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Alteration in chlorophyll fluorescence, lipid peroxidation and antioxidant enzymes activities in hybrid ramie (*Boehmeria nivea* L.) under drought stress

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Abstract

Ramie (*Boehmeria nivea* L.) is one of the major foreign exchange earning crops in China due to the high quality textile of its fine fiber. However, seasonal drought has been becoming a major limiting factor for the fiber yield, especially under global climate change. In this study, the physiological response of ramie hybrid to different drought stress levels was investigated to evaluate its drought resistance. Ramie hybrid is two-line hybrid ramie variety developed by male sterile line and a restorer line. Ramie hybrid varieties have higher yield potential and lower production cost compared to conventional varieties. A pot experiment was carried out in a greenhouse at Chongqing, China. Hybrid ramie cultivar was subjected to four water levels (75%-80%, 60%-65%, 45%-50% and 30%-35% of field capacity), which were represented as the well-watered, mild, moderate and severe drought stress conditions. These treatments were conducted at vigorous growth stage for 10, 15 and 20 days intervals. After imposition of the treatments, chlorophyll fluorescence, relative water contents (RWC), protein, proline, malonialdehyde (MDA) and enzymatic antioxidants were assessed on the 10^{th}, 15^{th} and 20^{th} day after treatments. Results indicated that progressive water stress caused a substantial decrease in maximum quantum efficiency of PSII photochemistry (Fv/Fm), quantum efficiency of open PSII reaction centers (Fv'/Fm'), quantum yield of PSII (Φ_{PSII}), photochemical quenching (qP) and electron transport rate of PSII (ETR) while non-photochemical quenching (NPQ) changed conversely. The improvement of the concentrations of MDA, proline and the activity of superoxide dismutase (SOD) and peroxidase (POD) were found in the intensified water stress, while RWC and soluble protein substantially reduced. At the beginning of stress, activities of catalase (CAT) increased but then decreased under severe drought stress. All above results showed that hybrid ramie plants responded to water limiting conditions by a strategy of changing physiological and biochemical parameters. However, these changes were closely related to the intensity and duration of the drought stress. In total, hybrid ramie was tolerant to drought and our results may help growers to improve the hybrid ramie cultivar production and management in the seasonal drought condition.

Keywords: Drought stress, chlorophyll fluorescence, relative water contents, protein, proline, malonialdehyde, antioxidant enzymes, hybrid ramie.
Abbreviations: CAT_catalase; ETR_electron transport rate of PSII; Fv/Fm_maximum quantum efficiency of PSII photochemistry; Fv'/Fm'_quantum efficiency of open PSII reaction centers; MDA_malonialdehyde; NPQ_ non-photochemical quenching; POD_ peroxidase; PSII_photosystem II; Φ_{PSII}_quantum yield of PSII; qP_photochemical quenching; RWC_relative water contents ; SOD_ superoxide dismutase.

Introduction

Water deficit is a serious agronomic problem and one of the most important factors limiting crop production worldwide. It is well known that drought stress impairs numerous metabolic and physiological processes in plants (Mahajan and Tuteja, 2005). Water stress could results in photo-inhibition that includes photo-damage to the photosynthetic apparatus causing irreversible inactivation of PSII. It also includes some photo-protective mechanisms causing slow and reversible reduction of the photosynthetic efficiency, or down-regulation of PSII and the dissipation excess excitation energy as heat. All these could cause changes in fluorescence parameters (Efeoğlu, 2009; Inamullah and Isoda, 2005). Water deficit induces generation of reactive oxygen species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-). These could disrupt the normal metabolisms of plants through oxidative damages to lipids, proteins and enzymes (Apel and Hirt, 2004; Shehab, et al., 2010). Drought-induced overproduction of ROS increases the concentration of MDA, which is considered as a marker for membrane lipid peroxidation. A decrease in membrane stability reflects the extent of lipid peroxidation, which is caused by ROS (Nayyar and Gupta, 2006). To minimize oxidative damage, plants have evolved a complex enzymatic and non-enzymatic antioxidant defense system (Yordanva et al., 2000), in which SOD, POD and CAT are most important antioxidative enzymes. Maintaining a higher level of antioxidative enzyme activities may contribute to drought induction by increasing the capacity against oxidative damage (Sharma and Dubey, 2005). Ramie (*Boehmeria nivea* L.), or 'Chinagrass', is one of major foreign exchange earning crops in China due to the high quality textile of its fine fiber. It is a perennial herbaceous monoecious flowering plant of the Urticaceae family. Ramie is a cross-pollinated cultivated plant with complex genetic background. Therefore, the offspring can be genetically segregated largely (Guo and Liu, 1989). Ramie conventionally reproduce by asexual means with vegetative organs include rhizome cuttings, stem cuttings, buds and shoots. However, these asexual propagation methods require tremendously intensive labor work, while pests and diseases are also easily transmitted to the offspring, which limits the expanding of ramie in larger area. Recently, ramie can reproduce with seeds by sexual means. Hybrid ramie is the commercial ramie crop from F_1 seeds of cross between two genetically dissimilar parents. There are some two-line hybrid ramie varieties developed by good male sterile line and restoration line (Si et al., 2000). The male sterile flowers in ramie displays on the male flower bud that is thin, small and uncracked compared to normal plants. The contents are very few and without pollen while the female flower growth is normal. The female flower opens when the male flower bud withers and falls off.

The two - line approach is based on the male sterility and the fertility restoration and involves two lines - the CMS (A) line and the restoration line (R) - for the commercial production of ramie hybrids. Ramie hybrids have the potential of yielding 20%-30% more than the conventional varieties (Zhao et al., 2008). However, seasonal drought is becoming a severe challenge in agriculture production in the Yangtze River Basin in China. It usually happens from June to August when is critical time for most crops, even worse, it tends to come along with high temperature. This climate phenomenon cannot be easily detected or explained by the average data of annual precipitation; however, it might be due to rainfall pattern shift as a result of global climate change. This extreme climate phenomenon has caused a great loss of yield for many crops including ramie. Early studies revealed that the drought tolerant ramie genotypes accumulated more proline and MDA, the activity of POD and CAT was higher than drought sensitive types (Jie et al., 2000; Liu et al., 2005). However, there were differences among genotypic varieties of ramie reproduced by asexual means exposed to drought stress. So far, there are not any reports on the influence of drought stress on hybrid ramie physiological behavior. Therefore, this study was undertaken to explore the alteration of hybrid ramie in chlorophyll fluorescence, RWC, soluble protein, proline, MDA and activities of antioxidant enzymes subjected to different watering regimes, in order to assess the resistance capacity of hybrid ramie to drought stress.

Results and discussion

Chlorophyll fluorescence

Fluorescence measurement as a non-intrusive method, allows the rapid assessment of quantum yield of electron flow through photosystem (PS) II. The method has been widely used for detecting water stress in plants, such as potato (Germ et al., 2007), Cassava (Oyetunji et al., 2007), canola (Kauser et al., 2006) and rice (Pieters and EI Souki, 2005). In our study, drought severely impaired PSII activity in the leaf of ramie plants and led to sustained decline in the Fv/Fm, Fv'/Fm', Φ_{PSII} , qP and ETR, while NPQ changed in opposite direction with intensified water stress (Table 1). Fv/Fm, Fv'/Fm', Φ_{PSII}, qP and ETR slightly (often non-significantly) decreased by 5.58%, 9.37%, 8.68%, 10.19% and 7.81% in mild water stress treatments while a comparatively larger reduction by 15.37% - 21.07%, 37.15% - 46.35%, 26.65% - 48.20%, 36.77% - 53.97% and 15.03% - 30.92% was detected in higher water stress treatments, respectively, as well as similar increase in NPQ. Nevertheless, the values of Fv/Fm significantly decreased for moderate and severe stress, whereas non-significant change for mild stress observed. This suggests the occurrence of the photo-damage of PSII under more severe water stress condition, while no photo-inhibitory or comparatively smaller photo-damage to the photosynthetic apparatus at mild stress condition observed. A significantly decrease of Fv/Fm also suggested a decrease in the maximum quantum efficiency of open PSII centers as well as an increase in energy dissipation as heat and increasing of photo-damage to the photosynthetic apparatus. Besides, the Φ_{PSII} significantly dropped at more progressive drought. Lu et al. (2001) and Sinsawat et al. (2004) reported that the quantum yield of electron transfer at PSII (Φ_{PSII}) was the product of the efficiency of the open reaction centers (Fv'/Fm') and the photochemical quenching (qP). Cornic and Fresneau (2002) found that photosynthetic carbon reduction and carbon oxidation cycles are the main electron sink for PSII activity during mild drought. Flagella et al. (1998) indicated that quantum yield of PSII (Φ_{PSII}), as related to Calvin cycle metabolism, is reduced only under drastic water deficit. It is long-term drought reduction in water concentration that led to considerable depletion of pea PSII core. Our results seemed to be in accordance with previous studies. The low Φ_{PSII} of drought stressed plants was the consequence of a lower efficiency of excitation energy trapping of PSII reaction centers, Fv'/Fm'. A significant decrease of the qP was observed in response to the drought treatment, which indicated that a larger percentage of the PSII reaction centers was closed at any time. This phenomenon expresses that the balance between excitation rate and electron transfer rate has changed, thus leading to a more reduced state of the PSII reaction centers (Efeoğlu et al. 2009). Moreover, ETR were significantly affected under more serious stress, suggesting that photosynthetic electron transport through PSII in higher water stress treatments was inhibited. As water stress intensified, a significant increase was observed in non-photochemical quenching (NPQ) in the leaves, thus denoting an increase in the energy dissipation through non-photochemical processes. This consequence could cause significant down-regulation of PSII activity. Changes in the fluorescence parameters observed in ramie agreed with those found in Maize (Efeoğlu et al. 2009) and soybean (Inamullah and Isoda, 2005) subjected to different watering regimes.

RWC, soluble protein, proline and MDA

Relative water concentration (RWC) is considered as a measure of plant water status, reflecting the metabolic activity in tissues. It is usually used as one of the most meaningful indexes for dehydration tolerance in wide variety of plants (Anjum et al., 2011). In this study, a linear reduction in RWC was detected with prolongation of varying moisture treatments. RWC dropped from 88.9%, 85.08%, 81.33%, and 77.66% on the 10^{th} day to 83.95%, 77.03%, 70.54% and 64.47% on the 20^{th} day under well-watered, mild, moderate and severe drought stress, respectively (Fig 1). This indicates that higher intensive and longer drought stress causes more reduction in RWC. Similar results were also documented in conventional cultivars in ramie under water deficit condition (Jie et al., 2000).

A steep reduction in soluble protein concentration was observed with prolongation of drought treatment in current study (Fig 2). With the aggravation of drought stress, the protein concentration showed obvious decreases with the rates of 10.62%-33.18% on the 10^{th} day, 10.81%- 41.42% on the 15th day, 16.05%-41.42% on the 20^{th} day compared with the well-watered control, respectively. Significant reduction in the concentration of protein occurred under moderate and severe drought stress after treatment, while mild drought did not cause significant decline on the 10th, 15th day after the stress treatments. This fact indicated that the effect was more pronounced in longer and higher drought stress in plants. Obviously, the amount of reduction in soluble protein was evidently related to drought intensity and drought duration. A similar change pattern in soluble proteins in wheat (Grudkowska and Zagdanska, 2010), pistachio (Abbaspoura et al., 2012), mulberry (Ren, 2009) and Norway spruce (Blodner et al., 2005) has been observed. In this study, the decreased Fv/Fm, Fv'/Fm', Φ_{PSII}, qP and ETR meant drought stress impaired PSII activity, which could cause photo-damage to the photosynthetic apparatus. The drought stress causes photosynthesis decrease and deficit to supply material for protein synthesis; therefore, protein synthesis will be dramatically reduced or even stopped (Havaux et al., 1987). On the other hand, the potential synthesis of chloroplast protein from their mRNA was influenced by water deficit, its effects on PSII protein metabolism, especially on the reaction centre proteins might account for the damage to PSII photochemistry (He et al., 1995). Proline accumulation is the first response of plants exposed to water deficit stress in order to reduce cell injury. In fact, proline can act as an osmoprotector of cytosolic enzymes and cellular structures (Abbaspoura et al., 2012). The MDA concentration is usually used to measure the extent of lipid peroxidation caused by oxidative stress (Smirnoff, 1993). In this study, the progression of drought stress resulted in remarkable increments by 23.61-84.41%, 24.73-81.45 and 25.96-78.60% in the concentrations of free proline and by 9.49-31.88%, 10.76-34.93% and 8.86-29.67% in the concentrations of MDA on the 10th, 15th, 20th day compared to the control, respectively (Fig 2). Consequently, both minimum proline and MDA concentrations were observed in 10d stress while maximum level in 20d stress. The change of these activities was strong evidence that the drought treatment actually could lead to oxidative stress. Nevertheless, mild water stress caused no significant difference in MDA concentrations but significant difference in proline concentrations. In other words, significant changes in free proline appeared at earlier drought stage than in MDA, which indicated that increased proline protected hybrid ramie plants from oxidative stress, lipid peroxidation and cell damage. Jie et al. (2000) reported that proline accumulation increased in drought resistant conventional varieties with the aggravation of water stress, while it initially improved, subsequently stopped increasing or even dropped in sensitive ones under serious drought stress. Liu et al. (2005) found that proline and MDA concentrations increased by 87-95% and 36.0-36.2% in the drought tolerant conventional cultivars, whereas by 115.3-195% and 46.8-67.8% in the drought sensitive ones under 55±1% FC, respectively. Compared to these conventional varieties, our result suggested that hybrid ramie was the more tolerant variety to moisture stress.

Activities of antioxidants enzymes

Plants have an internal protective enzyme-catalysed clean up system, which can protect the plants from reactive oxygen species (ROS) and guarantee the normal cellular function (Wang et al. 2003). SOD is a major scavenger of superoxide anion radical that catalyses the disputations of superoxide anion radical and results in the production of hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) (Smirnoff, 1993). CAT and POD play an essential role in scavenging for H_2O_2 toxicity. The combined action of CAT and SOD converts the toxic O_2^- and H_2O_2 to water and molecular oxygen (O_2), thus averting the cellular damage under unfavorable conditions likes drought stress (Reddy et al., 2000; Chaitanya et al. 2002). A link between tolerance to oxidative stress induced by water deficit and rise in antioxidant concentration in photosynthetic plants has been reported (Liu et al., 2011). This study presented a linear increment in SOD activities by 7.51-25.49%, 9.66-31.89% and 10.69-34.53% and POD activities by 11.52-51.29%, 10.61-44.92% and 16.65-61.85% on the 10th, 15th , 20th day, respectively (Fig 3) under mild, moderate and severe water deficit compared with the well water plants. The CAT activities was initially increased under stress condition and reached at peak on the 15th day after drought treatment and then found to be decreased on 20 days after drought treatment (Fig 3), and higher CAT activities was documented with the rate of 12.99-44.13%, 11.43-46.50% and 8.31-31.32% on the 10th, 15th, 20th day under mild, moderate and severe moisture deficit compared to the control. No significant changes were observed under mild stress, which mean drought induced effects on ramie was also related with drought intensity and drought duration, as well as a certain tolerance to the short time mild water stress for hybrid ramie. Nevertheless, in drought resistant conventional varieties POD activities enhanced while it initially enhanced, then dropped in sensitive ones with the aggravation of water stress (Jie et al., 2000). CAT activities increased less in the drought tolerant conventional cultivars (by 34.80-40.70%) than in the drought sensitive ones (by 59.20-77.60%) under 55±1% FC (Liu et al., 2005). From this point hybrid ramie was tolerant to drought stress.

Materials and methods

Plant materials and growth conditions

Pot experiment was carried out in a greenhouse at College of Agronomy and Biotechnology, Southwest University, Chongqing, China in spring 2011. The experimental area lies between latitudes 29° 49′ 32″ N, longitudes 106° 26′ 02″ E and altitude 220 m. The hybrid seedlings (Chuan Zhu 11, a two-line hybrid ramie variety developed by good male sterile line C9451 and restorer line R79-20) of ramie were provided by Dazhou Institute of Agricultural Sciences, Sichuan Province, China. On June 13, 2011 40-day-old nursery seedlings from the nursery beds in the fields were transplanted into each plastic pot (32 cm in diameter, 28cm in depth) filled with sandy loam soil containing organic matter 9.00 g kg^{-1}, total nitrogen 1.38 g kg^{-1}, total phosphorus 0.70 g kg^{-1}, total potassium 20.90 g kg^{-1}, alkali-hydro nitrogen concentration 230.29 mg kg^{-1}, available phosphorus 87.66 mg kg^{-1}, readily-available potassium 422.50 mg kg^{-1} and pH 6.73. Total weight of each pot was 14 kg after filling with soil and organic matter. The plastic pots were then shifted to greenhouse after transplanting the seedlings. The pots were arranged in a completely randomized design (CRD) with three replications of each experimental unit with 20 pots per treatment.

Drought treatments

Ramie plants were grown with normal water supply till the start of vigorous growth stage (about 45 days after transplanting), then were grouped in four drought treatments as follows: (1) 75% -80% FC (well-watered, CK), (2) 60%-65% FC (mild drought stress), (3) 45%-50% FC (moderate drought stress), (4) 30%-35% FC (severe drought stress). Field capacity (FC) is the maximum amount of water that soil can have by capillary action (Anjum et al., 2012). The field capacity of the pot soil was 23.70% (The pot soil was pre-tested for the water holding capacity by the oven-drying method). Moisture treatments were

Table 1. Parameters of chlorophyll fluorescence in dark-adapted leaves and in light-adapted leaves of hybrid ramie under different water regimes

Treatment	Fv/Fm	Fv'/Fm'	Φ_{PSII}	qP	NPQ	ETR
CK	0.807±0.023 a	0.576±0.026 a	0.334±0.023 a	0.756±0.036 a	10.90±0.17 c	61.96±1.90 a
MiDS	0.762±0.017 a	0.522±0.018 a	0.305±0.008 a	0.679±0.021 a	11.79±0.59 b	57.12±2.10 ab
MoDS	0.683±0.005 b	0.362±0.010 b	0.245±0.004 b	0.478±0.006 b	13.33±0.24 a	52.65±1.05 b
SDS	0.637±0.009 b	0.309±0.006 b	0.173±0.003 c	0.348±0.023 c	15.29±0.27 a	42.80±1.57 c

Values in the table are mean ± S.E (n = 3). Values followed by the same letter within columns do not differ significantly according to Newman–Keuls test ($\rho < 0.05$). CK: well-watered; MiDS: mild drought stress; MoDS: moderate drought stress; SDS: severe drought stress.

regularly monitored by TRIME-EZ/-IT (IMKO Micromodultechnik GmbH, Germany) according to Anjum et al., (2012). Each pot was weighed daily to maintain the desired soil water levels by adding appropriate volume of water, if necessary. After 20 days of imposition of different water regimes all plants were irrigated with normal water supply till harvest.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence measurement was determined after 18d of drought treatment following recommended procedures in the LICOR 6400 manual (LICOR Biosciences, Inc., Lincoln, NE) for dark-adapted leaves and light-adapted leaves. Chlorophyll fluorescence parameters were determined on the 6-7th fully expanded leaves from top, using an open gas exchange system (LI-6400; LI-COR, Inc., Lincoln, NE, USA) with an integrated fluorescence chamber (LI-6400-40 leaf chamber fluoremeter; LICOR). Leaves were dark-adapted for 120 min using dark-adapting leaf clips (LI-COR) for minimum fluorescence (Fo) and the maximum fluorescence (Fm) measurements. Maximum quantum efficiency of photosystem II (PSII) was calculated as: Fv/Fm = (Fm - Fo)/Fm. The energy harvesting efficiency of PSII in a light-adapted leaf was calculated as: F'v/F'm =(F'm − F'o)/F'm, where F'o is the minimal fluorescence in the light-adapted state, and F'm is the maximal value. Similarly the estimates of the relative quantum yield of photosystem II (Φ_{PSII}) can be calculated as: Φ_{PSII} = (F'm - Fs)/F'm using the steadystate parameter (Fs). Photochemical quenching is given by: qP = (F'm-Fs)/(F'm-F'o), non-photochemical quenching is also calculated as: NPQ = (Fs/F'm) - (Fs/Fm) (Hendrickson et al., 2004). The electron transport rate (ETR) was calculated by assuming a leaf absorption of 0.85 and a PSII : PSI ratio of 1:1 (ETR = PPFD × Φ_{PSII} × 0.85 ×0.5).

RWC, free proline and MDA assay

The ramie hybrid plants were sampled (6-7th leaf from top) after 10, 15 and 20d of drought treatment to assess the RWC, protein, proline, MDA and antioxidant enzymes activity. After washing, the leaves were frozen in liquid nitrogen and stored at -80°C until biochemical analysis. Free proline was measured as described by Bates et al. (1973), while the MDA concentration was measured according to the method of De Vos et al. (1991).

The relative water contents (RWC) was determined by recording the fresh weight (FW). The leaf samples were then immersed into the distilled water for 18 h for turgid weight (TW). Leaves were then dried at 70°C for 72 h to determinate the dry weight (DW). The RWC was calculated as: RWC = (FW - DW)/ (TW-DW) ×100.

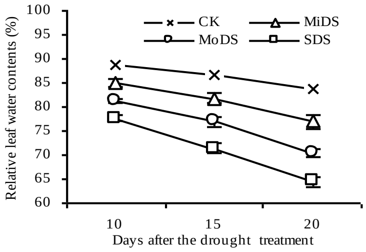

Fig 1. Relative water contents (RWC) in leaves of hybrid ramie under different soil water regimes. CK: well-watered; MiDS: mild drought stress; MoDS: moderate drought stress; SDS: severe drought stress.

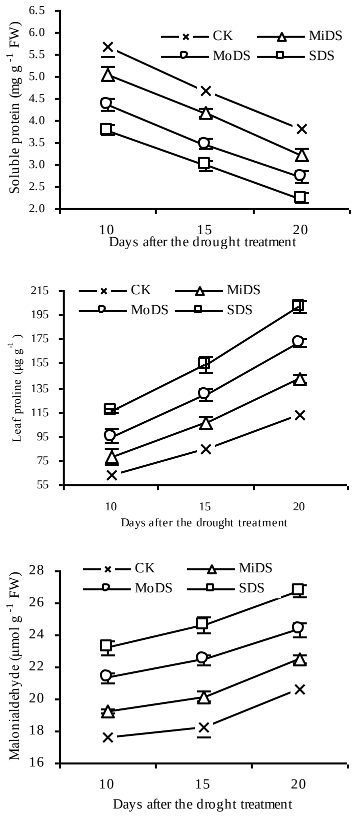

Fig 2. Concentration of soluble protein, proline and MDA in leaves of hybrid ramie under different soil water regimes. CK: well-watered; MiDS: mild drought stress; MoDS: moderate drought stress; SDS: severe drought stress.

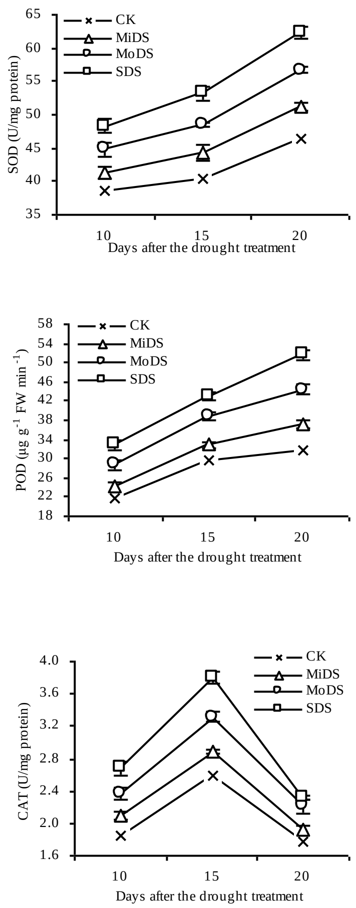

Fig 3. Concentration of superoxide dismutase activity (SOD), Peroxidase activity (POD) and Catalase activity (CAT) in leaves of hybrid ramie under different soil water regimes. CK: well-watered; MiDS: mild drought stress; MoDS: moderate drought stress; SDS: severe drought stress.

Soluble protein and antioxidant enzymes assay

Soluble protein concentrations were determined as described by Bradford (1976), using bovine serum albumin as a standard. Frozen leaf samples (0.5 g) were ground in liquid nitrogen with a mortar and pestle and homogenized in 10 ml 50 mM sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 11000 g for 20 min at 4°C. The supernatant was collected and used for antioxidant enzymes activity analysis. One millilitre of Bradford solution was added to 100 µl crude extract and absorbance recorded at 595 nm for estimation of total protein concentration. The protein concentration was calculated from a BSA standard curve.

The POD activity was determined by following the method of Upadhyaya et al. (1985). Both the SOD and CAT activity were assayed by ready kits provided by Nanjing Jiancheng Bioengineering Institute, China. Assay for SOD and CAT were carried out following the protocol mentioned with the detection kit. One unit of SOD activity was defined as the amount of enzyme required for 1 mg tissue proteins in 1 ml of a reaction mixture SOD inhibition rates to 50% as monitored at 550 nm while one unit of CAT activity was defined as 1 mg tissue proteins consumed 1µmol H_2O_2 at 405 nm sec^{-1}. CAT activity was expressed as enzyme units per mg of protein.

Statistical analysis

Data were processed by SPSS 16.0. The comparisons were tested after analysis of variance (one-way ANOVA) using Newman–Keuls test. Different letters indicate statistical difference at $\rho<0.05$.

Conclusion

This study showed that progressively intensified water stress resulted in a substantial change in chlorophyll fluorescence, RWC, protein, proline, MDA and antioxidant enzymes in hybrid ramie plants. These indicated that hybrid ramie plants responded to water limiting conditions by changing physiological and biochemical parameters. However, these changes were closely related with the intensity and duration of the drought stress. In total, hybrid ramie was tolerant to drought and our results might help growers to improve the hybrid ramie cultivar drought management.

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