In vitro germination and development of two endangered endemic Colombian orchids *Cattleya mendelii* and *Cattleya quadricolor*

Germinación y desarrollo *in vitro* de dos orquídeas amenazadas endémicas de Colombia, *Cattleya mendelii* y *Cattleya quadricolor*

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ABSTRACT

C*attleya mendelii* and *C. quadricolor* are endemic orchids from Colombia, which have been subjected to extraction from their natural environments for commercial purposes, becoming critically endangered. However, there is no sustainable management practice for the conservation of these species. The aim of this study was to establish a protocol of *in vitro* culture for both species. The effects of different combinations of GA_3 and NAA amending MS and KC culture media were assessed for the *in vitro* germination and plant growth. Plant development was assessed monthly over 120 days. A higher percentage of explant production for both species was observed with MS than KC. The higher GA_3 proportion of 1.5 µM resulted in enhanced germination, while the higher NAA concentration of 1.5 µM favored later stages of plant development. For instance, the number of roots and the length of roots and shoots were higher under incubation on MS that under KC for both species. *In vitro* germination for *C. mendelii* and for *C. quadricolor* was successful for their propagation and could be useful in future conservation programs for these species.

Keywords: Biotechnology, conservation, culture media, growth regulators, plant development.

RESUMEN

C*attleya mendelii* y *C. quadricolor* son dos orquídeas endémicas de Colombia que han sido sujetas a extracción de sus ambientes naturales para la venta, esto ha conducido a estas plantas a estar críticamente amenazadas. En la actualidad, no existe una práctica sustentable para el manejo de estas especies. El objetivo de este estudio fue establecer un protocolo de cultivo *in vitro* para ambas especies. El efecto de diferentes combinaciones de medios de cultivo MS y KC modificados con AG_3 y ANA fueron evaluados para la germinación y desarrollo *in vitro.* El desarrollo fue evaluado cada 30 días, durante 120 días. La mayor producción de explantes para ambas especies fue observada con MS. Mayor proporción de AG_3 de 1.5 µM resultó en el aumento de la germinación, asimismo mayores concentraciones de ANA de 1.5 µM favorecen los estadios tardíos del desarrollo vegetal *in vitro*. El número y la longitud de los brotes y las raíces fueron mayores cuando las plantas de ambas especies se cultivaron en medio MS. La germinación y desarrollo *in vitro* de *C. mendelii* and *C. quadricolor* en este estudio fue una aproximación exitosa para la propagación y puede ser considerada para programas de conservación de estas orquídeas.

Palabras clave: Biotecnología, conservación, desarrollo vegetal, medios de cultivo, reguladores del crecimiento vegetal.

INTRODUCTION

The genus *Cattleya* (Orchidaceae), native to Central and South America, occurs along mountain ranges, dry forests, and the transition to wet and cloudy hillsides and canyons, mainly on trees and rocks (Calderón 2007). C*attleya mendelii* Dombrain and *C. quadricolor* Lindl. are endemic to Colombia and have been traditionally kept in home gardens owing to their beautiful flowers. Additionally, the over-extraction from their habitat for sale at local and international markets has

led to an 80% reduction of their wild populations over the last century. As a result, these orchids have been recognized by the International Union for Conservation of Nature as a critically endangered species (Calderón 2007).

To date, there is no a sustainable management practice for these species of endangered orchids. In this respect, the *in vitro* propagation of vascular plants is a useful method that can be utilized for commercial or for conservation purposes as an alternative that might help in decreasing the extractive pressure on natural populations of endangered plants (Rubluo *et al*. 1993, Arditti & Krikorian 1996, Buyun *et al*. 2004, Lo *et al*. 2004, Santos-Hernández *et al*. 2005, Ávila-Díaz *et al*. 2009). However, the *in vitro* propagation of orchids requires a species-specific method for massive and rapid production of these plants (Arditti 1977, Colli & Kerbauy 1993, Shimura & Koda 2003).

Not only the form of nitrogen provided to the culture media but also the presence and concentration of some growth regulators can affect the rate of emergence and development of orchid seeds during *in vitro* culture (Ichihashi 1992, Chen *et al*. 2000, Park *et al*. 2002, Jawan *et al*. 2010). In consequence, medium optimization, which is a rapid approach for developing propagation protocols, can be useful for the massive production of these endangered *Cattleya* species. For these reasons, the aim of this study was to evaluate the effects of two culture media MS and KC, in combination with different concentrations of plant growth regulators NAA and GA_3 on *in vitro* germination and development of *Cattleya mendelii* and *C. quadricolor,* to establish a protocol for the *in vitro* propagation of both species to aid in their conservation.

MATERIALS AND METHODS

Plant material and establishment of *in vitro* culture

Closed capsules of *Cattleya mendelii* and *C. quadricolor* were obtained from the commercial nursery (Las Orquídeas, San Antonio del Tequendama, Colombia, 4º 34' 17" N, 74º 18' 45" W; 2000 masl), where the parental plants had been selected and cross pollinated by hand for this study. When the capsules started to turn yellow, they were harvested with sterile garden shears and transported to the *in vitro* culture laboratory of the Fundación Zoológico Santa Cruz, where they were kept during 1 day in the laminar flow hood until the start of the experiment.

While in the laminar flow hood, the capsules were disinfected by immersion in 90% ethanol (v/v) for five minutes, rinsed with sterile distilled water, and submerged in 5% sodium hypochlorite for 20 min, and rinsed with sterile distilled water. Each capsule was longitudinally dissected in a sterile Petri dish, where approximately half of the seeds were suspended in 70 mL of sterile distilled water. After shaking the seed suspension with a vortex, an aliquot of 1 mL, which contained ca. 50 seeds were placed uniformly in glass bottles containing the experimental culture medium.

Experimental treatments

Seed germination and seedling development were evaluated by planting the seeds in either KC (Knudson 1946) or MS (Murashige & Skoog 1962) culture media enriched with sucrose (30 g/L) and experimentally amended with various combinations of giberelic acid (GA_3) and naphthaleneacetic acid (NAA): 0/0; 0.5/1.5; 1.0/1.0; 1.5/0.5 µM respectively. For each experimental treatment, 25 mL of growth medium were added to a glass bottle of 120 mL in volume, in which the seeds were planted as described above.

The glass bottles were placed in a tissue culture room at 22 ± 2 ºC and a photoperiod of 12 h. Plant development was evaluated every 30 days and the number of individuals at the various stages were recorded as follows: germination (defined as the stage when the embryo emerges from the integument) at 30 days, protocorm-like bodies (the embryo is completely released from integument) at 60 days, shoots (the protocorm is differentiated) at 90 days, and seedlings (emergence of leaf blades, rhizomes) were recorded at 120 days.

After 120 days of culture the number of roots was counted and leaf and root length were measured with a vernier caliper (readable to 0.02 millimeters). The number of leaves was not counted because for these species the maximum number of leaves is fixed to one or two and does not respond to treatments at 120 days of *in vitro* culture (unpublished observations).

Data analysis

Data were analyzed under a factorial arrangement according to a fully randomized design. For each species, the stage of development and seedling growth were compared among medium and hormone treatment with two way ANOVAs followed by a *post hoc* Holm Sidak test (at $p < 0.05$). All analyses were conducted with Sigmastat 3.5 (Systat®, Richmond California, USA). Data are shown as means ± 1 S.E. for 10 glass bottles containing 50 seeds each that were prepared as described above.

RESULTS

Cattleya mendelii

For *C. mendelii* the MS medium promoted a higher seed germination than the KC medium (Table I; Fig. 1a). At 30 days of incubation, the highest germination rate of 95.2 ± 0.8% was observed for seeds grown on MS medium amended with 1.5 µM of GA_3 and 0.5 µM of NAA, while the lowest germination of 41.7 ± 1.2% was recorded for seeds incubated with no growth regulator enrichment in KC medium (Fig. 1a).

At 60 days of incubation, *C. mendelii* had the highest protocorm development when grown on MS than on KC (Table I; Fig. 1b). In particular, the highest survival at this stage of development, 94.6 ± 0.8%, was observed for plants growing in MS amended with 1.0 µM GA_3 and 1.0 µM NAA, while the lowest survival was recorded for plants incubated in KC without growth regulator enrichment.

At 90 days of incubation, a significant interaction was found between the medium and the growth regulator amendment for shoot yield of both orchids (Table I; Fig 1c). In particular, for *C. mendelii* the highest percentage of surviving plants with shoots was 97.1 ± 0.6% observed for individuals growing in MS amended with 0.5 µM GA_3 and 1.5 µM NAA.

At 120 days of incubation a greater seedling survival was observed for *C. mendelii* growing on MS than on KC (Table I; Fig. 1d). In particular, the highest seedling survival of 95.9 ± 0.9% was observed under incubation with MS amended with 0.5 µM GA_3 and 1.5 µM NAA, while the lowest survival of 60.3 ± 1.0% was recorded under incubation with KC with no growth regulator amendment.

Leaf length for *C. mendelii* was affected by the culture medium utilized (Table I; Fig. 2a). For individuals incubated in MS amended with 0.5 µM GA_3 and 1.5 µM NAA, a leaf length of 11.1 ± 0.5 mm and the presence of 4.1 ± 0.4 roots per plant with an average length of 9.6 ± 0.5 mm, were consistently higher than for plants incubated with other culture media (Fig 2b, c). Contrasting was the case of plants incubated with no hormonal enrichment in MS whose leaf length and root number and length only amounted to 5.0, 2.4, and 4.4 mm respectively.

Cattleya quadricolor

C. quadricolor showed significant differences on seed germination among the growth media (Table I; Fig. 1e). At 30 days of incubation, the highest germination of 84.3 ± 1.1% for seeds incubated in KC and 96.4 ± 0.6% in MS was observed for seeds incubated with 1.5 µM of GA_3 and 0.5 µM of NAA. The lowest germination of 54.2 ± 1.1% in KC and 59.3 ± 1.0% in MS medium was recorded for seeds incubated with no growth regulator amendment.

Statistical differences on protocorm survival were observed for *C. quadricolor* plants growing on either incubation medium (Table I; Fig. 1f). The highest protocorm survival of 95.7 ± 0.7% for plants grown in MS enriched with 0.5 µM GA_3 and 1.5 µM NAA. The lowest protocorm survival for *C. quadricolor* was recorded for plants grown on either medium without growth regulator amended.

The lowest yield of shoots for *C. mendelii* at 90 days of incubation was observed for plants grown in KC without growth regulators yielded 52.0 ± 0.8%. For *C. quadricolor*, the highest yield of 95.6 ± 1.1% was observed for plants incubated with MS amended with 0.5 µM GA_3 and 1.5 µM NAA. The lowest yield of shoots of 62.9 ± 1.1% was observed in MS amended with 1.5 µM GA_3 and 0.5 µM NAA.

Significant interaction was found between culture medium and growth regulator amendment for seedling survival of *C. quadricolor* (Table I; Fig 1h). The highest seedling survival of 94.4 ± 1.0% was recorded for plants incubated in MS enriched with 0.5 µM GA_3 and 1.5 µM NAA, while the lowest seedling survival of 64.5 ± 3.5% was observed for plants incubated in KC with no growth regulator amendment.

Seedling growth for this species was enhanced by the incubation in MS (Table I). Leaf length ranged from 5.1 ± 0.41 mm for plants incubated in either medium without hormonal amended to 10.2 ± 0.4 mm for seedlings grown in MS amended with 0.5 µM GA_3 and 1.5 µM NAA (Fig. 2d). The highest count of 4.2 ± 0.3 roots per plant was found for plants incubated with MS amended with 0.5 µM GA_3 and 1.5 µM NAA and KC amended with 1.0 µM GA_3 and 1.0 µM NAA (Fig. 2e). The lowest root number of 2.0 ± 0.3 was found for plants incubated in MS medium without hormonal amendment. Root length for seedlings of *C. quadricolor* averaged 8.9 ± 0.3 mm in KC and 9.2 ± 0.4 mm in MS medium with hormonal amended (Fig. 2f). It decreased by 33.7% for seedlings incubated in MS medium without hormonal amendment and by 51.7% for seedlings incubated in KC medium without hormonal amended.

DISCUSSION

While a high germination percentage was observed for both *Cattleya mendelii* and *C. quadricolor* under all treatments, MS was the best medium for this and all subsequent developmental stages. For germination, MS was best in combination with 1.5 µM GA_3 and 0.5 µM NAA, while for the remaining stages MS amended with 0.5 µM GA_3 and 1.5 µM NAA yielded higher survival. An exception was protocorm development for *C. mendelii,* whose largest number was observed for plants incubated in 1.0 µM GA_3 and 1.0 µM NAA in MS medium. Additionally, KC and MS were equally effective for leaf and root growth, as well as for root number of *C. mendelii* when amended with 0.5 µM GA_3 and 1.5 µM NAA for *C. quadricolor.* KC and MS media amended with 0.5 µM GA_3 and 1.5 µM NAA, showed differences in root number and leaf length.

Ammonium salts yield better *in vitro* growth than nitrates (Raghavan & Torrey 1964, Hew & Yong 2004). Seeds respond directly to the concentration of ammonium nitrate in the medium. For instance, when seeds germinate in a medium lacking NH_4, small protocorms are formed and further growth is suppressed (Raghavan & Torrey 1964, Kauth *et al.* 2008). Here, MS was a better culture medium for both species of *Cattleya* because its composition that includes ammonium nitrate provides the same proportion

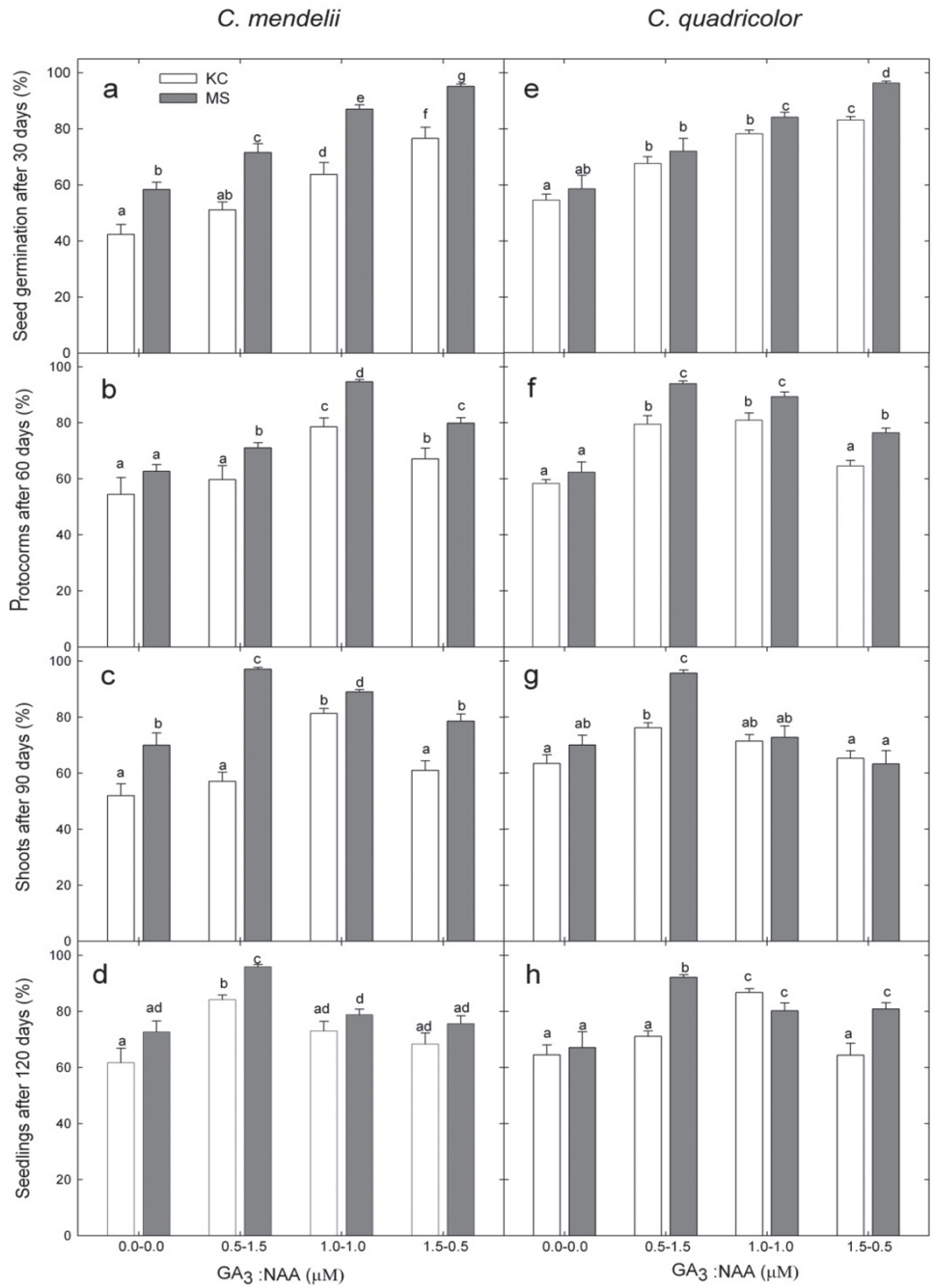

Figure 1. Survival for *Cattleya mendelii* (a-d) and *C. quadricolor* (e-h) at various developmental stages growing on KC (Knudson 1946; white bars) and MS (Murashige & Skoog 1962; grey bars) amended with various concentrations of GA_3 and NAA. Seeds that germinated (a, e) were counted at 30 days of incubation, while protocorms (b, f), were counted at 60 days of incubation. Shoots (c, g) were counted at 90 days of incubation and the ensuing seedlings (d, h) at 120 days of culture. Data are shown as mean ± S.E. (n = 10 glass bottles containing ca. 50 seeds each). For each panel, different letters indicate a statistical difference ($p < 0.05$) from Holm-Sidak test following 2-way ANOVAs.

Figura 1. Sobrevivencia de *Cattleya mendelii* (a-d) y *C. quadricolor* (e-h) en distintas etapas del desarrollo incubadas en los medios KC (Knudson 1946; barras blancas) y MS (Murashige & Skoog 1962; barras grises) enriquecidos con varias concentraciones de GA3 y NAA. Las semillas que germinaron (a, e) se contaron a los 30 días de incubación, mientras que los protocormos (b, f) se contaron a los 60 días de incubación. Los brotes (c, g) se contaron a los 90 días de incubación y las plántulas subsecuentes (d, h) a los 120 días de incubación. Se muestran los datos como promedio ± E.E. (n = 10 frascos de cultivo con ca. 50 semillas cada uno). Para cada panel, letras distintas indican diferencias estadísticas ($p < 0.05$) de un ANOVA de 2 vías seguido de una prueba de Holm-Sidak.

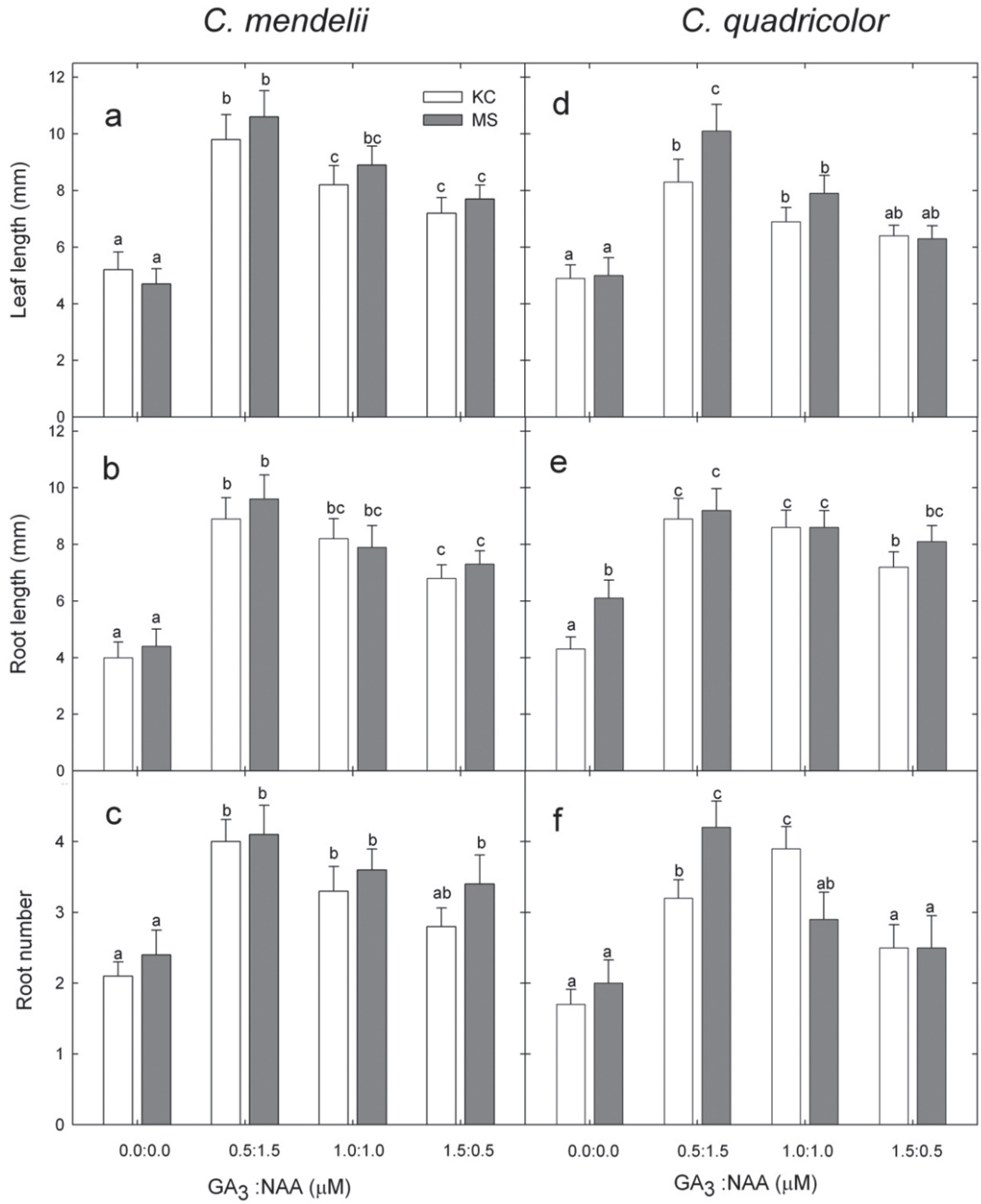

Figure 2. Seedling growth for *C. mendelii* (a-c) and *C. quadricolor* (d-f). Leaf length (a, d), root number (b, e) and root length (c, f) were determined at 120 days of incubation for seedlings growing KC (Knudson 1946; white bars) and MS (Murashige & Skoog 1962; grey bars) enriched with various concentrations of GA_3 and NAA. Data are shown as means ± S.E. (n = 10 glass bottles containing ca. 50 seeds each). For each panel, different letters indicate a statistical difference ($p < 0.05$) from Holm-Sidak test following 2-way ANOVAs.

Figura 2. Crecimiento de plántulas de *C. mendelii* (a-c) y *C. quadricolor* (d-f). La longitud de la hoja (a, d), número (b, e) y longitud (c, f) de raíces se determinaron a los 120 días de incubación para plántulas creciendo en los medios KC (Knudson 1946; barras blancas) y MS (Murashige & Skoog 1962; barras grises) enriquecidos con distintas concentraciones de GA_3 y NAA. Los datos se muestran como promedio ± E.E. (n = 10 frascos de cultivo con ca. 50 semillas cada uno). Para cada panel, letras distintas indican diferencias estadísticas ($p < 0.05$) de un ANOVA de dos vías seguido de una prueba de Holm-Sidak.

TABLE I. Two way ANOVAs of seedling growth and plant development for *Cattleya mendelii* and *C. quadricolor*.

TABLA I. ANOVA de dos vías del crecimiento de plántulas y desarrollo de *Cattleya mendelii* y *C. quadricolor*.

Cattleya mendelii

	SEED GERMINATION			PROTOCORMS			SHOOTS			SEEDLINGS			LEAF LENGTH			ROOT LENGTH			ROOT NUMBER		
	df	F	P	*df*	F	P	*df*	F	P	*df*	F	P	*df*	F	P	*df*	F	P	*df*	F	P
Medium	1	83.2	<0.001	1	23.8	<0.001	1	103	<0.001	1	14.9	<0.001	1	1.36	0.24	1	0.88	0.35	1	2.03	0.15
Growth regulator	3	52.7	<0.001	3	23.5	<0.001	3	23.8	<0.001	3	18.2	<0.001	3	47.2	<0.001	3	38.9	<0.001	3	10.9	<0.001
Medium × regulator	3	0.50	0.68	3	0.42	0.73	3	10.7	<0.001	3	0.38	0.76	3	0.86	0.86	3	0.39	0.75	3	0.20	0.89

Cattleya quadricolor

	SEED GERMINATION			PROTOCORMS			SHOOTS			SEEDLINGS			LEAF LENGTH			ROOT LENGTH			ROOT NUMBER		
	df	F	P	*df*	F	P	*df*	F	P	*df*	F	P	*df*	F	P	*df*	F	P	*df*	F	P
Medium	1	12.7	<0.001	1	37.3	<0.001	1	8.33	0.005	1	13.9	<0.001	1	4.89	0.03	1	8.36	0.05	1	0.10	0.751
Growth regulator	3	55.4	<0.001	3	62.2	<0.001	3	19.2	<0.001	3	13.2	<0.001	3	32.0	<0.001	3	43.9	<0.001	3	12.8	<0.001
Medium × regulator	3	1.22	0.30	3	2.04	0.11	3	4.62	0.005	3	7.21	<0.001	3	1.91	0.13	3	2.34	0.08	3	3.09	0.03

of ammonium and nitrate to seeds (Murashige & Skoog 1962). In contrast, the KC medium has a higher proportion of nitrate than ammonium (Knudson 1946). In consequence, considering that nitrogen uptake by roots is primarily mediated by the enzyme nitrate reductase, which assimilates nitrates the plants should have responded better to KC. However, the enzyme nitrate reductase is not expressed in orchid tissues until 60 days of *in vitro* germination making the MS medium better than KC medium for plants in early stages of development (Hew & Yong 2004).

Higher GA_3 to NAA ratios improved germination for both species incubated in either medium, because the direct effect on *in vitro* germination by the different GA_3 concentrations (Santos-Hernández *et al*. 2005). This phytohormone activates the α-amylase that mediates sugar absorption from the culture medium (Held & Piechulla, 2010). Thus an ammonium enriched medium such as MS enriched with high concentrations of GA_3 can yield very high germination responses as was the case in present work.

The later developmental stages (protocorms, shoots and seedlings) for both orchids, was generally enhanced by a higher proportion of NAA to GA_3. This is similar to the developmental responses of *Dendrobium* species, for which the number of shoots increases in response to the concentration of NAA (Parvin *et al.* 2009), and for *Grammatophyllum speciosum* cultivated in a medium enriched with NAA (Sopalun *et al.* 2010).

A higher proportion of NAA than GA_3 produced more and longer roots and longer leaves for both species grown in MS. For another Neotropical orchid, *Laelia speciosa*, GA_3 produces longer seedlings (Ávila-Díaz *et al.* 2009). Here, high concentrations of GA_3 resulted in shorter leaves and roots than those of plants incubated in medium without this phytohormone.

CONCLUSIONS

The combination of growth regulators and culture media utilized for the *in vitro* production of *Cattleya mendelii* and *C. quadricolor* from seeds was highly successful in this study, yielding vast number of seedlings of these endangered Colombian orchids. This method can be useful for producing plants for possible reintroductions into natural habitats and for commercial purposes in different markets, in order to decrease the extractive pressure facing natural populations.

ACKNOWLEDGMENTS

The authors are thankful to Fundación Zoológico Santacruz for technical and financial support.

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Recibido:03.03.15
Aceptado: 03.09.15