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Conidial vigor vs. viability as predictors of virulence of entomopathogenic fungi

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ABSTRACT

We tested the hypothesis that debilitated conidia exhibiting slow-germination (requiring > 16 h to germinate) are less virulent than vigorous conidia exhibiting fast germination (requiring ⩽ 16 h to germinate). Preparations of *Beauveria bassiana* s.l. strain CG 1027 with variable ratios of vigorous to debilitated conidia were assayed against third-instar larvae of *Spodoptera frugiperda*. As the proportion of debilitated conidia in test preparations increased, LC_{50} expressed in terms of total viable conidia increased, while LC_{50} expressed solely in terms of vigorous conidia remained constant, indicating that vigorous conidia were responsible for nearly all mortality observed in the assays. Larvae treated with conidia from low-quality batches (with high proportions of debilitated conidia) survived consistently longer than those treated with comparable doses of conidia from high-quality batches. These results confirm our previous hypotheses that inclusion of debilitated conidia in viability assessments can lead to overestimation of the quality (potency) of mycoinsecticide preparations and support our recommendation for use of short incubation periods for assessing viability whenever viability is relied upon as an indicator of product quality.

1. Introduction

Spore viability is widely relied upon as a measure of the quality of fungal inocula used in biological control research and applications (Jenkins and Grzywacz, 2000; Goettel et al., 2000). The most common approach in determining viability relies on direct microscopic observations of propagules incubated on a solid semi-synthetic substrate (Goettel and Inglis, 1997). Protocols generally call for incubation periods of ca. 24 h; however, methods based on vital staining or incubation in the presence of benzimidazole fungicides, which inhibit germ tube elongation, have been developed with claims of greater accuracy due to elimination of bias that results when germ tube growth from early-germinating conidia obscures inviable conidia on agar surfaces (see Goettel and Inglis, 1997). These techniques have, respectively, also enabled researchers to make very rapid assessments and provided flexibility in assessing large numbers of treatments/samples.

Vigor is a relatively recent term that relates to the strength of spore germination and germ tube growth, being strongly influenced by factors such as the fermentation system (type and amount of nutrients in the cell) and downstream processing (Jin et al., 1992). Speed of germination is one of the most commonly reported indicators of vigor. Studies have shown that long-term storage or even short periods of storage under unfavorable conditions of high relative humidity, temperature, and/or O_2 (factors that boost metabolic activity) have negative impacts on the speed of germination of *Beauveria bassiana* and *Metarhizium anisopliae* conidia (Alves et al., 1996; Faria et al., 2010; Lopes et al., 2013). Vigorous, non-stressed conidia of these fungi germinate within 18–24 h post-inoculation (hpi), whereas germination of storage-stressed (debilitated) conidia is delayed and asynchronous, occurring between 24 and 72 hpi (Faria et al., 2010).

High vigor expressed as fast germination is a desirable trait of entomopathogenic fungi and has been identified as a virulence factor in a number of host-pathogen associations (Al-Aidroos and Roberts, 1978; Hassan et al., 1989; Alves et al., 1996; Altre and Vandenberg, 2001). Correlations between germination speed and virulence, however, are not always evident (Chandler et al., 1993; Alves et al., 1996). In this work we investigated the hypothesis that calculations of doses based on viability assessments that measure vigor (doses based on numbers of fast-germinating conidia) are

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more consistent predictors of the potency of a conidial preparation than doses based on viability alone (doses based on total fast- + slow-germinating conidia). As a model, we used an isolate of *B. bassiana* sensu lato and third-instar larvae of *Spodoptera frugiperda* (Lepidoptera: Noctuidae).

2. Materials and methods

2.1. Preparation of conidial suspensions and germination assessments

Pure conidia of commercial strain CG1027 (also known as ESALQ PL63) of *B. bassiana* s.l., isolated from *Atta* sp. (Hymenoptera: Formicidae), were previously used in shelf-life experiments in which propagules were exposed to different temperatures and relative humidities for varying periods of time and then stored frozen (ca. −4 °C) until use in this study. The germination protocol followed recommendations by Lopes et al. (2013). Briefly, dry conidia were suspended in 0.05% Tween 80 and drop-inoculated onto agar plates (20 µl/drop). The inoculated plates were dried in a laminar flow hood for 15 min and then sealed with Parafilm and incubated at 25 °C. Percentages of vigorous conidia were determined by assessing viability after incubation on potato dextrose agar (PDA) for 16 h. Percentages of total viable conidia were determined via incubation on PDA + carbendazim (25 µg/L) for 48 h. Germination was assessed by direct microscopic observation at 400× magnification (without addition of stain/fixative solutions or coverslips); germinated conidia were identified as those with germ tubes longer than the width of an ungerminated conidium.

2.2. Bioassays with Spodoptera frugiperda larvae

Five-dose bioassays were performed with third-instar *S. frugiperda* (Lepidoptera: Noctuidae) larvae using batches of unformulated conidia with contrasting viabilities and vigor (see treatments, Table 1). Stock suspensions were prepared and serially diluted in 0.05% Tween 80 and then applied as 2-mL aliquots using a spray tower. Spray deposition was sampled during every application, and numbers of conidia per mm^2 were determined. Each assay included a control batch of larvae sprayed with Tween solution. Larvae were treated in groups (12 larvae/dose + control = 72 larvae/assay), and each assay was replicated 4 times (each replicate assay testing a conidial-batch subsample independently characterized with respect to the proportions of vigorous vs. total viable conidia and actual doses applied). Treated larvae were held individually in the cells of 24-well tissue culture plates (TPP Techno Plastic Products AG, Switzerland) containing corn leaves, and mortality was assessed daily for ten days. The experiment comprised three tests conducted on different dates over a period of nine weeks. In each test, assays were conducted with a batch of conidia with reduced vigor (variable across tests) and a "standard" batch with high vigor (see Table 1).

2.3. Statistical analyses

From each of the individual 5-dose assays, two estimates of LC_{50} were determined based on probit analyses with two alternative expressions of dose (dose classes): dose expressed in terms of vigorous conidia (numbers of germinated conidia counted in the 16-h viability assessments) vs. dose expressed in terms of total viable conidia (numbers of vigorous + slow-germinating conidia counted in the 48-h assessments). Within each test, data were analyzed by mixed-model ANOVA treating assay (nested within conidial batch) as a random effect and conidial batch quality (high vs. low) and dose class (vigorous vs. total viable conidia) as fixed effects. The mean alternative LC_{50} estimates for low-quality batches of conidia were ultimately compared to the corresponding estimates for the high-quality (standard) batches via single-degree-of-freedom *F*-tests, allowing for unequal variances (Welch's Test). The above-described ANOVAs were conducted with each log LC_{50} estimate unweighted or weighted by the inverse of its variance. The influence of debilitated vs. vigorous conidia on estimates of conidial batch potency was further illustrated via linear regression of LC_{50}s (inverse-variance weighted) on proportion conidia debilitated among total viable conidia. Finally, spray treatments within test were sorted into five dose categories, each including as many of the 12-larva treatment groups as possible within the discrete ranges shown in Table 2. Within-test daily mortality of larvae exposed to these dose categories was then analyzed by Cox proportional hazard regression for comparison of survival times ultimately expressed in terms of ST_{50} and/or ST_{30} (days until death of 50% or 30% of treated larvae).

Probit analyses were conducted using SAS PROC PROBIT (SAS 9.3, SAS Institute, Inc., 2012); all other analyses were conducted using JMP Pro 11.0.0 (SAS Institute, 2013). LC_{50} determinations were based on mortalities recorded at 7 days post-inoculation; however, monitoring was continued for an additional three days to increase accuracy of the survival time estimates.

3. Results

Bioassay results are presented in Table 1. Inverse-variance weighting had no effect on the hypothesis test conclusions. Each of the within-test, mixed-model ANOVAs revealed a highly significant interaction between dose class and batch quality (all *P*

Table 1
Effect of conidia quality on virulence of *Beauveria bassiana* isolate CG1027 against *Spodoptera frugiperda* larvae.

Quality/condition of conidia batch	Percent vigorous[a]	Total percent viable[b]	Vigorous/total viable ratio	LC_{50} (log LC_{50}) based on indicated dose class[c,d]		Probit regression line slope ±SE[d]
				Vigorous conidia (mm^2)	Total viable conidia (mm^2)	
Test 1. Healthy/vigorous vs. moderately stressed/debilitated batches of conidia						
High quality (healthy/vigorous)	83.3 ± 0.2	89.0 ± 0.9	0.935	587 (2.769 ± 0.154) a	628 (2.798 ± 0.154) a	2.682 ± 0.829 a
Low quality (debilitated)	44.5 ± 2.3	79.1 ± 0.8	0.563	1223 (3.087 ± 0.042) a	2180 (3.338 ± 0.043) b	2.318 ± 0.614 a
Test 2. Healthy/vigorous vs. highly stressed/debilitated batches of conidia						
High quality	90.1 ± 0.1	92.4 ± 0.3	0.975	417 (2.620 ± 0.018) a	428 (2.632 ± 0.017) a	2.319 ± 0.648 a
Low quality	22.8 ± 0.7	71.2 ± 1.0	0.321	420 (2.623 ± 0.048) a	1310 (3.117 ± 0.051) b	1.469 ± 0.187 a
Test 3. Healthy/vigorous vs. severely stressed/debilitated batches of conidia						
High quality	93.4 ± 1.0	95.7 ± 1.0	0.977	356 (2.552 ± 0.053) a	365 (2.562 ± 0.050) a	1.611 ± 0.316 a
Low quality	3.6 ± 0.6	64.9 ± 4.7	0.058	234 (2.369 ± 0.253) a	4341 (3.638 ± 0.284) b	1.159 ± 0.255 a

[a] Percent of conidia (mean ± standard error, $n = 4$) that germinated within 16 h.
[b] Percent of conidia (mean ± standard error, $n = 4$) that germinated within 48 h (total percent viable).
[c] Mean LC_{50} based on mortality recorded 7 days post-treatment. LC_{50}s obtained by back-transformation of mean log LC_{50} values (± values are standard errors, $n = 4$). Mean mortalities of control larvae sprayed with 0.05% Tween 80 in tests 1–3 were 4.2%, 14.6%, and 2.1%, respectively.
[d] Means within tests within columns followed by the same letter are not significantly different (Welch tests, $P < 0.05$).