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Debilitation in conidia of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* and implication with respect to viability determinations and mycopesticide quality assessments

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ABSTRACT

Germination of *Beauveria bassiana* (Bb) and *Metarhizium anisopliae* (Ma) conidia determined from a fast-rehydration (FR) protocol were compared to those obtained when dry conidia were subjected to slow rehydration (SR) by holding under high humidity conditions prior to aqueous suspension. Differences in viability estimates obtained using the FR vs. SR protocols increased markedly after conidia were exposed to various stress factors in storage (high a_w, temperature, and O_2 concentrations), with the SR protocol producing higher estimates of viability in all cases. After Bb conidia were stored under moist conditions for 21 days at 25 °C, the SR estimate of viability was >21% greater than the FR estimate. In jars flushed with different O_2 concentrations and stored at 50 °C for 34 days, proportional differences between protocols varied, depending on water activity, from 18–44% in jars flushed with 0% O_2 (100% N_2) to as high as 63–93% when treated with 21–22% O_2. For conidia stored over a broad range of moderate to high temperatures in the absence of O_2, SR–FR differences were ⩽9% at 25–40 °C but 30% at 50 °C. Germination of stressed Bb and Ma conidia increased substantially when incubation time on the germination substrate was increased from 24 to 72 h, whereas germination of non-stressed conidia showed little change. Conidia debilitated by stress were characterized by hypersensitivity to lethal imbibitional damage (damage that is mitigated by slow rehydration) and slow germination. Viability protocols that may provide more reliable assessments of overall mycopesticide quality are discussed.

1. Introduction

A considerable number of mycoinsecticides have reached the marketplace, and millions of hectares are treated annually with entomopathogenic fungi worldwide (Faria and Wraight, 2007). Despite recent advances, use of mycopesticides is proportionally limited when compared to their chemical counterparts, even in countries in which many mycopesticides are produced. The growing adoption of these microbial biocontrol agents depends on factors such as (1) development of better products, (2) development and implementation of truly integrated pest management strategies in which biological options are emphasized (Lomer et al., 1999; Thomas, 1999; Lacey et al., 2001), (3) the capacity of biopesticide manufacturers/retailers to maintain marketing and product-support teams, (4) cultural changes (acceptance by farmers of slow-acting, narrow-host-range products), and (5) sound, knowledge-based recommendations for product use. With respect to development of better products, this may encompass increased concentrations of active ingredients, more predictable shelf-lives under non-refrigerated conditions (Hong et al., 1997; Wraight et al., 2001), improved shipping and handling characteristics, greater UV-tolerance (Inglis et al., 2001), and ultimately, greater efficacy and reliability under field conditions (Lacey et al., 2001).

Development of mycoinsecticides and assessment of their quality rely to a great extent on viability determinations, and numerous techniques for measuring viability are available (Goettel and Inglis, 1997). Germination protocols with pre-incubation regimes including slow rehydration of dry conidia in high humidity environments have been shown to prevent imbibitional damage and thereby boost germination percentages when compared to fast rehydration of dry conidia (Moore et al., 1997; Magalhães and Boucias, 2004; Faria et al., 2009). In some cases, fast rehydration and longer incubation times achievable through use of media amended with a hyphal-growth inhibitor (benomyl) have been recommended (Milner et al., 1991; Meikle et al., 2003). In fact, Burges (1998) warned of the risks involved in performing viability counts after incubation

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for only 24 h in view of reports of substantial proportions of slow-germinating conidia in stored formulations. After seven years of storage at 6 ± 2 °C, for example, viability of fast-hydrated *Beauveria bassiana* conidia was 0% after incubation for 24 h but 100% after 72 h (Alves et al., 1996). For dried *M. acridum* (formerly identified as *M. anisopliae* var. *acridum*) stored in oil for 37 months at 17 °C, germination counts were 47% and 79% after incubation for 24 and 48 h, respectively (Moore et al., 1995).

After initiating studies on shelf-life of *Beauveria* and *Metarhizium* conidia we observed that germination counts following slow rehydration were usually higher than counts following fast-rehydration protocols, especially after storage under harsh conditions. Faced with uncertainties as to which protocol to rely on, we decided to launch a more detailed investigation. This study employed several stress factors, including temperature, water activity (a_w), and oxygen concentration to alter the physiological condition of *B. bassiana* and *M. anisopliae* conidial powders. Viability was then quantified using two recommended techniques incorporating rapid vs. slow rehydration. The principal objective of this study was to enhance our understanding of differences between germination protocols and increase awareness of important considerations when choosing among techniques designed to assess conidial quality. An additional objective included characterization of storage conditions promoting conidial longevity.

2. Materials and methods

2.1. Source of fungi

Fresh batches of a technical *B. bassiana* (Bb) product based on aerial conidia of isolate GHA were provided by Laverlam International Corporation (Butte, MT, USA). Aerial conidia of the isolate CB-10 (=ARSEF 7981 or CG 858) of *M. anisopliae* (Ma; classified according to Bischoff et al., 2009) were lab-produced on cooked rice (Faria et al., 2009) and stored for 14 months at −20 °C until the start of experiments.

2.2. Germination counts

For viability determination through the slow-rehydration (SR) protocol, a small portion of conidial powder (0.15–0.2 mg) was picked up on the tip of a spatula, transferred to a glass Petri dish, and incubated at 25 °C for 24 h in a 24-cm internal diameter glass "desiccator" with water-saturated atmosphere. The preceding treatment will hence be referred to as the SR protocol. The hydrated conidia were then added to a screw-cap glass vial containing glass beads and 7 mL of 0.05% Lutensol® (Ethoxylated Tridecyl Alcohol, BASF Corporation, Florham Park, NJ, USA), possessing a hydrophilic–lipophilic balance number of 10 (Jin et al., 1999). The temperature of water + surfactant was not controlled, but it was in the range 20–25 °C throughout. The suspension was agitated for 10 min on a wrist-action shaker (Burrel Scientific, Pittsburgh, PA, USA), and 10-μl aliquots were plated on 1 × 1 × 0.3 cm blocks (1 droplet/block) of yeast extract agar-based solid medium (YEA) (Meikle et al., 2003) amended with 0.005 g L^{-1} of benomyl (Bonide Chemical Co., Yorkville, NY, USA). Viability of conidia was also assessed through a conventional protocol of suspending usually dry conidia in water + surfactant and plating directly onto YEA immediately after removal from storage containers, omitting the slow-rehydration step. This treatment will be referred to as the fast-rehydration (FR) protocol. Following inoculation in both cases, the glass slides containing the agar blocks were incubated in parafilmed Petri dishes at 25 °C in darkness, and germination counts were performed 24 h post-inoculation (p.i.). Conidia were considered to have germinated when a germ tube was microscopically visible at 400× magnification with phase-contrast illumination. A minimum total of 200 conidia were examined in several microscope fields for each replicate suspension of each experimental treatment. Temperatures in incubators were monitored with digital data loggers (Hobo®, Onset Computer Corporation, Bourne, MA, USA). Reported temperatures were ±0.5 °C for 25 °C and ±1 °C for all other temperatures.

2.3. Effects of water activity on conidial viability

Constant equilibrium relative humidities (ERHs) were attained in airtight glass jars (0.95 L) with rubber-lined metallic lids (Ball®, Jarden Corporation, Muncie, IN, USA) modified to include a 13-mm rubber septum. In each of four replicate jars, 1.0 g of Bb conidial powder with initial water activity (a_w) of 0.274 contained in aluminum foil dishes was stored for 21 days at 25 °C over the desiccant calcium sulphate (eight-mesh indicating Drierite, W.A. Hammond Drierite Co., Xenia, OH, USA), distilled water, or saturated salt solutions (NaOH, $NaNO_2$, NaCl, and K_2SO_4). Respiratory activity of conidia was indirectly measured via determinations of O_2 and CO_2 concentrations at end of the experiment. Five-hundred μl of "air" was retrieved from each jar with a gas-tight syringe (model 1750, Hamilton Company, Reno, NV, USA) and injected into a gas chromatograph (Varian Aerograph, Walnut Creek, CA, USA) equipped with a thermal conductivity detector. Readings were compared against a commercial standard containing 6.96% O_2 and 4.91% CO_2 balanced with N_2 (Airgas East Headquarter, Salem, NH, USA). Following the gas chromatograph measurements, each jar was opened and the sample was quickly transferred to a water activity meter (LabMaster-a_w, Novasina, Pfäffikon, Switzerland) set to 25 °C. Germination after slow vs. fast rehydration was then determined as described in the previous section.

2.4. Effects of carbon dioxide and oxygen on conidial viability

Water activity of Bb conidial samples (0.6 g/sample) held in 3.4 cm diameter × 1.1 cm plastic cups (Novasina, Pfäffikon, Switzerland, code 4-1110601) was standardized by storage at either 10 or 25 °C in glass jars containing NaOH for a 48 h period. Water activity was measured and the samples were immediately transferred to 125-mL glass jars (Ball®, Jarden Corporation, Muncie, IN, USA) and sealed with metallic lids possessing rubber septa. Conidial a_w following 48-h incubation over NaOH and just before transfer to glass jars was 0.078 (10 °C) and 0.090 (25 °C) for the CO_2 experiments, and in the 0.084–0.090 range (25 °C) for the O_2 experiments. Lab relativity humidity (RH) during transfer of samples was in the mid 20's and low 40's during the first and second set of experiments with each gas, respectively. Samples were flushed for 40 min at 40 mL/min flow rate with variable CO_2 concentrations (0–100%) or O_2 concentrations (0–22.4%) balanced with N_2; additional samples were flushed with compressed air. Concentration of O_2 in every jar following flushing was checked to make sure ambient air had been successfully removed from storage containers. Each treatment was replicated four-fold, and both experiments were repeated on a different date. A preliminary short-term experiment at high storage temperatures revealed that the described containers were not completely sealed, as indicated by slow increases in O_2 concentration. Further investigation showed that O_2 ingress was largely prevented by sealing each 125-mL jar inside a 0.95-L jar containing the same mixture of gases. Using this double-container set up, glass jars were incubated at 50 °C for 34 days, and viabilities, a_w, and O_2 concentrations were determined as previously described (O_2 measured as a leakage indicator).

2.5. Effects of temperature on conidial Metarhizium viability

In a time-course experiment, samples of dry Bb conidia (0.6 g/sample) possessing initial a_w of 0.090 ± 0.001 were placed in 125-mL glass jars and flushed with 100% N_2 as described above. Using the double-container set up, storage was carried out at 25, 40, and 50 °C. O_2 concentration in jars, a_w, and germination counts were determined at all temperatures after storage for 15, 29 and 61 days. Four jars were used for each temperature/time treatment. O_2 concentration, a_w, and germination counts were determined as mentioned earlier. As in all previous experiments, samples were assessed destructively.

2.6. Relationship between conidial debilitation and imbibitional damage

This experiment was performed with debilitated vs. non-debilitated conidial powders of Bb and Ma. Debilitated (stressed) conidia were produced by incubation at 50 °C in jars with saturated $NaNO_2$ solution (64.4% ERH); debilitation incubation times were 13 h for Ma and 48 h for Bb. Non-debilitated conidia remained in storage at −20 °C. Four replicate samples of each species/treatment combination (0.6 g/sample) were then held over Drierite for 3 days at 25 °C to standardize a_w (procedure previously shown to dehydrate conidial powders to a a_w of ca. 0.021). Conidia were then slow-rehydrated for 24 h prior to immersion in water + surfactant at 34 °C, or fast-rehydrated (immersed in water + surfactant without previous slow hydration) at either 25 °C (Ma) or 0.5 °C (Bb). Fast rehydration at ca. 25 °C is known to cause significant imbibitional damage in dry *Metarhizium* conidia (Moore et al., 1997; Faria et al., 2009). Healthy (non-stressed) GHA conidia, on the other hand, are highly sensitive to imbibitional damage only at very low temperatures (Faria et al., 2009). Thus, for Bb the same procedure was followed except that the water + surfactant solution was set to 0.5 °C (using an ice bath) instead of 25 °C. Following all treatments, viabilities were measured after incubation for 24, 48, and 72 h at 25 °C. Based on the previously-described germination rates, two additional parameters were derived. The overall viability, representing what we consider to be the best estimate of total living (potentially viable) conidia in a test powder, was defined as viability following slow rehydration, immersion (imbibition) at 34 °C, and incubation for 72 h. The difference between overall viability and viability recorded following fast rehydration at 34 °C and incubation for 24 h represents the percentage of debilitated conidia in a conidial powder and includes conidia that are not capable of rapid germination and not capable of germinating unless gently (slowly) rehydrated.

2.7. Statistical analyses

Jars flushed with CO_2 and/or N_2 in which considerable leakage took place (final O_2 concentration >2%) and jars flushed with O_2 in which the final concentration was approx. 2% higher or lower than the target concentration were excluded from statistical analyses. Percent germination data were arcsine transformed to ensure normality and homogeneity of variances and examined using 2- or 3-way analysis of variance (ANOVA). Means were compared by the Tukey–Kramer HSD test and considered to be statistically different at the 5% significance level. All data analyses were performed using the JMP statistical package (SAS Institute Inc., Cary, NC, USA). The experiment investigating the relationship between conidial stress and imbibitional damage incorporated a nested or repeated measures design (estimates of germination over time were derived from subsamples of treated samples), and data were therefore analyzed using multivariate ANOVA (MANOVA), an analytical approach that does not depend on the sphericity assumption (Zar, 1999). Significance of interaction terms was tested using several alternative multivariate tests, including Wilk's Lambda and Pillai's Trace; results reported herein are those generated by the Pillai's Trace option. Means separation following MANOVA was conducted as recommended by Stevens (2002). The JMP multivariate analysis platform provides estimates of the degree of deviation from the sphericity assumption, reporting both the Greenhouse–Geisser (G–G) and Huynh–Feldt (H–F) estimations of the epsilon parameter. Tukey's HSD was considered valid for means testing in all cases as the mean of the G–G and H–F epsilon values exceeded 0.7 (see Stevens, 2002). The Tukey–Kramer option was accessed via the JMP standard least squares, random effects platform (standard univariate ANOVA, split plot design).

3. Results

3.1. Effect of conidial water activity

After storage in the glass jars with Drierite, distilled water, or the various saturated salt solutions for 21 days at 25 °C, water activities of conidia ranged from 0.021 to 0.972, equivalent to 2.1–97.2% in terms of equilibrium relative humidity ($a_w \times 100$) (Table 1). Final O_2 concentrations were negatively correlated ($r = -0.752$, $P < 0.0001$) and CO_2 concentrations positively correlated ($r = 0.986$, $P < 0.0001$) with a_w. In jars with moist conidia ($a_w \geqslant 0.957$), CO_2 reached over 14%, and O_2 dropped to ca. 1%. In jars with dry conidia ($a_w \leqslant 0.070$), low concentrations of CO_2 were not detectable, because very small CO_2 peaks were overshadowed by larger adjacent nitrogen peaks, which in the standard gas had an 18-fold greater concentration compared to CO_2. Two-way ANOVA indicated highly significant main effects of a_w and germination protocol ($F_{1,28} = 878.5$, $P < 0.0001$; $F_{1,28} = 56.5$, $P < 0.0001$, respectively) and a strong $a_w \times$ germination protocol interaction ($F_{4,28} = 9.7$, $P < 0.0001$). As a_w of the conidial samples increased from 0.021 to 0.743, the proportional difference between the estimates of viability from the SR vs. FR protocols increased from 3% to 21% (Table 1). When a_w increased from 0.743 to 0.957, viability dropped to <1%, regardless of the germination protocol.

3.2. Effect of carbon dioxide and oxygen

3.2.1. Carbon dioxide

Following CO_2 (balanced with N_2) storage at 50 °C and 0.098 a_w for 34 days, ANOVA (excluding the air storage treatment) revealed no interaction between germination protocols and CO_2 concentrations ($F_{3,24} = 1.1$, $P = 0.36$) (Fig. 1a). Mean germination determined from the FR protocol (average over the different CO_2 treatments) was 12% points lower than the estimate from the SR protocol (80% vs. 92%) (protocol main effect $F_{1,24} = 128.8$, $P < 0.0001$). ANOVA indicated a marginally significant effect of CO_2 concentration on germination (CO_2 main effect $F_{3,24} = 3.1$, $P = 0.044$); however, mean germination across CO_2 concentrations varied only from 90–95% following SR and 76–82% following FR and did not show a significant trend ($r = 0.177$, $n = 32$, $P = 0.33$). Compared to storage in a CO_2 atmosphere, storage in air resulted in markedly lower germination rates (<40% following FR and <82% following SR) and an increase in the difference between the mean FR vs. SR estimates (from 12% to 42% points).

Similar results were recorded following CO_2 storage at the higher a_w of 0.143, i.e., there was no germination protocol × CO_2 concentration interaction ($F_{3,21} = 1.0$, $P = 0.40$) and no effect of CO_2 concentration ($F_{3,21} = 0.82$, $P = 0.49$) (Fig. 1b). Also, as observed at the lower a_w, the effect of germination protocol was highly significant ($F_{1,21} = 249.5$, $P < 0.0001$); however, storage under wetter conditions resulted in lower viability throughout and a

Table 1
Viability of *Beauveria bassiana* conidia estimated by two different germination protocols following storage at different water activities for 21 days at 25 °C.

Treatment	Final conidial water activity, a_w	Final O_2 (%) in jar	Final CO_2 (%) in jar	Germination (%)[a]		Proportional difference between viability estimates[b]
				Slow rehydration (SR protocol)	Fast rehydration (FR protocol)	
Control	–	–	–	97.9 ± 1.01 a	97.5 ± 0.54 a	0.4 ± 0.67 a
Drierite	0.021 ± 0.0003	19.1 ± 0.42	Not detected[c]	97.8 ± 0.32 a	94.9 ± 1.42 ab	2.9 ± 1.57 b
NaOH	0.070 ± 0.0003	19.1 ± 0.46	Not detected[c]	97.1 ± 0.38 a	94.6 ± 0.43 ab	2.6 ± 0.55 ba
$NaNO_2$	0.636 ± 0.0030	18.7 ± 0.36	0.4 ± 0.04	97.6 ± 0.47 a	92.1 ± 0.66 b	5.6 ± 0.89 b
NaCl	0.743 ± 0.0024	17.3 ± 0.04	2.5 ± 0.12	95.3 ± 1.33 a	75.0 ± 2.73 c	21.3 ± 2.76 c
K_2SO_4	0.957 ± 0.0015	1.0 ± 0.10	14.1 ± 0.27	0.8 ± 0.83 b	0.2 ± 0.17 d	–
H_2O	0.972 ± 0.0012	1.1 ± 0.10	14.2 ± 0.21	0.0	0.0	–

[a] Germination percentages (means ± standard errors) within columns followed by the same letter are not significantly different (Tukey–Kramer HSD, $\alpha = 0.05$).
[b] Proportional differences (means ± standard errors) followed by the same letter are not significantly different (Tukey–Kramer HSD, $\alpha = 0.05$).
[c] CO_2 not detectable at concentrations below approximately 0.2%.

substantially greater difference between the two germination protocols (mean difference of 35% points at 0.143 a_w vs. 12% points at 0.098 a_w). After storage in air, germination following the FR protocol was 1.3% compared to 41.5% following the SR protocol, a proportional difference much greater than that observed at the lower a_w (97% vs. 13%).

In the first CO_2 experiment, average a_w for conidia flushed with compressed air was 0.098, a value similar to that recorded in the CO_2-flushed samples and equivalent to 4.3% moisture content according to isotherms established for this isolate (Faria et al., 2009). In the second experiment, however, the final a_w in the air treatment was higher than that measured in the CO_2 treatments (0.163 a_w or 5.7% moisture content, vs. 0.143 a_w or 5.3% moisture content in the CO_2-flushed samples). This higher moisture level likely contributed to the extreme difference in germination following the two germination protocols.

3.2.2. Oxygen

In experiments in which the effect of O_2 (balanced with N_2) was assessed, residual levels of O_2 in jars flushed with 100% N_2 was in

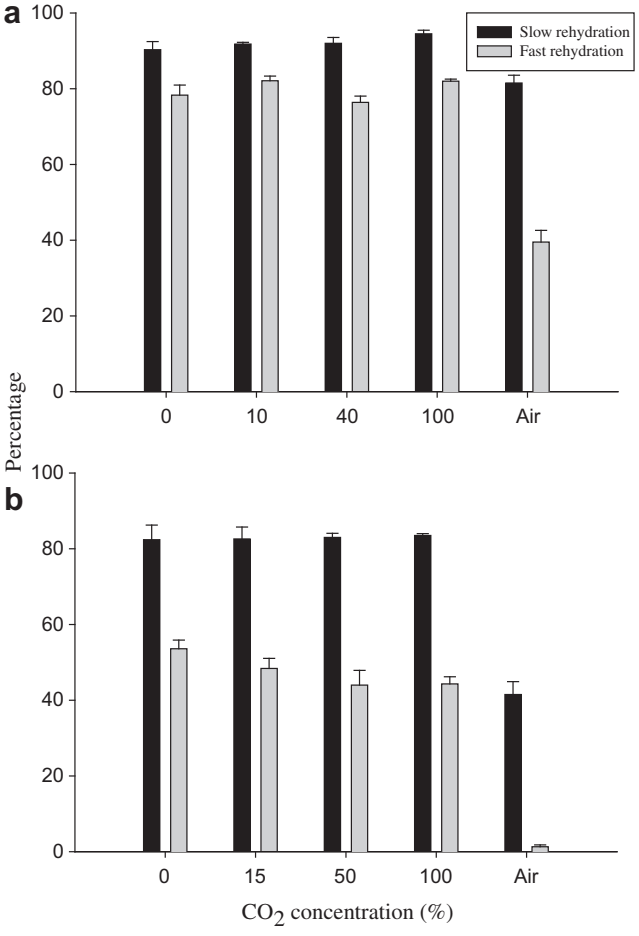

Fig. 1. Effects of CO_2 concentration on percent germination (means ± standard errors) of *Beauveria bassiana* strain GHA conidia following either the fast- or slow-rehydration germination protocol after storage at 50 °C for 34 days. (a) Conidia with 0.098 ± 0.005 a_w in all treatments. (b) Conidia in all CO_2 treatments with 0.143 ± 0.002 a_w.

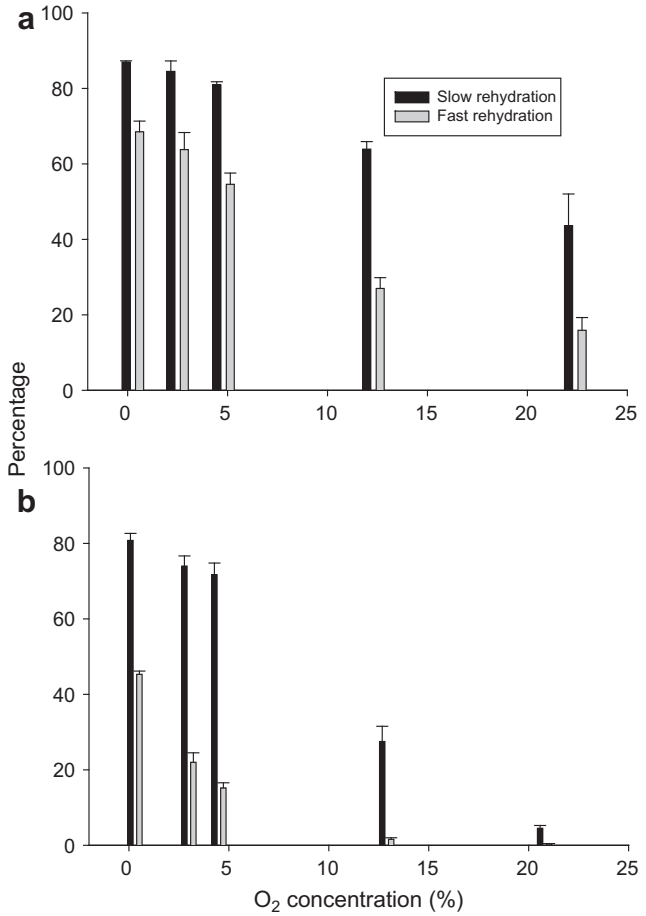

Fig. 2. Effects of O_2 concentration on percent germination (means ± standard errors) of *Beauveria bassiana* strain GHA conidia following either the fast- or slow-rehydration germination protocol after storage at 50 °C for 34 days. (a) Conidia with 0.100 ± 0.006 a_w. (b) Conidia with 0.147 ± 0.005 a_w.

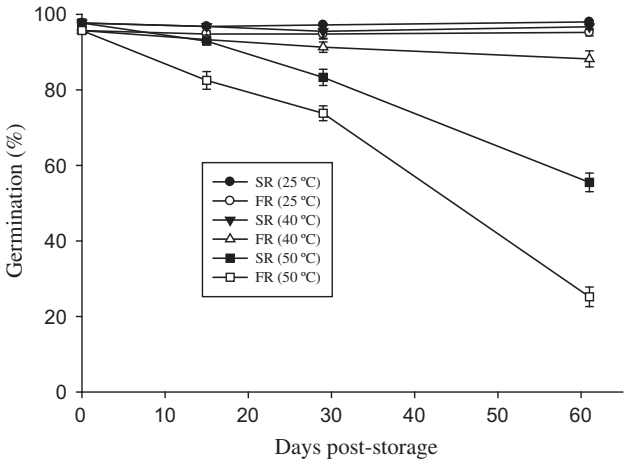

Fig. 3. Effects of temperature on percent germination (means ± standard errors) of *Beauveria bassiana* strain GHA conidia following either the slow-rehydration (SR) or the fast-rehydration germination protocol (FR). Samples gas-flushed with 100% N_2.

the 0.2–0.5% range, similar to residual levels in the CO_2 experiments. In the first experiment, with conidia at 0.100 a_w, ANOVA revealed no significant germination protocol × O_2 concentration interaction ($F_{4,26} = 1.1$, $P = 0.39$) (Fig. 2a). Both main effects were highly significant; mean germination decreased from 78% to 30% following storage in atmospheres with 0.3% vs. 22.4% O_2, respectively ($F_{4,26} = 58.3$, $P < 0.0001$), and germination following the FR protocol averaged 27% points lower than the SR protocol ($F_{1,26} = 118.6$, $P < 0.0001$).

In the second experiment with conidia at a higher a_w (0.147), a strong interaction was noted between germination protocol and O_2 concentration ($F_{4,28} = 20.2$, $P < 0.0001$) (Fig. 2b). The proportional difference between the germination estimates increased with increasing O_2 concentration (FR estimates were 21–25% lower than the SR estimates at 0.3–3% O_2 vs. 58–63% lower at 13–21% O_2). Both main effects were also highly significant (germination protocol $F_{1,28} = 657.5$, $P < 0.0001$; O_2 $F_{4,28} = 329.9$, $P < 0.0001$), and the negative effects of O_2 on conidial germination were more pronounced than observed under the drier conditions ($a_w = 0.100$) of the first test.

3.3. Effect of temperature

Plots describing the time-course of SR and FR germination counts in an N_2-rich atmosphere are shown in Fig. 3. A three-factor ANOVA (time, temperature, and germination protocol) indicated significance of all main effects and 2-way interactions (all P values $\leqslant 0.0001$) as well as the 3-way interaction ($F_{6,76} = 3.0$, $P = 0.01$). Increases in both temperature and time were associated with increasing proportional differences between the estimates of germination generated by the alternative protocols (temperature × germination protocol interaction $F_{2,76} = 12.3$, $P < 0.0001$). After storage for 2 months, mean FR germination counts were 3% lower than the SR counts at 25 °C and 55% lower at 50 °C.

3.4. Relationship between conidial stress and imbibitional damage

3.4.1. Beauveria bassiana

Germinability of conidia was dramatically affected by the short-term exposure to the high-stress conditions of 50 °C/64.4% ERH (Table 2). Germination rates of debilitated conidia were not only substantially lower than germination rates of non-debilitated conidia across all comparable pre-germination treatments (stress main effect: $F_{1,18} = 1046$, $P < 0.0001$), but the repeated measures ANOVA also indicated highly significant 2-way interactions of stress × germination protocol ($F_{2,18} = 28.1$, $P < 0.0001$) and stress × incubation time ($F_{2,17} = 22.3$, $P < 0.0001$). FR had a much greater negative effect on stressed conidia than on non-stressed conidia, and stressed conidia germinated more slowly than non-stressed conidia. The 3-way interaction was not significant ($F_{4,36} = 1.65$, $P = 0.18$). In the remaining analyses, data from the stressed vs. non-stressed conidial populations were analyzed separately.

Analysis of results with the non-stressed conidia indicated no rehydration protocol × time interaction ($F_{4,18} = 0.43$, $P = 0.79$). The main effect of incubation time was also insignificant; germination did not vary with time, regardless of the rehydration protocol (time main effect: $F_{2,8} = 0.29$, $P = 0.75$). On the other hand, germination protocol had a significant effect on viability assessments ($F_{2,9} = 182.4$, $P < 0.0001$). Germination following FR at 34 °C was consistently lower than germination following SR, but the mean difference was small (97.5% vs. 95.5%) and not statistically significant according to the Tukey test. In contrast, FR at 0.5 °C produced a significant 46% reduction in viability compared with SR.

In the case of the stressed conidia, germination increased with incubation time across all rehydration treatments (Table 2). The increases were more or less consistent across germination protocols, and this result was reflected in a marginally insignificant germination protocol × time interaction ($F_{4,18} = 2.5$, $P = 0.081$). The main

Table 2
Effects of conidial quality and rehydration protocol on germination of *Beauveria bassiana* strain GHA conidia, subjected (48 h at 64.4% RH and 50 °C) or not to stressful conditions during storage.

Incubation time (h)	Germination (%)[a]			Debilitated conidia (based on germination recorded after SR and FR at 34 °C)		
	Slow rehydration (SR protocol followed by immersion at 34 °C)	Fast rehydration (FR protocol)				
		34 °C	0.5 °C	Conidia killed by imbibitional damage (%)[b]	Surviving conidia exhibiting delayed germination (%)[c]	Total debilitated conidia (%)[d]
Non-stressed conidia						
24	97.3 ± 0.5a	95.5 ± 1.0a	50.1 ± 4.0b	2.0 ± 1.4	0.0 ± 0.9	2.0 ± 1.2
48	97.8 ± 0.4a	95.1 ± 0.6a	52.9 ± 4.7b			
72	97.5 ± 0.5a	95.5 ± 1.2a	51.6 ± 4.2b			
Stressed conidia						
24	55.6 ± 3.4bc	13.6 ± 2.2e	0.5 ± 0.4f	34.3 ± 6.0	29.9 ± 5.6	64.2 ± 2.6
48	69.8 ± 3.6ab	29.9 ± 3.8d	3.5 ± 1.2f			
72	77.8 ± 2.2a	43.5 ± 3.9 cd	3.8 ± 0.6f			

[a] Germination percentages (means ± standard errors) within each stress treatment (stressed or non-stressed) followed by the same letter are not significantly different (Tukey–Kramer HSD, $\alpha = 0.05$).
[b] Conidia unable to germinate at 34 °C unless slowly rehydrated (difference between SR/34 °C/72 h, the overall viability, and FR/34 °C/72 h germination).
[c] Conidia not killed by imbibitional damage that exhibited delayed germination (difference between FR/34 °C germinations at 72 vs. 24 h).
[d] Sum of items 2 and 3 above [equivalent to the difference between the overall viability (SR/34 °C/72 h) and FR/34 °C/24 h viability].

Table 3
Effects of conidial quality and rehydration protocol on germination of *M. anisopliae* (isolate CB-10), subjected (13 h at 64.4% RH and 50 °C) or not to stressful conditions during storage.

Incubation time (h)	Germination (%)[a]			Debilitated conidia (based on germination recorded after SR and FR at 34 °C)		
	Slow rehydration (SR protocol followed by immersion at 34 °C)	Fast rehydration (FR protocol)				
		34 °C	25 °C	Conidia killed by imbibitional damage (%)[b]	Surviving conidia exhibiting delayed germination (%)[c]	Total debilitated conidia (%)[d]
Non-stressed conidia						
24	91.3 ± 1.0abc	81.3 ± 1.9cd	49.9 ± 2.0e	10.2 ± 2.3	5.0 ± 1.3	15.3 ± 2.0
48	93.6 ± 0.8ab	86.6 ± 2.9bc	70.9 ± 2.1d			
72	96.5 ± 0.5a	86.3 ± 2.3bc	72.0 ± 5.3d			
Stressed conidia						
24	28.3 ± 10.4	0.0 ± 0.0	0.1 ± 0.1	43.5 ± 5.2	41.5 ± 2.6	85.2 ± 2.8
48	70.3 ± 4.9a	27.4 ± 3.6c	11.5 ± 1.9e			
72	85.2 ± 2.8b	41.5 ± 2.6d	20.1 ± 1.5ce			

[a] Germination percentages (means ± standard errors) within each stress treatment (stressed or non-stressed) followed by the same letter are not significantly different (Tukey–Kramer HSD, $\alpha = 0.05$).
[b] Conidia unable to germinate at 34 °C unless slowly rehydrated (difference between SR/34 °C/72 h, the overall viability, and FR/34 °C/72 h germination).
[c] Conidia not killed by imbibitional damage that exhibited delayed germination (difference between FR/34 °C germinations at 72 vs. 24 h).
[d] Sum of items 2 and 3 above [equivalent to the difference between the overall viability (SR/34 °C/72 h) and FR/34 °C/24 h viability].

effects of both incubation time and rehydration protocol were highly significant ($F_{2,8} = 39.1$, $P < 0.0001$; $F_{2,9} = 328.3$, $P < 0.0001$, respectively). In contrast to the results with non-stressed conidia, there was a significant negative effect of FR at 34 °C; after 72 h, germination of conidia subjected to FR was 44% compared to 78% for conidia subjected to SR (Table 2). Few conidia survived FR at 0.5 °C. In addition, 30% of the stressed conidia required >24 h to germinate following FR (13.6% vs. 43.5%).

3.4.2. *Metarhizium anisopliae*

Overall results with Ma (Table 3) were remarkably similar to those with *B. bassiana*, except that in the case of the non-stressed conidia, the main effects of both incubation time and germination protocol were significant ($F_{2,8} = 22.3$, $P = 0.0005$; $F_{2,9} = 60.6$, $P < 0.0001$, respectively), and the germination protocol × time interaction was significant, though only marginally ($F_{4,18} = 3.1$, $P = 0.041$). ANOVA of the data from the stressed conidia was complicated by the minimal germination observed within 24 h after FR with consequently zero or near-zero variance. Excluding the 24-h data, the main effects were highly significant ($P \leqslant 0.0002$), and there was no evidence of a 2-way interaction ($F_{2,8} = 0.5$, $P = 0.60$). Large proportions of the conidia exposed to the stressful storage conditions exhibited slow germination and high sensitivity to FR. Following SR, 57% of conidia required >24 h to germinate, and only 42% and 20% of conidia remained viable after FR at 34 and 25 °C, respectively.

4. Discussion

In the present study, viability of a highly stressed Ma conidial powder determined following fast rehydration was 0% after incubation for 24 h and >40% after 72 h; germination reached 85% following slow rehydration and incubation for 72 h. These observations highlight the great extent to which germination protocols may impact results of experiments involving entomopathogenic fungi. The ungerminated conidia of both Ma and Bb observed after incubation for 72 h appeared dead (not swollen) and, therefore, it is very likely that the full potential (overall) germination rates reported here are close to actual values. We have shown that exposure of Bb and Ma conidia to unfavorable (stressful) storage conditions of high a_w, temperature, and/or O_2 (factors that boost metabolic activity) results in both increased sensitivity to imbibitional damage during fast rehydration and reduced capacity for rapid germination (within 24 h). Slow, vapor phase rehydration of conidia and incubation for a prolonged period enabled many stressed conidia to successfully germinate. In all experiments, increasing the level of stress (i.e., increasing a_w, temperature, or O_2 concentration) resulted in increasing proportions of affected conidia. We refer to these conidia as stressed or "debilitated" conidia, which include conidia expressing delayed germination and conidia with increased susceptibility to imbibitional damage. There likely exists an additional category of debilitated conidia: those conidia alive at the time of rehydration but unable to initiate germ tube formation. Such conidia could clearly be characterized as moribund (approaching death) and might be detectable through use of fluorescent staining procedures not attempted in the present study (Schading et al., 1995).

The likely mechanism underlying imbibitional damage during rehydration of dry cells was unveiled two decades ago (Crowe et al., 1989), and there is abundant evidence supporting the existence of a gel-to-liquid crystalline phase transition when the phospholipids in dry cell membranes are rehydrated. Leakage of cell contents takes place when dry membranes are plunged into water at a temperature below the phase transition temperature (generally referred to as the melting temperature, T_m). Imbibitional damage can be avoided by slowly rehydrating dry cells in a water–vapor saturated atmosphere, or by immersion in water at a temperature >T_m (Crowe et al., 1992; Faria et al., 2009). For the dry, non-debilitated Bb powder, it is clear that T_m is below 34 °C, since both the FR and SR germination protocols performed at this imbibition temperature generated equally high estimates of viability. A T_m of <34 °C is in accord with values known for other microorganisms; it is well known, for example, that fast rehydration of dry *Saccharomyces cerevisiae* cells should be performed using water warmed to 38–42 °C (Echigo et al., 1966; van Steveninki and Ledeboer, 1974).

Regarding the high-viability (non-debilitated) Ma powder, 24% (96.5−72.0) of conidia were susceptible to imbibitional damage at 25 °C, whereas 65% (85.2−20.1) were susceptible following exposure to stress. For Bb conidia fast-rehydrated at 0.5 °C, conidial mortality due to imbibitional damage was 46% in a non-stressed powder vs. 74% in a stressed powder [at this low temperature, but not at 25 °C, non-debilitated Bb conidia are quite susceptible to imbibitional damage (Faria et al., 2009)]. It has been shown that stress induces production of fatty acids by de-esterification of membrane phospholipids and that this process may drive up T_m

(McKersie et al., 1989; van Bilsen and Hoekstra, 1993; van Bilsen et al., 1994).

Imbibitional damage is an irreversible physical process (Hoekstra and van der Wal, 1988), which instantaneously kills sensitive unicellular organisms, as previously shown in studies of pollen (Hoekstra, 1984) and conidia of entomopathogenic fungi (Faria et al., 2009). This fact explains the few significant differences in viability observed across incubation times following fast rehydration of non-stressed conidia at the low, imbibitional damage-inducing temperatures. In low-quality (stressed) powders, due to presence of conidia expressing delayed germination, germination rates tended to increase with increasing incubation time and the trends were similar, regardless of the rehydration protocol. Thus, the populations of conidia that survived imbibitional damage exhibited patterns of delayed germination very similar to those of conidia that were protected from imbibitional damage by the slow-rehydration protocol (Tables 2 and 3). This interesting result (insignificant or only marginally significant interaction between incubation time and rehydration protocol) supports a hypothesis that the underlying causes of delayed germination and increased sensitivity to imbibitional damage are very different and exhibit independent action.

Delayed germination has been associated with diverse factors, such as incompatibility with formulants and other chemicals (Alves et al., 1998; Mohan et al., 2007; Faria et al., 2009), UV radiation (Zimmermann, 1982; Moore et al., 1993; Braga et al., 2001; Ghajar et al., 2006), temperatures either below or above the optimal range following inoculation on nutrient media (Luz and Fargues, 1997; Devi et al., 2005), exposure to substrates with low water activities (Luz and Fargues, 1997; Milner et al., 1997; Andersen et al., 2006), short-term exposure of aqueous conidial suspensions to heat before inoculation on suitable media (Zimmermann, 1982; Rangel et al., 2005; Fernandes et al., 2008), and long-term storage at above-freezing temperatures (McClatchie et al., 1994; Moore et al., 1995; Alves et al., 1996; Magalhães and Boucias, 2004). With respect to long-term storage, delayed germination for Bb isolate 447 was first noticed after a 30 months-storage of conidial powders at 6 ± 2 °C (Alves et al., 1996). After seven years at this temperature, germination of unformulated conidia was reported to be 0% after incubation for 24 h but 100% after 72 h. In contrast, delayed germination was not observed for conidia stored frozen for the same period of time. Delayed germination was also observed for conidia of isolate IMI 330189 of *M. acridum* dried with silica gel and kept in oil, for which delayed germination was noticed after 18 months of storage at either 8 or 17 °C (Moore et al., 1995). After 37 months, ca. 30% of conidia stored at 17 °C did not germinate within 24 h but did so within 48 h, whereas at 8 °C viability was higher, and ca. 21% of living conidia did not germinate within 24 h. Dry conidia of *M. acridum* isolate CG 423 showed 21% viability at 24 h but 88% at 48 h after 6 months at ca. 25 °C (Magalhães and Boucias, 2004).

Delayed germination is a physiological phenomenon that becomes more pronounced as conidia age naturally or which can be expressed within a very short period of exposure to stressful conditions during storage. The effect of storage on retarded germination was briefly discussed by Burges (1998), who attributed delayed germination of dry conidia to the "extra time taken for recovery from physiological dehabilitation [sic] in storage." A deeper understanding of the physiological processes associated with delayed germination is clearly needed. Despite similar appearances of conidia comprising a population, the population may indeed be highly heterogeneous (Gottlieb, 1978). Intra-populational heterogeneity in conidia, including differences in age and maturity (Moore et al., 1996) and cold tolerance (Daoust and Roberts, 1983) have been shown or suggested.

We believe that the nature of delayed germination observed in our studies is fundamentally the same as that described in previous storage studies, the only difference being that while this condition took many months or even years to develop under low-temperature regimes, it was expressed after just a few hours of storage under conditions that boosted metabolic activity. Reactions in (hydrated) conidia are primarily enzymatic (Gottlieb, 1978), and increases in either temperature or water availability are expected to boost metabolic rates (Weber et al., 1999; Willcock and Magan, 2001). When both conidial a_w and temperatures are in the upper tolerable extreme, especially in the presence of O_2, onset of delayed germination would be greatly shortened. Other studies corroborate this hypothesis; for example, Fernandes et al. (2008) reported delayed germ tube emergence from Bb conidia following suspension in water and exposure to 45 °C for 1 h. Also, following exposure of *Metarhizium* sp. (formerly identified as *M. anisopliae*) conidia in aqueous suspension to 45 °C for 30 min, germination percentages after 24 and 48 h on agar medium were 6% and 82%, respectively (Zimmermann, 1982). Likewise, Rangel et al. (2005) observed similar results with *M. robertsii* isolate ARSEF 2575 (segregated from *M. anisopliae* in a genomic reclassification by Bischoff et al., 2009) following aqueous exposure to 45–47 °C for 3 h, with germination percentages varying from <10% after 24 h to about 50% after 48 h; for *M. acridum* the difference was less pronounced, ranging from about 80–95%. Finally, Luz and Fargues (1997) reported a considerable increase in the proportion of Bb conidia expressing delayed germination after storage at 35 °C vs. 20–30 °C.

The germination patterns observed for Bb conidia following incubation in jars with different ERHs, in which increasing ERH led to lower viabilities (following both SR and FR), is in agreement with results previously reported for this fungus (Clerk and Madelin, 1965; Sandhu et al., 1993). Elevated release of CO_2 in jars was related to high conidial water activities, demonstrating that respiration can be greatly stimulated under such conditions, resulting in increased occurrence of debilitated conidia (as indicated by pronounced differences in results between fast vs. slow rehydration protocols). When CO_2 concentrations were assessed through storage under a high-temperature regime, no negative effects of this gas on germination percentages were observed. The benefits of replacing air with N_2 or CO_2 in terms of extended longevity under storage has been previously reported for beneficial fungi (Clerk and Madelin, 1965; Teshler et al., 2007). We have further demonstrated that conidial viability is prolonged in low-O_2 atmospheres, independent of CO_2 concentration. In this study, presence of even low concentrations of O_2 was detrimental to viability, and increasing O_2 concentrations yielded increasing proportions of debilitated conidia. The importance of O_2-free environments for enhancing mycopesticide shelf-life was highlighted by Jin et al. (1999), and we have now demonstrated a link between O_2-deprived atmospheres and reduced numbers of debilitated conidia. Due to methodological limitations, the lowest O_2 concentration tested in our study ranged from 0.2% to 0.5%, representing the residual O_2 in jars following prolonged flushing with pure nitrogen at a gentle flow rate. This value is in agreement with average residual O_2 of 0.26% following gas flushing of foil bags (Teshler et al., 2007). In all gas experiments, viabilities were higher and numbers of debilitated conidia were lower for the drier conidia (0.100 a_w or 4.3% moisture content vs. 0.143 a_w or 5.3% moisture content). The results reveal that even seemingly small variations in a_w can substantially impact conidial longevity. Optimal a_w for prolonged longevity of entomopathogenic fungi is reported to lie in the range of 0.11–0.14 (11–14% ERH) at room temperature, corresponding to ca. 5% moisture content (Hong et al., 1998, 2001). The fact that in our study 0.098–0.100 a_w supported greater longevity than 0.143–0.147 a_w may be an indication that, under near-anaerobic storage conditions, desirable a_w values for prolonged longevity may be lower than previously thought. Although the same procedure was used in both O_2 experiments, relative

humidity conditions in the lab during transfer of samples to jars just before gas flushing were not constant, resulting in variable conidial water activities (and longevities). This fact highlights the potential importance of environmental conditions in production facilities during mycopesticide processing.

As previously discussed, the germination protocol based on slow rehydration in many cases led to substantially higher germination than the fast rehydration protocol, as did extension of the incubation period beyond 24 h; however, the higher counts from these methods included large numbers of debilitated conidia (those viable conidia unable to germinate within 24 h or exhibiting hypersensitivity to imbibitional damage). It is our belief that inclusion of debilitated conidia in counts could lead to misleading assessments of potential efficacy and that germination protocols based on fast rehydration (at a temperature that does not cause imbibitional damage in non-debilitated conidia) and short incubation times ($\leqslant$24 h) are more likely to reflect the "true" quality of conidia (see Appendix A). Indeed, preliminary bioassays with 2nd-instar *Spodoptera exigua* larvae indicated that debilitated conidia from our treatments were significantly less virulent than non-debilitated conidia (unpublished data). It is well documented that rapid germination is an important virulence factor in conidia of entomopathogenic fungi, particularly when applied against rapidly developing (frequently molting) insect larvae or against insects in habitats where environmental conditions are only marginally or intermittently favorable for infection (Vey and Fargues, 1977; Al-Aidroos and Roberts, 1978; Hassan et al., 1989; Samuels et al., 1989; Vandenberg et al., 1998). Clearly, viability assessments using fast rehydration protocols should be performed with warm water when working with fungal species or strains whose healthy (non-debilitated) conidia are inherently susceptible to imbibitional damage, such as Ma (Faria et al., 2009). Further investigations are warranted. Finally, standardization of initial water activity would make germination estimates from different laboratories more comparable. In our lab, drying of small amounts of conidia (0.6 g or less) in glass jars with ca. 30 g of Drierite for three days at 25 °C has proven adequate for producing consistently low water activities.

Only 2% of high-quality Bb conidia with very low water activity were susceptible to imbibitional damage at 34 °C when not subjected to previous slow rehydration, and delayed germination was not recorded (Table 2), suggesting that non-stressed, dry Bb conidia are capable of rapid water uptake and germination in moist environments. The vast majority of non-debilitated Ma conidia were also able to germinate rapidly without prior slow rehydration when inoculated onto a moist substrate. The imbibitional damage (9.9%) recorded for the non-stressed Ma powder (Table 3) may have been caused by the use of a much older batch compared to a relatively fresh Bb powder.

James and Jaronski (2000) observed that Bb conidia in preparations with high vs. low overall viabilities following storage were equally virulent against nymphs of *Bemisia tabaci* biotype B and concluded that the quality of the surviving propagules had not decreased with storage time. Doses had been adjusted for viability using germination counts recorded after fast rehydration and incubation for 18 h on an agar medium. These findings support our hypothesis that germination protocols based on fast rehydration and short incubation times may provide the best estimates of fungal quality.

Although the processes of conidial debilitation can be slowed by optimal drying, storage temperatures, and packaging systems (factors that extend shelf-life), virulence of debilitated conidia requires further investigation. Slow germination and hypersensitivity to imbibitional damage could severely compromise the ecological fitness of fungal biocontrol agents. Developing methods that minimize expression of these negative traits and adopting germination protocols that allow for exclusion of severely debilitated conidia are of paramount importance.

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Appendix A. Standard germination protocol for quality assessment of hydrophobic conidia (exclusion of debilitated conidia)

Stressed (debilitated) conidia exhibit hypersensitivity to imbibitional damage and delayed germination. These conidia may be less virulent than their non-stressed counterparts, and in assessing the overall quality of a technical conidial powder or mycopesticide formulation, it may be desirable to exclude these conidia from viability counts. In order to exclude propagules hypersensitive to imbibitional damage, dry conidial samples should be directly plunged into a surfactant solution adjusted to a temperature that does not cause imbibitional damage in dry, non-stressed conidia. To exclude slow-germinating propagules, incubation time should be established as the minimal time required for non-stressed conidia of the test isolate to reach maximum germination. Although additional studies are needed, a basic protocol follows:

A.1. Standardization of initial water content

Conidia (0.6 g or less) held in small plastic cups should be dried for 3 days at 25 °C in 125-mL glass jar with sealed lid. Each jar should contain ca. 30 g of the desiccant calcium sulphate (eight-mesh indicating Drierite, W.A. Hammond Drierite Co., Xenia, OH, USA). This procedure has been shown to result in conidial water activity between 0.019 and 0.030 (in equilibrium with 1.9–3.0% relative humidity). Other desiccants such as silica gel may also be used. We believe that skipping this step would not significantly alter the results when working with powders that have been stored under reasonably dry conditions (a_w < 0.30, equivalent to moisture content <8%).

A.2. Suspension preparation

Dry conidial sample (ca. 0.1 mg picked up on the tip of spatula) should be added to 10 mL of water containing any commonly used surfactant such as Tween 80. A minimum of four suspensions should be prepared. If the same suspensions will be used for concentration determinations via hemacytometer counts, use of a surfactant with a hydrophilic–lipophilic balance between 8 and 10, such as Silwet L-77 or TDA (polyoxyethylene tridecyl ether) at 0.2% is recommended, as surfactants with this balance produce optimal suspensions for counting (Jin et al., 2008, 2009). Vials with the surfactant solution and ca. 1 g of 2 mm glass beads, should be equilibrated at 33–34 °C immediately prior to addition of conidia. Solutions equilibrated at lower temperatures may be used for fungi like *B. bassiana* strain GHA that are insensitive to imbibitional damage at room temperatures.

A.3. Plating technique

After agitation for a few minutes, 10-μl droplets of conidial suspension (approximately 10^5 conidia/mL) should be immediately applied onto a 1.0 × 1.0 cm blocks of the desired agar-based benomyl-free germination medium on microscope slides (1 droplet/block, no spreading necessary). The microscope slides with the inoculated agar blocks should then be placed in Petri dishes, sealed with parafilm, and incubated in darkness at 25 °C. Alternatively, droplets of conidial suspension may be applied to agar medium in standard Petri dishes. Commonly used germination media include potato dextrose agar and Sabouraud dextrose agar supplemented with 1% yeast extract. Addition of gentamycin or other antibiotics may be required when working with contaminated conidial preparations. Optimal incubation time will be fungal species- and strain-specific. Recommended incubation time is 16–18 h for isolate GHA of *B. bassiana* and ca. 20 h for isolate CB-10 of *M. anisopliae*, but times may differ according to medium.

A.4. Counting

After the desired incubation time, at least 200 conidia should be counted for each replicate suspension through phase-contrast microscopy, although other microscopic techniques and staining (other than vital staining) can be used. Conidial samples from replicated suspensions should be plated individually on agar blocks. Number of replicates should be sufficient to proved desired minimum standard errors, coefficients of variation, or confidence intervals.

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