Organogenesis in Carnation

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Abstract. Shoot regeneration in carnation (*Dianthus caryophyllus* L.) was influenced by genotype, explant source, and plant growth regulator balance. Plants were regenerated from petals, calyxes, nodes, internodes, and leaves, but only petals, calyxes, and nodes were regenerative from all three cultivars examined ('Scania', 'Improved White Sire', 'Sandra'). Maximum proliferation was achieved with petals on Murashige and Skoog medium supplemented with 0.05 μM TDZ and 0.5 μM NAA. Shoot initiation originated from cells near vascular regions and perhaps from epidermal cells in petals and via organogenic callus from other explants. There was no evidence of chimera separation from petals or callus, but somaclonal variants (3.3%) were observed involving petal hue and plant dwarfness. Unstable color patterns were observed in tissue-cultured regenerants of 'Scania' and 'Improved White Sire' similar in type and frequency to propagules derived from cuttings; none were observed for tissue-cultured or cutting-derived plants of 'Sandra'. Chemical names used: N-phenyl-N'-1,2,3 -thiadiazol-5-ylurea [thidiazuron (TDZ)]; 1-naphthaleneacetic acid (NAA).

Organogenesis refers to the de novo differentiation of callus or other tissues into adventitious meristems that can form stems, leaves, roots, and, ultimately, plants (Pillai and Hildebrandt, 1969). Organogenesis can be exploited for genetic transformation, for chimera separation, and as a rapid means of plant propagation if frequency of somaclonal variation is low. In carnation, organogenesis can occur from various explants, including hypocotyls (Petru and Landa, 1974), internodes (Radojevic et al., 1989), leaves (Takeda, 1978), and petals (Gimelli et al., 1982, 1983, 1984a, 1984b; Kakehi, 1979; Leshem, 1986). In this study, we have compared regeneration potential of several explants (petals, calyxes, nodes, internodes, and leaves) from three carnation cultivars;

Materials and Methods

Tissue culture protocols. Stock plants of 'Scania', 'Improved White Sire', and 'Sandra' were grown in a greenhouse maintained at 16/10C (day/night) under natural light with supplemental light provided from incandescent bulbs to interrupt winter nights. Explants were disinfested for 30 sec in 80% ethanol, and surface sterilized for 30 min in a 0.5% (v/v) solution of NaOCl containing a few drops of Tween 20. Tissues were rinsed four to five times with sterile distilled water. Floral buds (10 to 12 mm in diameter) were separated into calyx and individual petals. The petals were explanted either entire or trisected (Fig. 1), and the calyx was cut into pieces (5 x 5 mm). Nodal segments were dissected to remove axillary buds and explanted on the agar, whereas internodal segments from the top 46 cm of stock plants were cut into pieces 10 mm long and explanted horizontally on the medium. Young leaves were cut into 1-cm² pieces and explanted both ad- and abaxially on the culture surface.

Explants were first cultured in 60 x 15-mm petri plates containing 10 ml of initiation medium consisting of Murashige and Skoog (1962) salts (Sigma, St. Louis), 87.6 mM sucrose, 26.6 μM glycine, 0.3 μM thiamine-HCl, 4.1 μM nicotinic acid, 2.4 μM pyridoxine-HCl, 0.6 mM inositol; and 1.0 g casein hydro-

Fig. 1. Excised carnation petals before explanting; trisected petal (left), entire petal (right).

lysate and 6 g agar (Sigma) per liter. The medium was supplemented with 0.0, 0.5, or 5.0 μM TDZ and 0.0, 0.5, or 5.0 μM NAA, and the pH adjusted to 5.8 before autoclaving for 20 min at 120 kPa. Cultures were kept at 25C in a growth room with a 16-h photoperiod provided by cool-white fluorescent lights (160 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density) until the rooting and acclimatization stages. Viable callus from leaves, internodes, nodes, and calyxes was then subculture in 70 x 50-mm-diameter glass (baby food) jars containing regeneration medium (MS medium supplemented with 0.5 μM TDZ and 5.0 μM NAA).

Rooting and acclimatization. Regenerated shoots were rooted on half-strength MS medium without plant growth regulators. Rooted shoots were then transferred to individual cells in plastic trays containing a greenhouse potting mix [1 soil :2 peat :2 perlite (by volume) plus 60 mg each of superphosphate and magnesium sulfate/kg, adjusted to pH 6.0]. Trays were enclosed within clear polyethylene bags and transferred to a growth chamber with 12-h days (25C) and nights (16C) with light provided by cool-white fluorescent bulbs (95 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Plastic covers gradually were removed over 1 month and the trays, after being completely uncovered for several days in the growth chamber, were transferred to the greenhouse. Plants that survived acclimatization were transplanted after 5 to 10 weeks and grown to flowering, receiving daily irrigation with a fertilizer solution containing 200 mg each N and K/liter (pH 6.8).

Results and Discussion

Regeneration of shoots. Petals, explanted on day 0, showed enlargement on day 1 and continued to expand until about day

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Table 4. Effects of cultivar, explant source, NAA, and TDZ concentration on shoot formation from carnation explants.

NAA (μM)	TDZ (μM)	Shoot formation (%) ^z				
		Petals ^y	Calyxes ^x	Nodes ^w	Internodes ^v	Leaves ^u
0.0	0.0	0	0	0	0	0
	0.5	100	0	10	0	0
	5.0	85	0	0	0	0
0.5	0.0	86	0	0	0	0
	0.5	77	33	0	0	0
	5.0	93	0	0	0	0
5.0	0.0	85	0	10	0	0
	0.5	92	33	10	0	67
	5.0	77	0	0	0	0
<i>Improved White Sim</i>						
0.0	0.0	0	0	0	0	0
	0.5	73	0	10	0	0
	5.0	72	0	0	0	0
0.5	0.0	58	0	10	0	0
	0.5	76	0	0	0	0
	5.0	87	0	0	0	0
5.0	0.0	46	0	10	0	0
	0.5	70	67	10	13	0
	5.0	92	0	0	0	0
<i>Sandra</i>						
0.0	0.0	0	0	0	0	0
	0.5	23	0	10	0	0
	5.0	35	0	0	0	0
0.5	0.0	0	0	0	0	0
	0.5	30	0	0	0	0
	5.0	56	0	20	0	0
5.0	0.0	0	0	0	0	0
	0.5	23	33	0	0	53
	5.0	31	0	0	0	0

^zShoot regeneration differences between cultivars and explant types are significantly different at $P = 0.01$ based on χ^2 analysis of presence or absence of shoots.

^ySixteen days in culture, trt n = 10-19.

^vEighty days in culture, trt n = 3.

^wForty-Fo days in culture, trt n = 10.

^uEighty days in culture, trt n = 15.

^xFifty days in culture, trt n = 15.

7. On day 3, the 'Scania' and 'Sandra' petals, which were white in the bud, turned red and pink, respectively; 'Improved White Sire' petals remained white. On day 5, petal bases of all cultivars were green. Shoot formation was first visible from 'Improved White Sire' petals on day 9, from 'Scania' petals on day 12, and from 'Sandra' petals on day 35. Shoot proliferation and growth in 'Improved White Sire' and 'Scania' continued as the petals slowly senesced and turned brown. All petals appeared limp and necrotic by day 28.

Shoot regeneration from petals depended on cultivar and TDZ-

Table 2. Effects of cultivar, NAA, and TDZ concentration on the number of shoots formed per carnation petal explant after 29 days. Each treatment contained 15 explants.

NAA (μM)	Mean no. of shoots/explant $\pm$ SE		
	TDZ (μM)		
	0.0	0.5	5.0
<i>Scania</i>			
0.0	0.0 $\pm$ 0.0	9.4 $\pm$ 1.8	10.4 $\pm$ 2.2
0.5	8.1 $\pm$ 2.2	15.0 $\pm$ 2.8	13.2 $\pm$ 1.9
5.0	3.6 $\pm$ 1.5	13.4 $\pm$ 2.3	14.1 $\pm$ 2.0
<i>Improved White Sim</i>			
0.0	0.0 $\pm$ 0.0	15.0 $\pm$ 3.9	13.9 $\pm$ 3.2
0.5	7.5 $\pm$ 1.6	14.2 $\pm$ 4.0	19.7 $\pm$ 1.9
5.0	3.6 $\pm$ 1.3	18.2 $\pm$ 5.0	20.0 $\pm$ 3.5
<i>Sandra</i>			
0.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2	0.5 $\pm$ 0.2
0.5	0.0 $\pm$ 0.0	0.7 $\pm$ 0.4	1.2 $\pm$ 0.4
5.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.3	1.0 $\pm$ 0.5

Fig. 2. Shoots developing from 'Scania' petal base after 14 days in culture.

Table 3. Effects of cultivar, petal sectioning, and section position on shoot proliferation of excised carnation petals after 25 days in culture^z.

Petal treatment	Shoot proliferation	
	Explants (%)	Mean shoots/explant ^y
	<i>Scania</i>	
Entire		
Basal end	50	27.6 $\pm$ 7.5
Trisection		
Apical	0	---
Mid	0	---
Basal	75	9.9 $\pm$ 2.8
<i>Improved White Sim</i>		
Entire		
Basal end	75	15.0 $\pm$ 2.0

petals is regenerative (Alzate et al., 1990; Gimelli et al., 1982, 1983, 1984a, 1984b; Kakehi, 1979; Leshem, 1986).

Light and scanning electron microscopy (Figs. 3 and 4) indicated that the shoot primordium developed near the petal vascular region, including bundle sheath cells, or closely related mesophyll cells (Fig. 4A), and perhaps from epidermal cells on

the perimeter of the petal base (Fig. 4B). Shoots were not formed from preformed meristematic cells. The first observed event was the formation of an "amorphous layer" on the basal edge (Fig. 4A), followed by the formation of proliferating callus (Figs. 3B and 4 A, B), and finally shoot initiation (Figs. 3 C, D, and 4 C, D).

Callus formed on the cut surfaces of all explant types from days 8 to 11. Shoot regeneration depended on genotype and TDZ-NAA balance (Table 1). All cultivars developed shoots from calyces and nodes, but only 'Improved White Sire' developed shoots from internodes and only 'Scania' and 'Sandra' developed shoots from leaves.

Although organogenesis was obtained from all explants studied, only petals proved to be a constant, dependable, and prolific source of shoots for all cultivars (Table 1). Regeneration from petals tended to be optimum from medium supplemented with a combination of 0.5 μM TDZ and 0.5 μM NAA. Shoot formation appeared to arise directly from petal tissue, but SEM suggested that a transient callus phase was involved. Organogenesis was sporadic in all other explants (i.e., calyces, nodes, internodes, and leaves) and only occurred directly from callus.

Several techniques improved shoot regeneration from petals. The most shoots were obtained by culturing petals at 30C on medium containing 60 g sucrose and 1 g casein hydrolysate/liter, 0.05 μM TDZ, 0.5 μM NAA, and that was solidified with 8 to 10 g agar/liter (data not presented). However, many treatments that maximized shoot proliferation also increased vitrification. Taking both proliferation and vitrification into consideration, the optimum protocol used to maximize unvitrified shoot production from petals was as follows: Temperature,

Fig. 3. Histology of organogenesis in petals: (A) cross section of petal base at day 0; (B) cellular mass emerging from vascular bundle at day 8; (C) shoot initiation from cellular mass showing apical dome and expanding leaves at day 10; (D) apical dome with several leaf primordia at day 20. Bar = 300 μm .

Table 4. Effect of propagation method on carnation flower phenotypes.

Floral phenotype	Frequency (% of plants)			Stability (% of flowers/variant plant)					
	Tissue cultured			Petal		Callus ²		Cuttings	
	Petal	Callus ²	Cuttings	No. ^y	%	No. ^y	%	No. ^y	%
<i>Scania</i>									
	n = 81	n = 8	n = 7	n = 1444		n = 103		n = 174	
All white	0.0	0.0	0.0						
All red	0.0	12.5	0.0						
Unstable red	100	87.5	100						
Hue	3.7	0.0	0.0	64	15.6	---	---	---	---
Tinged	100	87.5	100	1444	77.4	102	79.4	174	62.1
Flecked	69.1	37.5	57.1	1153	10.3	40	7.5	83	12.1
Blotched	4.9	0.0	14.3	158	3.2	---	---	28	3.6
Sectored	17.3	12.5	14.3	287	5.9	23	8.7	22	4.5
Striped	7.4	12.5	28.6	169	3.6	25	8.0	54	3.7
Malformed	3.7	12.5	0.0	79	35.4	23	4.3	---	---
<i>Improved White Sim</i>									
	n = 52	n = 16	n = 20	n = 659		n = 301		n = 136	
All red	0.0	0.0	0.0						
All white	61.5	37.5	45.0						
Unstable white	38.5	62.5	55.0						
Hue	0.0	12.5	0.0	---	---	43	7.0	---	---
Tinged	36.5	56.3	25.0	342	14.6	164	15.9	37	27.0
Flecked	1.9	0.0	5.0	26	3.8	---	---	2	50.0
Blotched	0.0	0.0	5.0	---	---	---	---	10	20.0
Sectored	0.0	0.0	10.0	---	---	---	---	19	15.8
Striped	1.9	18.8	40.0	7	14.3	61	4.9	62	16.1
Malformed	0.0	0.0	0.0	---	---	---	---	---	---
<i>Sandra</i>									
	n = 13	n = 12	n = 5	n = 153		n = 56		n = 100	
All pink	100.0	100.0	100.0						
Unstable pink	0.0	0.0	0.0						

¹Includes plants from leaves, internodes, nodes, or calyx.²No. flowers that showed floral variation.

15 to 20°C; agar, 8 to 10 g·liter⁻¹ sucrose, 30 g·liter⁻¹; casein hydrolysate, 0 to 0.5 g·liter⁻¹ cytokinin, 0.05 µM TDZ; auxin, 0.5 µM NAA; culture vessel, baby food jars or Magenta GA7.

Somaclonal variation. A total of 182 tissue culture-derived plants that produced 2716 flowers were evaluated for plant growth habit and flower morphology (Table 4) for 1 year. One plant derived from a 'Scania' petal was variant in growth habit with a compact, bushy form and shiny, nonglaucous leaves.

Individual flowers were examined for variations in hue, color pattern, and form in plants derived from tissue culture and cuttings (controls). Color pattern variants were visually classified as tinged, a discoloration of petals, flecked, small off-color irregular spots on the petals; sectored, a clearly defined petal sector of a variant color; striped, short streaks of a variant color traversing the petals; or blotched, similar to flecking except that flowers had larger off-color irregular patches dispersed across

had one cream and 20 white flowers, whereas the nodal variant had two cream and 20 white flowers. None of the 'Scania' or 'Improved White Sire' controls were variant for pigment hue.

Stability differences in flower patterns were noted between the three cultivars: 'Sandra' was very stable, and no variants were observed in either tissue-cultured or control plants, whereas 'Scania' and 'Improved White Sire' were unstable for tissue culture-derived and control plants. There was no convincing evidence that variant phenotypes were more frequent in tissue culture-derived plants than in vegetatively propagated plants from cuttings (Table 4). Many of the floral instabilities observed in tissue-cultured 'Scania' and 'Improved White Sire' plants also were noted in parental controls at similar frequencies.

There were two classes of malformed flowers from tissue culture-derived plants: those from flowers initiated in vitro, and those from flowers initiated ex vitro. Buds that were induced in

respect to predominant flower color, i.e., tissue-cultured 'Scania' were red with some small white spots, 'Improved White Sire' were white with small red spots, and 'Sandra' were all pink. No tissue culture-derived plants were obtained in which all flowers were of a variant color. The instability of petal color in 'Scania' and 'Improved White Sire' was similar for petal-derived, callus-derived, and vegetatively propagated plants. 'White Sire' is a periclinal chimera of 'William Sire' (Johnson, 1980; Pereau-Leroy, 1974; Sagawa and Mehlquist, 1957), and although 'Improved White Sire' is derived from 'William Sire', there are no reports confirming that it also is a periclinal chimera. However, an occasional completely red flower was noted in 'Improved White Sire' stock plants derived from cuttings. Assuming that 'Improved White Sire' is a periclinal chimera with L_1 being white and L_2 and L_3 being red, the expected phenotypes from tissue culture could be either 1) all-white flowers derived from L_1 , 2) white flowers with red spots derived from a combination of L_1 and L_2 , or 3) all-red flowers derived from L_2 or L_3 . In our study, there was no evidence of complete chimeral separation in 'Improved White Sire' from either petals or callus as would have been expected from a periclinal chimera. Our observations of red flower reversion and lack of chimeral separation from organogenesis may be reconciled with one of the following assumptions: 1) 'Improved White Sire' is not a periclinal chimera and the red flowers produced by cuttings were due to a mutation. The floral patterns of 'Improved White Sire' may be a result of genetic instabilities, perhaps transposons or highly mutable genes; 2) 'Improved White Sire' is a periclinal chimera, but the L_1 and L_2 layers are genetically white and only the L_3 layer is genetically red. The lack of chimeral separation in 'Improved White Sire' may be explained if L_3 tissue is not involved in organogenesis. The red flowers observed for some 'Improved White Sire' cuttings could be due to adventitious buds derived from L_3 .

Further studies will be needed to determine if petal regeneration could be used for a propagation system or if any of these organogenic systems can be used for genetic transformation or recovery of interesting genetic variants from different histogenic layers.

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