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A protocol for determination of conidial viability of the fungal entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae* from commercial products

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ABSTRACT

Techniques for directly determining conidial viability have widespread use but also have limitations for quality control assessments of formulated mycoinsecticides, especially in emulsifiable oil. This study proposes a new method based on adaptations of already established protocols that use the direct viability method to make it more economical and accurate, thus enabling its use in the evaluation of formulated products. Appropriate parameters and conditions were defined using products based on *Beauveria bassiana* and *Metarhizium anisopliae* in the forms of pure conidia, fungus-colonized rice, ground fungus-colonized rice and oil dispersion. The established protocol, named *ESALQ*, consists of the transfer of 150 μL of a suspension containing about 0.7 and 1×10^6 conidia/mL onto Rodac® plates with 5 mL of potato dextrose agar culture medium + 5 mg/L of Pentabiotic® and 10 μL/L of Derosal® (Carbendazim) and subsequent counting of germinated and non-germinated conidia. For the ground fungus-colonized rice and oil dispersion formulations, prior to transferring the fungal suspension to the medium, rice should be decanted and the oil removed, respectively. This method was compared with another direct viability method and with the *Colony-forming unit* (*CFU*) and *Fluorescence* viability methods, comparing the accuracy obtained using the coefficient of variation (CV) of the analysis of each method. The results showed that in addition to the ease of application, the developed method has higher accuracy than the other methods (with a CV up to seven times lower than in the *Standard* method and up to 32 times lower than *CFU*). The *CFU* method underestimated the concentration of viable conidia in most of the tested fungal forms, and in the emulsifiable oil products, these values were 54% lower for *B. bassiana* and 84% lower for *M. anisopliae*. The adaptations and standardizations proposed in the *ESALQ* method showed effective improvements for routine quality assessment of mycoinsecticides.

1. Introduction

The standardization of inexpensive and fast methods for evaluating the viability of fungal conidia is of great interest, especially for studies with biological pest control agents (Firstencel et al. 1990; Stentelaire et al., 2001). Conidial viability has been the main parameter used to determine the effects of various environmental factors on entomopathogenic fungi, including solar radiation, dehydration and water stress, extreme temperatures, pH, vigor and imbibition damage of conidia (Zimmerman, 1982; Jackson et al., 1991; Hallsworth and Magan, 1999; Rangel et al., 2004; Devi et al., 2005; Faria et al., 2009; Faria et al., 2015).

This parameter is also used by manufacturers of entomopathogenic fungi-based biopesticides to evaluate the quality of the formulated and non-formulated products, and *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Cordycipitaceae) and *Metarhizium anisopliae* (Metsch.) Sorok. (Ascomycota: Clavicipitaceae) are the most frequently used entomopathogenic fungi worldwide (Faria and Wraight, 2007). However, there are significant differences in the protocols used for conidial viability tests, and few studies aimed to measure the effectiveness of the different methods to allow research institutions and companies to select the best method for the quality control of their products (Jimenez and Gillespie, 1990; Jenkins and Grzywacz, 2000; Lopes et al., 2013).

In Brazil, 75% of the existing products based on entomopathogenic fungi are marketed in the form of pure conidia or technical concentrates (fungus-colonized substrates), and only 25% of the products are formulated; all such formulations are of the oil dispersion type (Michereff Filho et al., 2009), a very different reality from what is observed in the rest of the world (Faria and Wraight, 2007).

Some methods for determining the germination of fungal propagules are based on measuring the activity of dehydrogenase enzymes

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using tetrazolium salts (Sutherland and Cohen, 1983; Meier and Charvat, 1993; Stentelaire et al., 2001). Other staining methods include the combined use of the fluorochrome propidium iodide (PI) and fluorescein diacetate (FDA), examining, after treatment with these substances, the viable and non-viable conidia with the aid of a fluorescence microscope. These methods provide a more rapid assessment of conidial viability but are relatively more expensive and difficult to use for a large number of assessments in the short term (Firstencel et al., 1990; Jimenez and Gillespie, 1990), mainly because the reactants faint away over time and can be negatively affected by natural luminosity.

Some simple and inexpensive methods can be used, but they also have some limitations and drawbacks regarding their application. For example, the determination of the density of colony-forming units (CFUs) may have its accuracy influenced by weighing and pipetting errors as the results are directly linked to the amount of sample used (Goettel and Inglis, 1997; Jenkins and Grzywacz, 2000; Stentelaire et al., 2001).

Another commonly used method is direct counting, which is based on counting the viable and non-viable conidia/spores under a light microscope after incubation of a conidial suspension plated on a nutrient medium containing agar (semi-solid) for a period of time and at a predetermined temperature (Goettel and Inglis, 1997; Alves and Pereira, 1998). However, although this method is more applicable, there are still many aspects that can be improved for greater reproducibility and to facilitate assessments.

Milner et al. (1991) have suggested the addition of the selective fungicide Benlate® (Benomyl, DuPont Ltd.) to the culture medium in viability tests to provide a more uniform germination of *M. anisopliae* without impairing the viability. Due to the fungistatic properties of this product at certain concentration, fungi can remain incubated for a longer period, allowing comparative germination studies between different isolates or even to evaluate the same sample at different reading times. This active principle can even be used in assays for assessing the effects of imbibition in conidia of fungi, such as *B. bassiana* and *M. anisopliae*, as performed by Faria et al. (2010).

Due to these advantages, Benomyl began to be used by many authors in viability tests, including for making comparisons between isolates subjected to different environmental conditions or maintained for different storage periods. Benomyl no longer has approval for use in many countries. Therefore, it is necessary to select a new registered and commercially available fungicide that presents a similar effect when mixed with the culture medium and that can be used for assessing conidial viability via the direct counting method.

The currently available viability methods have limitations for products formulated in oil. It is important to propose some adjustments so that the same viability test can be used for different types and formulations of mycopesticides. It is noteworthy there is lack of studies aiming to compare the accuracy of the existing methods and to propose improvements for the methods used for stability assessment of products formulated in oil or in the form of technical concentrate.

Therefore, the objectives of this work were to develop a simple, economical and standardized method for determining the conidial viability of the entomopathogenic fungi *B. bassiana* and *M. anisopliae*, to compare its accuracy with other methods, and to validate the proposed method using different fungal biopesticides marketed in Brazil.

2. Materials and methods

This study was divided into two stages: i) development of the method called *ESALQ* for direct viability assessment, with the selection of a new commercial fungicide for use in tests of conidia germination; and ii) comparison of the accuracy of the developed method (named *ESALQ*) with other viability assessment protocols, and too the *ESALQ* method validation performed with mycopesticides marketed in Brazil.

Initially, several preliminary experiments were performed seeking alternatives to standardize and improve some aspects of the direct counting method, such as determining the lowest amount of culture medium, selecting a new fungicide for use in the analyses, selecting small plates to reduce the space used during incubation, and reducing errors resulting from the choice of fields to count and the time spent with the evaluation by delimiting predefined fields to count under a microscope.

Afterward, parameters related to the standardization of the protocol were assessed through experiments with different types of culture medium, effect of antibiotic use and validation of the newly selected fungicide. The *ESALQ* method is described later.

In the second stage, the *ESALQ* method was compared with the *CFU* (Goettel and Inglis, 1997) and *Fluorescence* (Firstencel et al., 1990) methods, considering as the more accurate methods those with viability data with lower coefficients of variation (CVs). Later on, the *ESALQ* method validation was performed with commercial products based on entomopathogenic fungi, adopting the direct counting method (henceforth referred as "*Standard*") as the comparison standard, according to Alves and Pereira (1998).

2.1. Source of fungi

Two entomopathogenic fungi were tested in the experiments that evaluated the parameters for the *ESALQ* method and in the comparisons between the *ESALQ* × *CFU* and *ESALQ* × *Fluorescence* methods: *B. bassiana* strain ESALQ-PL63 (*B. bassiana*) and *M. anisopliae* strain ESALQ-1037 (*M. anisopliae*), both from the collection of entomopathogens of the Insect Pathology and Microbial Control Laboratory (ESALQ/University of São Paulo, Piracicaba, SP, Brazil). These fungi were produced and provided by the company Koppert Brasil Ltd. (formerly Itaforte Bioprodutos Ltda©; Itapetininga, SP, Brazil), in the form of pure conidia (technical material, TC), two kinds of technical concentrate (fungus-colonized rice, TK1 and ground fungus-colonized rice, TK2), and emulsifiable oil formulation (oil dispersion, OD) (classification from Faria and Wraight, 2007), all from the same production batch, with moisture content of 7% (*w*/*w*), measured at the final phase of production. The company uses the process of fermentation in solid natural substrate (pre-cooked moistened rice), commonly employed in Brazil for the production of aerial conidia. The OD products were formulated in soybean oil with 5% emulsifier. The TC, TK1 and TK2 products were stored until use at −20 °C, and the OD formulation was stored at 4 °C. The products were removed from storage and gradually brought to room temperature (25 °C) before being used. The concentration was determined through serial dilution (0.05% Tween 80 solution) using an improved Neubauer hemocytometer (Alves and Moraes, 1998).

Commercial products based on different isolates of the fungi *B. bassiana* and *M. anisopliae* were used to *ESALQ* method validation, comparing the methods of direct assessment (*ESALQ* × *Standard*). Seven products based on *B. bassiana* and nine products based on *M. anisopliae* marketed in Brazil were used (TC or TK1 or TK2 or OD). To perform the tests, the products were removed from storage and gradually brought to room temperature (1 h at 8 °C, followed by 1 h at 18 °C), and the concentration was determined as described above.

2.2. Parameters for viability assessment

2.2.1. Selection of culture medium

Four culture media were tested: (a) Potato dextrose agar prepared in the laboratory using the broth of potato tubers (Alves et al., 1998) (PDA); (b) Commercial PDA (Difco®) (referred as PDAcom); (c) Sporulation medium (SM) (Alves et al., 1998) and (d) Water agar (2% *w*/*v*) (WA). The culture media were poured in Petri dishes, and the fungi were inoculated as described in *ESALQ* method for each preparation and then incubated in a growth chamber (26 ± 2 °C, 14 h photophase)

for 18 h until the evaluation. Each treatment (i.e., culture medium) consisted of three replicates, separated by each fungal preparation (TC, TK1, TK2, OD), with a total of 48 plates per fungus. The entire assay was repeated twice over time on different days.

2.2.2. Influence of antibiotics on conidial viability

Two experiments were conducted using the product Pentabiotic® (Fort Dodge Animal Health® Ltd., Brazil), which contains the substances benzatine benzylpenicillin (3,000,000 UI), procaine benzylpenicillin (1,500,000 UI), potassium benzylpenicillin (1,500,000 UI), streptomycin (1.250 mg) and dihydrostreptomycin base (1.250 mg). The antibiotic was added to molten PDA medium after autoclaving. The fungi were inoculated as described in *ESALQ* method for each preparation and were incubated in a growth chamber (26 ± 2 °C, 14 h photophase) for 18 h. In the first experiment, the effects of the antibiotic (5 mg/L of medium) were evaluated for all fungal preparations and compared to the 'control' medium without antibiotic. Three replicates were prepared per treatment for each fungal preparation (TC, TK1, TK2, OD), with a total of 24 plates per fungus, and the whole assay repeated twice at different times.

In the second experiment, the effect of different antibiotic concentrations on the germination of pure conidia (TC) was evaluated. The concentrations evaluated were 0 (blank control), 15, 40 and 75 mg/L of medium. Three replicates were prepared per treatment, totaling 12 plates per fungus, also repeated twice over time.

2.2.3. Influence of a fungistatic product on conidial viability

In previous experiments the fungicide Derosal® 500 SC (Bayer CropScience, a.i. Carbendazim - Methyl benzimidazol-2-ylcarbamate) was selected as an alternative to the formerly used Benlate® 500 (DuPont, a.i. Benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate)), which is no longer marketed in Brazil and many other countries. These two fungicides were compared to demonstrate the equivalence of their function in slowing down the germ tube growth. The products were added to the culture medium after autoclaving as follows: PDA medium (control); PDA medium with Benlate® (10 μL/L); and PDA medium with Derosal® (10 μL/L). The fungi were inoculated as described in *ESALQ* method onto each culture medium and incubated in a growth chamber (26 ± 1 °C, 14 h photophase) for 28 h until evaluation. The control treatment was assessed after 18 h, preventing the overgrowth (did not contain the fungicide). Three replicates were made per treatment within each fungal preparation (TC, TK1, TK2, OD), resulting in 36 plates per fungus, repeated twice at different times.

The effects of different concentrations of Derosal® on the viability of conidia (TC) were also addressed. The product was added to the culture medium at concentrations of 0 (control), 10, 30 and 60 μL/L, and the fungi were inoculated following the above mentioned protocol and incubated at 26 ± 1 °C, 14 h photophase for 28 h until the evaluation. The control treatment was evaluated after 18 h (did not contain the fungicide). Three replicates were used per treatment, with a total of 12 plates per fungus, and the entire experiment repeated twice on different occasions.

2.3. Protocol for the direct count method for the determination of conidial viability — ESALQ

The protocol was developed based on recommendations proposed by Alves and Pereira (1998) and Goettel and Inglis (1997), seeking primarily to optimize the time and material spent for analysis and to reduce errors in the assessments as well as standardizing parameters that are not always considered in other protocols. Based on our experiments, the following steps were established for the method.

2.3.1. Dilution and preparation of the suspensions

2.3.1.1. Technical material (TC) (pure conidia). Three independent 0.1-g samples of the product were taken and added to 10 mL of sterile deionized water + surfactant Tween 80 (0.05%). This suspension was sonicated for 3 min. Next, serial dilutions were conducted in 10 mL of sterile deionized water + surfactant Tween 80 (0.05%), vortexing for 30 s at each dilution. The number of conidia was counted in a hemocytometer, and the suspension was standardized to a final concentration about 0.7 and 1×10^6 conidia/mL.

2.3.1.2. Technical concentrate (TK1) (fungus-colonized rice). Three independent 5-g samples of the product were taken. Each sample were added to 50 mL of sterile deionized water + surfactant Tween 80 (0.05%) and, before shaking, a brush or glass beads were used to remove the conidia that are attached to the surface of the rice grain. This suspension was sonicated for 3 min. Next, serial dilutions were made as usual, vortexing for 30 s at each dilution. Next step regarding serial dilution and conidia counts follow the same procedure as mentioned above.

2.3.1.3. Technical concentrate (TK2) (ground fungus-colonized rice). Three independent 1-g samples of the product were taken and added to 10 mL of sterile deionized water + surfactant Tween 80 (0.05%). This suspension was sonicated for 3 min and vortexed for 30 s. Serial dilutions were conducted, vortexing for 30 s at each dilution. The number of conidia was counted in a hemocytometer, and the suspension was standardized to a concentration about 0.7 and 1×10^6 conidia/mL. Before plating, the suspension was left to decant for 1 min in order to decrease the amount of milled rice residues that are transferred to the plates along with the conidia.

2.3.1.4. Oil dispersion (OD). Three independent 1-mL samples of the product were taken, transferred to microcentrifuge tubes (Eppendorf type, 1.5 mL), and centrifuged for 5 min at 2500 rpm (573.1 ×*g*) (modified from Daoust et al., 1983). After centrifugation, the supernatant was discarded, and the tubes containing the conidia on the bottom were inverted on filter paper for 15 min to eliminate the remaining oil. After this period, 100 μL of emulsifier solution were added per tube, where the concentration may vary depending on the emulsifier used, mixing with the aid of the micropipette tip. In the tests presented below, 100 μL of the pure product Solub'oil® (General Chemicals and Service Ltd., Brazil) was used, but other products such as Silwet® L77 could be also employed. The aim of this surfactant is to remove the oil residue from conidia. It should be noted that it is necessary to use emulsifiers that do not affect the fungal viability. The conidia were resuspended in 1 mL of sterilized water + Tween 80 (0.05%) and vortexed for 2 min. The suspension was sonicated for 3 min. After this process, 1 mL of the suspension was transferred to tubes containing 9 mL of sterile water + Tween 80 (0.05%), followed by serial dilutions for counting in a hemocytometer, vortexing for 30 s at each dilution. The suspension was standardized to a concentration about 0.7 and 1×10^6 conidia/mL.

2.3.2. Plating (for all product types)

Petri dishes of the Rodac™ type (Replicate Organism Detection and Counting, 60 × 10 mm; Falcon Plastics Division of B-D Laboratories Inc., Los Angeles, CA, USA) were used containing 5 mL of culture medium (PDA). The plates were arranged on the flat surface of the laminar flow chamber and inoculated with 150 μL of fungal suspension (concentration about 0.7 and 1×10^6 conidia/mL) dropwise, covering up the four quadrants in the center of the plate. After evaporation of free water, the plates were closed and incubated for germination in growth chamber (26 ± 1 °C under 14 h photophase).

2.3.3. Evaluation (for all product types)

The viability assessments were undertaken with the direct count of viable and non-viable conidia after 20–22 h, under a light microscope

at 400× magnification, focusing on a field in the center of each quadrant, without choosing or changing the location display. Four scores were taken including more than 200 conidia per plate on average. Viable conidia are considered those that have germ tubes longer than their diameters.

2.4. Comparison of conidial viability assessment methods

2.4.1. Comparison between the ESALQ × CFU methods

For each method, three independent samples were removed from the products (1 g of TC, 5 g of TK1, 1 g of TK2 and 1 mL of OD of each sample). The samples were then diluted and enumerated in a hemocytometer as described above. The *CFU* method was performed in accordance with Alves and Moraes (1998), using PDA in Petri plates (90 × 15 mm) that received 0.1 mL of the serially diluted suspensions. The suspensions were spread over the entire surface of the culture medium using a sterile Drigalski spatula. The sub-samples of three dilutions obtained in the experiments of this study were quantified. Five replicates were prepared per sample, with 15 plates per method in each fungal preparation, with a total of 120 plates per fungus.

The plates were incubated at 26 ± 2 °C under 14 h photophase. The plates used for the *ESALQ* method were evaluated after 28 h. Those used for the *CFU* method were evaluated after 3 days by counting and marking the colonies at the bottom of the plates and incubating them again for confirmation for 7 more days, using plates with dilutions that delivered 30–300 colonies per plate.

2.4.2. Comparison between the ESALQ × Fluorescence methods

For each method, three independent samples were removed from the products (1 g of TC, 5 g of TK1, 1 g of TK2 and 1 mL of OD of each sample). The samples were then diluted and enumerated in hemocytometer as described above. The *Fluorescence* method was performed in accordance with Firstencel et al. (1990). Five replicates were prepared per sample, with 15 plates or slides per method in each fungal preparation, totaling 120 plates per fungus.

For the *Fluorescence* method, the stock solution of fluorescein diacetate fluorochrome (FDA) (Sigma Chemical Co.) was prepared in acetone at 4 mg/mL; the stock solution of propidium iodide (PI) (Sigma Chemical Co.) was prepared in deionized water at 3 mg/mL. The solutions were stored at 4 °C in lidded glass jars and covered with aluminum foil to limit exposure to light (Jones and Sneft, 1985). The FDA and PI working solutions were prepared just prior to testing, using 90 μL of the FDA stock solution and 60 μL of PI diluted in 5 mL of deionized water, keeping them on ice during use. These solutions were used for about 1 h, and after this time, they were discarded and replaced with new working solutions. Each slide was prepared in the dark by adding 1 μL of the fungal suspension and an equal amount of the working solution, mixing with the aid of the micropipette tip and covering it with a coverslip.

Assessments with the *ESALQ* method were performed as described in the protocol. For assessments with the *Fluorescence* method, at least 100 conidia were counted on each slide, in different fields of view, using 1000× magnification in a Carl Zeiss Axioskop 50® microscope equipped with an epifluorescence accessory with mercury lamp (100 W), an exciter filter (450–490 nm; blue light) and a 520-nm barrier filter in combination with a dichroic mirror (FT-510).

2.4.3. Comparison between the ESALQ × Standard methods — method validation

This assay was conducted for validating the *ESALQ* method and comparing it with the method proposed by Alves and Pereira (1998) (herein called *Standard*), which is widely used in Brazil for the analysis of commercial entomopathogenic fungi and assessments of experimental tests.

Three independent samples were removed from the products (1 g of TC, 5 g of TK1, 1 g of TK2 and 1 mL of OD of each sample) and were diluted and enumerated in a hemocytometer. Five replicates were prepared per sample, with 15 plates per method, for each product.

In the *Standard* method, PDA plates (90 × 15 mm) were used. For this method, samples of TC, TK1 and TK2 were prepared after dilution, plating and spreading 100 μL of the suspension with the aid of a sterile Drigalski spatula. For the OD formulation, the products were usually not diluted in water for plating, and 3 μL was directly applied over the surface of the culture medium and spread it well with a spatula so that the conidia came into contact with the medium. Incubation was performed in a growth chamber (26 ± 2 °C, 14 h photophase) for 18 h until analysis. Assessments were performed by counting at least 200 conidia in different fields of view, using 400× magnification.

Assessments with the *ESALQ* method were performed as described in the protocol for each type of product/formulation (TC, TK1, TK2, OD) after 28 h incubation due to the presence of the fungistatic agent in the medium.

2.5. Statistical analysis

All experiments were performed using a completely randomized design, and the normal distribution of the data was confirmed with the Shapiro-Wilk test. Initially, the data were analyzed descriptively (mean, standard error of the mean, standard deviation, variance of the assay, coefficient of variation of the assay), and the viability percentage data were subjected to analysis of variance (ANOVA). In the experiments evaluating the method parameters, means were compared using the Tukey–Kramer HSD test ($P < 0.05$). In the comparisons between the methods, mean viability percentages were compared using the T-test ($P < 0.05$).

In the experiment *ESALQ* × *CFU*, data were converted from CFU to viable conidia/g or viable conidia/mL of fungus, allowing the comparison between the two assessment methods. Analyses were performed using the statistical software SAS v. 9.1.3 (SAS Institute, 2002/2003).

3. Results

3.1. Method parameters

3.1.1. Selection of the culture medium

Culture media did not affect the germination of *M. anisopliae* conidia regardless of the fungal preparation (TC, TK1, TK2 and OD) ($P > 0.05$). For *B. bassiana*, conidial germination did not differ significantly in the media lab-made PDA, commercial PDA and Sporulation Medium. However, the Water Agar medium was less suitable for *B. bassiana* germination in TC ($F = 72.9$, $P < 0.0001$, CV = 1.7%) and TK2 ($F = 22.9$, $P < 0.0001$, CV = 2.1%), as viability decreased in about 9% and 7%, respectively (Table 1).

3.1.2. Influence of antibiotics on conidial viability

The addition of 5 mg/L of Pentabiotic to PDA did not affect the germination of *B. bassiana* and *M. anisopliae* in any of the tested preparations ($P > 0.05$). The viability percentages for *B. bassiana* in the treatments in the presence or absence of Pentabiotic® were, respectively, TC = 89.2 ± 1.0 and 87.4 ± 1.0; TK1 = 91.1 ± 0.9 and 92.7 ± 2.4; TK2 = 82.5 ± 1.8 and 80.6 ± 0.7; OD = 84.5 ± 1.8 and 83.4 ± 1.5. For *M. anisopliae* in culture medium with and without the addition of Pentabiotic®, respectively, these values were TC = 91.5 ± 1.0 and 92.8 ± 1.1; TK1 = 83.4 ± 0.6 and 86.3 ± 1.7; TK2 = 54.9 ± 1.5 and 52.7 ± 0.3; OD = 86.1 ± 0.5 and 85.1 ± 1.0 (with CVs lower than 3.5% in all analyses for both fungi).

In the experiment with different Pentabiotic® concentrations, for both *B. bassiana* and *M. anisopliae*, no effects were observed on spore germination (TC) ($F = 3.44$, $P > 0.05$, CV = 1.9%; $F = 1.07$, $P > 0.05$, CV = 2.6%, respectively). For *B. bassiana*, the viability percentages in treatments with 0, 15, 45 and 75 mg/L were 93.6 ± 1.5, 91.9 ± 0.9, 90.9 ± 1.1 and 88.9 ± 0.4, respectively. For *M. anisopliae*, these values

Table 1
Conidial viability (% germination) of *Beauveria bassiana* and *Metarhizium anisopliae* in different culture media (26 ± 2 °C under 14 h photophase).

Fungi	Types[1]	Culture media[2]				CV (%)
		PDA	PDAcom	SM	WA	
Beauveria bassiana	TC	93.4 ± 1.2a	92.4 ± 0.5a	94.1 ± 0.8a	84.0 ± 0.7b	1.7
	TK1	88.7 ± 0.7a	85.9 ± 1.2a	89.9 ± 1.8a	88.1 ± 3.1a	3.8
	TK2	89.1 ± 0.8a	88.8 ± 0.9a	87.1 ± 0.9a	81.6 ± 1.7b	2.1
	OD	76.8 ± 1.0a	78.8 ± 2.3a	82.4 ± 0.5a	78.7 ± 3.0a	4.4
Metarhizium anisopliae	TC	89.6 ± 2.0a	89.0 ± 0.2a	92.7 ± 1.3a	90.8 ± 1.6a	2.8
	TK1	81.2 ± 1.3a	78.0 ± 0.5a	83.4 ± 1.2a	79.8 ± 1.6a	2.5
	TK2	54.4 ± 0.8a	57.6 ± 5.1a	63.1 ± 1.7a	59.8 ± 1.7a	8.4
	OD	91.3 ± 0.3a	92.9 ± 0.3a	89.9 ± 0.1a	92.6 ± 1.3a	1.3

Means (±SEM) followed by the same letter in the line do not differ significantly according to the Tukey–Kramer HSD test (α = 0.05).
[1] Technical-grade active ingredients or formulation types: pure conidia (TC), fungus-colonized rice (TK1), ground fungus-colonized rice (TK2) and emulsifiable oil formulation (OD).
[2] PDA, Lab-made Potato dextrose agar; PDAcom, Commercial PDA, Difco®; SM, Sporulation medium (Alves et al., 1998); WA, 2% Water agar.

were 88.0 ± 0.7, 86.0 ± 1.9, 87.2 ± 1.1 and 89.2 ± 1.2, respectively. The addition of the antibiotic Pentabiotic® to the culture medium in the viability tests, even at a concentration of 5 mg/L, was effective in controlling bacterial contamination, with no occurrence of these contaminants in any of the performed experiments.

3.1.3. Influence of fungistatic on conidial viability

The germination of *B. bassiana* and *M. anisopliae* conidia in the presence of the fungicide Benlate® did not differ statistically ($P > 0.05$) from germination in the medium containing Derosal® in any of the preparations assessed (Table 2), suggesting that the latter constitute a proper substitute of the former, or either one can be used. For *B. bassiana* in TK2 and OD, the germination percentages in treatments amended with Derosal® were 4.3% and 9.7% higher than the values measured in the control treatment without any fungicide, respectively ($F = 9.2$; $P = 0.0147$, CV = 2.3% and $F = 14.6$; $P = 0.0049$, CV = 2.7%). In TK1, a difference was also observed among treatments ($F = 8.4$; $P = 0.0181$, CV = 1.9%). For *M. anisopliae*, no difference was found among treatments in any of the assessed preparations.

Tests with different concentrations of the fungicide Derosal® showed that at concentrations of 30 μL/L and 60 μL/L, conidial germination of *B. bassiana* (TC) was reduced about 6% and 8% compared to control, respectively ($F = 8.6$; $P = 0.0026$, CV = 3%) (Table 3). For *M. anisopliae*, no Derosal® concentration significantly differed to the control, although in the treatment with 60 μL/L ($F = 3.7$; $P = 0.0438$, CV = 3.4%), viability was slightly lower than in treatment with only 10 μL/L. The results indicated that the fungicide at a concentration of 10 μL/L of medium did not reduce the viability of these fungi and can be recommended for use at this concentration.

Table 2
Conidial viability (% germination) of *Beauveria bassiana* and *Metarhizium anisopliae* in Potato dextrose agar (PDA) medium with and without the addition of 10 μL/L of the fungicides Benlate® and Derosal® (26 ± 2 °C and 14 h photophase).

Fungus	Types[1]	Culture media[2]			CV (%)
		PDA	PDA + Benlate®	PDA + Derosal®	
B. bassiana	TC	94.1 ± 1.0a	92.7 ± 0.7a	94.9 ± 0.4a	1.3
	TK1	84.9 ± 1.1b	90.4 ± 0.8a	86.7 ± 1.0ab	1.9
	TK2	85.6 ± 0.3b	89.6 ± 0.3a	89.9 ± 1.9a	2.3
	OD	76.2 ± 1.5b	82.1 ± 1.6a	85.9 ± 0.3a	2.7
M. anisopliae	TC	91.0 ± 0.8a	89.5 ± 0.7a	89.0 ± 0.1a	1.2
	TK1	81.7 ± 0.2a	79.8 ± 2.0a	80.9 ± 1.6a	3.2
	TK2	54.0 ± 1.9a	49.7 ± 1.7a	52.2 ± 1.7a	5.9
	OD	91.3 ± 0.3a	93.6 ± 0.9a	91.6 ± 1.1a	1.6

Means (±SEM) followed by the same letter in the line do not differ significantly according to the Tukey–Kramer HSD test (α = 0.05).
[1] Technical-grade active ingredients or formulation types: pure conidia (TC), fungus-colonized rice (TK1), ground fungus-colonized rice (TK2) and emulsifiable oil formulation (OD).

3.2. Comparison of conidial viability assessment methods

3.2.1. Comparison of the ESALQ × CFU methods

Major differences were detected for all preparations of *B. bassiana* and *M. anisopliae* across data obtained in the assessments with the *ESALQ* method and with the indirect *CFU* method. In all cases, the *ESALQ* method proved to be more accurate, depicting lower variations among replicates and bearing lower CV values within assays (Table 4). In the *ESALQ* method, the CV was lower than 6% for all preparations of tested fungi. In the *CFU* method, these values were very high, with values for the TC of *B. bassiana* (79.7%) and the OD formulation of *M. anisopliae* (86.3%) being the highest. For both fungi, the major differences were observed between the methods for OD, with the CVs of the *CFU* method being 29 times higher than the one observed for the *ESALQ* method in *B. bassiana* and 28 times higher in *M. anisopliae*.

When the germination values obtained with the two methods were converted into the number of viable conidia/g or/mL, compared to the quantification previously performed in a hemocytometer, the *CFU* method had inconsistent results, demonstrating also that this method is not suitable to quantify these mycopesticides. For some formulations of fungi, the method significantly underestimated the conidia concentration: it was 59% lower in TC and 62% lower in OD for *B. bassiana*. For *M. anisopliae* in OD, values were underestimated by 93%. Comparing the viability percentages obtained using the two methods, only in TK1 for *B. bassiana* not significant differences between these methodologies were observed ($F = 0{:}17$; $P = 0.6996$; CV = 11.2%).

3.2.2. Comparison between the ESALQ × Fluorescence methods

It was not possible to perform assessments using the *Fluorescence* method for the fungi in OD due to the inability to properly mix the staining solution with the oil of the formulation. In TK1 and TK2, other structures (starch/rice particles), in addition to the conidia, were also stained by the reagents, which hindered visualization and counting. These findings demonstrate the limitations of the *Fluorescence* method for formulated products.

Table 3
Conidial viability (% germination) of *Beauveria bassiana* and *Metarhizium anisopliae* pure conidia in culture medium with different concentrations of Derosal® (26 ± 2 °C and photophase of 14 h).

Derosal® (μL/L of medium)	Fungi	
	Beauveria bassiana	Metarhizium anisopliae
0	92.2 ± 1.5a	90.4 ± 2.1ab
10	91.8 ± 0.5a	90.9 ± 1.7a
30	86.1 ± 1.9b	88.6 ± 1.1ab
60	84.5 ± 1.0b	84.5 ± 0.9b
CV (%)	3.02	3.43

Means (±SEM) followed by the same letter in the column do not differ significantly according to the Tukey–Kramer HSD test (α = 0.05).

Table 4
Comparison between the direct counting (*ESALQ*) and colony-forming unit (*CFU*) methods to determine the conidial viability of *Beauveria bassiana* and *Metarhizium anisopliae* in different types of commercial products (26 ± 2 °C and 14 h photophase).

Fungi	Types[1]	Total number of conidia[2]	Method	No. of viable conidia or CFU	% viable conidia or CFU (CV) [3]	T-test*
Beauveria bassiana	TC	9.1×10^{10}/g	ESALQ	$8.2 (\pm 0.12) \times 10^{10}$/g	90.7 ± 3.2 (3.2)	S
			CFU	$3.8 (\pm 1.3) \times 10^{10}$/g	41.0 ± 11.3 (79.7)	$t = 14.01$
						$P = 0.0201$
	TK1	4×10^{9}/g	ESALQ	$3.2 (\pm 0.06) \times 10^{9}$/g	81.3 ± 1.1 (4.3)	Ns
			CFU	$3.3 (\pm 3.3) \times 10^{9}$/g	83.0 ± 7.9 (29.3)	$t = 0.17$
						$P = 0.6996$
	TK2	4.1×10^{9}/g	ESALQ	$3.2 (\pm 0.08) \times 10^{9}$/g	78.5 ± 1.1 (5.6)	S
			CFU	$2.5 (\pm 1.9) \times 10^{9}$/g	48.0 ± 11.3 (36.8)	$t = 7.99$
						$P = 0.0475$
	OD	1.8×10^{9}/mL	ESALQ	$1.6 (\pm 0.02) \times 10^{9}$/mL	91.9 ± 0.6 (2.4)	S
			CFU	$6.9 (\pm 1.8) \times 10^{8}$/mL	38 .0± 12.3 (71.9)	$t = 26.63$
						$P = 0.0067$
Metarhizium anisopliae	TC	5.4×10^{10}/g	ESALQ	$4.9 (\pm 0.06) \times 10^{10}$/g	90.9 ± 0.7 (3.1)	S
			CFU	$5.9 (\pm 1.1) \times 10^{10}$/g	109.0 ± 13.3 (50.7)	$t = 0.74$
						$P = 0.4380$
	TK1	2.6×10^{9}/g	ESALQ	$2.1 (\pm 0.03) \times 10^{9}$/g	79.5 ± 0.8 (3.1)	S
			CFU	$2.7 (\pm 3.2) \times 10^{9}$/g	101.0 ± 20.7 (27)	$t = 178.10$
						$P = 0.0002$
	TK2	1.1×10^{9}/g	ESALQ	$5.3 (\pm 0.01) \times 10^{8}$/g	47.9 ± 0.7 (5.3)	S
			CFU	$1.0 (\pm 2.2) \times 10^{9}$/g	92 .0± 8.3 (48.9)	$t = 10.52$
						$P = 0.0316$
	OD	2.6×10^{9}/mL	ESALQ	$2.4 (\pm 0.03) \times 10^{9}$/mL	91.2 ± 0.7 (3.0)	S
			CFU	$1.9 (\pm 0.6) \times 10^{8}$/mL	7.4 ± 20.7 (86.3)	$t = 321.43$
						$P < 0.0001$

Means (±SEM) of number of viable conidia/g or mL, and percentage of germination presented (±SEM) (*T-test: significant — S, not significant — ns); CV = coefficient of variation of the test.
[1] Technical-grade active ingredients or formulation types: pure conidia (TC), fungus-colonized rice (TK1), ground fungus-colonized rice (TK2) and emulsifiable oil formulation (OD).
[2] Calculation based on the initial concentration obtained from counting in a hemocytometer.

Regarding the viability percentages obtained using the two methods, only TK1 for *M. anisopliae* was not significantly different ($F = 0.24$; $P = 0.6306$; CV = 3.7%) (Table 5). For TC, TK1 and TK2 of *B. bassiana* and TC of *M. anisopliae*, the viability values obtained were higher in the *ESALQ* method. For TK2 of *M. anisopliae*, the viability obtained using the *Fluorescence* method was 6.5% higher.

Table 5
Comparison between the direct counting (*ESALQ*) and *Fluorescence* methods to determine the conidial viability of *Beauveria bassiana* and *Metarhizium anisopliae* (26 ± 2 °C and 14 h photophase).

Fungi	Type[1]	Method	% germination (CV)	T-test*
Beauveria bassiana	TC	ESALQ	93.8 ± 0.6 (2.1)	S
		Fluorescence	88.5 ± 0.8 (3.1)	t = 25.83
				$P < 0.0001$
	TK1	ESALQ	87.4 ± 0.9 (3.5)	S
		Fluorescence	83.6 ± 0.9 (3.5)	$t = 7.98$
				$P = 0.0112$
	TK2	ESALQ	77.7 ± 0.9 (3.9)	S
		Fluorescence	73.8 ± 1.0 (4.3)	$t = 7.82$
				$P = 0.0119$
Metarhizium anisopliae	TC	ESALQ	92.8 ± 0.8 (2.9)	S
		Fluorescence	85.8 ± 1.1 (4.2)	$t = 26.14$
				$P < 0.0001$
	TK1	ESALQ	78.0 ± 1.1 (4.6)	Ns
		Fluorescence	78.7 ± 0.9 (3.6)	$t = 0.24$
				$P = 0.6306$
	TK2	ESALQ	67.9 ± 1.0 (4.6)	S
		Fluorescence	74.4 ± 1.3 (5.7)	$t = 14.90$
				$P = 0.0011$

Means (±SEM) presented for the germination percentages obtained in the two methods tested (*Test-t: significant — s, not significant — ns); CV = coefficient of variation of the test.
[1] Technical-grade active ingredients or formulation types: pure conidia (TC), fungus-colonized rice (TK1), ground fungus-colonized rice (TK2).

3.2.3. Comparison between the ESALQ × Standard methods — method validation

Comparing the *ESALQ* method with the *Standard* method, numerically lower CV values were found for all of the assessed products for both *B. bassiana* and *M. anisopliae* using the former method. Overall, for both fungi, the products with the largest variations in the CV within assays, regardless of the method used, were those in the form of TK2, with the highest values being observed in the analysis of a *M. anisopliae* product, with CVs of 65.6% in the *Standard* method and 65.1% in the *ESALQ* method (Table 6). Conidial viability in this product was extremely low (<1.3%).

Regarding the germination percentages, significant differences were observed between the methods used for some products. The germination percentages recorded in OD preparations estimated by the *Standard* method exhibited 6.3% and 32.7% lower values than those estimated by the *ESALQ* method for *B. bassiana* and *M. anisopliae*, respectively (Table 6).

4. Discussion

In this study, the standardization of steps generally disregarded in other protocols for determining the viability of entomopathogenic fungi was proposed. The method called *ESALQ* consists of plating conidia on PDA culture medium supplemented with antibiotics and the fungicide Derosal® at a concentration of 10 μL/L on Rodac™ plates. One of the main limitations of the existing methods for determining viability is the wide variability of results. The *ESALQ* method has advantages over the other methods to which it was compared in this study, particularly the smaller variation among replicates (i.e., greater accuracy), which reflects in consistently lower CVs.

The use of PDA medium is suggested because it is a simple and inexpensive growth substrate, with widespread use, in addition to being quite translucent on the plates, making the visualization of conidia easier. PDA medium is widely used for viability testing, as Francisco et al. (2006) had already demonstrated that this medium was one of the most suitable to promote the germination of conidia of

Table 6
Comparison between two direct methods for assessing the viability of the fungi *Beauveria bassiana* and *Metarhizium anisopliae* from different commercial products (26 ± 2 °C and 14 h photophase).

Fungi	Product[1]	% germination (CV)		T-test*
		Method		
		STANDARD	ESALQ	
Beauveria bassiana	TC	85.9 ± 0.85 (3.1)	89.6 ± 0.81 (2.8)	S $t = 9.33$ $P = 0.0068$
	TK1	87.2 ± 1.87 (6.7)	86.9 ± 0.87 (3.2)	NS $t = 0.17$ $P = 0.6880$
	TK1	33.5 ± 1.5 (17.6)	25.1 ± 0.43 (6.7)	S $t = 18.97$ $P = 0.0004$
	TK2	71.6 ± 1.09 (4.9)	80.9 ± 0.82 (3.2)	S $t = 46.90$ $P < 0.0001$
	TK2	43.2 ± 1.7 (12.5)	44.8 ± 0.76 (5.4)	NS $t = 0.76$ $P = 0.3950$
	TK2	32.4 ± 2.4 (29.3)	15.3 ± 0.86 (17.9)	S $t = 19.49$ $P = 0.0003$
	OD	85.6 ± 0.96 (4.3)	91.9 ± 0.58 (2.4)	S $t = 30.15$ $P < 0.0001$
Metarhizium anisopliae	TC	82.5 ± 0.8 (3.25)	79.4 ± 0.8 (3.21)	S $t = 9.64$ $P = 0.0061$
	TC	94.4 ± 0.4 (1.4)	94.9 ± 0.3 (1.1)	NS $t = 0.85$ $P = 0.3691$
	TK1	61.5 ± 3.4 (21.5)	68.9 ± 0.6 (3.2)	NS $t = 3.12$ $P = 0.0880$
	TK1	84.9 ± 1.6 (5.8)	89.9 ± 0.7 (2.6)	S $t = 6.63$ $P = 0.0191$
	TK1	62.9 ± 0.67 (4.2)	66.6 ± 0.6 (3.2)	S $t = 10.18$ $P = 0.0051$
	TK1	90.5 ± 0.7 (2.3)	92.1 ± 0.5 (1.6)	NS $t = 3.95$ $P = 0.0623$
	TK2	52.8 ± 1.0 (6.1)	47.9 ± 0.7 (5.3)	S $t = 15.66$ $P = 0.0009$
	TK2	1.29 ± 0.2 (65.6)	0.96 ± 0.2 (65.1)	NS $t = 0.51$ $P = 0.4844$
	OD	58.5 ± 0.8 (3.6)	91.2 ± 0.7 (3.0)	S $t = 26.19$ $P < 0.0001$

Means (±SEM) presented for the germination percentages obtained in the two methods tested (*T-test: significant — s, not significant — ns); CV = coefficient of variation of the method.

[1] Technical-grade active ingredients or formulation types: pure conidia (TC), fungus-colonized rice (TK1), ground fungus-colonized rice (TK2) and emulsifiable oil formulation (OD).

entomopathogenic fungi. Dextrose and proteins available in this medium enable that even conidia of isolates with small endogenous nutritional reserves germinate by obtaining the necessary nutrients from the culture medium. Nevertheless, other culture media can be used, except for Water-Agar. While culture media richer in nutrients offer better conditions for the germination of fungi (Iskandarov et al., 2006), nutritionally poor media such as WA medium negatively affect fungal viability (Ibrahim et al., 2002; Lane et al., 1991).

The addition of 5 mg/L of Pentabiotic® to the culture medium was sufficient to inhibit the growth of contaminants in all performed tests, even in formulated products, without influencing the conidial viability. The recommendation of the addition of antibiotics in the culture medium is particularly important for products such as TK2, as this product may contains particles along with the milled rice, often showing contamination in the assessment plates. Other antibiotics could likely be used because the compounds usually used as decontaminants in culture media, such as Penicillin, Tetracycline, Streptomycin, Erythromycin and Chloramphenicol, have a very specific bioactivity against bacteria (Black, 2002). For specific quality control assessments, antibiotic should be avoided because it may hide the contamination present in material evaluated.

The fungicide Benlate® (Benomyl) has been used as a supplement of the culture medium for standardizing the germination of *B. bassiana* conidia (Inglis et al., 1996) and *M. anisopliae* (Milner et al., 1991). Its mode of action in fungi is achieved by inhibiting one of the stages in mitotic cell division, leading to the normal formation of the germ tube but preventing the further development of hyphae, and consequently the overgrowth of the fungi on nutrient agar media (Goettel and Inglis, 1997). However, this product is no longer marketed, and the pure active principle is more difficult to obtain as well as more expensive. In this study, it was demonstrated that the fungicide Derosal® (also from the chemical group Benzimidazole) at concentration of 10 μL/L of medium (0.005 g of a.i. per liter of medium) may be used instead of Benlate®, because it showed the same fungistatic properties. This product can be easily found on the market and it has demonstrated here to be useful in fungal viability analysis by allowing longer incubation period without hindering the assessments. In the absence of the fungicide, it is commonly to find profuse hyphal growth, which prevents confirming the germination of each conidium individually due to the overlapping of germ tubes and hyphae over the non-germinated conidia on the plate (Goettel and Inglis, 1997). The use of fungicide makes standardization possible for a number of studies comparing different isolates over time. This standardization can be observed in tests on the effects of fungicides on the viability of *B. bassiana* in TK2 and OD, where it was observed that viability was higher in the presence of fungicides than in the control free of fungicide. This result is most likely related to the incubation periods, which took 18 h in the control and 28 h in treatments with fungicide. The possibility of a longer incubation period in the presence of the fungicide allows conidial viability to be more uniform, which may result in higher viability percentage values. However, conidia that take too long to germinate may have low vigor (Lopes et al., 2013; Faria et al., 2015). Based on routine assessments in our laboratory, it is recommended that the plates be assessed 20 to 22 h after inoculation with conidia. The assessment period can be adjusted depending on the fungal species and *B. bassiana* and *M. anisopliae* can be assessed even after 28 h; however, this incubation period is not possible for *Isaria fumosorosea* (Ascomycota: Cordycipitaceae), for example, because the growth of hyphae hinder the assessments.

With respect to comparisons between methods and to the validation of the *ESALQ* method using commercial products containing *B. bassiana* or *M. anisopliae* in different forms and formulations, the *ESALQ* method showed greater accuracy when compared to other methods for assessing conidial viability. This lower variability is likely associated with the fact that the *ESALQ* method enables a more uniform deposition of conidia due to the dropwise transfer of suspension on the surface of the culture medium when compared with the more uneven spreading obtained with the Drigalski spatula in the *Standard* method. In the *Standard* Method, the spreading provided by the spatula may accumulate conidia in certain regions of the culture medium surface and, when the objective lens focuses in these regions for counting, the visualization may be hampered by the growth and overlapping of germ tubes. The need to arbitrarily locate points with appropriate conidial concentrations during the counting makes the process more time consuming and can interfere with the final analysis, requiring the selection of fields for assessment in these cases.

Counting in the *ESALQ* method is performed by focusing anywhere within the quadrants defined on the Rodac™ plate, with uniform spreading and conditions for assessment, and the counting of conidia may be performed without changing the field of view.

Differences in results among the *CFU* method and the *ESALQ* method were highly expressive, and the *ESALQ* method showed lower variability. The *CFU* method may present results heavily influenced by weighing errors. Even using a precision scale and taking the maximum possible care, slight variations among samples may result in large differences in the final results. These variations occur because when using the *CFU* method, the results obtained are generated from colony counting data, thereby leading to large variations among replicates, i.e., this method has a lower accuracy and reproducibility.

A major limitation of the *CFU* method regards the assessments of formulated products, mainly those formulated in emulsifiable oil. As observed in this study, this method underestimates the final concentration of viable conidia in this type of product, most likely because conidia that remain clumped after plating form only one colony (Jenkins and Grzywacz, 2000; Stentelaire et al., 2001).

Some companies that sell fungi with the growth substrate (e.g., TK2) advocate the use of the *CFU* method for product analyses as they claim the existence of other vegetative structures in these products, which could also form colonies but would be undetected using the direct method. These structures were observed for *M. anisopliae*, but not for *B. bassiana*, values above 90% of *CFU*/total number of conidia by the *CFU* method and 48% of viable conidia/total number of conidia by the *ESALQ* method were observed for *M. anisopliae*, suggesting the presence of other structures (such as hyphae) that could form colonies and are not quantified using direct assessment methods. However, infective structures that should be evaluated in these products would be conidia.

Analysis of the results of the *Fluorescence* method indicated that it was very accurate in evaluating pure conidia (TC) of the assessed isolates of *B. bassiana* and *M. anisopliae*, supporting the recommendations made by other authors (Firstencel et al., 1990; Schading et al., 1995). However, although little variation was apparent for both TK1 and TK2, the assessments of these products proved to be much more laborious than when conducted using the *ESALQ* method. Other particles in the products are stained with the reagent, hindering visualization and the distinction between viable and non-viable conidia. For both fungal products in OD, the assessment using fluorochromes was not possible because the oil made the mixing of the reagent difficult, thus preventing its contact with most conidia. Thus, the use of the *Fluorescence* method is not recommended for routine analysis of products formulated in oil or containing much production waste (inert particle), such as the TKs. Besides, *Fluorescence* method might not be suitable for all fungi (Firstencel et al., 1990; Jimenez and Gillespie, 1990), and fluorochromes require greater care in manipulation because they are toxic and should be kept in the dark during preparation and use (e.g. the reagents lose their staining properties when exposed to light).

Overall, the greatest shortcomings found in the viability assessments of entomopathogenic fungi occurred with products formulated in oil. This observation was true mainly for species with small and round conidia (such as *B. bassiana*) once the oil may form tiny droplets on the surface of the culture medium that are difficult to distinguish from non-germinated conidia (Goettel and Inglis, 1997). In addition, some conidia may be encapsulated within larger oil droplets, thus preventing them from coming into contact with the culture medium, which may delay or even inhibit the onset of the germination process, causing this conidium to be mistakenly considered non-viable. Therefore, a major step was included in the *ESALQ* method that consisted of the removal of nearly all of the oil before plating, which facilitates visualization of conidia at the time of assessment.

For ground fungus-colonized rice (TK2), the large quantity of rice particles with mycelia may disrupt the visualization of conidia at the time of microscopic assessment. The *ESALQ* method suggests waiting 1 min for sedimentation of the particulate material prior to plating by pipetting to facilitate the assessment.

Other benefits of using the newly developed method compared to the other tested methods is the economy of culture medium and physical space due to the smaller size of the plate used for assessment. In the *Standard* method, three times more culture medium is used for each repetition, and in the case of *CFU*, in addition to the longer incubation period (from 5 to 10 days), the amount of culture medium required is four to five times greater than that used in the *ESALQ* method.

The results presented in this study underscore a method developed for determining the conidial viability of entomopathogenic fungi, in pure conidia or in commercial products, is effective and easy to apply and generally provides higher accuracy than the other traditional methods.

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