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EFFECT OF OSMOTIC STRESS ON PROLINE ACCUMULATION, PHOTOSYNTHETIC ABILITIES AND GROWTH OF SUGARCANE PLANTLETS (*SACCHARUM OFFICINARUM* L.)

SURIYAN CHA-UM* AND CHALERMPOL KIRDMANEE

National Center for Genetic Engineering and Biotechnology (BIOTEC)
National Science and Technology Development Agency (NSTDA) 113 Thailand Science Park,
Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand

Abstract

Disease-free sugarcane plantlets derived from meristem cutting were photoautotrophically grown on the MS medium and subsequently exposed to 0, 100, 200, 300 or 400 mM mannitol for 7 days. Osmotic pressure in the culture medium was increased with increase in mannitol concentration, causing low water use efficiency (WUE) (r^2 = 0.88) and chlorophyll degradation (r^2 = 0.92). Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total carotenoids (C_{x+c}), concentrations in the osmotic stressed leaves decreased, especially in 400 mM mannitol treatment, degrading 44, 81 and 72%, respectively when compared to control. In contrast, proline content in osmotic stressed plantlets was accumulated and peaked at 2,236.75 μmol g^{-1} FW in 300 mM mannitol treatment. The WUE and chlorophyll degradation were correlated with maximum quantum yield of PSII (F_v/F_m) (r^2 = 0.75) and photon yield of PSII (Φ_{PSII}) (r^2 = 0.83), respectively. The F_v/F_m and Φ_{PSII} in drought acclimatized plantlets decreased, when non-photochemical quenching (NPQ) reached. The reduction of Φ_{PSII} was positively related to net-photosynthetic rate (NPR) (r^2 = 0.85) as well as the proline content and NPQ (r^2 = 0.81). The NPR, stomatal conductance (G_s) and transpiration rate (E) in osmotic stressed plantlets were significantly dropped, leading to growth reduction (r^2 = 0.95). The basic knowledge of osmotic stressed responses may further be applied as effective indices for drought tolerance in sugarcane breeding program.

Introduction

Water limitation is one of the most important factors to reduce agricultural crop production, which is related to global climate changes, especially drought and heat stress (Ciais *et al*., 2005). Drought stress (water deficit or low water availability) is a major abiotic problem, widely distributed worldwide over 1.2 billion ha in rainfed agricultural land (Chaves & Oliveira, 2004; Kijne, 2006; Passioura, 2007). The drought environment has been reported as key factor to limit plant growth and development prior to the loss of productivity, especially of crop species (Bray, 1997; Chartzoulakis *et al*., 2002; Yordanov *et al*., 2003; Reddy *et al*., 2004; Blum, 2005; Neumann, 2008; Shao *et al*., 2008). There are many plant defense responses to water deficit such as transcription factors, water channels/transporters, hormonal regulation, osmoregulation and detoxification systems (Valliyodan & Nguyen, 2006; Seki *et al*., 2007; Shinozaki & Yamaguchi-Shinozaki, 2007; Cattivelli *et al*., 2008). Proline accumulation in drought stressed plants is one of the vital compatible solutes to function in cellular osmotic adjustment and scavenge detoxify oxidants (Delauney & Verma, 1993; Yamada *et al*., 2005; Valliyodan & Nguyen, 2006; Seki *et al*., 2007). There are many ways to enhance on proline accumulation such as Δ^1-pyrroline-5-carboxylate synthetase (P5CS) overexpression, proline dehydrogenase (ProDH) antisense suppression and exogenous proline application for drought tolerant propose (Kavi Kishor *et al*., 1995; de Ronde *et al*., 2000; Simon-Sarkadi *et al*., 2000; Kavi Kishor *et al*., 2005; Yamada *et al*., 2005; Ashraf & Foolad, 2007; Ali *et al*., 2008).

*Correspondence author: Tel.: 662-5646700; Fax.: 662-5646707; E-mail address: suriyanc@biotec.or.th

In drought conditions, water availability in supporting materials such as soil, vermiculite, perlite and peat-moss, is restricted, thereby causing low water use efficiency (WUE) in plant cells (Blum, 2005; Bloch *et al*., 2006; Costa *et al*., 2007; Shao *et al*., 2008). Low WUE is a primary effect on plant responses to water deficit conditions, leading to biochemical changes, including decreased Rubisco (ribulose-1,5-bisphosphatase carboxyase/oxygenase) activity and photochemical efficiency, enhanced accumulation of stress metabolites (proline, glycinebetaine, polyamine, glutathione, polyamines, sugars, sugar alcohols and α-tocolpherol), increased antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase); reactive oxygen species (ROS) reduction and physiological changes ***i.e.*** loss of membrane stability, reduced leaf water potential, pigment degradation, decreased stomatal conductance, reduced internal CO_2 concentration, NPR reduction and growth inhibition prior to plant death (Yordanov *et al*., 2003; Chaves & Oliveira, 2004; Reddy *et al*., 2004; Cattivelli *et al*., 2008; Shao *et al*., 2008). Sugarcane (*Saccharum officinarum* L.) is a member of Poaceae family which produces and accumulates sugar in the stem for sugar production in tropical and subtropical regions (Cordeiro *et al*., 2007). Sugarcane is a high biomass producer and it consumes a large amount of water and takes a long time (6-8 months) for plant growth and development prior to harvesting (Allison *et al*., 2007). Water management is an important factor for sugarcane plantation to achieve maximum yield, especially in arid and semiarid zones (Robertson *et al*., 1999; Wiedenfeld, 2000; Inman-Bamber & Smith, 2005; Singh *et al*., 2007). The aim of this study was to investigate the biochemical and physiological responses of sugarcane plantlets to water deficit using mannitol under *In-vitro* photoautotrophic conditions.

Materials and Methods

Plant materials: Disease-free sugarcane shoots (*Saccharum officinarum* L. cv. K84-200) derived from meristem cutting (Cha-um *et al*., 2006a) were propagated on the MS medium (Murashige & Skoog, 1962) containing 8.88 μM benzyl adenine (BA), 3% sucrose and 0.25% Phytagel® for 6 weeks. The multiple shoots were elongated on the MS medium without plant growth regulators for 4 weeks, then the single shoots were excised and the roots were induced on MS medium supplemented with 2.46 μM indole butyric acid (IBA) for 2 weeks. Plantlets were cultured *In vitro* under conditions of 25±2°C ambient temperature, 60±5% relative humidity (RH) and 60±5 μmol m^{-2} s^{-1} photosynthetic proton flux density (PPFD) provided by fluorescent lamps (TDL 36 W/84 Cool White 3350 Im, Philips, Thailand) with a 16 h d^{-1} photoperiod. Then, the sugarcane plantlets were transferred to MS sugar-free liquid medium (photoautotrophic condition) using vermiculite as supporting material for 7 days. The number of air-exchanges in the glass vessels was adjusted to 2.32 h^{-1} by punching a hole on plastic cap (∅ 1 cm) and covering the hole with a microporous filter (0.20 μm of pore size; Nihon Millipore Ltd., Tokyo, Japan). The plantlets were subsequently cultured in a plant growth incubator with the same conditions as previously mentioned and CO_2 enrichment at 1,000±100 μmol mol^{-1}. Mannitol (osmotic stress) concentrations in the culture medium were adjusted to 0, 100, 200, 300 or 400 mM for 7 days. Photosynthetic pigments, proline contents, chlorophyll a fluorescence, net-photosynthetic rate (NPR) and growth characters were measured.

Data measurements: Osmolarities of culture medium containing varying concentrations of mannitol were measured, according to Lanfermeijer *et al*., (1991) using an osmometer. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll and total carotenoids (C_{x+c}) concentrations were determined following the methods of Shabala *et al*., (1998) and Lichtenthaler (1987), respectively. One hundred milligrams of leaf material were collected, placed in a 25 mL glass vial, added with 10 mL of 95.5% acetone and blended with a homogenizer. The Chl_a, Chl_b, and C_{x+c} concentrations were measured using an UV-visible spectrophotometer. A solution of 95.5% acetone was used as a blank. Pigment degradation percentage was calculated as:

$$\text{Pigment degradation (\%)} = \left[1 - \frac{\text{Salt treatment}}{\text{Control}}\right] \text{ X } 100$$

Proline content from leaves was extracted according to the method of Bates *et al*., (1973). One hundred milligrams of leaf tissues were ground in liquid nitrogen. The homogenate powder was mixed with 1 mL aqueous sulfosalicylic acid (3% w/v) and filtered through filter paper (Whatman #1). Extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 mL of glacial acetic acid and 20 ml of 6 M H_3PO_4) and incubated at 95°C for 1 h. The reaction was terminated placing on an ice bath. The reaction mixture was vigorously mixed with 2 mL toluene. After warming at 25°C, the chromophore was measured on spectrophotometer (DR/4000, HACH, Loveland, Colorado, USA) at 520 nm. L-proline (Fluka, Switzerland) was used as a standard.

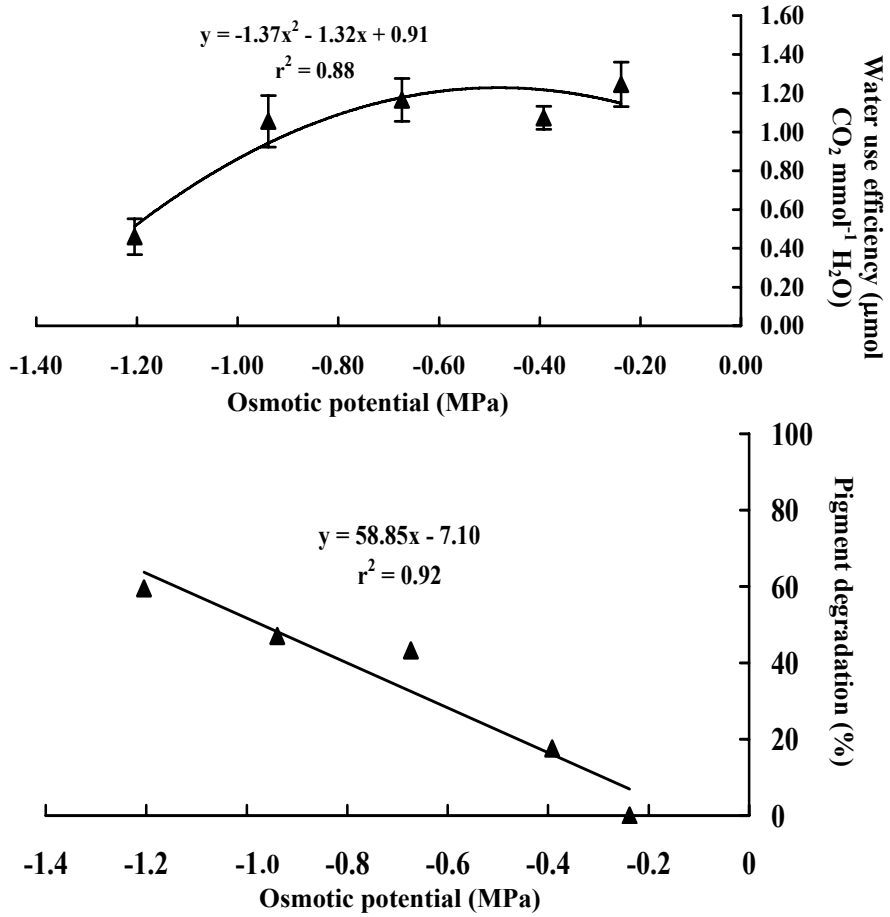
Chlorophyll *a* fluorescence emission from the adaxial surface of leaf was monitored with a Fluorescence Monitoring System (FMS 2; Hansatech Instruments Ltd., Norfolk, UK) in the pulse amplitude modulation mode, as previously described by Loggini *et al*., (1999) & Maxwell and Johnson (2000).

The net-photosynthetic rate (NPR), transpiration rate (E; mmol m^{-2} s^{-1}) and stomata conductance (G_s; µmol H_2O m^{-2} s^{-1}) were measured using Infra-red Gas Analyser (IRGA; Model Portable Photosynthesis System LI 6400, LI-COR® Inc, Lincoln, Nebraska, USA). The E and G_s were measured continuously monitoring H_2O of the air entering and existing in the IRGA headspace chamber. Water use efficiency (WUE) of acclimatized plantlets was calculated by the ratio of NPR to E (Cha-um *et al*., 2007).

Fresh and dry-weights, shoot height, root length and leaf area of sugarcane plantlets were measured as described by Cha-um *et al*., (2006b). Sugarcane plantlets were dried at 110 °C in a hot-air oven (Model 500, Memmert, Buchenbach, Germany) for 2 days, and then incubated in desiccators before measurement of the dry weight. Leaf area of plantlets was measured using a Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd., Burwell, Cambridge, UK).

$$\text{Water used efficiency (WUE)} = \frac{\text{Net photosynthetic rate (NPR)}}{\text{E}}$$

Experimental designs: The experiment was setup in a Completely Randomized Design (CRD) with six replicates and four plantlets per replicate. The mean values were compared by Duncan's New Multiple Range Test (DMRT) and analyzed by SPSS software (SPSS for Windows, SPSS Inc., Chicago, USA). The correlations between physiological and biochemical parameters were evaluated by Pearson's correlation coefficients.



Water use efficiency (µmol CO_2 $mmol^{-1}$ H_2O)
Pigment degradation (%)

Fig. 1. Relationship between osmotic potential in the culture medium and water use efficiency (WUE) (A), osmotic potential in the culture medium and chlorophyll degradation (%) of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Error bars represent ± SE.

Results

Water deficit of sugarcane *In-vitro* plantlets was established using mannitol in the medium to control the osmotic potential (ψ_s) or water available in the root zone. The osmotic pressure in the culture medium containing mannitol was reduced, leading to low water use efficiency (WUE) (r^2 = 0.88) and pigment degradation in the osmotic stressed plantlets (r^2 = 0.92) (Fig. 1). Chlorophyll a (Chl_a), chlorophyll b (Chl_b), toltal chlorophyll (TC) and total carotenoids (C_{x+c}) in osmotic stressed plantlets were significantly dropped, especially in the extreme water deficit treatments (300-400 mM mannitol). In mild drought conditions (100-200 mM mannitol), the Chl_a and Chl_b contents in the leaf tissues were maintained, while the TC and C_{x+c} contents were significantly reduced (Table 1). The Chl_a, Chl_b, TC and C_{x+c} contents in the sugarcane plantlets grown under 400 mM mannitol were more degraded 44, 81, 60 and 72% when compared to those control plantlets. In contrast, proline content in osmotic stressed plantlets increased, relating to mannitol concentrations in the culture medium and it was peaked at 2,236.75 µmol g^{-1} FW in the plantlets treated

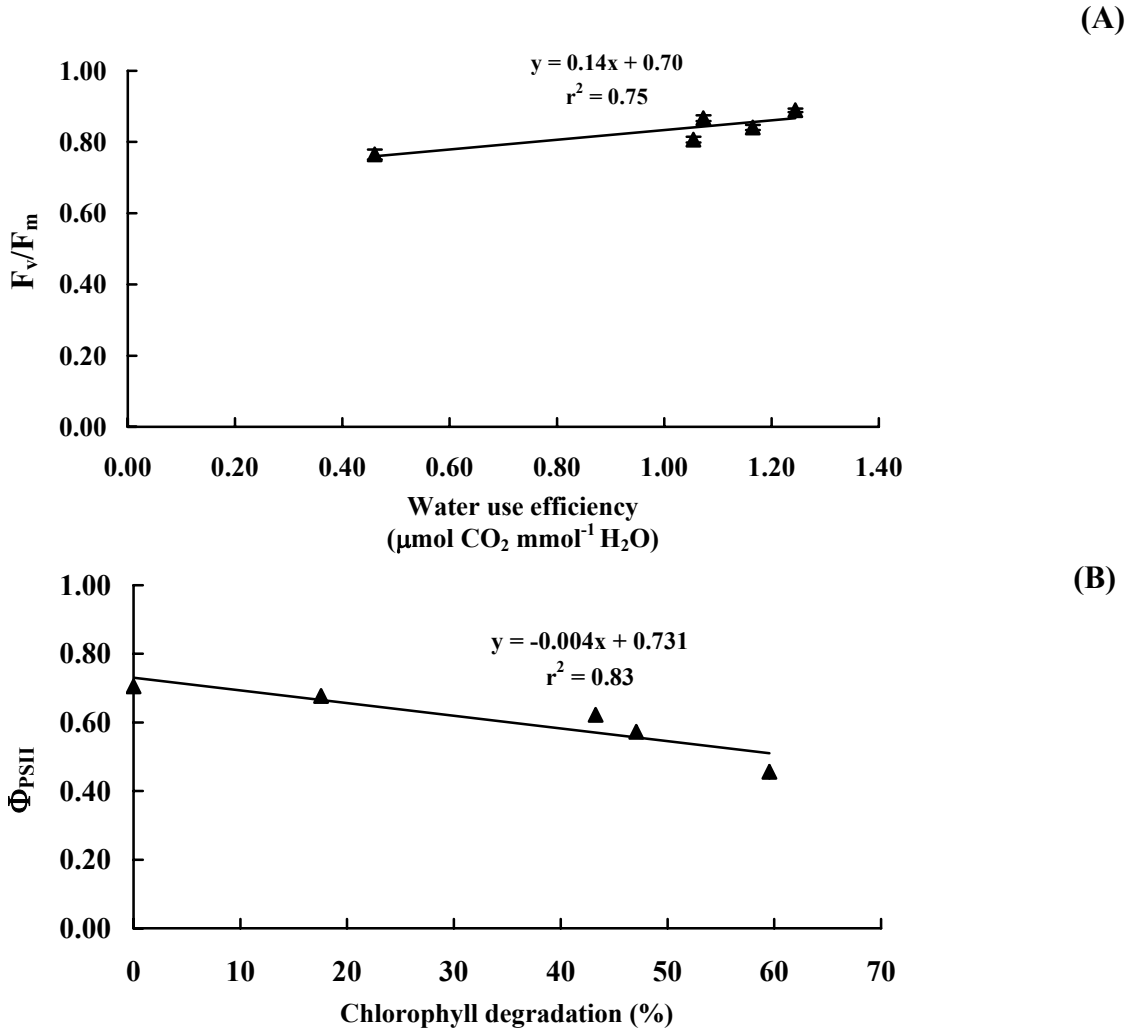


Fig. 2. Relationship between water use efficiency (WUE) and maximum quantum yield of PSII (F_v/F_m) (A), chlorophyll degradation (%) and photon yield of PSII (Φ_{PSII}) (B) of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Error bars represent ± SE.

with 300 mM mannitol. The WUE reduction and pigment degradation in drought acclimatized plantlets were strongly related to maximum quantum yield of PSII (F_v/F_m) (r^2 = 0.75) and photon yield of PSII (Φ_{PSII}) (r^2 = 0.83), respectively (Fig. 2). The F_v/F_m and Φ_{PSII} in osmotic stressed plantlets significantly decreased depending on mannitol concentrations in the culture medium, while non-photochemical quenching (NPQ) was increased (Table 2). The results showed that the Φ_{PSII} was positively correlated with net-photosynthetic rate (NPR) (r^2 = 0.85), as well as the proline content was positively related to NPQ (r^2 = 0.81) (Fig. 3). The NPR, stomatal conductance (G_s) and transpiration rate (E) in osmotic stressed plantlets at 300 mM mannitol drastically declined when compared to those of control plantlets (Table 2). In addition, there were strong relationships between

biochemical and physiological parameters in the osmotic stressed plantlets (Table 3). The Chl_a, Chl_b, C_{x+c}, F_v/F_m, NPR, G_s and E parameters showed the positive correlation, whereas NPQ and proline demonstrated negative relationships (Table 3). The NPR reduction in osmotic stressed plantlets was positively related to growth inhibition ($r^2 = 0.95$) (Fig. 4). Growth performances, fresh and dry weights and leaf area, in drought acclimatized plantlets were expressed in the similar pattern as the pigment degradation and photosynthetic reduction (Table 4). The leaf area was a sensitive parameter in drought stressed sugarcane, which was significantly reduced when exposed to water deficit. In addition, fresh and dry weights in mild-drought acclimatized plantlets (100-200 mM mannitol) were maintained better than those in extreme drought conditions (300-400 mM mannitol).

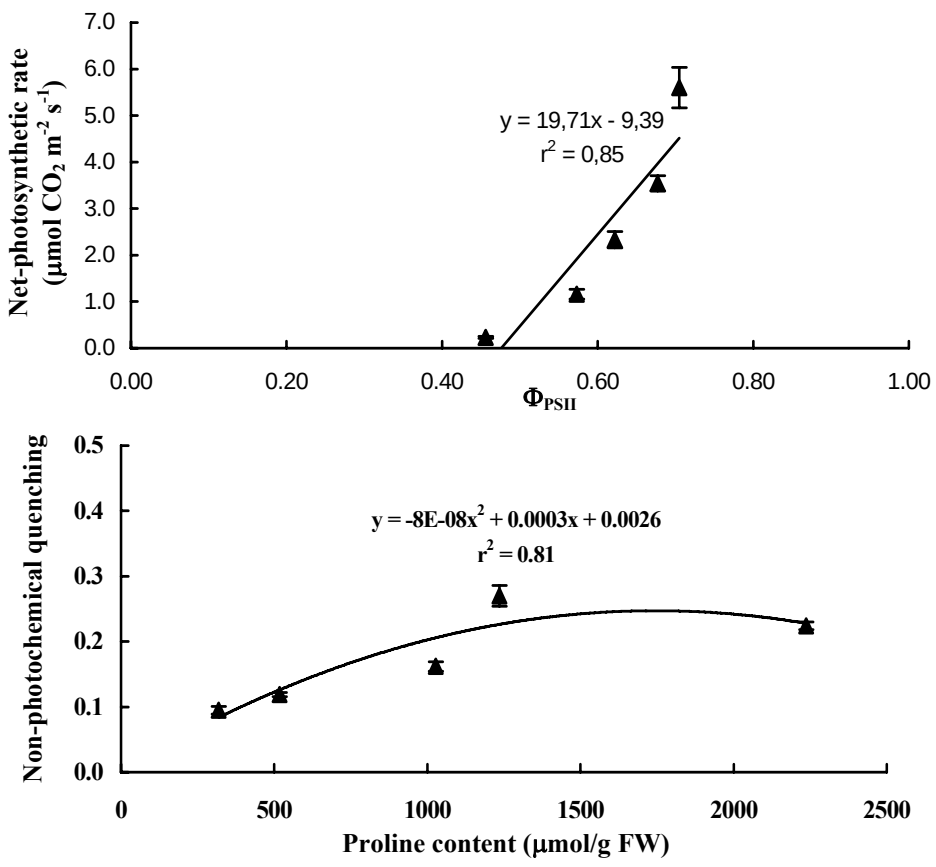


Fig. 3. Relationship between photon yield of PSII (Φ_{PSII}) and net-photosynthetic rate (NPR) (A), proline and non-photochemical quenching (NPQ) (B) of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Error bars represent ±SE.

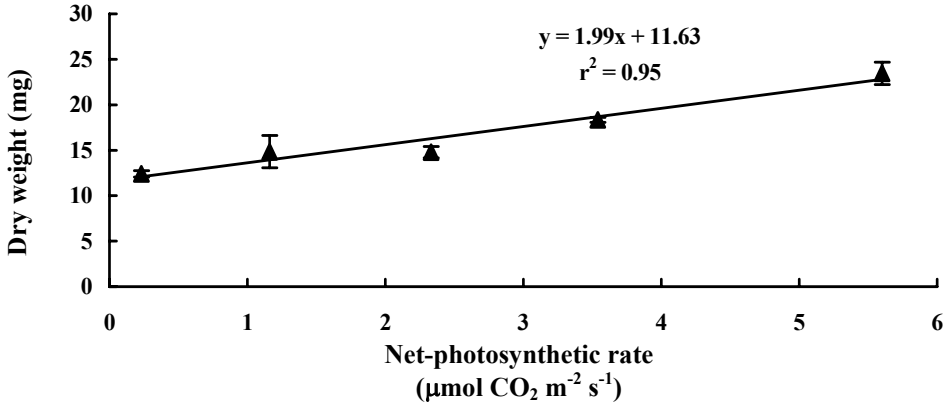


Fig. 4. Relationship between net-photosynthetic rate (NPR) and dry weight of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Error bars represent ± SE.

Table 1. Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC), total carotenoids (C_{x+c}) and proline contents of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Different letters in each column show significant difference at $p \leq 0.01$ (**) by Duncan's New Multiple Range Test (DMRT).

Mannitol (mM)	Chl_a (μg g^{-1}FW)	Chl_b (μg g^{-1}FW)	TC (μg g^{-1}FW)	C_{x+c} (μg g^{-1}FW)	Proline (μmol g^{-1}FW)
0 (Control)	231.08a	170.36a	401.44a	79.28a	318.34c
100	213.42a	117.50ab	330.92b	58.73b	517.34c
200	163.73b	64.00bc	227.73c	37.92c	1027.16b
300	159.53b	52.96c	212.49c	33.13c	2236.75a
400	129.68b	32.73c	162.41c	21.94d	1235.02b
ANOVA	**	**	**	**	**

Table 2. Maximum quantum yield of PSII (F_v/F_m), photon yield of PSII (Φ_{PSII}), non-photochemical quenching (NPQ), net-photosynthetic rate (NPR), stomatal conductance (G_s) and transpiration rate (E) of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Different letters in each column show significant difference at $p \leq 0.01$ (**) by Duncan's New Multiple Range Test (DMRT).

Mannitol (mM)	F_v/F_m	Φ_{PSII}	NPQ	NPR (μmol CO_2 m^{-2} s^{-1})	G_s (mol m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})
0 (Control)	0.889a	0.705a	0.095d	5.60a	0.79a	4.5a
100	0.867b	0.677b	0.119d	3.54b	0.64b	3.3b
200	0.841c	0.622c	0.162c	2.33c	0.42c	2.0c
300	0.807d	0.573d	0.224b	1.16d	0.12d	1.1d
400	0.765e	0.456e	0.270a	0.23d	0.08d	0.5d
ANOVA	**	**	**	**	**	**

Table 3. Relationship between physiological and biochemical parameters of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Significant levels at $p \leq 0.01$ are represented by ** using Pearson's correlation coefficients.

Parameters	Chl_a	Chl_b	C_{x+c}	PRO	F_v/F_m	NPQ	NPR	G_s	E
Chl_a	-	-	-	-	-	-	-	-	-
Chl_b	0.931**	-	-	-	-	-	-	-	-
C_{x+c}	0.893**	0.896**	-	-	-	-	-	-	-
PRO	-0.664**	-0.680**	-0.719**	-	-	-	-	-	-
F_v/F_m	0.861**	0.802**	0.915**	-0.679**	-	-	-	-	-
NPQ	-0.849**	-0.773**	-0.903**	0.717**	-0.958**	-	-	-	-
NPR	0.895**	0.871**	0.957**	-0.741**	0.940**	-0.901**	-	-	-
G_s	0.878**	0.865**	0.935**	-0.841**	0.937**	-0.941**	0.930**	-	-
E	0.892**	0.881**	0.973**	-0.771**	0.943**	-0.916**	0.962**	0.975**	-

Table 4. Growth characters, fresh weight (FW), dry weight (DW) and leaf area (LA) of sugarcane plantlets acclimatized under drought condition (mannitol) for 7 days. Different letters in each column show significant difference at $p \leq 0.01$ (**) by Duncan's New Multiple Range Test (DMRT).

Mannitol (mM)	FW (mg)	DW (mg)	LA (mm^2)
0 (Control)	147.6a	23.5a	1058a
100	134.8a	18.3b	935b
200	95.2b	14.8bc	782c
300	67.6c	14.8bc	485d
400	56.0c	12.4c	346e
ANOVA	**	**	**

Discussion

The osmotic control in the root zone of plant cultivation using mannitol and polyethylene glycol (PEG) solution has been well established in crop species *i.e.,* sugarcane (Errabi *et al*., 2006; Errabi *et al*., 2007), rice (Ahmad *et al*., 2007; Liu *et al*., 2007; Lefèvre *et al*., 2001), cowpea (Costa *et al*., 2007), alfalfa (Safarnejad, 2008), lentil (Yupsanis *et al*., 2001), three grass species (van den Berg & Zeng, 2006), maize (Ashraf *et al*., 2007) and halophyte species *i.e. Sevium portulacastrum* (Slama *et al*., 2007), *Cantaurea ragusina* (Radić *et al*., 2005; Radić *et al*., 2006), *Suaeda salsa* and *Kalanchoe claigremontiana* (Kefu *et al*., 2003). In the present study, the osmotic pressures of the culture medium declined consistently with increase in mannitol concentration, leading to low WUE in sugarcane plantlets grown under water deficit conditions. Similar results were demonstrated where the relative water content in the callus tissues was positively decreased with 0 (-0.4 MPa), 100 (-0.62 MPa), 200 (-0.84 MPa) and 300 mM mannitol (-1.08 MPa) contained in MS medium (Errabi *et al*., 2006; Errabi *et al*., 2007). The total chlorophyll and total carotenoid pigments in the leaf tissues of extreme water deficit were

degraded by 60 and 72%, respectively. Reduction in WUE in water-deficit sugarcane directly affects on photosynthetic pigment degradation, leading to reduce water oxidation in photosystem II defined by F_v/F_m and Φ_{PSII}, especially under extreme drought conditions (Nable *et al*., 1999; Robertson *et al*., 1999; de Silva & de Costa, 2004; Inman-Bamber & Smith, 2005; Smit & Singels, 2006; Silva *et al*., 2007; Shao *et al*., 2008). The chlorophyll degradation and chlorophyll a fluorescence diminution in sugarcane varieties CP72-1210, CP92-675, H99-295 and TCP02-4624 cultivated under drought condition reduced by 19.4 and 7.6%, respectively (Silva *et al*., 2007). In addition, the primary response of drought stressed sugarcane plantlets was osmotic adjustment through proline accumulation, which is well established in many plant species (Raymond & Smirnoff, 2002; Errabi *et al*., 2006; Ahmad *et al*., 2007; Errabi *et al*., 2007). In this study, proline content reached to maximum in the drought acclimatized plantlets under 300 mM mannitol (-0.94 MPa) and then dropped. In sugarcane varieties, R570 and CP59-73, the water content in calli treated with mannitol induced osmotic stress was significantly dropped with increase in mannitol concentration, whereas proline content was accumulated (Errabi *et al*., 2006). The proline accumulation in drought-stressed plants may play a role as osmolyte to maintain the organelles, resulting in the greenish leaf when exposed to water deficit condition (Yamada *et al*., 2005; Sankar *et al*., 2007; Safarnejad, 2008). Moreover, the sugarcane plantlets grown under extreme drought environments showed pigment damages, low F_v/F_m and NPR reduction, causing growth inhibition of sugarcane plantlets. There are many reports which show physiological and morphological changes such as leaf water potential, stomatal conductance, leaf area and productivity in sugarcane in response to drought stress, which are used as potential and rapid tool for screening for drought tolerance (Nable *et al*., 1999; Robertson *et al*., 1999; de Silva & de Costa, 2004; Inman-Bamber & Smith, 2005; Smit & Singels, 2006; Silva *et al*., 2007), especially under *In vitro* environments.

In conclusion, sugarcane variety K84-200 was very sensitive to water deficit (Ψ_s <- 0.67 MPa), as it had a maximum pigment degradation, low photosynthetic abilities and maximum growth reduction. The relationship between biochemical and physiological characters and growth of osmotic stressed plantlets was found to be positive in this investigation. They could be applied as effective indices for screening elite sugarcane varieties for drought tolerance.

Acknowledgements

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