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RESEARCH ARTICLE

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Responsibility of non-stomatal limitations for the reduction of photosynthesis—response of photosynthesis and antioxidant enzyme characteristics in alfalfa (*Medicago sativa* L.) seedlings to water stress and rehydration

Abstract Water stress by polyethylene glycol (PEG)–6000 solution ($\psi_s = 0.2$ MPa, stress time: 48 h, rehydration time: 48 h) was performed in leaves of two alfalfa cultivar (Long-Dong and Algonquin) seedlings. Gas exchange parameters, chlorophyll fluorescence parameters, activity of antioxidant enzyme and photosynthetic pigment concentrations were measured to investigate the available photosynthetic and antioxidant enzyme response to variable water conditions as well as stomatal and non-stomatal limitations to photosynthesis. The results showed that non-stomatal limitations were responsible for the reduction of photosynthesis during water stress. At the beginning of water stress (12 h), water was lost and then the stomata closed rapidly, which resulted in a decrease of transpiration, net photosynthesis and CO_2 diffusion. Therefore, when intercellular CO_2 concentration and carboxylation efficiency decrease, water use efficiency and value of stomatal limitation would increase. However, the decline of net photosynthetic rate was faster than transpiration rate. At the same time, the maximal photochemical efficiency, potential activity of PSII reaction center and photochemical quenching of chlorophyll fluorescence declined significantly, the activity of antioxidant enzyme increased rapidly and the photosynthetic pigment concentrations changed slightly. The results also indicated that, at the initial period of stress, neither oxidative stress nor membrane lipid peroxidation was induced, nor were photosynthetic structures damaged, but photosynthetic functions were partly inhibited. Therefore, the stomatal limitation and non-stomatal limitations had the same responsibility for the reduction of photosynthesis. At the mid-late stage of water stress, net photosynthetic rate, stomatal conductance, maximal photochemical efficiency, potential activity of PSII reaction center and photochemical quenching of chlorophyll fluorescence decreased linearly with the decline of the relative water content. And the relative electron transport rate, the effective quantum yield of PSII photochemistry and photosynthetic pigment concentrations declined continually. The activity of antioxidant enzymes maintained at a higher level but malondialdehyde accumulated gradually with prolonging of water stress. Simultaneously, the non-photochemical quenching of chlorophyll fluorescence increased obviously after water stress for 24 h. The remarkable decline of light saturated point of photosynthetic electron transport, that is, the initial point of photo-inhibition, was observed in advance. Therefore, non-stomatal limitations dominated the changes of a series of physiological and biochemical reactions during mid-late period of water stress. After 48 h rehydration, all the parameters except intercellular CO_2 concentration in Long-Dong recovered obviously but incompletely, which resulted from severe oxidative injury and photo-inhibition induced by water stress even though photo-protection was triggered during water stress in alfalfa leaves. Alfalfa seedlings were sensitive to water stress and there were certain differences between cultivars.

Keywords activity of antioxidant enzyme, alfalfa, chlorophyll fluorescence parameters, photosynthesis, stomatal and non-stomatal limitations, water stress, rehydration

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1 Introduction

Drought stress and its detrimental effects on plants in both natural and agricultural environments are receiving increasing

attention (Verslues et al., 2006). Water deficit can reduce photosynthesis chiefly by inhibiting growth and development of plants and their yield (Lu and Zhang, 1999; Flexas et al., 2004). On the contrary, plants can resist drought stress by morphological changes, e.g. by increasing size of the root system or reducing leaf area (Irigoyen et al., 1992), and by changing physiological and biochemical processes such as antioxidant defense systems, solute accumulation, etc. (Yin et al., 2005; Lei et al., 2006).

In the past years, researchers concluded that the reduction of photosynthesis in severe drought-stressed crops and forests was due to stomatal limitation or non-stomatal limitations (Shan and Chen, 1998; Meng et al., 2004). Marques da Silva and Arrabaca (2004) reported that stomatal limitation to CO_2 diffusion was the main reason for the decrease in photosynthesis on C_4 grass *Setaria sphacelate*. Lal and Edwards (1996) on C_4 species *Amaranthus cruentus* and *Zea mays* and Foyer and Harbinson (1997) on maize got the similar results. However, by studying four types of C_4 grasses, Ghannoum et al. (2003) pointed out that non-stomatal limitations were responsible for drought-induced photosynthetic inhibition. Most researchers indicated that the importance of stomatal limitation or non-stomatal limitations to the reduction of photosynthesis were mainly dependent on the degree of drought stress. During initial stress stage, the decrease in photosynthesis resulted from lower CO_2 supply to the carboxylating enzymes, as a consequence of stomatal closure. And with progress of water stress, the destruction of chlorophyll, serious disorganization of chloroplast's ultrastructure and enzyme inactivation, and even the production of subsequent photo-inhibition might be induced. In this situation, the non-stomatal limitations were the main reason for the reduction in photosynthesis (Sairam and Saxena, 2000; Sayed, 2003).

Simultaneously, in the whole photosynthetic process, the light-energy utilization of PSII was regulated through photochemical reaction, thermal dissipation and chlorophyll fluorescence (Chl F). The photochemical reaction and thermal dissipation caused a response of the quenching of variable Chl F that was related to PSII photosynthetic electron transport and the primary electron acceptor (Q_A) bounding together with production of active oxygen (Xu et al., 2002; Peng and Lin, 2003), under rather natural or water stress conditions. Therefore, photosynthetic changes were closely bound up with oxidative stress induced by water deficit. It is necessary to understand, systematically and integrally, the mechanism of photosynthesis, antioxidant systems and effect of stomatal or non-stomatal limitations under water deficit if there are few reports on the response of Chl F and antioxidant enzyme to variable water circumstances—from natural water circumstance to water stress and even subsequent rehydration (RH) in some pasture grasses like alfalfa. Moreover, the related literature in this field of alfalfa can be hardly found. In addition, plants subjected to water stress might create a special mechanism of repair after rehydration, but, concerning alfalfa, it has not been reported so far.

Alfalfa (*Medicago sativa* L.), with high yield and quality, is one of the forage grasses distributed worldwide. In arid and semi-arid areas, water supply of alfalfa depends mainly on natural precipitation (Wang, 2005). Therefore, water shortage limits growth and development of alfalfa. Some researchers reported that a good adapting ability to water stress should be reflected from morphology of roots, osmoregulation and antioxidant ability, etc. (Guo et al., 2002; Han and Wang, 2005). In addition, Wang et al. (2006) reported that alfalfa might have a series of responses to salinity, cold or diseases. Moreover, less is known about the possible and conjunct responsive mechanism of photosynthesis, especially for Chl F in alfalfa to variable water supply (from drought to rehydration). Two cultivars—Long-Dong and Algonquin, known as major pasture plants in the loess plateau in the northwest of China, are used in the present study. Long-Dong is a native cultivar of China and Algonquin, with higher yield, was introduced from Canada. Algonquin needs more water and fertilizer for normal development. The objective of the present study was to investigate the responsive mechanism of gas exchange parameters, Chl F characteristics, and activity of antioxidant enzymes on alfalfa leaves in a condition of fluctuating water supply.

2 Materials and methods

2.1 Plant material and growth conditions

Seeds of two alfalfa cultivars (Long-Dong and Algonquin) were sterilized in a 0.1% (w/v) $HgCl_2$ solution for 30 min, then put in a climatic chamber at 25°C until the cotyledons were fully expanded. Seedlings were transferred to 17 cm × 16 cm plastic barrels (15 seedlings per barrel) and placed in a growth chamber (model: PGV-36, Canada) with day/night rhythm: 12/12 h, temperature: 23/18°C, light intensity: 260 μmol/m^2·s, relative humidity: 65%. Half-strength Hoagland nutrient solution (pH: 5.5–6.0) was used to culture the seedlings. Every 3 days, culture medium was completely renewed. Water stress was applied with PEG–6000 (ψ_s: –0.2 MPa; and stress time: 48 h; subsequent rehydration time: 48 h) when the 7–8th leaf of the plants had emerged (30 days old). In the control, plants were grown in 1/2-strength Hoagland nutrient solution without PEG—6000.

2.2 Gas exchange parameters

Net photosynthetic rate (*Pn*), stomatal conductance (*Gs*), intercellular CO_2 concentration (*Ci*) and transpiration rate (*Tr*) in the top second fully expanded leaf of alfalfa were measured with LI-6400 portable photosynthetic system (LI-COR Company, USA). Carboxylation efficiency (*CE*) was the ratio of *Pn* to *Ci*, water use efficiency (*WUE*) was the ratio of *Pn* and *Tr* and the value of stomatal limitation (*Ls*) was $1-Ci/Ca$ (here, *Ca* was the CO_2 concentration in the atmosphere, 390 ± 5 μmol CO_2/mol).

2.3 Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters in the top second fully expanded leaf of alfalfa were measured using an imaging—pulse amplitude modulation (PAM) (Walz, Effeltrich, Germany). All samples were dark-adapted for 20 min prior to fluorescence measurements. The intensity of saturation pulse was 6 000 μmol/m^2·s and pulse length was 0.8 s. Light intensity during the measurement was 204 μmol/m^2·s and the actinic irradiance was natural light. After the first saturation pulse, we obtained the minimal fluorescence (F_o) and maximal fluorescence (F_m), then calculated potential activity of the PSII reaction center (F_v/F_o) and maximal photochemical efficiency of PSII reaction centers (F_v/F_m) based on variable fluorescence (F_v) = $F_m - F_o$. With applications of a saturating pulse once every 20 s, we obtained the steady-state fluorescence, photochemical quenching (q_P) and non-photochemical quenching (q_N). Rapid light curves of the relative electron transport rate (*ETR*) and the effective quantum yield of PSII photochemistry (*YIELD*) measurement were obtained through the application of a series of saturation pulses under increasing actinic irradiance (0, 4, 24, 59, 204, 334, 414, 504, 604, 714, 834, 964, 1 104 and 1 254 μmol/m^2·s).

2.4 Enzyme extraction and assay

The activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were calculated according to Gao (2000) and Zhang and Kirham (1994) with some modifications. 0.5 g leaf tissue material from the top second to fourth fully expanded leaf was ground in a mortar with pestle (0°C) containing a small amount of sand and 5.0 mL grinding media consisting of 50 mmol/L phosphate butter solution (PBS, pH = 7.8) and 1% PVP, followed by a 15 min centrifugation at 10 000 × g and 4°C. The supernatant extract was collected and stored at 4°C for all enzyme assays.

2.5 Measurements of MDA, *RWC* and photosynthetic pigments

Malondialdehyde (MDA) content was determined according to Zhang and Kirham (1994). Half a gram material from the top second to fourth fully expanded leaf of alfalfa were ground in a mortar in 5.0 mL of 5% (w/v) trichloroacetic acid, and followed by centrifugation at 4 000 × g for 10 min at 4°C. The supernatant extract (2.0 mL) was collected and mixed with 2.0 mL thiobarbituric acid. Samples were boiled for 10 min. After cooling down, samples were measured spectrophotometrically at wavelengths of 532, 600, and 450 nm, respectively.

Relative water contents (RWC) = $(W_f - W_d)/(W_t - W_d) \times 100$,

where W_f, W_d and W_t were fresh weight, dry weight and saturated weight of leaves, respectively.

Photosynthetic pigment concentration was determined according to Gao (2000). Samples of 0.1–0.2 g from the top second to fully expanded leaf of alfalfa were cut to pieces and soaked in 10 mL solution (ethanol: acetone: distilled water = 4.5 : 4.5 : 1) until the materials turned white (more than 24 h). Spectrophotometrical measurement was conducted at 663, 646, and 470 nm, respectively. Here, Chl a = $12.21 \times A_{665} - 2.81 \times A_{649}$; Chl b = $20.13 \times A_{649} - 5.03 \times A_{665}$; Total Chl = Chl a + Chl b; Carotenoid = $(1\,000 \times A_{470} - 27 \times$ Chl a $- 104 \times$ Chl b)/229.

2.6 Statistical analysis

The measurement of gas exchange parameters and chlorophyll fluorescence parameters was preformed in a growth chamber. Analysis of variance, *F*-test and Duncan's multiple comparisons in all parameters were carried out with stocks analyse software (SAS) system for Windows V8. Regression analysis was preformed between gas exchange parameters or chlorophyll fluorescence parameters and *RWC* by the soft of Sigmaplot 8.0 for Windows 2000. Quadratic and logarithmic models were applied for the light curve of *ETR* and *YIELD*.

3 Results

3.1 Effects of water stress and rehydration on gas exchange parameters (*Pn*, *Gs*, *Ci* and *Tr*)

Net photosynthetic rate (*Pn*) and stomatal conductance (*Gs*) in alfalfa leaves decreased significantly to 43.01% and 72.42% of the control in Algonquin, and to 34.83% and 24.11% in Long-Dong, respectively, after 12 h of water stress ($F = 186.40$ and 36.15, $P < 0.05$; Fig. 1 I, II). Compared with the control, intercellular CO_2 concentration (*Ci*) and transpiration rate (*Tr*) declined 13.90% and 33.91% in Algonquin and 16.63% and 70.02% in Long-Dong, respectively, when they were subject to water stress for 12 h ($F = 7.98$ and 53.35, $P < 0.05$; Fig. 1 III, IV). *Pn*, *Gs*, *Ci*, and *Tr* all declined continually as water stress progressed but *Ci* and *Tr* increased after 48 h of water stress. As a result of the different sensitivity of *Pn*, *Tr*, *Gs*, and *Ci* to water stress, water use efficiency (*WUE*) was increased gradually as water stress progressed and decreased after 48 h of water stress. The value of stomatal limitation (*Ls*), however, increased at the beginning of water stress (12 h), and then decreased with prolonging of water stress. On the contrary, carboxylation efficiency (*CE*) was decreased remarkably to 48.34% and 39.05% of the control in Algonquin and Long-Dong, respectively, after being water stressed for 12 h, and then declined.

The effects of water stress did not release completely after rehydration as indicated by the fact that *Pn*, *Gs*, *Tr*, and *Ci* were 42.01%, 29.06%, 31.42%, and 98.82% of controls in Algonquin, and 44.39%, 38.61%, 39.28%, and 94.73% in Long-Dong, respectively. On the other hand, *Pn*, *Gs*, *Tr*, and *Ci* did not show any difference between cultivars ($F = 0.07$,

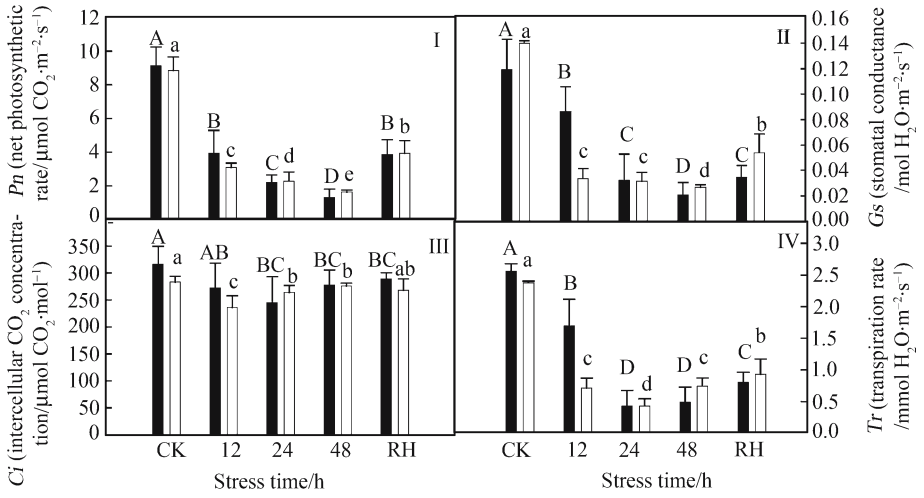


Fig. 1 Effect of water stress and rehydration on *Pn* (I), *Gs* (II), *Ci* (III) and *Tr* (IV) in Algonquin (-■-) and Long-Dong (-□-) Notes: the error bars represent standard deviation, and means with the same letter are not significantly different ($P<0.05$) where the capital letters stand for Algonquin and the small letters stand for Long-Dong. The same as below.

Table 1 Effect of water stress and rehydration on values of *Ls*, *CE* and *WUE* of alfalfa leaves

Stress time /h	*Ls*		*CE* /μmol·m^{-2}·s^{-1}		*WUE* /(μmol CO_2·mmol H_2O·m^{-2}·s^{-1})	
	Algonquin	Long-Dong	Algonquin	Long-Dong	Algonquin	Long-Dong
0	0.21±0.05 C	0.29±0.07 c	29.00±2.65 A	33.47±4.22 a	3.19±0.25 C	3.90±0.37 b
12	0.32±0.03 B	0.41±0.08 a	14.02±2.94 C	13.07±0.90 b	3.28±1.00 C	4.39±0.58 b
24	0.39±0.11 A	0.34±0.10 b	8.94±1.37 D	8.62±2.28 c	5.09±1.51 A	5.26±0.60 a
48	0.30±0.04 B	0.31±0.09 bc	4.662±1.84 A	5.89±0.50 c	2.82±0.74 C	2.21±0.29 c
RH	0.28±0.06 B	0.33±0.02 b	16.22±3.62 B	14.75±3.53 b	4.78±0.36 B	4.28±0.72 b

Notes: each value was the mean ($n\geqslant5$)±SE. The means with the same letter are not significantly different ($P<0.05$) where the capital letters stand for Algonquin and the small letters stand for Long-Dong. The same as below.

0.08, 0.02 and 0.01, $P>0.05$, respectively) in response to water stress. *Ls*, *WUE* and *CE* also did not recover completely after rehydration. It indicated that stomatal limitation controlled the decline of *Pn* and *Ci* after 12 h of water stress, and dark reaction of photosynthesis was affected. Non-stomatal limitations, however, were the main reason of decline of *Pn* and increase of *Ci*. Long-Dong did not significantly differ from Algonquin in the stomatal regulation mechanism under water deficit.

3.2 Effects of water stress and rehydration on activity of PSII

Alfalfa leaves subjected to water stress showed a consecutive decreasing pattern on the maximal photochemical efficiency of PSII reaction center (F_v/F_m) and the potential activity of PSII reaction center (F_v/F_o) ($F = 73.95$ and 139.38, $P < 0.05$; Fig. 2). Particularly, after 12 h of water stress, F_v/F_o and F_v/F_m decreased 25.08% and 5.24% in Algonquin, and 31.09% and 7.74% in Long-Dong, respectively, compared with controls. After 48 h of water stress, F_v/F_o and F_v/F_m declined to 21.75% and 60.56% of the controls in Algonquin and to 42.47% and 82.06% in Long-Dong, respectively. The recovery of 28.33% and 48.68% on F_v/F_o as well as 10.29% and 19.43%

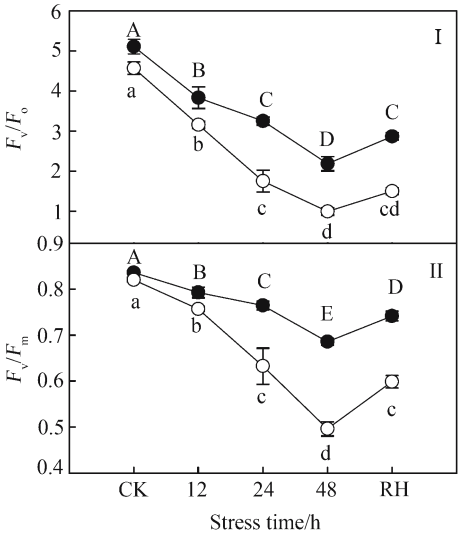


Fig. 2 Effects of water stress and rehydration on activity of PSII (F_v/F_o and F_v/F_m) in Algonquin (-●-) and Long-Dong (-○-)

on F_v/F_m in Algonquin and Long-Dong, respectively, was observed after rehydration. That is, water stress affected obviously the light-energy usage of PSII and the open extent of PSII reaction center from the earlier stage of water stress. At

the same time, F_v/F_o and F_v/F_m exhibited more obvious changes in Long-Dong leaves than those in Algonquin ($F =$ 120.12 and 115.46, $P < 0.01$), which indicated that there was a stronger adaptability to water deficit in Long-Dong.

3.3 Effects of water stress and rehydration on chlorophyll fluorescence quenching

Remarkable decline of 12.05% and 13.81% on the photochemical quenching of Chl F (q_P) was observed in Algonquin and Long-Dong leaves, respectively, when subjected to water stress for 12 h ($F = 9.19$, $P < 0.05$, Fig. 3). Then, continual decline as water stress progressed and obvious recovery of 82.79% and 86.06% of control, respectively, in Algonquin and Long-Dong after rehydration on q_P emerged. Unlike q_P, significant increase on the non-photochemical quenching of Chl F (q_N), more than 18.25% and 20.36% in Algonquin and Long-Dong, respectively, was shown in 24 h of water stress ($F = 2.88$, $P < 0.05$, Fig. 3), but incomplete recovery after rehydration on q_N also emerged. It was found that q_P declined to 73.73% and 73.29% of the controls and q_N increased by 198.13% and 44.26% compared with the controls when being stressed for 48 h in Long-Dong and Algonquin, respectively. In addition, q_N increased by 135.55% and 10.12% of the controls in Long-Dong and Algonquin after rehydration, respectively. Therefore, there was no difference in q_P ($F = 4.25$, $P = 0.05$) but there did exist different changes on q_N ($F =$ 18.48, $P < 0.05$) between the two cultivars. This suggested that photochemical capability of the PSII reaction center was affected from the beginning of water stress, but the self-protecting mechanism of alfalfa—dissipating surplus light-energy by heat energy—was increased in 24 h of water stress. Long-Dong might have stronger heat dissipating ability to water deficit than Algonquin.

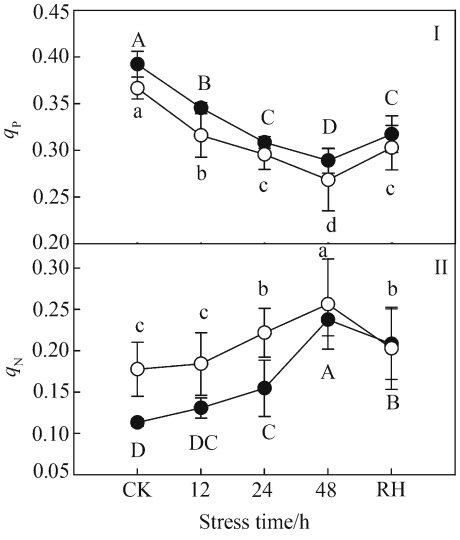
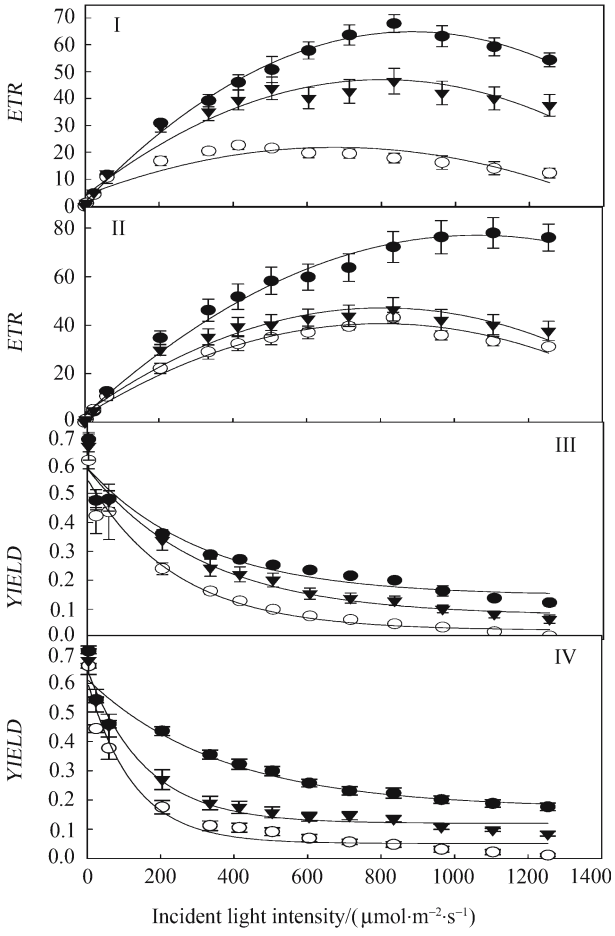


Fig. 3 Effects of water stress and rehydration on chlorophyll fluorescence quenching (q_P and q_N) in Algonquin (-●-) and Long-Dong (-○-)

3.4 Effects of water stress and rehydration on photosynthetic electron transport of PSII

As shown in Fig. 4, the relative electron transport rate (*ETR*) and the effective quantum yield of PSII photochemistry (*YIELD*) values were decreased obviously when subjected to water stress and did not recover to the level of control after rehydration under every active radiation of photosynthesis (*PAR*). Moreover, the decline of the top point of the light curve of *ETR* and the turning point of the light curve of *YIELD* was observed, which decreased the threshold of the saturated point of photosynthetic electron transport. At the same time, it also proved the coming of photo-inhibition in advance because the corresponding *PAR* of the top point was the initial



Notes: I) ●: $y = -8E-05x^2 + 0.1415x + 2.3326$, $R^2 = 0.9936$, ▼: $y = -6E-05x^2 + 0.1034x + 5.1532$, $R^2 = 0.9512$, ○: $y = -4E-05x^2 + 0.0487x + 5.356$, $R^2 = 0.8235$; II) ●: $y = -6E-05x^2 + 0.1364x + 4.0692$, $R^2 = 0.9875$, ▼: $y = -6E-05x^2 + 0.1036x + 5.0336$, $R^2 = 0.9608$, ○: $y = -6E-05x^2 + 0.0916x + 3.7200$, $R^2 = 0.9790$; III) ●: $y = -0.0935 \ln(x) + 0.8222$, $R^2 = 0.9744$, ▼: $y = -0.1053 \ln(x) + 0.8425$, $R^2 = 0.9721$, ○: $y = -0.1103 \ln(x) + 0.7986$, $R^2 = 0.9757$; and IV) ●: $y = -0.0913 \ln(x) + 0.8495$, $R^2 = 0.9670$, ▼: $y = -0.1099 \ln(x) + 0.8594$, $R^2 = 0.986$, ○: $y = -0.1156 \ln(x) + 0.8158$, $R^2 = 0.9918$.

Fig. 4 Response curves of *ETR* and *YIELD* to light intensity under water stress 0 hour (-●-), 48 hours (-○-) and rehydration 48 hours (-▼-) in Algonquin and Long-Dong

light intensity of photo-inhibition. The change patterns of *ETR* and *YIELD* could be expressed by the formulae: $y = ax^2 + bx + c$ (y: *ETR*, x: *PAR*) and $y = a\ln(x) + b$ (y: *YIELD*, x: *PAR*, $x \neq 0$), respectively.

The initial light intensity of photo-inhibition in creatine kinase (CK), water stress treatment and rehydration treatment were 883, 608, and 861 μmol/m^2·s in Algonquin and 1 136, 763, and 863 μmol/m^2·s in Long-Dong, respectively. On the other hand, the decline of *YIELD* values suggested a kind of necessary protective mechanism—the increase in heat dissipation in higher plants was induced (Dušan, 1999; Lu and Zhang, 1999; Zhang, 1999). These indicated that the photosynthetic dark-response was affected obviously by water stress, which weakened the activity of photosynthetic electron transport, and then brought the decline of *ETR* and *YIELD*, even *CE*, as well as damaged PSII reaction center.

3.5 Effects of water stress and rehydration on antioxidant enzyme activity

The activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) significantly changed from the earlier stage of water deficit ($F = 1\,264.97$; 6 104.67 and 801.53, $P < 0.05$), which, as shown in Fig. 5, were 0.94, 1.40, and 1.02 times of the controls in Algonquin and 1.25, 1.15, and 1.12 times in Long-Dong, respectively. After 48 h of water stress, the activity of SOD increased to the maximum—3.04 times of control in Long-Dong and just 2.27 times in Algonquin.

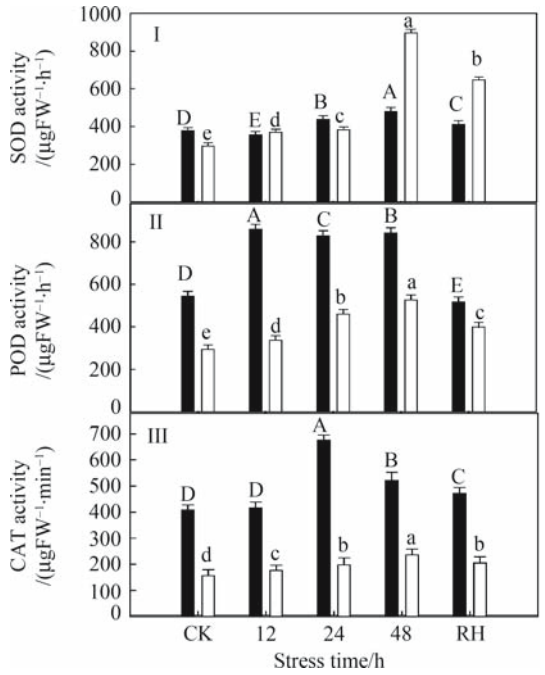


Fig. 5 Effects of water stress and rehydration on SOD (I), POD (II) and CAT (III) activity in Algonquin (-●-) and Long-Dong (-○-)

In Algonquin, the activity of POD and CAT maintained higher values during the period of stress after reaching the maximum (POD was 859.33 unit/gFW·h at stress 12 h and CAT was 675.62 unit/gFW·min at stress 24 h). In Long-Dong, the activity of POD and CAT increased gradually along with water stress and reached the maximum after 48 h (526.20 unit/gFW·h and 236.06 unit/gFW·min).

The activity of SOD, POD, and CAT in Long-Dong differed from that in Algonquin—SOD activity was stronger in Long-Dong while POD and CAT activities were stronger in Algonquin. Simultaneously, the obvious and incomplete decrease of the activities of SOD, POD, and CAT was also observed after rehydration in both cultivars (1.09, 0.95 and 1.16 times of control in Algonquin and 2.19, 1.36 and 1.31 times in Long-Dong, respectively). The different change of SOD, POD, and CAT activities might result from different sensitivities of Algonquin and Long-Dong to water stress.

3.6 Effects of water stress and rehydration on *RWC* and MDA

At earlier stage of water stress, relative water content (*RWC*) descended sharply, from 88.01% to 67.08% in Algonquin and from 85.49% to 74.22% in Long-Dong, and then declined continually up to 55.27% and 54.33% in Algonquin and Long-Dong, respectively, at the time of water stress for 48 h ($F = 375.14$, $P < 0.05$), which might have resulted from water loss or solute accumulation or both.

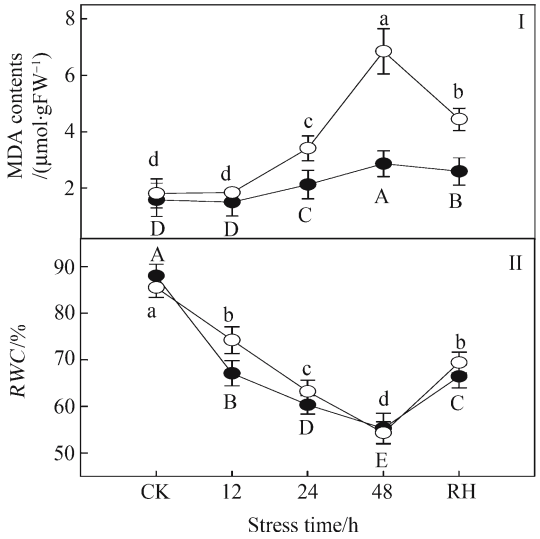


Fig. 6 Effects of water stress and rehydration on MDA contents and *RWC* in Algonquin (-●-) and Long-Dong (-○-)

Malondialdehyde (MDA) content showed slight and unobvious change at 12 h of stress, subsequently, rapid increase in Long-Dong and gentle increase in Algonquin in 24 h water stress, and accumulated (1.81 times and 3.78 times of the controls in Algonquin and Long-Dong, respectively) at the end of water stress ($F = 3856.37$, $P < 0.05$). Combined with

that shown in Fig. 5, it appeared that there was no accumulation of excessive active oxygen and production of the lipid peroxidation at 12 h of water stress. However, in the process of water stress, the lipid peroxidation was induced at the end. After rehydration, MDA content exhibited slight decrease in Algonquin (by just 90.65 % from 100% at water stress for 48 h) and dramatic decrease in Long-Dong (by 64.82% from 100% at stress 48 h), which meant that the antioxidative abilities in Algonquin and Long-Dong were different. At the same time, *RWC* increased obviously to 66.43% and 69.38% in Algonquin and Long-Dong, respectively.

3.7 Effects of water stress and rehydration on photosynthetic pigment concentration

Photosynthetic pigment concentrations were slightly increased at water stress of 12 h, which might be explained, at least partly, by rapid water loss. Subsequently, photosynthetic pigment (Chl a, Chl b, Total Chl and Carotenoid) concentrations declined gradually as water stress progressed (Table 2). Simultaneously, an increase ratio of Chl a/Chl b and a decrease ratio of Total Chl/Carotenoid were observed from control values of 4.44 and 5.05 to values of 5.04 and 4.83 at water stress for 48 h in Algonquin and from control values of 5.28 and 5.95 to values of 5.76 and 5.50 at water stress for 48 h in Long-Dong, respectively.

As a result of rehydration, photosynthetic pigment concentrations in Algonquin showed a small increase, but not in Long-Dong, because water stress was inclined to have more severe effects on chlorophyll concentration in Algonquin. However, the ratio of Chl a/Chl b and Total Chl/Carotenoid did not recover completely, which were 4.80 and 4.83 in Algonquin, and 4.94 and 5.68 in Long-Dong, respectively. Yao and Xie (2005) reported that the increase of Chl b concentration was caused by oxidation and transformation of Chl a, and carotenoid could replace Chl a to a certain extent to absorb and transport light-energy to the PSII reaction center and scavenge reactive oxygen under water stress. Hence, the decrease of carotenoid was one of the reasons of the reduction of Chl a. On the other hand, the decline of photosynthetic pigment concentrations was due to the increase of degradation rate or the decrease of synthesis rate of chloroplast (Smimoff and Colombe 1988).

4 Discussion and conclusions

The indirect effects of water stress were photo-inhibition and oxidative injury (Flexas et al., 2006). The data in this study indicated that progressive water stress induced a series of changes of photosynthesis and oxidative characteristics in leaves of alfalfa. At the early stage of water stress, water was lost rapidly, which resulted in a sharp decrease of *RWC*, and then, the stomata closed and CO_2 diffusion from the atmosphere to the site of carboxylation reduced. As a consequence, *Ci* and *CE* declined obviously, which was the main cause for the decrease of photosynthesis (Shan and Chen, 1998; Flexas et al., 2006). An initial decline of *Ci* was in accordance with an initial limitation of photosynthesis due to a lower stomatal conductance. However, in the present study, *Pn* was more severely affected than *Tr* (Fig. 1 I, IV). It was not in accordance with Irigoyen et al. (1992) who thought that *Tr* was affected more than *Pn* in 3-month-old alfalfa leaves. At the same time, F_v/F_o, F_v/F_m and q_P were reduced obviously and *WUE* has a lower increase in both cultivars, which indicated that the decrease of *Pn* was in line with the decline of photosynthetic capacity, that is, CO_2 fixation, ribulose-1,5-bisphosphate (RUBP) carboxylation, inorganic phosphorus transform, the open extent of PSII reaction center, and photosynthetic electron transport might be inhibited to a certain extent (Parry et al., 2002; Bota et al., 2004). Non-stomatal limitations, at least partly, were in the position to affect the reduction of photosynthesis in alfalfa leaves at water stress of 12 h though *Ls* was increased. Nevertheless, water stress had no obvious effect on q_N, that is, the photo-protection mechanism of alfalfa leaves was insensitive.

On the other hand, SOD, CAT, and POD activities increased significantly at water stress of 12 h (Fig. 5), which suggested that the transformation of super-oxidative anion was affected, in other words, the transport of photosynthetic electrons might be inhibited. It was identical with the response of F_v/F_o, F_v/F_m and q_P. What is more important is that MDA did not accumulate and photosynthetic pigment concentrations changed slightly because of rapid water loss in both cultivars at the earlier phase of water stress, which would be beneficial to the resistance improvement to drought adversity. Therefore, no oxidative stress and membrane lipid peroxidation were

Table 2 Effects of water stress and rehydration on photosynthetic pigment concentrations (mg·g^{-1} DW)

Cultivar		Stress time/h				
		0	12	24	48	RH
Algonquin	Chl a	1.60 ± 0.048 B	1.82 ± 0.020 A	1.46 ± 0.045 C	1.26 ± 0.073 D	1.44 ± 0.046 C
	Chl b	0.36 ± 0.004 B	0.43 ± 0.003 A	0.31 ± 0.006 C	0.25 ± 0.003 D	0.30 ± 0.004 C
	Total Chl	1.97 ± 0.045 B	2.26 ± 0.062 A	1.77 ± 0.038 C	1.50 ± 0.056 D	1.74 ± 0.034 C
	Carotenoid	0.39 ± 0.006 AB	0.41 ± 0.002 A	0.37 ± 0.010 B	0.34 ± 0.009 C	0.36 ± 0.008 B
Long-Dong	Chl a	2.06 ± 0.056 a	2.13 ± 0.019 a	1.83 ± 0.079 bc	1.67 ± 0.031 c	1.78 ± 0.073 bc
	Chl b	0.39 ± 0.002 b	0.41 ± 0.001 a	0.35 ± 0.009 b	0.29 ± 0.001 c	0.36 ± 0.005 b
	Total Chl	2.44 ± 0.067 b	2.54 ± 0.098 a	2.21 ± 0.034 b	1.98 ± 0.097 c	2.16 ± 0.059 bc
	Carotenoid	0.41 ± 0.002 ab	0.42 ± 0.011 a	0.39 ± 0.019 b	0.36 ± 0.005 b	0.38 ± 0.006 b

Note: Each value was the mean ($n \geqslant 3$) ± SE.

induced and photosynthetic structures were less damaged though photosynthetic functions were decreased at initial stress. In other words, stomatal limitation and non-stomatal limitations may cooperate with each other to reduce photosynthesis at the beginning of water deficit. It was inconsistent with Marques da Silva and Arrabac (2004) and Ghannoum et al. (2003), but was in accordance with Berkowitz (1998).

With prolonging of water stress and the continual decrease of *RWC*, *Pn*, *Gs*, F_v/F_o, F_v/F_m and q_P declined linearly (Fig. 7 I, II, V, VI and VII). At the same time, a decline was also observed in photosynthetic pigment concentrations, *CE*, *Ls*, *ETR* and *YIELD*. However, q_N and MDA content increased persistently even though antioxidant enzymes (SOD, POD and CAT) stayed at higher activity. *Ci* increased slightly, which was related to the reduction of *CE*. Of course, the supply of carbon to carboxylation enzymes could be limited because of the physical structure of the intercellular spaces due to leaf shrinkage (Lawlor and Cornic, 2002). Significantly, the presence of the light saturated point of photosynthetic electron transport and of the initial point of photo-inhibition was observed in advance (Fig. 4), so severe water shortage might appear at mid-later stage of water stress. Many authors, such as Grieu et al. (1995) on F_v/F_m in *Trifolum repens* and Damatta et al. (1997) on *YIELD* in coffee, did not find changes under water stress. Marques da Silva and Arrabaca (2004) found a decrease on q_N in C_4 grass *Setaria sphacelata*. Our results, in accordance with most researchers, confirmed that the water stress could bring an obvious decrease to F_v/F_m (Campos, 1998; Eickmerier et al., 1993) and *YIELD* (Eickmerier et al., 1993) and a remarkable increase to q_N. Simultaneously, the different changes of antioxidant enzyme activity in Algonquin and Long-Dong were shown. A different antioxidative mechanism between Algonquin and Long-Dong might exist. Zhu et al. (2004) reported a similar result in different kinds of soybean seedlings. Rubioa et al. (2002) reported the increase of SOD activity in transgenic alfalfa leaves subjected to water stress. However, Irigoyen et al. (1992) reported that SOD activity was unchanged and POD activity declined gradually during water stress in alfalfa leaves. Our results could be explained by the reduction of the absorption of light and the utilization of photochemical energy of the PSII reaction center, the inhibition of status alternation of oxidation-deoxidization of Q_A (Primary quinone electron acceptor of photosystem II), even a part deactivation of PSII reaction center (Berg et al., 1997; Sayed, 2003) and the damage of photosynthetic structures, i.e. the damage of photosynthetic pigments, especially to Chl a (Table 2). It implied that the water stress increased the sensitivity of photo-inhibition (Irigoyen et al., 1992) and the non-stomatal

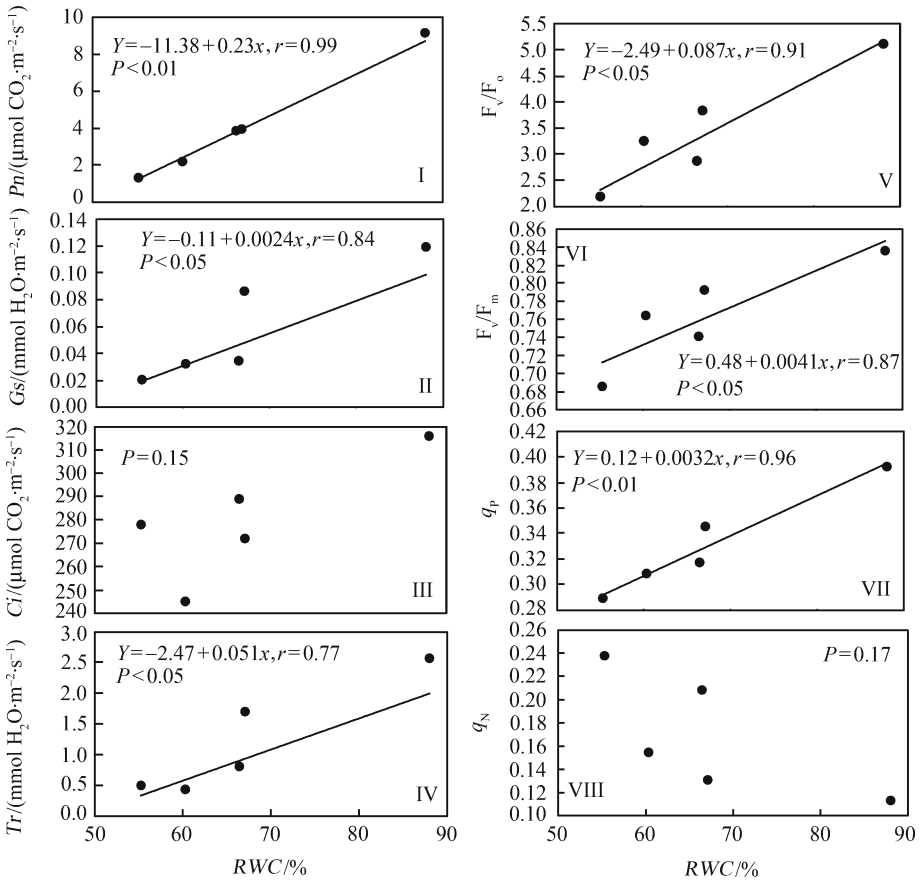


Fig. 7 Regression analysis of *RWC* and *Pn* (I), *Gs* (II), *Ci* (III), *Tr* (IV), F_v/F_o (V), F_v/F_m (VI), q_P (VII) and q_N (VIII) under water stress and rehydration in Algonquin and Long-Dong.

limitations were more and more vital to weaken photosynthesis with the progress of water stress.

After 48 h of rehydration, alfalfa produced a series of physiological and biochemical mechanisms of repair and made the production and scavenging of active oxygen balanced again, which resulted in the obvious recovery of all parameters. However, they did not reach the level of controls (expect *Ci* in Long-Dong). Therefore, oxidative injury and photo-inhibition induced by water stress could result in a severe damage in the structures and functions of PSII reaction center, which could not be released completely by rehydration. The resistance and endurance to water stress in alfalfa leaves might be weak even though abundant water was supplied again, after water stress treatment.

Compared with Algonquin, Long-Dong leaves showed a more rapid closure of the stomata, i.e., *Gs* decreased to 72.42% of the control in Algonquin and 24.11% in Long-Dong, respectively, after 12 h of water stress. After rehydration, *Pn*, *Gs*, *Tr*, and *Ci* in Long-Dong had better recovery than those in Algonquin (1.06 to 1.43 times of Algonquin, Fig. 1). Leaves exhibited more obvious decline in F_v/F_o and F_v/F_m by 21.75% and 60.56% of the control in Algonquin and 42.47% and 82.06% in Long-Dong when stressed for 48 h, and milder changes in Chl a of Long-Dong leaves than in Algonquin during water deficit is shown in Table 2. The q_N showed more obvious increase in Long-Dong than in Algonquin by 198.13% and 44.26%, respectively, compared with the controls when stressed for 48 h. In the meantime, the initial light intensity of photo-inhibition under water stress in Long-Dong (763 μmol/m^2·s) was higher than that in Algonquin (608 μmol/m^2·s). Long-Dong leaves had a significantly stronger self-protection ability to water deficit than Algonquin. The open extent of PSII reaction center, the photosynthetic electron transport, and photo-system structure were less damaged in Long-Dong than in Algonquin. Therefore, Long-Dong had a stronger adaptability to fast water deficit than Algonquin.

In summary, photosynthesis and activity of antioxidant enzyme were affected severely by mild water stress in the leaves of alfalfa even though the probable photo-protective mechanism existed—the increase of q_N with the progress of water stress and the decrease of *YIELD* with the increase of *PAR*. At the beginning of water stress, photosynthetic functions and structures in the leaves of alfalfa were influenced slightly and stomatal limitation and non-stomatal limitations might cooperate with each other to reduce photosynthesis. As water stress was prolonged, the oxidative injury and photo-inhibition became the main cause that would affect the photosynthetic process. At the same time, non-stomatal limitations began to dominate the change of physiological and biochemical reaction. After rehydration, no complete recovery occurred in alfalfa leaves. Accordingly, it suggested that alfalfa seedlings were sensitive to drought stress.

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