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ANTIOXIDANT ENZYMES ACTIVITY IN EMBRYOGENIC AND NON-EMBRYOGENIC TISSUES IN SUGARCANE

Actividad de enzimas antioxidantes en tejidos embriogénicos y no embriogénicos de caña de azúcar

MARINA MEDEIROS DE ARAÚJO SILVA[1], M. Sc.; CLÁUDIA ULISSES[2], Ph. D.; MARIA JAISLANNY LACERDA E MEDEIROS[1], M. Sc.; MANUELA MARIA CAVALCANTE GRANJA[3], M. Sc.; LILIA WILLADINO[2], Ph. D.; TEREZINHA CAMARA[2], Ph. D.

[1] Universidade Federal de Pernambuco. Recife, Brasil. marinamedeirosas@yahoo.com.br; jaislanny@yahoo.com.br

[2] Universidade Federal Rural de Pernambuco. Recife, Brasil. claulisses@hotmail.com; willadino.lilia@gmail.com; teca.camara@gmail.com

[3] Universidade Federal de Viçosa. Viçosa, Brasil. manukagranja@yahoo.com.br

Corresponding author: Marina Medeiros de Araújo Silva, Rua Dom Manoel de Medeiros, 52171-900, Recife, PE, Brasil. marinamedeirosas@yahoo.com.co

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ABSTRACT

The objective of this work was to induce direct somatic embryogenesis from segments of immature leaves of the RB872552 variety of sugarcane and to correlate this morphogenic event with oxidative stress. Two previously described protocols were utilized for the induction of somatic embryogenesis in sugarcane with different supplementations of the culture medium and different incubation conditions. For the conversion of embryos into plants was used MS medium without phytoregulators. Histological analyses and activity of antioxidant enzymes were also conducted for the embryogenic and non-embryogenic tissues. The formation of somatic embryos was obtained in 81 % of the explants with the combination of regulators 2,4-D (2,4-dichlorophenoxyacetic acid)and BAP (6-benzylaminopurine) when incubated under 16 h photoperiod. With regards to the antioxidant enzymes, there was increased activity of peroxidase and an increase in the soluble protein content in embryogenic tissues, whereas lower activities of polyphenol oxidase and catalase appeared in these tissues compared to non-embryogenic tissues. It could be inferred that oxidative stress plays an important role in the induction of somatic embryogenesis in sugarcane.

Keywords: 2,4-dichlorophenoxyacetic acid (2,4-D), oxidative stress, *Saccharum officinarum* L., somatic embryogenesis.

RESUMEN

El objetivo de este trabajo fue inducir la embriogénesis somática directa en segmentos de hojas jóvenes en la variedad de caña de azúcar RB872552, e investigar la correlación de este evento morfogénico con el estrés oxidativo. Se utilizaron dos protocolos de inducción de embriogénesis somática, previamente descritos para caña de azúcar, con distintas suplementaciones en el medio de cultivo y en las condiciones de incubación. Para la conversión de embriones en plantas se utilizó el medio MS sin fitoreguladores. Se realizaron además análisis histológicas y de actividad enzimática antioxidante en tejidos embriogénicos y no embriogénicos. La formación de embriones somáticos ocurrió en 81 % de los explantes, con la combinación de los reguladores de crecimiento 2,4-D (ácido 2,4-diclorofenoxiacético) y BAP (6-bencilaminopurina) e incubación bajo un fotoperiodo de 16 h. Respecto a las enzimas antioxidantes, se observó un incremento en la actividad de peroxidasa y en el contenido de

proteínas solubles en los tejidos embriogénicos, mientras que la polifenoloxidasa y la catalasa presentaron valores más bajos de actividad en estos tejidos, en comparación con los no embriogénicos. Se podría inferir que el estrés oxidativo juega un papel importante en la inducción de la embriogénesis somática en caña de azúcar.

Palabras clave: ácido 2,4-diclorofenoxiacético (2,4-D), embriogénesis somática, estrés oxidativo, *Saccharum officinarum* L.

INTRODUCTION

The cultivation of sugarcane covers tropical and subtropical regions on more than 80 countries, with Brazil being the largest producer. Because of this prominent position in the global economy, the cultivation of sugarcane is frequently entering into genetic improvement programs, and sugarcane's *in vitro* propagation has facilitated the fast multiplication of new varieties and has ensured the production of disease and virus-free plantlets (Behera and Sahoo, 2009; Snyman *et al.*, 2011).

Somatic embryogenesis (SE), a morphogenic system in which somatic cells generate embryos without the fusion of gametes, is highlighted amongst the various micropropagation techniques by allowing the generation of a large number of propagules and the regeneration of transformed plants (Elmeer, 2013). The expression of the embryogenic potential is dependent on the interaction between different factors such as the genotype and developmental stage of the explant (Féher, 2008) as well a son factors related to ambient growth, including the composition of the culture medium and the action of the growth regulators (Gaj, 2004). Another factor that has been increasingly recognized lately is oxidative stress (Carvalho *et al.*, 2009; Zavattieri *et al.*, 2010), which is defined as an imbalance in the redox state of the cells with an overproduction of the oxidant compounds (Bhattacharjee, 2012). Changes in the activities of the antioxidant enzymes have been correlated with an embryogenic response from somatic tissues, suggesting that oxidative stress may induce morphogenic processes (Pasternak *et al.*, 2002; Blazquez *et al.*, 2009; Zhang *et al.*, 2010; Ma *et al.*, 2012). In the initial phases of SE, various genes related to the oxidative stress are induced, which leads to the hypothesis that this is an extreme response to the stress conditions in cells cultivated *in vitro* (Karami and Saidi, 2010). The regeneration of sugarcane plants from SE began with studies by Ho and Vasil (1983), and has been described for different explants and a high number of varieties (Gill *et al.*, 2004; Lakshmanan, 2006; Taparia *et al.*, 2012; Nawaz *et al.*, 2013). However, knowledge about the biochemical events that occur when somatic cells become able to produce embryos is scarce; additional studies are necessary to determine these patterns during morphogenic development to provide subsidies for the optimization of *in vitro* propagation systems based on this technique.

The objective of this work was to induce direct SE in sugarcane (RB872552) and to investigate the activity of the enzymes involved in antioxidative metabolism in embryogenic and non-embryogenic tissues.

MATERIALS AND METHODS

Somatic Embryogenesis Induction

For direct somatic embryogenesis (DSE) induction, the explants utilized were segments of immature leaves of sugarcane (variety RB872552) cultivated in a temporary immersion system (TIS). The culture media described by Geetha and Padmanabhan (2001) and Ali *et al.*, (2007) were employed, using MS salts and vitamins (Murashige and Skoog, 1962), without coconut water, and supplemented with 2 % or 3 % sucrose (w/v) and 13.6 µM 2,4-D combined with 2.2 or 1.1 µM BAP, respectively. The media were jellified with 6 g L^{-1} agar and the pH adjusted to 5.8 prior to autoclaving (121 °C, 20 min).

After inoculation, the explants were kept in a growth room at 25 ± 2 °C with a photoperiod of 16h and a luminous intensity of 47 µmol m^{-2} s^{-1}. Forty days after incubation in the DSE induction media, the somatic embryos were separated from the explant and transferred to test-tubes containing MS medium without growth regulators to favor conversion into plants.

The experiment was realized in a completely randomized design, with two treatments and ten replicates per treatment, with each replicate being constituted by six test-tubes containing one explant. The rate of somatic embryo formation (RSEF= number of responsive explants/number of inoculated explants) was evaluated, and the DSE efficiency (E_{emb}) was obtained for each treatment. The DSE efficiency was estimated by considering the number of plants regenerated from the explants that formed embryos and were able to be transferred to conversion medium. The data were transformed ($\sqrt{x+0.5}$) and then submitted to variance analysis. The averages from the treatments were compared by Tukey test, at 5 % probability.

Histological Analysis

To obtain anatomical cuts, explant samples containing somatic embryos were fixed in FAA50 (formaldehyde: glacial acetic acid: 50 % ethanol, in a 1:1:8 proportion), dehydrated in an ascendant ethanolic series and infiltrated in paraffin, according to Johansen (1940). The material was sectioned with a 5 µm thickness using a rotary microtome. For the preparation of blades, the cuts of the sugarcane somatic embryos were submitted to a coloring process with safranin/astra blue (Kraus and Arduin, 1997). Images of these cuts were obtained via an Olympus CH30 photomicroscope and captured with a VD 480 optimedical program.

Biochemical Analysis

Biochemical analysis was performed for both explants that formed somatic embryos and those that did not (embryogenic- EE and non-embryogenic- NE, respectively). The samples were collected after they were placed in DSE induction media for 40 days and were subsequently frozen in liquid nitrogen

and stored at low temperatures (-20 °C). The protein extract was obtained by homogenization of 0.1 g of vegetal tissue in 4 mL of a 0.1 M sodium phosphate buffer (pH 6.5) with 0.05 g of polyvinylpyrrolidone (PVP). The homogenate was then centrifuged at 10,000 g and 4 °C for ten min, and the supernatant was separated and used for spectrophotometric determination of the soluble protein content (Bradford, 1976) and antioxidant enzymes activity according to the methods of Fatibello-Filho and Vieira (2002) for determination of peroxidase (POD, EC 1.11.1.7), Kar and Mishra (1976) for polyphenol oxidase (PPO, EC 1.14.18.1) and Berrs and Sizer (1952) for catalase (CAT, EC 1.11.1.6).

The data were submitted to ANOVA, arranged in a 2 x 2 factorial scheme (DSE induction treatments x explant aspects), and the averages were compared by Tukey test, at 5 % probability.

RESULTS

Somatic Embryogenesis Induction

From the third day of growth, all of the inoculated explants displayed oxidation, mainly in the extremities. However, the occurrence of oxidation did not constitute a limiting factor for the formation of somatic embryos from the leaf tissue of the explant after eight days. After 40 days of culture in the DSE induction media, the formation of somatic embryos (Figs. 1A and B) was verified to be higher in explants cultivated in the medium described by Ali *et al.*, (2007) in comparison to the explants cultivated in the medium described by Geetha and Padmanabhan (2001), with RSEF values of 0.816 and 0.416, accounting for more than 81 % and 41 % of responsive explants, respectively (Table 1).

After 15 days from the transfer of the embryos to the conversion medium without growth regulators, chlorophyllous regions and the beginning formations of leaves appeared and were preceded by tissue oxidation. The complete plants that were regenerated were recorded 60 days after the inoculation in conversion medium (Fig. 1D). 47 plants were regenerated from 19 explants that formed embryos when cultured in the medium described for Geetha and Padmanabhan (2001), and 254 plants were regenerated from the 41 embryogenic explants obtained in the medium described by Ali *et al.*, (2007). The embryogenic efficiency (E_{emb}) was 6.19 and 2.47 (Table 1), respectively.

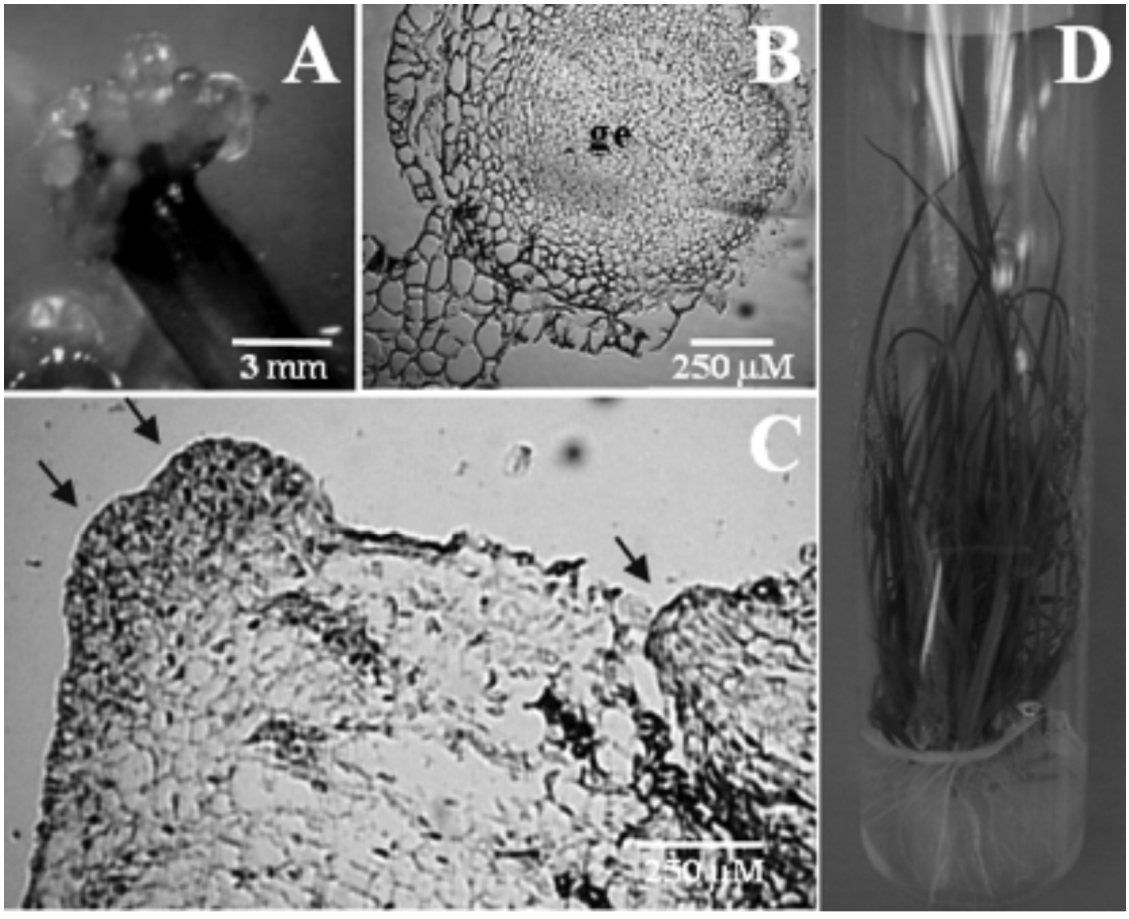

Figure 1. Somatic embryogenesis in *S. officinarum* (RB872552). A) Somatic embryos formed from segments of young leaves after 40 days of culture, B) histological seccion of embryo at globular stage (ge), C) histological seccion of embryogenic explant showing formation of pre-embryogenic masses (arrows), D) *in vitro* regenerated plants after 60 days of growth regulators suppression. Photographs Marina Medeiros de Araújo Silva.

Table 1. Rate of somatic embryo formation (RSEF) and embryogenic efficiency (E_{emb}) in *S. officinarum* (RB872552) explants after 40 days of culture in DSE induction media.

DSE induction treatments	IFE	E_{emb}
13.6 µM 2,4-D + 2.2 µM BAP + 2 % sucrose (Geetha and Padmanabhan, 2001)	0.416 b	2.47 b
13.6 µM 2,4-D + 1.1 µM BAP + 3 % sucrose (Ali *et al.*, 2007)	0.816 a	6.19 a

Means followed by different letters are significantly different ($p < 0.01$). CV(%) = 10.44.

These results show the feasibility of DSE use in the RB872552 variety of sugarcane, with embryo conversion into plants in a period of 90 to 100 days after *in vitro* explant inoculation.

Histological Analysis

The longitudinal section of the sugarcane explants showed the formation of pre-embryogenic masses (Fig. 1C) and somatic embryos at a globular stage (Fig. 1B) directly from the superficial region of the explant, namely, the epidermal cell layer. The nuclei of pre-embryogenic cells were strongly stained, while the cytoplasm was less stained when compared to the nuclei. Nearly all of the cells present in pro-embryos and early globular embryos show typical characteristics of meristematic cells in active division, including a reduced size, a dense cytoplasmic content, and a high nucleus/cytoplasm ratio.

Biochemical Analysis

The soluble protein content and the activity of the antioxidant enzymes were significantly different between the embryogenic (EE) and non-embryogenic (NE) explants, as shown in Figure 2. Higher levels of soluble proteins were found in the EE in comparison with the NE (Fig. 2A). In respect of antioxidant defense system, the polyphenol oxidase (PPO) enzyme activity was superior in the NE explants (Fig. 2B), suggesting a larger presence of phenolic compounds in these tissues. Peroxidase (POD) activity increased in the EE (Fig. 2C), while catalase (CAT) exhibited lower activity in these tissues (Fig. 2D). Furthermore, POD and CAT activity in embryogenic and non-embryogenic explants showed significant interaction among the studied treatments (Table 2). The activity of these key enzymes in the removal of hydrogen peroxide (H_2O_2) was superior in the EE explants from the treatment described by Geetha and Padmanabhan (2001) and in the NE explants from the treatment described by Ali *et al.*, (2007). This finding suggests that the cytokinins reinforce the enzymatic mechanisms for ROS elimination.

DISCUSSION

An efficient and reproducible regeneration system is essential need for producing micropropagated plantlets and to obtain transgenic plants through genetic engineering (Nawaz *et al.*, 2013). Many protocols have been reported in sugarcane tissue culture, mainly via organogenesis and indirect somatic embryogenesis (Snyman *et al.*, 2011). Nevertheless, our results confirm the feasibility of two culture media described for induction of direct somatic embryogenesis.

The results described in this work are similar to those presented by Ali *et al.*, (2007) on DSE induction in CP-77,400 and BL-4 varieties of sugarcane, obtaining an embryo formation of 90 % from leaf explants. The response of the RB872552 variety also confirms the efficiency of 2,4-D in SE induction. However, it was observed that the ratio of auxin/cytokinin (2,4-D/BAP) interfered with the embryogenic response. The RSEF and the E_{emb} increased proportionally when the 2,4-D/BAP ratio doubled (from 6.18 to 12.36), that is, when the BAP concentration was halved. In other species, an inhibiting effect of BAP on the formation of somatic embryos was observed. In leaf explants of *Primulina tabacum*, the formation of somatic embryos only occurred when BAP concentrations (either from isolated sources or in combination with other cytokinins) in the culture medium were reduced from 5 µM to 1 µM (Ma *et al.*, 2010). In addition to the combination of the growth regulators, the sucrose concentration was another factor that may have affected the success achieved with the employment of the methodology proposed by Ali *et al.*, (2007). Sucrose is the carbohydrate source most used in SE, and in general, the achievement of embryos in monocots and dicots is accomplished by the addition of 3 % of this disaccharide to the medium volume. Variations in the concentration of this sugar affect the osmotic conditions and the plant metabolism *in vitro*, affecting the initiation and differentiation processes of the embryos (Pereira *et al.*, 2003; Mengarda *et al.*, 2009; Yaseen *et al.*, 2013).

The embryogenic efficiency (E_{emb}) of 6.19 (Table 1) proves the higher efficacy of the culture medium described by Ali *et al.*, (2007), which resulted in the formation of approximately triple the quantity of plants compared the medium described by Geetha and Padmanabhan (2001). The regeneration of plants from somatic embryos in a medium without growth regulators was another benefit of the tested methodology. The regeneration of plants from indirect somatic embryogenesis in sugarcane varieties Co.J. 83 and Co.J. 86 was only possible in a conversion medium with 2.2 µM BAP (Gill *et al.*, 2004).

Histological study showed the formation of pre-embryogenic masses and somatic embryos at a globular stage directly from the superficial region of the explants. Similar structures were also observed by Garcia *et al.* (2007) from the growth of immature leaves of S. officinarum in a medium containing picloram or 2,4-D and by Desai *et al.* (2004) using immature

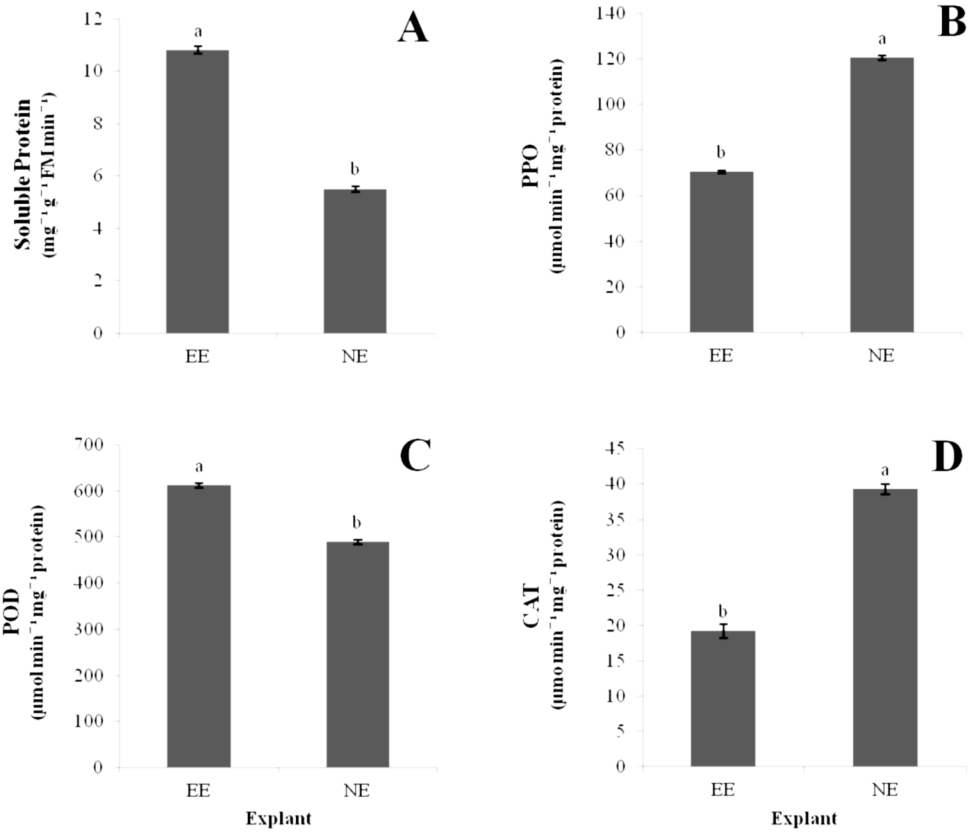

Figure 2. Biochemical analysis in embryogenic (EE) and non-embryogenic (NE) explants of *S. officinarum* 40 days after DSE induction. A) Soluble protein content, B) PPO activity, C) POD activity, D) CAT activity. (N = 5 ± standard error). Different letters are significantly different (p< 0.05).

Table 2. Peroxidase and catalase activity in embryogenic (EE) and non-embryogenic (NE) explants from the S. officinarum (RB872552) DSE induction treatments.

DSE induction treatments	POD (µM $min^{-1}mg^{-1}$ protein)		CAT (µM $min^{-1}mg^{-1}$ protein)	
	Explant aspect			
	EE	NE	EE	NE
13.6 µM 2,4-D + 2.2 µM BAP + 2 % sucrose (Geetha and Padmanabhan, 2001)	637.23 aA	402.93 bB	23.31 aB	32.35 bA
13.6 µM 2,4-D + 1.1 µM BAP + 3 % sucrose (Ali *et al.*, 2007)	587.55 bA	575.85 aA	15.10 bB	46.22 aA

Means followed by different letters are significantly different (p<0.01). CV(%) = 13.79. Lowercase letters for collums; capital letters for rows.

inflorescence segments grown in a medium supplemented with different growth regulators. This study also revealed that the cells present in pre-embryogenic masses and globular embryos were typical meristematic ones with a high nucleus/cytoplasm ratio, suggesting an increased ability in division of these cells with an increased in the synthesis of the nucleic acid and proteins, which provide energy for further development of the somatic embryos (Vasil, 1982; Ma *et al.*, 2012). The biochemical parameters were significantly different between the embryogenic (EE) and non-embryogenic (NE) explants (Fig. 2). Higher levels of soluble proteins were found in the EE in comparison with the NE (Fig. 2A). Higher metabolic activity in the embryogenics cultures of *S. officinarum*, with increases in the soluble protein concentrations as well as in the free proline and proteolytic activity, was also observed by Nieves *et al.* (2003). In relation to antioxidant enzymes, the PPO activity was superior in the NE explants. The increase in PPO activity in the first four weeks of the *in vitro* cultivation favors oxidation of tissues and the subsequent loss of morphogenic capacity and cellular death (Tang and Newton, 2004). Based on this fact, the high PPO activity may indicate higher oxidative stress levels in the non-

embryogenic explants, a situation that may have stopped the formation of embryos. The occurrence of stress may lead the somatic cells to change their morphogenic route and to differentiate; however, if the severity of the stress exceeds the capacity to mitigate the damage caused by reactive oxygen species (ROS) excess, the stress may cause totipotency loss and cellular death (Bray *et al.*, 2000; Fehér *et al.*, 2003). POD activity increased in embryogenic tissues, whereas CAT exhibited lower activity. In the search for possible markers for SE in Eurycoma longifolia, it was verified that the specific activity of POD was generally higher in embryogenic calluses in relation to non-embryogenic (Hussein *et al.*, 2006). Blazquez *et al.*, (2009) have identified a reduced activity of CAT in the early stages of embryogenesis in *Crocus sativus* induced with 2,4-D and BAP. This decline in CAT also preceded the appearance of somatic embryos in *Lycium barbarum* (Cui *et al.*, 1999) and *Gladioulus hybridus* (Gupta and Datta, 2004). According to Ma *et al.* (2012), the low activity of CAT most likely allows the increase in hydrogen peroxide (H_2O_2) levels, which promotes the formation of somatic pro-embryos.

In many plants, it has been demonstrated that antioxidant enzymes play an important role in somatic and zygotic embryogenesis (Kormuták *et al.*, 2003; Blazquez *et al.*, 2009; Sershen *et al.*, 2012). Until now, however, little information has been gathered about the role of redox metabolism in somatic embryogenesis (Ma *et al.*, 2012). POD and CAT can be considered "morphogenesis markers" (Konieczny *et al.*, 2008), as they are responsible for regulating H_2O_2 intracellular levels; a ROS can act as a secondary cellular messenger capable of inducing genic expression and protein synthesis and of promoting somatic embryogenesis (Agrawal and Purohit, 2012).

POD and CAT activity was superior in the EE explants from the treatment described by Geetha and Padmanabhan (2001) and in the NE explants from the treatment described by Ali *et al.*, (2007) (Table 2). The treatment described by Ali *et al.* (2007) has half of the BAP concentration the other treatment, which corresponds to twice the auxin/cytokinin ratio. Szechynska-Hebda *et al.*, (2007), studying the oxidative stress in the callus regeneration of Triticum aestivum, suggested that phytohormones may regulate the synthesis of some antioxidant enzymes and that because of this association between the endogenous level of phytohormones and the activity of enzymes, changes in the exogenous content of growth regulators can significantly influence the interactions that lead to morphogenic response. They also concluded that the cytokinins reinforce the enzymatic mechanisms for ROS elimination. Therefore, the differences in enzymatic activity observed in this work can be related to the distinct BAP concentrations utilized.

In *in vitro* growth, the uncommon supply of growth regulators is among the stress-inducing factors that may lead to an imbalance in the relation between antioxidant and pro-oxidant compounds (Gaspar *et al.*, 2002; Camara and Willadino, 2005). The response of the plant to stress can converge into cellular dedifferentiation, in addition to promoting genetic reprogramming, leading to changes in genic transition that are necessary so that somatic cells can acquire an embryogenic state (Zavattieri *et al.*, 2010; Grafi *et al.*, 2011). It emphasizes, therefore, the importance of studies related to SE, mainly in the comprehension of stimuli and ideal conditions that lead to induction, as well as the importance of knowledge about the mechanisms by which the growth regulators and stress factors are involved in the regulation and control of this process to increase the morphogenic efficiency for the production of superior plants on a large scale and the regeneration of genetically modified plants.

In summary, direct somatic embryogenesis in the RB872552 variety of sugarcane is obtained through the balance of auxin/cytokinin (2,4-D/BAP), and modifications in the activity of the antioxidant enzymes confirm the regulation of oxidative stress in the induction of this morphogenic route.

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