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Asexual Propagation of Four Medicinal Greek Endemic Plants of Lamiaceae Family With Conservation Priority From The Collection of The Balkan Botanic Garden of Kroussia, N. Greece.

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Abstract

Conservation of endemic rare-threatened plants and sustainable exploitation of biodiversity with emphasis on medicinal-aromatic plants and plants with horticultural/ornamental value can be achieved through ex situ conservation activities. For this purpose, propagation experiments with cuttings were performed on four local Ionian endemic species with conservation priority, *Stachys ionica* Halácsy, *Teucrium halascyanum*, *Thymus holosericeus* Čelak and *Thymus plasonii* Adamović (all Lamiaceae). For propagation, softwood tip cuttings (3-5 cm) were cut at early autumn from mother plants collected from the wild and maintained in open-air mother plantations. For experimentation, the base of cuttings was immersed for 1 min in solutions of four concentrations of IBA (0, 1000, 2000 and 4000 ppm). Cuttings were placed on a peat:perlite (1:3) substrate in the bench of greenhouse heated mist system. Most suitable treatment for *T. halascyanum* (3 ½ weeks) proved to be 1000 ppm IBA (32.13 roots 1.72 cm long, 100% rooting). Accordingly, 2000 ppm IBA gave 100% rooting for both *S. ionica* (28.5 roots 1.56 cm long,) (three weeks) and *Th. holosericeus* (4.4 roots 1.76 cm long) (seveneeks). *T. plasonii* cuttings treated with 2000 ppm IBA gave 85.71% optimum rooting with 8.67 roots 1.78 cm long.

Indexing terms/Keywords: Cuttings, *ex situ* conservation, Greek Flora, propagation protocols, rooting, *Stachys ionica*, *Teucrium halascyanum*, *Thymus holosericeus*, *Thymus plasonii*.

Introduction

In Europe, the conservation status of more than half of the habitats and species listed in the Annexes of the EU Habitats Directive is classified as unfavorable, and the target of halting biodiversity loss by 2010 was not achieved [1].Therefore, ex situ conservation constitutes an increasingly important supplementary tool for protection and in situ conservation of target-plants [2-5].

Stachys L. is one of the largest genera of the Lamiaceae family [6, 7]. Some species are annual, biennial, perennial, sub-shrub or shrubby and are found in rocky regions and mountain steppes [8, 9]. *Stachys* species are mainly distributed in the warm temperate regions of the Mediterranean and southwest of Asia [7, 8]. Due to the high content of secondary compounds, *Stachys* species are used in traditional medicine for the treatment of cardiac disease and are incorporated into anti-inflammatory drugs, analgesics and anticonvulsants [10]. The aerial parts are used in antispasmodic, diuretic, asthmatic, rheumatic and contained antibacterial and antioxidant compounds [11]. *Stachys ionica* Halácsy is a regional endemic of some Ionian Islands (Kefalonia, Zakynthos, Ithaka, Lefkada, Echinades), included in the Natura 2000 IPS and assessed as rare (R) [12]. *S. ionica* is a range-restricted Greek endemic chamaephyte that grows on cliffs, rocks, walls, ravines and boulders [13, 14].

Teucrium halacsyanum Heldr., an endemic species of Flora Hellenica, can be considered as an important element of the Ionian floristic region, since its main distribution area includes all major Ionian Islands (Zakynthos, Cephalonia, Lefkada, Kerkyra) and some of the western and southern coasts of Sterea Hellas (Prefecture of Aetolia-Akarnania). It is a characteristic obligate chasmophyte found mostly on limestone cliffs [15]. The genus

Teucrium belongs to the family Labiatceae and contains more than 300 perennial herbs, shrubs and sub-shrubs mainly native to Mediterranean region and south western Asia [16].Vegetative propagation by stem cuttings has been successfully applied to other *Teucrium* species (*T. divaricatum, T. polium, T. chamaedrys, T. flavum, T. scorodomia*) [17-20]. The effects of exogenous auxin on adventitious root formation in several *Teucrium* species has already been documented [20, 21].

The thyme belongs to the Lamiaceae family, genus Thymus, from which, there are possibly more than five hundred species due to the uncountable possibilities of hybridization and mutation [22]. Plants originating from the propagation of seeds can present distinct physical-chemical characteristics into a culture. The vegetative propagation is important because it provides the maintenance of the characteristics of the mother plant, standing out via stakes propagation, for the small quantity of material necessary and for the speed to obtain the seedlings [22].

Used for thousands of years in traditional medicine, the effects of *thymus* species in medicine are wide, from antimicrobial and anti-inflammatory to possible treatment for dementia or oncological pathologies [23]. *Thymus holosericeus* Čelak, an uncommon element of sclerophyllous vegetation is a regional endemic of some Ionian Islands (Kefalonia, Zakynthos, Lefkada), included in the Natura 2000 IPS and assessed as rare (R) [12]. *Th. holosericeus* is a range-restricted Greek endemic chamaephyte that grows on xeric Mediterranean phrygana and grasslands [13, 14].

Thymus plasonii Adamović is assessed as Endangered [IUCN criteria A2c, B2a,b(iii)] due to: small area of occupancy, small population size (<8000 individuals) and high probability of habitat alteration / destruction imposed by human activities (urbanization, overgrazing, construction works, invasion by many alien plants *Ailanthus altissima, Symphyotrichum squamatum, Diplotaxis tenuifolia, Solanum elaeagnifolium* etc.) [24]. *Th. plasonii* is a range-restricted Greek endemic chamaephyte that grows on temperate and sub-Mediterranean grasslands [13, 14].

Various auxins have been shown to improve overall rooting percentages, hasten root initiation, increase the number and quality of roots [25] and promote the development of uniform roots [26]. However, the responses to different types and concentrations of auxins vary among plant species and are affected by genotypes [27].

The aim of the study was to investigate the propagation possibilities of four local Ionian endemic plants of Lamiaceae family (*S. ionica, T. halascyanum, Th. holosericeus, Th. plasonii*) through softwood top cuttings. Thus, this study aimed to develop a method of vegetative rescue of these species under conservation priority through the induction of rooting after treating cuttings with different IBA concentrations.

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 four species was conducted. Softwood tip cuttings of 3-5 cm were taken during early-mid autumn from mother plants developed inside the greenhouse (3-4 cm for *S. ionica*, 3-5 cm for *T. halascyanum, Th. holosericeus* and *Th. plasonii*). The effect of the auxin indole-3-butyric acid (IBA) at four different concentrations (0, 1000, 2000, and 4000 ppm) on root formation was tested. Cuttings after immersion for 1 min in solutions of different IBA concentrations 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 weather conditions. Relative humidity was approximately 70-85% (mist). Experiments lasted for 3 weeks for *S. ionica*, 3 ½ weeks for *T. halascyanum* and seven weeks for both *Th. holosericeus* and *Th. plasonii* followed a randomized design with 7 replications per treatment for *S. ionica, Th. holosericeus* and *Th. plasonii*, and for *T. halascyanum* 9 replications per treatment. At the end of the experimental period, different for each species, the number of roots per rooted cutting and root length were measured. Rooting was expressed as %. Produced rooted plants from all 3 species were then transplanted in pots of 0.33 Lt (8x8x7 cm) and subsequently in 2.5 Lt containing a mixture of peat (Klasmann, TS2), perlite and soil (2:1:1 v/v). Plants were maintained at the nursery with the aim of creating adequate initiation material for future experiments on sexual or asexual propagation.

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≤0.05.

Results

Vegetative propagation of *S. ionica* by cuttings was successfully achieved within three weeks. All IBA concentrations (1000-4000 ppm) increased the number of roots (28.5-38.29) compared to the control (1.69 roots/rooted cutting). Rooting was 100% when cuttings treated with 2000 ppm IBA with respect to the IBA-untreated cuttings (85.71%). IBA gave similar root lengths (1.57-1.86 cm) to the control (1.69 cm). Symptoms of browning from the base to the top and subsequent necrosis were observed only in cuttings treated with 4000 ppm IBA (71.43%). Therefore, the best rooting treatment for *S. ionica* cuttings was 2000 ppm IBA (38.29 roots/rooted cutting, 1.86 root length, 100% rooting) (Table 1 and Fig. 1).

Table 1. Effect of IBA concentration (0, 1000, 2000 and 4000 ppm) on root number/rooted cutting, root length, rooting and necrosis percentages (%) in *S. ionica* cuttings (three weeks).

IBA (ppm)	Rooting (%)	Root number	Root length (cm)	Necrosis (%)
Control	85.71 b	12.17 ± 3.29 a	16.92 ± 3.09 a	0 a
1000	85.71 b	28.83 ± 3.33 b	15.65 ± 1.23 a	0 a
2000	100 c	38.29 ± 6.94 b	18.61 ± 1.43 a	0 a
4000	28.57 a	28.50 ± 5.35 b	15.56 ± 0.39 a	71.43 b
P-values	0.000***	0.010**	0.612 Ns	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. Ns: P ≥ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

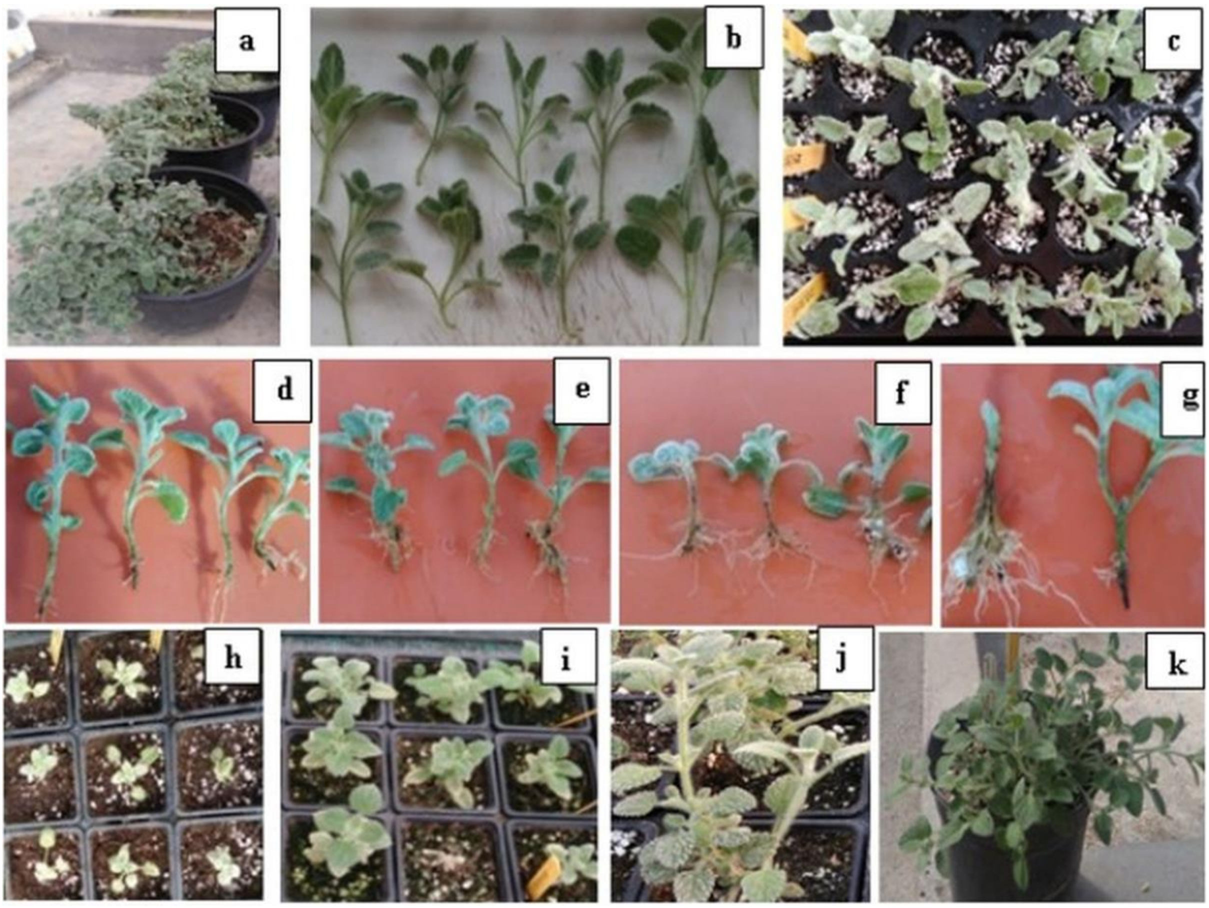

Fig 1. Asexual propagation of *S. ionica* in early-autumn: (a) mother plants, (b) cuttings prior to experimentation (c) cuttings in mist (all treatments), (d) control,(e) 1000 ppm IBA, (f) 2000 ppm IBA, (g) 4000 ppm IBA, (h-j) vegetative growth of transplanted rooted cuttings into 0.33 Lt pots; h – day 1, i – day 29, j – day 60, and (h) into 2.5Lt pots after three months.

In *T. halascyanum* cuttings, rooting was optimum (100%) with 1000 ppm IBA. Root numberwas not affected due to IBA application (24.13-32.89 roots/rooted cutting) in relation to the control (24.83 roots). Root length was negatively influenced, approximately by 0.5 cm when cuttings were treated with 4000 ppm IBA. Thus, 1000 ppm IBA (32.13 roots 1.72 cm long, 100% rooting) was the optimum treatments for rooting of cuttings after 3.5 weeks in autumn season (Table 2and Fig. 2).

Table 2. Effect of IBA concentration (0, 1000, 2000 and 4000 ppm) on rooting percentage (%), root number/rooted cutting and root length in *T. halascyanum* cuttings (3 ½ weeks).

IBA (ppm)	Rooting (%)	Root number	Root length (cm)
Control	85.71 a	24.83 ± 1.78 a	17.29 ± 0.76 b
1000	100 c	32.13 ± 4.96 a	17.15 ± 1.67 b
2000	90 b	32.89 ± 6.57 a	13.73 ± 1.20 ab
4000	88.89 b	24.13 ± 4.48 a	12.58 ± 1.05 a

P-values	0.000***	0.427 Ns	0.018*

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. Ns: P ≥ 0.05, * P ≤ 0.05, *** P ≤ 0.001.

Fig. 2. Asexual propagation of *T. halascyanum* in early-autumn: (a) mother plants, (b) cuttings prior to experimentation (c) cuttings in mist (all treatments), (d) control, (e) 1000 ppm IBA, (f) 2000 ppm IBA, (g) 4000 ppm IBA, (h) vegetative growth of transplanted rooted cuttings into 0.33 Lt pots after 15 weeks, and (i, j) into 2.5Lt pots after one and Four weeks, respectively.

Regarding *Th. holosericeus* cuttings, the best rooting performance within 7 weeks was the 2000 ppm IBA (8.67 roots 1.76 cm long, 100% rooting percentage), which was the only treatment with no appearance of necrotic cuttings. Root length was significantly enhanced in all IBA treatments (1.61-1.76 cm). The application of 1000 and 4000 ppm IBA even though gave higher root numbers (8.67-9 roots/rooted cutting), caused browning and necrosis symptoms to the majority of the cuttings (57.14% and 85.71%, respectively). No root formation occurred in the control IBA-untreated cuttings (Table 3 and Fig. 3).

Table 3. Effect of IBA concentration (0, 1000, 2000 and 4000 ppm) on root number/rooted cutting, root length, rooting and necrosis percentages (%) in *Th. holosericeus* cuttings (Seven weeks).

IBA (ppm)	**Rooting (%)**	**Root number**	**Root length (cm)**	**Necrosis (%)**
Control	0 a	0.00 ± 0.00 a	0.00 ± 0.00 a	100 d
1000	42.86 c	8.67 ± 1.28 c	17.63 ± 3.07 b	57.14 b
2000	100 d	4.14 ± 1.12 b	17.57 ± 4.91 b	0 a
4000	14.26 b	9.00 ± 0.00 c	16.11 ± 0.00 b	85.71 c
P-values	0.000***	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.

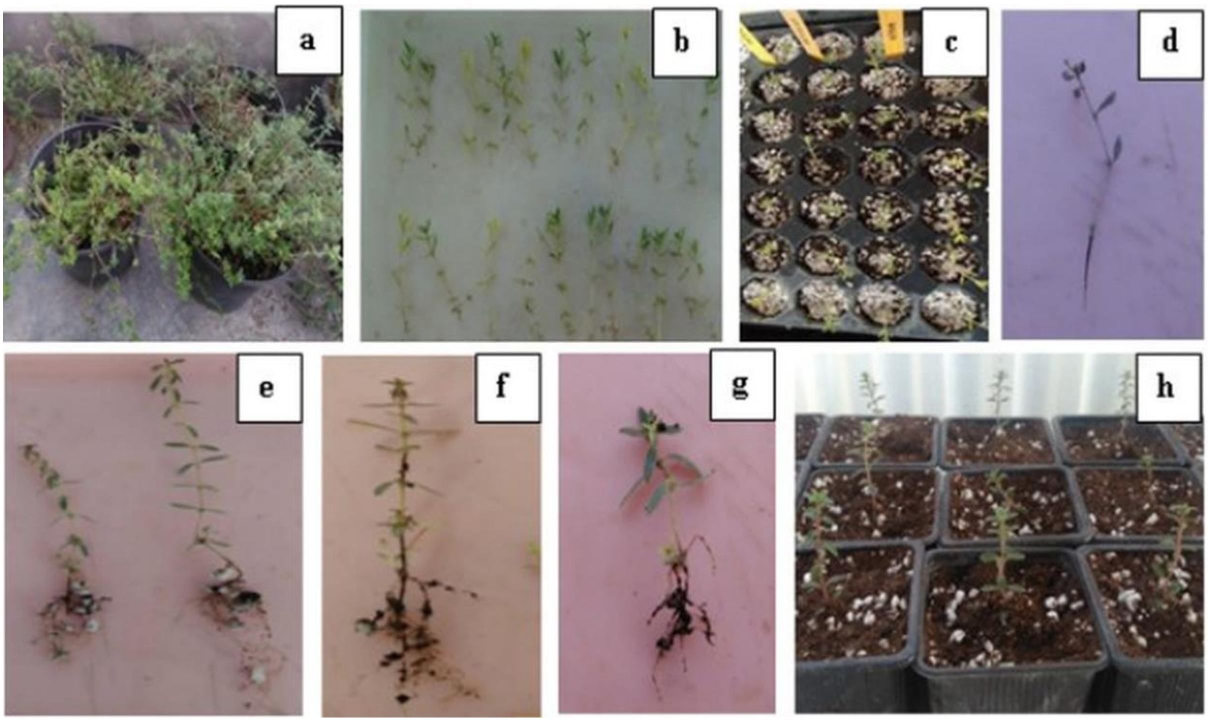

Fig. 3. Asexual propagation of *Th. holosericeus* in early-autumn: (a) mother plants, (b) cuttings prior to experimentation (c) cuttings in mist (all treatments), (d) control,(e) 1000 ppm IBA, (f) 2000 ppm IBA, (g) 4000 ppm IBA, and (h) vegetative growth of transplanted rooted cuttings into 0.33 Lt pots after one week.

Regarding *T. plasonii* cuttings, the best rooting treatment was 2000 ppm IBA (8.67 roots 1.78 cm long, 85.71% rooting percentage). Root length was not affected due to increasing IBA concentrations (1.78-2.15 cm) compared to control (1.74 cm). IBA at 4000 ppm gave the higher root number (12 roots/rooted cutting) but the highest necrosis percentage (57.14%). The highest rooting (85.71%) and lowest necrosis percentages (14.26%) were obtained with 2000 ppm IBA, differing to the control's (57.14% rooting, 42.86% necrosis) (Table 4 and Fig. 4).

Table 4. Effect of IBA concentration (0, 1000, 2000 and 4000 ppm) on root number/rooted cutting, root length, rooting and necrosis percentages (%) in *Th. plasonii* (7 weeks).

IBA (ppm)	Rooting (%)	Root number	Root length (cm)	Necrosis (%)
Control	57.14 b	7.13 ± 0.67 a	17.42 ± 3.39 a	42.86 c
1000	80.00 c	7.13 ± 0.74 a	20.12 ± 2.26 a	20.00 b
2000	85.71 d	8.67 ± 1.00 a	17.84 ± 1.56 a	14.26 a
4000	42.86 a	12.00 ± 0.75 b	21.45 ± 0.23 a	57.14 d
P-value	0.000***	0.000***	0.521 Ns	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 $P \leq 0.05$. Ns: $P \geq 0.05$, *** $P \leq 0.001$.

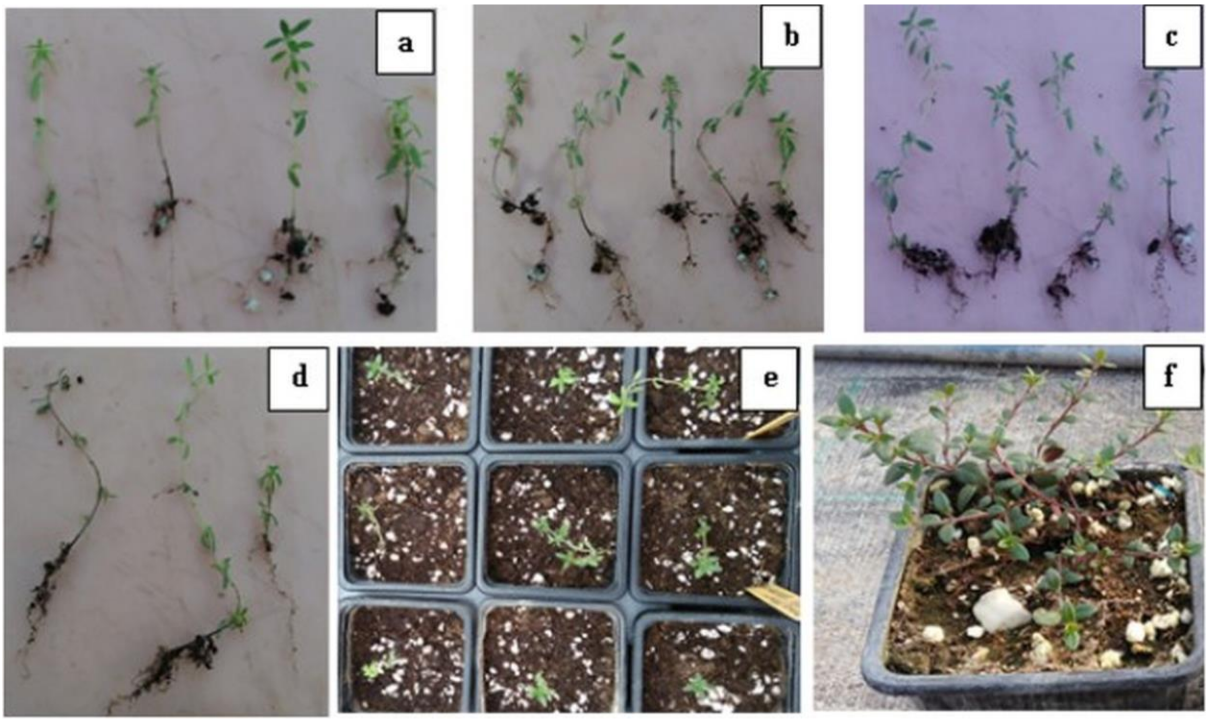

Fig. 4 Asexual propagation of *Th. plasonii* cuttings: **(a)** Control, **(b)** 1000 ppm, **(c)** 2000 ppm, **(d)** 4000 ppm IBA, **(e and f)** transplantation and vegetative growth of rooted cuttings into 0.33 Lt pots after 3 days and three months, respectively.

Discussion

In Lamiaceae family, a lot of species had stems able to form roots in cases of contacts with soil or water which enables the propagation with cuttings to be performed [28]. The macropropagation techniques play an integral part of improvement programs and have been explored for the propagation of many economically valuable species particularly the rare, endangered, and threatened plant species [29].

Auxins have been reported as central mediator of organ developments by promoting cell division, cell elongation, and cell differentiation [30]. In the present study with *S. ionica*, rooting of cuttings was 100% with 2000 ppm IBA. Similarly, in a previous conducted study, propagation success up to 80-100% was recorded for the same taxon (*S. ionica*) [31]. IBA has been reported to markedly increase adventitious root formation in many species [32-34]. However, with respect to the studied *S. ionica*, the highest applied IBA concentration of 4000 ppm was toxic because it caused browning and necrotic symptoms to the majority of the cuttings (71.43%). In another *Stachys* species (*S. arvensis*), rooting success of cuttings ranged from 60 to 80% [31].

In the current study employing *T. halascyanum*, the immersion of cuttings in solutions of different IBA concentrations stimulated their rooting ability (89-100%). Propagation success 60-80% by using cuttings in the same taxon to ours (*T. halacsyanum*) has also been reported [31]. IBA also positively affected root formation of *Teucrium* species. Rooting 100% was recorded for studied *T. halascyanum* cuttings treated with 1000 ppm IBA. Similar findings have been reported in other *Teucrium* species, including *T. divaricatum* (4000 ppm IBA: 100% rooting, 24.3 roots 1.0 cm long), *T. polium* (4000 ppm IBA: 88% rooting, 13.1 roots 0.9 cm long) and *T. chamaedrys* (2000 ppm IBA: 83% rooting, 19.3 roots 1.4 cm long) [20].

In relation to *T. halacsyanum* under experimentation, all IBA treatments led to an increase in rooting percentage of cuttings, whereas no substantial differences were observed in the number of roots. Different findings but up to a point were obtained in *Teucrium frutescens*, where the application of IBA improved both rooting percentage and root number as compared to untreated control [35]. In this study, 4000 ppm IBA had an adverse effect on root length of *T. halacsyanum* cuttings. The negative effect of high IBA concentrations on root length of cuttings could be attributed to an increase in ethylene production by plant cells as a reaction [33]. IBA treatments improved vegetative propagation of *T. halacsyanum* under study by softwood top cuttings within 3.5 weeks in autumn, compared to control. In accordance with our findings, in *Teucrium capitatum*, stem tip cuttings and bearing axillary shoots rooted at higher percentages (70-90%) after dipping in solutions with 1000-3000 mg/l IBA than in the control [36]. In the current study regarding *T. halacsyanum*, rooting response during autumn was high (85-100%) even in the control untreated cuttings. On the other hand, IBA treatments enhanced rooting of *T. fruticans* stem tip cuttings exhibiting a low rooting response during spring (40% rooting) even applying exogenous auxin [21].

In *Th. holosericeus* cuttings under study, 100% rooting was exhibited by the use of 2000 ppm IBA. In consistency with our findings, the vegetative propagation of other thymus species (*T. capitatus, T. serpyllum* and *T. vulgaris*) was also improved (increased rooting) when softwood cuttings treated with 500 ppm IBA solution [18]. A previous conducted study in the same taxon (*Th. holosericeus*) revealed 80-100% rooting of cuttings [31]. Similar findings have been reported in other thymus species including *Th. plasonii* (4000 ppm IBA: 100% rooting, 13.1 roots 1.5 cm long), *Th. longicaulis* subsp. *chaubardii* (4000 ppm IBA: 100% rooting, 12.4 roots 2 cm long) and *Th. degenii* (2000 ppm IBA: 100% rooting, 13.1 roots 1.5 cm long) [20]. However, no root formation occurred in the control-untreated *Th. holosericeus* cuttings within the seven -weeks experimental period inside the mist. The formation of roots on cuttings treated with auxins was important for survival, as cuttings in the control that did not form roots wilted and died [37]. Moreover, usage of rooting products has been reported as necessary to guarantee the rooting of cuttings for earlier transplanting [38].

The exogenous application of IBA generally improves rooting in thymus species [18]. In the current study with *Th. plasonii*, the two intermediate IBA concentrations (1000 and 2000 ppm) resulted concurrently in augmented rooting and diminished percentage of cuttings with browning and necrotic symptoms appearance. According to a previous conducted research study, asexual propagation of softwood cuttings of *Th. holosericeus* was successfully performed in 15 days where 0.2% IBA gave 80% rooting percentage with 13.1 roots/cutting and 1.5 cm root length [31]. In *Th. plasonii* under study, a decline in rooting potential and more intensified browning and gradually necrotic symptoms from the base towards the top of the cuttings were evident in the control and 4000 ppm IBA. Similar results were recorded for *Th. holosericeus*, in which the control (0% IBA) showed only 45% rooting and treatment with 0.4% IBA caused side effects and rooting was inhibited [31]. In the present experiment regarding *Th. plasonii* cuttings, 80 – 85.71% were the higher rooting percentages obtained after a seven-weeks period in early-mid autumn. However, the combination of GIS data with previous experience on rooting of other species of the same genus raised propagation success of *Th. holosericeus* nearly by 90% (from 45 to 80% rooting) revealing that early spring was the appropriate season for the rooting trials since greenhouse temperatures emulate those in its natural environment [20]. In accordance with our results for *Th. plasonii*, basal cuttings of *Thymus satureioides* treated with auxin IBA had a low average rate of rooting (11-25%) after eight weeks, besides, the hormone treatment significantly improved the rooting rate of summit cuttings (control: 48% and 91% with 500 ppm IBA) [39].

The presence of leaves on cuttings has been shown to stimulate root initiation in many species [40]. Leaves are important because they promote root formation and manufacture carbohydrates via photosynthesis [41]. In all four plant species under experimentation (*S. ionica, T. halascyanum, Th. holosericeus* and *Th. plasonii*), softwood top cuttings along with their upper leaves and stems were used. Rooting is stimulated by high levels of available carbohydrates provided by the leaves. Therefore, for cuttings to form new shoot and root tissues, it is important that they are supplied with appropriate amounts of light and water, since both are required for photosynthesis [42].

CONCLUSIONS

Vegetative propagation protocols for four locals Ionian endemics of Greece with conservation priority were optimized with subsequent development of plants using softwood top cuttings as source material. The results of this study showed that IBA is suitable for the mass propagation of the cuttings. Rooting 100% was achieved for the three out of the four species, *S. ionica, T. halascyanum* and *Th. holosericeus*, and up to 86% for *Th. plasonii*. The best rooting treatment for *S. ionica*, *Th. holosericeus* and *Th. plasonii* was 2000 ppm of IBA while for *T. halascyanum* 1000 ppm of IBA. Asexual propagation by cuttings was successfully performed within three weeks for *S. ionica*, 3 ½ weeks for *T. halascyanum* and seven weeks for both *Th. holosericeus* and *Th. plasonii* during autumn. The optimized protocols can be used to develop healthy and profuse root system with shoot bud development and further proliferation. This propagation method can play key role in rapid supply of quality planting material and help in minimizing the current challenge of their conservation. Through this method is viable the production of sufficient resources for habitat rehabilitation and for research on the medicinal properties of these important plants. Applied research, therefore, focuses on the development of propagation protocols allowing the re-introduction of species into their natural environment and facilitates their sustainable exploitation for high-added value products.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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