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## RESEARCH ARTICLE

# CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES AND PROTEIN PROFILEDURING DIFFERENT STAGES OF SOMATIC EMBRYOGENESIS IN SUGARCANE (*SACCHARUM OFFICINARUM* L.) VAR 93V297

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**ABSTRACT**

The present study describe about direct somatic embryogenesis and investigations on antioxidant enzyme activities during somatic embryogenesis in sugarcane. Somatic embryos were produced directly from the cut end of spindle leaf explants on MS medium (Murashige and Skoog's, 1962) containing different concentrations of 2, 4- dichlorophenoxyacetic acid (2, 4-D) alone or in combination with Kinetin (Kn). Maximum number (41.07±0.89) of somatic embryos were observed on MS medium augmented with 5.0mg/l 2, 4-D + 0.5mg/l Kn. Activities of antioxidant enzyme viz., Ascorbate peroxidase, Peroxidase, Catalase and Superoxide dismutase wasestimated during developmental stages (10, 20, 40 and 50 days) of somatic embryogenesis. Increased levels of APX and POX activity was observedduring initiation of embryos and small increase in activity till heart shaped and torpedo stage embryo formation, while SOD and CAT activitiesshown low levels of activity at embryo initiation stages andincreased steadily during heart and late heart-shaped embryo formation. This indicates tissue specific activation of these enzymes had occurred at different stages of embryo development. Protein profile shown one specific protein band with molecular weight of 27.4 kD appeared during globular, heart and torpedo stages of embryogenesis. However these bands disappeared during germination of embryos. And protein with molecular weight 36.8 kD was appeared from heart to germination stage of SE. therefore the present study may help in identification of specific proteins and role ofantioxidant enzymes during different stages of somatic embryo induction in sugarcane.



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## INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) belongs to family Poaceae. It is a major agronomical and commercial crop of India.Due to frequent genetic variations of the crop, many genetic improvement programs and micropropagation for large scale production has facilitated the fast production of disease and virus-free plantlets (Behera and Sahoo, 2009; Snyman *et al*., 2011; Silva *et al*., 2014). Somatic embryogenesis is the complex developmental processes in plants. In general, production of somatic embryos occurs from the somatic cells without fusion of gametes that helps in large scale propagation of true-to-type plants (Bhojwani and Razdan, 1996; Elmeer, 2013). Somatic embryogenesis (SE) is considered as the stress signaling responses, which facilitated by auxins that causes the embryogenic competence of the cells (Manivannan *et al*., 2015). Induction of somatic embryosis dependent on different factors viz., genotype and age of the explant (Feher, 2008), factors related to ambient growth conditions, composition of the culture medium and the interaction of explants with plant growth regulators (PGR) (Gaj, 2004), during this process cells may undergo various molecular and biochemical changes (Singla *et al*., 2007). Supplementing PGRs either induces somatic embryogenesis directly from the explants or from the embryogenic callus. It is evident from several reports that the synthetic auxin, 2,4-D is an efficient and effective PGR for somatic embryo induction (Raju *et al*., 2013) and stress signaling inducedby 2,4-D trigger the expression of specific genes and proteins responsible for SE (Mihaljevic *et al*., 2011). Hence, 2,4-D either alone or in combination with other PGR is frequently used in SE in many plants (Rathore *et al*., 2015). In the complex process ofSE several signaling molecules are involved. Among the signaling molecules, the reactive oxygen species (ROS) i.e., $H_2O_2$ enabledsignaling plays vital role in SE (Zhang *et al*., 2010; Kairong *et al*., 1999). In addition $H_2O_2$ acts as a major hallmark of oxidative stress, therefore plants have buildup the defensive system over excess levelsof

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$H_2O_2$by triggering specificdefensive antioxidant enzymes (Christou *et al*., 2014) and oxidative stress related genes are expressed during initial phases of somatic embryogenesis (Karami and Saidi, 2010). Antioxidant enzymes such asSOD, APX, POXand CAT are primarily associated withelimination of $H_2O_2$ (Tiryakioglu *et al*., 2006). The correlation of ROS and physiological processduring *in vitro* studies have beenreported for decades however still the role of primary metabolism remains ambiguous. There are relatively very few reports on the changes in protein profile during somatic embryogenesis particularly in sugarcane and Proteins are the key indicators of cellular differentiation, which can be used as genetic markers for identification of specific stages of embryogenesis (Menendez *et al*., 1994).

## MATERIALS AND METHODS

### Explants' preparation and culture conditions

For direct induction of somatic embryos sugarcane var. 93v297 tops measuring 10-15cm were excised from 3-4 months old plants. About 3-4 outer developed leaf whorl was removed manually till a spindle of 8-10 cm long and 1 cm diameter of 6-7 layers (Spindle) were obtained. Innermost whorl 3-4 layered young spindles were cut into small segments (1.0-1.5 cm) and used as explants. These spindle explants were sterilized as described by Arjun and Rao (2015). Then these explants were inoculated aseptically on MS medium supplemented with different concentrations (1.0- 6.0 mg/l) of 2, 4-D alone or in combination with Kn (0.5-1.5 mg/l). All cultures were incubated at 16h light and 8h dark photoperiod, providing a quantum flux density of 30lmols$^{-1}$m$^{-2}$by cool-white-fluorescent bulbs at 25 ± 2 $^0$C.

### Antioxidant enzyme assay

SOD activity was estimated by following the protocol ofGiannopolitis and Ries (1977)by monitoring the inhibition of photochemical reduction of nitrobluetetrazolium (NBT). The reaction mixture was irradiated for 14 min and absorbance read at 560nm with non-irradiated sample (blank). Activity of CAT was assayed by following the modified procedure of Claiborne (1984), the rate of decrease of absorbance at 240nm (decomposition of $H_2O_2$). The reaction mixture consisted of distilled water, 0.059M $H_2O_2$ and enzyme extract (50µl) in a final volume of 3ml. POX was assayed by the method of Evans (1968) after the oxidation of guaiacol to tetraguaiacol and theabsorbance was recorded at 470 nm. APX assay was assayed using Nakano and Asada (1981) method. The reaction mixture containing 0.2mM EDTA, 0.5mM ascorbic acid and 0.25mM$H_2O_2$. The reaction was initiated at 25 $^0$C by the addition of $H_2O_2$and enzyme extract (50µl) the decrease in absorbance at 290nm for 1min was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.8mmol/l/cm. Antioxidant enzyme activities were performed after the visibility ofinitiation of somatic embryosusing the standard chemicals (Himedia) and some minor changes in substrate volume, enzyme concentration and pH were done as it is essential to obtain the optimum activity of the enzymes.

### Statistical analysis

Thirty explants for *in vitro* studies and five cultures for antioxidant enzyme assay were taken and three replications per study were maintained. The data obtained was subjected to statistical analysis ANOVA to test the standard error (SE) and level of significance with Tukey-Kramer multiple comparison test using instat graphpad software.

## RESULTS AND DISCUSSION

### Induction and development of somatic embryos

Somatic embryos induced directly from the explants and found all over the surface of the spindle leaf explant within 40 days of culturing on MS medium supplemented with variable (3.5mg/l to 5.0mg/l) concentrations of 2, 4-D alone or in combination with Kn (Table 1). High frequency (89%) (Fig. 1a & b) and maximum number (41.07±0.89) SE were observed on MS medium containing 5.0mg/l 2, 4-D + 0.5mg/l Kn. Increasing the concentration of Kn decreased the number of somatic embryos. Figure 1 depicts the different stages of somatic embryos viz., Globular (Fig. 1c), heart (Fig. 1d) and torpedo (Fig. 1e) shaped embryos.

### Activities of POX, CAT, SOD and APX during somatic embryogenesis

Somatic embryo induction in sugarcane occurs through a precise series of morphogenic events. Kairong *et al*., (2002) suggested that SE is an unusual process that had established a link between ROS and cell differentiation. Surplus levels of ROS produced byendogenous auxin promotes dedifferentiation in cells (Pasternak *et al*., 2002; Correa-Aragunde *et al*., 2006) and these can affect the redox homeostasis, normal physiological and cellular activities in plants (Manivannan *et al*., 2015). In order to maintain the balance of intracellular ROS content, plants developed defensive antioxidative system to control increasing concentration of ROS. Concordantly, during the present study the activities of antioxidant enzymes such as SOD, APX, CAT and POX were rationally increased during the initiation stages of somatic embryo induction (Fig: 2). Activity of POX and APX were higher than SOD and CAT during globular stage of embryo development. POX content declined sharply from 27.17 to 14.09 units/mg protein during morphological changing stages and activity was least on 50 days cultures, similar observation is reported in *Cicer* (Ghanti *et al*., 2009). Contrary to this, Kormutak *et al*. (2003) reported 3-folds increased POX activity in silver fir culturesduring maturation stages of somatic embryos. POX is essential for maintaining the size and shape of protoderm cells during somatic embryogenesis (Cordewener *et al*., 1991) and it also plays an important role during lignin formation, there by keeps the cell wall of the embryos rigid (Whetten *et al*., 1998) and also help to reduce cellwall plasticity (Goldberg *et al*., 1986). APX content was increased during initiation of SE and reached maximum at 20 day cultures (24.47 µmol min$^{-1}$ mg$^{-1}$ protein) and the activitydeclined as increase in incubation period and developmentof somatic embryos, it reached least 14.12 µmol min$^{-1}$ mg$^{-1}$ protein in 50 days cultures. Activity of CAT and SOD increased in the globular stagesand reached maximum 17.12 units/mg protein and 21.02µmol/min/mg protein respectively in torpedo shaped embryos (40 days cultures) but the activity decreased (12units/mg protein and 12 µmol/min/mg protein)as increase in the incubation period of cultures to 50 days. POX and CAT are significantantioxidant enzymes responsible for scavenging$H_2O_2$ produced under various stress conditions and helps in overcoming the oxidative stress related damage (Willekens *et al*., 1995).

**Table 1. Effect 2, 4-D alone and in combination with Kn in somatic embryogenesis**

| Growth Regulators (mg/l) | | Frequency of SE induction | Number of SE after 40 days |
|---|---|---|---|
| **2, 4-D** | | | |
| 4.0 | | 45 | 17.07±0.33 |
| 5.0 | | 75 | 25.33±1.56 |
| 6.0 | | 68 | 19.67±0.98 |
| **2, 4-D** | **Kn** | | |
| 5.0 | 0.5 | 89 | 41.07±0.89 |
| | 1.0 | 87 | 37.67±0.33 |
| | 1.5 | 86 | 31.06±0.17 |

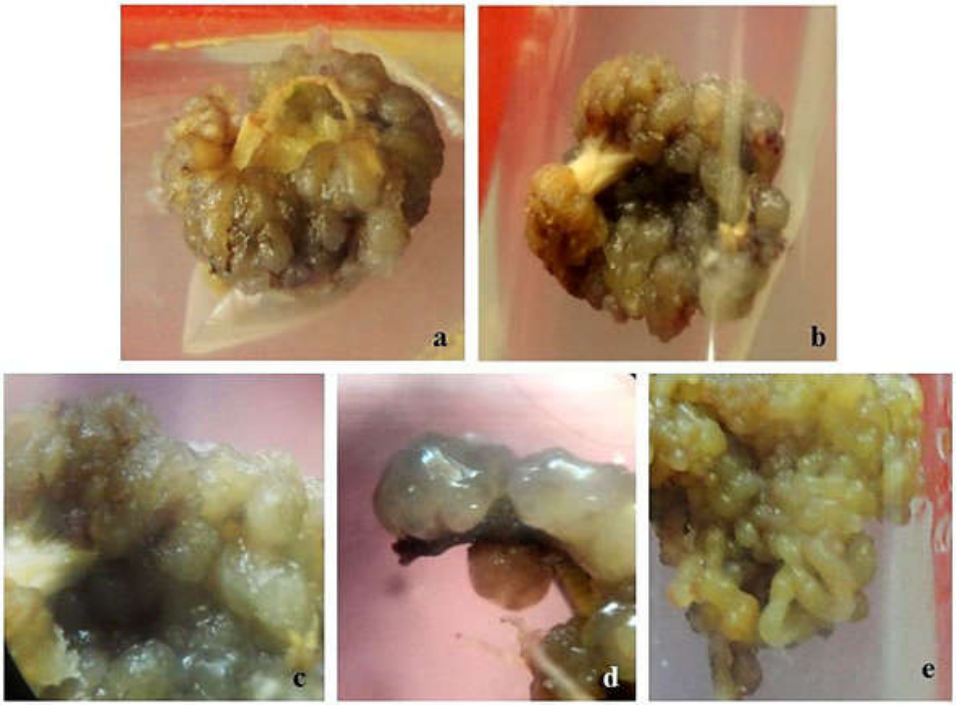


**Fig. 1. Direct somatic embryo induction from spindle leaf explant a. SE obtained MS + 5.0mg/l 2, 4-D + 0.5mg/l Kn, b. SE obtained MS + 5.0mg/l 2, 4-D + 1.0mg/l Kn, c, d & e. morphological development of somatic embryos c. globular stage embryo, d. heart shape embryo and e. Torpedo stage embryo**

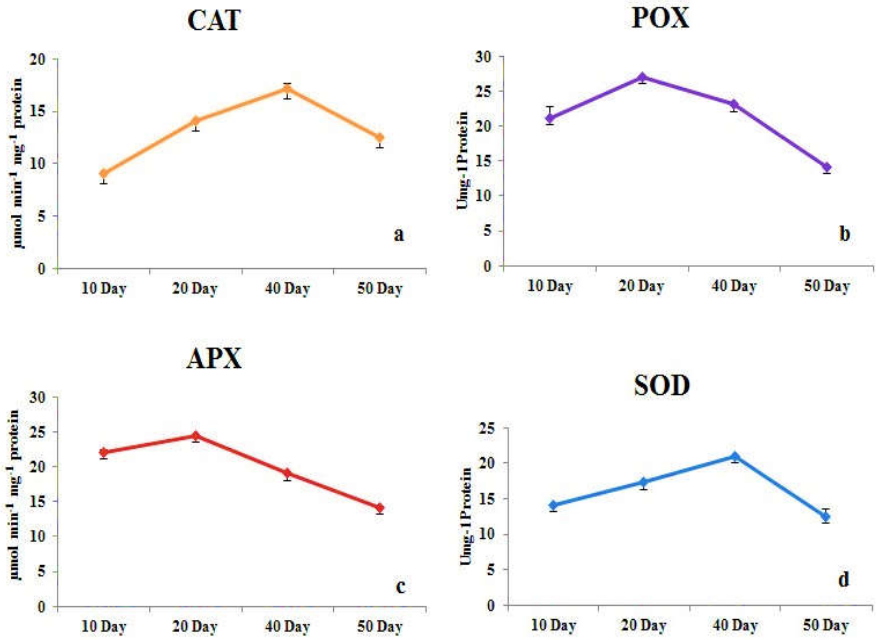


**Fig. 2. Antioxidant enzyme activity during different stages of somatic embryogenesis a. Catalase, b. Peroxidase, c. Ascorbate peroxidase and d. Superoxide dismutase**

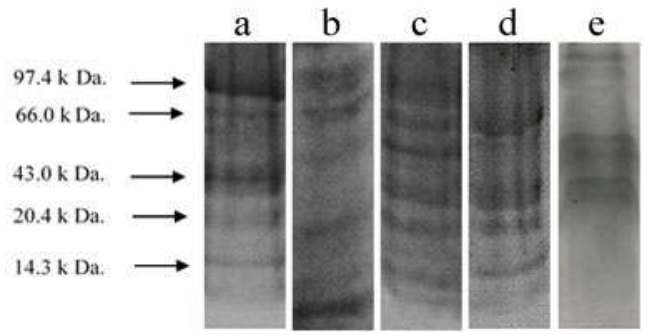


**Fig. 3. SDS-PAGE profile of proteins at different stages of somatic embryogenesis a. Protein ladder; b. Initiation of somatic embryogenesis and globular (12 Day cultures); c.Heartstage somatic embryos (25 day culture); d.torpedo stage somatic embryos (40 day culture) and e. Germination stage of somatic embryo (55 day)**

CAT have lower affinity for $H_2O_2$ (Dat *et al*., 2000) there by removes the bulk of $H_2O_2$ produced during β-oxidation of fatty acids (Bewley and Black 1994). Whereas POX has a higher affinity for $H_2O_2$ and allows scavenging of small amounts of $H_2O_2$ in more specific locations (Dat *et al*., 2000). From the results of the present study pertaining to higher activities of both POX and CAT during embryo developmental stage suggest that production of ROS is more at the early stages of somatic embryogenesis than at late stages. Similar observations were also made by Bagnoli *et al*. (1998) in chestnut. The key enzyme SOD dismutase the superoxide to $H_2O_2$. In the present investigation increase in SOD activity is consistent with the early and later stages of somatic embryogenesis and gradually decreasedas increase in incubation and later stages of somatic embryos. Similar observation reported in *Lyciumbarbatum* (Kairong *et al*., 2002). This suggests that $H_2O_2$ capable of inducing gene expression at particular time and causes protein synthesis, which can acts as a cellular second 'messenger'during somatic embryogenesis (Zavattieri, 2009). Moreover the lower level of $H_2O_2$ at the later/germinating stages of somatic embryos could be due to the emergence of meristematic and vascular tissues (Tian *et al*., 2003).

**SDS-PAGE gel electrophoresis during somatic embryogenesis**

Biochemical and molecular changes during somatic embryogenesis have been reported recently in many species such as peanut (Roja Rani *et al*., 2005), Chick pea (Ghanti *et al*., 2009), *Accasellowiana* (Gabriela *et al*., 2014). However as per our knowledge very few reports (Blanco *et al*., 1997; Reis *et al*., 2015) are available with sugarcane. Our study involves the protein profile pattern during different stages of somatic embryogenesis viz., Initiation (10 day), Heart (25 day), torpedo (40 day) and germination stages (55 day). Single dimension protein profile revealed the presence of different bands at different stages of SE and which were confirmed by comparing with protein ladder. Total five bands were observed during initiation of somatic embryo (15 days cultures) with molecular weight of 96.7, 75.9, 50.8, 27.4 and13.6 kD. Many significant difference in protein bands was observed as progress in embryogenesis (Fig. 3). Proteins with molecular weight of 27.4 kD was present in globular, heart and torpedo stages of embryos and 50.8 kD molecular weight protein band was present in globular, heart stage and disappeared during torpedo and germination stages of SE. 18.3 kD molecular weight protein band was observed in heart and torpedo stage embryos. 36.8 kD molecular weight protein band appeared from heart stage and remained till germination of embryos. On the other hand, three extrabands were observed from globular, heart and germinating stage embryos i.e., molecular weight of 96.7, 75.9 and 13.6kD during globular, 86.8, 68.2 and 15.1 kD from heart stage and 119.8, 104.8 and 58 kD from germination stage, while single protein band with molecular weight 52 kD is observed in torpedo stage embryos. The present result agrees the result of Ghanti *et al*., 2008 as they have reported the disappearance of few proteins at germinating stage of embryo. 27.4 kD protein was common in globular, heart and torpedo stage embryos and absent in germinating embryos similarly few protein bands were distinct in germinating embryo. This may suggestthat some proteins express at particular time of growth and play key role during the process of embryo development (Fellers *et al*., 1997; Ghanti *et al*., 2008). Some specific proteins which are very unique at particular stage of embryo development viz., in globular stage 96.7, 75.9 and 13.6 kD, in heart stage 86.8, 68.2 and 15.1 kD, in torpedo stage 62.9 kD. Therefore these specific proteins can be used as marker proteins for identification of development stages of SE. Similar observation was reported in Pea nut (Roja Rani *et al*., 2005) and Chick pea (Ghanti *et al*., 2008).

**Conclusion**

Our present results demonstrate the importance of $H_2O_2$ during the induction and developmental stages of somatic embryogenesis along with the modulation in antioxidant enzyme activities and activation of stage specific enzymes or proteins during differential stages of somatic embryogenesis. Therefore, we can affirm that these proteins/ enzymes can also be used as markers for identification of induction, development, and germination of somatic embryo in sugarcane.

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