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Does Sodium Nitroprusside Alleviate Water Deficit Stress in *Impatiens walleriana* Shoots Grown In Vitro?

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Abstract: *Impatiens walleriana* is a decorative horticultural plant species. Commercial production requires that the plants be brought to market, often accompanied by reduced water content during transport. Drought significantly affects metabolic processes in plants. The effects of polyethylene glycol (PEG)-induced water deficit on shoots of *I. walleriana* were investigated using different mechanisms at the biochemical level. In addition, the potential water deficit-ameliorating effect of sodium nitroprusside (SNP) was tested. Shoots of *I. walleriana* were initially grown on MS media supplemented with SNP (50, 100 and 250 μM). After pre-treatments, shoots were further transferred to media supplemented with PEG_{8000} (3%) and/or SNP (50, 100 and 250 μM). Water deficit conditions increased proline, photosynthetic pigments, malondialdehyde (MDA), H_2O_2, total phenolic content and antioxidant activity. In addition, PEG-induced water deficit increased superoxide dismutase (SOD) and peroxidase (POX) activities but decreased catalase (CAT) activity. SNP did not significantly affect photosynthetic pigments and total phenolic content but increased proline accumulation, MDA and H_2O_2 content, especially when applied simultaneously with PEG. Moreover, none of the investigated SNP pretreatments significantly altered the activities of SOD, POX, and CAT in *I. walleriana*. The results indicate that exogenous application of SNP effectively alleviated water deficit stress in shoots of *I. walleriana* grown in vitro.

Keywords: impatiens; drought stress; polyethylene glycol; sodim nitroprusside; reactive oxygen species; antioxidant enzymes; proline; polyphenols; photosynthetic pigments

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1. Introduction

Under natural conditions, drought implies the lack of water in the substrate caused by insufficient atmospheric precipitation or an intense plant transpiration process [1]. Drought is an abiotic stressor that negatively affects all developmental stages of the plant organism, including tissue dehydration, inhibition of cell elongation, differentiation and division, and reduction of basic physiological and metabolic processes, causing visible morphological changes [2]. The sensitivity of plant species to drought depends on their genotype, ontogenetic stage, additional stress-inducing factors, and the intensity, frequency, and duration of drought [3]. To increase drought resistance, plants respond through a complex network of interactions at all levels of organization. The accumulation of osmoprotectants, proteins with a protective role, and various products of secondary metabolism underlies plants tolerance to drought [4]. The effects of numerous stress factors, including drought, lead to increased production of reactive oxygen species (ROS) and disruption of cellular homeostasis [5]. In terms of cell membrane structure and function, lipid peroxidation is one of the best-studied markers of ROS action [6]. The balance between non-enzymatic and enzymatic antioxidants is crucial for the elimination of ROS. Non-enzymatic antioxidants such as soluble sugars, alcohols, glycine-betaine and organic acids, proline and

phenolic compounds play an important role in osmoprotection under various abiotic stresses [7–9]. As antioxidants, proline and phenolic compounds can eliminate free radicals or prevent the production of ROS [10,11]. Superoxide dismutases (SOD), peroxidases (POX), ascorbate peroxidases (APX), catalases (CAT), dehydroascorbate reductases (DHAR), monodehydroascorbate reductases (MDHAR) and glutathione reductases (GR) represent the main classes of enzymatic antioxidants [12].

Nitric oxide (NO), formally known as nitric monoxide, has long been recognised as a multifunctional signalling molecule in many physiological and pathological processes in animal and plant organisms. Because NO is a colourless, gaseous compound, with a half-life of only a few seconds, it does not require a carrier to cross the plasma membrane and diffuse rapidly to intracellular targets [13]. Recently, the role of NO in numerous developmental processes, including seed germination, root formation, gravitropism, mitochondrial functionality, photosynthesis, senescence, stomatal movements, and plant maturation, has been investigated [14]. In addition, NO participates in mitigating various abiotic stresses such as drought, salt, heat, UV light and metal ions [15]. The most used NO donor is sodium nitroprusside (SNP). This sodium salt comprises ferrous iron complexed with nitric oxide and five cyanide ions. Since NO is a gaseous molecule and direct exposure is technically difficult, NO donors that form transition metal-NO complexes are usually applied exogenously in vitro. The application of SNP improved photosynthetic rate membrane stability, reduced malondialdehyde (MDA) and H_2O_2 content, increased proline accumulation, and improved activity of antioxidant enzymes in drought-stressed plants [16]. Since SNP is a water-soluble salt, it is readily taken up by plant roots. As a NO donor, SNP can potentially improve plant tolerance both in vitro and ex vitro. Many studies also suggest that the application of SNP in the field/greenhouse could improve plant growth and development [17–19].

The genus *Impatiens* (Balsaminaceae) encompasses one of the largest genera of flowering plants [20]. The best-known plant species of this genus is *Impatiens walleriana*, a herbaceous annual plant with fleshy, succulent leaves and various coloured flowers [21]. Due to its decorative properties and long flowering period, from early spring to late autumn, *I. walleriana* is considered a very popular horticultural species worldwide [22]. Lack of water leads to a rapid decrease in cell turgor and tissue dehydration. Accordingly, insufficient water supply in the substrate during cultivation and especially during transport may have an unfavourable effect on growth and development and the decorative properties of *I. walleriana*.

Polyethylene glycol (PEG) with higher molecular weight (4000–8000) is a non-phytotoxic and non-penetrating, non-ionic water-soluble polymer that lowers the osmotic potential of nutrient solutions [23,24]. PEG stimulates water deficit, so it is very suitable for inducing irrigation limitation simulating the drought under controlled experimental conditions [25,26]. To our knowledge, only a few reports describe PEG-induced drought stress in two species of the genus *Impatiens*, two conducted under ex vitro and one under in vitro conditions. The first report described that PEG-induced drought stress altered the morphology and nutrient concentration of hydroponically grown *I. walleriana* plants [27], while the other report described the effect of PEG-induced drought stress on the germination rate, root length, enzymatic activity, and MDA content of *I. balsamina* [28]. The responses of *I. walleriana* shoots grown under PEG-induced physiological drought in vitro and the possible effect of exogenous salicylic acid as a stress-ameliorating agent have been described only recently [21].

The alleviating role of SNP in water deficit has also been investigated in numerous plant species [29]. Considering that the growth and development of *I. walleriana* is highly dependent on water presence in the substrate, the use of SNP under PEG-induced water deficit stress in *I. walleriana* has been investigated for the first time. Therefore, in the present work, the effect of SNP on the potential mitigation effect of PEG-induced water deficit stress conditions in *I. walleriana* shoots grown in vitro was presented at the biochemical level.

2. Materials and Methods

2.1. Plant Material, Growth Media, Culture Conditions and Treatments Pattern

Shoot explants used as primary plant material in this work, derived from mother stock cultures of *I. walleriana* (cultivar "Xtreme Scarlet") plants, were cultured on Murashige and Skoog (MS) medium [30] containing 30 gL^{-1} sucrose, 100 mgL^{-1} myo-inositol and 7 gL^{-1} agar. *I. walleriana* shoot explants (~2 cm long, Figure 1) were grown on MS medium without plant growth regulators or MS media supplemented with SNP at different concentrations (50, 100 and 250 µM) for three weeks. After these pretreatments, *I. walleriana* shoots were further transferred to fresh MS nutrient media supplemented with PEG_{8000} (3%) and/or SNP (at the respective concentrations) for one-week treatment (Figure 1). All media tested were adjusted to pH 5.8 with NaOH/HCl and autoclaved at 121 °C for 25 min. In vitro cultures were grown at 25 ± 2 °C and under a long-day photoperiod (16/8 h light/dark) provided by "Tesla" white fluorescent lamps (65 W, 4500 K), with a photosynthetic photon flux density of 47 µmol s^{-1} m^{-2}.

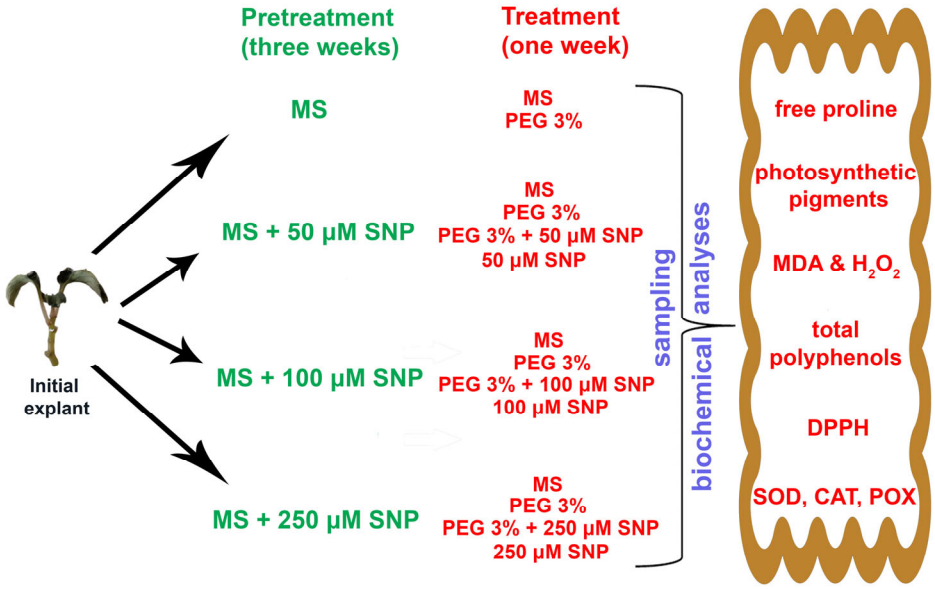

Figure 1. Experimental design including different pre-treatments and treatment patterns.

2.2. Analyses of Biochemical Parameters during Different SNP Treatments

For all biochemical analyses (free proline content, total polyphenol content and antioxidant activity, photosynthetic pigments, MDA and H_2O_2 content, as well as the activity of antioxidative enzymes), leaves with petioles were collected from in vitro grown *I. walleriana* shoots after a total of four weeks of experimentation. All plant material was deep frozen (−80 °C) before analyses.

2.2.1. Protective Metabolite: Free Proline Content

Free proline content was determined by ninhydrin (2,2-dihydroxyindane-1,3-dione) reaction according to the modified method of Carillo and Gibon [31]. Shoots of *I. walleriana* (250 mg) were homogenised in liquid nitrogen, and methanol extraction of free amino acids was carried out. After centrifugation, the supernatant was mixed with Chloroform and water. The aqueous phase was re-extracted with Chloroform, separated, and evaporated to dryness. The samples were resuspended in water, and the plant sample (or proline standard) was mixed with ninhydrin reagent. The samples were further incubated in a water bath at 100 °C, then cooled and diluted in ethanol. The absorbance of the yellow reaction product was measured at 350 nm. Background absorbance was corrected with reactions using ethanol instead of ninhydrin, in parallel for each sample as a blank. Proline content was determined from the standard curve. Results are expressed as µM/g

of sample fresh weight. Spectrophotometric determination of free proline was performed using an ELISA Micro Plate Reader (LKB 5060–006, Winooski, VT, USA).

2.2.2. Oxidative Damage: Photosynthetic Pigments, MDA and H_2O_2 Content

Photosynthetic pigment content was determined using 96% ethanol, as proposed by Lichtenthaler [32]. *I. walleriana* leaves (20 mg) were immersed and heated in a water bath at 70 °C. After incubation, the absorbance of photosynthetic pigments (at 470, 648, and 664 nm) was measured using a UV–visible spectrophotometer (Agilent 8453, Life Sciences, Santa Clara, CA, USA). Results are expressed as mg/g of sample fresh weight.

The level of lipid peroxidation was measured through MDA content according to the procedure described by Heath and Packer [33]. Shoots of *I. walleriana* (100 mg) were homogenised in liquid nitrogen. Extraction was performed with 0.1% triChloroacetic acid (TCA). The obtained homogenate was centrifuged at 4 °C for 10 min, and 20% TCA and 0.5% 2-thiobarbituric acid (TBA) were added to the supernatant. The reaction mixture was first heated at 95 °C for 30 min in a water bath and immediately cooled on ice. After centrifugation at 4 °C for 10 min, the absorbance of the supernatant was measured at 532 and 600 nm. Results are expressed as μM/g of sample fresh weight.

The content of H_2O_2 was determined according to Velikova et al. [34]. As for MDA determination, shoots of *I. walleriana* (150 mg) were homogenised in liquid nitrogen and extracted with 0.1% TCA. The homogenate was centrifuged at 4 °C for 15 min. The supernatant obtained was mixed with 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI. The absorbance of H_2O_2 was measured at 390 nm, and the results are expressed as μM/g of sample fresh weight. Spectrophotometric determination of MDA and H_2O_2 was performed using an ELISA Micro Plate Reader (LKB 5060–006, Winooski, VT, USA).

2.2.3. Nonenzymatic Antioxidants: Total Polyphenol Content and Antioxidant Activity of Secondary Metabolites

Total polyphenol content was determined using the Folin-Ciocalteu reagent (FC test) based on the method of Singleton et al. [35]. Shoots of *I. walleriana* (200 mg) were homogenised in liquid nitrogen and extracted in 96% ethanol. The extracts were further incubated at room temperature for 60 min and then centrifuged for 15 min. The obtained supernatant was further incubated together with FC reagent at room temperature in the dark. After 90 min, the blue-colored complex was quantified spectrophotometrically at 765 nm. Gallic acid (GA) was used as a standard phenol.

Antioxidant activity based on the concentration of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) radical was determined in *I. walleriana* shoots prepared the same way as for the FC test. The free radical scavenging capacity was determined by the DPPH assay described previously [36,37]. The reaction mixture containing the DPPH reagent solution was prepared using methanol and the supernatant of the sample. After 60 min at room temperature and under dark conditions, the degree of DPPH radical reduction was determined by measuring the absorbance at 520 nm.

Total polyphenol content (expressed as mM GA/g of sample fresh weight) and antioxidant activity (expressed as % of DPPH reduction) were described in detail by Đurić et al. [38]. Spectrophotometric determination of non-enzymatic antioxidants was performed using an ELISA Micro Plate Reader (LKB 5060–006, Winooski, VT, USA).

2.2.4. Enzymatic Antioxidants: SOD, CAT and POX Activity

Total soluble protein extracts for all enzyme assays were prepared by grinding the leaves (1 g) in liquid nitrogen and homogenising in extraction buffer containing 50 mM Tris pH 8, 1 mM EDTA, 30% glycerol, 1.5% polyvinylpolypyrrolidone, 10 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were centrifuged for 5 min to spin down the solids and then stored at −80 °C until use. According to Bradford, protein concentration was determined from a standard curve made using bovine serum albumin solution [39].

SOD activity was determined spectrophotometrically using a slightly modified method by Beyer and Fridowich [40]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM ethylenediaminetetraacetic acid, 260 mM methionine, 1.5 mM nitroblue tetrazolium Chloride (NBT) and 0.04 mM riboflavin. For each sample, six dilutions were prepared. The reaction mixture was pre-illuminated for 2 min, and then SOD activity was determined spectrophotometrically at 540 nm, using an ELISA Micro Plate Reader (LKB 5060–006, Winooski, VT, USA).

The spectrophotometric assay for CAT activity was carried out by the method described by Aebi [41]. While POX activity was determined according to Kukavica and Veljović-Jovanović [42], both methods were described in detail by Milošević et al. [43]. The spectrophotometric assay for CAT activity is based on the measurement of the decrease in absorbance at 240 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 20 mM H_2O_2 and enzyme extract. The kinetics of CAT activity was measured every 20 s for 3 min, where one unit of CAT activity was defined as the amount of enzyme that degrades 1 µmol of H_2O_2 in 1 min.

The reaction mixture for determination of POX activity contained 0.05 M potassium phosphate buffer (pH 6.5) and 1 M pyrogallol as enzyme substrate. The reaction was started by the addition of 30% H_2O_2 after the first 20 s. The POX catalysed the oxidation of pyrogallol with H_2O_2 to purpurogallin, which was monitored by absorbance determination at 430 nm. Measurements of CAT and POX activities were performed using a UV-visible spectrophotometer (Agilent 8453, Life Sciences, Santa Clara, CA, USA).

The activities of all antioxidant enzymes are expressed as U/mg protein. Measurements for all antioxidant enzyme analyses were repeated three times.

2.3. Statistical Analysis

The effects of fourteen SNP pretreatments/treatments on different biochemical parameters of *I. walleriana* shoot explants were evaluated after four weeks of culture using standard two-factor analysis of variance (ANOVA). Statistical analyses were performed using Statistica 10 software (StatSoft, Hamburg, Germany). Determination of all analysed parameters was performed on three biological samples per treatment. In addition, the absorbance values of all supernatants were measured three times for each sample. The results are presented as means ± standard errors. Comparisons between means were made using Fisher's LSD (least significant difference) post-hoc test calculated at a confidence level of $p \leq 0.05$.

3. Results

To investigate whether SNP acts as a stress-ameliorating agent during PEG-induced water deficit, *I. walleriana* shoot explants were grown in vitro for four weeks on fourteen combinations of SNP pretreatments and PEG and/or SNP treatments. Pretreatments with 50, 100 and 250 µM SNP in combination with MS treatment did not alter the morphology of *I. walleriana* shoots (Figure 2, first row). Treatment with PEG had a negative effect on the growth of SNP-pretreated shoots (Figure 2, second row). Moreover, treatment with PEG after MS pretreatment reduced the usual growth compared to all combinations where MS treatment was investigated. The growth of SNP-pretreated *I. walleriana* shoots was favoured by the simultaneous application of PEG and SNP, compared with PEG alone (Figure 2, third row). Unexpectedly, pretreatments with 50, 100 and 250 µM SNP followed by treatments with the same SNP concentrations enhanced growth of *I. walleriana* shoots compared to MS treatment (Figure 2, fourth row).

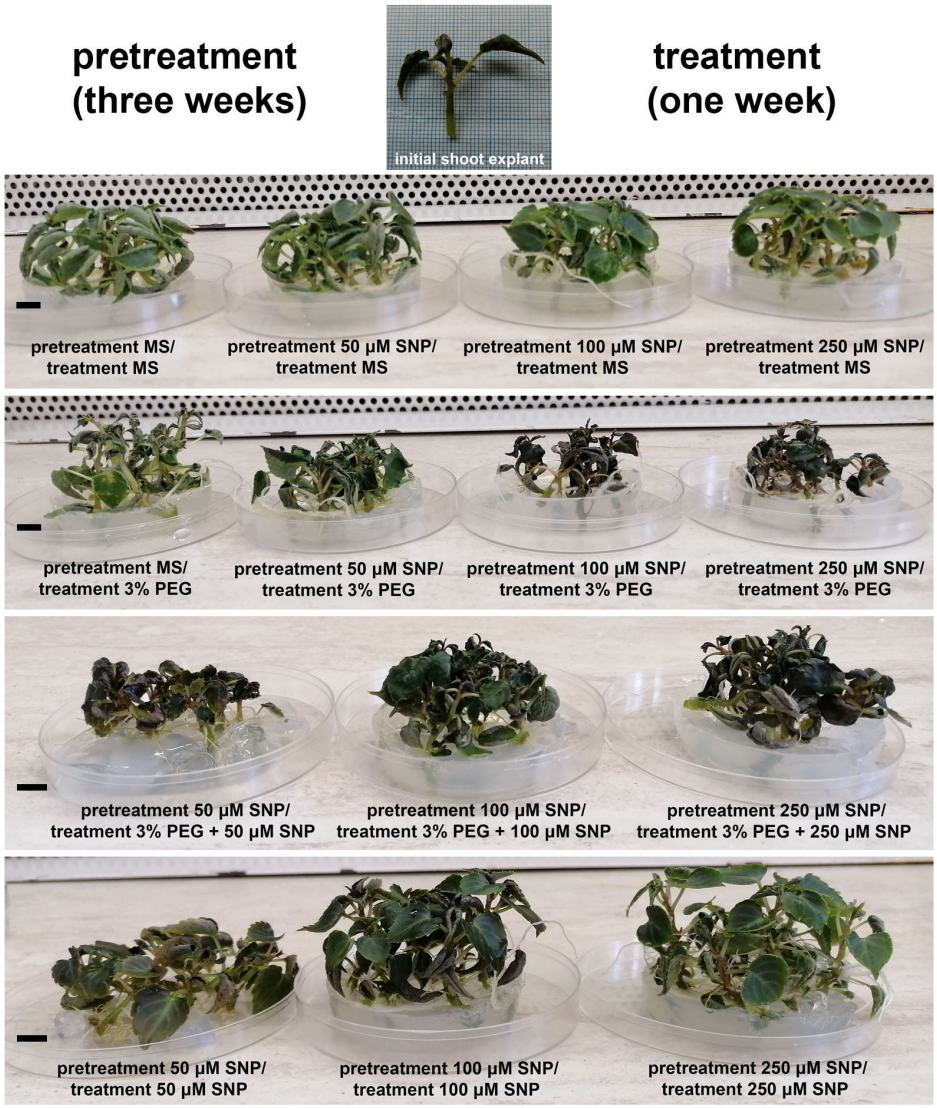

Figure 2. The effect of different SNP pretreatments and PEG and/or SNP treatments on growth and morphology of *Impatiens walleriana* shoots after four weeks in vitro. Bars indicate 10 mm.

3.1. Free Proline Content

In the control group of shoots, proline content was significantly increased under water stress conditions compared to non-stressed conditions (Figure 3). A similar pattern was observed in pretreatments with 50, 100, and 250 µM SNP. After pretreatment with 50 and 100 µM SNP, proline content in shoots grown on MS medium was similar to that in control, *I. walleriana* shoots grown under water deficit conditions on PEG. On the other hand, proline content in shoots subjected to pretreatment with 250 µM SNP was similar to that in the non-stressed control shoots. Pretreatment with all SNP concentrations did not significantly alter proline content in shoots treated with PEG compared to control water-stressed conditions. The combination of pretreatment and treatment with a single SNP treatment at each concentration did not alter proline content compared to control shoots grown under non-stressed conditions. The combination of PEG and SNP at all concentrations in the treatments significantly increased proline content compared to both control groups. The same pattern was observed in pretreatments with 100 and 250 µM SNP. After treatment with MS, proline content was similar to control shoots treated with PEG (after 100 µM SNP treatment) and non-stressed control shoots (after 250 µM SNP treatment).

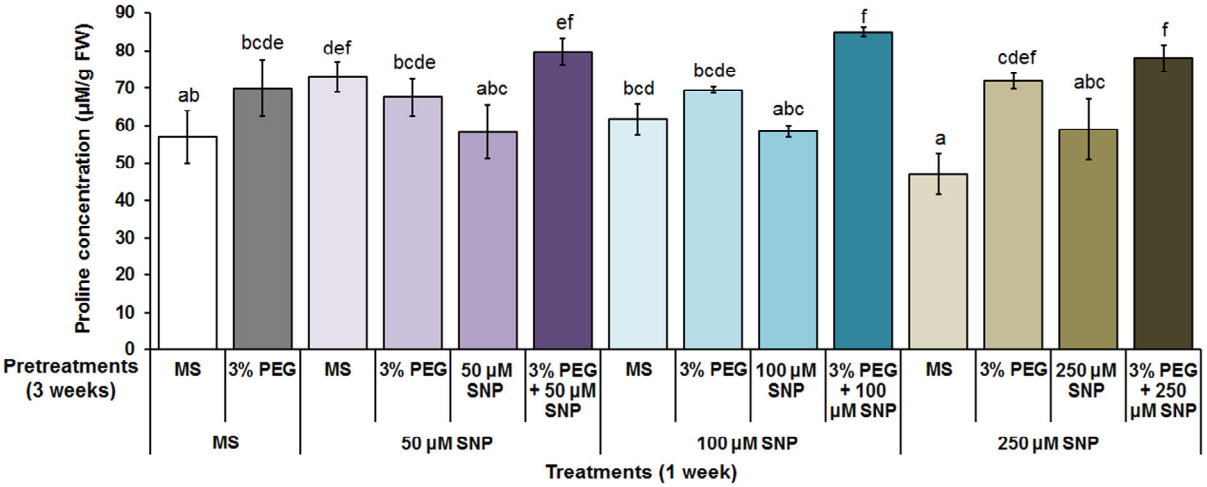

Figure 3. The effect of different SNP pretreatments and PEG and/or SNP treatments on proline content in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

3.2. Photosynthetic Pigments, MDA and H_2O_2 Content

In control groups of *I. walleriana* shoots pretreated with MS alone, it was found that PEG slightly increased total Chlorophyll (Chl) content (Figure 4). Pretreatment with 50 µM SNP significantly increased total Chl content only when PEG was used as treatment. The combination of PEG and 50 µM SNP in treatment decreased the total Chl content compared to both control groups. Pretreatment with 100 µM SNP increased total Chl content after the three treatments applied (MS, PEG and 100 µM SNP) compared to both control groups. Pretreatment with 250 µM SNP also significantly increased total Chl content in two treatment groups when MS or PEG were used. Interestingly, in each experimental group, treatment with PEG and SNP reduced total Chl content.

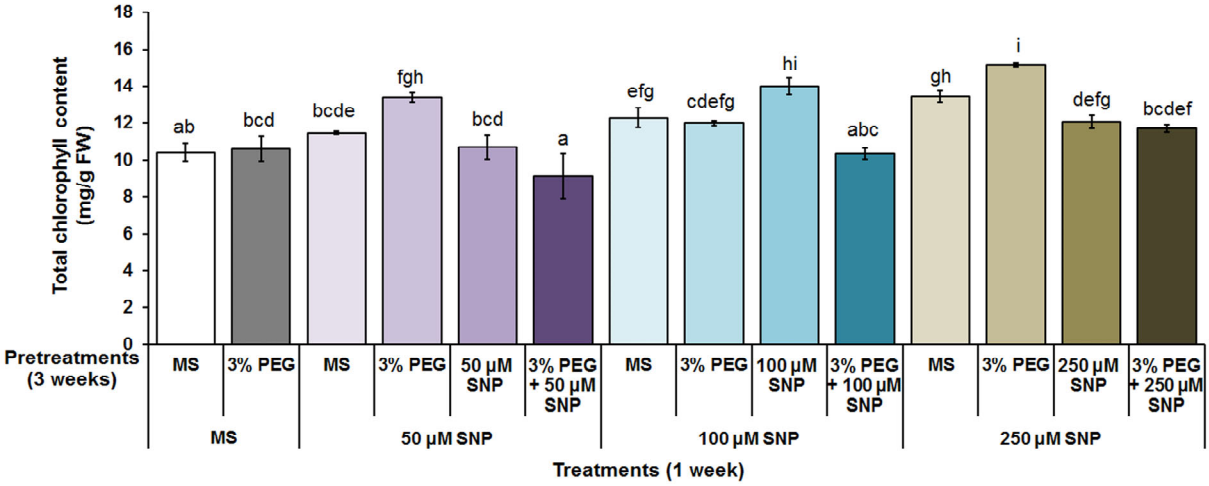

Figure 4. The effect of different SNP pretreatments and PEG and/or SNP treatments on total Chlorophyll content in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

Analysis of carotenoid content showed that approximately the same values were obtained in both control groups of *I. walleriana* shoots pretreated with MS and treated with MS or PEG (Figure 5). Pretreatment with 50 µM SNP significantly decreased carotenoid

content only after treatment with PEG compared with both control groups. In addition, a slight decrease in carotenoid content was observed in *I. walleriana* leaves after treatment with the combination of PEG and SNP. A moderate increase in carotenoid content was observed only after treatment with 50 µM SNP. Pretreatment with 100 µM SNP significantly decreased carotenoid content when the same SNP concentration was used for treatment. All other treatments did not significantly change the carotenoid content compared with the control groups. In contrast to all other SNP concentrations, pretreatment with 250 µM SNP decreased total carotenoid content in *I. walleriana* leaves after all four applied treatments, with the greatest reduction of carotenoid content after PEG treatment.

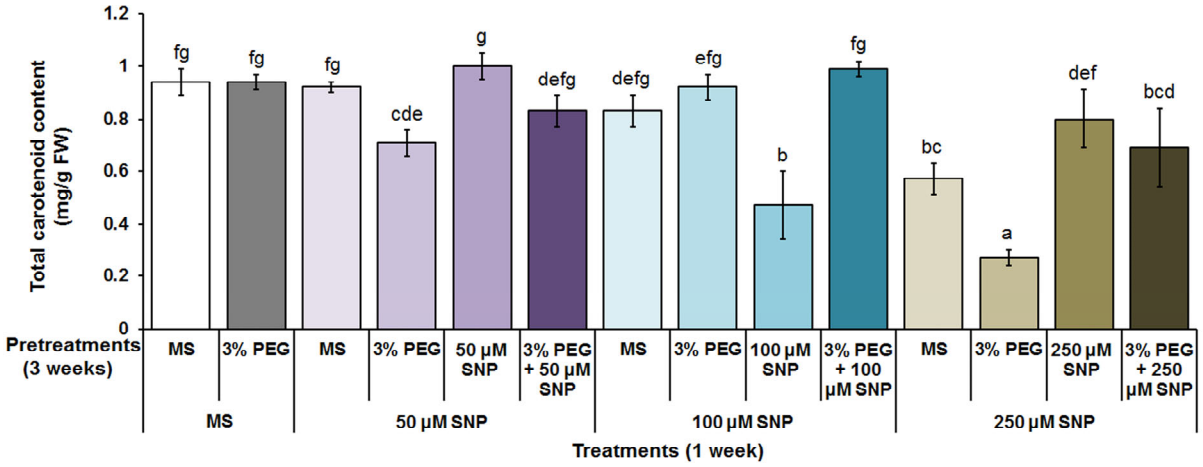

Figure 5. The effect of different SNP pretreatments and PEG and/or SNP treatments on total carotenoid content in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

Only a small increase in MDA content was detected in two control groups of *I. walleriana* shoots (Figure 6). In general, all SNP pretreatments increased MDA content in all experimental groups. After the first pretreatment with 50 µM SNP, lipid peroxidation was similar in shoots grown on MS and medium supplemented with PEG. Treatment with 50 µM SNP decreased MDA content but was still significantly higher than in both control groups. The combination medium containing 50 µM SNP and PEG increased MDA content to the highest value. The pretreatments with 100 and 250 µM SNP showed the same trend. A slight increase in MDA content was observed in shoots grown on MS medium compared to the control groups. The addition of PEG significantly increased MDA content. Treatments with 100 and 250 µM SNP, alone or in combination with PEG, decreased the level of lipid peroxidation, but MDA content was still higher than in both control groups.

Treatment with PEG in the control group of *I. walleriana* shoots increased H_2O_2 content (Figure 7). Pretreatment with 50 µM SNP increased H_2O_2 content only after the simultaneous application of PEG and SNP. Similar to the changes in MDA content, the same trend was observed for H_2O_2 content after pretreatments with 100 and 250 µM SNP. In *I. walleriana* shoots grown on MS medium, H_2O_2 content was similar to the control groups. Treatment with PEG significantly increased H_2O_2 content, while treatments with 100 and 250 µM SNP decreased H_2O_2 content. The highest H_2O_2 content, after pretreatment with 50 µM SNP, was observed in shoots grown on medium supplemented with a combination of PEG and 100 or 250 µM SNP.

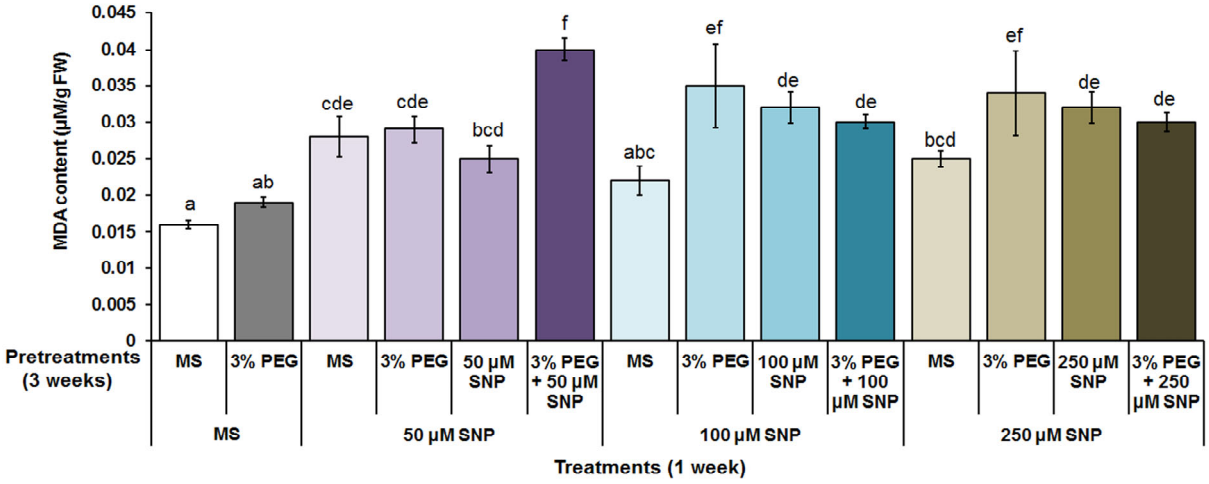

Figure 6. The effect of different SNP pretreatments and PEG and/or SNP treatments on MDA content in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

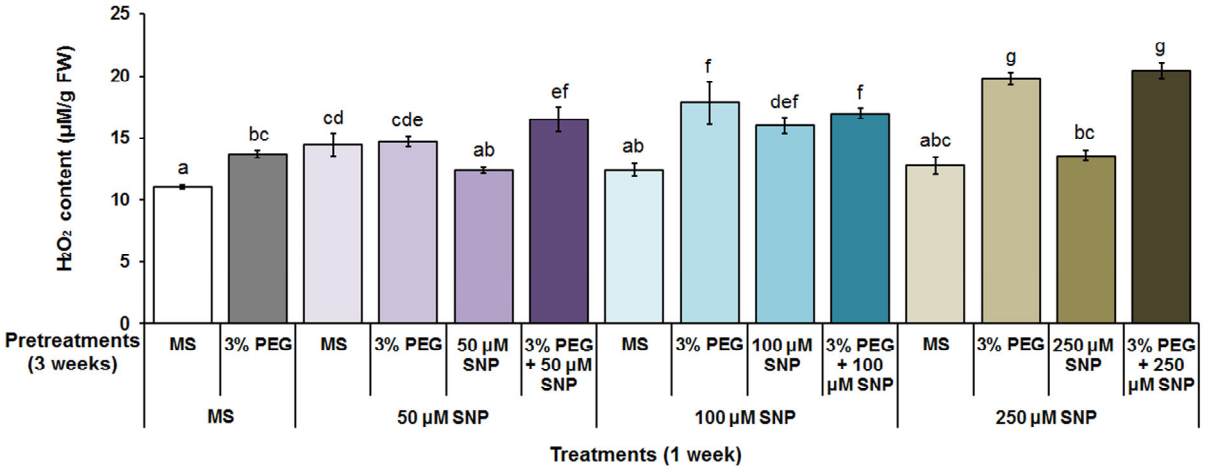

Figure 7. The effect of different SNP pretreatments and PEG and/or SNP treatments on H_2O_2 content in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

3.3. Total Polyphenol Content and Antioxidant Activity

Control shoots of *I. walleriana* exposed to PEG had increased total phenolic content (Figure 8). Interestingly, the total phenolic content did not change significantly after most SNP pretreatments and treatments compared to both control groups. Accordingly, increased phenolic content was detected after pretreatment with 50 µM SNP in shoots grown on MS or PEG-supplemented MS medium. In addition, a significant increase in total phenolic content was detected after pretreatment with 250 µM SNP in shoots grown only on MS medium supplemented with PEG.

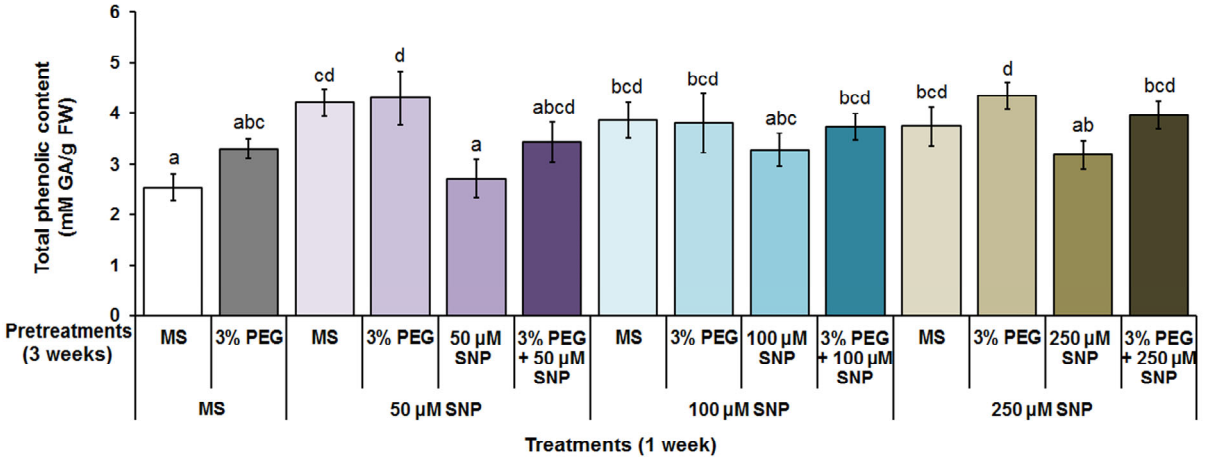

Figure 8. The effect of different SNP pretreatments and PEG and/or SNP treatments on total phenolic content in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

During the control treatment, PEG-induced water deficit increased antioxidant activity in the shoots of *I. walleriana* (Figure 9). It is very important to note that DPPH activity was significantly increased after all applied SNP pretreatments compared to both control groups.

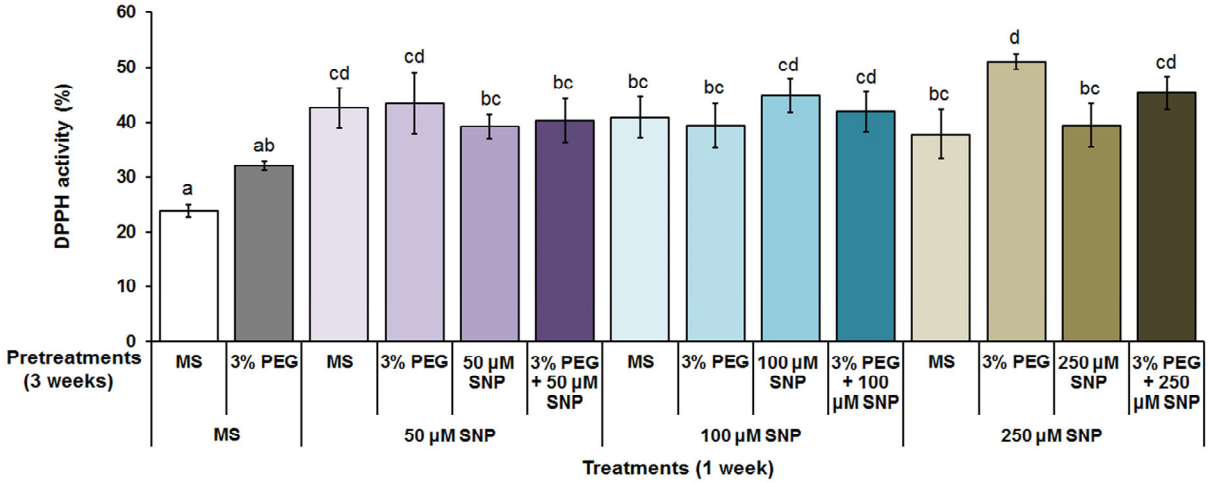

Figure 9. The effect of different SNP pretreatments and PEG and/or SNP treatments on DPPH activity in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

3.4. Superoxide Dismutase, Catalase and Peroxidase Activity

In control plants, SOD activity was slightly increased in water deficit-stressed shoots (Figure 10). After SNP pretreatments, the SOD activity in shoots grown on MS medium was similar to that in shoots grown under both control conditions. Also, SOD activity did not change significantly in shoots of *I. walleriana* treated with SNP only, compared to both groups of control shoots. It was also found that PEG-imposed water deficit, alone or with SNP, significantly decreased SOD activity after all SNP pretreatments compared to control.

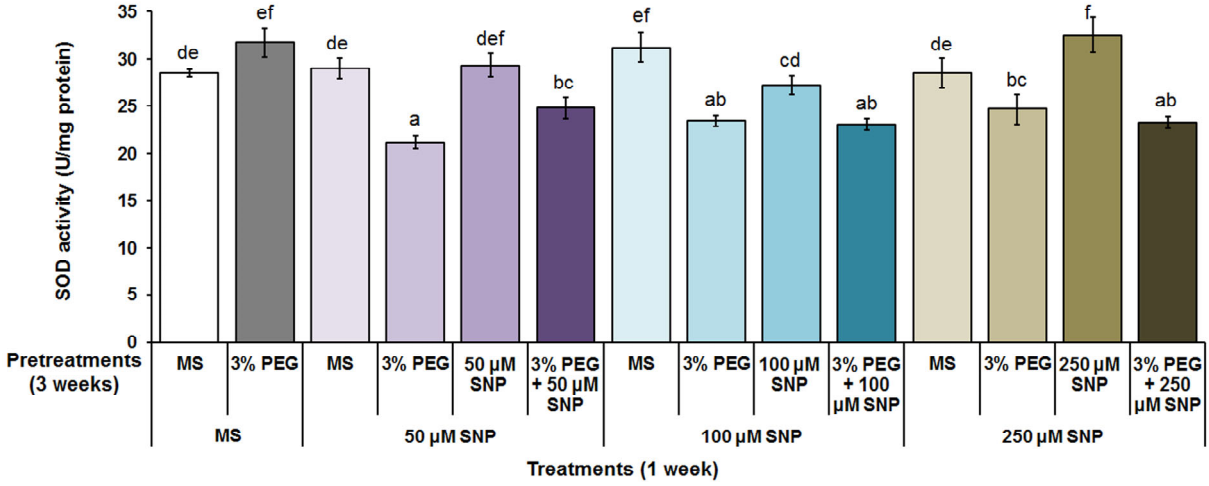

Figure 10. The effect of different SNP pretreatments and PEG and/or SNP treatments on SOD activity in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

I. walleriana shoots grown under PEG-imposed water deficit had significantly lower CAT activity than control shoots grown on MS (Figure 11). SNP pretreatments maintained CAT activity in shoots grown on MS as in both control groups of shoots, except after 100 µM SNP pretreatment, where CAT activity was significantly decreased. PEG-imposed water dificit also decreased CAT activity in shoots pretreated with all applied SNP concentrations. It was also noted that SNP concentrations were positively correlated with increased CAT activity in shoots treated with SNP only. The media containing PEG and SNP (50 and 100 µM) decreased CAT activity compared to both control groups of shoots. Only treatment with 250 µM SNP, alone or with PEG, increased CAT activity compared to other applied treatments, but this increase was still at the level of CAT activity detected in control shoots grown on MS.

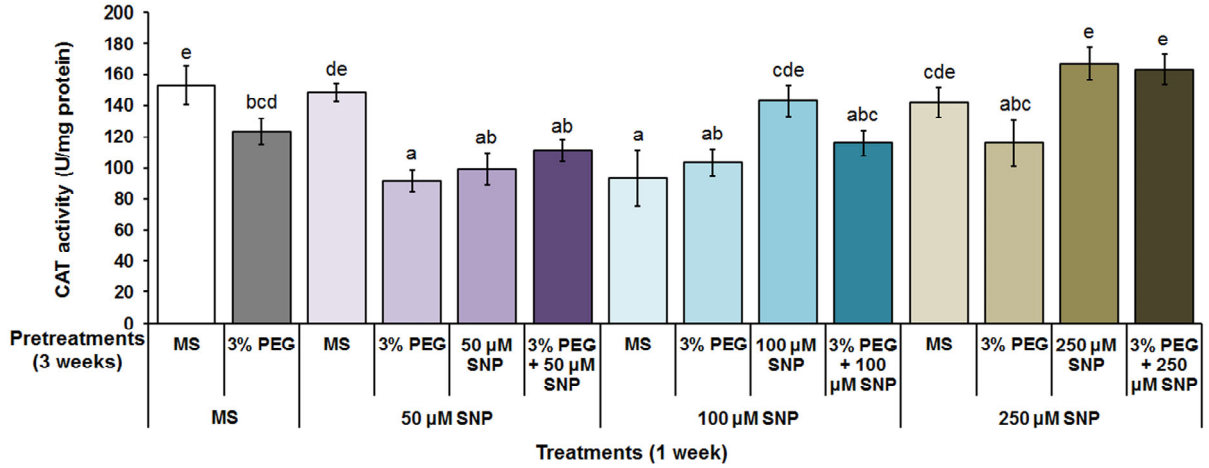

Figure 11. The effect of different SNP pretreatments and PEG and/or SNP treatments on CAT activity in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

PEG-imposed water deficit conditions significantly increased POX activity under control conditions (Figure 12). In shoots grown on MS, SNP pretreatments did not significantly alter POX activity compared with the corresponding control group. Also, the activity of

POX remained unchanged after all SNP pretreatments in *I. walleriana* shoots grown on PEG-imposed water deficit. On the other hand, POX activity was significantly decreased in shoots treated with only 50 and 100 µM SNP compared with the control groups. Further treatment, which implied the combination of PEG and SNP, did not significantly change POX activity compared to control groups.

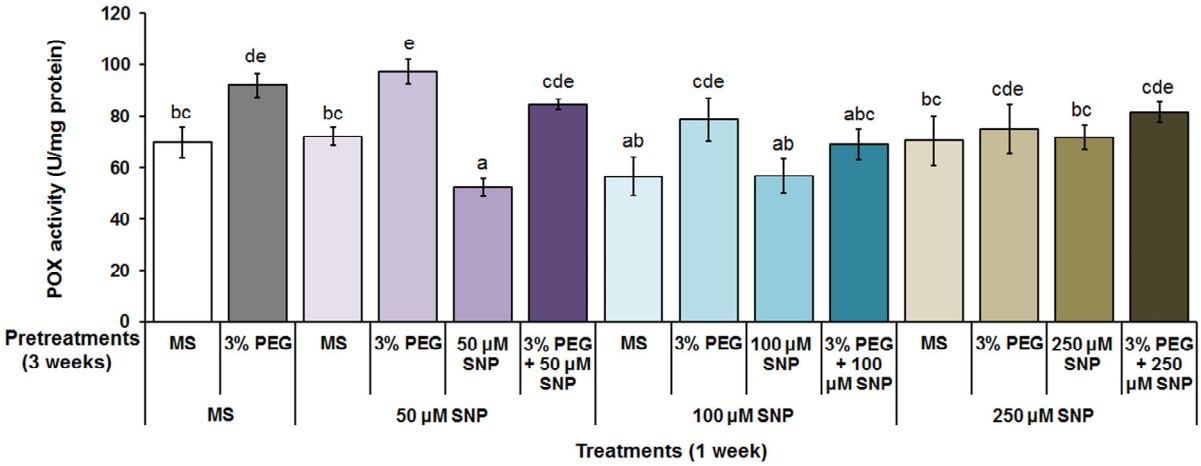

Figure 12. The effect of different SNP pretreatments and PEG and/or SNP treatments on POX activity in *I. walleriana* shoots grown under PEG-induced water deficit. Data represent means ± standard errors. Means marked with different letters are significantly different from control according to the LSD test and can be compared among each other ($p \leq 0.05$).

4. Discussion

Drought is one of the most common problems in agriculture, horticulture and forestry worldwide and ultimately affects crop productivity, resulting in reduced plant growth and development. In nature, lack of water in the substrate and intense transpiration are usually associated with the effects of high temperatures. Prolonged and intense drought can cause death even in the most tolerant plant species [44]. Accordingly, various techniques are used to produce commercially important plant species whose productivity may be limited by drought [45]. One of the most readily applicable and profitable techniques is the use of various pretreatments to overcome drought, including reactive oxygen-nitrogen-sulphur species, which can effectively influence plant tolerance to various abiotic stresses [46]. This work investigated how SNP, a widely used NO donor, affects various biochemical parameters of *I. walleriana* shoots grown in vitro under PEG-induced water deficit.

Osmoprotectants include compounds such as amino acids, sugars, alcohol sugars, organic acids and ions that allow more efficient water uptake from the substrate to the roots. The proline indicates different types of abiotic stress [47]. Water deficit conditions increased proline content in water-stressed *I. walleriana* shoots compared to those grown on MS medium. Previous investigations showed that PEG-treated *I. walleriana* shoots also accumulated cellular proline [21]. Increased accumulation of proline under PEG-induced water stress was also found in numerous plant species, including *Vitis vinifera* [48], several cultivars of sugarcane [49], and *Vanilla planifolia* [50]. Existing literature data show that exogenous application of SNP promoted water stress resistance through proline accumulation in safflower and lime [51,52]. The results presented in this work showed that SNP increased proline accumulation in shoots only after its simultaneous application with PEG. Increased proline content due to water deficit could be related to increased activity of different enzymes regulating the proline biosynthesis [53].

Since photosynthesis is directly dependent on photosynthetic pigments, the content of total Chl and carotenoids was studied. Decreased Chl content, due to impaired biosynthesis due to tissue water deficiency, was found in apple and cherry [54], blueberry [55] and many other plant species. Interestingly, PEG-induced water deficit did not significantly

alter the total Chl content in shoots of *I. walleriana* (Figure 4). It should be noted that increased Chl content during dehydration is an unusual phenomenon. However, a few reports of unchanged or even increased Chl content during water deficit stress induced by PEG in highly water stress-tolerant grass *Bouteloua gracilis* [56] and rice cultivars [57]. Drought also affects the biosynthesis and metabolism of carotenoids in plants [54,58]. Previously, it was reported that carotenoid content decreased significantly under drought stress conditions compared to the well-watered control [59,60]. However, our study showed that the carotenoid content of *I. walleriana* shoots remained quite stable after PEG-induced water deficit (Figure 5). This observation agrees with the recent results in a sensitive and drought-tolerant *Thymus* species, where drought did not alter the carotenoid content [61]. Since Chloroplast damage usually results in reduced carotenoid content, it is quite possible that unchanged pigment content during drought indicates ROS scavenging by antioxidants, which could explain better drought resistance [62]. According to the available literature data, dissociation of SNP releases iron and thus prevents Chlorosis of tissues by increasing Chl content in plants [14]. Accordingly, all SNP pretreatments increased Chl content in *I. walleriana* shoots grown on MS and 3% PEG. In contrast, pretreatments followed by treatments with SNP only did not affect Chl content compared to both control groups of *I. walleriana* shoots. The same observation was made in sunflower plants grown under drought stress [61]. It is well known that Chloroplasts play a key role in plant physiology due to various metabolic processes in membrane complexes. In addition to photosynthesis, Chloroplasts also synthesize amino acids, fatty acids, and various secondary metabolites. The altered Chl content in shoots of *I. walleriana* under PEG-induced water deficit can be explained by other mechanisms that involve Chloroplasts but exclude the process of photosynthesis. As in the case of Chl, carotenoid content in sunflower plants grown under water deficit stress was not affected by SNP [63]. SNP pretreatments decreased or did not significantly alter carotenoid content compared to both control groups of *I. walleriana* shoots. The effect of SNP on photosynthetic pigment content depends on several factors, including the applied SNP concentration, various stress conditions, and the plant species itself.

Lipid peroxidation is the reaction of oxygen with unsaturated lipids, producing a wide range of oxidation products [64]. A secondary product of lipid peroxidation, MDA, and its reaction with thiobarbituric acid has been used for many years as a suitable biomarker for determining lipid peroxidation. Normally, MDA content increases significantly during drought [65,66]. In the present study, MDA content increased slightly under PEG-induced water deficit compared to *I. walleriana* shoots grown on MS medium (Figure 6). Numerous studies have shown that the application of NO reduced oxidative damage, which was confirmed by reduced MDA content in PEG-stressed cultivars of rapeseed [67], two Indian mustard genotypes [68] and two watermelon genotypes [69]. Our results showed that, in general, all SNP pretreatments increased MDA content in *I. walleriana* shoots under PEG-induced water deficit. Although exogenous NO treatment under drought stress often reduces lipid peroxidation in plants, it is also known that NO can cause nitrosative stress under different abiotic stress conditions [70]. However, it is quite possible that oxidative stress was accompanied by nitrosative stress in the *I. walleriana* shoots studied.

Another expression of negative stimuli during drought is the formation of H_2O_2 in plant tissues. Drought stress resulted in higher H_2O_2 content in wheat [71], apple and cherry cultivars [54], sugar beet [72] and many others. The results obtained in this work showed that water limitation caused by PEG led to a significant increase in H_2O_2 content in *I. walleriana* shoots (Figure 7). As in the case of MDA, there are literature data describing the inhibitory effect of SNP on H_2O_2 production in *Malus hupehensis* [73], *Allium hirtifolium* [74], and pea seeds [75]. The results of this work show that all SNP pre-treatments and MDA increased H_2O_2 content in water deficit-stressed *I. walleriana* shoots. The highest H_2O_2 levels were observed in shoots grown on media supplemented with PEG and SNP. This result confirmed the hypothesis that increased MDA and H_2O_2 content induced nitrosative stress and enhanced the deleterious effects on plant tissues, previously described by Santisree et al. [76].

Polyphenols, the largest group of secondary metabolites, play an important role in plant growth and development during abiotic stress [77]. As antioxidants, polyphenols are very important for the removal of ROS forms and the prevention of damage to macromolecules and membranes [78]. A recent review reported increased accumulation of phenolic compounds during dehydration in many plant species [3]. The increased total phenolic content in control *I. walleriana* shoots exposed to PEG indicated a positive regulation of polyphenol biosynthesis and their role in neutralizing the negative effects of dehydration manifested by oxidative stress (Figure 8). It is known that dehydration positively regulates many genes involved in the biosynthetic pathways of polyphenolic components to increase their concentration during the stress period [78]. The accumulation of phenolic compounds (phenol, flavonoid, and anthocyanin) during drought and SNP application was reported in cotton plants [79], wheat [80], and marjoram [18]. It was interesting to note that SNP pretreatments and treatments did not significantly alter total phenolic content compared to either or both control groups of *I. walleriana* shoots. Phenolic compounds are known to reduce lipid peroxidation by scavenging hydroxyl radicals, which may be due to the activation of phenylamino lyase gene expression [81]. Considering that the total phenolic content did not change significantly, this could explain why the MDA content in *I. walleriana* shoots increased during the PEG-induced water deficit.

The antioxidant capacity is usually measured by the reduction degree of DPPH radicals. It is often used as a fast, simple, and reliable method [82]. Secondary metabolites react with radical forms as antioxidants and protect macromolecules from oxidation processes. Increased activity of antioxidants during dehydration was found in tomato [83], melinjo [84], sweet leaf [85] and many other plant species. The results showed that PEG-induced water deficit also increased antioxidant activity in *I. walleriana* leaves (Figure 9). Under stress conditions, plant cells always try to establish an intracellular balance between ROS production and antioxidants. At the same time, however, normal metabolic processes produce free radicals that must be removed by antioxidants. It is known that NO can act as an antioxidant in plant tissues. The beneficial effect on oxidative balance and antioxidant activity has been demonstrated in peach fruits and rice leaves [86]. It is important to note that all SNP pretreatments significantly increased DPPH activity in *I. walleriana* leaves. Although these results do not fully correlate with the total phenolic content, they suggest that *I. walleriana* shoots utilize the antioxidant properties of some other secondary metabolites as a protective mechanism during tissue dehydration.

The activities of antioxidant enzymes are often analysed in the context of various abiotic and biotic stress factors. Although SOD acts as the first in defense against ROS, other enzymatic components, including CAT and POX, also act as part of the antioxidant defense system [87]. In most cases, an increased concentration of ROS forms in cells increases the activity of antioxidant enzymes to establish homeostasis. However, the activity of some enzymes may remain unchanged or even decrease with increasing concentrations of ROS. Increased activity of SOD and POX during PEG-induced dehydration was found in numerous plant species, such as *Triticum aestivum* [88], *Triticum turgidum* [89], and *Cyclocarya paliurus* [90]. The results presented in this work showed that under control conditions, the activity of SOD was slightly increased in *I. walleriana* shoots during water deficit (Figure 10). Also, PEG-imposed water deficit conditions significantly increased POX activity in control conditions (Figure 12). On the other hand, in some plant species, reduced CAT activity was detected in response to water deficit stress conditions [91–93]. Accordingly, the activity of CAT was significantly reduced in *I. walleriana* shoots grown under PEG-imposed water deficit, compared to control shoots grown on MS (Figure 11). Such a reduction of CAT activity in *I. walleriana* shoots could be due to an inhibition of CAT synthesis or an altered assembly of enzyme subunits under water deficit.

The prevalent NO donor SNPs function as signaling molecules in plants and regulate the expression of many defense enzymes. Moreover, NO-mediated responses under drought conditions were mainly dependent on the upregulation of genes encoding antioxidant enzymes such as SOD, POX, and CAT [16]. Available literature data suggest that the

application of SNP [94,95] did not significantly depend on the physiological state of the plant [96] or even inhibited the activity of antioxidant enzymes [97]. The present study showed that all investigated SNP pretreatment, generally, did not significantly change SOD and POX activities in *I. walleriana* shoots. There were only two exceptions during the imposed water deficit when PEG alone or with SNP significantly decreased SOD activity in all SNP-pretreated shoots compared to control. The activity of POX also decreased significantly in shoots treated with all SNP concentrations compared to both control groups.

Interestingly, all SNP pretreatments applied did not significantly alter or decrease CAT activity. Only treatment with PEG and 250 μM SNP together increased CAT activity. However, this increase was also observed in control shoots grown on MS. All these findings suggest that SNPs can negatively affect enzymatic antioxidants during water deficit stress. These adverse effects appear to depend on several factors, including plant species, the type of antioxidant enzyme and the type of abiotic stress applied.

5. Conclusions

The presented results showed that PEG-induced water deficit had deleterious effects on the shoots of *I. walleriana*. All biochemical parameters analysed confirmed an increase in oxidative stress. These observations indicate that *I. walleriana* shoots are highly sensitive to water deficit in the substrate. The drought-ameliorating potential of SNP in *I. walleriana* shoots grown in vitro was tested by exogenous supplementation of growth medium with SNP. The results showed that the values of analysed parameters increased or remained unchanged after the PEG-induced water deficit. In an overall assessment, our results demonstrate for the first time that SNP has beneficial effects on biochemical parameters only when applied together with PEG, but not individually, to in vitro grown *I. walleriana* shoots.

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