Journal of Fruit and Ornamental Plant Research Vol. 20(2) 2012: 127-133
DOI: 10.2478/v10290-012-0022-8

# NEW BASAL MEDIA FOR PROTOCORM-LIKE BODY AND CALLUS INDUCTION OF HYBRID *Cymbidium*

## Short communication

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(Received August 29, 2012/Accepted September 26, 2012)

A B S T R A C T

High frequency protocorm-like body (PLB) production from hybrid *Cymbidium* Twilight Moon 'Day Light' has been developed through a new medium, Teixeira *Cymbidium* (TC) medium. Two new TC media containing variable amounts of macro- and micronutrients and other additives, inspired by Winarto and Teixeira (WT) medium for *Anthurium* and Murashige and Skoog (MS) basal medium were used to induce PLBs and callus. Control medium was research- and industry-standard Vacin and Went (VW) medium. The first TC medium, $TC_{PLB}$, could induce significantly more PLBs than on VW while high levels of macronutrients in the second TC medium, $TC_{CALLUS}$, and MS were required to induce callus. All PLB induction media contained 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (KIN), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar while callus-induction media were identical, except that KIN was substituted by thidiazuron (TDZ). Basal medium had a significant effect on PLB and callus formation. This protocol could be used to induce PLBs and callus from other *Cymbidium* species or cultivars.

**Key words**: basal medium, PLBs and callus, Teixeira *Cymbidium* (TC) medium, Winarto and Teixeira (WT) medium

**Abbreviations**: MS, Murashige and Skoog; 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

Plant tissue culture and its ability to regenerate and propagate plants *in vitro* are primarily dependent on the culture medium. Culture medium is a source of inorganic nutrients (micro- and macro-elements, etc.) and organic compounds (e.g., vitamins, etc.), and in most cases requires a carbohydrate (most often sucrose) to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. Photoautotrophic micropropagation provides carbon dioxide ($CO_2$) to substitute other carbon sources (Kozai et al., 2005). Plants often require trace amounts of organic compounds such as vitamins, and plant growth regulators, or PGRs, to stimulate organogenesis while the extent and quality of that response depends on the levels supplied, although this tends to be genotype-dependent (George et al., 2007; Niedz and Evens, 2007).

In the history of plant tissue culture, the formulae of several basal media is often based on the names of the scientists who have explored and developed them, including, for example, Murashige and Skoog (MS, 1962), Vacin and Went (VW, Vacin and Went, 1949), Gamborg's (Gamborg et al., 1968) and White's (White, 1943), which have been successfully established for the tissue culture of various plants and explants, and research objectives, and usually the best way to search for an ideal medium is to test already established media. In a previous study on C*ymbidium* micropropagation, Teixeira da Silva et al. (2005) tested 14 media formulations and found four patterns of development associated with the medium type: group 1 media tended to enhance callus and PLB formation; group 2 media promoted PLB formation with very little callus; group 3 media promoted good callus formation but very few PLBs); group 1 media produced a small amount of both callus and PLBs. Translated, for hybrid *Cymbidium* PLB formation, a high nutrient medium base such as MS contains minerals at high concentrations, developed originally for tobacco which can withstand strong levels of nutrients *in vitro*, and a weaker nutrient medium is required, ideally VW. Ironically, Wimber in 1963 and Morel in 1964, in their pioneering work, were the first ever to tissue culture *Cymbidium*, using MS medium. Indeed this was the first orchid to ever be tissue cultured.

The discovery of an appropriate basal medium is an important way to address the development of plants *in vitro*, e.g. Winarto-Teixeira (WT) medium developed by Winarto et al. (2011) for *Anthurium*.

This study concentrated on formulating new basal media suitable for inducing PLBs and callus for hybrid *Cymbidium*.

## MATERIAL AND METHODS

### Chemicals and reagents

All 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) originally developed spontaneously 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 α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (KIN), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA) (Teixeira da Silva et al., 2005). Callus induction and proliferation medium ($VW_{CALLUS}$) was similar to $VW_{PLB}$, except that thidiazuron (TDZ) was used instead of KIN (Teixeira da Silva and Tanaka, 2006). 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 $\mu mol/m^2/s^1$ 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.

Two new PLB-induction media, Teixeira *Cymbidium* (TC) medium, TC-1 and TC-2, inspired by but different to Winarto-Teixeira (WT) medium for *Anthurium* (Winarto et al., 2011), were tested against the industry standards, VW and half-strength MS (Tab. 1). In all of these media, PGRs, sucrose and growth conditions were identical to $VW_{PLB}$. The exact same four media indicated in Table 1 were then tested for callus induction, with all conditions identical for $VW_{PLB}$, except that KIN was substituted by TDZ ($VW_{CALLUS}$; Teixeira da Silva and Tanaka, 2006).

**Morphogenic analyses**

The number of PLBs formed per PLB segment as well the percentage of PLB segments that formed callus were measured. All measurements were made after 45 days in culture.

**Statistical analyses**

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment (i.e., each medium). All experiments were repeated in triplicate (n = 30, total sample number per treatment). 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® ver. 6.12 (SAS Institute, Cary, NC, USA), following Westfall et al. (1999).

Table 1. Two Teixeira *Cymbidium* (TC) basal medium compositions (TC-1 and TC-2), modified half-strength Murashige and Skoog (MS) and Vacin and Went (VW) media tested for PLB formation and callus induction in half-PLB culture of hybrid *Cymbidium* Twilight Moon 'Day Light'

| Medium component | Basal medium compositions tested | | | |
|---|---|---|---|---|
| | TC-1 | TC-2 | 1/2 MS | VW |
| Macronutrients | | | | |
| $NH_4NO_3 \cdot 4H_2O$ | 500 | 700 | 825 | 500 |
| $KNO_3$ | 500 | 1000 | 600 | 525 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | - | 392.5 | - | - |
| $Ca_3(PO_4)_2$ | - | - | - | 200 |
| $MgSO_4 \cdot 7H_2O$ | 150 | 200 | 195 | 250 |
| $CaCl_2$ | 300 | - | 220 | - |
| $NaH_2PO_4 \cdot H_2O$ | 200 | - | - | - |
| $KH_2PO_4$ | 150 | 165 | 85 | |
| Micronutrients | | | | |
| $H_3BO_3$ | 5.7 | 6.2 | 6.2 | - |
| KI | 0.65 | - | 0.83 | - |
| $MnSO_4 \cdot 4H_2O$ | 15.5 | 2.85 | 16.9 | 7.5 |
| $ZnSO_4 \cdot 7H_2O$ | 7.5 | 5.35 | 10.6 | - |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.2 | 0.2 | 0.25 | - |
| $CuSO_4 \cdot 5H_2O$ | 0.02 | 0.025 | 0.025 | - |
| $CoCl_2 \cdot 6H_2O$ | 0.02 | 0.025 | 0.025 | - |
| $Na_2EDTA \cdot 2H_2O$ | 37.3 | 37.3 | 37.3 | - |
| $Fe_2SO_4 \cdot 7H_2O$ | 27.5 | 27.5 | 27.5 | 25 |
| Vitamins | | | | |
| Glycine | - | - | 2.0 | - |
| *Myo*-inositol | 110.0 | 50.0 | 100.0 | - |
| Nicotinic acid | - | 0.5 | 0.5 | - |
| Pyridoxine-HCl | - | 0.3 | 0.5 | - |
| Thiamine-HCl | 0.5 | 1.0 | 0.1 | - |

All values in mg/l. See text for all other media constituents

## RESULTS AND DISCUSSION

TC-1 and VW could effectively form PLBs while TC-2 and 1/2 MS formed callus effectively. Consequently TC-1 was named as $TC_{PLB}$ while TC-2 was named as $TC_{CALLUS}$. This indicates that high concentrations of macronutrients together with KIN stimulate PLBs while low levels of macronutrients with TDZ stimulate callus (Tab. 2, 3 and Fig. 1).

In hybrid *Cymbidium*, a total of 14 basal media were tested for their ability to promote organogenesis from PLBs (Teixeira da Silva et al., 2005). The inclusion of tryptone in the medium improves callus regeneration and

Table 2. Effect of medium (TC) composition on PLB formation from half-PLB culture of hybrid *Cymbidium* Twilight Moon 'Day Light'

| Medium composition (TC) | Percentage of explants forming *neo*-PLBs [%] | Number of PLBs per explant | Fresh weight [mg] of PLB explant + *neo*-PLBs |
|---|---|---|---|
| VW (control) | 100 a | 7.8 a | 485 b |
| TC-1 | 100 a | 8.3 a | 526 a |
| TC-2 | 82 ab | 2.6 b | 128 c |
| 1/2 MS | 76 b | 1.8 b | 107 c |

Notes: Mean values followed by the same letter in the same column are not significantly different based on DMRT (P = 0.05). See text for media constituents. n = 90

Table 3. Effect of medium (TC) composition on callus formation from half-PLB culture of hybrid *Cymbidium* Twilight Moon 'Day Light'

| Medium composition (TC) | Percentage of explants forming callus [%] | Fresh weight [mg] of callus |
|---|---|---|
| VW (control) | 16 b | 21 b |
| TC-1 | 23 b | 16 b |
| TC-2 | 84 a | 64 a |
| 1/2 MS | 91 a | 56 a |

Notes: Mean values followed by the same letter in the same column are not significantly different based on DMRT (*P* = 0.05). See text for media constituents. n = 90

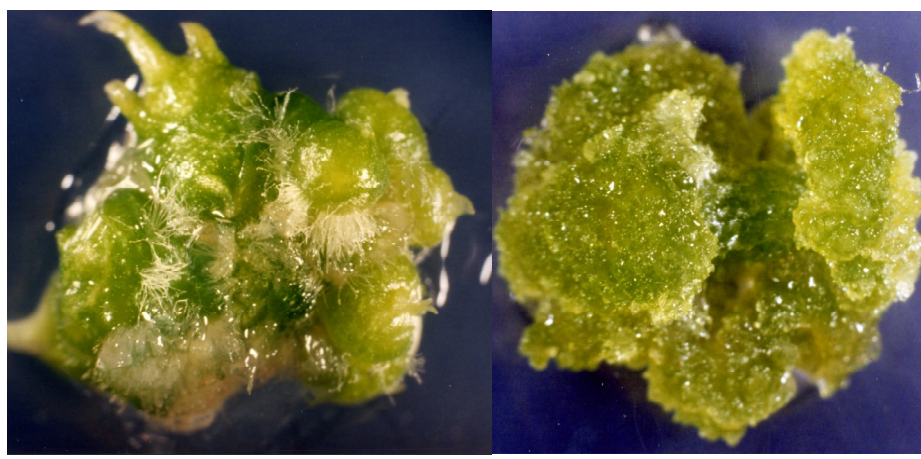

**Figure 1**. (Left) PLB induction on TC-1 ($TC_{PLB}$); (Right) Callus induction on TC-2 ($TC_{CALLUS}$)

proliferation (Huan et al., 2004). Namely, VW medium and similar media with weak concentrations of macro- and micro-nutrients (TC-1 or $TC_{PLB}$ in this study), were necessary for PLB induction and formation while higher levels of these nutrients were required for callus formation, namely MS, 1/2MS and TC-2 (i.e., $TC_{CALLUS}$). Winarto et al. (2011) developed, though extensive media testing, a new medium, Winarto-Teixeira (WT) medium, for *Anthurium*. Several studies, including this study, have indicated that basal medium is key to the success of a tissue culture protocol. For example, MS medium was a *sine qua non* basal medium for lily (*Lilium longiflorum*) (Nhut et al., 2002) and strawberry (*Fragaria* × *ananassa*) (Sutan et al., 2010).

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# NOWE POŻYWKI PODSTAWOWE DO INDUKCJI TWORÓW PROTOKORMOWYCH I KALUSA U HYBRYDY *Cymbidium*

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S T R E S Z C Z E N I E

Wysoką częstotliwość tworzenia się tworów protokormowych (protocorm-like bodies, PLBs) u hybrydy *Cymbidium* Twilight Moon 'Day Light' uzyskano dzięki nowej pożywce – Teixeira *Cymbidium* (TC). Dwie nowe pożywki TC zawierające zróżnicowaną ilość makro- i mikroelementów oraz innych dodatków, w oparciu o pożywkę Winarto i Teixeira (WT) dla *Anthurium* oraz pożywkę podstawową Murashige i Skoog'a (MS), zostały użyte do indukcji tworów PLB i kalusa. Pożywkę kontrolną stanowiła pożywka Vacin'a i Went'a (VW) uznawana za standard w pracach badawczych i przemyśle. Pierwsza pożywka, $TC_{PLB}$, była w stanie indukować istotnie więcej tworów PLB niż pożywka VW, natomiast do indukcji kalusa potrzebne były wysokie zawartości makroelementów w pożywkach $TC_{CALLUS}$ i MS. Wszystkie pożywki do indukcji tworów PLB zawierały 0,1 mg/l kwasu α-naftalenooctowego (NAA) i 0,1 mg/l kinetyny (KIN), 2 g/l tryptonu i 20 g/l sacharozy, zestalone 8 g/l agaru Bacto, a pożywki do indukcji kalusa były identyczne, z tym że KIN zastąpiono tidiazuronem (TDZ). Pożywka podstawowa miała istotny wpływ na powstawanie PLB i kalusa. Opracowana procedura może być wykorzystana do indukcji PLB i kalusa u innych gatunków lub odmian *Cymbidium*.

**Słowa kluczowe**: pożywka podstawowa, twory protokormowe (PLBs) i kalus, pożywka Teixeira *Cymbidium* (TC), pożywka Winarto i Teixeira (WT)