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Gelling Agent Affects Hybrid *Cymbidium* Plantlet Growth

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ABSTRACT

The choice of gelling agent impacted the growth and development of hybrid *Cymbidium* Maria 'Music Hour'. Gellan gum resulted, in general, in better plant growth parameters than liquid medium, Bacto agar and oatmeal agar. The number of roots was highest on Gellan gum as was the fresh and dry mass of shoots and roots although more leaves were produced on Bacto agar. These results point towards the need to test the agar source prior to growth of hybrid *Cymbidium* plantlets, since this medium substrate can strongly affect the outcome of an experiment.

Keywords: Gelling agent, *Cymbidium*, plantlet growth
Abbreviations: MP, "Miracle Pack"® culture system; **NAA**, α-naphthaleneacetic acid; **PLB**, protocorm-like body; **PGR**, plant growth regulator; **VW**, Vacin and Went

INTRODUCTION

The choice of gelling agent and the use of solid versus liquid medium are two of the most basic requirements for successful plant tissue culture (reviewed by Cameron 2008).

Cymbidium tissue culture is effective from the induction of protocorm-like bodies (PLBs). PLBs can themselves be induced naturally to form shoots (Teixeira da Silva and Tanaka 2006), without any change in medium, if left on the same medium. Even though medium formulation (Teixeira da Silva *et al*. 2005), biotic (Teixeira da Silva *et al*. 2006b) and abiotic factors (Teixeira da Silva *et al*. 2006a) affect PLB and callus formation from PLBs, the effect of gelling agent on plantlet growth and development has never been studied. In a previous and related study, Teixeira da Silva and Tanaka (2009) showed how the choice of gelling agent could affect the outcome of PLB formation and callus formation in hybrid *Cymbidium*, most likely due to their different physical properties (Prakash *et al*. 2004). Those findings spurred us further to investigate the effect on the next developmental level, namely shoot and root development *in vitro*. This is the focus of this study.

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* Maria 'Music Hour' (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) 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). All media were adjusted to different pHs (listed below for each gelling agent) with 1 N NaOH or HCL prior to autoclaving at 100 KPa for 17 min. PLB 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 (Plant Lux, Toshiba Co., Japan). 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 (Huan *et al*. 2004; Huan and Tanaka 2004).

Shoots were induced directly from PLBs. Longitudinally-bisected PLBs, 50 in total and 3-4 mm in diameter, were cultured in 300-ml flasks containing 120 ml of nutrient enriched-modified VW medium supplemented with 20 g l^{-1} sucrose, 50 g l^{-1} banana homogenate, 0.1 ml l^{-1} "Micro Health" (Bio U, Japan) and 0.5 g l^{-1} activated charcoal. After 3 months cultured, uniform shoots with approximately the same stem diameter size, 3 leaves (5 mm long) and no roots served as initial treatment explants.

Five shoots were cultured per 300-ml flask in 120 ml of solid medium or 200 ml of liquid medium in the case of the "Miracle Pack"® culture system (MP) experiments. Each treatment was repeated in triplicate.

Gelling agent

In order to test the effect on *Cymbidium* plantlet growth, three gelling agents and liquid medium were selected, as detailed next.
Bacto agar (control): 8 g l^{-1}, pH 5.3, Difco Labs, USA;
Gellan gum (Gelrite®): 2 g l^{-1}, pH 5.5, Merck & Co., USA;
Oatmeal agar: 72.5 g l^{-1}, pH 5.5, Sigma-Aldrich;
Liquid medium: no gelling agent, pH 5.3, sugar-free medium, CO_2 enrichment (3000 μmol mol^{-1} 24 h^{-1} d^{-1}). MP, as described by Tanaka *et al.* (1999), was used as the culture vessel.

Morphogenic analyses

The growth of plantlets (i.e., shoots and roots) was assessed. The following parameters were measured after 90 days: plant height (PH), root length (RL), SPAD value of leaf, number of leaves and roots, fresh and dry weight (FW and DW) and the FW/DW ratio of

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Short Communication

Table 1 Effect of gelling agent on hybrid *Cymbidium* Maria 'Music Hour' plantlet growth.

Gelling agent	PH	RL	SPAD *	Number of		Fresh mass of		Dry mass of		DWS/FWS	DWR/FWR
				Leaves	Roots	Shoots	Roots	Shoots	Roots		
Liquid	9.5 a	1.4 c	49.0 b	6.0 d	3.2 d	644.5 c	308.9 d	539.0 c	168.6 c	0.84 c	0.54 b
Gellan gum	9.4 a	4.0 a	49.6 ab	7.0 b	7.1 a	748.1 a	1388.3 a	680.7 a	784.7 a	0.92 a	0.57 ab
Bacto agar	7.3 b	3.6 b	52.0 a	7.5 a	6.1 b	697.8 b	987.8 c	635.5 b	571.9 b	0.90 ab	0.59 a
Oatmeal agar	9.5 a	3.5 b	42.2 c	6.5 c	5.3 c	679.7 bc	1124.1 b	609.4 b	622.2 b	0.90 b	0.56 ab

Means within a column followed by the same letters are not significantly different at $P < 0.05$ by Duncan's multiple range test

* Estimated chlorophyll content in the third leaf, counted from top downward, of the plantlet by SPAD chlorophyll meter

DWS, dry weight of shoot; **DWR**, dry weight of root; **FWS**, fresh weight of shoot, **FWR**, fresh weight of root; **RL**, root length; **PH**, plant height; **RL**, root length

Fig. 1 Growth of *Cymbidium* Maria 'Music Hour' plantlets on different gelling agents. (**A**) From left to right: Control (liquid medium on rockwool base in MP), Gellan gum, Bacto agar, oatmeal agar. (**B**) Growth of plantlets on oatmeal agar showing light-green leaves.

roots and shoots. DW was established after drying the shoots/roots in newspaper bags placed in a dry oven for 30 min at 105°C then 48 hrs at 60°C.

Chlorophyll content of the third leaf counting downwards from the plantlet apex was measured by a chlorophyll meter (SPAD-502, Minolta Co., Japan) and reported as the SPAD value (Teixeira da Silva *et al.* 2005).

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD). Data was subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's multiple range test (DMRT) using IRRISTAT version 3.0.

RESULTS AND DISCUSSION

The choice of gelling agent and/or solid vs liquid medium affected the organogenic outcome of hybrid *Cymbidium* Maria 'Music Hour' plantlet cultures (**Table 1, Fig. 1A**). Gellan gum resulted, in general, in better plant growth parameters than liquid medium, Bacto agar and oatmeal agar. The number of roots was highest on Gellan gum as was the fresh and dry mass of shoots and roots although more leaves were produced on Bacto agar. Interestingly, Gellan gum formed more PLBs that oat meal agar and potato dextrose agar in another hybrid *Cymbidium* (Teixeira da Silva and Tanaka 2009).

The chlorophyll content of the third leaf of *Cymbidium* plantlets grown in oatmeal agar was lowest among all treatments. The leaves were light green (see **Fig. 1B**) when the SPAD value of other treatments was no significantly different and the leaf color was dark green as uniform *Cymbidium* plant's leaf quality. The oatmeal agar based medium also strongly inhibited the initiation of new leaf and root compared to other gelling agents.

Conversely, in almost all plant growth parameters (except for PH and SPAD value), non-gelling agent (liquid medium), resulted in inhibited the growth of *Cymbidium* plantlets compared to the Gellan gum and Bacto agar treatments. In our study, photoautotrophic culture (CO_2 enriched-condition at 3000 µmol mol^{-1} $24h^{-1}$ d^{-1}, sugar-free medium, using MP as culture vessel) resulted in lower growth of *Cymbidium* plantlet than in the heterotrophic cultures.

The type of gelling agent strongly affected adventitious shoot regeneration capacity and the water content of *Tagetes* shoots (Jain *et al*. 2001; Modi *et al*. 2009). In *Dianthus*, as the agar concentration increases, so the number of hyperhydric shoots decreases (Casanova *et al*. 2008). In addition to reducing hyperhydricity, increasing the agar concentration can drastically reduce the shoot 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), *Pyrus* (Kadoka and Niimi 2003) and *Scrophularia yoshimurae* (Tsay *et al*. 2006). In this study, for *Cymbidium* plantlets, we did not observe hyperhydricity in any gel- or liquid-based media.

Agar is the most commonly used gelling agent in plant tissue culture (according to Babbar and Jain 1998), although Gellan gum or Gelrite®, a polymer of glucuronic acid, rhamnose, glucose and *O*-acetyl moieties (Scholten and Pierik 1998) is also a popular choice. 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 from 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-effective 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.

Although it is difficult to pin-point the possible reasons as to why different gelling agents might affect plant organogenesis, Beruto and Curir (2006) suggested that the level of impurities might be a contributing factor, as demonstrated for *Ranunculus asiaticus* shoots grown in three commercial agars.

REFERENCES

Babbar SB, Jain N (1998) 'Isubgol' as an alternative gelling agent in plant tissue culture. *Plant Cell Reports* **17**, 318-322

Beruto M, Curir P (2006) Effects of agar and gel characteristics on micropropagation: *Ranunculus asiaticus*, a case study. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II), Global Science Books Ltd., Isleworth, UK, pp 277-284

Cameron SI (2006) Plant tissue culture gelling agents and supports: History, development and function. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol V), Global Science Books Ltd., Isleworth, UK, pp 171-190

Casanova E, Moysset L, Trillas MI (2008) Effect of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. *Biologia Plantarum* **52**, 1-8

Chauvin J-E, Marhadour S, Cohat J, Le Nard M (1999) Effects of gelling agents on *in vitro* regeneration and kanamycin efficiency as a selective agent in plant transformation procedures. *Plant Cell, Tissue and Organ Culture* **58**, 213-217

George EF (1996) *Plant Propagation by Tissue Culture (Part 2) In Practice*, Exegetics, Basingstoke, 479 pp

Henderson WE, Kinnersley AM (1988) Corn starch as an alternative gelling agent for plant tissue culture. *Plant Cell, Tissue and Organ Culture* **15**, 17-22

Huan LT, Takamura T, Tanaka M (2004) Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid. *Plant Science* **166**, 1443-1449

Huan LT, Tanaka M (2004) Callus induction from protocorm-like body segments and plant regeneration in *Cymbidium* (Orchidaceae). *Journal of Horticultural Science and Biotechnology* **79**, 406-410

Ichihashi S, Hiraiwa H (1996) Effect of solidifier, coconut water, and carbohydrate source on growth of embryogenic callus in *Phalaenopsis* and allied genera. *Journal of the Orchid Society of India* **10**, 81-88

Ishii Y, Takamura T, Goi M, Tanaka M (1998) Callus induction and somatic embryogenesis of *Phalaenopsis*. *Plant Cell Reports* **17**, 446-450

Jain A, Kantia A, Kothari SL (2001) *De novo* differentiation of shoot buds from leaf callus of *Dianthus caryophyllus* L. and control of hyperhydricity. *Scientia Horticulturae* **87**, 319-326

Jain N, Babbar SB (2005) Guar gum and isubgol as cost-effective alternative gelling agents for *in vitro* multiplication of an orchid, *Dendrobium chrysotoxum*. *Current Science* **88**, 292-295

Kadoka M, Niimi Y (2003) Effects of cytokinin types and their concentration on shoot proliferation and hyperhydricity in *in vitro* pear cultivar shoots. *Plant Cell, Tissue and Organ Culture* **72**, 261-265

Modi P, Sinha A, Kothari SL (2009) Reduction of hyperhydricity in micropropagated French marigold (*Tagetes patula* L.) plants by modified medium parameters. *Floriculture and Ornamental Biotechnology* **3**, 40-45

Prakash S, Hoque MI, Brinks T (2004) Culture media and containers. *Low Cost Options for Tissue Culture Technology in Developing Countries. Proceedings of a Technical Meeting organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture*, 26-30 August 2002 Vienna, Austria, pp 29-40. Available online: http://www-pub.iaea.org/MTCD/publications/PDF/te_1384_web.pdf

Scholten HJ, Pierik RLM (1998) Agar as a gelling agent: chemical and physical analysis. *Plant Cell Reports* **17**, 230-235

Sorvari S (1986) The effect of starch gelatinized nutrient media in barley anther cultures. *Annales Academiae Scientiarum Fennicae* **25**, 127-133

Tanaka M, Yap DCH, Hew CS (1999) The physiology of *Cymbidium* plantlets cultured *in vitro* under conditions of high carbon dioxide and low photosynthetic photon flux density. *Journal of Horticultural Science and Biotechnology* **74**, 632-638

Teixeira da Silva JA, Chan MT, Sanjaya, Chai ML, Tanaka M (2006a) Priming abiotic factors for optimal hybrid *Cymbidium* (Orchidaceae) PLB and callus induction, plantlet formation, and their subsequent cytogenetic stability analyses. *Scientia Horticulturae* **109**, 368-378

Teixeira da Silva JA, Giang DDT, Tanaka M (2005) Photoautotrophic micropropagation of *Spathiphyllum*. *Photosynthetica* **44**, 53-61

Teixeira da Silva JA, Giang DTT, Chan M-T, Sanjaya, Norikane A, Chai M-L, Chico-Ruíz J, Penna S, Granström T, Tanaka M (2007) The influence of different carbon sources, photohetero-, photoauto- and photomixotrophic conditions on protocorm-like body organogenesis and callus formation in thin cell layer culture of hybrid *Cymbidium* (Orchidaceae). *Orchid Science and Biotechnology* **1**, 15-23

Teixeira da Silva JA, Singh N, Tanaka M (2006b) Priming biotic factors for optimal protocorm-like body and callus induction in hybrid *Cymbidium* (Orchidaceae), and assessment of cytogenetic stability in regenerated plantlets. *Plant Cell, Tissue and Organ Culture* **84**, 119-128

Teixeira da Silva JA, Tanaka M (2006) Embryogenic callus, PLB and TCL paths to regeneration in hybrid *Cymbidium* (Orchidaceae). *The Journal of Plant Growth Regulation* **25**, 203-210

Teixeira da Silva JA, Tanaka M (2009) Impact of gelling agent and alternative medium additives on hybrid *Cymbidium* protocorm-like body and callus formation. *Floriculture and Ornamental Biotechnology* **3**, 56-58

Teixeira da Silva JA, Yam T, Fukai S, Nayak N, Tanaka M (2005) Establishment of optimum nutrient media for *in vitro* propagation of *Cymbidium* Sw. (Orchidaceae) using protocorm-like body segments. *Propagation of Ornamental Plants* **5**, 129-136

Tsay H-S, Lee C-Y, Agrawal DC, Basker S (2006) Influence of ventilation closure, gelling agent and explant type on shoot bud proliferation and hyperhydricity in *Scrophularia yoshimurae* – a medicinal plant. *In Vitro Cellular and Developmental Biology – Plant* **42**, 445-449

Turner SR, Singha S (1990) Vitrification of crabapple, pear and gum on gellan gum-solidified culture medium. *Horticultural Science* **25**, 1648-1650

Vacin E, Went FW (1949) Some pH changes in nutrient solutions. *Botanical Gazette* **110**, 605-613

Zimmerman RH, Bhardwaj SV, Fordham IM (1995) Use of starch-gelled medium for tissue culture of some fruit crops. *Plant Cell, Tissue and Organ Culture* **43**, 207-213