



***In vitro* selection for drought tolerance in two elite sugarcane (*Saccharum* spp.) genotypes**

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ABSTRACT

Abiotic stresses, especially increased salinization of water resources and global scarcity of water, are the major causes of crop losses world-wide. Therefore, an *in vitro* study was conducted to observe the effect of osmotic stress induced by an osmoticum, polyethylene glycol (PEG), Mol. Weight 6000 on callogenesis and subsequent regeneration in sugarcane (*Saccharum* spp.) cv. CoJ 83 and CoH 119. Callus induction was observed by placing leaf roll segments (~ 1.5 cm long) of sugarcane cv. CoJ 83 and CoH 119 on solid Murashige and Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid (2,4-D; 4 mg/l and 5.5 mg/l for CoJ 83 and CoH 119, respectively) + Kinetin (Kin; 0.5 mg/l) + 560 mg/l proline + 30 g/l sucrose + 8 g/l agar. Subsequent regeneration from callus cultures was observed on solid MS + Kin (0.5 mg/l) medium. Significant callus induction and regeneration was observed in both the varieties, but with the addition of PEG (6000) into the callus induction medium, regeneration efficiency was reduced in both the cultivars. Further, the biochemical parameters like estimation of total soluble sugars and total proline content were also analysed. The results indicated that CoH 119 variety of sugarcane is more tolerant to drought stress as compared to CoJ 83 variety.

Key words: Abiotic, Callogenesis, Osmotic stress, Polyethylene glycol, Proline, Total soluble sugars

Sugarcane (*Saccharum* spp., 2n = 80 to 205), a C4 grass, belongs to tribe Andropogoneae, family Poaceae, order Poales and class Monocotyledoneae. Sugar industry is the second largest agro-based industry in India next to cotton, and India is the second largest producer of sugarcane next to Brazil with a production of about 342 million tonnes (FAOSTAT 2011). Abiotic stresses, such as drought and high salinity are the environmental factors limiting plant growth and productivity of crops. Plants have adapted to respond to these stresses at the molecular, cellular, physiological and biochemical levels, enabling them to survive under unfavourable environmental conditions. Various adverse environmental stresses induce the expression of a variety of genes in many plant species (Xiong *et al.* 2002, Shinozaki *et al.* 2003 and Bartels and Sunkar 2005). However, sugarcane, being a typical glycophyte, exhibits stunted growth or no growth under salinity, with its yield falling to 50% or more of its true potential (Subbarao and Shaw 1985). Besides this, salinity in the root zone decreases sugar yield through its effect on

both biomass and juice quality of sugarcane (Lingle and Weigand 1996).

In south-western districts of Punjab, salinity is the major abiotic stress which inhibits plant growth and reduces plant productivity due to water deficit, ion toxicity and nutritional imbalance resulting in considerable yield loss (Kaushal *et al.* 2013). It requires 10-15 years to complete the selection cycle and to get an improved sugarcane variety for commercial cultivation in sugarcane. This long duration causes a major bottleneck in breeding programmes (Siddiqui *et al.* 1994). Further, drought and salinity are quantitatively inherited traits making genetic improvement through breeding in sugarcane a rather difficult step. On the other hand, *in vitro* selection at cellular level can be carried out for some traits by growing cells from cell suspensions and calli on a medium supplemented with elevated levels of various biotic and abiotic stress factors (only the variant cells survive). Using this technique, many million cells (potential plants) can be screened in a single petri dish, which is practically difficult, if not impossible, to be adopted at the whole-plant level in the field (Gosal and Kang 2010). In response to various abiotic stresses like dehydration and heat stress, plants resort to various adaptations like accumulation of compatible solutes like sugar and sugar alcohols; and zwitterionic compounds (i.e. carrying a positive and negative charge). Former class includes sugar alcohols like mannitol, pinitol, sorbitol etc, and oligosaccharides

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such as trehalose and fructans. The latter class includes amino acids such as proline and quaternary ammonium compounds, such as glycine betaine.

Polyethylene glycol (PEG) is a non-permeable, non-toxic and non-metabolized compound which has been used for many cell and tissue culture studies to stimulate the osmotic stress (Ben-Hayyim 1987, Adkins *et al.* 1995, El-Tayeb and Hassanein 2000, Abdel-Rahman and Widholm 2010 and Wani *et al.* 2010). Polyethylene glycol (PEG-6000) is produced by the interaction of ethylene oxide with water, ethylene glycol, or ethylene glycol oligomers. Thus, the present investigation was carried out to study the inherent tolerance of calli and calli regenerated plants to osmotic stress induced by including PEG in the medium, which could serve as a base-line prior to screening of the drought-tolerant transgenic sugarcane plants.

MATERIALS AND METHODS

The research work was carried out in Tissue Culture and Genetic Transformation laboratories at School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana during 2011-13.

Spindles of 6 cm length and about 1.5 cm diameter were obtained by removing outer mature leaves from apical stem portions of sugarcane cv. CoJ 83 and CoH 119 [Fig 1(A)]. These were first washed in commercial detergent 'teepol' (2%) followed by washing in running tap water for 10-15 minutes to remove the detergent. Thereafter, the spindles were surface sterilized with 1% bavistin (BASF, India) followed by sterilization under laminar air flow cabinet with 0.1% mercuric chloride for 8-10 minutes with mild agitation and then rinsed thrice with sterile distilled water. Preparation of explants was done by removing outer 2 or 3 leaf whorls and the nodal portions from spindles. Innermost leaf whorls were cut transversely into 1-1.5 cm long segments.

Inoculations were performed under aseptic conditions. Rim and sides of the test tubes were flamed before each inoculation. For callus induction, leaf roll segments (1-1.5 cm long) of sugarcane cv. CoJ 83 and CoH 119 were placed on solid Murashige and Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid (2,4-D; 4 mg/l for CoJ 83 and 5.5 mg/l for CoH 119 + Kinetin (Kin; 0.5 mg/l) + 560 mg/l proline + 30 g/l sucrose + 8 g/l agar. After inoculations, the cultures were incubated at $25 \pm 2^\circ\text{C}$ in an air conditioned incubation room in total dark conditions. One explant was inoculated in each test tube.

After callus induction from the leaf roll segments, the one-week old embryogenic calli obtained from leaf roll segment were transferred on callus proliferation medium (MS + 2,4-D; 2.5 mg/l + Kin; 0.5 mg/l + 560 mg/l proline + 30 g/l sucrose + 8 g/l agar) supplemented with different concentrations (0.5%, 1.0%, 1.5% and 2.0%) of polyethylene glycol (PEG-6000). After three weeks, the analysis of embryogenic calli was carried out.

The 7-day old calli obtained from leaf roll segment were transferred on the callus proliferation media

supplemented with different concentrations of PEG-6000 (0.5%, 1.0%, 1.5%, 2.0%) for three weeks and then shifted on regeneration medium [MS + Kin (0.5 mg/l)]. Calli were incubated at $25 \pm 2^\circ\text{C}$ temperature with 16 hr light and 8 hr dark photoperiod. Percentage of regeneration was determined by the following formula; Number of regenerated calli/Total number of calli $\times 100$.

Proline estimation was carried out using the method devised by Chinard (1952). 0.25 g of calli was homogenized in 5 ml of 3% aqueous sulphosalicylic acid with pestle in a mortar. Mixture was centrifuged at 4000 rpm for 20 min at 24°C . 2 ml of supernatant was mixed with 2 ml of ninhydrin reagent stored at 4°C (1.25 g of ninhydrin was mixed with 30 ml of glacial acetic acid and 2 ml of 6mM phosphoric acid (pH = 1.0) with agitation until it was dissolved). Above mixture was kept in a boiling water bath for 1 hr followed by putting it in an ice bath for termination of the reaction. Added 4 ml of toluene and absorbance was taken at 520 nm using toluene as a blank. Proline concentration ($\mu\text{g/g}$ fresh weight) was determined from a standard curve prepared with 11 standard concentrations (0-50 μg) of L-proline.

Estimation of total soluble sugars (TSS) from calli was carried out using the method devised by Dubois *et al.* (1956). 0.5 g of calli was homogenized with 10 ml of 80% ethanol (v/v) in a borosil flask (30 ml) with a rod. Extraction of TSS was carried out once with 80% ethanol (v/v) followed by complete extraction with 70% ethanol (v/v) twice on a hot water bath. The alcoholic extract was combined every time by decanting in a 100 ml beaker. 0.5 ml of the above extract was taken in a test tube and mixed with 0.5 ml of distilled water alongwith blank. After addition of 1 ml of phenol, addition of 5 ml of sulphuric acid was made directly on the solution so that the contents are mixed and heated due to exothermic reaction. Tubes were swirled on a vortexer and kept for 20 minutes at room temperature. Absorbance was made at 490 nm and TSS were estimated using glucose as standard. The standard curve was prepared using 11 standard concentrations (0-100 μg) of glucose.

RESULTS AND DISCUSSION

Callus cultures were established in cv. CoJ 83 and CoH 119 by placing leaf roll segments on callus induction medium. Both the varieties, viz. CoJ 83 and CoH 119 exhibited excellent callus induction and regeneration. In variety CoJ 83, callus induction was observed on MS + 2,4-D (4 mg/l) + Kin (0.5 mg/l) + 560 mg/l proline + 30 g/l sucrose + 8 g/l agar, whereas in variety CoH 119, maximum callus induction was observed on MS + 2,4-D (5.5 mg/l) + Kin (0.5 mg/l) + 560 mg/l proline + 30 g/l sucrose + 8 g/l agar. After 2-4 days of culturing, swelling and unwhorling of leaf roll segments took place. After 12-14 days of culturing, callus initiation was observed and embryogenic callus was observed after ~36-40 days of culturing. Subculturing of primary calli was carried out after 42 days and the subcultured calli were maintained on callus proliferation medium, i.e. MS + 2,4-D (2.5 mg/l) + Kin (0.5

mg/l). After 14 days, the secondary calli obtained were divided into small fragments (~45-50 mg fresh weight) which were cultivated in separate test tubes on media containing different concentrations of PEG (0.5%, 1.0%, 1.5% and 2.0%) for 21 days. It was observed that necrosis and yellowing of callus occurred within three weeks of culturing on medium supplemented with 2.0% (w/v) PEG. Similar results were observed by Martinez *et al.* (2005) on medium supplemented with PEG for studying PEG induced water stress in *Atriplex halimus* L.

During the present investigation, PEG (6000) was used to study the inherent tolerance of calli in two cv. of sugarcane. The perusal of data suggested that the mean plant regeneration percentage decreased with increase in PEG concentration was seen: from 88.35% (control) to 8.75% (in calli shifted from medium containing 2.0% (w/v) PEG) in cv. CoJ 83 and from 82.63% (control) to 26.53% (in calli shifted from medium containing 2.0% (w/v) PEG) in cv. CoH 119. Also it was observed that although the mean per cent regeneration of CoH 119 is less than CoJ 83, however CoJ 83 variety was more sensitive to drought as compared to CoH 119. This indicated that CoH 119 variety is hardy as compared to CoJ83. In an earlier study conducted by Wani *et al.* (2010) in rice, mean plant regeneration capacity was decreased to 0% with medium supplemented with 2.0% (w/v) PEG in varieties PAU 201 and PR 116.

Proline concentration ($\mu\text{g/g}$ fresh weight) was determined from calli after 3 weeks of culture under aseptic conditions. In both the varieties, accumulation of free proline content noticed upon PEG treatment. In CoJ 83, proline content increased upto $192.95 \mu\text{g/g}$ fr. wt., while in CoH119, proline content reached to $110.2 \mu\text{g/g}$. Results indicated that proline content in calli increased with stress induced by PEG-6000 and was very high at 2.0% PEG concentration, where the mean plant regeneration was decreased to a drastic level in both the varieties of sugarcane. This might be due to the fact that in stressed plants, proline accumulation has a protective function as it may reduce stress induced cellular acidification or prime oxidative respiration to provide energy needed for recovery (Hare and Cress 1997). In sugarcane plants subjected to drought and heat stress, accumulation of large quantities of free proline could be used as an index for screening the genotypes for abiotic stress tolerance (Wahid and Close 2007, Gomathi *et al.* 2011 and Gomathi *et al.* 2013). Proline concentration was also increased in potato cultivars upon induction of salt stress in a study conducted by Jaarsma *et al.* 2013.

The aim of the present study was to further examine the effect of stress induced by PEG-6000 on total soluble sugars content (TSS). Data on TSS content indicated that in CoJ 83, TSS increased to $74.3 \mu\text{g/g}$ and upto $46.41 \mu\text{g/g}$ in CoH 119 from $2.26 \mu\text{g/g}$ and $1.13 \mu\text{g/g}$, respectively at control. Significant increases in TSS content upon drought stress treatment has been earlier reported by Mohammadkhani and Heidari (2008) indicating their role in osmotic adjustment during dehydration and upon heat stress treatment in sugarcane by several workers (Wahid

and Close 2007, Yuan *et al.* 2010 and Gomathi *et al.* 2013).

Salinity and water stress reduce the ability of plant to take up water and decrease growth rate, photosynthesis rate and stomatal conductance of plants (Shabani *et al.* 2013). Therefore, the results in the present study indicated that PEG, if included in the culture medium, can be used to carry out the screening of drought tolerant lines in sugarcane. Further, differential genotypic response was noted in callus ability to proliferate and regenerate plantlets under various concentrations of PEG-6000 for cv. CoJ 83 and CoH 119.

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