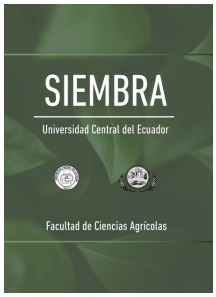
Parameters for the germination of elite fungal biocontrol strains



Parámetros adecuados para la evaluación de germinación de cepas élite de hongos

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Siembra 11 (1) (2024): e5631

Received: 09/10/2023 Revised: 14/11/2023 Accepted: 15/01/2024

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Abstract

The marketing of microbial pesticides has increased exponentially in recent years. Almost 70 % of the products marketed use as active ingredient conidia of biocontrol fungi, and one of the most frequently used criteria to evaluate the quality of these products is conidial germination test. Specifically, this methodology is used to select and assess the viability and vigor of elite strains with biological potential. Some of the factors that influence conidial germination over time, include the selection of culture medium, temperature, incubation time, and the addition of germination synchronizing agents. The optimization for each elite strain allows the characterization of a microorganism behavior over time, ensuring its biological activity and effective management as a biological resource for commercial purposes. The main objective of this study was to identify the optimal conditions (culture media, incubation time, and synchronizing agent concentration) for the germination of three elite strains: *Trichoderma koningiopsis* (Th003), *Lecanicillium lecanii* (Vl026), and *Metarhizium robertsii* (Mt004), which are active ingredients in biopesticides. As a result, the optimal conditions selected are as follows: agar-water medium with 0.0003 % benomyl for Th003 with an incubation period of 18 hours, agar-water medium with 0.0003 % benomyl for Vl026 with an incubation period of 24 hours, and PDA agar supplemented with 0.0003 % benomyl for Mt004 with an incubation period of 18 hours. These conditions will allow a precise evaluation of the germination of the biological control agents ensuring over time their viability and vigor as active ingredients in biopesticides.

Keywords: conidia, quality, biopesticides, viability

Resumen

La comercialización de plaguicidas microbianos ha aumentado de manera exponencial en los últimos años. Casi el 70 % de los productos que se comercializan utilizan como principio activo conidios de hongos biocontroladores y uno de los criterios más utilizados para evaluar la calidad de estos productos es la prueba de germinación de conidios. Específicamente, se utiliza para la selección y la evaluación de la viabilidad y vigor de cepas élite con potencial biológico. Algunos de los factores que influyen

SIEMBRA
https://revistadigital.uce.edu.ec/index.php/SIEMBRA
ISSN-e: 2477-5788
Frequency: half-yearly
vol. 11, issue 1, 2024
siembra.fag@uce.edu.ec
DOI: https://doi.org/10.29166/siembra.v11i1.5631

en la germinación de los conidios incluye la selección del medio del cultivo, la temperatura, el tiempo de incubación y la adición de sustancias sincronizadoras de germinación. La optimización para cada cepa élite permite estimar el comportamiento del microorganismo, lo que garantizará su actividad biológica y eficacia, al ser un recurso biológico con fines comerciales. El objetivo de este trabajo fue seleccionar las condiciones óptimas (medios de cultivo, tiempo de incubación y concentración de sustancia sincronizadora) para la germinación de tres cepas élite *Trichoderma koningiopsis* (Th003), *Lecanicillium lecanii* (Vl026) y *Metarhizium robertsii* (Mt004), las cuales son principios activos de bioplaguicidas en proceso de comercialización. Como resultado se seleccionó el medio de cultivo de agar agua con benomil al 0,0003 % con un tiempo de lectura de 18 h para Th003 y de 24 h para Vl026. Para la cepa Mt004, el medio de cultivo que favoreció la germinación sincrónica de los conidios fue agar PDA suplementado con benomil al 0,0003 % con un tiempo de lectura de 18 h. Estas condiciones permitirán a través del tiempo realizar una correcta evaluación de la germinación de los agentes de control biológico y garantizar su viabilidad y vigor como principio activo de bioplaguicidas.

Palabras clave: conidios, calidad, bioinsumos, viabilidad

1. Introduction

The commercialization of fungal-based microbial biopesticides has increased in recent years and accounts for 10-15 % of the total biopesticide market, which is estimated to reach $6.6 billion by 2022 (Pathan et al., 2019). Most of the fungi produced commercially for use in integrated pest and disease management programs belong to the order Hypocreales, with *Trichoderma* spp., *Metarhizium* spp., *Isaria* spp., *Lecanicillum* spp., *Paecilomyces* spp., and *Beauveria* spp.. standing out (Miranda-Hernández et al., 2017).

Approximately 60 % of all fungal biopesticides are products whose active ingredient is *Trichoderma* spp. species, which are available in different formulations. This is due to their diverse control mechanisms against plant pathogens such as *Botrytis cinerea*, *Fusarium* sp., *Phytophthora* sp., *Rhizoctonia solani* and *Sclerotium rolfsii* (Mukhopadhyay & Kumar, 2020; Rodríguez-León et al., 1999). In the specific case of *Trichoderma* spp. it uses multiple mechanisms to exert biological control against phytopathogens, such as mycoparasitism, production of secondary metabolites with antibiotic activity, competition for nutrients and space, and induction of systemic response in plants. In addition, lately multiple investigations reported that *Trichoderma* exerts growth promoting effects in plants, thus contributing to their growth and development. Within these mechanisms, it helps the degradation of organic matter, increasing the availability of nutrients for plants and improving the absorption of minerals such as MnO_2, Zn, Ca^{2+}, Mg^{2+}, K and P, as well as increasing the content, conferring benefits to the health and nutrition of plants (Saldaña-Mendoza et al., 2023; Zaki et al., 2020).

In the case of entomopathogenic fungi, the most advanced, and most easily available on the market, biopesticides belong to two genera: *Beauveria* spp. and *Metarhizium* spp. (Ascomycota: Hypocreales) (Faria & Wraight, 2007; McGuire & Northfield, 2020), due to their wide distribution, host diversity and high persistence in the environment (Brunner-Mendoza et al., 2019). *Metarhizium anisopliae* and *Beauveria bassiana* are two species of entomopathogenic fungi that have been widely studied for their use in biological control. Both species are found in nature and have been reported to act on a wide range of insect pests, making their application versatile. In addition, these fungi are also known for their high virulence, and ability to survive in the environment (Diego-Nava et al., 2023; Kala et al., 2020).

Currently, the standardization of economical and quick methods to evaluate the quality of agricultural biopesticides is of great interest. Specifically, quality control can be applied at different stages of bioinput production: such as in the purchase of raw materials and inputs, in the production process and in the finished product. The implementation of quality control at each stage ensures maximum efficiency in the field, as well as compliance with safety requirements (Santos Díaz et al., 2022). Microbiological quality control focuses on the identification, authentication, quantification and viability of the active ingredient, and the determination of contaminant content. The viability of the active principles of fungal-based biopesticides (conidia, cells, blastospores) can be evaluated by plate count or conidial germination; the latter parameter, is one of the most widely used to determine the quality of filamentous fungal-based biopesticides and, in some cases, allows evaluating the effects of environmental conditions such as ultraviolet radiation, extreme temperatures, and storage conditions (Oliveira et al., 2015; Stentelaire et al., 2001). Conidia are the active ingredient of almost 75 % of biopesticides available on the market worldwide (Zaki et al., 2020). Because of this, the protocol used to evaluate the germination of conidia is usually the most common technique in the process of quality control of filamentous fungi-based products. However, there are differences within protocols and, thus, it is necessary

to determine the most suitable characteristics in order to implement the best method by type of microorganism. Some of the factors that influence the germination of conidia of biocontrol fungi are the pH, the composition of the culture medium, the availability of water, the hydration process of the product, the incubation temperature and the use of germination synchronizing substances (Alves et al., 1996; Faria et al., 2010; Le Grand and Cliquet, 2013; Oliveira et al., 2015).

In summary, the procedure described by Inglis et al. (2012) is followed: the sample to be evaluated is diluted in serial dilutions in base 10, the dilution with an approximate concentration of 107 conidia mL^{-1} is sown in an appropriate culture medium and the inoculated Petri dishes are incubated for approximately 18 to 24 h; then, both the germinated and non-germinated conidia are quantified by microscopic observation (Inglis et al., 2012). In this sense, several authors recognize the need to establish critical variables in the methodology, such as: the selection of the culture medium, the incubation time, the way of sowing, the use of dyes, or the addition of substances that synchronize the germination process, among others (Lopes & Faria, 2019; Faria et al., 2010; Hsia et al., 2014; Oliveira et al., 2015). With the purpose of contributing to the standardization processes of quality control of biopesticides based on filamentous fungi, the objective of this study was to select the appropriate culture media, incubation time and concentration of germination synchronizing substance for the elite strains *Trichoderma koningiopsis* (Th003), *Lecanicillium lecanii* (Vl026) and *Metarhizium robertsii* (Mt004). These were selected from previous work because of their biological characteristics (Cotes Prado, 2018), and because they are active ingredients of biopesticides marketed for agricultural use. Additionally, it is important to mention that they belong to the most used genera in the development of agricultural bioinputs.

2. Materials and Methods

2.1. Elite Strains of filamentous fungi

The filamentous fungi *T. koningiopsis* Th003, *M. robertsii* Mt004 and *L. lecanii* Vl026 evaluated in this study were provided by the Agrosavia Microorganism Germplasm Bank [BGMA] located in Mosquera, Colombia, and were registered in the National Registry of Biological Collections [RNC] under the number RNC:129.

2.2. Culture Media Selection, Incubation Times, and Synchronizing Substance Concentration for Germination Test

For *T. koningiopsis* and *M. robertsii*, water agar [AA] and potato dextrose agar [PDA] culture media without and with benomyl (germination synchronizing substance) were evaluated at concentrations of 0.0003 % and 0.0005 %. For *L. lecanii* Vl026 germination was evaluated in culture media AA and agar malt extract [EM], with and without benomyl at the same concentrations. Reading times of 18 and 24 h were also evaluated. For this, from 5-day-old sporulated cultures of Th003, Vl026 and Mt004, a scraping was performed with 5 mL of Tween® 80, then filtered and serial dilutions were made up to 10^{-2} (dilution that allowed counting at least 300 conidia per Neubauer chamber). Two counts were performed for each strain and the final concentration of $1x10^{7}$ mL^{-1} conidia was adjusted, which allows a statistically representative conidia count to be performed, according to Santos Díaz et al. (2022) and Oliveira et al. (2015). Of each microorganism, 100 µL were seeded in the in the specific culture media and inoculated Petri dishes were incubated at 25 ± 2 °C for 18 and 24 h. At each evaluation time, the count of germinated and non-germinated conidia was performed by microscopic observation with the objective 40×. A conidium was considered germinated when the germinal tube was equal to or greater than the length of the same conidium (Permadi et al., 2020). The germination percentage was calculated according to equation [1] (Santos Díaz et al., 2022).

$$Germination\ (\%) = \left[\frac{\sum germinated\ conidia}{\sum Conidia\ without\ germinate + \sum Germinated\ conidia}\right] \times 100 \quad [1]$$

The experimental design was completely randomized with a 2x2x3 factorial arrangement and four repeated measurements over time. The experimental unit was defined as a petri dish with two readings and a total of 8 data (n=8) per treatment. For the statistical analysis, a multifactorial analysis of variance with a maximum-order interaction of 1 was used, using the STATGRAPHICS Centurion XVI version 16.1.03 program. For the analysis of the normality and homoscedasticity of the results, the Shapiro Wilks (95 %) and Bartlett (95%) tests

were performed, respectively. Once these principles were demonstrated, an ANOVA analysis of variance and a Tukey mean comparison test (95 %) were performed. Data that did not comply with the principles of normality and homoscedasticity were analyzed with a nonparametric Kruskal-Wallis test (95 %). Finally, for the selection of the germination test conditions, a decision matrix was designed following the hierarchical model. The criteria were: germinal tube formation and conidium morphology classified according to Table 1. The favourable conditions for each micro-organism corresponded to the treatment with the highest score for each selection criterion.

Table 1. Decision matrix for the selection of germination conditions for elite strains.

Criterium	Subcriterium	Clasification
Germinal tube formation	Adequate/Suitable size for reading	+++
	Long germinal tube	++
	Hyphal formation	+
Conidium morphology	Healthy conidia	++
	Deformed conidia	+

3. Results and Discussion

For the three elite strains evaluated, it was validated that the use of enriched media such as PDA and EM favors the germination of conidia compared to the use of basal culture media such as AA. This was to be expected, considering that these are enriched media, and they are commonly used for conidia growth. However, it is common for the use of this type of media to cause asynchronous germination of conidia, generating the need to select alternative media that allow a correct evaluation of the probability of successful establishment of conidia and their germination (Gilchrist et al., 2006). This is why the addition of substances such as benomyl at concentrations below 0.001 % allows the conidia germination process to be synchronous, precise and reproducible. The analysis of the culture medium and incubation time factors on the germination of the conidia of the evaluated fungi showed that for the fungus *T. koningiopsis* Th003, the culture medium has a statistically significant effect on the conidia germination ($F_{5,95}$ = 15,73; p = 0,000). In contrast, the culture medium selected for the fungi *M. robertsii* Mt004 (F5.95 = 8.99; p = 0.000) and *L. lecanii* Vl026 (F5.95 = 47.56; p = 0.000) and the variable incubation time (F5.95 = 9.50; p = 0.003 and F5.95 = 26.30; p = 0.000), showed a significant effect on the germination result of the conidia of the two fungi respectively. When evaluating the different culture media with *T. koningiopsis* Th003, results showed that in PDA and AA the average germination percentages (n=8) achieved after 18 h of incubation at 25 °C were 100 % and 97.24 %, respectively, and there were no significant differences between the two media evaluated.

The germination results obtained in this study suggest that in the case of Th003, the choice of culture medium (either enriched, or basal) and the substance used to induce synchronized germination are critical elements that significantly influence the accurate assessment of the biological potential of the biocontroller agent. This result aligns with Jackson's (1997) findings, which reported that the nutritional conditions during fungal growth and sporulation influence the accumulation of endogenous reserves, enabling newly formed spores to maintain their biological characteristics. In this sense, it is likely that the inoculated conidia maintained reserves to achieve their germination on the two media evaluated, but the addition of benomyl, even at low concentrations, did not allow the correct development and elongation of the germinal tube; probably, because benomyl inhibits the polymerization of tubulin, and acts on a point of the fungal metabolism that affects the cell division process (Castellanos González et al., 2022). Although no differences were observed in the percentage of germination achieved over time for the results of this study, it is crucial to avoid the introduction of variables that may interfere with the biological potential performance of the biocontroller fungus. Therefore, these results allow us to select the appropriate concentration of benomyl for the evaluation of the synchronous germination of the elite strain, and to be able to identify any abnormality of the fungus. In relation to the evaluation time intervals, no significant difference was identified in terms of the count of germinated conidia between 18 and 24 hours of incubation in any of the media analyzed. These results suggest that the range of incubation time evaluated allowed an adequate reading of a number of conidia that statistically ensure their viability, which

corresponds to one of the crucial aspects to guarantee the desired biological activity of the microorganisms used as active ingredient of bioinputs for agricultural use (Daryaei et al., 2016).

To evaluate the germination of *L. lecanii* Vl026 conidia, EM malt extract and AA water agar were used alone and supplemented with benomyl. In the EM agar medium with and without the addition of benomyl in the doses of 0.0003 % and 0.0005 %, a germination of 100 % was achieved from 18 h of incubation, similar to that obtained with AA without benomyl. This result is consistent with what was reported by Francisco et al. (2006), who evaluated the germination of an isolate of *L. lecanii* in different culture media, and reported an average germination rate greater than 99 % in AA, PDA and Sabouraud-Dextrose Agar medium. These results suggest that this microorganism has the ability to maintain an adequate nutritional reserve to ensure germination and growth of the fungus, even when cultured in minimal, or basal media such as AA (Francisco et al., 2006).

In contrast, germination of conidia in the AA medium supplemented with benomyl in the two doses caused a significant decrease in the number of conidia that germinated, and this reduction was directly proportional to the concentration of benomyl supplemented to the medium. As the concentration of benomyl increased, a decrease of 10 % and 17 % in the number of germinated conidia was observed at 18 and 24 h of incubation, respectively (Table 2). In contrast to the observations with Th003, reading time significantly affected the number of germinated conidia at 18 and 24 hours in the AA medium supplemented with benomyl, adjusted to concentrations of 0.003 % and 0.0005 %, respectively. This result showed the negative effect of the addition of benomyl at both concentrations in the AA medium and validates the influence of synchronizing substances on the viability of conidia (Francisco et al., 2006; Meirelles et al. 2023).

Table 2. Germination (%) of conidia of elite fungal strains evaluated in culture media with or supplemented with benomyl and incubation time.

	Germination (%)			
Culture Medium	**Incubation Time (h)**	***T. koningiopsis* Th003**	***L. lecanii* Vl026**	***M. robertsii* Mt004**
PDA	18	100 ± 0.00	NA	99.17 ± 2.36
	24	100 ± 0.00	NA	97.44 ± 5.48
PDA+0,0003% benomyl	18	82.09 ± 7.08	NA	99.05 ± 2.13
	24	88.45 ± 2.30	NA	98.47 ± 3.25
PDA+0,0005% benomyl	18	94.42 ± 3.81	NA	90.26 ± 6.93
	24	62.95 ± 9.90	NA	99.17 ± 1.78
AA	18	97.24 ± 2.25	100 ± 0.00	100 ± 0.00
	24	98.68 ± 3.72	100 ± 0.00	90.28 ± 4.23
AA+0,0003% benomyl	18	93.49 ± 4.04	90.48 ± 3.22	97.04 ± 1.54
	24	94.32 ± 1.46	98.67 ± 0.90	95.19 ± 5.31
AA+0,0005% benomyl	18	69.07 ± 7.89	82.94 ± 5.21	95.14 ± 1.65
	24	90.33 ± 1.35	92.91 ± 2.06	74.64 ± 12.00
EM	18	NA*	100	NA
	24	NA	100	NA
EM+0,0003% benomyl	18	NA	100	NA
	24	NA	100	NA
EM+0,0005% benomyl	18	NA	100	NA
	24	NA	100	NA

* NA: Germination was not evaluated in this culture medium.

For the fungus *M. robertsii* (Mt004), germination in AA at the two reading times (18 and 24 h) was equal to 100 % (Table 2). However, results showed that the germinal tube was found to be more than twice the size of the conidium and, in some observation fields, the formation of hyphae was recorded. When AA was supplemented with 0.0003 % benomyl at 18 h of incubation, 97.0 % germination was obtained and 95.19 % at 24 h of incubation, these values did not present significant differences between them ($F_{5.95} = 9.50$; $p = 0.0027$). In AA with 0.0005 % benomyl, values of 95.14 % were present at 18 h, and at 24 h of incubation germination decreased to 73.88 %. In this culture medium, an adequate conidia size was observed at 18 h, however, after 24 h of incubation, the conidia presented some type of deformation. This is possibly due to the fact that the concentration of 0.0005 % of benomyl generated a fungicidal effect, but not fungistatic compared to the concentration of 0.0003 %. It is necessary to take into account that the mode of action of this product is to inhibit the stages of mitotic cell division, which leads to the normal formation of the germinal tube, but prevents the subsequent development of hyphae and, consequently, the excessive growth of fungi in nutritious agar media, but in high concentrations it generates deformation and subsequently the death of the conidium (Oliveira et al., 2015).

M. robertsii in PDA agar showed a homogeneous behavior in the three culture media evaluated, with germination percentages above 90.52 % (Table 2). In PDA without supplementation, germination was greater than 97%, but the conidia presented germination where the hyphae developed and, therefore, the counting process was not adequate, since there was overlap between the germinated tubes (Santos Díaz et al., 2022). In PDA with 0.0003 % benomyl, an adequate germinal tube size was evidenced at the two reading times (18 and 24 h); in addition, the morphology of the conidia was not affected, allowing an adequate count with germinations greater than 98.47 %. In PDA supplemented with 0.0005 %, germination increased as the incubation time increased, since at 18 h a germination of 90.52 % was obtained, and this value reached 99.17 % at 24 h of incubation. This culture medium has been widely reported as suitable for the evaluation of the viability of *M. robertsii* strains (Afifah et al., 2020; Rangel et al., 2005; Santos Díaz et al., 2022), since the fungus easily assimilates carbon sources from the environment as it is compatible with this active ingredient (Yáñez & France, 2010).

Table 1 presents the decision matrix, in which the evaluation criteria selected were the formation of the germinal tube (specifically the size of the germinal tube), and the morphology of the conidia. For the strains *T. koningiopsis* Th003 and *L. lecanii* Vl026, AA supplemented with 0.0003 % benomyl was selected, since synchronous germination percentages greater than 98 % were obtained, and the germinal tube formation was of an adequate size for reading (equal to, or greater than conidium). In the case of the *M. robertsii* Mt004 strain, the selected culture medium was PDA supplemented with 0.0003 % benomyl (Table 3).

Table 3. Decision matrix for the selection of culture media and germination conditions for elite strains.

Elite strain	**Selection criteria**	**Culture media**					
		AA	**AA + 0.0003 (benomyl)**	AA + 0.0005 (benomyl)	PDA	PDA + 0.0003 (benomyl)	PDA+ 0.0005 (benomyl)
Th003	Germinal tube formation	++	+++	++	+	+	+
	Morphology of the conidia	++	++	+	++	++	+
Elite strain	**Variable**	AA	**AA + 0.0003 (benomyl)**	AA + 0.0005 (benomyl)	Agar EM	EM + 0.0003 (benomyl)	EM + 0.0005 (benomyl)
Vl026	Germinal tube formation	++	+++	++	+	+	+
	Morphology of the conidia	++	++	+	++	++	+
Elite strain	**Variable**	AA	AA + 0.0003 (benomyl)	AA + 0.0005 (benomyl)	PDA	**PDA + 0.0003 (benomyl)**	PDA+ 0.0005 (benomyl)
Mt004	Germinal tube formation	++	++	++	++	+++	+
	Morphology of the conidia	++	++	+	++	++	+

For the selection of the reading time, a comparison of Kruskal Wallis means was made at 95 %, where for Th003 no significant differences were found ($p > 0.000$), so the selected reading time was 18 h. For the Vl026 strain, germination at 24 h was significantly higher compared to that obtained at 18 h, so the longest incubation

time was selected. For the *M. anisopliae* strain Mt004 in the selected culture medium, there were no significant differences between the evaluation times, so the 18 h of incubation was selected for adequate reading (Figure 1).

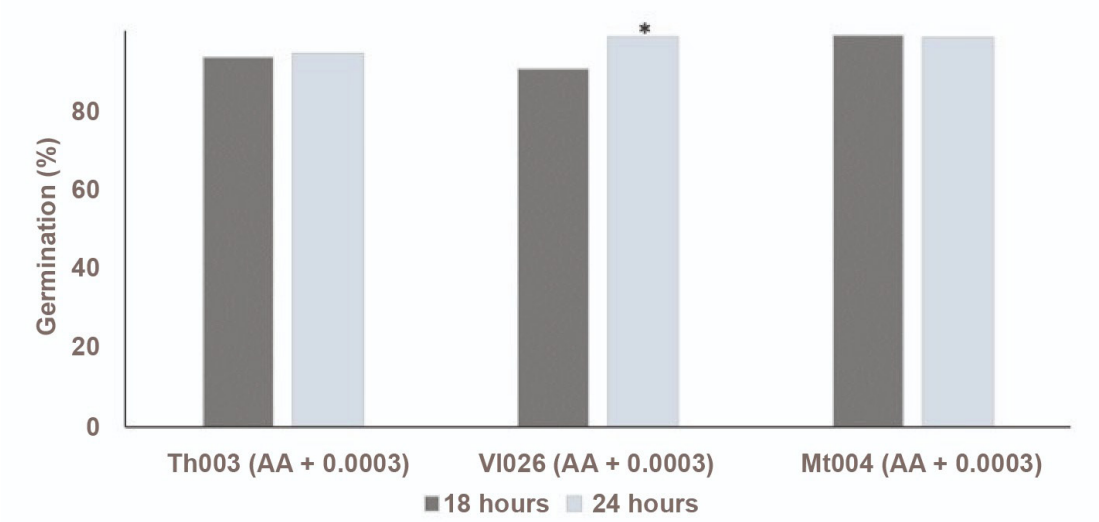

Figure 1. Comparison of incubation times (18 and 24 h) for the selected culture medium for the elite strains *T. koningiopsis* Th003, *L. lecanii* Vl026, and *M. robertsii* Mt004. * indicates significant differences, according to Kruskal Wallis test (α = 0.05).

4. Conclusions

The selection of the culture medium, incubation time and concentration of germination synchronizing substance for the different genera of fungi with potential as biocontrol agents is an essential strategy to ensure a reliable evaluation of conidia germination, thus ensuring the consistency and effectiveness of the results obtained.

The use of AA medium supplemented with 0.0003 % benomyl favored the synchronous germination of the conidia of *T. koningiopsis* (Th003) and *L. lecanii* (Lv026) from 18 h of evaluation.

Synchronous germination of *M. robertsii* conidia (Mt004) was achieved using PDA medium supplemented with 0.0003 % benomyl.

The addition of benomyl adjusted to 0.0005 % in AA and PDA caused abnormalities during germinal tube elongation, suggesting the appropriate use of a dose of 0.0003 % for the germination evaluation of elite fungal conidia.

Acknowledgements

The authors thank the Colombian Corporation for Agricultural Research for funding this project.

Contributor roles

- Liz Uribe-Gutiérrez: conceptualization, formal analysis, investigation, methodology, writing – review & editing.
- Adriana Santos-Díaz: conceptualization, formal analysis, investigation, methodology, writing – review & editing.

Ethical implications

Ethics approval not applicable.

Conflict of interest

The authors declare that they have no affiliation with any organization with a direct or indirect financial interest that could have appeared to influence the work reported.

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