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ALTERATIONS TO PLBS AND PLANTLETS OF HYBRID *CYMBIDIUM* (ORCHIDACEAE) IN RESPONSE TO PLANT GROWTH REGULATORS

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Abstract: A previous study examined, in detail, the morphological response of hybrid *Cymbidium* Twilight Moon 'Day Light' protocorm-like bodies (PLBs) to 26 plant growth regulators (PGRs). In this study, flow cytometric analyses of the PLBs derived from several of these PGR treatments revealed changes in the ploidy of PLBs while the ploidy of plant leaves remained constant. The SPAD value of leaves of plants derived from PGR treatments changed significantly. The choice of PGR must be accompanied by careful scrutiny of the possible resulting changes to morphology and physiological parameters.

Keywords: flow cytometry; plant growth regulator; PLB; SPAD; Teixeira *Cymbidium* (TC) medium; thin cell layer

Introduction

Cymbidium (Orchidaceae) is a *de facto* model orchid genus in terms of *in vitro* development [HOSSAIN & al. 2013; TEIXEIRA DA SILVA, 2013a, 2013b]. The protocorm-like body (PLB) is an effective propagule for *in vitro* clonal micropropagation and is a *de facto* somatic embryo [TEIXEIRA DA SILVA & TANAKA, 2006). A previous study examined the effect of 26 plant growth regulators (PGRs) on the resulting production of new PLBs, including the development of new PLBs, or *neo*-PLBs, their fresh weight, with darkness generally having a more negative effect than light [TEIXEIRA DA SILVA, 2014a]. Using that study's experimental protocol, PLBs derived from treatment of 26 PGRs. In addition, the SPAD value of the leaves of plantlets derived from these treatments was measured, with the objective of understanding whether treatment with PGRs has any downstream effects on plant growth and development.

Materials and methods

Basal protocols, reagents, plant material

All basic protocols for the establishment of *in vitro* cultures, PLB induction and proliferation, and *neo*-PLB induction followed, broadly, [TEIXEIRA DA SILVA & al. 2005, 2006a, 2006b] for hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan). Teixeira *Cymbidium* (TC) medium 1 [TEIXEIRA DA SILVA, 2012a] was the basal medium used in this study, supplemented with 0.1 mg/l NAA, 0.1 mg/l Kin, 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA) after adjusting pH to 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. As indicated

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in TEIXEIRA DA SILVA (2014a), all chemicals and reagents, including PGRs, were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless specified otherwise. PLBs were kept on 40 ml medium in 100-ml Erlenmeyer flasks (10 PLBs/flask), double-capped with aluminium foil, and cultures were placed at 25 °C, under a 16-h photoperiod with a light intensity of 45 μmol/m^2/s provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan).

Plant growth regulator-derived treatments

Three groups of PGRs from a previous study [TEIXEIRA DA SILVA, 2014a], or from other studies referenced within that study, that induced some form of a growth response *in vitro*, in terms of PLBs, were employed (see abbreviations list and Tab. 1 footer for full definition) (all at 1 mg/l): 6 cytokinins (Ads; BA; 2iP; Kin; TDZ; *m*T), 6 auxins (2,4-D; IBA; NAA; picloram; TRIA; PG) and 6 other growth substances (ABA; dicamba; GA_3; JA; MeJA; SA). The control was PGR-free TC medium (i.e., excluding NAA and Kin).

Growth parameters assessed

Neo-PLBs that were 60 days old, prior to the development of shoot tips [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013], were used. Ploidy was determined in 6-day-old PLBs, as described next, and based on TEIXEIRA DA SILVA & TANAKA (2006) and TEIXEIRA DA SILVA & DOBRÁNSZKI (2014). Shoots that formed from PLBs were rooted individually on PGR-free Hyponex medium solidified with 7 g/l agar, as described in TEIXEIRA DA SILVA & al. (2006a, 2006b) and TEIXEIRA DA SILVA & TANAKA (2006). Well-rooted plantlets (i.e., shoots that had developed a robust root system) and had developed at least 6 fully developed leaves, were used to assess SPAD readings (chlorophyll content), based on TEIXEIRA DA SILVA & al. (2007) and TEIXEIRA DA SILVA & DOBRÁNSZKI (2014), in the third leaf (counting downward from the apex), with a chlorophyll meter (SPAD-502, Minolta, Japan).

Flow cytometry

PLBs or the leaves of plantlets (0.5 cm^2 of fresh material) were chopped in a few drops of nucleic acid extraction buffer (Partec Cystain UV Precise P, Germany) to isolate nuclei, then left to digest on ice for 5 min. Based on the protocol suggested by Mishiba and Mii (2000), the nuclear suspension was then filtered through a 30 μm mesh size nylon filter (CellTrics®) and five volumes of Partec Buffer A (2 μg/ml 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton X-100, pH 7.5). After leaving this mixture at room temperature for 5 min, nuclear fluorescence was measured using a Partec® Ploidy Analyser with diploid barley (*Hordeum vulgare* L.) cv. 'Ryufu' serving as the internal control. Each biological sample (PLBs, leaves) was repeated in triplicate, and relative fluorescence intensity of the nuclei was analysed (coefficient of variation between samples $< 3\%$; minimum of 5000 nuclei for each sample).

Statistical analyses

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate. Data was subjected to analysis of variance with mean separation by Tukey's multiple range

test using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

Results and discussion

Visually, PLBs formed under control conditions (Fig. 1A) or after exposure to BA (Fig. 1B) do not reveal visible differences. However, flow cytometry of *neo*-PLBs indicates that TDZ, mT, 2,4-D, IBA, picloram, TRIA, PG, ABA, GA_3, and MeJa induced some level of endopolyploidy (Tab. 1; Fig. 2), which is not unusual for *Cymbidium* [FUKAI & al. 2002; TEIXEIRA DA SILVA, 2014b] and other orchids [TEIXEIRA DA SILVA & al. 2014], even under control growth conditions, and is usually associated with the external layer of cells in the PLB [TEIXEIRA DA SILVA & TANAKA, 2006]. However, the leaf tissue of plantlets derived from PLBs, once placed on ideal regeneration medium, displays no polyploidy ([TEIXEIRA DA SILVA & TANAKA, 2006]; this study, data not shown), suggesting that endopolyploidy may be an evolutionarily disadvantageous genetic system that is naturally selected against. The chlorophyll content, and thus photosynthetic ability of plantlets, that were derived from different PGR treatments (2iP, TDZ, 2,4-D, picloram, ABA, dicamba, JA, and MeJA) showed a significant decrease (relative to the control) in SPAD value. Although *neo*-PLBs may appear morphologically similar after exposure to a PGR treatment, this study indicates the importance of testing the resulting *neo*-PLBs cytologically, and also the physiological performance of plants.

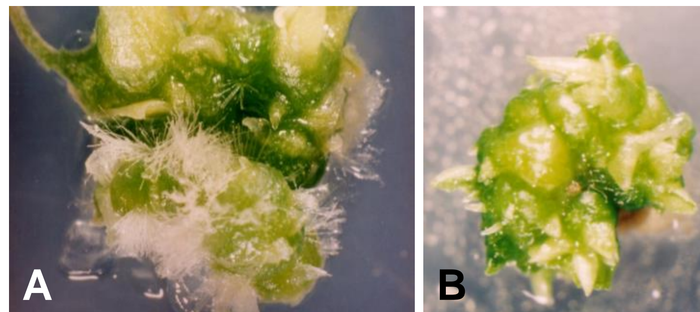

Fig. 1. *Neo*-PLB formation in hybrid *Cymbidium* Twilight Moon 'Day Light' under control conditions on TC medium [TEIXEIRA DA SILVA, 2012a] (A). The visual appearance of *neo*-PLBs in response to BA is similar, although productivity differs [TEIXEIRA DA SILVA, 2014a] (B)

Acknowledgement and conflicts of interest

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, N^6–[Δ2-isopentenyl] adenine (syn. 6(γ,γ-dimethylallylamino)purine); ABA, (±)-*cis*,*trans*-abscisic acid; Ads, adenine hemisulphate; BA, 6-benzyladenine (syn. BAP, 6-benzylaminopurine; Teixeira da Silva 2012b); dicamba, 3,6-dichloro-2-methoxybenzoic acid (syn. 3,6-dichloro-*o*-anisic acid); GA_3, gibberellic acid; IBA, indole-3-butyric acid; Kin, kinetin; MeJa, methyl jasmonate; NAA, α-naphthaleneacetic acid; PG, phloroglucinol; PGR, plant growth regulator; picloram, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; PLB, protocorm-like body; SA, salicylic acid; TC medium, Teixeira *Cymbidium* medium (TEIXEIRA DA SILVA, 2012a); TDZ, *N*-phenyl-N'-1,2,3-thiadiazol-5-yl-urea or thidiazuron; TRIA, 1-triacontanol (syn. melissyl alcohol or myricyl alcohol).

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Tab. 1. Flow cytometric analysis of *Cymbidium* Twilight Moon 'Day Light' PLBs derived from different PGRs (1 mg/l) after 60 days in TC medium. The SPAD value was calculated from the leaves (third fully developed leaf from the apex) of 6-month old plantlets derived from PGR treatments.

Treatment	PGRs	SPAD[1]	FC[2]
Control	None	41.1 a	92:8:0:0
Cytokinins	AdS	0	96:4:0:0
	BA	43.2 a	74:23:3:0
	2iP	33.7 b	71:27:t:0
	Kin	41.6 a	91:6:4:0
	TDZ	22.8 cd	64:31:2:t
	*m*T	35.8 ab	76:16:8:0
Auxins	2,4-D	25.7 cd	72:22:6:t
	IBA	0	90:8:2:0
	NAA	0	94:6:0:0
	Picloram	31.8 b	73:18:6:3
	TRIA	36.2 ab	72:24:4:0
	PG	0	80:16:3:1
Others	ABA[3]	19.7 d	71:28:1:0
	Dicamba[4]	26.9 c	91:9:0:0
	GA_3[5]	0	80:13:5:2
	JA[3]	30.8 b	88:12:0:0
	MeJa[3]	29.2 b	66:28:3:t
	SA[3]	42.1 a	92:6:t:0

Means followed by different letters within a column across all PGRs indicate significant differences at $P<0.05$ according to Tukey's multiple range test. SPAD = measurement of chlorophyll content on the third youngest leaf; all FC values represent 2C:4C:8C:16C relative ratios derived from three independent biological replicates; t = trace (≤2%).

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, N^6–[Δ2-isopentenyl] adenine (syn. 6(γ,γ-dimethylallylamino)purine); ABA, (±)-*cis*,*trans*-abscisic acid; Ads, adenine hemisulphate; BA, 6-benzyladenine (syn. BAP, 6-benzylaminopurine; see TEIXEIRA DA SILVA 2012b); dicamba, 3,6-dichloro-2-methoxybenzoic acid (syn. 3,6-dichloro-*o*-anisic acid); GA_3, gibberellic acid; IBA, indole-3-butyric acid; Kin, kinetin; MeJa, methyl jasmonate; NAA, α-naphthaleneacetic acid; PG, phloroglucinol; PGR, plant growth regulator; picloram, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; PLB, protocorm-like body; SA, salicylic acid; TC medium, Teixeira *Cymbidium* medium [TEIXEIRA DA SILVA, 2012a]; TDZ, *N*-phenyl-N'-1,2,3-thiadiazol-5-yl-urea or thidiazuron; TRIA, 1-triacontanol (syn. melissyl alcohol or myricyl alcohol)

[1] SPAD values of zero indicate that no plantlets could be derived from that treatment, thus SPAD could not be measured in leaves (these values were not included in the analyses.

[2] The 2C:4C value of leaves of plantlets derived from any treatment was not different (94:2 on average) and thus values are not presented.

[3] Also referred to as stress hormones.

[4] Also considered by some to be an auxin.

[5] Also referred to as a gibberellin.

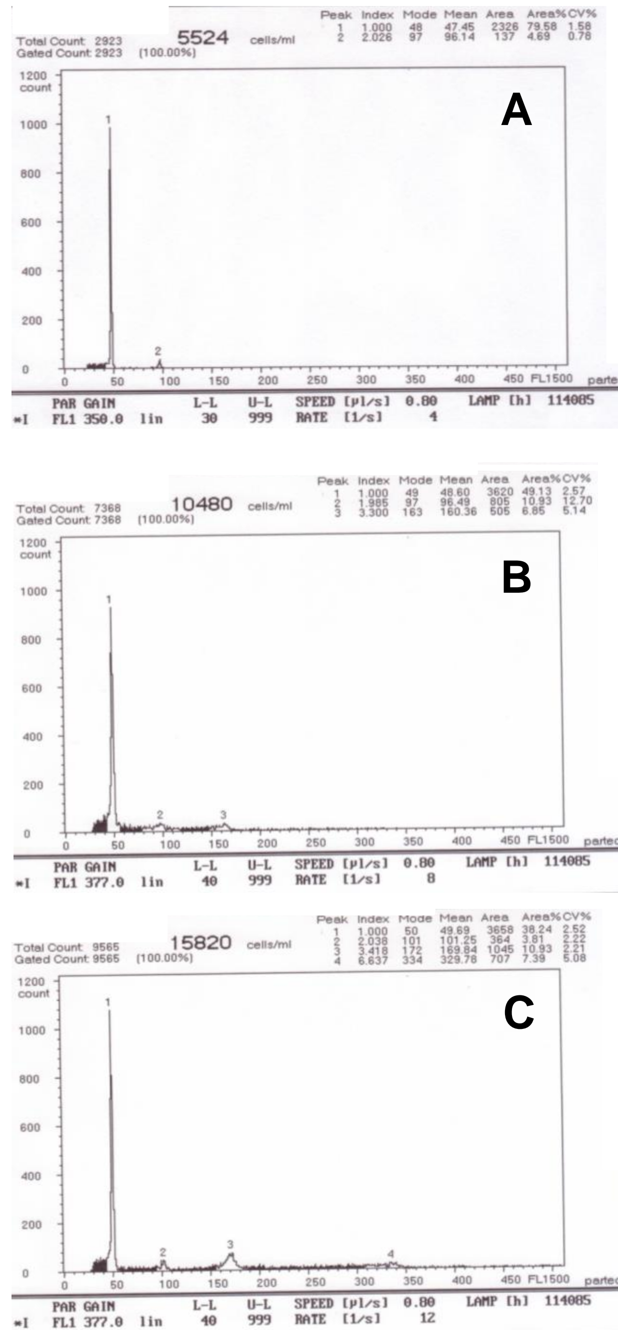

Fig. 2. Control versus endopolyploid *neo*-PLBs in hybrid *Cymbidium* Twilight Moon 'Day Light'. Control *neo*-PLBs on TC medium [TEIXEIRA DA SILVA, 2012a] (A). Endopolyploidy up to 8C in *neo*-PLBs in response to BA (B). Endopolyploidy up to 8C in *neo*-PLBs in response to picloram (C).