



Article

Exogenous Application of Melatonin Alleviates Drought Stress in *Ranunculus asiaticus* by Improving Its Morphophysiological and Biochemical Attributes

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Abstract: Melatonin (MT) controls various physiological functions and enhances plant drought tolerance. This study aimed to evaluate the effect of exogenous MT on the morpho-physiological and biochemical attributes of *Ranunculus asiaticus* under normal and drought conditions. A completely randomized design was used with two factors, drought stress and MT concentration (0, 50, 100, and 200 μ M), to assess the effect of foliar application of MT on *R. asiaticus* seedlings. The experiment was conducted with a total of two groups: the drought stress group and the control group. The foliar application of MT was carried out four times during the study period. The drought stress group exhibited considerably decreased shoot length by 26.0%, leaf number by 31.03%, leaf area by 62.2%, fresh and dry vegetative weights by 34.5% and 52.9%, respectively, total chlorophyll and carotenoid contents by 68.29% and 51.72%, respectively, and relative water content by 7.1%; early emergence of flower stalks was observed within 29 days; increased relative electrolyte leakage by 20.5% compared to well-watered plants. Conversely, the foliar application of MT notably increased growth parameters compared with their no-treatment counterparts. Foliar treatment with 200 μ M MT resulted in the most significant growth response in *R. asiaticus* under normal and drought stress conditions. Moreover, under stressful conditions compared with no treatment, exogenously applied MT induced the appearance of flower buds 21 days early and increased relative water content by 6.4%, proline contents by 32%, and peroxidase activity by 58% while reducing electrolyte leakage by 14.3%. Regarding tolerance index percentages, higher peroxidase and proline contents indicated their suitability for use as markers for drought tolerance, supporting the effective role of exogenous MT in enhancing the adaptability of *Ranunculus* to drought stress.

Keywords: water deficit; pleiotropic molecule; growth; lipid peroxidation; osmolyte molecule; antioxidant enzyme



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1. Introduction

Ranunculus asiaticus L., commonly known as “butter cup,” is a perennial geophyte with tuberous roots. This species belongs to the *Ranunculaceae* family that is endemic to the Mediterranean basin and Asia Minor. Its flowers are dark red in color and terminal, and this annual crop is cultivated for use in the floral industry [1]. The seeds and tuberous roots of the *R. asiaticus* plant can be used to propagate new plants [2]. This plant has recently become more widely cultivated because of the emergence of numerous hybrids [3]. Floral industries compete with other water-intensive industries, including agriculture, urban management, and human consumption, because of water shortages, especially in arid and semiarid regions. Because of this, floral industries must use water efficiently in order to conserve resources [4].

Water-stressed regions often attribute declining flower production to drought, one of the most widely spread adverse environmental factors [5–7]. The most effective water-management strategies include the study of drought tolerance in plants to select more-suitable plants and determine their water requirements. Water stress arises when physical and environmental factors lead to water scarcity for plant growth, which subsequently inhibits plant development and flower output by affecting the chlorophyll and water content [8]. Research on plant responses to drought-induced stress reveals that they are dependent on morphophysiological parameters, such as photosynthesis, water status, and biochemical changes [9,10]. The mechanisms underlying the drought stress responses of plants are extraordinarily complex and vary considerably with plant species, development stage, and duration of water shortage [11]. Severe drought-induced stress significantly impacts the physiological and biochemical functions of plants. The most deleterious effects of drought stress include reduction in the relative water content and water potential of leaves, loss of turgor, cell size reduction, decrease in photosynthetic pigments, and disturbance of different metabolic processes [12]. Moreover, responses can include stunted growth and partial or total plant death [13]. A severe lack of water can lead to an imbalance in the redox constituents of cells, i.e., the accumulation of reactive oxygen species (ROS) overwhelms the antioxidant defenses. This in turn leads to a cascade of oxidative damage that eventually impairs plant growth and development [14] and reduces plants' fresh and dry weight [15]. ROS production is considered harmful to cells as it can result in electron leakage, lipid peroxidation, membrane deterioration, and nucleic acid and protein damage [16]. Furthermore, it is suggested that active oxygen species, antioxidative enzymatic reactions, and the accumulation of osmolytes such as proline are appropriate markers of plants to drought stress [10,17,18]. Commonly, plant hormones regulate plant growth and enhance resistance [5]; hence, identifying the possible growth promoters and their processes is critical for strengthening agricultural drought tolerance.

Melatonin (MT; N-acetyl-5-methoxytryptamine), a low-molecular weight indole-based molecule, is present in all organisms and is thought to serve as a hormone in certain plant species. MT plays a variety of physiological roles in plants, including in growth, photosynthesis, biological cycles, germination of seeds and roots, and osmoregulation [19]. MT also contributes to the maintenance of ion homeostasis [20]. Exogenous MT was demonstrated to be absorbed by plants and to exert positive effects under several biotic and abiotic stress factors [21–23]. MT has been extensively studied for its possible role as a plant growth promoter; its mode of action involves cooperation with other chorismate-derived phytohormones, such as indole-3-acetic acid (IAA) [24] and salicylic acid [25]. Because of its role in scavenging ROS and as a modulator of the production and activity of antioxidants (both enzymatically and nonenzymatically or by altering the expression of genes involved in many physiological mechanisms), MT may mitigate the detrimental consequences of oxidative damage to proteins, lipids, and nucleic acids [19,26]. Under drought conditions, MT improved the germination percentage of rapeseed seedlings and increased the leaf area as well as fresh and dry weights of roots and shoots [22]. In *Moringa oleifera* L., MT improved photosynthetic pigments (IAA), phenolic and element contents, and antioxidant enzyme systems, and it decreased malondialdehyde (MDA) [12]. In maize plants, MT increased the relative water content (RWC), leaf water content, and relative saturation deficit, and it enhanced antioxidant activities, such as peroxidase (POD) and proline accumulation [27]. In tomato plants, the application of MT resulted in a stronger root system, which decreased their susceptibility to water deficit stress. This was evidenced by reduced membrane injury, presumably due to the activation of antioxidant enzymes and their related genes, which initiated ROS scavenging [28]. The drought resistance of tea seedlings significantly improved with MT application, as evidenced by reduced membrane damage, increased proline, total protein, and sugar content, and increased CAT and POD activity [29].

Nonetheless, to the best of our knowledge, no study has evaluated the relative impact of MT in improving *R. asiaticus* tolerance against drought stress. The present study

examined the possible implications of foliar MT treatment at various concentrations in promoting drought tolerance in *R. asiaticus* under drought conditions based on alterations in morphological, physiological, and biochemical attributes. The main objective of the study under the two levels of irrigation was to evaluate the influence of MT foliar application on the morphological response of *R. asiaticus*, including the emergence of flower buds, and to assess the impact on drought tolerance through pigment content (chlorophyll and carotenoids), RWC, proline accumulation, electrolyte leakage (EL), and POD activity. The drought tolerance index (DTI%) was also determined to identify the parameter(s) that could be used as an indicator of drought tolerance.

2. Materials and Methods

2.1. Material and Conditions for Planting

Pot experiments were conducted under uniform environmental conditions at a research greenhouse at the Floriculture and Dendrology Department of The Hungarian University of Agriculture and Life Science (Budapest, Hungary). The average day and night temperature of the greenhouse was maintained at 20 °C–15 °C with 60% relative humidity and a 14/10 h day/light photoperiod. Healthy seedlings of *R. asiaticus* (Oázis Gardening, Budapest-Pasarét) with four to five true leaves (5–6 cm in size) were transplanted 30 days after sowing into plastic pots measuring 9 × 9 × 10 cm. One plant was placed in each pot, and 80 plants were allocated to each group, with a total of 160 plants per hypothesis that were divided into two groups: the well-watered group and the drought group, with each group further subdivided into four subgroups that were treated with different concentrations of MT (20 plants/subgroup). The growing medium in each pot comprised a uniform mixture of Klassmann TS3 Baltic peat (chemical components presented in Table 1) supplemented with 3 kg/m³ Osmocote Exact Potassium Dominant (Scotts, NSW, Australia) and 1 kg/m³ (soluble carbonate). The planting process was initiated on 1 October 2021, and harvesting was performed 150 days later.

2.2. Experimental Design and Irrigation Treatments

After transplantation, plant irrigation was conducted according to standard irrigation techniques until the plants reached the eighth true-leaf stage and used 100% of field potential. Thereafter, 30 days after the transplants were established, the irrigation treatments were initiated. Using two way-ANOVA with the application of completely randomized design (RCD), irrigation and MT foliar application were considered two independent variables. The first factor, i.e., irrigation treatment, included two irrigation levels: well-irrigated, wherein the moisture content of the wet soil was maintained between 50 and 12 kiloPascal (kPa), and drought-stressed, wherein the moisture content was maintained between 18 and 20 kPa. This was combined with foliar MT application at four different concentrations: 0, 50, 100, and 200 µM at 45, 60, 75, and 90 days after *R. asiaticus* planting, respectively. Tensiometer readings were obtained daily using a Blumat Digital PRO Plus instrument (Blumat GmbH & Co. KG, Telfs, Austria) to assess the changes in soil moisture and soil water capacity (kPa) for each plant; the tensiometers were placed at a depth of 5–8 cm. The MT doses were selected as per previously published studies by Zhang et al. and Bidabadi et al. [8,30]. MT was purchased from Thermo Fisher Scientific (Geel, Belgium) and prepared according to the protocol published by Li et al. [31] by dissolving the solute in ethanol and then diluting it with Milli-Q water (ethanol/water (*v/v*) = 1/10,000), followed by application using a manual pump.

2.3. Morphological Characteristics

After the fourth foliar treatment within 2 weeks, 10 plant samples per treatment group were randomly selected and gathered to estimate the following vegetative growth parameters: plant height (cm) measured from the medium surface to the shoot apex using a meter rod, the number of leaves per plant counted manually, fresh and dry vegetative

weights (g) (shoots and leaves), leaf area (cm²) measured using a leaf area meter (Area Meter 350, ADC Bioscientific Ltd., UK) [32], and flower bud emergence (days from planting).

Table 1. Klassmann TS3 Baltic peat chemical components.

Substrate Type	Peat Moss
pH	6
EC (cm/mmhos)	0.4–0.5
Humidity %	50–65
Organic matter (OM)%	>90
Macroelement (1 kg/m ³)	
N:P:K	14:10:18
Microelement (mg/kg)	
Zn	32.45
Cu	15.6
Cd	0.42
Pb	15.28
Mo	0.10
Ni	8.75
Cr	0.70
Hg	<0.01
As	0.112
Co	1.11
Cl	<0.2
Na	<0.1

2.4. Photosynthetic Pigments Analysis

The photosynthetic pigments in the fresh leaves (0.1 g) from each treatment group were measured after crushing and extraction in 80% acetone. The homogenates were centrifuged at 14,000× g for 5 min, and 1.5 mL of the supernatant was used to analyze the leaf pigments. A spectrophotometer (Genesys 10S UV-VIS Spectrophotometer, USA) was used to measure the amount of light absorbed at 644 nm, 663 nm for total chlorophyll, and 480 nm for carotenoids [33].

The total chlorophyll and carotenoid contents were determined using the following equations:

$$\text{Total chlorophyll (mg g}^{-1}\text{) F.W} = [20.2 (A_{644}) + 8.02 (A_{663})] \times V/W$$

$$\text{Carotenoid (mg g}^{-1}\text{) F.W} = 5.01 \times A_{480} \times V/W$$

2.5. RWC in Leaves

RWC content was assessed using the method of Turk and Erdal [34]. The following formula was used to calculate the RWC with five replicates:

$$\text{RWC} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100.$$

A portion of the leaves was taken from the fifth leaf of the *Ranunculus asiaticus* plant for RWC analysis. The initial fresh weight (FW) of the leaf sample was determined upon sampling. The leaf fragment was then placed in distilled water overnight to allow it to rehydrate. After rehydration, the turgid weight (TW) of the leaf was determined. The

rehydrated leaf was then dried in an oven at 75 °C for a period of 24 h to estimate its dry weight (DW).

2.6. Electrolyte Leakage

Using the method used by Turk and Erdal [34], EL was evaluated by first placing 0.5-cm diameter disks taken from the leaves into tubes containing 40 mL of distilled deionized water at 10 °C for 24 h. On the following day, the initial electrical conductivity (EC1) of the solution was determined using a solution analyzer (Cole-Parmer Instrument Co., Chicago, IL, USA). To extract the total electrolytes, the samples were autoclaved for 20 min at 121 °C. After incubating the samples at 21 °C overnight, the final conductivity of the dead tissues (EC2) was determined. The proportion of EL was calculated of the solution of the dead tissue for each treatment using a previously published formula [35]:

$$\text{EL (\%)} = \text{EC1/EC2} \times 100.$$

2.7. POD Activity Determination

The enzyme extract was obtained by homogenizing 1 g of leaf sample with 2 mL of phosphate buffer (pH 7.0) using a pre-cooled mortar. The mixture was then centrifuged at $12,000 \times g$ for 20 min at 4 °C. The supernatant was used to measure the activity of POD. The activity was determined by following the procedure described by He et al. [36]. The reaction mixture was made up of 2.9 mL of 50 mM phosphate buffer (pH 5.5), 1 mL of 0.6 M hydrogen peroxide, 1 mL of 50 mM guaiacol, and 0.1 mL of enzyme extract. The reaction was incubated at 37 °C for 15 min and then stopped by adding 2 mL of 20% (v/v) trichloroacetic acid. The change in absorbance caused by the oxidation of guaiacol was measured at 470 nm.

The activity of POD was calculated as follows:

$$\text{POD activity Ug}^{-1} \text{ FW} = \Delta A_{470} \times V_t / W \times V_s \times 0.01 \times t$$

where ΔA_{470} represents the time for the change in absorbance, V_t is the total volume of the reaction mixture, W is the sample fresh weight, V_s is the volume of the crude enzyme, and t is the reaction time (min).

2.8. Proline Content

Following a previously described method [37], fresh leaf samples were ground, and 0.5 g samples were homogenized in 10 mL of 3% aqueous sulfosalicylic acid. The previous mixture was centrifuged at 4 °C for 10 min at 14,000 rpm. Subsequently, 200 μL of the supernatant was placed in test tubes containing 200 μL of acidic ninhydrin solution and 200 μL of glacial acetic acid. The tubes were immersed for 1 h in a 90 °C water bath, and the process was stopped by placing the tubes in an ice bath. Thereafter, 4 mL of toluene was added into the reaction mixture. Then, the mixture was vortexed for 20 s. After separating the toluene and aqueous phases for at least 20 min in the dark at room temperature, the toluene phase was carefully collected into test tubes, and absorbance of the colored solutions was read on spectrophotometer at 520 nm using a spectrophotometer (Genesys 10S UV-VIS Spectrophotometer, Rochester, NY, USA). The concentration of proline was calculated on a fresh-weight basis using a standard curve constructed using known concentrations of proline.

2.9. DTI

The DTI was determined as a percentage (%) for each of the analyzed traits, as indicated in [38], with a minor change in the symbols used as follows: $\text{DTI} = (T^* \text{ drought} / T^* \text{ cont}) \times 100$, where $T^* \text{ drought}$ represents the average traits value under the stress of a water deficit, and $T^* \text{ cont}$ represents the average traits value under well-watered conditions [9].

T^* (Drought and Control): all studied parameters.

2.10. Statistical Analyses

The current study adopted a completely randomized design. A two-way MANOVA followed by UNIANOVA was used to analyze the variables with Bonferroni correction for all dependent variables, with two between-factor levels: (1) treatment (irrigation and dry) and (2) MT concentrations (0, 50, 100, and 200 μM). In turn, a one-way MANOVA was used to analyze DTI characteristics.

Assumption treatments: The normality values of the residuals for most of the dependent variables were accepted using the Kolmogorov–Smirnov test ($p > 0.05$), with the exception of the cases of number of leaves, height DTI, number of leaves DTI, and EL DTI; these were accepted by testing skewness and kurtosis, wherein the absolute value of both measures was <1 [39]. Finally, normality was violated for the Carotenoid variable, and Log (value) data transformation was used.

The homogeneity of variances was assessed using Leven's F test and was satisfied ($p > 0.05$) for most of the dependent variables, whereas those of plant height, area/leaf, DW, RWC DTI, and Carotenoids DTI were violated ($p < 0.005$). However, the violated values of homogeneity were not serious and were accepted by the variance ratio test ($F = \text{Max variance}/\text{Min variance}$) whenever the max sample size/min sample size ratio was <1.5 and the max var/min var ratio was <6 [40]. Consequently, Tukey's post hoc test was used for factor-level comparisons [41,42]. The pairwise within-subject effect was compared using the Bonferroni method. All statistical procedures were performed using the IBM SPSS27 software [43].

3. Results

One- and two-way MANOVA overall tests revealed a highly significant multivariate main effect of factors (Wilk's lambda < 0.001). Furthermore, in the case of the performed two-way MANOVA, the interaction effect of the factor levels was significant (Wilk's lambda < 0.001) [44]; therefore, we compared the irrigation levels separately in terms of the hormone concentration levels, and then we compared the hormone concentration levels separately regarding the irrigation levels. A subsequent univariate ANOVA for different variables (Bonferroni correction) indicated significant differences for all individual variables >0.05 [45]. A post-hoc test was performed for all significant variables under the MT concentration factor and irrigation level effects. Different letters indicate different groups (Tukey/Games–Howell test, $p < 0.05$). Lowercase letters represent comparisons of MT concentrations under fixed treatments, whereas uppercase letters represent comparisons of irrigation levels under fixed MT concentration levels.

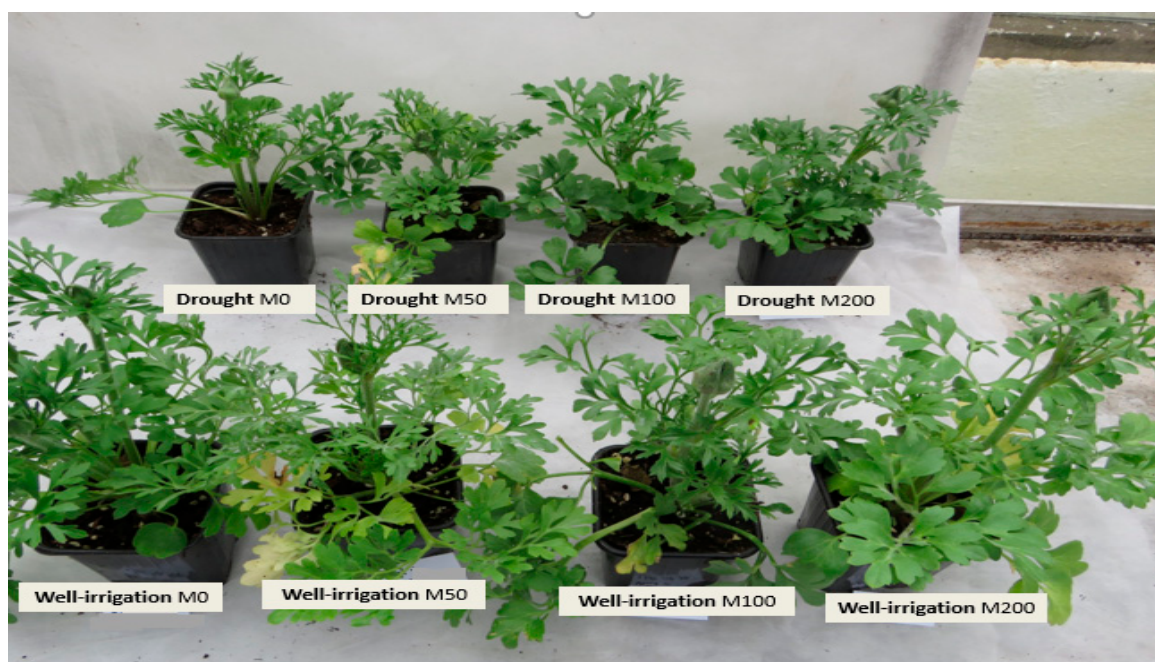
3.1. Impact of MT on Morphology under Drought Conditions

The findings of this study demonstrated that morphological parameter values decreased significantly under drought stress. However, MT treatment considerably affected plant development and growth. Under well-irrigated conditions, foliar spraying treatment with MT (0, 50, 100, and 200 μM) substantially improved fresh and dry vegetative weights, shoot length, leaf number, and leaf area compared with no treatment (W0MT). In untreated/stressed plants (D0MT), shoot length decreased to 13.97 cm, leaf number decreased to 4.60, leaf area decreased to 18.15 cm^2 , and shoot FW and DW decreased to 11.92 g and 1.47 g, respectively, compared with well-watered plants (W0MT). Compared with untreated/drought-stressed plants (D0MT), plants subjected to foliar spraying of MT (50 MT treatment) showed minor improvement in all vegetative traits, whereas those subjected to 100 MT and 200 MT treatments showed favorable effects on plant development in the form of dramatically increased shoot length (to 16.78 cm and 17.09 cm, respectively), leaf number (to 5.73 and 5.93, respectively), leaf area (to 28.78 cm^2 and 29.56 cm^2 , respectively), shoot FW (to 15.92 g and 17.01 g, respectively), and shoot DW (to 1.85 g and 2.01 g, respectively) (Table 2; Figure 1). These effects were observed in a concentration-dependent manner.

Table 2. Effects of exogenous melatonin application on the vegetative growth traits of *R. asiaticus* plants with/without drought stress.

Treatments		Shoot Length (cm)	No. of Leaves	Area/Leaf (cm ²)	Fresh Weight (g)	Dry Weight (g)
Effect of MT (μM) under well-irrigated conditions	0	18.87 ± 0.18 bA	6.67 ± 0.49 bA	47.97 ± 0.05 dA	18.19 ± 0.39 cA	3.12 ± 0.19 bA
	50	19.07 ± 0.27 bA	7.33 ± 0.49 aA	54.49 ± 0.11 cA	18.15 ± 0.15 cA	3.12 ± 0.07 bA
	100	21.49 ± 0.35 aA	7.67 ± 0.49 aA	55.31 ± 0.08 bA	18.82 ± 0.26 bA	3.17 ± 0.14 bA
	200	21.55 ± 0.34 aA	7.73 ± 0.46 aA	55.60 ± 0.18 aA	21.73 ± 0.27 aA	3.12 ± 0.19 aA
Effect of MT (μM) under drought conditions	0	13.97 ± 0.47 cB	4.60 ± 0.51 cB	18.15 ± 0.20 dB	11.92 ± 0.17 dB	1.47 ± 0.06 dB
	50	15.75 ± 0.37 bB	5.20 ± 0.56 bB	21.61 ± 0.16 cB	13.99 ± 0.09 cB	1.63 ± 0.09 cB
	100	16.78 ± 0.30 aB	5.73 ± 0.70 abB	28.78 ± 0.22 bB	15.92 ± 0.13 bB	1.85 ± 0.06 bB
	200	17.09 ± 0.39 aB	5.93 ± 0.46 aB	29.56 ± 0.17 aB	17.01 ± 0.10 aB	2.01 ± 0.12 aB

MT (μM) (exogenous melatonin) at different concentrations: 0, 50, 100, and 200 μM. Well-irrigated: plants under well irrigation conditions; Drought: plants under drought conditions. The values represent the mean ± standard deviation of at least 10 replicates. Lowercase letters: comparison of MT concentrations under fixed irrigation treatments; uppercase letters: comparison of the irrigation treatment groups under fixed MT concentration levels (Tukey/Games–Howell test, $p < 0.05$).

**Figure 1.** Morphological changes in *R. asiaticus* plants under well-irrigated and drought stress conditions after exogenous MT treatment.

MT (exogenous melatonin) at different concentrations (M0, M50, M100, and M200); 0, 50, 100, and 200 μM, respectively. Well-irrigated, plants under well irrigation conditions; Drought, plants under drought conditions.

Moreover, considering our findings, drought stress induced flower bud emergence early. Thus, the flower bud emergence time of the control plants (D0MT) occurred 29 days early, followed by 23, 19, and 21 days early for the D50 MT-, D100 MT-, and D200 MT-treated plants, respectively, compared to properly watered plants with or without MT application (Figure 2).

3.2. Changes in Photosynthetic Pigments

The effects of MT foliar applications at different doses (0, 50, 100, and 200 μM) on photosynthetic pigments in *R. asiaticus* plants subjected to drought stress (soil moisture, 18–20 kPa) are presented in Figure 3A,B, which reveal a dramatic decrease in the photosynthetic pigment

components of leaves (total chlorophyll (Chl) and carotenoids (Car)) compared with well-irrigated plants (no stress and no MT treatment). The rate of the decrease of the chlorophyll and carotenoid content of leaves in non-MT-treated plants under drought stress was estimated at 68.29% and 51.72%, respectively, compared with non-stressed plants without MT treatment. Regarding the foliar application of MT, the data showed that the spraying of MT on leaves (particularly 200 μ M MT) had a substantial impact on the chlorophyll and carotenoid content of the leaves in both conditions. Under drought circumstances, the chlorophyll and carotenoid content increased by 75 and 50%, respectively, in the 200 μ M MT plants compared to the non-MT-stressed plants (D0MT).

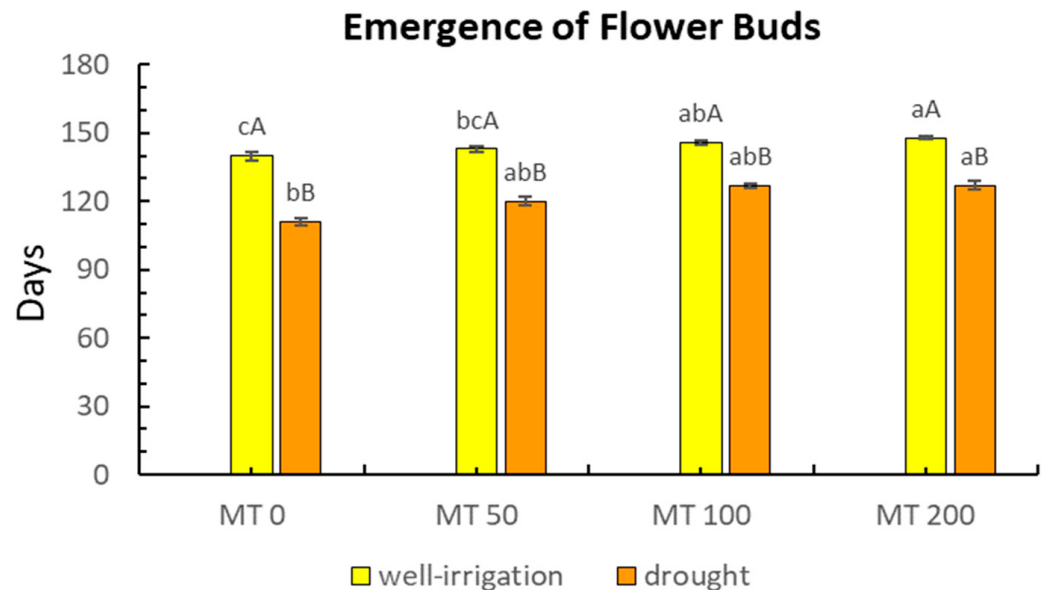


Figure 2. Effect of exogenous melatonin on the emergence of flower buds in *R. asiaticus* plants cultivated under well-irrigated and drought stress conditions. MT0, well-watered and drought-stressed plants without MT treatment; MT50, MT100, and MT200, treatment with MT at different concentrations (50, 100, and 200 μ M, respectively) under well-irrigated and drought circumstances. Lowercase letters: comparison of MT concentrations under fixed irrigation treatments; uppercase letters: comparison of the irrigation treatment groups under fixed MT concentration levels (Tukey/Games–Howell test, $p < 0.05$). The values represent the mean $\pm$ standard deviation of at least 10 replicates.

3.3. Exogenous MT Alters the RWC and Proline Content under Drought Stress

Plants' accumulation of osmolytes is an efficient defense mechanism against drought stress-induced osmotic stress [46]. Therefore, the plant's water stress levels can be evaluated by measuring the specific accumulated solutes. The relative water content (RWC) of the tissues of a plant is the most reliable indicator of its water status and capacity for survival under stressful conditions. The significant reduction of the RWC observed under drought stress was mitigated by MT treatment. Untreated/stressed plants (D0MT) showed a considerable decrease (7.1%) in RWC compared with untreated plants that had received adequate watering (W0MT). In contrast, MT-treated plants exhibited significantly improved RWC under stressful conditions. The optimum improvement in RWC was noted at 200 μ M MT, with a 6.4% increase in RWC values compared with nontreated/drought-stressed plants (D0MT) (Figure 4A).

Proline is a compatible solute that acts as a protective membrane solute and can maintain water balance, enhance cytoplasmic osmotic pressure, and protect cells during dehydration. The current investigation demonstrated that the proline content in *R. asiaticus* plants under drought stress was higher than those of well-irrigated plants (Figure 4B). In general, proline was impacted by drought stress and MT. Under drought stress conditions, the proline content in MT (50 μ M)-treated plants was slightly increased

by 17.8% compared to stressed plants without MT treatment (D0MT). In addition, plants treated with MT (100 and 200 μM) under stressful conditions showed significant increases in proline content (by 28.6% and 32.1%, respectively) compared with untreated/stressed plants (D0MT). Furthermore, the application of MT (0, 50, 100, and 200 μM) under non-stressed conditions led to a gradual slight increase in the proline content of well-irrigated plants (W0MT), which was less compared to plants subjected to drought conditions.

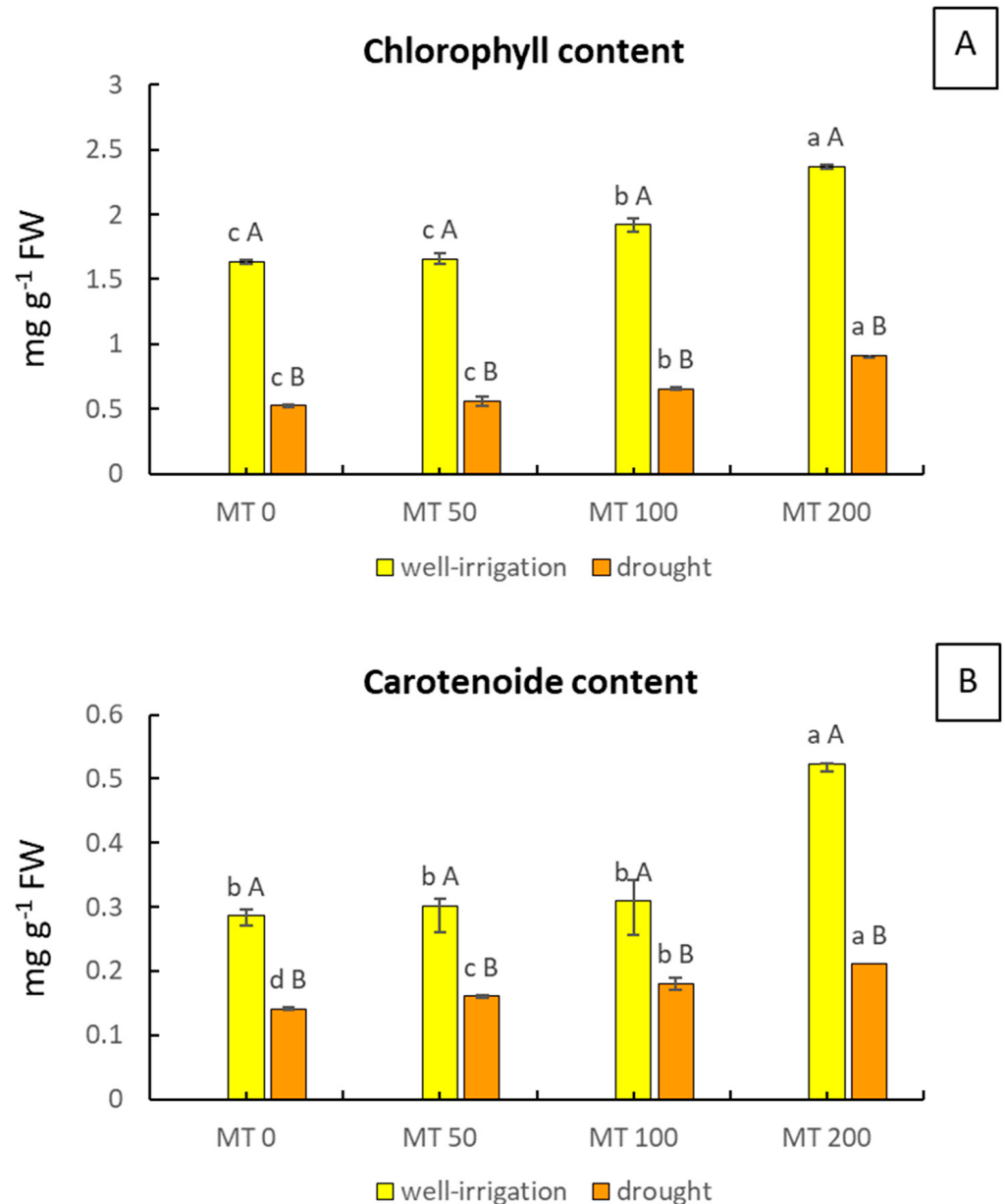


Figure 3. Effect of exogenous melatonin on the total chlorophyll (mg g^{-1} , FW) (A) and carotenoid (mg g^{-1} , FW) (B) content of *R. asiaticus* under drought stress conditions. (A) Chlorophyll content in leaves (mg/g fresh weight). (B) Carotenoid content in leaves (mg/g fresh weight). MT0, well-watered and drought-stressed plants without MT treatment; MT50, MT100, and MT200, treatment with MT at different concentrations (50, 100, and 200 μM , respectively) under well-irrigated and drought conditions. Lowercase letters: comparison of MT concentrations under fixed irrigation treatments; uppercase letters: comparison of the irrigation treatment groups under fixed MT concentration levels (Tukey/Games-Howell test, $p < 0.05$). The values represent the mean $\pm$ standard deviation of at least five replicates.

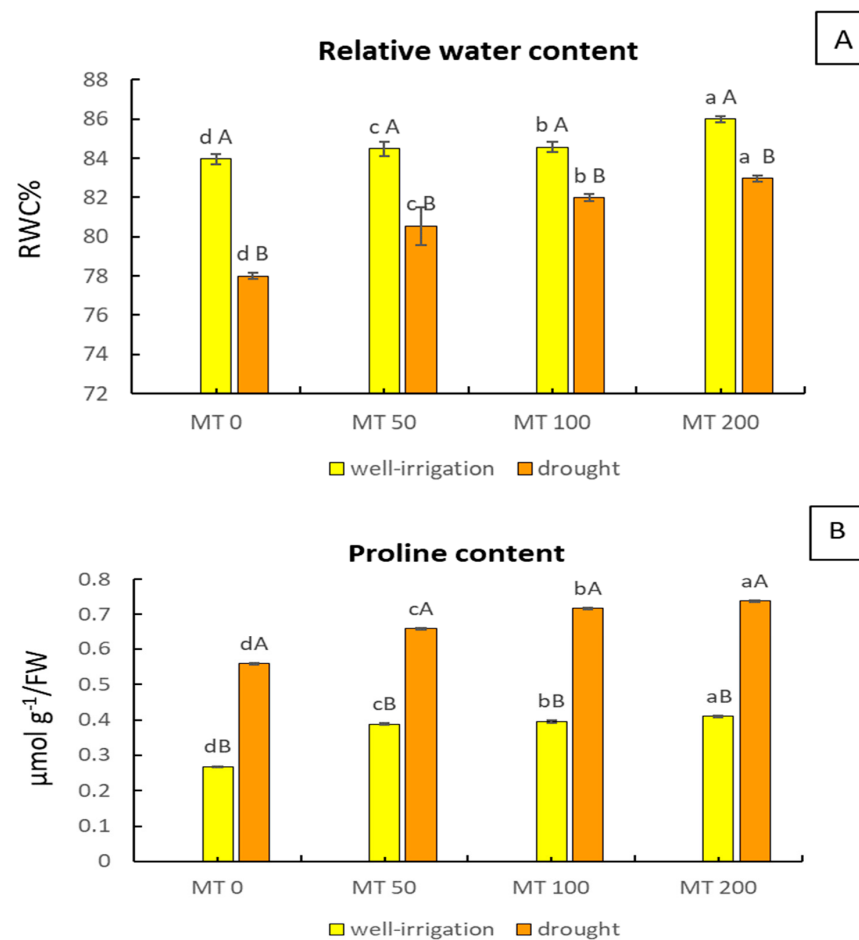


Figure 4. Effect of exogenous melatonin on the relative water content (%) (A) and proline content ($\mu\text{mol g}^{-1}/\text{FW}$) (B) of *R. asiaticus* under drought stress conditions. Lowercase letters: comparison of MT concentrations under fixed irrigation treatments; uppercase letters: comparison of the irrigation treatment groups under fixed MT concentration levels (Tukey/Games-Howell test, $p < 0.05$).

These results suggest that proline in the leaves of *R. asiaticus* plants adopts various pathways to counteract the negative effects of drought. Hence, applying exogenous MT may rectify these alterations and provide enhanced protection.

MT0, well-watered and drought-stressed plants without MT treatment; MT50, MT100, and MT200, treatment with different MT concentrations (50, 100, and 200 μM , respectively) under non-stressful and stressful conditions. The values represent the mean $\pm$ standard deviation of at least five replicates. Lowercase letters: comparison of MT concentrations under fixed irrigation treatments; uppercase letters: comparison of the irrigation treatment groups under fixed MT concentration levels (Tukey/Games-Howell test, $p < 0.05$).

3.4. MT Modulates the Activity of Peroxidase Enzymes and Checks EL

Drought stress increases the generation of ROS, which, in excess, can damage the permeability of cell membranes, and EL was used as an indicator of this oxidative damage [47]. The relationship between EL and POD activity was observed in our experiments (Figure 5A,B) to evaluate the function of POD in protecting membrane structure. As an activator of antioxidant enzymes, MT defends plants from oxidative stress [48]. This investigation tested the effect of drought stress on the POD activity of antioxidant enzymes in *R. asiaticus* plants with or without MT treatment (Figure 5A). Compared to MT-treated plants, the absence of MT significantly decreased POD activity in plants under both well-irrigated and drought conditions. Moreover, the POD activity of plants under well-watered conditions was lower than that detected in drought-stressed plants in a concentration-dependent manner. The POD activity of

untreated/stressed plants (D0MT) increased by 64.6% compared to untreated/well-irrigated plants (W0MT). Under non-stressed conditions, MT treatment (MT50 and MT100) led to a gradual increase in antioxidant enzyme activity (41.2% and 94.1%, respectively), followed by a slight decrease in the presence of MT200 (70.6%), which was still higher than that observed in nontreated plants (W0MT). Conversely, after exposure to drought stress, MT-treated plants (MT50, MT100, and MT200) exhibited a progressive increase in POD activity (16.6%, 45.8%, and 58.3%, respectively) compared to untreated plants (D0MT). The effects of water deficit and exogenous MT on membrane integrity and EL are illustrated in Figure 5B. The stressful conditions significantly enhanced EL. Compared with non-MT stressed plants, plants exposed to MT at concentrations of 50, 100, and 200 μM showed a substantial reduction in EL by 5.7%, 7.7%, and 14.3%, respectively. These findings indicate that foliar MT application at 200 μM was more successful at scavenging ROS buildup.

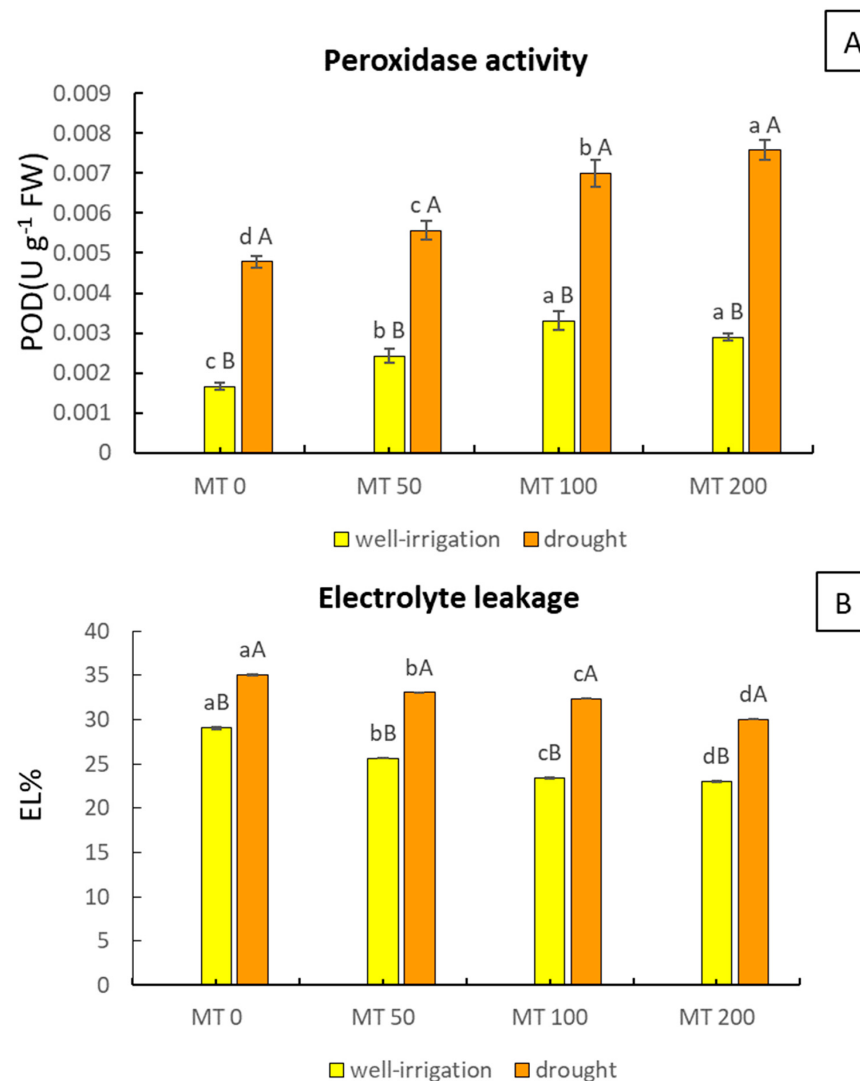


Figure 5. Effect of exogenous melatonin on peroxidase activity [POD (U g⁻¹ FW)] (A) and electrolyte leakage [EL (%)] (B) in *R. asiaticus* plants subjected to drought stress conditions. MT0, well-watered and drought-stressed plants without MT treatment; MT50, MT100, and MT200, treatment with different MT concentrations (50, 100, and 200 μM , respectively) under non-stressful and stressful conditions. The values represent the mean $\pm$ standard deviation of at least five replicates. Lowercase letters: comparison of MT concentrations under fixed irrigation treatments; uppercase letters: comparison of the irrigation treatment groups under fixed MT concentration levels (Tukey/Games–Howell test, $p < 0.05$).

3.5. DTI

Table 3 depicts the DTI of the investigated plants as a percentage of all examined variables between the non-MT-treated plants grown under well-irrigated and drought stress conditions during this study. In addition, the DTI% demonstrates that POD activity was the trait most sensitive to drought conditions, with a value of 282.35% compared to the DTI% associated with other traits. Proline content was the second most sensitive trait to drought with a value of 207.40%, followed by EL (120.49%). In addition, the remaining traits exhibited DTI values < 100% and >50% with the exception of carotenoids, shoot dry weight, leaf area, and total Chl, which demonstrated the lowest responsiveness to drought conditions with values of 49.12%, 47.20%, 37.84%, and 32.00%, respectively. Thus, the most sensitive traits to drought stress under application and non-application of MT are total chlorophyll content and leaf area because they were reduced by more than 60 percent.

Table 3. Drought tolerance index (DTI%) of all investigated *R. asiaticus* characteristics.

Traits	DTI (%)
Shoot length	74.02 ± 2.83 ef
Leaf number	69.37 ± 9.35 ef
Leaf area	37.84 ± 0.39 gh
Shoot fresh weight	65.53 ± 1.73 f
Shoot dry weight	47.20 ± 3.48 g
Time of flower bud emergence	79.31 ± 2.30 ed
Total chlorophyll content	32.00 ± 1.02 h
Carotenoid content	49.12 ± 0.58 g
Relative water content (RWC)	92.90 ± 0.45 d
Proline content	207.40 ± 0.30 b
Peroxidase activity (POD)	282.35 ± 10.53 a
Electrolyte leakage (EL)	120.49 ± 0.72 c

The values represent the mean ± standard deviation of at least five replicates. Different letters indicate significantly different groups (post hoc Tukey's test, $p < 0.05$). Lowercase letters: comparison of the tolerance index of all estimated traits under drought stress without MT application.

4. Discussion

Water deficiency is a serious environmental stressor that restricts agricultural growth and productivity, especially in arid and semiarid regions. It can have various physiological, anatomical, and morphological impacts on plants [49]. In our study, the impact of drought stress on *R. asiaticus* was significant and led to a decrease in all growth attributes as the stress level increased. This resulted in early flowering (as shown in Table 2 and Figures 1 and 2). The restricted growth seen under drought stress is a morphological response of plants to prevent water loss by reducing the transpiration area [50]. Previous related studies [12,51–53] demonstrated that drought stress reduced the desirable characteristics of mung bean, flax, sunflower, and *M. oleifera* plants and decreased their water-retaining ability; the authors attributed these alterations to drought-induced diseases and ROS formation [12,54]. Plant height reductions that were observed after exposure to drought conditions (Table 2) may be explained by the decrease in cell elongation, turgor, volume, and cell growth [55]. Water deficiency reduces the amount of water in the shoots; thus, this triggers osmotic stress and the inhibition cell development and division, resulting in stunted plant growth as a whole [56,57]. In addition, a decrease in leaf area under stress reduces water loss and carbon assimilation, degrades the pigments used in photosynthesis, and affects and reduces photosynthesis, thereby negatively impacting plant growth [58,59]. MT is a plant hormone that was shown to have dual functions in plants, including promoting growth [60–62] and protecting against abiotic stress [12,59–62]. MT regulates the biochemical and physiological functions in plants [12,22,63–65] and acts in trace amounts [15]. In the current study, MT treatment gradually enhanced vegetative growth traits under non-stress conditions. Additionally, the adverse effects of drought stress on the traits of *R. asiaticus* were mitigated in our study, resulting in improved growth performance (as shown in Table 2 and

Figure 1). Similar impacts were noted in 100 and 200 μ M MT-treated plants on enhanced shoot length and number of leaves compared to untreated/drought-stressed plants. At the same time, higher fresh and dry weights were recorded in 200 μ M MT-treated than in 100 and 50 μ M MT-treated/drought-stressed plants, indicating that exogenous MT application might mitigate the detrimental effects of drought stress on *R. asiaticus* by enhancing their morphophysiological characteristics. This is in agreement with Imran et al. [62], Sadak et al. [66], and Altaf et al. [10], who stated that exogenous MT accelerated the growth of soybean, lupin, and tomato plants, respectively, under drought conditions. This implies that high MT levels may allow plants to endure longer stress conditions, thereby significantly increasing their yield [63]. MT, an indoleamine, shares IAA's metabolic precursor, which may explain its influence on plant growth and development [67]. Moreover, Han et al. [60] demonstrated that the exogenous administration of MT to soybean plants induced a greater level of abscisic acid accumulation and increased their resistance to drought conditions, indicating that MT and other hormones may interact in this manner. Exogenous MT upregulates the expression levels of components related to defense; for instance, an Arabidopsis transcriptome analysis in response to MT demonstrated that auxin-responsive genes were up- and downregulated [22,68].

Nevertheless, in our study, stressed plants exhibited early flower bud emergence compared to plants that had received adequate irrigation (Figure 3). Furthermore, flower buds started to develop early to alter the growth of the plants in response to stressful circumstances [23,69]. Even in harsh environments, plants may be able to perpetuate their species in this manner [70]. Previous research also showed that shoots under drought stress develop flower buds earlier and on shorter stems than plants subjected to less stressful conditions [71]. In Arabidopsis, exogenous MT application results in delayed flowering [72]. The exogenous supplementation of tissues with MT led to a delay in flower bud emergence time because of the upregulation of the flowering locus C (FLC), which in turn suppressed the transcription of the flowering locus T (FT) [73,74].

Photosynthetic pigments and chlorophyll play crucial roles in vital physiological processes and in transmitting and absorbing solar energy [75]. Their reduction due to drought stress, which dehydrates mesophyll cells and inhibits the enzymes involved in glucose metabolism, may lead to a decrease in photosynthesis and damage to the pigments [76,77]. The present results reveal that drought stress significantly ($p < 0.05$) decreases plant pigment contents in *R. asiaticus* (Figure 3A,B). Meanwhile, foliar MT at different levels increases these pigments' content under normal and drought conditions.

Furthermore, carotenoids are essential pigments that act as photoprotectants and safety valves and have antioxidant properties that keep chlorophyll levels stable [59,78]. In our investigation, MT-treated *R. asiaticus* plants exhibited an increase in photosynthetic efficiency under stress. Further, 200 μ M MT was found to be the best concentration for inhibiting pigment breakdown (Figure 3A,B) and increasing carotenoid content, which could protect against the degradation of chlorophyll, resulting in the maintenance of higher chlorophyll content in MT-treated plants compared to untreated plants [74]. Similar outcomes regarding the improvement of chlorophyll levels were documented in *R. asiaticus*, rice (*Oryza sativa*), *M. oleifera*, and melon (*Cucumis melo* L.) under abiotic stress after exogenous MT treatment [12,23,60,79]. This effect is attributed to the antioxidant properties of MT and its impact on the genes that encode the chlorophyll-degrading enzymes chlorophyllase, pheophorbide (an oxygenase), and red chlorophyll catabolite reductase [74].

In many plant species, relative water stress is regarded as a quick and reliable signal of stress level that is closely linked to physiological changes at the leaf and entire-plant levels [18,80]. Furthermore, maintaining a high RWC is a well-known strategy in breeding programs for drought resistance and is essential to maintain plant metabolism [81]. Therefore, a decreased RWC indicates turgor loss and lower water availability for cell growth. The variance in RWC observed in drought-stressed plants is attributable to the varying capability of plants to absorb water or the capacity of stomata to reduce water loss [82]. The significant difference in the RWC detected between well-irrigated and drought-stressed

plants in our study demonstrated that plants were subjected to adequate water stress (Figure 4A). In addition, the increased RWC after MT application is likely attributable to the enhanced water absorption due to the safeguarding of the membrane [79,83]. It was demonstrated that MT can improve the thickness of a plant's cuticle, which in turn limits water loss. MT treatment was shown to improve drought tolerance in plants by keeping their turgor and water ratio stable [84].

One of the main objectives of this study was to investigate the effect of drought stress on the accumulation of certain chemicals such as proline in leaves and their impact on drought-tolerance enhancement. Proline can detoxify plants, act as an antioxidant against abiotic stresses, or stabilize macromolecules under dehydration stress by scavenging free radicals, buffering cellular redox potential [85] and maintaining the integrity of plasma membranes [86]. Under stressful conditions, the water status of the plant was found to be negatively correlated with proline accumulation in plants that did not receive MT treatment. However, with the application of MT, both proline and relative water content (RWC) showed significant increases (Figure 4A,B); this result is in agreement with Eisa et al. and Zhang et al. [23,87]. A negative relationship between RWC and proline accumulation appears to be associated with a diminished effect of proline on osmotic adjustment [81]. Moreover, MT acts as an antioxidant; thus, it prevents proline breakdown [20]. In this study, the highest proline level was detected in drought-stressed plants compared to well-watered plants. Meanwhile, exogenous MT treatments yielded a considerable but gradual increase in proline levels under drought conditions with increasing concentrations of MT; in particular, treatment with 200 μ M MT was the most effective (Figure 4B). Similar results were reported by Li et al. [24], Kamiab [65], and Jiang et al. [88].

The primary function of MT in plant stress tolerance is the enhancement of antioxidant defense mechanisms, which are well-known endogenous scavengers of ROS and antioxidants [48]. In the present study, we measured the activity of POD as an antioxidant enzyme and demonstrated that different POD levels were observed in plants under normal or drought conditions treated with or without MT application. MT-treated stressed plants showed notably higher POD activity that gradually increased with MT concentration, and 200 μ M MT had the highest POD activity (Figure 5A). This outcome is in agreement with previous studies obtained for different plant species, such as *Brassica napus* [22], pansy [18], and tomato [10], in which MT treatment during drought stress reduced oxidative damage and restored damaged cellular membranes. Moreover, MT, which has a strong long-distance signal, can be transported from treated plant tissues to distant untreated tissues via vascular bundles, resulting in abiotic stress tolerance [48]. Additionally, the administration of MT increases transcription, reduces ROS buildup by scavenging ROS, and promotes activity levels of antioxidant enzymes [89,90].

Conversely, the stability of the cell membrane or EL has long been recognized as a marker of abiotic cell damage [91]. The loss of K^+ due to drought stress is irreversible. This leakage can be attributed to a loss of membrane integrity, which reduces plants' ability to retain K^+ [92]. In this research, minimizing the EL in plants treated with MT afforded a protective effect toward membrane damage under drought conditions (Figure 5B).

Studies reported by Rodriguez et al. [93] demonstrated that MT could control the antioxidant enzyme system and improve enzyme activity in plants directly or indirectly. Similarly, soybean plants stressed by a water deficit and treated with exogenous MT exhibited alleviation of oxidative damage in leaves via an increase in POD activity and a reduction of EL levels [62], which is in contrast to the levels recorded under untreated drought conditions. In the present work, 200 μ M exogenous MT was the best concentration to ameliorate oxidative damage in leaves, suggesting that exogenous MT significantly preserved the cell membrane against oxidative stress during drought conditions (Figure 5B). In addition, the various doses led to distinct patterns of enhanced antioxidant enzyme activity.

Depending on the DTI% values, the characteristics investigated in this study can be classified into three categories. The first category consists of the variables having a

DTI% > 100%, as observed for POD, proline, and EL; the second category includes variables that showed a DTI% >50% and <100%, such as the time of flower bud emergence, shoot FW, number of leaves, and shoot length, in decreasing order. The final category includes the variables with DTI% values <50%, as observed for carotenoid content, shoot dry weight, leaf area, and chlorophyll. Based on the high DTI% values observed for POD enzyme activity, leaf proline content, and EL, it is conceivable to use these characteristics as obvious markers of response to drought stress in *R. asiaticus* plants. Several previous works have debated the use of DTI as a drought-response indicator in *Malva sylvestris*, *Althea rosea*, *Callistephus chinensis*, *Rudbeckia hirta* [9], and *Helianthus annuus* [94], in which stress could be assessed based on the stress index of various traits, such as plant height, dry matter, root length, and relative root volume and density. To the best of our knowledge, this is the first study that computed DTI% for a wide variety of *R. asiaticus* plant features. The results of this study may prove helpful for future studies of plant physiology and breeding.

5. Conclusions

This study revealed that the MT-induced improvement in drought stress tolerance in *R. asiaticus* plants was associated with biochemical changes and might be an effective practice under stress conditions. Our data shows evidence supporting the theory that exogenous MT application in *Ranunculus* seedlings enhances the performance of the antioxidant defense system by diminishing the generation of ROS, as demonstrated by POD activation and decreased EL. Furthermore, it can improve osmotic regulation capability by increasing osmolyte accumulation and maintaining water status to reduce drought-related damage. MT treatment also enhances the amount of carotenoid content, which acts as a nonenzymatic antioxidant, resulting in a higher level of total chlorophyll content being maintained and thereby strengthening vegetative growth parameters under drought conditions. Considering the DTI% values, POD enzyme activity and leaf proline content could indicate the drought stress response of *Ranunculus* plants. Overall, MT improved morpho-physiological and biochemical characteristics, as well as *R. asiaticus*' tolerance to a certain extent, under drought stress when applied at the optimal dose of 200 μ M. To achieve maximum drought tolerance, further studies using different MT concentrations are warranted.

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