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GROWTH REGULATORS, SUCROSE AND POTASSIUM IN THE GROWTH AND BIOCHEMICAL ACTIVITY OF *Curcuma longa* L. MICROPROPAGATED

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ABSTRACT - *Curcuma longa* L. is a plant widely used for its pharmacological and medicinal properties, however, does not have a conclusive micropropagation protocol. The objective was to evaluate how growth regulators, sucrose and potassium influence the growth and biochemical activity of *Curcuma longa* seedlings grown *in vitro*. Shoots were inoculated in culture medium supplemented with 6-benzylaminpurine - BAP (8.88 and 17.76 µM), Kinetin - KIN (0.92 and 2.16 µM) and naphthalene acetic acid - NAA (2.16 and 7.20 µM), potassium iodide KI (25 and 50 µM) and sucrose (30 and 60 gL^{-1}). Growth regulators at the lowest concentration and 30 gL^{-1} sucrose promoted increases in growth of *C. longa*. Antioxidant activity was high in all treatments. The activity of the enzymes catalase and ascorbato peroxidase were increased in the two treatments that contained the highest concentrations of growth regulators. It is concluded that the addition of growth regulators such as cytokinins and auxins are fundamental to increase the number of leaves and growth of shoots in *C. longa*. Since the best concentrations are in the range of 4.44 to 8.88 µM of (BAP) and 2.16 of NAA. The addition of supplemental potassium in the culture medium is not necessary, and the beneficial effects of doubling the usual sucrose concentration are nullified if other constituents of the medium are altered. The antioxidant activity and enzymes had their activity altered, especially in treatments that did not contain growth regulators.
Keywords: Turmeric, Zingiberaceae, culture medium, antioxidant, enzymes.

REGULADORES DE CRESCIMENTO, SACAROSE E POTÁSSIO NO CRESCIMENTO E ATIVIDADE BIOQUÍMICA DE Curcuma longa L. *MICROPROPAGADA*

RESUMO - *Curcuma longa* L. é uma planta muito utilizada por suas propriedades farmacológicas e medicinais, no entanto não possui um protocolo de micropropagação conclusivo. Objetivou-se avaliar como os reguladores de crescimento, sacarose e potássio influenciam no crescimento e atividade bioquímica das plântulas de *Curcuma longa* cultivadas *in vitro*. Brotações foram inoculadas em meio de cultura suplementado com 6-benzilaminopurina - BAP (8,88 e 17,76 µM), Cinetina - KIN (0,92 e 2,16 µM) e ácido naftalenoacético - ANA (2,16 e 7,20 µM), iodeto de potássio KI (25 e 50 µM) e sacarose (30 e 60 gL^{-1}). Os reguladores de crescimento na menor concentração e 30 gL^{-1} de sacarose promoveram incrementos no crescimento de *C. longa*. A atividade antioxidante foi elevada em todos os tratamentos. A atividade das enzimas catalase e ascorbato peroxidase foram incrementadas nos dois tratamentos que continham as maiores concentrações de reguladores de crescimento. Conclui-se que a adição de reguladores de crescimento como as citocininas e auxinas são fundamentais para incrementar o número de folhas e crescimento dos brotos em *C. longa*. Sendo que as melhores concentrações estão na faixa de 4,44 a 8,88 µM de (BAP) e 2,16 de ANA. A adição de potássio suplementar no meio de cultura não é necessária e os efeitos benéficos da duplicação da concentração usual de sacarose são anulados se outros constituintes do meio são alterados. E a atividade antioxidante e as enzimas tiveram sua atividade alterada, principalmente nos tratamentos que não continham reguladores de crescimento.
Palavras-chave: açafrão-da-índia, Zingiberaceae,meio de cultura, antioxidante, enzimas.

INTRODUCTION

Curcuma longa L., plant of Asian origin, popularly known as turmeric has been widely used in folk medicine, as well as in the manufacture of medicines, cosmetics, food coloring (AGGARWAL et al., 2007; SUETH-SANTIAGO et al., 2015) and also in landscape projects (PINTO and GRAZIANO, 2003). Its medicinal properties have gained prominence mainly as neoplasms

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inhibitor (FADUS et al., 2017; RIVERA-MANCÍA, 2018), mainly due to curcuminoids, the major compounds in essential oils, which have the potential to inhibit the proliferation of some types of cancer cells (LIU and HO, 2018). In addition, this species has antioxidant and anti-inflammatory properties (ZHAO et al., 2017; FADUS et al., 2017; RIVERA-MANCÍA, 2018).

The planting and formation of new areas are carried out through rhizomes (CECÍLIO FILHO et al., 2004); therefore, the propagative method is asexual with low yield. This method presents some limitations, because the rhizome is used for the manufacture of dyes and also the raw material for the extraction of essential oil. In this case, there is a reduction in the marketed part. In addition, conventional planting presents other limiting factors such as dormancy of rhizomes that do not emit new shoots between May and September, and contamination by pathogens that cause harm to producers (MIACHIR et al., 2004; HAQUE and GHOSH, 2018).

The culture of plant tissues has been shown to be a promising alternative, in order to increase the multiplication rate of seedlings, ensure the absence of pathogens and reduce production time (GEORGE et al., 2008). With the establishment of a protocol *in vitro* the seedlings of *C. longa* could be produced from this technique, thus all the production of rhizomes would be available for commercialization. Other benefits can be obtained from the elaboration of this protocol, such as obtaining secondary compounds *in vitro* from the use of elicitors (TRETTEL et al., 2017; TRETTEL et al., 2018).

To carry out the micropropagation process the first step is to establish an ideal means of cultivation. The main formulations use the MS medium (MURASHIGE and SKOOG, 1962). Therefore, it is necessary to establish concentrations of growth regulators, sucrose or even the addition of some nutrient. The composition of the medium changes the plant physiology and morphology, mainly due to the stress caused, due to the concentrations used in the cultivation protocol, resulting in increase or decrease of secondary compounds, as well as causing oxidative damages (GILL & TUTEJA et al. 2010) that may lead to explant death (MATKOWSKI, 2008).

For *C. longa* studies reveal that the addition of growth regulators in the culture medium has been beneficial for plant growth (MOHANTY et al., 2013; ANTONIAZZI et al. 2016), however the establishment of the regulatory concentration remains inconclusive. Moreover, it is expected that with the alteration of the formulation of the medium, the morphogenic responses do not necessarily tend to be the same. For example, usually the recommendation of sucrose in the MS medium is 3% for some species of the Zingiberaceae family can reach up to 6% (MOHANTY et al., 2014; FERRARI et al., 2016). Thus, the development pattern can be changed due to the combination of different concentrations of regulators and sucrose.

Growth regulators, cytokinins and auxins are the most used classes in tissue culture and can influence both plant morphology and physiological responses, as demonstrated by Antoniazzi et al. (2016), in their trials with *C. longa* that verified that the addition of auxins and cytokinins to the MS culture medium promoted an increase in the fresh mass of shoots as well as an increase in the number and dry mass of roots *in vitro*. Sucrose is the most used carbon source in the vast majority of micropropagation protocols, as it works as a carbon skeleton, since *in vitro* plants do not perform photosynthesis (GEORGE et al., 2008).

Another aspect that has been little studied with *C. longa* from *in vitro* cultivation was potassium supplementation in the culture medium. Potassium is a very important macro nutrient for the performance of various enzymes, homeostatic equilibrium and opening and closing of the stomata (AMTMANN and ARMENGAUD, 2009). Thus, it is believed that supplementation of this nutrient would be beneficial to the growth of *C. longa* seedlings.

The micropropagation protocols also lack of studies biochemists of the plants under conditions of different formularizations of the medium. These approaches for plants of *C. longa* are relevant because they demonstrate two situations, the first, how the plant reacts to cell damage and oxidation of biomolecules (BONACINA et al., 2017). In this case, the defense systems are activated by changing the action of compounds with antioxidant activity and free radical-removing enzymes, mainly superoxide dismutase (SOD), ascorbato peroxidase (APX), and catalase (CAT) (GILL and TUTEJA, 2010). And the second, how different supplements can contribute to the increase in antioxidant activity that aims to annul free radicals.

As such, the objective of this work was to evaluate how growth regulators, sucrose doses and potassium supplementation influence the growth, mineral composition and biochemical activity of the *Curcuma longa* seedlings cultivated *in vitro*.

MATERIAL AND METHODS

Obtaining explants and inoculation *in vitro*

Rhizomes of *C. longa* were collected in the medicinal garden of Campus 2 of the *Universidade Paranaense* (UNIPAR), Umuarama (Paraná), in December. These were washed in running water, dried in a laboratory environment without temperature and humidity control. Cracked, withered and disease-like rhizomes have been eliminated. At the time of installation of the tests, shoots emitted were used, which were removed with the aid of a scalpel. Then they were standardized for the length of approximately 1.5 ± 0.3 cm.

In laminar flow, the asepsis of the shoots was performed in a 2% sodium hypochlorite solution for 20 min. with agitation. Then, the shoots were washed with sterile water three times. The explants were inoculated in transparent glass flasks with 350 mL capacity, containing MS culture medium (MURASHIGE and SKOOG, 1962) supplemented with 6.5 g L^{-1}agar (Kasvi®) and pH adjusted to 5.8 before autoclaving. The experiment was set up in a completely randomized design, with five treatments, five

replications and four bottles per plot, totaling 100 parcels per trial. The treatments used in this trial are described in Table 1.

TABLE 1 - Concentrations of sucrose, growth regulators and potassium iodide, added to the MS medium in the growth of *Curcuma longa*.

Treatments	Sucrose (g L^{-1})	Regulators (µM)			K iodide (µM)
		BAP	KIN	NAA	
T1	30	8.88	0.92	2.16	25
T2	60	8.88	0.92	2.16	50
T3	30	17.76	1.38	7.20	25
T4	60	17.76	1.38	7.20	50
T5	30	0	0	0	0

Growth conditions and biometric and physiological assessments

The explants were kept for 90 days in a growth room, at 25±2ºC, photoperiod of 24 h of light (TRETTEL et al., 2017; TRETTEL et al., 2018) and light intensity of 2000 Lux (measured by means of lux meter device), obtained using light emitter diodes (LEDS) lamps from the brand Blumenau, LED T8 10W 6,000K, 100-240V-50/60Hz, power factor: ≥0.92 (high FP).

The evaluated characteristics were: number of shoots, number of roots, number of leaves, length of shoots (mm) and longitudinal diameter of the base (mm), dry biomass of shoots and roots, chlorophyll index. The length and diameter of the seedlings were obtained with the aid of a digital caliper. For the measurement of dry biomass, the plant material was separated and packed in paper bags, then taken to an air circulation oven, at 65°C for 4 days until a constant mass was obtained. The chlorophyll index was obtained using a chlorophyll meter of the brand chlorofilog®, model CFL 1030, turmeric leaves collected randomly from the flasks were positioned horizontally.

Determination of macro and micronutrients

Shoots (leaves plus stem) from each treatment were collected at the end of the experiment and dried in an oven at 65°C until constant biomass. After that, the plant material was sent to the Plant Tissue Laboratory at the University of São Paulo (USP). Nitrogen (N), phosphorus (P), Mg (magnesium), potassium (K), calcium (Ca) and sodium (S) (MALAVOLTA, 1980) were determined. All analyzes were performed in duplicate.

Antioxidant activity by the DPPH method

To obtain the extract, 1g of fresh leaves was weighed, the preparation of the extract followed the methodology proposed by (WATERHOUSE, 2002). The antioxidant activity was based on the extinction of the absorption of the 2,2-diphenyl-1-picryl hydrazyl radical (DPPH 60 µM) proposed by Rufino et al. (2009). The readings taken on the Beckman 640 B spectrophotometer (515 nm) with computer system were monitored every 30 minutes for a total of four readings, where the absorbance reduction was observed until stabilization. Three technical replicates and three biological replicates were used. The results were expressed as percentage of free radical sequestration (% SRL), according to the Equation 1:

$$aa = \frac{[(Ac-Am)]\times 100}{Ac} \quad \text{(Equation 1)}$$

Where:
aa = antioxidant activity (%)
Ca = control absorbance;
As = absorbance of the sample.

Enzyme activity

To obtain the enzymatic extract, 200 mg of leaf tissue were macerated in liquid nitrogen and homogenized in 1.5 mL of extraction buffer, composed of 400 mM of potassium phosphate (pH = 7.8), 1.0 mM EDTA and 200 mM ascorbic acid. The homogenate was centrifuged at 12.000 g, for 15 min at 4ºC and then the supernatant was collected (BONACINA et al., 2017).

SOD (EC 1.15.1.1)

The activity of the enzyme superoxide dismutase (SOD) was measured by its ability to inhibit photoreduction of nitroblue tetrazolium (NBT) as described by Giannopolitis and Ries (1977). The reaction (200 µL) consisted of KPO_4 buffer (pH = 7.8) 100 mM, 1 mM EDTA, 120 mM L-methionine, 750 µM NBT, 20 µM of riboflavin and 20 µL of the crude samples extract. The reading was taken at 560 nm, in which one unit of SOD (U) activity was defined as the amount of enzyme required to inhibit 50% reduction in NBT. SOD activity was expressed as U SOD g^{-1} MF min^{-1}.

CAT (EC 1.11.1.6)

Catalase enzyme activity (CAT) was performed according to the methodology proposed by Havir and McHale (1987). The reaction, (200 µL), consisted of KPO_4 buffer (pH = 7.0) 100 mM, H_2O_2 125 mM, H_2O autoclaved and 20 µL of crude extract from the samples. Activity was determined by the degradation of H_2O_2 within 1 min at 260 nm. Enzyme activity was quantified using the molar extinction coefficient 36 M^{-1} cm^{-1} (ANDERSON et al., 1995) being expressed in µmol H_2O_2 g^{-1} MF min^{-1}.

APX (EC 1.11.1.11)

The activity of ascorbate peroxidase enzyme (APX) was performed according to the methodology proposed by Nakano and Asada (1981). The reaction, (200 µL), consisted of KPO_4 buffer (pH = 7.0) 200 mM, 5 mM ascorbic acid, 1mM H_2O_2, H_2O autoclaved and 20 µL of crude extract from the samples. Activity was determined by the degradation of H_2O_2 in the range of 1 min at 290 nm. Enzyme activity was quantified using the molar extinction coefficient, 28.0 mM^{-1} cm^{-1}. APX activity was expressed as µmol ascorbic acid g^{-1} MF min^{-1}.

All enzymes were evaluated using 96-well flat bottomed elisa plates. Three technical replicates and three biological replicates were used. The equipment used was the UV-VIS spectrophotometer, Espectra Max Plus with SoftMax Pro 6.5.1 program.

Statistical Analysis

The measurements of biometric characteristics, antioxidant activity and enzyme were subjected to the normality test, according to Shapiro Wilk. In sequence, they were submitted to the analysis of variance (ANOVA) a p≤ (0.05) and the averages compared by means of the Tukey test (p≤0.05) using software SISVAR 5.6 (FERREIRA, 2011).

RESULTS AND DISCUSSION

For the characteristics number of leaves, length and dry matter of shoots there were significant differences (p≤ 0.05) (Table 2). For the number of leaves, a reduction of approximately 50% was observed in the control treatment (6.75), when compared to T1, which contained regulators - 8.88 µM (BAP), 0.92 (KIN), 2.16 (NAA) and addition of potassium (25 µM). In the control there was also a reduction in the growth of shoots of 28.77mm also compared to T1 (Table 2). For dry biomass of shoots, the highest average was verified with double the concentration of regulators and addition of potassium (T3), not differing statistically from the others. In the other treatments, the averages obtained were statistically equal (Table 2).

TABLE 2 - Concentrations number of shoots (NS), number of leaves (NL), number of roots (NR), chlorophyll index (CHL), length of shoot (LS), diameter of base (DAB), dry matter shoot (DMS), root dry matter (RDM) as a function of sucrose, growth regulators and K iodide being added to the medium MS of seedlings of *Curcuma longa* cultivated *in vitro* for 90 days.

Treatments	NS	NL	NR	CHL	LS (mm)	DAB (mm)	DMS (g)	RDM (g)
T1	1.25 ± 4 a*	11.50 ± 3a	9.50 ± 1a	22.41 ± 4 a	91.61 ± 11a	5.60±2 a	0.46 ± 0ab	0.47 ± 0 a
T2	2.00±1 a	14.25 ± 5a	7.50±1 a	25.85 ± 6 a	89.85 ± 8 a	6.74±1 a	0.44±0 ab	0.48±0 a
T3	1.50±1 a	14.50±6a	8.00±2 a	24,48±4 a	86.79 ± 6 a	5.27±1 a	0.59±0 a	0.41±0 a
T4	1.00±1 a	10.00±2a	7.50±1 a	23.59 ± 2 a	79.54 ± ab	5.64±1 a	0.53±0 ab	0.52±0 a
T5	0.50±2 a	6.75±3a	7.25±1 a	18.60±5 a	62.84 ± 17b	5.97±0 a	0.36±0 ab	0.46±0 a
CV (%)	8.50	7.20	26.73	19.93	13.19	17.32	20.29	21.34

*averages followed by the same letter in the column do not differ by Tukey test, at 5% probability of error. T1 = 30 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 25 µM K iodide, T2 = 60 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 50 µM K iodide, T3 = 30 g L^{-1} sucrose + 17.76 µM (BAP) + 1.38 (KIN) + 7.20 (NAA) and 25 µM K iodide, T4 = 60 g L^{-1} sucrose + 17.76 µM (BAP) + 1.38 (KIN) +7.20 (NAA) and 50 µM K iodide, T5 = addition of 30 g L^{-1} of sucrose and absence of regulators.

During treatments T1 to T4, which maintained the same concentrations of growth regulators, but with double the concentrations of sucrose and potassium iodide, no gain was seen in the characteristics evaluated (Table 2). Initially, it was thought that doubling the dose of these constituents in the medium could bring benefits to the growth of *C. longa* seedlings, but this hypothesis was not confirmed. In this case, it was only demonstrated that the use of growth regulators was necessary to stimulate the number of leaves and the length of shoots, being more important in this case than the addition of sucrose and potassium.

As for the role of growth regulators, there is a consensus among researchers about the need for use in the development *in vitro* of Zingiberaceae (ABDELMAGEED et al., 2011; JALA, 2012; MOHANTY et al. 2014), 0.92 KIN and 2.16 NAA (DAS et al., 2013; MOHANTY et al., 2013; ANTONIAZZI et al., 2016). The determining question was to establish which concentration should be used for each species. The results of this test, demonstrate values between 4.44 to 8.88 µM (BAP) were ideal. Concentrations above this average have shown few benefits for seedling growth. From these values, the increase in shoots would occur through the subcultures, since the Zingiberaceae have a wide responsive capacity to this method (MELLO et al., 2000; YUSUF et al., 2011; JALA, 2012).

Therefore, combining the use of growth regulators in the recommended ranges the subcultures seems to be a more suitable process for the production of seedlings *in vitro* (MELLO et al., 2000). There is no research demonstrating how regulators act on *C. longa* in gene expression, however studies carried out mainly with *Arabidopsis thaliana* indicate that cytokinins have the ability to stimulate genes involved in the organogenesis process such as *WIND1*, *BBM* and *AP2/ERT* (TANK and THAKER, 2011) and auxins stimulate *STM* and *WUS* both involved in the formation of shoots (NEELAKANDAN and WANG, 2012). Genomic sequencing and studies *in silico* of Zingiberaceae species will facilitate research in this sense, since this prior information is necessary to understand which gene families act in the development of plants.

In a second hypothesis, it was thought that the duplication of the usual sucrose and the addition of K to the medium could effectively contribute to the plants of *C. longa*. Other studies have shown that double the sucrose concentration was positive for Zingiberaceae (JALA, 2012; MOHANTY et al., 2014; FERRARI et al., 2016). However, little was discussed about the different patterns of morphogenetic response when constituents of the medium were changed, such as the concentration of salts, antioxidants, vitamins and regulators. The beneficial effect of double sucrose in this assay was canceled due to the modification of the basal formulation of the MS medium. Similar results were verified for different species of Zingiberaceae and revealed that when the amount of regulators was changed, the beneficial effect of the sucrose dose was canceled (BEJOY et al., 2010; MOHANTY et al., 2014).

Probably the imbalance between potassium and sucrose resulted in the responses of the growth characteristics of the seedlings of *C. longa*. The increase in K^+ levels can cause development problems (ANDRIOLO et al., 2010), among them the smallest growth due to homeostatic imbalances, which may have caused damage to the plasma membrane (AHIRE et al., 2013). In the absorption process, one nutrient can influence the other, given the interactions that can occur (SILVA and TREVISAN, 2015). Yet absorption does not depend only on its availability, but on its concentration, as a result of competition or association of different cations. Thus, the addition of potassium did not result in an increase in the mass and length of the seedlings, since the amount of this nutrient in the medium is able to supply the need for *C. longa*.

The nitrogen values remained higher than 30 g Kg^{-1} until T3, after which there was a reduction in the assimilation of this nutrient by the plant results that are also similar for potassium (Table 3). The literature points out that an imbalance in the levels of potassium can impair the absorption of calcium and magnesium (ANDRIOLO et al., 2010; SILVA and TREVISAN, 2015) this was only verified in the T5 that presented a higher concentration of calcium (Ca) in contrast less availability of Mg. This fact is related to its very similar chemical properties, such as the degree of valence and mobility, causing competition for adsorption sites (SALVADOR et al., 2011). However, Ca seems to have been favored by K, as it showed a higher amount of Ca in the leaves in all treatments suggesting a synergistic action between these nutrients.

TABLE 3 - Macro and micronutrients quantified in the shoots of *Curcuma longa* in the presence of different concentrations of sucrose, growth regulators and K iodide, after 90 days of cultivation *in vitro*.

Treatments (g kg^{-1})	N	K	Ca	Mg	S	P
T1	35.10±3.06	42.70±2.16	3.35±0.49	1.64±0.00	1.89±0.00	4.0±0.13
T2	30.50±2.61	43.50±6.49	6.20±1.03	1.70±0.33	1.65±0.37	4.1±2.61
T3	35.23±0.66	45.90±2.16	5.40±0.30	2.50±0.11	2.10±0.24	4.0±0.66
T4	28.45±1.88	36.72±0.00	5.89±0.10	2.0±0.19	2.10±0.53	3.66±1.88
T5	29.50±2.65	39.79±6.49	7.13±1.85	1.45±0.50	2.0±0.54	4.1±2.65

T1 = 30 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 25 µM K iodide, T2 = 60 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 50 µM K iodide, T3 = 30 g L^{-1} sucrose + 17.76 µM (BAP) + 1.38 (KIN) + 7.20 (NAA) and 25 µM K iodide, T4 = 60 g L^{-1} sucrose + 17.76 µM (BAP) + 1.38 (KIN) + 7.20 (NAA) and 50 µM K iodide, T5 = addition of 30 g L^{-1} of sucrose and absence of regulators.

The antioxidant activity increased 23.09% in T3 (Figure 1). Treatments four, five (T4 and T5) showed similar averages with no statistical difference between them. Treatments one and two showed intermediate antioxidant activity between 18.23 and 19.83%. The method used to check the antioxidant activity DPPH can react with phenolic compounds, as well as with aromatic acids (BORGES et al., 2011). In this case, these compounds are a reaction of plants to stress situations caused mainly by the generation of free radicals (BARTOSZ, 1997; TRIANTAPHYLIDÈS and HAVAUX, 2009). All treatments in some way caused stress on the seedlings of *C. longa*. What was verified by the high antioxidant activity.

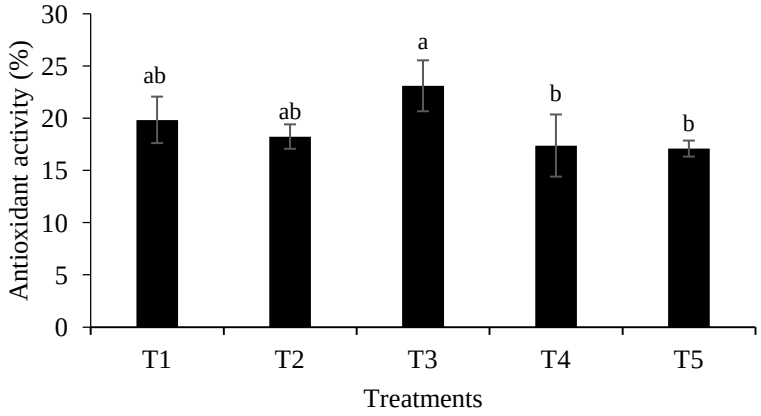

FIGURE 1 - Antioxidant activity (%) of leaves *Curcuma longa* as a function of sucrose, growth regulators and K iodide concentrations. *Average followed by the same letter in the column do not differ by Turkey's test, at 5% error probability. T1 = 30 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 25 µM K iodide, T2 = 60 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 50 µM K iodide, T3 = 30 g L^{-1} sucrose + 17, 76 µM (BAP) + 1.38 (KIN) + 7.20 (NAA) and 25 µM K iodide, T4 = 60 g L^{-1} sucrose + 17.76 µM (BAP) + 1.38 (KIN) + 7.20 (NAA) and 50 µM K iodide, T5 = addition of 30 g L^{-1} sucrose and absence of regulators.

The next studies may contemplate which substances could be providing this antioxidant protection. Studies with this purpose in plant tissue culture and with plants of the Zingiberaceae family are scarce. However, there are numerous studies carried out with leaves and rhizomes of *C.* long from crops, which point to curcuminoids (curcumin, dimethoxycurcumin and bisdimethoxycurcumin) as the main polyphenols in this species (MASUDA et al., 2002; SYED et al., 2015) and may occupy up to 3% of the dry biomass of rhizomes (KUMAR et al., 2006).

As for free radical-removing enzymes, the highest APX activity (2.50 mM) was found in T3, which contained 30 g L^{-1} sucrose, 17.76 µM (BAP), 1.38 µM (KIN), 7.20 µM (NAA) and 25 µM of K iodide, followed by control treatments and four (Figure 2), however there were no statistical differences between these treatments. Lower concentrations of the regulators hardly resulted in activity for this enzyme, which dropped dramatically in T1 and T2.

Similar results were verified for the CAT enzyme. In this case, treatment four showed greater activity (0.5 mM). The control treatments, two and three, showed close activities around (0.3 mM). Again, the lowest activity was seen in T1 (Figure 2), which was the only one that showed a statistical difference. SOD activity among the three enzymes was the one with the highest averages. Close to 1200 U.SOD in T3, T4 and T5 and above these values in T1 and T2, however, statistical differences were not observed.

Failure to add regulators as well as double the concentrations of treatment one resulted in increased activity of the enzymes. This demonstrated that both the absence and the excess of regulators can cause stress in the seedlings of *C. longa*. As shown in Table 2, in the control treatment the seedlings were impaired in growth and number of leaves. The greater activity of APX and CAT in these treatments demonstrated that they must be the result of greater production of free radicals caused by the stress suffered by seedlings.

In the case of SOD, other assays with *C. longa* have revealed that generally this enzyme showed higher averages compared to the others. This fact can probably be associated with the action of this enzyme, which is the first line of plant defense of plants against radicals. A single SOD unit was able to cancel the action of several singlet oxygen $O_2^{\bullet}$. From this reaction, there is formation mainly of the peroxide radicals H_2O_2 which are reduced to H_2O and a radical oxidized mainly by APX and CAT (SCHIEBER and CHANDEL, 2014; DEMIDCHIK, 2015).

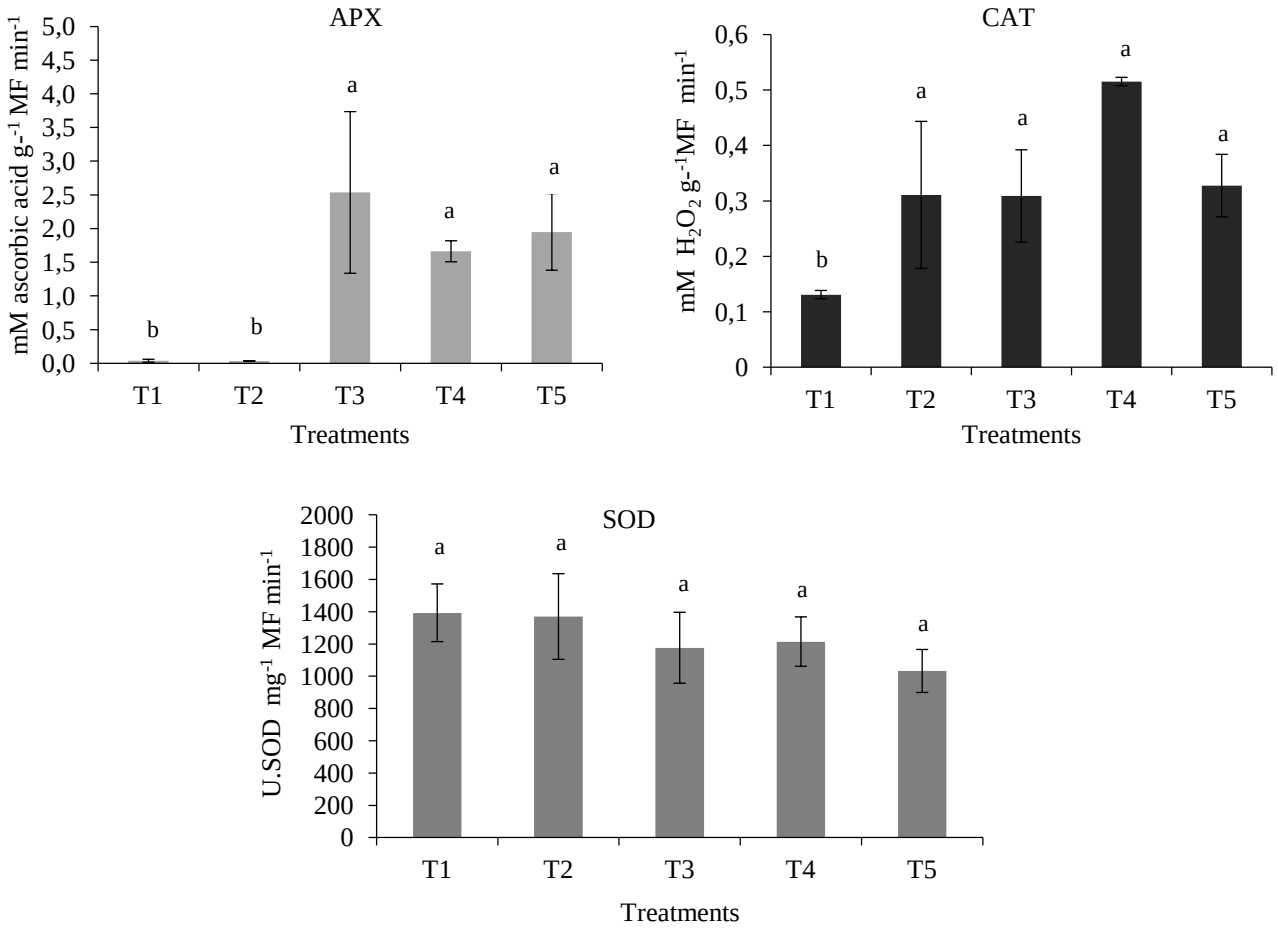

FIGURE 2 - Activity of antioxidant enzymes (APX, CAT and SOD) of leaves *Curcuma longa* due to different concentrations of sucrose, growth regulators and K. iodide *Averages followed by the same letter in the column do not differ by the Tukey test, at 5% probability of error. T1 = 30 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 25 µM K iodide, T2 = 60 g L^{-1} sucrose + 8.88 µM (BAP) + 0.92 (KIN) + 2.16 (NAA) and 50 µM K iodide, T3 = 30 g L^{-1} sucrose + 17.76 µM (BAP) + 1.38 (KIN) + 7.20 (NAA) and 25 µM K iodide, T4 = 60 g L^{-1}sucrose + 17.76 µM (BAP) + 1.38 (KIN) +7.20 (NAA) and 50 µM K iodide, T5 = addition of 30 g L^{-1} sucrose and absence of regulators.

In this way, growth regulators were essential in the multiplication of this species, which will contribute to the increase in the supply of seedlings. The usual dose of sucrose as well as the amount of potassium in the culture medium are also factors that do not need to be modified. The protocol used proved to be efficient and easy to propagate. A next step that could contribute to increasing the supply of seedlings would be the use of bioreactors, which has shown promise for a large number of plants. In this way, this protocol could be used to adapt immersion time and seedling growth conditions.

CONCLUSIONS

The addition of growth regulators such as cytokinins and auxins are fundamental to increase the number of leaves and growth of shoots in *Curcuma longa*. The best concentrations are in the range of 4.44 to 8.88 µM (BAP) and 2.16 NAA.

The addition of supplemental potassium in the culture medium is not necessary and the beneficial effects of doubling the usual sucrose concentration are nullified if other constituents of the medium are altered.

And antioxidant activity and enzymes had their activity altered, especially in treatments that did not contain growth regulators.

REFERENCES

ABDELMAGEED, A.H.A.; FARIDAH, Q.Z.; NORHANA, F.M.A.; JULIA, A.A.; KADIR, M.A. Micropropagation of *Etlingera elatior* (Zingiberaceae) by using axillary bud explants. **Journal of Medicinal Plants Research**, v.5, n.18, p.4465-4469, 2011.

AGGARWAL, B.B.; SUNDARAM, C.; MALANI, N.; ICHIKAWA, H. Curcumin: the Indian solid gold. **Advances in Experimental Medicine and Biology**, v.595, n.1 p.1-75, 2007.

AHIRE, M.L.; LAXMI, S.; WALUNJ, P.R.; KISHOR, P.B.K.; NIKAM, T.D. Effect of potassium chloride and calcium chloride induced stress on *in vitro* cultures of *Bacopa monnieri* (L.) Pennell and accumulation of medicinally important bacoside. **African Journal of Plant Biochemistry and Biotechnology**, v.23, n.4, p.366-378, 2013.

AMTMANN, A.; ARMENGAUD, P. Effects of N, P, K and S on metabolism: new knowledge gained from multi-level analysis. **Current Opinion in Plant Biology**, v.12, n.3, p.275-283, 2009.

ANDERSON, M.D.; PRASAD, T.K.; STEWART, C.R. Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. **Plant Physiology**, v.109, n.4. p.1247-1257, 1995.

ANDRIOLO, J.L.; JÄNISCH, D.I.; SCHMITT, O.J.; DAL PICIO, M., CARDOSO, F.L., ERPEN, L. Doses de potássio e cálcio no crescimento da planta, na produção e na qualidade de frutas do morangueiro em cultivo sem solo. **Ciência Rural**, v.40, n.2, p.237-242, 2010.

ANTONIAZZI, D.; FERRARI, M.P.S.; BARBOSA A.N.; SILVEIRA, F.A.S.; PIO, L.A.S.; PASQUAL, M.; MAGALHÃES, H.M. Growth regulators, DNA content and anatomy *in vitro*-cultivated *Curcuma longa* seedlings. **African Journal of Biotechnology**, v.15, n.32, p.1711-1725, 2016.

BARTOSZ, G. Oxidative stress in plants. **Acta Physiologia e Plantarum**, v.19, n.1, p.47-64, 1997.

BEJOY, M; DAN, M.; ANISH, N.P.; GEORGE, G.A.; RADHIKA, B.J. Induction of multiple shoots in *Amomumhy poleucum* Thwaites - a threatened wild relative of large cardamom. **The Society for Advancement of Horticulture**, v.12, n.1, p.46-49, 2010.

BONACINA, C.; TREVIZAN, C.B.; STRACIERI, J.S.; TIAGO B.; GONCALVES, J.E.; GAZIM, Z.C.; SOUZA, S.G.H. Changes in growth, oxidative metabolism and essential oil composition of lemon balm (*Melissa officinalis* L.) subjected to salt stress. **Australian Journal of Crop Science**, v.11, n.12, p.1665-1674, 2017.

BORGES, L.L.; LÚCIO, T.C.; GIL, E.D.S.; BARBOSA, E.F. Uma abordagem sobre métodos analíticos para determinação da atividade antioxidante em produtos naturais. **Enciclopédia Biosfera**, v.7. n.12, p.1-20, 2011.

CECÍLIO FILHO, A.B.; SOUZA, R.J.D. Caracterização dos estádios fenológicos da cúrcuma, em função da época e densidade de plantio. **Horticultura Brasileira**, v.17, n.3, p.244-247, 1999.

CECÍLIO FILHO, A.B.; SOUZA, R.J.D.; FAQUIN, V.; CARVALHO, C.M.D. Época e densidade de plantio na produção de cúrcuma. **Ciência Rural**, v.34, n.4, p.1021-1026, 2004.

DAS, A.; KESARI, V.; RANGAN, L. Micropropagation and cytogenetic assessment of Zingiberspecies of Northeast India. **Biotech**, v.3, n.6, p.471-479, 2013.

DEMIDCHIK, V. Mechanisms of oxidative stress in plants: from classical chemistry to cell biology. **Environmental and Experimental Botany**, v.109, [s.n.], p.212-228, 2015.

FADUS, M.C.; LAU, C.; BIKHCHANDANI, J.; LYNCH, H.T. Curcumin: Anage-old anti-inflammatory and anti-neoplasticagent. **Journal of Traditional and Complementary Medicine**, v.7, n.3, p.339-346, 2017.

FERRARI, M.P.S.; ANTONIAZZI, D.; NASCIMENTO, A.B.; FRANZ, L.F.; BEZERRA, C.S.; MAGALHÃES, H.M. Evaluation of new protocols to *Curcuma longa* micropropagation: a medicinal and ornamental specie. **Journal of Medicinal Plants Research**, v.10, n.25, p.367-376, 2016.

FERREIRA, D.F. Sisvar: a computer statistical analysis system. **Ciência e Agrotecnologia**, v.35, n.6, p.1039-1042, 2011.

GEORGE, E.F.; HALL, M.A.; KLERK, G.J. **Plant propagation by tissue culture.** v.1, Netherland: editor Springer, 2008. 501p.

GIANNOPOLITIS, C.N.; RIES, S.K. Superoxide Dismutases: I. Occurrence in Higher Plants. **Plant Physiology**, v.59, n.2, p.309-314, 1977.

GILL, S.S.; TUTEJA, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiology and Biochemistry**, v.48, n.12, p.909-930, 2010.

HAQUE, S.M.; GHOSH, B. Micropropagation of *Kaempferia angustifolia* Roscoe - an aromatic, essential oil yielding, underutilized medicinal plant of Zingiberaceae family. **Journal of Crop Science and Biotechnology**, v.21, n.2, p.147-153, 2018.

HAVIR, E.A.; MCHALE, N.A. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. **Plant Physiololgy**, v.84, n.2, p.450-455, 1987.

JALA, A. Effects of NAA BA and sucrose on shoot induction and rapid micropropagation by trimming shoot of *Curcuma longa* L. **International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies**, v.17, n.4, p.101-109, 2012.

KUMAR, G.S.; NAYAKA, H.; DHARMESH, S.M.; SALIMATH, P.V. Free and bound phenolic antioxidants in amla (*Emblica officinalis*) and turmeric (*Curcuma longa*). **Journal of Food Composition and Analysis**, v.19, n.5, p.446-452, 2006.

LIU, H.T.; HO, Y.S. Anticancer effect of curcumin on breast cancer and stem cells. **Food Science and Human Wellness**, v.7, n.2, p.134-137, 2018.

MALAVOLTA, E. **Elementos de nutrição mineral de plantas.** v.1,São Paulo: Editora Agronômica Ceres.1980. 251p.

MASUDA, T.; TOI, Y.; BANDO, H.; MAEKAWA, T.; TAKEDA, Y.; YAMAGUCHI, H. Structural identification of new curcumin dimers and their contribution to the antioxidant mechanism of curcumin. **Journal of Agricultural and Food Chemistry**, v.50, n.9, p.2524-2530, 2002.

MATKOWSKI, A. Plant *in vitro* culture for the production of antioxidants-a review. **Biotechnology Advances**, v.26, n.6, p.548-560, 2008.

MELLO, M.O.; AMARAL, A.F.; MELO, M. Quantifying the micropropagation of *Curcuma zedoaria* Roscoe. **Scientia Agricola**, v.57, n.4, p.703-707, 2000.

MIACHIR, J.I.; ROMANI, V.L.M.; AMARAL, A.F.C.; MELLO, M.O.; CROCOMO, O.J.; MELO, M. Micropropagation and callogenesis of *Curcuma zedoaria* Roscoe. **Scientia Agricola**, v.61, n.4, p.427-432, 2004.

MOHANTY, P.; BEHERA, S.; SWAIN, S.S.; BARIK, D.P.; NAIK, S.K. Micropropagation of *Hedychium coronarium* J. Koenig through rhizome bud. **Physiology and Molecular Biology of Plants**, v.19, n.4, p.605-610, 2013.

MOHANTY, S.; PARIDA, R.; SAHOO, S.; NAYAK, S. *In vitro* conservation of nine medicinally and economically important species of Zingiberaceae from eastern India. **Proceedings of the National Academy of Sciences, India Section B:** Biological Sciences, v.84, n.3, p.799-803, 2014.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Physiologia Plantarum**, v.15, n.43, p.473-497, 1962.

NAKANO, Y.; ASADA, K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. **Plant and Cell Physiology**, v.22, n.5, p.867-880, 1981.

NEELAKANDAN, A.K.; WANG, K. Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. **Plant Cell Reports**, v.31, n.4, p.597-620, 2012.

PINTO, A.C.R.; GRAZIANO, T.T. Potencial Ornamental de Cúrcuma. **Ornamental Horticulture**, v.9, n.2, p.99-109, 2003.

RIVERA-MANCÍA, S., TRUJILLO, J.; CHAVERRI, J.P. Utility of curcumin for the treatment of diabetes mellitus: evidence from preclinical and clinical studies. **Journal of Nutrition & Intermediary Metabolism**, v.14, [s.n.], p. 29-41, 2018.

RUFINO, M.; FERNANDES, F.; ALVES, R.; DEBRITO, E. Free radical-scavenging behaviour of some north-east Brazilian fruits in a DPPH system. **Food Chemistry**, v.114, n.2, p.693-695, 2009.

SALVADOR, J.T.; CARVALHO, T.C.; CORRÊA L.L.A. Relações cálcio e magnésio presentes no solo e teores foliares de macronutrientes. **Revista Acadêmica:** Ciência Animal, v.9, n.1, p.27-32, 2011.

SCHIEBER, M.; CHANDEL, N.S. ROS function in redox signaling and oxidative stress. **Current Biology**, v.24, n.10, p.453-462, 2014.

SILVA, M.L.S.; TREVIZAM, A.R. Interações iônicas e seus efeitos na nutrição de plantas. **Informe Agronômico**, v.1, n.149, p.10-16, 2015.

SUETH-SANTIAGO, V.M.S.; PERON, G.; DECOTÉ-RICARDO, D.; LIMA, M.E.F. Curcumina, o pó dourado do açafrão-da-terra: introspecções sobre química e atividades biológicas. **Química Nova**, v.38, n.4, p.538-552, 2015.

SYED, H.K.; LIEW, K.B.; LOH, G.O.K.; PEH, K.K. Stability indicating HPLC-UV method for detection of curcumin in *Curcuma longa* extract and emulsion formulation. **Food Chemistry**, v.170, n.1, p.321-326, 2015.

TANK, J.G.; THAKER, V.S. Cyclin dependent kinases and their role in regulation of plant cell cycle. **Biologia Plantarum**, v.55, n.2, p.201-212, 2011.

TRETTEL, J.R.; GAZIM, Z.C.; GONÇALVES, J.E.; STRACIERI, J.; MAGALHÃES, H.M. Effects of copper sulfate ($CuSO_4$) elicitation on the chemical constitution of volatile compounds and the *in vitro* development of Basil. **Scientia Horticulturae**, v.234, n.14, p.19-26, 2018.

TRETTEL, J.R.; NASCIMENTO, A.B.; BARBOSA, L.N.; MAGALHÃES, H.M. Volatile essential oil chemical composition of basil (*Ocimum basilicum* L. "Green") cultivated in a greenhouse and micropropagated on a culture medium containing copper sulfate. ***In Vitro* Cellular & Developmental Biology - Plant**, v.53, n.6, p.631-640, 2017.

TRIANTAPHYLIDÈS, C.; HAVAUX, M. Singlet oxygen in plants: production, detoxification and signaling. **Trends in Plant Science**, v.14, n.4, p.219-228, 2009.

WATERHOUSE, A.L. Wine phenolics. **Annals of the New York Academy of Sciences**, v.957, n.1, p.21-36, 2002.

YUSUF, N.A.; ANNUAR, M.S.; KHALID, N. Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. **African Journal of Biotechnology**, v.10, n.7, p.1194-1199, 2011.

ZHAO, S.; YANG, J.; HAN, X.; GONG, Y.; RAO, S.; WU, B.; LIANG, S. Effects of nanoparticle-encapsulated curcumin on HIV-gp120-associated neuropathic pain induced by the P2X 3 receptor in dorsal root ganglia. **Brain Research Bulletin**, v.135, [s.n.], p.53-61, 2017.