mauritius)	–	–	*B. dorsalis, S. frugiperda*

Data on target pests for the fungal isolates are provided in Niassy et al. (2012), Ekesi et al. (2007), and Akutse et al. (2020).

sterile 0.05% Triton-X-100 and the concentrations were microscopically enumerated using an improved Neubauer hemocytometer (Marienfeld, Lauda-Königshofen, Germany).

The suspensions were then adjusted to 3×10^6 conidia/ml using sterile 0.05% Triton-X-100. An aliquot (0.1 ml) was spread-plated onto SDA (for *M. anisopliae*) or PDA (for *B. bassiana*) in Petri dishes in four replications/isolate, and incubated at 25 ± 2°C and 0:24 L:D. After 18 hr, cultures were stained with 2 ml of lactophenol cotton blue (Hardy Diagnostics, Santa Maria, CA) and covered with three glass microscope coverslips (22 × 22 mm). Three hundred conidia per Petri dish were randomly counted. A conidium was considered viable when its germination tube was at least twice longer than its width by microscopic examination (×400 magnification). Once viability of above 75% was confirmed, the conidia were harvested from 21-d-old fungal cultures of each isolate, and their concentrations were determined using the above-described procedure. For each isolate, a uniform concentration of 1×10^8 conidia/ml was prepared for the subsequent bioassays.

Source and In Vitro Maintenance of *Apis mellifera*

Brood frames were obtained from *A. mellifera* colonies maintained in standard Langstroth hives at *icipe* apiaries, Nairobi, Kenya (S 1°13′17.51″ E 36°53′45.18″). Colonies were headed by naturally mated queens and were first established to be healthy using colony strength metrics described by Medrzycki et al. (2013) and Delaplane et al. (2013). Six colonies were selected and brood frames containing mature pupae of worker bees (red-eye stage) estimated to emerge in 1–3 d were collected. Frames were placed in modified wooden emerging cages (30 × 5 × 20 cm) and incubated at 0:24 L:D in a 406 liter high precision biological oxygen-demand (BOD) incubator (MIR-554, PHC Holdings Corporation, Tokyo, Japan) at *icipe*. To promote the emergence of bees, incubator temperature was calibrated to 34.5°C, and RH adjusted to 70–80% as suggested by Williams et al. (2013).

Newly emerged adult *A. mellifera* bees were transferred into sleeved Perspex cages (18 × 14 × 14 cm) using a soft camel brush at 24 hr intervals. Each cage received equal numbers of bees from different colonies and was replicated four times for each treatment. Caged bees were provided ad libitum with 50% (w/v) sugar solution and 0.5 g of bee-collected pollen, and maintained at 32°C and 70–80% RH one day before the bioassays. The bioassays were conducted in November 2019 with 30 bees per cage collected from three colonies and repeated in February 2020 with 35 bees per cage collected from the remaining three colonies.

Source and In Vitro Maintenance of *Meliponula ferruginea*

Six colonies of *M. ferruginea* were obtained from the *icipe* meliponary at Nairobi, Kenya, and had originally been sourced from Kakamega forest, Kenya (N 0°17′18.00″ E 34°51′13.19″). Before selection, each colony (≈3,000 adult bees) was visually checked for the absence of any pathogens and pests, the presence of an egg-laying queen bee, and at least seven brood combs containing eggs, larvae, or pupae. Brood combs with pupae of worker bees projected to emerge within one week were collected and placed in well-ventilated sterile 0.5 liter plastic cages and maintained in the BOD incubator calibrated to 30°C, and 60–70% RH as suggested by Dorigo et al. (2019). Combs were maintained in 0:24 L:D to facilitate the emergence of new adults.

An equal number of newly emerged bees from source colonies were transferred every 24 hr into sleeved Perspex cages using a

soft camel brush. Caged bees were provided ad libitum with 70% (v/v) honey–water solution and 0.5 g of pollen obtained from *M. ferruginea* colonies. Four replications were made for each treatment and bees were acclimatized to caging conditions (30°C, 60–70% RH and 0:24 L:D) one day before the bioassays. The bioassays with *M. ferruginea* were carried out in April 2020 with 30 bees per cage collected from three colonies and repeated in July 2020 with 35 bees per cage collected from the remaining three colonies.

Exposure of *Apis mellifera* and *Meliponula ferruginea* to Fungal Isolates

Bees were indirectly exposed to the six fungal isolates alongside the control. Whatman filter papers (18 × 14 cm) were sprayed with 10 ml of either sterile 0.05% Triton-X-100 (control) or isolate (1×10^8 conidia/ml) using a micro-spray tower (Potter Precision Laboratory Spray Tower, Burkard Manufacturing Co., Hertfordshire, England) at a pressure of 10 PSI. Filter papers for the controls were first sprayed, followed by each isolate suspension in four replications. Before and after each spray, the tower spraying chambers, contamination arena, and cuvettes were sterilized with 70% ethanol and rinsed with sterile water. Sprayed filter papers were air-dried for 10 min and introduced in the bottom of the cages. Caged bees were allowed to walk over the filter papers for 10 min. Five bees per cage were randomly sampled for conidial acquisition assessment and the remaining bees were transferred into clean Perspex cages (*A. mellifera*) or 0.5 liter plastic cages (*M. ferruginea*) lined inside with paper towels.

Assessment of Conidial Acquisition and Survival of Fungus-Exposed Bees

Bees for conidial acquisition assessment were suspended singly in 1 ml of sterile 0.05% Triton-X-100 and vortexed for 3 min at 700 rpm. Dislodged conidia were enumerated using an improved Neubauer hemocytometer. The remaining exposed and caged bees were maintained and fed based on the above-described protocols for each bee species. The survival of bees was monitored at 24 hr intervals for 10 d. Dead bees were removed from the cages and surface-sterilized by the passage in 3% sodium hypochlorite for 1 min, followed by 70% ethanol for 3 min, and three times rinsing in sterile water for 1 min. Surface-sterilized cadavers were singly placed in 95 mm (diameter) × 15 mm (height) plastic Petri dish lined inside with moistened filter paper. Cadavers were incubated at 25 ± 2°C in 0:24 L:D and mycosis was recorded from incubated cadavers after 2–7 d postinoculation by observing any growth of fungus on the surface using a microscope. Mortality due to fungus was confirmed through the presence of green- and white-colored mycelium for *M. anisopliae* and B. *bassiana*, respectively, on the surface of the cadavers, and identity established by comparing with mother cultures. In addition, when in doubt, slides were prepared from mycelial outgrowth and conidia to confirm fungus identity.

Statistical Analysis

Data analyses were performed using R software (R Core Team 2020). Conidial acquisition data were log-transformed and subjected to a linear mixed effect model implemented in the *lme4* package (Bates et al. 2015) with the *lmer* function. The tower spray treatment acted as a random factor. Means were separated using the *lsmeans* package (Lenth 2015) with the Tukey *P*-value adjustment method.

Daily percentage mortality was corrected by adjusting the treatment mortality with control mortality using Abbott's correction (Abbott 1925):

$$\text{adjusted daily mortality (\%)} = \frac{\text{treatment mortality} - \text{control mortality}}{100 - \text{control mortality}}$$

Adjusted mortality was subjected to probit regression using the *ecotox* package (Hlina 2020). This analysis provided the estimates for lethal time-response mortality to 10% (LT_{10}) and 25% (LT_{25}) of the population, 95% fiducial limits (FL), and regression slopes. Differences in LT were assessed by comparing the LT estimates and the overlapping 95% FL at $\alpha = 0.05$.

Bee survival data were analyzed using the *survival* package (Therneau and Lumley 2020) and *survminer* package (Kassambara et al. 2020). Cox mixed effect regression model implemented in the *coxme* package (Therneau 2020) was used to model bee survival. In this model, cage membership was used as a random factor. Separation of means was performed using the *lsmeans* package with Bonferroni-adjusted *P*-values. Survival curves were generated using the Kaplan–Meier estimator.

Mycosis was subjected to a generalized linear mixed effect model with logistic distribution using the *glmer* function from *lme4* package. The cage membership was used as a random factor. Means were separated using the *multicomp* package (Hothorn et al. 2008) with Tukey-adjusted *P*-values. Pearson's correlation analysis was used to establish relationships between conidia acquisition and LT_{10} and mycosis.

Results

Conidial Acquisition by *Apis mellifera* and *Meliponula ferruginea*

All fungus-exposed bees acquired conidia, while no conidia were detected in the control bees (Table 2). Therefore, controls were omitted from the analysis. *Apis mellifera* acquired significantly more conidia than *M. ferruginea* ($\chi^2 = 232.00$, df = 1, $P < 0.0001$).

Table 2. Conidial density (conidia per bee) after exposure to six entomopathogenic fungal isolates (1×10^8 conidia/ml)

Fungal isolate		*Apis mellifera*	*Meliponula ferruginea*
		Mean (±SE[a] × 10^4)	Mean (±SE × 10^4)
Metarhizium anisopliae	ICIPE 7	8.03 ± 0.01 b	1.85 ± 0.01 a
	ICIPE 20	12.97 ± 0.06 c	2.11 ± 0.02 a
	ICIPE 62	9.49 ± 0.03 b	2.00 ± 0.03 a
	ICIPE 69	7.03 ± 0.01 b	2.28 ± 0.03 a
	ICIPE 78	7.25 ± 0.03 b	1.90 ± 0.01 a
Beauveria bassiana	ICIPE 284	2.83 ± 0.05 a	1.14 ± 0.01 a

Means within columns with the same letters are not significantly different at $\alpha = 0.05$ according to the Tukey test. For each species, $n = 40$ bees per treatment and replications = 8.

[a]SE = standard error.

For *A. mellifera*, conidial acquisition differed significantly among isolates (χ^2 = 98.04, df = 5, P < 0.0001) but not between experiments (χ^2 = 0.34, df = 1, P = 0.56) or among experiment-isolate interactions (χ^2 = 3.70, df = 5, P = 0.60). Conidial acquisition by *A. mellifera* was highest when exposed to ICIPE 20 and lowest when exposed to ICIPE 284. On the other hand, for *M. ferruginea*, no significant difference in conidial acquisition was detected among isolates (χ^2 = 9.16, df = 5, P = 0.10), between experiments (χ^2 = 0.0016, df = 1, P = 0.97) or among experiments-isolate interactions (χ^2 = 3.45, df = 5, P = 0.63).

Time-Response Mortality of *Apis mellifera* and *Meliponula ferruginea*

The lethal time-response mortality to 10% (LT_{10}) and 25% (LT_{25}), and the corresponding fiducial limits and regression slopes of the fungus-exposed caged bees are presented in Table 3. The LT_{10} estimates for *A. mellifera* were shorter in treatments with ICIPE 7, ICIPE 20, ICIPE 69, and ICIPE 284 than in treatments with ICIPE 62 and ICIPE 78. LT_{25} estimates for *A. mellifera* were the shortest in treatments with ICIPE 7, followed by ICIPE 20 and ICIPE 69 treatments, and longest in treatments with ICIPE 62, ICIPE 78, and ICIPE 284.

In the bioassays with *M. ferruginea*, LT_{10} estimates were shortest in treatments with ICIPE 7 and ICIPE 69, followed by ICIPE 62, and longest in treatments with ICIPE 20, ICIPE 78, and ICIPE 284. However, LT_{25} estimates were the shortest in treatments with ICIPE 69, followed by ICIPE 7, ICIPE 62, and ICIPE 78 treatments, and longest in treatments with ICIPE 20 and ICIPE 284.

Postexposure Survival of *Apis mellifera* and *Meliponula ferruginea*

The 10-d postexposure survival of *A. mellifera* and *M. ferruginea* is summarized using the Kaplan–Meier survival curves (Fig. 1). Survival was significantly different between bee species (χ^2 = 29.46, df = 1, P < 0.0001), with overall *A. mellifera* survival (73.5%) being lower than overall *M. ferruginea* survival (85.5%) in all bioassays. Survival of *A. mellifera* was not significantly affected by bioassays (χ^2 = 2.65, df = 1, P = 0.10) but was significantly affected by treatments (χ^2 = 31.14, df = 6, P < 0.0001). There were no interactions between bioassays and treatments (χ^2 = 0.97, df = 6, P = 0.99). Compared to the controls (88.6%), a significant reduction in *A. mellifera* survival was detected after exposure to ICIPE 7 (73.2%, Z = –4.40, P = 0.0002), ICIPE 20 (73.6%, Z = –4.24, P = 0.0005), and ICIPE 69 (75.0%, Z = –4.13, P = 0.0008), and the corresponding corrected mortalities caused by these isolates were 16.9%, 17.4%, and 15.3%, respectively. However, there was no significant reduction in the survival of *A. mellifera* ($P \geq 0.06$ after exposure to ICIPE 62, ICIPE 78, and ICIPE 284. The survival of *A. mellifera* after exposure to these three isolates were 80.5%, 80.2%, and 84.1%, respectively. For *M. ferruginea*, no significant differences were detected in the survival between bioassays (χ^2 = 0.07, df = 1, P = 0.80), among treatments (χ^2 = 7.21, df = 6, P = 0.30) or their interactions (χ^2 = 1.03, df = 6, P = 0.98). Survival of fungus-exposed *M. ferruginea* ranged between 80.9–89.1%, while survival of fungus-free *M. ferruginea* was 90.9%.

Fungal Mycosis on *Apis mellifera* and *Meliponula ferruginea*

Mycosis of fungus-exposed *A. mellifera* and *M. ferruginea* is presented in Fig. 2. Mycosis was relatively low in fungus-exposed *A. mellifera* (<18.5%) and *M. ferruginea* (<11.7%), and no

Table 3. Estimates of lethal times (LT_{10}, LT_{25}) of fungal isolates (1 × 10^8 conidia/ml) against *Apis mellifera* and *Meliponula ferruginea*

		Apis mellifera			*Meliponula ferruginea*		
Fungal isolate		Slope[a] ± SE	LT_{10} (+95% FL[b])	LT_{25} (+95% FL)	Slope ± SE	LT_{10} (+95% FL)	LT_{25} (+95% FL)
Metarhizium anisopliae	ICIPE 7	5.0 ± 0.1	7.4 (7.1, 7.7) a	8.7 (8.4, 9.1) a	2.3 ± 0.1	8.9 (8.1, 10.2) a	12.8 (11.0, 16.1) ab
	ICIPE 20	4.7 ± 0.1	7.6 (7.0, 8.1) a	10.0 (8.4, 9.9) ab	2.1 ± 0.1	13.1 (10.9, 17.9) b	19.6 (15.1, 31.6) b
	ICIPE 62	4.9 ± 0.1	10.7(9.7, 12.8) b	12.6 (11.1, 16.8) c	2.2 ± 0.1	9.3 (8.3, 10.9) ab	13.7 (11.6, 17.8) ab
	ICIPE 69	4.0 ± 0.1	8.1 (7.7, 8.5) a	9.9 (9.4, 10.7) ab	2.6 ± 0.1	8.7 (8.0, 9.5) a	12.0 (10.8, 13.9) a
	ICIPE 78	4.4 ± 0.1	10.5 (9.9, 11.4) b	12.7 (11.7, 14.4) c	2.1 ± 0.1	10.7 (9.5, 13.9) b	15.9 (13.2, 21.4) ab
Beauveria bassiana	ICIPE 284	6.2 ± 0.1	8.9 (8.1, 9.7) a	12.7 (11.7, 18.7) c	2.3 ± 0.1	13.8 (11.5, 18.9) b	19.9 (15.4, 31.8) b

LT_{10} or LT_{25} followed by the same letters do not differ significantly at α = 0.05 according to the degree of overlaps in 95% FL. n = 220 bees per treatment and replications = 8.

[a]Probit regression model ± standard error (SE).

[b]FL = fiducial limits.

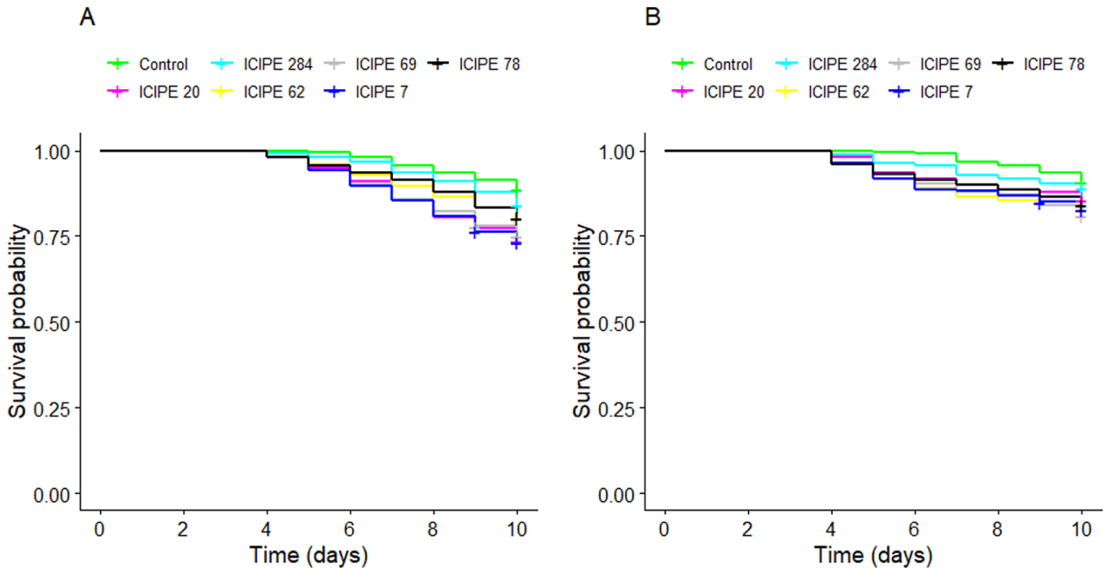


Fig. 1. Kaplan–Meier survival curves for *Apis mellifera* (A) and *Meliponula ferruginea* (B) exposed to 1 × 10^8 conidia/ml of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78), and *Beauveria bassiana* (ICIPE 284) isolates. n = 220 bees per treatment. '+' indicates right censorship.

mycosed insects were recorded in the controls. No significant differences in mycosis between bioassays with *A. mellifera* (χ^2 = 0.61, df = 1, P = 0.44) or *M. ferruginea* (χ^2 = 1.26, df = 1, P = 0.26) were observed. However, significant differences in mycosis of *A. mellifera* were detected among isolates (χ^2 = 39.21, df = 5, P < 0.0001), with ICIPE 7 and ICIPE 20 causing the highest mycosis, followed by ICIPE 69, while ICIPE 62, ICIPE 78, and ICIPE 284 caused the lowest mycosis. In bioassays with *M. ferruginea*, no significant differences in mycosis were detected among isolates (χ^2 = 5.59, df = 5, P = 0.23). None of *M. ferruginea* exposed to ICIPE 284 exhibited mycosis.

Correlation of Conidial Acquisition with Pathogenicity of Fungi

The LT_{10} estimates for fungus-exposed bees were correlated with conidial acquisition. In bioassays with *A. mellifera*, conidial acquisition and LT_{10} had a strong negative correlation in treatment with ICIPE 69 (R = –0.84, P = 0.009), weak negative correlations in treatments with ICIPE 7 (R = –0.18, P = 0.67), ICIPE 20 (R = –0.36, P = 0.38), ICIPE 62 (R = –0.27, P = 0.52), and ICIPE 78 (R = –0.36, P = 0.38), and a weak positive correlation in treatments with ICIPE 284 (R = 0.29, P = 0.49). Conversely, conidial acquisition and mycosis had strong positive correlations in treatments with ICIPE 7 (R = 0.89, P = 0.03) and ICIPE 20 (R = 0.84, P = 0.009), but weak positive correlations in treatments with ICIPE 69 (R = 0.61, P = 0.11), ICIPE 62 (R = 0.04, P = 0.93), ICIPE 78 (R = 0.09, P = 0.84), and ICIPE 284 (R = 0.05, P = 0.90).

In bioassays with *M. ferruginea*, conidial acquisition in treatments with ICIPE 69 correlated strongly and positively with mycosis (R = 0.78, P = 0.023) and weakly and negatively with LT_{10} (R = –0.53, P = 0.18). No significant correlations of conidial acquisition with LT_{10} were confirmed in treatments with ICIPE 7 (R = –0.43, P = 0.29), ICIPE 20 (R = –0.47, P = 0.24), ICIPE 62 (R = –0.18, P = 0.67), ICIPE 78 (R = –0.01, P = 0.97), and ICIPE 284 (R = 0.14, P = 0.74). Similarly, no significant correlations of conidial acquisition with mycosis were confirmed in treatments with ICIPE 7 (R = 0.50, P = 0.21), ICIPE 20 (R = 0.45, P = 0.27), ICIPE 62 (R = 0.42, P = 0.30), and ICIPE 78 (R = 0.03, P = 0.95).

Discussion

Entomopathogenic fungi are promising biocontrol agents against several devastating pests (Shah and Pell 2003, Maina et al. 2018, Akutse et al. 2020). The commercialized entomopathogenic fungi used in this study were considered safe according to their ecotoxicological dossiers, which were obtained in 48-hr oral bioassays with *A. mellifera* incubated at 25 ± 2°C and 50–70% RH. However, their effect on *A. mellifera* for a longer duration and through contact exposure under beehive simulated conditions (30 ± 2°C, 60–80% RH) remained unknown. Besides, assessment of toxicity on stingless bees is not part of registration requirements. The present study compared the effect of fungal-based biopesticides under development and already commercialized on key African insect pollinators, *A. mellifera*, and *M. ferruginea*, under laboratory conditions.

The efficacy of an entomopathogenic fungus is determined by its ability to adhere, germinate, penetrate and colonize the body of the host insect (Maina et al. 2018). The behavior of insects may determine the actual sites of adherence and penetration (Butt and Goettel 2000). In this study, we considered the realistic situation where bees visit flowers of crops sprayed with entomopathogenic fungi and therefore may be exposed through conidial adhesion on tarsi using inoculated filter paper (Butt and Goettel 2000). Both *A. mellifera* and *M. ferruginea* acquired conidia (1.1 × 10^4–1.3 × 10^5 conidia/bee) when exposed for 10 min to surfaces sprayed with 1 × 10^8 conidia/ml of isolates of *M. anisopliae* and *B. bassiana*. The tested concentration used in the study is considered effective to enable detection of any likely harmful effect of the fungus on experimental insects (Quesada-Moraga et al. 2006). However, for field application, commercialized entomopathogenic fungi may be formulated at a higher concentration (1 × 10^9 conidia/ml).

Conidial acquisition by *A. mellifera* was higher than *M. ferruginea*. Such disparity could be explained by their characteristic foraging behavior and morphological traits. In the field, Putra and Kinasih (2013) observed that the Eastern honey bee *Apis cerana* L. tends to spend less time on individual flowers and had higher pollination efficiency compared to the stingless bee *Trigona iridipennis* Smith (Hymenoptera: Apidae). Similar behavior was confirmed in caged conditions where *A. mellifera* exhibited considerably quicker movements over the fungal-treated surface and,

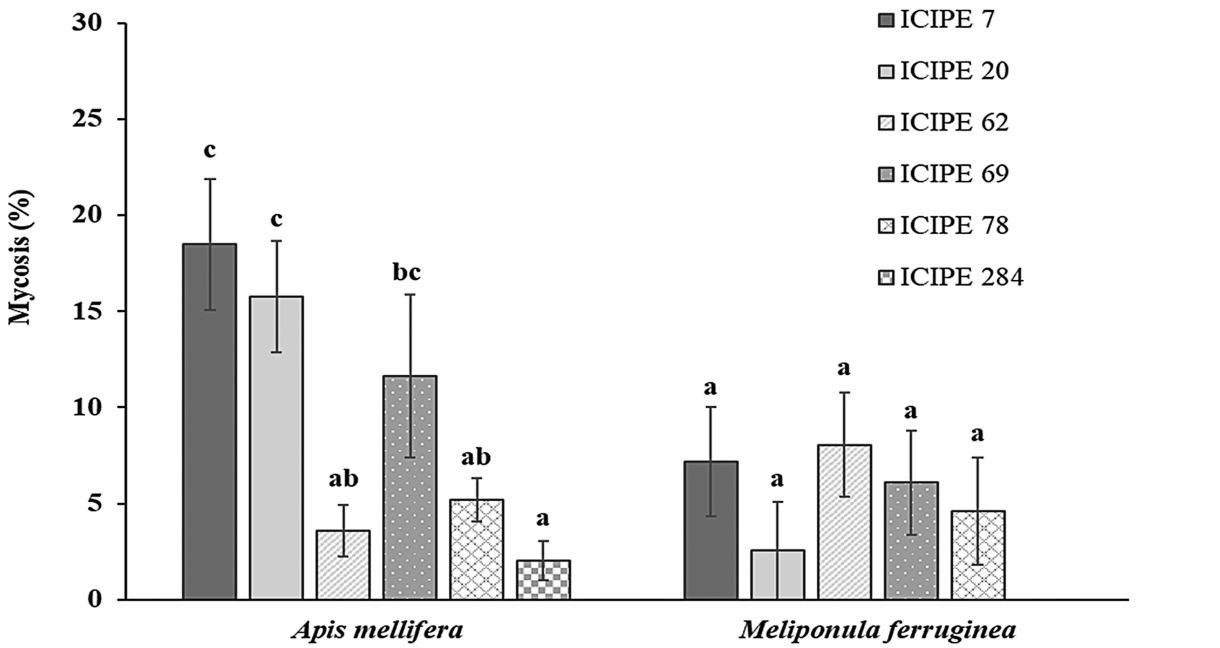


Fig. 2. Mycosis of bees after 10 d of exposure to 1 × 10^8 conidia/ml of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78), and *Beauveria bassiana* (ICIPE 284) isolates. Error bars represent the standard errors. For each species, different letters above error bars indicate significant differences in mycosis ($P < 0.05$) according to Tukey.

therefore, collected more conidia than *M. ferruginea*. Although with no direct reference to *M. ferruginea*, Kajobe (2006) observed that *A. mellifera* collects more and diverse pollen grains than the stingless bees *M. bocandei* and *M. nebulata*, and he indicated such differences could be correlated to their morphological variability such as body size. Generally, an *A. mellifera* worker bee has a total body length of 14.4 mm (surface area: 651 mm^2) (Adeoye et al. 2020) while a *M. ferruginea* worker bee has a total body length of 7.5 mm (surface area: 177 mm^2) (Eardley 2004), and this morphological variability could have accounted for the observed difference in conidial acquisition between the two bee species.

Unlike *M. ferruginea*, conidial acquisition by *A. mellifera* significantly differed among isolates, and this variability could be ascribed to conidial hydrophobicity, surface attachment cues such as adhesins in *Metarhizium* spp. (Liu et al. 2003, Mora et al. 2017) and lectin-binding proteins in *Beauveria* spp. (Wanchoo et al. 2009). Conidia of *M. anisopliae* are larger (8.5 µm length and 2.8 µm width) than conidia of *B. bassiana* (2.1–2.6 µm diameter) (Liu et al. 2003) and, therefore, the conidia of *M. anisopliae* were readily collected by the bees, especially *A. mellifera*. Additionally, conidial attachment is dependent on the fungus-specific cuticular composition of the exposed insect such as hydrocarbon epitopes (Greenfield et al. 2014), and the lack of variations in conidial acquisition indicates that *M. ferruginea* probably lacks these cues for the tested isolates.

A detected reduction in the survival of fungus-exposed *A. mellifera* can be linked to the caging of small groups of bees under laboratory conditions. Being social insects, bees caged in small groups may not express adequate allogrooming, which is normally present in their natural setting, and this may have artificially reduced their survival due to fungal infection. Alves et al. (1996) confirmed that confinement of small groups of *A. mellifera* worker bees secluded from their queen under artificial conditions renders them more stressful and consequently more vulnerable to *B. bassiana* and *M. anisopliae*.

The survival of both *A. mellifera* and *M. ferruginea* was not statistically different between the first and second bioassays, indicating that the susceptibility of bees at the seasons at which they were collected had no impact. Compared to the control (11.4% mortality), ICIPE 7, ICIPE 20, and ICIPE 69 caused a significant reduction in the survival of *A. mellifera* by 15.3–17.4%. Our findings agree with studies by Espinosa-Ortiz et al. (2011) demonstrating low mortality (< 12.7%) of caged *A. mellifera* after 10 d of exposure to 1 × 10^7 conidia/ml of certain isolates of *M. anisopliae* and *B. bassiana*. Butt et al. (1994) observed that direct spraying *A. mellifera* with two virulent isolates of *M. anisopliae* at low concertation (1 × 10^7 conidia/ml) resulted in low mortality (29–35%), however, when sprayed with high concertation (1 × 10^{10} conidia/ml), high mortality (>94.0%) with short LT_{50} (4.4–8.5 d) and almost 100% mycosis were recorded in 14-d bioassays. Potrich et al. (2018) exposed *A. mellifera* workers on smooth surfaces inoculated with *M. anisopliae* (1.0 × 10^9 conidia/ml), which resulted in a reduction of survival to 0% 128 hr postexposure. Colombo et al. (2020) also reported a significant reduction in *A. mellifera* survival after exposure to surfaces sprayed with 1 × 10^8 conidia/ml of *M. anisopliae* (12.5%) and *B. bassiana* (50.0%) in 6-d bioassays.

The tested isolates did not affect the survival of *M. ferruginea*. To our knowledge, this is the first report on the effect of fungal biopesticides on an Afrotropical stingless bee, specifically *M. ferruginea*. However, previous studies on 10–20-d bioassays with neotropical stingless bees indicated that some isolates of *B. bassiana* and *M. anisopliae* (1 × 10^5–1 × 10^9 conidia/ml) caused low mortality (<40.0%) to *M. beecheii, S. mexicana*, and *T. angustula* (Toledo-Hernandez et al. 2016) and significant survival reduction (<69.1%) of *M. scutellaris* (Conceição et al. 2014).

Generally, *M. ferruginea* was less susceptible to the isolates compared to *A. mellifera*. Although we could attribute this difference to conidial acquisition between the two species, their susceptibility to entomopathogenic fungi can also be linked to several other factors. For instance, Bull et al. (2012) and Hamiduzzaman et al. (2012) interrelated the low susceptibility of *A. mellifera* to *M. anisopliae* and *B. bassiana* with the upregulation of immune-related antimicrobial peptide genes including *abaecin*, *defensin-2*, and *hymenoptaecin*.

Bull et al. (2012) demonstrated that young (nursing) bees are very tolerant of fungi due to differential expression of 35 related antimicrobial genes compared to old (forager) bees, which expressed only 2 of these genes.

Conidial acquisition strongly correlated with LT_{10} and mycosis of *A. mellifera* after exposure to ICIPE 7, ICIPE 20, and ICIPE 69, and with mycosis of *M. ferruginea* after exposure to ICIPE 69. The effect of these isolates could be attributed to their genetics and general efficacy (Akutse et al. 2020, Gao et al. 2020). Reportedly, the generalist entomopathogenic fungi commonly possess a couple of virulence genes such as subtilisin-like *Pr1* genes (Gao et al. 2020). In particular, ICIPE 7, ICIPE 20, and ICIPE 69 are highly pathogenic to diverse pest groups, which could be related to the possession of chitinase *chi2* and *chi4* genes, and additional genes for toxin production and conidiation (Niassy et al. 2013).

Under laboratory conditions, we observed that the mortality caused by isolates did not exceed 17.4% for *A. mellifera* or 11.0% for *M. ferruginea*. However, in field conditions, the effects of the entomopathogenic fungi on bees would be lower compared to the observed values in the laboratory for the following reasons. First, the efficacy of the fungi is likely to be reduced by several adverse environmental conditions (Abbaszadeh et al. 2011). Secondly, honey bees and stingless bees inherently regulate their central nest temperatures to a typical range of 32–36°C (Jarimi et al. 2020) and 31–32°C (Jones and Oldroyd 2006), respectively, and these temperatures may restrict the performance of most entomopathogenic fungi (Alves et al. 1996, Davidson et al. 2003). Thirdly, these bees are social insects with sophisticated grooming and hygienic behaviors to detect and remove unusual materials, including fungus-related materials from other bees and eventually from the hives (Gliński and Buczek 2003). Studies investigating the impact of *Metarhizium* sp., *Beauveria* spp., and *Hirsutella thompsonii* Fischer (Hypocreales: Ophiocordycipitaceae) in the beehive showed that they did not cause any lethal effect on adult *A. mellifera*, their broods, queen fecundity, or colony development (Kanga et al. 2002, 2009; Meikle et al. 2007, 2008).

Our findings from contact toxicity in 10-d bioassays with *A. mellifera* and *M. ferruginea* exposed to 1×10^8 conidia/ml show that the tested isolates are nontoxic (<25% mortality) to bees according to the IOBC classification (Sterk et al. 2000). Therefore, these isolates can be safely implemented in the management of pests of pollinator-dependent crops. We consider high conidial acquisition coupled with laboratory conditions or cage membership may have stressed the bees, and probably accounted for the detectable effect of ICIPE 7, ICIPE 20, and ICIPE 69 on *A. mellifera*. Therefore, the three isolates may need a further assessment on hive colonies where bees are arguably less stressed. The interactions of bee pollinators and biopesticides can also be limited by careful timing of biopesticide application to avoid peak foraging periods and/or improving 'lure and infect' application techniques.

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