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Research paper

Responses of the model crop plant *Ocimum basilicum* L. grown hydroponically to combined hypoxia and salinity

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ABSTRACT

Basil (*Ocimum basilicum* L., cv Genovese) cultivated under hydroponic conditions was subjected to salinity, hypoxia, and combined stress for varying lengths of time to assess growth, metabolic, and antioxidant responses. Analyses demonstrated that stress type was the primary factor influencing plant responses, with treatment duration playing a secondary modulatory role. Growth parameters revealed organ-specific adaptations, with hypoxia increasing water content mainly in roots and salinity enhancing water retention in aerial organs. The combined stress contributed to the maintenance of tissue hydration. Photosynthetic pigments, soluble sugars, and proteins were found to be significantly affected, with protein accumulation being higher in aerial organs under combined stress and in roots under hypoxia. Growth and photosynthetic responses differed markedly among stress conditions. At T_4, shoot dry biomass under hypoxia was approximately 1.5-fold higher than under control conditions and 8-fold higher than under salinity, while chlorophyll a increased approximately 5-fold compared with T_1. Conversely, under combined hypoxia and salinity, chlorophyll a decreased by approximately 95% from T_1 to T_4. Antioxidant enzyme activities were also strongly modulated by treatment and exposure duration; notably, root CAT activity under the combined treatment at T_4 was approximately 10-fold higher than under control conditions. Phenolic compounds accumulated predominantly under hypoxia and were associated with increased antioxidant capacity. Overall, these findings indicate distinct and time-dependent physiological and biochemical responses of basil to salinity, hypoxia, and their combination.

1. Introduction

Given the rising global population and the increasing demand for food, the sustainable use of water resources has become increasingly important (Ramegowda and Senthil-Kumar, 2015). In this context, hydroponic system has emerged as an innovative and sustainable agricultural approach (Bugbee, 2003), allowing standardized plant production under controlled conditions and improving the use of water, nutrients, and space while supporting crop quality and economic returns (Son et al., 2020). Basil (*Ocimum basilicum* L.), an annual aromatic herb belonging to the Lamiaceae family, is mainly cultivated for its aromatic leaves, which are highly consumed fresh or processed, and valued for its bioactive compounds with wide applications in food, pharmaceutical and cosmetic industries (Corrado et al., 2021; Fedeli et al., 2025). Indeed, the cultivation of high-value crops such as basil (*Ocimum basilicum* L.), has rapidly expanded in recent years (Sapkota et al., 2019), creating novel commercial opportunities for basil growers (Aghamirzaei et al., 2024). Further, basil cultivation in soilless systems has received increasing attention, compared with conventional field agricultural practices. However, despite these advantages, hydroponic cultivation may expose the root system to environmental constraints, particularly inadequate oxygen availability and salinity.

Inadequate oxygen availability in the root zone can lead to hypoxic conditions and adversely affect root metabolism and overall plant function (Bunyuth and Mardy, 2024). Hypoxic conditions are characterized by aerobic respiration and mitochondrial oxidative phosphorylation inhibition (Bailey-Serres and Voesenek, 2008), resulting in reduced ATP production and a metabolic shift toward fermentative

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pathways (Colmer et al., 2020; Behr et al., 2021). This energy limitation has been demonstrated to reduce root development, alter membrane stability, and compromise water and nutrient uptake, ultimately leading to impaired shoot growth, leaf expansion, and photosynthetic efficiency in plants (Salvatierra et al., 2020; Feng et al., 2022). Furthermore, it is recognised that prolonged hypoxia promotes the build-up of toxic metabolites, such as reactive oxygen species (ROS), thus instigating oxidative stress and cellular damage (Colmer and Voesenek, 2009).

Salinity of the nutrient solution is a significant challenge in basil hydroponic culture. Elevated salt concentrations could generate an osmotic stress (Tarchoune et al., 2012; Bernstein et al., 2010), thereby limiting water availability, and also ionic stress due to the accumulation of Na^+ and Cl^- ions. The disruption of ionic homeostasis, interference with the uptake of essential nutrients such as K^+ and $Ca^{2+}$and impairment of key physiological and biochemical processes are all consequences of this (Azimzadeh et al., 2024).

In hydroponic systems, root hypoxia and salinity frequently co-occur, especially under long-term cultivation, creating a complex stress environment. The combined action of these factors has been shown to exacerbate energy deficiency, ionic imbalance, and oxidative stress. This, in turn, has been demonstrated to lead to stronger physiological and metabolic disturbances (Cohen et al., 2021; Zandalinas et al., 2021).

Although salinity and hypoxia have been extensively investigated as individual abiotic stresses, considerably less information is available on plant responses when these stresses occur simultaneously, particularly in hydroponically grown basil. The available information remains limited on the response of hydroponically grown basil to the combined effects of oxygen deficiency and salinity over an extended period, especially regarding growth, metabolic, and antioxidant responses. Characterizing these responses under individual and combined stress conditions may provide a better understanding of how basil responds to multiple abiotic constraints in hydroponic systems.

Therefore, the present study aimed to investigate the individual and combined effects of salinity and root hypoxia on growth performance, metabolic adjustments, and antioxidant defense mechanisms in hydroponically grown basil (*Ocimum basilicum* L.). We hypothesized that stress type and exposure duration would differentially affect the physiological and biochemical responses of basil and that combined salinity–hypoxia stress would trigger a coordinated redox-regulatory response characterized by enhanced antioxidant enzyme activities and increased accumulation of phenolic compounds.

2. Material and methods

2.1. Plant material and growth conditions

The experiment was conducted on basil (*Ocimum basilicum* L., cv Genovese) plants under controlled conditions in January and February 2025. Previous studies have shown that basil responses to salinity are cultivar-dependent, with cv. Genovese exhibiting relatively greater salt tolerance, associated with better water-use efficiency, maintenance of photosynthetic functionality, and antioxidant responses (Barbieri et al., 2012; Mancarella et al., 2016). Basil seeds were germinated in petri dishes for 10 days in the dark at 25°C. The resulting seedlings were transferred into six-litre crystallizers containing the basal nutrient solution prepared as described in Table 1 (Saglio and Pradet, 1980) to establish them under the hydroponic experimental conditions. The nutrient solution had a pH of 6.4, an electrical conductivity of 1.1 mS cm^{-1} , and a temperature of 24°C.

The crystallizers were aerated by a network of capillaries providing an air flow of 400 mL min^{-1} and maintained in a growth chamber at 27°C, with a 16/8 h light/dark photoperiod and 70% relative humidity. Plants were cultivated under artificial light with a photosynthetic photon flux density (PPFD) of approximately 250 μmol m^{-2} s^{-1} . Before stress imposition, plants were maintained under these hydroponic conditions for 21 days. Salinity and hypoxia treatments were subsequently applied to 22-day-old plants at the four-leaf stage. All four treatments were initiated simultaneously under the same environmental conditions.

Table 1
Composition of the basal nutrient solution used in the hydroponic experiment (Saglio and Pradet, 1980).

Compounds	Stock solution (g L^{-1})	Solution S (mg L^{-1})
KNO_3	70.8	283
NH_4NO_3	60.0	240
$Ca(NO_3)_2$	153	612
KH_2PO_4	34.0	136
K_2HPO_4	4.30	17.4
NaCl	2.92	11.7
$MgSO_4{\cdot}7\ H_2O$	39.3	157
H_3BO_3	371	1.48
$MnSO_4{\cdot}H_2O$	380	1.52
$ZnSO_4{\cdot}7\ H_2o$	287	1.50
$CuSO_4{\cdot}5\ H_2O$	6.25	25.0
$(NH_4)_6\ MO_7O_{24}{\cdot}4\ H_2O$	12	49.4
Iron (verofer)	50	100

The experimental setup followed a design including the combined effects of salinity and hypoxia at four different harvest times. **T_1**: harvest on 7 days after treatment application; **T_2**: harvest on 14 days after treatment application; **T_3**: harvest on 21 days after treatment application; **T_4**: harvest on 28 days after treatment application. The basal nutrient solution was used for all experimental treatments, with NaCl and/or aeration conditions modified according to the treatment. The nutrient solution was completely renewed every three days in all four treatments. For the control and hypoxia treatments, freshly prepared basal nutrient solution without NaCl was used at each renewal, whereas for the salinity and combined hypoxia–salinity treatments, freshly prepared nutrient solution containing 50 mM NaCl was used. Following each renewal, the corresponding aeration conditions were maintained according to the treatment. Four treatments were established: (*i*) control (**C**), consisting of the basal nutrient solution without added NaCl under aerated conditions; (*ii*) hypoxia (**H**), consisting of the basal nutrient solution without added NaCl under restricted aeration; (*iii*) salinity (**S**), consisting of the basal nutrient solution supplemented with 50 mM NaCl under aerated conditions; and (*iv*) combined hypoxia and salinity (**H+S**), consisting of the basal nutrient solution supplemented with 50 mM NaCl under restricted aeration. For the salinity and combined hypoxia–salinity treatments, NaCl was dissolved directly in freshly prepared nutrient solution to obtain a final concentration of 50 mM and thoroughly mixed to ensure homogeneous distribution. At each three-day renewal, the freshly prepared nutrient solution was supplemented with NaCl to maintain the target concentration of 50 mM. The concentration of 50 mM NaCl was selected to induce measurable physiological and biochemical responses while maintaining plant viability throughout the 28-day experimental period.

Under control and salinity conditions, the nutrient solution was continuously aerated using a multi-outlet aquarium air pump (NS 350) connected to two silicone capillary tubes per crystallizer. The airflow rate was measured using a rotameter and adjusted to approximately 400 mL min^{-1} for each aerated crystallizer. For the hypoxia and combined hypoxia–salinity treatments, aeration was completely removed at the beginning of treatment exposure by removing the two silicone capillary tubes and was not restored throughout the treatment period. Dissolved oxygen (DO) concentration was measured every day directly in the nutrient solution using a Dissolved Oxygen Benchtop Meter equipped with an optical DO probe (opdo®, HI6421). The probe was calibrated using a Na_2SO_3 solution to establish the zero-oxygen point, and DO concentration was expressed as mg O_2 L^{-1} . Under the experimental conditions, DO concentration was ca. 9.0 mg O_2 L^{-1} under aerated conditions and ca. 2.0 mg O_2 L^{-1} under hypoxic conditions. Plants were exposed to these conditions throughout the treatment

period, and measurements were performed at four sampling times after treatment initiation. At each sampling time, morphological parameters, secondary metabolites, and antioxidant enzyme activities were assessed (Fig. 1).

Plants were randomly assigned to the four experimental treatments. Each treatment consisted of nine independent biological replicates ($n = 9$). Each individual plant was grown in a separate 6-L crystallizer containing an independent nutrient solution and was considered one experimental unit. Thus, measurements obtained from different plants represented independent biological replicates.

2.2. Fresh and dry biomass

Plants are separated into aerial organs (AO) and roots (R). Their fresh biomass (FW) is determined using a precision balance (Mettler AE 160). They are placed in an oven (Memmert) at 60 °C for 3–4 days until their dry mass (DW) stabilises.

2.3. Water status

To assess the state of hydration of the various plant organs during the applied treatments. Water content was determined following the method reported in Shuyskaya et al. (2022) by the difference between the mass of fresh matter (FW) and that of dry matter (DW) referred to the mass of DW and expressed in g H_2O g^{-1} :

$$WC = ((FW - DW)/DW)$$

2.4. Determination of photosynthetic pigments

An amount (0.1 g) of fresh leaves (fully developed, healthy leaves) was homogenised with 1.5 mL of acetone (80% V/V). The extract was centrifuged at 15,000 rpm for 10 min. The absorbance of the samples was recorded by spectrophotometer (BIOBASE, BK-V1600PC) at wavelengths of 663 nm, 645 nm and 470 nm. The amounts of chlorophyll and carotenoid were expressed in mg g^{-1} FW following the equation reported by Lichtenthaler, (1987).

2.5. Total soluble sugar content

To assess the sugar content, the method reported by Irigoyen et al. (1992) was used. To prepare the extract, 100 mg of fresh shoots and roots was crushed in 1 mL of 95% ethanol. The resulting homogenate was then centrifuged at 3500 rpm for 10 min. The ethanolic extract obtained (0.1 mL) was mixed with 3 mL of anthrone reagent and the samples were kept at 100 °C for 10 min. After cooling, their absorbance was read at 625 nm. The amount of TSS was calculated using the standard glucose curve and expressed as mg g^{-1} FW.

2.6. Determination of soluble proteins

For the determination of total proteins, 0.8 g of fresh shoots and roots was homogenised at 4 °C in 1 M Tris-HCl (pH 6.8) using a mortar. The homogenates were centrifuged twice at 4 °C for 30 min at 13,000 g. 50 µL of supernatant with 200 µL of Bradford reagent and 750 µL of H_2O was added. Protein concentration was determined according to the Bradford (1976) method, using bovine serum albumin (BSA) as a standard, and the absorbance was measured at 595 nm using a spectrophotometer (BIOBASE, BK-V1600PC).

2.7. Antioxidant enzymes activities

500 mg of fresh shoots and roots was extracted in 2 mL of potassium phosphate buffer (50 mM), pH 7.0, (1 mM) EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). This was followed by centrifugation at 12,000*g* for 30 min at 4 °C, and the supernatant was separated to assess the activity of antioxidant enzymes (Sudhakar et al., 2001).

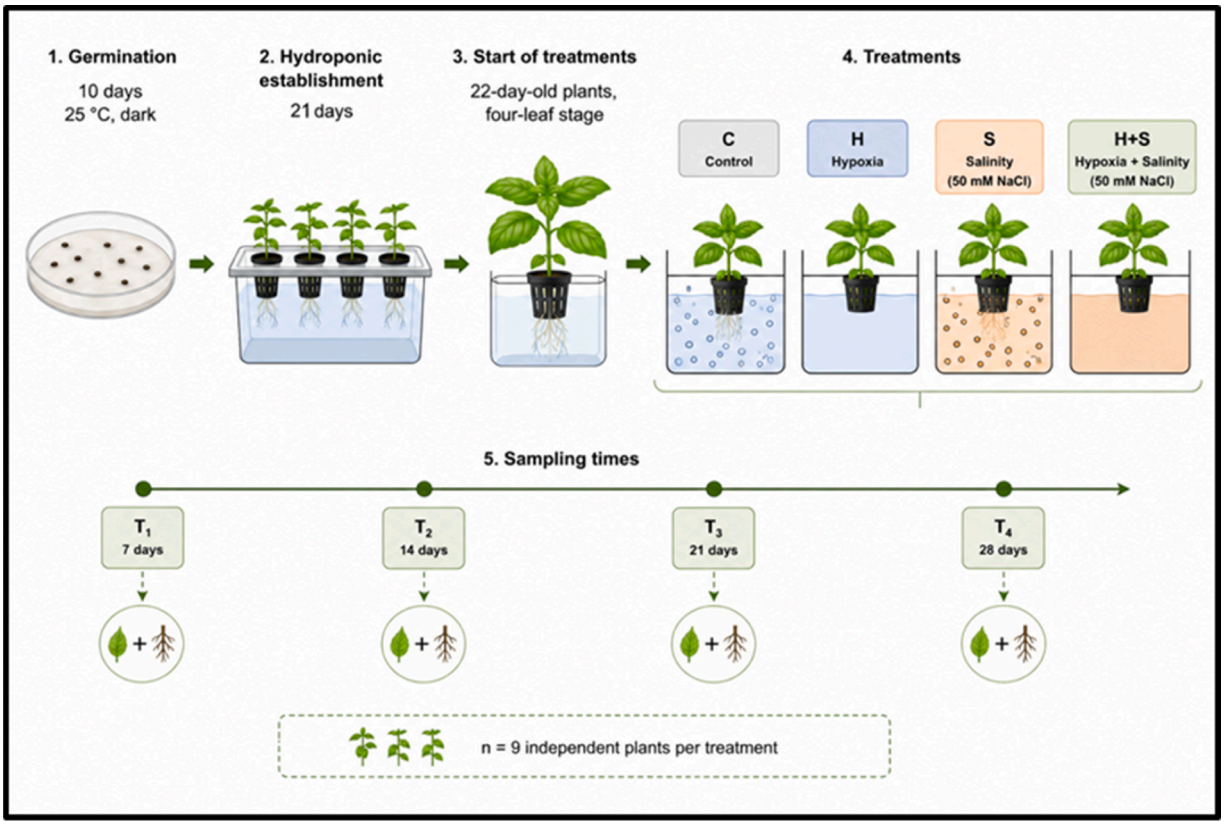


Fig. 1. Schematic representation of the experimental design.

2.7.1. Catalase (CAT)

The method of Aebi (1984) was used to measure CAT activity. The reaction mixture consisted of phosphate buffer (0.1 M, pH = 7), H_2O_2 (60 mM) and 10 µL of extract. CAT activity was recorded for 1 min at λmax = 240 nm using a UV–visible spectrophotometer, referred to one enzyme unit/g MF (U g^{-1} FW).

2.7.2. Superoxide dismutase (SOD)

SOD activity was measured by assessing the ability of the plant extract to suppress the reduction of nitro bleutetrazolium chloride (NBT) (Beauchamp and Fridovich, 1971). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH = 7), 13 mM methionine, 75 µM NBT, 0.1 M EDTA, 2 µM riboflavin and 50 µL of enzyme extract. The tubes were placed under a fluorescent lamp for 10 min at a distance of 20 cm and the reaction was stopped by switching off the lamp. And SOD activity was expressed as follows unit of enzyme per g fresh weight (U g^{-1} FW).

2.7.3. Ascorbate peroxidase (APX)

The activity of ascorbate peroxidase was measured using the method of Nakano and Asada (1987) with slight modifications. The reaction mixture consisted of 10 µL of enzyme extract, phosphate buffer (50 mM, pH = 7), H_2O_2 solution (5 mM) and ascorbic acid (0.25 mM). The absorbance of the prepared extract was read for 1 min at λmax = 290 nm using UV–visible spectrophotometer and APX activity was expressed as enzyme unit per g fresh weight (U g^{-1} FW).

2.7.4. Glutathione peroxidase (GPX)

GPX activity was determined according to (Cakmak, 1994) with modifications. The reaction mixture was consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.7 mM H_2O_2, 35 mM guaïacol and 15 µL of enzyme extract. And activity was estimated at 470 nm and was expressed as enzyme unit per g fresh weight (U g^{-1} FW).

2.7.5. Glutathione reductase (GR)

The activity of glutathione reductase (GR) was assayed according to the procedure followed by Hasanuzzaman et al. (2012). A reaction mixture was prepared using distilled H_2O, buffer solution (0.1 M K-P buffer, pH 7.0, containing 1 mM EDTA, 1 mM GSSG, and 0.2 mM NADPH), and 10 µL enzyme extract. The decrease in absorbance was monitored at 340 nm for 1 min. The activity was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

2.7.6. Glutathione stransferase (GST)

Glutathione S-transferase (GST) activity was analyzed following the methods described in Hasanuzzaman et al. (2017) using the reaction mixture containing GSH solution (1.5 mM GSH in 100 mM Tris–HCl buffer pH 6.5), distilled H_2O, 20 µL 1 mM H_2O_2 and 20 µL enzyme solution. The absorbance was recorded at 340 nm, and the extinction coefficient used for calculation was 9.6 mM^{-1} cm^{-1}.

2.8. Total polyphenol, total flavonoid, proanthocyanidins, flavanols content and antioxidant activity

Aerial organs were dried at room temperature. Once dry, 20 mg was treated with 20 mL methanol, the most polar solvent. Extraction was carried out using ultrasound at 30 °C for one hour. The extract was filtered, the solvent was evaporated by rotavapor (BUCHI RE 111) and the dry residue was recovered.

2.8.1. Total polyphenols content

The determination of total polyphenols in the plant extract was estimated by its blue color in the presence of the Folin-Ciocalteu reagent in an alkaline medium (Oktay et al., 2003). Phenols reduce the mixture of phosphotungstic acid and phosphomolybdic acid to tungsten and molybdenum oxide. The intensity of the blue coloration was measured at 760 nm using a spectrophotometer (BIOBASE, BK-V1600PC). A test sample of 125 µL of diluted extract (1 mg of dry residue in 1 mL of methanol) was added to 500 µL of distilled water and 125 µL of Folin-Ciocalteu reagent. After vigorous shaking and resting the mixture for 3 min, 1250 µL of a 7% $CO_3(Na)_2$ solution was added and the mixture was adjusted to 3 mL with distilled water. The tube was allowed to stand for 90 min at room temperature in the dark, and the absorbance was measured at 760 nm. A standard range was prepared with gallic acid and the phenolic compound contents were expressed in mg gallic acid equivalents per gram of dry matter (mg g^{-1} DW).

2.8.2. Flavonoids content

Flavonoids were measured using the method of Dewanto et al., (2002), based on the formation of a complex between flavonoids and aluminum chloride (Djeridane et al., 2006). The intensity of the orange color indicates the level of flavonoid content in the plants. 75 µL of a 5% $NaNO_2$ solution was added to 250 µL of diluted extract. After 6 min incubation at room temperature, 150 µL of a freshly prepared solution of aluminum chloride ($AlCl_3$) was added to the mixture. After a 5 min rest at room temperature, 500 µL of sodium hydroxide (NaOH, 1 M) was added to the mixture and the final volume was brought to 2500 µL with distilled water. The absorbance of this preparation was measured at 510 nm. The flavonoid content of the extracts was then expressed as mg catechin equivalents per gram of dry matter (mg g^{-1} DW).

2.8.3. Proanthocyanidins content

Proanthocyanidins were determined by butanol-HCL using the method of Maksimovic *et al.,* (2005). An aliquot of 0.5 mL of extract was added to 3 mL of butanol-HCL (95:5) and 0.1 mL of iron sulphate. After homogenisation, the mixture was incubated at 90 °C for 1 h. The absorbance of this preparation was measured at 530 nm. The proanthocyanidin content was determined by reference to a standard catechin range.

2.8.4. Flavanols content

Flavanols were measured using the method of Huang et al. (2007). 2 mL of extract was added to 2 mL of ethanolic solution of $AlCl_3$ (5%) and 0.5 g $NaNO_2$ were added. After 2 h 30 min incubation at 20 °C. Absorbance was measured at 440 nm and the standard used is quercetin (1.8 mg mL^{-1}) and the results were expressed as mg Eq of quercetin /g DW.

2.8.5. Antioxidant activity evaluation

The scavenging effect or antiradical power of the plant extracts studied with respect to the DPPH radical was assessed using the method described by Molyneux (2004). A 0.5 mL volume of the DPPH solution prepared in methanol was mixed with 1 mL of extract solution at different concentrations. After 30 min of incubation in the dark at room temperature, the absorbance was read at 517 nm. The percentage of inhibition (PI%) was calculated using the following equation:

$$PI\% = [(\quad A_{control} - A_{extract})/A_{control}]x100$$

The reducing power of an extract was evaluated by the redox reaction between the extract and transition metal ions, particularly iron (Huang et al., 2005). 200 µL of the extract was therefore added to 500 µL of 200 mM sodium phosphate buffer (pH = 6.6), 2.5 mL of potassium ferricyanide $K_3[Fe(CN)_6]$ (1%) and incubated at 50 °C for 20 min. 2.5 mL of trichloroacetic acid (10%) was added and centrifuged (12, 000 rpm) for 5 min. The reaction mixture contained 500 µL of supernatant, 500 µL ultrapure H_2O and 100 µL ferric chloride. The absorbance was read at 700 nm.

2.9. Statistical analysis

To test for significant effects of treatment, exposure period, and their interaction, permutational analyses of variance (PERMANOVA) were

performed using the model *response* ~ *Treatments* * *Periods*. Analyses were performed in RStudio using the adonis2 function of the vegan package. Post-hoc pairwise comparisons were performed using the pairwisePermutationTest function of the rcompanion package, with *p*-values adjusted for multiple comparisons using the false discovery rate (FDR) method. Compact letter displays used to indicate significant differences among groups were generated using the *cldList* function, with a significance threshold of $\alpha = 0.05$.

3. Results

3.1. Dry weight, water content in basil leaves and roots

Shoot biomass was significantly affected by treatment and sampling time (Figs. 2, 3A). No significant changes over time were observed under control, salinity, or combined hypoxia and salinity, whereas under hypoxia shoot biomass increased significantly, reaching at **T₄** approximately twice the value recorded at **T₁**. Comparisons among treatments showed that at **T₁** and **T₂** shoot biomass under hypoxia was approximately fivefold higher than under salinity, while control and combined hypoxia and salinity showed intermediate values and did not differ significantly from the other treatments. A similar pattern was observed at **T₃**, when shoot biomass under hypoxia remained approximately fivefold higher than under salinity. At **T₄**, hypoxia resulted in the highest shoot biomass, approximately 1.5-fold higher than the control and eightfold higher than salinity, whereas combined hypoxia and salinity did not differ significantly from the control.

Root biomass showed fewer significant differences than shoot biomass (Figs. 2, 3B). No significant temporal changes were detected within any of the four treatments, and no differences among treatments were observed at **T₁**. From **T₂** onward, root biomass under control and hypoxia was approximately twofold higher than under salinity, whereas combined hypoxia and salinity showed intermediate values. At **T₃**, hypoxia resulted in approximately threefold higher root biomass than salinity, while no significant difference was observed between hypoxia and the control. At **T₄**, most comparisons among treatments were not statistically significant.

Water content in shoots was significantly affected by treatment and sampling time (Fig. 4A). At **T₁**, plants exposed to hypoxia showed approximately sixfold higher shoot water content than plants under control, salinity, or combined hypoxia and salinity. No significant temporal changes were subsequently detected under hypoxia, control, or combined hypoxia and salinity. In contrast, under salinity, shoot water content increased significantly over time and at **T₄** was approximately sevenfold higher than at **T₁**.

Root water content showed a different temporal response (Fig. 4B). No significant changes over time were detected under hypoxia or combined hypoxia and salinity. In contrast, at **T₄** root water content under salinity and control conditions was approximately sixfold and fourfold higher, respectively, than at **T₁**. Comparisons among treatments showed that at **T₂** root water content under salinity was approximately 70% lower than under control conditions. At **T₃**, plants exposed to combined hypoxia and salinity showed approximately twofold lower root water content than control plants. At **T₄**, the highest root water content was observed under hypoxia, although the difference among treatments was not statistically significant.

3.2. Photosynthetic pigments

Chlorophyll *a* content showed distinct temporal patterns among treatments (Table 2). No significant temporal changes were detected under control conditions. In contrast, at **T₄** chlorophyll *a* under hypoxia was approximately fivefold higher than at **T₁**, whereas salinity and combined hypoxia and salinity resulted in decreases of approximately 70% and 95%, respectively, relative to **T₁**. Comparisons among treatments showed that at **T₂** chlorophyll *a* under hypoxia was approximately twofold higher than under control conditions. At **T₃**, hypoxia resulted in significantly higher chlorophyll *a* content than control, salinity, and combined hypoxia and salinity. The same pattern was observed at **T₄**, when hypoxia showed the highest chlorophyll *a* content among all

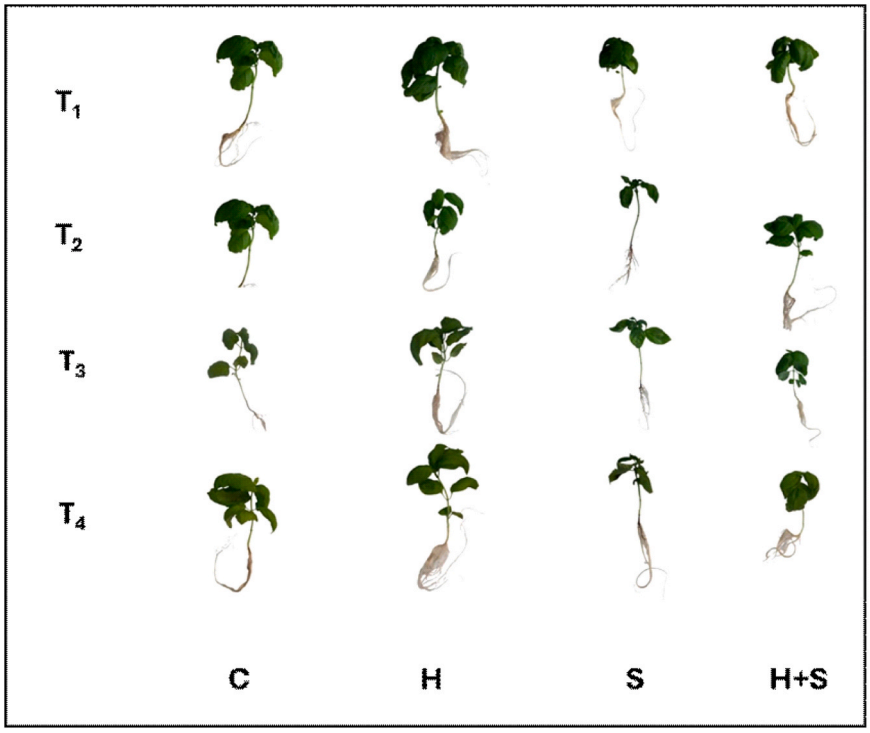


Fig. 2. Representative images of basil plants (*Ocimum basilicum* L.) exposed to different treatments (C: Control treatment, H: Hypoxia treatment, S: Salinity treatment, H+S: Combined treatment), during 28 days (T_1: harvest on 7days after treatments application; T_2: harvest on 14days after treatments application; T_3: harvest on 21 days after treatments application; T_4: harvest on 28 days after treatments application).

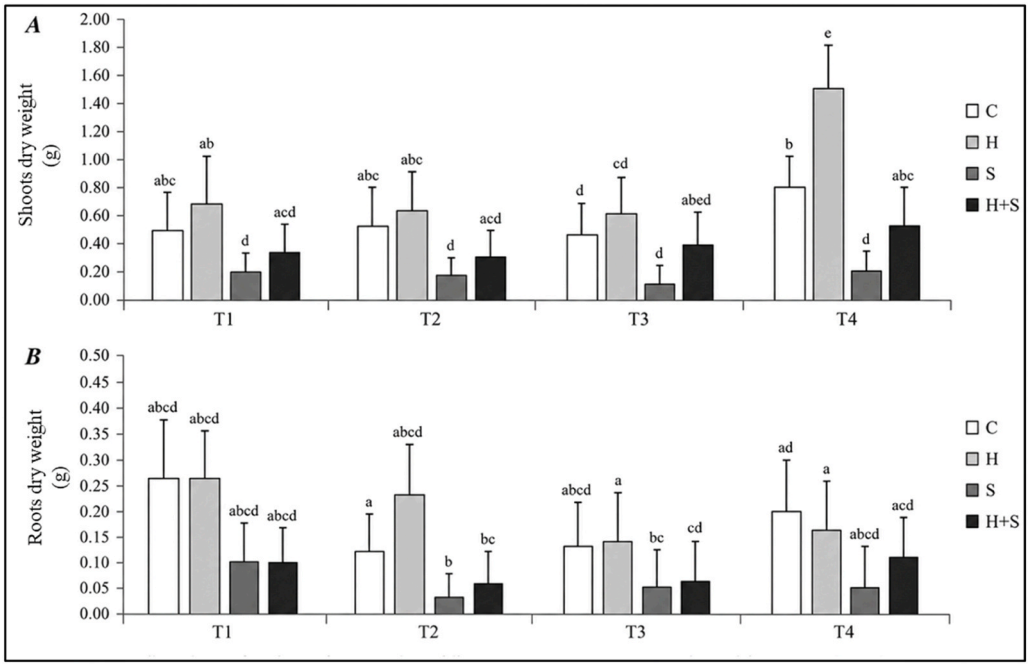


Fig. 3. Shoot (**A**) and root (**B**) dry weight under different treatments (**C**, **H**, **S**, and **H**+**S**) measured at four harvest times (T_1, T_2,T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S**: plants grown with salinity treatment; **H** + **S**: plants grown with ypoxia and salinity treatment combined. Bars represent mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

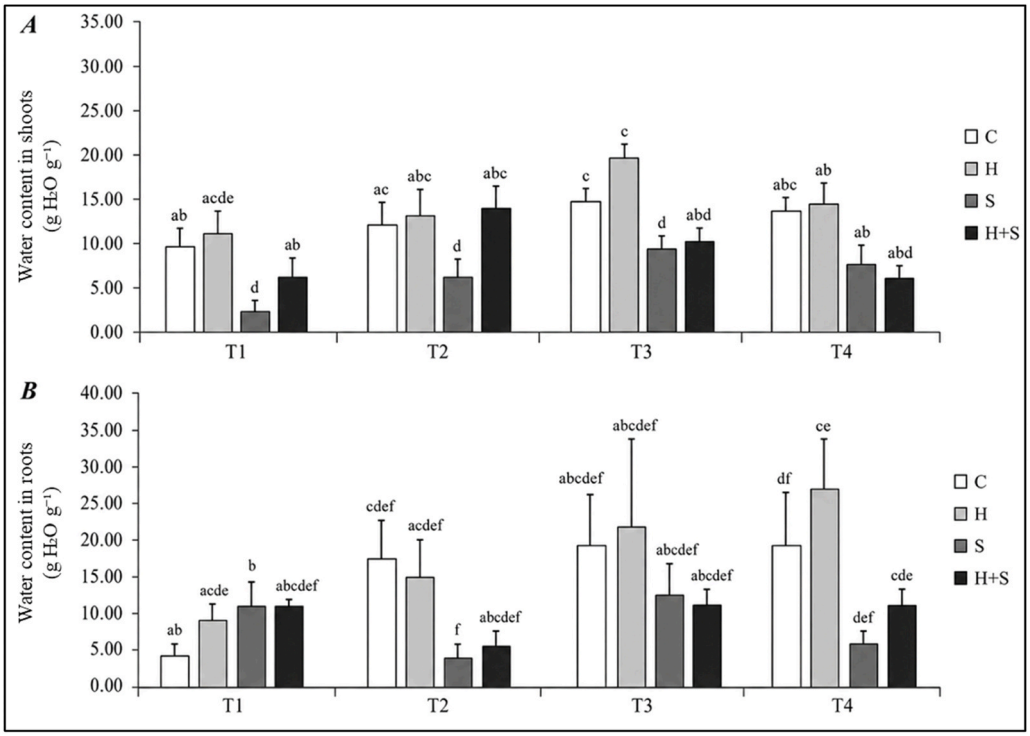


Fig. 4. Shoot (**A**) and root (**B**) water content under different treatments (**C**, **H**, **S**, and **H**+**S**) measured at four harvest times (T_1, T_2,T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S**: plants grown with salinity treatment; **H** + **S**: plants grown with hypoxia and salinity treatment combined. Bars represent mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

treatments.

Chlorophyll *b* did not change significantly over time under control, hypoxia, or salinity, whereas under combined hypoxia and salinity its content at **T₄** was approximately 76% higher than at **T₁**. Comparisons among treatments showed that hypoxia resulted in the highest chlorophyll *b* content at **T₁**, **T₂**, and **T₃**. At **T₄**, however, the highest chlorophyll

Table 2
Photosynthetic pigments content (mg g^{-1} FW) under different treatments (**C**, **H**, **S**, and **H+S**) measured at four harvest times (T_1, T_2, T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S**: plants grown with salinity treatment; **H + S**: plants grown with hypoxia and salinity treatment combined. Results expressed as mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

Chl *a*					**Chl T**				
	T_1	T_2	T_3	T_4		T_1	T_2	T_3	T_4
C	0.033 ± 0.019^{abcdef}	0.029 ± 0.008^{acf}	0.034 ± 0.006^{af}	0.051 ± 0.014^{a}	**C**	0.138 ± 0.011^{abc}	0.121 ± 0.005^{c}	0.113 ± 0.017 ^{h}	0.124 ± 0.009^{c}
H	0.019 ± 0.008^{abc}	0.057 ± 0.006^{be}	0.084 ± 0.017^{beg}	0.111 ± 0.004 ^{g}	**H**	0.241 ± 0.006^{d}	0.241 ± 0.006^{d}	0.214 ± 0.009^{e}	0.348 ± 0.006^{i}
S	0.057 ± 0.007^{be}	0.048 ± 0.010^{bef}	0.070 ± 0.007^{b}	0.018 ± 0.004 ^{cd}	**S**	0.174 ± 0.020^{abe}	0.136 ± 0.009^{ac}	0.160 ± 0.003^{b}	0.045 ± 0.002 ^{g}
H + S	0.064 ± 0.011^{be}	0.049 ± 0.009^{bef}	0.040 ± 0.021^{abcdef}	0.002 ± 0.009^{d}	**H + S**	0.210 ± 0.052^{abcde}	0.091 ± 0.023^{cfg}	0.078 ± 0.006 ^{f}	0.255 ± 0.010^{d}
Chl *b*					**Carotenoids**				
	T_1	T_2	T_3	T_4		T_1	T_2	T_3	T_4
C	0.105 ± 0.014^{abd}	0.092 ± 0.012^{acf}	0.079 ± 0.023^{abcf}	0.072 ± 0.011^{cf}	**C**	0.010 ± 0.002^{ab}	0.018 ± 0.002 ^{cd}	0.019 ± 0.002 ^{cd}	0.025 ± 0.002 ^{f}
H	0.221 ± 0.014^{de}	0.221 ± 0.014^{de}	0.129 ± 0.008^{b}	0.236 ± 0.007^{eh}	**H**	0.021 ± 0.008^{abcdefg}	0.030 ± 0.07^{cdfg}	0.024 ± 0.005^{cdfg}	0.033 ± 0.005 ^{g}
S	0.116 ± 0.015^{ab}	0.087 ± 0.003^{acf}	0.089 ± 0.004^{acf}	0.027 ± 0.002 ^{g}	**S**	0.017 ± 0.001^{ce}	0.012 ± 0.002^{ab}	0.025 ± 0.003^{dfg}	0.016 ± 0.002^{bce}
H + S	0.145 ± 0.042^{abcd}	0.041 ± 0.029^{cfg}	0.038 ± 0.023^{fg}	0.257 ± 0.008 ^{h}	**H + S**	0.017 ± 0.001^{ce}	0.011 ± 0.002^{a}	0.012 ± 0.002^{abe}	0.024 ± 0.002 ^{f}

b content was recorded under combined hypoxia and salinity, with significant differences compared with control, hypoxia, and salinity.

Total chlorophyll content was significantly affected by treatment and sampling time. No significant temporal changes were observed under control conditions. Under hypoxia, total chlorophyll content increased by approximately 45% from **T_1** to **T_4**, while an increase of approximately 20% was recorded under combined hypoxia and salinity. In contrast, salinity resulted in an approximately 75% decrease at **T_4** compared with **T_1**. At **T_2**, **T_3**, and **T_4**, hypoxia resulted in the highest total chlorophyll content, with significant differences compared with control, salinity, and combined hypoxia and salinity.

Carotenoid content varied significantly over time under control, salinity, and combined hypoxia and salinity, whereas no significant temporal changes were detected under hypoxia. At **T_1**, carotenoid content under salinity and combined hypoxia and salinity was approximately 50% higher than under control conditions. At **T_2**, in contrast, carotenoid content under these two treatments was approximately 35% lower than under the control. At **T_3**, combined hypoxia and salinity resulted in the lowest carotenoid content, whereas salinity produced values approximately 31% higher than the control. At **T_4**, hypoxia resulted in the highest carotenoid content, with values approximately 32% higher than the control, 94% higher than salinity, and 31% higher

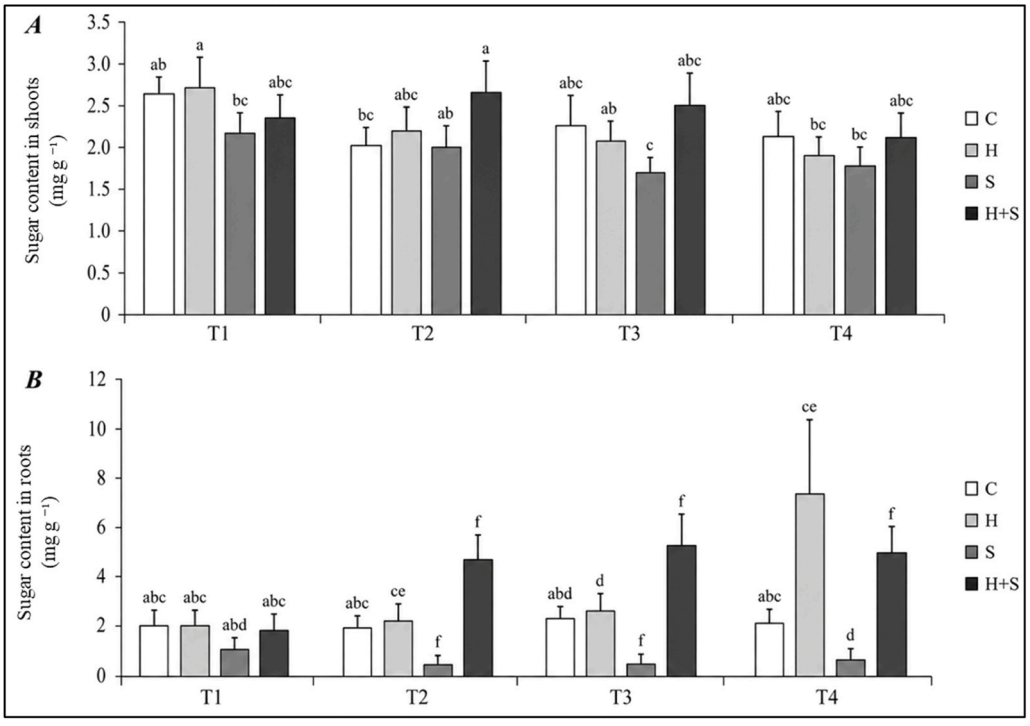


Fig. 5. Soluble sugars content in shoots (**A**) and in roots (**B**) under different treatments (**C**, **H**, **S**, and **H+S**) measured at four harvest times (T_1, T_2, T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S**: plants grown with salinity treatment; **H + S**: plants grown with hypoxia and salinity treatment combined. Bars represent mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

than combined hypoxia and salinity.

3.3. Total soluble sugars content

Total soluble sugar content in shoots was not significantly affected by treatment or sampling time (Fig. 5A), and no significant differences among treatments were detected at **T₁**, **T₂**, **T₃**, or **T₄**.

In roots, total soluble sugar content showed treatment- and time-dependent differences (Fig. 5B). Under hypoxia, the content increased significantly at **T₂**, reaching values approximately 20% higher than at **T₁**. Under combined hypoxia and salinity, total soluble sugar content increased over time and at **T₄** was approximately twofold higher than at **T₁**. No significant differences among treatments were detected at **T₁**. At **T₂**, total soluble sugar content under hypoxia was approximately sixfold higher than under salinity, whereas combined hypoxia and salinity resulted in values approximately 13-fold higher than salinity and twofold higher than hypoxia. At **T₃**, combined hypoxia and salinity again resulted in significantly higher total soluble sugar content than salinity and hypoxia. At **T₄**, the highest content was recorded under hypoxia, with significant differences compared with all other treatments.

3.4. Protein content

Shoot protein content did not change significantly over time under control or combined hypoxia and salinity (Fig. 6A). Under salinity, however, protein content decreased significantly at **T₄**, reaching values approximately 31% lower than at **T₁**. Comparisons among treatments showed that at **T₁** protein content under salinity and combined hypoxia and salinity was approximately 54% and 62% higher, respectively, than under control conditions. At **T₂**, the highest protein content was recorded under combined hypoxia and salinity, with significant differences compared with salinity and hypoxia, and a similar pattern was observed at **T₃**. At **T₄**, hypoxia resulted in the highest protein content, with values approximately 73% higher than the control and 48% higher than salinity, while no significant difference was detected between hypoxia and combined hypoxia and salinity.

Root protein content did not change significantly over time under control, salinity, or combined hypoxia and salinity (Fig. 6B). Under hypoxia, however, protein content increased significantly and at **T₄** was approximately 24% higher than at **T₁**. At **T₁**, **T₂**, and **T₃**, root protein content under hypoxia was significantly higher than under all other treatments. At **T₄**, hypoxia again resulted in the highest protein content, with values approximately 60% higher than the control and fourfold higher than salinity.

3.5. Antioxidant enzymes

Antioxidant enzyme activities showed distinct responses according to treatment, sampling time, and plant organ (Table 3).

In shoots, CAT activity under combined hypoxia and salinity increased significantly over time, reaching at **T₄** approximately fourfold the value recorded at **T₁**. At **T₃**, CAT activity under hypoxia and combined hypoxia and salinity was approximately sevenfold and 20-fold higher, respectively, than under control conditions. At **T₄**, combined hypoxia and salinity resulted in the highest CAT activity, with values approximately 80-fold higher than under control conditions, sixfold higher than under salinity, and twofold higher than under hypoxia.

Shoot SOD activity also varied among treatments and sampling times. Under combined hypoxia and salinity, SOD activity at **T₄** was approximately twofold higher than at **T₁**. At **T₁**, salinity resulted in approximately twofold higher SOD activity than combined hypoxia and salinity, whereas at **T₂** SOD activity under the combined treatment was approximately 70% lower than under salinity. At **T₃** and **T₄**, this pattern was reversed, with combined hypoxia and salinity resulting in approximately twofold higher SOD activity than control and hypoxia.

Shoot APX activity under combined hypoxia and salinity increased significantly over time and at **T₄** was approximately twofold higher than at **T₁**. At **T₁**, hypoxia resulted in significantly higher APX activity than

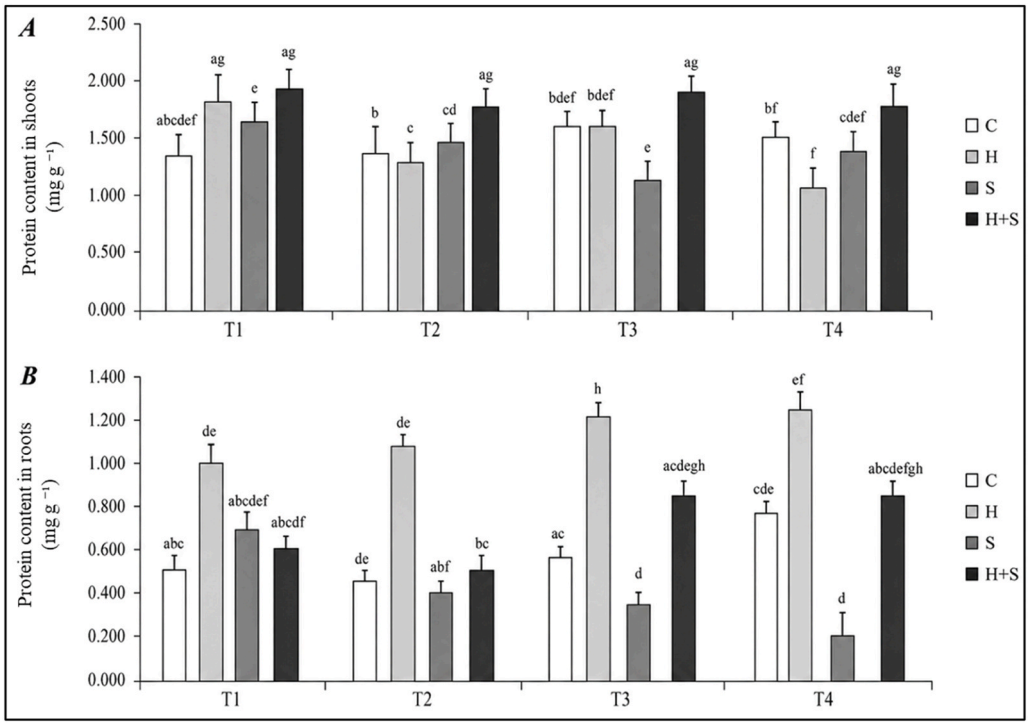


Fig. 6. Proteins content in shoots (**A**) and in roots (**B**) under different treatments (**C**, **H**, **S**, and **H+S**) measured at four harvest times (T_1, T_2,T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S**: plants grown with salinity treatment; **H + S**: plants grown with hypoxia and salinity treatment combined. Bars represent mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

Table 3
Antioxidant enzymes activity under different treatments (**C**, **H**, **S**, and **H+S**) measured at four harvest times (T_1, T_2, T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S** plants grown with salinity treatment; **H + S**: plants grown with hypoxia and salinity treatment combined. Results expressed as mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

		Shoots				*Roots*			
		T_1	**T_2**	**T_3**	**T_4**	**T_1**	**T_2**	**T_3**	**T_4**
CAT	**C**	0.80 ± 0.26^{ab}	0.98 ± 0.26^{abd}	0.50 ± 0.13^{b}	0.21 ± 0.66^{ab}	1.23 ± 0.^{26a}	1.53 ± 0.39^{ac}	1.47 ± 0.31^{a}	1.26 ± 0.77^{ac}
	H	4.40 ± 0.96^{c}	3.86 ± 0.32^{c}	4.22 ± 0.21^{c}	7.36 ± 2.28^{ce}	3.00 ± 1.21^{abcd}	5.5 ± 1.03^{abcde}	8.74 ± 1.03^{e}	11.47 ± 1.85^{ef}
	S	3.12 ± 1.22^{acd}	1.32 ± 0.30^{ad}	0.85 ± 0.74^{abd}	2.91 ± 0.74 ^{cd}	5.22 ± 0.30^{b}	3.26 ± 1.08 ^{cd}	4.20 ± 1.08^{bd}	23.51 ± 13.91^{abcdef}
	HS	3.60 ± 1.86^{abcd}	3.50 ± 1.72^{abcd}	15.26 ± 3.05^{ef}	16.70 ± 3.05 ^{f}	0.80 ± 0.23^{a}	3.50 ± 0.26^{d}	5.34 ± 0.26^{d}	14.70 ± 2.33 ^{f}
		T_1	**T_2**	**T_3**	**T_4**	**T_1**	**T_2**	**T_3**	**T_4**
SOD	**C**	0.59 ± 0.34^{abcde}	0.74 ± 0.07^{ac}	0.58 ± 0.24^{cde}	0.44 ± 0.12^{de}	0.12 ± 0.07^{a}	0.70 ± 0.14^{cde}	0.58 ± 0.23^{ade}	0.51 ± 0.22^{ad}
	H	0.95 ± 0.14^{abc}	0.66 ± 0.19^{cde}	1.16 ± 0.09^{b}	0.99 ± 0.24^{abcef}	0.83 ± 0.07^{bcde}	1.47 ± 0.48^{bcdef}	1.08 ± 0.56^{abcdef}	0.62 ± 0.31^{abcde}
	S	1.72 ± 0.46^{abf}	1.69 ± 0.57^{abcf}	0.91 ± 0.14^{abc}	1.11 ± 1.11^{abcdef}	1.38 ± 0.27^{bf}	1.68 ± 0.25 ^{f}	1.58 ± 0.13 ^{f}	1.81 ± 0.17 ^{f}
	H + S	0.64 ± 0.18^{cde}	0.18 ± 0.13^{d}	1.39 ± 0.37^{abcf}	1.57 ± 0.17 ^{f}	1.55 ± 0.39^{bcf}	1.84 ± 0.15 ^{f}	1.67 ± 0.31 ^{f}	1.47 ± 0.39^{bced}
		T_1	**T_2**	**T_3**	**T_4**	**T_1**	**T_2**	**T_3**	**T_4**
APX	**C**	1.36 ± 0.53^{a}	0.60 ± 0.20^{a}	1.50 ± 1.07^{af}	1.07 ± 0.86^{a}	0.33 ± 0.12^{a}	0.74 ± 0.18^{a}	0.49 ± 0.17^{a}	1.18 ± 0.87^{ac}
	H	14.04 ± 2.31^{b}	3.18 ± 0.23^{ef}	8.53 ± 1.16^{bcdg}	13.99 ± 2.58^{bg}	2.38 ± 0.45^{bc}	3.17 ± 1.06^{bcf}	4.27 ± 0.89^{bdf}	6.15 ± 0.50^{de}
	S	7.14 ± 0.78 ^{cd}	5.29 ± 1.35^{cde}	6.37 ± 0.61^{c}	4.89 ± 2.71^{acdef}	0.56 ± 0.13^{a}	7.42 ± 1.45^{de}	0.85 ± 0.76^{ac}	0.67 ± 0.13^{a}
	H + S	6.08 ± 0.29^{c}	6.64 ± 0.42 ^{cd}	8.56 ± 0.82^{dg}	13.80 ± 0.91^{b}	6.48 ± 0.84^{de}	5.50 ± 0.96^{def}	5.82 ± 0.16^{d}	8.09 ± 1.03^{e}
		T_1	**T_2**	**T_3**	**T_4**	**T_1**	**T_2**	**T_3**	**T_4**
GPX	**C**	1.32 ± 0.21^{a}	0.85 ± 0.07^{bc}	1.29 ± 0.04^{a}	0.72 ± 0.04^{c}	0.07 ± 0.06^{ab}	0.11 ± 0.04^{ab}	0.15 ± 0.10^{abc}	0.11 ± 0.02^{ab}
	H	1.45 ± 0.14^{a}	1.07 ± 0.28^{abc}	1.47 ± 0.18^{a}	1.18 ± 0.29^{abc}	0.31 ± 0.18^{abcd}	0.50 ± 0.25^{abcd}	0.48 ± 0.28^{abcd}	0.57 ± 0.16^{d}
	S	1.08 ± 0.53^{abc}	1.08 ± 0.37^{abc}	1.30 ± 0.55^{abcd}	1.91 ± 0.73^{abcd}	0.09 ± 0.06^{ab}	0.04 ± 0.04^{b}	0.10 ± 0.03^{ab}	0.08 ± 0.04^{ab}
	H + S	1.62 ± 0.22^{ad}	1.74 ± 0.31^{ad}	1.33 ± 0.24^{ab}	2.46 ± 0.33^{d}	0.37 ± 0.15^{acd}	0.38 ± 0.15 ^{cd}	0.41 ± 0.10 ^{cd}	0.53 ± 0.31^{abcd}
		T_1	**T_2**	**T_3**	**T_4**	**T_1**	**T_2**	**T_3**	**T_4**
GR	**C**	3.68 ± 0.56^{a}	4.00 ± 1.09^{a}	3.61 ± 0.93^{a}	3.42 ± 0.97^{a}	0.52 ± 0.09^{ab}	0.56 ± 0.19^{abe}	0.26 ± 0.10^{a}	0.43 ± 0.08^{ab}
	H	12.18 ± 4.95^{abcd}	10.34 ± 4.62^{abcd}	4.06 ± 1.26^{a}	10.48 ± 2.41^{bc}	16.35 ± 2.47^{c}	0.43 ± 0.09^{ab}	0.54 ± 0.02^{b}	0.32 ± 0.19^{ab}
	S	12.00 ± 4.73^{abcd}	8.70 ± 0.12^{b}	2.67 ± 1.22^{a}	16.36 ± 2.25 ^{cd}	2.64 ± 0.15^{d}	1.58 ± 0.88^{abdef}	0.39 ± 0.16^{ab}	0.55 ± 0.01^{b}
	H + S	11.70 ± 2.86^{bc}	12.74 ± 1.82^{c}	2.52 ± 0.62^{a}	20.21 ± 1.04^{d}	3.87 ± 1.87^{abdef}	2.50 ± 0.91^{def}	1.35 ± 0.17 ^{f}	0.60 ± 0.12^{be}
		T_1	**T_2**	**T_3**	**T_4**	**T_1**	**T_2**	**T_3**	**T_4**
GST	**C**	0.55 ± 0.28^{a}	0.39 ± 0.43^{a}	0.21 ± 0.10^{a}	0.32 ± 0.20^{a}	0.81 ± 0.12^{abc}	0.36 ± 0.05^{d}	0.49 ± 0.27^{abdf}	0.71 ± 0.33^{abcdf}
	H	0.61 ± 0.41^{a}	0.26 ± 0.16^{a}	0.34 ± 0.22^{a}	0.5 ± 0.20^{a}	0.63 ± 0.12^{abd}	0.50 ± 0.14^{abd}	0.32 ± 0.22^{bdf}	0.73 ± 0.20^{abc}
	S	0.26 ± 0.24^{a}	0.65 ± 0.34^{a}	0.47 ± 0.11^{a}	0.34 ± 0.23^{a}	0.83 ± 0.10^{ac}	0.96 ± 0.23^{abce}	0.95 ± 0.25^{abce}	0.045 ± 0.12 ^{f}
	H + S	6.92 ± 2.52^{b}	4.98 ± 1.44^{b}	3.77 ± 1.08^{b}	5.81 ± 0.91^{b}	4.31 ± 1.72^{ce}	1.68 ± 0.29^{e}	4.55 ± 2.59^{abcdef}	5.22 ± 2.34^{abcde}

salinity and combined hypoxia and salinity. From **T_2** to **T_4**, however, the highest APX activity was consistently recorded under combined hypoxia and salinity, with significant differences compared with the other treatments.

For shoot GPX, no significant differences among treatments were detected at **T_1**, **T_2**, or **T_3**. At **T_4**, combined hypoxia and salinity resulted in the highest GPX activity, approximately 100% higher than under hypoxia and approximately twofold higher than under control conditions.

Shoot GR activity did not change significantly over time under control, salinity, or hypoxia. Under combined hypoxia and salinity, GR activity increased significantly, reaching at **T_4** values approximately 72% higher than at **T_1**. At **T_1**, GR activity under the combined treatment was approximately twofold higher than under control conditions, while at **T_2** it was approximately twofold higher than under both control and salinity. No significant differences among treatments were detected at **T_3**. At **T_4**, the highest GR activity was recorded under combined hypoxia and salinity.

Shoot GST activity was not significantly affected by sampling time but differed among treatments. At all four sampling times (**T_1–T_4**), the highest GST activity was recorded under combined hypoxia and salinity and was significantly higher than under control conditions.

In roots, CAT activity increased significantly over time under both hypoxia and combined hypoxia and salinity, reaching at **T_4** approximately threefold and 17-fold, respectively, the corresponding **T_1** values. At **T_1**, salinity resulted in approximately threefold higher CAT activity than the control and sixfold higher activity than combined hypoxia and salinity. At **T_2**, CAT activity under the combined treatment was approximately twofold higher than under both control and hypoxia. At **T_3**, hypoxia resulted in approximately fivefold higher CAT activity than the control and twofold higher activity than both salinity and the combined treatment. At **T_4**, combined hypoxia and salinity produced the highest CAT activity, approximately tenfold higher than the control.

Root SOD activity was significantly influenced by treatment. At **T_1**, **T_2**, **T_3**, and **T_4**, salinity resulted in significantly higher SOD activity than control conditions.

Root APX activity under hypoxia increased significantly over time and at **T_4** was approximately twofold higher than at **T_1**. At **T_1**, combined hypoxia and salinity resulted in approximately fivefold higher APX activity than the control, threefold higher activity than hypoxia, and approximately fivefold higher activity than salinity. At **T_2**, salinity produced the highest APX activity, approximately ninefold higher than the control, twofold higher than hypoxia, and 1.5-fold higher than the combined treatment. At **T_3** and **T_4**, combined hypoxia and salinity resulted in the highest APX activity, with significant differences compared with all other treatments.

For root GPX activity, combined hypoxia and salinity resulted in approximately fourfold higher activity than the control at **T_1**. At **T_2**, GPX activity under hypoxia and combined hypoxia and salinity was approximately ninefold and fourfold higher, respectively, than under salinity. At **T_3**, combined hypoxia and salinity resulted in approximately threefold higher GPX activity than both control and salinity. At **T_4**, the highest GPX activity was observed under hypoxia, reaching values approximately fivefold higher than under salinity and threefold higher than under control conditions.

Root GR activity showed no significant temporal changes under control or combined hypoxia and salinity. In contrast, at **T₄** GR activity under hypoxia and salinity was approximately 97% and 78% lower, respectively, than at **T₁**. A general decrease in GR activity was observed from **T₂** onward across treatments.

Root GST activity was influenced by both sampling time and treatment. At all four sampling times (**T₁–T₄**), combined hypoxia and salinity resulted in the highest GST activity, with values significantly higher than under control conditions.

3.6. Antioxidant compounds and activity

Total polyphenol accumulation showed different temporal responses among treatments (Fig. 7A). No significant changes over time were detected under control or combined hypoxia and salinity. Under hypoxia, total polyphenol content increased significantly and at **T₄** was approximately 8% higher than at **T₁**. In contrast, salinity resulted in an approximately 50% decrease at **T₄** compared with **T₁**. Comparisons among treatments showed that hypoxia resulted in the highest polyphenol content at all four sampling times (**T₁–T₄**).

Flavonoid content did not change significantly over time under salinity or combined hypoxia and salinity (Fig. 7B). Under hypoxia, flavonoid content increased significantly, reaching at **T₄** values approximately 30% higher than at **T₁**. An increase was also observed under control conditions, with values at **T₄** approximately twofold higher than at **T₁**.

Proanthocyanidin content showed no significant overall effect of sampling time (Fig. 7C), but significant differences among treatments

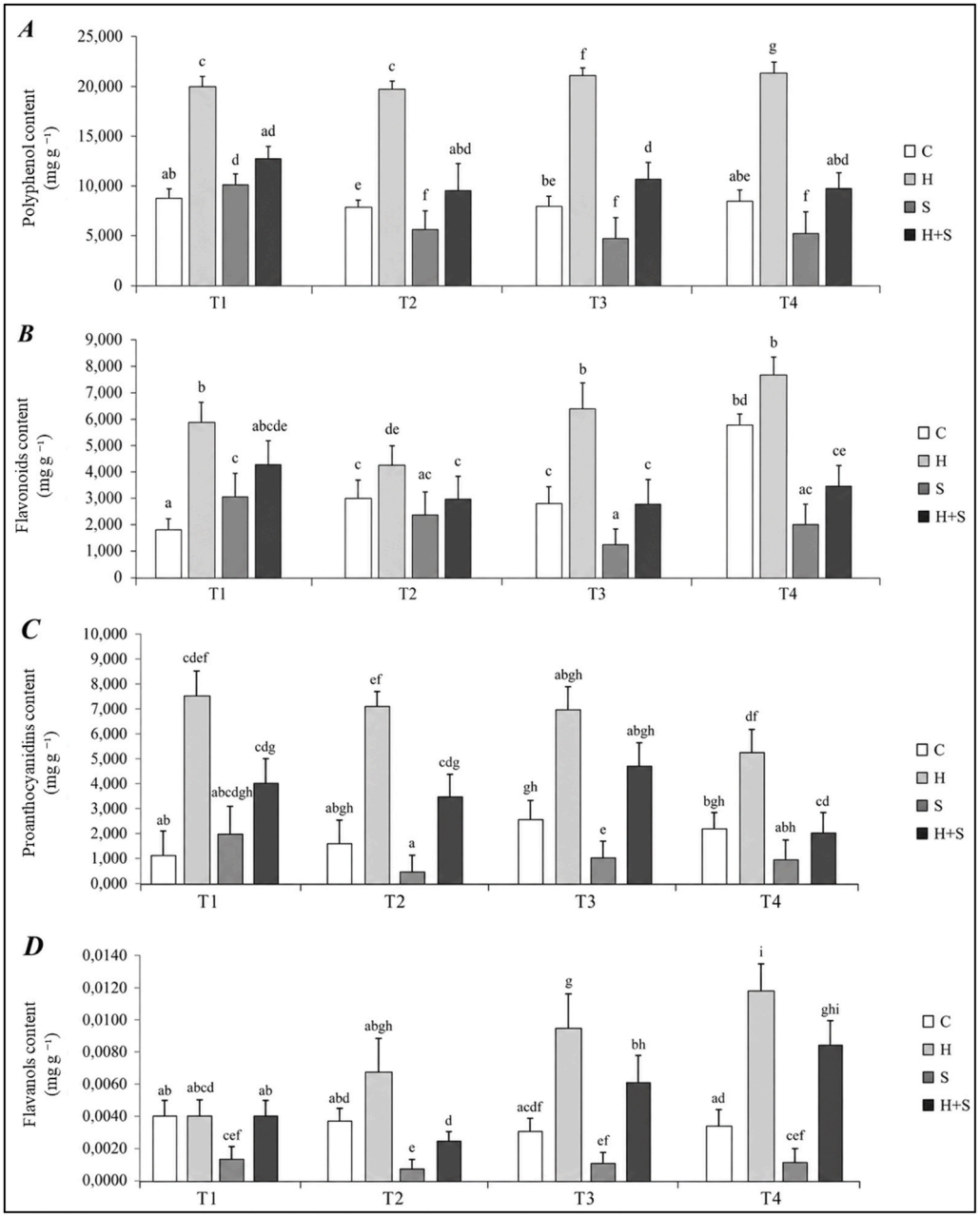


Fig. 7. Polyphenols content (**A**), flavonoids content (B), proanthocyanidin content (**C**), flavanols content (**D**) under different treatments (**C**, **H**, **S**, and **H**+**S**) measured at four harvest times (T_1, T_2,T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S**: plants grown with salinity treatment; **H** + **S**: plants grown with hypoxia and salinity treatment combined. Bars represent mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

were observed. At **T₁** and **T₂**, hypoxia resulted in approximately tenfold higher proanthocyanidin content than salinity and threefold higher content than the control. At **T₃**, proanthocyanidin content under hypoxia was approximately twofold higher than under salinity and threefold higher than under combined hypoxia and salinity. At **T₄**, hypoxia again produced the highest values, approximately twofold higher than the control, fivefold higher than salinity, and 1.5-fold higher than combined hypoxia and salinity.

Flavanol accumulation followed a broadly similar pattern, with hypoxia resulting in the highest accumulation in aerial organs (Fig. 7D). Combined hypoxia and salinity also resulted in slightly higher flavanol levels than salinity alone.

Antioxidant activity, assessed using DPPH and FRAP assays (Table 4), was significantly affected by treatment, whereas no significant effect of sampling time was detected. At **T₁**, the lowest DPPH IC_{50} value was recorded under hypoxia (0.04 mg mL^{-1}), followed by combined hypoxia and salinity (0.05 mg mL^{-1}), control conditions (0.22 mg mL^{-1}), and salinity (0.30 mg mL^{-1}). A similar pattern was observed at **T₂**, **T₃**, and **T₄**, with hypoxia consistently resulting in the lowest DPPH IC_{50} values and therefore the highest radical-scavenging activity. In contrast, the FRAP assay showed a different treatment-dependent pattern, with the highest numerical values generally recorded under salinity.

4. Discussion

In favorable environments, plants can easily obtain the nutrients they need to survive. However, they cannot escape when resources are limited or competition is high (Bo-Moon and Yamauchi, 2025). To overcome this, they have evolved adaptive defense strategies that often involve shifting from growth metabolism to resilience metabolism (Cirillo et al., 2021). The results of our experiment highlight the different responses of the basil cultivated in hydroponic system, to the stress induced by oxygen deficiency, salinity, and their combination.

4.1. Physiological parameters

The contrasting biomass responses observed among treatments indicate that salinity and hypoxia affected basil growth differently. Specifically, salinity was associated with the greatest growth limitation, particularly in comparison with hypoxia. This response is consistent with the osmotic and ionic constraints imposed by NaCl, which can restrict water and nutrient acquisition and consequently limit biomass production (Rashidi et al., 2020). Similar reductions in growth and shoot biomass have also been reported in hydroponically grown basil exposed to increasing NaCl concentrations (Corrado et al., 2021).On the other hand, hypoxia resulted in higher shoot biomass than the control under some exposure conditions. Although this response was unexpected under oxygen-limited conditions, it indicates that basil growth was differently affected by oxygen limitation and salinity under the experimental conditions considered. The mechanisms underlying the increased shoot biomass under hypoxia remain unclear and were not directly investigated in the present study. Recent evidence indicates that plant responses to hypoxia are highly context-dependent and involve substantial adjustments in energy metabolism, growth regulation, and carbon utilization rather than a uniform inhibition of growth (Van Veen et al., 2025). Therefore, the higher shoot biomass observed under hypoxia in the present research may reflect a specific response of basil to the intensity and duration of oxygen limitation imposed under the hydroponic conditions.

The combination of the two treatments (**hypoxia + salinity**) resulted in average growth, as compared with the saline and hypoxic treatments. Consequently, in term of growth, the combination stimulated growth at **T_1**, **T_2**, **T_3** and **T_4** of treatment. This suggests that hypoxia can alleviate the negative effects of salinity. Which may indicate that these changes in biomass provided the first key insight into the growth defense in basil plants cultivated in hydroponic, exposed to salinity, hypoxia, and also showed that it's combination is related to a complex effects on plants growth resulted by ionic imbalances (Barrett-Lennard and Shabala, 2013). These results are similar to those found in (Lifschitz et al., 2025) study on *Panicum coloratum* where biomass production was affected negatively under combined treatments than separately. The different magnitude of the biomass response between shoots and roots further indicates an organ-specific response to the imposed stresses. This distinction is particularly relevant under root-zone hypoxia because roots are directly exposed to reduced oxygen availability, whereas responses in aerial organs arise from the integration of local root responses with whole-plant metabolic and physiological regulation (Isayenkov et al., 2026; Ateeq et al., 2026).

4.2. Water content

The contrasting responses of shoot and root water content indicate that the effects of salinity and hypoxia on the water status were organ-specific rather than uniform across the whole plant. This distinction is physiologically relevant since roots are directly exposed to changes in both the osmotic environment and oxygen availability of the nutrient solution, whereas shoot hydration reflects the integrated response of root water acquisition, transport, and water loss from aerial tissues (Cai et al., 2026; Zhao et al., 2025).

The phenomenon is not observed in the shoots as it is in the roots. In the shoots, salinity treatment increase water content in comparison to the other treatments, our results are consistent with those of previous studies in Alam et al. (2022). This response may be associated with osmotic adjustment mechanisms activated under salinity, which promote the accumulation of inorganic ions and compatible solutes in shoot tissues. This osmotic regulation enhances water retention in aerial organs, which allows the maintenance of cell turgor and leaf hydration despite external osmotic constraints. Furthermore, the regulation of stomata by salinity can reduce transpiration rates (Boussora et al., 2024), thereby minimising water loss from leaves and contributing to higher water content in aboveground tissues. However, because osmotic potential, stomatal conductance, and transpiration were not measured in the present study, the higher shoot water content cannot be attributed to a specific water-regulation mechanism. In conditions of oxygen deficiency, the impact of hypoxia on root water content in basil plants was found to be significant. Changes in membrane properties represent an important component of plant acclimation to abiotic stress, and

Table 4
Antioxidant activity measured by DPPH test (IC50) and FRAP test (CE) under different treatments (**C**, **H**, **S**, and **H+S**) measured at four harvest times (T_1, T_2, T_3, and T_4). **C**: control plants; **H**: plants grown with hypoxia treatment; **S**: plants grown with salinity treatment; **H + S**: plants grown with hypoxia and salinity treatment combined. Results expressed as mean ± standard deviation. Different letters indicate significant differences among treatments within each harvest time according to PERMANOVA analysis ($p \leq 0.05$).

		T_1	T_2	T_3	T_4
DPPH	**C**	0.22 ± 0.06^{abcd}	0.08 ± 0.03^{cefg}	0.06 ± 0.04^{efg}	0.11 ± 0.02^{ce}
	H	0.04 ± 0.03^{efg}	0.05 ± 0.05^{cefg}	0.03 ± 0.01^{fg}	0.007 ± 0.00 ^{g}
	S	0.3 ± 0.09^{ab}	0.4 ± 0.2^{abcdef}	0.4 ± 0.08^{a}	0.2 ± 0.02^{bd}
	H + S	0.05 ± 0.06^{cefg}	0.09 ± 0.01^{ce}	0.09 ± 0.08^{cdefg}	0.07 ± 0.05^{cefg}
FRAP	**C**	0.017 ± 0.00^{ab}	0.016 ± 0.00^{ag}	0.017 ± 0.00^{abf}	0.011 ± 0.00 ^{cd}
	H	0.011 ± 0.00^{cde}	0.012 ± 0.00^{cdeg}	0.010 ± 0.00^{c}	0.009 ± 0.00^{e}
	S	0.1 ± 0.07^{abcdefghi}	0.1 ± 0.09^{abcdefghi}	0.13 ± 0.01 ^{h}	0.07 ± 0.00^{i}
	H + S	0.018 ± 0.00^{abf}	0.025 ± 0.00^{bf}	0.020 ± 0.00^{abdfg}	0.019 ± 0.00 ^{f}

stress-induced remodeling of membrane lipid composition has been reported as an adaptive mechanism under different environmental constraints (Zhao et al., 2024). Root-zone hypoxia directly constrains oxygen availability to root tissues and can alter energy metabolism and several physiological processes involved in root function. Recent evidence emphasizes that the response to low oxygen involves coordinated changes in energy status, redox regulation, and cellular metabolism (Van Veen et al., 2025). These processes provide a physiological context for the changes in root water content observed here, although the mechanisms controlling water uptake and retention were not directly assessed in the present study.

Overall, the contrasting water-content patterns between shoots and roots indicate that water status was differentially modulated by salinity, hypoxia, and their combination. Rather than indicating a uniformly improved hydration status under combined stress, these results point to distinct organ-specific responses whose magnitude changed with stress condition and exposure duration.

4.3. Pigments content

The contrasting pigment profiles among treatments indicate that salinity and hypoxia affected the photosynthetic pigment system differently. The marked decline in chlorophyll content under salinity is consistent with the sensitivity of the photosynthetic apparatus to ionic and osmotic stress and with previous observations in hydroponically grown basil, where increasing salinity impaired growth and altered photosynthetic performance (Corrado et al., 2021).

Variations in total chlorophyll content as an important component of photosynthetic apparatus, are indicative of the plant's capacity to maintain photosynthetic efficiency in response to environmental constraints (Yan et al., 2024). Under combined hypoxia and salinity treatment, the significant increase from $\mathbf{T_1}$ to $\mathbf{T_4}$ that leads to a partial recovery of Chl content suggests the activation of protective mechanisms, allowing so basil plants to preserve photosynthetic functionality despite adverse conditions. Contrary to what was found in the work of Zeng et al., (2013), on barley, where under combined stress decreased chlorophyll content more than under salinity alone.

Carotenoids have been demonstrated to be crucial for dissipating excess excitation energy and protecting chlorophyll molecules from photooxidative damage, especially in circumstances where stress limits carbon assimilation (Young, 1991; Strzalka *et al.,* 2003). Our results showed that carotenoid content was strongly influenced by treatments, periods, and their interaction, with the highest contribution of the treatment effect. Thus, under hypoxia, at $\mathbf{T_4}$, the highest level of carotenoids was observed, following by under salinity alone and after the combining these two constraints (hypoxia + salinity), contrary to which is find in the study in Mahmoud *et al.*(2021) where the combination or each treatment alone was used, but under these treatments they found a reduction in carotenoid content.

This provides further evidence to support the hypothesis that hydroponic basil grown under combined stress may induces a more coordinated pigments acclimation response resulting in an intermediate profile rather than salinity simple additive effect.

4.4. Total soluble sugars

Soluble sugars play multiple roles in plant responses to abiotic stress, serving as metabolic substrates and contributing to cellular osmotic and redox regulation (Peng et al., 2026). Their relevance may become particularly important under hypoxia, when restricted mitochondrial respiration increases the dependence of cells on glycolysis and fermentative metabolism for ATP production (Wang et al., 2025).

In our study, total soluble sugar (TSS) content in shoots was not significantly affected by the applied treatments throughout the 28 day experiment period. The limitation of carbohydrate availability as a consequence of a decline in photosynthesis could be also another reason for decrease in TSS (Goicoechea et al., 2005). In contrast, soluble sugar content increased in the roots under some stress conditions, whereas no significant treatment effect was detected in the shoots. These results indicate a different response of soluble sugar content between aerial organs and roots under the applied stress conditions. However, carbohydrate transport and allocation between organs were not directly investigated in the present study. Additionally, hypoxic conditions are known to limit mitochondrial respiration, reducing carbohydrate utilisation and leading to sugar accumulation in root tissues (Van Veen et al., 2025).

4.5. Proteins content

The contrasting protein responses observed between shoots and roots indicate that protein metabolism was differently modulated according to plant organ and stress condition. Furthermore, under hypoxic stress, alterations in protein content are indicative of metabolic reprogramming in response to oxygen deficiency (Zahra et al., 2021). The presence of limited oxygen availability has been demonstrated to exert a constraining effect on mitochondrial respiration. This, in turn, has been shown to induce anaerobic metabolism and the synthesis of stress related proteins. Proteins are implicated to facilitate glycolysis, fermentation and redox regulation (Mustroph et al., 2010). In this context, the higher root protein content observed under hypoxia may reflect a marked adjustment of root metabolism to the low-oxygen environment, although the specific proteins involved were not identified in the present study. Salinity stress has been shown to induce ionic and osmotic imbalance, which in turn stimulates the synthesis of protective proteins such as chaperones, osmoprotective enzymes, and antioxidant-related proteins. However, it has been confirmed that prolonged exposure to salinity can result in impaired protein synthesis, a phenomenon that can be attributed to reduced photosynthetic efficiency and nitrogen assimilation. This observation provides a plausible explanation for the variable protein response observed in response to salinity alone demonstrated by Athar et al. (2022).

In our study, protein accumulation exhibited a tissue specific response to stress conditions. The relatively high protein content observed in shoots under combined stress and in roots under hypoxia indicates that the biochemical response differed between organs rather than occurring uniformly throughout the plant. This distinction may reflect the different physiological environments experienced by roots and aerial tissues: roots were directly exposed to low oxygen and/or NaCl in the nutrient solution, whereas shoot responses resulted from whole-plant adjustments to root-zone stress. Because only total soluble protein content was measured, however, the functional identity of the proteins responsible for these changes remains unknown.

4.6. Antioxidant enzymes, compounds and activity

The antioxidant enzyme responses observed in basil were strongly dependent on treatment, exposure duration, and plant organ, indicating that redox regulation under salinity and hypoxia was not uniform. This is consistent with the central role of antioxidant enzymes in controlling ROS homeostasis under abiotic stress, where changes in ROS production and scavenging depend on both the type and intensity of the stress imposed (Rajput et al., 2021; Xu et al., 2025).

In the leaves, the combined treatment induced marked changes in CAT, SOD, APX, GPX, GR and GST activities, that the antioxidant enzyme response was strongly affected by the combined stress condition. The important effects of treatment and time founded with most enzymes show that antioxidant responses depend a lot on time. This coordinated response is an indicative of the interdependent roles of these enzymes within the ROS scavenging cascade. Enzymes such as SOD and CAT reacted quickly during the early stress phases from $\mathbf{T_1}$, while APX, GPX, GR, and GST were more strongly induced at later stages at $\mathbf{T_3}$ or $\mathbf{T_4}$, the early activation of (SOD) allow to catalyze the

dismutation of superoxide radicals into hydrogen peroxide, which is then detoxified by CAT in peroxisomes and by APX in chloroplasts and the cytosol, using ascorbate as an electron donor. The ascorbate-glutathione (AsA-GSH) cycle, involving APX, GR and GST, which plays a pivotal role in maintaining cellular redox balance by regenerating the reduced ascorbate and glutathione (Freire et al., 2024). The marked increase in GR activity further promotes the regeneration of reduced glutathione (GSH), which is essential for maintaining detoxification processes and protecting cellular components from oxidative damage (Blokhina et al., 2003; Lee and Lee, 2000). This sequential activation is characteristic of adaptive stress responses, whereby plants progressively strengthen their antioxidant capacity in response to ongoing stress (Sachdev et al., 2021).

In roots, a significant increase in antioxidant enzyme activity was also observed, with profiles comparable to those seen in the leaves. This suggests that responses to oxidative stress are systemic and involve inter-organ signalling mechanisms. In hydroponic systems, roots are exposed directly to conditions of oxygen deficiency, which limit mitochondrial respiration and lead to increased ROS (reactive oxygen species) production due to disruption of the electron transport chain. However, the observed activation of enzymes indicates that the oxidative stress response is not exclusively attributable to hypoxia, but rather to the combined effects of salinity-induced ionic imbalance and oxygen limitation. The combined treatment resulted in the highest enzyme activities in the roots, followed by hypoxia and then salinity alone. This finding highlights a cumulative or possibly synergistic effect of multiple stress factors on root redox metabolism (Rajput et al., 2021; Mishra et al., 2023).

The gradual increase in antioxidant enzyme activity over time suggests the establishment of both immediate and long-term acclimatization mechanisms. Initially, the accumulation of ROS functions as a signalling molecule, thereby triggering the transcriptional activation of genes associated with antioxidant defense. Prolonged exposure leads to sustained enzymatic protection and metabolic reprogramming, indicating that the plant adjusts its redox network to maintain cellular homeostasis (Bailey-Serres and Voesenek, 2008; Cannea and Padiglia, 2025). Overall, antioxidant enzyme activities showed treatment- and time-dependent responses, with the combined treatment producing marked increases in several enzymes at specific sampling times. However, increased antioxidant enzyme activity should not necessarily be interpreted as a direct proxy for enhanced stress tolerance, since antioxidant responses are closely interconnected with ROS signalling and may vary according to stress conditions and exposure (Xu et al., 2025).

Furthermore, research indicated that secondary metabolism in basil is highly responsive to abiotic stress conditions (Ahmed et al., 2015). Among the applied stresses, hypoxia was the most effective in stimulating the accumulation of phenolic compounds in aerial organs, followed by the combined stress. Conversely, salinity often induced a weaker response and, in some cases at $\mathbf{T_1}$, $\mathbf{T_2}$, $\mathbf{T_3}$ and $\mathbf{T_4}$, resulted in metabolite levels comparable to or even lower than those of the control. This reveals that oxygen deficiency serves as a potent stimulus for the activation of the phenylpropanoid pathway, a process that is likely to be initiated by stress-induced redox imbalance and altered carbon allocation (Abiko et al., 2012). The combined stress also promoted phenolic accumulation, although to a lesser extent than hypoxia alone. This response may be indicative of a compromise between growth, antioxidant defense, and energy demand, as plants under multiple constraints must balance resource allocation between primary and secondary metabolism (Haddadi et al., 2016). Flavonoids, flavanols and proanthocyanidins exhibited similar trends, and these compounds have been associated with antioxidant functions, including ROS scavenging and metal chelation, in previous studies (Mahajan et al., 2020).

The DPPH and FRAP assays revealed different patterns of antioxidant activity among treatments. The lower DPPH IC_{50} values observed under hypoxia coincided with the higher accumulation of phenolic compounds under this treatment, whereas the FRAP assay showed a different response.

Overall, the changes in phenolic compounds, antioxidant enzyme activities, and in vitro antioxidant assays show that basil modulated both enzymatic and non-enzymatic components of its antioxidant response under the different stress conditions. The integrated response has been demonstrated to contribute to oxidative balance and stress tolerance, particularly under conditions of hypoxia and combined stress (Zandalinas et al., 2020).

5. Conclusion

This study provides new insights into the physiological and biochemical responses of hydroponically grown basil to salinity, hypoxia, and their combination. Stress type was a major factor influencing plant responses, while exposure duration further modulated these effects. Salinity negatively affected basil growth, whereas hypoxia induced marked physiological and biochemical changes. Under combined hypoxia and salinity, basil showed changes in tissue hydration together with coordinated antioxidant enzyme and metabolic responses. These findings highlight the importance of considering both salinity and oxygen availability in hydroponic cultivation and provide a basis for further studies on plant responses to multiple abiotic stresses. From a practical perspective, our results show that both salinity and oxygen availability in the root zone need to be considered when growing basil hydroponically. The different responses observed under salinity, hypoxia, and their combination suggest that nutrient-solution aeration and salinity can influence plant performance in different ways, particularly when root-zone conditions are not optimal. Although further studies are needed, these findings may help guide the management of hydroponic basil and support future work comparing the responses of different cultivars to combined abiotic stresses.

CRediT authorship contribution statement

Abir Arfaoui: Writing – original draft, Formal analysis, Conceptualization. **Riccardo Fedeli:** Writing – original draft, Investigation. **Jamila Ben Salem:** Investigation. **Elisa Balestri:** Investigation. **Stefano Loppi:** Writing – review & editing, Supervision. **Samira Aschi-Smiti:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2026.106466.

Data availability

Data will be made available on reasonable request by the corresponding author.

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