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DIRECT ORGANOGENESIS AND LEAF-ANATOMY MODIFICATIONS *IN VITRO* OF *NEOREGELIA CONCENTRICA* (VELLOZO) L.B. SMITH (BROMELIACEAE)

JOÃO PAULO RODRIGUES MARTINS[1*], EDILSON ROMAIS SCHMILDT[2], RODRIGO SOBREIRA ALEXANDRE[2], EVARISTO MAURO DE CASTRO[1], THAÍS FURTADO NANI[1], MARINÊS FERREIRA PIRES[1] AND MOACIR PASQUAL[1]

[1]*Federal University of Lavras, Biology Department, Lavras, MG, Brazil*
[2]*Federal University of Espírito Santo, Agricultural and Biological Sciences Department, São Mateus, ES, Brazil*
[*]*Corresponding g author's e-mail: jprmartinss@yahoo.com.br; + 55353829-1783*

Abstract

Tissue culture can contribute in the multiplication of several species with commercial interest, like the bromeliads. It was aimed to evaluate cytokinins and its concentrations in the multiplication and leaf structure of *Neoregelia concentrica* (Vellozo) L.B. Smith. Previously *In vitro*-established *N. concentrica* plants were inoculated in MS medium supplemented with 6-benzylaminopurine (BAP) or Kinetin (KIN) with concentrations 0.0, 5.0, 10.0 and 15.0 µM. For the anatomic analyses tree plants of each treatment were randomly sampled at 60-day growth. Significant differences were verified in the evaluated characteristics due to the treatments. The raise in cytokinin concentrations induced a higher percentage and average number of explants with shoots. BAP provided higher averages when compared to KIN. The cytokinin use modified the epidermal structure and induced a larger thickening of the water-storage and chlorophyll parenchymas. The use of 15.0 µM BAP was efficient in the *In vitro* multiplication and in the leaf tissue development of *N. concentrica.*

Key words: Bromeliad, Cytokinin, *In vitro* multiplication, Tissue culture.

Introduction

Bromeliads are originated almost exclusively in the American continent and around 50% of the known species are found in Brazil. Most of the Brazilian species are endemic in the Atlantic Forest (Ceita *et al*., 2008; Ribeiro *et al*., 2009), a threatened biome with approximately 8% of its original area preserved (Myers *et al*., 2000).

These plants present ecology importance for being resource sources for the wildlife. They serve as shelter and also hold fleshy fruits, nectar and water (stored between the leaves) (Balke *et al*., 2008). Bromeliads also have commercial value as an ornamental plant, due to the beauty of its leaves and flowers (Vesco *et al*., 2011). For this reason, illegal gathering has been carried out in natural environments aiming income supplement. It is threatening some species with extinction (Negrelle *et al*., 2012).

Therefore, the mass multiplication of bromeliads rises as an economical and ecologic viable option. However, propagation through side shoots results in a limited number of plants, and they may be contaminated by *Fusarium* sp (Feuser *et al*., 2003). According to Guerra & Vesco (2010), the employment of plant tissue culture techniques is potentially important in a wide propagation of species of this family, as verified by Silveira *et al*., (2009) with *Neoglaziovia variegata* (Arr. Cam.) Mez, Santos *et al*., (2010) with *Acanthostachys strobilacea* (Schult. f.) Klotzsch, Huang *et al*., (2011) with *Guzmania*, Vesco *et al*., (2011) with *Billbergia zebrina* (Herbert) Lindley and Silva *et al.,* (2012) with *Nidularium innocentii* Lem. and *Nidularium procerum* Lindm.

Among the factors able to influence *In vitro* propagation, cytokinins are considered the most important factor, as they affect shoot induction, besides the significant effects related to the histological modification in plant tissues (Magyar-Tábori *et al*., 2010). Although these modifications may be observed in different plant organs, leaf is the most important, since the structural and/or physiological alterations can significantly affect the acclimation chance of success (Majada *et al*., 2000; Zobayed *et al*., 2001).

The objective was to evaluate the effects of the cytokinin types and concentrations in the multiplication and leaf structure of *Neoregelia concentrica*, during *In vitro* growth.

Material and Methods

***In vitro* establishment:** *Neoregelia concentrica* (Vellozo) L.B. Smith fruits were collected from adult plants grown in green house and the seeds were extracted mechanically. They were submitted to disinfestation in ethanol 70% for one minute and sodium hypochlorite (50% commercial solution and 2.5 activated chlorine) for 10 minutes. Subsequently, the seeds were washed 3 times in autoclaved distilled water, to remove the disinfesting solution excess and inoculated in test tubes containing 10ml of MS medium (Murashige & Skoog, 1962) with half of the original concentration, supplemented with $30gL^{-1}$ sucrose and solidified with $7gL^{-1}$ agar. The medium pH was adjusted to 5.8 before autoclaving at 120ºC for 20 minutes. After inoculation in horizontal laminar flow cabinet the plant material was kept in growth room at 27±2ºC with a 16-hour photoperiod, under fluorescent lamps providing 25.2µmol $m^{-2}s^{-1}$ photosynthetic photon flux.

Shoot multiplication: Obtained plants, (180-day old), were inoculated in test tubes containing 10 mL stationary liquid MS medium supplemented with $10mgL^{-1}$ citric acid, $30gL^{-1}$ sucrose and plant growth regulator 6-benzilaminopurine (BAP) or kinetin (KIN) with concentrations 0.0, 5.0, 10.0 and 15.0µM. The medium pH was adjusted to 5.8 before autoclaving at 120ºC for 20 minutes. After inoculation in horizontal laminar flow cabinet the plant material was kept in a growth room at 27±2ºC with a 16-hour photoperiod, under fluorescent lamps providing 25.2µmol m^{-2} s^{-1} photosynthetic photon flux.

The experimental design was completely randomized in factorial arrangement (two cytokinin types x four concentrations), with four repetitions, each parcel composed by five test tubes. In total 20 explants per treatment.

The evaluation was performed at 60-day subcultivation. The analyzed phytotechnical features were: Budding (%), shoot average number, longest-leaf average length (cm), fresh- and dry-mass (mg) of the budding. To obtain dry mass, the plant material was previously kept in forced ventilation oven at 65ºC until stabilization. All obtained data were submitted to analysis of variance and the averages were compared through Tukey's test at 5% probability and regression analysis.

Anatomy analysis: To perform anatomic characterization, tree plants from each treatment were sampled randomly at 60-day growth and fixed in a FAA (formaldehyde, acetic acid and ethanol 50%, proportion 0.5:0.5:9, v/v) for 48 hours, followed by conservation in ethanol 70% (Johansen, 1940).

Cross-sections were performed in the median region of the third completely expanded leaf in the rosette central region with aid of a manual microtome. Those were cleared with sodium hypochlorite 3% (v/v) and posteriorly stained with an astra blue and safranin solution, using glycerin 50% to assemble the sliders.

The cross-sections were observed in an optical microscopy coupled to a digital camera to capture images. The photomicrographs were used to measure the anatomy characteristics using the software UTHSCSA-Imagetool® calibrated with microscopy ruler.

The experimental design was completely randomized in factorial arrangement (two cytokinin types x four concentrations), with three repetitions per treatment. It was analyzed thickness of the following anatomic characteristic: adaxial and abaxial epidermis (μm), water-storage parenchyma (μm) and chlorophyll parenchyma (μm).

All the data were submitted to analysis of variance and the measures were compared by Tukey's test at 5% probability and regression analysis.

Scanning electron microscopy analysis: To characterize the leaf epidermis, tree plants from each *In vitro* multiplication treatment were used. The analyses were standardized using the fourth completely expanded leaf from the rosette central region. The material, previously fixed in FAA solution, was washed tree times, for ten min, in cacodylate buffer and post-fixed in osmium tetroxide 1% for, at least, 1 hour at room temperature. Subsequently, the material was washed tree times in distilled water, cut in smaller fragments, dehydrated in acetone gradient (25%, 50%, 75%, and 100%) and took to a critical point drying (CPD030). The leaf fragments were fixed on *stubs*, which were submitted to gold vaporizer (SCD 050) to posterior scanning electronic microscopy analysis in device MEV-LEO-EVO40. The analyses were carried out in the median region of the leaf abaxial epidermis.

Results and Discussion

Shoot multiplication: The different cytokinin used concentrations influenced in distinct ways the organogenesis response of *N. concentrica.* The budding (%) presented quadratic-growing model with the concentration raise, independent on the cytokinin type employed, acting positively in the break of apical dominance and in the induction of adventitious shoots (Fig. 1). The employment of cytokinins in the *In vitro* shoot induction is well documented, as in the papers of Pompelli & Guerra, 2005; Silva *et al*., 2009; Silveira *et al*., 2009; Jafari *et al*., 2011; Khan *et al*., 2011 and Wu *et al.,* 2012. However, in concentrations above ideal, it may occur an inhibitory effect, decreasing the organogenic-response frequency (Arumugam *et al*., 2009), which agrees with the results obtained, in which concentrations above 11.68 μM of the studied cytokinins were harmful to the budding frequency in explants of *N. concentrica* (Fig. 1).

When BAP was used in the medium, compared to KIN, it was observed a larger budding in the explants of *N. concentrica* (Table 1). The lower *In vitro* multiplication rates of *N. concentrica* in the media where it was employed KIN, may be related to the activity of enzymatic systems in the cleavage of furfuryl group. An example is the cytokinins oxidase/dehydrogenase which catalyzes the cytokinin degradation (Schmülling *et al*., 2003; Frébort *et al*., 2011) decreasing or inhibiting the organogenesis response.

The average shoot number presented a significant interaction for each of the cytokinin types and concentrations. The highest shoot number was presented by the explants grown in BAP supplemented media, when compared with KIN (Table 2). Silva *et al*., (2009) and Silveira *et al*., (2009) verified a higher shoot number in *Vriesea scalaris* E. Morren and *N. variegata* respectively, when BAP was employed at the media, if compared to KIN, likewise what we verified with *N. concentrica.* However, this is not common to all plant species, as observed in *Artemisia amygdalina* Decne (Rasool *et al.,* 2013), in which the use isolated BAP was not efficient in the formation of new shoots.

The lowest values were observed in the exogenous-cytokinin-free media (0.0 μM). They had 51.2 and 10.2 times lower in relation to the explants cultivated at 15.0 μM of BAP and KIN, respectively. This evidences the plant growth regulators importance to *In vitro* multiplication of the studied bromeliad (Table 2). *In vitro* organogenesis consists in several factors, among them in the differentiated-cell dedifferentiation followed by redifferentiation. This process may be stimulated by the exogenous application of plant growth regulators (Magyar-Tábori *et al*., 2010). The dedifferentiation (competence) makes the cell capable to respond to hormonal signs to organ induction (determination) (Sugiyama, 1999; Howell *et al*., 2003). According to Gahan & George (2008), the adventitious-shoot formation can be induced by exogenous cytokinins from dedifferentiated cells, which corroborates with the results obtained with *N. concentrica.*

When the average number of shoots was evaluated in relation to BAP and KIN concentrations, a linear growing model was observed for both plant growth regulators (Fig. 2). Pompelli & Guerra (2005) also observed a higher number of shoots in *Dyckia distachya* due to the cytokinin concentration raise. The average values were higher in BAP supplemented media, compared to KIN.

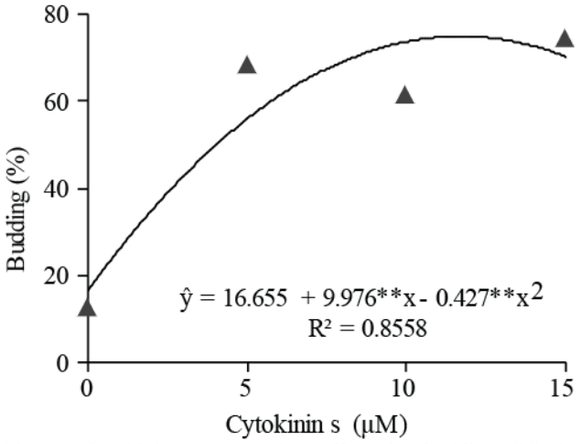

Fig. 1. Cytokinin concentration effect for budding (%) in explants of *Neoregelia concentrica* (**significant coefficient at 1% through t test).

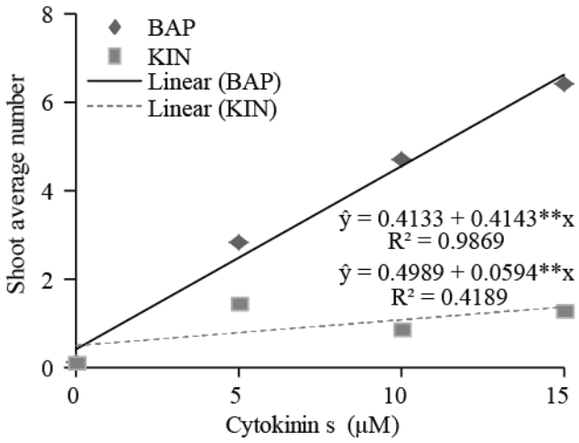

Fig. 2. Effect of the cytokinin types and concentrations on the shoot average number in *Neoregelia concentrica* explants. (**significant coefficient at 1% through t test).

Table 1. Effect of the cytokinin types in the budding (%) of *Neoregelia concentrica* subcultured *In vitro* for 60 days.

Cytokinins	Budding (%)
BAP	66.66 a*
KIN	41.56 b

*Averages followed by the same letter in the column do not differ between themselves according to Tukey's test at 5% probability

Table 2. Effect of the cytokinin types and concentrations on the shoot average number of *Neoregelia concentrica* subcultured *In vitro* for 60 days.

Concentrations (µM)	Shoot average number	
	BAP	KIN
0.0	0.125 a*	0.127 a
5.0	2.833 a	1.450 b
10.0	4.725 a	0.900 b
15.0	6.400 a	1.300 b

*Averages followed by the same letter in the column do not differ between themselves according to Tukey's test at 5% probability

The average length of the largest leaf was higher on plants cultivated with KIN when compared to BAP, regardless the concentrations (Table 3). Cytokinins are known for promoting cell division and expansion. However, the ideal type and concentration to stimulate shoot formation and aerial growth vary according to the plant species (Widiyanto *et al*., 2008). Some studies already showed that KIN is effective to stimulate axillary shoots with longer aerial parts, but it is ineffective to shoot multiplication. On the other hand, BAP is more effective in the induction of shoots with a shorter aerial part (Sinha *et al*., 2000; Rajeswari & Paliwal, 2006). The smaller length of the aerial part of shoots inducted with BAP may be due to toxic effects of ethylene, usually produced in explants grown in media with high concentrations of this cytokinin (Thomas *et al*., 2004; Zulfiqar *et al*., 2009).

The budding fresh mass had a linear model as cytokinin concentration increased, regardless the type (Fig. 3A). Cytokinins play a key-role in sintesis of DNA and plant cell division, providing multiple shoot induction (Kieber & Schaller, 2010; Khan *et al*., 2011) and consequently increase the explant fresh mass due to the increase on diferentiated cell in the shoots.

The budding dry mass displayed interaction of the factors. The highest average was obtained using 15.0 µM BAP, to other concentrations the dry matter average was similar to both cytokinins (Table 4). BAP can increase plant efficiency in converting sugar in dry matter (Gollagunta *et al*., 2004). Since it can regulate important physiological parameters which determine biomass distribution and formation through central genes of the primary metabolic routes. Thus they play a role in the development coordination of the aerial part through information related to nutrients availability (Schmülling, 2004).

When the budding dry mass was evaluated regarding the concentrations, only BAP subcultivated plants differed from each other, presenting a linear model as the concentrations raised (Fig. 3B), indicating a higher stimuli to cell plroliferation with this type of cytokinin.

Anatomical analysis: In this research, at growth conditions, *N. concentrica* leaves in frontal view presented tetracytic stomata with hypostomatic distribution and with trichome scales in both sides of epidermis, irregularly distributed. In transversal section the species presented unstratified epidermis, water-storage parenchyma formed by non-chlorophyll cells with thin walls, chlorophyll parenchyma with isodiametric cells, collateral vascular bundles, usually with larger bundles alternating with smaller bundles, and surrounded by fibers. The mesophyll has dorsiventral organization (Fig. 4). Those leaf characteristics are usual in bromeliads and have already been described in other species of this family (Benzing, 2000; Faria *et al*., 2012; Mantovani *et al*., 2012), as trichome scales in both epidermis, stomata restricted to abaxial epidermis and water-storage parenchyma.

The cytokinin types and concentrations employed influenced the analyzed anatomic characteristics (Fig. 4). The cytokinin type used during *In vitro* multiplication can influence the leaf tissue development; induced differentiated responses which can compromise the survival of *In vitro* propagated plants in the acclimation phase (Namli & Ayaz, 2007).

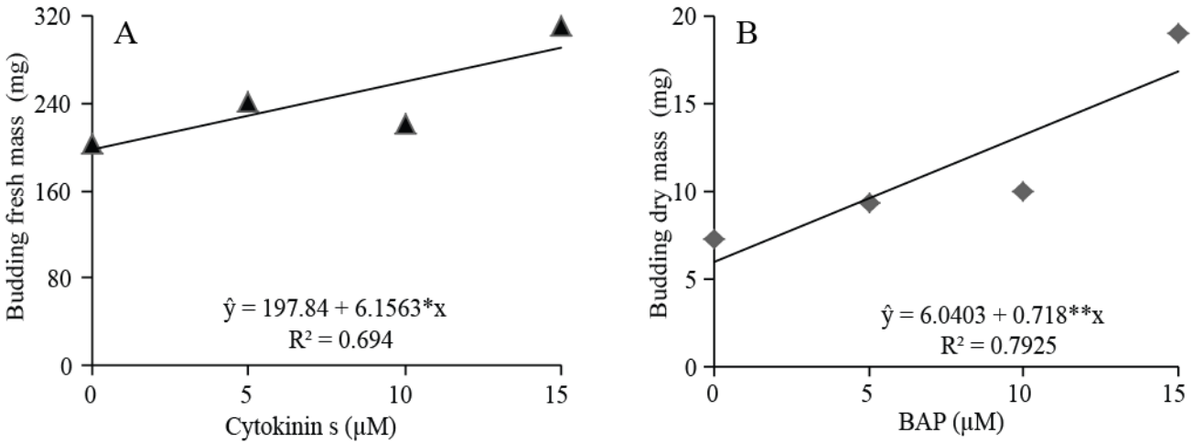

Fig. 3. Budding fresh (A) and dry (B) mass of *Neoregelia concentrica* at 60 days according to cytokinin concentrations (*Significant coefficient at 5% and **significant coefficient at 1% through t test).

Table 3. Influence of cytokinin type in the longest-leaf average length at 60-day *In vitro* subcultivation.

Cytokinins	Longest-leaf average length (cm)
BAP	2.485 b*
KIN	2.948 a

*Averages followed by the same letter in the column do not differ between themselves according to Tukey's test at 5% probability

Table 4. Effects of the cytokinin types and concentrations in the budding dry matter of *Neoregelia concentrica* sub cultivated *In vitro* for 60 days.

Concentrations (µM)	Budding dry matter (mg)	
	BAP	KIN
0.0	7.2 a*	7.6 a
5.0	9.4 a	10.7 a
10.0	10.0 a	9.7 a
15.0	19.0 a	10.4 b

*Averages followed by the same letter in the column do not differ between themselves according to Tukey's test at 5% probability

At 60-day growth trichome scale formation was observed in all the treatments (Fig. 4 R-S). This structure is important for being responsible for water and nutrients absorption, besides contributing in the light excess reflection, which can cause photo inhibition. (Benzing, 2000; Martin *et al*., 2013). The trichomes are connected to the epidermis by one or more cells placed in a uniseries structure, called stalk (Mantovani *et al*., 2012).

Concerning to the stomata complex, a larger opening in the stomata was verified in treatments employing cytokinins in the growth medium, when compared to control. The openings were less elliptical when the plants were cultivated with 15.0 µM BAP (Fig. 4I-P). Alterations in stomata shape directly affect its functionality, the more elliptical shape is characteristic of functional stomata, while more spherical shape is frequently associated to less functional stomata (Khan *et al*., 2003). This morphology is related to humidity of *In vitro* environment since the *N. concentrica* plants were cultivated in hermetic-sealed test tubes and immersed in liquid medium, besides the use of cytokinins in the growth medium, what can stimulate the opening of stomata, as verified by Gokhale and Bansal (2009). Similar results were observed in other plant species propagated *In vitro,* and often associated to high relative humidity and low irradiance during *in vitro* growth (Ivanova & Staden, 2010; Jausoro *et al*., 2010; Saéz *et al*., 2012a, 2012b).

Modifications in epidermal cells were observed. The employment of cytokinins induced cells with larger sinuosity and smaller cell elongation when compared to plants grown in medium free of plant growth regulator, especially at treatment with 15.0 µM BAP (Fig. 4I-P). Stoyanova-Koleva *et al*., (2012) obtained similar results in *Orthosiphon stamineus* Benth., observing larger sinuosity in leaf epidermal cells when the plants were cultivated with BAP in the medium, comparing to control (plant growth regulator-free). The low sinuosity of cell wall may be important for being related to adaptive characteristics against excess loss of water (Krauss, 1948). Cell size is related with hormone role and balance, cytokinins imply in regulation of cell proliferation (Werner *et al*., 2003) and auxins regulate cell proliferation and elongation (Jurado *et al*., 2010), agreeing with what was observed in *N. concentrica*, media with higher cytokinin concentration (hormone unbalance) induced a higher cell proliferation, however with little elongated cells.

Among the *In vitro* multiplication treatments, only the cytokinin concentrations influenced the epidermis thickness regardless the employed type. The thickness of adaxial and abaxial epidermis presented decreasing linear model as the concentrations raised (Fig. 5A and 5B, respectively). The organ size is determined by the cell number and size, it involves two fundamental processes: cell proliferation and expansion (Horiguchi *et al*., 2006; Horvath *et al*., 2006). These processes are controlled by plant hormones, which are known by playing an important role in cell cycle (Kurakawa *et al*., 2007; Jiang *et al*., 2012). In leaf epidermis, cytokinins regulate cell division and expansion, inducing smaller and less expanded epidermal cells due to delay the beginning of cell differentiation (Holst *et al*., 2011),what can explain the epidermal thickness decrease at growth media using exogenous cytokinin, compared to the control.

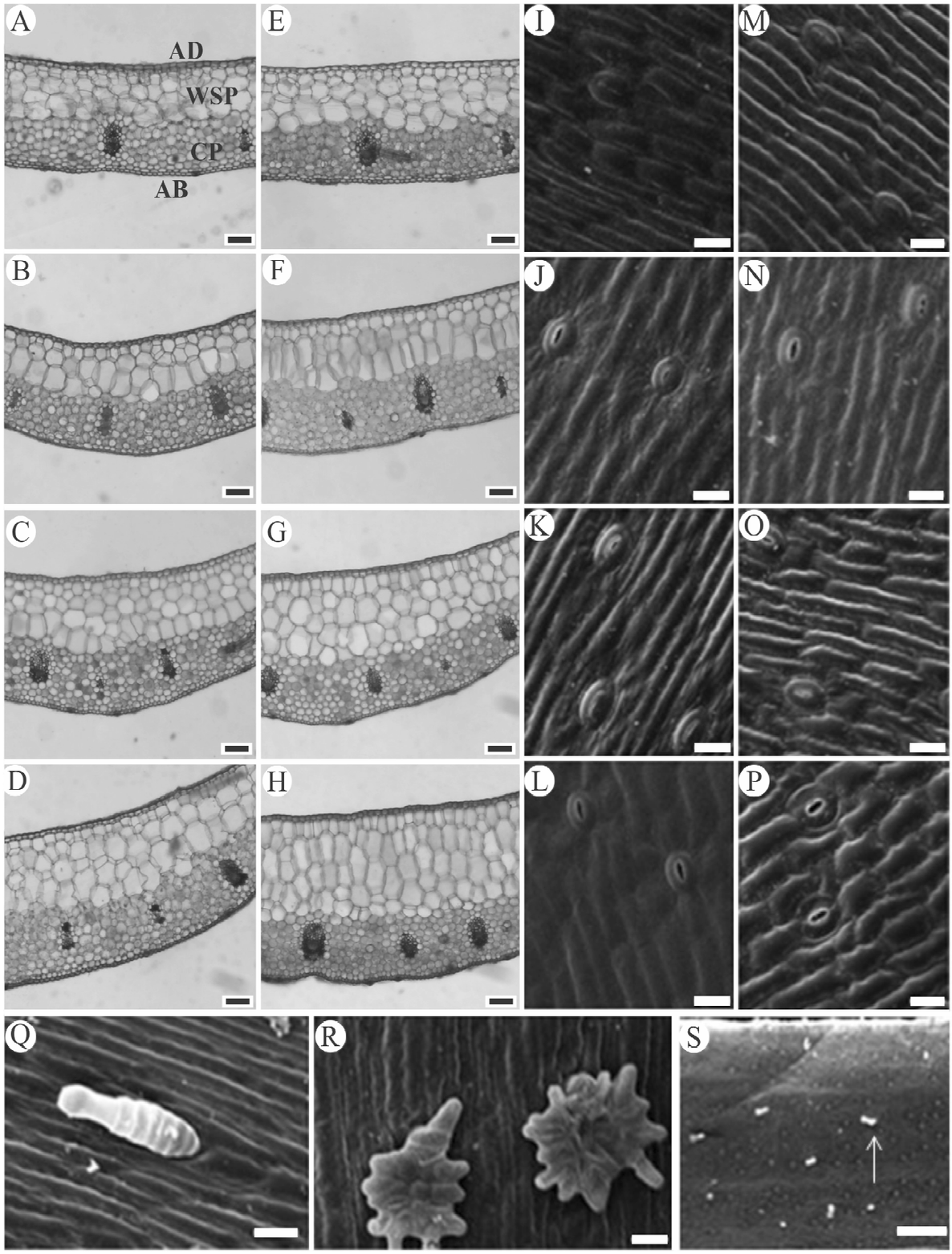

Fig. 4. Leaf sections of *Neoregelia concentrica* after 60 days subcultivation in media with different cytokinin types and concentrations. Transversal sections: KIN (A – 0.0, B – 5.0, C – 10.0, D – 15.0 µM) and BAP (E – 0.0, F – 5.0, G – 10.0, H – 15.0 µM). Abaxial paradermic sections: KIN (I – 0.0, J – 5.0, K – 10.0, L – 15.0 µM) and BAP (M – 0.0, N – 5.0, O – 10.0, P - 15.0 µM). Q – Trichome scale forming. R – Trichome scales. S – Frontal view of abaxial epidermis highlighting the trichome (arrow) irregular distribution. Adaxial epidermis (AD), Abaxial epidermis (AB), Water-storage parenchyma (WSP), Chlorophyll parenchyma (CP). Bar = 100 µm (A-H); Bar = 20 µm (I-Q); Bar = 30 µm (R); Bar = 200 µm (S).

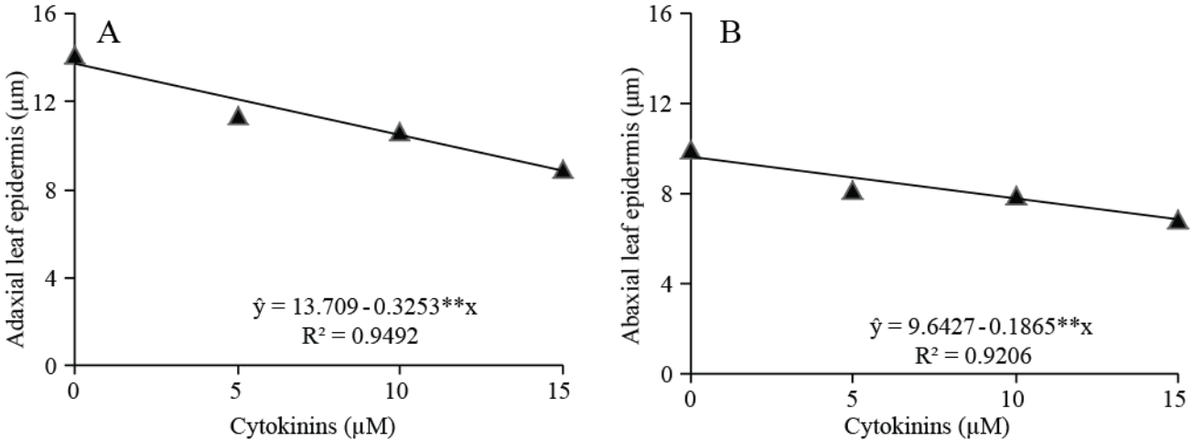

Fig. 5. Cytokinin concentration effect on adaxial (A) e abaxial (B) leaf epidermis in *Neoregelia concentrica* subcultivated for 60 days (**significant coefficient at 1% through t test).

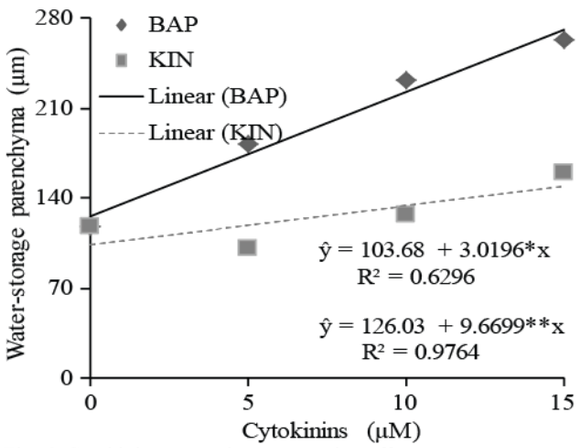

Fig. 6. Cytokinin-type and -concentration effects on water-storage parenchyma thickness in *Neoregelia concentrica* leaves during *In vitro* growth (*significant coefficient at 5% through t test).

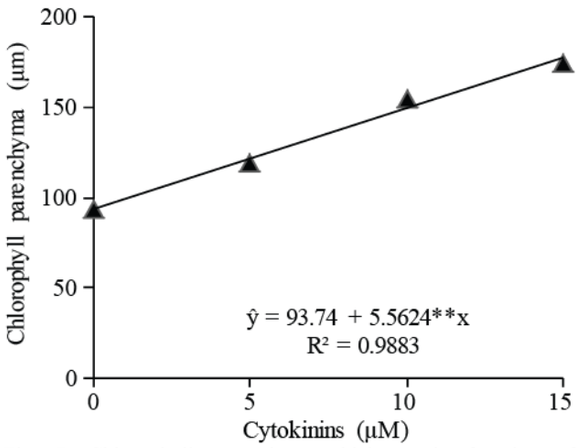

Fig. 7. Chlorophyll parenchyma thickness in function of cytokinin concentration in *Neoregelia concentrica* during *In vitro* growth (**significant coefficient at 1% through t test).

Table 5. Cytokinin-type and -concentration effect on the water-storage parenchyma thickness in *Neoregelia concentrica* subcultured *In vitro* for 60 days.

Concentrations (µM)	Water-storage parenchyma (µm)	
	BAP	KIN
0.0	118.01 a*	117.77 a
5.0	182.35 a	101.12 b
10.0	231.55 a	126.88 b
15.0	262.54 a	159.52 b

*Averages followed by the same letter in the column do not differ between themselves according to Tukey's test at 5% probability

Table 6. Cytokinin-type effect on chlorophyll parenchyma thickness in leaves of *Neoregelia concentrica*.

Cytokinin	Chlorophyll parenchyma (µm)
BAP	158.66 a*
KIN	112.26 b

*Averages followed by the same letter in the column do not differ between themselves according to Tukey's test at 5% probability

The water-storage parenchyma thickness presented significant interaction with both cytokinin types and concentrations, with a positive linear model in function of concentration raise for both cytokinin types (Fig. 6). This higher thickening is related to intense cell division, confirming the role of those plant growth regulators (Noh *et al*., 2010).

In each concentration it was verified that plants cultivated with BAP had superior results than the ones observed with KIN (Table 5). The water-storage parenchyma is the responsible for the hydric maintenance of bromeliads and it protects the chlorophyll region from intense light, besides favoring the photosynthetic process (Brighigna *et al*., 1984). This tissue can have great importance at the time of acclimation, avoiding dehydration of *In vitro* propagated plants during the first days after plantation, contributing to the achievement of high survival rates (Barboza *et al*., 2006). Thus, the employment of 15.0 µM BAP would be favorable to *In vitro* multiplication of the studied bromeliad.

The chlorophyll parenchyma thickness was influenced by the cytokinin types and concentrations, although these factors acted independently. This tissue thickening presented linear model with the cytokinin

concentration raise (Fig. 7). The chlorophyll parenchyma is the tissue responsible for the photosynthesis, its thickening is fundamental for more CO_2 fixation (Castro *et al*., 2009) contributing to the autotrophy of the plants after transfer to *ex vitro* environment. Conventional *In vitro* growth advocates the addition of high sucrose concentrations to the growth medium and low irradiance. These factors can induce anatomical changes which difficult the transition from the heterotrophic to the autotrophic metabolism. This difficulties can cause losses during acclimation (Seon *et al*., 2000; Kozai & Kubota, 2001; Arigita *et al*., 2002), making the use of 15.0 µM BAP potentially important in the chlorophyll parenchyma thickening and, possibly, greater photosynthetic efficiency in leaves of *In vitro N. concentrica.*

For this characteristic the greater thickening was obtained when BAP was employed to the growth media, comparing to KIN (Table 6). BAP application is also important at the organelle level of the chlorophyll parenchyma. There are evidences showing it can promote assimilate storage, which are imported from leaf parts to phloem companion cells, under the form of starch and lipids inside the chloroplasts, contributing to the maintenance of the sucrose concentration gradient in the system of conduction among donors and leaf parts (Paramonova *et al*., 2002).

In conclusion, we demonstrate that the cytokinin type can influence the organogenic response and the leaf anatomy of *Neoregelia concentrica,* being advised the use of BAP in the supplementation of culture medium. In contrast, the use of KIN is not indicated for *in vitro* multiplication of *N. concentrica.*

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