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Lettuce (*Lactuca sativa*) Uptake and Phytotoxicity of 1,2,4-Triazole Reduced by Symbiotic Fungi

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ABSTRACT

Soil contamination with nitrification inhibitors (NIs) has received a lot of attention over the past years due to their potential toxic effects on plant and soil systems. However, not many actions have been taken to control and prevent such NI contaminations of soils. This study aimed to investigate the effects of symbiotic fungi in reducing the uptake of the NI 1,2,4,-triazole (TZ) in lettuce (*Lactuca sativa* L. cv. Siyahoo) plants at the Mohaghegh Ardabili University field. For this purpose, three symbiotic fungi (*Serendipita indica*, *Glomus etunicatum* and *G. mosseae*) applied separately, and four doses of TZ (0, 5, 50, and 100 mg kg^{-1} soil) were used in a completely randomized design with four replications. The TZ concentration in plant leaves were quantified by high-performance liquid chromatography (HPLC) analysis. Exposure to TZ at 50 and 100 mg kg^{-1} caused a significant ($P \leq 0.01$) decrease in root colonization by fungal symbionts, chlorophyll content, and leaf fresh/dry weights. Plants colonized with symbiotic fungi had lower levels of TZ compared to non-inoculated plants, which were mostly accompanied by significant reductions in leaf concentrations of the oxidative stress indicators (malondialdehyde and hydrogen peroxide). In particular, lettuce plants inoculated with *S. indica* had 65% and 75% lower TZ levels (at 50 and 100 mg kg^{-1}, respectively) compared to the corresponding non-inoculated plants. The study highlights the adverse impact of TZ on plant growth and health, and underscores the potential of symbiotic fungi as effective measures to alleviate the phytotoxic effects of TZ.

Introduction

In modern agriculture, nitrogen (N) is utilized to enhance crop yield and efficiency (Reeves et al., 1986). However, not all available N is absorbed by plants, potentially leading to the leaching of nitrate (NO_3^-), formed from ammonium (NH_4^+), posing a significant threat to the environment (Grizzetti, 2011; Heydarnajad Giglou et al., 2024). Nitrification inhibitors (NIs) are substances that hinder the nitrification process, extending the retention time of ammonium N in the soil, increasing fertilizer use efficiency, and mitigating the impact of N fertilizer application on climate and the environment (Wang et al., 2019).

Crop growth and high productivity are ensured by the high use of nitrogen (N) fertilizers that pollute the environment, especially when N is in the nitrate form. Ammonium is oxidized to nitrate by nitrogen, but the roots can release biological nitrification inhibitors (BNI). Increased BNI protection reduces residual nitrogen loss and, under many conditions, increases plant nitrogen uptake. BNI reduces absorption in the presence of (1) low ammonium, (2) high soil ammonium, (3) rapid nitrification or slow ammonium uptake by plants, and (4) low accumulation or (5) absorption of rapid nitrification. Antibacterial inhibitors are more easily absorbed by

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humans than bactericides. However, some nitrification is necessary to increase the availability of ammonium and nitrate transport systems. The release of increased BNI should be accompanied by improved ammonium absorption. BNIs can reduce N, but in general they have a positive effect on the environment by increasing N retention significantly (Kuppe et al., 2024).

Nitrification inhibitors suppress the activities of various microorganisms, including ammonia-oxidizing bacteria/archaea, complete ammonia-oxidizing bacteria, and nitrite-oxidizing bacteria (Taylor et al., 2017; Huang et al., 2021; Qiao et al., 2015). Different NIs, such as dicyandiamide (DCD), 3,4-dimethylpyrazolephosphate (DMPP), and pyrazole compounds like 3-methylpyrazole (3-MP) and triazoles such as 1,2,4-triazole (Bundestag, 2017), are utilized in agriculture. Most studies have primarily focused on the effectiveness of NIs in reducing NO_3^- leaching, with limited research on their impact on plants and soil (Min et al., 2021), resulting in an underestimation of their pollution and toxicity.

Toxicity studies on NIs, such as DMPP in clover (*Trifolium repens*) (Rodrigues et al., 2018) and DCD in lettuce (*Lactuca sativa*) (Zerulla et al., 2001; Padash et al., 2022), have been conducted. For instance, Zerulla et al. (2001a) observed that the application of 1.5 kg ha^{-1} of DCD in lettuce caused severe leaf edge burning. Furthermore, Li et al. (2020) found that the use of 1,2,4-triazole (TZ), which disrupts nitrification, negatively affected aerobic and methane-oxidizing heterotrophic bacteria (Li et al., 2020). The control of NIs' toxic effects in the soil is critical for preserving the environment and ensuring the safety of food products.

One approach to mitigate the harmful effects of NIs and prevent their entry into the nutrient cycle is through their immobilization in the soil and plant roots using plant-microorganism bioremediation technologies (Padash et al., 2022). The use of microorganisms for reducing soil and plant pollution is cost-effective and environmentally friendly, preserving the physical, chemical, and biological characteristics of the soil (Soleimani et al., 2011). The utilization of symbiotic fungi to alleviate the negative effects of NIs was pioneered by Padash et al. (2022), who reported that the inoculation of lettuce plants with *Serendipita indica*, *Glomus etunicatum*, and *G. mosseae* reduced the concentration of DCD in the leaves, highlighting the significant impact of symbiotic fungi on reducing DCD toxicity. The presence of mycorrhizal fungi around the roots led to substantial pollutant degradation, suggesting that fungal inoculation of roots holds promise as a method to remediate pollution. For example, inoculation of rye (Secale cereale) plants by *Glomus mosseae* reduced petroleum hydrocarbons pollution, especially pyrene (Yu et al., 2011), and inoculation of alfalfa plants with *G. etunicatum* and *G. mosseae* removed more than 98% of phenanthrene and 88% of pyrene (Gao et al., 2011). The results of experiments in the field of NIs toxicity indicate the potential accumulation of NIs in the soil.

Although various research has focused on controlling DCD toxicity with symbiotic fungi, there are still knowledge gaps surrounding Nis’ toxicity in soil and plants. Considering the economic importance and high consumption of lettuce as a leafy vegetable, the present study was conducted with the aim of reducing the effects of TZ toxicity by using arbuscular mycorrhizal fungi (*G. etunicatum* and G. mosseae) and root endophyte (*S. indica*).

Material and methods

Field description and soil sampling

The research was conducted in 2021-2022 at the Mohaghegh Ardabili University field in Ardabil, Northwest Iran, under semi-arid climate conditions. Soil was collected from the 0-25 cm depth of the field, using a stainless auger. The collected samples were placed in plastic bags, transferred to the laboratory, air-dried, and passed through a 2 mm mesh sieve before undergoing physiochemical analysis.

Soil analyses

Soil chemical and physical properties were assessed using the soil analysis method outlined by Rowell (1994). Soil pH and electrical conductivity (EC) were determined using a 1:5 w/v soil-to-water ratio suspension. Organic matter (OM) content was established by oxidizing the soil organic fraction with potassium dichromate ($K_2Cr_2O_7$) and sulfuric acid (H_2SO_4) (Theng et al., 1999). Available potassium (K^+) was measured via flame photometry, while total N (N) and available P (Olsen-P) were determined using colorimetry with a UV–Vis spectrophotometer (HACH Company, Loveland, USA) (Liu et al., 2019). Available cadmium (Cd) was extracted using a solution containing 0.005 mol L^{-1} diethylene triamine pentaacetic acid (DTPA), 0.01 mol L^{-1} calcium chloride, and 0.1 mol L^{-1} (pH 7.3) triethanolamine (TEA) (Rowell, 1994).

The soil particle fractions (sand, silt, and clay percentages) were determined using the pipette method (Rowell, 1994). The soil exhibited the following physical and chemical properties: 68% sand, 20% silt, 12% clay, 1.2% organic matter, 0.05% total N, 7 mg kg^{-1} available P (Olsen method), 35 mg kg^{-1} available K (Flame Photometer), 0.7 mg kg^{-1} total Cd, with a pH of 7.3, and an electrical conductivity (EC) of 1.3 dS m^{-1}.

Applying the nitrification inhibitor 1,2,4,-triazole (TZ)

1,2,4-triazole powder, manufactured by Merck in Germany, was added to 1 kg of air-dried soil samples to create concentrations of 5, 50, and 100 mg kg^{-1} soil dry weight (DW), following the method described by Maftoun and Sheibany (1979). The soil amended with inhibitors was thoroughly mixed to achieve a homogeneous concentration of 1,2,4-Triazole in the soil. N was applied in the form of $(NH_4)_2SO_4$ from Merck in an aqueous solution to reach a concentration of 40 mg kg^{-1} soil, as outlined by Maftoun and Banihashemi (1981).

Plant cultivation

Lettuce seeds (*Lactuca sativa* L. cv. Siyahoo) were provided from the Seed Bank Institute of Karaj (Iran) and were sown in trays containing peat moss. When the seedlings reached the two-leaf stage (after 10 d), they were transferred to pots with NIs and inoculated with the fungi.

Preparation of the fungal inoculums

Serendipita indica was cultured in Petri dishes containing the Hill and Käfer (2001) medium. The plates were then placed in a growth chamber at 29 ± 1 °C in the dark for a duration of two weeks. Subsequently, one fungal plug with a diameter of 10 mm was positioned 1 cm below the roots of 10 d old lettuce seedlings. Non-inoculated treatments received equivalent weights of autoclaved mycorrhizal inoculum. The mycorrhizal inoculums, including *Glomus mosseae* and *G. etunicatum*, were prepared at the Department of Soil Science, University of Tabriz, following the method outlined by Aliasgharzadeh et al. (2001).

45 d after sowing, lettuce shoots were harvested, and the roots were separated from the soil substrate using tap water and a net to collect all the fine roots. The shoots were rinsed with distilled water, wiped with tissue paper, and dried at 75 °C for 48 h to determine dry weight.

Fungal symbiotic determination

Root segments, each 1 cm long, were separated, weighed, and preserved in 50% ethanol to determine root fungal colonization. The staining method described by Phillips and Hayman (1970) was employed to assess root colonization by fungi. The roots were immersed in a 10% KOH solution and heated for 5 min, followed by washing with water. Subsequently, the root samples were acidified with 1% HCl for 1 min, submerged in a 20% trypan blue staining solution, and heated for 10 min. From the stained samples, 30 root segments (each 1 cm long) per plant were cut and examined for fungal colonization using a light microscope (Olympus BH-2) at ×20 magnification. Root colonization was determined using the gridline intersection method (Giovannetti and Mosse, 1980). The percentage of root colonization per plant was calculated by dividing the total number of colonized root fragments (with arbuscules, vesicles, or hyphae) by the total number of root pieces examined, multiplied by 100.

Measurement of TZ in lettuce leaves

The concentration of TZ in leaf tissues was measured following the method outlined by Blondel et al. (2018), with slight modifications. Approximately 1 g of fresh leaf material from the youngest fully expanded leaf was used, weighed, and homogenized in 30 mL of deionized water using a shaker for 30 min. The resulting homogenate was filtered through Whatman paper No. 42. Subsequently, 5 mL of the obtained filtrate was acidified with 1% formic acid (0.2 mL) and left to stand for 30 min before centrifugation at 4800 rpm for 5 min to remove precipitated material and adjust the pH to match the conditions of high performance liquid chromatography (HPLC). The concentration of TZ in the acidified supernatant was determined using a Waters 2695 HPLC (AGILENT America Company) equipped with an action-H guard column (30 × 4.6 mm internal diameter; ID). The separation was achieved using three mobile phases: water/methanol (97/3 v/v) as phase A, methanol as phase B, and 2% formic acid in deionized water as phase C, at a flow rate of 0.25 mL min^{-1}. Detection was performed at 210 nm using a UV spectrophotometric detector (Blondel et al., 2018) with a flow rate of 0.6 mL min^{-1}.

Measurement of average number of stomata

Selected youngest fully expanded leaves were used to determine the number of leaf stomata through scanning electron microscopy (SEM).

Measurement of leaf chlorophyll and carotenoid contents

For this purpose, 100 mg of fresh leaf tissue was completely ground and homogenized with 10 mL of 80% acetone, then centrifuged at 4 °C for 10 min at 6000 rpm and then the supernatant was separated. The solution at wavelengths of 470, 645, and 663 nm was read by a spectrophotometer (HACH Company, Loveland, USA) (Biehler et al., 2010). Then the content of Total chlorophyll and carotenoid was calculated in terms of mg g^{-1} fresh weight of the tissue.

Measurement of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)

The concentration of MDA in leaves was determined following the method outlined by Heath and Packer (1968). Briefly, 0.1 g of fresh leaf sample was powdered and mixed with 500 mL of 0.1% trichloroacetic acid (TCA). The resulting extract was then centrifuged at 15,000 rpm for 10 min. Subsequently, 250 µL of the centrifuged solution was combined with 750 µL of a solution containing MDA, consisting of 0.5% TCA and 20% trichloroacetic acid (TBA). The mixture was heated in a water bath at 95 °C for 30 min and immediately cooled in ice. Afterward, it was centrifuged again for 15 min at 15,000 rpm. The absorbance of this solution was measured using a spectrophotometer at 532 nm, and the absorbance of other non-specific pigments at 600 nm was determined and subtracted from the obtained value. To calculate the MDA content, an extinction coefficient of 155 M^{-1} cm^{-1} was used. The concentration of H_2O_2 was determined using the method by Sergiev et al. (1997) with slight modifications. In this method, 0.2 g of fresh leaf sample was powdered and mixed with 3 mL of 0.1% TCA, and the resulting extract was centrifuged at 12,000 rpm for 15 min. Subsequently, 0.5 mL of the supernatant was combined with 0.5 mL of 0.2 M phosphate buffer solution and 1 mL of 1 M potassium iodide.

The absorbance of this final solution was read using a spectrophotometer at a wavelength of 390 nm. Finally, the concentration of H_2O_2 was expressed in micromoles per g of fresh leaf weight, with an extinction coefficient of 28 mM^{-1} cm^{-1}.

Statistical analysis

The experimental design was a factorial randomized design, including three fungal treatments (*G. etunicatum*, *G. mosseae*, and *S. indica*), and four doses of TZ (0, 5, 50, and 100 mg kg^{-1} soil) with four replicates per treatment. A two way analyses if variance (ANOVA) was conducted on all experimental data using IBM SPSS version 23 software (Chicago, USA). The variance was analyzed with respect to the main factors (symbiotic fungi and TZ) and their interaction. The differences between means were determined using Duncan's Multiple Range Test at 0.05 and 0.01 probability levels.

Results

Root colonization by symbiotic fungi

The results of comparing the effects of TZ on the colonization percentage of lettuce roots inoculated with the symbiotic fungi S. indica, G. etunicatum, and G. mosseae (Fig. 1) revealed that as the TZ concentration increased, the root colonization significantly decreased. Notably, the highest level of colonization was related to plants treated with S. indica, whereas the lowest level of root colonization was observed in plants treated with 100 mg kg-1 TZ with S. indica, G. etunicatum, and G. mosseae fungi. The substantial reduction in the percentage of colonization at a concentration of 100 mg kg-1 is consistent with the observation in Figure 2, where the root ends of plants treated with 100 mg kg-1 TZ exhibited a spherical morphology, possibly creating unfavorable conditions for fungal colonization and development.

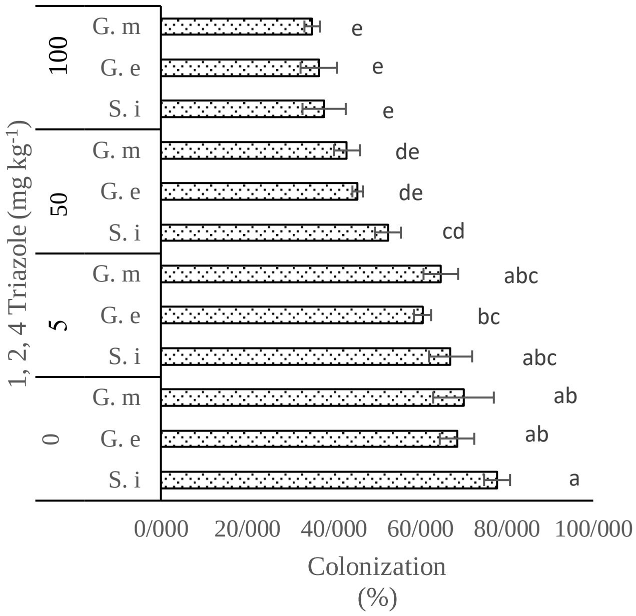

Fig. 1. The effect of different concentrations of 1,2,4-triazole (TZ) on colonization of lettuce roots by *Serendipita indica* (S. i), *Glomus mosseae* (G. m) and *Glomus etunicatum* (G. e) (n = 4, ± SD).

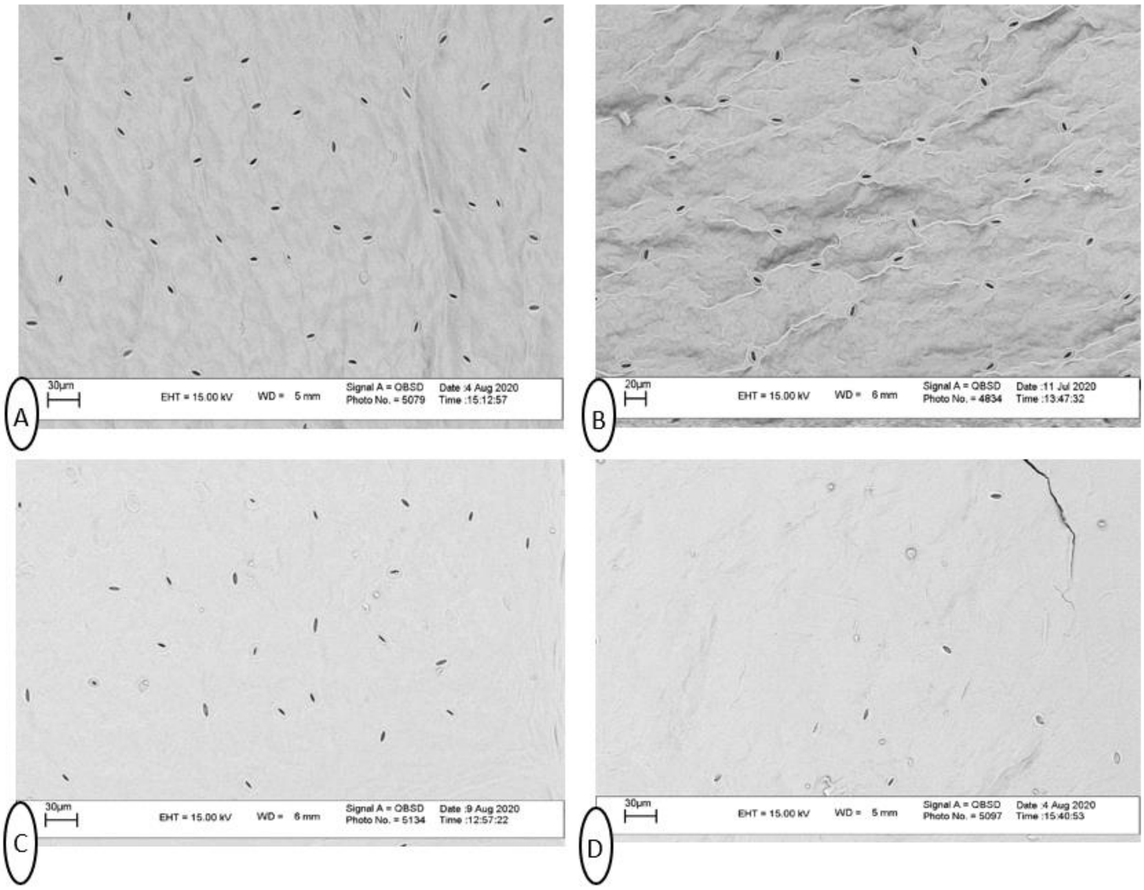

Fig. 2. The effect of different concentrations of 1,2,4-triazole on the number of leaf stomata (A) Control; (B) 5 mg kg^{-1}; (C) 50 mg kg^{-1}; (D) 100 mg kg^{-1} TZ.

Leaf and root characteristics

The application of TZ in concentrations of 50 and 100 mg kg^{-1}, resulted in toxicity symptoms in lettuce leaves, 19 and 15 d after sowing, respectively. These symptoms presented as leaf chlorosis, evident throughout the plant, leading to a gradual decrease in leaf greenness (Fig. 3). However, these symptoms were notably less pronounced in plants inoculated with *S. indica*, *G. etunicatum*, and *G. mosseae* compared to non-inoculated plants.

The impact of TZ on the number of leaf stomata has not been investigated previously. This study observed that the presence of a high concentration of TZ in the culture medium resulted in a reduction in the number of stomata in lettuce leaves. The results regarding the effect of TZ on the number of leaf stomata are presented in Table 1 and Figure 3. It was found that TZ at concentrations of 50 (C) and 100 (D) mg kg^{-1} caused a significant ($P \leq 0.01$) decrease in the number of leaf stomata.

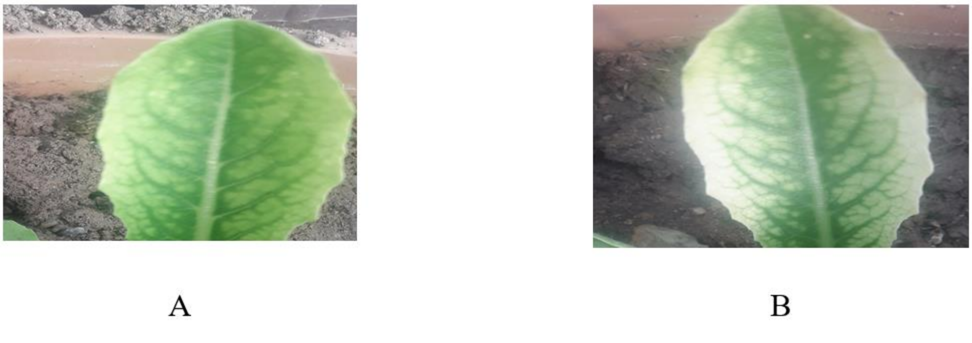

Fig. 3. Phytotoxicity symptoms (chlorosis) in leaves of lettuce plants subjected to 1,2,4-triazole (TZ) at concentrations of (**A**) 50 mg kg^{-1}; (**B**) and 100 mg kg^{-1}.

Table 1. The effect of different concentrations of 1,2,4-triazole (5, 50, and 100 mg kg^{-1}) on the number of leaf stomata (n = 4, ± SD). Different letters represent significant differences at $P \leq 0.01$.

	1,2,4-triazole (mg kg^{-1})			
	Control	5	50	100
Number of stomata	$42^a \pm 1.32$	$35^b \pm 1.87$	$22^c \pm 2.12$	$8^d \pm 1.8$

In the current experiment, while studying the impact of symbiotic fungi on reducing TZ toxicity, we observed that the effect of a TZ concentration of 100 mg kg^{-1} on plant roots was quite severe. The assessment of lettuce roots (Fig. 4) revealed that a concentration of 100 mg kg^{-1} TZ had led to the development of spherical root tips.

Fig. 4. The effect of high concentration of 1,2,4-triazole (100 mg kg^{-1} soil) on root morphology in lettuce.

Leaf fresh and dry weights

The interaction effect of symbiotic fungi and TZ on the dry weight index of lettuce leaves was found to be significant ($P \leq 0.01$). Comparing the mean effects of different TZ concentrations on the dry and fresh weights of lettuce under the colonization of symbiotic fungi *S. indica*, *G. etunicatum*, and *G. mosseae* (Fig. 5), the highest fresh and dry weights were observed in the treatment with *S. indica* fungus alone, without the presence of TZ.

As shown in Figure 5, plants treated with symbiotic fungi exhibited improved growth even in the presence of high TZ concentrations, although this growth differed significantly from the plants without TZ treatment.

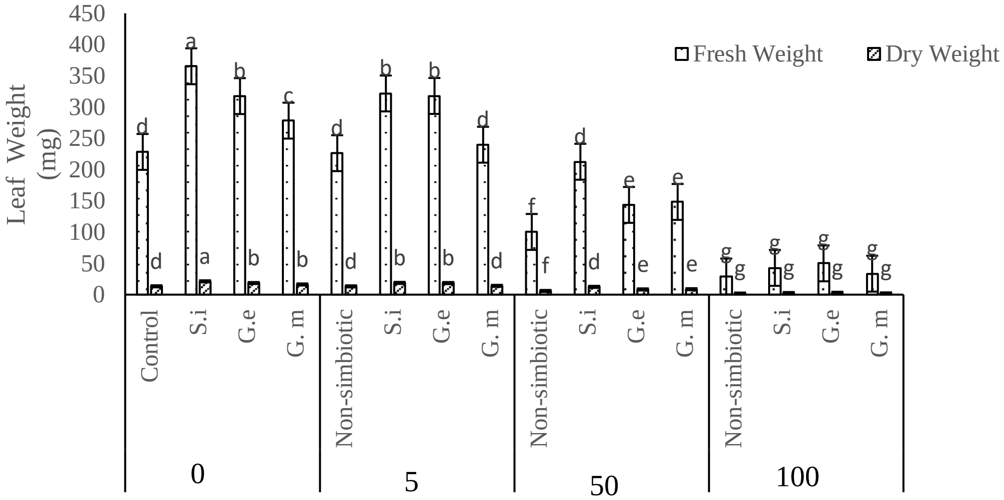

Fig. 5. The effects *Serendipita indica* (S. i), *Glomus mosseae* (G. m) and *Glomus etunicatum* (G. e) on leaf dry and fresh weight of lettuce plants subjected to different concentrations of 1,2,4-triazole (TZ) (n = 4, ± SD).

Total chlorophyll and carotenoids contents

According to the results regarding the effect of TZ on the total chlorophyll and carotenoid contents, it was found that as the TZ concentration increased, the total chlorophyll content in lettuce leaves began to decrease (Fig. 6). Conversely, with increase in TZ concentration, carotenoid content increased (Fig. 7).

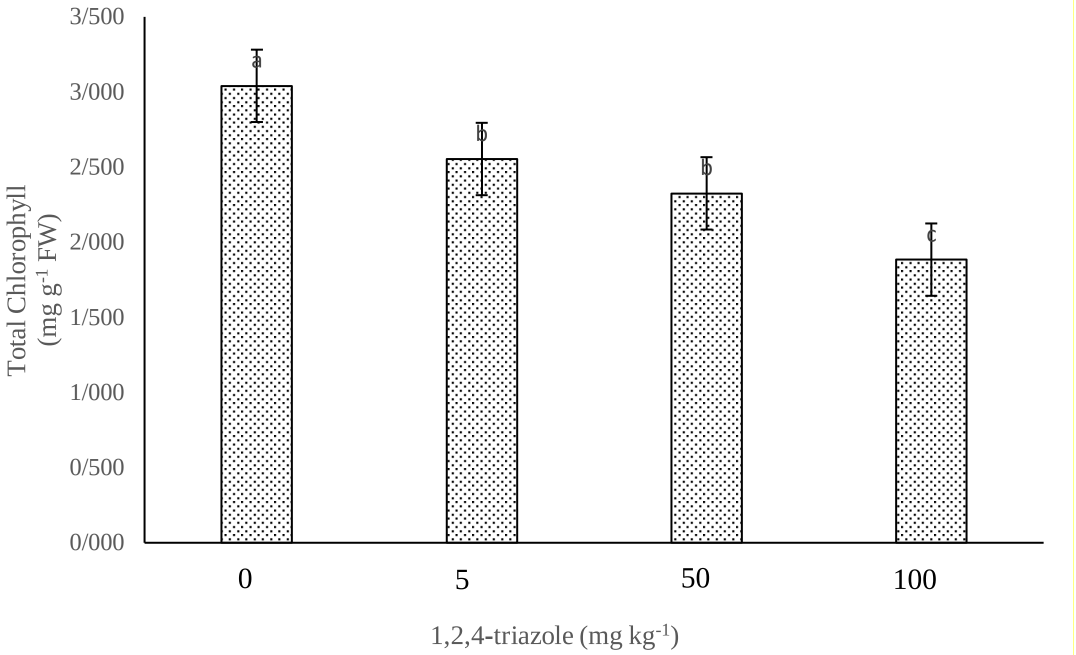

Fig. 6. Effects of different concentrations of 1,2,4-triazole (TZ) on total chlorophyll contents of lettuce plants (n = 4, ± SD).

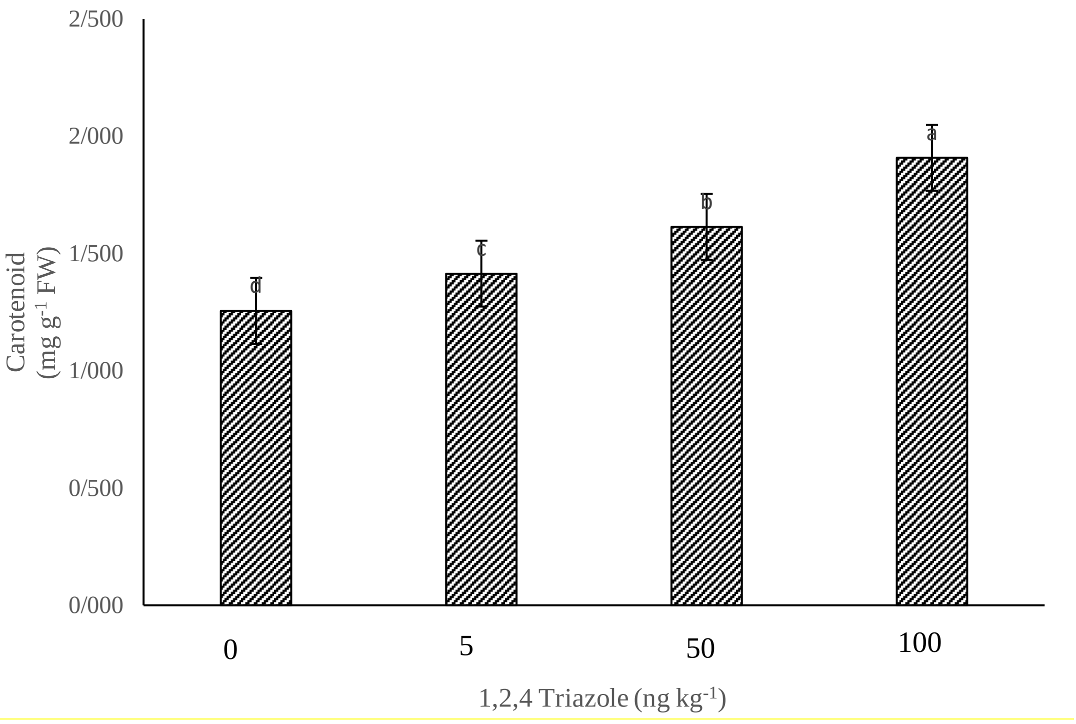

Fig. 7. Effects of different concentrations of 1,2,4-triazole (TZ) on carotenoid contents of lettuce plants (n = 4, ± SD).

Leaf concentrations of 1,2,4-triazole

The presence of symbiotic fungi *S. indica*, *G. etunicatum*, and *G. mosseae* significantly ($P \leq 0.001$) reduced the concentration of TZ in lettuce leaves (Fig. 8). In the 5, 50, and 100 mg kg^{-1} TZ treatments, the TZ concentrations in leaves of plants inoculated with *S. indica* were, respectively, 90, 50, and 75% lower than those in the leaves of non-inoculated plants. Furthermore, TZ concentrations in leaves of plants inoculated with *G. etunicatum* at 5, 50, and 100 mg kg^{-1} of TZ were 60%, 40%, and 76% lower, respectively, than those in the leaves of plants without symbiotic fungi. The concentrations of TZ in leaves of plants inoculated with *G. mosseae,* at 5, 50, and 100 mg kg^{-1} of TZ treatments were 40, 22, and 37% lower, respectively, compared to those of non-inoculated plants.

These results indicate that TZ accumulates in lettuce leaves (Fig. 8), and the symbiotic fungi effectively reduce this accumulation. Notably, *S. indica* was more effective in reducing TZ accumulation in leaves, compared to *G. etunicatum* or *G. mosseae*. The reduction of pollutants toxicity by symbiotic fungi has been well documented. The HPLC analysis results related to TZ accumulation in leaves of lettuce plants treated with 100 mg kg^{-1} TZ are shown in Figure 9. The effectiveness of *S. indica* (Fig. 9D) and *G. etunicatum* (Fig. 9C) in reducing TZ accumulation is evident.

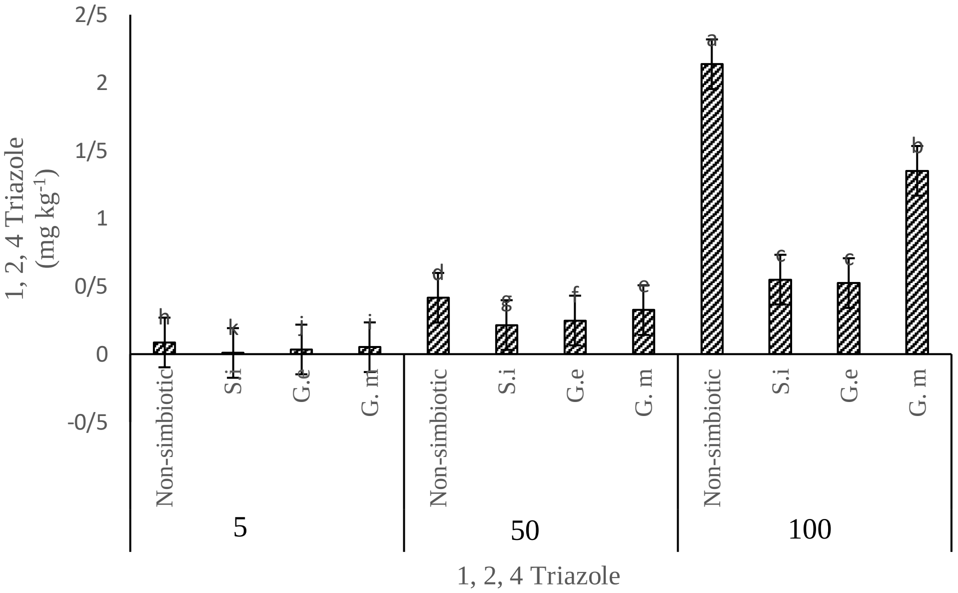

Fig. 8. Effects of inoculation with *Serendipita indica* (S.i), *Glomus mosseae* (G.m) and *Glomus etunicatum* (G.e) on accumulation of 1,2,4-triazole in lettuce leaves (n = 4, ± SD).

Malondialdehyde (MDA) concentration in leaves

The interaction between symbiotic fungi and TZ significantly ($P < 0.01$) affected the MDA concentration, with the highest MDA concentration observed in the 100 mg kg^{-1} of TZ treatment. Conversely, the lowest concentration of MDA was found in plants treated with *S. indica* without TZ (Fig. 10). Inoculation with *S. indica* reduced the leaf MDA concentration by 18% when compared to control plants without the TZ treatment. However, the use of 100 mg kg^{-1} TZ in non-inoculated plants increased the MDA concentration by 67% compared to the untreated control plants (Fig. 10). These results demonstrated that symbiotic fungi, *S. indica*, *G. etunicatum*, and *G. mosseae*, effectively reduced the concentration of MDA in lettuce leaves, even in plants treated with high TZ doses (50 and 100 mg kg^{-1}).

Hydrogen peroxide (H_2O_2) concentration

The interaction between symbiotic fungi and different concentrations of TZ significantly ($P < 0.01$) affected the H_2O_2 concentration of leaves (Fig. 11), with the highest level recorded at 100 mg kg^{-1} TZ in plants without symbiotic fungi. In contrast, the lowest concentration of H_2O_2 was observed in plants inoculated with *S. indica* without TZ. The use of TZ at 100 mg kg^{-1} increased the H_2O_2 production in non-inoculated plants by 58% (Fig. 11).

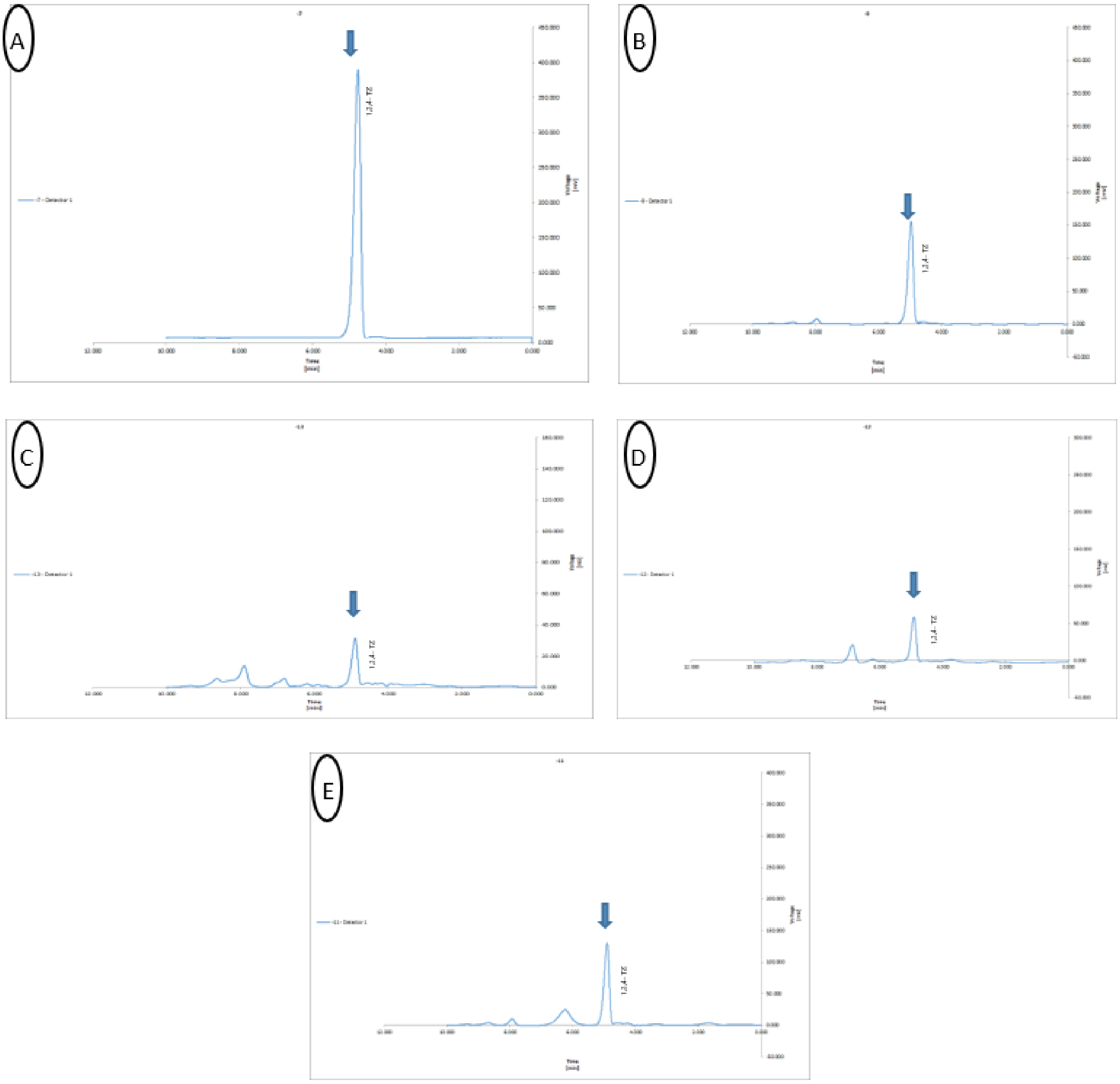

Fig. 9. HPLC graphs related to the 1,2,4-triazole (TZ) contents in leaves of lettuce plants treated with 100 mg kg^{-1} TZ as influenced by symbiotic fungi. (**A**) Standard; (**B**) 100 mg kg^{-1} TZ (non-inoculated control); (**C**) 100 mg kg^{-1} TZ + S. indica; (**D**) 100 mg kg^{-1} TZ + *G. etunicatum*; (**E**) 100 mg kg^{-1} TZ + *G. mosseae*.

Discussion

To the best of our knowledge, there has been no prior study addressing the mitigation of TZ symptoms in plants using fungal symbionts. However, the toxicity of NIs, such as DCD, which induces leaf chlorosis, has been previously reported in clover (genus *Trifolium*) (Macadam et al., 2003), lettuce (Padash et al., 2022). Although the reduction in the colonization percentage under TZ treatment was more pronounced compared to DCD, indicating the higher toxicity of TZ. The impact of NIs on root colonization has been a subject of debate. Some studies have reported that the use of NIs leads to an increase in NH_4^+ levels in the soil. García et al. (2008) noted that an increased NH_4^+ concentration in the soil strongly affects the growth and reproduction of symbiotic fungi in roots and interferes with spore germination. Furthermore, NH_4^+ can induce swelling of root cells, leading to cell wall cracking (Marschner, 2011), ultimately reducing root colonization. The effects of NIs, such as DCD, on plant roots have been investigated in a prior study (Padash et al., 2022). However, there has been no report on the effect of TZ on root morphology in any previous study.

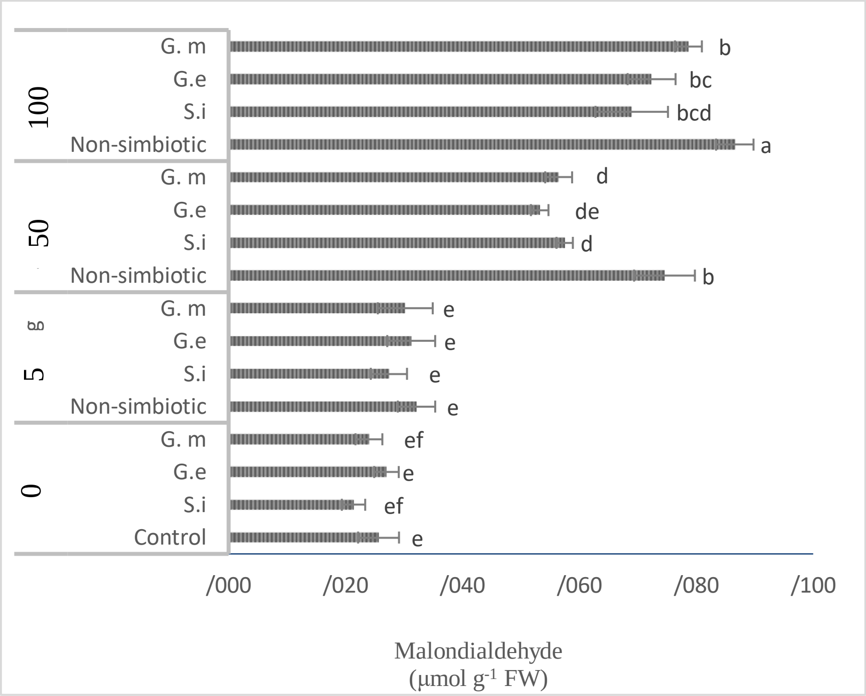

Fig. 10. Effects of *Serendipita indica* (S. i), *Glomus mosseae* (G. m) and *Glomus etunicatum* (G. e) on the concentration of malondialdehyde (MDA) in leaves of lettuce plants under different concentrations of 1,2,4-triazole (TZ) (n = 4, ± SD). Different letters indicate significant differences (P ≤ 0.01).

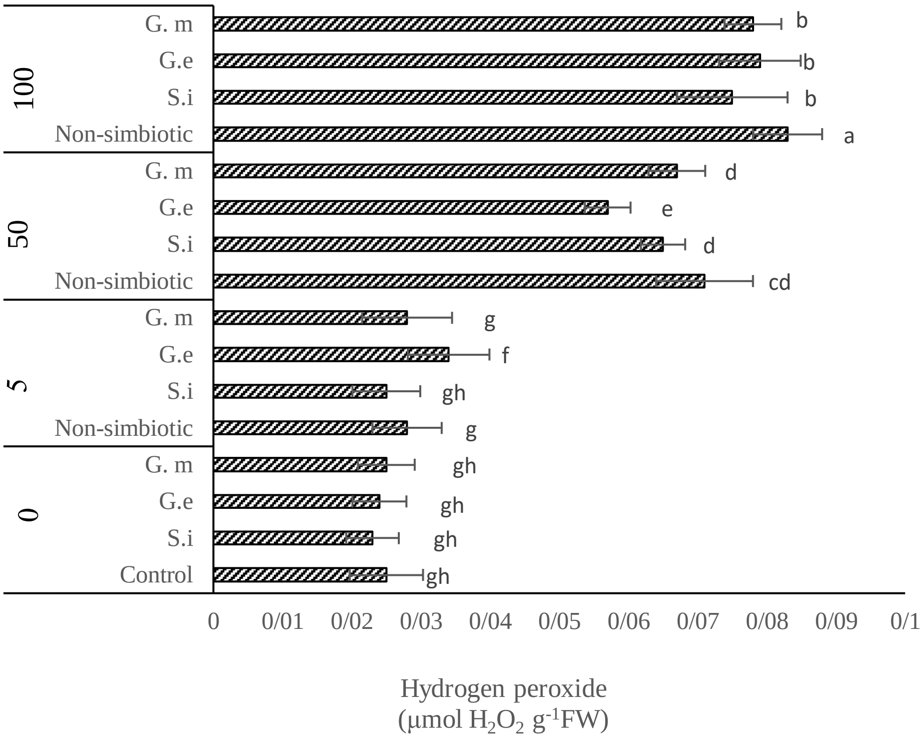

Fig. 11. Effects of *Serendipita indica* (S. i), *Glomus mosseae* (G. m) and *Glomus etunicatum* (G. e) on the concentration of hydrogen peroxide in leaves of lettuce plants under different concentrations of 1,2,4-triazole (n = 4, ± SD). Different letters indicate significant differences (P ≤ 0.01).

In a previous study on NIs (DCD), Padash et al. (2022) concluded that the DCD application reduced the growth of lettuce roots, consequently affecting the reduction in fresh and dry plant weight. As depicted in Figure 4, the roots exposed to high TZ concentrations exhibited uneven growth and morphological deformation. Therefore, based on the findings of this experiment, it can be inferred that the primary reason for the reduction in leaf dry and wet weights at the concentration of 50 and 100 mg kg^{-1} was diminished root growth. Additionally, the stress mitigation effects of symbiotic fungi were noteworthy.

In general, except for the concentration of 5 mg kg^{-1} of TZ, other TZ concentrations (50 and 100 mg kg^{-1}) led to a significant decrease in leaf weight. The use of high concentrations of NIs can lead to their continuous accumulation in the soil, resulting in adverse effects such as a decrease in plant yield (Zerulla et al., 2001). The endophytic fungus *S. indica* was shown to enhance the nutrients uptake in lettuce plants by stimulating the root growth and development, thereby increasing the yield (Padash et al., 2016).

Photosynthesis-limiting factors under stress conditions can be categorized into two main groups: stomatal and non-stomatal. Among the non-stomatal limiting factors, it appears that the decrease in chlorophyll content, as one of the non-stomatal limiting factors, is attributed to the effects of chlorophyllase, peroxidase, and phenolic compounds, ultimately leading to chlorophyll decomposition (Silva et al., 2007). A reduction in total chlorophyll content has been previously reported in lettuce plants under DCD toxicity (Padash et al., 2022). Moreover, the increase in carotenoid content in the presence of NIs may be linked to the reduction of chlorophyll (Padash et al., 2022), as carotenoid levels tend to rise in the absence of sufficient chlorophyll. Aranda et al. (2019) reported that mycorrhizal fungi significantly reduced the concentration of anthracene in plants. Additionally, it was demonstrated that mycorrhizal fungi reduce the toxicity of polycyclic aromatic hydrocarbons in *Medicago sativa* (Zhou et al., 2013). Pollutants like TZ are likely absorbed into the cell walls of symbiotic fungi, thereby reducing their uptake by the plant (Lenoir et al., 2016).

Malondialdehyde serves as the end product of membrane lipid peroxidation, and reflect the extent of cell membrane damage in stressed plants (Dhanda et al., 2004). In this study, plants treated with 100 mg kg^{-1} of TZ (without symbiotic fungi) exhibited the highest concentration of MDA than other plants, indicating a more significant cell membrane damage. A study by Qiu et al. (2013) supports our findings as they reported an increase in MDA concentration in Chinese cabbage when using fertilizers containing DCD compared to organic fertilizers. The results of this experiment on the effect of symbiotic fungi and TZ on the MDA concentration indicated that symbiotic fungi, especially *S. indica*, can decrease the MDA generation in plants (Fig. 9). Despite the extensive research on the effect of symbiotic fungi on MDA concentrations, especially under stress conditions, no previous study has investigated the MDA production in plants subjected to symbiotic fungi and TZ stress. However, the decrease in MDA production in lettuce by symbiotic fungi under TZ stress is likely due to their ability to limit TZ transfer to lettuce shoots (Figs. 8 and 9).

In general, the symbiotic fungi, including *S. indica*, *G. etunicatum*, and *G. mosseae*, effectively reduced the concentration of H_2O_2 in lettuce leaves, even in plants treated with high dose of TZ (50 and 100 mg kg^{-1}). Oxygen is an essential element in aerobic metabolic processes, but when incompletely reduced, it produces free ROS (reactive oxygen species). ROS, such as H_2O_2 and hydroxyl radicals, are generated when plants are exposed to abiotic stresses (Pigna et al., 2010). Similar to the effects we observed in TZ-treated lettuce plants, high concentrations of DCD (50 and 100 mg kg^{-1}) increased H_2O_2 production in lettuce plants. However, H_2O_2 levels decreased significantly in the presence of symbiotic fungi, indicating that symbiotic fungi are very effective in reducing ROS production in plants. The reduction in H_2O_2 production in plants treated with TZ and symbiotic fungi (Fig. 11) is probably due to the decrease in TZ uptake (Figs. 8 and 9).

Conclusions

The application of TZ caused a significant decrease in physiological indicators of the plant such as dry and fresh weights, root colonization, root growth, and total chlorophyll content. On the other hand, the inoculation of plant roots with symbiotic fungi increased these parameters in TZ-treated plants. Upon examining the concentration of TZ in leaves of the lettuce plants colonized with symbiotic fungi, it was evident that symbiotic fungi effectively reduced the TZ in inoculated plants. Interestingly, TZ also had a negative effect on root colonization by symbiotic fungi. However, even with a low percentage of colonization, symbiotic fungi were able to reduce the concentration of TZ in lettuce leaves to some extent. High TZ concentrations cause severe damage to plants, which was mitigated by fungal symbionts. Overall, the results of this study showed that TZ can negatively affect the growth and physiological parameters of lettuce plants, and the use of symbiotic fungi can partially alleviate the negative effects of TZ. However, extensive research is needed to fully understand the mechanisms of TZ phytotoxicity and the beneficial effects of symbiotic fungi to mitigate TZ toxicity.

Data availability
All data generated or analyzed during this study are included in this published article.

Conflict of Interest
The authors indicate no conflict of interest in this work.

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