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Micropropagation and Establishment of *Cypripedium* Lady Slipper Orchids

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Abstract

Root tip explants from mature, blooming size *Cypripedium* explanted in Steele-modified Harvais medium containing potato extract were incubated with the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and N^6-benzyladenine (BA) or kinetin but not 2,4-D or BA or kinetin alone, produced callus. If callus remained in contact with plant growth regulators multiple shoots and/or short roots developed. In contrast, if calli were reflasked into plant growth regulator-free media, then most calli produced plantlets that phenotypically resembled seed-derived seedlings. Plantlets resembling normal seedlings established readily following vernalization for 3-5 months whereas plantlets with multiple shoots and short roots grew more slowly with lower survival.

INTRODUCTION

Cypripedium hybrids, members of a genus of temperate terrestrial "ladyslipper" orchids have become popular garden plants. Like hybrids of many plant species, individual members of a grex show great variability. The ability to produce large numbers of genetically identical plants has made *Phalaenopsis, Cattleya* and *Cymbidium* hybrids valuable commercially; the value of micropropagated *Phalaenopsis* have an annual value of more than $200 million wholesale in the United States each year (Griesbach, 2002). Several studies have reported limited success with micropropagation of *Cypripedium* using protocorms in flask (Lee and Lee, 2003; Shimura and Koda, 2004), or seedlings still in flask. Tomita (1998) and Jo et al (2001) used root tip explants from in-flask *Cypripedium* seedlings, incubated with combinations of 2,4-D, picrolam and BA to stimulate callus and plantlet formation. *Cypripedium* node bud explants of in-flask seedlings have also been used (Hoshi et al, 1994). These methodologies are not useful for horticultural production since it is not possible to know how the flowers will appear in mature plants before collection of explants. Only one preliminary report was found in which mature C*ypripedium* were used as the source of tissue explants. Chu and Mudge (1996) used dormant lateral buds as the source of explant material. While this methodology has merit for horticultural production it is not ideal since very large plants with many rhizome buds are required to avoid killing a valued cultivar. Since *Cypripedium* rhizomes expand slowly, growth of plants to such size requires many years. In contrast, even young mature plants have 10-20 roots, making root tip an easy, nondestructive tissue source.

This study reports our initial results on micropropagation of *Cypripedium* using root tip explants of mature, blooming size *Cypripedium.*

MATERIALS AND METHODS

Plant Material

Cyp. reginae, *Cyp. parviflorum* var *parviflorum*, *Cyp. candidum*, *Cyp.* x*andrewsii*, *Cyp. macranthos*, *Cyp.* Gisela,*Cyp.* Peter, and *Cyp.* Sabine were mature, seed-derived plants that had bloomed the season of root tip collection were obtained from the Gardens at Post Hill LLC.

Explantation and Tissue Culture

Root tips were harvested in June or July following flowering, sterilized in a solution of sodium hypochlorite, and 0.5 mm cross section slices were placed in culture on Steele-modified Harvais medium (Steele, 1995) containing potato homogenate, 10 g/l, and growth regulators to induce callus formation. Calli were transferred to Malmgren medium (Malgmren 1996) containing10 g/l potato homogenate. Cultures were incubated at 22 -25°C in complete darkness.

Establishment of Plantlets

Once plantlets formed shoots and roots and stopped growth, they were removed from medium, washed thoroughly and stored moist in sealed plastic bags at 2-5 °C for 3-5 months in the dark to simulate winter ("vernalization"). Plantlets were planted out in a 1:1 mixture of fired, expanded clay (Turface®) and fired, expanded shale (Permatill®) in trays in ebb and flow hydroponics on June 1 and grown under 70% shade cloth until dormancy in the Fall. Trays were flooded daily at 11 AM, 2 PM and 5 PM with water that contained Dyna-Gro Grow® fertilizer 7-9-5 diluted 1:1000 in the water.

Statistical Analysis

Differences in percentages of explants forming calli were compared using Fishers Exact test. Differences in rhizome mass were compared using Student's *t*-test for unpaired observations with Welch correction for unequal standard deviations.

RESULTS AND DISCUSSION

Induction of Callus Formation in Root Tip Explants

When *Cyp. reginae* root tip explants were cultured in Harvais medium without potato extract, no callus formation was observed (Table 1). This is consistent with our observations of Cypripedium seed germination and growth. In the absence of potato extract or pineapple juice, the complex additives in Harvais and Malmgren medium, respectively, protocorm growth is never observed. In contrast, in the presence of potato extract and 2,4-D and BA or kinetin, callus formation was observed over a 16 week observation period (Table 1). None of the growth regulators were active by itself, but a combination of 2,4-D with either BA or kinetin supported callus formation. The optimum concentration of 2,4-D was 10µM and the optimum concentration of BA or kinetin was 1 µM. With this ratio of auxin to cytokinin, callus formation was observed in 70-100% of root tip explants.

Promotion of Differentiation of Callus into Plantlets

Callus was apparent by week 12 in nearly all explants that formed callus. If callus was left in medium containing plant growth regulators, shoots and roots began to appear. In many cases there were multiple shoots and many short (< 2 cm length) roots (Figure 1, Right panel, plantlet on left). This phenotype is quite different from in-flask *Cypripedium* seedlings, which have single shoots and long (> 5 cm) roots, about 90% have 3 roots , about 10% have 4 roots. In an attempt to control shoot and root number, in another experiment we removed calli the week they were first observed and placed them in media from which auxin or cytokinin or both were absent. During 12 weeks of observation in these media, it was observed that if both auxin and cytokinin were present, many calli did not differentiate, and those that did formed either multiple shoots, or many short roots, or plantlets with multiple shoots and many short roots (Table 2). Absence of only cytokinin resulted in no callus differentiation, absence of only auxin resulted in many calli forming single shoots but no roots, and absence of both auxin and cytokinin resulted in most calli forming single shoots and long roots, in 14/16calli, plantlets that resembled typical seedlings formed (Figure 1, Right panel, plantlet on right compared to a normal seedling, Left panel).

Applicability of Micropropagation Protocol to Hybrids and Species from Different Sections

The genus *Cypripedium* is divided into 11 Sections. Most horticulturally important species and hybrids belong to Sections Cypripedium, Macrantha, and Obtusipedila. We examined the ability of our micropropagation protocol to support callus formation from root tip explants in species and hybridsfrom each of theseSections. As reported in Table 1, in *Cypripedium reginae* from Section Obtusipetala, a ratio of 2,4-D:kinetin of 10:1 µM resulted in 90% of explants forming calli (Figure 2).

In two Section *Cypripedium* species, *Cyp. parviflorum* and *Cyp.candidum* and a hybrid, *Cyp.* x*andrewsii* (*Cyp. parviflorum* x *Cyp. candidum*), 2,4-D:kinetin of 10:1 µM resulted in callus formation in 80%, 90% and 90% of explants, respectively (Figure 2). In contrast, in a Section Macrantha species, *Cyp. macranthos*, only 20% of explants formed calli (Figure 2). Two hybrids with one Section Cypripedium and one Section Macrantha parent, *Cyp.* Gisela (*Cyp. parviflorum* x *Cyp. macranthos*) and *Cyp.* Sabine (*Cyp. kentuckiense* x *Cyp. tibeticum*) were intermediate in formation of calli, 60% and 50%, respectively (Table 2). A hybrid of two Section Macrantha species, *Cyp*. Peter (*Cyp. macranthos* x *Cyp. yunnanense*) formed calli as poorly as the Section Macrantha species, 20%.

Growth of Micropropagated Plantlets after Deflasking

At the time of planting out groups of 50 plantlets, rhizome mass in *Cyp. reginae* seedlings was 149 $\pm$ 25 mg mean + SEM), in *Cyp. reginae* micropropagated plantlets with single shoots and roots >5 cm in length was 135 $\pm$ 24 mg, and in *Cyp. reginae* micropropagated plantlets with multiple shoots and multiple roots <5 cm in length was 162 $\pm$ 34 mg. At the time of dormancy in Fall, shriveled stems and leaves were removed from rhizomes. Rhizome mass in in seedlings was 361 $\pm$ 61 mg (N=48), micropropagated plantlets with single shoots and roots >5cm in length was 348 $\pm$ 54 mg N=47), and micropropagated plantlets with multiple shoots and multiple roots <5 cm in length was 212 $\pm$ 25 mg (N=37) ($P<0.0004$ compared to seedlings and $P<0.0006$ compared to micropropagated plantlets with single shoots and roots >5 cm in length.

CONCLUSIONS

In the current study we have defined a general protocol useful in the micropropagation of *Cypripedium* hybrids. Under optimal conditions callus formation occurred in most root tip explants. Under differentiation conditions that resulted in micropropagated plantlets that resembled natural seedlings we observed nearly 100% establishment of these plantlets. Additional work is required to make the method adequately efficient in hybrids from all Sections within *Cypripedium*.

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Literature Cited

Chu, C.-C. and Mudge, K.W. 1996. Propagation and conservation of native lady's slipper orchids (*Cypripedium acaule*, *Cypripedium calceolus*, and *Cypripedium reginae*). In: Allen, C.(ed). North American Terrestrial Orchids. Propagation and Production. North American Native Terrestrial Orchid Conference, Germantown MD, pp. 107-112.

Griesbach , R.J. 2002. Development of Phalaenopsis Orchids for the Mass-Market. In: Janick J and Whipkey A (eds) *Trends in Crops and New Uses*, ASHS Press, Alexandria VA.

Hoshi, Y.,Kondo,K. and Hamatami, S. 1994. In vitro seed germinationof four Asiatic taxa of *Cypripedium* and notes on the nodal micropropagation of American *Cypripedium montanum*. Lindleyana 9:93-97.

Jo, S., Nishimura, N., Okazawa,Y. 2001. Micropropagation of Cypripedium yatabeanum through root tip culture in vitro. Abstracts of the7th Asia Pacific Orchid Conference.

Lee, Y. and Lee, N. 2003. Plant regeneration from protocorm-derived callus of *Cypripedium formosanum*. In Vitro Cell Dev. Biol. Plant 39:475-479.

Malmgren S. 1996. Orchid propagation: theory and practice.). In: Allen, C.(ed). North American Terrestrial Orchids. Propagation and Production. North American Native Terrestrial Orchid Conference, Germantown MD, pp. 63-71.

Shimura, H. and Koda, Y. 2004. Micropropagation of *Cypripedium macranthos* var. *rebunense* through protocorm-like bodies derived from mature seeds. Plant Cell Tiss, Org. Cult. 78:273-276.

Steele, W.K. 1995. Growing *Cypripedium reginae* from seed. Am. Orch. Soc. Bull.64:382-391.

Tomita, M. 1998. Picrolam-induced plantlet regeneration of *Cypripedium calceolus* through root tip culture in vitro. Abstracts of Toyohashi 98. Meeting of the International Plant Propagators Society.

Tables

Table 1. 2,4-D and BA or Kinetin support callus formation in Cyp. reginae root tip explants.

Treatment Group			Callus formation
2,4-D, µM	BA, µM	Kinetin, µM	Experiment1, Experiment2
With potato extract			
0	-	-	0/10, 0/10 a*
0	1	-	2/10, 1/10 a
0	10	-	1/10, 2/10 a
0	-	0.1	2/10, 1/10 a
0	-	1	3/10, 2/10 b
10	10	-	3/10, 4/10 b
10	1	-	7/10, 8/10 c
1	-	0.1	4/10, 6/10 b
10	-	0.1	5/10, 5/10 b
1	-	1	4/10, 6/10 b
10	-	1	8/10, 10/10 c
Without potato extract			
10	10	-	0/10 a
10	1	-	0/10 a

* Values within column followed by the same letter are not significantly different at P=0.05 using Fisher's Exact test.

Table 2. Removal of callus from media containing plant growth regulators affects shoot, root and plantlet formation.

Treatment Group	N	Structures present at the end of 12 weeks following			
		Callus	Shoots	Roots	Plantlets
Auxin + cytokinin	16	7	Multiple=9	< 5 mm = 5	5
Cytokinin removed	15	15	0	0	0
Auxin removed	16	4	Single=12	0	0
Auxin + cytokinin removed	16	2	Single=9 Multiple=5	<5 mm = 4 >5 mm = 10	14

Calli were removed the week they were first observed and reflasked into media with or without auxin (2,4-D) and/or cytokinin (kinetin) for 12 weeks following reflask. Structures formed by each callus are enumerated.

Figures

Figure 1. Left Panel: A seed-derived deflasked seedling of *Cypripedium reginae*. Right Panel: Deflasked root tip-derived plantlets of *Cypripedium reginae*.

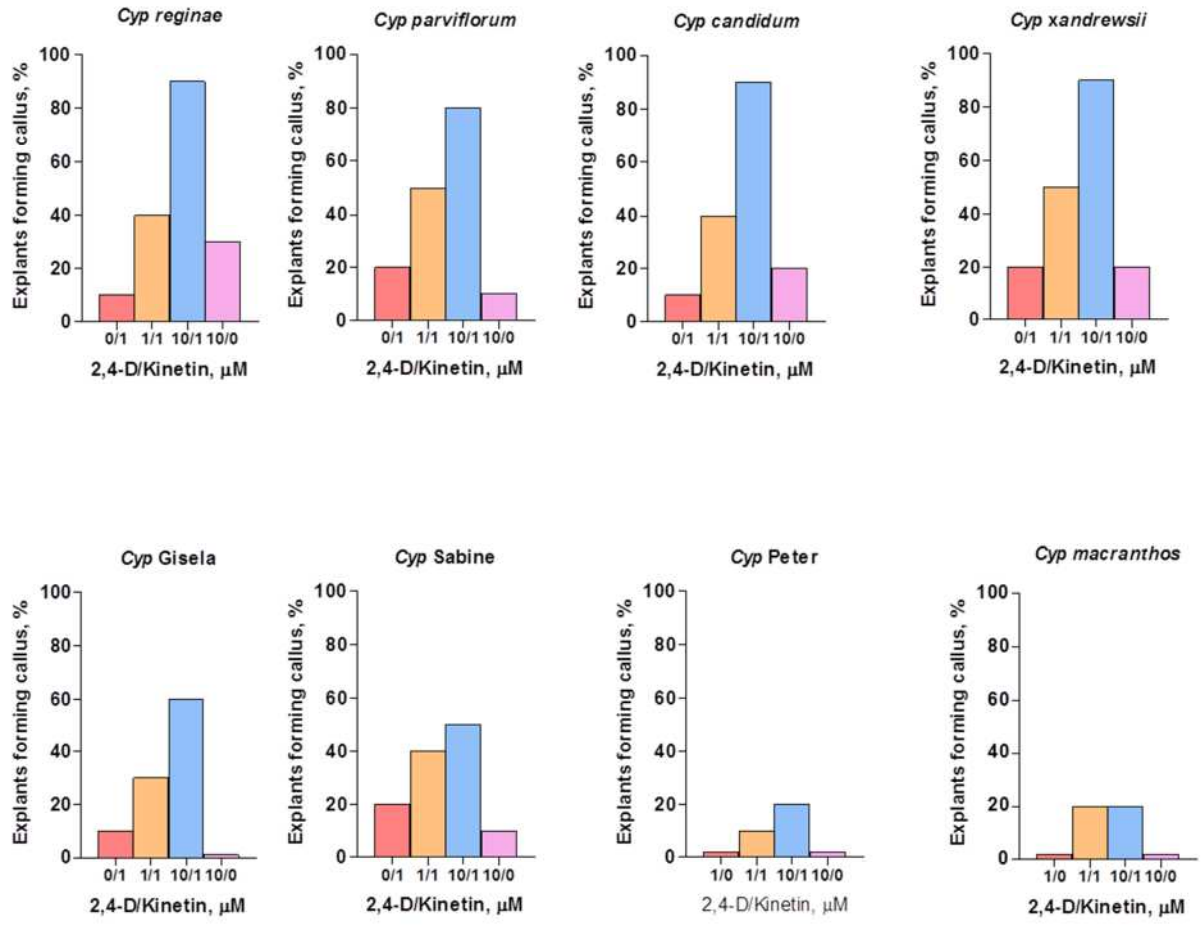

Figure 2. Percent of root tip explants (n=10, each group) forming calli in *Cypripedium* species and hybrids from different Sections.