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Original Paper

Asymbiotic germination, initial development *in vitro* and acclimatization of *Cyrtopodium paludicolum* Hoehne, a Brazilian Savanna orchid species

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Abstract

Cyrtopodium paludicolum is a terrestrial orchid species, native to Brazil, whose natural propagation is jeopardized by the intensive collection from the wild and is threatened by agricultural expansion in Cerrado areas. In light of that, this investigation aimed at studying the *in vitro* germination and early development of *C. paludicolum* as influenced by culture medium, sucrose and growth regulators as well as its micropropagation by using dark-grown stem segments. A protocol for its acclimatization is also detailed. The effects of Murashige & Skoog (MS), Knudson C (KC) and Vacin & Went (VW) media on the in vitro germination and initial development were tested. The influence of different concentrations of BA, NAA, and of sucrose on plant multiplication and growth were evaluated. The possibility of using etiolated stem segments for micropropagation was also assessed. Acclimatization was accomplished in two phases by using three different substrates. The results showed that VW was the best medium for germination whereas for seedling formation KC was the most advantageous since they were healthy and vigorous. Sucrose at 2% favored the greatest seedling growth and development. Shoot and root proliferation and development were best promoted in the presence of 2.28/2.28 and 0.57/0.57 µM BA/NAA, respectively. The use of etiolated stem segments for micropropagation was effective. Successful acclimatization was accomplished by initially growing plants in community pots containing a 3:1 (v/v) mix of Bioplant and dried *Sphagnum* moss followed by their transfer to individual pots containing a 2:1 (v/v) mix of Bioplant and Ouro Negro substrates.

Key words: Cerrado, culture media, growth regulators, protocorm development, sucrose.

Resumo

Cyrtopodium paludicolum é uma espécie de orquídea terrícola e nativa do Brasil cuja propagação natural está comprometida por coletas indiscriminadas em seu ambiente natural e sofre riscos de ameaça pela expansão agrícola em áreas de Cerrado. Assim, o presente trabalho teve como objetivo estudar a germinação e o desenvolvimento inicial *in vitro* de *C. paludicolum* bem como verificar a possibilidade de micropropagar a espécie a partir de segmentos caulinares estiolados. Um protocolo para a aclimatização também foi desenvolvido. Foi testada a influência dos meios de cultura Murashige & Skoog (MS), Knudson (KC) e Vacin & Went (VW) no processo de germinação e crescimento inicial. Foram testados os efeitos da adição de diferentes concentrações de BA, ANA e de sacarose na multiplicação e crescimento *in vitro* da espécie. A aclimatização foi realizada em duas fases utilizando-se três diferentes substratos. O meio VW proporcionou a maior germinabilidade das sementes enquanto o meio KC foi o que promoveu a melhor formação de plântulas saudáveis e vigorosas. A concentração de 2% de sacarose foi a mais favorável para o crescimento e desenvolvimento das plântulas. A melhor proliferação e desenvolvimento de brotos e raízes foi obtido na presença de 2.28/2.28 e 0.57/0.57 µM BA/NAA, respectivamente. O uso de segmentos estiolados foi eficaz

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para a micropropagação. A aclimatização foi realizada com sucesso crescendo inicialmente as plantas em potes comunitários contendo os substratos Bioplant e esfagno seco na proporção de 3:1 (v/v) seguido da transferência para recipientes individuais contendo Bioplant e Ouro negro na proporção de 2:1 (v/v).
Palavras-chave: Cerrado, meios de cultura, reguladores de crescimento, desenvolvimento de protocormos, sacarose.

Introduction

Orchidaceae is one of the largest plant families, with over 28,000 accepted species spanning 763 genera (Christenhusz & Byng 2016), and accounting for approximately 7% of all Angiosperms. Although more abundant and diversified in tropical forests, especially in Asia and the Americas, it is widely distributed throughout the world. Orchidaceae species are predominantly herbaceous and occur in nature as epiphytes, hemiepiphytes, lithophytes, terrestrials or even saprophytes (Suzuki *et al.* 2009).

In spite of their great diversity orchids are threatened, mainly due to a decrease in their natural habitats, indiscriminate collection from the wild and commercialization (Moreira *et al.* 2007; Zhang *et al.* 2018). For that reason, their multiplication in nurseries and laboratories is essential to reduce the risk of extinction. Due to their slow development, conventional vegetative propagation methods do not provide for rapid population growth. Therefore, *in vitro* propagation has been extensively used to propagate orchid species for both commercial and conservation purposes (Sopalun *et al.* 2010).

Several scientific investigations, carried out in different countries, have emphasized the use of *in vitro* techniques as a means to conserve orchid species. These studies describe seed germination (Ponert *et al.* 2013, Lemes *et al.* 2016, Yeung 2017) as well as culture of nodal segments and shoot apices (Ket *et al.* 2004; Yan *et al.* 2006; Vasudevan & Van Staden 2011) and rhizomes *in vitro* (Paek & Yeung 1991; Nayak *et al.* 1998; Sheelavantmath *et al.* 2000). However, it is important to highlight that, considering the vast number of orchid species, there is not a comparable amount of scientific publications regarding their *in vitro* seed germination.

The most adequate conditions for orchid seed germination and, consequently, seedling growth *in vitro* vary according to the genus and species (Suzuki *et al.* 2009; Abrão *et al.* 2014; Ferreira *et al.* 2017), which demonstrates the importance of studies regarding culture medium composition, especially its mineral nutrients, carbohydrate concentration and hormonal makeup. Such studies would allow for a better understanding of the physiological processes involved in the development of these species.

Another relevant factor that can affect the *in vitro* performance of plants is light availability. Plants present different and opposite phenotypic characteristics when developed in the presence or absence of light. Seedlings that are grown in darkness undergo a special type of development known as skotomorphogenesis (etiolation). Such seedlings develop elongated scale-like leaves, and distant internodes, and are unable to accumulate chlorophyll (Rodrigues *et al*. 2017). These characteristics are particularly interesting in the case of orchids because they usually exhibit very short internodes and etiolation makes it easier to divide the stems into segments containing an axillary bud which can be used as explants for micropropagation (Ferreira *et al.* 2011).

In the Brazilian Savanna, locally called Cerrado, the Orchidaceae family is one of the richest in terms of biodiversity. In this vegetational complex they are predominantly terrestrial (43.5%) and epiphytic (38.7%) species, and the remaining ones are climbing, rupicolous, aquatic and saprophytic (Barros *et al.* 2015). However, in the State of Tocantins in particular, increasing human activities such as agriculture, animal husbandry and flooding caused by the construction of several hydroelectric power plants, have the potential to seriously endanger several orchid species (Ferreira *et al.* 2018), including terrestrial ones. Picolotto *et al.* (2017) reported that the natural propagation of *Cyrtopodium paludicolum*, a native terrestrial orchid species object of the present study, is jeopardized by the intensive collection from the wild (due to its exuberant beauty), and is seriously threatened by agricultural expansion in that vegetation complex. In nature, its propagation occurs primarily by means of seed germination.

In light of the above, the present investigation aimed at studying the *in vitro* germination and early development of *C. paludicolum* as influenced by culture medium, sucrose and growth regulators as

well as its micropropagation by using dark-grown stem segments. A protocol for its acclimatization is also described.

Material and Methods

Plant species and material

Cyrtopodium paludicolum Hoehne is a terrestrial orchid that commonly occurs in permanently wet areas of the Brazilian Savanna (Cerrado). According to Barros *et al.* (2015), this species is native to Brazil (but not endemic) and naturally occurs in the States of Tocantins, Goiás, Mato Grosso do Sul, Mato Grosso, Minas Gerais, São Paulo and Paraná, and in the Federal District. The species blooms from December to April (Hall *et al.* 2013). Flowers of the species are shown in Figure 1. A voucher specimen of this species is deposited in the Tocantins Herbarium (HTO) under record number 2,158.

Five mature fruits of *C. paludicolum* prior to dehiscence and collected from different plants growing in the wild in a swampy area near the Agua Suja River in Porto Nacional (10°26'33" S, 48°24'16" W), Tocantins, Brazil, were the source of seeds for the present study. Seeds were removed from the fruits and thoroughly mixed in a beaker. Approximately one-tenth of the seed mix was removed and immersed in deionized and autoclaved water for 30 minutes (aqueous suspension of seeds). Subsequently, seeds were surface sterilized with 50 mL of a 15% (v/v) commercial bleach (2.5% active chlorine) in deionized/autoclaved water solution for 10 min., followed by three 15-minute rinses in 50 mL deionized/autoclaved water. After the last rinse, 40 mL of the water were removed and a 10 mL aqueous suspension of seeds was ready for inoculation onto the culture media.

Culture media and growth conditions

The effects of three culture media on *in vitro* germination were studied: Murashige & Skoog (1962) used at two different concentrations of its macronutrients (full- and half-strength) [MS; ½MS], Knudson C (1946) [KC], and Vacin & Went (1949) [VW]. They were all supplemented with 0.4 mg L^{-1} thiamine, 100 mg L^{-1} myo-inositol and 2% sucrose. The pH of the media was adjusted to 5.85 ± 0.01, and 0.2% Phytagel (Sigma Co., USA) was added to the media before autoclaving at 121°C and 105 kPa for 15 min. There were eight replicates for each culture medium. Each replicate consisted of a 100-mL glass jar (covered with plastic transparent lids) containing 40 mL of culture medium onto which 250 µL of the aqueous suspension of seeds mentioned above (containing ca. 350 seeds) was inoculated. The cultures were kept in a growth room at 27 ± 1°C under a 16-hour photoperiod provided by cool-white fluorescent lamps (Empalux, Brasil) at 30–35 µmol m^{-2} s^{-1}.

Germination and protocorm development

The germination analysis was carried out according to Suzuki *et al.* (2009). Seeds were considered germinated when swollen embryos (protocorm phase) were visible on the surface of the media. Material from four replications for each treatment were placed on microscope slides (three slides per replication) and analyzed with the use of a dissecting microscope. The counts encompassed all protocorms on each slide, which were gridded to aid in counting.

Ninety days after the onset of germination, the individuals contained in three replications were evaluated in relation to the stages of protocorm development (morphological characteristics). Four different developmental stages were considered according to Suzuki *et al.* (2009): stage 1 - swollen green embryos (protocorm phase); stage 2 - protocorm bearing one leaf; stage 3 - protocorm bearing two or more leaves; stage 4 - seedling with leaves and one or more roots. Four microscope slides per replication containing protocorms/seedlings

Figure 1 – Flowers of *Cyrtopodium paludicolum*. Scale bar = 1 cm. Composition based on Orchid Hunter

were prepared and analyzed by means of a dissecting microscope. The percentages of individuals obtained for each developmental stage and for each replication were multiplied by 1, 2, 3 and 4 (weights) according to their respective stages, so that the growth index could be calculated according to Spoerl (1948), as modified by Ferreira *et al.* (2017). The growth index of each replication was the sum of all stages of their individuals.

Effects of growth regulators and sucrose on multiplication and development

The effects of different concentrations of benzyladenine [BA] and naphthaleneacetic acid [NAA] (in combinations of 0, 0.57 and 2.28 µM) and of sucrose (at concentrations of 0, 1, 2, 3, 4, 5 and 6%) on plant multiplication and growth were also evaluated. The culture medium used was KC because it provided the best growth index. Seedlings aged 120 days and 1.5 ± 0.5 cm in length (which had all roots removed) grown *in vitro* were used as explants in the experiments. There were five replicates for each treatment. Each replicate consisted of 250-mL glass jars filled with 60 mL of culture medium (covered with plastic transparent lids) containing five explants (n=25). The experimental conditions were the same as described for the germination experiment. The results were analyzed according to the following variables: number of shoots and roots produced per inoculated explant, length of the longest shoot (measured from the base of the plant to the apex of the longest leaf) and root as well as dry mass of shoots and roots after 120 days of culture.

Micropropagation from dark-grown stem segments

In order to verify the possibility of *in vitro* multiplication by using etiolated stem segments, plants originated from *in vitro* germination were grown on KC medium supplemented with 0.4 mg L^{-1} thiamine, 100 mg L^{-1} myo-inositol and 4% sucrose according to Ferreira *et al.* (2011). Vigorous plants aged 150 days and 2.0 ± 0.5 cm in length were transferred to 300-mL glass flasks containing 90 mL of culture medium (covered with plastic transparent caps); these were kept under dark conditions for 180 days at 25 ± 1ºC. After that period 70% of the plants produced etiolated stems, some of which were sectioned into 1-cm segments containing at least one axillary bud. These segments were subsequently transferred to the same culture medium except for the fact that sucrose concentration was reduced to 2% and that two growth regulator combinations were used: a) 2.28 µM BA and 0.57 µM NAA, and b) 4.56 µM BA and 1.14 µM NAA. The control treatment consisted of growth regulator-free culture medium. Ten 90-mL glass flasks containing 40 mL of culture medium covered with plastic transparent caps were used. Ten etiolated stem segments were inoculated per flask. Flasks were kept in a growth room under the same environmental conditions described for the previous experiments. The results were evaluated 60 days after transferring the etiolated stem segments to the presence of light considering the following variables: percentage of segments that originated shoots and roots as well as length and dry matter of shoots and roots.

Acclimatization

For the process of acclimatization 150-day-old plants (bearing roots, leaves and pseudobulbs) were taken from culture vessels, thoroughly washed in running water for removal of growth medium, and transferred into six community pots (transparent plastic containers with covers - 16 cm × 12 cm basal width) each containing 10 plants. The substrate was composed of a mix of Bioplant (Bioplant Agrícola Ltda., MG, Brazil) and ground dried *Sphagnum* moss (Terra Brasil Jardinagem, SP, Brazil) tested at two ratios (3:1 and 3:2, v/v). Pots were kept for 120 days in a growth room with the same experimental conditions described for the germination experiment. Four weeks after transferring plants to the community pots their covers were removed. They were watered daily until the substrate reached the saturation point and plants were sprayed with 50 mL of deionized water once a day (phase 1). The results were analyzed according with the following parameters: survival percentage, average increment in height (measured from the base of the plant to the apex of the longest leaf) and average number of shoots, leaves and roots produced per plant (phase 1). After 120 days, 60 plants randomly selected from those that survived the first acclimatization phase were transferred to individual plastic pots (7 cm height × 6 cm basal diameter, perforated at the base) containing a substrate composed of Bioplant and Ouro Negro (Ouro Negro Ltda., GO, Brazil) at 2:1 (v/v) [phase 2]. Growth conditions were the same as described for phase 1 and plants were watered daily until the substrate reached the saturation point. Evaluation was based on survival percentage,

average increment in height and number of shoots and leaves produced, and was carried out after 120 days of growth. Following that, the surviving plants were transferred to a shade-house with 75% retention of solar radiation flux and were watered daily.

Experimental design and statistical analysis

The experiments were set up in a completely randomized experimental design. Analysis of Variance (ANOVA) was used to evaluate germination and acclimatization, and the means were compared by the Tukey test at the 5% probability level. Percentage results were arcsine transformed to normalize variation. The experiments were repeated twice. The Sisvar 5.6 (Ferreira 2011) software package was used for the statistical analysis.

Results and Discussion

Germination and protocorm development

The first evidence of the germination of *Cyrtopodium paludicolum* seeds was detected 15 days after seed inoculation onto the culture media. The time needed for the germination of orchid seeds varies greatly among species. In *C. saintlegerianum* germination took place 20 days after inoculation (Rodrigues *et al.* 2015). Ferreira *et al.* (2018) reported that *in vitro* germination of *Catasetum macrocarpum* seeds started 15 days after sowing. Seeds of *Cattleya forbessi* germinated 30 days after *in vitro* inoculation (Schneiders *et al.* 2012). *In vitro* germination of *C. bicolor* seeds occurred about 15 days after inoculation (Suzuki *et al.* 2010), whereas those of *Alatiglossum fuscopetalum* (Ferreira *et al.* 2017) germinated within ten days after *in vitro* sowing. Pedroza-Manrique *et al.* (2005) reported that seeds of *Comparettia falcata* germinated 40 days after *in vitro* inoculation. Therefore, germination responses do vary among different genera or even within species of the same genus.

In terms of germination, no significant differences (P<0.05) were observed among VW, ½ MS and KC media (Tab. 1). Soares *et al*. (2020) also reported no significant differences among these culture media for *Cattleya lundii* and *C. nobilior* but for *Brassavola tuberculata* ½ MS provided the best germination response. The lowest germination rate in the present study was verified on full-strength MS medium, which significantly inhibited this process. This could be related to the fact that *C. paludicolum* naturally occurs in areas where soils are hydromorphic which are, in general, not well developed and poor in nutrients (Reatto, 2008; Coringa *et al.* 2012). Thus, it is possible that low-nutrient media such as VW, ½ MS and KC have promoted higher germination rates by providing conditions similar to those where this species is commonly found. Similar results were observed by Caramaschi (2001) in *C. cristatum* (also a terrestrial orchid), whose seeds exhibited higher germination rates on VW than on MS. For *C. punctatum*, a terrestrial or rupicolous orchid species, Dutra *et al.* (2009) reported that VW also provided a much better germination response (26.1%) when compared to ½ MS and KC (12.1 and 10%, respectively). Suzuki *et al*. (2010) also verified the highest germination percentage of *Cattleya bicolor* seeds on VW when compared to KC and MS. Ferreira *et al*. (2018) reported that *Catasetum macrocarpum* seeds germinated better on KC and VW than on MS and ½ MS. In contrast, *Hadrolaelia tenebrosa* germinated better on KC (Suzuki *et al.* 2009) and *Dendrobium tosaense* seeds only geminated on MS, with no protocorm formation on KC and VW (Lo *et al.* 2004). These data indicate that different culture media provide diverse germination responses depending on the orchid species.

The different developmental stages of *C. paludicolum* observed during the first 90 days of culture (after germination) are shown in Figure 2. Based on the growth index (Tab. 1) KC was the best medium for protocorm development although significant differences (P<0.05) were not detected when this medium was compared with VW and ½ MS. Fráguas *et al.* (2003) also reported that KC

Table 1 – Germination percentages and growth indexes of *Cyrtopodium paludicolum* on Murashige and Skoog (MS and ½ MS), Knudson (KC), and Vacin & Went (VW) media.

Culture media	Germination (%)	Growth index
VW	92.12 a	270.81 a
½ MS	91.99 a	284.21 a
KC	90.16 a	286.11 a
MS	49.85 b	160.01 b

Values followed by the same letter (in rows) are not significantly different according to the Tukey's test at 5% probability.

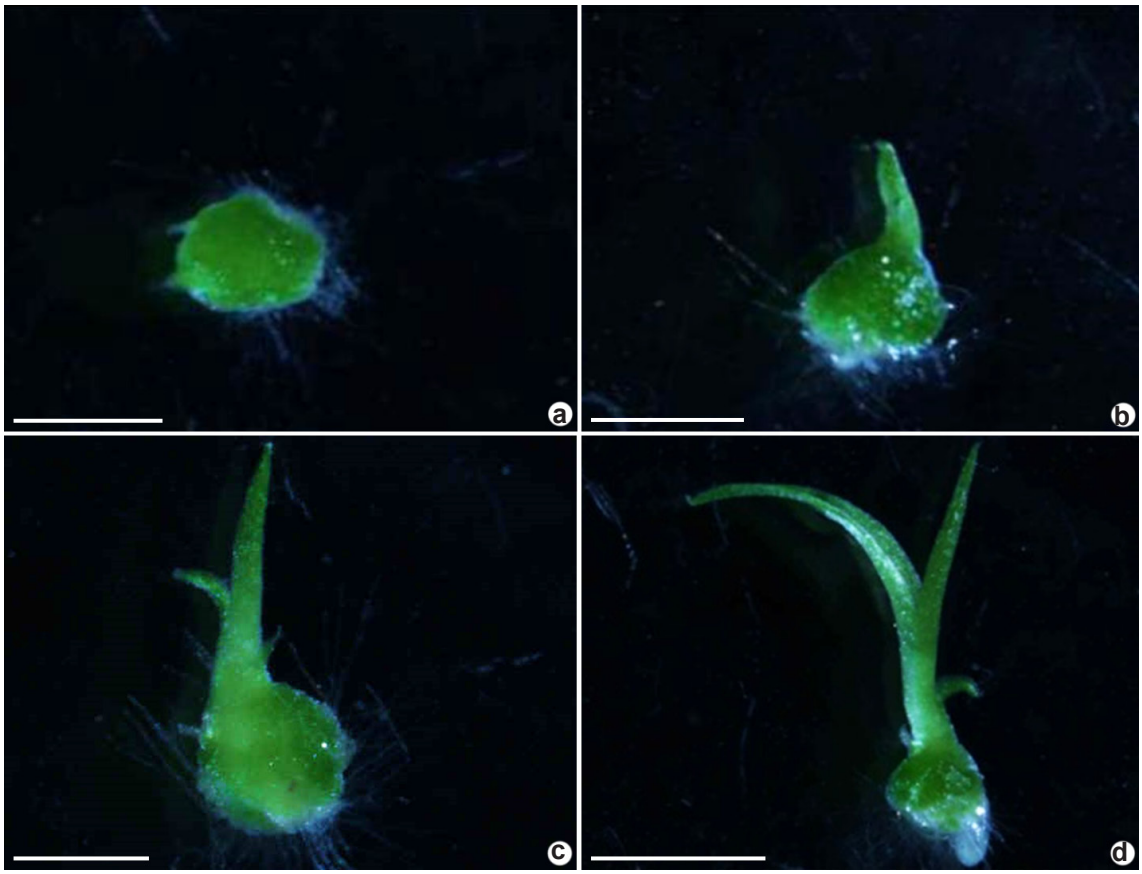

Figure 2 – Developmental stages in *Cyrtopodium paludicolum* protocorms. a. Stage 1 – swollen green embryos (protocorm phase). b. Stage 2 – protocorm bearing one leaf. c. Stage 3 – protocorm bearing two leaves. d. Stage 4 – seedling with leaves and one root. Scale bars = 2 mm.

was the best medium for the initial development of the seedlings of the hybrid *Cattleya labiata x Laelia itambana*. On the other hand, Stewart & Kane (2006) found that VW was the most effective medium for protocorm development of *Habenaria macroceratitis*. Suzuki *et al.* (2009) and Ferreira *et al.* (2018) also showed that the highest index growth of *Hadrolaelia tenebrosa* and *Catasetum macrocarpum*, respectively, were promoted by VW medium.

As was verified for germination, MS also inhibited protocorm development significantly. Again, this can be related to the fact that under natural conditions this species grows in nutrient-poor environments. Although MS is one of the most commonly used media for *in vitro* propagation of ornamental species, for some orchids a reduction in salt concentration favors protocorm and seedling development. In *C. paludicolum* a 50% reduction of the MS macronutrients provided a 77.6% increase in the growth index. Contrasting results were reported by Lo *et al.* (2004) for *Dendrobium tosaense*, whose protocorms exhibited greater development when grown on MS and ½ MS than on KC and VW media. The nutritional requirements of *D. tosaense* are probably higher than the other species mentioned including *C. paludicolum*, especially in terms of nitrogen since MS is four times richer in this nutrient than VW and MS (total nitrogen in MS = 60.06 mM, in KC = 16.04 mM, in VW = 12.77 mM and in KC = 16.04 mM).

Effects of sucrose and growth regulators on multiplication and development

The results related to the effects of different sucrose concentrations on the *in vitro* development of *C. paludicolum* after 120 days of culture are displayed in Table 2. Marked and significant differences were observed among the treatments. In general, sucrose-free culture medium did not

Table 2 – Effects of sucrose concentration on the multiplication and development of *Cyrtopodium paludicolum* after 120 days of *in vitro* culture. NS = number of shoots; LSL = longest shoot length; NR = number of roots; LRL = longest root length; SDM = shoot dry matter; RDM = root dry matter.

Sucrose (%)	NS	LSL (cm)	NR	LRL (cm)	SDM (g)	RDM (g)
0	0.28 b	0.38 c	1.04 c	1.54 b	0.102 c	0.004 d
1	0.52 a	2.05 a	4.09 ab	11.14 a	0.207 a	0.355 b
2	0.56 a	0.82 b	5.19 a	12.90 a	0.166 b	0.536 a
3	0.20 b	0.80 b	3.88 b	11.79 a	0.101 c	0.329 b
4	0.20 b	0.80 b	2.52 c	11.57 a	0.061 c	0.092 c
5	0.20 b	0.76 bc	2.44 c	11.65 a	0.068 c	0.071 c
6	0.04 b	0.06 d	2.48 c	11.68 a	0.102 c	0.145 c

Values followed by the same letter (in rows) are not significantly different according to the Tukey's test at 5% probability.

favor multiplication and development of this orchid species. Shoot proliferation was highest at 1 and 2% sucrose. Lower and higher concentrations of sucrose inhibited shoot formation, although significant differences (P<0.05) were not detected among most of the concentrations assessed. In *Miltonia flavescens* an increase in carbohydrate concentration favored shoot formation and the greatest values were detected at 4.5% sucrose (Besson *et al*. 2010). For *C. paludicolum* 4% sucrose caused a decrease in shoot proliferation. Regarding shoot length, 1% sucrose provided significantly longer shoots (P<0.05) when compared to the other treatments. In *Cattleya violacea* Galdiano Junior *et al.* (2013) verified that 2–3% sucrose concentrations were most favorable for shoot growth in length. In *C. paludicolum* an increase in sucrose concentration above 1% caused a significant inhibition (P<0.05) of this variable. In relation to shoot dry matter 1% sucrose was the most favorable treatment. Concentrations equal to or above 3% as well as the absence of sucrose significantly inhibited this parameter. Ferreira *et al.* (2017), studying the effects of the same sucrose concentrations on the *in vitro* development of *Alatiglossum fuscopetalum*, also reported that the absence of this carbohydrate was detrimental to shoot dry matter accumulation, but did not detect any significant differences among the other five concentrations assessed (1–6%), although 2% sucrose turned out to be the most favorable concentration.

In terms of root formation, the best result was observed at 2% sucrose, although no statistically significant differences were detected among 1, 2 and 3%. Higher concentrations as well as the absence of sucrose inhibited root formation. No significant differences (P<0.05) were verified for root growth in length among the sucrose concentrations used (except for the control treatment), even though 2% provided slightly better results when compared to the other treatments. Galdiano Junior *et al.* (2013) verified that root proliferation and growth in length in *Cattleya violacea* were greater when explants were cultivated at 2 and 3% sucrose. These results differ from those observed in *C. paludicolum*, whose values for these variables were greater at 1 and 2%. The results presented herein for these two variables also differ from those reported by Sorace *et al.* (2008) for *Oncidium baueri*, which was favored by 4% sucrose. Regarding root dry matter the best concentration was 2% sucrose. The absence of this carbohydrate and concentrations equal to or above 4% significantly inhibited dry matter accumulation. Similar results were also reported by Ferreira *et al.* (2017) for *A. fuscopetalum*.

Upon studying the effects of sucrose on the *in vitro* development of orchids, Rego-Oliveira *et al.* (2003) [*Oncidium varicosum*], Moreira *et al.* (2007) [*Laelia purpurata* var. *venosa x Cattleya warneri*] and Dignart *et al.* (2009) [*Cattleya walkeriana*] verified that 6, 2 and 3% sucrose, respectively, were the most favorable concentrations for the majority of the variables analyzed. Together these results reveal that developmental responses to different sucrose concentrations vary widely among species. In the case of *C. paludicolum*, it is possible that the sucrose concentration above 3% might have affected the osmotic potential of the culture medium and caused a decrease in water and nutrient uptake, as was stated by Fráguas *et al*. (2003) and Besson *et al*. (2010), thus hindering growth and development. Based on the results

Table 3 – Effects of benzyladenine (BA) and naphthaleneacetic acid (NAA) on the multiplication and development of *Cyrtopodium paludicolum* after 120 days of *in vitro* culture. NS = number of shoots; LSL = longest shoot length; NR = number of roots; LRL = longest root length; SDM = shoot dry matter; RDM = root dry matter.

BA/NAA (μM)	NS	LSL (cm)	SDM (g)	NR	LRL (cm)	RDM (g)
0/0	0.32 c	1.17 c	0.014 c	3.32 c	7.98 b	0.049 d
0/0.57	1.08 c	3.82 b	0.026 c	5.68 b	12.30 a	0.394 b
0/2.28	0.68 c	2.76 c	0.021 c	6.56 b	13.60 a	0.390 b
0.57/0	3.32 b	4.10 b	0.111 b	9.16 a	8.25 b	0.477 a
0.57/0.57	3.32 b	5.36 a	0.110 b	10.12 a	8.40 b	0.460 a
0.57/2.28	2.16 b	6.66 a	0.116 b	7.68 b	9.03 b	0.370 b
2.28/0	5.16 a	5.06 a	0.218 a	7.14 b	4.33 cd	0.217 c
2.28/0.57	5.32 a	6.27 a	0.253 a	7.88 b	5.14 c	0.180 c
2.28/2.28	5.24 a	6.84 a	0.252 a	7.76 b	3.17 d	0.167 c

Values followed by the same letter (in rows) are not significantly different according to the Tukey's test at 5% probability.

obtained it is feasible to infer that concentrations between 1 and 2% are most appropriate for the *in vitro* multiplication and development of *C. paludicolum* shoots.

The results regarding the effects of BA and NAA on the multiplication and initial development of *C. paludicolum* after 120 days of culture are shown in Table 3. The best combinations for shoot production were those containing 2.28 μM BA, even though no significant differences (P<0.05) were detected among them. The results also revealed that in the absence of a cytokinin, shoot formation was repressed. The presence of increasing auxin concentrations did not significantly affect shoot proliferation, although 0.57 μM provided better results than 2.28 μM. Contrasting results were reported by Ferreira *et al.* (2018) for *Catasetum macrocarpum*, where the presence of auxin in the culture medium inhibited shoot formation. In terms of shoot growth in length, the addition of BA to the culture medium was also beneficial for this parameter. NAA alone did not favor shoot length when compared to treatments containing BA. In the presence of this cytokinin, increasing amounts of NAA were advantageous for shoot length, although no significant differences (P<0.05) were observed among the cytokinin and auxin concentrations used. The best result was detected when BA and NAA were used at 2.28 μM. A few studies have also shown that orchid shoot growth in length can benefit from the combination of an auxin and a cytokinin such as BA, as was reported by Diaz & Alvarez (2009) for *E. mariae*, by Roy *et al*. (2011) for *V. coerulea*, and by Rosas & Garcia (2011) for *O. tigrinum*. On the other hand, for *C. saintlegerianum* the presence of BA in the medium inhibited shoot length (Rodrigues *et al.* 2015). For shoot dry matter accumulation the best results were obtained when BA was used at 2.28 μM, independently of NAA.

The addition of 0.57 μM BA and 0.57 μM NAA was the most effective treatment for root formation (Tab. 3), which showed that the combined action of a cytokinin and an auxin favored root production. However, higher NAA concentrations inhibited this variable. It is also worth mentioning that no significant difference was observed between 0.57/0.57μM BA/NAA and 0.57 μM BA alone. This result reinforces the fact that the presence of BA in the culture medium is important to enhance root formation in *C. paludicolum*. Similar results were reported by Rodrigues *et al.* (2015) for *C. saintlegerianum*. For root growth in length the best treatment was 2.28 μM NAA, but it did not significantly differ from 0.57 μM. This outcome is in accordance with the classical role of auxins in promoting cell expansion which contributes to longitudinal growth. In *Cattleya bicolor* Souto *et al.* (2010) reported that 2.6 μM NAA was the concentration that best stimulated the growth of roots in length after 360 days of culture *in vitro*. Regarding root dry matter the most effective treatments were BA alone at 0.57 μM or in combination with 0.57 μM NAA. These

results show that for *C. paludicolum* BA, at low concentrations, favors dry matter accumulation in roots and that an increase in NAA concentration in the presence of BA causes a decrease in this variable. A greater number of roots as well as an increase in their dry matter is especially important for the acclimatization phase because plants will be more capable of taking up water and nutrients from the substrate which, in turn, can provide higher survival rates.

Micropropagation by using etiolated stem segments

After 180 days of dark incubation plants produced etiolated chlorophyll-free stems clearly exhibiting nodes and internodes which could be easily divided into segments containing at least one axillary bud (Fig. 3a-c). These characteristics, commonly observed in plants grown in the absence of light, result from the inhibition of chloroplast development and a decrease in the levels of photosynthetic pigments (Taiz & Zeiger 2004). Approximately 20 days after being transferred to the presence of light, each bud developed into a new shoot (Fig. 3d) which eventually rooted and gave rise to a new plant. Root formation initiated about 30 days after transfer of dark-grown stem segments to the presence of light. Shoots and roots were formed in all treatments after transfer to the presence of light. No significant differences (P<0.05) were observed in terms of the percentage of stem segments that regenerated shoots and roots. In the growth regulator-free medium, 90% of the etiolated stem segments formed shoots and roots,

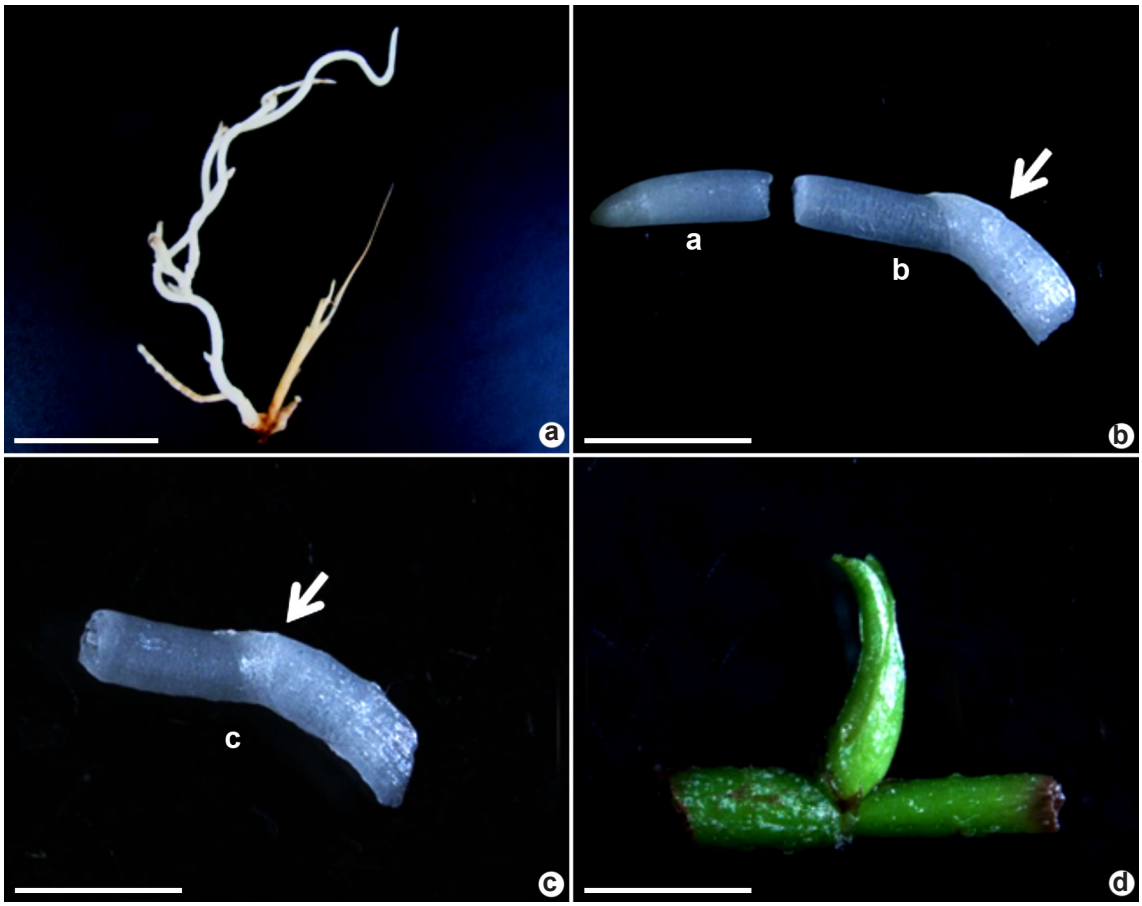

Figure 3 – Micropropagation of *Cyrtopodium paludicolum* by using dark-grown stem segments – a. etiolated stems after 180 days of incubation in the dark; b-c. sequence of an etiolated stem which was divided into segments (a, b and c). The apical segment is discarded and the other two are transferred to the presence of light; d. green shoot formed 20 days after transfer of a dark-grown stem segment to the presence of light. Arrows in b and c indicate nodal regions with a scale-like leaf protecting an axillary bud which will give rise to a new shoot. Scale bars = 4 cm (a); 0,5 cm (b, c, d).

whereas in media enriched with 2.28/0.57 and 4.56/1.14 μM BA/ NAA 85% of these segments produced shoots and roots. Similarly, Ferreira *et al.* (2011) reported that the addition of a cytokinin and an auxin to the culture medium did not stimulate the formation of shoots in etiolated stem segments of *Dendrobium* Second Love. Ramos & Carneiro (2007) also verified that different combinations of BA and NAA did not favor the *in vitro* formation of shoots of the hybrid *Cattleya x mesquitae* by using dark-grown stem segments.

The results concerning the growth of shoots and roots of *C. paludicolum* regenerated after the transfer of etiolated stem segments to the presence of light are shown in Table 4. The best results were obtained in the absence of growth regulators. In fact, except for shoot dry matter, all other variables exhibited significant better results (P<0.05) in the growth regulator-free medium. Probably, the endogenous levels of the regulators in the etiolated stem segments were at an optimal concentration for the development of the axillary buds and the consequent formation and growth of shoots and roots. Soares *et al.* (2010) also reported that the addition of BA to the culture medium was not beneficial for shoot proliferation in *Laelia crispata* subjected to etiolation. Different results were obtained by Ramos & Carneiro (2007) with the hybrid *Cattleya x mesquitae*. These authors verified that a combination of BA and NAA was beneficial for shoot length.

Acclimatization

The process of acclimatization refers to a set of procedures by which plants produced under controlled *in vitro* conditions are progressively transferred to a natural environment. Many individuals do not survive this process primarily because their root systems are scantily branched, are easily damaged during transfer and do not bear root hairs which make roots poorly functional in terms of water and nutrient absorption (Silveira *et al.* 2013).

In relation to the acclimatization of *C. paludicolum* (Fig. 4), the use of the substrates Bioplant and *Sphagnum* at the ratios 3:1 and 3:2, coupled with the irrigation regime employed was effective for plant survival since over 95% of the plants on average were alive at the end of the first acclimatization phase (Tab. 5). No significant difference (P<0.05) was detected between the two ratios. Ferreira *et al.* (2018) also reported that during the first acclimatization phase of *Catasetum macrocarpum*, no marked differences were observed when Bioplant and *Sphagnum* were used at two distinct ratios. The choice of these two components as well as Ouro Negro, used in the second phase, was due to the fact that they are easily available in the local market at reasonably low prices and because they are commonly used by orchid growers in the region. Sousa *et al.* (2015) verified that survival of *Brassavola tuberculata* plants during acclimatization was highest (75%) when they were grown on *Sphagnum* when compared to a mix of 1:1 *Sphagnum* and coconut fiber. These authors associated the higher survival percentage to the physical characteristics of the substrate (*Sphagnum*) such as water-holding capacity and porosity. Macedo *et al.* (2014) reported that the average survival percentage of *B. tuberculata* during acclimatization upon using *Sphagnum*, coconut fiber and charcoal, individually, was 80%.

Colombo *et al.* (2005), upon testing the effects of *Sphagnum*, ground coconut, coconut fiber and fern fiber for the acclimatization of the hybrid *Cattleya* Chocolate Drop *x* (*C. guttata* Lindl. *x L. tenebrosa* Lindl.), verified that *Sphagnum* was the substrate that promoted the highest plantlet survival (72%). Muller *et al.* (2007) reported

Table 4 – Growth of shoots and roots of *Cyrtopodium paludicolum* originated after transfer of dark-grown stem segment to the presence of light in the presence of BA and NAA. SRR = Shoot and root regeneration; LSL = longest shoot length; LRL = longest root length; SDM = shoot dry matter; RDM = root dry matter.

BA/NAA (mM)	SRR (%)	LSL (cm)	LRL (cm)	SDM (g)	RDM (g)
0/0	90 a	7.11 a	8.82 a	0.065 a	0.069 a
2.28/0.57	85 a	3.28 b	1.47 b	0.060 a	0.041 b
4.56/1.14	85 a	3.05 b	0.99 b	0.057 a	0.024 b

Values followed by the same letter (in rows) are not significantly different according to the Tukey's test at 5% probability.

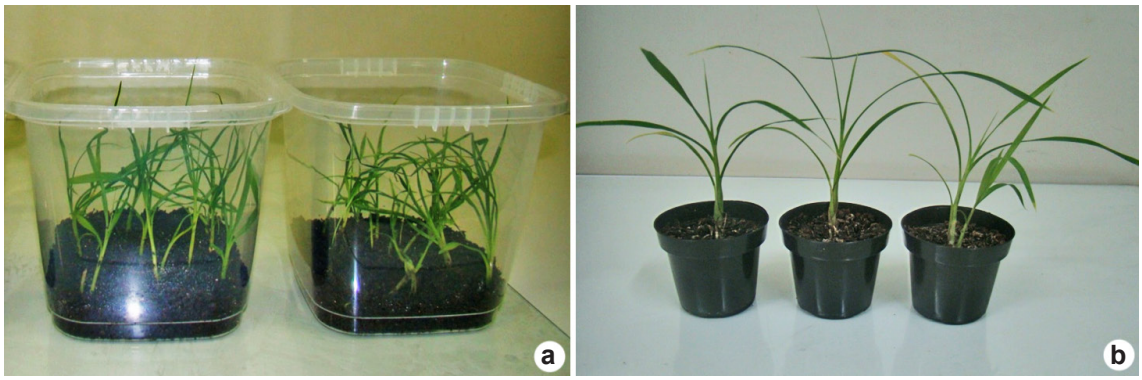

Figure 4 – Acclimatization of *Cyrtopodium paludicolum* – a. individuals in community pots (16 cm height × 12 cm basal width) 90 days after transfer from the *in vitro* culture medium; b. *C. paludicolum* plants at the second acclimatization phase 90 days after transfer to individual plastic pots (7 cm height × 6 cm basal diameter).

Table 5 – Influence of two different Bioplant and *Sphagnum* ratios on *Cyrtopodium paludicolum* survival and growth during the first acclimatization phase.

	Bioplant : *Sphagnum*	
Variables	3:1	3:2
Survival (%)	96.6 a	95.0 a
Height increment (cm)	11.9 a	9.1 b
Number of shoots/plant	2.9 a	1.3 b
Number of leaves/plant	5.2 a	4.3 b
Number of roots/plant	4.5 a	3.1 b

Values followed by the same letter (in lines) are not significantly different according to the Tukey's test at 5% probability.

that for *Miltonia flavescens*, an epiphytic orchid, ground coconut was the substrate that provided the highest survival percentage of plantlets (87.6%). They also mixed ground coconut with Plantmax, a substrate relatively similar to Bioplant, but survival decreased. Since *C. paludicolum* is a terrestrial orchid, it possibly requires substrates that are richer in nutrients when compared to epiphytic species. Thus, it is necessary to associate *Sphagnum* with a more complex substrate such as Bioplant which provides higher mineral content and extra water-holding capacity.

Regarding the growth variables (Tab. 5), the results revealed that the 3:1 (Bioplant and *Sphagnum*) ratio exhibited significantly better results (P <0.05) for all parameters evaluated. In contrast, Sousa *et al*. (2015) reported that in *B. tuberculata* a greater number of roots were formed when plants were grown in unmixed *Sphagnum*. This difference can be possibly related to the fact that substrate porosity is higher when *Sphagnum* is used unmixed, which would provide better conditions for root formation in epiphytic orchid species. In the case of *C. paludicolum*, a terrestrial orchid that naturally grows in wet areas, a substrate capable of holding greater amounts of water such as Bioplant seems to be more beneficial to root formation and development. In any case, a greater number of roots and leaves is important for the subsequent phases of acclimatization because plants will take up water and nutrients from the substrate more efficiently and have a larger photosynthetic area which, in turn, can grant higher survival rates.

In the second acclimatization phase, when plants were transferred to individual pots (Fig. 4b) and *Sphagnum* was substituted for Ouro Negro, the survival rate reached 96.66%. The substitution of *Sphagnum* for Ouro Negro, a much coarser substrate, aimed to provide a mix where roots could be exposed to rougher hardening conditions. This would eventually help plants adapt better when transferred to the soil in the natural environment. The average increment in height was 10.84 cm. The average number of shoot and leaves produced per plant was 1.2 and 3.5, respectively. During this phase leaf fall was observed, especially around 30-50 days after transfer to the individual pots. We believe that this abscission resulted from the stress of caused by the transfer to individual pots: roots were moved and sometimes exposed during transfer, the environmental conditions changed (decrease in humidity) and plants were not sprayed

daily with deionized water. It is possible that the decrease in the number of shoots produced per plant in this phase as compared to phase 1 might have resulted from a reduced photosynthetic rate due to the smaller number of leaves per plant. The plants were transferred to a shade house and 95% survived after three months. Dutra *et al.* (2009) also reported high survival rates for *C. punctatum* at greenhouse conditions. Further studies involving the reintroduction of these plants to their natural environment will be carried out in the near future.

In conclusion, the results obtained showed that VW was the most beneficial medium for *C. paludicolum* seeds, whereas for seedling formation, based on the growth index, protocorms should be transferred to KC medium 30 to 40 days after germination. On this medium seedling development was optimal and healthy and vigorous plants were formed. Culture medium should be enriched with 2% sucrose for best plant growth and development. Shoot and root proliferation and development were promoted when seedlings were grown in the presence of 2.28/2.28 and 0.57/0.57 μM BA/NAA, respectively. The use of etiolated stem segments for micropropagation was verified as very effective. Regarding the process of acclimatization, we recommend that 150-day-old plants be initially transferred to community pots containing a 3:1 (v/v) mix of Bioplant and *Sphagnum*. After 120 days they can be transferred to individual pots containing a 2:1 (v/v) mix of the substrates Bioplant and Ouro Negro. Four months later plants are ready to be transferred to nursery conditions.

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