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# Application of Antioxidant Enzyme Activity and Biochemical Characterization in Static and Suspension Cultures of *Withania somnifera* L. towards Food Technology

*Satyajit Kanungo*, Monika Kumari and Santi Lata Sahoo*

Biochemistry and Molecular Biology Laboratory, P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar, 751004, Odisha, India.

**Abstract**

*Withania somnifera* (L.) Dunal is an erect evergreen shrub commonly known as Ashwagandha. It is widely used in Ayurvedic, and in the traditional pharmacopeia system of India. It is one of the major ingredients in many formulations prescribed for a variety of musculo-skeletal conditions including arthritis and rheumatism. In the present study, variations in the quality and quantity of proteins and antioxidant enzymes were evaluated biochemically and enzymatically from the static and suspension cultures of *Withania somnifera* L. The nodal segments provided the maximum callusing of 90.25±0.06% with (1mg/l) BAP and (2mg/l) Kn of 2, 4-D. The static and suspension cultures were taken for the analysis of total soluble protein, and screened for antioxidant enzyme activity [catalase (CAT), superoxide dismutase (SOD) and guaiacol peroxidase (GPX)]. The protein content (1.2016 µg/µl) was found to be higher in static culture samples (0.870 µg/µl) than the protein obtained from the suspension culture. The antioxidant enzyme activity (CAT, SOD and GPX) was higher in the static culture samples (301.01± 0.42, 198.92 ± 0.29, 103.75 ± 0.11 nkat/mg of protein) than in the suspension culture. Specific activity staining of isoenzyme pattern exhibited three isoforms (CAT 1, CAT 2 and CAT 3) in the static culture samples but CAT 1 was absent in the samples extracted from the suspension cultures. In case of SOD, four bands (SOD 1, SOD 2, SOD 3 and SOD 4) were found in both samples whereas the intensity of GPX activity was found to be more in the static culture; however, both samples exhibited three isoforms (GPX 1, GPX 2 and GPX 3). Supplementation of the required nutrients along with phytohormones under *in vitro* conditions might be an enhancing factor to yield antioxidant enzymes in the static culture samples.

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Correspondence to:
Dr. Satyajit Kanungo
Tel: 00919438733453
Email: satya_9bt@yahoo.com

## 1. Introduction

Medicinal plants are of great importance to the health of individuals and communities. Human beings may not survive on this earth for long without plants because their products and active constituents play an important role in our day-to-day life. The medicinal value of plants lies in some molecules present in them that produce a definite physiological action on human body. Herbal medicines have received much attention as sources of secondary metabolites since they are environment friendly, and considered as time tested and relatively safe for human use [1]. Therefore, it is the need of the hour to search the potent herbal medicinal plants with an aim to validate their ethnomedicinal use. Research works on medicinal

plants also suggest that plants need to have some urgent attention unless they will be threatened [2].

*W. somnifera* is a short shrub (35–75 cm) with a central stem from which branches extend radially in a star pattern and are covered with a dense thick, small oval leaves. It is cultivated in many of the drier regions of India, such as Madhya Pradesh, Punjab, Sindh, Gujarat and Rajasthan. The plant grows from sea level to an altitude of 1500 meters [3]. The anti-inflammatory and immune modulatory properties of *W. somnifera* root extracts are likely to contribute to the chemo-preventive action.

Callus tissue is an essential material in plant cell culture system. When it is introduced into a liquid medium and agitated, the cells disperse throughout the liquid to form a cell suspension culture. Such cells are, in theory, totipotent, and should also have a potential to synthesize any of the compounds normally associated with the plant [4]. The newly formed cells are dispersed into the liquid medium, and become clusters and aggregates. These cells can exhibit much higher rates of cell division than cells in static culture. Thus, cell suspension offers advantages when rapid cell division or many cell generations are desired, or when more uniform treatment application is required [5]. Plant tissue culture technology holds great promise for micropropagation, conservation and enhancement of the natural levels of valuable secondary plant products. *In vitro* conservation can be possible through micropropagation, which allows making numerous clones of the plant by applying tissue culture techniques [6].

Oxidation is a chemical reaction that produces free radicals such as hydroxyl radical (OH) and superoxide anion ($O_2^-$), and reactive oxygen species in the cells include hydrogen peroxide ($H_2O_2$) [7]. In turn, these radicals and reactive oxygen species can start chain reactions like lipid peroxidation, or by oxidizing DNA or proteins [8], produce oxidative stress, which is thought to contribute for the development of a wide range of diseases including Alzheimer's [9, 10], Parkinson's syndrome [11], rheumatoid arthritis [12], and neuro degeneration in nerve cells [13]. Damage to DNA can cause mutations and possibly cancer, if not corrected by DNA repair mechanisms [14, 7] while damage to proteins causes enzyme inhibition, denaturation and protein degradation [15]. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions being oxidized themselves [8]. Antioxidants are widely used as ingredients in dietary supplements or as natural dietary compounds [16]. Dietary antioxidants include ascorbate, tocopherols, carotenoids and bioactive plant phenolics. The health benefits of fruits and vegetables are largely due to the antioxidant compounds and other phytochemicals, some with greater antioxidant properties [17]. This study aimed at exploring the protein aqueous bioactive metabolites and examining the antioxidant enzyme activity [catalase (CAT), superoxide dismutase (SOD) and guaiacol peroxidase (GPX)] to find out the expression of isoenzyme both in the static and suspension cultures of *W. somnifera.*

## 2.Materials and Methods

### 2.1. Plant and surface sterilization

The nodal and apical explants were collected from about the one year old healthy plant of *W. somnifera* from the botanical garden of P.G. Department of Botany, Utkal University, Bhubaneswar, Odisha, India. For the purpose of callogenesis study, young shoots were harvested during the month of January to April. The explants were washed thoroughly under running tap water for 30 min, followed by immersing in a 5% (v/v) solution of detergent (Labolene, Qualigens, India) for 20 min. They were subsequently agitated in 0.1% Bavistin (fungicide by BASF India Ltd.) for 15 min, followed by rinsing with distilled water to remove the traces of Bavistin. After washing, the explants were taken into the laminar air flow chamber, where the explants were sterilized in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for another 2 min, followed by rinsing with sterile double distilled water. This step was repeated for three times. Afterwards, the surface-decontaminated explants were implanted in MS [18] medium containing conical flasks.

### 2.2. Culture media and conditions

MS medium was supplemented with 3% (w/v) sucrose, and gelled with 0.8% (w/v) agar bacteriological grade (Himedia, Mumbai, India). The medium's pH was adjusted to 5.8 ± 0.2 before being autoclaved at 121°C for 15 min. All the cultures were maintained at 24 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps of 2×40W (Phillips, India) with 60 to 65% relative humidity.

### 2.3. Callus initiation and maintenance of static culture

Callus initiation in the MS medium was observed with supplementation of 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and Kn (Kinetin; Sigma, USA) ranging from 0.5 to 4.0 mg $L^{-1}$. The initiated calli were observed for their morphological changes with an interval of two weeks. The calli ware subsequently sub-cultured in the same medium after an interval of two weeks, and by this way, the static cultures were maintained for further analysis.

### 2.4. Establishment of suspension culture

50 ml of the MS liquid medium supplemented with 2, 4-D (1mg/L) was inoculated with crushed callus in 250ml culture flask in sterilized and fully aseptic environment, inside a laminar air flow. The callus was grinded by using a sterile pipette. A portion of the callus ware transferred with sterile forceps into the

onical flask containing the culture medium. Initially, the weight of the callus was recorded, and the flasks were placed into the culture room on an orbital shaker (80 rpm) until an adequate quantity of the callus was generated with a particular cell density [19].

**2.5. Extraction from callus and suspension culture**

Two months old calli derived from the nodal explants ware weighed and dried by wrapping them in perforated aluminum foils and keeping at 40°C for 72 hours in an oven [20].

The final weight of the fifteen-day old culture flasks containing the healthy cells/cell clumps was subjected to quantification of callus biomass. Solid materials (callus) were separated by filtration using a Whatman's No. 1 filter paper, and dried by wrapping in perforated aluminum foil at 40°C for 72 hours in an incubator. The dried weight was recorded for analysis of the biomass [20, 21].

**2.6. Quantitative estimation of protein**

The fresh tissue samples were homogenized in pre-chilled mortar and pestle with 50 mM potassium phosphate buffer (pH 7.8), 50 mMethylene diamine-tetra acetic acid (EDTA), 2 mMphenyl methyl sulfonyl fluoride (PMSF), and 10% (w/v) insoluble polyvinyl pyrrolidone (PVP). The homogenates were centrifuged at 14000 rpm for 20 min at 4°C, and the supernatants were processed for estimation of protein and antioxidant enzymes. Quantitative estimation of protein was done according to the method of [22] by measuring the absorbance at 750 nm, using bovine serum albumin as a standard, and the quantity of estimated protein was expressed as mg/g $^{-1}$ dry weight (g.d.w.).

**2.7. Assay and in-gel activities of antioxidant enzyme**

CAT activity was measured in a reaction mixture (3 ml) containing 100 mM sodium phosphate buffer (pH 6.8, 2 ml), 30 mM $H_2O_2$ (0.5 ml), and 0.5 ml enzyme extract according to the protocol of [23]. The decrease in absorbance due to hydrogen peroxide depletion was recorded at 240 nm by Hitachi model 200-20 UV-VIS spectrophotometer. Catalase activity was calculated by using the extinction coefficient of 40 M/cm$^{-1}$ for $H_2O_2$ at 240 nm, and was expressed as nKat moles of $H_2O_2$ decomposed per second/ml$^{-1}$of enzyme protein. After native polyacrylamide gel electrophoresis, the gel was stained by the procedure of [24]. The electrophoresed samples in the gel were incubated in 0.01% $H_2O_2$ (v/v) for 10 min, and subsequently developed in 2% $FeCl_3$ and 2% $K_3FeCN_6$ solution for 10 min. The principle involves the reaction of $H_2O_2$ with potassium ferricyanide (III) by reducing it to ferrocyanide (II). The peroxide is oxidized to molecular $O_2$. $FeCl_3$ reacts with ferrocyanide (II) to form stable, insoluble Prussian blue pigment. Catalase signaled its location by scavenging $H_2O_2$ causing transparent bands on the gel.

The activity of SOD was assayed according to the procedure of [25]. The reaction mixture was prepared by mixing 1.110 ml of 50 mM phosphate buffer (pH 7.4), 0.075 ml of 20 mM L-methionine, 0.040 ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride, and 0.1 ml of 50 µM EDTA. To this mixture, 100 µl of enzyme extract (50 µg protein) and 80 µl of riboflavin (50 µM) were added. The cocktail was mixed and illuminated for 10 min in an aluminum foil coated wooden box containing two 20 W-Philips fluorescent lamps fitted parallel to each other. Equal amount of phosphate buffer was added to the control tube instead of the sample. The sample and its respective control were run together. After 10 min of exposure, 1mL of Greiss reagent (prepared freshly by mixing equal volume of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-1-napthyl ethylene diamine) was added into each tube, and the absorbance was immediately measured at 543 nm. The activity was calculated as nKat/mg -1 of protein. The specific activity staining of SOD was performed as per the classical method of [26]. The gel was completely submerged in a freshly prepared staining buffer containing 50 mM phosphate buffer, 0.1 ml EDTA, 28 mM TEMED, 0.003 mM riboflavin, and 0.25 mM nitroblue tetrazolium for 30 min in dark condition. Thereafter, the gel was placed on an illuminated glass plate until the bands become visible.

Peroxidase activity was measured in a reaction mixture (3 ml) containing 100 mM potassium phosphate buffer (pH 7.0, 2.8 ml), 10 mM $H_2O_2$ (50 µl), 0.018M guaiacol (50 µl), and 100µl enzyme extract, according to the method of [27]. The increase in absorbance due to the formation of tetra-guaiacol was recorded at 436 nm. GPX activity was calculated by using the extinction coefficient of 26.6 M$^{-1}$cm$^{-1}$ for $H_2O_2$ at 436 nm and was expressed as nKat/mg$^{-1}$ of protein. The 10% gel was stained by the procedure of [28]. Then it was immersed in 0.018 M guaiacol for 30 min at room temperature rinsed twice in double distilled water, and immersed in 0.015% of $H_2O_2$ in 1% glacial acetic acid till the development of dark brown bands.

**2.8. Statistical analysis**

The percentage of antioxidant enzyme activities was the mean of independent experimental replicates (n=5). Means, standard deviations and standard errors were calculated for each treatment.

## 3. Results and discussion

### 3.1. Callogenesis

In W. somnifera, callus initiation was observed at the cut surfaces of apical [Fig. 1 A-B] and nodal explants [Fig. 1 C-D] in the MS medium supplemented with different concentrations of hormones such as 2, 4-D in combination with Kn and BAP [Table 1]. Initially, 0.5 mg L-1of 2, 4-D, Kn and BAP was added to the MS medium, but the callus generation was low (32.21±0.43%) and the color was found to be white [Fig. 2 B]. However, significant development of the callus (90.25±0.06%) was observed with the medium supplemented with 2, 4-D (2.0 mg L-1), Kn (1.0 mg L-1) and BAP (1.0 mg L-1). Subsequent periodical sub-culturing changed the color of the callus's partial pigmentation [Fig. 2 A&C]. It was also seen that when the concentration of the hormones such as 2, 4-D, Kn and BAP (2.5 mg L-1) was further increased in the MS medium, retardation in callus growth (57.72±0.18) was observed with the development of phenolics [Fig. 2 D]. Hence, it was concluded that by increasing or decreasing of the hormone concentration in MS medium (except for 2.0 mg L-1of 2, 4-D, 1.0 mg L-1 of Kn and 1.0 mg L-1 of BAP) provided less yield in terms of callusing.

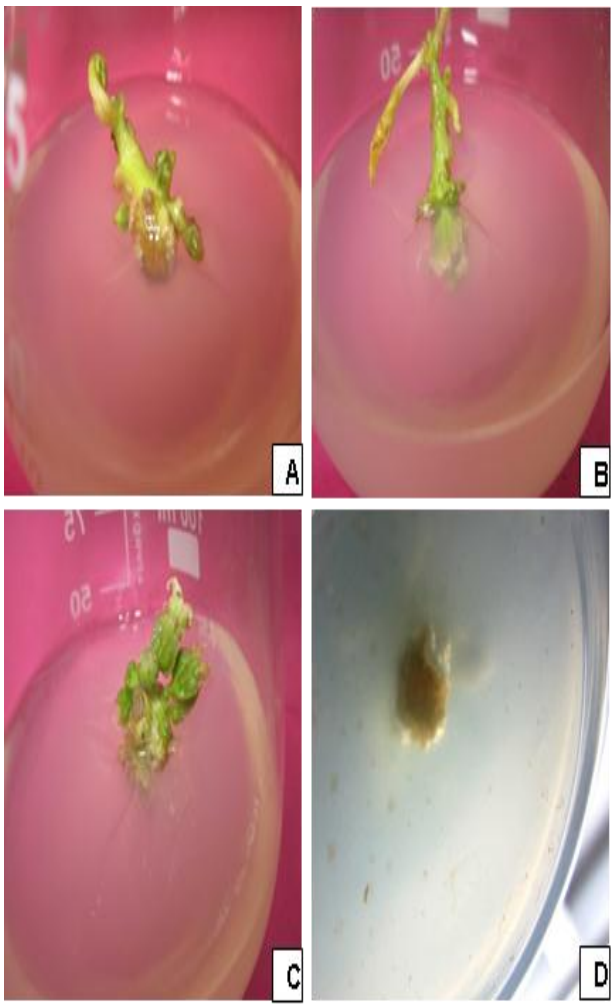


**Figure 1.** Callus initiation in *W. somnifera:* (A) & (B) - White callus from nodal explants, (C) – Callus from apical bud explants, (D) - Dorsal view of green callus from apical bud explants.

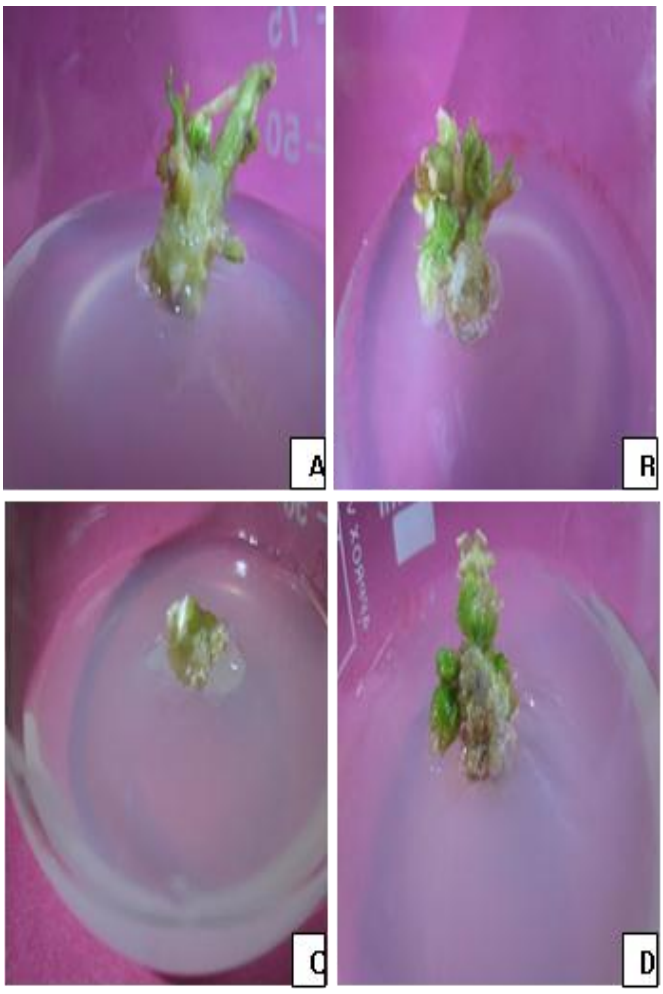


**Figure 2.** Callusing in *W. somnifera*: (A) – Whitish green callus from nodal explants, (B) – White Callus from apical bud explants, (C) – Whitish green callus from nodal explants, (D) - Profuse whitish brown callus from apical bud explants.

**Table 1.** Impact of different concentrations of auxin and cytokinin on callus induction in *W. somnifera* from apical and nodal explants in MS medium

| Growth regulators ($mg\ L^{-1}$) | | | Percentage of callus induction (Mean± SEM)* | Type of callus (Color) | Degree of callusing at the end of 4 weeks(#) |
|---|---|---|---|---|---|
| 2,4-D | KINETIN | BAP | | | |
| 0.5 | 0.5 | 0.5 | 32.21±0.43 | White | + |
| 0.75 | 0.75 | 0.75 | 42.17±0.13 | White | + |
| 1.0 | 1.0 | 1.0 | 60.02±0.68 | Whitish Green | + |
| 1.25 | 1.25 | 1.25 | 62.53±0.44 | Whitish Friable | ++ |
| 1.5 | 1.5 | 1.5 | 63.07±0.56 | White and Friable | + |
| 1.75 | 1.75 | 1.75 | 64.60±0.22 | Green | +++ |
| 2.0 | 1.0 | 1.0 | 90.25±0.06 | Whitish Green | ++++ |
| 1.0 | 2.0 | 1.0 | 78.49±0.23 | Brownish | ++ |
| 1.0 | 1.0 | 2.0 | 69.28±0.25 | Whitish Brown | ++ |
| 2.5 | 2.5 | 2.5 | 57.72±0.18 | Brown | + |

(#) Degree of callusing: + = Poor; ++ = Fair; +++ = Good; ++++ = Very good

*: The differences in mean values among the treatments are statistically significant ($P \leq 0.05$). Data represent the mean of 5 replicates for each treatment. Data were recorded after 4 weeks of culture. SEM: Standard error of mean.

In *W. somnifera*, generation of callus was found to be maximum (90.25±0.06%) with the synergistic activity of 2, 4-D (2.0 mg $L^{-1}$), Kn (1.0 mg $L^{-1}$) and BAP (1.0 mg $L^{-1}$). Similar observation was earlier reported by Raha and Roy [29] with the same hormone in *Holarrhena antidysenterica*. Callusing was observed at the basal cut end of the explants in the present study. Formation of basal callus is a common observation in tissue culture [30], perhaps due to the action of accumulated auxin at the basal cut end as cell proliferator, especially in the presence of cytokines [31]. Basal callus formation is often seen in plants showing strong apical dominance [32]. The auxins were found to be the best for callus proliferation and growth. Among the auxins, 2, 4-D was better for increase in callus biomass and total antioxidant content. 2, 4-D was also reported as the most effective auxin in various medicinal plants [33,34]. On the other hand, a higher concentration of 2, 4-D is inhibitory as it produces low amounts of callus.

At high concentrations of 2, 4-D, white color, friable texture, and lack of potentiality of callus regeneration are exhibited [32]. In the present study, the callus color was changed with higher concentrations of 2, 4-D that might be due to the accumulation of phenolic compounds in the cells. Accumulation of phenols in the cytoplasm leads to the cells oxidation followed by polymerization and formation of oxidized products appearing in brown or dark brown [35].

### 3.2. Study of cell biomass in suspension culture

The biomass study of the suspension culture indicates the growth of the cells in the presence of liquid media, and the impact of artificial culture conditions. In the present investigation, the growth of the suspended cells was observed within fifteen days of study [Table 2]. In the cell biomass study, it was noticed that the weight of the medium containing the culture has increased by a minimum of 1 gram during fifteen days.

**Table 2.** Study of callus biomass during suspension culture (15 days)

| Flask no. | Initial weight of flask without callus in gram (g) | Weight of flask with callus (g) | Final weight of flask with callus after 15 days (g) | Growth |
|---|---|---|---|---|
| 1 | 210.31 | 211.62±2.32 | 212.91±2.62 | 1.27±0.45 |
| 2 | 202.59 | 204.620±4.36 | 206.00±1.62 | 1.38±0.41 |
| 3 | 208.95 | 210.107±1.67 | 211.563±0.94 | 1.45±0.13 |
| 4 | 196.82 | 197.552±6.45 | 198.41±1.77 | 0.86±0.25 |
| 5 | 208.30 | 209.317±5.32 | 210.00±0.65 | 0.69±0.05 |
| 6 | 202.6 | 204.052±2.67 | 205.98±1.48 | 1.92±0.67 |

The data represent mean ± SEM of replicates (n=5).

The suspension culture system offers many advantages for examining the metabolic role of nutrients and their utilization in plants. In order to use the suspension culture system for examinations, a stable morphology and synchronous growth of cells are required [36]. MS liquid medium containing 2.0 mg $L^{-1}$ of 2, 4-D hormone exhibited the maximum growth of calli. After 7 days in suspensions, globular and larger calli were originated. Similar observation was earlier obtained by Fujimura and Komamine with the same hormone [37].

### 3.3. Protein content in static and suspension cultures

1.2016 µg of protein was found in 1µl of static culture, and 0.870 µg of protein was extracted from 1µl of the suspension culture. Hence, it was conformed that the less yield of protein provided by the suspension culture might be due to clumping of the suspended cells.

### 3.4. Antioxidant enzyme assay

The antioxidant activity of CAT, SOD and GPX enzymes ware assayed spectrophotometrically. For this purpose, they were electrophoresed to study their iso-enzyme pattern by characteristic staining of the gel from the static culture and suspension culture samples of *W. somnifera*. In all cases, enzymes activity was found to be the maximum in the static culture (callus) extracts compared to the suspension culture samples. The maximum amount of CAT activity (301.01 ± 0.42nkat/ mg) was obtained from the static callus extracts whereas (280.63 ± 0.14nkat/ mg) was found in the samples extracted from the suspension culture. The callus tissue synthesizes higher amounts of SOD (198.92 ± 0.29nkat/ mg) compared to the suspension culture samples (152.13±1.02nkat/ mg). Furthermore, the value became lower in the case of GPX like all the other types of antioxidant enzymes evaluated in *W. somnifera* in the static (103.75 ± 0.11nkat/ mg) and suspension (84.08±0.97nkat/ mg) cultures [Table 3]. Iso-enzyme patterns were studied by native-PAGE and specific activity staining for CAT, SOD and GPX. In the case of CAT, three isoforms including CAT 1, CAT 2 and CAT 3 were observed from the callus samples; however, CAT 1 disappeared from the suspension cultures [Fig. 3A]. It was also noticed that the intensity of bands varied from sample to sample; for instance, in the case of SOD, four bands (SOD 1, SOD 2, SOD 3 and SOD 4) were found in both samples [Fig. 3B]. The intensity of GPX activity was found to be more in the callus culture, but both samples exhibited three isoforms including GPX 1, GPX 2 and GPX 3 [Fig. 3C].

**Table 3.** Evaluation of CAT, SOD and GPX assays from the callus and suspension culture samples of *W. somnifera*L.

| Type of enzyme assay | Enzyme activity (nkat/ mg of protein) (mean ± SEM) | |
|---|---|---|
| | Callus culture | Suspension culture |
| CAT | 301.01± 0.42 | 280.63 ± 0.14 |
| SOD | 198.92 ± 0.29 | 152.13 ± 1.02 |
| GPX | 103.75 ± 0.11 | 84.08 ± 0.97 |

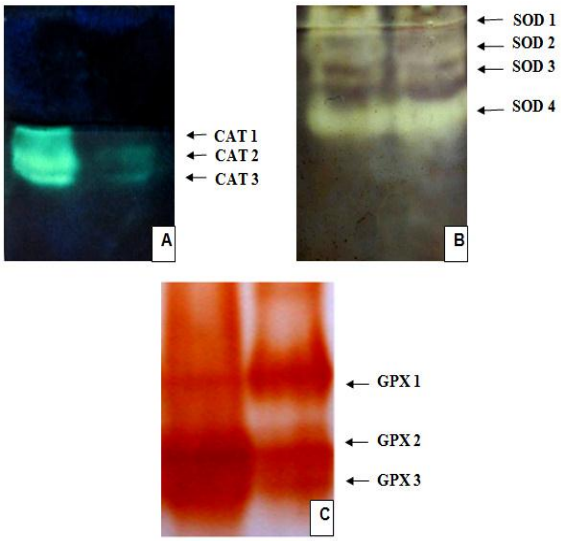


**Figure 3.** Activity staining of CAT (A), SOD (B) and GPX (C) from the callus and suspension cultures of *W. somnifera* L. Lane 1 (LHS): Iso-enzyme pattern in the callus extracts; Lane 2 (RHS): Iso-enzyme pattern in the suspension culture extracts, respectively, in each photograph.

In this study, generation of callus with the combination of auxins and cytokines exhibited enhancement in antioxidant activity. It was observed that the calli, which was obtained *in vitro*, showed significant amount of antioxidant enzymes (like CAT, GPX and SOD) in *Brassica nigra* plant [38, 39, 40]. Also it has been reported that in *W. somnifera*, the *in vitro* grown leaves contain significant amounts of antioxidant enzymes (like SOD, CAT and GPX) compared to the *in vivo* grown leaves. *In vitro* cultures may be considered as a model system for studies on the physiological effects of the stimulators and inhibitors of plant growth and development [28].

## 4. Conclusion

The findings of the study suggest that increased SOD, CAT and GPX activities were obtained in the static culture than in the suspension culture. Changes of iso-enzyme patterns determine the ability of plants to survive under stress conditions.

## 5. Acknowledgment

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## 6. Conflict of interest

The authors do not have any conflict of interest.

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