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Optimizing *Trichoderma longibrachiatum* Pellets with Spent Mushroom Substrate: A Study on Conidial Viability and Shelf Life

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Abstract

Spent mushroom substrate (SMS), a byproduct of mushroom production, is commonly used as growth media in plant nurseries and horticulture. This study aimed to develop *Trichoderma longibrachiatum* pellets using SMS to extend conidial shelf life and enhance efficiency for biological control. *T. longibrachiatum* strains isolated from paddy field soils in Phayao and Chiang Rai provinces were formulated into pellets using SMS and diatomaceous earth (DE) across four formulations. Each formulation contained viable conidia of *T. longibrachiatum* at a concentration of 2×10^7 conidia/mL of distilled water, with varying ratios of SMS and DE. The pellets were characterized based on weight, diameter, water solubility, and conidial shelf life after storage at 4°C for 60 days. Formulations 2–4 showed a significant increase in weight compared to formulation 1, depending on the percentage of DE. The average pellet diameters ranged from 9.40±0.27 mm (formulation 1) to 10.89±0.65 mm (Formulation 4). Water solubility tests revealed significant differences among the formulations ($P<0.05$). formulation 1 had the longest dissolution time (11.12±0.89 min), while formulation 4 had the shortest (0.26±0.08 min). All formulations maintained conidial viability when cultured on *Trichoderma* selective media (TSM). Based on water solubility and conidial viability, formulation 4 emerged as the optimal formulation for potential use in biological control applications in agriculture.

Introduction

Biological control refers to the use of living organisms, such as antagonistic microorganisms, to manage pests and diseases. Historically, pest and disease control in agriculture has largely relied on chemical-based pesticides due to their convenience and effectiveness. However, the extensive use of these chemicals has led to significant issues, including environmental contamination, decreased biodiversity, pathogen

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resistance, and risks to human health (Zhu et al., 2018). As a result, biological control has gained importance, driven by consumer demand for reduced pesticide use (Martinez et al., 2023; Symondson et al., 2002). In particular, pesticide pollution poses serious health risks, especially to farmers who are in direct contact with these chemicals (Tang et al., 2021).

Among biological control agents, *Trichoderma* species are widely used to combat plant pathogens and promote plant growth (Martinez et al., 2023; Hermosa et al., 2012). *Trichoderma* spp. are highly effective antagonistic fungi, commonly employed in organic farming due to their adaptability to various environmental conditions. These fungi are characterized by their bright green conidia, which are produced asexually and dispersed through conidiophores (Gams & Bissett, 1998). They exert direct biological control through mechanisms such as mycoparasitism, competition, and antibiosis, and indirect control through the induction of systemic resistance and growth promotion in host plants. *Trichoderma* spp. can rapidly colonize and penetrate plant pathogenic fungi, secreting enzymes that inhibit their growth. The efficacy of *Trichoderma* depends on several factors, including soil pH, water retention, temperature, and heavy metal presence (Mukhopadhyay & Kumar, 2020). *Trichoderma* is now widely used in agriculture as a biological control agent. There are many species of *Trichoderma,* but 20 species of the genus *Trichoderma* such as *T. harzianum, T. koningii, T. viride, T. atroviride, T. pseudokoningii, T. longibrachiatum, T. hamatum, T. polysporum* and *T. reesei* are the most important species that act as potential antagonists (Monaco et al., 1991).

Since *T. harzianum* was first registered by the U.S. Environmental Protection Agency in 1989 for plant pathogen control (Fravel, 2005), *Trichoderma* species have become the most widely commercialized biological control agents globally. Commercial formulations of *Trichoderma* products are now available in various forms, including powders, granules, microgranules, encapsulated granules, and liquid emulsions, to improve convenience for farmers to prepare (Martinez et al., 2023). However, challenges remain due to the limited shelf life of these formulations and the potential for uncontrolled germination during storage (Kumar et al., 2014). Fresh *Trichoderma* cultures can be difficult to prepare and may be less effective compared to chemical pesticides. Additionally, some farmers lack awareness of *Trichoderma's* mode of action, which may hinder its adoption. Thus, enhancing the stability, usability, and effectiveness of *Trichoderma*-based products is crucial.

Spent mushroom substrate (SMS), a byproduct rich in organic matter and nutrients, has been widely used in horticulture and plant nurseries. Research indicates that integrating SMS with *Trichoderma* can improve crop yields and reduce plant diseases (Kumar et al., 2022). Therefore, to enhance the long-term effectiveness of *Trichoderma*, the development of suitable storage methods and user-friendly formulations is essential. This research aims to develop *Trichoderma longibrachiatum* pellets from SMS to extend conidia shelf life and improve biological control efficacy.

Materials and methods

1. Strain of *T. longibrachiatum*

Trichoderma longibrachiatum was locally isolated from the paddy field soil in Phayao and Chiang Rai provinces, Thailand and classified based on molecular techniques. It is the most prevalent *Trichoderma* species in these regions. The spent mushroom substrate (SMS) was prepared according to the methodology outlined in petty patent number 2303002039. SMS, the residual substrate after harvesting mushroom fruiting bodies, was sterilized by autoclaving and passed through a 1 mm sieve prior to use.

2. Preparation of *T. longibrachiatum* pellets

The *Trichoderma* strain was cultured on Difco™ potato dextrose agar (PDA) (Difco, Becton, Dickinson and Company, USA) culture medium for 7 days at room temperature (27–28 °C). Conidia of *T. longibrachiatum* were collected from the surface of colonies on a PDA plate by washing with distilled water. The concentration of conidia was determined with a hematocytometer before pellet preparation.

Diatomaceous earth (Sigma-Aldrich, Merk, Germany) was used to collect and protect stored conidia of *T. longibrachiatum* from the surface of colonies on a PDA plate by using a cell scraper (CorningÆ, Merk, Germany), then stored at 4°C in sterile paper bags before being dried and refrigerated at –20°C until further use. Potato starch (Sigma-Aldrich, Merk, Germany) was incorporated to achieve a homogeneous formulation.

Four pellet formulations were prepared, each containing SMS, *T. longibrachiatum* conidia, diatomaceous earth, and potato starch (see Table 1). The SMS, diatomaceous earth, and potato starch were autoclaved and air-dried before use. Pellet formation followed a patented method, wherein the SMS was mixed with diatomaceous earth and starch before adding *T. longibrachiatum* conidia suspended in distilled water. The final conidia concentration was adjusted to 2×10^9 cells/100 mL. The ingredients were homogenized, then formulated into 10 mm diameter pellets (1 g/pellet) using conventional pill-making machines. The pellets were stored at 4°C in sterile paper bags, with 100 pellets/bag.

Table 1 Compositions of pellets

Formulations	Spent mushroom substrate (% w/w)	Diatomaceous earth (% w/w)	Potato starch (% w/w)	Conidia of *T. longibrachiatum* in distilled water (2×10^7 cells/mL)
F1	90	-	10	100
F2	80	10	10	100
F3	70	20	10	100
F4	60	30	10	100

Remark: Each formulation contains 100 pellets (weight: 1.00 g/pellet).

3. Characterization and physical properties of *T. longibrachiatum* pellets

Ten *T. longibrachiatum* pellets were randomly selected from each formulation for characterization. Pellet shape was documented using photography, and the diameter of the dried pellets, after 60 days of storage at 4°C, was measured with a vernier caliper. The weight of the pellets was determined using an analytical balance. Ten pellets from each formulation were weighed immediately after formulation and again after 60 days of storage at 4°C.

The physical properties of the pellets, including water solubility, were assessed based on the solubility of the carrier material, diatomaceous earth. As diatomaceous earth is generally insoluble and inert, its use in formulations can significantly impact on the water solubility of *Trichoderma* products (Chen et al., 2023). To determine solubility, each pellet was dissolved in 1,000 mL of distilled water in a graduated cylinder, and the dissolution time of the pellet carrier was recorded over a 20-min period.

4. Effect of conidia viability on *Trichoderma* selective media

The viability of *T. longibrachiatum* conidia in the pellets was evaluated through an in-vitro recovery test. Pellets from each formulation were assessed both immediately after formulation and after storage at 4°C for 10, 20, 30, 45, and 60 days. Each pellet was placed in the center of a Petri dish containing *Trichoderma* selective media (TSM).

The plates were incubated at room temperature (27–28°C), and the growth and development of *T. longibrachiatum* were observed and photographed. All assays were performed in triplicate. After 2–3 days of incubation, the colonies on the TSM plates were sub-cultured onto potato dextrose agar (PDA) plates to confirm the identity of *T. longibrachiatum.*

5. Statistical analyses

Data were statistically analyzed using one-way analysis of variance (ANOVA). Significant differences among the parameters were determined using Duncan's Multiple Range Test (DMRT) at a significance level of $P<0.05$.

Results and discussion

1. Characterization of the *T. longibrachiatum* pellets

The *T. longibrachiatum* was used for pellets formulation. The pellets contain spent mushroom substrate, diatomaceous earth (DE), starch and conidia of *T. longibrachiatum*. The characteristics of the *T. longibrachiatum* pellets from four formulations showed that all of the *T. longibrachiatum* pellets appeared to be spherical shape and the surface of the pellets was a little rough depending on the percentage of DE in the formulation process (Fig.1).

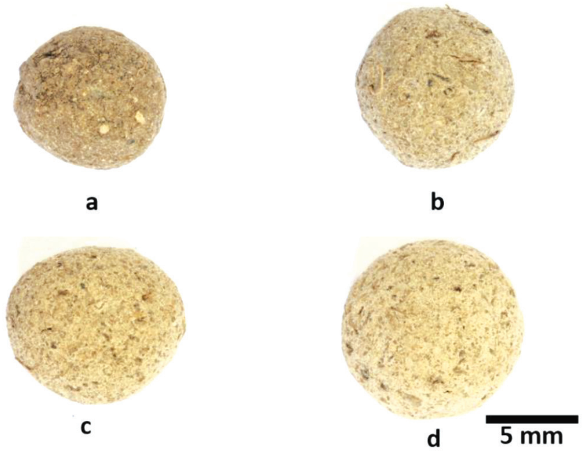

Fig. 1 Characteristics of *T. longibrachiatum* pellets (a: formulation 1, b: formulation 2, c: formulation 3 and d: formulation 4)

Previous studies have demonstrated the important impact of *Trichoderma* based on *Trichoderma* spore suspension (Küçük & Kıvanç, 2005; Kawicha et. al., 2020; Martinez et al., 2023). Based on these studies, the importance of *Trichoderma* conidia suspension on plant growth promotion, defense response and stress tolerance depends on the conidia viability and dose responses of *Trichoderma* spore. Several types of formulations have been developed and have shown a long shelf life when stored at 4-10°C for 6 months up to 1 year depending on the formulation. So, storage of conidia of *Trichoderma* at 4°C showed a greater extension of conidia viability than at room temperature. Therefore, in this study, *T. longibrachiatum* pellets were stored at 4°C and characterized conidia viability for 6 months (Fig. 2).

After 60 days, conidia viability was assessed for each formulation. The pellet color varied depending on the DE content. Formulation 1 (F1), which lacked DE, changed from black to dark brown after 60 days, while formulations 2–4 changed from black to shades of brown and pale brown (Fig. 2). The DE is used as a preservative in grains and in combination with other seed protectants (Ziaee et al., 2021). Several studies have been conducted to combine DE with entomopathogenic fungi to enhance conidial attachment (Abdelgaleil et al., 2021). The decrease in color intensity of formulation 2–4 was due to an increase in the percentage of DE in the formulation. So, optimization of the DE factor is important. The formulation of 40% DE in pellets was difficult to formulate due to its fragility. In this study, the maximum percentage of DE in their formulation process was 30% because it affected the hardness of pellets. The formulation of DE tested in this study apparently had a non-toxic effect on conidia viability similar to that of Luz et al. (2012), who studied the effect of DE on the *in vitro* germination of *Metarhizium anisopliae* conidia without affecting viability.

The weight of the pellets was measured with a digital balance. Ten pellets from each formulation were randomly measured after being formulated and then measured again 60 days after storage at 4°C as presented in Table 2. The results revealed that the pellets of formulations of 1–4, showed no significant differences between the formulations. After being stored at 4°C for 60 days, the results showed a decrease in the average weight of the pellets in all formulations. The reason for

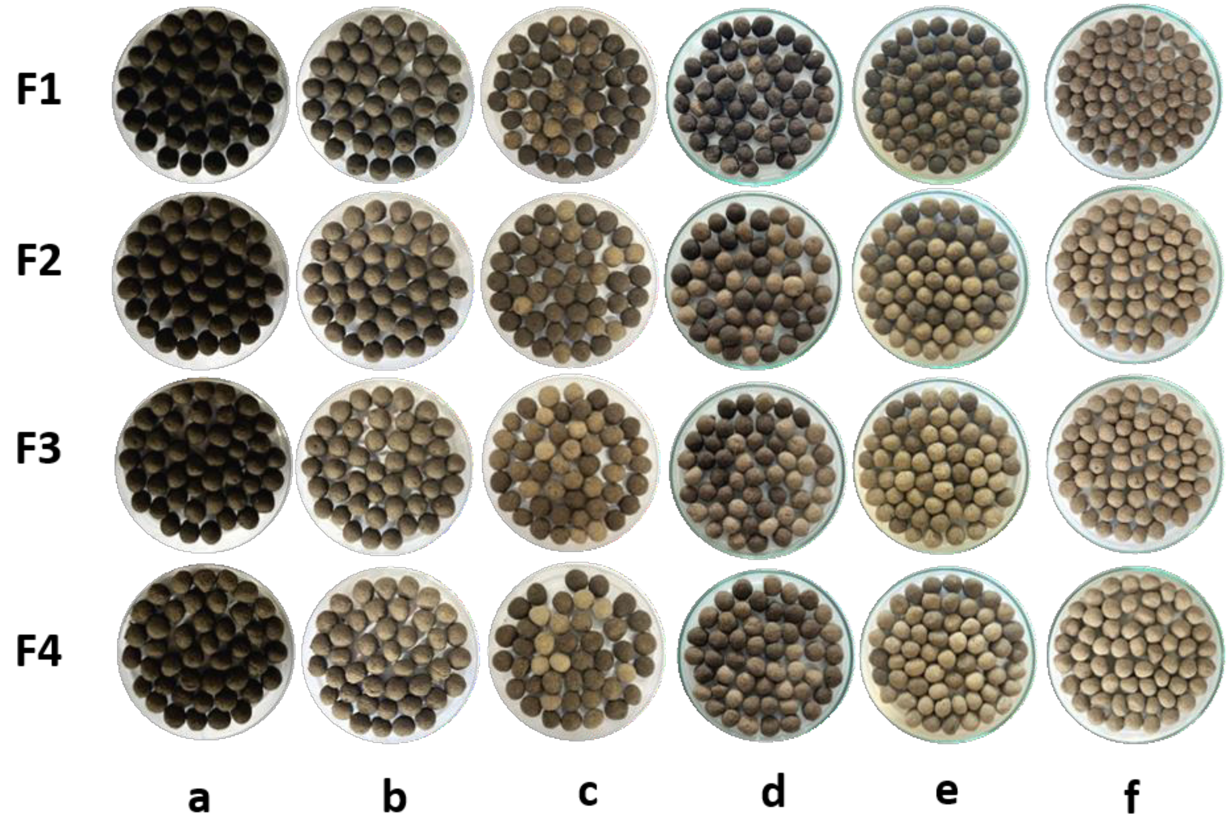

Fig. 2 Characteristics of *T. longibrachiatum* pellets (a: after formulation, b-f: after formulation and storage at 4°C for 10, 20, 30, 45, 60 days, respectively)

these decreases in weight is caused by the evaporation of distilled water in the formulation process. The average weight of *T. longibrachiatum* pellets from formulation 1 was 0.33±0.02 g, showing a significant decrease in pellet weight when compared to the other formulation. This is due to formulation 1 not having a combination of DE in the formulation process. The weight of pellets from Formulations 2-4 showed a significant increase in weight when compared to formulation 1, depending on the percentage of DE ($P<0.05$).

Pellet diameter was measured after 60 days of storage (Table 2). The average diameter for Formulations 1–4 was 9.40±0.27 mm, 10.37±0.32 mm, 10.46±0.48 mm, and 10.89±0.65 mm, respectively. Formulation 4 had the largest diameter, while formulation 1 was significantly smaller than the others ($P<0.05$). This indicates that DE percentage influences both pellet weight and size.

To evaluate the pellets' physical properties in field conditions, water solubility tests were conducted. Solubility is crucial for conidia release and growth in the environment, particularly in rice fields. Ten pellets from each formulation were dissolved in 1,000 mL of distilled water, and dissolution time was recorded. Significant differences in water solubility were observed across formulations ($P<0.05$). Formulation 1 exhibited the longest dissolution time (11.12±0.89 min), while formulations 2, 3, and 4 dissolved in progressively shorter times (1.32±0.14 min, 0.33±0.18 min, and 0.26±0.08 min, respectively). Formulation 4 demonstrated the fastest dissolution, indicating its potential as the optimal formulation for *T. longibrachiatum* pellets. These findings are consistent with those of Sukyai et al. (2020), who demonstrated that water solubility significantly affects the stability of *Trichoderma* products.

Table 2 Physical properties of pellets

Formulations	Weight (g)		Diameter size after storage 60 days (mm)	Water solubility (min)
	After being formulated into pellets^{ns}	After storage 60 days		
F1	1.01±0.04	0.33±0.02^{a}	9.40±0.27^{a}	11.12±0.89^{a}
F2	1.03±0.04	0.35±0.04^{ab}	10.37±0.32^{b}	1.32±0.14^{b}
F3	1.04±0.06	0.36±0.02^{b}	10.46±0.48^{b}	0.33±0.18^{c}
F4	1.05±0.06	0.37±0.04^{b}	10.89±0.65^{c}	0.26±0.08^{c}

Remark: The values are mean ± standard deviation. Means followed by different letter (s) in the same column differ significantly at 5% level of probability, ns: non-significant at 5% level of probability.

2. Characteristics of *T. longibrachiatum* pellets on conidia viability

The conidia viability of *T. longibrachiatum* pellets was assessed by culturing on *Trichoderma* selective media (TSM) for 10, 20, 30, 45, and 60 days at room temperature (27–28°C). Colony morphology was compared to a control plate with purified *T. longibrachiatum* culture (Fig. 3a). The results demonstrated rapid conidia growth, with colonies reaching 8–9 cm in diameter within 3–5 days. Initially, colonies appeared as fluffy white tufts with crusts, which gradually transitioned into light green to dark green, and the reverse side of the colonies turned pale greenish-yellow by day 7–8 (Fig. 3b).

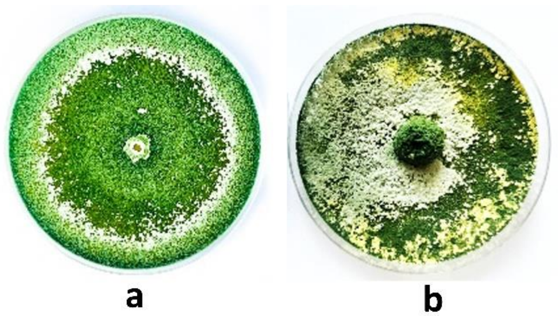

Fig. 3 Character of *T. longibrachiatum* pellet on PDA (a) and TSM (b) media

Following initial growth on TSM, selected colonies were transferred to potato dextrose agar (PDA) plates. On PDA, the conidia from all pellet formulations demonstrated rapid growth at all time points (10, 20, 30, 45, and 60 days) at room temperature (27–28°C), matching the growth patterns of the control culture. This confirmed that all formulations preserved maximum conidia viability for up to 60 days (Fig. 4).

Conidia viability is a key parameter in evaluating the effectiveness of carrier substances used in biological control agents. These carriers must support high survival rates over time, maintain conidia dormancy/viability during extended storage, and be non-toxic to the cells (Martinez et al., 2023). The conidia viability assessment method was adapted from Francisco et al. (2006). Using PDA as a growth medium is advantageous because it promotes conidial germination and facilitates easy visualization of the conidia (Oliveira et al., 2015).

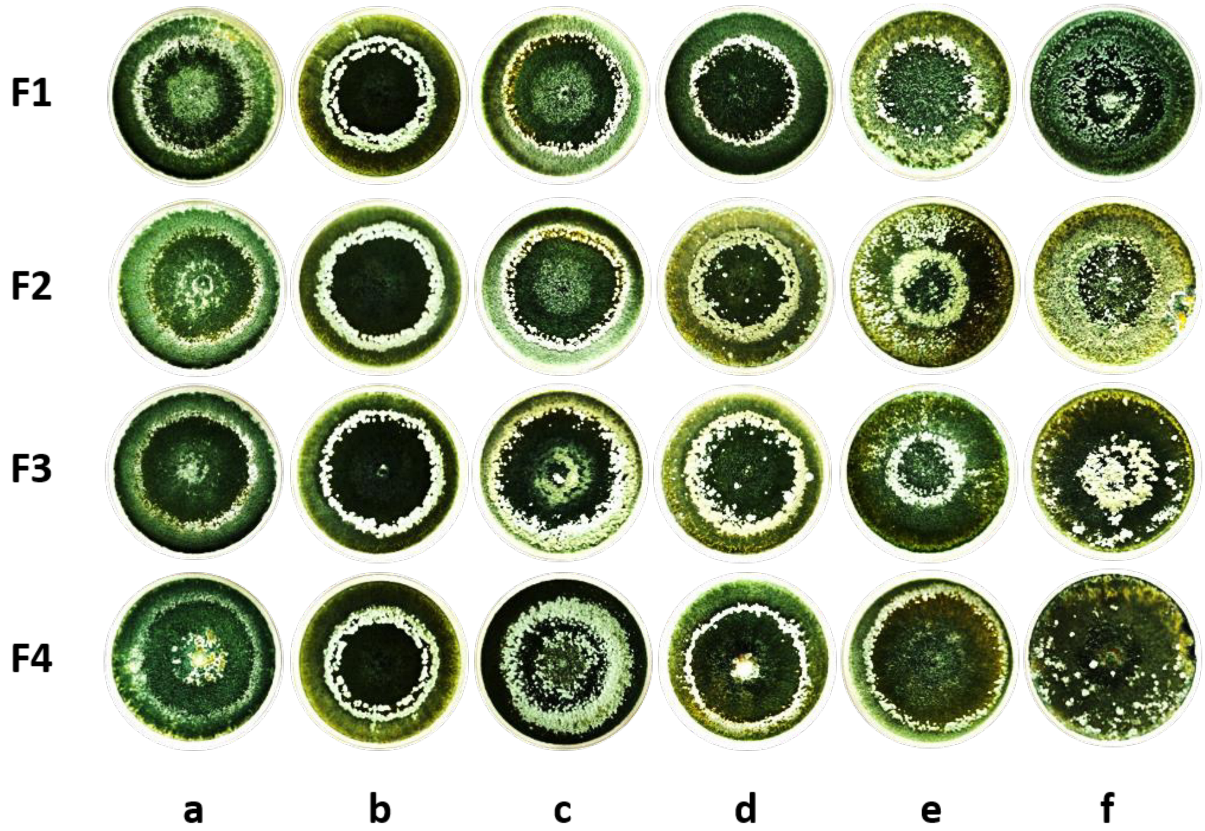

Fig. 4 Colony patterns of *T. longibrachiatum* on PDA from four pellets formulations. (a: after formulation, b-f: after formulation and storage at 4°C for 10, 20, 30, 45, 60 days, respectively)

Conclusion

T. longibrachiatum pellets can be formulated using spent mushroom substrate and diatomaceous earth (DE) in ratios of 0%, 10%, 20%, and 30% (w/w), respectively. These pellets maintain conidia viability for up to 60 days when stored at 4°C. Based on water solubility and conidia viability, formulation 4 was identified as the optimal formulation for pellet production. The ability of *T. longibrachiatum* pellets to maintain conidial properties and extend shelf life makes them a promising method for enhancing plant growth and controlling diseases in agricultural settings.

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