J. Appl. Hort., 3(1):3-12, January-June, 2001

The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth

J.A. Teixeira da Silva* and S. Fukai

Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa, 761-0795, Japan,
**Author for correspondence. E-mail: jaimetex@lycos.com*

Abstract

Agrobacterium-mediated plant genetic transformation requires a two-step process for its success: selection and regeneration of transformed tissues, and the elimination of the transformation vector, *Agrobacterium*. This study uses carbenicillin (CA), cefotaxime (CF) and vancomycin (VA) singly, or in combination, to eliminate *Agrobacterium tumefaciens* LBA4404 and AGLO growing on *Agrobacterium*-favouring (LB) and plant-favouring (MS) media, at transgenic plant selection levels (10 or 25 µg/ml kanamycin for chrysanthemum and tobacco, respectively). The three antibiotics differed in their capacities to eliminate *Agrobacterium i.e.,* bacterial threshold survival levels (TSLs), depending on the strain, medium and light conditions. Plant TSLs differed from those for *Agrobacterium*, and were cultivar, species and light dependent, with CA>VA>CF in terms of phytotoxicity. Since over 90% of plant transformation experiments use *Agrobacterium* as the transformation vector, with most of these containing an aminoglycoside antibiotic degrading gene, such as *nptII* or *hptII*, the morphogenic reaction of these two economically important plant species to these antibiotics has relevance to the various sectors involved with genetic transformation.

Key words: antibiotic selection, chrysanthemum, *Dendranthema grandiflora*, *Nicotiana tabacum*, phytotoxicity, shoot regeneration capacity (SRC), threshold survival level (TSL), tobacco

Introduction

Agrobacterium-mediated plant genetic transformation studies often utilize selective agents for the elimination of the bacterium, allowing for the survival and regeneration of transformed cells or tissues on a negative (*e.g.,* antibiotics) or positive (*e.g.,* mannose) selection medium. Selective agents can be used singly or in combination, the latter option reducing the possibility that the *Agrobacterium* acquire resistance to the agent.

The successful elimination of *Agrobacterium* (following sufficiently long co-cultivation periods to transform cells) from regeneration media in transformation protocols is important for the successful recovery of transgenic cells and tissues. Different antibiotics effectively eliminate *Agrobacterium*, the most common being cefotaxime (CF), carbenicillin (CA), vancomycin (VA) and timentin (Nauerby *et al.*, 1997). The former two were shown to inhibit the regeneration of *Agrobacterium*-inoculated plant tissues, while timentin (a mixture of ticarcillin and clavulanic acid) is used as an effective means of eliminating bacteria after infection, without phytotoxic effects (Boase *et al.*, 1996). The choice of selective agent is vital for the successful selection of transformed cells from non-transformed cells. CA and CF have a broad spectrum of activity against both Gram-positive and Gram-negative organisms, and function by blocking the cell wall mucopeptide biosynthesis, by inhibiting the cross-linking of peptidoglycan by binding and inactivating of transpeptidases; CF is a cephalosporin antibiotic with a broad spectrum of activity, low eukaryote toxicity, and effective at low doses (Mathias and Boyd, 1986), making it particularly attractive as a selective agent in plant tissue cultures. VA, in contrast, has a relatively narrow spectrum of activity and is bactericidal against some Gram-positive bacteria (Pollock *et al.*, 1983). The use of timentin is presently restricted in Japan to medical research only, and has thus not been included in this study.

Numerous groups have tackled the genetic transformation of chrysanthemum for over one decade (Table 1), with success only being obtained in select and easy-to-micropropagate varieties and cultivars. Some of the reasons hampering the rapid advancement of GM chrysanthemum lies in cultivar and protocol dependence. Each cultivar has a specific reaction to tissue culture and genetic manipulation, and no one protocol appears to be successful for a wide range of cultivars. We have shown in separate studies that factors also affecting the shoot regeneration capacity (SRC) of control and transgenic chrysanthemum plantlets are: the type of gene introduction method, the source and size of explant, the bacterial strain in *Agrobacterium*-mediated transformation, the selective agent used for GM plant selection, and its concentration. A wide range of cultivars has been the target of chrysanthemum genetic transformation using various explant sources and selective agents (Table 1), albeit with limited GM plant production.

This study focuses on three of the most commonly utilized antibiotics for the elimination of *Agrobacterium* in tissue culture: CF, CA and VA. Selection and regeneration in *Agrobacterium* transformation experiments have four limiting factors: 1) the plant, 2) the *Agrobacterium*, 3) the agent for selecting transgenic plant cells, and 4) the agent for eliminating the *Agrobacterium*. Successful transformation lies in the fine-point control and

Table 1. Studies involving Agrobacterium-mediated transformation of chrysanthemum (*D. grandiflora*)

Principal cultivar(s) + others	Source	Strain	Antibiotic	Concentration‡	Reference
Indianapolis White Giant #4	Stem	LBA4404	VA	100-300	Lemieux *et al.*, 1990
Parliament	Leaf	LBA4404,A281	CA	50	van Wordragen *et al.*, 1991
Des Moul*	Leaf	LBA4404	TI	500	Ledger *et al.*, 1991
Parliament	Leaf	LBA9402	CF; VA	125; 200	van Wordragen *et al.*, 1992a
1610, Parliament + 5	Leaf	LBA4404,A281,Ach5	CF; VA	250; 400	van Wordragen *et al.*, 1992b
1610, Parliament + 5	Leaf	LBA4404	CF; VA	250; 400	de Jong *et al.*, 1993
Carillon	Leaf, stem	EHA101,Ach5,C58,Bo542	CF	500	Renou *et al.*, 1993
Super White	Stem	LBA4404,C58 + 5 + 1**	CF	500	Lowe *et al.*, 1993
White Snowdon + 3	Leaf	B6S3	CF; TI	200; 500	Pavingerová *et al.*, 1994
8382, 89100, 89124	Flower	LBA4404,AGLO	CF; VA	250→125; 400→200	de Jong *et al.*, 1994
Hekla, Iridon, Polaris	Leaf	EHA105,Ach5,A281,Chry5	CA	500	Urban *et al.*, 1994
White Snowdon	Leaf	B6S3	CF; TI	200; 500	Benetka and Pavingerová, 1995
1581 + 9	Stem	AGLO	CF; VA	250→125; 400→200	Fukai *et al.*, 1995
Parliament + 4	Leaf	A281,GV3101,C58,CBE21	CF	500→250	Dolgov *et al.*, 1997
Peach Margaret	Leaf	LBA4404	TI	500	Boase *et al.*, 1998a
Peach Margaret + 2 + 1†	Leaf	LBA4404,EHA105 + 2MOG	TI	500	Boase *et al.*, 1998b
Hekla, Iridon, Polaris	Leaf	EHA105	CA	500	Sherman *et al.*, 1998
Yamabiko	Stem	C58,MP90	CF	250	Takatsu *et al.*, 1998
Shuhou-no-chikara	Leaf	EHA101	CF	250→100	Shinoyama *et al.*, 2000
Kanseisetsu	Stem	EHA101	CA	500	Shirasawa *et al.*, 2000
Hybrid†	Stem	EHA101	CF; VA	500→250; 125→100	Tosca *et al.*, 2000
Iridon	Leaf	EHA105	CA	500	Zheng *et al.*, 2001

‡ = µg/ml; **=*A. rhizogenes*; * *D. indicum*; † = *D. zawadskii*; CA = carbenicillin; CF = cefotaxime; TI = ticarcillin; VA = vancomycin.

balance of these four. Chrysanthemum, a globally important floricultural crop, and tobacco, a physiologically and genetically important model plant are used in conjunction with the developmentally controllable qualities of thin cell layers (TCLs; Tran Thanh Van, 1973) to demystify the growth-promotive or growth-inhibiting nature of CA, CF and VA. This study envisages showing the importance that the selective agent and its corresponding concentration have on maximizing SRC, while minimizing phytotoxicity and explant mortality, with the objective of obtaining GM chrysanthemum and tobacco plantlets.

Materials and methods

Trial 1: Effect of antibiotics on *Agrobacterium* control: LBA4404 harbouring plasmid pKT2 (nos-L-*nptII* (wild); 35S-LEI-*uid*A; Kirin Breweries, Inc.) and AGL0 carrying pKT3 (35S-L-*nptII*; nos-LEI-*uid*A; Kirin Breweries, Inc.) were used. *A. tumefaciens* LBA4404 and AGLO were cultured in 20 ml LB medium for 16-20 h at 27ºC. 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, pre-optimized *Agro*infection conditions. Bacteria were plated (10 µl) onto LB plates supplemented with 25 combinations (CA:CF:VA) of filter-sterilized antibiotics (Table 2), with or without 10 or 25 µg/ml kanamycin (non-phytotoxic chrysanthemum and TOB selection levels, respectively, following *Agro*infection), and placed in light or dark conditions identical to those of plant cultures in Trial 2.

Trial 2: Effect of antibiotics on plant morphogenesis: Transverse TCLs (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) from stem internode tissue of *in vitro* 'Lineker' (LIN; spray-type) and 'Shuhou-no-chikara' (SNC; standard-type) chrysanthemum (*Dendranthema grandiflora* (Ramat.) Kitamura), as well as tobacco (*Nicotiana tabacum* Samsun SS) were placed at 25ºC (light and dark) for a 16 h photoperiod (40 $\mu molm^{-2}s^{-1}$) on optimized shoot regeneration medium (MSs: MS + 2 mg/l benzyladenine (BA) + 0.5 mg/l α-naphthalene acetic acid (NAA) + 40 g/l sucrose; Fukai *et al.*, 1987) containing 0, 25, 50, 100, 250, 500, 1000 or 2000 µg/ml of one of the following filter-sterilized antibiotics: cefotaxime (CF, Claforan®), vancomycin (VA), carbenicillin (CA), or 6 CA:CF:VA combinations at either 50, 100, 200 or 400 µg/ml (Table 3), with (10 or 25 µg/ml kanamycin for chrysanthemum and TOB, respectively) or without (control) selection. New medium was made fortnightly. Shoots derived from any medium were harvested and placed on Hyponex® (soluble fertilizer, N:P:K = 6.5:6:19; 3 g/l) medium containing 20 g/l sucrose, and plantlets subcultured three times were maintained under a 16 h photoperiod (40 $\mu molm^{-2}s^{-1}$) at 25ºC. Chrysanthemum plantlets were acclimatized and maintained in the greenhouse under LD conditions, and placed in SD conditions for flower induction.

Morphological (plant) and plaque (*Agrobacterium*) scoring: All explants were scored for the amount of shoots (*i.e.,* shoot regeneration capacity, SRC), callus, explant survival (ES) and explant fresh weight after 60 d in culture. Plant threshold survival level (TSL), defined as the physiological state of the tTCL in

which no morphogenic development occurs, was also determined. Greenhouse-acclimatized chrysanthemum plants were checked for vegetative and flowering normality: plantlet height, number of leaves, total fresh weight, and the number of disk and ray florets per flower head.

The number of colony forming units (CFUs) and bacterial TSLs were calculated after 4 d and 14 d (period of medium renewal in plant cultures) in culture (selective 10 or 50 µg/ml kanamycin LB and MS). One cm^2 of non-distinguishable (*i.e.,* solid) plaque bacterial growth was standardized at 100 CFUs/cm^2 (or 1 PFU/mm^2), with an overgrown petri dish being πr^2 x 100 CFUs = 5600 CFUs (*i.e.,* the absolute maximum), where r = 4.25. Per cent bacterial growth thus was recorded with 1% = 56 CFUs.

Histological observation of morphology: Explants from all treatments were observed under light microscopy and scanning electron microspcopy (SEM) to observe shoot formation as well as any morphological changes arising from the treatments. For SEM, samples were fixed in FAA. Samples were then dehydrated in an ethanol series (50-100% EtOH for at least 6 h each), critical point-dried, sputter-coated with Pt 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 control (0 µg/ml) and a high level (250-500 µg/ml) of each antibiotic by chopping in a few drops of Partec Buffer A (2 mg/l 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 and 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 solution A for 1 min. Three samples were measured, and relative fluorescence intensity of the nuclei was analyzed when the CV 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 each per treatment. Data in Trial 1 was analysed for significance by ANOVA with the mean separation by Duncan's multiple range test.

Results

Bactericidal effect: All three antibiotics could effectively control *Agrobacterium* LBA4404 and AGLO growth, with CA>VA>CF in bactericidal effect (Table 2). AGLO was more resistant than LBA4404 at any concentration of any antibiotic, and growth was more pronounced on LB medium as opposed to MS medium. Growth of both strains was higher under dark conditions than under light conditions. TSLs for LBA4404 were 200 µg/ml CA on MS or LB, light or dark conditions, while for AGLO it was >400 µg/ml. When CF was used, the TSL was at 400 and >400 µg/ml for LBA4404 and AGLO, respectively,

Table 2. Effect of CA, CF and VA on LBA4404 and AGLO growth on both MS and LB media (kanamycin 10 µg/ml)

CA:CF:VA†	MS*								LB*							
	LBA4404				AGLO				LBA4404				AGLO			
	Light		Dark		Light		Dark		Light		Dark		Light		Dark	
	4d	14d	4d	14d	4d	14d	4d	14d	4d	14d	4d	14d	4d	14d	4d	14d
0:0:0	60	100	80	100	80	100	90	100	75	100	85	100	90	100	100	100
50: 0:0	8	90	15	100	25	100	35	100	15	95	20	100	20	100	35	100
100: 0:0	0	2	0	4	0	4	0	8	5	100	10	100	5	100	40	15
200: 0:0	0	0	0	2t	0	0	0	6t	0	0	0	0	0	0	0	<1
400: 0:0	0	0	0	0	0	0	0	2t	0	0	0	0	0	0	0	6t
0:50:0	15	100	15	100	25	100	20	100	10	100	20	100	20	100	30	100
0:100:0	5	90	5	100	15	100	20	100	12	100	18	100	25	100	25	100
0:200:0	<1	10	<1	6t	3	15	5	20	5t	5	1	8t	<1	2t	7t	5
0:400:0	0	0	0	2t	0	0	0	<1	0	0	0	6t	0	0	0	<1
0:0:50	20	100	35	100	30	100	35	100	15	100	20	100	<1	100	20	100
0:0:100	0	100	5	100	10	100	15	100	0	100	10	100	0	100	7	100
0:0:200	0	100	0	100	0	100	<1	100	0	100	0	100	0	100	5	100
0:0:400	0	0	0	<1	0	0	0	5	0	0	0	5	0	0	0	5
50:50:0	<1	100	1	100	0	100	2	100	0	100	6	100	0	100	8	100
100:100:0	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
200:200:0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50:0:50	0	0	0	4t	0	<1	0	2	0	0	0	<1	0	1	0	4
100:0:100	0	0	0	0	0	0	0	3t	0	0	0	0	0	0	0	<1
200:0:200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0:50:50	0	7	0	6	0	2	0	7	0	20	0	30	3	25	2	45
0:100:100	1	90	2	100	3	100	8	100	4	100	6	100	10	100	15	100
0:200:200	0	0	0	1	0	3t	<1	15	0	0	0	2	0	2	1	18
50:50:50	0	0	0	<1	0	0	8t	6	0	0	0	<1	0	6t	3	12
100:100:100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200:200:200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

† =µg/ml; Growth measured as % surface area of a single petri dish (1%=56 CFUs); t (1-10) = trace amounts (# CFUs)

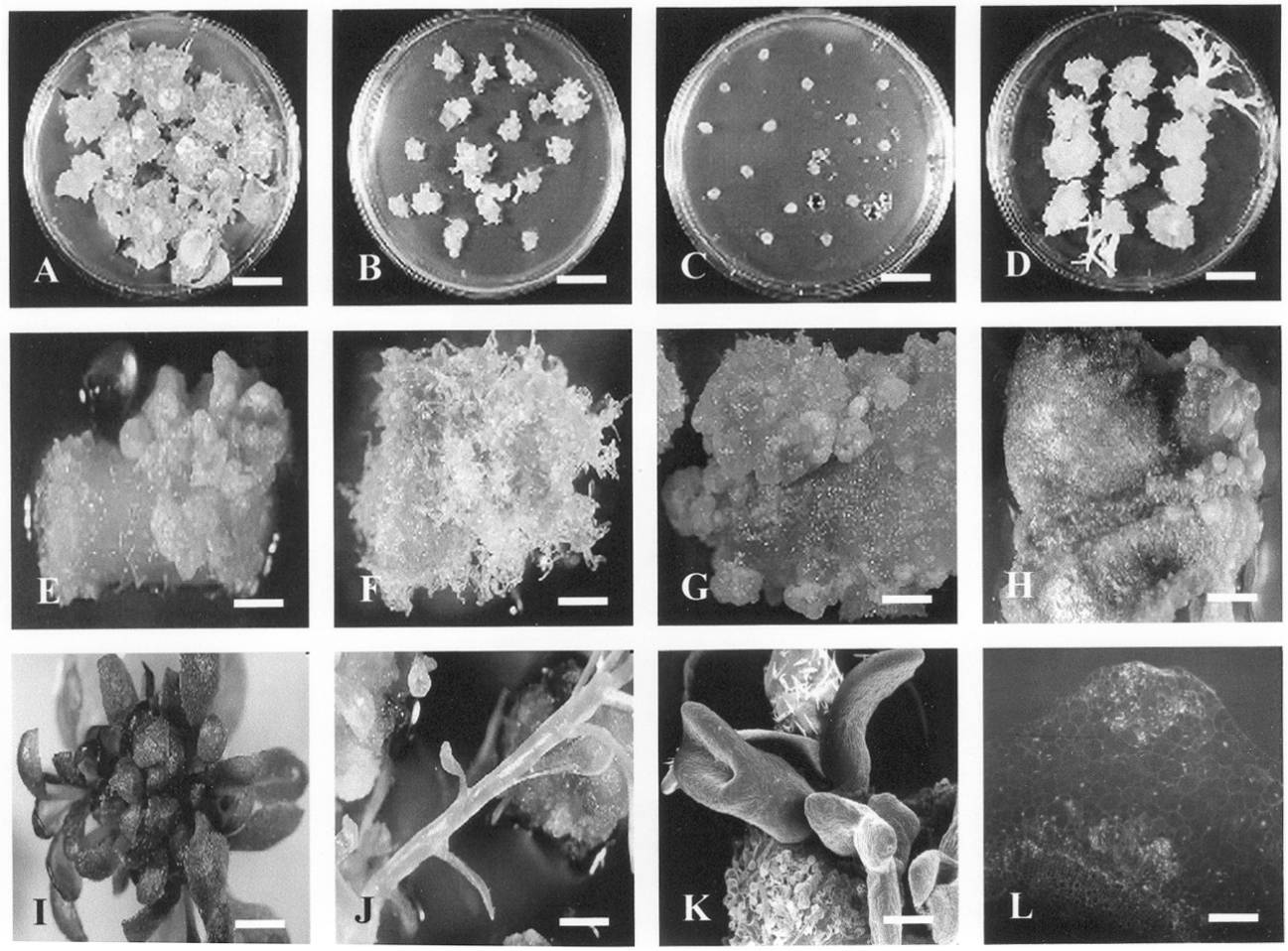

Fig. 1. Morphogenic reaction of chrysanthemum and tobacco to aminoglycoside antibiotics (AAs). Tobacco stem tTCL reaction to CA at (0 μg/ml, control; **A**), (250 μg/ml; **B**) and (1000 μg/ml; **C**) in the light and 100 μg/ml in the dark (**D**). Shoot bud primordium formation from SNC stem tTCL on non-selective medium (**E**); elongated tobacco cells on CA at 100 μg/ml in the dark (**F**); non-regenerable shoot primordia on SNC stem tTCL at 500 μg/ml CF (**G**); shoot primordia on SNC leaf explant at 100 μg/ml VA in the light (**H**); deformed SNC shoots (multiple shoots) on 50 μg/ml VA + 50 μg/ml CA (**I**); highly elongated SNC shoots on 0 μg/ml antibiotic (control) in the dark (**J**); SEM of shoot primordia formation on control SNC stem tTCL in the light (**K**); light microscope section showing showing normal shoot primordia production from the pericycle and callus production on the epidermis/sub-epidermis on SNC stem tTCL in the light at 100 μg/ml CF (**L**). Scale bars: 10 μm (**L**); 50 μm (**K**); 250 μm (**E-H**); 500 μm (**I**,**J**); 1 cm (**A-D**).

independent of the medium and light conditions. The TSL was >400 μg/ml for both strains when VA was used alone, independent of the medium and light conditions.

When CF and VA were used together, the antibactericidal effect was exponentially greater in the short-term (4 d after treatment), but growth on LB or MS media was as high as when the equivalent concentration of either antibiotic was used singly (Table 2). When CA was however used in a double combination with either CF or VA, then there was a considerable decrease in *Agrobacterium* growth over the short-term (4 d) and long-term (14 d) compared to the single antibiotic applications at the same concentration, independent of the medium, or light conditions. The use of CA, CF and VA together proved to be highly effective in the elimination of *Agrobacterium*, even when each was used at a low concentration (<100 μg/ml).

The presence of 50 μg/ml kanamycin eliminated almost 40-60% more *Agrobacterium* than when 10 μg/ml was used on MS (data not shown).

Effect on plant morphogenesis and phytotoxicity: Results of our studies reveal that the use of any of the three antibiotics, CA, CF or VA result in phytotoxicity in chrysanthemum and TOB, with an increase in the concentration of a single antibiotic, and with a parallel shift in the morphogenic capacity (SRC) of tTCLs (Fig. 1I; Fig. 2A,B,C), initially forming shoots at an antibiotic concentration of 0 μg/ml, to forming excessive callus (Fig. 1F) at higher concentrations (> 250 μg/ml), especially with CF and VA. CA>VA>CF in terms of phytotoxicity (decreasing TSL, Table 3 and ES, Fig. 2) for LIN, SNC and TOB. TSLs were established at 250, 500 and 1000 μg/ml CA for LIN, SNC and TOB TCLs, respectively in the light, but at 100, 250 and 500 μg/ml in the dark (Table 3). For CF, the TSLs were at 2000, >2000 and >2000 μg/ml for LIN, SNC and TOB in the light, but at 500, 500, 1000 μg/ml in the dark (Table 3). For VA, TSLs were at 1000, 2000 and 2000 μg/ml in the light, and 1000, 1000 and 2000 μg/ml in the dark (Table 3). In these cases MS medium was non-selective, that is 0 μg/ml kanamycin was used.

Double or triple antibiotic combinations were more phytotoxic (lower fresh shoot and callus weight) than if the antibiotics were utilized alone. Any combination containing CA was more

phytotoxic than if either CF or VA were used individually, and any double combination involving CA and VA at >100 µg/ml (data not shown) or triple combination involving >100 µg/ml was highly phytotoxic (Table 3; Fig. 2A,B,C for LIN, SNC and TOB, respectively).

The use of CA, CF or VA had a significantly (P<0.05) negative impact on the SRC of LIN and SNC with CA>VA>CF in terms of negative strength (Fig. 2A,B,C). The further addition of 10 µg/ml kanamycin to any CA, CF or VA concentration further reduced or eliminated the SRC. In TOB the same negative SRC-impacting trend could be observed for CA and VA, but the SRC was higher at 50 µg/ml CF, and significantly (P<0.05) higher at 250 µg/ml CF as compared to control tTCLs. The presence of CF could, however, not counter the negative SRC-inhibiting effect of 10 µg/ml kanamycin.

Despite a reduction of the SRC in LIN, SNC and TOB to 0, there was not complete tTCL explant death, as seen by the >0 ES values (Fig. 2A,B,C). The ES values (Fig. 2) and the TSL values (Table 1) together verify the growth-inhibiting or growth-promoting effect of CA, CF and VA when either alone, in combination, or together with the presence of kanamycin (10 or 50 µg/ml for chrysanthemum or TOB, respectively).

Flow cytometry and regeneration: FC results indicate that there exists a high level of genetic stability (high 2C : low 4C and no

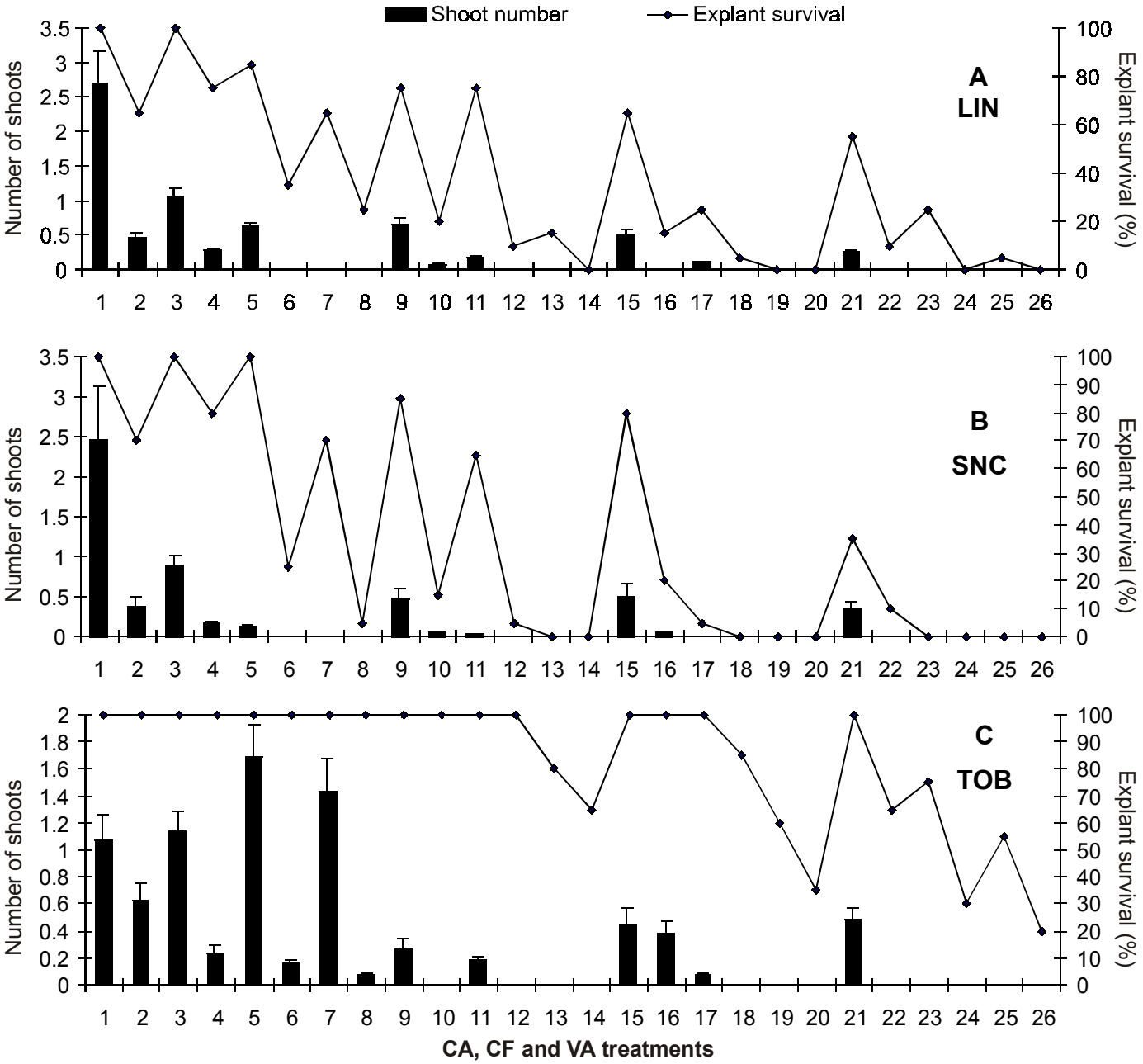

Fig. 2. Graphs showing the SRC and explant survival of LIN, SNC and TOB tTCLs in response to various CA, CF and VA treatments. Treatments (CA:CF:VA:kanamycin) in µg/ml: 1 (Control MSs); 2 (MSs + kanamycin 10 µg/ml); 3 (0:50:0:0); 4 (0:50:0:10); 5 (0:250:0:0); 6 (0:250:0:10); 7 (0:500:0:0); 8 (0:500:0:10); 9 (0:0:50:0); 10 (0:0:50:10); 11 (0:0:250:0); 12 (0:0:250:10); 13 (0:0:500:0); 14 (0:0:500:10); 15 (50:0:0:0); 16 (50:0:0:10); 17 (250:0:0:0); 18 (250:0:0:10); 19 (500:0:0:0); 20 (500:0:0:10); 21 (50:50:50:0); 22 (50:50:50:10); 23 (100:100:100:0); 24 (100:100:100:10); 25 (200:200:200:0); 26 (200:200:200:10).

divergence from the diploid state) in control chrysanthemum tTCLs and regenerated tissue (callus or shoot; Fig. 3A). No endoreduplication was registered (no >4C values), even when CA, CF or VA were applied at high (>500 µg/ml) concentrations (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 concentration, but a large increase in other C values (Fig. 3C). In general the presence of *Agro*infection resulted in an increase in the 2C and 4C values, independent of genotype. Chrysanthemum callus derived from any treatment (~50-100 µg/ml) with a high 2C

Table 3. Shoot and callus fresh weight of chrysanthemum and tobacco stem tTCLs in response to CA, CF and VA (single, double and triple combinations) after 60 days in culture

n=60		Light			Dark		
Antibiotic	**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	0	8.99±0.22 a	9.87±0.35 a	6.43±0.82 a	3.17±0.26 a	3.71±0.22 a	6.88±0.51 a
CA	25	5.83±0.17 b	8.88±0.63 b	6.03±0.58 a	3.03±0.41 a	3.62±0.28 a	5.98±0.76 b
	50	5.61±0.26 b	8.63±0.44 b	5.86±0.62 ab	0.61±0.18 b	1.13±0.28 b	5.86±0.62 b
	100	1.01±0.20 c	4.13±0.26 c	5.66±0.53 bc	0 b	0.22±0.07 c	1.01±0.24 c
	250	0 d	0.82±0.14 d	4.81±0.29 c	0 b	0 c	0.23±0.02 d
	500	0 d	0 e	0.14±0.03 d	0 b	0 c	0 d
	1000 t	0 d	0 e	0 d	0 b	0 c	0 d
CF	25	8.63±0.61 a	6.81±0.56 b	6.13±0.48 a	2.68±0.41 ab	3.21±0.28 a	6.61±0.84 ab
	50	8.01±0.83 b	7.12±0.55 b	5.99±0.26 ab	2.55±0.26 ab	2.76±0.43 b	6.92±0.67 a
	100	7.34±0.28 bc	5.24±0.48 c	5.63±0.46 b	2.62±0.31 ab	2.66±0.44 b	6.04±0.49 b
	250	6.73±0.29 c	5.53±0.28 c	1.39±0.14 c	2.22±0.34 b	0.69±0.13 c	4.43±0.62 c
	500	7.32±0.27 bc	4.49±0.38 d	0.99±0.20 cd	0 c	0 d	0.89±0.16 d
	1000	0.61±0.08 d	3.22±0.41 e	0.94±0.13 cd	0 c	0 d	0 e
	2000	0 d	1.78±0.26 f	0.62±0.14 d	0 c	0 d	0 e
VA	25	8.74±0.33 a	7.98±0.56 b	6.06±0.23 b	2.86±0.31 a	3.43±0.40 a	6.24±0.81 ab
	50	8.19±0.31 a	7.71±0.34 b	6.31±0.46 ab	2.91±0.64 a	3.52±0.51 a	6.42±0.49 a
	100	6.16±0.16 b	7.82±0.41 b	5.86±0.32 bc	1.62±0.47 b	1.83±0.34 b	5.74±0.26 b
	250	6.42±0.18 b	6.95±0.38 c	5.32±0.46 c	1.03±0.26 b	1.78±0.26 b	5.63±0.33 b
	500	4.31±0.16 c	5.33±0.61 d	4.14±0.32 d	0.31±0.11 c	0.42±0.12 c	1.22±0.17 c
	1000	0 d	1.22±0.28 e	0.67±0.14 e	0 c	0 c	0.14±0.04 d
	2000 t	0 d	0 f	0 e	0 c	0 c	0 d
CA:CF:VA	200:200:0	1.21±0.28 b	0.88±0.31 b	1.29±0.17 b	0.63±0.11 b	0.49±0.13 b	0.86±0.22 b
	200:0:200	0 b	0 b	0 b	0 b	0 b	0 b
	0:200:200	5.62±0.24 b	4.81±0.36 b	5.03±0.44 b	3.88±0.39 a	3.77±0.41 a	4.18±0.62 b
	50:50:50	2.61±0.13 b	1.23±0.34 b	2.02±0.36 b	1.31±0.22 b	1.09±0.17 b	1.78±0.28 b
	100:100:100	0 b	0 b	0 b	0 b	0 b	0 b
	200:200:200	0 b	0 b	0 b	0 b	0 b	0 b
Control§‡	0	0.03±0.00	0.03±0.00	0.05±0.01	0.03±0.00	0.03±0.00	0.05±0.01
Control‡	0	0.28±0.03 a	0.34±0.02 a	0.89±0.07 a	0.09±0.01 a	0.18±0.03 a	0.26±0.03 a
CA‡	25	0.09±0.01 ab	0.16±0.01 ab	0.41±0.06 b	0 a	0 a	0 a
	50	0 b	0 b	0.21±0.02 bc	0 a	0 a	0 a
	100 t	0 b	0 b	0 c	0 a	0 a	0 a
CF‡	25	0.36±0.04 a	0.42±0.05 a	1.01±0.12 a	0.12±0.03 a	0.22±0.07 a	0.31±0.08 a
	50	0.43±0.04 a	0.48±0.06 a	1.16±0.14 a	0.14±0.02 a	0.26±0.05 a	0.33±0.06 a
	100	0.13±0.02 ab	0.21±0.02 ab	0.34±0.05 b	0 a	0 a	0.11±0.02 a
	250 t	0 b	0 b	0 c	0 a	0 a	0 a
VA‡	25	0.14±0.04 a	0.22±0.03 ab	0.36±0.09 b	0.08±0.01 a	0.09±0.01 a	0.12±0.02 a
	50	0 b	0 b	0.11±0.02 bc	0 a	0 a	0 a
	100 t	0 b	0 b	0 c	0 a	0 a	0 a
CA:CF:VA‡	200:200:0	0.13±0.03 a	0.17±0.04 a	0.38±0.03 b	0	0.10±0.02 a	0.14±0.03 a
	200:0:200	0 b	0 b	0 b	0 a	0 a	0 a
	0:200:200	0.46±0.08 a+	0.61±0.07 a+	2.13±0.23 a+	0.22±0.04 a	0.27±0.05 a	0.88±0.14 a
	50:50:50	0.28±0.06 a	0.39±0.04 a	1.73±0.17 a+	0.13±0.03 a	0.16±0.03 a	0.24±0.04 a
	100:100:100	0 b	0 b	0 b	0 a	0 a	0 a
	200:200:200	0 b	0 b	0 b	0 a	0 a	0 a

*µg/ml; § Initial fresh weight of stem tTCL; ‡ 10 and 25 µg/ml kanamycin for chrysanthemum and TOB, respectively; All values in grams (minus initial fresh weight), mean ± SD; CA = carbenicillin, CF = cefotaxime, VA = vancomycin. Different letters within a column indicate significant differences for any one antibiotic or antibiotic combination (separately for no or selective kanamycin levels) at $P<0.01$ using Duncan's Multiple Test Range. t = threshold survival level (TSL) for LIN, SNC and TOB.

and/or 4C value had a high SRC, whereas callus with low 2C and/or 4C values (~500-1000 µg/ml), did not have an SRC, even when placed on non-selective MSs.

Endoreduplication was however evident in TOB callus and shoot cultures derived from any treatment (Fig. 3D), excepting controls (Fig. 3B). Leaf tissue exhibited no 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. Antibiotics, in combination with *Agro*infection reduced the 2C values much more than in chrysanthemum, but increased the 4C values more than in chrysanthemum. Any antibiotic treatment resulted in 8C values, stemming from the callus, since shoot material exhibited no 8C values. CA, CF or VA at or above 100 µg/ml stimulated endoreduplicated callus formation. TOB callus derived from any antibiotic treatment (at any concentration) with a high 2C and/or 4C with or without an 8C value could regenerate shoots, but callus having a low 2C and/or 4C values, together with high 8C value did not have an SRC, even when placed on non-selective regeneration medium.

Greenhouse acclimatization and flowering: Chrysanthemum plantlets derived from a number of the CA, CF and VA treatments, following subculture *in vitro* three times, resulted in 100% acclimatization with no different morphological flowering characteristics (Stem length, number of leaves, weight of cutting, number of ray and disk florets; Table 4) from control plants that were not subjected to any antibiotic treatment.

Discussion

Successful genetic transformation and SRC involve a close interaction between the level of selection of putatively transformed cells or tissues, and the level of control of the transforming vector/agent, *Agrobacterium*. Too low a control agent concentration results in excessive *Agrobacterium* growth with a resultant decrease in SRC; a moderate antibiotic concentration (usually species-dependent) reduces *Agrobacterium* growth to controllable levels and allows for non-phytotoxic growth and development with the subsequent harvesting of shoots; too high an antibiotic concentration results in both a decrease of *Agrobacterium* growth and plant regeneration, nearing their TSLs. TSLs and phytotoxicity, being closely related, can also be used interchangeably, with plant TSLs being genotype and light-dependent, while bacterial TSLs are strain and light-dependent.

Effect on bacteria: In our study, CA>VA>CF in controlling both LBA4404 and AGLO (Table 2). Elsewhere (Shackelford and Chlan, 1996) trials were conducted to determine the susceptibility of two *A. tumefaciens* strains, EHA101 and LBA4404, to ten different antibiotics, CA being the most effective against EHA101, and CF against LBA4404. Of 6 antibiotics (CA, CF, kanamycin, tetracycline, streptomycin, chloramphenicol, hygromycin B) tested, CF and CA were shown to be the most effective for eliminating *Agrobacterium* (Okkels and Pederson, 1988) but were not effective in bacterial control in *Brassica*, *Solanum* and *Rubus* cultures (Barrett *et al.*, 1997). Gentamycin at 250 µg/ml controlled up to 90% of systemic contamination in black pepper plants, and aminoglycosides were more active than penicillins (Meyer *et al.*, 1992). VA has been used for a long time to control crown gall and *Agrobacterium* spp. contamination (Boyle and Price, 1963). *Agrobacterium* in *Argyranthemum frutescens* shoot cultures was controlled in 67% or 61% of cultures when either VA or CF were used at 40 µg/ml, respectively, or in 47%, 58% or 53% of cultures when 40, 120 or 200 µg/ml of CA were used (Seyring, 1999). VA was more effective than CF, but less effective than CA in eliminating *Agrobacterium* (Table 2). VA and CF, when used alone at 1-4 or 4-16 µg/ml, respectively, were effective in the control of Gram-positive and Gram-negative bacteria in *Drosera*, *Spathiphyllum*, *Syngonium* and *Nephrolepis* shoot tip

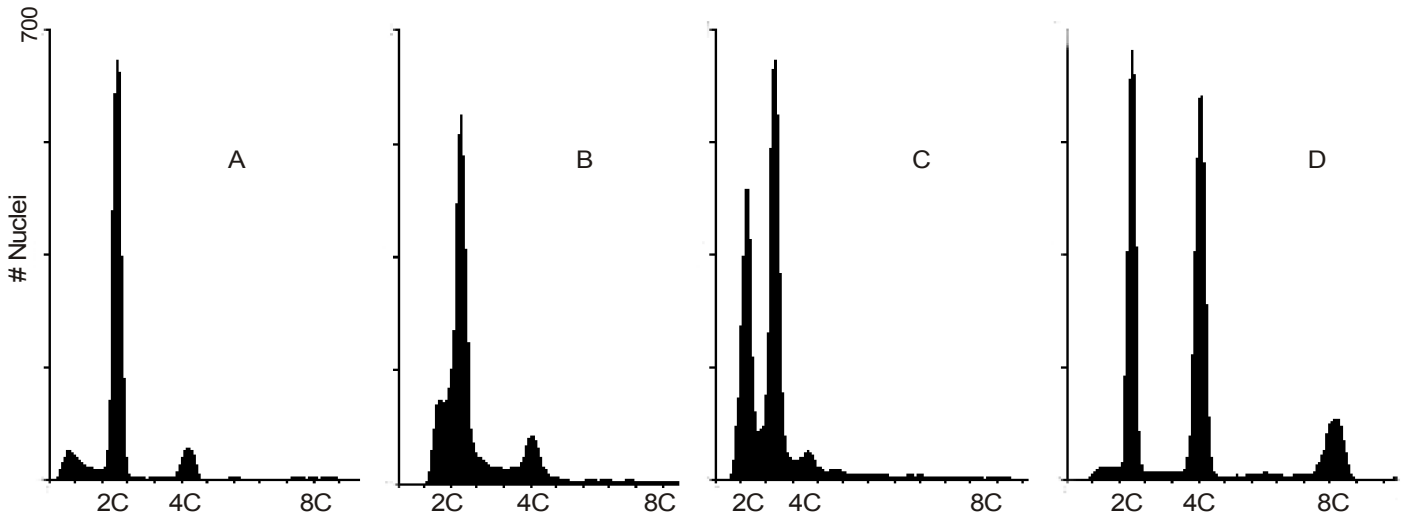

Fig. 3. Histograms showing ploidy levels of chrysanthemum (SNC) and tobacco (TOB) material on control and antibiotic-supplemented media showing relative 2C:4C:8C:16C (including S-phase cells) peaks. (**A**) Control SNC stem tTCL (light); (**B**) Control TOB leaf tTCL (light); (**C**) callus from SNC stem tTCL culture on 500 µg/ml CF (light); (**D**) callus from TOB stem tTCL culture on 1000 µg/ml CF.

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

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

cultures (Kneifel and Leonhardt, 1992). The exact degree of bactericidal effect that kanamycin (10 or 50 µg/ml for chrysanthemum and TOB tTCLs, respectively) had on cultures in this study was difficult to distinguish since kanamycin is necessary for the selection of putatively transformed cells in transgenic plant selection with either CA, CF or VA simultaneously utilized to eliminate the *Agrobacterium*. In any transformation study involving *Agrobacterium*, both bactericidal and selective agents are required, and must be used together to avoid formation of escapes and unnecessary explant loss due to infection.

The concept of using two antibiotics is more effective for bacterial elimination (Horsch and King, 1983; Leifert *et al.*, 1991), and CF has been used, in conjunction with VA, to effectively eliminate bacteria in *Pinus pinea* (Humara and Ordás, 1999), *Citrus aurantifolia* (Peña *et al.*, 1997) and several ornamentals (Leifert *et al.*, 1992). The presence of CF or rifampicin effectively eliminates many bacteria in apple at 250-1500 µg/ml or 50-200 µg/ml, respectively, although strong differences due to apple rootstock and strain were observed (Savela and Uosukainen, 1994). Triple antibiotic combinations (100 µg/ml each of CF, CA and mefoxin) were not sufficient to eliminate *Agrobacterium* in apple leaf explants (Hammerschlag *et al.*, 1997). Gram-negative bacteria in tansy culture were effectively controlled by CF, rifampicin and gentamicin (Keskitalo *et al.*, 1998). Double and triple antibiotic applications were shown to be highly effective in eliminating *Agrobacterium* at ~50 µg/ml, providing that CA was always present. The use of only CF with VA was only effective when each was used at >100 µg/ml (Table 2).

Effect on plants: CA, CF, VA and timentin have been extensively used to eliminate *A. tumefaciens* in *Dendranthema* transformation experiments (Table 1). In all papers, the effect of antibactericidal agent is not discussed, with rarely any inferences made from the presence of the selector agent for transformed cells and tissues. CF and VA are the two most commonly utilized antibactericidals, in rare cases used together, with timentin and CA used with a lower and similar frequency. Explant source and size differ from that used in this study, and levels utilized in other studies, such as 500 µg/ml CA, CF or VA are not phytotoxic (at least, not explicitly stated as such) in other studies, but are in our study. At these high concentrations (>100 µg/ml for CA, and ~250 µg/ml for CF or VA, Table 1), *Agrobacterium* is effectively eliminated, but there is a decrease in the SRC and ES (Fig. 2). The decrease in SRC is associated with a decrease in the 2C or 4C value of explant material (shoots or callus) and a simultaneous increase in DNA fragmentation (<2C in chrysanthemum; Fig. 3A vs. C) and polysomaty (~8C in TOB; Fig. 3B vs. D).

CA at 500 µg/ml was phytotoxic to both chrysanthemum and TOB, but LIN was more sensitive than SNC, with a TSL at 250 µg/ml in the light, or dark (Table 2). Even at 2000 µg/ml, CF was not phytotoxic to SNC and TOB, only to LIN, while VA was phytotoxic to all three at 2000 µg/ml. Phytotoxicity also occurred when double combinations of CA and VA were used (>200 µg/ml each), or when triple combinations at 100 µg/ml each were used. Double combinations of CA with CF, or CF with VA at < 100 µg/ml did not result in phytotoxicity (Table 3). The presence of kanamycin at 10 or 25 µg/ml, for chrysanthemum and TOB, respectively, with or without CA, CF or VA reduced the SRC and fresh weight (Table 2), and increased the phytotoxicity considerably. Phytotoxicity does not necessarily imply a complete loss of SRC, and only corresponds to low levels of ES (Fig. 2). CA>CF>VA in the negative impact on the rooting capacity and SRC of *A. frutescens* cultures when used at 40 µg/ml (Seyring, 1999). VA had a lower phytotoxic effect than CA but higher than CF (Table 3).

The higher levels of CA, CF or VA used in other chrysanthemum genetic transformation studies resulted in higher TSLs and lower phytotoxicity than if the same antibiotic were used in this study since explant size was significantly larger, and *Agrobacterium* strain was different (Table 1). The advantage of using TCLs in an experimental system is that, due to their reduced size and cell number, they are more medium-dependent. Results on their morphogenic response on a culture medium strongly reflect the effect of the medium and substances within it (such as CA, CF, VA and kanamycin), with minimal interference from endogenous substances.

The increase in SRC of *Pelargonium* (Boase *et al.*, 1996), tomato (Frary and Earle, 1996), larch (Levée *et al.*, 1997), tobacco (Nauerby *et al.*, 1997) and pine (Humara and Ordás, 1999) demonstrated by CF may be as a result of the PGR-effect after metabolism by plant cells (Mathias and Boyd, 1986; Holford and Newbury, 1992). The penicillins, CA and penicillin G stimulated callus growth and rhizogenesis, but had little impact on caulogenesis in *Antirrhinum majus* (Holford and Newbury, 1992), *Bouvardia ternifolia* (Robert *et al.*, 1989), *Daucus carota* (Biswas *et al.*, 1985; Chang and Schmidt, 1991), *Lemna minor* (Nickel and Finlay, 1954) and *Triticum aestivum* (Mathias and Boyd, 1986). CA was shown to inhibit somatic embryo development by 90% and tissue growth by 50% in Sitka spruce, and CF had no effect on tissue growth (Sarma *et al.*, 1995). In contrast, CF was shown to negatively impact root and shoot organogenesis in *Antirrhinum majus* (Holford and Newbury, 1992). CA was the least phytotoxic of streptomycin, rifampicin and CA in *Clematis*, *Delphinium*, *Hosta*, *Iris* and *Photinia* cultures (Leifert *et al.*, 1992).

At high concentrations, CF was phytotoxic to sugar beet growth, while at low concentrations it stimulated caulogenesis (Okkels and Pederson, 1988). CF was shown to enhance apple regeneration and shoot development at 250 µg/ml while CA at 500 µg/ml, alone or in combination with CF, induced abundant callus formation, inhibiting regeneration (Yepes and Aldwinckle, 1994). Callus cultures of *Hordeum* were improved by 80% when CF was used at 60-100 µg/ml (Mathias and Mukasa, 1987). CF was also shown to be phytotoxic to soybean and mungbean above 150 µg/ml (Kerven *et al.*, 1991). The stimulatory effect of CF in morphogenesis was also shown in apple (up to 200 µg/ml; Yepes and Aldwinckle, 1994), pear (Predieri *et al.*, 1989), *Pelargonium* (Barrett and Cassells, 1994), and several cereal crops (Mathias and Boyd, 1986; Eapen and George, 1990; Pius *et al.*, 1993). The higher the CA, CF or VA concentration, the greater the incapacity of tobacco callus to become morphogenic and regenerate into shoots or *de novo* callus, attributed to an increase in methylation of DNA in response to 500 µg/ml of the antibiotic (Schmitt *et al.*, 1997).

Hypermethylation is a defense reaction demonstrated by both animals and plants 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.

The negative impact on SRC and ES with an increase in CA, CF or VA concentration did not have a negative impact on the vegetative and flowering morphology of greenhouse-acclimatized chrysanthemum plants, with normal morphology and flowering being observed (Table 4). Flowers, which are the ultimate objective of growing and breeding chrysanthemum plants, were not affected by antibiotic treatments, even in plants derived from high antibiotic concentrations.

Acknowledgements

We wish to thank the Japanese Ministry of Education, Science and Culture (Monbusho) for a PhD scholarship to JATdS. Kind thanks are extended to Kirin Breweries, Inc., Japan for providing plasmid constructs.

References

Barrett, C. and A.C. Cassells, 1994. An evaluation of antibiotics for the elimination of *Xanthomonas campestris* pv. pelargonii (Brown) from *Pelargonium* x *domesticum* cv. 'Grand Slam' explants *in vitro*. *Plant Cell, Tiss. Org. Cult.*, 36:169-175.

Barrett, C., E. Cobb, R. McNicol and G. Lyon, 1997. A risk assessment study of plant genetic transformation using *Agrobacterium* and implications for analysis of transgenic plants. *Plant Cell, Tiss. Org. Cult.*, 47:135-144.

Benetka, V. and D. Pavingerová, 1995. Phenotypic differences in transgenic plants of chrysanthemum. *Plant Breed.*, 114:169-173.

Biswas, A.K., S. Mukherji, T.B. Jha and S.C. Roy, 1985. Regulation of growth and embryogenesis in cultured carrot (*Daucus carota* L.) tissue culture by penicillin. *Indian J. Exp. Biol.*, 23:154-159.

Boase, M.R., S.C. Deroles, C.S. Winefield, S.M. Butcher, N.K. Borst and R.C. Butler, 1996. Genetic transformation of regal pelargonium (*Pelargonium* x *domesticum* "Dubonet") by *Agrobacterium tumefaciens*. *Plant Sci.*, 121:47-61.

Boase, M.R., J.M. Bradley and N.K. Borst, 1998a. Genetic transformation mediated by *Agrobacterium tumefaciens* of florists' chrysanthemum (*Dendranthema x grandiflorum*) cultivar 'Peach Margaret'. *In Vitro Cell. Dev. Biol. – Plant*, 34:46-51.

Boase, M.R., R.C. Butler and N.K. Borst, 1998b. Chrysanthemum-cultivar-*Agrobacterium* interactions revealed by GUS expression time course experiments. *Sci. Hortic.*, 77:89-107.

Boyle, A. and R.M. Price, 1963. Vancomycin prevents crown gall. *Phytopathology*, 53:272-1275.

Chang, C.C. and D.R. Schmidt, 1991. Initiation and proliferation of carrot cells using a combination of antibiotics. *Planta*, 185:523-526.

de Jong, J., W. Rademaker and M.F. van Wordragen, 1993. Restoring adventitious shoot formation on chrysanthemum leaf explants following cocultivation with *Agrobacterium tumefaciens*. *Plant Cell, Tiss. Org. Cult.*, 32:263-270.

de Jong, J., M.J. Mertens, and W. Rademaker, 1994. Stable expression of the GUS reporter gene in chrysanthemum depends on binary plasmid T-DNA. *Plant Cell Rep.*, 14:59-64.

Dolgov, S.V., T.Y. Mitiouchkina, and K.G. Skryabin, 1997. Agrobacterial transformation of chrysanthemum. *Acta Hortic.*, 447:329-333.

Eapen, S. and L. George, 1990. Influence of phytohormones, carbohydrates, amino acids, growth supplements and antibiotics on somatic embryogenesis and plant differentiation in finger millet. *Plant Cell, Tiss. Org. Cult.*, 22:87-93.

Frary, A. and E.D. Earle, 1996. An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. *Plant Cell Rep.*, 16:235-240.

Fukai, S., Z. Chen and M. Oë, 1987. Cultivar differences in adventitious shoot formation from leaf segments of chrysanthemum (*Dendranthema grandiflora* (Ramat.) Kitamura). *Bull. Osaka Agric. Res. Centre*, 24:55-58.

Fukai, S., J. de Jong and W. Rademaker, 1995. Efficient genetic transformation of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) using stem segments. *Breed. Sci.*, 45:179-184.

Hammerschlag, F.A., R.H. Zimmerman, U.L. Yadava, S. Hunsucker, and P. Gercheva, 1997. Effect of antibiotics and exposure to an acidified medium on the elimination of *Agrobacterium tumefaciens* from apple leaf explants and on shoot regeneration. *J. Amer. Soc. Hortic. Sci.*, 122:758-763.

Holford, P. and H.J. Newbury, 1992. The effects of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum majus*. *Plant Cell Rep.*, 11:93-96.

Horsch, R. and K.A. King, 1983. A covert contaminant of cultured plant cells: elimination of a *Hyphomicrobium* sp. from culture of *Datura innoxia* (Mill.). *Plant Cell, Tiss. Org. Cult.*, 2:21-28.

Humara, J.M. and R.J. Ordás, 1999. The toxicity of antibiotics and herbicides on *in vitro* adventitious shoot formation on *Pinus pinea* L. cotyledons. *In Vitro Cell. Dev. Biol. – Plant*, 35:339-343.

Kerven, G.L., C.J. Asher, D.G. Edwards and B.Z. Ostatek, 1991. Sterile solution culture techniques for aluminium toxicity studies involving organic acids. *J. Plant Nutrit.*, 14:975-986.

Keskitalo, M., A. Pohto, M.L Savela, J.P.T. Valkonen, J. Simon and E. Pehu, 1998. Alterations in growth of tissue-cultured tansy (*Tanacetum vulgare* L.) treated with antibiotics. *Ann. Appl. Biol.*, 133:281-296.

Kneifel, W. and W. Leonhardt, 1992. Testing of different antibiotics against Gram-positive and Gram-negative bacteria isolated from plant tissue culture. *Plant Cell, Tiss. Org. Cult.*, 29:139-144.

Ledger, S.E., S.C. Deroles and N.K. Given, 1991. Regeneration and *Agrobacterium*-mediated transformation of chrysanthemum. *Plant Cell Rep.*, 10:195-199.

Leifert, C., H. Camotta, S. Wright, B. Waites, V. Cheyene and W.M. Waites, 1991. Elimination of *Lactobacillus plantarum*, *Corynebacterium* sp., *Staphylococcus sarprophyticus* and *Pseudomonas paucimobilis* from micropropagated *Hemerocallis*, *Choisya* and *Delphinium* cultures using antibiotics. *J. Appl. Bacteriol.*, 71:307-330.

Leifert, C., H. Camotta and W.M. Waites, 1992. Effect of combinations of antibiotics on micropropagated *Clematis*, *Delphinium*, *Hosta*, *Iris* and *Photinia*. *Plant Cell, Tiss. Org. Cult.*, 29:153-160.

Lemieux, C.S., E. Firoozabady and K.E.P. Robinson, 1990. *Agrobacterium*-mediated transformation of chrysanthemum. Integration of *in vitro* techniques in ornamental plant breeding. CPO Press, Wageningen.

Levée, V., M.A. Lelu, L. Jouanin, D. Cornu and G. Pilati, 1997. *Agrobacterium tumefaciens*-mediated transformation of hybrid larch (*Larix kaempferi* x *L. deciduas*) and transgenic plant regeneration. *Plant Cell Rep.*, 16:680-685.

Lowe, J.M., M.R. Davey, J.B. Power and K.S. Blundy, 1993. A study of some factors affecting *Agrobacterium*-transformation and plant regeneration of *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat.). *Plant Cell Tiss. Org. Cult.*, 33:171-180.

Mathias, R.J. and L.A. Boyd, 1986. Cefotaxime stimulates callus growth, embryogenesis and regeneration in hexaploid bread wheat (*Triticum aestivum* L. em Thell). *Plant Sci.*, 46:217-223.

Mathias, R.J. and C. Mukasa, 1987. The effect of cefotaxime on the growth and regeneration of callus from four varieties of barley (*Hordeum vulgare* L.). *Plant Cell Rep.*, 6:454-457.

Meyer, H.J., J. van Staden and S. Allen, 1992. The use of antibiotics to control systemic bacteria in *in vitro* cultures of *Piper nigrum* cv. Kuching. *South African J. Bot.*, 58:500-504.

Mishiba, K. and K. Mii, 2000. Polysomaty analysis in diploid and tetraploid *Portulaca grandiflora*. *Plant Sci.*, 156:213-219.

Nauerby, B., K. Billing and R. Wyndaele, 1997. Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. *Plant Sci.*, 123:169-177.

Nickel, L.G. and A.C. Finlay, 1954. Antibiotics and their effect on plant growth. *J. Agric. Food Chem.*, 2:178-182.

Okkels, F.T. and M.G. Pedersen, 1988. The toxicity to plant tissue and to *Agrobacterium tumefaciens* of some antibiotics. *Acta Hortic.*, 225:199-207.

Pavingerová, D., J. Dostál, R. Bísková and V. Benetka, 1994. Somatic embryogenesis and *Agrobacterium*-mediated transformation of chrysanthemum. *Plant Sci.*, 97:95-101.

Peña, L., M. Cervera, J. Juarez, A. Navarro, J.A. Pina and L. Navarro, 1997. Genetic transformation of lime (*Citrus aurantifolia* Swing.):factors affecting transformation and regeneration. *Plant Cell Rep.*, 16:731-737.

Pius, J., G. George, S. Eapen, and P.S. Rao, 1993. Enhanced plant regeneration in pearl millet (*Pennisetum americanum*) by ethylene inhibitors and cefotaxime. *Plant Cell, Tiss. Org. Cult.*, 32:91-96.

Pollock, H.M., J. Holt and C. Murray, 1983. Comparison of susceptibilities of anaerobic bacteria to cefemenoxime, ceftriaxone, and other antimicrobial compounds. *Antimicrob. Agents Chemother.*, 23:780-783.

Predieri, S., F. Fabbri and F. Malavasi, 1989. High frequency shoot regeneration from leaves of the apple rootstock M26 (*Malus pumila* Mill.). *Plant Cell, Tiss. Org. Cult.*, 17:133-142.

Renou, J.P., P. Brochard and R. Jalouzot, 1993. Recovery of transgenic chrysanthemum (*Dendranthema grandiflora* Tzvelev) after hygromycin resistance selection. *Plant Sci.*, 89:185-197.

Robert, M.L., M.R. Flores and V.M. Loyola-Vargas, 1989. Growth promoting activity of certain penicillins on cultured cells of *Bouvardia ternifolia*. *Phytochemistry*, 28:2659-2662.

Sarma, K.S., N.E. Evans and C. Selby, 1995. Effect of carbenicillin and cefotaxime on somatic embryogenesis of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). *J. Exp. Bot.*, 46:1779-1781.

Savela, M.L. and M. Uosukainen, 1994. Characterization of bacteria contaminating tissue cultures of apple rootstock 'YP'. *J. Appl. Bacteriol.*, 76:368-376.

Schmitt, F., E.J. Oakeley and J.P. Jost, 1997. Antibiotics induce genome-wide hypermethylation in cultured *Nicotiana tabacum* plants. *J. Biol. Chem.*, 272:1534-1540.

Seyring, M. 1999. Effect of antibiotics on elimination of *Agrobacterium tumefaciens* and the growth of shoot cultures of *Argyranthemum frutescens in vitro* and *in vivo*. *Gartenbauwissenschaft*, 64:177-182.

Shackelford, N.J. and C.A. Chlan, 1996. Identification of antibiotics that are effective in eliminating *Agrobacterium tumefaciens*. *Plant Mol. Biol. Reporter*, 14:50-57.

Sherman, J.M., J.W. Moyer and M.E. Daub, 1998. A regeneration and *Agrobacterium*-mediated transformation system for genetically diverse chrysanthemum cultivars. *J. Amer. Soc. Hortic. Sci.,* 123:189-194.

Shinoyama, H., M. Komano, Y. Nomura and T. Kazuma, 2000. Stable *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora* (Ramat.) Kitamura). *Bull. Fukui Agric. Exp. Station*, 35:13-21.

Shirasawa, N., T. Iwai, S. Nakamura and R. Honkura, 2000. Transformation and transgene expression of chrysanthemum (*Dendranthema grandiflora* (Ramat.) Kitamura). *Bull. Miyagi Pref. Agric. Res. Centre*, 67:15-20.

Takatsu, Y., H. Tomotsune, M. Kasumi and F. Sakuma, 1998. Differences in adventitious shoot regeneration capacity among Japanese chrysanthemum (*Dendranthema grandiflora* (Ramat.) Kitamura) cultivars and the improved protocol for *Agrobacterium*-mediated genetic transformation. *J. Jap. Soc. Hortic. Sci.*, 67:958-964.

Tosca, A., M. Delledonne, A. Furini, B. Belenghi, C. Fogher and P. Frangi, 2000. Transformation of Korean chrysanthemum (*Dendranthema zawadskii* x *D.* x *grandiflorum*) and insertion of the maize autonomous element *Ac* using *Agrobacterium tumefaciens*. *J. Genet. Breed.*, 54:19-24.

Tran Thanh Van, M. 1973. *In vitro* control of *de novo* flower, bud, root and callus differentiation from excised epidermal tissues. *Nature*, 246:44-45.

Urban, L.A., J.M. Sherman, J.W. Moyer and M.E. Daub, 1994. High frequency shoot regeneration and *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*). *Plant Sci.*, 98:69-79.

van Wordragen, M.F., J. de Jong, H.B.M Huitema and H.J.M. Dons, 1991. Genetic transformation of chrysanthemum using wild type *Agrobacterium* strains; strain and cultivar specificity. *Plant Cell Rep.*, 9:505-508.

van Wordragen, M.F., P.B.F. Ouwerkerk and H.J.M. Dons, 1992a. *Agrobacterium rhizogenes* mediated induction of apparently untransformed roots and callus in chrysanthemum. *Plant Cell, Tiss. Org. Cult.*, 30:149-157.

van Wordragen, M.F., J. de Jong, M.J. Schornagel and H.J.M. Dons, 1992b. Rapid screening for host-bacterium interactions in *Agrobacterium*-mediated gene transfer to chrysanthemum, by using the GUS-intron gene. *Plant Sci.*, 81:207-214.

Yepes, L.M. and H.S. Aldwinckle, 1994. Factors that affect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. *Plant Cell, Tiss. Org. Cult.*, 37:257-269.

Zheng, Z.L., Z. Yang, J.C. Jang and J.D. Metzger, 2001. Modification of plant architecture in chrysanthemum by ectopic expression of the tobacco phytochrome *B1* gene. *J. Amer. Soc. Hortic. Sci.*, 126:19-26.