

Use of meta-Topolin, an unconventional cytokinin in the *in vitro* multiplication of *Opuntia stricta* Haw.

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ABSTRACT

The erect prickly pear (*Opuntia stricta* Haw) is an important alternative used to feed ruminants during prolonged periods of drought. The objective of this research was to evaluate the effect of different types and concentrations of cytokinins on the morphophysiological aspects of *in vitro* multiplication of *O. stricta*. Segments of *O. stricta* were grown in liquid and semisolid Murashige and Skoog (MS) medium supplemented with 2.22 or 1.11 $\mu\text{M l}^{-1}$ of 6-benzylaminopurine (BAP) and 1.11 $\mu\text{M l}^{-1}$ meta-Topolin (mT). The experiment was conducted in a growth room at $25 \pm 2^\circ\text{C}$ over a period of 45 days. It were evaluated multiplication rate, shoot formation index, rooting and callus formation, fresh biomass, height, activity of antioxidant enzymes, and content of hydrogen peroxide and malondialdehyde. The experiment was carry out in a completely randomized design with six treatments and twelve replicates. The results showed that the consistency of the medium had influence on the shoots development and roots emission. The increase in BAP concentrations resulted in a reduction in the length and fresh biomass of the shoots and in the root system. The enzymatic activity and oxidative stress were inducted with the use of 6-benzylaminopurine. The micropropagation of *O. stricta* was improved with the use of mT, verified through shoot with better morphophysiological quality for acclimatization, with longer shoot length, rooting and reduction in the occurrence of hyperhydricity, a morphophysiological disorder that leads to large losses in micropropagation of this specie.

Keywords: antioxidant enzyme, hyperhydricity, *in vitro* stress, morphophysiological anomalies

Uso de meta-topolina, una citoquinina no convencional en la multiplicación *in vitro* de *Opuntia stricta* Haw.

RESUMEN

El nopal erecto (*Opuntia stricta* Haw) es una alternativa importante que se utiliza para alimentar a los rumiantes durante períodos prolongados de sequía. El objetivo de esta investigación fue evaluar el efecto de diferentes tipos y concentraciones de citoquininas en los aspectos morfofisiológicos de la multiplicación *in vitro* de *O. stricta*. Se cultivaron segmentos de *O. stricta* en medio de cultivo líquido y semisólido de Murashige y Skoog (MS) con 2.22 o 1.11 $\mu\text{M l}^{-1}$ de 6-bencilaminopurina (BAP) y 1.11 $\mu\text{M l}^{-1}$ de meta-Topolina (mT). El experimento se realizó en una sala de crecimiento a $25 \pm 2^\circ\text{C}$ durante un período de 45 días. Se evaluaron la tasa de multiplicación, el índice de formación de brotes, la formación de raíces y callos, la biomasa fresca, la altura, la actividad de las enzimas antioxidantes y el contenido de peróxido de hidrógeno y malondialdehído. El experimento se realizó con un diseño completamente al azar con seis tratamientos y doce repeticiones. Los resultados mostraron que la consistencia del medio de cultivo influyó en el desarrollo de brotes y en la emisión de raíces. El aumento en las concentraciones de BAP resultó en una reducción en la longitud y la biomasa fresca de los brotes y en el sistema radicular. La actividad enzimática y el estrés oxidativo se indujeron con

el uso de 6-bencilaminopurina. La micropropagación de *O. stricta* se mejoró con el uso de mT, verificado a través de brotes con mejor calidad morfofisiológica para la aclimatización con mayor longitud de brotes, enraizamiento y reducción en la aparición de hiperhidricidad, un trastorno morfofisiológico que conduce a grandes pérdidas de micropropagación de esta especie.

Palabras clave: anomalías morfofisiológicas, enzima antioxidante, estrés *in vitro*, hiperhidricidad

INTRODUCTION

The erect prickly pear (*Opuntia stricta* Haw.) is a forage of extreme importance for the Brazilian semi-arid region because it is resistant to prolonged drought periods, being an alternative for animal feeding in times of prolonged drought (Escobar *et al.*, 1986; Barbosa *et al.*, 2018). The Brazilian semiarid is characterized by erratic rains, with long periods of drought and high temperatures (Santos *et al.*, 2017).

Due to some problems in the sexual propagation (Escobar *et al.*, 1986), the production of seedlings of this specie for the distribution in the cooperatives has been done basically through the direct preparation of whole raquettes or through the technique of micro-cleavage, sectioning the cladodes and planting in beds until the development of rackets and transplantation for the field (Gava and Lopes, 2012). However, this technique presents some difficulties, for example, exposure of plant material to the attack of microbial pathogens and physical and temporal spatial limitation for the production of seedlings.

Micropropagation is an *in vitro* plant culture technique widely used for the production of large scale seedlings in a fast and safe way. This technique allows the production of plants identical to the parent plant, maintaining the genetic identity in short time space and physical environment (Zoghiami *et al.*, 2012; Singh, 2015). Silva and Souza (2017) confirm the effectiveness of the micropropagation for the production of seedlings of *O. stricta* for field planting.

In micropropagation, growth regulators play an important role in plant development. The cytokinins are a group of growth regulators that are related to the induction of cell division and regulation of the formation of new shoots processes (Cheng *et al.*, 2013; Gentile *et al.*, 2017). The type and concentration of cytokinins used in the culture medium are of

great importance for plant regeneration and multiplication during micropropagation (Aremu *et al.*, 2012). Many micropropagation systems use 6-benzylaminopurine (BAP) cytokinin because it is more efficient and economically viable (Werbrouck *et al.*, 1995; Rahman *et al.*, 2018). However, several morphophysiological disorders such as hyperhydricity have been attributed to the use of this regulator.

Hyperhydricity is a morphophysiological disorder in which the plant presents abnormal accumulation of water inside the cells or tissues, which results in a translucent appearance (Vasconcelos *et al.*, 2012). According to some authors (Quiala *et al.*, 2009; Lema-Rumińska and Kulus, 2014; Lázaro-Castellanos *et al.*, 2018), hyperhydricity is a highly relevant problem in the micropropagation of *Cactaceae*, because it can hinder the acclimatization stage.

In acclimatization it is essential to have seedlings with high growth pattern and uniformity because it will be used to plant new fields. Thus, the morphophysiological quality of the seedlings is extremely important for success in establishing the plants in the field (Silva and Souza, 2017).

The discovery of a new group of aromatic cytokines has been studied as an alternative to use BAP in order to reduce physiological disturbances caused by their excessive or prolonged use, as well as the reduction of oxidative stress in micropropagated plants (Bairu *et al.*, 2009; Amoo *et al.*, 2011; Aremu *et al.*, 2012; Naaz *et al.*, 2019). Considering the abnormal development of *O. stricta* on *in vitro* culture, the objective of this research was to evaluate the effect of different types and concentrations of cytokinins on the morphophysiological aspects of *in vitro* multiplication of *O. stricta*.

MATERIALS AND METHODS

The experiment and the biochemical analysis were carried out in the Plant Tissue Culture

Laboratory (PTCL) at the Federal Rural University of Pernambuco (UFRPE), Recife, Brazil.

Plant material

Shoots of erect prickly pear were obtained in the Biofactory Governador Miguel Arraes of the Center of Strategic Technologies of the Northeast (CETENE) in Recife, Pernambuco, Brazil.

In vitro multiplication

Shoots from Erect prickly pear (*O. stricta*), in the 2nd subculture, in semisolid multiplication medium containing Murashige and Skoog (1962) (MS) medium supplemented with 30 g l⁻¹ sucrose, 2.22 µM l⁻¹ BAP and 0.57 µM l⁻¹ 3-indoleacetic acid (IAA) were transferred to semisolid MS medium without BAP and cultured for 45 days. Explants of approximately 1.5 cm were obtained after excision in the apical and root regions of shoots, and it were used as explants.

The experimental treatments included different concentrations and cytokines types in the multiplication medium, composed by semisolid or liquid MS medium supplemented with 30 g l⁻¹ sucrose and 2.22 or 1.11 µM l⁻¹ BAP + 0.57 µM l⁻¹ IAA or 1.11 µM l⁻¹ 6-(3-Hydroxybenzylamino) purine (meta-Topolin, mT) + 0.57 µM l⁻¹ IAA. The control treatment was the medium containing 2.22 µM l⁻¹ BAP and gelled with agar. The pH of the all media was adjusted to 5.8 before autoclaving the liquid medium and before addition of the agar (Agar powder, for microbiology, Sigma-Aldrich) in the semisolid medium.

Two explants were inoculated on a 250 ml capacity vessel containing 20 ml of multiplication medium. At the moment of explants inoculation, the initial fresh biomass was determined with an analytical balance (Bel 120g mode M214Ail, resolution of 0.0001g) and after the period of shoots multiplication. The vessels were kept in growth room at 25±2 °C under cold white light (40 µM m⁻² s⁻¹) with 16 hours photoperiod over 45 days.

Shoots formation index (SFI), callus formation index (CFI), rooting index (RI) and

hyperhydricity index (HI) were determined by dividing the number of responsive explants among the number of total explants. The hyperhydricity index was evaluated according to the occurrence of characteristics translucent and vitreous shoots in each treatment.

Biochemical analysis

With the objective of to evaluate the level of oxidative stress in *O. stricta* plants in each treatment it were analyzed the enzymatic activity, the content of hydrogen peroxide (H₂O₂) and malondialdehyde (MDA). Ascorbate peroxidase (APX) and catalase (CAT) activity were determined using 0.1 g plant material that was triturated in liquid nitrogen with the addition ~0.01 g polyvinylpolypyrrolidone. This mixture was homogenized in 1 ml extraction buffer (pH 7.0) containing potassium phosphate, 1,4-dithiothreitol, and ethylenediamine tetra acetic acid. The homogenate was centrifuged at 10 000 g for 20 min at 4°C and the supernatant was collected for analysis. APX activity was determined according to the method proposed by Nakano and Asada (1981) at 290 nm with a 60 seconds interval. It was calculated based on the molar extinction coefficient for ascorbate (2.8 mM⁻¹ cm⁻¹) and expressed as µmol AsA mg⁻¹ protein min⁻¹. CAT activity was determined according to the methodology proposed by Beers and Sizer (1952) at 240 nm from the molar extinction coefficient for hydrogen peroxide (H₂O₂, 36 mM⁻¹ cm⁻¹), and expressed in µmol H₂O₂ mg⁻¹ protein min⁻¹.

In the measurement of hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) contents, 0.2 g of sample macerated in liquid nitrogen was used. Then, approximately 0.02 g Polyvinylpolypyrrolidone (pvpp) and 2 ml trichloroacetic acid (TCA) at 0.1% were added. The plant material was homogenized and centrifuged at 14000 g for 10 minutes at 4°C. The supernatant was used as a plant extract for the analysis. The levels of H₂O₂ were verified according to methodology proposed by Alexieva *et al.* (2001) and expressed as µM g⁻¹ FW. The MDA levels were measured according to Heath and Packer (1968) and expressed as nmol g⁻¹ FW.

Experimental design and statistical analysis

The experimental design was carry out in a completely randomized design with six treatments (BAP/2.22, BAP/1.11, mT/1.11, BAP/2.22, BAP/1.11 e mT/1.11) with twelve repetitions. The experimental unit was a vessels containing two explants. The variables analyzed were multiplication rate (determined by dividing the number of responsive explants among the number of total explants), shoot length (cm), fresh biomass (g), enzymatic activity, APX ($\mu\text{mol AsA mg}^{-1} \text{ protein min}^{-1}$), CAT ($\mu\text{mol H}_2\text{O}_2 \text{ mg}^{-1} \text{ protein min}^{-1}$), H_2O_2 ($\mu\text{mol g}^{-1} \text{ FW}$) and MDA ($\text{nmol g}^{-1} \text{ FW}$).

The statistical analyzes were performed using the ASSISTAT statistical program 7.6 beta. The data obtained were submitted to the Kolmogorov-Smirnov and Bartlett tests for confirmation of normality and variances homoscedasticity at 5% probability level ($p < 0.05$). The ANOVA was performed to determine significant differences between treatments, and when significant were submitted to Tukey test at 5% probability level ($p < 0.05$). Pearson correlations ($p < 0.05$) were performed among all variables.

RESULTS

The consistency in the culture medium significantly influenced shoot formation. The explants cultivated in liquid medium did not present enough shoot multiplication for the

biochemical analysis. There was no influence of cytokinin types and concentrations on the *in vitro* multiplication rate of erect prickly pear in semisolid medium. Nevertheless, shoots formed in medium with mT presented higher fresh biomass length and biomass than explants formed in the BAP treatments (Table 1).

The results of fresh biomass showed a significant positive correlation ($r = 0.59$, $P < 0.05$) with shoot height. The shoot length was reduced in BAP treatments. Fresh biomass had a tendency to decrease in the treatment with $2.22 \mu\text{M l}^{-1}$ BAP.

The use of mT reduced callus formation by 59% in relation to the control treatment stimulating the rooting and the shoots growth. Although, high concentrations of BAP in semisolid media caused rooting inhibition (Table 2). In all treatments with BAP, roots were formed from the explants. However, in the treatments containing mT, root formation was verified in the shoots (Figure 1).

It was observed the occurrence of explants that did not present shoots and showed deformation of the pods, modified leaf bases as found in palm (Areces-Mallea, 2017), especially in explants cultivated with BAP in liquid medium (Figure 1 AB). Many pods fell during the multiplication period and in the areola region there was callus formation, especially in treatments in liquid medium (Figure 1 A-C).

Table 1. Number of shoots per explant, height and fresh shoots biomass in *Opuntia stricta* Haw. *in vitro* grown with different types and concentrations of cytokinins in liquid and semisolid medium for 45 days.

Concentration/type of cytokinin ($\mu\text{M l}^{-1}$)	Medium	N° shoots/ explant	Shoots height (cm)	Fresh biomass of shoots (g)
BAP/2.22+IAA/0.57	liquid	-	-	-
BAP/1.11+IAA/0.57	liquid	-	-	-
mT/1.11+IAA/0.57	liquid	-	-	-
BAP/2.22 (Control)+IAA/0.57	semisolid	14.00 a	1.00 c	0.35 b
BAP/1.11+IAA/0.57	semisolid	12.88 a	1.40 b	0.50 ab
mT/1.11+IAA/0.57	semisolid	10.63 a	2.30 a	0.53 a

Media in the columns with the same letter do not show differences by the Tukey test ($p < 0.05$)

The explants cultured in liquid medium in the presence of mT showed an increase in shoot formation (47 and 52% in relation to the treatments with 2.22 and 1.11 $\mu\text{M l}^{-1}$ BAP, respectively) (Table 2). Regardless of the consistency of the nutrient medium, it was observed the occurrence of explants with hyperhydricity and callus formation (Table 2). In the semisolid medium with BAP, there was a higher frequency of hyperhydricity in the shoots, unlike the medium with addition of mT, where the branches showed uniformity and vigor (Table 1, Figure 1 DF).

The CAT activity did not differ among the evaluated treatments. Regarding APX, the enzymatic activity was lower in the treatment with 1.11 μM BAP (Table 3). Meanwhile, the highest levels of H_2O_2 and MDA coincided with the higher incidence of hyperhydricity and possible presence of betacyanin in BAP treatments, especially at high dose (2.22 μM). It was not possible to analyze the enzymatic activity and the contents of H_2O_2 and MDA in the shoots in liquid medium, mainly due to the low number of shoots and microbial contamination.

Table 2. Shoots formation index (SFI), callus formation index (CFI), rooting index (RI) and hyperhydricity index (HI) in *Opuntia stricta* Haw. *in vitro* cultivated in liquid and semisolid medium with different types and concentrations of cytokinins, during 45 days.

Concentration/type of cytokinin ($\mu\text{M l}^{-1}$)	Medium	SFI	CFI	RI	HI
BAP/2.22+IAA/0.57	liquid	0.20 b	0.80 a	1.00 a	0.93
BAP/1.11+IAA/0.57	liquid	0.18 b	0.85 a	0.74 ab	0.88
mT/1.11+IAA/0.57	liquid	0.38 b	0.41 ab	0.47 ab	0.89
BAP/2.22 (Control)+IAA/0.57	semisolid	0.67 ab	0.47 ab	0.27 b	0.93
BAP/1.11+IAA/0.57	semisolid	0.94 a	0.12 b	0.94 ab	1.00
mT/1.11+IAA/0.57	semisolid	0.67 ab	0.19 b	0.71 a	0.76

Media in the columns with the same letter do not show differences by the Tukey test ($P<0.05$)

Table 3. Activity of ascorbate peroxidase (APX), catalase (CAT), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in shoots of *Opuntia stricta* Haw. *in vitro* cultivated in liquid and semisolid medium with different types and concentrations of cytokinins for 45 days.

Concentration/type of Cytokinin ($\mu\text{M l}^{-1}$)	Medium	APX ($\mu\text{mol AsA/mg protein/min}^{-1}$)	CAT (nmol $\text{H}_2\text{O}_2/\text{mg protein/min}^{-1}$)	H_2O_2 ($\mu\text{mol/g FW}$)	MDA (nmol/g FW)
BAP/2.22+IAA/0.57	liquid	-	-	-	-
BAP/1.11+IAA/0.57	liquid	-	-	-	-
mT/1.11+IAA/0.57	liquid	-	-	-	-
BAP/2.22 (Control)+IAA/0.57	semisolid	5901.0 a	160.25 a	4.75 a	2.90 a
BAP/1.11+IAA/0.57	semisolid	4246.0 b	150.00 a	3.30 b	1.20 b
mT/1.11+IAA/0.57	semisolid	5359.5 a	150.50 a	3.97 ab	1.73 b

Media in the columns with the same letter do not show differences by the Tukey test ($P<0.05$)

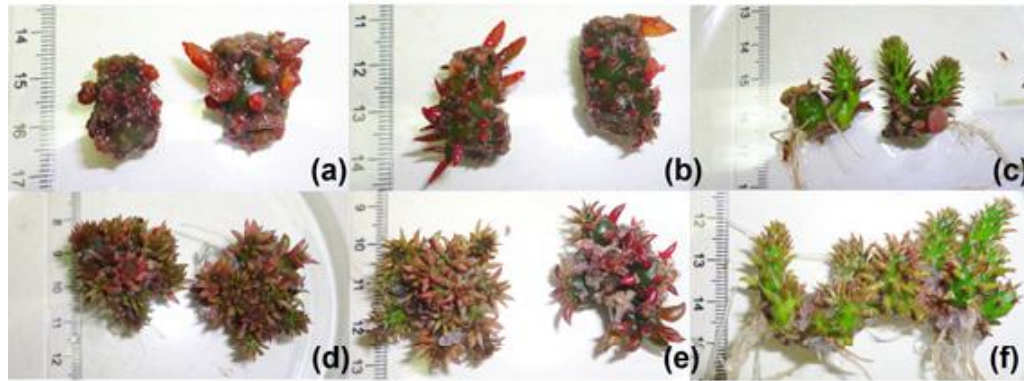


Figure 1. Aspects of *Opuntia stricta* Haw. explants cultured *in vitro* with different types and concentrations of cytokinins for 45 days. (a) Explants cultured in liquid medium containing $2.22 \mu\text{M l}^{-1}$ BAP. (b) Explants cultured in liquid medium containing $1.11 \mu\text{M l}^{-1}$ BAP. (c) Explants cultured in liquid medium containing $1.11 \mu\text{M l}^{-1}$ mT. (d) Explants grown on semisolid medium containing $2.22 \mu\text{M l}^{-1}$ BAP. (e) Explants grown on semisolid medium containing $1.11 \mu\text{M l}^{-1}$ BAP. (f) Explants grown on semisolid medium containing $1.11 \mu\text{M l}^{-1}$ mT.

DISCUSSION

The regeneration and multiplication of shoots during micropropagation can be affected by the type and concentration in the growth regulator used, especially the cytokinins, due to their importance in the process of cell division and organogenesis, responsible for inducing a greater number of shoots (Aremu *et al.*, 2017; Abd Aziz *et al.*, 2018). The cytokinin choice to be used in plants micropropagation is determined by their efficiency for inducing the proliferation, formation of healthy shoots, stimulating the acclimatization of plants (Bairu *et al.*, 2007). Since the 1980s, the cytokinins have importance in the *in vitro* *Opuntia* (*Opuntia amyclaea*) multiplication (Escobar *et al.*, 1986). In the present work, the efficiency of mT in shoots formation with superior morphophysiological quality was evident in relation to shoots regenerated in explants cultivated with BAP.

The reduction in shoot length of *O. stricta* may be related to the toxic effect of BAP on plants, due to the accumulation and the slow release of metabolites to other parts of the plant, causing inhibition of growth and rooting (Erig *et al.*, 2002; Bairu *et al.*, 2007; Aremu *et al.*, 2017). In this sense, Erig *et al.* (2002) observed a reduction in shoots height and fresh weight of *Rubus idaeus* L. due to the increase in BAP concentrations.

When *O. stricta* grown in semisolid medium with $2.22 \mu\text{M l}^{-1}$ BAP the shoots showed a rooting inhibition (Figure 1 D). This response may be related to the increase in the levels of the stable metabolite 6-benzyl-amino-9- β -D-glucopyranosylpurine (BAP9G), whose accumulation can cause root growth inhibition and lead to problems in plants acclimatization, according to the results of different investigations (Amoo *et al.*, 2011; Plaèková *et al.*, 2015).

As observed in this study, other authors confirmed the efficacy of mT in promoting rooting and reduction losses during the acclimation stage (Valero-Aracama *et al.*, 2009; Aremu *et al.*, 2012). When multiplication and rooting rates are satisfactory (Figure 1 C, Figure 1 F), it is possible to dispense the stage of shoots elongation before acclimatization in the natural environment (Csabai *et al.*, 2011). The increase in the shoots length in medium containing mT has already been described by other authors (Bairu *et al.*, 2007; Gentile *et al.*, 2017; Madzikane-Mlungwana *et al.*, 2017; Naaz *et al.*, 2019).

The excess of cytokinins may favor the occurrence of hyperhydricity, especially in culture with liquid medium with BAP (Figure 1 A-B). Hyperhydricity is considered a morphophysiological disorder that, depending on the intensity, may make the explants recalcitrant (Cruz *et al.*, 2009; Vasconcelos

et al., 2011). Among the factors stimulate the hyperhydric formation in shoots are the high humidity and the high concentration of growth regulators (Ziv, 2005; Lebedev *et al.*, 2018). Plants grown in static liquid systems are more susceptible to develop hyperhydricity because are immersed in the culture medium (Adelberg and Naylor-Adelberg, 2012). This condition hinders gas exchange and it can be detrimental to plant cells (Gao *et al.*, 2017).

In addition, several studies have reported a higher frequency of hyperhydricity in a variety of species grown in liquid medium (Tascan *et al.*, 2010; Balen *et al.*, 2012; Camargo *et al.*, 2019). For instance, in the micropropagation of *Telekia speciosa* (Schreber) with 5 μM I⁻¹ BAP or mT were recorded 50% and 5% of hyperhydricity in the shoots development, respectively (Csabai *et al.*, 2011). Amoo *et al.* (2011) report the effects of different types and concentrations of cytokinins in the *Barleria greenii* (Balkwill) micropropagation and emphasize the less mT toxic nature in relation to equimolar BAP concentrations, indicating its superiority in producing more shoots with reduction or total absence of abnormalities. Other authors also observed inhibition of shoots formation and a higher rate of abnormal sprouting with increasing BAP concentration, whereas treatments containing mT showed low abnormality rates (Bairu *et al.*, 2007; Wojtania, 2010; Amoo *et al.*, 2011; Aremu *et al.*, 2012). The results obtained in the present research are in agreement with the mentioned authors. These responses suggest that the structural difference between BAP and mT may have a profound impact on micropropagated plants (Bairu *et al.*, 2007), influencing the quality of the seedlings produced and possibly the survival during acclimatization on natural environment (Peixoto *et al.*, 2006).

The occurrence of abnormal characteristics in mT shoots grown (Figure 1 C, Figure 1 F) in the present work may be a consequence of the transition effects between BAP and mT (Amoo *et al.*, 2011), since the explants previously used already had exposed to the BAP. Hyperhydricity is affected by the source and concentration of cytokinin, but the frequency with which the phenomenon occurs is also related to the micropropagated species (Kadota and Niimi, 2003; Liu *et al.*, 2017). According to Amoo *et al.* (2011), there are

still some difficulties in the large-scale production of micropropagated plants, such as low multiplication rate, morphological abnormalities and inadequate rooting. These problems can be minimized by using the appropriate type and concentration of the growth regulator, especially the cytokinins, as they are related to cell division.

In the present work hyperhydric shoots showed intense purple coloration, mainly in the pods (Figure 1 A-B and D-E). A group of non-photosynthetic pigments, the betalains, is subdivided into betacyanins (purple-violet pigments) and betaxanthines (yellow pigments) (Soriano-Santos *et al.*, 2007). Red beet (*Beta vulgaris* L.) and *Opuntia* genus are the main sources of these pigments that are used as natural dyes (Georgiev *et al.*, 2008). According to Pavokovi and Krsnik-Rasol (2011), BAP is one of the components on the culture medium that stimulate the production of betacyanins. Some researchers claim that betacyanins can act in the kidnapping of reactive oxygen species (ROS), avoid their accumulation and protecting tissues and cells against damage caused by oxidative stress (Davies *et al.*, 2018).

The betalains have interest because of their antioxidant properties and recently the technical and commercial viability of several *in vitro* systems for commercial production of these pigments has recently been explored (Georgiev *et al.*, 2008; Pavokovi and Krsnik-Rasol, 2011). The higher staining intensity typical of the betacyanins in the hyperhydric shoots cultivated with BAP indicate the stress condition to which this plant material was exposed. These results allow to broaden the studies on plant stress and its relation with the production of metabolites by plants.

The recalcitrance of the hyperhydric explants corroborates the idea that ROS may be a possible link between oxidative stress and plant regeneration in tissue culture. Hyperhydricity compromises quality and impairs the acclimatization of micropropagated plants, where in commercial micropropagation, about 60% of plants may exhibit hyperhydric symptoms. The correlation of the phenomenon with the accumulation of ROS has been demonstrated by other authors and the present research suggests the existence of an oxidative stress linked to the condition

of hyperhydricity (Wu *et al.*, 2009; Tian *et al.*, 2015; Muneer *et al.*, 2018). The excess cytokinin and the reduction of gas exchange in the interior of the vessel to the external environment increased the stress condition, making it difficult to regenerate the tissues (Emara *et al.*, 2018).

Oxidative stress is characterized by the imbalance between ROS generation and the action of the antioxidant system (Mittler, 2002). The evaluation of the activity of antioxidant enzymes demonstrates that the enzyme APX was ahead in the line of defense against oxidative stress. The high APX activity in shoots cultivated at 2.22 BAP and 1.11 μM I⁻¹ of mT were related to high H₂O₂ levels in these treatments (Table 3), demonstrating the high affinity of this enzyme for the effective control of H₂O₂ (Cuypers *et al.*, 2016). On the other hand, the activity of CAT remained unchanged, confirming the information that the increase in H₂O₂ levels were not enough to cause adjustments in the activity of this enzyme.

Changes in redox metabolism in stressed plants has been evaluated by measuring the content of malondialdehyde (MDA), a lipid peroxidation product that has been used as a biochemical marker for evaluation of stress damage in plants (Wang *et al.*, 2003; Gülen *et al.*, 2008; Rubio-Wilhelmi *et al.*, 2011). The evaluation of MDA allows to determine the level of damage to cell membrane lipids and subcellular membranes. The main consequences of lipid peroxidation in cells are decreased fluidity, reduced membrane selectivity, damage in membrane proteins, inactivation of receptors, enzymes and ion channels. Plants exposed to stress exhibit increased levels of lipid peroxidation due to increased accumulation and generation of ROS (Gill and Tuteja, 2010). In the present work, the results showed that shoots cultivated with 2.22 BAP presented increases of 141 and 67% in MDA contents in relation to treatments with 1.11 BAP and mT, respectively. This result is indicative the change in redox reactions, suggesting a stress condition characterized by increased damage to the lipid membranes.

The results of this work indicate the possibility to use mT in the micropropagation of erect prickly pear in semisolid MS medium (Figure 1 F), with reduction in the occurrence of

hyperhydricity (Table 2). The increase in lipid peroxidation and the occurrence of hyperhydricity in the aerial part of *O. stricta* are indicative of stress that can lead to recalcitrance of the plant material. Thus, studies to improve the morphophysiological quality of micropropagated shoots and increasing the production of seedlings of *O. stricta* with socioeconomic interest, are important.

CONCLUSIONS

The results obtained in the present research suggest the use of mT in the *Opuntia stricta* Haw micropropagation. The occurrence of abnormalities *in vitro* culture compromises the production of seedlings, since the explants may become recalcitrant. The cytokinin type, concentration and nutrient medium consistency may alter regeneration capacity in *O. stricta* on *in vitro* culture.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Aziz AN, Tan BC, Othman RY, Khalid N (2018) Efficient micropropagation protocol and genome size estimation of an important cover crop, *Mucuna bracteata* DC. ex Kurz. Plant Cell Tissue and Organ Culture 132(2): 267-278; doi: 10.1007/s11240-017-1376-3
- Adelberg J, Naylor-Adelberg J (2012) Effects of Cytokinin on Multiplication and Rooting of *Aloe barbadensis* during Micropropagation on Agar and Liquid Medium. Journal of Medicinalley Active Plants 1(1): 1-5; doi: 10.7275/R5251G4V
- Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell and Environment

24(12): 1337-1344; doi: 10.1046/j.1365-3040.2001.00778.x

Amoo SO, Finnie JF, Van-Staden J (2011) The role of meta-topolins in alleviating micropropagation problems. *Plant Growth Regulation* 63(2): 197-206; doi: 10.1007/s10725-010-9504-7

Areces-Mallea AE (2017) *Leptocereus demissus* a New Species from Southwestern Hispaniola. *Cactus and Succulent Journal* 89(3): 115–121; doi: 10.2985/015.089.0303

Areces-Mallea AE (2017) *Leptocereus demissus* a New Species from Southwestern Hispaniola. *Cactus and Succulent Journal* 89(3): 115–121; doi: 10.2985/015.089.0303

Aremu AO, Bairu MW, Doležal K, Finnie JF, Van-Staden J (2012) Topolins: A panacea to plant tissue culture challenges? *Plant Cell, Tissue and Organ Culture* 108(1): 1-16; doi: 10.1007/s11240-011-0007-7

Aremu AO, Doležal K, Van-Staden J (2017) New cytokinin-like compounds as a tool to improve rooting and establishment of micropropagated plantlets. *Acta horticulturae* (1155): 497-504; doi: 10.17660/ActaHortic.2017.1155.73

Bairu MW, Jain N, Stirk WA, Doležal K, Van-Staden J (2009) Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. *South African Journal of Botany* 75(1): 122–127; doi: 10.1016/j.sajb.2008.08.006

Bairu MW, Stirk WA, Doležal K, Van-Staden J (2007) Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can meta-topolin and its derivatives serve as replacement for benzyladenine and zeatin? *Plant Cell, Tissue and Organ Culture* 90(1): 15-23; doi: 10.1007/s11240-007-9233-4

Balen B, Tkalec M, Štefaniæ PP, Vidakoviæ-Cifrek Ž, Krsnik-Rasol M (2012) *In vitro* conditions affect photosynthetic performance and crassulacean acid metabolism in *Mammillaria gracilis* Pfeiff. tissues. *Acta Physiologiae Plantarum* 34(5): 1883-1893; doi: 10.1007/s11738-012-0986-y

Barbosa ML, Silva TGFD, Zolnier S, Silva SMSE, Steidle-Neto AJ (2018) The influence of cladode morphology on the canopy formation of forage cactus plants. *Revista Caatinga* 31(1): 180-190; doi: 10.1590/1983-21252018v31n121rc

Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* 195(1): 133-140

Camargo SS, Rufato L, Magro M, Souza ALK (2019) Temporary immersion biorreactors: efficient technique for the propagation of the Pircinque strawberry. *Revista Brasileira de Fruticultura* 41(1): 00-00; doi: 10.1590/0100-2945(2019)102

Cheng ZJ, Wang L, Sun W, Zhang Y, Zhou C, Su HY, Li W, Sun TT, Zhao XY, Li XG, Cheng Y, Zhao Y, Xie Q, Zhang XS (2013) Pattern of Auxin and Cytokinin Responses for Shoot Meristem Induction Results from the Regulation of Cytokinin Biosynthesis by AUXIN RESPONSE FACTOR3. *Plant Physiology* 161(1): 240-251; doi: 10.1104/pp.112.203166

Cruz MAL, Silva ADC, Veiga CFM, Silveira V (2009) Biofábricas para produção de mudas por micropropagação: estratégia para o aumento da produtividade de cana-de-açúcar no Rio de Janeiro. *Inter Science Place* 1(5): 00-00

Csabai J, Nagy ZZ, Mándy AT (2011) *In vitro* shoot proliferation of *Telekia speciosa* (Schreb.) Baumg. Induced by different cytokinins. *Acta Biologica Hungarica* 62(4): 453–462; doi: 10.1556/ABiol.62.2011.4.10

Cuyppers A, Hendrix S, Amaral dos Reis R, De Smet S, Deckers J, Gielen H, Jozefczak M, Loix C, Vercamp H, Vangronsveld J, Keunen E (2016) Hydrogen Peroxide, Signaling in Disguise during Metal Phytotoxicity. *Frontiers in Plant Science* 7: 470; doi: 10.3389/fpls.2016.00470

Davies KM, Albert NW, Zhou Y, Schwinn KE (2018) Functions of Flavonoid and Betalain Pigments in Abiotic Stress Tolerance in Plants. In: Roberts JA, Evan D, McManus MT, Rose JKC (ed). *Annual Plant Reviews* pp. 1-41. John Wiley & Sons Ltd, Chichester, UK; doi: 10.1002/9781119312994.apr0604

- Emara HA, Nower AA, Hamza EM, El Shaib F (2018) Evaluation of Photomixotrophic Technique and Several Carbohydrate Sources as Affecting Banana Micropropagation. *International Journal of Current Microbiology and Applied Sciences* 7(10): 788-804; doi: 10.20546/ijcmas.2018.710.088
- Erig AC, De Rossi A, Fortes GRL (2002) 6-Benzilaminopurina e ácido indolbutírico na multiplicação *in vitro* da amoreira-preta (*Rubus idaeus* L.), cv. Tupy. *Ciência Rural* 32(5): 765-770; doi: 10.1590/S0103-84782002000500005
- Escobar AHA, Villalobos AVM, Villegas MA (1986) Opuntia micropropagation by axillary proliferation. *Plant Cell Tissue and Organ Culture* 7(3): 269-277; doi: 10.1007/bf00037744
- Gao H, Xia X, An L, Xin X, Liang Y (2017) Reversion of hyperhydricity in pink (*Dianthus chinensis* L.) plantlets by AgNO₃ and its associated mechanism during *in vitro* culture. *Plant Science* 254: 1-11; doi: 10.1016/j.plantsci.2016.10.008
- Gava CAT, Lopes EB (2012) Produção de mudas de palma-forrageira utilizando fragmentos de cladódios. Available in: <https://ainfo.cnptia.embrapa.br/digital/bitstream/item/63884/1/INT101.pdf>. Accessed 01/12/2018
- Gentile A, Frattarelli, Nota P, Condello E, Caboni E (2017) The aromatic cytokinin meta-topolin promotes an *in vitro* propagation, shoot quality and micrografting in *Corylus colurna* L. *Plant Cell Tissue and Organ Culture* 128(3): 693-703; doi: 10.1007/s11240-016-1150-y
- Georgiev V, Ilieva M, Bley T, Pavlov A (2008) Betalain production in plant *in vitro* systems. *Acta Physiologiae Plantarum* 30(5): 581-593; doi: 10.1007/s11738-008-0170-6
- Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48(12): 909-930; doi: 10.1016/j.plaphy.2010.08.016
- Gülen H, Cetinkaya C, Kadioglu M, Kesici M, Cansev A, Eriş T (2008) Peroxidase Activity and Lipid Peroxidation in Strawberry (*Fragaria X ananassa*) Plants Under Low Temperature. *Journal of Biodiversity and Environmental Sciences* 2(6): 95-100
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125(1): 189-198; doi: 10.1016/0003-9861(68)90654-1
- Kadota M, Niimi Y (2003) Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in *in vitro*. *Plant Cell, Tissue and Organ Culture* 72(3): 261-265; doi: 10.1023/A:1022378511659
- Lázaro-Castellanos JO, Mata-Rosas M, González D, Arias S, Reverchon F (2018) *In vitro* propagation of endangered *Mammillaria* genus (*Cactaceae*) species and genetic stability assessment using SSR markers. *In Vitro Cellular & Developmental Biology – Plant* 54(5): 518-529; doi: 10.1007/s11627-018-9908-z
- Lebedev V, Arkaev M, Dremova M, Pozdniakov I, Shestibratov K (2018) Effects of Growth Regulators and Gelling Agents on *Ex Vitro* Rooting of Raspberry. *Plants* 8(3): 1-10; doi: 10.3390/plants8010003
- Lema-Rumińska J, Kulus D (2014) Micropropagation of cacti-a review. *Haseltonia* 19: 46-63; doi: 10.2985/026.019.0107
- Liu M, Jiang F, Kong X, Tian J, Wu Z, Wu Z (2017) Effects of multiple factors on hyperhydricity of *Allium sativum* L. *Scientia Horticulturae* 217: 285-296; doi: 10.1016/j.scienta.2017.02.010
- Madzikane-Mlungwana O, Moyo M, Aremu AO, Plíhalová L, Doležal K, Van-Staden J, Finnie JF (2017) Differential responses to isoprenoid, N6-substituted aromatic cytokinins and indole-3-butyric acid in direct plant regeneration of *Eriocephalus africanus*. *Plant Growth Regulation* 82(1): 103-110; doi: 10.1007/s10725-016-0242-3
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plants Science* 7(9): 405-410; doi: 10.1016/S1360-1385(02)02312-9

- Muneer S, Park YG, Jeong BR (2018) Red and Blue Light Emitting Diodes (LEDs) Participate in Mitigation of Hyperhydricity in *In Vitro*-Grown Carnation Genotypes (*Dianthus caryophyllus*). Journal of Plant Growth Regulation 37(2): 370–379; doi: 10.1007/s00344-017-9733-3
- Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15(3): 473–497; doi: 10.1111/j.1399-3054.1962.tb08052.x
- Naaz A, Hussain SA, Anis M, Alatar AA (2019) Meta-topolin improved micropropagation in *Syzygium cumini* and acclimatization to *ex vitro* conditions. Biologia Plantarum 63(1): 174–182; doi: 10.32615/bp.2019.020
- Nakano Y, Asada K (1981) Hydrogen Peroxide is Scavenged by Ascorbate-specific Peroxidase in *Spinach Chloroplasts*. Plant and Cell Physiology 22(5): 867–880; doi: 10.1093/oxfordjournals.pcp.a076232
- Pavokovi D, Krsnik-Rasol M (2011) Complex Biochemistry and Biotechnological Production of Betalains. Food Technology and Biotechnology 49(2): 145–155
- Peixoto MJA, Carneiro MSS, Souza PZ, Diniz JDN, Souto JS, Campos FAP (2006) Desenvolvimento de *Opuntia ficus-indica* (L.) Mill em diferentes substratos, após micropropagação *in vitro*. Acta Sciences Animal Scientiarum 28(1): 17–20; doi: 10.4025/actascianimsci.v28i1.659
- Plaèková L, Hrdlička J, Smýkalová I, Cvečková M, Novák O, Griga M, Doležal K (2015) Cytokinin profiling of long-term *in vitro* pea (*Pisum sativum* L.) shoot cultures. Plant Growth Regulation 77(2): 125–132; doi: 10.1007/s10725-015-0044-z
- Quiala E, Matos J, Montalvo G, Feria M, Chávez M, Pérez AC, Alonso, NLP, Barbín R, Kowalski B (2009) *In vitro* propagation of *Pilosocereus robinii* (Lemaire) Byles et Rowley, endemic and endangered cactus. Journal of the Professional Association for Cactus Development 11: 18–25
- Rahman ZA, Ali MSM, Ghazalli MN, Awang K (2018) Optimization of Culture Media Formulations for Micropropagation of *Lepisanthes fruticosa*. Biosciences Biotechnology Research Asia 15(1): 51–58; doi: 10.13005/bbra/2607
- Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Begoña B, Rios JJ (2011) Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environmental and Experimental Botany, 72(2): 167–173; doi: 10.1016/j.envexpbot.2011.03.005
- Santos WM, Souza RMS, Souza ES, Almeida AQ, Antonino ACD (2017) Variabilidade espacial da sazonalidade da chuva no semiárido brasileiro. Journal of Environmental Analysis and Progress 2(4): 368–376; doi: 10.24221/jeap.2.4.2017.1466.368-376
- Silva MMA, Souza LM (2017) Respuesta de plantas micropropagadas de *Opuntia stricta* Haw en aclimatización y campo. Biotecnología Vegetal 17(2): 91-97
- Singh A (2015) Micropropagation of Plants. In: Bahadur B, Venkat Rajam M, Sahijram L, Krishnamurthy KV (eds). Plant Biology and Biotechnology pp. 329–346. Springer, New Delhi; doi: org/10.1007/978-81-322-2283-5_16
- Soriano-Santos J, Franco-Zavaleta ME, Pelayo-Zaldívar C, Armella MA (2007) Caracterización parcial del pigmento rojo del fruto de la Jiotilla. Revista Mexicana de Ingeniería Química 6(1): 19-25
- Tascan A, Adelberg J, Tascan M, Rimando A, Joshee N, Yadav AK (2010) Hyperhydricity and Flavonoid Content of *Scutellaria* Species *In Vitro* on Polyester-supported Liquid Culture Systems. HortScience 45(11): 1723–1728; doi: 10.21273/HORTSCI.45.11.1723
- Tian J, Jiang F, Wu Z (2015) The apoplastic oxidative burst as a key factor of hyperhydricity in garlic plantlet *in vitro*. Plant Cell, Tissue and Organ Culture 120(2): 571–584; doi: 10.1007/s11240-014-0623-0
- Valero-Aracama C, Kane ME, Wilson SB, Philman NL (2009) Substitution of benzyladenine with meta-topolin during shoot multiplication increases acclimatization of difficult and easy to acclimatize sea oats

- (*Uniola paniculata* L.) genotypes. Plant Growth Regulation 60(1): 43-49; doi: 10.1007/s10725-009-9417-5
- Vasconcelos AGV, Tomas LF, Camara TR, Willadino L (2012) Hiperidricidade: uma desordem metabólica. Ciência Rural 42(5): 837–844; doi: 10.1590/S0103-84782012000500013
- Wang Z, Pote J, Huang B (2003) Responses of Cytokinins, Antioxidant Enzymes, and Lipid Peroxidation in Shoots of Creeping Bentgrass to High Root-zone Temperatures. Journal of the American Society for Horticultural Science 128(5): 648–655; doi: 10.21273/JASHS.128.5.0648
- Werbrouck SPO, Jeugt BV, Dewitte W, Prinsen E, Onckelen HAV, Debergh PC (1995) The metabolism of benzyladenine in *Spathiphyllum floribundum* 'Schott Petite' in relation to acclimatisation problems. Plant Cell Reports 14(10): 662–665; doi: 10.1007/BF00232734
- Wojtania A (2010) Effect of meta-topolin *in vitro* propagation of *Pelargonium x hortorum* and *Pelargonium x hederæfolium* cultivars. Acta Societatis Botanicorum Poloniae 79(2): 101–106; doi: 10.5586/asbp.2010.013
- Wu Z, Chen L J, Long YJ (2009) Analysis of ultrastructure and reactive oxygen species of hyperhydric garlic (*Allium sativum* L.) shoots. *In Vitro Cellular & Developmental Biology - Plant* 45(4): 483–490; doi: 10.1007/s11627-008-9180-8
- Ziv M (2005) Simple bioreactors for mass propagation of plants. In: Hvoslef-Eide AK, Preil W (eds). Liquid Culture Systems for *in vitro* Plant Propagation pp. 79–93. Springer, Dordrecht; doi: 10.1007/s11240-004-6649-y
- Zoghalmi N, Bouamama B, Khammassi M, Ghorbel A (2012) Genetic stability of long-term micropropagated *Opuntia ficus-indica* (L.) Mill. plantlets as assessed by molecular tools: Perspectives for *in vitro* conservation. Industrial Crops and Products 36(1): 59–64; doi: 10.1016/j.indcrop.2011.08.006

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