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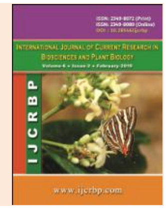

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Effect of indole-3-butyric acid application type and concentration on rooting efficacy in three endemic rare-endangered *Dianthus* species with conservation priority

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ABSTRACT

Efficient propagation of uniform starting material is a critical requirement for mass production of most ornamental plants. Conservation and protection of rare-threatened plants and sustainable exploitation of biodiversity can be achieved through ex situ conservation actions. Therefore, propagation experiments were carried out on three endemic Caryophyllaceae species with limited spread and conservation priority: *Dianthus ingoldbyi*, *Dianthus juniperinus* subsp. *bauhinorum* and *Dianthus fruticosus* subsp. *occidentalis*. Softwood top cuttings were used in the middle of winter for *D. fruticosus* (6-7 cm) and *D. juniperinus* (5-6 cm) and in early spring for *D. ingoldbyi* (4-5 cm). The base of cuttings was immersed for 1 min in solutions of four IBA concentrations (0, 1000, 2000 and 4000 ppm) and also dusted with 0.066% and 0.2% IBA (in powder form). The cuttings were placed in a peat: perlite (1:3) substrate on a heated greenhouse mist. The most appropriate treatment for *D. ingoldbyi* (7 weeks) was 2000 ppm IBA (15.07 roots3.06 cm long, 100% rooting). In *D. juniperinus*, 1000 ppm IBA gave the highest rooting (57.14%) with 39.75 roots 7.49 cm long (8 weeks). Rooting of *D. fruticosus* was more effective (45.33 roots 5.85 cm long, 28.57% rooting) with 1000 ppm IBA (8 weeks).

Introduction

The *Dianthus* genus is a member of the Caryophyllaceae, or pink, family and contains about 300 species. The genus is native to Europe, Asia, North Africa and the Arctic region, where one species is found (Tutin and Walters, 1993). The second edition of Flora Europaea (Tutin and Walters, 1993) lists 115 species, and 91 sub-species within 32 of these species. Seventy seven of the species listed are endemic to Europe. Recent studies have suggested that the Dianthus genus has one of the fastest rates of evolution in plants, possibly due to ployploidization (Weiss et al., 2002; Balao et al., 2009, 2011). Dianthus chromosomes number ranges from 30 (diploid) to 105 (Weiss et al., 2002).

Dianthus juniperinus subsp. *bauhinorum*is a range-restricted endemic of the Greek flora, distributed in

Kriti and Karpathos and the only chamaephyte that exists in habitats including cliffs, rocks, walls, ravines and boulders. It is an endemic plant of Crete, the only chasmophyte on the island that thrives in its natural environment at an altitude of 320-1750 meters. It is threatened with extinction. From IUCN, it is in the R category (rare) and is protected by the Greek law presidential decree 67/1981. This plant is found in small populations on rocky ecosystems throughout Crete and in the gorges of the White Mountains. It is a woody shrub that reaches up to 50 cm high. It also grows successfully at all altitudes and can withstand low temperatures up to - 15^{o}C during winter. Although it is not a coastal plant, it grows well in these areas because of its characteristic foliage. It has particular ornamental value due to its particular flower, its shape and the azure color of its branches, having a flowering period from early spring to summer. It can be planted in clusters or in combination with other plants in flower beds and rock gardens (Dimopoulos et al., 2013, 2016).

Dianthus fruticosus is an endemic plant of Greece found in rocky places of the Cyclades islands Serifos, Sikinos and Folegandros. It can be used as a bedding plant ideal for rocky gardens and Mediterranean roof gardens. It can also be a groundcover plant for slope stabilization and restoration of downgraded landscapes in the Mediterranean region (Papafotiou and Stragas, 2009). *D. fruticosus* subsp. *occidentalis* is a range-restricted Greek endemic, distributed in Ionian Islands, Kriti and Karpathos, and Peloponnisos (Dimopoulos et al., 2013, 2016).

Dianthus ingoldbyi is an endemic species of the genus growing only in some regions of the Balkan Peninsula and North-East of Greece (Reeve, 1967, Uysal et al., 1992, Başak and Güler, 2000, Trigas et al., 2007). It is a caespitose perennial 30 cm with a woody stock; stems puberulent below. Leaves 2mm wide, linear, acuminate, coriaceous. Flowers 2-5 together, sub-sessile. Epicalyx-scale 10-12, ovate, gradually acuminate. Calyx 15-17 x 2mm, tapering above the middle. Petal-limb 2-4mm, denticulate, whitish or greenish yellow (Tutin and Walters, 1993). According to Dimopoulos et al. (2013, 2016), this plant is a range-restricted hemicryptophyte of the East Mediterranean region (local endemic Thrace-Anatolia of Turkey) that grows in coastal habitats.

Industrialization, urbanization, tourism, excessive pasturing, agricultural activities and pollution are the main contributing factors threatening and endangering the future of rare plants (Kargıoğlu et al., 2008). Therefore, protection of endangered plants has been one of the most important goals for botanists in recent years (Dayan et al., 2013).

Carnation petals can be used as an ingredient for a tonic to perfume the skin (Pieroni et al., 2004), or can be crushed for oil used in perfumery (Lim, 2014). Carnation has been used in European traditional herbal medicine for coronary and nervous disorders (McGeorge and Hammett, 2002) and previously used to treat fevers (Bown, 1995; Lim, 2014).

Dianthus species such as *D. barbatus*, *D. chinensis*, *D. plumarius* and *D. gratinanopolitanus* were easily produced with cuttings (Galbally and Galbally, 1997; Hartmann et al., 2002). All carnations, but particularly the perpetual flowering varieties, can be propagated by cuttings with best success being achieved in late summer from short, sturdy, non-flowering side shoots (less than 10 cm long) (Jarrat 1988; McGeorge and Hammett 2002). Indole-3-butyric acid (IBA) has been the most common rooting hormone for general use because it is generally not phytotoxic over a wide concentration range and is effective in promoting rooting of a large number of plant species (Hartmann et al., 1990). Exogenous auxin application improves rooting efficiency and quality of stem cuttings, while IBA and NAA stimulate adventitious rooting in cuttings (Copes and Mandel, 2000).

Efforts were made to evaluate the proper application type (liquid solution/powder), and optimum concentration effects of the auxin "IBA" either in its pure form as solution diluted with ethanol as solvent or under the commercial powered formulation "Radicin", on rooting potential of cuttings in 3 selected endemic wild carnations of the Greek flora. The present study involved investigation of the individual effect of both IBA application type and concentration as well as their interaction and effectiveness in rooting ability of 3 Greek range-restricted, rare, endangered and prioritized *Dianthus* species for *ex situ* conservation, sustainable exploitation, protection of phytogenetic resources and prevention of biodiversity loss purposes.

Materials and methods

Plant material

All native plants maintained at the nursery of the Laboratory derived from natural populations as a result of botanic expeditions conducted at floristically important areas (e.g., National Parks, NATURA 2000 sites and other protected areas). For each plant, site specific information was kept (location, region, altitude, longitude and latitude) as well as a detailed habitat description. All plants collected, received immediate care in the nursery. They are designated as stock plants, planted at big containers or special places according to their needs, taken special treatments since they recover from transplantation shock.

Asexual propagation

Research on the asexual propagation of the three *Dianthu*s species was conducted. For *D. fruticosus* subsp. *occidentalis* and *D. juniperinus* subsp. *bauhinorum* softwood tip cuttings of 5-6 cm were taken in mid-winter (7/12/2017) and for *D.ingoldbyi* softwood tip cuttings of 4-5 cm in early-spring (16/03/2018) from mother plants maintained in pots inside unheated greenhouse from previous October.

The effect of the auxin IBA applied either as liquid solution or powder formulation (commercial name: Radicin) on root formation was tested. The base of the cuttings was either immersed for 1 min in solutions of four different IBA concentrations (0, 1000, 2000 and 4000 ppm) or overlapped with the commercial powder formulation Radicin containing 0.066% and 0.2% of IBA. The control treatment consisted of 50% of ethanol and 50% of distilled water. All the other IBA solutions (1000, 2000 and 4000 ppm) were prepared by using 50% of ethanol as a solvent for IBA reagent (Sigma-Aldrich, plant tissue culture tested, powder) and 50% of distilled water. Afterwards, cuttings were placed in propagation trays in a substrate of peat (Terrahum) and perlite (Geoflor) (1:3 v/v) and maintained at bottom heat benches in a plastic greenhouse. Soil temperature was kept at 18-21°C, air temperature was 15-25°C depending on weather conditions and relative humidity was 70-85% (mist).

Experiment in *D. fruticosus* subsp. *occidentalis* lasted for 8 weeks followed a randomized design with 6 treatments of 21 replications per treatment, 126 replicates in total. Experiment in *D. juniperinus* subsp. *bauhinorum* lasted for 8 weeks followed a randomized design with 6 treatments of 7 replications per treatment, 42 replicates in total. Experiment in *D.ingoldbyi* lasted for 7 weeks followed a randomized design with 6 treatments of 14 replications per treatment, 84 replicates in total. At the end of the experimental period for all three *Dianthus* species, the number of roots per rooted cutting and root length were measured. Rooting was expressed as %. Produced rooted plants were then transplanted in pots of 0.33 Lt (8x8x9 cm) and subsequently in 2.5 Lt pots containing a mixture of peat moss (Klasmann, TS2), perlite and soil (2:½:½ v/v). The successful survival percentage of rooted plants after each transplantation was recorded.

Statistical analysis

Analysis of variance (ANOVA) was performed with the SPSS 17.0 statistical package and mean separation with Duncan's Multiple Range Test. Significance was recorded at $P \leq 0.05$.

Results

Vegetative propagation of *D. fruticosus* subsp. *occidentalis* by cuttings was achieved within 8 weeks. No root formation occurred in the control-untreated cuttings. IBA at 1000 ppm gave more roots (45.33 roots/rooted cutting). Longer roots (5.39-5.85 cm) were obtained irrespective of IBA application type (solutions of 1000 and 2000 ppm concentrations, Radicin powder containing 0.066% and 0.2% IBA), however, 4000 ppm IBA solution decreased root length by 0.85-1.14 cm. The 28.57% of cuttings rooted with 1000 and 2000 ppm IBA, while all the other treatments gave lower rooting percentage (19.05%). Less roots (13 roots/rooted cutting) were obtained with 0.2% IBA (powder) and shorter roots (4.71 cm) with 4000 ppm IBA solution. Taking simultaneously all parameters into consideration (root number, root length, rooting %), the best treatment for the rooting of *D. fruticosus* subsp. *occidentalis* cuttings was 1000 ppm IBA solution (Table 1, Fig. 1a-1h).

Table 1. Effect of application type (solution, powder-formulation) and concentration of IBA (solution: 0-4000 ppm/ Radicin powder containing 0.066 and 0.2% IBA) on rooting of *D. fruticosus* subsp. *occidentalis*after 8 weeks in the mist (7/12/2017 – 6/2/2018).

IBA	Rooting percentage (%)	Root number	Root length (cm)
Control	0 a	0.00 ± 0.00 a	0.00 ± 0.00 a
1000 ppm	28.57 c	45.33 ± 8.79 d	5.85 ± 0.23 c
2000 ppm	28.57 c	24.33 ± 1.14 c	5.42 ± 0.24 c
4000 ppm	19.05 b	28.75 ± 2.07 c	4.71 ± 0.05 b
0.066%	19.05 b	19.00 ± 1.01 bc	5.74 ± 0.16 c
0.2%	19.05 b	13.00 ± 1.02 b	5.39 ± 0.23 c
P-values	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. *** $P \leq 0.001$.

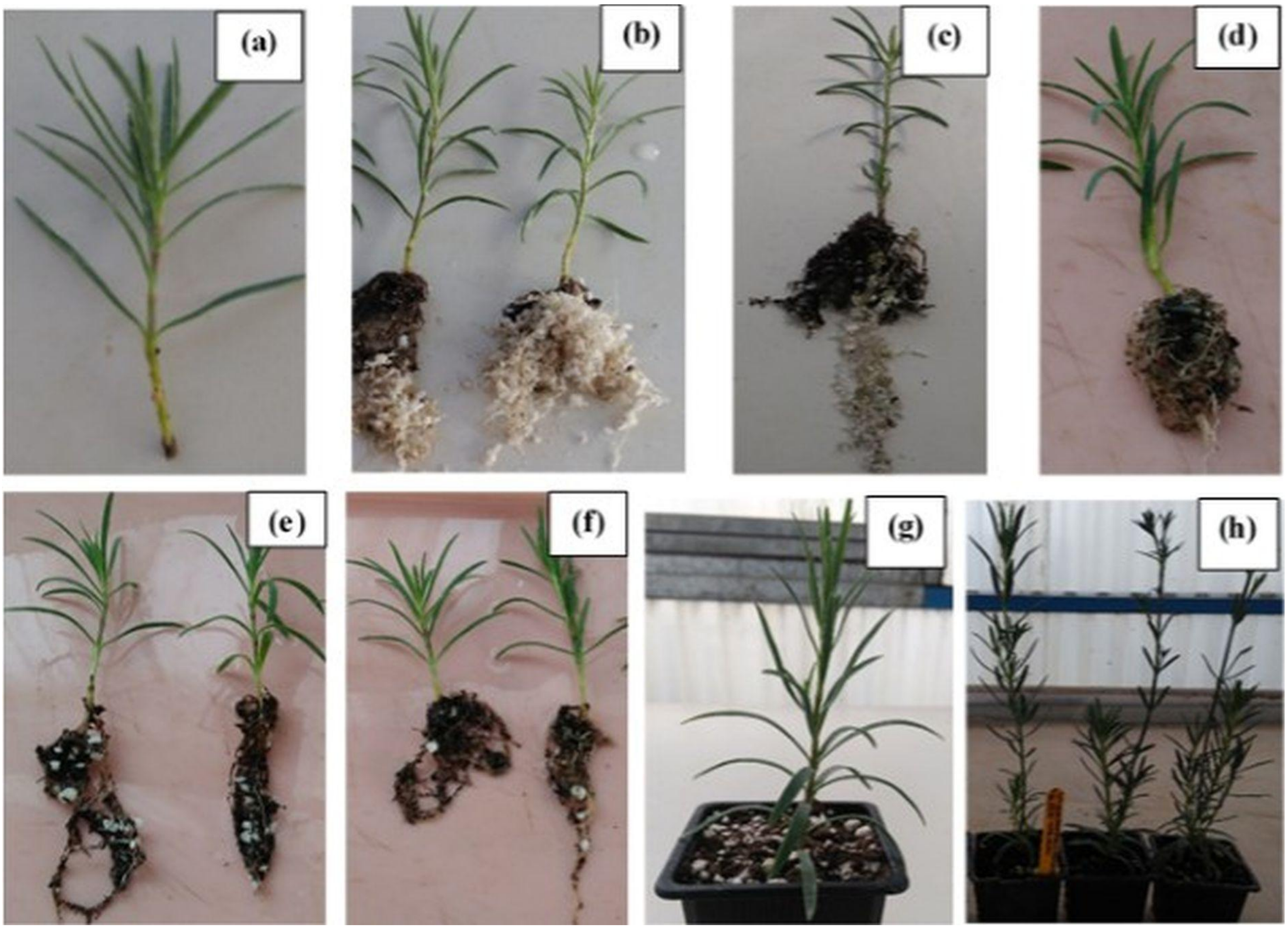

Fig. 1: Vegetative propagation of *D. fruticosus* subsp. *occidentalis* in mid-winter within a 8-week period (a) control, (b) 1000 ppm IBA solution, (c) 2000 ppm IBA solution, (d) 4000 ppm IBA solution, (e) 0.066% IBA (powder) and (f) 0.2% IBA (powder), (g, h) vegetative growth of rooted cuttings 10 and 30 days, respectively from their transplantation in pots of 0.33 Lt.

In *D. juniperinus* subsp. *bauhinorum* cuttings, the best rooting treatment was 1000 ppm IBA (57.14% rooting percentage, 39.75 roots 7.49 cm long) after 8 weeks during winter. No root formation was observed in the control treatment and in cuttings treated with 0.066% IBA (powder). IBA solutions of 1000 and 2000 ppm yielded similar root numbers and root lengths, which were higher among all the other treatments. The exposure of cuttings in 4000 ppm

IBA adversely affected rooting potential by diminishing root number (approximately 4-fold decrease), root length by 5.2-5.3 cm and rooting percentage by 1.5-2 times in comparison to the other two concentrations of IBA solutions (1000 and 2000 ppm). Cuttings treated with 0.2% IBA (powder) exhibited similar rooting percentage (25%) but higher root number and length (25.5 roots 6.45 cm long) with respect to 4000 ppm IBA solution (28.57% rooting, 9 roots 1.44 cm long). The application type of IBA influenced rooting performance of cuttings in a different way; IBA solutions especially 1000 and 2000 ppm gave better results than Radicin powder-formulation containing 0.066 and 0.2% of IBA (Table 2, Fig. 2a-2i).

Table 2. Effect of application type (solution, powder-formulation) and concentration of IBA (solution: 0-4000 ppm/ Radicin powder containing 0.066 and 0.2% IBA) on rooting of *D. juniperinus* subsp. *bauhinorum* after 8 weeks in the mist (7/12/2017 – 6/2/2018).

IBA	Rooting percentage (%)	Root number	Root length (cm)
Control	0 a	0.00 ± 0.00 a	0.00 ± 0.00 a
1000 ppm	57.14 d	39.75 ± 6.51 d	7.49 ± 0.24 d
2000 ppm	42.86 c	34.50 ± 0.98 d	7.63 ± 0.08 d
4000 ppm	28.57 b	9.00 ± 0.00 b	1.44 ± 0.00 b
0.066%	0 a	0.00 ± 0.00 a	0.00 ± 0.00 a
0.2%	25 b	25.50 ± 0.11 c	6.42 ± 0.05 c
P-values	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. *** P ≤ 0.001.

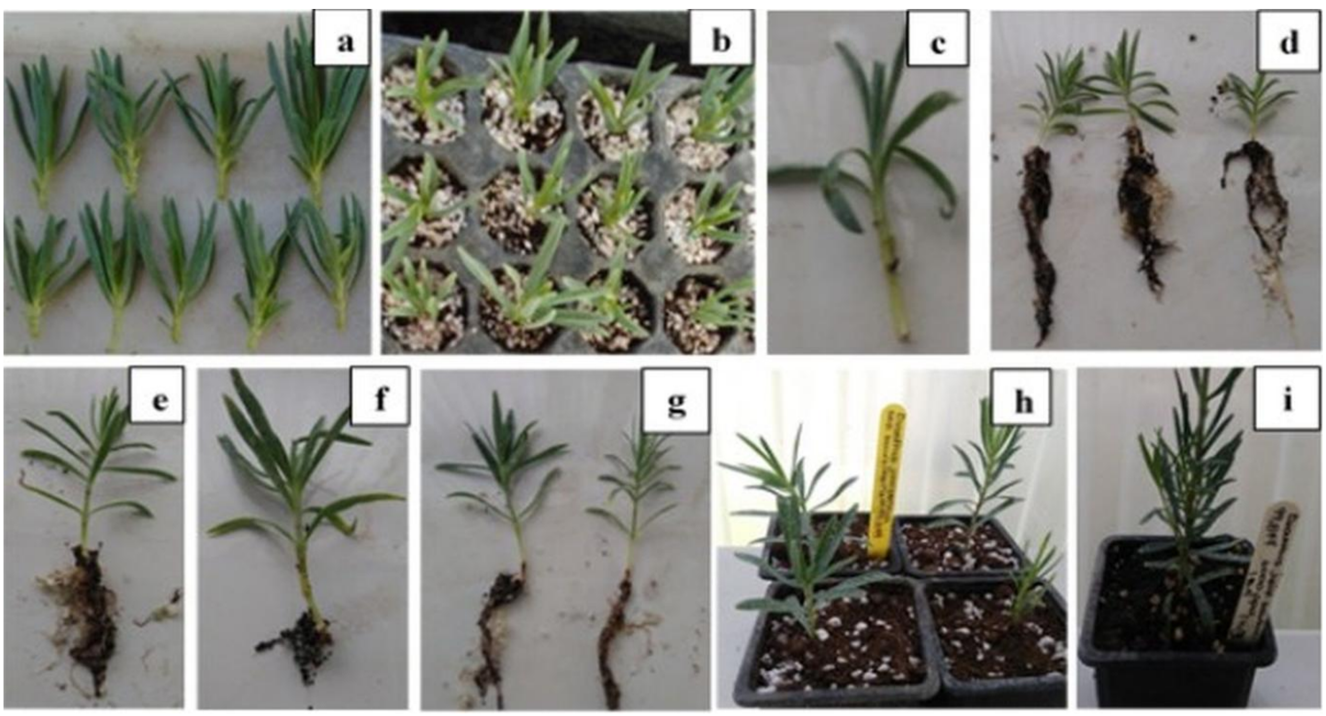

Fig. 2: Vegetative propagation of *D. juniperinus* subsp. *bauhinorum* in mid-winter within a 8-week period (a) cuttings prior to experimentation, (b) cuttings in multi-trays (all treatments, day 0) (c) control, (d) 1000 ppm IBA solution, (e) 2000 ppm IBA solution, (f) 4000 ppm IBA solution, (g) 0.066% IBA (powder), (h) 0.2% IBA (powder) and (i, g) growth of transplanted rooted cuttings in 0.33 Lt pots after 10 and 30 days, respectively.

Regarding *D. ingoldbyi* cuttings, the best rooting performance within 7 weeks was obtained with 2000 or 4000 ppm IBA (12.07-15.07 roots 3.06-3.32 cm long, 100% rooting percentage). The highest IBA concentration of 0.2% (Radicin, applied in powder form)was toxic causing browning and symptoms of necrosis to the 21.43% of cuttings with a simultaneous decline in their rooting potential (6.25 roots 2.15 cm long, 57.14% percentage of rooted cuttings) compared to IBA solutions (12-15.07 roots 3.06-3.33 long, 92.86-100% rooting) (Table 3, Fig. 3a-3g).

Table 3. Effect of application type (solution, powder-formulation) and concentration of IBA (solution: 0-4000 ppm / Radicin powder containing 0.066 and 0.2% IBA) on rooting of *D. ingoldbyi* after 7 weeks in the mist (16/03/2018 – 2/05/2018).

IBA	Rooting percentage (%)	Root number / rooted cutting	Root length (cm)	Necrosis Percentage (%)
Control	57.14 a	12.50 ± 1.56 c	2.34 ± 0.18 a	0 a
1000 ppm	92.86 b	12.00 ± 0.64 bc	3.33 ± 0.50 b	0 a
2000 ppm	100 c	15.07 ± 1.58 c	3.06 ± 0.29 ab	0 a
4000 ppm	100 c	12.07 ± 1.02 bc	3.32 ± 0.28 b	0 a
0.066%	92.86 b	8.62 ± 1.06 ab	3.37 ± 0.33 b	0 a
0.2%	57.14 a	6.25 ± 0.85 a	2.15 ± 0.21 a	21.43 b
P-value	0.000***	0.000***	0.016*	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. * P ≤ 0.05, *** P ≤ 0.001.

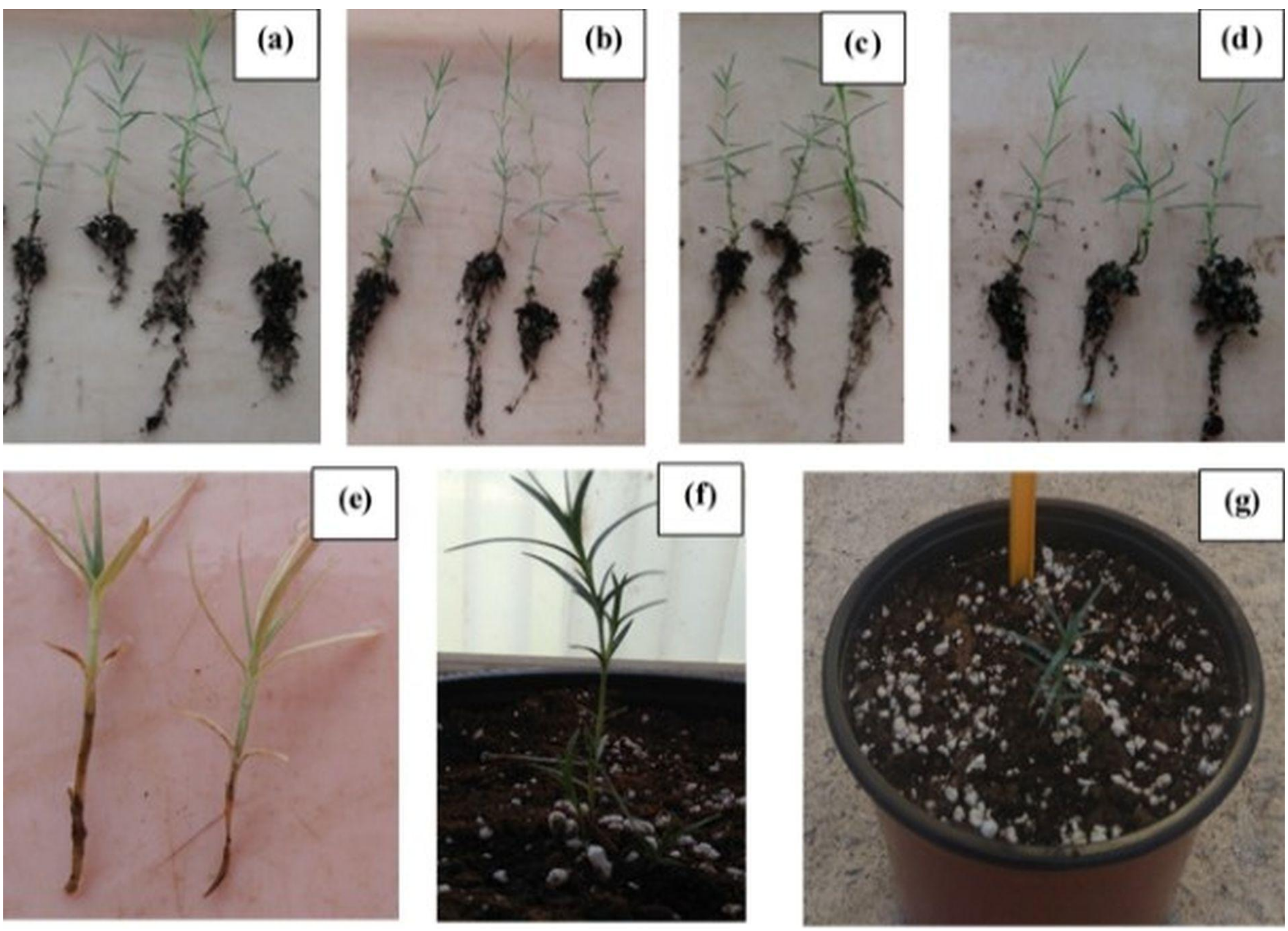

Fig. 3: Vegetative propagation of *D. ingoldbyi* in early-spring within a 7-week period (a) control, (b) 1000 ppm IBA solution, (c) 2000 ppm IBA solution, (d) 4000 ppm IBA solution, (e) 0.2% IBA (powder), (f, g) vegetative growth of transplanted rooted cuttings into 0.33 Lt pots at 10th day and (g) into 2.5 Lt pots after another 15 days.

Discussion

The investigation on the effect of IBA on rooting of different *Dianthus* species showed significant difference for rooting percentage, days to root initiation, root length and number of roots per cutting. Various media have been used successfully for growing potted plants and also for the rooting of cuttings. These media are naturally occurring organic materials such as coir dust, sawdust, sphagnum moss, cocopeat, etc. or other minerals such as vermiculite, perlite etc. These media enhance rooting in cuttings mainly by possessing a better water holding capacity, increased porosity and better nutritional levels over the conventional sand medium, which is normally used for the purpose. Besides these advantages, they generally carry very little pathogen load, and are very light in weight and hence easy to handle in greenhouse crop production.

IBA exerts different effects on plant growth and development, e. g. regulating response of plants against biotic and abiotic stresses or increasing plant yield, but primarily implicated in adventitious root formation and widely used commercially for the induction of adventitious roots (Normanly et al., 1995; Ludwig-Muller, 2000). In the present study with *D. fruticosus* subsp. *occidentalis*, rooting performance of cuttings after 8 weeks during winter period was best (28.57%) with 1000 ppm IBA solution. Similarly, in *Dianthus orientalis* Adams., the highest rooting rate (28%)was obtained from cuttings treated with 2000 ppm IBA and planted in a peat substrate than in perlite or peat + perlite ones, compared to the control untreated cuttings (2% rooting rate) (Hazar and Baktir, 2018). The stimulating effect of IBA on rooting potential of cuttings has previously been documented for other *Dianthus* species including *D. gratianopolitanus* 'Firewitch' (optimum: 750-1000 ppm IBA solution) (Pilon, 2006) and carnation cv. Guadina (optimum rooting percentage: 125 ppm IBA solution) (Khelwale et al.,2005).A previous study conducted in *D. fruticosus* subsp. *occidentalis* by Krigas et al. (2010) revealed intermediate percentages of propagation success ranging from 60 to 80% for softwood tip cuttings treated with different concentrations of IBA.

In the current study employing *D. juniperinus* subsp. *bauhinorum*, 1000 ppm IBA solution was the most appropriate treatment for rooting of the cuttings within an 8-week period during winter. In winter season, tip carnation (*Dianthus caryophyllus* L.) cuttings exhibited better results with respect to the length of roots and rooting percentage with 550 ppm NAA, whereas more roots were produced after treatment of cuttings with 550 ppm IBA (Kumar et al., 2006). In consistency with our findings, 200 ppm IBA proved to be the optimum rooting treatment for *D. caryophyllus* L. cv. Dona cuttings in terms of rooting percentage, root number, root length and percentage of establishment of rooted cuttings under polyhouse conditions (Renuka and Sekhar, 2014).

With respect to the third studied *Dianthus* species (*D. ingoldbyi*), rooting 100% was achieved after treatment of cuttings with 2000 and 4000 ppm IBA solution in spring after 7 weeks. Panahi and Morteza (2000), studied the effect of auxins on rooting of carnation cultivars (*D. caryophyllus* L.) and recorded highest number of roots per cutting with IBA at 100ppm. In the present study with *D. ingoldbyi* longer roots were obtained with 2000 ppm IBA and more roots per rooted cutting with 4000 ppm IBA solutions. Auxins are known to increase the cell division by increasing the level of endogenous cytokinins resulting in induction of more number of root primordial (Nanda and Kochhar, 1985). It appears likely that auxins initiate synthesis of structural enzyme proteins in the formation of adventitious roots thus increasing the root length through the process of acidification (Audus, 1963).

Related to the three *Dianthus* species in the current study, treatment of cuttings with IBA solutions was beneficial for enhancing their rooting potential, taking into consideration that no root formation occurred at least for *D. fruticosus* subsp. *occidentalis* and *D. juniperinus* subsp. *bauhinorum* without the application of IBA. The promoting effect of IBA on rooting is mainly due to its conversion to IAA in plant tissue (Epstein and Lavee, 1984). In the two studied *Dianthus species* (*D. fruticosus* subsp. *occidentalis* and *D. juniperinus* subsp. *bauhinorum*), however 4000 ppm IBA solution had an adverse effect on rooting performance of cuttings. Additionally, in the studied *D. ingoldbyi*, the highest IBA concentration of 0.2% (powder formulation) caused browning and necrotic symptoms to the 21.43% of cuttings with a simultaneous decrease in the length of roots. With increasing concentration, auxins can produce a variety of growth abnormalities within 24 hrs after treatment, including leaf epinasty, stem curvature, intensified green leaf pigmentation, and growth inhibition, changes followed by accelerated foliar senescence, chloroplast damage, destruction of membrane and vascular system integrity, necrosis, and plant death (Grossmann, 2000).

Absorption of auxin solutions at the base of stem cuttings can be influenced by auxin concentration and treatment duration, with increasing concentration and duration providing greater uptake (Howard, 1985a). In the present study, 1000 ppm IBA solution was the most appropriate rooting treatment for *D. fruticosus* subsp. *occidentalis* and *D. juniperinus* subsp. *bauhinorum* cuttings in winter period whereas IBA solutions of 2000 or 4000 ppm for *D. ingoldbyi* in spring. As with liquid formulations, the entry of auxin from the cut end of the cutting tends to elicit the most efficient rooting response, although epidermal applications of powder can be more effective if preceded by a solvent dip (Howard, 1985a). Absorption of auxin from powder formulations can be influenced by factors operating at the interface of the powder and the cutting stem (Howard, 1985b). In all three studied *Dianthus* species, rooting performance and vegetative growth of cuttings was better after treatment with IBA liquid solutions compared to its commercial powder formulation (Radicin). In accordance with our findings in *Dianthus* species, Ticknor (1981) examined rooting of cuttings of 10 woody ornamentals in response to one commercial talc formulation [Hormex 8 (0.8% IBA)] and two commercial liquid formulations [Dip 'N Grow and Wood's Rooting Compound both consisted of IBA + NAA combination] and observed that the treatment with the liquid formulations produced similar or better results than with the powder. The basal quick-dip and powder application methods of auxins tend to be most common, with the quick-dip generally considered to be the superior method of the two (Dirr and Heuser, 1987).

Conclusion

Rooting 100% was achieved in spring for *D. ingoldbyi* cuttings with 2000 ppm IBA (7 weeks). The 1000 ppm IBA treatment was found to be suitable for the rooting of the Cretan endemic *D. juniperinus* subsp. *bauhinorum* (57.14%) in autumn (8 weeks). Low rhizogenic capacity (28.57%) was found in autumn for *D. fruticosus* subsp. *occidentalis* (endemic Cyclades, Peloponnesus, Ionian Islands) cuttings treated with 1000 ppm IBA (8 weeks). It is necessary to apply rooting hormones for the vegetative propagation of endemic wild carnations with conservation priority. The success of rooting within different plant species even of the same genus depends on the application type of rooting hormone 'IBA' (solution, powder), the concentration of IBA, the season of year (autumn, spring), the juvenility and age of cuttings (younger and more tender or more mature and woody) as well as on the interaction of all the above mentioned factors. The development of specialized propagation protocols provides valuable material for applied research, constitute a valuable complementary tool for *in situ* conservation and stockpiling for the future sustainable and commercial exploitation of native plants.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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