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Research Article

Evaluation of various substrates for mass production and shelf life of *Trichoderma asperellum*

Rubal Kamboj, Saroj Yadav, J. A. Patil, Ajay Pal, Rohit Kumar

Abstract

Trichoderma species, which belong to the genus *Trichoderma* and the order Hypocreales within the Ascomycota phylum, are highly promising bio-control agents. They are commonly present in natural soil, decaying organic plant matter, and wood, demonstrating remarkable efficacy in combating various plant diseases, including those caused by parasitic nematodes. Being imperfect fungi, the formulation and shelf-life of *Trichoderma* species play vital roles in ensuring their successful utilization in commercial applications. The present study aimed to assess different substrates for mass production and shelf-life of two *Trichoderma asperellum* isolates, FbMi4 and FbMi6. The initial spore count of *T. asperellum* FbMi4 at three concentrations (30, 40, and 50 ml/100 g carrier) on the first day were 226×10^6, 255×10^6, and 291×10^6 spores/ml, respectively. Over a storage period of 60 days, the spore populations gradually decreased to 149×10^6, 169×10^6, and 192×10^6 spores/ml, with reductions in viability of 14.9%, 14.2%, and 14.6% for the respective concentrations. The study also found similar trends in *T. asperellum* FbMi6 and sorghum grains talc-based formulations. Neem cake, an organic amendment, outperformed potato dextrose broth in producing spores, even after 45 days of storage. Remarkably, the formulated solutions maintained a substantial number of viable spores for up to 60 days, indicating their potential for extended shelf-life and storage. These findings emphasize the significance of selecting appropriate substrates to achieve mass production and long-term viability of *Trichoderma* as an effective bio-control agent against various plant diseases, including those caused by parasitic nematodes.

Keywords formulation, fungal bio-agent, mass production, shelf life

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Introduction

The increasing awareness of the detrimental effects of chemical pesticides on crop ecosystems has sparked a rise in interest in using biological control methods to manage plant diseases. *Trichoderma*, a well-known fungal bio-control agent, offers numerous advantages compared to traditional approaches [1]. It has proven effective in combating various foliar and soil-borne plant diseases, including plant parasitic nematodes [2]. One of the major benefits of using *Trichoderma* as a bio-control agent is its positive impact on the environment and agriculture since it does not accumulate in the food chain and poses no threat to humans, animals, or other living organisms [3-5]. *Trichoderma* demonstrates several valuable properties, including its ability to act as an antifungal and nematicidal agent, as well as promoting plant growth and inducing plant defenses [6]. By stimulating systemic defense mechanisms and bolstering immune responses in plants, *Trichoderma* significantly enhances plant resistance.

This multifaceted approach makes it a highly effective bio-control agent. To ensure the commercial success of *Trichoderma* as a bio-control agent, it is essential to consider not only its bio-efficacy and shelf-life but also the availability and affordability of a suitable substrate for its mass multiplication [7-9]. A suitable substrate for multiplication is crucial to support its widespread application and adoption in agricultural practices [10].

Trichoderma species have been successfully cultivated on various grains and organic materials, such as maize, sorghum, pearl millet, wheat, wheat straw, used tea leaves, banana fruit bark, coffee husk, paddy-straw, diatomaceous earth, and earth granules impregnated with molasses [11]. *Trichoderma* is widely recognized for its effectiveness as an eco-friendly fungus that counteracts the negative effects of chemical pesticides [7]. High costs and hazardous impacts associated with chemical pesticides, biological control of plant diseases using *Trichoderma* has become a widely adopted and eco-friendly approach worldwide. To harness the potential of *Trichoderma* for improving crop health in commercial applications, it is essential to develop formulations with suitable carriers that allow the fungus to survive for an extended period [11]. One of the major challenges in utilizing *Trichoderma* as a bio-control agent is the large-scale production of inoculum. The successful commercial use of *Trichoderma* products heavily relies on their formulation and shelf life. The cost of raw materials for the commercial production of bio-control agents is another significant limitation that restricts their widespread use. To address this limitation, researchers have explored various substrates such as neem cake, vermicompost, farmyard manure, sorghum grains, and castor cake for the multiplication of *Trichoderma* [12-14].

In the present investigation, the focus was on screening different substrates to identify the most suitable ones for the multiplication of *Trichoderma asperellum* FbMi4 and FbMi6 under laboratory conditions. By finding cost-effective and efficient substrates for large-scale production, this research aims to contribute to the practical implementation of *Trichoderma* based bio-control strategies in agriculture. This study was carried out in the Department of Nematology under laboratory conditions in the premises of CCS Haryana Agricultural University, Hisar during 2021-2022.

Methodology

Isolation of fungal bio-agents

The fungal bio-agent *T. asperellum* was isolated from soil samples using a potatoes dextrose agar (PDA) medium. The agar plates were then incubated at 26°C for 4 days. After incubation, fungal colonies were selected, purified through streaking, and further incubated at 26°C for 7-8 days. Fungal bodies displaying green conidia were chosen, and microscopic observation confirmed their identity as *Trichoderma*. The isolated culture was preserved on PDA slants for future use and maintenance.

Preparation of the medium

Potato dextrose agar (PDA)

To culture, *T. asperellum*, Potato dextrose agar (PDA) medium was prepared using 200 g of peeled potatoes. These potatoes were cut into small pieces and boiled in 500 ml of water for 20 minutes. After boiling, the extract was collected in a beaker and filtered through a muslin cloth. Agar-agar (20 g) and dextrose (20 g) were added to the extract, and the volume was adjusted to 1000 ml using 500 ml of potato extract and 500 ml of distilled water. The resulting PDA medium was poured into conical flasks and test tubes, which were sealed with non-absorbent cotton plugs. The media-filled containers were sterilized in an autoclave at 121°C for 15-20 minutes to ensure sterility before use.

Preparation of potato dextrose broth (PDB)

Potato dextrose broth (PDB) was prepared and poured in conical flasks; except agar-agar, the same procedure was followed as in PDA preparation. A small quantity of inoculant was transferred to the conical flask containing potato dextrose broth (PDB). Flasks were sealed with cotton plug and kept in BOD at 25 ±2 °C temperature for seven days for fungal growth.

Preparation of PDB talc-based formulation
For the preparation of the bio-control formulation, PDB was used at a volume of 250 ml per 500 ml flask. A 3-day-old culture of *T. asperellum* was inoculated into the PDB medium and incubated at a controlled temperature of 26±2 °C for 15 days. The formulation was prepared by mixing biomass and medium with talc powder (used as a carrier) at concentrations of 30, 40, and 50ml/100g carrier. After drying the mixture, 500 mg of CMC per 100 g of carrier was mixed to enhance its binding capabilities. The final products were packaged in polythene bags and stored at a controlled temperature of 25±2 °C. To determine the population of *T. asperellum*, the formulated product underwent serial dilution technique. The product was diluted to achieve a concentration of 10^{-8}, and then 1 ml of the diluted solution was spread onto sterilized Petri plates containing PDA. The plates were rotated horizontally to ensure even distribution of the inoculum and subsequently incubated at 26±2 °C for 4 days to observe and record the spore count per ml. The efficacy and shelf life of these formulated products were assessed over 2 months.

Preparation of sorghum grain talc-based formulation
The process for formulating *T. asperellum* on sorghum grains was as follows: Sorghum grains were soaked overnight in a 2% dextrose solution. After draining the water, 500 g of sorghum grains were placed in polypropylene bags and autoclaved at 15 lbs pressure per square inch for 15 minutes to ensure sterility. Next, the autoclaved sorghum grains were inoculated with a 5 mm disc of *T. asperellum* and incubated at a controlled temperature of 28±1 °C for 15 days. During this incubation period, the biomass of sorghum grains containing *T. asperellum* was air-dried and then ground into a powdered form. The powdered sorghum grains with *T. asperellum* were mixed with talc powder at different ratios: 10g, 15g, and 20g per 100g of carrier. The formulations were dried at room temperature and 500 mg of Carboxymethyl Cellulose (CMC) per 100 g of carrier was added to improve binding. Finally, the formulated product was packed in polythene bags and stored at room temperature. The population of *T. asperellum* was periodically assessed using the serial dilution technique at 15-day intervals to monitor its effectiveness and viability over time.

Mass multiplication on organic amendments
The different organic amendments, including castor cake, neem cake, farmyard manure (FYM), and vermicompost, were placed in polypropylene bags and sterilized in an autoclave at 15 lbs pressure per square inch for 15 minutes. After sterilization, the bags were left at room temperature for 1 hour before being inoculated with a 5 mm disc of *T. asperellum*. The bags were then incubated at a controlled temperature of 28±1 °C for 15 days. Observations were recorded at 15-day intervals during the 2-month incubation period to monitor the population and viability of *T. asperellum*. The serial dilution technique was used to assess the population of *T. asperellum* in the organic amendments, providing valuable information on its effectiveness and survival over time.

Calculation of colony forming units (CFU) in stored formulations
CFU was quantified in various formulations by suspending serially diluted 1 g of powdered samples derived from different substrates on fresh PDA plates. Afterward, the plates were incubated at a controlled temperature of 26±2°C for 2-3 days. Each plate was replicated three times. The *T. asperellum* population counts on different substrates were repeated every 15 days for 60 days. Throughout the storage period, the powdered formulations were stored in sealed polythene bags at room temperature (25-30 °C).

Results and Discussion

Effect of PDB talc based formulation on shelf life of T. asperellum FbMi4 and FbMi6
Mass production of *T. asperellum* was carried to assess the effects of potato dextrose broth talc based formulation on the shelf life of both the isolates. Data (Table 1) indicated the shelf life and population decline (%) of *T. asperellum* FbMi4 in potato dextrose broth talc based formulations at three different concentrations *viz.*, 30, 40, and 50 ml. Evidently, the initial average number of spores at 30 ml was 226×10^{6} spores/ml and the number of spores gradually declined to 149×10^{6} after 60 days indicating a population

decline of 14.9%. Similar trends were observed in 40 and 50 ml concentrations. Initially average numbers of spores were 255×10^6 and 291×10^6 spores/ml in 40 and 50 ml concentrations, respectively which declined to 169×10^6 and 192×10^6 spores/ml after 60 days indicating a population decline of 14.2% and 14.6% respectively (Figure 1).

Table 1. Shelf life of *T. asperellum* FbMi4 and FbMi6 in PDB talc-based formulations

Storage period (Days)	Spores of *T. asperellum* FbMi4 (× 10^6)			Spores of *T. asperellum* FbMi6 (× 10^6)		
	30ml	40ml	50ml	30ml	40ml	50ml
1	226 (15.0)*	255 (16.0)	291 (17.0)	222 (14.9)	251 (15.8)	290 (17)
15	217 (14.7)	244 (15.6)	278 (16.7)	214 (14.6)	241 (15.5)	273 (16.5)
30	201 (14.2)	225 (15.0)	257 (16.0)	199 (14.1)	223 (14.9)	256 (16.0)
45	174 (13.2)	197 (13.0)	225 (15.0)	171 (13.1)	194 (13.9)	222 (14.9)
60	149 (12.2)	169 (13.0)	192 (13.9)	145 (12.0)	168 (13.0)	191 (13.8)
C.D. at 5%	(0.09)	(0.09)	(0.09)	(0.10)	(0.05)	(0.06)

*Figures in parenthesis are square root transformed

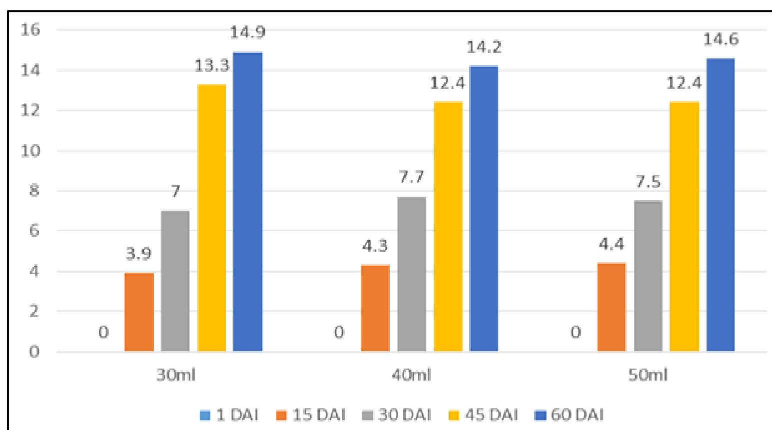

Figure 1. Population decline (%) of *T. asperellum* FbMi4 in PDB talc-based formulations

The average number of spores declined as the storage period increased. Data in Table 2 represent the initial spores of *T. asperellum* FbMi6. The average number of spores at 30 ml was 222×10^6 spores/ml and significantly reduced to 214×10^6 after 15 days indicating the population decline by 3.6% (Figure 2). The number of spores further declined and after 60 days of storage, the population decline reached to 145×106 spores/ml at 30 ml concentrations. A similar pattern was noted for both the 40 ml and 50 ml concentrations. The average spore counts after 60 days of storage were 168×10^6 and 191×10^6 spores/ml for 40 and 50 ml concentrations, respectively. It continued to decline indicating a reduction of 13.4%. A similar trend was observed at 50 ml concentration, the population decline was 13.9% when the storage period was 60 days.

Table 2. Shelf life of *Trichoderma asperellum* FbMi4 and FbMi6 in sorghum talc-based formations

Storage period (Days)	Spores of T. asperellum FbMi4 ($\times 10^6$)			Spores of T. asperellum FbMi6 ($\times 10^6$)		
	10g	15g	20g	10g	15g	20g
1	718 (26.8)*	823 (28.7)	965 (31.0)	711 (26.6)	812 (28.5)	953 (30.8)
15	694 (26.3)	787 (28.8)	914 (30.2)	687 (26.2)	774 (27.8)	902 (30.0)
30	656 (25.6)	741 (27.2)	855 (29.2)	644 (25.4)	727 (26.9)	846 (29.1)
45	595 (24.4)	677 (26.0)	793 (28.1)	581 (24.1)	666 (25.8)	783 (28.0)
60	529 (23.0)	596 (24.4)	715 (26.7)	517 (22.7)	583 (24.1)	704 (26.5)
C.D. at 5%	(0.06)	(0.08)	(0.05)	(0.12)	(0.16)	(0.07)

*Figures in parenthesis are square root transformed

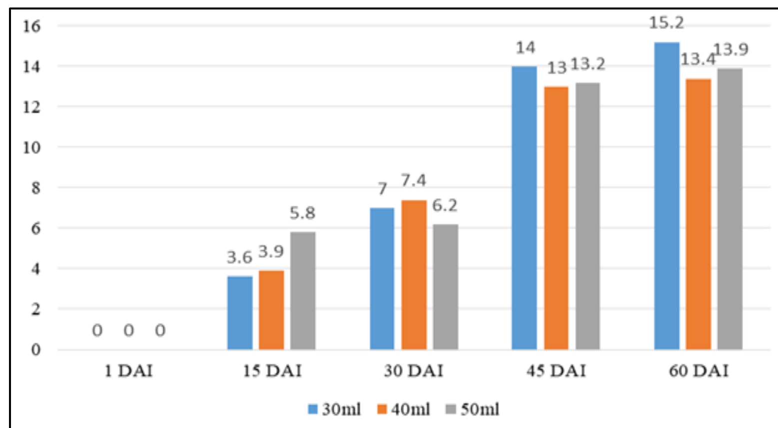

Figure 2. Population decline (%) of *T. asperellum* FbMi6 in PDB talc-based formulation

Shelf life of Trichoderma asperellum FbMi4 and FbMi6 in sorghum talc-based formulations

Shelf life of *T. asperellum* FbMi4 was investigated using talc-based formulations with three different concentrations: 10, 15, and 20 g per 100 g of talc. Data (Table 3) presents the shelf life and population decline (%) of *T. asperellum* FbMi4 in these sorghum talc-based formulations. On the first day, the average spore counts of *T. asperellum* FbMi4 in 10 g, 15 g, and 20 g of sorghum grains per 100 g of carrier were measured at 718×10^6, 823×10^6, and 965×10^6 spores/ml, respectively. It continued to decline as the storage period increased and after 60 days, the number of spores reached to 529×10^6, 596×10^6 and 715×10^6 spores/ml indicating a population reduction of 11, 11.9, and 13.8 % in 10, 15, and 20g, respectively. Table 4 presented data on the shelf life of *T. asperellum* FbMi6 in sorghum talc-based formulations. The results indicated an inverse relationship between the storage period and the average number of spores/ml across all three different concentrations. After 60 days of storage, the initial average spore count per ml at 10g concentration decreased from 711×10^6 to 517×10^8, indicating an 11% decline in population.

Table 3. Effect of different organic amendments on shelf life of *T. asperellum* FbMi4

Treatments	Number of spores/ml			
	15th Day	30th Day	45th Day	60th Day
T. asperellum FbMi4+ Neem cake	446 (21.1)*	363 (19.0)	241 (15.5)	112 (10.6)
T. asperellum FbMi4 + FYM	323 (18.0)	272 (16.5)	182 (13.5)	92 (9.6)
T. asperellum FbMi4 + Vermicompost	420 (20.5)	317 (17.8)	215 (14.7)	92 (9.6)
T. asperellum FbMi4 + Castor cake	425 (20.6)	330 (18.1)	215 (14.7)	104 (10.2)
Potato Dextrose Broth (check)	534 (23.1)	448 (21.1)	395 (19.9)	254 (15.9)
C.D. at 5%	(0.05)	(0.06)	(0.08)	(0.90)

* Figures in parenthesis are square root transformed

Table 4. Effect of different organic amendments on shelf-life of *T. asperellum* FbMi6

Treatments	Number of spores/ml			
	15th Day	30th Day	45th Day	60th Day
T. asperellum FbMi6 + Neem cake	441 (21.0)*	318 (17.8)	224 (15.0)	108 (10.0)
T. asperellum FbMi6 + FYM	318 (17.8)	261 (16.1)	172 (13.1)	88 (9.4)
T. asperellum FbMi6 + Vermicompost	411 (20.2)	312 (17.6)	213 (14.6)	87 (9.4)
Potato Dextrose Broth	521 (22.8)	437 (20.9)	386 (19.6)	249 (15.8)
C.D. at 5%	(0.06)	(0.06)	(0.06)	(0.11)

*Figures in parenthesis are square root transformed

Similarly, at 15 and 20g concentrations, the average spore counts reduced to 583×10^6 and 704×10^6 spores/ml, indicating population declines of 12.4% (Figure 3) and 10% (Figure 4) respectively. In this study, *T. asperellum* FbMi4 showed the highest average spore count, followed by *T. asperellum* FbMi6, both observed at 60 days after inoculation in 20g concentration of sorghum talc-based formulation.

Mass multiplication and shelf life of T. asperellum in different organic amendments

The present study was conducted to evaluate the effect of different organic amendments on the shelf life of *T. asperellum* FbMi4 and FbMi6. The observations were recorded on the 15th, 30th, 45th, and 60th day after inoculation. The data regarding the number of spores of *T. asperellum* per gram of each substrate is presented in Tables 3 and 4.

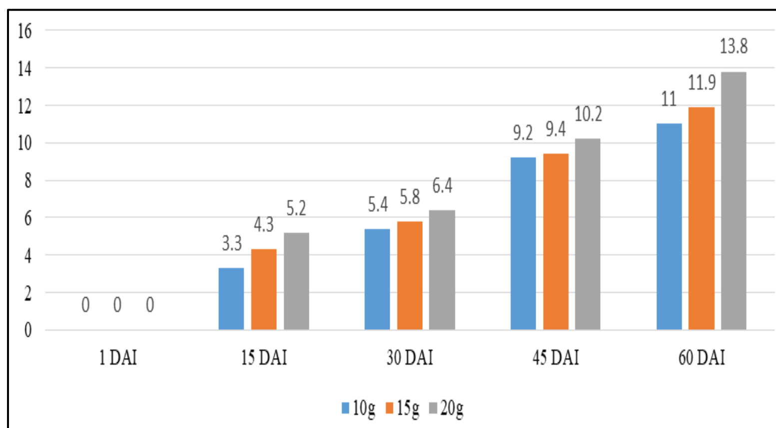

Figure 3. Population decline (%) of *T. asperellum* FbMi4 in sorghum talc-based formulations

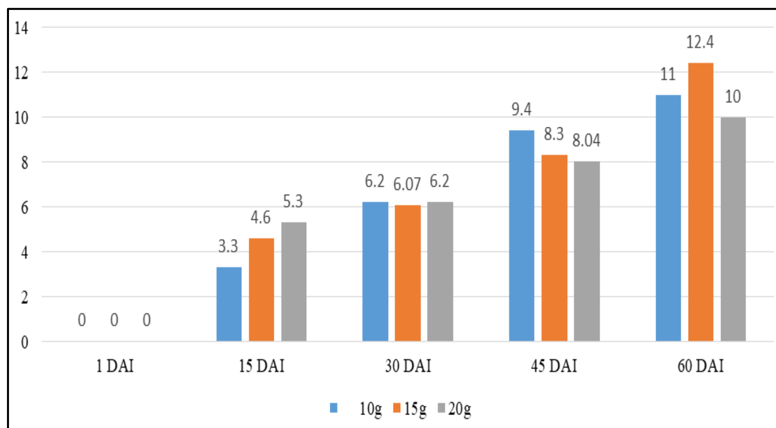

Figure 4. Population decline (%) of *T. asperellum* FbMi6 in sorghum talc-based formulations

The data revealed that among all the organic amendments, maximum and significantly higher numbers of spores were found in *T. asperellum* FbMi4 along with neem cake. Castor cake exhibited the second-highest spore count with 425 spores/ml followed by vermicompost and FYM. Similar trends were observed for *T. asperellum* FbMi6, where the spore counts per ml were 441, 318, 224, and 108/ml after 15, 30, 45, and 60 days, respectively. *T. asperellum* FbMi6 combined with castor cake showed comparable results. The average number of spores/ml was 112 in *T. asperellum* FbMi4 when used in combination with neem cake whereas in the case of FbMi6 along with neem cake, it became 108 spores/ml after 60 days.

The formulation and shelf life of any biological agent is critical for commercial application. Commercial uses of *Trichoderma* rely on the development of commercial formulations with appropriate carriers that allow *Trichoderma* to live for an extended time [15-17]. The current study was carried out to test different substrates for mass production of *Trichoderma* isolates. Data indicated that the average number of spores/ml was decreased 15 days after inoculation. After 60 days, the population of *T. asperellum* decreased drastically. At 210 days, there was a considerable decrease in *Trichoderma* population in talc, tea leaves, wheat bran, and sawdust materials [9]. The growth and sporulation of *Trichoderma* decreased gradually in talc-based formulations [18]. Shelf life of Trichoderma in talc-based formulations decreased steadily over time [19-23]. In the sorghum talc based formulation, the viable number of spores in the formulation was still higher in number as compared to PDB talc based formulation which indicated that the formulation can be stored further. Sorghum grains are believed to retain maximum shelf life due to their rich nutrient composition, well-distributed particles, and high water absorption

capacity [24]. In a study comparing different substrates for mass multiplication of *T. viride* and *T. harzianum*, sorghum grains supported the highest population of both *Trichoderma* species, while sawdust showed the lowest population [25]. Sorghum grains consistently outperformed other substrates in producing a larger population of *Trichoderma* species [11, 24]. The most favorable substrate for achieving the highest spore concentration, spore viability, and biomass production was found to be locally available millet [26]. Moreover, investigations on *Trichoderma* species revealed that their shelf life varied significantly depending on the substrate or medium type and storage temperature [27]. In the present study, the spores of *T. asperellum* FbMi4 and *T. asperellum* FbMi6 reached their peak numbers within 15 days but subsequently declined in all substrates used in the investigation. Indeed, several studies have investigated a wide range of substrates to facilitate the mass multiplication of bio-control agents [28]. These substrates include rice grain, sorghum grain, millet grain, cotton cake, mustard cake, wheat straw, cotton waste, maize, rice straw, sawdust, sugarcane bagasse, sugarcane ash, farmyard manure (FYM), and wheat bran [29-30]. The availability, cost-effectiveness, and suitability of these substrates vary depending on the local context and specific requirements of the bio-control agent being propagated. Understanding the interactions between different substrates and bio-control agents can lead to improved and efficient mass multiplication techniques, contributing to the widespread use of bio-control strategies in agriculture and pest management [31].

Conclusion

The study revealed that the sorghum talc-based formulation exhibited higher viable spore counts at 60 days after inoculation (DAI) when compared to PDB talc-based formulation for both *T. asperellum* FbMi4 and *T. asperellum* FbMi6. Among the various organic amendments tested, neem cake demonstrated the highest efficacy, showing the maximum number of spores in both isolates after 45 days. However, the spore count of both isolates declined significantly in all organic amendments as the storage period increased. These findings highlight the effectiveness of sorghum grains and neem cake as suitable substrates for producing viable inoculum of *Trichoderma*. This inoculum has the potential to be applied in the field to manage soil-borne plant pathogens that are challenging to control through conventional methods. For future research, it is crucial to explore the effectiveness of *Trichoderma* in controlling plant pathogens, develop formulations suitable for dry weather conditions with extended shelf life and field persistence, and scale up solid-state production systems with industry support. Widespread testing of bio-control technology in agricultural fields will further enhance the utilization of *Trichoderma* as a potent bio-control agent for managing plant diseases. These efforts are essential to advance bio-control practices and improve plant disease management in agriculture.

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