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RESPONSE OF HYBRID *CYMBIDIUM* (ORCHIDACEAE) PROTOCORM-LIKE BODIES TO 26 PLANT GROWTH REGULATORS

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Abstract

Teixeira da Silva J.A., 2014: Response of hybrid *Cymbidium* (Orchidaceae) protocorm-like bodies to 26 plant growth regulators [*Cymbidium* (Orchidaceae) hibrido į protokormą panašių kūnelių reakcija į 26 augalų augimo reguliatorius]. – Bot. Lith., 20(1): 3–13.

Plant growth regulators (PGRs) are one of the most effective means of controlling plant organogenesis *in vitro*. Hybrid orchid production relies on effective protocols to maximize clonal shoot output. This is best achieved when protocorm-like bodies (PLBs) are propagated. In a bid to deepen orchidologists' understanding of basic responses of *Cymbidium* to PGRs, this study aimed to establish the organogenic response of hybrid *Cymbidium* Twilight Moon 'Day Light' half-PLBs or PLB thin cell layers (TCLs) to a single application of PGRs (6 auxins; 7 cytokinins; 3 alternative PGRs), 3 herbicides or 7 growth inhibitors/retardants at 4 concentrations (1, 2, 4 or 8 $mg \cdot l^{-1}$) as well as a control (0 $mg \cdot l^{-1}$), both in the light and in the dark. The control (PGR-containing TC medium) performed best, but all auxins and growth inhibitors and retardants were toxic to *neo*-PLB formation, resulting in 100% death. A synthetic auxin (BSAA), a cytokinin (4-CPPU) and two herbicides (dicamba and picloram) were equally toxic. No auxins, TIBA, GA_3 or SA induced any organogenic response. 1 or 2 $mg \cdot l^{-1}$ 2,4-D or 1 $mg \cdot l^{-1}$ TDZ induced embryogenic callus, but 2–8 $mg \cdot l^{-1}$ 2,4-D resulted in abnormal shoots. TDZ induced direct multiple shoots. Only five remaining cytokinins (Ads, BA, Kin, ZR, 2iP) could form *neo*-PLBs, but always significantly less than the controls, independent of the explant used (half-PLBs or tTCLs) and light conditions (light vs darkness). These five cytokinins could be useful for *neo*-PLB induction of other *Cymbidium* hybrids. A new concept, the average cumulative value or ACV, is introduced.

Keywords: plant growth regulator, PLB, Teixeira *Cymbidium* (TC) medium, thin cell layer.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; 2,4,5-T – 2,4,5-trichlorophenoxyacetic 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); BNOA – β-naphthoxyacetic acid; BSAA – benzoselenienyl-3-acetic acid; 4-CPPU – *N*-(chloro-4-pyridyl)-*N*′-phenylurea (or forchlorfenuron); dicamba – 3,6-dichloro-2-methoxybenzoic acid (syn. 3,6-dichloro-*o*-anisic acid); GA_3 – gibberellic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; Kin – kinetin; MeJa, methyl jasmonate; NAA – α-naphthaleneacetic acid; 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; TCL – thin cell layer; TDZ – *N*-phenyl-N'-1,2,3-thiadiazol-5-yl-urea or thidiazuron; TIBA – 2,3,5-triiodobenzoic acid; TRIA – 1-triacontanol (syn. melissyl alcohol or myricyl alcohol); VW – Vacin and Went medium; ZR – zeatin riboside (syn. 9-(β-D-ribofuranosyl)-*trans*-zeatin or N^6-(*trans*-4-hydroxy-3-methyl-2-buten-1-yl)adenosine)

INTRODUCTION

Cymbidium (Orchidaceae), alongside two other orchids *Dendrobium* and *Phalaenopsis*, is rapidly emerging as a model plant due to the fine-scale understanding of its development *in vitro* (Hossain et al., 2013; Teixeira da Silva, 2013a, 2013b). Moreover, the protocorm-like body (PLB), which serves as an *in vitro* clonal propagule, is equivalent to the somatic embryo in orchids (Teixeira da Silva & Tanaka, 2006). *Cym-*

bidium is historically an important orchid since it was the first ever orchid to be propagated *in vitro*, although those studies used shoot tip culture, and not PLBs (Morel, 1960). In *Cymbidium*, as for several other orchid genera, the PLB developmental programme is influenced by several factors including, *inter alia*, the choice of plant growth regulator (PGR), culture conditions or explant used with PLBs forming new PLBs or *neo*-PLBs (Teixeira da Silva & Tanaka, 2006). Even though several basal media can support the induction and development of *Cymbidium* PLBs *in vitro* (Teixeira da Silva et al., 2006), Teixeira *Cymbidium* (TC) medium 1 (Teixeira da Silva, 2012a) was used in this study. In general, media with low levels of micro- and macronutrients best support the growth and development of *Cymbidium* PLBs, including Vacin and Went medium (Vacin & Went, 1969), although nutrient-rich media also allow for PLB induction and proliferation, making the choice of basal medium difficult to make unless rigorous genotype-independent testing is done. Hybrid *Cymbidium* Twilight Moon 'Day Light' is a well-studied cultivar *in vitro*, and since it responds well to *in vitro* manipulation, thus, served as the continued model plant in this study.

Most previous studies by the author using Twilight Moon 'Day Light' employed 0.1 $mg \cdot l^{-1}$ α-naphthaleneacetic acid (NAA) and 0.1 $mg \cdot l^{-1}$ kinetin (Kin) in combination to induce *neo*-PLBs, while a combination of thidiazuron (*N*-phenyl-*N*-1,2,3-thidiazol-5-yl urea; TDZ, with cytokinin-like properties) or 2,4-dichlorophenoxyacetic acid (2,4-D), usually at 0.01 $mg \cdot l^{-1}$, in combination with 0.1 $mg \cdot l^{-1}$ NAA, resulted in embryogenic callus formation, which, after developing, established PLBs, equivalent to somatic embryos (Huan et al., 2004; Huan & Tanaka, 2004; Teixeira da Silva & Tanaka, 2006). According to the author's estimates, as many as 50–60 underdeveloped *neo*-PLBs can form per PLB, although only 5–15 of these develop fully, depending on *in vitro* and medium-related biotic and abiotic conditions (Teixeira da Silva et al., 2006a, 2006b), most likely due to physical space limitations on the surface of a PLB. Callus, often friable, contains several dozen under-developed PLBs on the surface of explants (Teixeira da Silva & Tanaka, 2006), therefore, there is also interest in generating PLBs or shoots indirectly through callus. Studies on callus induction from PLBs tend to be scarcer due to its slow growth and necrotic tendency (Teixeira da Silva, 2013a). Callus could be induced from pseudobulb sections, rhizomes and roots of *Cymbidium ensifolium* or *C. sinense* seedlings, a terrestrial orchid species (Chang & Chang, 1998, 2000), with higher frequencies being reported by Huan & Tanaka (2004) in hybrid *Cymbidium* Great Flower 'Rainbow Drop' in the presence of 0.1–1 $mg \cdot l^{-1}$ NAA or 0.01-0.1 $mg \cdot l^{-1}$ l 2,4-D in conjunction with 0.1 or 0.01 $mg \cdot l^{-1}$ TDZ. Thin cell layers (TCLs), which tend to be more sensitive to changes in the medium and medium constituents (Teixeira da Silva, 2013b), were also used in this study. No other study on *Cymbidium* that tested the effect of PGRs used TCL explants.

Since PLB induction, formation and development in this hybrid *Cymbidium* is already quite well developed, most likely, as explained above, due to physical surface area restrictions, it would be impossible to increase the number of *neo*-PLBs formed per PLB explant, even if the number of primordial *neo*-PLBs can be maximized. Consequently, the objective of this study was to observe the response of Twilight Moon 'Day Light' half-moon PLBs (i.e., transversally dissected PLBs after removing the shoot tip) to a wider range of auxins, cytokinins, other PGRs and growth retardants, tested individually, than has already been reported in the literature (see a wider discussion in the Discussion section). The expectation is that a wide range of responses would be observed, but which could serve as a useful template for future studies on other *Cymbidium* hybrids or species (or even other orchid genera) that might be difficult to propagate effectively *in vitro*. The effect of brassinosteroids on *in vitro* organogenesis was not tested in this study. Initial trials indicated that PLBs responded differently in the light and in the dark, thus, both factors were also assessed in this study.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents, including PGRs, were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, the USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue-culture grade, unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) originally developed spontaneously from shoot-tip culture on VW agar medium without PGRs. These PLBs were induced and subcultured every two months on TC medium (Teixeira da Silva, 2012a), which served as the control medium in this study. TC medium contains 0.1 mg·l^{-1} NAA, 0.1 mg·l^{-1} Kin, 2 g·l^{-1} tryptone and 20 g·l^{-1} sucrose. TC medium was solidified with 8 g·l^{-1} Bacto agar (Difco Labs., the USA) and pH was adjusted to 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min (Teixeira da Silva et al., 2006; Teixeira da Silva & Tanaka, 2006). Light cultures were kept on 40 ml medium in 100-ml Erlenmeyer flasks, double-capped with aluminium foil, at 25°C, under a 16-h photoperiod with a light intensity of 45 µmol·m^{-2}·s^{-1} provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Two types of explants (10/flask) were used for *neo*-PLB induction and proliferation in all experiments: a) longitudinally bisected PLB (3–4 mm in diameter) segments (hereafter termed half-PLBs) and b) transverse thin cell layers (tTCLs) prepared according to Teixeira da Silva (2013b). Recommendations related to culture conditions, media and PLB induction, formation and proliferation were followed from the *Cymbidium* literature, specifically pertaining to medium formulation (Teixeira da Silva et al., 2006), abiotic factors (Teixeira da Silva et al., 2006a) and biotic factors (Teixeira da Silva et al., 2006b).

Plant growth regulators and growth inhibitors and retardants tested

The rationale was to assess whether a dose of any single PGR could induce organogenesis as or more effectively as PGR-containing TC medium, the control. Four broad groups were tested (see abbreviations list for details): auxins (2,4,5-T; BSAA; IAA; IBA; NAA; BNOA), cytokinins (Ads; BA; 4-CPPU; Kin; TDZ; ZR; 2iP), other PGRs (GA_3; SA; TRIA), herbicides (2,4-D; dicamba; picloram) and growth inhibitors and retardants (ABA; ancymidol; chlormequat; mepiquat; paclobutrazol; uniconazole; TIBA). Each PGR was tested separately in the light and in the dark.

Growth parameters assessed

Three parameters were assessed: 1) percentage of explants forming *neo*-PLBs, 2) the number of *neo*-PLBs per explant and 3) fresh weight (mg) of explant + *neo*-PLBs, after 45 days in culture. These parameters were assessed for both explants, namely half-PLBs and tTCLs, and for both light conditions, namely light and darkness. Usually, 30 days would only allow for the observation of premature *neo*-PLBs, 60 days would already result in the formation of shoot tips from *neo*-PLBs, while by 120 days shoots and roots will have fully developed, hence the choice was 45 days (Teixeira da Silva & Dobránszki, 2013). The PGRs listed in Table 1 that were able to elicit a quantitative response were ranked based on their average cumulative values (ACVs) across all four concentrations for both explants and for both light and dark as follows:

$$ACV_{PGR} = [\text{half-PLBs light } (C_1+C_2+C_3+C_4) + \text{ + tTCLs light } (C_1+C_2+C_3+C_4) + \text{half-PLBs dark } (C_1+C_2+C_3+C_4) + \text{tTCLs dark } (C_1+C_2+C_3+C_4)]/16 \quad (1)$$

where: ACV_{PGR} is the ACV of each PGR; C_1 = 1 mg·l^{-1}; C_2 = 2 mg·l^{-1}; C_3 = 4 mg·l^{-1}; C_4 = 8 mg·l^{-1}.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate (n = 30, total sample size per treatment). Data were subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, the USA). Significant differences between means were assumed at $p \leq 0.05$.

RESULTS

The ability of four groups of PGRs to alter or improve the organogenesis of a *Cymbidium* hybrid using two explant types (half-PLBs and PLB-derived tTCLs) under two environmental conditions (light vs continual darkness) was tested. The control, i.e. PGR-containing TC medium, performed best independent of the explant (Fig. 1A; Table 1), indicating that a single PGR is insufficient for inducing high levels of *neo*-PLB formation. All auxins and growth inhibitors and

Table 1. Effect of plant growth regulators in basal TC medium on *neo*-PLB formation from half-PLB or PLB tTCL culture of hybrid *Cymbidium* Twilight Moon 'Day Light' in the light and in the dark

Medium composition	PGR concentration ($mg \cdot l^{-1}$)	Explants forming *neo*-PLBs (%)		Number of *neo*-PLBs per explant		Additional fresh weight (mg) of PLB explant + *neo*-PLBs*	
		Light	Dark	Light	Dark	Light	Dark
Half-PLBs on: TC + PGRs (+ control)		100 a	79 b	8.3 ab	2.8	472 ab	285 b
PLB tTCLs on: TC + PGRs (+ control)		100 a	64 c	2.1 d	0.4	176 g	156 cd
Half-PLBs on: TC minus PGRs (- control)		100 a	41 de	1.2 de	1.4	277 e	128 d
PLB tTCLs on: TC minus PGRs (- control)		100 a	29 e	0.3 e	0.1	69 i	26 f
Half-PLBs (PGR-free TC) + Ads	1	100 a	52 d	2.3	1.1	184 g	96 de
	2	81 b	39 e	1.6	0.4	178 g	81 e
	4	76 b	22 f	0.7	0.1	132 gh	73 e
	8	48 cd	5 g	0.4	0.1	87 hi	54 ef
PLB tTCLs (PGR-free TC) + Ads	1	93 a	29 e	0.6	0.2	137 gh	109 de
	2	64 bc	22 f	0.2	0.1	126 gh	84 e
	4	28 de	11 fg	0.1	0.1	103 h	71 e
	8	7 f	0 g	0.1	0.1	68 i	56 ef
Half-PLBs (PGR-free TC) + BA	1	100 a	86 ab	4.7	2.2	443 b	301 ab
	2	100 a	73 bc	4.1	1.0	486 ab	326 a
	4	96 a	53 d	1.6	0.6	317 d	201 c
	8	73 bc	38 e	0.4	0.1	241 ef	176 cd
PLB tTCLs (PGR-free TC) + BA	1	91 ab	61 cd	0.8	0.2	208 f	171 cd
	2	87 ab	48 d	0.4	0.1	246 ef	183 c
	4	49 cd	36 e	0.1	0.1	147 gh	101 de
	8	13 ef	16 f	0.1	0.1	136 gh	74 e
Half-PLBs (PGR-free TC) + Kin	1	100 a	74 bc	3.2	1.8	483 ab	327 a
	2	93 a	61 cd	2.9	0.9	501 a	331 a
	4	57 c	42 de	1.6	0.2	361 cd	246 bc
	8	41 d	27 ef	0.8	0.1	237 ef	184 c
PLB tTCLs (PGR-free TC) + Kin	1	86 ab	57 cd	0.6	0.2	231 ef	176 c
	2	73 bc	52 d	0.4	0.1	244 ef	196 c
	4	56 c	41 de	0.1	0.1	156 gh	129 d
	8	27 e	24 ef	0.1	0.1	144 gh	96 de
Half-PLBs (PGR-free TC) + ZR	1	87 ab	68 c	3.7	0.3	381 c	154 cd
	2	79 b	59 cd	3.2	0.1	307 d	128 d
	4	61 c	32 e	0.9	0.1	218 f	94 de
	8	39 d	17 f	0.3	0.1	174 g	74 e
PLB tTCLs (PGR-free TC) + ZR	1	81 b	49 d	1.1	0.1	206 f	142 cd
	2	53 c	42 de	0.7	0.1	184 g	121 d
	4	29 de	31 e	0.2	0.1	139 gh	94 de
	8	6 f	4 g	0.1	0.1	98 h	68 ef
Half-PLBs (PGR-free TC) + 2iP	1	88 ab	91 a	6.1	4.8	496 a	302 ab
	2	76 b	84 ab	4.2	1.2	442 b	277 b
	4	54 c	63 cd	0.9	0.2	376 c	201 c
	8	22 e	31 e	0.3	0.1	248 ef	148 cd
PLB tTCLs (PGR-free TC) + 2iP	1	87 ab	79 b	1.1	0.1	278 e	161 cd
	2	71 bc	74 bc	0.4	0.1	239 ef	147 cd
	4	48 cd	51 d	0.1	0.1	174 g	106 de
	8	16 ef	26 ef	0.1	0.1	163 gh	77 e

Note: Mean values followed by the same letter in the same column (i.e., for each growth parameter, and for light and dark conditions) are not significantly different based on DMRT ($p = 0.05$). See text for media constituents. n = 90 (9 (3 × 3 blocks) Petri dishes × 10 for each treatment). PGR – plant growth regulator; PLB – protocorm-like body; TC – Teixeira *Cymbidium* medium (Teixeira da Silva, 2012a), includes 0.1 $mg \cdot l^{-1}$ α-naphthaleneacetic acid (NAA) and 0.1 $mg \cdot l^{-1}$ kinetin, 2 $g \cdot l^{-1}$ tryptone and 20 $g \cdot l^{-1}$ sucrose (see reference for modified micro- and macro-nutrients); tTCL – transverse thin cell layer. *A value of 54 mg or 12 mg was subtracted from mean values displayed as 54 mg and 12 mg represent the mean fresh weight of the starting material, i.e. half-PLBs or tTCLs, respectively

retardants (particularly the latter group of compounds, all of which were extremely toxic at even 1 mg·l^{-1} for both explant types and under both light conditions) were toxic to *neo*-PLB formation, resulting in 100% death (i.e. browning followed by necrosis) of explants (half-PLBs and tTCLs) at 8 mg·l^{-1} after 45 days (data not shown). One cytokinin, 4-CPPU, one synthetic auxin (BSAA) and two herbicides (dicamba and picloram) showed the same sensitivity and toxicity (i.e. no growth, browning and necrosis) even at 1 mg·l^{-1} for both explant types and under both light conditions (data not shown). Among the auxins, only in the case of 1 or 2 mg·l^{-1} NAA and 1 mg·l^{-1} BNOA, or among the alternative PGRs, in the case of 1 mg·l^{-1} TIBA, half-PLBs expanded, but no differentiation was observed. At 1 mg·l^{-1} GA_3 and SA, the explants did not change, i.e. they remained green, did not necrose and did not differentiate. The application of 1 or 2 mg·l^{-1} 2,4-D or of 1 mg·l^{-1} TDZ resulted in embryogenic callus induction (Fig. 1F) (confirming earlier reports by Huan & Tanaka (2004) and Teixeira da Silva & Tanaka (2006)), while higher concentrations of 2,4-D (2–8 mg·l^{-1}) resulted in hyperhydric, abnormal shoots (Fig. 1B). Only for TDZ was direct organogenesis observed, i.e. PLB → multiple shoots (Fig. 1D). In all other cases, PLB explants derived new PLBs or *neo*-PLBs, which, if left on TC medium indefinitely, developed shoots and roots (data not shown).

Only five remaining cytokinins Ads, BA, Kin, ZR, 2iP (Figs 1C, 1E) showed quantitative results with respect to *neo*-PLB formation. In all five cases, the performance – as assessed by three parameters: 1) percentage of explants forming *neo*-PLBs, 2) number of *neo*-PLBs per explant and 3) fresh weight (mg) of PLB explant + *neo*-PLBs – was always inferior to the performance of the controls, which contained optimized PGR concentrations in TC basal medium, namely 0.1 mg·l^{-1} NAA and 0.1 mg·l^{-1} Kin. The ACVs for positive control (TC + PGRs), negative control (TC – PGRs), Ads, BA, Kin, ZR and 2iP were 85.75%, 3.4, 272.3 mg, 67.5%, 0.75, 125.0 mg, 42.31%, 0.51, 102.4 mg, 63.75%, 1.04, 234.8 mg, 56.94%, 0.83, 252.6 mg, 46.06%, 0.70, 161.4 mg and 60.06%, 1.24, 239.7 mg for parameters 1, 2 and 3, respectively. The five cytokinins were ranked based on their ACVs across all four concentrations (positive and negative

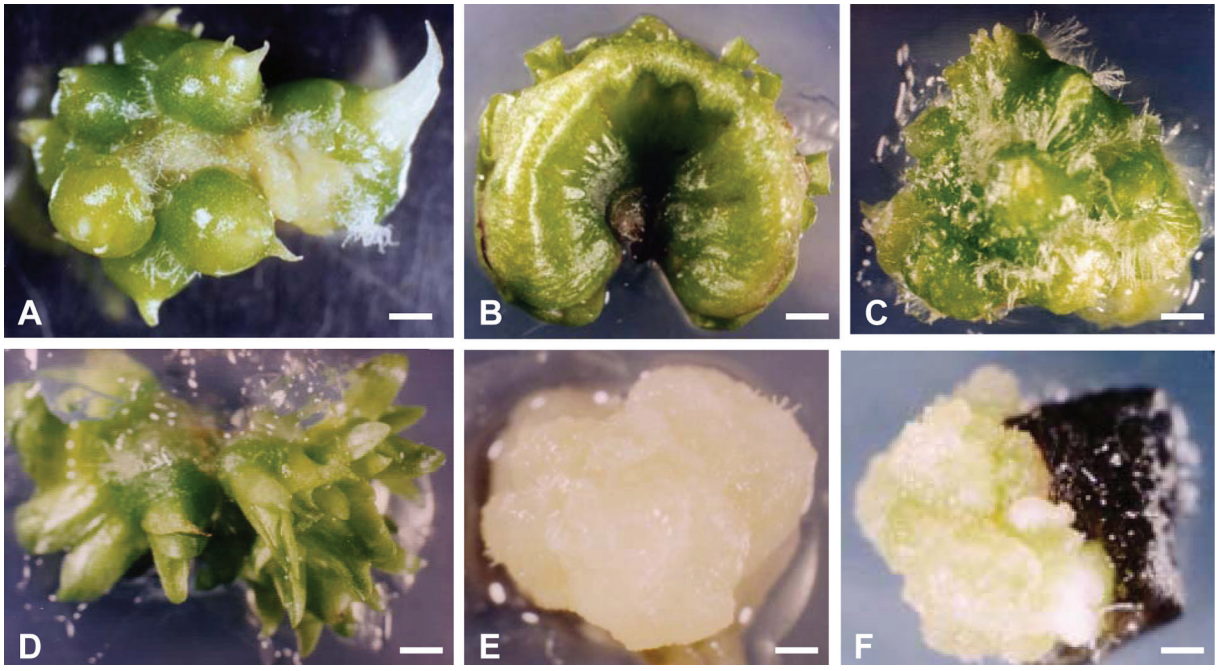

Fig. 1. The response of hybrid *Cymbidium* Twilight Moon 'Day Light' half-PLB explants to single doses of the selected plant growth regulators. A – *neo*-PLB formation in the control medium, i.e. Teixeira *Cymbidium* (TC) (Teixeira da Silva, 2012b) medium with 0.1 mg·l^{-1} NAA + 0.1 mg·l^{-1} Kin; B – deformed shoot formation in response to 2 mg·l^{-1} 2,4-D; C – *neo*-PLB formation in response to 2 mg·l^{-1} ZR; D – multiple shoot induction (the only case of direct organogenesis, except for 2,4-D) in response to 1 mg·l^{-1} TDZ; E – *neo*-PLB formation in response to 1 mg·l^{-1} 2iP. (F) – friable embryogenic callus formation in response to 1 or 2 mg·l^{-1} 2,4-D or 1 mg·l^{-1} TDZ; A–D = light; E, F = darkness; Bars: A, C = 1 mm; B, D, E, F = 0.1 mm

controls added for comparison) for both explants and for both light and dark culture conditions, i.e. for parameter 1: positive control > negative control > BA > 2iP > Kin > ZR > Ads; parameter 2: positive control > 2iP > BA > Kin > negative control > ZR > Ads; parameter 3: positive control > Kin > 2iP > BA > ZR > negative control > Ads (ACVs indicated for each PGR in Table 1). For example, the ACV for parameter 1 using Ads was 42.31%, calculated as follows (Eq. 1): [half-PLBs light (100 + 81 + 76 + 48) + tTCLs light (93 + 64 + 28 + 7) + half-PLBs dark (52 + 39 + 22 + 5) + tTCLs dark (29 + 22 + 11 + 0)]/16. An overall ranking of the ACVs for the three parameters reveals that positive control > 2iP > BA > Kin > negative control > ZR > Ads. In other words, relative to both controls, 2iP, BA and Kin stimulated more *neo*-PLB growth and development. The ACV is a novel, simple, yet informative parameter in plant tissue culture, the unit of which equals that of the parameter measured. Nonetheless, the ability of all five cytokinins to form *neo*-PLBs under light and dark conditions holds promise for alternative sources of *neo*-PLB induction for *Cymbidium* hybrids. It is possible that concentrations between 0.05 and 0.99 $mg \cdot l^{-1}$ would result in some organogenic response for several of the PGRs tested, but due to the inhibitory size of the already large experiment, such concentrations could not be (but should be) examined.

DISCUSSION

Cymbidium is rapidly turning into a model orchid, if not a model plant, at least for *in vitro* studies due to the fine-scale nature of experiments conducted on this genus. Consequently, this discussion will focus on studies that have employed *Cymbidium*, or other orchids, where the PGRs tested in this study have also been employed by other researchers. References related to unrelated genera (i.e. not related to orchids) were not referenced.

The use of PGRs to induce callus or *neo*-PLBs is not unique in *Cymbidium*. In terrestrial *Cymbidium ensifolium*, auxins stimulated rhizome growth, while cytokinins induced upright shoot formation (Lu et al., 2001). Shimasaki & Uemoto (1990) demonstrated that the apical meristems of *Cymbidium kanran* and *C. goeringii* or *C. forrestii* (Paek & Yeung, 1991) develop vigorous rhizomes *in vitro* at high NAA and 2,4-D concentrations; shoot formation from these rhizomes is accelerated when the auxin : cytokinin ratio is low; root formation occurred when both auxin and cytokinin were added, but only when potassium nitrate and ammonium nitrate were reduced. Incidentally, the ammonia to nitrate ratio also has a profound influence on *neo*-PLB formation in hybrid *Cymbidium* (Teixeira da Silva, 2013g). Studies on the clonal propagation of *Cymbidium* from leaf primordia (Lavrentyeva, 1986) concluded that: a) PLBs are larger when the cytokinin : auxin ratio is 1:1 or 2:1, b) the optimal size of explants is about 0.5 mm including 3–4 leaf primordia and c) PLBs arise at the base of leaf primordia. *Cymbidium* regeneration by vegetative buds, induced from young flower buds or inflorescences was achieved when NAA and BA were used at 1 mg l–1, and the production was enhanced when liquid medium was used (Kim & Kako, 1984). Important studies on the effects of PGRs on organogenesis of *Cymbidium* shoot apices *in vitro* (Kim & Kako, 1982, 1983) concluded that: a) auxins induced roots on shoot apex explants, but inhibited shoot development; b) the fresh weight of plantlets increased in the presence of high levels of 2,4-D, but roots were abnormal; c) NAA at 1 $g \cdot l^{-1}$ or 2,4-D at 0.1 $g \cdot l^{-1}$ could enhance plantlet formation and development; d) BA could enhance the formation of PLBs and shoots, but at high concentrations it inhibited root initiation; e) GA_3 and ABA had no effect; f) explants with three leaf primordia did not require an exogenous supply of PGRs for development; g) the production of PLBs was seasonal, with production beginning to increase in April, and reaching a peak in June; h) PLBs could form from both axillary and basal regions. The addition of NAA and BA enhanced the growth of PLBs, although BA was superior to NAA in *Cymbidium* (Fujii et al., 1999a). Studies on *Cymbidium* by Kukułczanka et al. (1987) concluded that the application of auxin to shoot tip cultures after 4 weeks of culture enhanced the growth of PLBs and plantlets, Kin increased the number of PLBs and GA_3 accelerated shoot growth but inhibited root development. Despite the positive effect of PGRs in *Cymbidium* organogenesis, Laneri (1990) and Hasegawa (1991) claimed that their presence caused phenotypic variations in the propagules. The use of 2,4-D (1–3 $mg \cdot l^{-1}$) produced only callus in *Cymbidium* (Ueda & Tori-

kata, 1968; Fonnesbech, 1972). Cytokinin-induced shoot or rhizome formation in *Cymbidium* rhizome cultures is common (Shimasaki & Uemoto, 1990). *Cymbidium eburneum* shoot cultures produced more PLBs (2.6 PLBs/PLB as opposed to 1.9 in controls) when in the presence of methyl jasmonate (MeJa) at < 1 µM (Shimasaki et al., 2003a). Nayak et al. (2002) found that the most responsive PGRs for *neo*-PLB formation from tTCLs in *Cymbidium aloifolium* are: ZR > BA > NAA > BA + NAA > Kin (in terms of percentage explants forming *neo*-PLBs and the number of PLB tTCLs forming *neo*-PLBs). In our study, a maximum of 28 *neo*-PLBs per PLB TCL formed. In contrast, Huan & Tanaka (2004), using another *Cymbidium* hybrid Great Flower 'Rainbow Drop', found that plain VW medium without any PGRs resulted in 90% of PLBs forming *neo*-PLBs. In Twilight Moon 'Day Light', the hybrid used in this study, never more than 2.1 or 0.3 *neo*-PLBs per half-PLB or tTCL, respectively formed when no PGRs were added (Table 1). However, 0.5 $mg \cdot l^{-1}$ NAA + 0.1 $mg \cdot l^{-1}$ TDZ resulted in most callus formation (40% of PLBs forming embryogenic callus), although 0.05 $mg \cdot l^{-1}$ 2,4-D + 0.01 $mg \cdot l^{-1}$ TDZ also could induce callus from 15% of PLBs (Huan & Tanaka, 2004). The use of coconut water, which often contains PGR-like substances, can result in as many as 90.2 *neo*-PLBs per PLB (Huan & Tanaka, 2004), and can improve the recovery of encapsulated PLBs when synseed alginate coat contains 10% (v/v) of coconut water (Teixeira da Silva, 2012e). However, subsequent studies on Twilight Moon 'Day Light' (Teixeira da Silva & Tanaka, 2006) indicated that initially (15–30 days), a high number of *neo*-PLBs per PLB could be observed, while embryogenic callus was in fact underdeveloped PLBs, thus, the correct and most accurate timing to represent a PLB count is 45 days (Teixeira da Silva & Dobránszki, 2013). Murashige & Skoog (1962) (MS) supplemented basal medium with 5.0 mM BA and 2.5 mM NAA resulted in most *neo*-protocorms per seed-derived protocorm (20.55) of *Cymbidium mastersii* (Mohanty et al., 2012). A BA+NAA combination was also effective for PLB formation of two *Cymbidium* hybrids, Nativity and Lapine Dancer (Fujii et al., 1999b), while the same two PGRs (1 $mg \cdot l^{-1}$ NAA + 2 $mg \cdot l^{-1}$ BA), together with a red light filter, maximized *neo*-PLB formation (approx. 12 per initial intact PLB) in *Cymbidium finlaysonianum* (Hamada et al., 2010). Half-strength MS medium containing 1 $mg \cdot l^{-1}$ BA and 2 $mg \cdot l^{-1}$ 2,4-D resulted in the highest somatic embryos per protocorm (34.4) of *Cymbidium bicolor*, although 2 $mg \cdot l^{-1}$ BA + 1 $mg \cdot l^{-1}$ NAA also resulted in somatic embryo (i.e. PLB) formation, but lower amounts, 26.0 (Mahendran & Bai, 2012). The addition of GA_3 or paclobutrazol reduced PLB formation in *Cymbidium kanran* and in hybrid *Cymbidium* Hiroshima Golden Cup 'Sunny Moon' (Shimasaki et al., 2002a).

Jasmonic acid, MeJa and salicylic acid (for hybrid Twilight Moon 'Day Light' and for *Cymbidium kanran* Makino) (Shimasaki et al., 2002b; Teixeira da Silva, 2012c, 2012d) as well as fungal elicitors, chitosan and hyaluronic acid (Nahar et al., 2011; Teixeira da Silva et al., 2013a) and ethylene inhibitors ($AgNO_3$, AVG, CEPA; silver nitrate, aminoethoxyvinylglycine and 2-chloroethylphosphonic acid, respectively; Teixeira da Silva, 2013c) were tested separately and were, thus, not included in this study. Moreover, activated charcoal and antioxidants, including ascorbic acid were assessed on *neo*-PLB formation (Teixeira da Silva, 2013d). Phloroglucinol (Teixeira da Silva et al., 2013a; Teixeira da Silva, 2013f) can be used as a chemical agent to promote root formation while magnetic fields can be used as a physical agent to manipulate the outcome of the organogenic response of *Cymbidium* PLBs (Van et al., 2012).

There exist no reports on the use of dicamba, a pre- and post-emergence herbicide for orchid tissue culture, while the use of picloram is limited to the development of suspension cultures in *Doritaenopsis*, leading to changes in ploidy level (Mishiba et al., 2001). No other study could be found for the use of other PGRs mentioned above in *Cymbidium* tissue culture.

Finally, regarding the ACV, the negative control may appear to have favoured the quantitative outcome of all three parameters only because the ACVs of all the cytokinins also took into consideration concentrations at which explants performed well (lower concentrations) or poorly (higher concentrations). However, when considering only the cytokinins, the ACV provides a simple measure for comparing or ranking the effectiveness of a group of factors, independently of the statistical analysis and also without the need for more complex methods such as cluster analysis. The reader is advised that the lower values registered by tTCLs relative to "regular" explant

types, the half-PLBs, does not necessarily reflect a poorer performance of the tTCLs. Rather, organogenesis is restricted by the actual explant size, surface area and volume (Teixeira da Silva, 2013b), and an adjustment (the Plant Growth Correction Factor; Teixeira da Silva & Dobránszki, 2011, unpublished data) that actually takes explant size, surface area and volume into consideration is actually required to make the quantitative outcome directly comparable.

CONCLUSIONS

After the mineral content of the basal medium, most likely the next important factor determining the organogenic outcome of hybrid *Cymbidium* growth *in vitro* is the choice of PGR.

Key findings of this study: 1 or 2 mg·l^{-1} 2,4-D or of 1 mg·l^{-1} TDZ induced embryogenic callus; 2-8 mg·l^{-1} 2,4-D resulted in abnormal shoots; TDZ induced direct multiple shoots; 5 cytokinins (Ads, BA, Kin, ZR, 2iP) formed *neo*-PLBs.

Predictably, wide variation was observed when a wide range of PGRs were used singly. However, different concentrations (higher and lower), combinations of PGRs, and from different commercial sources, need to be tested to induce callus or organogenesis, or to reduce toxicity.

The publication of negative results advances science because it serves to eliminate unnecessary or inconsequential possibilities. This, as well as the need to expand the choice of treatments was also highlighted in a recent study on regeneration in chrysanthemum petals in response to multiple PGRs (Teixeira da Silva, 2014).

Histological and cytogenetic analyses of regenerants should be conducted and the stability of important morphological characters such as flowering-related or pot-culture-related characteristics should be ascertained in acclimatization and greenhouse trials.

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CYMBIDIUM (ORCHIDACEAE) HIBRIDO Į PROTOKORMĄ PANAŠIŲ KŪNELIŲ REAKCIJA Į 26 AUGALŲ AUGIMO REGULIATORIUS

Jaime A. Teixeira da Silva

Santrauka

Augalų augimo reguliatoriai (AAR) yra viena iš veiksmingiausių augalų organogenezės *in vitro* valdymo priemonių. Hibridinių orchidėjų dauginimas vykdomas efektyviais metodais, besiremiančiais klonuotų ūglių išeigos didinimu. Tai geriausiai pasiekiama, kai yra dauginami į protokormus panašūs kūneliai (PPK). Siekiant pagilinti orchidologistų žinias apie pagrindines *Cymbidium* reakcijas į augimo reguliatorius, šio tyrimo tikslas buvo nustatyti *Cymbidium* hibrido Twilight Moon 'Day Light' organogenezinį atsaką, plonus ląstelių sluoksnius (PLS) iš PPK ar pusiau PPK paveikus augimo reguliatoriais (9 auksinais; 7 citokininais; 3 alternatyviais AAR) arba 5 koncentracijų (0, 1, 2, 4 arba 8 mg/l) 7 augimo inhibitoriais / retardantais tiek šviesoje, tiek ir tamsoje. Augimas buvo geriausias kontrolinėje terpėje (Tekseiros *Cymbidium* TC terpė su AAR), tačiau visi auksinai, augimo inhibitoriai ir retardantai buvo toksiški naujų PKK formavimuisi ir sukėlė 100 % žūtį. Sintetinis auksinas (BSAA), citokininas (4-CPPU) ir du herbicidai (dikamba ir pikloramas) buvo vienodai toksiški. Auksinai, TIBR, GR3 ar SR nesukėlė jokio organogenezinio atsakymo. Retardantas 2,4-D (nuo 1 iki 2 mg/l) arba 1 mg/l TDZ sukėlė kaliaus embriogenezę, o paveikus 2–8 mg/l 2,4-D atsirado nenormalių ūglių. TDZ sukėlė tiesius daugybinius ūglius. Tik likę 5 citokininai (Ads, BA, Kinetinas, ZR, 2iP) sugebėjo suformuoti *neo*-PPK, bet visada mažesnį skaičių nei kontrolinėje terpėje, tai nepriklausė nuo panaudoto eksplanto (pusės PPK ar PLS) ir apšvietimo sąlygų (šviesos ar tamsos). Šie 5 citokininai gali būti vertingi kitų *Cymbidium* hibridų *neo*-PPK indukcijai. Taip pat aptariama nauja vidutinių verčių (VV) įvertinimo koncepcija.