

## Influence of *Trichoderma harzianum* on growth, yield and quality of lettuce (*Lactuca sativa*) grown under a hydroponic system

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### Abstract

Lettuce is a valuable source of iron, vitamins, fiber, and phenolic compounds with health benefits. Enhancing its yield while preserving these compounds is essential for improving its nutritional value. The objectives of this study were to: (1) determine the optimal stage for *Trichoderma harzianum* inoculation (germination vs. seedling) and (2) evaluate its impact on growth, yield, and quality traits of lettuce cultivars grown under a Nutrient Film Technique (NFT) hydroponic system. Two greenhouse experiments were conducted using one Romaine cultivar ('Nebrase') and two Iceberg cultivars ('Lolo-Rossa' and 'Lolo-Bionda'). The results showed that the three inoculation approaches of *T. harzianum*; at the seed stage, transplant stage (by adding *T. harzianum* solution to the root system) or directly adding *T. harzianum* spores to the nutrient solution produced comparable effects on shoot and root growth. In the NFT system, results from the winter cycle (16-24 °C), showed no significant differences, as the conditions were non-stressful. However, during the summer cycle (28-35°C), which imposed heat stress, all cultivars inoculated at the seed stage exhibited significantly higher chlorophyll content (192 vs. 162  $\mu\text{mol m}^{-2}$ ), shoot fresh weight (134 vs. 91 g), leaf area (1170 vs. 840  $\text{cm}^2$ ), root fresh weight (25 vs. 12 g), and leaf number (30 vs. 24) compared to the non-inoculated control. These findings suggest that *Trichoderma* enhances lettuce resilience under heat stress conditions. In terms of cultivars, the Iceberg type 'Lolo-Rossa' consistently showed higher or comparable concentrations of total phenols (112-208  $\text{mg } 100 \text{ g}^{-1}$ ), flavonoids (251-505  $\text{mg } 100 \text{ g}^{-1}$ ), and antioxidant activity (38-46%) compared to the Romaine type 'Nebras'. Conversely, the Romaine type exhibited higher shoot and root biomass and larger leaf area. These findings highlight that *T. harzianum* inoculation, particularly at the seed stage, enhances heat stress tolerance and improves both yield and quality traits in hydroponically grown lettuce.

**Keywords:** flavonoids; NFT; soilless culture; stress; phenolic compounds

### Introduction

Lettuce is one of the main consumed leafy-green vegetables worldwide (Al-Karaki and Othman, 2023). According to the Food and Agriculture Organization (FAO), lettuce is produced in 105 countries with an

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estimated total harvested area of 1.3 million hectares (FAOSTAT, 2020). In 2020, the total lettuce production worldwide was about 27.6 million tons. The leading producers were China (14.3 million tons), the United States (4.4 million tons), India (1.1 million tons), and Spain (0.97 million tons). The market price for a metric ton of lettuce heads varied considerably, ranging from \$210 in Ecuador to \$4,453 in Latvia, with a global average of \$1,319.

Lettuce is characterized by a high water content (94-95%) and a low caloric value (Yang *et al.*, 2022). It is also a rich source of vitamins, minerals, and bioactive compounds such as polyphenols and carotenoids, which contribute to a range of health-promoting properties, including antioxidant, anti-inflammatory, anticancer, and antiviral activities, as well as protection against coronary heart disease (Kumar and Pandey, 2013; Yang *et al.*, 2022). According to Al-Karaki and Altuntas (2021), Romaine lettuce contains total phenolic compounds ranging from 1074 to 1322 mg per 100 g dry weight (DW), flavonoids from 541 to 726 mg per 100 g DW, and exhibits antioxidant activity ranging from 47% to 59%. In a separate study, Gan and Azrina (2016) reported that the ferric reducing antioxidant power (FRAP) of iceberg lettuce was 48 mM Fe per 100 g shoot fresh weight (SFW), while Romaine lettuce demonstrated a significantly higher FRAP value of 126 mM Fe per 100 g SFW.

Hydroponic systems are increasingly adopted worldwide to optimize growing conditions and ensure the precise delivery of water and nutrients to horticultural crops, including lettuce (Othman *et al.*, 2019; Putra and Yulianda, 2015). This soilless cultivation technique employs either liquid nutrient solutions or inert solid substrates such as coco peat, perlite, or rockwool as growth media (Othman *et al.*, 2024; Tüzel *et al.*, 2019; Tzortzakis *et al.*, 2022). The global market of hydroponics systems valued at \$9.5 billion in 2020 and is anticipated to reach \$17.9 billion by the year 2026 (MMRP, 2020). One of the principal advantages of hydroponic cultivation is the decoupling of plant growth from soil-related limitations, including soil-borne pathogens, salinity, suboptimal physical properties, and restricted nutrient availability (Tzortzakis *et al.*, 2022). Moreover, key aspects of the plant microenvironment—such as pH, nutrient concentration, and moisture levels—can be precisely regulated in hydroponic systems, thereby enhancing plant growth, yield, and overall production efficiency (Azizoglu *et al.*, 2021). Among water-based hydroponic systems, the Deep Water Culture (DWC) and Nutrient Film Technique (NFT) are the most commonly utilized methods for cultivating leafy greens such as lettuce. In these systems, seedlings are typically germinated in a solid medium before being transferred to the hydroponic setup. In contrast, substrate-based hydroponics—employing inert solid media—is predominantly used for fruiting vegetables such as tomatoes and peppers (Tüzel *et al.*, 2019). Despite their advantages, hydroponic systems, particularly closed systems like NFT, face key challenges related to nutrient management, product quality (leaf or fruit), and plant–microbe interactions, including the control of root-zone pathogens (Schwarz *et al.*, 2008). To optimize plant growth and yield in hydroponic cultivation, it is essential to carefully regulate the root and shoot microclimate parameters, select appropriate growing media, and manage microbial populations within the rhizosphere (Al-Ajlouni *et al.*, 2017; Othman *et al.*, 2019).

Fungi of the genus *Trichoderma* have been extensively utilized in crop production, particularly within soil-based cultivation systems. These fungi form beneficial symbiotic associations with host plants, often inducing systemic resistance through modulation of plant gene expression (Alfano *et al.*, 2007). Moreover, *Trichoderma* spp. can reduce the dependence on mineral fertilizers by enhancing soil microbial activity and nutrient availability. For instance, in tomato cultivation, the application of *Trichoderma* has been reported to improve rhizosphere microflora, increase nutrient availability, and decrease chemical fertilizer requirements by up to 25% (Cai *et al.*, 2015). Recently, the incorporation of *Trichoderma* species into hydroponic systems has attracted growing interest due to their potential to enhance plant growth, yield, and nutrient uptake. While traditionally known for their biocontrol efficacy in soil-based systems, *Trichoderma* spp. are now being explored for their biostimulant functions in soilless cultivation environments (Hernández-Huerta *et al.*, 2025).

Lettuce (*Lactuca sativa* L.) can be potentially grown worldwide. In recent years, hydroponic cultivation techniques have become increasingly important for lettuce production, particularly in the Mediterranean region. Promwee and Intana (2022) investigated the potential of *Trichoderma* spp. as a plant biostimulant in

hydroponically grown 'Green Oak' lettuce. Their study evaluated three strains of *T. asperellum* (NST-009, NST-028, and CBPin-01), and found that strain NST-009 successfully colonized both roots and leaves, exhibiting 100% survival. However, the efficacy of *Trichoderma* inoculation in hydroponic systems is not universally consistent and may vary in its effects on plant growth, yield, and quality. Gutiérrez-Chávez *et al.* (2025) demonstrated the positive effects of *T. asperellum* inoculation in a floating-root hydroponic system, where the application of strains TaMFP1 and TaMFP2 resulted in significant enhancements in lettuce performance. Compared to non-inoculated controls, plant height increased by 19.0%, root length by 25.7%, total fresh biomass by 76.4%, and total dry biomass by 82.6%. Additionally, leaf number and leaf area were elevated by 18.2% and 33.6%, respectively. These improvements occurred without any adverse effects on visual quality parameters, including leaf appearance and firmness.

A recent meta-analysis by Othman *et al.* (2024) evaluated the effectiveness of arbuscular mycorrhizal fungi (AMF) in hydroponic systems and revealed variable outcomes. Approximately 34% of the cases analyzed reported no positive effect on plant biomass or yield (29% showing no effect and 5% showing negative effects). Similarly, 37% of the studies found no improvement in fruit or leaf quality (16% with no effect and 21% with negative outcomes). In terms of nutrient accumulation in plant tissues, 60% of cases demonstrated no enhancement (47% with no effect and 13% with negative effects). The review emphasized that optimizing key variables—such as nutrient solution composition, timing of inoculation, and cultivar selection—can play a critical role in improving the efficacy of AMF in soilless cultivation systems.

Although the application of *Trichoderma* spp. in hydroponic systems has gained increasing interest, their effects on lettuce quality (particularly total phenolic content and radical scavenging activity) remain insufficiently investigated. Additionally, the optimal stage for *Trichoderma* inoculation (i.e., seed vs. transplant stage) in hydroponically grown lettuce has not been clearly defined. While previous research has primarily focused on the role of *T. harzianum* in promoting plant growth, the present study offers a novel contribution by simultaneously evaluating both the inoculation stage and method within a controlled hydroponic environment, under both stress and non-stress conditions. By linking these treatments to key quality traits, such as total phenolics, flavonoids, and antioxidant capacity, this study provides a more comprehensive understanding of how *T. harzianum* influences plant physiological responses beyond basic growth parameters.

We hypothesize that the timing of *T. harzianum* inoculation (seed vs. seedling stage) significantly influences the growth, yield, and quality traits of hydroponically grown lettuce and that early inoculation at the seed stage enhances physiological performance and antioxidant capacity, particularly under abiotic stress. The objectives of this study were: (1) to identify the optimal inoculation stage (germination vs. seedling) for *T. harzianum* application in hydroponically grown lettuce, and (2) to assess the effects of *T. harzianum* inoculation on growth, yield, and key quality attributes—namely phenolic content, flavonoid concentration, and antioxidant activity—in lettuce cultivated using NFT system.

## Materials and Methods

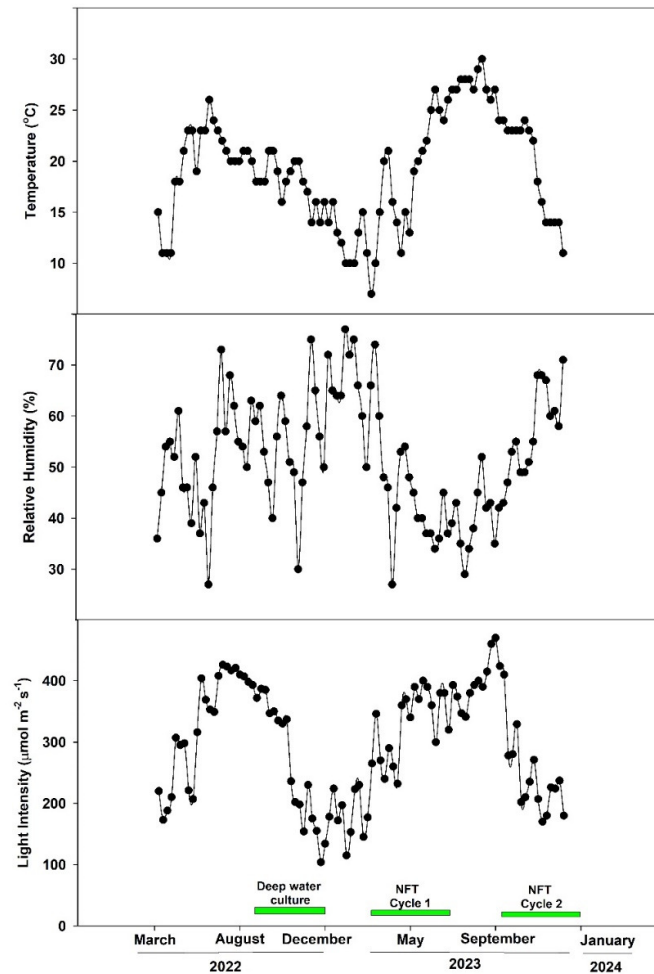
### *Study sites and plant material*

The study was conducted in the laboratories and glasshouse at the University of Jordan, Amman, Jordan. In both greenhouse experiments, lettuce seeds were sown in  $3.75 \times 3.75 \times 3.75$  cm rockwool slabs (Dk-2640; Grodan A/S, Hedehusene, Denmark). The seeded trays were incubated under greenhouse conditions at a temperature of  $22 \pm 3$  °C for a period of 20 days, during which a full-strength Hoagland nutrient solution was applied daily. Throughout the nursery stage, the average minimum and maximum temperatures were 18 °C and 25 °C, respectively. Relative humidity ranged from 40% to 60%, and the average photosynthetically active radiation (PAR) was 300  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

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Three lettuce cultivars were used in the study: one Romane type (Nebrase) and two Iceberg types ('Lolo-Rossa' and 'Lolo-Bionda'). Seeds of lettuce cultivars were obtained from Franchi Sementi (Franchi Seeds 1783, Italy). These cultivars are commonly cultivated in Jordan due to their nutritional value and adaptability to the Mediterranean climate. The fungal strain used for inoculation was *T. harzianum* (accession number MT626718), originally isolated from lemon leaves collected in the Jordan Valley in 2017. Environmental parameters including air temperature, relative humidity, and light intensity were monitored continuously throughout the experimental period using automated data logger systems, and the recorded weather data are presented in Figure 1.



**Figure 1.** Weather data during the study period, including temperature, relative humidity, and light intensity. Green lines represent the starting and the end of deep-water culture and nutrient film technique experiments (cycle 1 and cycle 2)

*Preliminary experiments*

The preliminary experiment aimed to detect the presence of *Trichoderma* in seeds of three lettuce cultivars, with the objective of identifying potential seed-borne fungal contaminants and ensuring the validity of subsequent inoculation assessments. Sixty seeds from each lettuce cultivar were incubated on 90-mm PDA (potato dextrose agar) plates at 25°C for seven days. Fungal and bacterial growth was assessed microscopically (B-Scope Trino E-plan, Euromex, Netherlands), with fungal identification based on spore morphology. Three fungal genera—*Aspergillus*, *Alternaria*, and *Trichoderma*—were identified, with *Aspergillus* being the most prevalent across all cultivars (Table 1). Bacterial colonies were more common in the Iceberg cultivars, particularly ‘Lolo-Rossa’, whereas the ‘Romane cultivar ‘Nebrase’ showed minimal bacterial presence.

**Table 1.** Total number of fungi and bacteria recovered from the three lettuce cultivars based on 60 seeds and three replicates per cultivar (Iceberg type: ‘Lolo-Bionda’ and ‘Lolo-Rossa’; Romane type: ‘Nebrase’).

Cultivar	Fungus					Bacteria	
	<i>Aspergillus</i>	<i>Alternaria</i>	<i>Trichoderma</i>	Total	%	Total	%
‘Lolo- Bionda’	0.0	1.0	0.0	1.0	0.03	6.0	0.375
‘Lolo- Rossa’	0.0	0.0	0.0	0.0	0.00	9.0	0.562
‘Nebrase’	27.0	0.0	7.0	34.0	0.97	1.0	0.062
<b>Total</b>	27.0	1.0	7.0	35.0	100	16	100

Following the initial experiment, a second trial was conducted to evaluate the effect of temperature on endophytic fungal recovery and seed germination, aiming to assess whether heat treatment could eliminate *Trichoderma* without impairing seed viability. Seeds from each cultivar were submerged in a water bath at 25, 35, 40, 45, 50, or 55 °C for 15 minutes (three replicates of 20 seeds per treatment). Treated seeds were plated on PDA and incubated at room temperature under an 18:6 light cycle. Germination began by day two, while fungal colonies emerged after 10 days. Results showed a significant cultivar × germination rate interaction, but temperature had no significant effect on fungal or bacterial recovery (Table 2). Heat treatment was therefore excluded from subsequent experiments to preserve seed viability.

**Table 2.** Analysis of variance (ANOVA) for the effect of different temperatures on the recovery of endophytic fungi and bacteria associated with lettuce (Iceberg type ‘Lolo-Bionda’ and ‘Lolo-Rossa’; Romane type ‘Nebrase’) seeds and the seed germination percentage

Cultivar	Temperature °C	Germination %	Total no. of seeds with fungi	Total no. of seeds with bacteria
	25 (control)	82.2 ab	2 a	3.11 ab
	35	71.1 b	4 a	2.67 abc
	40	78.89 ab	3.67 a	4.56 a
	45	77.8 ab	2.1 a	0.67 c
	50	86.7 a	2.89 a	2.89 abc
	55	55.6 c	1.67 a	2 bc
Lolo- Bionda		89.4 a	2.7 ab	3.4 a
Lolo- Rossa		70.6 b	1.28 b	1.94 a
Nebrase		66.1 b	4.17 a	2.56 a
Cultivar (C)		<.0001	0.01	0.17
Temperature (T)		0.0008	0.31	0.04
C×T		0.0003	0.06	0.36

Means (n = 4) in columns followed by different letters are significantly different at p < 0.05 according to least significant differences (LSD)

*Experiment I: Determining the optimal stage to inoculate T. harzianum on lettuce (germination vs. seedling)*

The DWC experiment was conducted from August to December 2022. *T. harzianum* inoculum was prepared from a 2-week-old *T. harzianum* PDA culture using wheat seeds as a carrier. The wheat seeds were soaked in water for 24 hours, autoclaved at 121°C and 15 psi, and inoculated with a *T. harzianum* spore suspension under aseptic conditions. The inoculated seeds were incubated in sterilized glass jars at 25 °C in the dark for two weeks, then dried under laminar flow, ground into powder, and stored in sterile jars. This powder was used to inoculate the lettuce plants at a concentration of  $1 \times 10^6$  spores/mL (Abu-Shanab *et al.* 2022).

*Trichoderma* application methods were tested using a DWC system under greenhouse conditions. The DWC was selected for its simplicity and ease of setup, enabling controlled evaluation of *T. harzianum* inoculation timing without the variability associated with continuous flow systems. Although NFT system may yield different plant responses due to their dynamic root environment, the pre-test served as a practical screening step to identify the optimal inoculation stage. The selected timing was then validated in the main experiment using the NFT system, in line with the study's primary objectives.

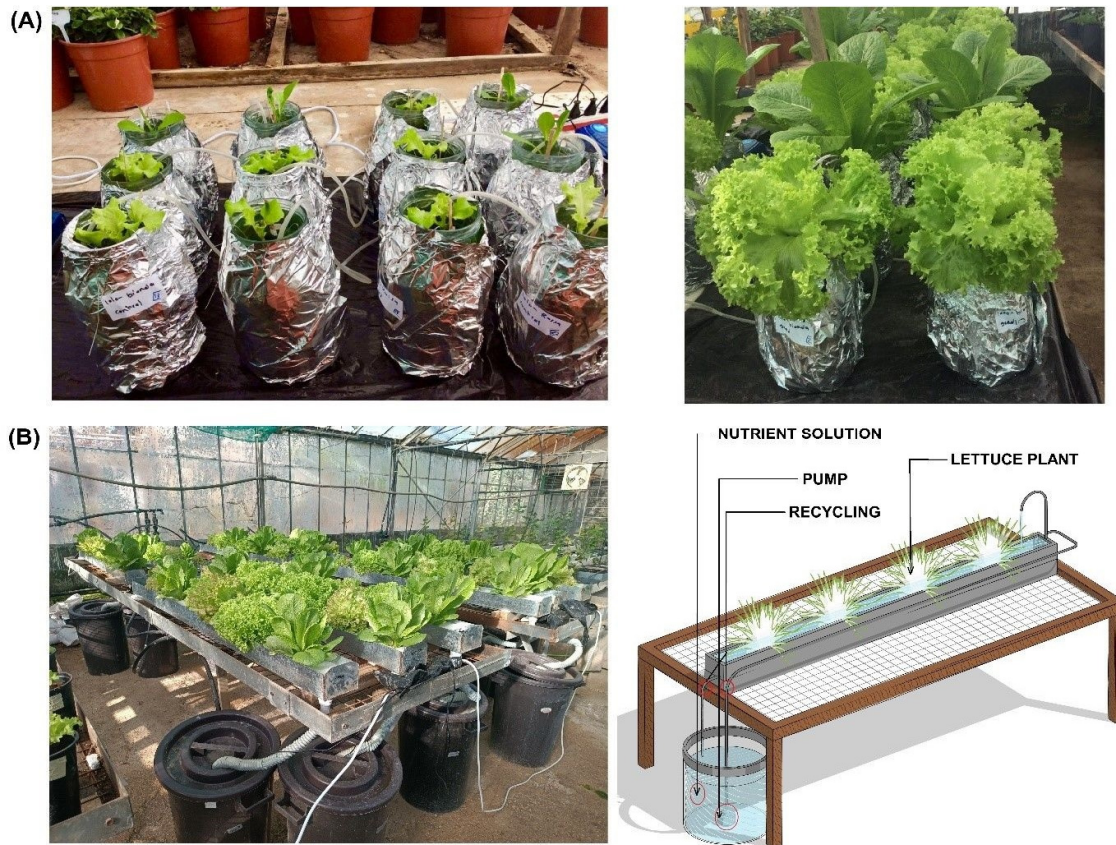
Four treatments were tested: a non-inoculated control and three *T. harzianum* application stages:

1. **Seed stage inoculation** - 1 mL of spore suspension ( $1 \times 10^6$  spores/mL) applied directly to each planting hole at sowing;
2. **Transplant stage inoculation** - 10 mL of spore suspension at  $1 \times 10^5$  spores/mL applied per plant two weeks after sowing (totaling  $1 \times 10^6$  spores per plant);
3. **Nutrient solution inoculation** -  $1 \times 10^6$  spores added per 2,400 mL of nutrient solution at the start of the experiment, using a diluted stock spore suspension.

Lettuce transplants, as described in the "Study Sites and Plant Material" section, were used in this experiment. Seeds were sown in rockwool slabs and incubated in a greenhouse for 20 days under controlled conditions ( $22 \pm 3$  °C, 40–60% RH, and  $300 \mu\text{molm}^{-2}\text{s}^{-1}$  light intensity), with daily application of full-strength Hoagland solution. All transplants were then transferred to 2,400 mL glass bottles filled with full-strength Hoagland solution (Figure 2a). Each bottle was equipped with an air stone for root aeration and wrapped in aluminum foil to inhibit microbial growth. The experiment was set up in a randomized complete block design (RCBD) with four replicates. Lettuce seedlings were grown in the DCW system for 50 days. Seventy days after sowing, chlorophyll content was measured using a chlorophyll meter (MC100; Opti-Science, NH, USA). Plants were then separated into shoots and roots for assessment of plant height, root length, leaf number and shoot and root fresh and dry weight. To confirm the presence of *Trichoderma*, root, stem, and leaf samples were aseptically collected and sequentially plated on PDA. Plates were incubated at 25-28°C for several days, and examined for typical *Trichoderma* colonies, which appear green or yellowish-green with a fuzzy texture.

*Experiment II: Effect of T. harzianum on growth, yield and quality of lettuce grown under hydroponic (NFT) system*

This experiment was carried out using NFT during the Spring (April-June) and Fall (October-December) of 2023. Lettuce transplants were used in this experiment, as outlined in the "Study sites and plant material" section. Lettuce cultivars were subjected to three treatments: (1) application of *T. harzianum* spore suspension ( $1 \times 10^5$  spores/mL) to germinated seeds on the day of sowing in trays, and (2) addition of *T. harzianum* inoculum to the nutrient solution at a rate of 0.05 g/L at transplanting (20 days after sowing) and (3) control transplants without inoculation. The seedling-stage inoculation was omitted from the NFT system because this treatment was initially evaluated in Experiment I under the DWC system. Based on those results, seed-stage inoculation proved to be the most effective and was therefore selected for further testing in the NFT experiment, in alignment with the study's primary objectives.



**Figure 2.** Hydroponic culture systems, (A) DWC after two and seven weeks after sowing, (B) NFT after seven weeks of transplanting

The management and incubation of seedlings prior to transplanting into the NFT system were conducted as described in the “Study sites and plant material” section. The NFT channels’ dimension measured 2 m in length, 0.155 m in width, and 0.15 m in depth, with six plants per channel at 0.33 m spacing. Each channel was connected to an individual 60 L nutrient solution tank, with a submerged pump (2,500 L/h) providing continuous circulation.

The experiment followed a split-block design with four replicates and two subsamples per replicate. Lettuce seedlings were grown in the NFT system for 55 days (Figure 2b), with chlorophyll content measured during the vegetative growth stage using a chlorophyll meter (MC-100 plus; Opti-Science, NH, USA). Plants were harvested at 70 days after sowing, separated into shoots and roots, and assessed for fresh and dry weight. Plant height, leaf number, and leaf area were measured for four randomly selected heads per treatment. The total leaf area ( $\text{cm}^2 \text{ plant}^{-1}$ ), was measured using leaf area meter (LI-3100; ADC BioScientific, Hoddesdon, UK).

For phenolic and scavenging analysis, fresh leaf samples (20 g) from each experimental treatment were extracted with 70% methanol. The extracts were filtered and the volume adjusted to 50 mL with 70% methanol. Antioxidant activity was assessed using of DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging assay. DPPH was prepared by dissolving 0.025 g in 50 mL of methanol. For each assay, 0.1 mL of plant extract was mixed with 3.7 mL methanol and 0.2 mL of the DPPH solution. The mixture was incubated in the dark for one hour, and absorbance was measured at 516 nm against a DPPH-methanol blank using a spectrophotometer. Radical scavenging activity was calculated as inhibition percentage (Williams *et al.*, 1995) using the formula:

$$\text{Inhibition\%} = [(\text{Absorbance of blank} - \text{Absorbance of sample}) / \text{Absorbance of blank}] \times 100$$

Total phenolic content was measured using the Folin-Ciocalteu method following Williams *et al.* (1995) with modifications by AlFayad and Othman (2024). In this assay, 0.2 mL of extract was mixed with 2.5 mL of 0.2 N Folin reagent, and left to stand for 5 minutes, followed by the addition of 2.0 mL of sodium carbonate solution. Samples were incubated at room temperature for two hours, and absorbance was measured at 750 nm against a methanol blank using a spectrophotometer. Results were expressed as gallic acid equivalents (mg GAE/100g of plant fresh weight).

Total flavonoid contents were determined using the aluminum chloride method as described by AlFayad and Othman (2024). In this assay, 0.2 mL of extract was mixed with 2 mL of distilled water and 0.15 mL of 15% sodium nitrate followed by incubation for six minutes. Afterward, 0.15 mL of 10% aluminum chloride (AlCl<sub>3</sub>) was added, and the mixture was incubated for another 6 minutes. Next, 2 mL of 4% sodium hydroxide (NaOH) was added, and the final volume was adjusted to 5.0 mL with distilled water. After a 15-minute incubation at room temperature, absorbance was measured at 510 nm using a spectrophotometer against a distilled water blank. Total flavonoid content was calculated using a catechin standard curve and expressed as catechin equivalents (mg/100g fresh weight).

#### Statistical analysis

Experiment I (deep-water culture) was arranged in randomized complete block design (RCBD) with two factors (*T. harzianum* inoculation stage and lettuce cultivars) and four replicates. Experiment II (NFT system) was arranged in split-block design with four replicates and two sub-samples within each replicate. Statistical analysis was performed using SAS software (Version 9.1 for Windows; SAS Institute, Cary, NC). Prior to conducting analysis of variance (ANOVA), data were assessed for normality and homogeneity of variances to verify compliance with ANOVA assumptions. ANOVA was then used to evaluate treatment effects and interactions, and mean comparisons were performed using the least significant difference (LSD) test at  $p < 0.05$ . Graphical representations of the results were generated using Sigmaplot (Version 14.0 for Windows; Systat Software, San Jose, CA).

## Results

### *Determining the optimal stage for T. harzianum Inoculation in Lettuce*

Table 3 shows the influence of different *T. harzianum* inoculation approaches on lettuce cultivars grown in DWC hydroponic system. Across all evaluated parameters-including chlorophyll content, shoot and root fresh and dry weights, leaf number, total water use, and colonization percentage-the three inoculation stages consistently resulted in values that were either significantly higher or statistically similar to the control, and never significantly lower. Apart from total water use and colonization percentage, no substantial differences were observed among the three inoculation stages for the measured traits.

Regarding cultivar performance, the Romane type ('Nebrase') had higher shoot and root components compared to Iceberg types 'Lolo-Bionda' and 'Lolo-Rossa'. Although Iceberg type 'Lolo-Bionda' and 'Lolo-Rossa' had lower chlorophyll content and water consumption than the Romane type 'Nebrase', the colonization percentages of *T. harzianum* remained similar across all cultivars.

**Table 3.** Growth parameters and colonization percentage of lettuce cultivars grown in DWC hydroponic system and inoculated with *T. harzianum* at different stages of cultivation.<sup>1,2,3</sup>

Inoculation stage	Chl ( $\mu\text{mol m}^{-2}$ )	SFW (g plant <sup>-1</sup> )	RFW (g plant <sup>-1</sup> )	LN (no. head <sup>-1</sup> )	SDW (g plant <sup>-1</sup> )	RDW (g plant <sup>-1</sup> )	TWU (mL cycle <sup>-1</sup> )	Colonization (%)
<b>Inoculation date (ID)</b>								
Control	115 a	136 b	21.1 b	20.8 b	6.27 b	0.79 b	2176 b	37.5 c
Seed	132 a	180 a	31.6 ab	24.1 ab	8.65 a	1.18 ab	2663 a	95.9 a
Transplant	136 a	174 a	36.8 a	25.2 a	8.40 a	1.32 a	2656 a	80.7 b
Addition to the nutrient solution after transplanting	136 a	153 ab	29.6 ab	22.0 ab	7.76 ab	1.05 ab	2041 b	94.5 a
<b>Cultivar (C)</b>								
'Lolo- Bionda'	81.4 b	161 b	35.1 b	20.1 b	7.49 b	1.38 a	2418 b	73.9 a
'Lolo- Rossa'	82.1 b	123 c	18.4 b	22.4 b	5.51 c	0.59 b	1748 c	80.2 a
'Nebrase'	226 a	199 a	35.9 a	26.6 a	10.3 a	1.29 a	2986 a	77.1 a
<b>ANOVA</b>								
ID	0.09	0.0015	0.06	0.01	0.01	0.06	<0.0001	<0.0001
C	<0.0001	<0.0001	0.0019	<0.0001	<0.0001	0.0001	<0.0001	0.52
ID × C	0.09	0.15	0.46	0.64	0.47	0.23	0.65	0.25

<sup>1</sup>Chlorophyll content (Chl), shoot fresh weight (SFW), root fresh weight (RFW), leaf number (LN), root dry weight (RDW), shoot dry weight (SDW), total water used (TWU), and colonization percentage

<sup>2</sup>Means in columns followed by different letters are significantly different at  $p < 0.05$  according to least significant differences (LSD)

<sup>3</sup> The DWC experiment was conducted between August and December 2022

#### *Effect of T. harzianum Application on Growth, Yield, and Quality of Lettuce Grown in a Hydroponic (NFT) System*

Two crop cycles were conducted using NFT system to evaluate the impact of *T. harzianum* inoculation, applied either at the seed sowing stage or by directly adding spores to the nutrient solution (Figure 4). During the first cycle, inoculation at the seed stage significantly enhanced several growth parameters, including chlorophyll content, shoot fresh weight, leaf area, root fresh weight, leaf number, and colonization percentage, compared to the control (Table 4). Moreover, seed-stage inoculation outperformed nutrient-tank inoculation in enhancing chlorophyll content, shoot fresh weight, and leaf area.

With regard to cultivar differences, the Romane type 'Nebrase' had higher chlorophyll content, leaf area, and root fresh weight than Iceberg types ('Lolo-Bionda' and 'Lolo-Rossa'), grown under NFT conditions. Interestingly, the inoculation treatment × cultivar interaction showed that only Nebrase consistently exhibited higher shoot and root fresh weight under both inoculation strategies—seed-stage and nutrient-tank inoculation—relative to its control (Table 5).

**Table 4.** Growth parameters and colonization percentage of lettuce cultivars inoculated with *T. harzianum* at different stages of cultivation under hydroponic (NFT) system <sup>1,2,3</sup>.

Inoculation stage	Chl ( $\mu\text{mol m}^{-2}$ )	SFW (g plant <sup>-1</sup> )	Leaf area (cm <sup>2</sup> )	RFW (g plant <sup>-1</sup> )	LN (no. Head <sup>1</sup> )	Colonization (%)
<b>Cycle 1</b>						
<b>Inoculation date</b>						
Control	162 b	90.9 b	839 b	11.8 b	23.9 b	54.4 b
Seed	192 a	134 a	1170 a	25.4 a	30 a	93.2 a
Addition to nutrient solution after transplanting	160 b	102 b	942 b	31.04 a	26.2 ab	100 a
<b>Cultivar (C)</b>						
'Lolo -Bionda'	113 b	135 a	1020 b	22.8 b	29.2 a	90.3 a
'Lolo -Rossa'	86.9 b	37.1 b	237 c	10.2 c	19.3 b	91.4 a
'Nebrase'	313 a	155 a	1695 a	35.3 a	31.6 a	95.6 a
<b>ANOVA</b>						
ID	0.02	0.004	0.0014	0.004	0.04	0.03
C	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.74
ID × C	0.14	0.01	0.59	0.0004	0.07	0.11
<b>Cycle 2</b>						
<b>Inoculation date</b>						
Control	112 a	221 a	1563 a	29.4 a	28.3 a	58.9 b
Seed	122 a	266 a	2156 a	36.9 a	31.6 a	87.8 a
Addition to the nutrient solution after transplanting	143 a	260 a	2074 a	38.3 a	33.8 a	100 a
<b>Cultivar</b>						
'Lolo -Bionda'	75.4 b	289 a	1614 b	41.5 a	26.1 b	80.0 a
'Lolo -Rossa'	89.2 b	141 b	730 c	18.0 b	27.8 b	81.1 a
'Nebrase'	225 a	316 a	3448 a	45.1 a	39.8 a	85.6 a
<b>ANOVA</b>						
ID	0.51	0.19	0.41	0.3	0.08	0.01
C	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.60
ID × C	0.44	0.04	0.47	0.03	0.5	0.03

<sup>1</sup>Chlorophyll content (Chl), shoot fresh weight (SFW), leaf area, root fresh weight (RFW), leaf number (LN) and colonization percentage

<sup>2</sup>Means in columns followed by different letters are significantly different at  $p < 0.05$  according to least significant differences (LSD)

<sup>3</sup>This experiment was conducted using the NFT during spring (April–June; Cycle 1) and fall (October–December; Cycle 2) of 2023

**Table 5.** Quality components (phenols, flavonoids, and antioxidant activity) of lettuce cultivars inoculated with *T. harzianum* at different cultivation stages. <sup>1,2,3</sup>

Inoculation stage	Phenols <sup>2</sup> (mg100g <sup>-1</sup> )	Flavonoids (mg100g <sup>-1</sup> )	Antioxidant activity (%)
<b>Cycle 1</b>			
<b>Inoculation date (ID)</b>			
Control	150 b	437 a	44.9 a
Seed	146 b	492 a	44.7 a
Addition to the nutrient solution after transplanting	193 a	513 a	43.2 a
<b>Cultivar (C)</b>			
'Lolo -Bionda'	146 b	516 a	42.4 a
'Lolo -Rossa'	208 a	505 a	46.1 a
'Nebrase'	135 b	420 a	44.4 a

<b>ANOVA</b>			
ID	0.02	0.14	0.78
C	<0.0001	0.08	0.35
ID × C	0.0004	0.0001	0.01
<b>Cycle 2</b>			
<b>Inoculation date</b>			
Control	78.8 b	170 a	35.4 a
Seed	85.9 ab	177 a	35.8 a
Addition to nutrient solution after transplanting	94.6 a	169 a	36.5 a
<b>Cultivar (C)</b>			
'Lolo-Bionda'	79.8 b	160 b	37.0 a
'Lolo-Rossa'	112 a	251 a	38.0 a
'Nebrase'	68.1 c	105 c	32.5 a
<b>ANOVA</b>			
ID	0.03	0.4	0.78
C	<0.0001	<0.0001	0.16
ID × C	0.01	0.0007	0.86

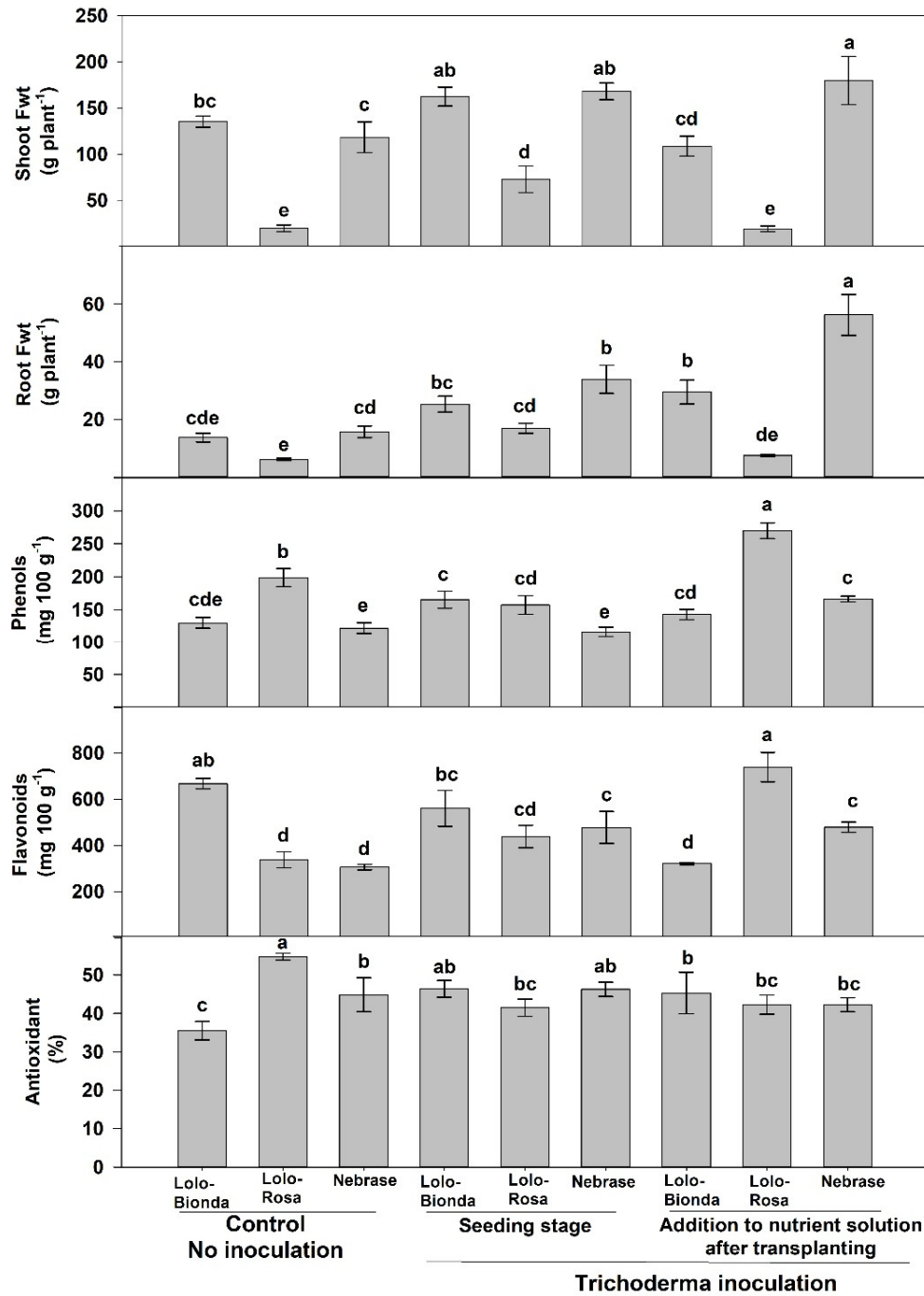
<sup>1</sup>Means in columns followed by different letters are significantly different at  $p < 0.05$  according to least significant differences (LSD)

<sup>2</sup>Total phenolic content was expressed as gallic acid equivalent (GAE) (mg GAE/100 g fresh weight basis) while total flavonoids content was expressed as catechin equivalent (CE) (mg CE/100 g fresh weight basis)

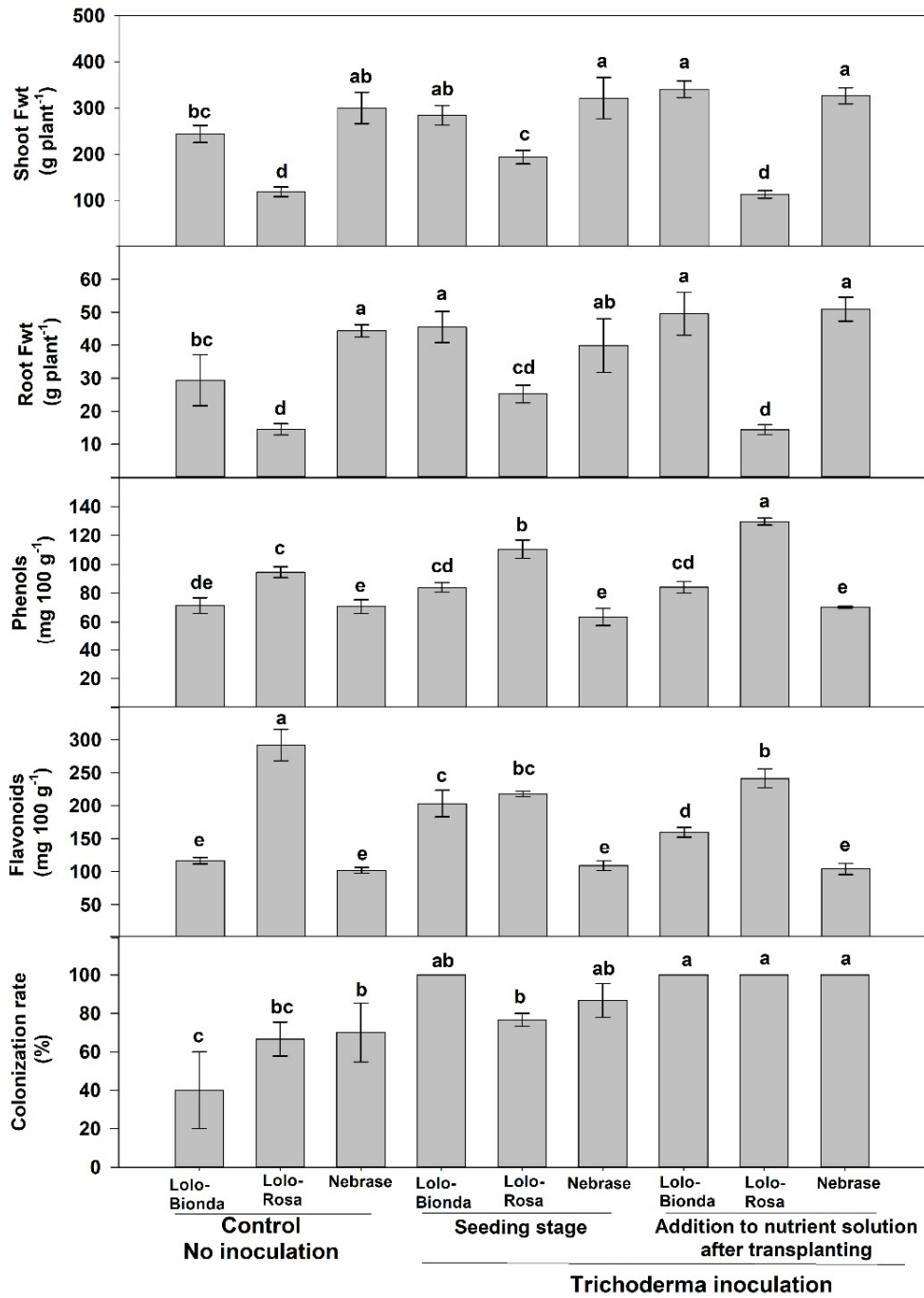
<sup>3</sup> This experiment was conducted using the NFT during spring (April–June; Cycle 1) and fall (October–December; Cycle 2) of 2023

The quality components of lettuce from different cultivars inoculated with *T. harzianum* at seed sowing stage, or by directly adding the inoculum to the nutrient solution after transplanting, are presented in Table 5. In cycle 1, total phenolic content was significantly higher in lettuce inoculated after the transplanting stage compared to both the control and the seed-stage inoculated plants. However, no significant differences were observed in flavonoid concentration or antioxidant activity among the treatments. Across all cultivars, flavonoid content and antioxidant activity were generally comparable, with the exception of total phenolic content (Table 5). The interaction between inoculation treatment and cultivar during the first cycle revealed that 'Lolo-Rossa' plants inoculated nutrient tank addition had significantly higher levels of both phenolics and flavonoids compared to the other treatments (Figure 3). Additionally, 'Lolo-Rossa' plants in the control treatment exhibited the highest antioxidant activity compared to inoculated counterparts (Figure 3).

In contrast to cycle 1, the response of lettuce cultivars to *T. harzianum* inoculation at different stages differed markedly in cycle 2 (Table 4). In this cycle, no significant differences were observed in chlorophyll content, shoot fresh weight, leaf area, root fresh weight, or leaf number between inoculated and control plants. However, colonization percentage was significantly higher in all inoculated treatments compared to the control. Among the cultivars, 'Nebrase' consistently outperformed 'Lolo-Rossa' in terms of chlorophyll content, shoot and root fresh weight, and leaf number (Table 4). 'Nebrase' also exhibited significantly higher chlorophyll content, leaf area, and leaf number than 'Lolo-Bionda'. The interaction between inoculation treatment and cultivar showed that 'Lolo-Rossa' consistently had the lowest shoot and root fresh weight across all treatments (Figure 4).



**Figure 3.** Shoot and root fresh weight, phenolic content, flavonoids, and antioxidant activity of lettuce cultivars inoculated with *T. harzianum* at different cultivation stages (Cycle 1)  
 Total phenolic content was expressed as gallic acid equivalent (GAE) (mg GAE/100 g fresh weight basis) while total flavonoids content was expressed as catechin equivalent (CE) (mg CE/100 g fresh weight basis). Means above bars followed by different letters are significantly different at  $p < 0.05$  according to least significant differences (LSD). This experiment was conducted using the NFT during spring (April–June; Cycle 1) of 2023.



**Figure 4.** Shoot and root fresh weight, phenolic content, flavonoids, and colonization rate of lettuce cultivars inoculated with *T. harzianum* at different cultivation stages (Cycle 2). Total phenolic content was expressed as gallic acid equivalent (GAE) (mg GAE/100 g fresh weight basis) while total flavonoids content was expressed as catechin equivalent (CE) (mg CE/100 g fresh weight basis). Means above bars followed by different letters are significantly different at  $p < 0.05$  according to least significant differences (LSD). This experiment was conducted using the NFT during fall (October–December; Cycle 2) of 2023.

With respect to leaf quality, inoculation via nutrient tank addition at the transplanting stage significantly increased phenolic content compared to the control (Table 5). However, total flavonoid content and antioxidant activity did not differ significantly between inoculated and non-inoculated plants. Among the cultivars, 'Lolo-Rossa' had the highest levels of phenols and flavonoids, with similar antioxidant activity compared to 'Lolo-Bionda' and 'Nebrase'. The interaction between inoculation treatment and cultivar indicated that both Iceberg-type cultivars ('Lolo-Bionda' and 'Lolo-Rossa') accumulated higher phenolic content when inoculated either at the seed stage or via nutrient tank addition (Figure 4). In contrast, the Romane-type 'Nebrase' showed no significant changes in phenolic content across treatments, suggesting a more stable response to *T. harzianum* inoculation.

## Discussion

### *The optimal inoculation stage*

The timing of *T. harzianum* inoculation—whether at the seed stage, transplanting stage, or via direct addition to the hydroponic nutrient solution—similar effects on plant biomass production in DWC system. Except for the non-inoculated control treatment, all inoculated lettuce plants grown in the DWC system showed similar shoot and root fresh and dry weights, indicating that the method of *T. harzianum* application did not significantly affect overall growth performance.

Water consumption of lettuce plants varied depending on the inoculation stage. Inoculation at the seed and transplanting stages resulted in approximately 22% higher water use compared to the control, likely due to increased biomass production in these treatments (Table 3). In contrast, inoculum addition directly to the nutrient solution tank after transplanting reduced water use by 6%, potentially due to limited early root development. These findings suggest that early-stage inoculation enhances both shoot and root development, which may increase the plants' water uptake demands during the growth cycle (de Carvalho *et al.*, 2020).

Interestingly, a 37.5% colonization percentage was observed in both the roots and shoots of non-inoculated control plants (Table 3), indicating the probable presence of seed-borne fungi, including *T. harzianum*. This finding suggests that commercial seeds may harbor endophytic or epiphytic fungal communities even without deliberate inoculation. However, the enhanced biomass observed in inoculated plants highlights that supplemental inoculation still confers additional growth benefits beyond natural colonization.

Despite similar growth outcomes across all inoculation methods, practical considerations influenced their suitability for commercial application. The direct application of liquid inoculum to individual cells was labor-intensive and thus less feasible for large-scale hydroponic systems. Given the comparable results between seed-stage and transplant-stage inoculation, these two methods were selected for further evaluation in subsequent experiments using the NFT system.

### *Effect of T. harzianum Application on Growth, Yield, and Quality of Lettuce Grown in a Hydroponic (NFT) System*

Hydroponics is a soil-free system that uses nutrient-rich solutions to support plant growth and reduce the environmental impacts of conventional farming (Othman *et al.*, 2019; Das *et al.*, 2020). Among these systems, NFT is commonly used for lettuce due to its efficient nutrient delivery, water-use efficiency, and suitability for year-round production in controlled environments (Frasetya *et al.*, 2021). During the first NFT cycle, lettuce plants inoculated with *Trichoderma* at the seed stage exhibited significantly higher chlorophyll content and growth traits (shoot fresh weight, leaf area, root fresh weight, and leaf number) compared to non-inoculated controls (Table 4).

*Trichoderma* species are widely recognized for their multifaceted benefits in agriculture, including enhancement of plant growth, nutrient uptake, and tolerance to abiotic and biotic stresses. These effects are

largely attributed to improved rhizosphere interactions and the production of bioactive compounds with growth-promoting properties (Abbey *et al.*, 2019; Zin and Badaluddin, 2020; Lorito *et al.*, 2010). Numerous studies have shown that *Trichoderma* spp. can improve plant morphology, biomass accumulation, and physiological functions under both optimal and stressful conditions (Abu-Shanab *et al.*, 2022; Halifu *et al.*, 2019; Wanjala *et al.*, 2023). For example, *T. harzianum* increased biomass production and lateral root development in cucumber (Contreras-Cornejo *et al.*, 2009), while *T. longipile* and *T. tomentosum* enhanced early growth in cabbage seedlings (Rabeendran *et al.*, 2000).

During cycle 1, lettuce plants exposed to high-temperature stress (28-35 °C) showed a more pronounced positive response to *Trichoderma* inoculation compared to those in cycle 2, which experienced cooler, non-stressful winter temperatures (16-24 °C).

This is supported by our results from the first cycle, where seed-stage inoculation significantly enhanced chlorophyll content, shoot fresh weight, leaf area, root fresh weight, leaf number, and colonization percentage relative to the non-inoculated control (Table 4). Considering that lettuce performs optimally within a temperature range of 7–24 °C (Mitchell *et al.*, 2002), these findings suggest that *Trichoderma* inoculation can enhance plant resilience and performance under heat stress conditions (Tyskiewicz *et al.*, 2022). *Trichoderma* species are known to thrive and remain metabolically active at 25–30 °C (Mukherjee and Raghu, 1997), suggesting that the cooler temperatures in cycle 2 may have reduced fungal activity and colonization efficiency, resulting in growth responses comparable to control treatments. Under optimal, non-stressful conditions, the beneficial effects of *Trichoderma* on plant growth and physiology appear less evident, reinforcing its role primarily as a stress-alleviating agent in hydroponic systems (Mukherjee and Raghu, 1997).

The application of *T. harzianum* also influenced the biochemical profile of lettuce, particularly phenolic compounds, which are crucial for plant defense and postharvest quality (Dai and Mumper, 2010; Hodges and Forney, 2003). Across treatments, total phenolic content was generally higher or at least comparable in inoculated plants, with the most notable increases observed when *T. harzianum* was applied at the transplanting stage. These findings align with reported phenolic ranges in lettuce (30–1322 mg/100 g DW), flavonoids (100–726 mg/100 g DW), and antioxidant activity (20–59%) (Al-Karaki and Altuntas, 2021; Al-Karaki and Othman, 2023). In this study, lettuce plants inoculated with *T. harzianum* consistently exhibited higher or comparable total phenols, flavonoids, and antioxidant activity compared to controls, particularly when *T. harzianum* added to the nutrient solution after transplanting. Interestingly, phenolic compound levels were generally higher in cycle 1 than in cycle 2, potentially due to a dilution effect caused by the larger shoot biomass in cycle 2, as previously described in plant nutrition studies (Leskovar and Othman, 2021; Jarrell and Beverly, 1981).

Cultivar-specific differences were also evident. The Iceberg-type cultivar ‘Lolo-Rossa’ consistently exhibited higher or similar concentrations of total phenols, flavonoids, and antioxidant activity compared to the Romaine-type ‘Nebrase’, a finding that contrasts with earlier reports suggesting Romaine cultivars generally possess higher phenolic content (Al-Karaki and Othman, 2023). The lower flavonoid content observed in the Nebrase cultivar during cycle 2 may again reflect a dilution effect, as this cultivar produced significantly greater shoot biomass and leaf area than ‘Lolo-Rossa’ and ‘Lolo-Bionda’. These observations underscore the potential for cultivar-dependent responses to *Trichoderma* inoculation and environmental conditions, highlighting the need for further investigation into genotype × environment × microbiome interactions in hydroponic systems.

## Conclusions

This study confirms that *Trichoderma* species, including *T. harzianum*, can occur naturally as an endophyte in commercial lettuce seeds and exert significant biostimulant effects, particularly under suboptimal conditions such as high-temperature stress. By evaluating both the timing (stage) and method of *T. harzianum* inoculation in a controlled hydroponic system, we demonstrated that *T. harzianum* consistently improved

critical growth parameters—such as chlorophyll content, shoot and root biomass, and leaf area—as well as quality-related traits including total phenolics, flavonoids, and antioxidant activity.

These findings support the potential of *T. harzianum* as a sustainable, climate-resilient tool for hydroponic crop production. However, the study also highlights important limitations. The persistence and viability of the fungal inoculum within the hydroponic solution were not monitored, leaving uncertainties about long-term colonization and efficacy. Additionally, potential interactions with native or opportunistic microbial communities (whether competitive or synergistic) were not assessed, which may influence colonization dynamics and plant response under commercial-scale conditions.

Future research should aim to address these limitations by incorporating microbial community profiling, evaluating inoculum stability and activity of *T. harzianum* across successive production cycles, and expanding the scope to include diverse crop species and varied environmental conditions. Furthermore, assessing the economic viability and cost-effectiveness of *T. harzianum* inoculation will be essential for its practical integration into large-scale hydroponic operations.

### Authors' Contributions

Conceptualization, Y.A.O. and K.M.A.; methodology, Y.O. and K.M.A.; software, L.A. and F.M.A.; validation, L.A. and F.M.A.; formal analysis, Y.A.O., K.M.A. and L.A.; investigation, Y.A.O., L.A. and K.M.A.; resources, Y.A.O. and K.M.A.; data curation, Y.A.O. and K.M.A.; writing—original draft preparation, L.A., Y.A.O. and K.M.A.; writing—review and editing, J.A.; visualization, L.A., J.A. and F.M.A.; supervision, Y.A.O. and K.M.A.; project administration, Y.A.O. and K.M.A.; funding acquisition, Y.A.O. and K.M.A.;

All authors have read and agreed to the published version of the manuscript.

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### Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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