agronomy

MDPI

Article

Toxicity of Tris(2-chloroethyl) Phosphate (TCEP) to Alfalfa's Root System: An Insight into TCEP's Damage to Morphology, Respiration, and Antioxidant Systems

Meijun Liu [1,2,3,*,†], Liangzhu Gong [1,2,3,†], An Yan [1,2,3], Wenjing Liu [1,2,3], Haojie Li [1,2,3] and Peiyi Guo [1,2,3]

[1] College of Grassland Science, Xinjiang Agricultural University, Nongda Road East 311, Urumqi 830052, China; zhu4340@126.com (L.G.); zryanan@163.com (A.Y.); 18097534969@139.com (W.L.); lhj13568100532@163.com (H.L.); 17765433651@163.com (P.G.)
[2] Key Laboratory of Grassland Resources and Ecology of Western Arid Region, Ministry of Education, Nongda Road East 311, Urumqi 830052, China
[3] Xinjiang Key Laboratory of Grassland Resources and Ecology, Nongda Road East 311, Urumqi 830052, China
* Correspondence: lebaby7@163.com
† These authors are co-first author.

Abstract

Tris(2-chloroethyl) phosphate (TCEP), as an organophosphate contaminant, poses a significant threat to the growth and development of plants, especially roots. This study aimed to clarify the mechanisms of TCEP's toxicity and damage to root systems, as well as the mechanisms of its damage to the respiration and energy metabolism of alfalfa root cells. The results showed that TCEP obviously affected the root length, root surface area, root volume, and root diameter of alfalfa. With increasing stress intensity, the total mitochondrial respiration rate and Cytochrome C Oxidase (COX) pathway respiration rate progressively declined, while the Alternative Oxidase (AOX) pathway respiration rate and its proportion of total respiration gradually rose. In addition, adenosine triphosphate (ATP) content and root vigor were significantly reduced. Moreover, with an increase in TCEP concentration, root superoxide anion radical content in alfalfa root cells was significantly elevated, while superoxide dismutase (SOD) and catalase (CAT) activities were significantly lowered, and ascorbate peroxidase (APX) and peroxidase (POD) activities were significantly enhanced. The present study indicated that respiration was disrupted, causing a lack of ATP in root cells under TCEP. Both the overproduction of reactive oxygen species (ROS) from the mitochondrial respiratory electron transport chain (mECT) and the deficiency of ROS-scavenging enzymes caused ROS accumulation, which led to the destruction of the cell membrane structure and exacerbated the disruption of the respiratory metabolism. The disruption of the conversion and reuse of energy by TCEP affected root growth and development.

Keywords: organophosphate; root morphology; respiratory metabolism; antioxidant; alfalfa

check for updates

Academic Editor: Marzia Vergine

Received: 1 October 2025
Revised: 20 October 2025
Accepted: 20 October 2025
Published: 25 October 2025

Citation: Liu, M.; Gong, L.; Yan, A.; Liu, W.; Li, H.; Guo, P. Toxicity of Tris(2-chloroethyl) Phosphate (TCEP) to Alfalfa's Root System: An Insight into TCEP's Damage to Morphology, Respiration, and Antioxidant Systems. *Agronomy* **2025**, *15*, 2483. https://doi.org/10.3390/agronomy15112483

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Tris(2-chloroethyl) phosphate (TCEP) is a commonly used organophosphorus compound that is widely used for its excellent flame-retardant properties [1]. However, it is very toxic and is easily enriched, which causes ecological and environmental safety issues [2–6]. Large amounts of TCEP have been detected in wastewater [7], sludge [8], and even in Lake Taihu [9]. TCEP readily accumulates in soil and is difficult to degrade [10]. Current studies primarily focus on elucidating the absorption, transport, and accumulation patterns of this substance within plants [11–14]. Organophosphorus esters (OPEs) can induce oxidative stress responses

in wheat (*Triticum aestivum* L.), thereby affecting its growth, development, and hormonal balance [15,16]. However, critical gaps remain in our understanding of the specific physiological mechanisms and toxicological principles governing their effects on root systems.

The root system serves as the primary organ for nutrient uptake in plants and is the first to sense and respond to the soil environment [17]; its growth and developmental status directly affect the aboveground biomass [18]. However, the morphological structure and physiological metabolism of roots are highly sensitive to environmental stress [19,20], which is closely linked to their inherent stress resistance [21]. Whether plants can adapt to TCEP stress by altering root morphology has yet to be demonstrated.

Respiration not only provides energy for root cells but also plays a crucial role in maintaining the redox balance within these cells; however, it is sensitive to stress [22]. Consequently, TCEP poses a direct threat to root growth and development by damaging root system conformation, inhibiting respiration, or inducing oxidative damage to root cells. The mitochondrial respiratory electron transport chain (mETC) in plants is divided into the cytochrome respiration pathway (CP) and the alternate respiration pathway (AP). In the CP, electron transfer is coupled with proton translocation, which promotes ATP production, whereas the AP lacks this property [23]. Whether TCEP stress interferes with CP and affects energy supply to the root system remains unclear. Furthermore, it has been shown that organophosphorus compounds induce oxidative stress in wheat, and AOX-catalyzed AP plays an important role in redox homeostasis [24]. When subjected to TCEP stress, it is uncertain whether the AP can mitigate stress-induced oxidative damage and thereby enhance plant resistance.

Alfalfa (*Medicago sativa* L.) is a perennial leguminous forage crop extensively cultivated in China. Its aboveground biomass and root system possess a high capacity for accumulating harmful substances, making it a valuable resource for ecological restoration [25,26]. In this study, Xinmu No. 4 was selected to investigate the changes in root morphology, respiration, ATP content, reactive oxygen species (ROS) production, and the responses of the antioxidant system under exogenous TCEP stress. The primary objective of this research is to elucidate the mechanisms underlying TCEP's toxicity and its detrimental effects on the plant root system. Furthermore, the findings of this study will contribute to a more accurate understanding of TCEP's environmental behavior and facilitate the assessment of its ecological risks in soil.

2. Materials and Methods

2.1. Test Materials

The test plant used in this study was "Xinmu No. 4" alfalfa (*Medicago sativa* L. Xinmu No. 4). The seeds were obtained from the College of Grass Industry at Xinjiang Agricultural University, while TCEP (98% purity) was sourced from Shanghai Aladdin Biochemical Science and Technology Co. (Shanghai, China).

2.2. Experimental Design

Due to its resistance to degradation, TCEP accumulates in the environment. To elucidate the mechanisms underlying TCEP's toxicity and its detrimental effects on plant root systems, concentrations of TCEP treatments were established at 0, 5, 10, 20, and 40 mg/L.

In this study, we employed a substrate culture method utilizing plastic pots measuring 10 cm in height and 10 cm in diameter. These pots were thoroughly washed and dried with deionized water before being filled with vermiculite to facilitate the assessment of the entire root system. Healthy alfalfa seeds were sown into the pots, and after sowing, a mixed solution containing the corresponding TCEP concentration and Hoagland's nutrient solution was applied bi-daily at a volume of 150 mL.

The experiment included four independent biological replicates (n = 5). Within each replicate group and TCEP concentration, 20 alfalfa seedlings were designated as independent statistical units for cultivation. Morphological analyses were conducted on roots from 15 to 18 randomly selected seedlings per group.

After 14 days of cultivation, alfalfa roots were harvested and rinsed with distilled water. High-resolution two-dimensional images were obtained using an Epson Expression 1200XL root scanner (Shanghai, China). Root geometric parameters—such as total root length, average diameter, surface area, and volume—were measured using WinRHIZO. The remaining root tissue was promptly immersed in liquid nitrogen for rapid cryopreservation.

2.3. Measurement Indicators and Methods

2.3.1. Root System Geometric Configuration Parameters

The plants were removed from their pots to wash the roots. A desktop scanner (Epson Expression 1200XL, Shanghai, China) was utilized to scan the alfalfa root systems for each treatment, and the images were stored on a computer for analysis of the planar geometrical configuration parameters of the root system, including root volume (RV), total root surface area (RSA), average diameter (AD), total root length (TRL), number of root tips, and number of nodes.

The cleaned root systems were scanned using the same desktop scanner (Expression 1200XL, Shanghai, China) to obtain digital images, which were subsequently saved to a computer. WinRHIZO 2019 root analysis software was then employed to analyze the root geometric configuration parameters, including total root volume (RV), total root surface area (RSA), average root diameter (AD), total root length (TRL), root tip count, and internode count, as described by Arsenault et al. in 2019 [27].

The drying method was utilized to determine the fresh weight of alfalfa roots by randomly selecting 20 alfalfa roots, rinsing them several times with distilled water, and measuring their fresh weight. Subsequently, the roots were placed in an oven at 105 °C for 30 min and then dried at 70 °C for 12 h. By weighing their dry weights, we calculated the specific root length (SRL) and specific surface area (SRS), where SRL (cm/g) = TRL/root biomass and SRS (cm^2/g) = RSA/root biomass [28].

2.3.2. Determination of Alfalfa Root Vigor and Cellular ATP Content

Root vigor was measured using the triphenyltetrazolium chloride (TTC) method, with differences in root vigor among treatment concentrations expressed as root reducing power. First, a standard curve was prepared. Roots of similar length and thickness were selected, and 0.1 g of the root system was cut off and placed in a 50 mL beaker. Then, 5 mL each of 0.4% TTC solution and 0.1 $mol \cdot L^{-1}$ phosphate-buffered solution (pH 7.0) were added. The mixture was thoroughly mixed to ensure that the root tip segments were fully immersed in the solution. The samples were incubated at 37 °C for 1 h to allow the root tip segments to develop color. After 1 h, 2 mL of 1 $mol \cdot L^{-1}$ H_2SO_4 solution was immediately added to terminate the reaction. The roots were removed, excess moisture was blotted with filter paper, and the roots were placed in a mortar. They were ground with 3–4 mL of ethyl acetate (without quartz sand). The extracted red TTF was carefully transferred to a graduated test tube, and the residue was washed with ethyl acetate 2–3 times until the washings were colorless. All washings were combined into the test tube and diluted to 20 mL with ethyl acetate. After thorough mixing, colorimetry was performed at 485 nm using ethyl acetate as the blank, and the OD value was recorded. The result was calculated using the standard curve [29].

ATP content was determined using an ATP bioluminescence assay kit purchased from Suzhou Keming Biotechnology Co. (Suzhou, China). Following the manufacturer's instructions, approximately 0.1 g of the sample was weighed, and 1 mL of the extraction

solution was added. The sample was homogenized in an ice bath, extracted by heating at 100 °C for 5 min, and centrifuged at 8000× *g* at 4 °C for 15 min, and the supernatant was collected for analysis. The supernatant was combined with the corresponding reagents from the kit. Subsequently, the ATP content was measured using the UV-1800 visible spectrophotometer T2600S (Youke, Shanghai, China) with the wavelength set to 700 nm. Enzyme activity was calculated according to the provided instructions [30].

2.3.3. Root Respiration Measurements

Under dark conditions, the oxygen consumption rate of root tissues was measured using an OXYTHERM oxygen electrode (Hansatech, UK). The procedure involved immersing 0.1 g (fresh weight) of root tissue in 2.6 mL of PBS buffer within a reaction cup. After the oxygen consumption rate stabilized, the total respiration rate (Rtotal, μmol/g/min) was calculated based on the linear decrease in oxygen concentration.

The oxygen consumption for COX respiration was assessed following the addition of a salicylhydroxamic acid (SHAM) solution at a working concentration of 20 mmol/L. The respiration rate of the AOX pathway was determined using the equation RAox = Rtotal − RCox.

2.3.4. Superoxide Anion (O^{2-}), Hydrogen Peroxide (H_2O_2) Content, Malondialdehyde (MDA), and Free Proline Content Determination

The contents of superoxide anion radicals, H_2O_2, MDA, and free proline in alfalfa roots were quantified using a kit obtained from Suzhou Keming Biotechnology Co. Following the manufacturer's instructions, 0.1 g of the sample was weighed, and 1 mL of extraction solution was added before homogenization in an ice bath. The homogenate was then centrifuged at 8000× *g* for 10 min at 4 °C. The supernatant was collected, and kit-specific reagents were added. Absorbance values for superoxide anion (O_2^-, 530 nm), hydrogen peroxide (H_2O_2, 415 nm), and malondialdehyde (MDA, ΔA = Δ532 − ΔA600) were measured using a UV–visible spectrophotometer (T2600S Youke, Shanghai, China). To determine proline content, 0.1 g of the sample was weighed, and 1 mL of extraction solution was added before homogenization in an ice bath. The homogenate was subjected to shaking extraction at 90 °C for 10 min, followed by centrifugation at 10,000× *g* for 10 min at 25 °C. The supernatant was collected, and kit-specific reagents were added. Proline content (PRO, 520 nm) was measured using the same UV–visible spectrophotometer T2600S (Youke, Shanghai, China). Enzyme activity was calculated according to the manual [31].

2.3.5. Determination of Antioxidant Enzyme Activity

The activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD) were measured using kits obtained from Suzhou Keming Biotechnology Co. Following the manufacturer's protocol, 0.1 g of the sample was weighed, and 1 mL of extraction solution was added. The mixture was then homogenized in an ice bath. Subsequently, the homogenate was centrifuged at 8000× *g* for 10 min at 4 °C. The supernatant was transferred, and the corresponding reagents from the kit were added. Absorbance values for SOD (450 nm), POD (470 nm), and CAT (240 nm) were measured at their respective wavelengths using the UV–visible spectrophotometer T2600S (Youke, Shanghai, China). Enzyme activity was calculated according to the provided instructions [31].

2.3.6. Data Analysis

The experimental data were organized using Excel 2010, plotted with Origin 2022, and statistically analyzed using one-way analysis of variance (ANOVA) with SPSS 20.0. Multiple comparisons were performed using Duncan's method, with significant differences indicated by different lowercase letters in the body graphs and tables ($p < 0.05$). A minimum of three biological replicates was conducted for each index determination.

3. Results

3.1. Effect of TCEP on Alfalfa Root Morphology

TCEP significantly affected the growth of alfalfa. The complete plant phenotype is shown in Supplementary Figure S1, while the isolated root system is shown in Supplementary Figure S2. The alterations in the root architecture of alfalfa under different concentrations of TCEP treatments are shown in Figure 1. Compared with the control, TCEP exposure significantly reduced the total root length (TRL) ($p < 0.05$), which progressively declined with increasing TCEP stress. At a concentration of 40 mg/L, TRL decreased by 25-fold relative to the control. The root surface area (RSA) was significantly reduced under 5 and 10 mg/L TCEP treatments compared to the control, and this difference became more pronounced with increasing stress intensity. Significantly reduced root volume (RV) was observed at TCEP concentrations of 5, 10, and 20 mg/L ($p < 0.05$), while at 40 mg/L, RV was 35.3-fold lower than that of the control. Average diameter (AD) was significantly reduced at 20 and 40 mg/L TCEP ($p < 0.05$), whereas no significant changes were observed ($p > 0.05$) at 5 and 10 mg/L.

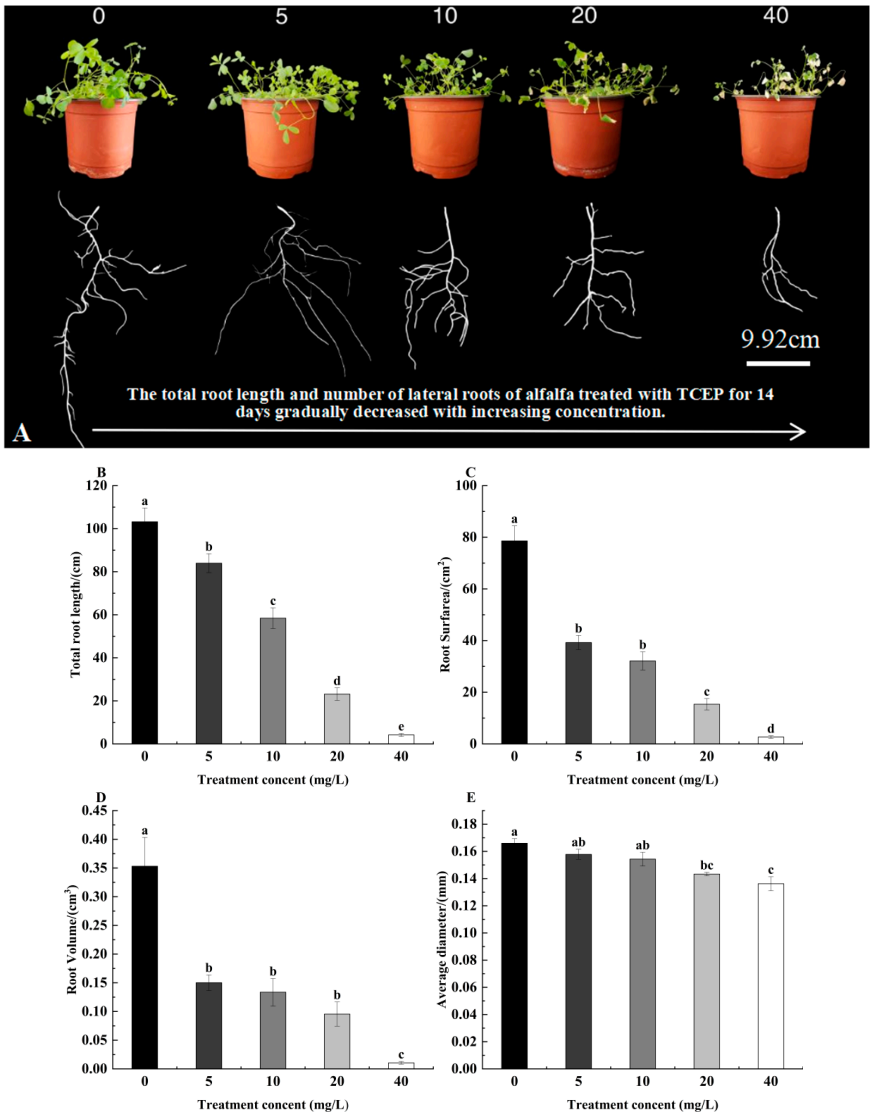


Figure 1. Effects of TCEP concentration on total root length (**B**), total root surface area (**C**), root volume (**D**), and average diameter (**E**) in alfalfa (**A**). Different letters indicate significant differences between different TCEP concentrations and the control group ($p < 0.05$).

3.2. Effect of TCEP on Root Biomass, Specific Root Length, and Specific Root Surface Area of Alfalfa Roots

The TCEP treatment significantly reduced alfalfa root biomass and affected alfalfa-specific root length and specific surface area ($p < 0.05$) (Figure 2). As the intensity of TCEP stress increased, both specific root length (SRL) and specific surface area (SRS) of alfalfa exhibited a gradual decline. Specifically, SRL decreased from a maximum of 504.235 cm/g to 33.057 cm/g, while SRS fell from 276.736 cm^2/g to a minimum of 21.035 cm^2/g under severe stress. This represented reductions of 15.25-fold for SRL and 13.15-fold for SRS compared to control (CK) values. SRL and SRS are critical morphological traits that influence the roots' ability to absorb water and nutrients, indicating that TCEP impacts water and nutrient uptake by altering alfalfa root morphology. Furthermore, with increasing treatment concentration, both the number of root tips and nodes showed a gradual decline. Under the 0 mg/L treatment condition, root tip and node counts peaked at 215.50 and 452.25, respectively. However, when the TCEP concentration was elevated to 40 mg/L, root tip and node counts decreased by approximately 3.33-fold and 3.53-fold, respectively, demonstrating a significant inhibitory effect ($p < 0.05$).

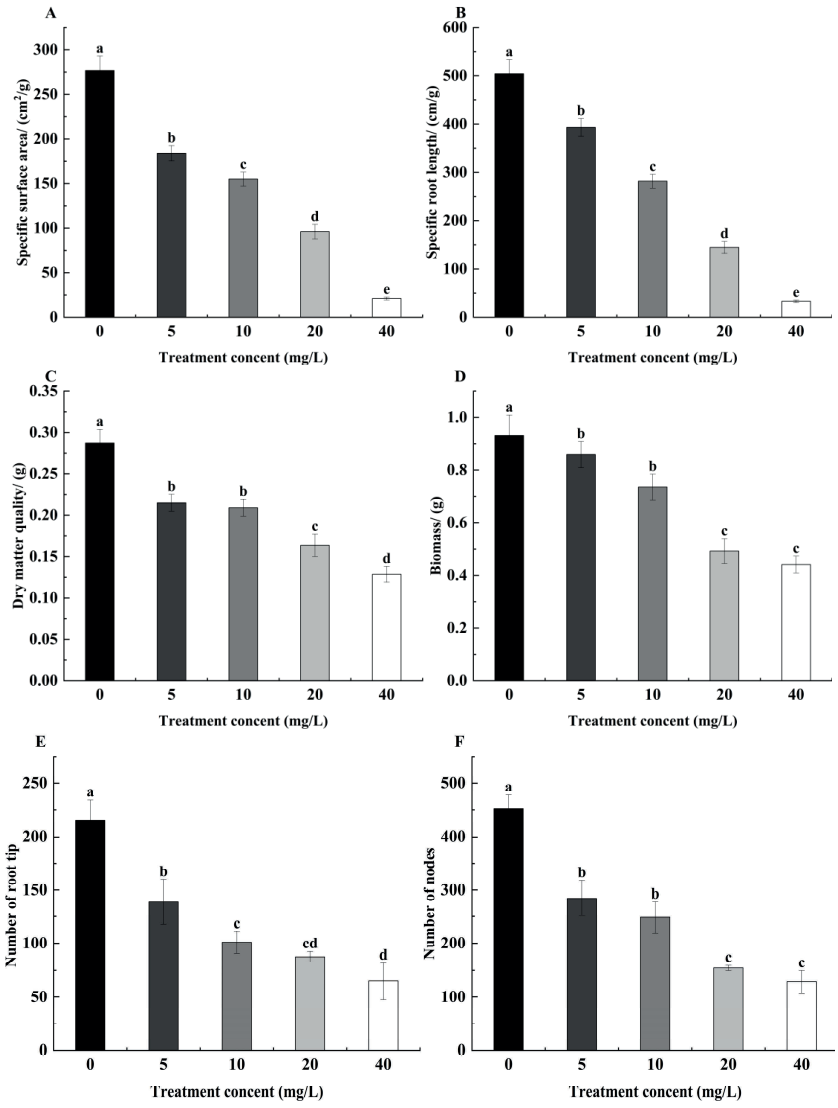


Figure 2. Effect of TCEP treatment on specific root surface area (**A**), specific root length (**B**), dry matter mass per plant (**C**), alfalfa root system biomass (**D**), root tip number (**E**), and node number (**F**). Different letters indicate significant differences between different concentrations of TCEP and the control ($p < 0.05$).

3.3. Effect of TCEP on Alfalfa Root Vigor, ATP Content, and Root Respiration

The results indicated that TCEP stress significantly reduced ATP content in alfalfa roots, as evidenced by a corresponding suppression of root vitality (Figure 3). Compared to the control group, TCEP treatment induced a concentration-dependent decline in root vitality ($p < 0.05$). Under severe stress conditions (40 mg/L), root vitality decreased by 94.4% relative to the control, which corresponds to only 5.6% of the control level. As stress intensity increased, both the total mitochondrial respiration rate and the CP respiration rate progressively declined ($p < 0.05$), while the AP respiration rate and its proportion of total respiration gradually increased ($p < 0.05$). The ratio of AP to total mitochondria respiration at 40 mg/L TCEP reached its peak ($p < 0.05$), indicating the most severe impairment of the mitochondrial electron transport chain (mETC). As the direct energy currency for cellular metabolism, ATP content exhibited a significant decline with increasing TCEP concentrations ($p < 0.05$). This indicates that TCEP disrupts the mitochondrial function of alfalfa roots, indirectly interfering with the activity of the mETC, inhibiting ATP synthesis, and ultimately depleting intracellular energy reserves.

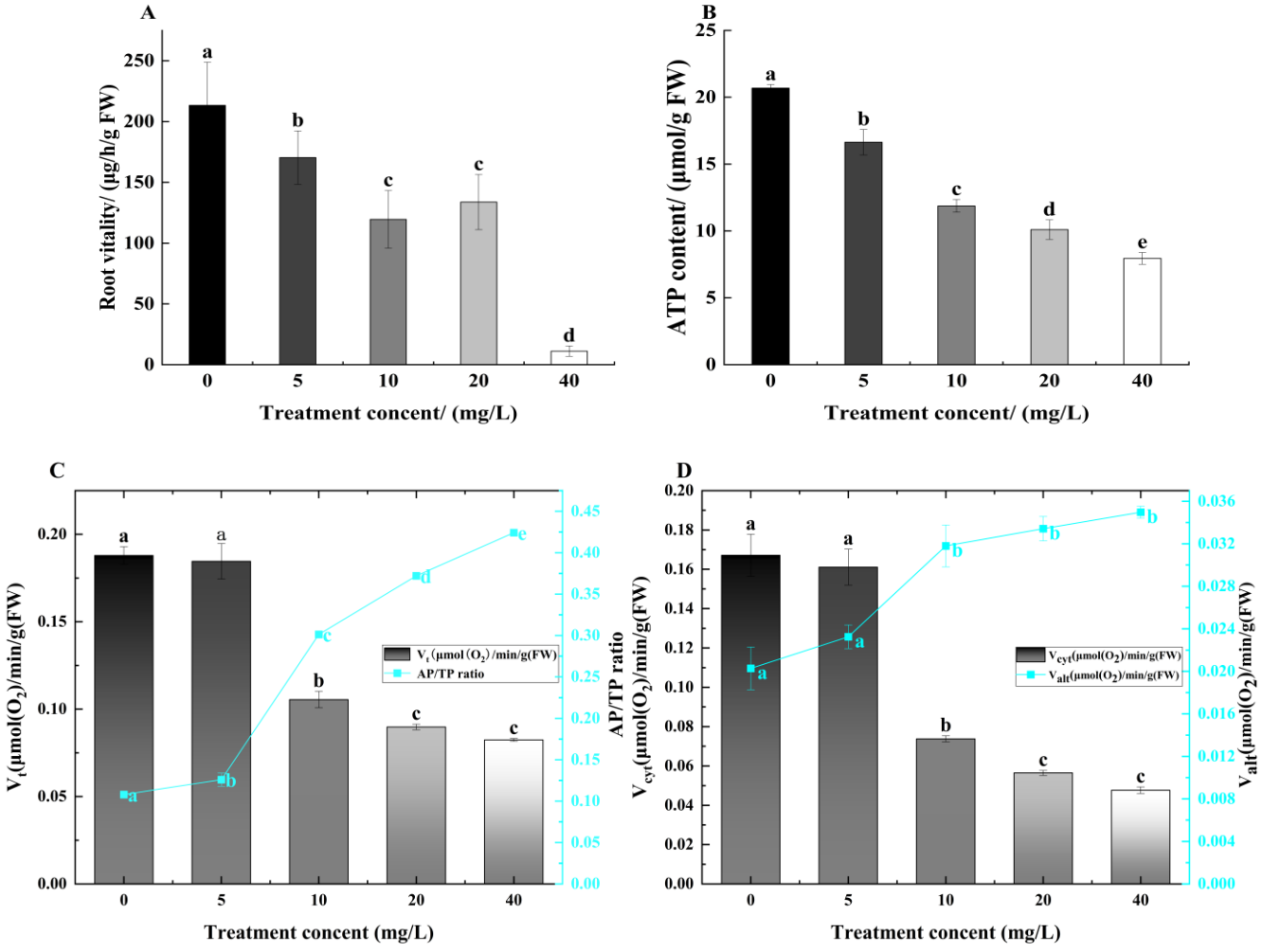


Figure 3. Influence of TCEP treatment on root activity (**A**), cellular ATP content (**B**), and root respiration in alfalfa (**C**,**D**). Different letters indicate significant differences between different concentrations of TCEP treatments and control ($p < 0.05$).

3.4. Effect of TCEP on Redox Balance in Alfalfa Roots

TCEP induces a substantial increase in superoxide levels, resulting in the disruption of cell membranes (Figure 4). Treatment with 5 mg/L TCEP significantly elevated the MDA

content in alfalfa roots compared to the control ($p < 0.05$). As TCEP stress intensified, the MDA content in alfalfa roots surged, reaching 20.8 μmol/g fresh weight (FW) at a TCEP concentration of 40 mg/L, which is 2.4 times higher than that of the control. Additionally, the free proline content, a key regulator of osmotic pressure in plant cytoplasm, significantly decreased at a TCEP concentration of 40 mg/L, although it remained higher than that of the control. This indicates that TCEP disrupts the structural integrity of alfalfa root cell membranes, leading to increased membrane lipid peroxidation under severe stress conditions. At a concentration of 5 mg/L, the H_2O_2 levels in the alfalfa root system were significantly higher than those in the control ($p < 0.05$). As the stress level increased, the H_2O_2 content in the treatment group progressively rose, reaching a peak of 52.56 μmol/g FW at a TCEP concentration of 40 mg/L. The changes in superoxide anion radical content in the alfalfa root system mirrored those of hydrogen peroxide, with TCEP treatment significantly increasing superoxide anion radical levels ($p < 0.05$). This suggests that the rapid accumulation of ROS in the alfalfa root system under TCEP stress leads to oxidative damage.

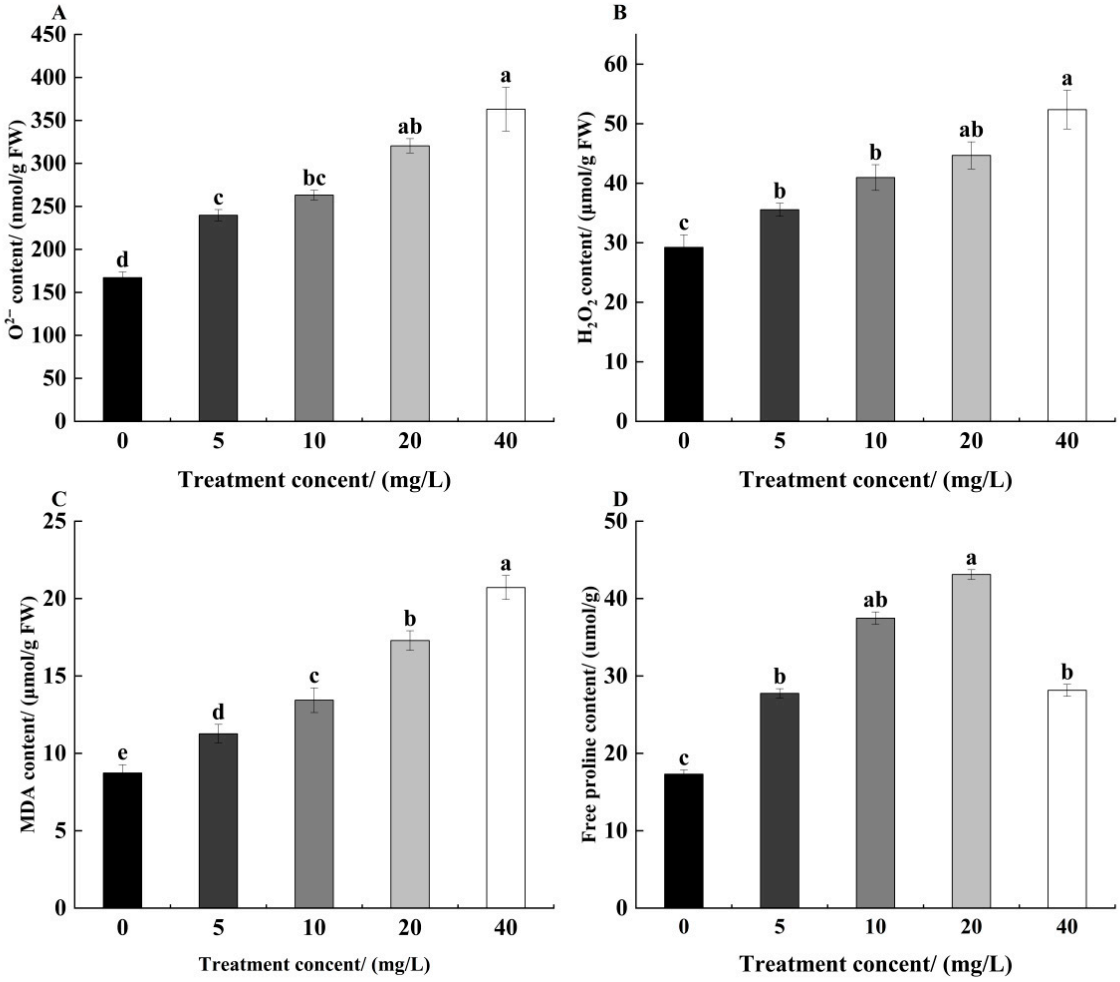


Figure 4. Effect of TCEP treatment on superoxide anion radical (**A**), hydrogen peroxide (**B**), malondialdehyde (**C**), and free proline content (**D**) in alfalfa roots. Different letters indicate significant differences between different concentrations of TCEP treatments and control ($p < 0.05$).

3.5. Effect of TCEP on Antioxidant Enzyme Activities of Alfalfa Roots

TCEP significantly reduced the activities of SOD and CAT, as illustrated in Figure 5. The antioxidant enzyme activities in alfalfa roots exhibited considerable changes under TCEP stress. Notably, SOD and CAT activities did not show significant differences under the

5 mg/L TCEP treatment ($p > 0.05$). However, as the concentration of TCEP increased, SOD activity significantly decreased compared to the control, while CAT activity also exhibited a substantial decline following treatments with 10, 20, and 40 mg/L TCEP. APX activity was significantly elevated at 10 mg/L ($p < 0.05$) and continued to rise with increasing TCEP concentrations, reaching its maximum activity at 40 mg/L. POD activity did not differ significantly from the control at lower TCEP concentrations, but it significantly increased at concentrations of 10 mg/L or higher ($p < 0.05$).

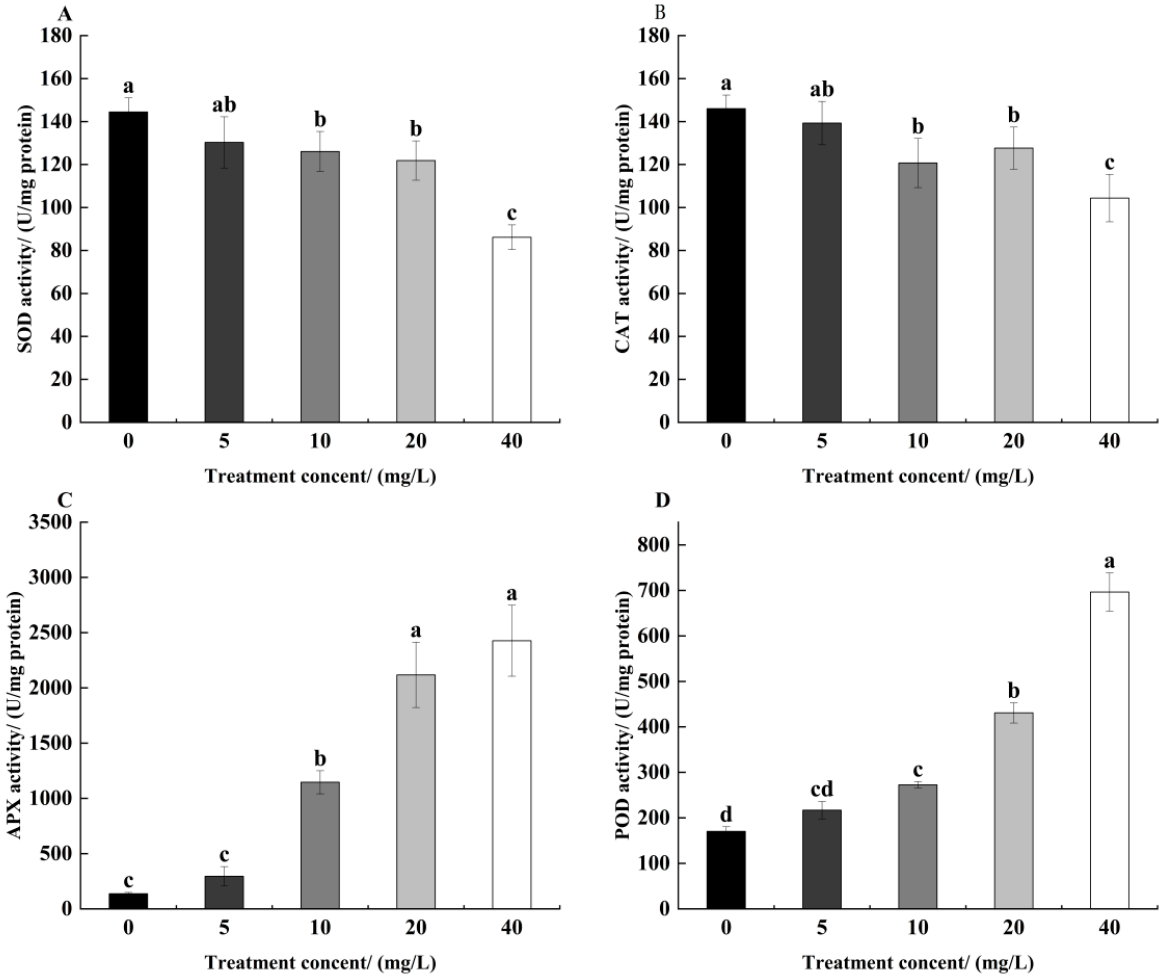


Figure 5. Effects of TCEP treatment on SOD (**A**), CAT (**B**), APX (**C**), and POD (**D**) activities in alfalfa roots. Different letters indicate significant differences between different concentrations of TCEP and the control ($p < 0.05$).

4. Discussion

TCEP is known to be environmentally bioaccumulative and persistent [2], exhibiting cytotoxic properties that can induce cell death [31]. The distribution and accumulation of TCEP have been documented [14,32–35], revealing its interference with normal physiological metabolism and the growth and development of the plant root system. Root morphology serves as a crucial phenotypic indicator of plant responses to environmental stress [36]. Exposure to TCEP surpassed the homeostatic regulatory threshold of the root system, inhibiting the total length, surface area, volume, and mean diameter of the alfalfa root system, which led to irreversible morphological and structural damage. Furthermore, TCEP may impair the water and nutrient uptake efficiency of the alfalfa root system, as evidenced by a significant reduction in specific root length, specific root surface area, number of root tips, and number of nodes [37].

Respiration is the sole pathway for providing the ATP energy required in root cells. Under normal conditions, the respiratory rate of the CP was higher than that of the AP, indicating that the production of a transmembrane plasmonic gradient via the CP drives ATP synthesis in alfalfa root cells [38]. The proper functioning of respiratory processes relies on the integrity of cell membranes. Under low-concentration TCEP stress, alfalfa maintains osmotic balance in root cells by increasing proline content, thus protecting cell membrane integrity. However, under high-concentration TCEP stress, despite a continued increase in proline, osmotic balance in alfalfa roots becomes disrupted, ultimately leading to cell membrane rupture.

The cellular respiration rate and ATP content significantly decrease following TCEP treatment compared to the control, indicating that TCEP disrupts the electron respiratory transport chain in cells, impeding ATP production and ultimately affecting root system growth.

The mitochondrial respiratory electron transport chain (mETC) serves as the primary site for the production of reactive oxygen species (ROS) in the root system [39]. TCEP disrupted the cellular respiratory electron transport, resulting in electron leakage and subsequent ROS production [40,41]. The AP is a respiratory pathway that enhances electron consumption, effectively reducing ROS production [42–44]. Although the cellular respiration rate of alfalfa roots was reduced, the proportion of AP in total respiration increased compared to the control, indicating that a self-defense mechanism was activated to diminish ROS production in the alfalfa root system under TCEP exposure. However, the results of this study reveal that TCEP treatment resulted in a significant accumulation of ROS in alfalfa roots. Numerous studies have demonstrated that excessive ROS accumulation can directly or indirectly oxidize biomolecules and damage cell membranes, consequently accelerating cell death and disintegration [41]. This suggests that the upregulation of AP in the root system is insufficient to alleviate the oxidative damage to the alfalfa root system under TCEP conditions.

In addition, the generation and scavenging of ROS in plant root cells are maintained in a dynamic equilibrium [45]. Under external stress, this balance is disrupted, leading to ROS accumulation, cellular damage, and an increase in harmful metabolites such as malondialdehyde (MDA) [46,47]. In the present study, TCEP induced oxidative stress in alfalfa root cells, disturbed the cellular redox balance, and aggravated oxidative damage. Plants possess a sophisticated antioxidant defense system to mitigate ROS levels and prevent oxidative injury, which includes enzymes such as CAT, SOD, and APX. In this investigation, the activities of APX and POD were elevated to alleviate ROS-induced oxidative damage in alfalfa root cells; however, this response was insufficient to fully protect the roots from TCEP-triggered oxidative damage.

5. Conclusions

TCEP exhibited high toxicity to the root system of alfalfa (Figure 6), inhibiting the mitochondrial electron transport and consequently reducing ATP production in root cells. The disruption of respiration caused by TCEP led to an overaccumulation of ROS, which was further exacerbated by the deficiency in the activity of ROS-scavenging enzymes. The excessive accumulation of ROS results in membrane peroxidation, thereby disrupting membrane structure and intensifying the impairment of mitochondrial electron transport. Ultimately, the damage inflicted by TCEP on respiration disrupts the conversion and reuse of energy in root cells, hindering root growth and development, and may even lead to the mortality of root cells.

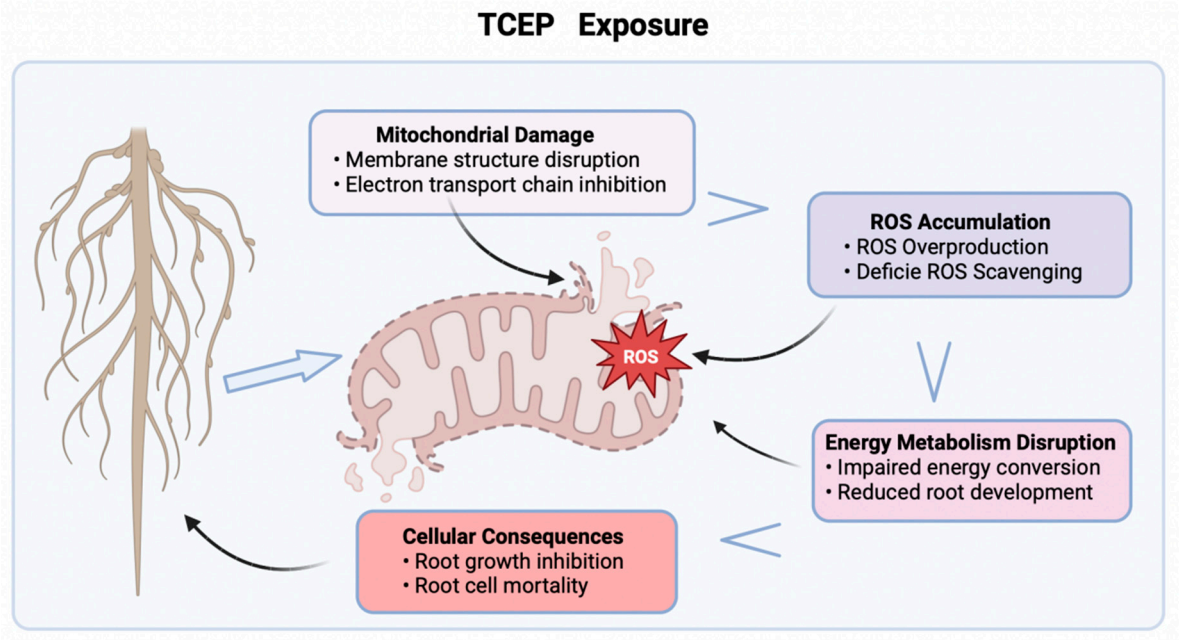


Figure 6. TCEP toxicity mechanism in alfalfa roots.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15112483/s1, Figure S1: Complete plant image; Figure S2: Isolated root system scan.

Author Contributions: Methodology, M.L. and L.G.; project administration, M.L.; investigation, M.L. and L.G.; funding acquisition, M.L.; conceptualization, M.L.; supervision, M.L.; validation, M.L., L.G., A.Y., W.L., H.L. and P.G.; writing—review and editing, M.L. and L.G.; data curation, A.Y., L.G., H.L. and W.L.; writing, L.G.; designed experiments, L.G.; formal analysis, L.G.; writing—original draft, L.G.; visualization, L.G., A.Y., W.L. and P.G.; validation, W.L., P.G. and H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Xinjiang Uygur Autonomous Region Tianshan Talents Young Top Talents, grant number 2024TSYCCX0032, and was funded by the National Natural Science Foundation of China, grant numbers 32360346 and 31860683.

Data Availability Statement: The original contributions presented in this study are included in the article; further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors sincerely thank the College of Grassland Science at Xinjiang Agricultural University for hosting and supporting this project within its facilities. We also express our gratitude to the Key Laboratory of Grassland Resources and Ecology in the Western Arid Region for its strong support of this work. The authors also acknowledge the Xinjiang Key Laboratory of Grassland Resources and Ecology.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

TCEP	Tris(2-chloroethyl) phosphate
COX	Cytochrome C Oxidase
AOX	Alternative Oxidase
SOD	Superoxide dismutase
CAT	Catalase
APX	Ascorbate peroxidase

POD	Peroxidase
mECT	Mitochondrial electron transport chain
ROS	Reactive oxygen species
OPEs	Organophosphate esters
ATP	Adenosine triphosphate
RV	Total root volume
RSA	Root surface area
AD	Average root diameter
TRL	Total root length
SRL	Specific root length
SRS	Specific surface area
TTC	Triphenyltetrazolium chloride
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
CP	Cytochrome respiration pathway
AP	Alternate respiration pathway

References

1. Wang, X.; Song, F. The neurotoxicity of organophosphorus flame retardant tris (1,3-dichloro-2-propyl) phosphate (TDCPP): Main effects and its underlying mechanisms. *Environ. Pollut.* **2024**, *346*, 123569. [CrossRef]
2. Li, S.; Wan, Y.; Wang, Y.; He, Z.; Xu, S.; Xia, W. Occurrence, spatial variation, seasonal difference, and ecological risk assessment of organophosphate esters in the Yangtze River, China: From the upper to lower reaches. *Sci. Total Environ.* **2022**, *851 Pt 1*, 158021. [CrossRef] [PubMed]
3. Wang, C.; Yuan, R.Y.; Wei, S.Q.; He, M.J. Occurrence, correlation, and partitioning of organophosphate esters in soil and tree bark from a megacity, Western China. *Environ. Sci. Pollut. Res. Int.* **2023**, *30*, 4359–4371. [CrossRef]
4. Zhao, Y.; Peng, M.; Liu, H.; Zhang, X.; Fu, D. Prepubertal Exposure to Tris(2-chloroethyl) Phosphate Disrupts Blood-Testis Barrier Integrity via Ferritinophagy-Mediated Ferroptosis. *Toxics* **2025**, *13*, 285. [CrossRef]
5. Paun, I.; Pirvu, F.; Chiriac, F.L.; Iancu, V.I.; Pascu, L.F. Organophosphate flame retardants in Romania coastline: Occurrence, faith and environmental risk. *Mar. Pollut. Bull.* **2024**, *208*, 116982. [CrossRef]
6. Hou, M.; Zhang, B.; Zhou, L.; Ding, H.; Zhang, X.; Shi, Y.; Na, G.; Cai, Y. Occurrence, distribution, sources, and risk assessment of organophosphate esters in typical coastal aquaculture waters of China. *J. Hazard. Mater.* **2024**, *465*, 133264. [CrossRef]
7. Astuti, M.P.; Notodarmojo, S.; Priadi, C.R.; Padhye, L.P. Contaminants of emerging concerns (CECs) in a municipal wastewater treatment plant in Indonesia. *Environ. Sci. Pollut. Res. Int.* **2023**, *30*, 21512–21532. [CrossRef]
8. Zhang, Y.; Zhao, B.; Chen, Q.; Zhu, F.; Wang, J.; Fu, X.; Zhou, T. Fate of organophosphate flame retardants (OPFRs) in the "Cambi® TH + AAD" of sludge in a WWTP in Beijing, China. *Waste Manag.* **2023**, *169*, 363–373. [CrossRef] [PubMed]
9. Zhang, W.P.; Zhang, Z.F.; Guo, C.S.; Lü, J.P.; Deng, Y.H.; Zhang, H.; Xu, J. Pollution Characteristics and Risk Assessment of Organophosphate Esters in Rivers and Water Body Around Taihu Lake. *Huan Jing Ke Xue* **2021**, *42*, 1801–1810. (In Chinese) [CrossRef]
10. Zhang, Q.; Wang, Y.X.; Jiang, X.X.; Xu, H.Z.; Luo, Y.Q.; Long, T.T.; Li, J.; Xing, L. Spatial occurrence and composition profile of organophosphate esters (OPEs) in farmland soils from different regions of China: Implications for human exposure. *Environ. Pollut.* **2021**, *276*, 116729. [CrossRef] [PubMed]
11. Liu, B.; Liu, S.; Li, J.; Ma, X.; Yu, Y. Occurrence of organophosphate esters (OPEs) in tobacco leaves across China: Spatial distribution, sources and exposure risk assessment. *Environ. Res.* **2025**, *279 Pt 2*, 121855. [CrossRef]
12. Wang, T.; Zhang, H.; Huang, C.; Ben, Y.; Zhou, H.; Guo, H.; Han, Y.; Zhang, Y.; Tong, P. Occurrence and potential risks of organophosphate esters in agricultural soils: A case study of Fuzhou City. *Southeast China J. Environ. Sci.* **2025**, *150*, 571–581. (In Chinese) [CrossRef] [PubMed]
13. Wang, Y.; Chen, R.; Zhang, Z.; Fu, Z.; Zhang, L.; Tan, F. Kinetics of uptake, translocation, and metabolism of organophosphate esters in japonica rice (*Oryza sativa* L.): Hydroponic experiment combined with model. *Sci. Total Environ.* **2024**, *951*, 175838. [CrossRef]
14. Lao, Z.L.; Wu, D.; Li, H.R.; Liu, Y.S.; Zhang, L.W.; Feng, Y.F.; Jiang, X.Y.; Wu, D.W.; Hu, J.J.; Ying, G.G. Uptake mechanism, translocation, and transformation of organophosphate esters in water hyacinth (*Eichhornia crassipes*): A hydroponic study. *Environ. Pollut.* **2024**, *341*, 122933. [CrossRef] [PubMed]

15. Liu, Q.; Liu, M.; Wu, S.; Xiao, B.; Wang, X.; Sun, B.; Zhu, L. Metabolomics Reveals Antioxidant Stress Responses of Wheat (*Triticum aestivum* L.) Exposed to Chlorinated Organophosphate Esters. *J. Agric. Food Chem.* **2020**, *68*, 6520–6529. [CrossRef] [PubMed]
16. Liu, S.; Wang, G.; Xing, Z.; Xue, H.; Wang, Y.; Wang, H.; Dong, X.; Chen, H.; Liu, Y. Stable Isotope and Multiomics Reveal Uptake, Translocation, and Transformation Mechanisms of Tris(2-chloroethyl) Phosphate in Wheat (*Triticum aestivum* L.). *J. Agric. Food Chem.* **2024**, *72*, 27797–27807. [CrossRef]
17. Peralta-Videa, J.; de la Rosa, G.; Gonzalez, J.; Gardea-Torresdey, J. Effects of the growth stage on the heavy metal tolerance of alfalfa plants. *Adv. Environ. Res.* **2004**, *8*, 679–685. [CrossRef]
18. Li, Z.; Dou, H.; Zhang, W.; He, Z.; Li, S.; Xiang, D.; Zhang, Y. The root system dominates the growth balance between the aboveground and belowground parts of cotton. *Crop Environ.* **2023**, *2*, 221–232. [CrossRef]
19. Li, S.; Wan, L.; Nie, Z.; Li, X. Fractal and Topological Analyses and Antioxidant Defense Systems of Alfalfa (*Medicago sativa* L.) Root System under Drought and Rehydration Regimes. *Agronomy* **2020**, *10*, 805. [CrossRef]
20. Luo, D.; Shi, J.Y.; Song, H.F.; Li, J.C. Effects of salt stress on growth, photosynthetic and fluorescence characteristics, and root architecture of *Corylus heterophylla* × *C. avellan* seedlings. *Chin. J. Appl. Ecol.* **2019**, *30*, 3376–3384. [CrossRef]
21. Huang, Q.L.; Zhang, M.M.; Li, C.S.; Li, B.Y.; Zhuo, S.L.; Yang, Y.S.; Chen, Y.D.; Zhong, A.N.; Liu, H.Y.; Lai, W.F.; et al. Response mechanism of water status and photosynthetic characteristics of *Cotoneaster multiflorus* under drought stress and rehydrated conditions. *Front. Plant Sci.* **2025**, *15*, 1457955. [CrossRef]
22. Vanlerberghe, G.C.; Martyn, G.D.; Dahal, K. Alternative oxidase: A respiratory electron transport chain pathway essential for maintaining photosynthetic performance during drought stress. *Physiol. Plant* **2016**, *157*, 322–337. [CrossRef] [PubMed]
23. Raghavendra, A.S.; Padmasree, K. Beneficial interactions of mitochondrial metabolism with photosynthetic carbon assimilation. *Trends Plant Sci.* **2003**, *8*, 546–553. [CrossRef]
24. Liao, Y.W.; Shi, K.; Fu, L.J.; Zhang, S.; Li, X.; Dong, D.K.; Jiang, Y.P.; Zhou, Y.H.; Xia, X.J.; Liang, W.S.; et al. The reduction of reactive oxygen species formation by mitochondrial alternative respiration in tomato basal defense against TMV infection. *Planta* **2012**, *235*, 225–238. [CrossRef]
25. Bora, K.S.; Sharma, A. Phytochemical and pharmacological potential of Medicago sativa: A review. *Pharm. Biol.* **2011**, *49*, 211–220. [CrossRef]
26. Zhong, S.Z.; Liu, X.J.; Ouyang, J.H.; Tu, X.J.; Song, W.Z.; Cao, W.; Tao, Q.B.; Sun, J. Effects of biochar and phosphorus fertilizer combination on the physiological growth characteristics of alfalfa in saline-alkali soil of the Yellow River Delta. *Chin. J. Grassl.* **2024**, *46*, 35–45. [CrossRef]
27. Arsenault, J.-L.; Poulcur, S.; Messier, C.; Guay, R. WinRHlZO™, a Root-measuring System with a Unique Overlap Correction Method. *HortScience* **2019**, *30*, 906D. [CrossRef]
28. Erktan, A.; Roumet, C.; Bouchet, D.; Stokes, A.; Pailler, F.; Munoz, F. Two dimensions define the variation of fine root traits across plant communities under the joint influence of ecological succession and annual mowing. *J. Ecol.* **2018**, *106*, 2031–2042. [CrossRef]
29. Altman, F.P. Tetrazolium salts and formazans. *Prog. Histochem. Cytochem.* **1976**, *9*, III-51. [CrossRef]
30. Jiang, N.; Yu, P.; Fu, W.; Li, G.; Feng, B.; Chen, T.; Li, H.; Tao, L.; Fu, G. Acid invertase confers heat tolerance in rice plants by maintaining energy homoeostasis of spikelets. *Plant Cell Environ.* **2020**, *43*, 1273–1287. [CrossRef]
31. Li, Z.; Jin, N.; Jin, L.; Wang, S.; Li, Y.; Sun, M.; Huang, S.; Xie, Y.; Meng, X.; Xu, Z.; et al. Use of silicon to protect tomato (*Solanum lycopersicum* L.) seedlings from low-calcium stress-derived oxidative damage. *Sci. Hortic.* **2025**, *349*, 114231. [CrossRef]
32. Antonopoulou, M.; Spyrou, A.; Giova, L.; Varela-Athanasatou, M.; Mouaimi, M.; Christodoulou, N.; Dailianis, S.; Vlastos, D. Flame-retardant Tris(2-chloroethyl) phosphate: Assessing the effects on microalgae, mussel hemocytes and human peripheral blood cells. *Environ. Res.* **2025**, *276*, 121512. [CrossRef]
33. Zhou, G.; Zhang, Y.; Wang, Z.; Li, M.; Li, H.; Shen, C. Distribution Characteristics and Ecological Risk Assessment of Organophosphate Esters in Surface Soils of China. *Toxics* **2024**, *12*, 686. [CrossRef] [PubMed]
34. Liu, Q.; Gao, H.; Yi, X.; Tian, S.; Liu, X. Root Uptake Pathways and Cell Wall Accumulation Mechanisms of Organophosphate Esters in Wheat (*Triticum aestivum* L.). *J. Agric. Food Chem.* **2022**, *70*, 11892–11900. [CrossRef]
35. Qin, Z.; Liu, L.Y.; Stubbings, W.A.; Wang, S. Analysis and subcellular distribution of organophosphate esters (OPEs) in rice tissues. *Environ. Sci. Pollut. Res. Int.* **2023**, *30*, 74021–74030. [CrossRef] [PubMed]
36. Gong, X.; Wang, Y.; Pu, J.; Zhang, J.; Sun, H.; Wang, L. The environment behavior of organophosphate esters (OPEs) and di-esters in wheat (*Triticum aestivum* L.): Uptake mechanism, in vivo hydrolysis and subcellular distribution. *Environ. Int.* **2020**, *135*, 105405. [CrossRef]
37. Wang, H.Q.; Zhao, X.Y.; Xuan, W.; Wang, P.; Zhao, F.J. Rice roots avoid asymmetric heavy metal and salinity stress via an RBOH-ROS-auxin signaling cascade. *Mol. Plant* **2023**, *16*, 1678–1694. [CrossRef]
38. Bauhus, J.; Khanna, P.R.; Menden, N. Aboveground and belowground interactions in mixed plantations of *Eucalyptus globulus* and *Acacia mearnsii*. *Can. J. For. Res.* **2000**, *30*, 1886–1894. [CrossRef]
39. Millar, A.H.; Whelan, J.; Soole, K.L.; Day, D.A. Organization and regulation of mitochondrial respiration in plants. *Annu. Rev. Plant Biol.* **2011**, *62*, 79–104. [CrossRef]

40. Lam, E.; Kato, N.; Lawton, M. Programmed cell death, mi- tochondria and the plant hypersensitive response. *Nature* **2001**, *411*, 848–853. [CrossRef]
41. Chen, G.; Zhang, S.; Jin, Y.; Wu, Y.; Liu, L.; Qian, H.; Fu, Z. TPP and TCEP induce oxidative stress and alter steroidogenesis in TM3 Leydig cells. *Reprod. Toxicol.* **2015**, *57*, 100–110. [CrossRef] [PubMed]
42. Van Aken, O.; Van Breusegem, F. Licensed to Kill: Mitochondria, Chloroplasts, and Cell Death. *Trends Plant Sci.* **2015**, *20*, 754–766. [CrossRef] [PubMed]
43. Del-Saz, N.F.; Florez-Sarasa, I.; Clemente-Moreno, M.J.; Mhadhbi, H.; Flexas, J.; Fernie, A.R.; Ribas-Carbó, M. Salinity tolerance is related to cyanide-resistant alternative respiration in Medicago truncatula under sudden severe stress. *Plant Cell Environ.* **2016**, *39*, 2361–2369. [CrossRef] [PubMed]
44. Maxwell, D.P.; Wang, Y.; McIntosh, L. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8271–8276. [CrossRef]
45. Van Aken, O.; Giraud, E.; Clifton, R.; Whelan, J. Alternative oxidase: A target and regulator of stress responses. *Physiol. Plant.* **2009**, *137*, 354–361. [CrossRef]
46. Espenes, A.; Press, C.M.; Dannevig, B.H.; Landsverk, T. Immune-complex trapping in the splenic ellipsoids of rainbow trout (*Oncorhynchus mykiss*). *Cell Tissue Res.* **1995**, *282*, 41–48. [CrossRef]
47. Xu, W.; Miao, Y.; Kong, J.; Lindsey, K.; Zhang, X.; Min, L. ROS signaling and its involvement in abiotic stress with emphasis on heat stress-driven anther sterility in plants. *Crop Environ.* **2024**, *3*, 65–74. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.