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The role of polyethylene glycol (PEG) pretreatment in improving sugarcane's salt (NaCl) tolerance

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Abstract: An important aspect of salt tolerance studies in different plants is the relationship between salt and water stress. In the present study calluses (60 days old) of 2 sugarcane cultivars (cv. SPF 234 and cv. HSF 240) were treated with 4 different salt concentrations, including a control (0 mM NaCl), after 1% polyethylene glycol (PEG) pretreatment for 5 days. Fresh weight, callus browning, and necrosis data were recorded at day 120. Biochemical parameters (soluble protein contents, and peroxidase, catalase, and superoxide dismutase activity) were also analysed. Regardless of PEG pretreatment, calluses subjected to salt stress had less fresh weight than the control; however, less necrosis was observed in PEG-pretreated callus cultures than in non-pretreated cultures subjected to the same salt concentration. PEG pretreatment enhanced the biosynthesis of soluble protein contents. Likewise, a general increase was observed in antioxidant enzyme activity in the PEG-pretreated callus cultures, as compared to the non-pretreated controls exposed to the same salt concentration. PEG pretreatment also increased the regeneration potential of the callus cultures of both sugarcane cultivars after NaCl treatment. These results suggest that PEG pretreatment could improve salt tolerance in these 2 sugarcane cultivars.

Key words: Antioxidant enzymes, in vitro, NaCl stress, polyethylene glycol, sugarcane

Abbreviations: Pretreated: P, Non-pretreated: NP

Introduction

Drought and salinity are becoming widespread in many regions, and may cause serious salinisation of more than 50% of all arable land by the year 2050 (Wang et al., 2003). It has been reported that over 800 million ha of land worldwide are salt-affected, either by salinity (397 million ha) or associated sodicity (434 million ha) (FAO, 2005). Although there is considerable difference in salinity tolerance, a salinity level of about 1.38 mΩ adversely affects many plant species (Alam et al., 2002). In recent years tissue culture has gained importance in the development of plants that are resistant to various abiotic stresses, as well as in elucidating the mechanisms operating at the cellular level by which plants survive under various abiotic stresses, including salinity (Jain, 2001). Plant tissue culture allows the control of stress homogeneity and the characterisation of cell behaviour under stress conditions, independent of the regulatory systems present at the whole plant level (Lutts et al., 2004).

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Sugarcane accounts for approximately 65% of world sugar production (Carson & Botha, 2002). Sugarcane, being a salt-sensitive crop, is affected in terms of cane quality when exposed to salt stress (Wahid et al., 1997). Hence, there is a constant need to develop new techniques, including plant tissue culture, for improving the salt tolerance of this crop. Salt stress causes water deficit, as a result of osmotic effects on a wide variety of metabolic activities in plants, and this water deficit results in oxidative stress because of the formation of reactive oxygen species. Reactive oxygen species that are the by-products of hyperosmotic and ionic stresses cause membrane dysfunction, which may lead to cell death (Bohnert & Jensen, 1996). Cells have a variety of defence mechanisms that protect against the harmful effects of reactive oxygen species. Antioxidant enzymes, including peroxidase, catalase, and superoxide dismutase, are the primary intracellular scavengers of reactive oxygen species (Uchida et al., 2002).

PEG (polyethylene glycol) has been used in vitro to induce water stress in plants (Ruf et al., 1967; Kaufman & Eckard, 1971). PEG is a non-penetrating inert osmoticum that lowers the osmotic potential of nutrient solutions, but it is not taken and is not phytotoxic (Lawlor, 1970). PEG stimulates water stress in cultured plant cells in the same way it does in the cells of intact plants.

The phenomenon of cross tolerance has also gained the attention of researchers in recent years. Cross tolerance refers to the exposure of tissue to a moderate stress that induces resistance to another stress (Genoud & Metraux, 1999); however, very little is known about how different stresses interact with each other. As salinity and water stress are 2 important stresses that affect crop productivity, the present study was conducted to test experimentally 2 main hypotheses with particular reference to 2 sugarcane cultivars: 1. Does PEG pretreatment of sugarcane callus cultures result in increased tolerance to osmotic stress, the main effect induced by salinity. 2. Does osmotic stress induced by PEG have any effect on the activity of antioxidant enzymes that may lead to greater salt tolerance in sugarcane.

Materials and methods

Uniform-sized leaf discs (5-8 mm in diameter × 2-3 mm thick) of 2 sugarcane cultivars (cv. SPF 234 and cv. HSF 240) derived from the young inner 2-3 whorls of leaves were used for callus induction. For callus induction and maintenance, MS (Murashige & Skoog, 1962) basal medium, with 30 g of sucrose L^{-1} and 13.5 µM 2,4-D solidified with 0.7% agar (Oxoid) and adjusted to pH 5.7, was used. All the cultures were maintained at 27 ± 2 °C with a 16-h photoperiod (35 mmol m^{-2} s^{-1}).

PEG pretreatment

For pretreatment 1% PEG (4000) was directly added to the callus maintenance medium and 60-day-old well-proliferating calluses were transferred to this medium for 5 days. The pretreated callus cultures were then transferred to callus maintenance medium containing different concentrations of NaCl, but lacking PEG.

NaCl treatment

For salt treatment of callus cultures 4 different NaCl concentrations were tested by adding separately to the MS medium. In addition to a control (0 mM NaCl), 1 sublethal NaCl concentration, 1 NaCl concentration above the sublethal level, and 1 NaCl concentration below the sublethal level were selected for each cultivar. Previous research on these sugarcane varieties in our lab (Munir and Aftab, unpublished) has shown that sublethal concentrations for SPF 234 and HSF 240 were 120 and 100 mM NaCl, respectively. Hence, the 4 salt levels tested on PEG-pretreated callus cultures of SPF 234 and HSF 240 were 0, 100, 120, and 140 mM NaCl, and 0, 80, 100, and 120 mM NaCl, respectively. For each NaCl treatment, 20 culture vessels (145 × 25 mm) were selected. A set of 20 × 4 non-pretreated culture vessels with the above-mentioned NaCl concentrations was used as non-pretreated controls.

Although it was difficult to quantify the level of callus necrosis, the following 5 categories based on visual observation were used during the present study: A: 81%-100%; B: 61%-80%; C: 41%-60%; D: 21%-40%; E: 0%-20%. PEG-pretreated and non-pretreated callus cultures were then analysed for fresh weight, soluble protein contents, and peroxidase, catalase, and superoxide dismutase activity at day 120. The callus

cultures were then transferred to an already-standardised regeneration medium (MS with 8.87 mM BAP and 0.5 mM TDZ) to study the effect of PEG pretreatment on regeneration potential.

Biochemical analysis

To extract proteins and enzymes, 1 g of tissue was crushed with 2 ml of 0.1 M phosphate buffer (pH 7.2) (13.6 g of KH_2PO_4 and 17.4 g of K_2HPO_4 in 1000 mL of distilled water). During crushing 0.1 g of polyvinyl polypyrrolidone (PVP) was added. The slurry was then centrifuged at 14,000 rpm for 10 min at 4 °C using a Sorvall RC-5B refrigerated super-speed centrifuge. The supernatant was used to estimate soluble protein contents and antioxidant enzyme activity. The biuret method of Racusen and Johnstone (1961) was adopted for the estimation of soluble protein contents. The reaction mixture was prepared by adding 2.0 mL of biuret reagent and 0.1 mL of supernatant. In the control test tube, 0.1 mL of distilled water was added instead of supernatant. Optical density was measured at 545 nm with a spectrophotometer (Hitachi U-1100). The quantity of protein was calculated from the standard curve for protein, which was prepared from bovine serum albumin and is expressed as mg g^{-1} of tissue.

During the present study peroxidase activity was assayed according to the method of Racusen and Foote (1965). The assay mixture for peroxidase contained 2.5 mL of 0.1 M phosphate buffer (pH 7.2), 0.2 mL of 1% guaiacol solution, 10 µL of crude enzyme extract, and 0.1 mL of H_2O_2 solution. The activity was estimated by measuring the absorbance at 470 nm and is expressed as mg g^{-1} of tissue.

For the estimation of catalase, a modified Beers and Sizer (1952) method was employed. Two buffer solutions (A and B) were prepared. Buffer A consisted of 50 mM potassium phosphate (pH 7.0), while buffer B was composed of a 0.036% H_2O_2 solution in 50 mM potassium phosphate buffer (pH 7.0). The reaction mixture consisted of 2.9 mL of buffer B and 0.1 mL of enzyme extract, while 3 mL of buffer A was used as a control. Change in the absorbance of the reaction solution was observed spectrophotometrically at 240 nm. To determine catalase activity the time required for A_{240nm} to decrease from 0.45 to 0.40 absorbance units was calculated. Catalase activity was then determined by the following formula:

$$\text{units mL}^{-1} \text{ of enzyme} = 3.45 \times \text{df min}^{-1} \times 0.1$$

where 3.45 corresponds to the decomposition of 3.45 µmol of hydrogen peroxide in a 3.0-mL reaction mixture producing a decrease in $A_{240\ nm}$ from 0.45 to 0.40 absorbance units, df is the dilution factor, min is the time in minutes required for A_{240nm} to decrease from 0.45 to 0.40 absorbance units, and 0.1 is the volume (mL) of enzyme used.

Superoxide dismutase (SOD) activity, the basis of which is its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), was determined using a slight modification of the method proposed by Maral et al. (1977). The reaction solution contained 2.0 mL of 1.0 mM sodium cyanide (NaCN), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA (ethylenediaminetetraacetic acid), and 2.0 µM riboflavin as a substrate. In the experimental tube, 5 µL of the enzyme extract was added. Experimental as well as control tubes were covered with black paper. The tubes were irradiated under light (two 40-W fluorescent lamps) for 10 min. The absorbance of both samples was then measured at 560 nm using a spectrophotometer. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of the photochemical reduction of NBT. SOD activity was expressed as units mg^{-1} of protein.

Statistical analysis

The experiment used a 4 × 2 factorial design, comprising 4 media and 2 pretreatments. Data were analysed using univariate analysis of variance (SPSS v.11). The data were transformed where required before applying the F test.

Results

Fresh weights

Figure 1 shows the changes in fresh weight of sugarcane callus cultures in response to PEG pretreatment at various NaCl concentrations. Although fresh weights of the sugarcane callus cultures (SPF 234 and HSF 240) were significantly affected by the different tested media, no significant change in fresh weights was recorded as a result of PEG pretreatment. At all the salt concentrations tested in each cultivar, the PEG-pretreated callus cultures

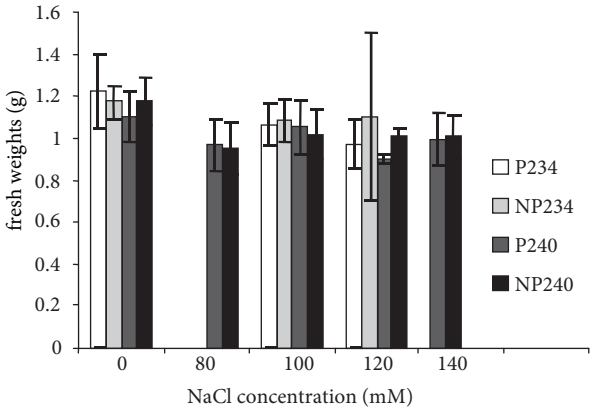

Figure 1. The effect of medium and/or PEG pretreatment on fresh weight in sugarcane callus cultures (cv. SPF 234 and cv. HSF 240) on day 120.

had higher fresh weight, as compared to the non-pretreated callus cultures, except at the 0 mM NaCl concentration.

Callus necrosis

Table 1 depicts the effect of salt stress on callus browning and necrosis in both sugarcane cultivars, according to callus necrosis scale A-E. It was observed that callus cultures became necrotic and brown when exposed to the medium containing salt. In the callus cultures of both sugarcane cultivars subjected to 0 mM NaCl, most of the callus cultures were not brown or necrotic, whereas some had very little browning and necrosis (category E). No change in callus necrosis was recorded at this salt level after PEG pretreatment and category E necrosis was observed in pretreated as well as in non-pretreated callus cultures treated with 0 mM NaCl. Category A necrosis was observed in non-pretreated callus cultures of cv. SPF 234 and cv. HSF 240 at the 120 and 140 mM NaCl concentrations, respectively, while pretreated callus cultures showed less necrosis (category B) at the same salt concentrations.

Soluble protein contents

It is evident from Figure 2 that soluble protein contents of the tested callus cultures were significantly affected both by the medium as well as PEG pretreatment. It was observed that callus cultures of cv. SPF 234 subjected to PEG pretreatment had higher soluble protein contents than those without PEG pretreatment at the same salt concentration. Likewise,

Table 1. The effect of medium and/or PEG pretreatment on sugarcane callus culture browning and necrosis (cvs. SPF 234 and HSF 240) on day 120.[a]

NaCl concentration (mM)	PEG pretreatment (1%)	Callus necrosis scales (A-E)[b]	
		SPF 234	HSF 240
0	_	E	E
	+	E	E
80	_	Not tested[c]	C
	+	Not tested[c]	C
100	_	C	C
	+	D	D
120	_	B	A
	+	D	B
140	_	A	Not tested[c]
	+	B	Not tested[c]

[a] Data on callus browning and necrosis are based on 20 replicates for each NaCl treatment.
[b] Callus necrosis (scale A-E); A: 81%-100%, B: 61%-80%, C: 41%-60%, D: 21%-40%, E: 0%-20%.
[c] Not tested, because according to the experimental design only 3 salt concentrations (sublethal NaCl level, and 1 concentration above and below the sublethal NaCl concentration), in addition to a control (0mM NaCl), were tested in each sugarcane cultivar.

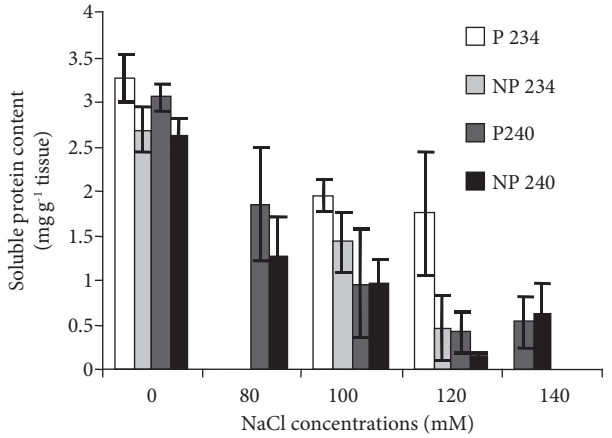

Figure 2. The effect of medium and/or PEG pretreatment on soluble protein contents in sugarcane callus cultures (cv. SPF 234 and cv. HSF 240).

for cv. HSF 240, although a decrease was observed in soluble protein contents of the callus cultures as the salt concentration increased (from 0 to 100, 120, or 140 mM NaCl), at all salt concentrations the soluble protein contents in PEG-pretreated callus cultures was higher than in the non-pretreated control at that particular salt concentration (Figure 2).

Peroxidase activity

Figure 3 shows that PEG-pretreated callus cultures of cv. SPF 234 had higher peroxidase activity than the non-pretreated callus cultures subjected to the same salt concentration. It may thus be suggested that PEG pretreatment followed by salinity stress stimulated the synthesis of the peroxidase enzyme in both sugarcane cultivars. For callus cultures of cv. HSF 240, a significant effect of PEG pretreatment on peroxidase activity was observed.

Catalase activity

Catalase activity in cv. SPF 234 is depicted in Figure 4. It is evident from the figure that only the medium had a significant effect on catalase activity at day 120, whereas PEG pretreatment had no influence on catalase activity in this cultivar.

A significant difference was also observed in the catalase activity in callus cultures of cv. HSF 240 as a result of PEG pretreatment. Catalase activity increased as the NaCl concentration increased, both in pretreated as well as in non-pretreated callus cultures (Figure 4).

Superoxide dismutase activity

A comparison of superoxide dismutase activity in pretreated and non-pretreated callus cultures of both sugarcane cultivars treated with various NaCl concentrations is depicted in Figure 5. At day 120 a significant increase in SOD activity was observed after PEG pretreatment at all the salt concentrations tested in cv. SPF 234 callus cultures (0, 100, 120, and 140 mM NaCl), whereas in cv. HSF 240 callus cultures no significant effect of PEG pretreatment on SOD enzyme activity was observed.

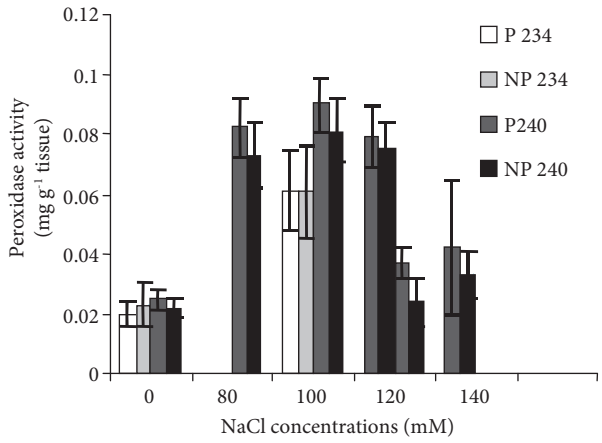

Figure 3. The effect of medium and/or PEG pretreatment on peroxidase activity in sugarcane callus cultures (cv. SPF 234 and cv. HSF 240) on day 120.

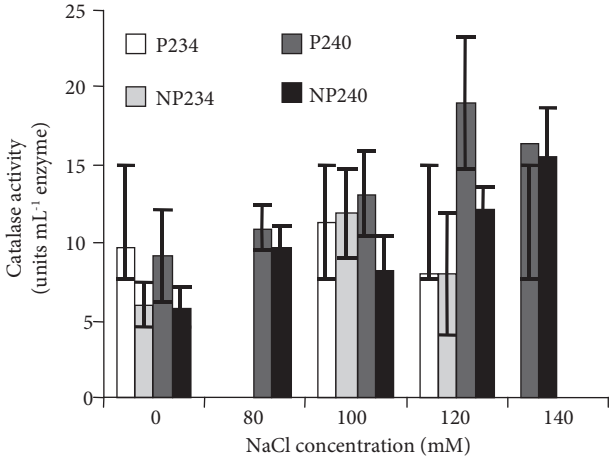

Figure 4. The effect of medium and/or PEG on catalase activity in sugarcane callus cultures (cv. SPF 234 and cv. HSF 240) on day 120.

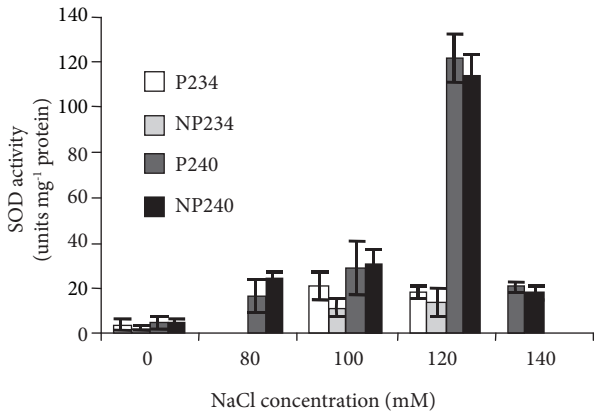

Figure 5. The effect of medium and/or PEG on superoxide dismutase activity in sugarcane callus cultures (cv. SPF 234 and cv. HSF 240) on day 120.

The effect of PEG on regeneration potential

Regeneration frequency of the non-pretreated callus cultures at the 0-mM NaCl concentration was 85%, while an 82% regeneration frequency was observed in PEG-pretreated callus cultures (Table 2). Similarly, a reduction (though greater than that observed for the previous NaCl concentration) was observed in the regeneration frequency of callus cultures at 100 mM NaCl when pretreated with 1% PEG. PEG pretreatment also increased the regeneration frequency of the callus cultures from 10% (the regeneration frequency in non-treated controls) to 13% at the 120-mM NaCl concentration.

Table 2 also shows that the salt-treated cv. HSF 240 callus cultures had regeneration potential up to the

Table 2. The effect of medium and/or PEG pretreatment on the regeneration potential of 120-day-old NaCl-treated sugarcane callus cultures (cvs. SPF 234 and HSF 240).[A]

NaCl concentration (mM)	PEG pretreatment (1%)	Regeneration frequency (%)		Number of shoots[B]		Shoot length[C] (cm)	
		Cultivars					
		SPF 234 234	HSF 240	SPF 234	HSF 240	SPF 234	HSF 240
0	_	85	76	42	44	0.96	0.95
	+	82	83	43	60	1	0.94
80	_	Not tested[D]	70	Not tested[D]	47	Not tested[D]	0.93
	+	Not tested[D]	83	Not tested[D]	72	Not tested[D]	0.94
100	_	82	75	54	54	0.89	0.88
	+	73	82	62	67	0.95	0.89
120	_	10	0	65	0	0.24	0
	+	13	0	64	0	0.28	0
140	_	0	Not tested[D]	0	Not tested[D]	0	Not tested[D]
	+	0	Not tested[D]	0	Not tested[D]	0	Not tested[D]

The given data were taken from 20 replicates per treatment.

[A] Data were recorded on day 30, after transferring 120-day-old calluses to the regeneration medium.

[B] Data for shoot number were transformed using √y (where y is the mean number of shoots) to normalise the data. Non-transformed mean values are presented.

[C] Data for shoot length were transformed using arcsine √y for cv. SPF 234 and 1/y for cv. HSF 240 (where y is mean shoot length) to normalize the data. Non-transformed mean values are presented.

[D] Not tested, because according to the experimental design only 3 salt concentrations (sublethal NaCl level, and 1 concentration above and below the sublethal NaCl concentration), in addition to a control, (0mM NaCl) were tested for each sugarcane cultivar.

100-mM NaCl concentration, with 82% and 75% regeneration in callus cultures with and without PEG pretreatment, respectively. PEG pretreatment increased the regeneration frequency from 76% to 83% in callus cultures treated with 0 mM NaCl. In callus cultures exposed to the 80-mM NaCl concentration, PEG-pretreated callus cultures had an 83% regeneration frequency, as compared to 70% in callus cultures not pretreated with PEG.

Discussion

A number of studies have shown the existence of cross-tolerance, i.e. exposure of a plant to a moderate stress condition induces tolerance to other stresses (Leshem and Kuiper, 1996). For example, salt stress has been reported to stimulate cold-hardiness in spinach seedlings (Ryu et al., 1995), water stress induced chilling resistance in rice (Takahashi et al., 1994), and heat stress increased tolerance to several abiotic stresses in plants (Sabehat et al., 1998).

Drought and salinity are often interconnected, and plant responses to salt and water stress have much in common. Salinity reduces the ability of plants to take up water, which quickly reduces the growth rate, as well as a number of metabolic changes identical to those caused by water stress (Munns, 2002). As a consequence, both salt and water stress often activate similar cell-signalling pathways (Shinozaki & Yamaguchi-Shinozaki, 2000; Knight & Knight, 2001); however, it has also been reported that salt stress at equivalent osmotic potentials is lethal, as compared to osmotic stress (Alam et al., 2002). Results of the present study indicate that, regardless of PEG pretreatment, calluses subjected to salt stress had less fresh weight than the control (Figure 1). It has been reported that both stresses inhibit the growth of various calluses (Ben-Hayyim et al., 1985). Rapid (essentially instantaneous) changes in plant growth rates have been observed with salt (Cramer & Bowman, 1991) and PEG stress (Chazen et al., 1995). Our results for fresh weights of sugarcane callus cultures after PEG and NaCl treatment are in agreement with these earlier findings in other plants.

It is evident from the results that PEG pretreatment increased the tolerance of sugarcane callus cultures to NaCl (Table 1). The callus cultures after PEG pretreatment had less necrosis than NaCl-treated callus cultures. Drought pretreatment induced by PEG was reported to increase tolerance to the osmotic effect, the main effect induced by salinity in a number of crops, e.g. parsley (Pill & Kilian, 2000), tomato (Chen et al., 2005), sunflower (Chojnowski et al., 1997), carrot, lettuce, and onion (Yeon et al., 2000), and pearl millet (Ashraf et al., 2003). On the other hand, other researchers did not establish a correlation between salt and PEG-induced water stress (Cayuela et al., 1996; Khajeh-Hosseini et al., 2003).

Though the actual mechanism of PEG-induced salt tolerance is not well understood, some suggest that specific proteins are induced by one particular kind of stress that is involved in the protection against other kinds of stresses (Pareek et al., 1995; Sabehat et al., 1998). Thus, environmental stresses may cause changes in soluble protein content as well as in the activity of various antioxidant enzymes. Figure 2 shows such changes in soluble protein contents in response to salt stress, as well as to PEG pretreatment in sugarcane callus cultures. It was observed in the present study that PEG pretreatment enhanced the levels of antioxidant enzymes (peroxidase, catalase, and SOD) in the callus cultures subjected to NaCl treatment, suggesting that non-toxic PEG stress may trigger a few (or perhaps more) biochemical changes (Figures 3-5). Such biochemical changes seem to enhance the antioxidant defence system in plants and increase their tolerance to different stress factors (Noctor & Foyer, 1998). Better growth indicators in the callus cultures subjected to elevated salt levels after PEG pretreatment in the present study support the above-mentioned viewpoint.

It was also observed during the present study that PEG pretreatment improved the regeneration potential of the salt-treated callus cultures (Table 2). The water stress imposed by PEG was quite beneficial to the maturation of somatic embryos. In white spruce osmotic treatment with 5.0%-7.5% PEG stimulated an increase in the maturation frequency (Attree et al., 1991). In carnation the highest percentage of embryo maturation occurred in MS medium supplemented with 1% PEG (Yantcheva et al., 1998). As the predominant regeneration pathway in our studies was through somatic embryogenesis, improved embryo maturation in the present study may have been

responsible for the observed enhancement in the regeneration potential of callus cultures cultured on stress-free regeneration medium. Hence, in the present study PEG pretreatment not only improved NaCl tolerance, but also increased the regeneration frequency of the treated sugarcane cultures. This further increased the potential to produce large numbers of salt-tolerant regenerants using the described procedure.

The present work thus highlights the role of PEG pretreatment in alleviating salt stress in both sugarcane cultivar callus cultures (cv. SPF 234 and cv. HSF 240), as indicated by the studied growth as well as biochemical parameters that indicated a relationship between osmotic and salt stresses. Furthermore, increased regeneration potential in the callus cultures also indicates the positive effect of PEG pretreatment in improving salt tolerance in sugarcane.

In conclusion, it can be suggested on the basis of our results that PEG pretreatment resulted in improving the growth of sugarcane callus cultures subjected to various salt concentrations. Hence, low-level PEG (1%) may help increase sugarcane's ability to cope with salt stress.

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