

Article

Exogenous Proline Modulates Physiological Responses and Induces Stress Memory in Wheat Under Repeated and Delayed Drought Stress

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Abstract: Drought stress negatively affects plant metabolism and growth, triggering complex defence mechanisms to limit damage. This study evaluated the effectiveness of a single foliar application of 1 mM L-proline (Pro) in winter wheat (*Triticum aestivum* L., cv. Bohemie) in two separate experiments differing in the time interval between application and drought—7 days (experiment 1) and 35 days (experiment 2). Net photosynthetic rate (A), transpiration rate (E), stomatal conductance (g_s), leaf water potential (Ψ_w), intrinsic water use efficiency (WUE_i), endogenous proline content (Pro), malondialdehyde content (MDA), and maximum quantum yield of photosystem II (F_v/F_m) were measured. In experiment 1, drought markedly reduced net photosynthetic rate, transpiration rate, stomatal conductance, and leaf water potential in both drought-stressed treatments, namely, without priming plants (S) and with Pro priming plants (SPro). Pro and MDA content increased under stress. Higher E and g_s in the SPro treatment indicated more effective stomatal regulation and a distinct water use strategy. Pro content was significantly lower in SPro compared to S, whereas differences in MDA levels between these treatments were not statistically significant. The second drought period (D2) led to more pronounced limitations in gas exchange in both S and SPro. Enhanced osmoregulation was reflected by lower Ψ_w ($S < SPro$) and higher Pro accumulation in S ($S > SPro$). The effect of exogenous Pro persisted in the form of reduced endogenous Pro synthesis and improved photosystem II protection. Rehydration of stressed plants restored all monitored physiological parameters, and Pro-treated plants exhibited a more efficient recovery of gas exchange. Experiment 2 demonstrated a long-lasting priming effect that improved the preparedness of plants for future drought events. In the SPro treatment, this stress memory supported more efficient osmoregulation, reduced lipid peroxidation, improved protection of photosystem II integrity, and a more effective restart of gas exchange following rehydration. Our findings highlight the potential of exogenous proline as a practical tool for enhancing crop resilience to climate-induced drought stress.

Keywords: proline; winter wheat; drought stress; stomatal regulation; stress memory; physiological priming



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1. Introduction

Under natural conditions, plants are frequently subjected to recurring periods of various combinations of environmental stressors, such as drought, extreme temperatures,

salinity, or pathogen attacks. In response to these stimuli, plants engage a broad spectrum of processes, encompassing physiological, biochemical, and molecular changes, which enable their adaptation and survival [1]. Drought is regarded as the most severe environmental stress [2]. In the context of climate change, this factor is gaining momentum and represents a serious threat to life on Earth, particularly with respect to maintaining food security for an ever-increasing global population [3].

Many reviews describe complex, cascade like resistance mechanisms, including (i) morphological changes (cuticle thickness, leaf shedding and rolling, and stomatal density) and adjustments in growth dynamics (changes in assimilate translocation, root-to-shoot ratio dynamics, root length increment, accelerated development, or, conversely, delayed senescence); (ii) modifications in water management (reduction in transpiration loss through altering stomatal conductance and distribution); (iii) osmotic and hormonal regulation and antioxidant and signalling mechanisms; and (iv) regulation of gene expression [4,5].

Preventing water loss through stomatal regulation, coordinated with the synthesis of protective and signalling molecules and the subsequent optimisation of photosynthetic processes, can be considered key responses [6]. Phytohormones such as abscisic acid (ABA), ethylene, salicylic acid (SA), and jasmonic acid (JA) influence hormonal signalling, with their levels and mutual interactions determining the effectiveness of the plant's response to stress [7]. Simultaneously, protective metabolites accumulate, particularly osmoprotectants such as proline, trehalose, and glycine betaine, along with antioxidants and secondary metabolites [8]. Sharma et al. [9] stated that osmoprotectants stabilise cellular structures and eliminate reactive oxygen species (ROS), maintain osmotic balance, protect enzymes and membranes from denaturation, regulate ion fluxes, and sustain enzyme activity under stress conditions. In this way, they contribute to the maintenance of metabolic homeostasis and enhance the overall resistance of plants to adverse conditions [9]. These compounds also contribute to osmotic adaptation, allowing plants to retain water and preserve cellular functions during drought or high salinity [10].

The amino acid proline (Pro) is an important multifunctional osmoprotective and protective molecule [11,12]. Its increased synthesis and accumulation is a response to abiotic stressors such as drought, salinity, and high and low temperatures [13]. Specifically, it functions as a scavenger of the hydroxyl radical, interacts with enzymes responsible for stress tolerance, protects protein structure and enzyme activity, maintains pH and redox balance, and supplements carbon, nitrogen, and energy sources [14]. The metabolism of proline involves its synthesis from glutamate or ornithine and degradation back to glutamate. The main synthetic pathway begins with the conversion of glutamate to Δ^1 -pyrroline-5-carboxylate (P5C) by the enzyme pyrroline-5-carboxylate synthetase (P5CS). P5C is subsequently reduced to proline by pyrroline-5-carboxylate reductase (P5CR). During degradation, proline is oxidised to P5C by proline dehydrogenase (ProDH) and then converted to glutamate by pyrroline-5-carboxylate dehydrogenase (P5CDH) [14,15]. The main genes involved in proline synthesis are *P5CS* (Δ^1 -pyrroline-5-carboxylate synthetase) and *P5CR* (pyrroline-5-carboxylate reductase). In proline degradation, key roles are played by the genes *ProDH* (proline dehydrogenase) and *P5CDH* (pyrroline-5-carboxylate dehydrogenase) [16,17].

Plant priming using chemical agents, as a first exposure to abiotic stress, can prepare plants for subsequent stress events [18]. Stress memory in plants is mediated by metabolomic (amino acids, sugars, etc.), proteomic (antioxidant and photosynthetic enzymes, etc.), transcriptomic (*WRKY*, *AREB*, etc.), and epigenetic mechanisms [19]. Crisp et al. [20] state that short-term memory (probably associated with increased metabolites or transcription factors) can persist for days or weeks, while memory based on epigenetic mechanisms may last for months and eventually be transmitted to the offspring. Epigenetic

changes play a significant role, i.e., modifications of DNA and histone proteins that alter chromatin structure and regulate gene expression without changing the DNA sequence itself [21]. Through modulation of gene expression, stress tolerance is enhanced [22,23], and at the same time, the ability of plants to “remember” stress and respond more effectively during subsequent stress episodes is reinforced [24,25]. Key epigenetic modifications include changes in DNA methylation, histone modifications, and the regulation by small non-coding RNAs, which can affect gene expression over the long term [19,26]. Changes in gene expression involve the transcriptional reprogramming of hundreds to thousands of genes associated with defence and recovery, leading to long-term adaptive changes in plants [27,28].

Numerous studies across various plant species have demonstrated the positive effects of foliar proline application on plants exposed to abiotic stress. For example, in maize, Khan et al. [29] reported that proline application (30 mM) enhanced plant growth and vitality. The combination of protective effects—including osmotic adjustment, reduced lipid peroxidation, and activation of antioxidant enzymes—explains the improved drought tolerance observed in proline-treated plants. Proline contributed to the maintenance of the photosynthetic apparatus of the flag leaf and increased both the number of ears and grains per ear [30].

Short-term responses of wheat to drought involve the activation of osmotic adjustment mechanisms, particularly the synthesis of proline, which acts as a compatible solute and enhances antioxidant enzyme activity, thus protecting the plant from oxidative stress [31,32]. These processes help maintain water balance and the stability of cellular structures, which are critical for survival under water deficit conditions [33,34]. In contrast, long-term responses involve complex physiological changes, including modifications in root system architecture, regulation of water use efficiency (WUE), and the expression of genes associated with drought tolerance [35]. Wheat plants develop deeper or more extensive root systems to access water more efficiently. These phenotypic adaptations, along with genes regulating proline metabolism, play a key role in long-term drought tolerance, although their specific functions and applications remain the subject of ongoing research [36].

In wheat, the most drought-sensitive stages include tillering, stem elongation, anthesis, and grain filling, with water deficit during these phases potentially reducing yield by up to 69% [37]. Exogenous Pro has repeatedly been shown to alleviate drought-induced damage and improve the physiological status of plants. For instance, applying 6 mM Pro after one week of growth under stress induced by 150 mM PEG-6000 increased endogenous Pro accumulation and enhanced CAT, APX, and GPX activities [38]. Foliar spraying with 150 mg·L⁻¹ Pro (~1.3 mM) onto the flag leaf at anthesis under 35% water holding capacity (WHC) improved chlorophyll, proline, glycine betaine, and phenolic contents while reducing MDA, grain number, and grain weight per spike [39]. Similarly, repeated application of 30 mM Pro during the first two weeks of drought (30% WHC) in four-week-old plants enhanced growth and physiological traits [40].

Based on current knowledge, proline priming predominantly leads to the suppression of the adverse effects of abiotic stresses on plants, while it may also contribute to the activation of mechanisms leading to the induction of stress memory [25,41,42]. Although many questions remain unanswered, exogenous application of proline is a potential technique to prevent the harmful effects of drought on plant growth and other physiological traits [43]. In our research, we focused on elucidating the effect of exogenous 1 mM L-proline on key physiological processes in wheat subjected to repeated periods of drought. In addition, the protective effect of exogenous proline under future drought stress was also investigated.

2. Materials and Methods

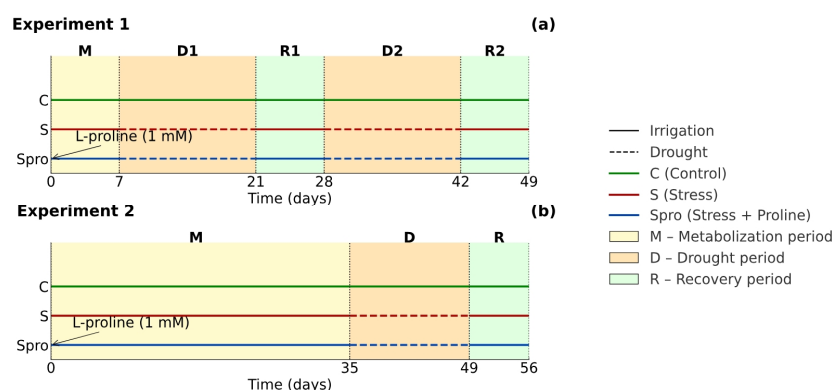
2.1. Experimental Design and Drought Stress Treatments

Winter wheat (*Triticum aestivum* L.), cv. Bohemie (Selgen Plc., Uhřetice, Czech Republic), was sown and cultivated in plastic containers (37 × 27 × 14 cm; 14 L). A total of 40 plants were grown in each container (2 plants in a 4 × 5 row spacing), corresponding to approximately 400 plants per square metre. The growing medium used was a commercial substrate (AGRO CS, Plc., Říkov, Czech Republic) with a pH of 5.0–6.5, composed of 80% white peat, 20% black peat, and 20 kg of clay per cubic metre. Nutrient content was 80–120 mg N L⁻¹, 22–44 mg P L⁻¹, and 83–124 mg K L⁻¹; the substrate particle size was 0–10 mm. Plants were grown in a controlled greenhouse environment under day/night temperatures of 20 ± 2 °C and 16 ± 2 °C, a natural light regime of 14 h light/10 h dark, relative humidity of 65–75%, and ambient CO₂ concentration and light intensity (GPS: 50°07'52.6" N, 14°22'14.8" E). Substrate moisture was maintained gravimetrically at approximately 75% of field capacity. The weight of the experimental container with substrate at 75% of field water capacity (FWC) was determined prior to the start of the experiment. The substrate was first air-dried, then fully saturated with water. After gravitational water was allowed to drain for 24 h, the weight corresponding to 100% FWC was recorded. Based on this value, the target weight representing 75% FWC was calculated and subsequently maintained using a gravimetric approach.

At the five-leaf stage (BBCH 15), two independent experiments were initiated. L-proline (1 mM; ≥99.5%, Merck KGaA, Darmstadt, Germany, CAS No. 147-85-3) was applied as a uniform foliar spray using a handheld pressure sprayer to induce plant priming at a rate equivalent to 100 L ha⁻¹ of spray solution. Drought stress was induced by withholding irrigation, allowing the substrate to dry progressively.

2.1.1. Experiment 1: Repeated Drought Stress in Two Periods

The experiment included three treatments: (i) control treatment (C; continuously irrigated throughout the experiment, with no priming); (ii) drought stress without priming (S); and (iii) drought stress with 1 mM Pro priming (SPro). The experimental timeline was as follows: priming period with Pro application (A); 7 days of metabolisation of the applied Pro (M); irrigation withheld for 14 days (first drought period, D1); re-irrigation for 7 days (first recovery period, R1); irrigation withheld again for 14 days (second drought period, D2); and re-irrigation for 7 days (second recovery period, R2) (Scheme 1a). Plant analyses and measurements were performed at the end of the D1, R1, D2, and R2 periods.



Scheme 1. Timeline of drought stress treatments in winter wheat. (a) Experiment 1: Repeated drought stress with two drought periods (D1 and D2) and two recovery periods (R1 and R2), following foliar application of 1 mM Pro in the SPro treatment. (b) Experiment 2: Delayed drought stress following a longer metabolisation period (M) and involving one drought period (D) and one recovery period (R). Solid lines indicate irrigation, and dashed lines indicate drought.

2.1.2. Experiment 2: Delayed Drought Stress

The experiment included three treatments: (i) control treatment (C; continuously irrigated throughout the experiment, with no priming); (ii) drought stress without priming (S); and (iii) drought stress with 1 mM Pro priming (SPro). The experimental timeline was as follows: priming period with Pro application (A); 35 days of metabolisation of the applied Pro (M); irrigation withheld for 14 days (drought period, D); and re-irrigation for 7 days (recovery period, R) (Scheme 1b). Plant analyses and measurements were performed at the end of the D and R periods. During the metabolisation period, the proline content was determined at weekly intervals in plants after exogenous application of 1 mM Pro.

2.1.3. Preparation of the Experimental Timeline (Scheme 1)

Based on a detailed description of the experimental design, a vector template was generated using the large language model platform ChatGPT (GPT-4o, OpenAI; accessed April 2025). The draft included the layout of two experimental variants, a time axis, and basic treatment labels. The file was then exported in SVG format and subsequently re-drawn, graphically adjusted, and finalised by the authors using Inkscape 1.3 (Free Software Foundation, Boston, MA, USA).

2.2. Leaf Water Potential

Leaf water potential (Ψ_w ; MPa) was determined from frozen leaf tissue. Samples were placed into 5 mL syringes, sealed with Parafilm, and stored at $-24\text{ }^{\circ}\text{C}$. After freezing, 10 μL of sap was extracted and applied onto a filter paper disc, which was then inserted into a C-52 sample chamber connected to a PSYPRO system (WESCOR Inc., Logan, UT, USA). Measurements were conducted in three technical replicates for each of five biological replicates (individual plants).

2.3. Leaf Gas Exchange

Gas exchange parameters, including net CO_2 assimilation (A ; $\mu\text{mol CO}_2\text{ m}^{-2}\text{ s}^{-1}$), stomatal conductance (g_s ; $\text{mmol H}_2\text{O m}^{-2}\text{ s}^{-1}$), and transpiration rate (E ; $\text{mmol m}^{-2}\text{ s}^{-1}$), were recorded in situ using a portable photosynthesis system (LCpro, ADC BioScientific Ltd., Hoddesdon, UK). Measurements were taken from the fourth or fifth fully expanded leaf between 9:00 a.m. and 12:00 p.m. under a photosynthetically active radiation (PAR) of $650\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$. Ambient CO_2 levels were maintained, and the temperature in the measurement chamber was set at $23\text{ }^{\circ}\text{C}$. Once steady-state conditions were reached (approximately 15 min), data were collected. One leaf per plant was measured in three biological replicates.

2.4. Intrinsic Water Use Efficiency (WUE_i)

Intrinsic water use efficiency (WUE_i) was calculated as the ratio of the net CO_2 assimilation rate (A ; $\mu\text{mol CO}_2\text{ m}^{-2}\text{ s}^{-1}$) to stomatal conductance (g_s ; $\text{mmol H}_2\text{O m}^{-2}\text{ s}^{-1}$), according to the following equation: $\text{WUE}_i = A/g_s$. The values of A and g_s were obtained simultaneously during in situ measurements of gas exchange using a portable photosynthesis system, LCpro (ADC BioScientific Ltd., Hoddesdon, UK), as described in Section 2.3. The resulting unit of WUE_i was expressed as $\mu\text{mol CO}_2\text{ mol}^{-1}\text{ H}_2\text{O}$.

2.5. Chlorophyll Fluorescence

Chlorophyll fluorescence parameters, including minimum fluorescence (F_0) and maximum fluorescence (F_m), were evaluated on dark-adapted leaves using a portable fluorometer (OS5p+, ADC BioScientific Ltd., Hoddesdon, UK). The fourth or fifth fully expanded leaf was dark-adapted for 20 min prior to measurement. A 1-second excitation pulse at 660 nm and a saturating light intensity of $3000\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ were applied. The max-

imum quantum efficiency of PSII (F_v/F_m) was calculated using the following formula: $F_v/F_m = (F_m - F_0)/F_m$. Measurements were taken from three biological replicates, with a total of five readings obtained across these replicates.

2.6. Proline Content

Free proline (Pro) concentration was quantified following the method of Bates et al. [44], with minor modifications. Leaf samples (0.5 g fresh weight) from all fully expanded leaves of each plant were immediately flash-frozen at $-80\text{ }^{\circ}\text{C}$, ground to a fine powder in liquid nitrogen, and extracted in 10 mL of 3% sulfosalicylic acid using a pre-chilled mortar and pestle. The resulting homogenate was then filtered through filter paper. Aliquots of 1 mL of the filtrate were mixed with 1 mL of acid ninhydrin solution and 1 mL of acetic acid, and the mixture was shaken for 20 min. Subsequently, the samples were heated at $95\text{ }^{\circ}\text{C}$ for 30 min, cooled in ice water, thoroughly mixed with 3 mL of toluene, and incubated for 20 min at room temperature. The samples were then stored at $4\text{ }^{\circ}\text{C}$ for 24 h, after which the upper toluene layer of the separation mixture was used to measure absorbance at 520 nm using a UV-vis spectrophotometer (Evolution 210, Thermo Scientific, Waltham, MA, USA). Pro content was determined in five biological replicates (individual plants) per treatment. The concentration was calculated from a standard calibration curve and expressed as mg g^{-1} FW (fresh weight).

2.7. Malondialdehyde Content

Malondialdehyde (MDA) levels were assessed using a modified thiobarbituric acid (TBA) assay [45]. Leaf samples (0.4 g fresh weight), prepared as a composite from all fully expanded leaves of each plant, were immediately flash-frozen at $-80\text{ }^{\circ}\text{C}$, ground to a fine powder in liquid nitrogen, and extracted in 80% ethanol. The resulting homogenate was centrifuged at 6000 rpm for 5 min. Two sets of 0.7 mL supernatant were prepared: one mixed with 0.7 mL of 0.65% TBA in 20% trichloroacetic acid (TCA) containing 0.01% butylated hydroxytoluene (BHT) and the second with 0.7 mL of 20% TCA with 0.01% BHT as a blank. Samples were incubated at $95\text{ }^{\circ}\text{C}$ for 25 min, cooled to room temperature, and centrifuged again at 6000 rpm for 5 min. Absorbance was measured at 440, 532, and 600 nm using a UV-vis spectrophotometer (Evolution 210, Thermo Scientific, Waltham, MA, USA). MDA concentration was determined in five biological replicates and expressed as $\mu\text{g g}^{-1}$ FW using an extinction coefficient of $155\text{ mM}^{-1}\text{ cm}^{-1}$.

2.8. Statistical Analysis

A one-way analysis of variance (ANOVA) was used to evaluate the effects of treatments on the measured variables. When the F-test indicated significance ($\alpha = 0.05$), treatment means were compared using Tukey's honestly significant difference (HSD) test. All statistical analyses were conducted using Statistica 13.5 (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Repeated Drought Stress in Two Periods (Experiment 1)

3.1.1. Gas Exchange

Measured values of CO_2 assimilation (A), transpiration rate (E), and stomatal conductance (g_s) are shown in Figure 1a–c. After the first drought period (D1), all three parameters were significantly reduced in stressed plants compared to the control. Pro application (SPro) led to a significant increase in stomatal conductance ($96.1\text{ mmol H}_2\text{O m}^{-2}\text{ s}^{-1}$) and transpiration ($2.04\text{ mmol H}_2\text{O m}^{-2}\text{ s}^{-1}$) compared to the treatment without Pro application (S). Differences in CO_2 assimilation between the S and SPro treatments were not significant (10.20 and $9.80\text{ }\mu\text{mol CO}_2\text{ m}^{-2}\text{ s}^{-1}$, respectively).

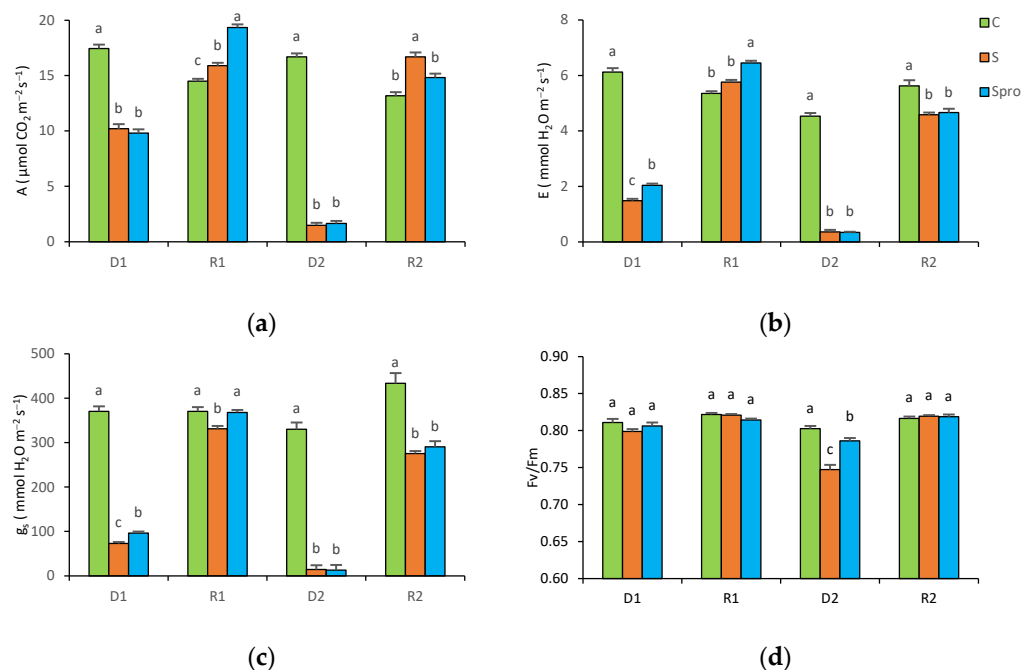


Figure 1. (a) Assimilation of CO_2 (A); (b) transpiration rate (E); (c) stomatal conductance (g_s); and (d) maximum quantum yield of photosystem II (F_v/F_m) measured in winter wheat plants (cv. Bohemie) during two drought periods (D1 and D2) and corresponding recovery periods (R1 and R2) in Experiment 1. Data represent mean values ($n = 3$) for three treatment variants: C (control, well-watered), S (drought stress without proline application), and SPro (drought stress with foliar application of 1 mM Pro). Error bars indicate standard error (SE). Different letters indicate statistically significant differences among treatments within each period (Tukey's HSD test, $\alpha = 0.05$).

After the first recovery period (R1), all monitored parameters (A , E , and g_s) recovered. Significant differences were found in CO_2 assimilation in the order $\text{SPro} > \text{S} > \text{C}$ (19.34, 15.89, and 14.49 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively). Pro application also significantly increased transpiration (6.45 $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) compared to both the control and the S treatment. A significantly lower stomatal conductance was measured in the S treatment (331.2 $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) than in the control (370.4 $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$).

The second drought period (D2) resulted in a marked decline in all gas exchange parameters (A , E , g_s) in both stressed treatments, with no significant differences between them. Compared to D1 values, average CO_2 assimilation decreased by 84.4% (1.56 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), transpiration by 79.5% (0.35 $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and stomatal conductance by 83.6% (13.5 $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$).

After the second recovery period (R2), a partial recovery of gas exchange parameters occurred. The highest CO_2 assimilation values were recorded in the S treatment (16.69 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), with no significant differences between the C and SPro treatments (13.17 and 14.82 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively). Transpiration and stomatal conductance in the stressed treatments did not reach the levels of control plants, and no significant differences were observed between the S and SPro treatments (Figure 1a–c).

3.1.2. Chlorophyll Fluorescence

After the first drought period (D1) and subsequent recovery (R1), no significant differences in the values of the maximum quantum yield of PSII (F_v/F_m) were observed in stressed plants (S and SPro) compared to control plants. During the D1 and R1 periods, F_v/F_m values ranged from 0.798 to 0.810 and from 0.814 to 0.822, respectively. The second drought period (D2) resulted in a significant reduction in F_v/F_m values in the stressed treatments. A significantly higher F_v/F_m value was recorded in the SPro treatment (0.786)

compared to the S treatment (0.747). After recovery (R2), F_v/F_m values in both stressed treatments increased to the level of the control plants (Figure 1d).

3.1.3. Leaf Water Potential

After the first drought period (D1), a significant reduction in leaf water potential was observed in the S variant (−2.13 MPa) compared to the control (−1.37 MPa). In the SPro treatment, a decrease in leaf water potential (−1.85 MPa) was also recorded; however, this difference was not statistically significant. During recovery (R1), leaf water potential in both stressed treatments increased to the level of the control plants (Figure 2a).

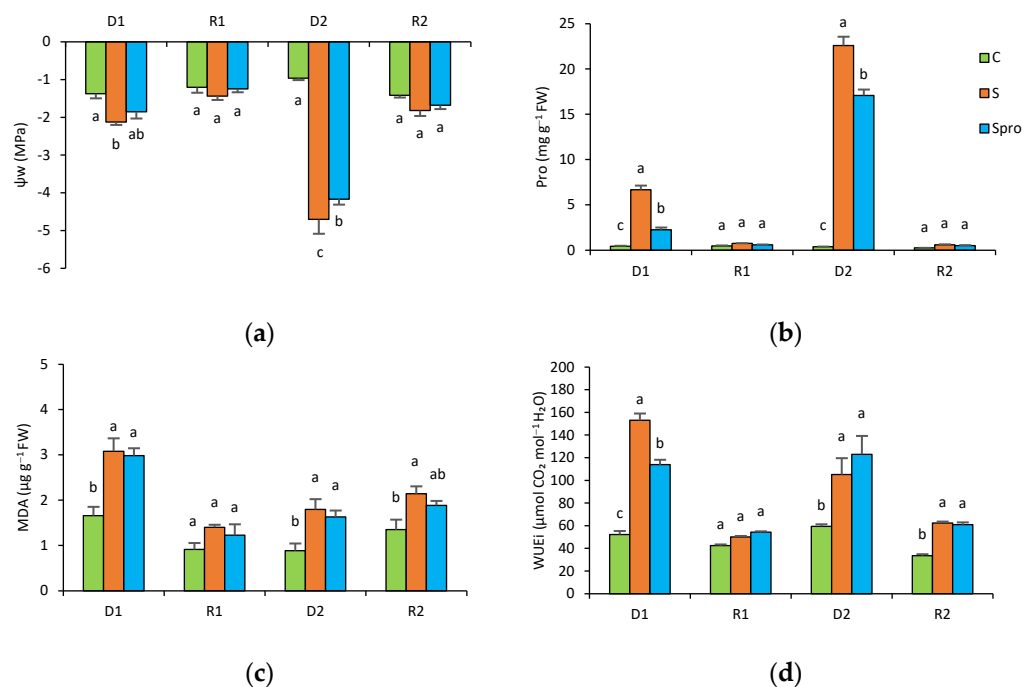


Figure 2. (a) Leaf water potential (Ψ_w); (b) free proline content (Pro); (c) malondialdehyde content (MDA); and (d) intrinsic water use efficiency (WUEi) in winter wheat plants (cv. Bohemie) measured during two drought periods (D1 and D2) and corresponding recovery periods (R1 and R2) in Experiment 1. Treatments include C (control, well-watered), S (drought stress without proline application), and SPro (drought stress with foliar application of 1 mM Pro). Data represent means $\pm$ standard error (SE), $n = 5$. Different letters indicate statistically significant differences among treatments within each period (Tukey's HSD test, $\alpha = 0.05$).

The second drought period (D2) caused a marked decrease in leaf water potential in both stressed treatments. The lowest values were observed in the S treatment (−4.70 MPa), followed by the SPro treatment (−4.17 MPa) and the control (−0.96 MPa). During the subsequent recovery (R2), leaf water potential was restored to the level of the control plants (Figure 2a).

3.1.4. Proline and MDA Content

The content of free Pro was significantly affected by stress and the application of exogenous L-proline. After the first drought period (D1), a significantly higher Pro content was determined in stressed plants without Pro application (S) (6.67 mg g^{-1} FW) compared to the SPro treatment (2.25 mg g^{-1} FW) and the control (0.43 mg g^{-1} FW). After the first recovery (R1), Pro content in the stressed treatments decreased to the level of the control (Figure 2b).

The second drought period (D2) induced a high accumulation of Pro in both stressed treatments, with significantly higher Pro levels in plants without Pro applica-

tion ($22.58 \text{ mg g}^{-1} \text{ FW}$), followed by the Spro treatment ($17.07 \text{ mg g}^{-1} \text{ FW}$), while Pro content remained low in the control ($0.37 \text{ mg g}^{-1} \text{ FW}$). During recovery R2, Pro content in both stressed treatments decreased to the level of the control plants (Figure 2b).

Drought stress significantly increased MDA content in stressed plants compared to the control. After the first drought period (D1), MDA levels reached $3.08 \mu\text{g g}^{-1} \text{ FW}$ in S and $2.98 \mu\text{g g}^{-1} \text{ FW}$ in SPro. However, differences between S and SPro were not statistically significant. After recovery (R1), MDA content decreased in all treatments, and no significant differences were found between treatments (Figure 2c).

During the second drought period (D2), MDA content in stressed plants again increased significantly compared to the control. Differences between S ($1.80 \mu\text{g g}^{-1} \text{ FW}$) and SPro ($1.63 \mu\text{g g}^{-1} \text{ FW}$) were not statistically significant. After the second recovery (R2), MDA levels in the SPro treatment returned to control levels, while the S treatment retained significantly higher MDA content than the control (Figure 2c).

3.1.5. Intrinsic Water Use Efficiency (WUE_i)

Intrinsic water use efficiency (WUE_i) was significantly affected by both stress and Pro application (Figure 2d). After the first drought period (D1), significant differences were observed between the treatments in the order $\text{S} > \text{SPro} > \text{C}$ (153.01 , 113.87 , and $52.21 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$, respectively). After the first recovery (R1), WUE_i values decreased to the level of the control plants.

The second drought period (D2) led to an increase in WUE_i in both stressed treatments. However, differences between the S and SPro treatments were not statistically significant, and the increase in WUE_i in the S treatment was lower than that observed during the D1 period. After recovery (R2), WUE_i values decreased in both S and SPro but remained significantly higher compared to the control (Figure 2d).

3.2. Delayed Drought Stress (Experiment 2)

3.2.1. Gas Exchange

After the drought period (D), a significant reduction in CO_2 assimilation, transpiration, and stomatal conductance was observed in the stressed plants (S and SPro) compared to the control plants. The lowest values of A and E were recorded in plants treated with proline (SPro), specifically $0.65 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ and $0.37 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively. In the S treatment, the values of these parameters were significantly higher ($3.11 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ and $0.80 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively). Stomatal conductance (g_s) was low in both stressed treatments, with no statistically significant difference between S and SPro.

After recovery (R), gas exchange parameters were restored in all treatments. The highest value of CO_2 assimilation was measured in the SPro treatment ($11.57 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), which differed significantly from both the S treatment ($8.32 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and the control ($9.02 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). Stomatal conductance (g_s) and transpiration (E) also reached the highest values in the Spro treatment ($240 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ and $5.62 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively), with statistically significant differences compared to the other treatments.

In the S, the value of E was significantly higher than in the control (4.76 vs. $3.57 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), while A and g_s were not statistically different between these treatments. Overall, gas exchange recovery was most pronounced in the SPro across all monitored parameters (Figure 3a–c).

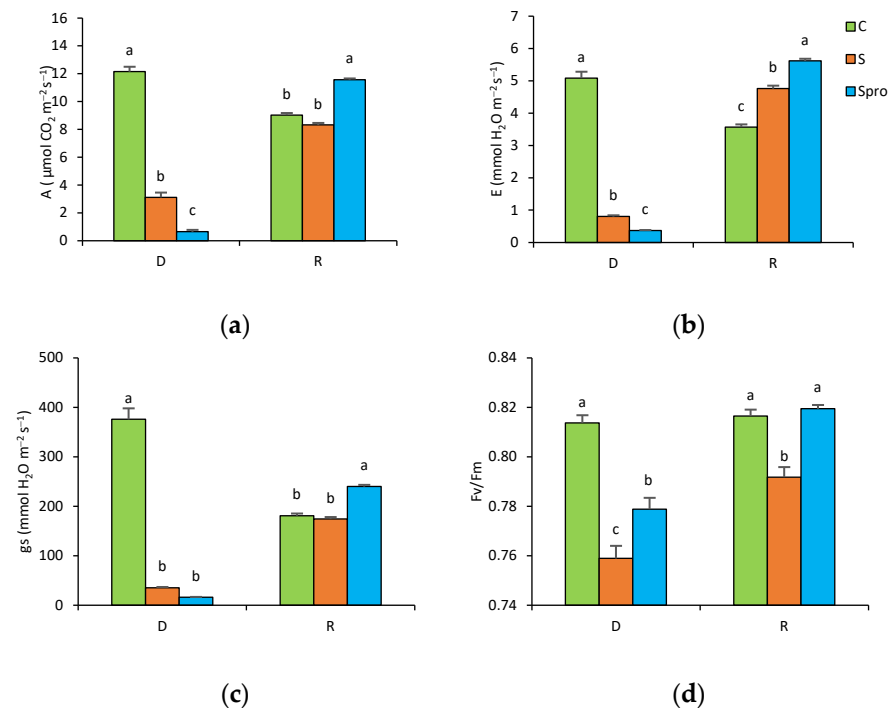


Figure 3. (a) Assimilation of CO₂ (A); (b) transpiration rate (E); (c) stomatal conductance (g_s); and (d) maximum quantum yield of photosystem II (F_v/F_m) measured in winter wheat plants (cv. Bohemie) during the drought period (D) and recovery period (R) in Experiment 2. Data represent mean values ($n = 3$) for three treatment variants: C (control, well-watered), S (drought stress without proline application), and SPro (drought stress with foliar application of 1 mM Pro). Error bars indicate standard error (SE). Different letters indicate statistically significant differences among treatments within each period (Tukey's HSD test, $\alpha = 0.05$).

3.2.2. Chlorophyll Fluorescence

After the drought period (D), a reduction in the maximum quantum yield of photosystem II (F_v/F_m) was observed in the stressed plants (S and SPro) compared to the control (Figure 3d). The values were recorded in the order $S < SPro < C$ (0.759, 0.779, and 0.814, respectively). Differences among all three treatments were statistically significant. After recovery (R), F_v/F_m values increased in all treatments. Values recorded in the SPro treatment (0.819) and C (0.816) were not statistically different, while a significantly lower value was observed in the S treatment (0.792).

3.2.3. Leaf Water Potential

During the drought period (D), leaf water potential (Ψ_w) significantly decreased in the stressed plants (S and SPro) compared to the control. The lowest value was recorded in the SPro treatment (-3.26 MPa), followed by the S treatment (-2.48 MPa), in comparison with the control plants (-1.42 MPa). After recovery (R), leaf water potential in the S treatment (-1.23 MPa) increased to the level of the control treatment (-1.18 MPa). In the SPro variant, leaf water potential remained lower (-1.58 MPa), with the difference compared to the other treatments being statistically significant (Figure 4a).

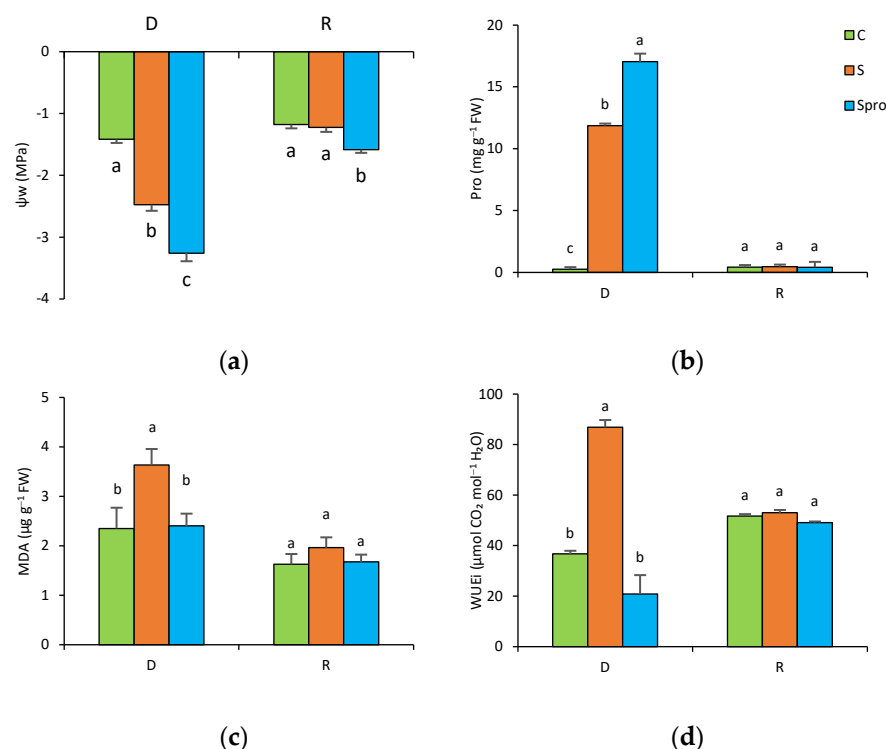


Figure 4. (a) Leaf water potential (Ψ_w); (b) free proline content (Pro); (c) malondialdehyde content (MDA); and (d) intrinsic water use efficiency (WUEi) in winter wheat plants (cv. Bohemie) during the drought period (D) and recovery period (R) in Experiment 2. Data represent mean values (Ψ_w , proline, and MDA: $n = 5$; WUEi: $n = 3$) for three treatment variants: C (control, well-watered), S (drought stress without proline application), and SPro (drought stress with foliar application of 1 mM Pro). Error bars indicate standard error (SE). Different letters indicate statistically significant differences among treatments within each period (Tukey's HSD test, $\alpha = 0.05$).

3.2.4. Proline and MDA Content

Table 1 shows the endogenous Pro content determined at weekly intervals in S and SPro* plants prior to drought stress exposure (*corresponds to the SPro treatment after stress induction, experiment 2). No significant differences in endogenous Pro content were observed between plants with and without proline application during the metabolisation period.

Table 1. Dynamics of free proline content (mg g^{-1} FW) in the leaves of winter wheat (*Triticum aestivum* L., cv. Bohemie) during a 35-day metabolisation period after foliar application of 1 mM Pro (SPro*) compared with untreated control plants (C). Values represent means $\pm$ standard error (SE), $n = 5$. Different letters at each time point indicate statistically significant differences between treatments according to Tukey's HSD test ($\alpha = 0.05$).

Treatments	Time (Days)					Mean
	7 d	14 d	21 d	28 d	35 d	
C	0.28 ± 0.03^a	0.42 ± 0.07^a	0.46 ± 0.06^a	0.37 ± 0.05^a	0.25 ± 0.02^a	0.36 ± 0.02^a
SPro *	0.38 ± 0.03^a	0.26 ± 0.01^a	0.49 ± 0.05^a	0.49 ± 0.05^a	0.43 ± 0.03^a	0.41 ± 0.03^a

* During the metabolisation period, SPro plants were grown under optimal conditions without stress and subsequently exposed to drought (drought period; D).

Drought (D) significantly increased proline content in stressed plants. The highest value was recorded in the SPro treatment (17.04 mg g^{-1} FW), followed by the S treatment (11.86 mg g^{-1} FW), while the control plants had minimal Pro content (0.25 mg g^{-1} FW) (Figure 4b). Differences among all treatments were statistically significant. After recovery (R), Pro content decreased in all treatments, and differences between them were no longer

statistically significant. The average content of free Pro after rewatering was 0.43 mg g^{-1} FW (Figure 4b).

MDA content significantly increased after the drought period (D) in plants without Pro application (S; $3.63 \text{ } \mu\text{g g}^{-1}$ FW) compared to the control ($2.35 \text{ } \mu\text{g g}^{-1}$ FW). MDA content in the SPro treatment ($2.40 \text{ } \mu\text{g g}^{-1}$ FW) was not statistically different from the control. After recovery (R), MDA levels decreased, and differences between the stressed treatments (S and SPro) and the control were not statistically significant (Figure 4c).

3.2.5. Intrinsic Water Use Efficiency (WUE_i)

After the drought period (D), a significant increase in WUE_i values was observed in the S variant ($86.84 \text{ } \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) compared to both the control treatment ($36.71 \text{ } \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) and the SPro treatment ($20.85 \text{ } \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$). After recovery (R), WUE_i values in S and SPro were comparable to the control, with no significant differences (Figure 4d).

4. Discussion

4.1. Repeated Drought Stress in Two Periods (Experiment 1)

Drought fundamentally affects plant morphology, physiology, and biochemistry. In line with this, CO_2 assimilation (A), stomatal conductance (g_s), and transpiration (E) decreased in both stressed variants, while intrinsic water use efficiency (WUE_i) increased due to a relatively greater limitation of g_s compared to A, as described by Lawson et al. [6]. Stomatal closure is a typical response to limited water availability, reducing water loss but also CO_2 uptake into the mesophyll, thereby decreasing photosynthetic capacity [46]. A decline in leaf water potential (Ψ_w) and increased malondialdehyde (MDA) levels indicated dehydration and oxidative damage to lipids, phenomena described by Seleiman et al. [4] and Chen et al. [8].

A single foliar application of 1 mM Pro prior to the first drought period mitigated some of the negative effects of water stress. Increased E and g_s values in the SPro treatment compared to S suggest more effective stomatal regulation and a distinct water use strategy that enabled the maintenance of turgor, as previously reported by Hosseinifard et al. [43]. Kaur and Asthir [11] demonstrated that proline improves osmotic balance in leaves and delays ABA-induced stomatal closure by stimulating H^+ -ATPase activity.

The higher g_s observed in the SPro treatment did not lead to an increase in A, which is consistent with the findings of Wang et al. [47], who reported that photosynthesis may also be limited by mesophyll CO_2 diffusion or reduced RubisCO activity. The significantly higher E in SPro compared to S may also contribute to more effective thermoregulation [18].

Water stress leads to an increase in endogenous Pro content in leaves [11]. Excessive Pro accumulation in stressed plants contributes to maintaining osmotic balance, optimising water uptake, and sustaining redox homeostasis, thereby reducing oxidative membrane damage [9]. In plants subjected to exogenous Pro application, we observed a significantly lower Pro content in leaves compared to untreated stressed plants. According to Szabados & Savaure [14], this reduction in Pro levels during stress in plants treated with exogenous Pro may be explained by feedback inhibition of the P5CS enzyme and rapid utilisation of proline via ProDH as a source of nitrogen and energy.

During water stress, enhanced ROS synthesis leads to an increase in MDA content, which signals lipid peroxidation [48]. In plants exposed to the first drought period (D1), we observed an increase in MDA levels; however, no statistically significant differences were found between treatments. Exogenous Pro is generally reported to reduce lipid peroxidation and thereby decrease MDA accumulation [17]. This effect was not observed in plants treated with exogenous Pro. In some cases, no change in MDA content was recorded

following Pro application, suggesting that Pro alone may not be sufficient to prevent lipid peroxidation [49]; therefore, based on our results, no clear conclusion can be drawn regarding MDA content in relation to the role of exogenously applied Pro. According to recent findings, Pro concentration alone is not a reliable indicator of stress tolerance or oxidative protection, as its effects depend primarily on metabolism and regulatory pathways [17]. As stated by Renzetti et al. [17], rather than its challenged osmotic function, it is the signalling role of Pro that plays a key part in plant adaptation, influencing redox balance, the activation of antioxidant enzymes, and the overall dynamics of the stress response.

After rehydration (R1), all monitored physiological parameters were restored in stressed plants. Water uptake increases leaf water potential and turgor, enabling stomatal reopening and the recovery of gas exchange, including CO₂ assimilation [46]. The rate of recovery depends on the extent of PSII damage and the efficiency of repair processes in the thylakoid membranes [50]. Antioxidant defence mechanisms and the elimination of ROS, associated with a reduction in MDA content, are also considered crucial [51]. Plants treated with Pro showed significantly higher gas exchange parameters. Exogenous Pro is known to accelerate the recovery of photosynthetic activity and enhance gas exchange traits [30]. After rehydration, endogenous proline levels in both S and SPro treatments decreased to control levels. Proline degradation is linked to energy and nitrogen metabolism, restoration of redox and osmotic balance, repair of cellular structures, and the reactivation of growth [14,52].

The second drought period (D2) resulted in more pronounced limitations of gas exchange in both S and SPro treatments. Stomatal closure and reduced transpiration represent primary adaptive mechanisms that help maintain cellular turgor [53]. Enhanced osmoregulation was reflected by a lower water potential (S < SPro) and greater Pro accumulation in the S treatment (S > SPro). Plants treated with exogenous Pro (SPro) maintained higher F_v/F_m values, suggesting reduced photochemical damage [54]. The lower MDA content during D2 compared to D1 may align with the findings of Lukić et al. [55], who reported that repeated stress enhances antioxidant activity, which can persist over several weeks.

The delayed recovery of g_s and E during R2, compared to the faster restoration of water potential, confirms that post-rehydration responses are not governed solely by stomatal control but also involve metabolic and photochemical repair processes. The inconsistency between the strong increase in A in the S treatment and the persistent mild limitation in F_v/F_m after rehydration is also described by Chaves et al. and Pinheiro & Chaves [46,51], who note that full recovery of photosynthesis requires coordinated restoration of stomatal regulation, photochemical efficiency, and biochemical capacity. It is known that repeated stress can induce short-term stress memory, based on metabolic and epigenetic modifications that facilitate a more efficient response to subsequent stress [24,56]. However, as pointed out by Crisp et al. [20], in many cases, a memory imprint is not established, as resetting and forgetting may be among the strategies plants employ to maximise growth under favourable conditions. The complexity of these processes is discussed in detail by Stacke et al. [57].

4.2. Delayed Drought Stress (Experiment 2)

During the metabolic phase (M) preceding the induction of drought stress (D) in experiment 2, endogenous Pro content was monitored in control plants and in those treated with exogenous Pro. Thirty-five days after a single foliar application of 1 mM Pro, basal Pro levels in the control (C) and proline-treated plants (SPro) did not differ. In other words, endogenous Pro content was comparable in C and SPro prior to the onset of stress, indicating the absence of residual applied Pro.

Following the subsequent drought period, significant differences were observed in Pro content and MDA accumulation between treatments. SPro plants accumulated 43% more endogenous Pro and exhibited 34% lower MDA levels compared to the S treatment. Hosseinifard et al. [43] reported that Pro priming can precondition the P5CS biosynthetic pathway for faster induction and prosynthesis. The positive effect of Pro priming has been explained by epigenetic mechanisms by Crisp et al. [20] and Liu et al. [41]. For instance, Ding et al. [58] described that *H3K4me3* epigenetic marks on the promoters of P5CS and SOD genes can persist after the initial stimulus has ceased, enabling more rapid re-induction of transcription. Additionally, lower water potential values were recorded in SPro plants. A decrease in water potential helps maintain cellular turgor and supports more efficient water uptake by roots [10].

Stomatal regulation in S and SPro was similar; however, significantly lower A and E values in SPro suggest different internal limitations. Ganie et al. [42] report that part of the photosynthetic limitation in primed plants may arise at the mesophyll level, for example, through restricted CO₂ diffusion or reduced RubisCO activity. In contrast, S plants had to initiate a similar activation de novo, which may have led to delayed responses and increased oxidative damage. As previously stated, S plants showed significantly higher MDA content and lower F_v/F_m values compared to SPro, indicating PSII damage and enhanced lipid peroxidation.

After rehydration (R), SPro plants showed faster and more complete recovery of A, g_s , and E than S. In the S treatment, these parameters remained slightly reduced. Increased F_v/F_m in SPro and lower residual MDA confirm more effective repair of photosystem II and detoxification of ROS, whereas S exhibited persistent mild photoinhibition. The degradation of accumulated Pro may have served as a source of carbon and nitrogen for the restoration of photosynthetic structures [14].

It is necessary to mention a methodological limitation arising from ontogeny: foliar application was performed at the BBCH 15 stage (fifth leaf fully developed), while the second drought-affected leaves formed up to the BBCH 31 stage. Although exogenous Pro was already degraded at the time of drought, epigenetic or physiological changes induced in the tissues may have persisted and influenced the overall plant response. The molecular transmission of such information between tissues, e.g., via RNA signals or hormonal pathways, is a well-documented phenomenon in plants [20].

5. Conclusions

A single foliar application of proline modulates plant responses to drought stress and their physiological performance at multiple levels. This effect did not manifest uniformly across all measured parameters but rather selectively modulated defence mechanisms and influenced the dynamics of stress recovery.

- (1) During the first stress period, proline was demonstrably involved in stomatal regulation, leading to enhanced gas exchange. Upon rewetting, more efficient water status and rapid resumption of photosynthetic assimilation were observed.
- (2) Repeated stress elicited a stronger response regardless of proline treatment. The effect of exogenous proline persisted in the form of reduced endogenous proline synthesis and improved protection of PSII.
- (3) A long-term priming effect was demonstrated, enhancing the preparedness of plants for subsequent drought. This stress memory supported more efficient osmoregulation, lower lipid peroxidation, improved protection of photosystem II integrity, and a more effective metabolic recovery after rehydration.

In our experiment, the priming effect persisted for more than 35 days after application, confirming the potential of exogenous proline as a practical tool to improve crop resilience

to climate-induced drought stress. However, many questions remain unanswered, highlighting the complexity of this issue and the need for further research. In addition to exploring metabolic and epigenetic aspects, it is also necessary to verify these mechanisms across different wheat cultivars and other crop species.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy15061370/s1>, Data source for tables.

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