South African Journal of Botany 85 (2013) 1–9

Contents lists available at SciVerse ScienceDirect

South African Journal of Botany

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

Effects of salt stress on ion content, antioxidant enzymes and protein profile in different tissues of *Broussonetia papyrifera*

Min Zhang [a], Yanming Fang [b], Yonghua Ji [a,*], Zeping Jiang [a,**], Lei Wang [a]

[a] Jiangsu Academy of Forestry, Dongshanqiao, Jiangning, Nanjing, Jiangsu, 211153, China
[b] College of Forest Resources and Environment, Nanjing Forestry University, 159 Longpan Road, 210037, China

ARTICLE INFO

Article history:
Received 17 May 2012
Received in revised form 11 November 2012
Accepted 14 November 2012
Available online 23 December 2012

Edited by P Berjak

Keywords:
Broussonetia papyrifera
Antioxidant enzymes
Protein profile
NaCl stress

ABSTRACT

Although some plant responses to salinity have been characterized, the precise mechanisms by which salt stress damages plants are still poorly understood especially in woody plants. In the present study, the physiological and biochemical responses of *Broussonetia papyrifera*, a tree species of the family, Moraceae, to salinity were studied. *In vitro*-produced plantlets of *B. papyrifera* were treated with varying levels of NaCl (0, 50, 100 and 150 mM) in hydroponic culture. Changes in ion contents, accumulation of H_2O_2, as well as the activities and isoform profiles of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in the leaves, stems and roots were investigated. Under salt stress, there was higher Na^+ accumulation in roots than in stems and leaves, and Ca^{2+}, Mg^{2+} and P^{3+} content, as well as K^+/Na^+ ratio were affected. NaCl treatment induced an increase in H_2O_2 contents in the tissues of *B. papyrifera*. The work demonstrated that activities of antioxidant defense enzymes changed in parallel with the increased H_2O_2 and salinity appeared to be associated with differential regulation of distinct SOD and POD isoenzymes. Moreover, SDS-PAGE analysis of total proteins extracted from leaves and roots of control and NaCl-treated plantlets revealed that in the leaves salt stress was associated with decrease or disappearance of some protein bands, and induction of a new protein band after exposure to 100 and 150 mM NaCl. In contrast, NaCl stress had little effect on the protein pattern in the roots. In summary, these findings may provide insight into the mechanisms of the response of woody plants to salt stress.

1. Introduction

Salinity is one of the major abiotic stresses, which has a major impact on plant production and productivity (Zhu, 2001). Excess amount of salt in the soil adversely affects plant growth and development (Sairam and Tyagi, 2004). High salt level causes ion imbalance and hyperosmotic stress in plants (Zhu, 2001), which has toxic effects on numerous biochemical processes. Salinity affects plants in many different ways including osmotic effects, which resulted from increased osmotic potential of the soil solution that makes the water in the soil less available for plants (Eraslan et al., 2007), specific-ion toxicity and secondary stresses such as oxidative damage (Zhu, 2001).

One of the biochemical changes occurring when plants are subjected to salt stress is the accumulation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•) (Van Breusegem et al., 2001). ROS can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Meloni et al., 2003), and damage membrane function (Gómez et al., 2004). Plants have evolved a series of antioxidant systems that protect them from these potential cytotoxic effects (Rahnama and Ebrahimzadeh, 2005), such as the system that reacts with active forms of oxygen and keeps them at a low level (e.g. superoxide dismutase, catalase and peroxidases), and the system that regenerates oxidized antioxidants (e.g. glutathione reductase and ascorbate peroxidase) (Smirnoff, 1993).

The existence of multiple molecular forms of antioxidant enzymes, and any changes they may undergo in response to various environmental signals imply potential roles for these isozymes in the detoxification of ROS (Pinhero et al., 1997). The isozymes could be used as a biochemical marker to study the tolerance of plant to stress (Ying et al., 2006). Many studies have been carried out to study the isozymes of plant correlated with their tolerance to stress by isozyme analysis. The altered antioxidant enzyme activities have been described in plant species under abiotic stresses including salinity (Kim et al., 2005; Elkahoui et al., 2005; Jebara et al., 2005; Rahnama and Ebrahimzadeh, 2005). In barley roots under salt stress, the activities of antioxidant enzymes increased significantly, among which CAT activity increased most drastically. And the significant increase in the

Abbreviations: APX, ascorbate peroxidase; BSA, bovine serum albumin; CAT, catalase; DHAR, dehydroascorbate reductase; DTT, dithiothreitol; GPX, guaiacol peroxidase; MDHAR, monodehydroascorbate reductase; PAGE, polyacrylamide gel electrophoresis; POD, peroxidase; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate, SOD, superoxide dismutase; TEMED, N,N,N′,N′-tetramethylethylenediamine.

* Corresponding author. Tel.: +86 25 52741622; fax: +86 25 52741620.
** Corresponding author. Tel.: +86 25 52745600.
E-mail addresses: zhangnju@163.com (Y. Ji), nmzhang@163.com (Z. Jiang).

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http://dx.doi.org/10.1016/j.sajb.2012.11.005

activities of the antioxidant enzymes in NaCl-stressed barley root was highly correlated with the increased expression of the constitutive isoforms as well as the induced ones (Kim et al., 2005). Study on antioxidant enzyme expression in *Catharanthus roseus* suspension cells in response to salt stress indicated that the antioxidant enzyme activity and isoenzymes were influenced by salt stress (Elkahoui et al., 2005). The same results were also found in common bean (*Phaseolus vulgaris*) nodules (Jebara et al., 2005) and potato seedlings (Rahnama and Ebrahimzadeh, 2005) under salt stress.

Protein synthesis can be affected by environmental stresses including water (Bewley et al., 1983) and salt stress (Hurkman and Tanaka, 1987; Sousa et al., 2003). Several salt-induced proteins have been identified (Singh et al., 1987; Fisher et al., 1994; Ali et al., 1999; Sousa et al., 2003), which belong to two distinct groups: salt stress proteins that accumulate only due to salt stress, and stress associated proteins, which accumulate in response to various environmental stresses including heat, cold, drought, waterlogging, etc. (Ashraf and Harris, 2004). Proteins accumulated in plants grown under saline conditions may provide a storage form of nitrogen which could be reutilized when stress is over (Singh et al., 1987), and they may play a role in osmotic adjustment (Qasim et al., 2003). These proteins may be synthesized *de novo* in response to salt stress or may be present constitutively at low concentration and increase when plants are exposed to salt stress (Pareek et al., 1997).

Broussonetia papyrifera, family Moraceae, is a large shrub or small tree. The bark of *B. papyrifera* is a source of fiber for making paper and cloth and the leaves, fruit and bark have a variety of traditional medicinal uses. Due to its fast growth this tree can rapidly colonize forest clearings and abandoned farmland. *Broussonetia papyrifera* is tolerant to drought and resistant to salt stress, making it a suitable woody plant for saline environments (Yang et al., 2009).

To gain insight into the mechanism underlying salt stress on woody plants, changes in antioxidant enzyme activities and isoenzymes of SOD, POD and CAT, as well as protein profiles in different tissues of *B. papyrifera* in response to NaCl treatment were investigated in the present study. And the tissue-specific responses of the antioxidant enzymes and protein expression to salt stress were analyzed.

2. Materials and methods

2.1. Plant culture and salt treatment

B. papyrifera shoot segments with axillary buds were used as explants. The explants were transferred to the shoot multiplication media composed of Murashige and Skoog (MS) basal salts, 1 mg L^{-1} 6-BA, 0.1 mg L^{-1} IBA, 30 g L^{-1} sucrose and 6.5 g L^{-1} agar, pH 5.8, and the cultures maintained at 25 ± 2 °C in a 16 h light and 8 h dark cycle, with an irradiance of 1500 lux. For root induction, half-strength MS medium supplemented with 0.5 mg L^{-1} IBA, 0.3 mg L^{-1} NAA, 30 g L^{-1} sucrose and 6.5 g L^{-1} agar, pH 5.8 was utilized, and the cultures were maintained as described above. Fifty *in vitro* regenerated *B. papyrifera* rooted plantlets of uniform size were planted into plastic pots filled with 500 mL of half-strength MS solution. A plastic sheet was placed over each pot to prevent evaporation. Plants were maintained in a growth chamber under controlled conditions. The nutrient solution was changed every other day. For salt treatment, four NaCl levels, 0 mM (with no NaCl added), 50 mM, 100 mM and 150 mM NaCl were established with three replicates. After five days of treatment the leaves, stems and roots were harvested.

2.2. Determination of ion contents

The harvested plants were quickly rinsed in deionized water to remove ions from the free space and the excessive water was dried with a paper towel. And the fresh weights of the roots, stems and leaves were harvested. Afterwards, the samples were oven-dried at 60 °C for 48 h and the dry weight was measured. The oven-dried ground material (0.5 g) was digested with 8 mL of sulfuric acid-hydrogen peroxide mixture. The concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+} and P^{3+} in the digests were determined with an inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Perkin Elmer Optimal 2100-DV).

2.3. Determination of H_2O_2

The H_2O_2 content was colorimetrically measured as described by Mukherjee and Choudhuri (1983).

2.4. Antioxidant enzymes activity assay

The activities of SOD, POD and CAT were determined spectrophotometrically with a Beckman DU 800 UV/VIS spectrophotometer. SOD activity was measured according to the protocol of Yu and Rengel (1999). One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of the photochemical reduction of NBT, and SOD specific activity was expressed as units per mg protein (Rodríguez et al., 2007). Specific POD activity was determined by measuring the oxidation of benzidine at 530 nm according to the previous report (Rahnama and Ebrahimzadeh, 2005). Total CAT activity was analyzed by measuring the consumption of H_2O_2 at 240 nm according to the method of Beers and Sizer (1952), and enzyme activity expressed as U mg^{-1} protein.

2.5. Enzyme extraction

Enzyme extractions were carried out at 4 °C according to the previously reported procedures (Pereira et al., 2002). Plant tissues were frozen in liquid nitrogen and ground with an ice-cold pestle and mortar, and then extracted in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DTT and 5% (w/v) insoluble PVP in the ratio of 1:3 (w/v). The homogenate was passed through four layers of cheesecloth and then centrifuged at 10,000 ×*g* for 30 min. The supernatant was collected and stored in small aliquots at −80 °C for SOD, POD and CAT analysis. Protein was determined by the method of Bradford (1976) using BSA as a standard.

2.6. Native gel electrophoresis and enzyme activity staining

The isozymes of antioxidant enzymes were visualized by non-denaturing polyacrylamide gel electrophoresis (native-PAGE) on a Mini-Protean III slab gel cell (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded onto each lane. And the gels were scanned with a ChemiDoc XRS imaging system (Bio-Rad) after the photochemical reaction.

Isozymes of SOD were separated on 10% polyacrylamide gel and stained according to Fornazier et al. (2002) with minor modification. After electrophoresis, the gels were washed extensively with distilled water and soaked in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tretazolium and 0.3% (v/v) N,N,N′,N′-tetramethylethylenediamine (TEMED), and incubated in the dark for 30 min at room temperature. After incubation, gels were exposed to the light for about 50 min. And then the gels were rinsed with distilled water and the colorless SOD bands were visible against a purple background.

POD isoenzymes were visualized by immersing the gels in 0.2 M acetate buffer (pH 4.8) containing 0.3% H_2O_2 and 4% benzidine in 50% methanol at room temperature until the brown color appeared (Van Loon, 1971).

For the staining of CAT, the method of Woodbury et al. (1971) was adopted. Briefly, the gels were rinsed with distilled water and then incubated in 0.3% H_2O_2 for 10 min. After being washed with distilled