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Adaptive photosynthetic and physiological responses to drought and rewatering in triploid *Populus* populations

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Abstract

Cuttings of *Populus cathayana* Rehd, originating from three triploid and one diploid populations with the same parents but different gamete origins, were used to examine physiological responses to drought stress and rewatering by exposure to three progressive water regimes. Progressive drought stress significantly decreased the leaf relative water content (RWC), photosynthesis, and chlorophyll fluorescence parameters, and increased the relative electrolyte leakage, malondialdehyde (MDA), free proline (Pro), and antioxidant enzymes, such as superoxide dismutase, peroxidase, and catalase, in the four populations evaluated. However, compared to the diploid population, triploid populations showed lower relative electrolyte leakage and MDA, higher RWC and Pro content, and more efficient photosynthesis and antioxidant systems under the same water regime. Our data indicated that triploid populations possessed more efficient protective mechanisms than that of diploid population with gradually increasing drought stress. Moreover, some triploid genotypes were less tolerant to water stress than that of diploids due to large intrapopulation overlap.

Additional key words: chlorophyll fluorescence gas exchange; malondialdehyde; poplar; population; stomatal limitation; water deficit.

Introduction

As one of the most important abiotic stressors, drought limits the survival of trees, plant growth, and forest productivity (Peng *et al*. 2011a). Plants show a wide range of adaptive changes in plant growth, cellular structure, and physico-biochemical responses under drought-stress conditions (Chaves *et al*. 2003). As the most direct effects, plants typically show reduced rates of growth and photosynthesis, which may involve alterations in carbon assimilation and metabolism (Souza *et al*. 2004). Upon water stress, the reduced assimilation may result from some integrated processes, such as an imbalance between the photochemical activity of PSII and electron transfer in the Calvin cycle, stomatal closure, and decreased activity of related photosynthetic enzymes (Lawlor and Cornic 2002, Chaves *et al*. 2003). In order to reduce transpiration in leaves and prevent excessive water shortage in tissues, the stomata close in response to drought stress (Jones and Sutherland 1991).

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Abbreviations: C_i – intercellular CO_2 concentration; CAT – catalase; CCI – relative chlorophyll content index; CK – control; CV – coefficient of variation; *E* – transpiration rate; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_v – variable fluorescence; F_v/F_0 – potential photochemical efficiency of PSII; F_v/F_m – maximal quantum yield of PSII photochemistry; FC – field capacity; FDR – first-division restitution; g_s – stomatal conductance; MDA – malondialdehyde; P_N – net photosynthetic rate; PCA – principal component analysis; PMR – post-meiotic restitution; POD – peroxidase; Pro – free proline; RMP – relative membrane permeability; ROS – reactive oxygen species; RSWC – relative soil water content; RW – rewatering; RWC – relative water content; SDR – second-division restitution; SOD – superoxide dismutase; TCA – trichloroacetic acid; triploid-F – the FDR population; triploid-S – the SDR population; triploid-P – the PMR population; WS – water stress treatments; WS_1 – mild water stress; WS_2 – moderate water stress; WS_3 – severe water stress; WUE – water-use efficiency; WW – well-watered.
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Drought stress often results in excessive production of reactive oxygen species (ROS), causing various damages in plants (Asada 1999). In order to maintain ROS contents, an antioxidative defense system, including both enzymatic antioxidants, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase, and glutathione reductase, and nonenzymatic antioxidants (such as carotenoids and ascorbate), is present to counteract ROS toxicity and maintain cell function integrity (Asada 1999, Reddy *et al*. 2004). Drought resistance in plants correlates with scavenging ROS and variation in the extent of osmotic adjustment (Tsugane *et al*. 1999). The synthesis and accumulation of some solutes, such as Pro, glycine betaine, and soluble sugars, can help cells maintain the intracellular balance of osmotic pressure and resist water deprivation and cellular dehydration (Bray 1997, Hare *et al*. 1998, Chaves *et al*. 2003).

Previous genetic studies have shown that polyploids are more or less tolerant to low soil moisture than diploids (Maherali *et al*. 2009). For example, tetraploid *Spathiphyllum* plants are more tolerant to drought than diploid plants and exhibit different morphological and anatomical structures following polyploidization (Van Laere *et al*. 2011). Xiong *et al*. (2006) reported that two hexaploid varieties of wheat showed the longest survival following drought, as well as the highest yields under drought conditions, similar to tetraploids; diploids were the least robust. However, Sugiyama (2006) found that diploid cultivars of *Lolium* had higher survival rates under drought conditions than tetraploid cultivars. Therefore, the present study used populations rather than individuals to understand the role of drought stress on the triploid genus *Populus*.

Due to the significant genetic diversity among poplars, their drought tolerance varies among populations, species, and genotypes (Zhang *et al*. 2004, Monclus *et al*. 2006). Poplars are sensitive to water deprivation, since they are naturally found in riparian areas where their roots can access water easily (Rennenberg *et al*. 2006, Koyama and Kielland 2011). However, triploid *Populus* is typically found in hostile growth environments. Thus, it is important to clarify the responses of triploid *Populus* to drought in order to select outstanding genotypes/individuals exhibiting drought tolerance. Physiological responses and the molecular basis of water deprivation and recovery have been examined in many naturally diploid populations/genotypes (Souza *et al*. 2004, Yin *et al*. 2004, 2005), but not in the triploid *Populus*. In this study, we evaluated three triploid *Populus* populations with different heterozygosities after exposure to high temperatures during the megasporogenesis period of female parents (Wang *et al*. 2010, 2012). We hypothesized that the triploid *Populus* populations might be more resistant to water deprivation than the diploid population. A comparative photosynthetic, protective enzymatic, and osmoregulatory analysis of the integrated physiological responses to drought stress and recovery were performed to assess the degree of genotypic variation and to increase our understanding of the physiological mechanisms in triploid *Populus* under water deprivation.

Materials and methods

Plant material, experimental design, and harvest: Plant materials used for this study included three synthetic allotriploid (2n = 3x = 57) and one diploid (2n = 2x = 38) progeny population. *Populus pseudo-simonii* × *P. nigra* 'Zheyin3#' (2n = 2x = 38) and *P. beijingensis* (2n = 2x = 38) were used as the female and male parents, respectively. According to our previous studies, high-temperature exposure was conducted during the megasporogenesis period of female buds to induce first- (FDR) and second-division restitution (SDR) 2n megaspores (Wang *et al*. 2010, 2012). The FDR and SDR triploid populations (triploid-F and triploid-S) were generated from megaspore chromosome doubling. Another allotriploid progeny population was induced by embryo sac chromosome doubling *via* post-meiotic restitution (PMR) 2n eggs (triploid-P) (Dong *et al*. 2014). Diploid progenies from the same cross combination with untreated buds were defined as the control population. All progenies were grown in an open field in Shunyi District, Beijing, China. A total of 30 genotypes of each population were randomly selected for storage in sand in Guan Xian County, Shandong Province, China, in December 2013. In March 2014, ten cuttings (approximately 15 cm in length, 1-year old) of each genotype were cultivated in pots (23 cm upper diameter × 18 cm lower diameter × 25 cm depth) filled with soil (clay:sand:vermiculite, 2:2:1, v/v/v). The cuttings were grown in a natural environment until July 2014. Six healthy cuttings of approximately equal height for each genotype were used for the water stress experiments.

The experiment followed a complete randomized design using three replications. During the progressive water stress treatments (WS), three water contents were established at 50–55% (WS_1), 30–35% (WS_2), and 10–15% (WS_3) of field capacity (FC), which were defined as mild, moderate, and severe water stress, respectively. The well-watered treatment (WW), which was maintained at 80–85% FC, was treated as the control group (CK). To ensure the target field capacity of the WS, a total of ten random selected pots were weighed every day. After drought treatment, the materials were rewatered (RW) to 80–85% FC. The entire experiment was performed for two weeks, and each watering stage lasted 3–4 d to obtain instantaneous responses under the continuing natural drought and rewatering conditions. The correlations between the sample harvesting date and the relative soil water content (RSWC) are shown in Fig. 1S (*supplement available online*) and some meteorological data are presented in Table 1S (*supplement available online*).

Three replications of each watering stage were performed, and the samples were harvested 3 d after rewatering. Every fourth to seventh fully expanded functional leaf from the top of the stem was immediately frozen in liquid nitrogen and stored at −80°C to determine enzymatic activities and physiological traits. No rainfall was recorded during the experiment.

RWC and relative membrane permeability (RMP): RWC was measured in the fourth leaf from the top following the method described by Boyer (1969) with minor modifications. Leaves were weighed immediately after harvest to determine the fresh mass (FM). The turgid mass (TM) was then recorded after the leaves were transferred to de-ionized water and maintained in a dim environment for 4 h. Dry mass (DM) was measured after drying at 80°C for 24 h. RWC was calculated using the following equation: RWC % = (FM – DM)/(TM – DM) × 100.

A total of 0.5 g of the fourth leaf, which was cut into discs (0.8 cm in diameter), was used to determine the RMP according to Xiao *et al*. (2009). The initial electrical conductivity (C_1) was measured using a conductivity instrument (*LC116, Mettler-Toledo Instruments Co. Ltd*, Zurich, Switzerland) after placing the discs in de-ionized water for 1 h in a dim environment. The samples were boiled for 30 min to achieve ion leakage, after which they were cooled to room temperature, and the second measurement of electrical conductivity (C_2) was obtained. RMP was calculated as follows: RMP (%) = $C_1/C_2 \times 100$.

Gas exchange and chlorophyll (Chl) fluorescence: Gas exchange was determined on the fourth to sixth functional leaf from the top of the stem, using the *LI-6400-02B* portable photosynthesis system (*LiCor-6400*, *LiCor*, Lincoln, NE, USA), during serious water stress (20–23 July, 2014). Photosynthesis measured before water stress was used as a control. The measurements were made on sunny days at 08:30 – 11:30 h, with maintenance of the photosynthetic photon flux density at 1,400 μmol(photon) m^{-2} s^{-1} and CO_2 concentration at 400 μmol mol^{-1}, a leaf temperature of 35 ± 1°C, a relative air humidity of 60–65%, and a flow of 500 μmol mol^{-1}. Photosynthetic parameters including net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) were simultaneously measured, recorded, and calculated according to von Caemmerer and Farquhar (1981). The water-use efficiency (WUE) was calculated as WUE = P_N/E (Bierhuizen and Slatyer 1965).

The Chl fluorescence parameters and relative Chl content index (CCI) were measured on the same leaves in the afternoon after photosynthesis measurements using the *MD-500* chlorophyll fluorescence spectrometer (*Yi Zong Qi Technology Co. Ltd.*, Beijing, China) and *CCM-200 Plus* Chl content meter (*OPTI-Sciences Inc*., USA), respectively. For fluorescence induction measurements, excitation and extinction wavelengths were 470 and 735 nm, respectively, and the intensity of the excitation was 3,400 μmol(photon) m^{-2} s^{-1}. During the measurements, the maximum (F_m) and minimum (F_0) fluorescence yields of the dark-adapted leaves were determined. The variable fluorescence (F_v) was calculated as $F_v = F_m - F_0$ (Roháček 2002), and the maximal quantum yield of PSII photochemistry (F_v/F_m) and the potential photochemical efficiency of PSII (F_v/F_0) were also calculated.

MDA and Pro: Lipid peroxidation was determined by measuring MDA content using the method described by Hodges *et al*. (1999) with minor modifications. A total of 0.5 g powdered leaf tissue was homogenized in 10 ml of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 × *g* for 10 min. Next, 2 ml 0.6% (w/v) thiobarbituric acid in 10% TCA were mixed with 2 ml of supernatant. The mixed solution was incubated in boiling water for 30 min, after which the reaction was stopped by placing in an ice bath. The mixture was centrifuged at 10,000 × *g* for 10 min, and the absorbance of the supernatant was measured at 532, 600, and 440 nm using a spectrophotometer (*Ultrospec 6300 pro*, Cambridge, England). MDA content was expressed in nmol per FM.

Pro content was assessed using the method of Bates *et al*. (1973) with minor modifications. The powdered leaf (0.3 g) was homogenized in 8 ml of 3% (w/v) aqueous sulfosalicylic acid solution and then centrifuged at 10,000 × *g* for 10 min. The supernatant (2 ml) reacted with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid for 1 h at 100°C before termination *via* ice bath. The mixture was then extracted with 4 ml of toluene. The absorbance was measured at 520 nm (*Ultrospec 6300 pro*, Cambridge, England). And Pro content was expressed in μg per FM.

Antioxidant enzymes: The extracts used for SOD (EC 1.15.1.1), POD (EC: 1.11.1.7), and CAT (EC: 1.11.1.6) measurements were prepared by homogenizing the leaf powder (1 g) in 10 ml of 0.1 mM phosphate buffer (pH 7.2) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant was used for enzymatic determination. The soluble protein content of the supernatant was monitored using *Coomassie Brilliant Blue G-250* according to Bradford (1976), using bovine serum albumin as a standard.

SOD activity was assayed according to the method of Giannopolitis and Ries (1977) by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. The amount of enzyme inhibiting 50% photochemical reduction of nitroblue tetrazolium was defined as 1 unit of SOD at 560 nm. POD activity was determined by the oxidation of guaiacol with the presence of H_2O_2 as described by Lin and Wang (2002). The absorbance of the mixture was measured at 470 nm by using a spectrophotometer (*Ultrospec 6300 pro*, Cambridge, England). POD activity was expressed in $mmol^{-1}$(product) min^{-1} g^{-1}(FM). CAT activity was determined using the method described by Carrillo *et al*. (1992). The reaction was started by the

addition of 0.1 mM phosphate buffer (pH 7.2) and 20 mM H_2O_2 to the enzyme extract. The breakdown of H_2O_2 at 240 nm was monitored using a spectrophotometer. CAT activity was expressed as mmol(H_2O_2) min^{-1} g^{-1}(FM).

Statistical analysis: Two-way analysis of variance (*ANOVA*), using the water stress treatments and populations as two independent factors, was applied to determine the differences in parameters (gas exchange, Chl fluorescence, RWC, RMP, MDA, Pro, and enzyme activities) between populations at each watering stage. The analyses were performed using the general linear model *ANOVA* procedure. If significance (p<0.05) was observed, then a *post hoc* comparison of the least significant difference (LSD) was used to determine the differences between populations. The within-population coefficient of variation (CV) was calculated as the standard deviation (SD) divided by the mean. *Pearson*'s correlation coefficients for all physiological traits of the genotypes overall were calculated to determine the correlations between any two traits under WW and WS_3. *SPSS 20.0* (*SPSS Inc*., Chicago, IL, USA) was used to perform the statistical tests and determine descriptive statistics. A principal component analysis (PCA) was performed on the trait values from four populations using *R* statistical software to represent physiological diversity under WW, WS_3, and RW. The original variables were homogeneous prior to PCA.

Results

Relative water content and electrolyte leakage: Both the RWC and electrolyte leakage were significantly affected by the populations, watering treatments, and the population × watering treatment interaction (Table 1). The RWCs of leaves from the four populations showed evident decreases under water stress (Fig. 1*A*). The RWCs slightly decreased under WS_1, but they were insignificantly different from WW. The RWCs decreased sharply and significantly under WS_2 and WS_3. Compared to the diploid population, the triploid populations (especially the triploid-F population) showed higher RWCs under WS_2 and WS_3. The electrolyte leakage, expressed as the RMP, increased with increasing water deprivation. The RMP of the stressed populations increased by 197, 123, 149, and 143% in diploid, triploid-F, -S, and -P populations under WS_3, respectively (Table 1). Both RWC and RMP in the four populations approximately recovered to their WS_1 levels 3 d after rewatering.

Photosynthesis and Chl fluorescence: The gas-exchange parameters in the four populations significantly decreased by water stress (Fig. 2). The effect of population was not observed under WUE (Table 1). F_v/F_m was significantly affected by the interaction between population and watering treatment (Table 1). The diploid population showed the greatest difference with triploid-F population compared to the other two triploid populations under WW. Reductions of the photosynthetic and Chl fluorescence parameters, excluding WUE and CCI, were lesser in the triploid populations than those in the diploid population (Table 1).

Malondialdehyde and free proline: Contents of MDA and Pro increased in the four populations with the severity of water stress, but there were apparent differences between the four populations (Fig. 3). The effects of population, watering treatment, and population × watering

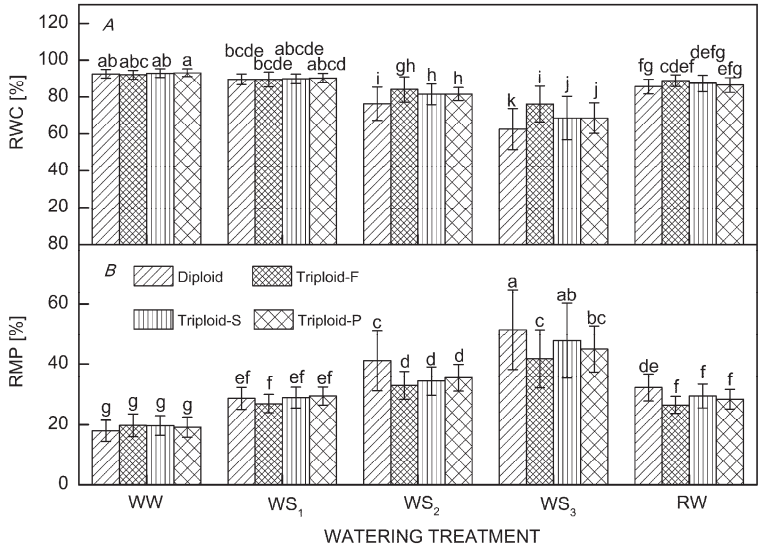

Fig. 1. Effect of watering on relative water content (RWC, %) (*A*) and relative membrane permeability (RMP, %) (*B*) in leaves from the four *Populus* populations. The bars indicate means ± SD (n = 3). *Different letters* on the bars indicate significant differences. WW – well-watered (80–85%); WS_1 – mild water stress (50–55%); WS_2 – moderate water stress (30–35%); WS_3 – severe water stress (10–15%); RW – rewatering (80–85%).

Table 1. Fold changes under severe water stress (WS_3) compared with well-watered (WW) conditions in the four populations. "+" mean the value of the parameter increased under WS_3, "–" mean value of the parameter decreased under WS_3. F_p – population effect; F_w – watering treatment effect; $F_{p \times w}$ – population × watering treatment interaction effect; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns – not significant; SD – standard deviation; P_N – net photosynthetic rate; g_s – stomatal conductance; C_i – intercellular carbon dioxide concentration; E – transpiration rate; WUE – water use efficiency; CCI – chlorophyll content index; F_v/F_m –maximal quantum yield of PSII photochemistry; F_v/F_0 – potential photochemical efficiency of PSII; RWC – relative water content; RMP – relative membrane permeability; MDA – malondialdehyde; Pro – free proline; SOD – superoxide dismutase; POD – peroxidase; CAT – catalase. Units: P_N [μmol(CO_2) m^{-2} s^{-1}]; g_s [mol(H_2O) m^{-2} s^{-1}]; C_i [μmol(CO_2) mol^{-1}]; E [mmol(H_2O) m^{-2} s^{-1}]; WUE [mol(CO_2) mol(H_2O)$^{-1}$]; RWC [%];RMP [%];MDA [nmol g^{-1} (FM)]; Pro [μg g^{-1}(FM)]; protein [μg ml^{-1}]; SOD [U g^{-1}(FM)]; POD [$mmol^{-1}$(guaiacol) min^{-1} g^{-1}(FM)]; CAT [$mmol^{-1}$(H_2O_2) min^{-1} g^{-1}(FM)].

Population		P_N	g_s	C_i	E	WUE	CCI	F_v/F_m	F_v/F_0	RWC	RMP	MDA	Pro	Protein	SOD	POD	CAT
		–	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+
Diploid	Mean	0.599	0.697	0.652	0.569	0.076	0.391	0.233	0.262	0.324	1.973	3.080	7.186	0.522	0.719	1.250	0.439
	SD	0.093	0.082	0.060	0.090	0.129	0.049	0.044	0.113	0.120	0.959	1.093	2.802	0.084	0.300	0.753	0.310
Triploid-F	Mean	0.585	0.652	0.642	0.516	0.124	0.407	0.133	0.234	0.173	1.226	2.141	10.960	0.445	1.611	1.458	0.680
	SD	0.080	0.071	0.058	0.079	0.255	0.059	0.058	0.083	0.105	0.800	0.712	3.541	0.090	0.759	0.881	0.452
Triploid-S	Mean	0.598	0.662	0.648	0.510	0.177	0.455	0.153	0.250	0.262	1.494	2.583	9.374	0.432	1.323	1.234	0.562
	SD	0.076	0.090	0.076	0.053	0.174	0.048	0.063	0.079	0.125	0.818	0.953	2.902	0.102	0.739	0.792	0.284
Triploid-P	Mean	0.593	0.618	0.642	0.524	0.154	0.470	0.173	0.241	0.264	1.432	2.433	8.391	0.459	1.160	1.555	0.498
	SD	0.090	0.100	0.081	0.047	0.143	0.049	0.053	0.067	0.089	0.645	1.344	2.573	0.100	1.105	1.179	0.247
F_p		***	***	*	*	ns	***	***	**	***	***	***	***	**	***	***	***
F_w		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
$F_{p \times w}$		ns	ns	ns	ns	ns	ns	***	ns	***	***	***	***	**	***	ns	ns

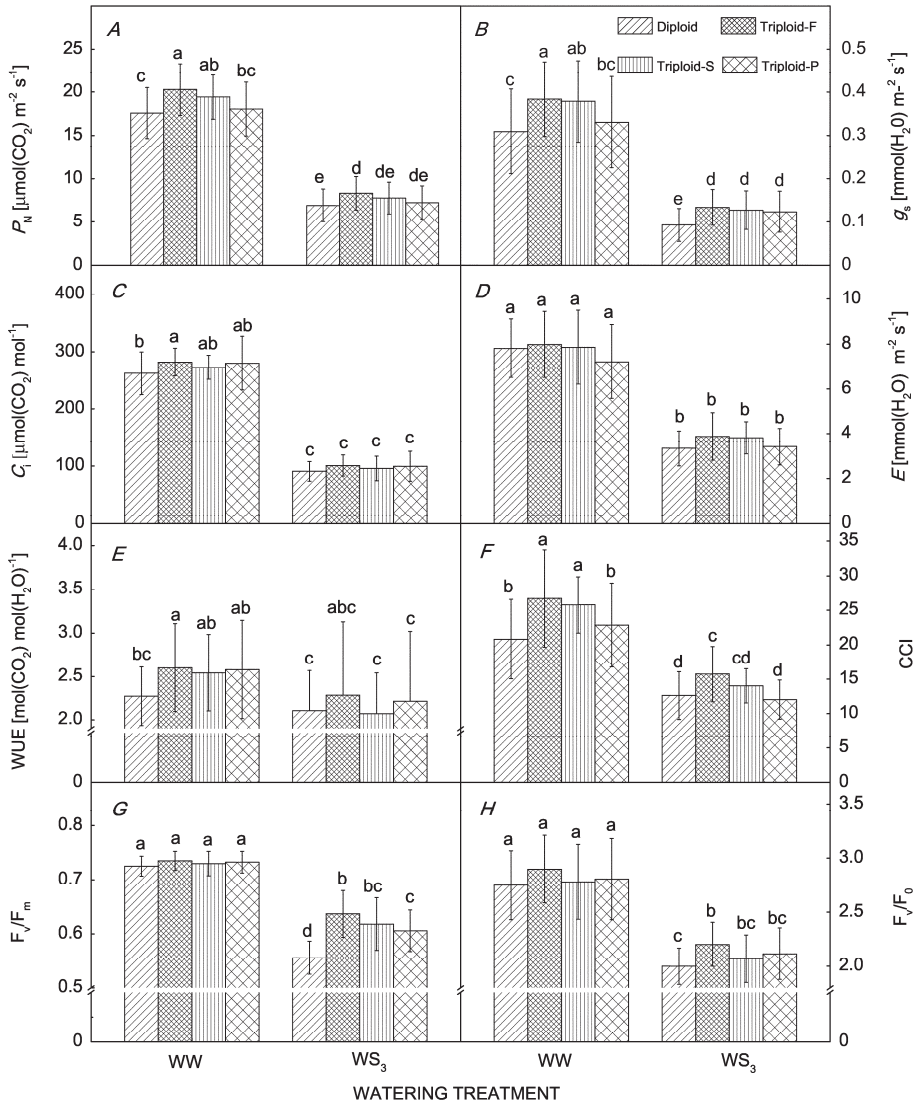

Fig. 2. Effect of watering on photosynthesis and chlorophyll fluorescence parameters in the four *Populus* populations. The bars indicate means ± SD (n = 3). *Different letters* on the bars indicate significant differences. WW – well-watered (80–85%); WS_3 – severe water stress (10–15%); P_N – net photosynthetic rate; g_s – stomatal conductance; C_i – intercellular carbon dioxide concentration; E – transpiration rate; WUE – water-use efficiency; CCI – chlorophyll content index; F_v/F_m – maximal quantum yield of PSII photochemistry; F_v/F_0 – potential photochemical efficiency of PSII.

treatments interaction showed significant differences (Table 1). In the diploid population, the MDA content increased by 308% under WS_3 compared with WW, while the increases were 214, 258, and 243% in the triploid-F, -S, and -P populations, respectively (Table 1). The greatest Pro accumulation was in the triploid-F population (11-fold increase compared to a 7.2-fold increase in the diploid population; Table 1). Significant differences in both MDA and Pro contents were observed under WS_2 and WS_3, while there were no differences under WS_1 (Fig. 3). MDA and Pro contents of the four populations recovered to their approximate concentrations seen under WS_1 and showed no differences 3 d after rewatering.

Antioxidative defense systems: Population and watering treatment significantly affected soluble protein content and antioxidative enzyme (SOD, POD, and CAT) activities (Table 1). The population × watering treatment interaction effect showed significance for soluble protein content and SOD activity, but not for POD or CAT activities (Table 1). The soluble protein contents of the four populations obviously decreased with increased water stress (Fig. 4*A*). It partially recovered after rewatering. The water shortage significantly activated the antioxidant system of leaves in the four populations. SOD, POD, and CAT showed similar variations with the intensified watering treatments and recovered to prior-stress levels after rewatering (Fig. 4*B–D*). However, compared to the diploid population, the triploid populations showed the higher enzyme activities under WS_3 (Table 1).

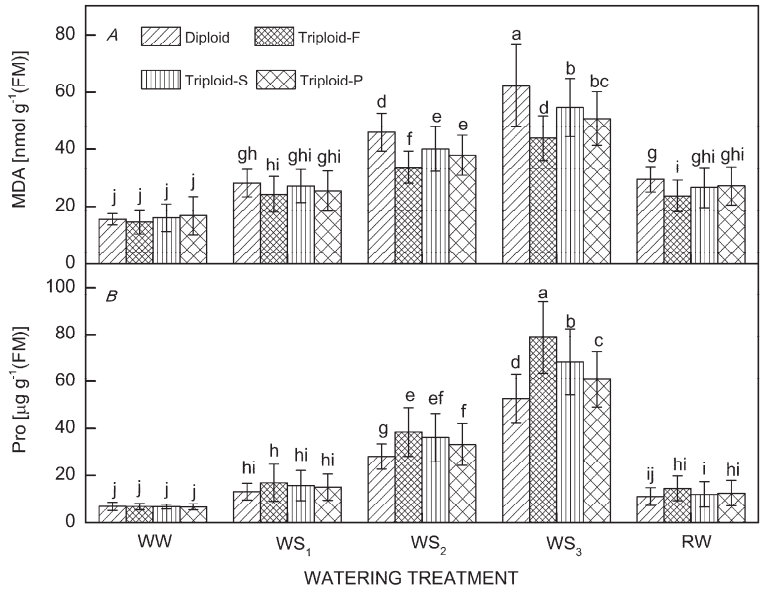

Fig. 3. Effects of watering on malondialdehyde (MDA) (*A*) and free proline (Pro) (*B*) contents in the four *Populus* populations. The bars indicate means ± SD (n = 3). *Different letters* on the bars indicate significant differences. WW – well-watered (80–85%); WS_1 – mild water stress (50–55%); WS_2 – moderate water stress (30–35%); WS_3 – severe water stress (10–15%); RW – rewatering (80–85%).

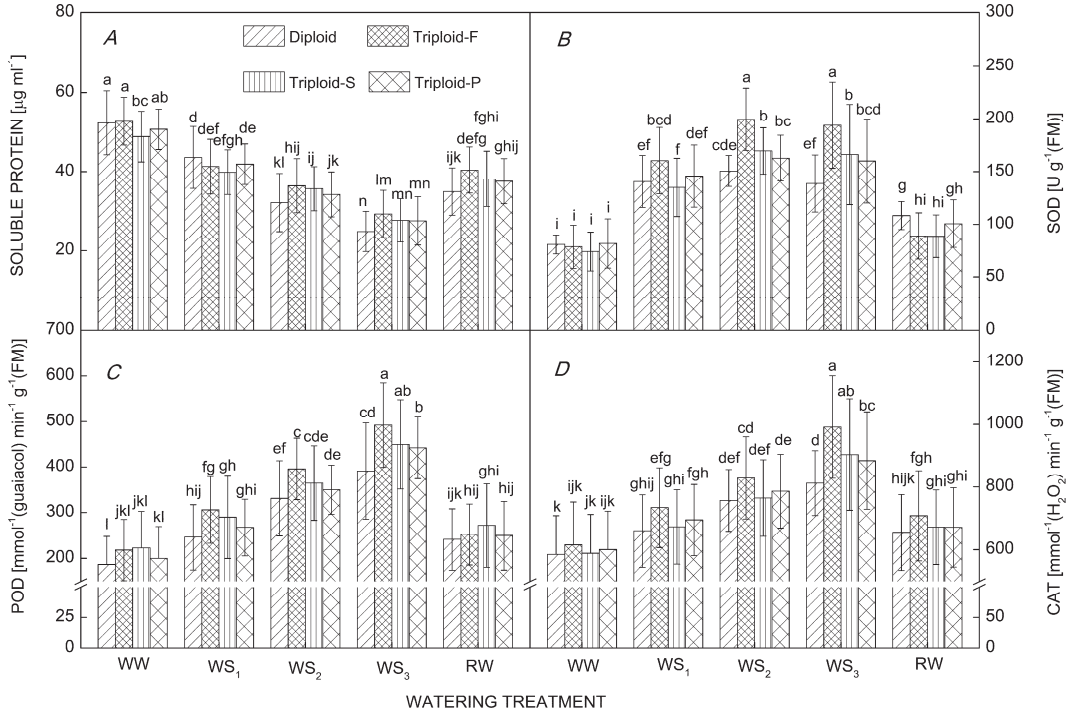

Fig. 4. Effects of watering on soluble protein content (*A*), superoxide dismutase (SOD) (*B*), peroxidase (POD) (*C*), and catalase (CAT) (*D*) activities in the four *Populus* populations. The bars indicate means ± SD (n = 3). *Different letters* on the bars indicate significant differences. WW – well-watered (80–85%); WS_1 – mild water stress (50–55%); WS_2 – moderate water stress (30–35%); WS_3 – severe water stress (10–15%); RW – rewatering (80–85%).

Coefficients of variation within populations, correlations among genotypes, and principal component analysis (PCA) of physiological responses: Variability was found within each population under WW and WS_3 (Table 2). POD and RWC had the highest and lowest CVs under WW, and triploid population had larger values than diploid in most of the parameters evaluated. The physiological traits of Chl fluorescence, cell membrane injury, and enzyme activities were not correlated under WW (Table 3). In addition, each two of photosynthetic parameters showed significant positive correlations (Table 3). However, significant correlations existed between photosynthesis and enzyme activities under WS_3, indicative of an obvious water stress response in the progenies (Table 3).

The scatter diagrams, derived from PCA under WW,

Table 2. Range (coefficient of variation) for physiological parameters under well-watered (WW) and severe water stress (WS_3) in the four populations. P_N – net photosynthetic rate; g_s – stomatal conductance; C_i – intercellular carbon dioxide concentration; E – transpiration rate; WUE – water use efficiency; CCI – chlorophyll content index; F_v/F_m –maximal quantum yield of PSII photochemistry; F_v/F_0–potential photochemical efficiency of PSII; RWC – relative water content; RMP – relative membrane permeability; MDA – malondialdehyde; Pro – free proline; SOD – superoxide dismutase; POD – peroxidase; CAT – catalase.

Parameter	Water treatment	Diploid	Triploid-F	Triploid-S	Triploid-P	Total
P_N [μmol(CO_2) m^{-2} s^{-1}]	WW	12.52–22.34 (16.7)	13.16–25.12 (14.7)	13.63–23.66 (13.2)	11.38–23.71 (17.1)	11.38–25.12 (16.3)
	WS_3	4.03–10.9 (27.2)	4.61–11.97 (23.3)	4.61–12.07 (23.6)	4.21–11.52 (26.7)	4.03–12.07 (25.7)
g_s [mol(H_2O) m^{-2} s^{-1}]	WW	0.149–0.508 (31.6)	0.156–0.524 (22.5)	0.190–0.583 (24.9)	0.154–0.522 (32.0)	0.149–0.583 (28.6)
	WS_3	0.031–0.183 (41.1)	0.058–0.212 (30.8)	0.043–0.225 (35.0)	0.063–0.218 (38.5)	0.031–0.225 (37.9)
C_i [μmol(CO_2) mol^{-1}]	WW	197.1–339.3 (14.2)	229.2–326.5 (8.5)	228.0–324.0 (7.7)	196.4–372.0 (16.7)	196.4–372.0 (12.5)
	WS_3	65.7–126.6 (18.7)	72.9–147.6 (18.0)	60.4–136.1 (22.1)	59.5–154.8 (26.1)	59.5–154.8 (21.7)
E [mmol(H_2O) m^{-2} s^{-1}]	WW	5.36–10.13 (16.6)	5.56–11.12 (18.3)	4.71–11.07 (20.8)	4.38–10.55 (22.7)	4.38–11.12 (19.8)
	WS_3	2.15–5.13 (23.2)	2.12–6.45 (27.4)	2.27–5.45 (18.8)	2.15–5.59 (23.8)	2.12–6.45 (24.2)
WUE [mol(CO_2) mol(H_2O)$^{-1}$]	WW	1.55–3.25 (14.9)	1.89–3.66 (19.4)	1.97–3.74 (17.3)	1.89–4.46 (21.9)	1.55–4.46 (19.3)
	WS_3	1.23–3.27 (22.0)	1.17–5.46 (37.0)	0.95–3.29 (22.7)	1.04–5.37 (36.2)	0.95–5.46 (30.7)
CCI	WW	8.27–30.10 (27.5)	15.53–42.17 (26.3)	15.60–32.93 (15.8)	15.07–37.77 (26.5)	8.27–42.17 (25.8)
	WS_3	5.21–19.74 (27.5)	8.23–23.93 (25.5)	8.27–18.62 (17.7)	7.99–18.26 (23.7)	5.21–23.93 (25.9)
F_v/F_m	WW	0.70–0.77 (2.5)	0.69–0.77 (2.4)	0.69–0.77 (3.1)	0.70–0.77 (2.7)	0.69–0.77 (2.7)
	WS_3	0.5–0.61 (5.5)	0.56–0.74 (6.8)	0.54–0.69 (7.9)	0.52–0.68 (6.3)	0.5–0.74 (8.3)
F_v/F_0	WW	2.07–3.63 (11.6)	2.37–3.55 (10.8)	2.09–3.55 (12.5)	2.01–3.60 (13.4)	2.01–3.63 (12.1)
	WS_3	1.73–2.34 (8.4)	1.89–2.62 (9.2)	1.75–2.55 (10.6)	1.74–2.64 (11.2)	1.73–2.64 (10.4)
RWC [%]	WW	88.54–99.33 (2.5)	86.47–96.96 (2.7)	89.63–98.66 (2.5)	89.82–97.26 (2.2)	86.47–99.33 (2.5)
	WS_3	38.57–80.33 (17.8)	52.06–90.36 (12.8)	31.87–86.72 (17.2)	48.33–80.43 (12.1)	31.87–90.36 (16.4)
RMP [%]	WW	12.31–25.10 (19.5)	14.42–29.00 (19.2)	14.05–31.88 (16.6)	13.74–28.51 (17.6)	12.31–31.88 (18.4)
	WS_3	32.06–71.3 (25.9)	30.08–64.75 (23.0)	33.41–88.54 (26.1)	30.12–63.96 (17.2)	30.08–88.54 (24.6)
MDA [nmol g^{-1}(FM)]	WW	12.41–19.84 (12.7)	9.12–26.77 (28.9)	8.49–27.46 (29.9)	7.06–29.35 (39.3)	7.06–29.35 (29.4)
	WS_3	41.23–98.96 (23.3)	28.27–58.51 (17.7)	36.49–78.9 (18.8)	29.55–66.75 (18.7)	28.27–98.96 (23.8)
Pro [μg g^{-1}(FM)]	WW	3.14–9.54 (25.0)	4.45–10.46 (19.9)	4.55–8.39 (15.5)	4.36–9.01 (18.7)	3.14–10.46 (19.9)
	WS_3	36.75–75.72 (19.8)	49.92–119.54 (19.2)	42.69–97.78 (20.5)	44.29–86.32 (19.3)	36.75–119.54 (24.6)
Protein [μg ml^{-1}]	WW	39.46–67.42 (15.4)	37.99–65.85 (11.4)	36.20–64.79 (13.1)	40.20–60.93 (10.1)	36.20–67.42 (12.9)
	WS_3	17.15–34.41 (20.4)	17.74–41.23 (20.5)	18.28–38.95 (19.9)	16.72–37.49 (22.2)	16.72–41.23 (21.4)
SOD [U g^{-1}(FM)]	WW	65.23–97.23 (11.2)	32.67–114.79 (26.0)	43.18–119.36 (24.9)	32.84–129.46 (28.6)	32.67–129.46 (23.6)
	WS_3	93.48–197.1 (19.6)	119.73–266.64 (21.0)	82.44–238.07 (28.6)	104.16–233.08 (24.9)	82.44–266.64 (26.6)
POD [$mmol^{-1}$(guaiacol) min^{-1} g^{-1}(FM)]	WW	55.20–293.50 (32.3)	95.60–366.70 (30.4)	90.85–415.20 (35.9)	67.70–356.20 (34.5)	55.20–415.20 (33.7)
	WS_3	172.65–595.87 (27.1)	345.05–698.95 (18.9)	283.1–653.9 (21.8)	317.65–535.35 (15.3)	172.65–698.95 (22.1)
CAT [$mmol^{-1}$(H_2O_2) min^{-1} g^{-1}(FM)]	WW	344.07–759.10 (21.0)	266.76–842.40 (22.2)	412.19–899.40 (21.0)	404.02–869.40 (20.3)	266.76–899.40 (21.0)
	WS_3	613.7–1,020.76 (12.8)	633.4–1,350.8 (16.5)	577.43–1,257.27 (19.7)	583.9–1,181.94 (17.6)	577.43–1,350.8 (18.3)

Table 3. Pearson´s correlation coefficients for pairs of physiological traits for all genotypes under well–watered (WW) (n = 120, *bottom left*) and severe water stress (WS_3) conditions (n = 120, *top right*) of *Populus*. P_N – net photosynthetic rate; g_s – stomatal conductance; C_i – intercellular carbon dioxide concentration; E – transpiration rate; WUE – water-use efficiency; CCI – chlorophyll content index; F_v/F_m –maximal quantum yield of PSII photochemistry; F_v/F_0 – potential photochemical efficiency of PSII; RWC – relative water content; RMP – relative membrane permeability; MDA – malondialdehyde; Pro – free proline; SOD – superoxide dismutase; POD – peroxidase; CAT – catalase.

	P_N	g_s	C_i	E	WUE	CCI	F_v/F_m	F_v/F_0	RWC	RMP	MDA	Pro	Protein	SOD	POD	CAT
P_N	1	0.835**	0.762**	0.378**	0.595**	0.609**	0.637**	0.529**	0.591**	–0.598**	–0.461**	0.662**	0.454**	0.661**	0.621**	0.496**
g_s	0.837**	1	0.728**	0.372**	0.460**	0.553**	0.624**	0431**	0.498**	–0.491**	–0.433**	0.587**	0.385**	0.607**	0.430**	0.372**
C_i	0.469**	0.545**	1	0.195*	0.512**	0.345**	0.652**	0.579**	0.517**	–0.582**	–0.451**	0.566**	0.518**	0.707**	0.553**	0.481**
E	0.542**	0.417**	0.137	1	–0.461**	0.248**	0.268**	0.136	0.229*	–0.303**	–0.221*	0.207*	0.209*	0.240**	0.206*	0.262**
WUE	0.317**	0.305**	0.250**	–0.602**	1	0.308**	0.366**	0.432**	0.368**	–0.310**	–0.255**	0.444**	0.282**	0.430**	0.409**	0.223*
CCI	0.824**	0.683**	0.403**	0.331**	0.353**	1	0.314**	0.130	0.247**	–0.136	–0.228*	0.393**	0.027	0.334**	0.263**	0.199**
F_v/F_m	0.005	0.028	0.101	–0.077	0.106	–0.027	1	0.705**	0.647**	–0.631**	–0.634**	0.694**	0.460**	0.744**	0.631**	0.614**
F_v/F_0	0.008	–0.020	0.106	–0.112	0.157	0.005	0.800**	1	0.487**	–0.554**	–0.423**	0.578**	0.497**	0.602**	0.549**	0.423**
RWC	0.020	–0.009	0.023	0.028	–0.041	0.092	–0.108	–0.080	1	–0.794**	–0.572**	0.557**	0.456**	0.598**	0.612**	0.490**
RMP	–0.043	–0.051	–0.008	–0.017	0.007	0.002	–0.102	–0.180*	–0.109	1	0.574**	–0.493**	–0.570**	–0.599**	–0.647**	–0.542**
MDA	–0.029	–0.046	0.002	–0.124	0.177	–0.043	0.113	0.029	0.044	–0.036	1	–0.519**	–0.449**	–0.545**	–0.495**	–0.424**
Pro	0.075	0.048	–0.016	–0.003	0.082	0.039	–0.027	–0.006	–0.028	–0.091	0.176	1	0.451**	0.746**	0.618**	0.501**
Protein	–0.036	–0.105	0.068	0.032	–0.065	–0.117	0.020	0.119	–0.029	–0.228*	0.023	–0.103	1	0.417**	0.496**	0.501**
SOD	0.028	–0.014	0.159	0.061	–0.027	0.007	0.057	0.073	–0.043	0.095	–0.036	–0.113	0.172	1	0.623**	0.57**
POD	0.188*	0.058	0.034	0.056	0.084	0.238**	–0.117	–0.147	0.031	–0.024	0.032	0.191*	0.088	–0.077	1	0.514**
CAT	–0.009	–0.089	0.001	0.026	–0.061	0.009	–0.115	–0.056	–0.068	–0.003	–0.116	0.175	0.224*	0.241**	0.173	1

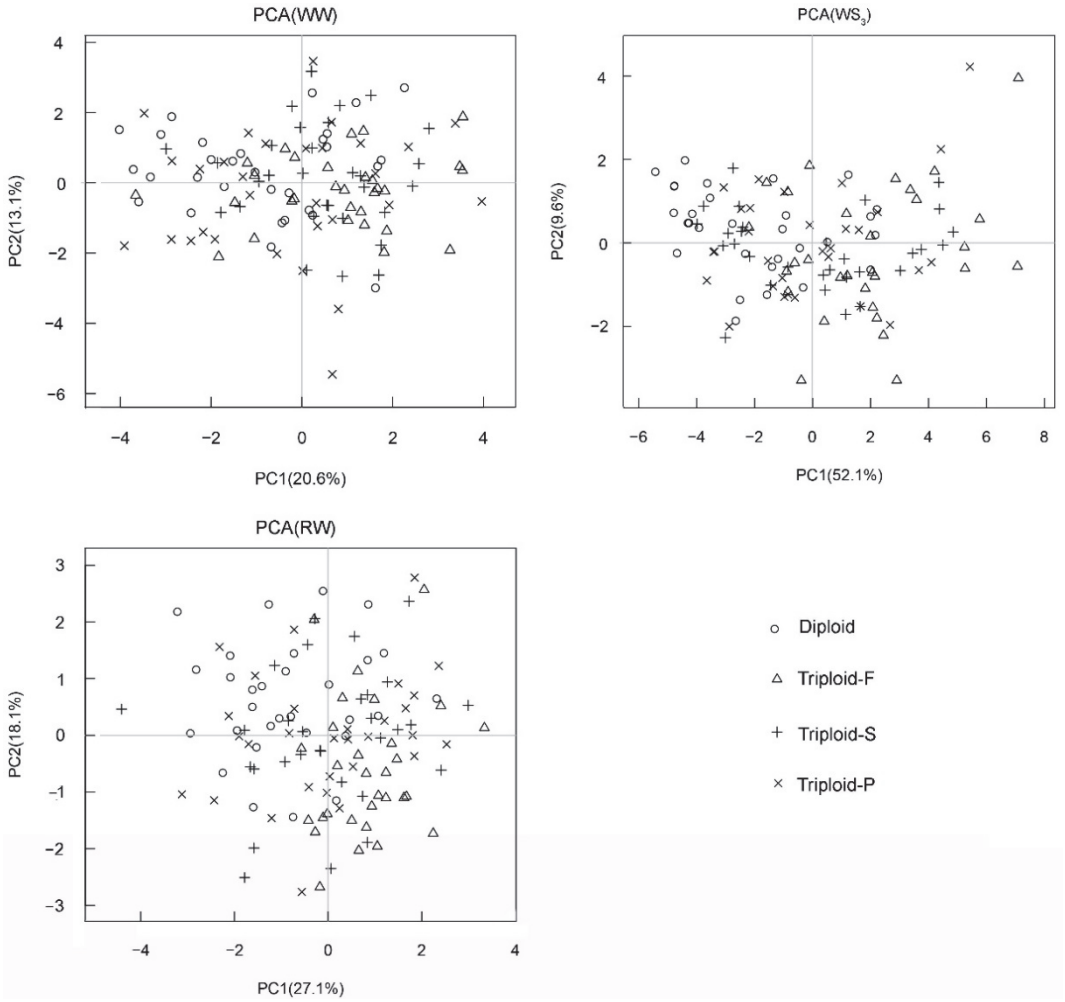

Fig. 5. The principal component analysis (PCA) results for well-watered (WW), severe water stress (WS_3), and rewatering (RW) conditions in 120 genotypes of *Populus*

WS_3, and RW conditions, showed a wide variability among the hybrid progenies following severe water stress and rewatering (Fig. 5). The first two principal components contributed 33.7, 61.7, and 45.2% of the diversity under WW, WS_3, and RW, respectively (Table 2S, *supplement available online*). P_N showed the highest variable loading in PC1, followed by g_s and CCI, under WW. *E* showed the highest variable loading in PC2, which had negative loadings for F_v/F_m and F_v/F_0. Under WS_3, the loadings of PC1 did not differ between variables, indicating that water stress influenced all physiological traits. Upon rewatering of the samples, RWC and RMP showed the highest variable loadings in PC1 and PC2, respectively.

Discussion

The adaptations of plants to water shortage conditions are comprehensive and complex processes widely affected by inherent drought tolerance mechanisms and external environmental conditions (Bray 1997). In this study, the physiological responses of four *Populus* populations, including three triploid populations with different 2n gamete origins and one diploid population, under continuous drought treatment were investigated. The leaf RWC is a common indicator of the sensitivity of plants to dehydration (Sánchez-Rodríguez *et al*. 2010). Compared to the diploid population, the three triploid populations showed significantly higher leaf RWCs under WS_2 and WS_3 (Fig. 1*A*), indicating that the triploid populations, especially triploid-F, exhibited better water usage than the diploid population under moderate and severe water stress. According to previous studies, drought-tolerant species/ genotypes show a lesser decrease in RWC under water stress compared to drought-sensitive species (Xiao *et al*. 2008, Yin *et al*. 2009).

Water stress significantly decreased photosynthesis,

Chl contents, and Chl fluorescence parameters in the four populations in our study (Fig. 2). Under WS treatment, the higher g_s was probably one reason for the higher P_N in the triploid populations, since stomatal limitation is coupled with photosynthetic reduction (Cornic 2000). Reducing g_s under WS is considered an adaptation mechanism to reduce water loss in plants, followed by a reduction in E due to stomatal control (Karimi *et al*. 2015). The present study showed that the triploid populations, compared to the diploid population, showed lesser decreases in g_s, E, and P_N under WS compared with WW (Table 1). This indicated that photosynthesis was more sensitive to water deprivation in diploids than in triploids. Nonstomatal factors limited photosynthesis at the WS_3 stage (Fig. 2). Under severe WS, the capacity of chloroplasts to fix CO_2 affects photosynthesis more than it does increased stomatal diffusive resistance (Bota *et al*. 2004). One of the factors affecting CO_2 fixation under drought is the inhibition of photochemical processes usually during severe water stress and at low RWC. Earlier studies showed that Chl contents typically declined under water stress because of their slow synthesis or rapid breakdown (Majumdar *et al*. 1991). However, our results showed that CCI decreased less in the diploid than that in the triploid populations (Table 1). Thus, the Rubisco and chlorophyllase activities may play important roles in the adaptability to water stress (Mihailović *et al*. 1997, Lawlor and Cornic 2002, Chaves *et al*. 2003, Bota *et al*. 2004). As an indicator of the photochemical efficiency of electron transport in PSII, Chl fluorescence can be used to indicate the extent of water stress damaging the photosynthetic apparatus (Roháček 2002). It has been shown that F_v/F_m is highly stable in many species in normal habitats but decreases sharply under biotic and abiotic stress conditions (Björkman and Demmig 1987). The F_v/F_m showed no differences under WW, while sharp decreases were observed under WS_3 (Fig. 2*G*), suggesting the damage of PSII. These data are consistent with those of other plant species, including *Triticum aestivum* L. (Kong *et al*. 2016). The reductions of F_v/F_m and F_v/F_0 were lesser in triploid populations than those in the diploid population under WS in our study (Fig. 2*G*,*H*), which indicated that the triploid populations, especially triploid-F, provided stronger photoprotection against water deprivation than that of diploid population.

Moreover, another potentially important mechanism of drought tolerance is osmotic adjustment, which can be achieved by the accumulation of compatible solutes, such as soluble sugars, Pro, and amino acids (Misra and Gupta 2005). Pro has been considered a major hydroxyl radical scavenger that protects plasma membrane integrity and prevents protein denaturation under drought stress (Ain-Lhout *et al*. 2001, Bartels and Sunkar 2005). In the present study, the contents of Pro significantly increased in the four populations under prolonged drought (Fig. 3*B*). It can be interpreted as a mechanism for lowering osmotic potential. Futhermore, diploids had the lowest RWCs under WS_2 and WS_3. However, the triploid-F population had higher contents of Pro than that of the diploid population under WS_2 and WS_3, which is indicative of a greater ability to scavenge free radicals in triploid compared to diploid populations. The significantly different levels of osmotic adjustment among the four populations may explain their different tolerance to drought.

Drought stress also resulted in serious damage of cellular membranes in all four populations, as indicated by electrolyte leakage and increased MDA contents after sustained water deprivation in our study (Figs. 1*B*, 3*A*). As an indicator of cell membrane damage, the magnitude of increase of the MDA content, a product of peroxidation by ROS, which affects phospholipids, has been used to determine the extent of damage caused by water deprivation (Xu *et al*. 2006, Saneoka *et al*. 2004). The significantly greater accumulation and increased rate of RMP and MDA in the diploid compared to triploid populations indicated that the triploid population exhibited less damage and more tolerance to drought. Drought-tolerant species including polyploid *Lonicera japonica* (Li *et al*. 2009) and *P. cathayana* Rehder accumulate less MDA and leak electrolytes (Xiao *et al*. 2008). The continuous increases in RMP and MDA under sustained water stress indicated that antioxidative protection was not sufficient to prevent cell membrane damage caused by ROS. Higher MDA contents under drought stress may result from the downregulated activity of antioxidative enzymes (Selote and Khanna-Chopra 2010), which was further supported in the present study by the enzyme activities and significantly negative correlation between MDA and enzyme activities under WS_3. In our study, enzymatic responses to ROS reflected ability to scavenge free radicals and to avoid damage from the oxidative stress caused by water stress in the four populations. There were significant differences in the activities of SOD, POD, and CAT between each stress stage (Fig. 4), but the higher enzyme rates in the triploid-F population (Table 1) suggested that triploid populations showed a stronger ability to resist to ROS and a greater tolerance to water deprivation. Increased enzyme activities have also been reported in wheat (Shao *et al*. 2005) and maize (Jiang and Zhang 2002).

Physiological variation was detected within the populations tested in this study (Table 2). Most parameters showed larger CVs under WS_3 compared with WW, indicating a genotype-based adaptive response to water stress. Triploid genotypes were more tolerant to water deficit than that of diploids in terms of peak values within populations. However, overlaps between populations were observed for each parameter (Table 2), indicating that some triploid genotypes were less resistant than the diploid genotypes. In *Avena* species, which have different ploidy types, some diploid species had a stronger tolerance to drought than tetraploid and hexaploid species (Peng *et al*. 2011b).

In conclusion, water stress caused a serie of adaptive

physiological effects in the four populations, including membrane damage, decreased RWC, photosynthesis, and chlorophyll fluorescence parameters, and increased antioxidant enzymatic activity. Notably, the effects of the physiological responses became increasingly obvious with increasing drought. The triploid-F population was more resistant to water deficits than the diploid population, followed by the triploid-S and triploid-P populations. The triploid-F population showed more substantial enhancement in the antioxidant enzyme activities, lesser membrane damage, and a lower decline in photoprotective activity and leaf RWC with increasing water stress intensity. However, some triploid genotypes were less resistant to drought than diploids due to large intra-population overlap.

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