In vitro propagation of Brazilian orchids using traditional culture media and commercial fertilizers formulations

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ABSTRACT. Some species of Brazilian orchids are practically extinct in their natural habitats. Large scale propagation of *in vitro* native orchids is a way of preserving them. Specific culture media for some species have already been described, but there are no studies on *Catasetum fimbriatum* and *Cyrtopodium paranaensis.* The aim of the present study was to establish *in vitro* propagation protocol of *C. fimbriatum* and *C. paranaensis* to reintroduce these species in natural habitat. The traditional culture media were: MS; MS modified with ½ macronutrients; MS modified with ¼ macronutrients, Vacin & Went and Knudson C. The commercial fertilizers formulations were: N.P.K (10-5-5) 2 mL L^{-1} and formulation N.P.K (10-30-20) 3 g L^{-1}. The data, assessed six months after the beginning of the experiment, consisted of: number of roots, main root length, pseudobulb diameter, plant dry weight, root dry weight and plant height. The best results of *C. fimbriatum* plant development were obtained using MS medium complete and MS medium modified with half of macronutrients for canopy length. For the number of roots, the best results were obtained using MS modified with half of macronutrients. The best results for *C. paranaensis* plant development were obtained using MS modified with half of the macronutrients for canopy length and MS modified with 1/4 of macronutrients for number of roots.

Key words: tissue culture, culture media, comercial fertilizers, *Catasetum fimbriatum, Cyrtopodium paranaensis,* orchids propagation.

RESUMO. Propagação *in vitro* de orquideas brasileiras utilizando meios de cultura tradicionais e formulações com fertilizantes comerciais. Algumas espécies de orquídeas brasileiras encontram-se praticamente extintas no seu habitat natural. Para que isto não ocorra, a propagação *in vitro* em larga escala é uma maneira de preservá-las. O estabelecimento de meios de cultura específicos para algumas espécies já foram descritos, porém não existem estudos para *Catasetum fimbriatum* e *Cyrtopodium paranaensis*. O objetivo deste estudo foi o estabelecimento de um protocolo de propagação *in vitro* para *C. fimbriatum* e *C. paranaensis* para a re-introdução das mesmas em seus habitats naturais. Os meios de cultura tradicionais utilizados foram: MS; MS modificado com ½ dos macronutrientes; MS modificado com ¼ dos macronutrientes, Vacin & Went e Knudson C. Os adubos comerciais utilizados foram: formulação N.P.K (10-5-5) 2 mL L^{-1} e formulação N.P.K (10-30-20) 3 g L^{-1}. Os dados avaliados após seis meses do início do experimento foram: número de raízes, comprimento da raiz principal, largura de bulbo, peso seco da parte aérea , peso seco das raízes e comprimento da parte aérea. Os melhores resultados para o desenvolvimento vegetativo e enraizamento de *C. fimbriatum* foram os meios MS com total e ½ dos macronutrientes para desenvolvimento da parte aérea, já para número de raízes o melhor meio de cultura foi o MS modificado com ½ dos macronutrientes. Para *C. paranaensis* o melhor resultado para o desenvolvimento vegetativo foi o apresentado no meio de cultura MS modificado com ½ dos macronutrientes e para número de raízes foi o MS modificado com ¼ dos macronutrientes.

Palavras-chave: cultura de tecidos, meio de cultura, fertilizantes comerciais, *Catasetum fimbriatum, Cyrtopodium paranaensis*, propagação de orquídeas.

Introduction

The Orchidaceae family is one of the largest of the Angiosperm families, consisting of about 700 genera and 35,000 species distributed throughout the world. Orchids are found in all the states of Brazil, and in greater quantity in the *Mata Atlantica* (Atlantic

Forest), which goes from *Pernambuco* state to *Rio Grande do Sul*. These plants are also found in abundance in riverside forest throughout the country, in the Savannah of Central Brazil, in the Amazon forest and even in the Northeastern tropical thorn bush forest (caatinga). There are more than 3,500 different Brazilian species, and the second major number of species is found in Colombia (Miller and Warren, 1996).

There are more than 50 species of *Catasetum* in the world and also a great variety of hybrids, where the flower can vary greatly in size and color. It is a tropical plant and generally easy to grow (Hersh, 1996). The *Cyrtopodium paranaensis* orchid is a tropical climate species typical of Brazilian hillsides. The *Cyrtopodium* genus is found in the Americas from Florida (USA) to Argentina (Meneses, 1995a, b).

There are few studies on the most suitable culture media for sowing native Brazilian orchids, and there is no information on the best maturity point of seeds and their sensitivity to sterilization and acclimatization processes (Stancato and Faria, 1996). Interest in Brazilian orchid species has increased sharply both in the domestic and external markets but the propagation of these plants has been unsuccessful, because there is little data available on their cultivation (Kerbauy, 1995).

Orchids have an immense biochemical, physiological and genetic diversity and there is a range of culture media designated for the multiplication of each species. The Knudson C (1946) is the most used medium for *in vitro* sowing and the formulas described by Vacin and Went (1949) and Murashige and Skoog (1962) are the most used for clonal propagation (Arditti e Ernest, 1990).

An ideal nutritive medium supplies the essential substances for tissue growth and controls the standard of *in vitro* development to a large extent. Because of this, several organic compounds are added to the culture medium to complement the substances biosynthesized by the cells and to supply the metabolic, energetic and structural requirements of the cell (Caldas *et al*., 1998).

Orchids can be rapidly multiplied by *in vitro* culture from sprout tips or meristems from *Cymbidium* orchids (Morel, 1960). This technique was used for several genera and found almost immediate practical application at the beginning of the 1970s. Currently, many commercial laboratories in Europe, North America and Southeast Asia annually produce millions of low cost of orchid plantlets using this methodology (Bornman, 1993).

In the last years, a large number of research about orchids culture media, specially with *Catasetum fimbriatum* were published (Peres *et al.*, 1999; Peres e Kerbauy, 1999; Majerowicz *et al.*, 2000).

The aim of the present study was to establish *in vitro* propagation protocol of *C. fimbriatum* and *C. paranaensis* to reintroduce these species in natural habitat.

Material and methods

The two Brazilian orchid species used in this study were *Catasetum fimbriatum* (Morren) Lindl. and *Cyrtopodium paranaensis* Schlt. The capsules of orchids with seeds inside were disinfected with 1.5% active sodium hypochlorite for 15 minutes. After disinfection, the capsules were washed in autoclaved water and opened with a scalpel. The seeds inside the capsule were collected and inoculated in the Knudson C germination medium within a blade flow chamber.

After seed germination, during the protocorm formation phase, 15 protocorms were inoculated per flask with the following treatments: T1) MS (1962); T2) MS modified with ½ macronutrients; T3) MS modified with ¼ macronutrients; T4) Vacin and Went (1949); T5) Knudson C (1946); T6) Commercial formulation N.P.K (10-5-5) 2 mL L^{-1}; T7) Commercial formulation N.P.K (10-30-20) 3 g L^{-1}. In all treatments 30 g L^{-1} of sucrose and 6 g L^{-1} of agar were added. In treatments T6 and T7, they were prepared with commercial formulations easily found in the market. These traditional culture media could be easily used in Bio-facture, as they are not difficult to be found, they do not cost a lot and because of the facility of its preparation when compared with the traditional ones.

Flasks of 250 mL capacity were used, with 50 mL of autoclaved culture medium each. The pH of each culture media was adjusted to 6.0 before putting in autoclave. These flasks were kept in a controlled environment at 25-27ºC and 16 hours light from a fluorescent lamp. The following characteristics were analyzed: root number, length of largest root, pseudo bulb diameter, canopy dry weight, root dry weight and canopy length. The assessments were made six months after the beginning of the experiment.

A complete randomized block design was used and there were five replications containing 15 protocorms per replication. The data obtained were submitted to analysis of variance and of the Tukey test.

Results and discussion

There were differences among the several culture media *in vitro* cultivation of *C. fimbriatum* for root number, length of largest root (cm), pseudo bulb diameter (cm), plant dry weight (mg), root dry weight (mg) and plant height (cm). The plants with the greatest growth of *C. fimbriatum* were observed in

treatments T1 (MS) and T2 (MS modified with ½ the macronutrients), both with 6.00 cm. The plants with lowest growth were observed in treatment T6 (N.P.K.- 10-5-5), with 1.80 cm (Table 1).

Table 1. Mean values for plant height, number of roots, largest root length, peudobulb diameter, plant and root dry weight of *Catasetum fimbriatum* six months after the beginning of the experiment. Universidade Estadual de Londrina, State of Paraná, 2003.

	Treatments	Plant height (cm)	Nº of roots	Main root length (cm)	Psuedobulb diameter (cm)	Plant dry weight (mg)	Root dry weight (mg)
T_1	MS	6.00 a[1]	2.24 b	5.00 c	0.25 c	26.20 bc	25.00 c
T_2	MS with ½ macronutrients	6.00 a	3.50 a	7.00 b	0.61 a	61.00 a	54.20 a
T_3	MS with ¼ macronutrients	3.30 c	2.12 bc	3.30 d	0.50 b	24.80 c	13.00 d
T_4	Vacin and Went	3.74 bc	1.60 c	1.52 e	0.20 c	7.60 dc	3.00 e
T_5	Knudson C	4.00 b	2.20 b	2.54 d	0.53 ab	46.20 ab	13.00 d
T_6	N.P.K (10-5-5)	1.80 d	1.60 c	8.04 a	0.25 c	1.40 d	34.00 b
T_7	N.P.K (10-30-20)	3.40 c	3.24 a	3.00 d	0.50 b	23.00 c	24.20 c
C.V %		7.14	12.80	11.70	10.90	28.53	18.50
DMS		0.60	0.60	1.00	0.10	21.00	9.00

[1] means followed by the same letter on the vertical do not differ by the Tukey test at 5% probability.

The plants with the greatest root number in *C. fimbriatum* were found in treatments T2 (MS modified with ½ the macronutrients) and T7 (N.P.K.- 10-30-20) with 3.50 and 3.24, respectively. The lowest root number were found in treatments T4 (Vacin and Went) and T6 (N.P.K.- 10-5-5), both with 1.60 (Table 1).

Peres *et al.* (1999) proposed that an endogenous balance from auxin to cytokinins is important to shoot formation in *C. fimbriatum.*

The plants with most satisfying root length were observed in T6 (N.P.K- 10-5-5) with 8.04 cm and the least in T4 (Vacin and Went) with 1.52 cm. The plants that show the greatest pseudo bulb diameter were those kept in treatment T2 (MS with ½ macronutrients) with 0.61 cm. Plants that show the least pseudo bulb diameter were those kept in treatments T1 (MS), T4 (Vacin and Went) and T6 (N.P.K.- 10-5-5) with 0.25, 0.20 and 0.25 cm, respectively (Table 1).

Culture media differed in the *in vitro* cultivation of *C. paranaensis* for number of roots, length of the largest root (cm), plant dry weight (mg), root dry weight (mg) and plant height (cm) (Table 2). Characteristic pseudo bulb diameter was not analyzed for *C. paranaensis* because they do not differ significantly.

Table 2. Mean values for plant height, number of roots, largest root length, plant and root dry weight, for *Cyrtopodium paranaensis,* in the different culture media 6 months after the beginning of the experiment. Universidade Estadual de Londrina, State of Paraná, 2003.

	Treatments	Plant height (cm)	Nº of roots	Main root length (cm)	Plant dry weight (mg)	Root dry weight (mg)
T_1	MS	6.64 b[1]	2.80 d	1.50 d	30.40 a	20.40 bc
T_2	MS with ½ macronutrients	8.70 a	2.76 d	1.80 cd	28.20 a	16.40 c
T_3	MS with ¼ macronutrients	5.10 c	4.76 a	3.60 b	17.0 b	41.40 ab
T_4	Vacin and Went	3.75 d	1.92 e	1.00 e	10.80 c	25.20 bc
T_5	Knudson	3.19 d	2.60 d	2.02 c	14.00 bc	15.20 c
T_6	N.P.K. (10-5-5)	1.91 e	4.00 b	4.30 a	16.80 b	53.60 a
T_7	N.P.K. (10-30-20)	3.70 d	3.40 c	3.80 b	19.00 b	50.00 a
C.V. %		11.73	8.31	6.71	13.28	28.26
DMS		1.11	0.52	0.34	5.20	4.40

[1] means followed by the same letter on the vertical do not differ by the Tukey test at 5% probability.

The plants having the greatest growth of *C. paranaensis* were observed in treatment T2 (MS modified with ½ macronutrients), with 8.70 cm. The plant with the lowest growth was observed in treatment T6, NPK (10-5-5), with 1.91 cm. The plants with the greatest root number in *C. paranaensis* were found in treatment T3 (MS modified with ¼ the macronutrients) with 4.76, while the lowest root number were found in treatment T4 (Vacin and Went) with 1.92 (Table 2). The plants with the most satisfying root length (cm) were observed in treatment T6 (NPK 10-5-5) with 4.30 cm, while plants that show the least were found in treatment T4 (Vacin and Went) with 1.00 cm.

Collins and Dixon (1992) cultivated the *Diuris longifoli* orchid *in vitro* in Vacin and Went culture (1949) and observed that the plantlets had excellent growth. However, in this study the orchids grew less in the same culture medium mentioned above.

The most adequate means presented for plant dry weight for *C. paranaensis* were for treatments T1 (MS), T2 (MS modified with ½ macronutrients) with 30.4 and 28.2 mg, respectively, and the least mean was for treatment T4 (Vacin and Went) with 10.8 mg. The greatest means for root dry weight were found in treatments T6 (N.P.K.- 10-5-5) and T7 (N.P.K.- 10-30-20) with 53.6 and 50.0 mg, respectively, and the least means were for treatments T2 (MS modified with ½ macronutrients) and T5 (Knudson C) with 16.4 and 15.2 mg, respectively.

The best growth culture media for *Laelia cinnabarina* Batem. orchid was MS medium modified with ½ macronutrients and complete medium Hoagland and Arnon (1950). However, more vigorous plantlets were obtained in the MS medium modified with ½ the macronutrients (Stancato and Faria, 1996).

When the two orchids *C. fimbriatum* and *C. paranaensis* were compared to the number of roots, there were differences among the culture media. The treatment with greatest root formation induction for *C. fimbriatum* was T2 (MS modified with ½ macronutrients) with 3.50 whereas for *C. paranaensis* the T3 (MS modified with ¼ macronutrients) was the best with 4.76.

The two orchids, *C. fimbriaum* and *C.*

paraneensis were different in plant height. The best treatments for *C. fimbriatum* were T1 (complete MS) and T2 (MS modified with ½ macronutrients), both with 6.00 cm. However for *Cyrtopodium paranaensis* the best treatment was T2 (MS modified with ½ the macronutrients) with 8.7 cm.

Kraus and Kerbauy (1992) compared different culture media for protocorm formation by roots explants of *Catasetum pileatum* orchid roots. The Murashige and Skoog (1962), Vacin and Went (1949) and Knudson C (1946) culture media were assessed and the best culture medium for protocorm regeneration was Murashige and Skoog. Majerowicz *et al* (2000) suggest that organic nitrogen and NH_{4+} are probably the most important nitrogen sources to *C. fimbriatum* plants.

The traditional MS media culture was more appropriate for the propagation *in vitro* of the two studied orchids when compared to the formulations containing the commercial fertilizers. This happened because the MS culture media is rich in macro and micronutrients while the commercial fertilizers have only N-P-K.

These results confirm that suitable culture media should be determined for each species and using more appropriate nutritive formulas can optimize the plant quality produced *in vitro*.

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