Scholars Journal of Agriculture and Veterinary Sciences OPEN ACCESS
Abbreviated Key Title: Sch J Agric Vet Sci
ISSN 2348–8883 (Print) | ISSN 2348–1854 (Online)
Journal homepage: https://saspublishers.com

Selection of Native Isolates of *Trichoderma* Spp on Different Natural Substrates for Mass Multiplication

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DOI: https://doi.org/10.36347/sjavs.2025.v12i10.001 | **Received:** 08.06.2025 | **Accepted:** 02.08.2025 | **Published:** 13.10.2025

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Abstract **Original Research Article**

The need to reduce the use of synthetic fungicides for crop pest control makes it necessary to evaluate and develop technologies that allow products to be obtained from microorganisms in an easy, economical, and effective way for their mass application in cultivated areas. This study was conducted to identify prominent strains of the genus Trichoderma and to determine suitable organic substrates for use in mass propagation. The yield and viability variables were established in a completely randomized experimental design (CRD). Native strains of *Trichoderma* spp T58, T38, T36, T52, T49, and FRAIII were evaluated, as were organic substrates such as sorghum, corn, 96% whole grain rice, 80% whole grain rice, 70% whole grain rice, and 50% whole grain rice. The mass production method was semi-industrial two-phase. The data were organized using Microsoft Excel software and then processed with the Infostat 2020 statistical package. The normality of the data and the homogeneity of the variances were analyzed. The comparison of means was performed using the Tukey test ($p < 0.005$). The organic substrates with the best spore yields were 96% whole grain rice, 80% whole grain rice, and 70% whole grain rice. The substrate with the best spore viability was the organic substrate based on 80% whole grain rice. The strain with the best yield was strain T49, and the strains with the best viability percentages were strains FRAIII, T36, and T38.

Keywords: Yield; viability; biphasic; strains; phytosanitary.

INTRODUCTION

Population growth leads to a growing demand for goods and services to meet basic needs. However, this phenomenon also generates a significant increase in the production of solid, liquid, and gaseous waste released into the environment. These wastes, derived from activities such as mining, agriculture, agribusiness, livestock farming, fuel stations, car washes, transportation, and various industries, can contain potentially toxic compounds. In the most critical cases, these residues have hazardous characteristics, including mutagenic and carcinogenic properties (Corrales, 2018).

For decades, conventional agriculture has resorted to the use of pesticides to control crop diseases, applying them directly to the soil, seeds, foliage, and fruits. While these products can offer agronomic benefits, their use also carries adverse effects, such as risks to human health, environmental pollution, and the development of resistance in target organisms. Faced with this problem, the need to develop sustainable alternatives has arisen, among which the incorporation of biological control agents stands out as a strategy for integrated pest management (Campos, 2018).

Some soils in the department of Chinandega were studied using toxicological analysis of toxaphene, and concentrations between 17,300 mg kg-1 dry weight and 38,000 mg kg-1 dry weight were found. These concentrations were the highest among other organochlorine pesticides, which showed concentrations below 500 mg kg-1 dry weight (Carvalho *et al*., 2011). Residues of intensively used organochlorine compounds are still found in Pacific soils, including toxaphene, dichlorodiphenyl trichloroethane, dieldrin, endrin, lindane, and endosulfan, among others, which have caused effects on human health, soil biota, and aquatic ecosystems (Corrales-Pérez 2018). In recent years, the genus Trichoderma has sparked considerable interest in the scientific community, being widely studied for its capacity as a biological control agent for soil-borne phytopathogens, its role in the decomposition of organic matter, and its potential as a plant growth promoter. Its versatility, adaptability, and ease of management have positioned Trichoderma as one of the most effective

antagonists in pest control. Furthermore, it represents a highly efficient technological alternative from a productive and economic perspective, especially in the formulation of high-quality bio fungicides for integrated pest management programs (Michel-Aceves *et al*., 2008).

The growing need to reduce the use of fungicides in phytosanitary control programs has driven the development of technologies that facilitate the production of bio inputs from microorganisms. These technologies must be accessible, cost-effective, and effective, allowing us to produce compounds with the quality and quantity required for large-scale application in agricultural systems.

"The production of fungal-based bio inputs on a commercial and industrial scale presents some drawbacks, such as the lack of knowledge of efficient alternative substrates, infrastructure, and the minimum necessary equipment; this situation has limited its development and use on a larger scale. There are different methods for reproducing Trichoderma; however, they are expensive. One of the most used substrates in mass production is whole grain rice, which is relatively expensive" (Michel-Aceves *et al*., 2008). In this context, the design of strategies to reduce production costs by identifying alternative organic substrates that promote high sporulation without compromising conidia viability becomes a priority.

Based on this problem, the objective of this study is to evaluate and select promising native isolates of the genus Trichoderma, using various organic substrates to optimize their mass reproduction.

MATERIALS AND METHODS

The research was conducted from May to October 2023 in the Biopesticides Laboratory of the National Agrarian University, located at km 12.5 of the Carretera Norte, Managua, Nicaragua, at the coordinates 12°08'52" North Latitude and 86°09'41" West Longitude. The study used quantitative experimental research methods, evaluating six native strains of Trichoderma spp. in six different organic substrates and different rice qualities to determine their effect on conidia viability and yield. Treatments were established in a completely randomized experimental design for both variables. A total of 36 treatments were evaluated, corresponding to the two factors: strains and organic substrates, with each factor having six levels. The inoculum was obtained from the National Agrarian University's biopesticide laboratory strains, preserved in a 25% acidified Potato Dextrose Agar culture medium at 40°C. For its use, it was reactivated and purified using a 25% acidified Potato Dextrose Agar culture medium. Various organic substrates were used, such as sorghum, corn, 96% whole grain rice, 80% whole grain rice, and 70% whole grain rice. These variables were evaluated through conidia production. The mass production method was a semi-industrial biphasic method (Monzón 2001). The methodology consisted of reproducing the inoculum in a synthetic solid culture medium to produce asexual reproduction structures. It was then multiplied in liquid matrices based on nitrogen, vitamins, phytohormones, and sucrose. These liquid matrices were agitated for 60 hours in a horizontal orbital shaker.

Mass multiplication began by placing 300 grams of each raw substrate in 10x6 inch heat-resistant bags with 50 ml of potable water. The bags were then sealed with staples and sterilized by moist heat at 1.5 bar pressure at 121oC for five minutes. Five bags were then inoculated with each strain for each substrate, depositing 20 ml of the fungal suspension in the liquid matrix with a spore concentration of 1.00E+08. The bags were placed in an incubation room at a temperature of 24°C. Once the fungus had colonized and germinated after five days, it was placed in plastic boxes trays measuring 42x21x71 cm. This process was done to increase the spore multiplication rates. The tray was then covered with black plastic to create a humid chamber. The incubation period was 72 hours. The trays were then uncovered and allowed to dry for 15 days until a humidity level of 5% was reached for subsequent viability and yield studies. Conidia samples from each strain and from each substrate were taken from the trays containing the five previously incubated bags. A total of five trays were collected for each treatment. Five random samples were taken for viability and yield studies. For the yield one gram was taken from each treatment and serial dilutions were made, this procedure was repeated three times and each repetition was considered a replicate, likewise for the viability the appropriate serial dilution was taken and sown in three Petri dishes, in each Petri dish they were deposited in five previously delimited points, in these points an aliquot of 20 µl of the suspension was deposited where the readings were made and a germinated conidia was considered those that were twice its length, this reading was carried out 22 hours after the test was established, in the case of the yield the readings were carried out 20 days after the trays were uncovered.

To evaluate yield, one gram of fungus was taken from the drying trays in a test tube containing nine ml of water. Serial dilutions (10^{-1},10^{-2}, $10^{-3,}$ 10^{-4} y10^{-5}) were performed. A 10^{-5} dilution was selected. A Neubauer chamber with a depth of 0.100 mm was used to count conidia, and a 20-microliter aliquot was placed in the counting chamber using a 10 to 100 µl micropipette (Monzón 2001). To homogenize the dilutions, a vortex mixer at 2400 rpm was used to separate the conidia from the substrate for 20 seconds. The conidia were then counted using an LW-Scientific light microscope with a 40X lens. Three replicates were performed per treatment. Three replicates were performed in each sample, where secondary frames were quantified. Five frames were quantified in each secondary frame, and the total number of conids found

was totaled. The spore yield/gram of substrate was calculated using the following formula:

Yield =Number of conidia observed × chamber factor × dilution factor Equation 1

Viability was determined by evaluating the average conidia germination time described by Monzón (2001). To set up the test, one gram of each substrate with its respective strain was taken and placed in a test tube containing 9 ml of sterile distilled water to perform serial dilutions of 10-1, 10-2, 10-3, 10-4, and 10-5. The 10-5 dilution was selected for this study. To perform this test, a 10-100 µl micropipette was used. A 20 µl aliquot of the suspension of each Trichoderma spp strain was taken into a 90 mm Petri dish marked with five circles on the back. The aliquot containing 1.5% water agar medium was placed in the dish. The inoculated Petri dishes were placed in a room at 24.5°C. Conidial germination was measured after 22 hours using a 40x LW-Scientific light microscope. To determine the viability of each isolate on its respective organic substrate, 10 microscopic fields were observed for each dish. A minimum of 200 conidia were observed on each dish, and conidia that reached twice its length was considered germinated.

The viability percentage will be determined using the following formula (French and Hebert, 1982). Used by (Castillo-Arévalo, 2025)

$$\text{Germinated conidia (\%)} = \frac{\text{Number of germinated conidias}}{\text{Total number of conidia}} \text{x100} \quad \text{Equation 2}$$

Table 1: Treatments evaluated

Organic substrates	**Evaluated strains**	**Treatments for viability**	**Treatments for yield**
96% whole grain rice	T58, T38, T36, T52, T49, FRAIII	6	6
80% whole grain rice	T58, T38, T36, T52, T49, FRAIII	6	6
70% whole grain rice	T58, T38, T36, T52, T49, FRAIII	6	6
50% whole grain rice	T58, T38, T36, T52, T49, FRAIII	6	6
Sorghum	T58, T38, T36, T52, T49, FRAIII	6	6
Corn	T58, T38, T36, T52, T49, FRAIII	6	6

The data were organized using Microsoft Excel and then processed using the Infostat statistical package, version 2020. Data normality and homogeneity of variance were analyzed for viability and yield variables. Means were compared using analysis of variance (ANOVA), and a comparison of means was performed according to Tukey's criterion (α=0.05).

RESULTS AND DISCUSSION

The analysis of variance (ANOVA) performed on the variable conidial yield of the strains on the substrates with an α=0.05 and 95% probability, whose model explained 97% of the variability in the data, which varied 12.30% from their mean (Table 1), indicates that there were significant differences between substrates ($p<0.0001$) and between strains ($p<0.0001$), and the strain*substrate interaction was significant ($p<0.0001$), showing that the type of substrate influences the multiplication of each strain (Table 1). Because the interaction was significant, an ANOVA was performed for each substrate with each Trichoderma spp. strain to determine spore production behavior on each substrate. In the factorial analysis, strain T49 presented the best yield, an average of 1.1E+13 spores per gram of fungus in substrate; on the contrary, strain T58 presented the lowest average yield, 3.56E+12 spores per gram of fungus in substrate (Figure 1).

Yield of Native Trichoderma spp. Strains on Different Substrates

Source of variation	**Sum of Square**	**Mean Square**	**Cuadrado Medio**	**Calculated F**	**p-value**
Model	1.69E+27	35	4.82E+25	63.06	<0.0001
Strains	5.49E+26	5	1.10E+26	143.56	<0.0001
Substrate	6.22E+26	5	1.24E+26	162.71	<0.0001
Strains*Substrate	5.17E+26	25	2.07E+25	27.03	<0.0001
Error	5.51E+25	72	7.65E+23		
Total	1.74E+27	107			
R^2: 0.97; Coefficient of Variation:12.3					

Spore yield behavior of native Trichoderma spp. strains on different organic substrates

Generally, all strains evaluated achieved sporulation greater than 3.6E+12; however, the strain that showed the highest spore production results was strain T49 with 1.1E+13 spores per gram of substrate. However, the strain that showed the lowest yield was strain T58, with spores per gram of substrate. This indicates that spore production depends on the strain type and may also be determined by inherent characteristics, even if they are from the same species. Allori, (2017), Given these similar results, it is evident that the spore yield of a given fungus depends on each strain.

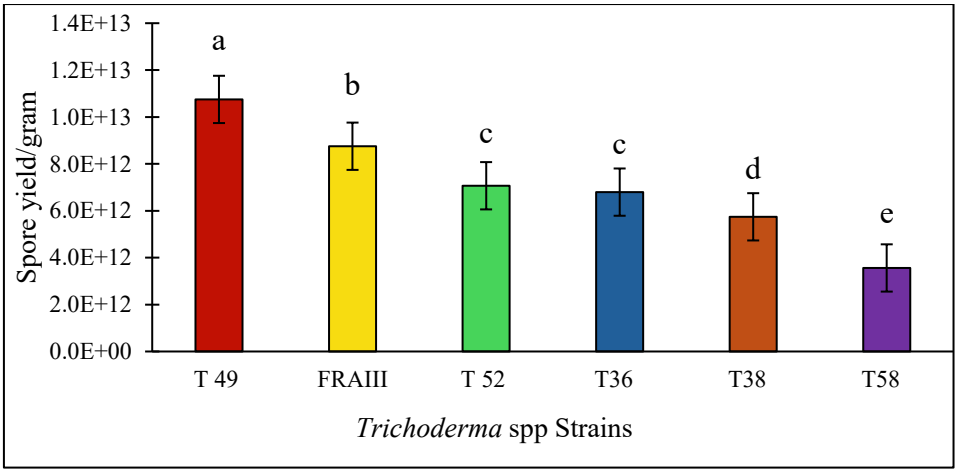

Figure 1: Minimum significant difference in average performance of native Trichoderma spp strains on different substrates according to Tukey's criterion.

Effect of Different Substrates on the Yield of Native Trichoderma spp. Strains

The analysis of variance for the spore yield variable in organic substrates showed significant differences with a high level of variability ($p<0.0001$). The average spore production per gram of fungus in the substrate varied, ranging from 2.9E+12 to 9.2E+12 spores per gram of substrate. This demonstrates that the nutritional composition of each substrate influences the number of spores produced. Some are more beneficial than others. However, all substrates proved suitable for mass production, based on the yields recorded.

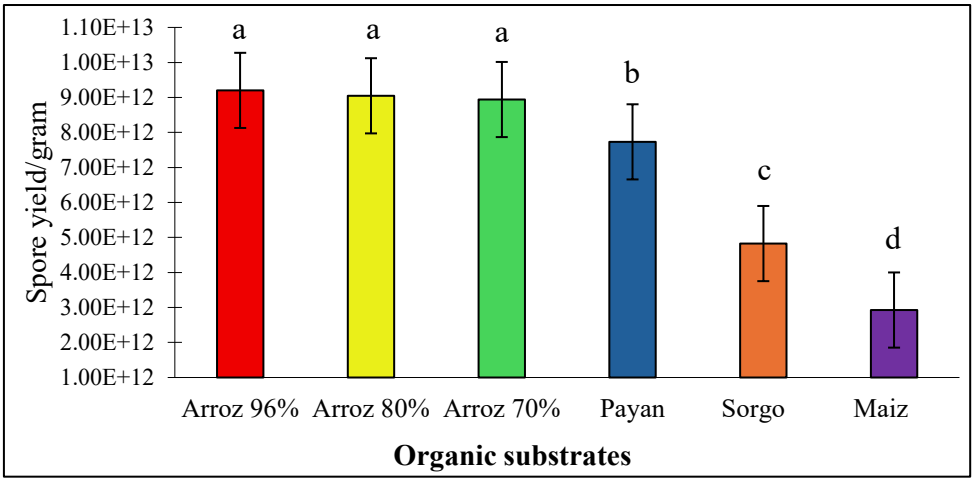

Figure 2: Average spore yield in substrates with different native strains of Trichoderma spp

The four rice-based substrates showed the best results. 96% of whole grain rice was the most favorable for the multiplication of the Trichoderma strains evaluated, with 9.2E+12 spores per gram of fungus in the substrate. This is due to the solidity of this rice and its integrity throughout the mass production process. The corn-based substrate showed the lowest spore yield, with 2.9E+12 spores per gram of fungus in the substrate. This could be due to the grain's hard, fibrous outer layer, also known as the hull. Romero *et al*. (2023) found this peculiarity when comparing several Lecanicillium isolates on various organic substrates, with corn found to be a substrate that discourages fungal spore multiplication.

Generally, the substrate that showed the highest spore production yields per gram of fungus was the 96% whole grain rice substrate. Likewise, 80% whole grain rice and 70% whole grain rice were statistically similar. However, the organic substrate that showed the lowest yield was the corn-based substrate. This indicates that spore production is determined by the substrate type and could also be influenced by the moisture absorption capacity and the adequate amount of nutrients, as well as its carbon-nitrogen ratio (Romero and Castillo-Arévalo, 2023). The growth and sporulation of the substrates evaluated vary in conidia production (Allori, 2017).

Comparative performance effect of six native Trichoderma spp strains on different substrates

The analysis of variance of the yield in number of conidia per gram of substrate indicates that there were significant differences between substrates ($p<0.0001$), between strains ($p<0.0001$) which indicates that the yield

is determined by the type of substrate and by the strain, the following figure shows the behavior of the strains in each substrate, which shows that the substrates that appear less humid discourage sporulation regardless of their nutritional content.

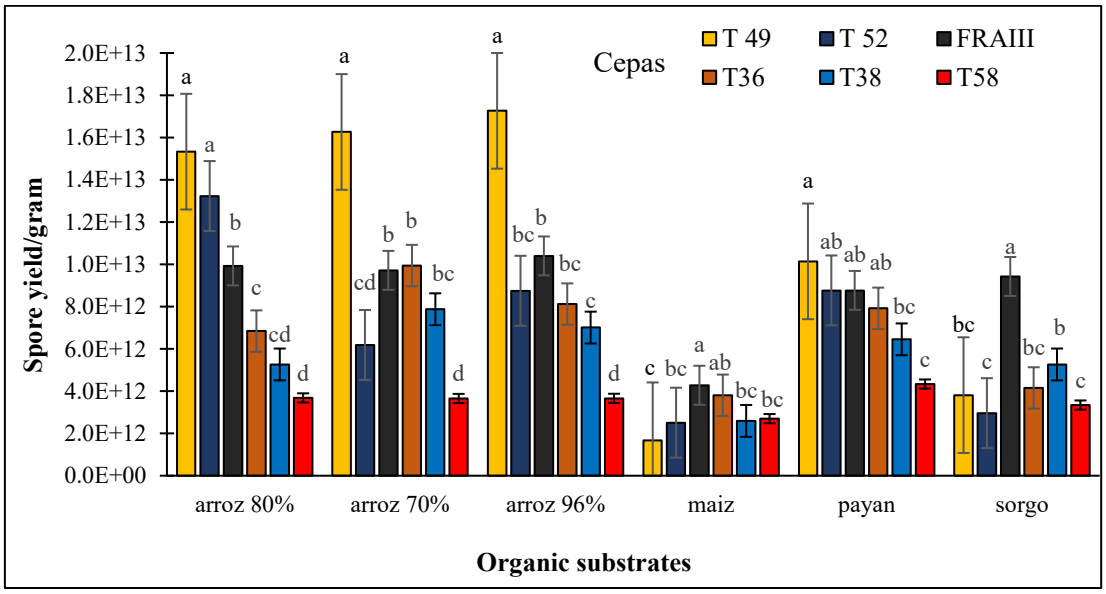

Figure 3: Comparison of spore yields of six native *Trichoderma* spp. strains in different organic substrates.

In our study, the best results were obtained from rice-based treatments of different qualities: 80% whole grain, 70% whole grain, and 96% whole grain. Payán rice (puntilla) even outperformed the other two substrates, sorghum and corn, making it an option for mass multiplication. These results demonstrate that the rice-based organic substrate is the best, based on its results in the study (Figure 3). One characteristic of a good substrate is its ability to absorb moisture throughout the mass production process (Romero, 2020).

The corn-based substrate showed the lowest average spore production per gram of fungus in the substrate. However, the yields found in corn are considered good. The lowest value was found in strain T49, with 1.7E+12 conidia per gram of fungus in the substrate. These results are similar to those found by Michel-Aceves *et al*. (2008), who evaluated different organic substrates and found rice to be one of the best. The native strain T49 showed the highest average yields in all substrates except corn and sorghum, demonstrating that it is a strain with good qualities for use in mass multiplication. Strains FRAII and T52 presented the second-best values and uniformity across all substrates evaluated. In this regard, Romero and Castillo-Arévalo (2023) demonstrated that different Lecanicillium isolates evaluated in different organic substrates had yields determined by the type of isolate and the substrate where they were multiplied. Similarly, Zeledón-Castro *et al*. (2023) corroborate this behavior in an in vitro study where they evaluated 37 native Trichoderma isolates under laboratory conditions, determining that multiplication depends on the type of Trichoderma spp isolate and the nutrient medium where it multiplies.

Spore Viability of Trichoderma spp. Strains on Different Substrates

Conidial viability of Trichoderma spp. strains ranged from 99.94% to 94.75%. The highest viability was recorded by strain FRA3, at 99.94%. The analysis of variance for conidial viability determined significant differences between strains ($p<0.0001$), indicating that the percentage of viability is determined by a particular strain and is an inherent characteristic of each strain (Table 1).

Table 1: Spore germination of Trichoderma spp. strains on substrates at 22 hours

Strains	% viability	Tukey Categories (0.005)
FRAIII	99.94	a
T36	99.93	a
T38	99.83	a
T 49	98.93	ab
T58	98.61	b
T 52	94.75	c

Means with a common letter are not significantly different ($p > 0.05$).

All strains had germination percentages greater than 94.75%. The FRAIII strain was the most promising in this study with a germination percentage of 99.94%. Likewise, strains T36 and T38 were statistically similar. Strain T52 had the lowest viability at 94.75%.

All substrates had viability greater than 96.13%, with 80% whole grain rice being the best substrate with 99.87%. It is evident that the type of substrate does influence viability, according to the study results. Bellino and Marroquín (2013) determined in their study that the best substrate in terms of viability was the sorghum-based substrate, with a viability value of 96%. These results are lower than the findings found in our present study, even though the sorghum-based substrate was the worst, maintaining conidia stability over the duration of the study. In another study conducted by Arévalo *et al*. (2017), they evaluated the optimization of substrates for Trichoderma conidia production and found germination percentage levels above 85%. These results are like those found in our research.

Effect of different organic substrates on conidia viability of Trichoderma spp. strains

All strains meet the requirement of a viability greater than 95% to be used for mass multiplication (Monzón 2001).

Table 2: Effect of organic substrates on the germination of Trichoderma spp strains

Organic substrates	% viability of conidia	Tukey Categories (0.005)
80% whole grain rice	99.87	a
50% whole grain rice	99.32	ab
Corn	99.14	ab
70% whole grain rice	99.12	ab
96% whole grain rice	98.41	b
Sorghum	96.13	c

The effect of different substrates on conidial viability of Trichoderma spp. strains varied and ranged from 99.87% to 96.13%. The analysis of variance performed for conidial viability determined significant differences between substrates ($p<0.0001$), indicating that the percentage of viability, as well as the strain, determines conidia viability (Table 2). Although statistical differences were recorded between the substrates, all possessed good attributes for use in mass multiplication.

CONCLUSIONS

The results show that spore yield is highly influenced by the strain type and substrate used. Strain T49 stood out for its high sporulation capacity, while rice-based substrates, especially the 96% whole grain rice, were the most effective. However, all substrates evaluated, and the different rice qualities managed to produce enough spores to be considered suitable for mass production. Regarding viability, all strains exceeded the quality standards for formulation processes with a viability greater than 94.75%. The FRAIII strain was the most promising with a viability of 99.94%, and the 80% whole grain rice substrate was the most favorable. These findings reinforce the importance of properly selecting both fungal isolation and the substrate to optimize biopesticide production. Furthermore, the study validates the use of biphasic semi-industrial methods as a viable and accessible alternative for Trichoderma spp. production.

Acknowledgments

The authors would like to thank the National Agrarian University for its research support, thanks to the funds provided by the Department of Agricultural Sciences.

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