

FOLIAR-SPRAYING OF PHYCOERYTHRIN NANO-SELENIUM ALLEVIATED SALT STRESS IN SOYBEAN SEEDLINGS VIS ENHANCING PHOTOSYNTHESIS AND ANTIOXIDANT FUNCTIONS

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ABSTRACT

Soil salinity stress, as one of the main abiotic stresses, affects agricultural production globally. In response to this, a novel phycoerythrin nano-selenium (PE-SeNPs) that comprised phycoerythrin 545 (Cr-PE545) for stabilizing the structure was successfully prepared to alleviate salt stress in the plant seedling. Aiming to evaluate their performance, the soybean seedlings (variety William 82) at three true leaves stage were randomly divided into four experimental groups, termed as CK (control), SS (salt stress group), PE (foliar application of 0.5 mM Cr-PE545), and PSe (foliar application of 0.5 mM PE-SeNPs), for which the CK was cultured in MS519 culture medium, and the SS, PE and PSe were cultured in MS519 supplemented with 50 mM NaCl. After 15 days, the efficacy of salt stress alleviation was analyzed by assessing indicators such as photosynthetic rate, chlorophyll content, and antioxidant enzyme activities. Compared to the salt-stressed group, in the PE-SeNPs treatment group, intercellular CO₂ concentration (Ci) decreased by 11.4%, and stomatal conductance (Gs), net photosynthetic rate (Pn) and total chlorophyll content increased by 101.4%, 191.38% and 198%, respectively; meanwhile, the superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities increased by 36.2%, 101% and 787%, respectively. The comprehensive analysis of mainly photosynthetic and physiological indicators highlighted that PE-SeNPs markedly help alleviate salt stress in soybean seedlings by promoting photosynthesis and scavenging intracellular reactive oxygen species. This work provides a beneficial foliar fertilizer for future potential applications in sustainable agriculture.

Keywords: Salt stress alleviation, Phycoerythrin, Phycoerythrin nano-selenium, Antioxidant activity, Enhanced photosynthesis

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INTRODUCTION

Selenium is a key active factor of enzymes with vital physiological functions. It boosts antioxidant enzyme activities, regulates growth, increases yield, improves quality, and enhances stress resistance in plants (Mushtaq *et al.*, 2022). However, the toxicity threshold of selenium is close to its threshold value for nutrition, plants are easily poisoned when it applied at higher dose (Gupta and Gupta, 2017). In recent years, nano-selenium has significantly lowered toxicity threshold, improving bioavailability and biocompatibility, becoming effective to expand its applications in the fields of functional food, pharmaceuticals, and agriculture because, compared to other selenium forms, it has unique antioxidant, antibacterial, and anticancer functions (Chen *et al.*, 2025). Notably, in order to reduce aggregation in the preparation of selenium nanoparticles, suitable templates or stabilizers are commonly required to enhance the stability of nano-selenium (Ndwandwe *et al.*, 2021). In addition to various polysaccharides commonly used, proteins represented as bovine serum albumin can be used as templates and stabilizers for the fabrication of nano-selenium to realize new functions (Chung *et al.*, 2020). Among them, the nano-selenium based on phycocyanin—a type of phycobiliprotein, has demonstrated significantly protective effects on cells, due to the valuable biological activities as a kind of natural pigment-protein (Jiang *et al.*, 2017).

As another type of phycobiliproteins, Cr-PE545 is a unique phycoerythrin with a stronger antioxidant property than phycocyanin, which is located in the thylakoid lumen of cryptophytes and acts as a light-harvesting antenna with a maximum absorption peak at 545 nm (Rathbone *et al.*, 2021). This natural pigment-protein with a molecular weight of approximately 60 kDa consists of four subunits ($\alpha_1\alpha_2\beta\beta$) that form a stable tetrameric structure and contains eight chromophores (Harrop *et al.*, 2014; Wang *et al.*, 2025a). Furthermore, previous studies have confirmed that the chromophores of phycoerythrin are crucial to scavenge free radicals effectively and exhibit strong antioxidant activity (Sato *et al.*, 2019). In addition, the newly established purification method of Cr-PE545, with more convenience, also provides a good foundation for its subsequent applications, especially the development of new nanomaterials (Pu *et al.*, 2025). However, a protocol for preparing nano-selenium using phycoerythrin has not been reported yet.

Currently, soil salinity stress, as one of the main abiotic stresses, causes osmotic stress, ion toxicity, oxidative stress, and secondary damage to plants, seriously affecting plant growth and yield and further limiting agricultural production (Al-Janabia *et al.*, 2024; Akram *et al.*, 2025). It is especially noteworthy that, under salt stress, reactive oxygen species (ROS) accumulate excessively in plants, which may eventually result in oxidative damage (Talaat *et al.*, 2022). Over the past decades, a series of functional nanomaterials, especially metal nanoparticles and selenium nanoparticles, have been shown to be effective in alleviating salt stress and enhancing salt tolerance in different crops (Fang *et al.*, 2023). Most notably, several studies indicated that nano-selenium, especially protein-bound nano-selenium, can mitigate salt stress through various mechanisms, including enhancing plant antioxidant capacity, regulating the accumulation of osmotic regulatory substances, and protecting photosynthesis and energy metabolism (Tsivileva, 2025).

Although recent research has demonstrated that exogenous application of selenium nanoparticles has beneficial effects on salt stress mitigation in soybean (*Glycine max*) seedlings, those nano-selenium do not contain any bioactive templates (Desouky *et al.*, 2024). As far as we know, the effects of nano-selenium containing phycoerythrin with antioxidant activity alleviating salinity stress are unclear. Thus, this study presents a new type of nano-selenium (PE-SeNPs) as a foliar fertilizer and subsequently examines its efficacy in improving the stress resistance of soybean seedlings and alleviating salt stress.

MATERIALS AND METHODS

Sample procurement: Sodium selenite (Na_2SeO_3), Ethylenediaminetetraacetic acid (EDTA), glutathione (GSH), hydrogen peroxide (H_2O_2), Nicotinamide adenine dinucleotide (reduced form) (NADH), Phenazine methosulfate (PMS), 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Nitro Blue Tetrazolium (NBT), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and ascorbic acid (Vc) were of analytical grade and purchased from Sinopharm (Shanghai, China). According to our previous study on purification of Cr-PE545, the analytical grade phycoerythrin (purity ratio $A_{545}/A_{280} = 13.66$) was obtained from unicellular cryptophyte algae of the *Rhodomonas salina* (Pu *et al.*, 2025). After the lyophilization, those proteins were stored at -20°C . All experiments were conducted in April 2025 at the School of Fisheries, Ludong University, China ($37^\circ31'11''\text{N}$, $121^\circ21'22''\text{E}$).

Preparation of PE-SeNPs: Firstly, 100 mg of Cr-PE545 lyophilized powder was dissolved in 8.75 mL of deionized water. Then, 250 μL of 50 mM Na_2SeO_3 solution was introduced, thoroughly stirring at room temperature in the dark. Subsequently, 200 μL of 30 mg/mL GSH solution was added at 10-minute intervals for a total of 5 times, and then continued to be darkly stirred for 1 hour at room temperature. Finally, after centrifugation (5000 rpm) and washing with deionized water three times, the sediment was collected for freeze-drying to obtain PE-SeNPs.

Characterization of PE-SeNPs

Particle size distribution and surface Zeta potential analysis: A laser particle sizer with a thrice repeated (Brookhaven, 90PlusPALS, USA) was employed to measure the features of particle size distribution and Zeta-potential of the materials, which provided evidence for the fabrication of PE-SeNPs.

Transmission Electron Microscopy (TEM): For the purpose of further confirming the successful preparation of PE-SeNPs, the morphology of these was observed by transmission electron microscopy (Thermo Fisher Scientific, Talos F200X G2, USA). Compared with the results from the laser particle sizer, the precise measurements and shapes of PE-SeNPs can be verified directly.

Fourier Transform Infrared (FTIR) spectroscopy: The FTIR of PE and PE-SeNPs was measured using the Fourier transform infrared spectrometer (Shimadzu, IRTracer-100, Japan). The analysis of characteristic peaks such as Se–O covalent bonds, pyrrole ring and amino groups can help identify the successful construction of PE-SeNPs.

UV-visible absorption spectroscopy: UV-visible absorption spectra were monitored by a UV-visible spectrophotometer (Shimadzu, UV-2600i, Japan) in the assessment of the characteristics of Cr-PE545, PE-SeNPs, in vitro antioxidant activity, photosynthetic pigment contents, activities of antioxidant enzymes and osmotic regulation index. More details are in the relevant sections.

Determination of the total reducing power of PE-SeNPs: In the beginning, 100 μ L of PE-SeNPs (or 100 μ L of vitamin C (Vc) solutions, as a positive control) at varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) were mixed with 1% potassium ferricyanide (100 μ L) and 0.2 mol/L phosphate buffer at pH 6.6 (100 μ L), followed by incubation at 50 °C for 20 min. In the event of terminating the reaction, all ten samples were added to 100 μ L of 10% trichloroacetic acid separately, and then centrifugation (5000 r/min for 10 min) was employed to obtain the supernatant. Afterward, 200 μ L of each collected supernatant was mixed with 200 μ L of deionized water and 0.1% FeCl₃, and then let it stand for 10 min. The absorbance was measured at 700 nm (Nwachukwu *et al.*, 2021).

Determination of the ability of PE-SeNPs to scavenge DPPH free radicals: Similarly, 2.0 mL of PE-SeNPs (or 100 μ L of vitamin C (Vc) solutions) at varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) were mixed with 0.002% DPPH solution (2.0 mL, dissolved in absolute ethanol) separately. The ten mixed solutions were incubated in the dark for 30 min and then centrifuged at 5000 r/min for 10 min. The absorbance of the obtained supernatants was recorded at 517 nm. Meanwhile, the varying concentrations of Vc served as a positive control; the scavenging activity was determined as follows: Scavenging activity (%) = $(1 - A_{\text{sample 517}} / A_{\text{control 517}}) \times 100$ (Nwachukwu *et al.*, 2021).

Determination of the chelating ability of PE-SeNPs for Fe²⁺: At first, the reaction mixture, containing 2.75 mL of deionized water, 0.05 mL of FeCl₂ (20 mM), 0.2 mL of ferrozine solution (5.0 mM) and 1.0 mL of samples that were PE-SeNPs at varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) or EDTA at varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL), respectively, was incubated for 10 min at room temperature. For all ten samples, the absorbance was measured at 562 nm. Using varying concentrations of EDTA as a positive control, the chelating ability was determined as follows: Chelating ability (%) = $(1 - A_{\text{sample 562}} / A_{\text{control 562}}) \times 100$ (Nwachukwu *et al.*, 2021).

Determination of the scavenging rate of hydroxyl radicals ($\cdot$ OH) by PE-SeNPs: Similar to the above, the varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) of the PE-SeNPs solution (or 100 μ L of vitamin C (Vc) solutions) (1.0 mL) were mixed with 2 mL reaction buffer (containing 2.67 mM deoxyribose, 0.2 M phosphate buffer (pH 7.4), and 0.13 mM EDTA) and ferrous ammonium sulfate (1.0 mL, 0.4 mM), respectively. Following adding 1.0 mL of H₂O₂ (10.0 mM) and incubating at 37 °C for 15 min, the ten prepared samples were added to 1.0 mL of 1.0% thiobarbituric acid and 1 mL of 2.0% trichloroacetic acid, separately; then, after boiling for 15 min, their absorbance was measured at 532 nm. Choosing the varying concentrations of Vc as a positive control, the scavenging activity of $\cdot$ OH was determined as follows: Scavenging activity (%) = $(1 - A_{\text{sample 532}} / A_{\text{control 532}}) \times 100$ (Nwachukwu *et al.*, 2021).

Determination of physiological indicators: At the start, 300 soybean seedlings (variety William 82) were grown hydroponically using MS519 culture medium, growing into the three true leaves. Among them, 120 seedlings were chosen for a follow-up salt stress experiment, based on having similar leaf sizes. The chosen 120 soybean seedlings were divided into four experimental groups following a Completely Randomized Design (CRD), where 30 plants were in each group: CK (control group), SS (salt stress group), PE (treatment group that sprayed 0.5 mM PE every day), and PSe (treatment group that sprayed 0.5 mM PE-SeNPs every day). The CK was cultured in MS519 medium; all three other groups were cultured in MS519 medium supplemented with 50 mM NaCl to induce salt stress. For each seedling, once a day, about 2 mL every time, spraying treatment enables the leaf surface to be evenly moist, and water droplets start to fall using a handheld sprayer (a nozzle aperture of 0.3 mm). The spraying time is set from 8:00 to 9:00 every morning. The CK group and the SS group were simultaneously sprayed with the same volume of deionized water to ensure consistent treatment conditions. Furthermore, in our previous pre-experiment, it was found that high-concentration nano-selenium could not effectively alleviate salt stress, in line with previous reports (Rady *et al.*, 2021). Therefore, we finally chose 0.5 mM PE-SeNPs as the suitable spraying concentration. After 15 days, for the above four experimental groups, each treatment group of soybean seedlings was separately harvested for analysis of physiological parameters. Then, the harvested soybean seedlings within the same treatment group were mixed as a set of pooled samples, thus constructing four sets of samples corresponding to CK group, SS group, PE group, and PSe group, respectively, prior to the determination of physiological indicators. For each group, subsequent experiments were randomly sampled thrice, following CRD. Then, each sample was subjected to three parallel tests. The mean of three parallel tests was used as the value of each sample. So, there will be nine testing values and three mean values for the independent group. Specifically, the values of Gs, Ci, Pn, chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoid (Car), and total photosynthetic pigment contents (TPC) were measured and the activities of SOD, POD and CAT, as well as the concentrations of superoxide anion (O₂ $\cdot^-$), malondialdehyde (MDA) and

free proline, were determined following previous reported methods with minor modifications (Duan *et al.*, 2025a; Liu *et al.*, 2025).

The Li-6400 portable photosynthesis meter (Li-Cor Company, USA) was used to measure Pn, Gs, Ci, and transpiration rate (Tr) between 9:00 and 11:00 a.m. Measurements were taken from the 3rd to 4th fully expanded true leaves. A red-blue LED light source was used with an intensity set to 1,000 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The leaf surface temperature during measurements was maintained at 20 °C. The Chlorophyll fluorescence was recorded with a portable plant fluorescence imaging system (FluorCam FC 1000-H, Photon Systems Instruments, Czech Republic).

Determination of Photosynthesis and Pigment Content: Chlorophyll a, chlorophyll b, and carotenoids were determined using 95% ethanol extraction in the dark (Lichtenthaler, 1987; Wellburn, 1994).

$$\begin{aligned} \text{Chl } a &= \frac{(12.72 \times A_{663} - 2.59 \times A_{645}) * V}{W} \\ \text{Chl } b &= \frac{(20.36 \times A_{645} - 5.19 \times A_{663}) * V}{W} \\ \text{Car} &= \frac{1000 * A_{470} - 1.63 \times \text{Chl } a - 104.96 \times \text{Chl } b}{245} * \frac{V}{W} \end{aligned}$$

V represents the volume of the extract, and W represents the sample mass, with water content removed to avoid interference.

Determination of SOD activity: In the beginning, 0.2 g of fresh soybean leaves (respectively from CK, SS, PE and PSe) was added into 2 mL of 5 mM PBS (pH 7.8) and ground, and then, after centrifuging (10,000 rpm) for 20 min at 4°C, the supernatant was collected as crude enzyme extract (termed as SLSS) for follow-up tests including SOD activity, POD activity, CAT activity and superoxide anion generation rate.

The reaction solutions were prepared with 50 mM PBS (pH 7.8), 130 mM methionine (MET), 750 mM nitro blue tetrazolium (NBT, protected from light), 0.1mM EDTA- Na_2 , and 0.02 mM riboflavin (protected from light). Three experimental groups were established: light control group, dark control group, and sample group. The light and dark control groups were prepared by adding 2 mL PBS, 0.3 mL MET, 0.3 mL NBT, 0.3 mL EDTA- Na_2 , 0.3 mL riboflavin and 0.25 mL distilled water. The light control group was incubated under 3000 Lux light for 20 min, while the dark control group was kept in darkness for the same duration. For the sample group, the reaction system contained 1.5 mL PBS, 0.3 mL MET, 0.3 mL NBT, 0.3 mL EDTA- Na_2 , 0.3 mL riboflavin, 0.5 mL SLSS and 0.25 mL distilled water, and was incubated at 3000 Lux for 20 min. All reactions were terminated by placing the samples in darkness for 10 min, followed by the determination of absorbance at 560 nm.

Determination of POD activity: The POD reaction solution was freshly prepared as follows: 28 μL guaiacol was added into 50 mL of 100 mM PBS (pH 6.0) and dissolved by heating. After cooling, 19 μL of 30% hydrogen peroxide (H_2O_2) was supplemented, and the mixture was refrigerated for immediate use. The control group consisted of 0.2 mL PBS (pH 6.0) and 3 mL POD reaction solution, while the sample group contained 0.2 mL SLSS mixed with 3 mL POD reaction solution. The absorbance change at 470 nm was immediately measured every minute for a total duration of three minutes.

Determination of CAT activity: Firstly, 60 mM PBS (pH 7.4) and 32.4 mM ammonium molybdate solution were prepared for follow-up tests. Subsequently, the CAT substrate solution was prepared freshly by diluting 0.2 mL of 30% H_2O_2 to 100 mL of the above PBS and then incubated in a 37 °C water bath. Three blank tubes (B1, B2, B3) and sample tubes were prepared. Tube B1: Mix 1 mL CAT substrate solution and 1 mL the above ammonium molybdate solution, then add 0.5 mL SLSS (do not reverse the adding order). Tube B2: Add 1 mL CAT substrate solution, 1 mL above ammonium molybdate solution and 0.5 mL 60 mM PBS (pH 7.4). Tube B3: Add 1.5 mL above ammonium molybdate solution and 1 mL 60 mM PBS (pH 7.4). For sample tubes: Mix 0.5 mL SLSS with 1 mL CAT substrate solution, incubate at 37 °C for 1 min, then add 1 mL above ammonium molybdate solution. After standing for 10 min, the absorbance was measured at 402 nm.

Determination of superoxide anion generation rate: At first, 50 mM PBS (pH 7.8), 10 mM hydroxylamine chloride solution, 17 nM sulfanilamide solution (dissolved in an acetic acid-water mixture, 3:1, V/V) and 7 nM 1-naphthylamine solution (acetic acid-water = 3:1, V/V) were prepared for follow-up tests. 1 mL SLSS, 0.5 mL of the above PBS and 1 mL of the above hydroxylamine chloride solution were mixed sequentially and then incubated at 25 °C for 20 min. Subsequently, the post-incubated solution was added to 1.0 mL of 1-naphthylamine and 1.0 mL of sulfanilamide, incubated for another 20 min in the same water bath. Finally, after adding 3.0 mL of diethyl ether, the mixture was centrifuged at 10,000 rpm for 5 minutes, and the supernatant was collected to measure its absorbance at 530 nm (Duan, *et al.*, 2025; Liu *et al.*, 2025).

Determination of proline content: After grinding 0.2 g of fresh soybean leaves in 2 mL of 3% sulfonated salicylic acid, the solution was centrifuged at 4°C (10,000 rpm, 20 min) to collect the supernatant. Then, 2 mL of the above supernatant

was mixed with 2 mL of glacial acetic acid and 2 mL of acidic indanone, heated in a water bath at 100°C for 30 min. After cooling to room temperature, 4 mL of toluene was added to it, shaking thoroughly, and then the upper organic phase was collected for analysis (Duan *et al.*, 2025b; Liu *et al.*, 2025).

Determination of malondialdehyde (MDA) content: Similarly, to prepare the test specimen, 0.2 g of fresh soybean leaves was ground in 2 mL of 10% trichloroacetic acid, and then, at 4°C, centrifugation was employed to obtain the supernatant (10,000 rpm, 20 min) for follow-up tests. After mixing the above supernatant with 2 mL of 0.6% thiobarbituric acid, the sample solution was heated in a 100°C water bath for 15 min, then centrifuged at 4000 rpm for 20 min. Finally, we collected the supernatant for the MDA content analysis (Duan *et al.*, 2025; Liu *et al.*, 2025).

Statistical Analysis: The treatments were analyzed in triplicate, following a CRD. The obtained data were subjected to one-way ANOVA, and Tukey's post-hoc tests were conducted for comparisons of distinct groups of samples, which were performed using IBM SPSS Statistics 24. All graphs were plotted using Origin 2018. The results of the experiments were expressed as the mean $\pm$ standard deviation (mean $\pm$ SD), and differences were considered significant at $p \leq 0.05$.

RESULTS

Characterization of PE-SeNPs complexes: In the visible region (450-750 nm), PE-SeNPs have a strong absorption at around 545 nm and a fluorescence emission peak at around 587 nm following excitation at 545 nm, both of which were similar to those of Cr-PE545. Considering that the energy transfer function of Cr-PE545 is closely related to its structure, the above results of unaltered optical activity also suggested that the structural integrity of Cr-PE545 was not changed much in PE-SeNPs (Figure 1A, B). As shown in Figure 1C, the SDS-PAGE analysis results of Cr-PE545 with a high purity show three bands at 9 kDa, 10 kDa, and 20 kDa, corresponding to the α_1 , α_2 , and β subunits, respectively, with scarcely any protein bands of impurity. In the infrared spectra, unlike Cr-PE545, PE-SeNPs exhibited a significantly characteristic peak at 1020 cm^{-1} , 1401.2 cm^{-1} and 1617.4 cm^{-1} , corresponding to Se–O bonds, pyrrole ring and amino groups, respectively (Figure 1D). Furthermore, as depicted in Figure 1E, the noticeable particles with well dispersibility at approximately 190 nm were observed using TEM, in agreement with the average hydrated particle size at 274.6 nm, and, in Figure 1F, the surface Zeta potential of Cr-PE545 and PE-SeNPs was at 17.45 mV and -62.87 mV, respectively, verifying the successful preparation of PE-SeNPs.

In vitro antioxidant activity analysis of PE-SeNPs: Given that nano-selenium typically exerts its biological effects through antioxidant activity, we subsequently evaluated the in vitro antioxidant activity of the prepared PE-SeNPs. As shown in Figure 2A, the total reducing power of PE-SeNPs increased with concentration and eventually stabilized at 3.2%. Vc possessed a strong reducing capacity (approaching 80%); in contrast, PE-SeNPs demonstrated a moderate but significant reducing ability. In the case of hydroxyl radicals, PE-SeNPs exhibited a remarkably high scavenging rate of 81.42% at 0.2 mg/mL, comparable to Vc at the same concentration (Figure 2B). The observed decrease in scavenging rate to 70.6% at higher concentrations (1.0 mg/mL) might be due to increased nanoparticle aggregation. Regarding DPPH radical scavenging activity (Figure 2C), PE-SeNPs showed a concentration-dependent increase, reaching 43.03% at 1.0 mg/mL. Although this was nearly half the scavenging rate of Vc (approximately 100%), it still reflects significant DPPH radical scavenging activity. Moreover, for Fe^{2+} chelation capacity (Figure 2D), PE-SeNPs exhibited a fluctuating trend, with chelation rates of 41.8% at 0.2 mg/mL and a peak of 60.29% at 0.8 mg/mL. In comparison, EDTA, a strong metal chelator, showed a rapid concentration-dependent increase, reaching 63.4% and 95.7% at 0.2 mg/mL and 0.8 mg/mL, respectively. These results indicate that PE-SeNPs possess notable Fe^{2+} chelation activity, reaching about two-thirds of EDTA's capacity at lower concentrations, thereby potentially mitigating metal-induced oxidative stress.

Enhanced photosynthetic performance after foliar spraying of PE-SeNPs: For the intensity of photosynthesis in soybean leaves, Gs and Pn are two closely related key indicators. As illustrated in Figure 3A, compared to the CK group, the SS group exhibited a 72.23% reduction in Gs, suggesting that the impaired stomatal opening directly prevented CO_2 from entering mesophyll cells normally, which brings a cascade of disadvantages. One of them is the higher intercellular CO_2 accumulation because they were limited to participate in photosynthetic reactions, which was in accord with the fact that Ci in the SS group increased by 28.3% in contrast to the CK group (Figure 3B). Furthermore, due to insufficient CO_2 supply, the dark reaction was suppressed partly, ultimately causing a significant decrease in Pn, which was confirmed by the results that are shown in Figure 3C, in which the net photosynthetic rate of the SS group decreased by 77.63% comparison with the CK group.

In contrast to the SS group, after spraying PE-SeNPs, the stomatal conductance of soybean seedlings doubled, indicating that it significantly restored stomatal conductance (Figure 3A). Considering that the unobstructed stomatal pathways not only facilitated external CO_2 uptake but also accelerated the consumption of Ci , the 11.4% reduce of Ci in the PSe group, as compared with the SS group, was quite reasonable, which was greater than that in the PE group (7.6%

decrease) (Figure 3B). Moreover, the restored balance between CO₂ supply and demand provided ample substrates for photosynthetic reactions, thus ultimately driving a substantial recovery in net photosynthetic rate. As depicted in Figure 3C, unlike the SS group, the Pn of the PSe group increased by 191.38% — a figure significantly higher than the 91% increase in the PE group, which should be attributed to the establishment of a positive feedback loop from stomatal conductance restoration to CO₂ supply-demand optimization, then photosynthetic efficiency recovery.

From another perspective, changes in crucial photosynthetic pigments content provide the supporting evidence for the aforementioned results in CO₂ exchange and photosynthetic efficiency. Then, we analyzed the content variation of Chl a, Chl b and Car. As demonstrated in Figure 3D, E, F, and G, in the comparison of the CK group, Chl a, Chl b, Car and TPC content in the SS group decreased by 74.4%, 75.3%, 9.2% and 74.6%, respectively, which is because salt stress disrupts chloroplast thylakoid membrane structure, leading to pigment degradation. After foliar application of PE-SeNPs, Chl a, Chl b, Car and TPC content in soybean seedling leaves increased by 207%, 173%, 10.6% and 198%, respectively, compared to the SS group.

As can be seen from Figure 3H, it can be inferred from the fluorescence signal images of qP, NPQ, and F_v/F_m that the signal intensities of the three indices were obviously reduced in the SS group versus the CK group and were enhanced to varying degrees in the PE group and the PSe group versus the SS group, especially those was higher in the PE-SeNPs-treated group than SS group and the PE group.

Aiming to give more detailed information about the photosynthetic reaction center (PS II), we examined some of the verifiable indicators such as the photochemical quenching coefficient (qP), non-photochemical quenching coefficient (NPQ), and maximum photochemical efficiency (F_v/F_m) — more accurate markers for PS II. As plotted in Figure 3I, the SS group's qP was 45.1% lower than the CK group. Following the foliar application of PE-SeNPs, the qP obviously increased by 24.6%, in contrast to the SS group; meantime, the NPQ in the SS group increased by 464% as opposed to the CK group (Figure 3J). As expected, the spraying of PE-SeNPs reduced NPQ by 65.96%. In addition, the F_v/F_m is also associated with the PSII function. Similar to the results of NPQ, the SS group's F_v/F_m was 39.8% lower than the CK group, while the PSe group's F_v/F_m was 64.13% higher than the SS group (Figure 3K).

Influence on the antioxidant system and osmotic regulation index via foliar application of PE-SeNPs: As demonstrated in Figure 4A, B, C, the activities of SOD, POD, and CAT (three antioxidant enzymes) in soybean leaves showed a significant reduction of 15.8%, 6%, and 55% in the SS group, as opposed to the CK group ($p \leq 0.05$). Meantime, compared to the SS group, for the PSe group, the activities of the above three antioxidant enzymes increased by 36.2%, 101% and 787%, respectively; for the PE group, the activities of the above three antioxidant enzymes increased by 20.4%, 2% and 140%, respectively. Additionally, compared with the CK group, the concentration of O₂⁻ increased noticeably in the other three groups; compared with the SS group, the superoxide anion production rate of soybean seedlings in the PE group and the PSe group decreased by 19.7% and 24.8%, respectively, which indicated that PE-SeNPs also exhibits a more pronounced effect (Figure 4D). Obviously, the antioxidant functions of PE-SeNPs in soybean seedlings are consolidated by these results. The results showed that the MDA content in the SS group, the PE group and the PSe group was significantly higher than that in the CK group (Figure 4E). However, compared with the SS group, the MDA content in soybean seedlings sprayed with PE-SeNPs decreased by 35.6%. Additionally, as depicted in Figure 4F, compared with the CK group, the content of free proline in soybean leaves increased by 145% under salt stress conditions. After spraying PE-SeNPs, the content of free proline in soybean leaves only rose to 94.8% of the control group (CK).

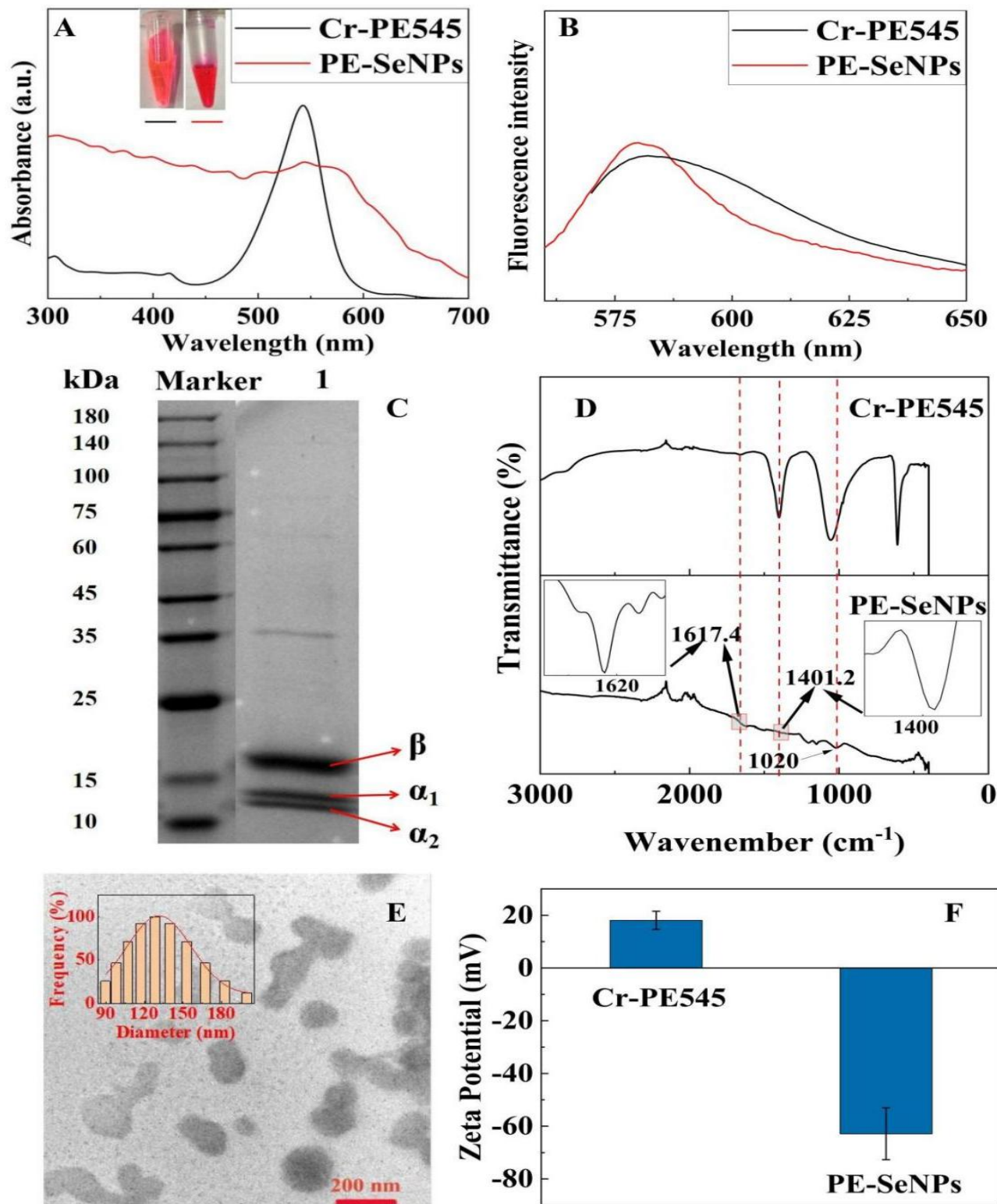


Fig. 1. Characterization of Cr-PE545 and PE-SeNPs. (A) UV-visible absorption spectra of Cr-PE545 and PE-SeNPs, (B) Fluorescence emission spectra of Cr-PE545 and PE-SeNPs, (C) SDS-PAGE of Cr-PE545, (D) FTIR spectra of Cr-PE545 and PE-SeNPs, (E) TEM images of PE-SeNPs (inset: size distribution histograms of PE-SeNPs), (F) Surface potential of Cr-PE545 and PE-SeNPs. Values represent mean $\pm$ SD ($n = 3$) using one-way ANOVA, Tukey's post-hoc test analysis.

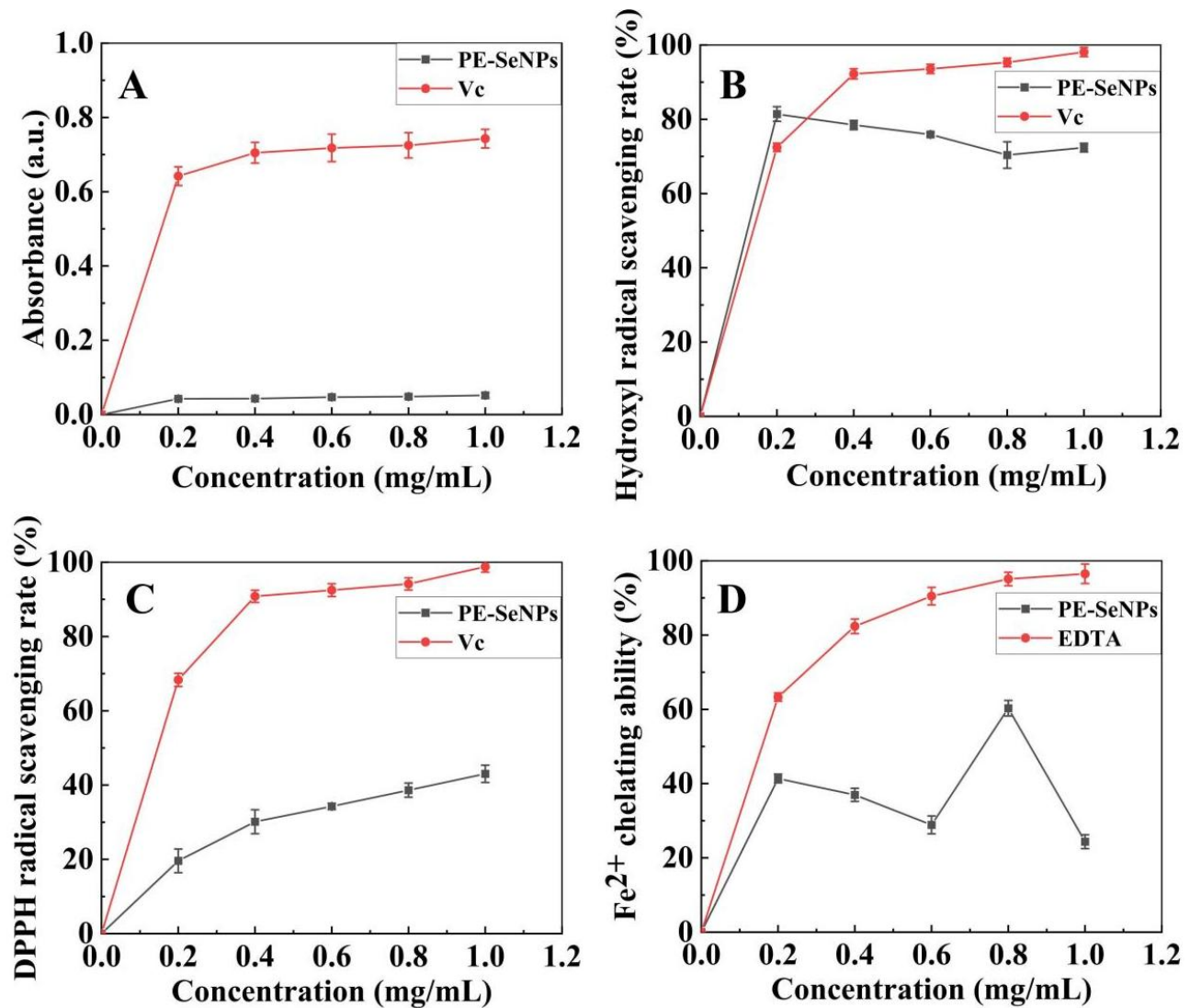


Fig. 2. In vitro antioxidant activity analysis of PE-SeNPs and controls. (A) Total reducing power of PE-SeNPs and Vc, (B) Hydroxyl radical scavenging capacity of PE-SeNPs and Vc, (C) DPPH radical scavenging rate of PE-SeNPs and Vc, (D) Fe²⁺ chelating capacity of PE-SeNPs and EDTA. Values represent means of three values $\pm$ SD (n = 3).

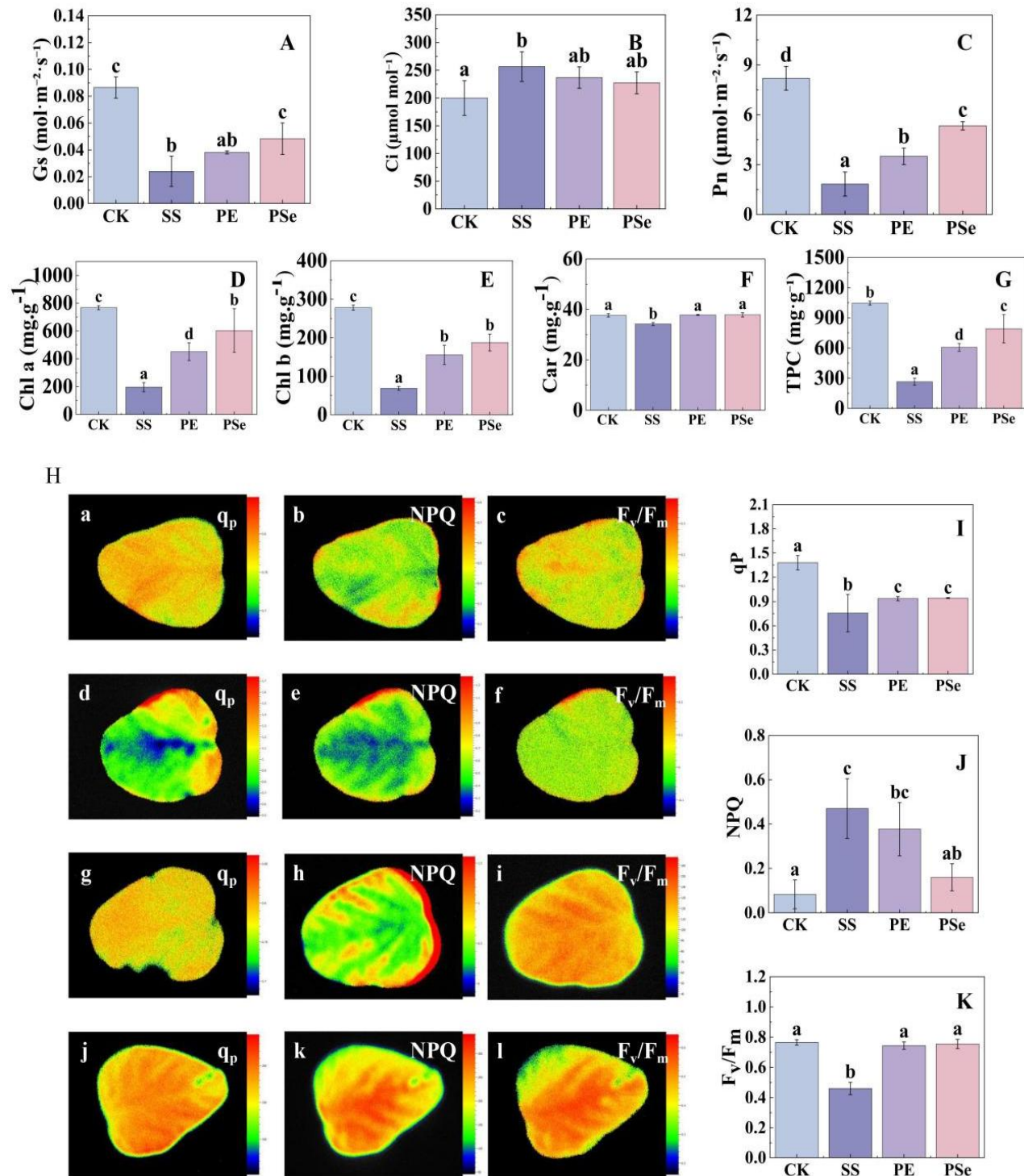


Fig. 3. Effect of PE-SeNPs and Cr-PE545 on photosynthetic performance in soybean seedlings under salinity stress: (A) Gs (Stomatal conductance), (B) Ci (Intercellular CO_2 concentration), (C) Pn (Net photosynthetic rate), (D) Chl a (chlorophyll a) content, (E) Chl b (chlorophyll b) content, (F) Car (Carotenoids) content, (G) TPC (Total photosynthetic pigment) content, (H) Chlorophyll fluorescence profiles (a-c: CK; d-f: SS; g-i: PE; j-l: PSe), (I) qP (Photochemical quenching coefficient), (J) NPQ (Non-photochemical quenching coefficient), (K) F_v/F_m (maximum photochemical efficiency). Values represent mean $\pm$ SD ($n = 3$) using one-way ANOVA, Tukey's post-hoc test analysis. Different lowercase letters in the figure indicate significant differences between groups ($p \leq 0.05$).

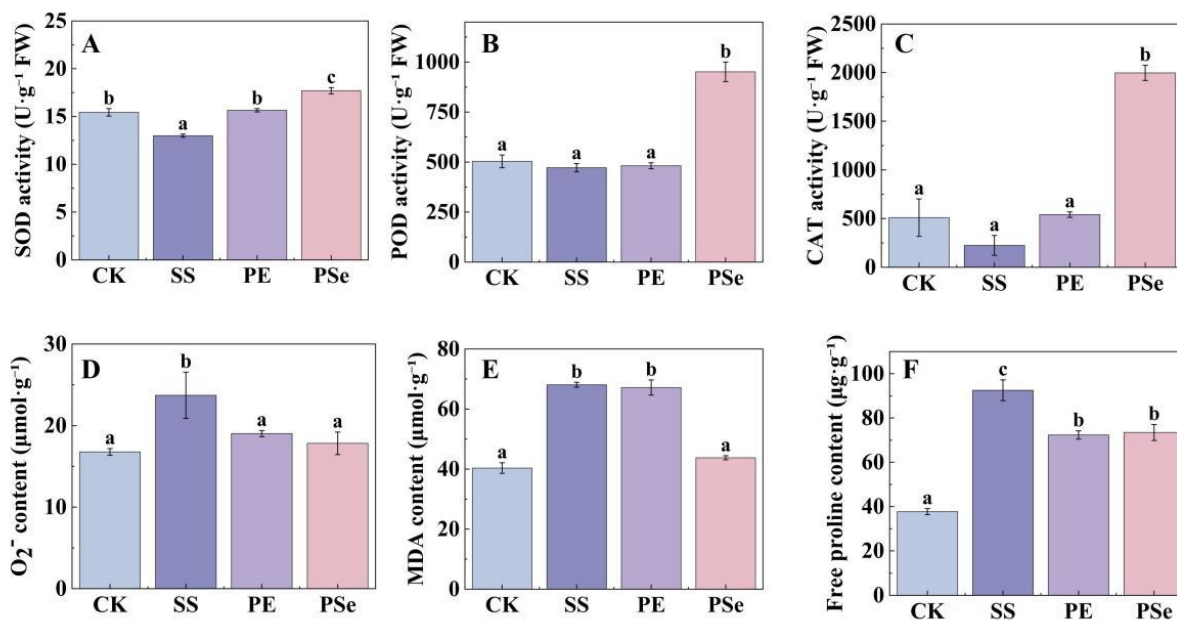


Fig. 4. Impact of PE-SeNPs and Cr-PE545 in soybean seedlings under salinity stress on (A) SOD (Superoxide dismutase) activity, (B) POD (Peroxidase) activity, (C) CAT (Catalase) activity, (D) Superoxide anion content, (E) MDA (Malondialdehyde) content, (F) Free proline content. Values represent mean $\pm$ SD ($n = 3$) using one-way ANOVA, Tukey's post-hoc test analysis. Different lowercase letters in the figure indicate significant differences between groups ($p \leq 0.05$).

DISCUSSION

The characterization data of PE-SeNPs complexes were recorded from various perspectives. Especially, in FTIR spectra, the Se–O characteristic peak at 1020 cm^{-1} that should be derived from Se–O covalent bonds between the nano-selenium and the hydroxyl or carboxyl side chains of Cr-PE545 confirms the presence of the PE-SeNPs complex (Vicas *et al.*, 2021). Additionally, the peaks at 1401.2 cm^{-1} and 1617.4 cm^{-1} are associated with the pyrrole rings and amino groups present in both of the PE and PE-SeNPs, indicating that the formation of selenium particles does not affect the pyrrole ring and amino groups of Cr-PE545 (Gibot and Goetz, 2021). Combining the obtained results of UV–visible absorption spectra, FTIR spectra and TEM, the PE-SeNPs were successfully prepared for subsequent applications.

For the PE-SeNPs, aforementioned *in vitro* antioxidant results strongly suggest their potential for enhancing photosynthesis and alleviating salt stress in soybean seedlings, likely by protecting chloroplasts and cellular membranes from oxidative damage, maintaining redox homeostasis, and preserving the integrity of photosynthetic apparatus under stress conditions (Desouky *et al.*, 2024). Based on current research, similar to other types of nano-selenium particles, PE-SeNPs should be capable of being directly absorbed by soybean leaves due to their nanoscale size and controlled-release properties, and subsequently, they gradually release selenium ions, thereby enhancing the plant's antioxidant capacity to achieve their efficacy in improving photosynthesis and alleviating salt stress (Wang *et al.*, 2025b).

Comparison with the CK group, the decreased Gs and Pn and increased Ci in the SS group show a chain reaction from stomatal closure to CO_2 supply-demand imbalance, then impaired photosynthetic efficiency (Wang *et al.*, 2025c). Following the foliar application of PE-SeNPs, by contrasting and analyzing the Gs, Ci and Pn, we suggested that the improvement of core photosynthetic indicators is positively correlated with the application of PE-SeNPs (Papanatsiou *et al.*, 2019; Wang *et al.*, 2022).

Furthermore, for the changes of Chl a, Chl b, Car and TPC contents, the degradation of pigments results in an insufficient light energy capture and further constrains photosynthetic electron transport and dark reaction efficiency. Combined with gas exchange impairment, it creates dual inhibition (insufficient light energy capture and CO_2 shortage), consistent with previous Pn findings (Shu *et al.*, 2012). After foliar application of PE-SeNPs, the incremental photosynthetic pigments could boost light energy capture and conversion efficiency, supplying more ATP and NADPH for dark reactions, collectively supporting the aforementioned Pn recovery, ultimately enhancing soybean seedlings' salt stress resistance (Abdelhameed *et al.*, 2025).

By analyzing the important indicators related to PS II, the lower qP in SS groups reflects a decrease in the opening of the PSII and the proportion of light energy allocated to photochemical reactions under salt stress, which directly correlates with insufficient light energy capture derived from reduced photosynthetic pigment content. According to current knowledge, those were able to diminish ATP and NADPH production and exacerbate the utilization limitations of dark reaction substrate (CO₂) (El-Nakhel *et al.*, 2022). The increased NPQ in the SS group indicates a relative surplus of light energy captured under salt stress, thus necessitating thermal dissipation to protect PSII. Following the foliar application, compared to the SS group, the obviously increased qP in the PSe group indicates more efficient restoration of the opening of the PSII and an enhanced allocation of light energy to photochemical reactions. Meanwhile, in the same group, the reduced NPQ hints at restoration of PSII function and enhancement of CO₂ utilization, thereby redirecting more light energy toward photochemical reactions, reducing excess light energy and improving light energy utilization efficiency. Herein, the multi-informational synthetic analysis results from the PS II, including qP, NPQ, and Fv/Fm, also verify PE-SeNPs's ability to effectively repair PSII damage and provide structural assurance for efficient photosynthetic reaction (Duan *et al.*, 2025).

In this case, the salt stress inhibits photosynthesis through a synergistic impairment of multiple pathways, including the stomatal closure, disruption of CO₂ supply-demand balance, reduce of light energy capture due to photosynthetic pigment degradation, and weakness of light-to-carbon conversion efficiency caused by PSII dysfunction, which collectively appears to be a reduction of net photosynthetic rate. Our results suggest that, after application of exogenous PE-SeNPs, the effective synergistic effects were established between restoring Gs more efficiently to optimize CO₂ supply, protecting photosynthetic pigments to enhance light energy capture, and restoring PSII function to enhance light energy conversion, which ultimately achieves a significant mitigation of salt stress of soybean seedlings, enhancing its salt tolerance.

Moreover, the increase in three antioxidant enzyme activities suggested that PE-SeNPs can significantly enhance the antioxidant capacity of soybean, and the Cr-PE545 seems to have a certain effect. According to current knowledge, salt stress leads to the production of a large amount of ROS, which causes lipid peroxidation damage in the plant membrane, and thus activates the antioxidant system (Talaat and Todorova, 2022). In this protection system, SOD is a key protective enzyme that eliminates O₂⁻, and CAT and POD convert H₂O₂ into H₂O and O₂²⁻, thereby preventing plant cells from being damaged by H₂O₂. Therefore, the activities of POD, SOD, and CAT are all reduced in soybean seedlings under salt stress, but the spraying of PE-SeNPs effectively increased the activities of POD, SOD, and CAT (Qu *et al.*, 2023). Additionally, compared to early report, in our work, the higher results of activities of POD, and CAT may further confirm that exogenous PE-SeNPs can significantly activate the antioxidant defense system of soybean seedlings (Osman *et al.*, 2021).

Previous finding has suggested that large amounts of superoxide free radicals induced by salt stress will cause membrane lipids to undergo peroxidation reactions and produce MDA, which reflects the degree of membrane lipid peroxidation in plants (Ji *et al.*, 2022). Thus, for the increase of MDA content in the other three groups compared with CK, we attributed this phenomenon to the fact that, under salt stress, the MDA content of soybean seedlings increases with the accumulation of ROS. Then, exogenous PE-SeNPs enhanced the antioxidant enzyme activity, which in turn leads to the elimination of ROS, thereby lowering the MDA content within the PSe group. From another perspective, for helping cells maintain osmotic pressure and water balance, osmotic regulatory substances such as free proline were synthesized under salt stress, the content of which can also represent the alleviation of the degree of salt stress (Spormann *et al.*, 2023). For soybean seedlings' leaves in the PSe group, the evidence of the decreased free proline validates the prediction from the alleviation of salt stress, consistent with the conclusion derived from the previous antioxidant effect analysis. Overall, these results further verify the antioxidant functions of PE-SeNPs for soybean seedlings, similar to a previous report (Desouky *et al.*, 2024). Although there are no reports on the potential toxicity of nano-selenium application, its long-term environmental safety still needs to be evaluated in the future.

Conclusions: In summary, a new type of phycoerythrin nano-selenium foliar fertilizer was prepared in this paper. In soybean seedlings, the proper application of PE-SeNPs can effectively alleviate the damage caused by salt stress and improve stress resistance by promoting photosynthesis, regulating osmotic balance and increasing the activities of POD, SOD, and CAT, thus directly or indirectly scavenging intracellular ROS and alleviating salt stress-induced damage. Those findings not only meet the demands of sustainable agriculture but also exhibit a promising application prospect in agricultural practices.

Conflict of Interest: None

Authors' Contribution Statements: Xiaodan Su: Conceptualization, Methodology, Writing - Original Draft. Xiangyu Lan: Validation, Data Curation. Changzhuo Zheng: Validation, Data Curation. Shuo Dong: Data Curation. Yang Pu: Funding Acquisition. Zhihong Tang: Writing - Review & Editing. Juan Ding: Formal Analysis, Writing - Review & Editing.

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