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# Influence of some parameters on the germination assessment of mycopesticides

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ABSTRACT

The substantial negative impact of some parameters on the germination of low-quality conidia (high proportion of slow-germinating propagules) was demonstrated, whereas for high-quality batches their effect was small or even absent. Germination was increased as the initial hydration status of conidia immediately prior to suspension preparation was increased, being ca. 33% and 80% for dehydrated *Metarhizium anisopliae* propagules (water activity $\leqslant$0.314) from low- or high-quality batches after an 18 h incubation period, respectively, and 63% and 95% for hydrated propagules (water activity = 0.933). Germination of low-quality propagules also increased as the time dry conidia were kept in aqueous suspension prior to inoculation onto culture media (15 min, 3 or 24 h) or the incubation time at 25 °C before counts (18, 48 or 72 h) was increased. Depending on treatment conditions, average germination of low-quality conidia varied from 53% to 98%. On the other hand, germination for high-quality conidia was always $\geqslant$94%. Regarding the relative humidity (RH) of the incubation atmosphere, the average germination rates for low-quality conidia on Potato Dextrose Agar (PDA) in Petri plates was 49%, while germination of these conidia on PDA blocks kept under lower RH inside plastic boxes was $\leqslant$23%. Use of lactophenol-staining and/or use of coverslips had a negative effect when germination assessment was performed for low-quality conidia, resulting in distorted counts or increased standard deviations compared to high-quality conidial batches. The occurrence of dislodged conidia (ungerminated conidia outside the inoculation zone due to hydraulic pressure exercised by addition of stains and/or coverslips added to the substrate by the time germination is assessed) was common place, whereas dislodged conidia were not seen in treatments with high-quality batches. This work underscores the importance of a number of parameters that anyone working with low-quality fungi needs to be cognizant of in their research.



## 1. Introduction

The commercialization of mycopesticides has increased dramatically worldwide in recent years (Harman et al., 2010; Li et al., 2010). Constant improvement in quality is a *sine qua non* condition to continue the expansion of these markets. The most used criterion to assess quality of mycoinsecticides is the concentration of active ingredient which is usually expressed as the total number of propagules per volume/weight and their viability (Jenkins and Grzywacz, 2000). Aerial conidia are the active ingredient in nearly 70% of the >170 commercial mycoinsecticides developed over the past four decades, 75% of which still in the market (Faria and Wraight, 2007). Protocols to assess conidial germination that are routinely used in R&D laboratories working with fungal pathogens are assumed to reflect conidial quality accurately. Some of the many factors that influence conidial germination of entomopathogenic fungi include the pH and composition of media (James, 2001; Ypsilos and Magan, 2005), water availability (Chandler et al., 1994, Luz and Fargues, 1997), age (Smith and Edgington, 2011), hydration level of the cells (Faria et al., 2010; Kassa et al., 2004), incubation temperature (Liu et al., 2003, Luz and Fargues 1997, Yeo et al., 2003), atmospheric RH during incubation (Kope et al., 2008, Luz and Fargues 1997), incubation time prior to assessing germination (Alves et al., 1996; Faria et al., 2010), temperature of water used in suspensions with imbibitional damage-sensitive conidia (Faria et al., 2009), and length of germ tube considered in assessments (Dantigny et al., 2006).

Many publications refer to germination protocols for assessment of fresh conidia produced under laboratory conditions, usually displaying elevated water content and viability (Milner et al., 1991; Braga et al., 2001; Andersen et al., 2006; Mohan et al., 2007). However, the usage of these and other protocols for aged samples or those exhibiting lower viabilities have not been deeply investigated. Recently, Faria et al. (2010) demonstrated that a substantial proportion of conidia exposed to stressful conditions become debilitated (low-vigor), showing slow germination and higher sensitivity to imbibitional damage compared to conidia from high-quality batches. In their study, high-quality *Beauveria bassiana* conidia exhibited similar germination on artificial medium by 24 and 72 h post-inoculation (hpi), and only ca. 2% of conidia were sensitive to imbibitional damage. On the other hand, germination for

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low-vigor conidia was14% and 44% for counts performed by 24 and 72 hpi, respectively, and >40% of dehydrated propagules were killed by imbibitional damage following immersion into water.

The ways that spores are obtained, stored, prepared, inoculated and grown have a substantial effect on the germination kinetics (Dantigny et al., 2006). Under some circumstances, quality of mycopesticides can be harmed by factors such as production process and drying of propagules (Guijarro et al., 2006; Jackson et al., 1997; Stephan and Zimmermann, 1998), or exposure to high temperatures during transportation or storage (Costa et al., 2002; Hong et al., 1999; Jenkins and Grzywacz, 2000). Therefore, germination assessment of conidial batches should be accurately performed to guarantee the type of precise conclusions needed for both basic and applied research. The main objective of this work was to investigate the effect on germination assessments of parameters not always considered in germination protocols (especially for low-quality conidia), such as the initial hydration status of conidia, the time conidia are kept in aqueous suspension prior to inoculation onto culture media, the incubation time before counts, and the use of lactophenol-staining and/or use of coverslips. We hope to alert the scientific community about the need for their standardization in order to allow reliable and comparable results to be obtained despite conidial quality. Toward this goal, conidia of the entomopathogenic fungus *Metarhizium anisopliae* were used as a model in the present study.

## 2. Materials and methods

### 2.1. Inoculum source and preparation of conidial suspensions

The experiments were conducted with *Metarhizium anisopliae* (isolate CG57) conidia, identified according to Bischoff et al. (2009). Conidia were produced on commercial Potato Dextrose Agar (PDA; Acumedia, Lansing, USA), although cooked rice kept inside plastic bags with a cotton plug on one corner (for gas exchange) was used for mass production whenever large amounts of propagules were needed. High-quality conidia were obtained following growth at 25 °C in darkness for 13–15 d. Low-quality batches (usually with 30–70% germination) were obtained by exposing high-quality conidial powders to 64.4% relative humidity (RH) for three days at 25 °C in a glass jar over saturated $NaNO_2$ solution (Faria et al., 2010). For germination assessments, conidial powders were suspended in 0.05% Tween 80, and, unless stated otherwise, final concentration ranged from $2.0 \times 10^5$ to $1.0 \times 10^6$ conidia/mL. Besides, conidial suspensions were homogenized for 2 min in a bath-type ultrasonic washer (Unique, model USC-1800, Indaiatuba, Brazil) before plating.

### 2.2. Effect of stain addition for germination assessment

Conidial suspensions were homogenized and 20 μL were immediately pipetted at the center of Petri dishes with fresh PDA (hereafter known as the "inoculation zone"; 1 droplet/inoculation zone, each being 0.4–0.5 in diameter), without spreading. Water activity of fresh PDA was shown to be in the 0.980–0.985 range. Inoculated dishes were sealed with parafilm following water evaporation from suspension droplets and then incubated at 25 °C in darkness for 18 h. At the time germination counts were performed, a drop of lactophenol cotton blue (50 μl) was or was not added to each inoculation zone. A total of 300 conidia in different microscopic fields at 400× magnification were counted in each inoculation zone in each of the four replicates, and viability of conidia in clumps was not assessed. In the control treatment, conidial suspensions were diluted to $2.0 \times 10^4$ conidia/mL, and germination examined for all conidia deposited on inoculation zones (n varied from 436 to 629), without use of stain. Coverslips were not used in any of the experiments. The experiment was performed twice, with conidia from high and low-quality batches.

### 2.3. Effect of coverslip addition for germination assessment

Two volumes (20 or 100 μL) of conidial suspensions were deposited onto each inoculation zone. Fifteen minutes after inoculation of suspension droplets, Petri dishes were parafilmed and then incubated at 25 °C in darkness for 18 h. For these experiments, no stain was used, and conidial assessment was carried out either by direct observation of the medium without a coverslip or after placing a coverslip (18 × 18 mm) directly onto the inoculated plate. Readings were performed in different regions of inoculation zones (center and border), as well as outside the inoculation zones for treatments in which coverslips were adopted and dislodgement of conidia (movement of ungerminated conidia to the outside of inoculation zones due to hydraulic pressure exercised by addition of stains and/or coverslips) was seen. A total of 300 conidia were counted per treatment in each zone in each of the four replicates, although in one case the number of conidia outside the inoculation zone was only 171. The experiment was replicated twice with low-quality batches.

### 2.4. Effect of incubation in different containers (preliminary experiment)

This set-up was used for one high- and one low-quality batch. A drop of conidial suspensions was applied per inoculation zone on PDA blocks (1.0 × 1.0 × 0.4 cm) (1 droplet/block, no spreading necessary) mounted on glass slides (1 block/glass slide). Prior to incubation, blocks were exposed or not to a 30 min drying cycle inside a laminar flow cabinet for evaporation of water from droplets. The temperature and RH inside the cabinet were monitored with a data logger (Hobo U10, Onset Computer Corp., MA, USA). For incubation, block-containing slides were transferred to plastic boxes (11 × 11 × 3 cm; 1 slide/box), with or without a humidified filter paper (3 mL of sterile $H_2O$ per piece of paper) covering the bottom of each box. Eight Hobo U10 data loggers, programmed to record temperature and RH every 30 min, were kept inside parafilmed boxes with or without humidified filter paper. Additionally, 20 μL of each suspension were inoculated onto PDA in Petri dishes, without spreading and without evaporation of water from suspension droplets. Parafilmed boxes and Petri dishes were incubated at 25 °C for 18 h. For viability assessment, neither coverslips nor lactophenol cotton blue were used, and 300 conidia per inoculation zone in each replicate were examined at 400× magnification.

### 2.5. Effects of immersion time and incubation time

Either high- or low-quality conidia were subjected to dehydration in glass vials with silica gel for 3 days at 25 °C. Under these conditions, dehydrated conidia are expected to reach water activities of ca. 0.03–0.08 (Pedreschi and Aguilera, 1997; Xavier-Santos et al., 2011). In glass tubes, conidia were suspended in 0.05% aqueous Tween 80 at 37 °C in order to avoid imbibitional damage. Following a 2 min exposure at this temperature, conidia were kept in immersion for different periods of time (15 min, 3 or 24 h) in incubators regulated to 25 °C. Then, suspensions were homogenized with ultrasound and 20 μL were deposited per inoculation zone in Petri dishes, which were left uncovered for 30 min to allow evaporation of the droplet, and then parafilmed and incubated at 25 °C in darkness. Regular PDA medium was used for germination counts at 18 hpi, whereas PDA amended with a fungistatic concentration of carbendazim (25 μg/L) was used for counts at 48 and 72 hpi.

### 2.6. Effects of the initial hydration status of conidia

Either high- or low-quality conidia were subjected to dehydration to different water activity levels in glass vials with silica gel, LiCl or $MgCl_2.6H_2O$. Conidia were also hydrated by exposure to saturated $K_2SO_4$ solution. Following 5 days at 25 °C, water activity of samples was measured with a water activity meter (LabMaster-aw, Novasina, Pfäffikon, Switzerland) set to 25 °C. Then, conidia were suspended in 0.05% aqueous Tween 80 at 25 °C and kept in suspension for 15 min. Inoculation onto regular PDA medium and incubation conditions were the same as for the previous experiment, and germination counts were performed at 18 hpi. As in the previous experiment, a total of 300 conidia per inoculation zone in each replicate were examined in various microscopic fields at 400× magnification, without use of lactophenol cotton blue or coverslips by the time germination was assessed. A conidium was considered to have germinated only if a germ tube was at least as long as the length of an ungerminated conidium. Besides, each experiment was conducted twice with conidia from high-viability batches, and twice with low-viability batches.

### 2.7. Statistical analyses

For all experiments, most mean germination percentages and standard deviations reported in the results are based on four counts. Subsamples were assigned to the various treatments in a randomized complete block design, and germination counts performed following different incubation times were recorded from independent samples (not repeated measures). Percent germination data were normalized by arcsine transformation prior to analysis of variance (ANOVA). Means were compared by Tukey-Kramer HSD (alpha = 0.05). ANOVAs and mean comparisons were performed using the JMP statistical software package (JMP Software, 2007). Whenever significant experiment x batch quality interactions were not found, data from experiments repeated on different dates were grouped.

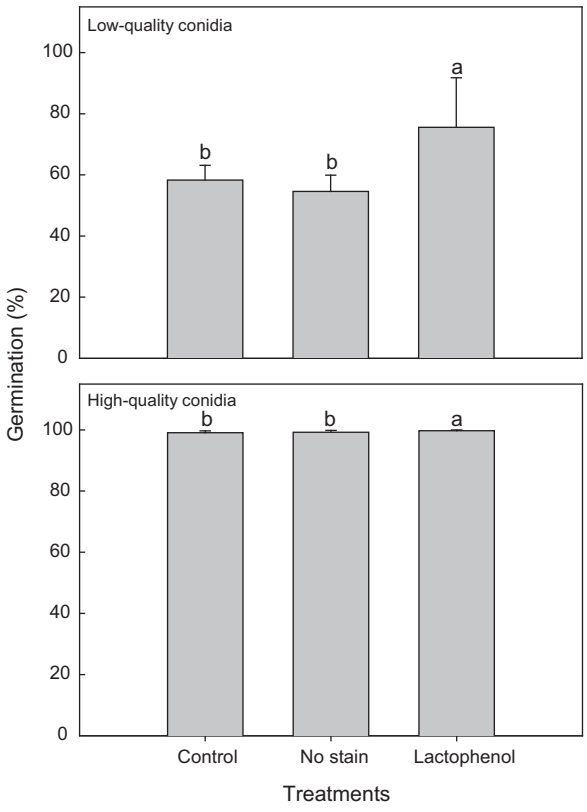


**Fig. 1.** Effect of stained mounting medium on germination counts (mean ± SD) by the time conidial viability is assessed. Treatments: 'Control' (inoculation of diluted suspension; all conidia per inoculation zone ($n$ = 436–629) were examined; neither a coverslip nor lactophenol cotton blue was added); 'No stain' (inoculation of concentrated suspension; 300 conidia counted per inoculation zone; neither a coverslip nor lactophenol cotton blue was added); 'Lactophenol' (inoculation of concentrated suspension; 300 conidia counted per inoculation zone; no coverslip; lactophenol cotton blue was used). Top graph: low-quality conidia; Bottom graph: high-quality conidia.

## 3. Results

### 3.1. Effect of stain addition for germination assessment

Three-way interaction (batch quality × use of stain × zone) was not significant ($F$ = 0.39; $d.f.$ = 1, 56; $P$ = 0.54). Marginally insignificant two-way interactions were observed for batch quality × zone ($F$ = 3.4; $d.f.$ = 1, 56; $P$ = 0.07) and use of stain × zone ($F$ = 3.3; $d.f.$ = 1, 56; $P$ = 0.08), whereas the batch quality × use of stain interaction was highly significant ($F$ = 16.4; $d.f.$ = 1, 56; $P$ = 0.0002). The main effect of zone (counts in center vs. border) was not significant ($F$ = 0.82; $d.f.$ = 1, 56; $P$ = 0.37). For high-quality conidia, germination means in the treatment without lactophenol (99.2 ± 0.60%) was only slightly lower than in the treatment with this stain (99.7 ± 0.22%; $F$ = 8.0; $d.f.$ = 1, 30; $P$ = 0.008). This difference was significant likely due to the small standard errors associated with the data (0.15 and 0.06, respectively), but it is biologically insignificant. On the other hand, germination for low-quality conidia in the treatment without lactophenol was considerably lower than in the treatment with lactophenol (54.6 ± 5.30% vs. 75.6 ± 16.19%; $F$ = 22.6; $d.f.$ = 1, 30; $P$ < 0.0001). When the control treatment was considered in the analyses, the treatment with stain was statistically higher than the two other treatments for either low- and high-quality conidia which, in turn, did not differ from each other (Fig. 1). Displacement of conidia to the outside of inoculation zones due to hydraulic pressure exercised by addition of lactophenol (Fig. 2) was quite common for low-quality batches. Germination outside inoculation zones varied from 0% to 0.3% for low-quality batches (data not shown) but, as expected, such leaks were not seen in experiments with high-viability conidia.

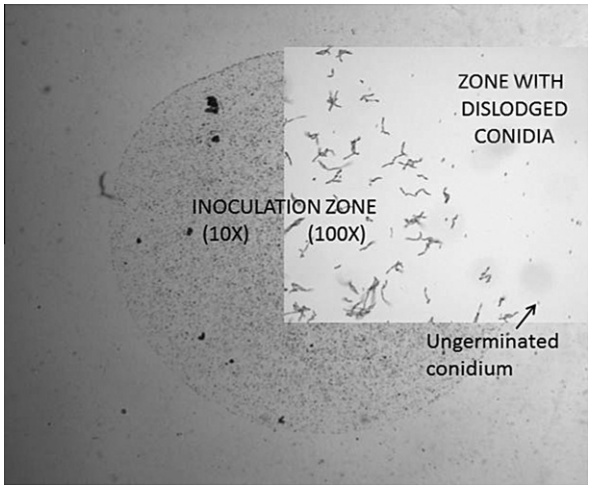


**Fig. 2.** Displacement of conidia to outside the inoculation zones due to hydraulic pressure exercised by addition of stain and/or usage of coverslips for germination assessment of conidia from low-quality batches. Magnifications were 10× (most of the inoculation zone and zone with dislodged conidia) and 100× (top right; portion of inoculation zone and zone with dislodged conidia).

### 3.2. Effect of coverslip addition for germination assessment

ANOVA of the data revealed lack of significant differences of the three-way (inoculation volumes × use of coverslip × zone) and all two-way interactions (all $P$ values $\geqslant 0.19$), as well as the main effect of inoculation volume (20 vs. 100 μL; $F = 2.3$; $d.f. = 1, 56$; $P = 0.13$) and zone (counts in center vs. border of inoculation zone; $F = 0.2$; $d.f. = 1, 56$; $P = 0.68$). On the other hand, use of coverslip had a significant effect on viability assessments ($F = 6.2$; $d.f. = 1, 56$; $P = 0.016$). Compared to the treatment without coverslip, use of coverslip was associated with higher overall germination counts (53.6% vs. 48.4%) and higher standard deviations (10.95% vs. 3.86%). Although significant differences among treatments were not seen within the inoculation zone, a trend of higher germination counts in treatments with coverslips was seen (Table 1). Besides, propagules dislodged by coverslips to outside the inoculation zone were ungerminated.

### 3.3. Effect of incubation in different containers (preliminary experiment)

Interaction between batch quality vs. treatment (incubation in plastic boxes with or without humidified filter paper) was significant ($F = 4.78$; $d.f. = 1, 52$; $P = 0.003$) and data were consequently analyzed independently for each batch type. For high-quality conidia, a two-factor ANOVA (drying prior to incubation, and relative humidity in plastic boxes) indicated lack of significance of all main effects and the 2-way interaction (all $P$ values $\geqslant 0.290$). When data from a standard treatment (Petri dishes with PDA) were considered in the analysis, significant differences were seen ($F = 3.72$; $d.f. = 2, 33$; $P = 0.035$). Overall germination counts for conidia in Petri dishes were marginally higher than for conidia kept in boxes with lower relative humidity (96.9 ± 1.62 vs. 93.6 ± 2.59). For low-quality conidia, the two-factor ANOVA revealed lack of significance for the 2-way interaction ($F = 1.19$; $d.f. = 1, 20$; $P = 0.29$). The main effects of drying prior to incubation in plastic boxes ($F = 7.2$; $d.f. = 1, 20$; $P = 0.014$) and the relative humidity inside boxes ($F = 11.4$; $d.f. = 1, 20$; $P = 0.003$) were significant. Drying of PDA blocks prior to incubation in boxes took place in a laminar flow at 29.4 ± 0.46 °C and 33.7 ± 1.32% RH, while conditions in the room outside the cabinet were slightly different (27.3 ± 0.22 °C and 37.2 ± 1.05% RH). Significant differences were also recorded when data from the control (Petri dishes with PDA) were considered in the analysis ($F = 30.02$; $d.f. = 2, 24$; $P < 0.0001$). Germination counts for low-quality conidia in Petri dishes were considerably higher than for conidia kept in boxes, with or without humidified filter paper, as shown in Table 2. Mean temperatures recorded by data loggers kept inside boxes in an incubator at 25 °C ranged from 24.8 to 25.0, independent of treatment, whereas RH was significantly affected by whether humidified filter paper was or was not put in the plastic boxes. In treatments with dry filter paper, RH 30 min after parafilm sealing of four boxes was 72–75% (similar to RH inside incubator, 72.1 ± 4.46% H), and rose to 90% RH at 3 h post-incubation and, finally, reached a maximum of 96–99% RH after 6–11 h. In treatments with humidified filter paper in parafilmed boxes, RH reached 88–89% after 30 min and was saturated at ca. 100% RH after 3-4.5 h. Despite elevated RH in boxes with humidified filter paper, water from droplets in inoculation zones had evaporated by the time germination was assessed, in contrast to the treatment in which parafilmed Petri dishes were used.

### 3.4. Effects of the immersion time and incubation time

The three-way interaction (batch quality × immersion time × incubation time), as well as all two-way interactions and main effects were significant (all $P$ values <0.0001) and, therefore, data were analyzed independently for each batch type. For conidia from high-quality batches, ANOVA with two main factors and all main effects were not significant (all $P$ values $\geqslant 0.970$). Germination counts were always in the 95–98% range, independently of immersion and incubation times. For low-quality conidia, the two-factor ANOVA revealed a significant 2-way interaction ($F = 22.4$; $d.f. = 4,$

**Table 1**
Occurrence of dislodged conidia and germination (%) for low-quality *Metarhizium anisopliae* conidia (isolate CG57) within and outside inoculation zones in tests with different inoculation volumes, and addition of coverslips when viability is assessed.

| Treatments | Occurrence of conidial displacement | Germination (%) | | |
|---|---|---|---|---|
| Volume/coverslips | | Outside inoculation zone | Center of inoculation zone | Border of inoculation zone |
| 20 μL/no coverslips | No | – | 48.7 ± 1.92 a | 44.3 ± 2.28 a |
| 20 μL/with coverslips | Yes (3/8) | 0.0 | 51.9 ± 12.55 a | 52.0 ± 8.80 a |
| 100 μL/no coverslips | No | – | 47.3 ± 2.48 a | 52.8 ± 3.73 a |
| 100 μL/with coverslips | Yes (3/8) | 0.0 | 54.1 ± 13.62 a | 56.4 ± 9.76 a |

Germination means (±SD) followed by the same letter are not significantly different according to Tukey-Kramer HSD ($\alpha = 0.05$).

**Table 2**
Effect of drying conidia prior to incubation on germination counts (mean ± SD) for high- and low-quality batches of *Metarhizium anisopliae* incubated at different atmospheric RH.

| Batch quality | Drying regime prior to incubation | Incubation conditions | | |
|---|---|---|---|---|
| | | Plastic box | | Petri dish |
| | | High RH | Very high RH | |
| High-quality | No drying | 94.3 ± 1.27 a | 94.4 ± 2.48 a | – |
| | With drying | 92.9 ± 3.39 a | 94.3 ± 2.78 a | – |
| | Control | – | – | 96.9 ± 1.61 a |
| Low-quality | No drying | 23.9 ± 3.93 bc | 29.1 ± 6.32 b | – |
| | With drying | 17.1 ± 3.39 c | 25.9 ± 4.80 b | – |
| | Control | – | – | 48.8 ± 4.97 a |

For each batch quality, germination means (±SD) followed by same letter are not significantly different according to Tukey-Kramer HSD ($\alpha = 0.05$). In the control treatment, conidial suspensions were inoculated onto PDA in Petri dishes and immediately sealed with parafilm, whereas in other treatments PDA blocks on glass slides were incubated in plastic boxes with or without humidified field paper on bottom. In plastic boxes with humidified filter paper (very high RH), RH reached 88–89% after 30 min and was saturated (ca. 100% RH) after 3–4.5 h, whereas in plastic boxes with dry filter paper, RH after 30 min was 72–75%, reaching 90% RH after 3 h and 96–99% RH after 6–11 h post-incubation. RH within Petri dishes was not monitored, although water activity of fresh PDA was in the 0.980–0.985 range.

**Table 3**
Effect of pre-inoculation hydration time (immersion time) and incubation time on germination (%) of *Metarthizium anisopliae* conidia.

| Batch quality | Immersion time | Incubation time (hpi) | | |
|---|---|---|---|---|
| | | 18 | 48 | 72 |
| High-quality | 15 min | 95.4 ± 3.41 a | 96.3 ± 2.42 a | 97.7 ± 2.19 a |
| | 3 h | 94.8 ± 2.64 a | 95.6 ± 1.70 a | 97.5 ± 2.25 a |
| | 24 h | 94.7 ± 3.46 a | 95.8 ± 3.47 a | 96.0 ± 3.69 a |
| Low-quality | 15 min | 53.4 ± 13.13 A b | 94.9 ± 2.18 A a | 96.5 ± 2.36 A a |
| | 3 h | 72.9 ± 10.36 B b | 96.3 ± 1.77 A a | 97.6 ± 1.49 A a |
| | 24 h | 94.3 ± 2.46 C a | 97.4 ± 0.89 A a | 98.1 ± 1.18 A a |

For high-quality conidia, germination means (% ± SD) followed by same letter are not significantly different according to Tukey-Kramer HSD ($\alpha = 0.05$). For low-quality conidia, due to significant immersion time × incubation time interaction, germination means (% ± SD) followed by the same capital letter in a column, or by the same lowercase letter in a row, are not significantly different according to Tukey-Kramer HSD ($\alpha = 0.05$).

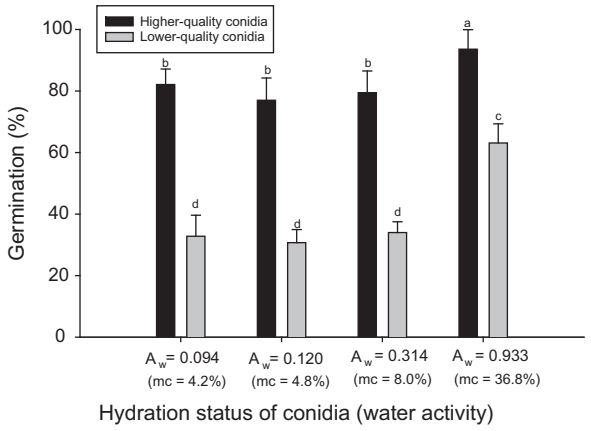


**Fig. 3.** Effect of dryness on germination of higher- and lower-quality *Metarhizium anisopliae* conidia. The germination counts were made at 18 h post-inoculation on PDA. Means (±SD) with the same letter are not significantly different according to Tukey-Kramer HSD ($\alpha = 0.05$). Moisture contents (mc) estimates based on Faria et al. (2009).

61; $P < 0.0001$). The main effects of immersion time ($F = 82.8$; *d.f.* = 2, 61; $P < 0.0001$) and incubation times ($F = 128.6$; *d.f.* = 2, 61; $P < 0.0001$) were also significant. As immersion time of conidia in tween prior to inoculation increased from 15 min to 24 h, germination counts at 18 hpi increased from 53.4 ± 13.13% to 94.3 ± 2.46% (Table 3). However, when immersion time was 15 min but incubation time was increased to 72 hpi, germination counts were 96.5 ± 2.36%.

### 3.5. Effects of the initial hydration status of conidia

Two-way batch quality × hydration status interaction was not significant ($F = 1.5$; *d.f.* = 3, 53; $P = 0.24$), whereas the main effects of batch quality ($F = 626.6$; *d.f.* = 1, 53; $P < 0.0001$) and dryness status (water activity; $F = 45.9$, *d.f.* = 3, 53; $P < 0.0001$) were highly significant. Both $a_w$ and conidial quality had significant impact on germination ($F_{3,53} = 45.91$; *d.f.* = 3, 53; $P < 0.0001$; $F = 626.55$; *d.f.* = 1, 53; $P < 0.0001$, respectively). As batch quality or hydration status of conidia prior to immersion in aqueous tween increased, germination counts increased significantly (Fig. 3).

## 4. Discussion

Many protocols currently used for high-quality conidial batches are inadequate to assess germination of batches with high proportion of dead or low-vigor conidia (with high proportion of slow-germinating propagules). Initial hydration status of conidia, duration of pre-inoculation hydration of conidial suspensions, atmospheric RH during incubation, duration of incubation, use of stained mounting medium and use of coverslips when germination is assessed, profoundly affect conidial viability counts when overall batch viability is poor (<ca. 70%). This finding requires protocols for accurate assessments of conidial quality to be standardized to guarantee reliable and comparable results for R&D experiments, as well as for monitoring quality of mycoinsecticides and possibly of other fungus-based products.

The influences of numerous other factors on germination assessments with entomopathogenic fungi were discussed in the Introduction. However, most parameters considered here have been ignored or given little attention because most previous studies focused on fresh conidia, usually obtained from artificial media cultivated in Petri dishes, being characterized by high initial water contents and high viabilities and germination speed. Standardization of the initial hydration status of conidia is an unusual practice whose importance was recently emphasized (Faria et al., 2010). These authors recommended adopting germination protocols that consider drying of conidia to water activity levels $\leqslant 0.30$ (a water content of ca. 8% or less) by using desiccants such as silica gel or calcium sulphate. As a matter of fact, we have demonstrated in this work that the initial dehydration of spore powders is an important step for germination protocols, and drying conidia to levels of water activity lower than ca. 0.30 prior to immersion in water yielded similar germination counts as previously hypothesized (Fig. 3). In studies with *M. robertsii* and *M. pingshaense*, germination delays for dry conidia compared to moist conidia varied from 4.6 to 9.0 h depending on the isolate (Xavier-Santos et al., 2011), underscoring the need for uniform protocols.

In our study, germination means for high-quality batches were $\geqslant 94\%$, independent of the duration of conidial hydration (immersion in water) immediately prior to inoculation, and the duration of incubation before assessing germination (Table 3). In turn, these parameters had a profound impact on overall germination of low-quality conidia. Low-quality conidial batches may still achieve high viability depending on germination protocol, even if their incubation is no more than 18 h at 25 °C. In general, immersion for either 3 or 24 h gave higher germination counts than for conidia kept immersed for only 15 min. Similarly, evaluations performed at 48 or 72 hpi produced higher germination than those performed at 18 hpi. Even if pre-testing immersion period was only 15 min, viabilities for low-quality conidial batches were 95% and 97% after 48 and 72 hpi, respectively (but only 53% at 18 hpi). Accelerated germination of *M. anisopliae* (*sensu lato*) conidia kept for 20 h in distilled water prior to inoculation onto solid medium was previously reported by Hassan et al. (1989). Virulence of low-quality unformulated conidia (0% and 100% germination rates at 24 and 72 hpi, respectively) toward *Diatraea saccharalis* (Lepidoptera: Crambidae) and *Solenopsis saevissima* (Hymenoptera: Formicidae)

was significantly decreased compared to fast-germinating conidia (100% germination at 24 hpi) (Alves et al., 1996), although the same trend was not seen for some formulated preparations. It is obvious that extensive comparisons between the virulence of low- and high-quality conidia is an area of study that needs urgent investigation.

Studies with saturated salt solutions and glycerol performed by Luz and Fargues (1997) showed the marked effect of atmospheric RH on *B. bassiana* germination and, even for treatments with high RH, viabilities for conidia exposed to a saturated atmosphere were higher than for conidia kept at 97% RH at 12 and 16 hpi, although significant differences were not seen from 20 hpi onwards. In our experiment, viability determinations for high-quality batches were not affected by incubation conditions (Petri dishes with PDA vs. PDA blocks in boxes with or without humidification with dampened filter paper), whereas viabilities for low-quality conidia kept in Petri dishes were higher than for conidia in boxes, probably due to the presence of a water film from droplets in the inoculation zones during the whole incubation period (not seen in boxes). A 30 min drying period inside a laminar flow cabinet prior to incubation of PDA blocks did not interfere with viability of high-quality conidia. However, the complete drying of water from inoculated conidial suspensions before incubation is recommendable since, besides avoiding accelerated germination of low-quality conidia in non-evaporated droplets (thereby inflating counts), drying also prevents leakage of ungerminated conidia from inoculation zones due to excessive shaking when dishes are manipulated during germination assessment (data not shown). Despite its common use (Goettel and Inglis, 1997), lactophenol cotton blue and other fixative agents applied to germination test plates may cause overestimation of counts, washing ungerminated conidia away from inoculation zones. As expected, the effect of coverslips alone was more subtle than the combined use of stain and coverslips as a consequence of lower hydraulic pressure. Although the lack of coverslips may lead to vapor condensation of microscope objectives, in our study most microscopic fields were very clean, and those exhibiting some degree of condensation could be rapidly cleaned by breaths of warm air. Additionally, coverslips (but not liquid fixatives) could be used for accurate determination of viability if, prior to inoculation, spore suspensions were spread on medium plates and liquid allowed to absorb into medium.

We have shown here that samples of low-quality conidia can be identified through adoption of germination protocols that embrace initial dehydration of propagules to low water contents, short pretesting immersion period, and incubation times enough to allow germination of only fast-germinating conidia (but capable of excluding low-vigor conidia from counts). However, a great deal of research is needed to elucidate the real role played by low-quality conidia of entomopathogenic fungi and other fungal agents in field conditions.

After many decades, most commonly followed germination protocols are not reliable to accurately determine viability of low-vigor fungal structures. The continued adoption of conventional protocols, in which important parameters considered in this study are usually disregarded, should be avoided in basic research in laboratories, especially those studies known to retard conidia germination (i.e., effect of UV and temperature). Additionally, it could result in increasing misreading and subsequent commercialization of low-quality mycopesticides, leading to undesirable results under field conditions. One challenge is to ensure that germination counts remain a useful and easy practical assessment or are replaced with another technique equally appropriate. In order to accurately determine conidial viabilities in basic and applied research projects, or monitor quality of mycopesticides for various reasons, scientifically based and standardized protocols should be adopted.

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## References

Alves, S.B., Pereira, R.M., Vieira, S.A., 1996. Delayed germination of *Beauveria bassiana* conidia after prolonged storage at low, above-freezing temperatures. Biocontrol Sci. Technol. 6, 575–582.

Andersen, M., Magan, N., Mead, A., Chandler, D., 2006. Development of a population-based threshold model of conidial germination for analysing the effects of physiological manipulation on the stress tolerance and infectivity of insect pathogenic fungi. Environ. Microbiol. 8, 1625–1634.

Bischoff, J.F., Rehner, S.A., Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101, 508–528.

Braga, G.U.L., Flint, S.D., Messias, C.L., Anderson, A.J., Roberts, D.W., 2001. Effect of UV-B on conidia and germlings of the entomopathogenic hyphomycete *Metarhizium anisopliae*. Mycol. Res. 105, 874–882.

Chandler, D., Heale, J.B., Gillespie, A.T., 1994. Effect of osmotic potential on the germination of conidia and colony growth of *Verticillium lecanii*. Mycol. Res. 98, 348–388.

Costa, E., Usall, J., Teixidó, N., Torres, R., Vinãs, I., 2002. Effect of package and storage conditions on viability and efficacy of the freeze-dried biocontrol agent *Pantoea agglomerans* strain CPA-2. J. Appl. Microbiol. 92, 873–878.

Dantigny, P., Bensoussan, M., Vasseur, V., Lebrihi, A., Buchet, C., Ismaili-Alaoui, M., Devlieghere, F., Roussos, S., 2006. Standardisation of methods for assessing mould germination: a workshop report. Int. J. Food Microbiol. 108, 286–291.

Faria, M.R.de, Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control 43, 237–256.

Faria, M., Hajek, A.E., Wraight, S.P., 2009. Imbibitional damage in conidia of the entomopathogenic fungi *Beauveria bassiana, Metarhizium acridum,* and *Metarhizium anisopliae*. Biol. Control 51, 346–354.

Faria, M., Hotchkiss, J.H., Hajek, A.E., Wraight, S.P., 2010. Debilitation in conidia of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* and implication with respect to viability determinations and mycopesticide quality assessments. J. Invertebr. Pathol. 105, 74–83.

Goettel, M.S., Inglis, G.D., 1997. Fungi: hyphomycetes. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, New York, NY, pp. 213–249.

Guijarro, B., Larena, I., Paloma, M., De Cal, A., 2006. Effect of drying on conidial viability of *Penicillium frequentans*, a biological control agent against peach brown rot disease caused by *Monilinia* spp. Biocontrol Sci. Technol. 16, 257–269.

Harman, G.E., Obregón, M.A., Samuels, G.J., Lorito, M., 2010. Changing models for commercialization and implementation of biocontrol in the developing and developed world. Plant Dis. 94, 928–939.

Hassan, A.E.M., Dillon, R.J., Charnley, A.K., 1989. Influence of accelerated germination of conidia on the pathogenicity of *Metarhizium anisopliae* for *Manduca sexta*. J. Invertebr. Pathol. 54, 211–219.

Hong, T.D., Jenkins, N.E., Ellis, R.H., 1999. Fluctuating temperature and the longevity of conidia of *Metarhizium flavoviride* in storage. Biocontrol Sci. Technol. 9, 165–176.

Jackson, M.A., McGuire, M.R., Lacey, L.A., Wraight, S.P., 1997. Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. Mycol. Res. 101, 35–41.

James, R.R., 2001. Effects of exogenous nutrients on conidial germination and virulence against the silverleaf whitefly for two hyphomycetes. J. Invertebr. Pathol. 77, 99–107.

Jenkins, N.E., Grzywacz, D., 2000. Quality control of fungal and viral biocontrol agents – assurance of product performance. Biocontrol Sci. Technol. 10, 753–777.

JMP Software, 2007. Version 7.0.SAS Institute, Cary, NC.

Kassa, A., Stephan, D., Vidal, S., Zimmermann, G., 2004. Production and processing of *Metarhizium anisopliae* var. *acridum* submerged conidia for locust and grasshopper control. Mycol. Res. 108, 93–100.

Kope, H.H., Alfaro, R.I., Levallée, R., 2008. Effects of temperature and water activity on Lecanicillium spp. conidia germination and growth, and mycosis of *Pissodes strobi*. BioControl 53, 489–500.

Li, Z., Alves, S.B., Roberts, D., Fan, M., Delalibera Jr., I., Tang, J., Lopes, R.B., Faria, M., Rangel, D.E.N., 2010. Biological control of insects in Brazil and China: history, current programs and reasons for their successes using entomopathogenic fungi. Biocontrol Sci. Technol. 20, 117–136.

Liu, H., Skinner, M., Brownbridge, M., Parker, B.L., 2003. Characterization of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for management of tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae). J. Invertebr. Pathol. 82, 139–147.

Luz, C., Fargues, J., 1997. Temperature and moisture requirements for conidial germination of an isolate of *Beauveria bassiana*, pathogenic to *Rhodnius prolixus*. Mycopathologia 138, 117–125.

Milner, R.J., Huppatz, R.J., Swaris, S.C., 1991. A new method for assessment of germination of *Metarhizium* conidia. J. Invertebr. Pathol. 57, 121–123.
Mohan, M.C., Reddy, N.P., Devi, U.K., Kongara, R., Sharma, H.C., 2007. Growth and insect assays of *Beauveria bassiana* with neem to test their compatibility and synergism. Biocontrol Sci. Technol. 17, 1059–1069.
Pedreschi, F., Aguilera, J.M., 1997. Viability of dry *Trichoderma harzianum* spores under storage. Bioprocess Eng. 17, 177–183.
Smith, C., Edgington, S., 2011. Germination at different water activities of similarly aged *Metarhizium* conidia harvested from ageing cultures. J. Stored Prod. Res. 47, 157–160.
Stephan, D., Zimmermann, G., 1998. Development of a spray-drying technique for submerged spores of entomopathogenic fungi. Biocontrol Sci. Technol. 8, 3–11.
Xavier-Santos, S., Lopes, R.B., Faria, M., 2011. Emulsifiable oils protect *Metarhiziumr obertsii* and *Metarhizium pingshaense* conidia from imbibitional damage. Biol. Control 59, 261–267.
Yeo, H., Pell, J.K., Alderson, P.G., Clark, S.J., Pye, B.P., 2003. Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and their pathogenicity to two aphid species. Pest Manag. Sci. 59, 156–165.
Ypsilos, I.K., Magan, N., 2005. Characterisation of optimum cultural environmental conditions for the production of high numbers of *Metarhizium anisopliae* blastospores with enhanced ecological fitness. Biocontrol Sci. Technol. 15, 683–699.