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Effect of nodules on dehydration response in alfalfa (*Medicago sativa* L.)

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ABSTRACT

Drought is a key abiotic stress that negatively affects growth and development as well as symbiotic nitrogen fixation in alfalfa (*Medicago sativa* L.). To understand whether nodulation would affect drought stress response in alfalfa, we analyzed the lipid peroxidation, activities of antioxidant enzymes including superoxide dismutase (SOD), and catalase (CAT), contents of superoxide anion radical, non-enzymatic antioxidants including reduced glutathione (GSH) and proline, total protein, and soluble sugar in dehydration-stressed alfalfa. Three-month-old alfalfa plants without nodule, with active nodules, or with inactive nodules were dehydrated for 0, 1, 2, 4, 6, 8, and 10 h. We found that roots and leaves from plants with nodules, especially with active nodules, showed less lipid peroxidation which was associated with higher CAT activities and higher levels of GSH. Roots and leaves with active nodules also accumulated less free proline and soluble sugar compared to plants without nodules, suggesting that proline and soluble sugar may have a limited role in osmotic adjustment in these plants. The results suggested that active nodules may have a positive effect on drought stress tolerance in alfalfa.

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1. Introduction

Drought stress, one of the major abiotic stresses, limits plant growth and production (Boyer, 1982). For legumes, drought stress not only limits plant production but also impairs nodule function (Ashraf and Iram, 2005; Clement et al., 2008). Studies have shown that drought stress negatively affected legume-rhizobia symbiosis, thus reducing the number of nodules (Ashraf and Iram, 2005). Drought stress also shortened longevity of nodules by inducing premature senescence of nodules (Puppo et al., 2005), and reduced the rate of nitrogen fixation in nodules (Sprent, 1971). When water is limited, transport of nitrogen-products away from the nodule was hindered (Ramos et al., 2003), also resulting in feed-back inhibition of nitrogen-fixing activity of root nodules (Ashraf and Iram, 2005).

Drought stress altered metabolic activities in nodules and plants (Clement et al., 2008). Nodulated alfalfa plants responded to temporary drought by osmotic adjustment, resulting in enhanced tolerance (Antolin and Sanchez-Diaz, 1992). Severe drought stress, however, reduced the leghemoglobin content in nodules and impaired nitrogen fixation activity (Figueiredo et al., 2008). One of the major consequences of altering metabolic activities in drought-stressed plants is a change in redox homeostasis, leading to oxidative stress via the production and accumulation of reactive oxygen species (ROS) (Franca et al., 2007). Legume nodules contain a variety of antioxidant enzymes and metabolites to prevent the formation of ROS and subsequent oxidative stress (Becana et al., 2001; Matamoros et al., 2003).

Alfalfa (*Medicago sativa* L.), an important legume forage plant worldwide, can be cultivated on marginal lands with a high yield and good quality due to its high-protein content. Its deep-root system can help avoid drought-induced damage in semi-dry lands (Moran et al., 1994). Alfalfa plants are able to fix nitrogen in symbiotic association with rhizobia. The interaction between alfalfa and rhizobia involves a complex molecular interaction that leads to the development of a novel plant organ, the root nodule (Gage, 2004).

Although the effects of drought on alfalfa growth and production, symbiotic interaction with rhizobia, and symbiotic nitrogen-fixation are relatively well studied, effect of nodulation on plant response to drought is poorly understood (Boisson-Dernier et al., 2001; Gage, 2004; Jones et al., 2007). In this study, we compared dehydration stress responses in alfalfa that had no nodule, with inactive nodules, or active nodules, by assessing the degree of lipid peroxidation, the changes in the content of superoxide anion radical, soluble sugar, total protein, non-enzymatic antioxidants, and the activities of antioxidant enzymes in roots and leaves.

Abbreviations: ROS, reactive oxygen species; RH, relative humidity; TBA, thiobarbituric acid; TCA, trichloroacetic acid; MDA, malondialdehyde; PBS, potassium phosphate buffer; PVP, polyvinylpyrrolidone; $O_2^{\bullet -}$, superoxide anion radical; SOD, superoxide dismutase; NBT, nitroblue tetrazoliun; EDTA-Na_2, ethylenediaminetetraacetic acid disodium salt; CAT, catalase; H_2O_2, hydrogen peroxide; GSH, reduced glutathione; DTNB, dithiobis-2-nitrobenzoic acid; LSD, least significant differences.

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2. Materials and methods

2.1. Plant culture and treatment

Seeds of alfalfa (*M. sativa* L. cv. algonquin) were surfaced sterilized with 70% ethanol for 5 min and rinsed 4–5 times with sterile water. The seeds were germinated on wet filter paper in Petri dishes in a dark growth chamber at 25/15 °C, 50/70% RH (day/night). Three-day-old seedlings were transplanted to plastic pots (7 by 31 cm) filled with sterilized sandy soil in the greenhouse. Before transplanting, the sandy soil was washed thoroughly with tap water to remove nutrients (Labidi et al., 2009). After transplanting, 2/3 of the seedlings were inoculated with *Rhizobium meliloti* strain Dormal, and 1/3 of the seedlings were not inoculated. The non-inoculated seedlings were watered with 1/4 strength Hoagland (Hoagland and Arnon, 1950) nutrient solution every day. The inoculated seedlings were divided into two groups. One group was watered with 1/4 strength Hoagland nutrient solution. These plants developed nodules without nitrogen fixation activity since the nitrogen in the nutrient solution inhibits the activity of *R. meliloti* strain Dormal. The other group was watered with N-free nutrient solution (Evans, 1974) daily. These plants developed functional nodules. Plants were cultured in the greenhouse with the average temperature of 30 ± 5 °C and 20 ± 3 °C, and the relative humidity of 55 ± 5% and 70 ± 5% during day and night, respectively. Ninety days after inoculation, the plants were subject to dehydration stress. Sand was first gently washed away from roots. The roots were immediately wrapped in a wet tissue paper (18 cm × 18 cm). The plants were then placed on a rack for dehydration stress. Since transpiration (photosynthesis) continued in shoots, which consumed the water in the tissue paper, plants started to wilt after a few hours. Leaves and roots were harvested before dehydration stress (0 h), and 1, 2, 4, 6, 8, and 10 h after dehydration stress. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. The experiment was repeated three times.

2.2. Analysis of lipid peroxidation

Lipid peroxidation was measured using a modified thiobarbituric acid (TBA) method (Puckette et al., 2007; Wang et al., 2009). Roots or leaves were ground in 5 mL of 5% (w/v) trichloroacetic acid (TCA) with a mortar on ice. The homogenate was centrifuged at 3000 × *g* for 15 min, and 2 mL of the supernatant fraction was mixed with 5 mL of 0.5% TBA in 5%TCA. The mixture was heated at 100 °C for 15 min, chilled on ice, and centrifuged at 3000 × *g* for 15 min. The absorbance of the supernatant was measured at 450, 532, and 600 nm. The concentration of malondialdehyde (MDA) was calculated as a measure of lipid peroxidation.

2.3. Determination of total protein and superoxide anion radical content and antioxidant enzyme activities

Roots and leaves of alfalfa plants were homogenized on ice with mortar and pestle in a 0.1 M potassium phosphate buffer (PBS) (pH7.8) within 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 × *g* for 15 min in a refrigerated centrifuge. The supernatant was collected and immediately used for enzyme assays. Total protein concentration was determined according to the Bradford (1976) using bovine serum albumin as a standard.

For the content of superoxide anion radical ($O_2^{\bullet -}$), Elstner's method (1976) was used with some modifications. One milliliter of the supernatant was mixed with 0.75 mL of 0.65 M PBS (pH7.8) and 1 mL of 10 mM hydroxylamine hydrochloride. The mixture was incubated at 25 °C for 20 min, then mixed with 1 mL of 17 mM sulfanilic acid and 1 mL of 7 mM α-naphthylamine, and incubated again at 30 °C for 30 min. The absorbance was measured at 530 nm using sodium nitrite as standard.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured by nitroblue tetrazoliun (NBT) method (Giannopolitis and Ries, 1977). The reaction mixture consisted of 1.5 mL of 50 nM PBS (pH7.8), 0.3 mL of 130 mM L-methionine, 0.3 mL of 750 μM NBT, 100 μM EDTA-Na_2, 0.3 mL of 20 μM riboflavin and 0.3 mL of enzyme extract. The test tubes containing the reaction mixture were kept for 30 min under 4000 l× (light intensity) at 25 °C. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction under the assay condition.

The activity of catalase (CAT, EC 1.11.1.6) was measured following the method of Maehly and Chance (1954) with some modifications reported by Raza et al. (2007). The final volume of the reaction mixture (3 mL) contained 50 nM PBS (pH 7.0) and 200 mM H_2O_2. The reaction was started by adding the enzyme extract, and change in absorbance of the reaction solution at 240 nm (reduction of H_2O_2) was read every 30 s. One unit of CAT activity was defined as an absorbance change of 0.01 unit min^{-1}.

2.4. Determination of non-enzymatic antioxidants

The level of reduced glutathione (GSH) was fluorometrically estimated (Hissin and Hilf, 1976) with some modifications. Roots or leaves were ground in 3 mL of 5% H_3PO_3. The homogenate was centrifuged at 14,000 × *g* for 10 min. The reaction mixture (3 mL), containing 2.6 mL of 150 mM PBS (pH 7.0), 0.2 mL of 0.1 mM dithiobis-2-nitrobenzoic acid (DTNB), and 0.2 mL of supernatant, was incubated at 30 °C for 5 min. The absorbance of the mixture was measured at 412 nm.

Proline content was determined spectrophotometrically following the description by Bates et al. (1973). Roots or leaves were homogenized with 10 mL of 3% sulfosalicylic acid, and the homogenate was centrifuged at 3000 × *g* for 20 min. Two milliliter of the supernatant was mixed with 2 mL of glacial acetic acid and 2 mL of acidic ninhydrin reagent, and boiled for 1 h. The tubes were cooled, and 5 mL of toluene were added, and the absorbance at 520 nm was determined.

2.5. Determination of soluble sugar content

To determine the soluble sugar content, a method described by Dreywood (1946) was followed. Roots or leaves were heated at 80 °C for 48 h to constant weight. The dry tissues were incubated in 80% ethanol at 80 °C for 30 min and centrifuged at 3500 × *g* for 10 min. Two milliliter of the supernatant was mixed with 5 mL of anthrone reagent was boiled for 10 min. The absorbance at 620 nm was determined.

2.6. Statistical analysis

All the assays described above were repeated at least four times on four biological replicates. The data were subjected to analysis of variance (ANOVA) to discriminate significant differences, and the least significant differences (LSD) of means were determined by using Ducan's test at the level of significance (defined as $\alpha = 0.05$).

3. Results

3.1. Changes in lipid peroxidation under dehydration

To investigate whether nodulation will change oxidative damage in alfalfa, we examined the content of MDA in alfalfa as an indicator of lipid peroxidation. Six types of tissues were examined and compared, roots without nodule (RNN), roots with inactive

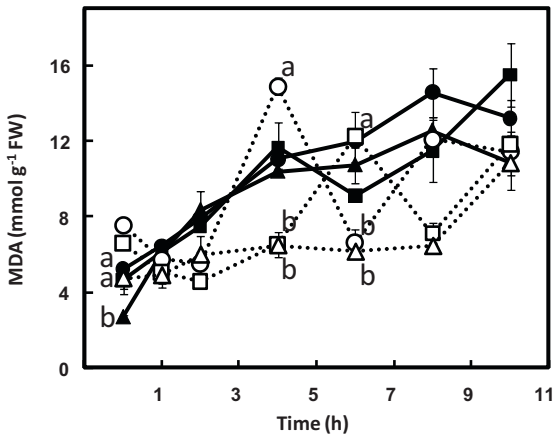


Fig. 1. Changes in the MDA level in roots and leaves of alfalfa without nodules (RNN = ▲; LNN = △), with inactive nodules (RIN = ●; LIN = ○), or with active nodules (RAN = ■; LAN = □) under dehydration stress. Three-month-old plants were subjected to dehydration stress, and tissues were harvested before dehydration stress (0 h) and 1, 2, 4, 6, and 10 h after dehydration for analysis. Data are shown as means ± SE of four independent experiments. Different letters indicate that the mean values are significantly different among the treatments ($\alpha = 0.05$). Letters on the left and right of the symbols indicate significant difference among root samples and among leaf samples, respectively. Some error bars are smaller than symbols.

nodules (RIN), roots with active nodules (RAN), leaves from the plants without nodule (LNN), leaves from the plants with inactive nodules (LIN), and leaves from the plants with active nodules (LAN). Roots and leaves started with the similar levels of MDA, except RAN showed the lowest MDA level. In general, the levels of MDA in both root and leaf tissues increased with time as dehydration stress progressed. It has to be noted that the changes were expressed on the basis of fresh weight and might be interfered by water loss itself. Therefore we emphasized the difference in response of the same type tissues under different treatments. Roots and leaves from the plants with nodules, whether the nodules are active or not, showed lower levels of MDA, especially at the late stages of dehydration. LAN showed the consistently lowest MDA level (Fig. 1).

3.2. Changes in the protein and superoxide anion radical content, and antioxidant enzyme activities under dehydration

Leaf tissues showed higher protein contents than root tissues. The protein content increased after dehydration treatment in both roots and leaves. The RNN tissues showed the greatest protein increase, up 28-fold, compared to that at 0 h. There was significant difference in the amount of protein among LNN, LIN, and LAN before dehydration and between RNN and RIN/RAN 6 h after dehydration (Fig. 2A). In both cases, the total protein content from LAN and RAN were at an intermediate level.

Dehydration stress resulted in an increase in $O_2^{\bullet -}$ radicals with time in both leaves and roots. RNN showed a great increase in $O_2^{\bullet -}$ radicals starting 4 h after dehydration, reaching twice as much as those in RIN and RAN (Fig. 2B). The $O_2^{\bullet -}$ radical content showed a steady increase in LNN starting 2 h after dehydration, with the steepest increase at 10 h after stress. The $O_2^{\bullet -}$ radical content in LAN and LIN tissues showed an alternating increase and decrease during the course of dehydration stress. Overall, it is difficult to judge whether the change in $O_2^{\bullet -}$ radical content in LAN and LIN is significantly different from that in LNN (Fig. 2B).

To better understand how alfalfa plants deal with oxidative stress under dehydration, we measured the enzymatic activities of catalase (CAT) and superoxide dismutase (SOD). As shown in Fig. 2C, there was no significant difference in the CAT activity among different leaf and root tissues before dehydration stress, except for LAN which showed much higher CAT activity than any other tissues. The CAT activity in LAN was higher than those in other tissues at 2 h after dehydration stress and then reached a similar level to LNN. Interestingly, the CAT activity in LIN showed a great increase 6 h after dehydration, remaining at the highest level compared to those in LNN and LAN at 8 and 10 h after stress. The CAT activity in roots was relatively constant during dehydration stress and was at similar levels among different root samples. One exception is that the CAT activity in RAN spiked at 6 h after dehydration stress.

The SOD activity is very similar among LNN, LIN and LAN before dehydration stress and during the first 2 h of dehydration. The SOD activity in LNN was enhanced greatly and was significantly higher

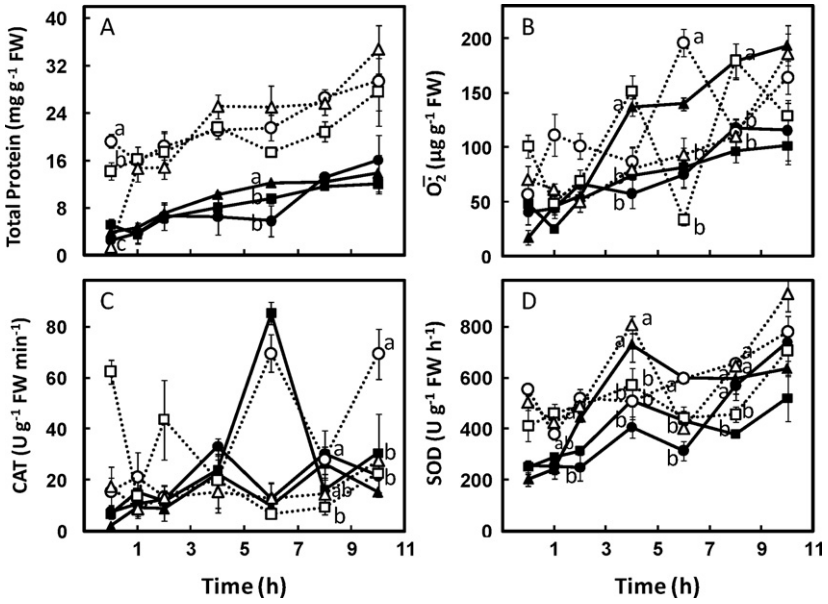


Fig. 2. Changes in the content of total protein (A) and superoxide anion radical (B), and the activities of CAT (C) and SOD (D) in roots and leaves of alfalfa without nodules (RNN = ▲; LNN = △), with inactive nodules (RIN = ●; LIN = ○), or with active nodules (RAN = ■; LAN = ○) under dehydration stress. Three-month-old plants were subjected to dehydration stress, and tissues were harvested before dehydration stress (0 h) and 1, 2, 4, 6, and 10 h after dehydration stress for analysis. Data are shown as means ± SE of four independent experiments. Different letters indicate that the mean values are significantly different among the treatments ($\alpha = 0.05$). Letters on the left and right of the symbols indicate significant difference among root samples and among leaf samples, respectively. Some error bars are smaller than symbols.

than LIN and LAN 4 h after dehydration stress. The SOD activity in LNN was also the greatest among all the tissues 10 h after dehydration stress. In LIN, the SOD activity showed steady increase starting 4 h after stress. The SOD activity in LAN, however, was relatively constant during stress, remaining the lowest compared to LNN and LIN during the last 6 h of stress (Fig. 2D). The SOD activity was lower in roots compared to leaves before dehydration stress. Little change in SOD activity was observed during the first hour of dehydration stress. RNN showed a great increase in SOD activity 2 h after dehydration stress, reaching the highest level 4 h after dehydration stress. RNN maintained high SOD activity at 6, 8 and 10 h after stress. RIN showed significantly lower SOD activity at 2, 4, and 6 h after dehydration stress but reached a SOD level similar to that in RNN at 8 and 10 h after dehydrations stress. RAN only showed a slight increase in SOD activity after dehydration stress and showed the lowest SOD activities at 8 and 10 h compared to RNN and RIN (Fig. 2D).

3.3. Changes in the GSH and proline content under stress

GSH and proline are reducing agents and play important roles in controlling oxidative stress associated with drought and dehydration stress. The GSH level in LIN and LAN was relatively constant during the first 4 h of dehydrates stress and then increased with time. LNN had a lower level of GSH (although not statistically different) before dehydration stress compared to LIN and LAN and showed a decrease in GSH level 1 h after dehydration stress, reaching a level significantly lower than that in LIN and LAN. The GSH level in LNN rose steadily starting 2 h after dehydration stress, reaching similar levels to those in LIN and LAN (Fig. 3A). The GSH levels are lower in roots than in leaves and also increased with time during dehydration stress. RNN started with the highest GSH level among all the root samples before dehydrates stress and showed no difference from other root samples until 8 h after dehydration stress, when it reached the lowest level of GSH compared to RIN and RAN. RAN showed a sharp increase in GSH level at 6 and 8 h after dehydration, reaching the highest level of GSH compared to RNN and RIN at 8 h after dehydration stress. However, the GSH level in RAN dropped sharply at 10 h after dehydration stress, reaching the same level as in RNN. RIN showed a steady increase in GSH during the stress and eventually reached the highest GSH in all the root samples (Fig. 3A).

Leaves and roots had similarly low levels of proline before dehydration and within the first 2 h of dehydration stress. The proline level increased with time afterward. A great increase in proline was observed in leaves at 8 and 10 h after dehydration stress (Fig. 3B). LAN, however, had a significantly lower proline level compared to LNN and LIN at 8 h after stress and remained the lowest level of proline 10 h after stress, although not statistically different, compared to LNN and LIN. The proline level in roots did not show a drastic increase as did in leaves and showed no difference among RNN, RIN, and RAN.

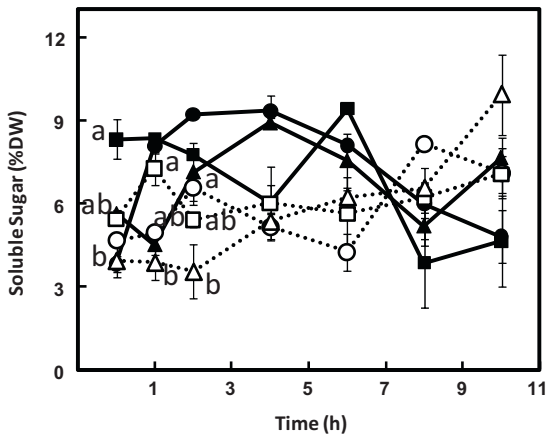


Fig. 4. Changes in the content of soluble sugar in roots and leaves of alfalfa without nodules (RNN = ▲; LNN = △), with inactive nodules (RIN = ●; LIN = ○), or with active nodules (RAN = ■; LAN = □) under dehydration stress. Three-month-old plants were subjected to dehydration stress, and tissues were harvested before dehydration stress (0 h) and 1, 2, 4, 6, and 10 h after dehydration stress for analysis. Data are shown as means ± SE of four independent experiments. Different letters indicate that the mean values are significantly different among the treatments ($\alpha = 0.05$). Letters on the left and right of the symbols indicate significant difference among root samples and among leaf samples, respectively. Some error bars are smaller than symbols.

3.4. Changes in the content of soluble sugar under stress

The soluble sugar content in RAN was the highest compared to that in other tissues but decreased rapidly after 2 h of dehydration stress. The level of soluble sugar in RAN became the lowest at the end of stress treatment. Both RNN and RIN showed an increase in soluble sugar content at the early stages of stress and then decreased 6 h after stress (Fig. 4). The soluble sugar content was the lowest in LNN and remained unchanged within the first 2 h of dehydration and increased with time 4 h after stress, reaching the highest among the leaf samples. The soluble sugar contents in LIN and LAN fluctuated during the course of stress treatment, reaching slightly higher levels at the end compared to those before dehydration stress. The soluble sugar content in RAN and LAN was the lowest among the root samples and leaf samples, respectively, toward the end of the stress treatment.

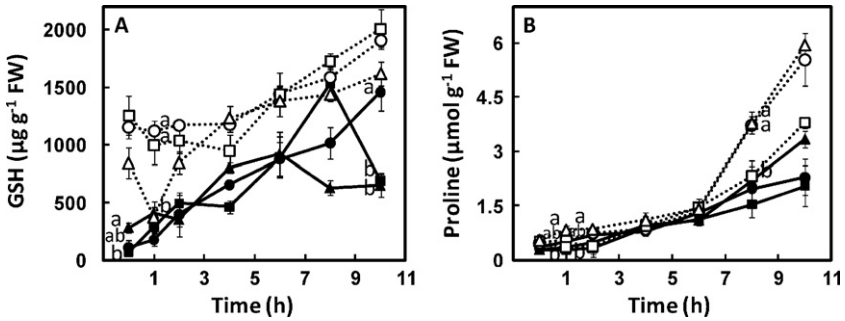


Fig. 3. Changes in the content of GSH (A) and proline (B) in roots and leaves of alfalfa without nodules (RNN = ▲; LNN = △), with inactive nodules (RIN = ●; LIN = ○), or with active nodules (RAN = ■; LAN = □) under dehydration stress. Three-month-old plants were subjected to dehydration stress, and tissues were harvested before dehydration stress (0 h) and 1, 2, 4, 6, and 10 h after dehydration stress for analysis. Data are shown as means ± SE f four independent experiments. Different letters indicate that the mean values are significantly different among the treatments ($\alpha = 0.05$). Letters on the left and right of the symbols indicate significant difference among root samples and among leaf samples, respectively. Some error bars are smaller than symbols.

4. Discussion

Lipid peroxidation in membrane is both a reflection and a measure of stress-induced damage at the cellular level (Demiral and Türkan, 2005). MDA is an end product of lipid peroxidation and has been used extensively as an indicator for free radical-caused membrane injury under various abiotic stress conditions (Alexieva et al., 2001). Our study found that the MDA content in the leaves and roots from the plants with nodules, whether the nodules are active or not, are usually lower than that from plants without nodules. Interestingly, the MDA level was the lowest in LAN and RAN before the stress and at the end of the 10 h-long dehydration stress. The results may reflect an increased ROS scavenging capacity and decreased damage to plasma membrane under dehydration conditions in these nodulated plants, suggesting that alfalfa plants with active nitrogen fixation may tolerate drought stress better.

The $O_2^{\bullet -}$ is one of the main sources of oxidative injury in plants (Scandalios et al., 1997). Our results showed that the $O_2^{\bullet -}$ content in nodulated roots (both RIN and RAN) was lower than RNN, especially at the late stages of the stress (4 h after stress). The $O_2^{\bullet -}$ content in leaves fluctuate greatly under stress and was also lower in LIN and LAN at the end of stress. These results provided the supporting evidence for the less membrane damage (lower MDA levels) in nodulated plants described above.

To cope with oxidative damage under extremely adverse conditions, plants have developed an antioxidant defense system that includes the antioxidant enzymes such as SOD and CAT (Foyer and Noctor, 2005) and the non-enzymatic defense systems such as proline and GSH (Dietz, 2005). SOD has a central role in the antioxidant defense network and increased SOD activity was correlated with increased protection from damage associated with oxidative stress (Asada, 1999). SOD, as a primary scavenger of superoxide anion radicals, can catalyze the conversion of the superoxide anion to H_2O_2 (Smirnoff, 2006). In this study, the SOD activity increased gradually after dehydration stress in all the tissues. The SOD activity in RAN and LAN, however, was generally lower than that in RNN and LNN. These results suggest that the increase in SOD activity in LNN and RNN may be required to reduce the higher $O_2^{\bullet -}$ in the tissue. However, the increase, despite higher SOD activity than those in tissues with nodulation, seemed insufficient to alleviate the $O_2^{\bullet -}$ level and therefore membrane peroxidation (MDA level). Alternatively, nodulated alfalfa may use other antioxidant systems than SOD to keep the $O_2^{\bullet -}$ level low under dehydration stress.

CAT is among the most effective antioxidant enzymes in preventing cellular damage (Scandalios, 1993). CAT converts H_2O_2 into water and oxygen. This enzyme does not require a reducing power and has a high reaction rate. So CAT plays an important role in scavenging ROS and maintaining the physiological redox status of organisms (Cho and Seo, 2005). Our data demonstrated the tissues with active nodules had higher level of CAT activity before dehydration stress and showed the greatest activity during dehydration response, such as in LAN at 2 h after dehydration and RAN at 6 h after dehydration. Although a higher CAT level was not maintained in LAN and RAN during the course of dehydration treatment, it is possible that the occasional increase of CAT may be sufficient to reduce the oxidative stress caused by H_2O_2.

GSH is the most abundant antioxidant in cell. GSH, acted as a primary antioxidant by scavenging free hydroxyl radicals and peroxyl radicals, is the major source of non-protein thiols in most plant cells (Shao et al., 2008). GSH is important for stress tolerance and symbiotic process (Riccillo et al., 2000). We noted that LAN and LIN, and RAN and RIN maintained higher GSH levels than LNN and RNN, respectively, at the late stages of dehydration stress. In another word, RNN and LNN were less able to sustain a high level of GSH under stress, which may again lead to greater oxidative stress and lipid peroxidation. RAN also showed a sharp drop of the GSH level 10 h after stress, implying plants were unable to maintain a high GSH level when stress became too severe.

Accumulation of proline is an important indicator of drought stress tolerance in higher plants (Paleg and Aspinall, 1981). Proline might act as a radical scavenger or a cellular osmotic regulator, conferring drought tolerance (Kavi Kishor et al., 1995; Manivannan et al., 2007). Our results showed that the proline content increased sharply 6 h after stress in both RNN and LNN, but the increase was much less in RAN and LAN. Our results were consistent with the report that free proline content significantly increased in *Vigna radiate* nodules under drought (Irigoyen et al., 2006). However, our results also suggested free proline might play a less significant role in antioxidation or osmotic adjustment in alfalfa that is active in nitrogen fixation. These plants may accumulate other type of amino acids such as the intermediates from nitrogen fixation for the osmotic adjustment purpose.

The increased proline content under stress was related to the decline in soluble proteins in plants (Ashraf and Iram, 2005). However, RNN and LNN in this study, however, had a higher or similar level of soluble protein compared to RAN and LAN, despite a higher level of free proline, suggesting the greater proline accumulation in these tissues may not be directly linked with the soluble protein metabolism.

There was a strong correlation between sugar accumulation and osmotic stress tolerance (Bartels and Sunkar, 2005). The hydroxyl group of sugar alcohols substitutes the $OH^{\bullet}$ of water to maintain the hydrophilic interactions with the membrane lipids and proteins, thus maintaining the structural integrity of the membranes (Mahajan and Tuteja, 2005). In this study, the soluble sugar content was higher in LAN and RAN compared to LNN and RNN, respectively, before dehydration stress. However, the pattern was reversed at the end of dehydrate stress, with higher sugar contents in LNN and RNN compared to LAN and RAN, respectively. The reverse pattern is due to a steady accumulation of soluble sugar in LNN compare to LAN and a less reduction of soluble sugar in RNN compared to RAN under stress condition. It was reported that the restriction of carbohydrate transport from leaves to nodule can inhibit nodule development (Singh and Singh, 2006). Nodules' specific activity appears to be limited by the ability of the nodule to cleave sucrose (Wang et al., 2007). Therefore, a lower soluble sugar in RAN under stress probably indicated that the active nodule consumed more soluble sugar in roots compared to RNN. An accumulation of soluble sugar in LNN suggested that photosynthesis continued under stress, while a constant but lower soluble sugar level in LAN compared to LNN suggest that carbohydrate or soluble sugar transport to the roots might be maintained under stress. Thus, it is possible that RAN and LAN may achieve osmotic adjustment through other mechanisms.

5. Conclusions

This study provided some supporting evidence for the notion that nodulation and nodule activities have a positive effect on drought response in alfalfa. Roots and leaves from plants with nodule, especially with active nodule, showed less lipid peroxidation, which was associated with higher CAT activities and higher levels of GSH. Roots and leaves with active nodule also accumulated less free proline and soluble sugar compared to the plants without nodule, suggesting proline and soluble sugar may have a limited role in osmotic adjustment in these plants or plants with active nodule may use a different mechanism in osmotic potential regulation. Further studies are needed to fully understand how nodulation affects drought response in alfalfa.

Acknowledgments

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References

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell & Environment 24, 1337–1344.

Antolin, M., Sanchez-Diaz, M., 1992. Photosynthetic nutrient use efficiency, nodule activity and solute accumulation in drought stressed alfalfa plants. Photosynthetica (Czech Republic).

Asada, K., 1999. The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Biology 50, 601–639.

Ashraf, M., Iram, A., 2005. Drought stress induced changes in some organic substances in nodules and other plant parts of two potential legumes differing in salt tolerance. Flora – Morphology, Distribution, Functional Ecology of Plants 200, 535–546.

Bartels, D., Sunkar, R., 2005. Drought and salt tolerance in plants. Critical Reviews in Plant Sciences 24, 23–58.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant and Soil 39, 205–207.

Becana, M., Dalton, D., Moran, J., Iturbe-Ormaetxe, I., Matamoros, M., Rubio, M., 2001. Reactive oxygen species and antioxidants in legume nodules. Physiologia Plantarum 109, 372–381.

Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C., Barker, D.G., 2001. Agrobacterium rhizogenes-transformed roots of Medicago truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Molecular Plant-Microbe Interactions 14, 695–700.

Boyer, J., 1982. Plant productivity and environment. Science 218, 443.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry 72, 248–254.

Cho, U., Seo, N., 2005. Oxidative stress in Arabidopsis thaliana exposed to cadmium is due to hydrogen peroxide accumulation. Plant Science 168, 113–120.

Clement, M., Lambert, A., Herouart, D., Boncompagni, E., 2008. Identification of new up-regulated genes under drought stress in soybean nodules. Gene 426, 15–22.

Demiral, T., Türkan, I., 2005. Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environmental and Experimental Botany 53, 247–257.

Dietz, K., 2005. 2 Plant thiol enzymes and thiol homeostasis in relation to thiol-dependent redox regulation and oxidative stress. Antioxidants and reactive oxygen species in plants, 25.

Dreywood, R., 1946. Qualitative test for carbohydrate material. Industrial & Engineering Chemistry Analytical Edition 18, 499–1499.

Elstner, E., Heupel, A., 1976. Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. Analytical Biochemistry 70, 616–620.

Evans, H., 1974. Symbiotic nitrogen fixation in legume nodules. Research Experiences in Plant Physiology. Springer-Verlag, New York, 294 C310.

Figueiredo, M., Burity, H., Martinez, C., Chanway, C., 2008. Alleviation of drought stress in the common bean (*Phaseolus vulgaris* L.) by co-inoculation with *Paenibacillus polymyxa* and Rhizobium tropici. Applied Soil Ecology 40, 182–188.

Foyer, C., Noctor, G., 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell and Environment 28, 1056.

Franca, M.B., Panek, A.D., Eleutherio, E.C.A., 2007. Oxidative stress and its effects during dehydration. Comp Biochem Phys A 146, 621–631.

Gage, D.J., 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiology and Molecular Biology Reviews 68, 280.

Giannopolitis, C., Ries, S., 1977. Superoxide dismutases: I. Occurrence in higher plants. Plant physiology 59, 309.

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74, 214–226.

Hoagland, D., Arnon, D., 1950. The water culture method for growing plants without soil. California Agricultural Experiment Station Circular 347, 32.

Irigoyen, J., Einerich, D., Sanchez-Diaz, M., 2006. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativd*) plants. Physiologia Plantarum 84, 55–60.

Jones, K.M., Kobayashi, H., Davies, B.W., Taga, M.E., Walker, G.C., 2007. How rhizobial symbionts invade plants: the Sinorhizobium – Medicago model. Nature Reviews Microbiology 5, 619–633.

Kavi Kishor, P., Hong, Z., Miao, G., Hu, C., Verma, D., 1995. Overexpression of P5CS increases proline production and confers osmotolerance in transgenic plants. Plant Physiology 108, 1387–1394.

Labidi, N., Mahmoudi, H., Dorsaf, M., Slama, I., Abdelly, C., 2009. Assessment of inter-varietal differences in drought tolerance in chickpea using both nodule and plant traits as indicators. Journal of Plant Breeding and Crop Science 1, 080–086.

Maehly, A.C., Chance, B., 1954. The assay of catalases and peroxidases. Methods of Biochemical Analysis 1, 357–424.

Mahajan, S., Tuteja, N., 2005. Cold, salinity and drought stresses: an overview. Archives of Biochemistry and Biophysics 444, 139–158.

Manivannan, P., Jaleel, C., Sankar, B., Kishorekumar, A., Somasundaram, R., Lakshmanan, G., Panneerselvam, R., 2007. Growth, biochemical modifications and proline metabolism in *Helianthus annuus* L. as induced by drought stress. Colloids and Surfaces B: Biointerfaces 59, 141–149.

Matamoros, M., Dalton, D., Ramos, J., Clemente, M., Rubio, M., Becana, M., 2003. Biochemistry and molecular biology of antioxidants in the rhizobia-legume symbiosis. Plant physiology 133, 499.

Moran, J., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R., Aparicio-Tejo, P., 1994. Drought induces oxidative stress in pea plants. Planta 194, 346–352.

Paleg, L., Aspinall, D., 1981. The physiology and biochemistry of drought resistance in plants.

Puckette, M.C., Weng, H., Mahalingam, R., 2007. Physiological and biochemical responses to acute ozone-induced oxidative stress in Medicago truncatula. Plant Physiology and Biochemistry 45, 70–79.

Puppo, A., Groten, K., Bastian, F., Carzaniga, R., Soussi, M., Lucas, M., De Felipe, M., Harrison, J., Vanacker, H., Foyer, C., 2005. Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. New Phytologist, 683–701.

Ramos, M., Parsons, R., Sprent, J., James, E., 2003. Effect of water stress on nitrogen fixation and nodule structure of common bean. Pesquisa Agropecüçria Brasileira 38, 339–347.

Raza, S., Athar, H., Ashraf, M., Hameed, A., 2007. Glycinebetaine-induced modulation of antioxidant enzymes activities and ion accumulation in two wheat cultivars differing in salt tolerance. Environmental and Experimental Botany 60, 368–376.

Riccillo, P., Muglia, C., De Bruijn, F., Roe, A., Booth, I., Aguilar, O., 2000. Glutathione is involved in environmental stress responses in Rhizobium tropici, including acid tolerance. Journal of Bacteriology 182, 1748.

Scandalios, J., 1993. Oxygen stress and superoxide dismutases. Plant Physiology 101, 7–12.

Scandalios, J., Guan, L., Polidoros, A., 1997. Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbour, New York.

Shao, H., Chu, L., Shao, M., Jaleel, C., Hong-mei, M., 2008. Higher plant antioxidants and redox signaling under environmental stresses. Comptes Rendus Biologies 331, 433–441.

Singh, B., Singh, G., 2006. Effects of controlled irrigation on water potential, nitrogen uptake and biomass production in Dalbergia sissoo seedlings. Environmental and Experimental Botany 55, 209–219.

Smirnoff, N., 2006. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytologist 125, 27–58.

Sprent, J., 1971. Effects of water stress on nitrogen fixation in root nodules. Plant and Soil 35, 225–228.

Wang, C.X., Saldanha, M., Sheng, X.Y., Shelswell, K.J., Walsh, K.T., Sobral, B.W.S., Charles, T.C., 2007. Roles of poly-3-hydroxybutyrate (PHB) and glycogen in symbiosis of Sinorhizobium meliloti with Medicago sp. Microbiology-Sgm 153, 388–398.

Wang, W., Kim, Y., Lee, H., Kim, K., Deng, X., Kwak, S., 2009. Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. Plant Physiology and Biochemistry 47, 570–577.