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Full Length Research Paper

Enhancement of salt tolerance in sugarcane by ascorbic acid pretreatment

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Ascorbic acid is a non-enzymatic antioxidant which plays an important role in the activation of biological defense mechanisms. The effect of 24 h ascorbic acid (0.5 mM) pretreatment was observed on subsequent growth and development of callus cultures as well as *in vitro*-grown plants of *Saccharum* sp. *hybrid* (cvs. HSF 240 and SPF 234). After pretreatment, callus cultures of HSF 240 were transferred to 80, 100 and 120 mM sodium chloride (NaCl) while those of SPF 234 were subjected to 100, 120 and 140 mM NaCl concentrations. The *in vitro*-grown plants were also transferred to the salt-containing media (0 to 160 mM; nine treatments) after ascorbic acid pretreatment. Ascorbic acid pretreatment to callus cultures resulted in more browning and necrosis. Pretreated *in vitro*-grown plants of both the sugarcane cultivars survived higher NaCl level and showed much less yellowing of leaves as compared to non-pretreated controls. Ascorbic acid pretreatment to *in vitro*-grown plants of cv. HSF 240 had a significant effect on catalase and peroxidase (POD) activities while in cv. SPF 234 a significant effect on root length, soluble protein contents as well as antioxidant enzyme activities was recorded. Hence both morphological as well as biochemical parameters studied during the present work suggested that ascorbic acid pretreatment of *in vitro*-grown sugarcane plants may enhance their salt tolerance.

Key words: Antioxidant enzymes, callus, protein content, salt stress.

INTRODUCTION

Soil salinity occupies a prominent place among the soil problems that threaten the sustainability of agriculture over a vast area in the world (Flowers 2004). Salinity affects the plant growth due to osmotic stress, nutritional or hormonal imbalance, specific ion (Na^+) toxicity or production of reactive oxygen species (Ashraf and Harris, 2004). Salinity is one of the main factors affecting economic yield of many crops including sugarcane crop of many countries of the world. It accounts for (Wahid, 2004). Sugarcane is an important industrial cash approximately 65% of the world sugar production (Carson and Botha, 2002). Besides sugar production, sugarcane generates numerous valuable by products like alcohol used by pharmaceutical industry, ethanol used as a fuel, bagasse used for paper and chip-board manufacturing and press mud used as a rich source of organic matter and nutrients for crop production. Keeping in view the economic importance as well as its vulnerability to salt stress, there is a need to develop strategies for the improvement of this plant for salinity tolerance.

During the recent years, cultured plant cells have been introduced as a convenient tool for research to elucidate the mechanisms operating at the cellular level by which plants survive under various abiotic stresses including salinity (Rains et al., 1980). This method also seems to support the traditional breeding strategies by producing plants with improved salt tolerance through selection of salt-tolerant cells in cultures and subsequent plant regeneration. Hence the prospects for the development of salt tolerance in sugarcane using tissue culture mean seem

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Abbreviations: CAT, Catalase; **NBT,** nitro blue tetrazolium; **POD,** peroxidase; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase.

quite promising.

During the stress conditions, free radicals mostly reactive oxygen species (ROS) are constantly produced. ROS are now considered signal molecules at non-toxic concentrations but at toxic concentrations they are also capable of injuring cells (Meloni et al., 2003). Plant cells have a variety of mechanisms for scavenging harmful effects of ROS including both enzymatic and/or non-enzymatic ones. There are many recent studies indicating elevated levels of antioxidants as a result of various environmental stresses (Hernandez et al., 2000; Sekmen et al., 2007). The antioxidant enzymes including peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) are mainly intracellular scavengers of ROS (Neto et al., 2006). Non-enzymatic factors include several small molecules that are antioxidant in nature such as alpha-tocopherol (vitamin E), ascorbic acid (vitamin C) and carotenoids (Sairam et al., 2002). Ascorbic acid, also known as Vitamin C, is an important antioxidant molecule that is widely utilized in cellular metabolism (Loewus and Helsper, 1982). Ascorbic acid also acts as primary substrate in the cyclic pathway for enzymatic detoxification of hydrogen peroxide; hence, it plays an important role in the activation of biological defense mechanisms (Arrigoni et al., 1979). Ascorbic acid has an additional role on thylakoid surface in protecting and regenerating oxidized carotenes and tocopherols, which are also helpful in protecting the plants against oxidative damage (Tappel, 1977).

There have been some reports indicating that the externally-applied ascorbic acid can be metabolized in tissues (Sapers et al., 1991; Gaddalah, 2000). Exogenous application of ascorbic acid and other biomolecules such as polyethylene glycol (PEG), sorbitol, mannitol, indole-3-acetic acid (IAA), and gibberelic acid were tested and regarded as 'short-cut method' for increasing the tolerance of different plants in particular members of Gramineae such as wheat against various abiotic stresses (Ashraf et al., 2003; Al- Hakimi and Hamada 2001; Wahid et al., 2007). In one recent study, pretreatment of ascorbic acid to callus cultures and *in vitro*-grown plants resulted in amelioration of salinity tolerance in potato (Sajid and Aftab, 2009) thus extending the potential usefulness of this approach to non-graminaceous plants under various culture conditions. The efficacy of exogenous application of ascorbic acid in sugarcane to mitigate salt stress has not been determined before. The similarities in general metabolism amongst graminaceous crops make this aspect of research quite exciting that may potentially reveal interesting comparisons to draw useful conclusions.

The above information thus served as an impetus for the current investigation. It aimed at finding out the role of ascorbic acid pretreatment in alleviation of salinity stress in *in vitro* sugarcane (*Saccharum* sp. *hybrid*, cvs. HSF 240 and SPF 234) cultures. In doing so, changes in both morphological as well as biochemical parameters such as proteins, POD, CAT and SOD activities associated with pretreatment and sodium chloride (NaCl) stress were studied during the present investigation. This work also aimed at providing species-specific information on sugarcane that may prove to be of applied value in the near future.

MATERIALS AND METHODS

During the present work, explants ranging from 5 to 8 mm in diameter (2 to 3 mm thick) derived from young inner two to three whorls of leaves were obtained from two sugarcane cultivars (HSF 240 and SPF 234). For callus induction, these explants were inoculated on MS (Murashige and Skoog, 1962) basal medium supplemented with 13.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D). Calluses were induced under dark conditions at 27 ± 2°C and shifted to 16-h photoperiod (35 µmol m^{-2} s^{-1}) at day 30.

Ascorbic acid pretreatment

Ascorbic acid pretreatment was given by shifting 60-day-old callus cultures to callus maintenance medium (as mentioned above) supplemented with 0.5 mM ascorbic acid for either 24 or 48 h. The selected concentration of ascorbic acid was based on Shalata and Neumann, (2001). Subsequently, such ascorbic acid-pretreated calluses were subcultured on the same medium lacking ascorbic acid but containing NaCl (as detailed below) after 24 h. Data for fresh weights, callus necrosis, soluble protein contents as well as antioxidant enzyme activities were collected at day 30.

To analyze the effect of ascorbic acid pretreatment on *in vitro*-grown sugarcane plants, 60-day-old regenerants from both the cultivars were transferred to MS basal medium containing 0.5 mM ascorbic acid for 24 h. The plants after the pretreatment were shifted to MS basal medium containing NaCl (0 to 160 mM) till the data collection at day 30.

NaCl treatment

During the present study, three different NaCl concentrations were selected for each sugarcane cultivar in addition to a control (0 mM NaCl). The three salt concentrations tested included one sublethal NaCl level, one above and one below the sublethal NaCl concentration for each cultivar. The sublethal NaCl concentrations as determined earlier (Munir and Aftab, unpublished data) were 100 and 120 mM NaCl for HSF 240 and SPF 234, respectively. Ascorbic acid-pretreated callus cultures of HSF 240 were shifted to the above-mentioned callus maintenance medium supplemented with 0, 80, 100 and 120 mM NaCl level. The callus cultures of SPF 234 were subjected to 0, 100, 120 and 140 mM NaCl level after ascorbic acid pretreatment. The experiment was performed with 20 replicates per treatment. A control (without ascorbic acid pretreatment) of 20 replicates for each NaCl treatment was also run. Ascorbic acid-pretreated and non-pretreated callus cultures were then analyzed for fresh weight, callus necrosis, soluble protein contents, POD, CAT and SOD activities at day 30.

To analyze the effect of ascorbic acid pretreatment on *in vitro* growth of plants, pretreated plants were shifted to MS basal medium supplemented with various NaCl concentrations (0, 20, 40, 60, 80, 100, 120, 140 and 160 mM; nine treatments). The data for fresh weights of plants, number of shoots per culture vessel, shoot length, numbers of roots per culture vessel and root length, soluble protein contents and antioxidant enzyme activities were recorded at day 30.

Callus necrosis being a qualitative parameter was difficult to express in quantitative terms, however, five categories (Scales A to E) as mentioned below were arbitrarily selected.

Table 1. Effect of medium and/or ascorbic acid pretreatment on fresh weights of sugarcane (cvs. HSF 240 and SPF 234) callus cultures maintained on MS medium supplemented with NaCl at day 30.

NaCl level (mM)	Ascorbicacid pretreatment[A] (0.5 mM)	Fresh weight of callus cultures at day 30 (g)[B]	
		HSF 240	SPF 234
0	–	1.26	1.24
	+	1.01	1.01
80	–	1.09	Not tested
	+	0.88	Not tested
100	–	1.05	1.06
	+	0.91	0.94
120	–	0.95	1.01
	+	0.84	0.90
140	–	Not tested	0.99
	+	Not tested	0.90
Effect of medium (with 3 and 152 df)		*	*
Effect of pretreatment (with 1 and 152 df)		*	*
Effect of medium x pretreatment (with 3 and 152 df)		NS	NS

[A] The + or - signs represent respective media supplemented with (+) or without (-) ascorbic acid.
[B] The results are based on 20 replicates for each treatment. Data were transformed using arcsin√y (where y is the value of fresh weight) to normalize the data. Non-transformed mean values are presented. *, NS Significant at 1% level (*) or non-significant (NS) according to F test with df mentioned against each. df, Degree of freedom.

Callus necrosis scales; A, 81 to 100; B, 61 to 80; C, 41 to 60; D, 21 to 40 and E, 0 to 20 callus necrosis. Ascorbic acid-pretreated and non-pretreated callus cultures were analyzed for fresh weights, soluble protein contents, POD, CAT and SOD activities at day 30. The ascorbic acid-pretreated and non-pretreated plants were also analyzed for fresh weights of plants, number of shoots/roots per culture vessel, shoot/root length, soluble protein contents and antioxidant enzyme activities at day 30.

Biochemical analysis

For the extraction of proteins and enzymes, samples were prepared by crushing 1 g tissue with 2 ml of 0.1 M phosphate buffer, pH 7.2. Biuret method of Racusen and Johnstone (1961) was adopted for the estimation of soluble protein contents. To determine POD activity (E.C 1.11.1.7), the method proposed by Racusen and Foote (1965) was employed. For the estimation of CAT (E.C 1.11.1.6), method of Beers and Sizer (1952) was employed with some modifications. The total SOD (E.C 1.11.1.6) activity expressed as units/mg of protein was measured by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to Maral et al. (1977). The details regarding above methods and modifications were the same as reported earlier (Munir and Aftab, 2009).

The effect of ascorbic acid pretreatment on callus cultures and plants was statistically analyzed by Univariate analysis of variance (SPSS Version 11).

RESULTS

Effect of ascorbic acid pretreatment on fresh weight, browning and necrosis in sugarcane callus cultures

None of the callus cultures of cv. HSF 240 survived whereas only 1% culture survival was observed for cv. SPF 234 after ascorbic acid pretreatment for 48 h (data not shown). Consequently, 48 h pretreatment of callus cultures with 0.5 mM ascorbic acid was no longer continued.

It is evident from the data given in Table 1 that both medium as well as ascorbic acid pretreatment for 24 h had a significant effect on fresh weights of callus cultures of both the sugarcane cultivars. A general reduction in fresh weights of callus cultures was recorded after ascorbic acid pretreatment.

The effect of ascorbic acid pretreatment on callus necrosis was recorded in terms of callus necrosis scales

Table 2. Effect of medium and/or Ascorbic acid pretreatment on callus browning and necrosis in sugarcane (cvs. HSF 240 and SPF 234) callus cultures maintained on MS medium supplemented with NaCl at day 30[a]

NaCl level (mM)	Ascorbic acid pretreatment [b] (0.5 mM)	Callus necrosis (Scales A - E)[c]	
		HSF 240	SPF 234
0	–	E	E
	+	C	C
80	–	C	Not tested
	+	A	Not tested
100	–	B	B
	+	A	A
120	–	A	C
	+	A	A
140	–	Not tested	C
	+	Not tested	A

[a] Data on callus browning and necrosis were based on 20 replicates for each NaCl treatment. [b] The + or - signs represent respective media supplemented with (+) or without (-) ascorbic acid. [c] Callus necrosis (Scales A - E); A, 81 to 100; B, 61 to 80; C, 41 to 60; D, 21 to 40; E, 0 to 20 % callus necrosis.

A to E (Table 2). Very little or no necrosis was observed in callus cultures of both sugarcane cultivars at 0 mM NaCl level. However greater necrosis was observed in ascorbic acid-pretreated callus cultures as compared to non-pretreated controls.

Effect of ascorbic acid pretreatment on soluble protein contents and antioxidant enzyme activities in callus cultures of sugarcane

Soluble protein contents of callus cultures

As evident from Table 3, ascorbic acid pretreatment had no significant effect on soluble protein contents in callus cultures of cv. HSF 240. It was though interesting to note that ascorbic acid pretreatment to callus cultures of cv. SPF 234 significantly decreased its soluble protein contents.

Peroxidase, catalase and superoxide dismutase activity of callus cultures

Ascorbic acid pretreatment to callus cultures had a significant effect on POD activity of callus cultures of both the cultivars at day 30 (Table 3). Generally a reduction was observed in POD activities of callus cultures after ascorbic acid pretreatment. Similarly no significant effect of ascorbic acid pretreatment was recorded on CAT activity in callus cultures of cv. HSF 240. However, the activity of CAT enzyme in callus cultures of cv. SPF 234 at day 30 was found to be significantly affected by both the medium as well as ascorbic acid pretreatment either alone or in combination. The data given in Table 3 indicate an increase in SOD activity with increasing NaCl concentration in the medium. It is also evident from the table that at all the NaCl levels tested for cv. HSF 240, ascorbic acid-pretreated callus cultures had greater SOD activity as compared to non-pretreated control except at 80 mM NaCl level. However, this increase was not significant statistically. Likewise in the other cultivar, no significant effect of ascorbic acid pretreatment on SOD activity was observed.

Effect of ascorbic acid pretreatment on fresh weight, browning and necrosis in *in vitro* grown plants of sugarcane

The data for fresh weights of plants, number of shoots per culture vessel, shoot length, numbers of roots per culture vessel and root length at day 30 are given in Table 4. It is evident from the data that fresh weights of both the sugarcane cultivars were significantly affected

Table 3. Effect of medium and/or ascorbic acid pretreatment on soluble protein contents, peroxidase, catalase and superoxide dismutase activities of sugarcane (cvs. HSF 240 and SPF 234) callus cultures maintained on MS medium supplemented with NaCl at day 30[a]

NaCl level (mM)	Ascorbic acid pre-treatment[b] (0.5 mM)	Soluble protein content (mg/g tissue)		POD activity (mg/g tissue)		CAT activity (units/ml enzyme)		SOD activity (units/mg protein)	
		HSF 240	SPF 234	HSF 240	SPF 234	HSF 240	SPF 234	HSF 240	SPF 234
0	–	3.85	3.54	0.02	0.02	5.4	3.23	0.79	1.80
	+	2.29	1.43	0.01	0.01	5.6	5.33	2.80	5.2
80	–	2.47	Not tested	0.08	Not tested	12.09	Not ested	10.64	Not tested
	+	0.77	Not tested	0.04	Not tested	11.33	Not tested	5.33	Not tested
100	–	1.5	2.67	0.08	0.07	17.16	11.10	25.79	15.47
	+	2.2	1.24	0.01	0.01	16	12	37	24.52
120	–	0.42	1.56	0.05	0.07	15.93	17.23	31.08	23.96
	+	0.47	1.67	0.05	0.02	16	12.67	36.03	19.33
140	–	Not tested	0.71	Not tested	0.06	Not tested	16.97	Not tested	32.41
	+	Not tested	0.37	Not tested	0.07	Not tested	8.33	Not tested	51.67
Effect of medium (with 3 and 40 df)		*	*	*	*	NS	*	*	*
Effect of pretreatment (with 1 and 40 df)		NS	*	*	*	NS	**	NS	NS
Effect of medium x pretreatment (with 3 and 40 df)		**	*	**	*	NS	**	NS	NS

[a] The results are based on 6 replicates for each treatment. [b] The + or - signs represent respective media supplemented with (+) or without (-) ascorbic acid. [*, NS] Significant at 1% level (*), 5% level (**) or non-significant (NS) according to F test. . df, Degree of freedom.

by the medium as well as pretreatment of ascorbic acid. It was also observed that non-pretreated *in vitro*-grown plants of cv. SPF could tolerate up to 100 mM NaCl level but the pretreated plants survived up to 160 mM NaCl concentration. A significant increase in the number of shoots per culture vessel as well as shoot length was observed after ascorbic acid pretreatment in both the sugarcane cultivars. For cv. HSF 240, no

Table 4. Effect of medium and/or ascorbic acid pretreatment on morphological growth parameters of *in vitro*-grown sugarcane plants (cvs. HSF and SPF 234) maintained on MS medium supplemented with different concentrations of NaCl at day 30[a]

NaCl level (mM)	Ascorbic acid pre-treatment[b] (0.5 mM)	Fresh weight (g)		Number of shoot/ culture vessel		Shoot length (cm)		Number of root/ culture vessel		Root length (cm)	
		HSF 240	SPF 234	HSF 240	SPF 234	HSF 240	SPF 234	HSF 240	SPF 234	HSF 240	SPF 234
0	-	0.45	0.50	9.3	10.8	8.7	9.17	7	6.1	3.05	4.0
	+	0.51	0.54	10.7	12.3	10.65	10.48	7.3	5.6	4.1	3.6
20	-	0.43	0.47	8.8	7.5	8.7	9.75	5.6	5.4	2.8	3.35
	+	0.45	0.51	9	8.9	9.7	10.78	5.6	6.2	3.4	2.90
40	-	0.35	0.40	5.6	6.5	6.5	7.35	5.7	5.4	3.7	3.8
	+	0.38	0.44	6	6.4	6.8	9.0	6.0	6.0	3.4	3.8
60	-	0.22	0.35	5.7	6.1	6.8	5.3	5.9	6.3	3.0	3.7
	+	0.33	0.45	8.5	7.6	8.2	6.7	6.0	5.8	2.7	2.7
80	-	0.23	0.31	4.2	3.5	6.9	5.9	5.8	6.4	3.8	4.0
	+	0.26	0.35	6.0	6.0	8.3	6.3	5.6	5.9	3.0	2.32
100	-	0.10	0.11	2.9	2.6	2.2	1.9	3.6	3.4	1.9	2.5
	+	0.16	0.20	3.4	3.3	3.3	2.8	4.2	3.9	2.6	3
120	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
	+	0.14	0.18	3.4	3.4	3.0	2.7	4.3	4.0	2.4	3.1
140	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
	+	0.11	0.14	3.1	3.2	2.7	2.4	3.9	3.4	2.1	2.8
160	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
	+	ND[c]	0.12	ND[c]	3.0	ND[c]	2.4	ND[c]	3.6	ND[c]	2.2
Effect of medium (with 5 and 135 df)		*	*	*	*	*	*	*	*	*	**
Effect of pretreatment (with 1 and 135 df)		**	*	**	**	**	**	NS	NS	NS	**
Effect of medium x pretreatment (with 5 and 135 df)		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a] The results are based on 20 replicates for each treatment. [b]The + or - signs represent respective media supplemented with (+) or without (-) ascorbic acid. [c]ND, Not determined. *, **, [NS] Significant at 1 % level (*), 5 % level (**) or non-significant (NS) according to F test. df, Degree of freedom.

significant effect of pretreatment was observed on number of roots per culture vessel as well as root lengths. Root lengths of plants of cv. SPF 234 were significantly affected by ascorbic acid pretreatment but no significant effect of pretreatment was recorded on number of roots

Table 5. Effect of medium and/or Ascorbic acid pretreatment on soluble protein contents, peroxidase, catalase and superoxide dismutase activities of *in vitro*-grown plants of sugarcane (cv. HSF 240 and SPF 234) maintained on MS medium supplemented with NaCl at day 30[a].

NaCl level (mM)	Ascorbic acid pre-treatment[b] (0.5 mM)	Soluble protein content (mg/g tissue)		POD activity (mg/g tissue)		CAT activity (units/ml enzyme)		SOD activity (units/mg of protein)	
		HSF 240	SPF 234	HSF 240	SPF 234	HSF 240	SPF 234	HSF 240	SPF 234
0	-	3.65	3.18	0.013	0.018	7.58	8.39	6.20	3.64
	+	3.35	3.90	0.021	0.017	11.55	11.25	7.95	12.79
20	-	2.60	3.07	0.016	0.031	14.86	13.32	15.68	15.26
	+	2.10	3.32	0.048	0.054	16.56	14.67	18.72	15.90
40	-	2.49	1.70	0.031	0.038	10.23	8.38	18.27	12.60
	+	1.86	2.32	0.036	0.054	13.68	13.31	21.33	17.24
60	-	1.76	1.73	0.045	0.032	15.61	15.94	16.25	16.56
	+	1.94	2.46	0.040	0.023	16.84	16.56	16.20	17.39
80	-	1.61	2.01	0.018	0.037	8.85	9.97	21.33	13.22
	+	2.27	2.30	0.030	0.061	13.74	14.23	23.18	18.24
100	-	0.09	0.04	0.018	0.027	3.47	5.35	10.58	9.44
	+	0.39	0.23	0.029	0.041	7.93	10.50	15.62	15.05
120	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
	+	0.24	0.20	0.027	0.035	7.12	8.7	14.47	14.27
140	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
	+	0.21	0.16	0.022	0.034	6.62	8.0	13.96	14.02
160	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]
	+	ND[c]	0.12	ND[c]	0.034	ND[c]	7.2	ND[c]	13.35
Effect of medium (with 5 and 75 df)		*	*	*	*	*	*	*	*
Effect of pretreatment (with 1 and 75 df)		NS	**	*	**	*	*	NS	*
Effect of medium x pretreatment (with 5 and 75 df)		NS	NS	NS	NS	NS	NS	NS	NS

[a] The results are based on 6 replicates for each treatment. [b] The + or - signs represent respective media supplemented with (+) or without (-) ascorbic acid. [c] ND: Not determined [*, NS] Significant at 1% level (*), 5% level (**) or non-significant (NS) according to F test.

per plant in this cultivar (Table 4).

Effect of ascorbic acid pretreatment on soluble protein contents and antioxidant enzyme activities in *in vitro*-grown sugarcane plants

Soluble protein contents of in vitro-grown plants

Data given in Table 5 indicate that ascorbic acid pretreatment had no significant effect on soluble protein contents of *in vitro*-grown plants of cv. HSF 240. However, the soluble protein contents of *in vitro*-grown plants of cv. SPF 234 significantly decreased with various salt treatments. A comparison of soluble protein contents of ascorbic acid-pretreated and non-pretreated plants indicated that ascorbic acid pretreatment had a positiveeffect on soluble protein contents in cv. SPF 234.

Peroxidase, catalase and superoxide dismutase activity of in vitro-grown plants

Table 5 also depicts a considerable increase in POD activity in the plants of cv. HSF 240 at all the salt levels after ascorbic acid pretreatment. The POD activities of ascorbic acid-pretreated or non-pretreated plants of cv. SPF 234 at 0 mM NaCl level were almost the same (0.017 and 0.018 mg/g tissue, respectively). A general increase in POD activities was recorded both by the medium composition as well as pretreatment at the other salt concentrations tested during the experiment. A significant effect of medium as well as pretreatment was observed on the CAT activities in plants of both the sugarcane cultivars. A comparison of CAT activities of *in vitro*-grown plants indicated that ascorbic acid pretreatment enhanced the CAT activity.

It is evident from Table 5 that only medium had a

significant effect on SOD activities of *in vitro*-grown plants of HSF 240. No significant effect of ascorbic acid pretreatment was recorded on SOD activities in this particular cultivar. Table 5 also indicates that there was a significant increase in SOD activity in the pretreated plants of SPF 234 as compared to non-pretreated controls. Unlike HSF 240, ascorbic acid pretreatment had a positive effect on SOD activities in this cultivar.

DISCUSSION

Apart from many other aspects, it has also been suggested that an important feature of salt stress is the generation of reactive oxygen species. ROS participate in multiple processes in plants and can damage other molecules and the cell structures of which they are a part (Mittler, 2002). In response to ROS, plants were reported to have produced higher amounts of enzymatic and non-enzymatic antioxidants, that is , SOD, CAT, POD, ascorbic acid and glutathione (Neto et al., 2006; Kusvuran et al., 2007).

Ascorbic acid is an important non-enzymatic molecule which is needed to activate biological defense mechanisms (Arrigoni and others, 1979). As an antioxidant, ascorbic acid has been found not only to react with hydrogen peroxide but also with O_2^-, OH^- and lipid hydroperoxides and thus enhances plant growth (Noctor and Foyer, 1998). Many workers estimated the potential of this compound in ameliorating and modifying the salt stress-induced changes in plants (Hamada, 1998; Janda et al., 1999). There are some indications that externally-applied ascorbic acid could be metabolized in tissues (Sapers et al., 1991). Although exogenous application of ascorbic acid or any other non-enzymatic antioxidant are of particular interest if the underlying mode of action of endogenous molecules is fully known, nonetheless, any positive response of plants towards salinity tolerance is also important since it offers amelioration of salinity tolerance at the lab level with a possibility of extension to the field conditions for which the methods may be worked out in times to come.

During the present study, ascorbic acid pretreatment was not proven to improve salt tolerance of callus cultures rather it caused more browning and necrosis of the callus tissues. Not always does the ascorbic acid pretreatment result in improved salinity tolerance, Afzal et al. (2005) have also reported that ascorbic acid pretreatment did not improve the seedling growth of wheat under normal (4 dS/cm) or saline (15 dS/cm) conditions.

On the other hand, it was observed that the exogenous supply of ascorbic acid to *in vitro*-grown sugarcane plants improved their salt tolerance as indicated by the studied morphological parameters. There is no prior report of ascorbic acid pretreatment given to callus cultures or *in vitro* plants of sugarcane. Ascorbic acid pretreatment on other plant tissues/organs such as leaves, however, has been reported in some plants. For instance, application of ascorbic acid to leaves was reported to increase the concentration of ascorbic acid in plants (Mozafar and Oertli, 1993). This increased supply of ascorbic acid was shown to enhance the tolerance level of sunflower plants to acid mist that was attributed to a greater accumulation of compatible solutes (Gaddalah, 2000). Shalata and Neumann (2001) reported that the addition of 0.5 mM ascorbic acid to the root medium, prior to salt-treatment for 9 h, facilitated subsequent recovery and long-term survival of 50% of the wilted tomato seedlings. Similarly, Al- Hakimi and Hamada (2001) observed that soaking of wheat grains in ascorbic acid also counteract the adverse effects of NaCl salinity on the wheat seedlings. Thus, it was suggested by these workers that additional supply of ascorbic acid to seedlings might decrease the build-up of active oxygen species and thereby increase resistance to salt-stress. The role of exogenous application of ascorbic acid in wheat has been reported by Athar et al. (2008). It was observed by these workers that ascorbic acid application to wheat seedlings counteracted the adverse effects of salt stress on growth of wheat by improving photosynthetic capacity of wheat plants against salt-induced oxidative stress. The role of ascorbic acid in amelioration of salinity tolerance in potato has been observed by Sajid and Aftab (2009). It was found that pretreatment with ascorbic acid to both salt treated plants and callus cultures of potato showed significant differences with respect to almost all of the studied growth and biochemical parameters. Though the response of callus cultures of sugarcane as observed during the present work was different from potato callus cultures but the results for *in vitro*-grown sugarcane plants were in line with these previous studies.

Results of the present investigation also indicated that in addition to increased necrosis a decrease in fresh weight was also observed in both ascorbic acid pretreated as well as non-pretreated callus cultures maintained on various NaCl levels. The concentration of ascorbic acid used during the present experiment as selected on the basis of available literature (Shalata and Neumann, 2001) could have been toxic to sugarcane callus cultures. Potentially, the duration of ascorbic acid pretreatment given to callus cultures could have been yet another important factor. Although NaCl stress resulted in reduced fresh weights but ascorbic acid pretreatment prior to transferring the plants to NaCl-containing medium significantly increased the fresh weights of the plants as compared to the non-pretreated control plants maintained at the same salt level.

It was also observed during the present study that ascorbic acid pretreatment resulted in decreased soluble protein contents as well as antioxidant enzyme activities in callus cultures. Contrarily, ascorbic acid pretreatment to the *in vitro*-grown plants resulted in greater soluble protein contents as well as increased activities of antioxidant enzymes POD, CAT and SOD. Plants with higher levels of antioxidant enzymes have more salinity tolerance because of resistance to oxidative damage, as

already explained by many workers (Rahnama and Ebrahimzadeh, 2005; Kusvuran et al., 2007). On the basis of observed antioxidant enzyme activities, it is inferred that ascorbic acid pretreatment did not apparently improve salt tolerance capacity of sugarcane callus cultures but what it certainly did improve was stress tolerance of *in vitro* sugarcane plants. This aspect is intriguing and justifies further work on sugarcane plants under greenhouse conditions. Why does ascorbic acid pretreatment improve salinity tolerance in sugarcane plants grown *in vitro*? What are the reasons that the same treatments have resulted in quite contrary results in sugarcane callus cultures? Can these findings be extended to greenhouse or field conditions in times to come? These are amongst several questions that perhaps are partially unanswered at this point in time.

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