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Increased heat tolerance afforded by oil-based conidial formulations of *Metarhizium anisopliae* and *Metarhizium robertsii*

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ABSTRACT

The thermotolerance of oil-based conidial formulations of *Metarhizium anisopliae* s.l. (IP 46) and *Metarhizium robertsii* (ARSEF 2575) were investigated. Conidia of IP 46 or ARSEF 2575 were suspended in different adjuvants and exposed to $45 \pm 0.2°C$ for 4, 6, 8 or 24 h; their viability was then assessed after 48 h incubation at $27 \pm 1°C$. Conidia heated in pure mineral or vegetable oil exhibited mean relative viability exceeding 70% after 8 h of heat exposure, whereas low germination (≤20%) was observed when conidia were heated in water (Tween 80® 0.01%), carboxymethyl cellulose gel or emulsifiable oils (Graxol® or Assist®) and exposed to heat for 6 or 8 h. In addition, conidia of IP 46 suspended in either pure mineral or canola oil and exposed to heat for 48 h had moderate viability, 57% or 41%, respectively. Unstable oil-in-water emulsions showed a higher percentage of conidia incorporated into oil micellae, while the stable emulsions had higher percentage of conidia outside the oil micellae. The thermotolerance of conidia formulated in stable emulsions, however, did not differ from that of conidia formulated in unstable emulsions. The present study highlights possibilities to alleviate the deleterious effects of heat stress towards *Metarhizium* spp. conidia applied for controlling arthropod pests and vectors through oil-based formulations.

Introduction

The entomopathogenic fungus *Metarhizium anisopliae* sensu lato (Hypocreales, Clavicipitaceae) is widely used for agricultural pest control as an alternative to the exclusive use of chemical pesticides (Ment et al., 2012; Zimmermann, 1993). Natural abiotic factors, however, may limit the potential of entomopathogenic fungi as active biological agents in arthropod control programs (Braga, Flint, Messias, Anderson, & Roberts, 2001; Fernandes et al., 2010; Fernandes, Rangel, Moraes, Bittencourt, & Roberts, 2008; Rangel, Braga, Anderson, & Roberts, 2005; Rangel, Fernandes, Dettenmaier, & Roberts, 2010), and heat is thought to be one of the most significant abiotic factors for its

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potentially deleterious effects on conidial germination, mycelial growth and sporulation (Fernandes et al., 2010; Ment et al., 2010; Polar et al., 2005; Rangel et al., 2010).

Identification of fungi naturally tolerant to heat may assist the selection of strains with potential for arthropod control under field conditions in strongly insolated tropical areas (Fernandes et al., 2008). Many studies have screened the thermotolerance of isolates of *Beauveria bassiana* s.l. (Devi, Sridevi, Mohan, & Padmavathi, 2005; Fernandes et al., 2008), *Isaria* spp. (Cabanillas & Jones, 2009), *Metarhizium acridum*, *Metarhizium robertsii* and *Metarhizium anisopliae* s.l. (Alves, Bateman, Gunn, Prior, & Leather, 2002; Fernandes et al., 2010; Rangel et al., 2005, 2010).

Besides the selection of thermotolerant strains, formulations are essential to develop biological pesticides with increased efficacy for pest control (Polar et al., 2005). The use of adjuvants is critically required to protect conidia against such adverse factors as high temperatures (Barreto et al., 2016; McClatchie, Moore, Bateman, & Prior, 1994), ultraviolet radiation (Bateman & Alves, 2000; Braga, Flint, Messias, et al., 2001; Braga, Flint, Miller, Anderson, & Roberts, 2001; Fernandes, Rangel, Moraes, Bittencourt, & Roberts, 2007; Moore, Bridge, Higgins, Bateman, & Prior, 1993), desiccation (Bateman, Carey, Moore, & Prior, 1993) and chemical fungicides (Lopes, Pauli, Mascarin, & Faria, 2011). In addition, formulation may improve the performance of fungi, particularly by favouring the dispersion and adhesion of conidia on the hydrophobic cuticle of susceptible arthropod hosts (David-Henriet, Pye, & Butt, 1998). The efficacy of various specialised types of conidial formulations has been demonstrated against different orders of insects (Lomer, Bateman, Johnson, Langewald, & Thomas, 2001; Luz & Batagin, 2005; Malsam, Kilian, Oerke, & Dehne, 2002) and ticks (Angelo et al., 2010; Camargo et al., 2012; Reis, Fernandes, & Bittencourt, 2008; Souza, Costa, Bittencourt, & Fagundes, 2009).

Conidia can be formulated with different adjuvants (Faria & Wraight, 2007), such as mineral oils derived from distilled fractionations of crude oil, vegetable oils extracted from seeds by pressing or using solvents (Mendonça, Raetano, & Mendonça, 2007) or emulsions of such oils (Peng & Xia, 2011). Emulsions are dispersions of two immiscible liquid phases. Oil-in-water emulsions are stabilised by the presence of emulsifying agents that reduce the interfacial and surface tension between oil and water (Frange & Garcia, 2009).

Polymerised cellulose gel has also been investigated as a promising adjuvant for fungal formulation and to facilitate application (Souza et al., 2009). Carboxymethyl cellulose (CMC) is an anionic water-soluble polymer derived from cellulose and marketed as a sodium salt. The advantage of the fungal formulation with CMC gel is possibly associated with its facilitation of conidial adhesion to the host, as well as its low toxicity, biocompatibility, biodegradability, low cost and high stability (Silva, Musical, Altmeyer, & Valentini, 2011; Souza et al., 2009). Moreover, this biopolymer can be used to develop microcapsules that increase ability of microbial biocontrol agents to withstand ultraviolet radiation (Durvasula & Forshaw, 2014).

The use of appropriate adjuvants may minimise the effects of extreme environmental temperatures on entomopathogenic fungi and concurrently may improve the efficacy of bioproducts for controlling arthropod pests and vectors. Despite the wealth of literature regarding the effect of heat on conidia prepared in different oil-based formulations (Alves et al., 2002; Barreto et al., 2016; Brooks et al., 2004; Mola & Afkari, 2012; Moore, Douro-Kpindou, Jenkins, & Lomer, 1996; Shimizu & Mitani, 2000; Stathers, Moore, &

Prior, 1993), there is still room for more research seeking novel oil-in-water emulsions using different oil types and emulsifiers. Moreover, to the best of our knowledge, there is little information on the thermoprotection properties of CMC gels to aerial conidia of biocontrol fungal agents. In the current study, the conidial thermotolerance of *M. anisopliae* s.l. IP 46 and *M. robertsii* ARSEF 2575 was investigated in different oil-based formulations and in aqueous preparations with the aim to simulate high temperatures during preparation of conidial suspensions for field spray applications (i.e. temperature of the water immediately before application). This study was conducted under the worst case scenario of high ambient temperature providing us a proof-of-concept basis for understanding how oil-based conidial formulations can benefit mycopesticides in tropical climate.

Material and methods

Fungal cultures

Two *Metarhizium* species were evaluated in the current study: (a) *M. anisopliae* s.l. IP 46 originated from a soil sample from Emas National Park, Goiás, Brazil (Rocha, Inglis, Humber, Kipnis, & Luz, 2012) and deposited at a laboratory research collection of entomopathogenic fungi in the Institute for Tropical Pathology and Public Health at the Federal University of Goiás (Goiânia, GO, Brazil). In addition, this fungus was further deposited and designated as strain CG 620 at the EMBRAPA Collection of Entomopathogenic Fungi (Cenargen, Brasília, Brazil); (b) *M. robertsii* ARSEF 2575 was originally isolated from naturally infected *Curculio caryae* (Coleoptera, Curculionidae) collected in South Carolina, USA, and is deposited at the United States Department of Agriculture – USDA–ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA).

    Both strains were cultured on potato dextrose agar medium (Difco Laboratories, Sparks, MD, USA) supplemented with $1 \, g \, L^{-1}$ yeast extract (Bacto™ Yeast Extract, Sparks, MD, USA) (PDAY) in Petri plates (80×10 mm) and incubated in the dark at $27 \pm 1°C$ for 15 d. Conidia were harvested with a microbiological loop, placed in Petri plates and held for 5 d at $5 \pm 1°C$ in a desiccator with activated silica gel in order to reduce the overall moisture content to $\leq 5\%$ (w/w).

Conidial formulations and heat tests

Dried conidia of IP 46 and ARSEF 2575 were formulated variously for heat tests conducted as follows. In a first set of experiments, conidia were suspended in pure mineral oil (Naturol®, Farmax Amaral Ltda, Divinópolis, MG, Brazil), emulsifiable mineral oil (Assist®, BASF, The Chemical Company, Duque de Caxias, RJ, Brazil), pure vegetal oil (Canola®, Caramuru Alimentos S.A., Itumbiara, GO, Brazil), emulsifiable vegetal oil (Graxol®, Agrária Indústria e Comércio Ltda., Jardinópolis, SP, Brazil), aqueous surfactant solution [Polyoxyethylene sorbitan monoleate (Tween 80®; Sigma Chemical Co., St. Louis, MO, USA) at 0.01% (v/v) previously heated up to 35°C in order to avoid imbibitional damage to dried conidia (Xavier-Santos, Lopes, & Faria, 2011)] or CMC gel [CMC 1.3% (w/v)] (EMFAL®, Empresa Fornecedora de Álcool Ltda., Duque de Caxias, RJ, Brazil). The pH of each solution (no addition of conidia) was measured at 27°C and

45°C with colour-fixed pH test strips (pH-Fix®, Macherey-Nagel, Germany) with wide range from 1 to 14, in 1 pH unit step. Aqueous conidial suspensions were quantified by haemocytometer counts, and concentration adjusted to 1×10^6 conidia mL^{-1}. Due to difficulties in making haemocytometer counts of conidia suspended in oil, these suspensions were prepared using the same weight of conidia used for preparation of aqueous conidial suspensions. Conidial suspensions were vigorously vortexed and 2-mL aliquots of each suspension transferred to a 100×16 mm glass screw-cap tube for further exposure to heat (45 ± 0.2°C) for 0 (control), 4, 6 or 8 h in water bath. The chosen high temperature, 45°C, is a threshold employed in previous studies to select heat tolerant fungal strains within *Metarhizium* spp. (Barreto et al., 2016; Fernandes et al., 2008; Rangel et al., 2005), because it does not cause death but induces heat stress in conidia of many entomopathogenic fungi.

In a second set of experiments, conidia suspended in pure mineral or vegetal oil were exposed to heat for longer periods: 0 (control), 8, 16, 24, 32, 40 or 48 h. In the third set of experiments, conidia were suspended in mineral oil-in-water emulsions [5%, 10% or 15%, with 5% Solub'Oil (General Chemicals and Service Ltda., Campo Mourão, PR, Brazil)], pure mineral oil or water (Tween 80®, 0.01% or Solub'Oil, 5%), and exposed to heat for 4 h.

In the fourth and last set of experiments for heat exposures, conidia suspended in 1 mL pure mineral oil were mixed with one of each surfactant: Tween 80® (at 10%, 25% or 50% v/v) or Solub'Oil (at 1%, 2.5% or 5% v/v); distilled water was then added to the suspension until completing 10 mL of final volume. The suspensions were vortexed and exposed to heat for 4 h. Twenty microliter aliquots of each formulation were also placed between slide and coverslip, and conidia (≥300) inside and outside oil micellae were quantified at 400× magnification; percentage of conidia encapsulated by oil micellae was calculated in relation to the conidia in the water phase.

Each of the four tests was conducted at least four times, in different days, and using new batches of conidia. Control conidial suspensions were not exposed to heat but held at 27 ± 1°C for equal periods as the other heated suspensions. Temperature was monitored with a HOBO H8 data logger (Onset Computer Corporation, Bourne, MA, USA).

Assessing effects of heat on conidial relative viability

After heat exposure, 1 mL of each sample was aliquoted into a screwed 15-mL centrifuge plastic tube (Gene, Ionlab Equip. Sup. Laborat. Hosp. Ltda., Curitiba, PR, Brazil) plus 9 mL of Tween 80® (0.05%, v/v) and 100 μL of Solub'Oil. Samples were then vigorously vortexed for 30 s and centrifuged for 5 min at 4200 rpm. The supernatant was discarded, and the process repeated to ensure the oil was removed (Oliveira, Pauli, Mascarin, & Delalibera, 2015). After removing the oil, the pellet of washed conidia was suspended in 1 mL Tween 80® (0.05%, v/v); then, each suspension was vortexed, and 20 μL inoculated in the centre of a Petri dish (35×10 mm) containing 8 mL PDAY plus 0.002% (w/v) benomyl (50% active ingredient; Benlate®, DuPont, São Paulo, SP, Brazil) (Braga, Flint, Miller, et al., 2001; Milner, Huppatz, & Swaris, 1991) and 0.05% (w/v) chloramphenicol (Officinal, Goiânia, GO, Brazil). The plates were incubated at 27 ± 1°C, in the dark, for 48 h. Two drops of cotton blue were applied with a Pasteur pipette over the inoculum in each plate, and viability (maximum germination) was immediately assessed at 400× magnification. A minimum of 300 random conidia per plate was evaluated, and the mean relative percent viability

calculated using the following equation: Relative viability (%) = $(W_t/W_c) \times 100$, where W_t is the number of germinating conidia, in each plate, exposed to heat for a period of time t, and W_c is the mean number of germinating conidia from the control group (not exposed to heat) (Braga, Flint, Miller, et al., 2001). Differences in conidial thermotolerance among formulations of the same fungal strain were determined by the analysis of variance (ANOVA), followed by Student–Newman–Keuls test to compare their means, with a significance level of 5% ($P < .05$). Data were checked for normality and homoscedasticity assumptions (i.e. based on Shapiro–Wilk's test and Bartlett's test) prior to one- or two-way ANOVA and did not require any transformation. In addition, t-Student test at $P < .05$ was used to compare means between mineral and vegetable oil treatments across exposure time.

Results

The pH of solutions used to formulate conidia varied from 4 to 8. Emulsifiable mineral oil (Assist®) and emulsifiable vegetable oil (Graxol®) had a pH of 4; pure vegetable oil (Canola®) and pure mineral oil (Naturol®) had pH of 7; Tween 80® 0.01% and CMC 1.3% had pH of 8. Additionally, pure Tween 80® and Solub'Oil had a pH of 7.

The mean relative viability of *M. anisopliae* s.l. IP 46 conidia varied significantly when suspended in different adjuvants (canola oil, mineral oil, aqueous surfactant solution, Graxol®, Assist® and CMC) and exposed to heat (45 ± 0.2°C) for 4 h ($F_{5,18}$ = 10.2; $P < .0001$), 6 h ($F_{5,18} = 20.6$; $P < .0001$) or 8 h ($F_{5,18} = 59.7$; $P < .0001$). Similar variation was observed with conidia of *M. robertsii* ARSEF 2575 exposed for 4 h ($F_{5,18} = 14.1$; $P < .0001$), 6 h ($F_{5,18} = 9.18$; $P = .0001$) or 8 h ($F_{5,18} = 9.5$; $P = .0001$) (Figure 1). Conidia of IP 46 or ARSEF 2575 suspended in pure mineral or vegetable oil presented mean viability rates exceeding 70% after 8 h of heat exposure, whereas virtually zero viability was observed when conidia were suspended in aqueous surfactant solution (Tween 80, 0.01% v/v) or in CMC gel preparation and exposed to heat for 6 or 8 h. Conidia of both IP 46 and ARSEF 2575 formulated with emulsifiable oils (Graxol® or Assist®) also exhibited low viability rates below 20% after 6 or 8 h of heat exposure (Figure 1).

Mean relative viability of IP 46 conidia suspended in pure canola oil and exposed to heat for 24, 32, 40 or 48 h did not differ significantly; conidial viability was 54.9%, 48.3%, 41.5% or 41.5%, respectively. These conidia exposed to heat for 24 h, however, had viability significantly reduced in comparison to conidia exposed to heat for 8 h; 54.9% and 84.9%, respectively, but did not differ from germination of conidia exposed to heat for 16 h, 68.3% ($F_{5,27} = 8.1$; $P < .0001$) (Figure 2). Conidia of IP 46 suspended in pure mineral oil revealed high relative viability for extended heat-treatment periods: 91.3% (8 h), 85.1% (16 h) or 75.0% (24 h), with no significant difference among them. Heat exposures for 40 or 48 h had mean viability rates of 52.6% and 57.3%, respectively; these values were significantly lower in relation to germination rates of heat-exposed conidia for 8 and 16 h ($F_{5,27} = 6.1$; $P = .0006$) (see Figure 2). By comparing relative viability trends (slopes) between canola and mineral oil, it was found that viabilities decreased at a similar rate for both oils across exposure time ($F_{5,54} = 0.3$, $P = .89$). However, the mineral oil afforded greater overall protection to conidia against heat
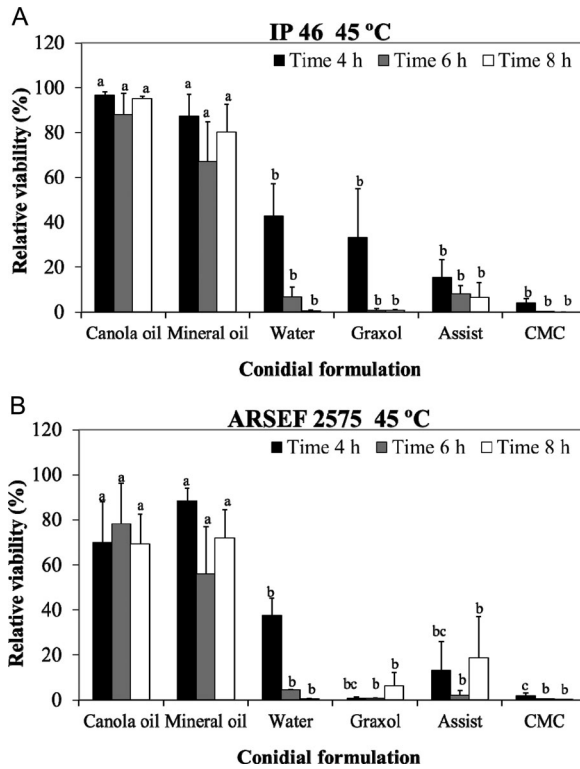
Figure 1. Relative viability (%) of (a) *M. anisopliae* s.l. (IP 46) and (b) *M. robertsii* (ARSEF 2575) conidia suspended in canola oil, mineral oil, water (Tween 80 0.01%), emulsifiable vegetable oil, emulsifiable mineral oil or CMC [CMC 1.3% (w/v)] exposed to $45 \pm 0.2°C$ for 4, 6 or 8 h, and incubated onto PDAY medium for 48 h at $27 \pm 1°C$ in the dark. Relative viability was calculated in relation to non-heated controls. Error bars are standard errors ($\pm$SE) of four independent trials. Bars (mean values) with the same lower-case letters in the same heat exposure period do not differ statistically ($P < .05$).
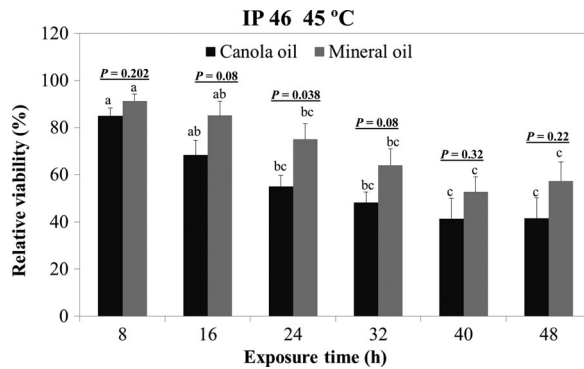

Figure 2. Relative viability (%) of *M. anisopliae* s.l. conidia (IP 46) suspended in canola oil or mineral oil, exposed to $45 \pm 0.2°C$ for 8, 16, 24, 32, 40 or 48 h, and incubated onto PDAY for 48 h at $27 \pm 1°C$ in the dark. Relative viability was calculated in relation to non-heated controls. Error bars are standard errors ($\pm$SE) of four independent trials. Bars (mean values) with the same lower-case letters within the same type of oil do not differ statistically ($P < .05$).
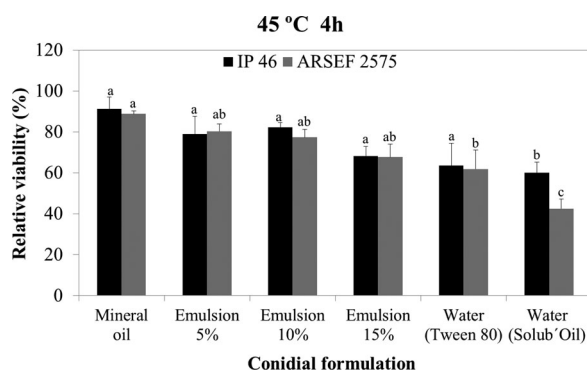
Figure 3. Relative viability (%) of *M. anisopliae* s.l. (IP 46) and *M. robertsii* (ARSEF 2575) conidia suspended in pure mineral oil, mineral oil-in-water emulsions with different oil concentrations [5%, 10% or 15% (v/v), with 5% Solub'Oil], water solutions [Tween 80˚, 0.01% (v/v) or Solub'Oil, 5% (v/v)], exposed to $45 \pm 0.2°C$ for 4 h, and incubated onto PDAY medium for 48 h at $27 \pm 1°C$ in the dark. Relative viability was calculated in relation to non-heated controls. Error bars are standard errors (±SE) of four independent trials. Bars (mean values) with the same lower-case letters within the same fungal strain do not differ statistically ($P < .05$).

than the vegetable oil ($F_{1,54} = 15.9$, $P = .0002$), specifically at 24 h exposure (*t*-Student test: $t = 2.38$, df = 10, $P = .038$).

Conidia of IP 46 or ARSEF 2575 suspended in standard aqueous solution containing Solub'Oil 5% were more susceptible to 4-h heat exposure ($45 \pm 0.2°C$) than conidia suspended in pure mineral oil; however, the mean viability of conidia heated in mineral oil-in-water emulsions (at 5%, 10% or 15% mineral oil) neither differed significantly from heated conidia suspended in aqueous solution with Tween 80˚ 0.01%, nor from heated conidia suspended in pure mineral oil (Figure 3). Conversely, the relative viability of ARSEF 2575 conidia suspended in aqueous surfactant solution containing Solub'Oil (5% v/v) was significantly inferior to the viability of their conidia suspended in mineral oil-in-water emulsions ($F_{5,30} = 9.74$; $P < .0001$).

Conidia of IP 46 or ARSEF 2575 suspended in 10% mineral oil-in-water emulsions with 10%, 25% or 50% of Tween 80˚ presented high instability when compared with oil-in-water emulsions prepared with different concentrations (1.0%, 2.5% or 5.0%) of Solub'Oil, which, in turn, provided high stability in terms of emulsion evenness. Unstable emulsions (prepared with Tween 80˚) showed high percentage of conidia inside the oil micellae (e.g. IP 46 with 90.3%, and ARSEF 2575 with 80.6%; both isolates at 50% Tween 80˚), whereas the stable emulsions (prepared with Solub'Oil) showed high percentage of conidia outside the oil micellae (e.g. IP 46 with 87.2% and ARSEF 2575 with 95.2%, both at 5% Solub'Oil) (Figure 4). The percentage of conidia encapsulated by oil micella was significantly different between the two emulsions (stable and unstable) investigated here [IP 46 ($F_{5,18} = 13.75$; $P < .0001$) or ARSEF 2575 ($F_{5,18} = 4.97$; $P = .0049$)]. Nevertheless, the thermotolerance of conidia in stable emulsions did not differ from that of conidia formulated in unstable emulsions [IP 46 ($F_{5,18} = 1.39$; $P = .2726$) or ARSEF 2575 ($F_{5,18} = 1.37$; $P = .2804$)] (Figure 4).
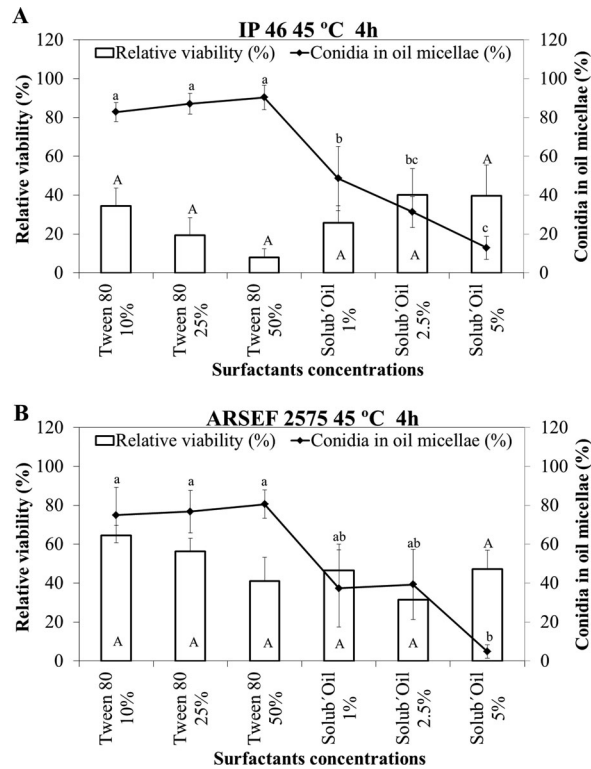
Figure 4. Relative viability (%) of (a) *M. anisopliae* s.l. (IP 46) and (b) *M. robertsii* (ARSEF 2575) conidia suspended in mineral oil-in-water emulsions [10% (v/v)] with different surfactant concentrations [Tween 80® (10, 25 or 50%); Solub'Oil (1.0, 2.5 or 5.0%)], exposed to 45 ± 0.2°C for 4 h, and incubated onto PDAY medium for 48 h at 27 ± 1°C in the dark. Percentage of conidia inside oil-in-water droplets in relation to conidia presented in water portion of each concentration of Tween 80® and Solub'Oil emulsions. Emulsions prepared with Solub'Oil were more stable than emulsions prepared with Tween 80® at room temperature, regardless the surfactant concentrations. Relative viability was calculated in relation to non-heated controls. Error bars are standard errors (±SE) of four independent trials. Open bars (mean values) with the same capital letters or line markers with the same lower-case letters do not differ statistically (*P* < .05).

Discussion

Different formulation strategies employed for entomopathogenic fungi have been proposed with the aim of increasing their efficacy as biological control agents against arthropod pests (Faria & Wraight, 2007). Fungal formulation can facilitate the application of bioproducts, as well as protect fungi from the detrimental effects caused by abiotic factors, including high temperatures (Barreto et al., 2016; Hedimbi et al., 2008; McClatchie et al., 1994; Oliveira et al., 2015). The present study demonstrated that conidia of *M. anisopliae* s.l. and *M. robertsii* formulated in vegetable or mineral oil were more tolerant to heat stress rather than unformulated conidia (i.e. conidia suspended in aqueous Tween 80 or Solub'Oil solution). Although reduction trends in relative conidial viability were similar to both types of emulsifiable oil, the mineral provided an overall better thermoprotection for these entomopathogens than the vegetable. As a matter of fact, it is well

documented that non-formulated conidia (suspended in aqueous Tween 80° 0.01%) of entomopathogenic fungi have limited viability or delayed germination when exposed to high temperatures (Devi et al., 2005; Fernandes et al., 2008, 2010; Keyser, Fernandes, Rangel, & Roberts, 2014; Rangel et al., 2005).

Mineral and vegetable oils are commonly used to control insect and fungal pests affecting agriculture or added to pesticides as adjuvant in oil-in-water emulsions to improve spraying performance and to increase absorption of the active ingredient by the target (Mendonça et al., 2007). Conidia of entomopathogenic fungi, such as *Metarhizium* spp., are lipophilic, suspending easily in oil (Bateman et al., 1993), and oil-based fungal formulations increase adhesion of conidia on host cuticle, and subsequently increase their performance against arthropod hosts under laboratory and field conditions (Camargo et al., 2012, 2014; Leemon & Jonsson, 2008; Luz, D'Alessandro, Rodrigues, & Fernandes, 2015; Reis et al., 2008; Rodrigues, Lobo, Fernandes, & Luz, 2015). Other advantages, yet less studied, related to oil-based formulations for entomopathogenic fungi regard their protective effects against chemical fungicides (Lopes et al., 2011) and imbibitional damage due to cold shock (Xavier-Santos et al., 2011).

Conidia of *M. anisopliae* IP 46 prepared in pure mineral or vegetable oil remained viable (ca. 50%; see Figure 2) even after 48 h of heat exposure. Conversely, conidia of both *Metarhizium* species experienced strong and moderate reductions in conidial viability when formulated with either an emulsifiable mineral oil (Assist°) or an emulsifiable vegetable oil (Graxol°), respectively (see Figure 1). Same trend of viability reduction was previously reported by Alves et al. (2002) when storing *M. anisopliae* conidia formulated in various emulsifiable adjuvant oils at 27°C for 40 weeks. These emulsifiable oil products are available commercially as agricultural insecticides and have additional constituents (not specified by the manufacturers, and reported as inert ingredients) to their formula; also, they were acid (pH 4) in comparison to the pure oils tested which had neutral pH (pH 7). These and possibly other unknown factors may have negatively impacted the viability of conidia in the emulsifiable oil suspensions tested because moderate or low mean germination of conidia not exposed to heat (control) was observed [76.7% (Graxol°) and 23.5% (Assist°); these data are not shown in Figure 1 because mean relative viability from conidial suspensions (calculated as mentioned in Section '*Assessing effects of heat on conidial relative viability*') was presented in detriment of percent mean germination]. On the other hand, mean germination of non-heated conidia prepared with pure oils, aqueous surfactant solution (Tween 80° 0.01%) or CMC was greater than 95%. These results are relevant in their indications that caution should be taken when mixing conidia of entomopathogenic fungi with ready-to-use emulsifiable oil formulations, since some components included in these commercial oils may be detrimental to conidial survival.

Extreme pH levels may change the membrane fatty-acid composition (Tamerler, Ullah, Adlard, & Keshavarz, 1998) and damage the primary and secondary structure of proteins of microbial cells (Magan, 2007). As reported previously, conidia of *M. robertsii* were significantly more sensitive to heat when produced on an acid medium (pH 4.59) rather than on an alkaline medium (pH 8.04) (Braga, Rangel, Fernandes, Flint, & Roberts, 2015).

Suspending conidia of both fungi in CMC gel did not confer heat protection to either *Metarhizium* species because their relative viability rates were as low as those achieved with conidia suspended in aqueous surfactant solutions. Despite the benefits that CMC

may offer for biopesticide formulations, its role in thermotolerance was shown here to be irrelevant for conidia of these two *Metarhizium* species.

The thermoprotection of conidia conferred by mineral oil-in-water emulsions may be related to the fact that dried conidia were suspended in pure oil prior to addition of the surfactant into the water for preparation of emulsions. Actually, the presence of most conidia inside (unstable emulsion) or outside (stable emulsion) the oil micellae in these emulsions did not change the susceptibility of conidia to heat stress. Essentially, emulsions are thermodynamically unstable systems, but depending on the surfactant used, the time required to separate the two immiscible phases (namely, oil and water) may be delayed or not (Frange & Garcia, 2009). In this context, Mendonça et al. (2007) reported that the value of the static surface tension is determined by the quantity and quality of the surfactant used in the formulation and not by the oil type (i.e. mineral or vegetable oil). In the current study, a more stable emulsion was obtained when Solub'Oil was used as surfactant regardless of the concentration tested. We suspect that a thin and persistent layer of oil coating single conidia was formed when oil-in-water emulsions were prepared with Solub'Oil at 5% (v/v), and this may have contributed somewhat to improving heat tolerance. Conidial suspensions prepared in stable emulsions appears to perform better than those in unstable emulsions, because the more stable the emulsion, the longer the homogeneity of the suspension, and this durability of emulsification consequently enhances the uniformity of applications and the overall performance of bioproducts for infecting the target hosts.

Conidia of IP 46 suspended in 10% mineral oil-in-water emulsion prepared with 5% Solub'Oil reached ca. 80% relative viability after heated for 4 h at 45°C (see Figure 3). In a different test, conidia of IP 46 suspended in 10% pure mineral oil-in-water emulsion with 10% Tween 80° or with 5% Solub'Oil and heated for 4 h at 45°C reached ca. 40% relative viability (see Figure 4(a)). Although experiments were conducted on different occasions with different purposes and, therefore, not compared statistically with each other, an apparent difference in results can be observed. We assume this difference may have been caused by the use of a different batch of products for conducting the fourth experiment (depicted in Figure 4) reported in this study, once the method is well established and at least four independent repetitions were conducted for each test.

In conclusion, the combination of dried *Metarhizium* spp. conidia with pure oils afforded substantial protection of conidia against heat stress, even when these oils were mixed with surfactants to prepare oil-in-water emulsions. Commercial emulsifiable oils and CMC gel did not protect conidia from high temperature. The preparation of stable oil-in-water emulsions seems to be a suitable formulation strategy that may not only enhance the efficacy of fungi by improving their spray dispersal and conidial adhesion on host cuticle, but also by increasing conidial tolerance to heat. All of these considerations may possibly improve the performance of *Metarhizium*-based mycopesticides in control programs of arthropod pests.

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Disclosure statement

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