

Bioaccumulation of Tire Wear Nanoparticles in Spinach (*Spinacia oleracea* L.) Leaves: Consequences for Metabolic Dysregulation and Growth Inhibition

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Abstract

Tire wear nanoparticles (TWPs) are emerging environmental contaminants with largely unknown effects on crops, necessitating an investigation into their uptake, toxicity, and impact on plant metabolism and metal accumulation. In this study, we characterized TWPs using dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDX), revealing their predominantly nanoscale size (average 284.6 nm), irregular morphology, and complex chemical composition, including organometallic phases and trace heavy metals. A controlled pot experiment was conducted to investigate the effects of soil and foliar-applied TWPs at various concentrations (0.05–0.5 mg/kg or mg/L) on spinach (*Spinacia oleracea* L.). Fluorescence microscopy confirmed internalization of TWPs through both root and leaf pathways. Low soil concentrations (0.05 mg/kg) transiently enhanced growth and metabolite accumulation, likely reflecting a hormetic response, whereas higher concentrations (≥ 0.25 mg/kg or mg/L) significantly suppressed biomass, leaf area, photosynthetic traits, and growth indices. TWPs induced oxidative stress, as indicated by elevated antioxidant enzyme activities at low doses and their decline at higher concentrations. Metal analysis revealed increased accumulation of Zn, Pb, and Cd in leaves, particularly under higher TWP exposure, highlighting the risk of metal contamination via nanoparticle-mediated transport. Overall, this study demonstrates that TWPs can enter plant tissues via both soil and foliar routes, disrupt morpho-metabolic processes, and facilitate heavy metal accumulation, underscoring their potential to impair crop productivity and pose risks to food safety in contaminated environments.

1. Introduction

Plastic pollution has become a global environmental concern, with plastic debris found in oceans, freshwater bodies, terrestrial ecosystems, and even the atmosphere. The rapid growth in plastic production and consumption has exacerbated this issue, with global plastic production estimated to have reached 400.3 million tons in 2023 (Yan et al., 2021). Because plastics are largely non-biodegradable, they persist in the environment for extended periods, fragmenting under physical, chemical, and biological processes into smaller particles known as microplastics (less than 5 mm) and nanoplastics (less than 100 nm) (Tsangaris et al., 2020). A significant contributor to this form of pollution, often overlooked in discussions of plastic debris, is tire wear material released through the friction of vehicle tires against road surfaces during driving, braking, or acceleration. These tire wear particles (TWPs) are subsequently transported into terrestrial and aquatic environments, primarily via road runoff (Sherlock et al., 2022; Wang et al., 2024).

Among various sources of microplastics, tire microplastics have been recognized as a major contributor to environmental pollution, with tire wear particles (TWPs) identified as their most abundant form (Luo et al., 2021; Wang et al., 2024). TWPs arise from the erosion of tire treads. They are chemically complex, comprising synthetic rubber polymers, fillers such as carbon black, oils, resins, sulfur compounds for vulcanization, and various performance-enhancing additives (Kang et al., 2025). This composition makes TWPs distinct from typical thermoplastics and of particular concern due to their potential to carry heavy metals and organic contaminants (Halle et al., 2020).

While considerable attention has been given to the impacts of microplastics such as polyethylene and polystyrene in aquatic systems, elastomers like tire-derived particles have received less focus, especially in terrestrial and agricultural contexts (Wagner et al., 2018). Yet, studies increasingly report that TWPs accumulate in soils through atmospheric deposition, irrigation with contaminated water, plastic mulching, biosolid applications, and runoff (Baensch-Baltruschat et al., 2020). Once in the soil, TWPs can alter their physical structure, microbial composition (Wei et al., 2024; Zeb et al., 2024), and enzymatic activity (Sheng et al., 2021), potentially affecting plant growth and ecosystem functions (Leifheit et al., 2022).

There is growing evidence that TWPs can penetrate plant tissues, disrupt water and nutrient uptake, and impair physiological functions (Castan et al., 2023). Micro- and nanoplastics have been shown to accumulate in plant roots and translocate to shoots and leaves, where they can interfere with photosynthesis, induce oxidative stress, and alter metabolism (Tsangaris et al., 2020; Li et al., 2020; Wang et al., 2025). Nanoplastics, in particular, pose greater risks than larger microplastics due to their smaller size, higher surface area, and greater reactivity, which enhance their mobility and interaction with plant cells. Moreover, TWPs often contain hazardous additives such as bisphenol A, phthalates, and heavy metals (e.g., Zn, Pb, Cd), which can leach into the environment and bioaccumulate in crops, raising concerns for food safety and human health (Santini et al., 2023; Wang et al., 2025).

Despite these risks, relatively few studies have examined how TWPs affect edible crops under controlled conditions, particularly regarding their uptake pathways (via roots or leaves), phytotoxicity, and potential to alter plant metabolism and heavy metal accumulation (Castan et al., 2022). Most existing studies focus on aquatic organisms, reporting impacts on growth, reproduction, feeding behavior, and metabolism, while terrestrial crop species remain under-investigated (de Souza Machado et al., 2020; Li et al., 2023). Furthermore, while it is known that TWPs can reach agricultural soils through multiple pathways, including the degradation of plastic mulch, irrigation, and atmospheric deposition, their interactions with crop plants and their implications for food security are poorly understood (Sommer et al., 2018).

Given this context, the present study addresses a critical knowledge gap by investigating the uptake, phytotoxicity, and morpho-metabolic impacts of TWPs on spinach (*Spinacia oleracea* L.), a widely consumed leafy vegetable. Specifically, we aimed to characterize TWPs in terms of their size, morphology, and chemical composition; assess their internalization through soil and foliar exposure; and evaluate their effects on spinach growth, leaf morphology, photosynthesis, antioxidant responses, and metal accumulation.

Our working hypothesis was that TWPs, depending on their concentration and mode of application, would be internalized by spinach, disrupt normal plant growth and physiology, and promote the accumulation of potentially toxic metals. This study provides novel insights into the environmental behavior of TWPs in terrestrial agroecosystems and their potential implications for crop productivity and food safety. The findings are expected to contribute to a better understanding of the ecological risks posed by tire-derived nanoparticles and inform strategies for managing micro- and nanoplastic contamination in agricultural settings.

2. Materials and Methods

A controlled pot experiment was conducted at the Botanical Garden of the Government College Women University Faisalabad, Pakistan. Spinach (*Spinacia oleracea* L. desi variety) seeds were sourced from the Ayub Agricultural Research Institute, Faisalabad. Pots (capacity 1 L) were filled with 0.8 kg of loamy-sandy soil mixture.

2.1 Synthesis and characterization of tire wear nanoparticles

Tire wear particles (TWPs) were generated from end-of-life automobile (car) tires. The tires were manually cut into small fragments and then coarsely ground using a mechanical grinder. The resulting material was further processed by ball milling, using balls in a ball-to-powder weight ratio of 10:1 to ensure efficient size reduction and particle uniformity.

The particle size distribution of TWPs was determined using dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, UK). Dried TWPs were suspended in deionized water and sonicated for 15 minutes to ensure homogeneity, dispersion, and minimize aggregation. The hydrodynamic diameter, polydispersity index (PDI), and diffusion coefficient were recorded at 25°C, with each sample analyzed in triplicate.

Functional groups on the TWPs were identified using FTIR spectroscopy in attenuated total reflectance mode (4000–400 cm⁻¹; resolution 4 cm⁻¹). Samples were air-dried, finely ground, and scanned three times to improve the signal-to-noise ratio.

Surface morphology and elemental composition were analyzed by scanning electron microscopy (SEM) combined with energy-dispersive X-ray spectroscopy (EDX) using a Zeiss Gemini 300 field emission SEM equipped with an Oxford Instruments X-MAX 80 mm² EDX detector. TWPs were ultrasonically dispersed in deionized water, mounted on boron-coated stubs, and examined at 500× magnification with 12 kV accelerating voltage. ZAF correction was applied during elemental quantification.

X-ray diffraction (XRD) patterns were recorded using Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) over a 2θ range of 5–80° with a step size of 0.02°. Finely ground powders were placed on low-background sample holders, and phases were identified using the ICDD PDF-4 + database.

2.2 Experimental design and TWP application

The experiment followed a completely randomized design with three replications. TWPs were applied through two modes: soil amendment and foliar spray. For soil application, TWPs were mixed thoroughly into the soil at concentrations of 0 (control), 50, 100, 250, and 500 mg/kg prior to sowing. For foliar application, TWPs were suspended in distilled water using sonication, and 0.01% Tween 20 was added as a surfactant. TWPs were sprayed on plants at the 5-leaf stage (20 days after sowing) with a handheld sprayer to ensure uniform coverage.

Plant sampling and trait evaluations were conducted at designated growth stages. Morphological and growth parameters were recorded at 15 and 30 days after foliar application (equivalent to 35 and 50 days after sowing, respectively). Gas exchange parameters, relative water content (RWC), and electrolyte leakage (EL) were assessed at 50 days after sowing, while biochemical analyses, including antioxidant enzyme activities and metabolite profiling, were performed at 45 days after sowing.

2.3 Morphological and growth measurements

At each sample, five plants per replicate were harvested. Shoots and roots were gently washed with distilled water, and leaf area was determined using the graph paper method. Fresh weights were recorded immediately, and dry weights were measured after oven-drying at 70°C for 48 h.

Growth indices were calculated as per Sandeep et al. (2016) and Shafiq et al. (2021).

$$\text{Leaf area index (LAI, cm}^2 \text{ leaf}^{-1}) = \frac{\text{Leaf area}}{\text{Ground area}}$$

$$\text{Leaf weight ratio (LWR, g g}^{-1}) = \frac{\text{Leaves dry weight}}{\text{Plant dry weight}}$$

$$\text{Specific leaf area (SLA, g g}^{-1}) = \frac{\text{Leaf area}}{\text{Leaf dry weight}}$$

$$\text{Leaf area ratio (LAR, cm}^2 \text{ mg}^{-1}) = \frac{\text{Leaf area}}{\text{Plant dry weight}}$$

$$\text{Net assimilation rate (NAR, g m}^{-2} \text{ d}^{-1}) = \frac{W_2 - W_1}{t_2 - t_1} \times \frac{\log_e L_2 - \log_e L_1}{L_2 - L_1}$$

where W_1 and W_2 are dry weights at t_1 and t_2 , and L_1 and L_2 represent the leaf area at t_1 and t_2 , respectively.

$$\text{Leaf area duration (LAD, day)} = \frac{L_1 + L_2}{2 \times (t_2 - t_1)}$$

$$\text{Crop growth rate (CGR, g m}^{-2} \text{ d}^{-1}) = \frac{W_2 - W_1}{p \times (t_2 - t_1)}$$

Where p is the ground area occupied by the crop.

$$\text{Absolute growth rate (AGR, cm day}^{-1}) = \frac{h_2 - h_1}{t_2 - t_1}$$

Where h_1 and h_2 represent the plant height at t_1 and t_2 , respectively.

$$\text{Relative growth rate (RGR, mg day}^{-1}) = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1}$$

2.4 Gas exchange parameters

Gas exchange measurements were performed 50 days after sowing between 12:00 and 14:00 h on the upper third of the fully expanded leaf using an infrared gas analyzer (IRGA). The parameters measured included photosynthetic rate (A), stomatal conductance (g_s), intracellular CO_2 concentration (C_i), and transpiration rate (E).

2.5 Pigments

Chlorophyll and carotenoid contents were determined by extracting 0.5 g fresh leaf tissue in 80% acetone and measuring absorbance at 663, 645, and 480 nm (Arnon et al., 1949).

$$\text{Chlorophyll a} = 12.7 (A_{663}) - \left[2.69 (A_{645}) \times \left(\frac{V}{1000 \times W} \right) \right]$$

$$\text{Chlorophyll b} = 22.9 (A_{645}) - \left[4.68 (A_{663}) \times \left(\frac{V}{1000 \times W} \right) \right]$$

$$\text{Total Chlorophyll} = 20.2 (A_{645}) + \left[8.02 (A_{663}) \times \left(\frac{V}{1000 \times W} \right) \right]$$

$$\text{Carotenoids} = 4.16 (A_{480}) - \left[0.89 (A_{663}) \times \left(\frac{V}{1000 \times W} \right) \right]$$

2.6 Antioxidant enzyme activities

Antioxidant enzyme activities were assayed using fresh leaf samples collected 45 days after sowing. 0.5 g of fresh leaf was homogenized with 10 ml of ice-cold phosphate buffer (0.1 M, pH 7.0) and frozen at -20°C for 24 h. Then, it was filtered and centrifuged (12,000 rpm, 15 min, 4°C).

Superoxide dismutase (SOD) activity was performed following the method of Beauchamp and Fridovich et al. (1971). The reaction mixture (3 mL) contained 2.6 mL of 50 mM phosphate buffer (pH 7.8), 0.1 mL of 75 μ M NBT, 0.1 mL of 13 mM L-methionine, 0.1 mL of 0.1 mM EDTA, 0.1 mL of 2 μ M riboflavin, and 0.1 mL of enzyme extract. The control contained all reagents except the enzyme extract, while the blank (dark control) lacked riboflavin and was kept in the dark. Reaction mixtures were exposed to fluorescent light (~ 4000 lux) for 10–15 min to initiate superoxide generation. Absorbance was recorded at 560 nm.

A peroxidase (POD) activity assay was performed using the method of Kuroda et al. (1990), which involved the guaiacol-H₂O₂ reaction. Absorbance was measured at 470 nm using a spectrophotometer. Catalase (CAT) activity was assessed by monitoring H₂O₂ decomposition (Chance and Maehly, 1955), and absorbance was measured at 240 nm.

2.7 Metabolites

Metabolite profiling was conducted 45 days after sowing. Flavonoid content (mg quercetin equivalent [QE] g⁻¹ fresh weight) was determined following the method of Shraim et al. (2021). 1 mL of plant extract (prepared in 80% acetone) was mixed with 0.5 mL of 2% AlCl₃ and 0.5 mL of 1 M sodium acetate. The mixture was incubated at room temperature for 10 minutes, allowing the formation of a pink complex, and the absorbance was recorded at 425 nm using a spectrophotometer. A standard calibration curve was generated using quercetin solutions (10–100 μ g mL⁻¹).

Total soluble sugars (mg g⁻¹ fresh weight) were estimated using the anthrone method described by Lowry et al. (1951). Plant extracts (1 mL) were mixed with 4 mL of freshly prepared anthrone reagent (0.26 g anthrone dissolved in 150 mL sulfuric acid). The mixture was heated at 90°C for 10 minutes, cooled to room temperature, and the absorbance was measured at 620 nm. Glucose was used as the standard, with a calibration curve prepared from solutions containing 0.1 g glucose per 100 mL of water and 0.5 g potassium tartrate.

Total soluble protein content (mg g⁻¹ fresh weight) was quantified following a modified method of Satpathy et al. (2020). The assay mixture consisted of 1 mL of plant extract and 1 mL of Solution C, which was prepared by mixing Solution A (sodium bicarbonate, potassium tartrate, and sodium hydroxide) with Solution B (copper sulfate in water). After incubation for 30 minutes, 0.5 mL of diluted Folin–Ciocalteu reagent (Solution D) was added, and absorbance was read at 750 nm. A standard curve of bovine serum albumin (0–0.2 μ g mL⁻¹) was used for protein quantification.

Total free amino acids (mg g⁻¹ fresh weight) were determined according to Hamilton and Van Slyke (1943). A reaction mixture comprising 1 mL of plant extract, 1 mL of 10% pyridine, and 1 mL of 2% ninhydrin was incubated in covered tubes at 100°C for 30 minutes. After cooling, the absorbance was measured at 570 nm. Quantification was done using a leucine standard curve (0–1 mg per 10 mL).

Total phenolic content (mg gallic acid equivalents [GAE] g⁻¹ fresh weight) was estimated using a modified Folin–Ciocalteu method (Genwali et al., 2013). Each assay contained 0.5 mL of plant extract, 2 mL of 1:10 diluted Folin reagent, and 4 mL of 7.5% sodium carbonate solution. The mixture was incubated at room temperature for 30 minutes, and absorbance was recorded at 765 nm. A standard curve was prepared using gallic acid solutions (50–500 mg L⁻¹).

2.8 Relative water content

Leaf relative water content (RWC) was measured at 50 days after sowing. Fresh leaf samples were weighed, rehydrated in distilled water for 24 hours at room temperature, and weighed again to obtain turgid weight. Samples were then oven-dried to a constant weight at 70°C for determination of dry weight. The RWC was calculated using the following formula (Reyes et al., 2018):

$$\text{RWC (\%)} = \left[\frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Turgid weight} - \text{Dry weight})} \right] \times 100$$

2.9 Electrolyte leakage

Electrolyte leakage (EL) was determined at 50 days after sowing using 1-cm leaf discs incubated in deionized water at room temperature for 24 hours (EC₁), followed by autoclaving at 120°C for 20 minutes (EC₂). Leakage was expressed as a percentage of total electrolytes (Lutts et al., 1996):

$$\text{EL (\%)} = 1 - \left[\frac{\text{EC}_1}{\text{EC}_2} \right] \times 100$$

2.10 Detection of TWP in plant tissues

Following Erdem et al. (2023), plant tissues (leaves and roots) were washed with distilled water and then homogenized in the same water. The homogenate (20 mL) was treated with 10% KOH and 30% H₂O₂, heated for 30 minutes, and then filtered through 0.45 µm cellulose acetate filters. Filtrate aliquots were stained with Rhodamine B, and excess dye was removed. Samples were visualized under a fluorescence microscope (ZEISS Vert.A1, Germany).

2.11 Heavy metals in leaves

Leaf samples were collected at harvest, thoroughly rinsed with deionized water to remove surface contaminants, and then oven-dried at 70°C until a constant weight was achieved. The dried leaf samples were finely ground and subjected to acid digestion following standard procedures. Briefly, 0.5 g of powdered leaf tissue was digested with a mixture of concentrated nitric acid (HNO₃) and perchloric acid (HClO₄) in a 4:1 ratio on a hot plate until the solution became clear. The digested samples were diluted with deionized water to a final volume of 50 mL and filtered through Whatman No. 42 filter paper. The concentrations of heavy metals, including Zn, Pb, Cd, Cu, Fe, Cr, As, Co, Ni, Mn, Ca, and Mg, were quantified using inductively coupled plasma optical emission spectrometry (ICP-OES; model: PerkinElmer Optima 8000).

2.12 Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) to assess the significance of treatment effects on measured parameters. When significant differences were detected ($p < 0.05$), means were compared using the least significant difference (LSD) test. Statistical analyses were performed using SPSS version 25.0 (IBM Corp.), and results are presented as mean $\pm$ standard error. A heat map of heavy metal accumulation in spinach leaves was generated using RStudio (R version 4.3.1) with the Complex Heatmap package. Data were standardized by Z-score normalization to enable comparison across metals and treatments.

3. Results

3.1 Characterization of tire wear nanoparticles (TWPs)

3.1.1 Particle size distribution

Dynamic light scattering (DLS) analysis of TWPs demonstrated a peak at 327.2 nm (Fig. S1). The particles were primarily in the nanoscale range, with an average hydrodynamic diameter of 284.6 nm and a polydispersity index (PDI) of 22.3%, indicating moderate heterogeneity within the suspension. The diffusion coefficient was calculated to be 1.7 µm²/s, while the measured transmittance of 25.3% indicated substantial light scattering by suspended particles. The size distribution peak appeared at 327.2 nm, accounting for 100% of the scattering intensity (SD: 151.4 nm). These results underscore the nanosized nature of the tire wear nanoparticle in suspension, with potential implications for environmental transport and bioavailability.

3.1.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis indicates the presence of various functional groups typical of tire wear nanoparticles (Fig. S2). A strong absorption at 2085.4 cm⁻¹ represents the nitrile (C $\equiv$ N) stretching mode, reflecting the degradation of synthetic rubber components. A weak band at 2111.5 cm⁻¹ was attributed to alkyne (C $\equiv$ C) stretching, while a band at 1996.0 cm⁻¹ indicated isothiocyanate (N = C = S) groups, likely from vulcanization additives. Aromatic (C = H) stretching was observed at 1828.3 cm⁻¹. Additionally, strong bands at 1731.3 cm⁻¹ and 1686.6 cm⁻¹ were assigned to carbonyl (C = O) and imine (C = N) groups, indicating oxidative degradation of the sample.

3.1.3 Scanning electron microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX)

SEM images at 500x, 1000x, and 4000x magnifications revealed TWPs with irregular, angular morphologies and coarse textures (Fig. S3a-c). Smaller fragments were found adhering to larger particles, indicating aggregation due to high surface energy. Cracks and voids were also observed, suggesting mechanical stress during formation. These structural features indicate a large surface area that can enhance the adsorption of pollutants.

EDX analysis (Fig. S3d) showed that carbon (60.35 wt%) was the dominant element, confirming the rubber-based composition of TWPs. Oxygen (14.22 wt%) suggested the presence of oxidized compounds or fillers. Zinc (9.37 wt%) and silicon (5.35 wt%) were present, consistent with tire additives. Sulfur (5.43 wt%), aluminum (1.78 wt%), calcium (2.74 wt%), and chlorine (0.75 wt%) likely originated from manufacturing additives or road dust. This elemental profile reflects the complex chemical composition of TWPs and their potential to interact with environmental pollutants.

3.1.4 X-ray diffraction (XRD)

XRD analysis of TWPs identified porphyrazine gallium chloride as the primary crystalline phase, accounting for 90.5% of the diffraction pattern (Fig. S4). This compound is a complex organometallic species that may originate from the high-temperature degradation of tire additives, such as pigments or stabilizers. Minor phases included cannizzarite (6.5%) and selenium (3.0%). The presence of lead (Pb)- and bismuth (Bi)-containing minerals (cannizzarite) suggests potential risks of heavy metal exposure. These crystalline phases, along with amorphous components, contribute to heterogeneous dissolution behaviors and metal release profiles, which can induce oxidative stress in plants and disrupt nutrient homeostasis.

3.2 Internalization of TWP in leaves via fluorescence microscopy

Fluorescence microscopy was employed to detect and visualize the internalization of tire wear nanoparticles in spinach leaf tissues using Rhodamine B dye. The control leaves exhibited negligible fluorescence, confirming the absence of nanoparticle accumulation or background signal (Fig. 1a). In plants grown in soil amended with 0.5 mg/kg TWP, moderate fluorescence was detected, indicating internalization and accumulation of nanoparticles following root uptake (Fig. 1b). The strongest fluorescence was observed in leaves subjected to foliar application (0.5 mg/L), reflecting substantial nanoparticle deposition or internalization through stomatal or cuticular pathways (Fig. 1c).

3.3 Biomass and growth dynamics

After 35 and 50 days of sowing, spinach growth traits showed clear variation depending on TWP concentration and application mode (Tables 1 and 2). In soil application after 35 days of sowing, 0.05 mg/kg significantly increased shoot length, shoot fresh weight, and shoot dry weight compared to other concentrations, while 0.5 mg/kg resulted in the shortest shoots and lowest shoot fresh weight (Table 1). Root length and biomass were also significantly higher at 0.05 mg/kg than at higher concentrations. After 50 days of sowing (15 days of foliar application), shoot length was significantly higher in the control and 0.05 mg/L treatments than in the 0.25 or 0.5 mg/L treatments, where marked reductions were observed. Shoot and root dry weights were significantly higher at 0.05 mg/L than at 0.5 mg/L, with the lowest values observed at this concentration.

Table 1
Growth traits of spinach after 35 days of sowing under different concentrations of tire wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar).

Treatment	Conc (mg/kg)	Root Length (cm)	Shoot FW (mg)	Root FW (mg)	Shoot DW (mg)	Root DW (mg)
Soil	0	9.96 ^d	413.3 ^{bc}	25.80 ^c	113.2 ^b	45.4 ^c
	0.05	14.63 ^a	568.7 ^a	70.86 ^a	290.5 ^a	99.5 ^a
	0.1	12.53 ^c	440.3 ^{bc}	53.60 ^b	222.5 ^{ab}	81.6 ^{ab}
	0.25	13.56 ^b	462.5 ^{bc}	65.16 ^a	218.2 ^{ab}	61.9 ^{bc}
	0.5	11.93 ^c	323.2 ^c	47.53 ^b	182.1 ^{ab}	51.3 ^c
	Anova (P value)	< 0.001	< 0.001	< 0.001	> 0.05	< 0.01
Foliar	0	13.80 ^a	347.8 ^a	53.83 ^{ab}	168.3 ^{ab}	67.4 ^b
	0.05	12.93 ^{ab}	334.2 ^a	62.66 ^a	201.5 ^a	88.7 ^a
	0.1	11.50 ^{abc}	307.8 ^a	46.10 ^d ^{bc}	158.5 ^b	67.7 ^b
	0.25	9.98 ^c	263.3 ^{ab}	33.13 ^d	137.8 ^{bc}	50.3 ^{bc}
	0.5	11.67 ^{abc}	176.3 ^b	46.83 ^{cd}	112.1 ^c	41.1 ^c
	Anova (P value)	< 0.05	< 0.05	< 0.01	< 0.05	< 0.01
Each value is a mean of 3 replicates followed by different letters indicating significant difference ($P \leq 0.05$) within an application mode using LSD.						

Table 2

Growth traits of spinach after 50 days of sowing under different concentrations of tire wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar).

Treatment	Conc (mg/kg)	Shoot Length (cm)	Root Length (cm)	SFW (mg)	RFW (mg)	Shoot DW (mg)	Root DW (mg)
Soil	0	19.64 ^a	15.43 ^{ab}	516.3 ^b	27.16 ^c	126.4 ^b	78.3 ^b
	0.05	15.16 ^b	16.06 ^a	639.1 ^a	53.03 ^b	310.4 ^a	156.8 ^{ab}
	0.1	14.1b ^b	14.76 ^{abc}	490.6 ^{bc}	57.46 ^b	275.8 ^a	184.4 ^a
	0.25	14.67 ^b	13.7 ^{bc}	521 ^{ab}	67.26 ^a	242.7 ^{ab}	99.4 ^b
	0.5	14.54 ^b	13.00 ^c	408.6 ^c	75.20 ^a	208.7 ^{ab}	83.4 ^b
	Anova (P value)	< 0.01	≤ 0.05	< 0.01	< 0.001	> 0.05	< 0.05
Foliar	0	13.67 ^b	14.90 ^a	380.2 ^a	67.30 ^a	196.2 ^{ab}	99.5 ^b
	0.05	16.60 ^a	14.13 ^a	410.5 ^a	58.50 ^a	226.2 ^a	110.3 ^a
	0.1	12.40 ^{bc}	13.67 ^a	357.7 ^{ab}	56.63 ^a	176.6 ^b	89.9 ^b
	0.25	11.00 ^c	11.86 ^b	268.0 ^{bc}	51.43 ^{ab}	161.4 ^{bc}	80.3 ^{bc}
	0.5	10.36 ^c	10.19 ^b	227.1 ^c	36.46 ^c	136.6 ^c	69.2 ^c
	Anova (P value)	< 0.01	< 0.001	< 0.01	> 0.05	< 0.01	< 0.01
Each value is a mean of 3 replicates, followed by different letters indicating significant difference ($P \leq 0.05$) within an application mode using LSD.							

After 35 days, soil-applied 0.05 mg/kg continued to promote significantly greater shoot and root fresh and dry weights compared to the control and higher doses, with 0.5 mg/kg showing consistently lower growth values (Table 2). After 50 days of foliar exposure, 0.05 mg/L significantly increased shoot length, shoot dry weight, and root dry weight relative to 0.25 and 0.5 mg/L, which produced significantly lower growth across traits.

After 35 days of soil application of 0.5 mg/kg TWP, spinach showed an 8.5% reduction in shoot length and a 21.8% reduction in shoot fresh weight compared to the control (Table 2). In contrast, foliar application of 0.5 mg/L TWP caused reductions of 7.0% in shoot length, 49.3% in shoot fresh weight, 33.4% in shoot dry weight, and 15.4% in root length. After 50 days of soil exposure, 0.5 mg/kg TWP led to a 26.0% decrease in shoot length, 20.9% in shoot fresh weight, and 15.7% in root length. Foliar application at 0.5 mg/L resulted in stronger declines, with 24.2% reduction in shoot length, 40.3% in shoot fresh weight, 30.4% in shoot dry weight, 31.6% in root length, 45.8% in root fresh weight, and 30.5% in root dry weight.

Spinach biomass indices, i.e., net assimilation rate (NAR), annual growth rate (AGR), relative growth rate (RGR), specific leaf weight (SLW), and leaf area duration (LAD) were calculated based on the biomass after 35 and 50 days of sowing (Fig. 2). All these traits showed a slight increase at the lowest concentration of TWP, as observed by the other growth and then increasing TWP showed decreasing trends. NAR by soil application was non-significant, and 0.5 mg/kg foliar application caused 28% reduction in NAR as compared to 0.05 mg/kg (Fig. 2a). The effect on AGR was non-significant (Fig. 2b). RGR by foliar application was non-significant, and 0.5 mg/kg soil application caused 15% reduction in NAR as compared to 0.05 mg/kg (Fig. 2c). The effect on SLW caused 5% reduction in soil as compared to control at 0.05 mg/kg, and significant at 0.05 mg/kg by foliar application (Fig. 2d). LAD was significantly reduced by 0.5 mg/kg as compared to control and 0.05 mg/kg (Fig. 2e).

3.4 Leaf morphology

The effects of TWP on spinach leaf morphology under soil and foliar applications are shown in Fig. 3. Soil applications of TWP at 0.05 mg/kg increased the leaf size compared to the control. However, at higher concentrations (0.1, 0.25, 0.5 mg/kg), leaf size progressively decreased, with 0.5 mg/kg showing the most pronounced reduction. Foliar application showed a similar pattern, with 0.05 mg/L increasing leaf size but causing visible damage (holes). With increasing concentrations (0.1, 0.25, 0.5 mg/L), leaf size decreased, and damage became more apparent at 0.05 mg/L. These results suggest that low concentrations of TWP may stimulate growth, while higher levels are detrimental to leaf development and health.

The effect of TWP on leaf area indices exhibited the same trend as growth, characterized by an initial increase followed by a reduction (Fig. 4). At lower concentration (0.05 mg/kg), leaf area index (LAI) and leaf area (LA), leaf area ratio (LAR), leaf weight ratio (LWR), and specific leaf area (SLA), showed slight increases compared to control. Higher concentration (0.5 mg/kg) led to a significant reduction in leaf indices after 35 and 50 days of sowing by foliar and soil TWP. This decline was more pronounced when TWP was applied through spraying, suggesting a greater sensitivity of leaves to direct contact with nanoparticles. After 50 days of 0.5 mg/kg TWP foliar exposure, reductions of 39.7%, 42.6%, 42.4%, and 23% were observed in LAI, LA, LAR, and SLA, respectively, compared to the control. At the same time, 0.5 mg/kg TWP soil exposure resulted in 20%, 21%, and 8% reductions in LAR, LWR, and SLA, respectively, compared to the control.

3.5 Photosynthesis pigments and photosynthesis

TWPs showed the same trend as biomass, but the effect is non-significant on all pigments (Fig. 5). Application of TWP significantly reduced photosynthetic traits in spinach, with the extent of reduction depending on both the mode of application and TWP concentration (Table 3). In soil-applied treatments, increasing TWP levels led to substantial declines in photosynthetic rate (Pn), transpiration rate (E), stomatal conductance (gs), and intercellular CO₂ concentration (Ci). At the highest soil-applied dose (0.5 mg/kg), Pn, E, gs, and Ci were reduced by 73.6%, 26.5%, 46.9%, and 48.3%, respectively, compared with the control.

Table 3

Photosynthesis traits of spinach after 50 days of sowing in response to different concentrations of tire wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar).

Treatment	Conc (mg/kg)	Pn (μmol CO ₂ m ⁻² s ⁻¹)	E (mmol H ₂ O m ⁻² s ⁻¹)	gs (mol m ⁻² s ⁻¹)	Ci (μmol mol ⁻¹)
Soil	0	21.4 ^b	2.23 ^a	0.32 ^a	404.3 ^a
	0.05	24.2 ^a	2.02 ^b	0.28 ^b	350.7 ^{ab}
	0.1	10.33 ^c	1.88 ^c	0.24 ^c	301.0 ^{bc}
	0.25	8.74 ^c	1.71 ^c	0.20 ^d	253.9 ^{bc}
	0.5	5.64 ^d	1.64 ^d	0.17 ^d	209.3 ^c
	Anova (F value)	< 0.001	< 0.001	< 0.001	< 0.05
Foliar	0	7.82 ^a	1.53 ^a	0.31 ^a	419.4 ^a
	0.05	8.84 ^a	1.35 ^a	0.267 ^b	355.7 ^{ab}
	0.1	7.88 ^a	1.07 ^b	0.214 ^c	294.1 ^{bc}
	0.25	6.24 ^{ab}	0.86 ^b	0.197 ^c	231.3 ^{bcd}
	0.5	4.71 ^b	0.54 ^c	0.17 ^d	167.4 ^d
	Anova (F value)	< 0.05	< 0.001	< 0.001	< 0.001
Each value is a mean of 3 replicates, followed by different letters indicating significant difference ($P \leq 0.05$) within an application mode using LSD.					

Similarly, foliar-applied TWP caused significant reductions in these traits, though the magnitude of decline was generally less pronounced than that observed with soil application. Foliar application of 0.5 mg/kg TWP resulted in reductions of 39.8% in Pn, 64.7% in E, 45.2% in gs, and 60.1% in Ci relative to untreated plants. Overall, the data indicate that TWP exposure has an adverse effect on photosynthetic performance, with soil application exhibiting stronger inhibitory effects than foliar application, particularly at higher concentrations.

3.7 Metabolites

The application of TWP had a significant impact on the biochemical attributes of the spinach plant under different concentrations via foliar and soil application treatments (Fig. 6). At the lowest concentration (0.05 mg/kg) of both soil and foliar TWP treatments, the accumulation of all metabolites was increased, but the significant increase in total soluble protein, total amino acids, total soluble sugars as compared to the control. However, as concentration increased to 0.25 and 0.5 mg/kg, a decline in all metabolites was observed. The soil application of 0.5 mg/kg TWP resulted in a 33.5%, 19.4%, 3.8%, and 2.7% decrease in total soluble proteins, total amino acids, phenolics, and soluble sugars, respectively, compared to the control. The foliar application of 0.5 mg/kg TWP resulted in a 32.2%, 31.7%, 8.8%, and 61% decrease in the above traits compared to the control.

3.8 Antioxidant assay

The enzymatic responses of spinach to TWP were assessed by analyzing the activity of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) through soil and foliar applications (Fig. 7). After a slight increase in all the enzyme activities at 0.05 mg/kg, a linear decrease in all enzyme activities was observed at 0.1 and above concentrations. POD activity showed a 0.39%, 0.1%, and 0.3% decline at 0.1, 0.25, and 0.5 mg/kg, respectively, by a soil application compared to the control (Fig. 7a). CAT activity showed a 8% and 5% decline at 0.25 and 0.5 mg/kg, respectively, by a soil application, and 3% decrease by 0.5 g/kg TWP as a foliar application (Fig. 7b). SOD activity declined by 12% at 0.5 mg/kg TWP by a soil application, and 9% and 6% decrease by 0.25 and 0.5 g/kg TWP as a foliar application (Fig. 7c).

3.9 Relative water content and electrolyte leakage

Exposure of both soil and foliar TWP affected the water retention and membrane stability of spinach leaves (Fig. 8). Relative water content (RWC) was significantly reduced by 7% at 0.5 mg/kg of TWP in soil, and 45% and 15% reduction at 0.25 and 0.5 mg/L of foliar TWP, as compared to control (Fig. 8a). Electrolyte leakage (EL) was significantly reduced by 13% at 0.5 mg/kg of TWP in soil, and 20% and 28% reduction at 0.25 and 0.5 mg/L of foliar TWP, as compared to control (Fig. 8b). So the foliar application caused more reduction in RWC and EL especially at 0.25 mg/L TWP.

3.10 Metal accumulation in spinach leaves

The concentrations of essential and non-essential metals in spinach leaves varied significantly depending on the application method and concentration of tire wear nanoparticles (Table 4; Fig. 9). Magnesium (Mg) concentration initially increased with increasing TWP concentration and then decreased, reaching a maximum at 0.1 mg/kg in soil and 0.25 mg/L in soil. Calcium (Ca) content showed no significant variation and remained stable across all treatments. Zinc (Zn) concentration fluctuated between 248.5 and 333.6 mg/kg by soil application of TWP, while it was significantly increased by 0.05, 0.1, and 0.25 mg/L foliar application of TWP, with a maximum at 0.05 mg/L. The iron (Fe) content increased at 0.05 mg/kg TWP in the soil and then decreased at higher concentrations. By foliar application of TWP, Fe content was significantly higher at 0.05, 0.25, and 0.5 mg/L as compared to the control.

Table 4

Concentrations of essential (Mg, Ca, Zn, Fe, Co, Mn, Ni, Cu) and non-essential metals (Cd, Pb, Cr, As) in spinach leaves after 50 days of sowing under different concentrations of tire wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar).

Treatment	Conc. (mg/kg)	Heavy metal (mg/kg dry weight)											
		Mg	Ca	Zn	Fe	Co	Mn	Ni	Cu	Cd	Pb	Cr	As
Soil	0	5019.5	330.6	333.6	92.1	101.4	47.5	70.5	1591.2	61.3	ND	98.9	251.7
	0.05	3560.0	328.5	248.5	110.5	1427.0	977.9	ND	1425.8	129.0	ND	110.9	329.2
	0.1	7879.0	338.5	331.0	57.8	616.9	478.2	ND	1502.6	132.8	ND	86.5	1023.3
	0.25	5122.0	330.0	257.3	27.9	197.0	139.0	ND	1623.1	100.7	ND	148.9	406.8
	0.5	7146.6	333.4	315.7	71.5	233.2	116.9	ND	1517.0	315.7	80.5	6.5	512.3
	LSD (0.5)	801.2	35.5	72.0	30.0	106.2	230.0	-	41.0	54.0	-	36.5	27.0
Foliar	0	3560.0	328.5	325.1	64.4	794.6	1030.4	4.2	1326.2	ND	ND	340.4	223.0
	0.05	4501.0	332.1	601.6	123.4	116.9	25.8	ND	1635.8	ND	13.0	310.3	294.8
	0.1	4371.0	330.7	373.9	57.1	159.7	42.1	ND	1722.3	0.1	23.9	149.5	47.0
	0.25	4939.0	348.8	489.3	176.9	81.3	45.6	ND	1673.7	103.7	ND	371.6	455.0
	0.5	4113.0	330.1	299.4	118.45	116.5	24.8	ND	1759.8	21.7	69.6	113.2	363.5
	LSD (0.5)	551.2	50.6	47.5	65.0	115.0	22.0	-	30.7	43.0	-	41.0	26.4
ND indicates not detected or values are less than the limit of detection by ICP-MS. Each value is the mean of three replicates.													

Cobalt (Co) and manganese (Mn) levels were markedly elevated at 0.05 mg/kg compared to the control and then decreased with increasing TWP concentration. Under foliar application of TWP, Co and Mn levels were continually decreased with increasing TWP concentration.

Nickel (Ni) was detected only under control. Copper (Cu) concentration was not affected by soil application of TWP, but was increased with increasing foliar concentration of TWP, with a maximum at 0.5 mg/L.

Cadmium (Cd) was detected in all treatments of soil application of TWP, with the highest accumulation at 0.5 mg/kg. For the foliar application of TWP, Cd was not detected in the control and at 0.05 mg/L, while it showed the highest concentration (103.7 mg/kg) at 0.25 mg/L of TWP. Lead (Pb) was not detected in soil treatments except at 0.5 mg/kg TWP (80.5 mg/kg). By foliar application of TWP, Pb was detected at 0.05, 0.1, and 0.5 mg/L of TWP, at which the Pb concentrations were 13, 23.9, and 69.6 mg/kg, respectively. Chromium (Cr) was highest at 0.25 mg/kg soil and 0.25 mg/L foliar application of TWP. Arsenic (As) content increased with TWP application, with the highest level observed at 0.1 mg/kg soil and 0.25 mg/kg foliar application of TWP.

4. Discussion

4.1 Physicochemical and structural properties of tire wear nanoparticles

The physicochemical characterization of tire wear nanoparticles (TWPs) in this study revealed properties with significant environmental and biological implications. Dynamic light scattering (DLS) analysis demonstrated that the TWPs suspension was predominantly composed of nanosized particles, with a single peak at 327.2 nm accounting for the entire scattering intensity. The average hydrodynamic diameter of 284.6 nm and a polydispersity index (PDI) of 22.3% indicated moderate heterogeneity within the suspension. These findings suggest that the size reduction and dispersion protocol applied, particularly ball milling and sonication, successfully produced relatively uniform nanoparticles. This shift toward a more homogeneous nanosized fraction implies a greater potential for mobility in environmental matrices, enhanced surface reactivity, and increased bioavailability (Kreider et al., 2010; Park et al., 2018; Lopez et al., 2023). The dominance of nanosized TWPs suggests that their behavior in environmental systems may differ markedly from that of larger tire-derived fragments (Shi et al., 2024). Nanosized particles are more likely to exhibit prolonged suspension in water, greater soil mobility, and higher rates of interaction with biota, including potential uptake by plants and microorganisms (Baensch-Baltruschat et al., 2020).

The chemical composition revealed by Fourier transform infrared (FTIR) spectroscopy complements the size findings by highlighting the surface chemistry of the TWP. The detection of peaks corresponding to triple bonds (2111.6 cm^{-1} , 2085.4 cm^{-1}) suggests the presence of alkynes or nitrile groups, likely derived from synthetic rubber components or formed during mechanical degradation (Klun et al., 2023). Strong absorptions associated with carbonyl groups (1998.0 , 1828.3 , 1731.3 cm^{-1}) point to oxidative modification of the polymer matrix, possibly due to thermal effects during wear or environmental aging. The peak at 1086.8 cm^{-1} indicates the presence of C–O or B–F bonds, consistent with the addition of additives or stabilizers (Huang et al., 2021). Although the spectrum lacked additional peaks indicative of advanced degradation, the presence of polar functional groups suggests that aged TWPs may have increased affinity for environmental contaminants, enhancing their role as vectors for co-pollutants or sites for microbial colonization.

X-ray diffraction (XRD) analysis provided further insight into the structural complexity of the TWPs. The predominance of porphyrinegalliumchloride (90.5%) highlights the presence of complex organometallic species, likely originating from tire additives or high-temperature transformations during abrasion (Dobroy et al., 2022). The identification of cannizzarite (6.5%) confirms the incorporation of lead and sulfur-containing phases, which is concerning given the known toxicity of lead in plants, including disruption of photosynthesis, water relations, and root development (Li et al., 2024). The presence of elemental selenium (3.0%) and bismuth compounds further points to a chemically diverse and potentially hazardous mixture. These elements can contribute to oxidative stress or interfere with nutrient balance in plants (Kumar et al., 2023; Zhou et al., 2021). Moreover, gallium-containing complexes may disrupt iron-mediated processes and mimic essential metals, potentially interfering with plant redox regulation and nutrient homeostasis (Li et al., 2024). Together, the size, surface chemistry, and structural composition of TWPs characterized in this study indicate a multifaceted potential for environmental persistence, reactivity, and biological impact.

4.2 Morphology and elemental composition of tire wear nanoparticles

The morphology and elemental composition of TWPs, as revealed by SEM-EDX analysis, provide critical insights into their environmental behavior and potential risks. Scanning electron microscopy revealed that the TWPs exhibited irregular, jagged edges and varied particle sizes, characteristics typical of particles generated through mechanical abrasion during tire-road contact (Adachi and Tainosho, 2004). The coarse texture and angularity of these particles imply a high surface area-to-volume ratio, which can enhance their chemical reactivity and capacity to adsorb environmental contaminants. Furthermore, the observed clustering of smaller fragments onto larger particles likely results from high surface energy, which promotes aggregation and may alter their environmental fate by influencing settling rates and transport dynamics in air, water, or soil systems.

Elemental mapping by EDX confirmed that carbon was the dominant component, consistent with the styrene-butadiene rubber matrix typical of most tire formulations (Rausch et al., 2022). The presence of oxygen may indicate surface oxidation processes or reflect the inclusion of oxygen-containing additives, such as silica or zinc oxide, which are incorporated into tires to enhance mechanical properties and aging resistance. The detection of zinc, sulfur, and silicon aligns with their well-established roles in vulcanization and as performance enhancers (Adachi and Tainosho, 2004; Klöckner et al., 2021; Lopez et al., 2023). These elements not only contribute to the structural integrity of tires but also represent potential sources of environmental contamination, as leaching from TWPs can release bioavailable metals into surrounding ecosystems (Jeong et al., 2022). Similar to the present results, several studies have detected the presence of other heavy metals, such as Pb, Cd, and Bi, in tire-wear particles (Klöckner et al., 2021).

Additionally, aluminum, calcium, and chlorine detected in the TWPs may originate from external sources, such as road dust, de-icing agents, or atmospheric deposition. This highlights the potential of TWPs to act as vectors for secondary pollutants. Given their chemical complexity, high surface reactivity, and persistence, TWPs are likely to contribute to long-term environmental contamination. They may pose ecotoxicological risks by interacting with co-occurring pollutants or facilitating their transport (Baensch-Baltruschat et al., 2020).

4.3 Fluorescence detection of tire wear nanoparticles in leaves

The fluorescence microscopy results provide insight into the entry and distribution of TWPs in spinach tissues via both soil and foliar exposure routes. In soil-treated plants, fluorescence detected in leaf tissues indicates uptake through the epidermis and subsequent movement via the xylem, suggesting vertical translocation toward the aerial parts (Li et al., 2021). This uptake route may disrupt water and nutrient transport if nanoparticles accumulate in xylem vessels (Wang et al., 2025). A stronger and more widespread fluorescence signal was observed in foliar-treated plants, likely due to nanoparticle penetration through stomata and epidermal layers (Wang et al., 2025). Stomatal openings, being natural entry points, allow direct access to internal leaf tissues, where TWPs can further migrate into the vascular system, including both xylem and phloem (Taylor et al., 2020; Wang et al., 2025). Once inside, nanoparticles interfere with cellular metabolism, block nutrient flow, and generate reactive oxygen species (ROS), leading to oxidative stress. This stress can damage chloroplasts, impair photosynthesis, and affect leaf physiology (Haung et al., 2021). The enhanced accumulation seen in foliar exposure suggests that direct contact with sensitive tissues increases the risk of cellular disruption (Sun et al., 2021).

4.4 Impacts on growth dynamics and biomass accumulation

The present study demonstrates that TWPs exert concentration- and application-dependent effects on spinach growth dynamics, with low concentrations eliciting transient stimulation and higher concentrations inducing clear toxicity. At the lowest tested soil concentration (0.05 mg/kg), TWPs appeared to promote shoot and root growth, suggesting a potential hormetic response. The growth stimulation observed at low TWP concentrations in our study may be linked to the plant's ability to metabolize TWP-derived compounds (Castan et al., 2022) or the presence of some nutrients such as zinc, sulfur, and calcium (Lopez et al., 2023). Castan et al. (2022) demonstrated that lettuce internalized and transformed TWP leachates into stable metabolites, which accumulated in the tissues. Such metabolic processing could explain the improved growth and metabolite levels we recorded at 0.05 mg/kg TWP, as low exposure may trigger mild stress responses or mimic nutrient effects. However, this growth-promoting effect was not observed consistently with foliar application, where low-dose TWPs produced minimal enhancement, indicating that foliar uptake and translocation may be more limited or that leaf exposure initiates defensive rather than growth-promoting responses.

At higher concentrations (≥ 0.25 mg/kg in soil or foliar treatments), significant reductions in shoot and root length, as well as biomass accumulation and key growth indices, were observed. The decline in absolute growth rate (AGR), relative growth rate (RGR), leaf area duration (LAD), specific leaf weight (SLW), and net assimilation rate (NAR) suggests that TWPs disrupt fundamental processes underlying biomass production. In particular, the reduction in NAR (by $\sim 36\%$) points to impaired photosynthetic efficiency, potentially due to damage to chloroplast structures or stomatal malfunction (Li et al., 2023). The suppression of AGR and RGR highlights reduced carbon assimilation and biomass partitioning efficiency, likely resulting from interference with carbon fixation or resource allocation (Lian et al., 2022). These effects were more pronounced under foliar application, implying that direct contact between nanoparticles and leaf tissues leads to greater physiological disruption, possibly through blockage of stomatal pores, oxidative damage to membranes, or interference with cellular metabolism (Chen et al., 2024).

The reduction in leaf area, leaf area index (LAI), and leaf area ratio (LAR) further illustrates that TWPs hinder leaf expansion and canopy development, key determinants of light interception and photosynthetic potential (Hossain et al., 2020). This is consistent with stress adaptation responses, where plants may restrict leaf expansion to conserve water or minimize further damage under adverse conditions (Ali et al., 2022). Such morphological changes were particularly severe following foliar exposure, likely because nanoparticle deposits on leaf surfaces more directly disrupt gas exchange and water regulation (Wang et al., 2025).

4.5 Physiological and biochemical responses to tire wear nanoparticles

Physiological and biochemical assessments revealed that TWP trigger concentration- and exposure-mode-dependent stress responses in spinach. The modest, non-significant changes in photosynthetic pigments at lower concentrations suggest that the photosynthetic machinery remained largely intact under mild exposure. However, the decline in carotenoids at higher doses indicates a compromised antioxidant defense system in chloroplasts. Carotenoids play a critical role in quenching reactive oxygen species (ROS); thus, their depletion reflects a state where oxidative stress exceeds the protective capacity (Zhen et al., 2023).

Metabolite profiling revealed that soil-applied TWPs at 0.1 mg/kg increased the levels of flavonoids, amino acids, and proteins, suggesting the activation of antioxidant and osmoprotective pathways that help plants counteract oxidative damage (Lian et al., 2020; Gao et al., 2019). Elevated flavonoid content reflects enhanced scavenging of ROS, while increases in amino acids and proteins indicate upregulation of stress-related metabolic processes. However, at higher concentrations, these protective responses were insufficient to prevent growth inhibition, consistent with previous findings that excessive nanoparticle exposure overwhelms plant defense systems and induces cellular damage (Dong et al., 2021).

The antioxidant enzyme profile further supports this interpretation. Superoxide dismutase (SOD) activity showed the strongest enhancement (24.1% at lower concentrations), highlighting its critical role in converting superoxide radicals to hydrogen peroxide, a less toxic form (Sun et al., 2018). Catalase (CAT) activity also increased (12.8%), aiding the breakdown of hydrogen peroxide, while peroxidase (POD) activity changed minimally, suggesting differential regulation of enzymatic defenses under nanoparticle stress. The limited POD response may reflect either early saturation of this pathway or its secondary role under the specific stress conditions induced by TWPs (Choudhury et al., 2013). At higher doses, the accumulation of toxic species likely surpassed the capacity of these enzymatic systems, contributing to oxidative damage and impaired growth (Lin et al., 2017).

Water status and membrane stability were also affected. A ~ 7% reduction in relative water content (RWC) indicates compromised water uptake or transport, potentially due to nanoparticle interference with root integrity or vascular function. An increase in electrolyte leakage (EL) of 13% reflects membrane lipid peroxidation and loss of ion homeostasis, hallmark symptoms of oxidative damage (Sharma et al., 2023). The stronger effects observed with soil-applied TWPs reinforce the role of roots as primary entry points for nanoparticles and sites of initial damage (Ma et al., 2023).

Together, these findings demonstrate that TWPs elicit complex physiological disruptions, including oxidative stress, impaired water relations, and metabolic alterations, ultimately leading to reduced growth and productivity. The severity of these effects depends on both concentration and exposure pathway, with foliar exposure imposing greater direct stress on photosynthetic tissues, while soil exposure results in more systemic impacts through root uptake and translocation.

4.6 Presence of heavy metals in TWP and accumulation in plant tissues

Our study demonstrated that exposure to TWPs resulted in differential accumulation of metals in spinach leaves, depending on the specific element, TWP concentration, and mode of application. The elevated accumulation of Pb and Cd at higher TWP doses is particularly noteworthy, as these metals were absent or undetectable in the control plants but reached significant levels following TWP exposure. Although Pb was not detected on the particle surface by EDX, XRD confirmed the presence of Pb-containing crystalline phases. This suggests that Pb, although not abundant on the surface, was present within the internal structures of TWPs and became bioavailable upon environmental release and subsequent plant exposure (Councell et al., 2004; Adachi and Tainosho, 2004). The substantial accumulation of Pb and Cd in leaves, particularly under higher soil and foliar treatments, highlights TWPs as carriers and facilitators of toxic metal entry into plant tissues (Adachi and Tainosho, 2004; Jeong et al., 2022).

In contrast, essential elements such as Co, Mn, and Ni generally decreased in plant tissues with increasing TWP concentration, despite their presence in the TWP matrix (e.g., sulfur and silicon detected by EDX, both of which may influence metal binding and bioavailability). The decline in these micronutrients suggests that TWPs may have disrupted normal nutrient uptake or caused competitive inhibition due to the release of toxic metals, leading to antagonistic interactions at the root-soil or leaf surface interface. For example, the excessive accumulation of Pb and Cd may have interfered with the transport of Co and Mn, as reflected by their reduced levels in treated plants. Overall, the combined metal profiling of TWPs (via EDX) and plant tissues indicates that the complex composition of TWPs, including both essential additives (Zn, Si, S) and hidden toxicants (Pb, Cd), influences not only metal availability but also the balance of nutrient uptake, with potential consequences for plant health and food safety.

Conclusion

This study highlights the potential risks posed by tire wear nanoparticles (TWPs) to crops. Detailed characterization confirmed that TWPs are predominantly nanosized, chemically complex, and capable of carrying heavy metals. Our findings demonstrate that TWPs can enter

spinach plants through both soil and foliar pathways, accumulate in leaf tissues, and disrupt growth, photosynthesis, and metabolic functions. Low concentrations showed minor stimulatory effects, likely due to stress adaptation or micronutrient contributions. However, higher concentrations significantly impaired biomass production, leaf development, and physiological processes, while promoting the accumulation of toxic metals such as Pb and Cd in plant tissues. These results highlight the importance of considering TWPs as an emerging pollutant in agricultural environments, with implications for crop health, productivity, and food safety. Further research is essential to understand their long-term impacts, interactions with soil and plant systems, and potential mitigation strategies.

Declarations

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: All authors declare that they have no conflicts of interest.

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Author contribution

Komal Zahra: Contributed to data collection, analysis, and interpretation. Sumera Anwar: Conceptualized and designed the study, supervised the research, and wrote the manuscript. Fahad Shafiq: Assisted in data analysis and interpretation. Shahbaz Khan: Provided expertise in plant physiology and contributed to the experimental design. Kahaf: Involved in data collection and laboratory experiments. Muhammad Ashraf: Provided guidance on the research methodology and contributed to manuscript editing.

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Figures

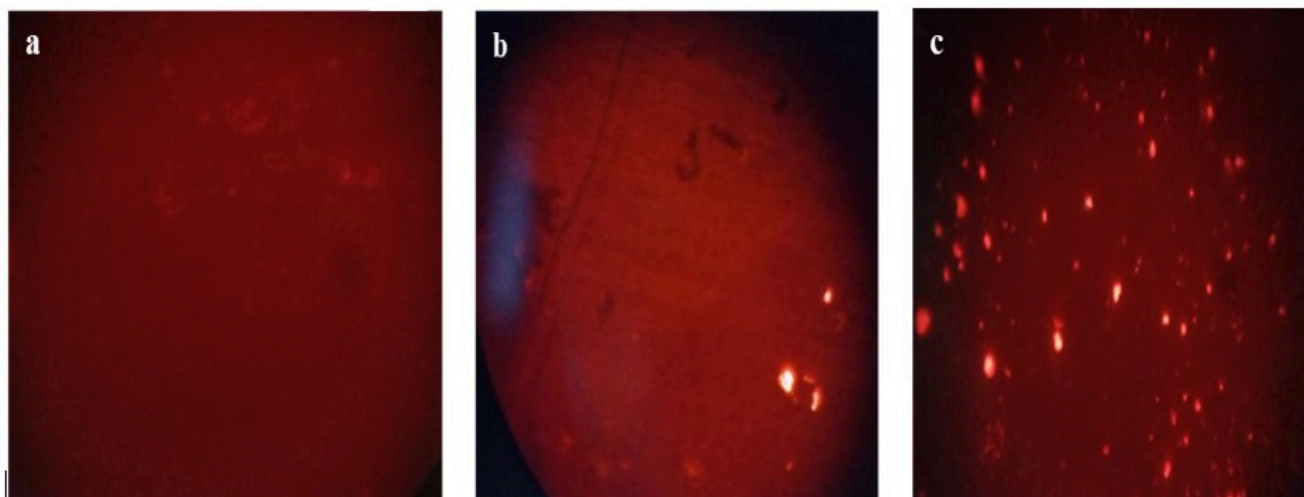


Figure 1

Fluorescence microscopic images to detect the presence of tire wear nanoparticles in spinach leaf samples stained with Rhodamine B dye. a) control (no application), b) roots treated with 0.5 mg/kg of tyre wear nanoparticles in soil, c) leaves treated with 0.5 mg/L tyre wear nanoparticles as foliar spray.

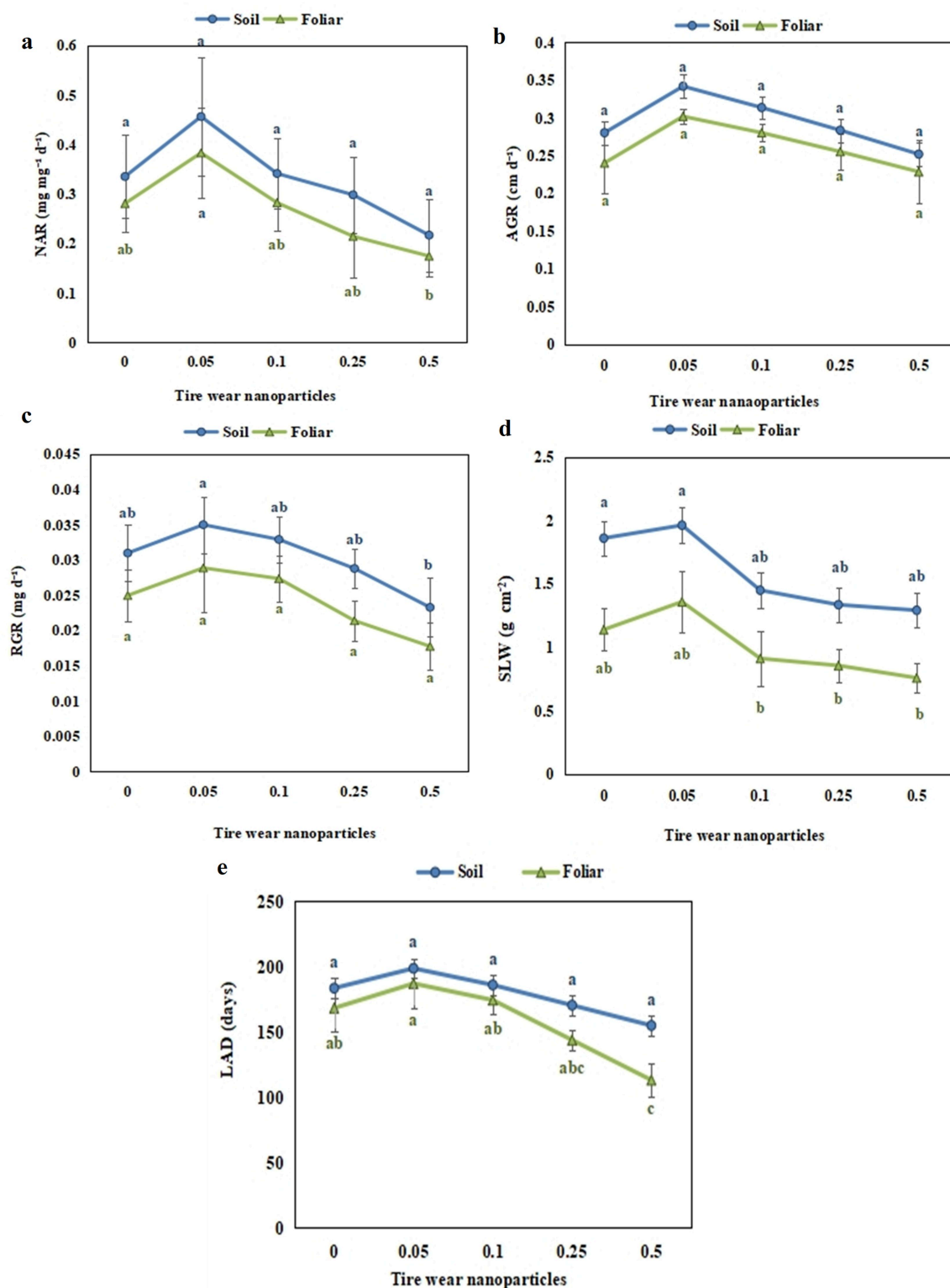


Figure 2

a) Net assimilation rate (NAR), b) Annual growth rate (AGR), c) Relative growth rate (RGR), d) Specific leaf weight (SLW), e) Leaf area duration (LAD) of spinach after 50 days of sowing of different concentrations (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar) of tire wear nanoparticles. Each value is a mean of 3 replicates $\pm$ standard error, followed by different letters indicating significant difference ($P \leq 0.05$) within an application mode using LSD.

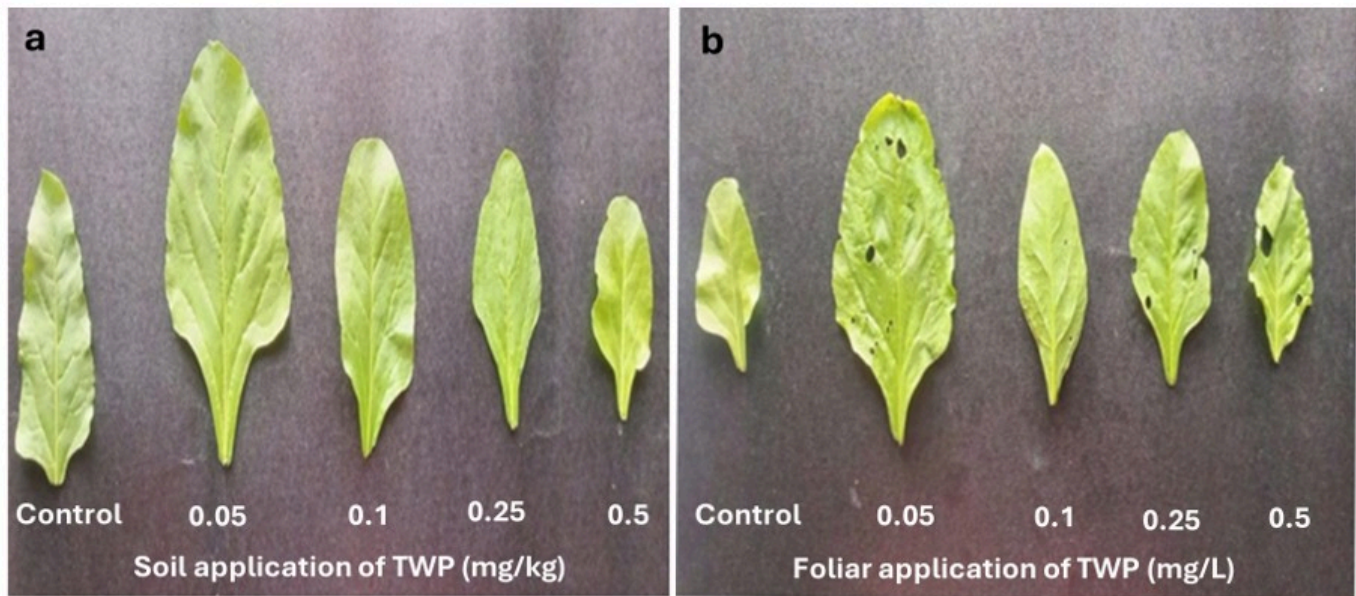


Figure 3

Leaf morphology of spinach after 45 days of sowing in response to tyre wear nanoparticles (TWP). (a) Representative leaves from plants subjected to soil application of TWP at 0 (control), 0.05, 0.1, 0.25, and 0.5 mg/kg. (b) Representative leaves from plants subjected to foliar application of TWP at 0 (control), 0.05, 0.1, 0.25, and 0.5 mg/L.

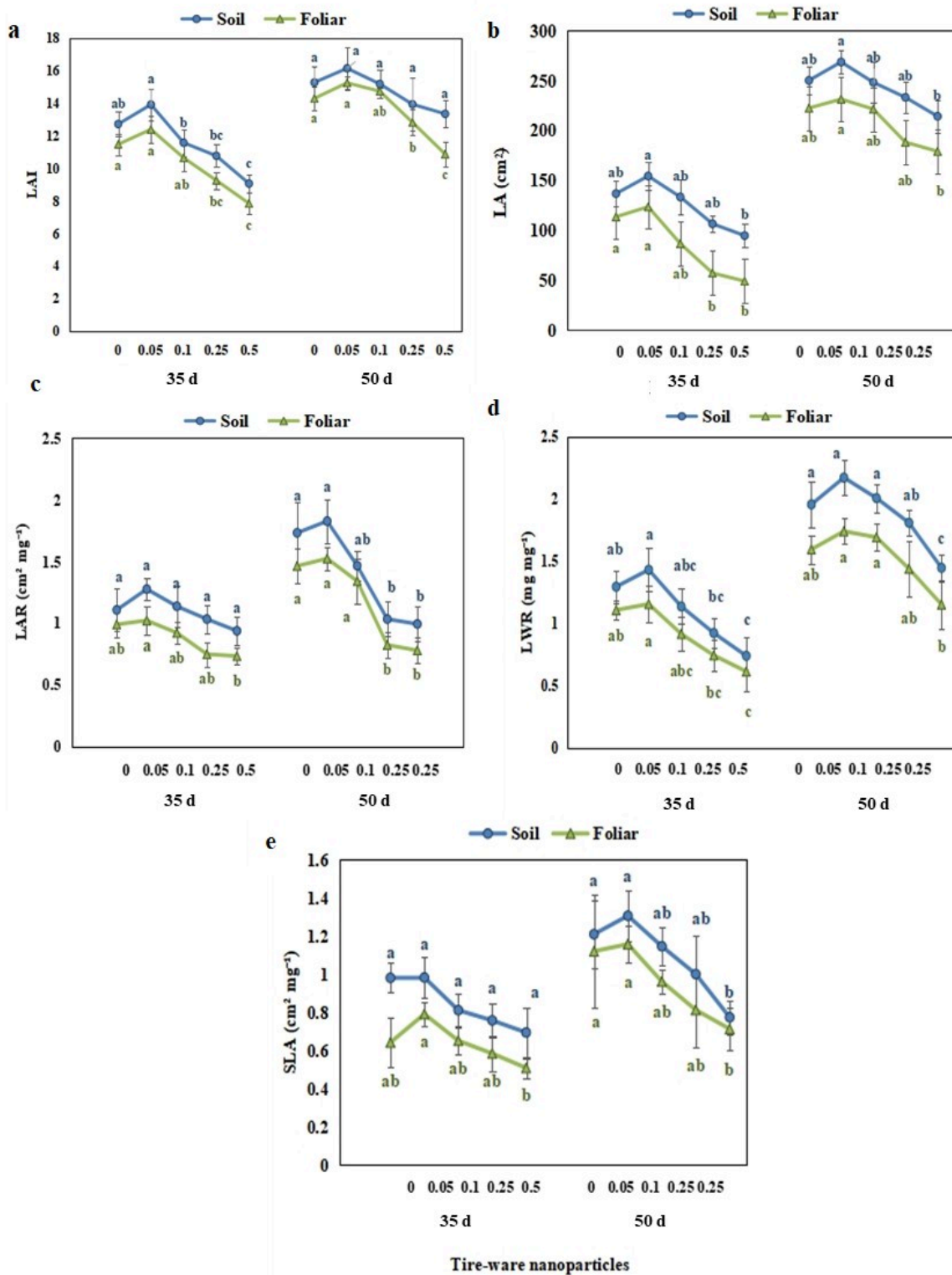


Figure 4

a) Leaf area index, b) Leaf area, c) Leaf area ratio, d) Leaf weight ratio, e) Specific leaf area of spinach after 35 and 50 days of sowing under different concentrations(0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar) of tire wear nanoparticles. Each value is a mean of 3 replicates $\pm$ standard error, followed by different letters indicating significant difference ($P \leq 0.05$) within an application mode using LSD.

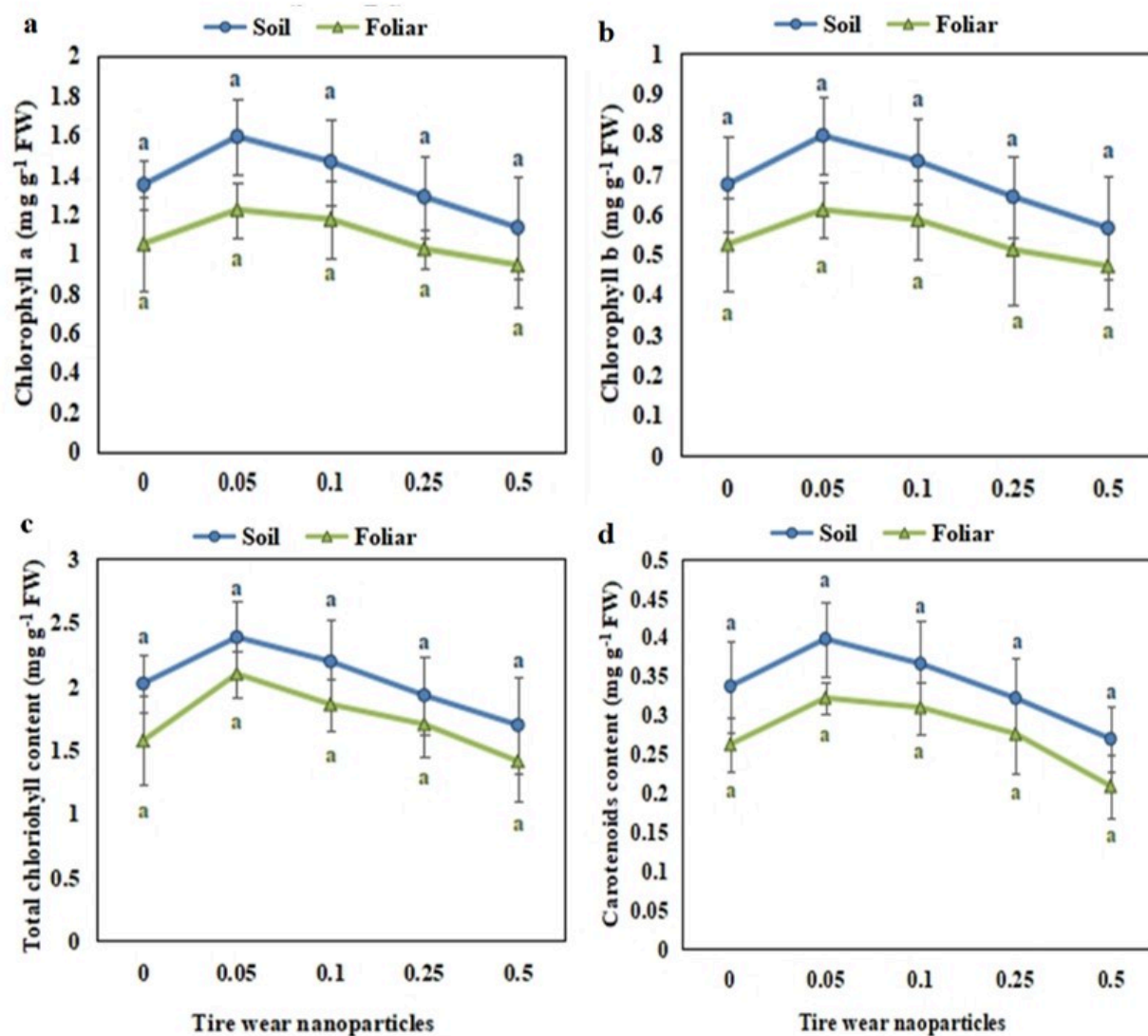


Figure 5

Chlorophyll a (a), chlorophyll b (b), total chlorophyll (c), and carotenoids of spinach after 45 days of sowing in response to different concentrations of tire wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar). Each value is a mean of 3 replicates ± standard error, followed by different letters indicating significant differences.

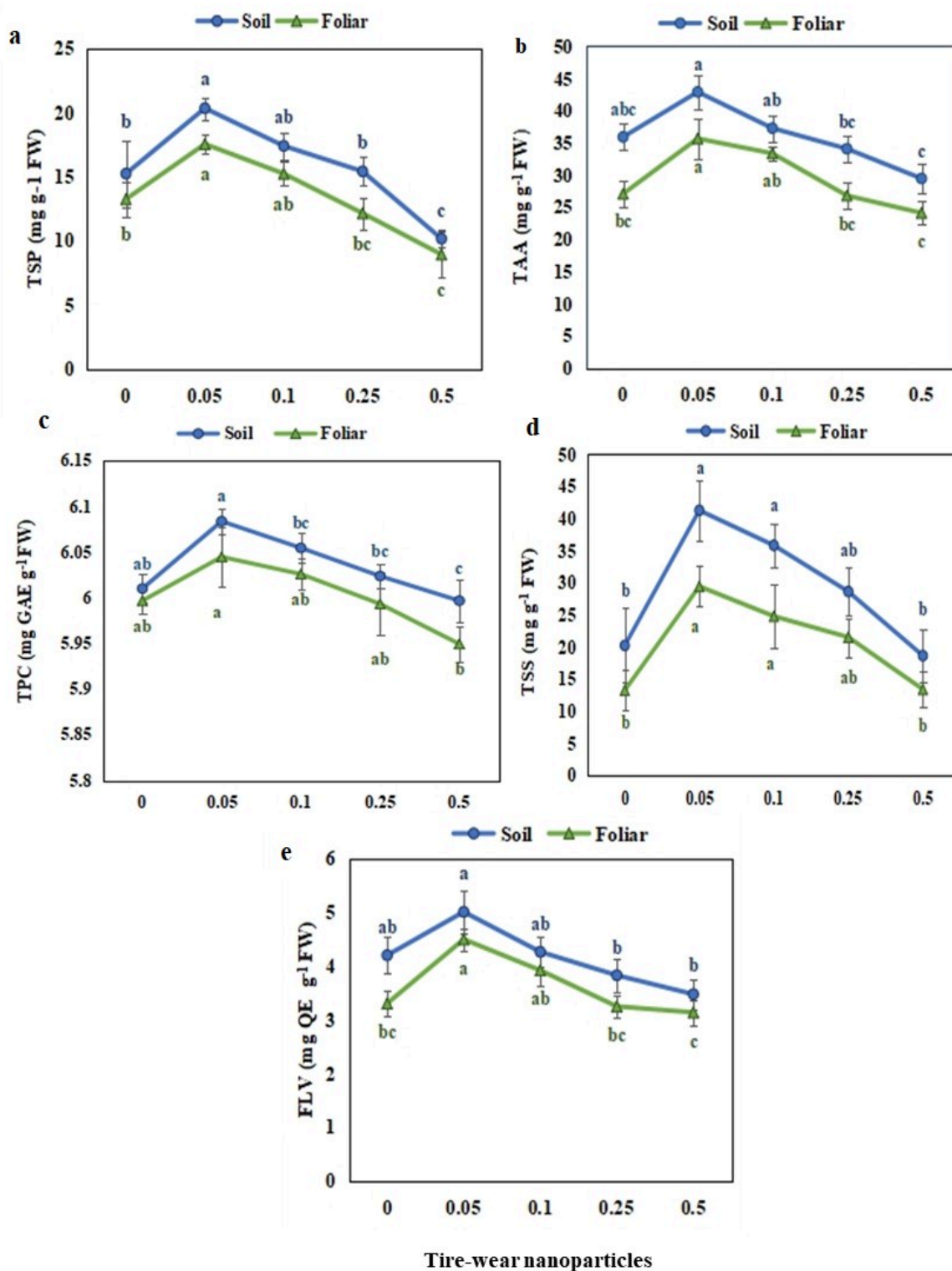


Figure 6

Metabolite content in spinach leaves in response to tyre wear nanoparticles. (a) Total soluble proteins (TSP), (b) Total free amino acids (TAA), (c) Total phenolics content (TPC), (d) Total soluble sugars (TSS), (e) Flavonoids (FLV) of spinach after 45 days of sowing in response to different concentrations of tyre wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar). Each value is a mean of 3 replicates $\pm$ standard error, followed by different letters indicating a significant difference.

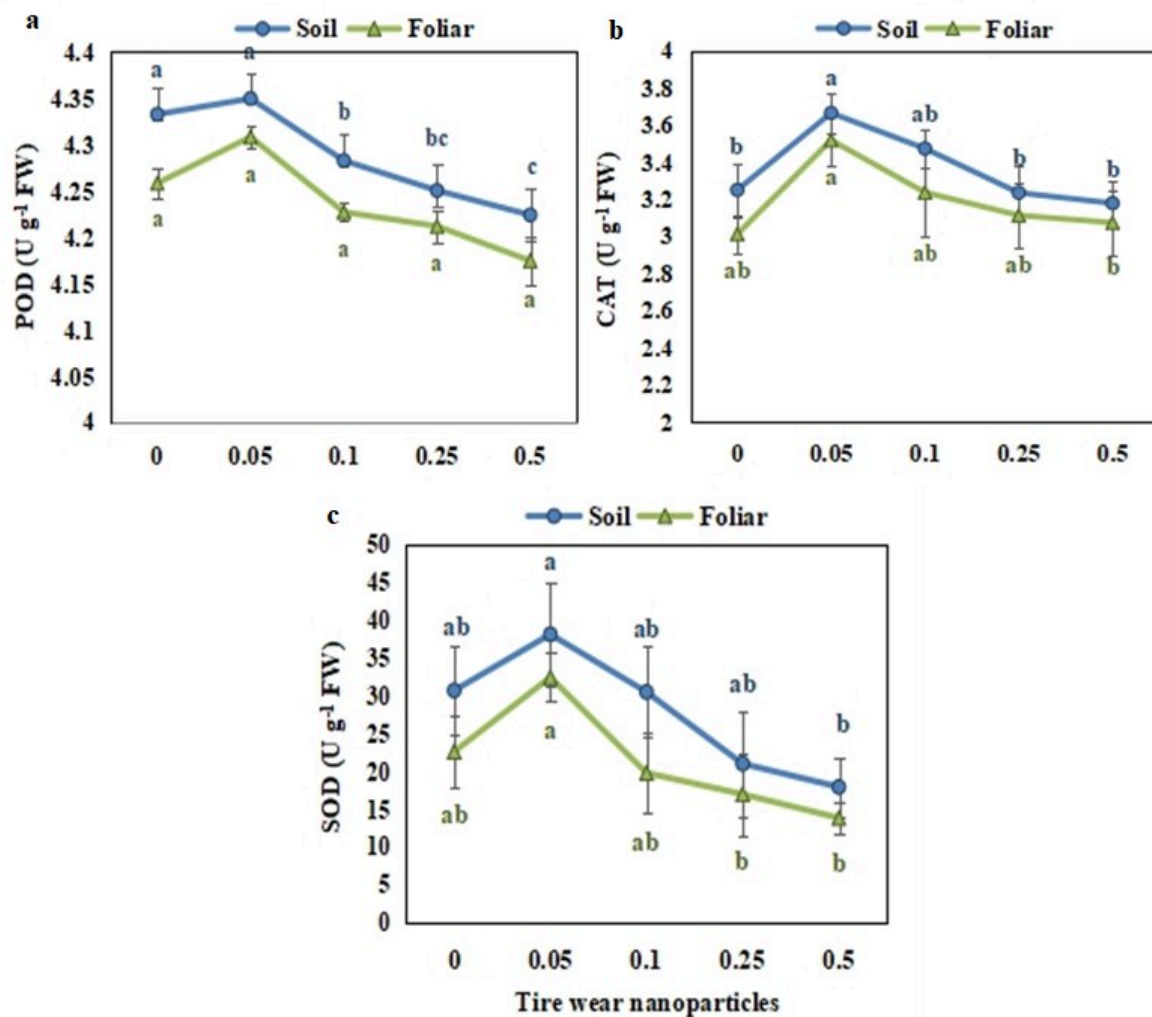


Figure 7

(a) Peroxidase (POD), (b) Catalase (CAT), (c) Superoxide dismutase (SOD) enzyme activities, of spinach after 45 days of sowing in response to different concentrations of tire wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar). Each value is a mean of 3 replicates $\pm$ standard error, followed by different letters indicating significant differences.

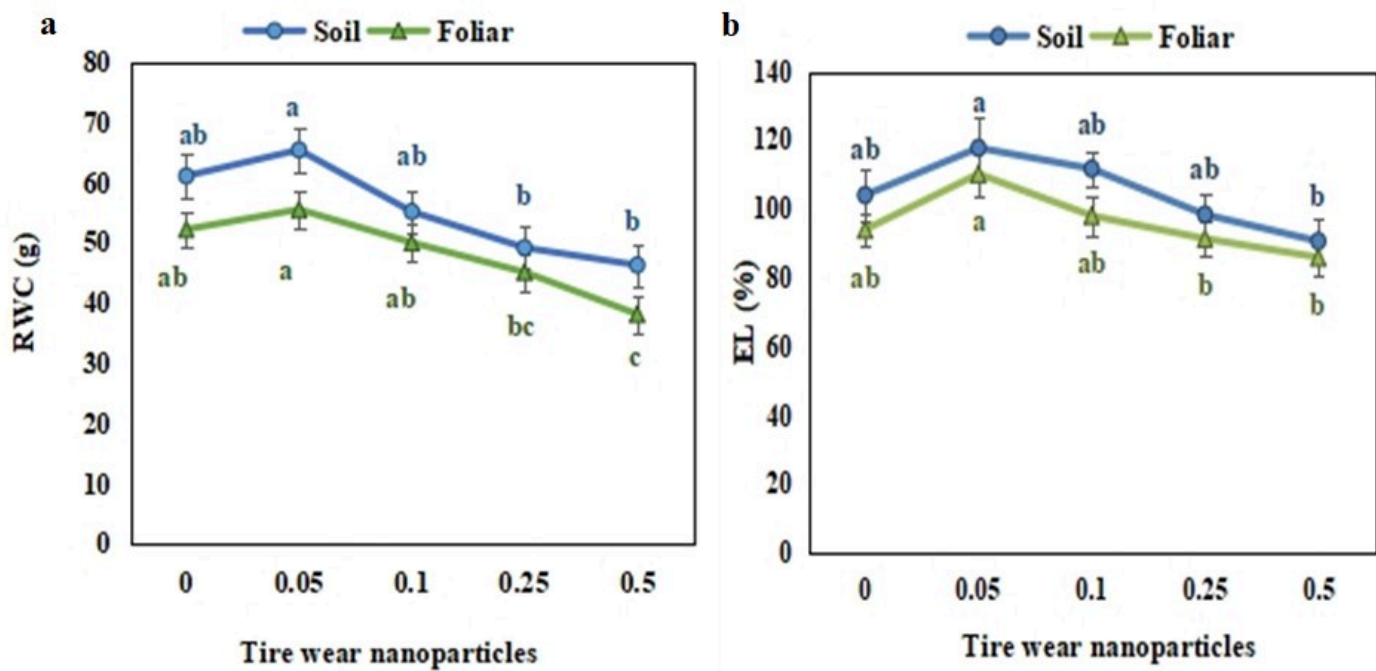


Figure 8

(a) Relative water content (RWC) and (b) Electrolyte Leakage (EL) of spinach leaves after 50 days of sowing in response to different concentrations of tire wear nanoparticles (0, 0.05, 0.1, 0.25, and 0.5 mg/kg for soil application, and 0, 0.05, 0.1, 0.25, and 0.5 mg/L for foliar). Each value is a mean of 3 replicates $\pm$ standard error, followed by different letters indicating significant differences.

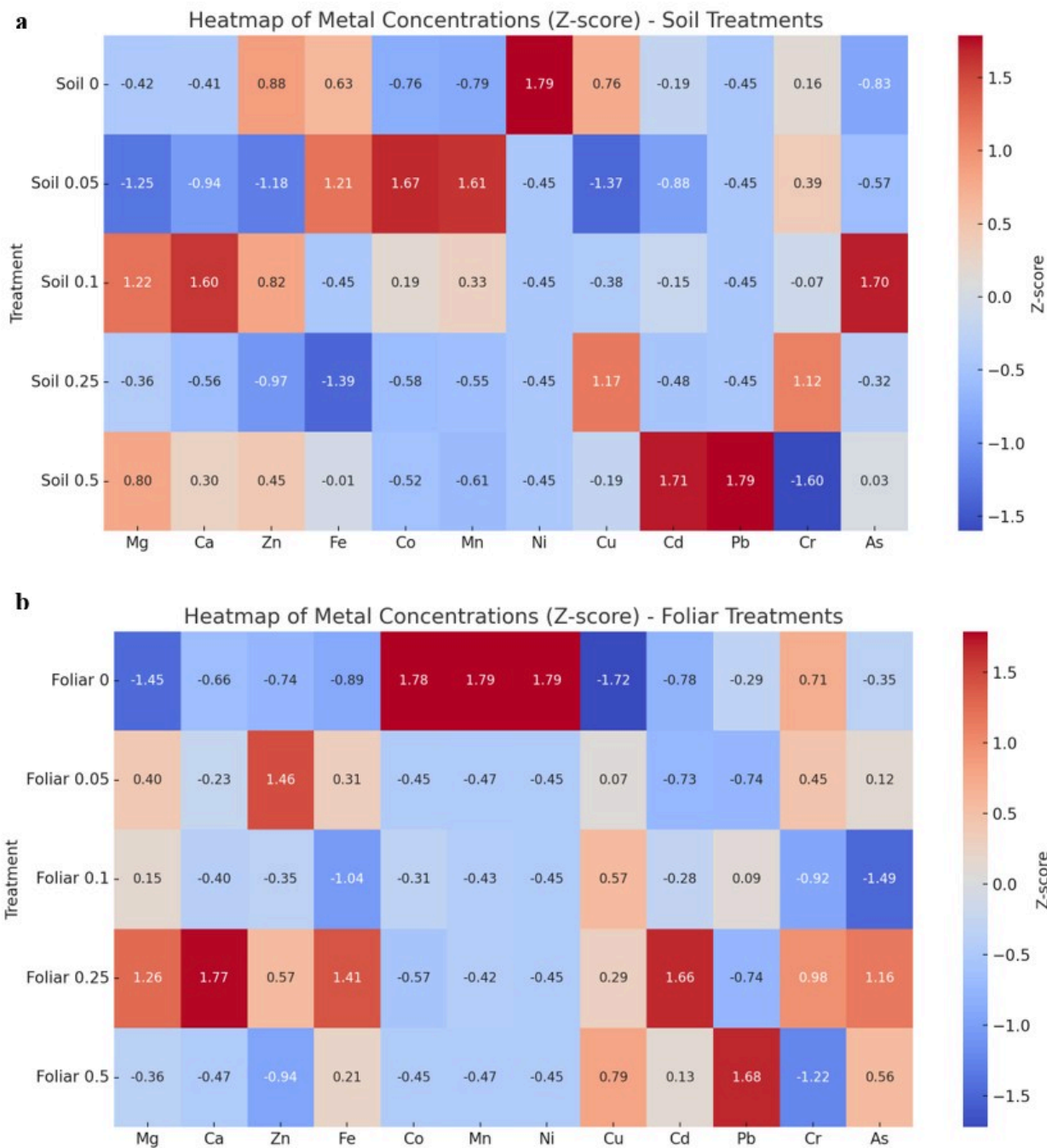


Figure 9

Heat map of heavy metal concentrations (Z-score normalized) in spinach leaves under different TWP treatments in a) soil and b) foliar. The color gradient represents standardized metal accumulation across treatments, with red indicating higher-than-average levels and blue indicating lower-than-average levels for each metal.

Supplementary Files

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