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Emulsifiable oils protect *Metarhizium robertsii* and *Metarhizium pingshaense* conidia from imbibitional damage

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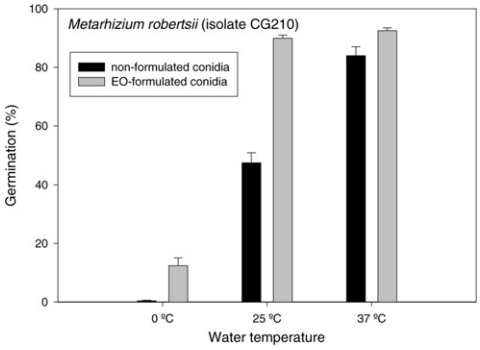
HIGHLIGHTS

- *Metarhizium robertsii* and *Metarhizium pingshaense* are sensitive to imbibitional damage (ID).
- Warm water prevents ID, but prolonged exposure harms conidia.
- Formulation with emulsifiable oils prevents damage to dry *Metarhizium* conidia.

GRAPHICAL ABSTRACT



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ABSTRACT

Imbibitional damage (ID) may take place when dehydrated cells are immersed in water and, within certain limits, damage to the plasma membrane is inversely proportional to the immersion temperature. Dried *Metarhizium anisopliae* conidia formulated in pure oil are not severely impacted when rehydrated in a cold agar-based substrate, whereas dried, unformulated conidia experience reduced viability when immersion temperatures ⩽25 °C. The primary objective of this study was to assess whether conidia of *Metarhizium robertsii* and *Metarhizium pingshaense* were sensitive to ID and, if so, determine whether oil dispersions (a formulation type in which fungal propagules are usually mixed with an emulsifiable oil prior to dilution in water) could be used to prevent this sensitivity. Unformulated *Metarhizium* conidia (isolates CG1091 and CG210) plunged into water equilibrated to different temperatures (0, 25, 31, 36, and 45 °C) experienced significant reduction of initial viability at 0 and 25 °C (and also 31 °C for one of the isolates) following a 2-min exposure time or after exposure to 45 °C for 60 min. Dry, unformulated conidia plunged into cold water or water equilibrated to 25 °C were physically harmed by ID, whereas viability of conidia following prolonged exposure to warm water was also harmed. Therefore, use of warm water in field operations was shown not to be an advisable strategy to alleviate ID, unless temperature and immersion time are critically monitored. On the other hand, mycopesticide formulation in an emulsifiable oil was shown to be an useful alternative to prevent ID on dehydrated *M. robertsii* and *M. pingshaense* conidia. Indeed, dried conidia formulated as an oil dispersion and plunged into water at either 0 °C or 25 °C showed significantly higher viabilities than unformulated counterparts.

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1. Introduction

The inundative use of entomopathogenic Hypocreales over the past decades has been recently discussed (Jaronski, 2010). The fungus *Metarhizium anisopliae* is commercially deployed in different countries seeking biological control of agricultural insect pests, especially those in Curculionidae, Scarabaeidae and Cercopidae (Faria and Wraight, 2007). The use of dry conidia is likely to be a trend for mycopesticides, due to advantages such as facilitation of harvesting and formulation, limited contamination, lower shipping and storage costs, and increased storability (Faria et al., 2009). Nevertheless, rapid water uptake (imbibition) by dry conidia causes severe damage to plasma membranes, especially in *Metarhizium* species, which are highly susceptible to this phenomenon (Moore et al., 1997; Faria et al., 2009). Methods of dramatically reducing this imbibitional damage (ID) include slow rehydration of dry propagules prior to immersion into water or their immersion into warm water (Crowe et al., 1989, 1992; Hoekstra et al., 1997), although neither method would be reasonable or feasible under field conditions. Alternatively, dry conidia may be formulated in pure oil for ultra-low-volume applications (Faria et al., 2009); nevertheless most available mycoinsecticides are recommended for water-based applications, such as technical products (which include fungus-colonized substrates and pure conidia), wettable powders and oil dispersions. Oil dispersion (OD), a formulation type characterized by conidia mixed with a water-immiscible fluid and emulsifiers (to render the mixture miscible in water), is intended for dilution in water before spraying. In some markets there is an increasing demand for oil dispersions, explained by ease of use, intense hydrophobicity of conidia-containing oil droplets and consequently greater rainfastness (Inglis et al., 2000), improved UV tolerance (Alves et al., 1998) and protection against incompatible water-soluble chemical pesticides (Lopes et al., 2011). OD has been mistakenly referred to as emulsifiable suspensions (ES) or emulsifiable concentrates (CE), formulation types which are linked to seed treatments and solutions (and not suspensions), respectively (FAO/WHO, 2002; Michereff Filho et al., 2009). For instance, a recent survey indicated that approximately one quarter of mycoinsecticides sold in Brazil are oil dispersions (Michereff Filho et al., 2009), as opposed to 10 years earlier when this formulation type was not commercially available. In the US/Europe, the major Hypocrealean mycoinsecticides are also oil dispersions (S. Jaronski, pers. commun.). Therefore, the primary objective of this study was to determine whether sensitive *Metarhizium* conidia can be protected from ID by emulsifiable oils.

2. Materials and methods

2.1. Source of fungi and growth conditions

Two Brazilian isolates of *Metarhizium*, obtained as liquid nitrogen-stored cultures from EMBRAPA's Collection of Entomopathogenic Fungi, were tested: CG210 (=ARSEF 760) of *Metarhizium robertsii* was originally isolated from the chysomelid *Cerotoma arcuata*, whereas isolate CG1091 of *Metarhizium pingshaense* was obtained from the curculionid *Cosmopolites sordidus*. Mass production was carried out under laboratory conditions using cooked rice as a substrate. Rice was soaked in distilled water (dH_2O) + chloramphenicol (500 mg/L) and then autoclaved for 20 min in polyethylene bags (150 g rice per bag). Each bag was inoculated with 10 mL of a fungal suspension (ca. 1.0×10^7 aerial conidia per mL of Tween 80 at 0.05% v/v), and then incubated at 25 ± 0.5 °C in darkness. Bags were opened after 2 weeks and substrate allowed to air dry for 24 h prior to harvesting. Aerial conidia were harvested by manually shaking the fungus-colonized substrate through a series of two sieves, 20 and 100 mesh. Conidia that passed through the 100-mesh sieve (150 μm pore size) were collected. The stock technical powders were stored at −20 °C.

2.2. Viability assessments: determination of minimum incubation time for dry and moist conidia

Conidial powders (ca. 0.1 mg) of the two fungal isolates were incubated in 150-mL hermetically sealed glass jars (Nadir Figueiredo, Sao Paulo, Brazil) containing either silica gel, saturated NaOH solution or distilled water for 3 days at 25 ± 0.5 °C. For viability determinations, conidial powders were transferred to glass tubes containing water (Tween 80 at 0.05% v/v) equilibrated to 37 °C in order to avoid imbibitional damage (Faria et al., 2009). Then, 20 μL droplets of suspension (ca. 2.0×10^5 conidia/mL) were pipetted onto $1 \times 1 \times 0.3$ cm blocks (1 droplet/block) of solid nutrient medium adjusted to pH 7.0 (Milner et al., 1991). Blocks were incubated on glass microscope slides in sealed Petri dishes in darkness at 25 °C. Following different incubation times (15, 18, 21, 24, and 27 h post-inoculation (h.p.i.)), samples were examined at 400× magnification in order to determine the minimum incubation time required to yield full germination counts. Conidia were considered germinated if a germ tube larger than conidial length was visible. Each treatment was replicated three times, and a minimum total of 200 conidia were examined in several microscope fields for each replicate suspension of each experimental treatment. In all experiments, germination counts were recorded from independent samples (not repeated measures).

2.3. Effect of water temperature and exposure time on dry conidia

Conidia were dried over silica gel for 3 days at 25 °C, then suspended in 0.05% aqueous Tween 80 at 0, 25, 31, 36, or 45 °C to a final concentration of ca. 2.0×10^5 conidia/mL. An ice-bath and a water bath were used for adjusting temperatures to 0 and 45 °C, respectively, whereas other treatments were kept in incubators (Marconi, model 404, Piracicaba, Brazil). Conidial suspensions were kept at the target temperatures for either 2 or 60 min, and then exposed to a 2-min cycle in a bath-type ultrasonic washer (Unique, model USC-1800, Indaiatuba, Brazil) to create homogeneous suspensions. For each fungal isolate (CG210 and CG1091), each treatment was replicated four times. Viability assessments were performed 27 h.p.i. following incubation of agar blocks in parafilmed Petri dishes in darkness at 25 °C as described for the previous experiment. Additionally, 20 μL of conidial suspensions, exposed to 45 °C water for either 2 or 60 min, were inoculated onto agar nutrient medium amended with the fungistatic compound carbendazim (Derosal®, Bayer, Sao Paulo, Brazil) at 50 μL/mL, and germination assessed 48 h.p.i.

2.4. Impact of oil dispersions on imbibitional damage mitigation

Conidia were dried over silica gel for 3 days at 25 °C. A portion was then mixed with the emulsifiable oil (EO) Natur'L Óleo (Stoller do Brasil, Cosmopolis, Brazil) to a final concentration of ca. 1.0×10^7 conidia/mL, and 1 mL of each suspension was subsequently mixed with 9 mL of water (0.05% Tween 80) held at 0, 25, or 37 °C for a 2-min exposure time. A germination protocol adapted from Oliveira (2009) allowed EO removal from conidial suspensions through use of the surfactant Solub'oil (General Chemicals, Campo Mourao, Brazil): conidial suspensions were mixed with the surfactant Solub'oil (100 μL per mL EO). The suspension was centrifuged at 1200 rpm for 5 min and the supernatant phase was discarded, whereas ca. 1 mL of conidia-containing pellet was added to 9 mL of 0.05% Tween 80 + 100 μL Solub'oil. The mix was centrifuged once more and the pellet was resuspended in 9 mL of

0.05% Tween 80 and plated on agar medium following a 2-min sonification cycle as previously mentioned. Blocks were incubated on glass microscope slides in sealed Petri dishes in darkness at 25 °C and viability was determined 24 h.p.i., as previously explained in Section 2.2. Each treatment was replicated four times and performed with one batch of isolate CG1091 and two batches of isolate CG210 produced on different dates (batches A and B).

2.5. Statistical analyzes

For all experiments, three to four replicate samples of conidia were prepared. Subsamples were assigned to the various treatments in a randomized complete block design. Percent germination data were normalized by arcsine transformation and analyzed by one- or two-way analysis of variance (ANOVA), depending on the experiment. Means were compared by Tukey–Kramer HSD or *t*-test ($\alpha = 0.05$). Statistical analyzes were performed using the JMP statistical software package (SAS Institute Inc., Cary, USA).

3. Results

3.1. Viability assessments: determination of minimum incubation times for dry and moist conidia

Initial quantity of water (water content) in conidia had a significant impact on germination rates of both fungal isolates (Fig. 1). By 15 h.p.i. germination rates were <5% for dry CG1091 conidia (3.8% and 4.8% for conidia dried over silica gel and NaOH, respectively) vs. 83% for moist conidia ($F_{2,6} = 45.2$, $P < 0.001$). Significant differences were also recorded at 18 ($F_{2,6} = 43.0$, $P < 0.001$) and 21 h.p.i. ($F_{2,6} = 31.0$, $P < 0.001$). No significant differences were observed by 24 h.p.i. ($F_{2,6} = 1.4$, $P = 0.30$), however, only a marginally significant difference was observed by 27 h.p.i. ($F_{2,5} = 6.3$, $P = 0.04$). Germination speed for conidia dried over silica gel and saturated NaOH solution did not differ during any tested incubation time (*P*-value ranging from 0.06 to 0.94). Maximum germination rates of (initially) dry CG1091 conidia, in the 90–91% range, were reached by 24 h.p.i., whereas germination rates for (initially) moist conidia reached 97% at the same incubation time. When comparisons were performed using similar initial moisture content, longer incubation times significantly increased conidial viabilities for all treatments. For conidia dried over silica gel, germination rate by 24 h.p.i. was 92% and except at 27 h.p.i. (90%) was significantly superior to other incubation times ($F_{4,9} = 96.7$, $P < 0.0001$). For conidia dried over saturated NaOH solution, germination rate by 24 h.p.i. was 91% and differed statistically from counts performed at 15 and 18 h.p.i. ($F_{4,10} = 52.1$, $P < 0.0001$); nevertheless, it was similar to germination by 27 h.p.i. Finally, germination rates for moist CG1091 conidia reached the 96–97% plateau in counts performed from 18 h.p.i. onwards, differing from count at 15 h.p.i. ($F_{4,10} = 11.2$, $P = 0.001$).

The initial water content of conidia had a similar dramatic impact on germination dynamics for isolate CG210 (Fig. 1). By 15 h.p.i. germination values were <25% for dry conidia (24.0% and 13.7% for conidia dried over silica gel and NaOH, respectively) vs. 85% for moist conidia ($F_{2,6} = 218.3$, $P < 0.0001$). Significant differences were also recorded at 18 ($F_{2,6} = 42.3$, $P < 0.001$), 21 ($F_{2,6} = 16.7$, $P < 0.01$) and 24 h.p.i. ($F_{2,6} = 92.0$, $P < 0.0001$), by which time germination of the initially dry conidia was in the 85–89% range vs. 99.5% for moist conidia. At 27 h.p.i. counts were not possible for moist CG210 conidia due to excessive hyphal growth, whereas germination of dry conidia reached 93–95%, with no difference observed between drying over silica gel or a NaOH saturated solution ($F_{1,4} = 0.77$, $P = 0.43$). Except for 15 and 24 h.p.i., in which germination speed for conidia dried over silica gel was slightly slower than for those dried over saturated NaOH solution ($F_{1,4} = 15.94$ and 22.13, and $P = 0.02$ and 0.01, respectively), differences between these two drying methods were not statistically significant (*P*-values $\geqslant 0.38$). For comparisons within similar initial moisture content, germination for conidia dried over silica gel was 93% by 27 h.p.i. and, except for 24 h.p.i., differed significantly from viabilities at other incubation times ($F_{4,10} = 42.1$, $P < 0.0001$). For conidia dried over saturated NaOH solution, germination by 27 h.p.i. was 95% and higher than all other incubation times ($F_{4,10} = 144.7$, $P < 0.0001$). Germination for moist CG210 conidia was 85% by 15 h.p.i. and reached the 96–99.5% plateau in counts performed from 18 h.p.i. onwards ($F_{3,8} = 11.4$, $P < 0.01$).

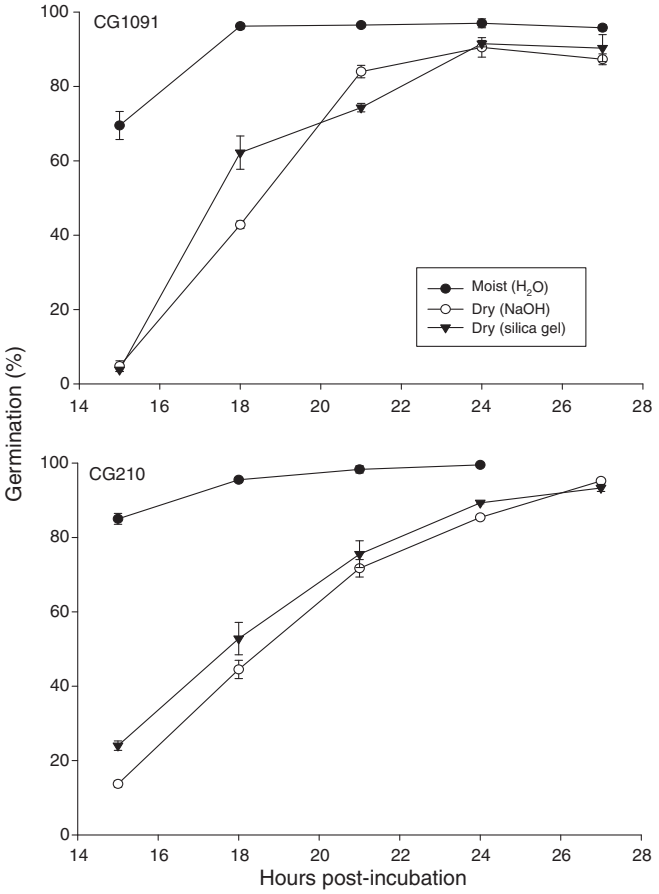

Fig. 1. Effect of initial water content of conidia and incubation times on germination rates (±standard error) of two *Metarhizium* isolates. Conidia were dried over silica gel or a saturated NaOH solution, or hydrated over water for 3 days at 25 °C before plating on solid nutrient medium followed by incubation at 25 °C. For isolate CG1091 (*M. pingshaense*), differences by 24 and 27 h.p.i were insignificant ($F_{2,6} = 1.4$, $P = 0.30$) or marginally significant ($F_{2,5} = 6.3$, $P = 0.04$), respectively. For isolate CG210 (*M. robertsii*), germination values for moist conidia were higher in all tested incubation periods (at 27 h.p.i., counts were not possible for moist conidia due to excessive hyphal growth).

Germination delay due to drying of CG1091 conidia over silica or saturated NaOH solution compared to moist conidia was calculated as 4.6 and 6.9 h, respectively, based on the time (15 h) required for initially moist conidia to reach 69.5% germination (cubic polynomial regressions not shown). For isolate CG210, germination delays following drying over silica gel or saturated NaOH solution were 7.6 and 8.8 h, respectively, based on the time (15 h) required for initially moist conidia to reach 85% germination. Based on time required for initially moist CG210 conidia to reach a germination of 95.5% (18 h), delay times were 8.8 and 9.0 h for conidia

dried over silica gel and NaOH, respectively. For both isolates, germination delays decreased from 21 h.p.i. onwards as predicted from Fig. 1.

3.2. Effect of water temperature and exposure time on dry conidia

An exposure time × water temperature treatment interaction was detected for isolate CG1091 ($F_{4,27} = 38.9$, $P < 0.0001$). The main effect of exposure time was highly significant ($F_{1,27} = 32.5$, $P < 0.0001$). Increasing exposure time from 2 to 60 min varied germination rates by averages of ⩽10% for conidia suspended in water equilibrated to ⩽36 °C, but germination counts were decreased by 79% for water at 45 °C (Table 2). The main effect of water temperature was highly significant ($F_{4,27} = 188.7$, $P < 0.0001$). Increasing water temperature from 0 to 36 °C increased germination rates by average of 86% (by 42% for 45 °C). Within the 2-min imbibition time, germination was significantly reduced at 0 and 25 °C ($F_{4,12} = 120.24$, $P < 0.0001$). When imbibition time was increased to 60 min, germination at 0 and 45 °C was significantly affected ($F_{4,15} = 116.63$, $P < 0.0001$).

Regarding isolate CG210, a two-way interaction (exposure time × water temperature) was also detected ($F_{4,27} = 40.6$, $P < 0.0001$). The main effect of exposure time was significant ($F_{1,27} = 15.7$, $P < 0.001$). Increasing exposure time from 2 to 60 min increased germination by averages of <9% for water ⩽36 °C, but germination counts were decreased by 62% for water at 45 °C (Table 2). The main effect of water temperature was highly significant ($F_{4,27} = 404.0$, $P < 0.0001$). Increasing water temperature from 0 to 36 °C increased germination rates by average of 95% (by 64% for 45 °C). Within the 2-min imbibition time, germination percentages were significantly reduced at 0, 25 and 31 °C compared to treatments in which water was warmed to 36 or 45 °C ($F_{4,15} = 323.27$, $P < 0.0001$). When imbibition time was increased to 60 min, germination percentages at 0, 25 and 45 °C were significantly affected ($F_{4,12} = 157.51$, $P < 0.0001$).

In the experiment in which incubation times following plating were investigated (27 vs. 48 h.p.i. at 25 °C), no significant differences were found for dry conidia of either fungal strain when they were previously exposed to water at 45 °C for 2 min ($F_{1,6} = 4.95$, $P = 0.07$ for isolate CG1091; $F_{1,5} = 3.51$, $P = 0.12$ for isolate CG210), as shown in Table 1. However, when exposure time was increased to 60 min, germination differed between incubation times for isolates CG1091 ($F_{1,6} = 8.46$, $P = 0.03$) and CG210 ($F_{1,5} = 7.11$, $P = 0.045$).

Table 1
Mean percent germination (±standard error) for isolates CG1091 (*M. pingshaense*) and CG210 (*M. robertsii*) following exposure of dry conidia to water at 45 °C for either 2 or 60 min.

Exposure time (min)	Isolate CG1091		Isolate CG210	
	Incubation time (h)	Germination (%)	Incubation time (h)	Germination (%)
2	27	81.1 ± 0.94 a	27	95.0 ± 0.37 a
	48	74.1 ± 2.46 a	48	92.2 ± 0.63 a
60	27	2.1 ± 0.30 a	27	33.3 ± 6.40 a
	48	0.5 ± 0.24 b	48	58.8 ± 3.10 b

For each fungal isolate and exposure time, averages within columns followed by the same letter indicate that no statistical difference was observed according to *t*-test ($\alpha = 0.05$). Viability at 27 h.p.i. following inoculation of conidial suspension onto agar medium, whereas for determinations at 48 h.p.i. medium was amended with the fungicide carbendazim.

Table 2
Influence of immersion temperature and exposure time on germination of dry *Metarhizium pingshaense* (isolate CG1091) and *M. robertsii* (isolate CG210) conidia.

Immersion temperature (°C)	Mean percent germination (±standard error)			
	Isolate CG1091		Isolate CG210	
	2 min	60 min	2 min	60 min
0	0 c	0 c	0 c	0 d
25	73.3 ± 1.00 b	80.8 ± 0.63 a	80.0 ± 1.33 b	83.9 ± 1.25 b
31	85.8 ± 1.79 ab	88.8 ± 3.77 a	82.8 ± 1.81 b	91.5 ± 0.60 ab
36	91.3 ± 2.18 a	81.1 ± 1.02 a	93.1 ± 0.80 a	96.3 ± 0.51 a
45	81.1 ± 0.94 ab	2.1 ± 0.30 c	95.0 ± 0.37 a	33.3 ± 6.40 c

Within each column, averages with different letters indicate significant differences among treatments (Tukey test, $\alpha = 0.05$).

3.3. Impact of oil dispersions on imbibition damage

A formulation type × water temperature treatment interaction was detected for isolate CG1091 ($F_{2,18} = 20.0$, $P < 0.0001$). The main effect of formulation type was highly significant ($F_{1,18} = 127.2$, $P < 0.0001$). When dry conidia were formulated with an emulsifiable oil prior to mixing with water, germination counts increased by an average of 21% compared to the unformulated conidia, although increases at specific temperatures were even greater (48% at 25 °C) (Table 3). The main effect of water temperature was highly significant ($F_{2,18} = 555.1$, $P < 0.0001$). Increasing water temperature from 0 to 37 °C increased germination rates by an average of 80%. Germination percentages for dry CG1091 conidia formulated as an oil dispersion before addition to water were highly significant compared to unformulated conidia at 0 °C ($F_{1,6} = 73.8$, $P < 0.0001$) and 25 °C ($F_{1,6} = 84.4$, $P < 0.0001$), and only marginally significant at 37 °C ($F_{1,6} = 6.2$, $P = 0.047$).

For isolate CG210, experiments were performed for two different batches, A and B. For batch A, results almost identical to those observed for isolate CG1091 were recorded (Table 3). A formulation type × water temperature treatment interaction was detected ($F_{2,18} = 4.5$, $P = 0.03$). The main effect of formulation type was highly significant ($F_{1,18} = 34.6$, $P < 0.0001$). Germination counts for EO-formulated conidia increased by an average of 21% compared to unformulated conidia, although increase as high as 43% was

Table 3
Influence of water temperature and formulation type (unformulated vs. EO-formulated conidia) on imbibitional damage in *Metarhizium pingshanese* (isolate CG1091) and *M. robertsii* (isolate CG210).

Water temperature (°C)	Mean percent germination (±standard error)					
	Isolate CG1091		Isolate CG210 (batch A)		Isolate CG210 (batch B)	
	Unformulated	EO-formulated	Unformulated	EO-formulated	Unformulated	EO-formulated
0	0 a	6.5 ± 0.74 b	0.4 ± 0.19 a	12.4 ± 2.65 b	8.1 ± 2.44 a	36.5 ± 0.72 b
25	32.9 ± 2.59 a	80.9 ± 0.48 b	47.4 ± 3.41 a	89.9 ± 1.10 b	52.4 ± 4.82 a	75.3 ± 2.51 a
37	79.0 ± 1.27 a	87.4 ± 1.04 b	84.0 ± 3.04 a	92.5 ± 0.95 a	66.1 ± 1.65 a	70.3 ± 2.21 a

Dry conidia were either plunged directly into water (0, 25 or 37 °C) or mixed with an emulsifiable oil (EO) prior to addition of water at different temperatures for a 2-min exposure time. Within each water temperature and fungal batch, averages (±standard error) with different letters indicate significant difference between treatments (*t*-test, $\alpha = 0.05$).

observed when water temperature was adjusted to 25 °C. The main effect of water temperature was highly significant ($F_{2,18}$ = 154.3, P < 0.0001). Increasing water temperature from 0 to 37 °C increased germination rates by an average of 82%. Germination percentages for dry conidia formulated as an oil dispersion before addition to water were significantly higher compared to unformulated conidia at 0 °C ($F_{1,6}$ = 12.1, P = 0.01) and 25 °C ($F_{1,6}$ = 39.4, P < 0.0001), but insignificant at 37 °C ($F_{1,6}$ = 1.3, P = 0.30). For batch B, a formulation type × water temperature treatment interaction was marginally insignificant ($F_{2,17}$ = 3.2, P = 0.07). The main effect of water temperature was highly significant ($F_{2,17}$ = 29.7, P < 0.0001) (Table 3), and increasing water temperature from 0 to 37 °C increased germination rates by an average of 46%. Germination counts for EO-formulated conidia increased by an average of 19% compared to unformulated conidia ($F_{1,17}$ = 15.1, P = 0.001). Germination of dry conidia formulated as an oil dispersion before addition to water was significantly higher compared to unformulated conidia at 0 °C ($F_{1,5}$ = 9.5, P = 0.03), marginally insignificant at 25 °C ($F_{1,6}$ = 4.41, P = 0.08) and highly insignificant at 37 °C ($F_{1,6}$ = 0.60, P = 0.47).

4. Discussion

Both *M. robertsii* and *M. pingshaense* conidia were shown to be sensitive to ID, as previously was demonstrated for *Metarhizium acridum* (Moore et al., 1997) and *M. anisopliae* (Faria et al., 2009). Formulation of ID-sensitive conidia in emulsifiable oils prevents physical damage to these fungal propagules during preparation of water-based suspensions. Dried, unformulated conidia of these fungi plunged into cold water or water equilibrated to 25 °C were affected by ID. On the other hand, dried EO-formulated conidia plunged into water at either 0 °C or 25 °C usually showed significantly higher viabilities compared to their unformulated counterparts, although the protective effect of oil dispersions (OD) at 0 °C was quite limited.

It has been shown that rice-grown *M. anisopliae* conidia with water activity ⩽0.333 (equivalent to water content ⩽8.2%) displayed germination percentages of 41% and 76% when plunged into water at 15 and 25 °C, respectively, whereas viabilities of moist conidia plunged in water at the same temperatures were >90% (Faria et al., 2009). Given the fact that *Metarhizium*-based mycoinsecticides may be sold as partially dry, unformulated products with ⩽6% water content (Jenkins et al., 1998; Cherry et al., 1999; Grimm, 2001), and that water temperatures are not controlled by farmers when fungal suspensions are prepared (and therefore, temperatures ⩽25 °C may be used), it is reasonable to assume that impact of ID on efficiency of *Metarhizium* conidia may help explain, at least partially, poor control of pests under field conditions. For instance, reports on the inefficiency of *Metarhizium*-based products with unspecified water content for control of spittlebugs are not uncommon (Carneiro, 1988; Dinardo-Miranda et al., 2004; Peixoto et al., 2009). Under field conditions, one should not expect farmers to slowly rehydrate dry mycoinsecticides or warm water prior to addition of dry mycoinsecticides, especially in large operations when the amount of fungal material is considerable. Low viabilities are also expected following broad adoption of active packaging for ID-sensitive conidia, in which fungal propagules may be dehydrated to water activity levels <0.03 due to action of moisture-absorbing sachets inside packages (Faria, 2009).

This study has also shown that use of warm water may be deleterious to conidia depending on temperature and exposure time. Although warm water can be used to mitigate imbibitional damage, attention should also be paid to exposure time. Whereas a 2-min exposure time was not detrimental when temperatures were ⩽45 °C, a 1-h exposure at 45 °C was enough to reduce initial viabilities by 62–79%, depending on isolate. The detrimental effect of immersion at 45 °C was measured in terms of conidial debilitation. As previously shown, conidia with generally lower viabilities tend to have a higher proportion of debilitated conidia, characterized by hypersensitivity to ID and slow germination (Faria et al., 2010). For isolate CG210, germination counts at 48 h.p.i. were significantly higher compared to 27 h.p.i. for conidia exposed to 45 °C for 1 h. reinforcing the fact that a proportion of conidia became debilitated. For isolate CG1091, the conidial powder had a lower quality than for isolate CG210, and exposure at 45 °C for 1 h killed the vast majority of conidia, which would explain the very low germination (⩽2.1%) for both incubation times. Whereas ID is physical damage with instantaneous lethal action on unicellular organisms, conidial debilitation is a physiological phenomenon usually associated to stressful conditions, such as exposure to elevated air (or suspension) temperatures.

It is clear that ways to cope with ID for sensitive conidia are very important, and proper formulation of conidia may be regarded as a feasible approach. The reasons for increasing adoption of ODs in mycopesticide formulation have been discussed elsewhere (see Section 1). Although OD may help prevent imbibitional damage in *Metarhizium* isolates, in many instances its adoption is not commercially attractive, because of technical reasons within mycoinsecticide companies (production process, availability of harvesting equipments, etc.) or for market-driven orientation. Therefore, development of additional formulations that confer ID-tolerance is required. Likewise, the effect of substrate and growth conditions on conidial susceptibility to ID is another important topic that needs detailed investigation, since we have observed that rice-grown conidia are significantly more sensitive to ID than PDA-produced conidia (M. Faria and colleagues, unpublished data). Rice-grown conidia dried to very low water activity levels usually show germination rates in the 65–75% range and >90% when immersed in water at 25 and 33–37 °C, respectively, according to this study and also Faria et al. (2009). The lower-than-expected germination percentages for unformulated conidia (33–52%, depending on conidial batch; see Table 3) could be explained by the fact that batches used in this particular experiment were low-quality ones, with highest germination percentages for each batch varying from 66% to 84% when water temperature was adjusted to 37 °C. As discussed before, low-quality conidial powders usually have a considerable proportion of debilitated conidia, which are more sensitive to imbibitional damage than "healthy" (non-debilitated) ones and, therefore, a greater proportion of *Metarhizium* conidia plunged at water regulated to 25 °C was likely killed by ID.

Drying conidia over silica gel and saturated NaOH solution have been reported to reduce water activity to 0.03 (Pedreschi and Aguilera, 1997) and 0.069 (3.5% mc) (Faria et al., 2009), respectively. In our study, germination speeds for conidia dried by these two treatments were usually the same. Based on previous experience, moist conidia used in our study were likely to have water activity >0.960 (>40% mc). Similar germination times for moist *Metarhizium* conidia from solid nutrient media with water activity ⩾0.99 were recorded by Andersen et al. (2006). We calculated that germination delays for dry conidia used in our study varied from 4.6 to 9.0 h compared to moist conidia, depending on the isolate. Ungerminated *Isaria fumosorosea* conidia were slightly less virulent than swollen conidia (harvested 8 h.p.i. of non-germinated aerial conidia in liquid medium) and significantly less virulent and showed greater LT_{50} than germinated conidia (harvested 13–17 h.p.i. in liquid medium) in bioassays with *Spodoptera frugiperda* (Fargues et al., 1994), reinforcing that germination speed is an important trait related to efficacy. Hassan et al. (1989) have reported that *Metarhizium* conidia soaked for 20 h in distilled water exhibited faster germination and appressorial formation, as well

as higher virulence toward *Manduca sexta* larvae, compared to freshly harvested, non-germinated conidia. Germination speed of dried submerged *M. acridum* conidia was retarded compared to fresh submerged spores (Kassa et al., 2004). On the other hand, in field experiments carried out at high humidity, *M. acridum* conidia dried over silica gel were as virulent toward the desert locust (*Schistocerca gregaria*) as moist conidia, and speed of kill was not affected (Moore et al., 1997). So, it is clear that the effect of drying of fungal propagules on virulence and speed of kill deserves further studies.

The implications of delayed germination of dried conidia for viability protocols pose great concern. Germination protocols proposed in the past did not take into account the standardization of initial water content of conidia, and incubation periods were usually established based on practicality (Goettel and Inglis, 1997; Jenkins et al., 1998). However, as demonstrated in this study, the initial water content has a dramatic impact on germination counts depending on time of assessment. Minimum (recommended) incubation times at 25 °C required for full germination of moist conidia was 18 h, and 24–27 h for initially dry conidia. Faria et al. (2010) have shown that viability assessments at longer than recommended incubation periods are undesirable, since debilitated conidia may be included in counts. For high-quality, moist conidia, germination counts performed at the minimum incubation time (e.g., 18 h.p.i.) are not expected to be significantly different from counts at 24 or 48 h.p.i. On the other hand, counts performed at longer than recommended incubation periods for low-viability batches are likely to be inflated by inclusion of debilitated conidia, erroneously suggesting that concentration of "healthy" active propagules in a given mycopesticide is greater than it actually is. An interesting piece of work that clarifies the lower virulence of debilitated conidia was carried out by Alves et al. (1996). In their shelf-life study with *Beauveria bassiana*, viability was 100% for unformulated conidia stored for 7 years either in a freezer or a refrigerator, although germination dynamics was quite different between these two treatments. For conidia kept in the freezer, viability was 100% in counts performed 24 h.p.i., whereas for those kept in the refrigerator the viabilities were 0% and 100% at 24 and 72 h.p.i., respectively. In other words, proportions of debilitated conidia in powders kept in freezer and refrigerator were nil (or minimal) and 100%, respectively. When bioassayed against *Diatraea saccharalis* and *Solenopsis saevissima*, mortality percentages and LT_{50}s considerably favored conidia stored in the freezer. Debilitated conidia killed 26% of treated 3rd instar *D. saccharalis* larvae with an LT_{50} of 12.6 days, whereas healthy conidia killed 100% of treated insects with an LT_{50} of only 3.7 days. Altogether, these results underline the importance of controlling incubation times in order to exclude debilitated conidia from counts, as well as standardizing the initial water content of conidia, preferably by drying, since debilitated conidia germinate faster if moist and, therefore, they could inflate viability counts even if minimum incubation times determined for dried conidia were used. This approach would help prevent the commercialization of low-quality mycopesticides, as previously discussed by Faria et al. (2010). According to these authors, optimal incubation times are species- and strain-specific, being 16–18 h for isolate GHA of *B. bassiana* and ca. 20 h for isolate CB-10 of *M. anisopliae* and, as shown in this study, about 24–27 h for isolates CG1091 (*M. pingshaense*) and CG210 (*M. robertsii*). However, it is important to point out that we did not use fresh conidia in this study, and although final germination counts were very high, minimum incubation times for fresh CG1091 and CG210 conidia could have been shorter.

The deleterious effect of rapid drying on subsequent conidial longevity was previously reported in studies with *M. acridum* (Hong et al., 2000). Although differences in full germination counts were not always significant, readings for conidia dried over silica gel were usually 7–10% less than for conidia hydrated in water vapor-saturated atmosphere, indicating that drying over desiccants may inactivate a small proportion of conidia. In studies under way in our laboratory, differences <5% have been seen for fresh, recently-produced batches of *Metarhizium* conidia (not published).

In conclusion, this work shows that ID is also an important phenomenon to species within the genus *Metarhizium* (*M. robertsii* and *M. pingshaense*) other than *M. anisopliae* and *M. acridum*, and it clearly reinforces the importance of standardizing viability protocols in order to avoid commercialization of low-quality mycoinsecticides. Moreover, we have shown the protection afforded by oil dispersions to ID-sensitive conidia, a practical approach that could be used under field conditions as opposed to slow rehydration or warming up of water before addition of dry *Metarhizium* propagules.

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