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Original Research

Combined Phytotoxicity of Microplastics and Lead on the Growth and Physio-Biochemical Characteristics of Tobacco (*Nicotiana tabacum*)

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Abstract

Microplastic and heavy metal contaminants have attracted global concern due to their ubiquitous presence and long-lasting persistence, yet little is known about their interactions on plant growth performance. Here, different concentrations of polyethylene (PE)-microplastics (MPs) and lead (Pb) were applied to soils to investigate their impacts on tobacco plants. Our results showed that tobacco plants grown in PE-MP- and Pb-contaminated soils displayed a significant reduction in leaf pigment content, photosynthetic efficiency, and plant biomass, as well as a remarkable increase in the contents of hydrogen peroxide, superoxide ions, and malondialdehyde. The Pb contents in plant roots decreased from 886 mg kg^{-1} to 765 mg kg^{-1} with increasing concentrations of PE-MPs, while the leaf Pb concentrations remained unaffected. The impaired photosynthetic performance in tobacco leaves was attributed to Pb stress, causing stomatal closure and PE-MP-induced nonstomatal limitation. Moreover, the coexistence of PE-MPs and Pb damaged the PSII reaction center, disturbed the electron transport process, and reduced photosynthetic efficiency. To alleviate oxidative damage, the contents of proline and soluble sugar, along with the antioxidant enzyme activities, underwent a significant increase in tobacco plants. This work offers valuable insights for addressing the challenges posed by the emergence of contaminants in agricultural production.

Keywords: polyethylene microplastics, heavy metal, synergistic effect, reactive oxygen species, oxidative damage, stress tolerance

Introduction

Plastic pollution is a growing global threat to both terrestrial and marine ecosystems due to its sluggish degradation rate, large application, and prolonged persistence [1]. Once discarded into the natural surroundings, plastic waste breaks down into smaller

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particles by physicochemical and biological forces, resulting in the generation of microplastics (MPs) when the plastic size is < 5 mm [2]. The wide application of plastic film residues, wastewater irrigation, organic fertilizer, and sewage sludge leads to large amounts of MP accumulation in agricultural soils [3]. Characterized by small particle size, large surface area, and high resistance to degradation, MPs are ubiquitous and persistent contaminants in agroecosystems that pose serious threats to crop productivity and human health [4, 5].

To date, numerous research investigations have reported the adverse impacts of MPs on plant growth and crop production. For example, polyethylene (PE) and polyvinyl chloride (PVC) MPs considerably reduced the root length, shoot weight, and chlorophyll (Chl) contents of lettuce, and the adverse effects increased with increasing microplastic concentration [6]. Moreover, Colzi et al. observed that soil contaminated with 40-50 μm PE, PVC, polypropylene (PP), and polyethylene terephthalate (PET) MPs significantly impaired the root system, reduce leaf size and Chl content, inhibit photosynthetic capacity, and decrease the concentrations of Mg^{2+}, Fe^{2+}, Cu^{2+}, and K^{+} in *Cucurbita pepo* L. [2]. Generally, MPs elicited detrimental impacts on plant growth through direct and indirect pathways. Once absorbed by plant roots and translocated to aboveground tissues, MPs can directly impact plant growth and crop production by blocking the seed pore [7], limiting water and nutrient uptake [8], inducing oxidative damage [9], generating genotoxicity and cytotoxicity [10], inhibiting root and shoot growth [11], disturbing metabolic systems [12], decreasing leaf chlorophyll content [13], and preventing photosynthetic capacity [14]. Furthermore, MPs can also indirectly impact plants by adversely affecting soil microbial community structure and function [15], suppressing the activities of soil microbiota and enzymes and altering soil physicochemical properties, such as soil aggregation [16], bulk density [17], water potential [18], and nutrient cycles [19].

In addition to the emerging organic contaminants, MPs, heavy metals, as inorganic pollutants, are another major environmental concern worldwide due to their nonbiodegradable nature, long-term persistence, high toxicity, and potential threats to food security and public health [20]. Lead (Pb) is the second most hazardous element after arsenic and is of great public concern [21]. Due to its high bioavailability to plants, Pb^{2+} can be easily transported and accumulate in edible parts of plants [22], resulting in phytotoxic effects on plant performance. To date, many studies have documented the hazardous effect of Pb on plant morphological [23], metabolic [24], physiological [25], and molecular processes [26]. Studies have revealed that excessive Pb^{2+} in plant tissues produces excessive reactive oxygen species (ROS) [27], increases membrane permeability [28], stimulates lipid peroxidation [19], induces leaf chlorosis [29], destroys chloroplast ultrastructure [30], damages genomic DNA [26], blocks root growth [31], retards seed germination [32], disturbs plant-nutrient relationships [33], suppresses plant photosynthesis efficiency [24], and inhibits respiration rates [23], resulting in considerable reductions in production and economic losses [34].

The large surface area, high hydrophobicity, and exceptional adsorption ability of MPs make them ideal carriers and transporters for other soil contaminants, including heavy metals in the environment [24, 35]. Recently, a few studies have investigated the joint toxicity of MPs and heavy metals on plant performance [15]. For example, a study performed by Tunali et al. found that PE-MPs combined with metal cations (e.g., Cu^{2+}, Zn^{2+}, and Mn^{2+}) led to greater inhibition of growth and Chl a concentration of Chlorella vulgaris than single metal ions or MPs alone [36]. Li et al. reported that compared to plants exposed to Cd alone, the application of PE-MPs remarkably intensified the negative effect of Cd stress on maize photosynthesis by inhibiting photosynthetic capacity and suppressing the expression of photosynthesis-related genes [34]. Although co-exposure to MPs and heavy metals has been confirmed to amplify their toxicity [37], the responses of plant growth and development to these contaminants are material type- and dose-dependent and plant species-specific [14, 38]. Knowledge of the combined phytotoxicity of PE-MPs and Pb on plant growth and physiological characteristics is still limited until now. In this study, PE was selected for testing since it is one of the most abundantly found microplastic polymers in both terrestrial and marine environments [39], and tobacco (*Nicotiana tabacum*) was chosen as the model crop. It is postulated that (1) the coexistence of PE-MPs amplified the accumulation of Pb^{2+} in tobacco tissues, and (2) PE-MPs and Pb elicit a synergetic pollution risk to the environment, potentially causing adverse impacts on the plant performance of tobacco. For the laboratory-scale experiments, tobacco was exposed to different concentrations of PE-MPs and Pb for four weeks to (1) explore the influences of PE-MPs on the uptake and accumulation of Pb^{2+} in tobacco shoots and roots, (2) reveal the toxicity mechanism of PE-MPs and Pb, individually and in combination, on the plant performance of tobacco, and (3) identify the possible antioxidative strategies for alleviating the oxidative damage caused by the co-contamination of PE-MPs and Pb. Our work will provide useful information on the toxicological behaviors between MPs and heavy metals in crop plants.

Methods and Materials

Preparation of Experiment Materials

Low-density PE microplastic particles with diameters ranging from 6.5 to 13 μm were obtained from Huachuang Chemical Co., Ltd. Before being

applied to the soil, the PE-MPs were sonicated in 0.1 M HCl solution, followed by distilled water to clean the surface metals. Tobacco seeds (c.v. Zhongchuan 208) were provided by Qingdao China Tobacco Seed Co., Ltd. (Qingdao, China), and the floating breeding technique was used for seedling cultivation. The surface soils (at a depth of 0-20 cm) used to set the experiments were sampled from unpolluted farmland and sieved through a 2 mm mesh. The following are the physicochemical properties of the sampled soils: soil pH 7.9, organic matter 13.4 g kg^{-1}, total N 81.7 mg kg^{-1}, available P 176.4 mg·kg^{-1}, available K 386.5 mg kg^{-1}, and available Pb 4.3 mg kg^{-1}.

Experiment Design

A two-factorial pot experiment was designed, including (1) five PE-MP doses, i.e., 0 (PE0), 250 (PE250), 500 (PE500), 750 (PE750) and 1000 (PE1000) mg kg^{-1} soil, and (2) two Pb concentrations, i.e., 0 (Pb0) and 500 (Pb500) mg kg^{-1} soil. Ten treatments with six replicates each were established. PE-MPs were thoroughly mixed into the soil to achieve the target doses. Pb was incorporated into the soil-MP blend by utilizing a solution of $Pb(NO_3)_2$. Subsequently, the soils underwent preincubation for four weeks to achieve equilibrium in the soil environment. When the third true leaf expanded, one healthy tobacco seedling was relocated into a plastic pot (16 cm in height and 12 cm in diameter) and cultivated in climate chambers. A total of 2.5 kg of PE-MP- and Pb-contaminated soil was added to each pot. The conditions of the climate chambers were set to maintain a light/dark cycle of 14 hours and 10 hours, respectively, along with a humidity of 70% and a photosynthetically active radiation level of 800 μmol m^{-2} s^{-1}. A total of 2.5 g of compound fertilizer containing N-P-K in a 15-15-15 ratio used as basal fertilization was applied to each pot to avoid nutrient deficiency, and deionized water irrigation was used every day to avoid water shortages.

Pigment Contents, Gas Exchange Parameters, and Chl Fluorescence

After 28 days of treatment, three healthy tobacco leaves in each treatment were randomly chosen to conduct the measurements of leaf SPAD values using a portable Chl metre (SPAD-502, Osaka 590-8551, Japan), gas exchange parameters including net photosynthetic rate (P_n), stomatal conductance (G_s), intercellular CO_2 concentration (C_i), transpiration rate (T_r), and water-use efficiency (*WUE*) using a portable photosynthesis system (LI-6400, Li-COR Inc., USA), Chl fluorescence parameters including minimal fluorescence (*Fo*), maximal fluorescence (F_m), maximum quantum yield of PSII photochemistry (F_v/F_m), quantum efficiency of PSII photochemistry (Φ_{PSII}), nonphotochemical quenching (*NPQ*), nonregulated (Φ_{NO}) and regulated (Φ_{NPQ}) thermal energy dissipation for the light-adapted state, and electron transport rate (ETR) using a portable chlorophyll fluorometer (PAM-2500, Walz, Germany).

The stomatal limitation (Ls) was determined utilizing the following equation [40]:

$$Ls = 1 - \frac{C_i}{C_a}$$

where C_i represents the internal CO_2 concentration and C_a represents the atmospheric CO_2 concentration.

Hydrogen Peroxide, Superoxide Ion, and Malondialdehyde Contents

The concentrations of hydrogen peroxide (H_2O_2) and superoxide ions ($O_2^{\bullet-}$) in fresh leaves of tobacco were analyzed based on the methods of Velikova et al. [41] and Yang et al. [42], respectively. The leaf malondialdehyde (MDA) contents were quantified by using the thiobarbituric acid (TBA) reaction [43].

Antioxidase Activities

Superoxide dismutase (SOD) was assayed using the nitrogen blue tetrazole (NBT) photochemical reduction method (LOD≈ 0.05-0.10 U mL^{-1}) [44]. Peroxidase (POD) was measured using the guaiacol method (LOD≈ 0.01-0.05 U mL^{-1}) [45]. Catalase (CAT) activity was determined using the ammonium molybdate method (LOD≈ 0.05-0.10 U mL^{-1}) [46]. Ascorbate peroxidase (APX) was performed based on the ascorbate oxidation method (LOD≈ 0.05-0.10 U mL^{-1}) [47]. The measurement of soluble sugar (SS) was conducted according to the anthrone-sulfuric acid colorimetric method (LOD≈ 0.05-0.10 U mL^{-1}) [48]. Proline (Pro) was assayed based on the ninhydrin colorimetric method (LOD≈ 1-2 μg mL^{-1}) [49].

Plant Biomass

After harvest, each plant was washed with deionized water and divided into two parts: shoot and root. The plant tissues were heated in a forced-air oven at 105 ◦C for half an hour and then dried at 65°C for 48 h. The dry weight was used to determine the shoot biomass (SB), root biomass (RB), gross biomass (GB), and root-to-shoot ratio (R/S).

Plant Pb Content

A ground sample of 0.1 g was digested in 10 mL of a 1:1 HNO_3:$HClO_4$ solution and heated in a high-pressure microwave system at 180°C for 120 min until the sample was completely digested. The solution was then diluted to a final volume of 20 mL with deionized water. An atomic absorption spectrophotometer (FAAS, AA-7000, Shimadzu, Japan) was applied to determine the plant Pb content (LOD≈ 0.01-0.02 μg mL^{-1}). The

following two formulas were applied to calculate the bioconcentration factor (BCF), which reflects the plants' ability to accumulate metals, and translocation factor (TF), which expresses the plants' ability to translocate metals, respectively:

$$BCF = \frac{Pb\ concentration\ in\ plant\ tissues}{Pb\ concentrarion\ in\ soil}$$

$$TF = \frac{Pb\ concentration\ in\ shoot}{Pb\ concentrarion\ in\ root}$$

Statistical Analysis

All statistical data analyses were conducted using IBM SPSS 18 software (SPSS, Chicago, IL, USA). The significant differences ($p<0.05$) between different PE-MPs treatments were analyzed by one-way ANOVA and followed by Duncan's post hoc test. Comparisons between Pb0 treatment and Pb500 treatment were performed using the Independent-Samples t-Test. Two-way ANOVA analysis was employed to analyze the significances of two factors (Pb and PE-MPs) and their interaction. Principal component analysis (PCA) was conducted by Origin 2022 software (Origin Lab, Massachusetts, USA). SmartPLS 3.0 was utilized for conducting structural equation modeling (SEM) in order to evaluate the potential effects of oxidative damage, enzymatic antioxidants, nonenzymatic antioxidants, and photosynthetic performance on the plant biomass of tobacco.

Results and Discussion

MPs and Pb are pervasive and emerging contaminants in terrestrial ecosystems, obtaining worldwide attention due to their co-presence and high toxicity [50, 51]; nevertheless, the understanding of their combined toxicity on terrestrial plants is still lacking. Evaluation of the biological responses of tobacco to combined PE-MPs and Pb stress will be helpful in enhancing the knowledge of the phytotoxicity of these two contaminants on tobacco growth performance and their potential risks to agricultural security.

Effects of Combined PE-MPs and Pb on the Plant Biomass of Tobacco

Among plant physiological and biochemical parameters, biomass is generally considered a morphological indicator that reflects the response of plants to environmental changes [52]. The changes in plant dry weights in different PE-MP and Pb treatments are shown in Table 1. Compared to the control (PE0 treatment), SB, RB, and GB significantly decreased by 7.1%~26.8% in the various PE-MP treatments. Under Pb-stressed conditions, compared to the PE0+Pb treatment, SB, RB and GB significantly decreased by 3.8%~26.7. Our present results showed that the shoot and root biomass of tobacco were markedly decreased by the addition of PE-MPs and Pb and had the lowest value when plants suffered combined PE-MPs and Pb stress (Table 1). The results were in line with the research of Yang and Gao, who reported that MPs derived from plastic film mulch seriously inhibited the growth of rice plants, resulting in a considerable decrease in plant height and biomass [53]. Song et al. also found that the biomass of *Hemerocallis fulva*, *Iris germanica* L., *Canna generalis*, *Pennisetum clandestinum*, and *Miscanthus sinensis* grown in soil containing 1000 mg kg^{-1} Pb decreased by 75.2%, 24.5%, 31.6%, 44.4%, and 26.2%, respectively, when compared to plants grown with no Pb [54]. Similarly, the dry weight of tartary buckwheat seedlings decreased from 1.72 g to 1.02 g when the plants were grown in a solution containing 80 mg L^{-1} polylactic acid MPs and 120 mg L^{-1} Pb^{2+} [55]. The phytotoxic mechanisms on the biomass of tobacco could be the cumulative physiological and metabolic perturbations caused by PE-MPs and Pb [51, 56].

The R/S ratio showed no noticeable difference in the PE250 and PE500 treatments, but significantly decreased by 8.0% in the PE750 treatment and 12.0% in the PE1000 treatment. Similarly, under Pb-stressed conditions, the R/S ratio was not affected by the PE250+Pb and PE500+Pb treatments but was significantly decreased by 10.7% in the PE750+Pb treatment and 17.9% in the PE1000+Pb treatment when compared to the PE0+Pb treatment. The two-way ANOVA in Table 1 shows that SB, RB, GB, and the R/S ratio of tobacco were all significantly affected ($p<0.001$) by PE-MPs and Pb, and RB and GB were remarkably influenced ($p<0.01$) by the interaction effect of PE-MPs and Pb. However, no significant interaction effect of those two factors was found on the SB and R/S ratio ($p>0.05$).

Effects of Combined PE-MPs and Pb on Plant Pb Uptake and Transportation

The Pb contents in different plant tissues of tobacco under different PE-MP and Pb treatments are shown in Table 2. When the plants suffered Pb stress, the Pb contents in the shoot and root of tobacco were 190 and 886 mg kg^{-1}, respectively. The high Pb contents in the roots may result from the creation of stable compounds between Pb and the carboxyl group, as well as glucuronic acid present in the cell wall of the root epidermis [57]. Consequently, the movement of Pb^{2+} ions was restricted or obstructed.

The content of Pb in the roots of tobacco was significantly decreased by PE-MP addition, while the shoot Pb content showed no marked difference among the different PE-MPs (Table 2). The results suggested that the application of PE-MPs had the ability to inhibit the amount of Pb^{2+} extracted by plant roots from the soils. Similar results were also obtained in the research

Table 1. The changes in tobacco dry weight under different PE-MPs and Pb treatments.

Treatment	SB (g)	RB (g)	GB (g)	R/S ratio
PE0	2.23±0.13 a	0.56±0.02 a	2.79±0.15 a	0.25±0.01 a
PE250	2.04±0.04 b	0.52±0.02 b	2.56±0.05 b	0.25±0.01 a
PE500	1.94±0.05 bc	0.49±0.02 b	2.43±0.07 bc	0.25±0.01 a
PE750	1.87±0.03 c	0.43±0.03 c	2.31±0.06 cd	0.23±0.01 b
PE1000	1.82±0.02 c	0.41±0.01 c	2.23±0.02 d	0.22±0.01 b
PE0+Pb	1.58±0.02 a	0.45±0.03 a	2.03±0.05 a	0.28±0.02 a
PE250+Pb	1.52±0.02 b	0.42±0.02 b	1.94±0.01 b	0.27±0.01 ab
PE500+Pb	1.49±0.01 c	0.39±0.01 bc	1.88±0.01 c	0.26±0.01 abc
PE750+Pb	1.44±0.01 d	0.36±0.01 c	1.80±0.01 d	0.25±0.01 bc
PE1000+Pb	1.40±0.02 e	0.33±0.01 d	1.73±0.01 e	0.23±0.01 c
PE-MPs	***	***	***	***
Pb	***	***	***	***
PE-MPs×Pb	ns	**	**	ns

Note: Values are the mean of three replicates. PE–PE: MPs treatment; Pb: Pb treatment; PE×Pb: interaction between PE-MPs and Pb. Different letters in the same column indicate significant differences ($p<0.05$) between different treatment groups according to Duncan's test. *** indicates $p<0.001$; ** indicates $p< 0.01$; * indicates $p<0.05$; ns indicates no significant differences.

conducted by Dong et al., who reported that both polystyrene and polytetrafluoroethylene MPs reduced arsenic (As), and the absorption of As decreased as the concentration of MPs increased [58]. The underlying mechanisms could be explained by the direct adsorption of heavy metals, the competition between metal ions and plastic particles for root adsorption sites, and the suppression of root functionality by MPs [58].

The calculated TF and BCF in the different PE-MP and Pb treatments were all lower than 1, indicating that less Pb was translocated to the aboveground parts and that PE-MPs played no role in the translocation of Pb from roots to shoots. The presence of PE-MPs had no significant effect on TF but markedly decreased BCF when tobacco plants were grown in soils containing 500 mg kg^{-1} Pb. Under Pb500 conditions, with the addition of PE-MPs, the BCF values decreased from 0.69 to 0.57. In this study, PE-MP addition caused a decrease in BCF, and the higher the concentration of PE-MP was, the lower the BCF value. This phenomenon can be attributed to the presence of MPs decreasing the bioavailability of soil Pb and restraining the absorption of Pb^{2+} by plant tissues [59].

Oxidative Damage Caused by PE-MPs and Pb Stress

The phytotoxicity of environmental pollutants is highly related to the production of ROS, including H_2O_2 and O_2^- [34, 60]. The high accumulation of ROS in plant tissues exceeds cell tolerance and leads to oxidative stress, protein degradation, RNA and DNA destruction, photosynthesis inhibition, and plant death [61]. In the present research, in the presence of PE-MPs, Pb ions, and their combination, the contents of H_2O_2 and O_2^- in tobacco leaves were much higher than those in the control (Fig. 1). Compared to PE0, H_2O_2 increased by 4.7~18.6%, O_2^- increased by 17.8~50.1%, and MDA increased by 1.8~43.7% in various PE-MP treatments. Furthermore, the addition of Pb exacerbated the oxidative damage caused by PE-MPs. Compared to Pb0, Pb500 significantly increased by the contents of H_2O_2 and O_2^- and MDA by 161.2~221.9%, 23.9~51.8%, and 102.9~146.3%, respectively.

Similar results were observed in studies of *Vicia faba* [62], maize [14] and tobacco [63] under MP-stressed conditions; rice [64], wheat [65] and triticale [66] grown in Pb-contaminated soils, as well as rapeseed [35] and buckwheat [67, 55] suffered combined MP and Pb stress. The toxicity caused by PE-MPs or Pb is reported to induce an increase in the level of MDA in the leaves of *Vallisneria natans* [68], lettuce [69], and maize [63], which is consistent with our results (Fig. 1c)). These results revealed that both PE-MPs and Pb stimulate lipid peroxidation generation and cause a direct oxidative burst and oxidative damage to the structure and function of the cell membrane in tobacco plants [14].

Table 2. Concentrations of Pb in shoots and roots of tobacco, bioaccumulation factor (BCF), and translocation factor (TF) of Pb under different soil PE-MPs and Pb treatments.

Treatment	Pb content in plant shoot (mg kg^{-1})	Pb content in plant root (mg kg^{-1})	TF	BCF
PE0	1.32±0.05 a	10.7±0.3 a	0.12±0.01 a	0.75±0.01 a
PE250	1.33±0.05 a	10.7±0.6 a	0.12±0.01 a	0.75±0.02 a
PE500	1.30±0.02 a	10.4±0.7 a	0.13±0.01 a	0.73±0.04 a
PE750	1.32±0.05 a	10.8±0.3 a	0.12±0.01 a	0.72±0.01 a
PE1000	1.37±0.01 a	11.1±0.5 a	0.12±0.01a	0.73±0.01 a
PE0+Pb	187.0±12.2 a	881.4±6.4 a	0.21±0.02 a	0.68±0.01 a
PE250+Pb	188.1±2.3 a	827.8±1.1 b	0.23±0.01 a	0.65±0.01 b
PE500+Pb	178.6±4.7a	816.5±4.9 b	0.22±0.01 a	0.62±0.01 c
PE750+Pb	172.8±10.9 a	807.9±1.3 b	0.21±0.01 a	0.60±0.02 cd
PE1000+Pb	174.0±5.6 a	766.8±13.4 c	0.23±0.01 a	0.57±0.01 d
PE-MPs	ns	***	ns	***
Pb	***	***	***	***
PE-MPs×Pb	ns	***	ns	*

Note: Values are the mean of three replicates. PE–PE: MPs treatment; Pb: Pb treatment; PE×Pb: interaction between PE-MPs and Pb. Different letters in the same column indicate significant differences ($p<0.05$) between different treatment groups according to Duncan's test. *** indicates $p<0.001$; ** indicates $p< 0.01$; * indicates $p<0.05$; ns indicates no significant differences.

Effects of combined PE-MPs and Pb on leaf chlorophyll contents and photosynthetic performance

The leaf chloroplast is the primary site of ROS production when plants suffer environmental stress [70]. Exogenous pollutants can trigger the generation of oxidative stress and lipid peroxidation, which damage the fine structure of chloroplasts and thylakoids [71] and block the synthesis of leaf Chl [72], resulting in photosynthetic pigment reduction [73]. In the present study, the contents of tobacco leaf pigments expressed by SPAD values in the PE-MP, Pb, and PE-MP+Pb treatments were significantly decreased (Fig. 2), indicating that these two contaminants destroyed photosynthetic pigment integrity and inhibited the biosynthesis of Chl. Previous studies exhibited similar conclusions in *Salvinia natans* [74], pepper [75], lettuce [69], and maize [63]. The reduction in leaf pigments was attributed to disturbances in tobacco metabolism [70], the activity of chlorophyllase, which catalyzes chlorophyll degradation [76], and the replacement of Mg^{2+} in Chl molecules [29].

Solar energy absorption, transport, and conversion by photosynthetic reactions is the basis for plant survival [77]. The changes in leaf gas exchange parameters under different PE-MP and Pb stress conditions are shown in Fig. 3. The values of P_n, G_s, T_r, and *WUE* were significantly inhibited by both PE-MPs and Pb stress ($p<0.05$), and their values decreased with increasing concentrations of PE-MPs, while the changes in C_i (Fig. 3c)) exhibited the opposite trend. Lower leaf pigment contents in the PE-MP- and Pb-exposed groups further suggested that photosynthesis was hindered. Indeed, our results showed that P_n, G_s, T_r, and *WUE* in tobacco leaves were markedly decreased by Pb stress, and the presence of PE-MPs exacerbated the phytotoxicity of Pb on photosynthetic performance (Fig. 3), resulting in photosynthetic efficiency inhibition, stomatal closure, transpiration block, and water utilization reduction. Similar results were observed in the research of Mohi Ud Din et al., who reported that 2 mM $Pb(NO_3)_2$ markedly decreased P_n, G_s, and T_r values while enhancing the value of C_i in leaves of rye, wheat, and triticale [66]. Gao et al. also showed that the presence of PE-MPs in the soil at 1.0 mg L^{-1} decreased P_n, G_s, and T_r and increased C_i in lettuce leaves compared to the control [69]. The reduced photosynthetic efficiency by Pb stress is attributed to the destruction of the chloroplast ultrastructure, inhibition of electron transport between photosystems, and suppression of the activities of key enzymes such as δ-aminolevulinic acid dehydratase and Rubisco [78, 79]. PE-MPs can directly affect plant photosynthetic performance by inhibiting RuBP carboxylation, blocking the PSII donor side, breaking electron transfer, and restraining redox reactions. Similar conclusions

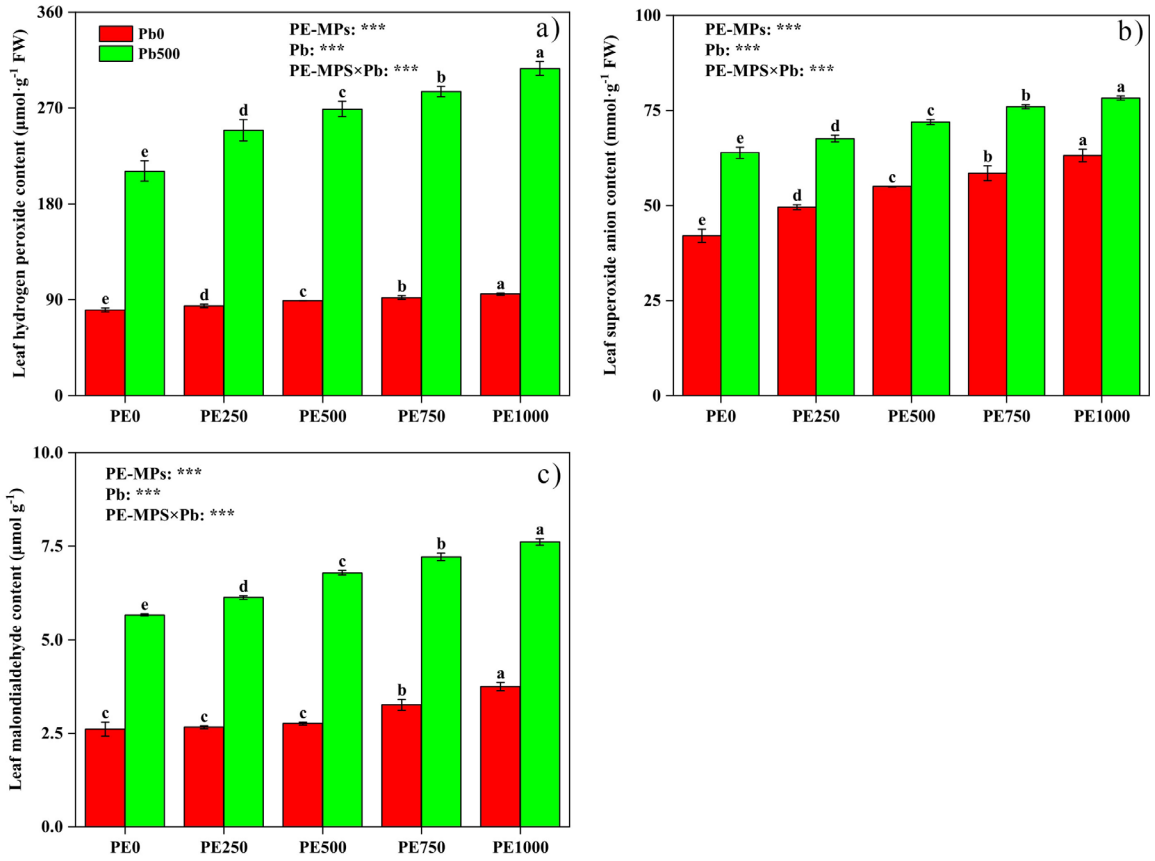

Fig. 1. Effects of PE-MPs and Pb treatment on a) the hydrogen peroxide, b) superoxide ion, and c) malondialdehyde contents in leaves of tobacco. Vertical bars represent ± SD of the mean (n = 3); different letters on the SD bars indicate significant differences among the PE-MPs treatments ($p<0.05$).

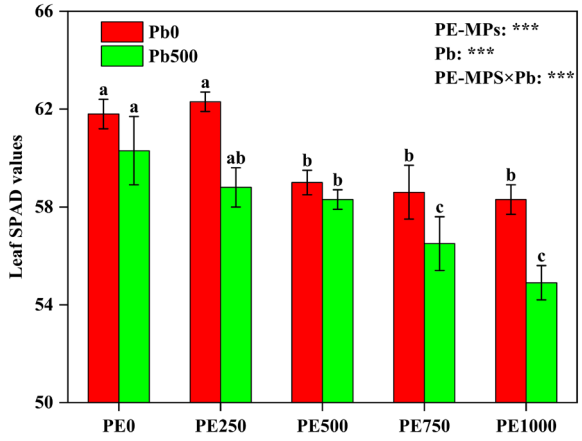

Fig. 2. SPAD values of tobacco leaves treated with different doses of PE-MPs and Pb. Vertical bars represent ± SD of the mean (n = 3); different letters on the SD bars indicate significant differences among the PE-MPs treatments ($p<0.05$).

were also supported by plants suffering phytotoxicity caused by MPs in different material types, such as PVC [80], polystyrene [58], and polypropylene (PP) [81]. Furthermore, stomata, which regulate the depletion and restoration of H_2O and CO_2, are the most crucial gas exchange systems. Plants controlling the efficiency of photosynthesis, respiration, and transpiration depend on the degree of stomatal opening. However, the abiotic stress caused by soil pollutants would result in the closure of the stoma, which was expressed by the reduced value of G_s [82]. The close stoma decreased the transpiration rate, thus resulting in reduced water use efficiency and nutrient absorption [83]. Ls in Fig. 3f) were remarkably inhibited by PE-MPs ($p<0.05$) but not significantly affected by Pb. On the other hand, the values of C_i and Ls in tobacco leaves were

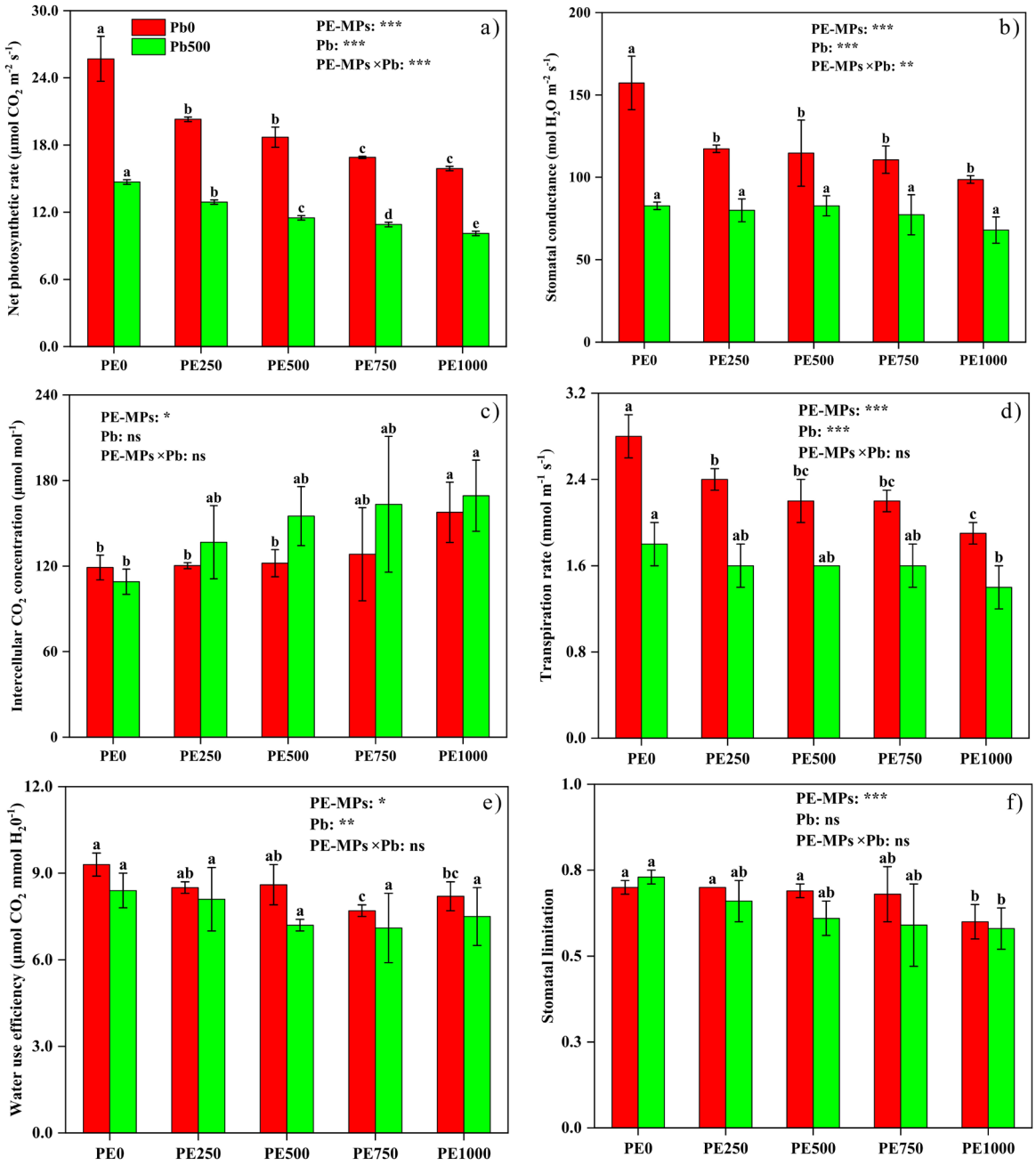

Fig. 3. Effects of PE-MPs and Pb toxicity on a) Net photosynthetic rate, b) Stomatal conductance, c) Intercellular CO_2 concentration, d) Transpiration rate, e) Water-use efficiency, and f) Stomatal limitation in leaves of tobacco. Vertical bars represent ± SD of the mean (n = 3); different letters on the SD bars indicate significant differences among the PE-MPs treatments ($p<0.05$).

significantly affected by exposure to PE-MPs, while Pb and PE-MPs+Pb treatments showed no remarkable influences ($p>0.05$, Fig. 3c)). The changes in C_i and Ls could be used to distinguish whether photosynthesis was controlled by stomatal limitation or nonstomatal limitation [40]. The increase in C_i and the enhancement of both G_s and Ls suggested that nonstomatal limitation regulates the photosynthetic rate under PE-MP-stressed conditions. As C_i and Ls were not remarkably influenced by Pb stress, while G_s showed a noticeable decrease, we speculate that Pb stress regulates the photosynthetic processes of tobacco by stomatal limitation. A similar conclusion was also found in studies on *Salvinia minima* Baker grown in 40 μM $Pb(NO_3)_2$ solution for 24 h [84].

Chl fluorescence is a convenient and efficient tool in exploring photosynthesis responses to environmental factors [85]. The change in Chl fluorescence indicators signifies the absorption, transmission, dispersion, and allocation of light energy in the processes of photosynthesis, especially photosystem II (PSII) photochemistry [86]. F_v/F_m, representing the effectiveness of the primary conversion of light energy within the PSII reaction centre, has been widely used to detect environmental stress-induced damage to PSII

[87, 88]. The effects of PE-MP and Pb treatments on Chl fluorescence parameters in tobacco leaves are presented in Table 3. All the Chl fluorescence parameters (with the exception of Φ_{NPQ}) were significantly influenced by the addition of PE-MPs. F_m, F_v/F_m, $\Phi_{PSII,}$ and ETR increased with increasing concentrations of PE-MPs, while F_o, *NPQ,* and Φ_{NO} showed the opposite trend. Under Pb0 conditions, compared to PE0, different PE-MP treatments decreased F_m, F_v/F_m, $\Phi_{PSII,}$ and ETR by 4.3~24.7%, 3.8~16.5%, 7.9~21.1%, and 2.8~19.1%, respectively, but increased F_o, *NPQ,* and Φ_{NO} by 7.1~19.4%, 12.0~64.0%, and 16.7~58.3%, respectively. This was verified by a substantial reduction in Φ_{PSII}, which indicates the actual photosynthetic efficiency. A reduction in the F_v/F_m ratio could be linked to restricted reoxidation of QA, which may also be connected to potential alterations in the photochemical activity of PSII [89]. Consequently, this leads to diminished electron transport from PSII to photosystem I (PSI) [84, 90]. The electron transport status of the PSII reaction centre is expressed by ETR, which can be depicted as the number and rate of light quanta absorbed during electron transport [91]. Furthermore, Pb stress exacerbated the inhibition of F_m, F_v/F_m, $\Phi_{PSII,}$ and ETR by PE-MPs and improved F_o, *NPQ,* and Φ_{NO}. Compared to Pb0, Pb500 significantly decreased F_m, F_v/F_m, $\Phi_{PSII,}$ and ETR in various PE-MP treatments by 21.6~30.8%, 22.8~31.8%, 31.4~36.7%, and 27.4~52.7%, and markedly increased F_o, *NPQ,* and Φ_{NO} by 25.5~31.7%, 17.1~42.9%, and 72.2~116.7%, respectively. The decline in ETR is a result of competitive inhibition of the water-splitting reaction caused by attachment to the oxygen-evolving complex [92]. The reduction in F_v/F_m and ETR suggested that both PE-MPs and Pb caused PSII damage [93] and Calvin cycle disturbance [94]. The changes in Φ_{PSII}, Φ_{NO}, and Φ_{NPQ} reflect the allocation of absorbed light energy by leaf pigments [95]. In our present study, low Φ_{PSII} and high Φ_{NO} values were found in different PE-MP and Pb treatments, while Φ_{NPQ} showed no marked change ($p>0.05$), indicating that the yield of PSII photochemical reactions under PE-MP- and Pb-stressed conditions was predominantly dominated by nonregulated quenching, while xanthophyll cycle-mediated energy dissipation played a less important role [96]. Furthermore, the value of *NPQ* was significantly increased by exposure to PE-MPs, Pb, and PE-MPs+Pb. This result suggested that tobacco plants have the ability to dissipate excess light energy into the air in the form of heat, thereby preventing the formation of ROS in leaf chloroplasts and effectively safeguarding photosynthetic structures from light-induced damage [97, 98].

Table 3. Effects of PE-MPs and Pb treatments on Chl fluorescence parameters in leaves of tobacco.

Treatment	F_m	F_o	F_v/F_m	*NP*Q	Φ_{PSII}	Φ_{NO}	Φ_{NPQ}	ETR
PE0	2837±21 a	607±8 d	0.79±0.01 a	0.25±0.05 c	0.38±0.04 a	0.12±0.01 d	0.50±0.04 a	361±12 a
PE250	2716±133 a	650±46 c	0.76±0.01 b	0.28±0.03 bc	0.35±0.05 ab	0.14±0.01 c	0.51±0.04 a	351±3 a
PE500	2432±137 b	665±9 bc	0.73±0.02 c	0.31±0.01 bc	0.34±0.04 ab	0.16±0.01 b	0.50±0.03 a	331±11 b
PE750	2205±22 c	697±9 ab	0.67±0.01 d	0.34±0.02 b	0.31±0.01 ab	0.18±0.01 a	0.50±0.01 a	311±7 cd
PE1000	2134±50 c	725±6 a	0.66±0.01 e	0.41±0.04 a	0.30±0.01 b	0.19±0.01 a	0.51±0.01 a	292±16 d
PE0+Pb	1978±32 a	762±24 d	0.61±0.01 a	0.32±0.02 b	0.26±0.04 a	0.26±0.01 c	0.49±0.05 a	262±23 a
PE250+Pb	1880±81 b	815±8 c	0.57±0.02 b	0.40±0.08 ab	0.24±0.02 ab	0.26±0.03 c	0.50±0.03 a	252±17 a
PE500+Pb	1807±49 b	868±6 b	0.52±0.02 c	0.43±0.08 a	0.23±0.01 abc	0.29±0.02 bc	0.49±0.03 a	228±15 a
PE750+Pb	1686±13 c	918±14 a	0.46±0.01 d	0.48±0.04 a	0.21±0.01 bc	0.31±0.03 ab	0.48±0.04 a	183±26 b
PE1000+Pb	1674±15 c	926±7 a	0.45±0.01 d	0.49±0.03 a	0.19±0.03 c	0.34±0.01 a	0.47±0.03 a	138±10 c
PE-MPs	***	***	***	***	***	***	ns	***
Pb	ns	***	***	***	***	***	ns	***
PE-MPs×Pb	ns	***	**	ns	ns	ns	ns	***

Note: Values are the mean of three replicates. PE–PE: MPs treatment; Pb: Pb treatment; PE×Pb: interaction between PE-MPs and Pb. Different letters in the same column indicate significant differences ($p<0.05$) between different treatment groups according to Duncan's test. *** indicates $p<0.001$; ** indicates $p< 0.01$; * indicates $p<0.05$; ns indicates no significant differences.

Effects of Combined PE-MPs and Pb on Antioxidant Enzyme Activities

To survive, plants have developed a series of antioxidant defense systems to avoid plant cells suffering oxidative damage by eliminating ROS and regulating osmotic pressure [57]. SOD, CAT, POD, and APX are four key antioxidant enzymes in scavenging $O_2^{\cdot-}$ and H_2O_2 [99]. SOD first catalyzes $O_2^{\cdot-}$ into H_2O_2 and O_2, and then the H_2O_2 molecules are broken down into H_2O and O_2 by the catalysis of CAT, POD, and APX [100]. Increased activities of SOD, CAT, POD, and APX have been widely observed in wheat [66], *Spirodela polyrhiza* [101] and *Peganum harmala* L. [102] subjected to Pb stress, *Vicia faba* [62], *Vallisneria natans* [103] and *Solanum photeinocarpum* [59] grown in MP-contaminated soils, and buckwheat [55] under combined MP- and Pb-stressed conditions. Our results also showed a noticeable increase in SOD, CAT, POD, and APX activity in tobacco leaves under PE-MP and Pb stress conditions (Table 4), indicating that tobacco plants have a cellular defense mechanism against oxidative damage [104]. On the other hand, Pb stress significantly increased the activities of POD, CAT, and APX and the SS and Pro contents, while the SOD activity markedly decreased. In addition to increasing the activities of antioxidant enzymes against oxidative stress, nonenzymatic antioxidants such as Pro and SS are critical in maintaining osmotic and cell turgor equilibrium [105]. In this study, tobacco plants accumulated significant amounts of Pro and SS to adjust osmotic regulation under PE-MP- and Pb-stressed conditions (Table 4), which is consistent with the studies of Lamhamdi et al. [106] and Pirzadah et al. [107]. The results suggest that tobacco plants are capable of alleviating oxidative damage by enhancing the biosynthesis of osmolytes and carbohydrates to eliminate ROS [108].

Comprehensive Analysis

To further assess the potential influences of PE-MPs and Pb on the plant growth of tobacco, PCA and SEM were conducted in Fig. 4 and Fig. 5, respectively. PCA is a well-established, unsupervised multivariate statistical technique that has been widely used to distinguish the differences in controlled treatments and cluster experimental traits with similar characteristics [63]. In our study, the results of PCA in Fig. 4 did not clearly separate the differences between different PE-MP and Pb treatments, suggesting that these two contaminants posed a combined stress on tobacco plants. Additionally, the study parameters, including GB, SB, RB, P_n, G_s, F_v/F_m, F_m, ETR, T_r, Φ_{PSII}, Φ_{NPQ},

Table 4. Effects of PE-MPs and Pb on the activities of antioxidant enzymes and the contents of soluble sugar and proline in leaves of PE-MPs.

Treatment	SOD activity (U g^{-1} protein FW)	POD activity (U g^{-1} protein FW)	CAT activity (U g^{-1} protein FW)	APX activity (U g^{-1} protein FW)	SS (μg g^{-1} FW)	Pro (μg g^{-1} FW)
PE0	460.2±16.8 c	8662±200 d	356±9 d	2.23±0.15 d	82.2±5.7 c	42.2±1.3 d
PE250	462.3±2.7 bc	8909±55 c	361±1 cd	2.35±0.05 d	83.6±0.4 c	42.7±0.4 cd
PE500	470.2±2.0 abc	9155±56 b	369±4 bc	2.76±0.10 c	87.9±2.0 bc	44.4±1.1 c
PE750	477.3±4.0 ab	9538±58 b	375±3 b	3.17±0.15 b	93.3±1.2 b	47.1±1.5 b
PE1000	485.6±4.3 a	9624±166 a	395±5 a	3.63±0.15 a	103.0±6.4 a	51.2±1.2 a
PE0+Pb	339.8±15.1 c	9623±126 c	360±2 e	2.67±0.06 d	123.8±9.9 e	58.5±3.3 e
PE250+Pb	375.7±8.6 b	9804±193 bc	378±5 d	2.77±0.06 cd	148.2±2.4 d	64.0±1.6 d
PE500+Pb	389.1±5.1 b	11002±587 b	407±6 c	2.87±0.06 c	177.0±4.1 c	71.4±1.2 c
PE750+Pb	416.3±5.4 a	12722±999 a	432±7 b	3.23±0.06 b	196.7±5.8 b	74.9±1.5 b
PE1000+Pb	430.7±5.4 a	12751±1005 a	462±5 a	3.66±0.09 a	224.8±12.2 a	81.3±1.1 a
PE-MPs	***	***	***	***	***	***
Pb	***	***	***	***	***	***
PE-MPs×Pb	***	***	***	***	***	***

Note: Values are the mean of three replicates. PE–PE: MPs treatment; Pb: Pb treatment; PE×Pb: interaction between PE-MPs and Pb. Different letters in the same column indicate significant differences ($p<0.05$) between different treatment groups according to Duncan's test. *** indicates $p<0.001$; ** indicates $p< 0.01$; * indicates $p<0.05$; ns indicates no significant differences.

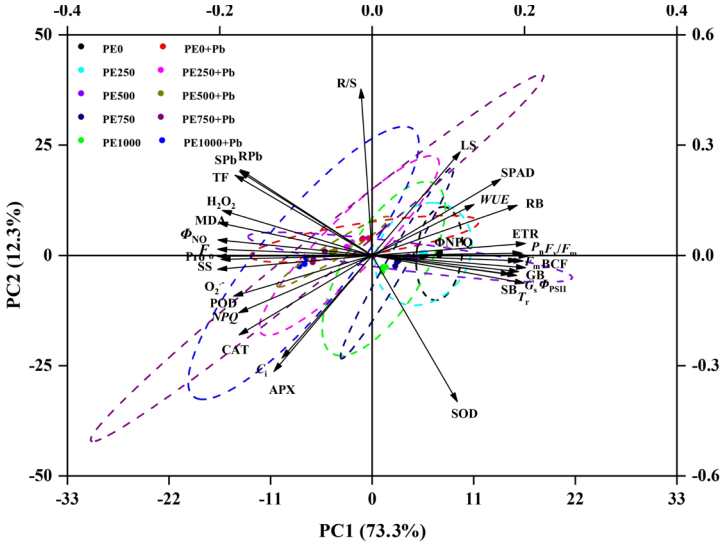

Fig. 4. Biplot of first (PC1) and second (PC2) principal components of 32 evaluated traits. APX - Ascorbate peroxidase, CAT - Catalase activity, C_i - Intercellular CO_2 concentration, ETR - Electron transport rate, F_o - Minimal fluorescence, F_m - Maximal fluorescence, F_v/F_m - Maximum quantum efficiency of PSII photochemistry in dark-adapted state, GB - Gross biomass, G_s - Stomatal conductance, H_2O_2 - Hydrogen peroxide, Ls - Stomatal limitation, MDA - Malondialdehyde content, *NPQ* - Non-photochemical quenching, $O_2^{\cdot-}$- Superoxide ion, POD - Peroxidase, P_n - Net photosynthetic rate, Pro - Proline, RB - Root biomass, SB - Shoot biomass, R/S - Root to shoot ratio, SOD - Superoxide dismutase, SPAD - SPAD value, T_r - Transpiration rate, *WU*E - Water use efficiency, Φ_{PSII} - Effective PSII quantum yield, Φ_{NO} - Effective PSII quantum yield, Φ_{NPQ} - Effective PSII quantum yield.

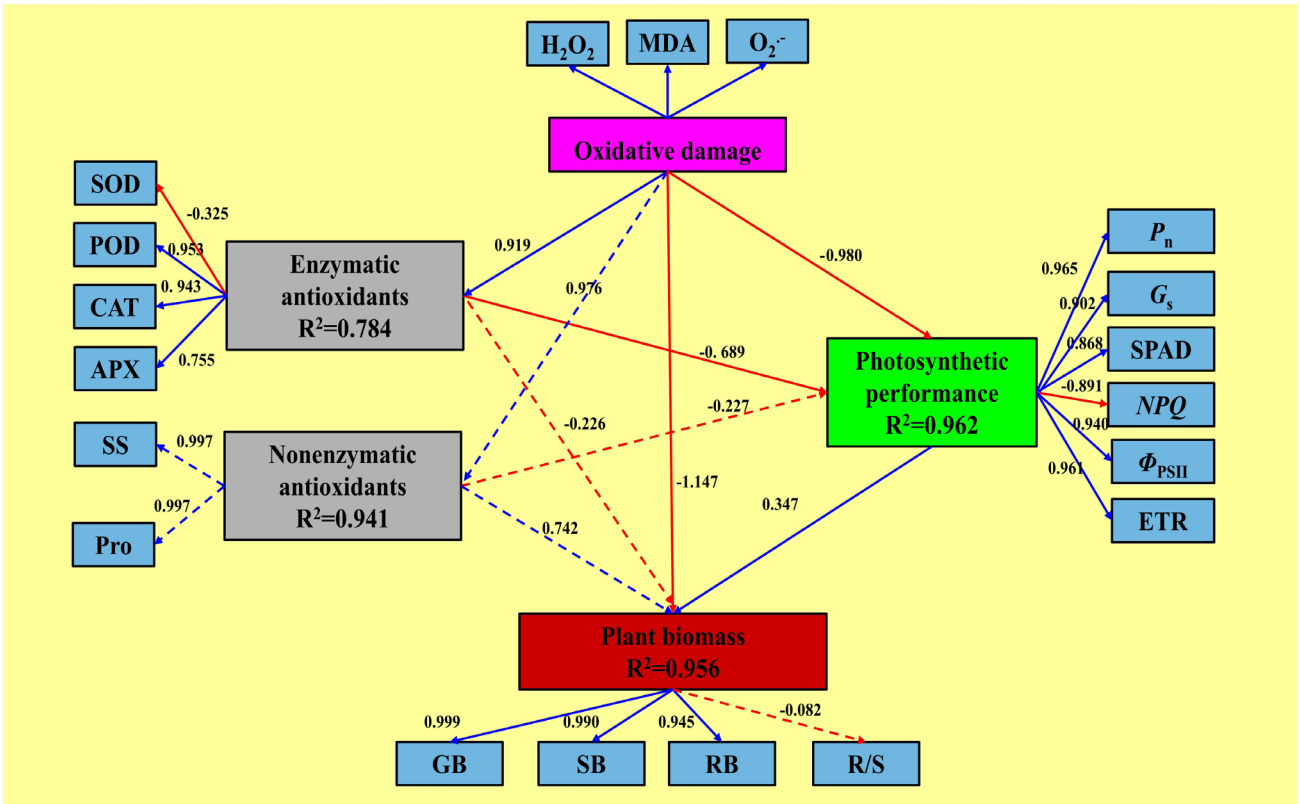

Fig. 5. Structural equation model explaining the plant growth of tobacco. Solid line arrows indicate significant paths; dotted line arrows indicate non-significant paths; blue line arrows indicate positive relationships; red line arrows indicate negative relationships; values associated with the line represent standardized path coefficients. The R^2 numbers within boxes denote the proportion of variance that could be explained by the corresponding variable in the structural equation model. APX - Ascorbate peroxidase, CAT - Catalase activity, ETR - Electron transport rate GB - Gross biomass, G_s - Stomatal conductance, H_2O_2 - Hydrogen peroxide, MDA - Malondialdehyde content, *NPQ* - Non-photochemical quenching, $O_2^{\cdot-}$ - Superoxide ion, POD - Peroxidase, P_n - Net photosynthetic rate, Pro - Proline, RB - Root biomass, SB - Shoot biomass, R/S - Root to shoot ratio, SOD - Superoxide dismutase, SPAD - SPAD value, Φ_{PSII} - Effective PSII quantum yield.

WUE and SPAD, were positioned along the positive axis of PC1, while the other research variables, such as H_2O_2, MDA, Pro, SS, O_2^-, *NPQ*, POD and CAT, fell along the negative axis of PC1. The findings revealed that PE-MPs and Pb caused severe oxidative stress on the photosynthetic performance and biomass of tobacco, and the plants can trigger self-protective mechanisms to alleviate oxidative damage by regulating antioxidant defense systems. SEM was employed to identify the direct and indirect influences of oxidative damage, enzymatic antioxidants, nonenzymatic antioxidants, and photosynthetic performance on the biomass of tobacco plants. According to the findings of the structural equation model in Fig. 5, oxidative damage, enzymatic antioxidants, nonenzymatic antioxidants, and photosynthetic performance explained 95.6% of the plant biomass. The calculated standardized path coefficients reflected that oxidative damage had a negative and direct effect (path coefficient = -1.147, $p<0.05$) and photosynthetic performance had a positive and direct impact (path coefficient = 0.347, $p<0.05$) on plant biomass and quality, while the effect of enzymatic antioxidants and nonenzymatic antioxidants on plant biomass was indirect. Furthermore, the impact of enzymatic antioxidants on photosynthetic performance was found to be significant (path coefficient = -0.689, $p<0.05$), implying that the role of enzymatic antioxidants in determining plant biomass is exercised through the control of the plant's photosynthetic performance.

Conclusions

In this work, we studied the potential impacts of PE-MPs, Pb, and their combination on the physiological and biochemical characteristics of tobacco plants. The results showed that PE-MPs and Pb alone caused serious oxidative stress and lipid peroxidation in tobacco plants, leading to a significant reduction in leaf pigments, photosynthetic efficiency, water utilization, and dry matter accumulation. Furthermore, the coexistence of PE-MPs and Pb exerts synergistic toxicity on the plant growth of tobacco. The limitation of photosynthesis by Pb was controlled by stomatal closure, while the suppression of photosynthesis by PE-MPs was dependent on nonstomatal limitation. Changes in Chl fluorescence parameters showed that PE-MPs and Pb posed dual damage to the PSII reaction center of tobacco and inhibited the electron transport process, resulting in a low light energy conversion efficiency. The reduced yield of PSII photochemistry under PE-MP- and Pb-stressed conditions was predominantly dominated by nonregulated quenching mechanisms. Furthermore, PE-MPs reduced the Pb contents in plant tissues, and the absorption of Pb decreased as the concentration of MPs increased. To counteract redundant ROS and alleviate oxidative damage caused by PE-MPs and Pb, both enzymatic antioxidants (SOD, POD, CAT and APX) and nonenzymatic antioxidants (Pro and SS) were activated by tobacco plants. The results of PCA further confirmed that PE-MPs and Pb posed a combined phytotoxicity to tobacco plants. Based on the SEM results, the reduction in plant biomass was directly and significantly associated with the increase in oxidative damage and the decrease in photosynthetic performance, while enzymatic antioxidants showed an indirect effect. In summary, the findings elucidate both the individual and synergistic phytotoxicity of MPs and heavy metals on plant growth, offering insights into the potential ecological risks of emerging pollutants to agricultural ecosystems.

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Conflict of Interest

The authors declare no conflict of interest.

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