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EFFECT OF POLYAMINE TYPE AND CONCENTRATION ON *IN VITRO* PROPAGATION AND *EX SITU* CONSERVATION OF *SIDERITIS RAESERI* BOISS & HELDR. SUBSP. *RAESERI*

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ABSTRACT: Polyamines (PAs) are considered plant growth regulators influencing several physiological and developmental processes. In the present study, the effects of the 3 PAs; putrescine (put), spermidine (spd) and spermine (spm) on micropropagation of *Sideritis raeseri* Boiss & Heldr subsp. *raeseri* were investigated. Shoot number was maximum (5.75-6.17) with 5-50 μM spd, shoot multiplication percentage (91.67%) with 10 μM spd or 25 μM put, and shoot length (30.14 mm) with 1 μM spm. Root number was higher with 500 μM spd (51 roots/rooted explant), root length (25.41 mm) with 100 μM spd, and rooting percentage (91.67%) with put (500, 1000 μM) or spd (1, 10, 50 μM). Among the 3 PAs, spd gave better results regarding shoot and root number, whereas spm enhanced shoot and root length to a greater extent than spd or put. Therefore, 10 μM spd was the most suitable PAs type for shoot proliferation of explants whereas 1 μM spm for shoot elongation. Spd at 50 μM was the best for root induction and 100 μM spm for root elongation. The *ex vitro* survival percentage of rooted microplants was high, 80-95%. An efficient micropropagation protocol was established for *S. raeseri* Boiss & Heldr subsp. *raeseri* using PAs.

KEYWORDS: *Ex Situ* Conservation, Germplasm Preservation, Greek Mountain Tea, Micropropagation, Polyamines, *Sideritis raeseri* Boiss & Heldr subsp. *raeseri*

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1. INTRODUCTION

In Greece, *Sideritis raeseri* Boiss & Heldr. is distributed in central, north-central and northern parts of the country (typically in the range of Pindos) or in neighbouring areas with Albania and the FYR of Macedonia [1]. *S. raeseri* Boiss & Heldr self-sows in Parnassus, Timfristos (Velouchi) and other mountains of Aetolia, Doris and Fthiotida [2]. *S. raeseri* Boiss & Heldr. subsp. *raeseri* is a range-restricted Balkan hemicryptophyte, distributed in North Central Greece, North Pindos, South Pindos and Sterea Ellas, growing in high mountain vegetation [3,4]. Recent reports show that sterols, coumarins, terpenoids, glycosides and flavonoid aglycones are among the main constituents of species that belong to the *Sideritis* genus [5,6]. Several *Sideritis* species exert different biological properties including inflammatory [7,8] and antioxidant activities [9,10,11], originated from their constituents and extracts. Furthermore, the *Sideritis* genus is rich in a number of therapeutically useful compounds and bioactive substances exhibiting antimicrobial [12,13], antiviral [14] and anti-ulcer effects [15]. A number of studies, both *in vitro* and *in vivo* have shown that *S. raeseri* Boiss. & Heldr. performs hypotensive and vasodilatory activities [16], as well as vasoprotective and gastroprotective effects [17]. Micropropagation of *S. raeseri* was studied as a first step for introduction in horticultural practice. Because of its beneficial properties, its over-collection in recent years in Greece resulted in population reductions in many areas. Several species of the genus are considered rare and endangered. Cultivation of *Sideritis* could be a good way for Greek farmers, both by supplying the internal market and covering the needs of the demanding markets abroad, since it is a unique product. Endangered, threatened and rare types have positively been grown and preserved by micropropagation as high number of development and small loads on number of preliminary plants and space. Additionally, plant tissue culture recognized as the most effective method for improving the crops by producing somaclonal and gametoclonal variations. The micropropagation method has a huge potential for manufacturing the plants with higher quality, separated from efficient variations in well-adapted high yielding genotypes that have good disease confrontation and stress acceptance dimensions [18]. Polyamines (PAs) are molecules that are responsible for different plant developmental processes. They control several cellular processes including DNA replication, cell division, protein synthesis, flower development, *in vitro* flower induction, fruit development, senescence, abiotic and biotic stress responses, and secondary metabolism [19]. Recent studies have indicated that PAs also affect the formation of plant architecture, such as internode elongation [20], root branching [21] and shoot apical dominance [22]. Previous studies have shown that exogenously applied PAs (e.g., put) can result in increased shoot growth, callus formation, and root elongation [23]. The exogenous PAs treatment would trigger proliferation and growth of plant cells leading to adventitious shoot formation [24]. Because of their presence in meristematic and growing tissues [25], PAs can also be considered, together with cytokinins, juvenility markers. Aliphatic PAs such as putrescine (put), cadaverine, spermidine

(spd) and spermine (spm) are low molecular mass polycations present in all living organisms [26]. PAs can be classified into two categories based on their biological effect [27]. The first comprises put and cadaverine, which stimulate cell elongation and root formation, like auxins and gibberellins. The second includes spd and spm, which similar to cytokinins, control cell division, organogenesis and plant senescence. PAs levels have been found to be higher in juvenile tissues than in adult ones [28]. Furthermore, it has been reported [29] that exogenous application of put may reduce the production of unwanted ethylene and enhance morphogenesis. Because of its bioactive compounds, this species-subspecies may comprise a potentially useful source for the development of herbal or botanical drug products. Thus, the present study was undertaken to verify the potential effects of the 3 PAs (put, spd and spm) on *in vitro* shoot proliferation and rooting of *S. raeseri* establishing an efficient micropropagation protocol, as a preliminary step for further experimentations focused on culinary and pharmaceutical industry.

2. MATERIALS AND METHODS

2.1 Plant Material and Culture Conditions

The effect of the PAs put, spd and spm was studied in *in vitro* experiments employing the *Sideritis raeseri* Boiss & Heldr. (Greek mountain tea of Velouchi or Parnassus). The experimental material was shoot tip explants (1.5-2.5 cm long) from previous *S. raeseri in vitro* cultures. For the initial establishment of the plant material *in vitro* apex meristems were cut and removed from the mother plants maintained in a peat:perlite (1:1) substrate in pots under unheated-greenhouse conditions. For the disinfection of the collected plant material, shoot tips were soaked in 70% ethanol for 1 min followed by 2% NaOCl solution for 15 min under continuous stirring. The successfully established explants were sub-cultured every 4 weeks until a sufficient amount of plant material to be concentrated. Three experiments were conducted. In the first experiment, the effect of put (Sigma-Aldrich) applied exogenously at 11 concentrations (0, 1, 2.5, 5, 10, 25, 50, 100, 250, 500 μM and 1 mM) was studied for enhancing *in vitro* shoot multiplication and rooting of *S. raeseri* shoot tip explants. In the second experiment, the effect of spd (≥ 98%, Sigma-Aldrich) applied exogenously at 7 concentrations (0, 1, 5, 10, 50, 100, 500 μM) was studied for improvement of micropropagation of *S. raeseri* explants. In the third experiment, spm (Sigma-Aldrich) was tested at 7 concentrations (0, 1, 5, 10, 50, 100, 500 μM) for stimulating shoot and root regeneration under *in vitro* conditions, simultaneously. All PAs concentrations were applied in combination with 2.69 μM NAA (Sigma-Aldrich). The nutrient medium used for the second and third experiment with spd and spm, respectively was the full MS (Murashige and Skoog) [30] supplemented with all the essential macronutrients, micronutrients, vitamins and amino acids, whereas for the first experiment with put, the ½ MS medium was used reduced by 50% in macro- and microelements. The culture medium was also supplemented for all 3 experiments with 30 g L^{-1} sucrose (Duchefa Biochemie). In the first experiment with put, 3 g L^{-1} of Phytagel (Sigma-Aldrich) was used as a gelling agent

while for the second and third experiment with spd and spm, respectively, 3 g L^{-1} Gelrite (Duchefa Biochemie). The pH of the medium in all 3 experiments was adjusted to 5.8 before adding the gelling agent and afterwards the medium was sterilized at 121 ºC for 20 min. The initial experimental plant material was shoot tip explants, 1.5-2.5 cm long that were transferred into Magenta vessels for plant tissue culture (Baby food jars, autoclavable, reusable, 62.4 mm × 95.8 mm, size: 200 mL), each containing 35 mL of the MS medium. Magenta^{TM} B-caps were used for covering the vessels. All cultures were maintained in a growth chamber. The chamber was programmed to maintain 16h light duration (40 μmol/m^2/s) supplied by cool white fluorescent lamps and a constant temperature of 22 ± 2 ºC. The first experiment with put included 11 treatments with 20 replications (explants)/treatment whereas the second and third experiment with spd and spm, respectively, included 7 treatments with 15 replications/treatment. In all 3 experiments, 4 shoot tip explants were inserted in each Magenta vessel. After 8 weeks of culture for all 3 experiments, measurements were taken regarding shoot proliferation and rooting attributes such as shoot number/explant, shoot length, root number/rooted microcutting, root length, shoot multiplication and rooting percentages (%). In addition, callus formation, vitrification and necrosis percentages were recorded (%).

2.2 Statistical Analysis

The experiment was completely randomized and analyzed by ANOVA (Analysis of Variance) using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, USA) at $P \leq 0.05$, according to Duncan's multiple range test ± S.E. in order significant differences among the treatments to be established. The main effect of PAs type, PAs concentration and their interaction was performed according to 2-way ANOVA and General Linear Model.

2.3 *Ex Vitro* Acclimatization of *In Vitro* Rooted Plantlets

Plantlets with well-developed shoots and roots were removed from the glass test tubes, washed thoroughly with tap water and transferred to an enriched peat (Terrahum): perlite (Perflor) (1:1 v/v) soil substrate. The rooted microplantlets were transferred into multi-point discs. Then these trays were placed in a nylon table bench tunnel with adjustable relative humidity or misting system, initially, in the first week with 65-72% relative humidity and the second week with 55-62%. After two weeks, the trays with the plants were transferred to one of the benches of the greenhouse (50 ± 5% relative humidity) for two more weeks, wherein watered was applied by sprinkling. After this period, the plants were transplanted into pots of 0.5 L, transferred to the nursery outside greenhouse, where the acclimatization of the plants was completed. After 8 weeks following transplantation, the adjusted plants were transferred to pots of larger capacity, 1.5 L, filled with enriched white peat moss (TS2, Clammann):perlite (Perflor):sand (2:2:0.5 v/v) soil substrate and maintained in the greenhouse. Finally, after 12 weeks from the initial transition of the *in vitro* rooted plantlets to the *ex vitro* environment, their survival percentage was recorded.

3. RESULTS AND DISCUSSION

3.1 Effect of PAs Type and Concentration on *in vitro* Shoot Proliferation of *S. raeseri*

Put promoted the production of multiple shoots increasing the relative percentage from 30% (control) (Fig. 1a) to 33.33-91.67% (Fig. 1b-1k). The maximum absolute value of shoot number/explant (3.08) was recorded with 25 μM put in the ½ MS medium. The elongation of shoots was highest (approximately 21 mm), when explants treated with 2.5 or 250 μM put. The highest shoot multiplication percentage (91.67%) was observed with 25 μM put. The phenomenon of hyperhydricity in plant tissues was evident in all treatments including the control to the 25-66.67% of explants. Put accentuated necrosis/browning symptoms by increasing the relative percentage from 10% (control) to 16.67-50% (Table 1). Shoot number/explant and shoot multiplication percentage were increased when the explants were treated with 10 or 50 spd, achieving 6.17 shoots/explant (doubled, compared to the control) and 83.33-91.67% shoot multiplication. Spd did not have an effect on shoot length (21.01-29.51 mm) in relation to the control (24.14 mm). However, shoot length was not differentiated significantly due to 5 μM spd application. Neither necrotic explants nor vitrification were observed in the spd-untreated microshoots. Spd (1-500 μM) caused symptoms of hyperhydricity and necrosis to the 8.33-50% of explants. The lowest vitrification and necrosis percentages (8.33%) were recorded in the presence of 5 and 10 μM spd, respectively. Therefore, the best results in terms of shoot number/explant (6.17) and shoot multiplication percentage (83.33-91.67%) were exhibited with 10 or 50 μM spd (Table 1). In relation to control (Fig. 3a), spm (1-500 μM) did not promote nor inhibited shoot regeneration in terms of shoot number and shoot elongation (Fig. 3b-3g). The maximum absolute value of shoot number/explant (3.69) and shoot length (30.14 mm) were recorded when the explants were treated with 1 and 5 μM spm, accordingly. In addition, spm (1-500 μM) negatively influenced the explants ability to proliferate by reducing the shoot multiplication from 75% (contol) to 41.67-58.33%. Neither vitrified nor necrotic explants were observed in the control treatment. However, spm caused hyperhydricity and necrosis/browning symptoms to the 16.67-33.33% and 41.67-91.67% of explants, respectively (Table 1). Among the 3 PAs, regarding shoot proliferation, spd applied at 5-50 μM gave higher shoot number (5.75-6.17) compared to the control (2.5-3.17 shoots/explants). Spm (1 μM), on the other hand exhibited the greater shoot length (30.14 mm). Shoot multiplication percentage was higher (91.67%) by adding 25 μM put or 10 μM spd to the culture medium. Vitrification problem was less intense and observed only to the 8.33% of explants when 5 μM spd were applied. Necrotic symptoms were apparent to the 8.33% of explants when the culture medium was fortified with either put (25, 500 μM) or 10 μM spd (Table 1).

Table 1: Effect of PAs; putrescine (put) (0-1000 μM), spermidine (spd) (0-500 μM) and spermine (spm) (0-500 μM) combined with 2.69 μM NAA on shoot number/ explant, shoot length, shoot multiplication, vitrification and necrosis percentages (%) in *S. raeseri* Boiss & Heldr. ssp. *raeseri*

Treatments (μM)		Shoot number	Shoot length (mm)	Shoot multiplication (%)	Vitrification (%)	Necrosis (%)
put	0	2.50 ± 0.42 abc	14.69 ± 0.54 a	30.00 b	50.00 g	10.00 b
	1	2.50 ± 0.29 abc	15.62 ± 0.52 ab	58.33 f	25.00 d	25.00 d
	2.5	1.83 ± 0.22 a	21.11 ± 1.11 bcdef	33.33 c	50.00 g	33.33 e
	5	2.58 ± 0.30 abc	17.88 ± 1.26 abcd	58.33 f	66.67 i	33.33 e
	10	2.33 ± 0.21 abc	20.36 ± 1.24 abcdef	66.67 g	50.00 g	25.00 d
	25	3.08 ± 0.21 abc	18.20 ± 0.68 abcde	91.67 j	50.00 g	8.33 b
	50	2.67 ± 0.32 abc	16.61 ± 0.81 abc	75.00 h	33.33 e	16.67 c
	100	2.83 ± 0.31 abc	18.02 ± 0.62 abcde	66.67 g	33.33 e	16.67 c
	250	1.67 ± 0.23 a	21.06 ± 1.17 bcdef	25.00 a	33.33 e	50.00 e
	500	3.00 ± 0.28 abc	17.49 ± 0.64 abcd	66.67 g	58.33 h	8.33 b
	1000	2.67 ± 0.27 abc	16.23 ± 0.93 ab	66.67 g	58.33 h	25.00 d
spd	0	3.17 ± 0.40 abcd	24.14 ± 2.07 efg	75.00 h	0.00 a	0.00 a
	1	4.83 ± 0.95 de	25.51 ± 2.39 fgh	75.00 h	16.67 c	16.67 c
	5	5.75 ± 1.28 e	29.51 ± 2.77 gh	66.67 g	8.33 b	33.33 e
	10	6.17 ± 0.89 e	21.01 ± 1.07 bcdef	91.67 j	50.00 g	8.33 b
	50	6.17 ± 0.93 e	22.48 ± 1.84 cdef	83.33 i	41.67 f	25.00 d
	100	4.00 ± 1.06 cd	22.65 ± 2.44 cdef	66.67 g	58.33 h	50.00 g
	500	2.08 ± 0.30 ab	23.44 ± 2.74 def	50.00 e	50.00 g	50.00 g
spm	0	3.17 ± 0.40 abcd	24.14 ± 2.07 efg	75.00 h	0.00 a	0.00 a
	1	2.00 ± 0.29 ab	30.14 ± 4.73 h	41.67 d	16.67 c	50.00 g
	5	3.69 ± 0.74 bcd	19.96 ± 2.03 abcdef	75.00 h	33.33 e	41.67 f
	10	2.67 ± 0.65 abc	20.64 ± 1.46 abcdef	50.00 e	25.00 d	66.67 d
	50	2.00 ± 0.28 abcd	16.72 ± 0.99 abc	58.33 f	25.00 d	91.67 e
	100	3.17 ± 0.66 abcd	24.83 ± 3.32 fgh	50.00 b	33.33 e	41.67 f
	500	2.75 ± 0.70 abc	20.96 ± 3.06 bcdef	41.67 d	33.33 e	66.67 d
P-values (2-way ANOVA)						
PAs type (A)		0.000***	0.000***	0.000***	0.000***	0.000***
PAs Conc.(B)		0.025*	0.010**	0.000***	0.000***	0.000***
A*B		0.000***	0.000***	0.000***	0.000***	0.000***

Means denoted by the same letter in each column for all 3 PAs in combination are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

In *S. raeseri*, put (1-1000 μM) stimulated the explants' ability to proliferate, but hardly modified the number of produced shoots. In the current study employing the Greek mountain tea of Velouchi or Parnassus, best results in terms of shoot number and shoot length were exhibited with 2.5 μM put, while shoot multiplication percentage was positively influenced due to 25 μM put. Put and spm did not improve the rate of produced new shoots from apricot leaves [31]. In *S. raeseri*, spd (10 or 50 μM) promoted the explants ability to multiply while spm (1-500 μM) did not favor nor inhibited

shoot multiplication percentage. In cucumber, spd but not put or spm enhanced the differentiation of multiple shoots from shoot tips [32]. In our study with *S. raeseri*, spm (1-500 μM) negatively affected shoot multiplication percentage. On the contrary, PAs promoted shoot regeneration from Passiflora leaves, cotyledons of *Brassica campestris* species and cucumber (*Cucumis sativus*) shoot tips [33]. In *S. raeseri*, 91.67% shoot regeneration was obtained in the presence of 10 μM spd, whereas spm did not differentiate shoot number and shoot length to a considerable degree.

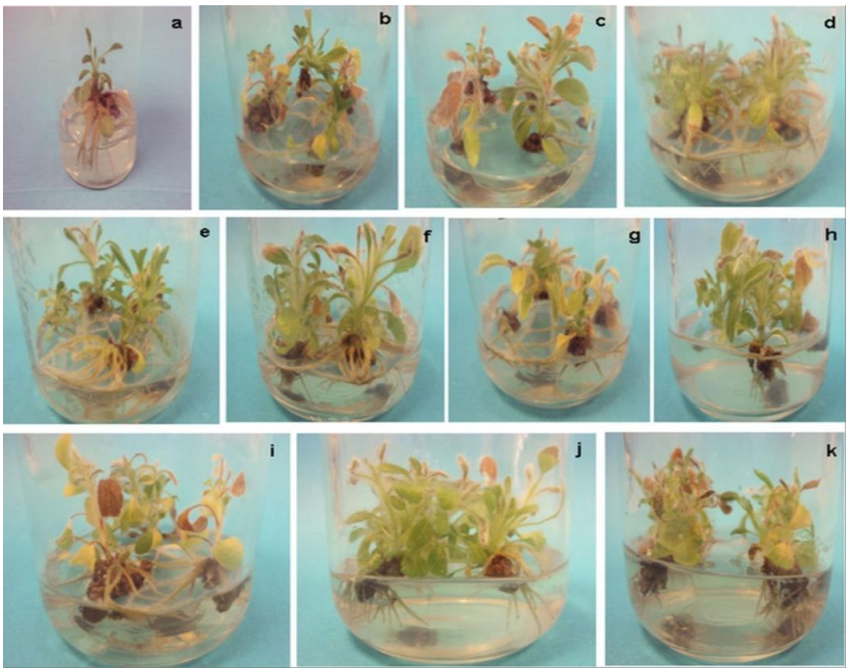

Figure 1: Effect of putrescine (put) concentration in a 2.69 μM NAA containing ½ MS (50% macro- and microelements) culture medium on micropropagation of *S. raeseri* shoot tip explants: (a) Control (put-free), (b) 1 μM put, (c) 2.5 μM put, (d) 5 μM put, (e) 10 μM put, (f) 25 μM put, (g) 50 μM put, (h) 100 μM put, (i) 250 μM put, (j) 500 μM put, (k) 1000 μM put

In *S. raeseri*, put (2.5-250 μM) stimulated shoot elongation, whereas spd and spm did not differentiate shoot length to a considerable degree. It has been reported that put applied exogenously increased shoot length in flax (*Linum usitatissimum* L.) [34], myrtle (*Catharanthus roseus* L.) [35], onion (*Allium cepa* L. cv. 'Giza 20') [36], artichoke (*Cynara scolymus* L.) [37] and bean seedlings *(Phaseolus vulgaris* L. cv. Giza) [38]. In wheat plants (*Triticum aestivum* var. Giza 168), foliar application of put (0.6-5 mM) increased shoot length and levels of endogenous phytohormones such as IAA, GA_3 and cytokinins but reduced plant height and endogenous level of the growth inhibitor ABA [39]. Another possible explanation for the positive effect of put on vegetative growth is attributed to the strengthening of cell division and cell expansion [40]. Stimulation of vegetative growth by put application is due to its effect in the synthesis of macromolecules on the PAs, which are known to increase the synthesis of nucleic acids and stimulate various processes related to the synthesis of proteins and promotion of cell division [41].

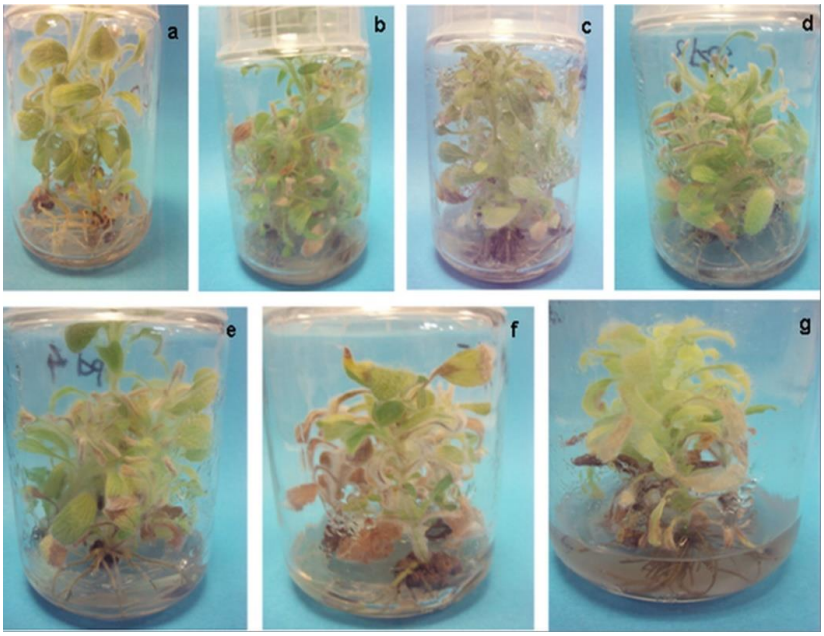

Figure 2: Effect of spermidine (spd) concentration in a 2.69 μM NAA containing full MS medium on micropropagation of *S. raeseri* shoot tip explants: (a) Control (spd-free), (b) 1 μM spd, (c) 5 μM spd, (d) 10 μM spd, (e) 50 μM spd, (f) 100 μM spd, (g) 500 μM spd

3.2 Effect of PAs Type and Concentration on *in vitro* Rooting of *S. raeseri*

Regarding rhizogenesis, only the highest applied put concentration of 1000 μM significantly increased the number of roots/rooted microcutting to 13.18. Put in the 1-500 μM concentration range stimulated shoot length to 15.32-22.35 mm (Fig. 1b-1k), from 10.95 mm (control) (Fig. 1a). The length of the roots was greatest (22.35 mm) in the presence of 25 μM put in the ½ MS culture medium. Significant increase in the rooting percentage from 70% (control) to 91.67% occurred when the explants were treated with 500 μM or 1 mM put. Callus induction was observed in all treatments and to the 91.67-100% of microcuttings (Table 2). Significant increase in the rooting percentage from 66.67% (control) (Fig. 2a) to 83.33-91.67% occurred when explants treated with 1-100 μM spd (Fig. 2b-2f). Supplementing the medium with 500 μM spd resulted in a 4-fold decrease of rooting, from 66.67 to 16.67%, and complete inhibition of callus induction (Fig. 2g). The highest applied spd concentration of 500 μM caused a 5-fold increase in root number from 10 (control) to 51, whereas lower spd concentrations (1-100 μM) did not have a significant effect. Spd at 5-50 μM enhanced root elongation from 15.23 mm (control) to 18.67-22.04, while when applied at 500 μM adversely affected this trait. Root length was greatest (22.04 mm) with 50 μM spd in the medium. Callus induction was slightly increased from 91.67 to 100% with 50 or 100 μM spd (Table 2). Root number/rooted microcutting (13.29) and root length (25.41) were greatest by incorporating 100 μM spm into the 2.69 μM NAA containing MS medium, in comparison to the control (10 roots of 15.23 mm in length). In this combination treatment, shoot elongation was stimulated, almost by 1 cm. Significant increase in the rooting percentage occurred when the explants were treated with 5

or 100 μM spm. Rooting percentage was highest (83.33%) when 5 μM spm were used. Callus induction was slightly increased from 91.67 to 100% in the 1-100 μM spd concentration range. Both rooting and callogenesis percentages were substantially diminished (approximately 2-2.5 fold decrease) in relation to control, in the presence of 500 μM (highest) spm concentration (Table 2).

Table 2: Effect of polyamines (PAs); putrescine (put) (0-1000 μM), spermidine (spd) (0-500 μM) and spermine (spm) (0-500 μM) combined with 2.69 μM NAA on root number/ rooted microcutting, root length (mm), rooting and callus induction percentages (%) in *S. raeseri*

Treatments (μM)		Root number	Root length (mm)	Rooting (%)	Callus induction (%)
put	0	9.14 ± 1.46 abcde	10.95 ± 0.58 ab	70.00 f	100 d
	1	10.13 ± 1.46 abcde	19.00 ± 0.87 ghi	66.67 e	91.67 c
	2.5	7.00 ± 0.88 ab	20.15 ± 0.80 hij	75.00 g	91.67 c
	5	10.00 ± 1.28 abcde	16.93 ± 0.63 fgh	75.00 g	91.67 c
	10	12.20 ± 1.46 def	19.72 ± 0.77 hij	83.33 h	91.67 c
	25	10.30 ± 1.08 abcde	22.35 ± 1.67 j	83.33 h	91.67 c
	50	10.40 ± 0.72 abcde	21.83 ± 1.26 ij	83.33 h	100 d
	100	7.57 ± 0.39 abc	18.89 ± 1.45 hij	58.33 d	100 d
	250	9.70 ± 1.15 abcde	15.88 ± 1.04 efg	83.33 h	91.67 c
	500	8.36 ± 0.87 abcd	15.32 ± 0.78 def	91.67 i	91.67 c
	1000	13.18 ± 1.27 ef	12.27 ± 0.62 abcd	91.67 i	91.67 c
spd	0	10.00 ± 1.37 abcde	15.23 ± 0.69 def	66.67 e	91.67 c
	1	9.33 ± 1.93 abcde	15.26 ± 1.10 def	91.67 i	91.67 c
	5	10.70 ± 1.57 bcdef	18.67 ± 1.18 ghi	83.33 h	91.67 c
	10	11.82 ± 1.85 cdef	21.21 ± 0.80 ij	91.67 i	91.67 c
	50	14.70 ± 1.93 f	22.04 ± 1.49 ij	91.67 i	100 d
	100	10.60 ± 1.58 abcdef	13.48 ± 1.02 abcde	83.33 h	100 d
	500	51.00 ± 0.00 g	12.16 ± 0.00 abcd	16.67 a	0 a
spm	0	10.00 ± 1.37 abcde	15.23 ± 0.69 def	66.67 e	91.67 c
	1	10.29 ± 0.79 abcde	13.59 ± 0.28 bcdef	58.33 d	100 d
	5	11.36 ± 1.63 bcdef	14.71 ± 1.31 cdef	83.33 h	100 d
	10	6.22 ± 0.82 a	10.21 ± 0.84 a	66.67 e	100 d
	50	9.00 ± 1.21 abcde	11.83 ± 0.77 abc	41.67 c	100 d
	100	13.29 ± 1.61 ef	25.41 ± 2.06 k	75.00 g	100 d
	500	11.50 ± 0.15 cdef	10.75 ± 0.08 ab	25.00 b	58.33 b
P-values (2-way ANOVA)					
Polyamine type (A)		0.000***	0.000***	0.000***	0.000***
Polyamine Conc. (B)		0.000***	0.000***	0.000***	0.000***
A*B		0.000***	0.000***	0.000***	0.000***

Means denoted by the same letter in each column for all 3 PAs in combination are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ***$P \leq 0.001$

In *S. raeseri*, all 3 PAs positively influenced rhizogenesis in terms of root number, root length and rooting percentage. PAs promoted early rooting and increased rooting percentage and root number in olive [42], hazel [28] and vitiligo [43]. In particular, root number of *S. raeseri* shoot tip explants was substantially augmented by 1 mM put, 500 μM spd or 100 μM spm. Spm favored rooting of bean microcuttings by increasing root number [44] and pretreatment of strawberry microcuttings with 1000 μM put led to increased root number [45]. Similarly, the pretreatment of chicory roots (*Cichorium intybus* L. cv. Lucknow Local) with 150 μM put increased the number of secondary and tertiary roots [46]. Root elongation of *S. raeseri* microshoots was significantly stimulated due to 1-500 μM put (optimum: 25 μM), 5-500 μM spd (optimum: 50 μM) or 100 μM spm. In bean cuttings (*Vigna radiata* L. cv. 105), 100 μM put increased root length [47] while in barley 1 μM put, spd or spm improved their root growth [48]. The elongation of detached rice roots at 25^{o}C under *in vitro* conditions was improved with 10-1000 μM put, whereas spd and spm had an inhibitory effect [49]. Our findings regarding root length of *S. raeseri* are not in line with those presented in *Arabidopsis thaliana* L., where 200-800 μM spd reduced root length whereas 1000-1400 μM spd resulted in complete inhibition of rooting [50]. In alfalfa seedlings (*Medicago sativa* L. cv. Siwa 1), the exogenous application of 10 μM put increased length of roots after 21 days of culture in a nutrient Hoagland solution [51]. In *S. raeseri*, the ability of the explants to root was remarkably promoted by all 3 PAs. In specific, 2.5-1000 μM put (optimum: 500-1000 μM), 1-100 μM spd and 5 or 100 μM spm were found to stimulate rooting rate. Enhancing was also the effect of the 3 PAs in other plant species, such as in the dwarf apple MM 106 rootstock, where put and spd promoted rooting (72-98%) [52], in *Tectona grandis* L. in which 160 mg/l put exhibited 70% rooting [53] and in *Fraxinus angustifolia* microshoots where 1 mM put or spd increased rooting from 76% to 100% while spm had an inhibitory effect [54]. However, there are also reports pointing out the negative effect or complete inhibition of rooting frequency due to spm application, such as in cherry (*Prunus avium* L.) [55], walnut (*Juglans regia* L.) [56], vitiligo (*Populus tremula* L. × *P. tremuloides* L.) [57] and strawberry (*Fragaria x ananassa* Duch.) [45]. PAs are implicated in root induction, increasing the activity of total peroxidases in the base of the explants, promoting the rapid growth of roots and increasing the rate of rooting [42]. The positive effect of exogenous PAs have been associated with low levels of endogenous PAs as it was found in olive shoots [52]. The possible mechanisms by which exogenously applied PAs can affect rooting remain unknown. One such mechanism may be the rapid catabolism of PAs, whose products (i.e. H_2O_2) could maintain low the concentration of endogenous auxins during the induction rooting phase. The formation of auxin-phenolic complexes, which could play an important role in rooting, could also be caused by the PAs [52].

Figure 3: Effect of spermine (spm) concentration in a 2.69 μM NAA containing full MS medium on micropropagation of *S. raeseri* shoot tip explants: (a) Control (spm-free), (b) 1 μM spm, (c) 5 μM spm, (d) 10 μM spm, (e) 50 μM spm, (f) 100 μM spm, (g) 500 μM spm

3.3 *Ex Vitro* Acclimatization of *In Vitro* Rooted Plantlets of *S. raeseri*

The survival percentage of the *in vitro* rooted plantlets originated from the put-enriched containing 2.69 μM NAA ½ MS medium (50% decrease in inorganic salts) after 12 weeks from their transition to the *ex vitro* environment of the unheated greenhouse was 80%. On the other hand, 90% survival rate was recorded when microplants derived from MS medium containing spd (1-500 μM). However, the highest *ex vitro* survival percentage (95%) was exhibited from plantlets rooted *in vitro* on MS medium fortified with 1-500 μM spm (Fig. 4a, 4b).

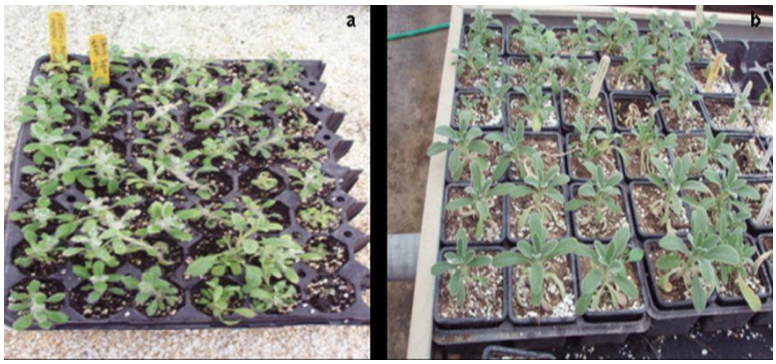

Figure 4: *Ex vitro* acclimatization and adaptation of the *in vitro* rooted *S. raeseri* plantlets to the greenhouse: (a) one week after planting of the plantlets in trays containing an enriched peat (Terrahum):perlite (Perflor) (1:1 v/v) soil substrate and their transfer in a nylon table bench tunnel with adjustable relative humidity or misting system, (b) 8 weeks from transplantation in bigger pots, filled with enriched white peat moss (TS2, Clammann):perlite (Perflor):sand (2:2:0.5 v/v)

4. CONCLUSION

Among the 3 PAs and taking simultaneously into consideration all shoot and rooting macroscopic characteristics, put was the least effective. Spd reinforced greater the explants ability to proliferate and form roots, while spm stimulated further shoot and root elongation. Therefore, the best protocol for the initial shoot proliferation stage of *S. raeseri* Boiss & Heldr subsp. *raeseri* explants is the use of 10 μM spd and for the following shoot elongation stage 1 μM spm. For root induction, 50 μM spd was optimum whereas for the following root elongation stage 100 μM spm gave better results. It was proven that PAs are stimulating agents in tissue culture systems constituting a very useful tool for future genetic transformation and breeding, obtaining improved characteristics. It seems that the different response among the 3 PAs to shoot proliferation and rooting attributes are dependent on PAs type, PAs concentration and their interactions.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

REFERENCES

1. Strid A, Tan T. Mountain Flora of Greece. Vol. II, Edinburgh University Press, pp. 974, 1991.
2. Goliaris A. Cultivation of the Mountain Tea. Ministry of Agriculture-Agritica. 1984; 16:29-31.
3. Dimopoulos P, et al. Vascular Plants of Greece: An Annotated Checklist. Englera 31. Freie Universität Berlin: Botanischer Garten und Botanisches Museum Berlin-Dahlem & Athens: Hellenic Botanical Society, 2013.
4. Dimopoulos P, et al. Vascular Plants of Greece: An Annotated Checklist. Supplement. Willd. 2016; 46(3):301-347.
5. Gil M, Ferreres F, Marrero A, Tomas-Lorente F, Tomas-Barberan FA. Distribution of Flavonoid Aglycons and Glycosides in *Sideritis* Species from the Canary Islands and Madeira. Phytochemistry. 1993; 34:227-232.
6. Pljevljakuši D et al. Chemical Properties of the Cultivated *Sideritis raeseri* spp. *raeseri* Boiss. & Heldr. Food Chem. 2011; 124:226-233.
7. Godoy A, De Las Heras B, Vivas M, Villar A. Anti-inflammatory Properties of a Lipid Fraction Obtained from *Sideritis javalambrensis*. Biol Pharm Bull. 2000; 23:1193-1197.

8. Güvenç A, Duman H. Morphological and Anatomical Studies of Annual Taxa of *Sideritis* L. (Lamiaceae) With Notes on Chorology in Turkey. Turk J Bot. 2010; 34:83-104.
9. Triantaphyllou K, Blekas G, Boskou D. Antioxidative Properties of Water Extracts Obtained From Herbs of the Species Lamiaceae. Int J Food Sci Nutr. 2001; 52:313-317.
10. Tunalier Z, Kosar M, Ozturk N, Baser HC. Antioxidant Properties and Phenolic Composition of *Sideritis* Species. Chem Nat Compd. 2004; 40:206-210.
11. Petreska J, et al. A potential Bioactive Phenolics of Macedonian *Sideritis* Species Used For Medicinal 'Mountain Tea'. Food Chem. 2011; 125:13-20.
12. Aligiannis N, Kalpoutzakis E, Chinou IB, Mitakou S, Gikas E, Tsarbopoulos A. Composition and Antimicrobial Activity of the Essential Oil of Five Taxa of *Sideritis* from Greece. J Agric Food Chem. 2001; 49:811-815.
13. Stagos D, et al. Correlation of Total Polyphenolic Content with Antioxidant and Antibacterial Activity of 24 Extracts From Greek Domestic Lamiaceae Species. Food Chem Toxicol. 2012; 50:4115-4124.
14. Abad MJ, Guerra GA, Bedoya del Olmo L, Bermejo P. Antiviral Activity of Medicinal Plants. Curr Top Phytochem. 2004; 6:113-123.
15. Gurbuz I, Ozkan A, Mine Y, Erdem KO. Anti-Ulcerogenic Activity of Some Plants Used in Folk Medicine of Pinarbasi (Kayseri, Turkey). J Ethnopharmacol. 2005; 101:313-831.
16. Kitic D, et al. Hypotensive, Vasorelaxant and Cardiodepressant Activities of the Ethanol Extract of *Sideritis raeseri* spp. *raeseri* Boiss. & Heldr. J Physiol Pharmacol. 2012; 63:531-535.
17. Brankovic S, et al. Spasmolytic Activity of the Ethanol Extract of *Sideritis raeseri* Boiss. & Heldr. ssp *raeseri* on the Isolated Rat Ileum Contractions. J Med Food. 2011; 14:495-498.
18. Brown DCW, Thorpe TA. Crop Improvement through Tissue Culture. World J Microbiol Biotechnol. 1995; 11:409-415.
19. Tun NN, et al. Polyamines Induce Rapid Biosynthesis of Nitric Oxide (NO) in *Arabidopsis thaliana* Seedlings. Plant Cell Physiol. 2006; 47:346-354.
20. Alcázar R, Garcia-Martinez JL, Cuevas JC, Tiburcio AF, Altabella T. Overexpression of *ADC2* in *Arabidopsis* Induces Dwarfism and Late-Flowering Through GA Deficiency. Plant J. 2005; 43:425-436.
21. Ben-Hayyim G, Damon JP, Martin-Tanguy J, Tepfer D. Changing Root System Architecture Through Inhibition of Putrescine and Feruloyl Putrescine Accumulation. FEBS Lett. 1994; 342:145-148.
22. Geuns JMC, Smets R, Struyf T, Prinsen E, Valcke R, Van Onckelen H. Apical Dominance in *Pssuipt*-Transformed Tobacco. Phytochemistry. 2001; 58:911-921.

23. Tang W, Newton R. Polyamines Promote Root Elongation and Growth by Increasing Root Cell Division in Regenerated Virginia Pine (*Pinus virginiana* Mill.) Plantlets. Plant Cell Rep. 2005; 24:581-589.
24. Martin-Tanguy J. Metabolism and Function of Polyamines in Plants: Recent Development (New Approaches). Plant Growth Regul. 2001; 34:135-148.
25. Torrigiani P, Altamura MM, Capitani F, Serafini-Fracassini D, Bagni N. De Novo Root Formation in Thin Cell Layers of Tobacco: Changes in Free and Bound Polyamines. Physiol Plant. 1989; 77:294-301.
26. Bagni N, Tassoni A. 2001. Biosynthesis, Oxidation and Conjugation of Aliphatic Polyamines in Higher Plants. Amino Acids. 2001; 20:301-317.
27. Kuznetsov VV, Rakitin VY, Sadomov NG, Dam DV, Stetsenko LA, Shevyakova NI. Do Polyamines Participate in the Long-Distance Translocation of Stress Signals in Plants? Russ J Plant Physiol. 2002; 49:120-130.
28. Rey M, Diaz-Sala C, Rodriguez R. Polyamines as Markers for Juvenility in Filbert. Acta Hortic. 1994; 351:233-237.
29. Bais HP, Sudha G, Ravishankar GA. Putrescine and Silver Nitrate Influence Shoot Multiplication, *In Vitro* Flowering and Endogenous Titers of Polyamines in *Cichorium intybus* L. cv. Lucknow Local. J Plant Growth Regul. 2000; 19:238-248.
30. Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. Physiol Plant. 1962; 15:473-497.
31. Petri C, Alburquerque N, Pérez-Tornero O, Burgos L. Auxin Pulses and a Synergistic Interaction between Polyamines and Ethylene Inhibitors Improve Adventitious Regeneration from Apricot Leaves and *Agrobacterium*-Mediated Transformation of Leaf Tissues. Plant Cell Tiss Organ Cult. 2005; 82:105-111.
32. Vasudevan A, et al. Leucine and Spermidine Enhance Shoot Differentiation in Cucumber (*Cucumis sativus* L.). In Vitro Cell Dev Biol-Plant. 2008; 4:300-306.
33. Shankar CU, Ganapathy A, Manickavasagam M. Influence of Polyamines on Shoot Regeneration of Sugarcane (*Saccharum officinalis* L). Egypt J Biol. 2011; 13:44-50.
34. El-Lethy SR, Ayad HS, Talaat IM. Physiological Effect of Some Antioxidants on Flax Plant (*Linum usitatissimum* L.). World J Agric Sci. 2010; 6:622-629.
35. Talaat IM, Bekheta MA, Mahgoub MH. Physiological Response of Periwinkle Plants (*Catharanthus roseus* L.) to Tryptophan and Putrescine. Int J Agric Biol. 2005; 7:210-213.
36. Amin AA, Gharib FAE, El-Awadi M, Rashad ESM. Physiological Response of Onion Plants to Foliar Application of Putrescine and Glutamine. Sci Hortic. 2011; 12:353-360.

37. El-Abagy HMH, Rashad ESM, Abdel-Mawgoud AMR, El-Greadly NHM. Physiological and Biochemical Effects of Some Bioregulators on Growth, Productivity and Quality of Artichoke (*Cynara scolymus* L.). Plant Res J Agric Biol Sci. 2010; 6:683-690.
38. Zeid IM. Response of Bean (*Phaseolus vulgaris*) to Exogenous Putrescine Treatment under Salinity Stress. Pak J Biol Sci. 2004; 7:219-225.
39. El-Bassiouny HM, Mostafa HA, El-Khawas SA, Hassanein RA, Khalil SI, Abd El-Monem AA. Physiological Responses of Wheat Plant to Foliar Treatments with Arginine or Putrescine. Aust J Basic Appl Sci. 2008; 2:1390-1403.
40. Cohen SS. A guide to the Polyamines. Oxford University Press, New York, 1998.
41. Willadino L, Camara T, Boget N, Claarols I, Santos M, Torne JM. Polyamine and Free Amino Acid Variations in NaCl-Treated Embryogenic Maize Callus from Sensitive and Resistant Cultivars. J Plant Physiol. 1996; 149:179-185.
42. Rugini E, Di Francesco G, Muganu M, Astolfi S, Caricato G. The Effects of Polyamines and Hydrogen Peroxide on Root Formation in Olive and the Role of Polyamines as an Early Maker for Rooting Ability. In: Altman A, Waisel Y. (eds). Biology of Root Formation and Development, Plenum Press, New York, 1997, pp. 65-73.
43. Hausman JF, Evers D, Kevers C, Gaspar T. Internal Controls of Root Induction of Poplar Shoots Raised *In Vitro*. J Appl Bot. 1997; 71:104-107.
44. Jarvis BC, Shannon PRM, Yasmin S. Involvement of Polyamines with Adventitious Root Development in Stem Cuttings of Mung Bean. Plant Cell Physiol. 1983; 24:677-683.
45. Tarenghi E, Carre M, Martin-Tanguy J. Effect of Inhibitors of Polyamine Biosynthesis and of Polyamines on Strawberry Microcutting Growth and Development. Plant Cell Tiss Organ Cult. 1995; 42:47-55.
46. Bais HP, Sudha G, Ravishankar GA. Putrescine Influences Growth and Production of Coumarins in Hairy Root Cultures of Witloof Chicory *(Cichorium intybus* L. cv. Lucknow Local*).* J Plant Growth Regul. 1999; 18:159-165.
47. Nag S, Saha K, Choudhuri MA. Role of Auxins and Polyamines in Adventitious Root Formation in Relation to Changes in Compounds Involved in Rooting. J Plant Growth Regul. 2001; 20:182-194.
48. Locke JM, Bryce JH, Morris PC. Contrasting Effects of Ethylene Perception and Biosynthesis Inhibitors on Germination and Seedling Growth of Barley (*Hordeum vulgare* L.). J Exp Bot. 2000; 51:1843-1849.
49. Lee TM. Polyamine Regulation of Growth and Chilling Tolerance of Rice (*Oryza Sativa* L.) Roots Cultured *In Vitro*. Plant Sci. 1997; 122:111-117.

50. Tassoni A, van Buuren M, Franceschetti M, Fornalè S, Bagni N. Polyamine Content and Metabolism in *Arabidopsis thaliana* and Effect of Spermidine on Plant Development. Plant Physiol Biochem. 2000; 38:383-393.
51. Zeid IM, Shedeed ZA. Response of Alfalfa to Putrescine Treatment under Drought Stress. Biol Plant. 2006; 50:635-640.
52. Naija S, Elloumi N, Ammar S, Kevers C, Dommes J. Involvement of Polyamines In The Adventitious Rooting of Micropropagated Shoots of the Apple Rootstock MM106. In Vitro Cell Dev Biol-Plant. 2009; 45:83-91.
53. Mendoza de Gyves E, Royani JI, Rugini E. Efficient Method of Micropropagation and *In Vitro* rooting of Teak (*Tectona grandis* L.) Focusing on Large-Scale Industrial Plantations. Ann For Sci. 2007; 64:73-78.
54. Tonon G, Kevers C, Gaspar T. Changes in Polyamines, Auxins and Peroxidase Activity during *In Vitro* Rooting of *Fraxinus angustifolia* Shoots: An Auxin-Independent Rooting Model. Tree Physiol. 2001; 21:655-663.
55. Biondi S, Dfaz T, Iglesias I, Gamberini G, Bagni N. Polyamines and Ethylene in Relation to Adventitious Root Formation in *Prunus avium* Shoot Cultures. Physiol Plant. 1990; 78:474-483.
56. Kevers C, Hausman JF, Faivre-Rampant O, Evers D, Gaspar T. Hormonal Control of Adventitious Rooting: Progress and Questions. J Appl Bot. 1997; 71:71-79.
57. Hausman JF, Kevers C, Gaspar T. Involvement of Putrescine in the Inductive Rooting Phase of Poplar Shoots Raised *In Vitro*. Physiol Plant. 1994; 92:201-206.
58. Rugini E. Involvement of Polyamines in Auxin and *Agrobacterium Rhizogenes*-Induced Rooting of Fruit Trees *In Vitro*. J Am Soc Hortic Sci. 1992; 117:532-536.