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Culture Vessel Affects Hybrid *Cymbidium* Protocorm-like Body and Callus Formation

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ABSTRACT

The number of protocorm-like bodies (PLBs) and embryogenic callus formed in hybrid *Cymbidium* Twilight Moon 'Day Light' is affected by the culture vessel (CV) used. Borosilicate test tubes (CV1), plastic and glass Petri dishes (CV2 and CV3), Whatman filter paper No. 1 membrane rafts (CV4), Milliseal®-covered jam jars (CV5), the Vitron™ (CV6) and 100-ml glass Erlenmeyer flasks (CV7, control) were tested. CVT7, which is the vessel conventionally used for the sub-culture and micropropagation of *Cymbidium* PLBs, resulted in 15.6 ± 1.15 PLBs per CV. CV2 and CV3 were as effective as CV7 (14.9 ± 0.95 and 15.8 ± 1.07, respectively) in PLB proliferation. Even though PLBs that formed in CV4 had higher fresh and dry weights, much fewer PLBs per CV were formed. In general, aerated CVs (CV5 and CV6) resulted in greater responsiveness of PLBs to callus formation, but differences were not significant. Although some laboratories have their established protocols for PLB proliferation, tests on the use of different CVs should be conducted prior to mass propagation since the choice of CV can affect material and running costs, the ease of multiplication and the quantitative output.

Keywords: embryogenic callus, orchid, PLB

Abbreviations: NAA, α-naphthaleneacetic acid; **PLB**, protocorm-like body; **PGR**, plant growth regulator; **TDZ**, thidiazuron (*N*-phenyl-N-1,2,3-thidiazuron-5'-ylurea); **VW**, Vacin and Went

INTRODUCTION

Wimber in 1963 and Morel in 1964 historically initiated the tissue culture of *Cymbidium* shoot tips, a culturally and economically important orchid genus, which also marked the historical beginning of plant *in vitro* tissue culture. *Cymbidium* tissue culture can be achieved by the culture of flower stalks, pseudobulbs, flower buds, shoot tips or protocorm-like bodies (PLBs); a few studies have reported the induction of callus in *Cymbidium*, either from PLB outer epidermal tissue (Begum *et al*. 1994b; Huan and Tanaka 2004a, 2004b; Huan *et al*. 2004), or inner PLB tissue (Begum *et al*. 1994a) in *Cymbidium* hybrids, or from pseudobulb sections, rhizomes and roots of seedlings of *C. ensifolium*, a terrestrial orchid species (Chang and Chang 1998). In the former studies callus induction was rapid, while in the latter it was slow. Studies on PLB formation in *Cymbidium* hybrids were extended to the use of PLB thin cell layers, conventional PLB segments and other explant types (Teixeira da Silva and Tanaka 2006) to test the effect of medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a) on PLB formation.

This study investigates the choice of culture vessel (CV) on the formation of PLBs from conventional PLB segments of epiphytic hybrid *Cymbidium* Twilight Moon 'Day Light', a popular hybrid. Since different CVs have different physical properties (Huang and Chen 2005), this parameter should be assessed for optimization of a tissue culture protocol.

MATERIALS AND METHODS

Chemicals and reagents

All plant growth regulators (PGRs) were purchased from Sigma-Aldrich (St. Louis, USA) and were of tissue culture grade. All other chemicals and reagents were of the highest analytical grade available and were purchased from either Wako (Japan) or Nacalai Tesque (Japan), unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) originated from shoot-tip culture on Vacin and Went (VW, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium or VW_{PLB}) every two months on modified VW supplemented with 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA) and 0.1 mg l^{-1} kinetin, 2 g l^{-1} tryptone and 20 g l^{-1} sucrose, and solidified with 8 g l^{-1} Bacto agar (Difco Labs., USA). The inclusion of tryptone in the medium improves callus regeneration and proliferation (Huan *et al*. 2004). Callus induction and proliferation medium (VW_{CALLUS}) was similar to VW_{PLB}, except that thidiazuron (TDZ) was used instead of kinetin. All media were adjusted to pH 5.3 with 1 N NaOH or HCL prior to autoclaving at 100 KPa for 17 min. 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). Longitudinally bisected PLB (3-4 mm in diameter) segments, 10 per flask, were used as explants for PLB induction and proliferation and for all experiments. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a) for PLB and callus induction, formation and proliferation.

Selection of culture vessel

In order to test the effect of CV on PLB and callus induction, formation and development, seven CVs were selected, in two separate experiments, the first to test the effect on PLB formation using VW_{PLB}, the second to test the effect on callus formation using

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Short Communication

Table 1 Effect of different culture vessels on *Cymbidium* Twilight Moon 'Day Light' PLB cultures and callus formation.

Culture vessel	Appearance	Explants forming callus (%)	No. PLBs/explant	*Neo* PLB fresh weight (mg)	*Neo* PLB dry weight (mg)
CV1	Normal	90 a	12.61 ± 1.08 b	642 ± 24 c	58 ± 6 c
CV2	Normal	90 a	14.93 ± 0.95 a	1077 ± 63 b	134 ± 9 b
CV3	Normal	90 a	15.86 ± 1.07 a	1049 ± 39 b	123 ± 6 b
CV4	Bloated and hyperhydric	24 b	3.64 ± 1.65 d	2068 ± 73 a	248 ± 13 a
CV5	Normal	96 a	9.64 ± 0.97 c	1081 ± 49 b	114 ± 9 b
CV6	Normal	96 a	9.87 ± 0.65 c	1023 ± 46 b	131 ± 6 b
CV7*	Normal	90 a	15.63 ± 1.15 a	1056 ± 33 b	136 ± 11 b

*= control; CV = culture vessel (see text for explanation of codes).
Data scored after 90 days and represent the mean ± SD (standard deviation) of at three replicates of $n = 20$ each, except for 60 replicates × $n = 1$. In each column, the values with different letters are significantly different ($P \leq 0.05$) according to DNMRT (Duncan's new multiple range test) or according to the χ^2 test ($P \leq 0.05$) for percentage values.

VW_{CALLUS}.
CV1: Borosilicate test tubes (25 cm tall, 15 ml of medium)
CV2: plastic Petri dishes (Falcon, 20 cm diameter, 15 ml of medium)
CV3: glass Petri dishes (20 cm diameter, 15 ml of medium)
CV4: Whatman filter paper No. 1 membrane rafts (filter sterilized filter paper wicks placed in 10 ml liquid medium in 100-ml glass Erlenmeyer flasks)
CV5: Milliseal®-covered jam jars (1 L, 25 cm tall, 50 ml of medium)
CV6: the Vitron™ (see e.g. Teixeira da Silva *et al*. 2007 for details, 108 ml of medium)
CV7 (control): 100-ml glass Erlenmeyer flasks (25 ml of medium)

Ten explants were placed per CV except for CV1, which contained one per tube. In all cases medium was solid medium except for CV4. The medium volume does not affect the outcome of the PLB or callus programmes (Teixeira da Silva *et al*. 2006a).

Morphogenic analyses

The number of PLBs formed per PLB segment as well the percentage of PLB segments that formed callus were measured. In the former, fresh weight of PLB masses were measured after 90 days while dry weight was established after drying the PLB masses in newspaper bags placed in a dry oven for 72 hrs at 60°C.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 20 replicates per treatment (except for CV1, which was 60 replicates). Data was subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's New Multiple Range test (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA) or by the χ^2 test for percentage values.

RESULTS

Culture vessel (CV) had a pronounced impact on the organogenic outcome of hybrid *Cymbidium* Twilight Moon 'Day Light' PLB cultures (**Table 1**). CVT7, which is the vessel conventionally used for the sub-culture and micropropagation of *Cymbidium* PLBs, resulted in the greatest number of PLBs per CV. CV2 and CV3 were as effective as CV7 in PLB proliferation. Even though PLBs that formed in CV4 had higher fresh and dry weights, much fewer PLBs per CV were formed. In general, aerated CVs (CV5 and CV6) did not produce more PLBs per explant but resulted in more callus formation, although differences were not significant (**Table 1**). Callus formation was poor in liquid culture (i.e. on membrane rafts) (CV4).

DISCUSSION

The choice of CV significantly affected the organogenic outcome of hybrid *Cymbidium* PLB proliferation experiments. This is not a surprising result considering that one of the drawbacks of a completely air-tight CV is poor air exchange, which often leads to the accumulation of ethylene within the CV (Read and Preece 2003) and which most affects the response of plant tissue culture *in vitro*. To improve this, different film types, ventilation vessels and accessories to CVs are available (Prakash *et al*. 2004), but a deeper discussion of these is beyond the scope of this manuscript.

Several studies have shown the advantages of using closures with filters or vented CVs, which allow gas exchange, increasing the photosynthetic capacity, the multiplication rate, and the survival of plants after transfer to *ex vitro* conditions (e.g. Tisserat and Silman 2000; Park *et al*. 2004; Tsay *et al*. 2006). Modi *et al*. (2009) showed how Petri dishes were superior to Erlenmeyer flasks and borosilicate test tubes in the shoot formation of French marigold (*Tagetes patula* L.). In our study, CV2 and CV3, the Petri dishes, produced as many PLBs and callus as Erlenmeyer flasks, but were clearly superior to test tubes (**Table 1**).

The increased availability of CO_2 by using CVs with filter may also influence the amount of photosynthetic pigments. *Nicotiana tabacum* plants grown in CVs with closures with microporous vents (better supplied with CO_2) had higher contents of chlorophyll *a*, *b* and β-carotene, higher photochemical activity of photosystem II and electron transport chain. Furthermore, plants grown under this condition had higher net photosynthetic rate, lower transpiration rate and stomatal conductance under *ex vitro* conditions than plants grown in tightly closed glass CVs (Haisel *et al*. 1999). Examples such as this are abundant throughout the literature. The water status of cultures is also influenced by the CV (Zimmerman 1995).

Culture vessel affected shoot bud induction and proliferation in *Magnolia* (De Proft *et al.* 1985), *Carica* (Magdalita *et al.* 1997) and *Gossypium* (Agrawal *et al.* 1997; Hazra *et al.* 2000); in the latter study the number of multiple shoots produced from *Gossypium* explants differed considerably when cultured in two different vessels that differed in shape, volume and quantity of medium: a larger culture vessel with more medium favoured the growth of shoots while in Modi *et al*. (2009)'s paper, the opposite was true. Jain *et al.* (2008) observed a pronounced effect of culture vessel on regeneration in *Stevia.*

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