Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

Journal of Plant Sciences 5 (2): 137-145, 2010
ISSN 1816-4951

Anti-Oxidation Profile in the Leaves of Maize Inbreds: Elevation in the Activity of Phenylalanine Ammonia Lyase under Drought Stress

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Abstract: In a basic investigation, the antioxidative responses of two maize inbreds, A-180 and A-619 were studied at their vegetative stage. The total antioxidant ability of the plant leaf materials was determined using Ferric Reducing Antioxidant Power (FRAP) assay. The results revealed that the total antioxidant capacity in the leaves of maize inbreds quickly increases during drought period, while it slowly reverts back to the normal level during recovery. Differential changes in the enzymatic antioxidation through redox enzymes Catalase, Peroxidase and Polyphenol oxidase were observed between both inbreds during drought period. The activity of Pheylalanine Ammonia Lyase (PAL), a key enzyme involved in biosynthesis of isoperonoid antioxidative compounds was found to be increased sharply during stress period in both inbred lines. The overall results suggested that isoperonoid compounds may be more responsible antioxidants in maize drought-challenged antioxidative system.

Key words: Antioxidant, drought, stress, maize, FRAP, enzyme activity

INTRODUCTION

One of the earliest observable aspects of a plant response to different types of environmental stresses is Oxidative burst, a rapid transient production of excessive amounts of Reactive Oxygen Species (ROS); such as superoxide anion O_2^-, hydrogen peroxide H_2O_2, singlet oxygen O^* and hydroxyl radical OH^* (Baker and Orlandi, 1995; Wojtaszek, 1997). The ROS toxicity in plant system may eventually cause membrane damages through the oxidation of polyunsaturated fatty acids or proteins that often lead to cell dead (Blokhina *et al*., 2003). Alternatively, ROS may be followed by multiple responses in transcription, translation, protein activity, metabolic changes and possibly programmed cell death (Desikan *et al*., 2001; Lee *et al*., 2007; Rentel *et al*., 2004; Yoda *et al*., 2006; Van Breusegem and Dat, 2006).

In contrast, plants have evolved defense antioxidative mechanisms to combat the danger posed by the presence of ROS (Asada, 2006; Halliwell, 2006). Increased levels of antioxidants involved in the detoxification process of ROS have been found in plants resistant to various types of environmental stresses (Shao *et al*., 2007; Mittler, 2002).

Antioxidative system in plants is grouped into two classes consisting of enzymatic and non-enzymatic types (Adam *et al*., 1995; Bestwick *et al*., 2001). The enzymatic antioxidation is carried out by a series of the redox enzymes generally catalyzing electron transfer to ROS using low molecular weight antioxidant compounds; e.g., ascorbate, tocopherol, glutathione, phenols and flavonoids as electron and proton donors (Shao *et al*., 2007; Mittler, 2002). Non-enzymatic antioxidation has been found to be carried out by some of the high molecular weight compounds mostly including a number of proteins that avidly scavenge ROS and protect plants structures and functions against oxidative damages (Okada and Okada, 1998).

Usually, oxidative system is controlled/balanced by antioxidative system during the normal growth and developments in plant system. Alternatively, it is well controlled for the better cope of plants with their different environmental stress situations (Asada, 2006; Halliwell, 2006).

Drought stress is a major abiotic factor that limits agricultural crop production. Like to other environmental stresses, it gives rise to the formation of reactive oxygen species which is often followed by the activation of antioxidative system in plants (Zhang and Kirkham, 1994). Plant cells were found to be protected following the activation of antioxidative system. A close relationship between antioxidant activity and drought stress tolerance have been already identified in various plants such as cotton, wheat, pea, oak tree, pine tree, sunflower and very recently it has been reported in gardencress pepperweed plant (Fischer and Maurer, 1976; Moran *et al*., 1994; Schwanz *et al*., 1996; Habibi *et al*., 2004; Ratnayaka *et al*., 2003; Saleh and Plieth, 2009). Although, plants commonly activate their total antioxidation machinery in response to drought stress situations, but it has been well shown that different plants exhibit different responses to stress in terms of various redox enzymes activities. For examples, in cotton and wheat plants, the activity of glutathione reductase activity has been found to be increased during drought stress (Fischer and Maurer, 1976); in pea plant, drought stress leads to pronounced decreases in the activities of catalase, dehydroascorbate reductase and glutathio reductase enzyme activities, but it results in the increase of peroxidase, superoxide dismutase and monodehydroascorbate reductse activities; in pendunculate oak and maritime pine trees, the activities of catalase and peroxidase enzymes have been found to be reduced during drought period under ambient CO_2, but their activities are increased under drought period at elevated CO_2 concentrations (Schwanz *et al*., 1996); in sunflower the activities of superoxide dismutase, catalase and glutathione peroxidase is increased in plants subjected to drought stress (Habibi *et al*., 2004); in gardencress pepperweed, the activities of catalase and peroxidase have been found to be reduced during drought stress period while superoxide dismutase and glutathione reductase activities have been shown to be increased (Saleh and Plieth, 2009).

Maize is an important crop in the world and it is well characterized by the multiplicity of its agro-industrial uses. In many areas, the productivity of this crop is limited due to drought stress. The improvement of its productivity in such areas requires understanding the mechanisms of tolerance of this plant to drought conditions.

The aim of this study was to determine the antioxidative status of the maize leaves during drought stress shifting and evaluate the relative activity of phenylalanine ammonia lyase under drought stress.

MATERIALS AND METHODS

Plant Growth and Treatments

The seeds of maize (inbred lines A-180 and A-619) were randomly (with no information about their drought resistance) provided by Dr. B. Baghban Kohnehrouz (plant genetic engineering laboratory, Department of Plant Breeding and Biotechnology, University of Tabriz, Iran). Seeds were surface sterilized using 1% NaOCl for 15 min and then rinsed three times in distilled water. Surface sterilized seeds were germinated and grown till seedling stage in petri plates. The seedlings with uniform sizes were transferred into experimental tubes containing 20 mL of modified Hoagland nutrient solution (Gholizadeh *et al*., 2007). They were allowed to grow under laboratory sun light condition (day to night period of 12: 12 h and

humidity of about 65%). For drought stress treatment of the test plants, the nutrient medium was completely withdrawn for 24 h. The experimental materials were taken as leaf blades and processed for experimental assessments. After stress period, plants were reverted back to the normal non-stressed conditions and allowed for recovery. The leaf materials were harvested as blades from the same plant and processed for further experiments.

Total Antioxidant Power Assay

The total antioxidant ability of the plant leaf materials was determined using ferric reducing antioxidant power (FRAP) assay of Benzie and Strain (1996). To 1 mL of plant extract in 0.1 M phosphate buffer (pH 7.0), 3 mL of FRAP reagent (10 mM TPTZ: tripyridyl triazine, 20 mM $FeCl_3$. $6H_2O$ and 300 mM sodium acetate buffer (pH 3.6) in the ratio of 1:1:10) was added and the reaction mixture was incubated at 37°C for 4 min. The assessment was carried out spectrophotometerically at A_{593}. Antioxidant potential of samples was determined against the standard curve of ferrous sulphate (Fe, 100-1000 μM). Ascorbic acid (100 μM) served as a positive control and BSA considered as negative control. FRAP values were calculated as follows:

FRAP value (μmol/100 mg) = A_{593} of test sample/A_{593} of standard×FRAP value (μmol/100 mg) of standard

The FRAP values of all test samples were presented as (μmol Fe^{II}/100 mg leaf fresh weight).

Enzyme Extraction

Two hundred milligram of leaf blades were homogenized in 1 mL of 0.1 M phosphate buffer, pH 7.0 containing 0.5 μL of β-mercaptoethanol and a pinch of polyvinyl polypyrrolidone (PVP). The homogenate was centrifuged at 12,000x g for 10 min and the supernatant was used for redox enzymes activity assay.

Assay of Catalase

Catalase activity was assayed according to the method of Sadasivam and Manickam (1992). The assessment was carried out spectrophotometrically by monitoring the changes in the OD values at 240 nm. Time required observing a decrease in OD from 0.45 to 0.40 was noted and used for calculating enzyme units. The activity of the enzyme was expressed as units per mg of soluble protein.

Assay of Peroxidase

The peroxidase activity was measured using the method as described by Sadasivam and Manikam (1992). One unit of the enzyme was considered as the amount of the enzyme that was responsible for the increase in OD value of 0.1 in 1 min at 436 nm. The activity of the enzyme was expressed as units per mg of soluble protein.

Assay of Polyphenol Oxidase

Method of Siddiq *et al.* (1992) was followed for the assay of polyphenol oxidase activity. The unit of enzyme activity was defined as the rate of changes in one unit absorbance per min at 420 nm. Enzyme activity was expressed as units per mg of soluble protein.

Assay of Phenylalanine Ammonia Lyase

Two hundred milligram of leaf material was homogenized in 2 mL of 25 mm borat buffer, pH 8.8 containing 2 μL β-mercaptoethanol and a pinch of polyvinyl polypyrrolidone (PVP). The homogenate was filtered through the cheese cloth, centrifuged at 12,000x g for 10 min and the supernatant was used for enzyme activity assay according to Sadisivam and Manickam (1992). One unit of the enzyme was defined as increase in absorbance of one unit per min. The activity of the enzyme was expressed as units per mg of soluble protein.

Statistics

Data points in the figures represent the Mean±SE of three individual treatments with two replications per treatment. Variance analysis was analyzed by ANOVA at $p \leq 0.05$. Values followed by different Latin letters in each assay on the graphs are statistically different ($p \leq 0.05$).

RESULTS AND DISCUSSION

Total Antioxidative Ability

Earlier studies have been shown that drought stress in plants induces the oxidative burst that is obviously followed by the activation of their antioxidative system (Zhang and Kirkham, 1994; Fischer and Maurer, 1976; Schwanz *et al*., 1996; Habibi *et al*., 2004; Saleh and Plieth, 2009). As maize plant has been well known as a rich source of redox potentials, we conducted present studies on the antioxidative responses of this plant under drought condition. In order to determine the total antioxidative status of test leaf samples, the ferric reducing antioxidant power (FRAP) method was used (Benzie and Strain, 1996). FRAP test is known as a simple and reproducible method used for the assessment of the total antioxidative capacities from different sources. The results showed that the total antioxidative capacity of maize A-180 leaves is sharply increased during stress period, so that the FRAP value is reached to 1.8 during stress period (Fig. 1a, b). Data showed that during recovery period the total antioxidative ability of the leaves is slowly fell down to the level of before stress stage. The total antioxidative status of the leaf tissues is reached to the level of before stress stage (~ 0.6 μmol Fe^{II}/100 mg) after four days of recovering. Variation in the total antioxidative status of the maize A-619 leaves was also showed similar pattern to A-180 (Fig. 1). The FRAP value was sharply increased to 1.1, but it was slowly declined to the level of before stress stage (~ 4.4 μmol Fe^{II}/100 mg) after four days of recovering. These results are in confirmation of already reported results in the case of gardencress plant (Saleh and Plieth, 2009).

Present results furthermore confirmed that drought stress induce the activity of antioxidative system that may contribute to drought resistance in maize. Usually, the early responses of plants to drought stress help them to survive for some time. Do the slow decline pattern in the antioxidative ability of both maize inbred lines, indicate that antioxidative system functions as an adaptive mechanism for drought tolerance in maize, it needs to be identified.

Redox Enzymes Activities

Antioxidative system in plants has been composed of several enzymatic and non-enzymatic components that are being active in a differential manner in response to drought stress in different plants. To explain the relative role of redox enzymes in the antioxidative system of drought-challenged maize, we assessed the activities of three enzymes catalase, peroxidase and polyphenol oxidase.

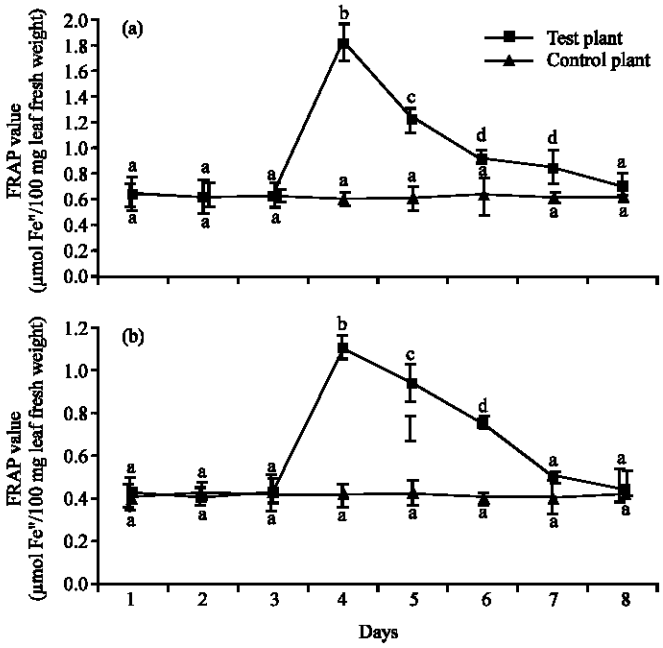

Fig. 1: Total antioxidation analysis by FRAP test. Leaf materials of the two maize inbreeds ((a) A-180 and (b) A-619) were processed for their total antioxidative ability using ferric reducing antioxidant power (FRAP) test at three different conditions including before stress, during stress and recovery periods. Non-treated plants were also taken as controls. The data were presented as the means of triplicates ($p \leq 0.05$). A sharp increase in antioxidation capacity of the leaves under one day drought stress and its slow decline during recovery period is an interesting result, indicating the more possible involvement of antioxidation strategy in maize drought resistance mechanism

The results revealed that during drought stress the activity of catalase show differential patterns in test plants (Fig. 2a-f). During 24 h stress period, it is increased about 0.003 units/mg soluble protein in maize A-180, while it is reduced about 0.002 in the case of A-619. During recovery, after 24 h, the activity of catalase is decreased up to 0.001 units, but still it remains higher than before stress point in maize A-180. But, the activity of catalase is come back to the level of before stress in A-619 inbreed (Fig. 2). The pattern of catalase activity in maize A-180 showed similarity to sunflower in which catalase activity is increased during drought stress (Habibi *et al.*, 2004). In contrast to A-180, the catalase pattern in A-619 inbreed was similar to those of gardencress pepperweed and pea plants (Saleh and Plieth, 2009).

The activity of peroxidase was found to be reduced about 0.001 units during stress period and reverted back to the level of before stress stage in maize A-180 (Fig. 2). In contrast, no changes in this enzyme activity were measured in the case of A-619 inbreed. The activity of peroxidase has been already reported to be remarkably increased in the case of pea, while its activity is highly decreased in gardencress pepperweed plant (Saleh and Plieth, 2009).

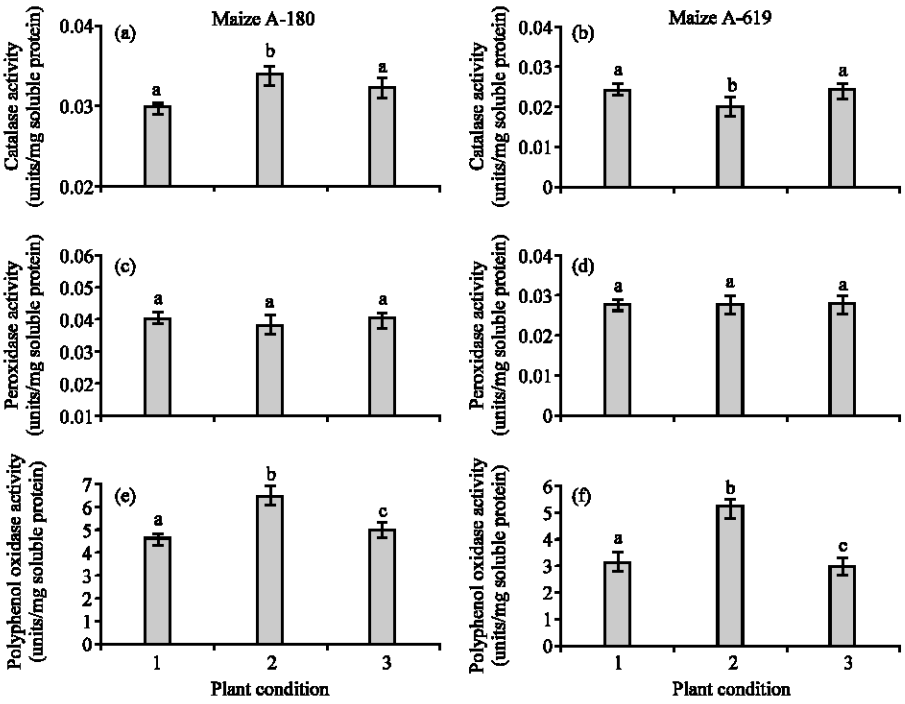

Fig. 2: (a-f) Enzymatic antioxidation assay. Maize leaf blades were processed for enzymatic antioxidative status as described in materials and methods. The assessments were carried out at three time points including 24 h before stress treatment (plant condition 1), 24 h after stress treatment (plant condition 2) and 24 h after recovery (plant condition 3). Enzymatic antioxidation status was analyzed in terms of the three redox enzymes activities including catalase, peroxidase and polyphenol oxidase. Data were presented as the means of triplicates ($p \leq 0.05$). Sharp increases and decreases in the enzymatic antioxidation activity in both inbreeds may suggest that these redox enzymes are not involved in adaptive drought resistance mechanism in maize plant

The activity of polyphenol oxidase is increased during drought stress in both maize inbreds (Fig. 2). Its activity is enhanced up to 6.4 units in maize A-180 when it is exposed to drought stress for 24 h. While, during one day recovery period, the activity of polyphenol oxidase is reduced to 5 units/mg soluble protein that is still remained higher than the level of before stress level. In A-619, polyphenol oxidase activity is increased up to 5.2 units during stress period, but it is reduced slightly lower than before stress stage during one day recovery.

The overall results revealed that the activities of catalase, peroxidase and polyphenol oxidase are sharply changed (increase or decrease) with drought stress conditional shifting. This is in contrast to the variation pattern in total antioxidation capacity, as the total antioxidative status of the leaf tissues are slowly declined during recovery period. Do the activities of catalase, peroxidase and polyphenol oxidase respond to drought stress in a deleterious manner in maize plant? The sharp variation pattern in their activities may help the plants to survive for a short period of drought stress.

Activation of Phenyl Propanoid Biosynthetic Pathway

The enzyme Phenylalanine Ammonia Lyase (PAL) catalyzes deaminating reaction of the amino acid phenyl alanine at the gateway from the primary metabolism into the important secondary phenylpropanoid metabolism in plants (Legrand *et al*., 1976; Hahlbrock and Scheel, 1989). Phenylpropanoid compounds not only fulfill various essential functions during plant development, but also they act as important protectants against various biotic and abiotic environmental stresses. The biosynthesis of PAL and accumulation of phenylpropanoid structures have been reported up on pathogenic attack including viruses, tissue wounding, UV irradiation, low temperatures, low levels of nitrogen, phosphate and iron (Dixon and Palva, 1995; Ritter and Schulz, 2004; Gholizadeh *et al*., 2004).

An increase in the activity of PAL enzyme has been recently reported in the cases of winter triticale and a drought resistant maize genotype (Hura *et al*., 2007, 2008). We conducted a part of present studies on PAL activity under drought stress in the other maize genotypes. Present results showed that the activity of PAL has sharp peaks during one day drought stress period in both test plants (Fig. 3a, b). It is increased from 4.3 to 8.1 and 4.7 to 9.2 units on first day of stress treatment in A-180 and A-619, respectively. Despite this, the variation pattern in PAL activity differs in both inbreds during recovery. In contrast to stress period, the activity of PAL was found to be slowly declined during recovery.

The overall pattern in the activity of the PAL enzyme showed similarity to the total anitioxidation pattern in maize plants under drought stress conditional shifting. May phenylpropanoids be more responsible compounds in drought-challenged maize plants?

Present results indicated that antioxidation system is operated during drought period in maize plants. But, the questions are these: (1) how much does antioxidation strategy help to drought resistance in maize and (2) may PAL be considered as a candidate for maize genetic engineering in drought resistance strategy in the future?

We predict that PAL can be a good candidate for antioxidative strategy in drought resistance in maize.

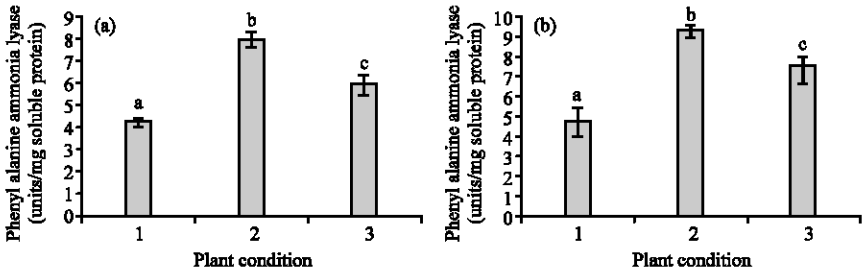

Fig. 3: (a, b) Assessment of the activity of phenylalanine ammonia lyase. Activation of propanoids biosynthetic pathway was assessed in terms of PAL activity analysis in both inbreeds as described in materials and methods section. Experiment was carried out at three different plant conditions including 24 h before stress treatment (plant condition 1), 24 h after stress treatment (plant condition 2) and 24 h after recovery (plant condition 3). Data were presented as the means of triplicates ($p \leq 0.05$). As the results show the activity of PAL is sharply increased during one day of drought period, while it is slowly reverted back at recovery period

ACKNOWLEDGMENT

The author of this paper is thankful to RIFS (Research Institute for Fundamental Sciences), University of Tabriz, Iran for funding of this research.

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