ORIGINAL ARTICLE

Antioxidative enzyme activities in calli during *in vitro* morphogenesis of *Sarpagandha* (*Rauwolfia serpentina* L.)

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Background: A reproducible and efficient regeneration system for production of uniform, elite, true to the type plantlets for large scale *in situ* and *ex situ* plantation is required to conserve the endangered medicinal plant *Sarpagandha* (*Rauwolfia serpentina* L. Benth. ex. Kurz). **Aims:** The aim of the present investigation was to assess antioxidative enzyme activities during the differentiation and regeneration of *Sarpagandha* and its role in catalytic scavenging of the reactive oxygen species for free radicals generated during *in vitro* morphogenesis. **Settings and Design:** The experiment was formulated using complete randomized design having 20 replicates per each treatment. Each experiment was repeated three times. **Materials and Methods:** The different explants of *Sarpagandha* were cultured aseptically in the Murashige and Skoog (MS) basal medium containing 3% sucrose (w/v), 0.7% agar and different concentration and combination of plant growth regulators. The antioxidative enzymes: catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), superoxide dismutase (EC 1.15.1.1) and guaiacol peroxidase (EC 1.11.1.7) were assayed employing standard biochemical procedure. **Statistical Analysis**: Data recorded on the different parameters were subjected to suitable statistical analysis based on the sample means of the various characters using SAS statistical software (SAS version 9.1, SAS Institute Inc. [Analysis of variance (ANOVA) was used to test statistical significance. The difference between significant treatments means and interactions were tested against C.D. at 5% ($P \leq 0.05$). **Results:** The maximum catalase (1.7 nkat/g fresh callus tissues) and superoxide dismutase activity (3.5 nkat/g fresh callus tissues) were observed in the shoot forming calli at the 4th week of culture. Shoot forming calli recorded the highest guaiacol peroxidase enzyme activity (2.6 nkat/g fresh callus tissues) at the 8th week of culture period whereas ascorbate peroxidase activity in the shoot forming calli at 1st week of culture recorded the maximum (0.3 nkat/g fresh callus tissues). Higher antioxidative enzyme activity was observed in shoot forming callus as compared to the non-shoot forming callus irrespective of enzymes studied. **Conclusions**: Higher enzyme activity was responsible for catalytic scavenging of the reactive oxygen species for free radicals and this could have led to a wholesome transformation of the component callus cells from an oxidized state to a more reduced state, the latter eventually become accountable for triggering the onset of embryogenesis.

Key words: Antioxidative enzyme activities, Callus, *in vitro* regeneration, *R. Serpentina*

INTRODUCTION

Rauwolfia serpentina L. Benth. ex. Kurz (family: Apocynaceae) is a woody perennial shrub, commonly known with different names; *sarpagandha*, snake root plant, chotachand, chandrika, etc. The roots of this plant have been used for centuries in ayurvedic medicines under the name *sarpagandha* and nakuli for the treatment of mental disorders. It has been stated that the drug is useful in epilepsy, sleeplessness and several other ailments.[1] Due to its medicinal values, the root of this plant has been popular both in India and Malaya-peninsula, from ancient times as an antidote to the stings of insects and poisonous reptile. It has also been used as febrifuge and stimulant to uterine contraction for insomnia and most of all for insanity.[2] It grows well in India, Pakistan, Sri Lanka, Burma and Thailand. In India, it is widely distributed in the sub-Himalayan tract from Punjab to Nepal, Sikkim and Bhutan. It is also found in the lower hills of Gangetic plains, Eastern and Western Ghats and Andamans.

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Its natural source is now almost exhausted due to indiscriminate, recurrent harvest and other anthropogenic pressure and activities. In Odisha, it is now mainly confined to some virgin valleys and hilly forest tracks. For the fulfillment of the present and future demand this rare medicinal shrub needs to be cultivated scientifically at a commercial scale. But its multiplication through seeds is difficult due to low germination percentage. Seed germination (15-20%) was observed in *Rauwolfia*, but only 10-13% plant development from the germinated seeds.[3] To cope up with alarming situation, plant tissue culture

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technique had been widely exploited for mass production of true to type, uniform, disease free plantlets. However, there exist three major limitations to the use of *in vitro* methods: (1) certain species are unresponsive (recalcitrant) to *in vitro* manipulation; (2) cultures maintained in the de-differentiated state for extended periods, lose their totipotent capacity and (3) there is an increased propensity for genetic instability in plants maintained *in vitro*. It is now widely accepted that free radical processes during *in vitro* cultures are associated with tissues development as well as cellular and genetic degeneration. Free radicals are a component of *in vitro* plant development. The application of exogenous antioxidants to plant cultures is used to stimulate morphogenetic responses and ameliorate deleterious oxidative stress. The present investigation was undertaken to study the activity of some antioxidative enzymes like catalase, ascorbate peroxidase, guaiacol peroxidase and superoxide dismutase during callus induction and its pivotal role during organogenesis.

MATERIALS AND METHODS

Plant Material

The *Rauwolfia* plants were grown in the herbal garden of Odisha University of Agriculture and Technology, Bhubaneswar, Odisha, India during the year (2008-11). The nodes, internodes and leaves of this plant were used as explants for the present *in vitro* culture. These explants were surface sterilized by soaking in 70% (v/v) ethanol for 1 min and then immersed in solution containing 1% (v/v) sodium hypochlorite and then washed for 20 min with Tween-20 solution (5–10 drops Tween-20 per litre of water). Then these explants were rinsed six times with sterile distilled water and then cultured onto Murashige and Skoog (MS) basal medium[4] containing 3% sucrose (w/v), 0.7% agar and different concentration and combination of plant growth regulators like 2,4-dichlophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA). The stock culture was sub-cultured at 20-30 days intervals. The calli were further transferred to the shoot induction medium for shoot regeneration. The regenerated shoots were excised and transferred to vessels containing 30 ml MS basal medium for root growth. MS basal medium with 3 different concentrations of NAA (0.2, 0.5 and 2.0 mg/l) and IAA (0.2, 0.5 and 2.0 mg/l) alone or in combination with 3% sucrose and 0.7% agar were tested for initiation, regeneration and growth of roots from cultured shoots. In this culturing stage, the levels of antioxidative enzyme synthesis were evaluated on different types of calli and their stages and culture condition.

Sampling for Biochemical Analysis

Explant derived callus was sub-cultured onto fresh medium for proliferation and further growth. At the first stage of callus induction, one set of callus tissues were transferred to the respective media for shoot regeneration, where the other set of callus was sub-cultured for further proliferation. At the end of each week callus samples of constant weight were harvested from proliferation medium as well as from regeneration medium separately and subjected to biochemical analysis.

Assay of Antioxidant Enzymes

The calli were homogenized under ice cold conditions with the pre-chilled mortar and pestle in the extraction buffer (pH 7.5). The extraction buffer used were 50 mM phosphate buffer for catalase, ascorbate peroxidase, guaiacol peroxidase and 50 mM Tris-HCl buffer containing 0.1 mM Ethylenediaminetetraacetic acid (EDTA) for superoxide dismutase. One ml homogenates were centrifuged at 12,000 rpm for 10 min at 4°C. The resultant supernatants were used for assay of enzymes after suitable dilution.

Catalase (EC 1.11.1.6)

The enzyme catalase was assayed as described by Abei.[5] The assay mixture consisted of 2.0 ml 0.1M phosphate buffer (pH 6.8), 0.5 ml 30 mM hydrogen peroxide and 0.5 ml diluted enzyme extract. The change in hydrogen peroxide concentration was recorded that 240 nm and catalase activity was calculated using extinction coefficient of 40 $mM^{-1}\,cm^{-1}$ for H_2O_2 at 240 nm. The activity is expressed in nanokatals (nkat), i.e. nmole of substrate used up per sec.

Ascorbate Peroxidase (EC 1.11.1.11)

The enzyme ascorbate peroxidase was assayed as described by Nakano and Asada.[6] The assay mixture consisted of 1.5 ml 0.1 M phosphate buffer (pH 6.8), 0.5 ml 3 mM ascorbate, 0.5 ml 12 mM hydrogen peroxide and 0.5 ml diluted enzyme extract. The change in absorbance was recorded at 290 nm. The ascorbate peroxidase activity was calculated using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ due to ascorbate depletion. The activity is expressed in nanokatals (nkat).

Superoxide Dismutase (EC 1.15.1.1)

The enzyme superoxide dismutase was assayed as described by Giannopolitis and Ries.[7] The assay mixture consisted of 2.5 ml Tris-HCl buffer (pH 8.9) containing 0.12 mM EDTA, 10.8 mM TEMED solution., 0.1 ml bovine albumin 3.3×10^{-3}(w/v), 0.1 ml 6 mM of nitroblue tetrazolium, 0.1 ml riboflavin 600 µm in 5 mM KOH and 0.2 ml enzyme extract. The initiation and termination of the reaction were done by turning the light on and off. The change in absorbance was recorded at 560 nm and its activity was expressed in units. One unit of SOD enzyme activity was defined as the amount that inhibited the nitroblue tetrazolium photo reduction by 50%.

Guaiacol Peroxidase (EC 1.11.1.7)

The enzyme guaiacol peroxidase was assayed as described by Kar and Feirabend.[8] The assay mixture consisted of

1.5 ml 0.1 M phosphate buffer (pH 6.8), 0.5 ml 30 mM guaiacol, 0.5 ml 30 mM hydrogen peroxide and 0.5 ml diluted enzyme extract. The change in absorbance due to tetraguaicol formation was recorded at 470 nm and its activity was calculated using extinction coefficient of 26.6 $mM^{-1}cm^{-1}$. The activity was expressed in nanokatals (nkat).

Data Analysis

Each *in vitro* experiment was replicated thrice with 20 explants per treatment. The frequency of shoot organogenesis and mean number of shoots per explant were determined at each stage on different media. Data recorded on the different parameters were subjected to suitable statistical analysis based on the sample means of the various characters.[9] All analytical determinations were repeated for three independent samples. Analysis of variance (ANOVA) was used to test statistical significance. The difference between significant treatment means and interactions were tested against C.D. at 5% ($P \leq 0.05$).[9] All the statistical calculation was done using SAS statistical software (SAS version 9.1, SAS Institute Inc., Cary, NC, USA)

RESULTS

The leaf and stem explant of *Rauwolfia serpentina* induced high-frequency callusing on modified Murashige and Skoog (MS) medium supplemented with 2.5 mg/l 2,4-D. Maximum regeneration of shoots from callus (90%) was observed in MS medium augmented with NAA (0.2 mg/l) and BA (1.5 mg/l). However, direct regeneration of shoots was recorded best in MS medium supplemented with BAP (2.5 mg/l). The maximum number of shoots per explant (6.5) was also highest in this phytohormone combination. Higher root induction from shoots was observed in MS medium supplemented with NAA (0.5 mg/l) [Figure 1].

The levels of antioxidants and the activities of antioxidant enzymes such as catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), superoxide dismutase (EC 1.15.1.1) and guaiacol peroxidase (EC 1.11.1.7) are investigated throughout the callus growth. The catalase activity was enhanced during the callus growth up to the end of 4th week followed by a steady decline till the termination of culture period (end of 8th week) both in the shoot forming and non-shoot forming cultures [Table 1]. The maximum catalase activity was observed in the shoot forming calli at the 4th week of culture (1.7 nkat/g fresh callus tissues). The non-shoot forming calli exhibited lower level of enzyme activity throughout the culture period as compared to the shoot forming calli.

The ascorbate peroxidase activity in the shoot forming calli at first week of culture recorded the maximum (0.3 nkat/g fresh callus tissues) and then gradual decrease of enzyme active was observed. In non-shoot forming calli, the activity

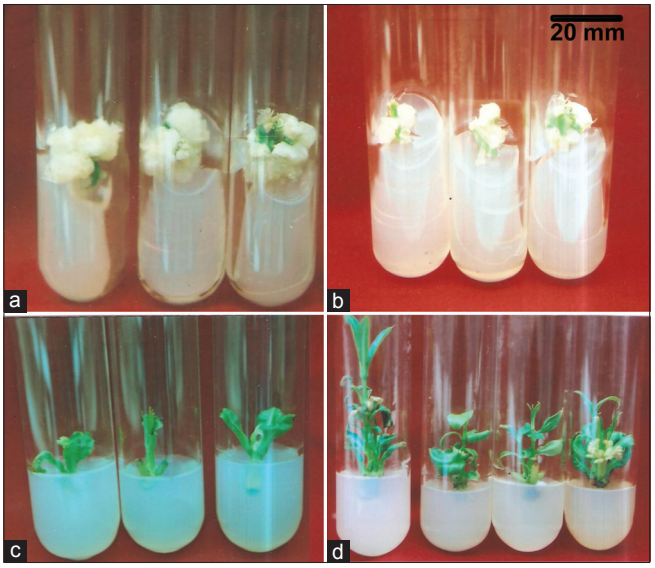

Figure 1: *In vitro* callus and multiple shoot induction from explant of *R. serpentina*, a = Shoot forming callus initiated from leaf explant on MS media supplemented with 2.5 mg/l 2.4-D mg/l IAA; b = Non-shoot forming callus initiated from leaf explant on MS media supplemented with 2.5 mg/l 2.4-D mg/l IAA; c = Shoot formation on nodal explants when cultured on MS medium supplemented with 2.5 mg/l BAP; d = Multiple shoot formation on nodal explants when cultured on MS medium supplemented with 2.5 mg/l BAP

Table 1: Antioxidative enzyme activity in the shoot forming and non-shoot forming calli nkat/g fresh callus tissues

Culture period (wk)	Catalase		Surperoxide dismutase		Ascorbate peroxidase		Guaiacol peroxidase	
	Shoot forming callus	Non-shoot forming callus	Shoot forming callus	Non-shoot forming callus	Shoot forming callus	Non-shoot forming callus	Shoot forming callus	Non-shoot forming callus
1	0.6	0.4	2.4	1.5	0.30	0.19	1.5	1.2
2	1.1	0.5	2.8	1.4	0.26	0.18	1.6	1.2
3	1.3	0.6	3.1	1.3	0.25	0.17	2.0	1.3
4	1.7	0.8	3.5	1.2	0.23	0.15	2.2	1.4
5	1.5	0.7	2.5	1.0	0.20	0.14	2.4	1.5
6	1.2	0.6	2.0	0.9	0.15	0.13	2.4	1.5
7	0.9	0.5	2.0	0.8	0.12	0.12	2.5	1.5
8	0.8	0.4	1.5	0.7	0.1	0.1	2.6	1.5
CD ($P = 0.05$)	0.12	0.08	0.21	1.1	0.05	0.03	0.18	0.09

Data shown were mean of the repeat experiments

of superoxide dismutase was decreased gradually till the termination of culture period (end of 8th week). On the other hand, in the shoot forming callus the activity of enzyme enhanced rapidly up to the end of 4th week of culture (3.5 nkat/g fresh callus tissues) and there after declined gradually till the end of 8th culture week (1.5 nkat/g fresh callus tissues) [Table 1 and Figure 1]. The shoot forming calli recorded higher superoxide dismutase enzyme activity as compared to the non-shoot forming calli throughout the culture period.

In shoot-forming calli, a high activity of guaiacol peroxidase was observed as compared to the non-shoot forming callus throughout the culture period. In shoot forming callus the activity of guaiacol peroxidase was enhanced up to the end of the culture period. The highest enzyme activity was observed at the 8th week of culture period (2.6 nkat/g fresh callus tissues).

DISCUSSION

Free radicals are associated with stress conditions and it is contributory factor in recalcitrance culture. Clearly, many products of secondary oxidative stress are cytotoxic and their production and interaction with macromolecules could actually promote genotoxicity and enzymatic dysfunction in culture systems. Oxidative processes may also have a direct role in *in vitro* development. Morphogenesis is a dynamic process controlled by the application of exogenous, plant growth regulators, which has capacity to alter primary oxidative metabolism and directly influence hormonal transduction pathways involving activated oxygen species. These activities also changed in relation to the culture condition, which modified callus differentiation. It also showed that the abilities of tissues to regenerate depended on the physiological state of cells and might be correlated with the production of reactive oxygen species.[8,10]

The callus induction from *in vitro* cultured explant and further differentiation of calli to shoots and roots depend on the composition of nutrients, vitamins and growth hormones in culture medium and culture conditions. During the culture period, the Superoxide radicals ($O_2^{\cdot-}$) and other activated oxygen species such as excited oxygen ($^1O_2$), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) are generated due to auxin accumulation.[11] The superoxide radicals and activated oxygen species are very highly reactive and in the absence of any protective mechanism could irreversibly cause cellular damages.[10] The armory of endogenous protective mechanisms include carotenoids, glutathione, ascorbate, a-tocopherol and several enzymes such as superoxide dismutase, catalase, ascorbate peroxidase which can effectively scavenge the toxic oxygen species as soon as these are formed in the cell compartments.

Shoot forming calli represent an auxin–deficient stress system and therefore, may generate free radicals which have recently been observed in calli initiated from rice embryos.[12] When the callus enters into the phase of embryogenesis, the constituent cells` may experience a switching over to a relatively more reduced state; the latter is expected to be associated with a decreased formation of reactive oxygen species or free radicals. This could explain for an increased activity of catalase, superoxide dismutase and ascorbate peroxidase in embryogenic calli compared to that without undergoing embryogenesis. Elevated levels of superoxide dismutase, catalase, and ascorbate peroxidase have also been correlated with increased level of oxygen stress resistance.[13,14] In the present investigation, shoot forming calli exhibited elevated catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), superoxide dismutase (EC 1.15.1.1) and guaiacol peroxidase (EC 1.11.1.7) activities as compared to non-shoot forming calli in all stages of growth which are in accordance with findings described earlier.[10-14] Changes in the antioxidant status of plant cultures, particularly those which are de-differentiated may be related to totipotent capacity. Manipulation of the oxidative status of plant cultures may provide a useful means of maintaining morphogenetic competence or overcoming recalcitrance and this may best be explored in relation to the application of exogenous hormones and cell signaling pathways. In other words, the higher order of enzyme activity calli placed on the embryogenesis medium was responsible for catalytic scavenging of the reactive oxygen species for free radicals. Perhaps, this could have led to a wholesome transformation of the component callus cells from an oxidized state to a more reduced state; the latter eventually become accountable for triggering the onset of embryogenesis.

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