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Physio-Biochemical Responses of Three *Aquilegia* Species Seedlings to Salt Stress

Lifei Chen †, Yuan Meng †, Dawei Jiang †, Fengrui Yang and Yunwei Zhou *

College of Horticulture of Jilin Agricultural University, 2888 Xincheng Street, Changchun 130118, China
* Correspondence: yunweizh@jlau.edu.cn
† These authors contributed equally to this work.

Abstract: Road deicing salts are widely used during winter in northern China, which makes it essential to choose proper salt-tolerant plant species in urban landscapes. Columbine (*Aquilegia*) is a herb with high ornamental and commercial values. This study evaluated three *Aquilegia* species (*A. oxysepala*, *A. parviflora*, and *A. viridiflora*) for salt tolerance by monitoring their germination state under different electrical conductivity (EC) of 0.1 dS·m^{-1} (distilled water), 1.0 dS·m^{-1}, 2.0 dS·m^{-1}, 3.0 dS·m^{-1}, 4.0 dS·m^{-1}, 5.0 dS·m^{-1}, 6.0 dS·m^{-1}, physio-biochemical responses to different EC of 0.3 dS·m^{-1} (tap water), 5.0 dS·m^{-1}, and 10.0 dS·m^{-1}. The germination and growth parameters, visual scores, dry weight, leaf stomatal conductance, photosynthetic rate, and chlorophyll contents of three species decreased under salt stress, which was opposite to the changes of electrolyte leakage, malondialdehyde, proline, and soluble sugar contents. Superoxide dismutase and peroxidase activity trend differently among species. These results showed that the germination threshold of three species was 6.0 dS·m^{-1}. *A. oxysepala* was the most salt-tolerant species, with a tolerance threshold of soil conductivity in 2.83 dS·m^{-1}, followed by *A. viridiflora* and *A. parviflora*. Therefore, *A. oxysepala* is suitable for planting as a ground cover in urban areas where deicing salt is applied.

Keywords: perennial; salt tolerance; gas exchange; visual score; osmotic adjustment

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1. Introduction

Previous research demonstrated that deicing salts could degrade irrigation water quality and increase soil salinity [1,2]. In the 2000s, 53 billion kg of deicing salt was applied to improve road accessibility in the US [2]. During the winter from 2009 to 2010, approximately three tons of salt was applied as a snow-melting agent in Beijing, to allow for smooth traffic. As a result, the salt content in soil from the ground surface was 0.11–0.29%, 1.83–4.83 times more than the control [3]. With surface runoff, soil infiltration and other actions, deicing salt flows to different surrounding soil areas, and is finally absorbed by plant roots [4]. Excessive toxic ions accumulated in the soil directly affect plant growth, and it is highly correlated with plant leaves damage and vitality decline [5]. With the increasing salt stress, choosing proper salt-tolerant plant species has been essential in horticultural breeding and agricultural plantations. To evaluate the irrigation water quality, total soluble salts (TSS) are measured by electrical conductivity (EC) [6], and it was widely applied in numerous studies to simulate the salt stress condition [7–9].

The effects of salt stress on plants are mainly divided into osmotic stress and ionic toxicity [10]. In the first stage, to prevent water loss, plants rapidly synthesize osmotic regulatory substances and regulate stomatal conductance, which is reflected in the change of biochemical index and volatility of photosynthetic parameters [11]. In the second stage, a substantial accumulation of toxic ions is the main cause of tissue damage, reflected in the visual quality and membrane protection system [12]. The main deicing salt ingredients are sodium chloride and calcium chloride [1]; their toxic effects are manifested in germination rate decline, growth inhibition, organic matter accumulation descending, and yield

reduction [12–15]. According to previous research, different species or cultivars have different abilities to endure salt stress. *Viburnum* × *burkwoodii* and *V.* × 'NCVX1' are tolerant to salinity, while *V. dilatatum* 'Henneke', *V. plicatum* var. *tomentosum* 'Summer Snowflake,' and *V. trilobum* are salt-sensitive [7]. *Phlox paniculata* 'John Fanick' and *P. paniculata* 'Texas Pink' showed varying degrees of damage [16]. A 'Crioula' guava rootstock is more salt-tolerant than 'Paluma' and 'Ogawa' [17].

Aquilegia L. was established by Linnaeus in 1953; it originated in Europe and North America. *Aquilegia* is a perennial ornamental plant that belongs to Ranunculaceae. There are about 70 species in this genus, which is now widely distributed in northern hemisphere countries [18]. *Aquilegia* could be used in civil engineering, yard and roadside gardening, and sightseeing establishments as bedding plants because of their elegant foliage shapes, unique floral characters, and well-adaptive features. Thus, *Aquilegia* has great application potential. Since the survival condition and adaptation of *Aquilegia* regarding salt tolerance are poorly reported, this study attempted to examine the physio-biochemical responses to simulated saltwater irrigation of three *Aquilegia* seedlings.

2. Materials and Methods

2.1. Plant Materials

A. viridiflora, *A. parviflora*, and *A. oxysepala* seeds were purchased from Situ Nursery (Shuyang, Jiangsu, China). All seeds were sown in a substrate with a volume ratio of turf: sand = 1:1 in 6 × 6 cm float trays on 10 May 2018. The seeds germinated in a greenhouse of Jilin Agricultural University, Changchun, China (43°48′ N, 125°24′ E). The annual mean, maximum, and minimum temperatures were 6.46 °C, 28 °C, and −20 °C, respectively. The mean illumination and relative humidity during the experiment were 6000 lux and 70.4%, respectively. Healthy seedlings with similar height and width were transplanted in 9 × 8 cm propagation cells with a drain hole in the bottom when the seedlings had four true leaves. The substrate was prepared with a volume ratio of loam: turf: perlite = 3:2:1. A total of 30 seedlings of each species were prepared for each treatment. All seedlings were well-irrigated with tap water until 15 July 2018.

2.2. Salt Treatments

2.2.1. Seeds Germination Salt Treatment

All *Aquilegia* seeds were sterilized using 0.1% $KMnO_4$ solution and then rinsed 3–5 times with distilled water. Since NaCl and $CaCl_2$ are the most common ingredients in deicing salts [1,9], treatment solution with electrical conductivity (EC) of 1 ± 0.1 dS·m^{-1} (EC 1), 2.0 ± 0.2 dS·m^{-1} (EC 2), 3.0 ± 0.2 dS·m^{-1} (EC 3), 4.0 ± 0.2 dS·m^{-1} (EC 4), 5.0 ± 0.2 dS·m^{-1} (EC 5), 6.0 ± 0.2 dS·m^{-1} (EC 6) in this study were prepared by adding NaCl and $CaCl_2$ in distilled water at a molar ratio of 2:1 [9]. All solution pH was adjusted to 5.8 ± 0.2 (Table 1). Distilled water was used as a control. Each petri dish (9 cm in diameter) contained two pieces of filter paper and 5 mL treatment solution; 30 seeds were placed in each petri dish. The germination condition was 20 °C, 12 h of light in an artificial climate incubator. The filter paper was changed every two days to maintain electrical conductivity stability. The germination seeds were counted every 24 h, and radicle length ≥ 2 times of seed diameter was taken as the standard germination count. The experiment terminated when the number of germinated seeds in each treatment did not change for two consecutive days.

2.2.2. Seedlings Salt Treatment

Treatment solutions with EC of 0.3 ± 0.1 dS·m^{-1} (control), 5.0 ± 0.2 dS·m^{-1} (EC 5), and 10.0 ± 0.2 dS·m^{-1} (EC 10) in this study were prepared by adding NaCl and $CaCl_2$ into tap water at a molar ratio of 2:1 [9]. All solution pH was adjusted to 5.8 ± 0.2 (Table 2). According to the phenotypic changes in the pre-experiment, the treatment solutions were set as the irrigation with a 10–20% leaching fraction in all treatments every five days from 16 July to 23 September 2018. The experiment ended when the plants died under EC 10 treatment. The leachate EC was measured after irrigation (Figure 1). Then, 50 mL of tap

water was irrigated to avoid confounding drought effects. This experiment was conducted from July to September, so the temperature and sun illumination were higher than at other times; all seedlings were planted in 9 × 8 cm propagation cells; therefore, although all seedlings were placed under shade, the water in the substrate soon evaporated. Increased amounts and frequency of tap water irrigation are needed to meet the requirement of regular growth. This might prolong the schedule of the experiment.

Table 1. The electrical conductivity (EC) and composition of control and salt solutions used in the study.

Treatment	EC ($dS \cdot m^{-1}$) [z]	NaCl (mM) [y]	$CaCl_2$ (mM) [y]
Control	0.1	—	—
EC 1	1.0	5.1	2.4
EC 2	2.0	10.7	5.5
EC 3	3.0	16.6	8.5
EC 4	4.0	22.0	11.3
EC 5	5.0	27.6	14.1
EC 6	6.0	34.2	17.5

[z] EC of control treatment and salt treatments, including salts and distilled water. [y] Calculated amount of salt (NaCl or $CaCl_2$).

Table 2. The electrical conductivity (EC) and composition of control and salt solutions used in the study.

Treatment	EC ($dS \cdot m^{-1}$) [z]	NaCl (mM) [y]	$CaCl_2$ (mM) [y]
Control	0.3	—	—
EC 5	5.0	22.1	11.3
EC 10	10.0	50.4	25.9

[z] EC of control treatment and salt treatments, including salts and tap water. [y] Calculated amount of salt (NaCl and $CaCl_2$).

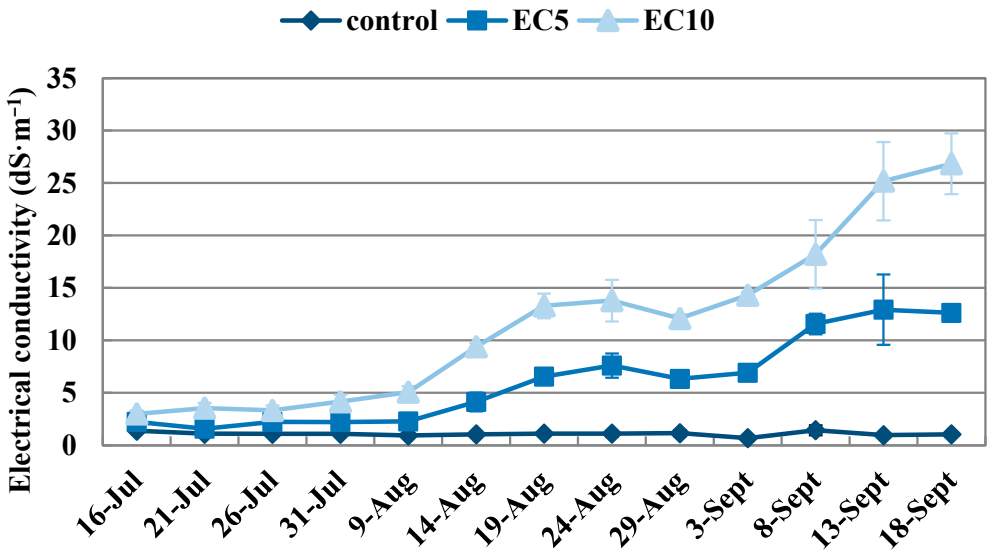

Figure 1. Leachate electrical conductivity (EC) from 16 July to 18 September 2018. Control, EC5, and EC10 correspond to the respective treatments of irrigation solution: tap water (control), EC of 5 $dS \cdot m^{-1}$, and 10 $dS \cdot m^{-1}$, respectively. Vertical bars represent standard error (SE).

2.3. Experimental Design and Statistical Analysis

This experiment had a split-plot design with different salt treatments as the main plots and different *Aquilegia* species as the subplot. All treatments were repeated three times. Tukey analysis of variance was performed at a significance level of $p < 0.05$. A two-way analysis was performed on the main and interaction effects. All statistical tests were conducted using SPSS (IBM SPSS Statistics 20, New York, USA).

2.4. Data Collection and Measurement

2.4.1. Germination Parameters

(1) Germination percentage (%) = (number of germinated seeds/total seeds number) × 100 [19].
(2) Germination potential (%) = (number of germinated seeds when the number of germinated seeds reached the peak/total seeds number) × 100 [19]
(3) Germination index = Σ(the number of germinated seeds on day t/the corresponding germination days) [19].
(4) Radicel length: At the end of the experiment, the average length of 10 radicles, which had been randomly selected, was measured with a ruler in millimeters [19].
(5) Vitality index = Σ(the number of germinated seeds each day/germination days) × radicle length [20].

2.4.2. EC Parameters

The EC of leachate was measured by portable EC meter-5061 (Sanxin Instrument Company, Shanghai, China) and EC meter (DDSJ-318; Shanghai Yidian Scientific Instrument, Shanghai, China). The final EC of rhizosphere soil was measured at the end of the experiment (water: soil = 5:1) [21].

2.4.3. Plant Growth

Growth parameters were measured as plant height, crown diameter, shoot dry weight (DW), and root DW. Plant height (cm) was the length from the pot rim to the tallest growth point of the seedlings. The crown diameter was the average width from two directions perpendicular to each other, which were randomly selected from the top of the plant. DW was measured after drying fresh tissues at 80 °C until DW was constant.

The visual quality of each plant was subjectively measured by specific scores. The damage state was divided into six levels: 5 for no visible damage, 4 for slight damage, 3 for less than 50% foliage injury, 2 for 50–90% foliage injury, 1 for over 90% foliage damage, and 0 for death [22].

All parameters were measured after the first two irrigations, one week before the end of the experiment, and then calculate the average value.

2.4.4. Photosynthetic Parameters

The third or fourth fully expanded leaves, which were counted upward from the soil surface, were taken as samples to measure the photosynthetic indexes. The photosynthesis (Pn), stomatal conductance (g_s), leaf transpiration rate (E), and intercellular CO_2 concentration (Ci) of the seedlings were measured with CIRAS-2 portable photosynthetic apparatus (PP system, Amesbury, MA, USA). The light intensity of LEDs was set as 1000–1400 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The photosynthetic indexes were measured after the first two irrigations, then the average value was calculated.

2.4.5. Biochemical Analyses

All biochemical parameters were measured after the first two irrigations and one week before the end of the experiment; the average value was then calculated. The chlorophyll content was determined using the alcohol and acetone method [23]. 0.05 g of fresh leaves tissue was immersed in 15 mL acetone: ethanol 1:1 solution. After the tissue faded and whitened completely, the absorbance density was measured at 665 nm and 649 nm.

Electrolyte leakage (EL) was measured by the conductivity method [24]. 0.2 g clean, fresh leaves tissue was immersed in 20 mL distilled water; then, after 8 min vacuuming, they were left to stand for 20 min before measuring the electrical conductivity. They were boil water bathed for 25 min and then measured the electrical conductivity again.

The malondialdehyde (MDA) content was measured through the method of trichloroacetic acid (TCA) and thiobarbituric acid (TBA) [25]. 0.2 g fresh leaves tissue was ground with 10 mL 5% TCA, and then after centrifugation for 10 min, 2 mL supernatant was boil water

bathed for 30 min with 2 mL 0.6% TBA. Supernatant absorbance density was measured at 532 nm, 600 nm, and 450 nm.

The soluble sugar content (mg/g) and proline content (μg/g) were measured using the anthrone method [26] and the ninhydrin method [27], respectively. To measure the soluble sugar content, 0.5 g fresh leaves tissue was boil water bathed for 20 min with 15 mL distilled water. Then 1 mL supernatant and 5 mL anthranone-sulfuric acid mixture were boil water bathed for 10 min. The absorbance density was measured at 620 nm. To measure the proline content, 0.5 g fresh leaves tissue was boil water bathed for 15 min with 5 mL 3% sulfosalicylic acid. Then 2 mL supernatant, 2 mL acetic acid, and 2 mL ninhydrin mixture were boil water bathed for 15 min. 5 mL toluene was added to extract proline. The absorbance density of the toluene solution was measured at 520 nm.

The superoxide dismutase (SOD) activity (U/g) was measured with the nitroblue tetrazolium (NBT) method [24]. To obtain the enzyme solution, 0.5 g of fresh leaves tissue was ground with 5 mL 0.05 mol·L^{-1} phosphate buffer solution (pH = 7.8). The mixture of enzyme solution, phosphate buffer solution, methionine, NBT, EDTA-Na_2, riboflavin, and distilled water was reacted for 10 min. The absorbance density of the mixture was measured at 560 nm. The amount of enzyme inhibiting 50% of NBT photoreduction was used as one enzyme activity unit (U), and SOD activity was expressed as the unit ratio of enzyme activity per gram of fresh tissue weight (U/g).

The peroxidase (POD) activity (U/g) was measured with the guaiacol method [27]. To obtain the enzyme solution, 1 g of fresh leaves tissue was ground with 5 mL 0.1 mol·L^{-1} phosphate buffer solution (pH = 6). The absorbance density of the enzyme solution mixed with guaiacol and distilled water was recorded every minute at 470 nm. The amount of enzyme with a 0.01 increase in absorbance value per minute at 470 nm was used as one enzyme activity unit (U), POD activity was expressed as the unit ratio of enzyme activity per gram of fresh weight (U/g).

Absorbance density was measured by a visible light spectrophotometer (722N, INESA, Shanghai, China). Electrical conductivity was measured by a conductivity meter (DDSJ-318; Shanghai Yidian Scientific Instrument, Shanghai, China).

3. Results

3.1. Germination Process

Seed germination of three *Aquilegia* species was inhibited under salt treatment (Figure 2). With the increase of the EC, the germination rate of three *Aquilegia* seeds decreased, and the initiation time of germination was delayed. For the control group, it took nine days to germinate, and the germination peak was approximately 12–14 days. For the *Aquilegia* seeds treated with EC 1, the initial germination time was delayed for 1–2 days, and the peak germination time was delayed for 1–3 days. None of the seeds germinated normally when the conductivity was 6 dS·m^{-1}.

3.2. Germination Parameters

With the increase of electrical conductivity, the germination rate, germination potential, germination index, radicle length, and vital index of three species of *Aquilegia* seeds were inhibited (Table 3). Salt treatments had an extremely significant impact on all indicators. Under the control conditions, the germination rate of *A. parviflora* was significantly lower than the other two species. With the treatment of EC 2, the germination rate of the three *Aquilegia* species decreased significantly compared to those in the control group. When the solution EC reached 3 dS·m^{-1}, the germination rate of *A. viridiflora* dropped significantly compared with control. The changes in germination potential of *A. parviflora* were not significant compared with the control until EC reached 5 dS·m^{-1}. However, all seeds' germination index, radicle length, and vital index decreased significantly under EC 2 compared to those in the control group. When the solution EC reached 6 dS·m^{-1}, all seeds could not to germinate.

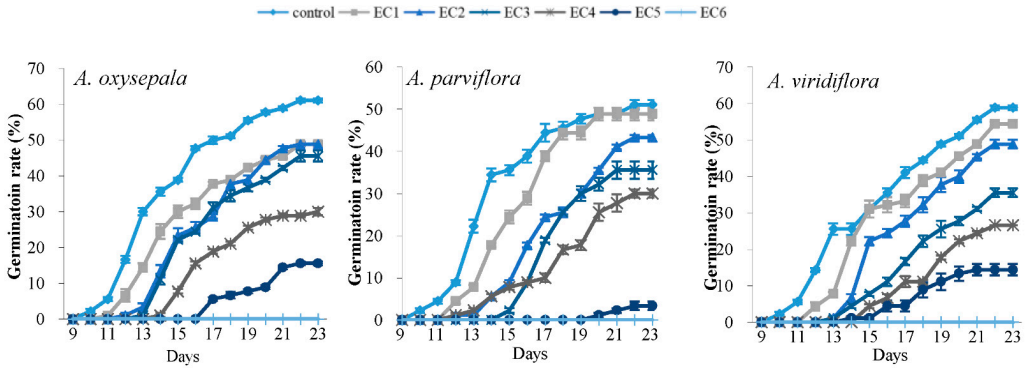

Figure 2. Average daily germination percentage of three *Aquilegia* seeds. Control, EC 1, EC 2, EC 3, EC 4, EC 5, and EC 6 correspond to the respective treatments of irrigation solution: distilled water (control), EC of 1 dS·m^{-1}, 2 dS·m^{-1}, 3 dS·m^{-1}, 4 dS·m^{-1}, 5 dS·m^{-1}, 6 dS·m^{-1}, respectively. Vertical bars represent standard error (SE).

Table 3. Germination rate, germination potential, germination index, radicle length, and vital index of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* under the treatments of distilled water (control) and salt solution at electrical conductivity (EC) of 1 dS·m^{-1} (EC 1), 2 dS·m^{-1} (EC 2), 3 dS·m^{-1} (EC 3), 4 dS·m^{-1} (EC 4), 5 dS·m^{-1} (EC 5), and 6 dS·m^{-1} (EC 6).

Variety	Treatments	Germination Rate (%)	Germination Potential (%)	Germination Index	Radicle Length (mm)	Vital Index
A. oxysepala	Control	61.1 Aa [x]	30.0 Aa	1.33 Aa	32.1 Aa	42.7 Aa
	EC 1	48.9 Ab	24.4 Aab	0.99 Ab	13.0 Bb	12.8 Bb
	EC 2	48.9 Ab	23.3 Aab	0.91 Abc	11.5 Ab	10.4 Abc
	EC 3	45.6 Ab	22.2 Aab	0.84 Ac	10.0 Abc	8.4 Abc
	EC 4	30.0 Ac	15.6 Ab	0.53 Ad	6.8 Abc	3.6 Ac
	EC 5	15.6 Ad	5.6 Ac	0.25 Ae	3.2 Ac	0.8 Ac
	EC 6	— [y]	—	—	—	—
A. parviflora	Control	51.1 Ba	22.2 Aa	1.10 Ba	29.6 Aa	32.6 Aa
	EC 1	48.9 Aa	17.8 Aa	0.97 Ab	23.6 Aa	22.7 Ab
	EC 2	35.6 Bb	17.8 Aa	0.64 Bb	14.2 Ab	9.2 ABc
	EC 3	35.6 Ab	18.9 Aa	0.61 Bb	11.5 Ab	6.9 Ac
	EC 4	30.0 Ab	16.7 Aa	0.52 Ab	9.9 Ab	5.1 Acd
	EC 5	3.33 Bc	1.1 Ab	0.05 Bc	0.7 Ac	0.31 Ad
	EC 6	—	—	—	—	—
A. viridiflora	Control	58.9 Aa	25.6 Aa	1.22 Aba	34.5 Aa	42.0 Aa
	EC 1	54.4 Aab	22.2 Aab	1.04 Aab	20.2 Ab	21.0 Ab
	EC 2	48.9 Ab	22.2 Aab	0.88 Ab	14.7 Abc	12.8 Ac
	EC 3	35.6 Ac	16.7 Aab	0.61 Abc	10.5 Ac	6.2 Ad
	EC 4	26.7 Ad	11.1 Ab	0.45 Ac	6.3 Ac	2.8 Bde
	EC 5	14.4 Ae	8.9 Ab	0.25 Ad	3.6 Ac	0.9 Ae
	EC 6	—	—	—	—	—
Treatments		*** [z]	***	***	***	***
Species		***	**	***	*	NS
Treatments × species		***	NS	**	**	NS

[x] According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. [y] No germinated seeds were observed. [z] Two-way analyze of main and interaction effects, *, **, ***, NS are the symbols for $p < 0.05$, $p < 0.01$, $p < 0.001$, not significant, respectively.

3.3. Rhizosphere Substrates

At the end of the experiment, the changes in arithmetic mean value in rhizosphere substrate EC were significant (Table 4). Treatments had an extremely significant impact on rhizosphere substrate EC (Table 5). The rhizosphere substrate EC of three species increased under salt stress conditions The final rhizosphere soil substrate EC under 5 dS·m^{-1} treatment was 3.51 to 3.86 times higher than the control, while the final rhizosphere soil substrate EC under 10 dS·m^{-1} treatment was 7.38 to 8.58 times higher than the control, indicating that salt accumulation occurred in the substrates. However, the difference in rhizosphere substrate EC among species in the same treatment was not significant (Table 4).

The substrate salt contents were 0.12%, 0.41%, and 0.89% for those irrigated with the control solution and salt solution with an EC of 5.0 and 10.0 dS·m^{-1}, respectively. At the end of this experiment, the final rhizosphere conductivity of three *Aquilegia* seedlings under EC 10 treatment was 2.83 dS·m^{-1}, 3.13 dS·m^{-1}, and 2.73 dS·m^{-1}, respectively.

Table 4. Electrical conductivity of growing medium (soil:water = 1:5) of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) or salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10).

Treatments	*A. oxysepala*	*A. parviflora*	*A. viridiflora*
Control	0.33 Ac [x]	0.37 Ac	0.37 Ac
EC 5	1.27 Ab	1.43 Ab	1.30 Ab
EC 10	2.83 Aa	3.13 Aa	2.73 Aa

[x] According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species.

Table 5. Two-way ANOVA analysis between rhizosphere EC, chlorophyll content, electrolyte leakage, MDA content, proline content, soluble sugar content, SOD activity, POD activity and principal component.

Principal Component	Rhizosphere EC	Chlorophyll Content	Electrolyte Leakage	MDA Content	Proline Content	Soluble Sugar Content	SOD Activity	POD Activity
Treatments	*** [z]	***	***	***	***	***	***	***
Species	NS	***	***	***	***	***	***	***
Treatments × Species	NS	***	***	***	**	***	***	***

[z] Two-way analysis of main and interaction effects, **, ***, NS are the symbols for $p < 0.01$, $p < 0.001$, not significant, respectively.

3.4. Plant Growth and Visual Scores

Plant height and crown diameter were inhibited by the salt treatments (Table 6). Treatments had highly significant impacts on plant growth and visual score. Plant heights of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* in EC 5 reduced by 17.5%, 18.5%, and 22.8%, respectively, compared to those in the control group. Plant heights were further reduced in EC 10, with 26.1%, 27.8%, and 27.4% reductions for *A. oxysepala*, *A. parviflora*, and *A. viridiflora*, respectively. Crown diameters of three species in EC 5 were reduced by 51.4%, 54.6%, and 62.1% compared to the control, respectively. In EC 10, the crown diameters of the three species were reduced by 52.3%, 55.7%, and 63.3% compared to the control, respectively.

Table 6. Plant height, crown diameter, and visual score of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) or salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10).

Variety	Treatments	Plant Height (cm)	Crown Diameter (cm)	Visual Score
A. oxysepala	Control	12.0 Aa [x]	21.6 Aa	5.0 Aa
	EC 5	9.9 Aa	10.4 Ab	3.6 Aab
	EC 10	8.9 Aa	10.3 Ab	2.4 Ab
A. parviflora	Control	11.7 Aa	22.1 Aa	4.8 Aa
	EC 5	9.5 Aa	10.0 Abb	2.7 Ab
	EC 10	8.4 Aa	9.8 Ab	1.0 Bc
A. viridiflora	Control	10.2 Aa	21.4 Aa	4.8 Aa
	EC 5	7.9 Aa	8.1 Bb	2.0 Ab
	EC 10	7.4 Aa	7.9 Ab	0.8 Bb
Treatments		*** [y]	***	***
Species		*	**	***
Treatments × Species		NS	NS	NS

[x] According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. [y] Two-way analysis of main and interaction effects, *, **, ***, NS are the symbols for $p < 0.05$, $p < 0.01$, $p < 0.001$, not significant.

During the experiment, the visual quality of all *Aquilegia* species changed remarkably (Table 6, Figure 3). The visual scores of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* in EC 5 were 3.6, 2.7, and 2.0, respectively. In EC 10, the visual scores of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* were further affected by salt stress. *A. oxysepala* and *A. viridiflora* had the highest and lowest visual scores in both stress treatments.

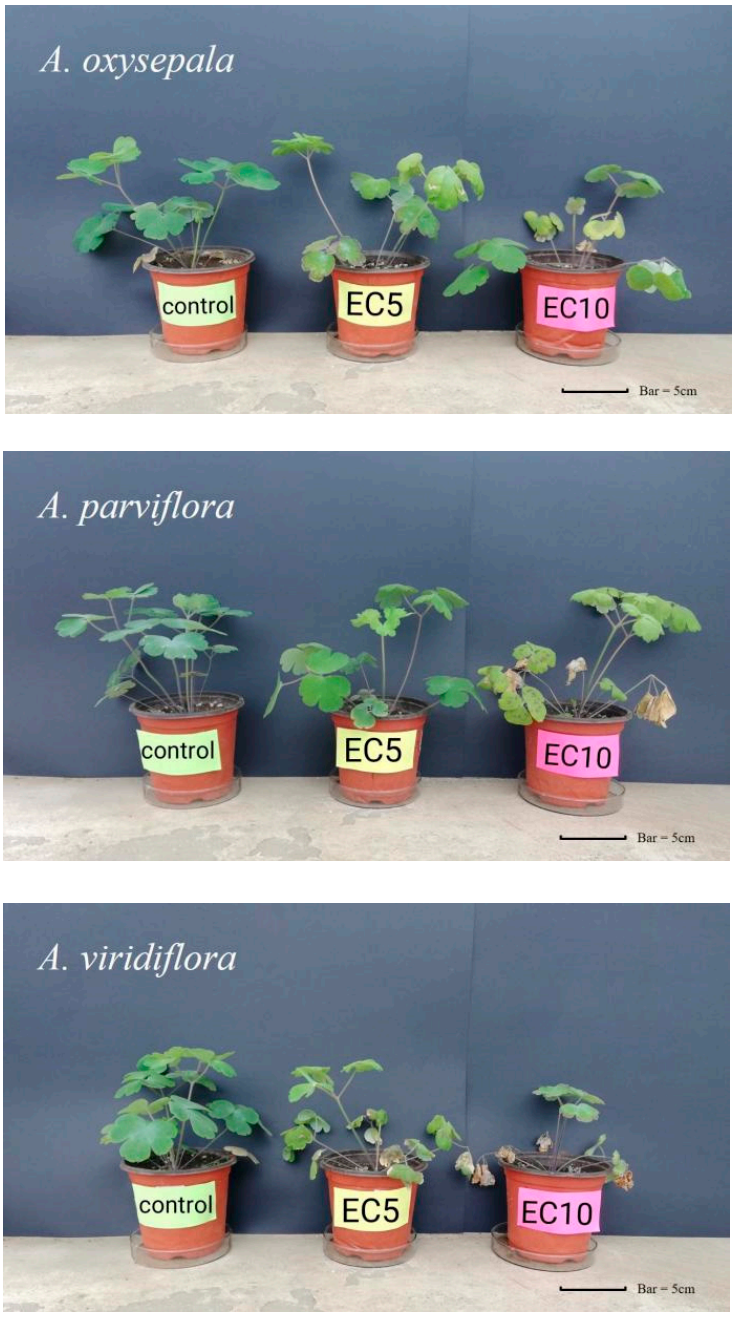

Figure 3. *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10) after 8 times.

The shoot DW was significantly affected by salt treatments (Table 7). Both treatments and species had an extremely significant impact on all DWs. In EC10, the shoot DWs of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* were reduced by 29.2%, 44.4%, and 37.4%, respectively, compared to those in the control. In EC10, the root DWs of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* were reduced by 78.9%, 87.3%, and 83.6%, respectively, compared to those in the control (Table 7). In EC10, the total DWs of *A. oxysepala*, *A. parviflora*,

and *A. viridiflora* were reduced by 56.7%, 68.3%, and 63.6%, respectively, compared to those in the control (Table 7). The decline in the shoot DW, root DW, and total DW of *A. oxysepala* were the smallest.

Table 7. Shoot dry weight (DW), root DW and total DW of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10).

Variety	Treatments	Shoot DW (g)	Root DW (g)	Total DW (g)
A. oxysepala	Control	1.3 Aa [x]	1.6 Aa	2.9 Aa
	EC 5	1.1 Ab	0.4 Ab	1.5 Ab
	EC 10	0.9 Ab	0.3 Ab	1.3 Ab
A. parviflora	Control	1.3 Aa	1.6 Aa	2.8 Aa
	EC 5	0.9 Bb	0.3 Bb	1.2 Bb
	EC 10	0.7 Bb	0.2 Bc	0.9 Bc
A. viridiflora	Control	1.1 Ba	1.4 Ba	2.5 Ba
	EC 5	0.8 Bb	0.3 Bb	1.1 Bb
	EC 10	0.7 Bb	0.2 Ab	0.9 Bb
Treatments		*** [y]	***	***
Species		***	***	***
Treatments × Species		*	**	*

[x] According to Tukey's variance and analy at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. [y] Two-way analysis of main and interaction effects, *, **, ***, NS are the symbols for $p < 0.05$, $p < 0.01$, $p < 0.001$, not significant.

3.5. Photosynthesis Parameters and Pigments

The photosynthesis parameters in the salt treatments changed differently (Table 8). Treatments had a significant impact on all photosynthesis parameters. The photosynthesis (Pn) and stomatal conductance (g_s) of all seedlings were reduced in EC 10 compared to those in the control group. However, the leaf transpiration rate (E) of *A. oxysepala* was not affected by the salt treatment. The changes in intercellular CO_2 concentrations (Ci) of all salt-stressed seedlings were similar, which increased in EC 5, then decreased in EC 10 compared to those in the control group.

Table 8. Net photosynthesis (Pn), stomatal conductance (g_s), leaf transpiration rate (E), and intercellular CO_2 concentration (Ci) of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10).

Variety	Treatments	Pn (μmol·m^{-2}·s^{-1})	g_s (mmol·m^{-2}·s^{-1})	E (mmol·m^{-2}·s^{-1})	Ci (μmol·mol^{-1})
A. oxysepala	Control	12.0 Aa [x]	562.3 Aa	8.4 Aa	306.7 Ab
	EC 5	9.7 Aa	379.3 Bb	9.3 Aa	319.7 Aa
	EC 10	6.8 Ab	311.7 Ab	8.1 Aa	294.3 Ac
A. viridiflora	Control	11.0 Aa	530.7 Aa	8.3 Aab	311.0 Aa
	EC 5	8.3 Aab	381.0 Bb	9.1 Aa	312.7 Aa
	EC 10	7.0 Ab	205.3 Bc	6.2 Ab	288.0 Ab
A. parviflora	Control	11.1 Aa	475.0 Aa	7.7 Ab	310.7 Ab
	EC 5	7.8 Ab	524.0 Aa	10.5 Aa	323.3 Aa
	EC 10	6.5 Ab	193.0 Bb	6.1 Ab	293.0 Ac
Treatments		*** [y]	***	**	***
Species		NS	NS	NS	NS
Treatments × Species		NS	***	NS	NS

[x] According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. [y] Two-way analysis of main and interaction effects, **, ***, NS are the symbols for $p < 0.05$, $p < 0.01$, $p < 0.001$, not significant, respectively.

The chlorophyll content changed differently among the three species (Figure 4). Treatments, species, and interaction all had a significant impact on chlorophyll content (Table 5). The chlorophyll contents of *A. oxysepala* and *A. parviflora* reduced significantly with increasing salt stress, which reduced by 32.97%, and 43.33% in EC 5, respectively, compared to those in the control group. However, *A. viridiflora* was different since its chlorophyll content reduced slightly and was remarkably higher than that of *A. oxysepala* and *A. parviflora* in EC 5 and EC 10. *A. parviflora* had the least chlorophyll content.

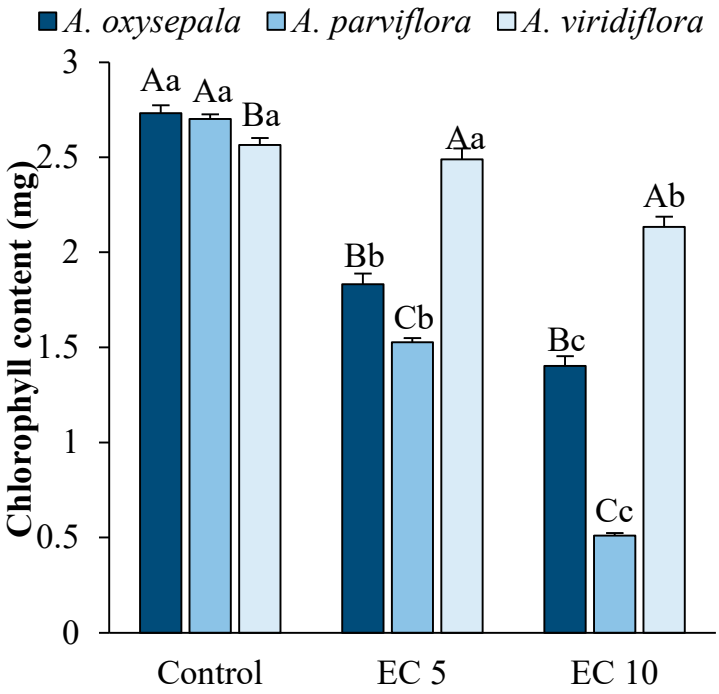

Figure 4. Chlorophyll (a+b) content of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10). According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. Vertical bars represent standard error (SE).

3.6. Electrolyte Leakage

As shown in Figure 5, all seedlings had an increasing EL. Treatments, species, and interaction all significantly impacted EL (Table 5). There was no significant difference among the three species in the control group; however, in EC 10, their EL increased by 525.3%, 572.8%, and 538.6%, respectively. The highest increase was observed in *A. parviflora*, and its EL was the highest in the salt treatments.

3.7. MDA and Proline Contents

MDA contents of all seedlings increased (Figure 6). Treatments, species, and interaction significantly impacted MDA content (Table 5). There was no obvious difference among the three species in the control group, while it was the opposite in EC 5 and EC 10; the highest increase was observed in *A. parviflora* in EC 10, which increased by 392.2% compared to that in the control. In contrast, the MDA content of *A. viridiflora* was steadier than the others. The proline contents of the three species increased (Figure 6), and that of *A. parviflora* was the highest in EC 5 and EC 10. Treatments, species, and interaction all had a significant impact on proline content (Table 5).

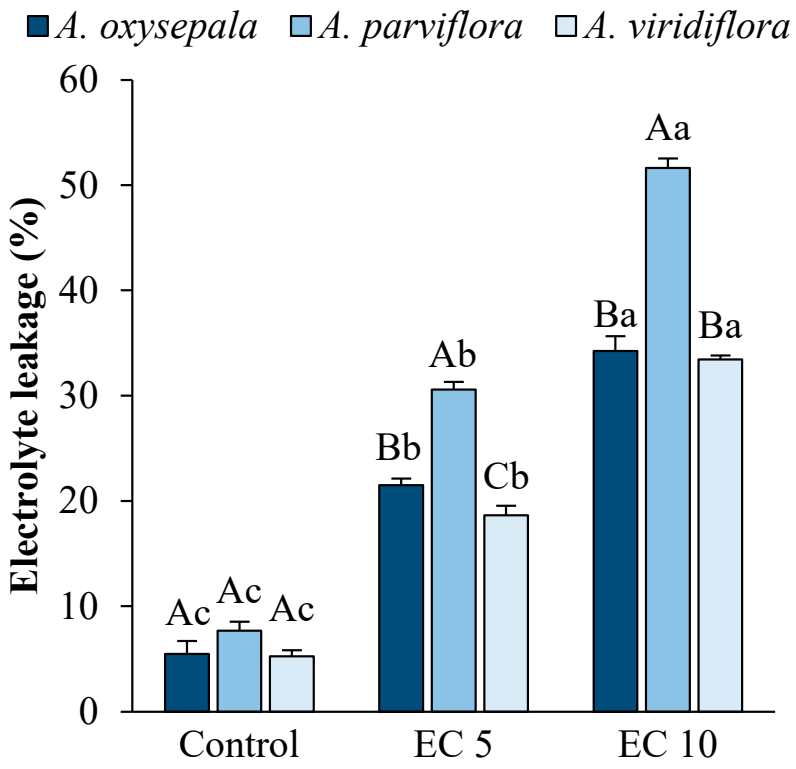

Figure 5. Electrolyte leakage of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10). According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. Vertical bars represent standard error (SE).

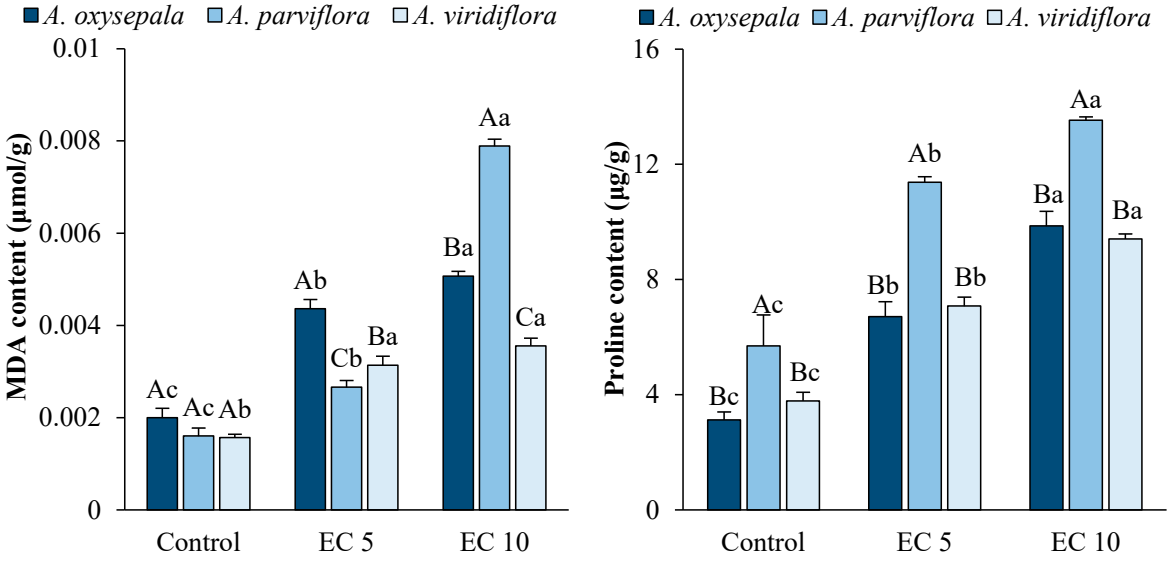

Figure 6. Malondiadehde (MDA) and proline contents of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10). According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. Vertical bars represent standard error (SE).

3.8. Soluble Sugar Content

In different salt treatments, the soluble sugar contents of the three species increased (Figure 7), and in EC 10, that of *A. viridiflora* increased the most, which increased by 118.3%, compared to the control. In contrast, the soluble sugar content of *A. parviflora* was steadier than the others. Treatments, species, and interaction all had a significant impact on soluble sugar content (Table 5).

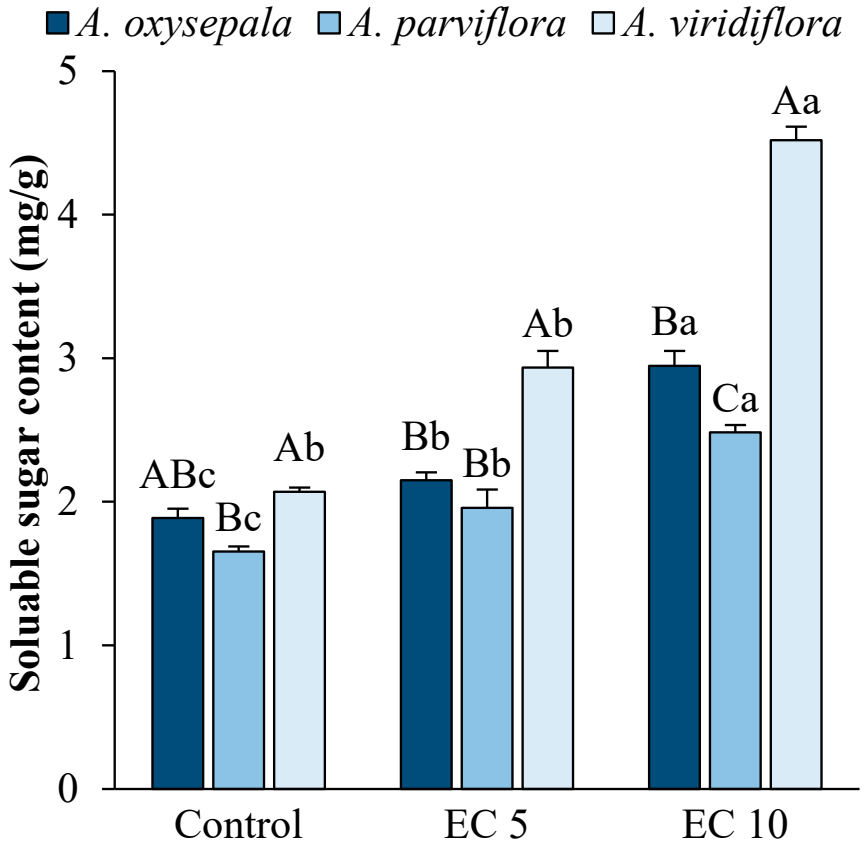

Figure 7. Soluble sugar content of *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 $dS{\cdot}m^{-1}$ (EC 5) and 10 $dS{\cdot}m^{-1}$ (EC 10). According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. Vertical bars represent standard error (SE).

3.9. Antioxidant Enzyme Activities

The changes in SOD were similar among the species (Figure 8); their SOD activities were more enhanced in EC 5 than in the control group, and were more restricted in EC10 than in EC 5. In EC 5, the SOD activity of *A. viridiflora* was the least, and there was no significant difference among the three species in EC 10. However, the POD activity was significantly different in salt treatments (Figure 8). In the stress treatment, the POD activity of *A. oxysepala* continuously decreased, while that of *A. parviflora* continuously increased. Only the POD activity of *A. viridiflora* did not change significantly in EC 5 and EC 10. Treatments, species, and interaction all had a significant impact on SOD and POD activities (Table 5).

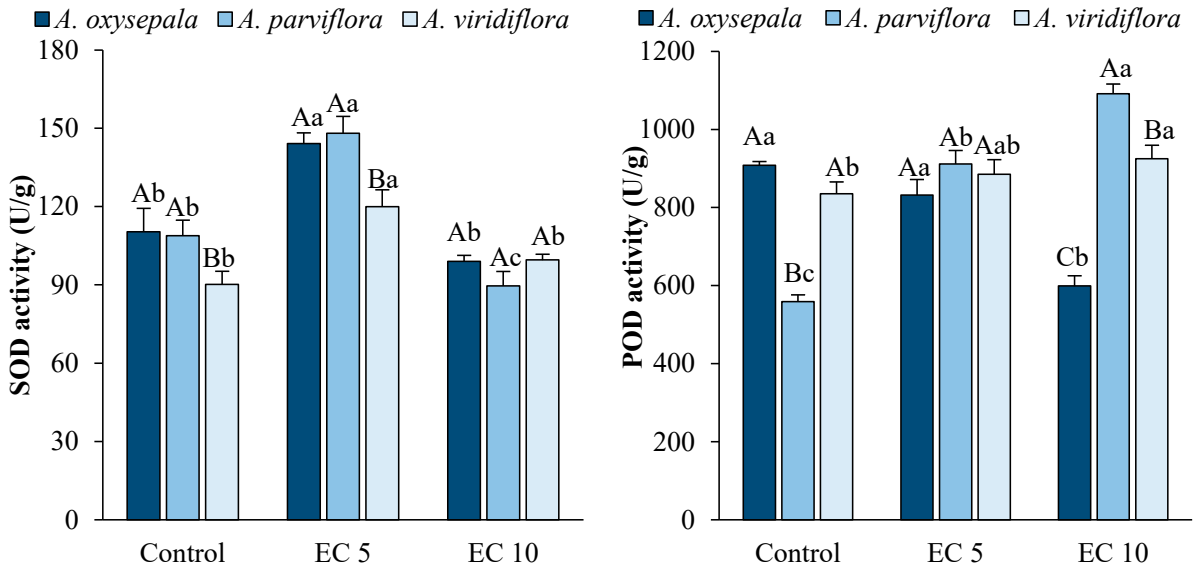

Figure 8. The antioxidant enzyme activity changes in *A. oxysepala*, *A. parviflora*, and *A. viridiflora* irrigated with tap water (control) and salt solution at an electrical conductivity (EC) of 5 dS·m^{-1} (EC 5) and 10 dS·m^{-1} (EC 10). According to Tukey's variance and analysis at $p < 0.05$, means with the same uppercase letters are not significantly changed among different species under the same treatment, and means with the same lowercase letters are not significantly changed among different treatments in the same species. Vertical bars represent standard error (SE).

4. Discussion

In the plant growth cycle, germination and seedling growth stages are the most sensitive to environmental stress [28]. Low osmotic potential caused by salt conditions prevents the seeds from absorbing water, which leads to dormancy lengthening or germination delay. Thus, avoiding germination under adverse conditions is an adaptation strategy to the environment [29]. Pakchoi (*Brassica chinensis*) [20], eggplant (*Solanum melongena*) [29], and hemp (*Cannabis sativa*) [30] were all inhibited under salt stress during the germination stage. Our research found that all *Aquilegia* seeds germination parameters decreased significantly under salt treatments. All seeds were unable to germinate when the solution EC reached 6 dS·m^{-1}. A previous study illustrated that the tolerance threshold of black cumin (*Nigella sativa*) seeds is 240 mM [31]. As well as *Aquilegia* black cumin also belongs to the Ranunculaceae family, which indicates that the salt tolerance varies with species.

In most situations, high salinity leads to osmotic stress and ion toxicity in plants, which is reflected in slow growth and wilting of leaves [32]. Similar conclusions were presented in recent studies, such as the plant heights of six ornamental grasses (*Acorus gramineus*, *Andropogon ternarius*, *Calamagrostis* × *acutiflora*, *Carex morrowii*, *Festuca glauca*, and *Sporobolus heterolepis*) all reduced when the EC of salt solution exceeded 5 dS·m^{-1}, and *S. heterolepis* was more stabilized than other species in different salt treatments, which indicated less salt-sensitive characteristics [33]. With the salt treatment in 5 dS·m^{-1} and 10 dS·m^{-1}, the growth of *Calendula officinalis* was restricted by the decline in the percentage of dry matter [34]. In the study of seven Texas Superstar® perennials, the plant height of *Ruellia* 'Katie Blue' was reduced by 50% in EC 5 compared the control group. In EC 5, the shoot DWs of *Phlox* 'Texas Pink', *Ruellia* 'Katie Blue', *Salvia* 'Henry Dueberg', and Mexican bush sage reduced by 44%, 18%, 12%, and 23%, respectively, compared to those in the control, group and their growth decreased in EC 10 [16]. In addition, excessive Na^+ accumulation is toxic to leaves, including cell metabolism disorder and tissue damage, manifested in necrosis, fading, scorching, and shedding [11]. For ornamental plants, it is crucial to maintain individual integrity and flawless leaves, as they are directly correlated with ornamental and commercial value [35]. Visual scores of four perennial ornamental plants: 'Angelina' (*Sedum rupestre*), 'Autumn Joy' (*S. telephium*), 'Blue Spruce' (*S. reflexum*),

and 'Blue Daze' (*Evolvulus glomeratus*), changed differently under salt conditions. 'Autumn Joy' and 'Blue Spruce' could sustain high visual quality and increased irrigation water electrical conductivity, indicating those two perennial ornamentals were more salt tolerant than the others [11]. All three Sego Supreme^{TM} (*Clematis fruticosa*, *Epilobium septentrionale*, and *Tetraneuris acaulis*) could sustain certain stress levels in high EC (10 dS·m^{-1}) [36]. In this study, the plant growth of three species was negatively affected by salt treatments. Plant height, crown diameter, and visual scores of *A. oxysepala* were the highest among the seedlings in each treatment. This result implied that the tissue damage and growth inhibition of *Aquilegia* were caused by toxic ions accumulation. Furthermore, in *A. oxysepala*, the decline in shoot DW, root DW, and total DW were the smallest, which indicated that *A. oxysepala* has a better salt tolerance than the other two species. According to González-Orenga, the shoot and root weight of *Thalictrum maritimum* did not change significantly until the NaCl concentration reached 300 mM treatment for 23 days. As well as *Aquilegia*, *T. maritimum* also belongs to Ranunculaceae family, which indicates various salt tolerance among species [37].

Salt stress also negatively affects the chlorophyll content, which is caused by chloroplast ultrastructure damage and chlorophyll degradation [38]. Similar results were found in previous studies: in the study of lettuce (*Lactuca sativa* L.), the chlorophyll content decreased significantly after salt treatment for six days, especially under the treatment of 400 mM NaCl [39], three *Echinacea* species (*E. purpurea*, *E. pallida*, and *E. angustifolia*) had less chlorophyll contents during salt stress [40]. In this study, the chlorophyll content of three *Aquilegia* seedlings decreased under salt stress, which revealed that the chloroplast structure was broken and chlorophyll degradation was caused by salt stress conditions. Salt stress often affects the chlorophyll content of more salt-sensitive herbaceous and woody landscape plants [41]. In this study, the leaf chlorophyll content of *A. viridiflora* was steadier than that of the other two species. Thus, *A. viridiflora* is relatively greatly adaptive to salt stress. In addition, *T. maritimum* is also a perennial geophyte of the Ranunculaceae family; after 23 days of 300 mM NaCl treatment, the total chlorophyll content declined by half compared with the control, revealing that it may be an adaptation strategy to the coastal conditions [37].

Furthermore, stomatal conductance (g_s) is the most significant feature in all photosynthesis parameters [42,43]. Stomates are switches for absorbing CO_2 and discharging H_2O. Hypertonic conditions could lead to the decline of osmotic pressure in stomata cells, which leads to stomatal closure. Meanwhile, the high content of Na^+ and Cl^- and low content of K^+ is one of the reasons for inducing stomatal closure [32]. The g_s of salt-tolerant durum wheat was higher than that of the salt-sensitive one during salt treatments [44]. Thus, it is reasonable to evaluate the salt tolerance with g_s. In this study, the g_s of *A. viridiflora* did not change significantly in the control and EC 5 treatment, nor in EC 5 and EC 10 for *A. oxysepala*. This result indicated that *A. viridiflora* could resist medium salt stress, while *A. oxysepala* could adapt to salt stress to some extent. In addition, the intercellular CO_2 concentration (Ci) decreased in EC 10, which indicated that the reduction in Pn was caused by stomatal limitation. The same result was found in the study of the *Avicennia marina* [45].

Salt stress exposure could enhance the reactive oxygen species (ROS) production in plants, including H_2O_2 (hydrogen peroxide), O_2^- (superoxide), and OH^- (hydroxyl radical). ROS overproduction could lead to lipid peroxidation [46]. Consequently, the aggravated membrane permeability was caused by severe salt stress. MDA was the final product of lipid peroxidation, which increased in this case. Similar conclusions were illustrated in previous studies. When the EC of the treatment solution was more than 16 dS·m^{-1}, the MDA content of *Sesuvium portulacastrum* increased remarkably compared to the control [47]. After exposure to 200 mM NaCl for 18 days, the MDA content of *Vetiveria zizanioides* increased significantly [48]. The MDA content of the salt-tolerant lotus 'Welcoming guests' was barely affected by salt stress, while the MDA content of the salt-sensitive lotus 'Hunan Lotus' increased significantly compared to the control. These results indicated that the membrane of 'Hunan Lotus' was easily affected by salt stress [49]. In this

study, the MDA contents of three species increased, and that of *A. parviflora* was the highest in EC 10, which indicated that its cell membrane was more fragile. In contrast, when exposed to salt stress, the MDA contents of some plant species, such as cauliflower [50] and radish cultivars [51] reduced. It is interesting to note that *T. Maritimum* was the opposite of *Aquilegia*; its MDA content reduced slightly under NaCl treatment compared with the control. *Aquilegia* and *T. Maritimum* belong to Ranunculaceae, from which we can infer that the change of MDA varies in different plant species or cultivars [37].

The cell membrane plays an essential role in the cell barrier. Its selective permeability can maintain the ion balance on both sides of the membrane, which is important in material transport and signal transduction. Under salt stress conditions, the excessive accumulation of ROS causes great damage to the membrane, including the enhancement of membrane permeability, recessive selective permeability function, and direct outflow of cell electrolytes [32]. EL reflects the cell membrane permeability directly. From previous studies, most plants, such as alfalfa (*Medicago sativa*) [52], black cumin (*N. sativa* L.) [53], and citrus trees cultivars (*Citrus aurantium* L., and *C. sunki* Hort. Ex Tan. × *P. trifoliate* L. Raf 'Swingle') [54], their EL increased under salt stress. The results showed that salt stress conditions were the reason the cell membrane broke and electrolytes were released from *Aquilegia*. Moreover, the EL of salt-sensitive rice 'Hitomebore' and 'IR28' was much higher than that of salt-tolerant rice 'Pokkali' [55]. MDA content and EL of *A. parviflora* were both the highest in EC 10, which indicated that *A. parviflora* was more sensitive to salt stress than the other two species.

Excessive accumulation of salt ions in the substrate leads to decreased soil osmotic potential, consequently increasing the osmotic difference between soil and the plant itself. To prevent water loss, plants produce specific organic solutes, such as proline, soluble sugar, and free amino acids, to adjust the osmotic pressure to balance the soil osmotic pressure [13]. Similar results were presented in recent studies: sunflower (*Helianthus annuus* L.) [56], carnation (*Dianthus caryophyllus*) [57], and lucerne (*M. sativa*) [58] had more soluble sugar and proline contents in salt stress than in control. However, different conclusions in evaluating the salt tolerance of different species or cultivars were reported in some studies. Salt-sensitive potato cultivar 'Concrod' had more proline content than the salt-tolerant potato cultivar 'Kennebec' [59], high level of proline accumulation was a specific feature of halophytes (*Th. halophila*) to tolerate salt stress [60]. Nevertheless, it would be inappropriate to evaluate the sensibility to salt stress, as the relationship between the proline content and salt tolerance was unclear [61]. Some researchers concluded that the proline is not the driving force of tolerance and is potentially associated with the antioxidant system in *Brassica* [62]. Further studies about the role of proline accumulation in salt stress are necessary. In this study, the soluble sugar content of all species increased, and the increase was the highest in *A. viridiflora*. The proline content of *A. parviflora* was the highest, which we can infer that both *A. viridiflora* and *A. parviflora* were easier affected by salt stress.

With the increasing ROS, the activity of enzymes, such as SOD and POD, was enhanced to detoxify the oxygen species [46]. Under salt stress conditions, SOD is the first defensive line against peroxidation damage. Associating with SOD, POD catalyzes H_2O_2 generated from oxidation resistance reaction into H_2O [32]. When the EC of the treatment solution was 0.3–12 $dS{\cdot}m^{-1}$, SOD activity of *S. portulacastum* increased significantly, while it decreased when the EC was over 16 $dS{\cdot}m^{-1}$ [47]. The SOD activity of the salt-tolerant lotus cultivars 'Welcoming Guests' was higher than that of salt-sensitive lotus cultivar 'Hunan Lotus'; however, the difference in POD activity between the two lotuses was not significant [49]. The POD activity of salt-sensitive rice cultivar 'Hitomebore' and 'IR28' was much higher than that of the salt-tolerant rice cultivar 'Pokkali' [55]. In this experiment, SOD activity of *A. viridiflora* was lower than that of others, while the difference among three species in EC 10 was not significant. However, the POD activity of *A. parviflora* increased continuously over time, which was higher than that of the others in EC 10. Combined with the conclusion of Dionisio-Sese [55], this result indicated that *A. parviflora* was much easier to be affected

by salt stress. In contrast, the POD activity of *A. oxysepala* declined over time, suggesting that it was more tolerant.

5. Conclusions

Under salt stress, *Aquilegia* synthesized osmotic adjustment substances to balance the osmotic potential. Due to toxic ion accumulation, membrane and photosynthetic systems were damaged, which activated the antioxidant defense system, and reflected in germination, growth inhibition and visual quality reduction ultimately. Germination thresholds of three *Aquilegia* species were determined. *A. parviflora* was easily affected in MDA, EL, and POD activity. *A. oxysepala* was more tolerant based on plant height, crown diameter, visual quality, g_s, and POD activity; it is also the most salt-tolerant, followed by *A. viridiflora* and *A. parviflora*. The tolerance threshold in soil conductivity of *A. oxysepala* is 2.83 $dS \cdot m^{-1}$. Consequently, *A. oxysepala* can be the best to be applied in deicing salt application areas as a ground cover.

Author Contributions: L.C., Y.M. and D.J. directed and performed the experiment, provided technical advice, analyzed data and drafted the manuscript together. F.Y. performed the experiments, analyzed data and edited manuscript. Y.Z. provided laboratories, instruments and equipment; edited and reviewed this manuscript; connected with all authors and involve them in major decisions about the publication. All authors read and approved the final manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the data involved in plant resources.

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