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Male and Female Plants of *Salix viminalis* Perform Similarly to Flooding in Morphology, Anatomy, and Physiology

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Abstract: *Salix viminalis* L., a dioecious species, is widely distributed in riparian zones, and flooding is one of the most common abiotic stresses that this species suffers. In this study, we investigated the morphological, anatomical, and physiological responses of male vs. female plants of *S. viminalis* to flooding. The results showed that the plant height and root collar diameter were stimulated by flooding treatment, which corresponded with higher dry weight of the stem and leaf. However, the dry weight of the underground part decreased, which might be due to the primary root having stopped growing. The little-influenced net photosynthesis rate (P_n) under flooding treatment could guarantee rapid growth of the aboveground part, while the unaffected leaf anatomical structure and photosynthetic pigment contents could ensure the normal operation of photosynthetic apparatus. Under a flooding environment, the production ratio of superoxide free radical ($O_2^{\cdot-}$) and malondialdehyde (MDA) contents increased, indicating that the cell membrane was damaged and oxidative stress was induced. At the same time, the antioxidant enzyme system, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), and osmotic adjustment substances, involving proline (Pro) and solute protein (SP), began to play a positive role in resisting flooding stress. Different from our expectation, the male and female plants of *S. viminalis* performed similarly under flooding, and no significant differences were discovered. The results indicate that both male and female plants of *S. viminalis* are tolerant to flooding. Thus, both male and female plants of *S. viminalis* could be planted in frequent flooding zones.

Keywords: willow; dioecious plant; gender difference; anatomical structure; gas exchange; antioxidant enzyme; osmotic adjustment

1. Introduction

Flooding is one of the primary abiotic stresses encountered by many plants, and it develops when the water content in the soil surpasses the field moisture capacity [1]. Soil flooding is usually caused by

heavy rainfall, poor soil drainage, and some irrigation practices [2]. The water layer above the soil can be shallow or deep, so partial or complete submergence can be induced [3]. The Intergovernmental Panel on Climate Change (IPCC) (http://www.ipcc.ch) reported that man-induced climate change will increase the frequency of heavy precipitations and tropical cyclone activity, and it is likely that the flood plains (i.e., lowlands), riparian zones, and cultivated lands will suffer from more frequent flooding events [4].

Soil flooding always restricts O_2 diffusion to plants, and thereby inhibits their aerobic respiration [5]. This is often accompanied by a decrease in soil pH and an accumulation of toxic soil substances [6,7]. Under low-oxygen conditions, plants usually have typical alternations in morphology, anatomy, and physiology to adapt to the anaerobic environment. At the morphological level, some adaptable plants under flooding conditions are often taller than their non-flooded comparisons, such as marsh dock (*Rumex palustris* Sm.) [8], rhodes grass (*Chloris gayana* Kunth), kleingrass (*Panicum coloratum* L.) [9], and Sentang (*Azadirachta excelsa* (Jack)Jacobs) [10]. On the contrary, there are also some plants that exhibit shorter stems as a result of the negative impact of the anaerobic environment on growth [11,12]. In addition, an obvious response of plants tolerant to flooding is the formation of adventitious roots, which could help to maintain normal function of water and nutrient uptake and thus relieve the harmful effect of flooding [12,13]. Besides, the generation of aerenchyma in tissues is the most common anatomical response, which could facilitate the transport of oxygen from shoots to roots [3].

Leaf gas exchange parameters are one of the most frequently studied physiological processes of plants under flooding. The photosynthetic rate of many plants has been shown to present a significant declining trend, especially for flooding-sensitive plant species [13–15]. However, flooding-tolerant plants could maintain a high photosynthetic rate or photosynthesis is basically not influenced [14]. The causes for inhibited photosynthesis can be complex, including declined stomatal closure, decreased chlorophyll content, destruction of chloroplast membrane structure, reduction of enzyme activity associated with photosynthesis, and so on [15,16].

Under a flooding environment, the production of reactive oxygen species (ROS), such as superoxide free radical ($O_2^{\cdot-}$), increases, which causes oxidative damage, lipid peroxidation of cell membranes, and irreversible metabolic dysfunctions, leading to cell death [17–19]. Malondialdehyde (MDA) is the final product of membrane lipid peroxidation, and the content of this substance is often used to assess the degree of oxidative damage of the cell plasma membrane [20,21]. However, the cellular ROS level can be regulated by a variety of antioxidant enzymes and nonenzymatic antioxidants [22]. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) activities are often activated when plants are subjected to flooding, indicating the efficient role of antioxidant enzymes in scavenging ROS [23,24].In addition, plants can also accumulate osmotic regulators, such as proline (Pro), to adjust the osmotic potential of cells, so that the stress pressure can be relieved [25].

Sexuality was precisely defined by Sachs for the first time, and since then, the study of sex in plants has been an interesting topic for researches [26]. Today, it is well established that dioecious plants account for 5.1% to 6.0% of angiosperm species (15,600 of 304,419 to 261,750) distributed in 987 genera and 175 families [27]. They play an important role in sustaining the stability and continuity of the structure and function of terrestrial ecosystems [28]. Because of different reproductive costs between male and female plants [29], gender (denoting the development of male and female individuals of plants) differences may be manifested under environmental stresses [30–32]. Previous studies reveal that gender differences vary greatly in plant species. It is shown that male plants of cathay poplar (*Populus cathayana* Rehd.) and yuannan poplar (*Populus yunnanensis* Dode.) are more resistant to stresses such as drought, salinity, and heavy metal [33–35]. However, the female plants of sea buckthorn (*Hippophae rhamnoides* L.) and dark-leaved willow (*Salix myrsinifolia* Salisb.) exhibit stronger tolerance to drought and UV-B stress, respectively [36,37]. So far, limited studies have been carried out to discuss gender-specific responses to flooding.

River systems are dominated by plenty of dioecious Salicaceae plants, which are frequently flooded [15,38]. Previous studies indicate that females of narrowleaf cottonwood (*Populus angustifolia*

James) are more tolerant to flooding than males, which could be related to the greater frequency of females in flood-prone sites [39,40]. *Salix viminalis* L., a species of *Salix*, Salicaceae, is predominant in riparian zones, and the female plants are more prevalent in wetter zones that are prone to suffer from flooding. However, whether the male and female plants of *S. viminalis* perform differently (i.e., the females are more tolerant to flooding than males) under flooding has not been documented. Although there has been research to examine the responses of *S. viminalis* to soil flooding [41], no studies have been conducted to discuss possible gender differentiation of *S. viminalis* under flooding. In this study, we investigated the gender-specific responses of *S. viminalis* in morphology, anatomy, growth, gas exchange, chlorophyll pigment, oxidative stress degree, osmotic adjustment, and enzymatic antioxidants to flooding stress. The objectives of this study were to: (1) explore how flooding affects the morphological, anatomical, and physiological processes of dioecious *S. viminalis,* and (2) assess whether differences exist between male and female plants of *S. viminalis* under flooding stress.

2. Materials and Methods

2.1. Plant Materials and Experiment Design

A male parent of *S. viminalis* from Heilihe nature reserve and a female parent from Saihanwula nature reserve were used for controlled intraspecific hybridization. In the F1 progeny containing 570 seedlings, 10 male and 10 female individuals were selected randomly. One-year-old branches were harvested to propagate cuttings from each individual on March 29th, 2018. The cuttings with a length of about 10 cm were grown in nutritive bowls filled with peat soil. Then, five male and five female clones were replanted in 16 cm diameter × 15 cm deep plastic pots on May 12th and the substrate was a 2:1 mixture of garden soil to peat soil. The plants were placed in a greenhouse of the Chinese Academy of Forestry Sciences in Beijing and were watered regularly until flooding application.

On June 10th, plants of similar height were selected for flooding treatment. The experimental layout was a completely randomized block design with two main factors (gender and watering regime). Two watering regimes were applied: well-watered treatment as control and flooding treatment. In the control, the plants were watered every day to maintain soil moisture at field capacity. In the flooding treatment, the pots were placed into a larger plastic bucket filled with tap water to 4 cm above the soil surface and watered every day to maintain the water level. During the flooding treatment, the morphological, photosynthetic, and physiological parameters were investigated at 7, 14, 21, 30, and 45 days. For the physiological parameters, the leaves were cut into pieces and mixed, put into the liquid nitrogen, and then stored at –80 °C. There were five blocks in this research, and the experiment was conducted during the gowning season from June 10th to July 25th, 2018. During this period, the precipitation is large and flooding events are more prone to occur.

2.2. Growth Characteristics (Height, Diameter, and Biomass)

The height and root collar diameter of each individual plant were measured at 0, 7, 14, 21, 30, and 45 days. The plant height was measured by a measuring tape with a precision of 0.1 cm, and the root collar diameter was evaluated by Vernier caliper with an accuracy of 0.01 cm. At the end of the experiment, the root, stem, and leaf were harvested and each component was dried to a constant weight and weighed, respectively.

2.3. Leaf Gas Exchange Parameters

Gas exchange parameters were measured in the 3rd~5th fully expanded and mature leaf from the top of the stem between 9:00 and 12:00 in the morning using the Li-Cor 6400XT photosynthesis measuring system (LI-COR, Lincoln, NE, USA) equipped with a red-blue light-emitting diode light source. The parameters included net photosynthesis (P_n), stomatal conductance (G_s), internal CO_2 concentrations (C_i), and transpiration rate (T_r). The optimal parameters were set as follows:

photosynthetic photon flux density of 1200 μmol m^{-2} s^{-1} and an approximately ambient CO_2 concentration of 400 μmol mol^{-1}.

2.4. Chlorophyll Pigment

About 0.20 g of leaf tissue, 0.2 × 0.2 cm in size, was immersed into 80% acetone (v:v) for 24 h, approximately under room temperature until all of the tissue turned white. Meanwhile, the tube was shaken at intervals. Then, the absorbance of the extracting solution was measured at 470, 646, and 663 nm. The content of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoid (Caro) were calculated by the equation described by Lichtenthaler [42].

2.5. Proline Content

Pro was extracted and measured according to the procedure by Bates et al. [43]. About 0.30 g of leaf tissue was immersed into 5 ml of 3% aqueous sulfosalicylic acid solution. After being extracted in a boiling water bath and filtered, 2.0 ml of glacial acetic acid and 2 ml of 2.5% acid ninhydrin were added into 2 ml of the supernatant. Then, the mixed solution was incubated in a boiling water bath. After the mixture was cooled, 5 ml of toluene was added and the absorbance was measured at 520 nm.

2.6. Production Ratio of Superoxide Free Radical, Malonaldehyde Content, Solute Proteins Content, and Autioxidant Enzyme Activities Determination

The frozen leaves were used to test the physiological indicators, including the production ratio of $O_2^{\cdot -}$, the MDA content, the solute proteins (SP) content, the SOD activity, the POD activity, the CAT activity, and the APX activity. All of these indicators were analyzed by applying the respective assay kit (Comin Biotechnology Co. Ltd., Suzhou, China).

2.7. Anatomical Structure Observation

The 5th fully expanded and mature leaf from the top of the stem was used for anatomical analysis. The same parts of the leaf were cut into 2 mm strips perpendicular to the main vein and immediately fixed in formalin, acetic acid, and alcohol solution (FAA) solution (70% alcohol/glacial acetic acid/40% formalin = 90:5:5, v/v) and stored at 4 °C. Then, the materials were, respectively, dehydrated with gradient ethanol of 75% (4 h), 85% (2 h), 90% (2 h), 95% (1 h), and 100% concentrations (two changes, 30 min). Next, they were immersed in mixed liquor with ethanol and xylene (10 min) and xylene (two changes, 10 min). Thereafter, the samples were embedded in paraffin by the Embedding Center (JB-P5, Wuhan Junjie Electronic Co., Ltd.), and subsequently crosscut into 4-μm-thick slices by a microtome (RM2016, Shanghai Leica Instrument Co. Ltd.). After dyeing in saffron for 2 h and solid green for 40 s, the cross-sections were observed and photographed using a NIKON ECLIPSE E100 microscope (Nikon Corporation, Japan).

2.8. Statistical Analysis

All data were analyzed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Before the ANOVAs, the data were checked for normality, as well as the homogeneity of variances using Shapiro-Wilk test and Levene's test, respectively. Individual differences among the means for each investigated time and each part of plant were identified by Duncan tests of one-way ANOVAs at a significance level of $p < 0.05$. Two-way ANOVAs were used to test the effects of gender, flooding, and their interaction, which was carried out using the multivariate General Linear Model procedure with Type III sum of squares.

3. Results

3.1. Gender-Specific Responses of Morphology to Flooding

All of the plants grew vigorously and no withering, defoliation, or death occurred during the study. At the end of experiment, about 3~5 leaves at the bottom of the plant under the flooding

treatment turned yellow. White hypertrophied lenticels and adventitious roots developed at the base of the submerged shoot after 3 and 5 days of flooding treatment, respectively. However, there were no obvious differences between male and female plants.

3.2. Gender-Specific Responses of Plant Growth to Flooding

S. viminalis plants continued to grow for both the control and flooding treatment groups during the experiment (Figure 1). Flooding did not significantly affect the plant height or root collar diameter before the first 21 days, but the two parameters for flooding treatment were significantly higher than the control at 30 and 45 days. However, the plant height and root collar diameter between male and female plants showed no obvious differences throughout the experiment (Figure 1). Besides, the growth parameters were not significantly affected by gender × flooding interaction.

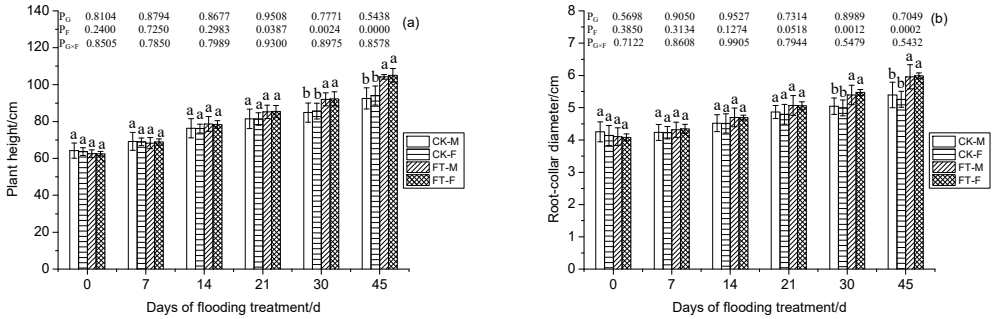

Figure 1. The plant height (**a**) and root collar diameter (**b**) of male vs. female *Salix viminalis* plants at different times of flooding treatment. Note: The data are the mean ± standard deviation (SD) from five replicates. Different small letters indicate significant differences between treatments at the same time point according to Duncan tests ($p < 0.05$). CK, control; FT, flooding treatment; M, male; F, female; P_G, gender effect; P_F, flooding effect; $P_{G\times F}$, gender and flooding interaction effect.

At the end of the flooding treatment, we found that the underground biomass of male and female plants was significantly decreased, including primary root and total root, in spite of the emergence of adventitious root, while the aboveground biomass (stem and leaf) was significantly increased (Figure 2). However, the total dry weight presented no significant differences between the control and the flooding treatment. Besides, the dry weight of each constituent and sapling had no significant differences between male and female plants. In addition, the dry weight was not significantly affected by gender × flooding interaction.

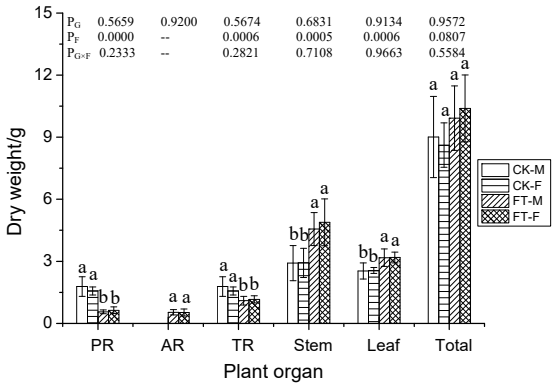

Figure 2. The dry weight of primary root, adventitious root, total root, stem, leaf, and sapling (total) of male vs. female *Salix viminalis* plants at the end of flooding treatment. PR, primary root; AR, adventitious root; TR, total root.

3.3. Gender-Specific Responses of Leaf Anatomical Structure to Flooding

The male and female plants displayed similar cross-section structures, including one-layer upper and lower epidermis, bi-layered compact palisade parenchyma, 2–3-cell-thick loose spongy parenchyma and transversal vascular bundles, etc. (Figure 3). After flooding treatment for 45 days, the anatomical structure of males and females were not affected significantly and palisade cells were still arranged regularly (Figure 3).

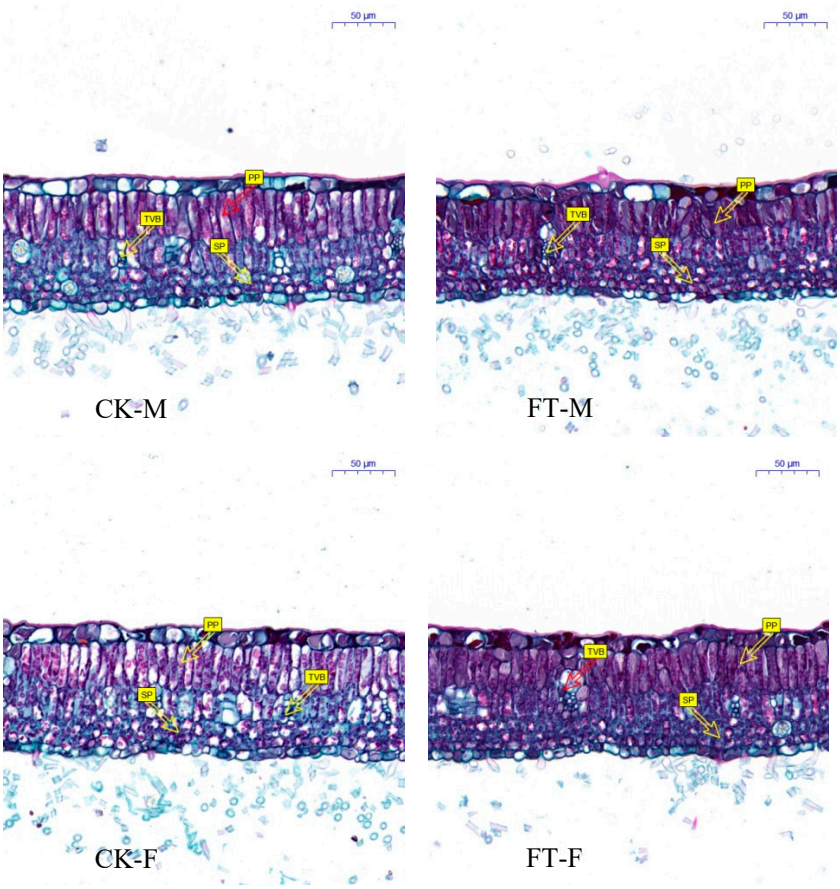

Figure 3. Leaf cross-sections of male vs. female *Salix viminalis* plants at 45 days of flooding treatment. Observations were done using th NIKON ECLIPSE E100 microscope at 20 × Magnification. *Scale bars* = 50 μm. PP, palisade parenchyma; SP, spongy parenchyma; TVB, transcurrent vascular bundle.

3.4. Gender-Specific Responses of Gas Exchange Parameters to Flooding

The gas exchange parameters, including P_n, g_s, C_i, and E, were not significantly affected by flooding throughout the experiment, although they fluctuated over time (Figure 4). The male and female plants of *S. viminalis* also presented no obvious differences. In addition, the gas exchange parameters were not significantly affected by gender × flooding interaction.

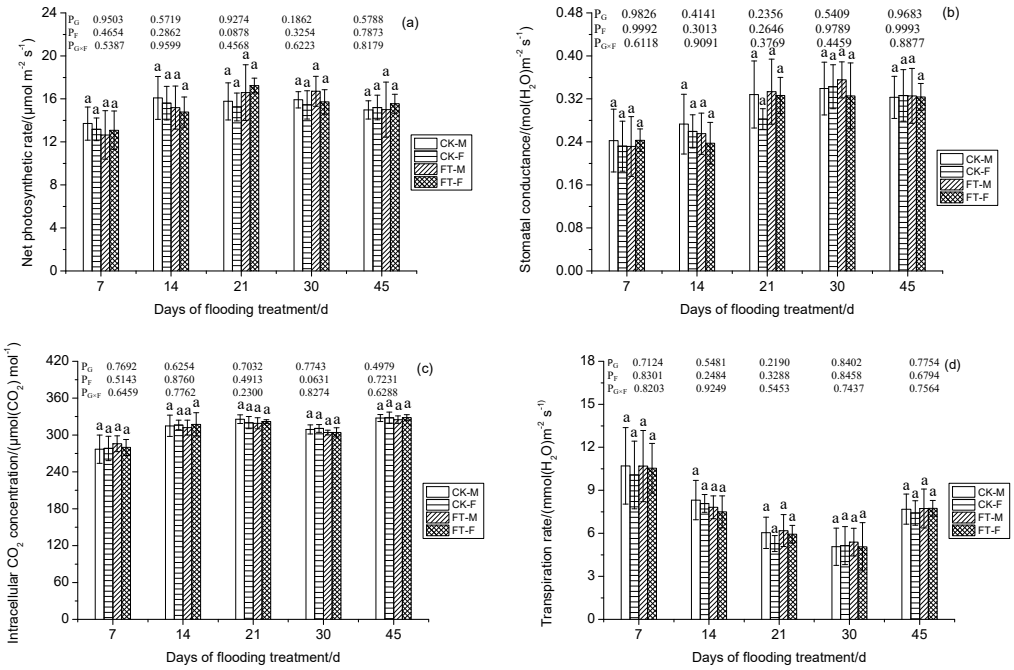

Figure 4. Net photosynthesis rate (P_n) (**a**), stomatal conductance (g_s) (**b**), intracellular CO_2 concentration (C_i) (**c**), and transpiration rate (E) (**d**) of male vs. female *Salix viminalis* plants at different times of flooding treatment.

3.5. Gender-Specific Responses of Chlorophyll Pigments to Flooding

The contents of Chl a, Chl b, and Car of *S. viminalis* plants were not significantly influenced by flooding treatment, and the three pigments also presented no significant difference between male and female plants (Figure 5). In addition, chlorophyll pigments were not significantly affected by gender × flooding interaction.

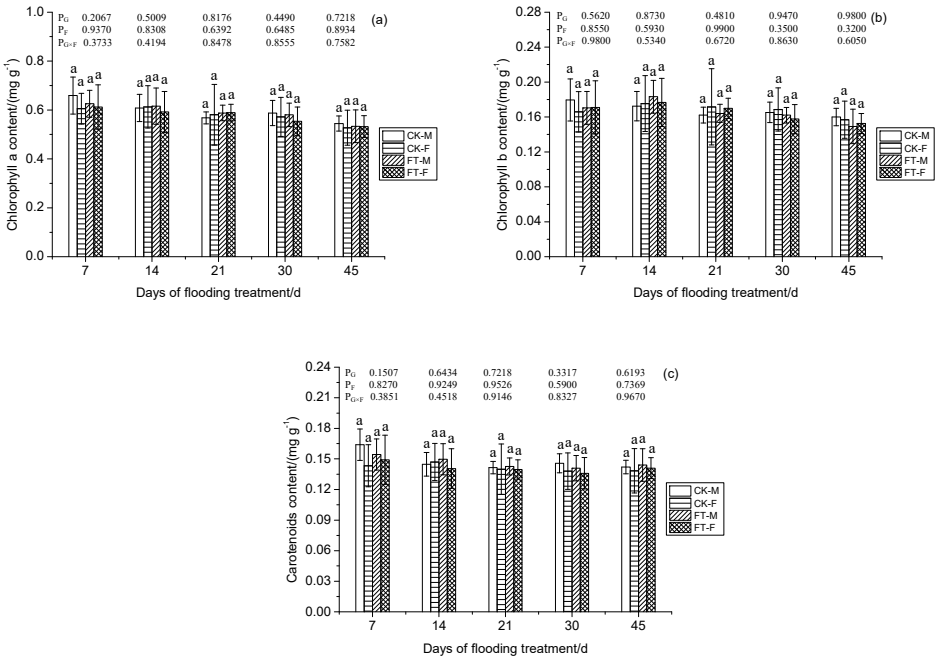

Figure 5. Chlorophyll a (Chl a) (**a**), Chlorophyll b (Chl b) (**b**), and Carotenoids (Car) (**c**) contents of male vs. female *Salix viminalis* plants at different times of flooding treatment.

3.6. Gender-Specific Responses of Superoxide Free Radical and Lipid Peroxidation to Flooding

Flooding treatment led to increases in the production ratio of $O_2^{\cdot-}$ and MDA contents of *S. viminalis* (Figure 6). The production ratio of $O_2^{\cdot-}$ under flooding treatment was significantly higher than that of the control after 14 days, while MDA contents increased significantly after 30 days of flooding treatment. However, both the production ratios of $O_2^{\cdot-}$ and MDA contents between male and female plants showed no significant differences. In addition, the two parameters were not significantly affected by gender × flooding interaction.

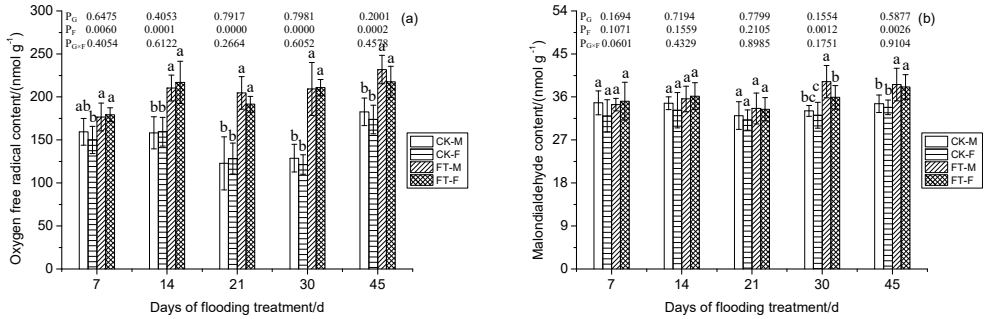

Figure 6. Production ratio of superoxide free radical ($O_2^{\cdot-}$) (**a**) and malondialdehyde (MDA) (**b**) contents of male vs. female *Salix viminalis* plants at different times of flooding treatment.

3.7. Gender-Specific Responses of Enzyme Activities to Flooding

Flooding treatment caused significant increases of SOD, POD, CAT, and APX activities in both male and female plants after 14 days, and the four enzyme activities were on the rise alongside the flooding treatment time (Figure 7). However, there were no significant differences between males and females of *S. viminalis*, neither in the control nor the flooding treatment. Besides, the enzyme activities were not significantly affected by gender × flooding interaction.

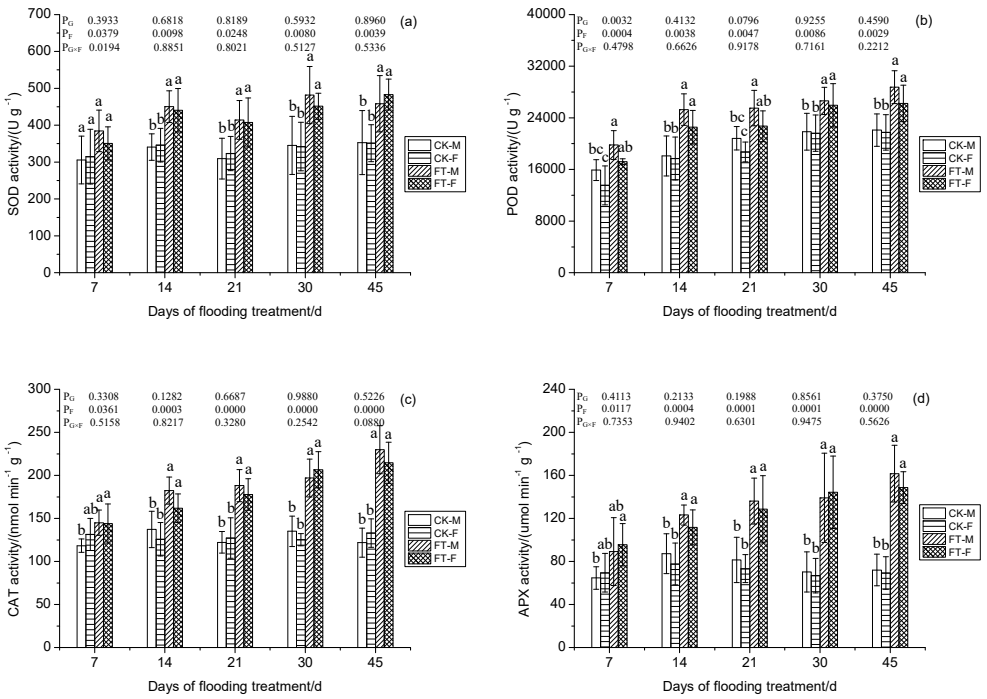

Figure 7. Superoxide dismutase (SOD) (**a**), peroxidase (POD) (**b**), catalase (CAT) (**c**), and ascorbate peroxidase (APX) (**d**) activities of male vs. female *Salix viminalis* plants at different times of flooding treatment.

3.8. Gender-Specific Responses of Osmotic Regulation to Flooding

Compared with the control, the flooding treatment increased Pro and SP contents significantly in *S. viminalis*, but there were no obvious differences between male and female plants (Figure 8). The Pro contents increased continually as time went on, while the SP contents presented a fluctuating variation throughout the experiment. Furthermore, the Pro and SP contents were not significantly affected by gender × flooding interaction.

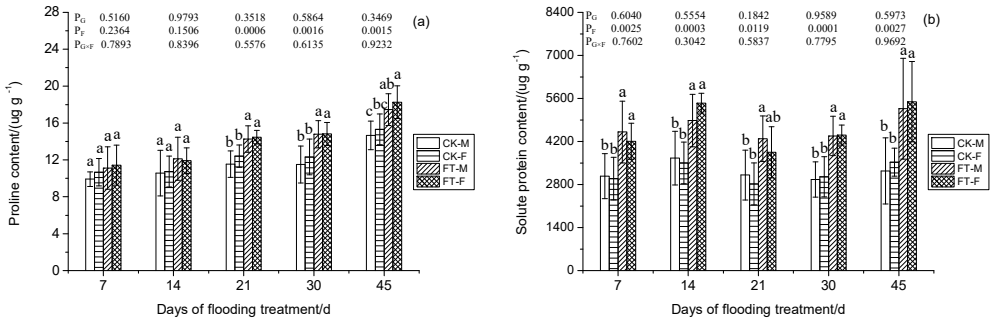

Figure 8. Proline (Pro) (**a**) and solute protein (SP) (**b**) contents of male vs. female *Salix viminalis* plants at different times of flooding treatment.

4. Discussion

4.1. Flooding Treatment Effects

4.1.1. Morphology, Growth, and Anatomical Structure

S. viminalis is a species of obligated riparian trees that are often distributed along streams and rivers and other wet areas [44]. In this research, we studied the response of males vs. females of *S. viminalis* in an artificial flooding treatment with water up to 4 cm above the soil surface. Hypertrophied lenticels and adventitious roots were induced after 3 days of flooding treatment, which are specific changes of flood-tolerant species [1,3]. The hypertrophied lenticels can contribute to air exchange between the stem and root of a plant and the atmosphere [1,45], while the adventitious roots can replace some functions of older damaged roots and help plants to uptake water and nutrients normally [24,46]. Symptoms of chlorosis and defoliation of leaves can also be induced by flooding treatment [1,47]. However, only yellow leaves of the flooded plants were observed in our study, which might indicate that *S. viminalis* is slightly damaged by long-time flooding treatment.

Flooding can bring about a reduction of plant height and root collar diameter, and the biomass of the leaf, stem, and root can also decrease [13,39,48,49]. In our study, flooding irritated the growth of the aboveground part, accompanied by taller plant height and thicker root collar diameter, which was consistent with the larger dry weight of the leaf and stem. However, the growth of the underground part was suppressed, along with a lighter dry weight of the root. Our results concerning morphology and biomass are consistent with previous findings of purpul osier (*Salix integra* Thunb.), *Salix variegate* Franch, and marsh bluegrass (*Poa leptocoma* Trin.) [38,50,51], which indicate that flooding has a negative effect on the root system, while it promotes the growth of the aerial part. The elongation of the stem might be an effective strategy for *S. viminalis* under our experimental conditions with a shallow water level, which might contribute to escaping from severe flooding [52–54]. In this study, morphological changes, including the emergence of hypertrophied lenticels and adventitious roots and accelerated aboveground growth, were conducive to adapting riparian zones for *S. viminalis*.

Anatomical characteristics are also important in improving the flooding tolerance of plants [55]. A previous study found that flooding-susceptible poplar displays an unstable anatomical structure in which the shape of palisade cells turns from long columns into circles [47]. However, the flooding-tolerant species still own regular palisade cells and are characterized by the presence of aerenchymatous

tissue [47,56]. In our study, the anatomical structure of both male and female *S. viminalis* was not influenced by flooding, which could ensure the stability of the photosynthetic structure. However, aerenchymatous tissue was not observed in the leaves of *S. viminalis*, which might be due to the fact that CO_2 could diffuse normally and so the formation aerenchymatous tissue was not initiated.

4.1.2. Photosynthesis

The photosynthetic rate generally decreases for non-flooding-tolerant species during the flooding period, and the earliest response is stomatal closure [15,49]. However, flooding-tolerant species can maintain a high level or even an unaffected photosynthetic rate [14,57]. Although the P_n of *S. viminalis* fluctuated over time, it was not significantly affected by flooding. The photosynthetic response of *S. viminalis* was in accordance with species of the same genus, such as *S. integra* cv. *qingpi* [1]. Usually, g_s has a positive correlation with P_n [58], which also applied to this study. The high g_s may increase gas exchange rates, as well as the total volumes, and hence plants assimilate more photosynthates for growth [59]. Also, the appearance of hypertrophied lenticels and adventitious roots in a short time is conducive to keeping the stoma open and maintaining an unaltered photosynthetic rate [60]. The pigment concentrations in the leaves of plants are closely associated with photosynthesis. Many previous studies have found that the Chl a, Chl b, and Car contents usually decline under flooding conditions, along with a reduction of photosynthesis [15,49,61]. However, the Chl a, Chl b, and Car contents in this study were unchanged, which could ensure the normal operation of photosynthesis. The observation of photosynthesis and chlorophyll indicate that *S. viminalis* were tolerant to flooding.

4.1.3. Oxidative Stress

Plants exposed to flooding conditions usually accumulate ROS, such as $O_2^{\cdot-}$ [62]. The ROS react with unsaturated fatty acids, which results in destruction of the membranes, while MDA is the final production of membrane lipid peroxidation [62,63]. We observed an enhanced production ratio of $O_2^{\cdot-}$ and MDA contents for both male and female *S. viminalis* plants under flooding treatment; the former and the later showed significant increases after 14 days and 30 days of treatment, respectively. Similar results have been shown in previous studies [19,64], and usually, the production ratios of $O_2^{\cdot-}$ and MDA contents are positively correlated with flooding treatment time [64,65]. The increased production ratios of $O_2^{\cdot-}$ and MDA contents can damage the membrane lipids and plants cells, break the cytomembrane structure, and cause oxidative stress [62,64,66]. Our results indicate that the flooding could induce oxidative stress and cause damage to the leaf cells of *S. viminalis*.

4.1.4. Antioxidant System

Plants have developed an antioxidant system, including a series of enzymes (SOD, POD, CAT, etc.) and substances to defend or alleviate the detrimental effect of ROS by metabolism under abiotic stresses [64]. Usually, the antioxidant enzyme activities are positively correlated with the self-protective ability of plants [67]. SOD is the first line of defense and it catalyzes the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2 timely and effectively in the cytosol, chloroplasts, and mitochondria [34]. SOD activity significantly increased after 14 days of flooding treatment in this study, suggesting that the detoxification of $O_2^{\cdot-}$ in *S. viminalis* is effective. POD, CAT, and APX can catalyze the decomposition of H_2O_2 to water and oxygen, eventually clearing up H_2O_2 in cells [23,34,64]. The three enzymes increased significantly in our study, which contributed to neutralizing the product H_2O_2 from the previous SOD. Similar result has been obtained in açai berry (*Euterpe oleracea* Mart.) [62] treated with flooding. The enhanced activities of antioxidant enzymes could increase the antioxidant ability of *S. viminalis*, and thus were helpful to reduce oxidative stress.

4.1.5. Osmotic Substances

Osmotic adjustment has been regarded as one of the most crucial protective mechanisms for plants to adapt to stressful environments, while Pro and SP are major osmotic substances [16,34]. Pro can act as

a cell protector and reducer of osmotic potential, which is involved in many cellular processes to keep a balance of ROS [19]. SP is also an important osmotic protective substance that plays a role in regulating osmotic potential [68]. Previous studies have found that flooding significantly increases Pro content in the leaves of pigeonpea (*Cajanus cajan* L. Millsp.) [69] and bambara groundnut *(Vigna subterranea* L. Verdc) [70], while accumulated SP has been discovered in *Acorus tatarinowii* Schott under flooding conditions [71]. Accumulated Pro and SP contents were observed in our study, indicating that osmotic adjustment was irritated and acted as an effective way for *S. viminalis* to respond to flooding. However, there is some dispute about the accumulation of Pro. An et al. [21] found that Pro accumulation does not contribute to maintaining water balance and it may be an indicator of injury for fig leaves under flooding condition.

4.2. Gender-Specific Responses

It is generally assumed that differences exist in males and females of dioecious plants due to different resource allocation, i.e., that females apply more resources in defense while males invest more resources in growth [72,73]. Gender differences usually appear or are greater under adverse conditions [74], and such differences exist in many plants, such as *Populus cathayana* [35], European aspen (*Populus tremula* L.) [75], and grey willow (*Salix glauca* L.) [76]. Gender-specific differences in flooding resistance vary in plant species, even when the plants are in the same genus. A previous study showed that males of eastern cottonwood (*Populus deltoides* Marsh) have better cellular defense mechanisms against damage caused by waterlogging stress, whereas females are more responsive to waterlogging stress [48]. In *S. variegate*, the males can adjust more flexibly their resource allocation, and so they are more tolerant to flooding [77]. However, the female plants of *P. angustifolia* are more tolerant to flooding, which is strengthened by the increased occurrence of female cottonwoods in streamside zones [39,40]. The favored occurrence of female plants in wetter sites is also apparent in some willows, and it is observed that female willows grow more vigorously than males [78,79].

In our study, flooding treatment caused morphological and physiological changes in *S. viminalis*, but male and female plants performed similarly and no significant gender-specific differences were discovered. This result is different from previous studies on gender differences of *S. viminalis*, which found that female plants may be more severely affected by fungal pathogens and high temperatures than males [80,81]. However, gender differences of *S. myrsinifolia* are not much affected by simulated climatic changes (enhanced CO_2 and temperature or their combination) at the pre-reproductive stage [73], and no specific difference was found in *P. angustifolia* in response to seasonal changes in water availability [82]. In our study, no obvious differences were found between the two genders, suggesting that similar patterns of morphology, anatomy, and physiology may be adopted by the two genders to co-exist in riparian zones, which is consistent with two dioecious riparian shrub species, namely, *Salix myrsinifolia* and *Salix lapponum* [83]. Besides, all plants survived 45 days of flooding with no leaf abscission, fast growth aboveground, unaffected photosynthesis, and strong antioxidant capacity and osmotic adjustment ability, which suggests that both male and female plants of *S. viminalis* are flood-tolerant. Thus, it is concluded that the greater frequency of females in riparian zones is not influenced by flooding, and other factors, such as demographic parameters, could also influence the spatial distribution of genders [84].To better understand the response of male and female *S. viminalis* to flooding, further studies at the level of molecular biology are needed.

5. Conclusions

The combination of morphological, anatomical, and physiological parameters can help us to better understand the gender-specific responses of *S. viminalis* to flooding. The present study suggests that the growth of plant height and root collar diameter is stimulated by flooding treatment, accompanied by increased dry weight of the aboveground part. The little-affected photosynthesis could guarantee the rapid growth of plants, while the unaffected leaf anatomical structure and photosynthetic pigment contents could ensure the normal operation of photosynthetic apparatus. However, the dry weight of

the underground part decreased at the end of the experiment, which might be owing to the fact that the primary root stopped growing. The flooding treatment caused oxidative stress in *S. viminalis*, but they could resist the stress by enhancing antioxidant enzyme activities and osmotic adjustment ability. Different from our expectation, gender-specific differences in *S. viminalis* were not obviously detected, and the male and female plants performed similarly to flooding. Comprehensive indicators indicate that both male and female plants of *S. viminalis* are tolerant to flooding, which would guarantee a sustainable population and maintain a stable riparian ecosystem. Thus, both male and female plants of *S. viminalis* could be planted in frequent flooding zones.

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