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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 2.0×10^5 to 1.0×10^6 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 2.0×10^4 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.