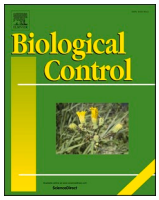
Biological Control 176 (2022) 105062

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon



Selecting thermal tolerant strains of entomopathogenic fungi to control *Ceratitis capitata* (Wiedeman) in tropical semi-arid conditions

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HIGHLIGHTS

- Thermal tolerance is an important characteristic of a biocontrol agent.
- Applying constant temperature can cause discharging efficient BCAs.
- Spore germination, mycelial growth, and insect mortality were different at constant and fluctuating temperature.
- Mildly thermotolerant strains under constant high temperature effectively killed adults *C. capitata* under fluctuating temperature.
- Field experiments in two contrasting seasons confirmed this result.

ARTICLE INFO

Keywords:
Thermal tolerance
Metarhizium anisopliae
Beauveria bassiana
Growth and virulence
Constant versus variable temperature

ABSTRACT

Although it is usual, using constant temperature to select thermo-tolerant entomopathogen (EF) strains could discard potentially efficient biocontrol agents (BCA). The objectives of this work were to select virulent EF against *C. capitata* and evaluate the effect of continual and fluctuating temperature on fungal development and virulence. Initial experiments compared the effect of constant temperatures (CT) (20, 25, 30, 35, and 38 °C) and simulated intraday temperature variation (SIV) (20–38 °C) over conidial germination, mycelial growth, and insect mortality. Temperatures $\geq$ 35 °C in the CT experiments significantly reduced conidial germination, and the strains of *Beauveria bassiana* *Bb*LCB81 and *Bb*LCB289 showed the highest conidial germination and mycelial growth. SIV also caused a significant effect, and *M. anisopliae* *Ma*LCB62 showed the highest conidial germination and mycelial growth. Applying EF strains as toxic baits in CT showed that the highest mortality occurred at 30 °C, and *Bb*LCB62 and *Bb*LCB289 showed the largest insect mortality at 38 °C. Survival analysis showed a slight increase in the average survival time (ST_{50}) at 30 °C. In SIV conditions, *Ma*LCB56 and *Bb*LCB289 were highly virulent. In a field cage experiment at a warmer temperature, *Bb*LCB62 was the most virulent strain according to the M-C test comparing survival curves. However, confirmed accumulated mortality on the 10th day was similar to the moderately tolerant *Bb*LCB289 (Tukey, $p < 0.05$). During winter, all EF strains showed similar mortality on 10th day (Tukey, $p < 0.05$), but a significant difference in the survival analysis. EF strains with mild temperature tolerance can be applied in seasons with high temperatures, and BbLCB62 and BbLCB289 were selected as potential biocontrol agents against *C. capitata*.

1. Introduction

Ceratitis capitata (Diptera: Tephritidae) Wiedeman originated from the Mediterranean region and distributed worldwide, producing direct and indirect damages to the fruits with severe economic losses (Rwomushana and Tanga, 2016). *C. capitata* is the most critical pest in the Brazilian mango production chain, reducing productivity and requiring pre and post-harvest treatments that increase production costs. Usually,

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https://doi.org/10.1016/j.biocontrol.2022.105062
Received 16 December 2021; Received in revised form 1 September 2022; Accepted 19 September 2022
Available online 24 September 2022

integrated management of *C. capitata* relies on field sanitation and insecticide sprayings for adult control or its application to the soil for larvae control (Dias et al., 2018). However, social concerns about food and environmental contaminants caused by insecticide motivated the search for biological approaches using macro and microorganisms (Paranhos et al., 2019; Toledo et al., 2017). Recent works have selected virulent entomopathogenic fungi (EF) strains against fruit flies to be applied using different strategies such as dispersant devices, soil drenching, and toxic baits (Gava et al., 2019; Navarro-Llopis et al., 2015; Toledo-Hernández et al., 2018).

In addition to virulence against target pests, an intrinsic tolerance to different abiotic stress is required when selecting efficient EF strains against pests in tropical conditions, mainly in semi-arid regions. Besides, semi-arid climates are known for a large daily thermal amplitude (Alvares et al., 2013), limiting EF activity in tropical and subtropical regions with dry summer. Low relative humidity (RH), high ultraviolet irradiation (UV), and high temperature are prevalent in the tropics, and the development of a biopesticide should address such constraints.

Conidial survival and initial steps of the infection process are highly dependent on environmental conditions. Borisade and Magan (2014), e. g., showed that high temperature (35–37 °C) significantly reduced the growth and sporulation of EF strains of different species. According to the authors, the deleterious effect of temperature was even higher when interacting with low water availability. Adding adjuvants to the formulations (UV protectants, humectants, dispersing agents) helps to reduce the effect of environmental stresses (Burges, 1998; de Oliveira et al., 2018; Santos et al., 2011). Oil formulations, for example, can increase conidial survival in low air RH, granting a more extensive survival period when the target pest can acquire a higher dose of the formulation from plant surfaces (Bateman et al., 1993; Wraight and Ramos, 2017).

Perhaps temperature is the most challenging environmental constraint to solve in the field. Even when it does not kill EF conidia, heat stress causes a delay in fungal growth and host infection (Keyser et al., 2014; Mwamburi et al., 2015). Nevertheless, variability in the tolerance of EF strains to temperature and UV has already been shown (Fernandes et al., 2007; Rangel et al., 2005). In fact, optimal temperature for conidia germination, mycelial growth, and infection progress can occur in a wide range of temperatures, a phenotypic trait that can be affected by a selective genetic selection occurring in the original environment of the EF strain (Fernández-Bravo et al., 2016; Ouedraogo et al., 1997; Rangel et al., 2005). Thus, the selection of EF strain should include temperature resistance as a desired characteristic in the early steps of the product development process (Borisade and Magan, 2014).

Temperature in fruit growing in tropical regions can peaks over 35 °C at summer, usually occuring some hours around 40 °C, which do not affect *C. capitata* population (Nyamukondiwa et al., 2010; de Oliveira et al., 2019). Most works on selecting thermal tolerant EF strains evaluated the effect of temperature on conidial germination and mycelial growth in constant temperature (Athanassiou et al., 2017; Bugeme et al., 2009), a condition that does not occur in nature. We hypothesized that even considering that such experiments inform the conidial death point and temperature threshold for mycelial growth (Rangel et al., 2005), the use of constant temperature to select tolerant strains could discard potentially useful biocontrol agents. Therefore, the objectives of this work were (1) evaluate the effect of continual and fluctuating temperature on fungal germination, growth, and virulence to *C. capitata*, and (2) to select virulent EF strains tolerant to the regimen of intraday temperature variation commonly observed in the Brazilian semi-arid region.

2. Materials and methods

2.1. Biological materials

1.a. Preserving EF strains and inoculum production.

Six local strains of *Beauveria bassiana* s. l. and *Metarhizium anisopliae s.l.* (Table 1) were cultivated in Sabouraud dextrose agar (SDA – glucose 40.0 g, yeast extract 20.0 g, peptone 10.0 g, agar 15.0 g, water 1.000 ml) and incubated at 28,0 ± 0.5 °C 14:10 h photoperiod for ten days. The strains were previously selected for virulence against *C. capitata* and reisolated from the insect cadavers (Table 1). Stocks of a work collection were preserved using the Castellani method by which mycelial disks collected from the outer limits of the colonies were transferred to flasks containing sterile distilled water at 5 °C (de Capriles et al., 1989). Propagules for the experiments were produced by scraping conidia from densely colonized plates using Triton X-100 0.05% w/v solution as a dispersant.

Large amounts of conidia were produced as described by Jaronski (2014). Briefly, conidial suspensions of each strain were inoculated in plastic bags containing autoclaved parboiled rice with 40% w/w humidity (10^5 conidia g^{-1}). After 15 days of incubation, the mixture of substrate and propagules were transferred to paper trays and dried in a forced-air drying oven at 35 °C until moisture content was approximately 10%. Conidia were extracted using a Mycoharvester (MH5, VBS Agriculture Ltd, Cornwall, UK).

1.b. Insect rearing.

The insects in these studies were reared using the methodology described by da Silva Neto et al. (2012). Briefly, the insects were initially obtained from pupae recovered from naturally infested fruits collected in local farms in 2015 and disposed into plastic trays containing vermiculite. Adults were maintained in transparent methacrylate cages (30 × 30 × 30 cm) and fed an artificial diet containing saccharose and hydrolyzed yeast (Biones®, Quatá, São Paulo, Brazil), and distilled water was offered *ad libitum*. Egg-laying occurred in a voile tissue in a lateral overture of the cages. The eggs were collected and seeded onto the artificial diet and incubated in a growth chamber settled to 25.0 ± 2 °C and RH 60 ± 10% and 12/12 h photoperiod. The artificial diet was prepared with (percentage weight/volume) distilled water (60.0%), sugarcane bagasse (13.4%), sugar (8.4%), soy flour (8.4%), beer yeast (8.4%), added with sodium benzoate (0.3%), and citric acid (2.0%). Larvae in the final of the 3rd instar (pre-pupae) were transferred to flasks containing vermiculite and disposed into methacrylate cages (30 × 30 × 30 cm) covered with organza and maintained in a growth chamber settled to the same conditions above.

2.2. Tolerance to high temperature in culture media

Effect of temperature on conidial germination was tested inoculating 100 μL of a suspension containing 10^6 conidia ml^{-1} of each strain in Petri dishes containing Sabouraud-dextrose agar plus yeast extract 1.0% w/v (SDAY). After plated, they were incubated in different incubators settled for constant temperatures (CT): 20, 25, 30, 35, 38, and 40 °C. Conidia germination was evaluated 16 h later in a microscope using

Table 1
Partial characterization of the EF strains of *M. anisopliae* s. l. and *B. bassiana* s. l. applied in the experiments.

EF Strains	Origin (Habita; isolation method; location)	Virulence to *C. capitata*
M. anisopliae LCB56	Soil; insect bait; Petrolina, PE	(Gava et al., 2019)
B. bassiana LCB62	Soil; culture medium; Juazeiro, BA	(Gava et al., 2019)
M. anisopliae LCB63	Soil; insect bait; Juazeiro, BA	Non-published
B. bassiana LCB81	*Spodoptera* sp. (Lep.); Petrolina, PE**	(Gava et al., 2019)
B. bassiana LCB289	Soil; culture medium; Petrolina, PE	(Leal et al., 2021)
M. anisopliae LCB312	Soil; culture medium; Petrolina, PE	(Gava et al., 2019)

* Insect bait technique using *Diatraea sacharalis* larvae in soil samples under natural vegetation of different locations in Brazil. ** Natural enzooty in maize production area in the experimental farm of Embrapa Semiárido.

400x magnification, counting at least 300 conidia in three different fields of each dish. Conidia were considered fully germinated when the germination tube reached double its diameter.

The effect of temperature on mycelial growth was tested by transferring discs (5 mm) obtained from the border of actively growing colonies of the EF strains (Davidson et al., 2003). The mycelial discs were deposited in plates containing SDA medium and incubated in the same conditions described above. Colony growth was measured using cardinal diameters previously drawn in the bottom of the Petri dishes every-two days until the colonies of one of the strains incubated at 25 °C reached the border of the plate (Ouedraogo et al., 1997). The experiment was conducted in triplicate and repeated twice using independent colonies for mycelial disc extraction.

2.2.1. Germination and growth rate in a simulated intraday temperature variation - SIV

In the second group of experiments, conidia and mycelium disks were applied to the SDA medium as described above. They were incubated in a growth chamber settled to simulate intraday air temperature variation between 20 and 38 °C, intraday thermal amplitude commonly detected during the summer in the tropics (Suplementary Fig. 1). Temperature and RH inside the incubator were monitored using a thermocouple coupled to a datalogger (Spectrum Tech., Aurora – USA). Conidia germination and mycelial growth were measured as described above. The experiments were conducted in triplicate and repeated twice using independent colonies for conidia and mycelial discs extraction.

2.3. Applying EF strains as toxic baits in controlled temperature

Water dispersible oil formulations were used in *in vivo* experiments. The formulations were prepared by adding pure conidia o each strain to commercial dispersible oil (Agrex'oil, Microquimica Tradecorp Ltda, Campinas - Brazil), achieving 10^9 conidia mL^{-1}. Formulation of each EF strain was mixed with a food lure solution (3% v/v) (Biofruit, Biocontrole Ltda. – Indaiatuba, Brazil) at a concentration of 10^7 conidia mL^{-1} and sprayed until runoff in the surface of mango leaves using a bench atomizer. After the runoff of the excess, the mango leaves were deposited inside transparent cages (20 × 20 × 20 cm). The control treatment received only the food lure solution plus water dispersible oil 1% v/v. Twenty pairs of 2 days old adults of *C. capitata* were transferred to each cage and kept in natural light for 16 h, aiming to obtain maximal feed activity usually occurring in the morning. After this period, the insects were transferred to laboratory cages previously disinfected and incubated in growth chambers settled for constant temperatures 20, 25, 30, 35, 38, and 40 °C. One control group without EF treatment was used in each temperature, and natural death was applied to correct mortality data.

The insects received water and diet *ad libitum* and were monitored for ten days. As a standard procedure, all insects that died in the first 24 h were discarded. Insect cadavers recovered from the cages were separated by sex and surface sterilized by rinsing in ethanol 70% for 30 s, then 30 s in a solution of sodium hypochlorite 1.0% (v/v), followed by three washes in autoclaved distilled water (ADW). The cadavers were maintained in Petri dishes with sterile filter paper moistened with ADW. The experiment was conducted in 6 repetitions using three different groups of insects divided into two cages per treatment each time.

2.4. Applying EF strains as toxic baits in SIV conditions

Healthy *C. capitata* adults were exposed to a mixture of a food lure and 1.0% v/v oil formulation of each strain's conidia (107 conidia mL-1). Control treatment also received only food lure plus oil 1% v/v. Application procedure and insect management were similar to those described in item 3. The cages were incubated in a growth chamber with temperature control settled to simulate an average summer day in the Brazilian tropical semi-arid region (20–38 °C). A reference treatment was maintained in a second growth chamber at 25 °C. In the SIV regimen, the growth chambers were settled to achieve temperatures from 20 to 38 °C, with 4 h of temperature at 35 °C per day and 2 h at 38 °C. Relative air humidity (RH) was 30% (day) to > 80% (night) (Supplemenatary Fig. 1). Procedures during the experiment and *post-mortem* processing were conducted as described above. The experiments were conducted in triplicate and repeated twice using different cohorts of insects each time.

2.5. Insect mortality in different seasons

Two experiments evaluated the influence of seasonal climate variation over the control efficiency of the selected EF strains *Bb*LCB62, *Ma*LCB63, and *Bb*LCB289 in field cages. The first experiment was conducted in September 2018, a period that the regional climate is characterized by high temperature and low RH (Supplementary Fig. 2). The second experiment was conducted in June 2019, a seasonal period with mild temperatures in the region (Supplementary Fig. 3). Climate variables were monitored using an automatic meteorological station (Campbell Sci., Logan – UT, USA) located at 100 m of the mango orchard.

Water dispersible oil formulations containing 10^9 conidia ml^{-1} were mixed in 3% w/v food lure solution, obtaining a tank mix with 10^7 conidia ml^{-1}. The suspension was sprayed in the leaves and branches of young mango plants (1.5 m height) disposed inside field cages (2.0 × 2.0 × 2.0 m) built with voile tissue. Mango plants were disposed 5 m from each other, and the field-cage borders were distant by three meters in the same treatment. At least one plant without treatment was maintained between cages for different treatments.

Fifteen pairs of 4 days old insects were transferred to each field-cage 30 min after spraying the preparations. The insects remained overnight into the field cages and were individually recovered using glass tubes and finally transferred to cages (0.5 × 0.5 × 1.0 m) covered with voile tissue. The cages remained suspended under mango trees canopy by wires covered by entomological glue. Water and artificial diet were provided *ad libitum*. This procedure was adopted to minimize the effect of predators and scavengers on sick insects and cadavers. Inside the plant canopy, the insects would be at least partially protected from UV radiation and tInsect death was monitored daily, and mortality data were registered. The experiment was conducted in a randomized design, using two different insect cohorts and four replications (two replications for each insect group).

2.6. Data processing and statistical analyses

2.6.1. Conidial germination and mycelial growth

Germination data did not fit to a normal distribution and were transformed using the equation $X'_{ij} = arcsen\left(\frac{X_{ij}}{100}\right)$, where X_{ij} and X'_{ij} represent the original and transformed data, respectively. Data of the mycelial growth rate were square-root transformed. The effect of temperature regimen on mycelial growth and conidia germination of EF strains and its interaction was analyzed using analysis of variance with Statistica for Window v.12 (StatSoft Inc.), followed by Tuckey's multiple comparison test ($p < 0.05$). A *t*-test ($p < 0.05$) procedure was used to evaluate the differences between temperature regimens and reference temperature for each EF strain.

The colony growth rate (GR) was obtained from the colony growth data by the equation: $GR = \frac{\sum_i^j \left(\frac{t_{i+1j} - t_i}{n}\right)}{N}$, where t_i is the colony diameter at different times along the experiment; n is the time between evaluations; N is the duration of the experiment. Data were submitted to analysis of variance and LSD test ($p < 0.05$) using Statistica for Windows v. 12 (StatSoft Inc.).

2.6.2. Insect mortality

Insect mortality data were submitted to natural death correction using the formulae of Schneider-Orelli's (Püntener, 1992). Proportional mortality data obtained at the end of the experiments did not showed normal distribution accorind to the Smirnoof-Kolmogor and Lilieford testsand were arcsine transformed and submitted to analysis of variance (ANOVA) and Tukey's multiple comparison test ($p < 0.05$) using Statistica for Windows V. 12 (StatSoft Inc.).

Mortality data registered during the experimental period were submitted to the Kaplan-Meier limit product procedure., Insect death events were coded 1, while 0 was applied to censored data (missing insects, undefined death cause) and to insects still alive at the end of the experiment. Mortality curves were compared to the control and each other using the Mantel-Cox (M-C) Chi-square (χ^2) test when indicated. Hazard ratio (HR) was estimated using the curve for the control as reference treatment, as also the average survival time (ST_{50}) was calculated for each treatment..

3. Results

3.1. Effect of temperature on germination and growth of fungal strains

3.1.1. Effect of constant temperature on EF conidial germination and mycelial growth

Conidial germination results under CT are shown as a proportion of germination obtained at 25 °C for each strain. However, data from 25 °C lacked variance and did not allow ANOVA or comparing results between temperatures. Average conidial germination for all strains was 80% of that observed at 25 °C (Fig. 1A) when incubated at 20 °C. There was no significant difference in conidial germination between EF strains until 30 °C. Temperature increase to 30 °C significantly reduced conidial germination for MaLCB312, while MaLCB63 and BbLCB289 slightly increased. The last strains showed the highest tolerance to 35 °C,swith conidial germination significantly higher than the others (Tukey's test; $p < 0.05$). Except for *M. anisopliae* *Ma*LCB312, most strains showed optimum germination at 30 °C (Fig. 2A). Meanwhile, conidia germination was negligible for all strains at 38 °C, and there was no germination at 40 °C (Fig. 1A).

Mycelial growth occurred at all temperatures except at 40 °C (Fig. 1B), and the colony growth rate (GR) was also negligible at 38 °C. There was a slight increase in GR between 20 and 30 °C for most strains, except for *Ma*LCB312, which was significantly lower than the others at 30 °C (LSD test; $p > 0.05$). There was a substantial reduction of GR at 35 °C for all EF for all strains; however, *Bb*LCB81 and *Bb*LCB289 showed significantly larger GR at this temperature by the Tukey's test ($p > 0.05$).

3.1.2. Effect of SIV on conidial germination and mycelial growth

The temperature inside the growth chamber during the experiments ranged from 20 to 38 °C (±2 °C) and relative air humidity (RH) between 50 and 90% (Supplementary Fig. 1). The average number of hours above 35 °C was 6.0 ± 0.5 h day^{-1}, with 2 ± 0.25 h day^{-1} at 38 °C. In this study, two different plates obtained from the inoculation of the same conidial suspensions under SIV treatment were evaluated at 16 (only 4 h after concluding simulated day temperature) and at 24 h (reaching a complete cycle of simulated day/night temperature). Germination count at 25 °C was performed only at 16 h because of excessive growth at 24 h.

There was a significant interaction between EF strains and temperature regimen on conidial germination ($F_{5;36} = 4.30$; $p < 0.05$). There was no significant difference among strains for conidial germination and mycelial GR at 25 °C. Nevertheless, all isolates showed lower germination at SIV than at 25 °C in the first evaluation (16 h) (Table 1). BbLCB62, BbLCB81, and BbLCB289 showed results similar to that obtained at 25 °C in counts done at 24 h.

Mycelial GR was less affected by SIV. However, there was a significant interaction between temperature regimen and strains for mycelial GR (F5;36 = 3.61; $p < 0.05$). While there was no significant difference among EF strains at a constant 25 °C (Table 1), MaLCB63 and BbLCB81 showed GR significantly lower at SIV.

3.2. Applying EF strains as toxic baits in controlled temperature

3.2.1. Effect of constant temperature on fungal virulence

Mortality in the control treatment at each temperature was generally lower than 10% until 30 °C, while at 35 °C it increased to 22.4%, and to 37% at 38 °C. All insects died in 3 days in all treatments at 40 °C, and data were discarded from further analysis. In this work, confirmed mortality occurred when insects showed signals of fungal exteriorization and conidiogenesis, while total mortality was the total number of death insects after natural mortality correction. GLM Anova showed that the

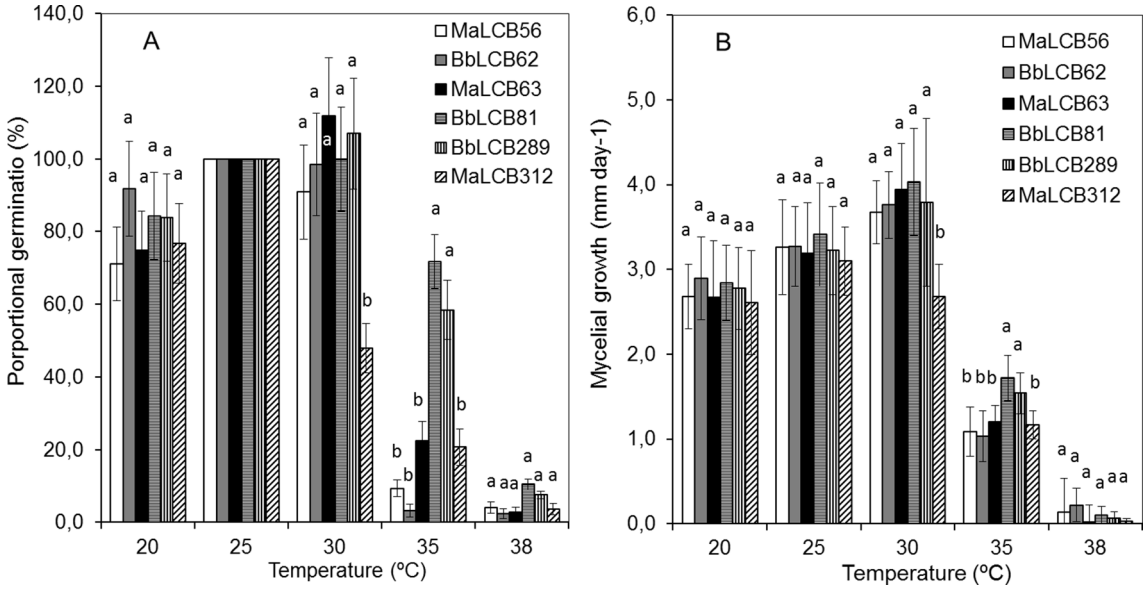

Fig. 1. Proportional germination of conidia (A) and mycelial development rate (mm day^{-1}) (B) of EF strains in SDA medium incubated in constant temperature. Columns showing similar letters are statistically similar according to the LSD test ($p < 0.05$). Data for germination at 25 °C were excluded from analysis since they did not have variance. Proportional germination was established as the percent germination relative to T = 25 °C.

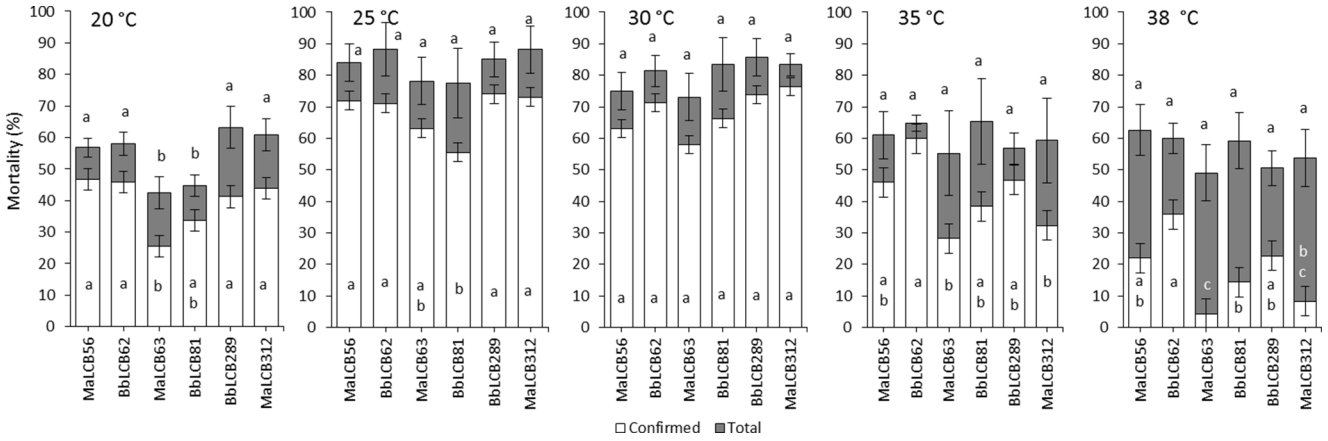

Fig. 2. Total and confirmed mortality of *C. capitata* (mean ± SE) treated with *M. anisopliae* and *B. bassiana* strains and incubated in constant temperature in chambers settled 20 to 38 °C. Total mortality was corrected for natural mortality using the formulae of Schneider-Orelli's using a control treatment for each temperature. Letters in the columns compare the total and confirmed mortality, columns with the same letter do not differ by the Tukey's test ($p < 0.05$). Results are shown as untransformed averages of corrected total and confirmed mortality caused by EF strains.

effect of the temperature was dependent of the EF strain ($F_{15;80} = 5.6$; $p < 0.01$). There was a significant effect of fungal strains over insect mortality at 20 °C both in confirmed ($F_{5;80} = 4{,}18$; $p < 0.01$) and total mortality ($F_{5;80} = 3{,}97$; $p < 0.05$) of *C. capitata*. There was no significant difference among EF strains in total mortality of *C. capitata* in almost all CT treatments, but *Ma*LCB63 and *Bb*LCB81 showed singnificantly lower mortality at 20 °C ($p < 0.05$) (Fig. 2). On the other side, a significant effect of strains over confirmed mortality of *C. capitata* was observed in all CT treatments ($p < 0.05$), except at 30 °C ($F_{5;80} = 2.21$; $p > 0.05$). Besides, differences among total and confirmed mortality increased strongly at higher temperatures, culminating at 38 °C when mortality was larger than 40% (Fig. 2).

There was an average reduction of 28.7% in confirmed mortality at 20 °C and 26.2% at 35 °C when compared to 25 °C. *BB*LCB81 showed the lowest confirmed mortality results among EF at 25 °C ($p < 0.05$), while *Ma*LCB63 and *Bb*LCB289 showed the highest reduction of confirmed mortality at 35 °C compared to 25 °C (Fig. 2). *Bb*LCB62 had the lowest reduction of confirmed mortality between the same temperatures, only 11.3%. There was no significant difference among EF strains on total mortality at 38 °C. However, confirmed mortality by the colonization of insect cadavers was significantly higher for *Bb*LCB62 by Tukey's test ($p < 0.05$), followed by *Ma*LCB56 and *Bb*LCB289 (Fig. 2).

3.2.2. *Fungal virulence in a simulated intraday temperature variation*

There was no significant effect of the interaction between EF strains and insect sex in mortality of *C. capitata* ($F_{1;55} = 1.172$; $P > 0.05$), and the data were pooled to analyze general mortality. There was a significant effect of strains over the confirmed mortality of the insects in the

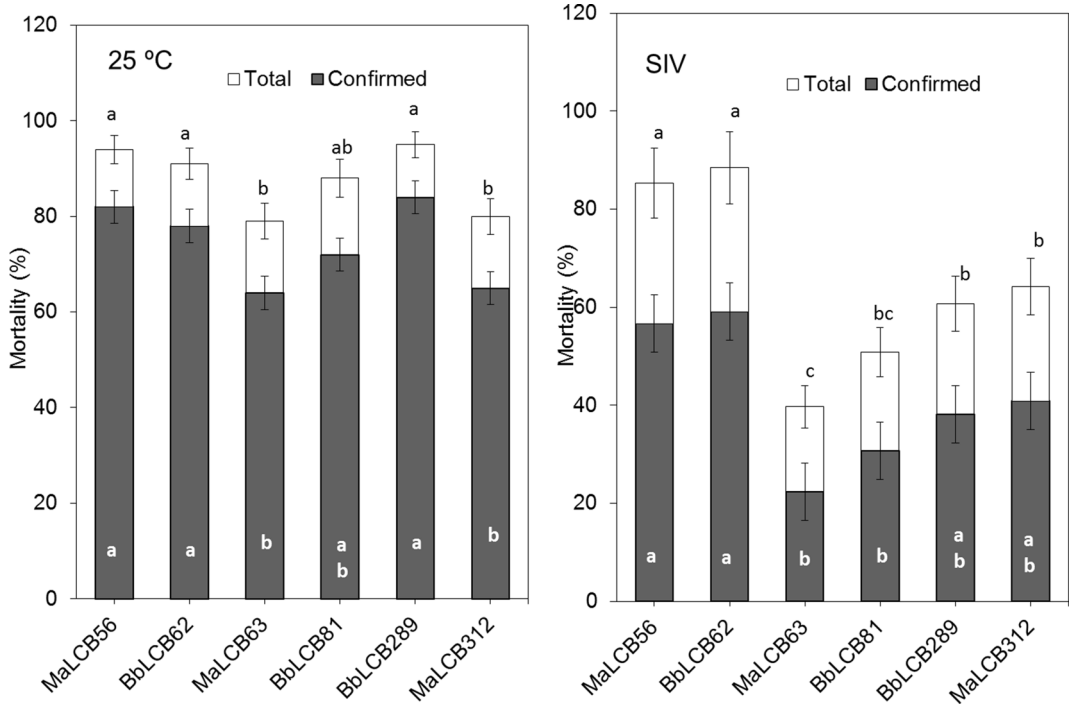

Fig. 3. Mortality of *C. capitata* (mean ± SE) treated with *M. anisopliae s.l.* and *B. bassiana* strains and maintained in incubation chambers settled to intra-day temperature fluctuations commonly found in tropical semi-arid regions (20 – 38 °C). Total mortality was corrected for natural death using the formulae of Schneider-Orelli's. Letters in black compare the total corrected mortality while letters in white compare the confirmed mortality; columns with the same letter do not differ by Tukey's test ($p < 0.05$).

two regimens of temperature (CT = 25 °C; SIV = 20–38 °C) after 10 days of the application of conidial suspensions ($F_{5;55} = 3.876$; $p < 0.05$). Fig. 3 shows that total mortality was higher than 80% to all strains in 25 °C, and *Ma*LCB56, *Bb*LCB62, and *Bb*LCB289 showed confirmed insect mortality significantly higher than the others according to the Tukey test ($p < 0.05$).

There was a significant effect of the temperature regimen to which the EF strains were submitted on insect mortality ($F_{5;55} = 4.26$; $p < 0.05$). *Ma*LCB63 and *Bb*LCB81 showed significantly lower confirmed mortality, according to Tukey's test ($p < 0.05$) (Fig. 3). On the other side, insects treated with *Ma*LCB56 and *Bb*LCB62 showed total and confirmed mortality statistically similar to each other and significantly higher than the other strains under SIV, while confirmed mortality was statistically similar to *Bb*LCB289 and *Ma*LCB312 (Tukey's test; $p < 0.05$). Meanwhile, the mortality of insects treated with *Ma*LCB63 was highly influenced by SIT, showing very low mortality when compared to CT. According to the M-C test in the Kaplan-Meier procedure in both experiments, SIV significantly affected EF strains virulence, increasing the TL_{50} obtained from the mortality curves (Table 3).

3.2.3. Insect mortality in different seasons in field cages

During the experiment conducted in September 2018 average T was 26.97 °C (18.04 – 36.27 °C), and the average RH was 53.21 (26.35 – 83.30%). There were 29 days with temperatures between 30 and 35 °C during 7.1 h per day in the period, and 4 days with 5.38 h of T higher than 35 °C (Table 4). September was also a dry period, showing only 10.39 h of RH higher than 60% per day, mainly at night (Supplementary Fig. 2). The average daily temperature increase was 1.28 °C h^{-1} on average (06:00–15:00), while temperature decrease was 0.91 °C h^{-1} (15:00–00:00) (Supplementary Fig. 2).

In June 2019, the average T was 28.31 °C (21.17 – 36.38 °C), and the average RH was 51.09 (24.08 – 80.6). The period presented 15.18 h with temperatures lower than 25 °C per day (Table 4). There were also 27 days with 8.41 h with RH higher than 80%, on average. Average T in the 10 days of the first repetition of the experiment was 24.46 °C (18.89 – 31.70), and average RH was 75.18% (34.55–96.90%), and in the second experiment average T was 25.10 °C (18.5–32.26) and RH 68.49 (33.62–95.20). Daily average increase of temperature was 0.98 °C h^{-1} (06:00–15:00), while the temperature decrease was 0.74 °C h^{-1} (15:00–00:00) (Supplementary Fig. 2).

Insect mortality caused by the EF strains was significantly affected by season ($F_{1;21} = 5.24$; $p < 0.05$). The maximum average corrected mortality of adults *C. capitata* infected in food bait in September was achieved by *Bb*LCB62 (74.8%), which was significantly higher than *Ma*LCB63 and statistically similar to *Bb*LCB289, according to the Tukey's test ($p < 0.05$) (Fig. 4). However, in the experiments conducted in June, all EF strains showed mortality statistically similar to each other by Tukey's test ($p > 0.05$).

The differences in virulence among strains in different seasons were confirmed by analyzing the mortality curve using Kaplan-Meier's procedure for corrected insect mortality (Fig. 4). Beneath predominantly higher temperatures and low humidity in September/2018, all mortality curves of EF were significantly different from the control (Table 5). *Bb*LCB62 was the most virulent strain, with mortality curves significantly different from *Ma*LCB63 and *Bb*LCB289 ($p < 0.05$; M-C $\chi 2$ test), resulting in a higher value of LT_{50}. Mortality risk (given by the hazard risk) for insects treated with *Bb*LCB62 and *Bb*LCB289 was 7.67 and 3.89 times higher than the mortality in the control respectively. *Ma*LCB63 showed 34.0% mortality at the end of the experiment, with a mortality risk of 3.31 (Table 5).

In June 2019, with predominant milder temperatures and higher RH, there was a highly significant difference between mortality curves of all EF and the control treatment ($p < 0.001$; M-C $\chi 2$ test) (Table 2). There was also an increase in insect death ratio, resulting in a lower TL_{50} compared to September. Only the mortality curves of *Bb*LCB62 and

Table 2
Conidia germination and mycelial growth rate of EF strains in SDA medium incubated at 25 °C and in incubation chambers settled to intra-day temperatures fluctuations (SIV) commonly found in tropical semi-arid regions (20 – 35 °C).

EF Strains	Germination (%)			Growth rate (mm day^{-1})	
	25 °C	SIV		25 °C	SIV
	16	16 h	24 h		
M. anisopliae LCB56	96.07 aA	34.69 bcB	71.12 bB	3.26 aA	3.07 aA
B. bassiana LCB62	97.37 aA	36.36 bcB	91.74 aA	3.27 aA	3.16 aA
M. anisopliae LCB63	89.47 aA	16.51 cB	74.911 bB	3.19 aA	2.94 aB
B. bassiana LCB81	97.50 aA	51.35 aB	84.26 abA	3.41 aA	3.03 aB
B. bassiana LCB289	92.40 aA	45.10 abB	83.87 abA	3.10 aA	2.89 aA
M. anisopliae LCB312	89.67 aA	48.62 abB	76.65 abB	3.21 aA	3.18 aA
Average	93.75 A	38.77B	80.43B	3.24 A	3.05 A

Small letters compare differences among EF strains, and numbers followed by the same letter do not differ by Tukey's test ($p < 0.05$). Capital letters compare the effect of temperature regimen on each EF strain by Student *t*-test ($p < 0.05$).

Table 3
Survival curve analysis according to Kaplan-Meier procedure in total mortality, curve comparison using Mantel-Cox test, and lethal time estimation for *C. capitata* (mean ± SE) exposed to mango leaves previously treated with toxic baits containing conidial preparations of *B. bassiana s.l.* and *M. anisopliae s.l.* strains and incubated at constant 25 °C (CT 25) and at simulated intraday temperature variation 20–38 °C (SIV).

	CT 25	SIV
Mantel-Cox test (Chi square)	8.52	10.37
df	5	5
P value	0.0036	0.0013
Average survival (ST_{50} - days)		
*M. anisopliae Ma*LCB56	4.2	6.0
*B. bassiana Bb*LCB62	4.0	6.0
*M. anisopliae Ma*LCB63	8.0	Undefined
*B. bassiana Bb*LCB81	6.3	10.0
*B. bassiana Bb*LCB289	4.4	7.0
*M. anisopliae Mf a*LCB312	6.5	8.5

Table 4
Incidence (number of days) and prevalence (average number of hours per day) of temperature and relative humidity specific intervals during field cage experiments using oil formulations of EF strains in warmer (September 2018) and mild (June 2019) seasons.

Climate variable	September 2018		June 2019	
	Incidence[1]	Prevalence[2]	Incidence	Prevalence
	Air temperature (°C)			
T < 20 °C	3.0	3.67	6.0	3.08
T 20–25 °C	30.0	7.55	30.0	12.10
T 25–30 °C	30.0	8.55	29.0	8.60
T 30–35 °C	29.0	7.10	12.0	7.54
T > 35 °C	4.0	5.38	0	0.00
	Day	Night	Day	Night
Taverage	32.88	23.25	29.71	21.82
Tmax	34.14	25.92	31.02	23.22
Tmin	30.7	21.32	27.34	20.56
	Relative humidity (%)			
RH %>80	4.0	2.12	27.0	8.41
RH 60–80%	30.0	8.27	30.0	8.00
RH 40–60%	30.0	9.00	24.0	8.85
RH < 40%	24.0	8.12	6.0	7.00
	Day	Night	Day	Night
RHaverage	35.41	68.74	49.58	84.59
RHmax	41.62	76.39	58.88	89.92
RHmin	31.78	56.65	43.64	78.33

[1] Number of days with temperature and RH in a given interval; [2] Average number of hours during the incidence period.

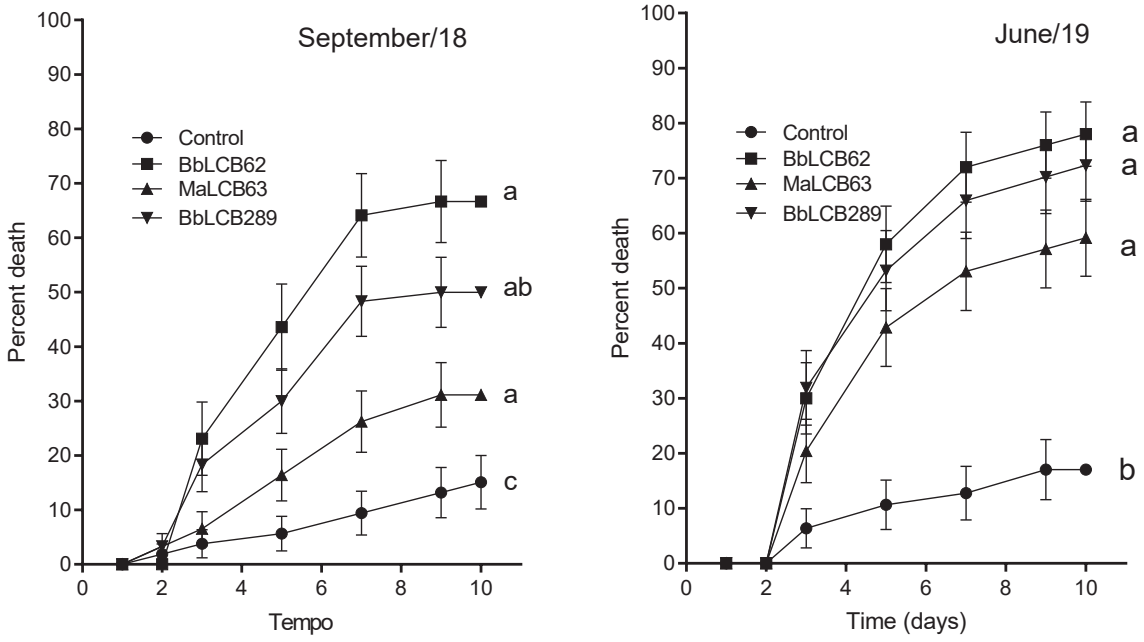

Fig. 4. Mortality curve of *C. capitata* adults (N = 120 insects per treatment) in field cage experiments conducted during summer (September 2018) and winter (June 2019). Letters in the graphics compare accumulated insect mortality between treatments at the end of the experiment. Treatments with the same letter did not differ by the Tukey test ($p < 0.05$).

Table 5
Analysis of the mortality curve of *C. capitata* in a field cage experiment conducted during summer 2018 (S) and winter 2019 (W). Mortality data were submitted to the Kaplan-Meier procedure, estimating the median survival of the insects. The curves were compared using the M-C [2] test ($p < 0.05$).

Comparison of Survival Curves	*χ^2	Degrees of freedom	P	†ST_{50}	¥HR
	September 2018				
Total	32.61	3	<0.0001		
*Bb*LCB62 × Control	28.52	1	<0.0001	7.0	7.67
*Ma*LCB63 × Control	4.31	1	0.038	ND	3.31
*Bb*LCB289 × Control	15.91	1	<0.0001	9.5	3.89
*Bb*LCB62 × *Ma*LCB63	14.06	1	<0.0001		3.62
*Bb*LCB62 × *Bb*LCB289	2.51	1	0.11		1.36
*Bb*LCB289 × *Ma*LCB63	5.06	1	0.024		1.99
	June 2019				
Total	39.21	3	<0.0001		
*Bb*LCB62 × Control	36.89	1	<0.0001	5.0	7.08
*Ma*LCB63 × Control	16.51	1	<0.0001	7.0	4.34
*Bb*LCB289 × Control	30.10	1	<0.0001	5.0	6.33
*Bb*LCB62 × *Ma*LCB63	4.186	1	0.041		1.40
*Bb*LCB62 × LCB289	0.250	1	0.617		1.08
*Bb*LCB289 × *Ma*LCB63	2.283	1	0.131		1.41

*Mantel-Cox Chi-square test; † Average survival time for 50% insects; ¥ Hazard ratio.

*Ma*LCB63 differed according to the M-C test ($p = 0.041$). The hazard ratio estimated by Kaplan-Meier's procedure was also higher than that obtained in September for all strains.

4. Discussion

All fungal strains used in this study were virulent to *C. capitata* when applied mixed with a food lure in laboratory conditions. Such results were expected since they were previously selected for their virulence to fruit fly adults (unpublished data). However, using EF as toxic bait for biological control of adult fruit flies in tropical regions requires an efficient BCA strain to survive and infect their target hosts in restrictive climate conditions. Some works showed that formulation could increase conidial tolerance to low humidity, high temperature, and UV (Barreto et al., 2016; Paixão et al., 2017). In most cases, such measures can only improve the efficiency of strains showing genetic resistance to abiotic stress (Burges, 1998).

4.1. Effect of temperature on EF strains germination and growth

Conidial germination and mycelial GR of the EF strains at different CT in this study are within the range of previous works using tropical strains (Ekesi et al., 1999; Ouedraogo et al., 1997; Rangel et al., 2005). In our study, CT higher than 30 °C reduced conidial germination and mycelial GR, but the results also showed significant variability among EF strains in the experimental conditions. These results corroborate those obtained by Mwamburi et al. (2015), who showed that constant temperatures between 30 and 35 °C killed the conidia or delayed their germination, even after transferring to 21 °C. The strain *Ma*LCB312 was highly sensitive to incubation at 30 °C, while *Bb*LCB81 and *Bb*LCB289 were the most tolerant in CT assays. The tolerance of these strains to temperature was higher than that reported by Dimbi et al. (2004) for *M. anisopliae* isolates. However, higher tolerance to temperature in EF strains from tropical and equatorial areas has already been shown (Rangel et al., 2005; Teja and Rahman, 2016).

In this study, most strains showed mycelial GR 12 to 23% higher at 30 °C than that in reference temperature (25 °C), except for MaLCB312. However, only BbLCB81 showed GR higher than 50% at 35 °C. Although a relationship between *in vitro* thermotolerance and control efficiency is still uncertain (Ouedraogo et al., 1997), many studies have shown a large variability among EF strains. On the other side, Fernandes et al. (2008) and Rangel et al. (2005) showed that conidial tolerance of EF strains originating from higher latitudes to temperature and UV radiation was significantly lower than those from tropical regions. Likewise, Ouedraogo et al. (1997) showed that, though most *Metarhizium* strains have had greater mycelial GR at 25 °C, three *M. anisopliae* strains were tolerant to 35 °C. While Fernández-Bravo et al. (2016) also showed that *B. bassiana* originally isolated from temperate regions mostly showed optimal incubation temperatures around 25 °C, with suboptimal growth at temperatures higher or lower than 25 °C, and none of the fungal isolates grew at 35 °C. However, the authors did not find an apparent relationship between thermal response between fungal genotype or leaf

and soil inhabitant EFs. Similarly, this work also did not find a relationship between habitat and thermotolerance since the EF strains BbLCB81, isolated from a lepidopteran host, and BbLCB289, a soil inhabitant, were highly tolerant to 35 °C. Besides, *M. anisopliae* LCB63, also a soil inhabitant, showed higher conidial germination and mycelial GR at 30 °C.

An assay was designed to evaluate the effect of fluctuating temperature regimen (ranging from 20 to 38 °C) on conidial germination and mycelia growth. A temperature of 38 °C was applied in the SIV treatment despite being highly detrimental to the EF strains in CT assays. Although SIV caused both a reduction of germination and GR and a delay in obtaining maximal results, the effect was lower than that in CT at 35 °C. These results corroborate those obtained by Keyser et al. (2014) and Mwamburi et al. (2015), who observed a "post-stress growth delay" effect of high temperature on *B. bassiana* strains. However, in both studies, the conidia were transferred to lower temperatures after exposure to high temperatures. A significant difference in germination between 16 and 24 h in SIV occurred independently of the EF strain, confirming the need for recovery from physiological damages caused by temperature before germination (Barreto et al., 2016).

The lack of significant differences among EF strains concerning mycelial growth in SIV showed that the effect of temperature was similar to all of them. However, GR of *Ma*LCB63 and *Bb*LCB81 differed from that reached at 25 °C, while the others did not. There are different possible effects of temperature on EF physiology, and perhaps the delay was caused by DNA repair, membrane recovery, and denaturation and synthesis *de novo* of structural proteins and enzymes (Lovett and St. Leger, 2015; Ma et al., 2015). Therefore, this result indicates that the thermal threshold for conidial germination and mycelial growth is a combination of temperature and time, specific for each EF strain (de Oliveira et al., 2018; Rangel et al., 2005). Together, the results in SIV showed that conidia germination was the more sensitive phase for a successful infection affected by temperature. Once it occurs, there is a lower effect of daily periods of high temperature over mycelial growth, or else it can be compensated by periods with adequate temperature in the night. It also increased the expectation about the effectiveness of selecting moderately thermotolerant but virulent strains to control *C. capitata*.

4.2. Effect of temperature on fungal virulence and control efficiency

Mortality of *C. capitata* adults was highly affected by EF strains in the CT experiment. The highest mortality was observed at 25 and 30 °C, showing a strong association with conidial germination and GR. A previous study also showed that CT higher than 30 °C significantly reduced mortality caused by *M. anisopliae* on *Ephestia khueniella* (Athanassiou et al., 2017). However, similar results were obtained by Alali et al. (2019), studying the variability of EF strains temperature tolerance and virulence against *Ephestia kuehiniella* among strains of *B. bassiana* originating from warm regions of Siria. In this study, the confirmed mortality of *C. capitata* adults at 35 and 38 °C was higher than expected than those obtained in the CT experiment on GR and conidial germination. Likely, EF conidia adhesion to microsites such as insects' spiracles, intersegmental folds, and mouthparts resulted in more significant infection once they can take advantage of humidity and body thermoregulation (Jaronski, 2010). A higher infection rate in mouthparts was expected, given EF's application as toxic baits with food attractants in fruit fly control.

Constant high temperatures affected insect survival, as shown by the difference of 40% between total and confirmed mortality at 38 °C and that no insect survived more than 3 days at 40 °C. Nyamukondiwa and Terblanche (2009) showed that the critical thermal maxima for *C. catpita* was around 42 °C. Likely long-term exposure to 40 °C temperature overwhelmed the thermoregulation mechanism of *C. capitata*. It also occurred at 38 and 35 °C, resulting in high insect mortality but low exteriorization and conidiogenesis. In fact, thermoregulation for tiny poikilotherms, such as fruit flies, is challenging, relying on behavior alteration and some metabolic and molecular responses (Kalosaka et al., 2009; Wojda, 2017). However, behavioral thermoregulation was limited to a reduced flying activity in CT experiments, which probably was inefficient and resulted in insect mortality. Constant temperature experiments allowed us to verify that *Bb*LCB62 was highly thermotolerant, while *Ma*LCB56 and *Bb*LCB289 were averagely virulent at higher temperatures; *Ma*LCB63, *Bb*LCB81, and *Ma*LCB312 were highly affected by high temperature.

All experiments in SIV started at a set point of 20 °C, from which it increased at a rate of 1.14 °C h^{-1} until 38 °C (06:00–13:00), and at a decrease rate of 0.87 °C h^{-1} (15:00–23:00). The temperature remained constant at 38 °C between 13 and 15:00, returning to 20 °C between 23 and 06:00. This ramping rate is relatively slow compared to previous work (Keyser et al., 2014; Nyamukondiwa and Terblanche, 2009; de Oliveira et al., 2018). However, it is ecologically relevant, representing an average summer day in the tropical semi-arid conditions of Brazil, and allows comparison to the field experiments.

Compared to treatment with CT 25 °C, SIV reduced insect mortality and increased TL_{50} of all strains. A recent work of Ghazanfar et al. (2020) also showed that fluctuating temperatures (15–35 °C) significantly reduced the virulence of *B. bassiana* ATCC74040 and even the toxicity of Bt toxins to *Heliothis virescens* and *Spodoptera littoralis*. In another study, fluctuating temperatures between 18 and 34 °C reduced the mortality of *Tryathoma infestans* treated with *B. bassiana* Bb10 (Lecuona et al., 2005). However, in this study, most strains showed confirmed mortality higher than that observed at 35 and 38 °C in the CT experiment. This result is highly related to the lower detrimental effect of SIV on EF germination and growth. SIV experiments detected three strain groups: a group highly thermotolerant with *Ma*LCB56 and *Bb*LCB62; an intermediate group formed by *Bb*LCB289 and *Ma*LCB312. The third group with *Ma*LCB63 and *Ma*LCB312 was susceptible to even a short exposure to high temperatures.

There was no difference in confirmed mortality among EF strains in the field cage experiments in June 2019, a period with a milder climate in the Brazilian semi-arid region (Supplement Fig. 2), while *Ma*LCB63 showed lower mortality in September. However, there was a significant difference among mortality curves of EF strains in both periods. The intermediary thermotolerant strain *Bb*LCB289 showed accumulated mortality statistically similar to the most tolerant *Bb*LCB62, confirming the results obtained in SIV experiments. Many studies show that higher temperatures can significantly influence host-pathogen interactions (Vega et al., 2009). Such responses may be caused by the low thermotolerance of the EF strains and immunological response of the insects (Sangbaramou et al., 2018). In fact, some insects search for increasing body temperature after infection by exposition to a heat source, known as "behavioral fever" (Inglis et al., 1996; Ouedraogo et al., 2004). In short, heat enhances host immune response, increases hemocyte counting, eliciting the expression of heat-shock proteins, phenoloxidase, and lysozyme activity (Mastore et al., 2019; Wojda, 2017). Added to the direct effect of heat on the EF, it helps to explain the results obtained in this work.

Although temperature commonly reaches over 40 °C during summer in the tropics, *C. capitata* population has not been affected by seasonal climate conditions, corroborating its large climate adaptation (Nyamukondiwa et al., 2010; de Oliveira et al., 2019). Soares et al. (2020), showed that its flutuaction has been shown more dependent of fruit host availability than of climate variation. The most effective strategy to reduce temperature damages to EF-based biopesticides in tropical conditions has been timing the spray in the evening or nightfall (Dolinski and Lacey, 2007). However, temperatures above 30 °C and RH lower than 60% commonly occur in the early evening in the tropics (Supplementary Figs. 1 and 2). Plant canopy can reduce the effect of UV caused by direct exposure to sunlight, while irrigation can increase RH under the canopy. However, it has little effect on temperature. In such conditions, the opportunity window offered by overnight periods is limited due to the time required for conidial germination and insect integument

penetration (Faria et al., 2015). It can be even more critical when infections result from the secondary acquisition of inocula from the plant surface when applied in toxic bait, as proposed in this work. Besides, EF strains highly susceptible to temperature will not succeed when used as toxic bait for controlling adults of fruit flies since they are more active during the daytime (Arredondo et al., 2018; Bayoumy and El-Metwally, 2017).

Temperature plasticity in EF strains may help buffer detrimental effects of temperature variation in the field, and it has received considerable attention over recent decades (Alali et al., 2019; Dimbi et al., 2004; Ekesi et al., 1999; Teja and Rahman, 2016). However, most studies evaluated *in vitro* germination and mycelial growth only at constant temperatures, allowing defining critical or threshold temperatures. To our knowledge, only a few evaluated insect death rates in SIV, and no work was performed in field conditions. In this study, there were discordant results between CT and SIV experiments regarding germination and growth of the strains, corroborating the hypothesis that these variables obtained *in vitro* have a poor correlation with field efficiency. While *Bb*LCB81 and *Bb*LCB289 showed promising results in CT experiments, they showed mycelial growth similar to the other strains in SIV experiments. Conidial germination also was less affected by fluctuating temperature, but *Bb*LCB62 and *Bb*LCB289 were the only to show results similar in 25 °C and SIV conditions.

Simulated intraday variation temperature experiments showed that *Ma*LCB56 and *Bb*LCB62 were highly virulent to *C. capitata* adults even in a simulated intraday temperature reaching 38 °C for 2 h. *Bb*LCB289 and *Ma*LCB312 caused slightly lower mortality in the same conditions, and the others showed significantly lower virulence. The field cage experiments confirmed the expectation that the mildly thermotolerant strains could perform in the field, mainly in the environmental conditions represented by irrigated fruticulture. From the results, we could conclude that a selection process considering only germination and mycelial growth at constant temperature could lead to the discharge of potentially virulent strains. Therefore, a more successful selection process could be approached by using 4 to 6 h under high temperatures and periods of mild temperature.

Funding information

This research was funded by Pernambuco State Research Foundation – FACEPE [Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco] Grant No APQ-0012-50.01/15, and Brazilian Agriculture Research Corporation – Embrapa [Empresa Brasileira de Pesquisa Agropecuária] 23.16.00.072.00 and MP2 020.10.06.013.00.

CRediT authorship contribution statement

Carlos Alberto Tuão Gava: Conceptualization, Methodology, Data curation, Software, Writing – original draft. **Clayton Moreira Leal:** Methodology, Data curation, Investigation. **Alicia Vieira de Sá:** Methodology, Data curation, Investigation. **Beatriz Aguiar Jordão Paranhos:** Conceptualization, Methodology, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2022.105062.

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