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Effect of synthetic auxins on *in vitro* and *ex vitro* bromeliad rooting[1]

João Paulo Rodrigues Martins[2], Edilson Romais Schimildt[3], Rodrigo Sobreira Alexandre[3], Breno Régis Santos[4], Gizele Cristina Magevski[3]

RESUMO

Efeito de auxinas sintéticas no enraizamento *in vitro* e *ex vitro* de bromélia

A cultura de tecidos pode contribuir na propagação de várias espécies de interesse comercial, como as bromeliáceas. Objetivou-se avaliar o tipo e a concentração de auxinas na rizogênese *in vitro* e *ex vitro* da bromélia *Neoregelia concentrica*. Brotos de *N. concentrica* foram induzidos em meio de cultivo com 15,0 µM de 6-benzilaminopurina, por 80 dias, com posterior subcultivo em meio isento de fitorreguladores, por 45 dias. Na rizogênese *in vitro*, os brotos foram cultivados em meios suplementados com ácido indol-3-butírico (AIB) ou ácido-naftaleno-acético (ANA), nas concentrações de 0,0 µM; 1,0 µM; 2,0 µM; 3,0 µM; e 4,0 µM. Para a rizogênese *ex vitro*, os brotos tiveram suas bases imersas, por 60 minutos, em solução de AIB ou ANA, nas concentrações de 0,0 µM; 5,0 µM; 10,0 µM; e 15,0 µM. Após imersão, os brotos foram plantados em bandejas plásticas contendo vermiculita. Ao final de cada método de rizogênese, foi realizada a análise de parâmetros fitotécnicos. Na rizogênese *in vitro*, observou-se maior número de raízes quando os brotos foram cultivados com concentrações superiores a 1,0 µM de ANA, em relação ao AIB. Contudo, a percentagem de enraizamento diferiu apenas aos 30 dias de cultivo *in vitro*, com maior indução de raízes nos brotos cultivados com ANA. Aos 60 dias, o enraizamento foi superior a 90% e estatisticamente semelhante em todos os tratamentos. Na rizogênese *ex vitro*, observou-se melhor formação do sistema radicular quando aplicados 5,0 µM de AIB, com as maiores médias para enraizamento e número de raízes.

PALAVRAS-CHAVE: *Neoregelia concentrica* (Vell.) L. B. Sm.; Bromeliaceae; cultura de tecidos; rizogênese.

ABSTRACT

The tissue culture can contribute to the propagation of several economic species, such as the bromeliads. This research aimed at evaluating the auxins type and concentration in the *in vitro* and *ex vitro* rhizogenesis of *Neoregelia concentrica* bromeliad. *N. concentrica* shoots were induced in a growth medium with 15.0 µM of 6-benzylaminopurine, for 80 days, followed by sub-cultivation in phytoregulator-free medium, for 45 days. In the *in vitro* rhizogenesis, the shoots grew in a medium supplemented with indole-3-butyric acid (IBA) or naphthalene-acetic acid (NAA), at the concentrations of 0.0 µM, 1.0 µM, 2.0 µM, 3.0 µM and 4.0 µM. In the *ex vitro* rhizogenesis, the bases of shoots were immersed, for 60 minutes, in IBA or NAA solutions, at the concentrations of 0.0 µM, 5.0 µM, 10.0 µM and 15.0 µM. After immersion, the shoots were planted in plastic trays with vermiculite. At the end of each rhizogenesis method, the phytotechnical parameters analysis was carried out. For the *in vitro* rhizogenesis, a higher number of roots were observed when the shoots were cultivated in concentrations higher than 1.0 µM of NAA, when compared to the IBA. However, the rooting rate differed only at 30 days after the *in vitro* growth, with a higher root induction in the shoots grown with NAA. At 60 days, the rooting rate was higher than 90% and statistically similar in all treatments. In the *ex vitro* rhizogenesis, a better formation of the rooting system was observed when 5.0 µM of IBA was applied, with higher rooting averages and number of roots.

KEY-WORDS: *Neoregelia concentrica* (Vell.) L. B. Sm.; Bromeliaceae; tissue culture; rhizogenesis.

INTRODUCTION

The Atlantic Forest biome, which is considered a hotspot, with nearly 8% of its original area preserved, houses several endemic bromeliad species (Myers et al. 2000, Ribeiro et al. 2009). These plants, especially species belonging to the Bromelioideae subfamily, are ecologically important for being sources of fleshy fruits, nectar and water (stored among leaves) and for sheltering mammals, amphibians, birds and insects (Balke et al. 2008).

Bromeliads also present important economic

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2. Universidade Federal de Lavras (UFLa), Departamento de Biologia, Lavras, MG, Brasil. *E-mail*: jprmartinss@yahoo.com.br.
3. Universidade Federal do Espírito Santo (UFES), Centro Universitário Norte do Espírito Santo, Departamento de Ciências Agrárias e Biológicas, São Mateus, ES, Brasil. *E-mails*: e.romais.s@gmail.com, rodrigosobreiraalexandre@gmail.com, gizelemagevski@hotmail.com.
4. Universidade Federal de Alfenas (Unifal), Instituto de Ciências da Natureza, Alfenas, MG, Brasil. *E-mail*: brenors@yahoo.com.br.

value as ornamental plants, due to the beauty of their leaves and flowers (Vesco et al. 2011). So, illegal extraction aiming income complementation has been carried out in natural environments, threatening some species with extinction (Negrelle et al. 2012).

The most common multiplication methods for bromeliads include seed propagation and lateral shoot division. However, these traditional methods are not appropriate for mass propagation (Carneiro et al. 1999), being the *in vitro* technique more suitable for reaching it (Guerra & Vesco 2010, Pickens et al. 2006).

A phytoregulator effect was observed in the *in vitro* morphogenesis of bromeliads, in *Neoglaziovia variegate* (Arr. Cam) Mez (Silveira et al. 2009), *Guzmania* 'Hilda' (Huang et al. 2011a), *Dyckia distachya* Hassler (Pompelli & Gerra 2005), *Vriesea gigantea* Gaudich. and *Vriesea philippocoburgii* Wawra (Droste et al. 2005). Thereby, these authors showed that tissue culture techniques are potentially important to the propagation of this taxonomic group. For the *Vriesea reitzii* Leme & Costa (Alves et al. 2006) and *Aechmea fasciata* Baker (Huang et al. 2011b) species, *in vitro* and *ex vitro* rhizogenesis capacity were observed from *in vitro* shoots.

Ex vitro rooting can contribute to the cost reduction of *in vitro* propagated plants (Phulwaria et al. 2012). *Ex vitro* rooted seedlings may present potential advantages, when compared to the *in vitro* ones, such as a better root system, easier adaptation and higher survival rate (Yan et al. 2010, Benmahioul et al. 2012, Phulwaria et al. 2013).

This study aimed at evaluating the effect of synthetic auxins types and concentrations on *in vitro* and *ex vitro* rhizogenesis of the *Neoregelia concentrica* (Vellozo) L. B. Smith bromeliad.

MATERIAL AND METHODS

The experiment was conducted at the Universidade Federal do Espírito Santo, in São Mateus, Espírito Santo State, Brazil, between September and November 2011.

In vitro growth and multiplication

N. concentrica plants previously established *in vitro* through seeds were inoculated in test tubes containing 10.0 mL of stationary liquid MS medium (Murashige & Skoog 1962), without paper support, supplemented with 10.0 mg L^{-1} of citric acid, 30.0 g L^{-1} of sucrose and 15.0 µM of 6-benzylaminopurine (BAP). After 80 days of growth, shoots were subcultivated, for 45 days, in 250.0 mL vials containing 20.0 mL of stationary liquid MS medium with no phytoregulators and supplemented with 30.0 g L^{-1} of sucrose. The pH medium was adjusted to 5.8, before autoclaving at 120ºC and 1 atm, for 20 minutes. After inoculation in laminar flow cabinet, the plant material was kept in a growth room, at 27 ± 2ºC and a 16-hour photoperiod, under fluorescent lamps providing 25.2 µmol m^{-2} s^{-1} of photosynthetic photon flux.

In vitro rooting

Shoots with approximately 2.0 cm length, from the *in vitro* multiplication stage, were individualized with the aid of a scalpel and inoculated in test tubes containing 10.0 mL of MS medium solidified with 3.5 g L^{-1} of agar and PH adjusted to 5.8, before autoclaving at 120ºC, for 20 minutes. This medium was supplemented with 10 mg L^{-1} of citric acid and concentrations of 0.0 µM, 1.0 µM, 2.0 µM, 3.0 µM and 4.0 µM of IBA (indole-3-butyric acid) or NAA (naphthalene-acetic acid), totalizing 10 treatments. After inoculation in laminar flow cabinet, the material was kept, for 60 days, in a growth room, at 27 ± 2ºC and a 16-hour photoperiod, under fluorescent lamps providing 25.2 µmol m^{-2} s^{-1} of photosynthetic photon flux.

The variables rooting (%) and average number of roots were evaluated at 30 and 60 days and the largest root length (cm) at 60 days.

The experimental design was completely randomized, in a 5x2 factorial scheme (auxin concentrations x types), with four repetitions per treatment and each plot comprising 8 test tubes, totalizing 32 explants per treatment. The obtained data were submitted to variance analysis (Anova) and the auxin-type factor averages were compared by using the Tukey test and the auxin concentration factor by regression analysis.

Ex vitro rooting

Shoots with approximately 2.0 cm length, from the *in vitro* multiplication stage, were individualized with the aid of a scalpel and washed in running water to remove the adhered growth medium. Afterwards,

their bases were immersed, for 60 minutes, in NAA or IBA, at concentrations of 0.0 µM, 5.0 µM, 10.0 µM and 15.0 µM, totalizing 8 treatments. Then, the material was planted in plastic trays with individual cells, containing vermiculite substrate, and kept in a greenhouse with intermittent mist (relative humidity of 70% and temperature of 30ºC). Every 20 days, a substrate supplementation was performed with MS ½ medium to provide the nutritional maintenance of the plants.

The evaluation process took place at 40 days of growth and the analyzed variables were rooting (%), largest root length (cm), average number of roots, volume (mm^3) and superficial area of the root system (mm^2). For the last two variables, millimetered pictures of individual roots were taken and measurements were performed with the aid of the Safira® software (Jorge & Rodrigues 2008).

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RESULTS AND DISCUSSION

The *in vitro* rooting of *N. concentrica* increased linearly at 30 days, with the concentration raise, independently of the auxin type used, acting positively over root formation. The highest auxin concentration resulted in rooting of 91.45% and its absence in 60.4% (Figure 1).

According to Carneiro et al. (1999), every shoot of *N. cruentra* (R. Graham) L. B. Smith rooted after transferred to the auxin-free medium. Arrabal et al. (2002) also observed root formation in *Cryptanthus sinuosus* L. B. Smith grown in auxin-free medium, showing that some species present higher rhizogenic potential than others. The same result was observed in pineapple plants (*Ananas comosus* L.), with every shoot rooted *in vitro*, in a medium with no growth regulator (Sripaoraya et al. 2003).

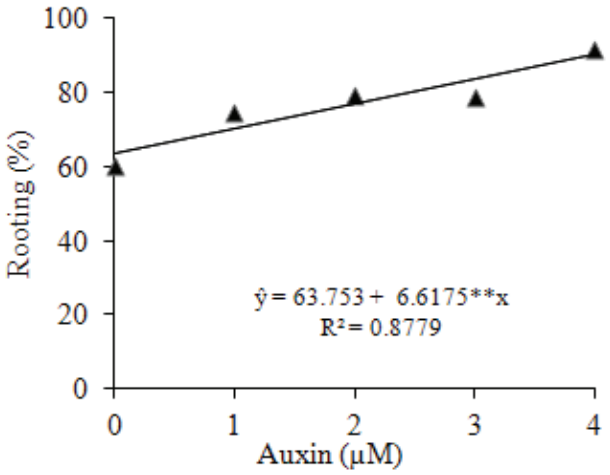

Figure 1. *In vitro* rooting of *N. concentrica* at 30 days, according to auxin concentration (São Mateus, ES, 2011).

When NAA was employed in the growth media, the rooting rate at 30 days was superior to the one with IBA, regardless of its concentration (Table 1). At 60 days, a high rate of root induction was observed in the explants, for both IBA and NAA, being statistically equal (Table 1). Mendes et al. (2008) obtained a higher *in vitro* rooting induction rate with *Vriesea cacuminis* L. B. Smith, when NAA was used in the growth media, if compared to IBA, similary to what was verified in *N. concetrica* at 30 days.

Table 1. Influence of the synthetic auxin type on the *in vitro* rooting of *N. concentrica* at 30 and 60 days (São Mateus, ES, 2011).

Concentration (µM)	Rooting (%)			
	30 days		60 days	
	IBA	NAA	IBA	NAA
0.0	60.9	60.4	91.3	91.2
1.0	68.3	81.2	100.0	97.0
2.0	65.6	93.3	91.5	100.0
3.0	59.7	100.0	92.0	100.0
4.0	83.9	96.4	91.5	100.0
Average*	67.7 b	86.2 a	93.2 a	97.6 a

* Averages followed by the same letter, in the line, for each evaluation date, do not differ among themselves, according to the Tukey test, at 5%.

The use of synthetic auxin aims at accelerating root formation and increasing rooting rate (Santos et al. 2010a), in accordance with the results obtained for *N. concentrica*, with a smaller root induction observed at 30 days of growth, when no exogenous auxin was employed, if compared to the other treatments (Figure 1).

The auxin-free medium presented rooting averages of 60% and 91%, respectively at 30 and 60 days (Table 1). This is probably due to the endogenous concentration of 3-indoleacetic acid (IAA), which managed to ensure the response. This natural auxin, which is naturally produced in the leaves and buds, moves to the low part of the plant, increasing its concentration at the base of the cut, along with other endogenous substances, stimulating root formation (Lone et al. 2010, Hartmann et al. 2011).

The number of roots presented significant interaction between the auxin types and concentrations. At 30 days, the highest number of roots was observed in media with concentrations higher or equal to 2.0 µM of NAA.

In this research, the initial absorption and accumulation of BAP in the shoot-induction medium, as well as its transfering to the rooting medium with 2.0 µM of NAA, may have provided a favorable hormonal balance to the root production. According to Mercier et al. (2003), cytokinins are necessary, together with auxins, to provide cellular division from the G1-S to the G2-S stage. At 60 days, the media containing NAA displayed higher averages than the ones with IBA (Table 2).

When the auxin concentration was evaluated for the number of roots, only the NAA-cultivated plants presented differences with the concentration increment, displaying linear and quadratic models, respectively at 30 and 60 days of *in vitro* cultivation (Table 2). Similar results were observed for *Billbergia rosea* Hortus ex Beer (Pardo et al. 2008), with an increase in the number of roots, when the NAA concentration increased during the *in vitro* cultivation.

No significant difference was verified for the highest average root length, in *N. concentrica* (Table 3). According to Poornima & Ravishankar (2007), auxins may have a negative effect on *in vitro* adventitious root growth, when plants remain in their presence all the time. This was not observed for the employed IBA and NAA concentrations in *N. concentrica*. Galvanese et al. (2007) concluded that the NAA concentration raise in the growth medium induced a smaller length in *Aechmea blanchetiana* (Baker) L. B. Smith.

Under *ex vitro* conditions, a survival rate of 100% was obtained, with root emission at the base of every *N. concentrica* shoot. Pompelli & Gerra (2005) also obtained high *ex vitro* rooting rates, working with the *Dyckia distachya* Hassler bromeliad, with rooting reaching more than 79% for shoots transferred directly to the substrate,

Table 3. Largest root length of *N. concentrica in vitro* shoots, according to synthetic auxin types and concentrations (São Mateus, ES, 2011).

Concentration (µM)	Largest root length (cm)*	
	IBA	NAA
0.0	2.345 a	2.350 a
1.0	1.972 a	2.197 a
2.0	2.275 a	2.415 a
3.0	1.990 a	2.177 a
4.0	2.147 a	2.132 a

* Averages followed by the same letter, in the line, for each evaluation date, do not differ among themselves, according to the Tukey test, at 5%.

Table 2. *In vitro* number of roots of *N. concentrica* at 30 and 60 days, according to synthetic auxin types and concentrations (São Mateus, ES, 2011).

Concentration (µM)	Number of roots			
	30 days		60 days	
	IBA	NAA(1)	IBA	NAA(2)
0.0	1.458 a	1.441 a	2.95 a	2.96 a
1.0	1.642 a	2.647 a	3.58 b	4.94 a
2.0	1.864 b	4.753 a	4.39 b	8.07 a
3.0	1.643 b	7.120 a	3.39 b	9.93 a
4.0	2.826 b	6.557 a	4.20 b	7.97 a

* Averages followed by the same letter, in the line, for each evaluation date, do not differ between themselves, according to the Tukey test, at 5%. (1) $\hat{y} = 1.5731 + 0.9026^{**}x$, $R^2 = 0.9026$; (2) $\hat{y} = 2.4641 + 4.122^{**}x - 0.6552^{**}x^2$, $R^2 = 0.9202$.

regardless of the auxin concentrations applied to the base.

The vermiculite used in the present experiment, as well as other substrates, such as pearlite, presents high macroporosity, what grants a high aeration and water-retention capacity, providing a better exploration of the root system and consequently a larger growth of the aerial part, uniformity and high seedling survival rate, especially during acclimatization (Costa et al. 2009, Ruta & Fortunato 2010). These substrate characteristics, associated to the endogenous IAA synthesized in the buds and young leaves, have probably ensured root induction in the *N. concentrica* explants, even in the absence of exogenous auxins.

The number of roots also found in non-treated explants is probably due to the supply of MS ½ medium, every 20 days. According to Santos et al. (2010b), the use of MS nutritive solution to irrigate *Acanthostachys strobilaceae* (Schult. F.) Klotzsch enabled a better growth rate of the root system, during acclimatization.

Abiotic factors, such as light (spectral quality or amount), also help to explain the root emission in non-treated explants, in the acclimatization process, for instance, the irradiance levels interfere in the accumulation of photoassimilates (Huang et al. 2011b). Some irradiance levels can increase the temperature, what can cause accumulation of metabolites, such as myo-inositol, glucose, fructose and sucrose, and may be related to the plant homeostasis maintenance, mostly in the initial phases of acclimatization (Mollo et al. 2011). Temperature also acts on the carbon fixation (CAM or C_3), as verified for *in vitro* pineapple plants (Nievola et al. 2005).

The number of roots in *ex vitro* cultivation presented interaction between auxin type and concentration. The explants grown in a medium with 5.0 µM of IBA reached a higher average to this variable (6.19 roots) than for NAA (Table 4). Better results with IBA, concerning *ex vitro* growth (Table 4), were probably obtained because this phytoregulator is metabolized in IBA aspartate and posteriorly combined with peptides. This combined form serves as this auxin storage, which is later gradually released, keeping the concentrations at the ideal levels, especially in the final stages of root formation (Rodrigues & Leite 2004).

As the concentration increased, the number of roots raised quadratically for IBA and linearly for NAA (Table 4). As observed for the *in vitro* rooting, *ex vitro* rooting is also affected by the auxin types and concentrations (Yan et al. 2010). Augusto et al. (2006) stated that the *ex vitro* rooting directly on substrate presents the advantages of decreasing the survival associated difficulties, during acclimatization and production, and reducing costs.

The auxin application at the base of cuttings or shoots, in raising doses, can produce stimulating effects on root induction to a peak from which auxin addition becomes inhibitory (Hartmann et al. 2011), what is consistent with the results obtained for IBA, with concentrations higher than 7.0 µM being harmful to root formation in *N. concentrica* (Table 4).

The largest root length in *ex vitro* cultivation also presented interaction among factors. The highest average was obtained with 5.0 µM of IBA, when compared to the same concentration of NAA. The other concentrations had similar results, regardless of the auxin type used (Table 5).

When the concentrations were evaluated according to the largest root length, only the shoots

Table 4. Number of roots of *ex vitro N. concentric*, according to synthetic auxin types and concentrations, after 40 days on vermiculite (São Mateus, ES, 2011).

Concentration (µM)	Number of roots*	
	IBA(1)	NAA(2)
0.0	3.507 a	3.502 a
5.0	6.190 a	3.220 b
10.0	4.930 a	3.940 b
15.0	3.190 b	4.675 a

* Averages followed by the same letter, in the line, for each evaluation date, do not differ among themselves, according to the Tukey test, at 5%. (1) $\hat{y} = 3.6806 + 0.6191^{ns}x - 0.0442^{**}x^2$, $R^2 = 0.8955$; (2) $\hat{y} = 3.1988 + 0.0848^{**}x$, $R^2 = 0.7448$.

Table 5. Largest root length of *ex vitro N. concentric*, according to the synthetic auxin types and concentrations, after 40 days on vermiculite (São Mateus, ES, 2011).

Concentration (µM)	Largest root length (cm)*	
	IBA(1)	NAA
0.0	3.8425 a	3.8675 a
5.0	4.5550 a	3.4475 b
10.0	3.8550 a	3.5150 a
15.0	3.4025 a	3.6975 a

* Averages followed by the same letter, in the line, for each evaluation date, do not differ among themselves, according to the Tukey test, at 5%. (1) $\hat{y} = 3.9255 + 0.1343^{*}x - 0.0116^{**}x^2$, $R^2 = 0.7977$.

treated with IBA differed from each other, following a quadratic model, as the concentration raised (Table 5), and the best IBA concentration was 5.13 µM.

According to Zietemann & Roberto (2007), root emission in larger number and length is fundamental, when the aim is seedling production in commercial scale. Moreover, a well formed root system increases the exploited soil area, promoting water and nutrients absorption, what enables a better seedling growth (Fracaro & Pereira 2004, Carvalho Júnior et al. 2009).

The volume and superficial area of the root system presented significant interaction among factors. When shoots were treated with 5.0 µM of IBA, the roots formed reached higher averages for these variables, when compared to the same concentration of NAA (Table 6).

In the concentration analyses for each auxin, the models were similar for volume and superficial area, concerning the root system. The model was quadratic for both increases in the IBA and NAA concentrations. However, the best results were obtained for IBA, with root system volume and area presenting higher values respectively at the concentrations of 4.88 µM and 5.24 µM (Table 6). The higher number of physiologically active roots, together with a larger root system area, promotes an increase in volume, which can be exploited, representing significant advantage, since it provides a greater adaptation of plants to the soil environment under adverse conditions, as well as for the nutrient absorption increase (Nibau et al. 2008, Rima et al. 2011).

For the external morphology of the root system formed *in vitro* and *ex vitro*, regardless of auxin type and concentration, different characteristics were verified between the rooting methods. *In vitro* roots were more fragile and did not present ramifications,

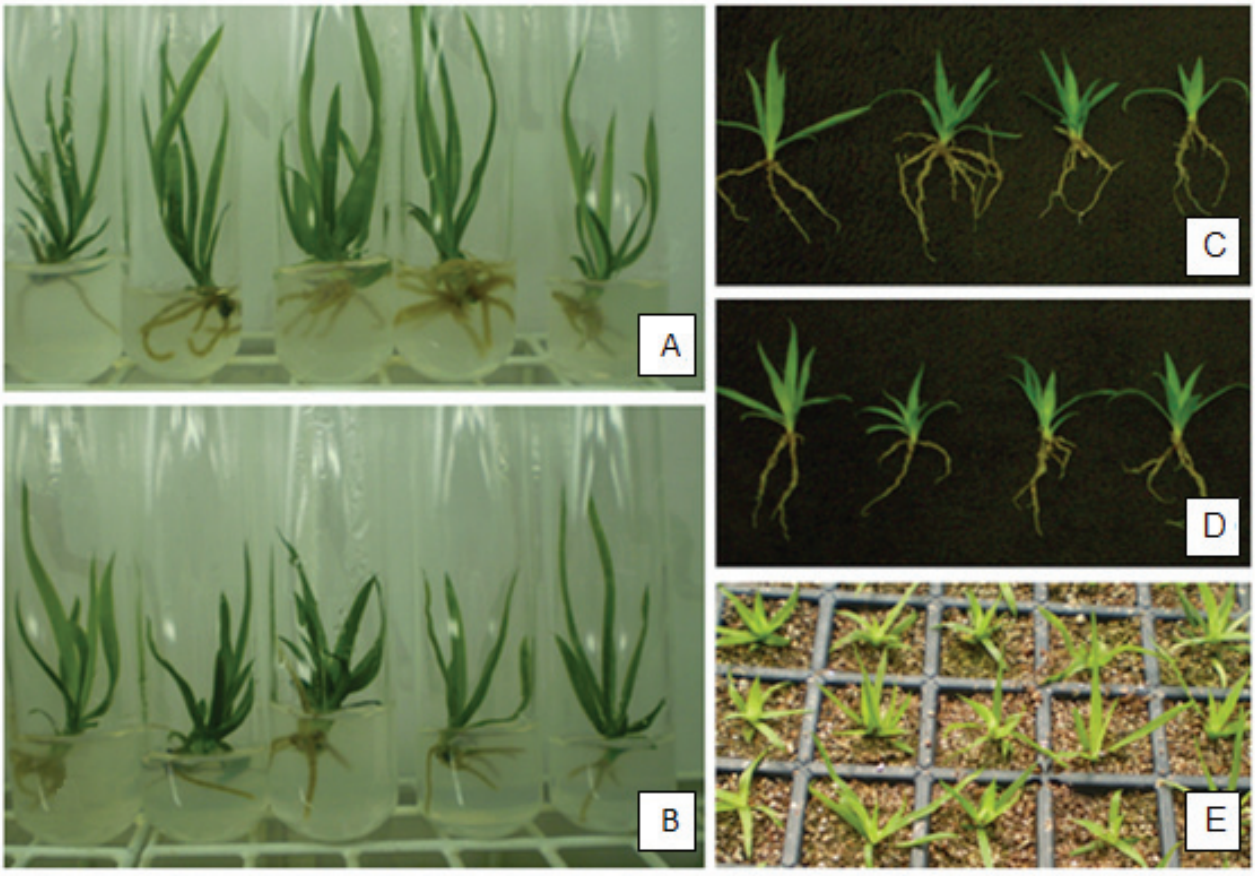

Figure 2. Morphological aspects of *N. concentrica* shoots rooted *in vitro* and *ex vitro* (São Mateus, ES, 2011). A) *In vitro* rooting with NAA (0.0 µM, 1.0 µM, 2.0 µM, 3.0 µM and 4.0 µM); B) *In vitro* rooting with IBA (0.0 µM, 1.0 µM, 2.0 µM, 3.0 µM and 4.0 µM); C) *Ex vitro* rooting with IBA (0.0 µM, 5.0 µM, 10.0 µM and 15.0 µM); D) *Ex vitro* rooting with NAA (0.0 µM, 5.0 µM, 10.0 µM and 15.0 µM), always from the left to the right; E) Plants in *ex vitro* conditions.

Table 6. Root system volume (mm^3) and superficial area (mm^2) of e*x vitro N. concentrica*, according to synthetic auxin types and concentrations, after 40 days on vermiculite (São Mateus, ES, 2011).

Concentration (μM)	Volume (mm^3)		Superficial area (mm^2)	
	IBA[(1)]	NAA[(2)]	IBA[(3)]	NAA[(4)]
0.0	116.26 a	115.96 a	378.89 a	375.14 a
5.0	200.26 a	69.92 b	689.53 a	265.42 b
10.0	86.54 a	83.98 a	297.94 a	284.47 a
15.0	43.89 b	109.51 a	208.08 b	376.61 a

* Averages followed by the same letter, in the line, for each evaluation date, do not differ among themselves, according to the Tukey test, at 5%. [(1)] $\hat{y} = 129.7 + 12.38^{**}x - 1.2665^{**}x^2$, $R^2 = 0.7241$; [(2)] $\hat{y} = 113.53 - 10.84^{ns}x + 0.7156^{**}x^2$, $R^2 = 0.9155$; [(3)] $\hat{y} = 429.09 + 41.994^{**}x - 4.0049^{**}x^2$, $R^2 = 0.6163$; [(4)] $\hat{y} = 372.36 - 29.809^{ns}x + 2.0185^{**}x^2$, $R^2 = 0.9851$.

while *ex vitro* roots were more resistant and with secondary roots (Figure 2). These characteristics were also observed in strawberry (*Fragaria* x *Ananassa* Duch.) (Borkowska 2001) and in *Siratia grosvenorii* (Yan et al. 2010). However, this is not common to all plant species, as observed in *Dickia distachya* (Pompelli & Gerra 2005), with limited and fragile *ex vitro* roots, as opposed to the more functional and numerous *in vitro* roots.

CONCLUSIONS

1. The auxin type influences on the rhizogenic response of *Neoregelia concentrica* shoots, being the NAA more indicated to *in vitro* rooting and the IBA to *ex vitro* rooting.
2. The growth medium supplementation with 3.0 μM of NAA was the most promising method for the *in vitro* rooting of *Neoregelia concentrica* shoots.
3. The application of 5.0 μM of IBA at the *Neoregelia concentrica* shoot base resulted in the best *ex vitro* radicular growth.
4. The *ex vitro* rooting method can be employed for *Neoregelia concentrica* shoots propagated *in vitro* aiming the decrease of costs and cultivation time.

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