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BRIEF COMMUNICATION

Activity of antioxidant enzymes and secondary metabolites during *in vitro* regeneration of *Sterculia urens*

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Abstract

The changes in the activities of antioxidant enzymes and amounts of proteins, phenols, and flavonoids in regenerating and non-regenerating calli during organogenesis of *Sterculia urens* were monitored. Maximum growth of calli and the most efficient regeneration of shoots occurred on Murashige and Skoog (MS) medium supplemented with 0.5 mg dm^{-3} benzylaminopurine (BAP) + 2 or 4 mg dm^{-3} naphtalene acetic acid (NAA). Peroxidase (POD), catalase, and superoxide dismutase activities increased in the regenerating calli but decreased in the non-regenerating calli. Six POD isoenzymes were detected. Protein content decreased in the non-regenerating calli and increased significantly during regeneration of shoots from callus. Total phenols and flavonoids increased in the non regenerating calli. SDS-PAGE analysis revealed a role of many proteins in organogenesis.

Additional key words: auxin, catalase, cytokinin, hydrogen peroxide, peroxidase, reactive oxygen species, superoxide dismutase.

In plants, reactive oxygen species (ROS), such as the superoxide radical, hydrogen peroxide, and singlet oxygen are constantly produced. The production of ROS has been associated with plant recalcitrance during *in vitro* culture (Benson 2000). The overproduction of ROS is defined as an oxidative stress and is a common phenomenon in plants when they are exposed to stresses like drought, salinity, extreme temperatures, high irradiance, or *in vitro* cultures (Price *et al*. 1989, Cassells and Curry 2001). Plants have developed a complex antioxidant system to protect themselves against oxidative damage (Larson 1988). Antioxidant protection systems include enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and polyphenol oxidase (PPO) as well as non-enzymatic antioxidants like phenols, α-tocopherol, carotenoids, *etc*. The role of ROS in plant growth and development is substantiated by the interplay of ROS with a number of phytohormones. Moreover, ROS have been implicated as second messengers in several plant hormone responses. Therefore, the study of the ROS production, oxidative stress, and the efficiency of antioxidants during different stages of organogenesis is of increasing interest. The objective of the present study was to compare the activities of enzymatic and non-enzymatic antioxidants and also content of a few secondary metabolites in regenerating and non-regenerating calli at various stages of shoot organogenesis in *Sterculia urens*. In addition, SDS-PAGE analyses of proteins and different POD isoenzymes were done.

Healthy seeds were collected, surface sterilized, and cultured on Murashige and Skoog (1962; MS) basal medium. After 10 - 15 d, cotyledonary nodes were excised and inoculated on MS basal medium supplemented with 0.5 mg dm^{-3} benzylaminopurine (BAP) + 2, 4, or 6 mg dm^{-3} naphtalene acetic acid (NAA). All cultures were incubated at temperature of 25 ± 2 °C, relative humidity of 60 %, a 16-h photoperiod, and irradiance of 40 - 50 μmol m^{-2} s^{-1}.

For biochemical analysis, about 1 g of regenerating (RC) and non-regenerating (NRC) calli of 15-d-old and 30-d-old cultures was homogenized separately with

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Abbreviations: BAP - benzylaminopurine; CAT - catalase; MS - Murashige and Skoog; NAA - naphtalene acetic acid, NRC - non-regenerating calli; PAGE - polyacrylamide gel-electrophoresis; POD - peroxidase, RC - regenerating calli; ROS - reactive oxygen species; SDS - sodium dodecyl sulphate, SOD - superoxide dismutase.
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2.0 cm^3 of pre-chilled phosphate buffer (0.1 M, pH 7.6) containing 0.1 mM EDTA in a chilled mortar and pestle. The homogenate was squeezed through double layered cheese cloth and centrifuged at 13 000 *g* and 4 °C for 15 min. The supernatant was used for the assay of enzymatic and non-enzymatic antioxidants. Cotyledonary node explants were used as a control.

The assay of SOD (EC1.15.1.1) was carried out by the method of Beauchamp and Fridowich (1971) based on the reduction of nitroblue tetrazolium (NBT) chloride. The CAT (EC 1.11.1.6) activity was assayed by the titrimetric method described by Radha Kissnan and Sarma (1963). The POD (EC 1.11.1.7) activity was assayed spectrophotometrically with *o*-dianisidine as hydrogen donor (Shannon *et al*. 1966, Sadasivam and Manickam 1996). Polyphenol oxidase activity was measured by the modified methods of Bateman (1962) and Sadasivam and Manickam (1996). For estimation of total soluble proteins and phenols, the supernatant from the above extract was precipitated with 200 g dm^{-3} trichloroacetic acid. The precipitate was used for protein estimation (Lowry *et al*. 1951) with bovine serum albumin as standard and the supernatant was used for phenol estimation according to Sadasivam and Manickam (1996). Total flavonoid content was measured by aluminum chloride assay described by Marinova *et al*. (2005).

For determination of protein profile of RC, NRC, and control samples, discontinuous sodium dodecyl sulphate (SDS)-PAGE was performed as described by Laemmli (1970) using 12 % (m/v) acrylamide. For isozyme separation, native PAGE was carried out by a modified method of Davis (1964) with a 10 % acrylamide gels at 4 °C without SDS and mercaptoethanol. Electrophoretic pattern of POD was obtained by staining the gels with benzidine according to Van Loon and Geelen (1971). The gels were immersed in 0.2 M acetate buffer (pH 4.8) containing 3 % (v/v) H_2O_2 and 4 % (v/v) benzidine in 50 % (v/v) methanol in dark at room temperature till brown bands appeared.

Statistical analyses were performed with general linear model (LCM) procedure of *SAS 9.2 software*. Data were subjected to *ANOVA* and differences among means were analyzed by Duncan's multiple range test at $\alpha = 0.05$. Each experiment was repeated at least three times.

Callus initiation started from cut ends within 7 - 10 d and shoot bud sprouting within 10 - 12 d on MS + BAP + NAA (Table 1). Of the three combinations, 0.5 mg dm^{-3} BAP + 4 mg dm^{-3} NAA gave maximum number of regenerating calli. The 15-d-old callus with sprouted shoots was taken as RC (Fig. 1*A*) and without sprouting as NRC (Fig. 1*B*). The 30-d-old callus with further increase in shoot number and shoot length was taken as RC (Fig. 1*C*) and without shoots but with increasing callus size as NRC (Fig. 1*D*). A gradual increase in POD, CAT, SOD, and PPO was observed in 15-d-old RC as well as NRC. In 30-d-old RC, PPO content gradually decreased but other three enzymes remained elevated. In 30-d-old NRC, POD activity further increased, SOD activity remained unchanged whereas CAT and PPO activities decreased (Table 2).

Protein content gradually increased during initial stages of RC but thereafter no prominent increase was observed. Phenol and flavonoid content was higher in NRC compared to RC. SDS-PAGE analysis revealed several distinct protein bands in RC and NRC but not in control samples (Fig. 2). Specifically, an accumulation of 18 to 13 kDa proteins was noted only in RC of 30-d-old calli. In non-denaturing PAGE, a total of 6 POD isoforms were detected (Fig. 3). POD1 was shown in control, POD2 in NRC and RC of 30-d-old calli, POD3, POD4, and POD 5 in all three samples, and POD6 in 15-d-old and 30-d-old NRC and RC. The amount of these isoenzymes was higher in 30-d-old calli when compared to 15-d-old calli. Very faint bands in control indicated low content of peroxidases.

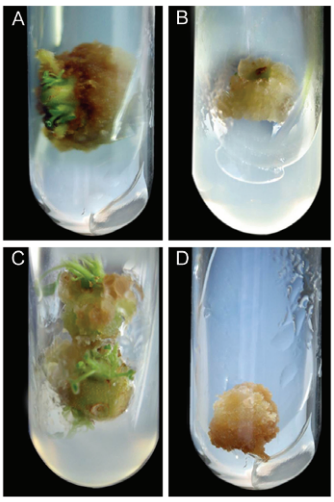

Fig. 1. Regenerating callus on MS + 0.5 mg dm^{-3} BAP + 2 mg dm^{-3} NAA (*A* - 15-d-old, *C* - 30-d-old) and non-regenerating callus on MS + 0.5 mg dm^{-3} BAP + 6 mg dm^{-3} NAA (*B* -15-d-old, *D* - 30-d-old).

Earlier, we reported multiple shoot regeneration of *S. urens* (Subhashini Devi *et al*. 2011) on MS medium with BAP or thidiazuron (TDZ). In this study, a combination of BAP and NAA was used in order to increase the frequency of callus formation. Callus induction was observed within 7 d on MS medium containing 0.5 mg dm^{-3} BAP + 4 mg dm^{-3} NAA. Similar results

Table 1. *In vitro* response of cotyledonary node explants producing non-regenerating calli (NRC) and regenerating calli (RC) on MS medium with 0.5 mg dm^{-3} BAP + different concentrations of NAA. Means ± SE, $n = 25$.

NAA [mg dm^{-3}]	RC [%]	NRC [%]
2	49.33 ± 1.33	37.33 ± 1.33
4	76.00 ± 2.31	18.33 ± 1.67
6	22.67 ± 1.33	50.67 ± 1.33

Table 2. Activities of antioxidant enzymes POD, CAT, SOD, and PPO [U mg^{-1}(protein) min^{-1}] and content of proteins, phenols, and flavonoids [mg g^{-1}(f.m.)] in the control, 15-d-old, and 30-d-old RC and NRC. Means ± SE, $n = 3$. Means within a column followed by the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's multiple range test.

	POD	CAT	SOD	PPO	Proteins	Phenols	Flavonoids
Control	24.7±0.32^{e}	285.6±0.34^{d}	1.1±0.00^{e}	5.7±0.06^{c}	70.0±0.57^{b}	51.0±0.57^{c}	254.3±0.66^{b}
15-d-old RC	223.7±0.46^{c}	416.8±0.56^{b}	2.7±0.06^{d}	9.0±0.03^{a}	101.0±0.57^{a}	50.3±0.33^{c}	301.6±0.88^{a}
30-d-old RC	556.7±0.62^{a}	488.5±0.77^{a}	4.5±0.06^{a}	3.5±0.04^{d}	101.6±1.20^{a}	51.6±0.88^{c}	251.6±0.88^{b}
15-d-old NRC	145.7±0.48^{d}	357.5±0.45^{c}	3.3±0.09^{c}	6.6±0.08^{b}	71.0±0.57^{b}	100.3±0.33^{b}	252.0±1.53^{b}
30-d-old NRC	377.6±0.44^{b}	221.2±0.47^{e}	3.7±0.05^{b}	3.3±0.04^{d}	61.6±0.88^{c}	121.0±0.57^{a}	302.0±1.53^{a}

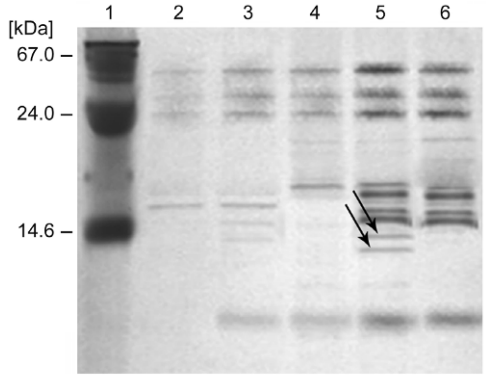

Fig. 2. SDS-PAGE pattern of proteins during regeneration of *S. urens*: 1 - molecular mass marker, 2 - control, 3 - 15-d-old regenerating callus (RC), 4 - 15-d-old non-regenerating callus (NRC), 5 - 30-d-old RC, and 6 - 30-d-old NRC. *Arrows* indicate specific bands in 30-d-old RC.

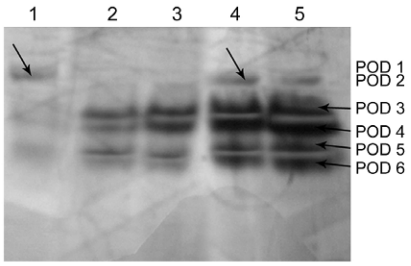

Fig. 3. Staining of POD isoenzymes: 1 - control, 2 - 15-d-old NRC, 3 - 15-d-old RC, 4 - 30-d-old NRC, 5 - 30-d-old RC. *Arrows* indicate specific POD isoforms.

where a combination of BAP with NAA gave highest frequency of callus formation were reported by Jana and Shekhawat (2012). Regenerating callus was observed at low concentrations of NAA while NRC at higher concentrations. The activities of all antioxidant enzymes were relatively high during the course of callus differentiation and regeneration when compared to cotyledonary node explants at the time of inoculation. Increased content of SOD, POD, CAT, and PPO in RC shows that these enzymes might affect content of endogenous phytohormones which consequently affected cell proliferation and differentiation. The POD and CAT activities in 15-d-old and 30-d-old RC with shoots were higher than those in NRC. Increase in POD and CAT activities during shoot organogenesis was reported in *Prunus* (Franck *et al.* 1995), *Solanum* (Kumar and Knowles 1993), and *Gladiolus* (Gupta and Datta 2003). SOD activity was also higher in RC than in NRC but not as prominently as POD and CAT activities. Increased activities of SOD and CAT were observed during callus differentiation in *Acanthophyllum sordidum* (Meratan *et al.* 2009) and in *Arachis hypogaea* (Zheng *et al.* 2005). SOD, POD, and CAT are known to play an important role in growth and differentiation (Gaspar *et al*. 1985, Jana and Shekhawat 2012, Mitrović *et al*. 2012) and their activity can be correlated with the process of differentiation during organogenesis. PPO activity increased during first 15 d of callus formation but then decreased in RC and remained unchanged in NRC. Increased activity of PPO during first 3 weeks followed by its decrease was reported in *Pinus strobus* (Tang and Newton 2005). Although PPOs are ubiquitous in angiosperms, their activities are induced in plant defense against pathogens and insects (Thipyapong *et al.* 2004) rather than by *in vitro* stress. The reports of Li and Steffens (2002) also show no role of PPO in callus differentiation. Our results suggest that the low H_2O_2 content might be involved in regulation of regeneration in *S. urens* which was confirmed by the results obtained by Gupta and Datta (2003), Jana and Shekhawat (2012), and Mitrović *et al*. (2012).

The total phenol content increased in 15-d-old and 30-d-old NRC but no change in RC was observed. These results suggest that accumulation of phenols might be inversely correlated with growth potential in *S. urens* callus. Similar findings were reported by Nakamura *et al.* (1998) and Arnaldos *et al*. (2001) during strawberry callus development. Flavonoids increased in 30-d-old NRC when compared to 15-d-old but decreased in RC. Similar results were observed during strawberry callus development (Arnaldos *et al.* 2001), *Momordica charantia* (Agarwal and Kamal 2007), and in *Hypericum mysorense* (Shilpashree and Rai 2009). The presence of BAP in MS medium was shown to induce production of flavonoids in *Hypericum perforatum* (Dias and Tomas 1998). The present study shows accumulation of flavonoids in callus cultures of *S. urens.* These results

suggest the possibility of production of some important secondary metabolites at this stage of *S. urens* cultures.

The organogenesis from the callus is a very complex process accompanied with expression of different genes and protein synthesis. A higher content of proteins in 30-d-old RC than in 30-d-old NRC was observed. Similar results were reported in sugarcane (Neves *et al*. 2003), *Arachis hypogaea* (Zheng *et al*. 2005), and *Asteracantha longifolia* (Panigrahi *et al*. 2007). Composition of POD isoenzymes can be used as potential marker system to characterize developmental pathway. As mentioned above, peroxidase zymogram in RC and NRC revealed heterogeneous banding pattern consisting of six distinct bands. Similar observations were reported in specific phases of *Crocus sativus* (Vatankhah *et al*. 2010) and *Tacitus bellus* (Mitrović *et al*. 2012) regeneration.

From the above findings, we conclude that oxidative stress developed during *in vitro* culture of *S. urens* due to low activities of POD, CAT, and SOD. Thus a direct relationship exists between increased activity of antioxidant enzymes and the presence of ROS in *S. urens*. The greater increase in POD, CAT, and SOD activities in RC than in NRC might suggest effective ROS scavenging including H_2O_2 in regenerated shoot. The roles of specific POD isoforms in RC and NRC suggest the fine regulation of the H_2O_2 content during shoot organogenesis in *S. urens*. Hence, a manipulation of the oxidative status of plant cultures may provide useful means of maintaining morphogenetic competence or overcoming recalcitrance.

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