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Salt Stress-induced Responses in Growth and Metabolism in Callus Cultures and Differentiating In Vitro Shoots of Indian Ginseng (*Withania somnifera* Dunal)

Farzana Sabir · Rajender S. Sangwan · Ritesh Kumar · Neelam S. Sangwan

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Abstract In vitro-grown shoots and calli of *Withania somnifera*, an important medicinal plant, were exposed to various types of salts under in vitro culture conditions. Membrane permeability, lipid peroxidation, and the antioxidant system increased in shoots as well as in unorganized callus tissues under all the three concentrations of KCl, NaCl, KNO_3, $NaNO_3$, and $CaCl_2$. The growth responses of shoots and callus cultures under various salt treatments revealed that the tissue could grow better under NaCl and KNO_3 compared to other salts and the in vitro shoots appeared healthy at 50 mM concentration of NaCl and KNO_3. The activity of antioxidant enzymes such as catalase (CAT), ascorbate peroxidase, guaiacol peroxidase, lipoxygenase, polyphenol oxidase, and glutathione reductase increased under salt treatments, especially at higher concentrations. The greatest activity increase was recorded for peroxidases, whereas CAT was the least responsive. Only two isoforms, Mn-superoxide dismutase (Mn-SOD) and Fe-SOD, could be visualized in callus tissue while Cu/Zn-SOD was absent. Diaphorase 4 was totally missing in callus tissue and was detected only in shoots. Phenolics accumulated at all the concentrations of the salts tested as an induced protective response. The higher concentration of $CaCl_2$ produced maximum increases in antioxidants and enzymatic activities compared to other salts. Thus, for *W. somnifera* the presence of excess calcium in the growing medium is most deleterious compared to other salts. Results also suggest that the nonenzymatic and enzymatic antioxidant systems of both the tissues played a primary role in combating the imposed salt stress.

Keywords Antioxidant enzymes · Ashwagandha · Calcium chloride · Indian ginseng · In vitro callus and shoot cultures · Sodium chloride and sodium nitrate · Potassium nitrate

Introduction

Plants encounter several abiotic and biotic stresses in their natural surroundings. The abiotic stresses such as low temperature, drought, and high salinity are common unfavorable conditions that adversely affect plant growth and crop production. Soil salinity is a major constraint to crop production because it limits crop yield and restricts use of land previously uncultivated. Plants respond to salinity stress by inducing several morphological, physiological, and metabolic responses (Zhao and others 2009). Salt stress is caused by various ions, mainly Na^+ and Cl^-, which can be transported into and out of cells. Salinity is also expressed by oxidative stress (Zhu 2001; Panda and Upadhyay 2003) as rapid and transiently active reactive oxygen species (ROS) such as the superoxide radical, hydroxyl radical, and singlet oxygen, which accumulate under both types of stresses. ROS interact with a wide range of molecules causing a series of responses, including pigment oxidation, lipid peroxidation, membrane destruction, protein denaturation, and DNA mutation (Mittler 2002). To mitigate and repair the damage initiated by

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F. Sabir · R. S. Sangwan · R. Kumar · N. S. Sangwan (✉)
Central Institute of Medicinal and Aromatic Plants (CIMAP-CSIR), P.O. CIMAP, Lucknow 226015, India
e-mail: nss.cimap@gmail.com

various ROS, plants have evolved specific protective mechanisms of antioxidants. Antioxidants can be divided into two classes: the low-molecular-mass nonenzymatic free radical scavengers and enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). SOD is a major scavenger of O_2^- and its enzymatic action results in the formation of H_2O_2 and O_2 (Wang and others 2004). POD decomposes H_2O_2 by oxidation of cosubstrates such as phenolic compounds and/or antioxidants, whereas CAT breaks down H_2O_2 into water and molecular oxygen (Mittler 2002). Plants grown under salinity also accumulate compatible solutes such as proline to function as osmotic buffers lowering the cellular osmotic potential to sustain water absorption from saline solutions and to restore intracellular ion homeostasis (Zhu 2001); they also exhibit ROS scavenging properties although the underlying mechanism is not yet clear (Xiong and Zhu 2002). Apart from these responses, salinized plants also display morphological and developmental adjustments (Sam and others 2003).

Withania somnifera is a very important plant of the traditional Indian system of medicine and has recently gained a lot of attention the world over because of its protective role in neurodegeneration and repair of synapses (Sangwan and others 2004). Optimized agricultural practices for this plant are available but the management and responses of *W. somnifera* under salt stress conditions are not known. As the plant is hardy and reported to grow under various kinds of agroclimatic regions, it will be useful to know if the plant is able to tolerate various salts as well. Tissue culture techniques are unique tools for studying the physiological effects of salt at the cellular level under a controlled environment and for studying plant responses to salt stress on a smaller scale with clear visual symptoms. The in vitro cultures from plants have been shown to be effective in showing responses to stress, even at different stages of development (Zhao and others 2009). Salt-tolerant lines of several plants have been isolated using in vitro techniques and their decreased regeneration potential with increasing salt levels has been noticed (Vijayan and others 2003; Woodward and Bennet 2005; Zhao and others 2009). Thus, in vitro cultures could provide a convenient and alternative way to test the salinity responses under controlled conditions. The purpose of the present study was to study the physiological and biochemical responses of *W. somnifera* to different salts and to activate adaptive metabolic responses using cultures as a responding system. The in vitro-grown shoots and calli of Indian ginseng were the two independent and structurally different cellular systems used to analyze the growth and antioxidant and phytochemical responses under various salt stress treatments. The study will help understand how *W. somnifera* grows under specific salt treatments.

Materials and Methods

In Vitro Cultures and Salt Treatments

Actively growing callus and shoot cultures of *W. somnifera* were generated and maintained as reported earlier (Sabir and others 2008). The stress conditions were imposed by the addition of chlorides of sodium, potassium, and calcium (NaCl, KCl, and $CaCl_2$) and nitrates of sodium and potassium ($NaNO_3$ and KNO_3) to the media at different concentrations (50, 100, and 200 mM). In case of callus cultures, lower concentrations (25 mM, 50 mM and 100 mM) of salts were utilized for imposing stress conditions. All the analyses were performed in the control tissue as well as in the treated tissues. The graphical comparisons were performed using Sigma Plot 9.0 software (Sigma-Aldrich, St. Louis, MO, USA).

Measurements of Relative Water Content

Relative water content (RWC) of the control shoots as well as the shoots with the different salt treatments was estimated as earlier (Sangwan and others 1994). RWC was calculated by using the formula RWC (%) = (FM − DM)/TM − DM × 100.

Chlorophyll Estimation

Chlorophyll *a*, chlorophyll *b*, carotenoids and total chlorophyll were extracted and estimated from fresh leaves, following the method of Lichtenthaler and Wellburn (1983).

Proline Measurement, Reducing Sugar, and Phenolics Estimation

Proline content was determined according to the method of Bates and others (1973) and calculated using the formula (μg proline in extract/115.5)/g sample = μmol proline g^{-1} FW. Total reducing sugar and phenolics were estimated in the equivalent amounts of sample tissues from control and stressed tissues using the methods of Nelson (1944) and Ksouri and others (2007), respectively. The total phenolics of extracts were measured by using the Folin–Ciocalteu reagent method. Folin–Ciocalteu's phenol reagent (1.5 ml, 10% v/v) (Sigma) and 1.2 ml (7.5% w/v) Na_2CO_3 were added to 0.3 ml of sample extract. The reaction mixture was thoroughly mixed and incubated in the dark for 30 min and the absorbance was measured at 750 nm. The value of total phenolics was expressed in terms of mg gallic acid equivalents per mg fresh tissue. Total reducing sugars were extracted from the fresh tissues of control as well as treated tissues in 80% ethanol. Appropriate aliquots were taken from the supernatants and treated with Nelson's reagent to give a blue color which was read at 560 nm (Nelson 1944).

Determination of Malondialdehyde Concentration

MDA was measured according to the method of Dionisio-Sese and Tobita (1998) and the MDA concentration was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Antioxidant Enzyme Assay

The shoot and callus tissues were homogenized in equal volumes of 100 mM Na-phosphate buffer (pH 6.5) containing 5.0% (w/w) insoluble polyvinylpolypyrrolidone and the homogenates were centrifuged at 12,000*g* for 30 min at 4°C. The supernatants obtained were used for enzyme assays. Protein content of the extracts was determined according to Lowry and others (1951) using bovine serum albumin as a standard. Catalase (CAT, EC 1.11.1.6) and peroxidase (POD, EC 1.11.1.7) were assayed by using the method of Chance and Maehly (1955). The specific activity of ascorbate peroxidase (Asc-POD, EC1.11.1.11) was determined by following the method of Nakano and Asada (1981), and polyphenol oxidase (PPO) activity was assayed by the method of Kumar and Khan (1982). Glutathione reductase (GR, EC 1.6.4.2) was assayed following the method of Smith and others (1988).

Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) of the control and treated shoots and callus cultures was performed under nondenaturing conditions using a Mini-Protean III electrophoresis cell (Bio-Rad, Hercules, CA, USA) at 4°C. Staining of isozymes was performed for POD (Shimoni 1994) and SOD (Beauchamp and Fridovich 1971). Amyl esterase (EST), glutamate oxalo-acetate transaminase [GOT (EC 2.6.1.1)], and diaphorase [DIA (EC 1.6.4.3)] were also analyzed in control and salt-treated tissues. The gel was stained for EST isoform and DIA isoforms (Sangwan and others 2003).

Withanolide Extraction

Withanolide extraction from the cultures and analysis of the extracts were performed as detailed earlier (Sangwan and others 2004; Sabir and others 2008).

Results and Discussion

Growth Parameters

Salt stress, like many other abiotic stresses, inhibits plant growth. Slower growth is an adaptive feature for plant survival under stress because it allows plants to rely on multiple resources (for example, building blocks and energy) to combat stress. Most of the salt treatments affected the growth of shoots (Supplementary Table 1; Fig. 1a) and calli negatively (Supplementary Table 2;

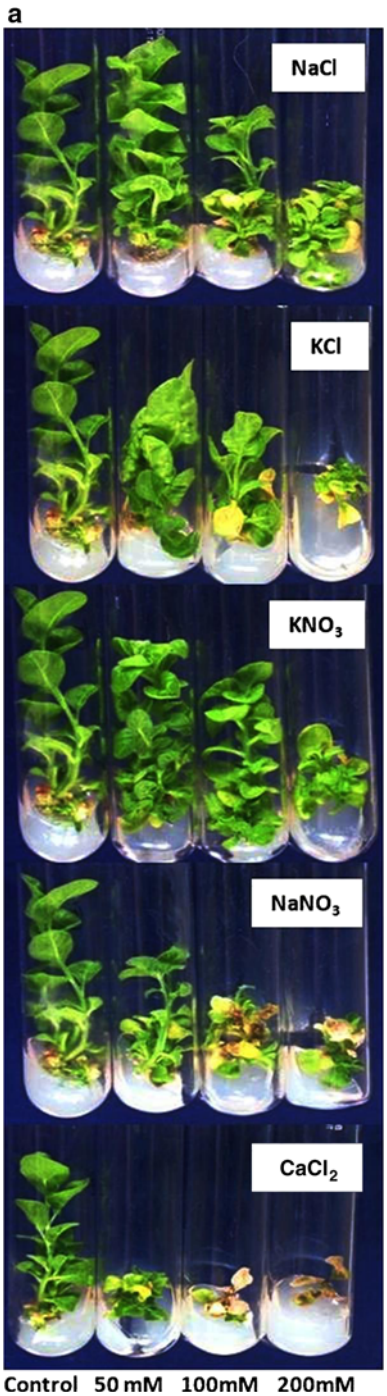

Fig. 1 **a** Effect of salts on the growth responses of in vitro grown shoots of *W. somnifera*. Salts used are NaCl, KCl, KNO_3, $NaNO_3$ and $CaCl_2$ at 50 mM, 100 mM, and 200 mM respectively. **b** Effect of salts on the growth of in vitro grown callus cultures of *W. somnifera*. Salts used are KNO_3, $NaNO_3$, NaCl, KCl, and $CaCl_2$ at 25 mM, 50 mM, 100 mM respectively

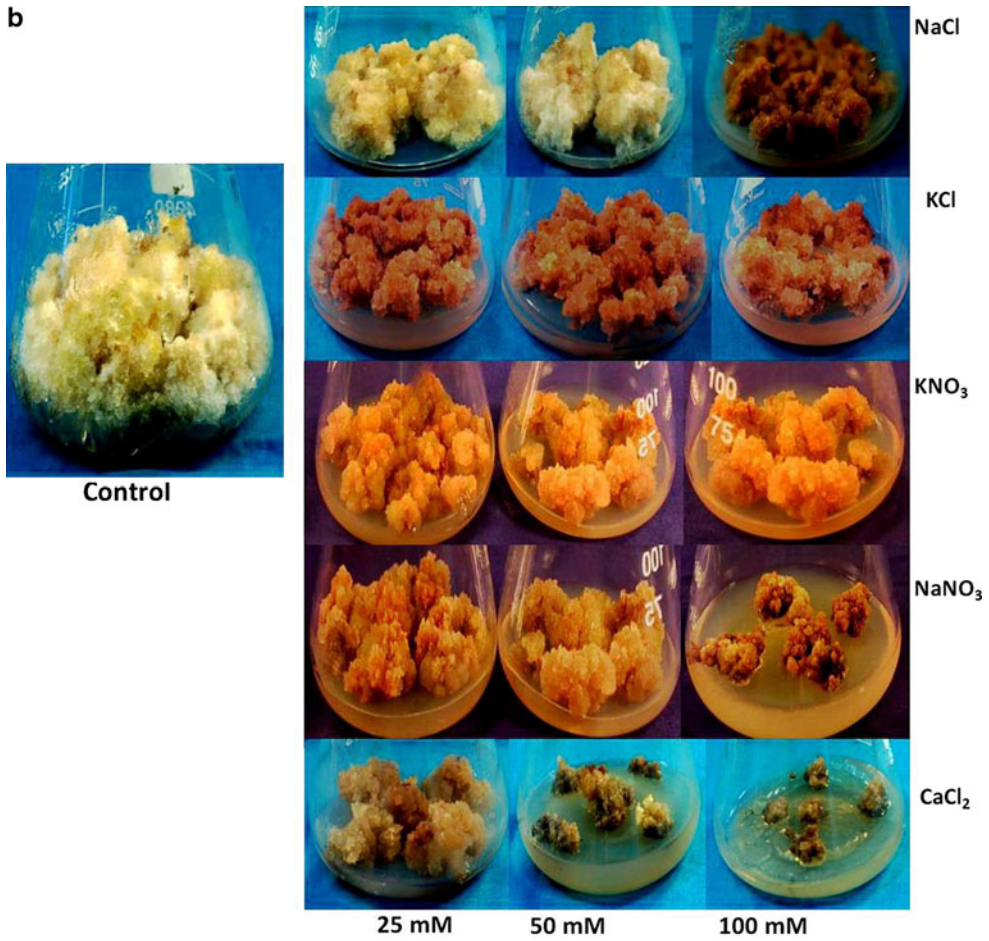

Fig. 1 continued

Fig. 1b). There was a marked change in the appearance of shoots and calli as well (Fig. 1a, b). Stressed shoots appeared chlorotic and stunted in growth and became necrotic at higher concentrations of salt, especially $CaCl_2$ which was the most damaging salt; beyond 100 mM the callus could not survive (Supplementary Table 2) and shoots also almost died (Fig. 1a). NaCl and KNO_3 caused the least damage. However, shoots supplemented with 50 mM concentration of NaCl, KCl, and KNO_3 were relatively greener, healthier, and bigger (Fig. 1a). All the salts at 200 mM caused the highest stress levels (Supplementary Table 1). Calli turned dark brown under NaCl and $NaNO_3$ treatments (Fig. 1b). The inhibition in dry weight accumulation of callus and shoot tissues was recorded more at the higher concentrations of both NaCl and $NaNO_3$ than other salt treatments. Callus tissue had more moisture content than shoots and thus exhibited a lower dry weight. Salt stress influences primarily the water content by making it less available to the cells. The molecular mechanism of this response is almost analogous to the water/drought and cold/chilling stress conditions. In all these traumatic circumstances, not enough water is available to the cells. Therefore, as the first response, the cell tries to save the available water by avoiding active growth. The chemical potential of the saline solution initially establishes a water potential imbalance between the apoplast and the symplast that leads to turgor decrease, which if severe enough can cause growth reduction (Sangwan and others 1994; Bohnert and others 1995). Growth comes to a halt when turgor is reduced below the threshold of the cell wall. Cellular dehydration begins when the water potential difference is greater than can be compensated for by turgor loss. This growth cessation finally leads to the low dry weight accumulation (Molassiotis and others 2006).

Relative Water Content, Chlorophyll Content, and Proline Content

Relative water content of the salt-treated shoots was found to decrease with increasing salt concentration (Table 1). The photosynthetic pigments such as chlorophyll *a*, *b*, total chlorophyll ($a + b$), and carotenoid content declined in the shoot cultures with increasing salt concentration (Table 1). The decrease was pronounced at the highest concentration

Table 1 Effect of salts on the growth responses, biochemical parameters, and antioxidant activities in in-vitro-grown shoots of *W. somnifera*

Treatment parameters	Control	NaCl			KCl			KNO_3		
		50 mM	100 mM	200 mM	50 mM	100 mM	200 mM	50 mM	100 mM	200 mM
Chlorophyll *a* (mg/g FW)	2.21 ± 0.055	1.47 ± 0.093	0.96 ± 0.026	0.48 ± 0.028	1.53 ± 0.038	1.05 ± 0.035	0.31 ± 0.055	1.68 ± 0.040	1.55 ± 0.040	0.92 ± 0.043
Chlorophyll *b* (mg/g FW)	1.30 ± 0.020	0.58 ± 0.023	0.40 ± 0.012	0.27 ± 0.0150	0.64 ± 0.030	0.44 ± 0.020	0.17 ± 0.018	0.68 ± 0.047	0.64 ± 0.022	0.42 ± 0.015
Total chlorophyll (mg/g FW)	3.51 ± 0.15	2.05 ± 0.1500	1.36 ± 0.050	0.75 ± 0.060	2.17 ± 0.060	1.49 ± 0.047	0.48 ± 0.074	2.36 ± 0.0500	2.19 ± 0.047	1.34 ± 0.064
Total carotenoid (mg/g FW)	0.58 ± 0.023	0.52 ± 0.012	0.38 ± 0.018	0.25 ± 0.011	0.57 ± 0.022	0.49 ± 0.028	0.19 ± 0.017	0.53 ± 0.025	0.52 ± 0.032	0.38 ± 0.022
%RWC	95.3 ± 4.20	90.5 ± 4.0	85.1 ± 3.2	58.2 ± 2.1	89.1 ± 3.0	82.3 ± 3.2	72.2 ± 2.0	91.1 ± 4.5	87.2 ± 4.4	74.3 ± 3.3
Total reducing sugar (mg/g FW)	0.74 ± 0.072	0.71 ± 0.067	0.84 ± 0.083	1.5 ± 0.074	0.51 ± 0.045	0.58 ± 0.042	0.67 ± 0.066	0.52 ± 0.067	0.67 ± 0.088	1.12 ± 0.15
Total phenolics (mg/g FW)	0.34 ± 0.066	0.33 ± 0.043	0.38 ± 0.053	0.68 ± 0.062	0.31 ± 0.023	0.35 ± 0.072	0.34 ± 0.046	0.12 ± 0.045	0.16 ± 0.041	0.30 ± 0.047
Total withanolide (mg/g DW)	7.7 ± 0.23	8.6 ± 0.34	5.06 ± 0.15	3.06 ± 0.10	5.98 ± 0.14	6.94 ± 0.23	5.1 ± 0.12	6.98 ± 0.34	3.32 ± 0.10	3.38 ± 0.098
Proline (μmol proline/g FW)	1.05 ± 0.045	1.72 ± 0.038	1.86 ± 0.036	2.03 ± 0.047	1.98 ± 0.05	2.07 ± 0.047	2.06 ± 0.051	0.96 ± 0.08	1.67 ± 0.078	1.88 ± 0.070
CAT (U/mg protein)	0.0071 ± 0.00015	0.0061 ± 0.00045	0.0074 ± 0.00036	0.011 ± 0.00052	0.0068 ± 0.00032	0.0077 ± 0.00028	0.0075 ± 0.00035	0.0045 ± 0.0002	0.005 ± 0.0002	0.0017 ± 0.00015
G-POD (U/mg protein)	0.068 ± 0.016	0.071 ± 0.018	0.16 ± 0.032	0.71 ± 0.013	0.31 ± 0.021	0.32 ± 0.012	0.27 ± 0.033	0.068 ± 0.0083	0.086 ± 0.01	0.071 ± 0.0088
ASc-POD (U/mg protein)	0.28 ± 0.020	0.23 ± 0.040	0.40 ± 0.026	0.72 ± 0.030	0.48 ± 0.025	0.52 ± 0.023	0.53 ± 0.036	0.32 ± 0.0280	0.36 ± 0.046	0.35 ± 0.036
PPO (U/mg Protein)	0.031 ± 0.011	0.078 ± 0.0076	0.12 ± 0.0156	0.24 ± 0.0087	0.11 ± 0.0058	0.17 ± 0.015	0.23 ± 0.014	0.057 ± 0.011	0.094 ± 0.017	0.12 ± 0.011
LOX (U/mg protein)	0.037 ± 0.004	0.076 ± 0.002	0.097 ± 0.005	0.11 ± 0.005	0.071 ± 0.0034	0.085 ± 0.004	0.091 ± 0.0031	0.018 ± 0.0011	0.031 ± 0.0043	0.044 ± 0.008
GR (U/mg protein)	0.033 ± 0.0030	0.035 ± 0.0057	0.055 ± 0.0030	0.11 ± 0.0088	0.026 ± 0.0045	0.043 ± 0.0038	0.081 ± 0.0044	0.044 ± 0.0022	0.053 ± 0.0027	0.071 ± 0.0033

Treatment parameters	Control	$NaNO_3$			$CaCl_2$		
		50 mM	100 mM	200 mM	50 mM	100 mM	200 mM
Chlorophyll *a* (mg/g FW)	2.21 ± 0.055	2.04 ± 0.040	0.87 ± 0.030	0.58 ± 0.025	0.66 ± 0.080	0.56 ± 0.060	0.13 ± 0.020
Chlorophyll *b* (mg/g FW)	1.30 ± 0.020	0.793 ± 0.040	0.38 ± 0.015	0.28 ± 0.032	0.29 ± 0.047	0.26 ± 0.014	0.112 ± 0.010
Total chlorophyll (mg/g FW)	3.51 ± 0.15	2.83 ± 0.081	1.26 ± 0.040	0.87 ± 0.042	0.95 ± 0.061	0.82 ± 0.050	0.23 ± 0.056
Total carotenoid (mg/g FW)	0.58 ± 0.023	0.57 ± 0.018	0.36 ± 0.027	0.28 ± 0.020	0.34 ± .012	0.33 ± 0.009	0.12 ± 0.015
%RWC	95.3 ± 4.20	86.3 ± 2.1	78.1 ± 3.3	48.6 ± 2.2	78.3 ± 2.1	68.4 ± 1.5	62.2 ± 2.3
Total reducing sugar (mg/g FW)	0.74 ± 0.072	0.97 ± 0.10	1.54 ± 0.05	1.64 ± 0.063	1.1 ± 0.12	1.18 ± 0.058	1.94 ± 0.072
Total phenolics (mg/g FW)	0.34 ± 0.066	0.32 ± 0.078	0.34 ± 0.051	0.82 ± 0.05	0.54 ± 0.043	0.70 ± 0.063	1.57 ± 0.083
Total withanolide (mg/g DW)	7.7 ± 0.23	4.41 ± 0.45	4.35 ± 0.38	1.71 ± 0.30	5.46 ± 0.34	traces	Traces
Proline (μmol proline/g FW)	1.05 ± 0.045	1.8 ± 0.085	1.86 ± 0.094	2.02 ± 0.08	1.96 ± 0.074	1.81 ± 0.064	1.78 ± 0.045

Table 1 continued

Treatment parameters	Control	$NaNO_3$			$CaCl_2$		
		50 mM	100 mM	200 mM	50 mM	100 mM	200 mM
CAT (U/mg protein)	0.0071 ± 0.00015	0.0051 ± 0.00035	0.0077 ± 0.0002	0.0098 ± 0.0006	0.0038 ± 0.0005	0.0047 ± 0.00023	0.0068 ± 0.00046
G-POD (U/mg protein)	0.068 ± 0.016	0.067 ± 0.028	0.11 ± 0.026	0.56 ± 0.018	0.26 ± 0.0083	0.31 ± 0.016	0.42 ± 0.024
ASc-POD (U/mg protein)	0.28 ± 0.020	0.28 ± 0.0400	0.32 ± 0.032	0.50 ± 0.045	0.11 ± 0.0134	0.31 ± 0.026	0.58 ± 0.028
PPO (U/mg Protein)	0.031 ± 0.011	0.087 ± 0.012	0.11 ± 0.011	0.13 ± 0.016	0.068 ± 0.0048	0.13 ± 0.0087	0.40 ± 0.011
LOX (U/mg protein)	0.037 ± 0.004	0.056 ± 0.0021	0.07 ± 0.0032	0.10 ± 0.0024	0.11 ± 0.0023	0.13 ± 0.0041	0.16 ± 0.003
GR (U/mg protein)	0.033 ± 0.0030	0.045 ± 0.0055	0.075 ± 0.0032	0.034 ± 0.0055	0.051 ± 0.0055	0.071 ± 0.0076	0.17 ± 0.0081

The data presented are mean ± SE of ten replicates obtained from two independent experiments

of all salts (200 mM). The most pronounced effect was observed with KCl, which retarded the accumulation of all the pigments, whereas $CaCl_2$ at its lowest concentration (50 mM) exerted a similar effect. The chlorophyll content is directly related to the growth and productivity of the plant. The resulting decrease in chlorophyll pigments under high salt concentration treatment might be the consequence of interference of salt concentration in chlorophyll synthesis, which in turn depends on sufficient iron balance. The presence of excess salt ions creates an imbalance in ion homeostasis which possibly restrains the iron to the protoprophyrin molecule resulting in decrease in the synthesis of chlorophyll (Agastian and others 2000). Insufficient chlorophyll content might lead to the malfunction of the photosystem under stress conditions and could increase leakage of electrons to O_2, resulting in a decline in total CO_2 fixation (Woodward and Bennett 2005; Molassiotis and others 2006).

Enhanced accumulation of proline in shoots treated with salts at all concentrations was observed. A noticeable increase in the proline content at even the lowest concentration (50 mM) was observed in treated tissue compared to the control (Table 1). In the case of calli, the accumulation of proline did not increase significantly compared to the control except at a higher concentration of $CaCl_2$, where there was an abrupt increase in the proline accumulation. With all the salt treatments, shoots exhibited a higher accumulation of proline compared to calli (Table 2). Plants have evolved diverse strategies of acclimatization and avoidance to cope with adverse environmental conditions. Interestingly, among various compatible solutes there are many reports of enhanced proline accumulation during stress conditions (Woodward and Bennett 2005; Molassiotis and others 2006). In the literature, the role of proline in stressed plants is often explained by its property as an osmolyte and its ability to balance water stress (Sangwan and others 1994). The first response of cells during salt stress is the imbalance of osmotic potential due to excess salt. To adjust the osmotic potential of the cell, a suitable osmolyte or osmoprotectant is required, which accumulates in the cytosol. Proline acts as a suitable osmoprotectant and accumulates in high concentrations without disturbing the intracellular milieu and attempts to preserve the activity of enzymes (Bohnert and others 1995). Proline has also been shown to protect plants against singlet oxygen- and free radical-induced damage (Alia and others 1991). Due to its action as a singlet-oxygen quencher and scavenger of $OH^{\bullet}$ radicals, proline is able to stabilize protein, DNA, and membranes. Accumulation of proline-rich proteins, and particularly proline residues in proteins, provides additional protection against oxidative stress. In addition, other possible positive roles of proline under stress have also been proposed, including regulation of cytosolic pH and of the

NAD/NADH ratio (Alia and others 1991), and it stores energy that can be rapidly broken down and used when the plant is relieved of stress (Hare and others 1998). Also, proline may play a role in flowering and development as both a metabolite and a signal molecule. Although there is a growing consensus that proline has special importance throughout the reproductive phase (from flower transition to seed development), a general agreement on the molecular and genetic mechanisms in which proline is involved is though yet to be established (Mattioli and others 2009).

Lipid Peroxidation

Lipid peroxidation was expressed as the level of malondialdehyde (MDA) content, which is a decomposition product of polyunsaturated fatty acid (PUFA), often considered a reflection of lipid peroxidation. In the present experiment, shoots and calli of *W. somnifera* treated with different salts showed a definite pattern of increase in the MDA content with increasing salt concentration (Tables 1, 2). KNO_3 showed the least peroxidation of lipids, whereas $CaCl_2$ exhibited the highest peroxidation values in both shoot and callus tissues (Tables 1, 2). These observations are in agreement with the hypothesis that the amount of MDA content is a direct sign of oxidative stress caused by damage to the lipid molecule of the cell membrane (Erturk and others 2007). An increase in lipid peroxidation was also observed in suspension culture *C. roseus* under salt stress (Elkahoui and others 2005). Lipid peroxidation in cells can be initiated enzymatically and nonenzymatically by the lipoxygenase enzyme and free radicals of ROS, respectively (Shewfelt and Purvis 1995; Elkahoui and others 2005).

Total Reducing Sugar and Phenolics Content

Polyphenol and total reducing sugar content increased under salt stress conditions in both shoots and undifferentiated calli (Tables 1, 2). In calli, total reducing sugar content was very low; however, its accumulation increased at higher concentrations of salts, especially NaCl and $NaNO_3$, whereas KNO_3 resulted in the least accumulation of total reducing sugar (Tables 1, 2). This increase might be due to the adjustment of cells to maintain their osmoticum, which was perturbed under salt stress. Sugar is also considered an osmoprotectant and reported to accumulate during salinity (Kovacik and others 2009). Soluble sugars (namely, sucrose, glucose, and fructose) that are altered by water deficit and salinity may also act as signaling molecules under stress (Chaves and Oliveira 2004) and also interact with hormones as part of the sugar-sensing and signaling network in plants. Phenolic compounds constitute a large group of organic compounds that are widely distributed in plants and exhibit a broad spectrum of biological activities (Balasundram and others 2006).

In the present study, phenolic content varied from the undifferentiated calli cells to the organized shoot tissues (Tables 1, 2). Shoots contained more phenolics than calli and further accumulated more phenolics than callus tissue under various salt treatments. Under KNO_3 treatment, both shoots and calli had a much higher accumulation of total phenolics. This finding is in agreement with previous reports (Agastian and others 2000; Muthukumarasamy and others 2000; Ksouri and others 2007). It is believed that the degree of oxidative cellular damage in plants exposed to stress is controlled by the ability of the plant to protect against oxidative agents (Oueslati and others 2009). Salt tolerance appears to be favored by an enhanced antioxidative capacity to detoxify ROS (Bohnert and others 1995; Kwon and others 2002). Plants vary widely in their phenolic composition and content, with both genetics and environment affecting the type and level of phenolic compounds (De Abreu and Mazzafera 2005). Increased total phenolics content was observed with moderate saline levels in red peppers (Navarro and others 2006). Phenolic compounds exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Balasundram and others 2006). Phenolics have been regarded as ubiquitous secondary metabolites, believed to function in plants to regulate responses to stress (Lattanzio and others 2009).

Antioxidant Enzyme Activities

In shoot cultures, the activity of Asc-POD was the highest, followed by PPO and G-POD. GR and CAT were recorded as the least responsive enzymes under stress conditions in the shoots (Table 1). In callus tissue, G-POD had the highest activity, followed by Asc-POD (Table 2), and low activity was recorded for PPO and GR, with the lowest activity recorded for CAT. The undifferentiated tissue level of calli had higher activity of G-POD and Asc-POD than the shoot tissues. On the other hand, the PPO activity in shoots and callus cultures was almost equal in controls, but it showed an increase under salt treatments (Tables 1, 2).

The overall pattern of CAT enzyme showed a marked increase in activities in tissues treated with various salts. This increase was clearly distinct in callus tissue compared to shoots. Shoot tissue already had higher CAT activity in controls, whereas in calli this enzyme was barely present. CAT activity in callus and shoot tissues of controls was recorded as the lowest compared to that of other antioxidant enzymes (Tables 1, 2). The highest CAT activity was recorded under $CaCl_2$ treatment and the lowest with KNO_3 and $NaNO_3$.

Table 2 Effect of salts on the growth responses, biochemical parameters, and antioxidant activities in callus cultures of *W. somnifera*

Treatments parameters	Control	NaCl			KCl			KNO_3		
		25 mM	50 mM	100 mM	25 mM	50 mM	100 mM	25 mM	50 mM	100 mM
Total reducing sugar (mg/g FW)	0.14 ± 0.063	1.13 ± 0.12	1.63 ± 0.14	3.40 ± 0.23	0.28 ± 0.18	1.17 ± 0.095	1.64 ± 0.19	0.17 ± 0.085	0.18 ± 0.088	0.31 ± 0.077
Total phenolics (mg/g FW)	0.044 ± 0.010	0.18 ± 0.020	0.17 ± 0.017	0.60 ± 0.040	0.015 ± 0.0058	0.028 ± 0.0066	0.16 ± 0.012	0.035 ± 0.00090	0.064 ± 0.0096	0.70 ± 0.026
Proline (μmol/g FW)	0.167 ± 0.038	0.23 ± 0.031	0.27 ± 0.030	0.31 ± 0.058	0.15 ± 0.042	0.21 ± 0.048	0.22 ± 0.050	0.16 ± 0.036	0.164 ± 0.031	0.29 ± 0.071
CAT (U/mg protein)	0.00073 ± 0.0004	0.0022 ± 0.0003	0.0107 ± 0.001	0.013 ± 0.0005	0.0093 ± 0.0004	0.0103 ± 0.0009	0.0112 ± 0.0005	0.0027 ± 0.0003	0.0045 ± 0.0002	0.0050 ± 0.003
G-POD (U/mg protein)	2.08 ± 0.32	5.55 ± 0.41	11.60 ± 0.40	11.45 ± 0.37	3.83 ± 0.30	3.66 ± 0.35	4.86 ± 0.22	4.10 ± 0.41	5.04 ± 0.35	4.63 ± 0.54
ASc-POD (U/mg Protein)	0.81 ± 0.18	2.71 ± 0.30	3.62 ± 0.41	4.45 ± 0.30	1.72 ± 0.25	2.36 ± 0.13	2.68 ± 0.58	1.71 ± 0.17	1.20 ± 0.17	1.84 ± 0.28
PPO (U/mg protein)	0.06 ± 0.0010	0.058 ± 0.0015	0.062 ± 0.0010	0.063 ± 0.0011	0.053 ± 0.0013	0.075 ± 0.0011	0.068 ± 0.0010	0.055 ± 0.0020	0.042 ± 0.0022	0.043 ± 0.0021
LOX (U/mg protein)	0.064 ± 0.0060	0.068 ± 0.0045	0.090 ± 0.0060	0.146 ± 0.0040	0.074 ± 0.0060	0.069 ± 0.0070	0.083 ± 0.0051	0.054 ± 0.0072	0.067 ± 0.0063	0.081 ± 0.0090
GR (U/mg protein)	0.043 ± 0.0042	0.078 ± 0.0033	0.055 ± 0.0032	0.060 ± 0.0033	0.055 ± 0.0025	0.053 ± 0.0017	0.046 ± 0.0047	0.044 ± 0.0014	0.028 ± 0.0013	0.023 ± 0.0031

Treatments parameters	Control	$NaNO_3$			$CaCl_2$		
		25 mM	50 mM	100 mM	25 mM	50 mM	100 mM
Total reducing sugar (mg/g FW)	0.14 ± 0.063	0.24 ± 0.12	2.50 ± 0.097	5.08 ± 0.25	0.17 ± 0.091	1.00 ± 0.18	2.74 ± 0.28
Total phenolics (mg/g FW)	0.044 ± 0.010	0.014 ± 0.0083	0.038 ± 0.010	0.042 ± 0.022	0.033 ± 0.019	0.048 ± 0.015	0.17 ± 0.038
Proline (μmol/g FW)	0.167 ± 0.038	0.033 ± 0.018	0.049 ± 0.028	0.088 ± 0.033	0.17 ± 0.062	0.15 ± 0.048	2.16 ± 0.048
CAT (U/mg protein)	0.00073 ± 0.0004	0.0052 ± 0.0003	0.0054 ± 0.0006	0.0071 ± 0.0002	0.0065 ± 0.0006	0.0098 ± 0.0011	0.021 ± 0.001
G-POD (U/mg protein)	2.08 ± 0.32	6.76 ± 0.44	6.27 ± 0.16	5.35 ± 0.51	4.27 ± 0.28	4.40 ± 0.58	9.80 ± 0.16
ASc-POD (U/mg Protein)	0.81 ± 0.18	1.48 ± 0.13	1.67 ± 0.13	2.43 ± 0.18	3.93 ± 0.30	4.25 ± 0.32	7.54 ± 0.78
PPO (U/mg protein)	0.06 ± 0.0010	0.048 ± 0.0021	0.036 ± 0.0023	0.042 ± 0.002	0.07 ± 0.003	0.053 ± 0.0012	0.043 ± 0.0015
LOX (U/mg protein)	0.064 ± 0.0060	0.070 ± 0.0080	0.073 ± 0.0030	0.106 ± 0.0070	0.054 ± 0.0080	0.063 ± 0.0085	0.175 ± 0.0060
GR (U/mg protein)	0.043 ± 0.0042	0.045 ± 0.0021	0.041 ± 0.0014	0.024 ± 0.0025	0.068 ± 0.0035	0.058 ± 0.0028	0.097 ± 0.0040

Data presented are mean ± SE of ten replicates of two independent experiments

G-POD had the highest activity among all other antioxidant enzymes in callus tissues. There was a substantial increase in activity under NaCl and $CaCl_2$ treatment, whereas other salt treatments also influenced the activity positively from control tissue (Tables 1, 2). Shoot tissues exhibited lower activity than callus tissues, which also was increased around sixfold in the presence of NaCl and $NaNO_3$ (Tables 1, 2).

PPO activity in shoots and calli was almost equal to that in control tissue and was as high as 8 times greater in shoots compared to control in the extreme salt treatment, whereas in callus culture an indefinite pattern of PPO activity was observed under salt stress (Tables 1, 2). The increase of PPO in shoot cultures might be due to the increase in phenolic content accumulation in salt-stressed shoots.

In both tissues, GR activity levels were almost similar in untreated tissue and were stimulated under salt treatment in shoots (Tables 1, 2). The highest activity was observed in $CaCl_2$ treated shoots. Contrary to this, in callus culture an indefinite pattern of activity was recorded for different salt stress treatments at various concentrations. An elevated level of GR under salinity stress has been reported (Desing and others 2007). Increased GR activity in the root and leaf pairs of *C. roseus* under the stress of NH_4NO_3 and KNO_3 has also been reported (Elkahoui and others 2005).

Tissue-specific Isozyme Profiling

Isozymic profiling of SOD, G-POD, and G-POD, GOT, DIA, and EST enzymes was performed on native gel to distinguish the effect of various salt stresses on both shoot and callus tissues. Three isozymes of esterase (EST1, EST2, and EST3) were detected on gel. In the case of shoot cultures, EST1 isozymic activity appeared as a zone, whereas four distinct sub-isoforms could be detected in callus tissue. It is noticeable that a lower concentration of KCl-stressed callus responded altogether differently from the callus tissue. The EST3 band was clearly detected in the shoots but was faint and barely detectable in callus tissue (Fig. 2). Four isoforms of diaphorase (DIA1, DIA2, DIA3, and DIA4) were detected in tissues under various salt treatments. In shoot cultures, barely visible and very faint bands of DIA1 and DIA2 were observed, whereas the band was detected in callus tissue treated with higher concentrations of NaCl, KCl, and KNO_3. Whereas DIA2 was detected in all callus tissue samples, DIA3 was visible as a zone of isozymic activity and was expressed in almost similar concentrations in all shoot samples. DIA4 was totally missing in callus tissue and was detected in only some samples of shoots (Fig. 2).

Three isoforms of G-POD were visualized on native gel. In the shoots, G-POD3 was present in clusters of many sub-isoforms, whereas it remained a single band in callus tissue (Fig. 2). In shoots, the enzyme activity pattern was in agreement with the quantification assay of the enzymes. On the other hand, in callus tissue the intensity of G-POD1 and G-POD2, but not G-POD3, remained similar at all salt concentrations.

Three isozymes of SOD; Mn-SOD, Fe-SOD, and Cu/Zn-SOD1, were detected on native gel. The overall activity of the enzymes was higher in calli than in shoots on gel as evidenced by the brighter bands. Only Mn-SOD and Fe-SOD could be visualized whereas Cu/Zn-SOD was absent in callus tissue. This isoform is generally attributed to the chloroplast organelle (Wang and others 2004), which is missing or poorly developed in undifferentiated callus tissue. That may be the reason for the absence of this isoform of SOD in callus tissue. Chloroplasts generally contain Cu/Zn-SOD and, in a number of plant species, Fe-SOD (Kwon and others 2002). The chloroplastic Cu/Zn-SOD is associated with the thylakoid membrane, whereas Fe-SOD is associated with the chloroplastic stroma (Kwon and others 2002). The intensities of the Mn-SOD and Fe-SOD clusters remained similar in response to type and concentration of salt in shoots, whereas Cu/Zn SOD1 was detected only in salt-stressed shoots and was especially more intense with higher concentrations of $NaNO_3$. There are reports that support the minimal involvement of Mn-SOD in oxidative stress management as it remained similar to various stress conditions (Kwon and others 2002). On the other hand, changes in sub-isoforms of the Fe-SOD cluster were detected in calli. Five sub-isoforms were observed in calli in which bands of Fe-SOD5 were detected only under the $NaNO_3$ and $CaCl_2$ stress (Fig. 2).

Two isoforms of GOT; GOT1 and GOT2, were discernible in both shoot and callus tissue and had similar patterns. Both GOT1 and GOT2 were barely noticeable in KNO_3, $NaNO_3$, and $CaCl_2$ in both tissue levels, whereas these were clearly visible in KCl-treated calli (Fig. 2).

The overall route involved in coping with the salt stress in shoots seems to be connected with the chloroplast. Due to salinity, chlorophyll pigment synthesis is hindered which causes inefficient photosynthesis. Although in vitro shoots are not completely autotrophic for their food from photosynthesis, to some extent they are actively involved in the normal functioning of the chloroplast. An inadequate chloroplast mechanism generates more ROS, which remain tightly regulated in normal-functioning cells. This leads to disturbance in the equilibrium of ROS. The ROS O_2^- is catalyzed to H_2O_2 by SODs, a family of metalloenzymes that catalyze the dismutation of O_2^- to H_2O_2 by CAT, ascorbate peroxidase, and several PODs all catalyze the subsequent breakdown of H_2O_2 to water and oxygen, differing in their requirement for reaction conditions and compartments of cells. In shoots chloroplast H_2O_2 is

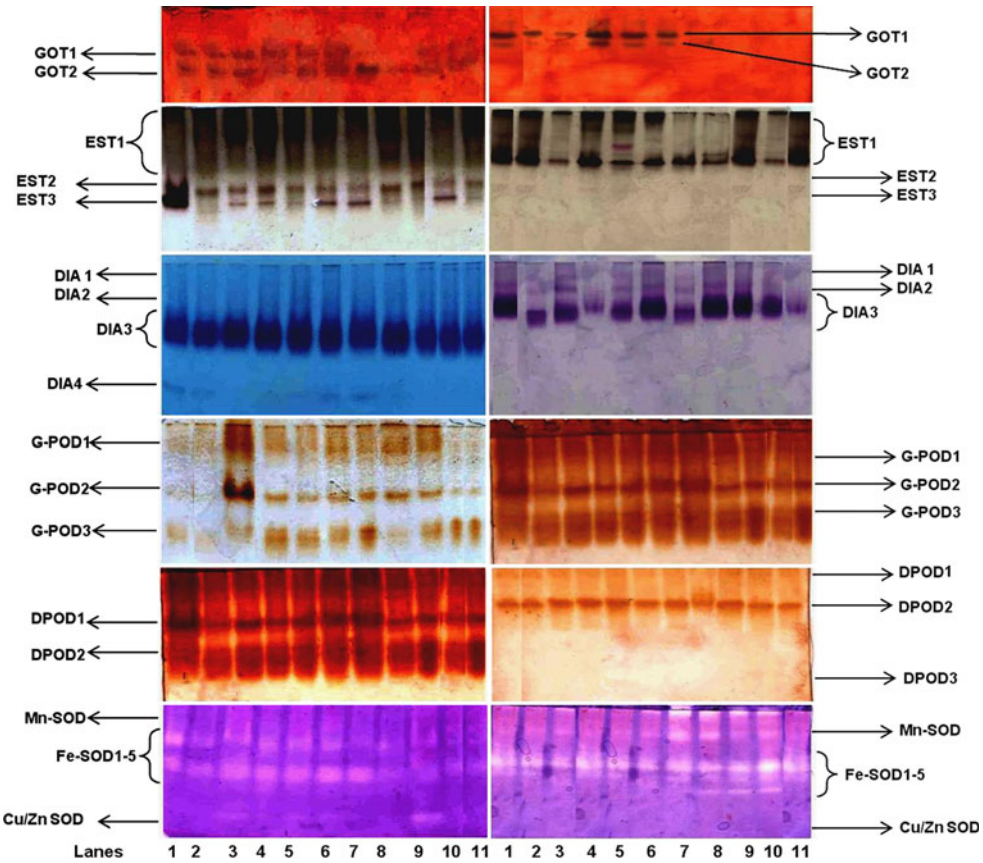

Fig. 2 Effect of salts on the enzyme activities of EST, DIA, GOT, G-POD, DPOD, and SOD of in vitro-grown shoot cultures (*left panels*) and callus cultures (*right panels*) of *W. somnifera*. Lanes in the *left panel*: Lane 1: Control; Lane 2: 100 mM NaCl; Lane 3: 200 mM NaCl; Lane 4: 100 mM KCl; Lane 5: 200 mM KCl; Lane 6: 100 mM KNO_3; Lane 7: 200 mM KNO_3; Lane 8: 100 mM $NaNO_3$; Lane 9: 200 mM $NaNO_3$; Lane10: 100 mM $CaCl_2$; Lane 11: 200 mM $CaCl_2$. and for callus cultures (*right panel*) of *W. somnifera*. Lanes in the ge1 lane: 1 Control; Lane 2: 50 mM NaCl; Lane 3: 100 mM NaCl; Lane 4: 50 mM KCl; Lane 5: 100 mM KCl; Lane 6: 50 mM KNO_3; Lane 7: 100 mM KNO_3; Lane 8: 50 mM $NaNO_3$; Lane 9: 100 mM $NaNO_3$; Lane10: 50 mM $CaCl_2$; Lane 11: 100 mM $CaCl_2$

reduced by POD using ascorbate as an electron donor. Oxidized ascorbate is then reduced by reactions that are catalyzed by GR (EC 1.6.4.2) in a series of reactions known as the Halliwell–Asada pathway. Results suggest that the conversion of O_2^- to H_2O_2 in the chloroplast is mediated by SODs, mainly Mn and Fe-SOD isoforms. Salinity induces the new isoform of Cu/Zn-SOD1 in shoot tissues. This dismutation of O_2^- to H_2O_2 in calli seemed to be related to Mn-SOD and Fe-SODs. This H_2O_2 then gets eliminated by CAT, POD, and Asc-POD depending upon the cellular compartments and conditions. In callus tissue, removal of H_2O_2 appeared to be highly associated with G-POD and then Asc-POD as the activity increased excessively in salt-stressed shoots. There is some involvement of CAT in the elimination of H_2O_2. The increase in Asc-POD activity left oxidized ascorbate, which finally was reduced by GR, detected as higher in salt-stressed shoots. In shoot cultures scavenging of H_2O_2 is not mainly due to CAT, except at higher concentrations of NaCl and $NaNO_3$. As a consequence of its sensitivity to environmental conditions, early loss of CAT was suggested to be a signal of antioxidant defenses. Involvement of Asc-POD in managing the salinity in shoots appears clearer and finally leads to the increase of GR (Fig. 2).

Withanolide Accumulation

Total withanolide content increased with 50 mM NaCl and declined with all other salt treatments (Table 1). Treatment with $NaNO_3$ at all concentrations resulted in the lowest accumulation of withanolide. The affects of KCl and KNO_3 on withanolide biosynthesis response was almost similar. An increase in secondary metabolites has been reported in the literature under various unfavorable circumstances (Cheng and others 2007). By being the most profuse and structurally diverse group of plant secondary metabolites, terpenoids play an important role in plant–insect, plant–pathogen, and plant–plant interactions (Cheng and others 2007). The physiological role of withanolides in the plant itself is still unknown, but there are several reports of involvement of secondary metabolites in defensive mechanisms. We also believe that the situation might be different when real plant–plant and plant–environment roles come into play, unlike the shoots and calli which remained under protected sterile conditions by having less plant–environment interaction. Under in vitro culture conditions with organized and unorganized tissue systems, the major role under stress conditions is played by cellular enzymatic and nonenzymatic antioxidant systems. The data from our

experiments show that various salt treatments provoked oxidative stress in calli and in vitro-grown shoots, as shown by the increase in lipid peroxidation, in spite of the induction of antioxidant enzymes. Increases in antioxidant activities and expression of specific new forms are also responses to the cellular damage provoked by NaCl. Probably, this increase in enzymatic and nonenzymatic antioxidants helped the tissue handle the deleterious effects of salt for the duration of the treatment and also reduced the severity of stress thus allowing cell growth to happen and tissues to live longer. The appreciable increase in total phenolics content under salt stress conditions indicates that this may be an inductive response under salt stress. Whereas withanolides have been shown to be associated with the membrane (Sangwan and others, unpublished data), the less prominent withanolide-related changes under salt stress may be suggestive of a protective and membrane stabilizing yet noninductive role for withanolide as a secondary metabolite in *W. somnifera* under in vitro salt stress conditions. Phenolics accumulated at all the concentrations of the salts tested as an inductive protective response of in vitro differentiated and undifferentiated tissue of *W. somnifera*. The increases in antioxidant enzyme activities were greatest in the presence of $CaCl_2$ compared to other salts. Thus, for *W. somnifera* the presence of excess calcium in the growing medium is most deleterious as revealed by the culture studies. *W. somnifera* could grow better in the presence of NaCl and KNO_3, whereas the presence of $CaCl_2$ is most undesirable for growth. The studies presented here provide an impetus for conducting growth adaptability responses of *W. somnifera* under field conditions in the presence of different salts.

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