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Accumulation of Stinging Nettle Bioactive Compounds as a Response to Controlled Drought Stress

Mia Dujmović [1], Nevena Opačić [2,*], Sanja Radman [2], Sanja Fabek Uher [2], Sandra Voća [1] and Jana Šic Žlabur [1]

[1] Department of Agricultural Technology, Storage and Transport, University of Zagreb Faculty of Agriculture, Svetošimunska Cesta 25, 10000 Zagreb, Croatia
[2] Department of Vegetable Crops, University of Zagreb Faculty of Agriculture, Svetošimunska Cesta 25, 10000 Zagreb, Croatia; sradman@agr.hr (S.R.)
* Correspondence: nopacic@agr.hr

Abstract: As the impact of global warming intensifies drought effects, plants need to adapt to drought and other climate change-induced stresses through various defense mechanisms. One of them is the increased synthesis of bioactive compounds (BCs), which helps plants overcome adverse environmental conditions. This effect can be used in sustainable controlled cultivation as a tool for the nutritional improvement of crops, so this study focused on growing stinging nettle (*Urtica dioica* L.) for human consumption in a controlled environment. Since nettle can be consumed as a green leafy vegetable due to its nutritional value, the aim of this study was to determine the content of BCs (ascorbic acid, phenolic compounds, and pigments) and antioxidant capacity of nettle leaves grown under different drought stress conditions in an ebb and flow hydroponic system. During the experiment, plants were treated with a nutrient solution adjusted for nettle cultivation for 1 hour and then exposed to three different drought intervals: 24, 48, and 96 h. During the 48 h drought interval, the plants accumulated the highest amounts of total phenolic content and total non-flavonoid content (400.21 and 237.33 mg GAE/100 g, respectively), and during the 96 h drought interval, the nettle accumulated the highest amount of ascorbic acid (96.80 mg/100 g fw). The highest antioxidant capacity was recorded during the 24 and 48 h treatments (2435.07 and 2444.83 µmol/TE, respectively) according to the ABTS and during the 48 h treatment (3773.49 µmol/TE) according to the FRAP assay. The obtained results show that different drought stress durations caused by the absence of nutrient solutions can have a positive effect on the accumulation of nettle BCs.

Keywords: *Urtica dioica* L.; climate change; drought stress; phenolic compounds; ascorbic acid; antioxidant capacity; sustainable cultivation; ebb and flow hydroponic system

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1. Introduction

Global warming and climate change are increasing the risk of droughts, making them longer, more frequent, and more severe, which has a significant influence on plants [1]. Drought stress is a form of abiotic stress that occurs when plants experience a prolonged period of water deficit and is one of the main factors limiting plant productivity in agriculture [2,3]. One of the major challenges for modern agriculture is to deal with the negative effects of climate change, such as drought. To cope with these problems, food production systems are turning to sustainable controlled cultivation that does not have harmful effects on the planet [4].

Drought can have different impacts on plants, affecting their growth, development, morphology, physiology, and biochemical composition, including altering the content of bioactive compounds (BCs) [5,6]. Since drought stress limits the availability of water, which is essential for plant growth and development, it can have several effects: lower yields, reduced cell turgidity, decreased nutrient uptake, reduced photosynthetic activity, altered

hormonal balance, induction of oxidative stress, and others [7–11]. To maintain normal physiological functions, molecular adaptation mechanisms occur at the metabolic and gene levels [12,13]. Such mechanisms can significantly change the nutritional value as well as the biological effects of plants. These responses aim to minimize water loss and maintain cellular functions under limited water availability. One of the most common responses to drought is the production of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide radicals, which are highly reactive molecules that can cause oxidative damage to plant cells [7,14]. Antioxidant defense systems can overcome or mitigate these negative effects and are often accompanied by alterations in the production and accumulation of BCs. Thus, drought stress can significantly affect the content and profile of BCs [15,16]. In favor of the above, studies have shown that plants grown under stressful conditions are often richer in nutrients than the same plants grown under optimal conditions [17,18].

Drought is one of the major challenges of global open-field crop production. Since agriculture significantly contributes to climate change [19], in order to reduce the negative consequences on the environment, it is necessary to turn to sustainable agricultural practices such as hydroponic ebb and flow cultivation. The ability to use less fertilizer, water, and energy makes this agricultural system environmentally friendly [20]. It involves periodic flooding and draining of the nutrient solution, which allows the application of controlled drought stress [21]. It is also important to emphasize that this technique eliminates contamination with heavy metals, nitrates, and pesticides that normally accumulate in the soil and ensures uniform plant material thanks to the possibility of controlling abiotic factors [22]. Choosing the appropriate cultivation method can contribute to the improvement of nutritional quality and the accumulation of bioactive substances in the plant material [22].

BCs are products of different metabolic pathways of plants, and their synthesis is influenced by abiotic and biotic factors. It has been found that different stress factors can intensify and enhance the synthesis of BCs [23]. Numerous studies show that plants subjected to deliberately induced stress and growth environment modifications such as drought, salinity, and temperature stress can increase the accumulation of some BCs (carotenoids, AsA, phenolics, anthocyanins, and glucosinolates) [24–29]. Some plant responses to the application of controlled stress also showed that water stress increased the levels of phenolic compounds in *Hypericum brasiliense* Choisy [24]; drought increased the glucosinolate concentration of *Tropaeolum majus* L. [26]; hot and cold water treatment increased the concentration of vitamin C, chlorophyll b, lycopene, *p*-coumaric and ferulic acid, and glucosinolates of young broccoli [30]; total phenols, carotenoids, ferulic and *p*-coumaric acid of broccoli seedlings were induced by high temperature [31]; induced mechanical stress resulted in a significantly higher content of major antioxidants in lettuce and green chicory [32]; and controlled nutrient solution management had a positive effect on total phenolic and flavonoid content, AsA, and pigment content of nettle grown in a floating hydroponic system [33].

The nutritional value of stinging nettle (*Urtica dioica* L.) derives from numerous BCs that possess antioxidant activity, such as ascorbic acid (AsA), polyphenols, pigments, and minerals [34]. These compounds have great health importance for the human organism because they can pair electrons from free radicals, activate antioxidant enzymes, and inhibit oxidases and carcinogenic compounds [35–37]. For its BCs, fresh stinging nettle leaves can be consumed as a green leafy vegetable, in cooked dishes, and as a source of medicinal substances. Despite its rich nutritional composition, nettle is rarely cultivated, and information on the effects of drought stress on nettle leaf phytochemistry is lacking.

Most research on nettle focuses on wild-collected plant material [38–40], but hydroponic cultivation offers many advantages and can be a great tool for producing nettle leaves of known origin, rich in BCs, and safe for consumption [22]. Since nettle is rarely cultivated, it is important to emphasize that there is no detailed information on the ebb and flow cultivation conditions and the influence of different drought intervals on BC composition. Most research studies on drought stress mainly provide data on the influence of longer

water deficit periods (6 to 18 days) on various crops [5,41,42]. This type of experiment tends to show whether shorter dry periods, which do not disturb the morphological characteristics and do not cause wilting or irreversible damage to the plant tissue of nettle, can have a significant effect on the BC content, thus improving the nutritional quality of nettle leaves. Since nettle naturally grows in moist habitats, it is important to determine how it can withstand moderate drought stress that may occur as a result of climate change. Therefore, the aim of this study was to define adaptation to moderate drought conditions and to determine the direct influence of 3 different irrigation regimes, i.e., drought stresses for 24, 48, and 96 h (1, 2, and 4 days, respectively), on the content of selected BCs in nettle leaves. The interval of 24 h was set as a control irrigation interval, while 48 h and 96 h each represented moderate droughts, with different durations. This research is the first step in defining the conditions for controlled nettle cultivation in the ebb and flow system.

Using sustainable techniques with low environmental impact helps to preserve the environment and reduce the damage of climate change. Understanding the influences of drought on plants is crucial for devising strategies to enhance their resilience, improve quality, and mitigate the negative impacts of water scarcity on ecosystems and agriculture.

2. Materials and Methods

2.1. Plant Material

The experiment was conducted in a heated greenhouse at the Department of Vegetable Crops, University of Zagreb Faculty of Agriculture, in Croatia in 2022. Plants were cultivated on three tables (6 m^2 each) using the hydroponic ebb and flow technique. Nettle seeds were sown on January 20 in polystyrene containers with 40 potsfilled with a commercial substrate (Klasman Potgrond H). Approximately 5 seeds were sown per pot, placed on the tables, and regularly irrigated. The beginning of sprouting was noted on February 6. When the plants reached the appropriate size and density, on April 11, they were subjected to different drought and irrigation intervals. Plants were treated with a nutrient solution for 1 h and then exposed to 3 different drought treatments lasting 24, 48, and 96 h. Mature plants were manually mowed on May 10 at the pre-flowering stage. After the yield was measured, leaves were cleaned, separated from the stems, and chemically analyzed at the Laboratory for Quality Analysis of Agricultural Products of Plant Origin at the University of Zagreb Faculty of Agriculture.

2.2. Cultivating Conditions

From April 11, plants were irrigated with a nutrient solution and subjected to different drought treatments. The nutrient solution according to the Johnson recipe was used and prepared with the following salts: KNO_3—250.99, KH_2PO_4—142.7, $Ca(NO_3)_2 \times 4H_2O$—501.5, $MgSO_4 \times 7H_2O$—256.25, FeEDTA 13%—12.8, H_3BO_3—1.32, $CuSO_4 \times 5H_2O$—0.026, $MnSO_4 \times 4H_2O$—0.79, $ZnSO_4 \times 7H_2O$—0.11, and $Na_2MO_4 \times 2H_2O$—0.018 mg/L (EC 1.5 mS/cm, pH 5.8—6.2). The pH of the solution was regulated by adding 56% HNO_3.

Throughout the entire plant growth and development process, the abiotic factors of the greenhouse air (from February 6 to May 10) and the nutrient solution (from April 11 to May 10) were regularly monitored. Figure 1 shows the values of temperature and relative air humidity measured during nettle cultivation. Air temperature and RH were measured using a tabletop thermohygrometer (Agrologistika d.o.o., Čakovec, Croatia). Air temperature values ranged from 4.5 to 40.6 °C with an average value of 22.3 °C, while relative humidity ranged from 32 to 53% with an average value of 41.4%. A multiparameter instrument (Hanna instruments HI98194, Nușfalău, Romania) was used to measure the pH, EC values (mS/cm), and DO (mg/L) of the nutrient solution. The pH averaged 6.7, the EC value averaged 1.5 mS/cm, and the average value of DO in the nutrient solution was 4.6 mg/L, as shown in Figure 2.

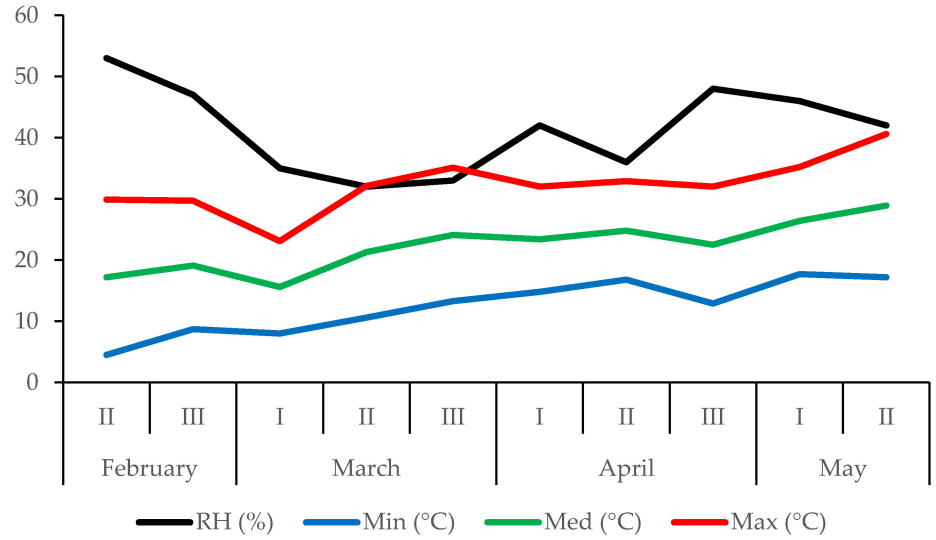

Figure 1. Abiotic factors of the greenhouse during the cultivation period; RH—relative humidity, Min—minimum temperature, Med—medium temperature, Max—maximum temperature. I, II, and III—first, second, and third 10 days of the month, respectively.

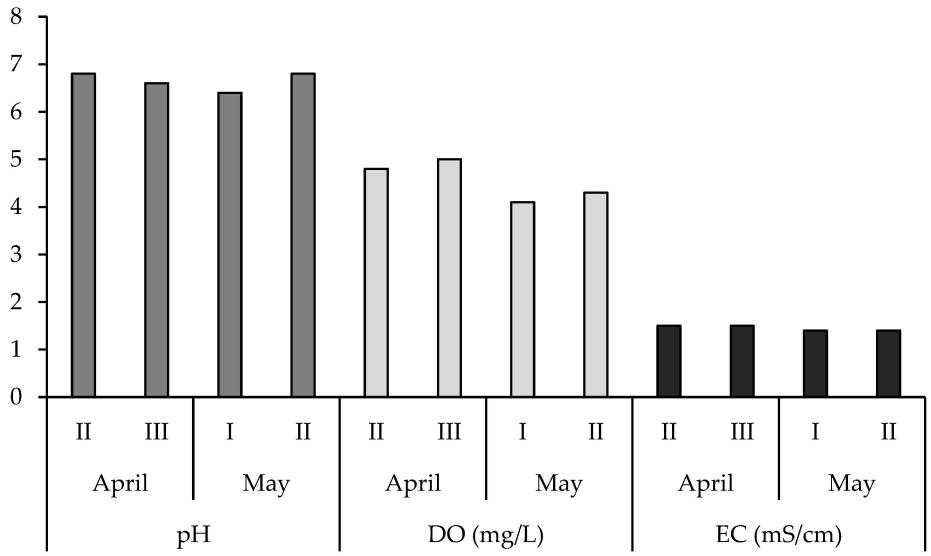

Figure 2. Abiotic factors of the nutrient solution during the cultivation period; DO—dissolved oxygen, EC—electrical conductivity.

2.3. Determination of Physico-Chemical Properties

Chromaticity parameters (L*, a*, b*, C, and h°) were determined according to the CIELab method using a colorimeter (ColorTec PCM+, PCE Instruments, Southampton, UK), where the L* value represents the lightness (0–50 indicates dark and 51–100 indicates light) and a* and b* values represent chromaticity (red to green and blue to yellow, respectively) [43]. The total dry matter content (DM, %) of stinging nettle leaves was determined by drying in an oven at 105 °C to constant mass, and the total acid content (TA, %) was determined by potentiometric titration with sodium hydroxide solution (c = 0.1 mol/L) according to the Association of Officiating Analytical Chemists (AOAC) [44].

2.4. Determination of the Ascorbic Acid Content

According to the standard AOAC method [45], titration with 2,6-dichloroindophenol (DCPIP) was used for the determination of the ascorbic acid (AsA) content. Approximately

4 g of the samples were homogenized with 100 mL of 2% (*v/v*) oxalic acid and then filtered through Whatman filter paper, and 10 mL of obtained filtrate was titrated with freshly prepared DCPIP until the appearance of pink stable coloration. The final quantitative measure of AsA content was expressed as mg/100 g fresh weight and calculated according to Equation (1):

$$\text{AsA (mg/100 g fw)} = (V \times F/D) \times 100 \quad (1)$$

where V is the DCPIP volume (mL), F is the DCPIP factor, and D is the sample mass in the filtrate (g).

2.5. Determination of Total Phenolic and Individual Phenolic Compounds

The spectrophotometric method with the Folin–Ciocalteu (FC) reagent was used for the determination of phenolic compounds, as described by Ough and Amerine [46]. Extracts were prepared by heating 10 g ± 0.01 of finely chopped leaves and 40 mL of 80% EtOH (*v/v*), to a boiling point with reflux, for 10 min. The process was repeated with another 50 mL of 80% EtOH (*v/v*). The solutions were filtered through Whatman filter paper into a 100 mL volumetric flask and made up with 80% EtOH (*v/v*), and obtained extracts were used for the determination of total phenolic (TPC), total flavonoid (TFC), and total non-flavonoid (TNFC) content and for the ABTS and DPPH assays as well.

The chemical reaction for TPC determination was prepared by mixing 0.5 mL ethanolic sample extract, 30 mL distilled water, 2.5 mL previously diluted FC reagent (1:2, *v/v*), and 7.5 mL saturated sodium carbonate solution in a 50 mL volumetric flask. The mixture was shaken well and made up to the mark with distilled water. After incubation for 2 h at ambient temperature, absorbances were determined at 750 nm with a spectrophotometer (Shimadzu, 1900i, Kyoto, Japan). Distilled water was used as a blank, and gallic acid was used as an external standard (concentrations 0–500 mg/L). The final TPC was expressed as milligrams of gallic acid equivalents per 100 g fresh weight (mg GAE/100 g fw) based on the equation of the gallic acid standard curve.

To determine the TNFC (phenolic acids, tannins, stilbenes, lignans, etc.), reaction mixtures were prepared by mixing 10 mL of the sample extract, 5 mL of HCl in EtOH (1:4, *v/v*), and 5 mL of formaldehyde (p.a.) and were blown with nitrogen. After incubation for 24 h in a dark place at ambient temperature, the solutions were filtered through Whatman filter paper, and the same aforementioned reaction with FC reagent and spectrophotometrically measurements were conducted. As an external standard, gallic acid was used, so TNFC was expressed as milligrams of gallic acid and equivalents per 100 g fresh weight (mg GAE/100 g fw). TFC was mathematically calculated as the difference between TPC and TNFC and expressed as milligrams of catechol equivalents per 100 g fresh weight (mg CTH/100 g fw).

Besides total phenolic compounds, some selected individual phenols were determined by liquid chromatography. For this purpose, the HPLC method (high-performance liquid chromatography) defined by Otles and Yalcin [47] was used to analyze caffeic acid, coumaric acid, ellagic acid, ferulic acid, and naringin. The extraction process was set up as follows: 1 g ± 0.01 of finely chopped leaves was mixed with 10 mL of 80% MeOH (*v/v*), homogenized with a laboratory homogenizer (IKA, UltraTurax T-18, Staufencity, Germany), and left for 30 min at 50 °C in an ultrasonic bath (Bandelin RK 103H, Berlin, Germany) to complete the extraction process. Before injection into the vials, the obtained extracts were filtered through Whatman paper and Chromafil PA filter. As an external standard for calibration of the method, a mixture of commercial standards of caffeic acid, coumaric acid, ellagic acid, ferulic acid, and naringin (Sigma Aldrich, Steinheim, Germany) was used. The solutions were prepared by dissolving the standards in HPLC-grade methanol to make stock solutions of 500 µg/mL, which were then used to prepare solutions (2–100 µg/mL) for the standard curve. Analysis of the phenolic compounds was conducted on a NUCLEOSIL 100-5 C18 column (5 µm, 250 × 4.6 mm i.d., Macherey-Nagel, GmbH, Düren, Germany) using an LC Nexera (Shimadzu, Kyoto, Japan) equipped with a photodiode array and fluorescence detector (PDA-RF). The phenolic standard solution mixtures and methano-

lic extracts were injected in duplicates using an autoinjector with an injection volume of 20 µL. Operation conditions were the same as those set up by Repajić et al. [40] with minor modifications. A gradient elution of two solvents was used: solvent A (3% formic acid in HPLC-grade water (*v/v*)) and solvent B (3% formic acid in HPLC-grade acetonitrile (*v/v*)). The gradient elution program started with 90% of solvent A and was reduced to 60% at 25 min. This was followed by a solvent A concentration of 30% at 30 min and 90% for the next 35 to 45 min. During a total run time of 45 min, a constant flow rate of 0.9 mL/min and a temperature of 23 °C were used. Qualitative identification was performed by comparing obtained retention times with the retention times of purchased commercial standards, and quantitative analyses were performed by calculating the calibration curves from the mixture of standards (Table 1). The final concentrations of individual phenols were expressed as mg/L. Phenolic compounds present in each sample during the different drought stress intervals are shown in chromatograms (Figure 3).

Table 1. Calibration curves equations of the individual phenolic standards mixtures.

Standard	Calibration Curve Equation	R^2 Value
Caffeic acid	y = 13159.9x + 12112.7	0.9998
Coumaric acid	y = 2551.11x + 2349.01	0.9999
Ellagic acid	y = 33829.1x − 6862.97	1.0000
Ferulic acid	y = 27461.5x − 90059.3	0.9870
Naringin	y = 3941.28x − 30329.7	0.9914

2.6. Determination of Pigment Compounds

Pigment compounds were determined by following the Holm [48] and Wettstein [49] method. Total chlorophylls (TCh), chlorophyll a (Chl_a), chlorophyll b (Chl_b), and total carotenoids (TCa) were measured spectrophotometrically (Shimadzu, 1900i, Kyoto, Japan) at wavelengths of 662 (Chl_a), 644 (Chl_b), and 440 nm (TCa), using acetone (p.a.) as blank. For extract preparation, the amount of 0.3 ± 0.01 g of fresh leaves was homogenized (laboratory homogenizer IKA, UltraTurax T-18, Staufencity, Germany) with a total volume of 15 mL of acetone (p.a.), which was added in 3 repetitions. The obtained acetone extracts were filtered through Whatman filter paper, and measured absorbances were used to quantify the pigment compounds by using Holm–Wettstein Equations (2)–(5). The final contents were expressed in mg/g.

$$\text{Chl\_a} = 9.784 \times \text{A662} - 0.990 \times \text{A644 (mg/L)} \quad (2)$$

$$\text{Chl\_b} = 21.426 \times \text{A644} - 4.65 \times \text{A662 (mg/L)} \quad (3)$$

$$\text{TCh} = 5.134 \times \text{A662} + 20.436 \times \text{A644 (mg/L)} \quad (4)$$

$$\text{TCa} = 4.695 \times \text{A440} - 0.268 \times \text{TCh (mg/L)} \quad (5)$$

2.7. Determination of Antioxidant Capacity

The same ethanolic extract used for phenolic compound determination was also used for antioxidant capacity determination through two spectrophotometric methods, ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (ferric reducing antioxidant power).

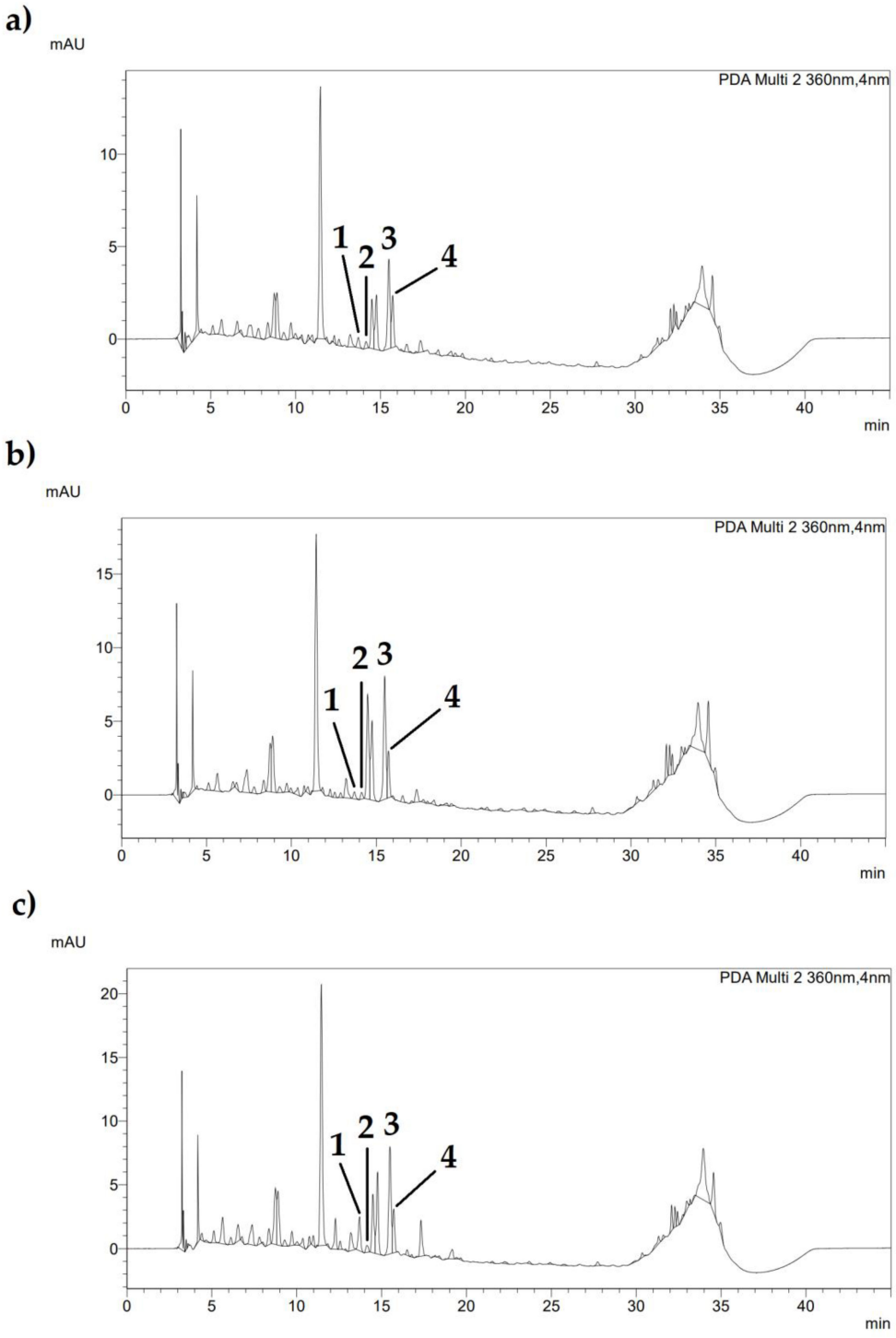

Figure 3. HPLC chromatogram of the individual phenolic compounds of fresh stinging nettle leaves recorded at 360 nm, cultivated under different drought stress intervals of (**a**) 24, (**b**) 48, and (**c**) 96 h drought treatment; 1—coumaric acid, 2—ellagic acid, 3—naringin, 4—ferulic acid.

The free radical scavenging capacity of the plant samples was determined by ABTS assay according to Miller et al. [50]. The method is based on the capacity of antioxidants to scavenge $ABTS^{\bullet+}$ radical cations, which is manifested as solution decolorization. For preparing an $ABTS^{\bullet+}$ radical cation solution, 88 µL of a 140 mM $K_2S_2O_8$ and 5 mL of a 7 mM ABTS solution were mixed and left to stand for 16 h in the dark at ambient temperature. From this reaction solution, a 1% working solution in 96% ethanol (*v*/*v*) was prepared. The absorbance was set up to 0.70 ± 0.02 at 734 nm. The amount of 2 mL

of 1% ABTS$^{\bullet+}$ radical cation solution and 160 µL of ethanolic extract were pipetted into a cuvette and left to incubate for 5 min at ambient temperature. Trolox was used as a standard and 96% ethanol as a blank control. Absorbances were measured at 734 nm with a spectrophotometer (Shimadzu, 1900i, Kyoto, Japan), and the final results were expressed as µmol TE/L (Trolox equivalents).

The ferric reducing antioxidant power (FRAP) of the ethanolic extracts was determined according to the method of Benzie and Strain [51]. In the presence of the sample antioxidant, Fe^{3+}–TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) is reduced to Fe^{2+}–TPTZ, and reduced iron is correlated with the amount of antioxidants and measured at 593 nm on a spectrophotometer (Shimadzu, 1900i, Kyoto, Japan). The FRAP reagent contained 75 mL of 300 mM acetate buffer (pH 3.6), 7.5 mL of 10 mM TPTZ reagent, and 7.5 mL of 20 mM $FeCl_3 \times 6H_2O$. It was freshly prepared, and 6240 µL of the FRAP reagent was mixed with 720 µL of water and 80 µL of ethanolic extract samples. The reaction mixtures were incubated in a water bath at 37 °C for 5 min. For the blank, a similar reaction was prepared, but instead of extracts, 80% EtOH (*v*/*v*) was used. To draw a calibration curve, Trolox was used as a standard, and antioxidant activity was expressed in µmol TE/L.

2.8. Statistical Analysis

The greenhouse experiment was laid out according to the randomized block design in three repetitions, and all laboratory analyses were performed in triplicate. All results were analyzed with one-way analysis of variance (ANOVA) using a general linear model procedure (PROC GLM) in SAS 9.4. software [52]. Mean values were compared using the *t*-test (LSD) and considered different at $p \leq 0.05$., while different superscript letters in the tables indicate significant statistical differences between the different drought treatment intervals.

3. Results

The values obtained for aboveground nettle yield (kg/m^2) during treatments of 24, 48, and 96 h of drought are presented in Figure 4. There were significant differences among the tested treatments. The highest yield was obtained under the most frequent nutrient solution irrigation period (24 h of drought).

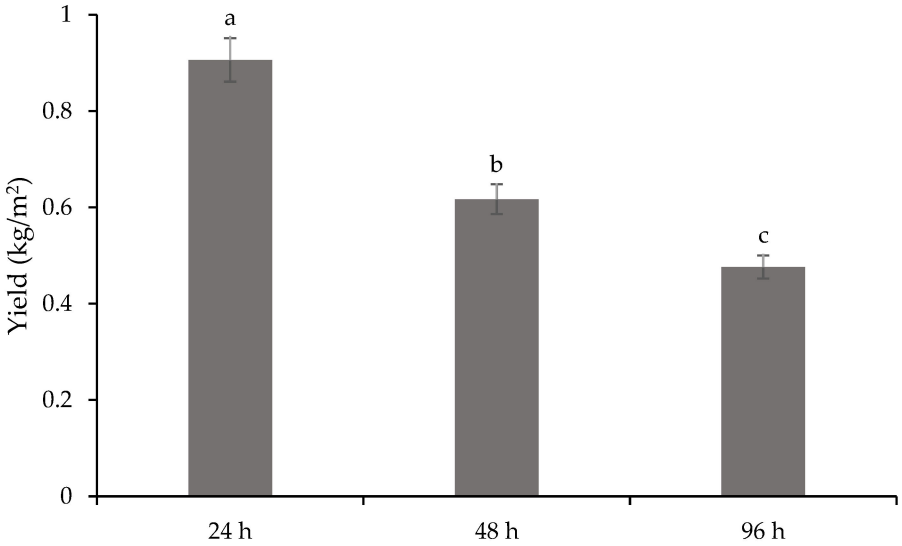

Figure 4. Yield of fresh aboveground stinging nettle cultivated under different drought intervals. Different letters indicate significant differences between mean values.

Table 2 shows the effects of drought on chromaticity parameters. All L* values below 50 indicate darker leaves, with the measured average being 42.44. Negative a* values show the presence of green color, with no change in green color among treatments. The average a* value was 14.73. Positive b* values show the presence of a yellow color. During the

treatment of 96 h, the highest b* value was recorded, which was higher by around 22% than in the other treatments. Analysis of variance showed that drought treatments had no significant effects on L* and a* values but strongly affected the b* value.

Table 2. Chromaticity parameters of fresh stinging nettle leaves cultivated under different drought intervals.

Treatments	L*	a*	b*	C*	h°
24 h	41.34 ± 1.45	−14.65 ± 0.56	23.17 ^{b} ± 1.85	27.41 ^{b} ± 1.85	122.35 ^{a} ± 1.13
48 h	41.77 ± 1.68	−13.91 ± 1.00	22.29 ^{b} ± 2.07	26.28 ^{b} ± 2.26	121.99 ^{a} ± 0.99
96 h	44.22 ± 0.98	−15.62 ± 0.32	27.69 ^{a} ± 0.44	31.79 ^{a} ± 0.49	119.44 ^{b} ± 0.44
ANOVA	NS	NS	0.0137	0.0169	0.0148
LSD	2.8008	1.3804	3.2414	3.4151	1.8092

L*—lightness; a*—green–red color; b*—blue–yellow color; C*—chroma; h°—hue; NS—non-significant. Results are expressed as mean ± standard deviation. Different letters indicate significant differences between mean values.

The results of the total dry matter content (DM) and total acid content (TA) of nettle leaves grown under different irrigation intervals are presented in Table 3. The different intervals had a significant influence on DM. The highest DM was recorded under intervals of 48 (21.5%) and 96 h (21.28%), which was about 13.5% higher than during the shortest (24 h) interval. On the other hand, the treatments did not affect the TA content, and values ranged from 0.24 to 0.29% of TA.

Table 3. Total dry matter content (DM %) and total acid content (TA %) of fresh stinging nettle leaves cultivated under different drought intervals.

Treatments	DM (%)	TA (%)
24 h	18.85 ^{b} ± 0.43	0.29 ± 0.03
48 h	21.50 ^{a} ± 0.56	0.24 ± 0.01
96 h	21.28 ^{a} ± 0.64	0.26 ± 0.06
ANOVA	0.0018	NS
LSD	1.0957	0.0769

Results are expressed as mean ± standard deviation. Different letters indicate significant differences between mean values.

The results of the analyzed bioactive compounds are shown in Tables 4–6. Statistical analysis of variance showed significant differences in AsA content between all varied treatments (Table 4). The highest content of AsA, 96.8 mg/100 g fw, was recorded during the longest (96 h) irrigation regime, while the lowest value was noted during the shortest interval (24 h treatment). The content of analyzed phenolic compounds (total phenolics—TPC, non-flavonoids—TNFC, and flavonoids—TFC) also differed significantly depending on the irrigation regime, as shown in Table 4. During the 48 h treatment, the highest TPC (400.21 mg GAE/100 g fw) and TNFC (237.33 mg GAE/100 g fw) were recorded, while the highest values for TFC were observed during both 24 h and 48 h irrigation intervals. Variance analysis showed that the controlled drought intervals had a significant effect on all phenolic compound contents.

As for individual phenols, values of the identified compounds are noted in Table 5. Naringin was dominant among the tested compounds, with an average value of 7.91 mg/L. The values obtained for ferulic and ellagic acid did not statistically differ and were present with an average amount of 4.21 mg/L and 1.18 mg/L, respectively. Coumaric acid was predominant in the longest dry period (6.81 mg/L), which was around 8 times higher than during the other two regimes, while caffeic acid was not detected in any sample. The results suggest that the different treatments had a significant influence only on coumaric acid.

Table 4. Ascorbic acid and phenolics compound content of fresh stinging nettle leaves cultivated under different drought intervals.

Treatments	AsA (mg/100 g fw)	TPC (mg GAE/100 g fw)	TNFC (mg GAE/100 g fw)	TFC (mg CTH/100 g fw)
24 h	73.01 ^{c} ± 2.63	328.06 ^{b} ± 0.51	166.01 ^{c} ± 0.90	162.05 ^{a} ± 0.40
48 h	79.52 ^{b} ± 3.60	400.21 ^{a} ± 1.41	237.33 ^{a} ± 1.35	162.88 ^{a} ± 2.76
96 h	96.80 ^{a} ± 2.48	308.45 ^{c} ± 0.52	173.61 ^{b} ± 0.56	134.84 ^{b} ± 0.63
ANOVA	0.0002	≤0.0001	≤0.0001	≤0.0001
LSD	5.8844	1.8354	1.9781	3.2987

AsA—ascorbic acid; TPC—total phenolic content; TNFC—total non-flavonoid content; TFC—total flavonoid content. Results are expressed as mean ± standard deviation. Different letters indicate significant differences between mean values.

Table 5. Individual phenolic compound content of fresh stinging nettle leaves cultivated under different drought intervals.

Treatments	Caffeic Acid (mg/L)	Coumaric Acid (mg/L)	Ellagic Acid (mg/L)	Ferulic Acid (mg/L)	Naringin (mg/L)
24 h	nd	1.15 ^{b} ± 0.10	0.79 ± 0.18	4.19 ± 0.05	7.88 ± 0.01
48 h	nd	0.61 ^{b} ± 0.32	1.23 ± 0.47	4.22 ± 0.03	7.89 ± 0.07
96 h	nd	6.81 ^{a} ± 1.94	1.52 ± 0.22	4.23 ± 0.07	7.96 ± 0.01
ANOVA	-	0.0010	NS	NS	NS
LSD	-	2.2702	0.6293	0.102	0.0793

nd—not determined; NS—non-significant. Results are expressed as mean ± standard deviation. Different letters indicate significant differences between mean values.

Table 6. Pigment compound content of fresh stinging nettle leaves cultivated under different drought intervals.

Treatments	Chl_a (mg/g)	Chl_b (mg/g)	TCh (mg/g)	TCa (mg/g)
24 h	0.85 ^{a} ± 0.01	0.50 ^{a} ± 0.02	1.34 ^{a} ± 0.03	0.23 ^{a} ± 0.01
48 h	0.64 ^{c} ± 0.01	0.26 ^{c} ± 0.01	0.90 ^{c} ± 0.01	0.23 ^{b} ± 0.01
96 h	0.71 ^{b} ± 0.01	0.42 ^{b} ± 0.01	1.13 ^{b} ± 0.01	0.22 ^{c} ± 0.01
ANOVA	≤0.0001	≤0.0001	≤0.0001	0.0004
LSD	0.0143	0.0242	0.0387	0.0036

Chl_a—chlorophyll a; Chl_b—chlorophyll b; TCh—total chlorophylls; TCa—total carotenoids. Results are expressed as mean ± standard deviation. Different letters indicate significant differences between mean values.

The content of chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophylls (TCh), and total carotenoids (TCa) was determined from a group of pigment compounds, and the results are presented in Table 6. Analysis of variance showed that the tested factor had a significant effect on pigment content. Additionally, according to the statistical analysis, significant differences were observed in the content of all pigments in the leaves during all treatments. The levels of Chl_a, Chl_b, TCh, and TCa were significantly higher in nettle leaves grown under the shortest irrigation interval (24 h) with the amounts of 0.85, 0.50, 1.34, and 0.23 mg/g, respectively.

The results of the antioxidant capacity of the studied samples exposed to different drought regimes are presented in Table 7. The values ranged from 2410.72 to 2435.07 µmol TE/L according to the ABTS and from 3114.24 to 3773.49 µmol TE/L according to the FRAP assay. Considering the results of the antioxidant capacity, it can be noticed that there was no statistical difference between 24 h and 48 h when using the ABTS assay. Using the FRAP assay, the highest antioxidant capacity was obtained under the influence of the intermediate irrigation interval of 48 h (3773.49 µmol TE/L), which was 21% higher than during the shortest drought period. A significant influence of controlled irrigation on the antioxidant capacity of nettle leaves was recorded.

Table 7. Antioxidant capacity of fresh stinging nettle leaves cultivated under different drought intervals.

Treatments	ABTS (μmol TE/L)	FRAP (μmol TE/L)
24 h	2435.07 ^{a} ± 4.23	3114.24 ^{c} ± 51.31
48 h	2444.83 ^{a} ± 4.57	3773.49 ^{a} ± 39.22
96 h	2410.72 ^{b} ± 12.78	3220.97 ^{b} ± 40.84
ANOVA	0.0058	≤0.0001
LSD	16.4	88.134

Results are expressed as mean ± standard deviation. Different letters indicate significant differences between mean values.

4. Discussion

One of the major agricultural tasks is to provide enough food through sufficient crop yields. Yield can be affected by many abiotic and biotic factors, but water availability is one of the critical conditions that can cause various challenges to agricultural production [53]. The results of this study show that the highest yield (0.91 kg/m^2) was accomplished when the plant received the most water (Figure 4). During the shortest drought period (24 h), the yield was 47% higher than during 48 h of drought and even 90% higher than during 96 h of drought. Based on obtained data, it can be concluded that prolonged unavailability of a nutrient solution leads to greater yield reduction. Droughts, with their complex patterns, are consistently related to negative impacts on crop yield on a global scale [54], and many studies recorded the same effect of reducing crop yield under drought stress [55–58]. Since water is essential for plants, physiological processes are disrupted when water supply is insufficient, resulting in reduced plant growth and development. Drought conditions decrease yield through reduced photosynthesis, nutrient deficiency, inhibition of cell division, and elongation [59,60]. Some of these effects may be responsible for yield reduction in nettle leaves. Under drought conditions, yield reduction is expected, so it is necessary to optimize and control drought stress in order to avoid large losses in agricultural production.

Visual perception plays a key role in the selection of nutritious and healthy foods, and color can serve as an indicator of food quality or defects. Customers associate different fruits and vegetables with their specific colors, which provide information about the condition of the plant material. The color of leaves can also be specific to different plant species and is related to pigment composition, mainly chlorophylls [61]. A change in the specific color and appearance of multi-colored spots may indicate disease [62], nutrient deficiency [63], or stress effects. In addition to various morphological changes, typical drought stress symptoms in plants include leaf yellowing [6], as was also observed in this research, where the leaves under the influence of the longest drought period (96 h) were the yellowest. To measure and define the color and color differences of nettle leaves, the CIELAB scale was used, and the results indicated darker leaves with the presence of green and yellow color, while drought treatments affected only the intensity of the yellow color (Table 2).

Total dry matter content (DM) represents the basic chemical composition of the raw material, i.e., the content of all components, such as proteins, carbohydrates, minerals, and BCs, except water [64]. Thus, plant materials with higher DM values possess higher nutritional quality. The obtained DM values in the nettle leaves ranged from 18.85 to 21.50% (Table 3), and plants irrigated for 48 and 96 h were more prone to DM accumulation than those with more frequent irrigation (every 24 h). Thus, longer dry periods positively affected DM accumulation, showing about 14% higher DM content than during the shortest dry period. This confirmed the expectation of the lowest DM content during the most frequent irrigation period due to the highest water availability. The increased accumulation of DM content may be due to several reasons. In general, the DM of plant tissues can be affected by various factors, including ecological and agronomic conditions and cultivation systems [65–67]. Water stress can have a positive or negative effect on the content of total DM in plant material, which depends on the length of the stress periods as suggested by the

results of this study. The trend of longer dry periods significantly increasing the DM content in leafy vegetables was confirmed by Mogren et al. [68]. Additionally, Soltys-Kalina et al. [5] confirmed that prolonged dry periods can lead to a decrease in the water content of leaves, i.e., an increase in DM content. Since drought results in temporary salt accumulation in the root zone as a consequence of the drying of the substrate [69,70], it is likely that under the influence of drought, an accumulation of nutrients occurred in the substrate. These nutrients are used for the production of DM, so it can be assumed that salt accumulation could result in an increase in DM during both 48 and 96 h periods. DM can also be affected by abiotic factors, and its accumulation is strongly correlated with water transpiration [71]. An average RH of 41.4% in the greenhouse during the cultivation period may have slowed down transpiration, causing high DM values during all treatments. This is one reason why cultivation in controlled conditions might take precedence over open-field-produced or collected plant material. Regardless of the influence of the investigated treatments, the obtained DM values are very close to those obtained for floating hydroponically cultivated nettle [72,73] and can be considered high compared to other leafy vegetables [74,75].

Organic acids naturally occur in vegetables as products of cellular metabolism and contribute to the sensory properties and durability of fresh raw materials [76]. The results of the present study showed that different absences of water had no effect on TA content in fresh nettle leaves (Table 3). Usually, the presence of TA in plant material can depend on environmental conditions, the type of plant tissue, and plant maturity [77]. Organic acids are key components in mechanisms that some plants use to better adapt to environmental stress [78]. Thus, it can be assumed that due to the stress caused by the deficiency of water, nettle synthesized an equal amount of organic acids during all treatments in order to preserve the plant metabolism.

AsA is an important compound with antioxidant properties found in various plant tissues including fruits and leaves [79,80], where it plays a crucial role in plant defense mechanisms against oxidative stress caused by various environmental factors, including drought [81]. On the other hand, AsA is an essential nutrient with numerous beneficial health properties required for the normal functioning of the human organism [82,83]. As previously shown in some other studies [14,55,84–87], the results of this research also confirm that drought can have a significant influence on the AsA content in plants. In this study, AsA content increased with the prolongation of dry periods, with the lowest value recorded during the shortest and the highest value during the longest dry period (Table 4). During the 96 h treatment, nettle leaves accumulated even 33% more AsA than during the 24 h regime. Regardless of the highest measured value, all nettle samples grown in the hydroponic ebb and flow system under drought treatments of 24 to 48 h can be considered a rich source of AsA, especially when compared to other studies on wild collected nettle leaves [88,89], other leafy vegetables [90–92], and the recommended daily intake (RDI) of vitamin C (according to the Institute of Medicine) [93]. Drought can both decrease [14,86] or increase AsA content in different agricultural crops, but most studies have shown that drought stress generally leads to an accumulation of AsA in plants. Accordingly, the enhancement of AsA levels affected by drought was established in several studies [55,84,85,87]. Drought stress triggers various adaptive responses in plants, including the activation of antioxidant defense mechanisms [94]. Drought can cause increased ROS (reactive oxygen species) production, which leads to oxidative stress [7,95]. AsA plays a crucial role as an antioxidant by scavenging ROS, neutralizing its harmful effects, and protecting plant cells by maintaining cellular redox homeostasis and protecting various cellular components (proteins, lipids, and DNA) from oxidative damage [79,96]. Therefore, under drought conditions, plants may allocate more AsA towards antioxidant defense, which was the case in the present study. Oxidative stress induced by adverse environmental conditions (drought and salinity) leads to lipid peroxidation with free radicals attacking and damaging cell membranes [15,97]. This, in turn, increases AsA production in order to overcome the negative effects. Another way to deal with environmental inconveniences and oxidative stress is to stimulate the biosynthesis of antioxidant enzymes, such as

superoxide dismutase, catalase, or glutathione peroxidase, whose activity can be affected by AsA [96,98]. On the other hand, drought can also disrupt normal AsA (nonenzymatic antioxidant) metabolism and affect the activity of other enzymes responsible for AsA biosynthesis, leading to changes in its content [98], as in the present study. Apart from the fact that the specific effect of drought on AsA content can vary depending on plant species, nutrient availability, duration, and severity of drought, different plant tissues may respond differently to drought stress. The results of this study show that nettle leaves grown under stress caused by 24 to 96 h of the absence of nutrient solution are a valuable source of AsA.

Phenolics are a diverse group of compounds found in plants that play various roles in their growth and development and are crucial in secondary metabolism as defense molecules [99]. In addition to plant protection, phenolic compounds have great application potential in human health, acting as antimicrobial, anti-inflammatory, and antitumor agents [100]. Drought stress can affect the concentration of phenolic compounds in plant tissues as suggested by some other studies [41,42,101–106] but also confirmed by the results of this research (Table 4). The results show that the irrigation interval of 48 h caused the highest levels of TPC and TNFC, with the TPC content being 22% higher than during the shortest treatment (irrigation interval of 24 h). Results for TNFC show that during the same 48 h treatment, non-flavonoid compounds were around 37% more abundant than during the longest drought treatment (96 h) and even 43% higher than during the shortest drought period (24 h). As for TFC, the 24 and 48 h treatments had the same effect on flavonoid accumulation, which was around 21% higher than during the longest irrigation interval (96 h). Drought can stimulate the biosynthesis of phenolic compounds in plants as a response to stressful conditions. Increased phenolic production is considered to be a protective mechanism against oxidative stress that enhances plant defenses [94]. In some cases, total phenolic content may increase as a result of drought-induced stress responses [42,87,101,102,105,106]. However, in other cases, prolonged drought stress may lead to a decrease in phenolic content due to various physiological and metabolic changes in the plant [41,103,104]. Moreover, different plant tissues, such as leaves, stems, and roots, may respond differently to drought stress in terms of phenolic production and accumulation [6,15,56,85,100]. The obtained results of the present study suggest that a moderate disruption of nutrient solution supply for 48 h is optimal to increase the accumulation of total phenolic compounds in nettle leaves.

Various individual phenolic compounds were recorded in nettle, depending on plant origin [107,108]. The presence of caffeic, coumaric, ellagic, and ferulic acid and naringin has been previously noted in nettle [38,47,107–110], other leafy vegetables such as spinach, lettuce, basil, Pakchoi, and Chinese cabbage [111,112], and leaves of other plant species as well [111–115]. These compounds play important roles in plant organisms: caffeic acid is involved in stress tolerance [116], coumaric acid modulates plant growth and metabolism [112], ellagic acid has a protective role [115], ferulic acid is found widespread in plant cell walls [117], and naringin is involved in leaf development [118]. Apart from the fact that all selected individual phenols have a role in plant development, they also show positive effects on human health and many pharmacological activities [100,111,112,115,118]. Of the individual phenolic compounds investigated, coumaric acid was the most dominant in the irrigation interval of 96 h. The increase in the content of coumaric acid was almost 8-fold higher compared to the other treatments. Drought-induced treatments had no influence on other individual phenolic compounds (ellagic and ferulic acid and naringin), and caffeic acid was not detected at all. This goes in favor of different phenolic compounds responding differently to drought stress. Moreover, depending on the plant species and their specific stress response mechanisms, different individual phenolic compounds may be synthesized or accumulated. For example, levels of caffeic, coumaric, and ferulic acids in *Vitis vinifera* L. leaves decreased significantly under drought stress [41], while the levels of coumaric and caffeic acids were greater in water-stressed leaves of *Ctenanthe setosa* (Rosc.) [101]. This, as well as the results of the present study, confirms that some plants may increase the production of certain phenolics, while others may decrease or

maintain their levels. Apart from the fact that the specific effects of drought on phenolic content can depend on plant species and organs, it is important to note that they can vary among plant developmental stages, genotypes, environmental conditions, and durations of drought [103,104,119]. In addition, due to the complexity of plant phenolic metabolism and interaction with other stress factors, it is challenging to generalize the precise influence of drought on the phenolic content of plants [100,120].

Photosynthetic pigments are molecules that play a crucial role in photosynthesis and are vital for the proper development and growth of plants. The two primary pigment groups involved in photosynthesis are chlorophylls and carotenoids [121]. They also manifest numerous beneficial biological functions and health benefits for the human organism [61,122]. In nettle leaves grown under different irrigation intervals, all pigments were most abundant in the 24 h interval (Table 6). The lowest Chl_a, Chl_b, and TCh contents were found during the 48 h treatment, and the values were about 25, 48, and 33% lower than during the 24 h treatment, respectively. The lower leaf chlorophyll content in treatments with reduced irrigation was also confirmed in experiments by other authors [55,123–125]. In general, drought stress inhibits the photosynthetic rate. When water becomes limited, plants may close their stomata to reduce water loss through transpiration. This also reduces the availability of CO_2 for photosynthesis. As a result, plants may reduce the synthesis of chlorophyll molecules because they are not needed in large quantities under reduced photosynthetic activity [9,126–128]. This reduction in chlorophyll content can also result in a visible symptom known as chlorosis, in which the leaves appear yellow or pale green due to lower chlorophyll levels. This can be correlated with the chromaticity parameter b*, where leaves under a 96 h irrigation interval were the yellowest (Table 2). Drought stress can also alter the ratio of different chlorophyll molecules (a and b) within the plant, increasing the ratio of chlorophyll a to chlorophyll b. This adjustment helps to maintain a more efficient energy transfer process and optimize the limited available light energy [129,130]. In the present study, this trend was recorded between the first and second treatments, where the Chl_a to Chl_b ratio increased by 1.5-fold as drought prolongated from 24 to 48 h. Carotenoids are accessory pigments that cooperate with chlorophylls and help protect chlorophyll molecules from excessive light energy [131,132]. During drought stress, plants may increase the production of carotenoids as a defense mechanism against oxidative stress caused by high light intensity and limited water availability as shown by some studies [85]. However, the results of the present study showed a different trend, with the highest carotenoid content detected during the shortest treatment, which was then slightly increased by prolonging irrigation regimes (Table 6). Although reductions in chlorophyll content can decrease photosynthetic rates and thus limit plant energy production and growth [133], the increased production of other BCs can help protect the plant from excessive light and oxidative damage, which could improve its survival chances under drought conditions. The specific responses of plant pigments to drought can vary depending on plant species, the severity and duration of the drought, and other environmental factors [6]. This study confirmed, as other experiments had previously shown, that drought conditions can have significant effects on plant pigment composition, with drought stress often leading to a decrease in total chlorophyll content in plants.

Antioxidants are any compounds that help protect cells from damage caused by free radicals [36,134]. All chemical compounds analyzed in this study (AsA, pigments, and phenols) are considered to have antioxidant properties. As previously confirmed, under drought stress, plants often respond by increasing the production of antioxidants because a drought-induced water deficit can lead to an imbalance between the production of ROS and the plant's ability to scavenge and neutralize them [7,105,135]. Compounds with antioxidant activity also protect lipids from peroxidation, preventing cell membrane damage caused by drought-induced oxidative stress [134]. Drought stress also triggers the activation of various antioxidant enzymes in plants, as previously mentioned, which scavenge and convert harmful ROS into less reactive or non-toxic forms [135]. In this study, two different assays for the determination of antioxidant capacity were used because antioxidant

systems can be both hydrophilic and lipophilic. Considering the values determined for nettle leaves under different irrigation intervals, it can be noted that the highest values were recorded during the 24 and 48 h treatments according to the ABTS assay, but these values were only about 1.2% higher than during the 96 h treatment. Higher antioxidant capacity values detected by the FRAP assay during longer irrigation intervals (48 and 96 h) are most likely due to the accumulation of antioxidant compounds in response to oxidative stress conditions. These results are in agreement with those of AsA and TNFC, for which the highest values were also determined during longer dry periods (48 and 96 h). It is necessary to emphasize that regardless of the statistical differences between irrigation intervals, high antioxidant capacity values were detected during all treatments, highlighting that nettle leaves are a potent antioxidant source, even under moderate drought conditions.

5. Conclusions

The conducted research provides additional data on the production of nettle in a sustainable ebb and flow hydroponic system under controlled drought stresses. The results show that drought encouraged the increased production of some BCs, especially AsA. The highest AsA and coumaric acid contents were recorded during the longest drought period (96 h), while the highest DM, TPC, and TNFC were determined during either 48 or 96 h of nutrient solution absence. The shortest treatment (24 h) was suitable for achieving the highest yield and the best accumulation of pigments. In general, the results suggest that at the expense of yield, the nutritional quality of nettle leaves can be significantly improved under the influence of moderate drought. Apart from the fact that the task of agriculture is to produce a sufficient amount of food, it is very important that this food is nutritionally rich, so it is necessary to find a balance in the application of controlled drought in order to meet the needs of yield and quality. The ebb and flow system with the possibility of controlled irrigation intervals and abiotic factors can be used as an efficient and sustainable practice for the production of nettle leaves rich in BCs. Further studies on the effects of longer drought on nettle phytochemistry, simulating severe climatic stress, are recommended. Understanding the influence of drought on BC levels in plant tissues is important for both plant physiology research and agricultural practices, as it can provide insights into the mechanisms of plant responses to water stress and help in the development of drought-tolerant crop varieties, which is also important in the context of climate change.

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