# THE EFFECTS OF EXOGENOUS MELATONIN APPLICATION ON GROWTH RATE PARAMETERS AND BIOACTIVE COMPOUNDS OF SOME SPINACH CULTIVARS (*SPINACIA OLERACEA* L.) GROWN UNDER WINTER CONDITIONS

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**Abstract.** The present study was carried out in order to reveal the effects of exogen melatonin (50MT: 50 µM - 100 MT: 100 µM) treatment on the growth rate characteristic and bioactive compound content variations of three spinach cultivars (Acosta-ACO; Anlani-ANL, and Matador-MTR, respectively) grown under cold conditions. While MTR was the richest variety in terms of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content, ANL was the richest variety in terms of Chl a to Chl b ratio, and xanthophyll content. Ascorbic acid and protein concentration were stimulated by MT in all three variants, but ANL had the highest value. While the proline content of cultivars decreased with only 50 MT in ACO when compared to the control, GB content decreased in MT doses. RWC increased with MT in ANL, whereas it caused to a decrease in RWC of 100 MT in ACO and 50 MT in MTR. MDA concentration was at a lower level and decreased only in ACO with 50 MT when compared to the control, while the $H_2O_2$ concentration was higher at MT doses in all three species. MT treatment stimulated SOD activity in ACO and ANL and increased POD activity in all three variants. Considering the growth characteristics such as root/stem length, leaf characteristics, and biomass, exogenous MT doses had positive effects on all three cultivars. These findings suggest that foliar MT treatment can attenuate cold damage by enhancing the enzymatic and non-enzymatic antioxidant capacity of spinach seedlings, as well as the growth rate.
**Keywords:** *bioactve compounds, growth, melatonin, spinach*

## Introduction

Plants frequently suffer critical conditions such as drought, salinity, and extreme temperatures in their environment. Low temperature, or cold stress, is one of the abiotic stress factors suppressing the growth and development, productivity, survival, and environmental spreading of plants (Pırınc and Alas, 2021). From a general perspective, it can be divided mainly into two groups: the chill stress occurring at temperatures between 0 and 15 °C without forming any ice crystal in plant cells and the freezing stress occurring below 0 °C and resulting in ice formation within plant parts (Banerjee et al., 2017). In many countries, leafy vegetables such as lettuce, spinach, parsley, and cabbage are subjected to low temperatures below 10-13 °C during the early development stage of shoots and leaves and significant yield losses occur (Turk and Erdal, 2015; Bałabusta et al., 2016) because early development phases of these vegetables' underground and aboveground organs are sensitive to water deficiency arising from the ice crystals due to low temperatures in cells and soil solution. Many studies showed that stress might cause several adverse effects on cell division and expansion, the growth rate of shoots and roots, leaf expansion, photosynthesis, water transport, allocation of nutrients, transpiration, respiration, and crop yield (Arnao and Hernández-Ruiz, 2017). Moreover, cold stress can result in an imbalance between light absorption and light quality, which are necessary for the productivity of the Calvin-


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Benson cycle (Ding et al., 2017). Besides that, it can also alter the physical and chemical structure of cellular membranes by changing their fluidity, which leads to excessive toxic compound accumulation, especially reactive oxygen species (ROS). The reactions, in which ROS accumulation increases the most in plant tissues, take place in the electron transport chain in the thylakoid and mitochondrial membranes (Jahns and Holzwarth, 2012; Barand et al., 2020). The ultimate result of ROS accumulation is often oxidative stress causing cell death, as well as organ senescence (Bałabusta et al., 2016; Kaya and Doganlar, 2019). However, to reduce the consequences of oxidative stress, or to improve its damage, plants have enzymatic and non-enzymatic defensive systems. While the enzymatic system consists of catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), the non-enzymatic system includes pigment molecules, secondary metabolites, osmolytes (Zhang et al., 2019; Faisal et al., 2022). Exogenous melatonin treatments were reported to promote the synthesis of photosynthetic pigments and, consequently, improve the fresh/dry weight, growth shoot, root, leaf expansion, and yield and quality (Turk and Erdal, 2015; Liang et al., 2018). Due to the similarity of the chemical structure of melatonin to that of auxin, it was emphasized that it plays a role in reactions where auxin is effective, leading to a rise in the growth rate. On the other hand, it can efficiently contribute to the protection of DNA and cell membrane damage, inhibit peroxide reactions by scraping ROSs, decrease lipid peroxidation, and improve oxidation resistance (Barand et al., 2020; Küçükyumuk, 2021). Therefore, the exogenous application of MT started to be used in improving the plant defense mechanisms under abiotic stress conditions for different agricultural cultivars (Wittayathanarattana et al., 2022). Spinach is one of the vegetables that are rich in minerals, polyphenols, amino acids, vitamins, carotenes and chlorophylls, enzymes, fiber, and water content but low in carbohydrates and fats. It is consumed both fresh and in industrially processed forms (Steingrover et al., 1986; Pırınc and Alas, 2021). Given the data of TUIK (2021), the spinach cultivation area in Turkey equals approximately 160 thousand decares, whereas the amount in the world was reported to be 949,820 ha. Considering the global spinach production, China ranks first with 85%, followed by the United States with 384,669 t, Japan with 226,382 t, and Turkey with 225,174 t (TUIK, 2021). Major factors playing a role in the decrease of spinach production are drought, salinity, and cold stress. The main objectives of the study are to determine (1) the effects of melatonin application on growth parameters, (2) the effects on chemical content in leaves, and (3) the dose of melatonin that can be used in spinach cultivation in Acosta, Anlani, and Matador varieties under cold conditions.

## Materials and methods

### *Description of spinach cultivars*

Acosta F1 (ACO), Anlani F1 (ANL), and Matador (MTR) spinach cultivars were used as study materials in the present study. Climatic characteristics of Kastamonu province (*Table 2*) were used in the selection of cultivars and the cultivars were selected among sinter cultivars or those resistant to cold climate. Moreover, importance was paid to characteristics such as suitability to greenhouse growing and open-area growing, growth rate, resistance to rupture, adaptation to stress conditions, and marketability. All three cultivars are adaptable to winter conditions and have strong structure and high leaf numbers (infoveg.turkey@syngenta.com; Seven, 2017; Turfan, 2017).


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### *Experimental design*

The present study was carried out between 11.11.2021 and 03.27.2022 in a covered study area with three open sides. In the study, balcony-type pots (60 × 18 × 16 cm) were used for the cultivation of plants; 10-12 L (2:1 peat-sand) soil mixture (*Table 1*) was filled into the pots and the seeds were sown in two rows. This study was designed with a randomized plot design with 3 replications. The application setup for each species was set as (1) control (C:0), (2) 50 MT (50 µM melatonin), and (3) 100 MT (100 µM melatonin). The MT doses applied in the study were selected by considering the germination capacity of the seeds, the fresh-dry weight and plant height parameters of the seedlings, and the results of the preliminary study, which determined the MT concentrations that caused an average 50% increase (Zhanget al., 2019; Ahmad et al., 2020). Seeds were irrigated three times a week until germination and seedling stage with 4-5 leaves. After the stage of 4-5 leaves, melatonin (MT: N-Acetyl-5-methoxytyptamine, CAS number: 73-31-4) was applied using foliar spray twice a week for 6 weeks. The control group plants were sprayed with pure water only. After the applications were terminated, the plants were removed from the soil without causing any damage to their roots, and the soil particles were cleaned from the seedlings by using pure water. Dehumidified plants on a blotter were used in measuring the root length, stem length, leaf length (a whole leaf), leaf blade length, leaf blade width, number of leaves per plant, and fresh weight of a whole plant, as well as the dry measurements.

The characteristics of the soil used in the experiment are presented in *Table 1.* The soil pH was 6.45, and conductivity 0.60. The nutrient contents in the soil sample were found to be (in mg $kg^{-1}$) 26850 for Mg, 3522 for P, 24030 for S, and 14880 for K.

***Table 1.*** *Some physical and chemical properties of experimental soil*

| | pH | N | P | K | Mg | Ca | S | Mn | Fe | Ni | Zn | Cu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | mg $kg^{-1}$ | | | | | | | | | |
| **Soil** | 6.44 | 0.65 | 3522 | 14880 | 26850 | 97850 | 24030 | 990.0 | 46130 | 142 | 145 | 58.2 |

The average climate values recorded during the study are shown in *Table 2*. It was determined that the monthly mean relative humidity in the district was approximately 58-90%. As seen in *Table 2,* while the mean temperature ranged between -1 and 20 °C during the study period; the lowest temperature ranged from -14-3 °C, and the highest temperature from 6-20 °C. The average level of precipitation ranged between 31.5 and 44.6 mm, and humidity between 58-90% (*Table 2*).

***Table 2.*** *Climatic data of the experimental area for 2021-2022*

| Months | Temperature (°C) | | | Mean precipitation (mm) | Mean relative humidity (%) |
|---|---|---|---|---|---|
| | Mean | Minimum | Maximum | | |
| November | 6 | 3 | 20 | 31.5 | 68 |
| December | 9 | -2 | 14 | 44.6 | 90 |
| January | -1 | -14 | 6 | 40.5 | 75 |
| February | 0 | -11 | 8 | 35.3 | 68 |
| March | 4 | -1 | 13 | 39.3 | 58 |


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### *Morphological measurements*

Seedling height, root length, leaf length and width were measured using a ruler. The fresh weights of a whole seedlings were measured using a precision scale, and the numbers of leaves were determined by counting the leaves. For the leaf surface area measurement of spinach varieties, the leaves were separated one by one and then placed on a printer surface and the measurements were made with the help of LeafArea 2.0.5.0, 2016 program.

### *Physiological and biochemical analysis*

Relative water content (RWC as %) of spinach cultivars was determined following the method described by Kaya et al. (2003). The chlorophylls, carotenoids, and xanthophyll were homogenized using ethanol and the estimations were made using the methods described by Kukric et al. (2012) and Chang et al. (2013). The proline content of leaves was determined using Bates' method (1973) and GB content was determined using Grieve and Grattan's method (1983), whereas the protein content was calculated using Bradford's method (1976) and total nitrate level was determined following the method by Cataldo et al. (2008). Malondialdehyde content (MDA) was estimated following the original method by Lutts et al. (1996). Hydrogen peroxide content ($H_2O_2$) was measured spectrophotometrically by using the method introduced by Velikova et al. (2000).

### *Enzymatic antioxidant activity*

The superoxide dismutase (SOD) activity was determined as its capacity to inhibit the photochemical reduction of nitro-tetrazolium blue chloride (NBT) at 560 nm (Çakmak, 1994). The catalase (CAT) activity was determined by monitoring the degradation of $H_2O_2$ at 240 nm in 2 min against a supernatant-free blank (Bergmeyer, 1970). The peroxidase (POD) activity was determined by following the method described by Lee and Lin (1995).

### *Statistical analysis*

Analysis of variance (ANOVA) was applied for analyzing the differences in the chemical composition of the leaf and growth parameters of spinach cultivars using the SPSS program (Version 11 for Windows). Following the results of ANOVAs, Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$ was used for significance.

## Results

### *Change in chlorophylls, carotenoids, and xanthophyll content*

The amount of Chl a ranged from 0.366 to 0.438 mg, Chl b from 0.129 to 0.468 mg, the total chlorophyll from 0.495 to 0.871 mg, and the total carotenoids from 10.79 to 14.19 mg in cultivars, respectively (*Table 3; Fig. 1a*). The ratio of chlorophyll a to chlorophyll b varied between 0.86 and 2.84 (*Fig. 1a*). The xanthophyll concentration ranged from 1.99 mg to 3.78 mg and generally increased compared to the control. However, doses of 50 MT in ACO and 100 MT in MTR reduced the xanthophyll content (*Fig. 1b*).


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***Table 3.*** *Changing of chlorophyll pigment of spinach cultivars (chlorophyll a, chlorophyll b, total chlorophyll, ratio of chlorophyll a to chlorophyll b)*

| | | Chlorophyll a mg $g^{-1}$ | Chlorophyll b mg $g^{-1}$ | Total chlorophyll mg $g^{-1}$ | Ratio of Chl a to Chl b |
|---|---|---|---|---|---|
| Acosta | Control | 0.411 ± 0.001c | 0.385 ± 0.001b | 0.796 ± 0.002c | 1.07 ± 0.001f |
| | 50 MT | 0.403 ± 0.001d | 0.468 ± 0.001a | 0.871 ± 0.002a | 0.86 ± 0.003g |
| | 100 MT | 0.416 ± 0.001c | 0.323 ± 0.001e | 0.739 ± 0.001d | 1.29 ± 0.005d |
| Anlani | Control | 0.438 ± 0.001a | 0.396 ± 0.001b | 0.834 ± 0.001b | 1.11 ± 0.002e |
| | 50 MT | 0.426 ± 0.001b | 0.342 ± 0.001d | 0.768 ± 0.001cd | 1.25 ± 0.006d |
| | 100 MT | 0.425 ± 0.001b | 0.223 ± 0.001f | 0.648 ± 0.001e | 1.91 ± 0.002c |
| Matador | Control | 0.366 ± 0.001e | 0.129 ± 0.001h | 0.495 ± 0.001g | 2.85 ± 0.010 |
| | 50 MT | 0.389 ± 0.001e | 0.147 ± 0.001h | 0.536 ± 0.001f | 2.65 ± 0.010b |
| | 100 MT | 0.406 ± 0.001d | 0.162 ± 0.001g | 0.569 ± 0.001f | 2.50 ± 0.011b |
| F. | | 1469.57 | 31747.31 | 25298.54 | 14037.38 |
| Sig. | | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

*Means indicated with different letters within same column are significantly different (P < 0.05); 50 MT:50 µM Melatonin, 100 MT: 100 µM Melatonin

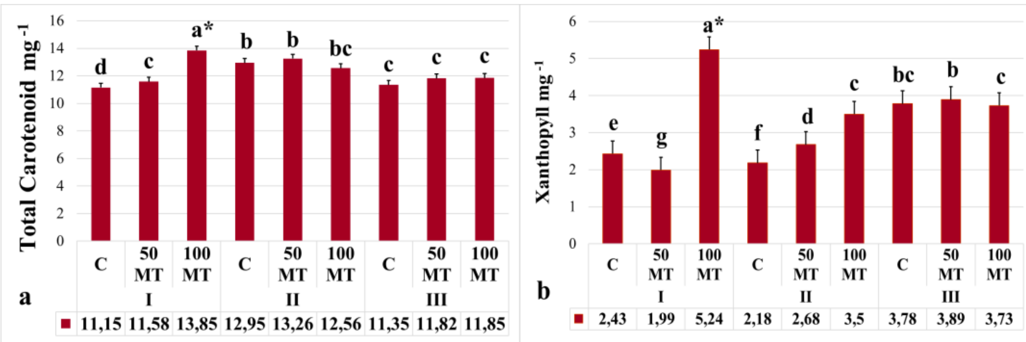


***Figure 1.*** *Change in total carotenoid and xanthophyll of spinach cultivars. C: Control, I: Acosta, II: Anlani, III; Matador; 50 MT: 50 µM Melatonin, 100 MT: 100 µM Melatonin*

### *Change in nitrogenous compounds*

Total nitrate amount in spinach cultivars varied between 17.45 mg and 50.77 mg, and MT applications increased nitrate levels in cultivars compared to control (*Table 4*). The amount of proline, glycine betaine, and soluble proteins, which are important osmolytes, is generally higher in all cultivars at MT doses than those of control groups (*Table 4*). However, MT applications caused a decrease in the amount of glycine betaine content in MTR (*Table 4*).

### *Change in ascorbic acid content and RWC level*

The level of ascorbic acid was higher in all cultivars treated groups than in control plants. Compared to the control, the highest values were obtained with 100 MT (24.31%) in ACO and 50 MT (21.28%) in AL, respectively (*Fig. 2a*). The RWC level showed a Partial increase in both MT doses in the MTR variety, while it showed a slight decrease in ACO by 100 MT and ANL by 50 MT (*Fig. 2b*).


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***Table 4.*** *Changing of nitrogenous compounds of spinach cultivars (total nitrate, proline, glycine betaine, total soluble protein)*

| | | Total nitrate mg $g^{-1}$ | Proline µmol $g^{-1}$ | Glycine betaine µg $g^{-1}$ | Total soluble protein mg $g^{-1}$ |
|---|---|---|---|---|---|
| Acosta | Control | 42.33 ± 0.07c | 72.52 ± 0.04c | 166.70 ± 0.15b | 19.59 ± 0.01b |
| | 50 MT | 47.80 ± 0.11b | 69.91 ± 0.04d | 168.80 ± 0.13b | 22.79 ± 0.01a |
| | 100 MT | 50.77 ± 0.11a | 80.27 ± 0.08b | 172.49 ± 0.15a | 20.30 ± 0.02b1 |
| Anlani | Control | 45.74 ± 0.08c | 63.78 ± 0.05e | 153.13 ± 0.08d | 11.23 ± 0.01d |
| | 50 MT | 37.62 ± 0.13d | 87.33 ± 0.08a | 163.06 ± 0.68c | 18.44 ± 0.01bc |
| | 100 MT | 39.76 ± 0.12d | 74.78 ± 0.10c | 164.38 ± 0.17bc | 17.48 ± 0.02c |
| Matador | Control | 17.45 ± 0.08f | 62.32 ± 0.08e | 160.59 ± 0.06c | 17.88 ± 0.02c |
| | 50 MT | 35.75 ± 0.01d | 75.16 ± 0.08c | 148.95 ± 0.12e | 20.37 ± 0.02b |
| | 100 MT | 21.57 ± 0.05e | 81.20 ± 0.20b | 160.01 ± 0.06 | 19.25 ± 0.02b |
| F. | | 16001.28 | 7915.358 | 851.715 | 18459.26 |
| Sig. | | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

*Means indicated with different letters within same column are significantly different ($P < 0.05$); 50 MT:50 µM Melatonin, 100 MT: 100 µM Melatonin

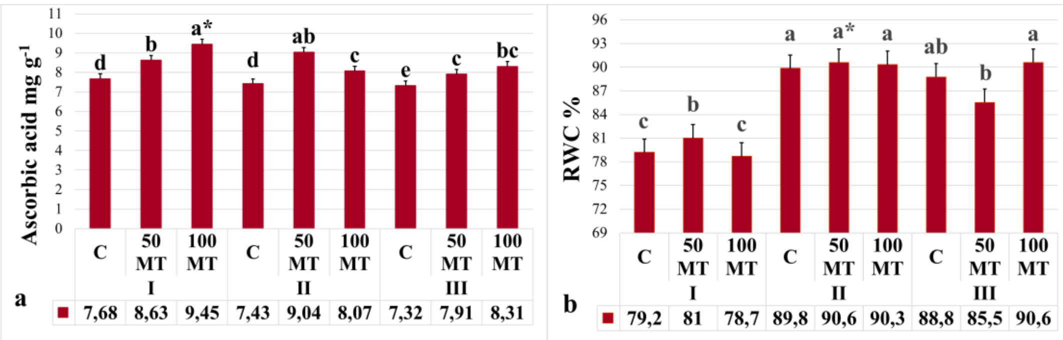


***Figure 2.*** *Changing of Ascorbic acid (a) and RWC (b) level of spinach cultivars. C: Control; I: Acosta; II Anlani; III: Matador; 50 MT:50 µM Melatonin, 100 MT: 100 µM Melatonin. *Means indicated with different letters within same column are significantly different ($P < 0.05$)*

### *Change of MDA and $H_2O_2$ content*

MDA content represents the degree of lipid peroxidation in cell membranes. Therefore, the higher the MDA content, means the cell damage is more serious. While 50 MT caused an increase in MDA content in ACO and ANL, both doses caused a rising in MDA content in MTR (*Table 5*). On the other hand, MT doses led to an increase in $H_2O_2$ concentration in all three cultivars compared to control (*Table 5*).

### *Change in antioxidant enzyme activities*

We compared the response of spinach cultivars MT application in terms of the activity of SOD and POD activities. SOD activity showed a significant increase in ACO and ANL, but it declined in MTR at MT doses (*Table 5*). POD activity in AO (5.13 EU) cultivar was found to be higher in all groups compared to the control group and reached the highest level at 50 MT (*Table 5*).


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***Table 5.*** *Changing of MDA and $H_2O_2$ concentration and POD and SOD activities of spinach cultivars*

| | | **MDA µmol g$^{-1}$** | **$H_2O_2$ nmol g$^{-1}$** | **POD EU Protein mg$^{-1}$** | **SOD EU Protein mg$^{-1}$** |
|---|---|---|---|---|---|
| Acosta | Control | 53.42 ± 0.21a | 174.39 ± 0.10d | 3.37 ± 0.02d | 17.05 ± 0.04e |
| | 50 MT | 84.15 ± 0.78a | 201.49 ± 0.10a | 5.13 ± 0.01a | 19.01 ± 0.10d |
| | 100 MT | 15.47 ± 0.16f | 182.23 ± 0.10c | 3.81 ± 0.01c | 32.44 ± 0.07b |
| Anlani | Control | 31.33 ± 0.16d | 153.47 ± 0.10g | 4.86 ± 0.01b | 24.57 ± 0.06c |
| | 50 MT | 37.73 ± 0.26c | 163.61 ± 0.06e | 4.96 ± 0.01a | 31.61 ± 0.07b |
| | 100 MT | 31.64 ± 0.20d | 156.52 ± 0.13f | 5.05 ± 0.01a | 37.40 ± 0.06a |
| Matador | Control | 27.74 ± 0.16e | 157.53 ± 0.17f | 3.21 ± 0.01e | 22.84 ± 0.03c |
| | 50 MT | 31.42 ± 0.10d | 182.67 ± 0.13b | 5.10 ± 0.02a | 21.87 ± 0.06c |
| | 100 MT | 33.52 ± 0.16d | 171.53 ± 0.12d | 5.10 ± 0.03a | 17.97 ± 0.07e |
| F. | | 466.312 | 18459.26 | 3027.161 | 12501.72 |
| Sig. | | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

*Means indicated with different letters within same column are significantly different ($P < 0.05$); 50 MT:50 µM Melatonin, 100 MT: 100 µM Melatonin

### *Change in growth rate parameter*

In terms of growth characteristics, exogenous MT applications were found to stimulate growth parameters in ACO, ANL and MTR spinach varieties. As shown in *Table 6*, MT applications increased root, shoot and leaf length in all three cultivars compared to control. However, the increase in root length is statistically significant only in ANL variety. Moreover, MT doses have led to an improvement in the leaf properties and fresh weight of a whole plant. Leaf surface area in leaf characteristics reached the maximum level at 50 MT dose in all three cultivars. The fresh weight of a whole plant was found to be the highest in ANL and MTR in 100 MT and in ACO at 50 MT compared to control (*Table 6*).

***Table 6.*** *Variations of growth rate parameters of spinach cultivars*

| **Cultivars** | **Group** | **Root length** | **Shoot length** | **Full leaf** | **Lamina length** | **Lamina width** | **Leaf area** | **Leaf** | **Fresh weight of full plant** |
|---|---|---|---|---|---|---|---|---|---|
| | | **cm** | | **cm$^2$** | | **Number** | | **g** | |
| Acosta | C | 10.29±0.29a | 7.00±0.1c | 6.54±0.2c | 4.11±0.1b | 3.11±0.11b | 15.50±0.15c | 8.00±0.21 | 2.12±0.15d |
| | 50 MT | 11.72±1.02a | 8.22±0.60b | 7.82±0.4b | 4.79±0.2a | 3.61±0.11a | 23.99±0.22a | 8.93±0.34 | 3.17±0.28c |
| | 100 MT | 10.72±0.6a | 8.29±0.2ab | 8.07±0.4b | 4.93±0.2a | 3.57±0.11a | 18.34±0.20b | 9.15±0.41 | 3.41±0.23b |
| Anlani | C | 7.36±0.7b | 8.25±0.4b | 7.59±0.3b | 5.04±0.2 | 3.40±0.14a | 15.34±0.17c | 8.07±0.25 | 4.42±0.37b |
| | 50 MT | 8.79±0.4b | 9.61±0.3a | 9.18±0.3a | 5.820.3a | 3.68±0.17a | 21.92±0.06a | 9.00±0.38 | 5.91±0.55a |
| | 100 MT | 9.29±0.40a | 9.04±0.3a | 8.25±0.3a | 5.11±0.2a | 3.54±0.48a | 21.54±0.13a | 8.29±0.33 | 4.61±0.17a |
| Matador | C | 9.29±0.33a | 6.82±0.3c | 5.63±0.3 | 3.89±0.2b | 3.18±0.12b | 11.56±0.18d | 8.14±0.28 | 3.07±0.24c |
| | 50 MT | 10.15±0.7a | 8.06±0.2b | 7.64±0.3b | 4.93±0.2a | 4.22±0.19a | 15.34±0.16c | 8.86±0.36 | 6.19±0.43a |
| | 100 MT | 10.09±0.6a | 7.07±0.2b | 6.04±0.2c | 4.32±0.2b | 3.62±0.11a | 14.85±0.23c | 8.79±0.26 | 4.79±0.37a |
| F | | 4.453 | 9.396 | 16.059 | 10.736 | 2.503 | 677.562 | 2.003 | 16.756 |
| Sig. | | 0.000 | 0.000 | 0.000 | 0.000 | 0.015 | 0.000 | 0.052 | 0.000 |

*Means indicated with different letters within same column are significantly different ($P < 0.05$); C: Control; 50 MT:50 µM Melatonin, 100 MT: 100 µM Melatonin


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## Discussion

### *Effect of MT pretreatment on the bioactive compounds of spinach cultivars*

#### *Pigment content*

Cold stress can yield irreversible changes in the photosynthetic apparatus, especially in the integrity of the thylakoid membranes, resulting in the suppression of enzyme activations responsible for chlorophyll biosynthesis and synthesis and leading to a reduction in the amount of chlorophyll (Fan et al., 2015; Lianget al., 2018). On the other hand, high-tolerance genotypes might alleviate or eliminate cold effects through defense mechanisms (Kıkı, 2022; Burgos et al., 2013; Qari et al., 2022). In this study, MT doses did not affect the protection of chlorophyll pigments against cold stress in ANL cultivar but showed a positive effect on the protection of pigment content in MTR (*Table 3; Fig. 1a, b*). In addition, 50 MT of ACO resulted in an improvement in chlorophyll b and total chlorophyll, whereas 100 MT caused a reduction. Cold stress accelerated the degradation of chlorophyll, possibly by damaging the grana membranes, and caused a decrease in its amount in ANL cultivar (*Table 3*). High pigment content in MTR might be related to the fact that this variety is more resistant to low temperatures, as well as the fact that MT doses protect the chloroplast membrane integrity, prevent the pigment systems from being injured by cold stress, and stimulate chlorophyll biosynthesis (Turk and Erdal, 2015). The fact that the total amount of carotenoids in MTR is higher than in other varieties confirms this result (*Fig. 1a*). In addition, xanthophyll content in ANL increased at both MT doses, while 100 MT in ACO and 50 MT in MTR stimulated xanthophyll content (*Fig. 1b*). According to the xanthophyll results, the high chlorophyll a content at high doses of xanthophyll indicates that this pigment protects chlorophyll a molecule from cold damage (Jahns and Holzwarth, 2012; Kıkı, 2022). Although xanthophylls are mainly bound to the proteins of pigment systems, a small fraction of xanthophylls is likely to be free into the thylakoid lipids, where they catalyze ROS scavenging and reduce lipid peroxidation (Verhoeven et al., 1999; Burgos et al., 2013; Chen et al., 2018). In this study, the fact that MDA is high at concentrations with high xanthophyll content strengthens this situation (*Fig. 1b; Table 5*). It is well known that carotenoid pigments play a major role in protecting the chlorophyll from photooxidative destruction (Liang et al., 2018). Melatonin was also reported to effectively protect the chlorophyll for barley, cucumber, pepper lettuce, spinach, and tomato seedlings exposed to cold stress (Korkmaz et al., 2017; Liu et al., 2015; Zhang et al., 2017; Chang et al., 2021; Zhou et al., 2021). Similar effects of melatonin treatment under cold conditions were cited by Fan et al. (2015), who reported that exogen melatonin treatments remarkably enhanced the photosynthetic pigment levels and the cold tolerance in bermudagrass. Debnath et al. (2018), Liang et al. (2017), and Ahmad et al. (2020), also revealed that MT foliar spray increased photosynthetic pigments under stress conditions depending on genotypes, MT dose, and stress severity. All authors cited that MT might delay leaf senescence by inhibiting chlorophyll degradation and improve photosynthetic efficiency by reducing ROS accumulation under cold conditions.

#### *Nitrogenous compounds content*

Vegetables, especially dark-colored leafy ones, are richer in nitrate (Fan et al., 2017). Nitrate is the main source of nitrogenous compounds including amino acids, proteins, enzymes, phytohormones, chlorophyll molecules, and secondary metabolites and it


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controls various physiological processes (Pratelli and Pilot, 2014; Pırınc and Alas, 2021). In this study, it was shown that the total nitrate level reduced only in ANL with 50 MT (*Table 4*). It was also found to be at the highest level in MTR with 100 and 50 MT (*Table 4*). On the other hand, there was a slight decrease in proline content in 50 MT for ACO, but it was higher in ANL and MTR at MT doses when compared to the control group (*Table 4*). MT contributed to the improvement of glycine betaine content in the first two cultivars but led to a decrease in MTR when compared to the control group (*Table 4*). Total soluble protein accumulation was stimulated by the exogen MT doses in all three cultivars in comparison to the control samples (*Table 4*). Nitrate accumulation in vegetables is influenced remarkably by environmental factors such as light intensity, low temperature, and salinity (Steingrover et al., 1986; Madebo et al., 2021). The findings about nitrate were in parallel with the literature, which revealed that low light availability in winter conditions increased nitrate accumulation. Santamaria et al. (2001) examining rocket and Seginer (2003) examining lettuce found an interaction between light intensity, nitrogen availability, and temperature on nitrate accumulation. Steingrover et al. (1986) showed that increases occurred in biomass and size in leaves as a result of increased nitrate and water intake and accelerated starch degradation during the nights in winter season. The increase in proline, glycine betaine, and protein levels in cold weather might be related to nitrate accumulation, the inhibition of denaturation of enzymes in amino acids, and the induction of protein accumulations with MT doses, which might have led to increased tolerance to cold stress injuries (Qari et al., 2022). Moreover, exogenous MT treatment might be useful in supplying plants with a metabolite (NO3) that is induced by/dependent upon low temperature and can be easily converted to amino acids. Proline, glycine betaine, and soluble protein are generally defined as osmoprotectants thanks to their osmotic adjustment properties under stress conditions (Yancey, 2005; Faısal et al., 2022). Apart from these, all of them, especially glycine betaine, may have shown a protective role in reducing intracellular ice crystals of spinach varieties (Banerjee et al., 2017). Similar effects of MT applications were reported by Debnath et al. (2018), Qiao et al. (2019), Madebo et al. (2021), that who showed melatonin treatments significantly increased amino acids and proteins levels in the tomato, cucumber, and wheat cultivars when compared to those of the control. Also, Fan et al. (2015), and Debnath et al. (2018) found that exogen MT induced proline, glycine betaine, and soluble protein accumulation in crops and, thus, delayed the leaf senescence.

*Ascorbic acid and RWC levels*

Ascorbic acid (vitamin C), which is a water-soluble molecule, is abundant in all cell compartments, including the cell wall. It plays an effective role in rapid cell expansion, detoxifying ROS, and strengthening stress tolerance (Foyer et al., 2020; Smirnoff, 2018). In this study, MT doses yielded an increase in ascorbic acid content in all three cultivars, especially in 50 MT for ACO and 100 MT for ANL, which remarkably increased the ascorbic acid content (*Fig. 2a*). The formation of ice crystals in apoplastic areas at low temperatures leads to significant changes in the physical and chemical structures of cellular membranes. This causes a reduction in the apoplastic water potential, as well as the stimulation of peroxidation chains in the membranes and the accumulation of toxic compounds such as MDA, aldehydes, ketones, hydrogen peroxide, superoxide, and singlet oxygen (Chen et al., 2018). In this study, there was no significant change in the proportional water content of the cultivars (RWC) when


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compared to the control, but it caused a decrease in 100 MT dose for ACO and 50 MT dose for MTR (*Fig. 2b*). The amount of water in the leaves reflects the plants' tolerance to low temperatures. Cold stress significantly decreases the plant's relative water contents (RWC); however, exogen MT treatment increase RWC by osmolytes, and limits the negative impacts of cold conditions (Yancey, 2005; Turk and Erdal, 2014). Also, MT application protects the stable structure of the membranes and maintains the level of RWC of cells (Arndt et al., 2015; Pu et a., 2021). As a matter of fact, the high levels of molecules such as proline, glycine betaine, and protein in this study confirm the literature (*Table 4*).

*MDA and $H_2O_2$ concentrations and antioxidant enzyme activities*

MT application increased the MDA concentration of varieties in general. However, ACO 100 MT application resulted in a decrease in MDA content (*Table 5*). MT application caused an increase in $H_2O_2$ concentration in all three cultivars (*Table 5*). In comparison to the control, the highest $H_2O_2$ was achieved with 50 MT in ACO and MTR (15.53-15.9%), respectively (*Table* 5). Similar to the present study, Turk and Erdal (2015), Kong et al. (2020) found that the amount of MDA increased at low temperatures and turgor/osmosis events were suppressed in cells. However, it was proven by many researchers that exogen chemical application might inhibit the formation of ice crystals in apoplastic sections, contributed to the maintenance of the stable structure of membrane lipids, and blocked the MDA and ROS accumulation in cells (Sun et al., 2018; Barand et al., 2020).

Many researchers reported that osmolytes and some minerals like K, Cl, Na, and enzymatic antioxidant compounds such as APX, CAT, SOD, and POD play an important role in preventing or ameliorating low temperature-induced oxidative injuries (Reiter et al., 2007; Zhang et al., 2019). In this study, MT doses stimulated SOD activity in ACO and ANL and raised POD activity in all three varieties (*Table 5*). SOD activity reached the highest level with 100 MT in ACO and ANL (90.17%-51.28%) (*Table 5*). POD activity, on the other hand, was at the highest level (56-58%) in MTR when compared to the control group at both doses (*Table 5*). These results suggest that foliar melatonin applications stimulated protection against low temperatures in spinach varieties and yielded an increase in growth and development (Fan et al., 2015, 2017; Kaya and Doganlar, 2019). Similar results were reported by a study concluding that exogenous melatonin application caused a decrease in MDA and $H_2O_2$ content and an increase in SOD and CAT activities in pepper seeds and stimulated the germination rate (Korkmaz et al., 2017). Moreover, Li et al. (2018), Sun et al. (2018), and Kong et al. (2020) reported that lipid peroxidation reactions in plants were inhibited by MT application and, additionally, CAT, APX, SOD, and POD enzyme activities were stimulated, and ROS synthesis was inhibited (Reiter et al., 2007; Zhang et al., 2019).

*Growth parameters of seedling*

Cold stress represses the proliferation of root, shoot, branching, and leaf due to a reduction in photosynthetic rate and nutrient and water uptake, as well as oxidative stress, and it causes a reduction in the final yield and quality of vegetables (Li et., 2018; Küçükyumuk, 2021; Baslak et al., 2021). As shown in *Table 6*, the length of the root, shoot, and whole leaf, leaf blade width and length, leaf surface area, the number of leaves per plant, and fresh weight of a whole plant increased in spinach cultivars by using MT


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applications (*Table 6*). Based on the growth parameters of spinach seedlings, it was observed that exogen melatonin treatment could offer protection against oxidative stress through enhancing pigments, nitrogenous compounds levels, and enzyme activities in comparison to the control groups (*Tables 3*, *4*, and *5*) (Liang et al., 2018; Madebo et al., 2021). Also, in terms of leaf characteristics, the larger leaf surface area and fresh weight of o whole seedlings with exogen MT may be associated with improved water contents under lower temperatures (Zhang et al., 2019; Ahmad et al., 2020). It was reported that the growth characteristics of plants were the most important parameters for cold stress. For instance, the roots contact the soil and absorb water from the soil, but these functions can be impacted by cold temperatures (Qiao et al., 2019). Similarly, it was proven that the length, width, and fresh weight of shoots and leaves were limited due to repressed cell division, activities of phloem and xylem, and photosynthetic rate under cold conditions, resulting in yield loss (Fan et al., 2015; Arnao and Hernández-Ruiz, 2017). Liang et al. (2018), Li et al. (2018), and Barand et al. (2020) found similar results; the length of shoot-root, their weight, leaf development, and biomass decreased under cold conditions, however, exogenous MT influenced the effects of low-temperature stress directly and indirectly and increased the productivity in vegetative organs. On the other hand, these results might be related to the synthesis of indole-3-acetic acid within the apical meristem of seedlings with MT doses (Du et al., 2020).

## Conclusion

While MTR was the richest variety in terms of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content, ANL was the richest variety in terms of Chl a to Chl b ratio and xanthophyll content. Ascorbic acid and protein concentration were stimulated by MT in all three variants, but ANL had the highest value. While the proline content of cultivars decreased with only 50 MT in ACO in comparison to the control, GB content decreased in MTR in MT doses. RWC increased with MT in ANL, whereas it led to a decrease in RWC of 100 MT in ACO and 50 MT in MTR. MDA concentration was lower and decreased only in ACO with 50 MT when compared to the control, while the $H_2O_2$ concentration was higher at MT doses in all three species. MT treatment stimulated SOD activity in ACO and ANL and increased POD activity in all three variants. From the aspect of growth characteristics such as root/stem length, leaf characteristics, and biomass, exogenous MT doses showed positive effects in all three cultivars. When all analysis results are evaluated together it can be said that melatonin application can contribute to rising spinach production under winter climates by increasing tolerance to cold conditions

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