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Growth and Physicochemical Changes of *Carpinus betulus* L. Influenced by Salinity Treatments

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Abstract: *Carpinus betulus* L. is a deciduous tree widely distributed in Europe with strong adaptation, and it plays a key role in landscaping and timbering because of its variety of colors and shapes. Recently introduced to China for similar purposes, this species needs further study as to its physiological adaptability under various soil salinity conditions. In this study, the growth and physicochemical changes of *C. betulus* seedlings cultivated in soil under six different levels of salinity stress (NaCl: 0, 17, 34, 51, 68, and 85 mM) were studied for 14, 28 and 42 days. The plant growth and gas exchange parameters were not changed much by 17 and 34 mM NaCl, but they were significantly affected after treatments with 51 ~ 85 mM NaCl. The chlorophyll content was not significantly affected at 17 and 34 mM salinity, and the relative water content, malondialdehyde content and cell membrane stability of *C. betulus* did not change obviously under the 17 and 34 mM treatments, indicating that *C. betulus* is able to adapt to low-salinity conditions. The amount of osmotic adjustment substances and the antioxidant enzyme activity of *C. betulus* increased after 14 and 28 days and then decreased with increasing salinity gradients, but the proline content was increased during the entire time for different salinities. The Na content of different organs increased in response to salinity, and the K/Na, Ca/Na, and Mg/Na ratios were significantly affected by salinity. These results suggest that the ability of *C. betulus* to synthesize osmotic substances and enzymatic antioxidants may be impaired under severe saline conditions (68 ~ 85 mM NaCl) but that it can tolerate and accumulate salt at low salinity concentrations (17 ~ 34 mM NaCl). Such information is useful for land managers considering introducing this species to sites with various soil salinity conditions.

Keywords: salinity; *Carpinus betulus*; morphological indices; gas exchange; osmotic adjustment substances; antioxidant enzyme activity; ion relationships

1. Introduction

Salinity stress is one of the main abiotic stresses affecting the growth of plants. Due to the influence of human population growth, increased industrial pollution, and improper irrigation, the existing salinized land in the world covers approximately 9.5×10^8 hm^2, approximately 22% of agricultural land worldwide [1,2]. The area of saline-alkaline land in China is approximately 9.9×10^7 hm^2, which accounts for 25% of the arable land in China [3]. Soil salinization has caused problems over vast areas in the world. In addition to soil salinity, de-icing salts have become a serious constraint for plant growth, particularly in cities [4]. Although de-icing salts help to keep pavements dry and safe during ice and snow, their extensive use can cause damage to plants along sidewalks, walkways, and driveways. Trees and shrubs can be injured by the dissolved salt that spreads into the soil. High levels of salinity will negatively affect the morphology, photosynthesis, metabolism, and physiological and biochemical processes of plants [5,6].

To resist salt stress, plants have evolved complex mechanisms to adapt to the living environment [7]. The mechanisms include osmotic adjustments by the accumulation of compatible solutes (proline, betaine, polyols, and soluble sugars), scavenging the reactive oxygen species (ROS) by increasing the activity of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), and maintaining a balance of intracellular ions by lowering the toxic concentration of ions in the cytoplasm. In addition to these responses, some plants can also change their morphological structure to adjust to saline conditions [8]. Plant species do vary in their sensitivity to salt damage [9,10]. Therefore, research of the changes in the morphology, physiological and biochemical processes of the plant in relation to the various salinity levels of the environment, the mechanisms involved in the plant response to salt stress, and the identification of trees more tolerant to salt for planting purposes, are of great significance [11].

Carpinus betulus L. (European hornbeam) is a well-known deciduous tree that originated in central Europe and Asia Minor as a dominant species in the forest canopy and has recently been introduced to China [12]. *C. betulus* is long-lived and has strong wood, which can tolerant a wide range of soil conditions, from coarse sand to clay, as well as acidic or alkaline soil pH levels [13,14]. These trees are very important landscaping trees in private and public green areas due to their rustic nature, beautiful shapes and strong adaptability, and they can readily be found in urban parks, gardens and along roadsides [15,16]. The wood of *C. betulus* is suitable for making pianos, violins, joinery, flooring, batons, pulleys, wooden gears and so on [14]. Recent studies have found remarkable antioxidant- and anticancer-related properties of *C. betulus* leaf extracts, making it a possible raw material for medicine [17]. The high ornamental and economic value of *C. betulus* makes it extremely popular all over the world. A considerable number of studies have been conducted related to *C. betulus*, including studies on breeding [18], seed biology [19], hybridization [20], heat resistance [21] and drought tolerance [22,23]. However, information on the response of *C. betulus* to salinity is scarce. Whether *C. betulus* can grow well in saline areas in China still remains unknown [24]. Therefore, the main objective of the study is to make a comprehensive assessment of the influence of salinity stress on *C. betulus* and to establish the mechanisms of adaptation it employs to tolerate salinity stress. We tested the hypothesis that the growth, biomass accumulation and leaf gas exchanges of the seedlings will decrease under the effect of salinity. We examine the growth and physicochemical changes of the seedlings in response to salinity stress to provide scientific data and findings related to the cultivation of *C. betulus* and to make better use of Carpinus species in landscaping.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The trials were conducted in the landscape experimental teaching center of Nanjing Forestry University in April 2013, in Nanjing (33°04′ N, 118°47′ E), Jiangsu Province, China, in a warm and humid subtropical monsoon climate, with annual rainfall of 1047 mm. The average annual, maximum and minimum temperatures are 15.7 °C, 40.7 °C, and −14 °C, respectively.

The *C. betulus* seeds were obtained from Hungary (imported by the China National Tree Seed Corporation). The seeds were treated with variable temperature stratification (23 °C, 30 days, then 5 °C, 4 months in moist sand) in November 2011 and sown in containers during March of 2012 to initiate growth. During March 2013, we selected well-grown seedlings, approximately 0.5 cm in diameter at ground level with 20-cm tall stems, and transplanted them into pots (10 cm in diameter and 15 cm tall) for uniform management. The experimental loamy clay soil was a mixture of equal amounts of soil, peat, vermiculite, and pearlite and had an acidic pH of 6.5. TFW-VI soil nutrient and moisture tester (TFW-VI, Wuhan, China) was used to determine the content of soil nutrients. The soil nutrient status was 32.05 mg g^{-1} total N, 12.15 ppm available P, 145 ppm available K, 687 ppm Ca, and 260 ppm Mg. Each pot had 500 g soil and one seedling. The potted plants received 0.5 L of Hoagland's nutrient solution at 2-week intervals. The potted plants were kept in a greenhouse under natural sunlight conditions at a temperature of 25 °C and relative humidity of 75%.

2.2. Salt Treatments and Experimental Design

Salt treatments were carried out when the seedlings were approximately 20 cm long with similar leaf number and leaf area, in April 2013. Each treatment had three replicates, and each replicate consisted of 25 random basins, with one seedling per pot. The pots were subjected to S0 (0 mM, control) and S1 ~ S5 (17, 34, 51, 68, and 85 mM) NaCl concentrations. Different salinity levels (S1 ~ S5) were developed by dissolving sodium chloride (NaCl) in distilled water as g L^{-1} and then recalculated into mM. The electrical conductivity of the substrates was 1.6 (S0), 3.3 (S1), 4.4 (S2), 6.2 (S3), 8.4 (S4) and 10.5 (S5) dS m^{-1}, respectively. In each pot, 200 mL of treatment water was applied based on the requirements for each treatment. To avoid osmotic shock, salt solutions were added in three equal parts on alternating days until the expected concentration was reached. Standard agronomic practices were adopted, and tree protection measures were carried out as necessary. To ensure the accuracy of the experimental design, we put a plastic tray at the bottom of the pot to retain any overflow solution, and this was placed back into the pot. The plants were monitored under the treatments for 42 days. After 14, 28 and 42 days of growth, measurements were carried out and samples collected for various physiological analyses, with three replicates for the measured parameters. Whole plants were harvested after 42 days of treatment.

2.3. Growth Parameters

Before the salt stress treatment, three seedlings were selected in each group to calculate their seedling height (H_0), diameter (D_0) and leaf number (L_0). At the final day of treatment, the height (H_1), diameter (D_1) and leaf number (L_1) were determined again. Height growth = $H_1 - H_0$; diameter growth = $D_1 - D_0$; leaf number increment = $L_1 - L_0$. After the treatment of 42 days of salt stress, the plants were separated into roots, stems and leaves, the leaf number was calculated, and the root length was measured; then, the samples were dried at 80 °C in an oven for 48 h, and their dry weights were recorded. The leaf area of each plant was determined using a portable leaf area meter (LI-3000C, LI-COR, Lincoln, NE, USA). Portions of these samples were frozen in liquid nitrogen stored at −80 °C.

2.4. Leaf Stomatal and Section Characteristics

Leaves of *C. Betulus* seedlings were collected at the final day of salinity treatment. The properly cleaned samples were cut into small pieces (approximately 5 × 5 mm) with a sharp blade. The excised leaves were placed in formalin acetic acid (FAA), dehydrated in a graded ethanol series of 30%, 50%, 70%, 90%, 95%, and 100% for 30 min each, penetrated with isoamyl acetate aldehyde, and dried in a critical point drying apparatus (K850, Emitech, London, UK). The tissues were mounted on stubs and coated with gold using an ion sputtering apparatus (E1010, Hitachi, Tokyo, Japan); then, the blade surface and cross-section were viewed under a scanning electron microscope (Quanta 200, FEI, Hillsborough, OR, USA), and the images were taken using the same instrument.

The stomatal density was calculated by the number of stomata per mm^2, and the stomatal size was reflected by the length between the junctions of the guard cells of each stoma. The number of open stomata, leaf thickness and palisade tissue thickness for each sample was measured under a photomicroscope system with a computer attachment.

2.5. Leaf Gas Exchanges and Chlorophyll Fluorescence Parameters

Photosynthetic parameters were determined in the third fully expanded leaves of each plant at the final day of salt treatments using a portable photosynthetic system (Ciras-2, Shanghai, China). The temperature was 25 °C, the intensity of the light was 1000 μmol $m^{-2}s^{-1}$, and the concentration of reference CO_2 was 380 μmol mol^{-1}. The parameters recorded included the net photosynthetic rate (*Pn*), transpiration rate (*Tr*), stomatal conductance (*Gs*), and intercellular CO_2 concentration (*Ci*). The water use efficiency (WUE_i) was calculated as follows: $WUE_i = Pn/Tr$. Chlorophyll fluorescence parameters were determined by the fluorescence leaf chamber of the Ciras-2 photosynthetic system.

The photochemical quantum efficiency (Φ_{PSII}), maximum photochemical efficiency of photosystem II (Fv/Fm), photochemical quenching parameter (qP), non-photochemical quenching parameter (NPQ), and electron transfer rate (ETR) were measured on dark-adapted leaves. Three seedlings were randomly determined for each treatment, and each seedling was measured 3 times.

2.6. Chlorophyll Content and Relative Water Content (RWC)

For the chlorophyll content of the leaves, chlorophyll was extracted from 4 ~ 5 pieces of fresh leaves with a mixture of acetone and 95% ethanol (*v*:*v* = 1:1), and the absorbance of the resulting extracts was measured by ultraviolet-visible spectrophotometry (Lambda25, PerkinElmer, Waltham, MA, USA) at wavelengths of 645 nm and 663 nm. The chlorophyll concentration (mg g^{-1}) was then calculated following the method of Porra et al. (1989) [25].

The relative water content (RWC) was measured according to Vivekanandan (2004) [26]. The fresh leaf tissues were weighed to obtain the fresh weight (W_1); then, they were placed in water and incubated at room temperature for 24 h to absorb water to reach a saturated state. The leaves were later taken out, blotted dry, and weighed to obtain the turgid weight (W_2). Finally, the tissues were dried at 80 °C for 24 h and weighed to obtain the dry weight (W_3). The relative water content (RWC) was then calculated according to the following formula:

$$\text{RWC} = (W_1 - W_3/W_2 - W_3) \times 100\% \quad (1)$$

2.7. Malondialdehyde Concentration (MDA) and Cell Membrane Stability

MDA content was measured as reported by Hodges et al. (1999) [27]. First, 0.3 g of leaves were ground at 4 °C in a mortar in 6 mL of a pH 7.8 phosphate buffer solution. The homogenate was centrifuged at 9000 rpm at 4 °C for 20 min. Then, 2 mL of an enzyme solution were homogenized in 3 mL of 0.5% thiobarbituric acid and 8% trichloroacetic acid, and the extracts were incubated at 100 °C for 20 min. After a brief passage in ice, the samples were centrifuged at 9000 rpm for 30 min at 4 °C, and the absorbance of the supernatant was measured at 532 and 600 nm.

The cell membrane stability was measured by the relative electrolytic conductivity (REC): 0.2 g sample of fresh leaves was rinsed three times with deionized water and incubated in hermetic tubes containing 20 mL deionized water for 5 h at 25 °C. The electrical conductivity of the leaf solution (S_1) was determined with a conductivity meter (DDS-307, Shanghai, China). Then, the tubes containing leaf samples were put in boiling water at 100 °C for 20 min to determine the electrical conductivity after release of all electrolytes (S_2). Leaf electrolyte leakage was determined according to Dionisio-Sese and Tobita (1998) [28] and calculated as follows:

$$\text{REC} = (S_1/S_2) \times 100\% \quad (2)$$

2.8. Soluble Sugars, Soluble Proteins, and Proline

Leaf soluble sugars content was measured by the method of Magné et al. (2006) [29]. First, 0.1 g of leaves was incubated in hermetic tubes containing 10 mL of deionized water, placed in boiling water for 30 min, cooled and filtered into a 25-mL volumetric flask that was then filled with distilled water. The reaction mixtures contained 0.5 mL of extract, 1.5 mL of distilled water, 0.5 mL of anthrone ethyl acetate and 5 mL of 98% sulfuric acid. Each tube was thoroughly shaken with the oscillator and immediately put into boiling water for 1 min. The absorbance of the supernatant was measured at 630 nm after cooling. Then, the value was put into glucose standard curve to find the appropriate content.

The content of soluble proteins was measured by the method of Coomassie brilliant blue G-250 staining. The reaction mixtures contained 0.1 mL of enzyme solution and 5 mL of Coomassie brilliant blue G-250 solution. The absorbance of the supernatant was measured at 595 nm after standing for 5 min. The leaf soluble proteins content was determined in the supernatant according to the method of Bradford (1976) [30] using bovine serum albumin (BSA) as the standard.

Proline was extracted and determined using the method of Steinert et al. (1990) [31]: Samples were homogenized with 3% sulfosalicylic acid and then centrifuged at 4000 rpm at 4 °C for 20 min. The supernatant was treated with acetic acid and acid ninhydrin and boiled in water for 1 h; then, the absorbance was measured at 520 nm. Proline was used to generate a standard curve.

2.9. Measurement of Antioxidant Enzyme Activities

Frozen leaves weighing 0.3 g were ground at 4 °C in a mortar in 6 mL of a pH 7.8 phosphate buffer solution. The homogenate was centrifuged at 9000 rpm at 4 °C for 20 min. The supernatant was collected as a crude enzyme extract for enzyme measurements and stored at 4 °C.

The SOD activity was analyzed according to Beyer et al. (1987) [32] with some modifications. The reaction mixture contained 1.5 mL of sodium phosphate buffer, 0.1 mL of enzyme solution, 0.3 mL of 130 mmol L^{-1} methionine, 0.3 mL of 20 μmol L^{-1} riboflavin, 0.3 mL of 100 μmol L^{-1} EDTA, 0.3 mL of 750 μmol L^{-1} nitroblue tetrazolium (NBT), and 0.5 mL of distilled water. The reaction was started by exposing the mixture to white fluorescent light for 15 min, and reduced NBT (blue color) was measured at 560 nm such that one unit of SOD activity caused 50% inhibition of NBT reduction per min.

The POD activity was determined according to Civello et al. (1995) [33]. The reaction mixtures contained 1 mL of enzyme solution, 3.8 mL of 0.3% guaiacol reaction solution, and 0.1 mL of 3% H_2O_2. The increase in absorbance (tetraguaiacol formation) was recorded at 470 nm every 1 min 3 times.

2.10. Measurements of the Sodium (Na), Potassium (K), Calcium (Ca), and Magnesium (Mg) Content

The roots, stems and leaves of seedlings were separately dried at 85 °C for 48 h. The dried samples were later digested with concentrated HNO_3 acid and $HClO_4$ acid (5:1 *v*:*v*). The Na, K, Ca, and Mg ion concentrations in the digested samples were measured using a plasma emission spectrometer (OPTIMA PE-4300DV, Waltham, MA, USA). Each test was repeated three times, taking the average.

2.11. Statistical Analysis

All data were analyzed by calculating the means and standard deviation (SD), using the one-way ANOVA, and the means were separated with Duncan's multiple range test at the 5% probability level using the SPSS statistical package version 22.0 (IBM Corp, Amonk, NY, USA).

3. Results

3.1. Changes in Growth under Increasing Levels of Soil Salinity

Growth inhibition and biomass reduction are the most sensitive physiological responses of plants under salt stress. The results showed that (Table 1) the reductions in growth became more pronounced with the increasing levels of salt in the soil; however, the root length, leaf area, dry weight of leaves were slightly higher under low salinity conditions (S1) than those in the control plants, which indicated that moderate (S3) and high (S4, S5) salt concentrations had obvious inhibitory effects on *C. betulus* but that the low concentration of salt treatment (S1) may promote the growth of the roots and leaves. The root/shoot ratio continued increasing with soil salinity, and the ratio of the treatment group was significantly higher than that of the control group.

Table 1. Effect of increasing levels of soil salinity on height growth, diameter growth, root length, leaf area, leaf number increment, dry weight of roots, stems and leaves, root/shoot ratio (R/S) and total biomass of *Carpinus betulus* L. after 42 days. S0 ~ S5 represents 0 ~ 85 mM NaCl respectively. The data in the table are the mean ± standard deviation (n = 3); different lowercase letters in each column indicate significant differences between treatments ($p < 0.05$).

Treatment	Height Growth (cm)	Diameter Growth (cm)	Root Length (cm)	Leaf Area (cm^2)	Leaf Number Increment
S0	4.89 ± 0.40 a	0.07 ± 0.008 a	18.55 ± 1.90 ab	10.48 ± 0.57 a	16.33 ± 3.06 a
S1	4.10 ± 0.72 ab	0.068 ± 0.004 a	20.04 ± 1.11 a	11.00 ± 0.98 a	14.33 ± 2.08 ab
S2	3.80 ± 0.60 b	0.058 ± 0.005 b	16.79 ± 0.80 bc	9.64 ± 1.52 ab	11.00 ± 2.65 bc
S3	2.50 ± 0.44 c	0.047 ± 0.005 c	15.23 ± 1.18 c	8.74 ± 1.08 abc	9.00 ± 2.00 cd
S4	1.97 ± 0.25 c	0.043 ± 0.006 cd	12.35 ± 1.16 d	7.52 ± 1.45 bc	7.67 ± 2.08 cd
S5	1.73 ± 0.25 c	0.037 ± 0.004 d	10.29 ± 1.56 d	6.81 ± 1.55 c	6.00 ± 1.00 d
Treatment	**Root (DW, g)**	**Stem (DW, g)**	**Leaf (DW, g)**	**Root/Shoot Ratio (R/S)**	**Total Biomass (DW, g)**
S0	0.16 ± 0.006 a	0.23 ± 0.008 a	0.27 ± 0.008 a	0.314 ± 0.002 e	0.66 ± 0.02 a
S1	0.16 ± 0.007 a	0.18 ± 0.012 b	0.28 ± 0.007 a	0.363 ± 0.005 d	0.62 ± 0.03 b
S2	0.14 ± 0.005 b	0.16 ± 0.007 c	0.21 ± 0.006 b	0.383 ± 0.002 c	0.52 ± 0.02 c
S3	0.13 ± 0.007 c	0.15 ± 0.010 c	0.16 ± 0.011 c	0.423 ± 0.006 b	0.43 ± 0.03 d
S4	0.12 ± 0.005 cd	0.13 ± 0.006 d	0.12 ± 0.008 d	0.476 ± 0.008 a	0.37 ± 0.02 e
S5	0.11 ± 0.006 d	0.11 ± 0.007 e	0.11 ± 0.007 d	0.482 ± 0.003 a	0.33 ± 0.02 e

3.2. Stomata Density, Size, and Leaf Section Characteristics

At the low salinity concentration (S1), the leaf stomata and leaf section characteristics of the *C. betulus* leaves were not significantly different from those with the S0 treatment (Table 2). However, with the increased salinity concentrations (S2 ~ S5), the stomata density of leaves decreased gradually. At a salt concentration of 85 mM, the stomata density was 65% of that of the control. Opened stomata and leaf thickness decreased with the increasing salinity. Only 17.66% open stomata were observed with the salinity concentration of 85 mM. The stomata shape, palisade and parenchyma tissues of *C. betulus* seedlings had different degrees of change under various levels of salinity (Figure 1a,b). The opening degree of the stomata decreased with the increase in salt stress, and the stomata formed only a thin seam at a salt concentration of S3. At S4, the guard cells were deformed, and the epidermal tissue was atrophied. In the S5 treatment, the stomata were nearly closed. The palisade tissue of *C. betulus* was elongated and arranged orderly in the control (Figure 1b, S0). The difference of leaf section characteristics under S1 and S2 salinity treatments was insignificant with the control, so we didn't attach the pictures. However, at the salinity concentrations of the S3–S5 treatments, the palisade and spongy parenchyma tissues were loosely arranged, and part of these tissues had started to shrink.

Table 2. The main effects of salinity on leaf stomata and leaf section characteristics of *C. betulus.* The parameters measured include stomata density, stomata length, opened stomata, leaf thickness, palisade tissue thickness, and palisade tissue thickness/leaf thickness. S0 ~ S5 represents 0 ~ 85 mM NaCl respectively. The data in the table are the mean ± standard deviation (n = 3); different lowercase letters in each column indicate significant differences between treatments ($p < 0.05$).

Treatment	Stomata Density (Number mm^{-2})	Stomata Length (μm)	Opened Stomata (%)	Leaf Thickness (μm)	Palisade Tissue Thickness (μm)	Palisade Tissue Thickness/Leaf Thickness (%)
S0	193.33 ± 16.04 ab	14.87 ± 1.70 a	98.04 ± 2.49 a	69.33 ± 4.21 a	19.30 ± 19.30 ab	27.79 ± 1.28 ab
S1	215.00 ± 15.00 a	15.70 ± 1.75 a	90.66 ± 2.14 a	67.89 ± 6.52 ab	20.32 ± 2.93 a	29.85 ± 2.01 a
S2	184.67 ± 19.43 bc	13.50 ± 1.70 ab	80.70 ± 1.66 b	64.26 ± 6.00 ab	19.10 ± 2.07 ab	29.70 ± 0.59 a
S3	158.67 ± 13.01 cd	11.60 ± 1.28 bc	68.57 ± 5.42 c	59.47 ± 5.39 b	16.13 ± 1.59 bc	27.12 ± 0.43 bc
S4	147.33 ± 13.01 de	10.57 ± 1.66 c	42.87 ± 2.00 d	57.75 ± 4.06 b	15.63 ± 1.95 bc	27.00 ± 1.52 bc
S5	125.67 ± 15.63 e	9.17 ± 1.25 c	17.66 ± 1.45 e	56.73 ± 7.91 b	14.13 ± 1.88 c	24.92 ± 0.20 c

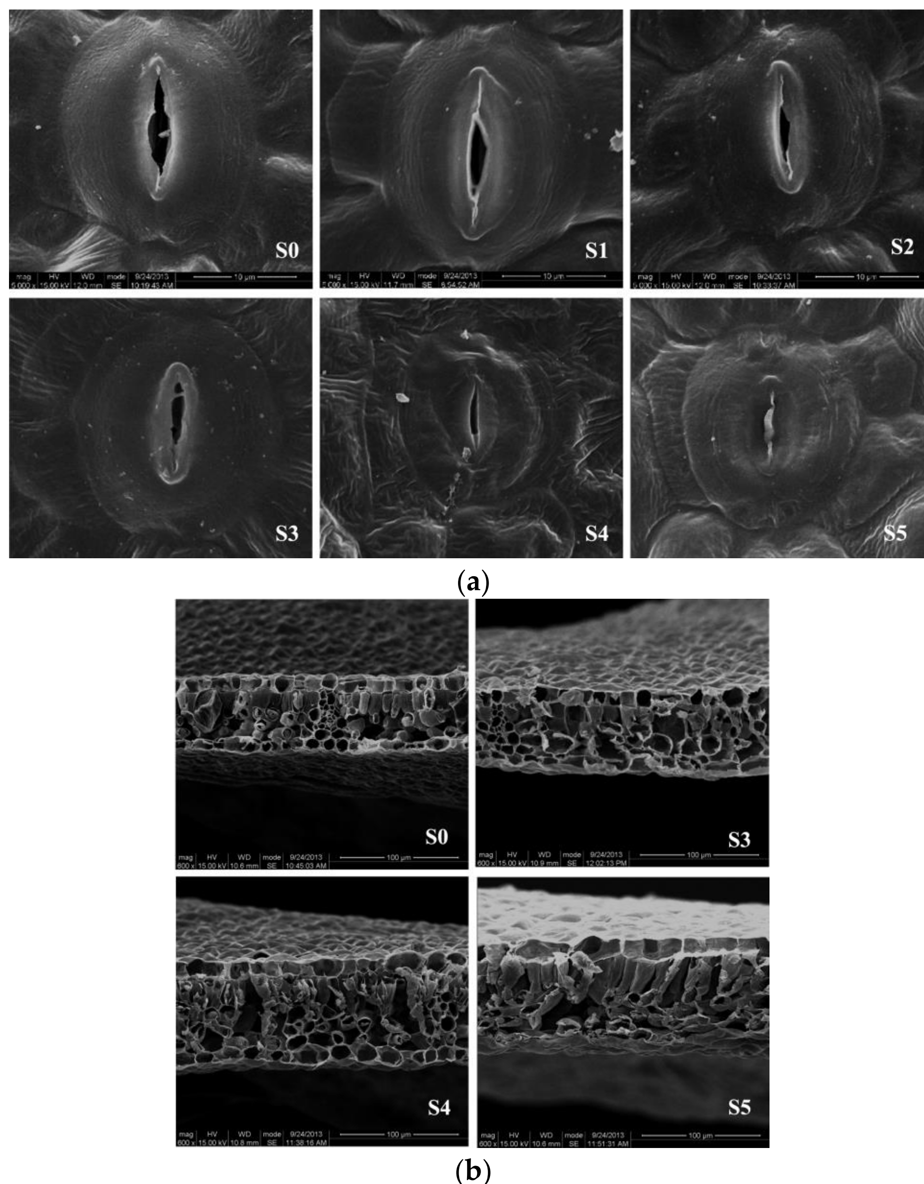

Figure 1. Stomata structure on the leaf surface (**a**), ×5000 and leaf section (**b**), ×600 of *C. betulus* under salinity treatments; S0 ~ S5 represents 0 ~ 85 mM NaCl respectively.

3.3. Leaf Gas Exchanges, and Chlorophyll Fluorescence Parameters

A significant reduction in the net photosynthetic rate was observed after 42 days of the S2 treatment (Table 3). The water-use efficiency (WUE_i) and stomatal conductance (*Gs*) of leaves decreased with increasing salt concentrations, with S2 ~ S5 NaCl having the most pronounced effect. The intercellular CO_2 (*Ci*) did not change significantly under S1 NaCl but did increase under the S2 ~ S5 salinity treatments. However, a significant increase in transpiration rates (*Tr*) occurred in the plants treated with S1 and S2 NaCl. Leaf fluorescence parameters, such as Φ_{PSII}, Fv/Fm, qP and ETR were not negatively influenced at less than S3 treatment, while only NPQ exhibited the most pronounced effect under all salinity treatments, which increased with the salt concentration.

Table 3. The main effects of salinity on gas exchange and chlorophyll fluorescence parameters of *C. betulus.* The parameters measured include the net photosynthetic rate (*Pn*), transpiration rate (*Tr*), water use efficiency (WUE_i), stomatal conductance (*Gs*), intercellular CO_2 concentration (*Ci*), photochemical quantum efficiency (Φ_{PSII}), maximum photochemical efficiency of photosystem II (*Fv/Fm*), photochemical quenching parameter (qP), non-photochemical quenching parameter (NPQ), and the electron transfer rate (ETR). S0 ~ S5 represents 0 ~ 85 mM NaCl respectively. The data in the table are the mean ± standard deviation (n = 3); different lowercase letters in each column indicate significant differences between treatments ($p < 0.05$).

Treatment	*Pn* (μmol m^{-2} s^{-1})	*Tr* (mmol m^{-2} s^{-1})	WUE_i	*Gs* (μmol m^{-2} s^{-1})	*Ci* (μmol m^{-2} s^{-1})
S0	6.47 ± 0.81 a	1.13 ± 0.09 bc	5.77 ± 1.02 a	76.67 ± 9.87 a	243.67 ± 9.45 e
S1	6.97 ± 1.12 a	1.38 ± 0.08 a	5.08 ± 1.07 ab	70.33 ± 8.02 ab	234.00 ± 20.66 e
S2	5.50 ± 0.70 b	1.28 ± 0.07 a	4.31 ± 0.52 bc	60.00 ± 5.00 bc	268.00 ± 11.79 d
S3	3.50 ± 0.30 c	1.11 ± 0.11 bc	3.16 ± 0.27 c	52.33 ± 8.39 cd	296.33 ± 4.04 c
S4	1.53 ± 0.50 d	1.00 ± 0.11 cd	1.52 ± 0.43 d	51.33 ± 7.57 cd	355.00 ± 14.53 b
S5	0.50 ± 0.20 d	0.88 ± 0.13 d	0.57 ± 0.25 d	44.67 ± 5.13 d	380.00 ± 13.53 a
Treatment	Φ_{PSII}	***Fv/Fm***	**qP**	**NPQ**	**ETR (μmol m^{-2} s^{-1})**
S0	0.49 ± 0.02 a	0.63 ± 0.03 a	1.03 ± 0.13 a	0.67 ± 0.04 f	255.48 ± 17.60 ab
S1	0.53 ± 0.07 a	0.59 ± 0.06 ab	0.97 ± 0.04 ab	0.77 ± 0.03 e	260.78 ± 25.76 a
S2	0.51 ± 0.03 a	0.54 ± 0.04 abc	0.90 ± 0.07 abc	0.88 ± 03 d	254.48 ± 12.81 ab
S3	0.47 ± 0.04 a	0.49 ± 0.09 abc	0.85 ± 0.10 bc	1.12 ± 0.04 c	226.42 ± 13.22 bc
S4	0.39 ± 0.05 b	0.45 ± 0.11 c	0.75 ± 0.10 cd	1.21 ± 0.03 b	206.02 ± 15.70 c
S5	0.36 ± 0.04 b	0.41 ± 0.06 c	0.60 ± 0.08 d	1.33 ± 0.03 a	200.28 ± 17.71 c

3.4. Chlorophyll Content, and Relative Water Content (RWC)

Chlorophyll is an essential molecule for the capture and transfer of energy in the photosynthetic machinery. The results showed that there were no significant differences between the control and S1 ~ S4 salinity after 14 and 28 days, but after 42 days, a significant reduction in chlorophyll concentrations was found under moderate (S3) and high (S4, S5) salinity treatments (Figure 2a). The water status of a plant is important for maintaining cellular and metabolic functions under salt stress. Under low salt stress (S1), the leaves maintained a higher RWC than those of the control, while a strong decrease was observed with S4 and S5 treatments after 14 days. However, it was significantly reduced after 28 and 42 days under different salt stress (Figure 2b).

3.5. Malondialdehyde Concentration (MDA) and Cell Membrane Stability

With the increased salinity treatments, the MDA content continued increasing, and the low salinity (S1, S2) treatments did not significantly affect the MDA content in leaves after 14 and 28 days (Figure 2c), while after 42 days, a significant increase in the MDA content occurred under S2~S5 salt concentrations. Cell membrane stability remained unchanged after 14 days under NaCl treatments less than S4 in leaves, but a significant increase was observed after 42 days under different treatments (Figure 2d).

3.6. Soluble Sugars, Soluble Proteins, and Proline

The soluble sugars content did not change significantly in leaves under S1 ~ S4 salinity treatments after 14 days of growth but continued increasing after 28 days under various salt stress, while after 42 days of growth, the soluble sugars content in high (S4, S5) salinity treatments was not significantly different than the control as well as plants treated with S1, while it was higher in S2 and S3 treatments (Figure 2e). The soluble proteins content was not significantly affected by salinity treatments after 14 and 28 days, but after 42 days, the content in S1was higher than the control, and a great reduction in protein content was found in S5 treatment (Figure 2f). We observed that the proline content in leaves

under S1 ~ S3 salt stress was not obviously increased after 14 days, however, after 28 and 42 days of growth, the proline content was significantly higher than that of the control group under S2 ~ S5 treatments (Figure 2g).

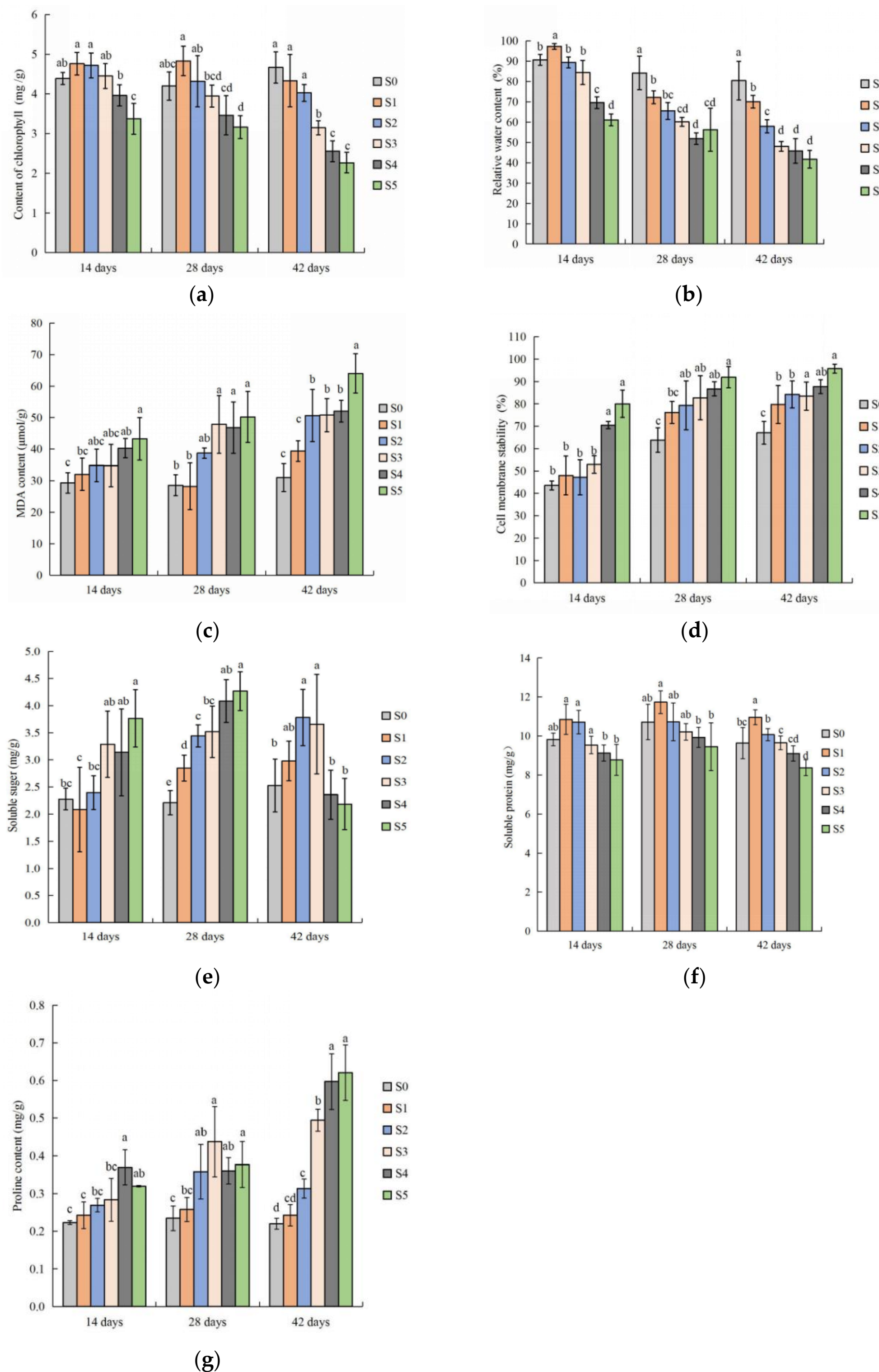

Figure 2. Chlorophyll content (**a**), relative water content (**b**), MDA content (**c**), cell membrane stability (**d**), soluble sugars (**e**), soluble proteins (**f**), and proline content (**g**) in leaves of *C. betulus* plants grown for 14, 28 and 42 days with increasing levels of soil salinity. Different lowercase letters indicate significant differences between treatments ($p < 0.05$), $n = 3$, S0 ~ S5 represents 0 ~ 85 mM NaCl respectively.

3.7. Antioxidant Enzyme Activities

The SOD activity increased at all concentrations of NaCl compared to that of the control plants after 14 and 28 days, as shown in Figure 3a. After 28 days, the SOD activity increased with increasing salt concentrations, with a peak value at S3. However, after 42 days, the SOD activity was higher at low salinity (S1) than the control and was not changed too much under S2 ~ S5 salt stress. Salt stress increased the POD activities in all treatments after 14 and 28 days (Figure 3b), while it was higher in S1 ~ S3, and POD was not obviously affected under high salinity (S4, S5) treatments than the control group after 42 days of stress.

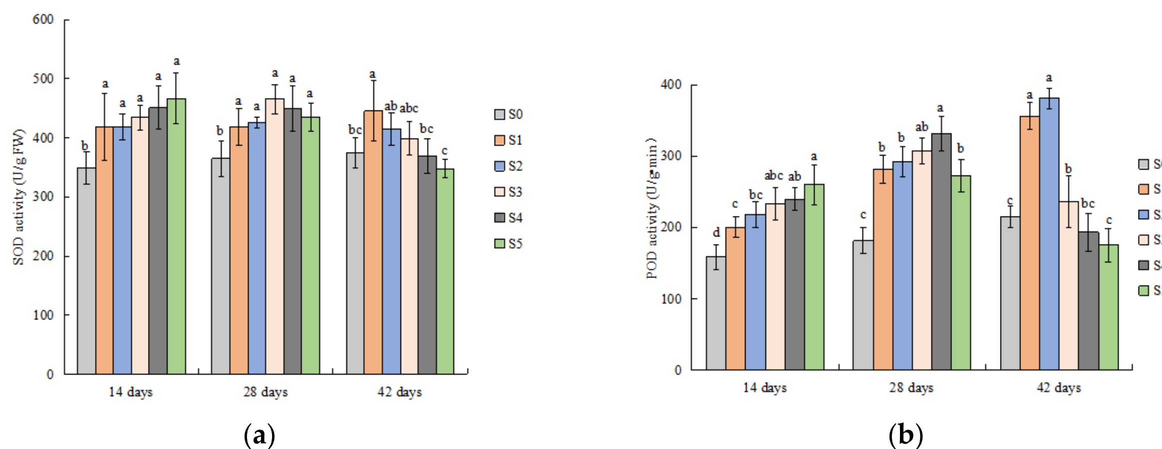

Figure 3. Effect of soil salinity on SOD activity (**a**), and POD activity (**b**) in leaves of *C. betulus* grown for 14, 28 and 42 days with increasing levels of soil salinity. Different lowercase letters indicate significant differences between treatments ($p < 0.05$), $n = 3$, S0 ~ S5 represents 0 ~ 85 mM NaCl respectively.

3.8. Sodium (Na), Potassium (K), Calcium (Ca), and Magnesium (Mg) Content of Various Organs

The Na concentration of the whole plant of *C. betulus* seedlings was increasingly pronounced with the increase in salinity concentration (Table 4). The Na content was significantly increased in stems and leaves under S2 ~ S5 salt treatments, while it was higher than the control in all salinity stress levels in the roots. In particular, the content of Na in the stem was higher than that in the roots and leaves and was significantly different from that of the control, reaching a maximum value at the concentration of S5, which was 20 times that of S0. The K concentration in leaves was significantly higher than that of the control under the salinity treatments, but the K content decreased in the roots. The concentrations of K ions in the roots and stems were lower than those in the leaves under all salt treatments. The Ca content increased in response to the salt treatment in leaves but remained unchanged in the roots and stems with NaCl concentrations less than S3. The Mg content increased significantly in stems, leaves and the whole plants in the S3 ~ S5 treatments, but it decreased in the roots. This result showed that the Ca content remained high in the stems, while the Mg content was the opposite and was higher in roots and leaves. The K/Na, Mg/Na, and Ca/Na ratios decreased significantly with salinity levels, which indicated that with the increased concentration of salt stress, the absorption of Na increased, while the absorption of other nutrients was relatively reduced. We also found that the ratio of each nutrient ion to Na in the leaves was greater than that of the roots and stems, indicating that the relative absorption of the leaves was higher than that of the roots and stems.

Table 4. Effect of different levels of soil salinity on Na, K, Ca and Mg content in roots, stems, and leaves of *C. betulus* seedlings. S0 ~ S5 represents 0 ~ 85 mM NaCl respectively. The data in the table are the mean ± standard deviation (n = 3); different lowercase letters in each column indicate significant differences between treatments ($p < 0.05$).

	Treatment	Na (mg g^{-1})	K (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})	K/Na	Ca/Na	Mg/Na
Root	S0	1.61 ± 0.05 e	7.92 ± 0.23 a	12.78 ± 0.36 b	4.18 ± 0.14 a	4.92 ± 0.04 a	7.59 ± 0.24 a	2.60 ± 0.11 a
	S1	3.61 ± 0.20 d	6.15 ± 0.28 b	12.43 ± 0.54 b	3.24 ± 0.24 bc	1.71 ± 0.15 b	3.45 ± 0.09 b	0.90 ± 0.02 b
	S2	4.75 ± 0.19 c	4.09 ± 0.16 cd	12.88 ± 0.40 b	2.89 ± 0.13 bc	0.86 ± 0.07 c	2.71 ± 0.07 d	0.61 ± 0.03 c
	S3	6.48 ± 0.27 a	3.85 ± 0.12 d	13.28 ± 0.60 b	2.77 ± 0.31 c	0.59 ± 0.01 d	2.05 ± 0.03 e	0.43 ± 0.05 d
	S4	5.44 ± 0.22 b	4.23 ± 0.08 c	16.29 ± 0.28 a	3.25 ± 0.38 bc	0.78 ± 0.05 c	3.00 ± 0.08 cd	0.60 ± 0.10 c
	S5	4.93 ± 0.25 c	4.39 ± 0.12 c	15.59 ± 1.11 a	3.32 ± 0.30 b	0.89 ± 0.06 c	3.17 ± 0.34 ab	0.67 ± 0.05 c
Stem	S0	0.85 ± 0.06 e	6.14 ± 0.24 b	30.22 ± 0.29 bc	2.18 ± 0.11 b	7.26 ± 0.71 a	35.68 ± 2.65 a	2.57 ± 0.22 a
	S1	1.75 ± 0.04 e	6.22 ± 0.17 b	25.17 ± 1.56 de	2.03 ± 0.09 b	3.56 ± 0.16 b	14.40 ± 1.22 b	1.16 ± 0.03 b
	S2	6.83 ± 0.12 d	3.83 ± 0.04 c	26.97 ± 0.35 de	2.01 ± 0.18 b	0.56 ± 0.01 c	3.95 ± 0.03 c	0.30 ± 0.03 c
	S3	9.83 ± 0.35 c	5.65 ± 0.17 b	33.30 ± 1.04 a	2.80 ± 0.09 a	0.58 ± 0.01 c	3.39 ± 0.04 dc	0.29 ± 0.01 c
	S4	15.87 ± 1.15 b	4.68 ± 1.24 c	31.16 ± 2.03 ab	3.01 ± 0.29 a	0.29 ± 0.06 c	1.96 ± 0.04 dc	0.19 ± 0.02 c
	S5	17.21 ± 0.40 a	7.30 ± 0.24 a	28.15 ± 1.43 cd	3.08 ± 0.18 a	0.42 ± 0.01 c	1.64 ± 0.07 d	0.18 ± 0.01 c
Leaf	S0	0.27 ± 0.03 e	7.25 ± 0.41 c	19.55 ± 0.50 d	3.47 ± 0.09 d	26.79 ± 2.52 a	72.30 ± 7.43 a	12.85 ± 1.35 a
	S1	0.45 ± 0.07 e	11.36 ± 0.22 a	22.01 ± 0.52 de	3.75 ± 0.11 bcd	25.49 ± 3.63 b	49.31 ± 6.25 b	8.38 ± 0.98 b
	S2	3.31 ± 0.64 d	9.22 ± 0.56 b	20.56 ± 1.94 cd	3.68 ± 0.30 cd	2.86 ± 0.57 c	6.36 ± 1.25 c	1.14 ± 0.23 c
	S3	9.08 ± 0.33 a	9.02 ± 0.30 b	24.29 ± 1.01 a	4.63 ± 0.20 a	0.99 ± 0.01 c	2.67 ± 0.02 c	0.51 ± 0.01 c
	S4	6.33 ± 0.07 b	8.60 ± 0.27 b	23.86 ± 0.94 ab	4.01 ± 0.23 bc	1.36 ± 0.04 c	3.77 ± 0.17 c	0.63 ± 0.04 c
	S5	5.92 ± 0.18 b	9.06 ± 0.21 b	24.32 ± 1.37 a	4.12 ± 0.26 b	1.53 ± 0.01 c	4.10 ± 0.11 c	0.70 ± 0.02 c
Whole plant	S0	2.73 ± 0.09 e	21.31 ± 0.55 b	62.54 ± 0.84 b	9.83 ± 0.25 ab	38.97 ± 2.81 a	115.93 ± 3.06 a	18.02 ± 1.19 a
	S1	5.81 ± 0.29 d	23.73 ± 0.54 a	59.61 ± 2.22 b	9.02 ± 0.42 bc	30.76 ± 3.93 b	67.16 ± 4.08 b	10.44 ± 0.94 b
	S2	14.89 ± 0.68 c	17.14 ± 0.70 d	60.41 ± 1.69 b	8.58 ± 0.26 c	4.28 ± 0.58 c	13.02 ± 2.04 d	2.04 ± 0.24 c
	S3	25.39 ± 0.25 b	18.52 ± 0.23 c	70.87 ± 0.58 a	10.21 ± 0.37 a	2.16 ± 0.01 c	8.11 ± 0.87 c	1.22 ± 0.05 c
	S4	27.64 ± 1.17 a	17.51 ± 1.13 cd	71.30 ± 2.72 a	10.27 ± 0.85 a	2.43 ± 0.10 c	8.73 ± 2.89 c	1.42 ± 0.15 c
	S5	28.07 ± 0.53 a	20.75 ± 0.52 b	68.06 ± 3.48 a	10.52 ± 0.53 a	2.85 ± 0.04 c	8.91 ± 3.96 c	1.55 ± 0.08 c

4. Discussion

The salinity stress can produce two kinds of stresses on plants: osmotic stress and ion toxicity [34], which cause the decline or injury of plant growth, photosynthetic ability, cytoplasmic membrane permeability and so on. Plants must be adapted to these two stresses to grow properly in saline conditions. Plants can change their morphological structure or enhance their own resistance by physiological and biochemical transformation to adapt to the adverse environment: for example, the protective effect of the accumulation of osmotic regulating substances and scavenging ROS by antioxidant enzymes. Different salinity stress levels may have various effects on plants, and the physiological and biochemical reactions in response to different salt stress levels of plants also differ.

In this study, we found that the salinity stress caused a prominent inhibition in the growth of *C. betulus* seedlings by the changes of height growth, diameter growth, the increment in leaf number, and dry weight of total biomass. A reduction in plant growth is a common effect of salt stress in other tree species, such as *Morus alba* [35] and *Melia azedarach* [36], and morphological changes and growth reductions provide visual evidence of the degree of injury caused by salt [37]. Salinity stress had a significantly higher inhibitory effect on the aboveground parts of *C. betulus* seedlings than on the belowground parts, which caused a significant increase in the root/shoot ratio. It is noteworthy that the growth parameters were significantly changed under S3 ~ S5 (6.2 ~ 10.5 dS m^{-1}) salt concentrations, which indicates that *C. betulus* cannot endure high salinity. According to Richards (1954) [38], sensitive crops do not thrive as the electrical conductivity of the soil is over 4 dS m^{-1}, and hence we can know that *C. betulus* is sensitive to salt stress.

Salinity stress will change the osmotic potential of roots, causing physiological drought in plants. Stomatae provide a channel for controlling the exchange of water and gas in plants, which has a direct effect on transpiration in plants. The characteristics of stomatae on the surfaces of the leaves, as well as their distribution, density, and open stomata, are greatly influenced by environmental moisture conditions. In this study, the stomata density, size, and leaf section characteristics were unchanged until the S2 treatment, while a significant decrease in those properties was found under the S3 ~ S5 treatments. The open stomatae and the degree of opening in *C. betulus* seedlings decreased with

increasing salinity, and the guard cells became deformed and shrank at high salt concentrations; these changes may produce negative effects on the photosynthetic efficiency of plants. Salt stress can lead to the closing of stomatae on leaf surfaces to different degrees and can reduce the size of the stomatal aperture [39]; a similar phenomenon has also been observed in *Fragaria ananassa* [40]. This change may be a physiological adaptation of plants to environmental stress, and our study also confirmed the observation. The leaf thickness, palisade tissue thickness and palisade tissue/leaf thickness ratio of *C. betulus* decreased significantly, the palisade and spongy parenchyma tissues became loosely arranged, and part of the group started to shrink when the salt concentration was over 51 mM. Some research found that the main reason for the reduction of leaf thickness under salt stress was that the length of palisade cells and the thickness of spongy parenchyma tissues decreased, and these changes caused an inhibition of photosynthesis and stunted the growth of seedlings [41]. The degree of the stomatal opening directly determines the transpiration and photosynthetic rates of plants [42]. The closing of stomatae can slow the rate of gas exchange, resulting in a decline in the photosynthetic and transpiration rates. These reasons may explain the reduction in the gas exchange parameters of *C. betulus* under salinity stress.

Soil salinity stress makes it difficult for plants to absorb nutrient elements (N, P), inhibits the synthesis of chlorophyll, accelerates the decomposition of chlorophyll, and reduces the absorption of light energy by chloroplasts. The chlorophyll content of *C. betulus* increased first and then decreased with the degree of salt stress. Some studies have postulated that low salt stimulation can increase the chlorophyll content in plants; for example, NaCl stress (8.5 ~ 34 mM) caused an increase in chlorophyll content in strawberry plants [43], and our study supported this idea. Stomatal regulation was adversely affected in plants under salinity stress [44]. In this study, the reduction of *Pn*, *Tr*, and WUE_i under moderate (S3) and high salinity (S4, S5) conditions may have been due to the negative regulation of stomatal conductance, while *Ci* was increased with the salt stress and negatively correlated with the other photosynthetic indices. It is generally believed that with the reduction of the photosynthetic rate in adversity, including porosity and non-stomatal factors, we can judge the main factor through the change trend of *Ci* [45], and the two factors can both exist under salt stress. In our study, the reduction of the photosynthetic rate of *C. betulus* contained the two factors. Of the different chlorophyll fluorescence parameters measured in *C. betulus* leaves in this study, we found that the NPQ was increased with salinity concentrations, and this effect may be a mechanism of *C. betulus* to protect the photosystems from damage caused by photoinhibition under salt stress [46]. The leaf chlorophyll fluorescence parameters were not significantly affected when subjected to S1 and S2 NaCl treatments, indicating that the electron transport and photosystems were not impaired, but a NaCl concentration over S3 caused severe damage to the plants, reducing the conversion and utilization efficiency of light energy by chloroplasts and inhibiting photosynthesis.

With the increase in the salt concentration after 28 and 42 days, the RWC of *C. betulus* leaves decreased significantly, which may be closely related to the accumulation of sodium ions in the leaves. Soil salinity can affect soil osmotic potential, which makes it difficult for the plant to absorb water and inhibits the growth of plants. Membrane damage is one of the main negative impacts of salinity stress on plants. The accumulation of MDA content in cells is the result of lipid peroxidation, which is often used to evaluate the membrane damage [47]. Cell membrane permeability can reflect the extent of lipid peroxidation caused by ROS and is also used as an important criterion for salt tolerance in the plants [48]. In this study, the content of MDA and cell membrane stability in the leaves of *C. betulus* seedlings increased as the period of salinity stress continued and reached a maximum at last, indicating that salinity stress caused damage to cell membranes and that the membrane protection system was destroyed under high salt concentrations. To relieve the oxidative stress, most plants can synthesize antioxidant enzymes to counteract the ROS in response to salt stress. SOD and POD can effectively eliminate the ROS and prevent the oxidative damage to the cell membrane [49]. In this study, the activities of the two enzymes increased after 14 and 28 days of different treatments, but a reduction in antioxidant enzyme activities was found at S3 ~ S5 NaCl stress after 42 days. This result

was similar to the result of *Quercus variabilis* seedlings [50]. Under low salt concentrations, the activity of protective enzymes in *C. betulus* was increased to scavenge the free radicals, but this ability was limited under moderate and high salt stress due to the decrease in protein synthesis, thus affecting the survival and growth of seedlings.

The ability of osmotic regulation is a key factor for plants to adapt to environmental stress. Osmotic regulation substances mainly include inorganic ions and some soluble organic substances, such as soluble sugars, soluble proteins, and proline [51]. Certain halophyte plants are resistant to salinity up to 200 mM NaCl or more, and can endure salinity by controlling the synthesis of organic 'compatible' solutes [52]. Soluble sugars are the product of photosynthesis, and the accumulation of soluble sugars can regulate the osmotic potential of plant tissue and help improve the resistance of plants to environmental stress. The results showed that the amount of soluble sugars in the *C. betulus* seedlings continuously increased before 28 days but changed insignificantly from that in the control as the salt concentration increased to S4 after 42 days; this result may be because of the decline of photosynthesis under high salt stress. Soluble proteins are materials involved in plant metabolism. The soluble proteins of *C. betulus* were negatively affected by high salinity treatments after 42 days, which was probably caused by the reduction in the assimilation of nitrogen substances or protein proteolysis induced by high salt levels. Proline plays an important role to maintain the osmotic pressure in salt-stressed plants. Reports have verified the function of proline in osmotic adjustment and cell structure protection, and a positive correlation has been observed between proline and tissue sodium content under salinity stress, as in where proline in rice (*Oryza sativa* L.) increased with salinity levels ($4 \sim 12$ dS m^{-1}) [53]. In *C. betulus* seedlings, the proline content continuously increased as the degree of salinity increased after 42 days' stress, which was of positive significance for the plant to adapt to the salt environment.

The separation distribution of ions is one of the important characteristics of plant salt tolerance. The accumulation of Na ions in photosynthetic tissues is extremely deleterious because it will interfere with K functions, including deactivating enzyme activity [54]. The normal activities of many cytosolic enzymes and the maintenance of membrane integrity are mainly decided by Na and K homeostasis in cells. The Na concentration of *C. betulus* significantly increased with the salinity concentration and mainly accumulated in the roots at low salt stress, thus reducing the damage caused by salt ions in the leaves. The K concentration in leaves was significantly higher than that of the control under salinity treatments. The Ca and Mg content changed minimally during the salt treatments. However, the K/Na, Mg/Na and Ca/Na ratios decreased significantly with salinity levels, which was mainly attributed to the increase in Na ions. These results indicated that *C. betulus* plants may be able to maintain ion homeostasis in the plants under low salt stress, while this capability is limited under moderate and high salt stress, causing the decline of biomass and photosynthesis.

5. Conclusions

This study showed that the growth and physicochemical parameters of *C. betulus* seedlings were inhibited under high salinity stress, including reduced gas exchange and increased MDA content, membrane permeability, and sodium accumulation, suggesting a limited degree of salt tolerance. We found that *C. betulus* seedlings can adapt to low salinity stress (S1, S2) by increasing the accumulation or synthesis of key osmotic adjustment substances and improving the activity of antioxidant enzymes and ionic homeostasis. However, the salt tolerance of *C. betulus* is limited under moderate (S3) and high salinity (S4, S5); the plant develops an insufficient resistance to the soil environments and has a difficult time growing. The inhibition of photosynthetic gas exchange, the restriction of the ability to synthesize osmotic adjustment substances and antioxidant enzymes, and the failure to restrict sodium exclusion may be the main reasons for the reduction of growth under high salinity levels for *C. betulus* seedlings. Therefore, *C. betulus* can be planted and popularized in low salinization areas, and its adaptability should be further observed.

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