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Article

Developing salinity tolerate trees for challenging sites and urban forests based on the inferences of physiological responses: using *Ulmus pumila* as an example

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Abstract: Elevated salinity is one of the major environmental limitation factors of plant growth and development and salinity stress compromises the production and survival of plantation and urban forests and agricultural crops in the arid, semi-arid, and intertidal zones. *Ulmus pumila*, a salt- indigenous tree species in Asia and is widely deployed in salt-affected areas in China, and *U.pumila* is promising for multi-varietal forestry in plantation and urban forests. The comprehensive mechanism of the intraspecific salt tolerance is still not clear yet. Here, we investigated the physiological responses of the salinity stress based on the antioxidant enzyme activities, osmotic adjustments, and gas exchange among salt-tolerant *U. pumila* genotypes for 100 days under five different NaCl levels (0%, 0.3%, 0.5%, 0.7%, and 0.9% w/v) with natural surroundings and rain shade at age-2. Salt stress decreased height (HR), ground diameter (DR), and dry weight (biomass) were significantly different among genotypes. HR and performance indices were positively correlated with photosynthesis rate (Pn), apparent mesophyll conductance (AMC), and chlorophyll (CHLL) with (r= 0.7 - 0.8 ***), but were negatively related to the free proline, sugar, and protein accumulation (r=-0.5 ~ -0.7***). We found that high accumulation of sugars and more activities of SOD enzyme in leaf tissue contribute to the osmotic adjustment and ROS scavenging system under salinity treatment; the sugar content and SOD activity play key roles in *U. pumila's* tolerance to salt stress, and are promising indicators for *U. pumila* species ex vitro selections. The *ex vitro* selection results align with the previous *in vitro* studies [37] and is promising for the MVF development.

Keywords: keyword 1; *Ulmus pumila* 2; *salt stress* 3; salt-tolerance 4; antioxidants 5; osmolytes 6; gas-exchange

1. Introduction

The global forestry sectors are facing multiple challenges in plantations such as the increasing demand for wood and fibre with high quality from shrinking forestland, emerging ecological and social risks due to climate change and deforestation [1]; plantation is more necessary at challenging sites for water soil conservation, land reclamation, carbon sequestration [2 ,3]; urban forests with climate and salinity stress-tolerated tree varieties are popular in municipalities especially for de-icing salt using cities [4-6]. Multi-varietal forestry (MVF) employs selected tree genotypes with desirable merits of commercial traits for operational scale production, which is built upon the sophisticated vegetative propagation techniques [7]. Urban forestry also adopted varietal deployment (e.g., vegetative reproduction) for horticultural application. In broad-leaf trees such as elms, MVF is promising as the result of the tissue culture and somatic embryogenesis technology for the landscape-scale deployment of elite varieties that are tolerant to abiotic stresses such as salinity [37]. MVF also enables the tree variety production from ex vitro selection for urban forest development.

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Soil salinity is a major abiotic stress factor challenges agriculture production and afforestation for long-term [8], and 99% of the world's flora cannot survive at such high salt surroundings [9]. Previous studies showed that 10% of the land area has been frequently affected by salinity worldwide, which included approx. 20 % of the world's irrigated land with significant crop yield losses [10,11]. Forests suffered from productivity losses due to high soil salinity in China [12], Canada [13], Australia [14], etc. Salinity tolerance of trees and crop varieties is necessary for restore the forest landscape and carbon sequestration [14,15]. The salt-tolerate plants that survive to reproduce in environments where the NaCl over 200 mM are called halophytes [16]. Excessive salinity (i.e., > 40 mM NaCl) in the soil will affect the growth and development of most plants [17,18].

A series of physiological and biochemical changes are involved during the plants' response to salt stress at the cell and tissue levels: osmotic stress, ionic stress, and oxidative stress; and these are the three main factors affecting the plants exposed to salinity [19,20]. Salinity stress imposes osmotic stresses and causes osmotic adjustment by the accumulation of osmotic regulation substances such as proline, sugar, and protein [21]. Salinity also causes the increasing reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) [22,23]. To scavenge ROS, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) neutralize the free radicals and reduce the potential cell damage caused by the oxidative stress [24,25]. For the whole individual plant, salinity stress will alter the ion homeostasis and cause an increase in Na^+ and Cl^- concentration and a decrease in K^+, Ca^{2+}, and N^{3+} content [26-28]. Salt accumulation damages the photosynthetic leaves while increasing the senescence rate [29]. For different plant species, several physiological responses to salinity qualitatively or quantitatively differentiate among the salt-tolerant and sensitive species or lines [30,31]. In purslane (*Portulaca oleracea*) and wheat (*Triticum aestivum*), the proline is an essential indicator that highly accumulated in salt-tolerant than in salt-sensitive cultivars [32,33]. While, opposite results were reported in Acacia spp. and Sorghum bicolor, and showed that the high accumulation of proline in leaves is not a key salt-tolerant indicator in these plants [31,34]. Similar results were also reported in different physiological reactions, such as antioxidant enzymes, soluble sugars, and amino acids [35,36]. Thus, the strategies of salt-resistant in plants were species-dependent that need to be studied in separate cases.

Siberian elm (*Ulmus pumila* L.) is an indigenous tree species to Asia and has often been considered for forest plantations in salt-affected areas in China due to its high resistance to drought, extreme temperatures, and salinity [37]. Thus, *U. pumila* plays an essential role in promoting revegetation and reducing wind erosion in the arid sandy [38,39] and steppe area [40,41]. Previous studies of *U. pumila* focused on the genetics and breeding due to its high tolerance to the Dutch elm disease [42-46]. More recently, the studies of programmed cell death and reactive oxygen species have been reported in *U. pumila* seeds [47-49]. The effect of salinity on Siberian elm has only been reported on the seedlings responses in photosynthetic characteristics in sand culture [50] and the activity of superoxide dismutase in leaves [51]. However, little is known about the physiological mechanism of salt-tolerant tissue-cultured *Ulmus pumila* under salt stress.

Our primary objectives of this study were to i) determine the salt effect on ex vitro of Siberian elm under nature surroundings, ii) find out the physiological salt-resistant strategy of *U. pumila* by comparing the different response mechanism between salt-tolerant and salt-sensitive *U. pumila* seedlings in the context of MVF. We hypothesized that the relatively longtime NaCl effect drives the physiological distinctions among two types of tissue-cultured varieties of *U. pumila* during the ex vitro test and selections.

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2. Materials and Methods

2.1. Plant material

The plant material used in this study were 1-year-old seedlings obtained from tissue culture. Five genotypes from gene mutation of *Ulmus pumila* (#1: original genotype No. '73001'; #2: original genotype name 'Mengxian'; #3: original genotype No. '80025', #5: original genotype No. '73006' and #6 original genotype No. '65225') and one hybrid genotype (#4: original genotype name 14×2 ④) were selected from our former study[37]. Among them, three of them were salt-sensitive genotype (#1, #2 and #3) and three genotypes were salt-tolerant (#4, #5 and #6).

2.2. Experimental setup and salt treatments

The study was carried out to examine the physiological changes and the different reactions between salt-tolerant and salt-sensitive elms when suffering the NaCl stress under the natural environment. Six genotypes were selected from the former study *in vitro* selection. The seedling materials used for this study initially obtained from the shoots tissue culture. For the first year, *in vitro* plants (about 200 plantlets per each) were transplants from the culture medium to seedbed in the greenhouse for acclimatization. Two months later, 120 seedlings with similar size (7 to 8 cm in height) of each genotype (720 seedlings in total) developed from the *in vitro* culture were selected and grown in 35 cm diameter pots filled with lemon soil (stylized with 0.27‰ of carbendazim) settled in the outdoor surrounding at the state nursery of Shandong Academic of Forestry, Jinan, China (117°28′ E, 36°44′ N). Seedlings were grown under a natural environment with well-cultivated conditions such as irrigation and disinsection before starting NaCl treatment. In the second year, 30 seedlings with similar growth condition of each genotype were selected and moved into rainproof installations (open area covered with transparent film) at the forestry test station of Shandong Agriculture University, Tai'an, China (116°20′ E, 35°38′ N).

The salt stress treatments were applied by immersing the pots with seedlings into different NaCl solutions. The treatment concentrations were 0% (control), 0.3%, 0.5%, 0.7%, 0.9% (w/v), which were equal to 51.3 mmol·L^{-1}, 85.5 mmol·L^{-1}, 119.7 mmol·L^{-1}, and 153.8 mmol·L^{-1} NaCl solution respectively. The NaCl represented low (0.3%), medium (0.5%, and 0.7%), and high (0.9%) salinity levels [27]. Based on the weather condition, pots with seedlings were soaked into different NaCl solution 6-10 hours per time and two to three times per week. To avoid the osmotic shock, for the higher salt treatment (0.7% and 0.9%), NaCl was added gradually over the period of one week. For the 0.7% and 0.9% treatments, the pots were immersed for three times consecutively into three NaCl solutions, i.e., 0.5%, 0.7%, and 0.9%, respectively. Every two weeks, the salt will be flushed from the soil to prevent soil build-up by adding fresh water from the top of the pots. Hoagland solutions (25%) were used as the fertilizer and were added to the solutions every two weeks. In addition to the water condition, all the experiment was taking out under natural surroundings.

Before and after the treatments, individual seedlings' height and ground diameter were determined (103 days in total). During the second month of NaCl treatments (55 days, before the mortality started), gas exchange, leaf concentrations of chlorophyll, proline, sugars, and proteins were measured and the activities of superoxide dismutase (SOD), peroxidase (POD) were determined. At the end of the experiment, total biomass

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without leaves (plants start dormancy early under higher salt stress) and sodium concentrations were measured. The visible injury was recorded two weeks before harvesting. The condition of each seedling (referred to as health level or health score) was assigned and given a different score (ranging from 0 to 10) according to the overall plant performance level: with 10 indicating the plants had no visible injury with large size, 9 indicating no injury but smaller size; 8 indicating very minor leaf chlorosis; 7, 6, 5, 4, 3, 2, and 1 indicating up to 20%, 20-30%, 30-50%, 50-60%, 60-70%, 70-80%, and 80-90%, of leaves with chlorosis, respectively, while 0 indicated plant mortality [52].

2.3. Measurements of the Free proline, chlorophyll, soluble protein, and sugar concentrations

Free proline concentrations were analyzed according to the method of Bates, *et al.* [53] with some modifications. Extracts were prepared from 0.5 g of fresh mature leaves (in the middle position of the main stem) with 5 ml 3% sulfosalicylic acid in a boiling water bath for 10 min. After cooling, the extract was filtered and 2 ml glacial acetic acid and 2 ml ninhydrin were added to 2 ml of the filtered extract. After 30 min of incubation in a water bath at 100°C, the mixture was cooled and 4 ml methylbenzene was added. The mixture was shaken for 30 s and centrifuged at 3000×g for 5 min prior to the spectrophotometric measurements. The absorbance of extracts was recorded at 520 nm and the concentrations of proline were determined from the standard curve prepared for pure proline (Sigma-Aldrich) according to Bates *et al*. [53].

Leaf chlorophyll was extracted from 0.2 g fresh mature leaves (both healthy and injured) with 4 ml of 80% acetone, 95% ethanol, and water (3:6:1, by volume) at 60°C for two hours. After cooling to the ambient temperature and centrifuging at 2500×g for 5 min, the absorbance of extracts was read at 645 and 663 nm to determine the chlorophyll a and b concentrations [54].

Soluble proteins were extracted from 0.2 g fresh mature leaves (in the middle position of the main stem) with 2 ml extraction buffer (10 mM Tris-HCl, 10% glycerol, 5% PVP, 1.0% Triton X 100) at pH 6.8. The protein extracts were centrifuged at 12000×g for 20min at 4°C before use, then the sample was analyzed with the spectrophotometer using the protein dye-binding method [55].

The tissue sugar concentrations were analyzed with the anthrone reagent method described by Sadasivam and Manickam [56]. Briefly, 0.5 g samples of mature leaves (in the middle position of the main stem) were extracted in 25 ml distilled water in a water bath (100°C) for 30 min twice. Anthrone (0.5 ml) in 5 ml H_2SO_4 was added to 50 ul of filtered extracts (with 1.95 ml distilled water). The absorbance was measured at 630 nm and the total soluble sugar concentration was calculated using the standard curve obtained for the glucose.

2.4. Antioxidant enzyme, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities

We prepared the enzyme assays as following, the 0.5 g fresh mature leaves were ground to a fine powder by using the liquid nitrogen; and then we produced the extracts with 3 ml of 50 mmol L^{-1} Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0), containing 1.0 mmol L^{-1} EDTA, 0.05 % (v/v) Triton X-100, 2% (w/v) PVP. The extracts were centrifuged at 4°C for 30 min at 20000×g and the supernatants were used to assay the antioxidant enzyme activities.

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The SOD activity was assayed by measuring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) [57,58]. One unit superoxide dismutase was defined as the amount of enzyme required to lead to a half percentage of inhibition of the rate of NBT reduction measured at 560 nm.

For the POD activity, changes in absorbance at 470 nm were measured every minute in the supernatants (obtained as described above) according to Civello, *et al*. [59]. A unit of peroxidase activity was expressed as the change in absorbance.

We measured the CAT activity measurement based on the homogenates that were centrifuged at 4°C for 30 min at 20000×g. The supernatants were estimated by measuring the decrease in absorbance due to the disappearance of H_2O_2 at 240 nm due to degradation of hydrogen peroxide using the method of Aebi [60].

2.5. Leaf gas exchange measurements

Net photosynthetic rate (Pn) ($\mu mol \cdot m^{-2} \cdot s^{-1}$), transpiration rate (Tr) ($mmol \cdot m^{-2} \cdot s^{-1}$), were measured on six plants per genotype per treatment combination using a CIRAS-2 portable photosynthesis system (PP Systems, Amesbury, MA, USA). Measurements were carried out at constant ambient conditions (the photosynthetic photon flux density was maintained at 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the leaf temperature kept at 28 °C and the CO_2 concentration maintain at 360 $\mu mol \cdot mol^{-1}$). All measurements were carried out between 09:00 and 11:00 am during continuous sunny days and the upper fully development leaves were used for the gas exchange measurements. Water use efficiency (WUE) was calculated as the quotient of the net photosynthesis and the transpiration rate, i.e., WUE = Pn / Tr [61].

2.6. Growth experiment set up and measurements

After 103 days, plant height and ground diameter were measured again to determine height and ground diameter growth rates; the dry weight of shoot and root were used as total biomass (for salt-sensitive genotypes, all growth data were collected after the death happened). Height relative growth rate (HR), ground diameter relative growth rate (DR) and the biomass were used as growth indicators. The height relative growth rate (HR) was determined as $\frac{(\text{Plant final height} - \text{Plant initial height})}{\text{Plant initial height}} \times 100\%$. The ground diameter relative growth rate (DR) was calculated as $\frac{(\text{Plant final ground diameter} - \text{Plant initial ground diameter})}{\text{Plant initial ground diameter}} \times 100\%$.

2.7. Data analysis

Growth parameters were measured in six genotypes (n=6) in each per treatment per genotype. The one-way ANOVA was carried out by the Data Processing System software (SPSS version 19, Chicago, IL, USA) and the means denoted by different letters were significantly different at p-Value <0.05 based on Duncan's multiple range tests. Principal Component Analysis (PCA) was carried out with the *prcomp()* function in R and the plot was made with the *fviz_pca_biplot()* in the *FactoMineR* and *factoextra* packages in R (R Core Team, 2013. http://www.R-project.org/).

There were two samples per treatment with three replications (n=6) measured for superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), free proline, total chlorophyll, malondialdehyde (MDA), soluble protein, sugar concentrations, and gas-exchange. The results were analyzed by a hierarchical analysis of variance (Nested ANOVA)

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using R v3.1.2 core functions [62] based on the degree of salt tolerance (salt-tolerant group and salt sensitive group). The significance level (α) was 0.05.

3. Results

3.1. Plant mortality

Salt-sensitive genotypes started dying at 0.7% NaCl after two months later. After 103 days of different concentration of NaCl, all plants of sensitive genotypes (#1, #2 and #3) were dead under the 0.9% NaCl. For the salt-sensitive genotype #1, #2 and #3, the mortality was 100%, 0% and 100% respectively under the 0.7% NaCl. On the contrary, the salt-tolerant genotypes (#4, #5 and #6) had zero mortality in all NaCl after 103 days.

3.2. Plant morphology

NaCl stress severely affected the morphology and induced plant injuries in both salt-sensitive genotypes (#1, #2, and #3) and salt-tolerant genotypes (#4, #5, and #6) in the middle and high NaCl after a period of three months' treatment. In low NaCl, the salt-tolerant genotypes were less affected (Table 1). When NaCl increased, the health level of salt-tolerant genotypes declined. In the 0.9% NaCl, the average level of salt-tolerant genotypes reached ~3, that 60% to 70% of leaves showed visible injuries.

The growth and visible injuries among the salt-sensitive genotypes were driven by low salt concentration. For Genotype #1, the score was 2 which meant that 70% to 80% of total leaves showed chlorosis or dead at 0.3% NaCl (Table 1). In genotype #2, the health score showed that 60%-70% of the plants' leaves were chlorosis or damaged at 0.3% NaCl (Table 1). The score in genotype #3 indicates that 50% to 60% of the visible injury was shown at 0.3% NaCl (Table 1). The score showed a declining tendency with increasing salt concentrations in NaCl (Table 1).

Table 1. Seedlings performance indices of six *U. pumila* genotypes subjected to different NaCl concentration treatments.

NaCl concentration	Score of plants healthy					
	#1	#2	#3	#4	#5	#6
0.0%	10.0 ±0.0	10.0 ±0.0	10.0 ±0.0	10.0 ±0.0	10.0 ±0.0	10.0 ±0.0
0.3%	2.0 ±0.0	3.0 ±0.0	5.0 ±0.0	6.2 ±0.3	5.0 ±0.0	5.0 ±0.0
0.5%	1.3 ±0.2	2.2 ±0.2	3.5 ±0.5	5.3 ±0.2	4.7 ±0.2	4.0 ±0.0
0.7%	0.0 ±0.0	1.3 ±0.2	0.0 ±0.0	4.3 ±0.2	4.0 ±0.0	4.0 ±0.0
0.9%	0 .0±0.0	0.0 ±0.0	0.0 ±0.0	3.3 ±0.2	2.5 ±0.2	3.3 ±0.2

Notes, means (n = 6) ± standard error.

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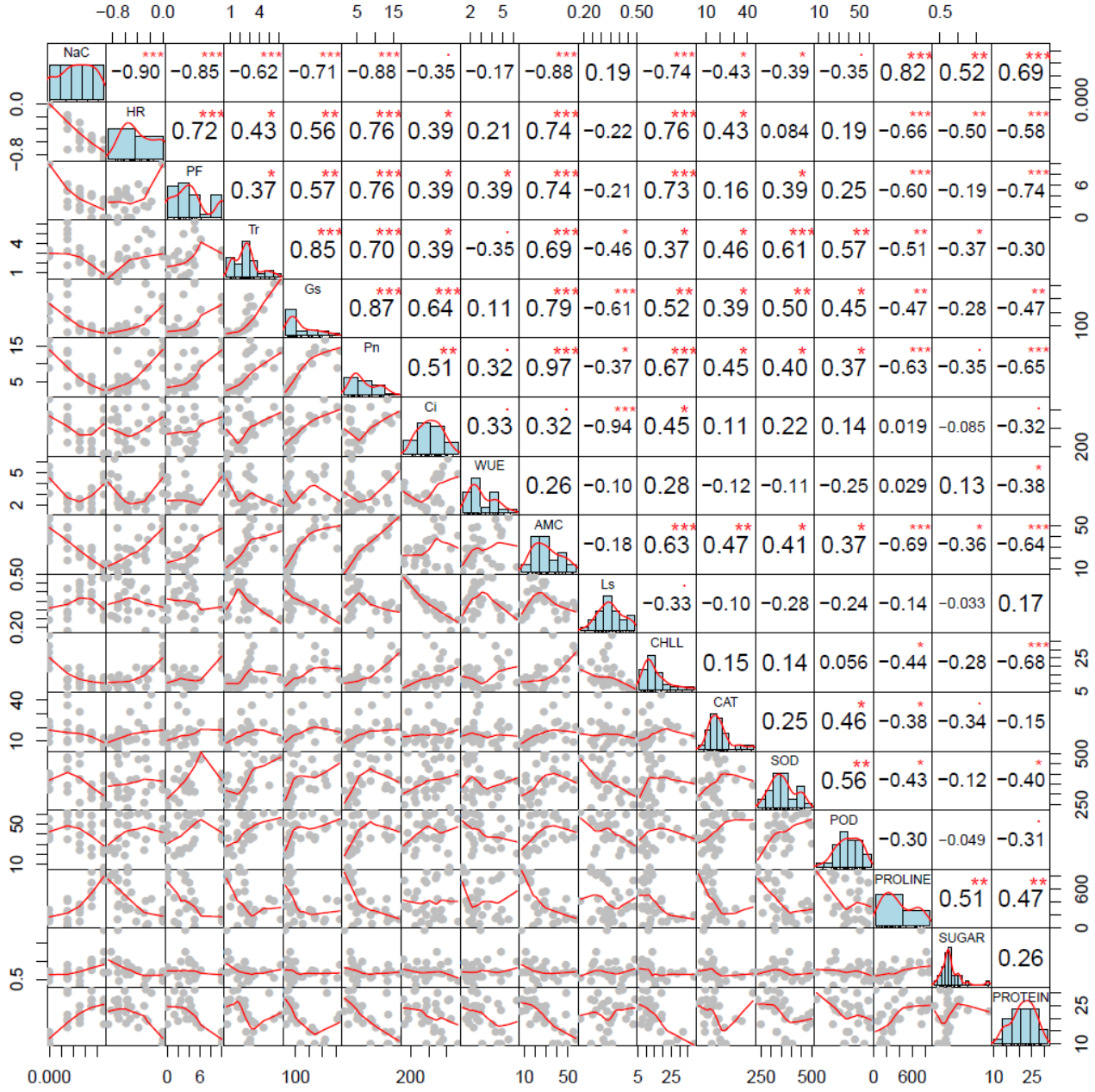

Figure 1. *Correlation* and histogram *of* physiological variables and NaCl treatment (NaC) including height growth rate (HR, %), performance *indices* (PF), *transpiration rate (Tr), Stomatal conductance* (Gs), average net photosynthesis rate (Pn), water use efficiency (WUE), internal carbon concentration (Ci), apparent mesophyll conductance (AMC), stomatal limitation percentage (Ls), chlorophyll *(CHLL), CAT, SOD, POD, Proline, sugar, and protein.* Pearson's correlation coefficients are in the right upper diagonal, the scatter plots are in the lower-left diagonal for the pairs of physiological variables, while the diagonal are the histograms of each variable. The p-values are denoted as following ***<0.0001, **<0.001,*<0.05. The first column of scatter plots and first row of correlation showed the relationship between NaC and all tested traits.

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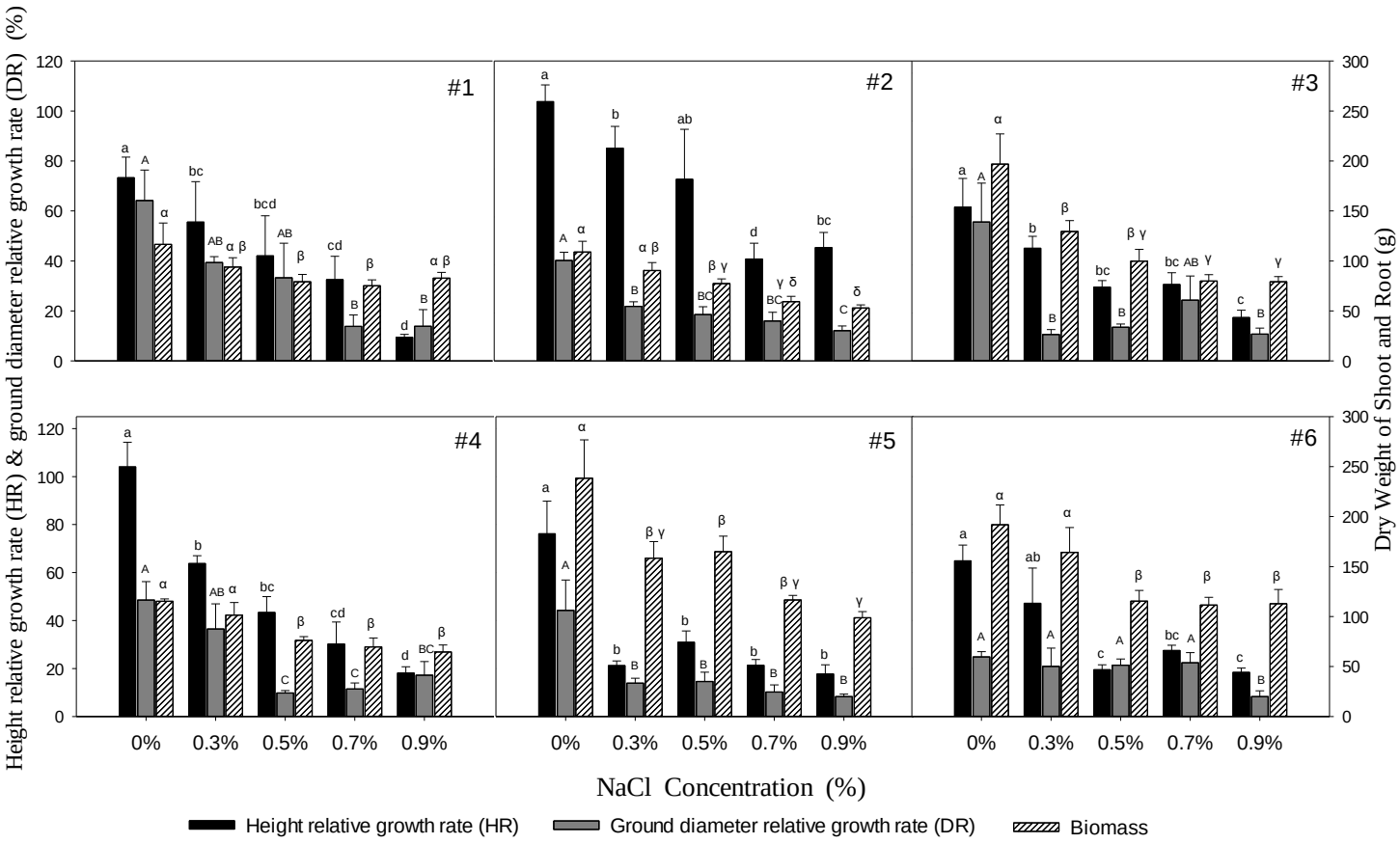

Figure 2. Height relative growth rate (HR), ground diameter relative growth rate (DR), and dry weight of shoot and root (biomass) in 6 genotypes of *U. pumila* subjected to different NaCl concentration treatments. Means (n = 6) ± SE are shown. Means followed by different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

3.3. Effects of NaCl on growth parameters

Height relative growth rate (HR), ground diameter relative growth rate (DR) and dry weight of shoot and root (biomass) decreased in the six studied genotypes with the increase in NaCl concentrations (Figure 2). Salt-sensitive genotypes had less affected on HR but more affected on DR and biomass compared with the salt-tolerant genotypes by the same concentrations of NaCl. Under low salt stress (0.3% NaCl), the HR, DR, and biomass in salt-sensitive genotypes were 23%, 55%, and 24% lower on average than the untreated control, respectively (Figure 1A, 1B, 1C). In salt-tolerant genotypes, the HR, DR, and biomass were 46%, 36% and 20% lower on average than the untreated control under low salt treatment, respectively (Figure 1D, 1E, and 1F). In the high salt treatment (0.9% NaCl), the HR, DR and biomass in salt-sensitive genotypes were 72%, 76%, and 47% lower on average than control, and 77%, 71%, and 48% lower on average than the control in salt-tolerant genotypes, respectively (Figure 2).

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Table 2. Nested ANOVA analysis of osmoregulation content, reactive oxygen species, gas exchange and sodium concentration in *U. pumila* between salt-tolerance and salt-sensitive group under NaCl treatment

Physiological indicator	Group F Value		Group: Genotypes F Value	
Sugar	1229.810	***	13.479	***
Free proline	329.720	***	698.79	***
Soluble protein	87.387	***	97.353	***
SOD	1648.780	***	60.004	***
POD	712.560	***	785.05	***
CAT	48.159	***	33.624	***
Chlorophyll	40.057	***	2425.341	***
Pn	9.736	**	32.2182	***
Tr	0.252	-	11.8733	***
WUE	0.050	-	2.2644	-

Notes, *** mean significant at 0.001 level; ** mean significant at 0.01 level; - mean not significant.

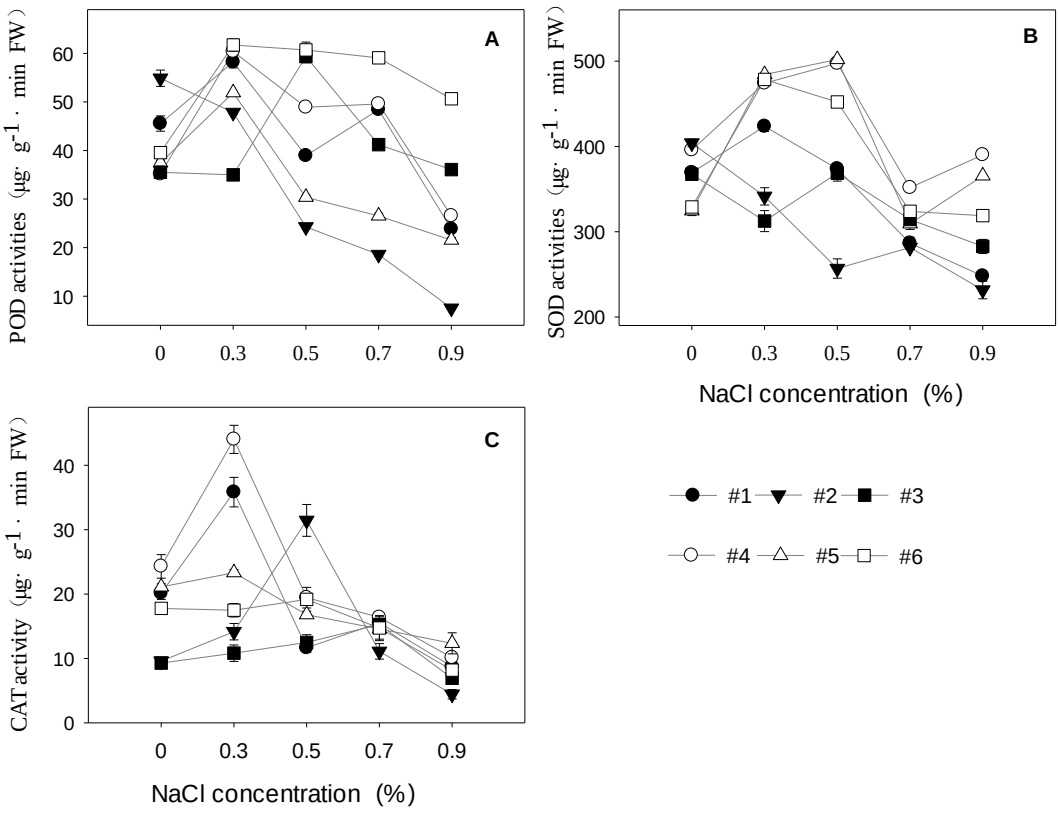

Figure 3. Changes in antioxidant enzyme activities subjected to NaCl treatments. Means (n = 3) ± SE are shown (A, POD activities; B, SOD activities; C CAT activities).

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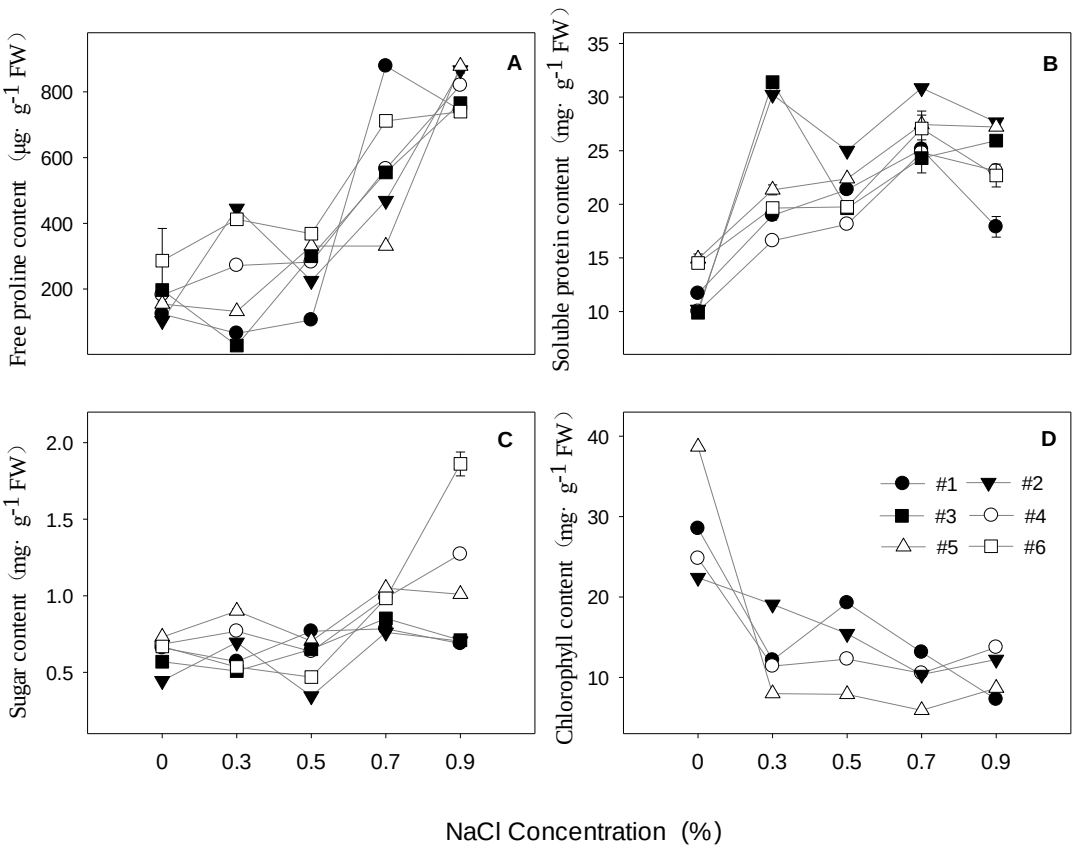

Figure 4. Changes in chlorophyll, soluble protein, sugar, and free proline subjected to treatments. Means (n = 3) ± SE are shown (a free proline content; b soluble protein content; c sugar content; d chlorophyll content in the NaCl treatments).

3.4. Nested ANOVA

We using the nested ANOVA to compare the difference of physiology parameters (SOD, POD, CAT, free proline, soluble protein, sugar, chlorophyll, and gas exchange indexes) between two groups (salt-sensitive genotypes and salt-tolerant genotypes). The result showed that the differences of SOD, POD, CAT, free proline, soluble protein, sugar, chlorophyll, net photosynthesis (Pn), between the salt-sensitive group and salt-tolerant group are significant (Table 2). While the result of transpiration rate (Tr), and water use efficiency (WUE) between the two groups are not significant (Table 2).

3.5. Antioxidant enzyme activities

NaCl drove the tissue peroxidase (POD) variation in different genotypes (Figure 3A). In genotype #1, the POD activity increased in 0.3% and 0.7% NaCl, decreased at 0.5% and 0.9% NaCl compared with the control. The POD activity decreased with the increase of NaCl concentrations in genotype #2. In genotype #3, the POD activity decline at 0.3%

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NaCl, increased at middle (0.5% and 0.7%) and high (0.9%) NaCl. For salt-tolerant genotypes, a sharp increase of POD activity was measured at 0.3% NaCl. In genotype #4, the POD activity increase in middle NaCl and a decline in the level below the control in high NaCl. The POD activity in genotype #5 decreased under rising NaCl concentrations (NaCl >0.5%). Salt treatment elevated the activities of the POD in genotype #6, at 0.9% NaCl, the POD activity was still 28% greater than in control.

Superoxide dismutase (SOD) activity kept at relatively higher concentrations in salt-tolerant genotype than salt-sensitive genotype under all NaCl (Figure 2B). In salt-tolerant genotypes, the activity of SOD enzyme increased in 0.3% and 0.5% NaCl. The increased SOD activity in salt-tolerant at 0.5% NaCl was 39% greater on average compared with untreated control. A slight decline in SOD activity was observed in salt-tolerant genotypes at 0.7% and 0.9% NaCl except genotype #5 (increased in 0.9% NaCl). On the contrary, NaCl stress decreased the SOD activity in most of the salt-sensitive genotypes (Figure 2B). In genotype #2, SOD activity decreased with the increasing NaCl concentrations. A similar reduction in SOD activity was shown in genotype #3 except a 0.34% increase than the control in 0.5% NaCl. The SOD activity in genotype #1 increased slightly in 0.3% and 0.5% NaCl and a sharp decline below the control at the 0.7% and 0.9% NaCl.

The catalase (CAT) activity in leaves increased in lower salt (NaCl 0.3% to 0.7%) treatments, decreased at higher salt (NaCl 0.7% to 0.9%) treatments (Figure 2C). In salt-tolerant genotypes, the activity of CAT activity reached the top in 0.3% or 0.5% NaCl which was 33.25% greater on average compared with untreated control. The CAT activity declined at 0.5% or 0.7% NaCl and dropped to the bottom in 0.9% NaCl. In 0.9% NaCl, the average of CAT activity in salt-tolerant genotypes was 51.22% lower compared with untreated control. In salt-sensitive genotypes (#1, #2 and #3), the activity of CAT enzyme reached the peak level in 0.3%, 0.5% and 0.7% respectively. The average of CAT activity in the peak was 88.02% greater compared with untreated control. The activity of CAT enzyme in salt-sensitive genotypes dropped to the bottom at 0.9% NaCl which was 50.18% lower compared with untreated control.

3.6. Proline tissue concentrations

Free proline (Pro) tissue concentration increased with the increasing salt treatment concentration in most of the genotypes, except for genotype #3 and #5 which decreased at 0.3% NaCl, and genotype #1 with a decrease at 0.3% and 0.5% of NaCl (Figure 3A). In 0.9% NaCl, all genotypes' tissue Pro reached the highest except genotype #1 (with the maximum proline levels in 0.7% NaCl). The concentration of proline in salt-sensitive genotypes averaged at 792$\mu g \cdot g^{-1}$ which was 5 times higher than control at 0.9% NaCl. In salt-tolerant genotypes, the average Pro concentration reached 813 $\mu g \cdot g^{-1}$ in 0.9% NaCl which was 3.3 times higher than the untreated control.

3.7. Soluble protein concentrations

Effects of NaCl increased the concentration of tissue soluble protein (SP) in all genotypes (Figure 3B). In salt-tolerant genotypes, the maximum SP tissue concertation was measured in 0.7% NaCl, the concentrations were 106% greater on average than the control. Similar

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patterns were found for salt-sensitive genotypes with the maximum proline levels induced by 0.7% NaCl, except for genotypes #3 which showed the highest SP levels in 0.3% (Figure 3B).

3.8. Sugar tissue concentrations

The tissue sugar levels in salt-tolerant genotype were higher than salt-sensitive genotype in higher NaCl (0.7% and 0.9% NaCl) (Figure 3C). Generally, the sugar concentration in salt-tolerant genotypes increased with the increasing NaCl except 0.5% NaCl concentration (which had a decrease of sugar content). The concentration of sugar in salt-sensitive genotypes decreased at 0.3% or 0.5% NaCl, the maximum values were measured in 0.7% NaCl. In 0.7% NaCl, the sugar concentration in salt-sensitive genotypes was 0.78 $mg \cdot g^{-1}$ (#1), 0.76 $mg \cdot g^{-1}$ (#2) and 0.85 $mg \cdot g^{-1}$ (#3), which was 46% higher on average than the control. In 0.9% NaCl, the average increases of sugar content in salt-sensitive genotype was 29% higher than the untreated control. While, in salt-tolerant genotype, the sugar concentration was 0.99 $mg \cdot g^{-1}$ (#4), 1.05 $mg \cdot g^{-1}$ (#5) and 0.98 $mg \cdot g^{-1}$ (#6) in 0.7% NaCl, and 1.27 $mg \cdot g^{-1}$ (#4), 1.01 $mg \cdot g^{-1}$ (#5) and 1.86 $mg \cdot g^{-1}$ (#6) in 0.9% NaCl which was 45% and 101% higher on average than the control, respectively.

3.9. Chlorophyll

Generally, total chlorophyll concentrations decreased with the increasing salt treatment concentrations in both salt-sensitive and salt-tolerant genotypes (Figure 3D). In 0.9% NaCl, the reduction of chlorophyll concentration averaged 65.8% in salt-sensitive genotypes, compared with about 50.5% for salt-tolerant genotypes at 0.9% NaCl.

3.10. Leaf gas exchange

Net photosynthesis rate (Pn) decreased with increasing of the NaCl treatments in both the salt-sensitive and the salt-tolerant genotypes (Table 3). The transpiration rate (Tr) increased at 0.3% NaCl, decrease at middle and high NaCl treatment of except genotype #3 (with a transpiration rate decline from 0.3% to 0.9% NaCl) (Table 3). The water use efficiency (WUE) showed a decrease in genotype #3, #4, and #5. In 0.9% NaCl concentration, an increase of (WUE) was measured in genotype #1, #2, and #6. The WUE in genotypes #1, #2 and #6 were lower in NaCl than the control. While for genotypes #3, #4, and #5 the WUE decreased in 0.3% to 0.7% NaCl increased in 0.9% NaCl.

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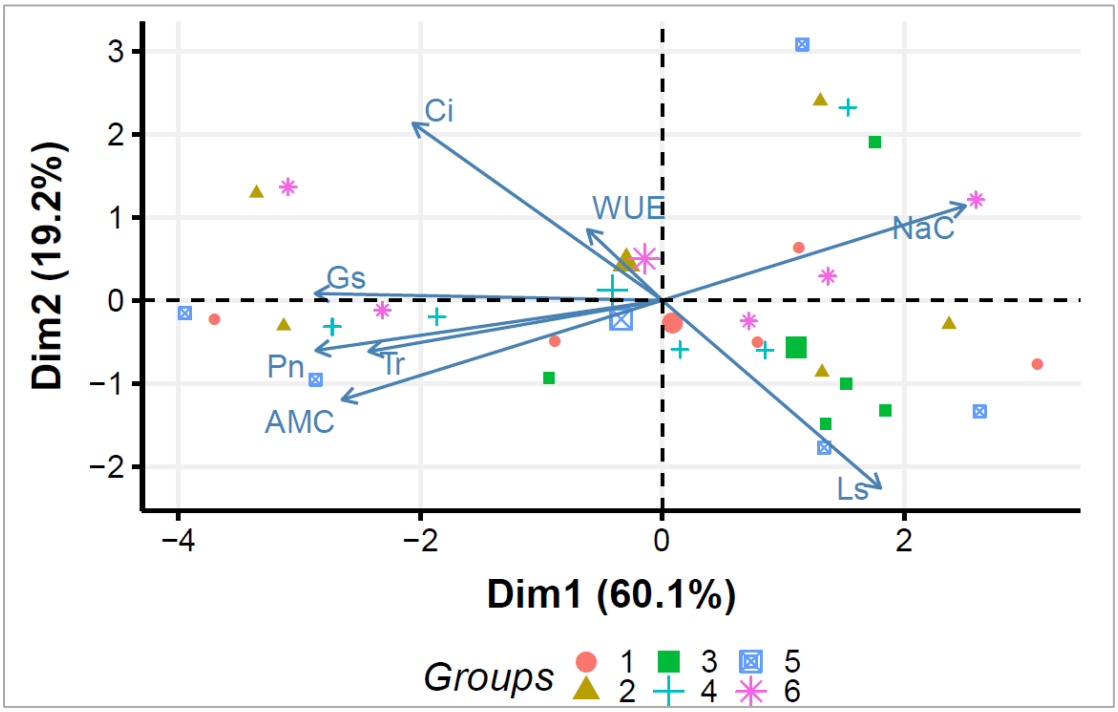

Figure 5. Principal component analyses (PCA) biplot of clonal average net photosynthesis rate (Pn), transpiration rate (Tr), water use efficiency (WUE), Ci (internal carbon concentration), AMC (apparent mesophyll conductance), Ls (stomatal limitation percentage), change in different NaCl concentration treatments (NaC) of 6 genotypes of *U. pumila* (color groups). NaC, NaCl treatment concentration. The principal components 1 and 2 (i.e., Dim 1 and Dim 2) explained 60.1% and 19.2% of total variance. WUE, Ls and Ci were not correlated with NaC gradient.

3.11. Phenotypic correlation and the correlations between NaCl treatment and traits

The phenotypic Pearson's correlation coefficients (Figure 1) showed that plant height growth, and performance indices were positively correlated with photosynthesis rate (Pn), AMC apparent mesophyll conductance), and chlorophyll (CHLL) with r ranging from 0.7 to 0.8 ***, but were negatively related to the free proline, sugar, and protein accumulation (r=-0.5- -0.7***). The proline was positively related to NaCl (0.82***), which was stronger than the relationship between NaCl and sugar or protein accumulation (r = 0.5-0.7 ***). However, growth performance, photosynthesis traits were negatively correlated with NaCl. Height, performance indices, stomatal conductance (Gs), AMC, and chlorophyll were negatively correlated with NaCl (r=-0.7 to -0.9***). SOD, POD, and CAT were negatively associated with NaCl (r = -0.4 *). WUE was negatively linked with NaCl but was not significant. A similar trend was shown in the PCA biplot in Figure 5 for multiple physiological traits and NaCl concentrations.

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4. Discussion

The effect of salt stress is manifested by changes in growth (promote or inhibition), morphology, and physiology, e.g., leaf abscission and death, increasing activates of superoxide dismutase enzyme, and declining photosynthetic capacity [8]. The survival rate is a reliable criterion to evaluate salt tolerance. Sodium chloride (NaCl) is the major cause of the inhibition or death to most of the glycophytes; as low as 20 mmol·L-1 (0.117%) NaCl could inhibit the growth of plants [63]. Here we found that the growth of *U. pumila* seedlings decreased with the jump of NaCl concentrations. When NaCl ≥ 0.7%, the severity of the reduction happened in both salt-tolerant and salt-sensitive plants (Figure 2). Regarding the salt-sensitive group, over 60% of the seedlings showed visible injury or leaf chlorosis show in 0.5% NaCl (Table 1) with the mortality rate reaching 100% at NaCl >0.7%. On the contrary, all the seedlings of salt-tolerant genotypes kept alive after the one-year period salt treatments, the visible injury and leaf chlorosis was between 60% and 70% under 0.9% NaCl (Table 1). Our findings confirmed with the feasibility of previous studies *in vitro* selection of salt-tolerant *U. pumila* [37].

Salt stress could lead to oxidative stress in plants by raising reactive oxygen species (ROS) rapidly [64]. ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), can cause cellular damages in plants [24]. To overcome the oxidative stress induced by salinity, the ROS scavenging system, such as SOD, CAT, and POD is activated and elevated to defend against oxidative stress [65]. The decrease of the ROS scavenging system often occurs when the salt concentration exceeds the threshold value [51]. Among these ROS scavenging systems, SOD is a key enzyme to maintain a normal physiological process and cope with the oxidative stress by converting O_2^- to O_2 and H_2O_2 rapidly [66]. In our study, salinity caused a sharp increase in the SOD activity in the seedlings of salt-tolerant genotypes (#4, #5, and #6) under lower NaCl (0.3%, 0.5%). Under higher NaCl, the SOD activity of salt-tolerant genotypes slightly dropped at the 0.7% salt concentration and rebounded at the 0.9% salt treatment (Figure 3B). On the contrary, SOD activity showed a declining trend in all salt-sensitive genotypes (#1, #2 and #3) (Figure 2B). Given the function of SOD, the salt-tolerant *U. pumila* genotypes are likely to possess a stronger O_2^- scavenging ability than the salt-sensitive genotypes. Our results are comparable to previous studies of crop genotypes, such as wheat (*Triticum aestivum*) [67], perennial ryegrass (*Lolium perenne*) [47] and maize (*Zea mays*) [68]. The product of SOD activity is hydrogen peroxide (H_2O_2), which can cause oxidative damage when the concentrations higher in the cell [69].

Various enzymes regulate H_2O_2 in plants' cell such as CAT and POD [70]. We found that the activity of CAT in salt-tolerant and salt-sensitive genotypes both increased in the lower NaCl and decreased at higher NaCl. The POD activity demonstrated a similar trend in both salt-tolerant and salt-sensitive genotypes under different NaCl concentration treatment (Figure 3A). The similar results agreed with the previous study of the *in vitro* selection of *U. pumila* [37] and soybean (*Glycin max*) [71].

By comparing the salt-tolerant and the salt-sensitive genotypes, we demonstrated no different tendency in the activity of CAT and POD enzyme (Figure 3B,C). In general, the over-reduction of the electron chain along with the lack of regeneration of the final electron acceptor in PSI (NADP+) favor electron transfer from ferredoxin to oxygen to form O_2^- (Mehler Reaction) in chloroplasts, which undergoes dismutation to H_2O_2 and O_2 that is catalyzed by the superoxide dismutase (SOD) [72]. The opposite reaction of SOD activity between salt-tolerant and salt-sensitive genotypes could indicate that the SOD activities have played a crucial protective role against the oxidative stress in salt-tolerant genotypes *U. pumila* caused by salt stress.

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Osmotic stress and ionic stress are the two major stresses due to high salinity [26]. Under salt stress, the accumulation of osmotic regulation substances such as free proline, sugar and soluble protein facilitate plants to 1) reduce the osmotic potential, 2) to increase the solutes accumulation and tissue solute concentration, and 3) to maintain tissue turgor and tissue growth [73]. Among various organic osmotica, sugars contribute up to 50% of the total osmotic potential in glycophytes subject to saline conditions [30,36]. The major role of sugar accumulation under salt stress is osmoprotection, osmotic adjustment, carbon storage and radical scavenging [35]. Elevated sugars contents in leaf tissue have been reported in numbers of research, such as *Beta vulgaris* [74], *Phaseolus vulgaris* [75], and *Lepidium crassifolium* [76]. In our study, the leaf sugar content in salt-tolerant genotypes increased dramatically under higher NaCl, which was doubled the concentration in 0.9% NaCl than the unsalted control (Figure 3C), while fluctuant trends were observed under different NaCl for the salt-sensitive genotypes. Thus, under higher NaCl, the sugars were highly accumulated in salt-tolerant *U. pumila* than the salt-sensitive genotypes fluctuant trends under different NaCl fluctuant trends under different NaCl. The similar result also reported in species such as sunflowers (*Helianthus annuus*) [77] and *Eruca sativa* [78]. The accumulated sugars content in leaves was a driving factor of survival rates of salt-tolerant genotypes under saline soil conditions such as the 0.7% and 0.9%.

The accumulation of soluble protein in the tissue under saline conditions affect osmotic adjustment and provides a storage form of nitrogen re-utilized later [36]. Increasing leaf soluble proteins have been observed under salt treatment in multiple species, such as mulberry (*Morus alba*) [79], chick-pea (*Cicer arietinum*) [80], and Maize (*Zea maize*) [81]. Previous studies also suggested that the leaf soluble protein was highly accumulated in the salt-tolerant than in salt-sensitive cultivars of sunflower (*Helianthus annuus*) [77], finger millet (*Eleusine coracana*) [82] and rice (*Oryza sativa*) [83]. In contrast, the soluble protein contents declines in response to salinity, e.g., in *Amaranthus tricolor* [84], *Raphanus sativus* [85] and mangrove (*Bruguiera parviflora*) [86]. Yet, there were no significant differences in leaf soluble protein content between salt-tolerant and salt-sensitive plants of safflower (*Carthamus tinctorius*) [77] and *Eruca sativa* [78]. We demonstrated that an increment of soluble protein content in all *U. pumila* genotypes under elevated NaCl (Figure 3B); and both salt-sensitive and salt-tolerant genotypes show a similar trend of the soluble protein content in leaves tissue. The accumulation of soluble protein in *U. pumila* is an inclusive physiological reaction of salt stress. The soluble protein accumulation in the leaf tissue fails to indicate the interspecific variation of salt tolerance of *U. pumila*, even though there is a positive correlation between NaCl and protein accumulation.

In addition to the role of osmotic adjustment, proline can scavenge free radicals, stabilize sub-cellular structures, and buffer cellular redox potential [87]. Proline accumulates extensively in leaf tissue under salt stress in plants, e.g., sorghum (*Sorghum bicolor*) [88], peanut (*Arachis hypogaea*) [88], olive trees (*Olea europaea*) [89], *Populus tomentosa* [90], and eucalypt genotypes (*Eucalyptus* spp.) [27]. We found that free proline content in leaves tissue increased with an increment of NaCl after two months treatment. Both the salt-sensitive and salt-tolerant genotypes expressed a similar trend in the change of proline content in *U. pumila* (Figure 3A) so that the accumulation of free proline in *U. pumila* is an general physiological incidence rather than the signitures due to the damage related to salt stress. Similar results were also reported in the comparative studies of sorghum (*Sorghum bicolor*) cultivars [31] and genotypes [91].

Salt stress also damages the chloroplast structure, reduces the chlorophyll leaf concentrations, net photosynthesis (Pn), and transpiration rate (Tr) [50]. In our study, the Pn rate and leaf chlorophyll content in *U. pumila* seedlings declined under the increasing NaCl (Figure 5, Figure 3D). Similar trends were reported *Calligonum mongolicum* [92], rice (*Oryza sativa*) [93] and chickpea (*Cicer arietinum*) [61]. In our study, both salt-tolerant and

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salt-sensitive genotypes show similar trends of change in the photosynthesis rate and leaf chlorophyll content under the salt stress without apparent differences among groups.

The change of Tr rate varied among different genotypes. For genotype #6, Tr increased at 0.3% and 0.5% NaCl and decreased at 0.7% and 0.9% NaCl. The Tr of #1, #2, and #5 seedlings increased at 0.3% NaCl and decreased at 0.5% to 0.9% NaCl. While for the genotypes #3, the Tr decreased with the increasing NaCl. The change of Tr expressed no common trends between the two groups or no significant difference between salt-tolerant and salt-sensitive genotypes (Table 2).

Water use efficiency (WUE) can describe the relationship between a unit of biomass produced per unit of water used [94]. In our study, no significant differences were shown between salt-tolerance and salt-sensitive genotypes. WUE was not associated with the NaCl. Similar results were reported in other species such as chickpea [61] that genetypic variation was expressed in terms of the WUE reaction under changing salt concentrations.

5. Conclusions

Salt-resistant strategies differ among plant species [36]. The comparative study of the salt-tolerant and sensitive species or genotypes improves the understanding of the salt-resistance behaviors and strategies during the salinity stress. The contrasting physiological changes demonstrated the specific salt-tolerant strategies given distinct SOD activities and sugar content between both three salt-tolerant *U. pumila* genotypes and three salt-sensitive *U. pumilas*. The high accumulation of sugars and elevated activities of SOD enzyme in leaf tissue contribute to the osmotic adjustment and ROS scavenging system and its role in salt-tolerant *U. pumila* against the salt stress. The results in the field *ex vitro* were in line with the findings in previous *in vitro* selections.

Supplementary Materials: Table S1 Net photosynthesis rate (Pn), transpiration rate (Tr), and water use efficiency (WUE) change in different NaCl concentration treatments of 6 genotypes of *U. pumila*.

Table S2. Principal component analysis (PCA) of NaCl treatment concentration (NaC) and photosynthesis variables.

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