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In Vitro Propagation of the Mount Parnitha Endangered Species *Sideritis raeseri* subsp. *Attica*

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Abstract: Over the past few decades, both wildfires and human-sparked fires have ravaged Mount Parnitha, destroying the mountain's unique natural ecosystem, applying pressure to its flora, and subjecting the vulnerable populations of *Sideritis raeseri* subsp. *attica* to excessive stress. The present study aims to establish an efficient micropropagation method starting from in vitro-grown seedlings. The in vitro germination study carried out during the production of seedlings revealed a higher germination rate (34.0% and 37.0%, respectively) at 20.0 °C and 25.0 °C. The in vitro-derived seedlings studied were used as the starting material for the establishment of various media. Murashige and Skoog (MS) media, hormone-free and containing 0.5 mg L^{-1} 6-benzyladenine (BA), led to the satisfactory (84.0–89.0%) establishment of plantlets. During the multiplication phase, the study used BA in conjunction with α-naphthaleneacetic acetic acid and four different cytocinins (BA; kinetin (KIN); 6-(γ-γ-dimethylallylamino) purine; zeatin) at low concentrations (0.5 mg L^{-1}). During the second subculture, a high multiplication index (7.3 and 6.4, respectively) was found for the hormone-free MS medium and the MS medium containing KIN at 0.5 mg L^{-1}. Hyperhydricity took place on the media supplemented with hormones. Rooting occurred on the half-strength MS medium (51.0%). After two months, the plants' survival rate stood at 100.0%, as did their ex vitro acclimatisation rate, which also registered at 100.0%. The present results could encourage not only the introduction of *S. raeseri* subsp. *attica* into the industry of floriculture as a new ornamental plant but also its ex vitro conservation.

Keywords: in vitro germination; ex vitro acclimatisation; GSI; micropropagation; tissue culture

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1. Introduction

Mount Parnitha is one of the ten national parks of Greece, located 35.0 km north of the metropolitan city of Athens [1]. It is part of the Natura 2000 Network (established by the European Union, Directive 92/43/EEC), a network of sites that are home to rare habitats and species. Mount Parnitha comprises three vegetation zones with 1093 plant species; ninety-three of these are Greek endemics [2]. Since ancient times, the vegetation of Parnitha has been under enormous human pressure brought about by anthropogenic factors, urbanisation, and fires. It is clear that, over time, the types of pressures and their temporal patterns, in conjunction with invasions and urbanisation [3], may eventually take their toll on Parnitha, leading to the extinction of some of the mountain's unique flora. Since 1913, there have been 438 fires on Mount Parnitha, which have ravaged the entire forest approximately six times (Figure 1A) [4–6].

Sideritis L. is one of the three largest genera of the Lamiaceae family. It includes more than 150 species of shrubs and annual or perennial herbs that are found mostly in the Mediterranean Basin [7] and it has long been known as an integral part of Greek flora. Its natural populations are threatened by cultivation in neighbouring fields with cultivars of unrelated resources [8]. The genus is used in homoeopathic medicine as a herbal tea and in folk medicine for the preparation of a variety of remedies. In Greece, there are only two taxa, i.e., *S. scardica* and *S. raeseri* Boiss. & Heldr. subsp. *raeseri*, which are cultivated on a

small scale for the preparation of mountain tea. Recently, the cultivation of *S. syriaca* subsp. *syriaca* and *S. clandestine* (Bory & Chaub.) Hayek subsp. *clandestina* has also begun [9].

Figure 1. (**A**) The slopes of Mount Parnitha after the widespread fires of 2007, 15 years ago; (**B**) mother plants of *Sideritis raeseri* subsp. *attica* on Parnitha; (**C**) flowering phase in July; (**D**) mother plants during seed collection.

S. raeseri Boiss. & Heldr. subsp. *attica* (Heldr.) Papan. & Kokkini [10] was placed on the list of vulnerable species [11] but its extinction risk (Figure 1B) has recently been updated, placing it on the list of the endangered species found on Mount Parnitha [12]. Small populations of the species can also be found on Mounts Kithairon and Pateras [13]. A hardy perennial that grows up to 80.0 cm, *S. raeseri* subsp. *attica* sprawls on rocky slopes at elevations over 1000.0 m. Its yellow flowers bloom from June to August on short, woody stems 30.0 cm long (Figure 1C,D). The species shows the potential for being a promising introduction as an ornamental plant into the landscape architecture and floriculture industries, as it is quite suitable for thematic gardens, rocky places, and degraded areas.

The members of the *Sideritis* genus have been studied for their antioxidants and their valuable components [14–16]. Moreover, they could be used as a natural source of antioxidants in the food industry and medicine [17,18]. The Cretan *S. syriaca* subsp. *syriaca* shows promising medicinal and cosmetic potential [19] and is grown locally in Crete as a pilot crop [20,21]. As to *S. raeseri*, commercial nursery growers provide farmers with plantlets that have been established mainly asexually and collected from populations found in the wild. Still, the origin and properties of this type of material remain uncharacterised [21]. The increased interest of nurseries and farmers in the propagation of aromatic plants in tandem with the intensive harvesting practices during the species' flowering stage constitutes a threat to the distribution of *S. raeseri*.

To the best of our knowledge, apart from a study of *S. raeseri* Boiss & Heldr subsp. *raeseri* starting from adult mother plants [22], there are no reports in the literature on the propagation of the present subspecies. *S. raeseri* subsp. *attica* has attractive, ornamental characteristics that make it an ideal candidate for commercial exploitation. It is precisely those characteristics that could be exploited in synergy with the development of in vitro propagation methods, leading to the introduction of suitable methods for the plant's conservation, in situ and ex situ alike. In this task, biotechnological methods are essential to developing successful in situ conservation strategies, as well as ex situ collections aimed at plant preservation [23–26]. Not only does seed propagation for in vitro use enhance genetic diversity leading to the selection of genotypes of high commercial value [27] but it also barely impacts the environment as it requires a small number of seeds to produce many

plants and does not involve harvesting plant parts, especially from endangered species [28]. Furthermore, in vitro seed propagation allows for quick shoot multiplication by small quantities of initial explant material derived from plants in the wild through germinated seeds and makes propagation independent of seasons. The practice of seed propagation accompanied by efficient clonal propagation strategies can meet the increased demands of the ornamental plant market. Moreover, it presents a challenge for researchers, including the authors of the present study, who wish to both exploit and conserve the *Sideritis* species via studies on in vitro propagation.

Therefore, the aim of the present study was to suggest an efficient protocol for in vitro propagation of *S. raeseri* subsp. *attica* starting from in vitro-grown seedlings. The effects of the nutrient medium and auxin concentration on in vitro rooting were tested. In addition, an assessment of seed germinability was carried out. Finally, the acclimatisation rate was tested targeting the exploitation of the species and its introduction as an ornamental plant into the floriculture industry.

2. Materials and Methods

2.1. Plant Material

In July 2020, we visited Mount Parnitha in Attica, Greece (38°09′59.3892″ N, 23°42′58.9932″ E), where we proceeded to collect fully matured seeds on the same day. The fully matured seeds came from native plants in the wild (Figure 1C,D). Our harvest was then transferred to our laboratory at the Agricultural University of Athens, where the seeds were stored in paper bags in the dark at room temperature, where the temperature stood at 25.0 °C and the relative humidity at 30.0%. In vitro germination and propagation did not take place until three months had elapsed after seed storage.

2.2. In Vitro Germination

We accomplished the seeds' surface sterilisation with 20.0% (*v*/*v*) commercial bleach (4.6% *w*/*v* sodium hypochlorite) for 10.0 min and then rinsed them three times (3.0 min/time) with sterile, distilled water. After disinfection, the seeds were sown in plastic Petri dishes (9.0 cm) with half-strength Murashige and Skoog (MS) media [29]. The seed cultures were incubated at 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, and 35.0 °C. Germination was defined as the appearance of a radicle at least 2.0 mm long and could be recorded daily in compliance with the rules of the International Seed Testing Association [30]. T_{50} (days) was defined as the days needed to reach 50% of the final germination percentage [31]. We used sixty seeds per treatment (four Petri dishes per treatment/15 seeds per Petri dish) and employed the Maguire formula [32], which measures the germination speed index (GSI) as

$$GSI = G1/N1 + G2/N2 + \ldots + Gn/Nn$$

in which G1, G2, and Gn = the number of normal seedlings measured during the first, second, and last counts; and N1, N2, and Nn = the number of sowing days during the first, second, and last counts.

2.3. Micropropagation

2.3.1. Establishment of Initial Cultures

Five days after we had finalised the seed germination stage, we transferred the seedlings (young plantlets) grown in vitro to a hormone-free MS medium (8.0 g L^{-1} agar), where they remained for forty days until the time we moved on to the next stage, the establishment of initial cultures. We initiated a subculture on a hormone-free medium, as well as on a medium containing 6-benzyladenine (BA) at 0.5 mg L^{-1}, with or without 4.0 g L^{-1} polyvinylpyrrolidone (PVP).

2.3.2. Multiplication

During the multiplication stage, node explants were cultured on a hormone-free MS medium or supplemented with BA at 0.1, 0.5, and 1.0 mg L^{-1} and α-naphthaleneacetic acetic acid (NAA) at 0.01 and 0.1 mg L^{-1} in various combinations (first subculture). The effect of four different cytokinins, i.e., BA, kinetin (KIN), 6-(γ-γ-dimethylallylamino) purine (2iP), and zeatin (ZEA), at 0.5 mg L^{-1} was tested on the second subculture. The MS medium and McCown woody plant medium (WPM) [33] were tested during the multiplication stage to control hyperhydricity using node explants from shoots deriving from the first and second subcultures.

2.3.3. In Vitro Rooting

Microshoots that were 1.5–2.0 cm long and produced by sub-culturing on various media during the multiplication stage were transferred for rooting onto half-strength MS media or half-strength WPM media (MS/2 and WPM/2, respectively) containing 0.0, 0.5, 1.0, or 2.0 mg L^{-1} indole-3-butyric acid (IBA). A completely randomised two-factor factorial design was used for each plant species (i.e., two types of nutrient medium × four IBA concentrations).

2.3.4. In Vitro Culture Conditions and Data Collection

In vitro cultures were carried out in Magenta GA-7 vessels (7.2 cm × 7.2 cm × 10.0 cm, Sigma-Aldrich), with four explants per vessel. The cultures were maintained at 25.0 °C. Cool-white, fluorescent lamps provided a 16 h photoperiod at 37.5 μmol m^{-2} s^{-1}. All media contained 30.0 g L^{-1} sucrose; were solidified with 8.0 g L^{-1} agar; had their pH adjusted to 5.7–5.8; and were autoclaved at 121.0 °C for 20.0 min. The collection of data took place after forty days of culture for the shoot regeneration and after twenty days for the rooting experiments. Data were collected on the shoot proliferation percentage, shoot number per explant, length of the shoots, number of nodes per shoot, lateral shoots, lateral shoot length, and number of nodes. The percentage (%) of hyperhydricity and callus were also recorded. The proliferation potential of the cultures, in other words, the multiplication index (MI) of each culture, was calculated by multiplying the percentage of explants that produced shoots by the mean number of shoots and mean shoot length per responding explant, then dividing by 0.6 (the length of each explant used for the sub-culture). Rooting percentages and root numbers and lengths were recorded during the rooting experiments.

2.3.5. Ex Vitro Acclimatisation

All rooted microshoots measuring 1.5–2.0 cm in length were thoroughly rinsed under running tap water. They were then placed into containers (500.0 mL, eight plantlets/container) that included both peat (pH 5.5–6.5, Klasmann-Delimann GmbH, Geeste, Germany) and perlite (particles diameter 1.0–5.0 mm, Perloflor, Isocon S.A., Athens, Greece) substrate 1:1 (*v*/*v*). In order to control the humidity, we covered all containers with plastic wrap (Sanitas; Sarantis S.A., Athens, Greece). Next, the containers were moved into a growth chamber for seven days (chamber temperature: 25.0 °C; 16 h cool-white, fluorescent light 37.5 μmol m^{-2} s^{-1}/8.0 h dark photoperiod). Next, the containers stayed uncovered for a period of seven days and then were transferred onto a heated glasshouse bench for another seven days (37°58′58.0″ N, 23°42′19.2″ E). It was at the end of this period that data on acclimatisation were recorded. After acclimatisation, the plants were transplanted into 500.0 mL plastic pots containing peat:perlite (1/1, *v*/*v*) and were fertilised monthly with 2.0 g L^{-1} complete water-soluble fertiliser (Nutrileaf 60, 20-20-20; Miller Chemical and Fertilizer Corp., Hanover, PA, USA). The last step, which took place two months later, involved the calculation of the plants' survival rate.

2.4. Statistical Analysis

For the purposes of our statistical analysis, we used a completely randomised design method. The experiments of seed germination had 4 replications of 15 seeds each,

i.e., 60 seeds per treatment. The seed germination results for each treatment are shown as the mean ± SE (standard error mean). As shown in the in vitro propagation data tables, the number of replicates per treatment differed among the experiments.

The significance of the results was tested by one-way ANOVA. In the case of in vitro rooting, two-way ANOVA was performed to find the statistically significant differences and possible interactions between the medium (MS/2, WPM/2) and IBA concentration (0.0, 0.5, 1.0, or 2.0 mg L^{-1}). The treatment means were compared using Tukey's test at $p \leq 0.05$ (JMP 14.0 software, SAS Institute Inc., Cary, NC, USA, 2013). Data on percentages were arcsine-transformed prior to the statistical analysis.

3. Results

3.1. Seed Germination

The disinfection method of the seeds resulted in a 90.0% success rate. The germination percentages of 34.0% and 37.0% proved to be similar at 20.0 °C and 25.0 °C, respectively (Table 1; Figures 2 and 3). The germination percentages at the cardinal germination temperatures of 15.0 °C and 30.0 °C were 17.0% and 7.0%, respectively, with no difference. T_{50} was completed in 16 days at 20 °C and 20 days at 25 °C; the seeds in those treatments had a higher GSI, which stood at 24.3 and 20.3, respectively (Table 1). Germination was completed in 32.0–35.0 days.

Table 1. In vitro germination of *Sideritis raeseri* subsp. *attica* seeds, T_{50}, times for full germination, and germination speed indexes (GSI) at the temperatures shown.

Temperature (°C)	Germination (%) ± SE †	T_{50} †† (Days)	Time for Full Germination (Days)	GSI
10.0	0.0 ± 0.0 c	-	-	-
15.0	17.0 ± 4.6 b	21.0	35.0	9.0 b
20.0	34.0 ± 5.3 a	16.0	33.0	24.3 a
25.0	37.0 ± 7.5 a	20.0	32.0	20.3 a
30.0	7.0 ± 6.5 b	30.0	32.0	1.7 b
35.0	0.0 ± 0.0 c	-	-	-
$F_{\text{one-way ANOVA}}$	*			*

† (SE): standard error; †† (T_{50}): time for 50% germination; *: significant at $p \leq 0.05$; mean (±SE) separation in columns by Tukey's test at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; $n = 4$, 15 seeds/Petri dish (total 60 seeds per treatment).

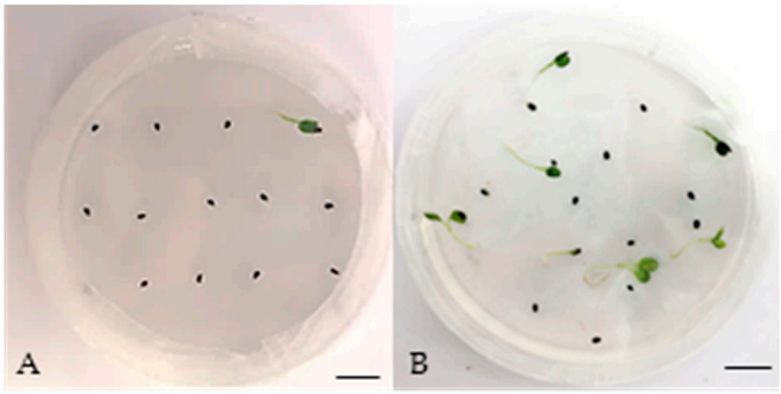

Figure 2. *Sideritis raeseri* subsp. *attica* seeds' germination in Petri dishes containing half-strength Murashige and Skoog media (MS) at 20.0 °C under 16.0 h light/8.0 h darkness: (**A**) after 9.0 days and (**B**) after 30.0 days of incubation. Bars represent a length of 1.0 cm.

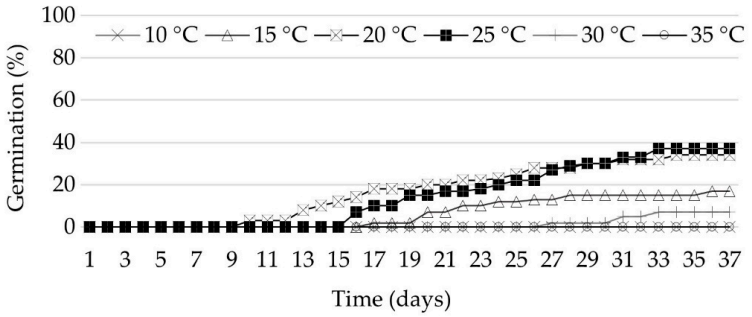

Figure 3. Germination time course curves of *Sideritis raeseri* subsp. *attica* seeds affected by temperature. Four Petri dishes (15 seeds/treatment) were used.

3.2. Micropropagation

3.2.1. Establishment of Initial Cultures

S. raeseri subsp. *attica* plantlets deriving from in vitro-grown seedlings were transferred to a hormone-free medium for further growth (initial culture). Forty days after the transfer, the plantlets had risen to 2.0–2.5 cm in height and showed vigorous growth of 5.0–7.0 leaves (Figure 4A,B). The establishment of initial cultures on MS media that was either hormone-free or supplemented with 0.5 mg L^{-1} was successful during a subsequent stage. The shooting percentage was exceptionally high and stood at over 84.0% (Table 2). Two shoots of similar heights were formed (1.1–1.4 cm) on the hormone-free media and the media supplemented with BA and presented no differences. On the other hand, on the media containing PVP, the shoots were the shortest, growing no higher than 0.6 cm (Figure 5A). A small number of short lateral shoots were formed. As to the plantlets themselves, 12.5–13.0% of them grew roots on the MS media that was hormone-free and the MS media that was supplemented with BA (Table 2, Figure 5A).

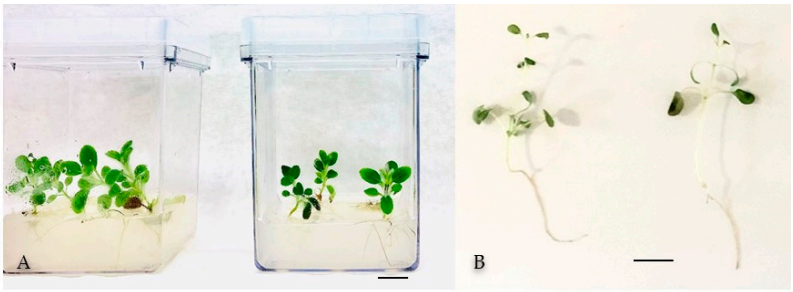

Figure 4. Forty-day-old young seedlings of *Sideritis raeseri* subsp. *attica* grown in vitro on half-strength MS media without any hormones (MS/2) (**A**) before and (**B**) during the subculture. Bars represent a length of 1.0 cm.

Table 2. Establishment stage: initial cultures of node explants excised from in vitro-grown, young seedlings of *Sideritis raeseri* subsp. *attica* on MS media without hormones (Hf) or supplemented with 6-benzyladenine (BA) at 0.5 mg L^{-1}, with or without polyvinylpyrrolidone (PVP) at 4.0 mg L^{-1}.

BA (mg L^{-1})	PVP (g L^{-1})	Shooting (%)	Shoot Number	Shoot Length (cm)	Node Number	Hyperhydricity	MI †	Lateral Shoot Number	Length of Lateral Shoots (cm)	Node Number of Lateral Shoots	Rooting (%)	Root Number	Root Length (cm)
-	-	89.0 a	2.2 a	1.1 a	4.2 a	-	3.6 ab	0.6 a	0.1 a	0.4 a	12.5 a	15.0 a	2.6 a
0.5	-	84.0 a	2.1 a	1.4 a	4.5 a	19.0 a	4.1 a	0.2 a	0.1 a	0.1 b	13.0 a	18.0 a	1.5 a
0.5	4.0	95.0 a	2.0 a	0.6 b	3.3 b	12.0 a	1.9 b	0.5 a	0.1 a	0.1 b	-	-	-
$F_{\text{one-way ANOVA}}$		ns	ns	**	**	ns	**	ns	ns	*	ns	ns	ns

† (MI): multiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6; *, **, significant at $p \leq 0.05$, $p \leq 0.01$, respectively; *F* values represented by ns indicate no significant differences at $p \leq 0.05$; mean separation in columns by Tukey's test at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; n = 45–60.

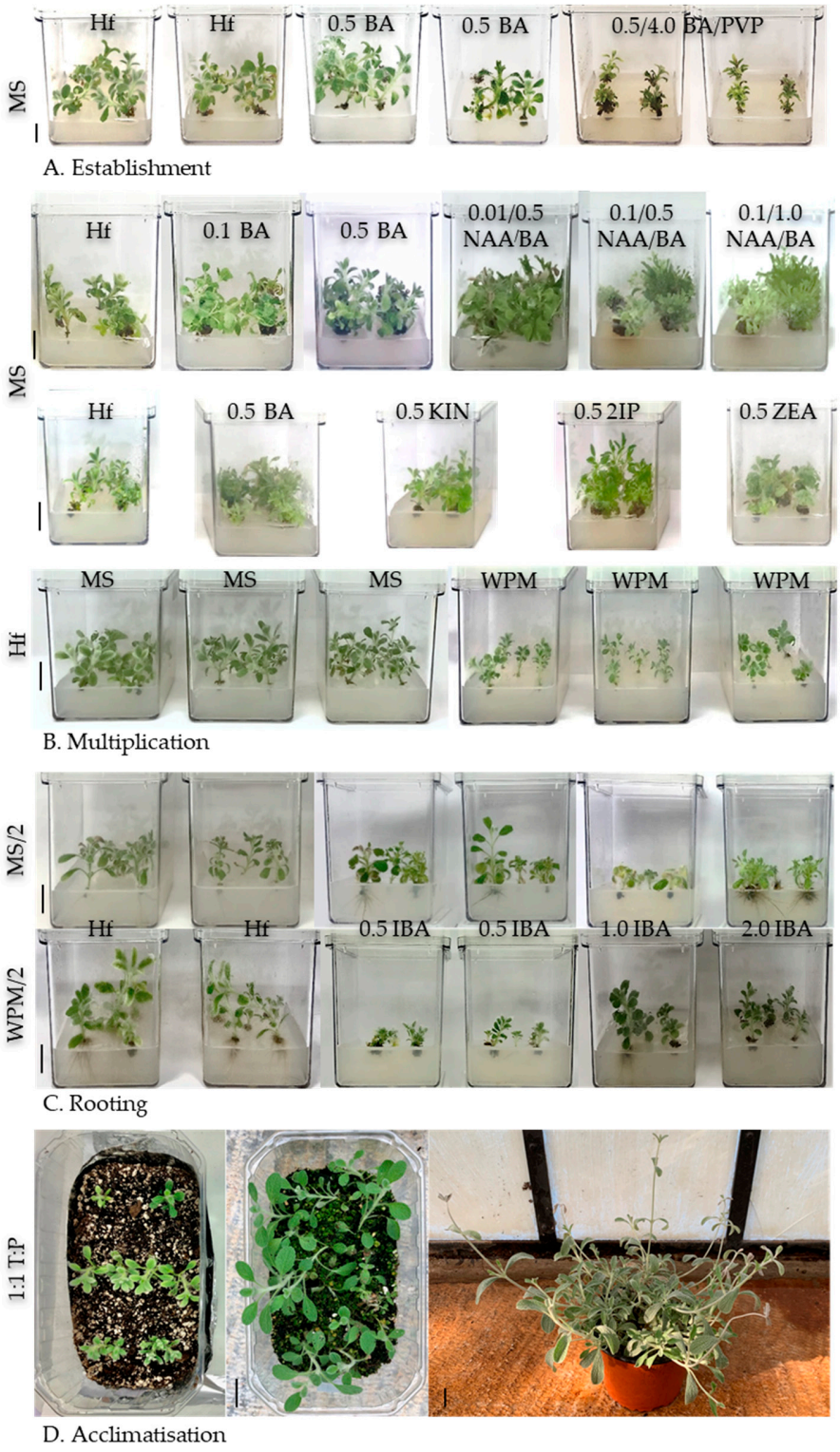

Figure 5. In vitro regeneration of *Sideritis raeseri* subsp. *attica*: (**A**) plantlets growing and root formation during the establishment of an initial culture of young seedlings after 40.0 days on various MS media; (**B**) multiplication stage and shoots forming on shoot explants after 40.0 days of culture on various MS media; (**C**) in vitro root formation after 20.0 days of culture on half-strength MS or WPM media (MS/2 or WPM/2, respectively) containing indole-3-butyric acid (IBA) at 0.0, 0.5, 1.0, or 2.0 mg L^{-1}; (**D**) plantlets during ex vitro acclimatisation on 1:1 T:P and two-month-old plants showing vigorous growth. Bars represent a length of 1 cm and the numbers represent the concentrations of phytohormones. (MS): Murashige and Skood medium; (Hf): hormone-free medium; (MS/2): half-strength MS.

3.2.2. Multiplication

Two subcultures were carried out on MS media containing (a) low concentrations of BA as well as NAA and (b) various cytokinins (BA, KIN, 2iP, ZEA) at 0.5 mg L^{-1}. Another subculture on a hormone-free MS medium and WPM media followed in order to eliminate the percentage of hyperhydricity recorded during the previous two subcultures. The first subculture presented a shooting percentage of over 78.0% in all treatments (Table 3; Figure 5B); no differences were observed in the lengths of the formed shoots and number of nodes and the shoot number registered higher (3.6 shoots/explant) on the 0.5/0.01 BA/NAA (mg L^{-1}) media. A small number of short, lateral shoots were formed and a higher MI (6.3) was calculated on the MS medium with 0.5/0.01 BA/NAA (mg L^{-1}) due to the formation of a higher number of shoots. Shoots were hyperhydric at a percentage of over 23.0% in all hormone-containing media with no differences.

Table 3. Effect of various concentrations of BA and α-naphthaleneacetic acetic acid (NAA) on MS media on shoot proliferation of *Sideritis raeseri* subsp. *attica* during the first subculture.

Hormone (mg L^{-1})		Shooting (%)	Shoot Number	Shoot Length	Node Number	Hyperhy-Dricity	Callus Formation	MI †	Lateral Shoot Number	Length of Lateral Shoots (cm)	Node Number of Lateral Shoots
BA	NAA										
-	-	81.0 a	2.1 b	1.1 a	3.9 a	6.0 b	23.0 c	3.1 b	0.3 b	0.2 a	0.5 a
0.1	-	78.0 a	2.4 b	1.2 a	3.2 a	32.0 ab	50.0 b	3.7 b	0.2 b	0.1 a	0.3 a
0.5	-	87.0 a	2.8 ab	0.9 a	3.2 a	23.0 ab	55.0 b	3.7 b	0.2 b	0.1 a	0.2 a
0.5	0.01	87.0 a	3.6 a	1.2 a	3.4 a	31.0 ab	54.0 b	6.3 a	0.6 a	0.1 a	0.3 a
0.5	0.1	92.0 a	2.6 ab	1.0 a	3.0 a	50.0 a	85.0 a	4.0 b	0.7 a	0.2 a	0.2 a
1.0	0.1	78.0 a	3.3 ab	0.8 a	4.5 a	20.0 ab	94.0 a	3.4 b	0.0 b	0.0 a	0.0 a
$F_{\text{one-way ANOVA}}$		ns	*	ns	ns	*	*	*	*	ns	ns

† (MI): multiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6; *, significant at $p \leq 0.05$; *F* values represented by ns indicate no significant differences at $p \leq 0.05$; mean separation in columns by Tukey's test at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; n = 45–50.

We used four different cytokines (BA, KIN, 2iP, ZEA) supplemented at low concentrations (0.5 mg L^{-1}) in the case of the second subculture. The shooting percentage was quite high, standing at over 78.5% regardless of the medium (Table 4, Figure 5B). The shoot production was higher on the MS medium supplemented with BA, with a 4.3 formation of shoots/explants, whereas the shoot length was higher (1.8 cm) on the hormone-free or KIN-supplemented media (Table 4). The range of hyperhydricity percentages was similar to the first subculture, i.e., 8.0–52.0%. It was higher for plantlets derived on 2iP-containing medium (52.0%). With regard to the MI, the hormone-free medium (7.3) and the media supplemented with BA or KIN (6.2 and 6.4, respectively) proved to be the most suitable.

Table 4. Effect of hormone-free MS medium and media supplemented with four different cytokinins, i.e., BA, kinetin (KIN), 6-(γ-γ-dimethylallylamino) purine (2iP), and (zeatin) ZEA, at 0.5 mg L^{-1} on shoot proliferation of node explants excised from in vitro-produced shoots during the first subculture of *Sideritis raeseri* subsp. *attica* seeds (second subculture).

Cytokinin (mg L^{-1})	Shooting (%)	Shoot Number	Shoot Length	Node Number	Hyperhy-Dricity	Callus Formation	MI †	Lateral Shoot Number	Length of Lateral Shoots (cm)	Node Number of Lateral Shoots
-	86.5 a	2.8 b	1.8 a	4.7 a	8.0 b	-	7.3 a	0.5 a	0.2 a	0.7 a
BA	78.5 a	4.3 a	1.1 b	4.2 ab	39.0 ab	62.0 ab	6.2 a	0.1 b	0.1 a	0.1 b
KIN	89.5 a	2.4 b	1.8 a	4.9 a	16.0 b	48.0 b	6.4 a	0.1 b	0.1 a	0.1 b
2iP	73.5 a	3.6 ab	1.2 b	4.0 b	52.0 a	63.0 a	5.3 ab	0.1 b	0.1 a	0.3 b
ZEA	81.5 a	3.4 ab	1.0 b	3.9 b	34.0 ab	88.0 a	4.6 b	0.1 b	0.1 a	0.3 b
$F_{\text{one-way ANOVA}}$	ns	***	***	***	*	*	*	**	ns	*

† (MI): multiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6; *, **, *** significant at $p \leq 0.05$, $p \leq 0.01$ $p \leq 0.001$, respectively; *F* values represented by ns indicate no significant differences at $p \leq 0.05$; mean separation in columns by Tukey's test at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; n = 45–50.

The subcultures on the hormone-free MS and WPM-supplemented media were successful across the board in terms of shooting formation, which stood at 100.0% (Table 5). The shoot numbers decreased and 1.9–2.0 shoots/explant of 1.5–1.7 cm in length formed. The MI registered at 5.0–5.4 (Table 5). Further, it was observed that the culture of explants on the hormone-free media thoroughly eliminated hyperhydricity (Table 5, Figure 5B).

Table 5. Effect of two different media, MS and McCown woody plant medium (WPM) without hormones, on the elimination of hyperhydration during the multiplication phase of *Sideritis raeseri* subsp. *attica*.

Medium	Shooting (%)	Shoot Number	Shoot Length (cm)	Node Number	Hyperhy-Dricity	Callus Formation	MI †	Lateral Shoot Number	Length of Lateral Shoots (cm)	Node Number of Lateral Shoots
MS	100 a	1.9 a	1.7 a	4.3 a	-	84.0	5.4 a	0.1 a	0.0 a	0.2 a
WPM	100 a	2.0 a	1.5 a	4.0 a	-	-	5.0 a	0.1 a	0.0 a	0.1 a
$F_{\text{one-way ANOVA}}$	ns	ns	ns	ns	-	-	ns	ns	ns	ns

† (MI): multiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6; *F* values represented by ns indicate no significant differences at $p \leq 0.05$; mean separation in columns by Tukey's test at $p \leq 0.05$; mean values followed by the same letter are not significantly different at $p \leq 0.05$; n = 45–50.

Finally, a small number of short lateral shoots also formed during all multiplication phases (Tables 3–5; Figure 5A,B)

3.2.3. In Vitro Rooting and Ex Vitro Acclimatisation

Spontaneous rooting of the microshoots derived during the establishment of the initial culture took place at low percentages (12.5%) on the MS media that were either hormone-free or supplemented with 0.5 mg L^{-1} (Table 2, Figure 5A). Microshoots also rooted spontaneously during the multiplication stage at higher percentages (Figure 4B, data not shown). Two-way ANOVA showed that the interaction of the main factors, medium type, and IBA concentration (0.0, 0.5, 1.0, or 2.0 mg L^{-1}) was significant during the in vitro rooting phase (Table 6). A higher rooting percentage (51.0%) of plantlets was observed on the MS/2 medium containing 0.0 mg L^{-1} IBA (51.0%). Plantlets were observed forming longer roots (0.9 cm). The MS/2 media supplemented with 2.0 (mg L^{-1}) IBA showed a reduction in root formation (14.0%). However, the root number was higher on the WPM/2 containing 2.0 mg L^{-1} IBA (Table 6, Figure 5C). Rooted microshoots were successfully (100.0%) acclimatised ex vitro (Figure 5D). Once the plants were acclimatised, they were transplanted into pots. Their survival rate after the transfer was again 100.0% (Figure 5D).

Table 6. In vitro rooting of microshoots derived in the multiplication stage affected by the concentration of IBA (0.0, 0.5, 1.0, or 2.0 mg L^{-1}) and the type of medium (half-strength MS or half-strength WPM).

Treatments	Rooting (%)	Root Number	Root Length (cm)
IBA (mg L^{-1})			
0.0 (control)	- †	-	-
0.5	-	-	-
1.0	-	-	-
2.0	-	-	-
Medium			
MS/2	-	-	-
WPM/2	-	-	-

Table 6. *Cont.*

Treatments		Rooting (%)	Root Number	Root Length (cm)
Interaction (IBA × Medium)				
0.0 mg L^{-1} (control) ×	MS/2	51.0 a	2.3 ab	0.9 a
	WPM/2	32.0 ab	0.4 b	0.4 ab
0.5 mg L^{-1} ×	MS/2	23.0 ab	2.6 ab	0.3 b
	WPM/2	20.0 ab	2.7 ab	0.4 ab
1.0 mg L^{-1} ×	MS/2	20.0 ab	2.4 ab	0.3 ab
	WPM/2	20.0 ab	3.7 ab	0.3 ab
2.0 mg L^{-1} ×	MS/2	14.0 b	2.1 ab	0.2 b
	WPM/2	28.0 ab	6.6 a	0.4 ab
F_{IBA}		ns	ns	ns
F_{medium}		ns	ns	ns
$F_{IBA \times med}$		*	*	*

† When interactions are significant, factors are not considered and mean values are not shown; ns: non-significant; * denotes significant differences between means at $p < 0.05$, indicated by different letters in columns; mean separation in columns by Tukey's test at $p \leq 0.05$; n = 45–50.

4. Discussion

The present study's main aim focused on the regeneration of the endangered *S. raeseri* subsp. *attica* starting with young, in vitro-grown seedlings. Nevertheless, while focusing on regeneration, we carried out a preliminary assessment of the species' germinability as previous studies of other *Sideritis* species found a strong correlation between germination and the environmental conditions [34,35]. In the present study, the germination rate was low and did not exceed 40.0%. Very low germination rates were also recorded for *S. perfoliate* and *S. erythabtha*, whereas *S. stricta* seeds did not germinate at all [36]. The germination rates for *S. athoa* were 70.0–72.0% on the solid MS/2 at 20.0 °C and 25.0 °C [37]. As for *S. chamaedryfolia* and *S. pungens*, the germination rates registered at 74.0% and 99.0%, respectively [34]. Shtereva et al. [38] achieved very impressive germination rates (up to 98.0%) for *S. scardica* after pre-treatment (mechanical scarification) and treatment with gibberellic acid. The present study defined the cardinal temperatures as ranging from 15.0 to 30.0 °C, whereas a range of 20.0–25.0 °C was found to be the optimal temperature range for seed germination, as temperatures higher or lower than these in the range were found to decrease the germination rate to all-too-low percentages. To confirm our findings, Papafotiou and Kalantzis [37] indicated the same optimal temperature range for *S. athoa* and Estrelles et al. [34] reported the same temperature range as being optimal for *S. chamaedryfolia* and *S. pungens*, which are found in Iberian habitats. The usual range for the germination of Mediterranean species is between 15.0 and 20.0 °C [39–41]. However, Picciau et al. [42] found that the germination of species growing at higher altitudes necessitates no pre-treatment at relatively high temperatures (20.0 °C), a finding also confirmed by the present study. The impact of the environment during seed production proved powerful [39]. Not only does the environment influence the seed size, germination rate, and viability but it also causes variations even within the same genotype [43–45]. Endemic species are also closely correlated with the characteristics of their habitats [34] and native plants have their own strategies for optimising their evolution in their respective ecosystems [46]. Moreover, it has been found that the inducement of physiological dormancy in seeds of *S. serrata* seedbanks in the soil under high and prolonged thermoperiods is possible [47]. Thermo-dormancy is a secondary dormancy that plays a role in preventing seed germination during occasional summer rainfalls [48,49]. The seeds used in the present study were collected from plants in one day and it may be construed that a proportion of thermodormancy could not be induced during storage. The low germination rate could be attributed to climate change, which is characterised by high temperatures and frequent conditions of water storage. This is a climate issue that negatively impacts seed germination as it reduces seed viability and

germinability [50] and leads to the realisation that further studies should focus on the effects of environmental parameters, such as temperature and photoperiod, on seed quality and germination. The ecophysiology of endangered species is far more vulnerable than that of other, non-endangered endemic species.

With regard to the in vitro propagation of *S. raeseri* subsp. *attica*, our study employed PVP, which has been used before in a wide range (from 0.5 to 20 g L^{-1}) for the micropropagation of *S. raeseri* subsp. *raeseri* [51]. It has been found that phenolics are produced often during the establishment stage of in vitro propagation. The production of phenolics has been observed in the case of explants derived from ex vitro-grown plants, as well as in the case of young seedlings, i.e., *Acacia catechu* [52], *Stereospermum personatum* [53], and the *Sideritis* species [36,54]. PVP is an antioxidant commonly used in binding phenolics that prevents the browning of both explants and medium during that stage of in vitro propagation of many species [55,56] such as *Thymus bleicherianus* Pomel [57], *Aloe vera* [58,59], and rose cultivars [60]. It is used at a range of concentrations, usually 0.5 to 5.0 g L^{-1}. Sahini and Gupta [53] used PVP at 1.5 g L^{-1} to control browning in subcultures of stem nodes from in vitro-grown plants of *A. catechu* on BA-supplemented MS media. On the other hand, PVP at times tends to have some undesirable effects due to its absorption of plant growth regulators and nutrients from the plant tissue [59]. Confirming the findings of the last study mentioned above, the present research also found that the use of PVP at low concentrations inhibited shoot length, possibly due to the absorption of auxins. Sarropoulou and Maloupa [51] also used PVP at higher concentrations (10 g L^{-1} PVP), which negatively impacted shoot length. Our own findings lead us to believe that there is no need for further research on the effect of PVP at a lower concentration since no visible production of phenolics emerged.

The micropropagation method presented in this study could help in vitro techniques to address the conservation and exploitation of *Sideritis.* The hormone-free MS medium proved to be efficient for the initial establishment of in vitro cultures, providing shooting formation and elongation, which both increased through subcultures. The supplementing of low concentrations of cytocinins based on references on the Lamiaceae and other xerophytic plants species, which responded better on the MS media containing low concentrations of cytokinins (up to 0.5 mg L^{-1}), also proved beneficial [61]. BA or KIN at 0.5 mg L^{-1} and BA/NAA at 0.5/0.01 (mg L^{-1}) were more effective than the hormone-free MS medium and shoot production was higher. On the other hand, they resulted in the formation of hyperhydric shoots. The shoot length was higher on the hormone-free MS medium and a multiplication cycle on the hormone-free MS medium may have eliminated the hyperhydricity phenomenon. The same medium was also found to be suitable for *S. angustifolia* with a higher elongation [62].

IBA was the most efficient auxin for the in vitro induction of the rooting of *Sideritis stricta* [63], *S. scardica* [38], *S. athoa* [37], and certain other Lamiaceae species [64,65]. As was the case with *S. augustifolia,* in the present study, rooting took place on the hormone-free MS/2 media [62]. Further investigation is required to promote in vitro-induced rooting. Regarding the survival of acclimatised plants, which is the final step of a successful micropropagation protocol, the present study verified that acclimatisation was fully successful (100.0%). An equally high acclimatisation rate was also observed in the cases of *S. raeseri* [51] in Greece and *S. stricta* in Turkey [63].

Seed production is frequently employed as the starting material for commerce-oriented horticultural and aromatic plants and could meet the increasing commercial demand for the *Sideritis* species, which, in this way, could be introduced into the floriculture industry. In the meantime, seed production could result in the establishment of ex situ conservation methods in botanical gardens, arboretums, or other ex situ facilities [66]. It could also help with the reintroduction of the species into its natural habitat, a practice that is effective with very small populations [67]. Bioresource collections could also help to preserve endangered species such as *Sideritis* [68]. It is worth mentioning that *Origanum dictamnus* is another endangered species from Greece, which has found uses in the practices of Greek

ornamental horticulture [12]. The conservation of threatened plants and programs aimed at the restoration of natural habitats must be supported by the development of optimal germination protocols.

5. Conclusions

To the best of our knowledge, our study is the first to present an efficient in vitro protocol of *S. raeseri* subsp. *attica.* It is also a preliminary study on the seed germinability of this endangered species. Three-month-old seeds of *S. raeseri* subsp. *attica* germinated, registering average percentages. The cardinal temperatures for germination were defined as 15.0 °C to 30.0 °C. With regard to micropropagation, in vitro culture was established from seedling-origin shoot explants on a hormone-free MS medium. During multiplication, the MS medium supplemented with KIN at 0.5 mg L^{-1} proved to have a high shoot production and low formation of hyperhydric shoots. A multiplication cycle on the hormone-free MS medium eliminated the hyperhydricity phenomenon. Be that as it may, it is evident that more research is required to promote in vitro-induced rooting provided that acclimatisation proves to be 100.0% successful, as it did in our study. The present method, in tandem with suitable genetic markers, could contribute to commercial exploitation and conservation programs alike.

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