Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i1.3510

Article

Conidial reproduction of *Trichoderma asperelloides* in culture media and organic substrates

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Abstract

The use of plant extracts for disease control in the framework of sustainable agriculture is a promising alternative due to their high effectiveness, low cost, and non-polluting nature of the environment. This work aimed to evaluate the biological activity of the conidial reproduction of *Trichoderma asperelloides* in culture media and organic substrates. Four strains of *T. asperelloides* were evaluated in solid substrates of rice, corn, sorghum, wheat, cornstarch powder and oats with peel of yellow peach native to the region; 250 g was added in polyethylene bags with an aliquot of 15 ml of distilled water, with the fungus and in glass jars, 10 mycelial discs of 0.5 cm diameter were added per strain during 45 days of incubation; in addition, the growth of *T. asperelloides* was tested in culture media of 5% V8 vegetable juice, potato dextrose agar, sabouraud dextrose agar, 5 g of PDA with wheat powder supplement, 5 g of potato dextrose agar with pine sawdust supplement, 5 g of sabouraud dextrose agar with eucalyptus powder supplement, bacteriological agar and MacConkey agar during seven days of growth; to obtain the conidia, serial dilutions were made with six replications per culture medium, with a concentration of 1 x 10^6. One hundred percent conidial reproduction was obtained in organic substrates and 87.5% mycelial growth in culture media and strain 3 was shown to have the highest conidial production.

Keywords:

antagonist, concentration, growth, strain.

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Introduction

Plant diseases are responsible for significant crop losses worldwide and pose a threat to global food security (Bevacqua *et al*., 2019). The use of plant extracts for pest and disease control in the framework of sustainable agriculture is a promising alternative due to their high effectiveness, low cost and non-polluting nature of the environment (Rodríguez *et al*., 2000). For example, organic waste and by-products of agribusiness that are easily acquired and provide a high production of viable fungal conidia (Rai *et al*., 2023). Likewise, the development of new methodologies for the mass production of biocontrollers that reduce the cost of production and make possible a greater use of this antagonist in various crops (Arévalo *et al*., 2017).

Fungi are eukaryotic and heterotrophic microorganisms that require organic compounds for their nutrition since they secrete enzymes that help break down a wide variety of substrates to absorb the nutrients found in dead leaves and other organic materials found in the soil (Ipiales *et al*., 2021). A type of beneficial microorganisms in the soil are the fungi *Trichoderma* spp., which establish a mutually beneficial symbiosis in the roots of higher plants for the absorption of water and nutrients (Liang *et al*., 2021).

Commercial substrates allow the optimization for the reproduction of microorganisms, which are composed of cellulose, hemicellulose and lignin; these substrates include rice and wheat straw, cottonseed hulls, sawdust, wastepaper, leaves and sugarcane residues (López-Martínez *et al*., 2022). They are used for fungal reproduction (Akter and Sadia, 2020); in addition, the *Trichoderma* genus is often applied to improve crop health, to manage plant diseases, as a biofertilizer, bioremediator, and biocontrol agent (Bhandari *et al*., 2021).

Besides, encouraging results have been obtained both in substrates and in conidial reproduction in media rich in meat, glucose and protein sources (Antomarchi *et al*., 2023). This research aimed to evaluate the biological activity of conidial reproduction of *Trichoderma asperelloides* in different culture media and organic substrates. The hypothesis was that at least 50% of the culture media will show mycelial growth of *T. asperelloides* and effects on commercial organic substrates.

Materials and methods

Study location

This research work was conducted in the Laboratories of Microbiology, Edaphology, and Environmental Analysis of the Technological University of the Tarahumara, located in Guachochi, Chihuahua, Mexico (January 2023), in conjunction with the Agricultural Microbiology Laboratory of the Faculty of Agriculture of Valle del Fuerte of the Autonomous University of Sinaloa in Juan José Ríos, Sinaloa and the Department of Phytopathology of the Autonomous University of the West, Los Mochis Regional Unit, Sinaloa (April-June 2023).

Obtaining isolates

To obtain the four strains of *T. asperelloides*, those with the highest degree of inhibition were selected, which have already been previously identified as strains 3 and 5, obtained from the municipality of Morelos, Chihuahua, with a climatological variation of 12 to 49 °C (Porras *et al*., 2021) and strains 1 and 8, isolated from the municipality of Guachochi, Chihuahua, with a temperature between -6 and 17.5 °C (Alvarado *et al*., 2019).

Obtaining substrates

For the mass reproduction of *T. asperelloides* on solid substrates of commercial origin, we used rice (*Oryza sativa* L.) (Arévalo *et al*., 2017), corn (*Zea mays* L.), sorghum (*Sorghum* M.), wheat (*Triticum* L.), cornstarch powder (Feijóo-Vivas *et al*., 2021; López-Martínez *et al*., 2022) and oats with peel of yellow peach native to the region.

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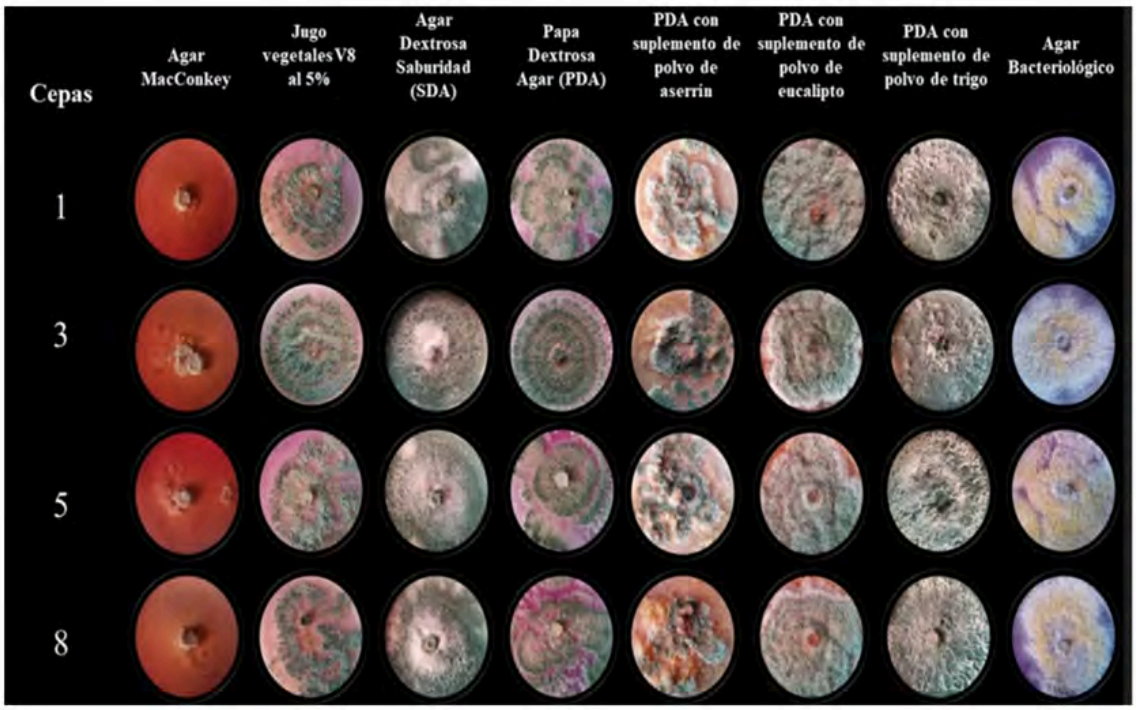
Culture media preparation

All strains were grown on 5% V8 vegetable juice (Camargo *et al*., 2021), potato dextrose agar (PDA), sabouraud dextrose agar (SDA) (Fletcher, 2019), 5 g of PDA with wheat powder supplement crushed in a mortar (Ruschioni *et al*., 2020), 5 g of PDA with pine sawdust supplement crushed in a mortar (Zhang *et al*., 2021), 5 g PDA with eucalyptus powder supplement crushed in a mortar with modifications (Ahmad *et al*., 2020), bacteriological agar (Rodrigues *et al*., 2019), MacConkey agar prepared under standard sterile conditions to avoid external contamination and according to the manufacturer's instructions (Figure 1) (Kyei *et al*., 2020); all media were subjected to a range of pH= 5.5 to 7, with alternation of light and darkness (14 and 10 h, respectively) for seven days in all culture media at a room temperature of 7 °C ±2 for bioassay 1 during winter (November) 2022 and at a room temperature of 17.5 °C ±2 for the bioassay in summer (May) 2023.

Figure 1. *In vitro* mycelial growth of *Trichoderma asperelloides* strains on culture media.



Substrate preparation

Gato's (2010) technique was considered in order to remove impurities from the substrates; for this purpose, the rice was placed in tap water for 24 h, then it was washed deeply until the starch was removed, then 250 g of that rice was placed in polyethylene bags and sterilized in an autoclave (brand: Zhejiang Top Instruments, model: YX-18LD) at 121°C for 60 min. For corn, it was ground to 1.3 g cm of grain and the sorghum was crushed until it was pulverized in a metal blender (brand: Waring, model: L.V.I.06714); glass jars wrapped in aluminum foil with plastic lids were used, where 250 g of both substrates was deposited (Vázquez *et al*., 2009).

In wheat, the seeds were superficially disinfected after 5 min of soaking in 1% sodium hypochlorite solution, followed by immersion in sterile water for 12 h, placed in glass jars with plastic lids, and sterilized by autoclave for 15 psi at 121 °C (Velmourougane *et al*., 2019). In the cornstarch powder substrate, Zhang *et al*. (2021) technique was followed with modifications; 30 g cornstarch powder was weighed in solid medium, mixed with boiling water with 80% relative humidity, deposited in a 250 ml glass container, and sterilized for 30 minutes with 15 psi at 121 °C.

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To prepare the oats with peel of yellow peach, 50 g of oats were blended until it was completely finely powdered. Then, 200 g of fresh peel of peach native to the region was taken, cut into small pieces of 5 cm, mixed, and stored in polyethylene bags for subsequent sterilization for 8 minutes with 15 psi at 121 °C.

Inoculation of *Trichoderma asperelloides*

The *T. asperelloides* strains were developed in potato dextrose agar (PDA) culture medium, at four days of growth (Arévalo *et al*., 2017), a scraping was performed with a bacteriological loop and a syringe was used to take a 15 ml aliquot of the mixture of distilled water with the antagonist, it was then injected with the substrate and left to rest for 45 days at 27 °C ± 2 with 14 h light and 10 h darkness, and the substrate was removed daily until obtaining the sporulation of the fungus (Cuenca *et al*., 2022); on the other hand, 10 mycelial discs of 0.5 cm in diameter per strain were added to the substrates contained in glass containers. The jars were incubated at the same temperature in complete darkness for 45 days (Vázquez *et al*., 2009).

Conidia counting through serial dilutions

To count the conidia, serial dilutions were made from one gram of substrate colonized with the fungus and were added to 9 ml of sterile distilled water; six replications were performed for each substrate; finally, the reading of six aliquots with a concentration of 1 x 10^6 was taken in the Neubauer chamber or hematocytometer (Arévalo *et al*., 2017).

To count the conidia in culture media, a scraping of the four strains of *T. asperelloides* previously grown in potato dextrose agar (PDA) was carried out in the eight culture media, with six replications with a concentration of 1 x 10^6, and the formula proposed by Bastidas (2018) was used: $V_2 = C_1XV_1/C_2$. Where: C1= initial concentration (known in the count); V1= initial volume (arbitrarily established when preparing the inoculum); C2= desired final concentration (depending on the study to be performed), and V2= final volume (unknown).

Statistical analysis

To carry out the analysis of the treatment with the highest conidial production in substrates and culture media, the design used was completely randomized with factorial arrangement with respect to the four strains of *T. asperelloides*. For the analysis of variance and the comparison of means, the Tukey-Kramer mean test was performed, with an alpha value of 0.05%, with six replications. These analyses were carried out using the JMP statistical package, version 9.0.1 [Statistical Analysis System, (SAS Institute Inc.), 2011].

Results and discussion

Conidial concentration of *Trichoderma asperelloides* in culture media

The difference between the treatments was determined according to the statistical results analyzed; the eight culture media were considered to obtain an average conidial production per strain. Strain 1 showed a conidial reproduction of 533 conidia ml^{-1} in the sabouraud dextrose agar (SDA) medium, with the highest spore production, whereas the MacConkey medium obtained the lowest conidial amount; for strain 3, 695 conidia ml^{-1} was visualized, and for strain 5, 627 conidia ml^{-1} since it quantified the PDA culture medium with wheat powder supplement with the highest number of conidia and the bacteriological agar with the lowest amount; finally, for strain 8, 183 conidia ml^{-1}, it was possible to visualize that the PDA with wheat powder supplement had the highest number of conidia and the SDA medium the lowest.

In strain 1, the daily radial growth obtained was 2.4 cm in PDA medium with wheat powder supplement in bioassay 1 and 2.325 cm with a standard deviation of 0.025 on average in PDA medium in bioassay 2 and MacConkey agar medium had the minimum growth of 0 cm in both

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bioassays; strain 3 showed 2.58 cm on average, the highest with the PDA medium with eucalyptus powder supplement in bioassay 1 and 2.36 cm in bacteriological agar in bioassay 2 and the medium with the lowest mycelial growth was MacConkey agar with 0 cm in both bioassays; for its part, strain 5 obtained 1.933 cm in PDA and PDA with sawdust supplement in bioassay 1 and 2.4 cm in bacteriological agar in bioassay 2 and the MacConkey agar medium had the minimum growth of 0 cm in both bioassays; finally, strain 8 showed 2.1 cm on average, the highest with the PDA medium with wheat powder supplement in bioassay 1 and 1.83 cm for the potato dextrose agar (PDA) medium in bioassay 2 and the medium with the lowest mycelial growth was MacConkey agar with 0 cm in both bioassays.

To test the *in vitro* development of *T. asperelloides* in the culture media for bioassay 1, significant growth of the fungus was observed in the culture media in PDA with wheat powder supplement (Figures 2 and 5), PDA with eucalyptus powder (Figure 3), potato dextrose agar and PDA with eucalyptus powder (Figure 4) and for bioassay 2, it was with potato dextrose agar (Figures 2 and 5) and bacteriological agar (Figures 3 and 4), so visible growth was recorded in three days; in contrast, in the rest of the culture media, growth was slower or even non-existent.

Figure 2. *In vitro* growth of *T. asperelloides* in culture media during seven days of development of strain 1. Different letters between rows indicate significant differences ($p \leq 0.05$).

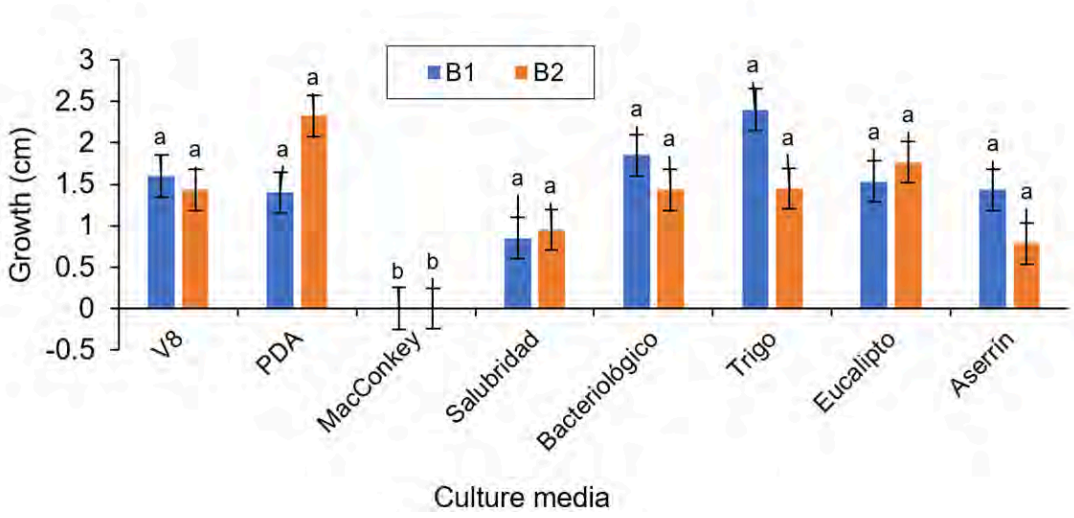

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Figure 3. ***In vitro*** **growth of** ***T. asperelloides*** **in culture media during seven days of development of strain 3. Different letters between rows indicate significant differences (*p*≤ 0.05).**

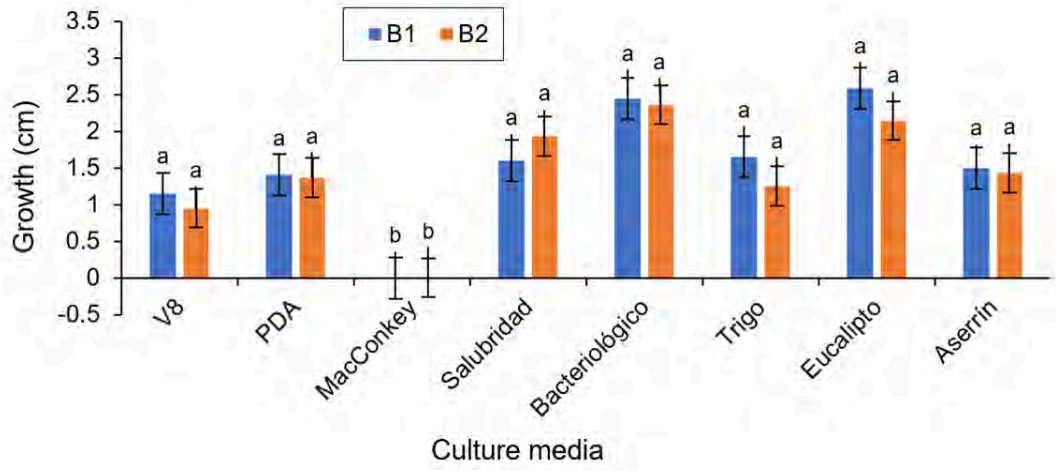

Figure 4. ***In vitro*** **growth of** ***T. asperelloides*** **in culture media during seven days of development of strain 5. Different letters between rows indicate significant differences (*p*≤ 0.05).**

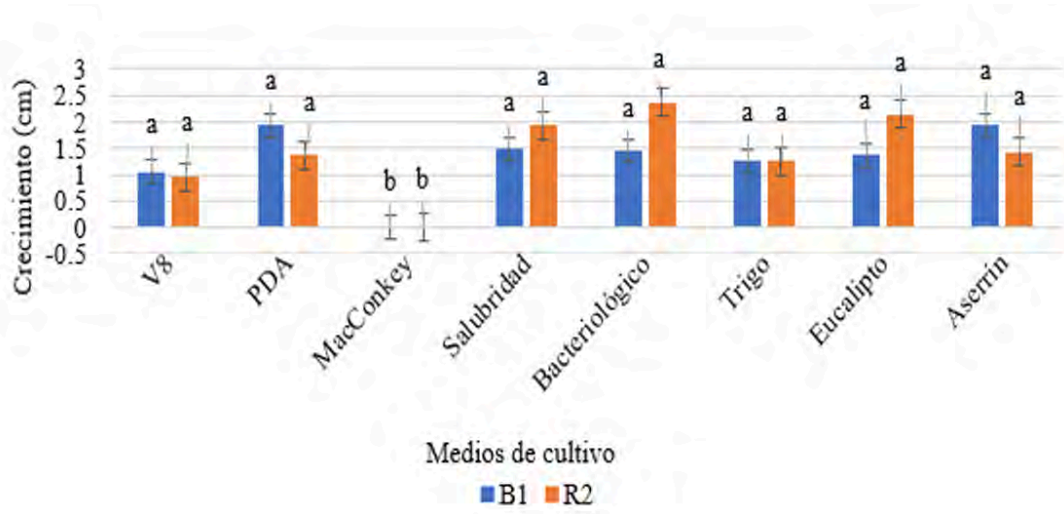

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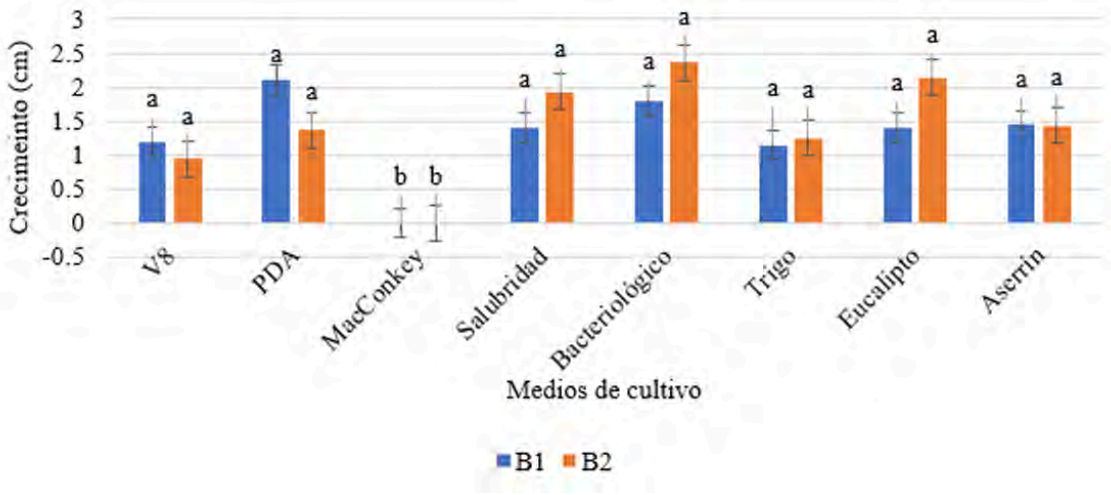
Figure 5. ***In vitro*** **growth of** ***T. asperelloides*** **in culture media during seven days of development of strain 8. Different letters between rows indicate significant differences (*p*≤ 0.05).**



The results are consistent with studies conducted by Shina *et al*. (2018), who evaluated five culture media [potato dextrose agar (PDA), potato carrot agar (PCA), carrot agar (CA), Czapek Dox agar (CDA) medium, and vegetable juice (V8)] by inoculating *T. viridae* and *T. harzianum*; in said study, it is reported that the highest radial growths were found in potato dextrose agar, whereas the lowest growth was recorded in vegetable juice; these results are consistent with those of the present research; a greater radial growth in potato dextrose agar and zero growth in the MacConkey medium were observed in both bioassays carried out.

On the other hand, studies carried out in Bangladesh reported the growth of *T. harzianum* in potato dextrose agar, modified potato dextrose agar, water agar, carrot agar, and corn meal agar, where the highest radial growth was found in potato dextrose agar (Jahan *et al*., 2013); these results coincided with those of the present study by demonstrating that the potato dextrose agar medium prevailed in most of the strains evaluated.

Thomas and Gangadhara (2017) also evaluated eight culture media (V8 juice agar, Richard's synthetic agar, sabouraud dextrose agar, oatmeal agar, corn meal agar, carrot dextrose agar, dextrose potato agar, and rye agar) for the growth of *Phytophthora capsici* during five days, in which sabouraud dextrose agar developed a larger diameter, with 69.5 mm of the colony, unlike the results in *T. asperelloides*, in which there was a radial growth of 0.7 and 1.9 cm in both bioassays in all the strains evaluated for sabouraud dextrose agar.

When evaluating the conidial production of the different strains of *T. asperelloides* in the municipality of Morelos, Chihuahua, climatological variables are considered as a key factor in the growth of the fungus as it was shown that strains 3 and 5 obtained the highest average number of conidia since the average temperature was 32 °C and the conidial reproduction in the different media varied from 1 x 10^6 to 1 x 10^9; in relation to the above, previous studies carried out in São Paulo, Brazil, evaluated the effect of specific factors on the production of conidia of *T. asperelloides*, *T. erinaceum*, *T. erinaceum* T-18, and *T. harzianum* and measured key variables, such as temperature and conidial production, in basic culture broth and the basal medium composed of 2 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$ and 0.02 g of $FeSO_4$. *T. asperelloides* showed 100% conidial production in seven days of growth, with a pH of 3.5 and an optimal temperature of 30 °C in both media, with a conidial production ranging from 1 x 10^8 to 2.6 x 10^8 (De Resende *et al*., 2020).

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Quantitative analysis of the mycelial growth of *Trichoderma asperelloides* in commercial organic substrates

In general, all substrates initially showed the formation of white mycelium of *T. asperelloides* on the vegetative material, later it turned to pine green; in the case of oats with peach peel, the color became blackish after 45 days; in sorghum, it took 22 days for the fungus to penetrate to carry out its invasion; in addition, clumps between 1 and 4.8 cm in diameter formed in cornstarch after 39 days (Figure 6).

Figure 6. Mass reproduction of *Trichoderma asperelloides* stored in polyethylene bags and glass containers of commercial organic substrates.

In the rest of the substrates, the presence of the fungus became evident between 12 and 18 days. The results indicate that conidial reproduction was more effective in rice grains and wheat seeds since the invasion was in its entirety in less time compared to the rest; these results are similar to those presented by Mulatu *et al*. (2021) when evaluating *Trichoderma* species under solid-state fermentation using 14 organic substrates after 21 days of incubation, in which it was found that a mixture of wheat bran and rice supports the maximum growth of *T. asperellum* (3.2×10^7 spores g^{-1} dry substrate) and *T. longibrachiatum* (3.5×10^7 spores g^{-1} dry substrate).

According to the statistical analysis, all substrates reached the ideal concentration of 1×10^6 conidia ml^{-1} with a purity of 100% in both those inoculated in polyethylene bags and those contained in glass; there were also concentration variations between substrates with respect to the different strains. Strain 3 was shown to have the highest concentration in all substrates; however, there was no significant difference between treatments (Table 1).

Table 1. Conidial concentration of *Trichoderma asperelloides* and purity of commercial substrates for agricultural use.

Substrate (treatment)	Number of strains		Concentration (conidia g^{-1} of substrate)
Rice	1	$1x10^8$	a
Corn	1	$1x10^6$	a
Sorghum	1	$1x10^6$	a
Wheat	1	$1x10^9$	a
Cornstarch	1	$1x10^8$	a
Oats with peach peel	1	$1x10^6$	a
Rice	3	$1x10^9$	a
Corn	3	$1x10^7$	a
Sorghum	3	$1x10^6$	a
Wheat	3	$1x10^9$	a
Cornstarch	3	$1x10^8$	a
Oats with peach peel	3	$1x10^6$	a

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Substrate (treatment)	Number of strains		Concentration (conidia g^{-1} of substrate)
Rice	5	$1x10^7$	a
Corn	5	$1x10^7$	a
Sorghum	5	$1x10^6$	a
Wheat	5	$1x10^8$	a
Cornstarch	5	$1x10^6$	a
Oats with peach peel	5	$1x10^6$	a
Rice	8	$1x10^7$	a
Corn	8	$1x10^7$	a
Sorghum	8	$1x10^6$	a
Wheat	8	$1x10^8$	a
Cornstarch	8	$1x10^6$	a
Oats with peach peel	8	$1x10^6$	a

Different letters between rows indicate significant differences ($p \leq 0.05$).

Compared to what was obtained by Cáceres and Galliani (2020), they evaluated organic substrates on *T. viridae* in sawdust, yellow grain corn, oatmeal, birdseed, dried lima bean husk, wheat grain, grape pomace and huarango pod, and their results were favorable for bean husks, with a higher concentration of 2 x 10^9 conidia g^{-1} in five days of incubation, and 100% purity was obtained in all substrates.

In addition, Perera *et al*. (2021) tested different treatments for the growth of *T. viridae*; they compared rice straw, wood sawdust, rice husk, and banana leaves for 36 days, their results were favorable as they had a significant effect of rice straw and sawdust on the development of *Trichoderma*; in this case, it was consistent with what was done in the present study, as rice grains were found to be the best source of mass reproduction and inoculum of *T. asperelloides*.

On the other hand, Silva *et al*. (2018) used the technique proposed in this study for the inoculation of *T. asperelloides* and *T. asperellum* on wheat granules with a concentration of 1 x 10^6 conidia ml^{-1} to determine mycoparasitism on *Sclerotinia sclerotiorum* and obtained results of 100% inhibition of the fungus on the substrate; nevertheless, studies carried out in Cuba have shown the massive reproduction of *T. harzianum* in white pine sawdust and there was 29.87% conidial reproduction on sugarcane bagasse (Antomarchi *et al*., 2023).

Nonetheless, there was a difference in this research since pine sawdust was only outstanding in strain 5 with the same PDA value, whereas López-Martínez *et al*. (2022) confirm corn cobs as the best substrate for the reproduction of *T. harzianum*, *T. harzianum*, *T. harzianum*, *Trichoderma* sp. and *T. longibrachiatum*, compared to the substrate in rice grains, the values were of 3.3 x 10^6 spores in rice and 1.74 x 10^6 in corn cob and it was shown that corn cobs are an ideal substrate for the reproduction of fungi without having to use a human food.

Conclusions

Of the eight culture media tested on four strains of *T. asperelloides* during both bioassays, potato dextrose agar, bacteriological agar, and PDA with wheat powder stood out with the highest growth value, and MacConkey medium did not show growth in the treatments; in addition, strain 3 was shown to have the highest conidial production by filling a 90 mm box of the different culture media in three days of experimentation.

All substrates showed viability for the conidial reproduction of the fungus and it was demonstrated that wheat seeds and rice grains are an ideal source for its growth at warm (17.5 °C ±2) and cold (7 °C ±2) temperatures; therefore the objective was met by confirming the biological effectiveness of *T. asperelloides* in commercial organic substrates since 100% conidial reproduction and 87.5% mycelial growth were obtained in culture media. It is recommended to test organic substrates in crops in the region either in controlled conditions or in the open field, adding them in a solid form on the soil; likewise, it is recommended to use the potato dextrose agar medium for conidial reproduction and thus be able to carry out the inoculations in liquid form.

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Acknowledgements

The first author thanks the National Council of Humanities, Sciences and Technologies (CONAHCYT), for its acronym in Spanish for awarding the scholarship for postgraduate studies, which allowed this research to be carried out, as well as the Autonomous University of the West, Technological University of the Tarahumara for supporting with the use of the equipment for the execution of this project and Brenda Guaderrama Villagran, who participated as a collaborator in the experimental work.

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Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i1.3510

Conidial reproduction of *Trichoderma asperelloides* in culture media and organic substrates

Journal Information
Journal ID (publisher-id): remexca
Title: Revista mexicana de ciencias agrícolas
Abbreviated Title: Rev. Mex. Cienc. Agríc
ISSN (print): 2007-0934
Publisher: Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias

Article/Issue Information
Date received: 01 October 2024
Date accepted: 01 February 2025
Publication date: 19 March 2025
Publication date: Jan-Feb 2025
Volume: 16
Issue: 1
Electronic Location Identifier: e3510
DOI: 10.29312/remexca.v16i1.3510

Categories

Subject: Articles

Keywords:

Keywords:

antagonist
concentration
growth
strain

Counts

Figures: 6
Tables: 1
Equations: 0
References: 34
Pages: 0