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THE EFFECT OF CARBON SOURCE ON *IN VITRO* ORGANOGENESIS OF CHRYSANTHEMUM THIN CELL LAYERS[1]

JAIME A. TEIXEIRA DA SILVA[2]

ABSTRACT

Carbon source is an indispensable factor for the development of an *in vitro* morphogenic program of chrysanthemum micropropagation. The choice of carbon source affects the qualitative and quantitative outcome, and also the response of thin cell layers when these are placed onto morphogenic (callus, root, shoot, somatic embryo) media. Threshold survival levels (TSLs) could not be obtained for sucrose, fructose or glucose. TSLs together with organ differentiation were, however, obtained for mannose (60-80 $g.L^{-1}$), xylose and lactose (40 $g.L^{-1}$) and cellulose (60-80 $g.L^{-1}$), making these carbon sources suitable as potential carbon sources for positive selection systems for chrysanthemum genetic transformation.

Key words: *Dendranthema* X *grandiflora*, phytotoxicity, regeneration capacity, threshold survival level.

RESUMO

EFEITO DE CARBONO NA ORGANOGÊNESE DE CAMADAS CELULARES FINAS DE CRISÂNTEMO

A fonte de carbono é factor indispensável no sucesso de um programa de desenvolvimento morfogênico *in vitro* de crisântemo. A escolha da fonte de carbono afeta a resposta qualitativa e quantitativa de camadas celulares finas, quando são colocadas em meios de desenvolvimento morfogênico (calo, raiz, eixo caulinar, embrião somático). Não se estabeleceram níveis de sobrevivência quando sucrose, frutose ou galactose foram usados, mas sim para manose (60-80 $g.L^{-1}$), xilose e lactose (40 $g.L^{-1}$), e celulose (60-80 $g.L^{-1}$), sugerindo o potencial dessas fontes de carbono em sistemas de seleção positivas em programas de transformação genética de crisântemo.

Palavras-chave: *Dendranthema* X *grandiflora*, fitotoxicidade, capacidade regenerativa, nível de sobrevivência.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; ES, explant survival; IAA, indole-3-acetic acid; NAA, α-naphthalene acetic acid; PGR, plant growth regulator; SE, somatic embryo; tTCL, transverse thin cell layer; TDZ, thidiazuron; TSL, threshold survival level.

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([2]) Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa, 761-0795, Japan. E-mail: jaimetex@lycos.com

1. INTRODUCTION

Sugars control the expression of many plant genes and their connection to metabolic and developmental processes is unequivocal (KOCH, 1996). Plant genetic transformation systems still require the use of antibiotics for the selection of cells or tissues that contain an antibiotic-degrading gene, such as *nptII* (kanamycin and other aminoglycoside antibiotics) and *hptII* (hygromycin), *inter alia*. This selection system is gradually being replaced by a positive (REED et al., 2001) selection system, since in the former there is the risk of resistance build-up to a selective substance by microbes, and a concern for potential negative environmental impacts (USFDA, 2002). Xylose, like mannose (a glucose epimer at the second carbon), is a carbohydrate that many plant species cannot metabolise, unless transformed by a xylose isomerase (EC 5.3.1.5) gene. Xylose isomerase converts xylose to xylulose, which is then metabolised through the pentose phosphate pathway. Mannose can be metabolised by phosphomannose isomerase (EC 5.3.1.8; *PMI*), which catalyses mannose-6-phosphate and fructose-6-phosphate (REED et al., 2001). Using the xylose isomerase gene as a selector gene, transformation efficiencies were shown to be higher in potato and tomato, but lower in tobacco compared to kanamycin-based selection systems (HALDRUP et al., 1998a,b, 2001). The *PMI* gene has already been successfully utilized for the genetic transformation of sugar beet, potato, oil seed rape and maize (JOERSBO, 2001).

This study investigates the use of various carbon sources that may be potential selective agents for positive selection systems. The rationale behind the experiments within this study lies in the fact that the plant cannot use or metabolise all carbon sources effectively, and thus these can be used as limiting factors to regeneration, growth and development. Using this principle it is possible to establish threshold survival levels (TSLs) in response to varying concentrations of different carbon sources, since when using a non-antibiotic marker gene, a low level of nutrient medium is utilized, making the explant highly dependent (or heterotrophic) on the carbon source. To further enhance medium-dependence of explants, thin cell layers (TCLs) are utilized. TCLs, derived from tissues or organs, are small in size, and are excised either a) longitudinally (lTCL), being thus composed of a few tissue types or b) transversally (tTCL), thus composed of several tissue types, which are, however, normally too small to separate, as in the case of chrysanthemum. In the TCL system, the morphogenic and developmental pathways of specific organs may be clearly directed and controlled (NHUT et al., 2003).

Chrysanthemum (*Dendranthema grandiflora* (Ramat.) Kitamura) cv. 'Shuhou-no-chikara' is an economically valuable ornamental crop that is difficult to transform (TEIXEIRA DA SILVA and FUKAI, 2002). Among other potentially important applications of chrysanthemum transformation, the most vital is that of engineering virus / viroid resistance (TEIXEIRA DA SILVA, 2003c). Furthermore, the shoot regeneration capacity of chrysanthemum is severely hampered by the presence of antibiotics in the selective caulogenic medium, which, despite optimisation for maximum shoot production in a caulogenic program, is disturbed at higher antibiotic concentrations (TEIXEIRA DA SILVA et al., 2003). Thus, the search for alternative selection systems using other carbon sources may benefit the outcome and efficiency of chrysanthemum genetic transformation. The aim of this study was test the applicability of various carbon sources to chrysanthemum transformation, and provide a simple and practical methodology of choosing appropriate positive selection agents by establishing growth and development threshold levels *in vitro* for all morphogenic programs.

2. MATERIAL AND METHODS

Plant material: *in vitro* and greenhouse culture conditions

In vitro plantlets of 'Shuhou-no-chikara' chrysanthemum (*Dendranthema* X *grandiflora* (Ramat.) Kitamura), a disbud-type cultivar (and the leading Japanese cut-flower market cultivar), were used for initial explant material. All *in vitro* cultures were maintained under a 16 h light period and 20 µmol / m^2 / s^1 (PGF lamps: Homo-Lux, National Electric Co., Tokyo, Japan) at 25 °C. tTCLs (~200-500 µm thick and 1-1.5 mm in diameter) containing all cell types, were prepared from stem internode tissue of *in vitro* mother-stock plantlets. Shoots, having at least 2 nodes and 4 leaves, regenerated from tTCLs were harvested and rooted in 1L plant boxes on Hyponex® (soluble liquid fertilizer, HYPONeX, Co. Ltd., Japan; N:P_2O_5:K_2O = 6.5:6:19; 3 $g.L^{-1}$) medium containing 20 $g.L^{-1}$ sucrose. Plantlets were sub-cultured twice and acclimatized to greenhouse conditions in Metromix® potting soil for two weeks, then re-planted at a density of four plants per pot, into 70:30 Masa (sandy) soil:organic compost under greenhouse conditions. Chrysanthemum plantlets were acclimatized at 90% relative humidity and maintained in the greenhouse under long-day (4 hour light induction from 10 p.m. to 2 a.m.) conditions before transfer to short-day (13-14 hours continual darkness) conditions for flower induction.

The normality of acclimatized plants was checked by observing vegetative (length of stem, number of leaves, mass) and flowering (number of ray and disk florets, flower colour) characteristics.

Effect of carbon source on morphogenesis (callogenesis, rhizogenesis, caulogenesis and somatic embryogenesis)

To test the effect of carbon source, treatment media were maintained at 25°C, both in the light (16 h light period, 20 $\mu mol/m^2/s^1$; PGF lamps: Homo-Lux, National Electric Co., Tokyo, Japan) and in the dark, without sucrose, but containing 0 (control), 20, 40, 60 or 80 $g.L^{-1}$ of one of the following: 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). In these treatments, together with control treatments in which carbon sources were not included, tTCLs were cultured on the following media under light (20 $\mu mol/m^2/s^1$; PGF lamps: Homo-Lux, National Electric Co., Tokyo, Japan) and dark conditions at 25 °C using a methodology described elsewhere (TEIXEIRA DA SILVA, 2003a): 1) callus induction: MS medium (mineral salts only; Murashige and Skoog, 1962) + 2 $mg.L^{-1}$ TDZ, 20 $g.L^{-1}$ sucrose; (2) root induction: MS + 1 $mg.L^{-1}$ NAA, 20 $g.L^{-1}$ sucrose; (3) shoot regeneration: MS + 2 $mg.L^{-1}$ BA, 0.5 $mg.L^{-1}$ NAA, 40 $g.L^{-1}$ sucrose and (4) somatic embryo induction: MS + 1 $mg.L^{-1}$ IAA or 2 $mg.L^{-1}$2,4-D, 20 $g.L^{-1}$ sucrose.

Morphological scoring

All explants were scored for the percentage of tTCLs forming shoots, roots, callus or somatic embryos, explant survival in the caulogenic program (shoots being the most important morphogenic product for micropropagation and genetic transformation) and total fresh mass (caulogenic program) after 6 weeks in culture. Plant threshold survival level (TSL), defined as the physiological state of the tTCL in which no morphogenic development (shoot, root, callus or somatic embryo) occurs, was also determined.

Histological analyses

Some of the explants from all of the treatments were observed under light microscopy and scanning electron microscopy. For the latter, samples were fixed in formalin: acetic acid: ethanol (70: 20: 10), dehydrated in an ethanol series (50-100% ethanol for at least 6 h each), critical point-dried, sputter-coated with platinum and viewed under a Hitachi-2150 SEM microscope.

Flow cytometry

Nuclei were isolated from about 500 mg of material (shoot or callus) by chopping in a few drops of Partec Buffer A, according to manufacturer's instructions. Nuclear fluorescence was measured using a Partec® Ploidy Analyser after filtering the nuclear suspension through 30 ìm mesh size nylon filter (CellTrics®) and adding five times of DAPI solution (2 $mg.L^{-1}$ 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% (w/v) PVP K-30, 0.1% Triton-X, pH 7.5; MISHIBA and MII, 2000) for 1 min. Three samples were measured, and relative fluorescence intensity of the nuclei was analysed when the coefficient of variation was <4%. A total of 2.500 nuclei were counted for any sample.

Statistical analyses

Experiments were organized according to a complete randomised block design (CRBD) with three blocks of n=20 each per treatment. Data were analysed for significance by ANOVA with the mean separation by Duncan's New Multiple Range test (DMRT).

3. RESULTS

Results reveal that the addition of different carbon sources to the culture media affected total fresh mass of chrysanthemum tTCLs and the quantitative production of callus (Figure 1 K), roots (Figure 1 B), shoots (Figure 1 A), and to a lesser extent, somatic embryos (Figure 1 E, L) after 6 weeks of incubation.

In vitro culture was best supported by sucrose (Table 1; Figure 2 O, P), although sucrose concentration higher than 60 $g.L^{-1}$ was inhibitory to shoot development. Sucrose can be substituted by glucose or fructose, which gave a similar high total fresh mass (Table 1), but showed significantly reduced ($P < 0.001$) callus, and root and shoot numbers (Figure 2 A, B and E, F for fructose and glucose, respectively) in both the light and the dark conditions. tTCL cultures showed the following gradient in the total shoot fresh mass according to the kind of carbon source: sucrose>glucose>fructose>>maltose>mannose>galactose>raffinose>sorbitol>turanose>starch˜cellulose>xylose˜lactose>mannitol (Table 1).

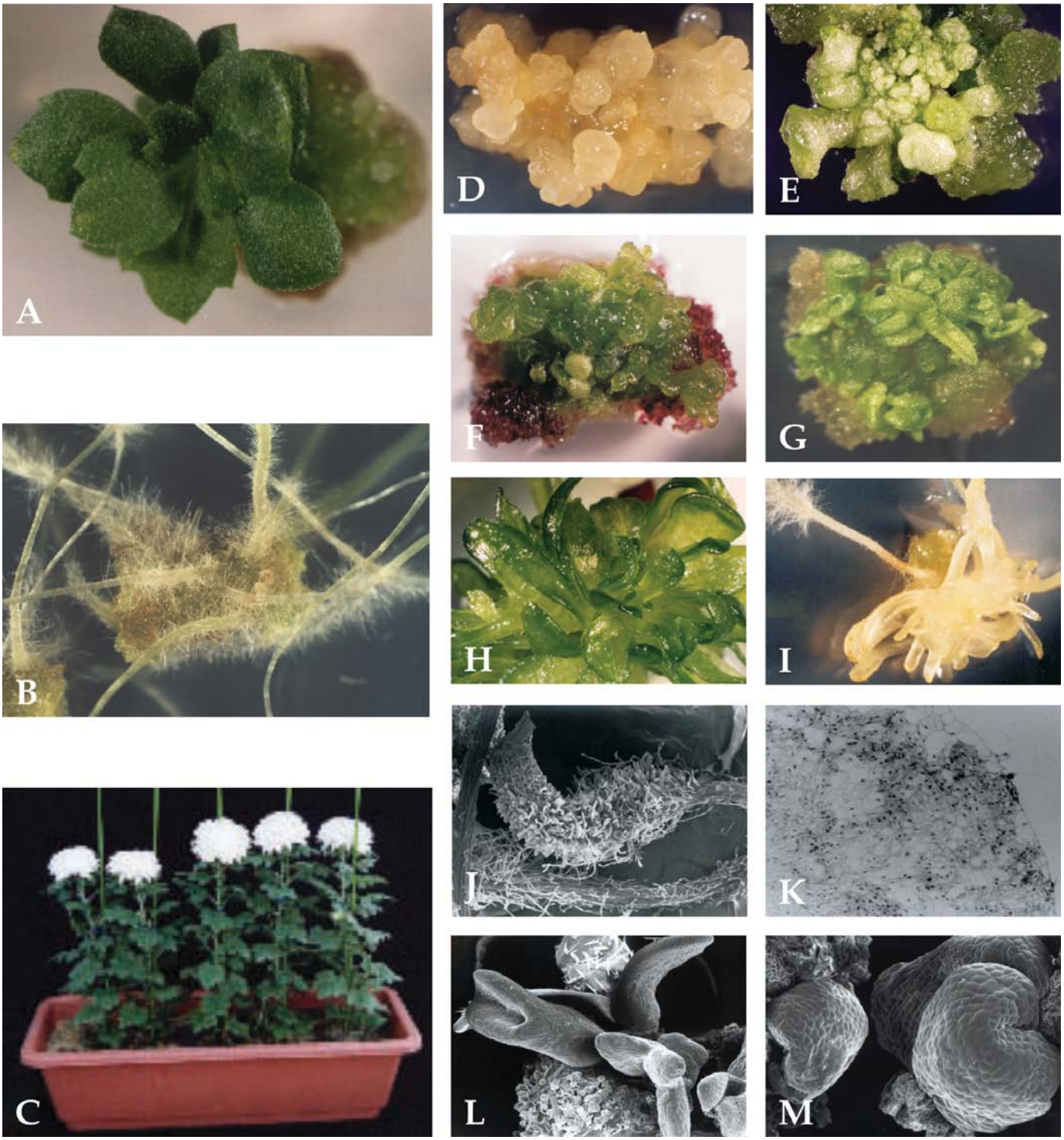


Figure 1. *In vitro* growth of chrysanthemum plantlets exposed to different carbon treatments. **A)** Control plantlets on BA- and sucrose-supplemented caulogenic medium; **B)** Control roots formed on NAA- and sucrose-supplemented medium; **C)** Greenhouse plantlets derived from different carbon-supplemented treatments; **D)** Abnormal somatic embryos formed on mannose-supplemented somatic embryogenic medium; **E)** Control somatic embryos on IAA- and sucrose-supplemented somatic embryogenic medium. Abnormal shoot formation on **F)** maltose- **G)** mannose- and **H)** raffinose-supplemented caulogenic medium; **I)** Modification in shoot morphology in the dark when on control caulogenic medium. **J)** Scanning electron microscopy of control root; **K)** callus (light microscope); **L)** shoots and **M)** somatic embryos Bar scale: 1 cm = 50 µm (J, L, M) 10 µm (K).

However, a similar gradient was not found when observing the different morphogenic programs (callogenic, rhizogenic, caulogenic, somatic embryogenic).

TSL is defined as the concentration of a carbon source at which any growth (including differentiation) is completely repressed. In principle, growth at concentrations much above the TSL can occur if the plant is endowed with a transgene allowing not only for the metabolic breakdown of the carbon source, but also its utilization in different growth and developmental programs. TSLs were obtained for galactose, glucose, mannose, xylose, lactose, turanose, raffinose, cellulose, starch, mannitol and sorbitol, but none were obtained for fructose, maltose or sucrose, in the latter three growth and / or differentiation occurring at or above 80 $g.L^{-1}$ of the carbon source (Table 1).

The effect of TDZ on callus formation occurred independently of the presence of a carbon source, and also independently of light conditions, although in quantitatively different amounts, although most (except for low levels for maltose and cellulose in both the light and the dark) carbon sources at 80 $g.L^{-1}$ inhibited callus formation completely (Figure 2), and resulted in tTCL death. In the case of mannitol or sorbitol, the presence of TDZ was insufficient to support the formation of callus at any concentration of these two carbon sources, suggesting a synergistic interaction between this cytokinin-like substance and the carbon source in the medium.

In stark contrast, the formation of roots (Figure 1 B) was highly dependent on the carbon source and on light conditions. Highest root-forming tTCLs occurred when sucrose was used as the carbon source, in both the light and the dark (Figure 2 O, P). Roots could also form, albeit in a lower number of tTCLs when fructose, galactose, glucose, mannose or maltose were utilized, in the light or the dark, except for mannose which produced no roots in the dark.

No roots were formed when xylose, lactose, turanose, raffinose, cellulose, starch, mannitol or sorbitol were used, in both the light and the dark, despite the use of IBA or coconut water, also strong stimulators of root formation in chrysanthemum (Teixeira da Silva, 2003b).

The formation of shoots (Figure 1 A) was also highly dependent on the carbon source and on light conditions. Highest shoot formation occurred when sucrose was utilized (Figure 2 O, P). The use of raffinose at any concentration produced shoots in at least 43% of the explants (Figure 2 S, T), but at higher concentrations (≥ 60 $g.L^{-1}$) these were abnormal.

Maltose also resulted in high shoot numbers (Figure 2 M, N), but these were abnormal (but not etiolated) when developed in the dark.

Smaller amounts of shoots were formed when either fructose, galactose, glucose or sorbitol were utilized as the carbon source, primarily in the light, while no shoots were formed when xylose, lactose, cellulose, starch or mannitol were used in either light or dark conditions (Figure 2).

Somatic embryogenesis (Figure 1 E) could be achieved at low concentrations, independent of the carbon source or light. A maximum of 4% of explants could form somatic embryos when glucose, sucrose or fructose were used, the differences not being significant ($P<0.001$), although light resulted in higher levels than in the dark. Of the explants with globular somatic embryos that were transferred to hormone-free, sucrose-supplemented (60 $g.L^{-1}$) MS medium, 100% formed multiple shoot clusters with rooting.

Flow cytometry results (Table 2) indicate that the highest 2C:4C ratios were obtained in carbon sources that are more readily metabolised by the plant such as sucrose, glucose and fructose, even when in the dark. Lower 2C:4C ratios were observed in more difficult to metabolise carbon sources, and this ratio remained low in both the dark and the light.

A low level of endoreduplication or polysomaty could be observed (8C values) in callus cultures derived from TCLs grown on mannose (light), maltose (light and dark) and raffinose (light and dark) supplemented media (Table 2). Leaf tissue of *in vitro* plants derived from any carbon source treatment did not demonstrate any endoreduplication, with 2C relative values being ≥94 in any case, independent of light or dark conditions. Plants that were acclimatized following the harvest of shoots derived from any carbon source treatment were not significantly different ($P<0.001$) in both vegetative growth and flower characteristics from control plants (Table 3; Figure 1 C).

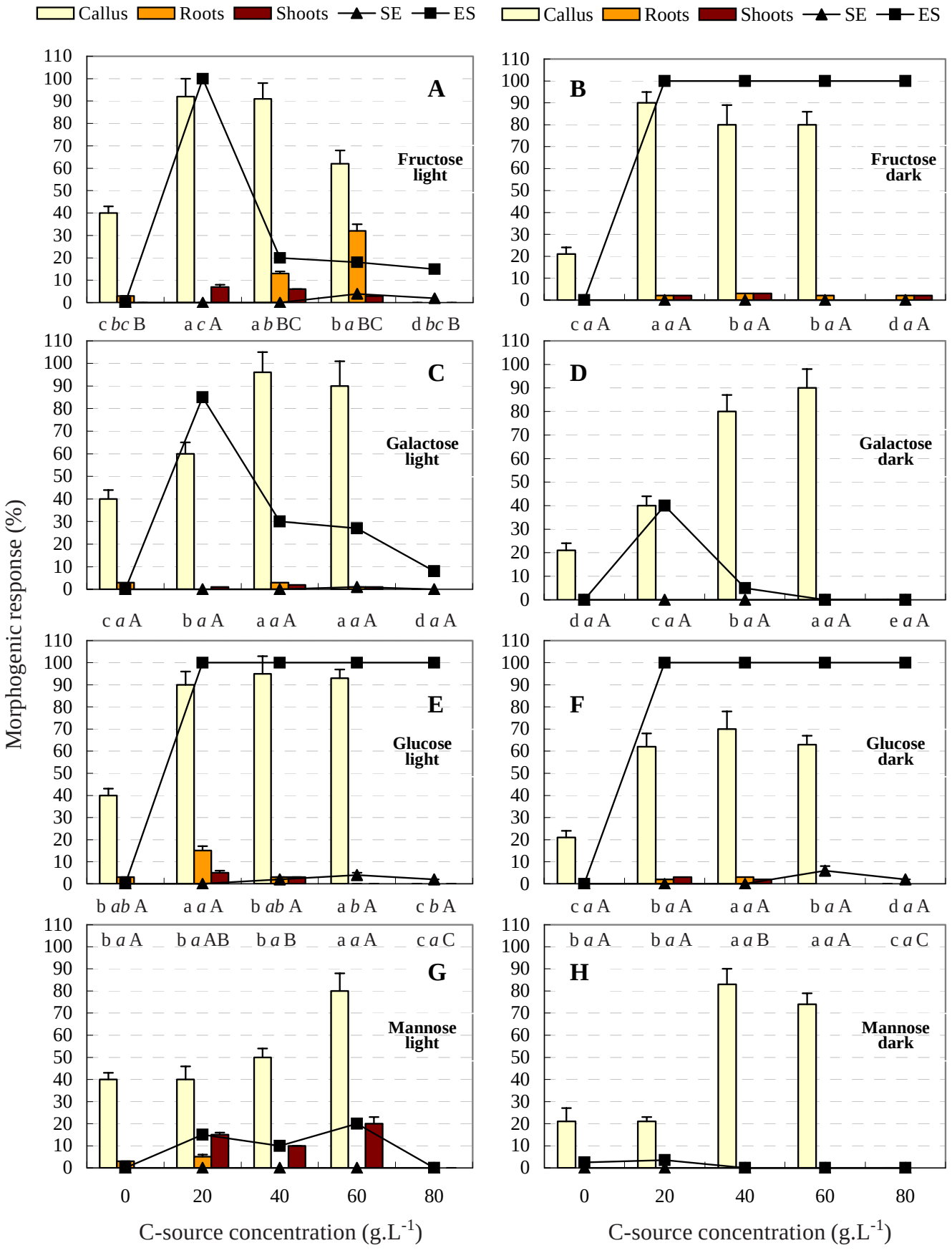
4. DISCUSSION

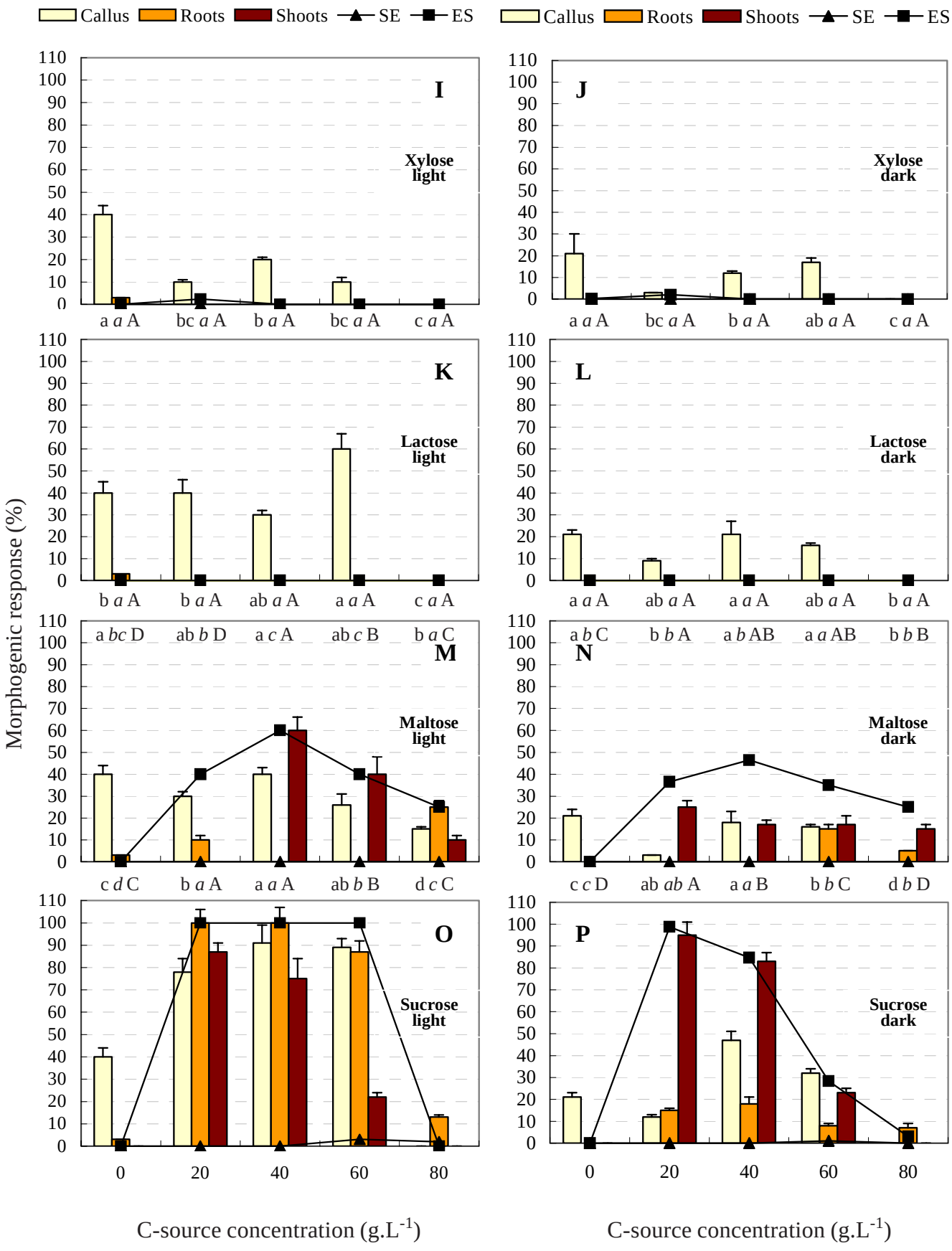
The applicability of a sugar as a selective agent in transformation systems depends on: 1) the capacity of the *in vitro* plant to metabolise, assimilate and thus utilize the carbon source at a low concentration and 2) the C-source to be "toxic" at higher concentrations, inhibiting thus normal development, defined here as the TSL. TSL levels could be achieved in 64% of the carbon sources tested (Table 1; Figure 2), suggesting their potential practical utilization, provided that genes coding for their respective degrading enzymes (Table 4) can be cloned into an established vector system.

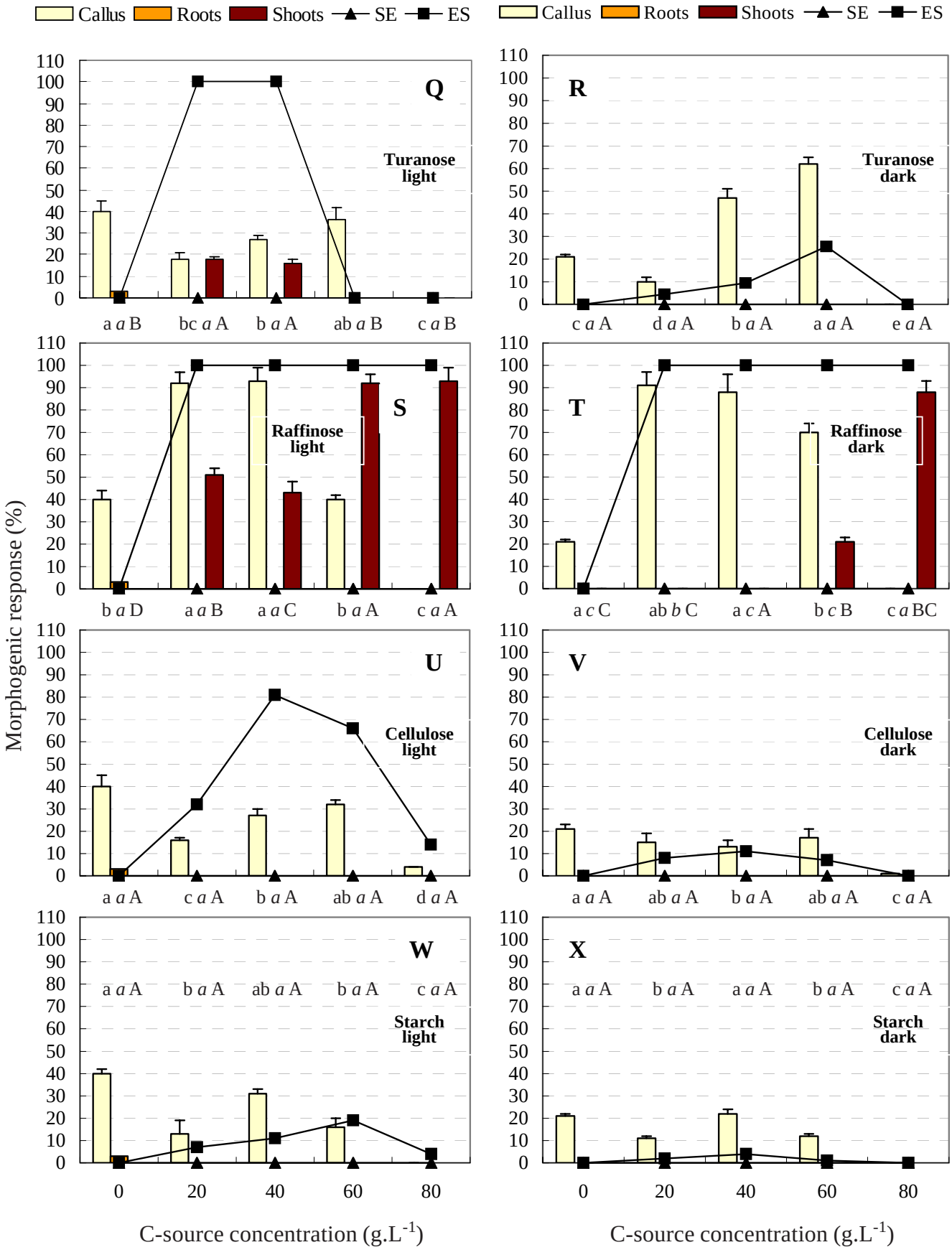
Table 1. Total fresh mass of chrysanthemum stem tTCLs (*n*=60) after 6 weeks in response to various carbon sources

C-source	$g.L^{-1}$	Light		Dark	
Control §	0	0.05±0.00		0.05±0.00	
Control *	40	6.87±1.35 a		3.71±0.82 a	
Control †	40	4.17±1.62 d		3.01±0.73 b	
Fructose	20	5.11±1.38 c		2.06±0.33 c	
Hexose	40	3.25±0.86 e		1.98±0.39 c	
	60	3.24±0.71 e		1.74±0.76 c	
	80	1.24±0.16 g		0.54±0.16 e	
Galactose	20	1.23±0.38 g		0.34±0.06 ef	
Hexose	40	1.57±0.81 fg		1.23±0.18 d	
	60	1.68±0.48 fg		0.26±0.02 ef	
	80	0.24±0.08 gh		0 f	‡
Glucose	20	5.47±0.32 bc		1.76±0.43 c	
Hexose	40	6.06±0.38 b		0.51±0.06 e	
	60	6.12±0.46 b		0.26±0.02 ef	
	80	3.41±0.38 de		0 f	‡
Mannose	20	1.31±0.06 g		0.26±0.06 ef	
Hexose	40	1.68±0.41 fg		0.16±0.04 f	
	60	1.28±0.26 g		0 f	‡
	80	0 h	‡	0 f	
Xylose	20	0.76±0.61 gh		0.62±0.31 e	
Pentose	40	0 h	‡	0 f	‡
	60	0 h		0 f	
	80	0 h		0 f	
Lactose	20	0.68±0.08 gh		0.26±0.02 ef	
Disaccharide	40	0 h	‡	0 f	‡
(*Glu* + *Gal*)	60	0 h		0 f	
	80	0 h		0 f	
Maltose	20	2.19±0.78 f		0.11±0.06 f	
Disaccharide	40	1.83±0.62 fg		0.16±0.04 f	
(*Glu* + *Glu*)	60	1.82±0.44 fg		0.15±0.02 f	
	80	1.21±0.57 g		0.28±0.09 ef	
Sucrose	20	6.03±1.54 b		2.29±0.23 bc	
Disaccharide	40	5.68±1.04 bc		0.26±0.02 ef	
(*Glu* + *Fru*)	60	5.49±0.34 bc		0.61±0.16 e	
	80	4.76±0.62 cd		0.42±0.13 ef	
Turanose	20	0.67±0.18 gh		0.14±0.02 ef	
Rare	40	0.31±0.07 h		0 f	‡
Disaccharide	60	0 h	‡	0 f	
	80	0 h		0 f	
Raffinose	20	1.67±0.28 fg		0.34±0.07 ef	
Trisaccharide	40	0.68±0.08 gh		0.26±0.02 ef	
	60	0.36±0.07 h		0.08±0.00 f	
	80	0 h	‡	0 f	‡
Cellulose	20	0.72±0.13 gh		0.32±0.06 ef	
Polysaccharide	40	0.68±0.08 gh		0.26±0.02 ef	
	60	0.62±0.27 gh		0 f	‡
	80	0 h	‡	0 f	
Starch	20	0.25±0.06 h		0 f	‡
Polysaccharide	40	0.18±0.03 h		0 f	
	60	0.12±0.05 h		0 f	
	80	0 h	‡	0 f	
Mannitol	20	0.09±0.01 h		0 f	‡
Sugar alcohol	40	0 h	‡	0 f	
	60	0 h		0 f	
	80	0 h		0 f	
Sorbitol	20	0.92±0.15 gh		0.11±0.03 ef	
Sugar alcohol	40	0.68±0.18 gh		0 f	‡
	60	0.81±0.15 gh		0 f	
	80	0 h	‡	0 f	

§ Initial fresh mass (mean g ± SD) of stem tTCL; * Full MS + sucrose 40 g/l; † = ½ MS + sucrose 40 g/l; ‡ = TSL; Different letters within a column indicate significant differences at $P<0.001$ using Duncan's New Multiple Range Test.


Callus
Roots
Shoots
SE
ES
Morphogenic response (%)
A
Fructose light
c bc B
a c A
a b BC
b a BC
d bc B
B
Fructose dark
c a A
a a A
b a A
b a A
d a A
C
Galactose light
c a A
b a A
a a A
a a A
d a A
D
Galactose dark
d a A
c a A
b a A
a a A
e a A
E
Glucose light
b ab A
a a A
b ab A
a b A
c b A
F
Glucose dark
c a A
b a A
a a A
b a A
d a A
G
Mannose light
b a A
b a AB
b a B
a a A
c a C
H
Mannose dark
b a A
b a A
a a B
a a A
c a C
0
20
40
60
80
C-source concentration (g.L-1)


Callus Roots Shoots SE ES
I
Xylose light
a a A bc a A b a A bc a A c a A
J
Xylose dark
a a A bc a A b a A ab a A c a A
K
Lactose light
b a A b a A ab a A a a A c a A
L
Lactose dark
a a A ab a A a a A ab a A b a A
M
Maltose light
a bc D ab b D a c A ab c B b a C
c d C b a A a a A ab b B d c C
N
Maltose dark
a b C b b A a b AB a a AB b b B
c c D ab ab A a a B b b C d b D
O
Sucrose light
P
Sucrose dark
Morphogenic response (%)
0 20 40 60 80
C-source concentration (g.L-1)
C-source concentration (g.L-1)


Callus
Roots
Shoots
SE
ES
Morphogenic response (%)
Q
Turanose light
a a B
bc a A
b a A
ab a B
c a B
R
Turanose dark
c a A
d a A
b a A
a a A
e a A
S
Raffinose light
b a D
a a B
a a C
b a A
c a A
T
Raffinose dark
a c C
ab b C
a c A
b c B
c a BC
U
Cellulose light
a a A
c a A
b a A
ab a A
d a A
V
Cellulose dark
a a A
ab a A
b a A
ab a A
c a A
W
Starch light
a a A
b a A
ab a A
b a A
c a A
X
Starch dark
a a A
b a A
a a A
b a A
c a A
0
20
40
60
80
C-source concentration (g.L-1)
C-source concentration (g.L-1)

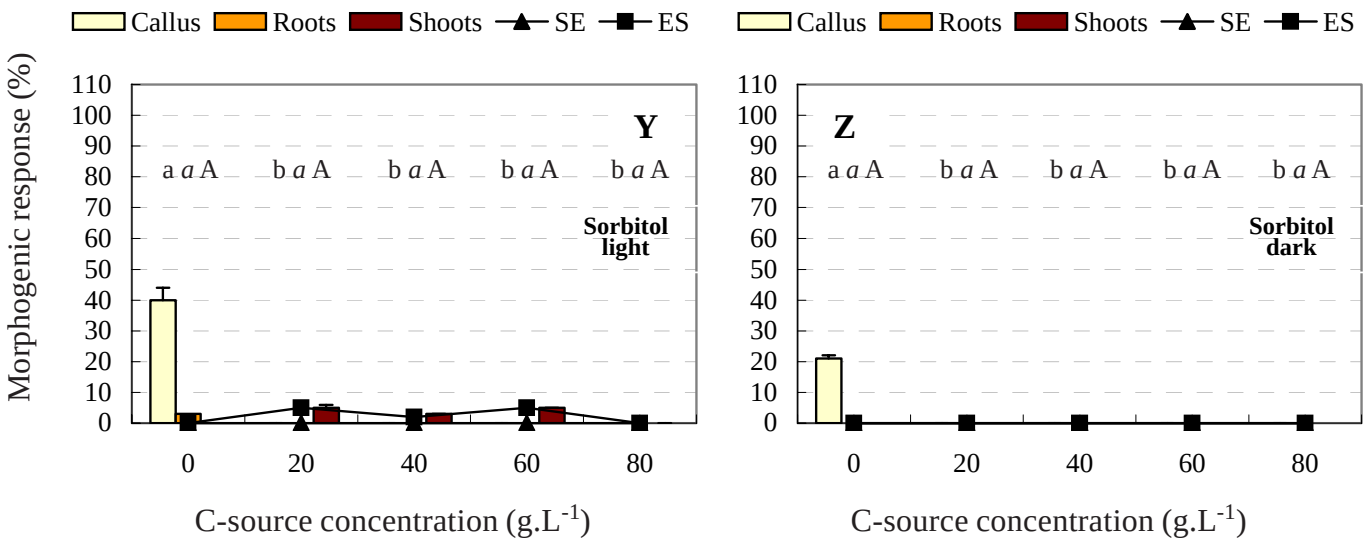


Figure 2. Morphogenic response of chrysanthemum tTCLs on callus, root, shoot and somatic embryo media to different carbon sources in the light and dark conditions. SE = somatic embryo; ES = explant survival. Sugar sources: fructose in the light (A) and dark (B); galactose in the light (C) and dark (D); glucose in the light (E) and dark (F); mannose in the light (G) and dark (H); xylose in the light (I) and dark (J); lactose in the light (K) and dark (L); maltose in the light (M) and dark (N); sucrose in the light (O) and dark (P); turanose in the light (Q) and dark (R); raffinose in the light (S) and dark (T); cellulose in the light (U) and dark (V); starch in the light (W) and dark (X); sorbitol in the light (Y) and dark (Z); Values for mannitol not represented since all morphogenic responses were 0; Different letters indicate significant differences at $P<0.001$ using Duncan's New Multiple Range Test: lower case, non italics (e.g. **a**) = % explants forming callus; lower case, italics (e.g. ***a***) = % explants forming roots; upper case, non italics (e.g. **A**) = % explants forming shoots.

Table 2. Ploidy ratios in 'Shuhou-no-chikara' tTCL-derived callus and *in vitro* plant leaf tissue from different carbon sources after 6 weeks culture

Carbon source	Light		Dark	
	Callus	Leaf	Callus	Leaf
Control §	97:1:0	96:1:0	92:6:0	95:1:0
Control *	92:7:0	98:1:0	89:8:0	98:1:0
Control †	94:4:0	98:0:0	91:3:0	97:1:0
Fructose	88:6:0	96:2:0	86:1:0	95:1:0
Galactose	87:7:0	97:2:0	78:1:0	nsf
Glucose	91:6:0	98:0:0	85:1:0	95:2:0
Mannose	76:4:2	96:2:0	69:2:0	nsf
Xylose	71:1:0	nsf	71:1:0	nsf
Lactose	70:1:0	nsf	60:1:0	nsf
Maltose	74:4:1	94:2:0	66:2:2	96:0:0
Sucrose	89:10:0	97:1:0	88:1:0	98:0:0
Raffinose	82:4:2	95:1:0	66:1:4	nsf
Cellulose	76:4:0	nsf	64:2:0	nsf
Starch	69:3:0	nsf	68:1:0	nsf
Mannitol	76:2:0	nsf	63:1:0	nsf
Sorbitol	79:4:0	94:2:0	68:1:0	nsf

§ Initial fresh mass of stem tTCL; * Full MS + sucrose 40 g/l; † = ½ MS + sucrose 40 g/l; Relative C ratios as 2C:4C:8C, measured after 2 weeks in culture; remaining C (i.e. 100% - other C values) = DNA fragments or other ploidy levels; nsf = no shoots formed.

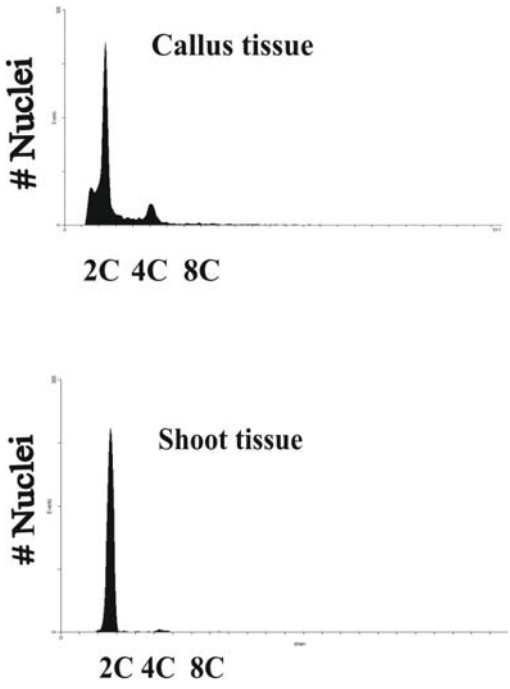


Table 3. Characteristics (mean ± SD) of greenhouse-acclimatized plantlets

Treatment	Length stem (cm)	# Leaves	Mass (g)	# Ray florets	# Disk Florets
Control	34.1±0.9 a	31.2±0.6 a	45.2±1.8 a	283.8±7.9 a	6.2±0.9 a
Carbon-plantlets	36.6±1.1 a	30.6±0.4 a	44.8±2.3 a	278.4±9.3 a	6.6±1.2 a

Different letters within a column are significantly different (P<0.001) using Duncan's New Multiple Range test.

Table 4. Potential enzymes for existing or future transformation systems

Carbon source	Common enzyme name	EC number
Fructose	Glucose-fructose oxidoreductase	1.1.99.28
Galactose	Galactose 1-dehydrogenase	1.1.1.120
Glucose	Glucose oxidase	1.1.3.4
Mannose	Mannose 6-phosphate isomerase	5.3.1.8
Xylose	Xylose isomerase	5.3.1.5
Lactose	Lactase	3.2.1.108
Maltose	Maltose phosphorylase	2.4.1.8
Sucrose	Sucrose á-glucosidase	3.2.1.48
Turanose	Unknown	-
Raffinose	Raffinose-raffinose á-galactotransferase	2.4.1.166
Cellulose	Cellulase	3.2.1.4
Starch	Glucan 1,4-á-glucosidase	3.2.1.3
Mannitol	Mannitol dehydrogenase	1.1.1.255
Sorbitol	L-iditol 2-dehydrogenase	1.1.1.14

When medium-dependent tTCLs (with limited sugar reserves and no defined sink-source) are used, heterotrophy or mixotrophy is forced by the addition of sugar to the culture medium (NHUT et al., 2003). Depending on the light intensity, temperature and carbon dioxide concentration in the Petri-dish air, *in vitro* plant growth and development can be totally dependent on an external source of carbohydrates (tTCL cultures in the dark) or partly covered their needs by limited photosynthesis (tTCL cultures in the light; NHUT et al., 2003).

TSLs could not be clarified when fructose (light or dark), galactose (light), glucose (light), maltose (light or dark) or sucrose (light or dark) were added to the different morphogenic program media, suggesting that since these carbon sources can be effectively utilized in growth (without inhibiting it at any "toxic" concentration), which continues to occur at or above 80 $g.L^{-1}$ (Table 1).

In-built metabolic systems thus are capable of effectively metabolising these carbohydrate sources, even at high concentrations, without reaching a saturation level, the TSL. Moreover, these carbon sources would not be suitable as selective agents in positive selection systems since both transgenic and control plants would grow equally well (not being easily distinguishable and leading to many escapes) at high concentrations of the carbon source. Although a TSL (80 $g.L^{-1}$) was established for galactose and glucose in the dark, the fact that some growth and differentiation occurred in the light would make them unsuitable candidates as selective agents in positive selection systems. The incapacity of tTCLs to use all carbon sources may result in cellular and DNA degradation resulting in increased variation in the ploidy (Table 2). A high 2C value with low 4C and 8C values indicates increased degradation products (MISHIBA and MII, 2000). A low 2C, with an increase in 4C or 8C values can be found in callus, suggesting the occurrence of polyploidy and/or endoreduplication.

Other carbon sources such as mannose, xylose, lactose, turanose, raffinose, cellulose, starch, mannitol and sorbitol could be effectively used as selective agents in positive selection systems since growth and/or differentiation is completely suppressed at low (20-60 $g.L^{-1}$) concentrations of the carbon source under light and dark conditions, the latter usually effecting a more negative impact, that is greater heterotrophy and/or reduced metabolism (Table 1; Figure 2).

The data within this study is encouraging for mannose and xylose since positive selection systems for them have already been established and can be applied to the genetic engineering of chrysanthemum, while for other carbon sources potential positive selection systems would still have to be devised (Table 4). Mannose and raffinose, however, despite exhibiting TSLs in both the light and the dark, and despite the former having an already existent positive selection system, both show abnormal shoot formation at ≥40 $g.L^{-1}$ (Figure 2). Mannose was also shown to negatively affect *Arabidopsis* root elongation and development (Baskin et al., 2001). Starch and mannitol completely inhibit any growth and / or differentiation in the dark, and despite having a reasonable TSL in the light, they may not be so suitable as selective agents in positive selection systems. Another factor determining the use of a carbon source as a selective agent in a positive selection system is cost.

Turanose, a rare disaccharide, can be costly as compared to other carbon sources, despite exhibiting TSLs in both the light and the dark (Table 1). Moreover, there is no available degrading enzyme (Table 4). Consequently carbon sources that may potentially serve as selective agents in positive selection systems (determined by both the existence of TSLs and normal morphology) are: xylose, lactose, cellulose and sorbitol.

Sucrose, like fructose and galactose were the only three hexoses with which somatic embryogenesis resulted (Figure 2 A-D, O, P). 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).

Starch is a homopolysaccharide that cannot be utilized by glycolysis before it is degraded into glucose, so the quantitative effect on any morphogenesis (callus, root, shoot or somatic embryo production) decreases when only glucose is used (Table 1; Figure 2) as a result of energetic requirements to degrade the polymer into glucose monomers. Starch is broken down into maltose by partial hydrolysis, and into glucose by full hydrolysis. In a similar manner, cellulose is partially reduced to cellobiose, then fully into glucose, explaining the better utilization of glucose than cellobiose by chrysanthemum tTCLs (Table 1; Figure 2) also due to energetic requirements. Glucose is reduced into sorbitol while mannose is reduced to mannitol, the sugar alcohols being reduced products from the aldehydes, also explain why mannose results in higher tTCL fresh mass and morphogenic reaction (callus, root, shoot) than when mannitol is used (Table 1; Figure 2).

Galactose, glucose and fructose (or levulose) are monosaccharides and should be more easily decomposed than sucrose, a disaccharide consisting of glucose and fructose. Galactose is an epimer of glucose, and its utilization in morphogenesis may depend on the endogenous levels of galactokinase (Hisajima and Thorpe, 1985). The same effect could be observed when glucose and fructose were joined together (sucrose>glucose+fructose>glucose>fructose; data nor shown). Fructose is an intermediary product of glucose catabolism, and the slow growth of tissue cultures on a fructose-containing medium is as 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 this study.

No study exists on the effect of turanose, a glycosylated fructose and rare disaccharide, on any aspect of plant tissue culture. The lack of any differentiation (and limited growth i.e. tTCL fresh mass increase) in both the light and the dark suggests that there are no native pathways existent within the plant to metabolise this carbon source. Both mannitol and sorbitol could not be used effectively in chrysanthemum callogenic, rhizogenic and somatic embryogenic programs and had limited effect on the successful outcome of the caulogenic program (Figure 2 W-Z).

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. Moreover, sugar alcohols are perceived by cells as chemical signals, with very high *in vitro* concentrations acting as chemical stress agents (Steinitz, 1999). When sugar concentrations in the plant increase, there is a repression of genes involved in mobilization of stored reserves and photosyntheis. 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 (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.

When output exceeds the plant's capacity to metabolise or export sugars (limited in the case of heterotrophic tTCLs), increasing sugar concentrations repress the same photosynthetic and / or metabolic genes.

The down regulation of photosynthetic genes (PEGO et al, 2000) may account for the TSLs and for the observable decrease in adventitious shoot formation, despite the continued increase in tTCL fresh mass (Table 1). The choice of carbon source for chrysanthemum *in vitro* culture, independent of the morphogenic program (callogenic, caulogenic, rhizogenic and somatic embryogenic) induced by tTCLs, has an effect on the quantitative and qualitative outcome of each program. In conclusion, of fourteen carbon sources tested, mannose and xylose showed the greatest potential to be used as selective agents in positive selection systems in chrysanthemum genetic transformation.

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