

The Effect of *Trichoderma harzianum* and its Mutant Isolate on Biological Control of *Pythium ultimum* in Lettuce (*Lactuca sativa* L.)

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In this study, we induced mutations in the genome of *Trichoderma harzianum* (NAS-T101) via gamma irradiation to enhance its antagonistic potential against *Pythium ultimum*. Efficacy testing of mutants and the wild type was conducted in a completely randomised design (CRD) with four replications in the greenhouse on lettuce (Grand Rapid cultivar) at the Nuclear Agriculture Research Institute collection (NSTRI, AEOI) in spring 2018. The experiments included plants treated with *T. harzianum* NAS-T101 (non-irradiated) and its selected mutant TM4 against the *P. ultimum* pathogen, and the control plant. Disease incidence (DI), morphological traits (length and dry weight of shoots and roots), and physiological traits (total protein, peroxidase, polyphenol oxidase, chlorophyll *a* and *b*, and malondialdehyde activity) were measured. The results showed that *T. harzianum* (NAS-T101 and TM4) significantly reduced DI and developed the plant root system. *T. harzianum* (NAS-T101 and TM4) treatments increased the accumulation of proteins of molecular weight associated with peroxidase and polyphenol oxidase enzymes, enhanced their activity, and led to a significant increase in chlorophyll *a* and *b*. The TM4 treatment showed a significant increase in antioxidant enzyme activities and protein accumulation compared to the control. This study showed that the mutant (TM4) was significantly more efficient at biocontrol of *P. ultimum* than its parental, un-irradiated isolate, and that lettuce plants treated with TM4 maintained morphological and physiological processes under disease conditions.

Keywords: *Trichoderma harzainum*, *Pythium ultimum*, biological control, gamma irradiation

Lettuce (*Lactuca sativa* L.) is a leafy plant in the Asteraceae family and has been cultivated in the Mediterranean region since 4500 BC. Lettuce is a source of essential vitamins, nutrients, and cellulose, which aid digestion, and there has been a growing public demand for organically grown lettuce (Fatouros *et al.* 2018). In agricultural pro-

duction, soil-borne pathogens pose significant challenges that can severely undermine crop yield and quality. Among these pathogens, *Pythium ultimum* is recognised as a major threat, particularly in the cultivation of lettuce. This oomycete pathogen causes damping-off and root rot diseases, which thrive in wet conditions and can lead to substantial

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economic losses in horticultural settings (Shirafkan *et al.* 2024a).

Management of *P. ultimum* in lettuce production often relies on integrated pest management (IPM) strategies that combine cultural practices, biological control measures, and chemical fungicides. The effectiveness of these approaches depends on understanding the pathogen's life cycle and environmental triggers (Schmidt *et al.* 2020). Notably, the efficacy of certain fungicides against *P. ultimum* varies, with some active ingredients proving more effective than others. For instance, metalaxyl has been observed to reduce damping-off severity in various crops, including lettuce (Agarwal *et al.* 2021; Mannai & Boughalleb-M'Hamdi 2021). However, reliance on chemical controls alone is not sustainable, and there is growing interest in biocontrol agents such as *Trichoderma harzianum*, which has shown potential to suppress *P. ultimum* either alone or in combination with other strategies (Ghoniem *et al.* 2021; Shirafkan *et al.* 2024b). The adoption of biological control agents has attracted attention as a viable alternative to chemical fungicides, aligning with the global movement towards more sustainable agricultural practices (Alizadeh *et al.* 2025).

T. harzianum, a filamentous fungus in the family Hypocreaceae, exhibits potent antifungal properties through diverse mechanisms of action and has been employed in agricultural practices to manage plant diseases (Alizadeh *et al.* 2024; Rostami *et al.* 2024b). Among its myriad functions, *T. harzianum* produces hydrolytic enzymes that degrade pathogen cell walls and engages in competitive exclusion, thereby inhibiting pathogen colonisation (Abbasi *et al.* 2016; Naseripour *et al.* 2017; Shahbazi *et al.* 2025). In addition to its antagonistic properties, *T. harzianum* is recognised for its ability to enhance plant growth by improving nutrient-use efficiency, particularly for phosphorus and micronutrients, thereby contributing to the overall health and resilience of plants (Rezalou *et al.* 2023; Petcu *et al.* 2023; Gharansaraei *et al.* 2025). Moreover, recent exploration of genetic modifications and induced mutations in *Trichoderma* isolates has opened new avenues for enhancing the efficacy of biocontrol agents. Techniques such as gamma irradiation have been employed to develop mutant isolates that ex-

hibit improved biocontrol capabilities and enhanced adaptability to environmental stressors (Soufi *et al.* 2021a; Abdul-Jabbar *et al.* 2025; Sorahinobar *et al.* 2025).

Despite the promising attributes of *T. harzianum*, there remains a substantial gap in understanding the specific effects of mutant isolates in targeted agricultural contexts. Therefore, the objective of this study is to investigate the potential to enhance the biological control activity of *T. harzianum* through induced mutations via gamma irradiation, with a focus on its effectiveness against *P. ultimum*. Further, this study aims to evaluate the morphological and physiological traits of lettuce treated with both wild-type and mutant isolates of *T. harzianum*, thereby contributing valuable insights into their applicability for sustainable agricultural practices.

MATERIAL AND METHODS

Biocontrol Agent Source

Trichoderma was isolated from soil samples collected from different parts of the Faculty of Agriculture Research Farm (Ferdowsi University, Mashhad, Khorasan state, Iran; latitude and longitude: 36.30587, 59.52787) by serial dilution on *Trichoderma*-selective medium (Soufi *et al.* 2021a). Identification of *Trichoderma* was based on morphological characters (Soufi *et al.* 2021a). ITS amplification was performed using the ITS1/ITS4 primers, and molecular identification was carried out as described by Soufi *et al.* (2021a). Based on molecular identification, the *Trichoderma* isolate was identified as *T. harzianum* and registered in the Gene Bank with NCBI accession code: MW718882.

Utilising γ -Radiation for Induced Mutation

After isolation and identification, washed spore suspensions of *T. harzianum* were irradiated in a cobalt-60 γ -irradiator at a dose rate of 0.23 Gy/sec at the Nuclear Agriculture Research School (NSTRI, AEOI). Dosimetry was performed using the Fickereference standard dosimetry system (Gamma-cell Issledovatel, PX-30). The dose range used to determine a suitable dose was 0, 50, 150, 200, 250, 300, 350, 400 and 450 Gy (Askari *et al.* 2024). After dose optimisation, irradiation

was performed at the optimised dose of 250 Gy (in three replicates) on the spore suspension (106 spores/mL) of *T. harzianum*. After irradiation, serial dilutions (10⁻⁶, 10⁻⁵, 10⁻⁴, 10⁻³, 10⁻², 10⁻¹) of the spore suspension were prepared, and 1 mL of each diluted sample was cultured on potato dextrose agar (PDA) and incubated for 72 h at 28°C (Soufi *et al.* 2021b). The selected dose was based on approximately 40–50% inhibition of *T. harzianum* (NAS-T101) spore germination on water agar (WA) medium for 24 hours (Ghasemi *et al.* 2019). Mutant isolates were sub-cultured seven times to ensure the immutability of morphological characteristics on Mandel's optimised medium (Soufi *et al.* 2021b).

Pathogenic Fungus Source

The pathogenic fungus *P. ultimum*, originally isolated from a lettuce host, was obtained from the plant protection laboratory (Shahbazi *et al.* 2025) of the Nuclear Agriculture Research School (NSTRI, AEOI).

In vitro Assay for Biocontrol

To select the *T. harzianum* mutant with the greatest potential to inhibit the pathogen's growth, the dual culture test was used. The antagonistic activity of *T. harzianum* (NAS-T101) and 26 mutants against *P. ultimum* was assessed using the dual culture technique. The extent of pathogen growth inhibition by *T. harzianum* (NAS-T101) and 26 mutants was estimated using the following formula (Abbasi *et al.* 2016).

$$I\% = (1 - C_n/C_o) \times 100$$

Where: C_n is the average diameter of the pathogen colonies in the presence of antagonist and C_o is the average diameter of the pathogen colonies in the control).

Greenhouse Evaluations and Morphological Traits

In vivo assay, sterile clay loam soil (consisting of 21% sand, 20% silt and 39% clay) was used, and greenhouse conditions were maintained with an average temperature of 20–22°C, a relative humidity of 65%, a photoperiod of 16 hours, and a photosynthetic photon flux density of 850–950 μmol/m²/s. The Grand Rapids lettuce seedlings, a pathogen-susceptible cultivar, were grown in sterile soil in 4 kg pots. For pathogen treatments, pots were inoculat-

ed with 2 g of *P. ultimum* inoculum 10 days before planting (Ghoniem *et al.* 2021). For biocontrol treatments, the topsoil was amended with 2 g of the respective *Trichoderma* isolate at planting time. Disease incidence (DI) and morphological traits were recorded 45 days after planting, using the method of Ghoniem *et al.* (2021). Morphological traits, including root length, stem length, plant diameter, number of leaves, plant fresh weight (yield), and dry weights of roots and stems, were measured for all treatments from the single-leaf stage to before the flowering stage, 45 days after planting. The greenhouse experiment was arranged in a completely randomised design (CRD) with four replications and included six treatment groups:

- *Control*: plants grown in sterile soil without pathogen or *Trichoderma* application.
- *Pathogen alone*: plants grown in soil infested with 2 g of *P. ultimum* inoculum (no *Trichoderma*).
- *Wildtype biocontrol alone*: plants treated with 2 g of *T. harzianum* NAST101 (wildtype) in sterile soil (no pathogen).
- *Mutant biocontrol alone*: plants treated with 2 g of *T. harzianum* TM4 (mutant) in sterile soil (no pathogen).
- *Wildtype + Pathogen*: plants grown in soil infested with *P. ultimum* and coinoculated with 2 g of *T. harzianum* NAST101.
- *Mutant + Pathogen*: plants grown in soil infested with *P. ultimum* and coinoculated with 2 g of *T. harzianum* TM4.

Measurement of Physiological Traits

To measure chlorophyll *a* and *b* by spectrophotometry, 0.5 g of fresh leaves were homogenised with 8 cc of acetone and 1 g of calcium carbonate, then centrifuged at 3,000–4,000 g for 15 minutes. 1 cc of the supernatant was added to a cuvette containing 9 cc of 80% acetone. The absorbance of the supernatant was measured at wavelengths A663 and A645 to estimate chlorophyll *a* and chlorophyll *b* contents, respectively, according to the standard spectrophotometric method, as described by Orojnia (2021).

$$\text{chlorophyll } a = (12.7 \times A_{663} - 2.69 \times A_{645}) \times V/1000 \times W$$

$$\text{chlorophyll } b = (22.9 \times A_{645} - 4.69 \times A_{663}) \times V/1000 \times W$$

Measurement of Malondialdehyde Activity (MDA)

To measure MDA, 0.5 g of fresh leaf tissue was homogenised with 5 mL of 1% trichloroacetic acid (TCA). The resulting homogeneous solution was centrifuged at 14,000–15,000 g for 25 minutes, and 1 mL of the supernatant was mixed with 4 mL of TBA-TCA (Sigma-Aldrich, Steinheim, Germany) and incubated at 60°C for 4 minutes (Ben Marie) (Orojnía *et al.* 2021).

$$\text{MDA} \left(\frac{\text{nmol}}{\text{mLFW}} \right) = (\text{A532} - \text{A600}) / 155000.10^5$$

Measurement of Polyphenol Oxidase Activity (POA)

Polyphenol oxidase activity was estimated with phosphate buffer and pyrogallol by Orojnía method (2021). Adsorption of Purpurogallin formed at 420 nm was measured.

Electrophoresis Test and Determination of the Molecular Weight of Enzymes Produced in Leaves

Protein content was estimated using bovine serum albumin (BSA) as the standard. Absorbance at 595 nm was measured with a spectrophotometer (Jenway, USA). Protein samples (50 mL) were extracted from plant leaves with acetone (50 mL) and stored at -70°C. The molecular weight of enzymes was determined by SDS-PAGE using a 5% polyacrylamide gel (accumulation) and a 12% gel (separator). Prior to electrophoresis, an equal volume of sample buffer (100 µL) containing 65 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% SDS (v/v), 5% 2-mercaptoethanol (v/v), and 0.2% aqueous bromophenol (w/v) was added to the protein sample (100 µL), the mixture was boiled for 5 minutes, and then applied to the gels. Proteins were separated at a constant voltage of 250 V in ready-to-run buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS (w/v), pH 8.3. The gels were stained with Coomassie Brilliant Blue R-250 in methanol-acetone. In methanol-acetic acid-water, the decomposition ratio was 1:1:8 (Askari *et al.* 2024).

Statistical Analysis

Statistical analysis was performed using SPSS Software v.26.0 (SPSS Inc., Chicago, IL, USA) with a one-way analysis of variance (ANOVA). Differences between data were assessed using Tukey's test at $p < 0.05$. All experiments were carried out in a completely randomised design, and the data were

expressed as the mean $\pm$ standard deviation (SD) of triplicate experiments.

RESULTS

Dual Culture Assay

Dual-culture assays revealed significant differences in the antagonistic activity of *T. harzianum* NAS-T101 (wild type) and its gamma-irradiated mutant isolates against *P. ultimum* (Table 1). All isolates listed in Table 1 are mutants derived from *T. harzianum* NAS-T101, obtained after gamma irradiation at 250 Gy. The isolates were coded TM1–TM26 based on colony morphology, growth characteristics, and antagonistic performance during preliminary screening. Among these, TM4 was selected for greenhouse experiments due to its superior pathogen inhibition and stable morphological traits.

The percentage of pathogen growth inhibition ranged from 31.22% to 69.32%, indicating substantial isolate-dependent variability. The mutant isolate TM4 exhibited the highest antagonistic activity, inhibiting *P. ultimum* growth by 69.32%, which was significantly higher than that of the parental isolate NAS-T101 (35.39%). Several other mutants, including TM5, TM6, TM9, and TM17, also showed relatively high inhibition (>60%), but TM4 consistently combined strong antagonism with rapid mycelial growth and stable sporulation. Rather than repeating all numerical values from Table 1, the results indicate that enhanced antagonistic activity was not strictly correlated with spore production or germination rate. For example, TM4 showed high inhibition despite moderate spore production, suggesting that qualitative factors such as enzyme secretion or secondary metabolite production play a major role in pathogen suppression.

Morphological traits, including colony colour and shape, varied among isolates. Mutants with petal-shaped colonies and darker green pigmentation, such as TM4, generally exhibited stronger antagonistic effects, which may reflect increased metabolic activity and enzyme secretion at the interaction zone with the pathogen. Overall, the dual culture assay clearly demonstrated that gamma irradiation generated substantial phenotypic diversity among *T. harzianum* mutants, with TM4 emerging as the

most promising candidate for further greenhouse evaluation.

The Table 1 provides a comparative analysis of various *Trichoderma* isolates, focusing on their morphological, reproductive, and antagonistic traits,

including germination time, colony characteristics, spore production, mycelial growth, pathogen inhibition capacity, and conidial dimensions. Below is an integrated interpretation of the results, organised by key functional and isolate-specific insights:

T a b l e 1

Morphological and antagonistic characteristics of *Trichoderma harzianum* NAS-T101 (wild type) and its gamma-irradiated mutant isolates (TM1–TM26) in dual culture assay after 72 h of incubation at 28°C. Mutant grouping was based on colony morphology, growth rate, and antagonistic performance against *Pythium ultimum*. Different lowercase letters within each column indicate significant differences at $p \leq 0.05$.

Isolate	Germination [h]	Colony shape	Color of colony	Spore production (cfu $\times 10^6$)	Mycelia growth [cm/day]	<i>P. ultimum</i> growth inhibition [%]	Conidial size [μ m]	
							Width	Length
<i>T. harzianum</i>	4.33 ^d ±0.58	Flat	Green light	2.58 ^a ±0.10	1.20 ^{f-h} ±0.07	35.39 ^{de}	5.08 ^a ±0.12	5.20 ^{f-h} ±0.02
TM1	5.67 ^{bc} ±0.58	Petal	Green	14.70 ^a ±0.18	0.98 ^{bc} ±0.05	45.05 ^{bc}	5.02 ^a ±0.01	5.10 ^{gh} ±0.02
TM2	6.67 ^{ab} ±0.58	Petal	Green light	6.17 ⁱ ±0.09	1.12 ^{c-g} ±0.02	40.55 ^d	5.02 ^a ±0.00	5.30 ^{d-g} ±0.02
TM3	5.67 ^{bc} ±0.58	Petal	Green light	1.85 ^p ±0.09	1.08 ^{c-c} ±0.05	31.32 ^c	5.02 ^a ±0.01	5.40 ^{c-f} ±0.02
TM4	4.67 ^c ±0.58	Flat	Dark Green	12.88 ^b ±0.08	2.55 ^a ±0.03	69.32 ^a	5.05 ^a ±0.06	5.10 ^{gh} ±0.02
TM5	5.67 ^{bc} ±0.58	Petal	Green light	5.65 ^{kl} ±0.09	1.12 ^{c-g} ±0.05	67.12 ^a	5.02 ^a ±0.01	5.51 ^{cd} ±0.01
TM6	4.33 ^d ±0.58	Petal	Dark Green	8.15 ^h ±0.02	1.20 ^{f-h} ±0.10	65.33 ^{ab}	5.02 ^a ±0.01	6.53 ^a ±0.01
TM7	5.67 ^{bc} ±0.58	Petal	Green light	3.87 ⁿ ±0.19	1.10 ^{d-f} ±0.00	39.32 ^d	5.02 ^a ±0.00	5.23 ^{f-h} ±0.02
TM8	5.67 ^{bc} ±0.58	Petal	Dark Green	11.60 ^d ±0.19	1.00 ^{b-d} ±0.03	40.05 ^d	5.02 ^a ±0.01	5.10 ^{gh} ±0.02
TM9	5.67 ^{bc} ±0.58	Petal	Green	5.20 ^m ±0.16	1.22 ^{gh} ±0.05	66.25 ^{ab}	5.02 ^a ±0.01	5.23 ^{f-h} ±0.02
TM10	6.67 ^{ab} ±0.58	Flat	Dark Green	8.84 ^g ±0.19	1.13 ^{c-g} ±0.07	45.12 ^{bc}	5.02 ^a ±0.01	5.50 ^{cd} ±0.15
TM11	6.00 ^{a-c} ±0.00	Flat	Dark Green	12.40 ^c ±0.19	1.30 ^{hi} ±0.10	49.33 ^{bc}	5.02 ^a ±0.00	5.84 ^b ±0.02
TM12	5.33 ^c ±0.58	Flat	Green	6.68 ⁱ ±0.15	1.47 ^j ±0.03	54.31 ^b	5.02 ^a ±0.01	5.21 ^{f-h} ±0.02
TM13	6.67 ^{ab} ±0.58	Flat	Green	4.03 ⁿ ±0.19	0.97 ^b ±0.03	31.22 ^c	5.02 ^a ±0.01	5.26 ^{c-g} ±0.02
TM14	6.67 ^{ab} ±0.58	Flat	Green light	5.46 ^{lm} ±0.19	1.15 ^{efg} ±0.08	48.45 ^{bc}	5.02 ^a ±0.01	6.01 ^b ±0.01
TM15	6.67 ^{ab} ±0.58	Flat	Dark Green	10.99 ^c ±0.25	1.23 ^{gh} ±0.07	47.25 ^{bc}	5.02 ^a ±0.00	5.10 ^{gh} ±0.02
TM16	6.67 ^{ab} ±0.58	Petal	Dark Green	9.15 ^f ±0.19	1.37 ^{ij} ±0.03	59.31 ^b	5.02 ^a ±0.01	5.47 ^{c-e} ±0.21
TM17	6.00 ^{a-c} ±0.00	Petal	Green	5.99 ^j ±0.19	1.08 ^{c-f} ±0.02	68.25 ^a	5.02 ^a ±0.01	6.61 ^a ±0.34
TM18	5.33 ^c ±0.58	Petal	Dark Green	11.49 ^d ±0.26	1.30 ^{hi} ±0.07	48.30 ^{bc}	5.02 ^a ±0.01	5.00 ^h ±0.02
TM19	6.67 ^{ab} ±0.58	Petal	Green	6.65 ⁱ ±0.19	0.85 ^a ±0.12	31.55 ^c	5.02 ^a ±0.00	5.20 ^{f-h} ±0.02
TM20	6.67 ^{ab} ±0.58	Flat	Green light	5.87 ^{jk} ±0.03	1.00 ^{b-d} ±0.07	45.33 ^{bc}	5.02 ^a ±0.01	5.10 ^{gh} ±0.02
TM21	7.00 ^a ±0.00	Petal	Dark Green	12.58 ^b ±0.08	1.35 ⁱ ±0.05	57.03 ^b	5.10 ^a ±0.15	5.57 ^c ±0.42
TM22	6.27 ^{ab} ±0.03	Petal	Green light	5.99 ^j ±0.09	1.32 ^{hi} ±0.00	52.50 ^{bc}	5.02 ^a ±0.01	5.44 ^{c-g} ±0.02
TM23	6.60 ^{ab} ±0.06	Flat	Dark Green	6.78 ⁱ ±0.05	1.37 ^{hi} ±0.02	58.33 ^b	5.03 ^a ±0.01	6.31 ^b ±0.01
TM24	6.58 ^{ab} ±0.08	Flat	Green light	9.20 ^f ±0.09	1.27 ⁱ ±0.05	48.13 ^{bc}	5.01 ^a ±0.00	5.12 ^{gh} ±0.02
TM25	6.07 ^{ab} ±0.05	Flat	Dark Green	5.47 ^{lm} ±0.15	1.15 ^{eg} ±0.04	40.52 ^d	5.02 ^a ±0.01	5.45 ^{c-e} ±0.21
TM26	6.09 ^{a-c} ±0.02	Flat	Green	5.37 ^{jk} ±0.03	1.23 ^{gh} ±0.05	44.33 ^{cd}	5.03 ^a ±0.01	6.41 ^a ±0.34

a. Germination Efficiency and Early Growth Dynamics

Germination time varied significantly across isolates, ranging from 4.33 hours (wild-type *T. harzianum* NAS-T101) to 7.00 hours (mutant TM21). Faster germination (e.g., 4.33–5.67 hours) is advantageous for rapid colonisation of plant roots or soil substrates, a critical trait for biocontrol agents competing against pathogens such as *P. ultimum*. Slower-germinating isolates (e.g., 6.67–7.00 hours) may prioritise resource allocation to stress tolerance or secondary metabolite production. Notably, isolates with shorter germination times (e.g., NAS-T101: 4.33 h) often exhibited lower spore production (2.58×10^6 CFU), suggesting a trade-off between establishment speed and reproductive output. Conversely, slower-germinating isolates such as TM21 (7.00 h) achieved higher spore counts (12.58×10^6 CFU), indicating divergent survival strategies among isolates. However, no strict correlation was observed between germination time and antagonistic performance, as the top-performing mutant TM4 (4.67 h germination, 12.88×10^6 CFU, 69.32% inhibition) combined rapid germination with high sporulation and strong pathogen suppression, demonstrating that these traits are not mutually exclusive in all isolates.

b. Spore Production and Reproductive Fitness

Spore production varied markedly among the gamma-irradiated mutants, ranging from 1.00×10^6 CFU (TM8) to 12.88×10^6 CFU (TM4). High sporulation (e.g., $>10 \times 10^6$ CFU in TM4) is critical for persistence in soil ecosystems and for large-scale biocontrol applications. However, spore production does not strictly correlate with pathogen inhibition. For instance, the high-sporulating mutant TM4 (12.88×10^6 CFU) exhibited the strongest antagonism (69.32% inhibition), whereas TM8, despite its moderate spore production (1.00×10^6 CFU), showed only 40.05% inhibition. This suggests that spore quantity alone does not determine biocontrol efficacy, which may depend more on metabolite diversity or enzymatic activity than on reproductive output.

c. Pathogen Inhibition and Antagonistic Potential

Pathogen inhibition varied widely, from 31.22% to 69.32%, with significant isolate-specific differ-

ences. The mutant isolate TM4, with 69.32% inhibition and high spore production (12.88×10^6 CFU), emerged as the top performer, likely due to potent antifungal metabolites (e.g., peptaibols, glucanases) or hyperparasitism. Notably, some isolates with high spore counts showed only moderate inhibition (e.g., TM21: 57.03% inhibition with 12.58×10^6 CFU), whereas others with moderate spore production achieved relatively strong inhibition (e.g., TM12: 54.31% inhibition with 6.68×10^6 CFU). This highlights the importance of isolate-specific metabolic profiles rather than reproductive capacity alone. Colony morphology did not consistently correlate with antagonism; for instance, the flat-colony mutant TM4 (69.32% inhibition) outperformed several petal-shaped mutants (e.g., TM21: 57.03%), indicating that morphological traits are not primary determinants of biocontrol efficacy.

d. Mycelial Growth and Colonisation Capacity

Mycelial growth rates ranged from 0.85 cm/day to 2.55 cm/day, with most isolates clustering around 1.00–1.47 cm/day. The outlier TM4, with a growth rate of 2.55 cm/day and the highest inhibition (69.32%), represents a potential elite isolate for rapid soil colonisation – a trait critical for pre-emptive pathogen suppression. Faster mycelial expansion enables quicker resource monopolisation and niche exclusion. However, growth speed did not consistently align with spore production or inhibition efficacy. For example, the fast-growing TM4 (2.55 cm/day) produced abundant spores (12.88×10^6 CFU), whereas slower-growing isolates such as TM18 (1.30 cm/day) still produced considerable spores (11.49×10^6 CFU). This underscores the ecological diversity of *Trichoderma* strategies, which balance colonisation speed, reproduction, and antagonism in an isolate-specific manner.

e. Conidial Morphology and Functional Implications

Conidial dimensions (length: 5.00–6.61 μm ; width: 5.01–5.10 μm) showed limited variation, suggesting conserved structural traits across isolates. Minor differences in length – for example, TM17 (6.61 μm) versus TM4 (5.10 μm) – may influence dispersal efficiency or adhesion to plant surfaces. Larger conidia might persist longer in harsh environments, whereas smaller ones could disperse

more readily. However, the absence of strong correlations between conidial dimensions and other traits (e.g., inhibition, growth rate, or sporulation) implies that spore morphology is not a primary determinant of biocontrol efficacy in this dataset.

f. Isolate-Specific Trade-Offs and Selection Criteria

The data highlight distinct trade-offs among *Trichoderma* isolates, necessitating strategic selection based on agricultural priorities. Isolates such as TM8 (11.60×10^6 CFU, 40.05% inhibition) prioritise high sporulation over direct antagonism, favouring reproductive fitness and soil persistence, whereas isolates like TM4 (12.88×10^6 CFU, 69.32% inhibition) excel in both pathogen suppression and spore production, demonstrating that high antagonistic activity and high sporulation are not mutually exclusive. Rapid colonisers such as TM4 (2.55 cm/day) combine fast growth with strong inhibition, while slower-growing isolates such as TM18 (1.30 cm/day) still maintain considerable spore output. Colony morphology further reflects functional adaptations: although petal-shaped colonies (e.g., TM21) were associated with moderate inhibition, the flat-colony TM4 showed superior performance, suggesting that metabolic versatility, rather than gross colony shape, governs biocontrol potential. These trade-offs emphasise the need to align isolate selection with specific environmental or operational demands and underscore the value of the gamma-irradiation approach in generating highly effective, multifunctional mutants such as TM4.

Morphological Traits

The study evaluated the impact of the *T. harzianum* isolate (NAS-T101) and its mutant (TM4) on lettuce growth parameters, including stem and root length, dry weights of stems and roots, and fresh weight (yield), in the presence of *P. ultimum*. The results highlight the dual role of *Trichoderma* in mitigating pathogen-induced growth suppression and acting as a biostimulant to enhance plant productivity and resilience (Table 2).

a. Growth Parameters Under Pathogen Stress

P. ultimum infection severely impaired plant growth, as evidenced by drastic reductions in stem length (55.13 cm vs. control: 276.63 cm), root dry weight (0.13 g vs. control: 1.99 g), and yield (0.45 g

vs. control: 1.96 g). These declines reflect the pathogen's destructive effects on nutrient uptake, root development, and overall plant vigour. The suppression of root biomass (0.13 g) and stem elongation (55.13 cm) underscores the pathogen's ability to disrupt physiological processes, likely through root rot or toxin production.

b. *Trichoderma*'s Biocontrol and Growth-Promoting Effects

Co-inoculation of *Trichoderma* isolates with *P. ultimum* significantly reduced pathogen damage. For instance, NAS-T101 with the pathogen restored stem length to 212.26 cm and root dry weight to 1.20 g, while the mutant TM4 isolate performed slightly better, achieving 245.38 cm stem length and 1.25 g root dry weight. This recovery suggests that *Trichoderma* directly antagonises the pathogen (e.g., through competition or antibiosis) and/or induces systemic resistance in the plant. Remarkably, *Trichoderma*-only treatments (without pathogen) exceeded control values, with NAS-T101 and TM4 boosting stem length to 289.77 cm and 294.35 cm, respectively, and root dry weight to 2.76 g and 2.89 g, respectively. These results highlight *Trichoderma*'s biostimulant properties, likely mediated by enhanced nutrient assimilation (e.g., phosphorus solubilisation), phytohormone modulation (e.g., auxin production), or improved stress tolerance.

c. Yield Enhancement and Isolate-Specific Efficacy

Yield metrics followed similar trends. Pathogen reduced yield to 0.45 g, but co-application with *Trichoderma* restored it to 1.68–1.99 g. *Trichoderma*-only treatments further increased yield to 2.51–2.69 g, surpassing the control (1.96 g). The mutant TM4 isolate consistently outperformed NAS-T101 across most parameters. For example, TM4 alone achieved the highest stem length (294.35 cm) and root dry weight (2.89 g), suggesting that mutagenesis may have enhanced its colonisation efficiency, metabolite production, or stress-priming capabilities. These isolate-specific differences could be attributed to genetic variations that influence symbiotic interactions or enzymatic activity.

Physiological and Biochemical Assays

The study evaluated the physiological and biochemical responses of lettuce plants to *T. harzianum*

T a b l e 2

The means comparison of the effect of *T. harzianum* (NAS-T101) and mutant TM4 on morphological characteristics of lettuce

Treatment	Length [cm]		Dry weight [g]		
	Stem	Root	Fresh weight	Stem dry weight	Root dry weight
Control (without pathogen)	54.67 ^a ±0.785	15.49 ^{ab} ±2.64	276.63 ^{ab} ±0.29	1.99 ^b ±0.20	1.96 ^b ±1.00
<i>P. ultimum</i> without <i>Trichoderma</i>	15.63 ^c ±0.70	3.96 ^c ±0.68	55.13 ^c ±0.21	0.13 ^b ±0.23	0.45 ^c ±0.56
<i>T. harzianum</i> (NAS-T101) with <i>P. ultimum</i>	43.60 ^b ±1.00	10.16 ^b ±2.84	212.26 ^b ±0.10	1.20 ^b ±0.18	1.68 ^b ±1.40
<i>T. harzianum</i> TM4 with <i>P. ultimum</i>	45.38 ^b ±1.80	11.72 ^b ±3.44	245.38 ^b ±0.80	1.25 ^b ±0.31	1.99 ^b ±0.97
<i>T. harzianum</i> (NAS-T101)	51.71 ^a ±1.50	17.32 ^a ±3.38	289.77 ^a ±0.20	2.76 ^a ±0.40	2.51 ^a ±1.16
<i>T. harzianum</i> T M4	54.45 ^a ±0.99	17.46 ^a ±2.53	294.35 ^a ±0.80	2.89 ^a ±0.23	2.69 ^a ±1.07

Note: a – c: lowercase letters of different text in a row show significant differences ($p \leq 0.05$)

isolates (NAS-T101 and its mutant TM4) under pathogen stress from *P. ultimum*, focusing on antioxidant enzymes, chlorophyll content, oxidative stress markers (MDA), and protein levels. The results highlight the dual role of *Trichoderma* as both a biocontrol agent against pathogens and a biostimulant that enhances plant health.

a. Antioxidant Enzymes and Oxidative Stress Mitigation

The pathogen *P. ultimum* significantly suppressed antioxidant enzyme activity when applied alone. Peroxidase levels fell from 4.41 U/g in the control to 1.67 U/g, and polyphenol oxidase (PPO) decreased from 26.65 U/g to 12.34 U/g, indicating compromised antioxidant defences. However, co-inoculation of *Trichoderma* isolates with the pathogen partially restored these enzymes. For instance, NAS-T101 and TM4 increased peroxidase to 2.75 U/g and 2.98 U/g, respectively, and PPO to 35.51 U/g and 32.34 U/g, respectively – values that matched or exceeded the control. Remarkably, *Trichoderma*-only treatments exhibited even higher enzyme activity (peroxidase: ~5.65–5.67 U/g; PPO: ~34.34–36.51 U/g), surpassing the control. This suggests that *Trichoderma* not only counteracts pathogen-induced suppression but also primes the plant's antioxidant system, potentially through systemic resistance or direct enzymatic activity. Concurrently, MDA levels – a marker of lipid peroxidation – rose sharply under pathogen stress (8.34 nmol/g

vs. control: 4.71 nmol/g), reflecting oxidative damage. *Trichoderma* application, whether with or without the pathogen, reduced MDA to 2.80–3.80 nmol/g, demonstrating its efficacy in mitigating oxidative stress, likely via enhanced scavenging of reactive oxygen species (ROS) by upregulated antioxidant enzymes.

b. Oxidative Stress and Plant Health

Although not explicitly shown in this dataset, the improved growth parameters under *Trichoderma* treatment align with previous findings of reduced oxidative stress (e.g., lower MDA levels) and enhanced antioxidant activity. Healthier root systems (evidenced by higher root dry weight) likely enhance water and nutrient uptake, indirectly supporting stem elongation and biomass accumulation. The significant increase in yield under *Trichoderma* treatments underscores its role in optimising photosynthetic efficiency and resource allocation.

c. Chlorophyll Preservation and Photosynthetic Enhancement

P. ultimum infection severely degraded chlorophyll content, reducing chlorophyll *a* to 8.23 mg/g (vs. control: 17.94 mg/g) and chlorophyll *b* to 6.04 mg/g (vs. control: 14.56 mg/g), indicative of photodamage or impaired nutrient uptake. Co-application of *Trichoderma* with the pathogen reversed this decline, elevating chlorophyll *a* to 20.14–21.18 mg/g and chlorophyll *b* to 18.49–19.54 mg/g, exceeding control levels. *Trichoderma*-only treatments

further boosted chlorophyll content (chlorophyll *a*: ~27.14–28.18 mg/g; chlorophyll *b*: ~20.54–21.49 mg/g), highlighting its biostimulant properties. This enhancement may stem from improved nutrient assimilation, stress tolerance mechanisms, or phytohormonal modulation by *Trichoderma*, which supports photosynthetic efficiency and overall plant vigour.

d. Protein Synthesis and Plant Resilience

The bands observed in densitometry showed an increase in protein content in lettuce leaves treated with *T. harzianum* (NAS-T101) and TM4 compared with the control (Table 3), which may reflect the expression of proteins weighing 90, 72, 49, 36, and 28 kDa (Figure 1). Pathogen infection drastically reduced protein content (1.14 mg/g vs. control: 3.30 mg/g), likely due to resource diversion towards defence responses or pathogen-driven degradation. *Trichoderma* application restored protein levels, with co-inoculated treatments reaching 4.14–4.16 mg/g and *Trichoderma*-only treatments achieving the highest values (4.94–5.16 mg/g). This increase suggests that *Trichoderma* can stimulate protein synthesis, potentially upregulating stress-related proteins (e.g., pathogenesis-related proteins) or improving nitrogen metabolism. Elevated protein content correlated with enhanced plant resilience, supporting plant growth and recovery under pathogen stress.

e. Isolate-Specific Efficacy and Mechanisms

Both NAS-T101 and TM4 isolates demonstrated robust biocontrol and biostimulant effects, though subtle differences emerged. Under pathogen challenge, the TM4 + *P. ultimum* treatment yielded marginally higher chlorophyll *a* (21.18 mg/g) than the NAS-T101 + *P. ultimum* treatment (20.14 mg/g). In the absence of the pathogen, the NAS-T101-alone treatment showed slightly lower protein content (4.94 mg/g) than the TM4-alone treatment (5.16 mg/g). These variations may reflect differences in colonisation efficiency, metabolite production, or interaction dynamics. The isolates likely act through multiple mechanisms: direct antagonism of *P. ultimum*, induction of systemic resistance (ISR), priming of antioxidant systems, and modulation of phytohormones (e.g., auxin, ethylene). Notably, *Trichoderma*-only treatments induced higher antioxidant enzyme activity and chlorophyll than the control, suggesting a "primed" state that prepares plants for stress even in the absence of the pathogen.

DISCUSSION

The study presented compelling evidence that both the wild-type *T. harzianum* NAS-T101 and its mutant isolate TM4 significantly enhanced biological control of *P. ultimum*, a notorious pathogen

T a b l e 3

The means comparison of the effect of *Trichoderma harzianum* (NAS-T101) and its mutant TM4 on physiological characteristics of lettuce

Treatment	Antioxidant enzymes (U/gr of leaf fresh weight)		Chlorophyll (mg/gr leaf fresh weight)		Malondialdehyde activity (nmol/g of fresh leaf weight)	Protein (mg/g of fresh leaf weight)
	Peroxidase	Polyphenol oxidase	<i>a</i>	<i>b</i>		
Control (without pathogen)	4.41 ^b ±0.09	26.65 ^b ±0.77	17.94 ^c ±0.72	14.56 ^c ±1.33	4.71 ^b ±0.05	3.30 ^b ±0.23
<i>P. ultimum</i> without <i>Trichoderma</i>	1.67 ^d ±0.07	12.34 ^c ±0.66	8.23 ^d ±1.67	6.04 ^d ±1.02	8.34 ^a ±0.04	1.14 ^c ±0.14
<i>T. harzianum</i> (NAS-T101) with <i>P. ultimum</i>	2.75 ^c ±0.22	35.51 ^a ±1.01	20.14 ^{bb} ±0.40	18.49 ^{ab} ±0.50	3.80 ^c ±0.02	4.16 ^{ab} ±0.76
<i>T. harzianum</i> TM4 with <i>P. ultimum</i>	2.98 ^c ±0.08	32.34 ^a ±0.06	21.18 ^{ab} ±1.34	19.54 ^{ab} ±1.52	3.14 ^c ±0.33	4.14 ^{ab} ±0.58
<i>T. harzianum</i> (NAS-T101)	5.67 ^a ±0.06	34.34 ^a ±0.9	28.18 ^a ±1.07	20.54 ^a ±1.52	2.84 ^d ±0.14	4.94 ^a ±0.04
<i>T. harzianum</i> T M4	5.65 ^a ±0.39	36.51 ^a ±1.70	27.14 ^a ±0.40	21.49 ^a ±0.50	2.80 ^d ±0.06	5.16 ^a ±0.06

Note: a–d: lowercase letters of different text in a row show significant differences ($p \leq 0.05$).

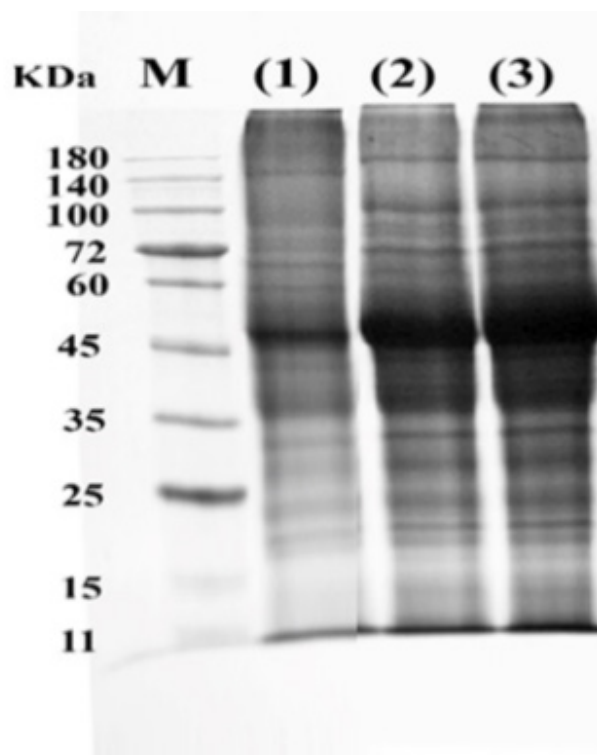


Figure 1. Protein profiles obtained by SDS-PAGE from lettuce leaf: (1) control plant, (2) plants treated with *Trichoderma harzianum* (NAS-T101) isolate, (3) plants treated with *T. harzianum* mutant isolate (TM4).

of lettuce. The results demonstrate that gamma irradiation-induced mutations offer a viable route to producing superior biocontrol agents that not only inhibit pathogen growth but also improve overall plant health. Specifically, the mutant TM4 exhibited a marked reduction in disease incidence, outperforming its parental isolate. This was accompanied by improved morphological and physiological traits in the treated lettuce, including increased root length, dry weight, and chlorophyll content. These improvements suggest that TM4's mechanisms extend beyond mere pathogen suppression, fostering plant resilience and potentially contributing to enhanced nutrient uptake and stress tolerance. Furthermore, the study illustrates the multifaceted role of *Trichoderma* species. Beyond their antagonistic properties, they serve as biostimulants that induce greater chlorophyll synthesis and antioxidant enzyme activity, ultimately leading to healthier, more robust plants. The results reinforce the need for a paradigm shift in agricultural practices towards sustainable methods that prioritise biocontrol agents and minimise reliance on chemical fungicides. By integrating such biological control strategies, grow-

ers can not only improve crop yields and quality but also promote eco-friendliness in agricultural systems.

This aligns with prior studies highlighting *Trichoderma* spp.'s capacity to inhibit soil-borne pathogens through mechanisms such as mycoparasitism, competition, and enzymatic degradation of pathogen cell walls (Sood *et al.* 2020; Kumar *et al.* 2023). The mutant TM4's enhanced efficacy mirrors findings by Afshan *et al.* (2023), who reported that UV/gamma irradiation mutagenesis amplifies *Trichoderma*'s antagonistic traits, likely due to increased production of lytic enzymes or secondary metabolites. Notably, the mutant's ability to reduce disease incidence from 94% in pathogen-challenged plants to 23% parallels the performance of commercial fungicides, as observed by Ghoniem *et al.* (2021) and Fatouros *et al.* (2018), positioning TM4 as a viable alternative to chemical controls in organic farming systems.

Beyond pathogen suppression, *T. harzianum* isolates acted as biostimulants, increasing chlorophyll content, protein synthesis, and antioxidant enzyme activity (peroxidase, polyphenol oxidase) while reducing malondialdehyde (MDA), a mark-

er of oxidative stress. These results corroborate the findings of Goharзад *et al.* (2020), who documented similar *Trichoderma*-induced enhancements in soybean. The increase in chlorophyll and carotenoids in lettuce treated with T.M4 may stem from hormonal modulation, particularly the upregulation of cytokinins and gibberellins, which regulate stomatal conductance and photosynthetic efficiency (Mazhabi *et al.* 2011; Hu *et al.* 2012). Furthermore, the induction of defence-related proteins (e.g., 36 kDa peroxidase, 72 kDa polyphenol oxidase) and the suppression of low-molecular-weight proteins (e.g., 16–17 kDa profilin) suggest that *Trichoderma* reprograms plant metabolism to prioritise stress resistance over allergenic protein synthesis, a finding consistent with Gawlik-Dziki *et al.* (2008) and Rostaminia *et al.* (2021). This metabolic shift not only mitigates pathogen damage but also improves nutritional quality, a critical consideration for leafy vegetables like lettuce.

Gamma radiation has emerged as a pivotal mutagenic tool for inducing genetic variability in *Trichoderma* isolates, yielding mutants with superior biocontrol traits. For instance, Rostami *et al.* (2024a) demonstrated that gamma-irradiated *T. harzianum* mutants exhibited enhanced antagonism against *Meloidogyne javanica* in tomatoes, underscoring the potential of radiation-induced mutagenesis to optimise fungal biocontrol agents. Similarly, Soufi *et al.* (2021b) reported that gamma irradiation of *T. aureoviride* significantly boosted its antagonistic activity against *Sclerotinia sclerotiorum*, highlighting the method's capacity to amplify isolate-specific pathogen suppression. These mutants often display elevated enzymatic activity; Abo-El-Seoud (2013) observed that low-dose gamma radiation stimulated *T. harzianum* and *T. viride* to degrade the pesticide oxamyl more efficiently, linking mutagenesis to both biocontrol and bioremediation potential. Moreover, Diep *et al.* (2020) found that gamma irradiation enhanced cellulase production in *Trichoderma* spp., improving their role in organic matter decomposition and soil health. Practical applications extend to integrated pest management, as Mohamed *et al.* (2023) showed that combining gamma-irradiated *Trichoderma* with entomopathogenic fungi synergistically suppressed *Galleria mellonella* infestations. However, the long-term ecological stability of

these mutants remains understudied, necessitating research into their environmental persistence and interactions, as proposed by Nandini *et al.* (2021). Further exploration of secondary metabolites in irradiated isolates, such as those detailed by Reino *et al.* (2007), could unlock novel biocontrol mechanisms. The mutant TM4's dual proficiency in antagonism and biostimulation highlights the potential of genetic and metabolic engineering to optimise *Trichoderma* isolates for multifunctional agricultural roles. However, as noted by Castillo *et al.* (2019), environmental factors such as soil microbiota, moisture and nutrient availability can modulate *Trichoderma* efficacy, necessitating field validation. For example, Petcu *et al.* (2023) demonstrated that *T. harzianum* combined with *Bacillus subtilis* enhanced maize resilience under field conditions, suggesting similar synergies could benefit lettuce production.

The functional diversity among *Trichoderma* isolates underscores the need for precision in isolate selection. While no single isolate excels across all parameters, phenotypic variability offers opportunities for tailored applications. For example, rapid colonisers such as TM4 (2.55 cm/day growth rate; 69.32% inhibition) are suited to pre-emptive biocontrol, monopolising ecological niches before pathogen establishment (Sood *et al.* 2020). Conversely, isolates with robust sporulation, such as TM21 (12.58×10^6 CFU), ensure sustained inoculum availability, which is critical for large-scale agricultural systems (Orojnias *et al.* 2021). This aligns with recommendations by Ramona *et al.* (2022), who advocated integrating high-sporulation *Trichoderma* isolates into seed coatings or soil amendments for prolonged disease suppression. Importantly, the mutant TM4 demonstrates that rapid colonisation and high sporulation are not mutually exclusive, as it combines a fast growth rate (2.55 cm/day) with the highest spore production (12.88×10^6 CFU) and the strongest pathogen inhibition (69.32%), making it a particularly promising candidate for integrated biocontrol strategies.

The molecular mechanisms underpinning *Trichoderma*-mediated resistance warrant further exploration. The upregulation of defence-related proteins (e.g., peroxidase, polyphenol oxidase) and the suppression of oxidative stress (reduced MDA) observed in this study suggest systemic acquired resis-

tance (SAR) and priming of plant immune responses, as described by Chervin *et al.* (2022) in grapevines. Furthermore, the reduction in profilin – a common allergen – highlights *Trichoderma*'s potential to enhance food safety, though this requires validation through allergenicity assays (Galletti *et al.* 2013). Future research should employ omics technologies (transcriptomics, metabolomics) to delineate the genetic and metabolic pathways driving isolate-specific efficacy, as proposed by Mohammadi *et al.* (2021) and Zhu *et al.* (2021). Such insights could guide the engineering of isolates with enhanced biocontrol and biostimulant traits, such as overexpression of chitinase genes or hormone biosynthesis pathways.

The economic and ecological implications of adopting *Trichoderma*-based strategies are profound. By reducing reliance on synthetic fungicides, farmers can lower production costs and minimise environmental contamination, aligning with global sustainability goals (Wang *et al.* 2018; Afshan *et al.* 2023). For instance, Ghoniem *et al.* (2021) demonstrated that *T. harzianum* combined with chamomile extract achieved disease control comparable to metalaxyl in beans, offering a cost-effective organic alternative. However, scalability challenges, such as the mass production of mutant isolates and field compatibility, must be addressed. Kumar *et al.* (2023) emphasised advances in fermentation technologies to optimise *Trichoderma* biomass production, while Bazghaleh *et al.* (2020) stressed the importance of host genotype compatibility to ensure consistent biocontrol outcomes.

Taken together, this study positions *T. harzianum*, particularly its mutant isolate TM4, as a versatile tool for sustainable lettuce cultivation. Its dual role in suppressing *P. ultimum* and enhancing plant health through antioxidant and biostimulatory mechanisms offers a proactive approach to crop management. However, moving from controlled experiments to field applications requires rigorous validation of isolate efficacy across diverse agronomic conditions. Integrating *Trichoderma* with complementary practices – such as organic amendments, resistant cultivars, and precision irrigation – could amplify its benefits, fostering resilient agricultural systems capable of withstanding biotic and abiotic stresses.

CONCLUSIONS

As we look towards future research directions, several key pathways can be explored. Firstly, additional studies should focus on the molecular basis of the enhanced antagonistic properties observed in the TM4 mutant. Employing omics technologies such as transcriptomics and metabolomics could illuminate the genetic and biochemical pathways that contribute to its superior performance against *P. ultimum*. This information may facilitate the engineering of *T. harzianum* isolates with even more refined traits for specific agricultural conditions. Secondly, investigating the long-term effects of using *T. harzianum* TM4 in field conditions is essential to assess its efficacy and economic viability. The scalability of production methods for the mutant isolate must also be addressed to ensure commercial applicability. Studies into the mass production of these mutants, including optimal growth conditions and formulation techniques, will be crucial for their adoption in the agricultural industry. Moreover, there is a need for comprehensive evaluation of interactions between different *Trichoderma* isolates and various host plant genotypes. Such studies could help identify compatible combinations that maximise biocontrol benefits while improving plant health in diverse environmental contexts. Additionally, integrating *T. harzianum* with other organic amendments or resistant plant varieties could create synergistic effects, further enhancing sustainability in crop management strategies. Finally, addressing the potential allergenic effects of *Trichoderma*-treated crops is a necessary step before these biocontrol methods can be widely adopted. Future research should include allergenicity assessments to ensure consumer safety and confidence in these biocontrol products. In conclusion, the promising outcomes of this study position *T. harzianum*, particularly its mutant TM4, as a crucial asset in the transition towards more sustainable and resilient agricultural practices. As research progresses, the effective deployment of tailored biocontrol strategies and their integration into conventional farming systems will be vital in achieving long-term food security while safeguarding environmental health.

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