Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating: 3.53
JMPS 2018; 6(6): 235-241

Received: 05-09-2018
Accepted: 06-10-2018

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Asexual propagation and *ex situ* conservation of *Hypericum empetrifolium* Willd. Subsp. *empetrifolium* (Hypericaceae), an East Mediterranean medicinal plant with ornamental value

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Abstract
Hypericum empetrifolium subsp. *empetrifolium* is a medicinal plant of conservation concern and ornamental value restricted to Greece, Albania, Western Turkey and Cyrenaica in Libya. For vegetative propagation in mid-autumn, shoot tip softwood cuttings (5-6 cm) were immersed for 1 min in solutions of 4 IBA concentrations (0, 1000, 2000 and 4000 mg L^{-1}). Cuttings were placed in propagation trays in a peat: perlite (1:3) substrate, under mist. For *in vitro* propagation, shoot tips explants were cultured in MS medium. The effect of BA alone and combined with auxins on shoot proliferation was studied. For *in vitro* rooting, different auxins (IBA, NAA, IAA) applied at different concentrations were tested. Highest rooting percentage for cuttings was noticed when using 1000 mg L^{-1} IBA (17.17 roots 2.84 cm long, 85.71% rooting) (6 weeks). The 0.1 mg L^{-1} BA + 0.01 mg L^{-1} IBA hormonal combination was the best for *in vitro* shoot proliferation, yielding 5.5 shoots/explant, 100% shoot formation and 5.42 shoot proliferation rate (4 weeks). Rooting *in vitro* was optimum with 0.1 mg L^{-1} IBA (3.22 roots 1.05 cm long, 56.25% rooting) (5 weeks). Acclimatization of rooted vitroplants was successful exhibiting survival rate up to 90%.

Keywords: Auxins, cuttings, germplasm conservation, micropropagation

Introduction
The Mediterranean Basin has been recognized as a hot spot for *Hypericum*, with more than 150 of the currently known 470 species occurring in this region [1]. *Hypericum* species contain a number of biologically active compounds, such as naphtodianthrones (hypericin and pseudohypericin), phloroglucinols (hyperforin and adhyperforin), flavonoids, procyanidins, tannins, essential oils, amino acids, phenylpropanoids, xanthones and other water-soluble components [2].

Hypericum empetrifolium Willd. subsp. *empetrifolium* (Hypericaceae) is a small evergreen shrub, restricted to Greece, Albania, Western Turkey and Cyrenaica in Libya [3, 4]. In Greece, this subspecies grows on xeric Mediterranean phrygana and grasslands, woodlands and scrubs, and it is distributed in East Aegean islands, Central Greece, Ionian Islands, Cyclades, Crete and Karpathos, North-East Greece, Peloponnese, Sterea Ellas and West Aegean islands [5, 6]. *H. empetrifolium* subsp. *empetrifolium* has been traditionally used in Greece and the Mediterranean Region as a medicinal plant showing antioxidant and antibacterial activity, anthelmintic and diuretic properties (flowers) or used externally as a wash to fasten the time needed to heal wounds, scalds, and herpes [7]. Naphthodianthrones and flavonoids have been identified in the crude extracts of its flowers [8] and in the composition of the essential oil [7]. Anti-inflammatory and analgesic effects of the polar extract of *H. empetrifolium* have been demonstrated *in vivo* in mice, although the active constituents have not yet been identified [3].

Apart from its medicinal value, *H. empetrifolium* subsp. *empetrifolium* can be used as a landscape plant of high ornamental value because of its impressive flowering; the yellow black-dotted flowers come in contrast with the dark green and dense foliage and the evergreen type of vegetation during winter. According to Davis [9], the flowering period is prolonged, extending from April to June, with numerous deep yellow flowers, fruiting in ovate-shaped capsules with oblique vesicles. Using this subspecies in Mediterranean parks, public or private gardens and archaeological sites could reinforce local biodiversity, maintaining the traditional character of the area [10, 11].

Propagation of *Hypericum* taxa (species and subspecies) is not very easy. The seeds of 59 *Hypericum* species have been recorded as orthodox, indicating that the life cycle of these species in the field may be related with deep seed dormancy [12]. Indeed, it has been found that the germination capacity of *Hypericum* species is very low, mainly due to the prolonged seed dormancy [13]. The vegetative or asexual propagation by cuttings is a simple and easily applied method of propagation offering true-to-type plants in a short time period and availability of superior material for large scale commercial plantations with quick productive gains [14]. This has been achieved mainly for the most famous medicinal plant of genus *Hypericum*, i.e. *H. perforatum* [15-19]. However, for each plant species, it is necessary to know the appropriate cutting collection period and the most effective concentration of the rooting hormones, with auxins being commonly used as root-promoting chemicals [20]. Micropropagation is an advanced vegetative propagation technique generating large numbers of genetically uniform and pathogen-free transplants in a limited time and space [15]. The *in vitro* plant regeneration of *H. perforatum* has been studied well, using as explants the whole seedling or their excised parts [15], hypocotyl sections [16, 18, 21], leaves [17] and leaf discs and stem segments [19], adventitious roots [22]. However, the survey of literature revealed than only one study has ever been reported on sexual and asexual propagation of *H. empetrifolium* subsp. *empetrifolium* [23]. The aim of this study was the investigation of asexual propagation methods for this promising *Hypericum* subspecies and the development of specific protocols in order to allow its use as an ornamental plant in urban and suburban areas, as well as a medicinal plant in the pharmaceutical industry.

Materials and Methods

Plant Material

Plant material was collected from the natural habitats of the selected subspecies from Fthiotida Prefecture in Sterea Ellas, near Lamia region (Central Greece) at an altitude of. 200m above sea level. Site-specific information was kept (location, region, altitude, geographical coordinates, habitat description). All collected plants were transferred to the nursery of the Balkan Botanic Garden of Kroussia-Laboratory for Protection and Evaluation of Native and Floricultural Species, Institute of Plant Breeding and Phytogenetic Resources, Hellenic Agtricultural Organization Demeter (Thermi, Thessaloniki) and received immediate care till they recover from transplantation shock. These plant individuals received the IPEN (International Plant Exchange) accession number GR-1-BBGK-99,782 and were the initial mother plants used for further experimentation.

Asexual Propagation by cuttings

In early-mid autumn, softwood top cuttings (5-6 cm) were cut from mother plants that were maintained inside the greenhouse (Thermi), and their base was immerged for 1 min in solutions of four IBA concentrations 0, 1000, 2000 and 4000 mg L^{-1}. 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, while air temperature was 15-25 °C, depending on local weather conditions. Relative humidity (RH) was over 80% (under mist). Experiments lasted for 6 weeks and followed a randomized design with 4 treatments and 7 replications per treatment. At the end of the experimental period, the number of roots per rooted cutting and root length were measured. Rooting was expressed as percentage (%). Produced rooted plants in late-autumn (mid-November) were then transplanted in pots of 0.33L and, subsequently in mid-winter (end of January), in 2.5L pots, all containing a mixture of peat (Klasmann, TS2), perlite and soil (2: ½: ½ v/v) substrate. The final survival percentage of the transplanted rooted plants after a 5-month period (end of April) was recorded.

Asexual propagation by *in vitro* culture

For the initial establishment of the plant material *in vitro*, during early-autumn juvenile shoot tips 1.5-2.5 cm long were cut and removed from mother plants maintained in a peat: perlite (1:1) substrate in pots -in open field. For the disinfection of the plant material, shoot tip explants were soaked in 70% ethanol for 1 min followed by 1.5% NaOCl solution for 20 min under continuous stirring and then washed into sterile distilled water for 4–5 times. The basal culture medium used for the initial establishment phase was the Murashige and Skoog (MS) [24] supplemented with 30 g L^{-1} sucrose (Duchefa Biochemie, The Netherlands), 0.25 mg L^{-1} benzyladenine (BA), 0.1 mg L^{-1} indole-3-butyric acid (IBA), 0.1 mg L^{-1} gibberrelic acid (GA_3) and solidified with 6 g L^{-1} Plant Agar (Duchefa Biochemie, The Netherlands). Successfully established explants were sub-cultured every 4 weeks and for 3 times in the above medium until a sufficient amount of plant material to be generated. The proliferated plant material was transferred into a hormone-free MS culture medium for 4 weeks prior to further experimentation.

For the shoot proliferation experiment, shoot-tip explants 1.5-2.5 long from the previous *in vitro* cultures were used. The effect of BA at 0.1 mg L^{-1} in combination with 3 different auxins; IBA, α-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA), all at 0.01 mg L^{-1} was studied. The MS was used as basal culture medium supplemented with 20 g L^{-1} sucrose and was solidified with 6 g L^{-1} Plant Agar. After 4 weeks of culture, the following measurements were recorded: shoot formation percentage (%), shoot number/explant, shoot length (cm), shoot proliferation rate and rooting percentage (%).

For the rooting experiment, plantlets 2.5-3.5 long from the previous *in vitro* cultures in a hormone-free MS medium were used. The effect of 3 different auxins was studied: IBA, NAA and IAA, each applied in 3 different concentrations (0, 0.1 and 0.25 mg L^{-1}). MS was used as basal culture medium as mentioned above. After 5 weeks of culture, the following measurements were recorded: rooting percentage (%), root number/rooted vitroplant, root length (cm), shoot formation percentage (%), shoot number/explant and shoot length (cm). The pH value of the culture media in both experiments was adjusted to 5.8 before adding the gelling agent and prior sterilization in autoclave at 121 °C for 20 min. Explants were cultured into Magenta vessels (62.4 mm × 95.8 mm, 200 ml) containing 35 ml of MS medium. All cultures were maintained in a growth chamber with 16-h photoperiod (40 μmol/m^2/s) under cool white fluorescent light and a constant temperature of 22 ± 2 °C.

Rooted plantlets were washed with tap water and transferred in propagation trays with a peat (Terrahum): perlite mixture 3:1. Trays were kept in the greenhouse under mist (over 80% RH and 50% light density (using a 50% shading net) for 10 days. Gradually, during the next days RH was reduced till normal greenhouse conditions and light density was increased uncovering the plants from the shading net.

Statistical analysis

Shoot proliferation experiment consisted of 5 treatments with 16 replications per treatment (4 vessels/treatment x 4

explants/vessel). The rooting experiment included 7 treatments where each value was the mean of 12 replicates (3 vessels/treatment x 4 explants/vessel). The experimental layout was completely randomized and ANOVA (Analysis of Variance) was carried out using the statistical package SPSS 17.0 (SPSS Inc, Chicago, Illinois, USA). To compare the means, the Duncan's multiple range test was used at $P \leq 0.05$. The rooting experiment was composed of 3 auxin types (IBA, NAA, IAA), 2 auxin concentrations (0.1 and 0.25 mg L^{-1}) and the control treatment (auxins-free). The main effect of factors (auxin type, auxin concentration) and their interaction were determined by the General Linear Model. Both experiments were repeated twice.

Results

Asexual propagation by cuttings

IBA positively affected rooting formation of *H. empetrifolium* subsp. *empetrifolium* cuttings. IBA at 1000 and 2000 mg L^{-1} concentrations raised the percentage of rooted cuttings in relation to the control (57.14%) reaching 85.71%. Cuttings treated with 1000 mg L^{-1} IBA produced longer roots (2.84 cm) with respect to control (2.08 cm). However, 4000 mg L^{-1} IBA adversely affected rooting potential of cuttings (42.86%, 3.67 roots 1.18 cm long,) compared to control (57.14% rooting, 15.75 roots 2.08 cm long) (Table 1, Fig. 1A-1E). After 5 months (end of April), 100% survival was recorded for rooted plants transplanted into 0.33L and subsequently into 2.5L pots (Fig. 1F, 1G).

Table 1: Effect of IBA concentration (0, 1000, 2000, 4000 mg L^{-1}) on rooting percentage (%), root number and root length in *H. empetrifolium* Willd. subsp. *empetrifolium* cuttings after 6 weeks.

IBA (mg L^{-1})	Rooting (%)	Root number	Root length (cm)
Control	57.14 b	15.75 ± 1.89 b	2.08 ± 0.10 b
1000	85.71 c	17.17 ± 2.54 b	2.84 ± 0.31 c
2000	85.71 c	14.67 ± 2.42 b	2.47 ± 0.18 bc
4000	42.86 a	3.67 ± 0.33 a	1.18 ± 0.14 a
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 $P \leq 0.05$, ***$P \leq 0.001$

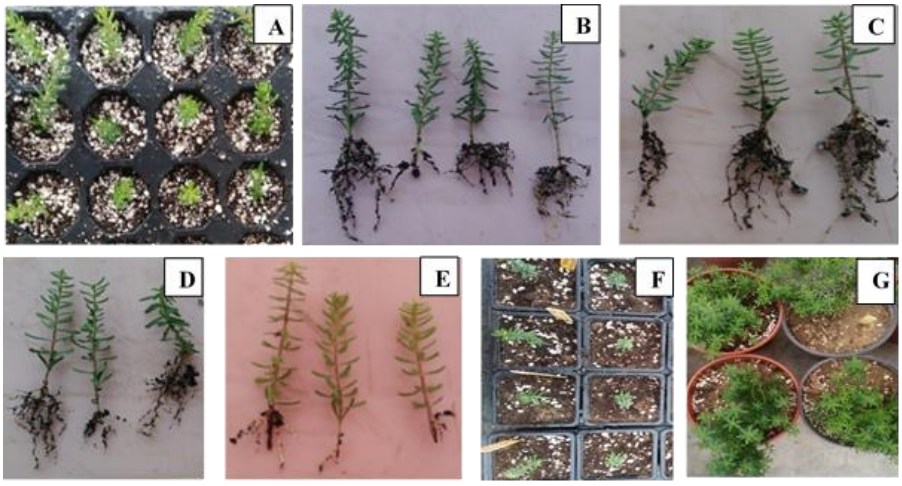

Fig 1: Asexual propagation of *H. empetrifolium* Willd. subsp. *empetrifolium*: (A) cuttings in Multiplate discs; (B) control; under (C) 1000 mg L^{-1} IBA, (D) 2000 mg L^{-1} IBA, (E) 4000 mg L^{-1} IBA; (F) vegetative growth of transplanted rooted cuttings into 0.33 pots (day 14) and (G) into 2.5L pots after 3 months.

Asexual propagation by *in vitro* culture

The percentage of successfully disinfected explants was 59%. During 3 continuous subcultures (4 weeks/each) in culture medium with plant growth regulators (PGRs), an adequate quantity of plant material was obtained (6-fold more produced explants within 3 months) (Fig. 2A, 2B). When explants were transferred in PGRs-free MS medium after 4 weeks of culture, a substantial enhancement was observed in vegetative growth and development of produced proliferated shoots (increase in height, number of buds, size, width and number of leaves per shoot, larger internodes and more dark green color) (Fig. 2C).

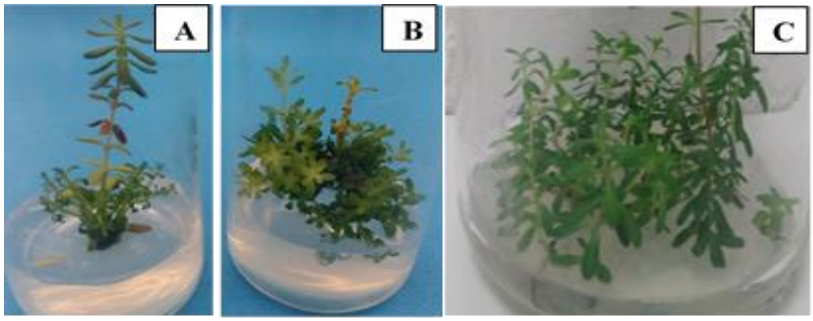

Fig. 2: *In vitro* shoot proliferation of *H. empetrifolium* subsp. *empetrifolium;* (A, B) MS medium containing 0.25 mg L^{-1} BA, 0.1 mg L^{-1} IBA, 0.1 mg L^{-1} GA_3 and (C) PGRs-free medium.

In the shoot proliferation experiment, the 0.1 mg L^{-1} BA + 0.01 mg L^{-1} IBA or IAA combinations increased the number of shoots (5.5-5.67 shoots/explant) in relation to control (1.08). The application of BA alone did not change shoot length significantly. However, the combined effect of BA + auxins caused an 1 cm-decrease in shoot length with respect

to control. The addition of BA alone or auxins' treatment (all 3 types) in culture medium raised both shoot proliferation rate (1.36-5.42) and percentage of explants with multiple shoot formation (58.33%-100%) (Fig. 3B-3E) compared to the control (0.11 and 8.33%, respectively). Shoot formation was 100% and shoot proliferation rate was highest (5.42) by adding 0.1 mg L^{-1} BA + 0.01 mg L^{-1} IBA to the medium (Fig. 3). Root formation (25%) was observed only in the control treatment (Table 2, Fig. 3A).

Table 2: Effect of BA alone and combined with auxins on shoot formation percentage (%), shoot number/explant, shoot length, shoot proliferation rate and rooting percentage (%) in *H. empetrifolium* subsp. *empetrifolium* (4 weeks).

Treatments (mg L^{-1})	Shoot formation (%)	Shoot number	Shoot length (cm)	Shoot proliferation rate	Rooting (%)
Control	8.33 a	1.08 ± 0.08 a	2.52 ± 0.24 b	0.11 ± 0.01 a	25 b
0.1 BA	58.33 b	2.17 ± 0.39 a	2.02 ± 0.37 ab	1.36 ± 0.24 b	0 a
0.1 BA + 0.01 IBA	100 d	5.50 ± 0.53 b	1.59 ± 0.19 a	5.42 ± 0.44 d	0 a
0.1 BA + 0.01 NAA	58.33 b	3.17 ± 0.71 a	1.58 ± 0.16 a	1.50 ± 0.38 b	0 a
0.1 BA + 0.01 IAA	91.67 c	5.67 ± 1.40 b	1.46 ± 0.14 a	4.20 ± 0.67 c	0 a
P-values	0.000***	0.000***	0.013*	0.000***	0.000***

Means ± 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 $P \leq 0.05$. *$P \leq 0.05$, ***$P \leq 0.001$

Fig 3: Effect of BA combined with auxins on *in vitro* shoot proliferation of *H. empetrifolium* subsp. *empetrifolium*: (A) control (B) 0.1 mg L^{-1} BA, (C) 0.1 mg L^{-1} BA + 0.1 mg L^{-1} IBA, (D) 0.1 mg L^{-1} BA + 0.1 mg L^{-1} NAA and (E) 0.1 mg L^{-1} BA + 0.1 mg L^{-1} IAA.

The addition of IBA increased rooting percentage during *in vitro* culture of *H. empetrifolium* subsp. *empetrifolium*. In the rooting experiment, IBA raised rooting percentage from 25% (control) to 43.75%-56.25% and increased the number of roots (3.22-3.43), whereas IAA gave same results with the control. No significant differences were noticed with the addition of IAA in comparison to the control, neither regarding rooting percentage (25% in both cases) nor at the number of roots formed (2-2.5), while NAA resulted in complete inhibition of rooting. Both IBA and IAA induced higher root lengths (0.71-1.05 cm) in relation to control (0.54 cm) (Table 3).

Additionally, in the rooting experiment, shoot number (2.25 shoots/explant) and shoot formation percentage (37.5%) were higher by the addition of 0.1 mg L^{-1} IBA into the culture medium. Shoot length was stimulated (3.04-3.23 cm) when the explants were treated with 0.25 mg L^{-1} IBA and 0.1 or 0.25 mg L^{-1} NAA, with respect to the control (2.26 cm). IAA gave similar shoot numbers and lengths to the control. Only IBA and IAA applied at 0.1 mg L^{-1} succeeded in raising the shoot formation percentage (31.25%-37.5%) in comparison to the control (12.5%). NAA, irrespectively of concentration (0.1 and 0.25 mg L^{-1}), caused a 2-fold decrease in the percentage of explants with shoot formation (6.25%), whereas IAA applied at 0.25 mg L^{-1} fully inhibited shoot proliferation (Table 3).

Table 3: Effect of auxins on rooting percentage (%), root number/rooted plant, root length (cm), shoot formation percentage (%), shoot number/explant and shoot length (cm) in *H. empetrifolium* subsp. *empetrifolium* (5 weeks *in vitro* culture)

Treatments (mg L^{-1})	Rooting (%)	Root number	Root length (cm)	Shoot Formation (%)	Shoot number	Shoot length (cm)
Control	25 b	2.50 ± 0.14 b	0.54 ± 0.01 b	12.5 c	1.31 ± 0.18 a	2.26 ± 0.19 a
0.1 IBA	56.25 d	3.22 ± 0.52 c	1.05 ± 0.07 d	37.5 e	2.25 ± 0.47 b	2.65 ± 0.18 abc
0.1 NAA	0 a	0.00 ± 0.00 a	0.00 ± 0.00 a	6.25 b	1.06 ± 0.06 a	3.23 ± 0.26 c
0.1 IAA	25 b	2.00 ± 0.13 b	1.00 ± 0.05 d	31.25 d	1.75 ± 0.32 ab	2.38 ± 0.20 ab
0.25 IBA	43.75 c	3.43 ± 0.34 c	0.71 ± 0.05 c	12.5 c	1.38 ± 0.31 a	3.04 ± 0.27 bc
0.25 NAA	0 a	0.00 ± 0.00 a	0.00 ± 0.00 a	6.25 b	1.06 ± 0.06 a	3.05 ± 0.23 bc
0.25 IAA	25 b	2.25 ± 0.17 b	0.98 ± 0.07 d	0 a	1.00 ± 0.00 a	2.88 ± 0.19 abc
P-values						
Auxin type (A)	0.000***	0.000***	0.000***	0.000***	0.042*	0.144 ns
Auxin Conc. (B)	0.000***	0.000***	0.000***	0.000***	0.021*	0.000***
(A)*(B)	0.000***	0.000***	0.000***	0.000***	0.138 ns	0.297 ns

Means ± 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 $P \leq 0.05$. ns: $P \geq 0.05$, *$P \leq 0.05$, ***$P \leq 0.001$

Forty (40) days after being planted in the greenhouse under mist and after gradually reduction of RH, the rooted plantlets survived up to 90%. The raised plants were maintained in normal greenhouse conditions (RH 35-45%, light density without shade nets) and were transferred in bigger pots allowing further growth (Fig. 4A, 4B). When plants reached the height of 10 cm (6 weeks after transplantation) (Fig. 4C) they were ready to continue growing in bigger (2.5 L) pots in typical outdoor environmental conditions, or readily available for cultivation in the field (Fig. 4D).

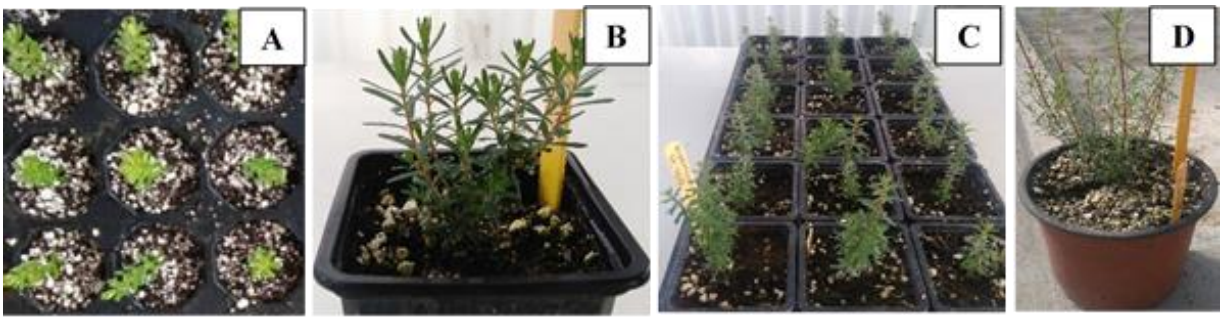

Fig 4: Acclimatization procedure of rooted *H. empetrifolium* subsp. *empetrifolium* vitroplants: (A) transition of *in vitro* rooted plants to *ex vitro* conditions in the mist (day 1); (B, C) vegetative growth of transplanted plants into 0.33 L pots maintained in the unheated greenhouse bench wherein watered by sprinkling after 2 and 6 weeks, respectively; and (D) growth of transplanted plants into 2.5L pots outside greenhouse after 4 weeks.

Discussion

The asexual propagation protocols developed for *H. empetrifolium* subsp. *empetrifolium* in this study should to be envisaged as: (a) contribution to Target 8 of the Global Strategy for Plant Conservation (''At least 75 percent of threatened plant species in *ex situ* collections, preferably in the country of origin, and at least 20 percent available for recovery and restoration programs of them included in recovery and restoration programs'') [25, 26], allowing future implementation across scales (local, regional, global); (b) critical step for rapid production of selected plant material upon request for a variety of reasons; (c) bridge connecting with its possible sustainable exploitation in the ornamental-horticultural or medicinal-cosmetic industries [27].

In the present study with *H. empetrifolium* subsp. *empetrifolium*, rooting of softwood top cuttings was optimum (85.71%) with 1000 mg L^{-1} IBA after 6 weeks. Our findings confirm the results achieved for the same taxon according to a recent study [28], who reported that 1000 and 2000 mg L^{-1} of IBA resulted in higher rooting percentages (72% and 73%, respectively), whereas the untreated apical stem cuttings had shown the lowest rooting performance (60%), regardless of season. The stimulating effect of IBA on vegetative propagation of *H. empetrifolium* subsp. *empetrifolium* (i.e. by cuttings in the current study) could be attributed to the activation of a number of successive physiological functions, involving polysaccharide hydrolysis activated under the effect of applied IBA, and as a result, increase of the content of physiologically active sugar, providing materials and energy for meristematic tissues and later for root primordia and roots [29]. Different propagation results were reported in other *Hypericum* species such as *H. reducium* [30], where stem tip cuttings rooted up to 70% even without the use of auxins (IBA, NAA) and *H. perforatum* [20], where stem tip cuttings rooted earlier and developed best when treated with 5 μM 2.4-D than with other auxins (IBA, NAA, IAA).

In this study with *H. empetrifolium* subsp. *empetrifolium*, the higher applied IBA concentration of 4000 mg L^{-1} influenced negatively the rooting ability of cuttings. Although auxins have been found to catalyze enzymatic reactions and thus increase the rate and quality of root production, in high concentrations they can have the opposite effect and retard or inhibit the formation of roots [31]. In general, the use of auxins stimulates rooting of different propagating materials, but the concentration to be used for this purpose routinely varies with the species studied, the maturity level of the propagating material used, the prevailing environmental conditions, as well as the mode of application of the PGRs to the plants [28-31].

The effects of auxins and cytokinins on *in vitro* shoot multiplication is known only for limited number of *Hypericum* species [15, 32]. With respect to *H. empetrifolium* subsp. *empetrifolium* studied here, BA application without auxins hardly affected the number and length of produced shoots. It has been reported that *H. retusum* shoots were successfully propagated after two subcultures in the presence of BA or kinetin (Kn) and among the two cytokinins, BA was more efficient than Kn in promoting shoot formation [33]. In contrast to our results, in *H. perforatum* seedlings [15] and *H. perforatum* leaf-explants [17], BA was found to be the most efficient cytokinin in shoot formation. Our findings support that 0.1 mg L^{-1} BA + 0.01 mg L^{-1} IBA was the best hormonal combination for shoot proliferation of *H. empetrifolium* explants (shoot number, shoot proliferation rate and shoot formation percentage). In accordance with our results, other studies in *H. foliosum* [32] and *H. canariensi* [34] also found that the highest number of shoots was obtained on media supplemented with BA and NAA.

Data from studies in *H. perforatum* [17, 35] support the use of auxins for *in vitro* rooting. However, the other reports indicated that PGRs were not necessarily needed for rooting in *H. erecta* [36], *H. perforatum* cv. 'Anthos' [16]; or rooting might be induced with or without auxins in *H. perforatum* [37]. In the current study, rooting of *in vitro* shootlets was optimum with 0.1 mg L^{-1} IBA, among the 3 applied auxin types (IBA, NAA, IAA) and the 2 concentrations (0.1 and 0.25 mg L^{-1}) tested. Similar findings to ours were reported also for *H. perforatum* [19] and *H. sinaicum* [38], indicating that shootlets rooted best on MS medium fortified with 1 mg L^{-1} IBA. Accordingly, the elongated shoots of *H. gaitii* were rooted maximum in full strength MS basal salts supplemented with 1.5 mg L^{-1} IBA [39]. In other *Hypericum* species, however, IAA proved to be the most appropriate auxin for *in vitro* rooting including *H. maculatum* applied at 1 mg L^{-1} [40] and *H. perforatum* at 2 mg L^{-1} [41].

Conclusion

To the best of our knowledge, no report is currently available on *in vitro* propagation of *Hypericum empetrifolium* subsp. *empetrifolium* starting from shoot tip explants as initial plant material, apart from the one reported by Akoumianaki-Ioannidou *et al*. [2], who established a micropropagation protocol for the same species-subspecies starting initially from *in vitro* germination of seeds. The 0.1 mg L^{-1} BA + 0.01 mg L^{-1} IBA hormonal combination was proved to be the most effective from those tested for the *in vitro* shoot proliferation phase and 0.1 mg L^{-1} IBA for the rooting phase. Regarding its vegetative propagation, rooting of softwood top cuttings in mid-autumn was affected by rooting hormone treatment and the rooting of cuttings was optimum with 1000 mg L^{-1} IBA (6 weeks). The results related to *H. empetrifolium* subsp.

empetrifolium propagated from cuttings and in tissue culture suggest that both techniques are feasible for producing selected plant material suitable either for reintroduction purposes and germplasm conservation, or for potential sustainable exploitation in the medicinal-cosmetic and ornamental-horticultural industries.

Acknowledgements
The postdoctoral research of Dr. Virginia Sarropoulou and the scientific publication was carried out within the framework "Strengthening of Postdoctoral Researchers" of the OP "Development of Human Resources, Education and Lifelong Learning", 2014-2020, which is being implemented from the National Scholarships Foundation (NSF) in Greece, and is co-funded by the European Social Fund and the Hellenic Republic.

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