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MORPHOLOGICAL AND BIOCHEMICAL RESPONSE TO OSMOTIC STRESS IN ALFALFA (*MEDICAGO SATIVA* L.)

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Abstract

Water stress is a serious environmental problem throughout the world which may be partially relieved by breeding cultivars that can tolerate low soil water potentials. Alfalfa (*Medicago sativa* L.) is normally grown in arid and semi-arid areas, so the investigation of alfalfa potential for drought tolerance and the proper selection of its drought tolerant accessions are very important in this context. Eight alfalfa accessions widely grown in Iran viz., Baghdadi, Nikshahri, Ghareh, Yazdi, Siriver, Sequel, Ranger and Kodi were assessed for drought tolerance at the germination and seedling stages. Osmotic stress was applied at different concentrations [zero (control), -0.4, -0.8 and –1.2 MPa] of PEG (polyethylene glycol) 6000. The data showed that the intensity of reduction in various growth attributes in different accessions was not the same in response to osmotic stress. The lowest reduction was observed in Yazdi and Nikshahri accessions and the highest in Ranger in most of the characters appraised due to simulated drought. In the second experiment, the selected accessions viz., Yazdi (osmotic tolerant), Nikshahri (moderate) and Ranger (osmotic sensitive) were grown in a hydroponic culture with different PEG 6000 concentrations. After 4 weeks, different characters such as root length shoot length, root/shoot length ratio, leaf area, leaf number, root and shoot dry weights, proline accumulation and concentrations of K^+ and Ca^{2+} were determined. The results showed that with increasing osmotic stress, most of the characters were decreased significantly. With increasing osmotic stress, proline accumulation and concentrations of Ca^{+2} and K^+ increased. There was a significant difference among the accessions in most of the attributes studied. In all cases, Yazdi was the most tolerant and Ranger was the most sensitive accession in response to osmotic stress.

Introduction

Adaptation of plants to water stress involves a number of morphological and physiological as well as biochemical changes (Delauney & Verma, 1993; Yordanov *et al.*, 2000). These adaptive mechanisms include traits, which promote the maintenance of high tissue water content, as well as those for promoting tolerance to low water availability. Osmotic adjustment is one such trait, which plays an important role in sustaining growth under water deficit conditions. Since osmotic adjustment is the decrease in osmotic potential by the active accumulation of organic as well as inorganic solutes within the cells, high concentrations of inorganic ions become detrimental to cellular metabolism and must be sequestered in the vacuole (Safarnejad *et al.*, 1996; Wensuo *et al.*, 2001). In order to keep osmotic balance, specific types of organic molecules (such as proline) are accumulated in the cytoplasm. These compounds are termed compatible solutes, because they can be accumulated in high concentrations without perturbing the normal physiological functions (Safarnejad *et al.*, 1996).

Osmolyte accumulation has been widely reported as a key putative mechanism for enhancing yields of crops subjected to drought stress (Heng-long *et al.*, 1999; Serraj & Sinclair, 2002; Gazanchian *et al.*, 2007; Ashraf & Foolad, 2007). One of the well known mechanisms reported by Serraj & Sinclair (2002) for beneficial crop yield responses to osmolyte accumulation was the maintenance of root development in order to reach water that may be available in the deeper soil profiles (Serraj & Sinclair 2002; Gazanchian *et al*., 2006). In plant species such as alfalfa, which are grown in arid environments, tolerance to water stress usually involves the development of low osmotic potentials (Santos-Diaz & Ochoa-Alejo, 1994). Analysis of alfalfa growth has shown that it responds to an onset of drought by a reduction in shoot and root elongation (Carter & Sheafer, 1983a, b; Hall, 1993), and the number of basal buds and shoots (Brown & Tanner, 1983; Perry & Larson, 1974). Brown & Tanner (1983) also found the reduced leaf area, internode length and proline is also known to play a vital role in plant drought tolerance and its levels are considerably increased in plants under drought stress. For example, dry matter production of alfalfa during the first 14 days after the stress was initiated.

Verslues & Sharp (1999) showed that proline concentration increases greatly in the growing region of maize (*Zea mays* L.) primary roots at low water potentials, largely as a result of an increased net rate of the proline deposition.

In a study it was found that declining vacuolar K^+ could inhibit root growth, because its accumulation in newly formed vacuoles drives cell expansion (Walker *et al*., 1998). Of the different essential nutrients K plays an important role in osmoregulation in plants subjected to water deficit conditions **(**Morgan, 1992; Marschner, 1995; Ashraf, 2004). The functions of K^+ in plant cells could be biophysical, such as osmoregulation or biochemical such as protein synthesis and enzyme activation (Walker *et al*., 1998).

The purpose of this study was the assessment of the most commonly grown alfalfa accessions for drought tolerance using some specific inorganic and organic solutes as selection criteria such as accumulation of some essential elements and proline.

Materials and Methods

Eight commonly grown alfalfa accessions in Iran viz., Baghdadi, Nikshahri, Ghareh, Yazdi, Siriver, Sequel, Ranger and Kodi were used for the present studies. Osmotic stress was applied with different concentrations [zero (control), -0.4, -0.8 and -1.2 MPa] of PEG 6000 following Michel & Kaufmann (1973) method. The seeds of each accession were carefully scarified and surface sterilized in 2% (v/v) Sodium hypochlorite solution for two minutes, washed with distilled water and dried at room temperature. The seeds of each accession were evenly spaced over five layer deep rafts of alkathene beads in plastic pots containing 300 cm^3 of 0.1 strength nutrient solution (Rorison in Hewitt, 1966) plus 4 mol m^{-3} $CaCl_2$, with different concentrations of PEG. Each accession was replicated four times in four separate containers for each concentration. The containers were placed in a growth room maintained at 25 ± 1ºC and relative humidity of 80% with 16 h photoperiod (80 µmol m^{-2} s^{-1} *PAR*). After two weeks, the germination percentage and rate, growth of radicles and plumules, fresh and dry weights of plumules and radicles were recorded.

The seeds of 3 selected accessions in experiment 1, viz., Yazdi, Nikshahri and Ranger were grown in nutrient solution (as in Experiment 1), without PEG, for 7 days and then transplanted individually into plastic containers (size 9 * 12 * 12 cm deep) containing nutrient solution. Twenty even size seedlings for each replicate were transplanted per generation per container on the Unilite sheet (plastofoam) with holes. Solutions comprised modified 0.1 strength nutrient solution (as in Experiment 1) with PEG 6000 at zero, -0.4, -0.8 and -1.2 MPa. The plants were grown for a further 30 days in a glasshouse in which temperatures ranged from 22-28ºC with 16 h photoperiod of natural daylight.

A randomized complete block design with four replicates was used under the same growth conditions as for the first experiment. After 30 days, data for root length, shoot length, root to shoot length ratio, leaf number, leaf area, and root and shoot dry weights (at 70ºC for three days), were recorded. Proline and concentrations of K^+ and Ca^{2+} were also determined in the shoot and root samples.

Proline estimation: Seedling tissue was frozen in liquid nitrogen and stored until required, after which time it was homogenized in methanol: chloroform: water (MCW 12:5:1 /V) using 0.2 g tissue per 2 cm^3 of MCW at room temperature and proline concentrations estimated using a modification of the method described by Singh *et al*., (1973), with the following modification. The homogenate was centrifuged at 5×1000 rpm for 5 min., in a Gallenkamp bench top centrifuge and the supernatant retained. The pellet was re-extracted in the same volume of MCW for 5 min. The two supernatants were pooled and separated into a lower chloroform and upper methanol water layer by adding 1.5 cm^3 water and 1 cm^3 chloroform to every 4 cm^3 of extract. The upper layer was used for proline estimation. Samples of 1.0 cm^3 were transferred to boiling tubes to which 1.25 cm^3 glacial acetic acid and 1.25 cm^3 of acid ninhydrin [1.25 g ninhydrin was warmed in 30 cm^3 glacial acetic acid and 20 cm^3 orthophosphoric acid (85%)] were added. The boiling tubes were heated in a boiling water bath for 65 min. and after cooling 5 cm^3 of toluene were added. The tubes were shaken on a whirlimixer and left for about 20 min., for the separation of the two layers (lower and upper layer). The absorbance of the upper (toluene) layer was measured at 515 nm against a toluene blank. The assay was calibrated with a standard solution of L-proline.

Estimation of K^+ and Ca^{2+}: For the analysis of K^+ and Ca^{2+}, 25-50 mg shoot or root samples were digested in concentrated Nitric acid (HNO3) (2 ml for shoots and 2 ml for roots). The concentrations of the digested samples were read in an atomic absorption spectrophotometer (Perkin Elmer 560).

Statistical analysis of data: Analyses of variance were carried out for three measurements on extracts from 3 independent experiments for each treatment and Bonferroni's method (Maxwell & Delaney, 1990) was used to compare the means.

Results

Growth measurement

Experiment 1. Assessment of alfalfa accessions: The mean data from experiment 1 are presented in Table 1. Considerable variation for drought tolerance was observed among the accessions. The germination percentage and germination rate were significantly reduced as PEG concentration increased ($p<0.001$), but accession Yazdi showed greater germination percentage at -1.2 MPa PEG treatment. As PEG concentration increased ($p<0.001$), the lengths of plumules and radicles of all accessions were significantly reduced, but the reduction was lower for Yazdi and higher for Ranger in comparison with the control (zero PEG) treatment. PEG also had a significant adverse effect on all biomass parameters except plumule and radicle dry weights. Increasing PEG concentration caused a significant reduction in plumule and radicle fresh weights, but this reduction was lower in Yazdi. There were significant differences among the accessions in all characters measured, and Yazdi was the most tolerant and Ranger the most sensitive accession.

Experiment 2. Second cycle of assessment: The mean data for leaf number, leaf area, shoot lengths, root lengths, root/shoot length ratio, shoot and root dry weights and root/shoot dry weight ratio are presented in Table 2 and Figs. 1, 2 and 3. Number and area of leaves were reduced with an increase in PEG. At -1.2 MPa PEG, the reduction in leaf number was 68.74% of the control in zero PEG for Yazdi and 75.7% and 100% for Nikshahri and Ranger, respectively. Shoot and root lengths were also reduced with an increase in PEG concentration (Figs. 1, 2). Shoot showed a nearly linear decrease in length in the presence of PEG (Fig. 1). Root length was reduced in all accessions in the presence of osmotic stress induced by PEG (Fig. 2).

Root/shoot length ratios increased with increasing PEG concentration. The increase was up to -0.8 MPa PEG for Yazdi and Nikshahri and -0.4 MPa PEG for Ranger. At -1.2 MPa PEG, the ratio was 2.29 compared with 1.31 at zero PEG for Yazdi. Ranger did not germinate at -0.8 and -1.2 MPa PEG.

Shoot and root dry weights were also significantly reduced ($p<0.001$) with increasing PEG concentration (Table 2; Fig. 3). Additionally, the tolerant accession, Yazdi, produced greater shoot and root dry weights at all PEG concentrations than the other accessions examined.

There was no significant difference in proline accumulation in the three accessions in the absence of PEG (Fig. 4). At -0.8 MPa PEG, proline accumulation increased in all accessions, but there was a significant increase in proline concentration in Yazdi accession ($p<0.001$). Proline accumulation of leaves in Yazdi increased 6-folds at -0.8 MPa PEG compared with that at zero PEG concentration (Table 3). At -1.2 MPa PEG, proline accumulation was higher in the shoots of accession Yazdi as compared to that in the other accessions (Fig. 4).

There was a significant difference between osmotic tolerant accession (Yazdi) and sensitive accession (Ranger) with respect to tissue K^+. However, with increasing osmotic stress, K^+ concentrations were increased in the shoots and decreased in the roots. Potassium concentration in the shoot showed greater increase in Yazdi and lower in Ranger. At -1.2 MPa PEG, the reduction in K^+ concentration in the root was 48.3% compared with the control.

Shoot Ca^{2+} concentration in the low osmotic stress (-0.4 MPa PEG) was increased in all accessions but in the high osmotic stress it was decreased.

Discussion

Although, tolerance of adult plants is important, tolerance to osmotic stress at the germination and seedling stages is highly desirable (Raghava Ram & Nabors, 1985; Larson & Bibby, 2004). This investigation indicated that the evaluation method used could effectively detect variation in germination and seedling emergence of alfalfa caused by PEG stress. Considerable variation in response to osmotic stress occurred among alfalfa accessions widely grown in Iran using hydroponic culture experiments. From the data of the assessment method, genetic variation seems to exist among accessions, which as it has been stated above is a requirement for a breeding program to improve osmotic tolerance. This is approved by the fact that the more tolerant accessions had greater shoot and root lengths and biomass production than the sensitive accessions.

The results showing consistency in the degree of osmotic tolerance of the accessions of alfalfa at different growth stages are in agreement with those of Noble *et al*., (1984) who developed a salt-tolerant line of alfalfa by selection at the adult stage. Similarly,

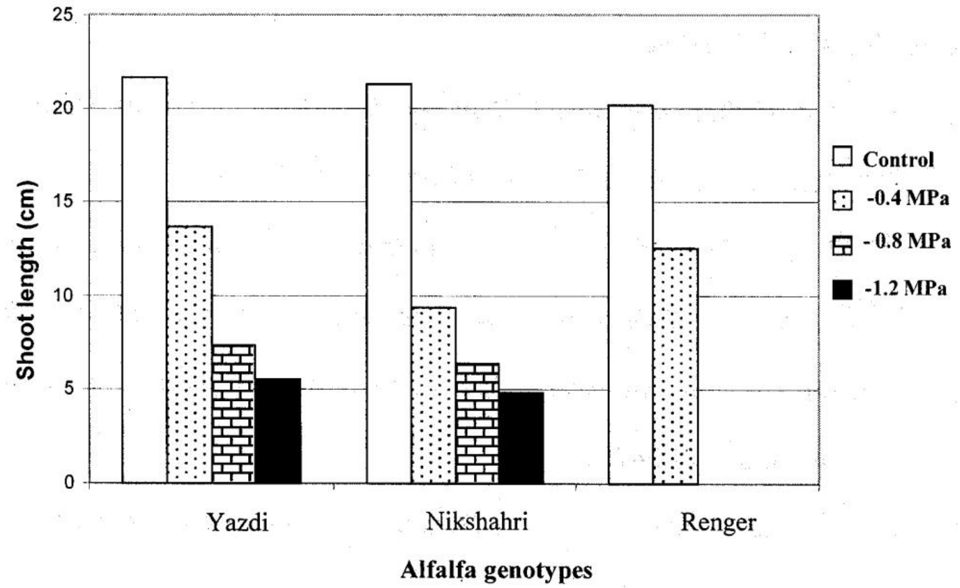

Fig. 1. Shoot length of 14-day-old seedlings of three *M. sativa* accessions in response to osmotic stress (PEG).

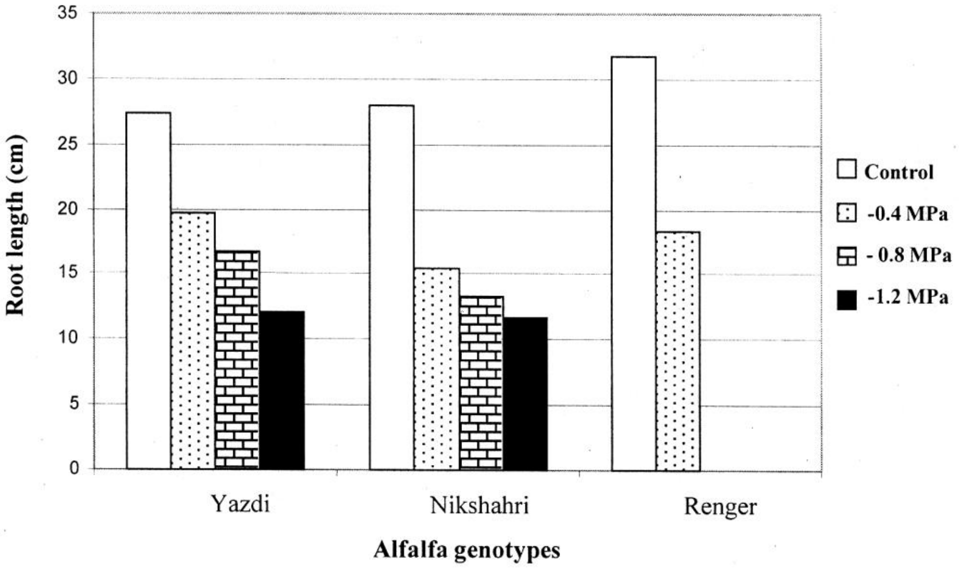

Fig. 2. Root length of 14-day-old seedlings of three *M. sativa* accessions in response to osmotic stress (PEG).

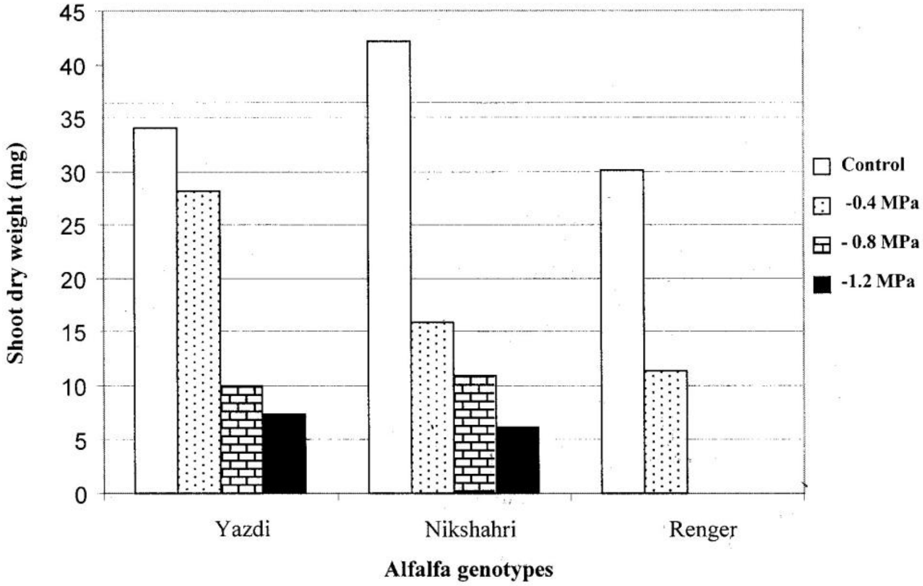

Fig. 3. Shoot dry weight of 14-day-old seedlings of three *M. sativa* accessions in response to osmotic stress (PEG).

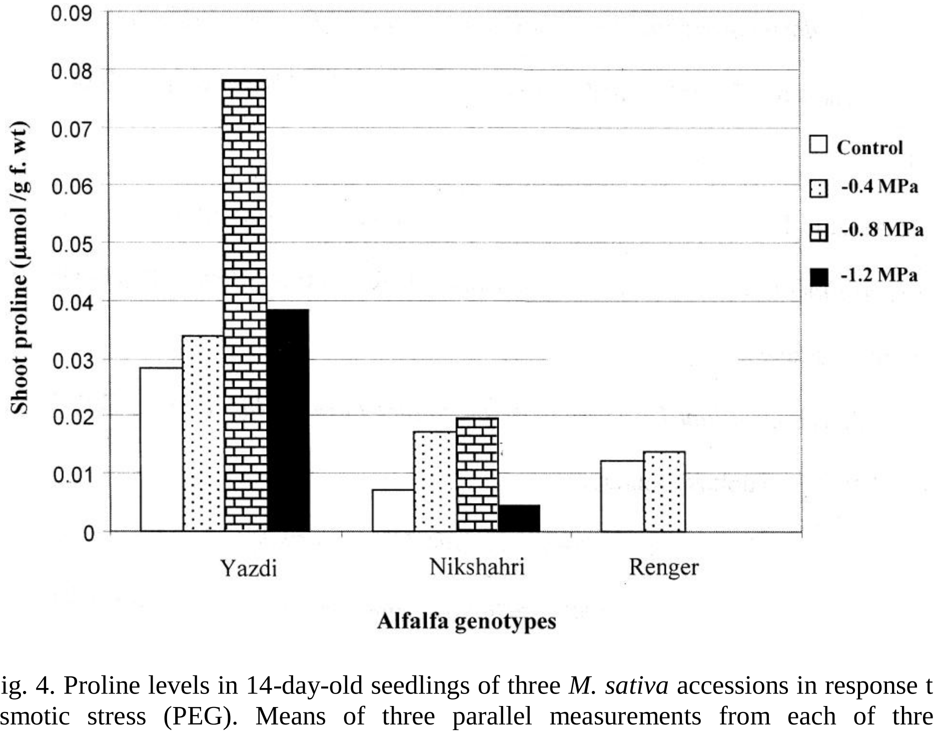

Fig. 4. Proline levels in 14-day-old seedlings of three *M. sativa* accessions in response to osmotic stress (PEG). Means of three parallel measurements from each of three independent extracts.

Ashraf *et al.* (1986) and Al-Khatib *et al.* (1993) developed a salt-tolerant line of the same species through mass selection at the seedling stage and this line was highly tolerant when tested at the adult stage. Analysis of the growth has shown that alfalfa responds to the onset of drought by a reduction in shoot and root elongation (Carter & Sheafer, 1983a, b; Hall, 1993), and the number of basal buds and shoots (Brown & Tanner, 1983; Perry & Larson, 1974). The development of water stress reduced leaf size, internode length, and dry matter production of alfalfa during the first 14 days after the stress was initiated (Brown & Tanner, 1983). Gazanchian *et al*., (2006) showed seedling emergence rate and root and shoot growth were decreased by limiting soil water content, while root-to-shoot length ratio (43%) was increased.

The data showed that proline accumulation increased with increasing drought stress, but its rate is different among accessions and organs. The PEG-induced increase in proline in alfalfa and in osmotic tolerant accession (Yazdi) indicated a positive correlation between proline accumulation and osmotic stress tolerance. Indeed, the accumulation of this amino acid may be a part of general response to adverse environmental conditions (Delauney & Verma, 1993: Ashraf & Harris, 2004). In fact, proline accumulation depends on the length of time and concentration of osmotic stress, the type of tissue, the age of the plant and the genotype (Safarnejad *et al.*, 1996; Ashraf & Foolad, 2007). Proline accumulation differs between cultivars adapted to certain growth conditions or regions, as well as within species more or less tolerant to drought (Heuer, 1994). In alfalfa plants, the most tolerant varieties exhibited the highest potential for proline accumulation in response to osmotic stress (Heuer, 1994) suggesting that it was involved in the osmotic adjustment of alfalfa plants during stress. In stress-shocked plants of accession Yazdi, the size of the amino acid pool was three to four times higher compared with that at control. Similar results were reported for salinity- drought- or osmotic-stressed plants and tissue cultures (Safarnejad *et al*., 1996; Galiba *et al*., 1989; Fougere *et al*., 1991). Following the 16 d water stress, both shoot dry weight and leaf width decreased up to 67% compared with the well-watered plants, whereas proline content increased up to 20-fold (Gazanchian *et al*., 2007). Proline is considered as a compatible solute (Samaras *et al*., 1995), an osmoprotectant (Okuma *et al.*, 2000) and may confer a protective effect by inducing stress-protective proteins (Khedr *et al*., 2003). Among amino acids, the accumulation of proline, is frequently reported in many plants or tissues in response to a variety of abiotic stresses (Gazanchian *et al*., 2007; Pedrol *et al*., 2000; Hare & Cress, 1997; Heng-long *et al.*, 1999). In maize primary root, for example, the proline level increased as much as a hundred fold under a low water potential (Voetberg & Sharp, 1991). The free proline level also increased from 4 to 40 times in pea in response to water stress (Francisco *et al*., 1998). This seems to indicate that proline may play a role in minimizing the damage caused by dehydration (Francisco *et al*., 1998).

Concentrations of K^+ and Ca^{2+} increased with drought stress and there was a significant difference among the accessions. K^+ concentration increased significantly in all accessions due to osmotic stress.

The results suggest that the accession Yazdi may tolerate severe drought stress due to low reduction in growth attributes and increased accumulation of proline and K^+ and Ca^{2+} in plant tissues.

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