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In vitro culture and acclimatization of *Cattleya xanthina* (Orchidaceae), an endangered orchid of the Brazilian Atlantic Rainforest

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Abstract

Cattleya xanthina is a Neotropical orchid endemic to the Brazilian Atlantic Rainforest, at high risk of extinction. In this paper, we investigated the effects of different culture media on *C. xanthina* as well as on their endogenous nitrogen status. Culture media studied: Knudson C (KC), Vacin and Went (VW), and Murashige and Skoog (MS), the latter used at two different concentration (full and half-strength; MS/2). After 180 days, plants were transferred to MS medium with different NAA and BA concentrations. In each treatment, biometric parameters were measured and the endogenous levels of photosynthetic pigments, total protein, nitrate and ammonium ions were quantified. Plants grown on KC medium had the lowest concentration of nitrogen but exhibited the greatest shoot development, production of photosynthetic pigments and total protein. Results of growth regulators showed that the highest concentration of auxin stimulated root development and the production of photosynthetic pigments, and that a higher concentration of cytokinin promoted protein synthesis and the development of shoots. Most successful acclimatization was obtained when a mixture of *Sphagnum* and *Pinus* bark was used as the substrate.

Key words: Auxin, conservation, cytokinin, *ex vitro*, nitrogen.

Resumo

Cattleya xanthina é uma orquídea neotropical endêmica da Mata Atlântica brasileira, com alto risco de extinção. Neste trabalho analisamos diferentes meios de cultura sobre o desenvolvimento de *C. xanthina*, e seu status de nitrogênio endógeno. Meios de cultura estudados: Knudson C (KC), Vacin & Went (VW) e Murashige & Skoog (MS), e o meio MS com metade da concentração de nutrientes (MS/2). Após 180 dias, as plantas foram transferidas para o meio MS com diferentes concentrações de NAA e BA. Em cada tratamento, foram analisados parâmetros biométricos e níveis endógenos de pigmentos fotossintéticos, proteína total, nitrato e íons de amônio. As plantas cultivadas em meio KC apresentaram menor concentração de nitrogênio, mas exibiram o maior desenvolvimento, produção de pigmentos fotossintéticos e proteína total. Os resultados dos reguladores de crescimento mostraram que a maior concentração de auxina estimulou o desenvolvimento radicular e a produção de pigmentos, e a maior concentração de citocinina promoveu síntese proteica e o desenvolvimento caulinar. A aclimatização mais bem sucedida foi obtida quando uma mistura de *Sphagnum* e casca de *Pinus* foi usada como substrato.

Palavras-chave: Auxina, conservação, citocinina, *ex-vitro*, nitrogênio.

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Introduction

Cattleya xanthina (Lindl.) Van den Berg is an endangered, epiphytic species that has been categorized as “under threat” by the Brazilian Ministry of the Environment (MMA 2014). According to Hoehne (1953), this species is endemic to the Atlantic Rainforest, occurring from southern Bahia to the Rio de Janeiro states. Human action is causing habitat destruction and subsequent population decline of orchid species. Furthermore, the fact that orchids go through a long vegetative period before reaching reproductive phase as well as their complex life-cycle increase the risk of extinction (Ferreira & Suzuki 2008). Thus, *in vitro* culture is an effective alternative to avoid orchid extinction, since it promotes increased germination rates and enables seedling production in short periods, which contributes to species conservation.

Previous studies related to other endangered orchids have demonstrated that the development of seedlings varies among species and is dependent, among other factors, on the culture medium (Dutra *et al.* 2008; Suzuki *et al.* 2009, 2010). Therefore, studies that investigate the importance of different nutrients, especially nitrogen, are very relevant to understanding the efficacy of the culture medium. Nitrogen can be assimilated by plants in the form of nitrate (NO_3^-) or ammonium (NH_4^+) ions. Generally, nitrate is the form of nitrogen most readily taken up by plants, although this may vary among species and may be influenced by environmental factors (Mengel & Kirkby 1978). According to Malavolta *et al.* (1997), the chlorophyll content present in plant tissues indicates the level of nitrogen absorbed. As it makes part of the chlorophyll molecule, nitrogen is critical to plant growth and any deficiency affects light absorption during photosynthesis, negatively influencing plant development (Cheng 2003).

Growth regulators have also been broadly used for the *in vitro* culture of orchids since they promote germination and development. Kuiper *et al.* (1988) affirm that nutrient availability can influence the synthesis and action of endogenous phytohormones, and nitrogen, in particular, can affect plant levels of auxin and cytokinin.

Population decline and the consequent threat to *Cattleya xanthina* in Brazil, besides the absence of studies concerning its propagation, confirm the importance of optimizing *in vitro* protocols for its germination and optimal development, allowing its conservation and reintroduction into natural environments.

Taking the above into consideration, this study aimed to analyze the influence of different culture media and growth regulators on the development of plants of *C. xanthina* as well as on their endogenous nitrogen status. Plant acclimatization was also performed.

Material and Methods

Plants and culture medium

Seedlings of *Cattleya xanthina* were produced *in vitro* by using seeds collected from eight fruits of plants belonging to the Orchid Collection of the Institute of Botany Research Center - Orchidarium of São Paulo state, Brazil. These seeds were grown in Murashige & Skoog medium and maintained under the same growing conditions of this paper.

The following culture media were used in the experiments: Knudson C (KC; Knudson 1946), Vacin & Went (VW; Vacin & Went 1949) and Murashige & Skoog (MS; Murashige & Skoog 1962); the latter was used at two different macro- and micronutrient concentrations (full and half-strength, MS and MS/2). All media were supplemented with 20 g.L⁻¹ sucrose (Vetec Química, Sigma-Aldrich, Rio de Janeiro, Brazil) and 0.4% bacteriological agar (Vetec Química, Sigma-Aldrich, Rio de Janeiro, Brazil), and the pH was adjusted to 5.8 (PA200, Marconi, Piracicaba, SP, Brazil). The media were autoclaved at 117.7 kPa and 121 °C for 15 min (Ibacli S.A., São Paulo, Brazil). Cultures were maintained in a growth room at 25 ± 2 °C and a photoperiod of 12 h at 30 μMm⁻² s⁻¹ PAR (GE Lighting, São Paulo, Brazil).

Experiment I (Influence of culture media)

In this experiment, 15 six-month-old seedlings (0.6 ± 0.1cm in length) originating from *in vitro* culture, had all roots removed and were placed in flasks containing KC, VW, MS and MS/2 media. Seven flasks per culture medium were used (n = 105). Data was collected after 180 days of culture.

Experiment II (Influence of naphthaleneacetic acid and benzyladenine)

In this experiment 15 plants (0.8 ± 0.1cm in length), originating from the MS medium of experiment I, had all roots removed and were transferred to flasks containing fresh MS medium supplemented with auxin (α-naphthaleneacetic acid - NAA) and cytokinin (6-benzyladenine - BA) used

in combinations of 0, 0.57 and 2.28 μM . There was a total of nine treatments and seven flasks were used per treatment ($n = 105$). Data were collected after 180 days of culture.

Biometric parameters

For both experiments described above, eight plants from each treatment were randomly selected and evaluated in terms of shoot and root (longest) lengths, number of leaves and roots, and fresh and dry mass of shoots and roots. The dry mass was determined after drying the plants at 60 °C to constant weight (Approximately one week).

Quantification of NH_4^+ and NO_3^-

Analysis of ammonium (NH_4^+) and nitrate (NO_3^-) was performed in plant material originated from Experiment I. Three 500 mg samples of fresh material (including shoots and roots, approximately 10 plants) were used for quantification and each sample was analyzed three times ($n = 9$). The fresh material was previously macerated in ultrapurified water, centrifuged at 12,000 rpm for 30 min and the supernatant was used for determination of NH_4^+ and of NO_3^- . Quantification of NH_4^+ was performed according to Weatherburn (1967) and as modified by Magalhães *et al.* (1992). Samples were analyzed in a spectrophotometer (U2M, Quimis, São Paulo, Brazil) at 625 nm. NH_4Cl was used to establish the standard curve. The method proposed by Cataldo *et al.* (1975), was used for NO_3^- determination. Absorbance was read at 410 nm. KNO_3^- was used as the standard.

Quantification of photosynthetic pigments

Quantification of chlorophyll and carotenoids was carried out according to Lichtenthaler (1987) in plant material originated from both experiments. Three 300 mg samples of fresh material (including shoots and roots, approximately six plants) were used for quantification and each sample was analyzed three times ($n = 9$). The fresh material was previously macerated in 80% cold acetone and filtrate, under low- light conditions. After filtration, the absorbance was read at 662 nm, 645 nm and 470 nm in a spectrophotometer.

Total protein quantification

Quantification of total protein was carried out according to Bradford (1976) in plant material originating from both experiments. Three 350 mg

samples of fresh material (including shoots and roots, approximately seven plants) were used for quantification and each sample was analyzed three times ($n = 9$). The fresh material was previously macerated in phosphate buffer, centrifuged at 12,000 rpm for 30 min. The supernatant were collected and combined with Coomassie Brilliant Blue G-250. The protein standard curve was obtained with Coomassie Brilliant Blue G-250 and bovine serum albumin. The absorbance was read at 595 nm in a spectrophotometer.

Acclimatization

Plants obtained from the original *in vitro* culture, 540 days of cultivation, with shoot length of about 2 cm and roots presence, were transferred to pots containing two different substrates: *Sphagnum* and a mixture of *Sphagnum* and *Pinus* bark (50:50 v/v). Five pots containing five plants were used for each substrate, the pots were maintained in greenhouse (combination of masonry and glass), with 80% of shading and a nebulization irrigation system one times per day. The plants received bi-weekly applications of the NPK fertilizer (Peters 20:20:20). Biometric data (number of leaves and shoot length) were collected after 180 days of cultivation. Root length and fresh and dry mass of shoots and roots were not collected as plants were subsequently used in a reintroduction program.

Statistical analysis

The results from the *in vitro* experiments were submitted to an analysis of variance (ANOVA) and the means were compared by the Tukey test at a 5% probability level. *Ex vitro* cultivation data were submitted to Fisher's analysis of variance by using the SPSS 20 software.

Results

Experiment I

After 180 days of culture (Fig. 1), the biometric parameters of *Cattleya xanthina* plants revealed that the greatest shoot length was obtained on KC medium (1.4 cm). However, there was no significant difference among the culture media assessed (Fig. 2a). Regarding average root length (Fig. 2a), plants grown on MS/2 and VW media showed the best results (1.9 and 1.8 cm, respectively). Leaf number was not influenced by culture medium (Fig. 2b), but MS inhibited root production.

There was no significant difference among the culture media in terms of shoot fresh mass (Fig. 2c), although the best result was obtained on KC medium. Root fresh mass was greater on VW medium (40.9 mg), followed by the MS/2 and KC media (26.7 and 25.3 mg, respectively) (Fig. 2c). KC medium showed higher shoot dry mass accumulation (4.3 mg) compared to MS, MS/2, and VW (2.6, 2.5, 2.2 mg, respectively)

(Fig. 2d). Root dry mass was greater on MS/2 medium (7.2 mg) in comparison with VW, KC and MS media (2.7, 2.6 and 0.8 mg, respectively) (Fig. 2d). Although MS exhibited the worst performance in terms of biometric parameters, plant survival was highest on this medium (data not shown).

The endogenous level of ammonium was highest in plants grown on KC medium (60.6



Figure 1 – a-d. Plants of *Cattleya xanthina* grown *in vitro* for 360 days in different culture media – a. KC medium; b. VW medium; c. MS medium; d. MS/2 medium. Scale bar = 1 cm.

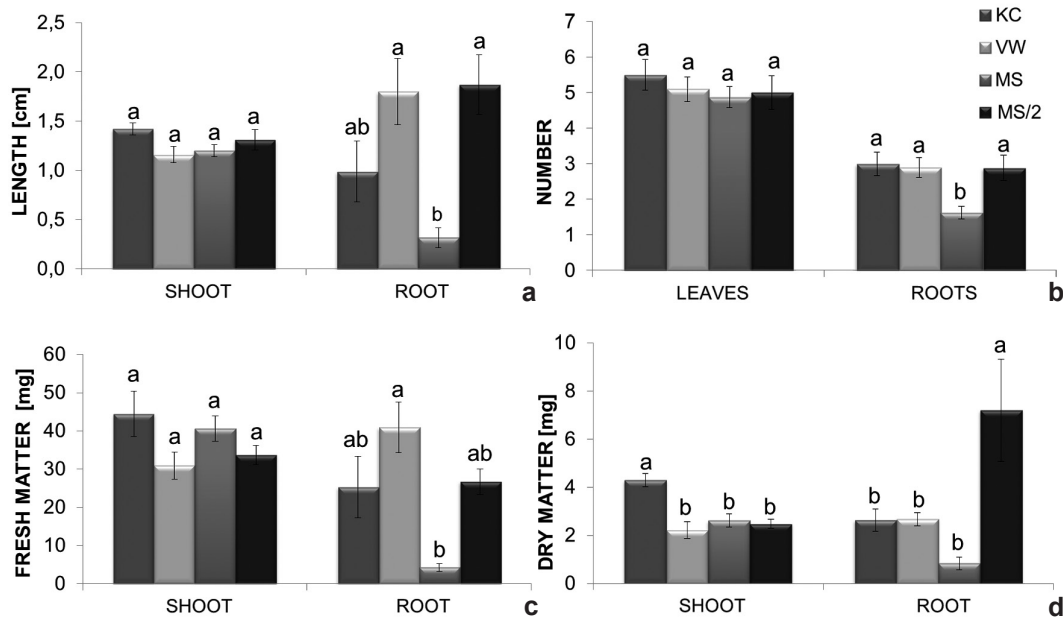


Figure 2 – a. Effects of different culture media on *Cattleya xanthina* shoot and longest root length. b. Number of leaves and roots. c. Fresh mass. d. Dry mass. Bars represent the standard error of the mean. Histograms with the same letters are not significantly different according to the Tukey’s test at a 5% probability level (n = 8).

$\mu\text{g}\cdot\text{g}^{-1}$ FM). Contrarily, on MS/2 medium plants exhibited the lowest level ($15.1 \mu\text{g NH}_4^+\cdot\text{g}^{-1}$ FM) (Fig. 3b). Plants grown on MS medium had higher nitrate levels in comparison with the other culture media (Fig. 3a), reaching $683.7 \mu\text{g}$

nitrate g^{-1} FM, while plants grown on VW, MS/2 and KC media had 221.8, 171.8 and $40.6 \mu\text{g}$ of nitrate $\cdot\text{g}^{-1}$ FM, respectively.

In general, the synthesis of photosynthetic pigments was stimulated by KC medium (227.1

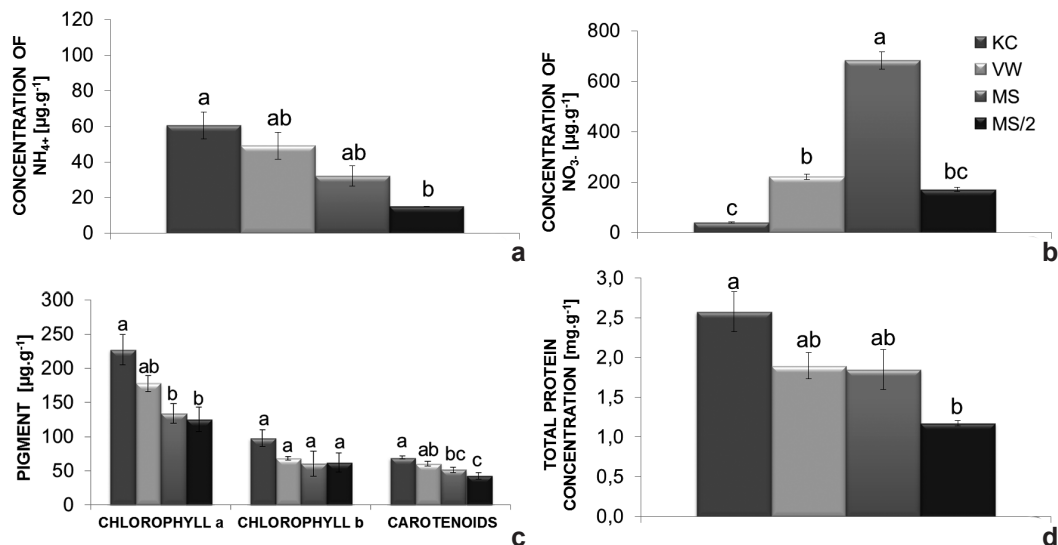


Figure 3 – a-d. Endogenous nitrogen levels of *Cattleya xanthina* plants grown on different culture media – a. nitrate; b. ammonium; c. photosynthetic pigments; d. total protein. Bars represent the standard error of the mean. Histograms with the same letters are not significantly different according to the Tukey’s test at a 5% probability level (n = 9).

μg of chlorophyll *a*. g^{-1} FM, 98.1 μg of chlorophyll *b*. g^{-1} FM and 69.5 μg of carotenoids. g^{-1} FM). Nevertheless, the amounts of chlorophyll *b* did not significantly differ among the culture media investigated (Fig. 3c).

The highest amount of total protein was found in plants grown on KC medium (2.5 mg g^{-1}), while plants on MS/2 medium had the lowest levels of total protein (1.2 mg. g^{-1}) (Fig. 3d).

Experiment II

The different combinations of benzyladenine and naphthaleneacetic acid promoted different root- and shoot-development responses. In the control treatment (hormone-free MS medium) plants exhibited shorter shoots and fewer leaves. Some treatments inhibited root formation (Fig. 4).

The shortest shoots (0.9 cm) were observed in the control treatment whereas the longest ones were



Figure 4 – a-i. Plants of *Cattleya xanthina* grown *in vitro* for 540 days on MS medium containing NAA and BA – a. MS; b. MS + 0.57 μM NAA; c. MS + 2.28 μM NAA; d. MS + 0.57 μM BA; e. MS+ 2.28 μM BA; f. MS + 0.57 μM NAA + 0.57 μM BA; g. MS + 0.57 μM NAA+ 2.28 μM BA; h. MS+ 2.28 μM NAA+ 0.57 μM BA; i. MS + 2.28 μM NAA+ 2.28 μM BA. Scale bar = 1 cm.

obtained on MS medium supplemented with 0.57 μM NAA + 2.28 μM BA (1.6 cm). Regarding root development, none of the treatments significantly differed from the control, although they varied between 0.2 and 0.6 cm in terms of length (Fig. 5a).

Leaf number was significantly influenced by naphthaleneacetic acid and benzyladenine. Plants grown on MS enriched with 0.57 μM NAA + 2.28 μM BA showed a greater number of leaves (9 leaves) when compared to the control treatment (5 leaves) (Fig. 5b). The greatest root production was obtained on MS supplemented with 0.57 μM NAA (approximately 3 roots per plant). Plants grown on MS enriched with 0.57 or 2.28 μM BA exhibited the smallest number of roots (± 0.8 root per plant) (Fig. 5b).

Shoot fresh mass was significantly affected by the action of plant hormones. MS medium enriched with 2.28 μM NAA + 0.57 μM BA provided the greatest fresh mass production (70.5 mg) followed by that supplemented with 0.57 μM NAA + 2.28 μM BA (61.4 mg) (Fig. 5c). The additions of 0.57 μM BA, 2.28 μM BA, 2.28 μM NAA and 2.28 μM NAA + 2.28 μM BA to the MS

medium were inhibitory (respectively 0.6, 1.2, 1.4 and 2.2 mg) to root fresh mass accumulation when compared to the control treatment (4.4 mg) (Fig. 5c). NAA at 0.57 μM NAA (12.4 mg) was the only treatment that surpassed the control.

MS supplemented with 2.28 μM NAA + 0.57 μM BA (6.2 mg) and 0.57 μM NAA + 2.28 μM BA (5.5 mg) (Fig. 5d) accounted for the best results in terms of shoot dry mass. Root dry mass was significantly greater on MS enriched with 0.57 μM NAA (1.4 mg) when compared with the control (0.5 mg). The lowest root dry mass production was verified in plants grown on MS supplemented with 2.28 μM BA (Fig 5d).

In general, the concentration of photosynthetic pigments was not significantly affected by the hormonal ratios of benzyladenine and naphthaleneacetic acid assessed. Plants of the control treatment presented the highest chlorophyll *a* level (172 $\mu\text{g}\cdot\text{g}^{-1}$) whereas those cultured on MS supplemented with 2.28 μM NAA + 0.57 μM BA exhibited the greatest amounts of chlorophyll *b* and carotenoids (87.6 $\mu\text{g}\cdot\text{g}^{-1}$; 66.1 $\mu\text{g}\cdot\text{g}^{-1}$, respectively). Plants grown on MS enriched with 2.28 μM BA showed the lowest

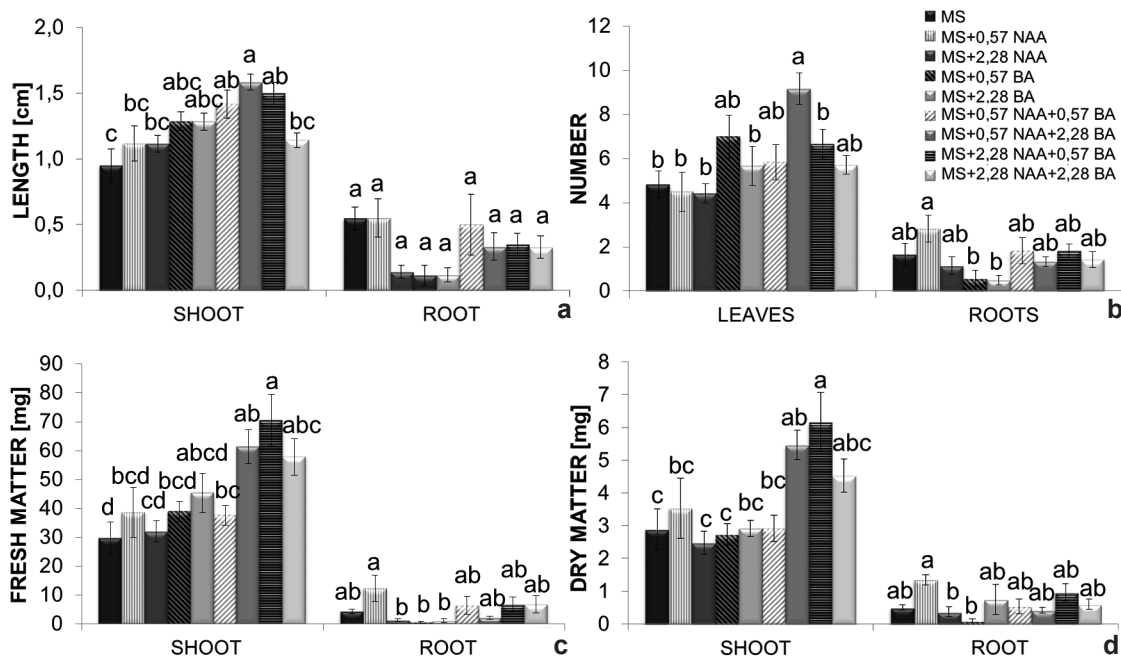


Figure 5 – a-d. Effects of benzyladenine (BA) and naphthaleneacetic acid (NAA); added to MS medium – a. on shoot and longest root length; b. number of leaves and roots; c. fresh mass; d. dry mass accumulation. Bars represent the standard error of the mean. Histograms with the same letters are not significantly different according to the Tukey’s test at a 5% probability level (n = 8).

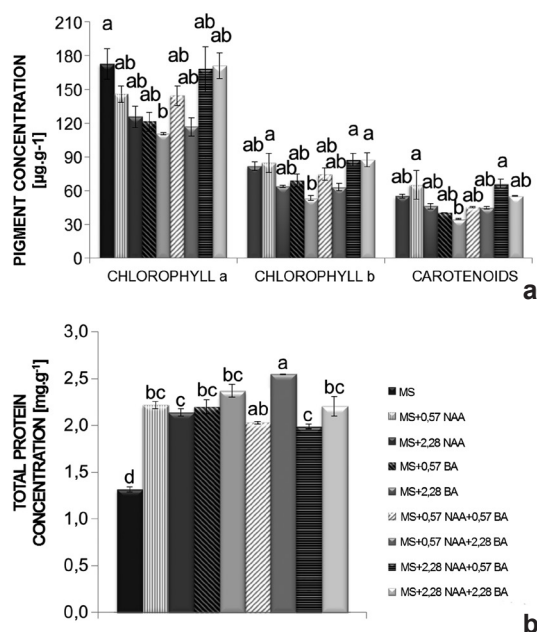


Figure 6 – a,b. Effects of benzyladenine (BA) and naphthaleneacetic acid (NAA) added to MS medium – a. on the amounts of photosynthetic pigments; b. on total protein. Bars represent the standard error of the mean. Histograms with the same letters are not significantly different according to the Tukey's test at a 5% probability level (n = 9).

concentrations of photosynthetic pigments: chlorophyll *a*, chlorophyll *b* and carotenoids (110.4; 53.3 and 44.7 µg.g⁻¹, respectively) (Fig. 6a).

Total protein concentration was stimulated by the presence of naphthaleneacetic acid and benzyladenine in the culture medium. Control plants had the lowest protein content (1.3 mg.g⁻¹). The plants produced more protein when grown on MS supplemented with 0.57 µM NAA + 2.28 µM BA and 2.28 µM BA (2.5 and 2.4 mg.g⁻¹, respectively) (Fig. 6b).

Acclimatization

The two substrates tested did not significantly differ in terms of plant survival percentage (*Sphagnum* [64%] and *Sphagnum* +*Pinus* bark [68%]). The average shoot length of plants grown in *Sphagnum* + *Pinus* bark (4.6 cm) was higher than that of plants grown in *Sphagnum* alone (3.4 cm) (Fig. 7a). No significant difference was detected between the two substrates in terms of leaf number (Fig. 7b).

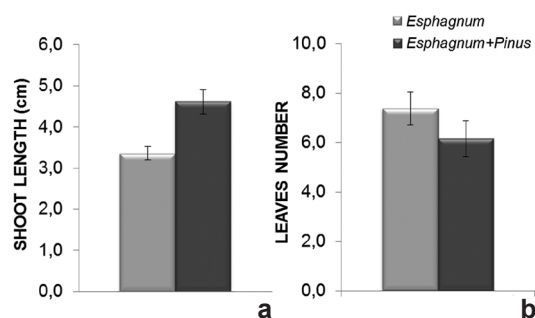


Figure 7 – a,b. Acclimatization of *Cattleya xanthina* – a. effects of two substrates on shoot length; b. leaf production. Bars represent the standard error of the mean and * indicates a significant difference according to the Fisher's test at a 5% probability level (n = 9).

Discussion

KC medium slightly stimulated shoot growth in length as well as leaf formation in comparison with the other assessed media. Root development was enhanced on KC, VW and MS/2 media, which have lower nutrient concentrations. MS medium inhibited root formation and growth in length, which could be related to its high nutrient concentration (Fig. 2a,b). Suzuki *et al.* (2009) also reported that MS inhibited root formation in *Hadrolaelia tenebrosa* (Rolfe) Chiron & V.P. Castro. In contrast, Rego-Oliveira & Faria (2005) verified that MS increased root length of *Catasetum fimbriatum* (C.Morren) Lindl. plants when compared to KC and VW.

Well-developed roots are of utmost importance during acclimatization because they provide the seedlings the absorptive capacity to survive (Aoyama *et al.* 2012). For this reason, before carrying out acclimatization, the reduction of medium nutrient concentration is generally suggested (Preece & Sutter 1991), as this enhances root formation and there is a greater chance of plant survival.

The greatest shoot fresh mass was obtained when plants were cultured on KC medium (Fig. 2c), whereas root fresh mass was highest on VW. The interaction of calcium and ammonium in the VW medium actively promoted fresh mass accumulation in *Dactylorhiza incarnata* (Dijk & Eck 1995), possibly due to the osmotic adjustment and water potential with lower calcium levels and higher amounts of ammonium (Shao *et al.* 2008). The same was observed for root fresh mass in *C. xanthina* (Fig. 2c). Shoot dry mass

was significantly higher on KC medium (Fig. 2d), which has also been observed in *Catasetum fimbriatum* (Rego-Oliveira & Faria 2005).

MS/2 provided the greatest root dry mass accumulation in *C. xanthina* whereas MS inhibited this parameter (Fig. 2d). Similar results were reported by Stancato *et al.* (2008) in *Laelia longipes* Rchb.f.. Studies with *Hadrolaelia tenebrosa* (Suzuki *et al.* 2009) also revealed that MS was extremely inhibitory to root fresh and dry mass. However, in *Cattleya bicolor* Lindl. Suzuki *et al.* (2010) verified that MS medium favored a greater root dry mass accumulation when compared to KC and VW. These results show again that the composition of the culture medium is extremely important for the development of orchids and that nutrient requirements vary among species. Therefore, it is of utmost importance to determine the most adequate nutritional demands for each species, especially for endangered ones.

The results obtained for the endogenous levels of NO_3^- and NH_4^+ confirm the efficient uptake of nitrogen by *C. xanthina* on the culture media investigated. KC and VW media have a higher ammonium/nitrate ratio (0.89, 1.46, respectively) whereas MS has the highest nitrate/ammonium ratio (1.9) (Fig. 3a,b). In the present study, the high concentration of nitrate observed during seedling development may be due to the transient accumulation of this nutrient in the vacuoles (Rufty *et al.* 1986; Mantovani *et al.* 2005). Later, nitrate is converted into ammonium which is assimilated by the plant.

The synthesis of photosynthetic pigments was promoted in plants cultivated on KC medium (Fig. 3c), which has a balanced nitrate/ammonium ratio (1:1). The slight stimulation of shoot-related parameters on KC medium could be a result of the higher chlorophyll *a* content in the tissues that possibly increased the photosynthetic rate. Chlorophyll plays the roles of absorbing light energy, activating the photosynthetic system and transforming it into chemical energy (Li *et al.* 2013), which promotes vegetative growth. Increased synthesis of photosynthetic pigments and proteins (Fig. 3c,d) also benefits plant acclimatization, since natural habitats have reduced amounts of nutrients (Pospóšilová *et al.* 1999). The presence of ammonium ions stimulates faster amino acid and protein synthesis as well as cell division and expansion (Jiménez 2001; Gallardo *et al.* 2003); results confirmed by the present study.

The efficiency of shoot growth of *C. xanthina* on MS enriched with $0.57 \mu\text{M}$ NAA + $2.28 \mu\text{M}$ BA (Fig. 5a) confirms that a higher concentration of cytokinin promotes stem cell division and stimulates shoot development (Lexa 2003) as well as nutrient mobilization (Lo *et al.* 2004). Auxin/cytokinin ratios favoring auxins tend to promote root development and those favoring cytokinins promote the development of shoots (Kefford & Goldacre 1961; Su *et al.* 2011; Ori *et al.* 2014).

Root formation was inhibited by the addition of BA alone, and was slightly stimulated on MS enriched with $0.57 \mu\text{M}$ NAA (Fig. 5b), which confirms the action of auxin on adventitious root formation by pericycle cells, responsible for initiating the processes of cell division and expansion (Casimiro *et al.* 2001). Similar results were observed in *Cyrtopodium glutiniferum* Raddi (Vogel & Macedo 2010); in this study all auxin treatments resulted in the production of plants with larger roots when compared to treatments containing cytokinin only. The proposition of Krouk *et al.* (2011), that increased endogenous auxin levels promote root development thus enhancing absorption of nutrients, including nitrogen, which has a primary role in the composition of the chlorophyll molecule, was apparently not effective in *C. xanthina* plants.

The higher levels of total protein in *C. xanthina* when cultured on MS enriched with $0.57 \mu\text{M}$ NAA + $2.28 \mu\text{M}$ BA (Fig. 6b), can be correlated with high rates of mitotic divisions (Silveira *et al.* 2004), which could subsequently be responsible for the stimulation of shoot growth in length and leaf production (Fig. 5a,b). Also, the mobilization of cytokinins in shoots might be promoted when there is plenty of available nitrogen. Therefore, higher levels of cytokinin in the culture medium could have enhanced the use of nitrogen, promoting cell division and thus protein synthesis (Krouk *et al.* 2011).

Plant acclimatization was best when plants were grown in *Sphagnum* with the addition of *Pinus* bark. *Cattleya xanthina* is found in dense, ombrophilous forests (Dutra *et al.* 2015) at altitudes varying from 500 to 900 meters above sea level (BFG 2018). Humidity rates are decreased at high altitudes which also occur when plants are transferred to *ex vitro* conditions, especially when *Pinus* bark is used in the substrate mix as it retains little moisture.

Overall, KC was the most efficient medium for the growth of *C. xanthina* under *in vitro*

conditions. It also stimulated the production of photosynthetic pigments and total protein. However, it caused high plant mortality when compared to MS. The addition of NAA and BA to MS medium was advantageous for *C. xanthina* plant development. Finally, the results showed that the concentration and form of nitrogen present in each culture medium are of utmost importance at every stage of plant development, providing active plant growth to ensure the urgent conservation of this orchid.

The present study establishes a propagation protocol for *C. xanthina*. The protocol followed with *in vitro* culture in KC culture medium for 180 days, transferred to MS medium with high level of auxin and low level of cytokinin, acclimatization in *Sphagnum* and *Pinus* bark with 80% shading, irrigation by nebulization and NPK fertilizer (20–20–20).

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