

Influence of culture media in viability test of conidia of entomopathogenic fungi

Influência de meios de cultura em teste de viabilidade de fungos entomopatogênicos

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ABSTRACT

This work aimed at investigating whether the culture medium used in viability tests affects the conidial germination of *Lecanicillium lecanii*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* isolates. The tests were performed on microscope slides containing one of the culture media: agar-water (AW), minimal medium (MM), potato-dextrose agar (PDA), potato-dextrose-1% yeast extract agar (PDAY), Sabouraud-dextrose-yeast extract agar (SDAY), and complete medium (CM). Three areas per slide were delimited and 0.05ml of a 5.5×10^5 conidia mL^{-1} suspension was applied to each area. One bioassay was performed for each isolate. Germination was determined after 15 hours of incubation at $26 \pm 0.5^\circ\text{C}$. The culture media influenced the germination of the species studied, verifying within and inter specific variations. CM and PDA provided the highest germination of *L. lecanii* isolates and the lowest was obtained on SDAY and AW. The germination of *B. bassiana* isolates was favoured by CM, PDA and PDAY media, a fact not observed in AW and MM. *P. fumosoroseus* isolates showed the highest germination on CM and PDA media and the lowest on SDAY. However, some isolates presented high germination on nutrient-poor media (AW and MM).

Key words: *Beauveria bassiana*, *Lecanicillium lecanii*, *Paecilomyces fumosoroseus*, germination, microbial control.

RESUMO

O presente trabalho objetivou investigar se meios de cultura utilizados em teste de viabilidade afetam a germinação de conídios de cinco isolados de *Lecanicillium*

lecanii, cinco de *Beauveria bassiana* e quatro de *Paecilomyces fumosoroseus*. Os testes foram realizados em lâminas de microscopia contendo um dos seguintes meios de cultura: Ágar-água (AA), Meio Mínimo (MM), Batata, dextrose e ágar (BDA), Batata, dextrose, ágar e 1% de extrato de levedura (BDAL), Sabouraud, dextrose, ágar e extrato de levedura (SDAL) e Meio Completo (MC). Delimitaram-se três áreas por lâmina e em cada uma aplicou-se 0,05mL de uma suspensão com concentração de $5,5 \times 10^5$ conídios mL^{-1} . Para cada isolado foi realizado um bioensaio, com seis tratamentos e cinco repetições. Avaliou-se a germinação 15 horas após incubação, a $26 \pm 0,5^\circ\text{C}$. Os meios de cultura influenciaram na capacidade de germinação das três espécies estudadas, ocorrendo variações inter e intraespecíficas. Verificou-se que os meios Completo e BDA proporcionaram as maiores porcentagens de germinação dos isolados de *L. lecanii*, sendo que as menores foram obtidas nos meios SDAL e AA. Os meios ricos em nutrientes (BDA, BDAL e Completo) favoreceram a germinação dos isolados de *B. bassiana*, o que não ocorreu com os meios pobres (AA e MM). Nos meios Completo e BDA foram obtidas as maiores porcentagens de germinação dos isolados de *P. fumosoroseus*. As menores porcentagens, por sua vez, foram obtidas no meio SDAL. Entretanto, alguns isolados apresentaram alta germinação em meios pobres em nutrientes (AA e MM).

Palavras-chave: *Beauveria bassiana*, *Lecanicillium lecanii*, *Paecilomyces fumosoroseus*, controle microbiano, germinação.

Bioinsecticides based on entomopathogenic fungi are currently commercialized, mainly using the

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species *Beauveria bassiana* (Bals.) Vuillemin, *Metarhizium anisopliae* (Metsch.) Sorokin, *Lecanicillium lecanii* Zare & Gams, and *Paecilomyces fumosoroseus* (Wize) Brown and Smith (LACEY et al., 2001). The viability of the fungus should be evaluated since it is an important component of the quality of the product (ALVES & PEREIRA, 1998). The virulence of these fungi has been correlated with the velocity of germination (HASSAN & CHARNLEY, 1989). In this respect, viability tests permit the evaluation of the germination capacity of conidia from its production to its field application.

Viability tests can be performed by spreading 10 μ L of a aqueous conidial suspension on Petri dishes containing a thin layer of potato-dextrose agar (PDA) medium (ALVES et al., 1998). Some researchers perform the test by placing the culture medium on microscope slides and adding drops of the conidial suspension (EKESI et al., 2001). The number of germinated conidia can be determined after 16 hours depending on the fungus and temperature (ALVES & PEREIRA, 1998).

Various culture media have been adopted to test conidial viability such as potato-dextrose-yeast extract agar, agar-water, Sabouraud-dextrose agar, and Sabouraud-maltose-yeast agar.

Certain treatments or the addition of chemical that are not required to support hyphal growth can activate germination (MOORE-LANDECKER, 1972). Physical or chemical environmental factors such as temperature, light, moisture, pH, substratum composition (MOORE-LANDECKER, 1972), O₂ and CO₂ (CARLILE et al., 2001), or any combination of these can activate spore germination. Therefore, composition of culture medium is an important factor to be considered.

The nutritional requirements for germination of entomopathogenic fungi are generally not complex (SMITH & GRULA, 1981), but various fungal species require different chemical, physical and nutritional conditions for optimal germination (CARLILE et al., 2001). In this context, the objective of the present study was to investigate whether the culture medium used in viability test influences the germination of isolates of three entomopathogenic fungal species.

The following species were studied: *Beauveria bassiana* (isolates JAB 6, JAB 7, AM 9, IBCB 7, and IBCB 66), *Paecilomyces fumosoroseus* (isolates JAB 12, IBCB 137, IBCB 201, and IBCB 148), and *Lecanicillium lecanii* (isolates ARSEF 6430, ARSEF 6431, ARSEF 6432, JAB 2, and JAB 45). These isolates have been deposited in the collections of Microbiology Laboratory and Laboratory of

Entomopathogens, Plant Production and Phytosanitary Departments respectively, Faculty of Agrarian and Veterinary Sciences, Sao Paulo State University.

The fungi were cultured on potato-dextrose-agar medium (PDA) for 7 days at 26 \pm 0.5°C under 14-hour photoperiod. A conidial suspension of each isolate was prepared adding 10ml of an aqueous 0.1% (v/v) Tween 80[®] solution to each fungus-containing plate. The surface of each colony was gently swept, the suspension formed was filtered and shaken in an electrical tube shaker. Conidial concentrations were estimated in a Neubauer chamber and adjusted to 5.5 x 10⁵ conidia ml⁻¹.

One bioassay was performed for each isolate, for a total of 14 assays. The following culture media (treatments) were used in each bioassay: 1% agar-water (AW), minimal medium (MM) (PONTECORVO et al., 1953), PDA, potato-dextrose-1% yeast extract agar (PDAY), Sabouraud-dextrose-1% yeast extract agar (SDAY) (ALVES et al., 1998), and complete medium (CM) (PONTECORVO et al., 1953, modified by AZEVEDO & COSTA, 1973).

The tests were performed using microscope slides disinfected with 70% alcohol. After the demarcation of three areas on the bottom surface, the slides were placed on Petri dishes, with a high relative humidity being maintained by two cotton pads moistened with distilled water. Two matches were placed in the horizontal position under each slide to prevent it from touching to touch the plate's bottom. Each slide's surface was covered with 4ml of culture medium and one drop (approximately 0.05ml) of the conidial suspension was placed in each area. After 15 hours of incubation at 26 \pm 0.5°C, the germination process was stopped by dribbling one drop of dye (1ml of a stock solution consisting of 1g methylene blue in 20ml lactic acid was added to 29ml lactic acid) on each area. One-hundred-and-fifty conidia, both germinated and non-germinated ones, were observed per area. Five slides (replications) were made for each treatment so that a total of 2,250 conidia per treatment could be observed and the percentage of viable conidia could be calculated.

A fully randomized design was adopted in all bioassays, including six treatments and five replications. The analyses were performed by the F test and means were compared by the Tukey test at a 5% probability error, using the ESTAT program (FCAV/Unesp, Exact Sciences Department).

Conidial viability analysis of *L. lecanii* isolates set showed that CM and PDA provided the highest germination percentages, while the lowest percentages were obtained with SDAY and AW medium

(Table 1). However, analysis of each isolate showed that the germination percentages obtained for CM and PDA were statistically equal to those observed for some of the other media.

The highest germination of *B. bassiana* conidia was provided by PDA, PDAY, SDAY, and CM (Table 1). For isolates of this fungus, nutrient-rich media favoured germination, while MM and AW medium, which possesses a lower nutritional value, promoted lower germination. The difference between media contributing or not to germination was more evident for *B. bassiana* than for *L. lecanii*. A markedly lower percentage of germination was observed for AM 9 isolate than for others.

B. bassiana requires a utilizable carbon sources to promote germination and a nitrogen source is needed for continued hyphal growth (SMITH & GRULA, 1981). Nevertheless, the authors suggested that conidia possess ample endogenous nitrogen reserves for synthesis of protein required for the germination process, since germination and some growth can occur in the absence of an exogenous

nitrogen source. Nutrient-rich media contain various carbon and nitrogen sources, a factor that might have been decisive in the observation of higher germination percentages.

The highest percentages for conidial germination of *P. fumosoroseus* isolates were obtained on CM and PDA and the lowest percentages on SDAY (Table 1). Furthermore, nutrient-poor (MM) or -free medium (AW) promoted high germination of IBCB 201 and IBCB 148 isolates, a fact also observed for some *L. lecanii* isolates. This observation suggests that these isolates are endowed with a good nutritional reserve. Some spores require an exogenous source of carbon or nitrogen for germination, while others do not require exogenous sources of nutrients but they utilize their reserve material such as carbohydrates, lipids or proteins (MOORE-LANDECKER, 1972). Genetic differences between isolates probably play a decisive role in this finding.

The results obtained with CM and PDA are probably due to their rich composition. CM includes an adequate sugar (glucose) and various macro- and

Table 1 - Conidial germination (%) of *Lecanicillium lecanii*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* isolates in some culture media at $26 \pm 0.5^\circ\text{C}^{1,2}$.

Fungus	Culture Media						C.V. ⁹
	AW ³	MM ⁴	PDA ⁵	PDAY ⁶	SDAY ⁷	CM ⁸	
<i>Lecanicillium lecanii</i>							
JAB 45	92.1 b	67.3 c	96.7 a	96.9 a	97.9 a	96.0 ab	3.35
JAB 2	93.3 c	97.1 ab	99.2 a	99.0 a	96.6 bc	98.6 a	3.11
ARSEF 6430	99.6 a	99.6 a	99.4 a	97.8 b	96.8 b	99.4 a	2.09
ARSEF 6431	99.5 ab	99.7 a	99.1 b	96.3 d	98.2 c	99.5 ab	1.40
ARSEF 6432	97.7 b	99.1 ab	99.4 a	99.1 ab	97.9 ab	98.5 ab	2.58
<i>Beauveria bassiana</i>							
JAB 6	83.2 c	89.3 b	98.2 a	98.0 a	97.7 a	97.5 a	2.56
JAB 7	98.3 bc	91.0 c	98.8 a	97.3 a	95.5 abc	95.7 ab	3.92
IBCB 7	87.5 c	90.3 bc	96.6 a	94.4 ab	97.3 a	97.6 a	4.45
AM 9	41.5 c	42.8 c	74.2 ab	74.0 ab	78.7 a	69.5 b	5.66
IBCB 66	76.4 b	74.3 b	94.9 a	94.3 a	95.4 a	94.5 a	4.12
<i>Paecilomyces fumosoroseus</i>							
JAB 12	92.1 c	93.3 b	98.6 a	98.8 a	97.9 a	96.0 ab	3.35
IBCB 137	93.3 c	97.1 ab	99.2 a	99.0 a	96.6 bc	98.6 a	3.11
IBCB 201	99.6 a	99.6 a	99.4 a	97.8 b	96.8 b	99.4 a	2.09
IBCB 148	99.5 ab	99.7 a	99.1 b	96.3 d	98.2 c	99.5 ab	1.40

¹Means showed in original value, but statistical analysis data were transformed in $\arcsin(x/100)^{1/2}$.

²Means followed by same letter on line are not significantly different at 5% of probability by Tukey test.

³Agar- water.

⁴Minimal medium.

⁵Potato, dextrose and agar.

⁶Potato, dextrose, agar and 1% yeast extract.

⁷Sabouraud, dextrose, agar and 1% yeast extract.

⁸Complete medium.

⁹Coefficient of variation (%).

micronutrient sources (inorganic salts, peptone, casein hydrolysate and yeast extract) required by the fungi, in addition to vitamins. PDA is a complex medium that includes dextrose and soluble starch and proteins. According to MOORE-LANDECKER (1972), under laboratory conditions, spore germination may be triggered by the addition of a diverse range of chemical agents including inorganic ions, carbohydrates, amino acids, lipids and vitamins, among others.

The germinative tubes of some isolates were much larger on any media than on others, indicating their faster germination. Although not measured specifically, a higher germination velocity was observed for the nutrient-rich media (PDA, PDAY, SDAY and CM), suggesting that the composition of culture medium can influence the germination velocity of conidium. Furthermore, due their rich composition, probably these media favour the growth of germinative tube.

Consistent information about the effect of the composition of the culture medium on conidial germination of entomopathogenic fungi is scarce on literature. The results of the present study showed that nutrient-rich media promote higher conidial germination; however, this was not a general rule, as observed for SDAY medium. For some isolates, nutrient-poor (MM) or -free medium (AW) can promote high germination. These differences are probably due to the genetic variability between isolates. Therefore, we conclude that culture media have an influence on the germination capacity of *B. bassiana*, *L. lecanii* and *P. fumosoroseus*. This influence differs among species, and varies within the same species from one isolate to another. Thus, this fact should be considered when performing viability tests, choosing the most adequate medium for the fungus under study.

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