

Effect of antioxidant protection system on regeneration potential of different chemotypes of *Withania somnifera* (L.) Dunal — A comparative analysis

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Received 11 September 2014; revised 02 June 2015

Withania somnifera (L.) Dunal, commonly called Indian Ginseng (*Ashwagandha*), is an important medicinal plant having a number of chemotypes which vary in their regenerative potentiality. In the present study, regenerative potentiality of four chemotypes of *W. somnifera* was compared using leaf explants. The study correlated that the chemotype (NIMTLI-130) with high total phenolic content (TPC), high H₂O₂ concentration and low proline content was less regenerative, whereas, the chemotype (NIMTLI-101) with lesser TPC, lower H₂O₂ concentration and high proline content was more regenerative. NIMTLI-130 showed higher SOD, GPX and APX activity compared to NIMTLI-101, but less CAT activity as well as low proline content and a weak detoxification effect resulting in lower regeneration. The study revealed that the difference in *in vitro* shoot organogenesis of different chemotypes largely depends on the antioxidant protection system of the plant involving non-enzymatic compounds and activities of antioxidant enzymes.

Keywords Antioxidant enzyme activity, Ashwagandha, Indian Ginseng, Proline content, Regeneration potential, Shoot organogenesis, Total phenolic contents

Withania somnifera (L.) Dunal (Fam. Solanaceae), commonly known as Indian Ginseng, locally, *Ashwagandha*, is an important medicinal plant used in Ayurvedic, Siddha and Unani Systems of medicine¹. Leaves and roots of this plant are widely utilized in the preparations of various herbal drugs for its biological activities, such as antibacterial, anti-inflammatory, antioxidative; and properties *viz.*, adaptogenic, anticonvulsant, antidiabetic, antiosteoporotic, antiproliferative, antistress, antitumor, cardioprotective, diuretic, hepatoprotective, hypocholesterolemic, hypoglycemic, immunomodulatory, radiosensitizing, thyroid stimulant, etc.²⁻⁵. The medicinal potential of the plant has been studied extensively against Alzheimer's disease and other central nervous system (CNS) related disorders, such as anxiety, catalepsy, depression, epilepsy and sleep⁶. The medicinal value of *W. somnifera* could be attributed to different bioactive molecules synthesized by them via mevalonate (MVA) (also called isoprenoid) pathway and non-mevalonate pathway (MEP)³.

In recent years, there has been a growing interest in the functional significance of reactive oxygen species (ROS) and the antioxidant response in growth, development and differentiation of plant cells⁷. The production of ROS has been associated with plant's recalcitrance towards regeneration during *in vitro* culture. The leaves, fruits and roots of *W. somnifera* possess antioxidant properties. Different chemotypes of *W. somnifera* differ in their regenerative potential. Therefore, it was proper to explore and correlate antioxidant enzyme activity, various compounds of antioxidant protection system with the regenerative potential of different chemotypes. Antioxidant protection involves nonenzymatic compounds such as carotenoids, ascorbic acid, α -tocopherol, glutathione, cysteine, phenolics and flavonoids and a number of enzymes including superoxide dismutase (SOD), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR)⁸. The amino acid proline is also a potent scavenger and balances the generation and elimination of ROS⁹. Proline has a direct positive effect on antioxidant enzyme system of the plants¹⁰. SOD is believed to play a crucial role in the antioxidant systems as it catalyses the dismutation of O₂⁻ into H₂O₂ and O₂¹¹. The conversion of H₂O₂ into water is carried

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out in cytosol and chloroplasts by ascorbate-glutathione cycle, which involves APX and GR¹². Whereas, GPX participate in a wide range of physiological processes including detoxification of H₂O₂, cell elongation, cell wall construction, plant responses to various stresses and an important role in cell growth and plant development¹³. Catalase enzyme plays an important role in acquisition of tolerance to oxidative stress in the adaptive response of cells by removal of electrons that can lead to the production of O₂⁻ free radical¹⁴. The antioxidant enzyme activities have been used as a parameter to evaluate efficiency of micropropagation protocol of saffron¹⁵. Reduced activity of antioxidant machinery has already been correlated with suppression of totipotency in plant protoplasts by Papadakis *et al.*¹⁵.

Being a member of Solanaceae, regeneration in *W. somnifera* is comparatively easy but different chemotypes differ in their withanolide content and also the regeneration potential.

An association between withanolide production and shoot organogenesis by *in vitro* cultures of *W. somnifera* has been reported by Sharada *et al.*¹⁶. Further, Jain *et al.*¹⁷ have shown a positive correlation between withanolide synthesis and morphological differentiation suggesting the synthesis was regulated in a tissue-specific way and organogenesis was the key regulatory factor which stimulates production of withanolides *in vitro*. The detection of higher content in differentiated cultures also points out that the enzymes responsible for biogenesis of withanolides *in vitro* might be optimally active under culture conditions. In the present study, an effort has been made to correlate the antioxidant enzyme activity, H₂O₂ concentration, TPC and proline content with the regeneration potential of four important chemotypes of *W. somnifera*.

Materials and Methods

Organogenesis in leaf segments

Four chemotypes of *Withania somnifera*, namely, NIMTLI-135, NIMTLI-130, NIMTLI-108 and NIMTLI-101, being maintained at CSIR-National Botanical Research Institute, Lucknow, were used for comparison of regenerative potential. Fully expanded leaves were excised from *in vitro* multiple shoots of all the chemotypes growing in Murashige and Skoog's¹⁸ basal medium supplemented with 0.5 mg/L benzyladenine (BA) and 0.25 mg/L indole-3-acetic acid (IAA). For organogenesis in leaf segments, kinetin (Kn) or BA (0.5, 1.0, 1.5 and 2.0 mg/L) along

with IAA (1.0 mg/L) were used as the growth regulators. The pH of all the media was adjusted to 5.8 before adding agar and autoclaved at 121°C for 20 min. All the media were solidified with 0.8% (w/v) agar. Cultures were incubated at 25 ± 2°C under 3 klux light through fluorescent tubes for 16-h light: 8-h dark photocycle at a light intensity of 50-60 μmol m⁻² s⁻¹.

Total phenolics

The contents of total phenolics were measured in leaf extracts of different chemotypes of *W. somnifera* collected from *in vitro* multiple shoots, according to Folin-Ciocalteu method¹⁹ following the preparation of the calibration curve, where 24, 75, 105 and 300 mg/L ethanolic gallic acid solution was mixed with 5 mL Folin-Ciocalteu reagent (diluted 10 fold) and 4 mL sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1 h at 20°C and the calibration curve was drawn. To the same reagent (Folin-Ciocalteu reagent & sodium carbonate) 1 mL of methanolic plant extract (10 mg/L) was mixed as described above and after 1 h the absorbance was measured. All determinations were performed in triplicates. Total phenolic contents in plant's methanolic extracts in Gallic Acid Equivalents (GAE) were measured by the formula:

$$C = c.V/m$$

where, C is total phenolic contents (mg/g of plant extract) in GAE; c, concentration of gallic acid deduced from the calibration curve (mg/L); V, volume of extract (1 mL); and m, dry weight (DW) of the plant material.

Measurement of H₂O₂

In vitro grown leaves of all the four chemotypes of *W. somnifera* were collected and used for measuring hydrogen peroxide content. The H₂O₂ content of the leaves was measured spectrophotometrically after reaction with potassium iodide (KI)²⁰. The leaves (500 mg FW) were crushed in 0.5 mL of 10³ mg/L trichloroacetic acid, 0.5 mL of 100 mM potassium phosphate buffer (pH 7.8) and 2 mL reagent i.e., 10³ mM KI, in fresh double-distilled water. After 1 h of incubation in the dark, the extract was centrifuged at 5000 rpm for 2 min. The absorbance of the supernatant was measured at 390 nm from the spectrophotometer. The blank consisted of 10³ mg/L TCA in the absence of leaf extract. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H₂O₂.

Proline assay

Proline was extracted according to the protocol of Singh *et al.*²¹. Leaf samples (100 mg) of different chemotypes of *W. somnifera* were ground in pure ethanol and placed into water bath at 95°C for 20 min. After incubation, samples were centrifuged at 14000 rpm for 5 min and the supernatant was collected into fresh tubes containing the soluble proline. A reaction mixture was prepared with ninhydrin 1% (w/v), acetic acid 60% (v/v) and ethanol 20% (v/v) protected from light. For proline estimation, 1 mL of reaction mixture was added with 0.5 mL of ethanol extract, mixed and heated at 95°C in the water bath for 20 min and centrifuged at 10000 rpm for 1 min. Supernatants were transferred into fresh tubes and absorbance was measured at 520 nm. For standard curve, proline solutions ranging from 0.04-1.0 mM were used.

Enzyme extraction

Fresh leaf (500 mg) samples of different chemotypes of *W. somnifera* were collected from *in vitro* multiple shoots and extracted for enzyme activity in 10 mM potassium phosphate buffer (pH 7.8), containing 1% PVP (w/v) and 0.5% triton X-100 (v/v) and 0.1 mM EDTA. The homogenization was done in liquid nitrogen in the ratio of 1:2 of leaf sample and buffer, respectively. The liquid form of homogenate was centrifuged at 10000 rpm for 30 min at 4°C (in 2 mL eppendorf). The supernatants was collected in clear eppendorf tube and stored at -80°C for subsequent analysis.

Protein determination

The total protein of different chemotypes of *W. somnifera* was determined by Bradford²² method using bovine serum albumin (BSA, Sigma Aldrich, USA) as a standard. Antioxidant enzyme assays were performed at room temperature and activity was measured on PerkinElmer, Lamda 35, UV/VIS spectrophotometer.

Antioxidant enzyme activity

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by nitrotetrazolium (NBT) photochemical assay according to Bayer and Fridovitch²³. The reaction mixture was prepared by mixing of 100 mM potassium phosphate buffer (pH 7.8), 10 mM L-methionine, 0.025% (v/v) Triton X-100 and 0.57 mM NBT. Aliquots (2 mL) of this mixture were delivered into small glass tubes,

followed by 0.02 mL of enzyme extract and 4.4% (w/v) riboflavin. Remaining volume was made up by autoclaved water. The mixture was mixed and then illuminated for 7 min in an aluminum foil-lined box, containing two 20 W florescent tubes. A control tube in which the sample was replaced by 0.02 mL of buffer was run in parallel and the optical density of all the tubes was measured at 560 nm on PerkinElmer, Lamda 35, UV/VIS spectrophotometer.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada²⁴. For the ascorbate activity of the tissue sample, an assay solution was prepared by mixing 50 mM potassium phosphate buffer, 5 mM ascorbate and 1 mM EDTA and 0.1 mL of the sample. At last, 1 mM hydrogen peroxide was added for starting the reaction. Reduction in ascorbate concentration was recorded by reading the absorbance at 290 nm continuously for 3 min.

Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was measured by monitoring the formation of tetraguaiacol from guaiacol²⁵. The reaction mixture contained 10 mM sodium phosphate buffer (pH 5.8), 1% guaiacol (v/v) and 100 mM H₂O₂. Due to oxidation of guaiacol, the absorbance increased and this absorbance was recorded at 470 nm every 10 s for 1 min.

Catalase (CAT, 1.11.1.6) activity was assayed by measuring the initial rate of H₂O₂ disappearance²⁶. Catalase assay reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.01 mL enzyme extract and 2 mM H₂O₂. The decrease in H₂O₂ was followed by a decline in absorbance at 240 nm.

Statistical analysis

Each tube or flask with 3-5 explants was taken as one replicate. Each experiment consisted of either 10 tubes or 5 flasks of each treatment and all experiments were repeated twice. Only the shoots with distinctly visible apical meristems were counted and the precociously sprouting axillary buds (observed in some treatments) were not taken into account for counting the number of shoots. At the time of the antioxidant experiment we had taken three replicate of each chemotype, and during the calculation, two closest readings of each sample were considered. Results were subjected to analysis of variance and significance test. The values of data for regenerated shoot buds and height of proliferated shoots are presented as mean ± SE of five replicate cultures. All statistical analyses were done using ANOVA-INDOSTAT software.

Results and Discussion

Organogenesis in leaf segments

It was observed that all the uppermost segments of leaves towards the tip were either less regenerative or they became yellow and later turned brown. The lower 2-3 segments were found regenerative and differentiated shoots within 4-5 weeks of incubation. The increase in morphogenetic ability of the leaves from the tip towards the base has already been reported in *Cajanus cajan*²⁷ and Asiatic hybrid lily²⁸ and explained by the fact that leaves reach maturity first at the distal part and subsequently basipetally towards the proximal part. Jain *et al.*¹⁷ have also observed that the presence of petiolar part along with lamina was essential for morphogenesis as no response was observed when lamina without petiolar part was cultured. Lower part of the leaves is directly in contact with the petiolar region. Due to which this part is highly dense with the hormone and other metabolite, which helps in the regeneration of the leaves²⁹.

The regeneration potential of leaf was evaluated in terms of number of shoots regenerated, percentage of responding cultures, time taken for shoot differentiation and condition of the explants in culture. The results are presented in Table 1. In our

preliminary studies, we found that shoot organogenesis in all the four chemotypes of *W. somnifera* responded best in the presence of BA as compared to Kn, therefore, BA was used for further experiments. Initially during 2nd week of incubation, compact nodular callus started developing mainly at the cut ends and margins of leaf lamina. These nodular callus developed shoot buds during 3rd week which were visible to the naked eye from 4th week of incubation. Shoot bud development was a nonsynchronous phenomenon, varied largely in different chemotypes when cultured on the same medium (Fig. 1 A-D). Lower concentration of BA (0.5 mg/L) was not at all effective to induce shoot organogenesis in any of the four chemotypes. The explants responded better in 1.0-1.5 mg/L BA but shoot differentiation was less in 1.0 mg/L BA as compared to 1.5 mg/L. Maximum response (85%) was observed in 1.5 mg/L BA in NMITLI-101, where a maximum of 18 shoot buds differentiated within 25 days of culture incubation (Table 1). BA and IAA had already been reported as the most effective PGR for shoot organogenesis in leaf segments of *W. somnifera*^{30,31}. On further increasing the concentration of BA (2.0 mg/L), although shoot organogenesis took place in all the chemotypes except NMITLI-130, but

Table 1—Chemotypic variation in shoot organogenesis in response to BA (1.0 mg/L IAA was common to all the treatments)

Chemotypes	BA (Conc. mg/l)	¹ Responding cultures (%) ± SE	² Time taken (days)	³ Avg. number of shoot buds/explant ± SE	⁴ Avg. height of shoots ± SE (cm)	⁵ General condition of the explants / regenerated shoots
NIMTLI-101	0.5	77.67±0.33	-	-	-	Swelling and growth of explants
	1.0	76.0±0.58	25	11.90±0.05	5.50±0.05	Shoots were fresh, green and healthy
	1.5	85.0±0.58	25	18.03±0.08	5.96±0.03	Shoots were fresh, green and healthy
	2.0	78.333±0.8	30	6.93±0.06	5.40±0.05	Shoots were associated with callus
NIMTLI-108	0.5	60.0±1.15	-	-	-	Swelling and growth of explants
	1.0	61.0±0.58	28	10.46±0.08	5.43±0.08	Shoots were fresh, green and healthy
	1.5	66.67±0.88	28	12.03±0.08	5.70±0.05	Shoots were fresh, green and healthy
	2.0	64.0±0.58	32	5.30±0.05	5.40±0.05	Shoots were associated with callus
NIMTLI-130	0.5	72.0±0.58	-	-	-	Swelling and growth of explants
	1.0	81.67±1.22	30	5.86±0.06	5.63±0.08	Shoots were fresh, green and healthy
	1.5	89.33±0.67	30	6.46±0.03	5.30±0.05	Shoots were fresh, green and healthy
	2.0	90.0±1.15	-	-	-	Large callus was formed
NIMTLI-135	0.5	74.67±0.33	-	-	-	Swelling and growth of explants
	1.0	77.0±0.58	32	8.0±0.11	5.63±0.08	Shoots were fresh, green and healthy
	1.5	83.0±0.58	32	8.33±0.08	5.30±0.05	Shoots were fresh, green and healthy
	2.0	70.0±0.58	35	4.70±0.10	5.46±0.03	Shoots were associated with callus

[*CD value at 5% & 1%; 3.022 and 6.924 for 1.0 mg/L BA; 5.731 and 13.219 for 1.5 mg/L BA; 3.351 and 7.726 for 2.0 mg/L BA; **CD value at 5% & 1%, 0.179 and 0.413 for 1.0 mg/L BA; 0.363 and 0.838 for 1.5 mg/L BA; 3.050 and 7.036 for 2.0 mg/L BA; ¹Response in the form of swelling, expansion, callus, or shoot organogenesis in leaf explants; ²Visibility of shoots with naked eye; ³Average of five explants; ⁴Average height of proliferating shoots after 60 days of culture incubation of leaf explants; ⁵After 5 weeks of culture incubation

the shoots formed were vitrified and associated with a large callus, which hindered/restricted their growth and development. Multiple shoots grew in height when the whole leaf explant, along with all the differentiated shoots were subcultured in a medium having lower concentrations of BA (0.5 mg/L) and IAA (0.5 mg/L, Fig. 2 A-D), however, the height of the regenerated shoots remained almost equal in all the chemotypes (Table 1). The analysis of variance of different chemotypes showed highly significant differences ($P \geq 0.01$) for number of regenerated shoot buds at all the three concentrations of BA. However, it showed nonsignificant differences for height of regenerated shoots at all the concentrations except for 1.5 mg/L BA (Tables 1 & 2). Similarly, the most regenerative (NMITLI-101) and the least regenerative (NMITLI-130) chemotypes on comparison showed significant differences ($P \geq 0.05$)

for number of regenerated shoot buds at all the concentrations of BA, however, height of regenerated shoots were significantly different ($P \geq 0.05$) only at 1.5 mg/L BA.

Total phenolic contents, H₂O₂ concentration, proline content and regenerative potential

Among different chemotypes of *W. somnifera*, NIMTLI-130 was having the highest TPC (0.248 mg/g), which decreased from NIMTLI-135 (0.237 mg/g) to NIMTLI-108 (0.190 mg/g), while NIMTLI-101 contained the lowest (0.079 mg/g) (Fig. 3A). The pattern of H₂O₂ concentration was also similar to that of TPC in all the chemotypes (Fig. 3B). On the contrary, proline content was measured highest in NMITLI-101 and the lowest in NMITLI-130 (Fig. 3C). For developing a correlation between regenerative potentiality and antioxidant system (TPC, proline

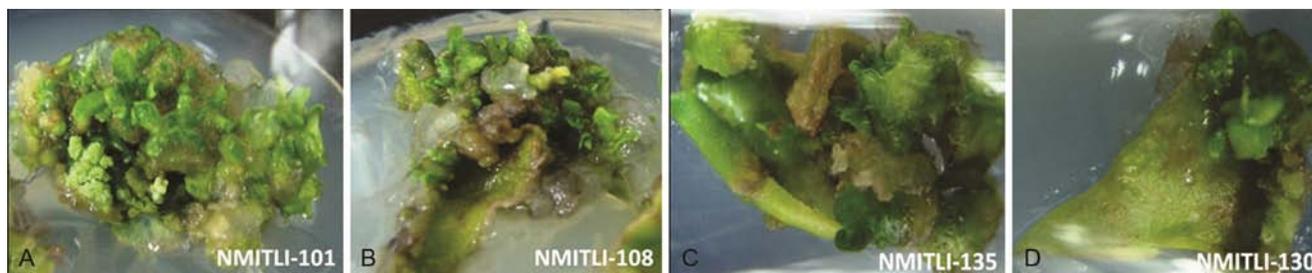


Fig. 1—Shoot organogenesis in leaf segments of different chemotypes of *W. somnifera* after 35 days of culture incubation; (A) NMITLI-101; (B) NMITLI-108; (C) NMITLI-135; and (D) NMITLI-130



Fig. 2—Proliferating shoots of different chemotypes of *W. somnifera* after 60 days of culture incubation; (A) NMITLI-101; (B) NMITLI-108; (C) NMITLI-135; and (D) NMITLI-130

Table 2—Analysis of variance (ANOVA) for the effect of different concentration of BA in shoot organogenesis in different chemotypes (NIMTLI-101, NIMTLI-108, NIMTLI-130 and NIMTLI-135).

Sources	df	Avg. number of regenerated shootbuds/explants			Avg. height of regenerated shoots		
		BA Concentration			BA Concentration		
		1.0 mg/L	1.5 mg/L	2.0 mg/L	1.0 mg/L	1.5 mg/L	2.0 mg/L
Replication	2	0.0158	0.0008	0.0108	0.0525	0.0008	0.0108
Treatments	3	21.3658**	78.0100**	4.8678**	0.0033	0.0300	0.3200**
Error	6	0.0236	0.0241	0.0186	0.0091	0.0108	0.0075

*Significant at 1%

content, and H_2O_2 concentration), the most regenerative (NMITLI-101) and the least regenerative (NMITLI-130) chemotypes have been discussed. It has been observed that NMITLI-130 with high TPC and high H_2O_2 concentration was less regenerative, whereas NMITLI-101 having lower TPC and lower H_2O_2 concentration showed more regeneration potential (Table 3). Between the chemotypes NMITLI-101 and NMITLI-130 statistical analysis were performed according to Fisher's LSD at 5% and 1% significance. Highly significant differences ($P \geq 0.01$) were found for TPC, proline content and H_2O_2 . It is well known in the literature that the higher phenolic compounds hinder growth and development and also the differentiation in tissue culture and therefore are inversely proportional to the regeneration potentiality³². The phenolic compounds of *Gossypium hirsutum* have been reported to attribute to regeneration potential of the plants³³. Higher

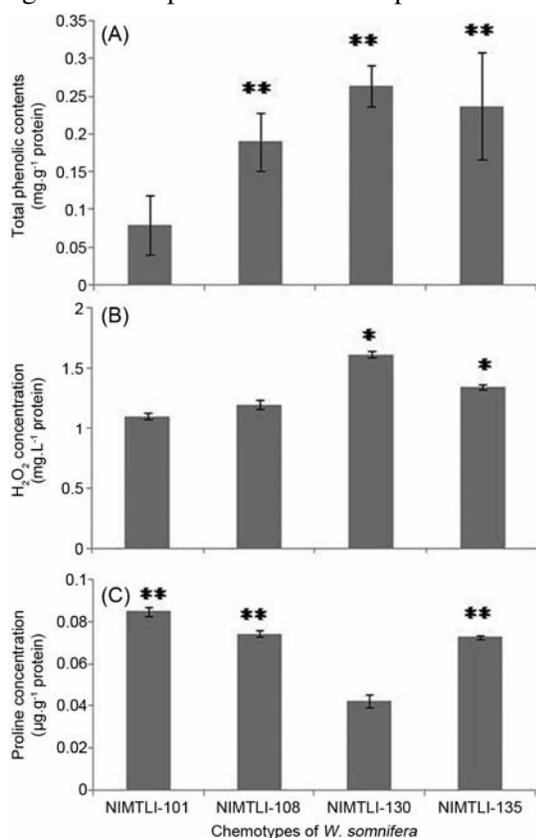


Fig. 3—Measurements of total phenolic contents and H_2O_2 concentration; (A) TPC; (B) H_2O_2 ; and (C) proline content. For measurement of TPC and H_2O_2 content, NMITLI-101 was taken as control, while for proline content, NMITLI-130 was used as control. Values are mean \pm SE where $P \geq 0.05$ (*) showing significant difference and $P \geq 0.01$ (**) highly significant differences (Fisher's LSD test).

proline content and its catabolism generates a ROS signal for the adaptive induction of endogenous stress defense and extends life span of *C. elegans* by >2 fold³⁴. It has been reported that high proline content provides protection to antioxidant system by scavenging the ROS produced³⁵ and has already been reported to enhance *in vitro* regeneration in *W. somnifera*³⁶ similar to our findings.

Antioxidant enzyme activity

In the present work, antioxidant enzyme activity was correlated with regenerative potential and further growth of the regenerated shoots in different chemotypes of *W. somnifera*. There were significant differences among chemotypes for antioxidant enzyme activity. The results are presented in Fig. 4 A-D. The SOD, GPX and APX activity was recorded as highest in NMITLI-130 $>$ NMITLI-135 $>$ NMITLI-108 and minimum in NMITLI-101 (Fig. 4 A-C). Only the most regenerative (NMITLI-101) and the least regenerative (NMITLI-130) chemotypes were discussed in detail. Between the chemotypes NMITLI-101 and NMITLI-130, statistical analysis was performed according to Fisher's LSD at 5% and 1% significance, and found highly significant ($P \geq 0.01$) for all antioxidant enzyme activities. During the study of different enzyme activity of *W. somnifera*, it was observed that all the enzyme activities were correlated to each other and also with TPC, H_2O_2 concentration and proline content of the plant. A significant relationship between SOD activity and TPC was observed in *W. somnifera*, which indicated that phenolic compounds are the major contributors to the antioxidant properties of the plant similar to other plants³⁷. Higher SOD activity in NMITLI-130 showed its role in dismutation of superoxide producing H_2O_2 and O_2 in the tissue. However, as this chemotype already has higher TPC and H_2O_2 concentration, it could not detoxify H_2O_2 further into water by GPX and APX, even at their higher activity level. This could create an imbalance

Table 3—Correlation coefficient analysis of four different chemotypes of *W. somnifera* for different parameters

	H_2O_2	TPC	Proline	APX	CAT	GPX	SOD
H_2O_2	1						
TPC	0.8638	1					
Proline	-0.9697	-0.7931	1				
APX	0.7910	0.6833	-0.9063	1			
CAT	-0.8246	-0.9814	0.7947	-0.7660	1		
GPX	0.9609	0.9473	-0.9453	0.8517	-0.9445	1	
SOD	0.9902	0.8065	-0.9916	0.8446	-0.7834	0.9446	1

in generation and metabolism of ROS leading to a variety of physiological challenges by disrupting redox homeostasis of cell³⁵. CAT activity and the proline concentration was comparatively low in chemotype NMITLI-130 (Fig. 4D), it could possibly lead to oxidative damage, which triggers programmed cell death ultimately leading to shoot browning/necrosis, a 'negative' consequence of it and resulted in lower regenerative potentiality in tissue culture³⁸. On the contrary, the maximum regeneration of shoots in chemotype NMITLI-101 may be explained by the higher activity of peroxisomal enzyme CAT, detoxifying the damaging effects of hydrogen peroxide³⁹ which decomposes H₂O₂ to water and O₂. The increased proline content, lower TPC and lower H₂O₂ concentration were mainly responsible for

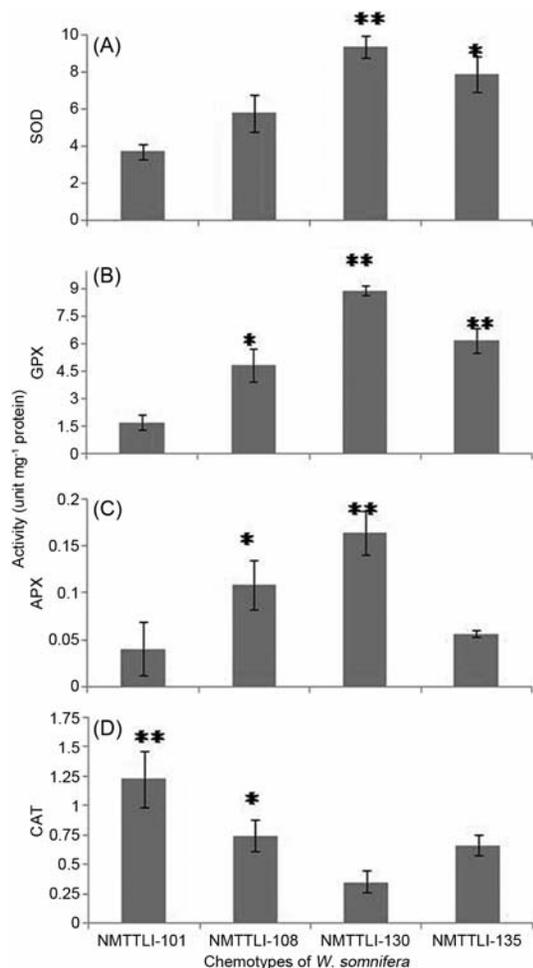


Fig. 4—Antioxidant enzyme activities in different chemotypes of *W. somnifera* (A) SOD; (B) GPX; (C) APX; and (D) CAT. For APX, GPX and SOD, NMITLI-101 was taken as control, while for CAT, NMITLI-130 was used as control. Values are mean \pm SE where $P \geq 0.05$ (*) showing significant difference and $P \geq 0.01$ (**) highly significant differences (Fisher's LSD test).

higher shoot regeneration in NMITLI-101. We have already discussed that the lower concentration of TPC promotes growth and differentiation in tissue culture, and therefore, are inversely proportional to the regeneration potentiality^{32,33}. There are reports available in literature where a decrease in SOD activity has been observed during shoot organogenesis in *Caladium bicolor*⁴⁰ and *Gladiolus hybridus* Hort.⁴¹. Decreased SOD activity was reported after addition of antioxidants in the regeneration medium of *Jatropha curcas* which could alleviate necrosis and increased shoot proliferation³⁸. But in contrast to this, reports are available in literature where increased SOD expression was supposed to be responsible for increased regenerative potentiality⁴². This is not true in the present case of *W. somnifera*, where among the four chemotypes, the one with lowest SOD, GPX and APX activity was most regenerative as in NMITLI-101. It has been reported in chrysanthemum that a decrease in the activity of a number of antioxidant enzymes that normally prevent the build-up of free radicals can be accounted for senescence in plants⁴³. A correlation coefficient analysis among different parameters of four chemotypes has been done and presented in Table 3. The analysis showed that all these parameters depend on each other to balance the antioxidant protection system by increasing or decreasing the activity of different enzymatic or nonenzymatic antioxidants.

In conclusion, the organogenesis was induced in *in vitro* leaves of four chemotypes of *W. somnifera* and an effort has been made to correlate this difference with the TPC, H₂O₂ concentration, proline content, and their antioxidant enzyme activity. The activity of four enzymes namely, SOD, GPX, APX and CAT was analyzed. It has been shown that the chemotype (NMITLI-130) with high TPC, high H₂O₂ concentration and low proline content was having lesser regeneration of shoots despite the higher activity of SOD, APX and GPX in that chemotype. In the same chemotype, CAT had a weak detoxification effect showing lesser activity. On the contrary, NMITLI-101 having low TPC, low H₂O₂ concentration and lower activity of SOD, GPX and APX had a strong detoxification effect with higher activity of CAT and high proline content.

Acknowledgement

The authors acknowledge the Department of Science and Technology, Government of India,

New Delhi, for financial support through a project no. GAP-231425.

References

- Andallu B & Radhika B, Hypoglycemic, diuretic and hypocholesterolemic effect of winter cherry (*Withania somnifera*, Dunal) root. *Indian J Exp Biol*, 38 (2000) 607.
- Rege AA, Ambaye YR & Deshmukh AR, *In vitro* testing of anti-HIV activity of some medicinal plants. *Indian J Exp Biol*, 1 (2010) 193.
- Singh G, Tiwari M, Singh SP, Singh S, Trivedi PK & Misra P, Silencing of sterol glycosyltransferases modulates the withanolide biosynthesis and leads to compromised basal immunity of *Withania somnifera*. *Sci Rep*, 6 (2016) 25562.
- Yadav CS, Kumar V, Suke SG, Ahmed RS, Mediratta PK & Banerjee BD, Propoxur-induced acetylcholine esterase inhibition and impairment of cognitive function: attenuation by *Withania somnifera*. *Indian J Biochem Biophys*, 47 (2010) 117.
- Gupta GL & Rana AC, Effect of *Withania somnifera* Dunal in ethanol-induced anxiety and withdrawal anxiety in rats. *Indian J Exp Biol*, 46 (2008) 470.
- Jain R, Kachhwaha S & Kothari S, Phytochemistry, pharmacology, and biotechnology of *Withania somnifera* and *Withania coagulans*: A review. *J Med Plants Res*, 6 (2012) 5388.
- Barba-Espin G, Diaz-Vivancos P, Job D, Belghazi M, Job C & Hernandez JA Understanding the role of H₂O₂ during pea seed germination: a combined proteomic and hormone profiling approach. *Plant Cell Environ*, 34 (2011) 1907.
- Van Buer J, Cvetkovic J & Baier M, Cold regulation of plastid ascorbate peroxidases serves as a priming hub controlling ROS signaling in *Arabidopsis thaliana*. *BMC Plant Biol*, 16 (2016) 163.
- Hasanuzzaman M, Alam MM, Rahman A, Nahar K & Fujita M, Exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxalase systems provides better protection against salt-induced oxidative stress in two rice (*Oryza sativa* L.) varieties. *Biomed Res Int*, 2014 (2014), 757219.
- Husaini AM & Abdin MZ, Overexpression of tobacco osmotin gene leads to salt stress tolerance in strawberry (*Fragaria x ananassa* Duch.) plants. *Indian J Exp Biol*, 7 (2008) 465.
- Abassi N, Kushad M & Endress A, Active oxygen-scavenging enzymes activities in developing apple flowers and fruits. *Sci Hort*, 74 (1998) 183.
- Chugh V, Kaur N, Grewal MS & Gupta AK, Differential antioxidative response of tolerant and sensitive maize (*Zea mays* L.) genotypes to drought stress at reproductive stage. *Indian J Biochem Biophys*, 50 (2013) 150.
- Gapper C & Dolan L, Control of plant development by reactive oxygen species. *Plant Physiol*, 141 (2006) 341.
- Olson KR, Gao Y, DeLeon ER, Arif M, Arif F, Arora N & Straub KD, Catalase as a sulfide-sulfur oxido-reductase: An ancient (and modern?) regulator of reactive sulfur species (RSS). *Redox Biol*, 12 (2017) 325.
- Papadakis AK, Siminis CI & Angelakis RKA, Reduced activity of antioxidant machinery is correlated with suppression of totipotency in plant protoplasts. *Plant Physiol*, 126 (2001) 434.
- Sharada M, Ahuja A, Suri KA, Vij SP, Khajuria RK, Verma V & Kumar A, Withanolide production by *in vitro* cultures of *Withania somnifera* and its association with differentiation. *Biol Plant*, 51 (2007) 161.
- Jain R, Sinha A, Jain D, Kachhwaha S & Kothari SL, Adventitious shoot regeneration and *in vitro* biosynthesis of steroidal lactones in *Withania coagulans* (Stocks) Dunal. *Plant Cell Tissue Organ Cult*, 105 (2011) 135.
- Murashige T & Skoog F, A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant*, 15 (1962) 473.
- Turkmen N, Sari F & Velioglu YS, Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chem*, 99 (2006) 835.
- Alexieva V, Sergiev I, Mapelli S & Karanov E, The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell Environ*, 24 (2001) 1337.
- Singh G, Tiwari M, Singh SP, Singh R, Singh S, Shirke PA, Trivedi PK & Misra P, Sterol glycosyltransferases required for adaptation of *Withania somnifera* at high temperature. *Physiol Plant*, (2017) doi: 10.1111/ppl.12563 (in press).
- Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72 (1976) 248.
- Beyer WF Jr & Fridovich I, Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal Biochem*, 161 (1987) 559.
- Nakano Y & Asada K, Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol*, 22 (1981) 867.
- Zheng X & Van Huystee R, Peroxidase-regulated elongation of segments from peanut hypocotyls. *Plant Sci*, 81 (1992) 47.
- Aebi H, Catalase *in vitro*. *Methods Enzymol*, 105 (1984) 121.
- Misra P, Direct differentiation of shoot buds from leaf explants of *Cajanus cajan* L. *Biol Plant*, 45 (2002) 347.
- Misra P & Kochhar S, Acclimatization of *in vitro*-raised Asiatic hybrid lily plants in subtropical climate and associated changes in stress proteins and antioxidant enzymes. *J Plant Biochem Biotechnol*, 17 (2008) 45.
- Zhou H, Li M, Zhao X, Fan X & Guo A, Plant regeneration from *in vitro* leaves of the peach rootstock 'Nemaguard' (*Prunus persica* × *P. davidiana*). *Plant Cell Tissue Organ Cult*, 101 (2010) 79.
- Pandey V, Misra P, Chaturvedi P, Mishra MK, Trivedi PK & Tuli R, *Agrobacterium tumefaciens*-mediated transformation of *Withania somnifera* (L.) Dunal: an important medicinal plant. *Plant Cell Rep*, 29 (2010) 133.
- Sivanandhan G, Thebora J, Kapil Dev G, Selvaraj N, Manickavasagam M & Ganapathi A, Effect of carbon and nitrogen sources on *in vitro* flower and fruit formation and withanolides production in *Withania somnifera* (L.) Dunal. *Indian J Exp Biol*, 53 (2015) 177.
- Abdelwahd R, Hakam N, Labhilili M & Udupa SM, Use of an adsorbent and antioxidants to reduce the effects of leached phenolics in *in vitro* plantlet regeneration of faba bean. *African J Biotechnol*, 7 (2008) 997.

- 33 Ozyigit II, Kahraman MV & Ercan O, Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.). *African J Biotechnol*, 6 (2007) 3.
- 34 Zarse K, Schmeisser S, Groth M, Priebe S, Beuster G, Kuhlow D, Guthke R, Platzer M, Kahn CR & Ristow M, Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal. *Cell Metab*, 15 (2012) 451.
- 35 Palmer AG, Liu Y, Adkins SM, Zhang X, Wu IL, Chen X & Lynn DG, The molecular language of semagenesis. *Plant Signal Behav*, 3 (2008) 560.
- 36 Ozden M, Demirel U & Kahraman A, Effects of proline on antioxidant system in leaves of grapevine (*Vitis vinifera* L.) exposed to oxidative stress by H₂O₂. *Sci Hort*, 119 (2009) 163.
- 37 Fatima N, Ahmad N & Anis M, Enhanced in vitro regeneration and change in photosynthetic pigments, biomass and proline content in *Withania somnifera* L. (Dunal) induced by copper and zinc ions. *Plant Physiol Biochem*, 49 (2011) 1465.
- 38 Misra P, Toppo DD, Gupta N, Chakrabarty D & Tuli R, Effect of antioxidants and associate changes in antioxidant enzymes in controlling browning and necrosis of proliferating shoots of elite *Jatropha curcas* L. *Biomass Bioenergy*, 34 (2010) 1861.
- 39 Soshinkova T, Radyukina N, Korolkova D & Nosov A, Proline and functioning of the antioxidant system in *Theilungiella salsuginea* plants and cultured cells subjected to oxidative stress. *Russian J Plant Physiol*, 60 (2013) 41.
- 40 Isah T & Mujib A, Studies on antioxidant enzymes activity during in vitro morphogenesis of *Caladium bicolor* Linn. *Int J Mod Cell Mol Biol*, 1 (2011) 1.
- 41 Gupta SD & Datta S, Antioxidant enzyme activities during in vitro morphogenesis of gladiolus and the effect of application of antioxidants on plant regeneration. *Bio Plant*, 47 (2003) 179.
- 42 Chatzidimitriadou K, Nianiou-Obeidat I, Madesis P, Perl-Treves R & Tsaftaris A, Expression of SOD transgene in pepper confer stress tolerance and improve shoot regeneration. *Electron J Biotechnol*, 12 (2009) 7.
- 43 Chakrabarty D, Verma AK & Datta SK, Oxidative stress and antioxidant activity as the basis of senescence in *Hemerocallis* (day lily) flowers. *J Horticult For*, 1 (2009) 113.