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Impact of Gelling Agent and Alternative Medium Additives on Hybrid *Cymbidium* Protocorm-like Body and Callus Formation

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ABSTRACT

The gelling agent and a selection of alternative medium additives impacted the number of protocorm-like bodies (PLBs) and percentage callus formed in hybrid *Cymbidium* Twilight Moon 'Day Light'. Gellan gum resulted in greater PLB production and callus formation than all other gelling agents tested which included agar, Bacto agar, phytagel, oatmeal agar, potato dextrose agar, guar gum, isubgol and corn starch. All of the alternative medium additives (full fat milk, Coca-cola®, coffee, green and Darjeeling teas) negatively impacted PLB production and almost completely suppressed callus formation, although tissue browning appeared to have been reduced by the presence of teas and coffee.

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

The most effective way to tissue culture *Cymbidium* is by the culture of protocorm-like bodies (PLBs). Callus has been induced in *Cymbidium* 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* (Chang and Chang 1998). In the former study callus induction was rapid, while in the latter it was slow. PLB formation in *Cymbidium* hybrids could also be achieved by the use of PLB thin cell layers and conventional PLB segments (Tei-xeira 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.

In a bid to continue the exhaustive examination of factors that influence PLB and callus formation in *Cymbidium*, this study investigates the choice of gelling agent and alternative medium additives on the formation of PLBs and callus from conventional PLB segments of epiphytic hybrid *Cymbidium* Twilight Moon 'Day Light', a popular hybrid. Since the former have different physical properties (Prakash *et al*. 2004), 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 Wako (Japan), unless specified otherwise.

Plant material, explants and culture conditions

Hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) PLBs, 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 2 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). Callus induction and proliferation medium (VW_{CALLUS}) was identical to VW_{PLB}, although thidiazuron (TDZ) replaced kinetin. All media were adjusted to different pHs (listed below) 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 45µmol 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 250 ml Erlenmeyer 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 other abiotic factors (Teixeira da Silva *et al*. 2006a) for PLB and callus induction, formation and proliferation

Gelling agent and alternative medium additives

In order to test the effect on PLB and callus induction, formation and development, seven gelling agents and five alternative medium additives 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 VW_{CALLUS}.

1. Experiment 1 (gelling agents tested):

Bacto agar (control): 9 g l^{-1}, pH 5.3, Difco Labs, USA
Agar: 8 g l^{-1}, pH 5.7, Wako
Phytagel: 2.5 g l^{-1}, pH 5.3, Sigma-Aldrich, USA
Gellan gum (Gelrite®): 2 g l^{-1}, pH 5.3, Merck & Co., USA
Oatmeal agar: 72.5 g l^{-1}, pH 5.3, Sigma-Aldrich
Potato dextrose agar: 72.5 g l^{-1}, pH 5.6, Nissui, Japan
Corn starch (local supermarket) + agar: 4 g l^{-1} each, pH 5.3

2. Experiment 2 (alternative medium additives tested):

No additives (control)

Full fat milk: Marunaka
Coca-cola®: stirred and shaken for 5 min
Coffee: Nescafé Excella (2 g l^{-1}), 500 mg in 250 ml, 3 min in boiling water then filtered with Whatman No. 1 filter paper
Green tea: Itoen, 1 bag 250 ml^{-1}, 3 min in boiling water
English tea: Darjeeling (Top Value), 1 bag 250 ml^{-1}, 3 min in boiling water

In both experiments 10 explants were placed per flask in 25 ml of medium the ideal medium volume (Teixeira da Silva *et al*. 2006a). The pH ranged from 5.3 to 5.7, which was shown not to influence PLB formation or callus induction (Teixeira da Silva *et al*. 2006a). In Experiment 2, all media were gelled with 8 g l^{-1} agar, pH 5.7 and 20 g l^{-1} sucrose was provided, except for the Coca-cola® treatment, where no additional sugar source was added.

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

The choice of gelling agent had a pronounced impact on the organogenic outcome of hybrid *Cymbidium* Twilight Moon 'Day Light' PLB cultures (**Table 1**). Noteworthy is the superior performance of Gellan gum in PLB formation while oat meal agar and potato dextrose agar performed very poorly. All the alternative medium additives resulted in poor PLB formation and almost complete suppression of callus formation (**Table 2**).

DISCUSSION

The choice of gelling agent significantly affected the organogenic outcome of hybrid *Cymbidium* PLB proliferation experiments (**Table 1**). In contrast, the rather alternative medium alternatives selected resulted in poor performance of PLB cultures and almost completely inhibited callus formation (**Table 2**).

The type of gelling agent strongly affected not only the adventitious shoot regeneration capacity in *Tagetes* explants but also the water content of shoots (Jain *et al*. 2001; Modi *et al*. 2009). In many studies it is often observed that as the agar concentration increases, so the number of hyperhydric shoots decrease, including *Dianthus* (Casanova *et al*. 2008). In addition to a reduction in hyperhydricity, increased agar concentration can drastically reduce the multiplication rate (George 1996). In the case of phytagel-solidified medium the highest number of hyperhydric shoots was found in various species e.g., *Malus* (Turner and Singha 1990) and *Pyrus* (Kadoka and Niimi 2003).

Agar is the most commonly used gelling agent in plant tissue culture (according to Babbar and Jain 1998), followed by Gellan gum or Gelrite®, a polymer of glucuronic acid, rhamnose, glucose and *O*-acetyl moieties (Scholten and Pierik 1998). Agar functions by binding water, thus the higher the agar concentration, the stronger the water is bound while Gelrite® requires the presence of cations for gelation. In general a low pH results in the non-setting of agar. The culture of *Phalaenopsis* leaf segments, obtained from shoots derived from flower-stalk cuttings cultured *in vitro* on Gelrite® promoted the formation of callus-derived PLBs more than when agar was used as the medium solidifying agent (Ichihashi and Hiraiwa 1996; Ishii *et al*. 1998). Henderson and Kinnersley (1988) found that the dry weight of tobacco and wild carrot cultures on corn starch was three times more than that on medium gelled with agar. Zimmerman *et al*. (1995) also found a mixture of corn starch and Gelrite to be suitable substitutes for agar in the cultivation of apple and red raspberry. Sorvari (1986) found the starches from barley, corn, potato, rice and wheat to all be suitable substitutes to agar for the culture of barley seeds, although the most effective was that form barley. 'Isubgol', which is derived form the mucilaginous husk derived from the seeds of *Plantago ovata*, was used as an alternative gelling agent to agar in the tissue culture and seed germination of *Syzygium cuminiii* and *Datura innoxia* (Babbar and Jain 1998) and was also as effective as guar gum in the cost-effetcive multiplication of *Dendrobium chrysotoxum* (Jain and Babbar 2005). Chauvin *et al*. (1999) noted how the choice of gelling agent affected the regeneration efficiency on selective medium in tulip, gladiolus and tobacco transformation experiments. Several cocoa-based additives

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

Gelling agent	Appearance of PLBs	Explants forming callus (%)	No. PLBs/explant	*Neo* PLB fresh weight (mg)	*Neo* PLB dry weight (mg)
Agar*	Normal	96 a	11.67 ± 1.48 b	1042 ± 27 c	98 ± 6 c
Bacto agar	Normal	96 a	14.43 ± 0.85 ab	1238 ± 47 b	124 ± 6 b
Phytagel	Normal	96 a	11.86 ± 0.97 b	1078 ± 41 c	97 ± 3 c
Gellan gum	Normal	96 a	18.74 ± 1.35 a	1783 ± 63 a	156 ± 9 a
Oat meal agar	Abnormal (shrunken)	20 c	4.34 ± 1.37 c	398 ± 35 d	33 ± 4 d
Potato dextrose agar	Abnormal (shape)	8 d	6.87 ± 0.85 c	511 ± 23 d	49 ± 5 d
Corn starch + agar	Normal	48 b	14.43 ± 1.35 ab	1356 ± 29 b	132 ± 8 b

* = control. Data scored after 90 days and represent the mean ± SD (standard deviation) of at three replicates of $n = 20$ each. 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.

Table 2 Effect of alternative medium additives on *Cymbidium* Twilight Moon 'Day Light' PLB cultures and callus formation.

Medium additive	Appearance of PLBs	Explants forming callus (%)	No. PLBs/explant	*Neo* PLB fresh weight (mg)	*Neo* PLB dry weight (mg)
Control*	Normal	96 a	11.67 ± 1.48 a	1042 ± 27 a	98 ± 6 a
Full-fat milk	Abnormal (small)	0 b	1.26 ± 0.25 ab	98 ± 11 b	9 ± 1 b
Coca-cola®	Abnormal (small)	3 b	11.86 ± 0.97 b	0 c	0 c
Coffee	Abnormal (small)	0 b	18.74 ± 1.35 a	0 c	0 c
Green tea	Abnormal (small)	6 b	4.34 ± 1.37 c	43 ± 9 bc	4 ± 4 bc
Daarjeeling tea	Abnormal (small)	6 b	6.87 ± 0.85 c	36 ± 8 bc	3 ± 2 bc

* = agar-based medium without any additives. Data scored after 90 days and represent the mean ± SD (standard deviation) of at three replicates of $n = 20$ each. 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.

were found to be suitable for the propagation of *Phytophthora palmivora* (Awuah and Frimpong 2002), but (to the authors' knowledge) no studies on the impact of teas, coffee and milk on plant cultures have never been examined. Although the results of our alternative media additives were not successful, they provide some hope and scope for future experiments.

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