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Foliar proline alleviates drought stress in peppermint *via* consistent growth and essential oil benefits, with stress-dependent regulation of ion balance and abscisic acid

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Abstract: This study examined whether exogenous proline (Pro, 10 mmol/L, foliar) uniformly alleviates drought stress in peppermint (*Mentha* × *piperita* L.) or whether its benefit intensifies with increasing stress severity. Plants were grown under four water field capacity (WFC) levels (95, 75, 50, 25%), with or without Pro, in a 4 × 2 factorial randomised design. Two-way ANOVA (Levene's test confirmed homogeneous variance) partitioned each variable into WFC and Pro main effects and their interaction, followed by Tukey's *HSD* ($P < 0.05$). Water deficit reduced growth and relative water content, increased osmolytes, disrupted ionic homeostasis (higher Na^+ and Na^+/K^+; lower K^+, Ca^{2+}, Mg^{2+}), activated antioxidant defences, and altered hormone levels (higher abscisic acid (ABA); lower indole-3-acetic acid (IAA), gibberellic acid (GA_3)). Pro significantly improved growth, water status, most osmolytes, antioxidants, IAA, GA_3, and essential oil (EO) yield, with no significant WFC × Pro interaction detected for these variables, providing no statistically detectable evidence that the magnitude of the effect differed among WFC levels. Pro's effect on Na^+, the Na^+/K^+ ratio, and ABA instead showed a significant WFC × Pro interaction ($P = 0.040$, 0.046, 0.001), intensifying under severe deficit. EO yield showed a biphasic response, rising at 75% and 50% WFC and falling at 25%, with Pro further increasing yield and menthol content. Pro thus acts mainly as a consistent protectant with no detected WFC × Pro interaction, alongside a distinct, stress-dependent role in ion and ABA regulation under severe drought.

Keywords: compatible solutes; phytochemical modulation; monoterpene profile; irrigation regime; metabolic reprogramming; nutrient partitioning

Drought stress is a major abiotic constraint that limits plant growth, physiological stability, and productivity under changing climatic conditions (Khan et al. 2025). Water deficit reduces cellular water potential, impairs photosynthetic efficiency, and promotes the accumulation of reactive oxygen species (ROS), leading to oxidative damage and metabolic disruption (Huang and Jin 2025, Shahhoseini et al. 2025). The severity of drought, commonly characterised by different water field capacity (WFC) levels, determines the magnitude of physiological and biochemical responses, including osmotic adjustment, antioxidant defence, hormonal regulation, and biomass allocation (Haghpanah et al. 2024, Formica et al. 2026).

Essential oils (EOs) are economically important secondary metabolites in aromatic and medicinal plants (Ozhuner 2025). Their biosynthesis depends on photosynthetic carbon supply, energy availability, and redox balance; therefore, drought can markedly affect both EO yield and composition (Jangpangi et al. 2025). Moderate

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water deficit may stimulate secondary metabolism and EO accumulation, whereas severe stress usually suppresses oil productivity due to reduced photosynthesis and oxidative constraints (Formica et al. 2026).

Peppermint (*Mentha* × *piperita* L.) is a valuable aromatic and medicinal plant widely cultivated for its EO, which is primarily composed of monoterpenes, including menthol, menthone, and related compounds (Ozhuner 2025). Recent studies on *Mentha* species confirm that drought intensity can alter physiological performance, biochemical activity, and EO-related traits (Araniti et al. 2024, Shahhoseini et al. 2025). Therefore, understanding how drought severity affects peppermint growth, EO yield, and EO composition is important for improving productivity and oil quality under water-limited conditions.

Proline (Pro) is a key compatible solute involved in plant adaptation to water deficit (Singh et al. 2025). It contributes to osmotic adjustment, membrane stabilisation, antioxidant regulation, and stress signalling (Khan et al. 2025, Pecka et al. 2025). By maintaining cellular hydration and metabolic stability, exogenous Pro may reduce drought-induced growth inhibition and indirectly support EO biosynthesis through sustained carbon assimilation and terpenoid metabolism (Jangpangi et al. 2025, Singh et al. 2025). Here, the term exogenous foliar application denotes Pro supplied externally *via* leaf spraying, as distinct from Pro synthesised endogenously through the ornithine and glutamate pathways under stress; foliar delivery allows cuticular and stomatal uptake, enabling exogenous Pro to supplement endogenous pools rapidly and influence osmotic and redox regulation before extensive *de novo* Pro synthesis occurs.

Although drought stress and Pro individually influence plant physiology and metabolism, whether their combined effect on peppermint differs significantly across water-deficit severity, or instead intensifies under more severe stress, has not been formally tested. This study assessed how water field capacity (WFC) levels of 95, 75, 50, and 25%, with or without Pro application, affected growth, water relations, osmolyte accumulation, ion balance, antioxidant activity, phytohormone levels, and EO yield and composition. We hypothesised that Pro would improve growth, water status, osmotic adjustment, antioxidant capacity, and hormonal balance under drought, based on Pro's established roles in osmotic adjustment, membrane stabilisation, and antioxidant regulation (Khan et al. 2025, Pecka et al. 2025, Renzetti et al. 2025, Singh et al. 2025). Because comparing Pro-treated and untreated plants separately at each WFC level cannot distinguish an effect that does not differ significantly across levels from one that depends on stress severity, this study used a full 4 × 2 factorial design in which the WFC × Pro interaction, not only the two main effects of WFC and Pro, was tested statistically for every measured variable.

MATERIAL AND METHODS

Plant material and growth conditions. A pot experiment was conducted in Egypt (30°06'N, 31°25'E) from January to March 2025. Peppermint (*Mentha* × *piperita* L.) seeds were supplied by the Ministry of Agriculture, Egypt. The material was supplied under this species designation, but the supplier could not certify a named cultivar, and cultivar-verified vegetative propagules were not available in sufficient uniform quantities for the experiment. Seeds were surface-sterilised with 70% (*v*/*v*) ethanol for 3 min and thoroughly washed with distilled water. Five seeds were planted in cylindrical pots measuring 50 cm in diameter and 50 cm in depth, each containing 10 kg of mixed soil (clay loam) and sand (soil/sand 2:1. After seedling emergence, plants were thinned to three uniform seedlings per pot. These seedlings were selected before treatment. Because the plants were seed-derived, genetic and chemotypic heterogeneity among experimental units cannot be excluded and is addressed as a limitation in the Discussion. Plants were grown under natural environmental conditions, with day/night temperatures ranging from 19.2 °C to 30.1 °C and relative humidity of 63–68%. All pots were irrigated daily to 100% water field capacity for 30 days after planting. Water field capacity was determined gravimetrically by saturating the soil, allowing excess water to drain for 24 h, and then weighing the soil. Phosphorus fertiliser was applied as a basal dose during soil preparation before planting at the recommended rate of 1.5 g P/pot in the form of KH_2PO_4. Nitrogen fertiliser was applied in two equal splits, the first at 15 days after planting and the second at 30 days after planting, coinciding with initiation of the water-regime treatments, at the recommended rate of 1.5 g N/pot as $CO(NH_2)_2$. The chemical and physical properties of the irrigation water and soil samples were analysed following Jackson (1973) and Cottenie et al. (1982), as presented in Tables 1 and 2.

Experimental design and water regime treatments. After 30 days of growth under well-watered

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Table 1. Chemical characteristics of the irrigation water

Parameter	pH	EC (dS/m)	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	HCO_3^-	Cl^-	SO_4^{2-}
			($mmol_+/L$)						
Value	7.21	0.62	2.85	1.42	1.65	0.48	2.15	2.36	1.92

Ion concentrations are expressed in $mmol_+/L$. EC – electrical conductivity

conditions, pots were arranged in a completely randomised design and assigned to eight treatment groups, each with five replicates (five pots per treatment; each pot, containing three uniform seedlings, was treated as one experimental unit). This corresponds to a 4 × 2 factorial arrangement (four WFC levels × two Pro levels) within a completely randomised design (CRD); the WFC × Pro interaction, together with the two main effects, was evaluated statistically for all measured variables. Four water regimes were imposed and are hereafter referred to as 95% WFC, 75% WFC, 50% WFC, and 25% WFC. During the experiment, these target levels corresponded to approximately 95–100, 75–80, 50–55, and 25–30% WFC, respectively, as determined by daily pot weighing and water replacement. The eight treatment combinations consisted of 95% WFC, 75% WFC, 50% WFC, 25% WFC, 95% WFC + Pro, 75% WFC + Pro, 50% WFC + Pro, and 25% WFC + Pro. Water-regime treatments began 30 days after planting and were maintained until harvest on 15 March 2025, resulting in a total stress duration of 45 days. Soil moisture was maintained by weighing the pots and adding the required amount of water to restore each treatment to its target WFC level. Because plant biomass constituted a small fraction of total pot weight relative to the soil and water content, increases in plant biomass over the 45-day treatment period were not separately corrected for gravimetric determination of pot weight. Pro-treated plants received the first foliar Pro application 5 days before the onset of water-deficit treatments, followed by weekly applications throughout the treatment period until harvest.

Proline application. Pro was applied as a foliar spray at a concentration of 10 mmol/L using L-proline (manufacturer: Sigma-Aldrich; purity ≥ 99%; St. Louis, USA). Pots assigned to Pro treatments were designated before the first application. The first foliar spray was applied 5 days before the start of water-regime treatments, at 25 days after planting, and applications were repeated weekly until the end of the experiment. For non-Pro treatments, plants were sprayed with an equal volume of distilled water at the same application times to serve as a foliar-spray control and to exclude potential effects of leaf wetting. No surfactant or wetting agent was added to either the Pro or the control spray solution. Spraying was carried out in the early morning using a hand sprayer until runoff to ensure uniform leaf coverage; because spraying continued until runoff, the volume applied varied slightly among pots according to canopy size rather than following a fixed volume/pot. This priming interval and weekly reapplication schedule were adopted to allow initial physiological priming before stress onset and to sustain elevated exogenous Pro levels throughout the stress period, following a repeated-application approach used to induce drought-stress memory in wheat (Pecka et al. 2025); this design, however, cannot statistically separate the contributions of initial priming, cumulative exogenous supply, and any stress-induced endogenous Pro synthesis, a limitation addressed in the Discussion.

Growth measurements. The shoot and root growth parameters of all treated and untreated plants were measured, namely shoot length, shoot fresh weight,

Table 2. Physical and chemical properties of the experimental soil

Category	Property	Value	Unit
Physical	sand	26	%
	silt	40	%
	clay	34	%
	texture class	clay loam	
Chemical	pH	6.92	
	EC	2.95	dS/m
	$CaCO_3$	1.85	%
	OC	0.19	%
Soluble ions	HCO_3^-	1.62	$mmol_+/L$
	Ca^{2+}	2.10	$mmol_+/L$
	Mg^{2+}	1.75	$mmol_+/L$
	K^{+}	1.97	$mmol_+/L$
	Na^{+}	1.58	$mmol_+/L$

EC – electrical conductivity; OC – organic carbon

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shoot dry weight, root length, root fresh weight, and root dry weight. Plants were harvested at the pre-flowering stage, at the end of the 45-day treatment period; all three plants per pot were harvested, and values were averaged per pot to give one replicate value per treatment. The dry weights (DW) of the shoots and roots were determined by placing them in bags and drying them in an oven set to 80 °C until the weight stabilised. Five replicates were used to determine the mean for each treatment.

Relative water content determination. RWC of both stressed and unstressed peppermint leaves was measured using the Schonfeld et al. (1988) method. Leaves were cut at the base with a scalpel, and fresh weights (FW) were measured immediately afterwards. The leaves were then soaked in distilled H_2O at room temperature for 24 h. The leaves' turgid weights (TW) were determined after blotting them dry with tissue paper. The leaves were dried in an oven at 80 °C for 48 h, and dry weights were measured. The RWC of all treatments was calculated as follows:

$$RWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$$

where: FW – fresh weight; TW – turgid weight; DW – dry weight.

Total chlorophyll measurement. Total chlorophyll content was determined spectrophotometrically using a methanol extraction, as described by Dere et al. (1998), with slight modifications. Fresh leaf tissue was homogenised in methanol, and the extract was stored in the dark to minimise pigment degradation. The homogenate was centrifuged, and the absorbance of the clear supernatant was measured at 666 nm and 653 nm using a spectrophotometer.

Organic osmolyte assessment. The ninhydrin-based colourimetric method was used to measure Pro levels in peppermint leaves across all treatments, following Lee et al. (2018). Fresh leaf tissue (0.5 g) was ground in 1% sulfosalicylic acid. The extract was centrifuged at 15 000 *g* for 5 min at 4 °C, and the supernatant was mixed with acidic ninhydrin reagent at a 1:2 ratio. The mixture was incubated at 95 °C for 30 min, and absorbance was measured at 510 nm with a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan). Pro concentration was quantified using a standard curve spanning 0–100 µg/mL.

Total soluble protein (TSP) was determined following the Bradford (1976) method. Fresh leaf tissue (0.5 g) was homogenised in phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, and 1.8 mmol/L KH_2PO_4; pH 7.2–7.4). The homogenate was centrifuged, and the supernatant was mixed with Bradford reagent and incubated for 30 min at room temperature. Absorbance was measured at 595 nm using a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan). Protein concentration was calculated using a bovine serum albumin standard curve ranging from 0 to 100 µg/mL.

Total soluble sugars (TSS) were extracted and measured as described by Yoshida et al. (1976). Dried leaf tissue was immersed in 10 mL of 80% ethanol at 25 °C and manually agitated overnight. For colour development, 0.1 mL of the alcoholic extract was mixed with 3.0 mL of freshly prepared anthrone reagent and heated in a boiling water bath for 10 min. After cooling, absorbance was measured at 625 nm using a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan). TSS concentration was estimated using a glucose standard curve ranging from 0 to 100 µg/mL.

Inorganic ion measurements. Na^+, K^+, Ca^{2+}, and Mg^{2+} contents were determined following the Wolf (1982) method. Dry leaf tissue (0.5 g) was digested overnight in 5 mL of H_2SO_4, then heated at 350 °C for 30 min in a digestion block. After cooling, 1 mL H_2O_2 was added, and the mixture was heated again for 20 min. This step was repeated until a clear solution was obtained. The digest was filtered and diluted to a final volume of 50 mL with distilled water. Na^+, K^+, Ca^{2+}, and Mg^{2+} contents were determined using a flame photometer (Jenway PFP-7, Bibby Scientific Ltd., London, UK), and concentrations were calculated from standard calibration curves ranging from 10 to 100 ppm.

Phytohormones estimation. Phytohormone levels were estimated in peppermint plants following Müller and Munné-Bosch (2011). Fresh leaves (0.2 g) were extracted with a solution of 1% acetic acid, 79% isopropanol, and 20% methanol. Samples were kept on ice for 30 min, sonicated for 10 min, and centrifuged at 13 000 rpm for 10 min at 4 °C. The supernatant was re-extracted and injected into an LC-MS/MS system (Agilent 1200 Infinity Series, Agilent Technologies, Santa Clara, USA) coupled to an Agilent 6430 triple-quadrupole mass spectrometer. An Agilent Eclipse Plus RRHD column (2.1 × 50 mm, 1.8 µm) was operated at a flow rate of 0.3 mL/min. The mass spectrometer switched between negative and positive ionisation modes based on analyte retention time, and multiple reaction monitoring was used to

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quantify abscisic acid (ABA), indole-3-acetic acid (IAA), and gibberellic acid (GA_3). Chromatograms were processed using MassHunter software, and calibration curves were applied to the integrated peak areas. Final concentrations were expressed as ng/g FW for IAA and GA3 and as µg/g FW for ABA.

Reduced glutathione and ascorbate estimation. Reduced glutathione (GSH) was determined according to Ellman (1959). Fresh peppermint leaves (500 mg) were homogenised in 15% metaphosphoric acid and centrifuged at 5 000 *g* for 30 min at 4 °C. The supernatant was mixed with phosphate buffer (100 mmol/L, pH 8.0) and 5,5'-dithiobis (2-nitrobenzoic acid). After incubation for 30 min, absorbance was measured at 412 nm using a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan). GSH concentration was calculated using a standard curve prepared from reduced glutathione.

Ascorbate (AsA) content was determined as described by Mukherjee and Choudhuri (1983). Fresh leaves were homogenised in 6% trichloroacetic acid and centrifuged at 5 000 *g* for 10 min. The supernatant was heated in a water bath for 15 min after adding thiourea and dinitrophenylhydrazine. After cooling, 80% H_2SO_4 was added, and absorbance was measured at 530 nm using a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan). AsA concentration was determined using an ascorbate standard curve.

Extraction and assay of antioxidant enzymes. To extract antioxidant enzymes, 1 g of fresh leaf tissue was homogenised in 50 mL of chilled phosphate buffer (100 mmol/L, pH 7.0) containing EDTA and 1% polyvinylpyrrolidone using a pre-chilled mortar and pestle. The homogenate was centrifuged at 15 000 *g* for 20 min at 4 °C, and the supernatant was used as an enzyme extract. Protein concentration in the enzyme extract was determined using the Lowry et al. (1951) method, with bovine serum albumin as the standard.

The ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated following Nakano and Asada's (1981) method. The assay involved a 1 mL reaction mixture containing 0.5 mmol of hydrogen peroxide, 0.5 mmol of ascorbic acid, 0.1 mL of enzyme extract, and potassium phosphate buffer (100 mmol/L, pH 7.0). Absorbance was monitored at 290 nm for 3 min using a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan).

The superoxide dismutase (SOD, EC 1.15.1.1) activity was evaluated using the method of Beyer and Fridovich (1987). The assay involved a 1.5 mL mixture containing 100 µL of enzyme extract, 100 µL EDTA, 13 mmol L-methionine, sodium phosphate buffer (50 mmol/L, pH 7.5), 75 µmol of nitroblue tetrazolium (NBT), and 60 µmol of riboflavin. Photochemical reduction of NBT was monitored at 560 nm. After a 15 min incubation, the light source was turned off.

Catalase (CAT, EC 1.11.1.6) activity was evaluated using the Aebi (1984) method, and absorbance was measured at 240 nm for 2 min using a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan). The calculation was performed with an extinction coefficient of 39.4 mmol/cm.

Glutathione reductase (GR; EC 1.6.4.2) activity was measured using the Foyer and Halliwell (1976) method. The assay mixture contained 0.1 mL enzyme extract, 0.5 mmol/L oxidised glutathione, 0.1 mmol/L NADP, and 100 mmol/L sodium phosphate buffer (pH 7.8). Absorbance was recorded at 340 nm for 2 min with a UV-visible spectrophotometer (UV-1900i Plus, Shimadzu Corporation, Kyoto, Japan) at an extinction coefficient of 6.2 mmol/cm.

Extraction and assay of essential oils. EO was obtained from dried peppermint leaves by hydrodistillation for 3 h using a Clevenger apparatus, following the method of Adams (2007). The EO yield was expressed as a percentage (*v*/*w*) based on DW. Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Shimadzu QP2010 ULTRA FID GC-MS system with a Restek Rxi-5 MS capillary column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness). Separation was achieved with helium as the mobile phase at a constant flow rate of 0.8 mL/min. The oven temperature started at 60 °C and increased to 280 °C at a rate of 4 °C/min. Key operating conditions included an injection port temperature of 290 °C and a GC-MS interface temperature of 300 °C. Electron impact ionisation at 70 eV was used for mass spectral data collection, with a mass-to-charge ratio range of 50–550 m/z. The ion source and detector were maintained at 250 °C and 150 °C, respectively. Components were identified by comparing retention indices and spectral patterns with reference data from the NIST and Wiley mass spectral libraries.

Statistical analysis. Data are presented as means ± standard errors (SEs), based on five independent pots per treatment for growth measurements and three independent pots per treatment for all other physiological, biochemical, ionic, hormonal, and essential-oil measurements. The experiment was

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Table 3. Complete two-way ANOVA summary for water field capacity and foliar proline effects

Trait	Unit	*n* per treatment	Error *df*	Levene *F*	Levene *P*	WFC *F*	WFC *P*	Pro *F*	Pro *P*	WFC × Pro *F*	WFC × Pro *P*	Signif. interaction	Interpretation
Proline	µmol/g FW	3	16	0.130	9.9450E-01	23.01	4.7896E-06	8.66	9.5624E-03	0.14	9.3463E-01	No	No statistically detectable WFC × Pro interaction
Total soluble protein	mg/g FW	3	16	0.205	9.7940E-01	63.47	4.2014E-09	9.20	7.9006E-03	0.81	5.0858E-01	No	
Total soluble sugars	mg/g DW	3	16	0.588	7.5630E-01	10.86	3.8898E-04	3.37	8.4908E-02	0.05	9.8495E-01	No	
Glutathione reductase	U/mg protein	3	16	0.341	9.2320E-01	14.51	7.9357E-05	3.98	6.3288E-02	0.20	8.9204E-01	No	
Superoxide dismutase	U/mg protein	3	16	0.559	7.7840E-01	31.63	5.8545E-07	16.86	8.2507E-04	0.62	6.1233E-01	No	
Catalase	U/mg protein	3	16	0.308	9.4000E-01	24.84	2.9169E-06	5.72	2.9455E-02	0.09	9.6489E-01	No	
Ascorbate peroxidase	U/mg protein	3	16	0.247	9.6610E-01	35.20	2.8171E-07	14.80	1.4243E-03	0.09	9.6550E-01	No	
Shoot length	cm	5	32	0.152	9.9240E-01	64.23	1.2316E-13	30.11	4.8061E-06	0.08	9.7025E-01	No	
Shoot fresh weight	g	5	32	0.560	7.8200E-01	39.71	6.7551E-11	34.08	1.7366E-06	0.33	8.0472E-01	No	
Shoot dry weight	g	5	32	0.346	9.2630E-01	37.93	1.1941E-10	26.30	1.3668E-05	0.77	5.1674E-01	No	
Na^+	mg/g DW	3	16	1.852	1.4550E-01	25.78	2.2891E-06	16.67	8.6739E-04	3.51	3.9819E-02	**Yes**	Pro response depended on WFC level
K^+	mg/g DW	3	16	0.238	9.6910E-01	14.77	7.1535E-05	11.22	4.0719E-03	0.25	8.6123E-01	No	No statistically detectable WFC × Pro interaction
Na^+/K^+ ratio		3	16	1.235	3.4080E-01	23.93	3.7212E-06	18.88	5.0090E-04	3.33	4.6103E-02	**Yes**	Pro response depended on WFC level
Ca^{2+}	mg/g DW	3	16	0.021	1.0000E+00	20.73	9.2864E-06	15.33	1.2325E-03	0.11	9.5153E-01	No	No statistically detectable WFC × Pro interaction
Mg^{2+}	mg/g DW	3	16	0.430	8.6950E-01	18.83	1.6832E-05	14.04	1.7568E-03	0.95	4.3784E-01	No	
Abscisic acid	µg/g FW	3	16	0.435	8.6590E-01	87.21	3.9552E-10	46.00	4.4015E-06	8.82	1.1081E-03	**Yes**	Pro response depended on WFC level
Indole-3-acetic acid	ng/g FW	3	16	0.582	7.6070E-01	58.17	7.9528E-09	15.55	1.1635E-03	0.78	5.2438E-01	No	
Gibberellic acid GA3	ng/g FW	3	16	0.158	9.9030E-01	21.28	7.8631E-06	6.50	2.1420E-02	0.18	9.0638E-01	No	
Essential oil yield	mL/100 g DW	3	16	0.555	7.8060E-01	66.24	3.0682E-09	50.50	2.4888E-06	2.09	1.4162E-01	No	
Relative water content	%	3	16	0.173	9.8740E-01	37.77	1.7315E-07	24.14	1.5584E-04	1.33	2.9893E-01	No	
Glutathione	nmol/g FW	3	16	0.176	9.8670E-01	43.36	6.5737E-08	8.99	8.5111E-03	0.14	9.3631E-01	No	No statistically detectable WFC × Pro interaction
Ascorbic acid	nmol/g FW	3	16	0.119	9.9590E-01	30.27	7.8741E-07	12.61	2.6634E-03	0.31	8.1782E-01	No	
Root length	cm	5	32	0.225	9.7660E-01	50.18	3.3546E-12	69.04	1.7104E-09	0.86	4.7247E-01	No	
Root fresh weight	g	5	32	0.418	8.8370E-01	58.00	4.9026E-13	47.96	7.6815E-08	0.13	9.4129E-01	No	
Root dry weight	g	5	32	0.781	6.0770E-01	67.41	6.3534E-14	84.98	1.5966E-10	1.21	3.2362E-01	No	
Total chlorophyll	mg/g FW	3	16	0.828	5.7910E-01	69.67	2.1110E-09	45.77	4.5350E-06	1.47	2.6050E-01	No	

WFC – water field capacity; Pro – foliar proline; *df* – degrees of freedom. Factor degrees of freedom: WFC = 3, Pro = 1, WFC × Pro = 3. Error *df* = 32 for growth traits ($n = 5$) and 16 for all other traits ($n = 3$). A nonsignificant interaction is interpreted as no statistically detectable evidence that the Pro response differed among WFC levels, not as proof of equality or additivity All Levene tests were nonsignificant ($P > 0.05$), supporting the homogeneity-of-variance assumption. FW – fresh weight; DW – dry weight

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analysed as a 4 × 2 factorial (WFC × Pro) in a completely randomised design. Homogeneity of variance across the eight treatment combinations was tested for every dependent variable using Levene's test; all tests were nonsignificant ($P > 0.05$). Treatment effects were therefore examined using two-way analysis of variance with Type II sums of squares, partitioning each dependent variable into the main effects of WFC and Pro and their interaction (WFC × Pro). Pairwise comparisons among the eight treatment combinations used Tukey's honestly significant difference (*HSD*) test at $P < 0.05$; combinations sharing a common letter in figures and tables do not differ significantly. Analyses were performed using IBM SPSS Statistics 29.0 (Armonk, USA) and SigmaPlot 10.0 (San Jose, USA). A complete ANOVA summary for all 26 measured variables, including replicate number, degrees of freedom, *F*-values, *P* values,

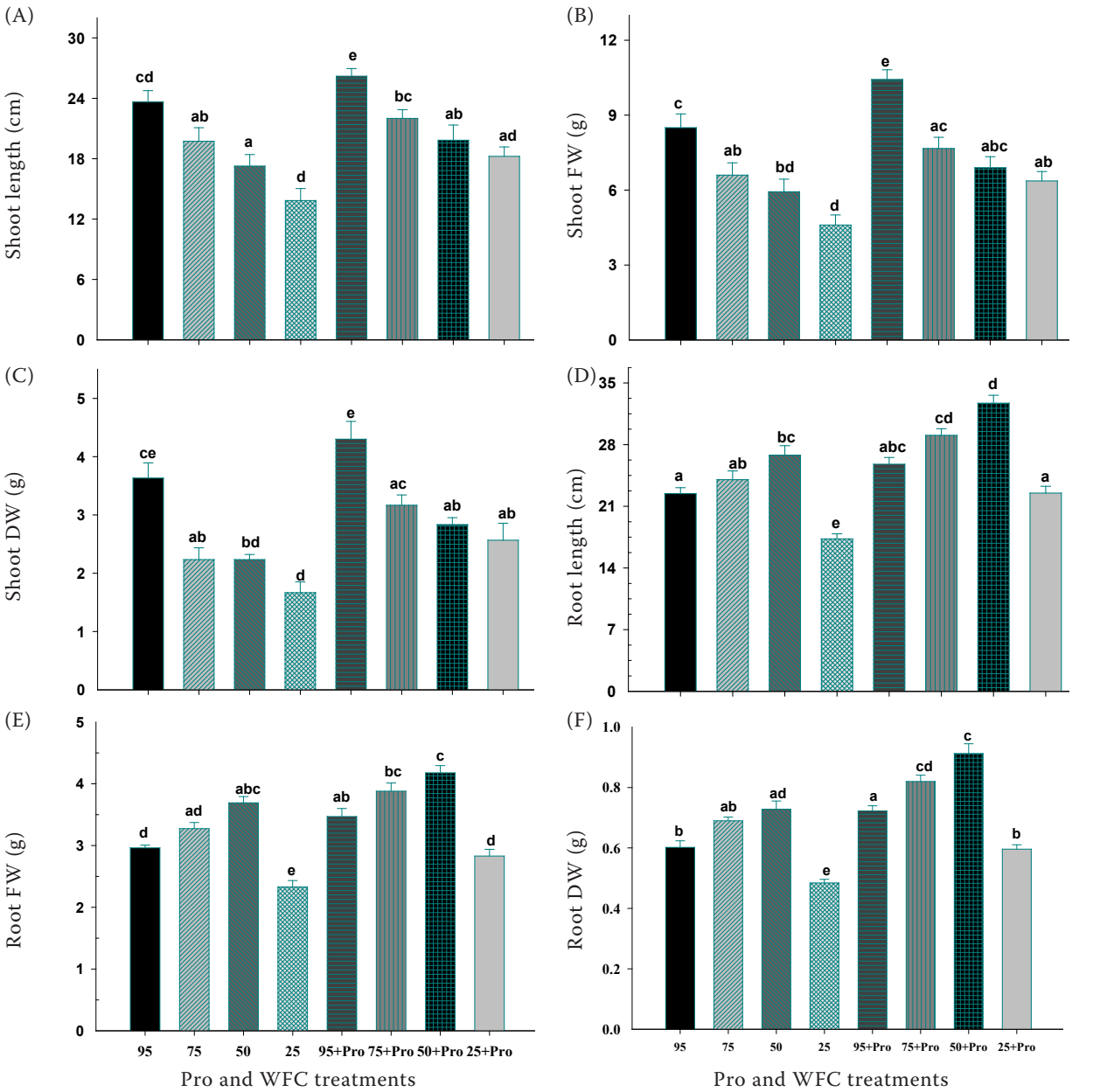

Figure 1. Impact of proline (Pro) application on growth under water deficit. (A) Shoot length; (B) shoot fresh weight (FW); (C) shoot dry weight (DW); (D) root length; (E) root fresh weight, and (F) root dry weight. Each column represents the mean of five replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at $P < 0.05$. WFC – water field capacity

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interaction tests, and Levene's tests, is provided in Table 3. A nonsignificant WFC × Pro interaction is interpreted only as an absence of statistically detectable interaction under the present design, not as proof that the Pro effect was equal, uniform, or additive at all WFC levels, particularly given the modest replication (n = 3) available for most endpoints.

RESULTS

Impact of the Pro application on growth under water deficit. Shoot growth was particularly sensitive to decreasing WFC (Figures 1A–C). Shoot length, fresh weight, and dry weight declined progressively (P < 0.05) as water availability decreased, with reductions of 41.5, 46, and 54%, respectively, at 25% WFC compared to the control. In contrast, root traits showed a more adaptive response under moderate stress (Figures 1D–F). Root length, fresh weight, and dry weight increased by 19, 25, and 25%, respectively, at 50% WFC, suggesting enhanced root development under moderate water limitation. However, severe stress at 25% WFC reduced these parameters by 23, 21, and 20%, respectively. Pro application significantly improved all six shoot and root growth variables (P < 0.05); no significant WFC × Pro interaction was detected for any of the six variables, providing no statistically detectable evidence that the magnitude of this improvement differed among WFC levels (Figures 1A–F).

Pro treatment improves RWC under water deficit. Relative water content (RWC) declined significantly (P < 0.05) with decreasing WFC, showing reductions of 36.5% and 48.4% at 50% and 25% WFC, respectively, relative to the control plants (Figure 2A). Pro-treated plants maintained higher RWC and exhibited smaller reductions of 17% and 28% under 50% WFC + Pro and 25% WFC + Pro treatments, respectively. Although this pattern suggests a proportionally larger Pro benefit under more severe stress, the WFC × Pro interaction for RWC was not statistically significant (P = 0.30); no significant WFC × Pro interaction was detected for RWC, providing no statistically detectable evidence that the magnitude of this benefit differed among WFC levels, rather than reflecting a confirmed stress-dependent interaction.

Impact of Pro external addition on water-stressed peppermint. Total chlorophyll content decreased significantly (P < 0.05) as WFC decreased, with reductions of 20.6, 44.9, and 55.9% at 75, 50, and 25% WFC, respectively, compared with the control (Figure 2B). Pro application significantly increased chlorophyll content at every WFC level (P < 0.05), by 15.0, 14.5, 48.2, and 47.3% at 95, 75, 50, and 25% WFC, respectively, relative to untreated plants at the same WFC level. No significant WFC × Pro interaction was detected for chlorophyll content (P = 0.26), providing no statistically detectable evidence that the magnitude of this Pro-associated increase differed among WFC levels.

Effect of Pro and different water-regime treatments on organic osmolyte accumulation. Organic osmolytes exhibited a clear, stress-dependent accumulation pattern (Figure 3). Pro, TSP, and TSS increased progressively (P < 0.05) with drought severity, reaching 68, 59, and 66%, respectively, at 25% WFC

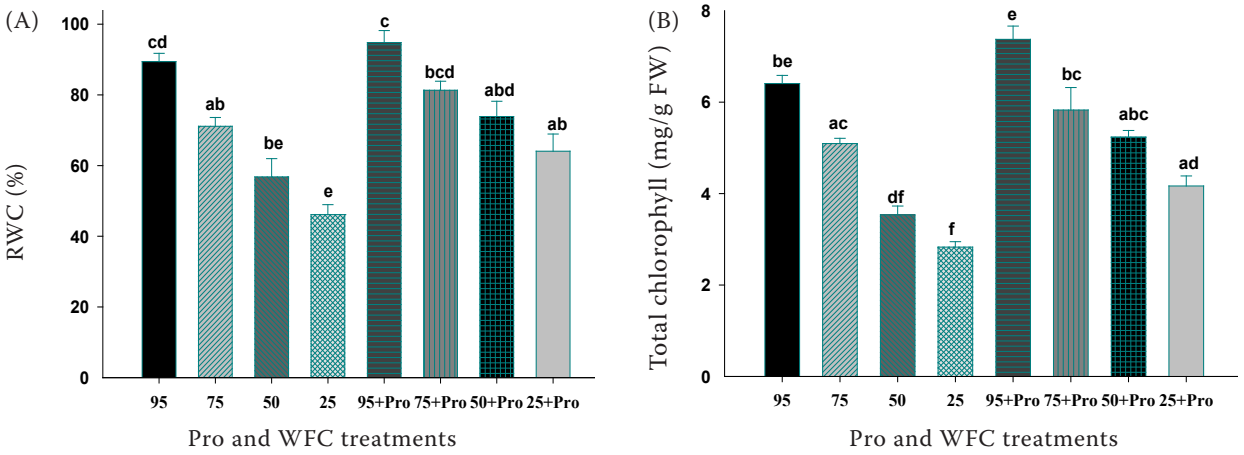

Figure 2. Proline (Pro) treatment affects (A) relative water content (RWC) and (B) total chlorophyll under water deficit. Each column represents the mean of three replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at P < 0.05. FW – fresh weight; WFC – water field capacity

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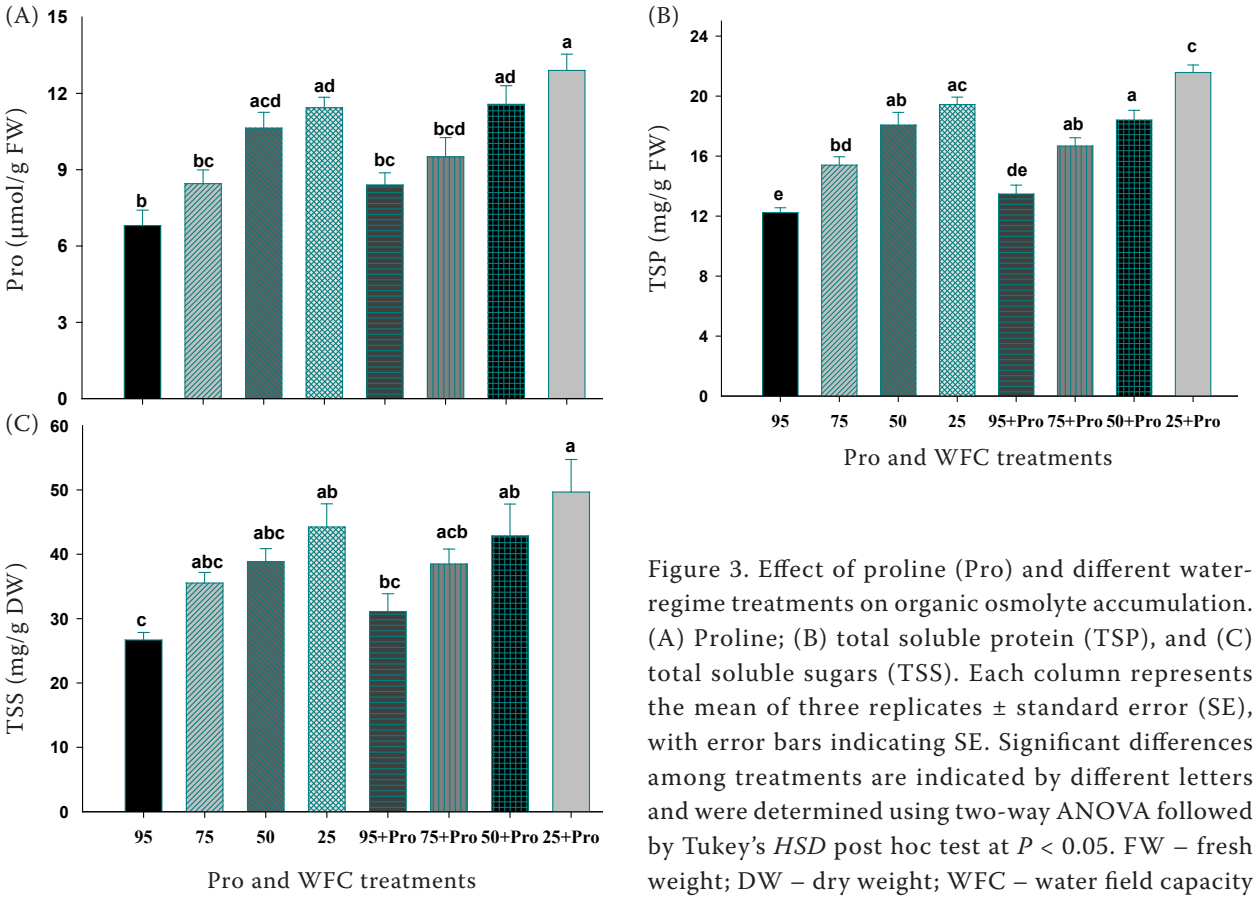

Figure 3. Effect of proline (Pro) and different water-regime treatments on organic osmolyte accumulation. (A) Proline; (B) total soluble protein (TSP), and (C) total soluble sugars (TSS). Each column represents the mean of three replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at $P < 0.05$. FW – fresh weight; DW – dry weight; WFC – water field capacity

relative to control values. Pro application significantly increased Pro and TSP content further ($P < 0.05$), by 90% and 76%, respectively, under 25% WFC + Pro compared with the control; TSS also increased numerically by 86% under the same comparison, but the main effect of Pro on TSS did not reach statistical significance ($P = 0.085$), so this increase should be regarded as a trend rather than a confirmed effect. No significant WFC × Pro interaction was detected for any of the three osmolytes, indicating that where Pro's effect is significant, it did not differ significantly across WFC levels.

Effect of Pro application on ionic homeostasis under water deficit. Water stress significantly altered ionic homeostasis in peppermint leaves (Figures 4A, C). Na^+ content and the Na^+/K^+ ratio increased markedly under water stress, with increases of 70.4% and 139%, respectively, at 50% WFC, and 131.5% and 276%, respectively, at 25% WFC compared with the control. Pro application limited these stress-induced increases, with smaller increases in Na^+ content and Na^+/K^+ ratio recorded under 50% WFC + Pro (31.5% and 50%, respectively) and 25% WFC + Pro (57.4% and 112%, respectively). For both Na^+ content and the Na^+/K^+ ratio, the WFC × Pro interaction was statistically significant ($P = 0.040$ and $P = 0.046$, respectively), confirming that Pro's mitigating effect on ionic imbalance is not uniform across WFC levels but becomes proportionally stronger as water deficit intensifies. This is the only pair of variables, aside from ABA (see phytohormone results below), for which a significant interaction was detected.

Water deficit also reduced K^+, Ca^{2+}, and Mg^{2+} levels. The strongest reductions occurred at 25% WFC, where K^+, Ca^{2+}, and Mg^{2+} decreased by 38.5, 53, and 37.6%, respectively, compared with the control (Figures 4B, D, E). The pro application partially restored these nutrient levels compared with untreated stressed plants, although they remained below control levels.

Non-enzymatic antioxidant response to Pro and various water regime treatments. GSH and AsA levels were significantly increased in peppermint leaves under water-deficit conditions (Figure 5). Compared with unstressed control plants, the greatest increases were recorded at 25% WFC, reaching 42% and 38% for GSH and AsA, respectively. Pro application further enhanced this response, increasing GSH and AsA levels by 50.5% and 47.3%, respectively, under 25% WFC + Pro.

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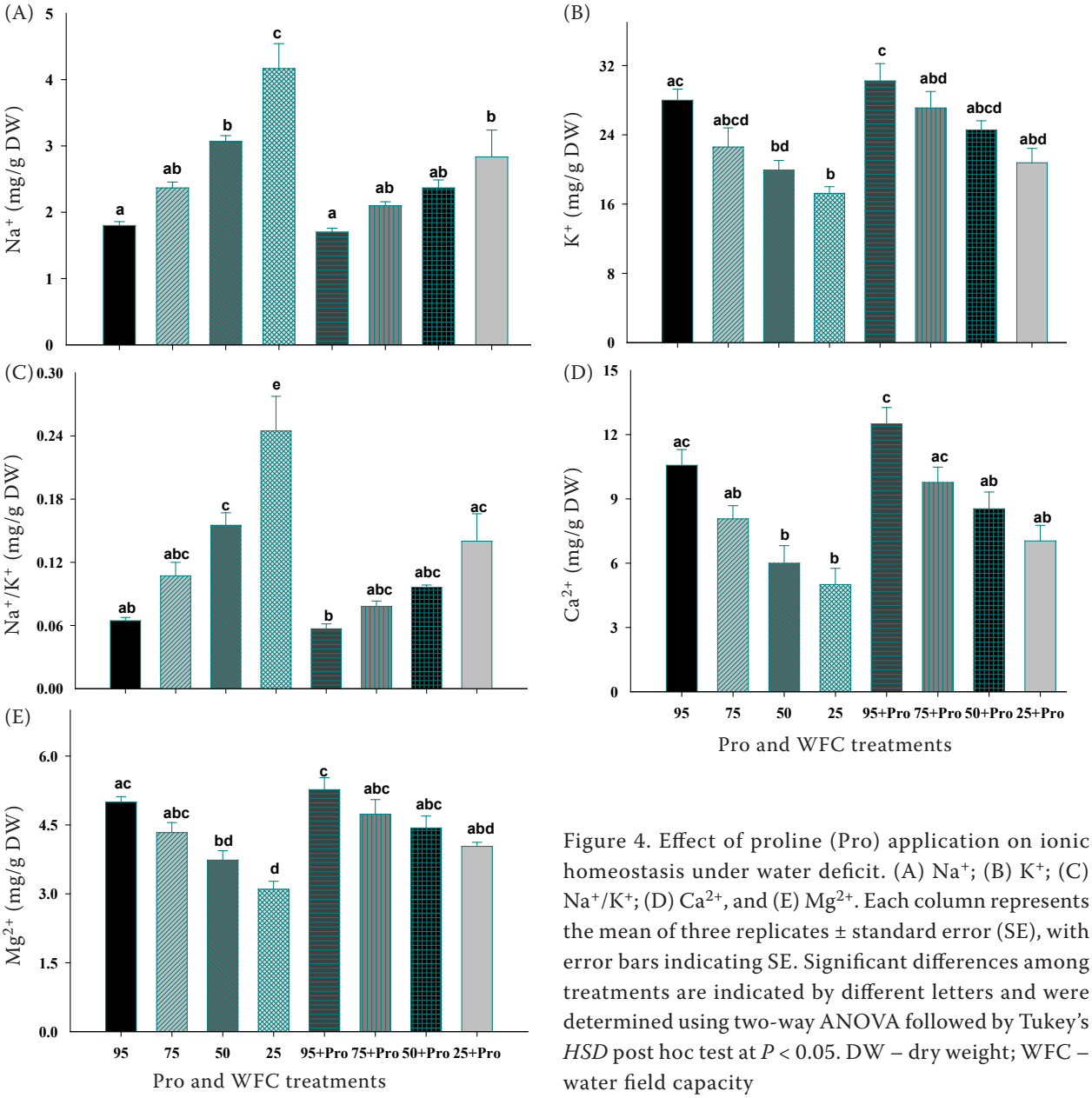

Figure 4. Effect of proline (Pro) application on ionic homeostasis under water deficit. (A) Na^+; (B) K^+; (C) Na^+/K^+; (D) Ca^{2+}, and (E) Mg^{2+}. Each column represents the mean of three replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at $P < 0.05$. DW – dry weight; WFC – water field capacity

Impact of the Pro and water deficit treatments on antioxidant enzyme activity. Water stress significantly elevated ($P < 0.05$) the activities of GR, SOD, CAT, and APX in peppermint leaves (Figure 6). Regarding untreated control plants, the activities of these enzymes increased by 53, 45, 47, and 44%, respectively, under 25% WFC. Pro application significantly increased SOD, CAT, and APX activities by 56, 59.8, and 57.6%, respectively, in the 25% WFC + Pro treatment ($P < 0.05$). GR activity was numerically higher under Pro at every WFC level (up to 60.7% at 25% WFC + Pro), but the main effect of Pro on GR did not reach statistical significance ($P = 0.063$); this should be reported as a tendency rather than a confirmed effect. No significant WFC × Pro interaction was detected for any of the four enzymes.

Pro application influences phytohormone levels in plants experiencing water deficit. Compared with the control, IAA and GA3 levels declined gradually ($P < 0.05$) as WFC decreased, with reductions of 62.2% and 53.9%, respectively, at 25% WFC (Figures 7A, B). Pro treatment significantly increased both IAA and GA3 ($P < 0.05$) at most WFC levels; no significant WFC × Pro interaction was detected for either hormone, providing no statistically detectable evidence that the magnitude of this increase differed among

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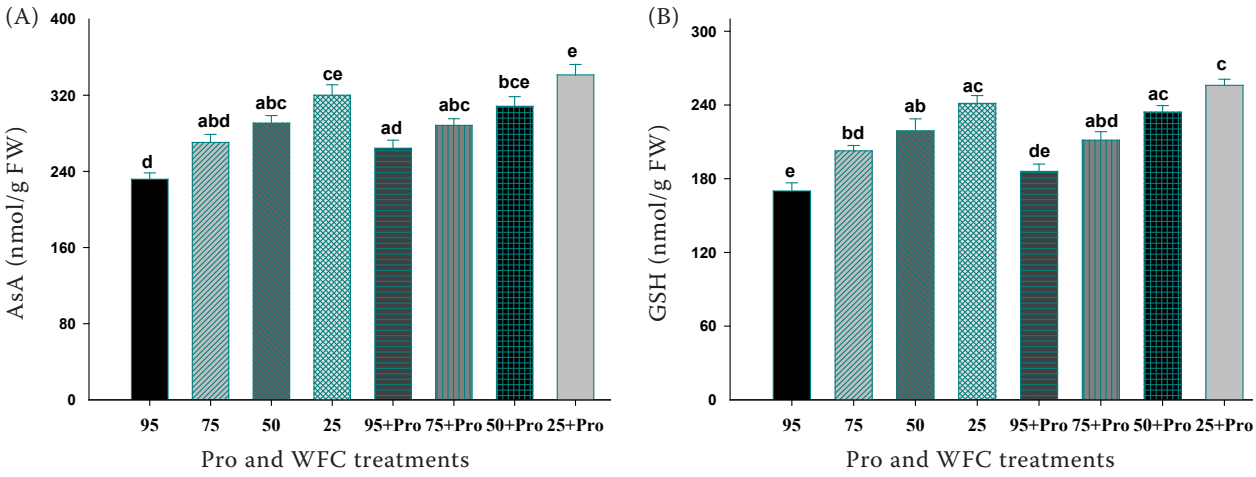

Figure 5. Non-enzymatic antioxidant response to proline (Pro) and various water regime treatments. (A) Ascorbic acid (AsA), and (B) glutathione (GSH). Each column represents the mean of three replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at $P < 0.05$. FW – fresh weight; WFC – water field capacity

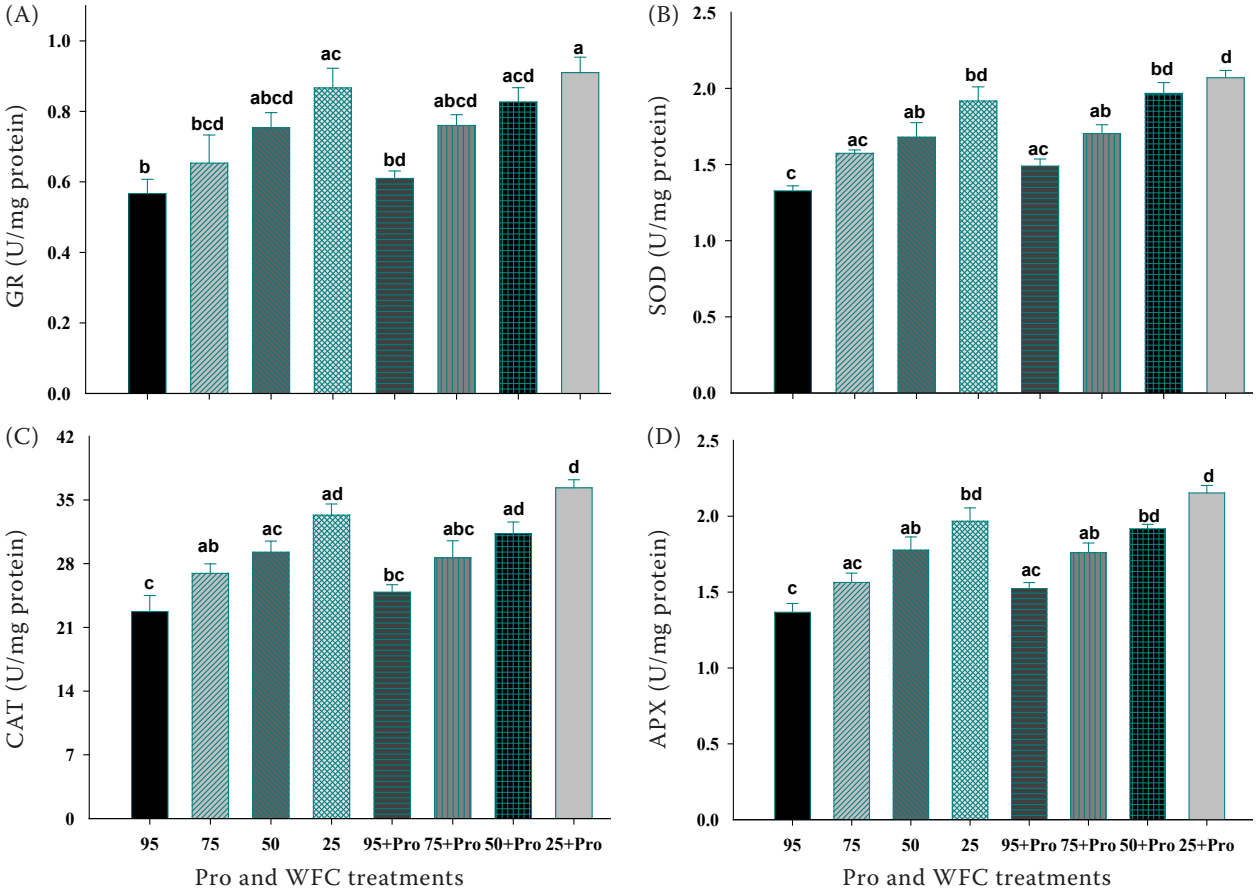

Figure 6. Impact of the proline (Pro) and water deficit treatments on antioxidant enzyme activity. (A) glutathione reductase (GR); (B) superoxide dismutase (SOD); (C) catalase (CAT), and (D) ascorbate peroxidase (APX). Each column represents the mean of three replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at $P < 0.05$. WFC – water field capacity

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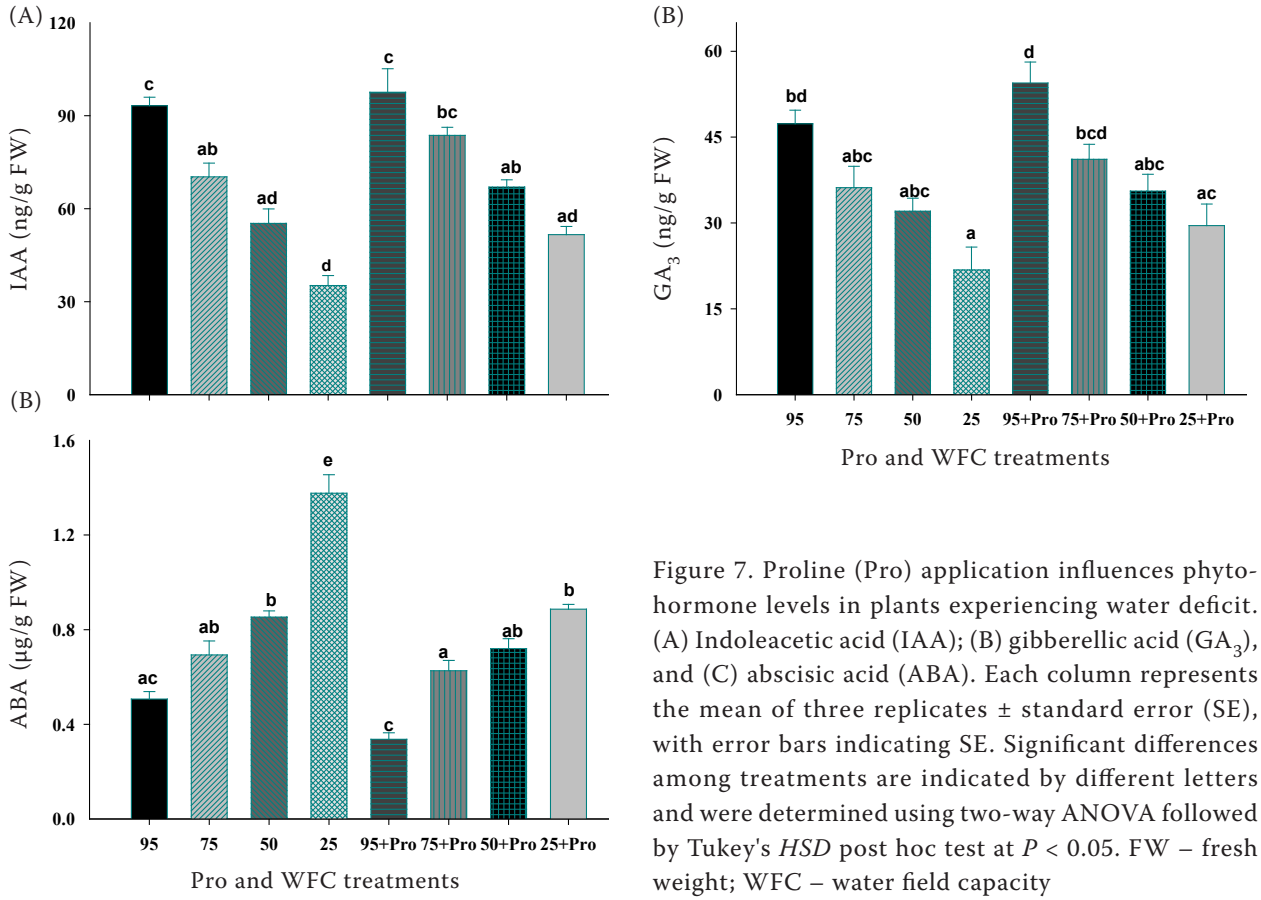

Figure 7. Proline (Pro) application influences phytohormone levels in plants experiencing water deficit. (A) Indoleacetic acid (IAA); (B) gibberellic acid (GA_3), and (C) abscisic acid (ABA). Each column represents the mean of three replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at $P < 0.05$. FW – fresh weight; WFC – water field capacity

WFC levels. ABA levels, in contrast, increased by 68.4% and 171.6% at 50% and 25% WFC, respectively (Figure 7C), and Pro treatment decreased ABA levels by 42% at 50% WFC and 75% at 25% WFC relative to untreated stressed plants. For ABA, the WFC × Pro interaction was statistically significant ($P = 0.001$), the strongest interaction detected among all measured variables, indicating that Pro's suppressive effect on ABA accumulation becomes disproportionately stronger as water deficit intensifies, rather than remaining constant across WFC levels.

Water deficit and Pro application significantly affected both essential oil yield and composition in peppermint. Menthol, the principal EO constituent, increased under moderate water deficit and peaked at 50% WFC + Pro (83%), compared with 72% in the control (Table 4). In contrast, severe stress at 25% WFC reduced menthol to 65% and was accompanied by marked increases in menthone and pulegone, indicating an unfavourable shift in monoterpene composition under extreme drought. The pro application mitigated this shift by enhancing the menthol-rich oil composition and reducing the accumulation of menthone and pulegone under stress. Minor constituents, including 1,8-cineole, limonene, β-caryophyllene, and β-pinene, showed relatively small variation among treatments.

Essential oil yield showed a biphasic response to water deficit (Figure 8). Moderate stress significantly increased EO yield by 13.9% at 75% WFC and by 16.7% at 50% WFC compared to control plants. In contrast, severe stress at 25% WFC decreased EO yield by 30.6%. Pro application significantly increased EO yield overall ($P < 0.05$), with the largest relative gains at 75% WFC + Pro (30.6%) and 50% WFC + Pro (38.9%), and a smaller apparent rescue at 25% WFC + Pro (reducing the yield decline to 5.6%). This pattern suggests a stronger Pro effect under severe stress, but the WFC × Pro interaction for EO yield was not statistically significant ($P = 0.14$); the apparent differences in Pro's benefit across WFC levels should therefore be described as a non-significant trend rather than a confirmed interaction.

DISCUSSION

Water deficit is among the most critical environmental challenges to the productivity of aromatic

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Table 4. Water deficit and proline (Pro) application significantly influenced essential oil composition in peppermint

Component	95% WFC	75% WFC	50% WFC	25% WFC	95% WFC + Pro	75% WFC + Pro	50% WFC + Pro	25% WFC + Pro
Menthol	72 ± 1.5^{c}	75 ± 1.6^{bc}	78 ± 1.8^{b}	65 ± 2.0^{d}	74 ± 1.4^{bc}	80 ± 1.7^{ab}	83 ± 1.9^{a}	72 ± 1.6^{c}
Menthone	12 ± 0.9^{c}	10 ± 0.8^{d}	8 ± 0.7^{e}	18 ± 1.2^{a}	11 ± 0.8^{cd}	7 ± 0.6^{ef}	6 ± 0.6^{f}	12 ± 0.9^{c}
Menthyl acetate	5 ± 0.4^{c}	6 ± 0.5^{bc}	7 ± 0.6^{b}	4 ± 0.4^{d}	6 ± 0.5^{bc}	8 ± 0.7^{a}	8 ± 0.7^{a}	6 ± 0.5^{bc}
1,8-cineole	3 ± 0.2^{a}	3 ± 0.2^{a}	3 ± 0.2^{a}	2 ± 0.2^{b}	3 ± 0.2^{a}	3 ± 0.2^{a}	3 ± 0.2^{a}	3 ± 0.2^{a}
Pulegone	2 ± 0.2^{c}	2 ± 0.2^{c}	2 ± 0.2^{c}	7 ± 0.6^{a}	2 ± 0.2^{c}	1 ± 0.1^{d}	1 ± 0.1^{d}	3 ± 0.3^{b}
Limonene	2 ± 0.2^{ab}	2 ± 0.2^{ab}	1 ± 0.1^{b}	2 ± 0.2^{ab}	2 ± 0.2^{ab}	1 ± 0.1^{b}	1 ± 0.1^{b}	2 ± 0.2^{ab}
Isomenthone	1.0 ± 0.1^{b}	0.5 ± 0.05^{c}	0.25 ± 0.03^{d}	1.5 ± 0.2^{a}	0.5 ± 0.05^{c}	0.25 ± 0.03^{d}	0.25 ± 0.03^{d}	0.75 ± 0.07^{b}
Neomenthol	1.0 ± 0.1^{b}	0.5 ± 0.05^{c}	0.25 ± 0.03^{d}	1.5 ± 0.2^{a}	0.5 ± 0.05^{c}	0.25 ± 0.03^{d}	0.25 ± 0.03^{d}	0.75 ± 0.07^{b}
β-Caryophyllene	1.0 ± 0.1^{a}	0.5 ± 0.05^{b}	0.25 ± 0.03^{c}	0.75 ± 0.07^{ab}	0.5 ± 0.05^{b}	0.25 ± 0.03^{c}	0.25 ± 0.03^{c}	0.5 ± 0.05^{b}
β-Pinene	1.0 ± 0.1^{a}	0.5 ± 0.05^{b}	0.25 ± 0.03^{c}	0.75 ± 0.07^{ab}	0.5 ± 0.05^{b}	0.25 ± 0.03^{c}	0.25 ± 0.03^{c}	0.5 ± 0.05^{b}
EO Yield (mL/100 g DW)	1.80 ± 0.08^{d}	2.05 ± 0.09^{c}	2.10 ± 0.10^{b}	1.25 ± 0.07^{f}	1.95 ± 0.08^{cd}	2.35 ± 0.11^{a}	2.50 ± 0.12^{a}	1.70 ± 0.09^{e}

Values are presented as means ± standard error (SE) of three replicates. Different letters within a row indicate significant differences among treatments at $P < 0.05$, as determined using two-way ANOVA followed by Tukey's *HSD* post hoc test. EO – essential oil; DW – dry weight; WFC – water field capacity

and medicinal plants, particularly amid increasing climate variability and limited irrigation resources. Its adverse effects largely stem from disrupted water relations, which in turn compromise osmotic balance, ion homeostasis, antioxidant capacity, hormonal regulation, and secondary metabolism, including essential oil biosynthesis (Haghpanah et al. 2024, Huang and Jin 2025). This is especially relevant in peppermint, where both biomass production and essential oil quality depend strongly on maintaining physiological stability under stress (Bistgani et al. 2024, Formica et al. 2026).

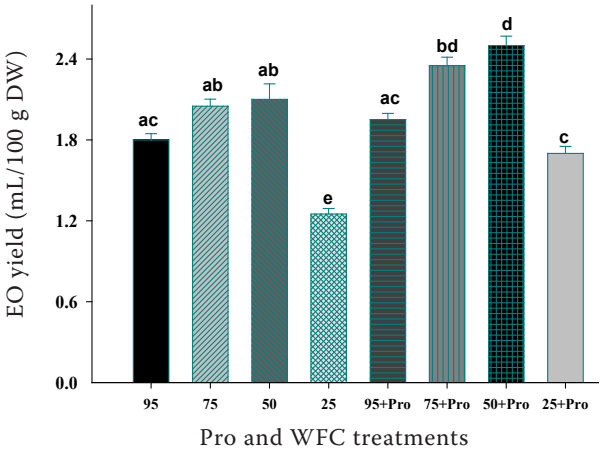

Figure 8. Proline (Pro) treatment enhanced essential oil (EO) yield in peppermint under different water-deficient conditions. Each column represents the mean of three replicates ± standard error (SE), with error bars indicating SE. Significant differences among treatments are indicated by different letters and were determined using two-way ANOVA followed by Tukey's *HSD* post hoc test at $P < 0.05$. DW – dry weight; WFC – water field capacity

In this study, water shortage severely hampered peppermint growth mainly by disrupting the plant's water balance. The strong link between decreased relative water content and lower biomass indicates dehydration was a primary factor constraining growth under intense stress. Reduced cellular hydration often limits photosynthesis, cell expansion, stomatal conductance, and metabolic processes in plants experiencing drought (Haghpanah et al. 2024, Huang and Jin 2025). Similar effects have been observed in aromatic and medicinal plants, where drought-related growth reduction is strongly tied to compromised water relations and diminished physiological functions (Araniti et al. 2024, Bistgani et al. 2024, Shahhoseini et al. 2025). Therefore, these results emphasise that preserving plant water status is essential for drought tolerance in peppermint.

Pro application significantly increased relative water content and most growth parameters; no significant WFC × Pro interaction was detected for RWC or for any of the six growth variables, providing no statistically detectable evidence that the magnitude of these benefits differed among WFC levels. The concurrent increases in proline and total soluble protein (total

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soluble sugars rose numerically but not significantly) indicate that osmotic regulation contributed consistently to maintaining cellular turgor and metabolic function under stress, regardless of stress severity (Khan et al. 2025, Pecka et al. 2025, Renzetti et al. 2025, Singh et al. 2025). Pro accumulation should still be interpreted cautiously, as it may reflect either stress severity or adaptive protection depending on species, stress level, and metabolic state (Haghpanah et al. 2024, Renzetti et al. 2025); here, the parallel improvements in water status, biomass, and EO yield, none showing a significant WFC × Pro interaction, support a protective rather than a purely diagnostic role for Pro-induced osmolyte accumulation.

Water deficit also disturbed ionic balance, increasing Na^+ and the Na^+/K^+ ratio while decreasing K^+, Ca^{2+}, and Mg^{2+}, changes that can compromise membrane stability, enzyme function, and nutrient transport under stress (Haghpanah et al. 2024, Elhakem 2025). Unlike most variables in this study, Pro's effect on ionic balance differed significantly across WFC levels: the WFC × Pro interaction was statistically significant for both Na^+ content and the Na^+/K^+ ratio (P = 0.040 and 0.046, respectively), meaning Pro's protective effect against ionic imbalance became proportionally stronger as water deficit intensified, rather than contributing a fixed offset at every WFC level. K^+, Ca^{2+}, and Mg^{2+} were also significantly improved by Pro, with no significant WFC × Pro interaction detected, providing no statistically detectable evidence that the magnitude of these benefits differed among WFC levels. Taken together, these patterns suggest that Pro's role in stabilising membranes and ion transport (Khan et al. 2025, Pecka et al. 2025) is most engaged precisely when ionic stress is more severe, rather than acting as a constant, stress-independent mechanism.

Water deficit also activated antioxidant defences, consistent with increased reactive oxygen species production under drought (Haghpanah et al. 2024, Shahhoseini et al. 2025). AsA, GSH, SOD, CAT, and APX all increased significantly under stress, and Pro further increased AsA, GSH, SOD, CAT, and APX activity; no significant WFC × Pro interaction was detected for any of these five, providing no statistically detectable evidence that the magnitude of this increase differed among WFC levels. GR was the exception: although GR activity was numerically higher under Pro at every WFC level, the Pro main effect on GR did not reach statistical significance (P = 0.063), and this should be reported as a tendency rather than a confirmed effect pending further replication. Antioxidant activation alone does not fully establish drought tolerance, since elevated antioxidant activity can also simply signal greater oxidative stress (Haghpanah et al. 2024); here, the combination of improved antioxidant capacity (with no detected WFC × Pro interaction) and improved growth and water status supports a protective, rather than a purely reactive, interpretation of Pro's antioxidant effect.

The observed hormonal changes indicate a shift from growth-driven to stress-adaptive metabolism under water deficit: ABA increased while IAA and GA3 declined, consistent with ABA-mediated stomatal closure and water conservation, alongside reduced auxin- and gibberellin-driven growth (Haghpanah et al. 2024, Huang and Jin 2025). Pro significantly increased both IAA and GA_3; neither hormone showed a significant WFC × Pro interaction, indicating this increase did not differ significantly across WFC levels. ABA showed the opposite pattern: Pro significantly decreased ABA accumulation, and this effect exhibited the strongest WFC × Pro interaction among all variables measured in this study (P = 0.001), indicating that Pro's ABA-suppressing action intensified as drought became more severe. Taken together with the ion-balance results above, this indicates that Pro's mode of action is not uniform across peppermint physiology: for growth, osmolytes, most antioxidant enzymes, IAA, and GA3, Pro contributes a consistent benefit that does not differ significantly across WFC levels, while for ABA signalling and ionic homeostasis specifically, Pro's benefit is stress-dependent and most pronounced under the most severe water deficit. These hormonal interpretations are based on the measured ABA, IAA, and GA_3 concentrations alone; the underlying signalling pathways, ion-transporter selectivity, and hormone-biosynthetic enzyme activities were not directly assessed and should be regarded as plausible explanations rather than demonstrated mechanisms. The magnitude of the measured IAA, GA_3, and ABA concentrations (tens of ng/g FW) falls within the range commonly reported for endogenous auxin and gibberellin levels in plant tissue, typically 10–100 ng/g FW for IAA and a similar order of magnitude for GA3, rather than the microgram range initially stated in an earlier draft; the unit has been corrected accordingly in the Methods (Khan et al. 2025, Huang and Jin 2025).

This study revealed a biphasic response in essential oil yield and composition to water deficit: moderate stress increased oil production, whereas

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severe stress reduced both yield and quality. This pattern supports the idea that mild abiotic stress can stimulate secondary metabolism, enhancing essential oil traits in medicinal and aromatic plants, whereas excessive stress hampers productivity due to physiological and metabolic constraints (Formica et al. 2026). In our research, moderate drought likely redirected carbon flux toward secondary metabolism, whereas severe drought likely limited primary metabolism and terpene biosynthesis due to reduced physiological capacity (Jangpangi et al. 2025, Formica et al. 2026). Similar stress-dependent effects have been observed in aromatic plants, where controlled stress increases phytochemical accumulation, but severe stress reduces biomass and metabolite yield (Bistgani et al. 2024, Formica et al. 2026).

Additionally, changes in the composition of peppermint essential oil further support this stress-dependent response. Moderate water deficit boosts menthol levels, while severe stress raises menthone and pulegone, indicating a negative shift in monoterpene makeup under extreme drought. These qualitative changes suggest that mild abiotic stress can enhance essential oil quality by altering the ratios of key volatile components, whereas more intense stress may disrupt biosynthetic pathways (Araniti et al. 2024, Formica et al. 2026). Consequently, the increased oil yield under moderate stress should be seen not just as a stress reaction, but as a deliberate metabolic adjustment that enhances both oil quantity and quality.

Across treatments, the essential oil was strongly menthol-dominant, with menthol accounting for 65–83% of the identified oil. This quantitative profile overlaps with menthol-rich *Mentha arvensis* cultivars, including Gomti and Shivalik, and with CIM-Kranti, whose menthol, menthone, and menthyl acetate proportions vary with cropping season, harvest timing, and drying conditions (Padalia et al. 2013, Bhatt et al. 2020). This comparison is chemical rather than taxonomic. It neither identifies the experimental material as *M. arvensis* nor assigns it to any named cultivar, because monoterpene proportions are influenced by genotype, developmental stage, irrigation regime, environment, harvest timing, and postharvest handling. The observed oil is therefore described only as a menthol-dominant chemotype of the tested seed-derived population.

Pro treatment also increased essential oil yield and enriched menthol content, with the largest numerical gains at WFCs of 75% and 50%. This effect may reflect improved water balance, ion distribution, antioxidant capacity, and carbon availability for secondary metabolism (Khan et al. 2025, Renzetti et al. 2025, Singh et al. 2025). However, the WFC × Pro interaction for EO yield was nonsignificant ($P = 0.14$), so the present analysis found no statistically detectable evidence that the magnitude of the Pro response differed among WFC levels. The larger gains under moderate stress should therefore be treated as a numerical trend, not as proof of a stress-specific or additive effect. The concurrent increase in menthol and decreases in menthone and pulegone indicate a more favourable monoterpene profile after Pro application, although terpene biosynthesis regulation was not measured directly and thus requires molecular validation.

Collectively, the present results show that foliar Pro alleviated drought injury in the tested peppermint population, but the statistical evidence does not support a single uniform mechanism. Significant Pro main effects were detected for growth, water status, chlorophyll, proline, total soluble protein, most antioxidant traits, IAA, GA_3, and EO yield, while the corresponding WFC × Pro interactions were nonsignificant. Thus, the present tests found no statistically detectable evidence that the magnitude of these Pro responses differed among WFC levels, but they do not prove equality or additivity. In contrast, significant interactions for Na^+, the Na^+/K^+ ratio, and ABA showed that Pro responses for these variables depended on water-deficit severity and were strongest under severe stress. Additional replication and molecular measurements are required to explain why ion regulation and ABA, but not most other endpoints, displayed stress-dependent interaction patterns.

Although this study provides comprehensive physiological, biochemical, hormonal, and phytochemical data, the specific molecular mechanisms remain unresolved. Gene-expression and enzyme-level analyses of Pro metabolism, ion transport, antioxidant regulation, ABA signalling, and monoterpene biosynthesis would be required to test the proposed pathways. Field trials across seasons and Pro concentrations are also needed before broader agronomic application. In addition, because Pro was applied repeatedly, both before and throughout the stress period, at a single concentration, the design cannot separate the contributions of initial priming, cumulative exogenous supply, and stress-induced endogenous Pro synthesis. Experiments comparing single and repeated applications across several Pro concentrations are needed to resolve these effects.

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A more fundamental, unresolved limitation concerns botanical and cultivar identity. The supplier provided the seeds as *Mentha* × *piperita* L. but could not certify a named cultivar, and no voucher specimen, molecular authentication, or cultivar-verified vegetative clone was available. Because the experiment used seed-derived plants, genetic and chemotypic heterogeneity may have contributed to the observed variation. The menthol-dominant GC-MS profile and its quantitative overlap with *M. arvensis* cultivars cannot retrospectively establish species or cultivar identity. This limitation is not resolved by the present study. Consequently, conclusions concerning essential-oil composition apply only to the tested seed-derived population supplied as *M.* × *piperita* and should not be generalised to a named peppermint cultivar or commercial chemotype. Confirmation requires repetition with voucher-documented, cultivar-verified, vegetatively propagated material and independent botanical or molecular authentication.

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