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PLANT REGENERATION FROM ROOT-TIP CULTURE OF *CYRTOPODIUM BRANDONIANUM* BARB. RODR. (ORCHIDACEAE)

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Abstract

A protocol for *in vitro* plant multiplication of *Cyrtopodium brandonianum* from root-tip culture was developed. Root tips were dissected from 150 day-old plants, obtained *in vitro* and were cultured on half-strength MS medium, supplemented with different concentrations of BAP, kinetin, zeatin, tidiazuron, and 2iP for induction of adventitious shoots. After 60-70 days of incubation, shoot differentiation could be distinguished. The half-strength MS medium supplemented with 0.5 mg l^{-1} TDZ showed the best response on shoot production, with, in average, 43% of the explants with shoots. Optimal rooting (20% of shoots with an average of 4.3 roots per explants), with no intervening callus was observed in medium containing half-strength MS medium, with 6% sucrose and 1 mg l^{-1} NAA.

Key words: *Cyrtopodium brandonianum, in vitro* plant regeneration, orchids, plant growth regulators, root-tip culture.

INTRODUCTION

The genus *Cyrtopodium* (Orchidaceae) is widely distributed in tropical and subtropical countries of Central and South America (Menezes 2000). In Argentina, 5 species of *Cyrtopodium* are known, including *C. brandonianum* Barb. Rodr., a terrestrial orchid that grows spontaneously in the grassland of the northwest of the Corrientes Province (Schinini et al. 2008). This orchid is popular among collectors due to its high ornamental value because of the beautiful flowers (Menezes 2000) (Fig. 1A), but its populations have decreased drastically over the past 10 years due to the depredation of many populations as well as the destruction of their habitats. The consequence is possible extinction of the species, and it provides justification for propagation and conservation of this valuable germplasm. *In vitro* culture can be an interesting technique for both propagation and germplasm conservation of this species.

Root-tip culture of orchids has attracted the attention of several investigators because it is a non-destructive technique in which the donor plants regenerate new roots in natural form. Also the availability of roots during the whole year makes them suitable explants for the cultivation *in vitro* (Kerbauy 1991, Park et al. 2003).There are very few reports on *in vitro* studies on species of the genus *Cyrtopodium.* Sánchez (1988) reported the regeneration of plants from root-tip cultures of *C. punctatum,* and Flachsland et al. (1996) developed a protocol for *in vitro* seed germination of *C. brandonianum*.

The objective of this study was to develop an efficient plant regeneration system by *in vitro* culture of root-tips, which can be utilized both with the purposes of multiplication and conservation of *C. brandonianum* plants.

MATERIALS AND METHODS

Plant material

In vitro plantlets of *Cyrtopodium brandonianum* Barb. Rodr. used as explants were raised by culturing seeds according to the protocol described by Flachsland et al. (2006). Briefly, isolated green capsules (Fig.1B) containing mature seeds (120 days after hand-pollination) were surface disinfected by immersion in 70% ethanol (5 min), and then in a solution of commercial bleach containing 2% NaOCl with two drops of Triton

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X-100® (15 min). The capsules were rinsed three times with sterile distilled water and the seeds were extracted and aseptically cultured by inoculating 450 ± 50 seeds onto 80 ml of seeds culture medium in 350 ml glass jars.

For seed germination, half-strength MS medium (Murashige and Skoog 1962), containing 3% sucrose, 2 g l^{-1} activated charcoal, and 25 g l^{-1} banana powder (SIGMA B-4032) was used.

Adventitious buds and shoot induction

After 150 days of culture, the root tips (1-1.5 cm in length) were isolated from plants of approximately 2-3 cm in hight (Fig. 1C) and aseptically cultured *in vitro* (10 root tips per jar) in 350 ml glass jars containing 80 ml of medium, which was half-strength MS medium supplemented with BAP (6-benzylaminopurine), KIN (6-Furfurilaminopurine), ZEA (6-[4-hydroxy-3-methyl-but-2-enylamino]purine), TDZ (1-phenyl-3-[1,2,3-thidiazol-5-yl]urea), or 2iP [(N^6-(2-Isopentenyl(adenine)] at 0.1, 0.5 and 1 mg l^{-1} or without plant growth regulators (control treatment).

All variants of the medium were adjusted to pH 5.8 with either KOH or HCl prior to the addition of 6 g l^{-1} agar (SIGMA A-1296). The variants of the medium were sterilized in autoclave at 1.46 kg cm^{-2} for 20 min. All the cultures were incubated in a growth room at a constant temperature of 27 ± 2°C with 14 h photoperiod (116 μmol m^{-2} s^{-1}) from cool-white fluorescent lamps.

Adventitious root induction

For root induction, the regenerated adventitious shoots of more than 1 cm in length were transferred (10 shoots per jar) to in 350 ml glass jars containing 80 ml of rooting medium.

The variants of the medium used for rooting of shoots consisted of either half-strength MS with sucrose at 3, 6, and 9% (control treatments) alone or supplemented with IBA (indole-3-butyric acid), NAA (α-naphthaleneacetic acid), or IAA (indole-3-acetic acid) at 1 and 3 mg l^{-1} (Table1). All the cultures were incubated in the same physical conditions described above.

Observations and statistical analysis

The experiments were conducted in a randomized complete block design with three replications. Each replication consisted of ten explants per flask, i.e. 30 explants per treatment. The data pertaining to mean percent of explants with the standard error (±SE) producing either calluses and shoots or calluses only were recorded after 90 days of culture. The percentage of rooting and the average number of roots per shoot were recorded after 90 days of culture. Data were subjected to analysis of variance (ANOVA) and comparisons of means were conducted using Tukey Multiple Comparison Test ($p < 0.05$).

RESULTS AND DISCUSSION

The earliest visible signs of callus growth from root tip explants were noticeable within 30 days of culture. In some treatments, root tips gradually enlarged and small, compact, white yellowish or green calluses were observed to grow on the explants after 45 days of incubation. On longer incubation (60-70 days), shoot differentiation could be distinguished (Fig. 1D). Shoot regeneration occurred in 10 out of 16 variants of the medium investigated (Fig. 2) but the percentage of explants which produced shoots was related to the type of cytokinins employed. There was no sign of shoots regeneration when root tips were cultured on medium without cytokinins or those containing any concentrations of KIN studied as well as the lowest concentration of ZEA or the highest level of 2iP. On the contrary, when TDZ was employed, shoots differentiation was enhanced. The half-strength MS medium supplemented with 0.5 mg l^{-1} TDZ showed the best response on shoots production, which occurred in 43% of the explants in average.

The experiments described above demonstrate the potential of *in vitro* shoots regeneration from root tips of *Cyrtopodium brandonianum.* Like species of *Cattleya* and *Oncidium*, this process was carried out through indirect organogenesis (Kerbauy 1991, 1993). The requirements of exogenous growth regulators are similar to the ones of other orchid plants in which shoots regeneration from root tips cultured *in vitro* was obtained by the addition of one cytokinins alone to the culture medium (Colli and Kerbauy 1993, Peres et al. 1999, Park et al. 2003). On the other hand, Sánchez (1988) accelerated the direct regeneration of plants of *Cyrtpodium punctatum* from root-tip culture adding coconut milk, a substance rich in cytokinins, to the basal medium.

Shoots formation from root tips was strongly affected by the composition of culture medium, particularly by the type and concentration of cytokinins.

The beneficial effects of TDZ, a potent cytokinin for plant tissue culture (Huetteman and Preece 1993), on *in vitro* plant propagation found in our work are in agreement with the results obtained in other genera of orchid such as, *Phalaenopsis* (Ernst 1994a, Chen and Piluek 1995, Chen et al. 2000) , *Doritaenopsis* (Ernst 1994a), *Cymbidium* (Nayak et al. 1997, Ernst et al. 1994b, Nayak et al. 1998, Chang and Chang 1998), *Dendrobium* (Nayak et al. 1997) and *Oncidium* (Chen et al. 1999, Chen and Chang 2000).

Although excised shoots were rooted (Fig. 1E) in 6 out of 21 variants of the medium investigated (Table 1), optimal rooting (20% of explants with an average of 4.3 roots per explant) with no intervening callus was observed within 60-70 days after transfer to medium containing 6% sucrose and 1 mg l^{-1} NAA. Asghar et al. (2011) have also achieved high rooting percentages

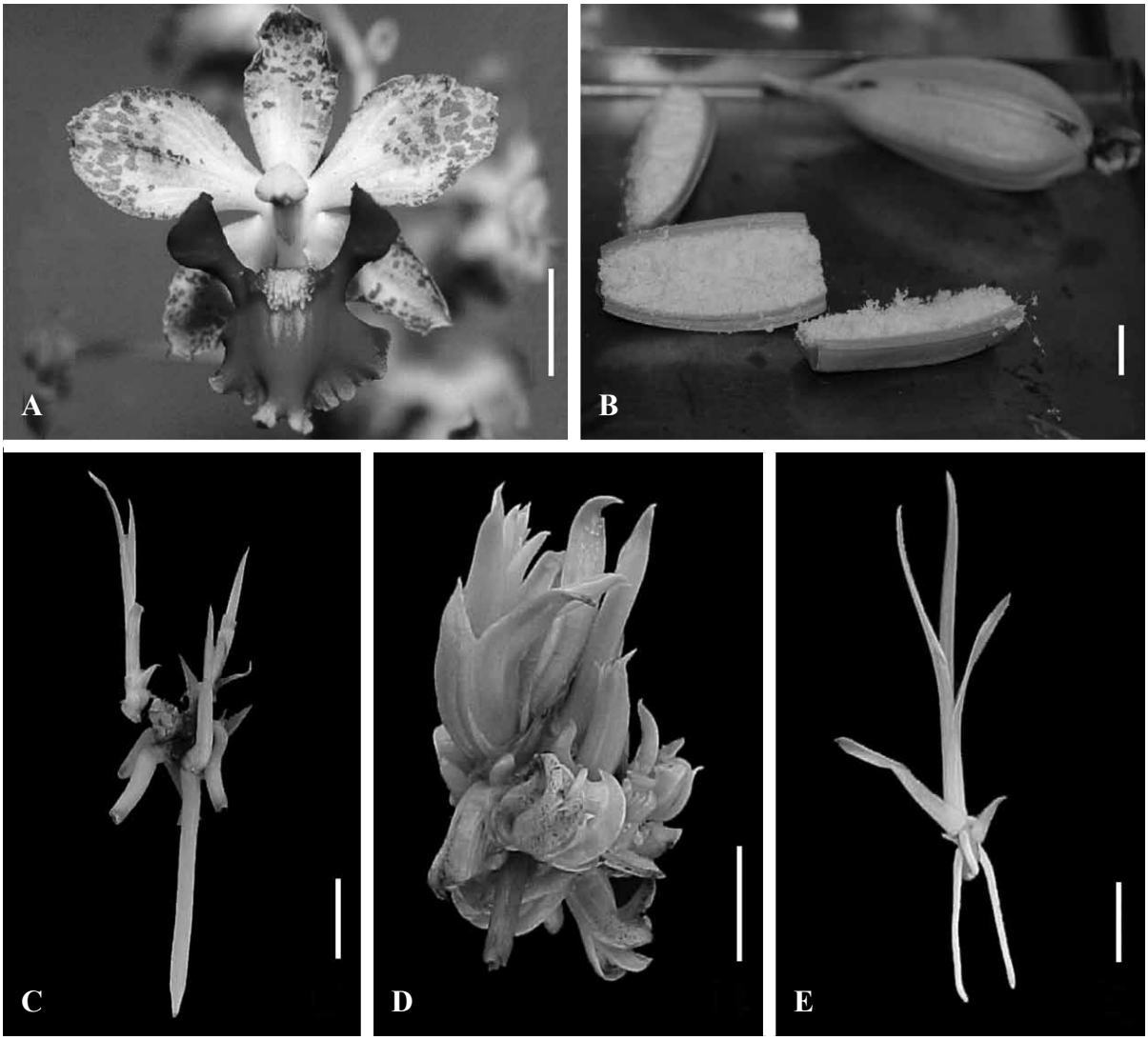

Fig. 1. Plant regeneration of *Cyrtopodium brandonianum* by *in vitro* root-tips culture. A) Flowers, B) Whole capsule and dissected portions, C) *In vitro* donor plant of root-tips, D) Multiple adventitious shoot differentiation from root-tip culture, E) Regenerated and rooted plant. Bars represent 1 cm.

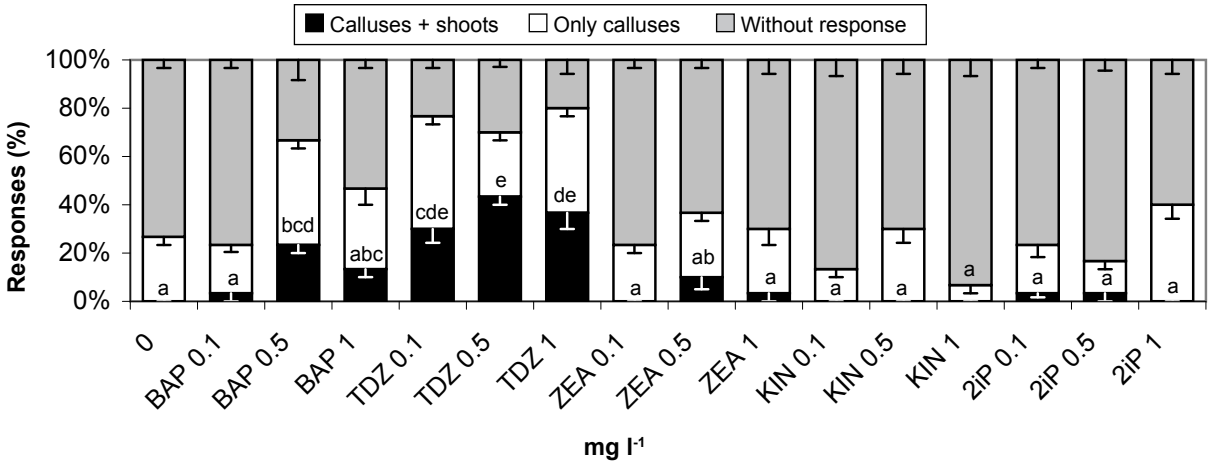

Fig. 2. Effect of different variants of culture medium on responding root-tips of *Cyrtopodium brandonianum* (Mean ± SE).

Table 1. Effect of different variants of culture medium on rooting response of *Cyrtopodium brandonianum.*

Half-strength MS medium supplemented with		Rooted shoots (%)	Mean number of roots
Sucrose (%)	Auxins (mg l^{-1})		
3	0	0 a	0 a
3	IAA 1	0 a	0 a
3	IAA 3	0 a	0 a
3	IBA 1	0 a	0 a
3	IBA 3	5.0 ± 2.9 ab	1.3 ± 0.2 b
3	NAA 1	5.0 ± 2.9 ab	1.2 ± 0.2 b
3	NAA 3	0 a	0 a
6	0	0 a	0 a
6	IAA 1	0 a	0 a
6	IAA 3	0 a	0 a
6	IBA 1	10.0 ± 2.9 b	1.2 ± 0.2 b
6	IBA 3	11.7 ± 4.4 bc	2.2 ± 0.2 c
6	NAA 1	20.0 ± 2.9 c	4.3 ± 0.3 d
6	NAA 3	5.0 ± 2.9 ab	1.3 ± 0.2 b
9	0	0 a	0 a
9	IAA 1	0 a	0 a
9	IAA 3	0 a	0 a
9	IBA 1	0 a	0 a
9	IBA 3	0 a	0 a
9	NAA 1	0 a	0 a
9	NAA 3	0 a	0 a

Means (± SE) within a column followed by the same letter are not significantly different according Tuckey's multiple comparison test at $p \leq 0.05$.

of buds of *Dendrobium nobile* (82.5 and 65%), supplementing the culture medium with 1 mg l^{-1} IBA or NAA, respectively. Some authors demonstrated *in vitro* rooting of the epiphytic orchid *Guarianthe skinneri* by the addition of IAA (Coello et al. 2010), however, it was not possible to promote the rooting of shoots of *Cyrtopodium brandonianum* using this auxin, regardless of sucrose concentration. The beneficial effect of high level of sucrose on rooting of shoots is not common in orchids, where generally rooting is induced with 2 or 3% sucrose (Arditti 2008). The percentage of shoots that formed roots and the number of roots per shoot varied significantly with different concentrations of sucrose and IAA, NAA or IBA. The medium without growth regulators did not promote root induction regardless of the sucrose concentration (Table 1). No roots were regenerated when 9% sucrose was employed.

In spite of the recognized limited morphogenetic ability of root meristem of higher plants, including orchids, the utility of root explants for micropropagation purposes is being increasingly realized due to their year round availability, low oxidation rate, and the ease with which they can be explanted (Chugh et al. 2009).

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