Aminoglycoside antibiotics affect the in vitro morphogenic response of chrysanthemum and tobacco

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Summary: Broadly the success of genetic transformation of plants requires non-chimeric selection of transformed tissues and its subsequent regeneration. With rare exceptions, most plant transformation protocols still heavily utilize antibiotics for the selection of transgenic cells containing an antibiotic-degrading selectable marker gene. The morphogenic capacity of *in vitro* chrysanthemum and tobacco stem and leaf explants change with the addition of aminoglycoside antibiotics (AAs). Of 6 antibiotics tested, phytotoxicity occurred at 10-25 and 50-100 μgml^{-1} in chrysanthemum and tobacco explants, respectively, depending on the size of the explant and the timing of application. The presence of light or darkness also had a significant effect. The use of transverse thin cell layers (tTCLs) in conjunction with high initial AA selection levels supported the greatest regeneration of transgenic material (adventitious shoots or callus) and the lowest number of escapes. Flow cytometric analyses demonstrate that regeneration can be predicted in both species, depending on the ploidy level of the callus. Endoreduplication was not observed in chrysanthemum, even at high AA levels, but occurred (8C or more) in tobacco callus, even at low AA concentrations (5-10 μgml^{-1}). The higher the AA level, the greater the DNA degradation and the lower the 2C and 4C values.

Key words: aminoglycoside antibiotic (AA), *Dendranthema X grandiflorum*, *Nicotiana tabacum*, phytotoxicity, polyploidy

Introduction

Plant genetic transformation studies often use aminoglycoside antibiotics (AAs) as selective agents for transgenic cells that contain an antibiotic resistance gene which encodes for an aminoglycoside 3'-*O*-phosphotransferase. This inactivates AAs by phosphorylation (*Coppoc*, 1996). Antibiotic-degrading selectable marker genes (and hence AAs) will continue to be used in the future because they are effective, and since new methods exist for their elimination from plants after transformation (*Yoder & Goldsbrough*, 1994), as occurs in the MAT vector system (*Ebinuma & Komamine*, 2001). Such methods might reduce the perceived biosafety hazards associated with the use of antibiotics in experimental biology. One of the problems hindering successful transformation protocols is balancing antibiotic concentration in order to achieve stringent selection while allowing regeneration to occur. Various AAs have been used at varying concentrations in chrysanthemum genetic transformation studies, but no study has specifically addressed the impact of AAs on growth/regeneration (*Table 1*).

Selection of transformed cells is based on the expression of the selectable marker gene, which produces an enzyme that confers resistance to a cytotoxic substance (often an antibiotic or a herbicide). The *nptII* (3'-*O*-phosphotransferase) gene, encoded by the *aph*A2 gene from transposon Tn5 of *Escherichia coli*, confers resistance to the AAs neomycin, G418 (*Bevan*, 1984), kanamycin (*Herrera-Estrella* et al., 1992), G418 (*Santerre* et al., 1984) and paromomycin (*Guerche* et al., 1987) while hygromycin B resistance gene (*hpt*, hygromycin phosphotransferase) from *E. coli* confers resistance to hygromycin (*Waldron* et al., 1985). The 6' gentamycin acetyltransferase (*gat*) gene confers resistance to gentamycin and other members of the AA family (*Gossele* et al., 1994). For control plants under normal conditions these AAs would otherwise prove to be toxic.

Thin cell layers (TCLs) comprise a system where the morphogenetic and developmental pathways of specific organs – derived from other specific or non-specific cells, tissues or organs – may be clearly directed and controlled (*Tran Thanh Van*, 1973). TCL explants are of a small 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, but which are normally too small to separate.

This study explores the negative effect that most AAs have on *in vitro* growth and morphogenesis (shoot and root formation) of chrysanthemum (one of the most important ornamental crop species globally) and tobacco (TOB; a model plant for the study of many physiological and genetic mechanisms). The effect of the AA concentration on plant morphogenesis and explant survival depends on the size of the explant, the choice of explant source, the timing of infection by *Agrobacterium tumefaciens* and selection pressure in genetic transformation experiments. These seek to maximize shoot regeneration, and eliminate shoot escape and shoot chimera formation. This study emphasizes the main limiting factors in any plant genetic transformation

Table 1 Aminoglycoside antibiotics (AA) used in chrysanthemum (D.X grandiflorum) genetic transformation studies

Principal Cultivar(s) + others	Source	AA	Selection	Iniotial Selection‡	Regeneration‡	Reference
Indianapolis White Giant #4	Stem	K	Early	25	25	Lemieux et al. 1990
Parliament	Leaf	K	Early	50	50-100	van Wordragen et al. 1991
Des Moul*	Leaf	K	Early	25	10	Ledger et al. 1991
Parliament	Leaf	K	Early	25	10	van Wordragen et al. 1992a "
1610, Parliament + 5	Leaf	K	Late	50	50	van Wordragen et al. 1992b
1610, Parliament + 5	Leaf	None	Late	–	–	de Jong et al. 1993
Carillon	Leaf, stem	KH	Late	K25 H5	K50 H10	Renou et al. 1993
Super White	Stem	K	Early	15–25	15-25	Lowe et al. 1993
White Snowdon + 3	Leaf	K	Late	100	50	Pavingerová et al. 1994
8382, 89100, 89124	Flower	K	Early	10–25	0	de Jong et al. 1994
Hekla, Iridon, Polaris	Leaf	K	Early	50	0'!50	Urban et al. 1994
1581 + 9	Stem	K	Early	10	0	Fukai et al. 1995
Iridon, Polaris + 4	Shoot tip	K	Early?	50	0	Yepes et al. 1994 §
Parliament + 4	Leaf	KH	Early	K10-50 H10-15	K10-50 H10-15	Dolgov et al. 1997
Peach Margaret	Leaf	K	Early	25	20	Boase et al. 1998a
Peach Margaret + 2 + 1†	Leaf	KS	Early	K25	K25	Boase et al. 1998b
Hekla, Iridon, Polaris	Leaf	P	Early	50	50	Sherman et al. 1998
Yamabiko	Stem	K	Early	50	10	Takatsu et al. 1998
Shuhou-no-chikara	Leaf	G	Early	20–30	20–30	Shinoyama et al. 1998
Kanseisetsu	Stem	H	Early	10–40	20	Shirasawa et al. 2000
Hybrid†	Stem	K	Early	100	100	Tosca et al. 2000
Iridon	Leaf	K	Late	50	50	Zheng et al. 2001

‡ = mgl^{-1}; *D. indicum*; † = *D. zawadskii* x *D. X grandiflorum*. GIM = Geneintroduction method (all *Agrobacterium tumefaciens*, except for § = Biolistics and Δ = *A. rhizogenes)*; AAs: G = G418; H = Hygromycin; K = Kanamycin; P = Paramomycin; R = Rifampicin. Selection: early (0–3 d), late (> 3d)

experiment are: the capacity of the plant material itself to grow, differentiate and develop under stress conditions, and the factors that enhance (or subdue) these processes, including the competitive growth with the infectious agent, *A. tumefaciens*.

Material and method

Trial 1: effect of aminoglycoside antibiotics on morphogenesis and threshold survival of untransformed explants

Stem internode tissue of *in vitro* cv. 'Lineker' (LIN) and cv. 'Shuhou-no-chikara' (SNC) chrysanthemum (*Dendranthema* X *grandiflorum* (Ramat.) Kitamura), as well as TOB (*Nicotiana tabacum* cv. Samsun SS) was cut transversally into 3-5 mm long concentric slices, 1–2 mm thick. These slices were then in turn cut in half, yielding semi-cylindrical explants. Explants (n=60) were placed on optimized shoot regeneration medium (MSs: MS + 2 mgl^{-1} benzyladenine (BA) + 0.5 mgl^{-1} α-napthalene acetic acid (NAA) + 40 gl^{-1} sucrose; *Fukai* et al., 1987) containing 0, 5, 10, 25, 50 or 100 mgl^{-1} of a single filter-sterilized AA: geneticin® (=G418), gentamycin, hygromycin B, kanamycin A, neomycin, or paramomycin (*Table 2*). Treatments were placed both in the light (16 h photoperiod, 40 $\mu molm^{-2}s^{-1}$) and in the dark, at 25 °C, and threshold survival levels (TSLs) were determined. A TSL is the level of an AA at which the tTCL (*Figure 1A*) does not undergo morphogenesis (callus or shoot formation). Shoots derived from any medium were harvested and placed on Hyponex® (soluble fertilizer, N:P:K = 6.5:6:19; 3 gl^{-1}) medium containing 20 gl^{-1} sucrose and three times subcultured plantlets were maintained under a 16 h photoperiod (40 $\mu molm^{-2}s^{-1}$) at 25 °C. Chrysanthemum plantlets were acclimatized and maintained in the greenhouse under long-day conditions, and placed in short-day conditions for flower induction. Greenhouse-acclimatized plantlets were checked for morphological (stem length, number of leaves, weight) and flowering normality (number of ray and disk florets, petal colour, flower head size).

Trial 2: effect of explant size and source on the strength of the AA impact on morphogenesis

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, lTCLs (~100–300 µm thick and 1–1.5 mm, 1–2 mm or 2–5 mm in diameter for LIN, SNC and TOB respectively) containing one or a few epidermal and subepidermal cell layers, and explants as used in Trial 1 were prepared from chrysanthemum and TOB stem internode tissue. In addition leaf tTCLs (~0.5–1 mm thick and 0.5–1 mm long) and explants (0.5 mm^2, 2–3 mm thick) were prepared from *in vitro* leaves of LIN, SNC and TOB, both leaf explant sizes containing midvein tissue.

All explants were placed on MSs containing 10 μgml^{-1} of any Trial 1 AA with or without infection by *A. tumefaciens* (LBA4404 harbouring plasmid pKT2 (nos-L-*nptII* (wild); 35S-LEI-*uid*A; L=Leader; E=enhancer; I=Intron; Kirin Breweries, Inc.). *A. tumefaciens* strain LBA4404 was cultured in 20 ml Luria Broth medium for

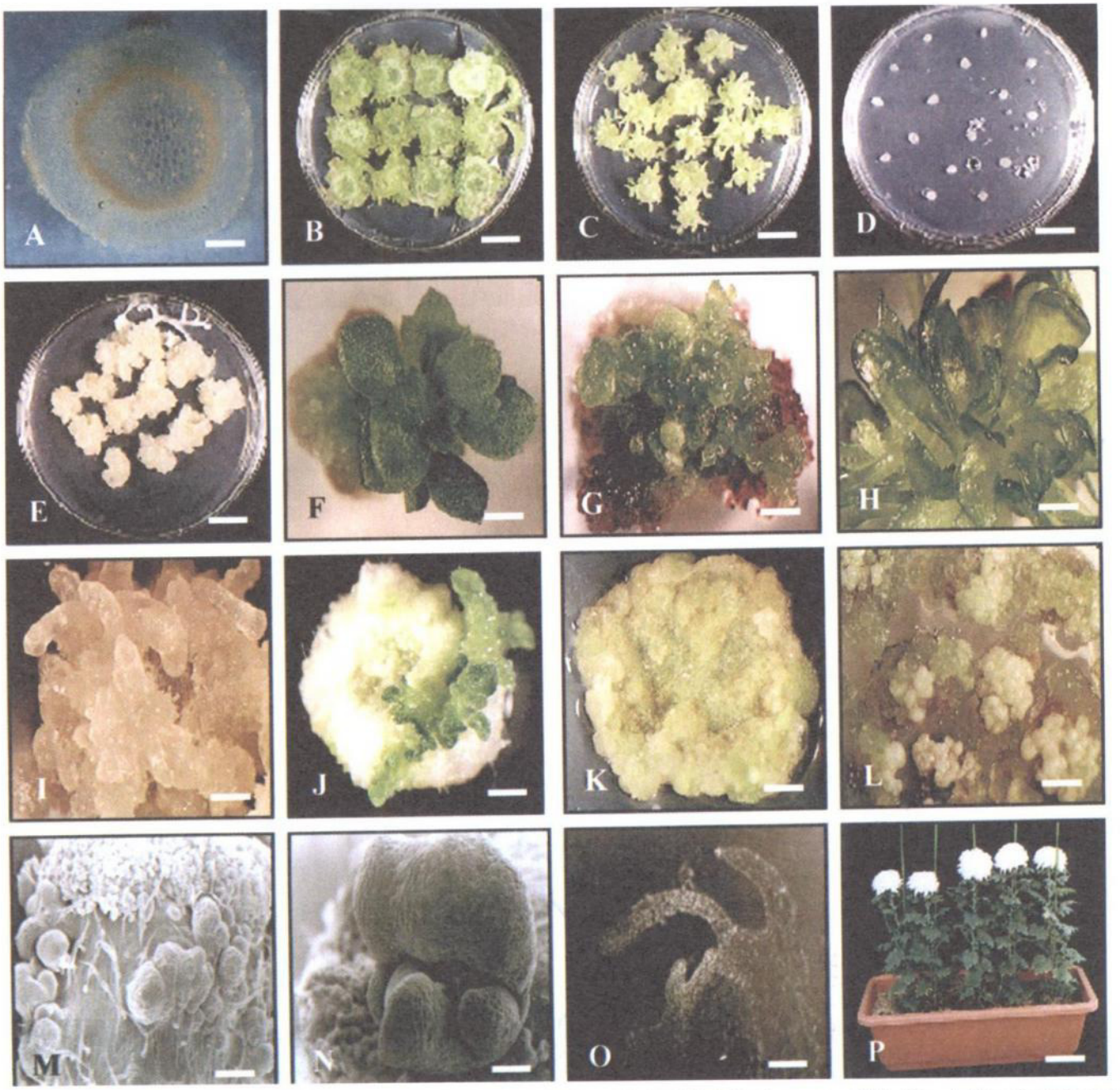

Figure 1 Morphogenic reaction of chrysanthemum and tobacco to aminoglycoside antibiotics (AAs). **A**) tobacco stem tTCL; (**B**) typical reaction tobacco to all AAs at (0 μgml^{-1}, control), (**C**) (10 μgml^{-1}) and (**D**) (100 μgml^{-1}) in the light and (**E**) 50 μgml^{-1} in the dark. (**F**) Shoot regeneration from SNC stem tTCL on non-selective medium; (**G**) non-regenerable shoot primordia on SNC stem tTCL at 10 μgml^{-1} with red callus (low 2C value); (**H**) deformed SNC shoots (hyperhydric) on 5 μgml^{-1} hygromycin; (**I**) highly elongated tobacco cells (meristematic and regenerable) on 10 μgml^{-1} gentamycin in the dark; (**J**) profuse white callus on tobacco stem tTCLs with normal shoot development at 10 μgml^{-1} kanamycin in the light; (**K**) *Agro*infected (pKT2) SNC leaf disks form meristematic callus that, upon high selection pressure (30 µg/ml kanamycin) form white (non-transformed) and green (putatively transformed) callus (**L**). (**M**) SEM of SNC stem explant on 5 μgml^{-1} kanamycin, showing normal shoot primordia and callus production on the cut surfaces; (**N**) SEM and (**O**) light microscope section through control SNC shoot primordium on non-selective MSs. (**P**) Shoots derived from AA treatments led to normal flowering that did not differ significantly from controls. Scale bars: 10 µm (**N**,**O**); 50 µm (**A**,**F**,**G**,**H**,**I**,**J**,**K**,**M**); 500 µm (**L**); 1 cm (**B**,**C**,**D**,**E**); 10 cm (**P**).

16–20 h at 27 °C. Hereafter, 1 ml of broth culture was centrifuged and then resuspended in 1 ml 10 mM glucose supplemented with 100 mM acetosyringone and adjusted to an OD_{540}=0.4–0.5. The bacterial suspension was applied at one drop per explant (~10 µl) and co-cultivated for 3–4 d. Inoculated explants were then selected on media containing 250 μgml^{-1} cefotaxime (Claforan®) for 1 week, then on media containing 125 μgml^{-1} cefotaxime and were subcultured bi-monthly, to eliminate *A. tumefaciens*.

Plantlets derived from these treatments were cultured and acclimatized in a greenhouse as described in Trial 1.

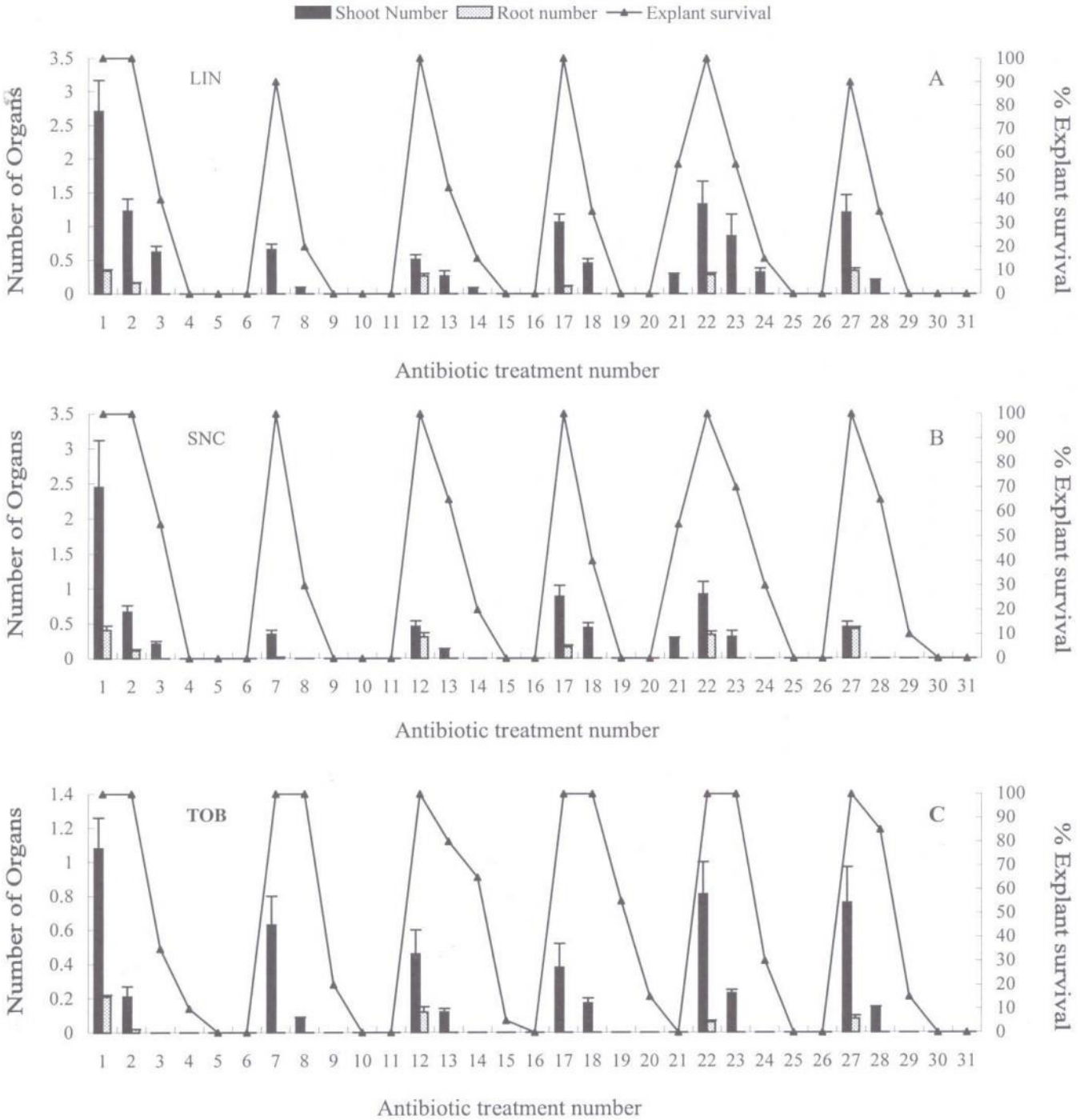

Figure 2 Graphs showing the morphogenic (i.e. root and shoot growth) reaction and tTCL explant survival in the light in response to aminoglycoside antibiotics at various concentrations. Treatments 1-31 on the X-axis correspond to treatments with the same number in *Table 2*.

Morphological scoring

All explants in both trials were scored for the amount (number and weight) of shoots, roots, callus, explant survival (ES) and explant fresh weight after 60 days in culture. Chrysanthemum plants were acclimatized in a greenhouse and checked for vegetative (plantlet height, number of leaves, total fresh weight) and flowering (number of disk and ray florets per flower head, flower colour, head size) normality.

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. For SEM, samples were fixed in FAA (formaldehyde:acetic acid: EtOH=5:5:90). Samples were then dehydrated in an ethanol series (50–100% EtOH for at least 6 h each), critical point-dried, sputter-coated

Table 2 Shoot and callus fresh weight of chrysanthemum and tobacco stem tTCLs in response to aminoglycoside antibiotics after 60 days in culture

n=60		Light				Dark		
Antibiotic	T#	Conc.*	LIN	SNC	TOB	LIN	SNC	TOB
Control§		0	0.04±0.00	0.05±0.00	0.08±0.01	0.04±0.00	0.05±0.00	0.06±0.01
Control	1	0	8.99±0.22 a	9.87±0.35 a	11.78±0.72 a	3.17±0.26 a	3.71±0.22 a	6.88±0.51 a
G418	2	5	0.59±0.02 c	0.61±0.04 cd	11.46±0.13 a	0.22±0.03 cd	0.34±0.04 cd	4.23±0.65 c
(Geneticin®)	3	10	0.28±0.06 cd	0.36±0.08 cd	1.31±0.02 f	0.09±0.01 d	0.11±0.01 d	0.34±0.06 g
	4	25	0 d	0 e	0.12±0.03 gh	0 d	0 d	0.06±0.00 h
	5	50	0 d	0 e	0 h	0 d	0 d	0 h
	6	100	0 d	0 e	0 h	0 d	0 d	0 h
Gentamycin	7	5	0.19±0.02 cd	0.28±0.02 d	10.94±0.63 a	0.26±0.03 cd	0.45±0.08 c	6.01±0.43 b
	8	10	0.07±0.00 d	0.11±0.01 de	7.71±0.55 b	0.09±0.00 d	0.09±0.01 d	6.46±0.08 ab
	9	25	0 d	0 e	0.72±0.13 fg	0 d	0 d	0.12±0.05 h
	10	50	0 d	0 e	0.09±0.01 gh	0 d	0 d	0.07±0.01 h
	11	100	0 d	0 e	0 h	0 d	0 d	0 h
Hygromycin B	12	5	1.28±0.18 b	1.86±0.21 b	6.19±0.42 c	1.02±0.21 b	1.34±0.17 b	6.21±0.66 ab
	13	10	0.60±0.04 c	1.62±0.14 b	3.15±0.21 d	0.42±0.09 c	0.98±0.21 bc	3.74±0.36 d
	14	25	0.23±0.01 cd	0.80±0.07 c	2.19±0.16 e	0.18±0.03 d	0.34±0.09 cd	2.64±0.25 e
	15	50	0 d	0 e	0.16±0.02 gh	0 d	0 d	0.18±0.03 gh
	16	100	0 d	0 e	0 h	0 d	0 d	0 h
Kanamycin A	17	5	0.36±0.03 cd	0.44±0.04 cd	11.26±0.69 a	0.27±0.07 cd	0.32±0.06 cd	5.77±0.08 b
	18	10	0.28±0.03 cd	0.34±0.02 d	8.21±0.62 b	0.09±0.01 d	0.18±0.03 cd	3.34±0.03 d
	19	25	0 d	0 e	0.89±0.07 fg	0 d	0 d	0.26±0.03 gh
	20	50	0 d	0 e	0.18±0.02 gh	0 d	0 d	0 h
	21	100	0 d	0 e	0 h	0 d	0 d	0 h
Neomycin	22	5	0.47±0.07 c	0.61±0.09 cd	11.63±0.78 a	0.39±0.09 c	0.56±0.11 c	6.06±0.43 b
	23	10	0.32±0.05 cd	0.42±0.05 cd	1.23±0.19 f	0.14±0.02 d	0.23±0.06 cd	0.87±0.12 f
	24	25	0.11±0.01 d	0.26±0.03 d	0.31±0.04 g	0 d	0.08±0.00 d	0.23±0.05 gh
	25	50	0 d	0.06±0.00 e	0 h	0 d	0 d	0.08±0.01 h
	26	100	0 d	0 e	0 h	0 d	0 d	0 h
Paramomycin	27	5	0.41±0.06 cd	0.81±0.13 c	11.03±0.56 a	0.32±0.06 c	0.46±0.08 c	5.86±0.61 b
	28	10	0.26±0.03 cd	0.66±0.08 cd	1.09±0.21 f	0.11±0.02 d	0.12±0.04 d	1.03±0.44 f
	29	25	0 d	0.12±0.02 de	0.23±0.04 gh	0 d	0 d	0.20±0.08 gh
	30	50	0 d	0 e	0 h	0 d	0 d	0.08±0.00 h
	31	100	0 d	0 e	0 h	0 d	0 d	0 h

* µg/ml; § Initial fresh weight of stem explant; T#=Treatment number; All values in grams (minus initial fresh weight §), mean ± SD; Different letters within a column indicate significant differences at P<0.01 using Duncan's Multiple Test Range.

with platinum and viewed under a Hitachi-2150 SEM microscope.

Flow Cytometry (FC)

Nuclei were isolated from about 0.5 cm^2 of the material (shoot and callus) derived from 60 day-old (from the initial point) material at control (0 μgml^{-1}) and threshold AA levels (10–25 or 50–100 μgml^{-1} for chrysanthemum and TOB, respectively) by chopping in a few drops of Partec Buffer A (2 μgml^{-1} 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton-X, pH 7.5; *Mishiba & Mii*, 2000). Nuclear fluorescence was measured using a Partec® Ploidy Analyser (PA) after filtering the nuclear suspension through 30 µm mesh size nylon filter (CellTrics®) and adding five times of Buffer A for 1 min. Three samples were measured, and relative fluorescence intensity of the nuclei was analyzed when the Coefficient of Variation was <4%. A total of 2500 nuclei were counted for any sample with minor adjustments made to peak areas deviating from this count.

Statistical analyses

Experiments were organized according to a complete randomized block design (CRBD) with three blocks of n=20 (explant number) each per treatment. Data was analysed for significance by ANOVA with the mean separation by Duncan's multiple range test.

Results

Effect of AA on morphogenesis and regeneration capacity

Results of our studies reveal that, without any exception, the addition of AAs to the culture medium negatively affects chrysanthemum and TOB shoot and callus fresh weight, even when *nptII*-containing *Agrobacterium* is used. An increase in concentration causes a decrease in shoot and callus fresh weight (*Figure 1B-D*). In all cases, an AA level above 10 μgml^{-1} caused a significant ($P<0.05$) reduction in shoot forming capacities (*Table 2*; *Figure 2*).

TSLs were established for LIN, SNC and TOB, respectively (*Table 2*) in each AA case at: 10, 10 and 25 μgml^{-1} for G418 (light and dark); 10, 10 and 50 μgml^{-1} for gentamycin (light and dark); 25, 25 and 50 μgml^{-1} for hygromycin B (light and dark); 10, 10 and 50 μgml^{-1} in the light and 10, 10 and 25 μgml^{-1} in the dark for kanamycin A; 25, 50 and 25 μgml^{-1} in the light and 10, 25 and 50 μgml^{-1} in the dark for neomycin; 10, 25 and 25 μgml^{-1} in the light and 10, 10 and 50 μgml^{-1} in the dark for paramomycin. Many AAs degrade faster when exposed to light, and this may explain the higher fresh weights and TSLs obtained in almost all AAs for both chrysanthemum and TOB in the light.

The negative impact of all AAs on chrysanthemum and TOB was genotype-independent. Shoot and callus (i.e. explant) fresh weights for all cultivars, and at almost all AA concentrations (except for LIN and SNC with gentamycin) were higher in the light than in the dark (*Table 2*) may be due to the capacity of explants to photosynthesize and assimilate carbon (sucrose from the medium) into biomass, despite the morphogenic-restrictive nature of the AA-containing medium.

Shoot and root formation was also severely inhibited in chrysanthemum and TOB by all AAs, at varying concentration (*Figure 2A-C*). The effect was genotype-dependent in chrysanthemum, with SNC being less affected than LIN in all AA treatments (*Figure 2A* vs. *2B*). Explant survival (ES) decreased exponentially with a linear increase in AA concentration (*Figure 2A-C*) for LIN, SNC and TOB, and the rapid loss mirrored that observed in shoot and callus fresh weight loss (*Table 2*).

Flow cytometry and polysomaty

FC results (*Figure 3*) indicate a high level of genetic stability in initial chrysanthemum explant and regenerated tissue (callus or shoot) from both control (*Figure 1F,N,O*; *Figure 3B*) and for (*Figure 3D*) treatments inoculated with *A. tumefaciens*. All material (callus or shoots) did not diverge from the diploid state, and no polysomaty (differing ploidy levels as a result of regular developmental pattern) was registered (seen by the lack of =8C values, *Table 3*), even when AAs were applied at higher (25–50 μgml^{-1}) concentrations of AAs (data not shown). At these high concentrations, there was a decrease in the 2C value with a subsequent slight increase in the 4C value at any AA concentration, but a large increase in the <2C (i.e. DNA degradation products from dead cells) values (*Figure 3C,D,H*). Leaf material always showed a high 2C and 4C value, indicating that the material had a greater amount of young tissue – having highly meristematic and actively dividing cells, being only 60d old – prone to division, even when AAs were added (*Table 3*). In general the presence of *Agrobacterium* resulted in an increase in the 2C and 4C values, independent of genotype and explant source. Chrysanthemum callus derived from any AA treatment (AAs at =5–10 μgml^{-1}) with a high 2C and/or 4C value could regenerate shoots, whereas callus with low 2C and/or 4C values (AAs at =10–25 μgml^{-1} and controls), together with high <2C values (*Figure 3C,D,H*) did not have a regeneration potential, even when placed on non-selective optimized regeneration medium.

Polysomaty was however evident in TOB callus cultures derived from any treatment (*Table 3*; *Figure 3E-G*), excepting controls (*Figure 3A*). Leaf tissue exhibited no

Table 3 Ploidy ratios in tTCLs from different sources with or without Agrobacterium after 60 days in culture

Antibiotic	ES	+ Agrobacterium		- Agrobacterium	
		SNC	TOB	SNC	TOB
Control§	Stem tTCL	94:2:0	92:6:0	98:2:0	95:2:0
Leaf tTCL	96:3:0	89:8:0	95:3:0	93:4:0	
G418	Stem tTCL	78:4:0	61:10:4	80:3:0	71:1:8
Leaf tTCL	84:6:0	66:11:3	82:2:0	73:4:8	
Gentamycin	Stem tTCL	67:3:0	58:11:4	64:1:0	68:2:6
Leaf tTCL	71:6:0	63:13:6	65:2:0	71:3:3	
Hygromycin B	Stem tTCL	76:1:0	69:2:1	86:1:0	83:1:1
Leaf tTCL	81:3:0	71:6:3	87:3:0	85:1:1	
Kanamycin A	Stem tTCL	70:2:0	60:9:3	66:3:0	69:1:4
Leaf tTCL	74:4:0	66:12:5	70:5:0	73:1:1	
Neomycin	Stem tTCL	79:1:0	64:3:2	73:2:0	73:1:3
Leaf tTCL	82:4:0	66:4:4	78:4:0	78:3:2	
Paramomycin	Stem tTCL	76:2:0	63:5:3	76:1:0	74:0:2
Leaf tTCL	79:3:0	68:7:3	78:3:0	76:2:1	

All measurements done with material on medium with 10 µg/ml of the respective antibiotic, except for the control § at 0 µg/ml; ES=Explant source; SNC='Shuhou-no-chikara', TOB=tobacco; Flow Cytometry relative C ratios as 2 °C:4 °C:8 °C.

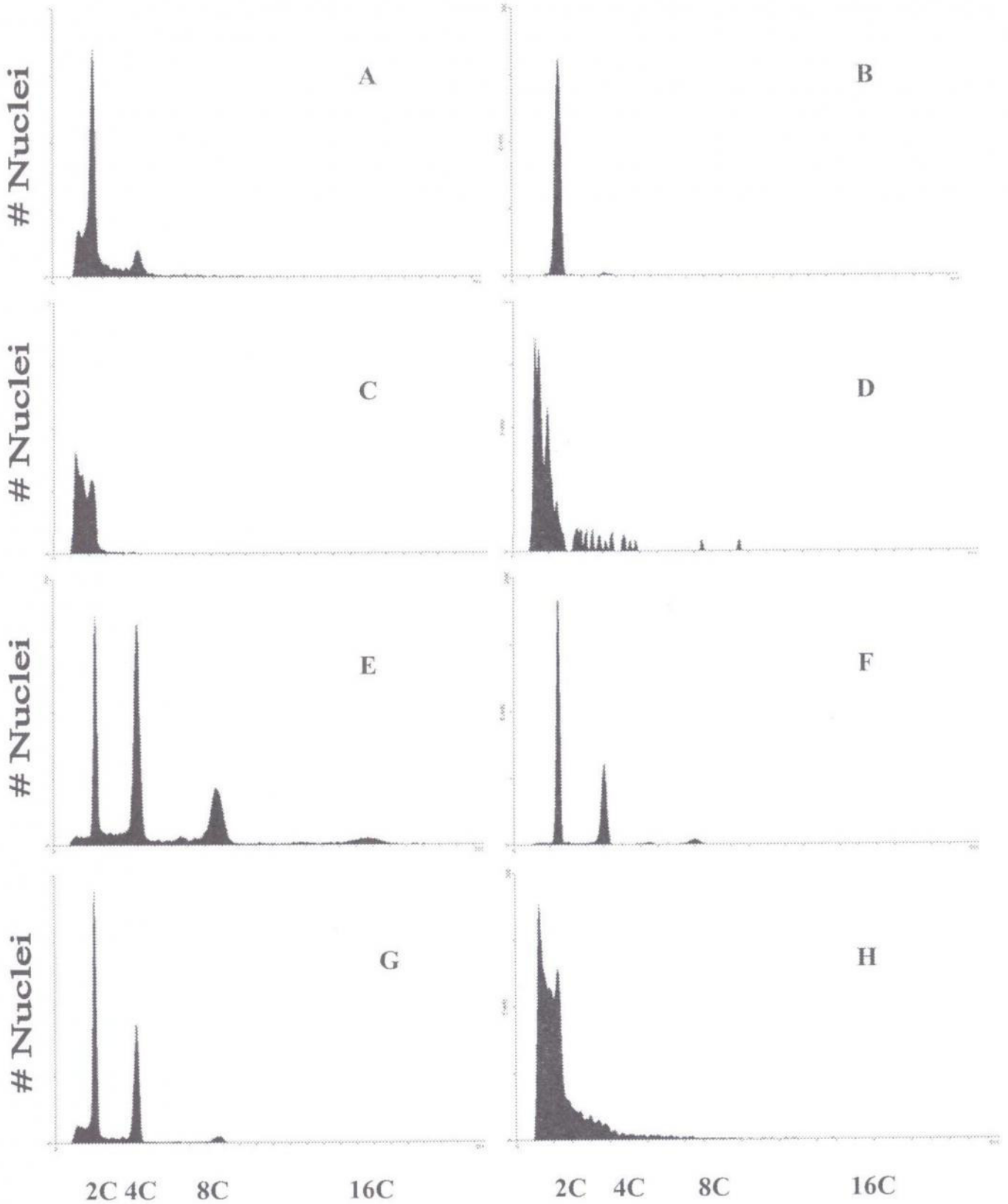

Figure 3 Histograms showing ploidy levels of different chrysanthemum (SNC) and tobacco (TOB) material on control and aminoglycoside antibiotic-supplemented media showing relative 2C:4C:=8C peaks. Large peaks before 2C are probably DNA degradation products derived from dead cells. (**A**) Control TOB leaf tTCL (light); (**B**) Control SNC stem tTCL (light); (**C**) SNC callus culture on 10 μgml^{-1} kanamycin (light); (**D**) SNC stem tTCL on 25 μgml^{-1} neomycin following inoculation with *Agrobacterium* (dark); (**E**) TOB stem tTCL on 50 μgml^{-1} gentamycin following inoculation with *Agrobacterium* (light); (**F**) TOB leaf tTCL on 25 μgml^{-1} G418 without *Agro*infection (light); (**G**) TOB stem tTCL on 50 μgml^{-1} paramomycin (light); (**H**) SNC stem tTCL on 25 μgml^{-1} neomycin (dark).

polysomaty despite some occurring in shoot apical meristems (data not shown). Trends were the same as those recorded above for chrysanthemum, but there were quantitative differences. AAs, in combination with *Agrobacterium* reduced the 2C values much more than in chrysanthemum, but increased the 4C values more than in chrysanthemum. Any AA treatment resulted in =8C values (8C and 16C), stemming from the callus, since shoot material exhibited no =8C values. Polysomaty of a higher order was also recorded (16C), and that of a lower order (<2C, the DNA degradation products). Degradation of DNA may have also contributed to such a high <2C value. In both chrysanthemum (*Figure 1G,H,K-M*) and TOB (*Figure 1B–D,I,J*), there was a decreasing gradient of negative effect by the AA on morphogenesis: gentamycin > kanamycin A > G418 > paramomycin > neomycin > hygromycin B. TOB callus derived from any AA treatment (at any concentration) with a high 2C and/or 4C with or without an 8C value (*Figure 3A*) could regenerate shoots, but callus having a low 2C and/or 4C values, together with =8C and/or <2C values (*Figure 3E*) did not have a shoot regeneration potential, even when placed on non-selective optimal regeneration medium (MSs), despite being able to form new callus.

Greenhouse acclimatization and flowering

Chrysanthemum plants derived from low or high AA concentration treatments, when subcultured three times on Hyponex® could be acclimatized at 100% (i.e. 100% survival), and following flower induction, plants did not differ from controls in all vegetative and flowering characteristics (*Table 4*).

The successful development of an efficient genetic transformation system for chrysanthemum and TOB requires an effective selection protocol such that the growth of non-transformed cells will be inhibited while that of transformed cells will result in callus or shoot formation. Presently shoot regeneration and genetic transformation are still problematic in chrysanthemum due to their genotype-dependent nature. For genotypes in which shoot regeneration is difficult, successful transformation becomes more difficult when AAs are utilized. Broadly two trends are observed: early selection (low or high AA levels) – immediately following the gene transfer method – or late selection (usually high AA levels) allowing a greater regeneration of shoots. The former tends to produce a low number of shoot regenerants and escapes, the latter a higher number of both (*Table 1*).

Marker genes are often used in experiments to select genetically modified plant cells at the initial steps of the transformation procedure. The most commonly used selective marker gene is the *nptII* gene, which encodes a phosphotransferase capable of phosphorylating AAs (kanamycin, G418, neomycin and paromomycin), and plant cells transformed with this gene can detoxify the AA in the selection medium. The selection and transformation efficiencies, however, are impacted by the biological activities of the different AAs (*Norelli & Aldwinckle*, 1993). Too high a concentration of an antibiotic may kill the non-transformed cells, thereby inhibiting regeneration of transformed cells (*Escandon & Hahne*, 1991). In contrast, insufficient levels of an antibiotic may result in overgrowth of non-transformed cells, inhibiting thus the regeneration and effective selection of transformed cells.

Table 4 Characteristics (mean±SD) of greenhouse-acclimatized plantlets derived from AA treatments * SNC; †LIN

Treatment	Length stem (cm)	# Leaves	Weight (g)	# Ray florets	# Disk Florets	Colour
Control	34.1±0.9	31.2±0.6	45.2±1.8	283.8±7.9	6.2±0.9	White*; pink†
AA-plantlets	36.6±1.1	30.6±0.4	44.8±2.3	278.4±9.3	6.6±1.2	White*; pink†

* SNC; † LIN

Discussion

Aminoglycoside antibiotics and genetic transformation systems

Antibiotic selection is a pre-requisite for the successful selection of transformed cells from among non-transformed cells in *Agrobacterium* transformation and particle bombardment of chrysanthemum and TOB. Transformed cells containing and expressing the *nptII* gene confer resistance to a number of members of the same AA family when on selective medium, and this fact has been explored in previous studies of chrysanthemum genetic engineering (*Table 1*). Results within this study show a negative impact that a number of AAs have on plant morphogenesis and shoot regeneration capacity (*Figure 1C,G,H,I*; *Figure 2*), even when below their TSLs (*Table 2*; *Figure 3C,D,H*).

Aminoglycosides are broad-spectrum antibiotics that act primarily by impairing bacterial protein synthesis through irreversible binding to prokaryotic ribosomes (especially the 16S and 30S ribosomal subunits), resulting in codon misreading (*Schlünzen* et al., 2001), and by creating fissures in the outer membrane of the bacterial cell (*Gonzalez & Spencer*, 1998). In plants, where most AAs are inactive or are weakly active, this translates into an inhibition of plasmid translation initiation, resulting in the white or "bleached" appearance of organs (*Figure 1L*). Aberrant proteins that may be inserted into the cell membrane may lead to altered permeability and further stimulation of AA transport (*Busse* et al., 1992) observed by the highly exponential decrease in fresh callus and shoot weight (*Table 2*), or ES and shoot and root formation (*Figure 2*) with a linear increase in AA concentration. At higher

concentrations, some AAs have been shown to alter the codon:anticodon matching, and the incorrectly charged tRNA results in a defective primary amino acid (*Coppoc*, 1996). It has generally been accepted that AAs are inhibitors of prokaryotic protein synthesis at concentrations of up to 25 μgml^{-1}, but that they may affect eukaryotic cells at higher concentrations, probably through non-specific binding to eukaryotic ribosomes and/or nucleic acids (*Takano* et al., 1996), such as observed in wheat 80S ribosomes that fail to elongate when cell cultures were exposed to mM amounts of G-418 (*Bar-Nun* et al., 1983). This is particularly true in TOB and for larger explant sizes in chrysanthemum where actively dividing cells with morphogenic capacity can regenerate on medium at higher concentrations (25–50 μgml^{-1}) of AA. Molecules that have a hydroxyl function at C-6' in place of an amino function, such as in kanamycin, gentamicin, G418/ geneticin® are effective inhibitors of eukaryotic protein synthesis (*Eustice & Wilhelm*, 1984), where their mode of action is to bind to the eukaryotic 80S ribosomal complex (*Bar-Nun* et al., 1983). This mode of action is evident in both chrysanthemum and TOB where TSLs are higher with neomycin and paramomycin (*Table 2*). Kanamycin is inactivated by phosphorylation, but gentamycin is resistant to these phosphorylases. Gentamycin was the AA with the strongest negative impact on morphogenesis (*Table 2*; *Figure 2*; *Figure 3E*). TSLs established for gentamicin, kanamycin and streptomycin were 10, 30–35 and 20–25 μgml^{-1}, respectively (*Coppoc*, 1996). Gentamycin was shown to reduce wheat cultures 25% more than kanamycin at 40 μgml^{-1} (*Smart* et al., 1995), but at 1–4 μgml^{-1} was the only AA effective in the control of Gram-positive and Gram-negative bacteria in *Drosera*, *Spathiphyllum*, *Syngonium* and *Nephrolepis* shoot tip cultures (*Kneifel & Leonhardt*, 1992).

Studies on Siberian elm showed that kanamycin was the most suitable AA for selection when the *nptII* gene was used as a selective marker gene, while neomycin, geneticin and paromomycin were inferior in their selective capacity in this order (*Kapaun & Cheng*, 1999). If an AA is used at high concentrations, transformed cells may die since toxic compounds are released from surrounding non-transformed cells (*Colby & Meredith*, 1990). Kanamycin was however shown to promote morphogenesis in TOB and carrot (*Owens*, 1979). High <2C FC values indicate AAs cause a large amount of genetic damage or DNA degradation at higher concentrations. The increase in 2C and 4C values demonstrated by *Agrobacterium* inoculation (*Table 3*) treatments (species, genotype and explant source-independent) may be attributed to cefotaxime that has a plant growth regulator effect after metabolism by plant cells (*Mathias & Boyd*, 1986; *Holford & Newbury*, 1992). The presence of cefotaxime could not serve, however, to counteract the phytotoxic effects of the AAs. *Schmitt* et al (1997) claimed that the higher the AA concentration, the greater the incapacity of the callus to become morphogenic and regenerate into shoots or *de novo* callus. This incapacity has been attributed to an increase in methylation of DNA in response to antibiotics, especially kanamycin and hygromycin. Hypermethylation is a defense reaction in both plants and animals in response to pathogen attack (*Schmitt* et al., 1997), such as a fungus, which produces antibiotics. An exogenously-applied dose of antibiotics would mimic a pathogen attack, and induce a defense response, hypermethylation, subsequent cell death and decreased regeneration capacities.

Kanamycin at 200 μgml^{-1} inhibited TOB regeneration when on a carrageenan medium, but with other gelling agents, regeneration was inhibited at 100 μgml^{-1} (*Chauvin* et al., 1999). Kanamycin was shown to inhibit regeneration completely at 5 μgml^{-1} in apple (*Yepes & Aldwinckle*, 1994), 20 μgml^{-1} in *Vitis* (*Colby & Meredith*, 1990) and 12.5 μgml^{-1} in mango (*Mathews & Litz*, 1990). Certain species such as walnut are very resistant to kanamycin due to the presence of endogenous non-specific kanamycin phosphotransferases (*Dandekar*, 1992) while others are extremely sensitive to it, like grape (*Gray & Meredith*, 1992) and *Rubus* (*Fiola* et al., 1990). In all the cases, explant size differed and thus the studies were not directly comparable. Nonetheless, explant size was an important factor to explant survival and selection and morphogenic response to AAs in LIN, SNC and TOB.

Effect of aminoglycoside antibiotics on plant morphogenesis

Phytotoxicity in chrysanthemum and TOB *in vitro* tTCL stem and leaf cultures occurred with all AAs, even when on an MSs medium with as low a level as 5 μgml^{-1} (*Table 2*; *Figure 2*). Higher levels (10–25 and 25–50 μgml^{-1} for chrysanthemum (*Figure 2A,B*) and TOB (*Figure 2C*), respectively) not only caused phytotoxicity, but where morphogenesis could occur, it was abnormal (hyperhydric, bleached, stunted or with altered morphology) suggesting that not only does the AA interfere with the photosynthetic photosystem I (*Horvath* et al., 2000), seen by interference in plasmid (chloroplast) functioning and subsequent tissue bleaching (*Figure 1L*), with ATP production in the mitochondria (*Joët* et al., 2001), and with DNA structure and integrity (low 2C values, and increased polysomaty for tobacco and <2C values for both TOB and chrysanthemum; *Figure 3*) and loss of cell morphogenic capacity (incapacity of callus forming on high AA concentration MSs to become morphogenic and regenerate; *Figure 1C,G,I*). Low levels (5–10 μgml^{-1}) of some AAs (kanamycin > G-418 > paramomycin) stimulated somatic embryogenesis in TOB when tTCLs were inoculated with *Agrobacterium* (data not shown).

The phytotoxicity of antibiotics has been shown in various plants, and studies to determine the effect of antibiotics on sugar beet growth showed that all antibiotics were toxic to regeneration but not to callus formation (*Okkels & Pederson*, 1988). Sugarbeet callus was inhibited by 50 μgml^{-1} geneticin, 150 μgml^{-1} gentamycin, 10 μgml^{-1} hygromycin and 150 μgml^{-1} kanamycin (*Catlin*, 1990). The

same could be observed for LIN and SNC at or above 10 μgml^{-1} when G418, gentamycin, kanamycin or paramomycin were used, and at 25 μgml^{-1} when hygromycin B or neomycin were used (*Table 2*; *Figure 2A,B*); in TOB, this translated into 25 μgml^{-1} for G418, neomycin and paramomycin, but 50 μgml^{-1} for gentamycin, hygromycin and kanamycin (*Table 2*; *Figure 2C*). At high concentrations, they were all phytotoxic, while at low concentrations hygromicin B stimulated caulogenesis. It has been shown that calcium reduces the effect of AAs in sugar beet, as it has the ability to interfere with the uptake of extracellular solutes and organic ions such as AAs (*Joersbo & Okkels*, 1996). Separate experiments showed that the addition of calcium at various concentrations has a further deleterious effect on chrysanthemum morphogenesis, thus not eliminating the negative impact of AAs (data not shown). In leguminous species, however, AA selection is inefficient (*Schroeder* et al., 1993). Paromomycin was shown to be effective in the selection of transgenic oats where neither G418 nor kanamycin had any effect (*Torbert* et al., 1995). Kanamycin, hygromycin and geneticin were all highly phytotoxic to onion cultures (*Eady & Lister*, 1998). These various studies, including our own, indicate that the growth-inhibiting nature of AAs are broad, affecting both monocots and dicots.

The loss of morphogenic capacity exhibited by LIN, SNC and TOB callus cultures can be slightly counter-acted by modifying the medium and pre-culture period. In both, the effect is the same as delaying selection, giving a greater chance for transformed cells to survive selection in a transformation experiment. Decreasing the co-culture period, if an *Agrobacterium* treatment, decreases the number of cells being infected and transformed, resulting in a lower transformation efficiency, higher escape and chimera formation, and ineffective selection at higher AA concentration levels, in the case of chrysanthemum. The use of filter paper (FP) has a similar AA effect-quenching effect and explants with or without a pre-culture period are able to regenerate callus and sometimes shoots when on FP medium than when on FP-less medium (data not shown). Regeneration and morphogenesis in plants can be induced in a well-defined reversible/irreversible manner by using the TCL system, a simplified system (*Tran Thanh Van*, 1973) that has been effectively utilized to induce a range of organs *in vitro* in ornamental and medicinal species.

Greenhouse acclimatization and flowering

Despite the loss of shoot regeneration capacity, and the negative impact of AA on morphogenic development, healthy plants could be regenerated from low or high AA concentration treatments, and could be acclimatized at a 100% success rate, with normal flowering being observed (*Table 4*). This has particular relevance since the final product of the growth and development process of *in vitro* chrysanthemum in control and transgenic plants is ultimately the flower.

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