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Nitrogen Metabolism and Photosynthesis in *Leymus chinensis* in Response to Long-term Soil Drought

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Abstract

Key enzyme activities related to nitrogen metabolism, gas-exchange, chlorophyll fluorescence, and lipid peroxidation were determined in *Leymus chinensis* (Trin.) Tzvel. plants under four soil moisture regimes (control: 75%–80% of field moisture capacity, mild drought: 60%–65%, and moderate drought: 50%–55% as well as severe drought: 35%–40%). Severe drought significantly decreased the key enzyme activities of nitrogen anabolism such as nitrate reductase (NR, EC 1.6.6.1), glutamine synthetase (GS, EC 6.3.1.2), and glutamate dehydrogenase (GDH, EC 1.4.1.2) but increased the key enzyme activities of nitrogen catabolism such as asparaginase (AS, EC 6.3.5.4) and endopeptidase (EP, EC 3.4.24.11), especially after long-term soil drought. Plant biomass, leaf-biomass ratio between the green leaf and total plant biomass, net photosynthetic rate, stomatal conductance, the maximal efficiency of PSII photochemistry, the actual quantum yield, and the photochemical quenching were significantly reduced by severe water stress. Plant malondialdehyde (MDA) concentration increased with the increase in water stress, particularly at the late-growth stage. Our results suggest that the key enzymes of nitrogen metabolism may play an important role in the photosynthetic acclimation of *L. chinensis* plants to long-term soil drought.

Key words: Asparaginase; Endopeptidase; Chlorophyll fluorescence; Gas exchange; Glutamate dehydrogenase; Glutamine synthetase; Lipid peroxidation; Nitrate reductase; Water stress.

Introduction

In China, grassland covers an area of about 400 million hectare, which is about 40% of the whole land area of China and 12% of the world's grassland. *Leymus chinensis* (Trin.) Tzvel. is a native perennial plant with rhizomes, good palatability, and high forage value. The grassland that it dominates spreads widely, from the southern Chinese loess plateau (about 107°E, 34°N) to northern Russian Baikal (107°40′E, 53°00′N) and from the Sanjiang plain of eastern China (135°05′E, 49°27′N) to Ulan Bator of Mongolia (106°53′E, 47°55′N) (Wang and others 1999). However, the grassland ecosystem has deteriorated as a

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result of climate change and land-use practices (for example, overgrazing, reclamation) during recent decades, which results in strong dust storms affecting human life and other ecological problems (Zhou and others 2002; Wang and Gao 2003).

Drought is a major factor constraining productivity of grass in many regions of the world. Grassland productivity is associated with the temporal and spatial distribution of precipitation (O'Connor and others 2001; Zhou and others 2002; Wang and Gao 2003). Drought adversely affects plant growth, leaf gas exchange, and chlorophyll fluorescence (Lu and Zhang 1999; Chaves and others 2003; Souza and others 2004; Xu and Zhou 2005a). Nitrogen is one of the most quantitatively important elements for plant growth and development, and it has great effects on cell growth and metabolism (Chen and others 2003). A large portion of nitrogen in the plant is allocated to the leaves throughout the life of the plant, and a large part of leaf nitrogen is invested in the photosynthetic apparatus (Makino and Osmond 1991). Photosynthetic capacity is closely associated with leaf nitrogen (Evans 1983; Llorens and others 2003); therefore, the percentage of leaf nitrogen may be used to estimate photosynthetic capacity.

At the whole-plant level, the effect of drought stress usually leads to a decrease in photosynthesis and growth, and it is associated with alterations in C and N metabolism (Chaves and others 2003; Raven and others 2004). Drought results in a decrease in leaf nitrogen content (Sinclair and others 2000). Drought stress and N limitation significantly reduce the net photosynthetic rate and Rubisco activity, but drought alone does not affect Rubisco activity (Heitholt and others 1991). However, decreases in Rubisco activity and net photosynthetic rates of plants in response to drought correlate with lower foliar N concentrations (Llorens and others 2003).

Nitrate reductase (NR), glutamine synthetase (GS), and glutamate dehydrogenase (GDH) are key enzymes associated with nitrogen metabolism (Lam and others 1996); they are also involved in photosynthesis and carbohydrate metabolism (Solomonson and Barber 1990; Sibout and Guerrier 1998). Glutamine synthetase incorporates photorespiratory and non-photorespiratory ammonium, it provides nitrogen for transport to maintain nitrogen status in plants (Suzuki and Knaff 2005), and it can be used as a marker for plant N status (Kichey and others 2006). A possible role of GDH is as an adaptive enzyme susceptible to environmental variables; its action depends on a carbon skeleton source (Lam and others 1996; Stitt and others 2002). Nitrate reductase and GS activities rapidly decrease under water stress (Somers and others 1983; Sibout and Guerrier 1998). The decreasing macromolecule hydration and increasing content in molecules acting as regulators were seen as major causes of the changes typically undergone by these key enzymes (for example, GS, GDH) in the assimilatory ammonia pathway when plants were water-stressed (Bourgeais-Chaillou and others 1992; Sibout and Guerrier 1998). Unfortunately, the effects of soil drought on the key enzyme activities related to nitrogen metabolism are still not well understood.

Increased production of reactive oxygen species (ROS), such as superoxide (O_2^{-1}), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH), is likely to occur during drought stress (Price and Hendry 1991; Sguerri et al 1993; Taylor and others 2004); this can induce lipid peroxidation in membranes (Price and Hendry 1991; Zhang and Kirham 1994). Lipid peroxidation is one of the symptoms most easily ascribed to oxidative stress, and it is often used as an indicator of oxidative damage under environmental stress (Foyer and Harbison 1994; Foyer and Noctor 2002; Hernández and Almansa 2002; Sofo and others 2004).

Malondialdehyde (MDA) is a marker for lipid peroxidation that has shown greater accumulation under environmental stresses (Hernández and Almansa 2002; Munné-Bosch and Alegre 2002; Sofo and others 2004). Therefore, in the present study, MDA content was used as an indicator for cellular membrane damage to assess the effect of water stress.

Plant density and productivity of *L. chinensis* decreases with decreasing precipitation (Wang and Gao 2003), and photosynthetic capacity is positively correlated with leaf N concentration (Niu and others 2003; Xu and Zhou 2005b; Chen and others 2005). However, the effects of long-term soil drought on photosynthesis and the key enzymes related to nitrogen metabolism have received little attention to date. Consequently, in the present study, we hypothesize that any reduction in photosynthesis under long-term soil drought is closely associated with key enzyme activities involved in nitrogen metabolism.

Materials and Methods

Plant Material and Experimental Design

Seeds of *Leymus chinensis* (Trin.) Tzvel. were obtained from a field grassland in Xilinhot, Inner Mongolia. The plant growth season in the region is from early May to mid-September, with the peak of plant growth during the summer period (from early

July to mid-August). The maximum plant height is around 0.4 m, the leaf area index is 1.5, and the aboveground biomass is 15 g m^{-2}.

The seeds were sterilized by 5% potassium permanganate solution for 8 min, then rinsed, and immersed in water for 7 days before they were put into a refrigerator at a temperature below 0°C. They were sowed into pots (0.56 l) wrapped with plastic film. Each pot was filled with 0.64 kg dry soil obtained at the same site the seeds were collected, and planted with a density of 6 plants per pot. In the chestnut-colored soil, the organic carbon concentration, total nitrogen, and available nitrogen were measured at 19.60 ± 0.18 g kg^{-1}, 4.18 ± 0.11 g kg^{-1}, and 89.46 ± 2.37 mg kg^{-1}, respectively. Total nitrogen concentration was determined by the standard macro-Kjeldahl procedure; the available nitrogen, by the alkaline hydrolysis diffusion procedure; and the carbon content, with the Walkley-Black method (Chen 1983). The type of the soil is castanozem, and its texture class is medium. The soil contained 29.0%, 31.2%, and 39.8% clay (< 0.005-mm particle diameter), silt (0.005–0.05 mm particle diameter), and sand (> 0.05 mm particle diameter). Field capacity (determined in the field 48 h after irrigation) was 25.3% gravimetric, the permanent wilting point was 6.0% (the moisture content of the soil at which plants wilt and fail to recover their turgidity when irrigated amply), and bulk density was 1.21 g cm^{-3}.

Soil water-withholding treatments were initiated at 30 days after sowing (plant initially tillering stage) in a sunlit greenhouse [Day/night, air temperature 26/19 ± 2°C, 13 h photoperiod (5:00–18:00 h)] to obtain better seedlings. The daily maximum photosynthetically active radiation (PAR) was around 1000 μmol m^{-2} s^{-1} above the plant canopy on a clear day, provided by sunlight and by a combination of cool-white fluorescent and incandescent lamps. The soil relative water contents (SRWC) were divided into four levels: control (75%–80% of field capacity), MID (mild drought, 60%–65%), MOD (moderate drought, 50%–55%), and SD (severe drought, 35%–40%) in parallel with −0.1, −0.4, −1.2, −2.3 MPa of plant water potential, respectively [measured with *WP4* Dew-point Potential Meter (Decagon Devices, Pullman, Washington, USA) at the sampled days]. Soil moisture levels were maintained with manual irrigation by weighing individual pots at 5:00 pm daily. Each target soil moisture level was achieved by decreasing water supply progressively about 20 days after the beginning of withholding water. SRWC decreased gradually from ample soil moisture (SRWC of 75%–80%) by 2–3 percentage points daily. In the experiment, we focused on the long-term drought effects, and the relevant parameters were sampled 20 days after the onset of water treatment because of limitations in the sampling amount.

Biomass Measurement

Biomass was measured at 20-day intervals after water-withholding treatments (samples of 6 plants per pot, three pots each treatment), and was dried at 80°C to constant weight, and then weighed.

Leaf Relative Water Content

The detached leaves (about 0.5 g fresh weight) were cut at about 9:00 am and weighed immediately to obtain fresh weight (FW). They were then placed in a beaker (25 ml) filled with water overnight. The next morning they were reweighed to obtain turgid fresh weight (TW). Dry weight (DW) was obtained after drying at 80°C for 24 h in a drying oven. The relative water content (RWC) could be calculated as $RWC = [(FW - DW)/(TW - DW)] \times 100$.

Leaf Water Potential

Leaf water potentials were measured at midday (11:30) on the youngest and fully expanded leaves. A *WP4* Dew-point Potential Meter (Decagon Device, Pullman, Washington, USA) was used for leaf water potential measurement, with six leaves for each treatment.

Leaf Gas Exchange

Six plants from each treatment were selected from different pots. Gas exchange parameters were measured on an attached youngest and fully expanded leaf 40–43 days after withholding water, always in the same order in which the treatments were measured.

The net photosynthetic rate per unit leaf area (*A*) and stomatal conductance (*Gs*) were measured using a 0.25-l chamber connected to a portable photosynthesis system (LI-6200, Li-Cor, Inc., Lincoln, NE, USA) under ambient temperature and irradiance. Readings were terminated after 30 s. The gas exchange parameters were calculated automatically using the software in the photosynthesis system. Water use efficiency (WUE) was calculated by dividing instantaneous values of *A* by *Gs*.

Chlorophyll Fluorescence

The youngest and fully expanded leaves were selected to determine the chlorophyll fluorescence

parameters 40 days after withholding water treatments. Three pots were measured in each treatment, and three to four leaves were measured for each pot. After a 30-min dark adaptation at room temperature (25°C), the parameters were measured by a fluorescence meter (PAM-2000, Walz, Effeltrich, Germany). The parameters were calculated according to the following formulae (Genty and others 1989): PSII photochemical efficiency: $F_v/F_m = (F_m - F_0)/F_m$, the actual quantum yield: $\Phi_p = (F_m' - F_S)/ F_m'$, photochemical quenching coefficient: $q_P = (F_m' - F_S)/ (Fm' - F_0')$, non photochemical quenching coefficient: $q_N = 1 - (F_m' - F_0')/(F_m - F_0)$.

Nitrogen Content

Nitrogen concentration in plant tissue was determined by the standard macro-Kjeldahl procedure (Nitrogen Analysis System, Büchi, Switzerland). All leaf dried samples were ground in a Wiley mill to a size small enough to pass through a screen with 1-mm openings, mixed thoroughly, then subsampled for nitrogen determinations. The fine-ground sample of 0.15 g, 8 ml H_2SO_4, 0.23 g K_2SO_4, and 0.07 g $CuSO_4$ was placed into Kjeldahl flasks, then digested. The ammonia was distilled from an alkaline medium and absorbed in boric acid. The ammonia was determined colorimetrically by 0.020 M H_2SH_4 solution.

Soluble Protein, Free Amino Acid Content, and the Activities of Key Enzymes

The youngest and fully expanded leaves were sampled at 20-day intervals after withholding water treatments. The leaf fresh samples were obtained at about 9: 00 am, and instantly frozen in liquid N for 1 min, then stored at −80°C for measurement of soluble protein and free amino acid (FAA) contents and the key enzyme activity assay. About 1 g of leaves were homogenized with 10 ml of 50 mM sodium phosphate, pH 7.8, containing 2 mM EDTA and 80 mM L-ascorbic acid. After the homogenate was centrifuged at 15,000 × *g* for 20 min, the supernatants were used to determine soluble protein, FAA content, and the activities of the key enzymes (Cruz and others 1970; Alvim and others 2001). Soluble protein and FAA contents in leaves were determined according to the methods of Bradford (1976) and Moore (1968), respectively.

The activity of nitrate reductase (NR, EC 1.6.6.1) was determined according to the procedure of Baki and others (2000). The reaction medium consisted (total volume 1 ml) of 50 mM sodium phosphate, pH 7.8; 10 μM FAD; 1mM DTT; 5 mM KNO_3; and 20 mM EDTA. The reaction was started by adding 200 μl extract and terminated after 5 min by adding 125 μl zinc acetate solution (0.5 M). The nitrite formed was measured colorimetrically by adding 750 μl of 1% sulfanilamide in 3 M HCl, and 750 μl of 0.02% N-naphyl-ethylenediamine hydrochloride; an absorption was determined at 546 nm.

The activity of glutamine synthetase (GS, EC 6.3.1.2) was determined by means of the synthetase reaction (Elliot 1953; Hadži-Tašković Šukalivić 1986). Briefly, the volume of the reaction mixture with 20 μM hydroxylamine and 100 μM Glu was 2.2 ml, including 0.5 ml of enzyme extract. Hydroxamate formation was measured in an assay mixture after 15 min at 30°C. The absorbance was measured at 540 nm.

The activity of glutamate dehydrogenase (GDH, EC 1.4.1.2) was determined in the aminating direction by following the change in the A_{340} (Loulakakis and Roubelakis-Angelakis 1990). One activity unit was determined as the amount needed to oxidize 1.0 μmol of NADH per min at 30°C.

The activity of asparaginase (AS, EC 6.3.5.4) was determined according to the methods of Huang and Chen (1985). The assay mixture contained 0.5 ml citrate buffer, 1 ml L-Asn, and 0.5 ml crude enzyme extract. Assays were run at 37°C for 20 min, and the reaction was stopped by adding 0.5 ml 15% trichloroacetic acid (TCA). The supernatant was added by 2 ml Nessler's reagent (the alkaline solution of potassium iodide and mercuric iodide). The change of absorbance in the super fraction was measured at 500 nm.

The activity of endopeptidase (EP, EC 3.4.24.11) was determined according to the procedure of Wittenbach (1979). Hemoglobin (0.05 M) was used as the substrate, and the assay mixture routinely contained 0.4 ml of 200 mM citrate buffer, 0.2 ml substrate, and 0.4 ml crude enzyme extract. Assays were run at 38°C for 1 h, and the reaction was stopped by adding 1 ml 10% TCA. The change of absorbance in the superfraction was determined at 280 nm.

Malondialdehyde (MDA) Estimate

At 20-day intervals after withholding water treatments, the youngest and fully expanded leaf material (200 mg) was homogenized in 2 ml 0.1% TCA solution. The homogenate was centrifuged at 15,000 × *g* for 10 min, and 0.5 ml of the supernatant was added to 1.5 ml thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 90°C in a shaking water bath for 20 min, and the reaction was stopped by placing the reaction tubes in an ice water

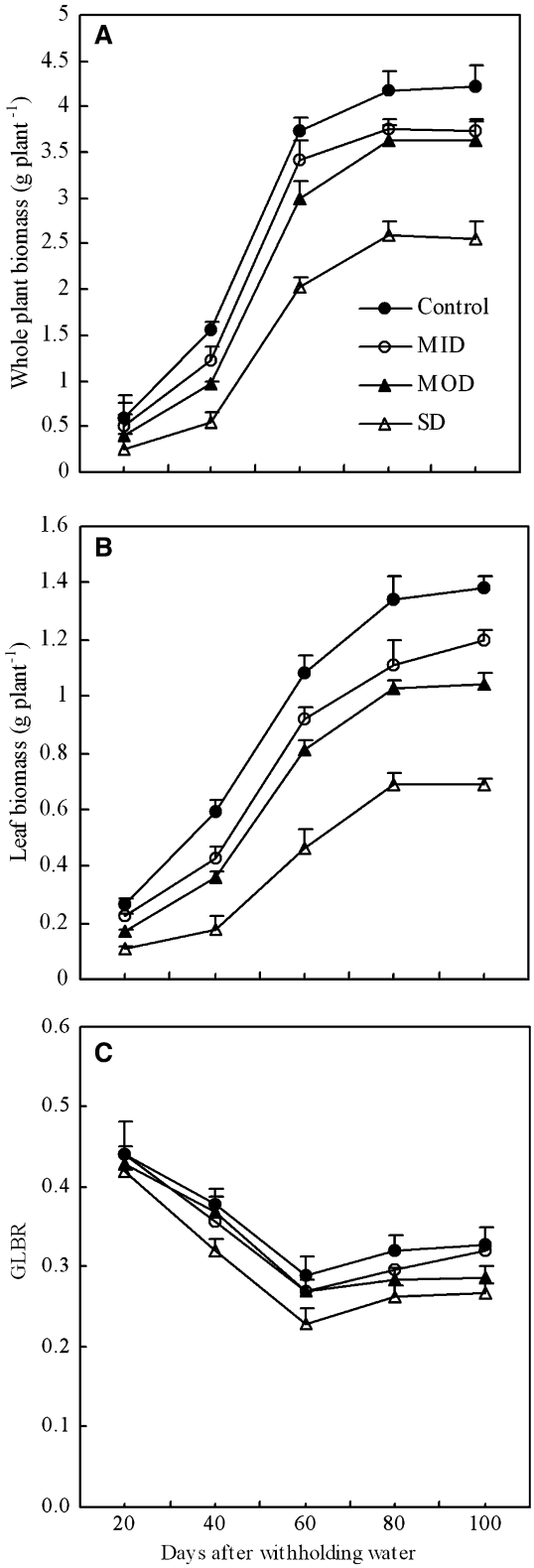

Figure 1. Changes in total plant (a) and green leaf biomass (b), and ratio between green leaf biomass and total plant biomass (GLBR, c) of *L. chinensis* under four soil moisture regimes [Control (75%–80% of field capacity), MID (mild drought, 60%–65%), MOD (moderate drought), SD (severe drought, 35%–40%)]. Values are means ± SE of three replications.

bath. Then, the samples were centrifuged at 10,000 $\times g$ for 5 min, and the absorbance of the supernatant was read at 532 nm (Hernández and Almansa 2002). The value for nonspecific absorption at 600 nm was subtracted. The amount of MDA was calculated from the absorption coefficient 155 mM^{-1} cm^{-1} (Cakmak and Horst 1991).

Statistical Analysis

The layout of the experiment was a randomized block design. All statistical GLM-ANOVA analyses were performed using SPSS 10.0 (SPSS for Windows, Chicago, IL, USA). Effects of soil moisture and time were analyzed using an one/two-way ANOVA. When significant according to the ANOVA, the comparisons between treatments were conducted with Duncan's multiple range test for mean significances ($p = 0.05$).

Results

Plant Growth

Figure 1a demonstrates the progression of plant growth during 100 days after withholding water treatments (DAW). The plants exhibited a similar change trend in plant growth over all soil water treatments. When plants were well watered, whole plant biomass increased gradually from 0.60 g at the beginning to 4.21 g plant^{-1} at 100 DAW. Severe drought (SD) substantially reduced plant biomass in relation to control plants from 0.26 g initially to 2.56 g plant^{-1} at 100 DAW. The difference in plant biomass between the control and SD was significant over the entire 100 days, and that between control and mild drought (MID) was significant after 80 DAW; it was not significant at 20 DAW, but it was significant after 40 DAW between control and MOD. There was a significant effect of drought × time according to a two-way ANOVA ($p < 0.01$).

There was a similar change trend in leaf growth as whole plant growth, but SD had a more profound effect on leaf biomass (Figure 1b). Leaf–biomass ratio (GLBR, ratio between the green leaf biomass and total plant biomass) decreased gradually before 60 DAW, thereafter, it maintained an approximately constant level until the end of the water-withholding treatments (Figure 1c). There was a significant effect of soil moisture on GLBR

($p < 0.05$) according to a one-way ANOVA. When compared to the well-watered treatment, mild and moderate drought had a minor effect on GLBR at 20 and 40 DAW, but severe drought generally caused GLBR to sharply decline throughout the water-withholding treatment, except at the beginning.

Changes in Leaf Relative Water Content and Water Potential

Figure 2a illustrates the progression of leaf relative water content (RWC) change during the whole water-withholding period. The plants exhibited a similar trend of RWC changes for water treatments overall. When plants were well watered, leaf RWC decreased gradually from 97% at the beginning to 85% at 100 DAW. Severe drought treatments substantially reduced leaf RWC from 92% initially to 78% at 100 DAW. The difference in leaf RWC between the control and SD was significant, but that between control and MID was not significant throughout the 100 days of withholding water treatment, and it was not significant at 20 DAW, but it was significant at 100 DAW between control and MOD.

There was a progressively decreasing trend of change in midday (11:30) leaf water potential, irrespective of soil water treatments (Figure 2b). When plants were well watered, leaf water potential decreased gradually from −0.85 Mpa at 20 DAW to −1.23 Mpa at 100 DAW. Severe drought treatments substantially decreased leaf water potential from −1.73 Mpa initially to −2.60 Mpa on 100 DAW. The reduction caused by MOD and SD was significant, especially at the late growth stage, indicating that the adverse effect of soil drought on leaf status was more profound with plant growth.

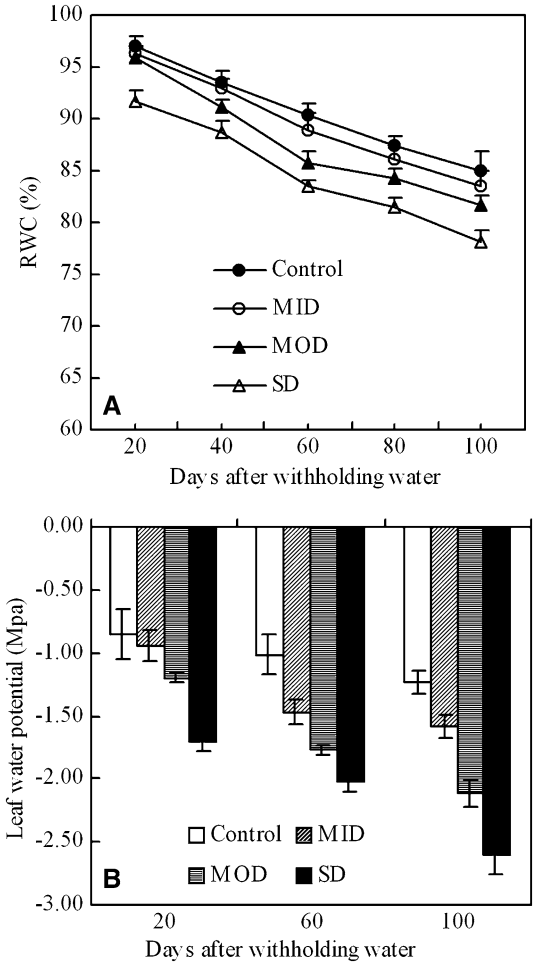

Figure 2. Changes in relative water content (RWC, a) and water potential (b) in leaves of *L. chinensis* under four soil moisture regimes. Values are means ± S.E. of three replications.

Gas Exchange and Chlorophyll Fluorescence

Table 1 shows the effects of soil moisture on leaf gas exchange parameters. As compared with control, MID had no significant effect on the parameters (ns), but MOD and SD significantly decreased net photosynthetic rate (A) by 37% and 56%, stomatal conductance (Gs) by 56% and 75%, respectively. Moderate and severe drought increased intercellular CO_2 concentration (C_i) by 2.3% and 9.2%. The intrinsic water use efficiency (WUE), dividing A by Gs, increased significantly with soil drought (Table 1).

We also determined the effects of soil moisture on leaf chlorophyll fluorescence (Table 2). As compared with control, MID had no significant effect on the parameters (ns), but SD significantly decreased the maximal efficiency of PS II photochemistry (F_v/F_m) by 9%, and the actual quantum yield (Φ_p) by 33% ($p < 0.05$), respectively. Based on the ANOVA, there was a slight effect of soil drought on the photochemical quenching (q_P). The effect of drought on non-photochemical quenching (q_N) was not significant ($p = 0.118$).

Leaf Nitrogen Content

As shown in Figure 3, when plants were well watered, leaf nitrogen contents (N) decreased gradually from 4.4% at the beginning to 3.4% at 100 DAW. Severe drought substantially reduced leaf N from 3.6% initially to 2.5% at 100 DAW. MID slightly stimulated leaf N level at 20 and 60 DAW but not at 100 DAW. As compared to control, both SD and MOD significantly reduced leaf N level for all 100 days.

Table 1. Leaf Gas-exchange Parameters of *L. chinensis* under Four Soil Moisture Regimes

Soil moisture	A (μmol m^{-2} s^{-1})	G_s (mol m^{-2} s^{-1})	C_i (μmol mol^{-1})	WUE (μmol mol^{-1})
Control	16.3 ± 0.98a	0.28 ± 0.07a	236 ± 6b	58.2 ± 2.3c
MID	15.1 ± 1.02a	0.20 ± 0.02a	231 ± 4b	75.5 ± 4.9b
MOD	10.3 ± 0.93b	0.12 ± 0.01b	241 ± 3ab	85.8 ± 5.1b
SD	7.2 ± 1.22c	0.07 ± 0.00c	257 ± 6a	102.9 ± 6.5a
p of ANOVA	0.013	< 0.001	0.045	0.044

A: photosynthetic rate (μmol m^{-2} s^{-1}), G_s: stomatal conductance of CO_2 (mmol m^{-2} s^{-1}), C_i: sub-stomatal CO_2 (μmol mol^{-1}), WUE: water use efficiency (μmol CO_2 mol^{-1} H_2O). Measured on 40–43 day after withholding water.
The data are expressed as mean ± S.E. for six independent determinations.
The values followed by the same letter are not significantly different between soil water treatments at $p < 0.05$ according to Duncan's multiple range tests.

Table 2. Leaf Chlorophyll Fluorescence Parameters of *L. chinensis* under Four Soil Moisture Regimes

Soil moisture	F_v/F_m	Φ_p	q_P	q_N
Control	0.79 ± 0.02a	0.35 ± 0.03a	0.46 ± 0.02	0.77 ± 0.02
MID	0.78 ± 0.02a	0.35 ± 0.02a	0.47 ± 0.02	0.77 ± 0.01
MOD	0.75 ± 0.02a	0.29 ± 0.01b	0.45 ± 0.01	0.77 ± 0.03
SD	0.71 ± 0.02b	0.24 ± 0.02c	0.41 ± 0.02	0.78 ± 0.03
p of ANOVA	0.029	0.010	0.070	0.118

F_v/F_m: the maximal efficiency of PSII photochemistry, Φ_p: the actual quantum yield, q_P: photochemical quenching coefficient, q_N: non-photochemical quenching coefficient. Measured on 40 day after withholding water. The data are expressed as mean ± SE for four independent determinations. The values followed by the same letter are not significantly different between soil water treatments at $p < 0.05$ according to Duncan's multiple range tests.

Soluble Protein and Free Amino Acid Contents

As shown in Figure 4a, at control and MID, leaf soluble protein content remained at a nearly constant level at 20 and 40 DAW and decreased gradually thereafter. For MOD and SD, it decreased gradually during the whole water-withholding treatment. The differences in leaf soluble protein contents were not significant between well-watered and MID, but the differences were significant between well-watered and MOD or SD during the entire water-withholding period, indicating that severe drought limits the accumulation of leaf soluble protein. FAA accumulation was stimulated by MID and MOD stresses at 20 and 40 DAW, and the stimulation disappeared at the end of withholding water (Figure 4b). Severe drought significantly reduced FAA content after 40 DAW, and MOD did after 60 DAW, but MID did not significantly affect it compared to controls.

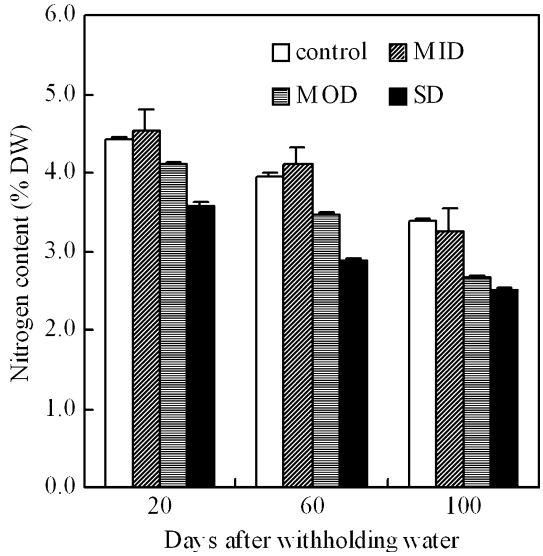

Figure 3. Changes in leaf nitrogen contents of *L. chinensis* under four soil moisture regimes. Values are means ± SE of three replications.

Nitrate Reductase, Glutamine Synthetase, Glutamate Dehydrogenase Activities

As expressed on the basis of dry weight (DW), when plants were well watered and subjected to MID, nitrate reductase (NR) activity remained at a relatively constant level at 20–40 DAW, decreased rapidly until 60 DAW, and leveled off thereafter (Figure 5a). For MOD and SD treatments, NR

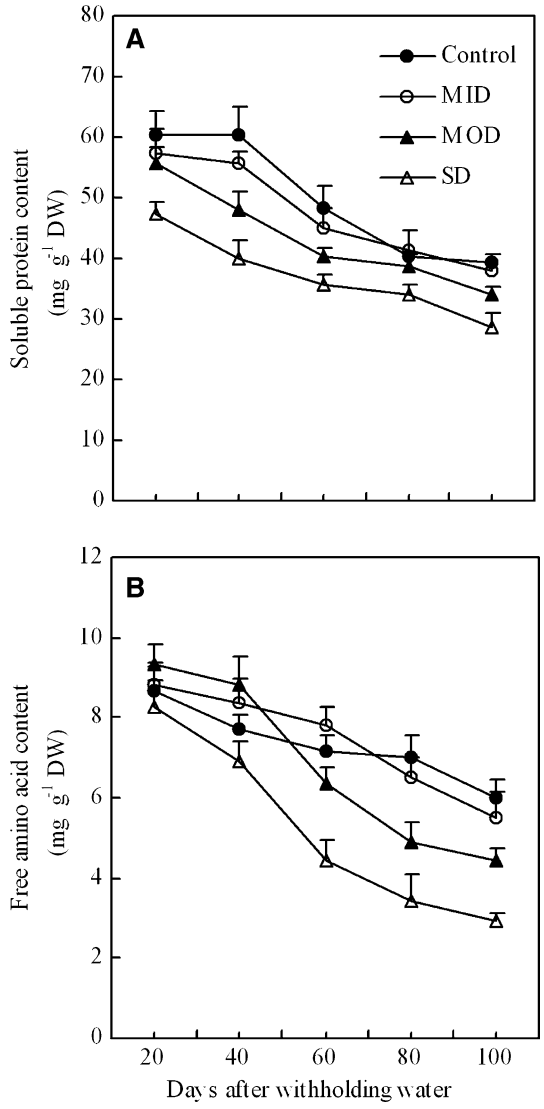

Figure 4. Changes of soluble protein (a) and total free amino acid (FAA; b) contents in leaves of *L. chinensis* under four soil moisture regimes. Values are means ± SE of three replications.

activity decreased gradually during the whole water-withholding period. The differences in NR activity were not significant between well-watered and MID, but were significant between well-watered and SD during all water-withholding periods, indicating that severe drought limits the NR activity.

There was a slightly decreased trend in the change of GS activity at sufficient soil water and MID treatments during the whole water-withholding period, but GS activity at MOD and SD sharply decreased with plant growth (Figure 5c). The difference in GS activity was not significant between well-watered and mild drought treatments, but it was significant between well-watered and MOD or SD after 40 DAW, indicating that severe drought inhibits GS activity. The changes in glutamate dehydrogenase (GDH) were similar to that of GS, except severe drought had a greater adverse effect (Figure 5e). As expressed on the basis of protein content, although control and MID had complex effects, MOD and SD significantly decreased the activities of the three key enzymes after 60 DAW (Figure 5b, 5d, and 5f).

Asparaginase and Endopeptidase Activities

Figure 6a shows the progression of asparaginase (AS) activity changes during 100 days after withholding water, as expressed on the DW basis. The AS activity increased gradually with plant growth. Soil drought increased AS activity during the whole water-withholding treatment. Compared to the control, SD significantly increased AS activity by 5% and 26%, at the starting point and the end of the water-withholding experiment based on dry weight, respectively. There was a similar change trend when the activity unit was expressed on the basis of protein content (Figure 6b), indicating that severe water stress clearly stimulates the catabolism of asparagines.

We also determined the effects of soil moisture on the changes of endopeptidase (EP) activity (Figure 6c and 6d). The trends of the changes in EP activities based on DW were similar to those expressed on the basis of protein content. Endopeptidase activity increased slightly under control and MID with plant growth, but it increased rapidly from 20 DAW to 60 DAW under MOD and SD. MID stress had a minor effect at the beginning (20 DAW), but thereafter, MOD, particularly SD, significantly increased EP activity until the end of the water-withholding experiment.

Malondialdehyde Contents

As shown in Figure 7, malondialdehyde (MDA) contents under soil water treatments overall were relatively constant from 20 to 40 DAW; thereafter, MOD and SD significantly increased it as compared with ample soil moisture, but control and MID only had a minor affect on MDA contents. The difference in MDA content was not significant between control and MID, but it was significant between control and SD during the whole water-withholding period. As compared with the control, MOD and SD increased MDA contents by 17% and 33% at the beginning, and 32% and 50% at the end of the water-withholding treatment, respectively; implicating that soil

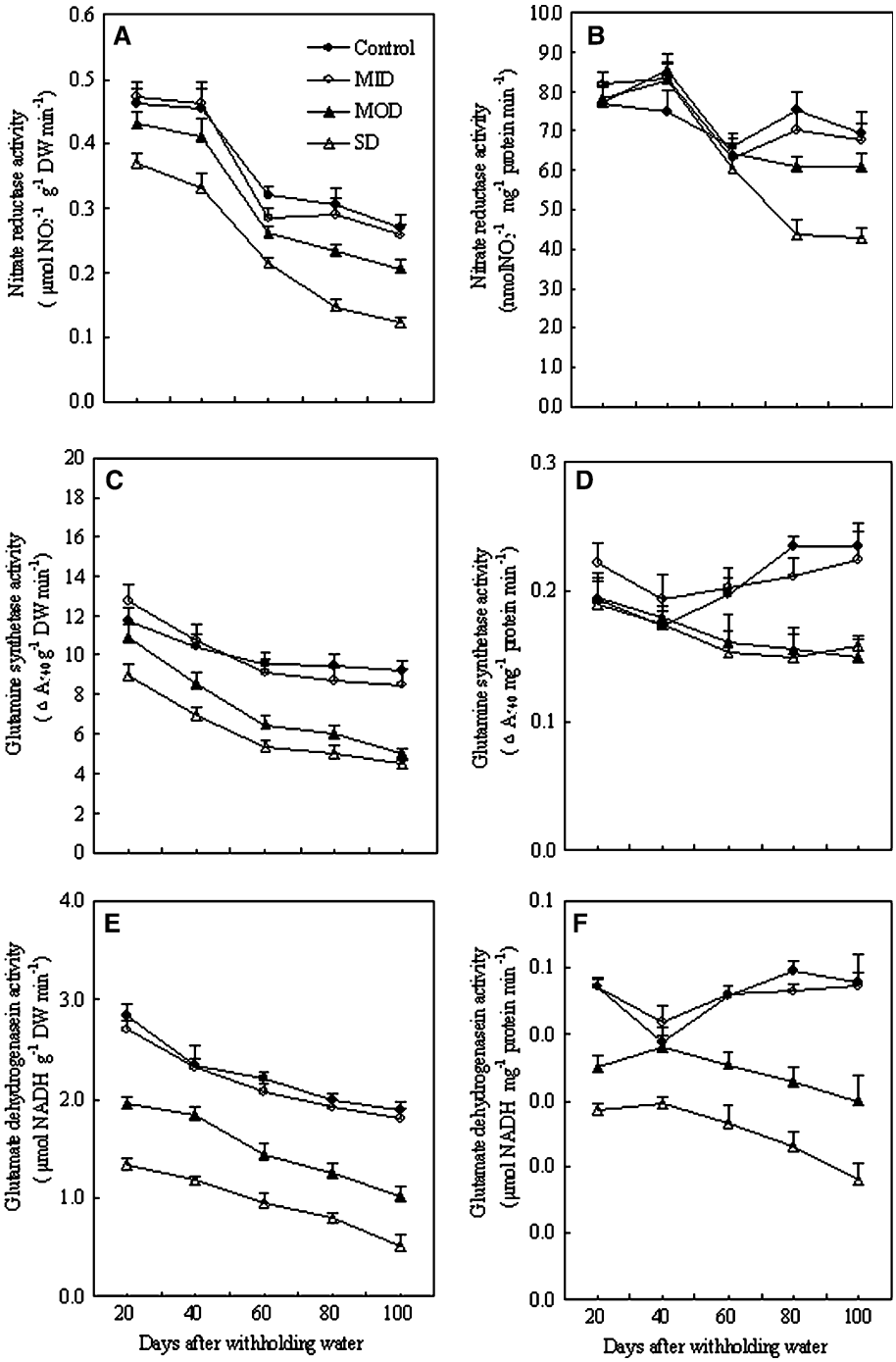

Figure 5. Changes of nitrate reductase (NR, a, b), glutamine synthetase (GS, c, d) and glutamate dehydrogenase (GDH, e, f) activities in leaves of *L. chinensis* under four soil moisture regimes, expressed on the basis of dry weight (a, c, e) and protein content (b, d, f). Values are means ± SE of three replications.

drought gradually accentuates injury to leaf cell membranes through lipid peroxidation with plant growth.

Discussion

Our results confirmed early reports that showed a decrease in leaf nitrogen content (N) (Sinclair and others 2000), activities of nitrate reductase (NR, Somers and others 1983) and glutamine synthetase (GS, Sibout and Guerrier 1998), as well as in photosynthesis (Kaiser 1987; Chaves and others 2003) when plants were subjected to severe drought (SD). The present study also showed that SD obviously reduced the activities of the key enzymes related to nitrogen anabolism, increased those of catabolism, enhanced lipid peroxidation, and adversely affected

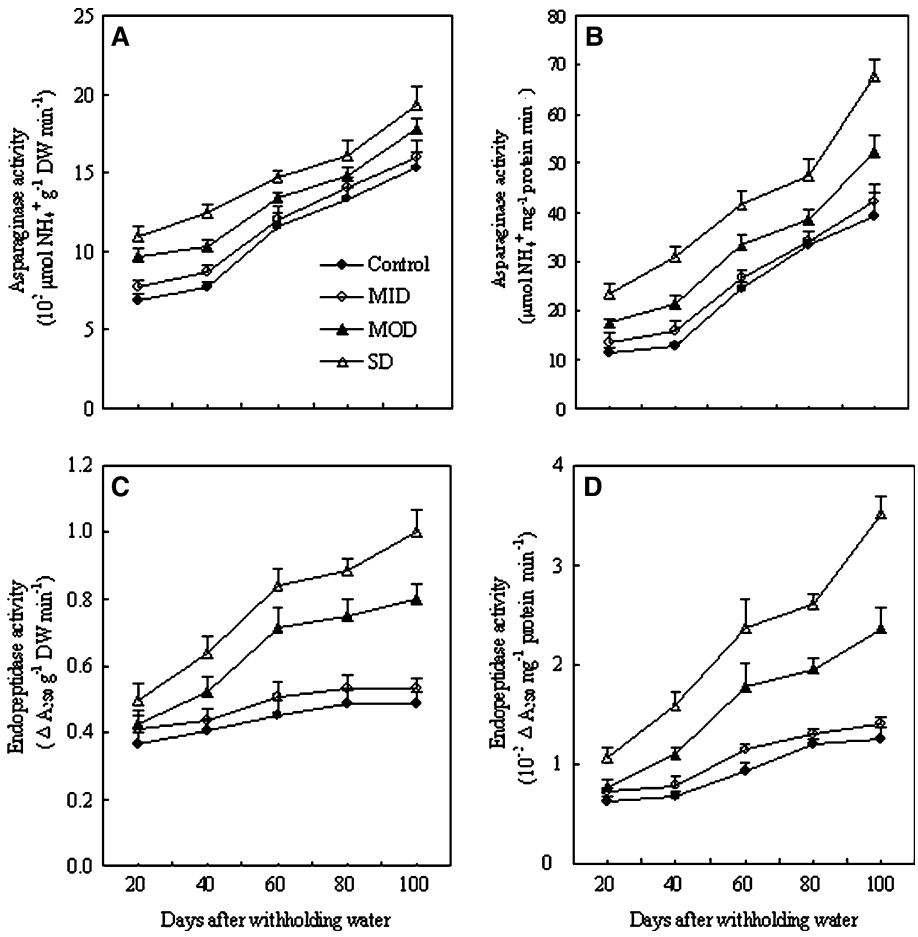

Figure 6. Changes of asparaginase (AS, a, b) and endopeptidase (EP, c, d) activities in leaves of *L. chinensis* under four soil moisture regimes, expressed on the basis of dry weight (a, c) and protein content (b, d). Values are means ± SE of three replications.

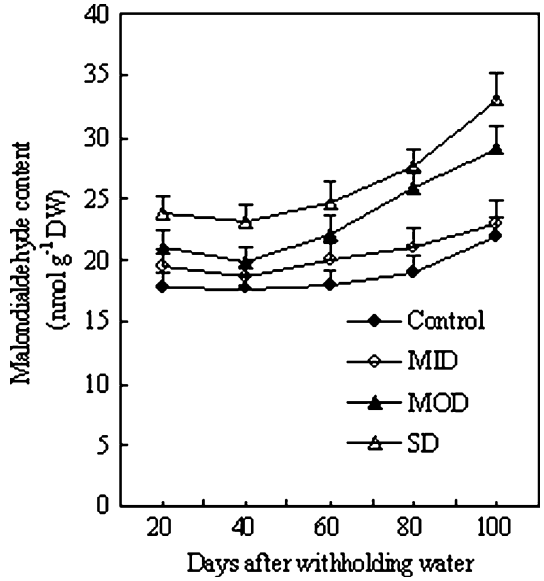

Figure 7. Changes of malondialdehyde (MDA) content in leaves of *L. chinensis* under four soil moisture regimes. Values are means ± SE of three replications.

chlorophyll fluorescence. The results suggest that the key enzymes of nitrogen metabolism may play an important role in the photosynthetic acclimation of plant to soil drought with plant growth.

Mild drought did not decrease plant photosynthesis (Heitholt and others 1991). As the plant is subjected to SD, however, components of the photosynthetic apparatus such as chloroplasts are injured, and photosynthetic capacity decreases, resulting in a decline in the net photosynthetic rate (A) (Kaiser 1987; Chaves and others 2003; Souza and others 2004; Marques da Silva and Celeste 2004), and the function of proteins related to plant photosynthesis (Srivali and Renu 1998), as well as an enhancement of peroxidation of mesophyll cell membranes (Zhang and Kirham 1994). The present results indicated that SD not only led to stomata closure, but also the increased intercellular CO_2 concentration (C_i), which was associated with a reduction in A and in the maximal efficiency of PS II photochemistry (F_v/F_m), as well as the actual quantum yield (Φ_p), implicating that, stomatal and non-stomatal limitation to photosynthesis at SD may occur simultaneously. Additionally, the

intrinsic water use efficiency (WUE) increased significantly with soil drought (Table 1), indicating that an increase in WUE from stomatal closure may outweigh a decrease in WUE due to inhibition of photosynthetic function under drought.

Leaf nitrogen content is important because it is associated with the CO_2 assimilatory capacity of crops, and in grasslands, it is an important determinant of forage nutritive value (Sinclair and others 2000). Photosynthetic rate and Rubisco activity increase with increasing leaf nitrogen. Thus, the percentage leaf N can be used to estimate photosynthetic capacity (Evans 1983; Llorens and others 2003; Takashima and others 2004). In elevated CO_2 studies, plants under N deficit also show low photosynthetic capacity (Ainsworth and others 2003; Bondada and Syvertsen 2005), suggesting leaf N plays a key role in photosynthetic acclimation to environmental variables. Leaf N decreased gradually with time, and with intensifying soil drought (Figure 3), agreeing with reports by Sinclair and others (2000), and Llorens and others (2003). This reduction in leaf N is closely associated with leaf senescence (Evans 1983; Kichey and others 2006; Xu and others 2006; Pommel and others 2006), which can be related to damage to the components of the photosynthetic apparatus, such as chloroplast thylakoid membranes (Kołodziejek and others 2003). In addition, soluble protein concentration could serve as an index for Rubisco protein and activity, because mean Rubisco protein concentrations in plant leaves comprise around 30%–50% of total soluble protein (Sicher and Bunce 1997; Long and others 2006). The decline in soluble protein content was also observed during drought, especially at later stages (Figure 4a). It is suggested that there is an N-deficient stress under severe soil drought, especially at later stages, and that it affects photosynthetic capacity.

Amino acid synthesis in photosynthesizing cells involves both carbon and nitrogen metabolism (Solomonson and Barber 1990; Lam and others 1996), and amino acids may act as feedback regulators of nitrate ion uptake and assimilation (Walch-Liu and others 2005). Reduction of nitrate ions by NR forms nitrite, which is further reduced to ammonia by nitrite reductase; the reductant is from photosynthetic electron transport (Lawlor 2002). Nitrogen reductase activity is decreased by water stress (Hsiao 1973; Kaiser 1987, Figure 5a and 5b). However, it is suspected that drought may have adverse effects on both NR expression and its activity (Mahan and others 1998; Baki and others 2000). Protecting NR activity through maintaining a constant protein and nitrogen content may improve plant growth under water stress (Singh and Usha 2003). Glutamine synthetase activity can be used as a marker to predict the N status of wheat (Kichey and others 2006). When poplar leaves were submitted to water stress for 18 h, GS activity decreased by about 50% (Sibout and Guerrier 1998). However, Venekamp (1989) reported that the activity of GS could be inhibited, but that of glutamate dehydrogenase (GDH) was stimulated by water stress. The inhibition in GS activity may be due to the lack of ATP under water stress; although the evidence is lacking, an alternative route of glutamate synthesis may be via GDH, an abundant enzyme linked to catabolic activities (for example, protein degradation) (Lawlor 2002). However, some studies provided evidence that GDH does not represent a significant alternate route for glutamate formation in plants (Suzuki and Knaff 2005). In the present study, SD markedly reduced leaf N and soluble protein contents, and the activities of three enzymes (NR, GS, and GDH) related to nitrogen anabolism and enhanced activities of two enzymes (asparaginase [AS] and endopeptidase [EP]) related to nitrogen catabolism, suggesting that the response of leaf N status and nitrogen metabolism to soil drought also may play an important role in decreasing photosynthetic capacity. Further research is needed on the complex role of these enzymes under environmental stress factors.

Nitrogen anabolism may couple with assimilation of other nutrient elements. For example, adenosine 5′-phosphosulfate reductase (APR) is inhibited by nitrogen source deficiency, indicating that sulfate reduction is regulated by nitrogen nutrition (Koprivova and others 2000). And sulfur (S) redistribution in soybean plants is strongly regulated by nitrogen availability (Sunarpi and Anderson 1997), although S is relatively immobile in plants as the proportion of S redistributed from leaf tissue is considerably smaller than that of N (Eriksen and others 2001). Utrillas and others (1995) reported that nitrogen, phosphorus, and S in a sward of *Cynodon dactylon* (L). Pers. showed low concentrations during summer drought. In beech ecotypes, phosphorus and phosphate (Pi) are decreased more under soil drought than N and S, which may be responsible for the lower phosphate mobility in the substrate due to lower water availability (Peuke and Rennenberg 2004). The decrease in the ratio of phosphate to phosphorus in tissues under water stress indicates the use of vacuolar phosphate pools for maintaining organic phosphorus homeostasis. Inadequate Pi supply to the chloroplast would limit ATP synthesis and RuBP regeneration under drought (Lawlor and

Cornic 2002). However, study of their metabolic mechanisms in relations to photosynthesis under drought may be required in the future.

Malondialdehyde (MDA) content did not obviously increase in some drought-stressed plants, showing better protection against oxidative stress under water-limited conditions (Parida and others 2004; Ramachandra Reddy 2004). However, in olive leaves, lipid peroxidation levels increased slightly at MID and they subsequently rose at SD (Sofo and others 2004), this result was confirmed in *L. chinensis* by our study. The enhanced oxidative stress is related to a reduction in photosynthetic capacity as plants age (Munné-Bosch and Alegre 2002). Reactive oxygen species (ROS) production increases under drought (Price and Hendry 1991; Sguerri and others 1993), and the decrease in the photochemical quenching coefficient (q_P) is accompanied by an increase in the formation of singlet oxygen ($^1O_2$), leading to an increase in photoinhibition (Foyer and Harbison 1994; Roháček 2002). Recently, Saneoka and others (2004) reported that MDA concentration decreases with increased N application in water-stressed bentgrass plants, indicating that higher levels of N nutrition may have contributed to drought tolerance in plants by preventing cell membrane damage. On the other hand, N deficit may decrease q_P, and increase susceptibility to photoinhibition (Lu and Zhang 2000). Thus, in the present experiment, an increase in MDA accumulation in parallel with a decline in the level of N nutrition suggested that lipid peroxidation and N deficit may, together, be responsible for a decrease in the photochemical capacity of PSII induced by SD.

Moderate stress refers to a lowering of relative water content (RWC) by more than 10 but less than 20 percentage points, and severe stress is a lowering of RWC by more than 20 percentage points (Hsiao 1973). In the present experiment, the change range of leaf RWC was relatively narrow under soil drought (Figure 2a). Soil moisture levels were maintained at 5:00 pm daily. Yang and others (2004) indicated that wheat plants subjected to water deficit could rehydrate overnight. Leaf material in the present experiment was sampled at 9:00 a.m. for the measurement of RWC, and at the same time for determining enzyme activities. Thus, there was less RWC change relative to leaf water potential at midday (Figure 2b). Moreover, the response of metabolic activity such as NR may be different in plants subjected to long-term-drought compared to those plants subjected to rapid dehydration (Kaiser 1987).

Finally, the sensitivity of the response to RWC depends on species (Hsiao 1973; Kaiser 1987; Lawlor 2002). For example, Lawlor (2002) suggested that the responses of mesophytes to RWC change are divided into two types. In the Type 1 response, potential photosynthetic rate (A_{pot}) is unaffected until a 20%–30% decrease in RWC occurs. However, in the Type 2, A_{pot} decreases linearly with decreasing RWC, showing progressive metabolic changes, probably related to limitation of ATP synthesis (Lawlor 2002). The present results indicated that the change in A , Gs, and F_v/F_m decreased linearly with loss of RWC, suggesting that this species seems to exhibit a Type 2 response. However, further investigation may be required concerning this issue.

Differences were observed in net photosynthetic rate and chlorophyll fluorescence as well as nitrogen content at different plant growth stages (Delgado and others 1994; McDonald and Paulsen 1997). Changes in concentrations of sugars and proline were different between mature cassava and expanding leaves under water deficit, and the magnitude of water stress effects depends on the stage of leaf development (Alves and Setter 2004). Hadži-Tašković Šukalivić (1986) also reported that the activities of GS and GDH in the developing maize kernel increases with grain filling. The present results showed that the activities of key nitrogen assimilation were weakened and lipid peroxidation of mesophylls cell was enhanced with plant development, suggesting that the grass plant may senesce at a late growth stage.

It is noted that, in the present experiment, *L. chinensis* were grown in pots over 4 months with a density of 6 plants per pot, without any fertilizer. The plants may become progressively N limited, even in well-watered treatments. The plants became progressively resource limited in a manner that compromises the ability of the experiment to address the drought effect. Thus, the soil drought affecting plant growth and metabolism may result from the resource limitation in the pot, especially at later growth stages. However, plants subjected to ample soil water absorbed more nitrogen from soil related to drought soil, consequently, drying soil may retain more N than wet soil (Heckathorn and Delucia 1994; Chen and others 2005). This may lead to more N limitation in ample soil moisture than drought conditions so that the leaf N of control treatments may retain relatively minor changes with plant growth, accordingly resulting in other relative parameter changes. Furthermore, drought can induce plant senescence, particularly at late plant growth stages (Evans 1983; Kichey and others 2006). Thus, nitrogen limitation and senescence may interact with drought, depending on plant

species (Chaves and others 2003; Pommel and others 2006). Nevertheless, further study is needed on the issue in larger experimental systems and in natural field conditions.

The present results indicate that severe soil drought accentuated the adverse effects of nitrogen metabolism by regulating the activities of key enzymes involved in nitrogen assimilation and catabolism, and led to cell membrane damage and the reduction of photosynthetic capacity, suggesting that the key enzymes of nitrogen metabolism would play a key role in the photosynthetic acclimation of plants to drought stress, particularly at late growth stages. Our findings that photosynthetic adaptation of *L. chinensis* to severe drought is negatively affected by N limitation and metabolism is of great physiological significance, because *L. chinensis* grows in regions with severe water scarcity combined with simultaneously high temperature during the growing seasons, and a co-occurrence of water deficits and N limitation is common in the grasslands (Chen and others 2005). Resistance of the grass to water stress must be increased to improve the characteristics related with key enzymes of N metabolism.

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