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Polyethylene Glycol (PEG)-Induced Dehydration Alters Enzymatic and Non-Enzymatic Components of the Antioxidant Defense System in *Nepeta nervosa* Royle ex Bentham

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Abstract: Plants have developed a variety of defense mechanisms that allow them to recognize and respond to specific stressors by triggering complex signaling networks that cause appropriate biochemical changes to overcome the stress. In this study, we subjected in vitro grown *N. nervosa* plants to PEG-induced dehydration stress for 1 day (1DPT), 3 days (3DPT) and 6 days (6DPT). Our study investigated antioxidant enzyme activities, including catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD), unveiling dynamic responses to PEG-induced water stress. CAT levels increased initially (1DPT) but declined with prolonged treatment; while POX activity significantly increased at 3DPT and 6DPT; and SOD, particularly the Mn-SOD3 isoform, demonstrated a substantial increase, emphasizing its role in the enzymatic free-radical scavenging activity. Furthermore, examination of the phenolic acid content revealed that rosmarinic acid (RA) was the predominant phenolic compound, followed by chlorogenic acid (CHLA), while ferulic acid (FA) and caffeic acid (CAFFA) were present in lower concentrations. Notably, PEG-induced dehydration significantly boosted RA content in *N. nervosa* plants at 3DPT. This increase highlights the plant's response to oxidative stress conditions and its role in non-enzymatic antioxidant defense mechanisms. These findings significantly contribute to our comprehension of *N. nervosa*'s adaptive strategies under PEG-induced dehydration stress, offering valuable insights into plant stress physiology within industrial and agricultural contexts.

Keywords: dehydration; polyethylene glycol; rosmarinic acid; antioxidant defense systems; *Nepeta nervosa*; in vitro culture; abiotic stress; antioxidant enzymes

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1. Introduction

All stages of a plant's life cycle (germination, growth, development and reproduction) are closely linked to and dependent on a combination of environmental factors. If one or more factors endanger the survival of the plant, these conditions are defined as stressful [1]. As sessile organisms, they have evolved a plethora of defense mechanisms that enable them to recognize and respond to specific stressors by triggering complex signaling networks that induce appropriate biochemical changes to overcome the stress [2,3]. As a result, various physiological changes occur in plants [4].

The first visible change due to water dehydration is the reduction in leaf area, which is accompanied by a decrease in cell expansion, thereby reducing the transpiration area [5]. After stress perception by membrane proteins, the most rapid physiological process is abscisic acid (ABA)-mediated stomata closure [6]. The ABA signal transduction system consists of protein phosphatases and kinases that induce the production of reactive oxygen species (ROS) [7]. Water stress also leads to reduced CO_2 fixation by the Calvin cycle,

resulting in an accumulation of reduction equivalents ($NADPH^+$ and H^+) that generate various ROS [8]. Incompletely-reduced oxygen species, including the well-studied singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), primarily relate to ROS [9,10], which on the one hand play an important signaling role in plants and control processes such as growth and development, while on the other hand triggering defense mechanisms against biotic and abiotic environmental stress.

The most important enzymes for scavenging ROS, including catalases (CAT), peroxidases (POX) and superoxide dismutases (SOD), form a highly efficient system for maintaining ROS homeostasis at various compartments of cell [11]. The first line of defense against ROS are the SOD, which eliminate $O_2^{\bullet -}$, forming H_2O_2 and O_2 [11]. Catalases are a group of metalloenzymes that catalyze the dismutation reaction of H_2O_2 to water and oxygen. Peroxidases are glycoproteins that catalyze the oxidation reaction of the substrate, resulting in water and an oxidized radical product [12].

Various polyphenolic compounds, including phenolic acids, are postulated to contribute to the non-enzymatic antioxidants [13], whose potential is mainly reflected in their highly effective ability to scavenge most oxidizing molecules involved in the production of free radicals in plants exposed to various environmental stresses [14–16]. The literature shows that water stress affects the production of phenolic compounds in plants and that this response can vary between and within species and plant parts [17,18]. Phenolic acids, including cinnamic acid derivatives, have a remarkably high antioxidant potential that is closely related to their molecular structure, which enables them to form stable phenoxyl radicals when they react with radical molecules [19,20]. The cinnamic acid derivatives, such as rosmarinic acid (RA), chlorogenic acid (CHLA), ferulic acid (FA) and caffeic acid (CAFFA), are particularly important due to their numerous health-promoting properties, such as anticancer, antimicrobial, antidiabetic, neuroprotective, hepatoprotective and anti-inflammatory effects [21–26]. The ability of cinnamic acid derivatives to scavenge free radicals, chelate prooxidant ions, prevent lipid peroxidation and bind to biomacromolecules has made them a valuable antioxidant used in the food industry [27–29]. Among these compounds, RA is found in many plant species, most notably in the Lamiaceae family which comprises specialized metabolite-rich aromatic plants. Currently, the most exploited species for RA isolation are *Salvia rosmarinus*, *S. officinalis*, *Mellisa officinalis*, *Ocimum basilicum* and *Mentha piperita* [30,31]. However, the production of this compound encounters several challenges, one of which is the extraction of highly pure RA, which is essential for various applications in industry. Obtaining RA from cultivated aromatic plants carries the risk of containing either externally-introduced impurities (i.e., pesticide or herbicide) or internal impurities originating from plant tissues (i.e., other specialized metabolites) [30–32]. Plant in vitro systems offer a valuable substitute for conventional field harvesting in overcoming the problem of external contaminants, in addition to other significant advantages such as controlled and enhanced biosynthesis, year-round production, genetic stability and scalability [33]. Selection of the most appropriate plant source with high contents of RA and significantly lower amounts of other groups of specialized metabolites could facilitate gaining high yields of highly pure RA. Previous studies have demonstrated a favorable compound ratio for RA derivation in *Nepeta nervosa* Royle ex Benth [34]. Plant species of the genus *Nepeta* (fam. Lamiaceae) are widely cultivated as horticultural and medicinal crops due to their high productivity of terpenes and phenolic compounds. Most species of this genus are characterized by the accumulation of the atypical monoterpenes, iridoids, both as aglycones (nepetalactones) and glycosides in their tissues, and only a few iridoid-lacking species are identified, among which is *N. nervosa* Royle ex Benth [34]. Moreover, *N. nervosa* has been shown to produce limited quantities and a poor diversity of other terpenoids [34]. However, this species has until now remained an unrecognized and underexploited candidate for RA isolation.

The present study uncovers the role of antioxidant molecules in the acclimation of *N. nervosa* plants cultivated in vitro to water deficit. This research aims to: (1) Explore antioxidative defense mechanisms during PEG-induced water stress to enhance understand-

ing of the physiology of plant adaptability to stress conditions; (2) Evaluate the potential of short-term PEG treatment in scaling up the production of phenolic acids, particularly high purity RA in *N. nervosa* plants. This is the first attempt to analyze the antioxidative mechanisms of *N. nervosa* under PEG-induced dehydration, which is a promising way to obtain RA extracts with a favorable compound ratio for industrial application.

2. Materials and Methods

2.1. Plant Material

Nepeta nervosa Royle ex Benth. (Lamiaceae) seeds were surface sterilized in 50% (*v*/*v*) commercial bleach in sterile deionized water (2% *w*/*v* active chlorine) for 2 min and thoroughly washed 5 times with sterile deionized water. Seeds were transferred into plastic Petri dishes (9 cm diameter) containing 25 mL of ½ MS culture medium, named basal medium (BM) [35], made with modifications as in [36]. The pH of the BM was adjusted to 5.8. The seeds germinated ten days after placing on BM medium. One-node stem segments of selected 4-week-old seedlings were used as explants for the multiplication of different *N. nervosa* genotypes. Sub-culturing was performed every 4 weeks on BM medium in 370 mL glass jars. Three months of multiplication of selected genotypes provided enough plant material for experiments. All the cultures were kept in a growth chamber at 25 ± 2 °C, under a 16/8 h light/dark regime with a photon flux density of 70 µmol/m^{-2} s^{-1}, provided by white fluorescent tubes.

2.2. Experimental Design

The PEG experimental setup was performed as reported by [36]. Briefly, five rooted *N. nervosa* plants, four weeks old, were placed on a metal stand in 6.5 × 6.5 × 10 cm Magenta vessels (Sigma-Aldrich, Darmstadt, Germany) containing 30 mL of the ½ MS modified liquid medium without sucrose ± water-soluble PEG 8000. The osmotic pressure of PEG solutions was −3 MPa. This was achieved by adding 500 g of water-soluble PEG 8000 into 1 L of ½ MS liquid media without sucrose (50% *w*/*v*) after the sterilization of the medium by autoclaving at 114 °C for 25 min and subsequent cooling to room temperature. Michel's [37] equation: $\Psi = 1.29\ [PEG]^2T - 140\ [PEG]^2 - 4[PEG]$; [PEG] = g PEG/g H_2O at T = +25 °C, was used to calculate the final water potential of the culture media with the addition of PEG.

After 1, 3 and 6 days of PEG treatment, leaves of the non-treated and PEG-treated plants were cut, collected, ground in liquid nitrogen and stored at −80 °C until further use. The results were obtained using three biological replicates. Each biological replicate represented a pool of leaf tissues collected from five plants grown in the single Magenta vessels.

The following formula was used to calculate the percentage of decrease or increase in some values:

$$\% = \pm(1 - T/C) \times 100$$

T is the parameter of treated plants, while C is a parameter of control ones, and a positive value indicates an increase, while negative indicates a decrease in parameters.

2.3. Determination of Relative Water Content (RWC)

The relative water content (RWC) of plants was performed as described in [36] according to the formula for calculating the RWC of plants: RWC (%) = [(FW − DW)/(TW − DW)] × 100. The fresh weight (FW) of both non-treated and PEG-treated plants was measured on the day of sampling. To obtain the turgid weight (TW), plants were further immersed in distilled water for 24 h, at room temperature, and measured after that. Dry weight (DW) was measured after plants' drying at 70 °C for 72 h. Each treatment was conducted in triplicate.

2.4. Preparation of Methanol Extracts

Powdered leaf tissue (about 50 mg) was transferred into Eppendorf tubes (2 mL) and soaked in 1 mL of 96% methanol. Samples were strongly mixed at vortex for 10 s, sonicated

in an ultrasonic bath (RK100, Bandelin, Berlin, Germany) for 15 min and further centrifuged at 10,000 g for 15 min at 4 °C. Supernatants were filtered through 0.2 µm syringe filters (Agilent Technologies, Santa Clara, CA, USA) and kept at 4 °C before analyses. Rosmarinic acid (RA), chlorogenic acid (CHLA), ferulic acid (FA) and caffeic acid (CAFFA) standards were obtained from Sigma-Aldrich, Darmstadt, Germany.

2.5. Metabolic Profiling of Targeted Phenolic Compounds

Chromatographic separations of phenolics in methanol extracts of *N. nervosa* plants were performed on a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) equipped with a diode array detector (DAD) and connected to a TSQ Quantum Access Max triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Basel, Switzerland). Elution was performed at 40 °C on Syncronis C18 column (100 × 2.1 mm) with 1.7 µm particle size (Thermo Fisher Scientific, Bremen, Germany). Gradient elution of the mobile phase, consisting of 0.1% formic acid in H_2O (A), and acetonitrile (B), was as previously reported [13], and the injection volume was set to 10 µL. The vaporizer temperature of the mass spectrometer equipped with a heated electrospray ionization (HESI) source was kept at 300 °C, and the ion source settings were as follows: spray voltage 4000 V, sheath gas (N_2) pressure 28 AU, ion sweep gas pressure 1.0 AU, auxiliary gas (N_2) pressure 10 AU, capillary temperature 275 °C and skimmer offset 0 V. Mass spectrometry data were acquired in negative ionization mode, and collision-induced fragmentations were performed using argon, with the collision energy (cE) set to 30 eV. Quantification of chlorogenic acid (CHLA), caffeic acid (CAFFA), ferulic acid (FA) and rosmarinic acid (RA) in samples was performed in a selected reaction monitoring (SRM) experiment of the mass spectrometer, tracking the two characteristic MS2 fragments for each compound. The stock standard solution of phenolic acids was prepared by dissolving 1 mg of each of the compounds in 1 mL methanol. The working solution, prepared by mixing the stock solutions to obtain the concertation of 20 µg/mL, was further diluted with methanol down to 5 ng/mL, to obtain other calibration levels. Regression was calculated for each of the calibration curves, which all showed a good linearity ($r = 0.999$, $p < 0.001$).

2.6. Determination of CAT, POX and SOD Activities

Detailed procedures for antioxidant enzyme extraction and activity determination were carried out as described by [38], with some modifications in measuring the concentrations of isolated soluble proteins. Instead of the Bradford [39] method, soluble protein concentrations were accurately measured using a Qubit® 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Bremen, Germany) by highly sensitive Invitrogen Qubit quantitation assays.

Native polyacrylamide gel electrophoresis (Native-PAGE) separation and activity staining of CAT (EC 1.11.1.6), POX (EC 1.11. 1.7) and SOD (EC 1.15.1.1) were described by [38], with some modifications. POX activity was visualized after the incubation of gels in staining solution containing 0.17% 4-chloro-naphthol and 0.11% H_2O_2 in 50 mM K-phosphate buffer pH 6.5. Multiscan FC (Thermo Fisher Scientific, Bremen, Germany) microplate reader was used for recording absorbance at 540 nm for quantification of SOD activity.

Imaging System iBRIGHT CL1500 (Invitrogen, Thermo Fisher Scientific, Bremen, Germany) was used to record gels with developed enzyme activities and measure the intensity of POX bands, while ImageJ 1.32 software (W. Rasband, National Institute of Health, Bethesda, Maryland, USA) was used to determine the intensity of CAT and SOD bands, as described by [38].

2.7. Statistical Analysis

The StatGraphics software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, Rockville, Maryland, USA) was used for the calculation of means and statistical errors in PEG-treated and non-treated plants, at 1, 3 and 6 days of treatment, while an

unpaired Students' *t*-test (Microsoft Excel for Microsoft 365 MSO (Version 2310 Build 16.0.16924.20054) 64-bit) was used to compare data, and a difference of $p \leq 0.05$ was considered significant.

3. Results and Discussion

Being an oxidative stress, water deficiency triggers the hyper-activation of non-enzymatic (phenolic compounds) and/or enzymatic ROS scavenging systems to prevent cellular damage and restore redox homeostasis. Consequently, levels of proteins responsible for ROS scavenging are found to be associated with the basal water stress tolerance [40]. A large amount of literature data has reported that abiotic stress leads to the increased production of ROS, which could increase production of plant defense secondary metabolites [41,42]. A substantial obstacle in the large-scale extraction of useful secondary metabolites arises from the low productivity of plants. This limitation stems from the fact that their content rarely exceeds 1% of dry weight [43]. Additionally, the yield of phenolic acids is subject to a multitude of influencing factors, including plant physiology, growth and development phases and pre- and post-harvest processes, as well as geographical and environmental factors. Much attention has been given lately to improving biosynthesis in Lamiaceae through the application of elicitors, chemical or physical tools (i.e., signal compounds and/or abiotic stress), and primarily on water stress [44–46], which is a common environmental phenomenon in the natural habitats of mints.

Herein, we investigated the impact of PEG-induced dehydration stress on enzymatic and non-enzymatic components of the antioxidant defense system of in vitro cultured *N. nervosa* plants and evaluated the potential of PEG treatment in scaling up the production of high-purity rosmarinic acid.

3.1. PEG-Induced Physiological Changes

Four-week-old in vitro grown *N. nervosa* plants, were exposed to PEG-induced dehydration stress for 1 (1 day PEG treatment—1DPT), 3 (3DPT) and 6 days (6DPT) (Figure 1B).

Tissue dehydration, turgor loss and reduction in leaf area (Figure 1B) and FW reduction (Figure 1C) in *N. nervosa* shoots were observed at all experimental points, which confirmed that the plants were experiencing water stress. When compared with respective non-treated *N. nervosa* (Figure 1A), the FW values of treated plants were significantly lowered by 39%, 67% and 68% at 1DPT, 3DPT and 6DPT, respectively (Figure 1C). These results are in accordance with the PEG-induced dehydration in *N. rtanjensis* and *N. argolica* ssp. *argolica* [36]. Likewise, Zulfiqar et al. 2021 [47] showed that shoot and root length, as well as the fresh and dry weight of sweet basil, were reduced under water stress.

The intensity of water stress in non-treated and PEG-treated plants was assessed by calculating the RWC (Figure 1D). This parameter is a useful indicator of the water status, as well as of the level of tolerance of plants to water stress. The results show that in *N. nervosa,* RWC values decrease significantly under the influence of PEG at all time-points of the experiment, which indicates that the PEG-treated plants experienced high-intensity water stress. The RWC values were significantly reduced (from 35% to 48% in comparison to the appropriate control) during the water-deficiency stress (Figure 1D). The water potential of the substrate was −3 MPa, which is a value that causes severe water stress in a number of plant species [48]. However, the RWC reduction was lower in *N. argolica* subsp. *argolica,* and especially in *N. rtanjensis* [36] than in *N. nervosa* plants, all exposed to PEG treatment under similar experimental conditions and water stress of the same intensity. Furthermore, the RWC values of non-treated *N. nervosa* plants (an average of 84%) were shown to be higher when compared to those in non-treated *N. rtanjensis* and *N. argolica* subsp. *argolica* plants (an average of 65% and 72%, respectively), which indicates structural and eco-physiological differences among related species. Moreover, it can be assumed that *N. nervosa* was less efficient in overcoming the dehydration-induced water stress under the current experimental conditions.

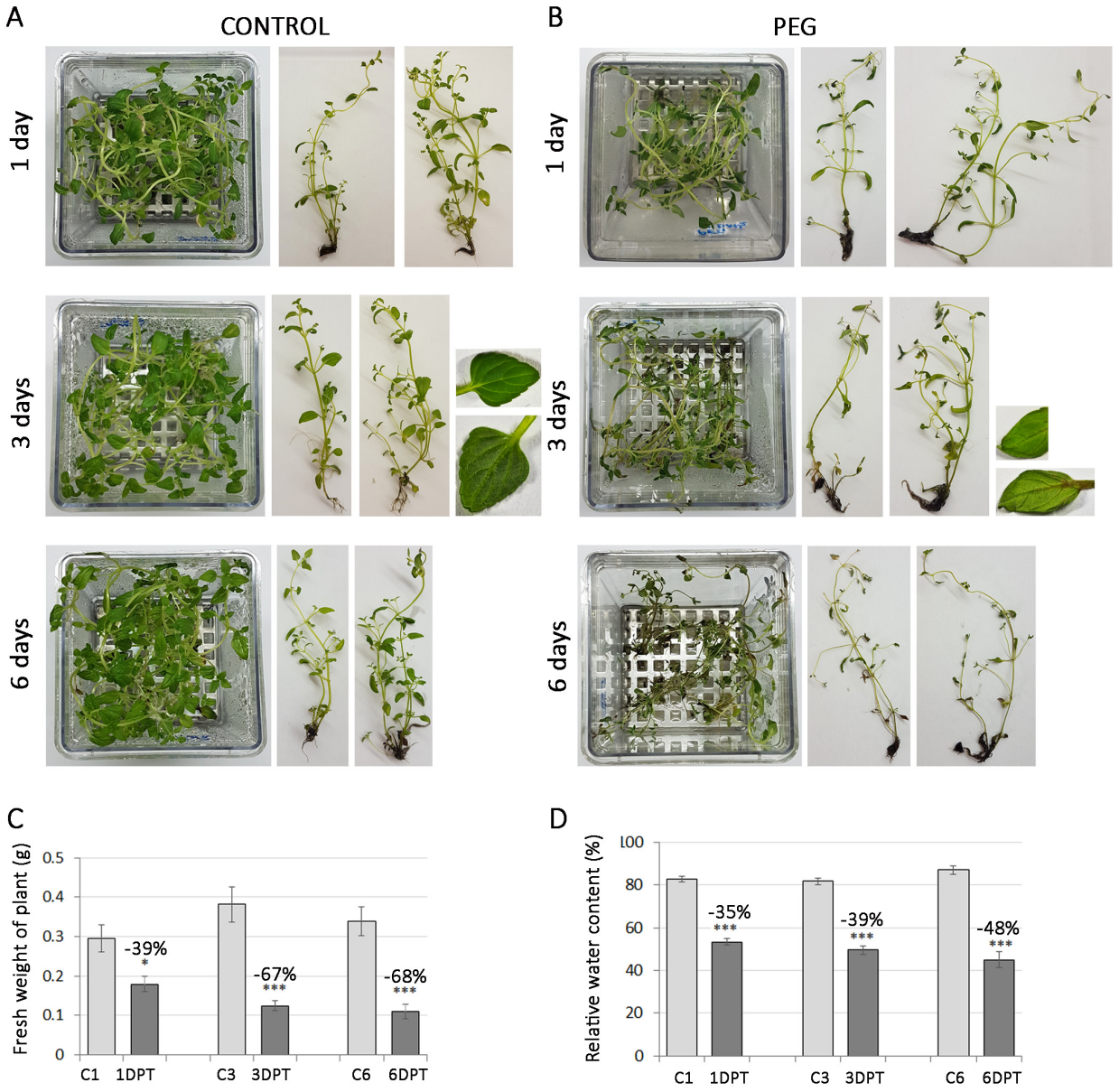

Figure 1. (**A**,**B**) Physiological changes in non-treated (control) and PEG-treated *N. nervosa* plants for 1, 3 and 6 days under in vitro conditions. (**C**,**D**) Parameters of dehydration stress: fresh weight (FW) of *N. nervosa* plants and their relative water content (RWC) were measured throughout 6 days of exposure to PEG-induced dehydration stress. *N. nervosa* plant immersed in distilled water for 24 h at room temperature to obtain turgid weight. PEG-treated *N. nervosa* plants (1DPT, 3DPT and 6DPT) are compared with their respective controls (C1, C3 and C6) at 1, 3 or 6 days of treatment. The results represent the mean ± SE of three biological replicates. Asterisks (*) indicate significant differences between PEG-treated and non-treated plants according to an unpaired *t*-test (* $p \leq 0.05$; *** $p \leq 0.001$). The numbers above the bars indicate the percent of reduction (−) relative to the control.

3.2. Content of Phenolic Acids

The chromatographic separation and quantification of phenolic compounds in samples was performed adopting an SRM (single reaction monitoring) experiment on the UHPLC/DAD/(±)HESI−MS^2 instrument operating in a negative mode. The analysis was targeted towards four phenolic acids belonging to the group of hydroxycinnamic acids (RA, CHLA, CAFFA, and FA), which were previously described as the most abundant phenolic compounds in *N. nervosa* [34]. *N. nervosa* is enriched with derivatives of CAFFA, including RA, which is structurally a dimer of CAFFA, as well as CHLA and nepetoidin

A or B (unpublished data). These compounds have previously been identified as abundant in other *Nepeta* species [34,49–52]. Peaks of CHLA, CAFFA, RA and FA were visible in UHPLC-DAD chromatograms at retention times of 5.18 min, 5.58 min, 6.98 min and 7.62 min, respectively (Figure 2A). The most abundant phenolic acid in *N. nervosa* leaves was RA, followed by CAFFA, while the observed concentrations of CHLA and FA were significantly lower. In parallel with phenolics, samples were also analyzed for the presence of iridoids (nepetalactone, nepetalactol 1,5,9-epideoxyloganic acid), and their concentrations were below the detection limits of the used analytical method. The obtained results are consistent with prior investigations on the constituents of *N. nervosa* extracts, which have consistently shown the absence of iridoids, while trace amounts of other terpenoid compounds were recorded [34,53,54]. Our findings further reinforce the hypothesis that *N. nervosa* represents a highly promising plant source for the extraction of RA, offering both a high yield and purity, due to its favorable quantitative and qualitative compound ratio.

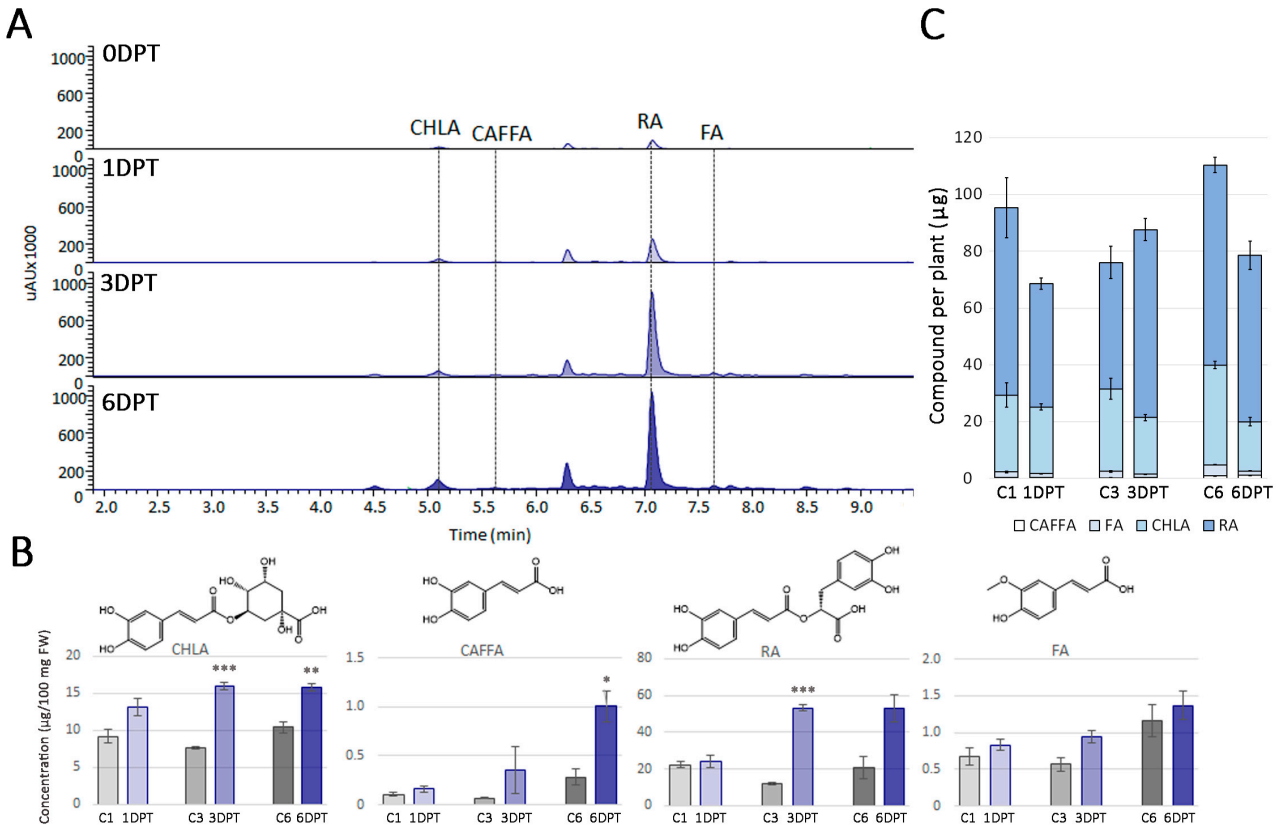

Figure 2. (**A**) Representative UHPLC/DAD chromatograms of the methanol extract of *N. nervosa* plants at the beginning of the experiment (0DPT), and one (1DPT), three (3DPT) and six (6DPT) days after the beginning of PEG treatment. (**B**) Content of phenolic acids: chlorogenic (CHLA), caffeic acid (CA), rosmarinic (RA) and ferulic (FA) in non-treated (grey bars) and PEG-treated (blue bars) *N. nervosa* plants for 1, 3 and 6 days of exposure. The results are expressed as µg/100 mg FW, and presented as the mean ± SE of three biological replicates. Asterisks (*) indicate significant differences between PEG-treated and non-treated plants according to an unpaired *t*-test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). The numbers above the bars indicate the percent of enhancement (+) relative to the control. (**C**) Overall productivity of *N. nervosa* PEG-treated (1DPT, 3DPT and 6DPT) and non-treated plants (C1, C3, and C6) grown in vitro. A total compound yield is expressed as the µg of compound per plant.

In non-treated plants, the concentration of RA was determined to be on average 18.27 µg/100 mg FW (~0.02% FW), whereas in treated plants at 3DPT, the content of RA significantly increased to 52 µg/100 mg FW (~0.05% FW). Thus, PEG-induced dehydration

led to an enhancement of RA amounts in *N. nervosa* plants from 9% (at 1DPT) to 355% (in 3DPT), in comparison to the respective controls (Figure 2B). Similarly to our findings, short-term PEG-induced water deficiency triggered a significant increase in RA content in *Salvia leriifolia* plantlets and calli grown in vitro [55]. Contrary to our results, severe drought stress did not affect the accumulation of rosmarinic acid in *Salvia milltiorrhiza* [44]. Furthermore, PEG showed negative effects on RA accumulation in *Thymus lotocephalus* shoots [56]. In summary, dehydration stress appears to modulate RA biosynthesis differently in related Lamiaceae species contingent upon the severity and duration of experimental conditions. A significant increase in the content of CHLA in *N. nervosa* was observed at 3DPT and 6DPT, while CAFFA content was significantly increased at 6DPT. No significant change in the amount of FA in response to PEG treatment was observed in *N. nervosa* plants (Figure 2B). These findings are consistent with expectations, as phenolic compounds play a vital role in regulating essential physiological functions in plants, providing resistance against a range of stress conditions. The pronounced antioxidant activity of phenolic compounds is attributed to their capacity to act as hydrogen atom donors and effectively scavenge free radicals, leading to the formation of less reactive phenoxyl radicals [57,58]. The formed radicals are more stable due to the delocalization of electrons and the existence of more resonant forms. Consequently, the stimulated accumulation of phenolic acids in response to oxidative stress conditions is also recorded in *N. pannonica* grown in the paraquat-enriched medium in vitro [51]. Likewise, in accordance with our results, PEG-induced dehydration elevated phenolic compound concentrations in several Lamiaceae species grown in vitro [59,60].

The quantitative assessment of 3DPT *N. nervosa* extracts suggests their potential value for industrial applications, as rosemary extracts containing RA within the range of 5–30% are widely recognized and utilized in the market as natural preservatives and antioxidants (Naturalin Bio-Resources Co., Ltd., Changsha City, Hunan Province, China). Furthermore, 3DPT induces no significant changes in the amounts of CAFFA and FA. Although CHLA is significantly elevated in the same PEG treatment, the chromatographic separation method adopted within the present study clearly distinguishes RA from CHLA, and can be isolated independently. Future research efforts should prioritize the identification of solvent systems that are both highly efficient and safety-conscious for the extraction of RA from *N. nervosa* samples, with potential applications across various industrial sectors.

Chlorogenic acid (9.04 µg/100 mg FW, an average value in non-treated plants) increased from 43% to 109% at 1DPT and 3DPT, respectively. Although the other two phenolic acids (FA and CAFFA) were detected at much lower values (about 0.80 µg/100 mg FW and 0.15 µg/100 mg FW, an average value in non-treated plants, respectively), PEG treatment also led to an increase in their content (66% FA at 3DPT, and 430% CAFFA at 3DPT). The largest increase in all detected phenolic acids occurred at 3DPT in comparison to the respective controls (Figure 2B). The highest concentrations of FA and CAFFA were observed at 6DPT, while the amounts of RA and CHLA accumulated at 3DPT appear to be maintained at 6DPT in treated plants. In the aerial parts of *Salvia officinalis,* a comparable halt in CAFFA to RA conversion was observed when contrasting mild and severe water-deficit conditions [61]. Ultimately, based on the growth parameters and phenolic compound production, it can be concluded that PEG-induced drought stress increased the overall productivity of *N. nervosa* plants after 3 days of exposure compared to the control, especially the RA yield (Figure 2C). Interestingly, the yields of FA and CHLA decreased. However, although greater phenolic compound accumulation was observed in PEG-treated plants (Figure 2B), their biomass decreased (Figure 1C), resulting in lower productivity at 1DPT and 6DPT compared to the controls (Figure 2C).

3.3. Antioxidant Enzymes Activity in N. nervosa Plants Influenced by PEG

Water-deficiency stress leads to the excessive production of ROS and oxidative damage, which induce the accumulation of osmolytes and the activation of antioxidant defense mechanisms [49,62]. Here, we analyzed the activities of three major antioxidative enzymes:

CAT, POX and SOD in the non-treated (C1, C3 and C6) and PEG-treated *N. nervosa* plants at 1DPT, 3DPT and 6DPT.

The in-gel assay unveiled the presence of four, two and one isoforms of CAT in non-treated *N. nervosa* plants at 1DPT, 3DPT and 6DPT, respectively. Conversely, in all PEG-treated *N. nervosa* plants, only one CAT isoform (CAT4) was detected at all experimental time points (Figure 3A,B). The spectrophotometric assay revealed a significant increase in CAT activity (29%) at 1DPT, whereas prolonged treatment for 3 and 6 days led to notable reductions in CAT activity (53% and 47% respectively) when compared to non-treated plants. The lowest CAT activity value (6.50 U/mg of total proteins) was recorded in *N. nervosa* plants at 6DPT (Figure 3C).

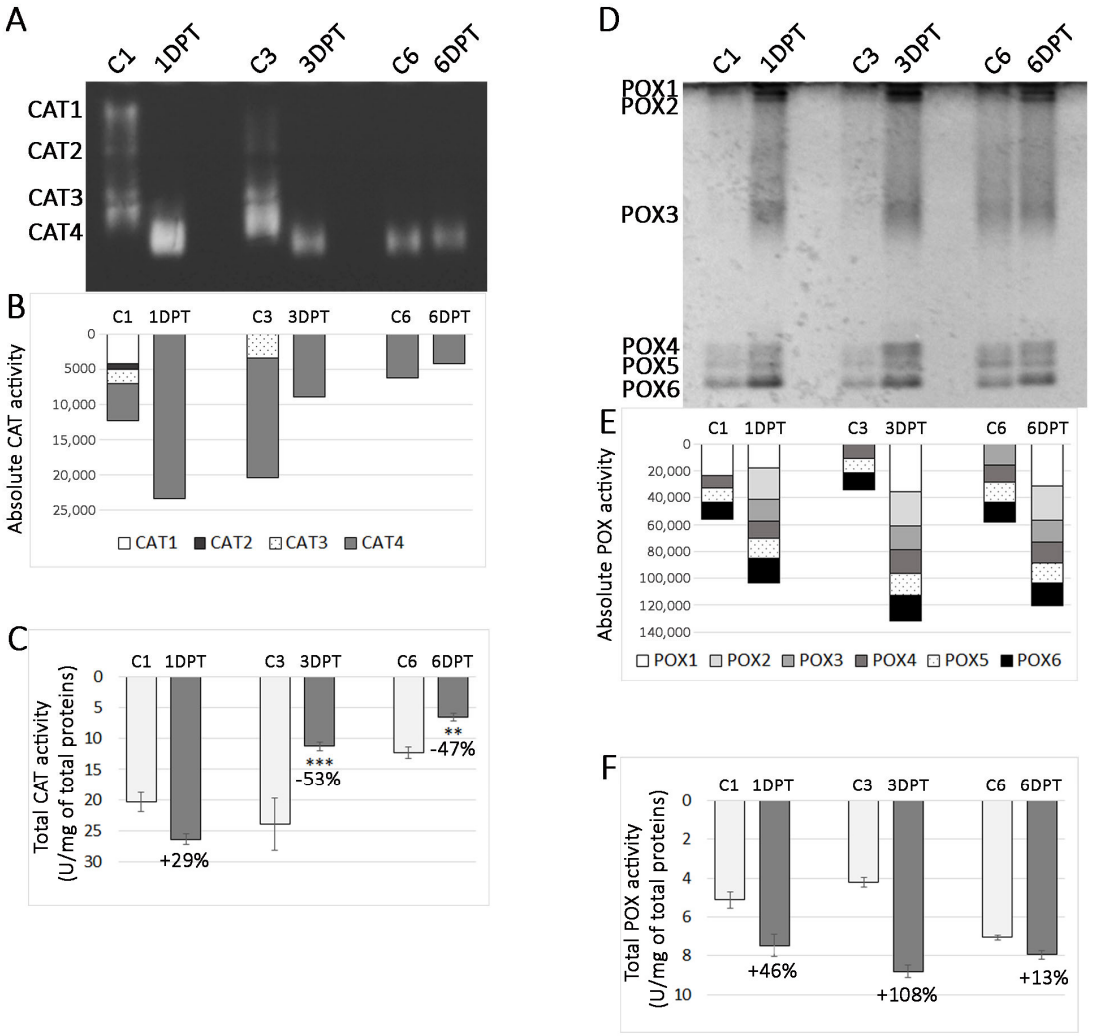

Figure 3. Catalases (CAT) and peroxidases (POX) activity in non-treated (C1, C3 and C6) and PEG-treated (1DPT, 3DPT and 6DPT) *N. nervosa* plants for 1, 3 and 6 days. (**A**) For the CAT assay, 5 µg of total soluble proteins extracted from the whole plant was loaded per lane on a 7% gel and separated by native-PAGE. (**B**) The detected activities were measured densitometrically by the Image J program and presented as absolute values. (**C**) Total CAT activity was measured spectrometrically. (**D**) For the POX assay, 20 µg of total soluble proteins extracted from the whole plant was loaded per lane on a 10% gel and separated by native-PAGE. (**E**) The detected activities were measured densitometrically by the Imaging System iBRIGHT CL1500 and presented as absolute values. (**F**) Total POX activity was measured spectrometrically. The results represent the mean ± SE of three biological replicates. Asterisks (*) indicate significant differences between PEG-treated and non-treated plants according to an unpaired *t*-test (** $p \leq 0.01$; *** $p \leq 0.001$). The numbers below the bars indicate the percent of enhancement (+)/reduction (−) relative to the control.

The staining of native-PAGE gels for POX activity revealed the presence of six isoforms in all PEG-treated *N. nervosa* plants, while non-treated plants exhibited three isoforms at 3DPT and four isoforms at the 1DPT and 6DPT (Figure 3D,E). Spectrophotometric measurement of POX activity demonstrated an increase ranging from 13% to 108% depending on the duration of PEG treatment. Specifically, the activity increased by 108% at 3DPT and by 13% at 6DPT compared to non-treated plants. The measured values of POX activity ranged from 7.47 to 8.82 U/mg of total proteins in *N. nervosa* plants subjected to PEG treatment for 1, 3 and 6 days (Figure 3F).

Staining native gels for SOD activity revealed the presence of four isoforms in both PEG-treated and non-treated *N. nervosa* plants at 1, 3 and 6 days (Figure 4A,B). Through the use of specific inhibitors (KCN and H_2O_2), Mn-SOD and Fe-SOD isoforms were distinguished (Figure 4D,E). Among the samples, one Fe-SOD and three Mn-SOD isoforms (Mn-SOD1, Mn-SOD2 and Mn-SOD3) were identified, with Mn-SOD3 exhibiting the highest activity in PEG-treated *N. nervosa* plants across all treatment durations (Figure 4A,B,D,E). The total SOD activities in non-treated plants remained similar in all experimental points, measuring approximately 5 U/mg of total proteins. However, in PEG-treated plants, the total SOD activities significantly increased, ranging from 78% to as high as 853% with the extension of the treatment duration from 1 to 6 days, compared to the non-treated control (Figure 4C).

Previous studies have extensively reported the efficient role of CAT and POX enzymes in effectively removing intracellular H_2O_2 by converting it into H_2O and O_2 [63]. In our study, we observed a significant increase in POX activity in the leaves of *N. nervosa* plants in all experimental points compared to the control. The observed pattern of POX activity correlates with the concentration of RA throughout the entire experiment. Interestingly, except for at 1DPT, CAT activity was significantly decreased upon PEG treatment, suggesting that H_2O_2 elimination is predominantly carried out by POX. This finding aligns with similar trends observed in wheat, where CAT activities increased during the early phase of drought stress but decreased with prolonged water-stress duration [64]. Moreover, [65] demonstrated an increase in CAT activity under PEG-mediated drought stress in the shoots and roots of two sunflower cultivars (Musala and Aydın), which is consistent with our results for *N. nervosa* PEG-treated plants at 1DPT. In *Nepeta pannonica* shoot cultures, the activities of the oxygen scavenging enzymes CAT and POX were progressively enhanced in response to oxidative stress induced by increasing concentrations of paraquat [51].

Superoxide dismutase is a well-known enzyme that plays a vital role in neutralizing harmful oxygen molecules ($O_2^{\cdot}$) by converting them into less reactive species such as H_2O_2 and H_2O. In our study, the activity of SOD, particularly the MnSOD3 isoform, was significantly elevated in the leaves of *N. nervosa* plants treated with PEG when compared to the control (up to an 8.5-fold increase at 6DPT). This substantial increase in SOD activity highlights the robust effort of the enzyme, particularly MnSOD3, in scavenging cellular free radicals. This finding aligns with previous research where PEG-induced water stress also resulted in increased SOD activities in sugarcane leaves [66].

The findings of our study reveal an interesting phenomenon in *N. nervosa* leaves, where PEG treatment leads to an increased number of POX and SOD isoforms compared to the corresponding controls. On the other hand, the number of CAT isoforms remains either the same or shows a lesser increase. Additionally, we observed that the most mobile isoforms of CAT, POX and SOD exhibited the highest activities in PEG-treated *N. nervosa* plants for 1, 3 and 6 days. This finding is significant because it indicates a dynamic response of antioxidative enzymes to PEG-induced water stress. The increased number of POX and SOD isoforms suggests an enhanced capacity to scavenge ROS and protect the plant cells from oxidative damage. Furthermore, the high activities of these isoforms in PEG-treated plants indicate their crucial role in combating ROS and maintaining cellular homeostasis under water-stress conditions.

These findings are consistent with previous research by [47], who demonstrated increased activities of CAT, SOD and POX in sweet basil leaves under drought stress.

Similarly, Sarker et al. 2018 [67] confirmed that CAT and SOD play important roles in detoxifying ROS in both sensitive and tolerant genotypes of *Amaranthus tricolor* under drought stress. Collectively, these studies emphasize the importance of antioxidative enzymes in mitigating the detrimental effects of drought stress and highlight their potential as targets for enhancing plant stress tolerance in agricultural practice.

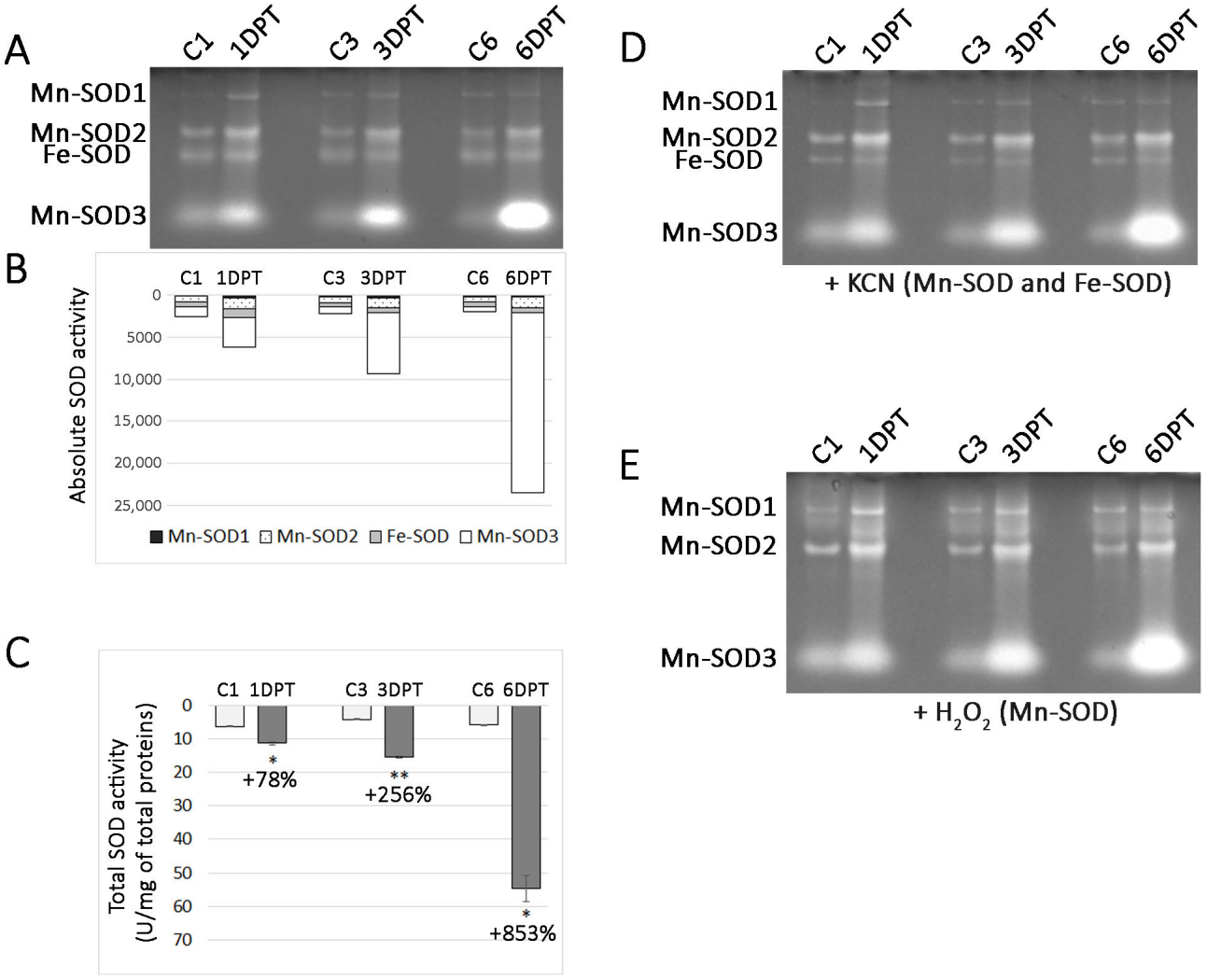

Figure 4. Superoxide dismutase (SOD) activity in non-treated (C1, C3 and C6) and PEG-treated (1DPT, 3DPT and 6DPT) *N. nervosa* plants for 1, 3 and 6 days. (**A**) For the SOD assay, 20 µg of total soluble proteins extracted from the whole plant was loaded per lane on 10% gel and separated by native-PAGE. (**B**) The detected activities were measured densitometrically by the Image J program and presented as absolute values. (**C**) Total SOD activity was measured spectrometrically. (**D**,**E**) Identification of SOD isoforms (Mn-SOD and Fe-SOD) based on differential sensitivity to inhibitors. The results represent the mean ± SE of three biological replicates. Asterisks (*) indicate significant differences between PEG-treated and non-treated plants according to an unpaired *t*-test (* $p \leq 0.05$; ** $p \leq 0.01$). The numbers below the bars indicate the percent of enhancement (+) relative to the control. The results represent the mean ± SE of three biological replicates.

4. Conclusions

In conclusion, our study on PEG-induced dehydration in *Nepeta nervosa* plants grown in vitro has unveiled a comprehensive understanding of both enzymatic and non-enzymatic antioxidant protective mechanisms. The enhanced enzymatic antioxidative defense mechanisms, particularly POX and SOD, provide insights into stress tolerance and plant adaptability. While CAT activity increased initially (1DPT) but decreased with prolonged treatment,

POX and SOD (especially the Mn-SOD3 isoform) activities exhibited a substantial increase at 3DPT and 6DPT, underscoring their roles in radical scavenging activity. Furthermore, the significant increases in RA at 3DPT and CHLA at 3DPT and 6DPT, along with CAFFA at 6DPT, have revealed the predominant non-enzymatic antioxidant components during water-stress conditions in *N. nervosa*. The increase in RA, a valuable phenolic compound, at 3DPT suggests the potential for scaling up its production for applications in pharmaceuticals, cosmetics and nutraceuticals. Overall, our findings contribute to the understanding of *N. nervosa* responses to dehydration and offer promising avenues for industrial applications related to phenolic compounds, antioxidative defense and plant stress physiology.

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