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Meta-topolin: an alternative for the prevention of oxidative stress in sugarcane micropropagation

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ABSTRACT - (*Meta*-topolin: an alternative for the prevention of oxidative stress in sugarcane micropropagation). The influence of two aromatic cytokinins (CKs), 6-benzylaminopurine (BAP) and *meta*-topolin (*m*T), on *in vitro* propagation and redox metabolism of sugarcane (*Saccharum* spp., variety RB98710) was investigated. Plants were cultured in Murashige and Skoog (MS) medium supplemented with *m*T (5 µmol.L^{-1}) or BAP (5 or 6.66 µmol.L^{-1}) for 40 days. The use of *m*T provided an increase in the multiplication rate and stem length of plants and shoots when compared to BAP. Shoots generated from the *m*T treatment presented low malondialdehyde (MDA) content and superoxide dismutase (SOD) activity, although they had higher hydrogen peroxide (H_2O_2) content. Thus, the H_2O_2 did not act as a stress marker, but it is related to plant growth and development processes.
Keywords: antioxidative enzymes, cytokinins, H_2O_2, *Saccharum* spp.

RESUMO - (*Meta*-topolina: uma alternativa para a prevenção do estresse oxidativo na micropropagação da cana-de-açúcar). A influência de duas citocininas aromáticas (CKs), 6-benzilaminopurina (BAP) e *meta*-topolina (*m*T) na propagação *in vitro* e no metabolismo redox da cana-de-açúcar (*Saccharum* spp., variedade RB98710) foi investigada. As plantas foram cultivadas em meio Murashige e Skoog (MS) suplementado com *m*T (5 µmol.L^{-1}) ou BAP (5 ou 6,66 µmol.L^{-1}) por 40 dias. O uso de *m*T proporcionou aumento na taxa de multiplicação e no comprimento do caule das plantas e brotações quando comparado ao cultivado com BAP. Os brotos gerados a partir do tratamento com *m*T apresentaram baixo teor de malondialdeído (MDA) e atividade de superóxido dismutase (SOD), apesar de apresentarem maior teor de peróxido de hidrogênio (H_2O_2). Assim, o H_2O_2 não atuou como marcador de estresse, mas tem relação com os processos de crescimento e desenvolvimento das plantas.
Palavras-chave: citocininas, enzimas antioxidantes, H_2O_2, *Saccharum* spp.

Introduction

Commercial-scale micropropagation of sugarcane (*Saccharum* spp.) is already a reality in the agro-technological sector. This process allows a quick multiplication of newly released varieties, production of uniform and disease-free plants, and high cane productivity and sugar yield (Dobhal *et al*. 2013, Hasner *et al*. 2019). The 6-benzylaminopurine (BAP) is an important aromatic cytokinin (CK) that is routinely utilized in micropropagation systems, and its effectiveness has been described to different species (Malá *et al*. 2013, Nower 2014, Gutiérrez *et al*. 2019).

Although of the benefits on multiplication, high concentrations or prolonged exposure to cytokinins (CKs) can lead to stress conditions that limit the *in vitro* micropropagation, due to the occurrence of morpho-physiological disorders (Razani *et al*. 2019). Thus, another aromatic CK, *meta*-topolin (*m*T) appears to be more advantageous in improving shoot proliferation while reducing or even alleviating some of the adverse effects encountered with the use of BAP (Aremu *et al*. 2012, Malá *et al*. 2013, Amoo *et al*. 2014).

There have been some studies on how artificial environment in culture *in vitro*, especially by the use

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of exogenous plant growth regulators (PGRs), affect antioxidative enzyme activity about plant tissue growth and multiplication (Díaz-Vivancos *et al*. 2011, Amoo *et al*. 2014, 2015). This occurs due to the increased production and accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) (Gupta 2010). Some studies have found that ROS play an important role in plant growth and development through cellular signaling (Carol & Dolan 2006, Baťková *et al*. 2008, Mabuchi *et al*. 2018, Smirnoff & Arnald 2018, Waszczak *et al*. 2018). This research had as objective to analyze the influence of *m*T and BAP on redox metabolism and *in vitro* propagation of sugarcane (variety RB 98710).

The variety RB98710 was developed by the Brazilian Interuniversity Network for the Development of Sugarcane Industry - RIDESA, and shows high sucrose content and agricultural production, as well as low fiber content and precocious maturation.

Material and methods

The study was carried out in the Plant Tissue Culture Laboratory (PTCL) at the Federal Rural University of Pernambuco, Recife, Brazil.

Plants of variety RB98710 previously established *in vitro* were cultivated in flasks containing 20 mL of liquid MS (Murashige & Skoog 1962) medium with 5 $\mu mol.L^{-1}$ of two different cytokinins (CKs) 6-benzylaminopurine (BAP) and *m*T (*meta*-topolin). A third treatment containing 6.66 $\mu mol.L^{-1}$ of BAP was used as a control since it is commonly used for sugarcane micropropagation. Cultures were incubated in a growth room at 25 ± 2 ºC and 16 h photoperiod (irradiance of 40 $\mu mol\ m^{-2}\ s^{-1}$ provided by white fluorescent lamps) for 40 days.

Growth and biochemical measurements were done in plants and shoots. The fresh weight and stem length of plants and shoots were expressed in terms of the increment (last - initial). The number of shoots per plant was also evaluated.

The content of H_2O_2 was analyzed according to Alexieva *et al*. (2001). Lipid peroxidation was measured in terms of MDA levels according to Heath & Packer (1968). Enzymatic activity analyses were carried out using 0.1 g of fresh leaf samples followed by homogenization in potassium phosphate buffer 100 mM (pH 7.5) containing 1 mM EDTA and 3 mM DTT (*threo*-1,4-Dimercapto-2,3-butanediol). The homogenate was centrifuged at 10.000 *g* and 4 °C for 15 minutes and the supernatant obtained was used for spectrophotometric quantification performed in triplicate. Total protein content was measured by the Bradford method (1976). The specific activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11) were measured according to Giannopolitis & Ries (1977), Havir & Mchale (1987) and Nakano & Assada (1981), respectively.

A completely randomized design was adopted, with three treatments and ten repetitions. The experimental unit consisted of a flask containing three plants. The data were analyzed by ANOVA and the means were compared by Tukey's test using the software Assistat v. 7.7 beta (Silva 2012).

Results and Discussion

Cytokinins (CKs) promoted different responses to plants and shoots growth (table 1). The fresh weight was highest in shoots cultured in the presence of 5 $\mu mol.L^{-1}$ *m*T and BAP. The use of *m*T promoted a higher stem length of plants and shoots and increased the number of shoots formation of shoots (table 1).

The H_2O_2 content was higher in plants cultivated with 5 $\mu mol.L^{-1}$ of BAP, whereas the shoots had higher concentration of H_2O_2 with 5 $\mu mol.L^{-1}$ of *m*T (table 2). Regarding lipid peroxidation, represented by MDA levels, the plants cultivated with *m*T had more MDA content when compared with plants cultivated to BAP (table 2).

However, in shoots, a lower MDA content was observed in those cultivated with *m*T. The highest number of shoots per plant induced by *m*T was connected with a low activity of antioxidant enzymes when compared to the usual concentration of BAP (6.66 $\mu mol.L^{-1}$). Among the antioxidant enzymes studied, SOD showed the lowest activity in plants and shoots (table 2). Despite presenting low SOD activity, the shoots displayed high content of H_2O_2 (table 2). The highest CAT activity in plants and shoots was observed on the 6.66 $\mu mol.L^{-1}$ concentration of BAP (table 2). In our study, the APX activity was higher in plants cultivated with BAP comparison to the medium with *m*T, but it was not statistically significant in shoots.

Shoots of *Pelargonium hortorum* growing on medium supplemented with *m*T, have had enhanced H_2O_2 production. This coincided with the higher activity of antioxidant enzyme and shoot formation (Wojtania & Skrzypek 2014).

In the shoot culture of *Cistus heterophyllus*, the addition of BAP induced a significant increase in the free radical scavenging capacity (López-Orenes *et al*. 2013) and thus indicating that the CKs are involved in the regulation of plant growth and development as well as a stress response.

Table 1 Fresh weight (g) and length (cm) of plants and shoots, and the number of shoots per plant in a variety of sugarcane (RB98710) cultivated *in vitro* with different cytokinins treatments (µmol.L^{-1}) for 40 days.

Cytokinin treatment	Fresh weight		Length		Shoot/Plant
	Plants	Shoots	Plants	Shoots	
5 *m*T	0.35 ± 0.02 a	0.08 ± 0.004 a	7.12 ± 0.30 a	6.31 ± 0.13 a	2.00 ± 0.13 a
5 BAP	0.27 ± 0.02 a	0.10 ± 0.006 a	1.15 ± 0.08 c	4.37 ± 0.08 c	1.33 ± 0.13 b
6.66 BAP	0.27 ± 0.04 a	0.04 ± 0.002 b	4.43 ± 0.63 b	5.25 ± 0.28 b	1.08 ± 0.15 b

Means ± SE (n = 10). Different letters indicate significant differences ($p < 0.05$).

Table 2**.** Content Contents of H_2O_2 (µmol g^{-1} FW) and MDA (nmol g^{-1} FW), and specific activities of SOD (U mg^{-1} protein), CAT (nmol H_2O_2 mg^{-1} protein min^{-1}), and APX (µmol AsA mg^{-1} protein min^{-1}) in sugarcane plants and shoots (RB98710) cultivated *in vitro* with different cytokinins treatments (µmol.L^{-1}) for 40 days.

	Cytokinin treatment	H_2O_2	MDA	SOD	CAT	APX
Plants	5 *m*T	6.90 ± 0.12 b	4.05 ± 0.20 a	0.52 ± 0.08 b	103.75 ± 5.58 b	332 ± 0.14 b
	5 BAP	7.52 ± 0.12 a	3.30 ± 0.15 b	1.66 ± 0.18 a	90.75 ± 7.26 b	482 ± 0.19 a
	6.66 BAP	6.75 ± 0.17 b	2.87 ± 0.06 b	1.59 ± 0.18 a	139.75 ± 4.55 a	465 ± 0.25 a
Shoots	5 *m*T	8.07 ± 0.37 a	1.40 ± 0.12 b	0.95 ± 0.15 c	81.25 ± 6.44 b	374 ± 0.10 a
	5 BAP	6.50 ± 0.23 b	1.45 ± 0.16 b	2.09 ± 0.08 b	100.25 ± 4.39 ab	378 ± 0.25 a
	6.66 BAP	6.85 ± 0.06 b	2.95 ± 0.10 a	3.36 ± 0.07 a	117.50 ± 5.07 a	378 ± 0.14 a

Means ± SE (n = 4). Different letters indicate significant differences ($p < 0.05$).

Responses of growth similar to showing the favorable effects of topolins in micropropagation have also been recorded in other species, such as *Scutellaria* spp. and *Pelargonium hortorum* (Aremu *et al*. 2012, Brearley *et al*. 2014, Naaz *et al*. 2019).

Superior multiplication rates were recorded in plants of two banana cultivars *in vitro*. The plants cultivated with *m*T and *m*TR (*meta*-topolin riboside) were major when compared to equimolar concentration BAP (Bairu *et al*. 2008).

The PGRs added into culture medium may cause changes in the physiology and biochemical of plant cells. Consequently, metabolic disturbances occur which promote the accumulation of ROS (Ozden & Karaaslan 2011). The increase in ROS levels can induce growth inhibition, leading to inferior quality plant formation (Gupta 2010). Thus, growth and morphogenic responses of plants and shoots sugarcane may be related to changing in the equilibrium of redox reactions caused by CKs. The active production of H_2O_2 in the apoplast, catalyzed by oxidases NADPH dependent (Petrov & Breusegem 2012) and peroxidases in the cell wall (Neill *et al*. 2002) is a prerequisite for normal development and growth of the plants (Petrov & Breusegen 2012). The apoplastic origin of the H_2O_2 can explain the increased content of this ROS in shoots cultivated with *m*T. The apoplastic pathway of H_2O_2 formation is related to cell growth and expansion. Once in which the shoots displayed higher stem length.

A lower MDA content observed in shoots cultivated with *m*T suggesting that cultivation with this CK induces better protection against oxidative damage. Also, it was confirmed that H_2O_2 accumulation is not always related to the peroxidation process.

For many years the H_2O_2 was described as an ROS capable of causing damage to the proteins, lipids, nucleic acids (Bienert *et al*. 2006) and supramolecular structures (Petrov & Breusegem 2012). In the last decade, however, it was demonstrated that the H_2O_2 may act as a signaling molecule involved in multiple physiological functions (Mitller *et al*. 2011, Smirnoff *et al*. 2018), including cell division (Livanos *et al*. 2012).

In contrast to results obtained this investigation, the enhanced of shoot formation capacity in *P. hortorum* cultivated with *m*T, coincided with higher levels of H_2O_2, and activities of CAT and POD (Wojtania &

Skrzypek 2014). Therefore, CKs also can alter the levels of antioxidant enzymes *in vitro* plants, for example, in *Crocus sativus* (Díaz-Vivancos *et al*. 2011). Enzyme SOD constitutes the first line of plant defense, converting superoxide anion radical ($O_2^{\bullet-}$) to H_2O_2, which is quickly metabolized by APX and CAT (Amoo *et al*. 2015). A low SOD activity and high content of H_2O_2 in shoots demonstrate that the dismutation of the $O_2^{\bullet-}$ is not the main source of H_2O_2 in the shoots sugarcane RB98710 when were cultivated with *m*T.

An increase in the activity of antioxidant enzymes with increased CK concentration was also reported in *Merwilla plumbea* (Amoo *et al*. 2015). According to López-Orenes *et al*. (2013), BAP stimulates the production of antioxidant compounds in cultivated shoots of *C. heterophyllus*, probably due to the stress originated by the addition of this phytoregulator in medium cultured. Obtained results indicated that CAT activity was the component of the antioxidative defense system involved in H_2O_2 detoxification that suffered more changes in response to the type and concentration of CK added in the culture medium.

Conclusion

Based on the data this research, *m*T can be used as a potential alternative to BAP to *in vitro* propagation of sugarcane (var. RB98710), since it positively influences the growth and quality of the plants. The production and detoxification of ROS are modified by CKs. The low SOD activity, the lower MDA content, and the best means for the growth parameters in the shoots cultivated with *m*T confirmed that H_2O_2 acted signaling in the processes of plant growth and development.

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