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EFFECT OF DIFFERENT SUBSTRATE–INGREDIENT MEDIA FORMULATIONS ON THE GROWTH AND SPORULATION OF *Trichoderma viride* AND *Trichoderma harzianum* UNDER *IN VITRO* CONDITIONS

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ABSTRACT

Developing cost-effective nutrient matrices is essential for the industrial mass production of fungal biocontrol agents. This study assessed fifteen custom agro-industrial substrate formulations (T_1–T_{15}) against standard potato dextrose agar (PDA; T_{16}) to determine their effects on the vegetative colonization and sporulation kinetics of *Trichoderma viride* and *Trichoderma harzianum in vitro*. Radial mycelial spread was recorded over six days, followed by viable colony-forming unit (CFU) assays and hemocytometer-based conidial counts at 13 days after inoculation (DAI).

Formulations T_6–T_{15} significantly outpaced standard PDA in early vegetative expansion, led by T_9 at 3 DAI in both *T. viride* (52.91 mm) and *T. harzianum* (60.05 mm). While complex matrices (T_1–T_5) exhibited an initial enzymatic adaptation lag, all experimental formulations matched or surpassed the control by day 6. *T. harzianum* demonstrated broader metabolic vigor, saturating Petri plates (90.00 mm) across ten media by day 4, compared to only two for *T. viride*. Sporulation assays at 13 DAI revealed an uncoupling between microscopic conidial yield and viable germinability. In *T. viride*, T_{14} provided optimal overall performance, combining near-maximal mycelial coverage with profuse, uncountable viable CFUs and substantial conidial density (56.33). For *T. harzianum*, twelve formulations achieved uncountable CFU yields, with T_1, T_8 and T_9 uniquely sustaining complete colonization alongside top-tier spore densities (≥87.67). These results demonstrate that tailored agro-industrial residues can replace synthetic media, offering a low-cost pathway to formulate robust, high-potency *Trichoderma* inoculants.

Key words : Agro-industrial residues, Biocontrol formulation, Conidiogenesis, Mass multiplication, *Trichoderma* spp.

Introduction

Biological control of soil-borne phytopathogens using antagonistic fungi such as *Trichoderma* is a cornerstone of sustainable disease management. These rhizosphere-competent fungi suppress pathogens through nutrient competition, mycoparasitism, antibiosis and induced systemic resistance (Harman *et al.*, 2004). However, commercial-scale bioformulation requires economical, high-yielding mass-multiplication substrates. Standard semi-synthetic media like Potato Dextrose Agar (PDA) rely on costly purified dextrose and lack the nutritional complexity needed for dense sporulation, often favoring thin vegetative growth over resilient propagule formation.

In filamentous fungi, transitioning from vegetative hyphal extension to conidiogenesis depends on the nature and carbon-to-nitrogen (C:N) balance of the growth matrix (Gao *et al.*, 2007). Readily assimilable sugars in jaggery fuel rapid initial hyphal elongation, whereas complex starches in sago, corn flour and potato starch require inducible enzymatic hydrolysis, sustaining prolonged growth. Combining these carbon sources with organic nitrogen from soybean and lentil flours, alongside B-complex vitamins and trace minerals from yeast extract, establishes a balanced nutrient profile that stimulates phialide differentiation and spore maturation.

Crucially, rapid radial colony expansion does not

guarantee high reproductive output, as abundant carbon can delay sporulation. Moreover, microscopic haemocytometer counts tally all physical conidia regardless of cytoplasmic maturity, whereas Colony Forming Unit (CFU) assays quantify only metabolically viable propagules capable of germination (Jin *et al*., 1996). Given the distinct substrate-utilization efficiencies of *T. viride* and *T. harzianum*, this study evaluated fifteen cost-effective substrates–ingredient solid media formulations against a standard PDA control under *in-vitro* conditions to characterize radial growth kinetics, compare microscopic and viable spore yields and identify standardized media for mass multiplication.

Materials and Methods

The research was executed at the Department of Plant Pathology, Government College of Agriculture, Dhule. Axenic stock cultures of *Trichoderma viride* and *Trichoderma harzianum* were obtained from the departmental culture repository and sustained on Potato Dextrose Agar (PDA) slants at 4°C, with periodic subculturing to maintain physiological viability.

Media formulation

Fifteen customized, cost-effective solid media formulations were evaluated alongside a standard control, Potato Dextrose Agar (T_{16}). The formulations integrated diverse carbon and organic nitrogen matrices including sago/tapioca pearl, lentil powder, soybean flour, corn flour, jaggery and potato starch. Yeast extract was amended to supply micronutrients, B-complex vitamins and essential growth cofactors. Technical-grade agar-agar (15 g/L) served as the structural solidifying base and chloramphenicol (50 mg/L) was incorporated across all formulations to arrest prospective bacterial contaminations. The treatment details are given in Table 1.

The experimental design followed a Completely Randomized Design (CRD) with three functional replications per treatment. Each medium was homogenized in distilled water and sterilized by autoclaving at 121°C and 1.1 kg/cm² pressure (15 psi) for 20 min. Approximately 20 mL of molten medium was aseptically dispensed into sterile 90 mm Borosil Petri plates under a laminar airflow cabinet. Following solidification, uniform 5 mm mycelial plugs cut from the actively expanding peripheral margins of 5-day-old *Trichoderma* cultures were inoculated at the center of each plate. Inoculated plates were maintained in a BOD incubator at 28 ± 1°C.

Assessment of Radial Growth Kinetics and Sporulation capacity

A separate experiment was conducted for each *Trichoderma* species. After testing the media, the formulations showing the highest growth and spore count were selected for further studies (standardized media).

The developmental biology of the fungi was tracked systematically. Radial mycelial expansion was measured dynamically from the third to the sixth day post-inoculation to capture early colonization speeds. Once the vegetative phase concluded, the reproductive output (sporulation efficiency) of each matrix was precisely quantified on the 13th day of incubation. This was achieved through dual methodologies: estimating the overall Colony Forming Units (CFU) and performing direct microscopic haemocytometer conidial counts.

Results and Discussion

Effect of different substrate–ingredient media formulations on the growth and sporulation of *Trichoderma viride* under *in vitro* conditions

The nutrient-matrix variations evaluated in this trial exerted a pronounced influence on the vegetative

Table 1 : Treatment details.

S. no.	Trt.	Ingredients
1	T_1	Tapioca (10g) + Lentil Powder (5g) + Soybean Flour (5g) + Yeast (3g) + KH_2PO_4 (1g) + K_2HPO_4 (1g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (1g) + $ZnSO_4 \cdot 7H_2O$ (20mg) + $MnSO_4 \cdot H_2O$(10mg) + $FeSO_4 \cdot 7H_2O$ (20mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2 .2H_2O$ (150mg) + Agar (15g) + Chloramophenicol (50mg)
2	T_2	Corn Flour (10g) + Lentil Powder (5g) + Soybean Flour (5g) + Yeast (3g) + KH_2PO_4 (1g) + K_2HPO_4 (1g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (1g) + $ZnSO_4 \cdot 7H_2O$ (20mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (20mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2 .2H_2O$ (150mg) + Agar (15g) + Chloramophenicol (50mg)
3	T_3	Jaggery (40g) + Lentil Powder (5g) + Soybean Flour (5g) + Yeast (4g) + KH_2PO_4(1g) + $K_2 HPO_4$ (1g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (1g) + $ZnSO_4 \cdot 7H_2O$ (20mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (20mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2 .2H_2O$ (150mg) + Agar (15g) + Chloramophenicol (50mg)
4	T_4	Potato Starch(10g) + Jaggery(20g) + Lentil Powder (5g) + Soybean Flour (5g) + Yeast (4g) + KH_2PO_4 (1g) + K_2HPO_4 (1g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (1g) + $ZnSO_4 \cdot 7H_2O$ (20mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$

Table 1 continued....

Table 1 continued....

		(20mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (150mg) + Agar (15g) + Chloramophenicol (50mg)
5	T_5	Potato Starch (10g) + Lentil Powder (5g) + Soybean Flour (5g) + Yeast (4g) + KH_2PO_4 (1g) + K, HPO_4 (1g) +$MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (1g) + $ZnSO_4 \cdot 7H_2O$ (20mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (20mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (150mg) + Agar (15g) + Chloramophenicol (50mg)
6	T_6	Tapioca (20g) + Lentil Powder (10g) + Soybean Flour (10g) + Yeast (3g) + KH_2PO_4 (1.5g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H$, O (15mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (100mg) + Agar (15g) + Chloramophenicol (50mg)
7	T_7	Corn Flour (20g) + Lentil Powder (10g) + Soybean Flour (10g) + Yeast (3g) + KH_2PO_4 (1.5g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (15mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (100mg) + Agar (15g) + Chloramophenicol (50mg)
8	T_8	Jaggery (40g) + Lentil Powder (10g) + Soybean Flour (10g) + Yeast (4g) + KH_2PO_4 (1.5g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (15mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2$.2H, O (100mg) + Agar (15g) + Chloramophenicol (50mg)
9	T_9	Potato Starch(15g) + Jaggery(20g) + Lentil Powder (10g) + Soybean Flour (10g) + Yeast (4g) + KH_2PO_4 (1.5g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (15mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (100mg) + Agar (15g) + Chloramophenicol (50mg)
10	T_{10}	Potato Starch(15g) + Lentil Powder (10g) + Soybean Flour (10g) + Yeast (4g) + KH_2PO_4 (1.5g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (15mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (100mg) + Agar (15g) + Chloramophenicol (50mg)
11	T_{11}	Tapioca (30g) + Lentil Powder (15g) + Soybean Flour (15g) + Yeast (3g) + KH_2PO_4 (1g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (10mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (200mg) + Agar (15g) + Chloramophenicol (50mg)
12	T_{12}	Corn Flour (30g) + Lentil Powder (15g) + Soybean Flour (15g) + Yeast (3g) + KH_2PO_4 (1g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (10mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (200mg) + Agar (15g) + Chloramophenicol (50mg)
13	T_{13}	Jaggery (40g) + Lentil Powder (15g) + Soybean Flour (15g) + Yeast (3g) + KH_2PO_4 (1g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (10mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (200mg) + Agar (15g) + Chloramophenicol (50mg)
14	T_{14}	Potato Starch (20g) + Jaggery(20g) + Lentil Powder (15g) + Soybean Flour (15g) + Yeast (3g) + KH_2PO_4 (1g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (10mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (200mg) + Agar (15g) + Chloramophenicol (50mg)
15	T_{15}	Potato Starch(20g) + Lentil Powder (15g) + Soybean Flour (15g) + Yeast (3g) + KH_2PO_4 (1g) + K_2HPO_4 (0.5g) + $MgSO_4 \cdot 7H_2O$ (0.5g) + KCl (0.5g) + $ZnSO_4 \cdot 7H_2O$ (10mg) + $MnSO_4 \cdot H_2O$ (10mg) + $FeSO_4 \cdot 7H_2O$ (15mg) + $CuSO_4 \cdot 5H_2O$ (1mg) + $CaCl_2.2H_2O$ (200mg) + Agar (15g) + Chloramophenicol (50mg)
16	T_{16} (Control)	Potato Dextrose Agar + Chloramophenicol (50mg)

colonization and reproductive behavior of *Trichoderma viride* across the six-day evaluation window (Table 2).

Early vegetative kinetics recorded at 3 days after inoculation (DAI) revealed that formulation T_9 supported the fastest initial expansion, reaching a radial diameter of 52.91 mm. Based on critical difference, treatment T_9 was statistically equivalent (*at par*) to T_{15} (49.89 mm) and T_{11} (49.69 mm). In turn, T_{15} shared parity with T_{11}, T_{12} (49.19 mm), T_{10} (48.68 mm), T_7 (48.08 mm) and T_{14} (47.87 mm). Extending the parity range, T_{11} remained statistically comparable to T_{12}, T_{10}, T_7, T_{14} and T6 (46.40 mm). The slowest initial growth was recorded in formulation T_3 (27.88 mm), which remained statistically on par with T_1 (30.36 mm).

Furthermore, treatment T_1 fell within the same significance bracket as T_2 (30.84 mm), T_4 (31.84 mm) and T_5 (32.09 mm). When benchmarked against the standard potato dextrose agar (PDA) control T_{16} (36.32 mm), media formulations T_6 through T_{15} clearly outpaced the control matrix, whereas formulations T_1 through T_5 lagged behind in early colony spread.

By day 4, vegetative proliferation accelerated markedly in several enriched matrices. Treatments T_{10} and T_{11} both achieved full plate colonization (90.00 mm).

Table 2 : Effect of different substrate–ingredient media formulations on the growth and sporulation of *Trichoderma viride* under *in-vitro* conditions.

S. no.	Treatment	*Linear Growth (mm)				*Spore Count at 13 DAI	
		Day 3	Day 4	Day 5	Day 6	CFU	Haemocytometer
1	T_1	30.36(5.51)	66.44(8.15)	90.00(9.49)	90.00(9.49)	19.67	47.00(6.86)
2	T_2	30.84(5.55)	56.25(7.50)	85.00(9.22)	90.00(9.49)	UC	91.00(9.54)
3	T_3	27.88(5.28)	53.12(7.29)	82.45(9.08)	85.20(9.23)	18.00	55.33(7.44)
4	T_4	31.84(5.64)	58.60(7.66)	84.91(9.21)	88.13(9.39)	UC	6.00(2.45)
5	T_5	32.09(5.67)	62.35(7.90)	79.53(8.92)	80.80(8.99)	UC	45.67(6.76)
6	T_6	46.40(6.81)	79.48(8.92)	90.00(9.49)	90.00(9.49)	14.00	10.67(3.27)
7	T_7	48.08(6.93)	74.37(8.62)	86.15(9.28)	86.61(9.31)	40.00	8.33(2.89)
8	T_8	46.13(6.79)	69.50(8.34)	88.00(9.38)	88.12(9.39)	43.67	58.67(7.66)
9	T_9	52.91(7.27)	86.04(9.28)	86.15(9.28)	86.96(9.33)	13.67	94.00(9.70)
10	T_{10}	48.68(6.98)	90.00(9.49)	90.00(9.49)	90.00(9.49)	47.67	5.00(2.24)
11	T_{11}	49.69(7.05)	90.00(9.49)	90.00(9.49)	90.00(9.49)	39.33	47.33(6.88)
12	T_{12}	49.19(7.01)	76.53(8.75)	87.01(9.33)	87.42(9.35)	9.00	32.67(5.72)
13	T_{13}	39.33(6.27)	68.95(8.30)	90.00(9.49)	90.00(9.49)	31.33	81.00(9.00)
14	T_{14}	47.87(6.92)	84.24(9.18)	90.00(9.49)	90.00(9.49)	UC	56.33(7.51)
15	T_{15}	49.89(7.06)	78.56(8.86)	86.25(9.29)	87.15(9.34)	28.33	71.00(8.43)
16	T_{16} (Control)	36.32(6.03)	55.52(7.45)	69.77(8.35)	75.62(8.70)	39.67	69.44(8.33)
	SEd	0.06	0.12	0.15	0.13	—	0.23
	CD 1%	0.24	0.48	0.58	0.51	—	0.91

Note: *Figures in parenthesis are square root transformed ($\sqrt{n+1}$) values
DAI: Days After Inoculation UC: Uncountable Count CFU: Colony Forming Unit

These top-performing formulations were statistically on par with treatments T_9 (86.04 mm) and T_{14} (84.24 mm). Formulation T_9, in turn, was statistically comparable to T_{14}, T_6 (79.48 mm) and T_{15} (78.56 mm). Formulation T_{14} also shared parity with T_6, T_{15} and T_{12} (76.53 mm). At the lower end of the continuum, T_3 exhibited the most sluggish growth (53.12 mm), remaining statistically at par with control T_{16} (55.52 mm), T_2 (56.25 mm) and T_4 (58.60 mm). Control T_{16} was similarly at par with T_2, T4 and T_5 (62.35 mm). Notably, except for formulation T_3, every tested medium surpassed the standard control formulation at this stage.

By the fifth day of incubation, six formulations T_1, T_6, T_{10}, T_{11}, T_{13} and T_{14} reached ceiling colonization at 90.00 mm. These six formulations formed an extensive statistical group that was at par with T_8 (88.00 mm), T_{12} (87.01 mm), T_{15} (86.25 mm), T_7 (86.15 mm), T_9 (86.15 mm), T_2 (85.00 mm), T_4 (84.91 mm), T_3 (82.45 mm) and T_5 (79.53 mm). Control T_{16} lagged significantly behind all custom media at 69.77 mm, sharing statistical parity only with T_5.

By day 6, T_2 joined the maximum-colonization group, resulting in seven formulations (T_1, T_2, T_6, T_{10}, T_{11}, T_{13} and T_{14}) maintaining complete plate coverage (90.00 mm). These treatments were at par with the remaining custom formulations: T_4 (88.13 mm), T_8 (88.12 mm), T_{12} (87.42 mm), T_{15} (87.15 mm), T_9 (86.96 mm), T_7 (86.61 mm), T_3 (85.20 mm) and T_5 (80.80 mm). The PDA control T_{16} registered the lowest linear growth across the entire test series (75.62 mm), demonstrating statistical parity solely with T_5.

Spore production dynamics measured at 13 DAI presented striking divergences between colony-forming potential and microscopic conidial density. Four formulations T_2, T_4, T_5 and T_{14} yielded profuse, uncountable (UC) colony populations, representing the highest viable propagule yield. Among the enumerable cultures, T10 supported the highest viable count (47.67 CFU), followed by T_8 (43.67 CFU), T_7 (40.00 CFU) and T_{11} (39.33 CFU). Media T_{12} showed the poorest viable propagule yield at 9.00 CFU. In comparison to PDA control T_{16} (39.67 CFU), higher viable yields occurred in T_2, T_4, T_5, T_{14}, T_{10}, T_8 and T_7, whereas the remaining formulations produced lower counts. Direct microscopic enumeration (Hemocytometer conidial counts) revealed that formulation T_9 stimulated the highest absolute spore density (94.00). The formulation T_9 was statistically equivalent (*at par*) to T_2 (91.00) and T_{13} (81.00). Formulation T_2 was also at par with T_{13}, while T_{13} shared

parity with T_{15} (71.00) and control T_{16} (69.44). At the lower tail, T_{10} produced the fewest conidia (5.00), which was statistically at par with T_4 (6.00) and T_7 (8.33). Treatment T_4 was also at par with T_7 and T6 (10.67). Formulations T_9, T_2, T_{13} and T_{15} clearly surpassed the control T_{16}, while all other media yielded lower conidial concentrations.

Effect of different substrate–ingredient media formulations on the growth and sporulation of *T. harzianum* under *in vitro* conditions

T. harzianum displayed a distinct pattern of nutrient responsiveness, characterized by rapid vegetative colonization across most custom formulations (Table 3).

At 3 DAI, early mycelial spread was led by treatment T_9, which recorded a radial diameter of 60.05 mm but it was statistically at par with T_{13} (55.93 mm). Formulation T_{13}, in turn, established a broad parity bracket comprising T_{15} (54.24 mm), T_{10} (54.10 mm), T_{11} (52.50 mm), T_8 (52.46 mm) and T_6 (51.07 mm). Conversely, formulation T_1 yielded the slowest initial spread (34.29 mm), remaining at par with T_2 (35.08 mm), T_5 (35.83 mm), T_4 (36.51 mm) and T_3 (37.85 mm). Comparing initial colony sizes to the PDA control T_{16} (42.10 mm), treatments T_6 through T_{15} grew substantially faster, whereas formulations T_1 through T_5 exhibited slower initial growth.

By day 4, *T. harzianum* had expanded across the entire Petri surface (90.00 mm) in ten distinct formulations: T_6, T_7, T_8, T_9, T_{10}, T_{11}, T_{12}, T_{13}, T_{14} and T_{15}. This leading cohort was closely followed by T_4 (79.25 mm), which was statistically at par with T_3 (78.67 mm). In contrast, control T_{16} recorded the slowest expansion rate (69.61 mm). The control was statistically on par with T_1 (70.83 mm), which in turn was at par with T_2 (72.96 mm) and T_5 (73.13 mm). Crucially, every custom formulation outperformed the PDA control on day 4.

On day 5, the group of formulations supporting complete 90.00 mm colonization expanded to twelve: T_3, T_5, T_6, T_7, T_8, T_9, T_{10}, T_{11}, T_{12}, T_{13}, T_{14} and T_{15}. These twelve treatments were statistically at par with the remaining three custom formulations: T_4 (89.83 mm), T_2 (89.45 mm), and T_1 (89.43 mm). Control T_{16} lagged behind at 85.69 mm, falling well short of every experimental medium.

By the terminal observation at day 6, all fifteen custom media formulations (T_1 through T_{15}) had achieved full plate coverage of 90.00 mm. Control T_{16} remained incomplete at 87.25 mm, establishing that all custom formulations supported faster overall colonization than the standard reference substrate.

Sporulation patterns for *T. harzianum* at 13 DAI showed twelve custom formulations (T_1, T_2, T_5, T_7, T_8, T_9, T_{10}, T_{11}, T_{12}, T_{13}, T_{14} and T_{15}) as well as the PDA control T_{16} developed uncountable (UC) colony densities. Among the three countable cultures, T_6 supported the highest count (90.00 CFU), followed closely by T_3 (86.22 CFU) and T_4 (86.14 CFU). Direct conidial tallies (Hemocytometer conidial counts) identified T_1 as the most prolific sporulation substrate, yielding 92.67 conidia. Formulation T_1 was statistically on par with T_8 (90.00), T_9 (87.67) and T_7 (76.67). Treatment T_8 was similarly at par with T_9 and T_7, while T_9 was at par with T_7 and T_{10} (72.67). The lowest spore count occurred in T_2 (4.67), which was statistically at par with T_{11} (5.33) and T_{15} (8.67). Evaluating these outputs against control T_{16} (48.56), formulations T_1, T_8, T_9, T_7, T_{10}, T_4 (65.33), T_{14} (63.00), T_{13} (62.67) and T_{12} (51.67) demonstrated clear reproductive gains, whereas T_6, T_5, T_3, T_{15}, T_{11} and T_2 suppressed conidial formation.

Developing cost-effective, nutrient-dense growth substrates is essential for industrial-scale bioformulation of *Trichoderma* biocontrol agents. The distinct growth and sporulation behaviors observed here highlight how carbon, nitrogen, and supplementary mineral sources steer fungal metabolism and differentiation.

Vegetative Colonization Dynamics and Mycelial spread

Across both fungal species, custom formulations designated T_6 through T_{15} consistently triggered faster initial colony growth than standard PDA. This trend peaked in formulation T_9, which established the highest initial mycelial spread in both *T. viride* (52.91 mm) and *T. harzianum* (60.05 mm) at 3 DAI. The rapid colony expansion supported by matrices such as T_9, T_{10}, T_{11} and T_{13} can be attributed to the ready availability of easily metabolizable carbon sources combined with an optimal carbon-to-nitrogen (C:N) ratio. Soluble carbohydrates fuel central carbon metabolism, driving adenosine triphosphate (ATP) synthesis and providing the carbon skeletons required for rapid cell wall biosynthesis during apical hyphal extension (Gao *et al*., 2007; Steyaert *et al*., 2010).

In contrast, treatments T_1 through T_5 supported comparatively slower early growth, often falling behind or matching the PDA control during the first 72 hours. This early lag likely reflects the complex, unhydrolyzed polymeric nature of the raw substrates in these mixes. When *Trichoderma* encounters recalcitrant agro-industrial residues, it must first synthesize and secrete an array of extracellular hydrolytic enzymes including cellulases, hemicellulases and chitinases to digest the

Table 3 : Effect of different substrate–ingredient media formulations on the growth and sporulation of *Trichoderma harzianum* under *in-vitro* conditions.

S. no.	Treatment	*Linear Growth (mm)				*Spore Count at 13 DAI	
		Day 3	Day 4	Day 5	Day 6	CFU	Haemocytometer
1	T_1	34.29(5.86)	70.83(8.42)	89.43(9.46)	90.00(9.49)	UC	92.67(9.63)
2	T_2	35.08(5.92)	72.96(8.54)	89.45(9.46)	90.00(9.49)	UC	4.67(2.16)
3	T_3	37.85(6.15)	78.67(8.87)	90.00(9.49)	90.00(9.49)	86.22	15.33(3.92)
4	T_4	36.51(6.04)	79.25(8.90)	89.83(9.48)	90.00(9.49)	86.14	65.33(8.08)
5	T_5	35.83(5.99)	73.13(8.55)	90.00(9.49)	90.00(9.49)	UC	28.67(5.35)
6	T_6	51.07(7.15)	90.00(9.49)	90.00(9.49)	90.00(9.49)	90.00	36.00(6.00)
7	T_7	47.99(6.93)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	76.67(8.76)
8	T_8	52.46(7.24)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	90.00(9.49)
9	T_9	60.05(7.75)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	87.67(9.36)
10	T_{10}	54.10(7.36)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	72.67(8.52)
11	T_{11}	52.50(7.25)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	5.33(2.31)
12	T_{12}	49.02(7.00)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	51.67(7.19)
13	T_{13}	55.93(7.48)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	62.67(7.92)
14	T_{14}	50.32(7.09)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	63.00(7.94)
15	T_{15}	54.24(7.37)	90.00(9.49)	90.00(9.49)	90.00(9.49)	UC	8.67(2.94)
16	T_{16} (Control)	42.10(6.49)	69.61(8.34)	85.69(9.26)	87.25(9.34)	UC	48.56(6.97)
	SEd	0.09	0.05	0.02	0.02		0.23
	CD 1%	0.36	0.19	0.10	0.10		0.91

Note: *Figures in parenthesis are square root transformed ($\sqrt{n+1}$) values.
DAI: Days After Inoculation UC: Uncountable Count CFU: Colony Forming Unit

matrix into assimilable monomeric sugars (Druzhinina *et al.*, 2011; Kubicek *et al.*, 2014). This necessary physiological adaptation phase delays visible hyphal extension.

Once these catabolic enzyme cascades were operational, however, vegetative growth accelerated rapidly. By day 5 and day 6, cultures on T_1 through T_5 had largely caught up with the rest of the cohort, reaching statistical parity with the fully colonized plates (90.00 mm). Both *Trichoderma* species consistently outpaced the PDA control on almost every custom formulation, confirming that standard synthetic media lack the complex nutrient diversity and micronutrient balance needed for maximal vegetative vigor (Sangle *et al.*, 2007; Singh *et al.*, 2014).

A clear inter-specific difference emerged from the growth curves: *T. harzianum* was consistently more robust and rapid in vegetative spread than *T. viride*. By day 4, ten separate custom media formulations had already reached full coverage (90.00 mm) in *T. harzianum*, whereas *T. viride* reached that ceiling in only two formulations (T_{10} and T_{11}). This faster colonization highlights the broader enzymatic machinery and higher substrate-utilization efficiency commonly documented in *T. harzianum*, traits that underpin its competitive saprophytic ability in rhizosphere colonization (Bissett, 1991; Harman *et al.*, 2004).

Sporulation Kinetics

A central finding of this investigation is the clear physiological uncoupling between vegetative biomass expansion, microscopic conidiogenesis, and viable colony emergence. In *T. viride*, treatment T_{10} yielded the fastest radial growth (90.00 mm by Day 4) and a respectable viable CFU yield (47.67 CFU), yet produced the lowest hemocytometer spore density (5.00). Conversely, T_9 generated the highest hemocytometer spore tally (94.00) while yielding a modest 13.67 CFU. In *T. harzianum*, treatment T_1 yielded poor early radial expansion (34.29 mm at Day 3) but supported both uncountable CFU viability and the highest hemocytometer count (92.67). Meanwhile, formulations T_2, T_{11} and T_{15} reached complete vegetative colonization and uncountable CFU levels, yet produced negligible hemocytometer spore tallies (< 9.00).

These divergent trends illustrate that high conidial counts under a hemocytometer do not guarantee high germination competence or viable CFU formation. Hemocytometer counts tally all physical conidia produced

in the matrix, regardless of viability, cell wall maturity or endogenous reserve content. In contrast, CFU assays measure only those propagules capable of surviving harvesting, suspension, and subsequent germination on re-isolation plates (Papavizas, 1985; Jin *et al*., 1996a).

The composition of the substrate directly shapes these reproductive dynamics. Conidiogenesis in *Trichoderma* is typically triggered by local nutrient depletion, carbon starvation, or specific light and physical cues (Schuster *et al*., 2012; Schmoll *et al*., 2016). Formulations rich in readily available, unbuffered sugars can maintain prolonged vegetative hyphal growth, thereby delaying or repressing the sporulation cascade. Conversely, substrates providing sustained, slow-release nutrient flows or those that experience balanced nitrogen depletion trigger robust conidiophore formation and yield mature, robust phialoconidia equipped with thick chlamydospore-like walls, dense lipid reserves and high viable germination rates (Munoz-Centeno *et al*., 1996; Lewis *et al*., 1998; Agosin *et al*., 1999).

The uncountable CFU values recorded in formulations such as T_{14} for *T. viride* and across twelve formulations for *T. harzianum*, suggest that these substrate matrices supplied an optimal balance of complex carbohydrates, bioavailable nitrogen and essential trace minerals (such as magnesium, zinc and iron). These nutrients are crucial cofactors for both conidiophore differentiation and the metabolic resilience of dormant spore reserves (Jackson *et al*., 1996; Steyaert *et al*., 2010).

Implications for Mass production and Formulation selection

From a commercial perspective, identifying a single production medium requires balancing rapid colonization, high spore yield, and long-term propagule viability. For *T. viride*, formulation T_{14} stands out as a premier candidate: it supported near-maximal vegetative spread (84.24 mm by day 4; 90.00 mm by day 5), produced uncountable viable CFUs and maintained a strong hemocytometer spore count (56.33). Formulations T_2 and T_9 also showed strong potential, with T_9 excelling in total conidial density and T_2 delivering high viable CFU numbers. For *T. harzianum*, formulations T_1, T_8 and T_9 proved exceptionally effective. Each supported complete vegetative colonization, produced uncountable viable colony-forming units and generated top-tier hemocytometer spore counts (92.67, 90.00 and 87.67, respectively).

Standardizing mass-multiplication media using these high-performing agro-industrial matrices offers a dual advantage: it significantly lowers raw ingredient costs relative to synthetic broths while yielding robust, stress-tolerant bio-inoculants capable of establishing effectively in agricultural soils.

Conclusion

Tailored substrate matrices markedly outpaced standard Potato Dextrose Agar in driving both vegetative colonization and reproductive output, though radial expansion rates routinely decoupled from conidiogenesis across both biocontrol agents. *T. harzianum* exhibited greater nutritional versatility and accelerated spatial colonization compared to *T. viride*, yet each species displayed distinct media dependencies for spore production. Notably, formulation T9 served as the most effective universal matrix by securing rapid early colony spread alongside peak microscopic conidial densities in both species. Beyond this dual performer, formulation T_{14} proved optimal for maximizing viable propagule yield in *T. viride*, whereas formulations T_1 and T_8 uniquely triggered maximal conidial induction in *T. harzianum*, establishing these custom substrates as high-efficiency replacements for conventional media in bioprocess scale-up.

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