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Use of iso-osmotic solution to understand salt stress responses in lentil (*Lens culinaris* Medik.)

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ABSTRACT

Lentil, an important source of protein for human consumption, is considered as a salt susceptible crop species. Thus the present study was carried out to evaluate the most important lentil physiological characters that induce salt tolerance. Salt stress results in osmotic stress immediately followed by ionic toxicity. We used iso-osmotic solutions with different kinds of osmotica i.e. salt (NaCl and KCl), and polyethylene glycol (PEG) to identify the specific response of osmotic stress and ionic toxicity. All of these altered seedling height, chlorophyll, malondialdehyde (MDA), hydrogen peroxide (H_2O_2), proline, reduced ascorbate (AsA) and total glutathione content, and ion uptake. Moreover, these osmotica also altered the activities of antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), except monodehydroascorbate reductase (MDHAR). Among different osmotica, only NaCl exhibited leaf chlorosis, reduction in K^+ and disruption in ion homeostasis, and increased MDA, H_2O_2 and proline content than iso-osmotic KCl and PEG, indicating susceptibility of lentil seedling to salt stress. Addition of Ca along with NaCl showed no chlorosis and improved K^+ content. These results demonstrate that prevention of Na-induced K depletion in root might enhance salt tolerance in lentil.

1. Introduction

Soil salinity affects 20% of the total global irrigated land and 33% of irrigated agricultural land which has led to severe decrease in crop production in those salt affected areas (Negrao and Tester, 2016). Moreover, it is predicted that a half of cultivable lands would be afflicted with salinity by 2050, since every year 10% of arable land are being affected by salinity for natural and anthropogenic reasons (Jamil et al., 2011). Total crop production rate may fall presumably with the increase of saline area, posing threat to food security since the requirement of food is assumed to increase by 70 to 110% for ever growing population by 2050 (Munns et al., 2012; Hasanuzzaman et al., 2016). Salt stress affects plant growth, immediately creating osmotic stress and later, ionic toxicity (Munns and Tester, 2008). Salinity-induced osmotic stress causes a reduction in leaf growth by inhibiting cell expansion and cell division. This is followed by chlorosis, necrosis and senescence of mature leaf as a result of ionic toxicity (Carillo et al., 2011); additionally, the excess amount of Na^+ disrupts ion homeostasis. Furthermore, it interferes with protein synthesis and enzyme activity because Na^+ can compete with K^+ for major substrate binding sites in the enzymes (Carillo et al., 2011; Shabala and Pottosin, 2014). To cope with osmotic stress, plants decline water loss by reducing stomatal conductance, leading plants to suffer from CO_2 unavailability required for the functioning of the Calvin Cycle. As a result, light absorption exceeds the demand required for photosynthesis and photorespiration, which subsequently boost reactive oxygen species (ROS) production. Accumulation of Na^+ and Cl^- at toxic levels also contributes to ROS production by interfering with repairing damage of photosystem II due to excess light (Allakhverdiev et al., 2002; Miller et al., 2010; Bose et al., 2014). Overproduced ROS causes oxidative damage to DNA, proteins and lipids, and in severe case, cell death occurs (Hasanuzzaman et al., 2013; Mishra et al., 2013). However, the antioxidant defense system of plants controls the excess ROS generation and prevents cell from damaging its vital constituents. The non-enzymatic antioxidants such as ascorbic acid, (AsA), glutathione (GSH), phenolic compounds, alkaloids, and α-tocopherols and enzymes such as catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) function together

Abbreviations: AO, Ascorbate oxidase; APX, Ascorbate peroxidase; AsA, Ascorbic acid; CAT, Catalase; CDNB, 1-Chloro-2,4-dinitrobenzene; DAB, Diaminobenzidine; DHA, Dehydroascorbic acid; DHAR, Dehydroascorbate reductase; DTNB, 5,5-Dithio-bis-(2-nitrobenzoic) acid; Gly, Glyoxalase; GR, Glutathione reductase; GSH, Reduced glutathione; GSSG, Oxidized glutathione; MDA, Malondialdehyde; MDHAR, Monodehydroascorbate reductase; MG, Methylglyoxal; PEG, Polyethylene glycol; Pro, Proline; ROS, Reactive oxygen species; SLG, *S*-D-lactoyl-glutathione; SOD, Superoxide dismutase; TBA, Thiobarbituric acid; TCA, Trichloroacetic acid.

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to maintain ROS below the level that causes cellular injury (Gill and Tuteja, 2010; Hasanuzzaman et al., 2012). Upregulation of the antioxidant system may provide tolerance to plants under salt stress condition by maintaining cellular redox homeostasis (Gill and Tuteja, 2010; Hasanuzzaman et al., 2011; Nahar et al., 2016).

Since the osmotic component of salt stress mimics drought stress, to thoroughly understand the mechanism of salt stress, it is necessary to differentiate between damages caused by the osmotic component and ionic component of salt stress. A rapid response due to osmotic stress is growth reduction, often occurs within minutes, and within a few days, ion specific damage appears on leaf showing necrosis, chlorosis and senescence because the excess amount of Na^+ accumulation in cytosol occurs at higher rate than the rate of compartmentalization of cell to reduce ion toxicity in the cell (Munns, 2002).

To dissect salinity-induced damages, different types of osmotica such as PEG, mannitol, sorbitol, KCl and NaCl are used. Iso-osmotic PEG-6000 and NaCl treatment during vegetative and reproductive stages could not affect the growth and yield of rice, except that K^+ content reduced under salt stress (Castillo et al., 2007). However, PEG-6000 caused greater growth inhibition of leaf and accumulation of ABA compared with NaCl (Chazen et al., 1995). Seed germination was considerably reduced in maize due to PEG-6000, but completely failed due to NaCl of similar osmotic potential (Mohammadkhani and Heidari, 2008). NaCl, but not iso-osmotic PEG resulted in the instability of photosystem-II (PSII) and the reduction of PSII energy conversion efficiency (Muranaka et al., 2002). In contrast, NaCl caused fewer damages on sorghum growth, Ca^{2+} metabolism and photosynthetic gas exchange than KCl (Wang et al., 1999). Interestingly, plant responses to these osmotica greatly differ with genotypes. For example, a lentil genotype, Pantelleria (PAN) has showed tolerance against NaCl whereas it showed susceptible response against PEG (Muscolo et al., 2015).

Lentil (*Lens culinaris)* is a beneficial crop in many ways: a cheap source of protein for human consumption, having the capability of nitrogen fixation and rich in metabolites which are of pharmacological importance (Sidari et al., 2008; Afzal et al., 2014). Among the glycophytes, lentil is very sensitive to salt like other legumes (Ashraf and Waheed, 1990). Therefore, lentil growth and development are negatively affected by salinity stress. Salinity can reduce lentil yield up to 50% by negatively affecting yield attributes (Ayoub, 1977; Golezani and Yengabad, 2012).

Development of salt tolerant genotype through breeding is an urgent task for breeders. Thus, identification of physiological attributes that confer salt tolerance may reduce time and increase the success rate of breeding program. Addressing this problem, in this experiment we differentiated the damages caused by osmotic and ionic component of salt stress. Thus we could find out the most appropriate physiological attributes only for salt tolerance in lentil.

2. Materials and methods

2.1. Plant materials and treatments

Lentil (*L. culinaris* Medik cv. BARI Lentil-7) seeds were collected from Bangladesh Agricultural Research Institute (BARI), Bangladesh. Lentil seeds were placed on 6-layered moistened filter paper in Petri dishes. Then the Petri dishes were transferred to a germination chamber for 72 h. Germinated seedlings were then transferred and grown in a growth chamber under an irradiance of 350 μmol (photon) m^{-2} s^{-1}, a temperature of 25 ± 2 °C, and a relative humidity of 65–70%. One Petri dish contained 35 seedlings. Hyponex (Tokyo, Japan) solution was diluted 5000-fold and applied as the nutrient solution. To create iso-osmotic stress according to Sosa et al. (2005), 100 mM NaCl, 100 mM KCl and 12% (*w*/*v*) PEG 6000 were prepared with the nutrient solution. To observe the effect of severe osmotic stress, 20% (*w*/*v*) was used. To determine the response of lentil seedling upon exposure to these osmotica, six-day-old seedlings were subjected to these iso-osmotic solutions. The control plants were grown in the Hyponex solution only. Nutrient solutions were changed every two days. Seedlings were harvested 4 days after treatment. To understand the response of these osmotica, picture was taken 6 days after treatment. To observe the protective role of Ca, 2 mM $CaCl_2$ was added along with 100 mM NaCl. The experiment was conducted using a completely randomized design with three replications. All chemicals used in this study were purchased from Wako, Japan.

2.2. Observation of seedling growth

To determine seedling growth, shoot length, root length, and fresh weight (FW) were measured. Fresh weight and length were expressed as mg seedling^{-1} and cm respectively.

2.3. Determination of water content

To determine water content (WC) of shoot, fresh weight (FW) and dry weight (DW) were measured. For DW, seedlings were oven dried at 80 °C until the weight became constant. Finally, WC was calculated using the following formula: $WC\ (\%) = \{(FW - DW)/DW\} \times 100$.

2.4. Determination of ion content

Sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) ion contents were determined according to Rahman et al. (2016a). Plant samples were oven dried at 80 °C for a period until weight becomes constant. 100 mg of dried root and shoot were ground and digested separately with an acid mixture, nitric acid and perchloric acid (5:1) at 70 °C for 48 h. Then mineral contents were measured by using an atomic absorption spectrophotometer (Hitachi Z-5000; Hitachi, Japan).

2.5. Determination of chlorophyll content

For determining chlorophyll (chl) content, a leaf sample (0.5 g) was homogenized with 10 ml of 80% acetone using a mortar and pestle. After centrifugation at 9000 ×*g* for 10 min, absorbance of supernatant was measured with a UV-spectrophotometer at 663 and 645 nm for chl *a* and chl *b* contents, respectively as described by Arnon (1949).

2.6. Determination of proline content

Proline (Pro) content was quantified according to a widely used method by Bates et al. (1973).

2.7. Determination of osmotic potential

To determine osmotic potential, leaves were homogenized using ice cold mortar and pestle followed by centrifugation at 12,000 ×g for 10 min. Plant extract was then used to determine osmolarity (c) using a K-7400 semi-micro osmometer according to Rahman et al. (2016b). Osmotic potential was calculated by converting osmolarity to osmotic potential using the following formula.

$$\psi_{\Pi}\ (\mathrm{MPa}) = -c\left(\mathrm{mOsmol\ kg^{-1}}\right) \times 2.58 \times 10^{-3}$$

2.8. Determination of electrolyte leakage

Electrolyte leakage was measured according to Dionisio-Sese and Tobita (1998). Shoot sample (0.2 g) was cut into smaller pieces and then placed in a test tube containing 15 ml distilled deionized water. Covering with caps, test tubes were heated at 40 °C for 10 min. After