HortScience 39(5):1083–1087. 2004.

Micropropagation of *Dianthus gratianopolitanus*

Margarita Fraga and Mertxe Alonso
Departamento de I+D, Fundación PROMIVA, Boadilla del Monte, 28660 Madrid, Spain

Philippe Ellul
Laboratorio de Células y Tejidos Vegetales, IBMCP-CSIC, 46022 Valencia, Spain

Marisé Borja[1]
Departamento de I+D, Fundación PROMIVA, Boadilla del Monte, 28660 Madrid, Spain; and Departamento de Bioquímica y Biología Molecular I, UCM, 28040 Madrid, Spain

Additional index words. Caryophyllaceae, shoot regeneration, tissue culture, virus elimination

Abstract. **Meristem culture and/or thermotherapy were used to eliminate viruses from ornamental *Dianthus gratianopolitanus* Vill. ('Spotti' and 'Frosty Fire') mother plants. Shoot tip, leaf, node, and ovary explants collected from greenhouse-maintained, virus-free plants were cultured in vitro for shoot initiation on Murashige and Skoog (MS) medium containing BAP, kinetin, or 2-iP with or without IAA or NAA. Culture of shoot tips in MS with 0.57 μM IAA and node explants in MS with 2.46 μM 2-iP is recommended for 'Spotti' cultivar. In 'Frosty Fire', optimum number of axillary shoots was obtained from shoot tip and node explants in MS without plant regulators. Leaves and ovaries were not adequate explants for *D. gratianopolitanus* micropropagation because none or only a low percentage of explants regenerated shoots. High levels of cytokinins increased the number of shoots per explant but also increased the production of aberrant phenotypes and induced hyperhydricity. Adventitious shoots rooted in vitro with auxins, but maximum rooting was 97% ex vitro without auxins. This study demonstrated that *D. gratianopolitanus* can be successfully micropropagated. Chemical names used: 6-benzyladenine (BAP); kinetin (KIN); 6-(γ,γ-dimethylallylamino)-purine (2iP); indole-acetic acid (IAA); indole-3-butyric acid (IBA); α-naphthaleneacetic acid (NAA); gibberellic acid (GA_3).**

Plants within the genus *Dianthus* are popular with all gardeners. Cultivars of *D. grationapolitanus,* with their dense sea-green or blue-green foliage, make excellent groundcovers for beds and borders (Jelitto and Schacht, 1990). They are commercially produced in both North America and Europe using shoot tip cuttings. This is a slow, season-bound process for the multiplication of new cultivars or elite material. There is a need for new releases of this species since *D. grationapolitanus* is susceptible to *Pythium*, *Alternaria,* and viral infections, including carnation latent carlavirus (CLV), carnation mottle carmovirus (CarMV), and carnation vein mottle potyvirus (CVMV) (Pallás et al., 1999; Sánchez-Navarro et al., 1999). Cucumber mosaic cucumovirus (CMV) has also been reported in *Dianthus* (Lovisolo et al., 1968).

Hyperhydricity is a common problem associated with micropropagation in *D. caryophyllus* (Leshem, 1983; Leshem and Shalev, 1988). Nevertheless, micropropagation of *D. caryophyllus* has been achieved using explants from shoot tips (Earle and Langhans, 1975; Johnson, 1980), stem segments (Roest and Bokelmann, 1981; Watad et al., 1996), leaves (Van Altvorst et al., 1992, 1994), axillary buds (Miller et al., 1991), nodes (Van Altvorst et al., 1995), ovules (Demmink et al., 1987; Sato et al., 2000), and petals (Kakehi, 1979; Nakano et al., 1994). Shoot regeneration was also induced from leaf segments of *D. chinensis* (Jethwani and Kothari, 1996) and mesophyll protoplasts of *D. superbus* (Kim and Lee, 1996). However, there are no reports describing micropropagation of *D. gratianopolitanus*. The objective of this study was to establish a rapid micropropagation system for commercial cultivars of *D. gratianopolitanus*.

Received for publication 18 June 2002. Accepted for publication 16 Apr. 2003. We thank Pino Lago, Adolfo García, Andrés Henández, Antonio Ortega, Yago Pérez, David del Pozo, José Antonio Segado, and Cristina Sesma for their technical assistance. This work was supported by a Ministerio de Ciencia y Tecnología grant (BIO-10000-13).
[1]To whom reprint requests should be addressed. Mailing address: Departamento de I+D, Fundación PROMIVA, Finca La Veguilla, Boadilla del Monte, 28660 Madrid, Spain.; e-mail imasd@promiva.com.

Materials and Methods

Plant material and virus elimination. Plants of *D. gratianopolitanus* 'Spotti' and 'Frosty Fire' were purchased from a local nursery and maintained under greenhouse conditions at 20 ± 4 °C day/16 ± 4 °C night. Plants exhibited viral symptoms (e.g., basal necrosis and leaf mottle) and 40 plants of each cultivar were analyzed for CMV, CarMV, CLV, TMV, tospoviruses (subgroups I, II, and III), and potyviruses with a direct antibody sandwich ELISA using commercially produced antibodies (AGDIA, Elkhart, Ind.).

None of the plants were positive for CMV, TMV, or the tospovirus screen. However, all plants were infected with CarMV, CLV, and potyvirus. Meristems (0.3 mm) from infected shoot tips were initiated in MS medium without growth regulators for 3 months. Elongated shoots (3 cm) were screened as before and found to be free of CarMV but infected with CLV and potyvirus. Two hundred plants from each cultivar were then cultured at 37 °C for 6 weeks. Meristems from the surviving plants were excised and some shoots were CLV-free, but all shoots were still infected with potyvirus. Therefore, CLV-free plants were cultured at 39 °C for 6 weeks. After 3 months of meristem culture from the surviving plants, a new ELISA test determined which cultivar shoots obtained were potyvirus-free. Clean shoots were rooted ex vitro, acclimatized in the greenhouse, and used as explants for the following experiments.

Explants and surface sterilization. Shoot tips (3 mm), 1.5-cm basal young leaf segments, the first seven primary single nodal segments (1 cm), and ovaries from virus-free plants grown in the greenhouse were used as explants. Ovaries were classified as closed (8–10 mm) or open (11–16 mm), according to size. Explants were surface disinfected by immersion in a 0.7% sodium hypochlorite solution with 0.5% Tween 20 (Sigma) for 15 to 20 min before three to five rinses in sterile, double-distilled water.

Tissue culture medium and conditions. Murashige-Skoog (MS, 1962) medium containing 3% sucrose was used for tissue culture. The medium pH was adjusted to 5.75 before addition of 0.7% agar-agar (Copanor, S.L. Spain) and autoclaving at 121 °C for 20 min. Explants (five/vessel) were cultured in 500-mL sterile glass vessels (V-580, Vicasa, Spain) containing 120 mL of medium. Cultures were incubated under 40–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 16-h photoperiod provided by MazdaFluor cool-white lamps at 24 ± 2 °C. All culture vessels were sealed with tape (W-101.300, Teixpac, S.A. Madrid).

Effects of plant growth regulators on shoot initiation and multiplication. The effects of three cytokinins [BAP (2.22 and 4.44 μM), kinetin (2.32 and 4.65 μM), 2iP (2.46 and 4.92 μM)], and two auxins [NAA (0.54 μM) or IAA (0.57 μM)] on shoot formation were evaluated by adding filter-sterilized plant growth regulators to autoclaved medium. Each experiment had three to five replicate cultures of five explants per treatment and was conducted three times. The number of nonhyperhydric shoots produced per explant, the number of nodes per shoot, the internode length, and the percentage of explants showing growth response were recorded after 3–5 weeks in culture for shoot tips and node explants and after 10–12 weeks in culture for flower ovary explants.

Root initiation. To study the effect of auxins on root initiation from micropropagated shoots, filter-sterilized IBA (2.46 and 4.92 μM), IAA (2.85 and 5.70 μM), or NAA (2.68 and 5.37 μM) were added to autoclaved medium with or without BAP (2.22 μM). Shoots with three nodes

(4–5 cm in length) were excised and transferred to root initiation medium. The percentage of shoots forming roots was quantified, as well as the number of roots per rooted shoot, and the root length was measured after 2 weeks.

Transfer to greenhouse conditions. Rooted or unrooted shoots were rinsed in water to remove all agar and transplanted into commercially available potting mix (Gramoflor, Holland) in B-28 plastic trays (Teku). Plantlets were acclimatized by placing them in a fog chamber set to 80% humidity with bottom heating at 21 °C for 2 weeks. Afterwards, plants were potted in M-11 (11 cm) plastic pots (I.P.S., Portugal) with the same potting mix and maintained at 20 ± 4 °C day/16 ± 4 °C night in a greenhouse.

Statistical analysis. A completely randomized design was used in all experiments. In the regeneration experiment, the percentage of explants with shoots and percentage of hyperhydric explants were analyzed using a multinomial logistic regression analysis for the main effects and for their interactions. A Pearson correlation analysis was conducted between the number of hyperhydric shoots and treatment. Data on shoots/explants, nodes/shoot, and internode length were subjected to analysis of variance (ANOVA) to determine the influence of explant type and growth regulator treatments on these variables. Mean roots/shoot and mean length/root were separated using Tukey's least significant difference (LSD) test at $P \leq 0.05$, after ANOVA. All the statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago).

Results and Discussion

While there are abundant reports on *D. caryophyllus* in vitro production using a wide variety of explants, micropropagation of *D. gratianopolitanus* has not been reported.

Virus elimination. The initial plants were infected with CarMV, CLV, and potyviruses (ELISA test). CarMV elimination was achieved in 100% of the shoots by 3 months of meristem culture. CLV elimination required a 37 °C thermotherapy treatment (86% of 'Spotti' and 65% of 'Frosty Fire' shoots survived) and an additional round of meristem culture where 43% of 'Frosty Fire' and 31% of 'Spotti' shoots were CLV-free. In order to obtain potyvirus-free shoots, further meristem culture from the plants surviving 39 °C thermotherapy (32% of 'Spotti' and 38% of 'Frosty Fire') was performed. Virus elimination was achieved in 92% and 88% of the 'Frosty Fire' and 'Spotti' cultivars, respectively. Acclimatized clean plants were used in all micropropagation experiments.

Effects of cultivar and explant type on D. gratianopolitanus shoot initiation. Shoot tips and nodes from 'Spotti' and 'Frosty Fire' cultivars regenerated shoots in all 21 media tested (Tables 1 and 2). Differential response to growth regulators was observed among the different explant types. Between 100% and 20% of shoot tip explants of both cultivars, with node explants ranging between 100% to 30% for 'Frosty Fire' and 90% to 50% for 'Spotti', produced multiple shoots. Regeneration from shoot tips and nodes was slower in 'Frosty Fire' than in 'Spotti', requiring two additional weeks in culture.

Leaf explants from either cultivar failed to regenerate in any of the 21 media. When open ovaries were used as explants, shoot regeneration was observed only for 'Spotti'

Table 1. Regeneration from shoot tip and node explants of *Dianthus gratianopolitanus* 'Spotti' after 3 weeks of culture on MS medium with different concentrations of growth regulators.

Growth regulators (μM)		Shoot tip explants			Node explants		
		Phenotype	%Explants with shoots	%Vitreous explants	Phenotype	%Explants with shoots	%Vitreous explants
NAA	BAP						
0	0	A	100	0	A	80	0
0	2.22	B	100	0	B	70	0
0	4.44	C	80	30	B	80	0
0.54	0	A	90	0	A	70	0
0.54	2.22	B	100	20	B	80	10
0.54	4.44	B	60	40	C	60	10
IAA	BAP						
0.57	0	A	80	0	A	80	0
0.57	2.22	B	100	0	C	70	10
0.57	4.44	C	90	10	C	70	20
NAA	KIN						
0	2.32	A	100	0	A	80	0
0	4.65	A	90	10	A	80	10
0.54	2.32	C	100	0	C	80	0
0.54	4.65	C	80	0	B	70	0
IAA	KIN						
0.57	2.32	C	70	10	C	70	0
0.57	4.65	B	90	20	C	80	0
NAA	2iP						
0	2.46	C	70	10	A	80	0
0	4.92	B	90	40	C	70	10
0.54	2.46	C	80	0	C	70	0
0.54	4.92	C	80	30	C	70	0
IAA	2iP						
0.57	2.46	A	90	40	A	90	0
0.57	4.92	C	60	40	C	80	0

Table 2. Regeneration from shoot tip and node explants of *Dianthus gratianopolitanus* 'Frosty Fire' after 5 weeks of culture on MS medium with different concentrations of growth regulators.

Growth regulators (μM)		Shoot tip explants			Node explants		
		Phenotype	%Explants with shoots	%Vitreous explants	Phenotype	%Explants with shoots	%Vitreous explants
NAA	BAP						
0	0	A	100	0	A	100	0
0	2.22	B	30	0	B	40	10
0	4.44	B	20	10	B	10	30
0.54	0	B	70	0	B	70	10
0.54	2.22	B	70	0	B	40	20
0.54	4.44	B	40	0	B	60	40
IAA	BAP						
0.57	0	A	90	0	A	100	0
0.57	2.22	B	40	0	B	60	0
0.57	4.44	B	60	0	B	30	10
NAA	KIN						
0	2.32	A	80	0	B	80	0
0	4.65	B	100	0	B	50	0
0.54	2.32	B	70	10	A	50	10
0.54	4.65	B	50	20	B	30	10
IAA	KIN						
0.57	2.32	B	10	20	B	70	0
0.57	4.65	B	60	20	B	50	10
NAA	2iP						
0	2.46	B	60	0	B	40	20
0	4.92	B	70	10	B	40	20
0.54	2.46	A	40	0	A	90	0
0.54	4.92	B	60	0	A	90	0
IAA	2iP						
0.57	2.46	A	60	10	B	50	0
0.57	4.92	B	80	0	B	60	0

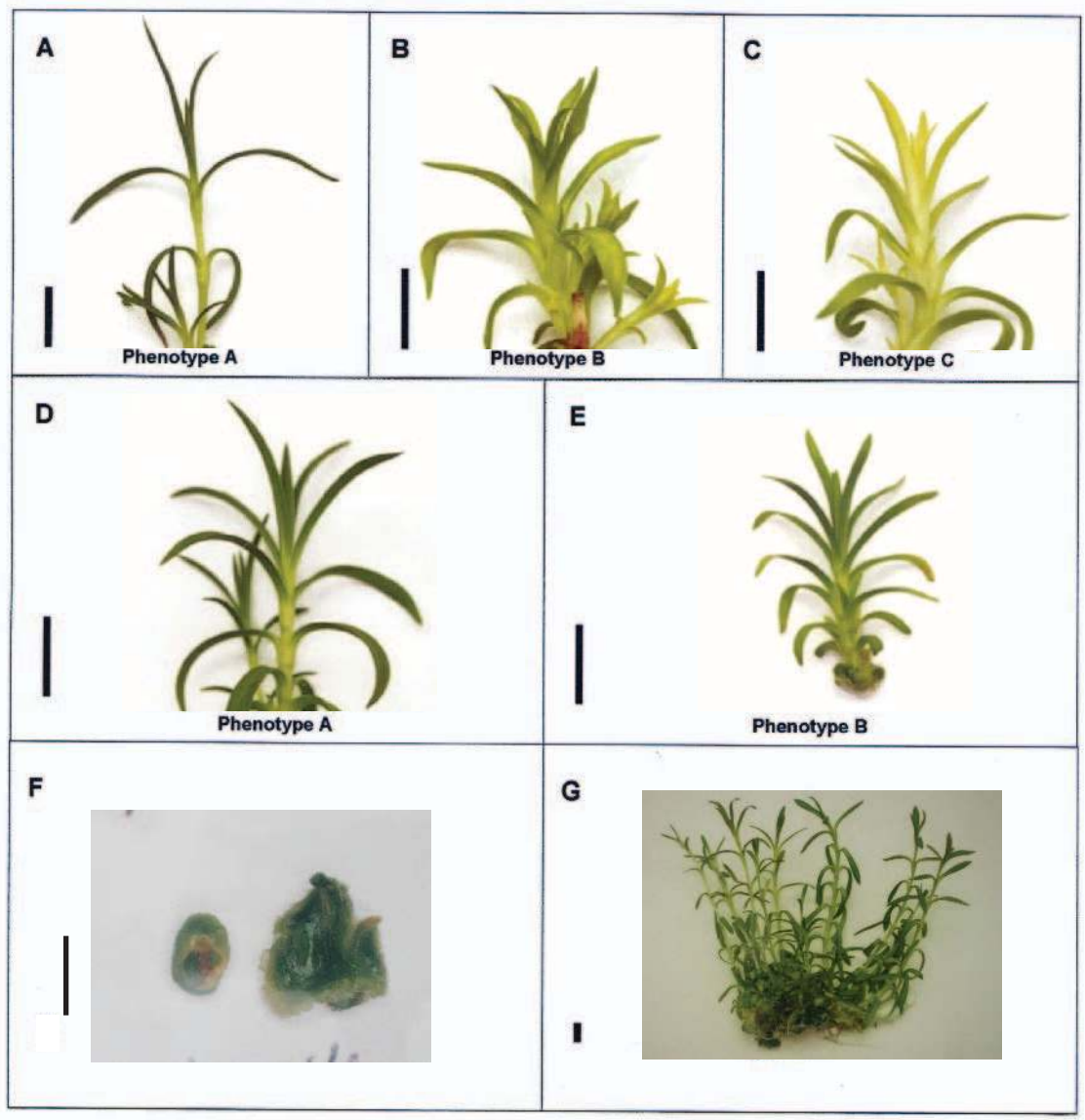

Fig. 1. Different phenotypes found in *Dianthus grationapolitanus* shoot tip regeneration: 'Spotti' after 3 weeks of culture: (**A**) Phenotype A, with green-greyish leaves and normal internode distance; (**B**) Phenotype B, with green-grayish leaves but short internode distance; and (**C**) Phenotype C, with yellowish leaves and/or necrotic shoots; and 'Frosty Fire' after 3 weeks of culture: (**D**) Phenotype A, with normal internode distance; and (**E**) Phenotype B, with short internode distance. 'Spotti' cultivar flower ovary regeneration: (**F**) after 3 weeks of culture and (**G**) after 10 weeks of culture. (**bar** = 1cm).

in the presence of 2.46 μM 2iP or a combination of 4.92 μM 2-iP and 0.57 μM IAA after 10 weeks of culture. On the other hand, for 'Frosty Fire', even though initial callus formation was observed with closed and open ovary explants after 12 weeks in media with 0.54 μM NAA, no shoots were obtained. Leaf and ovary regeneration protocols have been described for other *Dianthus* species (Demmink et al., 1987; Jain et al., 2001; Jethwani and Kothari, 1996; Miller et al., 1991; Roest and Bokelmann, 1981). However, our results showed that these explants were not suitable for *D. gratianopolitanus* micropropagation.

Phenotypic variation and hyperhydricity. Variation among the phenotypes was observed in the regenerated shoots when nodes and shoot tips were used as explants (Fig. 1). Out of the 21 media tested, only seven for 'Spotti' (Table 1) and five for 'Frosty Fire' (Table 2) allowed the regeneration of normal phenotype A shoots, with mean internode distance ranging from 9–13 mm (Fig. 1A) and 4–6 mm (Fig. 1D), respectively. Abnormal phenotypes were observed with all of the other growth regulator combinations. In 'Spotti', shoots with short internodes (mean internode distance, 2–7 mm) belonged to phenotype B (Fig 1B). Shoots assigned to phenotype C exhibited chlorosis and/or necrosis independently of the internode distance (Fig 1C), and they eventually dried and died. On the other hand, in 'Frosty Fire' only phenotype B shoots (internode mean distance, 1–3 mm) were observed (Fig.1E). For both cultivars, phenotype B shoots became less vigorous after several transfers, and failed to recover normal type A phenotype even if transferred to other media. These results clearly indicated that choice of medium was a key step in the regeneration of normal phenotype. Both phenotype B and C shoots were unable to root. Furthermore, from the media rendering phenotype A shoots, only two (MS and IAA 0.57 μM) were common for both cultivars (Tables 1 and 2). These results suggest that growth regulator requirements in *D. gratianopolitanus* are cultivar dependent and should be adjusted for each cultivar.

Hyperhydricity is another limiting step in plant tissue culture and is often severe among species in Caryophyllaceae (Cassells and Walsh, 1994; Mii et al., 1990). Logistic regression analysis for 'Spotti' (Table 3) indicated that the effect of explant type is significantly influenced by hyperhydricity. In this cultivar, hyperhydric shoots were less frequent when nodes were used as explant source (Table 1). On the other hand, the effect of cytokinins on shoot hyperhydricity was significant for both cultivars (Tables 3 and 4). Furthermore, our results showed that there was a positive correlation between the number of hyperhydric shoots and cytokinins BAP (Pearson correlation coefficient = 0.204) and 2-iP (Pearson correlation coefficient = 0.093) concentration. Jain et al. (2001) also reported that BAP promoted

hyperhydricity of *D. caryophyllus* organogenic callus and shoots even when combined with GA_3 and bactopeptone. On the contrary, Leshem (1986) reported that 5.37 μM NAA enhanced the propagation of *D. caryophyllus* shoot tips that developed into hyperhydric plantlets, while BAP had an opposite effect. In *D. gratianopolitanus* micropropagation, the addition of BAP to the medium increased hyperhydricity and stimulated the production of aberrant B and C phenotypes (Tables 1 and 2). Nevertheless, for both cultivars and explant types, treatments were found that resulted in normal shoots (phenotype A) without hyperhydricity.

Effects of plant growth regulators on D. grationapolitanus multiplication efficiency.

Taking into account the percentage of explants with shoots and the number of shoots per explant for each cultivar and explant type, the optimum regeneration media were selected. The addition of auxins was significant in the regeneration of 'Spotti' (Table 3), where 0.57μM IAA induced 3.6 shoots per explant in 80% of the shoot tip explants. Moreover, when nodes were used as explant source and cultured on 2.46 μM 2-iP medium, 2.3 shoots were regenerated in 80% of the explants with 3.9 axilary nodes available per shoot. Therefore, when the optimal media were used with each explant type and 10 initial virus-free plants, it was possible to regenerate after three multiplication transfers more than 240 and 3440 axenic shoots from shoot tips and node explants, respectively, giving a total of more than 3690 shoots in 9 weeks. Regeneration from shoot tip and node explants in MS without plant regulators is recommended for the multiplication of 'Frosty Fire'. Although the shoot formation rates are only 2.4 and 1.3 shoots/explant, respectively, 100% of the explants regenerated normal shoots. Considering that the number of nodes/shoot was 4.8 for node explants, with an analogous number of initial explants and multiplication transfers, more than 2400 shoots were regenerated, while only 130 shoots were obtained from shoot tip explants after 15 weeks.

The addition of cytokinins to the media increased the number of shoots per explant, but also promoted the production of aberrant phenotypes (Tables 1 and 2) with reduced internode length (Tables 3 and 4), making these shoots inadequate for *D. gratianopolitanus* micropropagation.

The interaction between cytokinins and auxins significantly affected *D. gratianopolitanus* multiplication (Tables 3 and 4), but a useful combination could not be found. For example, phenotype A plant predominated (4.2 shoots/explant in 'Spotti' and 4.3 in 'Frosty Fire') when media contained 2.46 μM 2iP and 0.57 μM IAA. However, the percentage of 'Frosty Fire' explants with shoots was very low (60%) and 40% of 'Spotti' and 10% of 'Frosty Fire' shoot tip explants were hyperhydric. The maximum number of 'Spotti' shoots was obtained when explants were incubated in medium containing 4.44 μM BAP and 0.54 μM NAA. Unfortunately, most shoots exhibited poor phenotype, and 40% of shoots were hyperhydric.

Table 3. Effects of explant type, auxins, and cytokinins on *Dianthus gratianopolitanus* 'Spotti' regeneration.

Source of variation	df	% Explants with shoots[z]	% Vitreous explants[z]	Shoots/ explant[y]	Nodes/ shoot[y]	Internode distance[y]
Explant type (E)	1	0.05^{ns}	17.95***	99.34***	27.47***	7.62**
Auxins (A)	2	1.70^{ns}	1.05^{ns}	10.62***	2.65^{ns}	1.00^{ns}
Cytokinins (C)	6	23.96***	31.00***	51.99***	2.33*	29.09***
E × A	2	1.96^{ns}	1.90^{ns}	5.84**	4.56*	1.90^{ns}
E × C	6	20.13**	8.88^{ns}	2.71*	1.45^{ns}	3.27**
A × C	12	10.08^{ns}	19.79^{ns}	2.65**	3.36***	2.36**

[z]Chi square values from logistic regression.
[y]F values from ANOVA.
$^{ns, *, **, ***}$Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 4. Effects of explant type, auxins, and cytokinins on *Dianthus gratianopolitanus* 'Frosty Fire' regeneration.

Source of variation	df	% Explants with shoots[z]	% Vitreous explants[z]	Shoots/ explant[y]	Nodes/ shoot[y]	Internode distance[y]
Explant type (E)	1	0.4^{ns}	2.58^{ns}	63.48***	0.02^{ns}	0.42^{ns}
Auxins (A)	2	0.96^{ns}	1.23^{ns}	0.75^{ns}	0.59^{ns}	0.55^{ns}
Cytokinins (C)	6	40.24***	14.55*	19.74***	4.10***	8.39***
E × A	2	1.38^{ns}	0.89^{ns}	3.19*	0.16^{ns}	3.68*
E × C	6	8.80^{ns}	10.7^{ns}	3.72**	0.87^{ns}	0.38^{ns}
A × C	12	20.22^{ns}	18.83^{ns}	2.72**	2.69**	1.73^{ns}

[z]Chi square values from logistic regression.
[y]F values from ANOVA.
$^{ns, *, **, ***}$Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively

Table 5. Rooting of *Dianthus gratianopolitanus* 'Spotti and 'Frosty Fire' after 2 weeks of culture on MS medium with different concentrations of growth regulators.

Growth regulators (μM)		'Spotti'			'Frosty Fire'		
		Phenotype	Mean no. roots/ rooted shoot[z]	Mean length/ root[z] (cm)	Phenotype	Mean no. roots/ rooted shoot[z]	Mean length/ root[z] (cm)
NAA	BAP						
0	0	A	3.9 a–c	1.8 e	A	3.3 a–c	1.9 d
2.68	0	A	6.0 bc	1.1 b–d	A	3.9 ab	0.3 a
2.68	2.22	B	1.5 a–c	0.2 a–c	B	1.5 ab	0.9 a–d
5.37	0	A	2.2 a	0.7 ab	A	4.3 a–c	0.4 ab
5.37	2.22	B	1.2 a–c	0.3 a–d	B	3.4 ab	0.5 ab
IAA	BAP						
2.85	0	A	3.3 ab	0.8 a–c	A	6.6 c	0.7 b
2.85	2.22	B	---	---	A	---	---
5.70	0	A	5.1 a–c	1.3 de	B	4.0 a–c	1.1 c
5.70	2.22	C	---	---	B	---	---
IBA	BAP						
2.46	0	A	3.5 a–c	1.6 de	A	4.3 a–c	1.7 d
2.46	2.22	B	---	---	B	1.3 a	0.3 ab
4.92	0	A	6.8 c	1.2 c–e	A	5.3 bc	0.7 a–c
4.92	2.22	B	1.5 a–c	0.2 a	B	1.0 ab	0.6 b

[z]Means in columns with the same letter are not significantly different according to LSD at $P \leq 0.05$.

Rooting and acclimatization. Even though ex vitro rooting was slower, ≈97% rooting was observed within 4 weeks for both cultivars. Maximum in vitro rooting was 80% for 'Spotti' shoots treated with 2.85 or 5.7 μM IAA or 2.46 μM IBA, and 70% for 'Frosty Fire' shoots treated with 2.46 μM IBA (Table 5). The mean number of roots per shoot was increased by IAA and IBA treatments. Statistical analysis indicated that there is a significant difference between cultivars, since 'Spotti' in vitro rooting percentage was better (data not shown). These results indicated that in *D. gratianopolitanus,* rooting is genotype dependant as described for *D. caryophyllus* (Kallak et al., 1997). The addition of 2.22 μM BAP to root induction medium inhibited the production of roots and, interestingly, as in the *D. gratianopolitanus* multiplication experiment, also affected the phenotype of the rooted plants (Table 5). The survival rate of tissue-cultured rooted *D. gratianopolitanus* plants was >99% under greenhouse conditions.

Six months after removal from tissue culture, plants appeared very uniform (Fig. 2) and flowered more abundantly than the original virus-infected plants. The present study enabled the successful in vitro multiplication of two *D. gratianopolitanus* commercial cultivars, 'Spotti' and 'Frosty Fire'.

Literature Cited

Cassells, A.C. and C. Walsh. 1994. The influence of the gas permeability of the culture lid on calcium uptake and stomatal function in "*Dianthus*" microplants. Plant Cell Tissue Organ Cult. 37:171–176.

Demmink, J.F., J.B.M. Custers, and J.H.W. Bergervoet. 1987. Gynogenesis to bypass crossing

Fig. 2. *Dianthus grationapolitanus* (**A**) 'Spotti' and (**B**) 'Frosty Fire' 6 months after removal from tissue culture.

barriers between diploid and tetraploid *Dianthus* species. Acta Hort. 216:343–344.

Earle, E.D. and R.W. Langhans. 1975. Carnation propagation from shoot tips cultured in liquid medium. HortScience 10:608–610.

Jain, A., A. Kantia, and S.L. Kothari. 2001. De novo differentiation of shoot buds from leaf-callus of *Dianthus caryophillus* L. and control of hyperhydricity. Sci. Hort. 87:319–326.

Jelitto, L. and W. Schacht. 1990. Hardy herbaceous perennials. Timber Press, Portland, Ore.

Jethwani, V. and S.L. Kothari. 1996. Phenylacetic acid induced organogenesis in cultured leaf segmens of *Dianthus chinensis*. Plant Cell Rpt. 15:869–872.

Johnson, R.T. 1980. Gamma irradiation and in vitro separation of chimeral genotypes in carnation. HortScience 15:605–606.

Kakehi, M. 1979. Studies on the tissue culture of carnation. V. Induction of redifferentiated plants from the petal tissue. Bul. Hiroshima Agr. Coll. 6:159–166.

Kallak, H., M. Reidla, I. Hilpus, and K. Virumäe. 1997. Effects of genotype, explant source and grown regulators on organogenesis in carnation callus. Plant Cell Tissue Organ Cult. 51:127–135.

Kim, J.C. and E.A. Lee. 1996. Plant regeneration from mesophyll protoplasts of *Dianthus superbus*. Plant Cell Rpt. 16:18–21.

Leshem, B. 1983. Growth of carnation meristems in vitro: Anatomical structure of abnormal plantlets and the effect of agar concentration in the medium on their formation. Ann. Bot. 52:413–415.

Leshem, B. 1986. Carnation plantlets from vitrified plants as source of somaclonal variation. HortScience 21:320–321.

Leshem, B. and P.D. Shalev. 1988. The effect of cytokinins on vitrification in melon and carnation. Ann. Bot. 62:271–276.

Lovisolo, O., M. Conti, and E. Luisoni. (1968). Su di un ceppo del virus del mosaico del centriolo (CMV) isolato du garofano. Phytopathol. Mediterranea 7(2/3):71–76.

Majada, J.P., F. Tadeo, M.A. Fal, and R. Sánchez-Tamés. 2000. Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. Plant Cell Tissue Organ Cult. 63:207–214.

Mii, M., M. Buiatti, and F. Gimelli. 1990. Carnation, p. 284–318. In: P.V. Ammirato, D.R. Evans, W.R. Sharp, and Y.P.S. Bajaj (eds.). Handbook of plant cell culture. McGraw-Hill, N.Y.

Miller, R.M., V. Kaul, J.F. Hutchinson, and D. Richards. 1991. Adventitious shoot regeneration in carnation (*Dianthus caryophyllus* L.) from axillary bud explants. Ann. Bot. 67:35–42.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Nakano, M., Y. Hoshino, and M. Mii. 1994. Adventitious regeneration from cultured petal explants of carnation. Plant Cell Tissue Organ Cult. 36:15–19.

Pallás, V., J.A. Sanchez-Navarro, M.C. Cañizares, and E. Cano. 1999. A new test of detection of carnation viruses. The routine of viruses affecting carnation crop comes into a new era. Floraculture Intl. Vol. 9, No. 5, p. 32–34.

Roest, S. and G.S. Bokelmann 1981. Vegetative propagation of carnation *in vitro* through multiple shoot development. Sci.. Hort. 14:357–366.

Sanchez-Navarro, J.A., M.C. Cañizares, E. Cano, and V. Pallas. 1999. Simultaneous detection of five carnation viruses by non-isotopic molecular hybridization. J. Virol. Methods 82:167–175.

Sato, S., N. Katoh, H. Yoshida, S. Iwai, and M. Hagimori. 2000. Production of doubled haploid plants of carnation (*Dianthus caryophyllus* L.) by pseudofertilized ovule culture. Sci. Hort. 83 (3–4):301–310.

Van Altvorst, A.C., H.J.J. Koehorst, T. Bruinsma, and J.J.M. Dons. 1992. Adventitious shoot formation from *in vitro* leaf explants of carnation (*Dianthus caryophyllus* L.). Sci. Hort. 51:223–235.

Van Altvorst, A.C. H.J.J. Koehorst, T. Bruinsma, J.J.M. Dons. 1994. Improvement of adventitious shoot formation from carnation leaf explants. Plant Cell Tissue Organ Cult. 37: 87–90.

Van Altvorst, A.C., S. Tancheva, and J. Dons. 1995. Cells within the nodal region of carnation shoot exhibit a high potential for adventitious shoot formation. Plant Cell Tissue Organ Cult. 40:151–157.

Watad, Abed A., A. Ahroni, A. Zuker, H. Shejtman, A. Nissim, and A. Vainstein. 1996. Adventitious shoot formation from carnation stem segments: A comparison of different culture procedures. Sci. Hort. 65:313–320.