

Physiological and molecular responses of broccoli to *Trichoderma viride* and fertilization

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Abstract

This study investigated the physiological and molecular responses of broccoli (*Brassica oleracea* var. *italica*) to the application of the biocontrol fungus *Trichoderma viride* in combination with nitrogen fertilizer as a chemical fertilizer and Vit-Org VG as an organic fertilizer. The aim was to evaluate the integrative effects of these treatments on plant growth, physiological performance, and gene expression related to growth and metabolic activity. A controlled experimental design was used with treatments including sole and combined applications of *T. viride*, nitrogen fertilizer, and Vit-Org VG. Results indicated that the combined treatments significantly enhanced vegetative growth parameters, including plant height, leaf area, and biomass accumulation, compared with individual applications and the control. Physiological analysis revealed improvements in chlorophyll content, photosynthetic efficiency, and nutrient uptake efficiency under integrated treatments. Molecular analysis using qRT-PCR demonstrated distinct temporal expression patterns of growth-related genes. *MDH* exhibited a rapid upregulation at 1 dpt followed by a decline, indicating early metabolic activation, whereas *BoTLP1* showed a progressive and sustained increase, reaching higher expression levels at later stages. Importantly, the combined treatment induced the highest expression levels of both genes across all time points, suggesting a strong synergistic regulatory effect on gene activity. Overall, the most pronounced improvements were observed in the combined treatment of *T. viride* with nitrogen fertilizer and Vit-Org VG, highlighting synergistic interactions that enhance both physiological performance and molecular regulation. These findings demonstrate the potential of integrating beneficial microbes with specific fertilization strategies to improve broccoli productivity in a sustainable manner.

Keywords: broccoli, nitrogen fertilizer, qRT-PCR, *Trichoderma viride*, Vit-Org VG

Introduction

Broccoli (*Brassica oleracea* var. *italica*) is a cruciferous vegetable of high nutritional and economic importance worldwide. It is rich in bioactive compounds, including dietary fiber, essential vitamins (A, C, and K), minerals, and secondary metabolites such as glucosinolates and phenols, which contribute to its antioxidant and health-promoting properties (Syed et al., 2023). These compounds exhibit antioxidant and anti-inflammatory effects and may reduce the risk of chronic diseases, including cardiovascular disorders and cancer, making broccoli a valuable functional food (Andrés et al., 2025). Sulforaphane further enhances cellular defense mechanisms and reduces oxidative stress (Syed et al., 2023).

Plant nutrition plays a key role in growth and yield. Nutrient availability, especially nitrogen, directly influences uptake efficiency, root development, and

biosynthesis of essential biomolecules (Colla et al., 2018). Nitrogen is essential for protein and chlorophyll synthesis, thereby enhancing photosynthesis, metabolism, and biomass accumulation (Xu et al., 2021).

In addition to chemical fertilization, bio-stimulants such as *Trichoderma* spp. improve plant growth and nutrient uptake while reducing dependence on pesticides (Guzmán-Guzmán et al., 2025). *Trichoderma viride* enhances nitrogen and phosphorus uptake and improves growth under nutrient-limited conditions (Song et al., 2024).

Organic fertilizers contribute significantly to sustainable agriculture by improving soil fertility, structure, and microbial activity, which enhances nutrient uptake and plant physiological performance (Etesami & Maheshwari, 2020). They also increase chlorophyll content and growth under stress conditions (Shen et al., 2023).

The integration of organic fertilizers with *T.*

viride provides a sustainable strategy to enhance crop productivity (Etesami & Maheshwari, 2020). These agronomic inputs can also influence gene expression, improving metabolic efficiency and stress tolerance (Zhang et al., 2022). Key genes such as *BoTLP1* and *MDH* play important roles in growth regulation. *BoTLP1* is associated with osmotic adjustment and defense-related pathways, while *MDH* is involved in the TCA cycle and energy production. *GAPDH* is used as a stable reference gene in qRT-PCR analyses (Li et al., 2023; Chen et al., 2021).

The combined application of biological, organic, and mineral fertilization may enhance gene expression, leading to improved physiological traits such as plant height, chlorophyll content, and biomass. Therefore, this study aimed to evaluate the synergistic effects of *T. viride*, organic fertilization, and nitrogen fertilizer on broccoli growth by analyzing physiological parameters and the expression of *BoTLP1* and *MDH* using qRT-PCR, with *GAPDH* as a reference gene.

Materials and Methods

Experimental Materials

The experiment was conducted in the experimental greenhouses of the College of Agriculture, Wasit University, Iraq (32.49726498667815, 45.842268296449376). Greenhouse conditions were maintained at an average daytime temperature of 27 °C and a nighttime temperature of 17 °C. The fungal isolate *Trichoderma viride* was obtained from the Microbiology Laboratory, Department of Plant Protection, College of Agriculture, University of Wasit, and used as a biological growth-promoting agent. Broccoli (*Brassica oleracea* var. *italica*) seeds were sourced from AGRO Seeds (Turkey). The organic fertilizer (Vit-Org VG) was obtained from Bionatura (Italy). Nitrogen fertilizer was applied in the form of urea (46% N) and purchased from local markets.

Preparation of *T. viride* Inoculum

Potato Dextrose Broth (PDB) was prepared by boiling 200 g of sliced potatoes to obtain an extract, followed by the addition of 20 g of dextrose. The mixture was heated until complete dissolution and then dispensed into 500 mL glass flasks. The flasks were subsequently sterilized by autoclaving at 121 °C under 15 psi for 20 minutes prior to use.

Thereafter, a *T. viride* inoculum was prepared in the microbiology laboratory by inoculating the PDB medium with three fungal discs (0.5 cm in diameter) obtained from 5-day-old cultures previously grown on Potato Dextrose Agar (PDA). The cultures were incubated

at 25 ± 2 °C for 5–7 days under continuous agitation using a rotary shaker to ensure uniform fungal growth. After incubation, the fungal suspension was collected directly from the liquid medium and filtered through sterile gauze to remove mycelial fragments. The spore concentration was determined using a hemocytometer and adjusted to 1 × 10⁶ spores/mL.

Soil and Pot Preparation

Two-kilogram capacity plastic pots were used and filled with a 1:1 (v/v) mixture of potting soil and peat moss. Prior to use, the soil mixture was thoroughly sieved to remove impurities and ensure uniformity.

The soil was sterilized by autoclaving at 121 °C under 15 psi pressure, and the sterilization process was repeated on the following day to ensure complete elimination of any microbial contamination. After sterilization, the soil was allowed to cool and then distributed into the pots.

Broccoli seeds were sown directly into the pots at a rate of three seeds per pot. Irrigation was applied as needed in greenhouse conditions, while maintaining optimal environmental conditions for germination and seedling growth, including suitable temperature and relative humidity.

Application of Treatments

Included five treatments: a control treatment, a bio-fungal treatment with *T. viride*, an organic fertilizer treatment (Vit-Org VG), a nitrogen fertilizer treatment (urea, 46% N), and a combined treatment including all of the aforementioned components.

Two days before pre-planting, the *T. viride* inoculum was applied at a rate of 100 mL per pot, while the organic fertilizer was prepared at a rate of 1 mL per 10 L of water, and 100 mL of this solution was applied to each pot, and the nitrogen fertilizer at 840 mg per pot. The combined treatment included all individual treatments applied together at their original individual doses without any change in concentration or volume (Combined treatment) and thoroughly mixing them with the soil. No treatment was applied to the control.

Physiological Measurements

Physiological measurements were conducted to evaluate the effect of treatments on plant growth 30 days after planting. These included plant height, fresh and dry weights of shoot and root systems, and leaf chlorophyll content.

Plant height was measured from the soil surface to the apex using a graduated ruler and expressed in

centimeters. Fresh weight was recorded immediately after harvest using a precision balance. Shoot and root systems were separated prior to weighing.

Samples were then oven-dried at 70 °C for 48 h until constant weight and weighed to determine dry biomass, after which dry weights were recorded. Leaf chlorophyll content was estimated using a SPAD chlorophyll meter and expressed in SPAD units.

Gene expression assay

Broccoli seeds were sown in pots containing sterilized soil as described in the previous section (2.5). When the seedlings reached 20 days of age and the true leaves had fully emerged, the previously described treatments were applied to the seedlings (2.5). Subsequently, one leaf was collected separately from each plant at 0, 1, 5, and 10 days post treatment (dpt) application, in three replicates. The leaf samples were immediately flash-frozen in liquid nitrogen and then stored at -80 °C for subsequent analyses.

Total RNA extraction, first-strand cDNA synthesis, and quantitative real-time PCR (qRT-PCR) were performed according to previously described methods (Alkooranee et al., 2015). Specific primers targeting *BoTLP1* and *MDH* genes were used, while *GAPDH* served as the internal reference gene for normalization **Table 1**.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD) with three replicates per treatment. Data were analyzed using analysis of variance (ANOVA) with GenStat software. Mean comparisons were performed using the least significant difference (LSD)

test at $P < 0.05$. The relative change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Results and discussion

Synergistic Effects of T. viride and Fertilization Regimes on Growth and Physiological Characteristics of broccoli

The results of greenhouse conditions demonstrated that the combined treatment (*T. viride* + Vit-Org VG + nitrogen fertilizer) significantly outperformed the control across all evaluated traits, exhibiting the highest values in plant height, shoot and root fresh and dry biomass, and chlorophyll content. This enhancement suggests a synergistic interaction among the applied factors, resulting in improved plant growth and physiological performance after 40 days of seed germination **Figure 1**.

The plant height (cm), fresh and dry weight of the shoots (g), fresh and dry weight of the root system, and chlorophyll content were measured in broccoli seedlings treated with the fungus *T. viride* 18 cm, 15.21 gm, 1.82 gm, 1.72 gm, 0.090 gm and 79.34, respectively, and for Vit-Org VG fertilizer treated reached 15 cm, 14.34 gm, 1.61 gm, 1.44 gm, 0.083 gm and 76.76, respectively, and when treated with nitrogen fertilizer reached 14 cm, 13.15 gm, 1.32 gm, 0.95 gm, 0.040 gm and 77.19, respectively, and reached 20 cm, 16.63 gm, 2.08 gm, 1.80 gm, 0.091 gm and 80.62, respectively when treated with combined treatment compared with control treatment reached 11 cm, 10.33 gm, 1.15 gm, 0.77 gm, 0.040 gm and 71.15, respectively (**Table 2**).

The increase in plant height and biomass observed under the *T. viride* treatment can be attributed to its role as a plant growth-promoting fungus. It

Table 1. qRT-PCR primers used in study

Gene name	Gene ID		Sequence (5'-3')
Thaumatococcus-like protein 1	BoTLP1	F	CCGTGTTGAAGACGGAGGAA
		R	CCAGTCTCTTGATAATCACAC
Malate dehydrogenase	MDH	F	CCGTAACGAAAGCACAAACA
		R	AAGGACCACTGAAATCACGAAC
housekeeping gene	GAPDH	F	CGCTTCCTCAACATCATCCCA

Table 2. Interactive effects of *T. viride*, organic fertilizer and nitrogen fertilizer, and their combined application on growth and physiological traits of broccoli plants

Treatment	Plant height cm	Shoot		Root		Chlorophyll Content SPAD
		Fresh weight gm	Dry weight gm	Fresh weight gm	Dry weight gm	
Control	11.0 d	0.77 d	0.040 c	1.15 e	10.33 e	*71.15 d
<i>T. viride</i>	18.0 b	1.72 ab	0.090 a	1.82 b	15.21 b	79.34 ab
Vit-Org VG	15.0 c	1.44 b	0.083 b	1.61 c	14.34 c	76.76 c
Nitrogen fertilizer	14.0 d	0.95 c	0.040 c	1.32 d	13.15 d	77.19 bc
Combined	20.0 a	1.80 a	0.091 a	2.08 a	16.63 a	80.62 a
L.S.D.	0.94	0.62	0.13	0.14	0.008	1.22

*Each value is the mean of three replicates (n = 3)

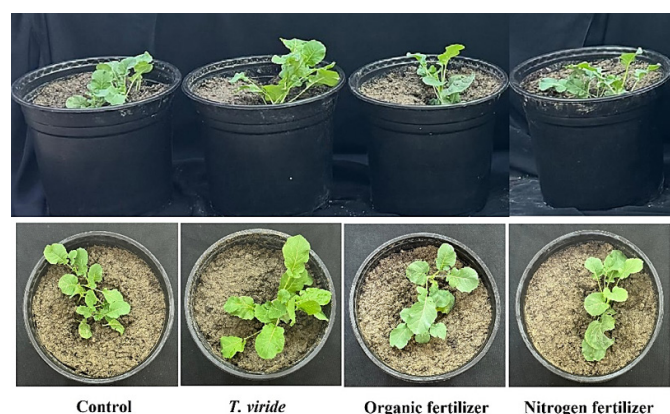


Figure 1. Synergistic effects of *T. viride*, organic and nitrogen fertilizers on broccoli growth and physiology

enhances nutrient uptake by improving root efficiency and secreting organic acids and enzymes that increase nutrient availability in the soil. Furthermore, it produces plant hormone-like compounds, such as auxins (IAA), which promote cell elongation (Vinale et al., 2008; Woo et al., 2022).

The application of organic fertilizer also contributed to the improvement of the studied traits due to its role in enhancing soil structure and increasing cation exchange capacity, as well as serving as a slow-release source of nutrients, thereby ensuring a continuous nutrient supply to the plant (Mthiyane et al., 2024). This explains the superior performance of this treatment compared to the control.

Nitrogen fertilizer (urea, 46% N) improved vegetative growth and chlorophyll content, owing to the essential role of nitrogen in the synthesis of amino acids, proteins, and chlorophyll, as it is a key component of the photosynthetic process (Barandalla et al., 2025). However, its effect was less pronounced than that of the combined treatment, indicating that nitrogen application alone does not maximize plant performance without integration with biological and organic inputs.

The increase in chlorophyll content observed across the treatments, particularly in the combined treatment, reflects enhanced photosynthetic efficiency, which is associated with improved nutrient availability and stimulation of physiological processes within the plant. Several studies have reported that *Trichoderma* spp. can enhance photosynthesis by improving plant nutritional status and mitigating stress conditions (Shoresh et al., 2010).

Gene Expression

The relative expression levels of *MDH* and *BoITLP1* genes in greenhouse conditions of broccoli plants were

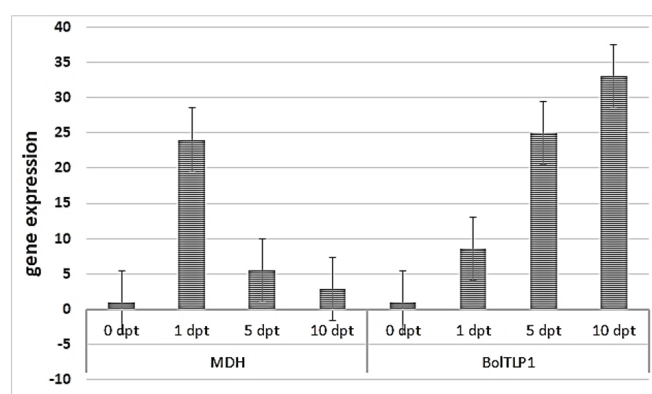


Figure 2. Gene expression levels in leaves of broccoli seedlings were treated at 0, 1, 5, and 10 dpt by *T. viride*. via qRT-PCR, using GAPDH as an internal reference

analyzed at different time points (0, 1, 5, and 10 days post-treatment, dpt) under four treatments: *T. viride*, Vit-Org VG, nitrogen fertilizer, and the combined treatment, using qRT-PCR with GAPDH as an internal reference gene.

Gene expression levels of broccoli treated with *T. viride*

The results presented in **Figure 2** demonstrated that the gene expression patterns of both *BoITLP1* and *MDH* in seedlings of broccoli leaves treated with *T. viride* were significantly affected by different treatments and measurement time periods, reflecting a comprehensive physiological response associated with growth stimulation.

Gene expression levels for the *BoITLP1* gene showed distinct time-dependent changes after treatment. Expression started at baseline on day 0 dpt (Fold change = 1), then gradually increased to 8.6-fold at 1 dpt, continued to rise to 25-fold at 5 dpt, and peaked at 33-fold at 10 dpt.

This pattern suggests a delayed but robust and sustained response in gene expression, indicating that this gene may be involved in long-term regulatory processes related to growth or ongoing physiological responses, rather than an immediate response. The high expression levels at later stages (5–10 dpt) may reflect the activation of regulatory pathways associated with plant growth reprogramming or enhanced metabolic efficiency. Taiz et al. (2015) demonstrated that growth-related genes often exhibit gradual and sustained expression patterns rather than rapid responses.

Furthermore, large changes in expression levels (up to 33-fold) indicate robust gene regulation, consistent with the findings of Bustin et al. (2009) on the subject of regulatory response genes that may show high amplification in relative expression when using $\Delta\Delta Ct$ analysis.

The *MDH* gene exhibited a completely different

pattern, also starting at a baseline level (1-fold at 0 dpt), then rising very sharply at 1 dpt to 24-fold, before declining to 5.5-fold at 5 dpt, and then to 2.9-fold at 10 dpt. The high increase after 1 dpt suggests that this activation was a short-term initial response aimed at supporting energy needs at the onset of the physiological response, followed by a gradual return to homeostasis.

This pattern reflects an early, rapid response followed by a gradual decline, a pattern common in genes associated with primary metabolism and energy production. The sharp rise at 1 dpt indicates an immediate activation of metabolic processes, particularly those related to the malic acid cycle and the energy cycle, where *MDH* plays a key role in the conversion of malate to oxaloacetate within the respiratory pathways (Taiz et al., 2015).

Overall, these results indicate that under *T. viride* treatment, *MDH* is involved in early metabolic adjustment, whereas *BoITLP1* plays a major role in sustained defense responses, with maximum activation observed at 5 dpt. Malate dehydrogenase is a key component of the tricarboxylic acid (TCA) cycle, contributing to energy production and maintaining redox balance—processes essential during periods of high biological activity and rapid growth (Feng et al., 2024). This early surge reflects the plant's ability to rapidly activate its metabolic pathways to meet the increased energy demands resulting from biotic and nutrient treatments.

This pattern reflects its functional association with proteins involved in growth-related physiological processes, including cellular development and metabolic regulation under stimulated conditions. Furthermore, the application of *T. viride* resulted in a pronounced increase in gene expression, especially for *BoITLP1*, which aligns with the known role of this fungus in enhancing plant physiological performance and promoting growth-related pathways (Alkooranee et al., 2015; Zhou et al., 2023).

Gene expression levels of broccoli treated with Vit-Org VG

The relative gene expression patterns of both *BoITLP1* and *MDH* in broccoli leaves showed a clear response to the Vit-Org VG organic fertilizer treatment across different time periods (0, 1, 5, and 10 days post-treatment, dpt).

For *BoITLP1* **Figure 3**, expression remained at a basal level at 0 dpt (1-fold), then increased to 4.6-fold at 1 dpt. A significant increase in expression was observed at 5 dpt, reaching 24.5-fold, the highest recorded value, before decreasing relatively at 10 dpt to 16.4-fold, while

remaining considerably higher than the control level, indicating sustained gene activation over a longer time period.

The *MDH* gene exhibited a different pattern (Figure 3), with expression increasing from a basal level (1-fold) at 0 dpt to 5.3-fold at 1 dpt, then peaking at 5 dpt (8.6-fold), before declining to 2.9-fold at 10 dpt, reflecting a transient, transient response.

These results indicate that Vit-Org VG treatment leads to integrated gene expression activation involving both an early metabolic response and ongoing gene regulation. The early and mid-level increase in *MDH* gene expression is consistent with its role in supporting metabolic processes and energy production during early growth stages (Igamberdiev & Eprintsev, 2016). In contrast, the gradual and robust increase in *BoITLP1* expression, particularly at 5 dpt, suggests its role in long-term regulatory processes associated with growth stimulation and improved plant physiological performance. Furthermore, the fact that both genes reach their highest expression levels at 5 dpt indicates that this period represents a critical phase in the plant's physiological adaptation to organic fertilization. Previous studies have shown that organic treatments and growth promoters lead to coordinated activation of genes involved in metabolism and cell regulation due to enhanced nutrient availability and the presence of growth-promoting compounds such as humic acids (Nardi et al., 2002; Taiz et al., 2015).

Overall, these results demonstrate that Vit-Org VG treatment contributes to a dual gene response, beginning with the activation of metabolic processes, followed by a phase of sustained gene regulation, reflecting efficient physiological adaptation of the plant to organic fertilization.

Gene expression levels of broccoli treated with nitrogen fertilizer

The relative gene expression of both *BoITLP1* and *MDH* in broccoli leaves **Figure 4** showed a clear response to nitrogen mineral fertilizer application across different time points (0, 1, 5, and 10 dpt).

For *BoITLP1*, expression started at a basal level at 0 dpt (1-fold), then increased significantly to 15.4-fold at 1 dpt, indicating an early and rapid response to the treatment. This was followed by a relative decrease in expression at 5 dpt (10-fold), before a marked increase to 27.7-fold at 10 dpt, the highest level recorded (Figure 4).

This biphasic pattern reflects a two-phase response: a rapid initial response followed by a delayed reactivation phase, which may be related to the reorganization of physiological processes due to nitrogen

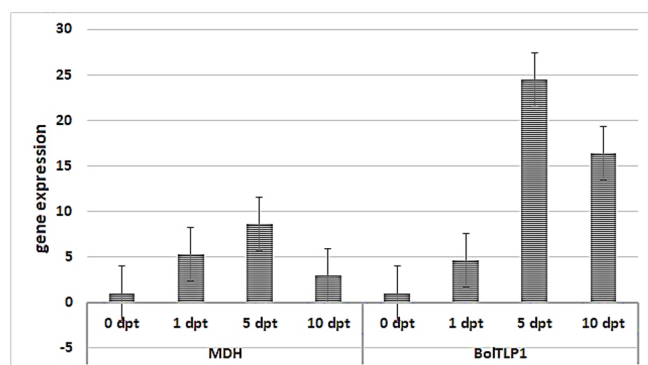


Figure 3. Gene expression levels in leaves of broccoli seedlings were treated at 0, 1, 5, and 10 dpt by Vit-Org VG. via qRT-PCR, using GAPDH as an internal reference

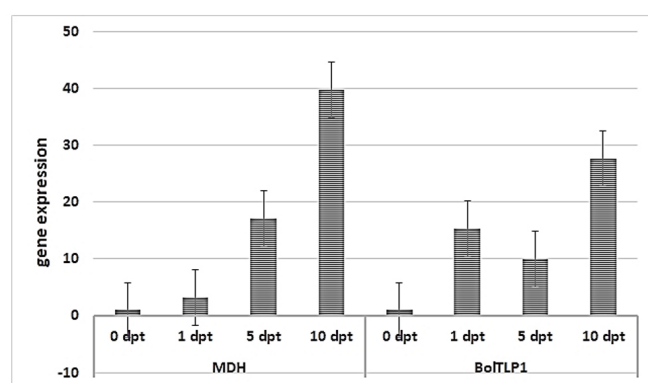


Figure 4. Gene expression levels in leaves of broccoli seedlings were treated at 0, 1, 5, and 10 dpt by Nitrogen fertilizer. via qRT-PCR, using GAPDH as an internal reference

availability. Nitrogen is known to play a pivotal role in regulating growth-related gene expression through its influence on protein and enzyme synthesis (Taiz et al., 2015). The *MDH* gene exhibited a more pronounced progressive pattern, with expression increasing from 1-fold at 0 dpt to 3.15-fold at 1 dpt, then to 17.1-fold at 5 dpt, reaching a very high peak of 39.8-fold at 10 dpt (Figure 4).

This substantial increase indicates a strong and sustained activation of metabolic processes associated with energy production. Since *MDH* is a key component of the citric acid cycle, its increased expression reflects the increased energy demand for growth resulting from nitrogen availability (Igamberdiev & Eprintsev, 2016). This suggests that nitrogen fertilization not only provides the plant with nutrients but also reprograms metabolic and gene activity. Nitrogen availability enhances the efficiency of photosynthesis and amino acid synthesis, which is reflected in increased enzyme activity and growth-related gene expression (Marschner, 2012).

Furthermore, the significant increase in *MDH*

expression at 10 dpt may indicate that the plant has reached an active growth phase requiring high energy levels, thus necessitating enhanced cellular respiration pathways. This pattern reflects activation at the inception and continuation of metabolic processes associated with the citric acid cycle (TCA cycle), indicating increased energy (ATP) demand during early and middle growth phases. Elevated *MDH* is directly associated with increased mitochondrial respiratory activity, an indicator of basal plant metabolism activation (Taiz et al., 2015.; Elsanosi et al., 2024).

Overall, these results suggest that nitrogen mineral fertilization leads to a strong and progressive activation of gene expression, particularly in genes associated with metabolism, along with the stimulation of complex regulatory responses, reflecting a highly efficient physiological adaptation of the plant to nutrient availability.

Gene expression levels of broccoli treated with combined treatment

The relative gene expression results for both *BoITLP1* and *MDH* in broccoli leaves **Figure 5** showed an exceptional response to the synergistic treatment of the probiotic and organic/nitrogen fertilization across different time periods (0, 1, 5, and 10 days post-treatment, dpt).

For the *BoITLP1* gene, expression was at a basal level at 0 dpt (1-fold), then increased significantly to 33-fold at 1 dpt, and continued to rise to 55-fold at 5 dpt, before peaking at 140-fold at 10 dpt. This pattern reflects a strong and sustained upward response, indicating long-term activation of regulatory processes associated with growth and physiological adaptation.

The *MDH* gene exhibited a different pattern, recording its highest expression level at 1 dpt (130-fold),

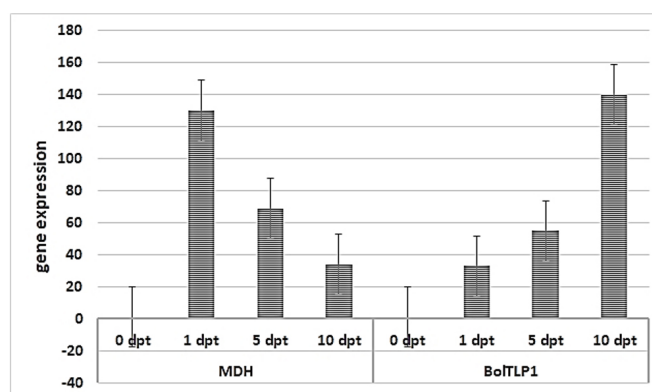


Figure 5. Gene expression levels in leaves of broccoli seedlings were treated at 0, 1, 5, and 10 dpt by Combined treatment. via qRT-PCR, using GAPDH as an internal reference

then decreasing to 69-fold at 5 dpt, and continuing to decline to 34-fold at 10 dpt, while remaining at elevated levels compared to the control. This pattern indicates a rapid and acute metabolic response in the early stages, followed by a gradual decline as activity persists.

These results suggest a functional integration between the early metabolic response and long-term gene regulation under the influence of synergistic treatment. The significant increase in *MDH* expression on day 1 reflects a strong activation of energy production pathways, which is expected due to the high availability of nutrients and growth-promoting substances, leading to increased energy demand to support physiological processes (Igamberdiev & Eprintsev, 2016).

In contrast, the sustained increase in *BoTLPI* expression up to day 10 suggests a role for this gene in regulating the long-term response and promoting sustained growth. This is attributed to the combined effect of, the biotic factor that stimulates biosignatures within the plant; the organic fertilizer that improves uptake efficiency and provides the plant with organic compounds; and the nitrogen fertilization that promotes protein and enzyme synthesis. This synergy leads to a comprehensive reprogramming of gene and metabolic activity within the plant.

The exceptionally high gene expression levels (up to 140-fold) reflect a strong amplifying response, which is often observed when multiple stimuli act in synergy, enhancing molecular signaling and activating a large number of biological pathways simultaneously (Nardi et al., 2002). Furthermore, the difference in peak timing between the two genes (1 dpt for *MDH* and 10 dpt for *BoTLPI*) confirms that the plant response occurs in two main phases: An immediate metabolic response phase to conserve energy (*MDH*) and A sustained gene regulation phase to support growth and adaptation (*BoTLPI*).

These findings are consistent with physiological concepts indicating that optimizing plant nutrition, especially when combining different sources, leads to integrated activation of metabolic and gene regulation pathways (Taiz et al., 2015; Marschner, 2012).

Interaction between bio-stimulation and nutrient supply, leading to enhanced coordination between early metabolic responses and later-stage growth-related processes. The higher expression levels observed for *BoTLPI* compared to *MDH* can be explained by the fact that TLPs are involved in protein accumulation and physiological regulation at later stages, whereas *MDH* primarily contributes to energy supply and metabolic intermediates required for sustaining these processes.

Recent studies have highlighted that TLPs are not only involved in stress-related responses but also play roles in plant development, symbiotic interactions, cell wall remodeling, supporting cell growth and growth regulation, making them important targets for improving crop performance (Taiz et al., 2015.; Buchanan and Gruissem., 2015). In parallel, enzymes such as *MDH* are closely linked to metabolic flexibility and redox homeostasis, which are essential for maintaining cellular function during periods of increased physiological activity (Feng et al., 2024). In general, the results indicated that the treatments led to a clear stimulation of growth-related metabolic activity, through increased gene expression of genes responsible for energy production and cellular regulation. Furthermore, the synchronized response of the two genes suggests an integration of metabolic pathways and molecular regulation during the physiological response (Bustin et al., 2009).

Overall, the combined treatment resulted in a coordinated and enhanced response, characterized by early metabolic activation *MDH* and sustained gene induction *BoTLPI*. The higher expression levels observed in this treatment compared to individual applications suggest a synergistic interaction between *T. viride*, Vit-Org VG, and nitrogen fertilizer, leading to improved broccoli performance and metabolic activity at the molecular level.

Previous research investigating the effects of biofertilizers on broccoli production reported that reducing mineral fertilizer inputs and replacing part of them with microbial inoculants did not lead to significant changes in soil chemical properties, microbial abundance, microbial activity, or crop yield when compared with conventional inorganic fertilization. The application of bacterial inoculants alone or in combination with fungal formulations also showed limited effectiveness in enhancing soil microbial communities or improving functional activity under the studied conditions. However, a positive association was identified between broccoli yield and soil nitrogen content, microbial activity, and CO₂ emissions, indicating the importance of soil biological processes in supporting plant productivity. Overall, these findings suggest that biofertilizers may contribute to reducing dependence on mineral fertilizers without negatively affecting yield, although their direct stimulatory effects may vary depending on environmental and management conditions (Ollio et al., 2024).

Conclusions

The combined application of *Trichoderma viride*, organic fertilizer, and nitrogen fertilizer significantly

enhanced broccoli growth and physiological performance. This improvement was associated with increased expression of *MDH* and *BoTLP1* genes, indicating an early metabolic response followed by sustained growth regulation. Overall, the integrated treatment showed a clear synergistic effect, highlighting its effectiveness in improving broccoli productivity at both physiological and molecular levels.

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