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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Innovative sustainable bioreactor-in-a-granule formulation of *Trichoderma asperelloides*

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Abstract

The advancement of fungal biocontrol agents depends on replacing cereal grains with low-cost agro-industrial byproducts for their economical mass production and development of stable formulations. We propose an innovative approach to develop a rice flour-based formulation of the beneficial biocontrol agent *Trichoderma asperelloides* CMAA1584 designed to simulate a micro-bioreactor within the concept of full biorefinery process, affording in situ conidiation, extended shelf-life, and effective control of *Sclerotinia sclerotiorum*, a devastating pathogen of several dicot agricultural crops worldwide. Rice flour is an inexpensive and underexplored byproduct derived from broken rice after milling, capable of sustaining high yields of conidial production through our optimized fermentation-formulation route. Conidial yield was mainly influenced by nitrogen content (0.1% w/w) added to the rice meal coupled with the fermentor type. Hydrolyzed yeast was the best nitrogen source yielding 2.6×10^9 colony-forming units (CFU)/g within 14 days. Subsequently, $G_{Control}$, $G_{Lecithin}$, $G_{Break\text{-}Thru}$, $G_{Bentonite,}$ and $G_{Organic\ compost+Break\text{-}Thru}$ formulations were obtained by extrusion followed by air-drying and further assessed for their potential to induce secondary sporulation in situ, storage stability, and efficacy against *Sclerotinia*. $G_{Control}$, $G_{Break\text{-}Thru}$, $G_{Bentonite}$, and $G_{Organic\ compost+Break\text{-}Thru}$ stood out with the highest number of CFU after sporulation upon re-hydration on water-agar medium. Shelf-life of formulations $G_{Control}$ and $G_{Bentonite}$ remained consistent for > 3 months at ambient temperature, while in $G_{Bentonite}$ and $G_{Organic\ compost+Break\text{-}Thru}$ formulations remained viable for 24 months during refrigerated storage. Formulations exhibited similar efficacy in suppressing the myceliogenic germination of *Sclerotinia* irrespective of their concentration tested (5×10^4 to 5×10^6 CFU/g of soil), resulting in 79.2 to 93.7% relative inhibition. Noteworthily, all 24-month-old formulations kept under cold storage successfully suppressed sclerotia. This work provides an environmentally friendly bioprocess method using rice flour as the main feedstock to develop waste-free granular formulations of *Trichoderma* conidia that are effective in suppressing *Sclerotinia* while also improving biopesticide shelf-life.

Key points

- *Innovative "bioreactor-in-a-granule" system for T. asperelloides is devised.*
- *Dry granules of aerial conidia remain highly viable for 24 months at 4 °C.*
- *Effective control of white-mold sclerotia via soil application of Trichoderma-based granules.*

Keywords Bioprotectant · Biocontrol agent · *Trichoderma* · Solid-state fermentation · White mold · Granule formulation

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Introduction

White mold, also known as *Sclerotinia* stem rot, caused by the ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most globally destructive diseases that affect soybean growth and yield (Boland and Hall 1994). It is endemic in approximately 28% of soybean production areas in Brazil, causing economic losses of up to US$ 1.47 billion annually (Lehner et al. 2017). This fungus infects the host plants' leaves, flowers, stems, and pods, forming resistant structures known as sclerotia, which can survive in soil or on crop debris for several years (Bolton et al. 2006). The management of *S. sclerotiorum* occurs at several stages of crop development and usually requires multiple methods, including biological control specifically targeting the remaining pathogen's sclerotia in soil, where *Trichoderma* is by far the most successful microorganism (Smolińska and Kowalska 2018; Faria et al. 2022a).

Trichoderma is a well-known genus of soil-borne filamentous fungi that displays multi-beneficial roles in agriculture, including broad biocontrol activity against several plant pathogens, promotion of plant growth, and mitigation of abiotic stresses in plants (Lorito et al. 2010; Hermosa et al. 2012; Rubio et al. 2017; Morán-Diez et al. 2020). Its application, the most sold fungal biofungicide for agriculture, has significantly increased in recent years (Bueno et al. 2020; Meher et al. 2020; Woo et al. 2023), as well as formulations based on them, which can potentially minimize the chemical fungicide/fertilizer inputs in crops (Woo et al. 2014, 2023).

Currently, *Trichoderma* spp. are produced by biphasic fermentation systems, i.e., initially, the inoculum is grown in liquid culture media being transferred to solid substrates (rice, barley, wheat, oat, or millet) for induction of asexual conidiation or simply sporulation that leads to a sheer number of aerial conidia, the main active ingredient for different types of formulation (Li et al. 2010; Jin and Custis 2011; Woo et al. 2014; Mascarin et al. 2019). In this system, the spores' harvesting is required to concentrate the fungal biomass for the formulation process (Faria and Wraight 2007; Li et al. 2010; Mascarin et al. 2019). However, metabolites that harbor antimicrobial properties and/or act as plant stimulants are inevitably lost during this process. Moreover, the solid residue after spore extraction must be appropriately disposed of, e.g., used to produce energy (Elias et al. 2022) or in composting. Growth substrates, generally rice grains, represent up to 50% of production costs (Stanbury et al. 2017). Thus, the increases in paddy rice cost in Brazil, which reached more than 100% between January 2020 and October 2023 (CEPEA - Centro de Estudos Avançados em Economia Aplicada 2023), directly impact the production of fungal biocontrol agents that rely on this cereal grain for their growth. Another concern resides in the social importance of rice as the primary source of energy and protein for more than half of the world's population (Bird et al. 2000).

Thus, agricultural byproducts (wastes) offer a valuable alternative to the use of rice grains, as it allows the reduction of production costs and ensures a noble destination for this residue, especially in Brazil, where many agricultural byproducts are plenty and inexpensive (Esa et al. 2013; Farinas 2015; Soccol et al. 2017). Among them, broken rice, a raw material for rice meal production, represents 10 to 15% of all rice processed. Due to its low cost (about 1/3 to 1/2 of the brown rice price), high availability, and good nutritional value (74% starch and 7% protein) (Liu et al. 2016), several technologies have been proposed to increase its use in the industry (Nakano et al. 2012; Ahmed et al. 2015; Myburgh et al. 2019), including biological control manufacturers (Bich et al. 2018; Jaronski 2014). Grinding broken rice into meal to use as substrate and carrier in formulations can become a simple, inexpensive, and waste-free alternative for developing new *Trichoderma*-based products using the full biorefinery concept, thus avoiding the conidia extraction step while keeping valuable metabolites in the final product.

To this end, we proposed in this study (i) to optimize *T. asperelloides* stationary fermentation in rice flour to sustain high conidial yields; (ii) to develop a rice flour-based granule formulation designed to simulate a bioreactor-in-a-granule prototype capable of supporting high conidiation, extended shelf-life, and effective biocontrol activity against sclerotia of *S. sclerotiorum*; and (iii) to study the influence of storage temperature on the shelf stability of such developed formulations. Considering that *Trichoderma* can hydrolyze starch into simple sugars (Schellart et al. 1976; Asis et al. 2021), the use of starchy products as rice flour for mass production and as a carrier in *Trichoderma*-based formulations may provide a competitive advantage to the competing native soil microbial community, corroborating the concept of a "bioreactor-in-a-granule" system contributing to a sustainable environment and economy.

Materials and methods

Microorganisms

Trichoderma asperelloides strain CMAA 1584 (GenBank accession ON542481), used throughout this study, was isolated from soil collected at Embrapa Environment Experimental Station (22°43′43″ S and 47°01′04″ W), Jaguariúna, SP, Brazil, and was selected based on its ability to control *S. sclerotiorum* (Rezende et al. 2020). For preservation, sporulated colonies were cut into 5-mm-diameter disks and transferred into cryovials containing 1.5 mL sterile solution of 20% (v/v) glycerol (Dinâmica®, Indaiatuba, Brazil)

Table 1 Values of factors and levels (low and high) of the experimental planning according to Plackett–Burman Design 12 (PBD 12)

Factors	Code	Level	
		−1 (low)	+1 (high)
Moisture (%)	X_1	40	60
Inoculum density (conidia g^{-1})	X_2	1×10^5	1×10^6
Substrate weight (g)	X_3	100	150
Fermentor type	X_4	Polypropylene bag	Erlenmeyer flask
Nitrogen content (% w/w)	X_5	0.1	1

prepared with double deionized water and stored at − 80 °C. Frozen stock cultures of this fungus served as the primary source of inoculum to grow it on Potato-Dextrose-Agar (PDA, Acumedia Manufacturers®, Michigan, USA) in Petri dishes (Pleion®, polystyrene, 90 × 10 mm, Barueri, Brazil) at 25 ± 2 °C with 12:12 h photoperiod for 10 days until fully sporulated. This fungal strain has been registered under the Brazilian genetic heritage—Sisgen—number A135E26.

The plant pathogen used in our bioassays was *S. sclerotiorum* strain CMAA 1105 (GenBank accession OM348513), obtained from the Collection of Microorganisms of Agricultural and Environmental Importance (CMAA). *S. sclerotiorum* CMAA 1105 was isolated from tomato plants (*Solanum lycopersicum*) in Jaguariúna, SP, Brazil (22°43′43″ S and 47°01′04″ W). The pathogen was grown on a PDA medium through induction of myceliogenic germination from surface-sterilized sclerotia, and the newly formed sclerotia were stored at 4 °C until use in bioassays.

Optimization of stationary fermentation in rice flour

The optimization of *T. asperelloides* stationary (solid-state) fermentation in rice flour was performed using the statistical experiment design of Plackett–Burman (PBD 12) with two levels (+1 and −1), which allowed to investigate the effect of the following five factors: substrate moisture (%), inoculum density (conidia g^{-1} of substrate), substrate weight (g), fermentor type (polypropylene bags and Erlenmeyer flasks), and nitrogen content (% w/w) (Tables 1 and 2). The experiments were independently repeated three times using different fungal batches.

The stationary (solid-state) fermentation process was carried out and compared based on two fermentation types: 1000-mL Erlenmeyer flasks (Pyrex®, Corning, Barueri, Brazil) versus polypropylene autoclavable bags (35 × 25 cm, 1 L; thickness 0.06 µm). *T. asperelloides* CMAA 1584 was grown on PDA medium at 25 ± 2 °C and 12:12 h photoperiod in a growth chamber. After 10 days, conidia were suspended in 10 mL of a sterile solution containing 0.04% polyoxyethylene sorbitan monooleate (Tween® 80, Synth, Diadema, Brazil). The concentration was adjusted with a hemocytometer under a phase-contrast microscope at × 400 magnification (DM 500, Leica Microsystems GmbH®, Wetzlar, Germany) to provide a final inoculum size of 1×10^5 and 1×10^6 conidia g^{-1} of substrate, according to the inoculum density level (Table 1).

In each trial, an autoclaved urea solution (20% C and 46.6% N, Hexapur®, Amsterdam, Netherlands), previously

Table 2 Effect of moisture, inoculum density, substrate weight, fermentor type, and nitrogen content on colony-forming units of *Trichoderma asperelloides* CMAA 1584 per gram (on wet basis) of rice flour (CFU g^{-1})

Trial	Factors					*T. asperelloides* yield (CFU g^{-1})
	X_1	X_2	X_3	X_4	X_5	
1	1	−1	1	−1	−1	6.7×10^7
2	1	1	−1	1	−1	1.2×10^9
3	−1	1	1	−1	1	2.6×10^7
4	1	−1	1	1	−1	1.1×10^9
5	1	1	−1	1	1	6.9×10^5
6	1	1	1	−1	1	1.8×10^7
7	−1	1	1	1	−1	3.5×10^8
8	−1	−1	1	1	1	8.3×10^7
9	−1	−1	−1	1	1	4.7×10^8
10	1	−1	−1	−1	1	7.5×10^7
11	−1	1	−1	−1	−1	5.0×10^8
12	−1	−1	−1	−1	−1	1.9×10^8

X_1 = moisture (%); X_2 = inoculum density (conidia g^{-1} of substrate); X_3 = substrate weight (g); X_4 = fermentor type (polypropylene bag and Erlenmeyer flask); X_5 = nitrogen content (% w/w)

adjusted to delivery 1 and 0.1% of the substrate weight in nitrogen, was added to the rice flour. The substrate moisture was adjusted to 40 and 60% with sterile deionized water (Table 1) and incubated for 14 days at ambient temperature (26 ± 2 °C) with 12:12 h of photoperiod. Afterward, 1 g of fungus-colonizing substrate was collected to determine the colony-forming units (CFU) by serial dilutions and plating 100 µL aliquots on Petri dishes containing PDA + 0.1% Triton X-100 (Synth®, Diadema, Brazil). Data were expressed as CFU g^{-1}.

Screening nitrogen sources

The impact of different nitrogen sources on the concentration of propagules was evaluated after optimizing the mass production of *T. asperelloides* cultivated in rice flour as the main feedstock. The non-significant factors were kept constant in levels that provided economy [moisture (40%), inoculum density (1×10^5 conidia g^{-1} of substrate), and substrate weight (150 g)]. The significant factors were kept constant at the levels that regarded the highest yield of CFU g^{-1} of substrate [nitrogen content (0.1%) and fermentor type (Erlenmeyer flasks)].

Five nitrogen sources were evaluated: autolyzed yeast (8.0% N, LysCell®, ICC, São Paulo, Brazil), ammonium sulfate (21.2% N, Vetec®, Duque de Caxias, Brazil), corn steep liquor (3.4% N, Ingredion®, Mogi Guaçu, Brazil), cottonseed flour (9.3% N, Pharmamedia®, ADM Co, Decatur, USA), and hydrolyzed yeast (6.6% N, Hilysis®, ICC, São Paulo, Brazil). The propagule production was evaluated after 14 days of incubation and expressed in CFU g^{-1}.

Mass production and granular formulations

Mass production of *T. asperelloides* CMAA 1584 was performed under optimized conditions in rice flour supplemented with hydrolyzed yeast at 0.1% (w/w) (Tables 3 and 4).

Table 3 Estimated values of the effects and *P* values for moisture, inoculum density, substrate weight, fermentor type, and nitrogen content on the colony-forming units (CFU) of *Trichoderma asperelloides* CMAA 1584 per gram (on wet basis) of rice flour

Variable	Effect value	*P* value
Moisture	155,632,778	0.347
Inoculum density	10,132,778	0.951
Substrate weight	− 125,488,333	0.447
Fermentor type	406,710,556	0.018
Nitrogen content	− 477,733,889	0.006

Table 4 Effect of various nitrogen sources on colony-forming units (CFU) of *Trichoderma asperelloides* CMAA 1584 per gram (on wet basis) of rice flour under optimized conditions

Nitrogen sources	*T. asperelloides* yield ($\times 10^8$ CFU g^{-1})
Hydrolyzed yeast	26.2 (± 4.5) a
Corn steep liquor	21.9 (± 4.2) ab
Autolyzed yeast	16.8 (± 3.0) ab
Cottonseed flour	8.7 (± 0.2) bc
Ammonium sulfate	2.6 (± 0.5) c

*Values represent means (± standard error) and when followed by the same letter do not differ significantly from each other (Tukey $P < 0.05$)

Five formulations were prepared using the inoculated rice flour, after mass production, and adding bentonite (Sigma-Aldrich®, St. Louis, MO, USA)—$G_{Bentonite}$, soy lecithin (Quimisul®, Joinville, Brazil)—$G_{Lecithin}$, Break-Thru S301 (Evonik®, Essen, Germany)—$G_{Break\text{-}Thru}$, and Break-Thru S301 with organic compost (Ribumin® C, Technes Agrícola, Guatapara, Brazil)—$G_{Organic\ compost\ +\ Break\text{-}Thru}$, as shown in Table 5. A formulation with only colonized substrate was used as a control treatment. Formulations were processed in an extruder cylinder (CL22, Do Cheff®, Erechim, Brazil) of 2.5 mm thickness to generate the granules (Supplemental Fig. S1). The material was air dried at room temperature inside an exhaust hood (Vidy®, Taboão da Serra, Brazil) equipped with three micro exhaust motors 25 cm above from the formulations (Elco do Brasil®, Taboão da Serra, Brazil). The granules were dried until reaching constant final moisture of 5 ± 1% (w/w), measured with a moisture analyzer (ID-200, Marte Científica®, São Paulo, Brazil).

Storage stability

Each granular formulation was stored in paper bags, without vacuum packaging, and under two conditions (fridge at 4 °C or ambient temperature) to evaluate shelf-life. An aliquot consisting of 1 g of each formulation was sampled at 1, 2, 3, 4, 6, 9, 12, and 24 months after storage and then mixed in a 9 mL sterile solution of 0.04% Tween® 80 (Synth®, Diadema, Brazil) followed by serial dilutions. Next, 100-µL aliquots of the final dilution were plated on Petri dishes containing 20 mL of PDA + 0.1% Triton X-100 (Synth®, Diadema, Brazil) and incubated for 48 to 72 h at 25 ± 2 °C and 12:12 h photoperiod until colonies were visually detectable. For each formulation and evaluation date (1, 2, 3, 4, 6, 9, 12, and 24 months), six independent replicates were performed for each interval, avoiding sample correlation over time. Results were expressed as colony-forming units per gram (CFU/g^{-1}) of dried granules.

Table 5 Composition of granular (G) formulations of *Trichoderma asperelloides* CMAA 1584

Formulations*	Rice (%)	Rice flour (%)	Break-Thru S301 (%)	Soy lecithin (%)	Bentonite (%)	Organic compost (%)
$G_{Control}$	50.0	50.0	–	–	–	–
$G_{Lecithin}$	47.5	47.5	–	5.0	–	–
$G_{Break\text{-}Thru}$	48.7	48.7	2.5	–	–	–
$G_{Bentonite}$	47.5	47.5	–	–	5.0	–
$G_{Organic\ compost + Break\text{-}Thru}$	26.0	26.0	5.0	–	–	43.0

*The final moisture for all formulation reached 5 ± 1% (w/w). Bentonite (Sigma-Aldrich®, St. Louis, MO, USA), soy lecithin (Quimisul® SC, Joinville, SC, Brazil), Break-Thru S301 (Evonik®, Essen, Germany), and organic compost (Ribumin® C, Technes Agrícola, Guatapara, SP, Brazil)

Conidiation of *T. asperelloides* granular formulations after 12 months of storage at 4 °C

Conidiation of *T. asperelloides* was evaluated following concept of a "bioreactor-in-a-granule" system. Samples of 0.1 g of each granular formulation storage at 4 °C were collected, gently ground in a pestle and mortar at room temperature (~ 25 °C) to a fine powder, and then spread over the surface of a 2% (w/v) water-agar medium (Kasvi®, São José dos Pinhais, Brazil) in Petri dishes (20 mL/plate). After incubation at 25 ± 2 °C under 12:12 h photoperiod for 10 days, the entire surface of the plate was washed with 10 mL of a sterile solution containing 0.04% of Tween® 80, and the number of CFU was determined, as previously described. Results were expressed in CFU g^{-1}. The initial CFU g^{-1} of all formulations was determined before incubation and subtracted from those obtained on the 10th day of incubation.

Effectiveness of *T. asperelloides* granular formulations against *S. sclerotiorum* sclerotia

Dark-pigmented mature *S. sclerotiorum* sclerotia were produced in 500-mL Erlenmeyer flasks containing carrots and cornmeal, according to Garcia et al. (2012). Each autoclaved flask received three 5-mm PDA disks of *S. sclerotiorum* mycelium, taken from the edge of a 7-day-old colony and incubated at 25 ± 2 °C. After 30 days of growth on the carrot-cornmeal substrate, mature sclerotia were removed, placed on absorbent paper inside a laminar flow chamber, left to dry for 24 h, and then kept in a refrigerator at 4 °C before being used in the bioassays. Twelve sclerotia were randomly placed in crystal clear polystyrene boxes (11 cm × 11 cm × 3.5 cm) (Gerbox®, J. Prolab Indústria Ltda., São José dos Pinhais, Brazil) containing 200 g of dystroferric dark red latosol previously autoclaved at 121 °C for 60 min on three consecutive days to evaluate the effectiveness of *T. asperelloides* formulation ($G_{control}$) against *S. sclerotiorum* sclerotia. The soil was then inoculated with $G_{Cont.}$ formulation of *T. asperelloides* in the concentrations of 5×10^4, 5×10^5, and 5×10^6 CFU g^{-1} of soil. The boxes were incubated at 25 ± 2 °C with a photoperiod of 12:12 h for 15 days. One of the controls was set up with sterile distilled water and other with conidia suspension of *T. asperelloides* in the concentration of 5×10^6 conidia g^{-1} of soil. Soft and disintegrated sclerotia, due to colonization by *Trichoderma* strains, were counted as degraded after slight pressure with a tweezer (Henis et al. 1983). After 15 days of incubation in those polypropylene boxes, all sclerotia were removed, surface-disinfested with ethanol (70%) and sodium hypochlorite (2%) for 2 min, and subsequently rinsed three times in sterile distilled water. Sclerotia was incubated on Neon medium for 7 days at 25 ± 2 °C to evaluate viability. Viable sclerotia presented the formation of a yellow halo (Napoleão et al. 2006). The test was carried out in a completely randomized design, with three treatments (inoculum size) and four replicates in addition to the control treatments.

After refrigerated storage (4 °C) for 24 months, five viable formulations ($G_{Control}$, $G_{Lecithin}$, $G_{Break\text{-}Thru}$, $G_{Bentonite}$, and $G_{Organic\ compost + Break\text{-}Thru}$) were assessed to evaluate their effectiveness in the concentration of 5×10^6 CFU g^{-1} of soil to inhibit the myceliogenic germination of sclerotia of *S. sclerotiorum* using the same methodology described before.

Statistical analysis

Data generated with Plackett–Burman experiments were analyzed with a Pareto diagram to select the meaningful factors at 5% probability level of significance. Variables were $\log_{10}$-transformed before analysis when necessary to meet homoscedasticity assumptions. Data were submitted to analysis of variance (ANOVA) and comparison of means by Tukey test ($P < 0.05$) to evaluate the effects of nitrogen sources on *T. asperelloides* yield, conidiation, and efficacy against *S. sclerotiorum*. Storage stability data were $\log_{10}(x+1)$ transformed and then fitted to a mixed linear model with normal distribution to assess the formulation, storage time, storage temperature, and their interaction terms on conidial viability, followed by comparison of means by

Tukey test ($P < 0.05$). Statistical analyses were performed using Minitab® software version 19.1 (https://www.malavida.com/en/soft/minitab/) and R statistical software (https://www.r-project.org/).

Results

Selection of critical variables to produce *T. asperelloides* in rice flour

Among the five parameters analyzed in the PBD 12 (Table 2), the nitrogen content and the fermentor type were statistically significant ($P < 0.05$) regarding the *T. asperelloides* yield. The nitrogen content ($P = 0.006$) added to rice flour negatively affected the substrate concentration (Table 3 and Fig. 1). The nitrogen content increase, from level − 1 (0.1%) to + 1 (1.0%) with all other conditions held constant, led to a yield reduction from 1.2×10^9 in trial 2 to 6.9×10^5 CFU g^{-1} in trial 5 (Table 2). Erlenmeyer flasks, used as fermentor type, proved to be more efficient than polypropylene bags ($P = 0.018$; Table 3 and Fig. 1), as the former was associated with a higher number of CFU g^{-1} from 6.7×10^7 in trial 1 to 1.1×10^9 in trial 4 (Table 2). These results are also illustrated in the Pareto diagram (Fig. 1), where bars stretching beyond the reference line are considered significant ($P < 0.05$).

The highest concentrations of CFU were observed in trials 2 (moisture = 60%, inoculum density = 1×10^6 conidia g^{-1} of substrate, substrate weight = 150 g, fermentor type = Erlenmeyer flask, nitrogen content = 0.1%) and 4 (moisture = 60%, inoculum density = 1×10^5 conidia g^{-1} of substrate, substrate weight = 100 g, fermentor type = Erlenmeyer flask, nitrogen content = 0.1%). Under these conditions, *T. asperelloides* produced, on average, 1.2×10^9 and 1.1×10^9 CFU g^{-1} of rice flour after 14 days of cultivation, respectively (Table 2). The lowest CFU production was observed in trials 5 (6.9×10^5 CFU g^{-1}) and 6 (1.8×10^7 CFU g^{-1}) (Table 2).

Screening of nitrogen sources for *T. asperelloides* production in rice flour

Hydrolyzed yeast, corn steep liquor, and autolyzed yeast produced the highest concentrations of *T. asperelloides* (2.62×10^9, 2.19×10^9, and 1.68×10^9 CFU g^{-1}, respectively) after 14 days of incubation, without significant differences among them ($P < 0.05$) (Table 4). Ammonium sulfate attained the lowest CFU yields of *T. asperelloides* g^{-1} from rice flour (2.6×10^8 CFU g^{-1}), differing significantly from the hydrolyzed yeast, corn steep liquor, and autolyzed yeast ($P < 0.05$) (Table 4), but similarly to the production using cottonseed flour (8.7×10^8 CFU g^{-1}).

Storage stability

All three factors together, formulation composition × storage time × temperature, significantly affected the conidial viability of *T. asperelloides* ($F = 267.24$, df = 52, 630, $P < 0.0001$; Fig. 2). Notably, all conidia-based granule formulations lost viability faster when stored at ambient temperature (25 ± 2 °C) but not under cold storage. Of particular interest, formulations $G_{Control}$ and $G_{Bentonite}$ showed the best shelf-life results at ambient temperature (25 ± 2 °C), with no significant drops ($P < 0.05$) of viability after 3 months. At time 0, the formulation $G_{Bentonite}$ had 1.98×10^8 CFU g^{-1}, and after 12 months of storage, the viability dropped to 1.04×10^6 CFU g^{-1}. The viability of $G_{Lecithin}$, $G_{Organic\ compost\ +\ Break\text{-}Thru}$, and $G_{Bentonite}$ formulations was kept high for 24 months when stored at 4 °C (Fig. 2). For these three formulations ($G_{Bentonite}$, $G_{Lecithin}$, and

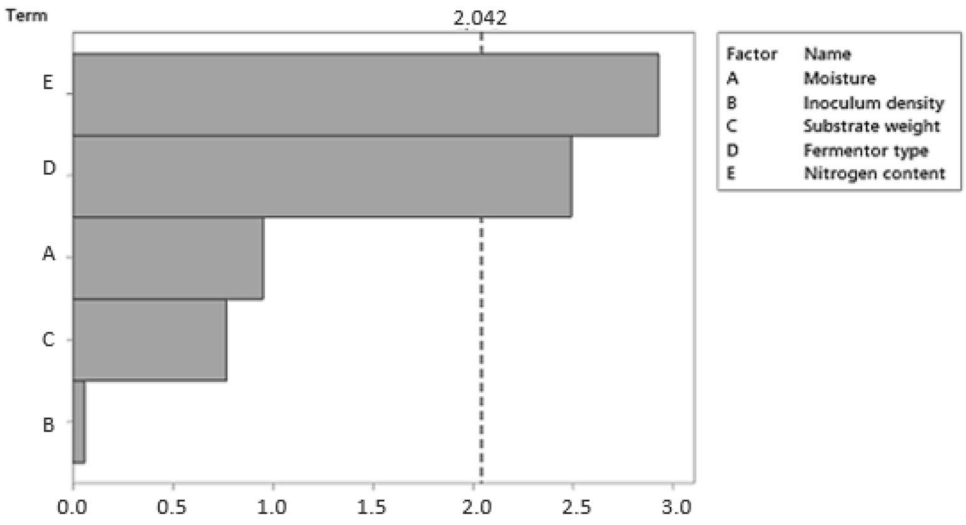

Fig. 1 Pareto diagram ($\alpha = 0.05$) demonstrating the significance of the moisture, inoculum density, substrate weight, fermentor type, and nitrogen content as predictors potentially affecting the viable spore production expressed in colony-forming units (CFUs) of *Trichoderma asperelloides* CMAA 1584 for medium optimization

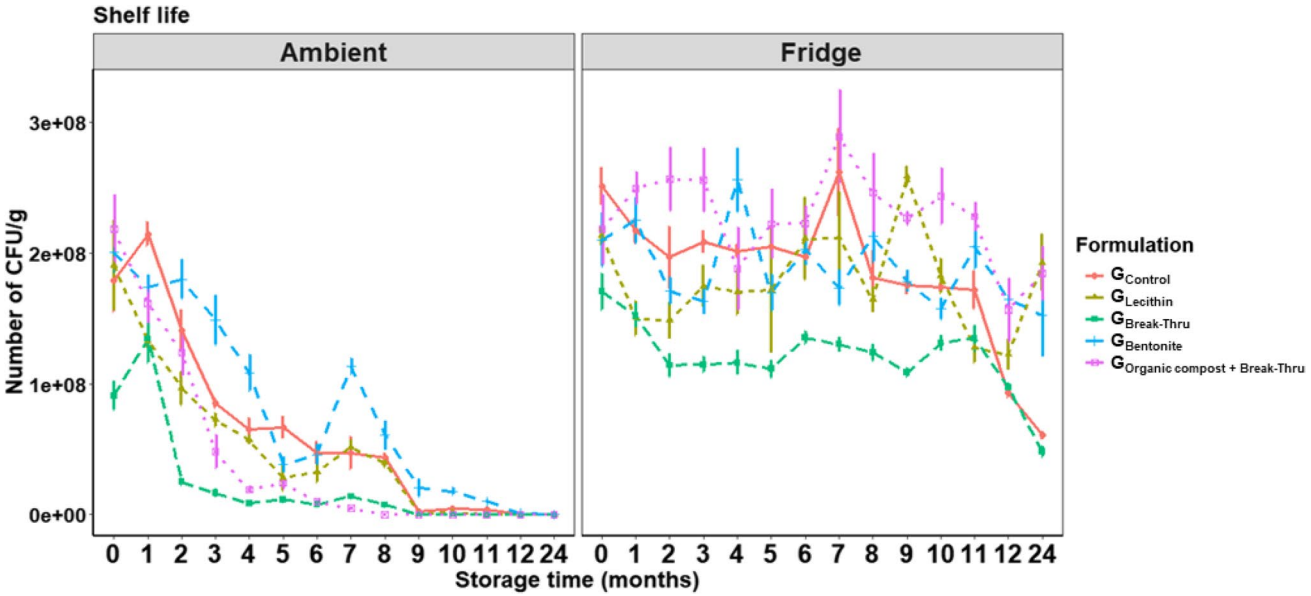

Fig. 2 Shelf-life of *Trichoderma asperelloides* CMAA 1584 formulations stored in the fridge (4 °C) (**A**) and at ambient temperature (25 ± 2 °C) (**B**) for 24 months. Trends represent means (± standard error, $n = 6$)

$G_{Organic\ compost + Break\text{-}Thru}$), the initial viability was 2.06×10^8, 1.48×10^8, and 2.09×10^8 CFU g^{-1} at time 0. After 24 months of fridge storage, the viability was 1.40×10^8, 1.87×10^8, and 1.79×10^8 CFU g^{-1}, respectively (Fig. 2).

All formulations did not retain conidial viability after 12 months of storage at room temperature. However, when kept under cold storage, formulations $G_{Lecithin}$, $G_{Bentonite}$, and $G_{Organic\ compost + Break\text{-}Thru}$ stood out by affording at 24 months higher than 1.5×10^8 CFU g^{-1} in comparison to the other formulations that exhibited less than 1×10^8 CFU g^{-1}. The $G_{Break\text{-}Thru}$ formulation did not show significant differences between storage temperatures and could be stored in both conditions for only 1 month (Fig. 2).

Conidiation of *T. asperelloides* granular formulations after 12 months of storage at 4 °C

The use of starchy compounds as main components in granular formulations of *T. asperelloides* resulted in CFU concentration increase in all formulations, as seen for $G_{Lecithin}$ (from 2.9×10^8 to 5.9×10^9 CFU g^{-1}) and for $G_{Break\text{-}Thru}$ (from 1.6×10^8 to 1.2×10^{10} CFU g^{-1}) (Table 6). It supports the hypothesis that polysaccharides like starch added to the formulation could be used as energy source by this fungus, increasing their viability. Among the five formulations, $G_{Break\text{-}Thru}$ stood out as the one with the highest yields ($P < 0.05$) (1.2×10^{10} CFU g^{-1}), followed by formulations $G_{Organic\ compost + Break\text{-}Thru}$, $G_{Control}$, and $G_{Bentonite}$, resulting in 7.3×10^9, 6.8×10^9, and 6.7×10^9 CFU g^{-1}, respectively (Table 6). The $G_{Lecithin}$ formulation afforded the lowest increments in CFU, increasing from 2.9×10^8 to 5.9×10^9 CFU g^{-1} (Table 6).

Effectiveness of *T. asperelloides* formulations against *S. sclerotiorum*

The number of soft and degraded sclerotia showed significant differences ($P < 0.05$) among the different concentrations of *T. asperelloides* applied. Increasing dosages provided linear increases for this variable, with mean values of 2.1, 8.3, and 23.0%, at 5×10^4, 5×10^5, and 5×10^6 CFU g^{-1} of soil, respectively (Fig. 3A). The conidia suspension (5×10^6 CFU g^{-1} of soil) of *Trichoderma* without formulation and $G_{Control}$ formulation showed similar effectiveness in

Table 6 Conidiation of granular formulation of *Trichoderma asperelloides* CMAA 1584 after 12 months of storage at 4 °C upon re-hydration in 2% (w/v) water-agar-medium and incubation for 10 days at 25 ± 2 °C and 12:12 h photoperiod

Formulations	Conidiation ($\times 10^8$ CFU g^{-1})
$G_{Control}$	68.2 (± 5.3) ab
$G_{Lecithin}$	59.1 (± 11.1) b
$G_{Break\text{-}Thru}$	119.5 (± 16.2) a
$G_{Bentonite}$	67.3 (± 14.0) ab
$G_{Organic\ compost + Break\text{-}Thru}$	73.6 (± 4.6) ab

*Values represent means (± standard error) and when followed by the same letter do not differ significantly from each other (Tukey $P < 0.05$)

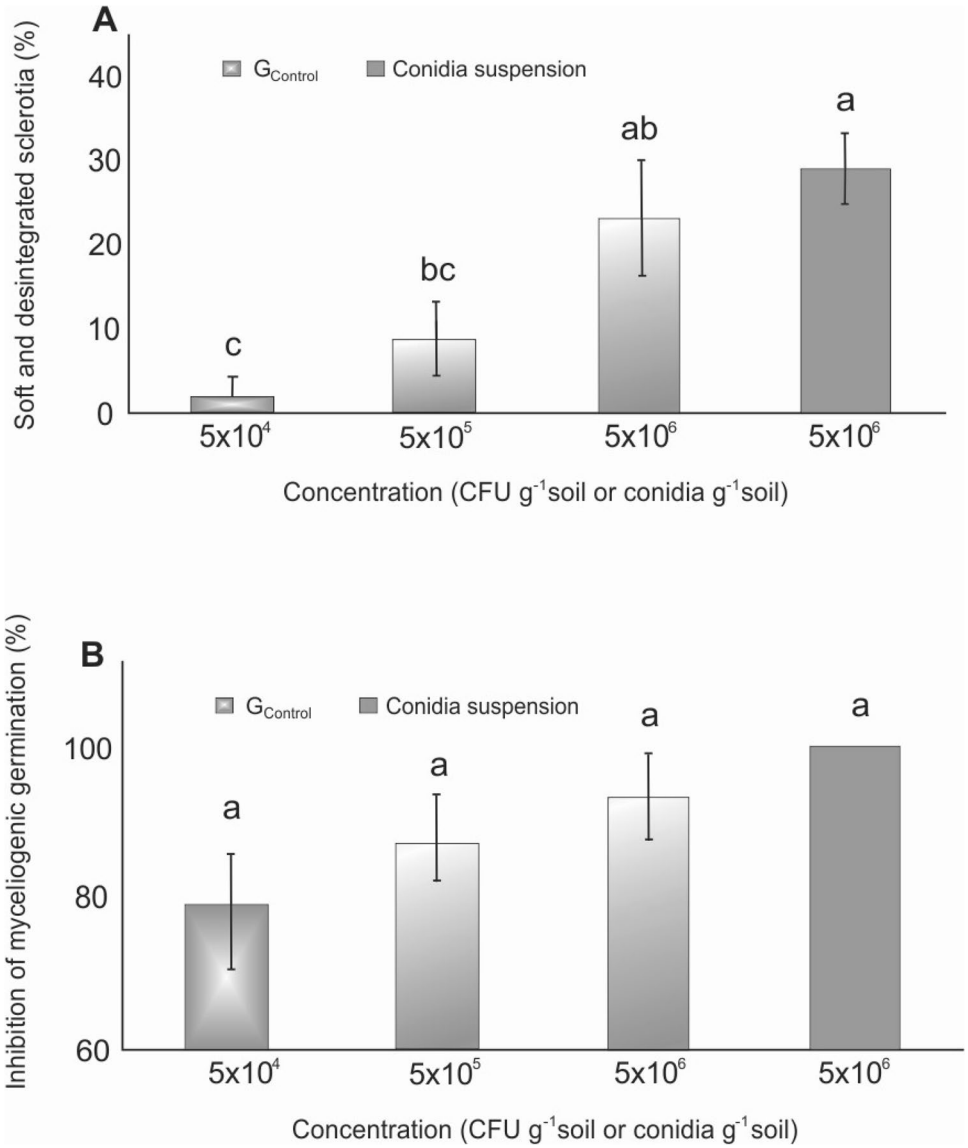

Fig. 3 Soft and disintegrated *Sclerotinia sclerotiorum* sclerotia (**A**) and inhibition of myceliogenic germination (**B**) by *Trichoderma asperelloides* CMAA 1584 at different concentrations applied to the soil. Untreated group (control) is not shown as all sclerotia were healthy and germinated 100%. Bars indicate the mean (± standard error, $n = 4$) and lettering indicates significant differences (Tukey test, $P < 0.05$)

disintegrated sclerotia (Fig. 3A). In addition, *T. asperelloides* formulated in granules ($G_{Control}$) inhibited the myceliogenic germination ($P < 0.05$) of *S. sclerotiorum* sclerotia (Fig. 3B). *T. asperelloides* at 5×10^4, 5×10^5, and 5×10^6 CFU g^{-1} of soil inhibited the myceliogenic germination of *S. sclerotiorum* sclerotia at 79.2, 87.5, and 93.7%, respectively (Supplemental Fig. S2 and Fig. 3B). No significant differences ($P < 0.05$) were observed across *Trichoderma*'s inoculum loads when formulations were applied to the soil surface (Fig. 3B). The high control level observed after applying the conidial suspension is probably due to the better infiltration of *T. asperelloides* conidia in the soil, initiating the infection process faster than the formulated fungus, characterized by a slow release activity.

After cold storage (4 °C) for 24 months, the *Trichoderma* from five formulations ($G_{Control}$, $G_{Lecithin}$, $G_{Break\text{-}Thru}$, $G_{Bentonite}$, and $G_{Organic\ compost\ +\ Break\text{-}Thru}$) evaluated at 5×10^6 CFU g^{-1} of soil significantly inhibited the myceliogenic germination of *S. sclerotiorum* sclerotia (Supplemental Fig. S3), resulting in 100% of effectiveness when compared to sclerotia treated only with water (mock control). This result suggests that these granular formulations of *T. asperelloides* maintained the fungus biocontrol activity after 1-year storage.

Discussion

In this study, a novel approach was developed to streamline the mass production and formulation of *Trichoderma* in a cost-effective and environmentally responsible manner following the full biorefinery approach, resulting in increased conidiation, extended shelf-life, and successful control of a destructive plant pathogen, *S. sclerotiorum*. This innovative

method revolves around a "bioreactor-in-a-granule" system, employing an economical agro-industrial byproduct, specifically rice flour, to generate fungal conidia. Accordingly, the entire fermentation product served as the primary feedstock for obtaining the desired formulation, adhering to the principles of a circular economy and full biorefinery, wherein waste is transformed into a value-added resource. Among the five critical factors scrutinized during the stationary fermentation of *T. asperelloides* grown in rice flour (as detailed in Table 1), it is concluded that the fermentor type and the nitrogen content in substrate played pivotal roles in achieving the highest conidia yields (refer to Table 3 and Fig. 1). Notably, the supplementation of nitrogen at a rate of 0.1% (w/w) resulted in the most substantial concentration of colony-forming units (CFU), underscoring the significance of this nutrient in facilitating fungal growth and conidia production.

Carbon, hydrogen, oxygen, and nitrogen are essential nutrients for microbial growth. Variations in nutrient sources and the carbon-to-nitrogen (C:N) ratio directly impact propagule yield, desiccation tolerance, and morphogenesis driving the formation of various propagule types (Jackson et al. 1991; Verma et al. 2007; Mascarin et al. 2018; Rezende et al. 2020). Nitrogen sources are particularly critical in culture media due to their high added value and nutritional importance for fungal metabolism and growth. However, costs can be reduced by utilizing a wide range of complex organic nitrogen compounds, mostly derived from agricultural commodities, such as vegetable or microbial proteins (Jackson 1997; Mascarin et al. 2018). We observed that complex organic nitrogen sources, including hydrolyzed yeast, corn steep liquor, autolyzed yeast, and cottonseed flour, yielded the highest conidia production when *Trichoderma* was cultivated in rice flour, surpassing the performance of inorganic sources like ammonium sulfate (Table 4).

An essential feature in microbial growth is related to the content of soluble amino nitrogen content and the total amount of free amino acids. Our study investigated four organic nitrogen sources containing 3.4 to 9.3% total N for supplementation in the rice flour substrate. Several other nutrients, such as carbohydrates, organic acids, trace metals, and vitamins, are also found in these organic complex compounds derived from agro-industrial byproducts, and they may play a role as driving factors for the highest yields observed in this study (Mascarin et al. 2018).

We observed impressive conidia yields under our optimized conditions. Specifically, hydrolyzed yeast, corn steep liquor, autolyzed yeast, and cottonseed flour provided yields of 2.6×10^9, 2.1×10^9, 1.6×10^9, and 8.7×10^8 colony-forming units (CFU) per gram, respectively (as detailed in Table 4). These results notably surpassed the findings of Cavalcante et al. (2008), Hewavitharana et al. (2018), and Muniz et al. (2018), who worked with different *Trichoderma* species and various agricultural byproducts as low-cost substrates in solid-state fermentation. In the context of fermentor type, we propose that the superior yields achieved in Erlenmeyer flasks can be attributed to the favorable surface-area-to-volume ratio. Erlenmeyer flasks, therefore, provided a proper surface-area-to-volume ratio, enabling enhanced gas exchange and, consequently, leading to more efficient heat dissipation, as depicted in Fig. 1 and Table 3, in contrast to the polypropylene bag system. Despite this, using Erlenmeyer flasks is not a viable alternative to produce *Trichoderma* on a large scale. However, an adaptation of this model for solid substrate fermentation is feasibly viable, as also reported by Dallastra et al. (2023) that devised a novel pilot-scale tray bioreactor for solid-state fermentation of fungal spores.

One of the primary challenges in developing fungi-based products, such as beneficial microorganisms, is ensuring their prolonged shelf-life (Faria et al. 2022b). Various factors, including the species of the microorganism (Hong et al. 2001), the type of propagules used (Cliquet and Zeeshan 2008), storage temperature (Hong et al. 1999), humidity (Hong et al. 2001), and the choice of packaging system (Faria et al. 2012), individually or collectively, play a critical role in determining the stability of fungal products on the shelf. As expected, room temperature storage was disadvantageous compared to cold storage for *T. asperelloides*, regardless of the formulation tested (Fig. 2). However, given that our formulations were neither vacuum-packaged nor stored under conditions of low relative humidity, a more pronounced decline in conidial viability was confirmed, albeit the opposite trend was seen when these fungal formulations were kept at cold storage. Three ($G_{Lecithin}$, $G_{Bentonite}$, and $G_{Organic\ compost\ +\ Break\text{-}Thru}$) out of five formulations tested here demonstrated excellent shelf stability during the cold storage of *T. asperelloides* conidia, even after 24 months. Implementing appropriate active atmosphere packaging methods, such as vacuum packaging or dual moisture-oxygen scavengers, promises to extend the shelf-life of *T. asperelloides* conidia even under room temperature conditions. This observation aligns with findings related to the sensitivity of other microorganisms, as seen for blastospores and aerial conidia of *Beauveria bassiana* to non-refrigerated storage conditions over several months (Faria et al. 2012; Mascarin et al. 2016). According to Swaminathan et al. (2016), the interplay among formulations, storage temperatures, and relative humidity underscores no universally suitable formulation for storage under all conditions. Notably, changes in relative humidity affect spore survival in some formulations more significantly than in others. They found that among the five formulations of *T. atroviride* tested, the one containing starch was the least influenced by high temperatures and humidity.

Starch is a multi-purpose additive in formulations of biological control agents (BCAs) (Vassilev et al. 2020), which, besides acting as an energy source for microbial cells (Lewis

and Papavizas 1985; Klaic et al. 2018), enhances the stability of the formulation (Przyklenk et al. 2017) by providing protection against thermal, oxidative, and osmotic stresses (Chan et al. 2011; Schoebitz et al. 2012). Starch, after gelation, provides an ideal viscosity for processing, which is adequate for granulation processes (e.g., extrusion, pelletization) as proposed in our research. Moreover, its high capacity of swelling (i.e., water adsorption) makes the medium prone to re-hydrate when in soil, favoring the microorganism growth (Ribeiro and Carmo 2019). The rice flour employed as a substrate and a carrier in granular formulations is not only a feasible substitute to rice grains, but also a waste-free alternative for developing new *Trichoderma*-based formulations within the concept of circular economy. It is important to consider that the utilization as a powder (flour) increases the interaction of rice starch with water, helping in the swelling and in gel formation processes (Klaic et al. 2018; Giroto et al. 2020). Incorporating the substrate (rice flour) in the formulation has the advantage of preserving cellular integrity by reducing mechanical stresses (from the extraction process, not necessary here) and reducing one operation before granulation, saving energy and time. It is noteworthy that, even after 24 months of storage at 4 °C, all formulations kept the effectiveness of *Trichoderma* in inhibiting myceliogenic germination of sclerotia, as well as in parasitizing and disintegrating the sclerotia of *S. sclerotiorum*. These results reinforce the formulation's overall protective effect, especially in terms of environmental stress and oxidation.

The $G_{Bentonite}$ and $G_{Organic\ compost + Break\text{-}Thru}$ formulations yielded the best viability results for storage stability under refrigerated conditions (Fig. 3). Bentonite, a type of clay, is known for its absorptive properties. In this formulation, bentonite might have served multiple purposes. Its porous structure could have helped in retaining moisture, providing a favorable environment for *Trichoderma* to maintain its viability. Additionally, it might have acted as a carrier for other components, aiding in the even distribution of nutrients. According to Stotzky and Burns (1982), soil with concentrations greater than 2% of montmorillonite (clay mineral phase of bentonite) significantly reduced fungal respiration, radial growth, and conidia germination. Montmorillonite is a highly hydrophilic particle with thixotropic properties in suspension, which favors its deposition in a very organized way, making thin films over moist surfaces (Pereira et al. 2012). Furthermore, Lavie and Stotzky (1986a), using scanning electron microscopy, showed that the clay particles were tightly bound to the hyphae of *Histoplasma capsulatum*, suggesting that the clays reduced respiration rate by adhering to the mycelial surface, thereby interfering with the movement of nutrients, metabolites, and gases across the mycelial wall. The reduction of fungal respiration seemed to have been indirectly impacted by montmorillonite through its absorption of an iron-transporting siderophore produced by the fungus (Lavie and Stotzky 1986b). Thus, results obtained in the storage stability of $G_{Bentonite}$ formulation may be due to similar effects in *T. asperelloides* propagules.

Young et al. (2006) reported that adding humic acids increased the survival and storage rates of *Bacillus subtilis* formulated in alginate beds. They can oxidize or reduce elements, photosensitize chemical reactions, and either enhance or retard the uptake of toxic compounds or micronutrients in plants and microorganisms (Nardi et al. 2002; Bacilio et al. 2003). Hence, the authors suggest that the humic acid once entrapped in the beads can benefit plants when the composite is applied to the soil and the microorganism by offering supplementary nutrients during encapsulation. This rationale is consistent with our findings for $G_{Organic\ compost + Break\text{-}Thru}$ formulation, which is rich in humic substances.

Given the growing interest in the use of BCAs and their delivery requirements, which means that the active propagules must reach the target or colonize the habitat (van Lenteren et al. 2018), the use of nutrient sources (or prebiotics) to microbial cells in formulations for the production of new infective propagules is very advantageous. Lewis and Papavizas (1985) obtained similar results in the conidiation of *Trichoderma* formulations by adding starch, in which the number of CFU g^{-1} of soil increased up to 100 times. Furthermore, according to Przyklenk et al. (2017), starch is not fully degraded by *Metarhizium brunneum* after 4 weeks of incubation in Petri dishes, indicating that it cannot only provide long-term nutrients in the field but also contribute to building up the inoculum density of the microorganism in the soil until its total consumption.

In summary, the stationary fermentation of *Trichoderma* spp. in rice flour substrate for its sporulation followed by granulation or extrusion as key component of the downstream processing to obtain a granular formulation offers a straightforward, cost-effective, and environmentally sustainable approach to creating innovative biopesticides using aerial conidia. Furthermore, it is imperative to conduct thorough investigations on the persistence of these formulations in real-world field conditions and assess their effectiveness against a wide range of plant pathogens to unlock the whole potential of this formulation method.

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Declarations

Ethics approval This article does not contain any studies with human participants or animals.

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