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NOVEL FACTORS AFFECTING SHOOT CULTURE OF CHRYSANTHEMUM (*DENDRANTHEMA* × *GRANDIFLORA*)

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Abstract

Teixeira da Silva J.A., 2014: Novel factors affecting shoot culture of chrysanthemum (*Dendranthema* × *grandiflora*) [Nauji veiksniai įtakojantys chrizantemų (Dendranthema × grandiflora) ūglių kultūrų auginimą]. – Bot. Lith., 20(1): 27–40.

Chrysanthemum (*Dendranthema* × *grandiflora* (Ramat.) Kitamura) continues to be one of the most important ornamental plants in the world. Although the tissue culture of chrysanthemum has been widely explored, several unexplored topics remain, and, in developing countries, there is always the constant search for reducing the cost of raising tissue cultured plants. In this study, by focusing on a leading market cultivar in Japan, 'Shuhou-no-chikara', alternatives to agar (as the gelling agent) and sucrose (as the carbon source) for chrysanthemum tissue culture were sought. Both Gellan gum and agar resulted in greater shoot and root production than all other gelling agents tested, including Bacto agar, phytagel, oatmeal agar, potato dextrose agar, barley starch and corn starch. All of the alternative liquid-based medium additives tested (low and full fat milk, Coca-cola®, coffee, Japanese green, Oolong and Darjeeling teas) negatively impacted plant growth, stunted roots and decreased chlorophyll content (SPAD value) of leaves. There was no difference between plants grown on medium with refined sucrose or table sugar, although poor growth was observed when stevia (*Stevia rebaudiana*) extract was used. Photoautotrophic micropropagation increased significantly the shoot mass relative to control plants, even when the density of plants was doubled. Aeration improved plantlet growth. The tetrazolium test was a simple, but effective essay to see the intensity and strength of root growth in different basal media. MDH activity decreased in the root+shoot extract of plants grown on most alternative media, but remained high on TCSGM (Teixeira's chrysanthemum shoot growth medium), Gellan gum, aerated and CO2-enriched cultures. A similar trend was observed for deaminating GDH, while an opposite trend was observed for aminating GDH activity. These experiments indicate that tissue culture research for chrysanthemum still provides a rich field for exploration with interesting and valuable results.

Keywords: agar, alternative gelling agents, milk, oatmeal agar, table sugar.

INTRODUCTION

Tissue-cultured plants undoubtedly cost more than plants derived by other methods of propagation and there is a constant need to explore alternatives to drive down costs (Purohit et al., 2011). Chemicals in micropropagation media are estimated to cost a little less than 15% of the total cost, but the cost of gelling agent per unit media is 73.53% (Prakash et al., 2004), although this estimate is likely to vary considerably in different countries around the world. Medium costs, to be realistic, are however, dwarfed by lighting and electricity costs, estimated at a ratio of 99: 1 (personal calculations), but dealing with tissue culture methods to reduce energy costs – a current global crisis – is the topic for another dis-

cussion, and beyond the focus of this paper. Several alternative low-cost gelling agents have been tested including sago powder, isabgol husk, guar gum, cassava flour, xantham gum (Maliro & Lameck, 2004; Jain & Babbar, 2005, 2006; Gour & Kant, 2011) and several other media alternatives tested on a hybrid orchid, *Cymbidium* (Teixeira da Silva & Tanaka, 2009; Van et al., 2012a), but which were also repeated to some extent for chrysanthemum in this study. The choice of gelling agent is one of the most basic requirements for successful plant tissue culture (reviewed by Cameron, 2008).

Photoautotrophic micropropagation is one possible means to reduce production costs and install automation-robotization of the micropropagation process by minimizing microbial contamination, and by increasing photosynthetic rate, growth and rooting *in vitro* and survival percentage *ex vitro* (Norikane et al., 2010). Tanaka (1992) and Hahn & Paek (2005) showed that photoautotrophic culture, compared with heterotrophic growth, resulted in improved growth parameters such as larger and more vigorous uniform *Phalaenopsis* plantlets. Teixeira da Silva et al. (2007b) showed that photoautotrophic conditions led to higher callus and protocorm-like body (PLB) fresh and dry weight, number of PLBs, and more robust hybrid *Cymbidium* plants. The Vitron (gas-permeable vessel created by Otsuka, Tokushima, Japan) was successful in the photoautotrophic micropropagation of *Spathipyllum* (Teixeira da Silva et al., 2006).

The tissue culture of chrysanthemum has been very well explored (Teixeira da Silva 2003, 2004a; Shinoyama et al., 2006; Teixeira da Silva et al., 2013), including through the use of thin cell layers (Teixeira da Silva et al., 2007a) and more recently the use of flower tissue (Teixeira da Silva, 2014), and thus a background will not be dealt with here. Chrysanthemum is traditionally micropropagated on solid-based, sucrose-supplemented media, but has also been propagated photoautotrophically or photomixotrophically under CO_2 enrichment, with reduced contamination levels and production costs (Mitra et al., 1998), while tissue-cultured plants performed just as well as hydroponic systems (Hahn et al., 1998). Despite this, the use of table sugar and a wide range of alternative medium additives and gelling agents have not yet been explored in chrysanthemum tissue culture, and this is the focus of this study.

MATERIALS AND METHODS

Chemicals and reagents

Unless specified otherwise, all chemicals and reagents were of tissue culture grade or the highest grade available. In all cases of morphological analyses and biochemical assays, except where specified otherwise, chemicals or reagents were purchased from Nacalai-Tesque (Kyoto, Japan), Wako (Osaka, Japan) or Sigma-Aldrich (St. Louis, MI, USA). Gellan gum (Gelrite®) was purchased from Merck (USA). The following were bought from local supermarkets in Takamatsu (Japan) (brand name indicated in parentheses): low and full fat milk (Meiji), Coca-Cola®, instant, granulated coffee (Nescafé, Nestlé), Japanese green tea (Itoh), Oolong tea (Suntory), Darjeeling tea (Tetley), sugarcane-derived table sugar (Mitsui Sugar Co. Ltd.; percentage sucrose unknown), and corn starch (A-Price). Stevia (*Stevia rebaudiana*) was a Chinese cultivar (high level of rebaudioside) grown under field conditions.

Plant cultures, explants and general media preparation

In vitro plant cultures and mother (donor) plants were established and amassed over several months and maintained as explained in detail in Teixeira da Silva (2005). In addition, to create sufficient stock for each replicate trial, after removing shoot tips for experiments, new stock was established using the remaining plantlets by subculturing nodal explants containing two nodes and at least one fully expanded leaf. Apical shoots 2–3 cm long with two fully open/developed leaves were harvested from actively growing cultures and used for tissue culture and regeneration experiments. Axillary shoots were not used. After adding 2 mg/l 6-benzyladenine (BA), 1 mg/l 1-naphthyleneacetic acid (NAA) and 20 g/l sucrose (no sucrose in photoautotrophic experiments) to basal Murashige & Skoog (MS; 1962) medium (including full-strength MS micronutrients, macronutrients and vitamins), all media were adjusted to pH 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 100 KPa for 15 min. This medium served as the control, following optimization in Teixeira da Silva (2005) for all experiments and will be referred to hereafter as Teixeira's chrysanthemum shoot growth medium (TCSGM) throughout the

remainder of the text. Shoots from all controls and treatments 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. The interface between lid and growth bottles (except for Vitron experiments) was sealed with double layers of Parafilm® (Pechiney Plastic Packing, Chicago, IL). All cultures were initiated simultaneously and tracked for 60 days.

Substrate, medium additive and photoautotrophic treatments

Five sets of experiments were established to test the effect of substrate; medium additives and CO_2 enrichment on chrysanthemum shoot growth. All experiments were repeated in triplicate, with 36 samples in total per treatment (12 × 3). In each experimental treatment, 12 shoots were placed in each 500-ml glass bottle (AsOne, Osaka, Japan), grown on 150 ml of solidified TCSGM, except for the photoautotrophic experiments, in which the Vitron was used and liquid TCSGM was employed. All media were set to pH 5.8. No additives were filter sterilized; all were autoclaved. All culture vessels from all treatments were placed under temperature and light conditions identical to those used for plantlet induction and proliferation (Teixeira da Silva, 2005).

Set A (alternative medium gelling agents). Oatmeal agar (30 g/l), agar, bacto agar, potato dextrose agar and phytagel (8 g/l), barley starch and corn starch (4 g/l), and gellan gum (Gelrite®) (2 g/l). Pre-experimental trials were conducted to test which concentration was firm, not rock-hard, nor jelly-like so as to support the growth of shoots and plantlets. The control was TCSGM.

Set B (innovative additives). Full and low-fat milk (100%, v/v), Oolong tea (50%, v/v), Coca-cola®, coffee, stevia extract, Japanese green, Oolong and Darjeeling teas (2%, v/v). Coca-cola® was shaken for 24 h prior to use in medium to de-gas the soft drink. A stock solution of teas, stevia extract and coffee was prepared. For coffee and stevia extract, 10 g/l (coffee powder or fresh stevia leaves) was added to boiling water, stirred well and then added to culture medium until a 2% (v/v) final concentration was obtained. Similarly, Japanese green tea and Darjeeling tea stocks were prepared by infusing five teaspoons of dry leaves or a tea-bag (approx. 5 g), respectively for 3 min in boiling water and then added to culture medium until 2% (v/v) final concentration was obtained. Oolong tea was purchased as a ready-made bottled tea and was used as such in media preparation. The control was TCSGM.

Set C (TC grade vs table sugar). Table sugar was added to medium at the same concentration as sucrose in the control, i.e. TCSGM. Sucrose costs 2200 JPN Yen/kg while refined table sugar (sugarcane-derived) costs 98 JPN Yen/kg (78 JPN Yen = 1 US$; September, 2012), i.e. 20 times higher cost.

Set D (aeration). Aeration was provided by container caps that were perforated in the centre with a hole of 4 mm in diameter and covered by a Milliseal® (Japan Millipore Co., Ltd, Tokyo, Japan) to increase the ventilation within the vessel. The control was glass flasks with TCSGM.

Set E (photoautotrophic micropropagation and plant density). Rockwool (RW) with 125 ml of cool autoclaved liquid TCSGM 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 Vitron when at room temperature (Van et al., 2012b). Either 12 or 24 shoots were inserted into the RW (i.e. single vs double density), evenly spaced. There were two sub-treatments (ST) (both densities tested for each):

ST1 (control): no CO_2-enrichment, with 20 g l^{-1} (w/v) sucrose-containing liquid TCSGM.

ST2: CO_2-enrichment (3000 µmol mol^{-1} 24 h^{-1} d^{-1}), sugar-free liquid TCSGM.

Morphological analyses

The growth and development of plants were evaluated after 60 days following culture initiation, i.e. plating shoot tips. Survival percentage, plantlet height (mm), total number of newly formed leaves (i.e. excluding the initial two leaves), and total plantlet net fresh weight (FW) were determined. Plantlet survival was calculated based on the percentage of plants being totally green. Plantlet growth was measured in mm counting from the shoot tip to the base minus the initial size of the initial shoot tip explants (i.e. 30 mm).

Physiological analysis

Chlorophyll content of the third leaf (still attached to the plant) 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., 2006). The SPAD value, which is reported in SPAD units, is highly correlated with chlorophyll content (R^2 = 0.89) and is thus a simple and effective way to reflect chlorophyll content (Coste et al., 2010).

Biochemical assays

The roots of 10 plants growing *in vitro* after 60 days on any treatments were immersed into liquid TCSGM medium containing 0.005% tetrazolium violet, whose 0.1% stock solution was filter sterilized (0.22 µm syringe filter, Millipore, USA), modified from the original idea of using such an assay to examine dehydrogenase activity in actively-growing roots in tissue culture (Kurzbaum et al., 2010). Purple colour intensity was scored (- = none; + = weak; ++ = strong; +++ = very intense) after 96 h. As a control, boiled chrysanthemum plants grown *in vitro* on TCSGM for 60 days, were used.

The activities of total malate dehydrogenase (NAD^+-MDH, EC 1.1.1.37) and of aminating (NADH-GDH) and deaminating (NAD^+-GDH) glutamate dehydrogenase (EC 1.4.1.2) were determined according to Kumar et al. (2000) and pertinent references therein, with modifications, as indicated next. All steps in the assays pertaining to extraction were performed on ice. For all assays, 200 mg of fresh roots and leaves, harvested from *in vitro* plantlets on day 45 and day 60 of *in vitro* culture. Since no roots formed until about day 20 (in the fastest treatments), no assays were performed until day 45. Thus, for all assays, shoots and roots of healthy, vigorous (5–6 cm tall) 60-day-old plantlets growing on TCSGM served as the control material.

For the total NAD^+-MDH activity assay, 200 mg of root+leaf tissue was homogenized in 5 ml of 100 mM Tris–HCl buffer (pH 7.8) containing 20 mM $MgCl_2$, 1 mM dithiothrietol, 1 mM EDTA and 1 mM PMSF (phenylmethanesulfonylfluoride) using a chilled pestle and mortar. The homogenate was then centrifuged at 4°C at 22.000 × *g* for 15 min. The supernatant was dialysed using a phenyl-sepharose column and using TMZP buffer (Wojciechowski & Kantrowitz, 2002) at 4°C for 3 h against the extraction buffer. The reaction mixture contained, in a total volume of 3 ml, 100 mM Tris–HCl buffer (pH 7.8), 20 mM $MgCl_2$, 1 mm EDTA, 0.1 mM NADH, 0.5 mM oxaloacetate and 200 ml enzyme. The reaction was initiated at 25°C when the enzyme was added and NADH oxidation was monitored at 340 nm in a spectrophotometer (Shimadzu MPS-2000, Tokyo, Japan). Enzyme-specific activity was expressed as nmol NADH oxidized s^{-1} mg $protein^{-1}$.

For the total NADH-GDH and NAD^+-GDH activity assay, 200 mg of root+leaf tissue was homogenized in 5 ml of 100 mM Tris–HCl buffer (pH 8.0) containing 3.3 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM DTT and 1 mM PMSF. The mixture was centrifuged at 22.000 × *g* for 10 min at 4°C. Supernatants were dialysed against the extraction buffer in cold for 4 h and served as the enzyme extracts. Aminating (NADH-GDH) and deaminating (NAD^+-GDH) activities of the enzymes were assayed based on the change in absorbance at 340 nm due to NADH oxidation or NAD^+ reduction. The assay medium for the amination reaction contained 76 mM Tris–HCl buffer (pH 8.1), 20 mM α-ketoglutarate, 150 mM $(NH_4)_2SO_4$, 0.2 mM NADH, 1 mM $MgCl_2$ and 200 ml enzyme in a total volume of 3 ml. NADH oxidation was followed by recording the change in absorbance at 340 nm in a spectrophotometer and enzyme-specific activity was expressed as nmol NADH oxidized s^{-1} mg $protein^{-1}$. The assay mixture for the deamination reaction consisted of 100 mM Tris–HCl buffer (pH 9.0), 0.6 mM NAD^+, 50 mM L-glutamate and 200 ml enzyme in a total volume of 3 ml and enzyme-specific activity was expressed as nmol NAD^+ oxidized s^{-1} mg $protein^{-1}$.

All assays were done at 25°C and in triplicate based on three different individual plants from separate flasks or Vitron, i.e. nine samples per assay.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD). Each experiment was repeated three times 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 Student's-*t*-Distribution (standard error, Excel 2010) were used to test for differences between means.

RESULTS AND DISCUSSION

Although the tissue culture of chrysanthemum is well-established and explored (Teixeira da Silva et al., 2013), surprisingly there are still gaps in our knowledge about the reaction of this ornamental to several factors *in vitro*. Even though these may have limited commercial interest, they are nonetheless interesting facts to explore and certainly advance our knowledge of how this ornamental responds *in vitro*. In this study, five sets of experiments were conducted to test for the response of leading (market-wise) Japanese chrysanthemum cultivar ('Shuhou-no-chikara') to the following aspects *in vitro*: agar type (i.e. culture support base); novel additives; plant density under photoautotrophic (Fig. 1) and heterotrophic conditions; aeration.

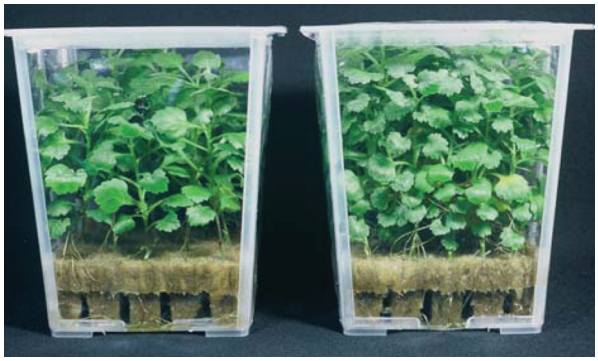

Fig. 1. Photoautotrophic micropropagation of *Dendranthema* × *grandiflora* 'Shuhou-no-chikara' under 3000 ppm CO_2 enrichment in an air-permeable vessel, the Vitron; right (24) = double the density of left (12 plants). Growth observed after 60 days

Photoautotrophic micropropagation is one possible way of increasing plant biomass under high CO_2 and high PPFD conditions, as has recently been shown with *Oncidesa* plantlets grown under super-enriched CO_2 conditions (Norikane et al., 2013). Photoautotrophic culture (CO_2-enriched condition at 3000 µmol/mol/24 h/d, sugar-free medium) resulted in lower growth of *Cymbidium* plantlets than in heterotrophic cultures (Van et al., 2012a), while Teixeira da Silva et al. (2007b) found photoautotrophic micropropagation to significantly improve shoot and root fresh and dry weight of another hybrid *Cymbidium*. Teixeira da Silva et al. (2006) also found that CO_2 enrichment in the Vitron improved the growth of *Spathiphyllum* plants.

Shoots grew best on gellan gum- and agar-based optimized shoot induction medium (TCSGM), while all other agar types resulted in much poorer performance (Table 1A) or even bushiness in the case of barley starch (Fig. 2D). All plants performed poorly on TCSGM when supplemented with full-fat or low-fat milk (Fig. 2E), Coca-Cola ®, coffee or tea (Japanese green, Oolong and Darjeeling teas) (Table 1B). Plantlets performed equally well whether TC grade sucrose or commercial table sugar was used (Table 1C). Aeration resulted in improved plantlet FW, but the highest value was with CO_2 enrichment (Table 1D). Photoautotrophic micropropagation resulted in a significant increase of the shoot mass and root mass (data not shown) relative to control plants, even when the density of plants was doubled (Table 1E). Tetrazolium indicates the actively-growing plant

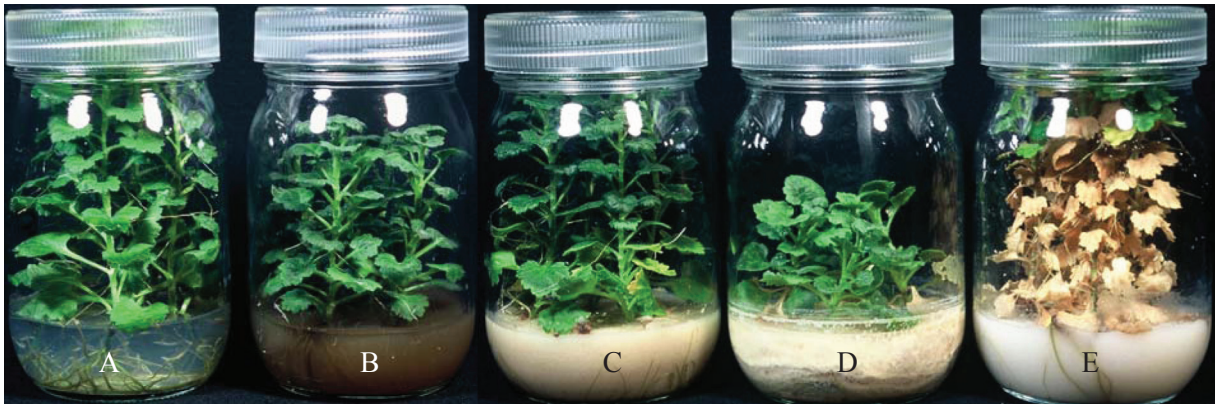

Fig. 2. *In vitro* micropropagation of *Dendranthema* × *grandiflora* 'Shuhou-no-chikara' on control medium (A), 2% (v/v) stevia extract (B), 1% (w/v) corn starch (C), 1% (w/v) barley starch (D) and 100% (v/v) full-fat milk (E), demonstrating the capacity to grow on media with different gelling agents, bases or additives, albeit with different growth responses. Growth observed after 60 days

Table 1. Effect of alternative liquid-based medium additives on chrysanthemum (*Dendranthema* × *grandiflora* (Ramat.) Kitamura) 'Shuhou-no-chikara' plantlet growth and development, measured after 60 days

Treatments*		Survival (%)	Plantlet height (mm)	No. new leaves	Plantlet net FW (mg)
Universal (i.e. for all treatments) control: Agar-based TCSGM*					
		100 a	108 a	24 b	800 b
A. Gelling agent					
Bacto agar		22 bc	12 cd	16 bc	237 d
Barley starch		43 b	8 cd	9 bc	481 cd
Corn starch		56 b	16 cd	20 bc	563 c
Gellan gum (Gelrite®)		100 a	112 a	28 b	1106 a
Phytagel		41 b	20 c	10 bc	531 c
Potato dextrose agar		4 c	0 d	0 c	15 e
Oatmeal agar		61 b	64 b	20 b	844 b
B. Alternative liquid-based medium additives					
Coca-cola®		0 c	2 d	0 c	Neg.
Coffee		0 c	0 d	0 c	Neg.
Milk	Full-fat	100 a	112 a	39 a	763 bc**
	Low-fat	100 a	24 c	12 bc	801 b**
Stevia extract		89 ab	38 bc	9 bc	1014 ab
Tea	Darjeeling	85 ab	58 b	12 bc	604 c
	Japanese green	87 ab	61 b	13 bc	586 c
	Oolong	78 ab	49 b	10 bc	529 c
C. Table sugar		100 a	99 a	21 b	916 ab
D. Aeration		100 a	104 a	36 ab	1086 a
E. + CO_2	12 plants	100 a	123 a	44 a	1282 a
	24 plants	100 a	116 a	40 a	1179 a

Note: Means within a column within each set of experiments (A, B, C, D, E) and for each plant character followed by the same letters are not significantly different to TCSGM* at $p = 0.05$ by Duncan's multiple range test, n = 108 per treatment (36 × 3 replicates × 3 repetitions). In all cases, 12 shoot tips were plated per culture vessel, except for Set E, in which 12 vs 24 plants were compared under photoautotrophic conditions. Neg. = negligible (not zero, but close enough). ** Except for about 10% of terminal new leaves, most leaves were dry and brittle.
FW – fresh weight,* – control based on Teixeira da Silva (2005), TCSGM – Teixeira's chrysanthemum shoot growth medium

tissues and dehydrogenase activity (Kurzbaum et al., 2010). Most of the treatments showed varying degrees of tetrazolium colour formation (Table 2), which interestingly seemed to follow a pattern mimicking the SPAD values, suggesting that root and shoot assays could be used in a unique manner to test plant viability in response to different factors *in vitro*, including stresses, etc. and opening up new vistas for the application of these assays more widely in plant tissue culture.

MDH activity decreased in the root+shoot extract of plants grown on most alternative media, but remained high on TCSGM, Gellan gum, aerated and CO_2-enriched cultures (Table 2). MDH activity was negligible (< 10 nmol NADH/NADPH oxidized s^{-1} mg $protein^{-1}$) in plants grown on several media, suggesting near death of the plants. Kumar et al. (2000) also noted a decrease in total and organelle-specific MDH activity in response to NaCl-induced stress in rice (*Oryza sativa*) *in vitro* cultures, while Sánchez-Rodríguez et al. (1977) noted a similar trend for *Casuarina* plants exposed to drought stress, while Jorge et al. (1997) also noted a similar trend for MDH activity of *Cereus peruvianus* plants exposed to sugar and temperature stress, suggesting that the trend for stress treatments *in vitro* is for MDH activity to decrease. An increase in aminating (NADH-GDH) activity was observed in the same media, where MDH activity decreased, while activity of deaminating enzyme (NAD^+-GDH) decreased concomitantly. Although KUMAR et al. (2000) noted an increase in aminating GDH activity with an increase in salt stress *in vitro*, no clear pattern or trend could be observed for deaminating GDH. GDH activity

Table 2. Effect of alternative liquid-based medium additives on chrysanthemum (*Dendranthema* × *grandiflora* (Ramat.) Kitamura) 'Shuhou-no-chikara' SPAD value and dehydrogenase activity, measured in leaves and roots, respectively after 60 days

Treatments*		SPAD value (SPAD units)	Tetrazolium colour**	MDH activity (nmol NADH/NADPH oxidized s^{-1} mg $protein^{-1}$)	NAD^+-GDH activity***
Universal (i.e. for all treatments) control: Agar-based TCSGM*					
		42.1 a	+++	84.2 b	18.2 a : 26.0 b
A. Gelling agent					
Bacto agar		22.2 b	+	16.3 c	6.8 b: 41.2 a
Barley starch		34.6 ab	++	Neg.	Neg.: 40.6 a
Corn starch		38.0 a	++	Neg.	Neg.: 38.9 a
Gellan gum (Gelrite®)		43.8 a	+++	76.2 b	20.8 a : 21.3 b
Phytagel		41.8 a	+++	Neg.	Neg.: 39.2 a
Potato dextrose agar		4.1 c	-	Neg.	Neg.: 40.0 a
Oatmeal agar		38.0 b	++	Neg.	Neg.: 36.2 a
B. Alternative liquid-based medium additives					
Coca-cola®		0 c	-	Neg.	Neg.: 42.0 a
Coffee		0 c	-	Neg.	Neg.: 43.9 a
Milk	Full-fat	12.4 bc	+	Neg.	Neg.: 41.0 a
	Low-fat	18.6 b	+	Neg.	Neg.: 38.6 a
Stevia extract		23.2 b	+++	Neg.	Neg.: 42.1 a
Tea	Darjeeling	33.1 ab	++	Neg.	Neg.: 39.6 a
	Japanese green	34.8 ab	++	Neg.	Neg.: 41.4 a
	Oolong	31.2 ab	++	Neg.	Neg.: 43.2 a
C. Table sugar		40.4 a	+++	74.8 b	22.9 a : 20.4 b
D. Aeration		42.6 a	+++	81.6 b	20.9 a : 21.6 b
E. + CO_2	12 plants	44.2 a	+++	101.3 a	21.1 a : 23.2 b
	24 plants	41.6 a	+++	98.6 a	19.6 a : 26.6 b
Tetrazolium control (boiled plants)			-		

Note: Means within a column within each set of experiments (A, B, C, D, E) and for each plant character followed by the same letters are not significantly different to TCSGM* at $P = 0.05$ by Duncan's multiple range test, n = 108 per treatment (36 × 3 replicates × 3 repetitions). In all cases, 12 shoot tips were plated per culture vessel, except for Set E, in which 12 vs 24 plants were compared under photoautotrophic conditions. Neg. = negligible (< 10 nmol NADH/NADPH oxidized s^{-1} mg $protein^{-1}$ for MDH activity and < 5 nmol NAD+ reduced s^{-1} mg $protein^{-1}$ for NAD^+-GDH activity).

* – control based on Teixeira da Silva (2005), TCSGM – Teixeira's chrysanthemum shoot growth medium, ** – purple colour intensity score measured after 96 h: - – none, + – weak; ++ – strong, +++ – very intense, *** – NADH-GDH activity (nmol NAD+ reduced s^{-1} mg $protein^{-1}$: nmol NADH oxidized s^{-1} mg $protein^{-1}$)

was shown to increase under stressful conditions: wheat in response to darkness (Lauriere & Daussant, 1983; Yamaya & Oaks, 1987), senescence in barley (Calle et al., 1986), NaCl-induced salinity in *Vigna radiata* (Gulati & Jaiwal, 1996), heavy metal (Cd and Ni) toxicity in *Silene italica* (Mattioni et al., 1997) and exposure to octane and benzene in *Lolium perenne* and *Medicago sativa* (Sadunishvili et al., 2009). Dehydrogenases are important in plant metabolism since they generate reducing power for various biosynthetic processes; they also support redox cycling in the cell, allowing the plant to sustain stress (Hare et al., 1998).

Most of the factors assessed in this study have been shown to affect the growth of plantlets *in vitro* of other species, although such factors have never been assessed for chrysanthemum. Some key studies and findings will be highlighted. The choice of gelling agent affected organogenesis in hybrid *Cymbidium* plantlet cultures in which gellan gum resulted, in general, in better plant growth parameters than Bacto agar and oatmeal agar, while the number of roots was the highest on gellan gum as was the fresh and dry mass of shoots and roots although more leaves were produced on Bacto agar (Van et al., 2012a). In that study, the chlorophyll content of

Cymbidium plantlets grown in oatmeal agar was the lowest among all basal medium treatments; finally, oatmeal agar-based medium strongly inhibited the initiation of new leaves and roots compared to other gelling agents. Gellan gum formed more PLBs than oat meal agar and potato dextrose agar in another hybrid *Cymbidium* (Teixeira da Silva & Tanaka, 2009).

Gelling agent is one of the most basic requirements for successful plant tissue culture (Cameron, 2008). The choice of gelling agent strongly affected adventitious shoot regeneration capacity and water content of French marigold (*Tagetes minuta*) shoots (Jain et al., 2001; Modi et al., 2009). Hyperhydricity of *Dianthus caryophyllus* shoots was indirectly proportional to agar concentration (Casanova et al., 2008), although shoot multiplication rate can be drastically reduced if the concentration is too high (George, 1996). *Malus x domestica* (apple) shoots became hyperhydric when cultured on phytagel-solidified medium (Turner & Singha, 1990), as did those of *Pyrus communis* (pear) (Kadoka & Niimi, 2003) and *Scrophularia yoshimurae* (Tsay et al., 2006). Van et al. (2012a) did not observe hyperhydricity in *Cymbidium* plantlets in any gel-based media. In this study, hyperhydricity was not observed, but plants grew poorly, relative to agar and gellan gum. Scholten & Pierik (1998) noted how seven commercial agar brands (1. Merck 1614, 2. Daishin (Brunschwig Chemie, Netherlands), 3. Difco Bacto, 4. MC 29 (LabM, UK), 5. BD (Becton Dickinson) grade A, 6. BD granulated and 7. BD purified) resulted in very different organogenic responses in rose (*Rosa hybrida* L. cv. 'Motrea') shoot length and weight and root number and weight, lily (*Lilium longiflorum* cv. 'Enchantment') bulblet formation on scale explants and cactus (*Sulcorebutia alba*) areole number. Dobránszki et al. (2011) showed that the rheological and diffusion properties of gelling agents affected shoot development in apple and black locus, or the incidence of hyperhydricity in *Aloe*, as shown by Ivanova & van Staden (2011).

Agar is most likely the most commonly used gelling agent in plant tissue culture (Babbar & Jain, 1998), although Gellan gum or Gelrite®, a polymer of glucuronic acid, rhamnose, glucose and *O*-acetyl moieties (Scholten & Pierik, 1998), is also a popular choice. The gelling properties of agar and gellan gum are very different. Agar functions by binding the water. Therefore, the higher the agar concentration, the more strongly water is bound. Gelrite®, however, requires the presence of cations for gelation; thus, in general, when medium pH is low, agar cannot set (Cameron, 2008; Morris et al., 2012). The gelling properties of the other gelling agents tested are unknown or are poorly publicized or tested. *Phalaenopsis* leaf segments obtained from shoots derived from flower-stalk cuttings cultured *in vitro* on a Gelrite®-solidified medium resulted in the formation of more callus-derived PLBs than when agar was used as the medium solidifying agent (Ichihashi & Hiraiwa, 1996; Ishii et al., 1998). When tobacco and wild carrot cultures were grown on medium gelled with corn starch, dry weight was three times higher than on medium gelled with agar (Henderson & Kinnersley, 1988). A mixture of corn starch and Gelrite® was a suitable substitute for agar in the *in vitro* cultivation of apple and red raspberry (Zimmerman et al., 1995). Starches from barley, corn, potato, rice and wheat were all suitable substitutes for agar in the culture of barley (*Hordeum vulgare* L.) seeds, although the most effective was that from barley (Sorvari, 1986). 'Isubgol' is a derived form of the mucilaginous husk isolated 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 & Jain, 1998). Isubgol was also as effective as guar gum in the cost-effective multiplication of an orchid, *Dendrobium chrysotoxum* (Jain & Babbar, 2005). The choice of gelling agent affected the regeneration efficiency on selective medium in tulip (*Tulipa* sp.), gladiolus (*Gladiolus* sp.) and tobacco transformation experiments (Chauvin et al., 1999). The level of impurities within a gelling agent may 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 (Beruto & Curir, 2006). Agar was better than sago powder, guar gum or isabgol husk as a media-solidifying agent for *Balanites aegyptiaca* and *Phyllanthus emblica* (Gour & Kant, 2011).

Gour & Kant (2011) conducted a cost analysis and showed that the cost of one litre of MS medium (including vitamins, 3% sucrose and 0.8% agar) as per the 2007-08 price-list issued by HiMedia Labora-

tories (India) was INR (Indian Rupees) 30.64 (USD 0.65; currency conversion rate: 1 USD = 47 INR). Out of the various components of MS media, micronutrients (which are used at ≤ 100 mg/l), macro-nutrients (which are used at >100 mg/l), vitamins, sucrose and agar contributes 4.73, 0.59, 0.78, 20.36 and 73.53% of the total cost, respectively. Thus, agar and sucrose are two major components of nutrient media that contribute the most towards the cost of media. These authors also claimed that media solidified with isabgol husk in place of agar could reduce the cost of gelling agents per unit media by ~44%. According to Prakash (1993), the use of common sugar in place of laboratory-grade sucrose reduces the cost of carbon source of the medium by 78–87%, when applied to ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) production. In a study by Gour & Kant (2011), table sugar could reduce the cost of sucrose for one unit of MS medium by ~93%. However, estimates made by Teixeira da Silva (unpublished) show that medium costs are minimal relative to electricity costs, with actual medium additives accounting for approximately 1–3% of total tissue culture costs, of which the gelling agent accounts for a fraction of that 1–3%. Thus, although Gour & Kant (2011) showed that gelling agent costs could be reduced by ~44%, this is relatively irrelevant in the total picture of a tissue culture protocol.

Bhattacharya et al. (1994) found that sago (from *Metroxylon sagu* Rottb.) and isubgol (from *Plantago ovata* Forsk.) were highly cost-effective gelling agents compared to agar for the micropropagation of chrysanthemum (*Dendranthema grandiflora* Tzvelev) plantlets. Due to poor access to such materials, which are primarily produced in India, these gelling agents were not compared in this study. Crude agar was as good as AR grade agar in multiplication in *Wrightia tomentosa* (Joshi et al., 2009). Jain & Babbar (2005) indicated that the cost of one litre of isabgol and guar gum medium is about 2.5–13 times less than different brands of agar. The advantage of both is that, being of plant origin, they are biodegradable and do not pose any threat to the environment once disposed after use. Guar gum, used as a gelling agent in the *in vitro* propagation of *Linum usitatissimum* and *Brassica juncea*, was found to be 8–80 times cheaper than agar and Difco bacto agar, respectively (Babbar et al., 2005).

Gour & Kant (2011) found sucrose to be the second largest factor contributing to the cost of a tissue culture medium. Raw sugar, table sugar and jaggary were used as alternative carbon sources in place of sucrose, each at 2% (w/v). Replacement of table sugar, jaggary and raw sugar could reduce the cost of the carbon source up to 95.19, 90.38 and 85.58%, respectively. Among these low-cost alternatives to sucrose, table sugar improved the *in vitro* rooting of microshoots more than all other carbon sources (75%), but was still worse than the 100% rooting response observed on sucrose-containing medium, although the authors suggested that for *in vitro* rooting of microshoots at a large scale, table sugar could be a good option to reduce cost. In our study, plantlet growth was equally good on TCSGM, whether TC-grade sucrose or table sugar were used. When stevia extract or Coca-Cola® was used as the alternative sugar (carbohydrate) sources, plantlets grew poorly. However, in chrysanthemum, other carbohydrates can influence rhizogenesis, caulogenesis (shoots) and somatic embryogenesis (Teixeira da Silva, 2004b), callus and PLB formation in *Cymbidium* hybrids (Teixeira da Silva et al., 2007b) and root induction in tree peony (*Paeonia suffruticosa* Andr.) (Wang et al., 2012).

The need for a specific carbohydrate is species- and stage-specific. Prakash et al. (2004) claimed local (Indian) sugar to be as good as high-grade laboratory sugar for the multiplication of banana (*Musa paradisica*). Maple syrup (from *Acer saccharum*) was used for the multiplication (at 50 g/l) and rooting (at 34 g/l) of cherry (*Prunus avium*) rootstocks from nodal segments and shoot tips, although the carbohydrate requirement for rooting of shoots also depended upon the availability of auxins, nitrogen and light (Bonga & Aderkas, 1992). In that study, even though maple syrup initially promoted pre-meristemoid formation, it was inhibitory thereafter. The multiplication of *Wrightia tomentosa* shoots was higher on sugar cubes- and agar-supplemented media than on media supplemented with jaggary (Joshi et al., 2009).

CONCLUSIONS AND STUDY RATIONALE

The tissue culture of plants, including ornamental plants, can be enriched if new alternative gelling agents and medium alternatives can be used to sub-

stitute current, conventionally accepted standards such as agar or sucrose. In this study, some alternative gelling agents and additives were used to assess the growth response of chrysanthemum plants *in vitro*. Interestingly, several of the trends are similar to those observed for another, unrelated, ornamental *Syngonium* (data not shown). The selection or rationale for the choice of these substances in this study is not random. A more in-depth explanation follows. Why was milk tested? Milk is a water-based daily consumed drink in many countries around the world and often much of it is wastefully discarded to manipulate milk prices (Food and water watch, 2010). The rationale for testing milk was thus to serve as a possible alternative substrate to water if the latter is not available. Initial trials with milk showed that it curdled *in vitro* after autoclaving, thus could serve as a "solidifying" agent or substrate. The use of milk may thus address two factors simultaneously: milk wastage and an alternative substrate. Tea and coffee are well-known antioxidants that have the ability of removing reactive oxygen species (ROS) from *in vivo* or *in vitro* environments (for example, Anissi et al., 2014; Korir et al., 2014), which would hypothetically lead to improved cellular survival and growth. Thus, they were tested in this study. Since Japan has a wide range of readily available and cheap teas (lower range at 0.7–0.9 US$/liter for ready-made tea or about 3 US$ for 50 tea-bags, which can make 1 liter per bag), several were tested in this study. Coffee is also a universally consumed beverage. Stevia has as much as 300-fold higher sweetness than sucrose (and is thus one of the market competitors) (Meireles et al., 2006). Although there is a good literature about the growth of stevia *in vitro*, there is no study yet that uses stevia as an extract *in vitro* to test the growth of any plant. This is the first such study in the plant science literature. Finally, another, cheap and popular drink available globally (every country except for Cuba and North Korea) is Coca-Cola® (http://en.wikipedia.org/wiki/Coca-Cola), which could serve, in a degassed form, as an alternative form of sucrose in plant *in vitro* cultures. The choice of Coca-Cola® was due to its wider (relative to other competing cola-based drinks) universality and not because the author is a fan of the product (or not a fan of the competitor products).

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CONFLICTS OF INTEREST AND DISCLAIMER

The author declares no conflicts of interest. The author does not specifically endorse any of the brands used in this study. These were used with the pure intention of scientific exploration in mind.

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NAUJI VEIKSNIAI ĮTAKOJANTYS CHRIZANTEMŲ (*DENDRANTHEMA* × *GRANDIFLORA*) ŪGLIŲ KULTŪRŲ AUGINIMĄ

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Santrauka

Chrizantema (*Dendranthema* × *grandiflora* (Ramat.) Kitamura) vis dar tebėra viena iš svarbiausių dekoratyvinių augalų pasaulyje. Nors chrizantemų audinių kultūra buvo plačiai naudojama, tačiau dar ne visos galimybės išsemtos ir išsivysčiusiose šalyse nuolatos ieškoma būdų, kaip sumažinti išlaidas auginant augalus iš audinių kultūrų. Šio tyrimo duomenimis, didžiausią dėmesį skiriant pirmaujančiai rinkos veislei Japonijoje 'Shuhou-no-chikara', buvo ieškoma alternatyvų agarui (standikliui) ir sacharozei (anglies šaltiniui). Tiek gelano guma, tiek ir agaras lėmė didesnę pumpurų ir šaknų produkciją negu kiti ištirti standikliai, įskaitant Bacto agarą, fitagelį, avižinį agarą, bulvių dekstrozės agarą, miežių ir kukurūzų krakmolą. Visi alternatyvūs skysti terpių priedai (riebus ir pusriebis pienas, Coca-cola®, kava, japoniška žalioji, Oolong and Darjeeling arbatos) neigiamai paveikė augalų augimą, stabdė šaknų augimą ir sumažino lapuose chlorofilo kiekį (SPAD vienetais). Auginant augalus terpėje su valyta sacharoze arba stalo cukrumi skirtumų nepastebėta, tačiau panaudojus stevijos (*Stevia rebaudiana*) ekstraktą augimas sumažėjo. Naudojant fotoautotrofinį mikrodauginimą, žymiai padidėjo ūglių masė, lyginant su kontroliniu variantu, netgi tuo atveju, kai augalų tankis dvigubai didesnis. Vėdinimas pagerino augalų augimą. Tetrazolio testas buvo paprasta, bet veiksminga priemonė, išryškinanti intensyvų ir stiprų šaknų augimą skirtingose pagrindinėse terpėse. Ekstraktuose, išskirtuose iš augalų šaknų ir ūglių, išaugintų daugelyje alternatyvių terpių, MDH (malato dehidrogenazės) aktyvumas sumažėjo, bet išliko aukštas išaugintų TCŪAT (Tekseiros chrizantemos ūglių auginimo terpėje), gelano guma aeruotose ir CO_2 praturtintose kultūrose. Panaši tendencija buvo stebima deamidinant GDH (glutamato dehidrogenazę), tuo tarpu priešinga tendencija pastebėta ją amidinant. Šie chrizantemų audinių kultūrų tyrimai suteikia naujų galimybių vykdyti tolimesnę metodinę paiešką ir gauti įdomių bei vertingų rezultatų.