BURSA ULUDAĞ ÜNİVERSİTESİ ZİRAAT FAKÜLTESİ DERGİSİ
Journal of Agricultural Faculty of Bursa Uludag University
e-ISSN 2651-4044
https://dergipark.org.tr/tr/pub/bursauludagziraat
http://www.uludag.edu.tr/ziraatdergi
Haziran/2025, 39(1), s. 1-22

ARAŞTIRMA MAKALESİ RESEARCH ARTICLE

Geliş Tarihi (Received): 25.05.2024
Kabul Tarihi (Accepted): 24.12.2024

Investigation of the Effects of Foliar Amino Acid, Melatonin and Potassium Applications on Spinach Cultivar under Water Stress[A]

Nezahat TURFAN[1*]

Abstract: The main objective of this study is to explore the potential benefits of exogenous supplements of asparagine (Asn), phenylalanine (Phe), melatonin (Mel), and potassium nitrate (KNO_3) in mitigating the effects of water deficiency (WD) on spinach. The improvement effects of four chemicals on WD-stressed seedlings were assessed by comparing growth rate traits, chlorophyll, secondary metabolites, nitrogenous compounds, mineral contents and variations in antioxidant enzyme activity. According to the results, the growth rate traits of seedlings were reduced with WD stress, but the exogenous supplements of Asn, Phe, Melatonin, and KNO_3 alleviated the suppressing effect of drought on growth parameters. Supplements of four stimulants led to a marked boost in the content of chlorophyll, lutein, anthocyanin, phenolic compounds, flavonoids, proline, and glycine betaine (GB) in the WD-stressed seedlings compared to the control. Moreover, these substances suppressed the generation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in seedlings under WD stress but caused a significant enhancement in polyphenol oxidase (PPO), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD) and superoxide dismutase SOD) activities. The WD stress reduced magnesium (Mg), calcium (Ca), manganese (Mn) and iron (Fe) levels, but boosted the accumulation of potassium (K), phosphorus (P), sulfur (S), nickel (Ni), copper (Cu) and zinc (Zn) levels. Considering all data, it can be said that an exogenous supply of Asn, Phe, Mel and KNO_3 to the Acosta spinach variety exposed to WD stress made a significant contribution to increasing yield and nutritional quality by preventing oxidative stress and strengthening enzymatic and non-enzymatic defences.

Keywords: Antioxidant, nutrition, spinach growth, stimulants, water stress.

[A] The study does not require approval from an ethics committee. The article has been prepared according to research and publication ethics.

[*] **Sorumlu yazar/Corresponding Author:** 1 Nezahat TURFAN Kastamonu University, Science Faculty, Biology Department, Kuzeykent, 37150, Kastamonu, Türkiye, ntrufan@kastamonu.edu.tr OrcID 0000-0002 5753-0390

Atıf/Citation: Turfan, N. 2025. Investigation of the Effects of Exogenous Amino Acid, Melatonin and Potassium Applications on The Responses of Acosta Spinach to Drought. *Bursa Uludag Üniv. Ziraat Fak. Derg*., 39(1), 1-22. https://doi.org/10.20479/bursauludagziraat.1489707

Yapraktan Amino Asit, Melatonin ve Potasyum Uygulamalarının Ispanağın Su Stresine Toleransındaki Etkilerinin İncelenmesi

Öz: Bu çalışmanın temel amacı, su eksikliği stresinin (WD) ıspanak üzerindeki etkilerini hafifletmede asparajin (Asn), fenilalanin (Phe), melatonin (Mel) ve potasyum nitrat (KNO_3) ekzogen uygulamalarının potansiyel faydalarını araştırmaktır. Bu dört kimyasalın kuraklık stresindeki fideler üzerindeki iyileştirici etkileri, büyüme hızı özellikleri, klorofil, ikincil metabolitler, azotlu bileşikler, mineral içerikleri ve antioksidan enzim aktivitesindeki değişimler karşılaştırılarak değerlendirilmiştir. Sonuçlara göre, WD uygulamasıyla ıspanak fidelerinin büyüme hızı özellikleri azalmış ancak Asn, Phe, Mel ve KNO_3 ilaveleri kuraklığın büyüme parametreleri üzerindeki baskılayıcı etkisini hafifletmiştir. Dört uyarıcının uygulaması, WD stresine maruz bırakılan fidelerde kontrole kıyasla klorofil, lutein, antosiyanin, fenolik bileşikler, flavonoidler, prolin ve glisinbetain (GB) içeriğinde önemli bir artışa yol açmıştır. Ayrıca bu uyarıcılar WD stresi altındaki fidelerde malondialdehit (MDA) ve hidroejen peroksit (H_2O_2) üretimini baskılamış ancak polifenol oksidaz (PPO), askorbat peroksidaz (APX), katalaz (CAT), proksidaz (POD) ve süperoksit dismutaz (SOD) aktivitelerinde önemli bir artışa neden olmuştur. WD uygulamasıyla magnezyum (Mg), kalsiyum (Ca), mangan (Mn) vedemir (Fe) düzeyleri azalırken, potasyum (K), fosfor (P), sülfür (S), nikel (Ni), bakır (Cu) çinko (Zn) düzeyleri artırmıştır. Veriler değerlendirildiğinde, su eksikliği stresine maruz kalan Acosta ıspanak çeşidine dışarıdan Asn, Phe, Mel ve KNO_3 verilmesinin, oksidatif stresi önleyerek, enzimatik ve enzimatik olmayan savunma mekanizmasını güçlendirerek verim ve besin kalitesinin artmasına önemli katkı sağladığı söylenebilir.

Anahtar Kelimeler: Antioksidant, besin, ıspanak kültürü, uyarıcılar, su stresi.

Introduction

Spinach (*Spinacia oleracea* L.*)* is a leafy crop rich in pigments, secondary production, nitrogenous compounds, vitamins, antioxidant enzymes, and minerals that benefit human health. It is a vegetable that is not selective in the climate, can grow in almost every region with its summer and winter cultivars, has a short vegetation period and is less costly than other vegetables (Gürel, 2024). Furthermore, spinach is a versatile crop that can be marketed as fresh or frozen and is even used to produce baby food. Due to the rising demand for nutritious, safe and economical food sources, the need for spinach production increases even more (Yaman Türkkan and Kibar, 2022). On the other hand, spinach cultivation, like other agricultural products, is not primarily affected by water deficiency, leading to approximately 65% of global yield loss (Hamed et al., 2025). Due to water taking up 80-95% of the fresh biomass of any species, the absence of its impairs morphological, physiological and biochemical functions, resulting in reduced crop productivity (Akpınar et al., 2021). According to researchers, the decrease in biomass is mainly due to water deficiency, which interrupts photosynthetic metabolism, inhibits the synthesis of pigments, and inhibits cell division in vegetative organs (Seymen et al., 2024). The most visible symptoms of water stress are the alterations in leaf characteristics such as yellowing, rolling, permanent wilting, early leaf senescence, and smaller sizes (Jafari et al., 2022). Additionally, water stress can trigger oxidative stress, which disturbs the stability of cellular structures and inhibits the accumulation of compatible solutes (Gilani et al., 2020; Jalali et al., 2020). Wen et al. (2017) and Akın and Kaya (2023) expressed that water stress caused a loss of viability, suppressed elongation, and prevented respiration in plant root cells. The breeding method still maintains its importance in crop production under water deficiencies. However, using bioactive substances to enhance plant resistance can boost yield and nutritional quality by activating various mechanisms, including pigments, osmolytes, secondary components, and antioxidant defences (Fang et al., 2022; Turfan et al., 2024). Recently, the use of amino acid (Kaya et al., 2013; Turfan, 2023b), melatonin (Sadak et al., 2020), and mineral fertilisers (Akşahin and Gülser, 2020; Kaya, 2024) has been considered a promising strategy to stabilise cellular membranes, adjust cytoplasmic solutes, and enhance water and mineral availability from the roots. These stimulants are easily absorbed by leaf tissues and used in biochemical reactions that regulate growth and development (Turfan et al., 2024; Hamed et al., 2025). Studies show that stimulant supply results in faster, stronger defence responses in crops compared to controls. Stimulants offer beneficial effects such as improving nutrient-water availability, enhancing photosynthetic gain, activating enzymatic and non-enzymatic defences, and boosting agronomic quality by promoting vegetative growth (Akşahin and Gülser, 2020; Turfan, 2023a). Asparagine (Asn), an essential amino acid in nitrogen metabolism and storage, serves as the phloem's primary transporter for reduced nitrogen due to its high nitrogen-to-carbon ratio (Kaya et al., 2013). It also plays a key role in sulfur absorption and the accumulation of compatible solutes within plant cells. Qu et al. (2019) and Soares et al. (2020) found that Asn regulates sugar utilisation in plant roots. Supplying Asn foliar to cotton under drought conditions enhanced growth by activating antioxidant defences (Akın and Kaya, 2023). Phenylalanine (Phe), an aromatic amino acid, serves as a substrate for numerous compounds and a precursor for secondary metabolites, including flavonoids, phenolic acids, anthocyanins, tannins, benzenoids, and phenylpropanoids, which contribute to plants' combat stress (Jiao et al., 2018). Studies by Aghaei et al. (2019) and Ramzan et al. (2023) displayed that exogenous phenylalanine boosts growth and antioxidant defence in drought-stressed mustard and cotton plants. Melatonin, a water- and fat-soluble molecule, protects cell membranes from oxidative stress (Turfan, 2023a). Exogenous Mel supply reduces MDA and ROS production in plants under drought conditions. It also enhances chlorophyll, secondary metabolites, and compatible solutes during stress (Zhang et al., 2016). Potassium (K) has multiple roles in metabolic processes, including maintaining cell turgidity, regulating plant-water relations, photosynthesis, ATP balance, nutrient uptake, phloem transport, and stress resistance (Zahoor et al., 2017). Studies showed that melatonin and KNO_3 mitigate drought-induced oxidative damage by activating defence systems. Exogenous melatonin has improved drought stress tolerance in cabbage (Zhang et al., 2016), moringa (Sadak et al., 2020), and cauliflower (El-Bauome et al., 2022). Similarly, the KNO_3 application boosted the yield and quality of spinach and potato under water-deficient conditions (Gilani et al., 2020; Bukhari et al., 2023). Under drought, the synthesis of some chemicals is suppressed while others are promoted, helping plants survive by protecting them from oxidative damage. These strategies could be more effective in drought-sensitive species, where exogenous supplements can strengthen adaptation mechanisms.

While each substance enhances resistance through similar mechanisms, they generally activate different pathways. Numerous studies have documented the response of crop species to drought conditions and the drought tolerance of other plants when supplemented with asparagine, phenylalanine, melatonin, and potassium. However, the specific modulatory roles of these four compounds in mediating drought responses in this species remain to be elucidated. Therefore, revealing four substances' similar and particular influences on adaptation mechanisms can provide significant clues to developing a better strategy in leafy vegetable cultivation under drought conditions. Also, like all other crops, spinach responds to drought conditions by altering its bioactive components' content and diversity, reflecting its nutritional quality (Ekinci et al.,2015). This event may be advantageous in stimulating drought tolerance and understanding the production mechanism of specific targeted compounds important in nutritional values. Therefore, investigating the effects of exogenous amino acids, melatonin and KNO_3 supplements on spinach under drought conditions may offer valuable opportunities to produce targeted, chemically rich spinach with diverse properties. The essential aim of this study is to compare the effects of exogenous asparagine, phenylalanine, melatonin and KNO_3 supplements on the morphological, physiological and biochemical responses to drought in the Acosta F1 spinach variety.

Materials and Methods

Description of experimental design

This experiment was conducted between 16 November 2022 and 16 March 2023 in plastic greenhouse conditions with three replications using balcony-type pots (70 x 18 x 17) as four pots for each replicate by a completely randomised design. A mixture of peat and perlite in a 3:1 ratio (v/v) was used for growing spinach seedlings, with each pot filled to a capacity of 16 L (Yaman Türkkan and Kibar, 2022). In the greenhouse, mean temperatures in November, December, January, February, and March are 8.8, 3.44, -0.8, 7.2 °C, and 16.38 °C, respectively. In addition, mean relative humidity values were 70, 75, 77, 68, and 72 per cent for five months. All cultivation continued for about five months, during which the minimum temperature was -14.8°C (January) and the maximum temperature was 16.39 °C (March). Acosta 1 spinach cultivar was used in the present study. It is a species that is tolerant of low temperatures, fragility, and diseases and suitable for greenhouse and open-area growing (Turfan, 2023a). Seeds were surface sterilised with sodium hypochlorite (1%) for 5 min, followed by thorough cleaning with distilled water. The soil's nutrient components, EC, and pH values are presented in Table 1.

Table 1. Some properties of the soil used in the experiments

	%					**mg kg^{-1}**					**dS m^{-1}**	
	Mg	**K**	**Ca**	**P**	**S**	**Fe**	**Mn**	**Zn**	**Cu**	**Ni**	**EC**	**pH**
Soil	26.85	14.88	79.00	3.35	2.40	4130	990.90	46.80	44.40	142.00	6.70	6.30

Table 2. Properties and concentrations of chemicals used in this study

Group	Concentration	Abbreviations
1	Without application	Control
2	Water deficiency (40%)	WD
3	L-Asparagine (2.6 mM)	Asn
4	Asparagine + Water deficiency	Asn+ WD
5	L-Phenyalalnine (3.4 mM)	Phe
6	Phenylalanine + Water deficient	Phe+WD
7	Melatonin (50 mM)	Mel
8	Melatonin + Water deficiency	Mel+WD
9	KNO_3 (50 mM)	KNO_3
10	KNO_3+Water deficient	KNO_3+WD

While the major element levels of the soil ranged from 2.40 (S) to 79.00% (Ca), trace elements ranged from 44.0 to 4130 mg. The soil's pH value was found to be 6.26, and its electrical conductivity (EC) was 6.7 dS m^{-1}.

After sowing, all pots were rinsed with nutrient solution (Hoagland and Arnon, 1950.) twice a week until L-asparagine (Asn), L-Phenylalanine, Melatonin (Mel), and KNO_3 applications were started. L-Asparagine (L-Asparagine monohydrate, Sigma, CAS: 5794-13-8), L-Phenylalanine (L-Phenylalanine, Sigma, CAS: 63-91-2), Melatonin (Melatonin Sigma CAS:73-31-4), and KNO_3 (KNO_3: 50 mM, Merck CAS: 7757-79-11) applications were started when the seedlings were at the 4-5 leaf stage. Water stress treatments were imposed on the plants for eight weeks, beginning four weeks after transplanting (at 4-6 leaves). Spinach plants were harvested in the ninth week. The chemicals to be applied from the leaves have been prepared by dissolving the determined concentrations in the nutrient solution (Hoagland and Arnon, 1950).

The application models of the study are given in Table 2. The doses of applied stimulants were defined by a preliminary trial performed with a vial. To this end, we observed concentrations that promote a noticeable increase in the shoot, root, and leaf lengths, as well as the fresh weight of seedlings for each stimulant to identify growth-stimulating doses.

The water shortage level was selected by determining the concentration that inhibited vegetative development in seedlings by around 40%. Drought stress was maintained through field capacity (40%). Initially, 1 kg of soil mixture (peat: perlite) was taken, and an adequate water level was added until it was fully saturated. After that, the pot was kept for 24 hours so that gravitational water could completely leak out through gravitational pull. Finally, 100 % field capacity (FC) was determined by using the equation (Özbek and Kaman, 2014).

FC (%) =Water Added-Water leached

Morphological measurement

Shoot and root lengths were measured using a measuring tape. Fresh (FW) and dry weight (DW) were taken using the digital weight balance. Samples were oven-dried at 60 °C for 72 h to record dry weight (Cemeroğlu, 2007).

Elemental analyses

For measurement of soil and leaf element (*Mg*, *P*, *S*, *K*, *Ca*, *Na*, *Cl*, *Al*, *Mn*, *Fe*, *Ni*, *Zn* and *Cu*) status, soil (taken before setting up experiments) and leaf samples were dried at 65 °C and powdered. These samples were used to determine the elemental analysis in Kastamonu University's Central Research Laboratory using the ICP-OES (SpectroBlue II) device.

Determination of pigment and secondary compounds

Measurements of leaf chlorophyll (SPAD) contents were performed using a SPAD device (SPAD-502, Minolta Corporation, Ltd., Osaka, Japan) from the very top of the leaf stem of 10 leaves per group, moving towards the tip of the leaf, and the chlorophyll content was determined by taking the average of the values obtained. The lutein contents were measured using the methods described by Kukric et al. (2012). For total phenolic estimation, Folin-Denis reagent was employed by using a double-beam UV-visible spectrophotometer (Folin and Denis, 1915). The absorbance was recorded at 660 nm, and the total phenolic content was calculated using the standard curve of 0.1mg mL gallic acid, which was expressed in mg g^{-1} dry weight. Total flavonoids were measured using Kumaran and Karunakaran's method (2006). A 500 mg leaf tissue was homogenised in 10 mL of 80% methanol solution. The reaction solution contained 4 mL deionised water and 1 mL extract, 0.3 mL of 5% $NaNO_2$. Further, 0.3 mL $AlCl_3$ and 2 mL NaOH were added. The absorbance was recorded at 510 nm. Polyphenol oxidase (PPO) activity of spinach tissue was performed by monitoring the increase in absorbance at 420 nm for 4-methyl catechol spectrophotometrically (Kumar et al., 2008). The assaying mixture (3 mL) contained 100 mM sodium phosphate buffer (pH 7.0), five mM 4-methyl catechol and 500 µL of crude extract. The total volume of the reaction mixture was 3.0 mL. One unit (U) of enzyme activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per minute. The total nitrate (TN) content was determined by using the salicylic acid method (Cataldo et al., 1975). The absorbance was measured at 410 nm, and the TN level was calculated using the equation obtained from the NO_3 standard curve. The NT was expressed as mg g^{-1}. The amount of proline was estimated using a standard curve, as suggested by Bates' method (1973). The proline content was expressed in µmol g^{-1} considering the molecular weight of the proline. The glycine betaine (GB) content was determined using the method introduced by Grieve and Grattan (1983). Measurement of malondialdehyde (MDA) content was employed by the Lutts et al. (1996) method. Firstly, 500 mg spinach samples were homogenized in 5 mL 5% trichloroacetic acid (TCA) and then centrifuged at 10 000 x g for 15 minutes. 4 mL of the supernatant was taken and 1 mL of 0.5% TBA (thiobarbituric acid) solution was added. The reaction mixture was incubated in boiling water (95 ºC) for 60 minutes. The absorbance of the samples cooled in an ice bath was noted at 532 nm and 600nm. The level of MDA was estimated as µmol g^{-1} using the extinction coefficient of 155 mM^{-1}. In the hydrogen peroxide (H_2O_2) assaying, the method described by Velikova et al. (2000) was followed. A 500 mg sample was extracted with 0.1% TCA. After centrifuging at 12 000 x g for 15 minutes, 0.5 mL phosphate buffer (pH 7.0) and 1 mL potassium iodide (KI) were added to the supernatant (0.5 mL). The absorbance was read at 355 nm and H_2O_2 concentration was calculated with the H_2O_2 standard curve.

Antioxidant Enzymes Activities

Antioxidant enzyme extraction of spinach samples was conducted with sodium phosphate buffer solution (50 mM, pH 7.6). 500 mg samples were homogenised in a 5 mL buffer containing 0.1 mM Na-EDTA (ethylenediamine tetraacetic acid disodium salt) and centrifuged for 15 min at 15 000 rpm at 4°C. The obtained supernatant was used in the measurements of antioxidant enzyme activities such as ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) (Zhang et al., 2006). The ascorbate peroxidase (APX) activity was determined by recording the decline in absorbance at 290 nm as ascorbate is oxidised in the presence of hydrogen peroxide (H_2O_2). Activity measurement was performed with a mixture consisting of 0.5 mM ascorbate, 100 mM sodium phosphate buffer (pH 7.0) and enzyme extract. The reaction was started by adding H_2O_2 to the indicated mixture. APX activity of samples was defined as mg protein^{-1} min^{-1}. The catalase (CAT) activity of samples was assayed by measuring the decomposition of H_2O_2 by the catalase enzyme present in the crude extract. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2, and 100 µL of crude extract. One unit (U) of CAT activity was expressed as the amount of enzyme that caused an absorbance change of 0.001 per minute under assay conditions. The peroxidase (POD) activity was determined by measuring the oxidation of guaiacol, in the presence of H_2O_2 by the peroxidase enzyme present in the enzyme extract. The reaction mixture contained 2 mL of buffer (composed of 400 µL of 8 mM guaiacol and 1600 µL of 100 mM sodium phosphate buffer, pH 6.4), 1 mL of 24 mM H_2O_2, and 0.5 mL of enzyme extract. Absorbance values were recorded twice at 30-s intervals at 470 nm. POD activity was expressed as U µl^{-1} min^{-1}. The superoxide dismutase (SOD) activity was determined by inhibition of the photochemical reduction of NBT (nitroblue tetrazolium). The reaction mixture (5 ml) consisted of 50 mM sodium phosphate buffer (pH 7.4), 130 mM methionine, nitroblue tetrazolium (750 µm), EDTA (100 µM), riboflavin (20 µM), and enzyme extract (100 µL). Each sample was illuminated with light (60 mol m^{-2} s^{-1}) for 15 minutes, and absorbance was then read at 560 nm. One unit (U) of SOD activity was expressed as a U mg^{-1} protein.

Relative water content (RWC)

RWC of leaves was measured using the method of Turfan et al. (2024). First, the fresh weight of approximately 1 cm leaf discs was recorded (Fresh Weight: FW). The leaf discs were then placed in a petri dish filled with deionised water and kept there for 4 hours. After noting the turgid weight (TW) of the samples, they were dried in an oven at 70°C for 48 hours. Finally, the dry weight was recorded, and the leaf RWC was calculated using the following formula: $RWC (\%) = [(FW - DW) / (TW - DW)] \times 100$ (I)

FW: Fresh weight, DW: Dry weight, TW: Turgid weight

Statistical Analysis

All experimental data related to the amino acids, melatonin and potassium supplements to the spinach seedlings under drought stress on growth rate traits, chemical constituents, and element accumulation were subjected to multiple analyses of variance (MANOVA) using SPSS statistical software (SPSS for Windows, Version 16).

Following the results of MANOVA, Tukey's honest significance difference (HSD) test (α= 0.05) was also applied.

Results

Variation of morphological traits in the spinach seedlings

The present experiment gives data from the growth rate traits obtained in Table 3. Shoot height ranged from 11.26 to 20.82 cm, root height from 9.24 to 14.99 cm, fresh weight of a plant from 5.15 to 10.90 g, and fresh weight of a leaf from 0.81 to 1.55 g, respectively.

Table 3. Variation of leaf growth rate parameters of spinach seedlings

	SH (cm)	**RH (cm)**	**P-FW (g)**	**L-FW (g)**	**LH (cm)**	**LBH (cm)**	**LW (cm)**	**Leaf number**
Control	14.98±0.13^{c}	12.85±12^{ab}	6.80±0.11^{c}	1.06±0.03^{c}	10.85±0.18^{b}	6.92±0.04^{b}	3.98±0.05^{b}	11.3±0.22^{b}
WD	11.26±0.13^{d}	9.24±0.09^{c}	5.15±0.08^{d}	0.81±0.02^{d}	9.54±0.10^{c}	5.82±0.05^{c}	3.50±0.07^{c}	9.8±0.14^{c}
Asn	17.96±0.20^{b}	14.25±0.08^{a}	10.10±0.14^{a}	1.39±0.02^{a}	12.20±0.13^{ab}	8.02±0.04^{a}	4.97±0.04^{ab}	13.1±0.24^{a}
Asn-WD	16.54±0.18^{b}	13.06±0.17^{ab}	7.83±0.09^{bc}	1.11±0.03^{b}	11.59±0.15^{b}	7.11±0.03^{b}	4.02±0.05^{b}	11.2±0.14^{b}
Mel	16.84±0.18^{b}	13.98±0.11^{a}	8.71±0.10^{b}	1.15±0.03^{b}	12.39±0.16^{ab}	7.49±0.09^{ab}	4.13±0.03^{b}	12.4±0.16^{ab}
Mel-WD	15.78±0.11^{bc}	12.98±0.09^{ab}	7.59±0.08^{bc}	1.11±0.03^{b}	12.00±0.08^{b}	7.11±0.05^{b}	3.91±0.03^{bc}	10.6±0.16^{b}
Phe	17.30±0.15^{b}	14.31±0.10^{a}	8.88±0.10^{b}	1.32±0.03^{ab}	13.84±0.10^{a}	7.85±0.12^{ab}	4.88±0.09^{ab}	12.8±0.20^{ab}
Phe-WD	16.02±0.10^{bc}	12.37±0.96^{b}	7.87±0.10^{bc}	1.18±0.02^{b}	12.28±0.11^{ab}	7.26±0.07^{ab}	4.50±0.09^{ab}	11.5±0.17^{b}
KNO_3	20.82±0.07^{a}	14.99±0.11^{a}	10.90±0.0^{6a}	1.55±0.04^{a}	14.01±0.05^{a}	8.65±0.11^{a}	5.10±0.02^{a}	13.4±0.17^{a}
KNO_3-WD	17.32±0.12^{b}	13.07±0.09^{ab}	7.98±0.07^{bc}	1.29±0.03^{ab}	12.07±0.16^{ab}	8.24±0.13^{a}	4.62±0.08^{ab}	11.3±0.16^{b}
F	424.07	27.604	323.224	191.82	86.50	138.98	148.17	45.04
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*: Means (±: n=10 in the same column for each trait in each group with the same lower-case letter are not significantly different by the MANOVA test at $P < 0.001$. WD: Water deficient, Asn: Asparagine, Mel: Melatonin, Phe: L-Phenylalanine; SH: Shoot height, RH: Root height, P-FW: Plant fresh weight, L-FW: Leaf fresh weight, LH: Leaf height, LBH: Leaf blade height, LW: Lamina width.

Water deficiency caused a 24.81% decrease in shoot development, a 27.61% decrease in root length and a 24.34% decrease in fresh weight compared to the control (P< 0.001). Fresh weight was in the range of 0.81 and 1.55 g, leaf height in the range of 9.54 and 13.84 cm, blade height in the range of 5.82 and 8.65 cm, and blade width between 3.50 and 4.88 cm. Leaf numbers ranged from 9.80 to 13.40, and they were found to be highest with just the KNO_3 supplement and the lowest with the WD (Table 3). Results exhibited that WD application led to a significant decline in growth rate traits, which was reflected in a reduction in shoot, root, and leaf characteristics (Table 3). On the contrary, foliar applications led to an increase in these parameters. However, the growth rates of seedlings exogenously supplemented with four substances under water stress were lower than those treated with just four stimulators.

Variation of pigment and secondary metabolite content

The effect of Asn+WD, Mel+WD, Phe+WD, and KNO_3+WD supplements on the pigment and secondary metabolites in spinach seedlings was summarised in Figure 1a-f.

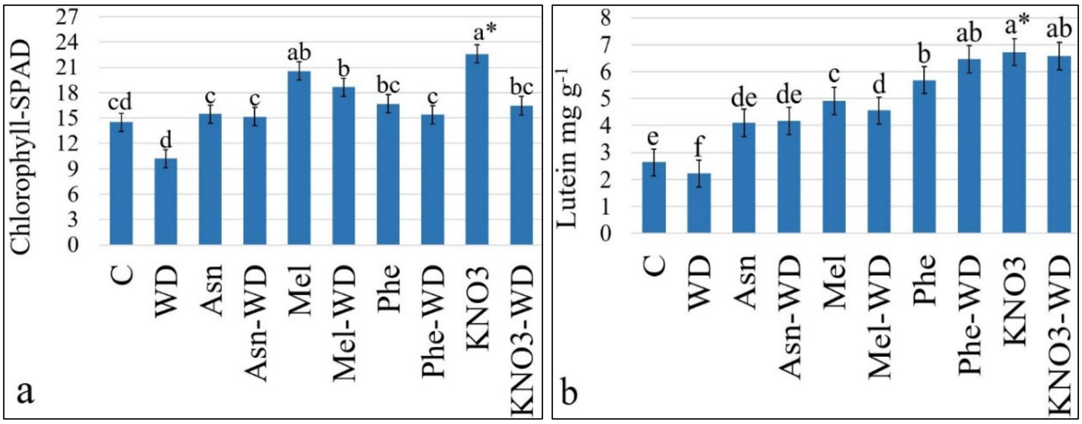

Figure 1. *Variation of chlorophyll (a) and lutein (b) in the seedling. C: Control, WD: Water deficiency, Asn: Asparagine, Mel: Melatonin, Phe: Phenylalanine. *: Means (±: n=3 in the same column for each trait in each group with the same lower-case letter are not significantly different by the MANOVA test at P < 0.001.*

The chlorophyll content (SPAD) of the seedlings varied between 10.22 and 22.59, with the lowest value in the WD-stressed group (29.61%) and the highest value in the just KNO_3 (55.62%) and Mel (41.76%)-applied seedlings (Figure 1a). The stimulants that most enriched the amount of chlorophyll in the WD-stressed group were Mel at 82%, followed by KNO_3 at 61.25%. Lutein content was the lowest in the control (2.64 mg), but it reached the highest values with the KNO_3 (6.59-6.73 mg) and the Phe-applied groups (4.46-5.26 mg) (Figure 1b).

Secondary metabolites, including anthocyanin, polyphenol, flavonoid, and also enzymes involved (PPO) in the synthesis of these compounds significantly altered (P<0.001) by WD and exogenous supplementations (Figures 2a-d). The WD stress caused a notable decrease in anthocyanin (38.78%), flavonoid (22.91%) and total phenolic compounds (26.36%) regarding the control. Contrarily, the highest incremental values of these compounds were achieved with just the Phe application (82.59%, 58.77%, and 82.00%, respectively).

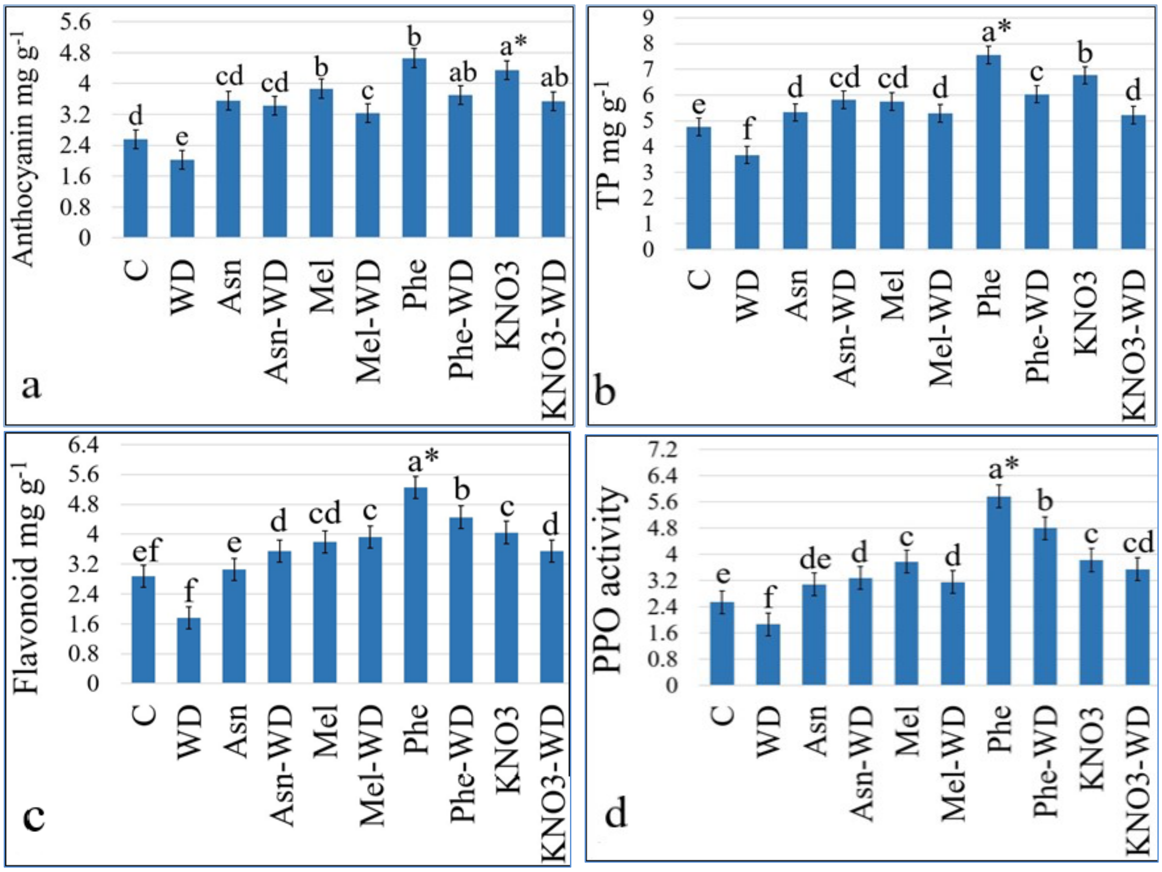

Figure 2. *Variation of anthocyanin (a), total phenolic (TP: b), flavonoid (c), and polyphenol oxidase activity (PPO: d) in the seedling. C: Control, WD: Water deficiency, Asn: Asparagine, Mel: Melatonin, Phe: Phenylalanine. *: Means (±: n=3 in the same column for each trait in each group with the same lower-case letter are not significantly different by the MANOVA test at P < 0.001.*

PPO activity in seedlings varied from 1.87 to 5.77 EU, significantly decreasing with the WD (26.36%). Enzyme activity peaked with the Phe supplement, followed by the Phe-WD group (Figure 2d).

Nitrogenous compounds

The total nitrate (NT) content of spinach seedlings ranged from 1.65 mg (Phe) to 4.89 mg (WD) (Figure 2a). The nitrate accumulation of seedlings ranged from 2.13 to 4.96 mg (WD-KNO3), proline from 8.70 (WD) to 17.88 (Asn), and GB from 1.71 (WD) to 4.35 mg (Asn), respectively (Figure 3a-d). The WD evoked the accumulation of nitrate, whereas it reduced the proline and GB contents. In addition, the four substances improved the amount of proline and GB in both unstressed and drought-stressed seedlings while significantly suppressing nitrate accumulation (Figure 3b-3c). Compared to the control, the highest proline and GB contents in the seedlings were found in the Asn group, while the lowest nitrate content was seen in the KNO_3 and the Phe groups. In this study, RWC levels were in the range of 58.66% (WD) and 90.46% (KNO_3) in seedlings (Figure 3d). With the supplementation of the four substances, there was a significant increase in RWC levels, even in those subjected to drought. In unstressed plants, the highest RWC level was recorded with Asn (26.00%) and Mel (28.44%), while the highest RWC level was obtained with KNO3 (24.51%) and Mel (17.60%) application in WD-stressed groups (Figure 3d).

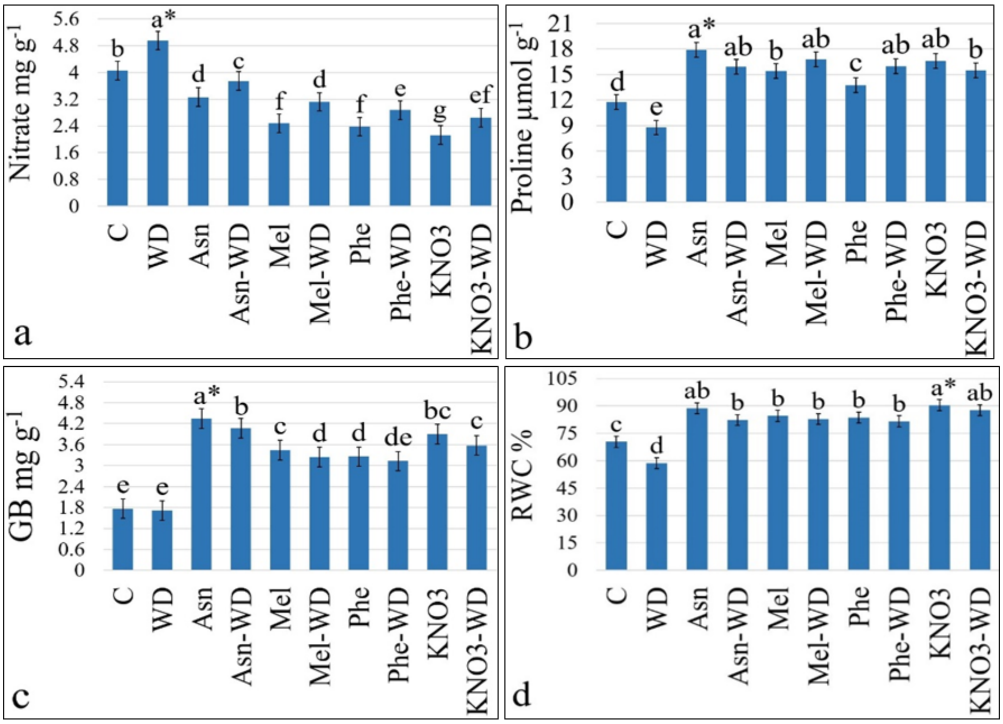

Figure 3. *Variation of total nitrate (a), proline (b), glycine betaine (c), and RWC (d) in seedlings. C: Control, WD: Water deficiency, Asn: Asparagine, Mel: Melatonin, Phe: Phenylalanine, GB: Glycinebetaine. *: Means (±: n=3 in the same column for each trait in each group with the same lower-case letter are not significantly different by the MANOVA test at P < 0.001.*

Variation of Oxidative Stress and Enzyme Activities

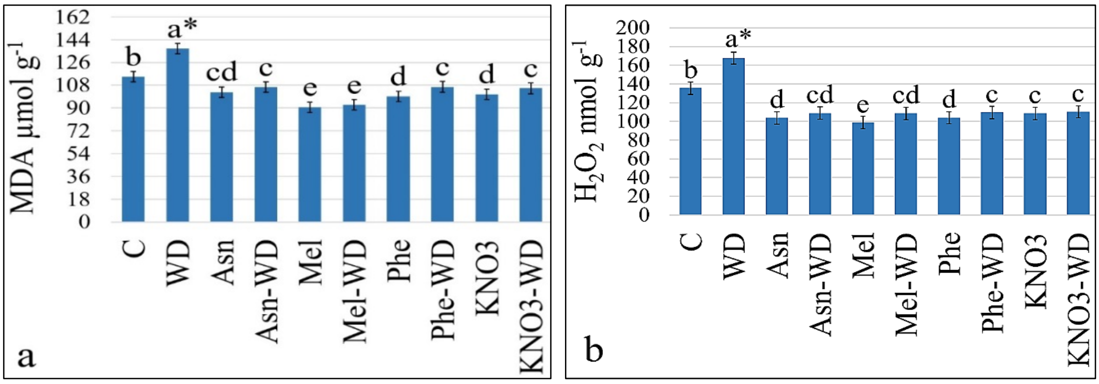

Figure 4. *Variation of MDA (a) and H_2O_2 (b) concentrations of seedlings. C: Control, WD: Water deficiency, Asn: Asparagine, Mel: Melatonin, Phe: Phenylalanine; *: Means (±: n=3 in the same column for each trait in each group with the same lower-case letter are not significantly different by MANOVA test at P < 0.001.*

In this study, there was a marked rise in MDA (19.50%) and H_2O_2 (23.70%) contents in the stressed seedlings compared to the control. In contrast, a considerable reduction occurred in the levels of MDA and H_2O_2 with just four supplements in the unstressed seedlings, especially in the Mel-applied (20.83, 27.16%) (Figure 4a-b). However, exogenous supplements to WD-suffered seedlings reduced MDA and H_2O_2 content compared to just stimulants applied groups.

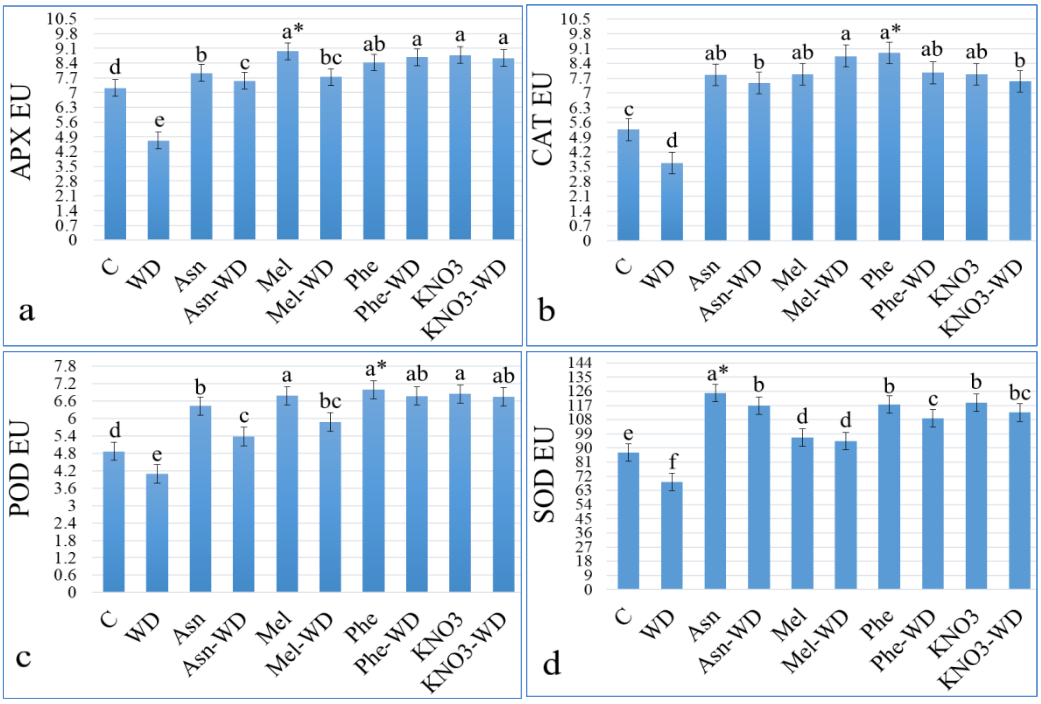

Figure 5. *Variations of APX (a), CAT (b), POD (c), and SOD (d) activities in spinach seedlings.*: Means (±: n=3 in the same column for each trait in each group with the same lower-case letter are not significantly different by the MANOVA test at P<0.001. C: Control, WD: Water deficiency, Asn: Asparagine, Mel: Melatonin, Phe: Phenylalanine*

Regarding antioxidant enzymes, the activity of APX, CAT, POD, and SOD ranged from 4.72 to 8.97 (WD-Mel), from 3.67 to 8.89 (WD-Phe), from 4.09 to 6.99 (WD-Phe), and from 64.49 to 124. 66 (WD-Asn) EU, respectively (Figure 5a, d). The WD stress caused a noticeable decline in the activity of four enzymes compared to the control group. In contrast, supplements with Asn, Phe, Mel, and KNO_3 led to elevated activity in four enzymes (Figure 5a, d). In seedlings subjected to WD, APX activity peaked in KNO_3-supplied plants at 8.64 EU. In contrast, CAT activity was highest in Mel-treated plants (8.75 EU), POD activity peaked with Phe treatment (6.78 EU), and SOD activity reached its maximum with Asn treatment (124.66 EU), as shown in Figures 5a-d.

Mineral Status of Samples

In this study, there was a significant alteration in the levels of the essential elements, such as Mg, S, P, K, and Ca, depending on applications (P<0.001). A notable reduction occurred with the WD in the Mg and Ca, while a noticeable boost was observed in the S, P, and K concentrations (Table 4). The highest Mg was found in Mel-supplied seedlings, but the highest S, K and Ca content was recorded with the KNO_3-provided group (Table 4). On the other hand, the lowest levels of Mg and Ca were obtained from the WD-stressed groups, the lowest K in the control, and the lowest S in just the Mel-provided groups (Table 4).

Table 4. Variation of P, K, Ca, Mg and S status of spinach seedlings (in mg kg^{-1})

Group	**P**	**K**	**Ca**	**Mg**	**S**
Control	9888±20^{b}	56455±140$^{\imath}$	44221±120^{c}	18466±120^{c}	4656±20^{e}
WD	12458±120^{a}	86444±140^{a}	38866±120^{d}	13646±120^{d}	5866±20^{a}
Asn	9246c±20^{d}	64322±140^{h}	46880±120^{bc}	22455±200^{b}	4876±20de
Asn-WD	9472±20^{c}	66368±140^{fg}	48456±120^{b}	21456±200^{b}	5234±20^{c}
Mel	8876±20^{e}	74522±140^{e}	45622±120^{c}	24366±200^{a}	5068±20^{d}
Mel-WD	9285±20^{cd}	68464±140^{g}	44764±120^{c}	22856±200^{b}	5428±20^{b}
Phe	9025±20^{d}	76478±140^{d}	47817±120^{b}	21648±200^{b}	4426±20^{f}
Phe-WD	9328±20^{cd}	72344±140^{f}	50432±130^{a}	20542±200b^{c}	4618±20^{e}
KNO_3	9144±20^{d}	82268±140^{b}	48764±120^{b}	21548±200^{b}	5542±20^{b}
KNO_3-WD	9466±20^{c}	78566±140^{c}	45345±120^{c}	20166±200^{bc}	5256±20^{c}
F	1417549	74707579	10154132	10595528	300759
P	<0.001	<0.001	<0.001	<0.001	<0.001

*: Means (±: n=3 in the same column for each trait in each group with the same lower-case letter are not significantly different by the MANOVA test at $P \leq 0.05$. WD: Water deficient, Asn: Asparagine, Mel: Melatonin, Phe: L-Phenylalanine

Table 5. Variation of Fe, Zn, Cu, Mn and Ni status of spinach seedlings (in mg kg^{-1})

Group	Fe	Zn	Cu	Mn	Ni
Control	622.8±20^{e}	78.7±4.6^{f}	13.8±1.5^{c}	156.5±14de	36.5±2.8^{d}
WD	586.6±20^{f}	84.2±5.2^{cd}	18.7±1.7^{a}	142.4±12^{e}	46.9±3.6^{a}
Asn	652.7±20^{c}	78.9±4.7^{f}	12.6±1.1^{d}	168.5 ±16^{c}	34.8±2.4^{e}
Asn-WD	644.4±20^{d}	70.6±4.4^{g}	15.3±1.3^{bc}	175.5±16^{bc}	38.9±2.8^{c}
Mel	653.8±20^{c}	84.4±5.2^{cd}	14.8±1.3^{c}	188.7±6^{a}	40.5±3.2^{b}
Mel-WD	648.2±20^{d}	86.8±6.2^{c}	16.9±1.4^{ab}	184.6±18^{ab}	38.8±2.8^{c}
Phe	678.6±20^{a}	90.6±6.4^{b}	15.4±1.4^{bc}	174.4±15^{bc}	40.4±3.7^{b}
Phe-WD	654.4±20^{c}	94.6±6.8^{a}	18.3±1.4^{a}	178.9±25^{b}	44.7±4.1^{ab}
KNO_3	654.8±20^{c}	84.4±5.2^{cd}	16.4±1.4^{b}	158.3±14^{d}	38.9±3.6^{c}
KNO_3-WD	644.7±20^{d}	82.7±5.2^{e}	18.6±1.4^{a}	165.7±15^{cd}	35.6±3.2^{d}
F	3411228	21148089	245543	83487	27641
P	<0.001	<0.001	<0.001	<0.001	<0.001

*: Means (±: n=3 in the same column for each trait in each group with the same lower-case letter are not significantly different by MANOVA test at $P < 0.05$. WD: Water deficient, Asn: Asparagine, Mel: Melatonin, Phe: L-Phenylalanine.

The results of micronutrient average values are given in Table 5. The highest and lowest values recorded for Mn, Fe, Ni, Cu and Zn (in mg kg^{-1}) were in the range of 142.4-188.7, 586.6-64.8, 35.6-46.9, 13.8-18.6 and 70.6-94.6, respectively. The lowest levels of those elements compared to the control were recorded with the WD-stressed groups, except Ni (the KNO_3-WD) (Table 5).

Discussion

Leafy plants may require considerable energy for cell expansion and differentiation activities; hence, water deficiency can spell a catastrophe for them at these stages (Haghighia et al., 2020). The results revealed that WD caused an essential decrease in shoot and root length, shoot and root fresh weight, and leaf development in spinach seedlings. Still, exogenous Asn, Phe, Mel, and KNO_3 supplements eliminated drought stress's adverse effects on these parameters (Table 3). However, the growth rates of seedlings treated with stimulant alone were higher than those of seedlings supplemented with stimulant along with drought and the control group seedlings. Also, among all groups, KNO_3 was the application that improved growth rates the most, followed by Asn and Phe, respectively (Table 3). Consistent with the results, Haghighia et al. (2020) in cabbage (Ekinci et al., 2015; Ors and Suarez, 2017) in spinach, Ramzan et al. (2023) in mustard, observed a reduction in the growth rate of seedlings by WD stress, in contrast to the control group. Likewise, while the improvement effect of the exogenous amino acid supplement on the growth traits in lettuce under water stress was shown by Yang et al. (2020) and Khan et al. (2019), a positive effect of potassium on spinach growth grown under drought was demonstrated by Gilani et al. (2020), and Bukhari et al. (2023). Further, an increased growth rate in the cucumber and Brassica with exogenous Mel application under drought conditions was reported by Zhang et al. (2013) and Mir et al. (2020). All researchers claimed that drought stress declines the growth traits of plants by inhibiting cell expansion with a lower cell turgor, reducing photosynthetic gain, and preventing chlorophyll synthesis. However, the use of exogenous enhancer supplements has been proven to be highly effective in alleviating the effects of drought damage by providing a boost to various plant activities such as cell development, photosynthesis, turgor/osmotic adjustment, and senescence delay (Soares et al., 2020; Seymen et al., 2024).

The agronomical qualities of vegetables depend on photosynthesis, which is driven by the efficiencies of photosynthetic pigments, the amount of which is affected by the light, temperature and water status of leaf tissues (Zahoor et al., 2017). Therefore, a more excellent pigment value means a healthier plant and a higher yield. In this study, drought-stressed seedlings had significantly lower chlorophyll and lutein levels than the control group (Figure 1a, 1b). However, the supplements of those stimulants to the WD-stressed seedlings resulted in an improvement in chlorophyll content. The stimulants that most improved the amount of chlorophyll in seedlings exposed to drought were Mel and KNO_3, while the amount of lutein was enriched by KNO_3 and Mel (Figure 1a-b). The decremental values in chlorophyll and lutein were associated with the breakdown of pigment molecules due to water stress, whereas the incremental values have been associated with the improving effect of the applied stimulants on pigment biosynthesis, nitrogen utilisation and antioxidant defence (Mir et al., 2020; Kaya and Shabala, 2023). The findings are consistent with previous studies that have recorded a comparable decline in chlorophyll and carotenoid under water deficiency. Similar observations for cabbage, lettuce, and spinach subjected to drought stress were reported by Haghighia et al. (2020), Jalali et al. (2020), and Turfan (2023b). However, the contribution of exogenous amino acids, Mel, and KNO_3 to the accumulation of chlorophyll and carotenoid under water stress has been demonstrated by the studies of Gilani et al. (2020) and Fang et al. (2022), who examined the effects of drought on spinach and sesame. Likewise, Jiao et al. (2018), Haider et al. (2021), and Turfan (2023a) observed enhanced chlorophyll and carotenoid with amino acids, Mel, and KNO_3 under natural conditions in many species.

The secondary products, such as anthocyanins, polyphenol, and flavonoids, contained in spinach contribute to the development of spinach under stress conditions by strengthening cell walls, inhibiting lipid peroxidation, and scavenging ROS (Aghaei et al., 2019). In this study, anthocyanin, flavonoid, and total phenol, as well as the activity of the PPO enzyme responsible for the synthesis of these compounds, were significantly reduced by water stress ($P<0.001$). However, exogenous supplementation of the four substances significantly promoted their accumulation (Figure 2a-d). The highest levels of these four substances in the WD and unstressed seedlings were reached with the Phe supplement, followed by the KNO_3 (Figure 2a-d). The decremental values of secondary metabolites under water-deficient conditions agreed with the results of Naz et al. (2020). Enhanced levels of secondary metabolites are consistent with previous observations by Haghighia et al. (2020) and Ramzan et al. (2023), who sprayed cabbage and mustard with amino acids exposed to the abiotic stresses. Similarly, the improvement effect of Mel on the secondary metabolism in moringa, cabbage, and lettuce subjected to water deficiency has been shown by Zhang et al. (2016) and Sadak et al. (2020) studies. In contrast, the enrichment effect of KNO_3 on those substances has been reported by Bukhari et al. (2023) and Turfan (2023a,b).

Underwater shortage conditions, plants may accumulate nitrate due to reduced utilisation for organic molecule synthesis (Chen et al., 2021). In this study, while drought application led to nitrate accumulation, its accumulation was, on the contrary, inhibited by exogenous Phe, Mel and KNO_3 supplements. However, there was a second increase in nitrate level with the WD-Asn supplements compared to the control (Figure 3a). Although the seedlings treated with Phe and KNO_3 had the lowest NT level, a significant decrease occurred in the groups exposed to the WD stress with these substances (Figure 3a). Similarly, Chen et al. (2021), Turfan (2023b), and Sadak (2022) observed that nitrate accumulation in drought-exposed plants was minimised by the application of biostimulants. One possible explanation for why Mel, Phe, and KNO_3 fertilisers reduced NT accumulation in spinach seedlings is that these substances promote nitrogen utilisation in the synthesis of pigments, amino acids, and other nitrogenous compounds (Jiao et al., 2018; Jalali et al., 2020). As another nitrogen source, proline and glycine betaine (GB) contribute to coping with drought damage by adjusting cell turgor or preventing osmotic stress, preventing the structures of membrane lipids, proteins and pigments from oxidation (El-Bauome et al., 2022; Turfan, 2023b). Therefore, increased osmolytes in the cells indicate drought resistance in plants (Ibrahim et al., 2020). In the study, the seedlings exposed to water deficiency were the poorest group in terms of both compounds (Figure 3a-3b). On the contrary, in unstressed seedlings, the group most affluent in proline and GB was that which supplied only ASN (Figures 3b-c). On the other hand, among the WD-stressed plants, the seedlings treated with Mel had the highest levels of proline, while the Asn-provided group exhibited the highest levels of GB (Figure 3b-3c). Consistent with this study, Zahoor et al. (2017) and Bahar et al. (2021) results also proved that proline accumulation is less in drought-sensitive plants, but this phenomenon was reversed with exogenous amino acids (cotton, spinach, moringa), melatonin (spinach) and KNO_3 (potato) supplements. Likewise, Turfan (2023a) suggested that water stress inhibited GB accumulation in plants significantly, while low temperature may promote its accumulation. Chen et al. (2021), Khan et al. (2019), and Min et al. (2021) have demonstrated that proline and GB contribute to improving the nutritional quality as well as the appearance of crops due to osmoprotectant and antioxidant properties under abiotic stresses.

The water deficiency adversely affects RWC levels of leaves, causing osmotic stress evoked by intracellular water deficiency, thereby suppressing water uptake and the accumulation of compatible solutes (Ahanger and Agarwal, 2017). In this study, the WD stress caused a critical reduction in the RWC ratio compared to the control (Figure 3d). The highest level was achieved by just the KNO_3, followed by the Asn-applied seedlings. In addition, RWC content was significantly increased in drought-stressed seedlings upon exogenous KNO_3 and Mel supplements (Figure 3d). In corroboration with our results, Ekinci et al. (2015), and Ahanger and Agarwal (2017) observed that decreased RWC in leafy vegetables under water stress caused severe losses in quality and yield by interrupting cell division, photosynthesis, and nitrogen-carbon metabolism. However, exogenous Asn (Qu et al., 2019), Phe (Ramzan et al., 2023), Mel (Mir et al., 2020), and potassium (Zahoor et al., 2017) applications have been declared to improve RWC levels of plants by accumulating osmolytes in the cells, controlling transpiration efficiency of leaves and promoting the growth of root cells. It has been suggested that four substances have played essential roles in the maintenance of RWC levels of leaves by regulating the uptake of water (Ahanger and Agarwal, 2017), inducing osmolyte accumulations (Soares et al., 2020), and also preventing membrane injuries (El-Bauome et al., 2022).

Although MDA and ROS are byproducts of normal metabolic reactions in plant cells, excessive buildup has also been considered a well-defined oxidative stress indicator. Both can lead to the degradation of proteins, enzymes, and DNA/RNA structures (Kaya and Shabala, 2023). In this study, the drought-stressed group exhibited the highest level of MDA and H_2O_2, while the Mel-provided groups displayed the lowest levels. While the inhibitory effect of these stimulants on MDA and H_2O_2 accumulation in unstressed plants was in the order of Mel, Phe, KNO_3 and Asn, their effects under water deficiency were in the order of Mel, Asn, Phe, and KNO_3 (Figure 4a-b). Consistent with the literature, high MDA and H_2O_2 levels were associated with cellular membranes' destruction and organic molecules' degradation due to oxidative stress caused by water deficiency. Previous studies displayed that WD stress resulted in a rise in MDA and H_2O_2 of spinach (Turfan, 2017), cabbage (Haider et al., 2021), and cauliflower (El-Bauome et al., 2022), which aligns with our findings. The lower MDA and H_2O_2 levels of the four substances are probably attributable to the higher osmolyte accumulation, production of secondary metabolites, and enzyme activity, which are promoted by the used substances. (Figure 4a-d). It has been shown by different studies that the induced activities of enzymes by antioxidant molecules permitted stressed plants to prevent ROS generation, resulting in an abatement of the injury of ROS-induced oxidative stress (Turfan et al., 2024).

Although water deficiency can excite oxidative stress, plants have evolved defence systems, including non-enzymatic (osmolytes, pigments, secondary metabolites) and enzymatic (APX, CAT, POD, and SOD) molecules, to cope with the destructive effect of its. Results revealed that the WD-stressed seedlings exhibited a lower ($P< 0.001$) activity of APX, CAT, POD, and SOD enzymes compared to the control (Figure 5a-d). On the contrary, with the four substances, a noticeable boost in the activity of the four enzymes was observed in both WD-applied and unstressed plants. While the enhancement effects of enzymes in unstressed seedlings were observed as Mel, Phe, KNO_3, and Asn in WD-applied seedlings, this order was determined as Phe, KNO_3, Mel, and Asn. Results of enzymes demonstrated that water deficient declined APX, CAT, POD, and SOD activities, but exogenous supplements of four stimulators work in line with the expected purpose by strengthening against drought by the antioxidant defence in spinach seedlings. In harmony with our results, Gilani et al. (2020), and Haider et al. (2021) reported decremental values in enzyme activity under water stress in maize, spinach, and lettuce. Moreover, all authors stated that the inhibitory effect of WD on enzyme activity could be ameliorated through amino acids, melatonin and potassium. This increment in APX, CAT, POD, and SOD may be due to these substances' role in enhancing antioxidants that neutralize ROS, associated with oxidative stress. Another study revealed that supplying amino acids and KNO_3 contributed to the inhibition of ROS, such as H2O2, O2*-, and OH, by preventing oxidative stress (Bahar et al., 2021; Turfan et al., 2024).

Underwater scarcity conditions, mineral deficiency or toxicity may occur in plants due to damage to plant roots and disruption of plant-water relations (Zhang et al., 2013). In this study, the WD reduced the Mg and Ca content but increased the S, P, and K content. In unstressed seedlings, Mg was the highest with the Mel and S, K, and Ca were the highest with the KNO_3 (Table 4). In the WD-stressed group, Mg reached the highest level with Mel, P with Asn, S with KNO_3 and Ca with Phe, respectively (Table 4). The high S, P, and K levels in seedlings under water restriction may be due to the interruption of protein and carbohydrate metabolism due to the adverse effects of drought stress (Ibrahim et al., 2020). As it is known, while P participates in the structure of sugars, amino acids and nucleic acids, the K element also plays a vital role in maintaining the osmotic balance in the leaves (Ahanger and Agarwal, 2017). Sulfur is the structural element of amino acids, vitamins and some enzymes. It has also been reported by Ibrahim et al. (2020) and Bahar et al. (2021) that these elements accumulate in the leaves because these metabolisms are blocked by water deficiency. A decrease in Mg content due to water restriction may be related to antagonism between K and Mg and a reduction in pigment synthesis in the leaf. A lower level of Ca with water shortage was associated with Ca being an immobile element. The low water content suppressed Ca absorption from the roots. Wen et al. (2017) and Haghighia et al. (2020) observed similar results by foliar spray of amino acid in maize under water stress and in peas and cabbage under water stress. The same responses with Mel and KNO_3 were cited by Mir et al. (2020), Haider et al. (2021), and Fang et al. (2022) in dark leafy species. Likewise, similar to this study, Bukhari et al. (2023) and Turfan (2023b) revealed no statistical difference between the P content of the dark leafy plants under water deficiency.

Trace element amounts, including Mn, Fe, Ni, Cu, and Zn, showed significant changes according to the applications. At the same time, Mn and Fe contents decreased with drought, Asn reduced Ni and Cu contents, and Zn contents were reduced by Phe-WD (Table 5). Variations in trace element levels in leaves may be due to the differences in the elements' mobility and the effect of the four supplied substances on the uptake of the elements (Turfan, 2023b). Additionally, differences in the interactions of amino acids with cations/anions may have affected trace element accumulation in seedlings (Turfan, 2025).

Conclusions

This study examined the impacts of exogenous supplements asparagine, phenylalanine, melatonin, and potassium nitrate on spinach seedlings under water stress, focusing on growth, bioactive chemicals, antioxidant enzymes, and mineral status. Results showed that WD negatively impacted growth traits, accumulations of pigments, secondary metabolites, nitrogenous compounds, and enzyme activity. However, supplements of Asn, Phe, Mel, and KNO_3 improved these attributes, even under stress. KNO_3 was the most effective in mitigating water stress, followed by Phe. When comparing enzyme activities in seedlings experiencing WD, it was observed that APX was activated by KNO_3 and Mel; CAT was activated by Mel and Phe; POD was induced by Phe and KNO_3; and SOD was stimulated by Asn and KNO_3. Also, Mel reduced MDA and H_2O_2 levels in WD-stressed seedlings, while Asn and KNO_3 improved RWC. The WD stressed plants had lower levels of Mg, Ca, Mn, and Fe but higher levels of K, P, S, Ni, Cu, and Zn.Additionally, seedlings under WD stress with stimulant provision displayed increased microelement content compared to those treated alone. In conclusion, growth rate, nutrients, and enzyme activity were increased in Acosta 1 spinach seedlings upon exogenous supplements of amino acids, melatonin, and KNO_3, even in groups stressed with WD. The KNO_3 and Phe were the most effective applications for drought resistance in spinach cultivation. The responses of any species to water stress reveal insights into their physiological mechanisms. Understanding how exogenous stimulants alter drought tolerance can help optimise agricultural production in water-limited areas and improve nutrient quality. Given the positive effects of KNO_3 and Phe, they are offered as exogenous supplements to enhance spinach cultivation and nutritional quality in arid and semi-arid regions.

Acknowledgements

The study does not require approval from an ethics committee. The article has been prepared according to research and publication ethics. Since the study has only one author, there are no conflicts of interest

References

Aghaei, K., Pirbalouti, A.G., Mousavi, A., Badi, H.N. and Mehnatkesh, A. 2019. Effects of foliar spraying of L-phenylalanine and application of bio-fertilisers on growth, yield and essential oil of hyssop [*Hyssopus officinalis* L. subsp. Angustifolius (Bieb.)]. *Biocatalysis and Agricultural Biotechnology,* 21: 101318.

Ahanger, M.A. and Agarwal, R.M. 2017. Potassium up-regulates antioxidant metabolism and alleviates growth inhibition under water and osmotic stress in wheat (*Triticum aestivum* L). *Protoplasm*, 254 (4): 1471-1486.

Ahmad, S., Su, W., Kamran, M., Ahmad, I., Meng, X., Wu, X., Javed, T. and Han, Q. 2020. Foliar application of melatonin delays leaf senescence in maise by improving the antioxidant defence system and enhancing photosynthetic capacity in semiarid regions. *Protoplasm*, 57(4):1079-1092.

Akın, S. and Kaya, C. 2023. Asparagine and nitric oxide jointly enhance antioxidant capacity and nitrogen metabolism to improve drought resistance in cotton: Evidence from long-term field trials. *Food and Energy Security 00*, e502.

Akpınar, A., Cansev, A. and Altınşeker Acun, D.Z. 2021. Responses of *Spinacia oleracea* L. cv. matador plants to various abiotic stresses such as cadmium metal toxicity, drought and salinity.*Journal of Agricultural Faculty of Bursa Uludag University*, 35(1):103-117.

Akşahin, V. ve Gülser, F. 2020. Bazı organik materyallerin ve inorganik gübrelerin çemen bitkisinin gelişimine etkileri. *Bursa Uludag Üniv. Ziraat Fak. Derg.,* 34(2): 255-266.

Bahar, A.A., Faried, H.N., Razzaq, K., Ullah, S., Akhtar, G. and Amin, M. 2021. Potassium-induced drought tolerance of potato by improving morphophysiological and biochemical attributes. *Agron*, 11(12): 2573.

Bates, L.S., Waldern, R.P. and Teare, I.D. 1973. Rapid determination of free proline for water stress studies. *Plant and Soil*, 39: 205-207.

Bukhari, S.A.B.H., Lalarukh, I., Amjad, S.F., Mansoora, N., Naz, M., Naeem, M., Bukhari, S.A., Shahbaz, M., Ali, S.A. and Marf, T.D. 2023. Drought stress alleviation by potassium-nitrate-containing chitosan/montmorillonite microparticles confers changes in *Spinacia oleracea* L.. *Sustainability*, 13: 9903.

Cataldo, D.A., Haroon, M., Schrader, L.E. and Youngs, V.L. 1975. Rapid colourimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Communications in Soil Science and Plant Analysis*, 6: 71-80.

Cemeroğlu, B. (2007). Gıda Analizleri. Gıda Teknolojisi Derneği Yayınları, 34, 168–171.

Chen, Z., Cao, X. and Niu, J. 2021. Effects of Melatonin on morphological characteristics, mineral nutrition, nitrogen metabolism, and energy status in alfalfa under high-nitrate stress. *Frontiers in Plant Sciences*, 12: 694179.

Ekinci, M., Ors, S., Sahin, U., Yildirim, E. and Dursun, A. 2015. Responses to the ırrigation water amount of spinach supplemented with an organic amendment in greenhouse conditions. *Communications in Soil Science and Plant Analysis*, 46(3): 327-342.

El-Bauome, H.A., Abdeldaym, E.A., Abd El-Hady, M.A.M., Darwish, D.B.E., Alsubeie, M.S., El-Mogy, M.M., Basahi, M.A., Al-Qahtani, S.M., Al-Harbi, N.A. and Alzuaibr, F.M. 2022. Exogenous proline, methionine, and melatonin stimulate growth, quality, and drought tolerance in cauliflower plants. *Agriculture*, 12(9):1301.

Fang, S., Yang, H., Wei, G., Shen, T., Wan, Z., Wang, M., Wang, X. and Wu, Z. 2022. Potassium application enhances drought tolerance in sesame by mitigating oxidative damage and regulating osmotic adjustment. *Frontiers in Plant Sciences*, 13:1096606.

Folin, O. and Denis, W. 1915. A colourimetric method for the determination of phenols (and phenol derivatives) in urine. *Journal of Biological Chemistry*, 22(2):305-308.

Gilani, M., Danish, S., Ahmed, N., Rahi, A.A., Akrem, A., Younis, U., Irshad, I. and Iqbal R.K. 2020. Mitigation of drought stress in spinach usıng ındıvıdual and combıned applıcatıons of salıcylıc acıd and potassıum. *Pakistan Journal of Botany*, 52(5): 1505-1513.

Grieve, C.M. and Grattan, S.R. 1983. Rapid assay for determination of water-soluble quaternary ammonium compounds. *Plant Soil*, 70:303-307.

Gürel S. 2024. Effects of kitchen waste compost and chemical fertilizer application on spinach (*Spinacia oleracea* L.) c.v. Matador plant growth and nitrate accumulation. *Journal of Plant Nutrition*, 47(8):1246-1261.

Haghighia, M., Saadata, S. and Abbeyb, L. 2020. Effect of exogenous amino acids application on cabbage growth and nutritional value under drought stress. *Scientia Horticulturae*, 272*:* 109561.

Hamed, H., Mahmoud, G.A. and Abeed, A.H.A. 2025. Unravelling growth and metabolic dynamics in drought-stressed spinach plants: exploring the contribution of biological gibberellin. *Scientia Horticulturae*, 340: 113924. https://doi.org/10.1016/j.scienta.2024.113924

Haider, S.A., Lalarukh, I., Amjad, S.F., Mansoora, N., Naz, M., Naeem, M., Bukhari, S.A., Shahbaz, M., Ali, S.A. and Marfo, T.D. 2021. Drought stress alleviation by potassium-nitrate-containing chitosan/montmorillonite microparticles confers changes in *Spinacia oleracea* L. *Sustainability*, 13: 9903.

Hoagland, D.R. and Arnon, D.I. 1950. The water-culture method for growing plants without soil. *California Agriculture Experiment Station Circular*, 347:1-32.

Ibrahim, M.F.M., El-Samad, G.A., Ashour, H., El-Sawy, A.M., Hikal, M., Elkelish, A., El-Gawad, H.A., El-Yazied, A.A., Hozzein, W.N. and Farag, R. 2020. Regulation of agronomic traits, nutrient uptake, osmolytes and antioxidants of maise as ınfluenced by exogenous potassium silicate under deficit ırrigation and semiarid conditions. *Agronomy*, 10:1212.

Jafari, M., Shahsavar, A.R., Talebi, M. and Hesami, M. 2022. Exogenous Melatonin protects lime plants from drought stress-ınduced damage by maintaining cell membrane structure, detoxifying ROS and regulating antioxidant systems. *Horticulturae*, 8: 257.

Jalali, M., Ghaffarian Mogharab, M.H., Nazary, H. and Zare, A.A. 2020. Uptake and nitrate accumulation affected by the partial replacement of nitrate-N with different sources of amino acids in spinach and lettuce. *Journal of Plant Physiology*, 9: 37-45.

Jiao, Y., Chen, Y., Ma, C., Qin, J., Nguyen, T.H.N., Liu, D., Gan, H., Ding, S. and Luo, Z.B. 2018. Phenylalanine as a nitrogen source induces root growth and nitrogen-use efficiency in *Populus canescens*. *Tree Physiol*, 38:66-82.

Kaya, C., Aydemir, S., Sonmez, O., Ashraf, M. and Dikilitas, M. 2013. Regulation of growth and some key physiological processes in salt-stressed maise (*Zea mays* L.) plants by exogenous application of asparagine and glycerol. *Acta Botanica Croatica*, 72(1):57-168.

Kaya, C. and Shabala, S. 2023. Melatonin improves drought stress tolerance of pepper (*Capsicum annuum*) plants via upregulating nitrogen metabolism. *Functional Plant Biology,* 51(1).

Kaya, G. 2024. Effects of drought stress on germination and seedling growth of seed primed with boron in spinach. *Journal of Agricultural Production*, 5(3), 201-207.

Khan, S., Yu, H., Li, Q., Gao, Y., Noman Sallam, B., Wang, H., Liu, P. and Jiang, W. 2019. Exogenous application of amino acids improves the growth and yield of lettuce by enhancing photosynthetic assimilation and nutrient availability. *Agronomy*, 9: 266.

Kukric, Z.Z., Topalic-Trivunovic, L.N., Kukavica, B.M., Matoš, S.B., Pavičic, S.S., Boroja, M.M. and Savic, A.V. 2012. Characterisation of antioxidant and microbial activities of nettle leaves (*Urtica dioica L.*). *Acta periodica Technologia*, 43:257-272.

Kumar, V.B.A., Mohan, T.C.K. and Murugan, K. 2008. Purification and kinetic characterisation of polyphenol oxidase from Barbados cherry (*Malpighia glabra* L.). *Food Chemistry*, 110: 328-333.

Kumaran, A. and Karunakaran, R.J. 2006. Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*. *Food Chemistry*, 97: 109-114.

Lutts, S., Kinet, J.M. and Bouharmont, J. 1996. Effects of various salts and mannitol on ion and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) callus cultures. *Journal of Plant Physiology*,149 (1-2): 86.

Mir, A.R., Siddiqui, H., Alam, P. and Hayat, S. 2020. Melatonin modulates photosynthesis, redox status, and elemental composition to promote the growth of *Brassica juncea*-a dose-dependent effect. *Protoplasma*, 257(6):1685-700.

Naz, H., Akram, N.A. and Kong, H. 2020. Assessment of secondary metabolism involvement in water stress tolerance of Quinoa (*Chenopodium quinoa* WILLD.) subjected to varying water regimes. *Pakistan Journal of Botany*, 52:1553-1559.

Ors, A. and Suarez, D.L. 2017. Spinach biomass yield and physiological response to interactive salinity and water stress. *Agricultural Water Management*, 190: 31-41.

Qu, C., Hao, B., Xu, X., Wang, Y., Yang, C., Xu, Z. and Liu, G. 2019. Functional research on three presumed asparagine synthetase family members in Poplar. *Genes*, 10(5): 326.

Ramzan, T., Shahbaz, M., Maqsood, M.F., Zulfiqar, U., Saman, R.U., Lili, N., Irshad, M., Maqsood, S., Haider, A., Shahzad, B., Gaafar, A.Z. and Haider, F.U. 2023. Phenylalanine supply alleviates the drought stress in mustard (*Brassica campestris*) by modulating plant growth, photosynthesis, and antioxidant defence system. *Plant Physiology* and *Biochemistry, 2*01:107828.

Sadak, M.S., Abdalla, A.M. and Abd Elhamid, E.M. 2020. Role of melatonin in improving growth, yield quantity and quality of *Moringa oleifera* L. plant under drought stress. *Bulletin of the National Research Centre*, 44: 1-13, 18.

Soares, E., Shumbe, L., Dauchot, N., Notte, C., Prouin, C., Maudoux, O. and Vanderschuren, H. 2020. Asparagine accumulation in chicory storage roots is controlled by translocation and feedback regulation of biosynthesis in leaves. *BioRxiv*, 228(3): 922-931.

Turfan, N. 2017. Effect of some abiotic stress factories on savrun spinach (*Spinacea oleracea* L.). *Turkish Journal of Agriculture - Food Science and Technology,* (6): 660-667.

Turfan, N. 2023a. The effects of exogenous melatonın applıcatıon on growth rate parameters and bıoactıve compounds of some spınach cultivars (*Spinach oleracea* L.) grown under wınter condıtıons. *Applied Ecology and Environmental Research*, 21(2):1533-1547.

Turfan, N. 2023b. The effects of exogenous tyrosine supplement on spinach (*Spinacia oleracea* L.) cultivation under lithium stress. *Medıterranean Agrıcultural Scıences*, 36(3): 101-108.

Turfan, N. 2025. Revealing the impact of L-cysteine and L-phenylalanine supplements on the yield, nutritional quality and storage of garlic. *Journal of Plant Nutrition*, 48:10, 1742-1763.

Turfan, N., Kibar, B., Davletova, N. and Kibar, H. 2024. Ameliorative effects of humic acid and L-tryptophan on enzyme activity, mineral content, biochemical properties, and plant growth of spinach cultivated in saline conditions. *Food Science & Nutrition, 12:* 8324-8339. *Food Science & Nutrition*, 12*:* 8324-8339.

Velikova, V., Yordanov, I. and Edreva, A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science*, 151 (1): 59-66.

Wen, Z.H., Li, H.G., Shen, J.B. and Rengel, Z. 2017. Maize responds to low shoot P concentration by altering root morphology rather than increasing root exudation. *Plant Soil*, 416:377-389.

Yaman Türkkan, Ö. and Kibar, B. 2022. Effects of different organic fertilizers on plant growth, yield, quality properties and element contents in spinach. *International Journal of Agriculture and Wildlife Science,* 8(2): 208-222.

Yang, X., Cui, X., Zhao, L., Guo, D., Feng, L., Wei, S., Zhao, C. and Huang, D. 2020. Exogenous glycine nitrogen enhances accumulation of glycosylated flavonoids and antioxidant activity in lettuce (*Lactuca sativa* L.). *Frontiers in Plant Science*, 8:2098.

Zahoor, R., Zhao, W., Abid, M., Dong, H. and Zhou, Z. 2017. Potassium application regulates nitrogen metabolism and osmotic adjustment in cotton (*Gossypium hirsutum* L.) functional leaf under drought stress. *Journal of Plant Physiology*, 215:30-*38.*

Zhang, L.L., Han, S.C., Li, Z.G., Liu, N. and Li, L.Y. 2006. Effects of the infestation by Actinote thalia pyrrha (Fabricius) on the physiological indexes of *Mikania micrantha* leaves. *Acta Ecologica Sinica*, 26 (5): 1330-1336.

Zhang, N., Sun, Q., Li, H., Li, X., Cao, Y. and Zhang, H. 2016. Melatonin improved anthocyanin accumulation by regulating gene expressions and resulted in high reactive oxygen species scavenging capacity in cabbage. *Frontiers in Plant Sciences,* 7:197.

Zhang, N., Zhao, B., Zhang, H.J., Weeda, S., Yang, C., Yang, Z.C., Ren, S. and Guo, Y.D. 2013. Melatonin promotes water-stress tolerance, lateral root formation, and seed germination in cucumber (*Cucumis sativus* L.). *Journal of Pineal Research*, 54(1):15-23.

Zhou, Z., Zhou, J., Li, R., Wang, H. and Wang, J. 2007. Effect of exogenous amino acids on Cu uptake and translocation in maie seedlings. *Plant and Soil*, 292: 105-117.