agronomy

MDPI

Article

Melatonin Mitigated Salinity Stress on Alfalfa by Improving Antioxidant Defense and Osmoregulation

Xiaoqian Guo [1,2], Yu Shi [1,2,3], Guanglong Zhu [2] and Guisheng Zhou [1,2,*]

[1] Jiangsu Provincial Key Laboratory of Crop Genetics and Physiology, Yangzhou University, Yangzhou 225009, China
[2] Joint International Laboratory of Agriculture and Agri-Product Safety, Yangzhou University, Yangzhou 225009, China
[3] Center for Chinese Urbanization Studies, Soochow University, Suzhou 215123, China
* Correspondence: gszhou@yzu.edu.cn

Abstract: Melatonin (MT) is a growth regulator and antioxidant that can resist peroxidation damage on plants caused by environmental stresses. In this study, the alleviation effects of melatonin on alfalfa under salt stress were investigated in terms of photosynthesis, antioxidant enzymes, and osmoregulation. The alfalfa seedlings were cultured in 200 mM NaCl Hoagland solution. Five levels of MT (0, 0.1, 0.2, 0.3, and 0.4 mM) were applied as a foliar spray. Generally, the foliar spray of MT increased root length, root surface area, height, leaf length and width, aerial and root biomass, SPAD readings, the content of proline and soluble protein, and the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Malonaldehyde (MDA) content was decreased by MT foliar spray. The beneficial effects of MT on alfalfa under salt stress were dosage-dependent, and excessive MT levels inhibited alfalfa growth. The alleviating effects of MT on salt stress were more pronounced at 0.3 mM MT. This study suggested that exogenous MT foliar spray at appropriate levels can ameliorate the adverse effects of salt stress on alfalfa seedlings.

Keywords: foliar spray; osmolyte; salt tolerance; antioxidase

Citation: Guo, X.; Shi, Y.; Zhu, G.; Zhou, G. Melatonin Mitigated Salinity Stress on Alfalfa by Improving Antioxidant Defense and Osmoregulation. *Agronomy* **2023**, *13*, 1727. https://doi.org/10.3390/agronomy13071727

Received: 4 May 2023
Revised: 20 June 2023
Accepted: 23 June 2023
Published: 27 June 2023

1. Introduction

Alfalfa (*Medicago sativa* L.) is one of the most vital leguminous forage crops planted in the world due to its strong nitrogen fixation capacity and high protein content [1]. At present, alfalfa is the forage crop with the biggest acreage in China, reaching about 3.77 million ha^{-1} [2]. It is a moderate salt-tolerant plant among the legume family. However, its production declines when the salt level in the soil exceeds a certain threshold [3,4]. Since several alfalfa-planting regions are affected by high salinity, the sensitivity of alfalfa crops to salinity is an issue attaching the attention of alfalfa producers and scientists [5].

Soil salinity is a major factor restricting crop productivity and quality [6]. Salinity inhibits cell division and expansion of plant growing points of the salt-stressed plants, resulting in stunted leaves and stems [7]. The number of leaves, leaf area, height, and dry weight of roots, leaves, and stems of salt-stressed alfalfa plants are significantly reduced, resulting in reduced crop yield [8]. In addition, salinity stress leads to the imbalances of water and ions and enhances oxidative stress by producing reactive oxygen species (ROS), which negatively affect hormonal and antioxidant balances and photosynthesis, causing a decline in plant growth [9]. Due to the importance of alfalfa and the increasing incidence of salinity stress, it is urgent to improve the salt tolerance of alfalfa plants for better and sustainable production.

Melatonin (MT, N-acetyl-5-methoxytryptamine) is a natural antioxidant produced by all living species, including animals, plants, and microbes [10,11]. It plays multiple roles in plant growth and resistance to abiotic stress [12]. Many studies have indicated that as a plant growth regulator, MT regulates germination, root development, floral

transition, fruit formation and maturation, and leaf senescence [13–17]. MT also mitigates the damages caused by abiotic stresses, such as salinity, drought, heavy metals, cold, ultraviolet radiation, alkali ions, and biotic stresses on higher plants [18]. Under high salt stress, exogenous melatonin allows plants to maintain robust roots, ameliorate growth inhibition, and enhance photosynthetic ability [19]. Hence, the application of exogenous melatonin to plants is considered an effective strategy for alleviating salt stress. However, there are few reports on the effects of MT on alfalfa under salinity stress.

The responses of plants to salinity, as well as to melatonin, varied in species types, genotypes, and growth stages [20]. "Chifeng" is an improved local alfalfa cultivar in the Chifeng area of Inner Mongolia and is one of the varieties widely planted in China. In a previous study, the effects of salinity on alfalfa "Chifeng" were investigated, and 200 mM was found to be a threshold salinity level for its growth and development. Based on this, the objectives of this study were to evaluate the mitigative effects of exogenous MT on alfalfa "Chifeng" seedlings under salinity stress and to identify the appropriate MT level for this alfalfa cultivar if the mitigating effects of MT are proved.

2. Materials and Methods

2.1. Study Site and Plant Materials

A controlled study was conducted in the incubator and the greenhouse of the Joint International Research Laboratory of Agriculture and Agri-Product Safety of the Ministry of Education of China, Yangzhou University, in 2021. The tested alfalfa cultivar is "Chifeng", which is an improved cultivar widely planted in China.

2.2. Experimental Design

The plump seeds of alfalfa with uniform size were selected and sterilized using 70% ethanol solution for 10 min. The sterilized seeds were placed incubated in Petri dishes with distilled water and germinated in an incubator (RX-1000GT, Changzhou, China) at 20 °C with a photoperiod of 8 h. After seven days of germination, the seedlings were wrapped in a sponge and fixed in the holes of foam plates, and then incubated in plastic pots with Hoagland nutrient solution (NSP1020, Beijing Coolaber Science and Technology Co., Ltd., Beijing, China) [21]. All the plastic pots were placed in the greenhouse for further growth. During the whole study, the plastic pots and foam plates were wrapped in tin foil to avoid light. The greenhouse was maintained at a temperature of 30/25 °C (day/night) with a photoperiod of 16 h, and the relative humidity was 45%.

On the 14th day after transplantation, all seedlings were directly subjected to 200 mM salinity treatment (200 mM mixed salt solution was prepared with NaCl and Hoagland nutrient solution; NaCl: A501218, Sangon Biotech Shanghai Co., Ltd., Shanghai, China). At 24 h after salt stress treatment, five levels of melatonin (CM7111, Beijing Coolaber Science and Technology Co., Ltd., Beijing, China), T1 (0.1 mM MT), T2 (0.2 mM MT), T3 (0.3 mM MT), T4 (0.4 mM MT), and CK (distilled water), were sprayed on seedlings respectively. The leaves were sprayed with melatonin every 12 h for a total of three times. Each treatment was repeated three times. During the whole study, the Hoagland solution was changed every seven days, and the electromagnetic air pump was used to ventilate so as to prevent roots from rotting.

2.3. Observations and Measurements

On the 7th, 14th, and 21st days after foliar spraying (DAF), six seedlings were randomly selected to measure plant height from each pot, and six leaves were randomly selected from all the intact leaves of these six seedlings to measure leaf length and leaf width, respectively. Then the seedlings were divided into two parts, the aerial part, and the root, to determine fresh weight. Root-related indexes were determined by a root analyzer. Subsequently, all the samples were dried in the oven at 180 °C for 30 min and then dried at 60 °C for 48 h to determine dry weight.

The SPAD readings were recorded using a hand-held chlorophyll meter (SPAD-502, Konica-Minolta, Tokyo, Japan) at the top, middle, and base of each leaf, and the average of SPAD readings of each pot was calculated. The intact leaves of ten seedlings were collected and soaked in liquid nitrogen for 15 min and stored in the fridge (−80 °C) for the determination of the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), and the contents of malonaldehyde (MDA), soluble protein and proline. The SOD activity was determined according to the method of Gao [22]. The CAT activity was determined using the method of Zou [23]. The POD activity was determined as described by Zhang and Zhai [24]. The MDA content was determined according to the method of Gao [22]. The content of soluble protein was determined using the method of Liu and Zhang [25]. The proline content was determined as described by Li [26].

2.4. Statistical Analyses

This study was arranged in a completely randomized design with three replications. Data were subjected to Microsoft Excel 2021 to calculate the average values. DPS 9.0 was used for a one-way analysis of variance, and the LSD test was used for multiple comparisons between different treatments ($p \leq 0.05$). Graphing function and Pearson correlation analysis were performed using Origin 2022b.

3. Results

Spraying melatonin significantly increased the root length of alfalfa seedlings, but root length was inhibited by the highest melatonin level (0.4 mM). The T3 (0.3 mM MT) treatment provided better beneficial effects on root length over three sampling dates than other treatments. The average root diameter under melatonin treatment was almost the same as that of the control group. On 21 DAF, the T3 treatment increased root length and average root diameter by 30.8% and 2.0%, respectively. The root surface area generally enhanced from 0 to 0.3 mM MT level and then declined at 0.4 mM MT level, despite there being a fluctuation on 7 DAF. The low MT level had no significant effect on the root surface area, while the root surface area at the T1 (0.1 mM MT) treatment was always higher than that of the control. Conversely, the root surface area at the highest MT level was always significantly lower than the control's ($p < 0.05$). The root surface area at T3 treatment remained at the highest level over the three growth stages of alfalfa ($p < 0.05$) (increased by 10.1%, 62.2%, and 37.4%, respectively) (Table 1).

The application of MT generally and significantly increased seedlings' fresh weight and dry weight. Although T1 had no significant effect on fresh aerial weight on 7 DAF, it did significantly enhance the aerial dry weight. Similar results also occurred with other MT treatments. All the biomass parameters reached the maximum readings at the T3 treatment over three sampling dates, and they were increased by 62.9%, 77.8%, 47.5%, and 86.7%, respectively, at T3 as compared with the control on 21 DAF. Despite the fact that the aerial weight and dry weight both significantly increased with the application of MT, the ratio of root to shoot also showed the same trend. This result indicated that the promotion of MT on the roots was greater than on the aerial part (Figure 1).

The application of MT had various effects on morphological characteristics (Table 2). The leaf width of alfalfa seedlings applied with MT was significantly higher than the control, but there was no significant difference among these four MT levels. Except for T1, the other three MT treatments remarkably increased leaf length to varied degrees. Conversely, only the T3 treatment significantly increased plant height at three sampling dates (36.0% on 7 DAF, 30.8% on 14 DAF, and 24.5% on 21 DAF, respectively). During 21 days after MT application, the growth of leaf width was faster than the other two parameters from 7 to 14 DAF, while from 14 to 21 DAF, the growth rate was affected by the application of MT.

Table 1. Effects of melatonin on root length, diameter and surface area of alfalfa plants under salt stress.

Days	Melatonin	Root Length (cm plant^{-1})	Root Average Diameter (mm plant^{-1})	Root Surface Area (cm^2 plant^{-1})
7 DAF	CK	24.3 ^{b}	0.184 ^{a}	4.17 ^{bc}
	T1	24.5 ^{b}	0.193 ^{a}	4.23 ^{b}
	T2	26.6 ^{a}	0.197 ^{a}	3.95 ^{cd}
	T3	27.3 ^{a}	0.193 ^{a}	4.59 ^{a}
	T4	21.2 ^{c}	0.189 ^{a}	3.79 ^{d}
14 DAF	CK	25.0 ^{c}	0.240 ^{a}	7.4 ^{c}
	T1	25.9 ^{c}	0.230 ^{a}	8.1 ^{bc}
	T2	27.8 ^{b}	0.233 ^{a}	9.7 ^{b}
	T3	29.9 ^{a}	0.238 ^{a}	12.0 ^{a}
	T4	23.2 ^{d}	0.232 ^{a}	5.1 ^{d}
21 DAF	CK	25.3 ^{d}	0.256 ^{ab}	10.7 ^{bc}
	T1	26.8 ^{c}	0.236 ^{ab}	12.2 ^{b}
	T2	29.8 ^{b}	0.220 ^{b}	12.5 ^{ab}
	T3	33.1 ^{a}	0.261 ^{a}	14.7 ^{a}
□	T4	23.3 ^{e}	0.229 ^{ab}	8.6 ^{c}

DAF: Days after foliar spraying. CK: distilled water, T1: 0.1 mM MT, T2: 0.2 mM MT, T3: 0.3 mM MT, T4: 0.4 mM MT. Within each sampling date, the data followed with different letters are statistically different at the 0.05 probability level.

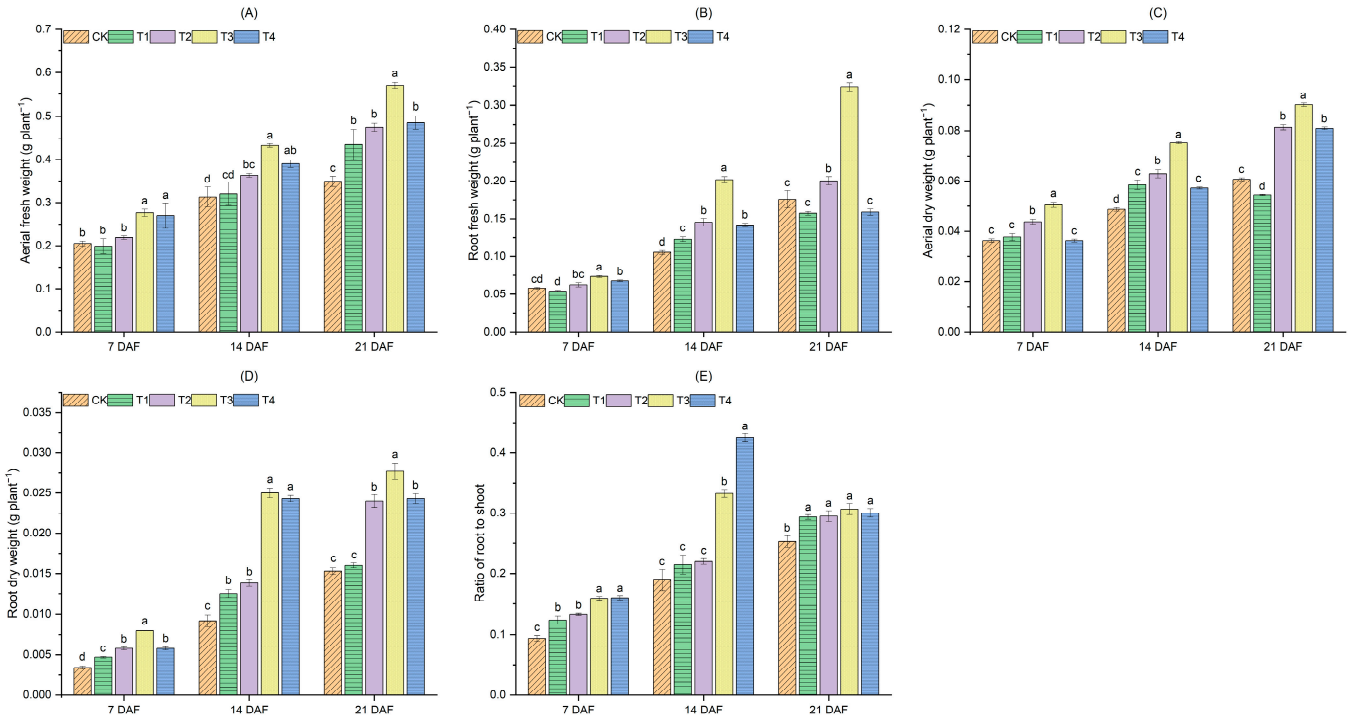

Figure 1. Effects of melatonin on fresh and dry biomass of alfalfa plants under salt stress. (**A**) Aerial fresh weight; (**B**) fresh root weight; (**C**) aerial dry weight; (**D**) root dry weight; (**E**) ratio of root to shoot. DAF: Days after foliar spraying. CK: distilled water, T1: 0.1 mM MT, T2: 0.2 mM MT, T3: 0.3 mM MT, T4: 0.4 mM MT. Within each sampling date, the data followed with different letters are statistically different at the 0.05 probability level.

Table 2. Effects of melatonin on height, leaf length, and width of alfalfa plants under salt stress.

Days	Melatonin	Height (cm)	Leaf Length (cm)	Leaf Width (cm)
7 DAF	CK	11.4 ^{b}	1.08 ^{b}	0.80 ^{b}
	T1	12.2 ^{b}	1.10 ^{b}	0.85 ^{ab}
	T2	12.6 ^{b}	1.12 ^{ab}	0.90 ^{ab}
	T3	15.5 ^{a}	1.22 ^{a}	0.95 ^{a}
	T4	12.0 ^{b}	1.08 ^{b}	0.93 ^{a}
14 DAF	CK	14.3 ^{c}	1.18 ^{c}	1.08 ^{b}
	T1	14.1 ^{c}	1.22 ^{c}	1.15 ^{ab}
	T2	15.1 ^{b}	1.37 ^{ab}	1.28 ^{a}
	T3	18.7 ^{a}	1.38 ^{a}	1.22 ^{ab}
	T4	14.8 ^{bc}	1.30 ^{b}	1.18 ^{ab}
21 DAF	CK	15.9 ^{b}	1.22 ^{b}	1.10 ^{b}
	T1	14.2 ^{c}	1.23 ^{b}	1.23 ^{a}
	T2	15.7 ^{b}	1.38 ^{a}	1.30 ^{a}
	T3	19.8 ^{a}	1.38 ^{a}	1.28 ^{a}
□	T4	16.1 ^{b}	1.48 ^{a}	1.22 ^{a}

DAF: Days after foliar spraying. CK: distilled water, T1: 0.1 mM MT, T2: 0.2 mM MT, T3: 0.3 mM MT, T4: 0.4 mM MT. Within each sampling date, the data followed with different letters are statistically different at the 0.05 probability level.

MT had a significant effect on the SPAD value of alfalfa seedlings (Table 3). SPAD readings were enhanced with the increase in MT level, reached the maximum at the T3, and then decreased. The highest MT level (T4) had lower SPAD reading but was still higher than the control. Although the T1, T2 (0.2 mM MT), and T4 (0.4 mM MT) all significantly increased SPAD value on 21 DAF, there was no remarkable difference among these three treatments. Compared with the control, the T3 treatment enhanced SPAD value by 27.2% on 7 DAF, 23.8% on 14 DAF, and 24.2% on 21 DAF, respectively. Over three sampling dates, SPAD readings did not change significantly with the growth of alfalfa seedlings.

Table 3. Effects of melatonin on SPAD readings and MDA content of alfalfa plants under salt stress.

Melatonin	SPAD Readings			MDA (nmol mg^{-1})		
	7 DAF	14 DAF	21 DAF	7 DAF	14 DAF	21 DAF
CK	33.8 ^{b}	36.6 ^{d}	37.6 ^{c}	49.1 ^{a}	73.3 ^{a}	65.8 ^{a}
T1	34.6 ^{b}	39.6 ^{c}	41.2 ^{b}	34.2 ^{b}	52.8 ^{b}	43.4 ^{c}
T2	35.7 ^{b}	41.3 ^{b}	41.8 ^{b}	19.7 ^{d}	42.1 ^{c}	41.1 ^{c}
T3	43.0 ^{a}	45.3 ^{a}	46.7 ^{a}	17.5 ^{e}	31.8 ^{d}	31.3 ^{d}
T4	40.2 ^{a}	40.5 ^{bc}	40.7 ^{b}	24.1 ^{c}	39.5 ^{c}	52.5 ^{b}

DAF: Days after foliar spraying. CK: distilled water, T1: 0.1 mM MT, T2: 0.2 mM MT, T3: 0.3 mM MT, T4: 0.4 mM MT. Within each sampling date, the data followed with different letters are statistically different at the 0.05 probability level.

The content of MDA was significantly affected by MT treatment (Table 3). With the application of MT, the MDA content showed a trend of decrease before the increase. The T3 treatment had the lowest MDA content at three sampling dates (17.5, 31.8, and 31.3 nmol mg^{-1} on 7, 14, and 21 DAF, respectively), followed by the T2 treatment. Although the MDA content at the T4 level was lower than that at the T2 level on 14 DAF, this reduction was not statistically significant. A similar situation was also observed between the T1 and the T2 treatments on 21 DAF.

MT treatment had significant and positive effects on the content of proline and soluble protein (Table 4). The highest proline and soluble protein content were found during the T3 treatment. These two parameters treated with T2 were a close second. On 7 DAF, there was no significant difference between T2 and T4 in promoting proline and soluble protein. Compared with CK, T3 increased proline and soluble protein by 24.9% and 55.3%, respectively, on 21 DAF. MT treatments showed a better beneficial effect on proline than on

soluble protein on 14 DAF, which was supported by the higher percentage increase, while the reverse situation was observed on 21 DAF.

Table 4. Effects of melatonin on proline and soluble protein content of alfalfa plants under salt stress.

Melatonin	Proline (μg g^{-1})			Soluble Protein (mg g^{-1})		
	7 DAF	14 DAF	21 DAF	7 DAF	14 DAF	21 DAF
CK	159.9 ^{d}	217.0 ^{d}	367.7 ^{d}	9.7 ^{d}	13.0 ^{d}	14.1 ^{d}
T1	210.0 ^{c}	358.8 ^{b}	402.3 ^{c}	11.7 ^{c}	16.1 ^{b}	17.0 ^{bc}
T2	240.4 ^{b}	384.0 ^{a}	431.9 ^{b}	13.6 ^{b}	16.0 ^{b}	18.1 ^{b}
T3	258.4 ^{a}	382.7 ^{a}	459.1 ^{a}	18.2 ^{a}	19.6 ^{a}	21.9 ^{a}
T4	228.7 ^{b}	255.8 ^{c}	413.4 ^{c}	14.1 ^{b}	14.5 ^{c}	16.3 ^{c}

DAF: Days after foliar spraying. CK: distilled water, T1: 0.1 mM MT, T2: 0.2 mM MT, T3: 0.3 mM MT, T4: 0.4 mM MT. Within each sampling date, the data followed with different letters are statistically different at the 0.05 probability level.

According to Figure 2, the application of MT generally significantly improved SOD, POD, and CAT activities over three sampling dates. Although CAT activity showed an increasing trend with increasing MT level on 7 DAF, the beneficial effects of MT on CAT were not statistically significant. Interestingly, the effects of MT on CAT increased with the passage of time. Among three antioxidant enzymes, the MT treatment affected SOD activity to the highest degree. On 21 DAF, T3 enhanced SOD activity by 11.3%, POD activity by 21.6%, and CAT activity by 26.1%.

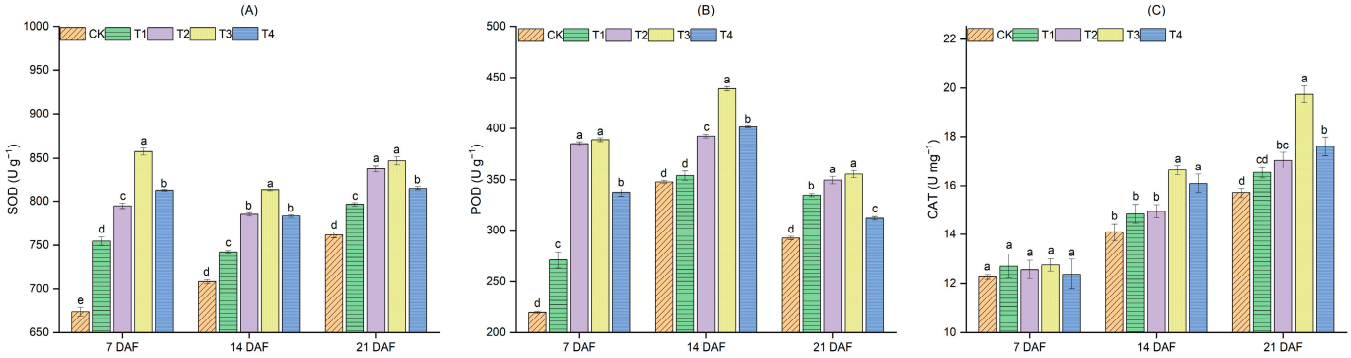

Figure 2. Effects of melatonin on SOD, POD, and CAT activities of alfalfa plants under salt stress. (**A**): SOD; (**B**): POD; (**C**): CAT. DAF: Days after foliar spraying. CK: distilled water, T1: 0.1 mM MT, T2: 0.2 mM MT, T3: 0.3 mM MT, T4: 0.4 mM MT. Within each sampling date, the data followed with different letters are statistically different at the 0.05 probability level.

The Pearson correlation analysis showed that MDA was negatively correlated with all other parameters, whether significantly or insignificantly, while positive correlations were observed within other parameters (Figure 3). Interestingly, leaf length had negative correlations with root length and root surface area, although these correlations were not significant. Except for root surface area and leaf width, aerial dry weight was significantly correlated with other parameters. Among all the measured physiological and biochemical parameters, the largest correlation coefficient (0.84) was obtained between aerial dry weight and SOD, followed by proline. Similar situations were also observed for root dry weight. All these results implied that the salt tolerance of alfalfa was strongly related to the antioxidant capacity and osmotic regulation.

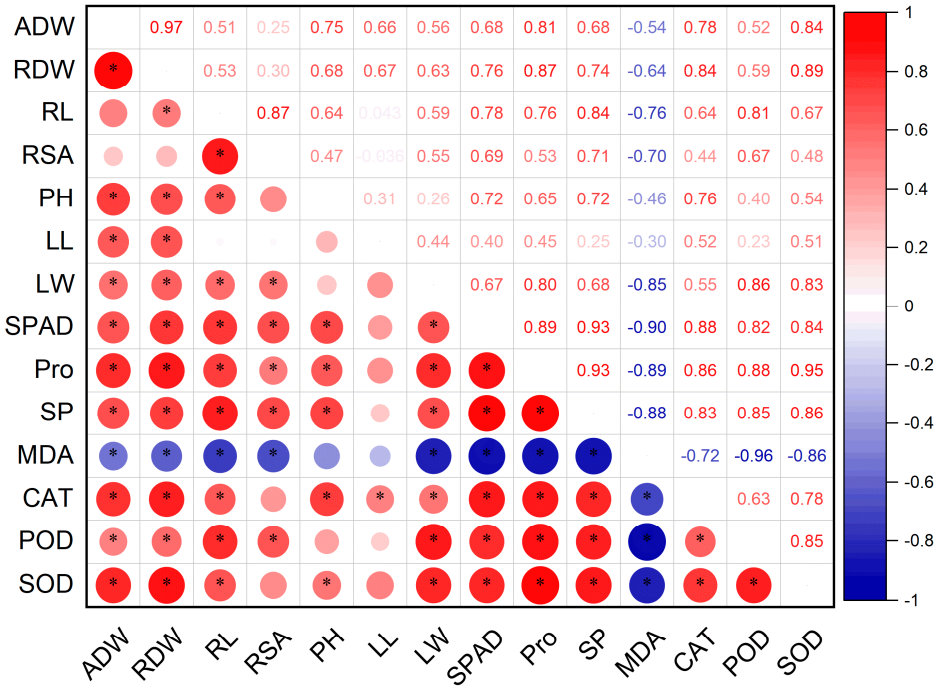

Figure 3. Correlation between measured parameters of alfalfa plants under salt stress. ADW: aerial dry weight; RDW: root dry weight; RL: root length; RSA: root surface area; PH: plant height; LL: leaf length; LW: leaf width; Pro: proline; SP: soluble protein. * Significant difference at $p \leq 0.05$. The numbers are correlation coefficients between the parameters.

4. Discussion

Given that high salinity stress leads to osmotic stress, ion toxicity, and the production of ROS [27], it is essential to improve plant salt tolerance for sustainable agriculture. Melatonin treatment was reported to enhance the salt tolerance of different crops [28–30]. In this study, the effects of melatonin on alfalfa were evaluated in terms of photosynthesis, antioxidant enzymes, and osmoregulation, and the hypothesis that exogenous MT could ameliorate the adverse effects of salt stress on alfalfa seedlings was proved.

In the present study, the application of MT increased root, shoot, and leaf attributes of alfalfa seedlings under salinity stress resulting in increased biomass (both fresh and dry weight). The promotions of MT treatments on alfalfa seedlings were dosage-dependent. However, the highest MT level inhibited root growth. Similar results were obtained by Kamiab, who reported that all the MT treatments except higher levels (125 and 150 µM) remarkably promoted growth characteristics under different salinity levels [31]. Among these measured morphological parameters, aerial dry weight had no significant correlation with leaf width, and a similar correlation was observed between root dry weight and root diameter. These results indicated that the increases in plant height, leaf length, and root length were the direct reason for the enhancement of aerial and root biomass. In this study, leaf and shoot attributes at the highest MT level (T4) were higher than those of the control, while root parameters had the reverse situation, suggesting that the root was more sensitive to MT than the leaf and shoot. This was also confirmed by the higher ratio of root to shoot, with more increase in root dry weight than in aerial dry weight.

Chlorophyll is a vital photosynthetic pigment. It can preliminarily judge leaf function under adverse conditions [32]. The results of this study showed that leaf SPAD readings of alfalfa seedlings treated with MT (especially at the T3 level) were higher than those of the control. On the one hand, melatonin can prevent chlorophyll from being degraded by enzymes such as chlorophyllase, peroxidase, and phenophytinase, and regulates pheophorbide a oxygenase [33]. On the other hand, melatonin can stimulate pigment synthesis by improving the genes participating in the biosynthesis pathway of carotenoids and chloro-

phylls [33–35]. The changes in photosynthetic pigments directly affect the photosynthesis rates in plants [36]. Increased leaf SPAD readings suggested that melatonin ameliorated the adverse effects of salt stress on photosynthesis.

The accumulation of osmotic substances plays a vital role in maintaining cell stability and protecting cells from being damaged by salinity stress [37]. In the present study, melatonin treatment enhanced the content of proline and soluble protein. The increase in soluble protein content could be due to the fact that melatonin can act as a scavenger of reactive oxygen species to decrease excess intracellular ROS and inhibit protein degradation or promote the synthesis of new proteins under salt stress [38]. It is well known that proline provides protection against salinity in plants by playing the roles of compatible osmolytes, enzyme protectant, free radical scavenger, cell redox balancer, cytosolic pH buffer, and subcellular structure stabilizer [39,40]. The increase in proline content might result from increased osmotic regulation and salt tolerance [37]. The additive effect of melatonin on these characteristics under stress conditions depended on the concentration, and an appropriate concentration of melatonin could mitigate the peroxidation damage to membrane lipids.

Lipid peroxidation is an indicator of cell membrane damage under stress conditions. The change in MDA content is generally considered a suitable indicator of oxidative damage, reflecting the stability of cell membranes [41]. Pearson correlation analyses showed that MDA was negatively correlated with all the other measured characteristics. The MDA content in this study was lower in the MT treatments than in the non-MT treatment, suggesting that MT application ameliorated the harmful effects of salt stress on alfalfa seedlings. These findings confirmed the previous reports of Wei et al., who reported that banana seedlings applied with 60 mM NaCl and 100 μM MT showed lower MDA content and membrane permeability than plants applied with only 60 mM NaCl [42].

Compared with normal growth conditions, the contents of free radicals and reactive oxygen species are usually enhanced under stress conditions [43]. SOD, in the antioxidant defense systems, is the first line of defense against oxidative stress, which reduces O_2^- to H_2O_2. CAT can then convert H_2O_2 to H_2O in plant cells [12]. The foliar spray of MT increased the activities of SOD, POD, and CAT in this study. Combined with MDA content, these results suggested that melatonin could eliminate ROS by inducing the activities of antioxidant enzymes, which helps to maintain the stability and integrity of cell membranes, reduce cell membrane damage, and recover from salt stress. However, CAT activity was not remarkably affected by lower MT level (T1), and all the MT levels had no significant effects on CAT activity on 7 DAF. This revealed that CAT activity was less sensitive to MT than SOD and POD. Pearson correlation analyses that among these three antioxidant enzymes, the coefficient between SOD and dry biomass was the largest also partially confirmed it.

The Pearson correlation analysis showed that the growth attributes of alfalfa were positively correlated with the contents of proline and soluble protein, as well as the activities of CAT, POD, and SOD. This indicated that the foliar spray of MT alleviated the adverse effects induced by salt on alfalfa by improving osmotic regulation and enhancing antioxidant ability so as to reduce the accumulation of ROS, maintain the permeability and integrity of cells, and reduce the decomposition of chlorophyll. The decrease in MDA content and the increase in SPAD readings of alfalfa treated with MT also confirmed this.

5. Conclusions

This study implied that the MT foliar application (particularly 0.3 mM) ameliorated the damage of salinity stress on alfalfa. The alleviation may be attributed to increased antioxidant capacity, osmotic regulation, photosynthesis, and decreased MDA content. These positive changes in the physiological and biochemical parameters of alfalfa resulted in increased morphological characteristics and ultimately showed an increase in biomass.

Author Contributions: Conceptualization, X.G., Y.S. and G.Z. (Guisheng Zhou); data curation, G.Z. (Guanglong Zhu) and G.Z. (Guisheng Zhou); formal analysis, X.G. and Y.S.; funding acquisition, G.Z. (Guisheng Zhou); investigation, X.G. and Y.S.; project administration, G.Z. (Guisheng Zhou); Supervision, G.Z. (Guanglong Zhu); writing—original draft, X.G.; writing—review and editing, X.G., G.Z. (Guanglong Zhu) and G.Z. (Guisheng Zhou). All authors contributed to the article. All authors have read and agreed to the published version of the manuscript.

Funding: This study is financially supported by China National Key R and D Program (2022YFE0113400).

Data Availability Statement: Data used in this article are present in the tables and figures.

Conflicts of Interest: The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Anower, M.R.; Peel, M.D.; Mott, I.W.; Wu, Y. Physiological processes associated with salinity tolerance in an alfalfa half-sib family. *J. Agron. Crop Sci.* **2017**, *203*, 506–518. [CrossRef]
2. Zhang, C.; Shi, S.; Wang, B.; Zhao, J. Physiological and biochemical changes in different drought-tolerant alfalfa (*Medicago sativa* L.) varieties under PEG-induced drought stress. *Acta Physiol. Plant.* **2018**, *40*, 25. [CrossRef]
3. Maas, E.V.; Grattan, S.R. Crop yields as affected by salinity. In *Agronomy Monograph No. 38*; Skaggs, R.W., van Schilfgaarde, J., Eds.; American Society of Agronomy, Inc.: Madison, WI, USA, 1999; pp. 55–108.
4. Nabizadeh, E.; Jalilnejad, N.; Armakani, M. Effect of salinity on growth and nitrogen fixation of alfalfa (*Medicago sativa* L.). *World Appl. Sci. J.* **2011**, *13*, 1895–1900.
5. Anower, M.R.; Mott, I.W.; Peel, M.D.; Wu, Y. Characterization of physiological responses of two alfalfa half-sib families with improved salt tolerance. *Plant Physiol. Biochem.* **2013**, *71*, 103–111. [CrossRef] [PubMed]
6. Ansari, M.; Shekari, F.; Mohammadi, M.H.; Juhos, K.; Végvári, G.; Biró, B. Salt-tolerant plant growth-promoting bacteria enhanced salinity tolerance of salt-tolerant alfalfa (*Medicago sativa* L.) cultivars at high salinity. *Acta Physiol. Plant.* **2019**, *41*, 195. [CrossRef]
7. Bansal, S.; Yin, X.H. Effects of salinity, growth condition, and soil type on shoot dry matter of Alfalfa (*Medicago sativa*)—A meta-analysis. *Commun. Soil Sci. Plan* **2021**, *52*, 201–211. [CrossRef]
8. Singh, K.N.; Chatrath, R. Salinity tolerance. In *Application of Physiology in Wheat Breeding*; Reynolds, M.P., Ortiz-Monasterio, J.J., McNab, A., Eds.; CIMMYT: Texcoco, Mexico, 2001; pp. 101–110.
9. Acosta-Motos, J.R.; Ortuño, M.F.; Bernal-Vicente, A.; Díaz-Vivancos, P.; Sánchez-Blanco, M.J.; Hernandez, J.A. Plant responses to salt stress: Adaptive mechanisms. *Agronomy* **2017**, *7*, 18. [CrossRef]
10. Dubbels, R.; Reiter, R.J.; Klenke, E.; Goebel, A.; Schnakenberg, E.; Ehlers, C.; Schiwara, H.W.; Schloot, W. Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography-mass spectrometry. *J. Pineal Res.* **1995**, *18*, 28–31. [CrossRef]
11. Zhao, D.; Yu, Y.; Shen, Y.; Liu, Q.; Zhao, Z.; Sharma, R.; Reiter, R.J. Melatonin Synthesis and Function: Evolutionary History in Animals and Plants. *Front. Endocrinol.* **2019**, *10*, 249. [CrossRef]
12. Azizi, F.; Amiri, H.; Ismaili, A. Melatonin improves salinity stress tolerance of Phaseolus vulgaris L. cv. Pak by changing antioxidant enzymes and photosynthetic parameters. *Acta Physiol. Plant.* **2022**, *44*, 40. [CrossRef]
13. Lei, Q.; Wang, L.; Tan, D.X.; Zhao, Y.; Zheng, X.D.; Chen, H.; Li, Q.T.; Zuo, B.X.; Kong, J. Identification of genes for melatonin synthetic enzymes in 'Red Fuji' apple (*Malus domestica Borkh. cv. Red*) and their expression and melatonin production during fruit development. *J. Pineal Res.* **2013**, *55*, 443–451. [CrossRef]
14. Park, S.; Back, K. Melatonin promotes seminal root elongation and root growth in transgenic rice after germination. *J. Pineal Res.* **2012**, *53*, 385–389. [CrossRef]
15. Tiryaki, I.; Keles, H. Reversal of the inhibitory effect of light and high temperature on germination of Phacelia tanacetifolia seeds by melatonin. *J. Pineal Res.* **2011**, *52*, 332–339. [CrossRef]
16. Wang, P.; Yin, L.; Liang, D.; Li, C.; Ma, F.; Yue, Z. Delayed senescence of apple leaves by exogenous melatonin treatment: Toward regulating the ascorbate-glutathione cycle. *J. Pineal Res.* **2012**, *53*, 11–20. [CrossRef]
17. Shi, H.; Wei, Y.; Wang, Q.; Reiter, R.J.; He, C. Melatonin mediates the stabilization of DELLA proteins to repress the floral transition in *Arabidopsis*. *J. Pineal Res.* **2016**, *60*, 373–379. [CrossRef]
18. Wang, Y.; Reiter, R.J.; Chan, Z. Phytomelatonin: A universal abiotic stress regulator. *J. Exp. Bot.* **2017**, *69*, 963–974. [CrossRef]
19. Arnao, M.B.; Hernández-Ruiz, J. Melatonin and its relationship to plant hormones. *Ann. Bot.* **2017**, *121*, 195–207. [CrossRef]
20. Baath, G.S.; Shukla, M.K.; Bosland, P.W.; Steiner, R.L.; Walker, S.J. Irrigation water salinity influences at various growth stages of *Capsicum annuum*. *Agric. Water Manag.* **2017**, *179*, 246–2533. [CrossRef]
21. Shiyab, S.M.; Shatnawi, M.A.; Shibli, R.A.; Smeirat, N.G.A.; Ayad, J.; Akash, M.W. Growth, nutrient acquisition, and physiological responses of hydroponic grown tomato to sodium chloride salt induced stress. *J. Plant Nutr.* **2013**, *36*, 665–676. [CrossRef]
22. Gao, J.F. *Plant Physiology Experiment Instruction*; Higher Education Press: Beijing, China, 2006; pp. 210–211. (In Chinese)
23. Zhou, Q. *Plant Physiology Experiment Instruction*; China Agriculture Press: Beijing, China, 2000; pp. 167–169. (In Chinese)

24. Zhang, Z.L.; Zhai, W.J. *Plant Physiology Experiment Instruction*; Higher Education Press: Beijing, China, 2005; pp. 123–124. (In Chinese)
25. Liu, Z.Q.; Zhang, S.C. *Physiology of Plant Resistance*; Agriculture Press: Beijing, China, 1994; pp. 371–372. (In Chinese)
26. Li, H.S. *Principles and Techniques of Plant Physiological and Biochemical Experiments*; Higher Education Press: Beijing, China, 2001; pp. 258–260. (In Chinese)
27. Sreenivasulu, N.; Grimm, B.; Wobus, U.; Weschke, W. Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica*). *Physiol. Plant.* **2000**, *109*, 435–442. [CrossRef]
28. Meftahizadeh, H.; Baath, G.S.; Saini, R.K.; Falakian, M.; Hatami, M. Melatonin-Mediated Alleviation of Soil Salinity Stress by Modulation of Redox Reactions and Phytochemical Status in Guar (*Cyamopsis tetragonoloba* L.). *J. Plant Growth Regul.* **2022**, *42*, 1118–1126. [CrossRef]
29. Park, H.-S.; Kazerooni, E.A.; Kang, S.-M.; Al-Sadi, A.M.; Lee, I.-J. Melatonin Enhances the Tolerance and Recovery Mechanisms in *Brassica juncea* (L.) Czern. Under Saline Conditions. *Front. Plant Sci.* **2021**, *12*, 593717. [CrossRef]
30. Ren, Z.; Shi, J.; Guo, A.; Wang, Y.; Fan, X.; Li, R.; Yu, C.; Peng, Z.; Gao, Y.; Liu, Z.; et al. Melatonin Mediates the Regulation of Morphological and Anatomical Traits in *Carex leucochlora* under Continuous Salt Stress. *Agronomy* **2022**, *12*, 2098. [CrossRef]
31. Kamiab, F. Exogenous melatonin mitigates the salinity damages and improves the growth of pistachio under salinity stress. *J. Plant Nutr.* **2020**, *43*, 1468–1484. [CrossRef]
32. Yao, X.; Zhou, M.; Ruan, J.; Peng, Y.; Ma, C.; Wu, W.; Gao, A.; Weng, W.; Cheng, J. Physiological and Biochemical Regulation Mechanism of Exogenous Hydrogen Peroxide in Alleviating NaCl Stress Toxicity in Tartary Buckwheat (*Fagopyrum tataricum* (L.) Gaertn). *Int. J. Mol. Sci.* **2022**, *23*, 10698. [CrossRef]
33. Sharif, R.; Xie, C.; Zhang, H.; Arnao, M.B.; Ali, M.; Ali, Q.; Muhammad, I.; Shalmani, A.; Nawaz, M.A.; Chen, P.; et al. Melatonin and Its Effects on Plant Systems. *Molecules* **2018**, *23*, 2352. [CrossRef]
34. Siddiqui, M.H.; Alamri, S.; Alsubaie, Q.D.; Ali, H.M. Melatonin and Gibberellic Acid Promote Growth and Chlorophyll Biosynthesis by Regulating Antioxidant and Methylglyoxal Detoxification System in Tomato Seedlings Under Salinity. *J. Plant Growth Regul.* **2020**, *39*, 1488–1502. [CrossRef]
35. Siddiqui, M.H.; Alamri, S.; Khan, M.N.; Corpas, F.J.; Al-Amri, A.A.; Alsubaie, Q.D.; Ali, H.M.; Kalaji, H.M.; Ahmad, P. Melatonin and calcium function synergistically to promote the resilience through ROS metabolism under arsenic-induced stress. *J. Hazard. Mater.* **2020**, *398*, 122882. [CrossRef]
36. Yang, X.; Han, Y.; Hao, J.; Qin, X.; Liu, C.; Fan, S. Exogenous spermidine enhances the photosynthesis and ultrastructure of lettuce seedlings under high-temperature stress. *Sci. Hortic.* **2022**, *291*, 110570. [CrossRef]
37. Chen, L.; Liu, L.; Lu, B.; Ma, T.; Jiang, D.; Li, J.; Zhang, K.; Sun, H.; Zhang, Y.; Bai, Z.; et al. Exogenous melatonin promotes seed germination and osmotic regulation under salt stress in cotton (*Gossypium hirsutum* L.). *PLoS ONE* **2020**, *15*, e0228241. [CrossRef]
38. Gao, W.; Feng, Z.; Bai, Q.; He, J.; Wang, Y. Melatonin-Mediated Regulation of Growth and Antioxidant Capacity in Salt-Tolerant Naked Oat under Salt Stress. *Int. J. Mol. Sci.* **2019**, *20*, 1176. [CrossRef]
39. Iqbal, N.; Umar, S.; Khan, N.A.; Khan, M.I.R. A new perspective of phytohormones in salinity tolerance: Regulation of proline metabolism. *Environ. Exp. Bot.* **2014**, *100*, 34–42. [CrossRef]
40. Alam, P.; Albalawi, T.H.; Altalayan, F.H.; Bakht, A.; Ahanger, M.A.; Raja, V.; Ashraf, M.; Ahmad, P. 24-Epibrassinolide (EBR) Confers Tolerance against NaCl Stress in Soybean Plants by Up-Regulating Antioxidant System, Ascorbate-Glutathione Cycle, and Glyoxalase System. *Biomolecules* **2019**, *9*, 640. [CrossRef]
41. Chen, Y.-E.; Mao, J.-J.; Sun, L.-Q.; Huang, B.; Ding, C.-B.; Gu, Y.; Liao, J.-Q.; Hu, C.; Zhang, Z.-W.; Yuan, S.; et al. Exogenous melatonin enhances salt stress tolerance in maize seedlings by improving antioxidant and photosynthetic capacity. *Physiol. Plant.* **2018**, *164*, 349–363. [CrossRef]
42. Wei, J.; Liang, J.; Liu, D.; Liu, Y.; Liu, G.; Wei, S. Melatonin-induced physiology and transcriptome changes in banana seedlings under salt stress conditions. *Front. Plant Sci.* **2022**, *13*, 938262. [CrossRef]
43. Esboei, M.M.; Ebrahimi, A.; Amerian, M.R.; Alipour, H. Melatonin confers fenugreek tolerance to salinity stress by stimulating the biosynthesis processes of enzymatic, non-enzymatic antioxidants, and diosgenin content. *Front. Plant Sci.* **2022**, *13*, 890613. [CrossRef]