Australian Journal of Crop Science AJCS
AJCS 5(1):55-60 (2011) ISSN:1835-2707

Response of proline, soluble sugars, photosynthetic pigments and antioxidant enzymes in potato (*Solanum tuberosum* L.) to different irrigation regimes in greenhouse condition

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Abstract

To evaluate the response of proline and soluble sugars content, chlorophyll a (CHLa), chlorophyll b (CHLb), total chlorophyll (TC), catalase (CAT) and ascorbate peroxidase (APX) activity in potato (*Solanum tuberosum* L.) leaves to different irrigation regimes at two growth stages, a greenhouse factorial experiment was conducted in a completely randomized design (CRD) with three replications. The factors consisted of four different irrigation regimes as 100% (I_0), 80% (I_1), 60% (I_2) and 40% (I_3) of field capacity and growth stages: 50% emergence to 50% flowering (GS_1) and 50% flowering to physiological maturity (GS_2). Fresh leaf tissues were used to determine proline and soluble sugars content, CHLa, CHLb, TC and CAT and APX activity. According to the results, irrigation regimes had significant effect on proline content, soluble sugars and catalase (CAT) activity, but no significant differences were detected among irrigation regimes for CHLa, CHLb, TC and APX activity. Limited irrigation increased proline concentration and total soluble sugars in leaves. None of studied traits were affected by growth stages. Interaction between irrigation regimes and growth stages was not significant for all studied traits. Result also indicated that the highest proline content (4.9µmol.g^{-1}FW), total soluble sugars (55.9 mg.g^{-1}FW) and CAT activity (12.7 µmol H_2O_2 min^{-1} g^{-1}FW) were related to irrigation at 40% of field capacity (I_3). It was concluded that proline and soluble sugar levels were increased in potato leaves under deficit irrigation regimes.

Key words: Ascorbate peroxidase activity; catalase activity; growth stages; osmoregulation; water deficit
Abbreviations: CAT-catalase; APX-ascorbate peroxidase; CHLa-chlorophyll a
CHLb-chlorophyll b; TC-total chlorophyll; θ-gravimetric water content

Introduction

Recent publications (FAO, 2004) have shown the importance of the potato (*Solanum tuberosum*, L.) as a global food crop, ranking fourth among others. Water deficit and salt stresses are global issues to ensure survival of agricultural crops and sustainable food production (Jaleel et al., 2007). Drought is the most important limiting factor for crop production and it is becoming an increasingly severe problem in many regions of the world (Passioura, 2007). Many investigations have demonstrated that potato is relatively sensitive to water deficit stress (Opena and Porter, 1999; Porter et al., 1999; Fabeiro et al., 2001). Relative water content (RWC), leaf water potential, stomatal resistance, transpiration rate and leaf and canopy temperature are important characteristics that influence plant water relations (Siddique et al., 2001). Under severe transpiration or water deficit, potato RWC is lower than that of many other crops (Loon, 1981). This might be one of the reasons for susceptibility of potato to water deficit. The values of RWC for irrigated potatoes is 80-100%, while for those who non-irrigated is 76-87% (Loon, 1981).Thornton (2002) and Shock (2004) found that potato are very sensitive to water deficit stress in all growth stages, especially tuber formation. Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing power such as ATP and NADPH. Both the chlorophyll a (CHLa) and b (CHLb) are prone to soil drying damages. However, carotenoids have additional roles and partially help the plants to withstand adversaries of drought (Farooq et al., 2009). Drought stress induced changes in the ratio of CHLa and CHLb and carotenoids (Farooq et al., 2009). The chlorophyll content decreased to a significant level at higher water deficits in sunflower (Kiani et al., 2008) and *Vaccinium myrtillus* (Tahkokorpi et al., 2007). Reactive oxygen species (ROS) are partially reduced forms of atmospheric oxygen. ROS typically result from the excitation of O_2 to form singlet oxygen (O_2) or

Table 1. Variance analysis for proline, Chlorophyll a (CHLa) , Chlorophyll b (CHLb) total chlorophyll (TC), soluble sugars content and catalase (CAT) and ascorbate peroxidase (APX) activity as effected by different irrigation regimes and growth stages

Source of variation	df	Proline	Chlorophyll a	Chlorophyll b	Total chlorophyll	Soluble sugars	CAT	APX
Irrigation regime (I)	3	22.29 **	0.88	0.55	1.51	2876.6 **	105.42 **	0.77
Growth stage (Gs)	1	0.38	0.16	1.14	0.49	1.89	12.76	0.005
I × Gs	3	2.8	0.46	0.52	2.59	42.79	7.67	0.002
Error	16	2.12	0.98	0.36	1.75	71.88	7.1	0.3

**, Significant at *P<0.01*

from the transfer of one, two or three electrons to O_2, and generation of a superoxide (O^{2-}), hydrogen peroxide (H_2O_2) or a hydroxyl (OH) radicals, respectively (Shigeoka et al., 2002). The cells are normally protected against ROS by the operation of an antioxidant defense system, comprised of enzymatic (superoxide dismutase, catalase, glutathione reductase, ascorbate peroxidase) and nonenzymatic (ascorbate, α-tocopherol, carotenoids, glutathione) components (Shigeoka et al., 2002). ROS production and consequently antioxidant enzymes activities is further enhanced when plants exposed to various abiotic stresses, such as drought (Rubio et al., 2002), salinity (Broetto et al., 2002) and low and high temperature (Pastori and Foyer 2002). Osmotic adjustment is a mechanism to maintain water relations under osmotic stress. It involves the accumulation of a range of osmotically active molecules/ions including soluble sugars, sugar alcohols, proline, glycinebetaine, organic acids, calcium, potassium, chloride ions and etc. Under water deficit and as a result of solute accumulation, the osmotic potential of the cell is lowered, which attracts water into the cell and helps with the maintenance of turgor. Potato responds to drought and salt stresses by accumulating proline which functions as an osmoprotector, osmoregulator and ROS scavenger (Benavides et al., 2000). Regarding to involvement of the above mentioned mechanisms in water deficit stress, an investigation was designated to study the effects of different irrigation regimes during two growth stages on total chlorophyll (TC), CHLa, CHLb, proline and total soluble sugars content and antioxidant enzymes activity (CAT and APX) in potato leaves.

Materials and methods

Certified seeds of *S. tuberosum* L. cv. Marfuna with uniform size (35–50mm) were used as experimental material during 2008, from April to August at the research greenhouse of Urmia University, Iran (37°32' N, 45°5' E; altitude, 1320 m). Potato tubers were planted in pots (25 cm diameter and 40 cm height) filled with 24 kg clay loam soil with a bulk density of 1.51 gcm^{-3}. The soil moisture content was measured gravimetrically on every alternate day immediately before each irrigation. Soil moisture contents (% θ, weight based) at field capacity (FC) and permanent wilting point were 17% and 3.0%, respectively. Until 30 days after planting, pots were watered equally then, irrigation treatments were applied as: 100% of field capacity (FC) (I_0), 80% of field capacity (0.8 FC) (I_1), 60% of field capacity (0.6 FC) (I_2), and 40% of field capacity (0.4 FC) (I_3). Pots were weighed daily and water added to each pot as lost weight of pots. Growth stages consisted of 50% emergence to 50% flowering (GS_1) and 50% flowering to physiological maturity (GS_2). Fertilizers were applied at rates of 150 N, 30 P, 220 K kg/ha according to the soil test analysis. All treatments were repeated three times in experimental pots in a factorial form based on complete randomized design. Greenhouses were kept as close as possible to the external air temperature (22-25ºC day and 19ºC night). The tubers were planted on 21 April, 2008 and emerged about three weeks later. They were harvested on 6 August, 2008.

Pigments extraction and estimation

The extraction of chlorophyll a and b and TC was carried out according to Gross (1991). At the end of each growth stages (GS_1 and GS_2), the fresh tissue of young and expanded leaves collected and freeze at -80ºC then, the leaves (0.25 g) were homogenized with 80% acetone. The optical density (O.D.) of the extracted chlorophyll was measured at 645 and 663 nm by using spectrophotometer PD-303. TC, CHLa and CHLb were calculated by the following formulae (Gross, 1991).

CHLa = (0.0127× OD663) – (0.00269× OD645)
CHLb = (0.0229× OD645) – (0.00468× OD663)
TC = (0.0202× OD645) + (0.00802× OD663)

Enzyme extraction and assay

One-tenth g of fresh foliar tissue (uppermost leaves taken at the end of two growth stages) was analyzed for enzymatic assays. Catalase activity (µmol H_2O_2 min^{-1} g^{-1} FW) was assayed by measuring the initial rate of hydrogen peroxide disappearance (Chance and Maehly, 1959). The reaction mixture contained 2.5 ml of 50 mM potassium phosphate buffer (pH 7.4), 0.1 ml of 1% hydrogen peroxide and 50 µl of enzyme extract. The homogenate was centrifuged at 15000 g for 15 min at 4 ºC and the supernatant was immediately used for the enzyme assay. The decrease in hydrogen peroxide was followed as a decline in optical density at 240 nm and the activity was calculated using the extinction coefficient of 36 mM cm^{-1} for hydrogen peroxide. Ascorbate peroxidase ($\mu molg^{-1}$ FW min^{-1}) activity was determined as described by Asada (2001). The reaction mixture contained 2.5 ml of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.2 ml of 1% hydrogen peroxide and 0.1 ml enzyme extract. The homogenate was centrifuged at 15000 g for 15 min at 4 ºC and the supernatant was used to measure enzyme activity. The hydrogen peroxide-dependent oxidation of ascorbate was

followed by monitoring the decrease in absorbance at 290 nm, using the extinction coefficient of 2.8 mM cm^{-1}.

Determination of proline and soluble sugars content

To determine the proline content, 0.5 g of dry leaves was homogenized with 5 ml of 95% ethanol. Above phase of filtrate was separated and its sediments were washed by 5 ml of 70% ethanol for two times and its above phase added to the previous over compartment. The mixture was centrifuged at 3500 g for 10 min at 4ºC and the supernatant was recovered and alcoholic extract kept in refrigerator at 4ºC (Paquin and Lechasseur, 1979). One ml of alcoholic extract was diluted with 10 ml of distilled water and 5 ml of ninhydrin (0.125 g ninhydrin, 2 ml of 6 mM NH_3PO_4, 3 ml of glacial acetic acid) and 5 ml of glacial acetic acid added then mixture placed in boiling water bath for 45 min at 100ºC. The reaction was stopped by placing the test tubes in cold water. The samples were rigorously mixed with 10 ml benzene. The light absorption of benzene phase was estimated at 515 nm using a PD-303 model spectrophotometer. The proline concentration was determined using a standard curve. Free proline content was expressed as µmol g^{-1} DW of leaves (Irigoyen et al., 1992). To measuring the content of soluble sugars, 0.5 g of dry leaves was homogenized with 5 ml of 95% ethanol. One-tenth ml of alcoholic extract preserved in refrigerator mixed with 3 ml anthrone (150 mg anthrone, 100 ml of 72% sulphuric acid,W/W). The samples placed in boiling water bath for 10 minutes. The light absorption of the samples was estimated at 625 nm using a PD-303 model spectrophotometer. Contents of soluble sugar were determined using glucose standard and expressed as mg g^{-1} DW of leaves.

Statistical analysis

All experimental data reported were averages of three replicates and the SE of the means was determined. Analysis of variance and Duncan's multiple range test were performed using SPSS software version 15.0.

Results and Discussion

As indicated by analysis of variance, irrigation regimes had significant effects on proline and soluble sugars content and CAT activity ($P < 0.01$), while no significant differences were detected for chlorophyll content and APX activity among irrigation regimes. Also, no considerable alteration was found in chlorophyll content at two growth stages. Interaction effects between the irrigation regimes and growth stages were not significant for all measured parameters (Table.1).

Proline content

Statistical analysis showed that proline content was highly affected ($P< 0.01$) by irrigation regimes, but not affected by growth stages and the interaction between growth stages and irrigation regimes (Table.1). The maximum proline content (4.94 µmol g^{-1} FW) obtained in the most restrictive irrigation regime I_3 (0.4 FC) (Fig.1). In general, proline content of leaves increased with the decline in irrigation water, suggesting that the production of proline is probably a common response of potato under drought conditions.

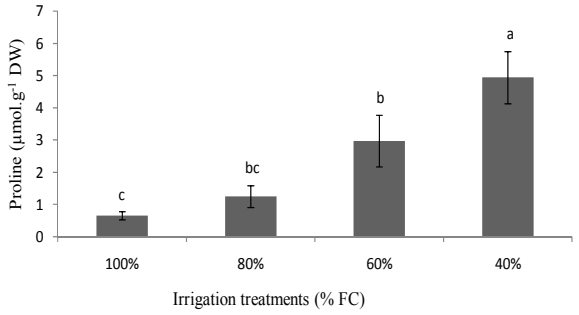

Fig 1. Effect of irrigation regimes on proline content in the leaves of *S. tuberosum* c.v. Marfuna. Values are the means ± SE of three replicates. Means with different letters are significantly different ($P<0.05$). Shoot samples were taken and measured at the end of two growth stages.

The role of proline in adaptation and survival of plants under drought stress reported by Watanabe et al (2000) and Saruhan et al (2006). Osmotic adjustment through the accumulation of cellular solutes, such as proline, has been suggested as one of the possible means for overcoming osmotic stress caused by the loss of water (Caballero et al., 2005). Proline is a non-protein amino acid formed in most tissues subjected to water stress and together with soluble sugars is readily metabolized following recovery from drought (Singh et al., 2000). Proline also serves as a sink for energy to regulate redox potentials, a hydroxyl radical scavenger (Sharma and Dietz, 2006), a solute that protects macromolecules against denaturation and as a means for reducing acidity in the cell (Kishor et al., 2005). Lobato et al (2008) reported that the accumulation of proline and free amino acids in soybean *(Glycine max cv. Sambaiba)* leaves were increased under water deficit 67 and 388.1%, respectively. Teixeira and Pereira (2006) indicated that proline content significantly increased in all potato organs in response to the stress conditions. This increase was more remarkable in roots and tubers than in the leaves. High levels of proline enable the plant to maintain low water potentials causing the accumulation of compatible osmolytes that allows additional water to be taken up from the environment, thus buffering the immediate effect of water deficit within the organism (Mousa and Abdel-Aziz, 2008).

Soluble sugars content

Soluble sugars content in leaves significantly increased under stress condition. The effect of growth stages and the interaction of growth stages by irrigation regimes were not significant for this trait (Table.1). The highest content of these osmolytes (55.99 mg g^{-1} DW) was found in leaves under irrigation regime 0.4 FC (I_3) ($P< 0.01$). Soluble sugars content in irrigation regime I_3 were 1.1, 2.5 and 5.1 times more than those of I_2, I_1 and I_0 irrigation regimes, respectively. The amount of soluble sugars decreased with the decline of irrigation water (Fig.2). Irrigation regimes I_0 and I_1 were significantly different while no difference was observed between I_2 and I_3. The accumulation of sugars in response to drought is quite well documented (Izanloo et al., 2008, Watanabe et al., 2000). Soluble sugars may function as a typical osmoprotectant, stabilizing cellular membranes and maintaining turgor pressure. Gene ontology

attributes such as proline and soluble sugar accumulations were highly enriched in the drought-up-regulated genes, suggesting that those metabolic pathways are important in responses to drought stress. Indeed, the importance of many of these pathways to drought tolerance has been empirically supported by transgenic experiments (Umezava et al., 2006).

Chlorophyll content

CHLa, CHLb and TC in different irrigation regimes were not significantly affected. The effect of growth stages and the interactive effect of irrigation regimes by growth stages on photosynthetic pigments were not significant as well (Table. 1). Obtained results regarding to chlorophyll content in the current study are similar to those obtained by Saltmarsh et al (2006). Yanqiong et al (2007) found that the content of chlorophyll, free proline and soluble sugars increase under drought stress. Manirannan et al (2007) detected a depression in CHL a and b and TC in *Helianthus annuus* L. under water stress. In this study the chlorophyll content remained unchanged under drought and this is similar to what was recorded by Jensen (1985) in onions. According to Poljakoff and Gale (1975), the ability to synthesize more chlorophyll under water stress is a good criterion for the species tolerant to drought. Teixeira and Pereira (2006) reported that chlorophyll content of potato leaves increased significantly in response to drought (5.6%). The decrease in chlorophyll content observed in the leaves collected from salt-stressed plants (Soussi et al., 1998) indicating that salt stress is more severe to potato plants than drought, where the chlorophyll content slightly increased, probably because of some water turgor loss (Bussis and Heineke, 1998). Khosravifar et al (2008) reported that in potato cultivar Agria, the maximum content of chlorophyll was 72.3% and its minimum content was 37.6% during the irrigation after 175 mm and 35 mm evaporation from Class A pan, respectively.

Protective enzymes activities

Catalase activity increased with the decrease of irrigation water ($P< 0.01$) but, ascorbate peroxidase activity remains unchanged (Table.1). Catalase activity increased in irrigation regimes 0.6 and 0.4 FC compared to FC and 0.8 FC. The I_2 and I_3 irrigation regimes had 56% and 65% higher CAT activity than the I_0 treatment (Fig.3). Drought, like other environmental stresses induces oxidative stress. To be able to endure oxidative damage under unfavourable conditions, plants possess both nonenzymatic antioxidants such as carotenoid, flavonoids, α-tocopherol, ascorbic acid and glutathione, and enzymatic antioxidants such as CAT and peroxidase (POX) (Smirnoff, 1993; Munné-Bosch and Alegre, 2000). These enzymatic and nonenzymatic antioxidants have been reported to accumulate under various environmental stresses (Acar et al., 2001), while comparatively higher activity of antioxidants have been shown in tolerant cultivars than those in sensitive ones (Reddy et al., 2004), indicating that higher antioxidant enzyme activity has a role in imparting tolerance against environmental stress. Mechanisms that reduce oxidative injury may play a secondary role during drought tolerance. CAT is only present in peroxisomes and it is indispensable for ROS detoxification

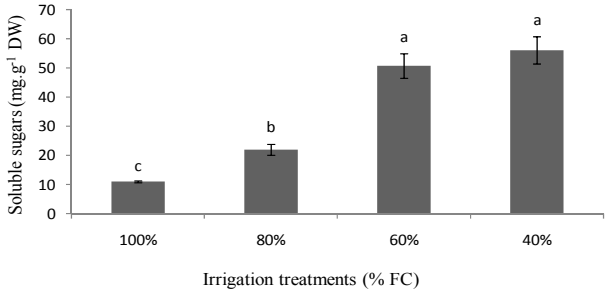

Fig 2. Effect of irrigation regimes on soluble sugars content in the leaves of *S. tuberosum* c.v. Marfuna. Values are the means ± SE of three replicates. Means with different letters are significantly different ($P<0.05$). Shoot samples were taken and measured at the end of two growth stages.

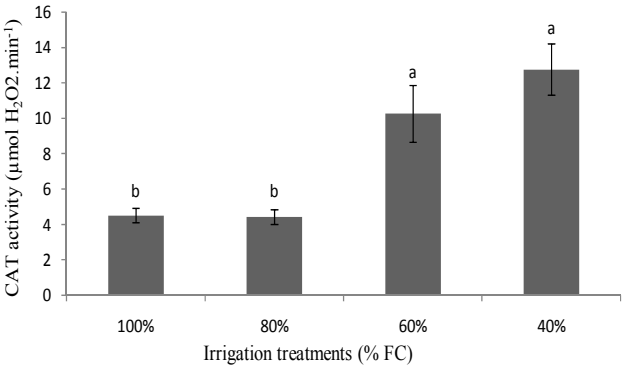

Fig 3. Catalase activity subjected to different irrigation regimes in the leaves of potato. Values are the means ± SE of three replicates. Means with different letters are significantly different ($P<0.05$). Shoot samples were taken and measured at the end of two growth stages.

during stress when high levels of ROS are produced. The balance between SOD, APX or CAT activities in cells is crucial for determining the steady-state level of superoxide radicals and H_2O_2 (Mittler, 2002). Increased CAT activity under water stress has been reported by Agarwal and Pandey (2003a) and Da et al., (2005). Benevides et al (2000) reported the enzymes responsible for hydrogen peroxide detoxification such as ascorbate peroxidase and catalase in *S. tuberosum*. However, they suggested that ascorbate peroxidase was likely to be more important than catalase in the ROS detoxification. Since hydrogen peroxide was also involved in peroxidase-mediated oxidative polymerization, which results in cell wall strengthening, the activation of peroxidase may have a protective role. However under abiotic stress causing hydrogen peroxide accumulation, this may be one of the factors that results in the inactivation of catalase (Velikova et al., 2000). It is suggested that the higher concentrations of catalase and ascorbate peroxidase might have removed the O2 radicals and its product H_2O_2 induced by water stress (Mousa and Abdel-Aziz, 2008). Under the conditions of the present study, it is concluded that the amount of the irrigation water influenced the

proline content, soluble sugars and CAT activity. Maximum proline content was recorded in the leaves of potato grown under most restrictive irrigation regime (I_3). Soluble sugars content was highest in I_3 treatment as well. Also high CAT activity was obtained by application of I_3 treatment. We are expanding the current investigation to different potato cultivars in greenhouse and field conditions to precisely study the above mentioned traits and other important traits involved in drought tolerance in potato.

Acknowledgment

Authors gratefully thanked to Urmia University for financial support of the work.

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