

Article

Selenium Nanoparticles Boost the Drought Stress Response of Soybean by Enhancing Pigment Accumulation, Oxidative Stress Management and Ultrastructural Integrity

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Abstract: Drought is a persistent and devastating obstacle to crop production, affecting both humanity and livestock. The application of selenium (Se) effectively mitigates various types of abiotic stresses and enhances crop yield under unfavorable conditions. However, our understanding of how nano-Se (nSe) alleviates drought stress (DS) in soybeans is still limited. To address this gap, our study focused on assessing the effectiveness of foliar nSe application during the reproductive stage of soybeans. Three concentrations of nSe were applied to plants grown in pots filled with clay loam soil, simulating DS conditions. Our findings reveal that nSe spraying significantly promoted the accumulation of above-ground dry biomass and enhanced relative water content (RWC) and photosynthetic pigment over alone-DS treatment. Furthermore, nSe application boosted the activity and contents of protective enzymes and osmolytes, resulting in a dose-dependent reduction in electrolyte leakage (EL), reactive oxygen species (ROS) accumulation, and malondialdehyde (MDA) content. Additionally, nSe improved stomatal characteristics and mesophyll cell ultrastructure, further mitigating the adverse effects of drought stress. These findings suggest the potential of nSe as an effective strategy to enhance soybean tolerance and potentially improve crop yields under drought conditions.

Keywords: water scarcity; nanoparticle; *Glycine max*; abiotic stress; climate change



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1. Introduction

Drought poses significant misfortune and is a persistent obstacle to crop production, as well as to the well-being of humanity and livestock alike [1]. Climate change further intensifies droughts by increasing their frequency, duration, and severity, thereby rendering the world increasingly arid and hotter [1]. China is renowned for its variable weekly, annual, and inter-annual precipitation patterns and temperature fluctuations, often leading to drought conditions. More seriously, the spatial distribution of water resources in China gradually decreases from southeast to northwest, and recently, the rainfall in the region has become significantly uneven due to global climate change [2]. Over the past decade, droughts have caused significant famine, averaging a grain loss of 39.2 billion kg and accounting for a 14.7% economic loss to GDP worldwide.

It is generally agreed that loss of turgor pressure, osmotic imbalance, and the generation of ROS promote lipid peroxidation and decrease leaf water potential in plants experiencing drought stress [3–5]. The decreased turgor pressure resulting from drought stress leads to a reduction in or cessation of growth by limiting cell extensibility and

expansion [6]. Additionally, drought stress disrupts stomatal characteristics and chloroplast organization through various mechanisms, including osmotic imbalance and the accumulation of ROS, which trigger lipid peroxidation, protein degradation, and nucleic acid damage [7].

Concurrently, drought stress alters various metabolic and physiological processes in plants, such as chlorophyll biosynthesis, nutrient and water uptake, and photosynthetic apparatus function, leading to growth arrest [4,8,9]. In maize plants, drought stress causes a decline in photosynthetic pigment content, relative water content, photosynthetic efficiency, hormonal imbalance, and ultrastructural changes in leaves, indicating a compromise in the plant's internal defense system [5,10]. In recent decades, numerous studies have been conducted to investigate drought tolerance mechanisms in plants, including osmotic stress adjustment, the activation of enzymatic and non-enzymatic antioxidant systems, and the modulation of plant hormones in various crop species [11–14]. Also, previous studies have focused on highlighting the drought tolerance mechanisms in different crop species using different techniques such as drought priming, management practices, organic and inorganic fertilizer supplementation, and phytoremediation, among others [10,14,15]. However, relatively few studies have explored the potential application of nanotechnology to understand drought tolerance mechanisms in soybean.

Selenium (Se) is an important micronutrient that plays a vital role in plant growth and development [16]. It increases photosynthetic pigments in plants [17], exhibits antioxidant activity [18], and is used in fertilizers and as a fungicide. Low doses of Se (0.5 mg kg^{-1}) amendment in soil promoted the height of rice seedlings, as well as their chlorophyll content, carbon dioxide assimilation, and antioxidant activities, and decreased the ROS level in rice tissue [19]. In addition, foliar spraying of selenate on tomato leaves increased the superoxide dismutase (SOD) activity and ascorbic acid (ASA) and glutathione contents (GSH), while the malondialdehyde content (MDA) decreased [20], suggesting the beneficial role of Se supplementation in different crop species under various abiotic stresses.

Nanotechnology has been shown to be a viable solution that both greatly increases global agricultural yields and effectively reduces the environmental impact of the current climate change scenario [21]. The use of nanoparticles (NPs) in agricultural systems is considered more sustainable, having low toxicity and being a cost-effective approach [22,23]. They can be used as fertilizers, pesticides, growth regulators, biosensors, antimicrobial agents, and plant mimics. Studies have proven that the targeted use of nanoscale micronutrients such as ZnO increased the nutrient uptake in sorghum [24]; ZnO in maize improved redox homeostasis [21]; Se in soybean enhanced the metal tolerance index [25]; and SiO₂ in rice alleviated rice blast fungus disease [26]. Recently, the supplementation of nSe protected the photosynthetic pigments and exhibited high levels of osmolytes and antioxidant activity in strawberry plants subjected to drought and salinity stress, leading to tolerance against these stresses [8,27]. But there is no study about the role of nSe on drought stress mitigation in the reproductive stage of soybean plants, making it necessary to explore the underlying mechanism.

Soybean stands as a crucial crop for global food security and sustainable development, owing to its dual functionality as a rich source of protein and oilseed. Commonly referred to as a “Miracle crop”, it boasts over 40% protein and 20% oil content. However, unfortunately, cultivated soybeans are vulnerable/sensitive to drought conditions, posing a significant challenge [28]. Despite this, our understanding of the mitigation mechanisms against drought stress in soybean plants, especially during the reproductive stage, remains limited, particularly when it comes to the application of NPs. In the current study, we applied nSe via spraying to assess its potential role in mitigating drought stress in soybeans. These findings contribute significantly to our understanding of the mechanisms that enable crop plants to tolerate drought stress, thereby advancing our knowledge in this crucial area.

2. Materials and Methods

2.1. Experimental Condition and Treatment of Plants

Seeds of soybean (cv. Huachun5) were surface sterilized using 1% NaClO and 30% ethanol for 10 min and rinsed 5 times with distilled water. The seeds were grown in pots filled with clay loam soil collected from farmland near the South China Agriculture University, Guangzhou, Guangdong, China. The soil properties were 36.3% clay, 22.8% sand, and 41.9% silt, along with 19.56 g kg⁻¹ of soil organic matter, 5.76 soil pH, 0.06 mg kg⁻¹ of selenium (Se), and available NPK of 112, 39, and 108.45 mg kg⁻¹, respectively. Each plastic pot size was 25 and 22 cm in height and diameter, respectively. Five seeds per pot were grown in a rain-protected chamber under natural light. Pots were watered daily until the reproductive stage of soybean. Prior to that, the seedlings were thinned and three uniform and healthy seedlings were left per pot. There were a total 45 pots, which were divided into 5 treatments and 9 replications. The five treatments included the control (CK), which was fully watered, drought stress (DS), and drought stress sprayed with 100 mg L⁻¹ of nSe (DS + nSe100), 150 mg L⁻¹ of nSe (DS + nSe150), and 200 mg L⁻¹ of nSe (DS + nSe200). Treatments started at the reproductive stage (R2 stage; open flower at one of the two uppermost nodes on the main stem with a fully developed flower). The nSe was sprayed twice on the corresponding pots at the beginning of DS and after 5 five days of DS imposition, and the plants were left for a further 7 days (until stage R3). The nSe was obtained from SIGMA Aldrich, Shanghai, with a average particle size of 20 nm in the solid state. The NPs were suspended in aqueous solution and the zeta potential was determined, which was −19 to 35 mV. The detailed characterization, such as EDS, XRD, and SEM, of nSe can be seen in our previous study [29]. Prior to every use, the nSe solution was thoroughly mixed and stirred in a water bath sonicator for 60 min to facilitate the suspension of NPs in the solution. Samples were collected for downstream analyses after the completion of treatments.

2.2. Measurement of Shoot Dry Matter Accumulation and Relative Water Content

To determine the dry weight accumulation, the shoot was excised at the base and subsequently dried in an oven maintained at 80 °C for three days. Following this, the dried shoot was precisely weighed using an electronic balance. On the other hand, the relative water content (RWC) was determined according to the mentioned method [30]: the highest fully expanded leaf on the main stem was weighed (FW), submerged in distilled water for 24 h (TW), and then dried at 70 °C for 72 h (DW). The RWC (%) was calculated using the following formula:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100. \quad (1)$$

2.3. Measurement of Light-Harvesting Pigment

One day before collecting samples at the R3 stage, the SPAD values were measured in the top fully expanded trifoliolate leaf using a chlorophyll meter (SPAD-502; Minolta, Tokyo, Japan). Three measurements were obtained from each plant, with a total of nine replications, and the data were then averaged. Photosynthetic pigment was determined by acetone methods [31]. For instance, 10 pieces of fresh leaf were cut and dipped in 80% acetone in a 10 mL tube and then placed overnight at room temperature in dark. The tubes were shaken several times to facilitate the removal of the green color of the leaves. When all the green color was extracted from the leaf samples, the tubes were centrifuged briefly, and measurements of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), and carotenoid contents were observed at wavelengths 470, 663, and 646 nm spectrophotometrically (model UV-2600, Shimadzu, Japan).

2.4. Observation of Stomata Aperture and Guard Cells by Scanning Electron Microscopy

For scanning electron microscopy (SEM) observation, fresh leaf samples from control and treated plants were collected and instantly fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered solution (PBS; pH 7) for 1–2 h, then post-fixed with 1.0% osmium tetroxide in the above-mentioned PBS. They were washed in PBS and dehydrated with different

ethanol concentrations (30–100%). Finally, the specimens were dried using liquid carbon dioxide and examined under the scanning electron microscope (SU8010, Hitachi, Japan).

2.5. Determination of Ultrastructural Changes by Transmission Electron Microscopy

For the analysis of ultrastructural characteristics, fresh leaf segments excluding the midrib were selected from all treatments. These segments were then incubated overnight in a solution of 2.5% glutaraldehyde diluted in PBS (100 mM, pH 7). Subsequently, the samples were rinsed three times with the same PBS. Post-fixation was achieved by immersing the samples in 1% OsO₄ for 2 h, followed by another rinse with PBS for 15 min.

To dehydrate the samples, they were exposed to a graded series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 15 min each. Subsequently, the samples were dehydrated twice with absolute acetone for 20 min each. After dehydration, the samples were embedded first in a 1:1 mixture of resin and acetone for 1 h, then in a 1:3 mixture for 3 h, and finally incubated overnight in pure resin. The prepared samples were ultra-thin sectioned using an ultra-microtome (Leica EM UC7, Germany). These sections were then stained with alkaline lead and uranyl acetate, mounted on copper grids, and observed under a transmission electron microscope (TEM, Jeol JEM-2100Plus, Japan) for ultrastructural analysis.

2.6. Quantification of Electrolyte Leakage, H₂O₂, O₂^{•−} Generation, and MDA Content

The electrolyte leakage (EL) was determined using the Dionisio-Sese and Tobita method [32]. The calculation was performed based on the following formula:

$$\text{EL\%} = (\text{EC1} - \text{EC0}) / (\text{EC2} - \text{EC0}) \times 100 \quad (2)$$

The H₂O₂ content (expressed in mol g^{−1} FW) was determined following the method described previously [33]. Initially, the leaf extract was diluted with 1 M sulfuric acid. Subsequently, NH₃ and a titanium reagent were added to the mixture. The optical density (OD) of the solution was then measured at 415 nm, as described earlier [34]. To calculate the H₂O₂ content, a standard curve was constructed using known concentrations of H₂O₂.

To assess the superoxide (O₂^{•−}) content, leaf samples were immersed in a solution containing 10 mM K-phosphate buffer (pH 7.4), 0.05% nitro blue tetrazolium chloride (NBT), and 10 mM sodium azide for 1 h. The mixture was then boiled at 85 °C for 15 min, cooled, and the OD was recorded at 580 nm, following the protocol described by [35].

The thiobarbituric acid (TBA) method was employed to measure lipid peroxidation in soybean tissue following the protocol described by Wu et al. [36] with minor modifications. Briefly, 300 mg of shoot sample was ground into a powder using a tissue homogenizer. The powdered sample was then mixed with 6 mL of 50 mM PBS (pH 7.8) and centrifuged at 12,000 × g at 4 °C for 15 min. The supernatant obtained was further mixed with 5% thiobarbituric acid (TBA) and trichloro-acetic acid (TCA), followed by incubation in a water bath for 10 min at 95 °C. The mixture was then quickly cooled on ice. Subsequently, the sample was centrifuged at 4800 × g for 10 min, and the supernatant was collected. To assess lipid peroxidation, the activity-specific and non-specific absorbance were measured at 532 nm and 600 nm wavelengths, respectively, using a UV spectrophotometer (model UV-2600, Shimadzu, Japan). The results were expressed as MDA content, a marker of lipid peroxidation.

2.7. Determination of Antioxidant Enzyme Activities

To assess antioxidant enzymatic activities, fresh shoot samples (0.3 g) were ground and homogenized in PBS (50 mM, pH 7.8) and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was then collected for further analysis.

For superoxide dismutase (SOD, EC 1.15.1.1) activity, a reaction solution containing 50 mM PBS (pH 7.8), 0.1 mM EDTA, 5.2 μM NBT, and 750 mM methionine was mixed with the supernatant and incubated for 10 min in light. The activity of SOD was measured spectrophotometrically at 560 nm, as suggested previously [36].

Peroxidase (POD, EC 1.11.1.7) activity was determined following the suggested methods [37]. Briefly, the supernatant was mixed with a reactant solution (300 mM H₂O₂, 1.5% Guaicol, 50 mM PBS) and the absorbance was recorded at 560 nm using a UV spectrophotometer.

Catalase (CAT, EC 1.11.1.6) activity was determined following the methods suggested by Wu et al. [38] by homogenizing 50 mM PBS (pH 7.8) with the supernatant and then adding 300 mM H₂O₂. The absorbance activity was measured at 240 nm for 30 s using UV spectrophotometry.

For ascorbate peroxidase (APX, EC 1.11.1.11) determination, the supernatant was mixed with a reaction solution containing PBS (50 mM, pH 7.8), H₂O₂ (300 mM), and ascorbic acid (7.5 mM), and the absorbance was recorded at 290 nm for one min using a UV spectrophotometer. On the other hand, glutathione reductase (GR, EC. 1.6.4.2) activity was measured following the previous method [38].

2.8. Measurement of Non-Enzymatic Antioxidants

The ascorbic acid (AsA) and glutathione pool (total glutathione, oxidized glutathione (GSSG), and reduced glutathione (GSH)) contents in soybean tissue were determined using the method described in [39] and [40], respectively. Briefly, 0.3 g shoot samples of soybean from all treatments were separately ground and mixed with 5 mL of 10% (*w/v*) trichloroacetic acid. The mixture was vortexed briefly and then centrifuged at 13,000× *g* for 20 min. The supernatant was collected and mixed with glutathione reductase (GR) and 5,50-dithio-bis (2-nitrobenzoic acid) (DNTB) to determine total glutathione. To measure GSSG, the enzyme extract (120 µL) was mixed with 10 µL of 2-vinylpyridine and 20 µL of 50% triethanolamine. Prior to these measurements, a standard curve was constructed using GSSG to calculate total glutathione and GSSG values. The reduced GSH level was estimated by subtracting the GSSG value from the total glutathione value.

2.9. Determination of Proline and Glycine Betaine Contents

The proline assay was performed according to the established protocol in [41]. Briefly, 200 mg of fresh shoot sample underwent homogenization in 2 mL of 3% sulfosalicylic acid solution. The homogenate was then centrifuged for 10 min at 10,000× *g*. Following centrifugation, the supernatant was discarded, and the pellet was washed twice with 3% sulfosalicylic acid solution. The proline content was determined by using the ninhydrin reagent and toluene extraction technique. The optical density (OD) of the sample was spectrophotometrically assayed at 520 nm, using toluene as the blank. The concentration was estimated by using a previously established standard curve.

The glycine betaine (GB) content was determined using the Grieve and Grattan [42] method. Briefly, 500 mg of fresh leaves was mixed with 20 mL of deionized water and agitated for 48 h at 25 °C. The mixture was centrifuged for 8 min at 12,000× *g* and 4 °C, then diluted with 2N sulfuric acid (H₂SO₄) (1:1 *v/v*). An aliquot of 500 µL was incubated on ice for 60 min, followed by the addition of 200 µL of cold potassium iodide–iodine (KI–I₂). After 16 h of storage at 4 °C, the samples were centrifuged again at 12,000× *g* and 4 °C for 12 min. The supernatant was discarded while keeping the samples on ice to separate the periodide complexes from the acid medium. The formed periodide crystals were dissolved in 9 mL of 1,2-dichloroethane and left for 2 h. The absorbance at 365 nm was measured using a UV spectrophotometer (model UV-2600, Shimadzu, Japan) and the GB content was expressed as µg g^{−1} FW.

2.10. Determination of MDHAR and GST Activities

The activities of monodehydroascorbate reductase (MDHAR) and glutathione S-transferase (GST) were assessed following the standard assay kit of Beijing Solarbio Science & Technology Co., Ltd., Beijing, China (Cat. Nos. BC0350 and BC0650, respectively).

2.11. Statistical Analysis

A factorial experiment was conducted in this study using a completely randomized design (CRD) with nine replicates. The collected data were analyzed with one-way ANOVA using R language version 4.3.1. Tukey's HSD test was applied to assess statistical significance at $p < 0.05$ among the treatments. Visualization of graphs were performed with OriginPro 2021.

3. Results

3.1. Above-Ground Dry Biomass and Relative Water Content

The above-ground biomass of soybean plants at the reproductive stage was evaluated following a different treatment (Figure 1a). Our findings indicate that, under CK conditions, the above-ground dry biomass was higher than that observed in all other treatment groups. When DS was applied, the dry biomass of stressed plants decreased significantly, with a relative decrease of approximately 65.5% compared to the CK. However, when plants were exposed to nSe at treatment concentrations of nSe100, nSe150, and nSe200, the dry biomass was significantly restored, particularly at the highest nSe concentration. Specifically, the biomass increased by 31.3%, 97.7%, and 137.8% under nSe100, nSe150, and nSe200 treatments, respectively, compared to the DS treatment.

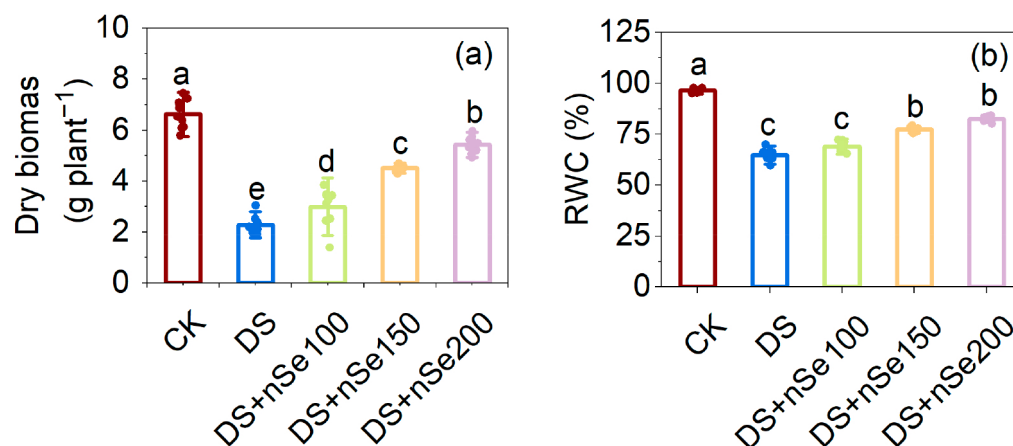


Figure 1. Effect of nSe on above-ground biomass (a) and relative water contents (b) of soybean after 12 days (R3) of drought stress imposed at R2 stage. The different treatment groups were control (CK), drought stress (DS), drought stress + 100 mg L⁻¹ nSe (DS + nSe100), drought stress + 150 mg L⁻¹ nSe (DS + nSe150), and drought stress + 200 mg L⁻¹ nSe (DS + nSe200). Different letters indicate significant differences (Tukey's test, $p \leq 0.05$).

RWC is a crucial indicator of a plant's survival capabilities and leaf water status. Our results demonstrated that, upon exposure to DS, the RWC decreased steadily compared to the CK plants (Figure 1b). For instance, the RWC of drought-stressed plants decreased to approximately 28.6% of that of CK plants. However, the spraying of nSe in a dose-dependent manner restored the RWC of drought-stressed plants. Specifically, the decreases in RWC were 28.5%, 19.8%, and 14.3% under nSe100, nSe150, and nSe200 treatments, respectively, compared to the CK plants.

3.2. Effect of nSe on Light-Harvesting Pigment Contents under Drought Stress

Upon the imposition of DS, the SPAD value rapidly declined by 59% compared to the CK treatment (Table 1). This drought-induced significant reduction in SPAD value, indicative of chlorophyll content, was partially mitigated by the foliar application of nSe treatments. When compared to plants solely exposed to DS, the decline in SPAD values was reduced to 48%, 27%, and 15% under treatments with nSe100, nSe150, and nSe200, respectively. Similarly, under DS conditions, the soybean plants grown in soils without the application of nSe exhibited a decrease in the contents of chl *a* (46%) and chl *b* (57%), as well

as carotenoids (43%), when compared to the CK plants (Table 1). However, the spraying of nSe dose-dependently led to an increase in the contents of chl *a*, chl *b*, and carotenoids of the drought-stressed plants. Specifically, the chl *a* content increased by 17%, 39%, and 57% under nSe100, nSe150, and nSe200 treatments, respectively. Similarly, the chl *b* content rose by 23%, 27%, and 34%, and carotenoid content increased by 5%, 23%, and 52% under the same concentration levels. The total chlorophyll contents (*a* + *b*) and *a/b* ratio were also higher under nSe foliar application over the DS treatment. These results indicate that the application of nSe treatments significantly attenuated the drought-induced decline in pigment contents compared to plants treated with DS alone.

Table 1. Effect of different concentrations of nSe on pigment contents under drought stress in soybean.

Treatments	SPAD	chl <i>a</i> mg g ^{−1} FW	chl <i>b</i> mg g ^{−1} FW	Car mg g ^{−1} FW	<i>a</i> + <i>b</i>	<i>a/b</i>
CK	26.11 ± 2.18 a	12.5 ± 1.16 a	3.38 ± 0.60 a	8.75 ± 0.58 a	15.88 ± 0.95 a	3.85 ± 0.02 a
DS	10.72 ± 3.71 c	6.69 ± 0.36 e	2.44 ± 0.34 b	5.01 ± 0.59 d	8.130 ± 0.43 e	2.72 ± 1.41 c
DS + nSe100	13.62 ± 3.52 c	7.84 ± 0.18 d	3.00 ± 0.08 a	5.29 ± 0.24 d	11.50 ± 0.14 d	2.41 ± 0.11 c
DS + nSe150	19.02 ± 2.77 b	9.29 ± 0.53 c	3.11 ± 0.25 a	6.14 ± 0.33 c	12.40 ± 0.33 c	3.02 ± 0.40 b
DS + nSe200	22.23 ± 2.18 ab	10.5 ± 0.27 b	3.26 ± 0.17 a	7.64 ± 0.44 b	13.54 ± 0.16 b	3.53 ± 0.28 ab

Data are means of 9 replicates (±SD). Different lowercase letters indicate significant differences between treatments based on one-way ANOVA followed by Tukey's test at *p* < 0.05.

3.3. Effect of nSe on Stomata Aperture and Morphological Traits under Drought Stress

To assess the impact of nSe foliar spray on stomatal aperture and morphological alterations under DS, we conducted SEM observations (Figure 2a–e). Our observations revealed that control plants exhibited well-apertured stomata with maintained shape. Conversely, DS drastically altered the stomatal behavior, resulting in deformed structures and a sunken, closed appearance. Additionally, DS led to a reduction in the length, width, and area of mesophyll cell stomata compared to the CK plants (Figure 2f–h). However, the spraying of nSe in drought-stressed plants facilitated stomatal aperture, organized the stomatal shape, and dose-dependently increased the length, width, and area of mesophyll cell stomata over the alone-DS treatment.

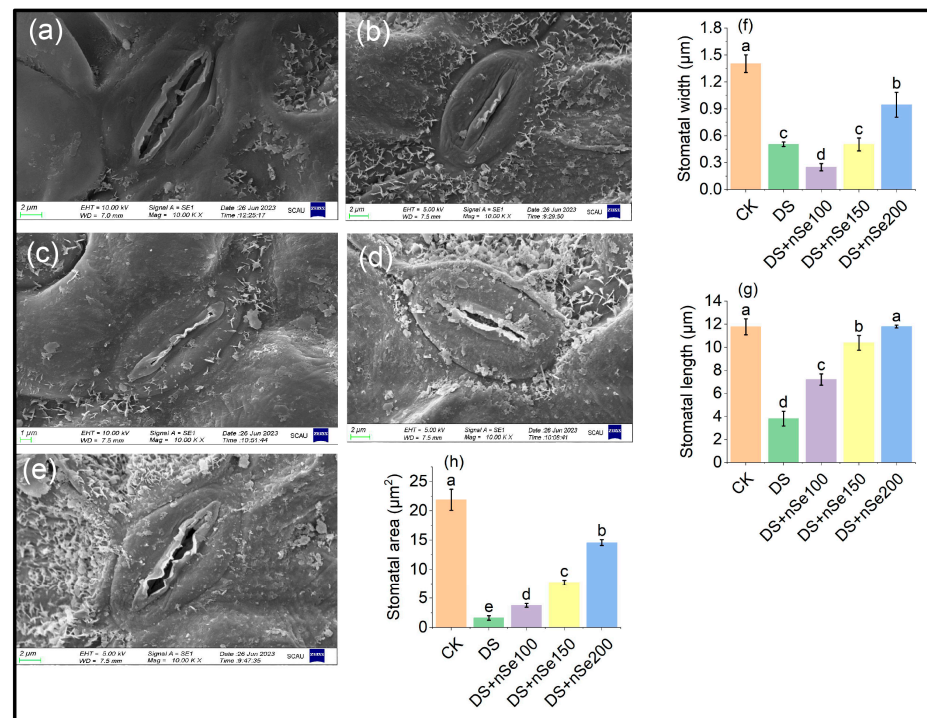


Figure 2. SEM observation of leaf epidermal surface and stomatal parameters of soybean at R3 stage under drought stress in response to different concentrations of nSe. (a) CK, (b) DS, (c) DS + nSe100,

(d) DS + nSe150, and (e) DS + nSe200. (f) stomatal width, (g) stomatal length, and (h) stomatal area. The different treatment groups were control (Ck), drought stress (DS), drought stress + 100 mg L⁻¹ nSe (DS + nSe100), drought stress + 150 mg L⁻¹ nSe (DS + nSe150), and drought stress + 200 mg L⁻¹ nSe (DS + nSe200). Different letters indicate significant differences (Tukey's test, $p \leq 0.05$).

3.4. Effect of nSe on Ultrastructural Changes under Drought Stress

Chloroplasts, the sites of chlorophyll existence, play a crucial role in plant photosynthesis. The ultrastructure of the mesophyll cell chloroplasts in drought-stressed plants is severely affected compared to CK plants (Figure 3a–e). Control plant mesophyll cell chloroplasts have a distinct shape, large starch grains (SGs), and well-organized grana (G) with large and round plastid globules (PGs), also known as osmiophilic granules. However, under drought conditions, the chloroplasts become distorted, with broken grana in the mesophyll cells. The most obvious alteration under DS is the disappearance of the plastoglobuli. However, under nSe200 treatment, the chloroplast regained its shape and was abundant with large plastid globules. Similarly, the starch granule retained its shape, and the grana were compact, resembling the control mesophyll cell.

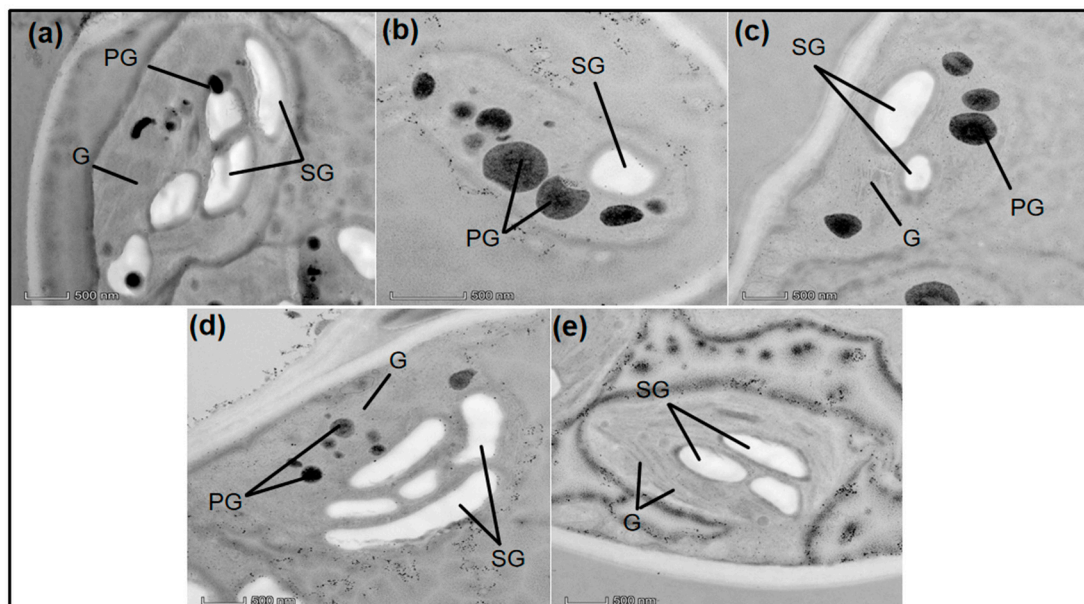


Figure 3. TEM observation showing the ultrastructural alteration of leaf chloroplast organelles of soybean at R3 stage under drought stress in response to different concentrations of nSe. (a) CK, (b) DS, (c) DS + nSe100, (d) DS + nSe150, and (e) DS + nSe200. The different treatment groups were control (Ck), drought stress (DS), drought stress + 100 mg L⁻¹ nSe (DS + nSe100), drought stress + 150 mg L⁻¹ nSe (DS + nSe150), and drought stress + 200 mg L⁻¹ nSe (DS + nSe200). SG; starch grain, PG; plastoglobuli, G; grana.

3.5. Effect of nSe Spraying on Electrolyte Leakage and ROS Accumulation under Drought Stress

Electrolyte leakage (EL) was measured in drought-stressed plants, both with and without the application of nSe (Figure 4a). The results indicated that DS caused an increase in ion leakage from leaf tissue compared to the control treatment, which was indicative of the induction of drought stress. However, the application of nSe reduced ion leakage in a dose-dependent manner in drought-stressed soybean plants. Specifically, under nSe treatment, the EL was reduced by 8%, 24%, and 35% compared to the drought treatment alone. Overall, all treatments resulted in lower EL compared to the drought treatment alone.

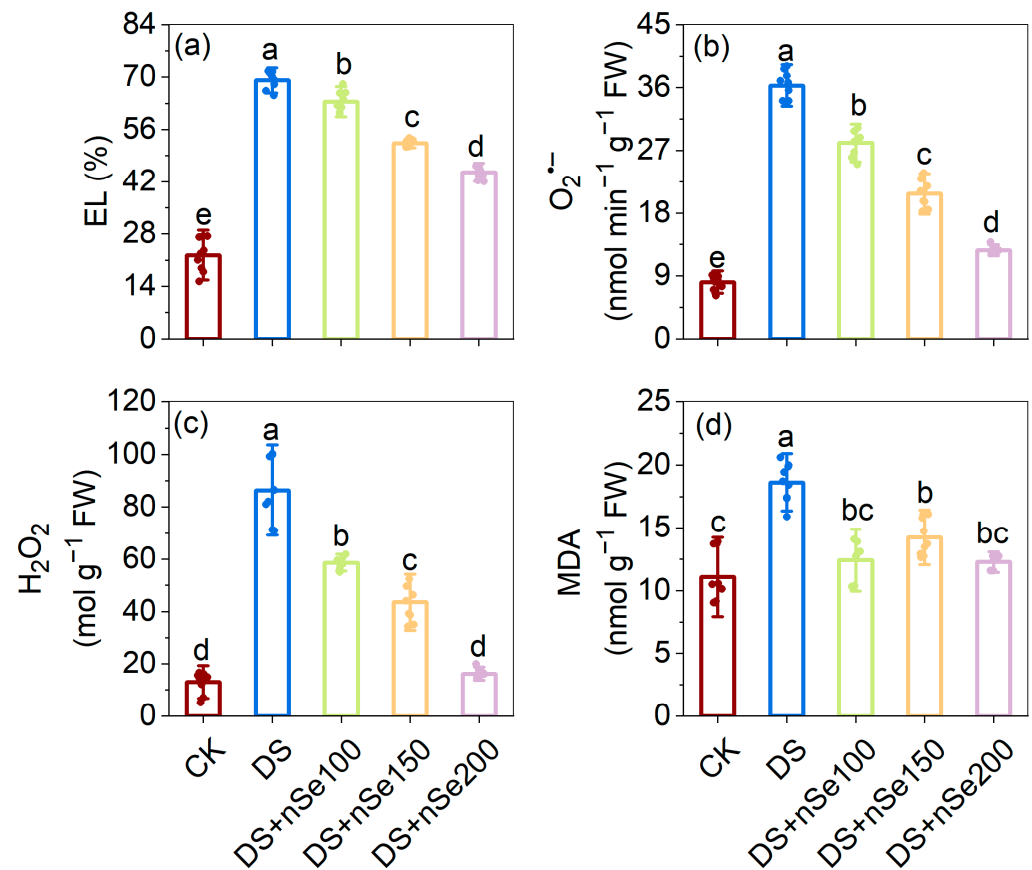


Figure 4. Effect of various concentrations of nSe on electrolyte leakage (a), superoxide content (b), hydrogen peroxide content (c), and MDA content (d) in leaves of soybean at R3 stage under drought stress. The different treatment groups were control (CK), drought stress (DS), drought stress + 100 mg L⁻¹ nSe (DS + nSe100), drought stress + 150 mg L⁻¹ nSe (DS + nSe150), and drought stress + 200 mg L⁻¹ nSe (DS + nSe200). Different letters indicate significant differences (Tukey's test, $p \leq 0.05$).

To investigate the accumulation of ROS in the shoots of soybean plants under DS treatment, and how this accumulation responds to nSe application, quantification of H_2O_2 and $O_2^{\bullet-}$ was conducted (Figure 4b,c). The results demonstrated that ROS accumulation occurred in soybean plants following the imposition of DS treatment. Notably, the DS plants exhibited significantly elevated levels of both H_2O_2 and $O_2^{\bullet-}$ compared to the CK plants. However, spraying with nSe dose-dependently reduced the contents of H_2O_2 and $O_2^{\bullet-}$ when compared to the alone-DS treatment. Notably, the foliar application of high doses of nSe treatment (200 mg L⁻¹) effectively reduced H_2O_2 accumulation to levels comparable to the CK. In addition to ROS, DS significantly induced the accumulation of MDA content (68% increased) relative to CK. However, the MDA concentration decreased under nSe application, with the highest decrease (34) observed with the highest dose of nSe treatment over the alone-DS treatment, followed by 33% and 17% decreases observed under nSe100 and nSe150, respectively (Figure 4d).

3.6. Effect of nSe Spraying on Antioxidant Enzyme Activities under Drought Stress

The results indicated that the foliar application of nSe treatments augmented the antioxidant enzyme activities of the drought-stressed plants compared to CK (Figure 5a–e). For example, DS-alone treatment slightly induced the activities of POD (68%), CAT (55%), and GR (60) compared to the CK plants. However, no significant modulation was found in the activities of SOD and APX enzymes compared to the control. Foliar application of nSe150 and nSe200 to drought-stressed plants significantly augmented the activities of these antioxidant enzymes. The high-dose nSe (nSe200) treatment on drought-stressed

plants significantly increased the activities of all these enzymes. Importantly, no significant differences in enzyme activities were observed between the DS treatment and the low-dose (100 mg L^{-1}) nSe treatment except APX activity (Figure 5a–e).

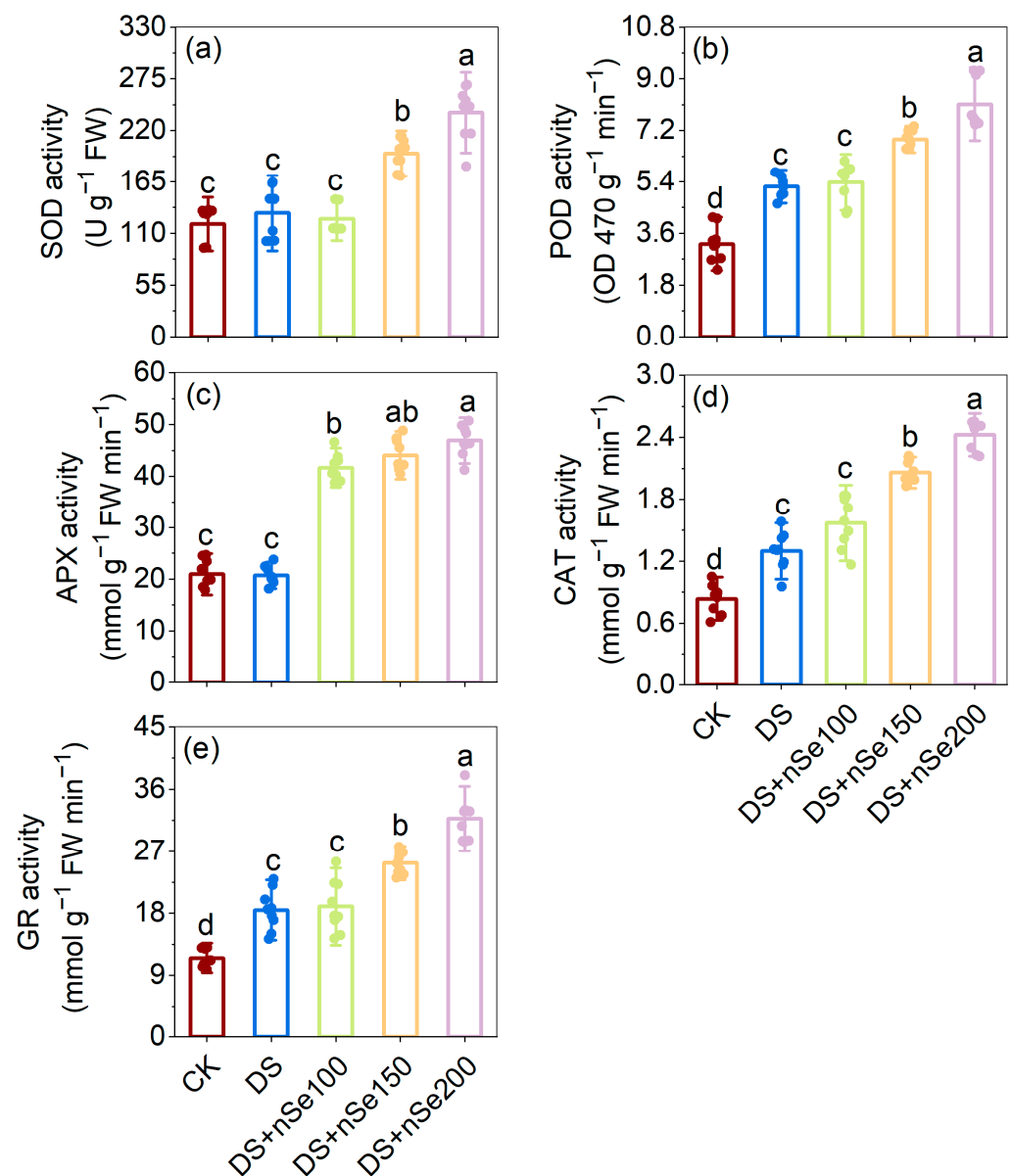


Figure 5. Effect of various concentrations of nSe on superoxide dismutase activity (a), peroxidase activity (b), ascorbate peroxidase activity (c), catalase activity (d), and glutathione reductase activity (e) in leaves of soybean at R3 stage under drought stress. The different treatment groups were control (CK), drought stress (DS), drought stress + 100 mg L^{-1} nSe (DS + nSe100), drought stress + 150 mg L^{-1} nSe (DS + nSe150), and drought stress + 200 mg L^{-1} nSe (DS + nSe200). Different letters indicate significant differences (Tukey's test, $p \leq 0.05$).

3.7. Effect of nSe on Glutathione Pool under Drought Stress

In response to drought stress, the impact of nSe treatment on the glutathione pool (GSH, GSSG, and ASA) was evaluated (Figure 6a–d). Compared to the CK, plants experiencing drought exhibited a decrease in reduced glutathione (GSH) and an increase in oxidized glutathione (GSSG) in soybean shoot tissue. Additionally, the GSH/GSSG ratio was lower under DS compared to other treatments. nSe treatment resulted in an increase in GSH content, along with the GSH/GSSG ratio, and a decrease in GSSG content compared to the drought treatment. Furthermore, ASA content increased slightly under DS compared

to the CK treatment (Figure 6d). However, spraying with nSe dose-dependently increased ASA content in soybean shoots compared to both the CK and DS treatments. Notably, a higher concentration of nSe had a more positive effect on the glutathione pool than a lower concentration in drought-stressed plants.

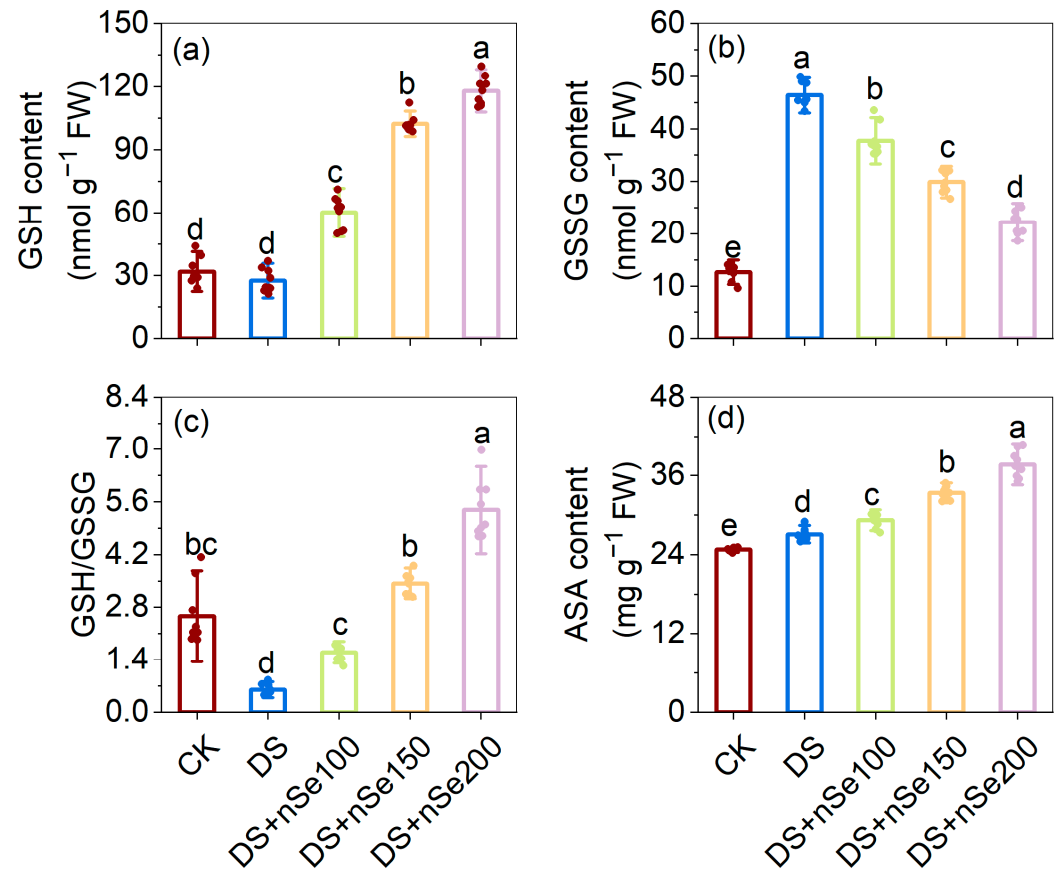


Figure 6. Effect of various concentrations of nSe on reduced glutathione content (a), oxidized glutathione content (b), reduced + oxidized glutathione content ratio (c), and ascorbic acid content (d) in leaves of soybean at R3 stage under drought stress. The different treatment groups were control (CK), drought stress (DS), drought stress + 100 mg L⁻¹ nSe (DS + nSe100), drought stress + 150 mg L⁻¹ nSe (DS + nSe150), and drought stress + 200 mg L⁻¹ nSe (DS + nSe200). Different letters indicate significant differences (Tukey's test, $p \leq 0.05$).

3.8. Effect of nSe on GST and MDHAR Enzyme Activities under Drought Stress

After drought stress and nSe treatments, both GST and MDHAR enzyme activities exhibited varying degrees of regulation. Specifically, DS slightly elevated the activities of these enzymes compared to the CK treatment (Figure 7a,b). In contrast, the spraying of nSe resulted in higher activities of both GST and MDHAR enzymes compared to both DS and CK. Notably, the highest concentration of nSe (200 mg L⁻¹) resulted in significantly higher activities of these enzymes compared to low concentrations.

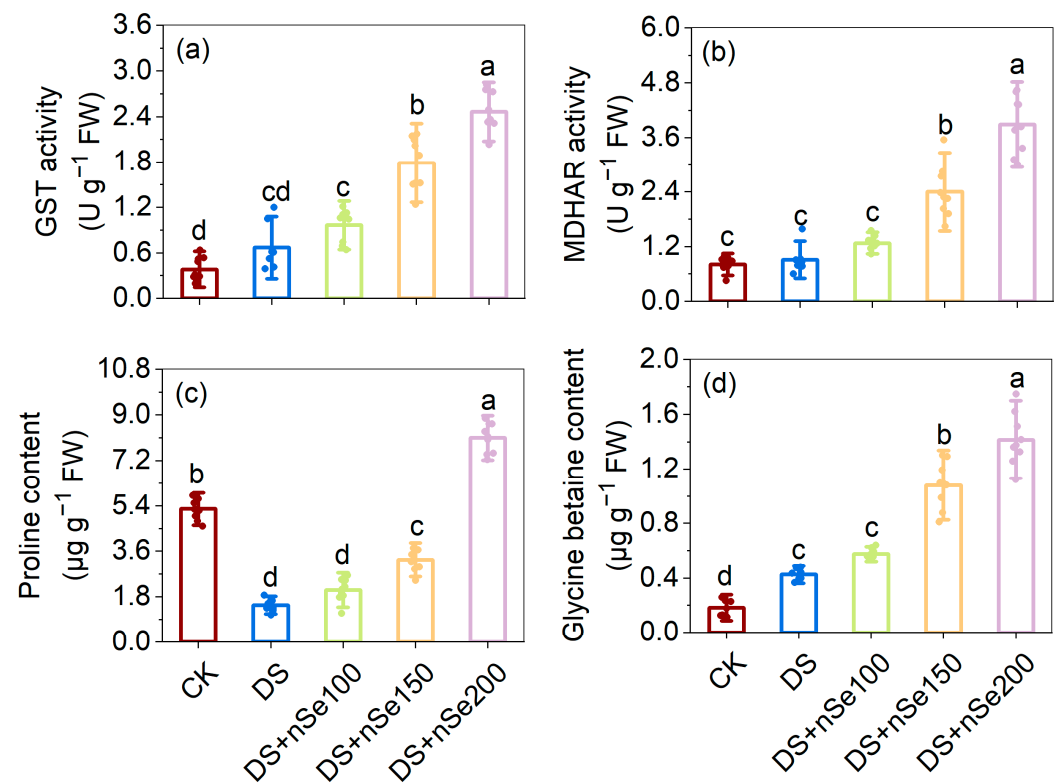


Figure 7. Effect of various concentrations of nSe on glutathione S-transferase activity (a), monodehydroascorbate reductase activity (b), proline content (c), and glycine betaine content (d) in leaves of soybean at R3 stage under drought stress. The different treatment groups were control (CK), drought stress (DS), drought stress + 100 mg L⁻¹ nSe (DS + nSe100), drought stress + 150 mg L⁻¹ nSe (DS + nSe150), and drought stress + 200 mg L⁻¹ nSe (DS + nSe200). Different letters indicate significant differences (Tukey's test, $p \leq 0.05$).

3.9. Effect of nSe on Proline and GB Contents under Drought Stress

The proline content was drastically reduced under drought treatment, with a decrease of up to 72% compared to the CK (Figure 7c). Similarly, exposure to nSe100 and nSe200 nSe concentrations resulted in proline reductions of 53% and 38%, respectively. However, the high concentration of nSe (200 mg L⁻¹) surprisingly led to a notable increase in proline content by 53% relative to the CK plants. Conversely, glycine betaine (GB) content positively responded to both DS and nSe treatments, with the exception of the nSe100 concentration, where the difference was not statistically significant from alone-DS treatment (Figure 7d). Notably, the highest increase in GB content was observed with nSe200 application.

3.10. Principal Component Analysis and Pearson Correlation

We further performed principal component analysis (PCA) to visualize the relationships between different physiological and biochemical parameters, as well as the effects of various NP applications on these parameters under DS conditions (Figure 8a). The PCA biplot results indicated that the first principal component (PC1) explained 53.7% of the variance, while the second principal component (PC2) explained 36.3% of the variance, together explaining 90% of the total variance in the data. The vectors for oxidative stress markers, such as H₂O₂, O₂^{•-}, MDA, GSSG, and EL, exhibited a negative correlation with NP application in response to drought treatment. These indicated potential membrane damage and impaired cellular integrity in the drought-treated plants. Conversely, the vectors for antioxidant enzymes, including SOD, POD, CAT, APX, GR, and osmolytes such as proline and GB, showed a positive correlation with NP treatments, particularly the 200-NP group. These observations indicated that NP exposure reduced oxidative stress and promoted the antioxidant defense system in the drought-treated plants.

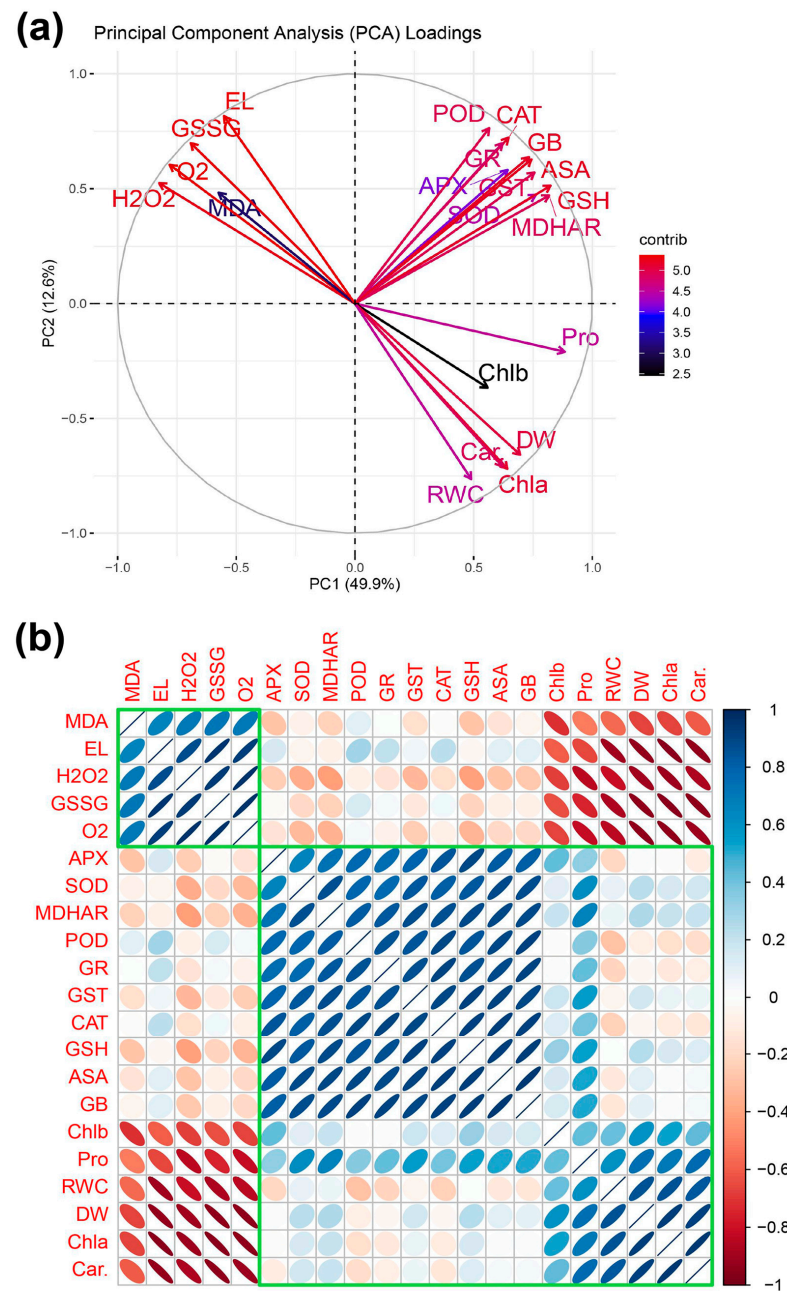


Figure 8. Loading plot for the first two principal components in the principal component analysis (PCA) for 21 measured traits of soybean tissue (a). Pearson's correlation analysis of the measured parameters under drought stress in response to foliar application of nSe treatment (b). Color scale from light green to dark red shows negative correlation, while green to dark blue shows positive correlation.

Pearson's correlation analysis was also performed to assess the relationship between physiological and biochemical parameters under DS and nSe treatments (Figure 8). The results demonstrated a positive correlation among the antioxidants CAT, SOD, POD, APX, GR, and GSH. These parameters also showed a positive correlation with proline, ASA, GB, GST, and MDHAR, while exhibiting a negative correlation with H_2O_2 , $O_2^{\bullet-}$, MDA, and GSSG (Figure 8b). Furthermore, DW, chl *a*, chl *b*, carotenoids, and RWC were negatively correlated with oxidative stress biomarkers.

4. Discussion

Given the current scenario of climate change and the global surge in demand for agricultural productivity, it is necessary and urgent to intensify the search for a climate-smart remediation approach that can address water scarcity. A number of studies have explored the effect of NPs on abiotic stress tolerance in plants, with the majority focusing primarily on the seedling growth stage. However, crop plants are more susceptible to drought stress during the reproductive and seed filling stages [15]. Additionally, while most studies have focused on the function of nSe in reducing the accumulation and toxicity of heavy metals in plants, there is insufficient research on the function and mechanism of nSe in soybean plants under drought stress at the reproductive stage. Previously, it was observed that the exogenous application of elemental selenium (Se) reduced drought stress in tomato plants by promoting growth and photosynthetic efficiency, while mitigating oxidative stress biomarkers and maintaining plasma-membrane integrity [43]. This finding indicated a positive role for Se in drought stress alleviation. In the present study, we employed nSe instead of elemental Se to investigate its impact on drought stress alleviation. To the best of our knowledge, this is the first study to apply nSe for mitigating drought stress during the reproductive stage of soybean.

It has been observed that drought stress has a negative impact on dry biomass by hindering cell elongation and cell division [7], regarded as the primary indicators of the induction of abiotic factors [44]. Consequently, this leads to a decrease in the length, height, and surface area of plant tissue exposed to drought conditions [45,46]. In this study, without the foliar spraying of nSe, DS resulted in a significant decrease in these traits due to the degradation of chloroplast pigments, thus disturbing the photosynthetic processes [47], resulting in growth and biomass reduction [21]. Furthermore, the enzyme chlorophyllase might have played a role in inhibiting or depriving chlorophyll synthesis and stimulating chlorophyll degradation, as suggested by study conducted in [48]. Additionally, carotenoids not only fulfill structural functions but also participate in non-enzymatic antioxidant activities, leading to ROS depletion, and play a crucial role in safeguarding plants from lipid peroxidation [49]. It can also be observed that there was a significant negative correlation between photosynthetic pigments and oxidative stress biomarkers (Figure 8). In contrast, the photosynthetic pigments such as chl *a*, chl *b*, and carotenoids, which are crucial for plant growth as they facilitate the synthesis of carbohydrates via photosynthesis, were attenuated by nSe spraying. In our previous study, nSe, both alone and in combination with ZnONPs, had a substantial effect on the chlorophyll content in soybean plants exposed to arsenic stress, leading to enhanced photosynthetic efficiency and dry biomass [29]. Consistent with these findings, our results suggest that Se has a beneficial role in enhancing drought tolerance in soybean plants, potentially through its ability to support photosynthesis and maintain cellular integrity, resulting in an increase in dry biomass. Taken together, the increase in light-harvesting and accessory pigments caused by nSe can be attributed to several factors, which include enhanced enzymatic activities, such as rubisco, chloroplast stabilization, increases in the uptake of essential metals involved in chlorophyll biosynthesis such as Fe and Zn, and the promotion of reduction processes in the biosynthesis pathway of photosynthetic pigments due to the low redox potential because of NP treatment.

Stomatal control serves as a crucial physiological factor for optimizing water utilization under drought conditions. Our SEM analysis revealed that DS significantly impairs stomatal aperture, evidenced by the shrinkage of guard cells along with a decrease in other stomatal parameters, such as stomatal length, width, and area, compared to nSe and CK treatments. Such changes in stomatal aperture in maize leaves under drought stress have been previously reported by Ahmad et al. [10] and were considered a primary response of plants to drought stress.

Furthermore, these alterations may stem from the accumulation of ROS and lipid peroxidation, leading to oxidative damage to cellular structures and functions upon exposure to drought stress. This damage deforms the stomatal aperture and compromises the integrity of guard cells [50]. Prior studies have established a link between alterations in

stomatal aperture, guard cell disruption, and the accumulation of oxidative stress biomarkers in maize [21] and *Arabidopsis* [51]. In other words, these findings are likely attributed to the accumulation of abscisic acid (ABA) under drought stress, which promotes stomatal closure to prevent water loss through leaf evapotranspiration [50]. In contrast, foliar application of nSe to soybean plants enhanced stomatal characteristics, as clearly reflected in the improved stomatal length, width, and area, as well as the health of guard cells. In summary, the observed increase in the RWC and dry biomass of soybean plants can be directly attributed to the improved stomatal traits. Similar phenomena have been observed in maize plants treated with ZnO-NPs in [21].

In addition to chlorophyll depletion under DS, the ultrastructural modifications in the chloroplast highlight the profound disturbances in metabolic functions and lipid composition caused by drought stress compared to CK conditions. Anjum et al. [52] reported that drought stress leads to the accumulation of lipid peroxidation, resulting in significant disorganization of chloroplasts and a subsequent depletion of chlorophyll content in maize. This disruption was further exacerbated by the increase in the number and size of plastoglobuli, which are degradation products responding to drought stress, causing degradation of grana thylakoids [53]. The observed reduction in cell size and narrowing of intercellular spaces clearly indicate that drought has a profound impact on leaf expansion. Another reason for chloroplast alteration maybe the osmotic imbalance generated by DS between stroma and chloroplasts [10]. This can be attributed to the deleterious effects of drought on turgor potential and cell wall plasticity. Previous studies have also reported a decline in chlorophyll content, attributed to both the denaturation of chlorophyll molecules and the inhibition of chlorophyll biosynthesis due to chloroplast membrane disruption [54].

Interestingly, foliar spraying with various concentrations of nSe effectively repaired the alterations in leaf mesophyll cell chloroplasts induced by DS. Specifically, under a high dose of nSe (200 mg L^{-1}), the chloroplast maintained its original shape, exhibiting large starch grains and fewer plastoglobuli, indicating relief from DS. The high content of chlorophyll and carotenoids observed under nSe application can be associated with the recovery of chloroplast function under drought conditions. In a similar context, Salam et al. [21] reported that ZnONPs improved the chloroplast shape and enlarged the size of starch grains in maize, implying a reduction in cobalt (Co) stress, which they linked with depletion of ROS in response to NP application. In accordance with our observation, a recent finding reported that nSiO repaired the leaf cell structural changes in wheat [55] induced by stress conditions.

Drought stress heavily perturbs ion distribution and homeostasis within the plant cell, inducing oxidative stress markers such as EL, $\text{O}_2^{\bullet-}$, H_2O_2 , singlet oxygen (O_2), and lipid peroxidation in plants. The accumulation of $\text{O}_2^{\bullet-}$, H_2O_2 , and O_2 can provoke a partial or severe oxidation of cellular components, inducing redox status changes, leading to damage to nucleic acid, lipids, and proteins [56], so continuous control of ROS and of their metabolism is decisive under stress conditions [57,58]. In this study, we reported a significant increase in EL and high generation of H_2O_2 and $\text{O}_2^{\bullet-}$ in drought-stressed plants, indicating that soybean plants are indeed susceptible to oxidative damage, evident from the elevated levels of MDA content. It is crucial to maintain ROS accumulation below a certain threshold to prevent lipid peroxidation, as otherwise this can subsequently result in EL [59].

In contrast, the concentration-dependent reduction in oxidative stress markers after nSe application suggests a positive role for nSe spraying in drought tolerance. These responses may be attributed to the high levels of ROS scavengers, including enzymatic and non-enzymatic antioxidants involved in the Asada–Halliwell pathway [60]. Similarly, declines in oxidative stress and lipid peroxidation and improvements in membrane stability and integrity by nSe and/or elemental Se application in *B. juncea* [60], soybean [29], and strawberry [27,61] have been established under different abiotic stressors.

The accumulation of osmolytes, particularly free proline, is a widespread phenomenon observed in plants under abiotic stress conditions [62], including drought [63]. Proline is a

well-known osmoprotectant usually regulated under stress conditions, and plays a vital role in ROS scavenging, as well as stabilizing proteins and the cell membrane [64]. In this study, the application of high concentrations of nSe spraying resulted in increased proline levels compared to DS alone. This elevation in proline potentially contributed to improved hydration status in the nSe-treated plants, evident from their higher RWC (Figure 1b). The higher proline content in the nSe-treated plants contributed to a lower osmotic potential, which, in turn, increased turgor pressure, leading to higher water potentials and improved water-holding capacity [57]. Ahmed et al. [63] also established the role of proline and GB in osmotic adjustment leading to the normalization of turgor pressure under drought and salinity stress in barley plants.

Moreover, DS caused a marked increase in GB content in plants treated with nSe compared to other treatments. The enhanced GB levels might have protected enzyme activity, particularly enzymes involved in sugar and amino acid metabolism [65], resulting in increased proline levels in the nSe-treated plants. Based on these findings, it is speculated that the soybean plants treated with nSe might have acquired better protection against DS compared to untreated plants, due to the elevated levels of GB and the osmotic protection provided by the higher proline content. Taken together, these increased levels of free proline and GB contributed to osmotic adjustment in the plants.

The ROS generated by abiotic stress in plants are primarily countered by the antioxidative defense system. This system initiates with SOD, which catalyzes the dismutation of $O_2^{\bullet-}$, converting it into H_2O_2 . The formed H_2O_2 is then reduced to H_2O either by the activity of CAT or through the Asada–Halliwell pathway (ASA–GSH antioxidant) along with POD and APX enzymes [61,65,66]. GSH and ASA act as reductants in these reactions, ultimately becoming oxidized themselves. In this study, a slightly increased activity of antioxidant enzymes such as SOD, CAT, GPX, POD, and APX, along with improved contents of ASA–GSH, play a beneficial role in counteracting oxidative stress under DS conditions alone. However, to accelerate the efficiency of the antioxidant defense system of crop plants under stressful conditions, they need exogenous boosters, as these defense systems can be overwhelmed and alone may be unable to maintain a balance between the generation and removal of ROS, potentially leading to oxidative injuries within plant cells [67]. Therefore, upon foliar application of nSe, the activities and content of enzymatic and non-enzymatic antioxidant, respectively, significantly increased dose-dependently, suggesting more efficient protection against oxidative toxicity induced by drought conditions.

In addition, the enhanced contents of GST along with the increased activity of MDHAR observed in soybean plants may contribute to a higher level of adaptation to drought stress when nSe is present compared to plants without nSe treatment. This is indicated by the lower accumulation of MDA in plants treated with nSe compared to those without nSe under drought stress conditions. The increased activity of APX and CAT upon spraying nano-Se and SiO by detoxifying H_2O_2 was also observed in strawberry under drought stress [61]. Mittler [68] found that NP application augmented ROS-dependent signaling pathways, which drive plant growth through facilitating chloroplast functionality and antioxidant activity. A similar protection mechanism against arsenic toxicity was reported by Farooq et al. [69] by imposing Se-NPs to suppress the oxidative stress toxicity in *B. napus*. Studies have shown that properties of nano-Se, such as antioxidant and biological activities, are inversely/directly proportional to the particle size and surface area to volume ratio of NPs and so are depend on plant species, duration of exposure, stress type, and methods of application [21,70–72]. Overall, this study presents compelling evidence that nSe serves as a significant strategic tool for mitigating drought stress in soybean plants.

5. Conclusions

In conclusion, this study demonstrates that nSe, through foliar treatment, can significantly enhance soybean resistance to DS in a dose-dependent manner, thereby stimulating plant tolerance. The application of nSe significantly reduced the adverse effects of DS by promoting photosynthetic pigments and dry biomass accumulation, as well as increasing

RWC and antioxidant defense mechanisms. This ultimately led to reduced oxidative stress, contributing to an integrated approach for enhancing soybean tolerance to drought. The application of nSe also led to enhanced stomatal characteristics, guard cell health, and chloroplast ultrastructure. These findings have further deepened our understanding of nanotechnology application in agriculture, particularly in regions prone to drought-like weather conditions. Future studies could explore the underlying molecular mechanisms of how nSe interacts with plant cells to enhance drought tolerance, as well as investigate the potential of nSe in other crop species facing similar challenges.

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