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# Sex-specific responses of *Populus deltoides* to combined salinity and calcium under waterlogging conditions

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## Abstract

In this study, we investigated the sex-specific ion uptake and physiological and biochemical responses to combined salinity and calcium treatments of male and female *Populus deltoides* under waterlogging conditions. Results indicate that both *P. deltoides* clones were sensitive to salinity and calcium. Under well-watered conditions, salinity stress could especially increase the Na content in female *P. deltoides* clones, whereas salinity and calcium could increase the K and Ca content in male clones. Waterlogging could evidently stimulate Na content in leaves under salinity, especially in female *P. deltoides* clones. However, waterlogging had no visible effects on the amount of Ca absorption between the two clones under abundant calcium conditions. The physiological and biochemical responses to combined salinity and calcium exceeded the effects of salinity or calcium individually. However, no significant difference was observed, hence indicating the similar responses of male and female *P. deltoides* clones. The lower Na content and Na:Ca ratio, and the higher K and Ca content in male clones suggest that the male clones could maintain ion homeostasis better than the female clones. The sex-specific differences in net photosynthetic rate, intercellular $CO_2$ concentration, effective quantum yield of photosystem II, photochemical quenching coefficient, non-photochemical quenching coefficient, photosynthetic electron transport rate, content of superoxide anions, hydroxyl radicals, $H_2O_2$, soluble protein, and activities *of superoxide dismutase* under certain conditions suggest that female *P. deltoides* clones are more sensitive to salinity, calcium, and the combination of both than their male counterparts.

*Additional key words:* combined abiotic stress, ion uptake, physiological responses, poplars, sexual dimorphisms.

## Introduction

*Populus deltoides* is widely used as a desirable tree species for the construction of riparian-protective forests in Europe, North America, and China due to its fast growth, strong post-flooding recovery, and strong tolerance to waterlogging stress and winter flood (Gladwin and Roelle 1998, Cao and Conner 1999, Rowland 2001, Yang *et al.* 2011, Miao *et al.* 2017). However, waterlogging and salinity stresses often simultaneously occur in estuary and intertidal zones. Thus, whether *P. deltoides* could be used in the construction of riparian-protective forests in these specific regions remains unknown. The morphological, physiological, and biochemical responses to summer waterlogging, summer flooding, winter flooding, spring waterlogging, and post-flooding recovery and the ability of *P. deltoides* to adapt to these types of stresses have been well elucidated (Regehr *et al.* 1975, Cao and Conner 1999, Cooper *et al.* 2003, Yang *et al.* 2011, Yang *et al.* 2014, Miao *et al.* 2017). However, the mechanisms by which *P. deltoides* adapts to combined waterlogging and salinity stress are still unknown.

Calcium functions as an essential nutrient and a secondary messenger; it maintains the integrity and structure of membranes and cell walls, regulates plant growth and development, and mediates complex responses toward various developmental and environmental cues (Rengel 1992, Cramer 2002, Srivastava *et al.* 2013). Studies on exogenous calcium alleviating salinity stress in plants have been well documented (Rengel 1992, Lopez

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*Abbreviations*: APX - ascorbate peroxidase; $c_i$ - intercellular $CO_2$ concentration; E - transpiration rate; ETR - photosynthetic electron transport rate; $F_0$ - minimum fluorescence; $F_v/F_m$ - variable to maximum fluorescence ratio corresponding to maximum efficiency of photosystem II; $g_s$ - stomatal conductance; MDA - malondialdehyde; $O_2^{\cdot-}$ - superoxide anion radical; $^{\cdot}OH$ - hydroxyl radical; $P_N$ - net photosynthetic rate; POD - peroxidase; PS - photosystem; qN - non-photochemical quenching; qP - photochemical quenching; SOD - superoxide dismutase; WUEi- instantaneous water use efficiency; Yield - effective quantum yield of photosystem II.

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and Satti 1996, Singh *et al*. 1999, Cramer 2002, Cabañero and Carvajal 2004, Murillo-Amador *et al*. 2006, Tuna *et al*. 2007, Tattini and Traversi 2009, Srivastava *et al*. 2013, An *et al*. 2014, Salahshoor and Kazemi 2016). Cramer (2002) described in detail the sodium-calcium interactions under salinity stress and suggested that these interactions could affect membrane properties, water and ion transport, nutrition, photosynthesis, and plant growth. However, different species, cultivars, and plant genotypes respond differently to supplemental Ca under salinity stress. Most species and genotypes respond positively to Ca application, whereas others respond negatively. In addition, Ca content and optimal Na:Ca ratio are important factors that improve salinity tolerance in plants; Ca content is usually in the range of 5 - 10 mM and Na:Ca ratio 10 - 20, depending on the salinity levels and genotypes (Cramer 2002, Srivastava *et al*. 2013). Therefore, whether *P. deltoides* is tolerant or sensitive to calcium and how it responds to exogenous calcium under saline conditions must be investigated.

Calcium alleviates damages brought about by waterlogging or flooding stresses in sweet potato (Lin *et al*. 2008), muskmelon (Gao *et al*. 2011), cucumber (He *et al*. 2012), soybean (Oh *et al*. 2014), and pepper (Yang *et al*. 2016). Oh *et al*. (2014) suggested that Ca might affect cell wall and hormone metabolisms, protein degradation/synthesis, and DNA synthesis in soybean roots under flooding stress. Yang *et al*. (2016) observed that exogenous Ca could alleviate waterlogging-induced damages to pepper plants by regulating the osmolyte content, antioxidant system activity, root respiration, and metabolism. However, responses to combined Ca and waterlogging stress need further research, especially in woody plant species. In particular, whether this combination is an independent process or causes additive effects must be determined. Seawater contains abundant sodium and calcium. Thus, the calcium content in estuary and intertidal zones is higher than in the riparian zones of freshwater rivers or lakes. The forests in estuary and intertidal zones often simultaneously encounter waterlogging, salinity, and excess of calcium. Although *P. deltoides* has good plasticity to waterlogging stress, its response to combined salinity and calcium under waterlogging condition must be defined.

*Populus* spp. are dioecious species. Different sexes and ages of poplars might employ different strategies to cope with waterlogging or flooding stress (Letts *et al*. 2008, Nielsen *et al*. 2010, Rood *et al*. 2010, Yang *et al*. 2011, Miao *et al*. 2017). Yang *et al*. (2011) and Miao *et al*. (2017) demonstrated that male *P. deltoides* clones have better waterlogging/flooding tolerance than female clones. However, Rood *et al*. (2010) and Nielsen *et al*. (2010) suggested that female *P. angustifolia* is more flood tolerant and successful in low and flood-prone sites than its male counterpart. Letts *et al*. (2008) reported that the photosynthetic gas exchange, leaf reflectance, chlorophyll fluorescence, and photosynthetic water-use efficiency of male and female *P. angustifolia* clones in riparian woodland showed no significant difference. Juvany and Munné-Bosch (2015) suggested that general conclusions about sex-related stress tolerance in plants are not plausible. Sexual dimorphism may be related to species-specific differences, specific life-history traits, and adaptability to natural habitats. Therefore, sexual dimorphism and the ongoing multifactorial environmental stress must be further understood. Although the sex-dependent responses of poplars to waterlogging stress have been compared, limited research has been conducted regarding the responses to combined salinity and calcium under waterlogging conditions. Consequently, the physiological mechanisms underlying sex-related differences in response to combined salinity and calcium stress under waterlogging conditions have not been fully understood.

*P. deltoides* is widely used in the Huaihe River and Yangtze River basin in China due to its fast growth rate and strong ability to overcome waterlogging stress. Male and female *P. deltoides* clones were used in this study to investigate sexual dimorphism when the plants are exposed to salinity, calcium, and combined salinity and calcium treatments under waterlogging condition. We hypothesized that sex-specific responses of *P. deltoides* to salinity, calcium, and combined salinity and calcium under well-watered and waterlogging conditions occurred, and the males would maintain ion homeostasis better than the females did. To confirm this hypothesis, the variations in ion uptake, photosynthesis, chlorophyll fluorescence, reactive oxygen species content, and antioxidant enzyme activities were analyzed.

## Materials and methods

**Plants and experimental design:** The male (*Populus deltoides* clone Juba) and female (*P. deltoides* clone Danhong) were full-sib clones resulting from a cross between *P. deltoides* 2KEN8 (♂, imported from Texas, USA) and *P. deltoides* 55/65 (♀, imported from Carbondale, USA). The female and male cuttings of *P. deltoides* originated from the artificial forests in Qianjiang (30°09′N, 121°31′E), Hubei Province, China. The cuttings were planted into 10-dm$^3$ plastic pots filled with 10 kg of homogenized soil (one cutting per pot) on 1 March 2015. After sprouting and growing for 2 months, *P. deltoides* seedlings with similar crown sizes and equal heights (about 60 cm) were selected for the experiments and placed in a natural environment with 1 261 mm mean annual rainfall, 1 494 mm annual evaporation, 80 % annual relative humidity, and 16.9 °C annual temperature.

The experimental layout was a randomized complete block design considering five main factors (sexual clones, watering regime, salinity, calcium, and combined salinity and calcium treatments). In the well-watered treatments, all pots were watered with fresh water or solutions every day, and excess water or solutions were allowed to drain through drainage holes into the dishes placed under the buckets. The excess water or solutions in the dishes were then re-used in the next watering to avoid loss of soil nutrients. For the salinity, calcium, and combined salinity and calcium treatments, 100 mM NaCl solutions, 10 mM $CaCl_2$ solutions, and mixture of both solutions were used for watering every other day, respectively. Fresh water was used for the other days. In the waterlogging treatments, the pots were watered with freshwater every day to 5 cm above the top soil. For the salinity, calcium, and combined salinity and calcium treatments, NaCl and/or $CaCl_2$ were added in accordance with the water content at the initial stage of waterlogging treatment. The final concentrations of NaCl and $CaCl_2$ were 100 and 10 mM, respectively. The pots subjected to salinity, calcium, and combined salinity and calcium treatments were watered with freshwater every day up to 5 cm above the soil during waterlogging. The plants were treated from 1 May to 15 May 2015. At the end of the 15-d treatment, gas exchange rate and chlorophyll fluorescence were measured, and fresh leaves were collected for analyses. Six replications with four cuttings each were used for each treatment.

**Determination of Na, K, and Ca content in leaves:** Sodium, potassium, and calcium were determined in accordance with the method of Williams and Twin (1960). The seventh mature leaves from the top of the plants were sampled. Powdered dry samples (0.2 g) were transferred into a dry and clean 100-$cm^3$ digestion vessel. A total of 10 $cm^3$ of di-acid ($HNO_3$:$HClO_4$ at the ratio of 2:1) mixture was added to the flask. The flasks were then heated at a temperature slowly raised to 200 °C. Heating was stopped when dense white fumes of $HClO_4$ were observed. The content of the flasks was boiled until it became clean and colorless. After cooling, the content was placed in a 50-$cm^3$ volumetric flask, which was filled up with deionized water to the marked spot. Na, K, and Ca content was measured from the digested leaf samples by using a flame photometer (*M410*, Sherwood, OR, USA). The concentrations were calculated using standard curves.

**Gas exchange measurements:** The fourth fully expanded leaves from six cuttings of each treatment were selected for measuring gas exchange parameters. Net photosynthetic rate ($P_N$), stomatal conductance ($g_s$), intercellular $CO_2$ concentration ($c_i$), and transpiration (E) were measured from 9:00 to 11:30 using a *LI-COR 6400* portable photosynthesis system (*LI-COR*, Lincoln, NE, USA). The photosynthetically active radiation was set to 1 400 μmol $m^{-2}\cdot s^{-1}$, provided by a *6400-02 LED* radiation source. The flow rate of air through the sample chamber was set at 500 μmol $m^{-2}$ $s^{-1}$, and the leaf temperature and relative humidity were maintained at 25 ± 0.8 °C and 50 %, respectively (Yang *et al*. 2011). Instantaneous water use efficiency ($WUEi = P_N/E$) was calculated.

**Chlorophyll fluorescence measurements:** The same leaves used for gas exchange were selected for the measurements of chlorophyll fluorescence parameters: maximum efficiency of photosystem (PS) II (variable to maximum fluorescence ratio $F_v/F_m$), effective quantum yield of PS II (Yield), non-photochemical quenching coefficient (qN), photochemical quenching coefficient (qP), and photosynthetic electron transport rate (ETR) using a pulse amplitude modulation chlorophyll fluorometer (*PAM 2100*, *Walz*, Effeltrich, Germany). The leaf samples were placed in darkness by covering them with aluminum foil for 30 min, followed by measurement of minimum fluorescence ($F_0$) at 250 μmol $m^{-2}$ $s^{-1}$ of photosynthetic photon flux density (PPFD) and maximum fluorescence ($F_m$) following a saturating pulse of actinic light of 2 400 μmol $m^{-2}$ $s^{-1}$ PPFD (Yang *et al*. 2011). The measurements were carried out between 8:00 and 11:00.

**Determination of reactive oxygen species (ROS) and malondialdehyde (MDA) content:** Detections of hydrogen peroxide ($H_2O_2$), superoxide radical anions ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$), and MDA content were based on spectrometric procedures described previously (Yang *et al*. 2011, 2015, Han *et al.* 2015).

**Determination of soluble protein content and antioxidant enzyme activities:** Approximately 1g of fresh samples were ground with liquid nitrogen and then homogenized in 10 $cm^3$ of 100 mM universal sodium phosphate extraction buffer as described by Han *et al.* (2015) and Yang *et al* (2015). The supernatant (0.5 $cm^3$) was stored at -80 °C until it was used for the determination of soluble protein and ROS content, and antioxidant enzyme activities. The soluble protein was quantified using the Bradford method (Bradford 1976). The antioxidant enzyme activities, including peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX), were determined spectrophotometrically in accordance with the manufacturer's instructions and as described by Yang *et al*. (2011, 2015) and Han *et al.* (2015). One unit (U) of SOD activity was defined as the amount of the enzyme inhibiting photoreduction of nitroblue tetrazolium by 50 %. One unit of POD activity was defined as the amount of the enzyme causing a change in absorbance of 0.01 at 470 nm. One unit of APX was defined as the amount of the enzyme causing a change in absorbance of 0.01 at 290 nm.

**Statistical analyses:** The results were expressed as means ± standard errors ($n = 6$). The *SPSS 16.0* software was used for statistical analysis. Post hoc multiple comparison from the general linear model was used to evaluate the effects of sex, waterlogging, salt, calcium, and their interaction, and the Tukey's test was employed to test the sex and treatment differences. Differences were considered significant at $P < 0.05$.

## Results

Significant visible damages, such as leaf chlorosis, leaf necrosis, or leaf abscission, were observed when plants were exposed to salinity, calcium, and combined salinity and calcium whether under well-watered or waterlogging conditions. However, all plants could survive well after being exposed only to waterlogging. No significant morphological variations were observed among the plants under all treatments. However, insignificant differences in morphological traits were found between female and male clones under the same treatment.

Under well-watered conditions, salinity and combined

Table 1. The variations of Na, K, and Ca content, and Na:Ca ratio in leaves of male and female *Populus deltoides*. Means ± SEs, $n = 6$; different letters indicate significant differences at $P < 0.05$ according to Tukey's test; * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$, ns - not significant difference. $F_S$ - sex effect, $F_W$ - waterlogging effect, $F_{Na}$ - salt effect, $F_{Ca}$ - calcium effect, $F_{Na\times Ca}$ - salt × calcium interaction, $F_{S\times W}$ - sex × waterlogging interaction, $F_{S\times Na}$ - sex × salt interaction, $F_{S\times Ca}$ - sex × calcium interaction, $F_{S\times Na\times Ca}$ - sex × salt × calcium interaction, $F_{W\times Na}$ - waterlogging × salt interaction, $F_{W\times Ca}$ - waterlogging × calcium interaction, $F_{W\times Na\times Ca}$ - waterlogging × salt × calcium interaction, $F_{S\times W\times Na\times Ca}$ - sex ×waterlogging × salt × calcium interaction.

| Water regime | Treatment | Sex | Na [mg g$^{-1}$(d.m.)] | K [mg g$^{-1}$(d.m.)] | Ca [mg g$^{-1}$(d.m.)] | Na:Ca ratio |
|---|---|---|---|---|---|---|
| Well-watered | control | female | 0.71±0.02 g | 9.93±0.11 d | 10.33±0.09 i | 0.07±0.0016 fgh |
| | | male | 0.45±0.01 g | 10.47±0.13 d | 10.88±0.14 i | 0.04±0.0007 gh |
| | NaCl | female | 2.18±0.08 e | 8.73±0.12 ef | 12.56±0.11 f | 0.12±0.0017 def |
| | | male | 1.48±0.01 f | 13.03±0.31 a | 14.15±0.27 d | 0.15±0.0051 de |
| | Ca | female | 0.60±0.02 g | 10.46±0.22 d | 12.08±0.21 fgh | 0.05±0.0011 gh |
| | | male | 0.86±0.06 g | 12.79±0.26 ab | 12.40±0.41 fg | 0.07±0.0045 fgh |
| | NaCl+Ca | female | 2.01±0.03 e | 11.92±0.06 c | 11.87±0.23 fgh | 0.17±0.001 d |
| | | male | 1.50±0.02 f | 12.33±0.21 bc | 14.19±0.25 d | 0.11±0.0026 efg |
| Waterlogging | control | female | 0.67±0.02 g | 12.88±0.28 ab | 20.38±0.23 b | 0.03±0.0007 h |
| | | male | 0.61±0.02 g | 13.16±0.11 a | 21.29±0.21 a | 0.03±0.001 h |
| | NaCl | female | 26.51±0.29 b | 6.60±0.14 j | 13.42±0.20 e | 1.98±0.0162 b |
| | | male | 25.44±0.46 c | 9.25±0.23 e | 13.58±0.25 de | 1.87±0.0661 c |
| | Ca | female | 0.90±0.01 g | 7.69±0.16 hi | 11.68±0.15 h | 0.08±0.0003 fgh |
| | | male | 0.95±0.07 g | 7.39±0.16 i | 12.13±0.08 fgh | 0.08±0.0055 fgh |
| | NaCl+Ca | female | 31.84±0.23 a | 8.51±0.10 fg | 11.78±0.13 gh | 2.08±0.0056 a |
| | | male | 24.54±0.31 d | 8.16±0.12 gh | 16.88±0.25 c | 1.89±0.0397 c |
| | | $P > F_S$ | 0.000*** | 0.000*** | 0.177 ns | 0.487 ns |
| | | $P > F_W$ | 0.000*** | 0.000*** | 0.000*** | 0.000*** |
| | | $P > F_{Na}$ | 0.000*** | 0.000*** | 0.003** | 0.000*** |
| | | $P > F_{Ca}$ | 0.000*** | 0.000*** | 0.000*** | 0.008** |
| | | $P > F_{Na\times Ca}$ | 0.000*** | 0.000*** | 0.000*** | 0.788 ns |
| | | $P > F_{S\times W}$ | 0.000*** | 0.000*** | 0.000*** | 0.120 ns |
| | | $P > F_{S\times Na}$ | 0.000*** | 0.000*** | 0.001*** | 0.325 ns |
| | | $P > F_{S\times Ca}$ | 0.000*** | 0.000*** | 0.000*** | 0.004*** |
| | | $P > F_{S\times Na\times Ca}$ | 0.000*** | 0.000*** | 0.000*** | 0.078 ns |
| | | $P > F_{W\times Na}$ | 0.000*** | 0.000*** | 0.000*** | 0.000*** |
| | | $P > F_{W\times Ca}$ | 0.000*** | 0.000*** | 0.000*** | 0.016* |
| | | $P > F_{W\times Na\times Ca}$ | 0.000*** | 0.000*** | 0.000*** | 0.689 ns |
| | | $P > F_{S\times W\times Na\times Ca}$ | 0.000*** | 0.000*** | 0.000*** | 0.000*** |

salinity and calcium treatments significantly increased the Na content to approximately threefold in female and male *P. deltoides* clones compared with their controls (Table 1), whereas calcium treatment exhibited no significant effect on the Na content of both clones. Thus, the Na content in salinity and combined salinity and Ca treatments was significantly higher than that in only Ca treatment for both clones. In addition, the female clones had significantly higher Na content than male clones after salinity and combined salinity and Ca treatments.

Salinity and combined salinity and Ca treatments under waterlogging conditions could substantially stimulate Na uptake in female and male clones. Both treatments increased the Na content to more than 12-fold in both clones compared with their individual controls under well-watered conditions. However, waterlogging and Ca treatment under waterlogging caused no effect on the Na content of both clones compared with their individual controls. In addition, the combined salinity and Ca treatment could significantly stimulate Na uptake in female clones but significantly inhibit the same process in male clones compared with salinity treatment under waterlogging. The female clones had significantly higher Na content than males after salinity and combined salinity and Ca treatments under waterlogging.

Under well-watered conditions, the salinity, Ca, and combined salinity and Ca treatments significantly increased the K content (Table 1) of male clones compared with the controls. Meanwhile, the K content in female clones was significantly increased by the combined salinity and Ca treatment, but significantly inhibited by salinity treatment, and not significantly affected by Ca treatment. No significant difference in K content was observed among male clones in different treatments. In addition, the male clones had significantly higher K content than the female clones after salinity and Ca treatments.

Table 2. Net photosynthetic rate ($P_N$), stomatal conductance ($g_s$), intrinsic water use efficiency (WUEi), transpiration rate (E), and intercellular $CO_2$ concentration ($c_i$) variations in male and female *Populus deltoides*. Means ± SEs, *n* = 6; different letters indicate significant differences at $P < 0.05$ according to Tukey's test. For explanation of abbreviations - see Table 1.

| Water regime | Treatment | Sex | $P_N$ [μmol m$^{-2}$ s$^{-1}$] | $g_s$ [mol m$^{-2}$ s$^{-1}$] | WUEi [μmol mmol$^{-1}$] | E [mmol m$^{-2}$ s$^{-1}$] | $c_i$ [μmol mol$^{-1}$] |
|---|---|---|---|---|---|---|---|
| Well-watered | control | female | 18.43±1.28 a | 0.59±0.11 a | 4.56±0.78 a | 4.33±0.92 de | 300.55±11.94 ab |
| | | male | 16.30±0.26 ab | 0.47±0.04 ab | 3.54±0.46 bc | 4.73±0.49 cde | 315.52±7.65 ab |
| | NaCl | female | 11.99±0.89 de | 0.63±0.04 a | 1.98±0.12 e | 8.10±0.26 a | 310.32±6.08 ab |
| | | male | 16.01±1.14 ab | 0.49±0.05 ab | 1.63±0.03 e | 7.41±0.67 ab | 315.80±3.79 ab |
| | Ca | female | 14.28±1.23 bcd | 0.58±0.04 a | 1.88±0.13 e | 7.61±0.44 ab | 312.84±6.56 ab |
| | | male | 13.97±0.16 bcd | 0.57±0.06 a | 1.70±0.07 e | 8.26±0.41 a | 320.20±4.56 a |
| | NaCl+Ca | female | 15.44±1.53 abc | 0.50±0.08 ab | 2.13±0.15 e | 7.28±0.76 ab | 299.64±3.71 ab |
| | | male | 14.68±0.86 bcd | 0.52±0.05 ab | 1.87±0.18 e | 7.89±0.29 ab | 312.30±9.45 ab |
| Waterlogging | control | female | 13.30±0.89 bcd | 0.47±0.06 ab | 3.74±0.13 ab | 3.58±0.35 e | 291.19±5.31 ab |
| | | male | 14.46±0.19 bcd | 0.48±0.05 ab | 3.41±0.36 bc | 4.33±0.41 de | 280.24±5.53 bc |
| | NaCl | female | 11.77±0.39 de | 0.26±0.07 c | 3.26±0.43 bcd | 3.78±0.67 e | 294.44±13.4 ab |
| | | male | 13.66±0.94 bcd | 0.34±0.02 bc | 2.64±0.13 cde | 5.19±0.37 cde | 296.04±6.75 ab |
| | Ca | female | 14.39±1.38 bcd | 0.46±0.08 ab | 2.26±0.26 de | 6.48±0.71 abc | 299.90±9.8 ab |
| | | male | 11.81±0.57 de | 0.36±0.06 bc | 1.97±0.11 e | 6.05±0.58 bcd | 303.10±8.72 ab |
| | NaCl+Ca | female | 12.52±1.49 cde | 0.24±0.08 c | 3.33±0.55 bcd | 3.99±0.84 e | 254.44±23.51 c |
| | | male | 10.08±0.39 e | 0.26±0.04 c | 2.60±0.4 cde | 4.03±0.51 e | 291.93±17.2 ab |
| | | $P > F_S$ | 0.023* | 0.314 ns | 0.008** | 0.243 ns | 0.093 ns |
| | | $P > F_W$ | 0.000*** | 0.000*** | 0.006** | 0.000*** | 0.000*** |
| | | $P > F_{Na}$ | 0.009** | 0.005** | 0.011* | 0.327 ns | 0.25 ns |
| | | $P > F_{Ca}$ | 0.03* | 0.309 ns | 0.000*** | 0.000*** | 0.816 ns |
| | | $P > F_{Na\times Ca}$ | 0.066 ns | 0.556 ns | 0.000*** | 0.000*** | 0.015* |
| | | $P > F_{S\times W}$ | 0.181 ns | 0.304 ns | 0.913 ns | 0.733 ns | 0.827 ns |
| | | $P > F_{S\times Na}$ | 0.711 ns | 0.422 ns | 0.912 ns | 0.998 ns | 0.312 ns |
| | | $P > F_{S\times Ca}$ | 0.444 ns | 0.712 ns | 0.518 ns | 0.666 ns | 0.241 ns |
| | | $P > F_{S\times Na\times Ca}$ | 0.827 ns | 0.654 ns | 0.512 ns | 0.714 ns | 0.386 ns |
| | | $P > F_{W\times Na}$ | 0.78 ns | 0.024* | 0.002** | 0.000*** | 0.527 ns |
| | | $P > F_{W\times Ca}$ | 0.995 ns | 0.411 ns | 0.365 ns | 0.232 ns | 0.714 ns |
| | | $P > F_{W\times Na\times Ca}$ | 0.015* | 0.404 ns | 0.149 ns | 0.497 ns | 0.291 ns |
| | | $P > F_{S\times W\times Na\times Ca}$ | 0.013* | 0.288 ns | 0.631 ns | 0.235 ns | 0.403 ns |

Waterlogging stimulated K uptake in female and male clones compared with the controls. However, the salinity, calcium, and combined salinity and Ca treatments under waterlogging could significantly inhibit K uptake in both clones. Several differences in K content were found in both clones after all three treatments under waterlogging. In addition, the male clones had a significantly higher K content than the female clone after salinity treatment under waterlogging.

Under well-watered conditions, the Ca content (Table 1) of female and male clones significantly increased in salinity, Ca, and combined salinity and Ca treatments compared with the controls. No significant differences in Ca content were noted among female clones under different treatments. In addition, the male clones had higher Ca content than the female clones in each treatment, and significantly higher Ca content was detected in salinity and combined salinity and Ca treatments.

Waterlogging stimulated Ca uptake in female and male clones compared with the controls. However, the salinity, Ca, and combined salinity and Ca treatments under waterlogging could not significantly affect Ca uptake in both clones compared with their individual controls under well-watered conditions. Differences in Ca content were observed among the treatments under waterlogging. In addition, the male clones had a significantly higher Ca content than the female clones in the combined salinity and Ca treatment under waterlogging, whereas no significant differences in Ca content were found between the clones in salinity and Ca treatments.

Under well-watered conditions, salinity and combined salinity and Ca treatments significantly increased the ratio of Na:Ca (Table 1) in female and male clones compared with their controls, whereas Ca treatment exhibited no significant effect on the ratio of Na:Ca in both clones. Thus, the values of the Na:Ca in salinity and combined salinity and Ca treatments was significantly higher than in Ca treatment alone for both clones. In addition, the female clones had significantly higher values of Na:Ca than male clones in combined salinity and Ca treatments.

Salinity and combined salinity and Ca treatments under waterlogging substantially increased the ratio

Table 3. Maximum efficiency of photosystem II ($F_v/F_m$), effective quantum yield of PS II (Yield), photochemical quenching (qP), non-photochemical quenching (qN), and photosynthetic electron transport rate (ETR) variations in male and female *Populus deltoides*. Values are means ± SE ($n = 6$); different letters indicate significant differences at $P < 0.05$ according to Tukey's test. For explanation of abbreviations - see Table 1.

| Water regime | Treatment | Sex | $F_v/F_m$ | Yield | qP | qN | ETR |
|---|---|---|---|---|---|---|---|
| Well-watered | control | female | 0.762±0.011 b | 0.618±0.028 abcd | 0.899±0.023 abcd | 0.280±0.043 efg | 67.333±3.383 a |
| | | male | 0.778±0.002 ab | 0.656±0.01 ab | 0.928±0.007 a | 0.231±0.031 efg | 65.000±1.155 abc |
| | NaCl | female | 0.780±0.005 ab | 0.613±0.024 abcd | 0.902±0.012 abc | 0.378±0.061 cde | 60.667±2.603 abcd |
| | | male | 0.775±0.008 ab | 0.528±0.019 fg | 0.865±0.011 defg | 0.574±0.024 ab | 52.333±1.764 ef |
| | Ca | female | 0.778±0.007 ab | 0.622±0.011 abcd | 0.906±0.009 abc | 0.334±0.017 def | 61.667±1.333 abcd |
| | | male | 0.779±0.007 ab | 0.648±0.008 ab | 0.920±0.009 ab | 0.305±0.036 defg | 64.333±0.882 abc |
| | NaCl+Ca | female | 0.788±0.005 ab | 0.639±0.005 abc | 0.908±0.004 abc | 0.311±0.012 defg | 63.333±0.333 abc |
| | | male | 0.788±0.005 ab | 0.647±0.021 ab | 0.914±0.01 abc | 0.293±0.039 efg | 64.333±2.186 abc |
| Water-logging | control | female | 0.778±0.007 ab | 0.653±0.011 ab | 0.920±0.005 ab | 0.196±0.034 fg | 65.000±1.155 abc |
| | | male | 0.783±0.004 ab | 0.663±0.017 a | 0.926±0.008 a | 0.183±0.071 g | 65.667±1.856 ab |
| | NaCl | female | 0.776±0.007 ab | 0.505±0.037 g | 0.848±0.022 g | 0.551±0.092 ab | 66.667±4.910a |
| | | male | 0.787±0.01 ab | 0.596±0.015 bcde | 0.896±0.007 abcde | 0.446±0.046 bcd | 59.000±1.528 bcde |
| | Ca | female | 0.788±0.001 a | 0.538±0.011 efg | 0.861±0.001 efg | 0.573±0.021 ab | 53.000±1.000 ef |
| | | male | 0.795±0.005 a | 0.583±0.022 cdef | 0.887±0.011 bcdef | 0.482±0.056 bc | 58.000±2.082 cde |
| | NaCl+Ca | female | 0.794±0.017 a | 0.499±0.021 g | 0.859±0.004 fg | 0.528±0.03 ab | 56.333±0.882 de |
| | | male | 0.801±0.006 a | 0.571±0.01 def | 0.878±0.009 cdefg | 0.648±0.041 a | 49.667±2.028 f |
| | | $P > F_S$ | 0.188 ns | 0.417 ns | 0.109 ns | 0.946 ns | 0.073 ns |
| | | $P > F_W$ | 0.019* | 0.000*** | 0.001*** | 0.000*** | 0.005** |
| | | $P > F_{Na}$ | 0.138 ns | 0.000*** | 0.000*** | 0.000*** | 0.003** |
| | | $P > F_{Ca}$ | 0.005** | 0.261 ns | 0.252 ns | 0.001*** | 0.001*** |
| | | $P > F_{Na\times Ca}$ | 0.694 ns | 0.000*** | 0.003** | 0.000*** | 0.018* |
| | | $P > F_{S\times W}$ | 0.556 ns | 0.261 ns | 0.284 ns | 0.313 ns | 0.845 ns |
| | | $P > F_{S\times Na}$ | 0.631 ns | 0.025* | 0.098 ns | 0.048* | 0.003** |
| | | $P > F_{S\times Ca}$ | 0.694 ns | 0.536 ns | 0.671 ns | 0.801 ns | 0.027* |
| | | $P > F_{S\times Na\times Ca}$ | 0.678 ns | 0.223 ns | 0.518 ns | 0.708 ns | 0.907 ns |
| | | $P > F_{W\times Na}$ | 0.499 ns | 0.055 ns | 0.277 ns | 0.076 ns | 0.372 ns |
| | | $P > F_{W\times Ca}$ | 0.616 ns | 0.000*** | 0.001*** | 0.000*** | 0.000*** |
| | | $P > F_{W\times Na\times Ca}$ | 0.861 ns | 0.838 ns | 0.419 ns | 0.918 ns | 0.018* |
| | | $P > F_{S\times W\times Na\times Ca}$ | 0.677 ns | 0.000*** | 0.005** | 0.013* | 0.273 ns |

of Na:Ca in female and male clones. Both treatments increased the values of Na:Ca to more than 20-fold in both clones compared with their individual controls under well-watered conditions. However, Ca treatment under waterlogging caused no effect on the ratio of Na:Ca of both clones compared with their individual controls. The female clones had significantly higher Na:Ca ratio than male clones after salinity and combined salinity and Ca treatments under waterlogging. The effects of sex, waterlogging, salt, Ca and their interaction are also shown in Table 1.

Under well-watered conditions, the salinity, Ca, and combined salinity and Ca treatments decreased $P_N$, $g_s$, and WUEi. The treatments significantly increased E but caused no effect on $c_i$ (Table 2) in female and male clones compared with their controls. No significant differences in $P_N$, $g_s$, WUEi, E, and $c_i$ were found among male or female clones in different treatments. In addition, the male clones had significantly higher $P_N$ than the female clones in salinity treatment, whereas no significant differences in $P_N$, $g_s$, WUEi, E, and $c_i$ were identified between both sexes in other treatments.

Waterlogging decreased $P_N$, $g_s$, WUEi, E, and $c_i$ in both clones compared with the controls. The salinity, Ca, and combined salinity and Ca treatments under waterlogging decreased $P_N$, $g_s$, and E, increased WUEi, and showed no effect on $c_i$ in both clones compared with their controls under well-watered conditions. Under waterlogging, significant variations in $P_N$ (combined salinity and Ca treatments in male clones), $g_s$ (salinity and combined salinity and Ca treatments in female clones), and WUEi (salinity and combined salinity and Ca treatments in female clones) were observed compared with their individual controls under well-watered conditions. In addition, the male clones had significantly higher $c_i$ content than the female clones after the combined salinity and Ca treatment under

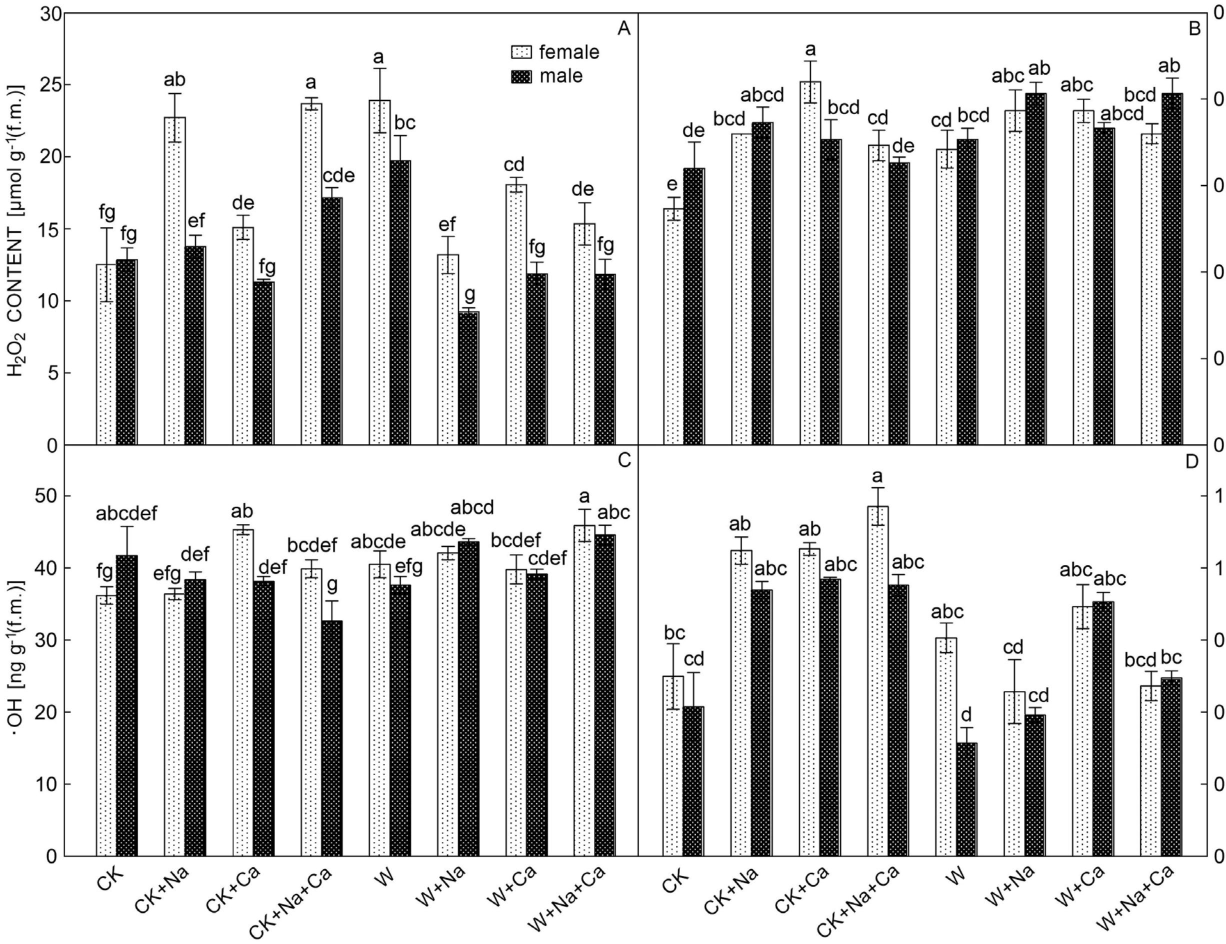

Fig. 1. Hydrogen peroxide ($H_2O_2$, *A*), superoxide radical ($O_2^{\cdot-}$, *B*), hydroxyl radical ($^{\cdot}OH$, *C*), and malondialdehyde (MDA, *D*) content in male and female *Populus deltoides*. CK - control treatment, CK+Na - salinity treatment, CK+Ca - calcium treatment, CK+Na+Ca - combined salinity and calcium treatment, W - waterlogging treatment, W+Na - salinity treatment under waterlogging, W+Ca - calcium treatment under waterlogging, W+Na+Ca - combined salinity and calcium treatment under waterlogging. Means ± SEs, *n* = 6; different letters above the columns indicate significant differences at $P < 0.05$ according to Tukey's test.

waterlogging, whereas no significant differences in $P_N$, $g_s$, WUEi, E, and $c_i$ were recognized between both sexes after other treatments under waterlogging. The effects of sex, waterlogging, salt, Ca and their interaction effects were also shown in Table 2.

Under well-watered conditions, salinity, Ca, and combined salinity and Ca treatments showed no significant effect on $F_v/F_m$, Yield, qP, qN, and ETR (Table 3) in female clones compared with the controls, whereas salinity treatment significantly decreased Yield, qP, and ETR and significantly increased qN in male clones. $F_v/F_m$, Yield, qP, qN, and ETR exhibited no significant difference among female clones under different treatments. The Yield, qP, and ETR of male clones in salinity treatment were significantly lower than those of their counterparts in Ca and combined salinity and Ca treatments, whereas the qN was significantly higher in salinity treatment than in the other two treatments. In addition, significant differences in Yield, qP, qN, and ETR between both clones were found only in salinity treatment. No significant differences in $F_v/F_m$, Yield, qP, qN, and ETR between both sexes were observed under other treatments.

Waterlogging showed no significant effect on $F_v/F_m$, Yield, qP, qN, and ETR in female and male clones compared with the controls. The salinity, Ca, and combined salinity and Ca treatments under waterlogging increased qN, decreased Yield, qP, and ETR; and showed minimal effect on $F_v/F_m$ in both clones compared with their individual controls under well-watered conditions. However, significant variations were observed in Yield (in both clones under Ca and salinity and Ca treatments), qP (in female clones all three treatments), qN (in both clones under Ca and combined salinity and Ca treatments), and ETR (in both clones under combined salinity and Ca treatment) under waterlogging. Slight differences in $F_v/F_m$, Yield, qP, qN, and ETR were found among male or female clones under the three treatments in waterlogging. In addition, the male clones had significantly higher Yield levels than the female clones after Ca and combined salinity and Ca treatments under waterlogging stress conditions and significantly higher qP levels than the females in Ca treatment. The effects of sex, waterlogging, salt, Ca and

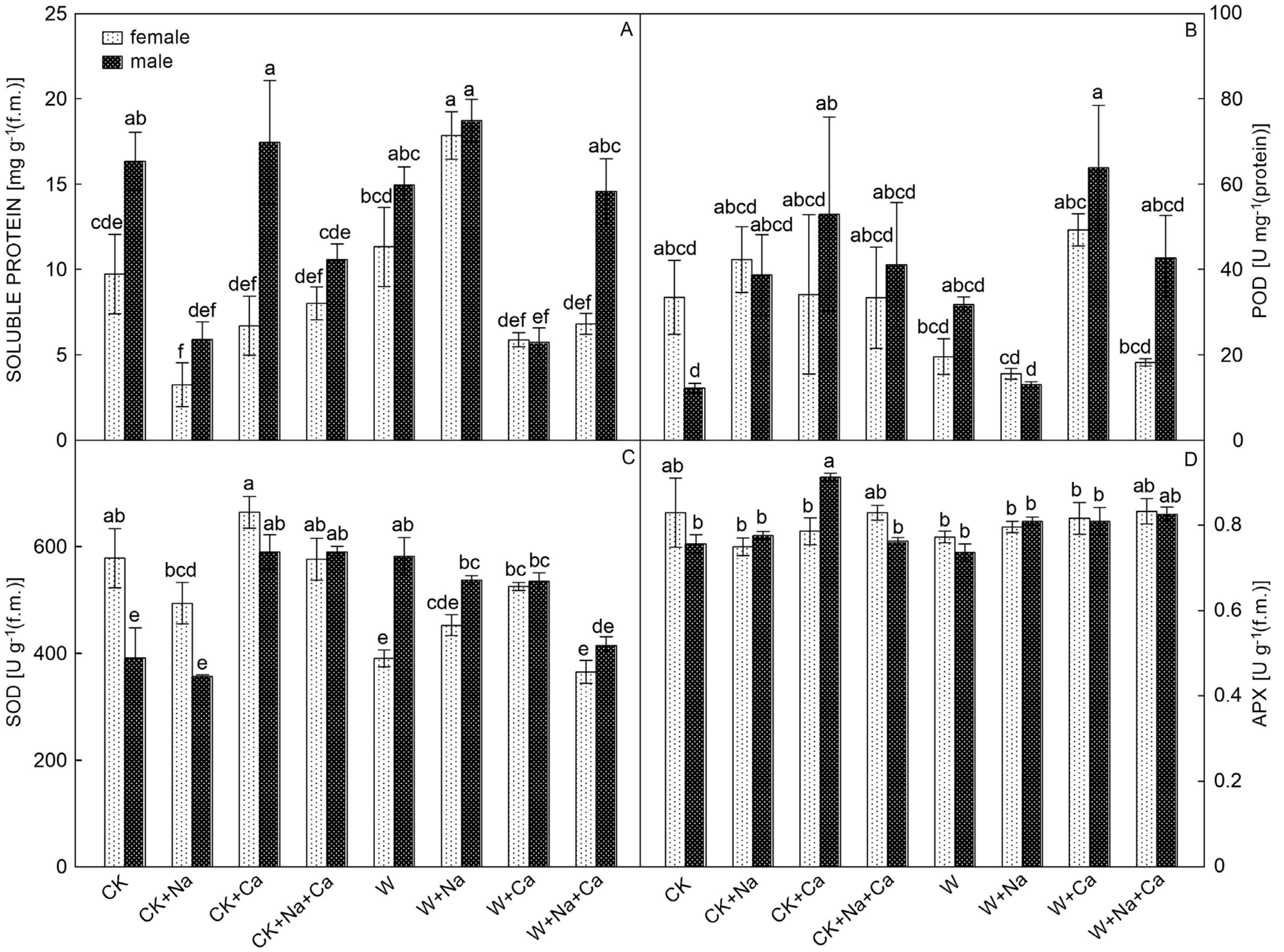

Fig. 2. Soluble protein content (*A*) and activities of peroxidase (POD, *B*), superoxide dismutase (SOD, *C*), and ascorbate peroxidase (APX, *D*) in male and female *Populus* deltoids. Means ± SEs, $n = 6$; different letters above the columns indicate significantly differences at $P < 0.05$ according to Tukey's test. For explanation of abbreviations - see Fig. 1.

their interaction effects were also shown in Table 3.

Under well-watered conditions, the salinity, Ca, and combined salinity and Ca treatments increased the contents of $H_2O_2$ (Fig. 1*A*), $O_2^{\cdot-}$(Fig. 1*B*), $\cdot$OH (Fig. 1*C*), and MDA (Fig. 1*D*) in female and male clones compared with their controls. The female clones had higher levels of $H_2O_2$, $O_2^{\cdot-}$, $\cdot$OH, and MDA than the male clones. In female clones, salinity treatment could significantly increase the levels of $H_2O_2$ and $O_2^{\cdot-}$; Ca treatment could significantly increase the levels of $H_2O_2$, $O_2^{\cdot-}$, and $\cdot$OH; the combined salinity and Ca treatment could significantly increase the levels of $H_2O_2$, $O_2^{\cdot-}$, and MDA. However, only the combined salinity and Ca treatment increased the level of $H_2O_2$ in male clones. Slight differences in the levels of $H_2O_2$, $O_2^{\cdot-}$, $\cdot$OH, and MDA were detected in both clones under the three treatments. In addition, the female clones had significantly higher levels of $H_2O_2$ than the male clones in all three treatments, significantly higher levels of $O_2^{\cdot-}$ in Ca treatment, and significantly higher levels of $\cdot$OH in Ca and combined salinity and Ca treatments than the male clones.

Waterlogging significantly increased the content of $H_2O_2$, $O_2^{\cdot-}$, and $^{\cdot}$OH in female clones but did not significantly affect the content of $O_2^{\cdot-}$ and $^{\cdot}$OH in male clones. All three treatments under waterlogging conditions had limited effects on the content of $H_2O_2$, $O_2^{\cdot-}$, $^{\cdot}$OH, and MDA in both clones compared with their individual controls under well-watered conditions. Under waterlogging, significant variations were observed in $H_2O_2$ (in both clones under salinity and combined salinity and Ca treatments), $O_2^{\cdot-}$ (in male clones under combined salinity and Ca treatment), $^{\cdot}$OH (in both clones under combined salinity and Ca treatment), and MDA (in female clones under salinity and combined salinity and Ca treatments) compared with their individual controls under well-watered conditions. Slight differences were noted in their content in all treatments under waterlogging. In addition, the female clones had significantly higher content of $H_2O_2$ than the male clones in all three treatments under waterlogging stress conditions. However, no significant differences between both sexes were identified in all treatments. The effects of sex, waterlogging, salt, Ca, and their interaction effects could be found in Table 1 Suppl.

Under well-watered conditions, the soluble protein content (Fig. 2*A*) significantly decreased after salinity treatment in both clones and after combined salinity and Ca treatment in male clones. No significant variations in the POD (Fig. 2*B*), SOD (Fig. 2*C*), and APX (Fig. 2*D*) activities were found when the female clones were exposed to all treatments. However, in male clones, significant increases in the POD and APX activities were observed after Ca treatment, whereas significant increases in SOD activities were induced during Ca and combined salinity and Ca treatments. Slight differences were noted in the soluble protein content and POD, SOD, and APX activities of female clones under the three treatments, whereas for the male clones, Ca treatment could induce significantly higher content of soluble proteins and APX activity than the other two treatments. In addition, the male clones had significantly higher soluble protein content and APX activity in Ca treatment than the female clones.

Waterlogging caused no significant effect on soluble protein contents and POD and APX activities in either female nor male clones. However, the SOD activities in male clones significantly increased under waterlogging stress but significantly decreased in female clones. Under waterlogging stress conditions, the soluble protein contents were significantly increased by salinity treatment of both clones, whereas the SOD activities in male clones were significantly increased by salinity treatment, significantly decreased by Ca treatment in female clones, and significantly decreased by the combined salinity and Ca treatment in both clones. Under waterlogging, minimal differences in SOD and APX activities were observed in both clones under all treatments. In addition, the male clones had significantly higher soluble protein content and SOD activity than the female clones after the combined salinity and Ca treatment under waterlogging. The effects of sex, waterlogging, salt, Ca and their interaction effects could be found in Table 2 Suppl.

## Discussion

Previous studies have suggested that specific *P. deltoides* clones and hybrids could survive well under 200 mM NaCl treatment (Bray *et al*. 1991, Fung *et al*. 1998, Singh *et al*. 2000). Specifically, 10 mM Ca is necessary for alleviating stress-induced damages in many plants (Cramer 2002, Cabañero *et al*. 2004, Murillo-Amador *et al*. 2006, Parvin 2016, Yang *et al*. 2016). A total of 20 mM Ca was used to enhance the germination, growth, and yield of plants under NaCl stress (Lopez and Satti 1996, Salahshoor and Kazemi 2016). However, leaf chlorosis, leaf necrosis, and leaf abscission, after 15-d 100 mM NaCl treatment or 10 mM Ca treatment suggested that female and male *P. deltoides* clones are sensitive to Na and Ca under well-watered or waterlogging conditions. Therefore, although *P. deltoides* is a desirable tree species for riparian-protective forests due to its strong waterlogging tolerance (Regehr *et al*. 1975, Yang *et al*. 2011, Miao *et al*. 2017), it is unsuitable for the construction of protection forests in estuary and intertidal zones with high concentrations of Na and Ca.

The combination of salinity and waterlogging stresses has a more significant influence on the growth, anatomical traits, antioxidative traits, and elemental toxicity of non-halophytes than one type of stress (Alhdad *et al*. 2013, Zeng *et al*. 2013, Haddadi *et al*. 2016). Na-Ca interactions *via* exogenous Ca application could directly regulate plant growth, photosynthesis, osmotic stress, mineral nutrition, and water and ion transport, thereby mitigating the adverse effects of salt-induced ionic toxicity (Rengel 1992, Lopez and Satti 1996, Singh *et al*. 1999, Cramer 2002, Girija *et al*. 2002, Cabañero and Carvajal 2004, Murillo-Amador *et al*. 2006, Tuna *et al*. 2007, Jian *et al*. 2009, Tattini and Traversi 2009, Salahshoor and Kazemi 2016, Srivastava *et al*. 2013, An *et al*. 2014). However, research rarely focused on the plant response to combined salinity and Ca stress under well-watered or waterlogging conditions. Salinity and Ca under well-watered conditions could increase the K and Ca content, ROS production, and antioxidant enzyme activities and decrease $P_N$, $g_s$, WUEi, Yield, and ETR. Further analyses of these physiological and biochemical traits suggested that salinity and Ca cause similar responses, and their combination is not more serious than individual effects. In addition, an independent process occurs under the combined effects of salinity and Ca. The lack of sharp variation in Na and Ca content in leaves suggests that the ionic toxicity from salinity and Ca possibly affected the root systems but not the aerial parts under well-watered conditions. Waterlogging could notably stimulate Na uptake in leaves under abundant NaCl. However, this condition had no evident effects on Ca uptake under abundant Ca. The decreased Na content and increased K and Ca content of male clones suggest that male clones can maintain ion homeostasis better than female clones under salinity, Ca, and combined salinity and Ca treatments (Cramer 2002).

The sex-specific morphological, physiological, biochemical, ultrastructural, transcriptional, and proteomic responses to waterlogging (Letts *et al*. 2008, Nielsen *et al*. 2010, Rood *et al*. 2010, Yang *et al*. 2011, Miao *et al*. 2017) and salinity (Chen *et al*. 2010, Chen *et al*. 2011, Jiang *et al*. 2012, Xu *et al*. 2015, Li *et al*. 2016) stresses in poplars have been well studied. The majority of studies concluded that females are more sensitive to salinity and usually experience greater negative effects than males. However, Robinson *et al*. (2014) suggested that no evidence of sexual dimorphism or differential resource investment strategies existed between males and females in mature leaves of mature *P. tremula* using whole-genome oligonucleotide microarrays and RNA-sequencing. McKown *et al*. (2017) also failed to detect sexual dimorphism in more than 1 300 individuals from *P. trichocarpa* and *P. balsamifera*. Our previous studies have also demonstrated that male *P. deltoides* clones develop better cellular defense mechanisms against waterlogging/flooding stress than their female counterpart, thereby making them less susceptible (Yang *et al*. 2011, Miao *et al*. 2017). In the present study, differences in certain parameters were insignificant between the two sexes under stressed conditions. However, sex-specific differences in $P_N$, $c_i$, Yield, qP, qN, ETR, $O_2^{\cdot-}$, $^{\cdot}OH$, $H_2O_2$, soluble protein content, and SOD activity under certain stressed conditions suggest that female *P. deltoides* clones are more sensitive to salinity, Ca, and combined salinity and Ca treatments than their male counterparts under well-watered or waterlogging conditions. The oxidative stress might limit the photosynthetic $CO_2$ assimilation and damage to photosystems.

In conclusion, both sexes of *P. deltoides* are waterlogging tolerant but sensitive to salinity and Ca. Under well-watered conditions, salinity stress could especially increase the Na content of female clones, whereas salinity and Ca could especially increase the K and Ca content in male clones. Waterlogging could remarkably stimulate Na uptake in leaves under salinity, especially in female *P. deltoides*. However, waterlogging had no effects on Ca uptake under abundant Ca. Salinity and Ca caused similar physiological and biochemical responses, and their combination had not more significant effect than single stresses, suggesting that independent processes occur under the combined effects of salinity and Ca. The lower Na content and higher K and Ca content in male clones suggested that males have better abilities to maintain ion homeostasis than females. The sex-specific differences in $P_N$, $c_i$, Yield, qP, qN, ETR, $O_2^{\cdot-}$, $^{\cdot}OH$, $H_2O_2$, soluble protein content, and SOD activity under certain stresses suggest that *P. deltoides* female clones are more sensitive to salinity, Ca, and combined salinity and Ca treatments than male clones either under well-watered or waterlogging conditions. This study sheds a new light on the possible construction of *P. deltoides* protected forests and increases the understanding of sexually dimorphic responses to combined multifactorial stresses.

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