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In vitro cultures for micropropagation, mass multiplication and preservation of an endangered medicinal plant *Sideritis scardica* Griseb

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ABSRACT: In vitro methods have been widely used in the conservation of threatened plants in recent years and it is clear that this trend is increasingly being applied to more plant species at risk of extinction. Herewith, an efficient protocol has been developed for mass-propagation of *Sideritis scardica* from apical buds and internode stem explants and conservation in natural habitats. Murashige and Skoog basal medium containing 2 mg L^{-1} zeatin + 0.2 mg L^{-1} indole-3-acetic acid (IAA) and 0.25 mg L^{-1} zeatin + 0.2 mg L^{-1} indole-3-acetic acid were the best for shoot proliferation. The use of both types of explants was beneficial for micropropagation. Elongated shoots were successfully rooted in the MS half-strength basal medium supplemented with 0.2 mg L^{-1} IAA + 2 mg L^{-1} indole-3-butyric acid + 0.5 mg L^{-1} gibberellic acid. Propagation and growth of plantlets under greenhouse conditions were managed successfully. The *in vitro* propagation of *S. scardica* may be applicable for other *Sideritis* species and for other economically important medicinal plants as well.

Key words: Pirin mountain tea, Mursalitsa tea, seed germination, *in vitro* response, micropropagation, *ex vitro*

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INTRODUCTION

The genus *Sideritis* (family Lamiaceae) taxonomically is a complex group which comprises more than 150 annual and perennial herbs and small shrubs, widely distributed in the Mediterranean area, Canary and Madeira Islands (Obón de Castro & Rivera-Núñez 1994; Gonzales-Burgos *et al.* 2011). *Sideritis* species are usually calcicolous and heliophilous plants growing in dry and semiarid areas. They are potentially a rich source of bioactive compounds. It has long been known that herbs and their extracts have antimicrobial, anti-inflammatory, and antioxidant activities. Also, the major secondary metabolites of medicinal interest are diterpenes, flavonoids, and essential oils. The latter are stored in glandular hairs, and consist of mainly monoterpenes, sesquiterpenes, diterpenes, triterpenes, sterols, flavones, coumarins and phenylpropanoids which vary according to the species (e.g. Barberán *et al.* 1985; Palomino *et al.* 1996; Koleva *et al.* 2003; Tunalier *et al.* 2004; Ivancheva *et al.* 2006; Petreska *et al.* 2011; Todorova & Trendafilova 2014).

In Bulgaria, the genus *Sideritis* is represented by four species - *S. scardica*, *S. syriaca* L. (*S. taurica* Steph. Ex Wild), *S. montana* L. and *S. lanata* L. The most widely-used as an important medicinal plant is *S. scardica* Griseb, giving a tea known as the Pirin Mountain tea or the Mursalitsa tea. It is distributed in the mountainous regions of Southern Bulgaria from 1000 m to 2200 m.a.s.l. (Slavyanka, Pirin, and Rhodope Mts) (Ivanov *et al.* 1977; Assenov 1989; Petrova & Vladimirov 2010). The area of distribution of *S. scardica* is closely linked to the Balkan Peninsula. The species is a Balkan endemic encountered in Southwest Albania, Greece, Macedonia, Bulgaria and Turkey (Heywood 1972; Assenov 1989; Strid & Tan 1991; Yordanova & Apostolova 2000). *Sideritis scardica* Griseb. is traditionally used in folk medicine as a herbal tea, because of its pharmaceutical effects to

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treat the symptoms of various ailments, such as cough (antitussive effect), common cold (sedative and analgesic effects), gastrointestinal disorders etc. (Koleva *et al.* 2003; Ivancheva *et al.* 2006; Kostadinova *et al.* 2008) and for this reason it has been intensively collected from natural habitats for years. Nowadays, the distribution of *S. scardica* is quite limited, and the species has been included in the Red Book of Bulgaria as an endangered plant (Evstatieva 2012). It is under governmental protection and its collection from native habitats is prohibited. Seeds collected from native populations exhibit dormancy and have a low germination ability under laboratory and natural conditions (about 5%) (Evstatieva & Koleva 2000). Excessive exploitation of this valuable medicinal plant has seriously lowered the state of its natural reserves and reproductive capacity. Thus, inappropriate methods of collection and insufficient recovery periods have destroyed the normal sexual and vegetative reproduction of native populations of the species (Petrova & Vladimirov 2010). The restoration capacity of native plants is quite limited due to the rather low seed germination rate and coefficient of vegetative reproduction. Plants need from 4 to 6 years for optimal development and maximum accumulation of biologically-active substances in the rhizomes. There has been no evidence of spontaneous vegetative reproduction (Evstatieva *et al.* 1990; Todorova *et al.* 2012).

Despite the great interest in *Sideritis* species and extensive research on their phytochemistry, plant biotechnology methods have been little exploited so far. Cultivation of valuable species in experimental conditions is an important approach to multiply and conserve critical plant genotypes. *In vitro* multiplication techniques for endangered plant species make reintroduction into the natural environment possible (Wochock 1981) and reduce the risk of extinction (Pence 1999; Nadeem *et al.* 2000; Chandra *et al.* 2006). Among aromatic medicinal plants, *Sideritis* species have a great potential to benefit from biotechnological techniques.

Tissue culture of *Sideritis* species has been reported on several occasions (Sánchez-Gras & Segura 1987, 1988, 1997; Garcia-Granados *et al.* 1994; Faria *et al.* 1998; Erdağ & Yürekli 2000; Uçar & Turgut 2009; Papafotiou & Kalantzis 2009; Danova *et al.* 2013). However, a detailed protocol for micropropagation, rooting and reintroduction of the regenerated plants into natural conditions (*ex vitro*) has still not been developed. The present investigation proposes an efficient protocol for *in vitro* micro-propagation of *S. scardica* Griseb from seedling explants.

MATERIAL AND METHODS

Initial plant material. Tissue cultures were initiated from seeds of *S. scardica* which were kindly provided by the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia. Plants were collected from their natural stands in the Central Rodopi Mountains, Bulgaria.

Sterilization of the seeds. *S. scardica* seeds were washed with liquid soap and running tap water prior to use. Then they were surface-sterilized applying three alternative protocols: i/ soaking in 70% ethanol for 5 min; ii/ soaking in 70% ethanol for 3 min followed by 15 min in 20% v/v commercial bleach, whose active component was sodium hypochlorite (ACE -Procter & Gamble Co., USA, containing 4.85% active chlorine) and, iii/ soaking in 70% ethanol for 1 min followed by immersion in 0.1% mercuric chloride ($HgCl_2$) for 10 min. A drop of Tween-20 (Sigma-Aldrich) was added to each sterilized solution. Each procedure was followed by a triple rinse in autoclaved distilled water for 5 min, 10 min and 15 min (Table 1).

***In vitro* seed germination.** To stimulate the germination response, sterilized seeds were treated with 0.01 M solution of gibberellic acid (GA_3) for 24 h. To maximize seed germination, part of the seeds were subjected to scarification (mechanical injuring of the seed coat without injuring the internal seed material) prior to culturing. They were germinated aseptically in glass tubes containing 10 ml MS (Murashige & Skoog 1962) basal medium supplemented with 25, 50 and 100 mg L^{-1}

Table 1. Methods of sterilization of *Sideritis scardica* seeds

Variant	Pre-sterilization		Proper sterilization		Final stage of sterilization	Non-infected
	Disinfectant	Exposure time, min	Active ingredient	Exposure time, min		seeds, %
i	70% ethanol	5			sterile water	13.2
ii	70% ethanol	3	20% commercial bleach + 1 drop of Tween 20	15	sterile water	100.0
iii	70% ethanol	1	0.1% mercuric chloride + 1 drop of Tween 20	10	sterile water	35.7

gibberellic acid (GA_3), 30 g L^{-1} sucrose, 6 g L^{-1} plain agar (Duchefa NL) and 500 mg/l casein hydrolyzate (Table 2). The glass tubes were sealed with foil and incubated in a growth chamber at 25±1°C in the dark for two weeks and then under cool white fluorescent light (lamps FL-36 W^{-1}, Tungsram, Hungary, with a photon flux density (PFD) of 70 μmol m^{-2} s^{-1}) on 16/8 h (light/dark) photoperiod. To establish the optimal medium for germination, percentage of germinated seeds was recorded after imbibition for 14, 28 and 42 days. The resultant *in vitro* seedlings were cultivated on various nutrient media for further development.

Multiplication of the shoot. Apical buds and internode stem segment explants obtained from 42-day-old seedlings were used to establish shoot cultures. Explants were cultured on the following media; namely MS fortified with various concentrations (mg L^{-1}, w/v) of cytokinins: N^6-benzylaminopurine (BAP), 6-(g,g–dimethylallyl amino) purine (2iP), zeatin, kinetin, either individually or in combination with auxins: indole-3-acetic acid (IAA), a-naphthyl acetic acid (NAA) (Table 2). Medium without growth regulators was used as the control. The media pH was adjusted to 5.7 before autoclaving at 121°C for 20 min. All culture media contained 3% sucrose (w/v) and solidified

Table 2. Composition of culture media for *in vitro* seed germination, shoot development multiplication and rooting of *Sideritis scardica* Griseb.

Media variants	Concentration of phytoregulators (mg/L)								
	BAP	Zeatin	2-iP	Kinetin	IAA	NAA	IBA	GA_3	Casein hydrolysate
MSG 0								0	0
MSG1								25	500
MSG2								50	500
MSG3								100	500
MSD0	0	0	0	0	0	0			
MSD1	2.0				0.2				
MSD2	1.0				0.1				
MSD3	1.0					0.1			
MSD4		2.0			0.2				
MSD5		1.0			0.2				
MSD6		1.0				0.1			
MSD7		0.25			0.2				
MSD8			3.0		0.3				
MSD9				0.1		0.1			
MSD10				0.1		1.0			
½ MSR 0					0	0	0	0	
½ MSR1							0.1	0.5	
½ MSR2							0.5	0.5	
½ MSR 3							1.0	0.5	
½ MSR 4							2.0	0.5	
½ MSR 5					0.2		2.0	0.5	
½ MSR 6						0.5		0.5	
½ MSR 7						1.0		0.5	

MSG – media for germination; MSD – media for development, shoot formation and multiplication of plant regenerants; MSR –rooting medium

with 0.6% agar. Explants were placed vertically in glass tubes (150 x 25 mm) containing 20 ml culture medium and plugged tightly with foil to reduce desiccation of the medium. Cultures were maintained initially in the dark at 25 ± 1°C to reduce the browning problem. Each treatment consisted of 40 explants and was replicated three times, representing a total of 120 observations per treatment. After three-week sub-culturing for shoot development, the number of shoots per explant and shoot length were recorded.

Rooting and transfer of plantlets to soil. Shoots regenerated from explants were excised and individually transferred to MS medium with ½-strength salts and sucrose (½MS) supplemented with various concentrations of auxins: IAA, indole-3-butyric acid (IBA), NAA and GA_3 (Table 2) for rooting induction, at pH 5.8. Each rooting treatment consisted of 40 explants replicated 3 times, representing a total of 120 observations per treatment. The frequency of rooting, length of roots, average number of plantlets and propagation rate was recorded after 3 weeks of culture. After 21 day in rooting medium, the rooted shoots were removed from the culture medium and the roots were washed in sterile distilled water to remove all traces of agar. The plantlets were then transferred to plastic pots (5 cm diameter) containing garden soil mixed with peat and perlite (2:1:1) (v/v/v) under controlled growth chamber conditions (20 ± 1°C, 16-h photoperiod, 70-90% relative humidity and 70 µmol m^{-2} s^{-1} light intensity). After 6 weeks, the potted plants were placed under greenhouse conditions for acclimatization and hardening. After one month of acclimatization, plants were placed in the field.

Culture conditions and statistical analysis. Cultures were maintained in a growth chamber at 20-22 °C in darkness for two weeks (both for germination and shoot multiplication) and then under 16-h photoperiod provided by cool white fluorescent tubes with a PFD of 70 µmol m^{-2} s^{-1} for organogenesis induction and 90 µmol m^{-2} s^{-1} for plant adaptation with 55-60% and 70-90% relative humidity, respectively. Explants were sub-cultured every 4 weeks.

Data were collected after 30 and 50 days for multiple shoot induction and rooting respectively. Data are presented as mean ± SE of 40 explants per treatment, replicated three times, and calculated using Sigma Plot 3.1 (Systat Software Inc., SSI).

RESULTS

This is the first overall protocol that has been developed for micropropagation of *S. scardica*, a Balkan endemic species. The optimum method of sterilization of seeds is a critical stage in introducing plants to tissue culture. The effectiveness of sterilization may be dependent on the type, concentration and kind of treatment with the sterilizing agent (Roxas *et al.* 1996; Tomaszewska-Sowa & Figas 2011). The percent of non-infected seeds of *S. scardica* was very low after pre-sterilization by 70% ethanol alone (Table 1). Considerably more non-infected

Table 3. Effect of GA_3 and scarification on seed germination and length of seedlings of *Sideritis scardica*

Medium	GA_3 mg L^{-1}	After 28 days		After 42 days	
		Seed germination, %	Length of seedlings, cm	Seed germination, %	Length of seedlings, cm
AD	0	11.4	7.4 ± 0.03	17.1	15.3 ± 0.10
MSG0	0	30.9	14.6 ± 0.09	53.1	15.7 ± 0.09
MSG1	25	61.6	20.4 ± 0.07	76.5	21.0 ± 0.10
MSG2	50	76.3	16.9 ± 0.10	97.8	17.6 ± 0.06
MSG3	100	87.8	18.4 ± 0.09	91.6	19.2 ± 0.08
MSG0 + SC*	0	40.6	17.8 ± 0.08	62.7	15.9 ± 0.09
MSG1 + SC	25	68.8	20.4 ± 0.06	74.5	21.5 ± 0.10
MSG2 + SC	50	89.1	20.1 ± 0.07	94.8	19.5 ± 0.09
MSG3 + SC	100	94.3	21.1 ± 0.09	96.9	22.0 ± 0.11

*SC – scarification
Means are given for 40 measurements and the experiment was replicated three times;
Values are mean ± SE

seeds were found after sterilization by a combination of 70% ethanol and 0.1% mercuric chloride. Seeds that were sterilized by 70% ethanol and 20% commercial bleach were not infected (Table 1). Thus, the best method to sterilize seeds of *S. scardica* was to disinfect them in 70% ethanol for 3 min, and then in 20% commercial bleach for 15 min. 100% sterile seeds, capable of regenerating explants, were obtained with this method.

Seeds germinated on all the media tested (Table 3). They were germinated for up to six weeks of culture, giving normal roots, shoots and leaves. The percentage of germinated seeds varied in different media. MSG2 and MSG3 media supplemented with 50 and 100 mg L^{-1} GA_3 resulted in 97.8% and 91.6% seed germination after 42 days. However, in the control variant and in MSG0, seed germination percentage was quite low. Scarification of the seeds had little effect on seed germination as germination rates were similar in all the treatments, with or without scarification (Table 3).

The apical bud and stem explants obtained from *in vitro*-grown seedlings were cultured on 10 variants of MS media, either alone or supplemented with BAP, zeatin, 2iP or kinetin (Table 4). Shoot development in at least 40% of explants was established on all the media tested, with the maximum shoot development of 87.3% obtained in apical bud explants with MSD4 medium supplemented with zeatin and IAA. Maximum shoot development in stem segment explants (78.6%) was obtained with

Table 4. Morphogenic response of apical bud and stem segment explants of *S. scardica* to different concentrations of cytokinins.

Medium	Shoot development, %	Shoot number/explant	Shoot length, mm
Apical buds			
MS control	44.1	1.20 ± 0.05	0.5 ± 0.10
MSD1	67.0	1.55 ± 0.16	10.0 ± 0.51
MSD2	49.2	1.25 ± 0.05	0.5 ± 0.09
MSD3	47.8	1.12 ± 0.15	0.5 ± 0.08
MSD4	87.3	2.67 ± 0.24	14.0 ± 0.12
MSD5	76.7	2.40 ± 0.21	13.0 ± 0.10
MSD6	40.0	1.30 ± 0.14	10.0 ± 0.55
MSD7	68.3	2.66 ± 0.12	16.0 ± 0.19
MSD8	54.1	1.78 ± 0.38	12.3 ± 0.45
MSD9	58.7	1.40 ± 0.15	11.0 ± 0.10
MSD10	60.2	1.68 ± 0.40	15.0 ± 0.87
Stem segments			
MS control	40.5	1.24 ± 0.08	0.8 ± 0.95
MSD1	78.6	1.50 ± 0.20	10.0 ± 0.86
MSD2	44.6	buds	up to 2.0
MSD3	46.1	buds	up to 3.0 - 4.0
MSD4	84.0	3.00 ± 0.15	18.6 ± 0.13
MSD5	73.6	1.90 ± 0.22	16.5 ± 0.12
MSD6	46.7	1.67 ± 0.20	12.2 ± 0.30
MSD7	70.8	2.95 ± 0.09	18.5 ± 0.13
MSD8	62.5	1.60 ± 0.24	14.0 ± 0.12
MSD9	48.7	1.65 ± 0.37	12.5 ± 0.28
MSD10	51.6	1.70 ± 0.25	13.0 ± 0.80

Data (x ± SE) were collected after 28 days from three independent experiments each with 40 replicates. See Table 2 for definition of MSD media hormone additions.

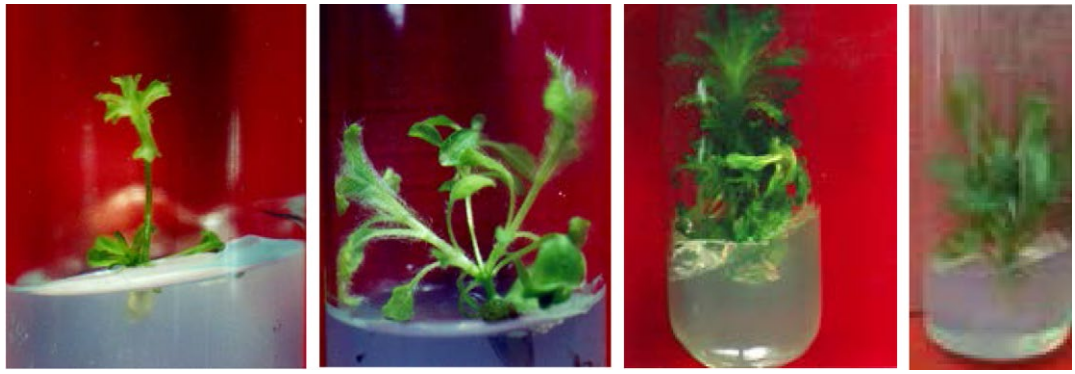

Fig. 1. Regeneration response of *in vitro* seedlings of *S. scardica* – A) regenerant from stem internode segments; B) regenerant from apical buds; C) plantlets after 28 days of cultivation; D) rooted plantlets

Table 5. Root formation and acclimatization of regenerated plantlets of *S. scardica*.

Medium	No of regenerants	Rooting, (%)	Root length, (cm)	Plantlet height (cm)	Propagation rate
½ MSR 0	150	34.0	1.10 ± 0.09	1.80 ± 0.13	2.30 ± 0.12
½ MSR 1	160	70.6	1.45 ± 0.07	4.50 ± 0.07	4.26 ± 0.11
½ MSR 2	160	68.5	1.22 ± 0.09	4.22± 0.09	3.75 ± 0.12
½ MSR 3	160	82.7	1.95 ± 0.08	4.75 ± 0.09	4.75 ± 0.10
½ MSR 4	180	90.0	2.14 ± 0.06	4.97 ± 0.08	5.20 ± 0.11
½ MSR 5	160	98.2	2.55 ± 0.05	5.84 ± 0.11	6.57 ± 0.09
½ MSR 6	150	18.0	0.5 ± 0.18	0.8 ± 0.16	1.2 ± 0.08
½ MSR 7	150	22.0	0.8 ± 0.15	1.0 ± 0.14	1.5 ± 0.09

Data (x ± SE) were collected after 50 days from three independent experiments with 40 replicates.
See Table 2 for definition of ½ MSR media hormone additions.

MSD1 medium . Images of typical explant responses to regeneration are show in Fig. 1 A, B. From each bud, a single shoot developed with shoot length varying from 0.5 mm (control variant) to 16.0 mm (MSD7) medium supplemented with zeatin and IAA after 30 days (Fig. 1 C). Of the combinations, MS medium supplemented with zeatin and IAA (MSD4) was the most effective, providing the highest shoot regeneration capacity of both types of explant associated with a high number of shoots per explant (2.67 and 3.00 mean shoots/explant from apical bud and stem explants, respectively). Stem segments showed similar responses, with two exceptions: on MSD2 and MSD3 media supplemented with BAP alone or in combination with IAA, the explants formed only buds even after 40 days. Once organized, the shoot buds continued development into small green shoots with primordial leaves.

Half-strength MS supplemented with NAA, IBA, IAA and GA_3 in different combination induced roots after 30 days (Fig. 1 D). Both types of explant source resulted in a high number of rooted plants (Table 5). However, the best rooting response was obtained on medium MSR5 supplemented with 0.2 mg L^{-1} IAA + 2 mg L^{-1} IBA + GA_3 with a rooting percentage of 98.2%. ½ MSR5 also gave the highest root length (2.55± 0.05), longest plantlets

Fig. 2. Micropropagated plants of *S. scardica* in pots after 60 days

Fig. 3. *Ex vitro* plants of *S. scardica*

(5.84 ± 0.11) and highest propagation rate (6.57 ± 0.09), indicating that MSR5 was the most suitable medium for production of rooted plantlets of *S. scardica*. NAA suppressed rooting (Table 5). Of the three auxins tested, IBA was superior in terms of rooting percentage and number of roots per shoot when the medium was supplemented with GA_3. In 30 days, four or five roots developed from each shoot with root length ranging from 0.5 cm (MSR6) to 2.55 cm (MSR5). Plantlets with fully expanded leaves and well-developed roots were successfully hardened off inside the environmentally controlled growth chamber in the selected planting substratefor 6 weeks (Fig. 2). During acclimatization, a high survival rate (about 85%) was observed in rooted shoots and cultivation *ex vitro* (Fig. 3).

DISCUSSION

The germination of seeds of *S. scardica* is poor under natural conditions (Evstatieva & Popova 1998). Investigation of seed germination of several *Sideritis* species (Estrelles *et al.* 2010; Yankova-Tsetkova *et al.* 2013) has revealed a strong correlation between germination and environmental conditions. Using tissue culture techniques to study regeneration ability of three *Sideritis* species, Uçar & Turgut (2009) reported that germination ratio was very low. In the present study, germination was observed in all seeds, whether treated with GA_3 alone or after scarification, as demonstrated by germination rates of 97.8% and 91.6% on MSG2 and MSG3 media after 42 days (Table 3). There were, however, no significant differences in percent seed germination after scarification compared with non-scarified seeds. Thus treatment with GA_3 and casein hydrolysate had some effect on *S. scardica* seed germination. These results are in good agreement with results of Estrelles *et al.* (2010), Yankova-Tsetkova *et al.* (2013) and Gümüşçü (2014) Thus, the effects of GA_3 and casein hydrolysate on seed germination rate (Table 3) probably allow *S. scardica* to be added to the list of species with seed germination stimulated by gibberellic acid.

Multiple shoots were cultured from apical buds and stem segment explants of *Sideritis scardica* on MS solid media supplemented with different concentrations of plant growth regulators (Table 2). Of all the media used, the best response was observed on MSD4 and MSD7 media (Table 4) Effects of PGRs and vitamins in the medium on shoot induction in a number of *Sideritis* species have been studied by Çördük & Aki (2011), Faria *et al.* (1998) and Erdag & Yürekli (2000). Our results demonstrate that zeatin 2 mg L^{-1} + 0.2 mg L^{-1} IAA (MSD4) induced the highest percentage of shoot proliferation from apical explants; the combination of 2 mg L^{-1} BAP + 0.2 mg L^{-1} IAA (MSD1) was the most responsive in stem explants (Table 4) Shoot induction by zeatin from different explants has been reported by other authors in peppermint (Ghanti *et al.* 2004), olive (Ali *et al.* 2009), high bush blueberry (Eccher & Noe 1988, Ostrolucká *et al.* 2004), and grapevine (Gousssard 1982). Furthermore, zeatin was shown to be less inhibiting than 2iP, especially at higher doses (Eccher & Noe 1988). In our case, the use of 2iP in combination with IAA did not improve the morphogenetic capacity of explants. Similar results were reported by Papafotiou & Kalantzis (2009), where low shoot production on medium supplemented with 2iP was found with *in vitro* propagated *Sideritis athoa*. A comparison of apical and stem explants cultured on different media indicated that both types of explant were responsive to different media in a similar way. This demonstrates that both types of explant of *S. scardica* (apical buds and stem segments) are able to produce proliferating shoots. Although the medium composition influenced significantly the mean number of shoots formed per explant, the proliferation rate was low, ranging from 1.12 to 2.67 shoots per apical bud explant and from 1.24 to 3.00 shoots per stem explant (Table 4).

Sufficiently-developed plants were transferred to eight rooting half-strength media differing in the presence of auxins (IBA, IAA and NAA) (Table 5). *In vitro* medium composition significantly affected rooting ability (number of roots and root elongation). Half-MSR5 medium supplemented with IAA (0.2 mg/L), IBA (2.0 mg/L) and GA_3 (0.5 mg/L) resulted in 98.2% rooted plants. In this study with *S. scardica* Griseb. In contrast, NAA had no effect on rooting capacity. Thus, on ½ MSR6 and ½ MSR7 media (NAA + GA_3) rooting was less than in the control medium (only 18.0 and 22.0% for ½ MSR6 and ½ MSR7 respectively, compared with 34.0%, Table 5). Therefore, medium MSR5 with IAA and IBA was optimal for culture of *S. scardica*. Our results support the conclusion of other authors of the role of auxins for tissue culture (Sánchez-Gras & Segura 1988, 1997; Marki 2000).

It was found (Evstatieva & Koleva 2000; Todorova *et al.* 2012) that, after re-introduction of *in vitro*-propagated *Sideritis* plants into the wild, at least two and preferably three years' development was before examining

their phytochemical and biological activities. This period was most appropriate for harvesting and producing material suitable for medical purposes from *in vitro*-propagated *Sideritis* spp. Reintroduced plants obtained in this experiment were one year after planting in natural conditions. They grew very well (Fig. 3). Nevertheless, phytochemical analysis is needed to assess the qualitative and quantitative composition of secondary metabolites produced by these regenerated *Sideritis scardica* plants.

CONCLUSION

The detailed procedure described here offers a potential system for mass-propagation of *S. scardica* from seedling explants and conservation in natural habitats. MS media containing 2 mg L^{-1} zeatin + 0.2 mg L^{-1} IAA (MSD4) and 0.25 mg L^{-1} zeatin + 0.2 mg L^{-1} IAA (MSD7) were the best for shoot proliferation. Both apical bud and stem explants were useful for micropropagation. MS basal medium supplemented with 0.2 mg L^{-1} IAA + 2 mg L^{-1} IBA + 0.5 mg L^{-1} GA_3 was the best for root induction. The *in vitro* propagation of *S. scardica* may be applicable for other *Sideritis* species as well as for other economically important medicinal plants.

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Botanica SERBICA

REZIME

In vitro kultura, mikropropagacija, masovno umnožavanje i očuvanje ugrožene lekovite biljke *Sideritis scardica* Griseb

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In vitro metode se sve više upotrebljabaju u konzervaciji ugroženih biljaka i postoji trend da se više primenjuju na biljke u opasnosti od istrebljenja. Ovde se daje protokol koji je razvijen za masovnu propagaciju vrste *Sideritis scardica,* poče iz vršnih pupoljaka i internodija te protokol razvijen za konzervaciju u prirodnim staništima. Murashige Skoog osnovni medijum (MS) sa dodatkom 2 mg L^{-1} zeatina + 0.2 mg L^{-1} indol-sirćetne kiseline i 0.25 mg L^{-1} zeatina + 0.2 mg L^{-1} indol-sirćetne kiseline su se pokazali kao najbolji za proliferaciju izdanaka. Izduženi izdanci su se uspešno ukorenjavali na MS/2 bazalnom medijumu sa dodatkom 0.2 mg L^{-1} indol-sirćetne kiseline + 2 mg L^{-1} indol-buterne kiseline + 0.5 mg L^{-1} giberelne kiseline. Propagacija i rast dobijenih biljčica u uslovima staklenika bile su uspešne. Protokol *in vitro* uspostavljen za *S. scardica* može biti primenjen i na druge vrste roda *Sideritis* a potencijalno i na druge ekonomski važne lekovite biljke.

Ključne reči: pirinski čaj, klijanje semena, *in vitro* odgovor, mikropropagacija, *ex vitro*