Indian Journal of Natural Rubber Research, **16** (1 & 2) : 45-52, 2003

ABIOTIC STRESS INDUCED OVER-EXPRESSION OF SUPEROXIDE DISMUTASE ENZYME IN TRANSGENIC *HEVEA BRASILIENSIS*

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Sobha, S., Sushamakumari, S., Thanseem, I., Rekha, K., Jayashree, R., Kala, R.G., Jayasree, P.K., Asokan, M.P., Sethuraj, M.R., Dandekar, A.M and Thulaseedharan, A. (2003). Abiotic stress induced over-expression of superoxide dismutase enzyme in transgenic *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research*, **16** (1 & 2) : 45-52.

Abiotic stress induced over-expression of superoxide dismutase (SOD), catalase and peroxidase enzymes were studied in the transgenic embryogenic calli of *Hevea brasiliensis*, clone RRII 105. Two month old calli were transformed using *Agrobacterium tumefaciens* harbouring the binary vector containing neomycin phosphotransferase (*npt*-II) for kanamycin resistance as the marker gene for selection, β-glucuronidase (GUS) as the reporter gene and the sequence coding for SOD enzyme under the control of FMV 34S promoter. The transformed calli were proliferated in modified Murashige and Skoog medium fortified with hormones 2,4-D (4.5 µM), BA (2.2 µM) and NAA (1.1 µM). Over-expression of SOD, peroxidase and catalase enzymes in response to abiotic stresses like water stress, osmotic stress and different light regimes in transgenic embryogenic callus cultures were determined. Water stress was induced by the addition of different concentrations (0.2-1.0%) of phytagel and osmotic stress using polyethylene glycol (PEG), mannitol and sorbitol (2-10%) in the culture medium. More than 50% over-expression of SOD was observed when 0.4% phytagel was added to the medium and 40% over-expression was obtained when the culture medium was supplemented with 4.0% PEG. Catalase and peroxidase were also over-expressed correspondingly.

Key words: Genetic transformation, *Hevea brasiliensis*, Superoxide dismutase, Transgenic callus.

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INTRODUCTION

The recent developments in genetic modification techniques have opened new avenues for the production of fertile transgenic plants with increased yield, stress tolerance and resistance to pathogens. Although, genetic transformation and stable integration of foreign genes have been successful in many cereal crops and herbaceous annual plants, the success in perennial tree crops is rather limited (Dandekar *et al.*, 1988; James *et al.*, 1989; Ueno *et al.*, 1996). In *Hevea brasiliensis*, *Agrobacterium tumefaciens* as well as particle bombardment mediated genetic transformation and subsequent plant regeneration have been reported earlier (Arokiaraj *et al.*, 1994 and 1998; Jayashree *et al.*, 2000).

Active oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are produced in aerobic organisms as a consequence of oxygen metabolism (Asada and Takahashi, 1987). Green plant tissues may produce more active oxygen species than animal tissues, because plants generate oxygen during photosynthesis and consume it during respiration. Further, because of their growth under high light intensities and a high cellular concentration of dioxygen, plants are subjected to oxidative stress. Increased antioxidant enzyme activities have been reported in response to heat and light conditions that cause sunscald in vegetables, fruits and flowers (Rabinowitch and Sklan, 1980).

Superoxide dismutase (SOD) [EC 1.15.1.1.] is the first enzyme involved in the detoxifying process of the active oxygen species. SOD enzymes are a class of metallo enzyme, which are known to accelerate the spontaneous dismutation of superoxide radical to hydrogen peroxide and molecular oxygen (Fridovich, 1986). Peroxidase and catalase enzymes eliminate the hydrogen peroxide. Thus the combined action of these enzymes convert superoxide radical and hydrogen peroxide to water and molecular oxygen.

$$(O^{\cdot}_2) + (O^{\cdot}_2) + 2(H^+) \xrightarrow[SOD]{} H_2O_2 + O_2$$

In plants, the formation of superoxide and other active oxygen species is accelerated as a consequence of various abiotic stresses like water, salinity, high or low light intensities (Scandalios, 1990). The accumulation of active oxygen species, particularly superoxide anion, causes a process of oxidative deterioration that ultimately leads to cell death (Thompson *et al.*, 1987). Therefore, protection of the cells from the damaging effects of free radical is highly essential for their survival in the aerobic environment. It is reported that in *H. brasiliensis* plants experiencing drought stress, there is an increased diversion of photosynthetic electrons away from the carbon to molecular oxygen leading to enhanced production of reactive oxygen species and free radicals which hastens leaf senescence (Jacob and Karaba, 2000). Das *et al.* (1998) reported that tapping panel dryness (TPD), a physiological disorder in *H. brasiliensis* is associated with a higher level of free radical accumulation and a reduced level of SOD activity.

The present paper reports genetic transformation of *H. brasiliensis* for the over-expression of SOD for enhancing the efficiency of scavenging superoxide radicals to confer tolerance to environmental stress and TPD. The over-expression of the free radical scavenging enzymes SOD, peroxidase and catalase in response to induced abiotic stresses like water stress, osmotic stress and different light regimes in normal and transgenic callus cultures were studied.

MATERIALS AND METHODS

Genetic transformation and development of transgenic calli

Hevea (clone RRII 105) calli from immature anthers were developed following the method developed by Jayasree *et al.* (1999). Yellow friable calli were used for *Agrobacterium* mediated genetic transformation. The plasmid vector used was pDU 96-2412 that contains *uidA* as reporter gene, *npt* II as selectable marker gene and *Hevea* Mn-SOD gene under the control of FMV 34 S promoter (Fig1). The 702 nucleotide Mn - SOD cDNA was obtained by RT-PCR of mRNA isolated from *H. brasiliensis* using primer sequences corresponding to a previously published sequence of *Hevea* Mn- SOD (Miao and Gayner, 1993). The binary vector pDU 96-2412 was inserted in the *Agrobacterium* strain EHA 101 to create a functional vector for transformation experiments.

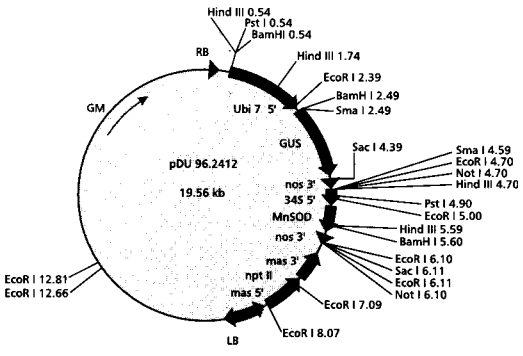

Fig. 1. Plasmid vector pDU96-2412 for the expression of HbMnSOD

Two month old calli were infected with the *Agrobacterium* strain. The infected calli and bacteria were co-cultivated for 72 hours in darkness, air dried in the laminar flow

hood and placed on MS (Murashige and Skoog, 1962) basal medium, supplemented with hormones 2-4 D (4.5 µM), BA (2.2 µM), cefotaxime (500 mg/l) to prevent the overgrowth of *Agrobacterium* and kanamycin (300 mg/l) for selection of transformed cell lines. The transgenic cell lines were separated after checking GUS expression. The transformed calli were proliferated by subculturing in modified MS medium supplemented with B_5 vitamins (Gamborg *et al.*, 1968), 3% sucrose, 500 mg/l casein enzymatic hydrolysate, 300 mg/l glutamine and hormones 2, 4-D (4.5 µM), BA (2.2 µM) and NAA (1.1 µM). The transformed calli were periodically subcultured in the same medium at 30 day intervals and embryogenic calli were obtained after six months. The pH of all media was adjusted to 5.6 before autoclaving at 121°C for 15 min.

DNA was isolated from transgenic as well as control calli by CTAB method (Doyle and Doyle, 1990) for Southern hybridisation. 10 µg of genomic DNA was digested with the restriction enzyme *Sac* I and the fragments were separated in 1% agarose gel. A 2.31 bp *Eco* R I fragment containing the GUS coding region of the plasmid vector was used as the positive control and the DNA from the non-transgenic callus digested with *Sac* I was used as the negative control. Blots were prepared by transferring them to nylon membranes (Hybond N^+, Amersham) by capillary blotting. The membrane was hybridized with ^{32}P dCTP labelled GUS probe, synthesized using multiprime labeling kit (Amersham). Hybridization was performed for 16 hrs and the membrane was washed twice at room temperature in 2X SSC and 0.1% SDS followed by two washes (15 min each) at 65°C in 0.1X SSC and 0.1% SDS. Blots were exposed to X-ray film overnight at -70°C.

Induction of stress

The transgenic embryogenic calli (confirmed by GUS staining and Southern hybridization) were used for the induction of stress keeping non-transgenic (normal) calli as control. Water stress was induced by subculturing the callus in media containing different concentrations (0.2- 1.0%) of phytagel and osmotic stress was induced by subculturing in media containing different concentrations of either polyethylene glycol (PEG, molecular weight 6000), mannitol or sorbitol (2-10%) in hormone free half strength MS media. The cultures were maintained in darkness at 25±1°C and were subcultured at 30 day intervals. For determining the effect of light on enzyme activity, the cultures were kept in two light regimes, continuous light and complete darkness for 30 days. The enzyme analyses were carried out after stress induction for one month. The experiment was repeated thrice using 10 replicate samples for each treatment.

Enzyme preparation

Transgenic and control embryogenic calli (0.5 g) were homogenised in a mortar and pestle after freezing in liquid nitrogen and the fine powder were resuspended in 5.0 ml phosphate buffer (0.1 M, pH 7.0). The resulting slurry was filtered through cheesecloth and the filtrate was centrifuged at 8000 X g at 4°C for 10 minutes. The supernatant was used for the assay of superoxide, peroxidase and catalase enzymes activity.

Enzyme assay

Superoxide dismutase enzyme activity was estimated following the method of Kakkar *et al.* (1984). The assay mixture contained 1.2 ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml phenazine methosulphate (186 µM), 0.3 ml nitroblue tetrazolium (300 µM), 50 µM enzyme preparation and distilled water in a total volume of 3.0 ml. The reaction was started by the addition of 0.2 ml of β-NADH (780 µM), allowed to continue for 90 sec at 30°C and then stopped by adding 1.0 ml acetic acid. Colour intensity of the chromogen in the reaction

mixture was measured at 560 nm in a Beckman UV spectrophotometer. The reaction mixture devoid of enzyme that served as control developed maximum colour, which decreased with increasing concentration of enzyme. One unit of enzyme activity was defined as the enzyme concentration required to reduce the optical density (at 560 nm) of chromogen by 50% in one minute under the assay conditions and was expressed as specific activity in milli units per minute per mg protein. Since the assay was done for 90 sec, a factor of 2/3 was applied for calculating units.

Peroxidase activity in the sample was estimated according to Addy and Goodman (1972). The reaction mixture contained 2.5 ml phosphate buffer (0.1 M, pH 6.5), 0.5 ml guaicol (10 µM) and 10 µM enzyme preparation. The blank consisted of all the components of the reaction mixture except hydrogen peroxide, which was replaced by water. The reaction was started by adding 0.2 ml H_2O_2 (20 µM). The quantity of tetraguaicol formed during the reaction was measured in a spectrophotometer at 420 nm for two minutes at 15 sec. intervals. Enzyme activity was calculated from the increase in absorbance at 420 nm after the addition of hydrogen peroxide. One unit of enzyme activity was defined as the change in OD per min per mg protein and expressed as specific activity.

The catalase activity was estimated spectrophotometrically by measuring the initial rate of disappearance of hydrogen peroxide at 240 nm by the method of Machly and Chance, (1954). The reaction mixture contained 2.5 ml phosphate buffer (0.01 M, pH 7), 0.5 ml H_2O_2 (30 µM) and 0.2 ml of enzyme extract. The reaction was started by the addition of H_2O_2 and the decrease in hydrogen peroxide was followed as a decline in OD at 240 nm. The activity was expressed in units where one unit of catalase converts one millimole of H_2O_2 per minute per mg protein.

The protein content in the samples were estimated following Lowry *et al.* (1951) after precipitating the protein with 10% trichloroacetic acid. Each experiment was repeated thrice in RBD and the data was analysed statistically.

RESULTS AND DISCUSSION

The callus clumps growing in the presence of kanamycin (300 mg/l) were GUS positive and represent the minimum number of putative transformation events. The GUS positive and control calli proliferated in modified MS medium. Yellow friable embryogenic calli were formed after subculturing for six months in the same medium. Presence of *uid*A gene was detected in the transgenic callus by southern hybridization with GUS probe and its stable integration was confirmed by digesting the DNA with *Sac* I enzyme. Hybridisation with GUS probe produced bands, with more than 4.5 kb size representing the DNA fragments containing the GUS gene and a part of the plant genomic DNA located outside the left T-DNA border (Fig. 2). Each band that hybridized to genomic DNA represent a single copy of the transgene.

Analysis of the transgenic and normal embryogenic calli provided interesting insights into the expression of free radical scavenging enzymes, particularly superoxide dismutase, in response to abiotic stress. The transgenic calli in which water stress was induced with 0.4% phytagel exhibited more than 50% increase in SOD activity. On further increasing the phytagel concentration the SOD activity started declining in transgenic as well as in control calli. However, compared with the control, at all concentrations tried, the transgenic calli exhibited a higher SOD activity (Table 1).

The role of SOD in the protection of plants against oxidative stress is well documented (McKersie *et al.*, 1993; Van Camp *et al.*, 1996; McKersie *et al.*, 1999). The present

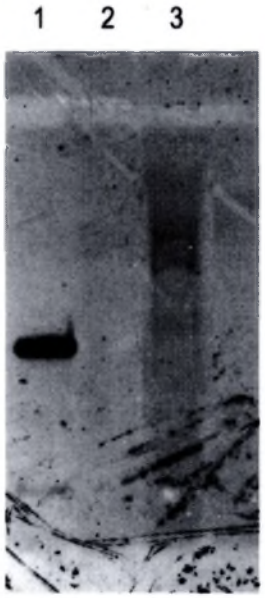

Fig. 2. Southern hybridisation with GUS probe; Lane 1 : 2.3 kb *Eco* R I digested fragment which contains the GUS region of the plasmid pDU96.2412 (positive control); Lane 2 : Nontransgenic DNA (negative control); Lane 3 : DNA isoloated from transgenic callus digested with *Sac* I enzyme

Table 1. **Effect of phytagel induced stress on SOD activity**

Phytagel conc. (%)	SOD activity (m.units/min/mg protein)	
	Normal	Transgenic
0.2	9.15	10.07
0.4	9.88	16.78
0.6	8.42	12.95
0.8	7.23	11.17
1.0	6.59	8.95

CD(AB)=0.89
A: Types of callus B: Phytagel concentration

results indicate the over-expression of SOD probably as an indicator of stress-induced accumulation of superoxide and the increased enzyme activity protected the cells from the damaging effect of O_2^-. However, at and above 0.8% phytagel concentration the enzyme activity decreased and browning of the tissues occurred in transgenic as well as in normal calli. Probably the enzyme level at this concentration may not be adequate to scavenge the O_2^- effectively and free radical accumulation might have caused cell damage which led to the browning of the tissue and finally to cell death. Gupta *et al.*, (1993) also reported the over- expression of SOD in transgenic tobacco plants and protection against oxidative stress caused by exposure to high light and low temperature.

The SOD expression observed was similar when osmotic stress was induced with different concentrations of PEG ranging from 2-10%. The highest enzyme activity in transgenic callus was observed with 4% PEG, which is more than 40% higher than in the control (Table 2). On further increasing the PEG concentration, the SOD activity decreased both in the transgenic and normal cultures (Table 2). The difference in SOD specific activities of transgenic and control tissues due to water and PEG stress were found to be statistically significant. No significant difference in SOD activity was observed when different concentrations of mannitol or sorbitol were used to impart stress. Further, no significant difference in the enzyme activities were observed between the cultures maintained in complete darkness or in continuous light for 30 days (Data not shown).

Table 2. **Effect of PEG induced stress on SOD activity**

PEG conc. (%)	SOD activity (m.units/min./mg protein)	
	Normal	Transgenic
2	10.77	10.83
4	11.23	16.27
6	9.38	15.07
8	7.54	12.78
10	5.83	11.80

CD(AB)=0.84
A: Types of callus B: PEG concentration

The peroxidase activity was also found to be higher in calli over-expressing SOD. This may be due to the increased SOD activity during induced stress, which led to the accumulation of hydrogen peroxide. Accumulation of H_2O_2 is toxic to cells and the ex-

tent of damage depends on how efficiently the H_2O_2 is eliminated by the peroxidase and catalase enzymes. The observed increase in peroxidase and catalase activity may be due to the increased substrate availability. The highest level of peroxidase activity was observed at the phytagel (0.4%) and PEG (4.0%) concentrations in which the SOD activity was maximum (Table 3). On further increasing the phytagel and PEG concentrations the peroxidase activity decreased. However, in the non transgenic calli a drastic reduction in peroxidase activity was observed at 6% PEG concentration followed by an increase at 8 % (Table 4).

Table 3. **Effect of phytagel induced stress on peroxidase activity**

Phytagel conc. (%)	Peroxidase activity (units/min/mg protein)	
	Normal	Transgenic
0.2	9.53	11.80
0.4	15.83	22.18
0.6	11.69	17.71
0.8	11.35	16.34
1.0	9.69	12.23

CD (AB) =1.62
A: Types of callus B: Phytagel concentration

Table 4. **Effect of PEG induced stress on peroxidase activity**

PEG conc (%)	Peroxidase activity(units/min/mg.protein)	
	Normal	Transgenic
2.0	9.72	11.28
4.0	12.49	15.19
6.0	9.95	14.03
8.0	12.39	12.86
10.0	7.79	8.21

CD(AB) =1.37
A: Types of callus B: PEG concentration

Catalase also plays an important role in eliminating the harmful effects of free radicals in biological systems. A reduction in the activity of this enzyme could lead to the accumulation of O_2^- and H_2O_2, which in turn helps the formation of hydroxyl radical, which is highly toxic. Catalase also eliminates H_2O_2 by converting it to molecular oxygen and H_2O. In the present study, the trend of catalase activity was similar to that of SOD and peroxidase. Catalase activity was higher at 0.4 % phytagel and 4 % PEG concentrations (Tables 5 & 6). For other concentrations the difference was not statistically significant.

Table 5. **Effect of phytagel induced stress on catalase activity**

Phytagel conc. (%)	Catalase activity (units/min/mg protein)	
	Normal	Transgenic
0.2	0.28	0.37
0.4	0.40	0.49
0.6	0.30	0.46
0.8	0.26	0.45
1.0	0.22	0.38

CD (A)=0.028. CD (B)=0.045
A: Types of callus B: Phytagel concentration

Table 6. **Effect of PEG induced stress on catalase activity**

PEG conc. (%)	Catalase activity (units/min/mg protein)	
	Normal	Transgenic
2.0	0.29	0.43
4.0	0.36	0.49
6.0	0.20	0.39
8.0	0.28	0.37
10.0	0.22	0.33

CD (A)=0.028. CD (B)=0.045
A: Types of callus B: PEG concentration

The results indicated that the proportionate increase in catalase activity in transgenic tissues plays an important role in the stress protection provided by SOD over-expression. The mechanisms to detoxify oxygen free radicals are varied. An enhanced activity of SOD, catalase, peroxidase and glutathione reductase associated with reduced cell damage was observed by Li and Van-Staden (1998) when maize callus cultures were subjected to water stress. In the present study, proportional to the over- expression of SOD, the other two enzymes were

also over-expressed. The elevated SOD, peroxidase and catalase enzyme activities in response to abiotic stress suggests that the over- expression of SOD enzyme influences the expression of other free radical scavenging enzymes to provide protection against oxidative stress in normal as well as transgenic callus cultures.

ACKNOWLEDGEMENT

The authors wish to extend their gratitude to Dr. N.M. Mathew, Director, Rubber Research Institute of India for constant support and encouragement given during this study. The authors also wish to thank Mr. Ramesh B. Nair for help rendered in statistical analysis.

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