

# Filter Paper Type Affects the Morphogenic Programs and Buffers the Phytotoxic Effect of Antibiotics in Chrysanthemum and Tobacco Thin Cell Layers

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**Abstract.** Filter paper types significantly affected the growth, development and differentiation of chrysanthemum and tobacco stem thin cell layers (TCLs) from in vitro plantlets. Three different filter paper types, normally with varied uses in plant biology, showed varying morphogenic-altering and antibiotic-buffering capacities. Advantec #2 and Whatman #1 significantly stimulated root, shoot and callus formation while Whatman #3 inhibited them, as compared to TCLs placed directly on agar. Filter paper buffered the phytotoxic effect of antibiotics kanamycin and cefotaxime, substances commonly used in genetic transformation experiments, up to as much as 50%, independent of species or genotype. In both 'Lineker' and 'Shuhou-no-chikara' chrysanthemum cultivars, Advantec #2 and Whatman #1 filter papers stimulated embryogenesis but in tobacco all three filter paper types significantly reduced embryogenesis and explant survival.

Filter paper is extensively used by plant tissue culturists as a base for explant support in many solid and semi-solid media, being commonly used in seed germination (Emmerich and Hardegee, 1991; Jones et al., 1991; Rahman et al., 2000) and moisture retention (Griffith et al., 1991) studies. Filter paper has also been used for plant seed toxicity tests (PSTT; Wang, 1993), as a sealing material for in vitro culture vessels (Han et al., 1995), and as a base for *Rhizobium* (Griffith et al., 1991) and fungal mycelium growth analyses (Rodrigues et al., 1997). In addition, the filter paper disk method has long been used in the study of antimicrobial activities of root extracts in vitro (Sharma et al., 1989). Despite the wide use of filter paper in tissue culture, this is the first report that truly addresses its direct effect on plant tissue culture and in vitro growth and morphogenesis.

Thin cell layers (TCLs) allow for the directed control of morphogenic and developmental pathways of shoots, roots, flowers or somatic embryos derived from other specific or nonspecific cells, tissues or organs when the milieu in which they are placed is also controlled (Tran Thanh Van, 1973). This capacity comes from the size and medium-dependent nature of TCLs with minimal interference from internal factors and growth substances. TCLs, being small in size are excised either: 1) longitudinally (ITCL), being composed of a few tissue types; or 2) transversally (tTCL) consisting of several tissue types which are normally too small to separate, and are explants that have been successfully used in the propagation of numerous ornamental species (Le and Nhut,

2000). Due to the sensitive nature of TCLs (due to their size and restricted cell number) to media manipulations, they provided a suitable means to detect the effect of antibiotics on plant morphogenesis and explant survival as affected by the presence or absence of filter paper, even if the latter is not commonly used in plant cell, tissue and organ culture.

This study examines the effect of filter paper on in vitro growth and morphogenesis of tobacco (*Nicotiana tabacum*) and chrysanthemum [*Dendranthema ×grandiflora* (Ramat.) Kitamura] tTCLs while focusing on the buffering effect it has on the phytotoxicity of antibiotics. This study further explores the effect that filter paper has on chrysanthemum (an important global floricultural crop) and tobacco (a model plant) in vitro growth and morphogenesis (callus, shoot, root or somatic embryo formation) when placed on specific morphological program (callogenic, caulogenic, rhizogenic or somatic embryogenic) media.

**Plant material:** In vitro and greenhouse culture conditions. Shoots (at least 4 cm in height) derived from any in vitro medium (control and treatment plants) were harvested and placed in 1-L polycarbonate plant boxes (CB-3; Iuchi, Japan) containing 100 mL of Hyponex medium (soluble liquid fertilizer, Hyponex, Japan; 6.5N–6P<sub>2</sub>O<sub>5</sub>–19K<sub>2</sub>O; 3 g·L<sup>-1</sup>) supplemented with 20 g·L<sup>-1</sup> sucrose, and maintained under a 16-h photoperiod (40 μmol·m<sup>-2</sup>·s<sup>-1</sup>) using plant growth fluorescent lamps (PGF; 45 μmol·m<sup>-2</sup>·s<sup>-1</sup>, Homo-Lux, National Electric Co., Tokyo) at 25 °C. Control plants from which TCLs were derived were maintained on the same medium and under the same conditions. Chrysanthemum plantlets were acclimatized at 90% relative humidity and maintained in the greenhouse under long-day (natural daylight with a 4-h light interval from 10:00 PM to 2:00 AM) conditions before transfer to short-day (13–14 h continual darkness) conditions for flower induction. Acclimatized plantlets were checked for morphological and flowering normality.

**Explant preparation and treatment.** Stem internode tissue of in vitro 'Lineker' (LIN) and 'Shuhou-no-chikara' (SNC) chrysanthemum, as well as tobacco 'Samsun SS') tTCLs (≈200–500 μm thick and 1–1.5 mm, 1–2 mm or 2–5 mm in diameter for LIN, SNC and tobacco respectively) containing all cell types were prepared from internode stems of 6–8 cm high in vitro plantlets (≈30-d-, 45-d-, and 60-d-old plantlets for tobacco, LIN and SNC, respectively). To test the effect of filter paper on growth and morphogenesis, sterilized rings, 9 cm in diameter, of three filter paper types [Advantec #2 (A#2); Whatman #1 (W#1); medium fast filtering speed]; and Whatman #3 (W#3; chromatography paper)] were placed a posteriori on 25 mL of different autoclaved morphogenic program media [Table 1; for tobacco (Le and Nhut, 2000); for chrysanthemum (Teixeira da Silva, 2001)] in 9.5-cm wide petri dishes (Sekisui, Japan). Control tTCLs were placed directly onto 25 mL of agar (i.e., without filter paper) of the same morphogenic media formulated to induce specific morphogenic programs.

To test the effect of filter paper on the phytotoxic reaction of tTCLs to antibiotics, tTCLs were placed on optimized caulogenic

Table 1. Morphogenic programs in chrysanthemum and tobacco.

Plant	Programme	Sucrose (g·L <sup>-1</sup> )	PGR <sup>2</sup> (on MS) (mg·L <sup>-1</sup> )	L/D	DT	# / tTCL <sup>3</sup>
Tobacco	Callogenic	Sucrose 30	IBA 3 K 0.1	L	8–10	---
	Caulogenic	Sucrose 30	IBA 1 K 10	L	10–12	2.4
	Rhizogenic	Sucrose 10	IBA 10 K 0.1	L/D	10–20	6.5
	Somatic embryogenic <sup>4</sup>	Sucrose 60	IAA 3	L/D	30–40	2.1
Chrysanthemum <sup>5</sup>	Callogenic	Sucrose 40	TDZ 1	L/D	16–20	---
	Caulogenic	Sucrose 40	BA 2 NAA 0.5	L	25–35	1.3
	Rhizogenic	Sucrose 20	NAA 1	L/D	30–40	6.7
	Somatic embryogenic <sup>4</sup>	Sucrose 60	IAA 2	L	45–60	1.2

<sup>2</sup>PGR = Plant growth regulator.

<sup>3</sup>Mean number of organ ± standard deviation.

<sup>4</sup>Any developmental stage; BA = Benzyladenine; IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; K = Kinetin (6-Furfurylaminopurine); NAA = α-Naphthylacetic acid; TDZ = Thidiazuron; DT = Differentiation time (days).

<sup>5</sup>SNC.

<sup>6</sup>Globular.

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program medium (Table 1) containing 10 or 50  $\mu\text{g}\cdot\text{mL}^{-1}$  filter-sterilized kanamycin A, and 250 or 500  $\mu\text{g}\cdot\text{mL}^{-1}$  filter-sterilized cefotaxime for chrysanthemum and tobacco, respectively, with or without filter paper. All treatments were placed in the light (16-h photoperiod, 40  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , PGF lamps) at 25 °C.

**Scoring of morphological response.** All explants were scored for the number of shoots or roots, and fresh weight of callus and ES after 60 d in culture. Greenhouse-acclimatized SNC plants ( $n = 15$ ) were checked and compared for vegetative and flowering characteristics (plantlet height, number of leaves, total fresh weight, and the number of disk and ray florets per flower head) using the experimental design as outlined below when flowers were fully open ( $\approx 60$  d after acclimatization).

**Histological analyses.** Explants from all treatments were observed under light microscopy and scanning electron microscopy (SEM) to observe shoot formation as well as any histological changes arising from the treatments. SEM samples were fixed in FAA (formalin : acetic acid : 70% ethanol = 5:5:90), dehydrated in an ethanol series (50% to 100% EtOH for at least 6 h each (Fukuda et al., 1997), critical point-dried, sputter-coated with Pt and viewed under a Hitachi-2150 SEM microscope (Tokyo).

**Flow cytometry.** Nuclei were isolated from  $\approx 0.5$  g of in vitro plant material (shoot and callus) derived from 60-d-old control and cefotaxime-treated plants (10 and 50  $\text{g}\cdot\text{mL}^{-1}$  kanamycin for chrysanthemum and tobacco, respectively, or 100  $\mu\text{g}\cdot\text{mL}^{-1}$  cefotaxime for both) by chopping the sample in a few drops of Partec Buffer A [ $2 \mu\text{g}\cdot\text{mL}^{-1}$  4,6-diamidino-2-phenylindole (DAPI), 2 mM  $\text{MgCl}_2$ , 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton-X, pH 7.5; Mishiba and Mii, 2000]. Nuclear fluorescence was measured using a Partec Ploidy Analyser (Partec GmbH, Germany) after filtering the nuclear suspension through a 30- $\mu\text{m}$  mesh size nylon filter (CellTrics) and adding five aliquots of Partec Buffer A for 1 min. Three samples were measured for each treatment, and relative fluorescence intensity of the nuclei analyzed when the coefficient of variation was  $< 4\%$ . A total of 2500 nuclei were counted for any sample.

**Experimental design and statistical analyses.** Experiments were organized according to a randomized complete-block design (RCBD) with three blocks of 20 replicates per treatment. The effects of filter paper type and antibiotic on four programs (shoot, root, callus, somatic embryo) were separately analysed for each cultivar (tobacco, LIN, SNC), and 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 version 6.12 (SAS Institute, Cary, N.C.).

## Results and Discussion

Morphogenesis (adventitious shoot or root, callus, somatic embryos) derived from tTCLs has been well defined in both tobacco and

chrysanthemum (Table 1, Fig. 1), and in both were significantly affected by the use of filter paper in this study. In both plant species, use of A#2 or W#1 resulted in higher amounts of any organ than when tTCLs were directly plated onto agar medium (Table 2). When W#3 was used, root and shoot formation was significantly inferior to controls and to the use of A#2 or W#1 (Table 2) in tobacco. A similar trend was observed for both *Dendranthema* cultivars in the case of shoot and root formation. It can thus be concluded that A#2 and W#1 improved root and shoot morphogenesis while W#3 resulted in lower amounts of somatic embryogenesis in any species, and reduced explant survival in the root, shoot, somatic embryo (Table 2) and callus (Table 3) programs for both tobacco and chrysanthemum.

The use of shoots, followed by callus, is the primary means of generating transgenic chrysanthemum plants (Teixeira da Silva and Fukai, 2002a). The ability to increase the number of shoots per tTCL, especially when on an antibiotic selective medium would increase the chances of obtaining genetic transformants. The number of shoots formed per tobacco tTCL was greater when A#2 or W#1 filter paper were used, while W#3 resulted in lower shoot number per tTCL than controls (Table 2; Fig. 2). This trend was the same when either kanamycin or cefotaxime were used even though the number of shoots was significantly lower (Fig. 2), with a corresponding decrease in explant survival (Fig. 3). In standard propagation medium (Table 1) fewer adventitious shoots were formed when tTCLs

were placed directly on agar in LIN and SNC, respectively. In both chrysanthemum cultivars, the number of shoots formed per SNC tTCL was higher when either A#2 or W#1 filter paper were used, while W#3 resulted in a lower shoot number per tTCL than controls (tTCLs plated directly onto agar medium; Table 2, Fig. 2). In LIN, however, the number of shoots formed per SNC tTCL was higher than controls when A#2, W#1, W#3 filter paper were used. A similar trend was found for both LIN and SNC, even though the number of shoots was lower when either kanamycin or cefotaxime were used (Fig. 2), with a corresponding decrease in explant survival (Fig. 3). The use of W#3 significantly reduced not only the shoot, root and somatic embryo number per tTCL in both tobacco and chrysanthemum, but also decreased the ES in almost every case, except for shoot formation in LIN, where the difference was insignificant.

A similar trend was observed for the rhizogenic program (Table 2). The control of rhizogenesis (Teixeira da Silva, 2002) is only now beginning to be explored in chrysanthemum as an alternative to shoots in the regeneration of transgenic plants. An increase in the amount of root production in both chrysanthemum and tobacco, stimulated by the use of A#2 and W#1 might be beneficial for the mass production of adventitious roots for secondary metabolite production (Palic et al., 2002).

Somatic embryogenesis is poorly documented for chrysanthemum, with low rates being attributed to explant source and cultivar-dependence. Use of filter paper decreased the number of somatic embryos formed per

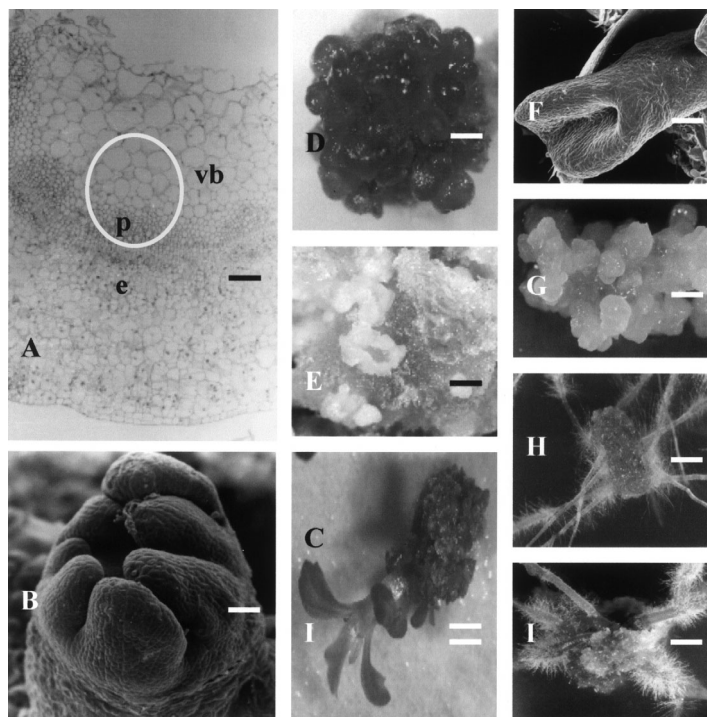


Fig. 1. In vitro growth of SNC chrysanthemum tTCLs. (A) C/S through a tTCL highlighting the vascular bundle (vb), parenchyma (p) and epidermis (e); (B) SEM and light microscopy (C) of shoot from tTCL culture on A#2 on caulogenic medium; (D) Somatic embryos induced in the light and dark (E); (F) SEM of somatic embryo on A#2 filter paper overlaying IAA-supplemented medium; (G) embryogenic callus on SNC tTCLs on filter paper-free, TDZ-supplemented caulogenic medium; (H) root formation in the dark on filter paper-free medium; and (I) root formation in the light on tTCLs on filter paper-free, NAA-supplemented rhizogenic medium; Bar scale: 1 cm = 50  $\mu\text{m}$  (A, C, E, F); 200  $\mu\text{m}$  (B, D, G, H, I).

tTCL, especially when W#1 or W#2 were used (Table 2).

Callus is occasionally used as a source of indirect shoot or root regeneration, especially when the formation of these organs is severely hampered by the presence of antibiotics (Teixeira da Silva and Fukai, 2002b). Among tobacco tTCLs plated directly onto agar, the use of either kanamycin or cefotaxime at 50 or 500 mg·L<sup>-1</sup>, respectively, resulted in a significant decrease in tTCL fresh weight within the callogenic program (Table 3) with a corresponding decrease in explant survival (Fig. 3). Use of filter paper inhibited callus fresh weight while the use of A#2 and W#1 resulted in greater callus fresh weights when medium contained kanamycin (Table 3), suggesting that filter paper had a buffering influence on the phytotoxicity of antibiotics. Use of W#3 resulted in the lowest callus fresh weight (Table 3). In both LIN and SNC chrysanthemum tTCLs plated directly onto agar, the use of either kanamycin or cefotaxime at 50 or 500 mg·L<sup>-1</sup>, respectively, resulted in a significant decrease in the tTCL fresh weight within the callogenic program (Table 3). As in tobacco, the use of filter paper resulted in reduced callus fresh weights compared to no-antibiotic controls. The use of A#2 and W#1 resulted in greater callus fresh weights over no filter paper controls for both kanamycin and cefotaxime treatments (Table 3). However, the difference with the latter was not statistically significant. The use of W#3 resulted in reduced callus fresh weights compared to controls, A#2

and W#1, independent of the antibiotic and the presence of filter paper (Table 3).

Both morphogenic response (root, shoot, somatic embryo, Fig. 2, Table 2; callus, Table 3) and explant survival (Table 3, Fig. 3) decreased with or without the presence of antibiotics when W#3 was used, suggesting that W#3 was detrimental to tobacco or chrysanthemum tissue culture and failed to buffer the effect of antibiotics. This may have repercussions in genetic transformation experiments where negative selection systems (using antibiotics) usually negatively impact the normal morphogenesis of explants, and where we would wish to maximize the amount of organs formed even in the presence of selection.

The effect of antibiotics on the rhizogenic and somatic embryogenic programs was not tested since the primary organs desired in genetic transformation experiments are usually the shoot and callus.

A 100% explant survival rate was observed in all programs for both tobacco and chrysanthemum with the use of A#2 or W#1, or when tTCLs were plated directly onto agar medium (Tables 2 and 3). Explant survival was reduced when W#3 was utilized (Tables 2 and 3, Fig. 2). This trend with respect to the filter paper type is constant, even when kanamycin and cefotaxime were used (Fig. 2). The qualitative results for explant survival, and organ (callus, adventitious shoot and roots, somatic embryos) formation were the same whether light or darkness were utilized, but quantitatively inferior in dark treatments, for both tobacco and

chrysanthemum, independent of the presence or absence of filter paper or antibiotics (data not shown).

Flow cytometry analysis of shoots and callus revealed that chrysanthemum (both LIN and SNC) callus had a lower 2C: 4C ratio than shoots (Fig. 4, A vs. B) derived from the same treatment (controls, filter paper or antibiotics). In tobacco, shoots derived from any treatment (controls, filter paper and antibiotics) had a high 2C: 4C ratio, occasionally demonstrating 8C values, that is endoreduplication (Fig. 4C), especially at high kanamycin or cefotaxime concentrations. Tobacco callus material, however, derived exclusively from high kanamycin or cefotaxime concentration treatments (with or without filter paper) resulted in both 8C and 16C polysomaty (Fig. 4D).

Greenhouse SNC chrysanthemum plants and all characteristics of flowers were not different between control plants and plants derived from the A#2 filter paper treatment (Table 4).

Our results demonstrated that filter paper has both a growth-promotive and growth-inhibiting effect on morphogenesis in tobacco and chrysanthemum (both cultivars) depending on the type of filter paper used. In both species, A#2 and W#1 filter papers promoted the development of adventitious roots and shoots (to differing degrees of significance) more than tTCLs placed on rhizogenic and caulogenic program media without filter paper (Tables 2 and 3, Fig. 2). These morphogenic programs were negatively affected when W#3 was used,

Table 2. Influence of explant support material on the morphogenic response of Tobacco and Chrysanthemum tTCLs.

Explant support material	Morphogenic response program					
	Shoot organogenesis		Rhizogenesis		Somatic embryogenesis	
	No. shoots/tTCL	Explant survival	No. roots/tTCL	Explant survival	No. embryos/tTCL	Explant survival
<i>Tobacco (Nicotiana tabacum 'Samsun SS')</i>						
Agar	2.43 c	100 a	6.50 b	100 a	2.14 a <sup>z</sup>	100 a
Advantec #2	4.47 a	100 a	8.13 a	100 a	1.12 c <sup>z</sup>	100 a
Whatman #1	4.03 b	100 a	7.85 a	100 a	1.61 bc <sup>z</sup>	100 a
Whatman #3	1.93 d	93 b	3.15 c	87 b	1.74 b <sup>z</sup>	74 b
<i>Dendranthema ×grandiflora 'Lineker'</i>						
Agar	1.43 b	100 a	3.16 c	100 a	1.01 b <sup>y</sup>	100 a
Advantec #2	2.41 a	100 a	4.63 a	100 a	2.13 a <sup>y</sup>	100 a
Whatman #1	2.22 a	100 a	4.02 b	100 a	2.01 a <sup>y</sup>	100 a
Whatman #3	1.61 b	98 a	3.04 c	92 b	0.84 b <sup>y</sup>	67 b
<i>Dendranthema ×grandiflora 'Shuhou-no-chikara'</i>						
Agar	1.26 bc	100 a	1.88 b	100 a	1.22 b <sup>y</sup>	100 a
Advantec #2	2.37 a	100 a	4.01 a	100 a	1.96 a <sup>y</sup>	100 a
Whatman #1	1.99 b	100 a	3.77 a	100 a	1.54 ab <sup>y</sup>	100 a
Whatman #3	1.14 c	88 b	1.67 b	96 a	0.86 c <sup>y</sup>	64 b

<sup>z</sup>Any developmental stage; Different letters within a column (i.e., within a morphogenic program) are significantly different ( $P \leq 0.05$ ) for any one species/cultivar according to Duncan's multiple range test.

<sup>y</sup>Globular.

Table 3. Influence of explant support and antibiotic type and concentration on morphogenesis of Tobacco and Chrysanthemum tTCL's.

Explant support material	Chrysanthemum																	
	Tobacco						Lineker						Shuhou-no-chikara					
	Control		Kanamycin*		Cefotaxime*1		Control		Kanamycin*		Cefotaxime*1		Control		Kanamycin*		Cefotaxime*1	
	FW	ES	FW	ES	FW	ES	FW	ES	FW	ES	FW	ES	FW	ES	FW	ES	FW	ES
Agar	11.78 a	100 A	1.26 g	8 B	6.43 c	48 AB	8.99 a	100 A	0.36 f	5 B	2.63 d	31 A	9.87 a	100 A	0.44 g	3 B	6.81 d	28 A
Advantec #2	9.62 b	100 A	3.17 d	15 A	9.43 b	51 A	7.24 b	100 A	1.42 e	21 A	4.04 c	36 A	8.67 b	100 A	0.86 f	28 A	7.72 c	26 AB
Whatman #1	9.43 b	100 A	2.87 de	12 AB	9.26 b	41 B	6.99 b	100 A	1.21 e	16 AB	3.72 c	34 A	8.23 b	100 A	0.66 fg	24 A	7.37 cd	20 B
Whatman #3	2.68 e	46 B	0.45 h	13 AB	2.01 f	14 C	2.82 d	29 B	0 g	0 B	0.48 f	3 B	2.46 e	33 B	0 h	0 B	0 h	0 C

Fresh weight (FW) values in mg·L<sup>-1</sup>; \* = 10 and 50 mg·L<sup>-1</sup> for chrysanthemum and tobacco, respectively; \*1 = 250 and 500 mg·L<sup>-1</sup> for chrysanthemum and tobacco, respectively; ES = % Explant (tTCL) survival; Different letters within a column are significantly different ( $P \leq 0.05$ ) for any one species/cultivar according to Duncan's multiple range test.



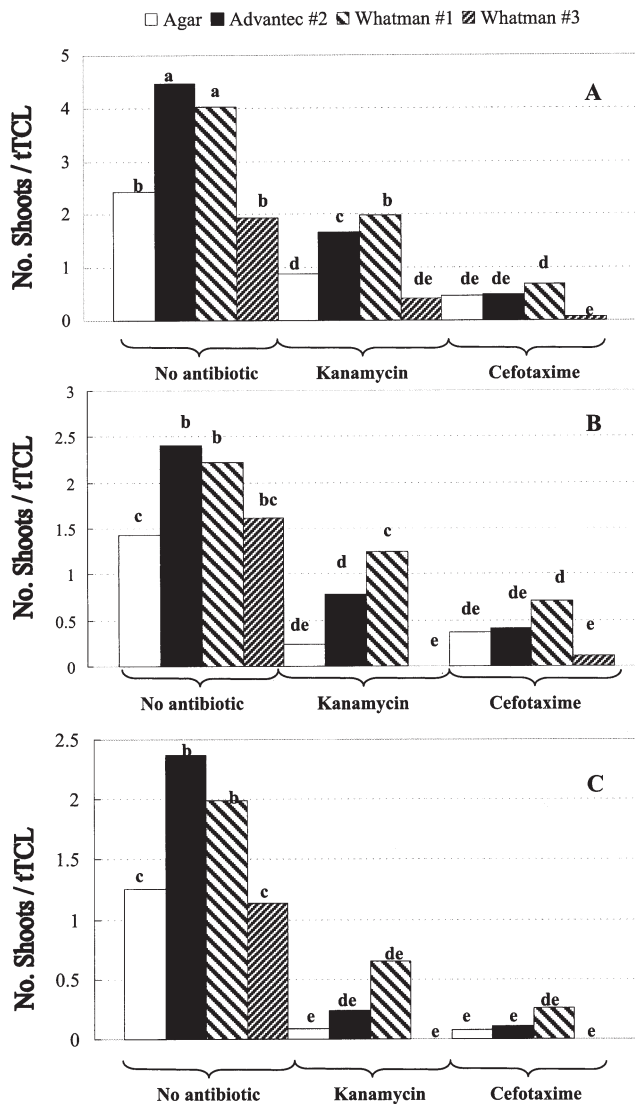


Fig. 2. Number of shoots per tobacco (A) and chrysanthemum (LIN, B; SNC, C) tTCLs on control (antibiotic-free) and antibiotic (kanamycin or cefotaxime)-containing caulogenic medium after 60 days in culture. Bars within graphs with similar letters are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

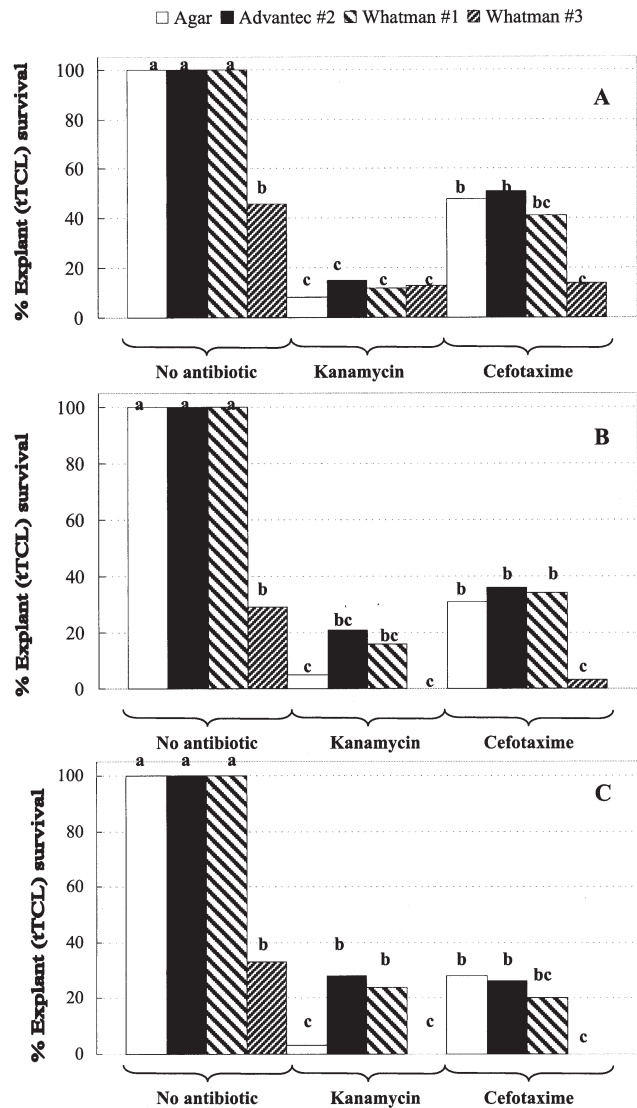


Fig. 3. Percentage of explant survival of tobacco (A) and chrysanthemum [LIN, (B); SNC, (C)] tTCLs on control (antibiotic-free) and antibiotic (kanamycin or cefotaxime)-containing caulogenic medium after 60 d in culture. Bars within graphs with similar letters are not significantly different according to Duncan's multiple range test ( $P = 0.05$ ).

as was explant survival (Tables 2 and 3, Fig. 3), perhaps as a result of the physical-chemical properties of filter paper. The retention, absorption capacities, or both, of the different filter papers may play an important role in the permeability of plant growth regulators, ions, and also antibiotics from the agar to the explant. Low explant survival values (Tables 2 and 3, Fig. 2) for when W#3 was used, independent of the morphogenic program and the species, suggests that explant mortality is a function of the physical and chemical properties of the filter paper rather than the explant, since even an increase in relative humidity did not result in increased ES (data not shown).

Despite the use of filter paper in many tissue culture experiments a definitive study analysing its effect on any plant species' in vitro growth and morphogenesis is lacking. Reports discussing the direct effect of filter paper on any aspect of plant tissue culture were not found and were explored for the first time in this experiment. This study further clearly

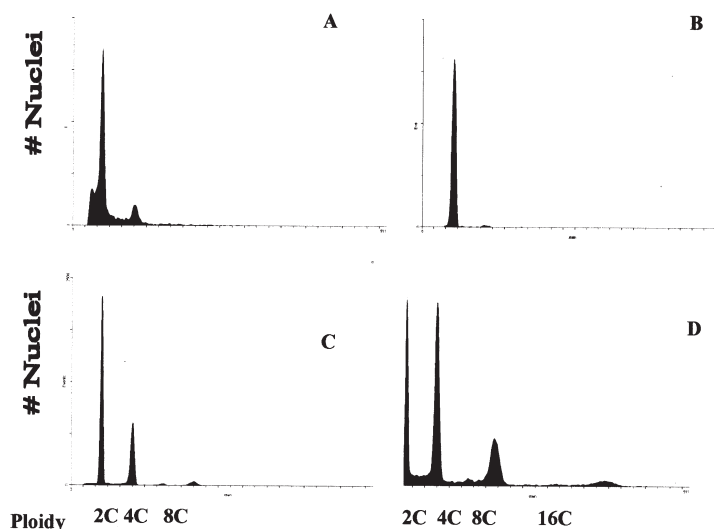


Fig. 4. Histograms of flow cytometric analysis showing the number of nuclei in various tissues corresponding to 2C, 4C, 8C, and 16C DNA content as estimated by DAPI staining of nuclear DNA. Chrysanthemum (SNC) callus (A) and shoot (B), and tobacco shoot (C) and callus (D) material derived from tTCL culture.

Table 4. Characteristics of greenhouse-acclimatized plantlets (n=15).

Treatment	Length stem (cm)	# Leaves	Wt (g)	# Ray florets	# Disk florets
Control	34.1 a	31.2 a	45.2 a	283.8 a	6.2 a
A#2-plantlets	36.6 a	30.6 a	44.8 a	278.4 a	6.6 a

Different letters within a column are significantly different ( $P \leq 0.05$ ) according to Duncan's multiple range test.

demonstrates the necessity to test the use of filter paper in both conventional in vitro tissue culture and micropropagation practices, as well as in transformation experiments, since the choice of filter paper can significantly affect the morphogenic response of explants, and the sensitivity to both plant growth regulators and antibiotics, still frequently utilized in genetic transformation studies. As is the case of almost any genetic transformation study, the chance of obtaining a genetic transformant increases with the capacity to produce propagules. In the case of chrysanthemum, where transformation is possible through various gene introduction methods (Teixeira da Silva and Fukai, 2002a) and where transient transgene expression can be increased/maximized by optimising the physical parameters, the capacity to increase the production of propagules (primarily adventitious shoots, but also roots, callus and somatic embryos) by the addition of A#2 or W#1 would surely enhance the chance of success, especially where organs are derived from a single-cell origin, and where the initial explant (TCL) is small.

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