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The effect of different cytokinins on chlorophyll content and morphological features of *in vitro Nidularium* 'Kertész Jubileum'

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Summary: During in vitro multiplication of *Nidularium* 'Kertész Jubileum', 20 g/l sucrose, 5 g/l agar, 100 mg/l inositol, and different concentrations of benzyladenine (BA), benzyladenine-riboside (BAR), kinetin (KIN), meta-topolin (mT) were added to the MKC (Knudson, 1946) basal medium. Furthermore, 0.1 mg/l naphthaleneacetic acid was used to every medium. Number of shoots, length of leaves, number and length of roots, chlorophyll (a+b) content were examined and evaluated with Ropstat statistical software. As compared to the other cytokinin, significantly most shoots were obtained in the case of applying BA. Increasing of BA-concentration (as far as 2 mg/l) enhanced shoot number (from 10.92 to 19.26) but 4 mg/l BA resulted only 6.63 shoot. The less efficient cytokinin was KIN, in most cases no more than about 2 shoot was achieved. Regarding the length of leaves, the higher level of BA effected averagely the shorter leaves (from 24,46 to 7.31 mm). KIN effected significantly the longest leaves (43.4-61.29) in inverse proportion to the concentration. The same cytokinin resulted the most (and the longest) roots with the highest rooting percentages, but more KIN decreased the number and length of roots (from 7.95 to 4.4 and from 38.49 to 22.73 mm). There were no definite correlation between cytokinin concentration and chlorophyll (a+b) content, but the highest doses resulted decreasing (except of meta-topolin which leads to the lowest values). Summarizing, BAR effected the highest contents (mostly more than 1400 µg/g), particularly in the case of 1 mg/l (1807.3 µg/g).

Keywords: cytokinin, *Nidularium,* multiplication, rooting, chlorophyll content

Introduction

Nidulariums (a genus about 25 Southeast-Brazilian species and several hybrids) are typical rosette-formed bromeliads with hard, toothed, sword-shaped leaves and inconspicuous flowers, colorful bracts during flowering period (*Makara,* 1982).

Bromeliads are available as nice, variable, amazing indoor plants with wide tolerance. Although slow growing and long breeding-season, these plants can be cultured economically because of their high sale values and low labour costs. Micropropagation of bromeliads is not easy (due to the heavily infected naked buds), but more and more *in vitro* propagated plants were produced and sold (because this is the most efficient vegetative method of multiplying). Most species belonged to the *Bromelioideae* subfamily can be *in vitro* cultured only on liquid medium (tissue necrosis and destructions are easily appears in the case of too much aeration), but higher concentration of macroelements with different kind of natural floral stimulators (e.g. coconut milk) are suitable (*Tillyné & Honfi*, 2008).

Several taxa of bromeliad were used during *in vitro* trials, as *Aechmea fasciata* (*Zimmer & Pieper*, 1974; *Vinterhalter & Vinterhalter*, 1994), *Cryptanthus bromelioides var. tricolor* (*Mathews & Rao*, 1982), *Vriesea gigantea* and *V. philippocoburgii* (*Droste* et al., 2005), *V. fosteriana* (*Mercier & Kerbauy*, 1992), *V. reitzii* (*Rech Filho* et al., 2005, 2009), *Tillandsia cyanea* 'Anita' (*Pierik & Sprenkels*, 1991), *Ananas comosus* (*Khan* et al., 2004; *Be & Debergh*, 2006; *Hamad & Taha*, 2008; *Hamid* et al., 2013).

Only few taxa of *Nidularium* were cultured *in vitro* conditions. *Silva* et al. (2012) sowed sterilized *N. procerum* and *N. innocentii* seeds on MS (*Murashige & Skoog*, 1962) germination medium with 30 g/l sucrose, 0 or 6 g/l agar, 0.37 mg/g NAA and various concentrations of BA. In order to induce multiplication from leaf explants, proximal and distal part of leaves were placed on MS medium with 30 g/l sucrose, 0 or 7 g/l agar, 0.12 mg/g BA and different levels of NAA. For elongation, liquid or solid MS medium (with 7 g/l agar) containing diverse doses of GA_3 (which was added after or before autoclaving) and 30 g/l sucrose was used. There were no interaction between consistency of medium and BA concentrations, but *N. procerum* had higher multiplication rate than *N. innocenti*, especially in the case of 0.9 mg/l BA (14.9 shoots). *N. innocenti* produced only 3.3 shoots/explants on the best medium containing 1.8 mg/l BA. About the observation of the authors, *Paiva* et al. (2009) obtained the best results (5.75 shoot) on medium with 1 mg/l + 0.1 mg/l NAA in the case of *N. fulgens*. Shoot indication from leaf was low (*in vitro* multiplication was more suitable

for seedlings than leaf explants) and *N. innocentii* had lower values (maximum 20% shooting percentage, 2 shoot/explant on solid medium with 0.5 mg/l BA) than *N. procerum* (liquid medium + 0.25 mg/l NAA: 40%, solid medium + 5 mg/l NAA: 4 shoots). However this latter species produce fewer roots in lower percentages (1 root, 4% rooting on solid medium + 0.25 mg/l NAA) than *N. innocentii* (7–10.5 root, 40–70% rooting on liquid medium with 0.12–1 mg/l NAA). High rooting (96–100%) of both species was detected on medium with or without GA_3 during elongation, but more (8.5) roots and lower percentage (61.3%) of lateral shoots was obtained in the case of *N. innocentii* than *N. procerum* (3.4 and 80%).

Carvalho et al. (2013) placed seeds of an endemic, threatened species named *N. minutum* on ½ MS medium containing full-strength micronutrients, 0,1 mg/l thiamine, 100 mg/l myo-inositol, 5 g/l agar, 30 g/l sucrose. Plants were maintained under different temperatures for 3 or 6 months: 25±2 °C (control), 5±2, 10±2 and 15±2 °C. Several features were examined (e.g. length of roots and leaves, fresh or dry mass of shoots or roots, survival rate, chlorophyll a,b and carotenoid content) and datas were shown that higher temperatures resulted significantly heavier fresh and dry weights, longer leaves and roots. Most plants survived the trial (94–100%) except of the coldest treatment (5±2 °C). In most cases, higher values were achieved after 6 months; only contents of pigments were decreased excluding if the temperature was 15±2 °C. Plants which were cared in cooler climate (10 or 15 ±2 °C) have been able to adapt well for lower temperatures, which criteria was beneficial for cost-effective *in/ex vitro* storage or preservation. Not incidentally if reintroduction will be possible, cold-threatened plants can better tolerate extreme temperatures (from 2 to 30 °C) of their natural habitat.

Nidularium 'Kertész Jubileum' is a Hungarian cultivar with decorative yellow bracts, was bred for the 25th anniversary of the Kertész Co-operative (the breeder was József Retkes). A quick micropropagation method was established by *Jámborné* et al. (2003). The proliferation medium (½ MS) was contained with different levels of cytokinins (BA, KIN) + 0.05 mg/l IBA, 20–30 g/l sucrose. For *in vitro* rooting, similar medium was used with 0.25–1 mg/l NAA and 0 or 1 g/l activated charcoal (AC). The highest number of shoots (11.8) was obtained on medium with 1 mg/l BA + 0.05 mg/l IBA and KIN resulted significantly fewer shoots in the case of every concentration. Length of shoots was the largest (4 mm) on medium with 0.25 mg/l BA, although increasing of BA-concentration effected shorter shoots. The optimal sucrose-level was 20 g/l. During the next phase, 1.0 mg/l IBA resulted 83% rooting with averagely 4.4 root but values of rooting were decreased (77% and 2.6) on medium supplemented with 0.75 mg/l IBA. Furthermore, 1 g/l AC significantly dropped both of rooting percentage and the number of roots.

Materials and methods

In vitro plants were used during this trial in the laboratory of Department of Horticultural and Dendrology. For multiplication MKC (*Knudson*, 1946) basal medium with different concentrations and type of cytokinins (which were shown on *Table 1*) was used. Every medium contained 0.1 mg/l NAA, 100 mg/l inositol, 5 g agar, 20 g/l sucrose. The pH was adjusted 5.6 with KOH and autoclaving was done for 30 minutes on overpressure (10^5 Pa). Plants were illuminated by white light 40 µM/m/s using 16/8 light/dark cycles for 3 months. The temperature was 20–25 °C.

Table 1. Growth regulators of MKC (*Knudson,* 1946) medium used during *in vitro* multiplication of *Nidularium* 'Kertész Jubileum' plants

Medium	Cytokinin level	BA (mg/l)	**BAR (mg/l)**	**KIN (mg/l)**	**mT (mg/l)**	**NAA (mg/l)**
A1	1	0.25	–	–	–	0.1
A2	2	0.5	–	–	–	0.1
A3	3	1.0	–	–	–	0.1
A4	4	2.0	–	–	–	0.1
A5	5	4.0	–	–	–	0.1
R1	1	–	0.25	–	–	0.1
R2	2	–	0.5	–	–	0.1
R3	3	–	1.0	–	–	0.1
R4	4	–	2.0	–	–	0.1
R5	5	–	4.0	–	–	0.1
K1	1	–	–	0.12	–	0.1
K2	2	–	–	0.25	–	0.1
K3	3	–	–	0.5	–	0.1
K4	4	–	–	1.0	–	0.1
K5	5	–	–	2.0	–	0.1
M1	1	–	–	–	0.25	0.1
M2	2	–	–	–	0.5	0.1
M3	3	–	–	–	1.0	0.1
M4	4	–	–	–	2.0	0.1
M5	5	–	–	–	4.0	0.1

For determination of chlorophyll (a+b) content 100 mg leaf sample was used (4 fold/treatment). Leaves were destructed by a dash (approx. 0.5 g) of quartz sand and 10 ml acetone (80%) After 24 hour refrigeration absorbance of suspensions was measured by *Genesys 10vis* spectrophotometer at 644 and 663 nm wavelength. Chlorophyll content was calculated by the following formula (*Horváth* & *Erdei*, 2003):

$$\text{Chlorophyll (a+b) µg/g} = (20.2 \times A644 + 8.02 \times A663) \times V/w$$

Where: V = volume of tissue extract (10 ml)
w = fresh weight of tissue (0.1 g)
A = absorbency

Figure 1. Different morphological characteristics of *in vitro Nidularium* 'Kertész Jubileum' plants cultured on medium with different type of cytokinins (K:KIN, A:BA, M: mT)

Figure 2. *In vitro Nidularium* 'Kertész Jubileum' plants (left in worm's-eye view and right in profile)

Figure 3. Collected *in vitro Nidularium* 'Kertész Jubileum' plants before preparing tissue extract (left) and an acclimatized group after the trial (right)

All datas (chlorophyll a+b, length of shoots, leaves and roots, number of shoots and roots; collected from 20 kind of medium and 685 plant) were evaluated by Ropstat (*Vargha*, 2002, 2008) statistical software (one-way analysis of variance, p<0.1–0.01). *In vitro* and (after the experiment) acclimatized plants were shown on *Figure 1–3*.

Results and discussion

The effects of cytokinins on the number of shoots of in vitro multiplicated Nidularium 'Kertész Jubileum'

The highest number of shoots was developed when BA was added to the medium (as compare with the other medium, difference was significant in the case of 1 and 2 mg/l BA). Increasing of BA-level (until 2 mg/l) effected more and more shoots (from 10.92 to 19.26), but 4 mg/l BA had a negative effect on multiplication (with only 6.63 shoots). On the other hand, the highest concentration (4 mg/l) of BAR effected the most shoots (8,1), and 3–5.97 shoots were found on medium with 0.25–2 mg/l BAR. The fewest (1.65–2.03) shoots were achieved in the case of using KIN in every concentration (*Figure 4*). *Jámborné* et al. (2003) had similar results about the effect of KIN and BA on shoot-multiplication, although in that trial different kind of basal medium (½ MS instead of MKC) and lower level of NAA (0.05 mg/l) was used.

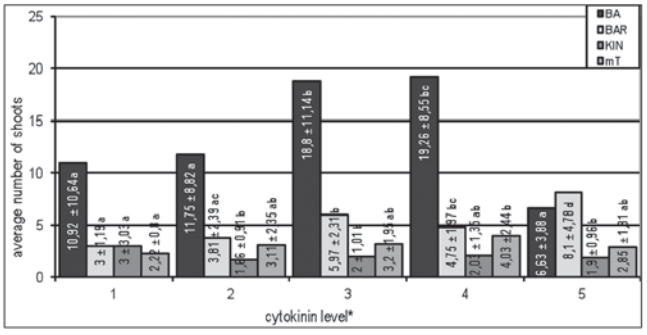

Figure 4. Number of shoots of *in vitro Nidularium* 'Kertész Jubileum' plants cultured on different kind of medium (*cytokinin levels were shown on ***Table 1***)

The effects of cytokinins on the length of leaves of in vitro multiplicated Nidularium 'Kertész Jubileum'

Higher BA doses effected shorter (24.46–7.31 mm) leaves. Similar tendency (with higher values: 26.32–10.68 mm) was obtained on medium with BAR. Plants developed significantly the longest leaves in the case of applying KIN, also higher concentrations resulted shorter leaves: 61.29–43.4 mm (*Figure 5*). Taking it all rounds, there were negative correlation between the number of shoots and the length of leaves. Analogous coherence was detected between shoot-length and -number (*Jámborné* et al., 2003).

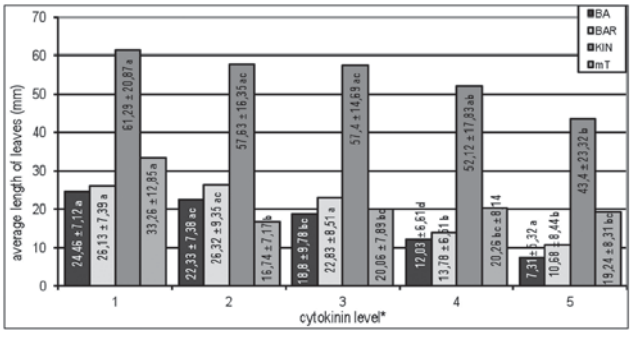

Figure 5. Length of shoots of *in vitro Nidularium* 'Kertész Jubileum' plants cultured on different kind of medium (*cytokinin levels were shown on ***Table 1***)

The effects of cytokinins on the number and length of roots of in vitro multiplicated Nidularium 'Kertész Jubileum'

Root number and -length was the highest (negative correlation with concentration was obtained) on medium supplemented with KIN (7.95-4.4 root and 38.49–22.73 mm length). Mostly not more than 2 root (to a maximum

10 mm) were found in the case of using the other cytokinins, particularly 1–2–4 mg/l BA resulted few (0.09–0.7) root (*Table 2*.).

The effects of cytokinins on percentage of rooting of in vitro multiplicated Nidularium 'Kertész Jubileum'

According to *Figure 6*, plants totally rooted (100%) on medium containing 0.25–0.5–1 mg/l KIN (0,12 and 2 mg/l resulted 95.23 and 83,33%). Similarly high value (96.77%) was achieved in the case of using 0.25 mg/l mT, but higher mT doses reduced rooting (51.42–33.3%). Similar tendency was detected when BA was added to the medium: 0.25 mg/l BA resulted 81,08%, although only 58.33–4.54% of plants developed roots in the case of more BA. The lowest maximum level of percentage (72.5%) was shown by BAR, however there were no as large differences of rooting % between BAR-bearing medium than the other type of cytokinins (except KIN). In most cases (especially more than 0.25 or 0.5 mg/l BA, BAR and mT) only approx. 40–50% of plants produced roots. Presumably 0.1 mg/l NAA was not enough sufficiency for better rooting. In maintenance of this opinion *Jámborné* et al. (2003) achieved higher percentage of rooting (77 and 83% in the case of using 0.75 or 1 mg/l NAA).

On the strength of cytokinin types (*Figure 7*), KIN resulted significantly the highest rooting percentage (95.71%), and BA had the lowest efficiency (44.93%).

Table 2. Number and length of roots, chlorophyll (a+b) contents of *in vitro Nidularium* 'Kertész Jubileum' plants from different kind of medium

Medium	Average number of roots	Average length of roots (mm)	Chlorophyll (a+b) content (µg/g)
A1 (0.25 mg/l BA)	3.27 ± 2.5 a	12.59 ± 10.4 a	1543.6 ± 18.35 abc
A2 (0.5 mg/l BA)	1.77 ± 2.24 b	6.5 ± 8.85 b	1150.3 ± 445.59 abc
A3 (1.0 mg/l BA)	0.7 ± 1.2 c	2.82 ± 5.01 bc	1202.2 ± 245.79 abc
A4 (2.0 mg/l BA)	0.7 ± 0.93 c	3.91 ± 5.03 b	1274.8 ± 204.43 abc
A5 (4.0 mg/l BA)	0.09 ± 0.42 c	0.45 ± 2.13 d	1030.4 ± 19.95 ac
R1 (0.25 mg/l BAR)	2.3 ± 2.76 a	8.77 ± 14.22 ab	1609.4 ± 15.78 bd
R2 (0.5 mg/l BAR)	1.71 ± 2.01 a	6.65 ± 7.74 ab	1437.2 ± 6.29 abc
R3 (1.0 mg/l BAR)	1.11 ± 1.83 a	4.11 ± 6.93 a	1807.3 ± 1.07 ad
R4 (2.0 mg/l BAR)	1.51 ± 1.8 a	11.51 ± 12.43 b	1427.3 ± 2.67 abc
R5 (4.0 mg/l BAR)	0.81 ± 1.07 b	4.24 ± 5.86 a	873.63 ± 1.47 bc
K1 (0.12 mg/l KIN)	7.95 ± 4.59 a	38.14 ± 31.61 ab	953.12 ± 71.21 abc
K2 (0.25 mg/l KIN)	6.26 ± 2.03 a	38.49 ± 28.48 a	1094.7 ± 233.45 abc
K3 (0.5 mg/l KIN)	6.25 ± 2.23 a	36.67 ± 18.59 ab	1217.4 ± 201.41 abc
K4 (1.0 mg/l KIN)	5.81 ± 2.12 a	33.79 ± 21.29 ab	788.29 ± 158.82 abc
K5 (2.0 mg/l KIN)	4.4 ± 4.02 b	22.73 ± 19.91 b	758.11 ± 158.36 abc
M1 (0.25 mg/l mT)	4.19 ± 3.48 a	20.77 ± 21.72 a	1004.2 ± 416.28 abc
M2 (0.5 mg/l mT)	0.88 ± 1.5 b	2.92 ± 4.71 b	1115.1 ± 287.5 abc
M3 (1.0 mg/l mT)	1.31 ± 1.87 b	4.6 ± 6.56 b	869.47 ± 152.65 abc
M4 (2.0 mg/l mT)	0.96 ± 1.25 b	5.19 ± 7.14 b	698.22 ± 183.33 abc
M5 (4.0 mg/l mT)	1.08 ± 2.15	3.7 ± 6.08 b	1027.1 ± 136.2 abc

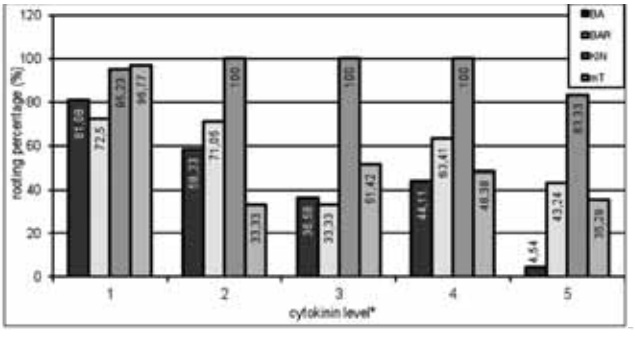

Figure 6. Rooting percentages of *in vitro Nidularium* 'Kertész Jubileum' plants cultured on different kind of medium (*cytokinin levels were shown on ***Table 1***)

The effects of cytokinins on chlorophyll (a+b) content of leaves of in vitro proliferated Nidularium 'Kertész Jubileum'

There were no definite coherence between chlorophyll (a+b) content of leaves and concentration of cytokinins, but the highest doses (except mT) effected decreasing (*Table 2*.). BAR led to the best results (with mostly more than 1400 µg/g) and the highest value (1807.3 µg/g) was detected in the case of using 1 mg/l BAR. On the other hand, the lowest contents (698.22–1115.1 µg/g) was achieved in leaves of plants maintained on medium with mT. In the case of BA application 0.25 mg/l resulted the highest level (1543 µg/g), although only approx. 1000–1200 µg/g chlorophyll (a+b) content were obtained if higher BA doses were used.

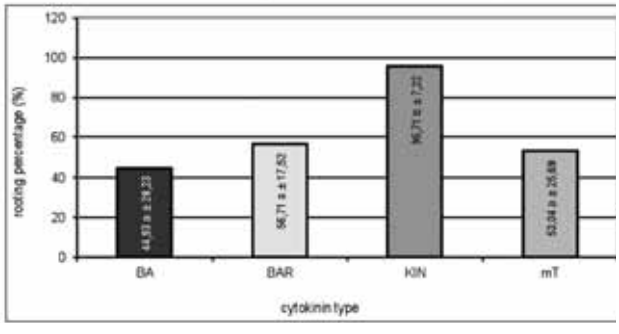

Figure 7. Rooting percentages of *in vitro Nidularium* 'Kertész Jubileum' plants cultured on different type of cytokinins

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