Propagation of Ornamental Plants
Vol. 00, № 0, 2000: 00-00

IN VITRO SHOOT PROLIFERATION, ROOTING, AND ACCLIMATIZATION OF FOUR DIVERSE *DIANTHUS PETRAEUS* W. ET K. GENOTYPES USING TDZ, NAA, AND IBA

Georgios Tsoktouridis[1]*, Katerina Grigoriadou[1], Evangelia Doua[1], Anna Nikolaidou[2], Georgios Menexes[3], and Eleni Maloupa[1]

[1]Laboratory for the Conservation and Evaluation of Native and Floricultural Species, Balkan Botanic Garden of Kroussia, Hellenic Agricultural Organization "Demeter", P. O. Box 60125, 570 01 Thermi, Thessaloniki, Greece,
*Fax: + 30 2310 478907, *E-mail: gtsok1@yahoo.co.uk
[2]Regional Laboratory for Agricultural Applications and Analysis of Fertilizers (P.E.G.E.A.L.) of Central Macedonia, Ministry of Rural Development and Food, Directorate of Research, Industrial Area, Sindos, 57400 Thessaloniki, Greece,
[3]Laboratory of Agronomy, School of Agriculture, Aristotle University, 541 24, Thessaloniki, Greece

Abstract

In vitro mass propagation was achieved for four diverse genotypes of the wild carnation *Dianthus petraeus*, originating from four different geographical areas in Greece. Adventitious shoot proliferation was induced on Murashige and Skoog (MS) medium supplemented with 0.5, 1, and 2 mg l^{-1} of TDZ in combination with 0, 0.1 and 0.5 mg l^{-1} of NAA, using explant material from *in vitro* maintained stock microshoots. Hyperhydricity of the proliferated microshoots was observed and overcome by transferring the hyperhydrous microshoots to full-strength MS medium without growth regulators for four weeks prior to *in vivo* acclimatization. The effect of plant growth regulators was investigated and the mean number of adventitious shoots was recorded throughout all the developmental stages of micropropagation. The supplement of 0.1 mg l^{-1} NAA in the growth medium is recommended for all *D. petraeus* genotypes but treatments with different amounts of TDZ produced variable amounts of adventitious shoots (~17-24) per explant, depending on the genotype. A significant number of proliferated shoots was observed after the exclusion of TDZ and NAA during hyperhydricity reduction treatment and a well developed root system formed using either NAA or IBA. The regenerated plantlets with well-developed root and shoot system were successfully acclimatized in greenhouse and exterior nursery conditions.

Key words: hyperhydricity, micropropagation, shoots proliferation, thidiazuron, wild carnation

INTRODUCTION

Carnation is an economically important crop in the ornamental industry and a significant research target is to select, propagate and breed new cultivars worldwide. Hence, there is a need for germplasm collection, *ex situ* conservation and *in vitro* gene bank preservation of the wild *Dianthus* spp. Many of the wild *Dianthus* species are threatened with extinction. The Red Lists of the International Union for Conservation of Nature (IUCN) list 75 species of endangered *Dianthus* (Walter and Gillett 1998).

In the framework of strategies to control and diminish biodiversity loss by 2020 according to the Global Strategy for Plant Conservation (Wyse-Jackson and Sutherland 2000), the Balkan Botanic Garden of Kroussia pioneers actions for *in situ* and *ex situ* conservation of native flora at the local, national, and international levels (Maloupa et al. 2008). However, the production of elite plants and the development of new cultivation protocols for new crops make still necessary the development of advanced *in vitro* propagation systems (Grigoriadou and Maloupa 2008).

Propagation via tissue culture gives opportunity for multiple adventitious shoots formation. For this reason mass propagation is essential for introducing new flower crops in ornamental horticulture; moreover, maintaining plant stock cultures *in vitro* is a very good tool for germplasm conservation of vulnerable wild endangered species.

Micropropagation of carnation is nowadays a routine procedure using numerous successful protocols found in the literature (Salehi 2006, Casas et al. 2010). These include supplement of various concentrations

Received: September 24, 2013 *Accepted: December 10, 2013*

of 1-Phenyl-3-(1,2,3,-thidiazol-5-yl)urea (TDZ) and α-naphtaleneacetic acid (NAA) for *Dianthus caryophyllus* (Paramesh and Chowdhury 2005), *D. chinensis* and *D. barbatus* (Nontaswatsri et al. 2008), and 35 cultivars of *D. caryophyllus* (Nontaswatsri et al. 2002). Various concentrations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) were used for *D. balbisii* (Berardi et al. 2006) and combinations of NAA, IBA, BAP, N^6-(2-Isopentenyl)adenine (2iP), and TDZ for *D. fruticosus* (Papafotiou and Stragas 2009) and *D. deltoides* (Popović et al. 2008). The *in vitro* use of BAP for *D. caryophyllus* regenerated shoots with hyperhydricity (Jain et al. 2001) and therefore supplement with other cytokinins like TDZ, 6-Furfurilaminopurine (kinetin), and 2iP is preferable for shoot proliferation.

Previous research on micropropagation of *D. petraeus* was conducted for the endemic Romanian genotypes (subsp. *simonkaianus*), using diverse combinations between NAA, IBA, IAA and BAP, kinetin, 2iP, TDZ (Miclăuş et al. 2003, Cristea et al. 2006).

The aim of this research was to study the effect of TDZ, NAA, and IBA on adventitious shoot proliferation, rooting, and acclimatization of four diverse *D. petraeus* genotypes, which are dissimilar in morphology and originate from four different geographical areas of Greece. In addition, a hyperhydricity reduction treatment, greenhouse acclimatization, and plant establishment outdoors, were investigated.

MATERIALS AND METHODS

Plant material

Wild plants of *Dianthus petraeus* were collected from nature, transferred to nursery, maintained in 9 l plastic pots, and acclimatized for several years at sea level. Four diverse genotypes of *D. petraeus* were selected from four different geographical regions of North Greece in the following locations: at the mountain of Paggeo, Kavala, at 1890 m, 05,2888 (#2888), the mountain of Athos, Agion Oros, Chalkidiki, at 1700 m, 06,3631 (#3631), the mountain of Falakro, Volakas, Drama at 1700 m, 99,1151, (#1151), and the mountain of Falakro, Volakas, Drama at 2000 m, 01,1471, (#1471).

Culture media and conditions

Shoot tips taken from *D. petraeus* pot plants were surface disinfected by 1.5% sodium hypochlorite solution containing 2 drops of Tween-20 per 100 ml solution for 15 min with shaking and then rinsed three times with sterile distilled water. All shoot tips were cultured on MS medium (Murashige and Skoog 1962), supplemented with 20 g l^{-1} sucrose. The medium pH was adjusted to 5.8 with NaOH or HCl before Phytagel (3 g l^{-1}) was added and autoclaved at 121°C for 20 min. The culture medium was supplemented with various amounts of NAA (0.1 and 0.5 mg l^{-1}), IBA (0.1, 0.5, and 1.0 mg l^{-1}) prior autoclaving, whereas only TDZ (0.5, 1.0, and 2.0 mg l^{-1}) was filter-sterilized (pore size at 0.2 μm) and supplemented into the autoclaved medium (when its temperature dropped to 55 ± 5°C). A total of 30 ml medium was dispensed in Magenta B-cap jars (200 ml in capacity). All cultures were maintained in a growth room at 22 ± 2°C, 16-h of cool white fluorescent light at photon flux density 40 μmol m^{-2} s^{-1}, daily and five shoot tips (~2 cm long) were cultured in each jar.

Selection of explant material

The experiments utilized shoot tips from *in vivo* growing pot-plants and *in vitro* produced ~4 to 6-week old plants of *Dianthus petraeus* #2888. The *in vitro* explants were derived from *in vitro* maintained as stock that was subcultured every 4-6 weeks, for three years. The MS medium was supplemented with various amounts of TDZ (0, 0.5, 1.0, and 2.0 mg l^{-1}) each combined with (0.1 mg l^{-1}) NAA. The experiment was carried out using a 4 × 2 factorial experiment based on a completely randomized design (CRD). Each treatment contained 3 replications of 5 explants (Table 1). The results were evaluated after 4 weeks.

Table 1. Effect of the PGRs and type of initial explant on the number of the formed adventitious shoots of *genotype* #2888.

Treatments		Type of explants	
TDZ (mg l^{-1})	NAA (mg l^{-1})	Explants *in vivo*	Explants *in vivo*
0.0	0.1	0.00 ± 0.00 a	1.67 ± 0.36 b
0.5	0.1	2.93 ± 0.84 a	16.20 ± 1.60 a
1.0	0.1	1.50 ± 0.70 a	16.47 ± 2.33 a
2.0	0.1	1.47 ± 0.55 a	16.87 ± 2.06 a

The means ± SE in the same column followed by different letters are statistically significant different according to Bonferroni's LSD criterion. The two-way interaction between type of explants and TDZ is significant at $p < 0.001$.

Effect of TDZ and NAA in adventitious shoot proliferation

Four to six week old, *in vitro* formed adventitious shoots, from genotypes #2888, #3631, #1151, and #1471 were used as initial explants. They were cultured on MS medium supplemented with various amounts of TDZ (0, 0.5, 1.0, and 2.0 mg l^{-1}) each combined with (0, 0.1, and 0.5 mg l^{-1}) NAA. The experiment for adventitious shoot proliferation consisted of 48 treatments according to a 4 × 3 × 4 factorial experiment based on a completely randomized design. Each treatment contained 3 replications of 5 explants (Table 2). The results were evaluated after 4 weeks.

Table 2. Effect of TDZ, NAA, and genotypes on the number of the formed adventitious shoots.

Treatments		*D. petraeus* genotypes			
TDZ (mg l^{-1})	NAA (mg l^{-1})	#1151	#2888	#1471	#3631
0.0	0.0	3.60 ± 0.43 e	1.27 ± 0.34 c	0.93 ± 0.34 e	0.07 ± 0.07 f
	0.1	3.87 ± 0.50 e	1.67 ± 0.36 c	0.60 ± 0.19 e	0.87 ± 0.40 f
	0.5	6.67 ± 0.27 e	1.00 ± 0.32 c	1.93 ± 0.33 e	0.47 ± 0.34 f
0.5	0.0	15.13 ± 1.47 bcd	9.73 ± 1.68 b	19.73 ± 1.53 ab	17.27 ± 1.98 b
	0.1	21.47 ± 2.00 a	16.20 ± 1.60 a	18.00 ± 1.07 b	15.00 ± 1.44 bc
	0.5	14.67 ± 1.49 cd	15.67 ± 1.32 a	10.87 ± 1.12 d	7.73 ± 0.79 e
1.0	0.0	19.80 ± 1.62 ab	9.20 ± 0.98 b	16.73 ± 2.35 bc	13.13 ± 1.99 bcd
	0.1	20.67 ± 1.37 a	16.47 ± 2.33 a	20.27 ± 1.09 ab	15.80 ± 1.89 b
	0.5	14.90 ± 1.08 bcd	17.40 ± 2.27 a	11.60 ± 1.29 d	9.20 ± 1.14 de
2.0	0.0	19.20 ± 2.10 abc	6.80 ± 0.76 b	24.40 ± 2.30 a	24.80 ± 1.88 a
	0.1	20.53 ± 1.92 a	16.87 ± 2.06 a	21.47 ± 1.59 ab	23.90 ± 1.05 a
	0.5	11.90 ± 1.11 d	15.07 ± 0.99 a	12.53 ± 1.26 cd	10.40 ± 1.53 cde
LSD Bonferroni = 5.12					

The means ± SE in the same column followed by different letters are statistically significant different according to Bonferroni's LSD criterion. The three-way interaction between genotype, TDZ, and NAA is significant at $p < 0.001$.

Reduction of hyperhydricity of regenerated shoots

Adventitious shoots (~2 cm long) were dissected from clusters of each of the above 48 treatments separately and placed on a MS medium without plant growth regulators (PGRs). These shoots were continued to proliferate shoots although they were cultured on a PGRs free medium and therefore, the number of shoots was recorded. The experiment consisted of 48 treatments according to a 4 × 3 × 4 factorial experiment based on a completely randomized design (CRD). Each treatment contained 4 replications of 5 explants. The results were evaluated after 4 weeks (Table 3).

Shoot proliferation during rooting

Four to six week old *in vitro* maintained shoots, were used as initial explants for genotypes 2888, #3631, #1151, and #1471. They were cultured on MS medium supplemented with NAA (0, 0.1, and 0.5 mg l^{-1}) and IBA (0, 0.1, 0.5, and 1.0 mg l^{-1}). The experiment consisted of 24 treatments according to a 4 × 6 factorial experiment based on a completely randomized design. Each treatment contained 3 replications of 5 explants (Table

Table 3. Effect of the persistence of PGRs and genotypes on the number of the formed shoots cultured on PGRs free MS medium; the explants derived from the *in vitro* maintained material treated with TDZ and NAA.

Treatments		*D. petraeus* genotypes			
TDZ (mg l^{-1})	NAA (mg l^{-1})	#1151	#2888	#1471	#3631
0.0	0.0	3.05 ± 0.34 d	1.20 ± 0.34 e	0.00 ± 0.00 d	0.00 ± 0.00 b
	0.1	2.30 ± 0.26 d	1.70 ± 0.45 e	0.25 ± 0.12 d	0.00 ± 0.00 b
	0.5	2.30 ± 0.36 d	2.30 ± 0.44 e	0.55 ± 0.20 d	0.00 ± 0.00 b
0.5	0.0	7.75 ± 0.89 abc	7.35 ± 0.94 cd	5.75 ± 0.52 bc	1.85 ± 0.52 ab
	0.1	5.95 ± 0.73 c	7.90 ± 1.04 bcd	5.85 ± 0.28 bc	0.95 ± 0.35 ab
	0.5	6.45 ± 0.47 bc	7.95 ± 0.76 bcd	5.75 ± 0.46 bc	0.30 ± 0.11 b
1.0	0.0	6.35 ± 1.04 c	8.30 ± 1.03 bcd	8.80 ± 0.65 a	1.95 ± 0.50 ab
	0.1	7.05 ± 0.75 abc	9.50 ± 0.98 abc	5.50 ± 0.67 c	1.95 ± 0.27 ab
	0.5	6.50 ± 0.48 bc	7.05 ± 0.88 d	5.15 ± 0.40 c	0.35 ± 0.15 b
2.0	0.0	8.65 ± 0.90 ab	6.20 ± 1.03 d	8.45 ± 0.92 a	3.10 ± 0.33 a
	0.1	6.90 ± 0.64 abc	9.65 ± 0.99 ab	7.80 ± 0.43 ab	2.90 ± 0.48 a
	0.5	9.15 ± 0.49 a	10.65 ± 1.15 a	5.40 ± 0.45 c	1.00 ± 0.21 ab
LSD Bonferroni = 2.26					

The means ± SE in the same column followed by different letters are statistically significant different according to Bonferroni's LSD criterion. The three-way interaction between genotype, TDZ, and NAA is significant at $p < 0.001$.

Table 4. Effect of NAA, IBA, and genotypes on the number of the formed shoots.

Treatments		*D. petraeus* genotypes			
NAA (mg l^{-1})	IBA (mg l^{-1})	#1151	#2888	#1471	#3631
0.0	0.0	3.60 ± 0.43 b	1.27 ± 0.34 ab	0.93 ± 0.34 cd	0.07 ± 0.07 a
0.1	0.0	3.87 ± 0.50 b	1.67 ± 0.36 ab	0.60 ± 0.19 d	0.87 ± 0.40 a
0.5	0.0	6.67 ± 0.27 a	1.00 ± 0.32 b	1.93 ± 0.33 bc	0.47 ± 0.34 a
0.0	0.1	3.47 ± 0.27 b	1.67 ± 0.32 ab	2.47 ± 0.55 b	0.13 ± 0.13 a
	0.5	4.67 ± 0.27 b	1.80 ± 0.50 ab	4.80 ± 0.17 a	0.07 ± 0.07 a
	1.0	3.60 ± 0.31 b	2.53 ± 0.51 a	4.87 ± 0.48 a	0.20 ± 0.20 a
LSD Bonferroni = 1.27					

The means ± SE in the same column followed by different letters are statistically significant different according to Bonferroni's LSD criterion. The two-way interaction between genotype and NAA/IBA treatments is significant at $p < 0.001$.

4). The number of proliferated shoots was measured after 4 weeks.

Statistical analysis

Data were analyzed with ANOVA for testing the significance of the main effects and interactions of the factors of interest. Means of treatments' combinations were compared with the Bonferroni's LSD multiple comparisons test (Toothaker 1993), after a significant two-way or three-way interaction at significance level $p < 0.05$ (Steel et al. 1997). All statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago III).

Greenhouse acclimatization and plant establishment

All *in vitro* rooted plantlets were carefully rinsed with tap water to remove the adhering medium and then planted in plastic trays filled with a peat : vermiculite : perlite : scolecite (2 : 2 : 1 : 0.5, v/v) mixture. The trays with the plantlets were placed in a greenhouse at 25 ± 4°C, equipped with a fog system supplying a 90% relative air humidity and 50% shading for two weeks. The relative humidity was reduced at 5% per day during the second week, while light intensity was gradually increased. The trays with the plantlets were maintained for another 2 weeks in a greenhouse at 25 ± 4°C, 55 ± 4% relative air humidity and direct sunlight. Then the plantlets were transplanted into 1 l plastic pots contained a mixture of soil : peat : vermiculite : scolecite (5 : 2 : 1 : 2, v/v) and transferred to exterior nursery conditions. A total of ~350 pots from each *D. petraeus* genotype were *in vivo* acclimatized and the number of successfully acclimatized plants was recorded one year later.

RESULTS AND DISCUSSION

Selection of explant material

An initial experiment for selecting the explants was employed to find the proper type of explants to use for *in vitro* mass propagation of *D. petraeus* genotypes. The highest mean number of proliferated adventitious shoots after surface disinfection of *in vivo* explants was 2.93 ± 0.84, in comparison to 16.87 ± 2.06 when *in vitro* explants were used (Table 1). However, there were no significant differences among the concentrations of TDZ within the range 0.5-2.0 mg l^{-1} and therefore the lowest concentration of 0.5 mg l^{-1} of TDZ was considered adequate for micropropagation of #2888. The most important observation was the disproportionate number of shoots produced per *in vivo* explant, compared to that produced when *in vitro* explants were used. The later presented a homogenous rate of shoot induction per explant for most of the treatments. According to the results of ANOVA the interaction between type of explants and TDZ concentration was significant ($p < 0.001$) (Table 1).

There are possibly many factors that influence the low number of proliferated shoots produced from the *in vivo* explants. The *in vivo* explants may be affected by sodium hypochlorite solution during disinfection and they were not responded well under *in vitro* conditions. Also, it was supposed that every explant derived from different developmental stage, age, and site position possibly contained unbalanced endogenous PGRs, which prevented the induction of adventitious shoots (Watad et al. 1996). In contrast, the *in vitro* explants grew within an appropriate aseptic environment therefore, they promoted the high numbers of proliferated adventitious shoots. The effect of the *in vitro* regenerative activity, due to concentrations of several endogenous PGRs and enzyme activities, has been reported in *Schlumbergera* and *Rhipsalidopsis* species during micropropagation, indicating that the endogenous cytokinin oxidase/dehydrogenase and total peroxide activity increased the adventitious shoot formation, during subculturing (Sriskandarajah et al. 2006).

Similar results were observed during micropropagation of the wild carnation *Dianthus henteri*, producing ~15 adventitious shoots per explant, which were derived from shoot tips of *in vitro* germinated seedlings

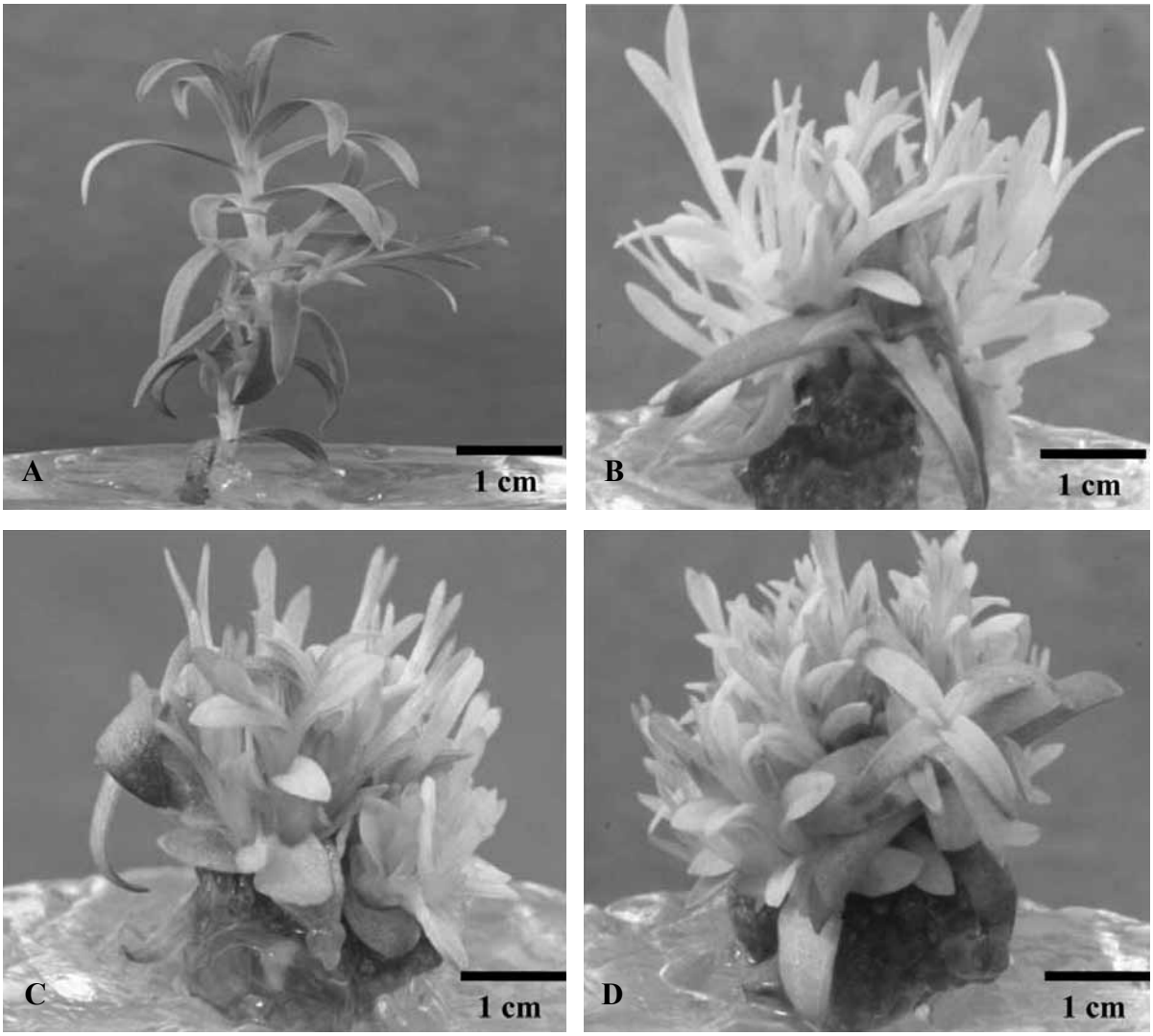

Fig. 1. Adventitious shoot proliferation of *in vitro* grown shoots of *Dianthus petraeus* 99,1151, on MS medium supplemented with 0.1 mg l^{-1} NAA and A) 0 mg mg l^{-1} TDZ, B) 0.5 mg l^{-1} TDZ, C) 1.0 mg l^{-1} TDZ, and D) 2.0 mg l^{-1} TDZ. Amorphous mass of green callus is observed at all TDZ treatments.

(Cristea et al. 2010). Another advantage of using *in vitro* explants is the low percentage of contaminated cultures. It was demonstrated that contamination usually leads to poor results in *Dianthus petraeus* ssp. *simonkaianus*, with losses reaching the 45% of *in vivo* cultures (Miclăuş et al. 2003).

Effect of TDZ and NAA in adventitious shoot proliferation

In vitro cultures of *D. petraeus* genotypes, cultured on medium without PGRs proliferated between 0.07 ± 0.07 (#3631) and 3.60 ± 0.43 (#1151) shoots, showing a ~3-fold more shoots per explant of #1151 than the other genotypes (Table 2).

The application of TDZ caused hyperhydricity, which was observed in treatments with 1.0 and 2.0 mg l^{-1}, producing adventitious shoots with shorter and thicker leaves than those from the treatment of 0.5 mg l^{-1} TDZ (Fig. 1). Hyperhydrated proliferated shoots are frequently observed in micropropagation of carnation (Watad et al. 1996, Miclăuş et al. 2003, Salehi 2006, Casas et al. 2010, Cristea et al. 2010) but these shoots turned to normal after cultivation on a PGRs-free MS medium.

An amorphous mass of green callus was also forming in all treatments with TDZ, especially at concentrations of 1.0 and 2.0 mg l^{-1}. Somaclonal variation was not macrometrically observed in none of the *D. petraeus* genotypes acclimatized *in vivo*. The occurrence of callus formation during micropropagation was also documented for *Dianthus petraeus* ssp. *simonkaianus* using various amounts of BAP (Miclăuş et al. 2003).

The study focused on the development of a micropropagation protocol for each of the Greek *D. petraeus*

genotype and therefore, application of as low amounts of plant growth regulators as possible, in order to produce an adequate number of proliferated shoots without hyperhydricity. The optimum amounts of growth regulators for #1151 and #2888 were 0.1 mg l^{-1} of NAA and 0.5 mg l^{-1} TDZ, producing 21.47 ± 2.00 and 16.20 ± 1.60 shoots respectively; for #1471 was 0.5 mg l^{-1} TDZ without NAA, producing 19.73 ± 1.53 shoots; and for #3631 was 2.0 mg l^{-1} TDZ without NAA, producing 24.80 ± 1.88 shoots.

Increasing the concentrations of TDZ had no significant effect on shoot production for genotypes #1151, #1471, and #2888 but significantly affected genotype #3631, producing more shoots at the concentration of 2.0 mg l^{-1} TDZ than 0.5 and 1.0 mg l^{-1} (Table 2) (Fig. 1). According the ANOVA results, the three-way interaction between genotypes, TDZ, and NAA concentrations was significant ($p < 0.001$) (Table 2).

Treatment for the reduction of hyperhydricity of regenerated shoots

In vitro shoot development of *D. petraeus* genotypes was investigated by transferring all the TDZ/NAA regenerated shoots to a PGRs free MS medium for four weeks. The genotypes #1151, #2888, and #1471 continued to proliferate shoots at significant levels, with a maximum number of 9.15 ± 0.49, 10.65 ± 1.15, and 8.80 ± 0.65 for the genotypes #1151, #2888 and #1471, respectively (Table 3). The genotype #3631 showed almost no effect in persistence of TDZ/NAA for continuing proliferation of microshoots, and produced a maximum mean number of 3.10 ± 0.33 (Table 3). According to the ANOVA results the three-way interaction between genotypes, TDZ, and NAA concentrations was significant ($p < 0.001$) (Table 3).

The differences concerning highest and the lowest numbers of proliferated shoots from the various TDZ/NAA treatments were not significant among the #1151, #2888, and #1471 genotypes but the three of them were significant with the genotype #3631. This differentiation confirms a genotype-specific effect for #3631, indicating either the possible existence of inhibitors for shoot proliferation, or the slow metabolic reactions for shoot induction during the four weeks of *in vitro* culture. Species-specific responses in growth regulators and nutrient supply during micropropagation have been reported in different wild carnations (Miclăuş et al. 2003) and therefore the application of a general micropropagation protocol in carnation (Salehi 2006) is expected to be impossible.

Hyperhydricity effects and callus development were not observed during the four weeks of culture and all shoots were homogenously developed into rooted plantlets. The elimination of hyperhydricity and shoot development in cultures without PGRs is a positive effect for *D. petraeus* micropropagation. The rapid elimination of hyperhydricity during subculturing on a PGR's free MS medium is very important for mass propagation of *D. petraeus* genotypes because it made possible to produce the maximum number of proliferated shoots, e.g. 24.80 ± 1.88 for genotype #3631 when TDZ at 2.0 mg l^{-1} was supplemented (Table 2).

Shoot proliferation during rooting

Throughout our experiments, it was quite interesting to score the continuation of adventitious shoot proliferation during the supplement of NAA and IBA in the medium for rhizogenesis. The genotype #1151 produced the highest number of shoots with the highest mean number reaching 6.67 ± 0.27, when NAA at 0.5 mg l^{-1} was supplemented, indicating an endogenous phytohormonal activity as a hypothesis (Table 4). The highest number of proliferated shoots for #1471 was 4.87 ± 0.48, 2.53 ± 0.51 for #2888, and 0.87 ± 0.40 for #3631, showing statistically significant differences among them, proving genotype-specific effects. The lowest numbers of proliferated shoots from the various NAA and IBA treatments were not significant among the #1471, #2888, and #3631 genotypes but the three of them were significant with the genotype #1151 (Table 4). According to the results of ANOVA the interaction between genotypes and NAA/IBA treatments was significant ($p < 0.001$) (Table 4).

Rooting of adventitious shoots of *D. petraeus* genotypes in this study was easily succeeded at 100% with or without the addition of NAA or IBA, but the addition of auxins promoted rooting for at least 7-10 days earlier than without NAA or IBA. The formation of a dense, well-branched root system was generally observed for almost all of the shoots treated with different concentrations of NAA or IBA (data not shown). Rooting of shoots in *D. caryophyllus* cultivars is easily succeeded with extended use of 1.0 to 2.0 mg l^{-1} of NAA and IBA (Salehi 2006), for *D. fruticosus* at 2 mg l^{-1} of IBA (Papafotiou and Stragas 2009), and for *D. deltoides* without addition of PGRs (Popović et al. 2008).

The experiments with NAA and IBA support the hypothesis of possible existence of different level of endogenous auxins, which synergistically act in shoot proliferation without the application of cytokinins. The fluctuation of the mean number of shoots proliferation at the various concentrations of NAA and IBA varied in each genotype, showing the different range of endogenous biochemical compounds and activation during micropropagation (Sriskandarajah 2006). These compounds are possibly dependent on the natural habitat and origin of the plant, characterizing the distinctiveness of each genotype, even though the four genotypes studied had almost the same phenotype.

Greenhouse acclimatization and plant establishment

The *in vivo* acclimatization of the rooted microplants of *D. petraeus* followed a standard acclimatization system developed in the nursery (Grigoriadou and Maloupa 2008). Results on the field indicate a mean survival rate of 90-100% for the four genotypes. These plants were successfully developed into the genuine natural characteristics of the stock mother plants (data not shown). It was feasible to observe a uniformity of the plants, as well as the uniqueness, developmental stages and growth of each genotype. The phenotypes of #1151, #2888, and #1471 appear to be almost identical, except the genotype #3631 showing a slight difference in growth development, and shape. Acclimatization of carnation microplants has been reported to be at high precedence; at 90% for *D. caryophyllus* cultivars (Salehi 2006), 92% for *D. deltoides* (Popović et al. 2008), and 70% for *D. fruticosus* (Papafotiou and Stragas 2009).

The acclimatization of *D. petraeus* plants proved to be a successful routine procedure with high survival rate. Therefore, detailed measurements of each developmental stage and growth of plants were assumed to be unnecessary for investigation at this point of our research.

Acknowledgements: We thank the personnel of the Laboratory of Conservation and Evaluation of the Native and Floricultural Species for their valuable contribution in maintaining the *Dianthus petraeus* plants during *in vivo* acclimatization. We also thank the editor and the anonymous reviewers who have contributed to improve the presentation of our article. The expenses of publication were covered from the company THEOFRASTOS - Liquid Fertilizers, (3-5 Velerefonti, GR-20100 Korinthos, Greece). This project was partially supported from the Greek State Scholarships Foundation as a Postdoctoral grant to Dr. Georgios Tsoktouridis.

REFERENCES

Berardi G., Roncasaglia R., Scaravelli D., Dradi G. (2006). *In vitro* propagation of *Dianthus balbisii* Ser. Subsp. *liburnicus* (Bartl.) Pign. by shoot tip culture. Acta Horticulturae, 725: 427-430.

Casas J. L., Olmos E., Piqueras A. (2010). *In vitro* propagation of carnation (*Dianthus caryophyllus* L.). *In:* Jain S. M., Ochatt S. J. (Eds). Protocols for *In Vitro* Propagation of Ornamental Plants. Methods in Molecular Biology, 589: 109-116.

Cristea V., Brummer A.T., Jarda L., Miclăuș M. (2010). *In vitro* culture initiation and phytohormonal influence on *Dianthus henteri* – a Romanian endemic species. Romanian Biotecnology Letters, 15: 25-33.

Cristea V., Pușcaș M., Miclăuș M., Deliu C. (2006). Conservative micropropagation of some endemic or rare species from the *Dianthus* genus. Acta Horticulturae, 725: 357-364.

Grigoriadou K., Maloupa E. (2008). Micropropagation and salt tolerance of *in vitro* grown *Crithmum maritinum* L. Plant Cell, Tissue and Organ Culture, 94: 209-217.

Jain A., Kantia A., Kothari S. L. (2001). *De novo* differentiation of shoot buds from leaf-callus of *Dianthus caryophyllus* L. and control of hyperhydricity. Scientia Horticulture, 87: 319-326.

Maloupa E., Krigas N., Grigoriadou K., Lazari D., Tsoktouridis G. (2008). Conservation strategies for native plant species and their sustainable exploitation: Case of the Balkan botanic garden of Kroussia, N Greece, *In:* Teixeira da Silva J. A. (Ed.). Floriculture and Ornamental Plant Biotechnology: Advances and Topical Issues, vol. V (4), Global Science Books, Isleworth, UK: 37-56.

Miclăuș M., Cristea V., Deliu C. (2003). Micropropagation on *Dianthus petraeus* W. et K. ssp. *simonkaianus* (Péterfi) Tutin. Contribuții Botanice, XXXVIII: 77-84.

Murashige T., Skoog F. (1962). A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Plant Physiology, 15: 473-497.

Nontaswatsri C., Fukai S., Touma T., Goi M. (2002). Comparison of adventitious shoot formation from node and leaf explants of various carnation (*Dianthus caryophyllus* L.) cultivars. Journal of Horticultural Science and Biotechnology, 77: 520-525.

Nontaswatsri C., Ruamrungsri S., Fukai S. (2008). Callus induction and plant regeneration of *Dianthus chinensis* L. and *Dianthus barbatus* L. via anther culture. Proceedings of the International Workshop of Ornamental Plants, 788: 109-114.

Papafotiou M., Stragas J. (2009). Seed germination and *in vitro* propagation of *Dianthus fruticosus* L. Acta Horticulturae, 813: 481-484.

Paramesh T. H., Chowdhury S. (2005). Impact of explants and gamma irradiation dosage on *in vitro* mutagenesis in carnation (*Dianthus caryophyllus* L.). Journal of Applied Horticulture, 7: 43-45.

Popović M., Grbić M., Marković M. (2008). Propagation of *Dianthus deltoides* L. by shoot culture. Bulletin of the Faculty of Forestry, 97: 209-220.

Salehi H. (2006). Can a general shoot proliferation and rooting medium be used for a number of carnation cultivars? African Journal of Biotechnology, 5: 25-30.

Sriskandarajah S., Prinsen E., Motyka V., Dobrev P., Serek M. (2006). Regenerative capacity of Cacti Schlumbergera and Rhipsalidopsis in relation to endogenous phytohormones, cytokinin oxidase/dehydrogenase, and peroxidase activities. Journal of Plant Growth Regulators, 25: 79-88.

Steel R. G. D., Torrie J. H., Dickey D. A. (1997). Principles and procedures of statistics: a biometrical approach. 3rd edn. Mc Graw-Hill, New York, 666 pp.

Toothaker L. (1993). Multiple Comparison Procedures. Sage Publications Inc., Newbury Park, California, 104 pp.

Walter K. S., Gillett H. J. (1998). 1997 IUCN Red List of Threatened Plants. Compiled by the World Conservation Monitoring Centre. Gland, Switzerland: IUCN – The World Conservation Union: 122-124.

Watad A. A., Ahroni A., Zuker A., Shejtman H., Nissim A., Vainstein A. (1996). Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. Scientia Horticulture, 65: 313-320.

Wyse-Jackson P. S., Sutherland. L. A. (2000). International Agenda for Botanic Gardens in Conservation. Richmond: Botanic Gardens Conservation International, London, 58 pp.