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Effect of Aminoglycoside Antibiotics on in-Vitro Morphogenesis from Cultured Cells of Chrysanthemum and Tobacco

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Successful genetic transformation of plants requires non-chimeric selection of transformed tissues and their subsequent regeneration. With rare exceptions, most transformation protocols still rely heavily on antibiotics for selecting transgenic cells that contain an antibiotic-degrading selectable marker gene. Here, the morphogenic capacity of in-vitro explants of chrysanthemum and tobacco stems and leaves (control and transgenic) changed with the addition of aminoglycoside antibiotics (AAs). In a test of 6 AAs, phytotoxicity occurred at concentrations of 10 to 25 and 50 to 100 μg mL^{-1} in chrysanthemum and tobacco explants, respectively. Light conditions as well as explant source and size also had significant effects. 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 revealed no endoduplication in chrysanthemum, even at high AA levels. However, this phenomenon was observed in tobacco calli (8C or more), even at low AA concentrations (i.e., 5 to 10 μg mL^{-1}).

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

In most plant genetic transformation studies, aminoglycoside antibiotics (AAs) are used as agents for selecting transgenic cells that contain an antibiotic resistance gene (*nptII*). This gene encodes an aminoglycoside neomycin-3'-O-phosphotransferase that inactivates AAs via phosphorylation (Coppoc, 1996). One of the problems hindering the success of such transformation protocols is balancing the antibiotic concentration to achieve stringent selection while allowing regeneration to occur. The *nptII* gene confers resistance to the AAs neomycin, G418 (Bevan, 1984), kanamycin (Herrera-Estrella et al., 1992), and paramomycin (Guerche et al., 1987). For control plants under normal conditions, these antibiotics would otherwise prove toxic. Several AAs have been used at varying concentrations in other chrysanthemum genetic transformation studies, but no research has specifically addressed their impact on growth and regeneration.

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 (Nhut et al., 2003). TCL explants are small-sized explants that are excised either a) longitudinally (lTCL), being thus composed of a few tissue types, or b) transversally (tTCL), thereby containing several tissue types, but which are normally too small to separate (Nhut et al., 2003).

This study explored the 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 (a model plant for the study of many physiological and genetic mechanisms). We examined the effect of the AA concentration on plant morphogenesis and explant survival, based on explant size and source, the timing of infection by *Agrobacterium tumefaciens*, and selection pressure. Our genetic transformation experiments were deemed successful only if they maximized shoot regeneration, eliminated shoot escape, and avoided shoot chimera formation.

MATERIALS AND METHODS

Effect of AAs on Morphogenesis and Threshold Survival Levels of Untransformed Explants

The experimental in-vitro plant materials included two cultivars of chrysanthemum (*Dendranthema* X *grandiflorum* (Ramat.) Kitamura), 'Lineker' (LIN) and 'Shuhou-no-chikara' (SNC) as well as one of tobacco

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Abbreviations: AA, aminoglycoside antibiotic; BA, 6-benzyladenine; LIN, 'Lineker'; NAA, α-naphthalene acetic acid; SNC, 'Shuhou-no-chikara'; TCL, thin cell layer; TSL, threshold survival level.

(*Nicotiana tabacum* cv. 'Samsun NN'). Stem internodal tissue was obtained by cutting the plantlets transversally into 3- to 5- mm-long concentric slices, 1 to 2 mm thick. These slices were then cut in half, yielding semi-cylindrical explants. Explants (n = 60) were placed on 25 mL of an optimized shoot regeneration medium (MSs: MS + 2 mg L^{-1} 6-benzyladenine (BA) + 0.5 mg L^{-1} α-naphthalene acetic acid (NAA) + 40 g L^{-1} sucrose; Teixeira da Silva, 2003) in Petri-dishes (9.5 cm wide; Sekisui, Japan) containing 0, 5, 10, 25, 50, or 100 mg L^{-1} of a single filter-sterilized AA: geneticin® (=G418), gentamycin, hygromycin B, kanamycin A, neomycin, or paramomycin (Table 1). Treatments were placed either in the light [16 h photoperiod, 40 µmol m^{-2} s^{-1} from plant growth fluorescent lamps (PGF; Homo-Lux, National Electric Co., Tokyo, Japan)], or in the dark, at 25°C. The threshold survival level (TSL) was determined as the level of an AA at which the tTCL (Fig. 1A) did not undergo morphogenesis. Shoots derived from any medium were then harvested and placed in 1-L polycarbonate plant boxes (CB-3; Iuchi, Japan) on 100 mL of a Hyponex® medium (soluble liquid fertilizer, HYPONeX, Co. Ltd., Japan; N:P_2O_5:K_2O = 6.5:6:19; 3 g L^{-1}) containing 20 g L^{-1} sucrose. These were subcultured three times and maintained under a 16-h photoperiod (40 µmol m^{-2} s^{-1}) at 25°C. The chrysanthemum plantlets were acclimatized and maintained in the greenhouse under long-day conditions, then treated to short-days (i.e., 13 to 14 h darkness with a 4-h light break from 10 p.m. to 2 a.m.) to induce flowering. These greenhouse-acclimatized plantlets were checked for normality

Table 1. Shoot and callus fresh weights of chrysanthemum (LIN, SNC) and tobacco stem tTCLs in response to AAs after 60 d of culture

n=60			Light			Dark		
Antibiotic	T#	Conc.*	'Lineker'	SNC	Tobacco	'Lineker'	SNC	Tobacco
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^{-1}; § Initial fresh weight of stem explant; T# = Treatment number; † = TSL; All values in grams (minus initial fresh weight §), mean ± SD; Different letters within a column indicate significant differences at $P < 0.05$ using Duncan's multiple range test.

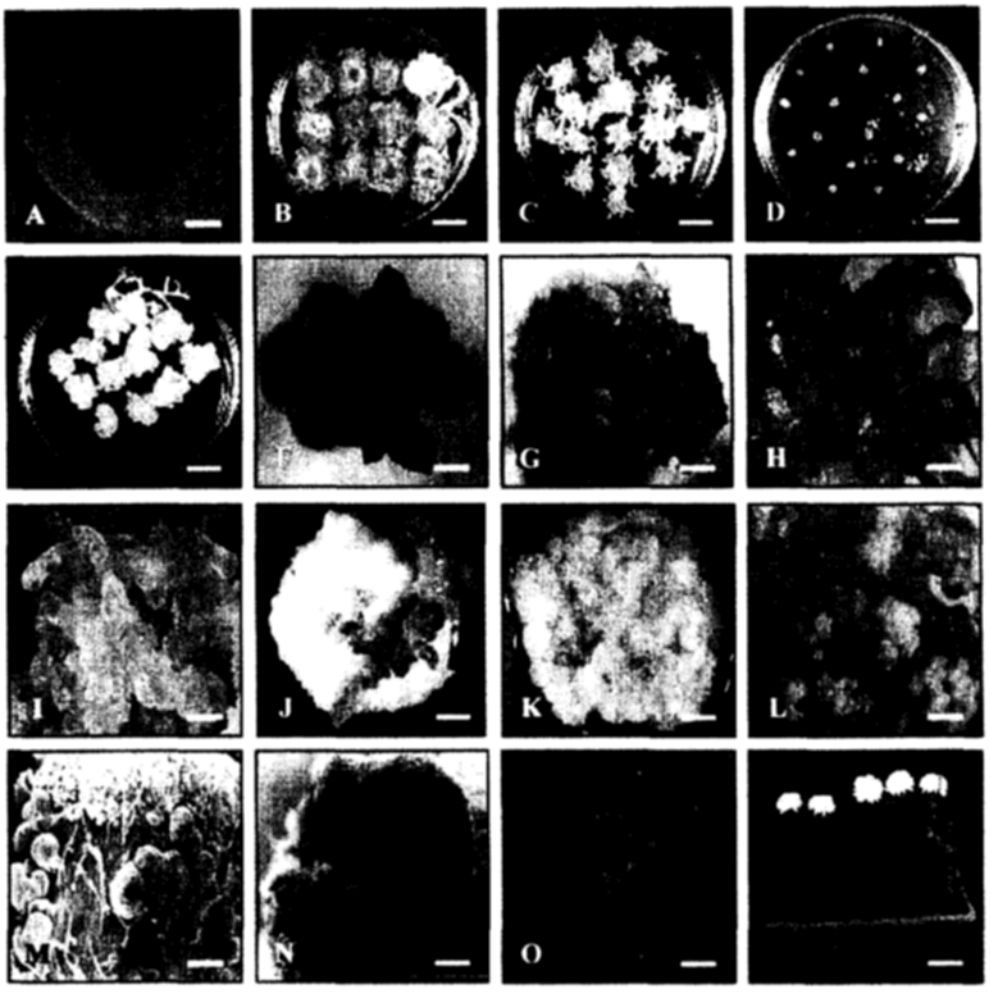

Figure 1. Morphogenic reaction of chrysanthemum and tobacco to aminoglycoside antibiotics (AAs). (A) tobacco stem tTCL; (B typical reaction by tobacco to kanamycin (but similar response observed in other AAs) at 0 µg mL^{-1}, control, (C) 10 µg mL^{-1} and (D) 100 µg mL^{-1} in the light, and (E) 100 µg mL^{-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 µg mL^{-1} G418 with red callus (low 2C value); (H) deformed SNC shoots (hyperhydric) on 5 µg mL^{-1} hygromycin; (I) highly elongated tobacco cells (meristematic and regenerable) on 10 µg mL^{-1} gentamycin in the dark; (J) profuse white callus on tobacco stem tTCLs with normal shoot development at 10 µg mL^{-1} kanamycin in the light; (K) *Agro*infected (pKT2) SNC leaf disks form meristematic callus that, upon high selection pressure (30 µg mL^{-1} kanamycin) form white (non-transformed) or green (transformed) callus (L). (M) SEM of SNC stem explant on 5 mg mL^{-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).

in their morphology (stem length, number of leaves, weight) and flowering properties (number of ray and disk florets, petal color, size of flower head).

Effects of Explant Size and Source on the Strength of the AA Impact on Morphogenesis

We used chrysanthemum and tobacco internode tissue derived from the stems of 6- to 8-cm long in-vitro plantlets (approximately 30, 45, or 60 d old for tobacco, LIN, and SNC, respectively). From these we prepared tTCLs (all ~200 to ~500 µm thick and 1.0 to 1.5 mm, 1.0 to 2.0 mm, or 2.0 to 5.0 mm in diameter for LIN, SNC, and tobacco, respectively) that contained all cell types; lTCLs (~100 to ~300 µm thick and 1.0 to 1.5 mm, 1.0 to 2.0 mm or 2.0 to 5.0 mm in diameter for LIN, SNC, and tobacco, respectively) containing one or a few epidermal and subepidermal cell layers; as well as explants. In addition, leaf tTCLs (~0.5 to 1.0 mm thick and 0.5 to 1.0 mm long) and explants (0.5

mm^2, 2.0 to 3.0 mm thick) were prepared from in-vitro leaves of LIN, SNC, and tobacco. Both types contained mid-vein tissue.

All explants were placed on MSs containing 10 μg mL^{-1} of any Trial 1 AA. These were then treated with or without infection by *A. tumefaciens* (LBA4404 harboring plasmid pKT2 (nos-L-*nptII* (wild); 35S-LEI-*uidA*; L = Leader; E = enhancer; I = Intron; Kirin Breweries, Inc., Japan). This strain was first cultured in 20 mL of Luria Broth medium for 16 to 20 h at 27°C, then 1 mL of the broth culture was centrifuged and re-suspended in 1 mL 10 mM glucose that was supplemented with 100 mM acetosyringone and adjusted to an OD_{540} = 0.4 to 0.5. The bacterial suspension was applied at one drop per explant (~10 μL) and co-cultivated for 3 to 4 d. Afterward, the inoculated explants were selected on media containing 250 μg mL^{-1} cefotaxime (Claforan®) for 1 week, then on media containing 125 μg mL^{-1} cefotaxime, where they were sub-cultured bi-monthly to eliminate the *A. tumefaciens*.

The plantlets derived from these treatments also were cultured and acclimatized in a greenhouse as described above.

Growth and Rooting of *Agro*infected Transgenic Plants on Different AA Selective Media

Twenty LIN, SNC, and tobacco tTCLs were placed on selective media in the light at AA TSLs (determined in Trial 1; see Table 2). Five LIN, SNC and tobacco transgenic plants were obtained via the methods of Teixeira da Silva and Fukai (2002a, 2002b), then placed on the same AA TSL levels of Hyponex® to determine their rooting scores. Transgenic lines were derived from *Agro*infection with pBI121 (in LBA4404), pKT2 (in AGL0), or pKT3 (in AGL0). These plasmids had been described previously by Teixeira da Silva and Fukai (2002a).

Morphological Scoring

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

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), then dehydrated in an ethanol series of 50 to 100% EtOH for at least 6 h each (Fukuda et al., 1997). They were then critical point-dried, sputter-coated with platinum, and viewed under a Hitachi-2150 SEM microscope (Tokyo, Japan).

Flow Cytometry

Nuclei were isolated from about 0.5 cm^2 of the material (shoot and callus) derived from 60 d-old (from the

Table 2. Ploidy ratios in tTCLs from different sources with or without *Agrobacterium* after 60 d of culture.

Antibiotic	ES	+ *Agrobacterium*		- *Agrobacterium*	
		SNC	Tobacco	SNC	Tobacco
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^{-1} of the respective antibiotic, except for the control § at 0 μg mL^{-1}; ES = Explant source; SNC = 'Shuhou-no-chikara'; Flow Cytometry relative C ratios as 2C:4C:8C.

initial point) material at control (0 μg mL^{-1}) and threshold AA levels (10 to 25 or 50 to 100 μg mL^{-1} for chrysanthemum and tobacco, respectively) by chopping in a few drops of Partec Buffer A [2 μg mL^{-1} 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, and 0.1% Triton-X (pH 7.5); Mishiba and Mii, 2000]. Nuclear fluorescence was measured using a Partec® Ploidy Analyser (PA) after filtering the nuclear suspension through a 30-μm mesh nylon filter (CellTrics®) and adding five volumes 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%. At least 2500 nuclei were counted for any particular sample.

Statistical Analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 20 replicates per treatment. The data were subjected to analysis of variance (ANOVA) with means separation ($P \leq 0.05$) by Duncan's new multiple range test (DMRT), using SAS® vers. 6.12 (SAS Institute, Cary, N-C, USA).

RESULTS

Effect of AA on Morphogenesis and Regeneration Capacity of Non-Transformed Plant tTCLs

Without exception, the addition of AAs to the culture medium negatively affected chrysanthemum and tobacco shoot and callus fresh weights, even when *nptII*-containing *Agrobacterium* was used. An increase in concentration caused a decrease in shoot and callus fresh weight (Fig. 1, B, C, and D). In all cases, an AA level above 10 μg mL^{-1} prompted a significant ($P < 0.05$) reduction in shoot forming capacities (Table 1; Fig. 2).

TSLs were established for LIN, SNC, and tobacco, respectively (Table 1) in each AA case at: 10, 10, and 25 μg mL^{-1} for G418 (light and dark); 10, 10, and 50 μg mL^{-1} for gentamycin (light and dark); 25, 25, and 50 μg mL^{-1} for hygromycin B (light and dark); 10, 10 and 50 μg mL^{-1} in the light, and 10, 10 and 25 μg mL^{-1} in the dark for kanamycin A; 25, 50 and 25 μg mL^{-1} in the light, and 10, 25 and 50 μg mL^{-1} in the dark for neomycin; and 10, 25 and 25 μg mL^{-1} in the light, and 10, 10 and 50 μg mL^{-1} in the dark for paramomycin. The negative impact of all AAs on chrysanthemum and tobacco was genotype-independent. Shoot and callus 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 1). This may have been a result of 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. Likewise, many Aas degraded faster when exposed to light.

Shoot and root formation was also severely inhibited in chrysanthemum and tobacco by all AAs, at varying concentrations (Fig. 2, A, B, and C). This effect was genotype-dependent in chrysanthemum, with SNC being less affected than LIN in all AA treatments (Fig. 2, A and B). Explant survival decreased exponentially with a linear increase in AA concentration (Fig. 2, A, B, and C) for LIN, SNC, and tobacco; this rapid loss mirrored that observed in the declining shoot and callus fresh weights (Table 1).

Effect of AA on Morphogenesis and Regeneration Capacity of Transformed Plant tTCLs and Rooting

As with the results from Trials 1 and 2, all AAs had a genotype-independent negative effect on tTCL morphogenesis and rooting capacity. However, the ordering of this impact differed, with G418 > kanamycin A > gentamycin > paramomycin > neomycin > hygromycin B (Table 3). All control (non-transformed) tobacco or chrysanthemum plants were able to root, the tTCLs derived from their stems showed 100% survival when on non-selective (no AA) MSs. In contrast, the same control plants could not root on selective Hyponex® nor did their tTCLs undergo morphogenesis when placed on MSs containing TSL concentrations (Table 1) of an AA (Table 3). The *Agro*infected controls on non-selective MSs had slightly lower explant survival levels (up to 7% mortality) as a result of the growth-inhibiting effect of *Agrobacterium*.

The rooting percentage for all transformed plants was 100% when they were placed on non-selective Hyponex®. Likewise, their tTCLs underwent morphogenesis (maximum 7% mortality) when placed on non-selective MSs. Transformed plants placed on selective Hyponex® showed low root induction rates, and their tTCLs had decreasing morphogenesis when placed on selective MSs; in both cases, G418 > kanamycin A > gentamycin > paramomycin > neomycin > hygromycin B. In this last instance, the inhibition of transformant rooting capacity and transformant tTCL morphogenic capacity was greater from AGL0 (both pKT2 and pKT3) than from LBA4404 (pBI121).

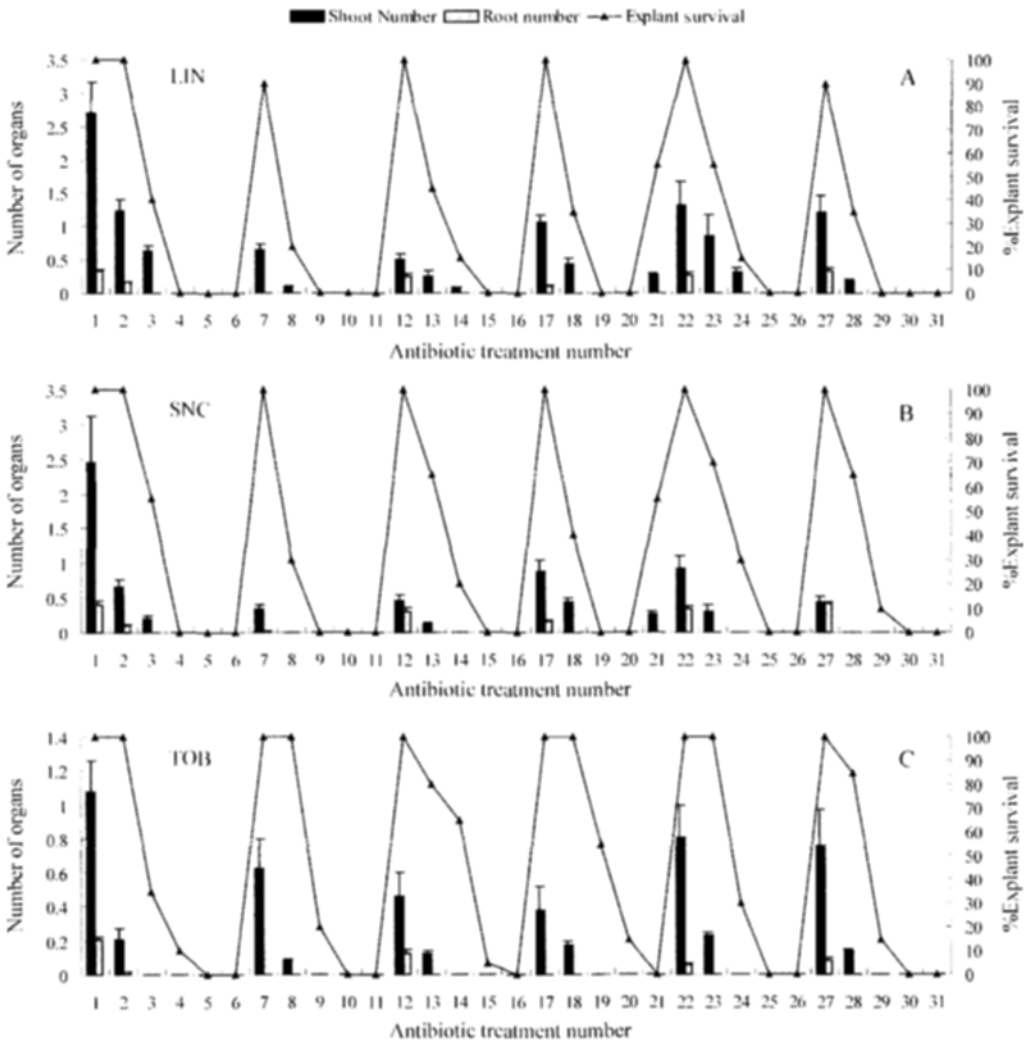

Figure 2. Graphs showing the morphogenic (i.e., root and shoot growth) reaction and tTCL explant survival in the light ir response to aminoglycoside antibiotics at various concentrations. Treatments 1 to 31 on the x-axis correspond to treatments o the same number in Table 2.

Flow Cytometry and Endoduplication

The results from our flow cytometry (Fig. 3) indicated a high level of genetic stability in the initial chrysanthemum explants and regenerated tissue (callus or shoot) from both the control and the treatments inoculated with *A. tumefaciens* (Fig. 1, F, N, and O; Fig. 3B). None of the materials (calli or shoots) diverged from the diploid state, and no endoduplication (i.e., differing ploidy levels as a result of a regular developmental pattern) was registered (as seen by the lack of $\geq$ 8C values; Table 2), even when AAs were applied at higher concentrations (25 to 50 µg mL^{-1}). At those levels, the 2C value decreased with a subsequent, slight rise in the 4C value at any AA concentration, while a large increase was recorded in the <2C values (i.e., DNA degradation products from dead cells; Fig. 3, C, D, and H). Leaf material always showed high 2C and 4C values, indicating that this young (60-d-old) tissue type had a greater amount of highly meristematic and actively dividing cells that were prone to division, even when AAs were added (Table 2). In general the presence of *Agrobacterium* resulted in an increase in the 2C and 4C values, independent of genotype and explant source. Shoots were regenerable from chrysanthemum calli derived from any AA treatment (AAs at $\leq$5 to 10 µg mL^{-1}) with high 2C and/or 4C values. In contrast, calli with low 2C and/or 4C values (AAs at $\leq$10 to 25 µg mL^{-1} and the controls), as well as those with high <2C values (Fig. 3, C, D, and H) had no regeneration poten-

Table 3. Effect of *Agrobacterium* plasmid on growth and rooting of transgenic plants after 30 d on different AA selective media.

		pBI121						pKT2						pKT3					
		Tobacco		LIN		SNC		Tobacco		LIN		SNC		Tobacco		LIN		SNC	
AA	+/–/C	ES*	Root*†	ES*	Root*†	ES*	Root*†	ES*	Root*†	ES*	Root*†	ES*	Root*†	ES*	Root*†	ES*	Root*†	ES*	Root*†
Control †	–	100	5	100	5	100	5	100	5	100	5	100	5	100	5	100	5	100	5
Control †'	–	100	5	100	5	100	5	95	5	98	5	97	5	93	5	97	5	95	5
G418	+	67	3	40	0	45	0	52	2	37	1	32	0	42	1	32	0	28	0
	–	98	5	98	5	98	5	93	5	95	5	95	5	93	5	95	5	97	5
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gentamycin	+	88	5	73	3	75	3	87	5	67	3	68	2	88	5	57	2	58	1
	–	95	5	97	5	97	5	97	5	93	5	95	5	93	5	93	5	95	5
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hygromycin B	+	92	5	93	4	91	4	87	5	88	5	91	5	88	5	87	5	90	5
	–	100	5	100	5	100	5	100	5	100	5	100	5	100	5	100	5	100	5
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kanamycin A	+	82	5	70	2	73	2	85	5	52	2	57	1	87	5	48	2	45	1
	–	97	5	98	5	97	5	95	5	98	5	97	5	93	5	97	5	95	5
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neomycin	+	93	5	97	3	98	2	91	5	90	5	93	5	90	5	88	5	92	5
	–	100	5	100	5	100	5	97	5	97	5	98	5	95	5	97	5	98	5
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paramomycin	+	90	5	93	3	95	2	88	5	90	4	88	4	90	5	88	5	92	5
	–	100	5	100	5	100	5	98	5	95	5	97	5	98	5	97	5	97	5
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* = % tTCLs surviving (i.e., not becoming necrotic), on MSs, $n=20$; *† = number of transformants forming roots on selective Hyponex®, $n=5$; † = antibiotic-free medium (MSs or Hyponex®) without *Agro*infection, †' = antibiotic-free medium (MSs or Hyponex®) with *Agro*infection; + (including the AA i.e. at TSL concentration), – (excluding the AA), C = control tTCL or non-transformant on selective medium with AA at TSL concentration. All *Agro*infection treatments also contain Cefotaxime® at 250 µg mL^{-1} to eliminate *Agrobacterium*.

tial, even when placed on a non-selective, optimized regeneration medium.

Endoduplication was evident in tobacco callus cultures derived from any treatment (Table 2; Fig. 3, E-G), except the controls (Fig. 3A). Leaf tissue exhibited no endoduplication, although some occurred in the shoot apical meristems. Trends were the same as those recorded for chrysanthemum, but without quantitative differences. Compared with the chrysanthemum, AAs, in combination with *Agrobacterium*, reduced the 2C values much more while increasing the 4C values in tobacco. Any AA treatment resulted in ≥ 8C values (8C and 16C), originating from the callus, since shoot material exhibited no ≥8C values. In addition, endoduplication was also observed at a higher order (16C), and a lower order (<2C, the DNA degradation products). Degradation of the DNA may have also contributed to such a high <2C value. In both chrysanthemum (Fig. 1, G, H, K, L, and M) and tobacco (Fig. 1, B, C, D, I, and J), AA almost always showed a decreasing gradient of negative effect on morphogenesis: gentamycin > kanamycin A > G418 > paramomycin > neomycin > hygromycin B. Tobacco callus derived from any AA treatment (at any concentration) with a high 2C and/or 4C, with or without an 8C value (Fig. 3A), could regenerate shoots. Nevertheless, calli with low 2C and/or 4C values, together with ≥8C and/or <2C values (Fig. 3E), had no shoot regeneration potential, even when placed on a non-selective, optimal regeneration medium (MSs); this despite being able to form new calli.

Greenhouse Acclimatization and Flowering

After being sub-cultured three times on Hyponex® and acclimatized in the greenhouse, the chrysanthemum plants derived from low or high AA concentration treatments showed 100% survival. Moreover,

Table 4. Characteristics (mean ± SD) of greenhouse-acclimatized plantlets derived from AA treatments.

Treatment	Stem length (cm)	# Leaves	Weight (g)	# Ray florets	# Disk Florets	Color
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†

*'Shuhou-no-chikara'; †'Lineker'.

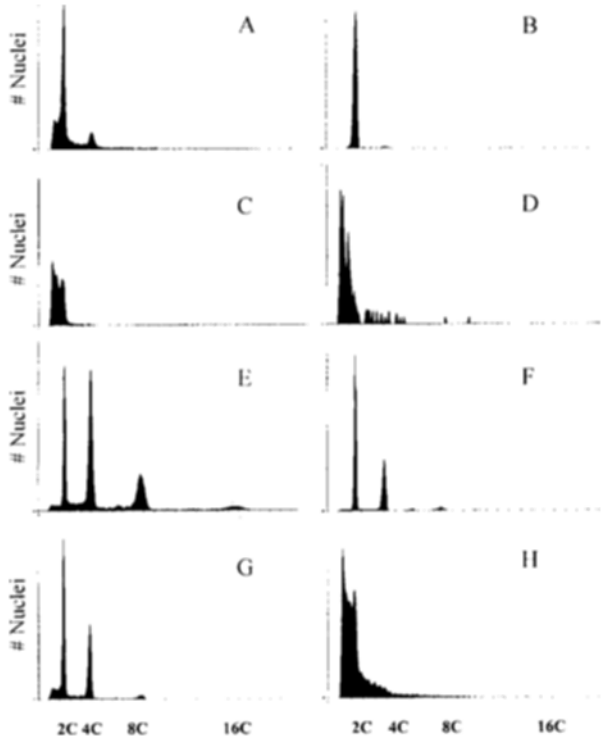

Figure 3. Histograms showing ploidy levels of different chrysanthemum (SNC) and tobacco material on control and aminoglycoside antibiotic-supplemented media showing relative 2C:4C: ≥8C -peaks. Large peaks before 2C are probably DNA degradation products derived from dead cells. (A) Control tobacco leaf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

following flower induction, plants did not differ from the controls in any of their vegetative and flowering characteristics (Table 4).

DISCUSSION

Aminoglycoside Antibiotics and Genetic Transformation Systems

For *Agrobacterium* transformation and particle bombardment of chrysanthemum and tobacco, antibiotic selection is a pre-requisite for the successful selection of transformed cells. Those cells that contain and express the *nptII* gene confer resistance to a number of members of the same AA family when on a selective medium; this gene is the most commonly used in chrysanthemum genetic transformation research. Most of those studies employ early selection, that is, transferring the explants onto a selection medium within 2 d of *Agro*infection. When kanamycin is used, a range of 10 to 100 μg mL^{-1} is claimed to result in effective initial selection. However, in many of those same experiments, the jkanamycin concentration is reduced to 10 μg mL^{-1}, or entirely eliminated, thereby resulting in chimerism. In our investigation, AAs had a negative impact on plant morphogenesis and shoot regeneration capacity (Fig. 1, C, G, H, and I; Fig. 2), even when below their TSLs (Tables 1 and 3; Fig. 3 C, D, and H). This AA effect was confirmed by the transformants, which had reduced rooting capacity on media containing TSL concentrations of various AAs, as well as by the decreased morphogenesis of tTCLs derived from them (Table 3).

When shoot regeneration is difficult, the use of AAs makes successful transformation even more challenging. Early selection (low or high AA levels), immediately following a gene-transfer method, is recommended over late selection (usually high AA levels) -- an approach that results in a lower incidence of chimerism in the former but greater regeneration of shoots in the latter (Teixeira da Silva and Fukai, 2002b).

The choice of *Agrobacterium* strain, plasmid type, and AA affect the capacity of tTCLs to differentiate, and for shoots to form roots (Table 3). Selection and transformation efficiencies, however, are impacted by the biological activities of the different AAs (Norelli and Aldwinckle, 1993). Too high a concentration of an antibiotic may kill the non-transformed cells, thereby inhibiting regeneration of transformed cells (Escandon and Hahne, 1991). In contrast, insufficient levels of an antibiotic may result in the overgrowth of non-transformed cells, thus inhibiting the regeneration and effective selection of transformed cells.

Aminoglycosides are broad-spectrum antibiotics that act primarily 1) by impairing bacterial protein synthesis through irreversible binding to prokaryotic ribosomes (especially the 16S and 30S ribosomal subunits), which results in codon misreading (Schlünzen et al., 2001), and 2) by creating fissures in the outer membrane of the bacterial cell (Gonzalez and Spencer, 1998). In plants, where most AAs are inactive or weakly active, this means that plasmid translation initiation is inhibited, resulting in the white or "bleached" appearance of organs (Fig. 1L). Aberrant proteins that may be inserted into the cell membrane can lead to altered permeability and further stimulation of AA transport (Busse

et al., 1992). This was manifested here in the highly exponential decrease in fresh callus and shoot weight (Table 1), or explant survival and shoot and root formation (Fig. 2) when our AA concentration was linearly increased. At higher levels, some AAs alter the codon: anticodon matching, and the incorrectly charged tRNA results in a defective primary amino acid (Coppoc, 1996).

AAs are generally considered to be inhibitors of prokaryotic protein synthesis at concentrations of up to 25 μg mL^{-1}, but may affect eukaryotic cells at higher concentrations, probably through non-specific binding to eukaryotic ribosomes and/or nucleic acids (Takano et al., 1996). This has been observed in wheat 80S ribosomes that fail to elongate when cell cultures are exposed to mM amounts of G-418 (Bar-Nun et al., 1983). Likewise, for tobacco and larger explant sizes in chrysanthemum, actively dividing cells with morphogenic capacity can regenerate on media at higher AA concentrations (25 to 50 μg mL^{-1}). Molecules that have a hydroxyl function at C-6' in place of an amino function, such as in kanamycin, gentamycin, G418/ geneticin®, are effective inhibitors of eukaryotic protein synthesis (Eustice and Wilhelm, 1984), where their mode of action is to bind to the eukaryotic 80S ribosomal complex (Bar-Nun et al., 1983). In our study, this mode of action was evident in both chrysanthemum and tobacco, where TSLs were higher with neomycin and paramomycin (Table 1). Whereas kanamycin is inactivated by phosphorylation, gentamycin is resistant to these phosphorylases and proved to be the AA with the strongest negative impact on morphogenesis (Table 1; Fig. 2; Fig. 3E). The TSLs established for gentamycin, kanamycin, and streptomycin are 10, 30 to 35, and 20 to 25 μg mL^{-1}, respectively (Coppoc, 1996). Although gentamycin can reduce wheat cultures by 25% more than kanamycin at 40 μg mL^{-1} (Smart et al., 1995), at 1 to 4 μg mL^{-1}, it is the only AA effective in the control of Gram-positive and Gram-negative bacteria in *Drosera, Spathiphyllum, Syngonium,* and *Nephrolepis* shoot tip cultures (Kneifel and Leonhardt, 1992).

In contrast, studies of Siberian elm have shown kanamycin to be the most suitable AA when the *nptII* gene is used as a selective marker gene, while neomycin, geneticin and paramomycin, in that order, are inferior in their selective capacity (Kapaun and Cheng, 1999). If an AA is used at high concentrations, transformed cells may die because toxic compounds are released from the surrounding, non-transformed cells (Colby and Meredith, 1990). Kanamycin has, however, been shown to promote morphogenesis in tobacco and carrot (Owens, 1979). High <2C FC values indicate that AAs cause a large amount of genetic damage or DNA degradation at higher concentrations. The increase in 2C and 4C values demonstrated here (Table 2) by the *Agrobacterium* inoculation treatments (i.e., species, genotype, and explant source-independent) may be attributed to cefotaxime, which has a plant growth-regulator effect after its metabolism by plant cells (Mathias and Boyd 1986; Holford and Newbury, 1992). However, the presence of cefotaxime could not counteract the phytotoxic effects of the AAs.

Schmitt et al. (1997) have 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 phenomenon has been attributed to an increase in the methylation of DNA in response to antibiotics, especially kanamycin and hygromycin. Hypermethylation is a defense response by both plants and animals under attack from pathogens, such as a fungus, that produce antibiotics (Schmitt et al., 1997). Therefore, an exogenously-applied dose of antibiotics would mimic a pathogen attack, thereby inducing a defense reaction, hypermethylation, subsequent cell death, and decreased regeneration capacities.

At 200 μg mL^{-1}, kanamycin can inhibit tobacco regeneration when on a carrageenan medium; with other gelling agents, regeneration is, nevertheless, inhibited at 100 μg mL^{-1} (Chauvin et al., 1999). This particular antibiotic has been shown to inhibit regeneration completely at 5 μg mL^{-1} in apple (Yepes and Aldwinckle, 1994), at 20 μg mL^{-1} in *Vitis* (Colby and Meredith, 1990), and 12.5 μg mL^{-1} in mango (Mathews and Litz, 1990). Certain species, such as walnut, are very resistant to kanamycin due to the presence of endogenous, non-specific kanamycin phosphotransferases (Dandekar, 1992). Other species, though, are extremely sensitive to it, including grape (Gray and Meredith, 1992) and *Rubus* (Fiola et al., 1990). However, in all those studies, explant sizes have differed, thus making those investigations not directly comparable.

Effect of Aminoglycoside Antibiotics on Plant Morphogenesis

In our experiments phytotoxicity in chrysanthemum and tobacco in-vitro tTCL cultures occurred with all AAs, even when on MSs media at concentrations as low as 5 μg mL^{-1} (Table 1; Fig. 2). Higher levels also caused phytotoxicity (10 to 25 and 25 to 50 μg mL^{-1} for chrysanthemum (Fig. 2, A and B) and tobacco (Fig. 2C), respectively). Moreover, when morphogenesis did occur at those levels, it was abnormal (i.e., hyperhydric, bleached, stunted, or with altered mor-

phology). This suggests that the AA interfered not only with Photosystem I (Horvath et al., 2000), as demonstrated by the hindrance of plastid (chloroplast) functioning and subsequent tissue bleaching (Fig. 1L), but also with ATP production in the mitochondria (Joët et al., 2001) and, with DNA structure and integrity (i.e., low 2C values and increased polysomaty for tobacco, and <2C values for both tobacco and chrysanthemum; Fig. 3). In addition, cell morphogenic capacity was lost (that is, calli forming on high AA-concentration MSs were incapable of becoming morphogenic and regenerating; Fig. 1, C, G, and I). Low levels (5 to 10 $\mu g\ mL^{-1}$) of some AAs (kanamycin > G-418 > paramomycin) stimulated somatic embryogenesis in tobacco when tTCLs were inoculated with *Agrobacterium*. The AA TSL, *Agrobacterium* strain, and plasmid type in our transformation experiments were important factors in determining explant survival and selection, as well as the morphogenic response to AAs in LIN, SNC, and tobacco (Table 3).

Antibiotic phytotoxicity has been demonstrated in various plant species. For example, in studies to determine their effect on sugar beet growth showed, all antibiotics have been found to be toxic to regeneration but not to callus formation (Okkels and Pederson, 1988). In particular, sugar beet callusing is inhibited only by 50 $\mu g\ mL^{-1}$ geneticin, 150 $\mu g\ mL^{-1}$ gentamycin, 10 $\mu g\ mL^{-1}$ hygromycin, or 150 $\mu g\ mL^{-1}$ kanamycin (Catlin, 1990). In our research, the same was observed for LIN and SNC at or above 10 $\mu g\ mL^{-1}$ when G418, gentamycin, kanamycin, or paramomycin was used, and at 25 $\mu g\ mL^{-1}$ when hygromycin B or neomycin was added (Table 1; Fig. 2, A and B). In tobacco, those levels were 25 $\mu g\ mL^{-1}$ for G418, neomycin, and paramomycin, but 50 $\mu g\ mL^{-1}$ for gentamycin, hygromycin, and kanamycin (Table 1; Fig. 2C). At high concentrations, all AAs were phytotoxic, while at low amounts, hygromycin B stimulated caulogenesis.

Calcium can reduce the effect of AAs in sugar beet by interfering with the uptake of extracellular solutes and organic ions, e.g., AAs (Joersbo and Okkels, 1996). However, the addition of Ca at various concentrations has a further deleterious effect on chrysanthemum morphogenesis, and does not eliminate the negative impact of AAs. In leguminous species, however, AA selection is inefficient (Schroeder et al., 1993). Paramomycin can be useful in the selection of transgenic oats whereas neither G418 nor kanamycin has any effect (Torbert et al., 1995). In contrast, kanamycin, hygromycin, and geneticin all are highly phytotoxic to onion cultures (Eady and Lister, 1998). Therefore, these various studies, including our own, indicate that the growth-inhibiting nature of AAs is broad, affecting both monocots and dicots.

The loss of morphogenic capacity exhibited by LIN, SNC, and tobacco callus cultures can be slightly counteracted by modifying the medium and pre-culture period (Teixeira da Silva and Fukai, 2002a). For both parameters, the effect is the same as with delayed selection, giving a greater chance for transformed cells to survive selection in a transformation experiment. In the case of chrysanthemum, however, shortening the co-culture period during *Agrobacterium* treatment decreases the number of cells being infected and transformed, resulting in lower transformation efficiency, higher escape and chimera formation, and ineffective selection at higher AA concentrations. The use of filter paper (FP) has a similar AA quenching effect. Explants, with or without a pre-culture period, are better able to regenerate calli and, sometimes, shoots when placed on an FP medium. Regeneration and morphogenesis also can be induced in a well-defined reversible/irreversible manner via a simplified technique with TCL (Nhut et al., 2003). This system can be effectively utilized to induce a range of organs in-vitro in ornamental and medicinal species.

Acclimatization and Flowering

Despite the loss of shoot regeneration capacity and the negative impact of AA on morphogenic development, we were able to regenerate healthy plants derived from either low or high AA concentration treatments. Acclimatizion was 100% successful, and normal flowering was observed (Table 4). This result has particular relevance because the ultimate goal with in-vitro chrysanthemums (either controls or transgenic plants) is to promote flowering.

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