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Research Paper

Differential accumulation 0f phyto-biochemical parameters in Etra (Calligonum comosum Herit) plants against water deficiency

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Abstract: Ertà (*Calligonum comosum*), a large perennial plant, is a dominant vegetation component of sandy deserts across central Asia. Photosynthetic pigments and biochemical characteristics of *C. comosum* in response to water deficiency were analyzed based on the photosynthetic pigments, chlorophyll a (Chl. *a*), chlorophyll b (Chl. *b*), chlorophyll stability index (CSI), total protein content (TPC), free amino acids (FAA), total soluble sugars (TSS) and proline (Pro) content. Drought stress was created by withholding irrigation on base of field capacity percent (FC %). The results indicated that drought stress significantly decreased the main photosynthetic pigments. Chl. *a*, Chl. *b*, total chlorophyll content [Chl. (*a*+*b*)], Chl. *a*/*b* ratio and CSI. Drought stress also affected biochemical parameters. FAA, TSS and Pro increased with increase in drought stress intensity. At all treatments, water deficiency induced a reduction in TPC. The findings indicate that Ertà plants were able to tolerate conditions of low water availability. Osmotic adjustment in leaves is an important mechanism enabling plants to cope with drought.

Keywords: drought stress, photosynthetic pigments, soluble sugars, proline, amino acids

Introduction

Caligonum species are dominant perennial shrubs in active sand dunes and stabilize sand fields in most desert areas (Tao, 2000; Dashti *et al*., 2011). They can exist in mobile sand dunes in condition of hard drought and suitable for revegetation of deserts (Zang, 1992). Ertà (*Calligonum comosum*), a large perennial plant, is a dominant vegetation component of sandy deserts across central Asia. This species is very broadly distributed in Middle Asia, the Middle East, Iran and Saudi Arabia (Pyankov *et al*., 1999; Ranjbarfordoei *et al*., 2013).

The recorded global climate changes have certainly generated ecological mutations which, particularly contributed to intensification of desertification process, especially affecting arid and semi-arid regions. Within these regions, aridity has a permanently determinative effect due to the precipitation deficit (Munns *et al*., 2006) accompanied with high water loss by evapotranspiration and salt-rich water irrigation of culture (Yamguchi and Blumwald, 2005).

Drought causes numerous physiological and biochemical changes in plants like reduced leaf size, stem extension, root proliferation, reduced water use efficiency (Farooq *et al.,* 2009), alteration in metabolic activities (Lawlor and Cornic, 2002), inhibition of enzymatic activities (Ashraf *et al.,* 1995), disturbances in solute accumulation (Khan *et al.,* 1999) or a combination of all these factors. Plants are able to limit water losses by closing their stomata, inhibition of photosynthetic enzyme activity and regeneration, and alteration in carbohydrate metabolic equilibrium (Flexas *et al*., 2004).

In response to drought stress, xerophytes induce tolerance mechanisms that contribute to adapt to osmotic stresses caused by severe water deficiency. A large number of metabolites are involved in this adjustment (Munns, 2002). Among these organic molecules that are often associated with plant drought tolerance are organic compounds such as soluble sugars and amino acids such as proline (Volaire *et al*., 1998). Under drought stress conditions, plants responds appear as accumulation of water soluble carbohydrates together with other compatible solutes such as amino acids (De Roover *et al*., 2000;). These osmolytes are used for maintaining leaf cell turgor, protecting membrane integrity and preventing protein denaturation (Xue *et al*., 2008).

Another plant response to drought stress is change in photosynthetic pigment content that play important roles in harvesting light. The content of both chlorophyll *a* and *b* changes under drought stress (Farooq *et al*., 2009). The aim of this study was to investigate some biochemical characteristics in Ertà (*C. comosum*) plants under different levels of drought stress. It is concluded that Ertà plants were able to tolerate conditions of low water availability. Further, osmotic adjustment in leaves is an important mechanism enabling plants to cope with extreme drought. a large perennial plant is a dominant vegetation.

Martial mad Methods

This study was carried out at the Faculty of Natural Resources and Earth Sciences, University of Kashan, Iran. The prevailing climate there is of the arid type (Kardavani, 1990). Average annual temperature is about 20°C. Total annual precipitation amounts to about 100 mm, with an uneven distribution throughout the year, while evaporation is 2500–2700 mm annually. Total radiation is 2900–3100h per year (Arbaby, 2010).

Plants: Seeds of Ertà were collected in November 2011 from typical dry areas of the Maranjab in Kashan County, Isfahan Province, Iran (34°00´ -34°10´ N, 51°27´-51°35´ E, 800–950 m a.s.l.). Seeds were sown on wet tissue paper in petri dishes. After germination, seedlings were put into small plastic pots (150 cm) and grown for about two months.

Thirty healthy seedlings of uniform height were chosen and transferred to a 6-L pot containing sand (gathered from sand dunes in the region), watered to field capacity (FC) each day. To ensure nutrient deficiency was not limiting, 120 kg ha^{-1} of N [$(NH_4)_2$ SO_4], 55 kg ha^{-1} of P (mineral superphosphate) and 85 kg ha^{-1} of K (K_2SO_4) were applied to each pot (Pascale *et al*., 2003). Seedlings were then grown in an open air under natural conditions for three months.

Drought treatment: Drought treatments were initiated five months after sowing. The soil water content (SWC) treatment was divided into five levels: control (100% of FC; at this level of FC, mean SWC was 32.3%), MID (mild drought, 80% FC), MOD (moderate drought, 60% FC), SD (severe drought, 40% FC) and ED (extreme drought, 20% FC) (Ranjbarfordoei *et al*., 2013). Plants were subsequently watered when the soil attained the desired level of SWC in each drought treatment. Irrigation interval was determined by the drought conditions of each treatment. SWC was monitored with the help of a WP4 dewpoint potentialmeter (Decagon Devices Inc., Washington, USA). The experiment was arranged in a completely random design with five replicates per drought treatment.

Phyto-biochemical measurements: Main photosynthetic pigments (chlorophyll *a* and *b*) were extracted from the leaves and estimated by the method of Arnon (1949). Half of a gram leaf material was ground with 10 ml of 80 per cent acetone and centrifuged at 2500xg for 10 minutes at 4°C. This procedure was repeated until the residue became colourless. The extract was transferred to a graduated tube and made up to 10 ml with 80 per cent acetone and assayed immediately.

Three milliliters aliquots of the extract were transferred to a cuvette and the absorbance was read at 645, 663 and 480 nm with a spectrophotometer (U-2001-Hitachi) against 80 per cent acetone as blank. Chlorophyll content was calculated using the formula of Arnon and expressed in milligram per gram dry weight (mg g^{-1} DW). The chlorophyll stability index (CSI) was determined according to Sairam *et al*. (1997) and calculated as follows:

CSI = (total chlorophyll under stress/total chlorophyll under control) × 100

Total soluble sugar was estimated by the method of Nelson (1944). Leaf samples were treated with 80 percent boiling ethanol for taking extractions (5 ml extract representing 1 g of tissue). Five readings for each sample were taken.

One ml of ethanol extract taken in the test tubes was evaporated in a water bath. To the residue, 1 ml of distilled water and 1 ml of 1 N sulphuric acid were added and incubated at 49 °C for 30 min. The solution was neutralised with 1 N sodium hydroxide using methyl red indicator. One ml of Nelson's reagent was added to each test tube prepared by mixing reagent A and reagent B in 25:1 ratio (Reagent A: 25 g sodium carbonate, 25 g sodium potassium tartarate, 20 g sodium bicarbonate and 200 g anhydrous sodium sulphate in 1000 ml; Reagent B: 15 g cupric sulphate in 100 ml of distilled water with 2 drops of concentrated sulphuric acid). The test tubes were heated for 20 min in a boiling water bath, cooled and 1 ml of arsenomolybdate reagent (25 g ammonium molybdate, 21 ml concentrated sulphuric acid, 5 g sodium arsenate dissolved in 475 ml of distilled water and incubated at 37 °C in a water bath for 48 h) was added. The solution was thoroughly mixed and diluted to 25 ml and measured at 495 nm in a spectrophotometer. The reducing sugar contents of unknown samples were calculated from glucose standard.

Free amino acids content was determined according to Moore and Stein (1948). One ml ethanol extract was taken in 25 ml test tubes and neutralized with 0.1 N sodium hydroxide using methyl red indicator. One ml of ninhydrin reagent was added (800 mg stannous chloride in 500 ml citrate buffer, pH 5.0, 20 g ninhydrin in 500 ml methyl cellosolve; both solutions were mixed). The contents were boiled in a water bath for 20 min, 5 ml of diluent solution (distilled water and n-propanol mixed in equal volume) was added, cooled and diluted to 25 ml with distilled water. The absorbance was measured at 570 nm in a spectrophotometer. The standard graph was prepared using leucine.

Free proline content was determined according to Gilmour *et al*., (2000). Seedling samples from each variety was homogenized in 3% (w/v) Sulphosalicylic acid 1 mL at room temperature and then stored at 4°C over night. The supernatant was added with acid ninhydrin and glacial acetic acid. The mixture was heated at 100°C for 45 min in a water bath. Reaction was then stopped by using an ice bath. The mixtures were extracted with toluene and measured at wavelength 519 nm. Proline concentration was determined using calibration curve and expressed as mg g^{-1}DW.

Protein content was determined according to Lowry *et al*. (1948). Fresh tissue weighing 0.5 g was macerated in 20 per cent trichloroacetic acid using mortar and pestle. The homogenate was then centrifuged at 600 rpm for 30 min and the supernatant was discarded. Five ml of 0.1 N NaOH was added to the pellet and it was centrifuged for 30 min. The supernatant was saved for the estimation of protein. To 0.5 ml of the extract, 5 ml of copper reagent 'C' was added (Reagent C: mixture of reagents A and B in the 50:1 ratio; Reagent A: 2 per cent Na_2CO in 0.1 N NaOH; Reagent B: equal volume of 1 per cent $CuSO_3$ and 2 per cent sodium potassium tartrate). The tubes were shaken well and allowed to stand in dark for 10 min at room temperature, 0.5 ml of properly diluted Folin-Ciocalteau reagent was added to the solution and mixed thoroughly. The absorbance was read at 500 nm in a spectrophotometer against an appropriate blank. Bovin serum albumin was used as the standard.

Results

The results in Tabl 1 show the effects of increasing drought stress on photosynthetic pigments in leaves at different drought stress levels. Drought stress caused a significant reduction in Chl *a* content when stress exceeded MOD, ranging from a reduction by 42% in SD to a reduction by 53% in ED compared to the control. A significant decrease in Chl *b* was observed at SD and it did not show a significant alteration with more increases in stress. At all treatments, drought stress induced a reduction in total chlorophyll content, but a significant reduction was initiated at MOD. Significant reduction in Chl (*a*+*b*) content initiated at MOD and continued with further increases in stress to SD. Although a decrease in Chl (*a*+*b*) was observed between SD and ED levels, it was not statistically significant. A decrease in CSI was observed with increasing drought stress. Significant reduction for this parameter started at MOD and followed to SD. It was highest at control level (100%) and lowest at ED (49%) (Table1).

Exposure of Ertà plants to the selected drought stress levels induced marked alterations in biochemical contents (Table 2). TPC did not change significantly with increasing drought stress levels from control to MID, but a further increase in drought stress led to a significant increase in the value of this parameter from about 38.60 at MOD to 18.30 at ED. An increase in FAA was observed with increasing drought stress. Significant increase for this parameter started at MOD (27.5 mg g^{-1}) and increased to ED (43 mg g^{-1}). Likewise, TSS increased with increasing drought stress, and all treatments were significantly higher compared to the control, with an increase of 18%, 63%, 94% and 124%, for MID, MOD, SD and ED, respectively (Table 2).

Table 1 Effects of different levels of drought stress on photosynthetic pigment components (PPC) in leaves of *C. comosum* (values are mean ± S.E., n =5)

PPC	Chl. *a* (mg g^{-1})	Chl. *b* (mg g^{-1})	Chl. (*a*+*b*) (mg g^{-1})	Chl. (*a*/*b*)	CSI (%)
SWC (FC %)					
Ctrl	13.71±1.73^{a}	6.53±0.72^{a}	20.24±2.11^{a}	2.10±0.13^{a}	100.00±4.41^{a}
MID	13.56±1.21^{a}	6.38±0.97^{a}	19.84±1.93^{a}	2.12±0.09^{a}	99.00±4.28^{a}
MOD	11.82±1.62^{a}	5.41±0.69^{a}	17.23±1.11^{b}	2.18±0.04^{a}	85.00±3.53^{b}
SD	7.88±1.10^{b}	3.80±0.54^{b}	11.68±1.27^{c}	2.08±0.07^{b}	58.00±2.47^{c}
ED	6.41±0.93^{b}	3.47±0.38^{c}	9.88±0.85^{c}	1.85±0.05^{c}	49.00±2.72^{c}

Different letters in each column show significant difference at $P < 0.05$ by Duncan's Multiple Range Test (DMRT). SWC: soil water content; FC: field capacity; Chl. *a*: chlorophyll *a*; Chl. *b*: chlorophyll *b*; Chl. (*a*+*b*): sum of chlorophyll *a* and *b*; CSI: chlorophyll stability index.

Table 2 Effects of different levels of drought stress on biochemical components (BC) in leaves of *C. comosum* (values are mean ± S.E., n =5)

BC	TPC (mg g^{-1})	FAA (mg g^{-1})	TSS (mg g^{-1})	Pro (μg g^{-1})
SWC (FC %)				
Ctrl	60.37±7.41^{a}	14.00±2.18^{a}	36.80±3.44^{a}	50.20±8.18^{a}
MID	52.34±8.22^{a}	17.70±3.47^{a}	43.32±4.56^{b}	60.12±7.57^{a}
MOD	38.60±5.68^{b}	27.50±3.10^{b}	60.15±6.81^{c}	71.80±9.11^{b}
SD	24.80±4.13^{c}	35.30±4.35^{c}	71.60±5.77^{d}	87.30±9.77^{c}
ED	18.30±3.83^{d}	43.00±4.18^{d}	82.52±6.64^{e}	94.40±10.65^{c}

Different letters in each column show significant difference at $P < 0.05$ by Duncan's Multiple Range Test (DMRT). SWC: soil water content; FC: field capacity; TPC: total protein content; FAA: free amino acids; TSS: total soluble sugars; Pro: proline content.

Conclusion

Leaf chlorophyll content is one of the most important factors in determining the photosynthesis rate and dry matter production. Our data indicate that Chl. *a* and Chl. *b* concentrations were decreased in response to drought stress. Reduction of pigments content, as a result of drought stress can be attributed to a decrease in chlorophyll synthesis or to an increase in chlorophyll degradation. Such an alteration has been considered as a typical symptom of drought stress (Hugo *et al*., 2008). Chlorophyll content [Chl. (*a*+*b*)] and Chl. *a*/*b* ratio of leaves are widely used to characterize the general state of the photosynthetic apparatus (Zhang *et al*., 2011). A decreased Chl. (*a*+*b*) and Chl. *a*/*b* ratio was also observed under drought stress

in our study (Table 1). A decline in these parameters suggests that the photosynthetic efficiency and PSII might be affected by drought stress. Similar results have been reported in the leaf chlorophyll content in other plants under water stress (Hugo *et al*., 2008; Nikolaeva *et al*., 2010; Wu *et al*., 2008). CSI showed a significant and negative relationship with the level of drought stress. Decreased CSI percentages in response to drought stress have been reported in chickpea (Rahbarian *et al*., 2011) and cotton (Mssacci, 2008).

The maximum TPC in leaves were recorded at control plants and decreased with the increased in drought stress. The drought stress injury causes damage to protein synthesizing mechanism (Shashi and Godara 2011). The possible reason for decreased protein content under water stress may be due to increased activity of protease and also it may be due to proteolysis or decreased synthesis or both. Leaf proteins undergo accelerated hydrolysis as drought stress develops. Similar findings were reported by and (Shashi and Godara (2011) in *Ziziphus mauritiana*.

Our study clearly demonstrated that increasing drought stress increased FAA content of *C. comosum* leaves. Similar results were obtained in sorghum (Yadav *et al*., 2005), okra (Beemarao *et al*., 2007), and *Arachis hypogaea* (Asha and Rao 2002). The accumulation of amino acids may be due to the hydrolysis of protein and also may occur in response to the changes in osmotic adjustment of their cellular contents (Greenway and Munns 1980). Free amino acid accumulation is more important to account for most of the changes in osmotic potential. The accumulation of free amino acids under stress indicates the possibility of their involvement in osmotic adjustment (Yadav *et al*., 2005) and osmotic adjustment is an important mechanism alleviating detrimental effects of drought stress (Morgan 1984).

Soluble sugar is an important constituent and source of energy for all living organisms. Plants manufacture this organic substance during photosynthesis and it is consumed during respiration. TSS concentration is, thus, indicative for the physiological activity of a plant and it determines the sensitivity of plants to drought stress. TSS concentration in leaves of *C. comosum* was strongly increased by an increase in drought stress (from 18% to 124%). Increased TSS concentrations in response to drought have been reported in *Atriplex halimus* (Martínez *et al*., 2004), oak (Épron and Dreyer 1996) and olive (Ennajeh *et al*., 2006). From the increasing trend in total soluble sugar content in stressed plants, one can say that sugars act as osmotica and/or protect specific macromolecules and contribute to the stabilization of membrane structures (Bratels and Sunkar 2005) or it can be attributed to the degradation of starch (Épron and Dreyer 1996).

Present study revealed that Ertà plants subjected to different levels of drought stress accumulate concentrations of Proline (from1.2 to 1.9 fold) than controls. Proline accumulation under drought stress was previously reported in wild jujube (*Ziziphus lotus*) (Maraghni *et al*., 2011), *Prunus persica* (Arndt *et al*., 200) and sorghum (Yadav *et al*. 2005). Increased proline in the stressed plants may be an adaptation the purpose of which is to overcome the stress conditions. Proline accumulates under stressed conditions supplies energy for growth and survival and thereby helps the plant to tolerate stress (Chandrashekar and Sandhyarani 1996). Proline accumulation in plants might have a scavenger function and act as an osmolyte. The reduced proline oxidase may be the reason for increasing proline accumulation.

This study revealed significant changes in all parameters measured, mostly at MOD and SD levels. Therefore, Chl. *a*, Chl. *b* and TPC suffered decrease under water restriction. However, TTS, FAA and Pro were increased due to osmotic adjustment process. It is concluded that Ertà plants were able to tolerate conditions of low water availability. Further, osmotic adjustment in leaves is an important mechanism enabling plants to cope with extreme drought.

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