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Original Scientific Paper

Micropropagation and *ex situ* conservation of three rare and endemic ornamental *Dianthus* taxa (Caryophyllaceae)

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ABSTRACT:
The conservation of numerous plant taxa of spontaneous flora which are endemic, endangered, vulnerable or rare is realized by *in situ* or *ex situ* methods. The biotechnology of *in vitro* cultures is an important part of these programmes. The aim of the study was the *in vitro* proliferation and rooting reactivity of three rare species of wild origin carnations (*Dianthus* genus) with limited distribution and conservation priority: the endangered and rare-restricted *D. ingoldbyi* (a local endemic from some regions of the Balkan peninsula, Turkey and North-East Greece), the Cretan local Greek endemic, range-restricted and endangered *D. juniperinus* subsp. *bauhinorum,* and the least concern or near threatened range-restricted Greek endemic of the Cyclades, Peloponnese and Ionian islands *D. fruticosus* subsp. *occidentalis*. In all *Dianthus* taxa, using shoot-tips as explants and NaClO as the sterilizing agent (2%) a 50-90% sterilization rate was reported. In *D. fruticosus* subsp. *occidentalis* (2 shoots/ explant 18.78 mm long) a 100% shoot regeneration rate was registered in the presence of the combination of 0.25 mg/L BA, 0.1 mg/L IBA, and 0.1 mg/L GA_3 (5 weeks), whereas *D. juniperinus* subsp. *bauhinorum* responded best (100% shoot proliferation, with 3.5 shoots, 22.38 mm long) in the presence of 0.5 mg/L BA + 0.1 mg/L NAA (4 weeks). *Dianthus ingoldbyi* yielded better performance i.e. 5.5 shoots/ explant 34.18 mm long but with a 28.33% hyperhydricity rate in the initial MS medium enriched with 0.25 mg/L BA, 0.1 mg/L IBA, 0.1 mg/L GA_3 as well as in the PGR-free MS medium during the subsequent shoot multiplication stage (100% shoot proliferation, 3.88 shoots 62.09 mm long, absence of hyperhydricity) (5 weeks). Among the three tested auxins (IBA, NAA, IAA), NAA at 0.25 mg/L induced better rooting (100%, 9.75 roots/rooted microshoot 32.47 mm long) in *D. juniperinus* subsp. *bauhinorum* (5 weeks), whereas both *D. fruticosus* subsp. *occidentalis* (5.48 roots 20.51 mm long) and *D. ingoldbyi* (6.89 roots 23.93 mm long) showed the best rooting results (100%) on the MS auxin-free medium (4 weeks). After 3-4 weeks, the survival rates of the *ex vitro* acclimatized plants in the heated greenhouse mist in a peat: perlite (1:1 v/v) substrate were 100%, 90% and 50% for *D. ingoldbyi, D. fruticosus* subsp. *occidentalis*, and *D. juniperinus* subsp. *bauhinorum*, respectively.

Keywords:
auxins, cytokinins, *in vitro*, endangered, wild carnations

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INTRODUCTION

The *Dianthus* genus is characterized by a high rate of endemic species and is of considerable phytogeographic importance due to the fact that over half of its species occur in small geographically restricted ranges. Nowadays, conservative actions regarding genetic resources are focused both on the economic importance of plants and on general efforts towards the protection of spontaneous gene funds, especially for those species which are endangered by man's destructive intervention. Many of the taxa included in the *Dianthus* genus can be found on national and European Red Lists (Ciocârlan 2000). In vitro biotechnology can successfully meet the needs for the conservation of these rare-endangered endemic species comprising not only the conservation of plant genetic resources, but also their management, characterization, and application (sustainable use) (Coelho *et al.* 2020).

Dianthus fruticosus L. subsp. *occidentalis* Runemark is a plant up to 30 cm tall, which thrives in crevices and limestones at altitudes of 0-200 m and blooms from July to October. It can be used as a ground cover plant to stabilize slopes and restore degraded landscapes in the Mediterranean region (Papafotiou & Stragas 2009). *Dianthus fruticosus* subsp. *occidentalis* is a range-restricted herbaceous perennial and chamaephyte Greek endemic, distributed throughout the Ionian Islands, Crete, Karpathos and the Peloponnese, growing on cliffs, rocks, walls, ravines and boulders, characterized as being of least concern (LC) or near threatened (NT) according to IUCN categories A&B (Taxonomy according to Botanika Chronika: Kefalonia) and is also protected by Greek legislation (Presidential Decree 67/1981) (Dimopoulos *et al.* 2016). *Dianthus ingoldbyi* Turill. is a local species of the genus which grows in some regions of the Balkan Peninsula, Turkey, and North-East Greece (Trigas *et al.* 2007). It is a caespitose perennial, 30 cm tall with a woody stock and stems puberulent below. *Dianthus ingoldbyi* is an important range-restricted local endemic hemicryptophyte of the Eastern Mediterranean region (present in North-East Greece), growing in coastal habitats, included in the Red Data Book of Greece, characterized as endangered (EN) according to both the national (Flora Hellenica) and overall IUCN category with conservation priority type 2, and is protected by Presidential Decree 67/1981 (Dimopoulos *et al.* 2016). *Dianthus juniperinus* subsp. *bauhinorum* (Greuter) Turland is a range-restricted local Greek endemic chamaephyte which grows in habitats such as cliffs, rocks, walls, ravines, and boulders, distributed in Crete and Karpathos, characterized as endangered (EN) according to the overall IUCN A&B category (Flora Hellenica) and is protected by Greek law (Presidential Decree 67/1981) (Dimopoulos *et al.* 2016).

Several studies concerning *in vitro* multiplication of some of Europe's endemic and/or endangered *Dianthus* taxa have been conducted: *D. pyrenaicus* Pourr. (Marcu *et al.* 2006); *D. giganteus* d'Urv., *D. alpinus* L., *D. ferrugineus* Mill., *D. gallicus* Pers. (Cristea *et al.* 2006); *D. petraeus* Waldst. & Kit. subsp. *simonkaianus* (Péterfi) Tutin (Micläuş *et al.* 2003); *D. nardiformis* Janka (Holobiuc *et al.* 2009); *D. henteri* Heuff. ex Griseb. & Schenk (Cristea *et al.* 2010) and *D. giganteus* d'Urv. subsp. *banaticus* (Heuff.) Tutin (Cristea *et al.* 2006). Adventitious shoot regeneration in carnations was affected by the culture environment, the combination of plant growth regulators and the ratio and type of explant (Casanova *et al.* 2008). The micropropagation of carnations is influenced by the type of cytokinin (BA, Kin, TDZ, 2-ip) and the concentration used (Brar *et al.* 1995; Ali *et al.* 2008). Regenerated shoots from axillary buds and shoot tips are true-to-type because their regeneration process does not involve the callus phase (Brar *et al.* 1995).

Regeneration from *in vitro* germinated seeds in the genus *Dianthus* is feasible, but may be difficult because these rare plants grow in inaccessible habitats. An alternative method is *in vitro* culture of different explants collected from native plants (Ali *et al.* 2008; Cristea *et al.* 2010; Holobiuc *et al.* 2010a).

The aim of this study was to elaborate an efficient micropropagation protocol for three endangered *Dianthus* taxa, evaluating the effect of different plant growth regulators on root regeneration in shoot-tip explants.

MATERIALS AND METHODS

Collection of plant material. The botanical collection was performed using a special permit from the Balkan Botanic Garden of Kroussia (BBGK), which is issued annually by the Greek Ministry of Environment and Energy. In the case of *D. fruticosus* subsp. *occidentalis*, the seeds and fresh-soft cuttings were collected in the middle of May 2006 from wild-growing populations found as maquis and chasmophyte vegetation on calcareous substrates in Myrtos (Altitude: 0 m, Latitude: N 38°20'50", Longitude: E 20°32'18") on the island of Kefalonia (Greece). In the case of *D. ingoldbyi*, the seeds and fresh-soft cuttings were collected in late August 2008 from wild-growing populations found as maquis in sandy and rocky habitats in Maroneia (Altitude: 20 m, N 40°52'07" E 25°31'47") in the Rhodope region (Greece). In the case of *D. juniperinus* subsp. *bauhinorum*, the seeds and fresh-soft cuttings were collected in the middle of May 1999 from wild-growing populations found in limestone crevices and ledges, at 200-1800 m on the island of Crete (Greece). The collected material (seeds and cuttings) for all three *Dianthus* species was transferred to the facilities of the BBGK in Thessaloniki (Thermi) and received an International Plant Exchange Network (IPEN) accession number (*D. fruticosus* subsp. *occidentalis*: GR-1-BBGK-06,3309, *D. ingoldbyi*: GR-1-BBGK- 08,5046-2 and *D. juniperinus* subsp. *bauhinorum:* GR-1-BBGK-99877).

For the purposes of desiccation, the seeds from the three *Dianthus* species were maintained for 80 days in a dark chamber at 15°C and relative humidity (RH) of 15% after their collection from the wild. For long-term storage (4-5 years), the seeds were stored in sealed containers within a seed bank at 4°C and RH <5%. The seeds were sterilised using Signum fungicide solution (0.1 g/ 100 ml ddH_2O) stirred for 30 min, followed by 70% ethanol for 1 min and 5% sodium hypochlorite (NaOCl) for 5 min and five rinses with sterile ddH_2O per each sterilisation agent. The seeds were placed under aseptic conditions inside a laminar flow hood in Magenta vessels (200 ml volume) containing 35 mL of MS medium enriched with 20 g/L sucrose and 6 g/L Plant Agar (pH: 5.8), and were transferred to a growth chamber at 22 ± 1°C adjusted to a 16 h photoperiod and 40 μmol/m^2/s light intensity.

The initial cuttings of the three *Dianthus* species collected from the wild were treated with 0.2% IBA (powdered formula under the commercial name Radicin) and they were placed for a 7-week period on a heated bench under mist at 19 ± 2°C and RH 80–90%. The rooted cuttings were then transplanted into a larger pot (0.33 L) containing a mixture of a more enriched peat moss (TS2, Klasmann): perlite mixture at a 3:1 v/v ratio. After four months, the plants were transplanted into a larger pot (2.5 L) containing a mixture of peat (Klasmann, TS2), perlite and soil (2:1: ½ v/v) to continue growing. Following this process, adequate stock material (mother plants) was established for further experiments, genetically identical to the origin wild plant. The excess plant material was planted in the grounds of the Balkan Botanic Garden of Kroussia at sea level in Thermi, Thessaloniki for long-term ex situ conservation purposes.

In the three *Diathus* species, the mother plants created via rooting of the collected cuttings constituted the initial material for disinfection and initial establishment in vitro of shoot-tip explants which were cut and removed from the mother plants. The choice of using shoot-tip explants from rooted cuttings derived via vegetative propagation rather than seedlings derived via the germination of seeds in vitro was based on the one hand on the production of clonal plants (asexually propagated), and on the other on the genetic variability imparted to the seedlings (sexually propagated). In the case of seeds, only their germination ability was evaluated.

I. *In vitro* culture establishment-disinfection (1st micropropagation) stage. In the case of *D. ingoldbyi*, in late-February, 1-1.5 cm long juvenile shoot tips were disinfected following immersion in a 70% ethanol solution for 1 min and subsequently in a 2% NaOCl solution for 10 min. For *D. fruticosus* subsp. *occidentalis*, 40 1-2 cm long shoot-tips were used as explants (mid October). The disinfection protocol used involved their immersion in a fungicide Signum solution for 30 min (0.07%) followed by a 70% ethanol (1 min) and 3% NaOCl solution (20 min). For *D. juniperinus* subsp. *bauhinorum*, 40 shoot-tip explants 1.5-2.5 cm long (mid October) were used for the initiation of the culture. The disinfection protocol used involved their immersion for 30 min in a fungicide solution [(0.070 g Signum/ 100 ml dd H_2O)] followed by a 70% ethanol solution (1 min) and afterwards a 3% NaOCl solution (15 min). The basal culture medium used in all three *Dianthus* species for the initial establishment stage after disinfection was the MS (Murashige & Skoog 1962) supplemented with 30 g/L sucrose, 0.25 mg/L 6-benzyladenine (BA), 0.1 mg/L indole-3-butyric acid (IBA), 0.1 mg/L gibberellic acid (GA_3), and 6 g/L Plant Agar (pH=5.8). The culture medium was sterilized in an autoclave at 121°C and 1.5 atm pressure for 20 min. After disinfection, an *in vitro* stock plant material for all three *Dianthus* taxa was successfully established. Further experiments were conducted in micropropagation stages (shoot proliferation and rooting) using different plant growth regulators (PGR's) categories (cytokinins, auxins), types and concentrations to establish the most effective propagation protocol for each *Dianthus* taxon.

II. *In vitro* shoot proliferation (2nd micropropagation) stage. In all three *Dianthus* taxa, the experimental material in this stage consisted of the shoot tip explants (1-2 cm long) derived from previous *in vitro* stock cultures sub-cultured successively every 4 weeks on a full strength PGR-free MS basal culture medium. In the case of *D. fruticosus* subsp. *occidentalis,* the individual effect of three cytokinin types, i.e. BA, kinetin (KN) and 2-isopentenyl-adenine (2-ip) each applied at a concentration of 0.25 mg/L simultaneously with three auxin types; IBA, a-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA), each applied at 0.1 mg/L on a MS medium enriched with 20 g/L sucrose and 6 g/L Plant Agar (pH:5.8) was studied (5 weeks). For *D. juniperinus* subsp. *bauhinorum*, the individual effect of cytokinin BA applied at two concentrations (0.25 and 0.5 mg/L) in combination with two different auxin types (NAA, IAA) at two concentrations (0.05 and 0.1 mg/L) on a MS medium enriched with 30 g/L sucrose and 7 g/L Plant Agar was studied (pH: 5.8) (4 weeks). In the case of *D. ingoldbyi*, a MS (PGR-free) basal medium supplemented with 20 g/L sucrose and 6 g/L Plant Agar (pH=5.8) (5 weeks) was used. The *in vitro* response was evaluated using three parameters: the rate of shoot multiplication (%) - the explants which regenerated/the total number of explants × 100, the mean number of shoots/explants, the mean shoot length (mm/initial explant) and the percentage of chlorosis (if applicable).

III. *In vitro* rooting (3rd micropropagation) stage. In the case of both *D. ingoldbyi* and *D. fruticosus* subsp. *occidentalis* the proliferated shoot tips were cultured on a MS PGR-free medium with the addition of 20 g/L sucrose and 6 g/L Plant Agar (pH: 5.8) for a 4-week culture

period. For *D. juniperinus* subsp. *bauhinorum*, the individual effect of three different auxin types (IBA, NAA, IAA) each applied at two concentrations (0.1 and 0.25 mg/L) on a MS PGR-free medium enriched with 30 g/L sucrose and 7 g/L Plant Agar (pH: 5.8) was studied after 5 weeks of culture. Magenta vessels (35 mL medium/ vessel) were used for rooting. Recordings were made for the rooting rate (%) - the number explants which rooted/ the total of inoculated explants × 100, the mean number of roots/rooted microshoot and the mean root length (mm).

IV. *Ex vitro* acclimatization of *in vitro* plantlets (4th micropropagation) stage. The rooted microshoots of all three *Dianthus* species, during spring for *D. ingoldbyi* and in autumn for both *D. fruticosus* subsp. *occidentalis* and *D. juniperinus* subsp. *bauhinorum,* were planted in multiple (N = 84) bags, in a soil mixture of peat (Terrahum) and perlite at a ratio of 1:1 v/v and transferred to an indoor heated misting system in the greenhouse (at 18-21°C base temperature, 15-25°C air temperature and 70-85% relative humidity for 3 weeks) in conditions of reduced light intensity (thermal curtains). After this period, the plants were transplanted in larger volume pots (0.33 L) in a substrate containing peat moss (TS2): perlite (Geoflor): soil (2: ½: ½ v/v) respectively and placed on a greenhouse bench (temperature range 17-24°C and RH 40%-85%) under conditions of a gradual decrease in RH (5%/day) and an increase in light intensity. After a 3-month further growth period, the plants were transplanted in larger volume pots (2.5 L) in a substrate containing peat (TS2): perlite: soil (2: ½: ½ v/v) and transferred to the natural environment (outside the greenhouse) in the nursery under a 50% shade net.

Statistical analysis. The experimental design was completely randomized. The means were subjected to analysis of variance (ANOVA) and compared using Duncan's multiple-range test ($P < 0.05$). For *D. juniperinus* subsp. *bauhinorum*, the first proliferation experiment included 13 treatments with 8 repetitions (explants)/treatment (4 explants/vessel × 2 vessels/treatment), while the second one included 9 treatments with 8 repetitions/treatment (4 explants/vessel × 2 vessels/ treatment). In the case of *D. juniperinus* subsp. *bauhinorum*, the rooting experiment included 7 treatments with 8 repetitions/treatment (4 explants/vessel × 2 vessels/treatment). For *D. fruticosus* subsp. *occidentalis*, the proliferation experiment included 8 treatments with 9 repetitions/treatment (3 explants/vessel × 3 vessels/treatment). In the case of both *D. fruticosus* subsp. *occidentalis* and *D. juniperinus subsp. bauhinorum,* the proliferation experiments were 3×3 factorial with three cytokinin types (BA, KN, 2-ip) and three auxin types (IBA, NAA, IAA). The main effect of the factors (cytokinin type, auxin type) and their interaction was determined by a general linear model/2-way ANOVA. For *D. juniperinus* subsp. *bauhinorum,* the rooting experiment was 3×3 factorial with three auxin types (IBA, NAA, IAA) and three auxin concentrations (0, 0.1 and 0.25 mg/L). The main effect of the factors (auxin type, auxin concentration) and their interaction was determined by a general linear model/ 2-way ANOVA. For *D. juniperinus* subsp. *bauhinorum,* the second shoot proliferation experiment was not multi-factorial and the means were subjected to one-way ANOVA.

RESULTS

***In vitro* seed germination.** In *D. fruticosus* subsp. *occidentalis*, 5-year old seeds (n=44) (based on their storage period in the seed bank at 4°C and RH <5%) yielded a maximum 56.67% germination rate after 50 days of culture on MS medium (t_{50} value: 22th day). In both *D. ingoldyi* (n=17) and *D. juniperinus* subsp. *bauhinorum* (n=50), 4-year-old seeds exhibited maximum germination rates of 94.12% and 70% after 6 days (t_{50}: 4th day) and 32 days of culture (t_{50}: 5th day) on MS PGR-free medium respectively.

***In vitro* culture establishment-(Disinfection stage).** In *D. fruticosus* subsp. *occidentalis*, after a 2-week period the disinfection protocol resulted in a 80-90% sterilization rate, i.e. only 10-20% of the explants were contaminated. In *D. juniperinus* subsp. *bauhinorum*, 82.5% sterilization after 2 weeks of culture was recorded following the disinfection protocol, while in *D. ingoldyi* 50% of the plant material was successfully disinfected. After 5 weeks of culture on the MS basal medium supplemented with 30 g/L sucrose, 0.25 mg/L BA, 0.1 mg/L IBA, 0.1 mg/L GA_3, and 6 g/L Plant Agar (pH=5.8), *D. fruticosus* subsp. *occidentalis* yielded a 100% shoot multiplication rate, 2 shoots per explant and 18.78 mm mean shoot length. Accordingly, *D. juniperinus* subsp. *bauhinorum* explants exhibited a 43.33% shoot multiplication rate, 1.13 shoots per explant and 17.5 mm mean shoot length. In the case of *D. ingoldyi*, a 100% shoot multiplication rate was recorded with the production of 5.5 shoots per explant and 34.18 mm mean shoot length, but with 28.33% of the explants showing mild hyperhydricity symptoms at the same time (data not shown).

***In vitro* shoot proliferation.** In the first proliferation experiment using *D. juniperinus* subsp. *bauhinorum,* among the 3 cytokinins (BA, KN, 2-ip), only BA applied alone and in combination with auxins (IBA, NAA, IAA) led to multiple shoot induction after 5 weeks of culture on MS medium, whereas the other 2 cytokinins did not have such an impact. In particular, the mean shoot numbers were higher (2.5-2.75 shoots/explant) with the addition of either 0.25 mg/L BA alone or 0.25 mg/L BA + 0.05 mg/L NAA with a non-significant difference, while the shoot multiplication rate was optimum (100%) un-

Table 1. Effect of three cytokinin types (BA, KN, 2-ip) each applied at 0.25 mg/L in combination with 3 auxin types (IBA, NAA, IAA) each applied at 0.05 mg/L on *in vitro* shoot proliferation in *Dianthus juniperinus* subsp. *bauhinorum* after 5 weeks of culture.

Treatments (mg/L)	Mean shoot number/ explant	Mean shoot length (mm)	Shoots multiplication rate (%)
Control (PGRs-free)	1.25 ± 0.25 b	10.63 ± 2.13 c	25 d
0.25 BA	2.75 ± 0.75 a	19.69 ± 1.07 abc	75 b
0.25 BA + 0.05 IBA	2.00 ± 0.58 ab	15.84 ± 3.37 abc	50 c
0.25 BA + 0.05 NAA	2.50 ± 0.65 a	17.50 ± 3.23 abc	75 b
0.25 BA + 0.05 IAA	2.00 ± 0.00 ab	14.38 ± 1.20 bc	100 a
0.25 KN	1.00 ± 0.00 b	25.00 ± 4.56 a	0 e
0.25 KN + 0.05 IBA	1.00 ± 0.00 b	17.50 ± 1.44 abc	0 e
0.25 KN + 0.05 NAA	1.00 ± 0.00 b	23.75 ± 3.75 ab	0 e
0.25 KN + 0.05 IAA	1.00 ± 0.00 b	18.75 ± 2.39 abc	0 e
0.25 2-ip	1.00 ± 0.00 b	16.67 ± 3.12 abc	0 e
0.25 2-ip + 0.05 IBA	1.00 ± 0.00 b	16.67 ± 3.12 abc	0 e
0.25 2-ip + 0.05 NAA	1.00 ± 0.00 b	20.00 ± 5.40 abc	0 e
0.25 2-ip + 0.05 IBA	1.00 ± 0.00 b	11.67 ± 1.18 c	0 e
p-values			
Cytokinin type (A)	0.000 ***	0.007 **	0.000 ***
Auxin type (B)	0.723 ^{ns}	0.077 ^{ns}	0.000 ***
(A) * (B)	0.845 ^{ns}	0.872 ^{ns}	0.000 ***

Means ± standard error with the same letter in each column denotes that there are no statistically significant differences according to Duncan's multiple range test at $P \leq 0.05$. $^{ns}P \geq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$.

PGRs - plant growth regulators; BA - 6-benzyladenine; IBA - indole-3-butyric acid; NAA - α -naphthalene acetic acid; IAA - indole-3-acetic acid; KN - kinetin; 2-ip - 2-isopentenyl adenine.

der the combined effect of 0.25 mg/L BA + 0.05 mg/L IAA. The best results in terms of the shoot multiplication rate (100%) and the mean shoot number (2 shoots/ explant) at the same time were recorded in the combined treatment of 0.25 mg/L + 0.05 mg/L IAA, whereas the mean values of the shoot length were greater (25 mm) with 0.25 mg/L KN followed by 23.75 mm obtained with 0.25 mg/L KN + 0.05 mg/L NAA without a significant difference (Table 1; Fig. 1A-M). In the subsequent (2nd) proliferation experiment conducted on *D. juniperinus* subsp. *bauhinorum,* a 100% shoot multiplication rate was obtained with the following 3 treatments: (A) 0.5 BA + 0.05 NAA, (B) 0.5 BA + 0.05 IAA and (C) 0.5 BA + 0.1 NAA (mg/L) after 4 weeks of culture on MS medium.

Table 2. Effect of BA (0.25, 0.5 mg/L) combined with two auxins (NAA, IAA) (0.05 and 0.1 mg/L) on *in vitro* shoot proliferation of *Dianthus juniperinus* subsp. *bauhinorum* (4 weeks of culture).

Treatments (mg/L)	Mean shoot number/ explant	Mean shoot length (mm)	Shoots multiplication rate (%)
Control (PGRs-free)	1.13 ± 0.13 d	14.38 ± 2.40 c	12.5 g
0.25 BA	2.50 ± 0.53 b	16.07 ± 1.56 bc	62.5 d
0.25 BA + 0.05 NAA	1.88 ± 0.52 bcd	12.81 ± 0.88 c	37.5 f
0.25 BA + 0.05 IAA	2.29 ± 0.25 bc	20.12 ± 2.26 ab	85.71 b
0.5 BA	2.00 ± 0.19 bcd	14.79 ± 1.34 c	75 c
0.5 BA + 0.05 NAA	3.75 ± 0.22 a	19.56 ± 0.73 ab	100 a
0.5 BA + 0.05 IAA	2.50 ± 0.13 b	16.04 ± 0.99 bc	100 a
0.5 BA + 0.1 NAA	3.50 ± 0.30 a	22.38 ± 0.48 a	100 a
0.5 BA + 0.1 IAA	1.50 ± 0.13 cd	14.38 ± 1.79 c	50 e
p-values	0.000 ***	0.000 ***	0.000 ***

Means ± standard error with the same letter in each column denotes that there are no statistically significant difference from each other according to Duncan's multiple range test at $P \leq 0.05$. ***$P \leq 0.001$.

PGR's - plant growth regulators; BA - 6-benzyladenine; NAA - α -naphthalene acetic acid; IAA - indole-3-acetic acid.

The mean shoot values were higher (3.75 and 3.5) in the case of treatments with 0.5 BA + 0.05 NAA and 0.5 BA + 0.1 NAA, respectively. The combined effect of 0.5 BA + 0.1 NAA gave the highest shoot length (22.38 mm). Even though the simultaneous application of 0.5 BA + 0.05 NAA resulted in effective shoot proliferation, it also caused mild hyperhydricity in 30% of the explants. Satisfactory proliferation but with the production of multiple lateral dwarf shoots (< 0.5 cm) was recorded when the medium was supplemented with 0.5 BA + 0.05 IAA. Among the different treatments, the 0.5 BA + 0.1 NAA combination resulted in a better appearance, robustness, vigor and vegetative growth of the explants (Table 2; Fig. 2A-I). Between the two proliferation experiments, the optimum shoot proliferation, in terms of the regeneration rate (100%), shoot number (3.5 shoots/ explant), mean shoot length (22.38 mm), culture period duration (4 or 5 weeks) and appearance or absence of vitrification at the same time, was obtained after 4 weeks of culture on MS medium enriched with 0.5 mg/L BA + 0.1 mg/L NAA (Tables 1 & 2).

In *D. fruticosus* subsp. *occidentalis,* the combined effect of 0.25 mg/L BA + 0.1 mg/L NAA substantially stimulated the growth of the explants (an average shoot

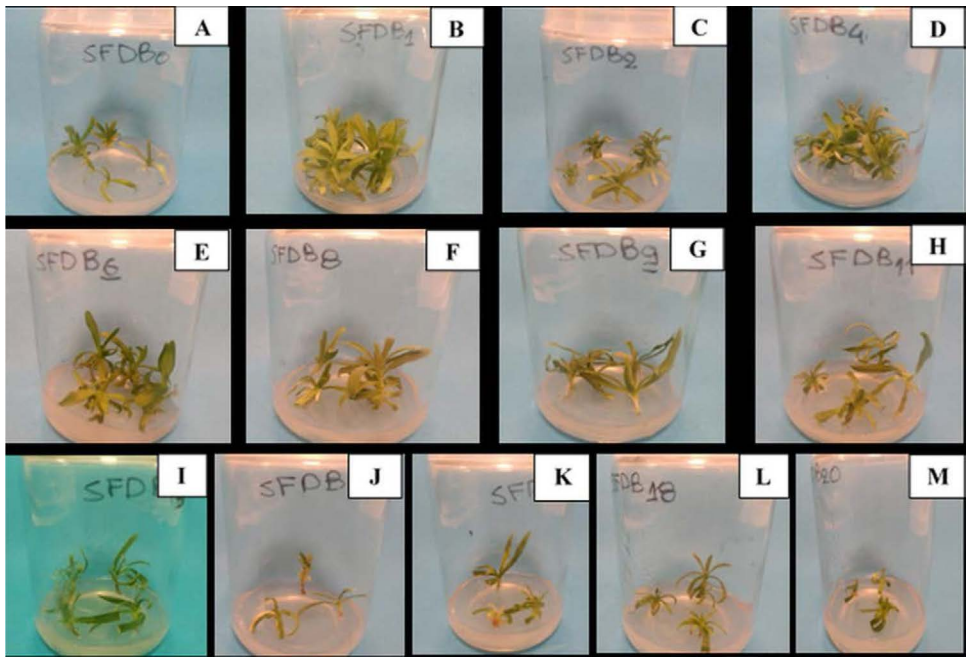

Fig. 1. *In vitro* shoot proliferation of *Dianthus juniperinus* subsp. *bauhinorum* after 5 weeks of culture in MS medium with different PGRs (mg/L) including control (PGR-free) **(A)**, 0.25 BA **(B)**, 0.25 BA + 0.05 IBA **(C)**, 0.25 BA + 0.05 NAA **(D)**, 0.25 BA + 0.05 IAA **(E)**, 0.25 KN **(F)**, 0.5 KN + 0.05 IBA **(G)**, 0.5 KN + 0.05 NAA **(H)**, 0.25 KN + 0.05 IAA **(I)**, 0.25 2-ip **(J)**, **(K)** 0.5 2-ip + 0.05 IBA **(K)**, 0.5 2-ip + 0.05 NAA **(L)** and 0.25 2-ip + 0.05 IAA **(M)**. Abbreviations: PGRs = plant growth regulators; BA = 6-benzyladenine; IBA = indole-3-butyric acid; NAA = α -naphthalene acetic acid; IAA = indole-3-acetic acid; KN = kinetin; 2-ip = 2-isopentenyl adenine.

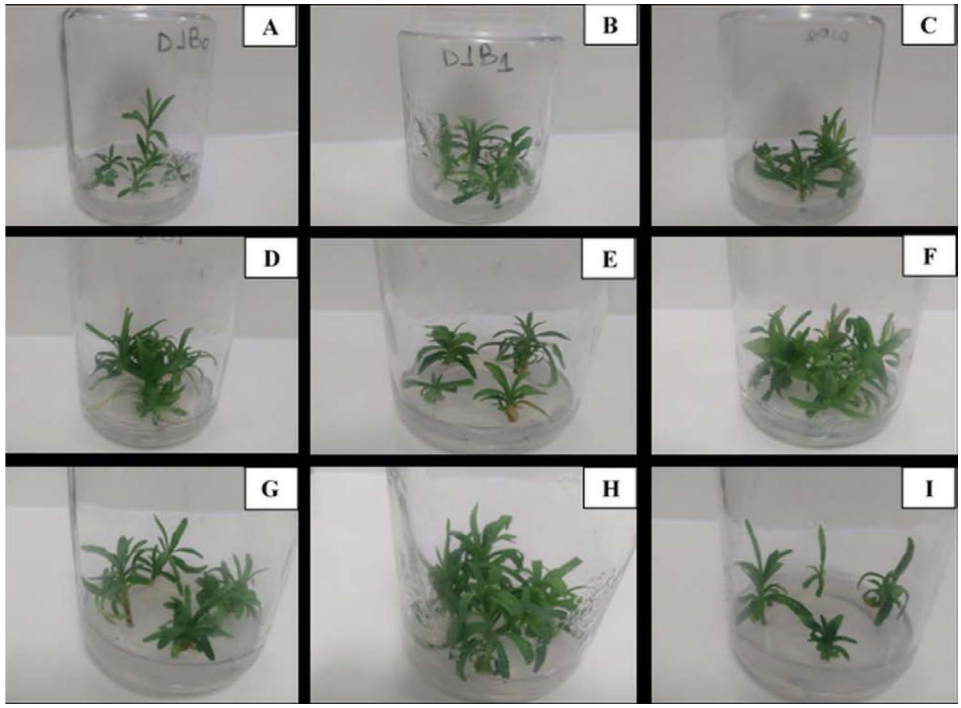

Fig. 2. *In vitro* shoot proliferation of *Dianthus juniperinus* subsp. *bauhinorum* after 4 weeks of culture in MS medium with different PGRs (mg/L) including control (PGR-free) **(A)**, 0.25 BA **(B)**, 0.25 BA + 0.05 NAA **(C)**, 0.25 BA + 0.05 IAA **(D)**, 0.5 BA **(E)**, 0.5 BA + 0.05 NAA **(F)**, 0.5 BA + 0.05 IAA **(G)**, 0.5 BA + 0.1 NAA **(H)** and 0.5 BA + 0.1 IAA **(I)**. Abbreviations: PGRs = plant growth regulators; BA = 6-benzyladenine; NAA = α -naphthalene acetic acid; IAA = indole-3-acetic acid.

length of 20 mm) by 0.5-1 cm and completely inhibited the occurrence of chlorotic explants, therefore this treatment was the optimum for the shoot elongation phase following the initial shoot induction (Table 3; Fig. 3B). Shoot multiplication (with a 33.33% rate) was achieved in the case of the treatment supplemented with 0.25 mg/L BA + 0.1 mg/L IBA, but with the simultaneous appearance of chlorosis in 66.67% of the explants. Among the 3 cytokinins (BA, KN, 2-ip) tested, 2-ip caused total chlorosis in 100% of the explants and did not allow multiple shoot induction (Table 3).

In *D. ingoldbyi*, after 5 weeks of culture in the proliferation medium consisting of the MS basal medium (PGR-free) enriched with 20 g/L sucrose and 6 g/L Plant Agar (pH: 5.8), 100% of the explants successfully multiplied with the production of 3.88 shoots per explant

Table 3. Effect of three cytokinin types (BA, KN, 2-ip) each applied at 0.25 mg/L with three auxin types (IBA, NAA, IAA) each applied at 0.1 mg/L on *in vitro* shoot proliferation of *Dianthus fruticosus* subsp. *occidentalis* after 5 weeks of culture.

Treatments (mg/L)	Mean shoot number/ explant	Mean shoot length (mm)	Shoots multiplication rate (%)	Chlorosis rate (%)
0.25 BA + 0.1 IBA	1.33 ± 0.24 a	13.33 ± 1.18 bc	33.33 a	66.67 b
0.25 BA + 0.1 NAA	1.00 ± 0.00 b	20.00 ± 3.54 a	0 b	0 c
0.25 BA + 0.1 IAA	1.00 ± 0.00 b	16.67 ± 1.18 ab	0 b	0 c
0.25 KN + 0.1 IBA	1.00 ± 0.00 b	15.00 ± 0.00 abc	0 b	0 c
0.25 KN + 0.1 NAA	1.00 ± 0.00 b	11.67 ± 1.18 bc	0 b	0 c
0.25 KN + 0.1 IAA	1.00 ± 0.00 b	10.00 ± 0.00 c	0 b	66.67 b
0.25 2-ip + 0.1 IBA	1.00 ± 0.00 b	12.50 ± 2.50 bc	0 b	100 a
0.25 2-ip + 0.1 NAA	1.00 ± 0.00 b	10.00 ± 0.00 c	0 b	100 a
p-values				
Cytokinin type (A)	0.159 ^{ns}	0.002 **	0.000 ***	0.000 ***
Auxin type (B)	0.159 ^{ns}	0.523 ^{ns}	0.000 ***	0.000 ***
(A) * (B)	0.113 ^{ns}	0.019 *	0.000 ***	0.000 ***

Means ± standard error with the same letter in each column denotes that there are no statistically significant differences according to Duncan's multiple range test at $P \leq 0.05$. ^{ns} $P \geq 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$ *** $P \leq 0.001$.

BA - 6-benzyladenine; IBA = indole-3-butyric acid; NAA = α -naphthalene acetic acid; IAA = indole-3-acetic acid; KN - kinetin; 2-ip - 2-isopentenyl adenine.

Table 4. Effect of three auxin types (IBA, NAA, IAA) each applied at 3 concentrations (0, 0.1 and 0.25 mg/L) on *in vitro* rooting of *Dianthus juniperinus* subsp. *bauhinorum* after 5 weeks of culture.

Treatments (mg/L)	Mean root number/ rooted microshoot	Mean root length (mm)	Rooting rate (%)
Control (auxins-free)	8.00 ± 0.19 ab	30.26 ± 0.05 ab	25 e
0.1 IBA	8.40 ± 0.30 ab	34.50 ± 2.42 a	62.5 b
0.25 IBA	4.00 ± 0.19 c	24.84 ± 0.35 bc	25 e
0.1 NAA	6.20 ± 0.58 bc	36.87 ± 2.94 a	62.5 b
0.25 NAA	9.75 ± 1.58 a	32.47 ± 3.43 a	100 a
0.1 IAA	5.25 ± 0.91 c	22.81 ± 2.01 c	50 c
0.25 IAA	9.00 ± 0.87 a	35.06 ± 1.65 a	37.5 d
p-values			
Auxin type (A)	0.139 ^{ns}	0.035 *	0.000 ***
Auxin concentration (B)	0.061 ^{ns}	0.769 ^{ns}	0.000 ***
(A)*(B)	0.000 ***	0.000 ***	0.000 ***

Means ± standard error with the same letter in each column denotes that there are no statistically significant differences according to the Duncan's multiple range test at $P \leq 0.05$. ^{ns} $P \geq 0.05$; * $P \leq 0.05$; *** $P \leq 0.001$.

IBA - indole-3-butyric acid; NAA - α -naphthalene acetic acid; IAA - indole-3-acetic acid.

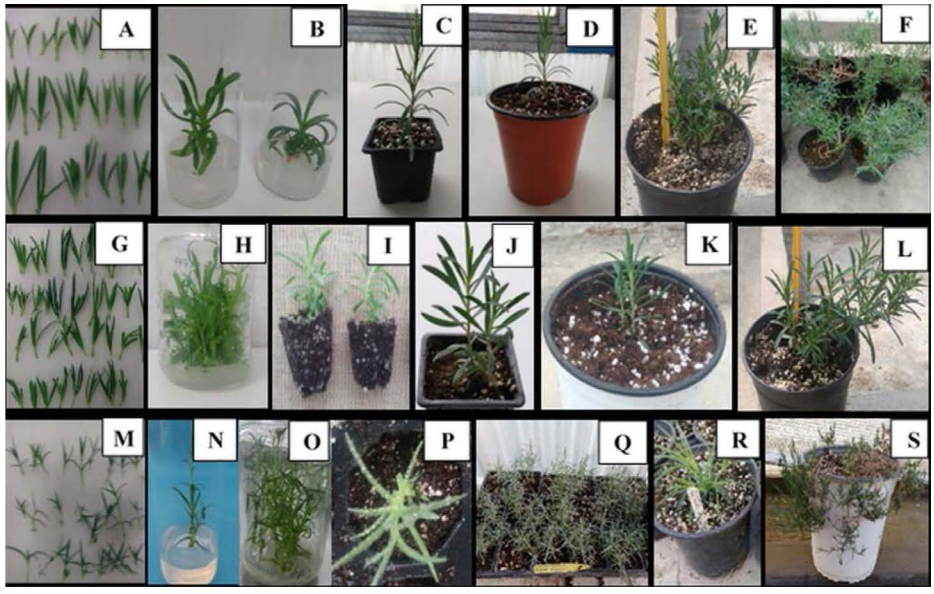

Fig. 3. Steps in the micropropagation protocols involving the disinfection, initial establishment, *in vitro* culture, multiplication, rooting, *ex vitro* acclimatization, and vegetative growth of three *Dianthus* species after transplantation to larger volume pots from the internal mist system, to a non-heated greenhouse bench and finally to the external environment in the nursery. *D. fruticosus* subsp. *occidentalis* **(A-F)**, *D. juniperinus* subsp. *bauhinorum* **(G-L)** and *D. ingoldbyi* **(M-S)**.

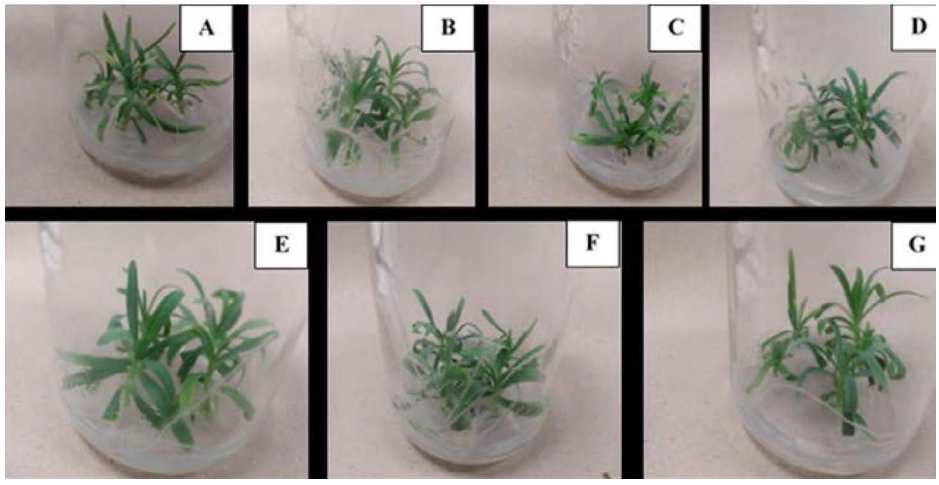

Fig. 4. *In vitro* rooting and proliferation of *Dianthus juniperinus* subsp. *bauhinorum* after 5 weeks of culture in MS medium with three auxin types applied in different concentrations (mg/L) including control (auxin-free) **(A)**, 0.1 IBA **(B)**, 0.25 IBA **(C)**, 0.1 NAA **(D)**, 0.25 NAA **(E)**, 0.1 IAA **(F)** and 0.25 IAA **(G)**. Abbreviations: IBA = indole-3-butyric acid; NAA = α -naphthalene acetic acid; IAA = indole-3-acetic acid.

and a 62.09 mm mean shoot length without the appearance of hyperhydricity (data not shown; Fig. 3N).

***In vitro* rooting.** In *D. juniperinus* subsp. *bauhinorum*, among the 3 auxins (IBA, NAA, IAA) used, NAA at 0.25 mg/L gave a 100% rooting rate, the maximum number of roots (9.75 roots/ rooted microshoot) and the best root development (32.47 mm length) (Table 4; Fig. 4A-G). In both *D. fruticosus* subsp. *occidentalis* (Fig. 3B) and *D. ingoldbyi* (Fig. 3O), a 100% rooting rate was obtained for shoot-tips cultured for 4 weeks in MS PGR-free medium supplemented with 20 g/L sucrose and 6 g/L Plant Agar (pH: 5.8). In *D. fruticosus* subsp. *occidentalis* 5.48 roots per rooted microshoot and a 20.51 mm mean root length were obtained, while *D. ingoldbyi* explants gave 6.89 roots per rooted microshoot and a mean root length of 23.93 mm (data not shown).

***Ex vitro* acclimatization of *in vitro* plantlets.** In *D. fruticosus* subsp. *occidentalis*, the survival rate of the rooted microshoots under *ex vitro* conditions initially under a mist regime, then inside a greenhouse and finally outdoors in the external environment of the nursery within pots for 2 months in spring (1/3/2019-1/5/2019) was high, at 90% (Fig. 3A-F). In *D. juniperinus* subsp. *bauhinorum*, a 50% survival rate was recorded for rooted microshoots maintained for 3 weeks in mist conditions and 93.75% 2 weeks after their transplantation to 0.33Lt pots on an unheated greenhouse bench (Fig. 3G-L). In *D. ingoldbyi*, the acclimatization and hardening of the *in vitro* rooted seedlings under *ex vitro* greenhouse conditions after 3 months was successful with a 100% survival rate (Fig. 3M-S).

DISCUSSION

***In vitro* shoot proliferation.** The need for the ideal cytokinin and its optimum concentration is often a critical success factor in establishing an efficient micropropagation protocol for any plant species. The response of the explants to different PGR's, applied either alone or simultaneously, varied among the three different *Dianthus* species and was taxon-dependent. It is widely known that a high cytokinin to low auxin ratio favours shoot proliferation, while a high auxin to low cytokinin ratio favours root formation, a principle considered to be the general model of organogenesis and applied in micropropagation protocols (George *et al.* 2008).

Genotype, proliferation rate and shoot elongation are the three main attributes influenced by cytokinin type, cytokinin concentration and their interaction (Gübbük & Pekmezei 2004). In *D. juniperinus* subsp. *bauhinorum,* among the 3 cytokinins (BA, KN, 2-ip), only BA applied alone and simultaneously with auxins (IBA, NAA, IAA) resulted in shoot proliferation, with the optimum results recorded (100%) using the 0.25 mg/L BA + 0.05 mg/L IAA combination (5/1 hormonal balance). Different results are reported in *D. petraeus* Waldst. & Kit. subsp. *simonkaianus* (Péterfi), where the best proliferation rate was achieved on the medium with 1/1 hormonal balance (0.5 mg/L kinetin and 0.5 mg/L NAA), while at 10/1 hormonal balance (0.5 mg/L BA and 0.05 mg/L NAA or 0.5 Kin and 0.05 mg/L NAA) kinetin was reported to be preferable to BA (Miclăuş *et al.* 2003). In the present study, in *D. juniperinus* subsp. *bauhinorum*, the treatment which yielded better results concerning the shoot multiplication rate (100%), shoot number (3.5 shoots/explant) and shoot development (22.38 mm) was: 0.5 mg/L BA + 0.1 mg/L NAA after 4 weeks of culture. Similar to our results, Kovác (1995) reported the highest shoot multiplication on MS medium supplement-

ed with 1 mg/L BA, while Cristea *et al*. (2010) found that a combination of 1 mg/L BA and 0.1 mg/L NAA at 10/1 ratio gave an optimum multiplication in *D. henteri*. When taking into consideration the shorter duration of the culture period (4 instead of 5 weeks), the combined effect of BA + NAA (5/1 cytokinin/auxin hormonal balance) is more appropriate for the shoot elongation stage (22.38 mm) (4 weeks) than the effect of cytokinin KN applied either alone at 0.25 mg/L or in combination with 0.05 mg/L NAA (23.75-25 mm) (5 weeks). The ability of cytokinins to induce the process of organogenesis has been found to be correlated with the regulation of auxin efflux (Pernisová *et al*. 2009).

In *D. juniperinus* subsp. *bauhinorum,* despite the combined effect of 0.5 mg/L BA and 0.05 mg/L NAA or 0.05 mg/L IAA being more effective in promoting shoot proliferation (100%), the appearance of vitrified explants with a 30% rate and production of multiple lateral dwarf shoots, was an undesirable effect. Cytokinins can induce hyperhydricity in many species, usually in a concentration-dependent manner and when other conditions in the culture system are not optimized (Ivanova & van Staden 2008). Sharma & Mohan (2006) reported that the cytokinin type is one of the most important factors which affected the rate of vitrification, so that using kinetin on the medium prevented vitrification, while the application of BA increased this phenomenon. According to Radojević *et al*. (1997), one of the possible causes of vitrification could be the impact of BA, especially at higher concentrations, as has already been proved for *D. caryophyllus* and *D. petraeus*. Thus, the 0.5 mg/L BA + 0.1 mg/L IAA combination was the best treatment for substantially enhancing the proliferation stage (100%) of *D. juniperinus* subsp. *bauhinorum* shoot-tip explants and preventing the vitrification problem. Buah *et al*. (2010) stated that different cytokinin types have different relative strengths which influence their effectiveness to enhance the initial shoot induction stage, and this differential ability could be attributed to four main factors including stability, mobility, conjugation rates and hormone oxidation. According to Rahman *et al*. (2006), BA is more effective than kinetin and 2-ip in shoot proliferation because the amount of BA conjugated in the medium is smaller than that in the other plant hormones or is not easily broken down and thus persists in the medium, permitting larger amounts of BA existing in their free or ionized form which can be readily available to plant tissues from the medium.

In *D. fruticosus* subsp. *occidentalis,* initial shoot induction and elongation were stimulated by the presence of 0.25 mg/L BA and 0.1 mg/L NAA in the MS medium. A previous study by Papafotiou & Stragas (2009) revealed that the best multiplication rate for *D. fruticosus* L. was achieved with a 5/1 balance of 2-ip (0.5 mg/L) and NAA (0.1 mg/L). In *D. fruticosus* subsp. *occidentalis,* among the 3 cytokinins, BA was the most effective, while 2-ip in the proliferation stage induced chlorosis in the explants. BA superiority in stimulating regeneration compared to other cytokinins such as kinetin and 2-ip has been proved in other *Dianthus* species (Ali *et al*. 2008), probably because BA is a chemically stable cytokinin in tissue culture while most other purine-type cytokinins are considered unstable (Klemš *et al*. 2000).

In *D. ingoldbyi*, multiple shoot formation was enhanced at a 100% rate (5.5 shoots/shoot-tip explant and 34.18 mm mean shoot length) within 5 weeks of culture in the MS medium containing the 3 basic PGR types (cytokinin, auxin, and gibberellins) including 0.25 mg/L BA, 0.1 mg/L IBA and 0.1 mg/L GA_3. In a previous study involving the same taxon, the best shoot proliferation rates (84.8%, 5.9 shoots per auxillary bud explant, 36 mm mean shoot length) were recorded after 30 days of culture in MS medium supplemented with 1 mg/L BA and 0.3 mg/L NAA (Arda *et al*. 2016). There are differences concerning the in vitro response of different *Dianthus* taxa using different combinations and PGR ratios. In *D. petraeus* W. et K. subsp. *simonkaianus* (Péterfi) Tutin 110 microshoots/apical explant (Micläuş *et al*. 2003) were obtained at 1/1 balance (BA 1 mg/L and IAA 1 mg/L) after 37 days of culture, while in *D. pyrenaicus* Pourr., a 2/1 ratio (0.5 mg/L BA and 1 mg/L NAA) produced 4.1 new shoots/explant (Marcu *et al*. 2006) after 70 days of culture.

***In vitro* rooting.** Regarding metabolism, auxins are rapidly absorbed by cells either through influx carrier proteins or through passive diffusion, resulting in direct conjugation or oxidation to inactive forms through enzymatic action within the cell, which in turn leads to the availability of a small portion of the supplied auxin in its free form. The capacity of root formation among the clones is dependent on the ease and timing of the hydrolysis of the conjugated to free auxin forms (George *et al*. 2008). In *D. juniperinus* subsp. *bauhinorum*, among the 3 auxins, NAA was the ideal type and 0.25 mg/L its optimum concentration in promoting the process of rhizogenesis exhibiting 100% rooting, a mean of 9.75 roots per rooted microshoot and mean root length of 32.47 mm after a 5-week culture period. Similar results are reported in *D. caryophyllus*, with 5 cm long shoots being moved to a rooting substrate, where the best response was on MS medium containing 1 mg/L NAA (Ali *et al*. 2008). Marković *et al*. (2013) reported that rooting of *D. serotinus* Waldst. et Kit shoots was most successful on half-strength MS medium with 0.5 mg/L NAA (76.7%), while the rooting percentage on MS medium with the same NAA concentration was slightly lower (68.3%). According to Hartmann *et al*. (2010), the positive impact of NAA on rooting induction could be related to the inhibitory activity of the enzyme IAA-oxidase, which in turn blocks the IAA degradation and increases its activity. The exogenous application of NAA is

known to positively influence the root formation process as it may increase the endogenous auxin, or may synergistically alter the actions or the endogenous synthesis of the plant tissue's naturally occurring auxin (IAA), or even raise the sensitivity of the plantlet to IAA, thus promoting rooting (Hartmann *et al*. 2002). Contradictory results related to the most effective auxin type in rooting other than NAA have been reported in *D. pyrenaicus* where 1 mg/L IAA was better than 1 mg/L NAA (73.2% rooting versus 11%) (Marcu *et al*. 2006) and 2 mg/L IBA in *D. fruticosus* (Papafotiou & Stragas 2009). The effect of the three auxins (IBA, NAA, IAA) each applied at 0.5 and 1 mg/L concentrations on the rooting of *D. gratianopolitanus* was neither positive or negative in terms of root number (Fraga *et al*. 2004). IBA is said to release IAA at a slow rate which in turn is more easily metabolized as a naturally occurring auxin in plants, while the stability and persistence of NAA in plant tissues can have an inhibitory effect on root growth (Epstein & Lavee 1984) altering the auxin: cytokinin ratio in the plant tissue to such a degree that the ratio becomes more favorable for additional root induction as observed in the case of NAA used for *D. juniperinus* subsp. *bauhinorum*. IAA is the most frequently encountered natural auxin, and is known to be more easily oxidised than the other natural IBA, or the synthetic auxin NAA (George *et al*. 2008). The rooting rate is a parameter of higher significance than root length for *ex vitro* plantlet establishment and acclimatization success, although longer roots allow better fixation of plantlets in the substrate and water-nutrient exploration (de Bona *et al*. 2011).

In this study, *D. fruticosus* subsp. *occidentalis* (5.48 roots/rooted microshoot of 20.51 mean length) and *D. ingoldbyi* (6.89 roots/rooted microshoot of 23.93 mean length) were better rooted (100%) after 4 weeks of culture in MS PGR-free medium. Consistent with our findings, in other *Dianthus* taxa, the rooting of microshoots was better stimulated on MS medium (full, half and ¼ strength) devoid of PGRs especially auxins, such as *D. nardiformis* (Holobiuc *et al*. 2010b) and *D. mainensis* (Erst *et al*. 2014). However, lower rooting percentages (20-40%) have been recorded in other *Dianthus s*pecies (*D. henteri, D. spiculifolius*, *D. giganteus, D. banaticus*) (Pop & Pamfil 2011). The promotion of rooting without applying auxins in the medium may be due to the sufficiently balanced production of endogenous auxin in the microshoot apex, transported basipetally to the cut surface (George *et al*. 2008).

***Ex vitro* acclimatization.** The successful acclimatization of regenerants is a critical step in determining the efficiency and cost effectiveness of any micropropagation protocol. *Ex vitro* survival and subsequent vegetative and root system growth may shorten the marketing cycle and accelerate the establishment of propagules under field conditions (Idris *et al*. 2015). In *D. fruticosus* subsp. *occidentalis* and *D. ingoldbyi*, the *ex vitro* survival rate of the rooted microshoots in a peat: perlite (1:1 v/v) mixture initially in the mist system and subsequently on the greenhouse bench during a 3-month period during spring was high, at 90-100%. High survival rates during the acclimatization and hardening process have been reported for other *Dianthus* species including *D. serotinus* (89%) in a 4:1 mixture of peat and sand (Marković *et al*. 2013), 97-100% in *D. deltoides* L. (Marković *et al*. 2013), and 61.5% in *D. superbus* subsp. *superbus* (Mikulík 1999). On the other hand, in *D. juniperinus* subsp. *bauhinorum* a lower survival rate was recorded after 3 weeks exposure in the mist system (50%) and a 93.75% survival rate at 2 weeks after transplantation and transfer to an unheated greenhouse bench. *In vitro* rooted plants were successfully acclimatized (82-97%), which corresponds to the results obtained for other *Dianthus* taxa *D. arenarius* subsp. *bohemicus* (85%), *D. gratianopolitanus* (over 99%) and *D. petraeus* subsp. *noeanus* (100%) (Kovác 1995; Radojević *et al*. 1997; Fraga *et al*. 2004), where the rate of acclimatized plants planted in a 1:1 peat and sand substrate mixture turned out to be the lowest (82.7%), while those planted in a 4:1 ratio of the same mixture exhibited 97% success. In different *Dianthus* taxa, different substrate mixtures are reported for the *ex vitro* acclimatization of *in vitro* rooted microshoots, such as sand: vermiculite (1:1), sand: neutral soil (1:1), and carbonate soil: vermiculite (2:1) (Erst *et al*. 2014).

CONCLUSION

In vitro propagation and acclimatization protocols for three *Dianthus* taxa (*D. ingoldbyi*, *D. juniperinus* subsp. *bauhinorum,* and *D. fruticosus* subsp. *occidentalis)* were elaborated for the purposes of biodiversity, their ex situ preservation and floricultural production. *In vitro* culture ensures micropropagated plants which can be used for reintroduction in natural habitats and the augmentation of existing populations, and to provide plant material for additional conservation studies for *ex situ* preservation. In all three *Dianthus* taxa, the MS medium enriched with the 3 main PGR groups; cytokinins, auxins and gibberellic acid is essential for the initial establishment of shoot-tip explants. In the subsequent shoot multiplication stage, 0.5 mg/L BA + 0.1 mg/L NAA for *D. juniperinus* subsp. *bauhinorum*, and 0.25 mg/L BA + 0.1 mg/L IBA + 0.1 mg/L GA_3 for *D. fruticosus* subsp. *occidentalis* proved to be the best combination treatments (100% rate). Among the three *Dianthus* taxa, *D. ingoldbyi* and *D. fruticosus* subsp. *occidentalis* respond better to rooting at a 100% rate without the addition of PGRs to the MS medium in contrast to *D. juniperinus* subsp. *bauhinorum* which exhibits 100% rooting with the exogenous application of NAA at 0.25 mg/L. The different direct organogenetic responses in terms of micropropa-

gation stages are dependent on genotype, the *Dianthus* taxon, and the types and concentration of PGRs combinations.

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REZIME

Botanica SERBICA

Mikropropagacija i *ex situ* očuvanje tri retka i endemična ukrasna taksona *Dianthus* (Caryophyllaceae)

Virginia Sarropoulou i Eleni Maloupa

Očuvanje brojnih biljnih taksona spontane flore koje su endemične, ugrožene, ranjive ili retke realizuje se *in situ* ili *ex situ* metodama. Biotehnologija *in vitro* kultura je važan deo ovih programa. Cilj istraživanja je bila *in vitro* proliferacija i reaktivnost ukorenjivanja tri retke vrste karanfila divljeg porekla (iz roda *Dianthus*) sa ograničenom distribucijom i prioritetom u zaštiti: ugrožen i ograničene distribucije *D. ingoldbyi* (lokalni endemit nekih delova Balkanskog poluostrva, Turske i SI Grčke), *D. juniperinus* subsp. *bauhinorum* lokalni grčki endemit sa Krita, ograničenog rasprostranjenja i ugrožen, i *D. fruticosus* subsp. *occidentalis*, grčki endemit Kiklada, Peloponeza i jonskih ostrva, ograničenog rasprostranjenja i sa LC-NT kategorijama ugroženosti. Kod svih istraživanih taksona iz roda *Dianthus*, korišćenjem vrhova izdanaka kao eksplantata i NaClO kao sredstva za sterilizaciju (2%) prijavljena je stopa sterilizacije od 50-90%. Kod *D. fruticosus* subsp. *occidentalis* (2 izdanka/ eksplant dužine 18,78 mm) registrovana je 100% stopa regeneracije izdanaka u prisustvu kombinacije 0.25 mg/L BA, 0.1 mg/L IBA, 0.1 mg/L GA3 (5 nedelja), dok je *D. juniperinus* subsp. *bauhinorum* najbolje reagovao (100% proliferacija izdanaka, sa 3.5 izdanaka, dužine 22.38 mm) u prisustvu 0.5 mg/L BA + 0.1 mg/L NAA (4 nedelje). *Dianthus ingoldbii* daje bolje performanse, tj. 5.5 izdanaka po eksplantatu dužine 34.18 mm, ali sa hiperhidricitetom od 28.33% u početnoj MS medijumu obogaćenom sa 0.25 mg/L BA, 0.1 mg/L IBA, 0.1 mg/L GA3 kao i u MS srednja bez PGR-a tokom sledeće faze razmnožavanja izdanaka (100% proliferacija izdanaka, 3.88 izdanaka dužine 62.09 mm, odsustvo hiperhidričnosti) (5 nedelja). Među tri testirana auksina (IBA, NAA, IAA), NAA pri 0.25 mg/L je indukovao bolje ukorenjavanje (100%, 9.75 korena/ukorenjeni mikroizdanak dužine 32.47 mm) kod *D. juniperinus* subsp. *bauhinorum* (5 nedelja), dok su *D. fruticosus* subsp. *occidentalis* (5.48 korena dužine 20.51 mm) i *D. ingoldbii* (6.89 korena dužine 23.93 mm) najbolje ukorenjeni (100%) na MS medijumu bez auksina (4 nedelje). Posle 3-4 nedelje, stope preživljavanja *ex vitro* aklimatizovanih biljaka u zagrejanoj magli staklenika u supstratu treset: perlit (1:1 v/v) bile su 100%, 90% i 50% za *D. ingoldbyi, D. fruticosus* subsp. *occidentalis*, i *D. juniperinus* subsp. *bauhinorum*, respektivno.

Ključne reči: auksini, citokinini, *in vitro*, ugrožen, divlji karanfili