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Original article

A fungal-based pesticide does not harm pollination service provided by the African stingless bee *Meliponula ferruginea* on cucumber (*Cucumis sativus*)

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Abstract – Stingless bees (Apidae: Meliponini) provide pollination services to crops and produce high–quality honey. The application of agrochemicals during the management of crop pests is an increasing threat to pollinators and the ecosystem services they provide. Biopesticides are considered as better alternatives; however, there is limited evidence of their impact on stingless bees. We evaluated the effect of the most widely used African fungal biopesticide (*Metarhizium anisopliae* ICIPE 69) on mortality, foraging behavior and pollination success of the African stingless bee *Meliponula ferruginea* under semi-field conditions. Colonies of *M. ferruginea* were introduced into four 24 m^2 greenhouse compartments containing blooming cucumber (*Cucumis sativus*) plants. Cucumber plants were sprayed with a suspension of the biopesticide alongside a sterile 0.05% Triton–100–X (control). The experiment was repeated three times during different cucumber growing seasons. Biopesticide application did not significantly affect *M. ferruginea* mortality, flight activity, flower visitation, pollen foraging, *C. sativus* fruit set or *C. sativus* yield. Forager bees acquired a high number of conidia (7,600 ± 54 conidia /bee) immediately after biopesticide application; however, a significant decline was observed in the subsequent days. Conidial persistence and viability on plant surfaces declined significantly with days. There was no correlation between conidial acquisition and pollen load by forager bees (657 ± 29 pollen/bee). This study demonstrates that *M. anisopliae* ICIPE 69 did not negatively impact *M. ferruginea* mortality, pollination behaviour and success, and can therefore be safely used in stingless bee–dependent crop systems.

Agrochemical / Foraging activity / Fruit set / *Metarhizium anisopliae* / Pollen load / Yield

1. INTRODUCTION

Cucumber (*Cucumis sativus* L.) is among the global–leading crops with a high dependency on insect pollination (Giannini et al. 2015; Klein et al. 2007). Moreover, this crop is susceptible to damaging insect pests including, fruit flies, whiteflies, aphids and spider mites (Kambura et al. 2018; Sharma et al. 2016). Whereas chemical insecticides have been successfully used in pest management, they have negatively affected nontarget organisms such as pollinators (Del Sarto et al. 2014; Henry et al. 2012). Hence, there is widespread interest in the research and development of safe alternatives such as biopesticides (Akutse et al. 2020; Glare et al. 2016; Kidanu and Hagos 2020).

The use of biopesticides is highly preferred due to their ecological bio–persistence, little to no ecotoxicity and lack of development of resistant

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pest populations (Kidanu and Hagos 2020; Maina et al. 2018; Thungrabeab and Tongma 2007). *Metarhizium anisopliae* ICIPE 69, registered by RealIPM (Thika, Kenya), is a widely used fungal biopesticide in sub–Saharan Africa (Akutse et al. 2020). It has been registered for use against fruit flies (*Ceratitis* spp.), the fruit tree mealybug (*Rastrococcus invadens* Williams), the Western flower thrips (*Frankliniella occidentalis* Pergande), the tomato leaf miner (*Phthorimaea absoluta* Meyrick) and the pea leaf miner (*Liriomyza huidobrensis* Blanchard) (Akutse et al. 2020). It is also about to be registered for controlling the false codling moth (*Thaumototibia leucotreta* Meyrick) and the bean pod borer (*Maruca vitrata* Fabricius) (Akutse et al. 2020).

Stingless bees are important wild and domesticated insects for pollination and production of high–quality honey in tropical and subtropical regions worldwide owing to their populous colonies, species richness and abundance in different ecosystems (Anguilet et al. 2015; Bafo 2019; Eardley and Kwapong 2013; Kiatoko et al. 2018; Quezada–Euán 2018). They visit flowers of about 90 crops while effectively pollinating 18 crops (Heard 1999; Slaa et al. 2006). They are preferred as pollinators because of their perennial colonies with high polylecty, ecological adaptability, floral constancy and effective forager recruitment, and are easy to be nested in hives, propagated, requeened and otherwise managed (Heard 1999). Compared to honeybees (*Apis* spp)., stingless bees are fifty times more species richer with over 500 species identified globally (Michener 2013, 2007). In Afrotropical regions, *Meliponula* sp. is the most biodiverse stingless bee genus (Eardley et al. 2010) and has been managed for the provision of hive products including honey, pollen and propolis (Bafo 2019; Eardley and Kwapong 2013; Kiatoko et al. 2016). Recently, *Meliponula* spp. are being used as pollinators to improve crop productivity (Asiko 2012; Kajobe 2006; Kiatoko et al. 2014). However, their populations are at risk due to application of agrochemicals (Lima et al. 2016), degradation of natural nesting and foraging habitats (Kiatoko et al. 2018), and pests and diseases (Bobadoye et al. 2016; Purkiss and Lach 2019). Biopesticides are gaining prominence in pest control, however, variable lethal and sublethal effects to stingless bees under laboratory conditions have been observed using fungal–based biopesticides (Conceição et al. 2014; Toledo-Hernandez et al. 2016), bacterial–based biopesticides (Araujo et al. 2019; Barbosa et al. 2015; Tome et al. 2015) and botanical extracts (Barbosa et al. 2015; Cunha Pereira et al. 2020). For instance, *Beauveria bassiana*–based biopesticides caused 35–85% mortality of the stingless bee (*Melipona scutellaris* Latreille) (Conceição et al. 2014), and *M. anisopliae*–based biopesticides caused 94%, 39% and 53% mortality of the stingless bees *Tetragonisca angustula* Latreille, *Scaptotrigona mexicana* Gue´rin–Meneville and *Melipona beecheii* Bennett, respectively, while *B. bassiana* caused < 30% mortality of these bee species (Toledo–Hernandez et al. 2016). Our laboratory studies demonstrated that *M. anisopliae* ICIPE 69 was slightly pathogenic to the stingless bee *Meliponula ferruginea* Cockrell (80.9–89.1% survival) and the Western honey bee (*Apis mellifera* L.) (73.2–84.1% survival) (Omuse et al. 2021a). The effects of biopesticides on survival, foraging behavior and success of the stingless bees remain unexplored under field conditions.

Understanding the effect of biopesticides on pollinators is critical in pest and pollinator management (IPPM) programs. Therefore, the objectives of the present study were (1) to assess the effect of *M. anisopliae* ICIPE 69 on *M. ferruginea* mortality, flight activity, foraging behaviour, and *C sativus* fruit set and yield; (2) to investigate conidial acquisition by *M. ferruginea* foragers and its effect on pollen load; and (3) to establish persistence and viability of *M. anisopliae* ICIPE 69 on flowers and leaves of *C. sativus* in the greenhouse.

2. MATERIAL AND METHODS

2.1. Study site and treatment setup

Three experiments were carried out during three crop growing seasons (April 2020–June 2020, September 2020–November 2020 and

December 2020–February 2021). Experiments were conducted in two 120 m^2 (8 × 15 m) greenhouses at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya (01°13′26″S 36°3′48″E, 1,600 m above sea level). Greenhouses were identically constructed and prepared using the same experimental design. In preparation for experiments, each greenhouse was partitioned with transparent insect–proof materials (0.26–mm mesh size) into five compartments, each measuring 24 m^2 (3 × 8 m). The 1st, 3rd and 4th compartments acted as buffers while the 2nd and 5th compartments were designated as either the biopesticide treatment or the control treatment (Figure 1). The buffers were included to limit the drifting of biopesticides during and after spray from the biopesticide treatment compartments to the control treatment compartments. Treatments were distributed in a randomized complete block design.

2.2. Biopesticide

A culture of the biopesticide *M. anisopliae* ICIPE 69 was obtained from the *icipe* microbial repository. This culture was mass–produced in sterile rice grains as fermentation substrate. Before application, conidia of the biopesticide were quantified and checked for viability as follows. The concentration of conidia in 0.1 g of the substrate was established by preparing a suspension in 10 mL of sterile 0.05% Triton–100–X followed by the enumeration of conidia using an improved Neubauer hemocytometer. A suspension of 1×10^6 conidia/mL was prepared and an aliquot (0.1 mL) was spread–plated on Sabouraud dextrose agar (SDA) (Oxoid, Hampshire, UK) in a plastic Petri dish in four replications and incubated at 25 °C and 0:24 light:dark (L:D) photo phase for 18 h. Each plate was stained with 2 mL lactophenol cotton blue, and three glass coverslips (22 × 22 cm) were placed on the

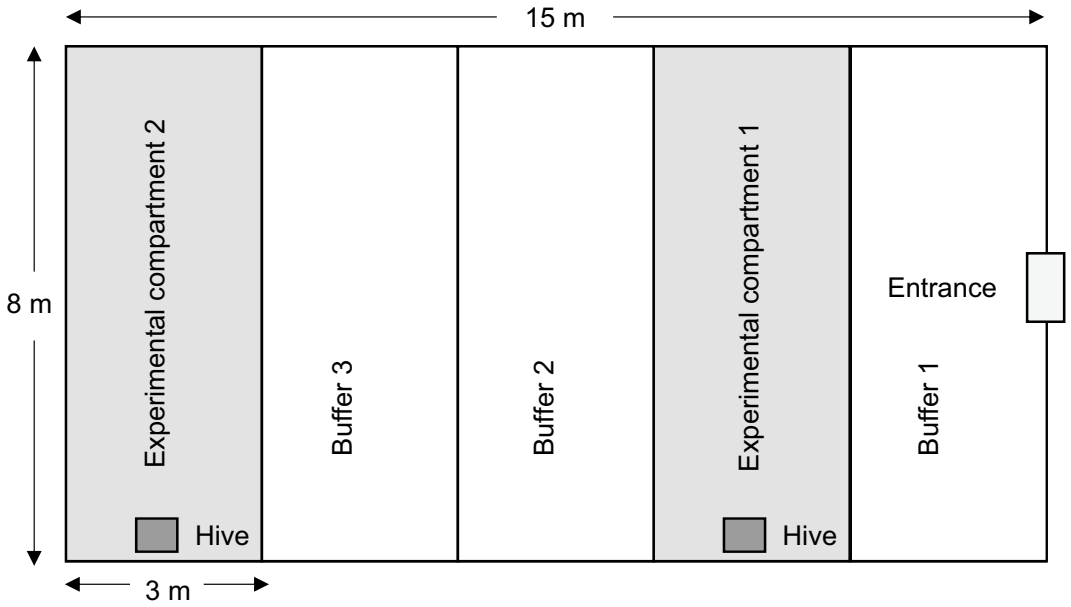

Figure 1. Treatment layout in greenhouses used to test the effects of a fungal-based biopesticide *Metarhizium anisopliae* on pollination of *Cucumis sativus*. Colonies of the stingless bee *Meliponula ferruginea* were introduced in the greenhouse to test if the biopesticide was detrimental to bees' survival, and if biopesticide application reduced their pollination service on cucumber. Buffers were used to separate treatment compartments. Experiments were conducted in three greenhouses maintained at similar conditions and were repeated three times during different cucumber growing seasons. The distance between treatment compartments was 6 m and plants were raised from seeds and maintained at the different experimental compartments following good agricultural practices.

culture surface. Conidial viability was assessed microscopically by randomly counting 100 visible conidia (germinated versus non–germinated) under each glass coverslip. Conidial viability equaled 87 ± 4% (mean ± standard error).

2.3. Bee colonies

Colonies of *M. ferruginea* were obtained from a meliponary established by *icipe* at Isiekuti around Kakamega Forest, Kenya and transported to *icipe*, Nairobi. Colonies were initially trapped from their natural nesting sites (tree trunks in the forests or human–made mud houses) and housed in the original Australian Trigona hive (30 × 28 × 20 cm). Each colony was visually checked for the absence of pests and pathogens; and the presence of an adequate number of bees (about 3,000 adult bees), at least one egg–laying queen, 7–14 combs with brood and eggs, and over 20 storage pots for honey and bee–collected pollen.

2.4. Crop

Cucumis sativus var. Ashley seeds (Simlaw Seeds Co., Nairobi, Kenya) were raised in plastic nursery trays filled with moist seedling propagation substrate (C1 R8089, Kekkilä Professional, Vantaa, Finland). Fourteen–day–old seedlings were transplanted singly in 23 L planting polybag containing a 50% mixture (v/v) of red soil (nitisols) and farmyard manure. Thirty polybags were placed in each treatment compartment and arranged in 10 rows and 3 columns. Polybags were spaced 0.8 m within rows and 0.9 m between rows according to good agronomical practices. The plants were watered daily via drip irrigation. Three vines per plant were trained to climb the 2.5 m high trellis and extra branches trimmed periodically.

2.5. Installation of *Meliponula ferruginea* colonies

Each treatment compartment received one *M. ferruginea* colony 4 weeks after transplanting of seedlings. Each colony was hung 1 m above the ground, 0.3 m from one side of the compartment and 1.5 m from the insect–proof materials (Figure 1). Two feeder plates were placed 2 m apart and 6 m from a colony, and contained either distilled water or propolis for the bees to drink and build colony structures, respectively. Clean 10–20 mm pebbles were placed in feeder plates containing distilled water to prevent bees from drowning. The water and propolis were replenished every day and every 5 days, respectively. Propolis was sourced from *M. ferruginea* colonies maintained at the *icipe* meliponary. Early introduction of *M. ferruginea* colonies before the application of treatments allowed the bees to acclimatize to greenhouse conditions and start to forage.

2.6. Application of treatments

The required amount of conidia was suspended in sterile 0.05% Triton–100–X at a ratio of 1×10^8 conidia/mL. A 16 L knapsack hand spray pump calibrated to apply 300 L/ha was loaded with 1.5 L of sterile 0.05% Triton–100–X (control) or biopesticide suspension. Cucumber plants in the control treatment were sprayed first, followed by those in the biopesticide treatment. Treatments were applied in the late evening (1830–1900 h) 16 days after the introduction of bee colonies, and at this time, bee colonies and feeders were temporarily removed from greenhouses.

2.7. Assessment of flight activity, foraging activity and mortality of forager bees

Flight activity and foraging activity were recorded based on approaches used by Golastra et al. (2012) and Cheng et al. (2018) when assessing the effect of chemical insecticides on *A. mellifera* in semi-field conditions. However, we made modifications to these approaches to accommodate the evaluation of a biopesticide on *M. ferruginea*. Prior to data collection, we observed that bees exhibited peak flight and

foraging activity around 1200–1500 h. Therefore, we recorded flight activity as the number of bees exiting and entering the hive colony at 5-min intervals for 30 min during 1330–1400 h and simultaneously across all treatment compartments. These observations were made every three days starting 6 days before until 18 days after treatment application (i.e., day –6, –3, 0, 3, 6, 9, 12, 15, and 18).

Foraging activity was observed during 1400–1430 h and simultaneously across all treatment compartments by counting forager bees visiting open flowers of three focal cucumber plants at 5 min intervals for 30 min. The three focal plants were those in the middle row across all treatment compartments. We recorded the temperature and the relative humidity (RH) inside greenhouses using a digital Thermo–hygrometer (HC520, Yueqing Xinyang Technology Co., Hubei, China) and counted open flowers produced by the three focal crops after the observation of foraging activity. Flight and foraging activity data were collected on the same sampling dates by trained personnel who were randomly assigned to each treatment compartment during each sampling date to avoid observer biases.

Twenty forager bees were collected from each treatment compartment during 1430–1500 h on day 0, 6, 12, and 18 after treatment application. Forager bees were gently captured when they were about to leave male flowers using clean and well–ventilated transparent 50-mL plastic vials. Forager bees were placed in 0.5-L plastic cages and provided ad libitum with 70% (v/v) honey–water solution and 0.5 g beebread. Caged bees were maintained in a biological oxygen demand incubator (MIR–554, PHC Holdings Co., Tokyo, Japan) calibrated to 30 °C, 60–70% RH and 0:24 L:D. Forager bee mortality was scored at 24 h intervals for 20 days. Dead bees were surface–sterilized by passage in 3% sodium hypochlorite (for 1 min) and 70% ethanol (for 3 min) and rinsed thrice in sterile water (for 1 min). Surface–sterilized cadavers were individually placed in plastic Petri dishes lined with moistened filter paper and incubated at 0:24 L:D and 25 ºC. Cadavers were monitored for 7 days by microscopic examination for possible mycosis (fungal outgrowth).

2.8. Assessment of fruit set and maturation, and yield

All the female flowers produced by the main cucumber vines in treatment compartments were tagged at the date of inception 9 days before until 9 days after treatment application. Flowers that did not develop into fruits 6 days after tagging were recorded and the fruit set was expressed as a percentage of tagged flowers that formed fruits. Fruits that reached their physiological maturity (14 days from tagging) were counted and expressed as a percentage of mature fruits resulting from the tagged flowers.

All physiologically mature fruits were harvested across all treatment compartments. The weight of individual fruits was obtained using an electronic weighing balance (UW6200H, Shimadzu Corporation, Kyoto, Japan) with 0.01 g readability. Fruits were then individually submerged in water in a 1,000-mL glass beaker, and the volume of displaced water was measured in a calibrated 1,000-mL glass cylinder.

2.9. Assessment of pollen load, and conidial acquisition and persistence

Five forager bees were individually collected from each treatment compartment using sterile 10-mL plastic vials. Forager bees were collected when they were about to leave male flowers after foraging for pollen 0, 3, 6, 9, 12, 15 and 18 days after treatment application. About 1 mL of 0.05% Triton–100–X was added in each vial, vortexed for 3 min at 700 rpm to dislodge pollen and conidia, and enumerated using an improved Neubauer hemocytometer.

From each treatment compartment, five male flowers and five leaves were plucked and individually placed in sterile 50 mL plastic vials on day 0, 3, 6, 9, 12, 15 and 18 after applying treatments. Each vial was loaded with 5 mL of sterile 0.05%

Triton–100–X and conidia were dislodged and quantified using the above–described procedure. For each sampling date, suspensions from leaves samples were pooled and tested for conidial viability using the procedure described in Sect. 2.6.

2.10. Statistical analysis

Data analyses were performed in R statistical software (R Core Team 2020). Prior to analysis, flight activity was expressed as the number of bees exiting and entering the hive colony/min and foraging activity was expressed as the number of bees/flower/min. Flight activity, foraging activity and fruit yield were analyzed using a generalized linear model (GLM) and conidial persistence on leaves was analyzed using Quasi–Poisson regression. Conidial persistence on flowers and conidial acquisition by forager bees were analyzed using a zero–inflated negative binomial regression implemented in the *pscl* package (Jackman 2020). Bee mortality, fruit set, fruit maturation and conidial viability were subjected to logistic regression. Post–hoc analyses were performed for parameters that showed significant differences using the *lsmeans* package (Lenth 2015) with the Tukey method for adjustment of p–values. The relationships between flight and foraging activity, bee conidial acquisition and pollen load, and fruit weight and volume were established using Pearson's correlation analysis. In the first experiment, bees in one of the control treatments started to forage near the end of the observation days and, therefore, were excluded from the analyses.

3. RESULTS

3.1. Flight activity, foraging activity and survival of forager bees

Flight activity varied significantly among experiments (Figure 2, $\chi^2 = 88.5$, df = 2, $p < 0.001$). In the first experiment, except between treatments ($\chi^2 = 0.1$, df = 1, $p = 0.8$), there was a significant difference in flight activity among observation dates ($\chi^2 = 111.2$, df = 8, $p < 0.001$) and an interaction between treatments and observation days ($\chi^2 = 39.9$, df = 8, $p < 0.001$). Flight activity in the biopesticide treatment was significantly lower ($p < 0.05$) than in the control treatment on day –3 and 12, but significantly higher ($p < 0.05$) than in the control treatment on day 18. In the second experiment, there were significant differences in flight activity between treatments ($\chi^2 = 11.1$, df = 1, $p = 0.001$) and among observation days ($\chi^2 = 86.4$, df = 8, $p < 0.001$), but no interaction ($\chi^2 = 8.8$, df = 8, $p = 0.4$). Flight activity was only significantly higher ($p < 0.05$) on day –3 in the biopesticide treatment than in the control treatment. In the third experiment, except between treatments ($\chi^2 = 1.7$, df = 1, $p = 0.2$), there was a significant difference in flight activity among observation days ($\chi^2 = 67.1$, df = 8, $p < 0.0001$), and an interaction between treatments and observation days ($\chi^2 = 18.0$, df = 8, $p = 0.02$). Flight activity was only significantly lower ($p < 0.05$) in the biopesticide treatment compared to the control treatment on day 3. For the first, second and third experiments, flight activity was 9.5 ± 0.6, 6.7 ± 0.9 and 5.1 ± 0.8 bees/min in the control treatment, and 9.8 ± 1.9, 8.2 ± 1.0 and 4.6 ± 0.8 bees/min in the biopesticide treatment, respectively.

Foraging activity varied significantly among experiments (Figure 3, $\chi^2 = 74.3$, df = 2, $p < 0.001$). In the first experiment, foraging activity varied significantly between treatments ($\chi^2 = 11.2$, df = 1, $p = 0.008$), among observation dates ($\chi^2 = 143.6$, df = 8, $p < 0.001$) and their interaction ($\chi^2 = 57.0$, df = 8, $p < 0.001$). In the second experiment, there was a significant difference in foraging activity between treatments ($\chi^2 = 44.4$, df = 1, $p < 0.001$), among observation days ($\chi^2 = 63.4$, df = 8, $p < 0.001$) but no interaction ($\chi^2 = 13.5$, df = 8, $p = 0.1$). In the third experiment, there was a significant difference in foraging activity between treatments ($\chi^2 = 29.7$, df = 1, $p < 0.001$), among observation days ($\chi^2 = 51.2$, df = 8, $p < 0.001$) and their interaction ($\chi^2 = 26.8$, df = 8, $p = 0.001$). The number of open flowers and the weather conditions inside greenhouses during afternoon varied across observation days (Figure 2). Across experiments, foraging activity

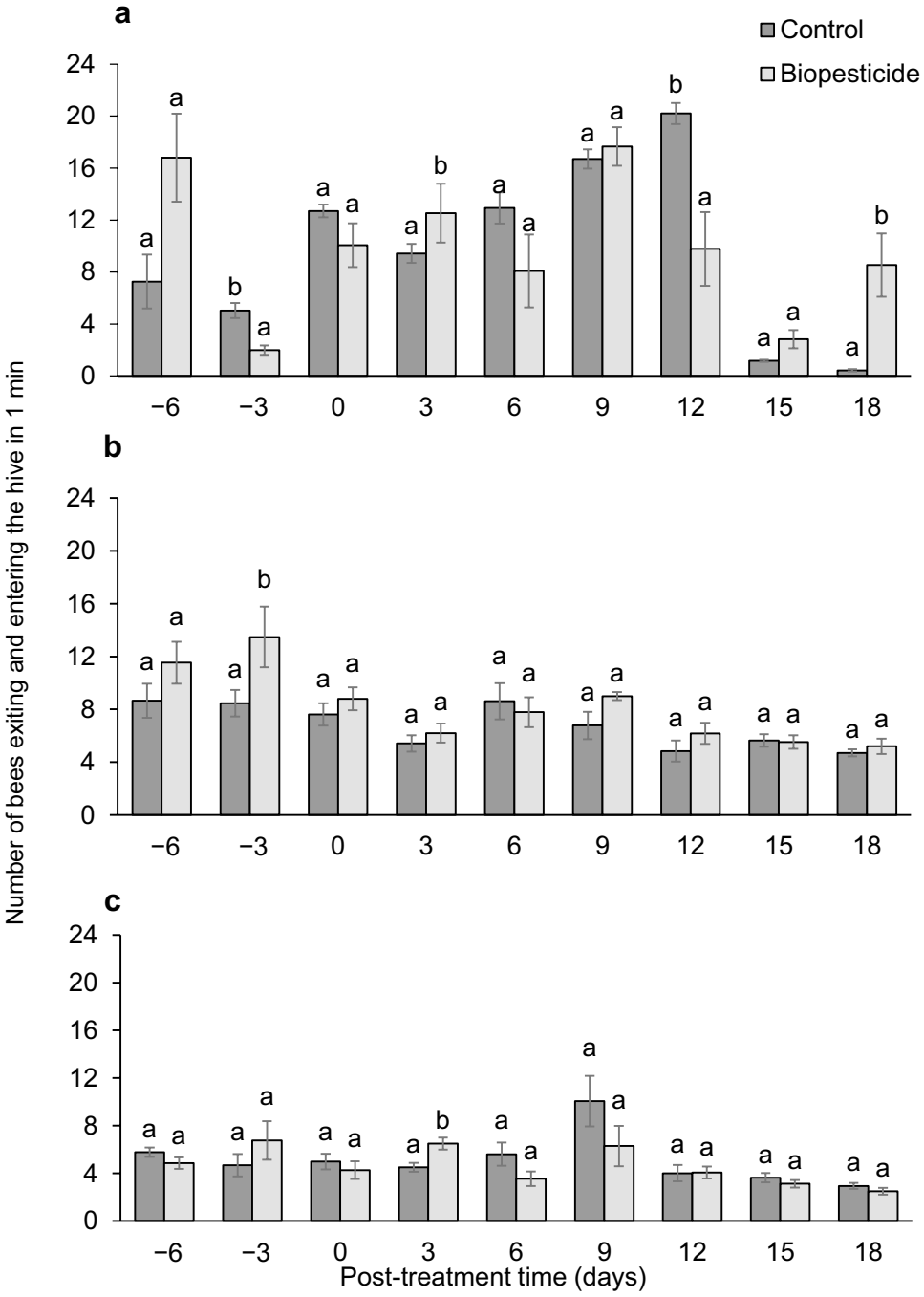

Figure 2. Flight activity of the stingless bee *Meliponula ferruginea* reared in greenhouses with *Cucumis sativus* treated with a fungal–based biopesticide *Metarhizium anisopliae*, during different seasons; **a**: April 2020–June 2020, **b**: September 2020–November 2020, **c**: December 2020–February 2021. Flight activity was measured as the number of bees exiting and entering hive colonies in treated (biopesticide) and non–treated (control) flowers, after every 5 min. The number of bees was checked before and after biopesticide application (day 0). Error bars represent standard errors. Different letters indicate statistical differences between columns on the same day of each graph at $\alpha = 0.05$ according to the Tukey post–hoc test.

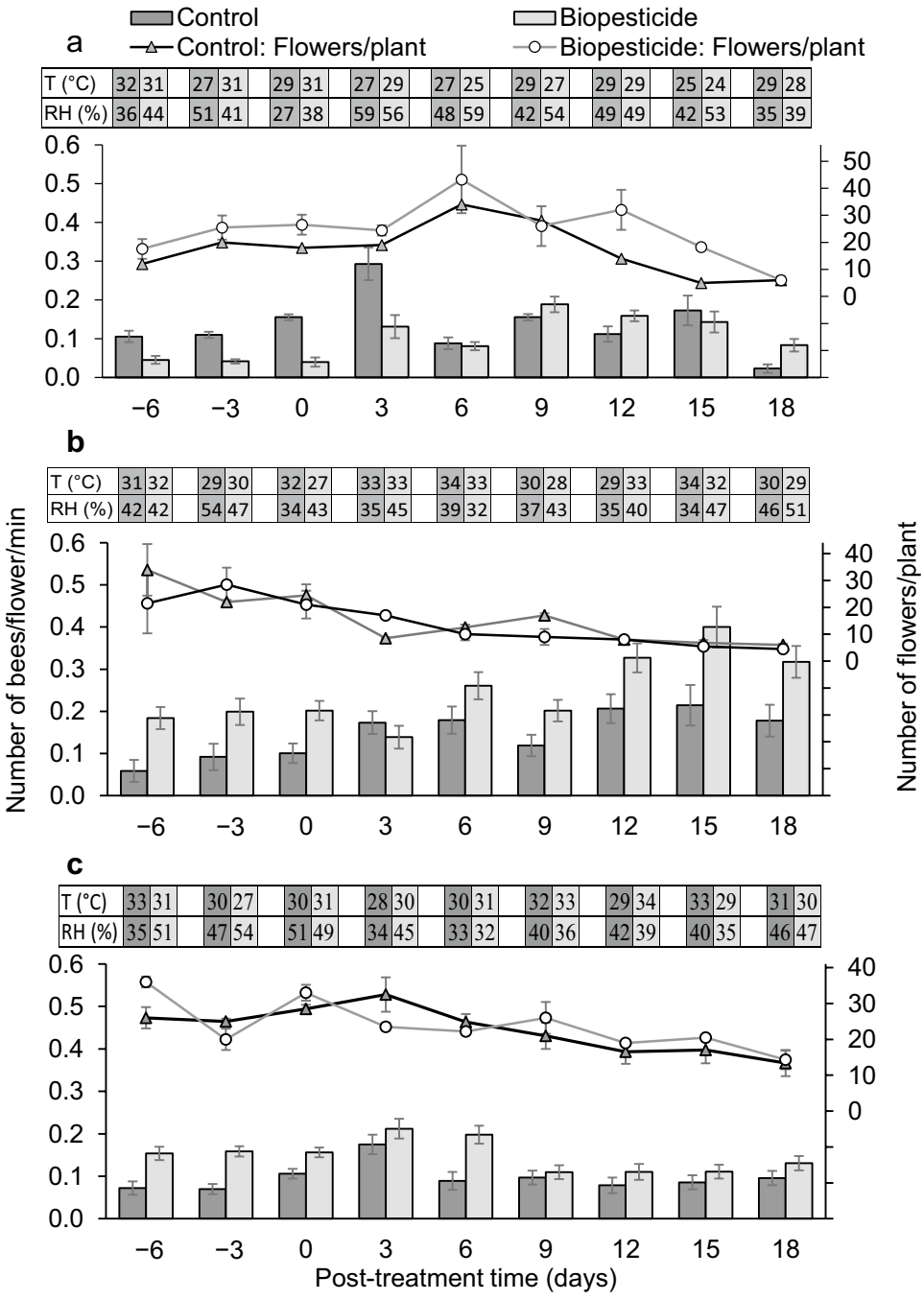

Figure 3. Foraging activity of the stingless bee *Meliponula ferruginea* reared in greenhouses with *Cucumis sativus* treated with a fungal – based biopesticide *Metarhizium anisopliae*, during different seasons; **a**: April 2020–June 2020, **b**: September 2020–November 2020, **c**: December 2020–February 2021. Foraging activity was measured by counting the bees foraging in treated (biopesticide) and non-treated (control) flowers, after every 5 min. The y–axis on the right shows the numbers of open flowers produced by an individual plant in the control and biopesticide treatment compartments. Provided in the table are the temperature (T) and relative humidity (RH) inside the greenhouse during the afternoon (1400–1430 h). The number of bees/flower/min and flowers/plant, as well as the temperature and RH, were checked before and after biopesticide application (day 0). Error bars represent standard errors.

correlated positively and strongly with flight activity (Pearson's correlation: $R = 0.1$, $p = 0.01$) and was significantly affected by number of open flowers ($\chi^2 = 143.5–160.7$, df = 18–19, $p < 0.001$), temperature ($\chi^2 = 17.7–66.7$, df = 7–9, $p \leq 0.01$) and RH ($\chi^2 = 69.2–101.6$, df = 13–15, $p < 0.001$). In the first, second and third experiments, foraging activity was 0.14 ± 0.02, 0.15 ± 0.03 and 0.10 ± 0.02 bees/flower/min in the control treatment, and 0.10 ± 0.02, 0.25 ± 0.03 and 0.15 ± 0.02 bees/flower/min in biopesticide treatment, respectively.

Forager bee mortality from either biopesticide or control treatments did not exceed 22% (Table I). Forager bee mortality was not statistically different among experiments ($F = 2.5$, df = 2, $p = 0.9$) and, therefore, data from experiments were pooled. No significant effects of treatments ($F = 2.8$, df = 1, $p = 0.1$), post–treatment days ($F = 2.8$, df = 3, $p = 0.07$) or their interaction ($F = 0.6$, df = 3, $p = 0.6$) were detected on forager bee mortality. None of the bee cadavers from the biopesticide treatment developed mycosis.

3.2. Fruit set and development, and yield

Results of fruit set and fruit maturation are presented in Table II. Fruit set was not significantly different across experiments ($F = 1.7$, df = 2, $p = 0.1$). Fruit set was not significantly affected by treatments ($F = 0.7$, df = 1, $p = 0.4$), observation days ($F = 1.9$, df = 5, $p = 0.1$) or their interaction ($F = 0.5$, df = 5, $p = 0.8$). Similarly, fruit maturation was not significantly different across experiments ($F = 0.5$, df = 2, $p = 0.6$). Fruit maturation was not significantly different between treatments ($F = 0.4$, df = 1, $p = 0.6$) but significantly differed among observation days ($F = 3.4$, df = 5, $p = 0.001$). There was no interaction between treatments and observation days on fruit maturation ($F = 0.5$, df = 5, $p = 0.8$).

The volume of a fruit was directly proportional to its weight (Pearson's correlation: $R = 1$, $p < 0.001$). Therefore, we analyzed the fruit weight dataset. Fruit weight in the second and third experiment was significantly higher compared to the first experiment (Table III, $\chi^2 = 175.1$, df = 2, $p < 0.001$). In the first experiment, except across the observation days ($\chi^2 = 11.5$, df = 5, $p = 0.04$), fruit weight was not significantly affected by treatments ($\chi^2 = 3.5$, df = 1, p = 0.06), nor was there an interaction between treatments and observation days ($\chi^2 = 2.5$, df = 5, $p = 0.8$). In the second experiment, fruit weight was not affected by treatments ($\chi^2 = 1.2$, df = 1, $p = 0.3$), observation days ($\chi^2 = 3.1$, df = 5, $p = 0.7$) or their interaction ($\chi^2 = 2.0$, df = 5, $p = 0.9$). Likewise, in the third experiment, fruit weight was not affected by treatments ($\chi^2 = 1.4$, df = 1, $p = 0.5$), observation days ($\chi^2 = 2.8$, df = 5, $p = 0.7$) or their interaction ($\chi^2 = 1.5$, df = 5, $p = 0.9$).

3.3. Conidial acquisition and persistence

No conidia were detected on cucumber plant surfaces or forager bees in control treatments. In biopesticide treatments, the number

Table I Mortality percentage (mean ± standard error) of the stingless bee *Meliponula ferruginea* after foraging on flowers of *Cucumis sativus* treated (biopesticide) or non-treated (control) with *Metarhizium anisopliae*. Experiments were conducted in greenhouses containing treated and non-treated flowers, and installed with colonies of *M. ferruginea*. Bee mortality outside the colonies was checked immediately after treatment application and at every 6 days, during 18 consecutive days

Post–treatment days	Control (%)	Biopesticide (%)
0	12.4 ± 1.2	15.8 ± 5.5
6	4.4 ± 1.9	10.8 ± 2.7
12	7.4 ± 2.0	6.7 ± 2.5
18	10.0 ± 4.5	14.2 ± 2.0

Table II Percentage (mean ± standard error) of fruit set and fruit maturation of *Cucumis sativus* treated (biopesticide) or non – treated (control) with *Metarhizium anisopliae*. Colonies of stingless bee *Meliponula ferruginea* were installed in greenhouses containing blooming *C. sativus*. Percentage of fruit set and fruit maturation was assessed within 3 – day intervals, starting 9 days before until 9 days after applying biopesticide

Post–treatment days	Fruit set (%)		Mature fruits (%)	
	Control	Biopesticide	Control	Biopesticide
−9 – −7	87.7 ± 2.5a	89.1 ± 2.8a	80.2 ± 4.0a	80.1 ± 4.0a
−6 – −4	93.6 ± 1.3a	91.0 ± 2.1a	87.6 ± 2.1b	87.2 ± 2.1b
−3 – −1	94.2 ± 1.2a	89.4 ± 4.7a	90.1 ± 2.3b	86.7 ± 2.3b
0 – 2	93.6 ± 2.1a	96.1 ± 2.6a	83.7 ± 4.8b	88.0 ± 4.8b
3 – 5	94.2 ± 1.2a	92.0 ± 1.5a	89.3 ± 2.2b	87.6 ± 2.2b
6 – 8	88.3 ± 0.7a	86.0 ± 6.4a	81.4 ± 1.5a	76.0 ± 1.5a

Same letters within columns indicate no statistical difference at $p > 0.05$ (the Tukey post hoc test)

of conidia after biopesticide application on cucumber plants declined significantly with observation days on leaves ($F = 7.6$, df = 6, $p < 0.001$) and flowers ($F = 45.8$, df = 6, $p < 0.001$) (Table IV). Similarly, the number of conidia adhering to the bees' body during foraging declined significantly with observation days (Table IV, F = 14.4, df = 6, $p < 0.001$). The number of conidia acquired by forager bees did not significantly affect the number of pollen they collected during foraging (Pearson's correlation; $R = 0.1$, $p = 0.4$). The number of pollen collected during observation days by forager bees was not statistically different (Table IV). However, conidial viability on cucumber leaves declined significantly with observation days (Figure 4, $F = 121.0$, df = 6, $p < 0.001$).

4. DISCUSSION

Pollinators visiting flowering crops have an ultimate effect on their productivity including, fruit set and fruit quality at maturation (Klein et al. 2007). In the present study, spraying blooming cucumber plants with biopesticides did not significantly affect *M. ferruginea* pollination behaviour (flight activity, foraging activity and pollen foraging) and cucumber productivity (fruit set, maturation and yield).

Table III Mean (± standard error) of 14 – day – old *Cucumis sativus* fruit weight (grams) in greenhouses installed with colonies of stingless bee *Meliponula ferruginea*. Greenhouse compartment containing blooming *C. sativus* were treated with biopesticide *Metarhizium anisopliae* or distilled water (control). The weight of fruit formed during 9 days before until 9 days after application treatment was recorded

Post–treatment days	April 2020–July 2020		September 2020–November 2020		December 2020–February 2021	
	Control	Biopesticide	Control	Biopesticide	Control	Biopesticide
−9 – −7	296 ± 15	257 ± 22	354 ± 13	358 ± 27	338 ± 15	371 ± 29
−6 – −4	289 ± 22	272 ± 22	366 ± 15	376 ± 21	363 ± 18	366 ± 21
−3 – −1	246 ± 22	234 ± 28	362 ± 36	359 ± 14	379 ± 42	372 ± 22
0 – 2	304 ± 21	242 ± 26	379 ± 19	353 ± 15	376 ± 24	380 ± 27
3 – 5	248 ± 20	253 ± 37	385 ± 83	336 ± 24	372 ± 22	378 ± 20
6 – 8	234 ± 24	196 ± 16	346 ± 31	311 ± 20	364 ± 2	376 ± 27

Table IV Mean (± standard error) of biopesticide's conidia retained on *Cucumis sativus* plant surfaces, and conidia and pollen collected by stingless bee *Meliponula ferruginea* foragers. Greenhouse compartment containing blooming *C. sativus* were installed with colonies of *Meliponula ferruginea* and treated with biopesticide *Metarhizium anisopliae* or distilled water (control). Conidial load and pollen load were assessed at each 3 days, consecutively, for 18 days after application of treatment

Days	Conidia/flower		Conidia/leaf		Conidia/bee		Pollen/bee	
0	215,350 ± 226	e	51,695,000 ± 9,000	d	7,600 ± 54	d	731 ± 74	a
3	80,880 ± 613	d	44,840,000 ± 9,000	d	5,100 ± 67	d	610 ± 74	a
6	6,700 ± 193	c	35,915,000 ± 13,000	bc	1,080 ± 64	c	581 ± 59	a
9	1,600 ± 60	b	25,635,000 ± 13,000	bcd	780 ± 41	bc	645 ± 44	a
12	500 ± 50	ab	14,225,000 ± 17,000	abc	80 ± 0	ab	607 ± 61	a
15	200 ± 15	ab	13,370,000 ± 9,000	ab	20 ± 0	a	687 ± 48	a
18	0	ab	7,190,000 ± 17,000	a	0	a	618 ± 55	a

Different letters within columns indicate significant difference at $p < 0.05$ according to the Tukey post hoc test

This study demonstrates that the detectable differences in flight activity and foraging activity were not caused by treatment application, i.e., the differences were evident before and after applying treatments, and were randomly distributed across experiments, between treatments and among observation days. Significant variations in flight activity and foraging activity among experiments could be associated with several number of factors. For instance,

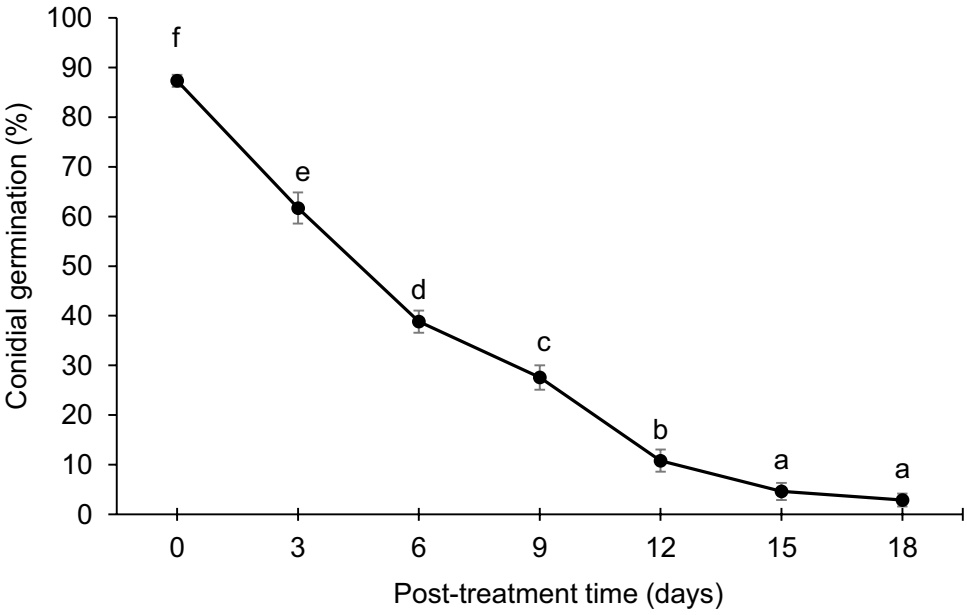

Figure 4. Conidial viability on the *Cucumis sativus* leaves after being sprayed with the fungal biopesticide *Metarhizium anisopliae*. Error bars represent standard errors. Different small letters above error bars indicate significant differences at $\alpha = 0.05$ according to the Tukey post hoc test.

Ferreira Junior et al. (2010) observed that flight activity and floral resource collection activity of the stingless bee *Melipona bicolor schencki* Gribodo varied according to environmental conditions and seasons. Our study was conducted at different seasons and flight activity was more intense in April 2020–June 2020 (first experiment) compared to September 2020–November 2020 (second experiment) and December 2020–February 2021 (third experiment), while foraging activity was more intense in September 2020–November 2020 compared to April 2020–June 2020 and December 2020–February 2021. The intensity of foraging activity across the three seasons matches the blooming trend in areas around Kakamega forest where the *M. ferruginea* colonies were sourced from. Additionally, the average number of open flowers/plant/day was highest in the first (7.3 ± 2.0) and third experiment (7.9 ± 2.0), and, therefore, the first and third experiments experienced low foraging activity compared to the second experiment, which had the lowest average number of open flowers (5.1 ± 1.2). However, differential flight activity could be ascribed to bees seeking floral resources, water and propolis, and bees removing waste out of the colony. Moreover, variations in flight activity and foraging activity among the observation days can be linked to different weather conditions (specifically temperature and RH) and the number of open flowers. In addition, although colonies of similar health were initially selected, differences in colony physiology among treatments may also account for some variation.

Stingless bee flight and foraging activity as observed here is consistent with the study by Visalakshy et al. (2019), who reported no significant reduction in pollination activity of the dwarf honeybee (*Apis florea* Fabricius), the Asiatic honeybee (*A. cerana* Fabricius), and the hoverflies *Eristalis aryorum* Fain and *Chrysomya megacephala* Fabricius visiting mango (*Mangifera indica* L.) flowers sprayed with *M. anisopliae*. Similarly, except for spinosad (a chemical compound derived from *Saccharopolyspora spinosa* Mertz and Yao), Challa et al. (2019) observed no significant difference in relative abundance, foraging rate or foraging speed of *A. cerana* pollinating oilseed brassica (*Brassica juncea* L.) sprayed with the fungal-based biopesticides *B. bassiana* and *Nomuraea rileyi* Farl, and the botanical azadirachtin.

Pollinators collect pollen and nectar from flowering plants as part of their essential food. In our study, forager bees collected pollen alongside conidia, which remained consistently high during the cucumber flowering period (657 ± 29 pollen/bee). The highest number of conidia ($7{,}600 \pm 54$ CFU/bee) was collected immediately after biopesticide application, which then declined by 67.3% every three days. However, conidial acquisition by forager bees did not affect their pollen foraging. Omuse et al. (2021a) showed that an adult *M. ferruginea* can acquire between 1.85×10^4 to 2.25×10^4 conidia of *M. anisopliae* isolates applied on filter paper. Our results indicate that *M. ferruginea* acquires much fewer conidia in semi-field conditions than in the laboratory.

Depending on the species of the target insects, different fungal–based biopesticides may target different stages of the pests (Koca et al. 2019) and the biopesticide used in this study has been demonstrated to infect nearly all stages of insects (Ekesi et al. 2007; Niassy et al. 2012). Laboratory studies indicate that the susceptibility of stingless bees depends on the dose, species and isolate of entomopathogenic fungi tested (Conceição et al. 2014; Toledo–Hernandez et al. 2016; Omuse et al. 2021a). Whereas in the laboratory, *M. anisopliae* ICIPE 69 caused *M. ferruginea* mortality and mycosis (Omuse et al. 2021a), these were not the case in the present semi-field study. As indicated above, bees acquired fewer conidia in the field than in the laboratory. In addition, conidial viability may have decreased due to environmental conditions under field settings.

In the field, the persistence and viability of fungal biopesticides may be reduced by many factors related to the treated crops and the environment (Abbaszadeh et al. 2011). In our study, cucumber plants sprayed with biopesticide

retained conidia but conidial density declined by 71.8% on flowers and 26.4% on leaves every three days. Leaves possessed a higher conidia retention capacity, arguably due to a larger surface area than flowers. Equally, conidial viability declined by 41.9% every 3 days. Relatively high temperatures and low RH in the early afternoon could have contributed to the drastic declines of conidial persistence and viability in the greenhouse. In field conditions, Jaronski (2010) observed that the conidial viability of the fungal biopesticide (*B. bassiana*) sprayed on melon (*C. melo* L.) plants reduced daily by 9–11% and 47% on the underside and upper leaf surfaces, respectively.

Our results showed that biopesticide application did not affect cucumber fruit set and fruit maturation. Cucumber fruit set was 91.1 ± 1.0% while fruit maturation was 84.8 ± 1.3%. Similarly, cucumber fruit yield (weight) was not different between the biopesticide and control treatments. However, fruit yield was significantly higher in September 2020–November 2020 and December 2020–February 2021 than in April 2020–June 2020. This variation could be related to weather conditions across growing seasons. April–June constitute the coldest months (17.6–19.3 °C) with 1.9–4.9 mm precipitation while September–November and December–February are among the hottest months (18.3–19.3 °C, 18.6–20.5 °C) with 0.8–3.7 mm and 1.3–2.2 mm precipitation, respectively, in Nairobi, Kenya (Merkel 2019).

It is worth noting that there are several groups of biopesticides with different toxicity to stingless bees. For instance, azadirachtin and spinosad can cause detrimental effects to adult workers of the stingless bee *Melipona quadrifasciata* Lepeletier (Barbosa et al. 2015). Azadirachtin and chlorantraniliprole (a botanical) are considered to have low toxicity but can impair flight activity, and potentially reduce foraging activity and colony survival of the stingless bees *Partamona helleri* Friese and *Scaptotrigona xanthotrica* Moure (Tome et al. 2015). The detrimental effect of *M. anisopliae* has also been shown on some stingless bees, especially *T. angustula* (Toledo-Hernandez et al. 2016).

We previously demonstrated that the fungal–based biopesticide *M. anisopliae* was nontoxic to *M. ferruginea* in a 10–day laboratory mortality bioassay (Omuse et al. 2021a). In the current study, we found no significant effect of *M. anisopliae* on *M. ferruginea* mortality, flight activity and foraging activity, and consequently on cucumber fruit set, maturation and yield. In addition, the tested biopesticide is unlikely to germinate and grow optimally inside the central brood areas of the honey bee and stingless bee colonies based on the predictive modelling (Omuse et al. 2021b). Our results validate the safety of *M. anisopliae* ICIPE 69 as an ecofriendly alternative for pest management in crop systems supported by pollination services of stingless bee (*M. ferruginea*), especially in IPPM programs. Future studies should focus on evaluating the effect of fungal–based biopesticides on other stingless bee species.

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AUTHOR CONTRIBUTION

All authors conceived this research and designed experiments; ERO, SN, TB participated in the design and interpretation of the data; ERO performed experiments and analyses; ERO wrote the paper and all authors participated in the revisions of it. All authors read and approved the final manuscript.

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

R software custom codes for data analyses for the current study are available from the corresponding author on reasonable request.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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