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Imbibitional damage in conidia of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium acridum*, and *Metarhizium anisopliae*

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ABSTRACT

When dried organisms are immersed in water, rapid imbibition may cause severe damage to plasma membranes; in unicellular organisms, such damage is usually lethal. This study investigated effects of water activity (dryness) of organisms and immersion temperature on imbibitional damage in three insect pathogenic fungi. Conidial powders of *Beauveria bassiana* (*Bb*), *Metarhizium anisopliae* (*Ma*) and *Metarhizium acridum* (*Mac*) were dried/hydrated to a broad range of water activities (a_w) (0.023–0.961) prior to immersion in water at 0.5–33 °C. Imbibitional damage in conidia of each fungus occurred rapidly, with no differences in viabilities observed following immersion for 2 vs. 60 min. Damage increased with decreasing water activity of the conidia and decreasing temperature of the immersion water. Dry ($a_w \leqslant 0.333$) *Metarhizium* spp. conidia were highly susceptible to imbibitional damage, with viability declining to ⩽5% after immersion at 0.5 °C and ⩽63% following immersion at 15 °C. Germination of the driest *Ma* conidia was reduced to 66% after treatment at 25 °C. In contrast, *Bb* was highly tolerant to damage, with significant reductions in viability (to levels as low as 43–65%) occurring only when dry conidia were immersed at 0.5 °C. Damage was prevented when conidia were slowly rehydrated by humidification prior to immersion and immersion temperature was increased to 33–34 °C; germination of all fungi was ⩾94% under these optimal conditions. However, immersion of the driest *Bb*, *Ma*, and *Mac* powders in warm water (33 °C) also resulted in high viabilities (95%, 89%, and 94%, respectively), and slow-rehydrated conidia also retained high viability (87%, 92%, and 83%, respectively) after immersion in ice-cold water (0.5 °C). Formulation of conidia in pure (non-emulsifiable) paraffinic oil provided considerable protection from imbibitional damage. This study underscores a need for establishing standard protocols for preparing aqueous suspensions of sensitive fungi for both research and commercial applications.

1. Introduction

Many fungi display the ability to survive profound dehydration (Burges, 1998; Crowe et al., 1992). In desiccated (anhydrobiotic) states, spores are able to survive extreme environments characterized by dry heat, freezing/thawing, and acid conditions (Griffin, 1994). With respect to fungal entomopathogens employed as microbial biological control agents, enhanced thermotolerance imparted by desiccation (Hedgecock et al., 1995; Moore et al., 1996; Chen et al., 2008; Connick et al., 1998) is particularly significant as it can substantially increase the shelf-lives of fungus-based biopesticide products (Burges, 1998; Wraight et al., 2001).

When dried organisms are immersed in water, rapid imbibition may cause severe damage to plasma membranes (Crowe et al., 1992; Hoekstra et al., 1999). In unicellular organisms, such injury usually leads to cell death (Nijsse et al., 2004). Numerous studies with various organisms, including seeds, pollens, and yeasts, have demonstrated that imbibitional damage increases with decreasing moisture content (dryness) of the organism subjected to rapid rehydration and decreasing immersion temperature. Imbibitional damage can be prevented either by using warm water for rehydration or by slowly rehydrating the organism in a humid atmosphere (vapor phase rehydration) prior to immersion in water (see Crowe et al., 1992).

Imbibitional damage and methods for its prevention have been studied in yeasts (van Steveninki and Ledeboer, 1974; Crowe et al., 1992), but we are aware of only one reference to imbibitional damage among filamentous fungi, which cites *Metarhizium acridum* (Driver & Milner) Bischoff, Rehner, & Humber conidia dried to 4–5% moisture content (Moore et al., 1997) (*M. acridum* was formerly identified as *Metarhizium anisopliae* var. *acridum*; see Bischoff et al., 2009). Viability of conidia of this fungus that were slowly rehy-

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drated by exposure to a water-saturated atmosphere for 5–40 min was >70%, whereas viability of conidia that were rapidly rehydrated by immersion in water was <25%.

Rehydration of conidia to levels of normal metabolism without membrane damage is of paramount importance for successful pest control using dry formulations of fungal pathogens. In-depth studies of imbibitional damage as a potentially important factor in the efficacy of mycopesticide products are lacking. The primary objective of this study was to quantify the effects of water activity and temperature as key factors determining severity of imbibitional damage in conidia of three commercially important species of entomopathogenic fungi. Broader objectives included increasing awareness and understanding of this phenomenon among mycopesticide researchers, producers, and users and assessing practical means of circumventing the problem. Toward the latter objective, an experiment was conducted to determine if oil formulation has potential to protect conidia from imbibitional damage.

2. Materials and methods

2.1. Source of fungi

Three fungi were tested: *Beauveria bassiana* (Bals.) Vuill. (*Bb*) strain GHA originally isolated from a chysomelid in USA, *M. anisopliae* (Metschn.) Sorokīn (*Ma*) isolate CB-10 (ARSEF 7981) from a cercopid in Brazil, and *M. acridum* (*Mac*) isolate IMI-330189 (ARSEF 3341) from an acridid in Niger. Unformulated conidia of *Bb* were obtained from Laverlam International Corp. (Butte, MT, USA). *Metarhizium* isolates were lab-produced using a commonly employed biphasic process in which culture is initiated in liquid and completed on a semi-solid substrate. In the initial phase, flasks containing 100 ml liquid medium (20 g glucose, 20 g yeast extract l^{-1}) (Jenkins and Prior, 1993) were inoculated with conidia and incubated on a rotary shaker (150 rpm) for 6 days at 22–25 °C. In the second phase, barley mixed with distilled water (dH_2O) was autoclaved for 20 min in polyethylene bags (100 g dry barley flakes + 60 ml dH_2O per bag). Each bag was then inoculated with 70 ml of liquid culture from the initial production phase. Bags were incubated at 25 °C in darkness, and aerial conidia were harvested after 2 weeks by manually shaking the fungus-colonized substrate through a series of two sieves, 20 and 100 mesh. Conidia that passed through the 100-mesh sieve (150 μm pore size) were collected. The three stock technical powders were stored at −20 °C.

2.2. Viability determinations

For viability determinations, 10 μl droplets of suspension comprising conidia suspended in water with a low concentration of surfactant (see below) or pure paraffinic oil were pipetted onto 1 × 1 × 0.3 cm blocks (1 droplet/block) of yeast extract agar-based solid medium (YEA) containing 0.5 g yeast extract, 100 mg gentamicin, 0.1 g Tween 80, and 16 g agar l^{-1} (Meikle et al., 2003), and incubated on glass microscope slides in sealed petri dishes in darkness at 25 °C. After the desired incubation time, a coverslip was applied, and the sample was examined at 400× magnification with phase-contrast illumination. Conidia were considered germinated if a germ tube of any length was visible. A minimum total of 200 conidia were examined in several microscope fields for each replicate suspension of each experimental treatment.

In all experiments, incubators providing experimental temperatures were continuously monitored with digital data loggers (Hobo®, Onset Computer Corp., Bourne, MA, USA). Reported temperatures were ±0.5 °C for 25 °C and ±1 °C for all other temperatures.

2.3. Initial tests to identify optimal procedures for assessing germination

Three different concentrations (0, 0.005, and 0.1 g l^{-1}) of benomyl (Bonide Chemical Co., Yorkville, NY, USA) were tested in YEA. Benomyl slows hyphal growth following germ tube emergence, allowing germination assessments over prolonged periods (Milner et al., 1991). Conidial samples of *Bb*, *Ma*, or *Mac* (0.6 g/sample) were collected from the stock powders and placed in 3.4 cm diam. × 1.1 cm plastic sample cups (Novasina, Pfäffikon, Switzerland). Water activity (a_w) was measured at 25 °C with an electronic meter (LabMaster-a_w, Novasina, Pfäffikon, Switzerland); a_w of the samples ranged from 0.150 to 0.243. From each sample, three subsamples (0.15–0.2 mg picked up on the tip of a spatula) were taken. One of these subsamples was maintained in its original dry state, while the other two were slow hydrated via incubation at 25 °C in a "desiccator" with a water-saturated atmosphere; one subsample was incubated for 40 min and the other for 24 h. Following these treatments, each of the three subsamples was transferred to a screw-cap glass vial (23 ml capacity) containing approximately 1 g of 2 mm glass beads and 7 ml of dH_2O (temperature not recorded) with 0.05% of the surfactant Lutensol® (Ethoxylated Tridecyl Alcohol, BASF Corporation, Florham Park, NJ, USA) possessing a hydrophilic–lipophilic balance number of 10 (Jin et al., 1999). Resulting suspensions (containing 1–3 × 10^6 conidia/ml) were agitated for 10 min with a wrist action shaker set at 6.7 oscillations/sec (Burrel Scientific, Pittsburgh, PA, USA), and viability was determined using blocks of YEA with the above-indicated range of benomyl concentrations. Germination was recorded 18 h post-inoculation (p.i.) for *Bb* and 20 h p.i. for both *Ma* and *Mac*. This experiment was repeated with conidia dried to the 0.021–0.023 a_w range using the desiccant calcium sulfate (eight-mesh indicating drierite®, W.A. Hammond Drierite Co., Xenia, OH, USA), and germination was recorded 18, 21, and 25 h p.i. for *Bb*, *Ma* and *Mac*, respectively.

In a third experiment, the above-described procedure was repeated with the following alterations. The 0.6-g samples of each fungus were initially dried at 25 °C for 5–9 days in hermetically sealed 125 ml glass jars (Ball®, Jarden Corp., Muncie, IN, USA) containing drierite. Distilled water plus 0.05% Lutensol (hereafter referred water + Lutensol) used in the fast-rehydration treatments was equilibrated at 25 °C. Following the fast- vs. slow-rehydration treatments, conidia were inoculated onto blocks of YEA containing 0.005 g l^{-1} benomyl (hereafter referred to as YEA + benomyl), and germination counts were performed 24, 48, and 72 h p.i.

2.4. Effect of fast rehydration time on viability

Conidial samples of each fungal isolate were placed individually in 125-ml glass jars containing drierite and stored at 25 °C for 10 days. After drying, subsamples of conidia were fast rehydrated into water + Lutensol previously equilibrated to 0.5 °C in an ice bath (methods otherwise as described above). Suspensions were manually shaken for 5 s and then returned to the ice bath and incubated for an additional 2 or 60 min. After treatment, each suspension was vortexed for 10 s at room temperature (23–24 °C), and a sample was plated on YEA + benomyl. Conidia were incubated for 48 h at 25 °C and germination was assessed.

2.5. Effects of conidial water activity and imbibition temperature on imbibitional damage

Conidial samples of the three fungal isolates were placed in hermetic 125-ml glass jars containing either dH_2O, drierite, or saturated salt solutions based on sodium hydroxide (NaOH), magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_20$), or sodium nitrite ($NaNO_2$) and