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How Does Choice of Substrate and Culture Conditions Affect the Growth and Development of *Cymbidium* cv. Green Planet 'Energy Star' Protocorm-like Bodies?

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Summary

The choice of gelling agent and culture condition affected the growth and development of hybrid *Cymbidium* Green Planet 'Energy Star' protocorm-like bodies (PLBs). A liquid medium culture system using a rockwool support in a Techno pot® under heterotrophic culture (sucrose-containing medium with non-enriched CO_2 conditions) resulted in improved plant growth compared with standard-agar, gellan gum and oatmeal agar used as substrate. Since the majority of explants (96.7 %) died in non-agitated liquid culture, a static liquid culture system is not recommended. *Cymbidium* PLBs showed superior properties (higher survival rate of explants, more PLBs, and higher total fresh weight and dry weight of *neo*-PLBs) when cultured under heterotrophic conditions than when cultured under photoautotrophic conditions.

Key words. *Cymbidium* – heterotrophic – photoautotrophic – protocorm-like body – substrate

Introduction

The choice of gelling agent and the use of solid *vs.* liquid medium are two of the most basic requirements for successful plant tissue culture (reviewed by Cameron 2008). In addition, the choice of culture vessel, the size and volume of the vessel and the density of the explants, as well as the culture conditions are several factors affecting the organogenic outcome of a tissue when cultured *in vitro*, especially in orchids which are in general more sensitive to culture condition parameters *in vitro* than other ornamentals (Teixeira da Silva and Tanaka 2009a).

Cymbidium can be effectively tissue cultured when using protocorm-like bodies (PLBs) as explants (Teixeira da Silva et al. 2006a). PLBs naturally form shoots (Teixeira da Silva and Tanaka 2006), even if left on the same PLB-inducing medium, although the organs from which other organs can or are derived appears to be strictly controlled, despite the attempt to manipulate multiple biotic and abiotic parameters *in vitro* (Teixeira da Silva and Tanaka 2006). Medium formulation (Teixeira da Silva et al. 2005b), biotic factors (Teixeira da Silva et al. 2006b), abiotic factors (Teixeira da Silva et al. 2006a), and choice of gelling agent all have a profound effect on PLB and callus formation as well as plantlet growth and development. Previously, Teixeira da Silva and Tanaka (2009a) showed how the choice of gelling agent or culture vessel (Teixeira da Silva and Tanaka 2009b) could affect the outcome of PLB, callus, or plantlet formation in the hybrid *Cymbidium*, most likely due to their various physical properties (Prakash et al. 2004).

Over two decades, the concept of photoautotrophic micropropagation has been proposed as a means to reduce production costs and install automation-robotization of the micropropagation process by minimal microbial contamination, increased photosynthetic rate, growth and rooting *in vitro* and survival percentage *ex vitro* (review by Kozai 1991; Norikane et al. 2010). Previous studies by Tanaka (1992) and Hahn and Paek (2001) on *Phalaenopsis* plantlet development concluded that photoautotrophic culture, compared with heterotrophic growth, resulted in improved growth parameters such as larger and more vigorous uniform plantlets. As observed in the development of *Cymbidium* PLBs, explants cultured under photoautotrophic conditions (sugar-free liquid medium, CO_2 enrichment and high light intensity) had poorer performance *in vitro* those cultured with sugar and non-CO_2 enrichment, low light intensity (Kubota et al. 1992). In contrast to the result reported by Kubota et al. (1992), Teixeira da Silva et al. (2007) showed that photoautotrophic conditions led to higher callus and PLB fresh and

dry weight, number of PLBs, and more robust hybrid *Cymbidium* plants.

In most of the above studies, half-PLBs (½-PLBs) or PLBs cut longitudinally (i.e. not along the equatorial plane) were used as explants, although whole PLBs or thin cell layers of the outer PLB layers can equally be used, albeit a lower regeneration capacity from the latter two explant types (Teixeira da Silva and Tanaka 2006).

Despite these widely revealing studies, the ability to induce PLBs on different substrates under photoautotrophic or heterotrophic conditions has never been examined for any orchid. This gap in the literature, and inspired by earlier findings, spurred us to investigate further, in this paper, whether the choice of substrate and culture conditions could affect PLB productivity.

Materials and Methods

Plant material, explants and culture conditions for PLB proliferation

Hybrid *Cymbidium* Green Planet 'Energy Star' (Bio-U, Japan) PLBs that originated from shoot-tip culture on Vacin and Went (VW) (Vacin and Went 1949) agar medium with no added plant growth regulators, were induced and ½-PLBs were subcultured (PLB induction and proliferation) every 2 months on modified VW supplemented with Nitsch's microelements (Nitsch and Nitsch 1967), 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA, Nacalai Tesque, Kyoto, Japan) and 0.1 mg l^{-1}kinetin (Wako Chemicals Ltd., Tokyo, Japan), 2 g l^{-1} tryptone (Bacto, Difco Laboratories, Sparks, MD), i.e. VWPLB (Teixeira da Silva et al. 2005a). All media were adjusted to pH 5.3 or 5.5 depending on substrate with 1 N NaOH or 1 N HCl prior to autoclaving at 100 KPa for 17 min. PLBs were cultured at 25 ± 1 °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), with or without CO_2 enrichment. All cultures were static, i.e. not shaken. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva et al. 2005a), 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).

Substrate, liquid medium and photoautotrophic treatments

In order to test the effect of the following parameters on *Cymbidium* PLB growth and development, five treatments were tested as follows:

Treatment 1. Agar (control): The medium tested was VW_{PLB} with 20 g l^{-1} sucrose, solidified with 8 g l^{-1} agar (Wako, Osaka, Japan), pH 5.3. Ten ½-PLBs were cultured in 40 ml medium in 100-ml Erlenmeyer flasks, double-capped with aluminum foil.

Treatment 2. Gellan gum (Gelrite®): The VW_{PLB} was solidified with 2 g l^{-1} Gellan gum (Merck & Co., USA), pH 5.5; all other conditions were identical to the control.

Treatment 3. Oatmeal agar: 30 g l^{-1} Oatmeal agar (Sigma–Aldrich, St. Luis, MO) was used as the gelling agent. The pH of the medium was adjusted to 5.5; all other conditions were identical to the control.

Treatment 4. Liquid medium: Techno pot® (Sumiron Co., Ltd, Osaka, Japan) (TP) with liquid VW_{PLB} (110 ml) and no gelling agent, pH 5.3. Ten explants (½-PLBs) were placed in each TP. Container caps were perforated in the centre with a hole 4 mm in diameter and covered by a Milliseal® (Japan Millipore Co., Ltd, Tokyo, Japan) to increase the ventilation ability of the vessel. The container was sealed with double layers of Parafilm® (Pechiney Plastic Packing, Chicago, IL).

There were two sub-treatments, namely:

* Treatment 4.1 TP vessel, CO_2-enrichment (3000 μmol mol^{-1} 24 h^{-1} d^{-1}), sugar-free liquid medium;
* Treatment 4.2 TP vessel, no CO_2-enrichment, with 20 g l^{-1} (w/v) sucrose liquid medium.

5. Treatment 5. Rockwool (RW):TP with 110 ml of cool autoclaved liquid VW_{PLB}, pH 5.3 poured evenly over a 20 (4 × 4) mm^2 joined-block RW base (Grodan® RW Multiblock™, AO 18/30, Grodiana A/S, Denmark) and no gelling agent. The RW was sterilized in a dry sterilizer at 150 °C for 2 h and placed in the TP when at room temperature (Van et al. 2012). One explant (½-PLB) was placed on top of each RW block, at ten ½-PLBs/TP (Fig. 1). There were 2 sub-treatments:

* Treatment 5.1. TP vessel, RW substrate, CO_2-enrichment (3000 μmol mol^{-1} 24 h^{-1} d^{-1}), sugar-free liquid medium.
* Treatment 5.2. TP vessel, RW substrate, no CO_2-enrichment, with 20 g l^{-1} (w/v) sucrose liquid medium.

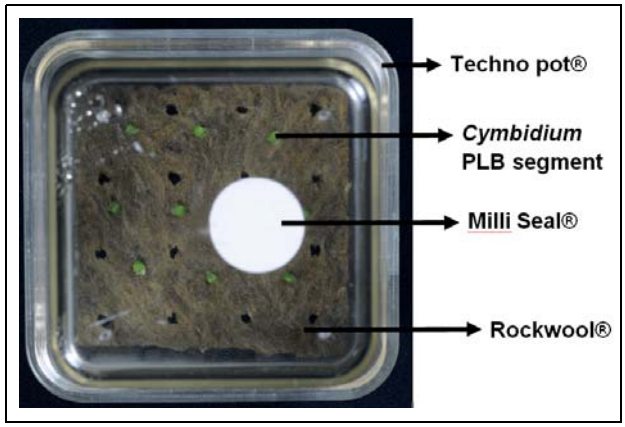


Fig. 1. Top view of Rockwool®-based Techno pot® system for „seeding" *Cymbidium* PLBs with a Milli Seal® to allow for improved aeration.

All culture vessels from all five treatments (and four subtreatments) were placed under temperature and light conditions identical to those used for *neo*-PLB induction and proliferation.

Morphogenic analyses

The growth and development of PLBs were evaluated after eight weeks following culture initiation of the explant (i.e., ½-PLB) as survival rate and total number of newly formed PLBs (i.e., *neo*-PLBs) per explant, total fresh weight (FW) and total dry weight (DW). DW was determined after material was dried in an oven at 105 °C for 30 min and then at 60 °C for 48 h. The survival rate of PLBs was calculated based on the number of explants forming PLBs as a percentage of the total number of explants. For total number of newly formed PLBs, only green PLBs > 3 mm in diameter with a round and uniform shape were counted.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD, n = 21). The entire experiment was repeated twice and each experiment had three replicates. For all parameters tested, data analyses were carried out using IRRISTAT version 3.0. Following one-way analysis of variance (ANOVA), Duncan's multiple range test (DMRT) at P = 0.05 and the Student's-*t*-Distribution (standard error, Excel 2010) were used to test for differences between means.

Results and Discussion

The type of substrate affected the organogenesis of *Cymbidium* cv. Green planet 'Energy Star' ½-PLBs (Table 1). More than 90 % of the initial ½-PLBs cultured on static-liquid medium under photoautotrophic or heterotrophic micropropagation conditions died, while explants that survived (< 10 %) induced abnormal *neo*-PLBs (Fig. 2A). The survival rate of the explants (½-PLBs) cultured on semi-liquid medium using RW as the substrate (97 and 93 % for heterotrophic and photoautotrophic culture, respectively) was higher than that on solid media (solidified using 82 % agar, 92 % Gellan gum or 55 % oatmeal agar). Under the same culture conditions and culture system, the growth and development of explants cultured on solid medium which was either solidified by agar, Gellan gum or oatmeal agar showed no significant differences except for total DW of newly formed PLBs (*neo*-PLBs) cultured on oatmeal agar medium, which was higher than the DW of other treatments (Fig. 2B). However, the ½-PLBs cultured on oatmeal agar had a shrunken appearance or could only induce weak or abnormal *neo*-PLBs. Most of the *neo*-PLBs turned yellowish, had an abnormal shape, and tended to die (Fig. 2C). Interestingly, in previous studies with another hybrid *Cymidium* Twilight Moon 'Day Light', explants cultured in Gellan gum formed more *neo*-PLBs than on oat meal agar, agar and potato dextrose agar (Teixeira da Silva and Tanaka 2009a). The ½-PLBs cultured on RW under heterotrophic micropropagation (photosynthetic photon flux (PPF) of 45 μmol m^{-2} s^{-1}, sugar-containing medium with no CO_2 enrichment) had significantly more *neo*-PLBs and total FW/DW of *neo*-PLBs than ½-PLBs cultured under photoautotrophic conditions (sugar-free medium and high CO_2 concentration of 3000 μmol mol^{-1}), and compared to those cultured on solid medium (Table 1, Fig. 2B, 2D). These results were similar to the results reported previously in another hybrid *Cymbidium* (Teixeira da Silva et al. 2007). Moreover, the *neo*-PLBs formed on the explants cultured on RW under heterotrophic conditions were healthy and had a uniform shape i.e., they were round, individually separated,

Table 1. Effect of choices of substrate and culture condition on *Cymbidium* cv. 'Green Planet' PLB growth and development.

Treatments*	Survival rate (%)	No of PLBs	Fresh weight (mg)	Dry weight (mg)
Agar (control)	82	10.18 b ± 1.30	211.58 b ± 16.93	14.77 b ± 1.55
Gellan gum	92	9.56 b ± 1.86	216.16 b ± 25.47	15.75 b ± 1.65
Oatmeal agar	55	11.09 b ± 5.29	233.72 b ± 102.28	18.16 a ± 6.93
Rockwool (VW liquid medium, sucrose+, CO_2–)	97	12.92 a ± 1.76	274.06 a ± 32.78	19.35 a ± 2.52
Rockwool (VW liquid medium, sucrose–, CO_2+)	93	4.03 c ± 1.00	61.18 c ± 7.55	2.88 c ± 0.28
VW Liquid medium (sucrose+, CO_2–)	10	N	N	N
VW Liquid medium (sucrose–, CO_2+)	0	N	N	N

Means within a column followed by the same letters are not significantly different at P = 0.05 by Duncan's multiple range test, n = 21 and Standard Error (SE).
VW: Vacin and Went, N: Data not available

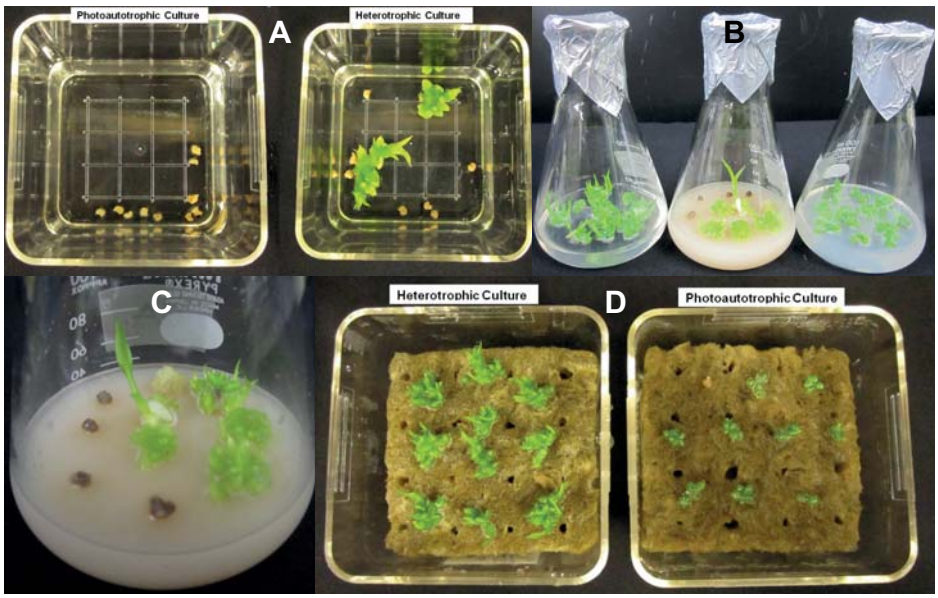


Fig. 2. (A): Growth of *Cymbidium* Green Planet 'Energy Star' PLB on liquid medium. From left to right: photoautotrophic culture, heterotrophic culture; (B): Growth of *Cymbidium* Green Planet 'Energy Star' PLB on different gelling agents. From left to right: Gellan gum, oatmeal agar, agar (control); (C): Growth of *Cymbidium* Maria Green Planet 'Energy Star' PLB on oatmeal agar showing unhealthy newly formed PLBs. (D): Growth of *Cymbidium* Maria Green Planet 'Energy Star' PLB on rockwool. From left to right: heterotrophic and photoautotrophic culture.

~3–5 mm in diameter and could regenerate plantlets earlier than the *neo*-PLBs derived from all other treatments (Fig. 2D). Kubota et al. (1992) also found that *Cymbidium* cv. 'Lisa rose' PLBs cultured under photoautotrophic micropropagation (high PPFD of 140 μmol m^{-2} s^{-1}, high CO_2 concentration of 2000 μmol mol^{-1}, sugar-free medium) had significantly fewer *neo*-PLBs and DW per unit PLB than PLBs cultured on sucrose-containing medium under low PPFD (20 μmol m^{-2} s^{-1}) and a low (i.e., ambient) CO_2 concentration (450 μmol mol^{-1}). Conversely, Honjo et al. (1988) reported that PLBs with a low sucrose concentration and CO_2 enrichment seemed healthier than that in non-photoautotrophic condition.

Although agar is believed to be the most commonly used gelling agent in plant tissue culture (Babbar and Jain 1998), particularly in orchid culture, Gellan gum or Gelrite®, a polymer of glucuronic acid, rhamnose, glucose and *O*-acetyl moieties (Scholten and Pierik 1998) are also popular choices. The way in which agar and Gellan gum work as a gelling agent is practically very different. Agar functions by binding water, thus the higher the agar concentration, the stronger the water is bound (Beruto et al. 1999). In contrast, Gelrite® requires the presence of cations for gelation (http://www.accurx.net/Gelrite_Spec_Sheet.pdf). Agar contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture vessels (Debergh 1983). When *Phalaenopsis* leaf segments obtained from shoots derived from flower-stalk cuttings were cultured *in vitro* on a Gelrite®-solidified VW medium, more callus-derived PLBs formed than when agar was used as the medium solidifying agent (Ichihashi and Hiraiwa 1996; Ishii et al. 1998). Similarly, the dry weight of tobacco (*Nicotiana tabacum*) and carrot (*Daucus carota*) cultures on corn starch was three times higher than on medium gelled with agar (Henderson and Kinnersley 1988) while a mixture of corn starch and Gelrite were suitable substitutes for agar in the *in vitro* cultivation of apple (*Malus pumila*) and red raspberry (*Rubus idaeus*) (Zimmerman et al. 1995). Sorvari (1986) found that starch from barley, corn, potato, rice and wheat were all suitable substitutes for agar in the culture of barley (*Hordeum vulgare*) seeds, although the most effective was that from barley. 'Isubgol' is derived from the mucilaginous husks derived from the seeds of *Plantago ovata* and is increasingly used as an alternative gelling agent to agar, for example in the tissue culture and seed germination of *Syzygium cuminiii* and *Datura innoxia* (Babbar and Jain 1998). 'Isubgol' was also as effective as guar gum in the cost-effective multiplication of *Dendrobium chrysotoxum* (Jain and Babbar 2005). Beruto and Curir (2006) suggested that the level of impurities within a gelling agent might contribute to the outcome of an organogenic pathway, as was demonstrated for *Ranunculus asiaticus* shoots grown in basal medium containing one of three commercial agars (Oxoid, Merck, and Roth).

However, recently, because of concerns about the high price of pure grade of agar, its non-toxic nature and the exclusivity of using this solidifying agent possibly resulting in over-exploitation of its resources, alternative substrates for agar, liquid, or semi-liquid culture systems have being discovered and explored (Sarifi et al. 2010). Alternative substrates for agar such as Bacto agar, phytagel, Gellan Gum, oatmeal agar, potato dextrose agar and corn starch were tested on the proliferation of *Cymbidium* PLB cv. Twilight Moon 'Day Light'; Gellan gum was superior to all other substrates in *neo*-PLB proliferation (Teixeira da Silva and Tanaka 2009a). In this study, we shared the same conclusion as that found by Teixeira da Silva et al.

(2006a) in which it was demonstrated that solid medium (solidified by agar or Bacto agar or Gellan gum) supported higher *Cymbidium neo*-PLB production than either liquid culture or membrane rafts (filter-sterilized filter paper wicks placed in 10 ml liquid medium in a 100-ml test tube) in terms of number of *neo*-PLBs produced.

Liquid culture systems have some advantages: culture conditions are much more uniform, media can be changed easily and the production cost is reduced compare to solid medium (Berthouly and Etienne 2005). *In vitro* liquid culture has been successfully used in the *in vitro* culture of conifers (Pramod and Roger 2005), rose (Tulecke 2006), *Chrysanthenum* (Hahn and Paek 2005), *Doritaenopsis* seedlings (Tsai and Chu 2008), *Phalaenopsis* (Park et al. 2000), and *Cymbidium* (Huan and Tanaka 2004). The major disadvantage of liquid medium is hyperhydricity, which is a severe physiological disorder (Berthouly and Etienne 2005; Tsai and Chu 2008) while static liquid medium has low aeration (Mehrotra et al. 2007). In the present studies, these are two strongly potential causes for the death of > 90 % of initial *Cymbidium* ½-PLBs cultured and for the hyperhydricity observed in the remaining < 10 %. Similar findings were found with *Cymbidium* PLB cv. Twilight Moon 'Day Light' (Teixeira da Silva, unpublished results), which are not in agreement with the results reported previously using the same cultivar **(**Huan and Tanaka 2004), suggesting that this topic needs to be further studied. One possible way of overcoming these inherent problems associated with liquid culture of PLBs, could be by shaking, temporary immersion in temporary immersion systems, as were shown to be successful for (Sanchéz et al. 2011), or the use of bioreactors, although the latter system tends to increase capital layout costs considerably, increase the risk of contamination and mechanical damage, or foam formation in bubble-aerated bioreactors (Berthouly and Etienne 2005).

Another popular choice is to create a semi-liquid or semi-solid medium. The use of cotton fiber support to create semi-liquid media has been reported in the micropropagation of *Dendrobium* (Puchooa 2004), banana (*Musa* AAA, Albany et al. 2005), *Chrysanthemum* (Hahn and Peak 2005) and potato (*Solanum tuberosum* L.) (Kanwal et al. 2006). Pieces of glass wool, nylon and filter paper were used as supporting bridges to create a semi-solid medium for the proliferation of *Chrysanthenum*, *Cattleya* (Adelberg et al. 1997), and *Cymbidium* PLBs (Teixeira da Silva et al. 2006a). The success of the Grodan RW block in this study as a novel substrate for PLB proliferation and *CyTPidium* micropropagation lends it as a potentially important alternative substrate that could substitute agar and Gellan gum as current main-stream substrates. RW is made from molten rock spun into fibers like cotton candy which ensures a good distribution of water and optimum aeration and water balance and the nutrient solution. Moreover, the blocks are tapered or V shaped to create an air space below to allow for air pruning. While the gel might not always solidify again, the RW can be heat sterilized after use and re-bagged thus saving cost for growers (Online information: http://www.hydroponics.net/c/118), although current supplies tend to be from Denmark, making import costs high. RW has already been applied as a very useful susbstrate for the micropropagation of difficult-to-root woody ornamentals such as *Amelanchier, Cercis canadensis*, cherry (*Prunus avium*), and apple (Chu and Mudge 1996) or for improving the growth of *Spathiphyllum* compared to gelling medium (Tanaka 1991). RW was also claimed was the most useful substrate for the production of strawberry (*Fragaria virginiana*) plants (Bartczak et al. 2007). In the micropropagation of orchids, RW and liquid medium system was used widely for the *in vitro* culture of *Phalaenopsis* and *Cymbidium* plantlet (Tanaka 1992; Van et al. 2011a, 2011b). In this study, we used a culture system that combined the advantages of RW (i.e., high aeration, strong water-holding capacity) to overcome the weakness of static liquid culture for the proliferation of *Cymbidium neo*-PLBs. The greatest survival rate, total number, FW and DW of *neo*-PLBs from 10 original ½-PLBs could be achieved in 2 months by using this RW-liquid medium-heterotrophic (sugar contained, no CO_2 enrichment) culture system, leading to *neo*-PLBs with a more uniform shape and size and from which only a single shoot arose (i.e. avoiding multiple shoot formation from PLB clusters commonly reported in the literature by other groups).

Finally, of extremely practical importance and interest, this system has been implemented in a commercial orchid tissue culture lab, BioU, Tokushima, Japan.

Conclusion

The growth and development of *Cymbidium* Green Planet 'Energy Star' PLBs was examined in different substrates (agar, Gellan gum, oatmeal agar, no-substrate (liquid medium), RW) and culture conditions (photoautotrophic and heterotrophic culture). The proliferation of *Cymbidium neo*-PLBs was best in the RW-liquid-TP culture system under heterotrophic culture.

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