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RESEARCH ARTICLE

In vitro selection and characterization of polyethylene glycol (PEG) tolerant callus lines and regeneration of plantlets from the selected callus lines in sugarcane (*Saccharum officinarum* L.)

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Abstract A system for in vitro selection of drought tolerant callus lines in sugarcane was developed. High molecular weight PEG was used as selective agent. Selected callus line grew better than non-selected callus when grown on different concentrations of PEG. The activity of antioxidant enzymes like CAT, POX, APX and SOD were high in selected callus than in non-selected callus. Osmolytes like proline and ascorbic acid were at higher levels in selected callus than in non-selected callus, however at higher concentrations (20–30 %) of PEG, levels of proline and ascorbic acid decreased. The frequency of organogenesis and number of plantlets decreased in selected callus than in non-selected callus. The results can be used for in vitro screening and manipulations of sugarcane for improvement of drought tolerance

Keywords Antioxidant enzymes · Callus · PEG · Selection · Proline · Sugarcane

Abbreviations

2,4-D	2,4-dichloro phenoxy acitic acid
BAP	6-Benzyl aminopurin
NAA	α-Napthalene acitic acid
Kn	Kinetin
CAT	Catalase
POX	Peroxidase
APX	Ascorbate peroxidase
SOD	Superoxide dismutase
MS	Murashige and Skoog
ROS	Reactive Oxygen Species

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Introduction

Drought continues to be a major setback for the production of crop plants. Drought is an environmental stress which causes important agricultural losses in arid and semiarid areas. Sugarcane requires 10–12 months from planting to harvesting and faces severe drought and other factors. Drought is one of the principal environmental stress which constraint sugarcane production (Begum et al. 2011). Increasing crop production in drought environment may be achieved through breeding crops that are more tolerant to drought. However conventional breeding efforts are time, cost and labor intensive. Biotechnology like tissue culture technology offer rapid alternative in crop improvement. In recent years, tissue culture based in vitro selection has emerged as a feasible and cost-effective tool for developing stress-tolerant plants. Plants tolerant to both the biotic and the abiotic stresses can be acquired by applying the selecting agents such as NaCl, for salt tolerance, PEG or mannitol, for drought tolerance (Errabii et al. 2008). In vitro selection for cells exhibiting increased tolerance to drought stress has been reported (Sabbah and Tal 1990; Santos-Diaz and Ochoa-Alejo 1994; El-Haris and Barakat 1998; Hassan et al. 2004; Errabii et al. 2006).In most of the cases PEG is used to induce water stress. PEG of high molecular weight has been long used as a non-penetrating non-ionic inert osmoticum lowering the water potential of nutrient solutions without being taken up or being phytotoxic (Hassan et al. 2004). Effect of NaCl and PEG on growth, Osmolytes accumulation and antioxidant defense in cultured cells of sugarcane is reported by Patade et al. (2011, 2012), however in their investigations no attempt is made to select PEG tolerant callus lines, characterization of

PEG tolerant callus lines and regeneration of plantlets from PEG tolerant callus lines. Musa (2011) used PEG as selection agent to screen some sugarcane varieties for drought tolerance; however the author has not attempted selection of callus lines for PEG (Drought) tolerance. Begum et al. (2011) has screened tissue culture raised sugarcane somaclones for drought tolerance using PEG as selection agent. The aim of the present investigation is to select callus lines tolerant to PEG and characterize calli lines with respect to growth Osmolytes accumulation and activity of antioxidant enzymes and compare with non selected callus lines. Hence in this investigation we report isolation and characterization of drought tolerant (PEG tolerant) callus lines and regeneration of plants from the selected callus lines in variety Co-86032. This is a high cane and sugar yielding variety (Jadhav et al. 1998).

Material and methods

Callus induction and in vitro selection

For the present investigation sugarcane variety Co-86032 was used. Callus from leaf sheath explants was initiated on Murashige and Skoog (1962) medium supplemented with 30 g/l sucrose 8 g/l agar and 2, 4-D 1 mg/l + Kn 0.5 mg/l. The medium was adjusted to pH 5.8 autoclaved at 120 °C and 15 lb pressure for 20 min. Cultures were maintained at 26 ± 1 °C under 30 μm^{-2} S^{-1} light provided by cool fluorescent light under 16 h light and 8 h dark period. After 4 weeks of growth calli were separated from the explants and 250±10 g of calli pieces were subcultured on the same medium for further proliferation. After 4 weeks of growth 250±10 g of calli pieces were transferred to MS medium, described above and additionally containing 20 % PEG (Molecular weight 6,000) to initiate selection shock. This concentration of PEG was chosen based on our previous report ((Jabeen 2007) which revealed that 20 % PEG decreased the growth of callus considerably and at 30 % death of all the calli occurred. Thirty calli were used for both control and selection medium. After sub cultures for 4 weeks, most of the calli become dark brown except for small groups of cells that remained light in colour. These sectors were sub cultured on the same medium for two passages of 4 weeks each. To test the tolerance of these sectors the calli were transferred to medium without PEG for one passages of 4 weeks each and again transferred to PEG supplemented medium for two cycles of 4 weeks each. Calli actively growing at this stage were considered as PEG tolerant and were used for characterization and regeneration of plantlets. Growth measurements in terms of fresh weight were taken 30 days after growth. Similarly for biochemical estimations and enzyme activity 30 days old callus was used. All the experiments were repeated thrice and the average value recorded and presented. Control calli were continuously sub cultured on MS medium without PEG. For regeneration of plants from both selected and non selected callus MS medium supplemented with 2 mg/l Kn + 1 mg/l BAP was used. For rooting of the plantlets derived from non-selected and selected callus, they were transferred on MS medium supplemented with 5 mg/l NAA+ 20 % PEG.

Estimation of proline

Free proline was estimated according to method described by Bates et al. (1973). Callus (500 mg) was homogenized in 3 % (w/v) aqueous sulfosalicylic acid and centrifuged at 1,000 g for 10 min. The filtered homogenate was reacted with equal volume each of acid ninhydrine and acetic acid at 100 °C for 1 h and the reaction was terminated on an ice bath. The reaction mixture was extracted with 4 ml toluene and mixed vigorously. The chromophore containing toluene was separated from the aqueous phase and warmed to room temperature. The absorbance of proline-ninhydrine product was recorded at 520 nm using toluene as blank. Proline content was expressed as μg^{-1} FW

Estimation of protein

Samples of frozen callus tissue were ground in Tris–HCl (60 mM, pH 6.8) at 4 °C, using pre-chilled mortar and pestle. The extract was centrifuged (4,000 g for 15 min at 4 °C) and the supernatant was used for protein estimation according to Bradford (1976)

Estimation of ascorbate

Ascorbic acid (As) was extracted in 5 % (m/v) methophosphoric acid with sand at 4 °C. The homogenate was then centrifuged at 3,000 g for 20 min at 4 °C ascorbate content was quantified in the supernatant as described by Sadasivam and Manickam (1992). An aliquot of 1 ml of the supernatant was mixed with 1 ml of 2 % Dinitrophenyl Hydrazine (DNPH) reagent. The reaction mixture was allowed to stand for 40 min at room temperature, then the absorbance was recorded at 540 nm, using ascorbic acid as standard.

Estimation of lipid peroxidation

Lipid peroxidation was determined in terms of Malonylaldehyde (MDA) content using the Thiobarbutric acid reaction (TBARS) according to Heath and Packer (1968). Callus tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle. Subsequently about l00mg of powder was homogenized in 5 ml of TCA (0.1 % w/v) and

centrifuged at 10,000 g for l0 min at 4 °C 1 ml of supernatant was mixed with 4 ml of 0.5 % TBA reagent in 20 % TCA. The mixture was heated at 95 °C for 30 min cooled over ice and centrifuged at 10,000 g for 10 min the absorbance of the supernatant was recorded at 532 nm and corrected for non specific absorbance at 600 nm. MDA content was calculated using an extinction coefficient (ε) of 155 mM ^{1} cm ^{1}and and expressed as μmol g^{-1} FW.

Estimation of catalase activity

Catalase activity was measured according to method of Chandlee and Scandalios (1984). One gram of frozen callus was homogenized in a pre chilled pestle & mortar with 5 ml of ice cold 50 mM phosphate buffer. The extract was centrifuged at 4 °C for 20 Min. at 12,500 X g. The supernatant was used for enzyme assay. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7.0) 0.4 ml 15 mM H_2O_2 and 0.1 ml of enzyme extract. The decomposition of H_2O_2 was followed by decline in absorbance at 240 nm (ε =36 mM^{-1} cm^{-1}). Catalase activity was expressed in unit mg^{-1} protein. One unit was defined as the amount of enzyme catalyzing the decomposition of 1 n mol H_2O_2 per m $^{-1}$ per mg^{-1} protien.

Estimation of peroxidase activity

Peroxidase activity was assayed by the method of Kumar and Khan (1982). Assay mixture contained 2 ml of 0.1 M Phosphate buffer (pH 6.8) 1 ml of 0.01 M Pyrogallol, 1 ml of 0.005 M H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5N H_2SO_4. The Purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5N H_2SO_4 at zero times. The activity was expressed in unit mg^{-1} protein. One unit (U) is defined as that amount of enzyme, which forms 1 mol of purpurogallin per minute per mg protein under the assay conditions.

Estimation of ascorbate peroxidase activity

The activity of APX was determined by measuring the decrease in absorbance at 290 nm and the amount of Ascorbate oxidized to dehydroascorbate was calculated from the extinction coefficient 2.8 (Nakano and Asada 1981). The reaction mixture (2 cm3) consisted of 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbic acid, 1.0 mM H_2O_2 and 0.2 ml enzyme extract. The activity of APX is expressed as units per m $^{-1}$ per mg^{-1} protien. One unit is defined as micromoles of ascorbate oxidized per m $^{-1}$ per mg^{-1} protien.

Estimation of Superoxide dismutase activity

Superoxide dismutase activity was estimated in terms of inhibition of the photochemical reduction of Nitroblue tetrazolium (NBT) as per the procedure of Beyer and Fridovich (1987). The reaction mixture (3 ml) consisted of 50 mM phosphate buffer (pH 7.8) and 0.1 mM EDTA, 14.3 mM methionine, 82.5 mM NBT and 2.2 mM riboflavin. The reaction was initiated by adding 100 μl of enzyme extract. The tubes were kept 30 cm below a light source (2×40 W fluorescent tubes) for 30 min. The reaction was stopped by switching off the light. The reduction of NBT was measured by monitoring the change in absorbance at 560 nm. The activity was expressed in terms of units mg^{-1} protein. One unit of enzyme was defined as the amount of enzyme that brings about 50 % inhibition of NBT under the assay conditions.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The values are mean ± SE for three samples in each group, P values ≤ 0.5 were considered as significant

Results and discussion

Selection of PEG tolerant callus and growth of the callus

Callus obtained from leaf sheath explants was transferred to MS medium supplemented with different concentrations (0–30 %) of PEG. It was noticed that increasing concentration of PEG resulted in progressive reduction and browning of the callus (Fig. 1a–f). However some pockets of callus were seen growing on 20 % PEG supplemented medium (Fig. 1d, g). These surviving pockets of calli were excised and subcultured on selection medium for two passages of 4 weeks each and were again transferred to medium without PEG for 4 weeks. These calli when transferred to 20 % PEG supplemented medium for another 4 weeks, were capable of growing on the selective medium. These callus lines were considered as PEG tolerant. The growth of the selected calli on medium without PEG showed lower growth than non-selected callus grown on PEG free medium as reported by Gangopadhyay et al. (1997) in tobacco; however selected callus grew better than the non-selected calli on various levels (5–30 %) of PEG (Table 1), however it was noticed that, the growth of selected calli too decreased at higher (25&30 %) concentration of PEG, but the selected calli showed significantly better growth ($p \leq 0.05$) than the non selected calli on medium supplemented higher concentrations

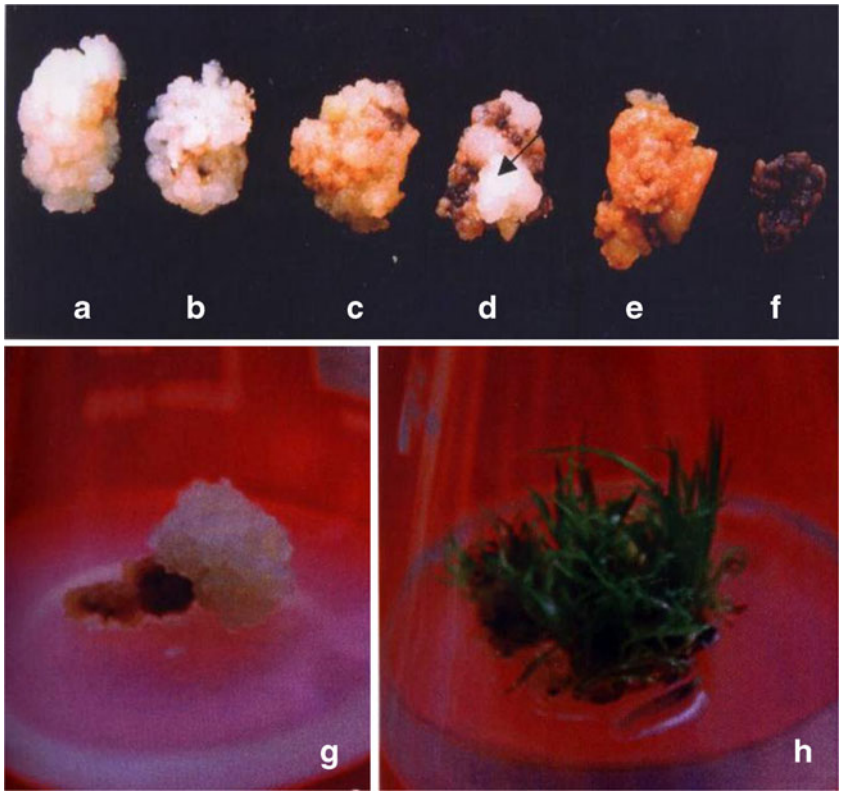

Fig. 1 **a.** Callus on medium without PEG. **b,c,d,e** & **f.** callus growing on 10,15,20,25 and 30 % PEG supplemented medium. Note progressive reduction in the growth of callus and browning of callus. **g.** Surviving callus on 20 % PEG supplemented medium. **h.** Regenerated plantlets on MS+ 2 mg/l Kn +1 mg/l BAP

(25&30 %) of PEG. Growth of the PEG tolerant calli is shown to be better than non selected calli in tobacco (Gangopadhyay et al. 1997) sunflower (Hassan et al. 2004). Addition of PEG to the medium produces osmotic stress (Kumar et al. 2011) and decreases the water potential (Aazami et al. 2010) which negatively effects growth. Decreased growth in the presence of PEG in the medium is reported in sugarcane (Errabii et al. 2008) sunflower (Hassan et al. 2004) *Sorghum* (Bhaskaran et al. 1985) tomato (Abdel-Raheem et al. 2007; Aazami et al. 2010).

Proline and protein accumulation

Proline and ascorbate levels in the selected calli were higher than the non selected calli when grown on different levels of PEG supplemented media (Table 2). Proline and protein content decreased with an increase in the concentration of PEG, however selected calli maintained higher levels of proline and protein than non-selected calli at any given concentration of PEG. This explains that proline accumulation accompanies survival and growth of callus under drought conditions. Shah et al. (2012) reported 17 fold increase in the proline content of 20 % PEG selected calli than the non selected calli of rice. A similar trend in proline levels is reported in tobacco (Gangaopadhyay 1997) sunflower (Hassan et al. 2004). Increase in the proline accumulation on exposing calli to PEG is reported in *Sorghum* (Bhaskaran et al. 1985) rice (Aqeel-Ahmad et al. 2007) Proline accumulation under water deficit has been mainly recognized as an osmotic agent (Handa et al. 1986).

Table 1 Growth response of non-selected and selected sugarcane callus on different concentrations of PEG

PEG (%)	Non selected callus		Selected callus	
	Fresh weight(mg)	Dry weight(mg)	Fresh weight(mg)	Dry weight(mg)
0	976±2.90^{f}	99±3.0^{f}	936±6.0^{d}	104±2.3^{a}
5	742±3.30^{e}	80±5.0^{e}	992±6.0^{e}	133±2.0^{a}
10	582±8.80^{d}	65±1.0^{d}	997±1.6^{e}	140±1.6^{b}
15	433±5.70^{c}	49±2.0^{c}	1015±2.0^{f}	148±3.0^{c}
20	243±8.8^{b}	30±2.0^{b}	964±3.0^{c}	154±3.0^{d}
25	142±5.7^{a}	18±4.0^{a}	894±3.0^{b}	181±1.2^{e}
30	138±4.6^{a}	16±4.0^{a}	652±4.0^{a}	164±2.3^{f}

Data represents average of three replicates; each replicate consists of 10 cultures Mean ± Standard error. Mean followed by the same superscript in a column is not significantly different at $P \leq 0.05$ levels

Table 2 Effect of PEG on Proline and Protein content of non-selected and selected callus of Sugarcane

Non selected Callus			Selected Callus	
PEG(%)	Proline(mg/g FW)	Protein (mg/g FW)	Proline(mg/g FW)	Protein(mg/g FW)
0	2.26±0.17^{b}	1.44±0.03^{d}	2.67±0.20^{b}	2.20±0.28
5	5.90±0.6^{c}	1.35±0.17^{d}	7.26±0.30^{d}	3.86±0.32
10	7.96±0.17^{d}	1.10±0.0^{d}	14.82±0.40^{g}	4.28±0.48
15	10.56±0.16^{e}	0.23±0.18^{a}	24.58±0.20^{h}	4.36±0.38
20	12.78±0.16^{f}	0.64±0.0^{c}	26.20±0.18^{i}	3.28±0.26
25	1.68±0.0^{a}	0.58±0.1^{b}	12.46±0.36^{f}	2.86±0.18
30	ND	ND	2.20±0.28	1.48±0.18

Data represents average of three replicates; each replicate consists of 10 cultures. Mean ± standard error. Mean followed by the same superscript in a column is not significantly different at $P \leq 0.05$ levels. *ND* Not detected

Selected calli exhibited osmotic adjustment in response to PEG stress through the synthesis of solutes like proline. A similar observation was made by Patade et al. (2012) in sugarcane callus cultures in response to PEG treatment. Increase in protein content in the PEG selected callus lines is reported in chili (Nath et al. 2005) and tomato (Srivastava et al. 1995).

Ascorbic acid content

Selected callus line accumulated more ascorbic acid when compared to non-selected callus line (Table 3). However ascorbic acid content in both the callus lines decreased at higher concentrations of PEG. At any given concentration of PEG ascorbic acid content was more in selected callus than in non-selected callus line. Ascorbate is shown to regulate different processes associated with growth and development, further it has been shown that it maintains the osmotic status of stressed tissue Prabha and Bharti (1980)). Lower levels of ascorbate at higher levels of PEG in non selected calli may be due to the necrosis of the calli with response to PEG treatment as evident by inhibited callus growth.

Lipid peroxidation

Oxidative damage to lipids was determined as lipid peroxidation in terms of amount of malondialdehyde (MDA). MDA content of tolerant callus was significantly ($p \leq 0.05$) less when compared to that of sensitive callus line (Table 3). Lipid peroxidation in response to NaCl and PEG stress is reported in cultured sugarcane cells (Patade et al. 2012), however they have not selected callus lines tolerant to PEG and hence a comparison with reference to lipid peroxidation is not available with cell lines showing tolerance to PEG. The results indicate that PEG tolerant callus line maintained membrane integrity when growing in drought environment, however sensitive calls line were unable to maintain membrane integrity under PEG stress, resulting in decreased growth and metabolic imbalance. PEG induced lipid peroxidation is also reported in other species like bean (Yaser et al. 2010) blueberry callus (Ming et al. 2009).

Table 3 Effect of PEG on MDA and Ascorbate content in non-selected and selected callus of Sugarcane

Non selected Callus			Selected Callus	
Con	MDA (μm/gFW)	Ascorbate(mg/gFW)	MDA(μm/gFW)	Ascorbate(mg/gFW)
0	12.88±0.40^{d}	23.42±0.17^{c}	8.28±0.36^{a}	23.28±0.28^{c}
5	12.59±0.80^{d}	34.58±0.20^{d}	8.26±0.38^{a}	38.42±0.36^{e}
10	14.34±0.12^{e}	67.67±0.16^{h}	10.46±0.42^{c}	74.28±0.28^{j}
15	15.46±0.11^{f}	75.98±0.28^{i}	10.28±0.36 ^{c}	89.62±0.52^{k}
20	18.45±0.16^{g}	20.26±0.38^{b}	9.58±0.52^{b}	58.64±0.72^{g}
25	24.59±0.02^{h}	16.38±0.42^{a}	9.52±0.56^{b}	42.28±0.86^{f}
30	ND	ND	ND	ND

Data represents average of three replicates; each replicate consists of 10 cultures. Mean ± Standard error. Mean followed by the same superscript in a column is not significantly different at $P \leq 0.05$ levels. *ND* Not detected

Table 4 Effect of PEG on antioxidant enzymes in non selected and selected callus of sugarcane

PEG	Non selected callus				Selected callus			
	Catalase U mg^{-1} protien	APX U mg^{-1} protien	POX U mg^{-1} protien	SOD U mg^{-1} protien	Catalase U mg^{-1} protien	APX U mg^{-1} protien	POX U mg^{-1} protien	SOD U mg^{-1} protien
0	2.81±0.3^{b}	8.62±0.9^{b}	20.42±1.2^{b}	31.48±1.4^{b}	2.94±0.7^{b}	8.92±0.8^{b}	22.64±1.8^{b}	34.82±1.6^{c}
5	2.98±0.6^{b}	8.78±0.8^{b}	22.46±1.6^{c}	34.56±1.6^{c}	4.26±0.2n^{c}	16.40±0.8^{c}	38.32±1.4^{e}	36.22±1.2^{d}
10	4.24±0.6^{c}	10.26±0.8^{c}	26.48±1.2^{d}	36.90±0.8^{d}	10.80±0.8^{d}	24.86±0.6^{e}	36.24±1.4^{d}	46.68±1.8^{f}
15	6.20±0.4^{d}	12.98±0.6^{d}	30.82±1.4^{f}	38.26±0.8^{e}	12.82±0.8^{e}	32.24±0.5^{f}	40.28±1.8^{f}	40.24±1.4^{e}
20	1.82±0.2a	6.24±0.4^{a}	18.26±1.7^{a}	20.42±1.2^{a}	3.86±0.4^{c}	19.28±0.4^{d}	30.20±1.6^{c}	32.42±2.0^{b}
25	ND	ND	ND	ND	1.48±0.2^{a}	4.26±0.9^{a}	16.46±1.9^{a}	22.48±2.2^{a}

Data represents average values of three independent experiments. Mean ± Standard error. Mean followed by the same superscript in a column is not significantly different at $P \leq 0.05$ levels. *ND* Not detected

Activity of antioxidant enzymes

Water stress (PEG stress) led to significant ($p \leq 0.05$) increase in the activity of the antioxidant enzymes like CAT, POX, APX and SOD. (Table 4) However the activity of these enzymes decreased in both selected and non selected calli at higher levels of PEG, however the activity of these enzymes was more in selected calli at any concentration of PEG. PEG induced increase in the activity of CAT, APX and SOD is reported in sugarcane callus cultures (Patade et al. 2011, 2012). Increased activity of antioxidant enzymes in callus derived from drought tolerant maiz cultivars is reported (Li and van Stadan 1998). Increased activity of these enzymes under PEG stress is also reported in callus cultures of tobacco (Bueno et al. 1998). Antioxidant enzymes play a vital role in conferring tolerance to drought and other abiotic stresses. ROS such as superactive radicals, Hyderogen peroxide and the hydroxyl radicals liberated during stress can cause lipid peroxidation resulting in membrane damage, protein degradation (Davies 1987) and enzyme inactivation (Fridovich 1986). Plants have endogenous mechanism to protect cellular and sub-cellular system from toxic effects of these radicals in the form of antioxidant enzymes such as CAT, APX, POX and SOD (Larson 1988). The higher activity of antioxidant enzymes could be the reason of better growth of selected calli than the non selected calli on PEG supplemented medium.

Regeneration of plantlets

For regeneration of plantlets callus of both non-selected and selected calli was transformed to MS medium supplemented with 2 mg/l kinetin and 1 mg/l BAP, where multiple shoots were observed within 10–18 days (Fig. 1h). In the non-selected callus organogenesis was observed within 10–12 days after inoculation whereas in selected callus organogenesis was observed only after 15–18 days. The frequency

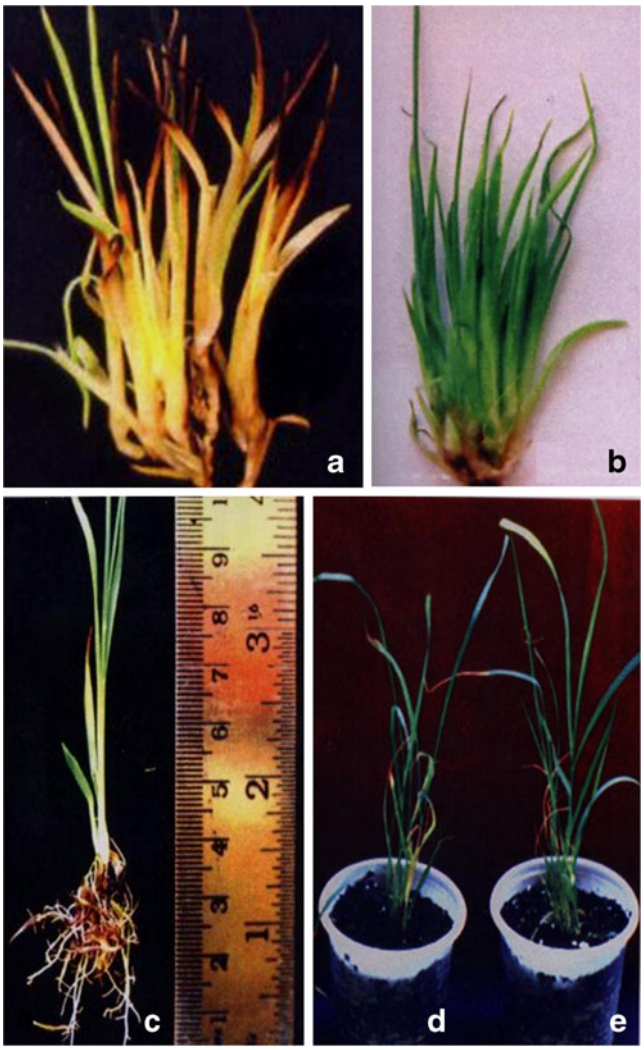

Fig. 2 **a.** Plantlets from non selected callus irrigated with 20 % PEG solution. Note browning of the plantlets. **b.** Plantlets from selected callus and irrigated with 20 % PEG solution. Note plantlets are green and healthy. **c.** Rooted plantlet on MS+ 20 % PEG + 5 mg/l NAA. **d & e.** Potted plantlets derived from non selected and selected callus respectively

of organogenesis was 100 % in non-selected calli it was reduced to 75 % in selected calli.). The number of shoots per calli was higher (98±0.15) in non selected callus when compared to selected calli (36±0.18).

For induction of roots elongated shoots about 4–6 cm in length, derived from non-selected and selected calli was transferred to rooting medium consisting of MS + 5 mg/l NAA+20 % PEG. It was noticed that plantets obtained from non-selected calli failed to root on PEG supplemented media whereas plantlets obtained from selected calli showed 86 % rooting within 10 days of transfer, each plantlet showing 10–12 roots (Data not presented). (Fig. 2c). Rooted plantlets were transferred to poly cups containing sand and soil (2:1) for hardening (Fig. 2d&e). A total of 26 healthily growing plants were recovered from PEG tolerant calli. To test the tolerance of these plants to PEG, 20 plants of both, plantlets obtained from non selected calli and selected calli were transferred to pot and after 20 days of growth in pot, were irrigated, with solution containing 20 % PEG, every alternate day for 20 days. It was noticed that plantlets regenerated from non selected calli turned brown and died within 10 days, whereas plantlets regenerated from PEG tolerant calli remained green and healthy (Fig. 2a & b). Plant regeneration from PEG tolerant callus lines is reported in rice (Siddeswar and Kavi Kishor 1989) tomato (Singh and Sharma 2008).

Conclusions

From the present investigation it can be concluded that PEG tolerant callus lines in sugarcane can be obtained by recurrent selection of callus on Peg supplemented medium and plantlets can be regenerated from the selected callus lines. It can also be concluded that proline, protein, ascorbic acid content and higher levels of the activity of antioxidant enzymes like CAT, POX, APX and SOD are responsible to maintain better growth of selected callus lines than the non-selected callus lines.

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