



Effects of Combined Drought and Salinity Stress on Oxidative Damage and the Antioxidant Enzyme System in Aronia (*Aronia melanocarpa* L.) 'Nero' under *In Vitro* Conditions

In Vitro Koşullarda Kombine Kuraklık ve Tuz Stresinin
'Nero' Aronya (*Aronia melanocarpa* L.) Çeşidinde
Oksidatif Hasar ve Antioksidan Enzim Sistemi Üzerine
Etkisi

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EFFECTS OF COMBINED DROUGHT AND SALINITY STRESS ON OXIDATIVE DAMAGE AND THE ANTIOXIDANT ENZYME SYSTEM IN ARONIA (*ARONIA MELANOCARPA* L.) 'NERO' UNDER *IN VITRO* CONDITIONS

ABSTRACT

This study investigated the effects of single and combined drought (10% PEG-6000) and salinity (100 mM NaCl) stress on oxidative injury, antioxidant defense, and physiological responses of aronia (*Aronia melanocarpa* L.) 'Nero' under *in-vitro* conditions. After 14 days, combined stress induced markedly stronger biochemical disruption than individual treatments. Malondialdehyde (MDA) increased from 8.4 nmol g⁻¹ FW in the control to 14.7 (PEG) and 16.2 (NaCl), reaching 24.8 nmol g⁻¹ FW under combined stress. Hydrogen peroxide (H₂O₂) rose from 3.2 to 11.6 µmol g⁻¹ FW in the dual treatment, indicating intensified reactive oxygen species accumulation. Antioxidant enzymes were strongly upregulated in response to stress. SOD activity increased from 62.4 to 138.6 U mg⁻¹ protein under combined stress, while CAT (21.8 U mg⁻¹ protein), APX (13.2 U mg⁻¹ protein), GR (9.7 U mg⁻¹ protein), and POD (29.4 U mg⁻¹ protein) also showed their highest activities in the dual treatment. Non-enzymatic antioxidants followed a similar pattern: proline increased nearly fivefold (1.8 to 8.7 µmol g⁻¹ FW), total phenolics rose from 5.4 to 13.8 mg GAE g⁻¹ FW, flavonoids reached 5.2 mg CE g⁻¹ FW, and DPPH scavenging activity increased from 38.6% to 64.3%. Despite enhanced antioxidant responses, oxidative markers remained elevated under combined stress. Relative water content declined from 91.4% to 61.3%, and SPAD values decreased from 42.5 to 28.9, indicating impaired water status and chlorophyll stability. Simultaneous osmotic and ionic stress, therefore, imposes a substantially greater redox and physiological burden than single stress exposure in aronia 'Nero'. Future studies should evaluate different stress intensities and exposure durations across various aronia cultivars and under field conditions, while also investigating the molecular and metabolic mechanisms underlying tolerance to combined stress.

Keywords: Combined Abiotic Stress, Oxidative Damage, Antioxidant Enzyme System, Phenolic Metabolism, *In Vitro* Culture.



IN VITRO KOŞULLARDA KOMBİNE KURAKLIK VE TUZ STRESİNİN 'Nero' ARONYA (*ARONIA MELANOCARPA L.*) ÇEŞİDİNDE OKSİDATİF HASAR VE ANTIOKSİDAN ENZİM SİSTEMİ ÜZERİNE ETKİSİ ÖZ

Bu çalışma, *in vitro* koşullarda aronya (*Aronia melanocarpa* L.) 'Nero' çeşidinde tekil ve kombine kuraklık (%10 PEG-6000) ile tuz (100 mM NaCl) stresinin oksi-datif hasar, antioksidan savunma sistemi ve fizyolojik yanıtlar üzerindeki etkilerini araştırmıştır. On dört günlük uygulama sonunda, kombine stresin tekil uygulama-lara kıyasla belirgin düzeyde daha güçlü biyokimyasal bozulmaya yol açtığı belir-lenmiştir. Malondialdehit (MDA) içeriği kontrolde 8.4 nmol g⁻¹ TA iken, PEG uy-gulamasında 14.7 ve NaCl uygulamasında 16.2 nmol g⁻¹ TA'ya yükselmiş, kombine stres altında ise 24.8 nmol g⁻¹ TA'ya ulaşmıştır. Hidrojen peroksit (H₂O₂) düzeyi ise 3.2 µmol g⁻¹ TA'dan çift stres uygulamasında 11.6 µmol g⁻¹ TA'ya yükselerek reaktif oksijen türlerinin belirgin birikimini göstermiştir. Antioksidan enzim aktiviteleri stres koşullarında güçlü biçimde artış göstermiştir. SOD aktivitesi kombine stres altında 62.4'ten 138.6 U mg⁻¹ protein'e yükselmiştir. Benzer şekilde CAT (21.8 U mg⁻¹ protein), APX (13.2 U mg⁻¹ protein), GR (9.7 U mg⁻¹ protein) ve POD (29.4 U mg⁻¹ protein) aktiviteleri de en yüksek değerlere kombine stres uygulamasında ulaşmıştır. Enzimatik olmayan antioksidanlar da benzer bir eğilim göstermiştir. Prolin miktarı yaklaşık beş kat artarak 1.8'den 8.7 µmol g⁻¹ TA'ya yükselmiştir. Toplam fenolik içerik 5.4'ten 13.8 mg GAE g⁻¹ TA'ya çıkmış, flavonoid içeriği 5.2 mg CE g⁻¹ TA'ya ulaşmış ve DPPH radikal süpürme aktivitesi %38.6'dan %64.3'e yükselmiştir. Bununla birlikte, antioksidan yanıtların artmasına rağmen oksida-tif belirteçlerin kombine stres altında yüksek kalması dikkat çekmiştir. Relatif su içeriği % 91.4'ten %61.3'e gerilemiş, SPAD değerleri ise 42.5'ten 28.9'a düşerek su durumu ve klorofil stabilitesinde belirgin bir bozulmaya işaret etmiştir. Elde edi-len bulgular, ozmotik ve iyonik stresin eş zamanlı uygulanmasının aronya 'Nero' bitkisinde tekil stres uygulamalarına kıyasla daha ağır bir redoks ve fizyolojik yük oluşturduğunu göstermektedir. Gelecekteki çalışmalarda farklı stres şiddetleri ve uygulama sürelerinin, farklı aronya çeşitleri ve tarla koşulları altında değerlendirilmesiyle birlikt kombine strese toleransın altında yatan moleküler ve metabolik mekanizmaların araştırılması önerilmektedir.

Anahtar Kelimeler: Kombine Abiyotik Stres, Oksidatif Hasar, Antioksidan En-zim Sistemi, Fenolik Metabolizma, *In Vitro* Kültür.



1. INTRODUCTION

Abiotic stress factors are among the primary environmental constraints limiting plant growth and agricultural productivity worldwide. Among these, drought and salinity are particularly critical, as their frequency and intensity have increased due to global climate change and unsustainable irrigation practices (Melino and Tester, 2023; An et al., 2025; Ray, 2025; Badavath and Saski, 2026; Mishra, 2026). In natural and agricultural ecosystems, plants are frequently exposed to multiple stress factors simultaneously rather than individually, and combined stress conditions often induce more complex and severe physiological responses than single stresses (Sánchez-Bermúdez et al., 2022; Dixit et al., 2024; Alptekin and Kunkowska, 2025; Jiang et al., 2025). Drought and salinity stress primarily disrupt cellular homeostasis by inducing osmotic imbalance and ionic toxicity. These disturbances lead to excessive production of reactive oxygen species (ROS), including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$) (Thakur and Garg, 2022; Saharaya et al., 2023; Yadav et al., 2024). Although ROS act as signaling molecules at controlled levels, their overaccumulation results in oxidative damage to lipids, proteins, and nucleic acids (Mittler et al., 2022; Sood, 2025; Wu et al., 2025; Ou et al., 2026; Zhu et al., 2026). Lipid peroxidation is one of the major consequences of oxidative stress, and malondialdehyde (MDA) content is widely used as a reliable indicator of membrane damage under stress conditions (Toyokuni et al., 2022; Aggarwal et al., 2025; Oliwa et al., 2026). To counteract oxidative damage, plants have evolved sophisticated antioxidant defense systems comprising enzymatic and non-enzymatic components. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and peroxidase (POD) play pivotal roles in detoxifying ROS and maintaining redox balance (Rane et al., 2022; Shehzad and Mustafa, 2023; ul Islam et al., 2023; Nagar et al., 2026). SOD catalyzes the dismutation of superoxide radicals into H_2O_2 , which CAT and APX subsequently scavenge, while GR maintains the reduced glutathione pool necessary for redox cycling (Foyer and Kunert, 2024; Noctor et al., 2024; Maiti et al., 2025). In addition to enzymatic antioxidants, non-enzymatic metabolites such as proline and phenolic compounds contribute significantly to stress tolerance. Proline accumulation functions in osmotic adjustment, ROS scavenging, and membrane stabilization under drought and salinity stress (Dubrovna et al., 2022; El Moukhtari et al., 2023; El Moukhtari et al., 2025). Phenolic compounds, synthesized via the phenylpropanoid pathway, possess strong antioxidant properties and are involved in stress signaling and defense responses (Wagay et al., 2023). Enhanced phenolic accumulation under abiotic stress has been reported in several fruit crops, suggesting a close relationship between secondary metabolism and stress adaptation (Sathi et al., 2022). Aronia is a fruit species characterized by exceptionally high levels of phenolic compounds and strong antioxidant capacity (Sidor and Gramza-Michałowska, 2019; Jurendić

and Ščetar, 2021). Owing to its rich anthocyanin and polyphenol content, it has attracted considerable attention as a functional food crop (Kulling and Rawel, 2008). While several studies have evaluated its phytochemical composition and antioxidant potential, limited information is available on its physiological and biochemical responses to combined abiotic stress (Nas et al., 2023; Ekinci et al., 2024). Most existing studies have focused on either drought or salinity stress individually, leaving a significant knowledge gap concerning the interactive effects of these stresses on oxidative balance and antioxidant defense mechanisms (Angon et al., 2022).

In vitro culture systems provide a controlled experimental platform to simulate abiotic stress conditions and to investigate plant responses with high reproducibility (Martins et al., 2018; Parween et al., 2025). The use of polyethylene glycol (PEG) to induce osmotic stress and sodium chloride (NaCl) to impose salinity stress *in-vitro* enables precise assessment of stress-induced oxidative responses without field-related environmental variability. Therefore, the present study aimed to evaluate the effects of combined drought (PEG-induced) and salinity (NaCl-induced) stress on oxidative damage, antioxidant activities, and phenolic metabolism in aronia 'Nero' under *in vitro* conditions. It was hypothesized that combined stress would induce a stronger oxidative response than single stresses and trigger coordinated modulation of enzymatic and non-enzymatic antioxidant defense systems.

2. MATERIAL AND METHOD

2.1 Plant Material and In-vitro Culture Conditions

Healthy, disease-free mother plants of aronia (*Aronia melanocarpa* L.) cv. 'Nero', obtained during the 2025 growing season, were used as plant material. Young shoot tips actively growing were selected as explants. After collection, shoot tips were subjected to a preliminary washing step under running tap water for 20 min. Surface sterilization was performed in a laminar airflow cabinet. Explants were first immersed in 70% (v v⁻¹) ethanol for 30 s, followed by treatment with 2% (v v⁻¹) sodium hypochlorite solution containing a few drops of Tween-20 for 12 min. Subsequently, explants were rinsed three times with sterile distilled water to remove residual sterilizing agents (George, 2008). Sterilized shoot tips were inoculated onto Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962). The culture medium contained 3% (w v⁻¹) sucrose and 0.7% (w v⁻¹) agar. The pH was adjusted to 5.8 using 1 N NaOH or 1 N HCl prior to autoclaving at 121°C for 20 min. Explants were pre-cultured for 4 weeks, and well-developed, and well-developed healthy plantlets were used for stress treatments. Subcultures were performed at four-week intervals. Cultures were maintained at 25 ± 2°C under a 16 h light / 8 h dark photoperiod with a light intensity of approximately 45 µmol m⁻² s⁻¹ provided by fluorescent lamps.

2.2 Stress Treatment

Four treatment groups were established: a non-stressed control, PEG-6000-induced drought stress, NaCl-induced salinity stress, and a combined drought and salinity stress treatment in which PEG-6000 and NaCl were applied simultaneously. Drought stress was imposed by adding 10% (w v⁻¹) PEG-6000 (Sigma-Aldrich, USA) to the culture medium to reduce its water potential. This concentration was selected based on literature reports and preliminary experiments indicating that it induces moderate osmotic stress under *in vitro* conditions (Lawlor, 1970; Michel and Kaufmann, 1973; Ferreira et al., 2025; Okumuş et al., 2025). Salinity stress was applied by supplementing the nutrient medium with 100 mM NaCl. In the combined stress treatment, 10% (w v⁻¹) PEG-6000 and 100 mM NaCl were added simultaneously to the medium. Stress treatments were maintained for 14 days under constant culture conditions. The experiment was conducted in a completely randomized design with three biological replicates, each consisting of at least 10 plantlets. For biochemical and physiological analyses, plantlets were randomly sampled at the end of the stress period, and all measurements were performed on the same day.

2.3 Determination of Oxidative Stress

All spectrophotometric analyses were carried out using a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) with quartz cuvettes of 1 cm optical path length. Measurements were performed at room temperature.

Malondialdehyde (MDA): Lipid peroxidation was determined according to Heath and Packer (1968). Approximately 0.5 g of fresh leaf tissue was homogenized in liquid nitrogen with 5 mL of 0.1% (w v⁻¹) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 x g for 15 min at 4°C. One milliliter of the supernatant was mixed with 0.5% (w v⁻¹) thiobarbituric acid (TBA) prepared in 20% (w v⁻¹) TCA. The mixture was incubated at 95°C for 30 min and then rapidly cooled in an ice bath. After centrifugation at 10,000 x g for 10 min, absorbance was measured at 532 nm, and nonspecific turbidity was corrected by subtracting the absorbance at 600 nm.

MDA concentration was calculated using the following formula:

$$\text{MDA (nmol mL}^{-1}\text{)} = [(A_{532} - A_{600}) / 155.000] \times 10^6$$

Results were expressed as nmol g⁻¹ fresh weight (FW).

Hydrogen Peroxide (H_2O_2): H_2O_2 content was determined following Velikova et al. (2000). Approximately 0.5 g of fresh leaf tissue was homogenized in 0.1% (w v⁻¹) TCA and centrifuged at 12.000 x g for 15 min. A 0.5 mL aliquot of the supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). The reaction mixture was incubated in the dark for 30 min. Absorbance was measured at 390 nm, and H_2O_2 concentration was calculated using a standard curve and expressed as $\mu\text{mol g}^{-1}$ FW.

Antioxidant Enzyme Assays (AEA): For enzyme extraction, 0.5 g of fresh leaf tissue was ground in liquid nitrogen and homogenized with 5 mL of ice-cold extraction buffer containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 1% (w v⁻¹) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15.000 x g for 20 min at 4°C, and the supernatant was used as the enzyme extract. All enzyme activities were normalized to protein content. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard. Enzyme activities were expressed as U mg⁻¹ protein.

Superoxide Dismutase (SOD): SOD activity was determined according to Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 2 μM riboflavin, and 0.1 mM EDTA. The mixture was exposed to fluorescent light for 15 min, and absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT reduction.

Catalase (CAT): CAT activity was determined according to Aebi (1984). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2 . The decrease in absorbance at 240 nm due to H_2O_2 decomposition was monitored for 1 min. Activity was calculated using an extinction coefficient of 39.4 mM⁻¹ cm⁻¹.

Ascorbate Peroxidase (APX): APX activity was measured according to Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and 0.1 mM H_2O_2 . The decrease in absorbance due to ascorbate oxidation was monitored at 290 nm.

Glutathione Reductase (GR): GR activity was determined following Foyer and Halliwell (1976). The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.5 mM oxidized glutathione (GSSG), and 0.12 mM NADPH. The decrease in NADPH absorbance was recorded at 340 nm.

Peroxidase (POD): POD activity was determined using a reaction mixture containing 50 mM phosphate buffer (pH 6.0), 20 mM guaiacol, and 40 mM H_2O_2 . The increase in absorbance due to tetraguaiacol formation was measured at 470 nm (Chance and Maehly, 1955).

Non-Enzymatic Antioxidants (NEA): Proline content was determined according to the method of Bates et al. (1973). The extract was reacted with acidic ninhydrin and glacial acetic acid, incubated at 100°C for 1 h, and after extraction with toluene, absorbance was measured at 520 nm.

Total Phenolic Content (TPC): TPC was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Briefly, 0.5 mL of methanolic extract was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent. After 5 minutes, 2 mL of 7.5% Na₂CO₃ was added, and the mixture was incubated for 30 minutes. Absorbance was measured at 765 nm.

Total Flavonoid Content (TFC): TFC was determined using the aluminum chloride (AlCl₃) colorimetric method. To 0.5 mL of methanolic extract, 1.5 mL of 95% methanol, 0.1 mL of 10% (w v⁻¹) AlCl₃ solution, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were added. The reaction mixture was incubated at room temperature in the dark for 30 minutes. Absorbance was measured at 415 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). A calibration curve was prepared using catechin standard solutions, and the results were expressed as mg catechin equivalent (mg CE g⁻¹ FW). All analyses were performed in triplicate.

DPPH Radical Scavenging Activity (DPPH-RSA): DPPH radical scavenging activity was determined according to the method of Brand-Williams et al. (1999). A 0.1 mM DPPH solution was prepared in methanol. One milliliter of plant extract was mixed with 3 mL of DPPH solution and incubated in the dark at room temperature for 30 minutes. After incubation, absorbance was measured at 517 nm. Methanol was used as the control instead of the extract.

The radical scavenging percentage was calculated using the following formula:

$$\text{Radical Scavenging Activity (\%)} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

Where: A_{control} = The absorbance value of the DPPH solution.

A_{sample} = The absorbance value of the reaction mixture containing the extract.

In addition, antioxidant capacity was expressed using a Trolox standard calibration curve as follows:

Antioxidant Capacity = μmol TE g⁻¹ FW

Where, the results were calculated as Trolox equivalents using the standard curve equation.

2.4 Determination of Physiological Parameters

Relative Water Content (RWC): Relative water content (RWC) of the leaves was determined according to the method of Barrs and Weatherley (1962). Fresh weights (FW) of randomly selected leaf samples from each treatment were measured immediately using an analytical balance. The leaves were then placed in Petri dishes containing distilled water and kept at room temperature for 4 h to obtain their fully turgid weight (TW). After measuring the turgid weight, the samples were dried in an oven at 70°C for 48 h until constant weight was reached, and the dry weight (DW) was recorded.

RWC was calculated using the following formula:

$$RWC (\%) = \left(\frac{FW - DW}{TW - DW} \right) \times 100$$

Where:

FW= fresh weight of the leaf, TW= fully turgid weight, DW= dry weight

The results were expressed as percentage (%).

Leaf Chlorophyll Content (SPAD): Leaf chlorophyll content was determined using a nondestructive method with a portable chlorophyll meter (SPAD-502 Plus, Konica Minolta, Japan). Measurements were taken from three different points on each leaf, avoiding the midrib, and the average value was recorded as SPAD units. At least 10 plantlets were evaluated per treatment group, and the results were expressed as mean $\pm$ standard error (SE). SPAD measurements are widely accepted as a rapid and reliable indicator of relative chlorophyll content in plants and are commonly used in stress physiology studies.

2.5 Statistical Analysis

All experiments were conducted using a completely randomized design with three biological replicates. Data were subjected to one-way analysis of variance (ANOVA) using SAS software (Version 9.4, SAS Institute Inc., Cary, NC, USA). When significant differences were detected, mean comparisons were performed using Tukey's multiple range test at the 0.05 probability level. Results are presented as mean $\pm$ standard error (SE). Differences were considered statistically significant at $p < 0.05$. In addition, hierarchical clustering and heat map visualization were performed to evaluate multivariate relationships among treatments and measured parameters. Heat map analysis was conducted using Python (Version 3.9) with the commonly used scientific libraries pandas, NumPy, seaborn, and matplotlib. Prior to analysis, data were standardized using Z-score normalization to eliminate scale differences among variables. The heat map was generated using the Euclidean distance and Ward's linkage methods.

3. RESULTS AND DISCUSSION

The effects of drought (PEG-induced), salinity (NaCl-induced), and their combined application on oxidative damage, antioxidant defense mechanisms, and physiological responses in aronia were evaluated under *in vitro* conditions. Significant variations were observed among treatments, indicating differential stress responses depending on the type and combination of abiotic stress factors. The results are presented and discussed below in relation to oxidative injury, enzymatic and non-enzymatic antioxidant responses, and physiological adjustments.

3.1 Oxidative Damage (MDA and H₂O₂)

As presented in Table 1, both PEG-induced drought and NaCl-induced salinity significantly increased lipid peroxidation compared to the control. MDA content increased from 8.4 nmol g⁻¹ FW in the control to 14.7 and 16.2 nmol g⁻¹ FW under PEG and NaCl treatments, respectively. The combined PEG + NaCl treatment resulted in the highest MDA level (24.8 nmol g⁻¹ FW), representing nearly a threefold increase relative to the control. This pronounced elevation indicates aggravated membrane lipid peroxidation under simultaneous osmotic and ionic stress conditions. A similar trend was observed for H₂O₂ accumulation (Table 1). Control plants exhibited 3.2 μmol g⁻¹ FW H₂O₂, whereas PEG and NaCl treatments increased H₂O₂ levels to 6.9 and 7.5 μmol g⁻¹ FW, respectively. The combined stress treatment caused a marked rise to 11.6 μmol g⁻¹ FW, indicating intensified oxidative pressure. The significant differences among treatments suggest that dual stress exposure amplifies ROS production beyond the levels induced by individual stresses. The concurrent increase in both MDA and H₂O₂ under combined stress demonstrates that aronia experiences substantially greater oxidative injury when exposed to simultaneous drought and salinity. The nearly threefold rise in MDA and the more than threefold increase in H₂O₂ relative to the control highlight the synergistic nature of combined stress. These findings suggest that the interaction between osmotic imbalance and ionic toxicity enhances ROS generation, exceeding the antioxidant defense system's basal detoxification capacity.

Table 1. Effects of single and combined stress treatments on oxidative damage parameters in aronia

Treatment	MDA (nmol g ⁻¹ FW)	H ₂ O ₂ (μmol g ⁻¹ FW)
Control	8.4 ± 0.6 ^c	3.2 ± 0.3 ^c
PEG	14.7 ± 1.1 ^b	6.9 ± 0.5 ^b
NaCl	16.2 ± 1.3 ^{ab}	7.5 ± 0.6 ^{ab}
PEG + NaCl	24.8 ± 1.9 ^a	11.6 ± 0.8 ^a

Values are mean ± SE (n = 3). Different letters within a column indicate significant differences at p < 0.05 according to Tukey's test.

3.2 Antioxidant Enzyme Activities (SOD, CAT, APX, GR, POD)

Stress treatments significantly influenced antioxidant enzyme activities in *Aronia melanocarpa* (Table 2). All stress applications induced marked increases in SOD, CAT, APX, GR, and POD activities compared to the control, indicating activation of enzymatic defense mechanisms in response to elevated oxidative pressure. SOD activity increased from 62.4 U mg⁻¹ protein in the control to 98.7 and 104.3 U mg⁻¹ protein under PEG and NaCl treatments, respectively. The highest SOD activity (138.6 U mg⁻¹ protein) was observed under combined stress, suggesting enhanced dismutation of superoxide radicals into H₂O₂. CAT activity followed a similar pattern, increasing significantly under stress conditions. While single stresses induced moderate elevations, the combined treatment resulted in the greatest CAT activity (21.8 U mg⁻¹ protein), indicating increased capacity for H₂O₂ detoxification. APX and GR activities were also significantly enhanced under stress treatments, reflecting activation of the ascorbate-gluthathione cycle. Notably, GR activity showed the greatest increase under combined stress, suggesting intensified redox cycling to maintain glutathione homeostasis. POD activity showed a strong increase across all stress treatments, with the highest level observed under the PEG + NaCl treatment. The coordinated upregulation of SOD, CAT, APX, GR, and POD indicates that aronia activates a comprehensive enzymatic antioxidant defense system under abiotic stress conditions.

Table 2. Effects of stress treatments on antioxidant enzyme activities in aronia

Treatment	SOD (U mg ⁻¹ protein)	CAT (U mg ⁻¹ protein)	APX (U mg ⁻¹ protein)	GR (U mg ⁻¹ protein)	POD (U mg ⁻¹ protein)
Control	62.4 ± 4.1 ^c	9.6 ± 0.7 ^c	4.8 ± 0.4 ^c	3.2 ± 0.3 ^c	12.5 ± 1.0 ^c
PEG	98.7 ± 6.5 ^b	15.3 ± 1.1 ^b	8.6 ± 0.6 ^b	6.1 ± 0.4 ^b	19.8 ± 1.4 ^b
NaCl	104.3 ± 7.2 ^{ab}	16.7 ± 1.3 ^{ab}	9.4 ± 0.7 ^{ab}	6.8 ± 0.5 ^b	21.5 ± 1.6 ^{ab}
PEG + NaCl	138.6 ± 9.3 ^a	21.8 ± 1.6 ^a	13.2 ± 0.9 ^a	9.7 ± 0.6 ^a	29.4 ± 2.1 ^a

Values are mean ± SE (n = 3). Different letters within a column indicate significant differences at p < 0.05 according to Tukey's test.

3.3 Non-Enzymatic Antioxidants

Marked alterations were detected in non-enzymatic antioxidant metabolites in response to single and combined stress treatments (Table 3). Proline concentration increased substantially under stress conditions, rising from 1.8 µmol g⁻¹ FW in control plants to 4.9 and 5.3 µmol g⁻¹ FW under PEG and NaCl treatments, respectively. The most pronounced accumulation was observed in the combined PEG + NaCl treatment (8.7 µmol g⁻¹ FW), corresponding to a nearly fivefold increase compared with the control. Such a sharp increase highlights the central role of proline in osmotic regulation and cellular protection under intensified dehydration and oxidative stress.

A consistent enhancement was also observed in total phenolic content (Table 3). Control samples contained 5.4 mg GAE g⁻¹ FW, whereas phenolic levels increased to 8.7 mg GAE g⁻¹ FW under PEG and 9.2 mg GAE g⁻¹ FW under NaCl stress. Under combined stress conditions, total phenolics reached 13.8 mg GAE g⁻¹ FW, representing more than a twofold increase relative to single stresses and approximately a 2.5-fold rise compared to the control. This substantial enrichment suggests a strong stimulation of phenylpropanoid metabolism as part of the plant's secondary defense strategy against excessive ROS accumulation. Flavonoid content followed a comparable pattern but was quantitatively less pronounced. While control plants exhibited 2.1 mg CE g⁻¹ FW, stress treatments elevated flavonoid levels to 3.6 and 3.9 mg CE g⁻¹ FW under PEG and NaCl, respectively. The combined treatment further increased flavonoid concentration to 5.2 mg CE g⁻¹ FW. Although the relative increase was lower than that observed for total phenolics, this accumulation reflects reinforcement of low-molecular-weight antioxidant defenses contributing to ROS neutralization and membrane stabilization. DPPH radical scavenging activity increased progressively across treatments, from 38.6% in the control to 55.4% (PEG) and 57.8% (NaCl). The highest activity was measured under combined stress (64.3%). Nevertheless, the proportional increase in DPPH activity was modest when compared with the substantial elevation in total phenolics, indicating that despite enhanced antioxidant metabolite synthesis, the severity of combined stress may partially offset the overall radical-scavenging efficiency.

Table 3. Effects of stress treatments on non-enzymatic antioxidant parameters in aronia

Treatment	Proline ($\mu\text{mol g}^{-1}$ FW)	Total Phenolics (mg GAE g ⁻¹ FW)	Flavonoids (mg CE g ⁻¹ FW)	DPPH (%)
Control	1.8 $\pm$ 0.2 ^c	5.4 $\pm$ 0.4 ^c	2.1 $\pm$ 0.2 ^c	38.6 $\pm$ 2.5 ^c
PEG	4.9 $\pm$ 0.4 ^b	8.7 $\pm$ 0.6 ^b	3.6 $\pm$ 0.3 ^b	55.4 $\pm$ 3.1 ^b
NaCl	5.3 $\pm$ 0.5 ^b	9.2 $\pm$ 0.7 ^b	3.9 $\pm$ 0.3 ^b	57.8 $\pm$ 3.4 ^b
PEG + NaCl	8.7 $\pm$ 0.7 ^a	13.8 $\pm$ 1.1 ^a	5.2 $\pm$ 0.4 ^a	64.3 $\pm$ 3.8 ^a

Values are mean $\pm$ SE (n = 3). Different letters within a column indicate significant differences at p < 0.05 according to Tukey's test.

3.4 Physiological Parameters (RWC and SPAD)

As presented in Table 4, both drought and salinity stress significantly reduced relative water content (RWC) compared to the control. Control plants maintained a high RWC value of 91.4%, whereas PEG and NaCl treatments decreased RWC to 74.6% and 72.8%, respectively. The combined PEG + NaCl treatment resulted in the lowest RWC (61.3%), representing approximately a 33% reduction relative to the control. This marked decline indicates severe impairment of water balance under simultaneous osmotic and ionic stress. Similarly, leaf chlorophyll content measured as SPAD values declined significantly under stress treatments (Table 4).

While control plants exhibited a SPAD value of 42.5, PEG and NaCl treatments reduced chlorophyll content to 35.8 and 34.6, respectively. Under combined stress, SPAD values decreased further to 28.9, suggesting enhanced chlorophyll degradation and impaired photosynthetic capacity. The reduction in RWC under stress conditions reflects diminished water uptake and increased cellular dehydration, particularly under combined stress. Concurrently, the decline in SPAD values suggests disruption of chloroplast integrity and possible oxidative degradation of photosynthetic pigments. These physiological impairments are consistent with the elevated MDA and H_2O_2 levels observed under combined stress, indicating that intensified oxidative damage negatively affects water status and photosynthetic performance in aronia.

Table 4. Effects of stress treatments on physiological parameters in aronia

Treatment	RWC (%)	SPAD
Control	91.4 ± 2.1 ^a	42.5 ± 1.8 ^a
PEG	74.6 ± 2.8 ^b	35.8 ± 1.5 ^b
NaCl	72.8 ± 3.0 ^b	34.6 ± 1.7 ^b
PEG + NaCl	61.3 ± 2.9 ^c	28.9 ± 1.6 ^c

Values are mean ± SE (n = 3). Different letters within a column indicate significant differences at $p < 0.05$ according to Tukey's test.

The present findings clearly indicate that combined drought and salinity stress induces more severe oxidative and physiological disruption in aronia than single stress treatments. The nearly threefold increase in MDA content and the substantial rise in H_2O_2 levels under combined stress are consistent with earlier reports demonstrating that simultaneous osmotic and ionic stresses intensify ROS generation beyond additive effects (Zandalinas et al., 2022; Mittler et al., 2025). Similar synergistic increases in lipid peroxidation have been documented in grapevine and tomato subjected to dual stress conditions, where MDA levels under combined stress significantly exceeded those observed under individual drought or salinity treatments (Zandi and Schnug, 2022; Hajam et al., 2023; Shajar et al., 2025). The magnitude of oxidative damage observed in the present study is consistent with the stress sensitivity previously reported for aronia, supporting the interpretation that combined stress imposes amplified membrane injury through enhanced lipid peroxidation and ROS overproduction (Mahmood and Pirlak, 2025).

In response to this oxidative burden, antioxidant enzyme activities were significantly elevated, reflecting activation of the enzymatic ROS-scavenging network. Enhanced SOD activity under drought and salinity stress has been widely reported as an early protective response that converts superoxide radicals into H_2O_2 (Mishra et al., 2023; Foyer and Kunert, 2024). The concurrent increases in CAT and APX activities observed here are consistent with previous findings indicating

coordinated detoxification of H_2O_2 under abiotic stress (Parvin et al., 2022; Nowicka et al., 2023). Notably, the pronounced upregulation of GR suggests intensified operation of the ascorbate-glutathione cycle, which is critical for maintaining redox equilibrium under stress conditions (Chauhan et al., 2022). Comparable activation of the GR-APX system under combined drought and salinity has been reported in strawberry and grapevine, where enhanced antioxidant enzyme activities were associated with partial mitigation of oxidative damage (Belal et al., 2023; Brown, 2025). However, despite this enzymatic reinforcement, oxidative markers remained significantly elevated under combined stress, indicating that ROS production likely exceeded the antioxidant machinery's detoxification capacity under severe dual stress.

The accumulation of non-enzymatic antioxidants further corroborates this stress-induced metabolic reprogramming. The nearly fivefold increase in proline under combined stress aligns with numerous studies demonstrating proline's dual function in osmotic adjustment and ROS scavenging under drought and salinity (Alvarez et al., 2022). Likewise, the substantial enhancement in total phenolics and flavonoids observed in this study reflects activation of the phenylpropanoid pathway, a well-established response to oxidative stress (Molodchenkova and Fannin, 2025). Enhanced phenolic accumulation under drought and salinity has been reported in several fruit crops, including strawberry, grapevine, and apple, suggesting that secondary metabolite biosynthesis constitutes a conserved adaptive strategy (Hasanuzzaman and Fujita, 2022). The observed increase in DPPH radical scavenging activity corresponds with elevated phenolic content, supporting the strong contribution of phenolics to antioxidant capacity (Swain et al., 2025). Nevertheless, the comparatively moderate proportional increase in DPPH activity relative to phenolic accumulation may indicate that severe combined stress partially constrains overall radical-scavenging efficiency, a phenomenon also reported under prolonged or high-intensity stress conditions (Raya et al., 2025; Sinha et al., 2025).

Physiological impairment, reflected by reduced RWC and SPAD values, provides functional evidence of stress severity. Decreased RWC under PEG and NaCl treatments confirms impaired water uptake and osmotic imbalance, consistent with findings in grapevine and tomato under similar stress regimes (Kissoudis et al., 2016; Gupta and Senthil-Kumar, 2017). The reduction in SPAD values suggests chlorophyll degradation and possible oxidative damage to the photosynthetic apparatus, which has been linked to ROS-mediated pigment breakdown under drought and salinity (Arora et al., 2016; Sachdev et al., 2021).

Importantly, the integrative heatmap analysis (Figure 1) provides a holistic visualization of these coordinated responses. The standardized Z-score representation clearly demonstrates a pronounced shift toward elevated oxidative markers and antioxidant activation under combined stress, while physiological parameters (RWC

and SPAD) display a strong inverse pattern. In this representation, red indicates values above the overall mean for each parameter, whereas blue indicates values below the overall mean; therefore, the high RWC and SPAD values in the control appear red, while their marked reductions under PEG + NaCl stress appear blue. Conversely, oxidative damage markers and antioxidant-related parameters are predominantly below the overall mean in the control but above it under combined stress. This contrasting color gradient between biochemical activation and physiological decline highlights the systemic nature of stress-induced disruption. The clustering pattern further indicates that the PEG + NaCl treatment forms a distinct response profile, separated from single-stress treatments, supporting the hypothesis that combined stress triggers a qualitatively amplified and integrated defense response rather than a merely additive effect. Thus, the heatmap not only confirms individual statistical trends but also illustrates the coordinated reprogramming of oxidative, enzymatic, and physiological pathways under dual stress exposure.

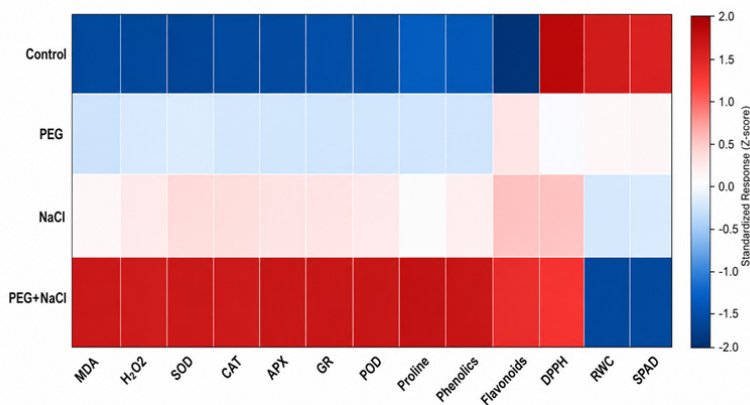


Figure 1. Heatmap illustrating the standardized responses (Z-score) of oxidative damage markers, antioxidant enzyme activities, non-enzymatic antioxidants, and physiological parameters in aronia under control, drought (PEG), salinity (NaCl), and combined (PEG + NaCl) stress conditions. Red indicates standardized values above the mean of each parameter, whereas blue indicates values below the mean. Before visualization, the data were standardized using Z-scores to enable comparisons among variables measured on different scales

CONCLUSION

Combined drought and salinity stress caused a pronounced disruption in oxidative balance and physiological performance in aronia 'Nero' under *in vitro* conditions. The marked increases in MDA and H₂O₂ clearly indicate intensified membrane lipid peroxidation and ROS accumulation when osmotic and ionic

stresses occurred simultaneously. Although both enzymatic and non-enzymatic antioxidant systems were substantially upregulated, the persistence of elevated oxidative markers under combined stress suggests that detoxification mechanisms were only partially effective under severe dual stress exposure. The sharp decline in relative water content and chlorophyll index further demonstrates that oxidative disturbance was accompanied by functional impairment at the physiological level. The coordinated elevation of antioxidant enzymes, together with enhanced proline and phenolic accumulation, reflects a complex defensive adjustment; however, the magnitude of oxidative injury under PEG + NaCl treatment indicates that cellular homeostasis remained under considerable strain. These findings emphasize that evaluating drought or salinity in isolation may underestimate the actual stress burden experienced under natural conditions. In polyphenol-rich species such as *A. melanocarpa*, metabolic plasticity contributes to stress adaptation, yet simultaneous exposure to multiple abiotic constraints can overwhelm protective capacity. Understanding such integrated stress responses is essential for predicting plant performance under increasingly variable climatic scenarios. Future studies should evaluate different stress intensities and exposure durations across various aronia cultivars and under field conditions, while also investigating the molecular and metabolic mechanisms underlying tolerance to combined stress.

Conflict of Interest

The author declares that there is no conflict of interest.

Ethics

This study does not require ethics committee approval.

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