

# 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)

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## ABSTRACT

It is common to utilize sucrose as a carbon source in the induction of *Cymbidium* PLB and callus, and as an energy source in the tissue culture of other orchids. This study found carbon sources to be clustered into 4 groups according to their suitability for PLB and/or callus induction: cluster A (high PLB and callus inducers): fructose, sucrose; cluster B (intermediate PLB and callus inducers): galactose, glucose, turanose (a rare sugar); cluster C (intermediate PLB and low callus inducers): lactose, maltose, starch, mannitol, sorbitol; cluster D (poor PLB and callus inducers): mannose, xylose, raffinose, cellulose. Plants regenerated from control PLB-derived shoots were cytologically (using flow cytometry) and genetically (using 10mer RAPD) identical, despite varying photosynthetic capacities. Callus exhibited high levels of polysomaty. Plants could root under photoauto-, photohetero- and photomixotrophic conditions, with the latter showing the highest photosynthetic capacity at almost all photosynthetic photon fluxes (0-300  $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Plants were acclimatized from all treatments with a 100% survival rate, although those derived from photomixo- and photoautotrophic conditions appeared (visually) to be much stronger, sturdier and more robust.

**Keywords:** carbohydrates, embryogenesis, flow cytometry, phytotoxicity, PLB, RAPD, rare sugar, regeneration capacity

**Abbreviations:** NAA,  $\alpha$ -naphthaleneacetic acid; PGR, plant growth regulator; PLB, protocorm-like body; PPF, photosynthetic photon flux; RAPD, random amplified polymorphic DNA; VW, Vacin and Went

## INTRODUCTION

Any heterotrophic plant culture requires the presence of a carbon source for its successful photosynthetic functioning. In addition to their essential roles as substrates in carbon and energy metabolism and in polymer biosynthesis, sugars have important hormone-like functions as primary messengers in signal transduction, and can act as morphogens, providing positional information to the cell cycle machinery and different developmental programs (Rolland *et al.* 2002). Sugars are also important signaling molecules, particularly during stress-related cellular events, and these may affect morphogenesis in plants, such as somatic embryogenesis, by themselves acting as a stress when applied at unnaturally high concentrations (reviewed in Penna *et al.* 2006). Among carbohydrates, sucrose has generally been used as the most suitable source of carbon and energy for growth of plant tissues (George 1993). Sucrose degradation is the first step for carbon utilization by plant cells and its breakdown is mediated by two enzymes, invertase and sucrose synthase, allowing the subsequent utilization of the hexoses produced.

*Cymbidium*, one of the commercially most important orchid genera, can be tissue cultured by shoot tips, flower stalks and flower buds, seeds, pseudobulbs, protocorm-like bodies (PLBs) or calli, and the most frequently, if not ex-

clusively used carbon source is sucrose, due to its low cost. *Cymbidium* tissue culture and biotechnology has recently been reviewed (Nayak *et al.* 2006) and several studies in a sequence of experiments aimed at improving the tissue culture of *Cymbidium* hybrids have examined factors such as basal medium and nutrient conditions (Teixeira da Silva *et al.* 2005), explant source and their organogenic/somatic embryogenic potential (Teixeira da Silva and Tanaka 2006a; Teixeira da Silva *et al.* 2006).

This study investigates the effects of carbon sources, including a rare sugar, turanose, on the frequency of PLB and callus induction from PLB transverse thin cell layers (tTCLs) in hybrid *Cymbidium* Twilight Moon 'Day Light'. The size and properties of tTCLs have been shown to be effective in the micropropagation of several ornamental plants (Teixeira da Silva 2004a; Nhut *et al.* 2006; Teixeira da Silva *et al.* 2007).

## MATERIALS AND METHODS

### Plant materials, culture conditions and acclimatization

Hybrid *Cymbidium* Twilight Moon 'Day Light' and Forest Garden #6 (BioU, Japan), the latter only in certain experiments, originating

from shoot-tip culture, were used. Twilight Moon 'Day Light' PLBs, which derived spontaneously from the base of shoot cultures on a banana-enriched medium, were subcultured every two months on modified Vacin and Went medium (1949) supplemented with 0.1 mg.L<sup>-1</sup> NAA and 0.1 mg.L<sup>-1</sup> kinetin, 2 g.L<sup>-1</sup> tryptone and 20 g.L<sup>-1</sup> sucrose (VW<sub>PLB</sub>), and solidified with 8 g.L<sup>-1</sup> Bacto agar (Difco Labs, USA). Callus was induced on callus induction and proliferation medium (VW<sub>CALLUS</sub>), similar to VW<sub>PLB</sub>, except that TDZ was used instead of kinetin (established protocol by the lead author). All media were adjusted to pH 5.3 with 1N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 mL medium in 100 mL Erlenmeyer flasks (10 PLBs per flask), double-capped with Al foil, at 25 ± 1°C, under a 16 h photoperiod with a light intensity of 45 μmol.m<sup>-2</sup>.s<sup>-1</sup> provided by plant growth fluorescent lamps (Homo Lux, Japan). PLB transverse thin cell layers (tTCLs), <1 mm thick, 3-4 mm in diameter, were used as explants for both PLB induction and proliferation, and callus induction. A total of 20 shoots (4-5 cm in length) derived from PLBs on any carbon source were transferred to 80 mL Hyponex<sup>®</sup> (6.5:6:19; 3 g.L<sup>-1</sup>) medium (5 shoots per bottle) and either 1% (w/v) of the same carbon source except for rare sugars, added at 0.01% (w/v), or 3% (w/v) sucrose in a glass bottle (75 x 130 mm), and placed at the same temperature and light conditions as described above. For acclimatization, individual *in vitro* plantlets were transferred to coarse pine bark (Pin Décor, France) and placed in a greenhouse for three weeks with daily watering, and no additional fertilization.

### Photoauto-, photohetero- and photomixotrophic micropropagation

Twenty-five shoots (4-5 cm in length) derived from control treatment PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (BioU, Japan) were transferred to an OTP<sup>®</sup> film culture vessel, the Vitron<sup>®</sup> (Fig. 1). Each shoot was embedded in a 25-hole rockwool multiblock, prior to which 200 mL VW<sub>PLB</sub> medium (without agar) was evenly distributed, and placed at the same temperature and light conditions as described above. CO<sub>2</sub> gas was supplied at a constant (24 h) super-elevated concentration (3000 ppm). In addition, a total of 20 shoots (4-5 cm in length) were transferred to 80 mL Hyponex<sup>®</sup> (6.5:6:19; 3 g.L<sup>-1</sup>) medium (5 shoots per bottle) or VW<sub>PLB</sub> medium with 3% (w/v) sucrose in a glass bottle (75 x 130 mm) containing a Milliseal<sup>®</sup>, with or without CO<sub>2</sub> enrichment, photohetero- or photoautotrophic, respectively or with both sucrose and CO<sub>2</sub> enrichment (i.e. photomixotrophic) and placed at the same temperature and light conditions as described above.

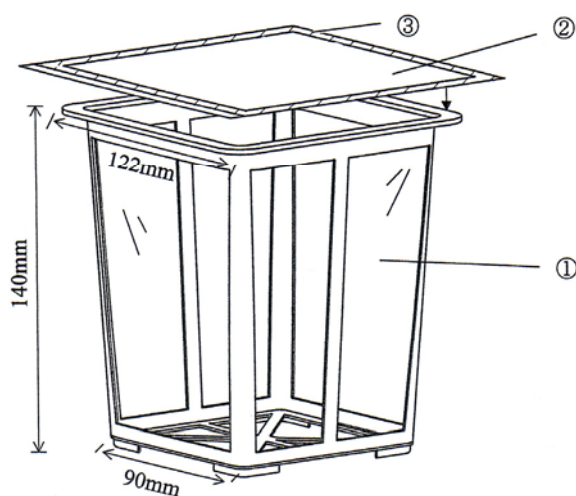


Fig. 1 Diagram of Vitron vessel. 1 = main frame, 2 = top seal film, 3 = adhesive area.

### Effect of carbon sources and rare sugar

In order to test the effect of carbon source on PLB and callus formation, PLB tTCLs were placed on VW<sub>PLB</sub> medium with different concentrations of carbon sources. The different carbon sources,

tested at 0.5, 1, 2 and 4% (w/v) were: monosaccharides (fructose, galactose, glucose, mannose, all hexoses, and xylose, a pentose); oligosaccharides (lactose, maltose, sucrose, all disaccharides, turanose, a rare disaccharide and raffinose, a trisaccharide); polysaccharides (cellulose, starch); polyols or sugar alcohols (mannitol, sorbitol).

### Morphogenic and photosynthetic analysis

Plantlet growth was quantified by the number of new leaves and roots, plant height, fresh and dry weight of shoots and roots. Chlorophyll content in the third leaf (counting downward from the top) of the plantlets was measured as the SPAD value by a chlorophyll meter (SPAD-502, Minolta, Japan). The photosynthetic rate of the leaves was measured using a LI-COR portable gas exchange system (LI-6400, LI-COR, USA). Measurements were performed at 25°C and the vapor pressure deficit at the leaf surface was maintained between 2.3 and 3.1 kPa. The CO<sub>2</sub> concentration in the sample chamber was set at 400 μL.L<sup>-1</sup>. Measurement of CO<sub>2</sub> uptake between the range of 0 μmol.CO<sub>2</sub>.m<sup>-2</sup>.s<sup>-1</sup> and 300 μmol.m<sup>-2</sup>.s<sup>-1</sup> was conducted using a built-in red light-emitting diode lamp.

### Scanning electron microscopy

Samples were fixed in 30% FAA II (90:5:5, 70% ethanol : formalin : acetic acid) for 2 d, 50% FAA II for 2 d and 70% FAA II for 2 d. Fixed specimens were dehydrated through an ethanol/acetone series (detailed in Teixeira da Silva *et al.* 2005), critical point dried, coated with Pt and examined under a scanning electron microscope (Hitachi S-2150, Japan).

### Flow cytometry

Nuclei were isolated from 0.25 cm<sup>3</sup> of the material (PLB and callus) derived from any treatment by chopping in a few drops of nucleic acid extraction buffer (Partec Cystain UV Precise P, Germany), digesting on ice for 5 minutes. The nuclear suspension was then filtered through 30 μm mesh size nylon filter (CellTrics<sup>®</sup>) and five times of Partec Buffer A (2 μg.mL<sup>-1</sup> 4,6-diamidino-2-phenylindole (DAPI), 2 mM MgCl<sub>2</sub>, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton-X, pH 7.5; Mishiba and Mii 2000) was added at room temperature for 5 minutes. Thereafter, nuclear fluorescence was measured using a Partec<sup>®</sup> Ploidy Analyser. Three samples were measured, and relative fluorescence intensity of the nuclei was analyzed when the Coefficient of Variation was <3%. A minimum of 5000 nuclei were counted for each sample. The internal standard was barley as defined in Teixeira da Silva *et al.* (2005).

### DNA extraction and PCR analysis

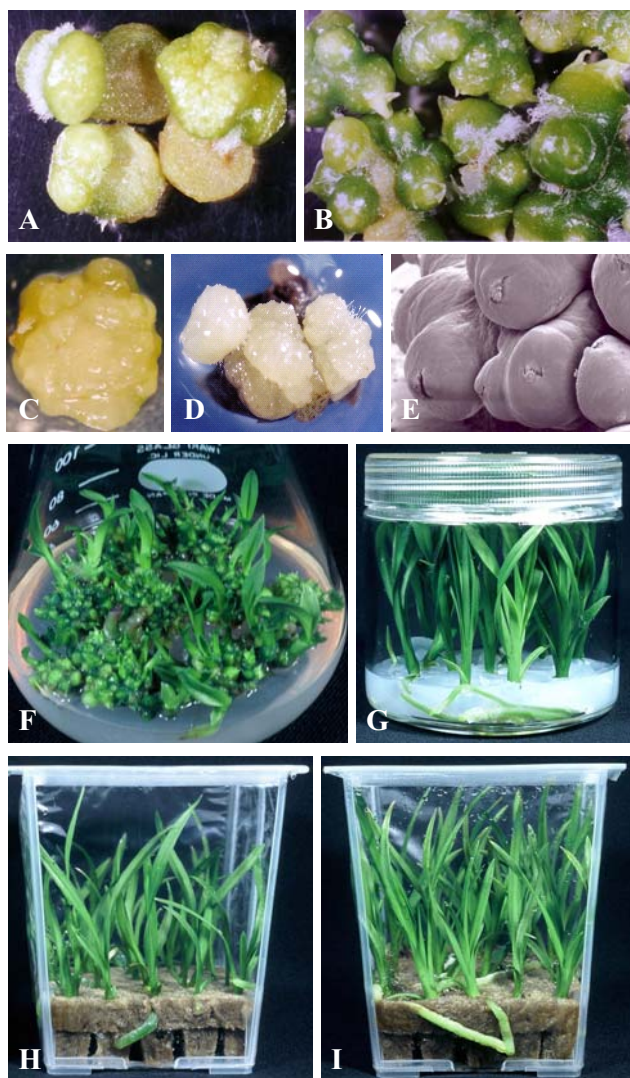
DNA was isolated from *in vitro* PLBs using Qiagen DNeasy<sup>®</sup> (U.S.A.) following manufacturer's instructions and following recommendations for orchid DNA extraction (Teixeira da Silva and Tanaka 2006b). DNA was quantified using a Shimadzu UV-1200 spectrophotometer, and purity established from 260:280 nm ratios.

A 10mer primer (B-10) was used as defined in Teixeira da Silva *et al.* (2006). Amplification conditions for B-10 were: 94°C for 10 min (hot-start) followed by 35 cycles of (94°C 30 s, 42°C 60 s, 68°C 30 sec: annealing) and 72°C for 10 min (extension) in a Perkin Elmer GeneAmp PCR System 2400 thermocycler. All cycling conditions included a hot start. An Invitrogen PCR Super-Mix High Fidelity PCR kit was used: 66 mM Tris-SO<sub>4</sub> (pH 9.1 at 25°C), 19.8 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.2 mM MgSO<sub>4</sub>, 220 μM of each dNTP, 1.1 units of recombinant *Taq* DNA polymerase. 50 μL reactions were performed containing 20 μM of the primer and 100 ng of target/template DNA. A total of 15 μL of PCR products were analysed three times by agarose gel electrophoresis on 1% SeaKem<sup>®</sup> GTG<sup>®</sup> agarose (BioWhittaker Molecular Applications, USA)/TAE. Gels were run with 5 μg.mL<sup>-1</sup> ethidium bromide. DNA was visualized using a 320 nm UV transilluminator, and photographed using Kodak 667 Polaroid film. For comparison, DNA was also extracted from unrelated controls, *Phalaenopsis* Galant Beau 'George Vazquez' grown on sucrose medium (Tanaka *et al.* 1988) and *Dendranthema grandiflora* 'Shuhou-no-chikara' grown on sucrose medium (Teixeira da Silva and Fukai 2003).

**Table 1** Subsequent organogenesis (leaves and roots) of PLB-derived shoots ( $n = 25$ ) derived from different carbon sources on growth, photosynthetic and cytogenetic parameters in *Cymbidium* Twilight Moon 'Day Light', 90 days after placing on Hyponex® medium (+ 3% sucrose).

Sugar (group and type)	Leaves				Leaf:Root* <sup>1</sup>		Roots				SPAD* <sup>2</sup>
	N <sub>o</sub>	FW	DW	FW:DW	N <sub>o</sub>	FW	DW	FW:DW			
<b>Hexose (Monosaccharides)</b>	fructose	6.2 ab	412.6 a	42.1 b	9.8 b	0.47 c	0.9 a	382.1 a	18.2 c	21.0 b	39.6 a
	galactose	6.6 a	418.9 a	38.6 bc	10.9 b	0.53 bc	0.8 a	369.4 a	17.9 c	20.6 b	28.2 b
	glucose	6.8 a	416.7 a	41.0 b	10.2 b	0.44 c	1.2 a	389.2 a	16.8 c	23.2 b	40.1 a
	mannose	5.9 b	399.6 a	42.2 b	9.5 b	0.45 c	1.2 a	347.9 ab	16.6 c	21.0 b	26.8 b
<b>Pentose</b>	xylose	4.2 c	287.3 b	21.1 c	13.6 ab	0.42 c	0.6 ab	221.7 c	6.8 d	32.6 a	25.8 b
	lactose	6.4 a	409.4 a	37.9 bc	10.8 b	0.39 c	0.4 b	196.2 c	7.1 d	27.6 ab	26.9 b
<b>Disaccharide</b>	maltose	6.2 ab	394.8 a	41.4 b	9.5 b	0.49 c	0.3 b	171.4 c	8.9 d	19.3 b	24.8 b
	sucrose	6.6 a	402.9 a	38.0 b	10.6 b	0.53 bc	1.1 a	367.5 a	18.6 c	19.8 b	39.2 a
<b>Rare disaccharide</b>	turanose	5.8 b	400.2 a	33.8 bc	11.8 ab	0.83 ab	0.7 ab	346.2 ab	24.2 b	14.3 c	19.9 c
<b>Trisaccharide</b>	raffinose	3.6 c	261.4 b	21.6 c	12.1 ab	0.77 ab	0.2 b	151.6 c	9.6 d	15.8 c	26.1 b
<b>Polysaccharide</b>	cellulose	3.9 c	263.8 b	20.8 c	12.7 ab	0.49 c	0.1 b	79.8 d	3.1 e	25.7 ab	27.2 b
	starch	6.6 a	414.6 a	42.7 b	9.7 b	1.13 a	1.2 a	361.4 a	42.1 a	8.6 d	26.6 b
<b>Polyol</b>	mannitol	6.1 ab	416.8 a	50.6 a	8.2 bc	0.92 a	1.1 a	322.4 b	36.2 a	8.9 d	28.1 b
	sorbitol	6.3 ab	404.2 a	51.7 a	7.8 c	0.71 b	1.3 a	346.8 ab	31.4 ab	11.0 cd	28.6 b
<b>FC*<sup>5</sup></b>			89:11:0:0* <sup>3</sup>				83:13:t:t* <sup>4</sup>				

Data presented as means; different letters within a column, and within a single treatment, indicate significant differences at  $P < 0.05$  according to Duncan's New Multiple Range test. Abbreviations: FW = fresh weight (mg), DW = dry weight (mg); FC = flow cytometry. \*<sup>1</sup> = (leaf FW:DW):(root FW:DW), or measurement of carbon partitioning; \*<sup>2</sup> = measurement of chlorophyll content on the 3<sup>rd</sup> youngest leaf; \*<sup>3</sup> = relative amounts; measurement from at least three leaves (mixture of tip and sheath) t = trace ( $\leq 2\%$ ); \*<sup>4</sup> = relative amounts; measurement from at least three roots (mixture of proximal tip and distal end) t = trace ( $\leq 2\%$ ); \*<sup>5</sup> = relative amounts; a minimum of three samples per explant source, t = trace ( $\leq 2\%$ ); all FC values = 2C:4C:8C:16C relative ratios.



**Fig. 2** *In vitro* growth and development of epiphytic hybrid *Cymbidium* Twilight Moon 'Day Light'. (A) PLB neo-formation on PLB tTCLs on control VWPLB; (B) PLB or (C) callus formation on VWPLB with 3% (w/v) sucrose; (D) callus formation by turanose, a rare disaccharide; (E) SEM of galactose-induced PLB cluster; (F) glucose-induced PLBs after 45 days on VWPLB. Development of Twilight Moon 'Day Light' shoots under (G) photoheterotrophic (3% (w/v) sucrose), (H) photoautotrophic (3000 ppm CO<sub>2</sub>-enrichment), or (I) photomixotrophic (3% (w/v) sucrose + 3000 ppm CO<sub>2</sub>-enrichment) conditions and subsequent rooting on Hyponex® in agar medium (G) or VWPLB on rockwool substrate (H, I).

## Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 20 replicates 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® vers. 6.12 (SAS Institute, Cary, NC, USA).

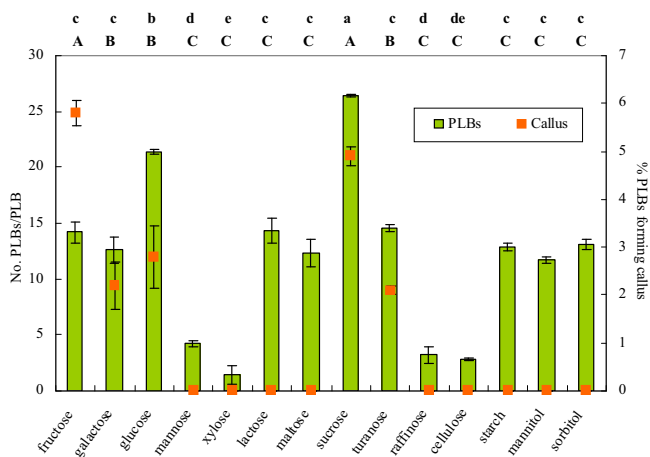
## RESULTS AND DISCUSSION

### TCLs as suitable explant sources

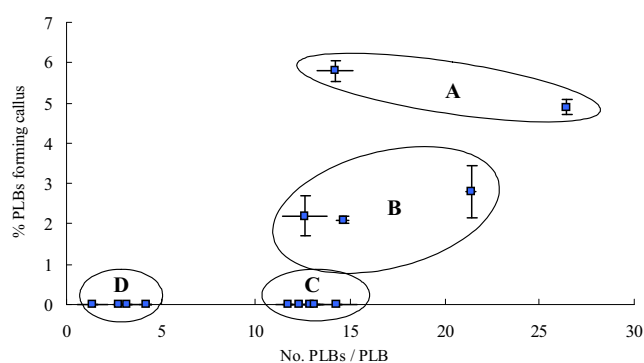
Traditional orchid tissue culture relies on the use of a carbon source for photoheterotrophic growth and micropropagation. Thin cross sections (TCSS) or thin cell layers (TCLs) of plant parts, designed to control morphogenesis more strictly than regular multi-tissue/organ explants (Teixeira da Silva 2004a; Nhut *et al.* 2006; Teixeira da Silva *et al.* 2007), especially from 6-7 week-old PLBs whose cells at this stage are highly meristematic, have been used to hasten and improve plant regeneration in other orchids, viz. *Aranda*, *Cymbidium*, *Rhynchostylis* and *Spathoglottis* (Tran Thanh Van 1974; Begum *et al.* 1994; Lakshmanan *et al.* 1995; Teng *et al.* 1997; Le *et al.* 1999). Huan and Tanaka (2004) reported that the induction of callus from PLB half-segments occurred within a month, but by using tTCLs, PLB initials could be visualized with the naked eye on the epidermal surface within 2 weeks (Fig. 2). Entire PLBs, or even PLB segments serve as sink tissues from which shoots emerge. The presence of such a large sink would be unsuitable for such a carbon-photosynthesis effect study. TCLs, on the other hand, rely almost exclusively on the medium as their sole carbon and nutrient sources.

According to the PLB- and callus-inducing capacities of different carbon sources (Table 1; Figs. 2, 3, 5, 6), four clusters could be obtained (Fig. 4): cluster A (high PLB and callus inducers): fructose, sucrose; cluster B (intermediate PLB and callus inducers): galactose, glucose, turanose; cluster C (intermediate PLB and low callus inducers): lactose, maltose, starch, mannitol, sorbitol; cluster D (poor PLB and callus inducers): mannose, xylose, raffinose, cellulose.

In *Dendranthema* tissue culture studies, TCL threshold survival levels could not be obtained for sucrose, fructose or glucose, and normal organ differentiation occurred even when high concentrations (up to 16% w/v) of these carbon sources were applied to culture media. In contrast, in the same study, growth of shoots, roots or (embryogenic) calli was inhibited at or above 4% (w/v) when either mannose, xylose, lactose or cellulose were used, making these carbon sources suitable candidates for positive selection systems in



**Fig. 3 Effect of carbon source on maximum PLB formation and callus induction.** Different letters (lower case, PLBs; upper case, callus) along a row indicate significant differences at  $P < 0.05$  according to Duncan's New Multiple Range test.



**Fig. 4 Clustering of carbon sources.** Cluster A (high PLB and callus inducers), from left to right: fructose, sucrose; cluster B (intermediate PLB and callus inducers), from left to right: galactose, turanose, glucose; cluster C (intermediate PLB and low callus inducers), from left to right: mannitol, maltose, starch, sorbitol, lactose; cluster D (low PLB and callus inducers), from left to right: xylose, cellulose, raffinose, mannose.

chrysanthemum genetic transformation (Teixeira da Silva 2004b). Higher levels were not tested since these would never be practically used in a tissue culture study or in micropropagation.

**Effect of carbon source**

**Table 1** shows the residual effect of initial carbon pulsing on subsequent growth parameters, whereas **Table 2** shows the capacity of plants to metabolize that carbon source for either PLB and/or callus initiation, and subsequent development of shoots derived from neo-formed PLBs induced by the same carbon sources. All hexoses (monosaccharides) and disaccharides produced high numbers of new leaves, as could starch and the polyols (**Table 1**) 90 days after shoots were placed on Hyponex® medium supplemented with 3% (w/v) sucrose. In the case of lactose, cellulose, starch and the polyols, mannitol and sorbitol, a substitution of sucrose for the same carbon source in the shoot development and rooting stages resulted in deteriorating numbers of leaves (**Table 2**), indicating that these carbon sources cannot be effectively metabolized, despite the recovery of shoots when sucrose is added in the shoot development and rooting stages (**Table 1**). Xylose and the trisaccharide, raffinose could not produce many new leaves, or roots, independent of whether the same carbon source was used throughout (**Table 2**), or whether sucrose was substituted at 3% (**Table 1**). SPAD values follow the physiological trend (**Tables 1, 2**), indicating that there is a direct link between the capacity to use a carbon source for PLB, callus, shoot or root production in *Cymbidium* and the functioning of the photosynthetic machinery.

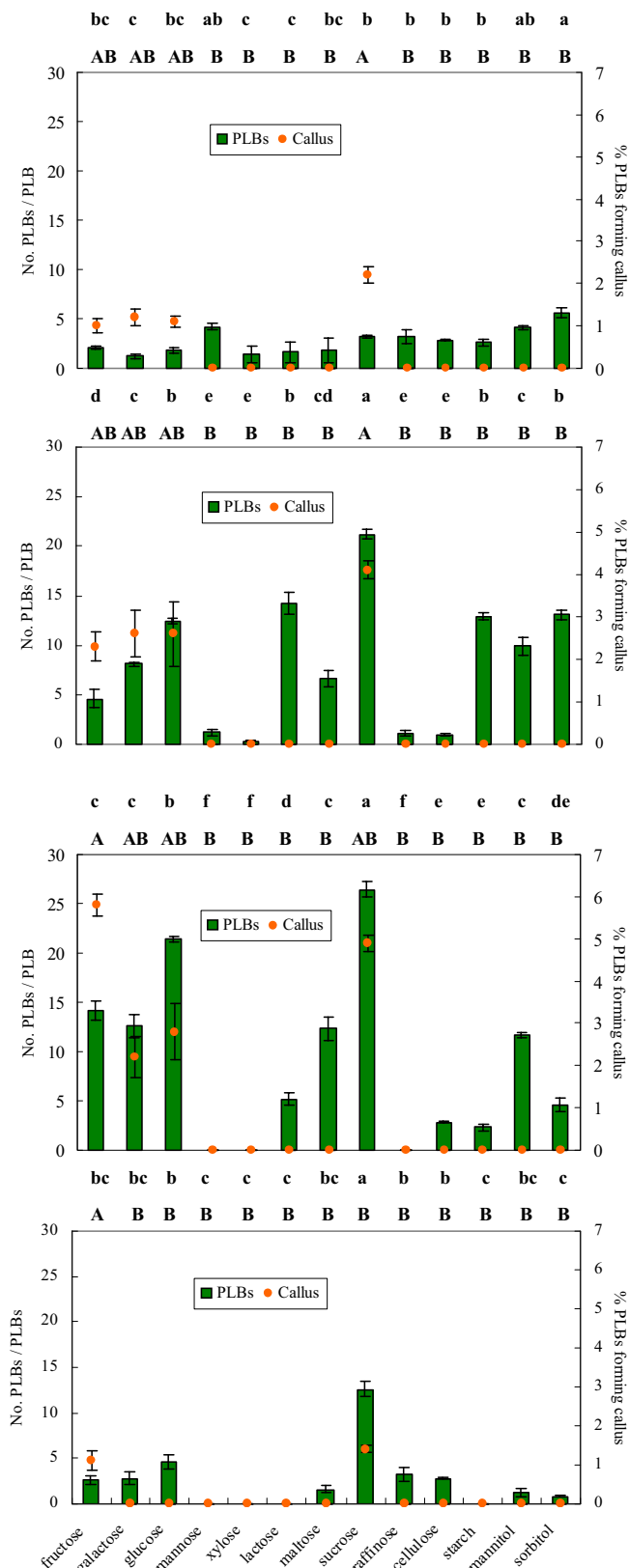
A review of the literature reveals that without exception, all orchid tissue culture and micropropagation studies used sucrose as the carbon source, although other carbohydrates such as glucose or fructose have been shown to be suitable in *Phalaenopsis* and *Dendrobium* (Hew *et al.* 1988); trehalose for PLB formation and high frequency plant regeneration in *Oncidium* (Jheng *et al.* 2006) and others, particularly galactose, were shown to be toxic (Ernst *et al.* 1971). A high sucrose concentration (5%) was shown to almost double the number of PLBs formed on a Hyponex® medium (Momose and Yoneda 1989). Pretreatment of mature PLBs with sucrose also increased the occurrence of single cell embryogenesis (Li *et al.* 2005). A high sucrose concentration (10%) or a high ABA concentration (10 mg.L<sup>-1</sup>) resulted in tolerance in *Spathoglottis plicata* protocorms, the former resulting in sucrose accumulation, the latter in dehydrin accumulation (Wang *et al.* 2003).

Galactose, glucose and fructose (or levulose) are monosaccharides and should be more easily metabolized (or less energy-consuming) than sucrose, a disaccharide consisting of glucose and fructose. Galactose is an epimer of glucose, and its function in morphogenesis may depend on the endogenous levels of galactokinase (Hisajima and Thorpe 1985). Results in **Table 1** indicate that any monosaccharide, except

**Table 2** Subsequent organogenesis (leaves and roots) of PLB-derived shoots ( $n = 25$ ) derived from different carbon sources on growth, photosynthetic and cytogenetic parameters in *Cymbidium* Twilight Moon 'Day Light', 90 days after placing on the same medium (+ 1% (w/v) of that carbon source).

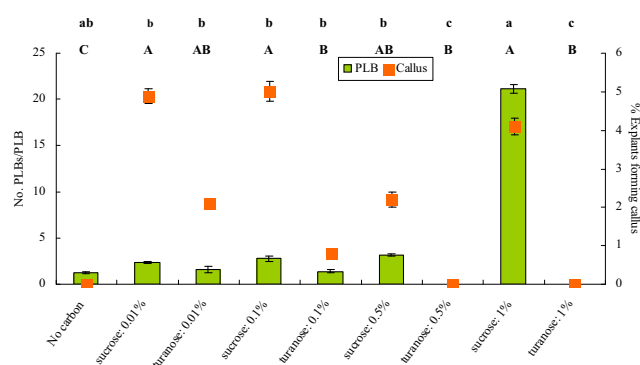
Sugar (group and type)	Leaves				Leaf:Root* <sup>1</sup>				Roots				SPAD* <sup>2</sup>
	No	FW	DW	FW:DW	No	FW	DW	FW:DW	No	FW	DW	FW:DW	
<b>Hexoses (Monosaccharides)</b>	fructose	6.7 a	399.8 a	38.2 a	10.5 a	0.50 c	1.4 a	421.6 a	20.1 a	21.0 a	39.6 a		
	galactose	5.4 ab	362.1 ab	37.4 a	9.7 a	0.75 c	0.7 ab	216.8 c	16.8 a	12.9 b	28.2 b		
	glucose	6.4 a	406.3 a	38.6 a	10.5 a	1.06 b	0.5 ab	188.7 c	19.1 a	9.9 b	40.1 a		
	mannose	5.7 ab	314.8 b	32.7 ab	9.6 a	0.91 b	0.5 ab	194.4 c	18.4 a	10.6 b	26.8 b		
<b>Pentose</b>	xylose	1.6 c	222.8 c	19.8 c	11.3 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	25.8 bc		
<b>Disaccharides</b>	lactose	1.4 c	228.6 c	20.1 c	11.4 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	26.9 b		
	maltose	5.7 ab	381.6 ab	37.6 a	10.1 a	2.20 a	0.2 b	84.9 d	18.6 a	4.6 bc	24.8 bc		
	sucrose	6.6 a	402.9 a	38.0 a	10.6 a	0.53 c	1.1 a	367.5 b	18.6 a	19.8 a	39.2 a		
<b>Rare disaccharide</b>	turanose	4.0 b	316.9 b	29.8 b	10.6 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	19.9 c		
<b>Trisaccharide</b>	raffinose	4.2 b	357.6 ab	32.4 ab	11.0 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	26.1 b		
<b>Polysaccharides</b>	cellulose	2.2 c	246.2 c	25.2 bc	9.8 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	27.2 b		
	starch	2.1 c	248.4 c	22.8 bc	10.9 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	26.6 b		
<b>Polyols</b>	mannitol	1.0 cd	207.2 c	19.7 c	10.5 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	28.1 b		
	sorbitol	4.1 b	356.9 ab	32.9 ab	10.8 a	1.0 b	0.0 b	0.0 e	0.0 b	0.0 c	28.6 a		
<b>FC*<sup>5</sup></b>		86:14:0:0* <sup>3</sup> (pooled)				81:12:5:t* <sup>4</sup> (pooled)							

All carbon sources added at 1% (w/v) except for rare sugars, added at 0.01% (w/v). Data presented as means; different letters within a column, and within a single treatment, indicate significant differences at  $P < 0.05$  according to Duncan's New Multiple Range test. Abbreviations: FW = fresh weight (mg), DW = dry weight (mg); FC = flow cytometry. \*<sup>1</sup> = (leaf FW:DW):(root FW:DW), or measurement of carbon partitioning; \*<sup>2</sup> = measurement of chlorophyll content on the 3<sup>rd</sup> youngest leaf; \*<sup>3</sup> = relative amounts; measurement from at least three leaves (mixture of tip and sheath) t = trace ( $\leq 2\%$ ); \*<sup>4</sup> = relative amounts; measurement from at least three roots (mixture of proximal tip and distal end) t = trace ( $\leq 2\%$ ); \*<sup>5</sup> = relative amounts; a minimum of three samples per explant source, t = trace ( $\leq 2\%$ ); all FC values = 2C:4C:8C:16C relative ratios.



**Fig. 5** Effect of mono-, di-, tri-, polysaccharides, and polyols on PLB and callus induction at 0.5% (A), 1% (B), 2% (C) and 4% (D). Different letters (lower case, PLBs; upper case, callus) along a row indicate significant differences at  $P < 0.05$  according to Duncan's New Multiple Range test.

for mannose (a hexose) or xylose (a pentose) resulted in significantly similar numbers of PLBs per PLB tTCL as when sucrose, a disaccharide, was used. A gradient effect could be observed when glucose and fructose were used alone, or in combination: sucrose > glucose + fructose > glucose > fructose (data not shown). Fructose, a phosphorylated product, and an intermediary product of glucose

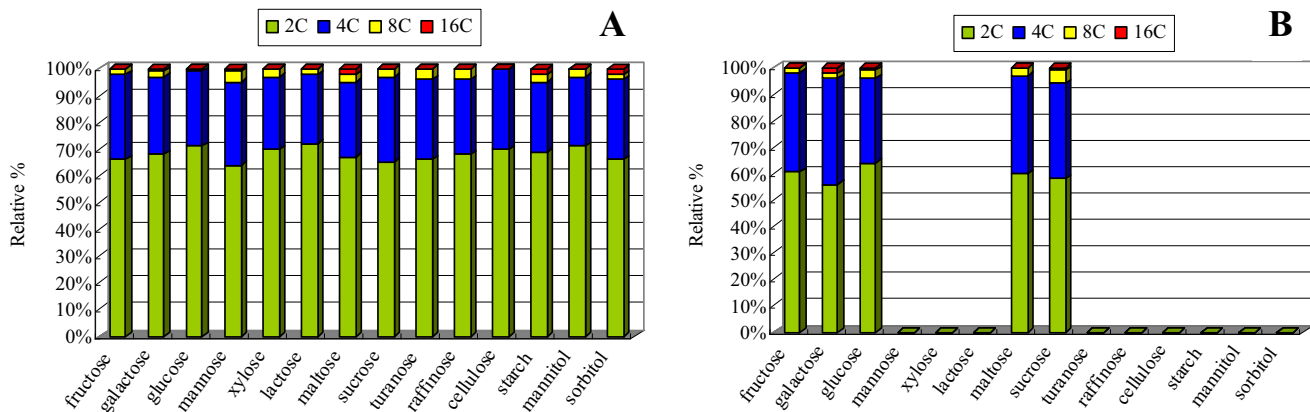


**Fig. 6** Effect of the rare sugar, turanose (with sucrose as positive control, no carbon as negative control) on PLB and callus induction. Different letters (lower case, PLBs; upper case, callus) along a row indicate significant differences at  $P < 0.05$  according to Duncan's New Multiple Range test.

catabolism, and the slow growth of tissue cultures on a fructose-containing medium is a result of the inhibition of glycolysis by fructose or its degradation products; glucose promotes root growth, fructose promotes shoot development and sucrose promotes both shoot development and root growth in asparagus (Li and Wolyn 1997), although this was not quantitatively discriminated as in our study. Exogenous carbohydrates play an important role as carbon and energy source and act as osmotic agents (Thorpe 1974; Brown *et al.* 1979), besides acting as signalling molecules (Smeeckens *et al.* 2000). In cashew tissue culture, a combination of fructose and maltose was found beneficial for the development of well-developed shoots for use in cashew micropropagation (Gemias and Bessa 2006). Incidentally, in microbes "in certain conditions" fructose is the preferred carbon source even before glucose and is metabolized through glycolysis as in the case of glucose, but the fructose metabolic pathway is shorter (Tom Granström, pers. obs.), although this mechanism may differ in plants. Kato-Noguchi *et al.* (2006) believe that a hexokinase-independent signal cascade may be triggered by the rare sugar, D-psicose, a fructose analogue, after the roots of lettuce seedlings were inhibited by this rare sugar (Afach *et al.* 2006). Akimitsu *et al.* (2006) believe that this "negative" growth-inhibiting property of some rare sugars could be used beneficially, e.g. in delaying flowering. Therefore, in where there is no difference in uptake, fructose is preferred over glucose, and its use is faster. Spinach somatic embryogenesis could only occur when glucose or fructose, and to a lesser extent, galactose were utilized, the optimum being at 29 mM of any of the three hexoses (Komai *et al.* 1996). Maltose was a superior carbohydrate/osmoticant during the critical stages of loblolly pine somatic embryo because it facilitated a natural osmotic environment, and trehalose had significant effect on PLB maturation and plantlet conversion (Pullman *et al.* 2003). Mannose negatively affected *Arabidopsis* root elongation and development (Baskin *et al.* 2001). Mannose also negatively affected PLB formation and induction (Figs. 3-5), and subsequent shoot and root production (Tables 1, 2). Lactose, made up of D-galactose and D-glucose, maltose, made up of two molecules of D-glucose, and cellulose, consisting of a series of D-glucopyranose rings, could not readily form PLBs, and when used as the sole carbon source, resulted in poor shoot and root growth and development (Table 2). Starch on the other hand, a polymeric form of D-glucose, was readily degraded by plants, and could support the growth of PLBs, shoots and roots (Table 2).

### Effect of polyols

Polyols (or sugar alcohols), the reduced form of aldoses and ketoses, accumulate in plants in response to abiotic stress (cold, water, or salt stress), thus demonstrating their pro-



**Fig. 7 Endopolyploidy in PLBs and callus.** Relative percentages of 2C, 4C, 8C, and 16C in PLBs (A) and callus (B) derived from media containing different carbon sources. Callus from carbon sources in which no ploidy values are shown were insufficient for sampling and/or replication.

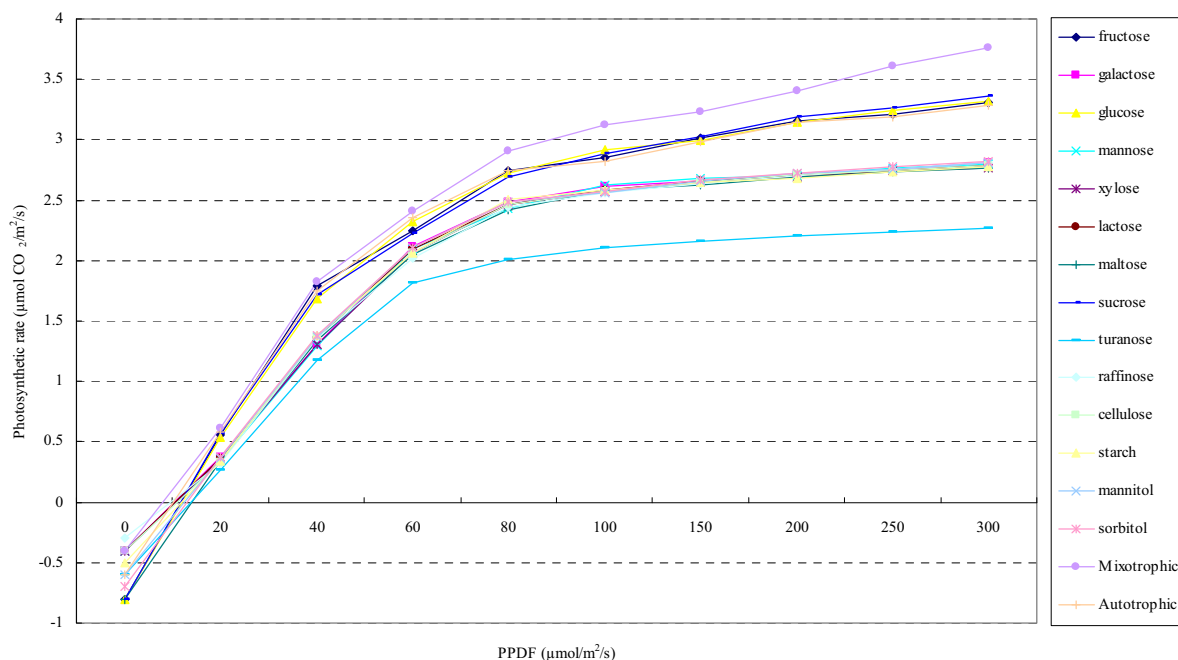
fective effects on metabolism and, like sucrose, are synthesized in autotrophic (source) leaves and transported inside the phloem to heterotrophic (sink) organs to sustain plant growth and development (Loescher and Everard 1996). Sugar alcohols in barley, tobacco and tomato induce molecular and physiological responses that do not belong to primary carbon metabolism, indicating that they are metabolised to some degree since they are perceived by cells as chemical signals, with very high *in vitro* concentrations acting as chemical stress agents (Steinitz 1999). The polyol *myo*-inositol, a constituent of coconut liquid endosperm, and involved in cell-wall biosynthesis, is often added to tissue culture media, where it was shown to improve *Cymbidium* growth (Fonnesbech 1972). The sugar alcohol mannitol is widely used to prepare isotonic media for the culture of orchid protoplasts. In the tissue culture of *Dendrobium* and *Darwinara*, sorbitol and mannitol were detected in the medium following one month PLB micropropagation (Kishi and Takagi 1997). PLBs have the capacity to metabolize both polyols (mannitol and sorbitol), and use them for the production of new PLBs, but cannot effectively use them for callus induction. In addition, despite the use of polyols in stress-mediated signaling, their constant presence as the primary carbon source resulted in poor plant development (Table 2), suggesting a limitation to their metabolism which is possible because no energy is de-

rived from xylitol. The proton gradient directly affects the ATP construction at mitochondrial membrane, therefore if there is no gradient, then there will be no ATP. In bacteria a high concentration of polyol, for example xylitol at 50 g/L will destroy the consistency of a microbial cell, perhaps through the destruction of the proton gradient across the membrane (Tom Granström, unpublished data).

#### Effect of rare sugar

The rare disaccharide, turanose, could support the limited production of PLBs and callus (Tables 1, 2; Figs. 3, 4, 6) and the minimal growth of shoots and roots (Tables 1, 2). The growth observed may, however reflect a positive residual effect of nutrients, PGRs and sucrose present in PLB induction medium, VW<sub>PLB</sub>. The subsequent physiology of plants was negatively affected: low SPAD values (Tables 1, 2), cellular ploidy (Fig. 7) and photosynthetic capacities (Fig. 8).

The use of rare sugars, monosaccharides that rarely exist in Nature (Granström *et al.* 2004), in plant tissue culture and molecular biology, although at an initial stage, has already yielded some interesting, and contrasting results. Model legume plant, *Lotus japonicus* seedlings were inhibited by D-psicose, and macro-array analysis revealed a concomitant increase in stress inducible protein in the roots or



**Fig. 8 Photosynthetic capacities of shoot leaves derived from *Cymbidium* Twilight Moon 'Day Light' plantlets grown on medium supplemented with different carbon sources (same as in Table 2), mixo- or autotrophic micropropagation.**

nodules (Nomura *et al.* 2004). D-Psicose was also shown to be an elicitor in plant defense, with seven defense genes being activated (Northern blot analysis) in *Citrus* sp. (Tajima *et al.* 2004). Ishida *et al.* (2004) showed that all growth parameters (except for root length) in strawberry, *Fragaria × ananassa* (and to a limited extent in the orchid, *Bletilla striata*) were stimulated in the range of 0.005-0.05% (w/v) of D-psicose, even greater than the application of D-glucose or D-fructose, two natural monosaccharides which play an important role as energy sources for growth and differentiation. Separate studies (Suzuki *et al.* 2004) on a range of monocots and dicots showed that the application of D-allose promoted shoot and root growth of *Brassica oleracea* between 10 µM and 0.1 mM, while 0.1 mM D-psicose inhibited root growth of all the plants tested. It was postulated that D-psicose adopts the furanose form, which might be the cause of etiolation and growth inhibition.

### CO<sub>2</sub>-enrichment, photomixo- and photoautotrophic micropropagation

Photoheterotrophic cultures, with or without a Milliseal® to promote gaseous exchange with the culture room, resulted in a low leaf:root fresh weight:dry weight ratio, indicating the preferential formation of roots (Table 3; Fig. 2). Contrastingly, both photomixotrophic culture (sucrose and CO<sub>2</sub>-enrichment) and photoautotrophic culture (CO<sub>2</sub>-enrichment only) resulted in a high leaf:root fresh weight:dry weight ratio, indicating the preferential formation of shoots (Table 3). Despite those differences in treatments, the SPAD value or photosynthetic capacity of plants under the various treatments did not alter (Tables 2, 3; Fig. 8).

Internal CO<sub>2</sub> gas concentration in *Cattleya* reached in excess of 2% during the day (Cockburn *et al.* 1979). *Cymbidium* (terrestrial C<sub>3</sub> plant) and *Dendrobium* PLBs could grow autotrophically (without exogenous sucrose) *in vitro* under high light and CO<sub>2</sub>-enrichment (Kirdmanee *et al.* 1992; Lim *et al.* 1992). Plantlet growth was greatly enhanced when 'Mokara Yellow' (*Arachnis hookeriana* × *Ascocenda* 'Madame Kenny') was placed at super-elevated (1%) CO<sub>2</sub> conditions (Gouk *et al.* 1999); particularly at 5% CO<sub>2</sub> a 170% increase in dry mass, a higher root:shoot ratio, 373% increase in leaf area, 75% increase in soluble protein, and a 12-90% and 27-90% decrease in Rubisco and PEP-carboxylase activity, respectively (Gouk *et al.* 1997). PLBs were produced from leaf bases of *Cymbidium* 'Burgundian Chateau', accumulation of endogenous free sugar reached peaks after 3-5 and 25 days in culture while starch synthesis reached a maximum 20 days after the start of culture (Arditti and Ernst 1993). A high photosynthetic photon flux (PPF), high CO<sub>2</sub> concentration and increased air exchange (i.e. photoautotrophic culture conditions), promoted growth and increased photosynthesis in *Phalaenopsis*, *Neofinetia*

*falcata*, *Cymbidium kanran* and *C. goeringii* (Kirdmanee *et al.* 1992; Hahn and Paek 2001). Increased growth of *Cymbidium* occurred in a low PFP-high CO<sub>2</sub> culture system, the Miracle Pack® (Tanaka *et al.* 1999). Tissues obtained from apical meristems of *Dendrobium* 'Multico White' could only use glucose, fructose, and sucrose as carbon sources; both growth and respiration rates increased with increasing sugar levels (Hew *et al.* 1988). Sucrose-phosphate synthase (SPS) is one of the key regulatory enzymes in carbon assimilation and partitioning in plants, playing a crucial role in the production of sucrose in photosynthetic cells. The high level expression of a full-length SPS cDNA encoding SPS from *Oncidium* 'Goldiana', *sps1*, in flowers suggests that it might play an important role in flowering; moreover growth under higher irradiance and elevated CO<sub>2</sub> leads to an accumulation of the *sps1* transcript in the photosynthetic leaves, associated with the leaf photosynthetic rate (Li *et al.* 2003). High PPF *in vitro* resulted in higher (38%) *Phalaenopsis* fresh weight, wider leaves and more roots than at low PPF (Konow and Wang 2001). Greenhouse *Phalaenopsis*, when exposed to stimulatory treatments such as low temperatures, high light intensity and CO<sub>2</sub> enrichment (1000-3000 ppm) at 20°C, increased the sucrose content of leaves 2-3 weeks after the start of treatment, reducing the days to spiking (Kataoka *et al.* 2004). Greenhouse studies on *Sophralaeliocattleya* (CAM) and *Cymbidium* (C<sub>3</sub>), the former with a greater potential to adapt to high photosynthetic fluxes than the latter, showed big fluctuations in activities of foliar antioxidative enzymes (SOD, APX, CAT) and a decrease in foliar chlorophyll content with an increasing radiant flux (Li *et al.* 2001). Photoautotrophic micropropagation was also successfully used in sweet potato (Giang *et al.* 2006). In our study SPAD values remained constant, independent of the use of CO<sub>2</sub> or of sucrose (photohetero- or photomixotrophic conditions, Table 3). The mykorrhizal orchid, *Neottia nidus-avis* is unable to catalyze photosynthesis despite having Chl *a* and xanthophyll, resulting in yellowish-brown leaves since part of the carotenoid absorption is shifted into the green spectral region (Menke and Schmidt 1976). In *Paphiopedilum*, in contrast, despite the reduced levels of stomatal conductance when irradiated with red light, this deficiency can be corrected (up to 77% more growth) when plantlets are irradiated with blue light (Zeiger *et al.* 1985).

### Cytogenetic stability

Flow cytometric analyses indicated that despite the use of different carbon sources, the 2C:4C:8C:16C ratios did not alter significantly, for both PLBs and callus (Figs. 7, 9). However in general callus produced higher levels of polysomaty than PLBs, although the application of the rare sugar, turanose resulted in increased endopolyploidy in

**Table 3** Subsequent organogenesis (leaves and roots) of PLB-derived shoots (*n* = 25) derived from different carbon sources on growth, photosynthetic and cytogenetic parameters in *Cymbidium* Twilight Moon 'Day Light' and Forest Garden #6, 90 days after transfer to Hyponex® medium (+ 3% sucrose).

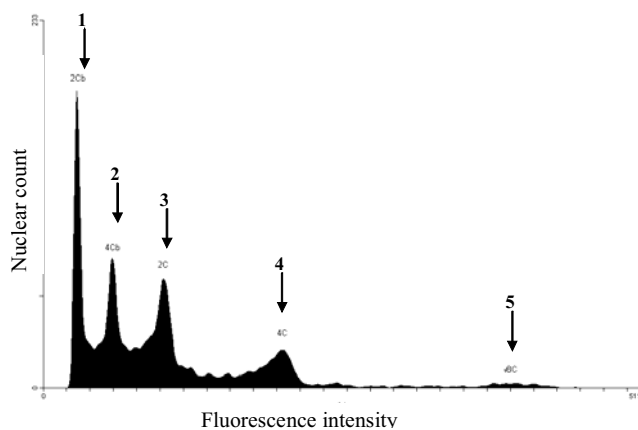
Treatment	Cultivar	Leaves				Leaf:Root <sup>*1</sup>		Roots			SPAD <sup>*2</sup>
		N <sub>o</sub>	FW	DW	FW:DW	N <sub>o</sub>	FW	DW	FW:DW		
Glass bottle (+3% sucrose) (-CO <sub>2</sub> )	TMDL	6.8 b	346.2 c	41.6 b	8.3 b	0.59 b	1.8 a	400.6 ab	28.6 b	14.0 a	38.8 b
	FG#6	6.2 b	301.6 c	39.8 b	7.6 b	0.58 b	1.1 b	326.4 b	24.7 bc	13.2 a	39.4 ab
Glass bottle (+3% sucrose) (Milliseal®-CO <sub>2</sub> )	TMDL	6.7 b	333.8 c	44.1 ab	7.6 b	0.52 b	1.6 ab	388.7 ab	26.8 b	14.5 a	40.1 b
	FG#6	6.4 b	311.9 c	39.6 b	7.9 b	0.56 b	1.2 b	344.6 ab	24.6 bc	14.0 a	39.2 b
Glass bottle (+3% sucrose) (Milliseal®+CO <sub>2</sub> )	TMDL	8.1 a	501.6 a	48.6 a	10.3 a	1.1 a	2.4 a	448.6 a	49.2 a	9.1 b	46.4 a
	FG#6	7.8 a	486.2 a	46.6 ab	10.4 a	1.0 a	1.9 a	389.2 ab	37.8 a	10.3 b	48.1 a
Vitron® (-3% sucrose) (+CO <sub>2</sub> )	TMDL	7.1 ab	456.8 ab	43.8 ab	10.4 a	1.1 a	1.4 ab	196.2 c	20.1 c	9.8 b	41.4 b
	FG#6	6.8 b	441.4 b	45.2 ab	9.8 a	0.9 a	0.8 b	188.7 c	17.6 c	10.7 b	42.1 ab

Data presented as means; different letters within a column, and within a single treatment, indicate significant differences at *P*<0.05 according to Duncan's New Multiple Range test. CO<sub>2</sub> concentration = 3000 ppm. Abbreviations: FW = fresh weight (mg), DW = dry weight (mg); FC = flow cytometry. <sup>\*1</sup> = (leaf FW:DW):(root FW:DW), or measurement of carbon partitioning; <sup>\*2</sup> = measurement of chlorophyll content on the 3<sup>rd</sup> youngest leaf; <sup>\*3</sup> = relative amounts; measurement from at least three leaves (mixture of tip and sheath) t = trace (≤2%); <sup>\*4</sup> = relative amounts; measurement from at least three roots (mixture of proximal tip and distal end) t = trace (≤2%); <sup>\*5</sup> = relative amounts; a minimum of three samples per explant source, t = trace (≤2%); all FC values = 2C:4C:8C:16C relative ratios.

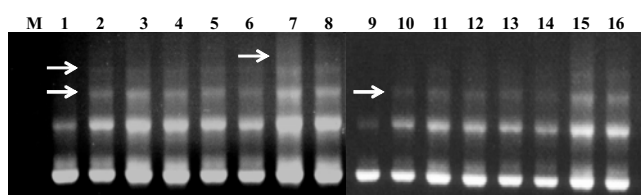
PLBs (Figs. 7, 9). Moreover, the 2C:4C ratio of both leaves and roots was high, but a trace amount of endopolyploid levels could be detected in the roots (Figs. 7, 9). Fukai *et al.* (2002) and Fujii *et al.* (1999) also showed high nuclear polyploidy in PLBs, roots and floral organs in *Cymbidium*.

RAPD banding for B-10 10mer primer showed no polymorphisms, despite the use of different carbon sources (Fig. 10). RAPDs using fifteen 10mer arbitrary primers allowed a total of 132 RAPD markers, 78% of which were polymorphic, to distinguish 36 *Cymbidium* cultivars (Obara-Okeyo and Kako 1998). The presence of three additional bands (not present in fructose and turanose) suggests alterations in the mitochondrial genome. RAPDs were also used to detect genetic differences in several *Cymbidium* in *in vitro* studies (Teixeira da Silva *et al.* 2005; Teixeira da Silva and Tanaka 2006a; Teixeira da Silva *et al.* 2006).

When sugar concentration in the plant increases, there is a repression of genes involved in the mobilization of stored reserves and photosynthesis. At the same time, genes required for metabolism and storage of carbon metabolites for future use are induced (Koch *et al.* 2000). Carbon metabolite (hexose)-mediated regulatory mechanisms regulate photosynthesis and provide the necessary integration with plant metabolism and the genes encoding them



**Fig. 9** Histogram showing polyploidy in hybrid *Cymbidium* Twilight Moon 'Day Light' PLB or callus. Arrows: (1) control diploid *Hordeum vulgare* (barley) 2C and (2) 4C; (3) *Cymbidium* 2C, (4) 4C, (5) 8C.



**Fig. 10** RAPD-PCR of epiphytic hybrid *Cymbidium* Twilight Moon 'Day Light' 3-month old *in vitro* plantlets using B-10 10mer primer to test for genetic stability. Lanes: (M) DNA marker (100 bp); (1) fructose, (2) galactose; (3) glucose; (4) mannose; (5) xylose; (6) lactose; (7) maltose; (8) sucrose; (9) turanose; (10) raffinose; (11) cellulose; (12) starch; (13) mannitol; (14) sorbitol; (15) *Phalaenopsis* Galant Beau 'George Vazquez' on sucrose medium (Tanaka *et al.* 1988); (16) *Dendranthema grandiflora* 'Shuhou-no-chikara' on sucrose medium (Teixeira da Silva and Fukai 2003). Arrows = carbon source-specific bands.

(Pego *et al.* 2000). Gene regulation by hexoses occurs when the depletion of sugars results in activation of gene expression and to an increase in photosynthetic capacity, explaining the higher photosynthetic capacities, as demonstrated by mono- and disaccharides when compared to other carbon sources (Fig. 8). When output exceeds the plant's capacity to metabolise or export sugars (limited in the case of heterotrophic tTCLs), increasing sugar concentration represses the same photosynthetic and/or metabolic genes. The down regulation of photosynthetic genes (Pego

*et al.* 2000) may account for the observable decrease in PLB formation, despite the continued increase in tTCL fresh weight (Table 1). In addition, new sugar sensing mechanisms exist for disaccharide analogs (turanose, a glycosylated fructose and rare disaccharide) that are not even membrane permeable (Ferne *et al.* 2001), explaining even its capacity to be metabolized, and used for PLB production.

## CONCLUSIONS

The choice of carbon source for *Cymbidium in vitro* culture, independent of the organogenic programme (PLB or callus) induced by PLB tTCLs, has an effect on the quantitative and qualitative outcome of each programme. Certain carbon sources, including rare sugars, have the potential to be utilized in a positive selection system in genetic transformation since organogenesis is limited when they are applied even at low concentrations. This has been demonstrated in chrysanthemum (Teixeira da Silva 2004b) and in *Cymbidium* (this study). Moreover, conventional glass-bottle heterotrophic micropropagation favours root formation on a sucrose-based medium, photoautotrophic propagation in the Vitron® (Fig. 1) favours shoot formation, without a compromise in cytogenetic or photosynthetic qualities. Preliminary results indicate that the trends observed in the growth and photosynthetic data of *Cymbidium* Twilight Moon 'Day Light' may represent a constant across various hybrids. Preliminary analyses of 8 other *Cymbidium* hybrids (Aroma Candle 'Hot Heart', Pretty Poetry 'Malachite', Alice Beauty 'No 1', Spring Night 'No 12', Dream City 'No 1', Call Me Love 'Snow Princess', Energy Star 'No 4', Sweet Moon 'No 2') indicated that the qualitative effect of these carbon sources may be universal, at least in *Cymbidium*.

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