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# Increased tolerance of *Beauveria bassiana* and *Metarhizium anisopliae* conidia to high temperature provided by oil-based formulations

Daian Guilherme Pinto de Oliveira[a,c,*], Rogerio Biaggioni Lopes[b], Janayne Maria Rezende[c], Italo Delalibera Jr.[c]

[a] Federal University of Technology/UTFPR, Biology Department, 85892000 Santa Helena, PR, Brazil
[b] Embrapa Genetic Resources and Biotechnology, P.O. Box 02372, 70770-917 Brasilia DF, Brazil
[c] ESALQ/University of São Paulo, Department of Entomology and Acarology, P.O. Box 9, 13418–900 Piracicaba, SP, Brazil

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ABSTRACT

The influence of the temperature of aqueous conidial sprays on conidial viability and virulence against *Diatraea saccharalis* was evaluated for pure conidia, rice + fungus (technical concentrates) and oil-based formulations of *Beauveria bassiana* s.s. and *Metarhizium anisopliae* s.s. under laboratory conditions. The fungal preparations were suspended in water and maintained at 26 °C, 36 °C and 46 °C for one, four and six hours. Conidial viability was determined by plating aliquots of each suspension onto PDA medium followed by incubation for 20–22 h and observing for viable conidia (germ tubes longer than diameter of conidia). Fungal virulence was determined by spraying suspensions onto third-instar larvae of *D. saccharalis*. In general, germination and virulence, particularly for unformulated conidia, were negatively affected by increases in water temperature and exposure time in suspension. However, the decrease in conidial viability in the oil-in-water emulsion was less than 7% for both species after 6 h of exposure at 36 °C, in contrast to reductions of 7–21% and 28–60% for the oil-free suspensions of *B. bassiana* and *M. anisopliae*, respectively. For the sprays of conidia in an oil-in-water emulsion previously exposed to elevated water temperatures for longer periods, the levels of insect mortality were higher than those of pure conidia or technical concentrates under identical conditions. Our results indicate that emulsifiable oil-based formulations can protect the conidia of both species of fungi from the adverse effects of high water temperatures before spraying in the field.

## 1. Introduction

Mycopesticides have been traditionally formulated and sold as products to be sprayed on insect pests in many crops (Faria and Wraight, 2007). Indeed, microbial products have received increased attention (Glare et al., 2012), leading to the emergence of companies worldwide. Nevertheless, few entomopathogenic fungi are commercially available in the market or have been used in the field (Faria and Wraight, 2007; Lacey and Goettel, 1995). For the mycoinsecticides registered in Brazil, nearly all are based on the fungi *Beauveria bassiana* s.l. (Bals.) Vuill. (Ascomycota: Cordycipitaceae) and *Metarhizium anisopliae* s.l. (Metsch.) Sorokin (Ascomycota: Clavicipitaceae) (Li et al., 2010; Michereff-Filho et al., 2009). However, products with low quality and inconsistent results in the field are frequently observed.

Unfavorable conditions in the field for fungal infection and survival partly explain the problem of low quality and inconsistent results. Solar radiation, humidity and temperature are the most important abiotic factors affecting the survival and infectivity of entomopathogenic fungi (Bugeme et al., 2008; Inyang et al., 2000). Therefore, many studies have been conducted to determine the effects of these abiotic factors on the biological parameters of the fungi *B. bassiana* and *M. anisopliae* in laboratory and field conditions (Braga et al., 2001; Brooks et al., 2004; Bugeme et al., 2008; Devi et al., 2005; Huang and Feng, 2009; Lazzarini et al., 2006; Moore et al., 1993; Perfetti et al., 2007; Rangel et al., 2004, 2005, 2008a, 2008b; Sosa-Gómez and Alves, 2000; Thompson et al., 2006; Yeo et al., 2003).

Appropriate formulations can improve the field performance of entomopathogenic fungi-based products considerably under unfavorable environmental conditions, increasing persistence and activity (Lacey et al., 2001; Hong et al., 2005; Jackson et al., 2010). Despite improved performance, in Brazil, most of the entomopathogenic fungi are used as unformulated technical concentrate (72.5%), and few formulations are commercially available (Faria and Wraight, 2007; Michereff-Filho et al., 2009).

* Corresponding author at: Federal University of Technology/UTFPR, Biology Department, 85892000 Santa Helena, PR, Brazil.
*E-mail address:* daiang@utfpr.edu.br (D.G.P.d. Oliveira).

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Some formulations provide protection against abiotic factors that are deleterious to conidia. For example, oil-based formulations can protect conidia against imbibitional damage (Xavier-Santos et al., 2011) and the detrimental effect of UV (Alves et al., 1998; Hedimbi et al., 2008; Moore et al., 1993) or chemical pesticides (Lopes et al., 2011). The negative effect of temperature on conidia viability has received particular attention. However, the effect of temperature on conidia formulations has been evaluated primarily in shelf-life studies (Alves et al., 2002; Hedgecock et al., 1995; Moore et al., 1995; Starthers et al., 1993; McClatchie et al., 1994) or with conidia diluted in mineral oil (Barreto et al., 2016; Alves et al., 2016), and little is known about the harmful effects of water temperature and exposure time in tank mixtures immediately before use.

In tropical environments, temperatures above 30 °C are very common during the spring and summer. This period is also typical for mycoinsecticide applications, and in the tank mixture exposed to the sun before spraying, has observed previously even above 50 °C can be reached (Oliveira et al., unpublished observations), which may interfere with conidial vigor and viability. Therefore, studies must be conducted to evaluate the effects of temperature and exposure time on the efficiency of suspensions of entomopathogenic fungi used for application after preparation of the tank mixture. Thus, we assessed the influence of temperature during the preparation and pre-application periods on the viability of the fungi *B. bassiana* and *M. anisopliae* as unformulated conidia and conidia formulated in emulsifiable oil and virulence on *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae).

## 2. Materials and methods

### 2.1. Source of fungi

*Beauveria bassiana sensu stricto* (s.s.) (based on EF1-α, unpublished data) strain ESALQ-PL63 and *Metarhizium anisopliae sensu stricto* (s.s.) (Rezende et al., 2015) strain ESALQ-1037, both originally isolated from ants and maintained in the Entomopathogenic Fungal Collection at the Insect Pathology Laboratory (ESALQ/University of São Paulo, Brazil), were used in the current study because they are the base of products marketed for control of *D. saccharalis* in Brazil. Dried conidia (technical material, TC), an emulsifiable oil formulation (oil dispersion, OD) and two technical concentrates (fungus-colonized rice, TK1, and ground fungus colonized rice, TK2) of both fungi were provided by Itaforte Bioprodutos Ltda., Itapetininga, SP, Brazil (now Koppert Brazil Ltd.). Unformulated concentrates and oil-formulated products were from the same production batch, with an approximate moisture content of 7%, scored at the final stage of the drying process. The company uses solid-state fermentation of grain (rice), which is commonly employed in Brazil for production of aerial conidia (Li et al., 2010). Oil dispersions were formulated in soybean oil with 5% emulsifier (proprietary formula). TC, TK1 and TK2 were stored at −20 °C and the OD at 4 °C until used. The products were removed from storage and gradually brought to room temperature (25 °C) by the time of the experiments. The concentration of conidia in each product was determined by counting the conidia in a Neubauer hemocytometer after serial dilution in water with Tween 80 (0.05%).

### 2.2. Insect rearing

*Diatraea saccharalis* was used in this work because in the laboratory it's quite susceptible to Bb and Ma fungi and is well-known model for virulence evaluations (unpublished data). The third-instar larvae used in the bioassays were obtained from a laboratory colony maintained on artificial diet (by Hensley and Hammond, 1968), following the rearing techniques described by Parra (1998), at controlled conditions (26 ± 1 °C, 70 ± 10% relative humidity, with a 14 h photophase).

### 2.3. Conidial viability assessment

Conidial viability for all preparations (TC, TK1, TK2 and OD) was assessed following the protocol described by Oliveira et al. (2015). Briefly, unformulated conidia (TC) and technical concentrate (TK) samples were directly mixed in deionized water + Tween 80 (0.05%) and vortexed. For the OD formulations, a modification was established to improve the evaluation. Samples of 40 mL of a suspension (originally 50 mL of OD in 950 mL of water) were transferred to centrifuge tubes (50 mL) with 100 μL of the surfactant Solub'oil™ (General Chemicals & Service Ltda., Brazil) and vortexed for 1 min. The mixture was then centrifuged for 5 min at 2500 rpm (573.1*g*) at 4 °C (Sorvall Centrifuge T-6000B; Thermo Fisher Scientific Inc.). One mL of the concentrated conidia suspension in the bottom of the tube was carefully pipetted for further dilution.

Conidial suspensions for all preparations were standardized to a final concentration of $1 \times 10^6$ conidia $mL^{-1}$. Rodac™ petri dishes containing 5 mL of potato dextrose agar (PDA) medium were inoculated with 150 μL of fungal suspension. After evaporation of free water, the plates were closed and incubated for germination at 26 ± 1 °C with a 14 h photophase. Viability was determined with direct counts of viable and non-viable conidia after 20–22 h under a light microscope at 400× magnification. Viable conidia had germ tubes that were longer than their diameters.

Survival curves were also determined hourly for TC and OD formulations up to 12 h, in order to evaluate the trends in decrease in conidial viability of both fungi with prolonged exposure. Four different suspensions from independent samples (replicates) of both formulated and unformulated conidia were prepared in water + Tween 80 (0.05%) at 22 °C. The suspensions were pipetted into 1.5 mL microcentrifuge tubes and maintained at 26 ± 1 °C, 36 ± 1 °C and 46 ± 1 °C for 12 h in full light. For the temperatures of 26 °C and 36 °C, conidial viability was scored after 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h. At 46 °C, assessments were conducted at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 7, 9 and 12 h after mixed in water. Conidial suspensions were inoculated and evaluated as described previously.

### 2.4. Diatraea saccharalis virulence assessment

Four independent suspensions of $1.25 \times 10^8$ conidia/mL (replicates) were prepared in 500 mL of water + Tween 80 (0.05%) at 22 °C for each unformulated concentrate and oil-formulation. The suspensions were prepared in Schott® culture flasks (1000 mL) and mixed vigorously for 20 min. Suspensions were split into 50 mL centrifuge tubes and incubated at temperatures of 26 ± 1 °C, 36 ± 1 °C and 46 ± 1 °C for 0, 1, 4 and 6 h in full light. After this period, independent samples were pipetted and inoculated in Rodac™ petri dishes for viability assessments as described above. Three plates were prepared per replicate and incubated for 28 h in incubator (26 ± 1 °C and a photophase of 24 h) until evaluated. At each assessment period, 2 mL of the same suspension used in the viability assessments was sprayed on groups of 50 third-instar larvae of *D. saccharalis* using a Potter's spray tower (Burkard Manufacturing, calibrated to 15 psi). For each treatment, insects were separated into 5 groups of 10 larvae each. Control treatments were water + Tween 80 (0.05%) or oil-in-water emulsion without conidia in the same proportions used in the OD formulation. Two min after spraying, larvae were transferred to plastic petri dishes (60 × 15 mm) containing filter paper in the lid and artificial diet as a food source (the same used in the colony, but without anticontaminants) and incubated at 26 ± 1 °C, with a photophase of 14 h. Mortality was evaluated daily and up to the 10th and 12th day for *B. bassiana* and *M. anisopliae*, respectively. Fresh insect diet was provided daily. Dead insects were placed in a moistened chamber to confirm sporulation by the fungi.

### 2.5. Statistical analyses

All experiments were performed using a completely randomized design (CRD), and analyses were conducted using the SAS statistical software package (SAS Institute Inc., 2003). A factorial arrangement (3 × 3) was performed for the experiments that assessed the effects of temperature and time of exposure on conidia. Data were analyzed by ANOVA for the presence of an interaction, with means tested by Tukey–Kramer HSD tests (α = 0.05). The means obtained at time 0 were compared with all other means by orthogonal contrasts (α = 0.05), and the correlation between conidial viability and larval mortality was obtained by Pearson's index (α = 0.05). Percent insect mortality was normalized by $\arcsin\sqrt{x/100}$ transformation, adjusted by the Schneider-Orelli formula and analyzed by ANOVA. Survival analysis was used to estimate the time to reach 50% conidia viability ($ST_{50}$), and log-rank testing with 5% probability was applied for comparisons between survival curves (R Statistical Software).

## 3. Results

### 3.1. Effects of exposure time and temperature on the viability and virulence of formulated and unformulated conidia of B. bassiana in a water suspension

Conidial viability was influenced by temperature and exposure time, and a two-way interaction was observed for each of the unformulated concentrates and also for the oil formulation [(TC, $F_{8,27}$ = 2904.3; P < .0001; CV = 4.5%), (TK1, $F_{8,27}$ = 667.4; P < .0001; CV = 8.1%), (TK2, $F_{8,27}$ = 927.7; P < .0001; CV = 6.7%), (OD, $F_{8,27}$ = 113.6; P < .0001; CV = 12.8%)]. Conidial viability decreased for the three unformulated conidia concentrates (TC, TK1, TK2) after 6 h in water at 26 °C (Table 1). By contrast, conidial viability did not decrease in the OD at this temperature. Conidia viability also decreased with time in TC, TK1 and TK2 at 36 °C, but conidia were not affected in the OD. At 46 °C, the death of conidia was rapid, with a 99.8%, 82.1% and 97.6% decrease in conidia viability after 4 h of exposure for TC, TK1 and TK2, respectively. The increase in water temperature also affected conidia germination in the OD with time; however, the effects on conidia were less negative at 46 °C after 4 h (59.1% reduction) and 6 h (66.5% reduction) compared with those in TC, TK1 and TK2 (Table 1).

Water temperature and exposure time of conidia in suspension also influenced the mortality of *D. saccharalis* larvae sprayed with all fungal preparations, and a two-way interaction was observed [(TC, $F_{8,27}$ = 6.08; P = .0013; CV = 12.3%), (TK1, $F_{8,27}$ = 3.63; P = .0171; CV = 11.1%), (TK2, $F_{8,27}$ = 4.7; P = .0052; CV = 13%), (OD, $F_{8,27}$ = 8.45; P = .0001; CV = 16.5%)]. The mortality of *D. saccharalis* was 83% by conidia in the OD formulation exposed to 26 °C for 6 h, whereas in the unformulated concentrates (TC, TK1 and TK2), mortalities ranged from 48% to 75%. Lower levels of insect mortality occurred when conidia were exposed to 46 °C for all treatments, and fungal efficacy decreased with the increase of exposure time for the conidia in water. After 4 h exposure of conidia to this temperature, mortality rates were less than 25% for the unformulated concentrates and 38.9% for the OD. No insect mortality was scored after 6 h of exposure to the TC and was less than 16% for TK1, TK2 and the OD after the identical exposure time (Table 2). Differently from the observed for the conidial viability, conidia endurance against temperature was not evident for any of the preparations in *in vivo* tests. However, the correlation between conidia viability and insect mortality was significant for all preparations as indicated by Pearson's analysis [(TC = 0.911, P = .0002), (TK1 = 0.747, P = .0130), (TK2 = 0.925, P = .0001), (OD = 0.951, P < .0001)].

### 3.2. Effects of exposure time and temperature on the viability and virulence of formulated and unformulated conidia of M. anisopliae in a water suspension

Similarly to the results observed for *B. bassiana*, *M. anisopliae* conidia were affected by water temperature and time in suspension for all preparations, and a two-way interaction was also observed [(TC, $F_{8,27}$ = 886.84; P < .0001; CV = 2.7%), (TK1, $F_{8,27}$ = 348.0; P < .0001; CV = 3.8%), (TK2, $F_{8,27}$ = 752.11; P < .0001; CV = 2.7%), (OD, $F_{8,27}$ = 24.33; P < .0001; CV = 9.8%)]. A water temperature of 26 °C caused a significant decrease in conidia viability for TC (11%), TK1 (12.5%) and TK2 (23.5%) after 6 h, in contrast to no reduction in the OD formulation at the identical temperature ($F_{2,9}$ = 0.08, P = .9188). The pattern was similar at 36 °C with no decrease in viability detected for the OD ($F_{2,9}$ = 0.14, P = .8702) but with reductions in viability of 14.9–21.7% and 28.1–60% for unformulated conidia after 4 and 6 h, respectively. The decrease in conidia viability

**Table 1**
Decrease of conidial viability (%) of different *Beauveria bassiana* and *Metarhizium anisopliae* preparations exposed for 1, 4 and 6 h in water suspension at 26 °C, 36 °C and 46 °C.

| Preparation[2] | Time | Viability decrease (%)[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Beauveria bassiana*[3] | | | Initial viability | *Metarhizium anisopliae*[3] | | | Initial viability |
| | | 26 °C | 36 °C | 46 °C | | 26 °C | 36 °C | 46 °C | |
| Pure conidia (TC) | 1 h | 1.3 ± 0.1 Bb$^{ns}$ | 1.2 ± 0.5 Bb$^{ns}$ | 3.2 ± 0.4 Ba | 94.2 ± 0.5[4] | 1.1 ± 0.4 Cb$^{ns}$ | 4.1 ± 0.7 Cb | 8.2 ± 0.4 Ba | 93.7 ± 0.8[4] |
| | 4 h | 2.6 ± 0.1 Bc | 6.9 ± 1.1 Ab | 99.8 ± 0.2 Aa | | 4.9 ± 0.3 Bc | 19.8 ± 0.7 Bb | 99.3 ± 1.1 Aa | |
| | 6 h | 4.6 ± 0.4 Ac | 7.2 ± 0.7 Ab | 99.3 ± 0.6 Aa | | 11.0 ± 0.8 Ac | 60.0 ± 1.7 Ab | 99.8 ± 1.1 Aa | |
| Technical concentrate (TK1) | 1 h | 2.5 ± 0.6 Ba$^{ns}$ | 3.0 ± 0.6 Ba | 5.2 ± 0.5 Ca | 90.5 ± 0.3[4] | 6.2 ± 0.9 Bc | 11.1 ± 0.4 Cb | 19.6 ± 0.1 Ba | 78.4 ± 0.3[4] |
| | 4 h | 4.6 ± 0.9 Bc | 10.5 ± 0.9 Ab | 82.1 ± 1.4 Ba | | 7.8 ± 1.1 Bc | 21.7 ± 1.8 Bb | 98.1 ± 1.5 Aa | |
| | 6 h | 8.8 ± 0.8 Ab | 11.1 ± 1.4 Ab | 94.5 ± 0.4 Aa | | 12.5 ± 0.9 Ac | 28.1 ± 0.9 Ab | 99.8 ± 1.2 Aa | |
| Technical concentrate 2 (TK2) | 1 h | 2.9 ± 0.4 Ba | 2.6 ± 1.2 Ca | 3.6 ± 0.6 Ba | 89.4 ± 0.8[4] | 0.0 ± 0.0 Ca$^{ns}$ | 0.0 ± 0.0 Ca$^{ns}$ | 1.2 ± 0.4 Ba$^{ns}$ | 73.2 ± 0.7[4] |
| | 4 h | 5.7 ± 0.9 ABc | 11.6 ± 0.7 Bb | 97. 6 ± 0.4 Aa | | 5.9 ± 0.6 Bc | 14.9 ± 0.6 Bb | 99.1 ± 0.6 Aa | |
| | 6 h | 8.8 ± 1.5 Ac | 21.5 ± 0.6 Ab | 99.3 ± 0.4 Aa | | 23.6 ± 0.7 Ac | 31.2 ± 0.8 Ab | 100.0 ± 0.8 Aa | |
| Oil Dispersion (OD) | 1 h | 0.0 ± 0.0 Aa$^{ns}$ | 0.3 ± 0.1 Aa$^{ns}$ | 1.0 ± 0.2 Ca$^{ns}$ | 90.0 ± 1.2[4] | 2.0 ± 0.6 Aa$^{ns}$ | 4.3 ± 0.6 Aa$^{ns}$ | 5.2 ± 0.8 Ca$^{ns}$ | 92.8 ± 0.3[4] |
| | 4 h | 0.6 ± 0.1 Ab$^{ns}$ | 4.8 ± 0.9 Ab$^{ns}$ | 59.1 ± 2.4 Ba | | 2.8 ± 0.8 Ab$^{ns}$ | 3.8 ± 0.3 Ab$^{ns}$ | 49.5 ± 5.3 Ba | |
| | 6 h | 3.3 ± 0.6 Ab$^{ns}$ | 3.0 ± 0.7 Ab$^{ns}$ | 66.5 ± 3.4 Aa | | 3.9 ± 1.3 Ab$^{ns}$ | 6.7 ± 1.6 Ab$^{ns}$ | 76.2 ± 2.8 Aa | |

[1] Relative germination was calculated in relation to non-exposed controls (Initial viability).
[2] Technical material (TC = pure conidia), technical concentrate (TK1 = rice + conidia, TK2 = ground rice + conidia,) and formulated in emulsifiable oil (OD).
[3] Within each fungal species and preparation, means ( ± SE) followed by the same lower letter in lines and capital letter in columns do not differ according to Tukey-Kramer HSD test (α = 0.05) in the presence of interaction. Coefficient of variation from tests (*Beauveria bassiana*: TC = 4.5%; TK1 = 8.1%; TK2 = 6.7%; OD = 12.8%; *Metarhizium anisopliae*: TC = 5.3%; TK1 = 7.5%; TK2 = 5.0%; OD = 17.3%).
[4] ($^{ns}$) Not significantly different compared to non-exposed controls (Initial viability) according to individual comparisons by orthogonal contrasts (α = 0.05).

**Table 2**
Confirmed mortality (%) of *Diatraea saccharalis* larvae caused by conidia of *Beauveria bassiana* or *Metarhizium anisopliae* maintained for 0, 1, 4 and 6 h in water suspension at different temperatures.

| Preparation[2] | Time | Mortality (%)[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Beauveria bassiana*[3] | | | Non-exposed control | *Metarhizium anisopliae*[3] | | | Non-exposed control |
| | | 26 °C | 36 °C | 46 °C | | 26 °C | 36 °C | 46 °C | |
| Pure conidia (TC) | 1 h | 72 ± 4.8 Aa$^{ns}$ | 59 ± 5.8 Aa$^{ns}$ | 49 ± 5.8 Aa$^{ns}$ | 62 ± 4.8[4] | 81 ± 7.1 Aa$^{ns}$ | 75 ± 11.2 Aa$^{ns}$ | 53 ± 11.6 Aa | 89 ± 4.9[4] |
| | 4 h | 80 ± 5.8 Aa$^{ns}$ | 52 ± 13.1 Aa$^{ns}$ | 18 ± 4.1 Bb | | 79 ± 12.4 Aa$^{ns}$ | 47 ± 2.4 Bb | 23 ± 7.3 Bc | |
| | 6 h | 57 ± 8.5 Aa$^{ns}$ | 44 ± 6.5 Aa | 0 ± 0.0 Bb | | 73 ± 8.0 Aa | 33 ± 2.0 Bb | 9 ± 3.7 Bc | |
| Technical concentrate (TK1) | 1 h | 71 ± 3.2 Aa$^{ns}$ | 51 ± 8.7 Aa$^{ns}$ | 70 ± 8.5 Aa$^{ns}$ | 70 ± 5.7[4] | 45 ± 2.9 Aa$^{ns}$ | 45 ± 2.9 Aa$^{ns}$ | 20 ± 4.1 Aa | 48 ± 22.2[4] |
| | 4 h | 54 ± 7.5 Aa$^{ns}$ | 40 ± 8.7 Aab$^{ns}$ | 19 ± 2.9 Bb | | 48 ± 11.8 Aa$^{ns}$ | 23 ± 5.3 ABa | 5 ± 2.9 ABb | |
| | 6 h | 48 ± 7.5 Aa$^{ns}$ | 35 ± 4.1 Aab | 10 ± 4.8 Bb | | 45 ± 5.0 Aa$^{ns}$ | 15 ± 2.9 Bb | 0 ± 0.0 Bc | |
| Technical concentrate 2 (TK2) | 1 h | 65 ± 8.7 Aa$^{ns}$ | 72 ± 2.5 Aa$^{ns}$ | 65 ± 6.5 Aa$^{ns}$ | 85 ± 6.5[4] | 38 ± 8.5 Aa$^{ns}$ | 35 ± 2.9 Aa$^{ns}$ | 30 ± 4.1 Aa$^{ns}$ | 43 ± 2.5[4] |
| | 4 h | 75 ± 6.5 Aa$^{ns}$ | 55 ± 8.7 Aa$^{ns}$ | 25 ± 6.5 Bb | | 33 ± 2.5 Aa$^{ns}$ | 43 ± 4.8 Aa$^{ns}$ | 0 ± 0.0 Bb | |
| | 6 h | 75 ± 9.4 Aa$^{ns}$ | 45 ± 6.5 Aab | 15 ± 2.9 Bb | | 40 ± 10.0 Aa$^{ns}$ | 23 ± 6.3 Aa | 0 ± 0.0 Bb | |
| Oil Dispersion (OD) | 1 h | 97 ± 2.5 Aa$^{ns}$ | 92 ± 4.8 Aa$^{ns}$ | 89 ± 4.1 Aa$^{ns}$ | 100 ± 0.0[4] | 88 ± 3.7 Aa$^{ns}$ | 84 ± 5.1 Aa$^{ns}$ | 82 ± 9.1 Aa$^{ns}$ | 92 ± 2.0[4] |
| | 4 h | 80 ± 7.9 Aa$^{ns}$ | 89 ± 5.8 Aa$^{ns}$ | 39 ± 10.4 Bb | | 85 ± 5.1 Aa$^{ns}$ | 94 ± 2.5 Aa$^{ns}$ | 50 ± 9.0 ABb | |
| | 6 h | 83 ± 6.5 Aa$^{ns}$ | 79 ± 6.3 Aa$^{ns}$ | 16 ± 2.9 Cb | | 74 ± 5.1 Aab$^{ns}$ | 85 ± 6.8 Aa$^{ns}$ | 40 ± 9.5 Bb | |

[1] Relative mortality was calculated in relation to non-exposed controls. Mortality was corrected by the Schneider-Orelli's formula.
[2] Technical material (TC = pure conidia), technical concentrate (TK1 = rice + conidia, TK2 = ground rice + conidia) and formulated in emulsifiable oil (OD).
[3] Within each fungal preparation, means ( ± SE) followed by the same lower letter in lines and capital letter in columns do not differ according to Tukey-Kramer HSD test (α = 0.05) in the presence of interaction. Coefficient of variation from tests (*Beauveria* bassiana: TC = 12.3%; TK1 = 11.1%; TK2 = 13.0%; OD = 16.5%; *Metarhizium anisopliae*: TC = 11.2%; TK1 = 13.1%; TK2 = 11.8%; OD = 15.6%).
[4] ($^{ns}$) Not significantly different compared to non-exposed controls (Initial viability) according to individual comparisons by orthogonal contrasts (α = 0.05).

approached 100% for TC, TK1 and TK2 after 4 h at 46 °C; however, only 49.5% of conidia were killed in the OD (Table 1).

Mortality rates of *D. saccharalis* by *M. anisopliae* were influenced by water temperature of the conidia suspension, with a significant interaction between both parameters [(TC, $F_{8,27} = 6.05$; P = .0013; CV = 11.2%), (TK1, $F_{8,27} = 3.58$; P = .0181; CV = 13.1%), (TK2, $F_{8,27} = 11.97$; P < .0001; CV = 11.2%), (OD, $F_{8,27} = 2.69$; P = .0466; CV = 15.6%)]. The efficacy of TC and TK1 decreased after 6 h in water at 36 °C, but the same exposure time did not affect the efficacy of the OD and TK2. Conidia of TC, TK1 and TK2 exposed to the highest temperature (46 °C) caused low levels of larval mortality for the technical grade preparations (≤22.5% after 4 h and ≤ 8.5% after 6 h of exposure). Despite the negative effect on conidia in the oil-based formulation (OD) at 46 °C after 6 h of exposure time, insect mortality reached 40% (Table 2). The protection afforded by OD was clearly demonstrated by the orthogonal contrast analyses between insect mortality by fresh OD formulation (92%) and exposed OD formulation for samples maintained at 36 °C for 6 h (85%) and at 46 °C for 1 h (82%). Additionally, high correlations between conidia viability and insect mortality for all preparations were indicated by Pearson's analysis [(TC = 0.925, P = .0001), (TK1 = 0.844, P = .0021), (TK2 = 0.903, P = .0003), (OD = 0.954, P < .0001)].

### 3.3. Survival curves (% decrease in conidial viability) of Beauveria bassiana and Metarhizium anisopliae for unformulated (TC) and oil-based formulated (OD) conidia

No difference in viability was observed between unformulated and formulated conidia of *B. bassiana* in a water suspension after 12 h of exposure at 26 °C ($F_{1,4} = 1.98$; P = .2319; CV = 2.18%) and only a marginal difference was detected for *M. anisopliae* for the same conditions ($F_{1,4} = 8.75$; P = .0416; CV = 2.98%). A significant decrease in conidia viability was noted at 36 °C for unformulated and formulated conidia for both species of fungi after 12 h of exposure [(*B. bassiana*, $F_{1,4} = 13.48$; P = .0214; CV = 0.69%) and (*M. anisopliae*, $F_{1,4} = 262.95$; P < .0001; CV = 1.06%)]; however, differences were less than 2% and 12%, respectively. A decrease in conidia viability of both fungi was clear with exposure to a high water temperature. The $ST_{50}$ was 5.45 h (5.34–5.56) and 5.58 h (5.47–5.69) for unformulated conidia of *B. bassiana* and *M. anisopliae* at 46 °C, respectively. A decrease in conidial viability was also observed in oil-in-water emulsions for both species at the identical temperature, and $ST_{50}$ values reached 6.08 h (5.96–6.20) and 6.35 h (6.22–6.48) for *B. bassiana* and *M. anisopliae*, respectively (Fig. 1). Thus, the OD provided protection against temperature when compared with unformulated conidia of *M. anisopliae* and *B. bassiana* at 46 °C ($\chi^2 = 20.4$; df = 1; P = 6.24e−6 and $\chi^2 = 17.3$; df = 1; P = 3.26e−5, respectively).

## 4. Discussion

The potential negative effects of high water temperatures on conidia after preparation of a tank mixture and before spraying have received little attention. In the present study, we examined the deleterious effects of water temperature and exposure time in water on formulated and unformulated conidia of *B. bassiana* and *M. anisopliae* and then on the ability of treated conidia to infect an insect host. Although the negative effect of high temperatures on formulated conidia has been previously described only in shelf-life studies (Alves et al., 2002; Hedgecock et al., 1995; McClatchie et al., 1994; Starthers et al., 1993) and for conidia diluted in mineral oil (Barreto et al., 2016; Alves et al., 2016), we are the first to report on the partial protection provided by emulsifiable oil to conidia against high temperatures in oil-in-water suspensions, with reductions in conidia death and with conidia maintaining infectivity.

Heat stress is well known to cause irreversible damage to conidia, e.g. *Metarhizium anisopliae* and *B. bassiana* conidia are severely affected when exposed to 45 °C for 4–8 h in a water suspension (Fernandes et al., 2008; Rangel et al., 2005). Although the thermotolerance of *M. anisopliae* is apparently higher compared to other species according to some studies (Horaczek and Viernstein, 2004; Rangel et al., 2005), in this study, the strains of *B. bassiana* and *M. anisopliae* tested had similar sensitivity when exposed to high temperatures. The thermal death point for thermotolerant *B. bassiana* isolates reported by Fernandes et al. (2008) was 46 °C after 6 h of exposure, which was very similar to our results of less than 6% and 2% survival of *B. bassiana* and *M. anisopliae* conidia, respectively, under identical conditions. Water temperatures ≤36 °C for up to 4 h in our study did not result in a large reductions in conidia viability. Actually, immersion in warm water (between 30 °C

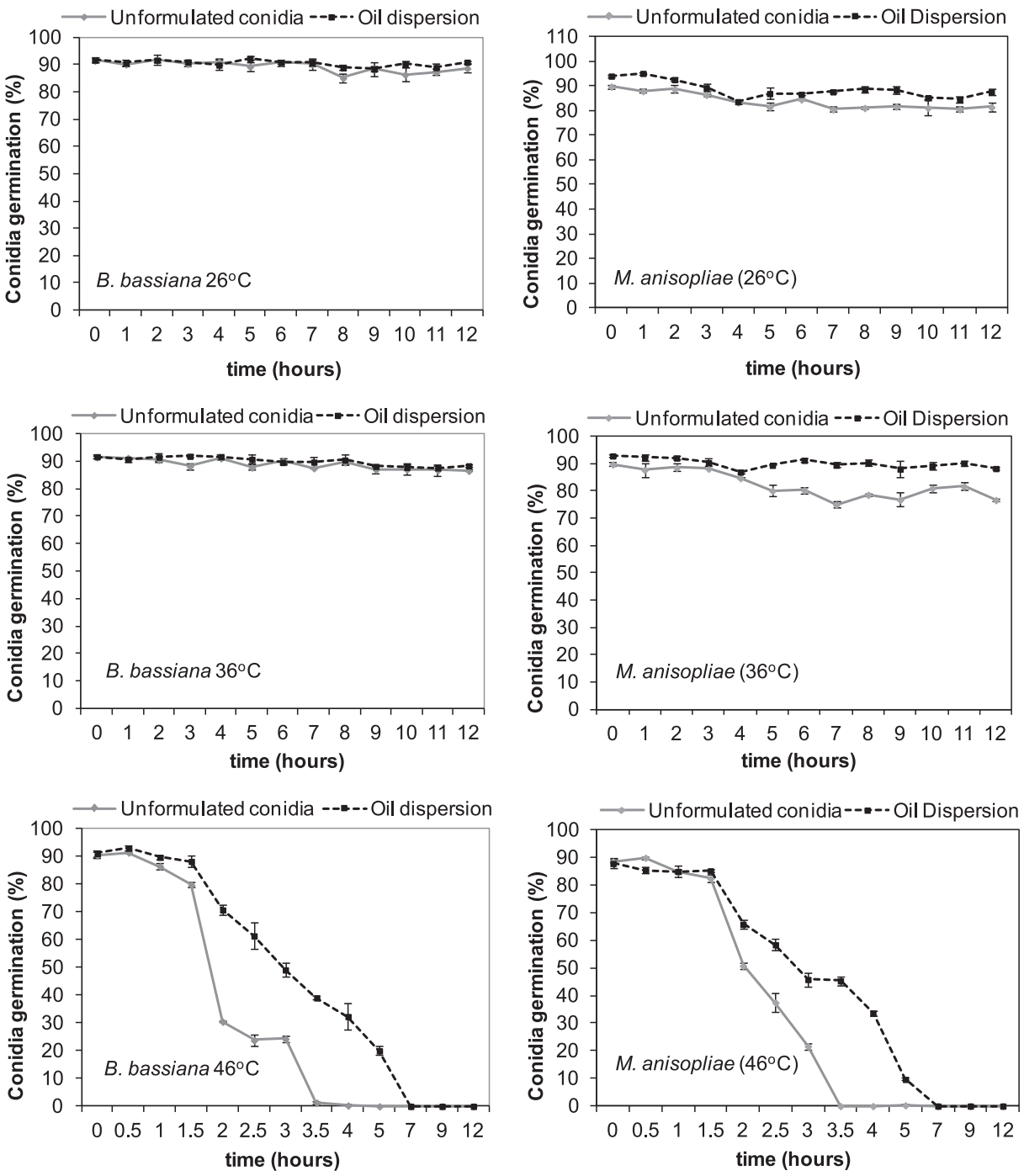


**Fig. 1.** Viability decrease (%) of *Beauveria bassiana* and *Metarhizium anisopliae* conidia maintained in water suspension at 26, 36 and 46 °C for up to 12 h prepared as pure conidia (▲) and in an oil-based formulation (■).

and 36 °C) is required to avoid imbibitional damage for very dry conidia (Faria et al., 2009).

For conidia of both species in a water mixture exposed for 1 h, we showed that temperature (26 °C and 36 °C) or preparation (TC, TK1 or TK2) did not affect insect mortality, although a slight reduction in conidial viability was observed in some treatments, particularly for the oil-free preparations. At 46 °C, only the conidia of *M. anisopliae* exposed for 1 h affected the mortality of *D. saccharalis* and only for TC and TK1 preparations. Similar to viability, fungal efficacy was not negatively affected at 26 °C, regardless of preparation or time of exposure in water (1, 4 or 6 h). After 4 h of exposure at 46 °C, both conidial viability and insect mortality decreased for all preparations, and damage to unformulated conidia was detected after 6 h at 36 °C. Therefore, to avoid decreases in product performance in the field, these results clearly indicate that the exposure times of tank mixture preparations be short and that water temperatures should not exceed 30 °C.

Water temperatures above 35 °C and long exposure times were used in our study to simulate extreme conditions faced by farmers during tank mixture preparations and the time gap before sprays are applied. Based on our previous observation, it is not uncommon in tropical regions for water temperatures to exceed 40 °C, for example, when mixtures are prepared during the afternoon on a sunny day for sprayers equipped with a black-color tank. The practice of mixing the product in water hours before spraying is also common in Brazil, which is a

strategy sometimes used by farmers with chemical pesticides to save time in the management of large crop areas. Under these conditions, an OD formulation can reduce the harmful effect of higher temperatures on conidia. Thus, in addition to the protection afforded by oil formulations under storage conditions (Daoust et al., 1983; Moore et al., 1995; Starthers et al., 1993), these formulations also protect conidia in oil-in-water suspensions against the heat.

Furthermore, it should be remembered that the oil and oil-in-water emulsions also enhance the efficacy of conidia in comparison to purely aqueous sprays, as was shown by Inglis et al. (1996) and Jenkins and Thomas (1996). They also enhance adhesion to foliar canopy in the face of rain (Inglis et al., 2000).

In water, conidia rapidly increase their moisture content and become more susceptible to heat stress, compared with dry-heat (Hedgecock et al., 1995; Hong et al., 1999; Morley-Davies et al., 1995; Rangel et al., 2005; Zimmermann, 1982). The conidia, which remain drier inside small oil bubbles in oil-in-water emulsions (Bateman, 1996; Ibrahim et al., 1999), are most likely protected from the heat by a thin layer of this hydrophobic compound. Oil formulations provide similar protection against chemical pesticides and imbibitional damage (Lopes et al., 2011; Xavier-Santos et al., 2011).

Based on the decrease in conidial viability of *M. anisopliae* at 36 °C, as shown in Table 1, conidial death was expected to increase with longer times of exposure. However, high viability of unformulated conidia was maintained after 12 h of exposure in water (Fig. 1). Differences in the quality of the batches and/or initial conidia moisture content might explain some of the differences observed between experiments. However, this type of difference was not observed at 46 °C, and the OD formulation provided significant protection to *M. anisopliae* and *B. bassiana* conidia under heat stress when compared directly with unformulated conidia (TC).

Our results addressed the importance of the water temperature and exposure time in a water suspension for *B. bassiana* and *M. anisopliae*-based mycopesticides before their use in the field, and we concluded that inappropriate handling conditions, particularly high water temperatures, must be recognized and avoided by biopesticide users. Temperatures of approximately 26 °C or exposure times in suspension not longer than 1 h are conditions that guarantee conidial viability during the tank mixing of unformulated products. Moreover, an oil-based formulation can be an important tool to manage heat stress on conidia under unfavorable conditions in sprayer containers.

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