Effects of different combinations of growth regulators for bud induction from seedlings of *Cattleya walkeriana* Gardner (Orchidaceae)

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ABSTRACT. The present study reports a research with different concentrations and combinations of auxins and cytokinins to activate bud development and to obtain multiplication of *Cattleya walkeriana* (Orchidaceae) without an intermediate callus stage. Seedlings of *C. walkeriana* were cultured on B5 medium containing 2% sucrose and solidified with 0.65% agar, with factorial combinations of the auxins 1-naphthaleneacetic acid (NAA), 3-indolbutyric acid (IBA), and cytokinins 6-benzylaminopurine (BA) and N-(2-furanyl-methyl)-1-purine-6 amine (Kinetin) at concentrations of 0.0, 0.5, 1.0, and 1.5 mg/L. The highest number of buds with elongated leaves occurred by cultivating seedlings on medium containing different concentrations of IBA and BA, while the lowest number was observed in NAA and KIN combination. IBA alone yielded better results at the three tested concentrations, when compared to the total absence of growth regulators. The higher frequency of bud induction using BA and IBA combination indicated that this combinations were more effective for *C. walkeriana in vitro* multiplication using seedlings as explants.

Key words: orchid, micropropagation, *Cattleya walkeriana*, growth regulators.

RESUMO. Efeito de combinações diferentes de reguladores de crescimento a partir da indução de botões de plântulas de *Cattleya walkeriana* Gardner (Orchidaceae). No presente estudo, foram investigadas diferentes combinações e concentrações de auxinas e citocininas para ativar o desenvolvimento de brotos e multiplicar plântulas de *Cattleya walkeriana* (Orchidaceae), sem estágio intermediário de calos. As plântulas de *C. walkeriana* foram cultivadas em meio B5, contendo 2% de sacarose, 0,65% de agar e combinações fatoriais das auxinas ácido naftalenoacético (NAA) e ácido 3-indolbutírico (IBA), e das citocininas 6-benzilaminopurina (BA) e cinetina (KIN), nas concentrações de 0,0; 0,5; 1,0 e 1,5mg/L. O maior número de brotos com folhas desenvolvidas foi formado nas plântulas cultivadas em meio contendo IBA e BA, enquanto o menor número de brotos foi observado na combinação NAA e KIN. Houve maior indução de brotos em meio contendo somente IBA (na ausência de citocininas) nas três concentrações. A maior freqüência de indução de brotos usando as combinações de BA e IBA indicaram que essa combinação foi mais efetiva para a multiplicação *in vitro* de *C. walkeriana,* usando plântulas como explantes.

Palavras-chave: orquídeas, micropropagação, *Cattleya walkeriana*, reguladores de crescimento.

Introduction

Cattleya walkeriana Gardner (Orchidaceae) is a tropical epiphytic orchid native to the central region of Brazil. It occurs currently in the states of *Goias, Mato Grosso, Minas Gerais* and *São Paulo*. It has been cultivated for commercial production since 1970 (Rao, 1977) and it is considered an endangered species (Alves, 1991). The factors contributing to its decline are: habitat destruction by human activities and commercial preference for its beautiful and colorful flowers.

Little is known about the specific requirements for asymbiotic germination or *in vitro* maintenance and multiplication of *C. walkeriana*, including the possible role of growth regulators in this process. The seedlings growing is often a slow process, due to the large time periods required for adult plants

formation. In recent years, certain requirements for asymbiotic germination have become apparent (Silva, 2000) and the effect of light radiation is important for shoot and root development and seedling growth (Islam *et al.*, 1999). However, to date, there is no report on seedlings *in vitro* multiplication using growth regulators.

The use of growth regulators has been reported for propagating other *Cattleya* genus species. *In vitro* multiplication of *C. aurantiaca* through shoot tip meristems culturing has been reported by Mauro *et al.* (1994), and root meristem culture has been used for multiplication of a *Cattleya* hybrid, i.e. *C. nobilior* Rchb. F. × *C. loddigesii* Ldl (Kerbauy, 1991). The present study reports the effects of different combinations of auxins and cytokinins for bud induction from *C. walkeriana* seedlings.

Material and methods

Seedlings (1.0-2.0 mm) with only one unexpanded leaf of *C. walkeriana* were used as explants. The seeds were *in vitro* germinated on Knudson-C medium (Knudson, 1946), modified by the addition of a micronutrient solution (Arditti *et al.*, 1982), 20% coconut water, 1 g/L peptone, and 0.1 mg/L NAA (Silva, 2000).

The seedlings were placed in flasks (10 cm height x 4 cm diameter) containing 15 mL of B5 macro- and micro-salts, vitamins (Gamborg *et al.*, 1968), and 2% sucrose, solidified with 0.65% agar, and the following combinations of the auxins 1-naphthaleneacetic acid (NAA), 3-indolbutyric acid (IBA), and the cytokinins 6-benzylaminopurine (BA) and N-(2-furanyl-methyl)-1-purine-6 amine (Kinetin); four experiments (BA and IBA, BA and NAA, Kinetin and IBA, and Kinetin and NAA) were replicated three times at each factorial combination, i.e., 0.0, 0.5, 1.0, and 1.5 mg/L (BA 1.0 mg/L = 4.40 μM; Kinetin 1.0 mg/L = 4.60 μM; NAA 1.0 mg/L = 5.37 μM; IBA 1.0 mg/L = 4.92 μM). Six explants (i.e. seedlings) were used per flask.

After the inoculation, cultures were incubated in a growth chamber (Fanen, mod 347 G) at 25 ± 1°C under 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by light radiation with cold-white fluorescent lights (PPF), under a 16-h photoperiod.

The average number of buds induced per explant was recorded for each auxin and cytokinin combination after 90 days of culture. Differences among treatments were tested by one-way analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparisons Test (The SAS System Copyright © 1999-2000 by SAS Institute Inc., Cary, NC, USA).

After 8 months, rooted seedlings were removed, freed of agar by washing in running tap water and planted in a vermiculite and fern fiber compost mixture (1:1). The pots were covered with polyethylene hoods, to prevent desiccation and to allow acclimatization. Room temperature was 26 ± 2ºC.

Results and discussion

Multiple bud formation from seedlings of *C. walkeriana* showed significant differences when the explants growing on medium with different types and combinations of growth regulators were compared (Table 1). The highest number of buds with expanded leaves occurred on media containing different concentrations of IBA and BA (mean = 2.0173) and the lowest number of buds was observed on NAA and KIN combination (mean = 1.2638). The effects of IBA x KIN (mean = 1.7673) and NAA x BA (mean = 1.6770) combinations on multiple bud induction are not significantly different when submitted to Tukey's Studentized Range Test.

Table 1. Variance analysis for multiple bud induction in *Cattleya walkeriana* seedlings cultured for 90 days in NAA x KIN, IBA x KIN, NAA x BA, and IBA x BA combinations and concentrations (0.0, 0.5, 1.0, and 1.5 mg/L), in a fully randomized design with three replicates and six seedlings per pot

Source of Variation	d.f.	Sum of Squares	Mean Square	*F* values
Hormone Concentration	15	46.2074	3.0804	4.68**
Hormone Combination	3	84.8428	28.2809	42.98**
Conc. X Comb.	45	107.0876	2.3797	3.62**
Error	1088	715.9444	0.6580	
CV (%)	48.24%			

** Significant at the 1% level of probability, *F* test

A differential effect of IBA at 1.0 and 1.5 mg/L was observed in cytokinin absence (Table 2). IBA alone produced better results at all concentrations, in comparison to total absence of growth regulators (controls), but NAA alone showed no significant effects on multiple bud production (Table 2).

In *Cymbidium sp*, another orchid genus, shoot formation was also suppressed when NAA was used (Begum *et al.*, 1994).

In *C. aurantiaca*, BA at 10.0 mg/L and NAA at 0.1 mg/L were used for shoot tip multiplication, and a medium supplemented with 10 mg/L NAA was used for development of plantlets with increased leaf and root biomass (Mauro *et al.*, 1994). BA and NAA combinations have been reported at different concentrations for shoot multiplication of others orchid species (George and Ravishankor, 1997). However, *in vitro* multiplication of orchids has been more frequently achieved using protocorms or

protocorms sections and embryogenic callus induction.

The higher frequency of bud induction using the BA and IBA combination (Table 2) indicated that this combination was more effective for *in vitro* multiplication of *C. walkeriana* using seedlings as explants. Roots and leaves development occurred in all explants, and although not reported in the present study, a differential effect of IBA at 1.0 or 1.5 mg/L concentration was evident for root and leaf growth and for pseudobulb development (Figure 1).

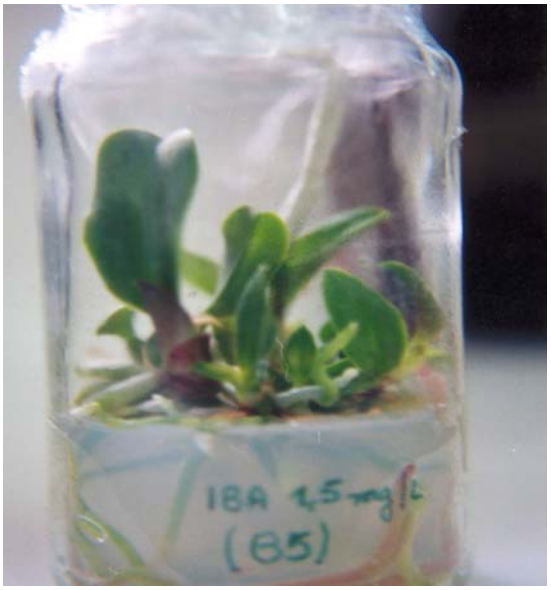

Figure 1. Roots and leaves development from multiple buds induced in *Cattleya walkeriana* seedlings, cultured 24 weeks on B5 medium, containing 1.5 mg/L IBA and no cytokinin (1.5 ×)

Continued growth after transplantation of *C. walkeriana* plants obtained from explants cultured in medium containing 1.0 or 1.5 mg/L IBA and no cytokinins yielded 12-month-old plants morphologically similar to adult plants (Figure 2), since a prominent pseudobulb is characteristic of adult plants.

Figure 2. *Cattleya walkeriana* plant (12 month-year old) developed from seedlings cultured with 1.5 mg/L IBA showing developed roots, leaves and pseudobulb growth, after *ex vitro* acclimation (Bar = 2 cm)

Table 2. *F* values and mean numbers of bud induction in *Cattleya walkeriana* seedlings at different treatments (T) with auxin (NAA and IBA) and cytokinin (KIN and BA) combinations in a fully randomized design, with three replicates and six seedlings per pot. Means followed by the same letter are not different by the Tukey's studentized range test

		NAA× KIN	IBA× KIN	NAA× 6-BA	IBA× 6-BA
F values		5.97**	5.97**	2.80**	3.59**
CV (%)		48.10%	44.07 %	60.85 %	41.10 %
Means	T1 (0.0 × 0.0)	1.0556 ± 0.1277^{e}	1.0556 ± 0.1836^{d}	1.0556 ± 0.2405^{c}	1.0556 ± 0.1958^{c}
	T2 (0.5 × 0.0	$1.2778 \pm 0.1277^{a,b,c}$	$1.9444 \pm 0.1836^{a,b,c,d}$	$1.3333 \pm 0.2405^{a,b,c}$	$1.9444 \pm 0.1958^{a,b,c}$
	T3 (1.0 × 0.0)	1.0556 ± 0.1277^{e}	$2.5556 \pm 0.1836^{a,b}$	$1.1667 \pm 0.2405^{b,c}$	$2.5556 \pm 0.1958^{a,b}$
	T4 (1.5 × 0.0)	1.0000 ± 0.1277^{e}	2.7778 ± 0.1836^{a}	1.0556 ± 0.2405^{c}	2.7778 ± 0.1958^{a}
	T5 (0.0 × 0.5)	$1.4444 \pm 0.1277^{a,b,c}$	$1.4444 \pm 0.1836^{c,d}$	$1.6667 \pm 0.2405^{a,b,c}$	$1.7222 \pm 0.1958^{b,c}$
	T6 (0.5 × 0.5)	1.0556 ± 0.1277^{e}	$1.6111 \pm 0.1836^{c,d}$	$1.8333 \pm 0.2405^{a,b,c}$	$2.2778 \pm 0.1958^{a,b}$
	T7 (1.0 × 0.5)	1.1111 ± 0.1277^{e}	$1.2222 \pm 0.1836^{c,d}$	$1.6667 \pm 0.2405^{a,b,c}$	$2.2222 \pm 0.1958^{a,b}$
	T8 (1.5 × 0.5)	$1.2778 \pm 0.1277^{a,b,c}$	$1.4444 \pm 0.1836^{c,d}$	$1.8889 \pm 0.2405^{a,b,c}$	$2.0000 \pm 0.1958^{a,b,c}$
	T9 (0.0 × 1.0)	$1.7778 \pm 0.1277^{a,b}$	$1.7222 \pm 0.1836^{b,c,d}$	$1.8333 \pm 0.2405^{a,b,c}$	$1.8333 \pm 0.1958^{a,b,c}$
	T10 (0.5 × 1.0)	$1.5000 \pm 0.1277^{a,b,c}$	$1.3333 \pm 0.1836^{c,d}$	$1.5000 \pm 0.2405^{a,b,c}$	$1.9444 \pm 0.1958^{a,b,c}$
	T11 (1.0 × 1.0)	$1.3333 \pm 0.1277^{a,b,c}$	$1.8333 \pm 0.1836^{b,c,d}$	2.4444 ± 0.2405^{a}	$2.1111 \pm 0.1958^{a,b}$
	T12 (1.5 × 1.0)	1.0556 ± 0.1277^{e}	$1.7222 \pm 0.1836^{b,c,d}$	$2.3333 \pm 0.2405^{a,b}$	$2.0000 \pm 0.1958^{a,b,c}$
	T13 (0.0 × 1.5)	1.8333 ± 0.1277^{a}	$1.8333 \pm 0.1836^{b,c,d}$	$1.8889 \pm 0.2405^{a,b,c}$	$1.8889 \pm 0.1958^{a,b,c}$
	T14 (0.5 × 1.5)	1.0556 ± 0.1277^{e}	$1.7778 \pm 0.1836^{b,c,d}$	$1.6667 \pm 0.2405^{a,b,c}$	$1.9444 \pm 0.1958^{a,b,c}$
	T15 (1.0 × 1.5)	$1.2222 \pm 0.1277^{a,b,c}$	$2.0000 \pm 0.1836^{a,b,c}$	$1.9444 \pm 0.2405^{a,b,c}$	$2.0000 \pm 0.1958^{a,b,c}$
	T16 (1.5 × 1.5)	$1.1667 \pm 0.1277^{b,c}$	$2.0000 \pm 0.1836^{a,b,c}$	$1.5556 \pm 0.2405^{a,b,c}$	$2.0000 \pm 0.1958^{a,b,c}$

** Significant at the 1% level of probability, *F* test

The use of IBA as an auxin for orchids *in vitro* culture has been reported only for a hybrid of *Cattleya* using root meristems as explants (Kerbauy, 1991). In *C. walkeriana*, a medium supplemented with IBA is important for the development of plants with increased root elongation and leaf expansion, and for pseudobulb induction.

Small and not significant callus proliferation was observed on the basal surfaces of only 1 or 2 of the explants cultured in media containing 1.0 and 1.5 mg/L IBA without Kinetin, 1.5 mg/L NAA and 0.5 and 1.0 mg/L Kinetin, and 1.5 mg/L IBA and 1.0 mg/L BA. Although callus production may represent a loss of shoot potential proliferation (Scowcroft and Ryan, 1986), the leaves and pseudobulbs of *C. walkeriana* were frequently formed on medium with higher auxin content. This is very important, since regenerants from callus tissues are not desirable in clonal propagation, due to the potential for somaclonal variation (Larking and Scowcroft, 1981). Thus, we trust that the results of the present study may facilitate future researches on growth regulator requirements for *in vitro* culture of *C. walkeriana* species.

References

ALVES, R.J.V. *Guia de campo das orquídeas da Serra de São José*. Brazil, 1991.

ARDITTI, J. *et al* . Orchid seed germination and seedling culture - a manual. *In*: ARDITTI, J. (Ed.). *Orchid biology*: reviews and perspectives II. New York: Cornell University Press, 1982. p. 243-270.

BEGUN, A.A. *et al*. Formation of protocorm-like bodies (PLB) and shoot development through *in vitro* culture of *Cymbidium* PLB. *J. Jpn. Soc. Hort. Sci.*, Tokyo, v. 63, p. 663-673, 1994.

GAMBORG, O.L. *et al*. Nutrient requirements of suspension cultures of soybean root cell. *Exp. Cell. Res.*, Orlando, v. 50, p. 151-158, 1968.

GEORGE, P.S., RAVISHANKAR, G.A. *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. *Plant Cell Rep.*, Berlin, v. 16, p. 490-494, 1997.

ISLAM, M. O. *et al*. Effects of quality on seed germination and seedling growth of *Cattleya* orchids in vitro. *J. Jpn. Soc. Hort. Sci*., Tokyo, v. 68, p. 1132-1138, 1999.

KERBAUY, G.B. *In vitro* conversion of *Cattleya* root tip cells into protocorm-like bodies. *J. Plant. Physiol.*, Jena, v. 138, p. 248-251, 1991.

KNUDSON, L. A new nutrient solution for the germination of orchid seed. *Am. Orchid Soc. Bull.*, West Palm Beach, v. 15, p. 214-217, 1946.

LARKING, P.J., SCROWCROFT, W.R. Somaclonal variation – a novel source of variability from cell cultures. *Theor. Apll. Genet.*, Berlin, v. 60, p. 197-214, 1981.

MAURO, M. *et al*. Influence of benzylaminopurine and alpha-naphthaleneacetic acid on multiplication and biomass production of *Cattleya auratiaca* shoot explants. *Lindleyana*, West Palm Beach, v. 9, p. 169-173, 1994.

RAO, N.A. Tissue culture in the orchid industry. *In*: RENERT, J.; BAJAJ, Y.P.S. (Ed.). *Applied and fundamental aspects of plant cell, tissue and organ culture*. Berlin: Springer Verlag, p. 44-69, 1977.

SCROWCROFT, W.R., RYAN, S.A. Tissue culture and plant breeding. *In*: YEOMAN, M.M. (Ed.). *Plant culture technology*. Oxford: Blackwell Scientific Publications, p. 67-94, 1986.

SILVA, C.I. *Aspectos reprodutivos, morfo-anatômicos, da germinação das sementes e do desenvolvimento inicial in vitro* de *Cattleya walkeriana* Gardner (Orchidaceae). 2000. (Monografia) - Universidade de Franca, Franca, 2000.

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