Environmental and Experimental Botany 86 (2013) 29–34

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

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

Peizhi Yang[1], Pan Zhang[1], Bing Li, Tianming Hu*

Department of Grassland Science, Northwest A & F University, Yangling, Shaanxi Province 712100, China

ARTICLE INFO

Article history:
Received 5 May 2010
Received in revised form 14 May 2011
Accepted 18 May 2011

Keywords:
Nodules
Alfalfa
Dehydration
Antioxidant system

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.

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.

* Corresponding author. Tel.: +86 13709124728; fax: +86 29 87092355.
E-mail address: hutianming@126.com (T. Hu).

[1] These authors contributed equally to this work.

0098-8472/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.envexpbot.2011.05.012

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