(RESEARCH ARTICLE)

CrossMark
click for updates

Effect of glycine betaine, polyvinylpyrrolidone and D-mannitol on micropropagation of *Sideritis raeseri* Boiss and Heldr. subsp. *Raeseri.*

Sarropoulou Virginia * and Maloupa Eleni

Hellenic Agricultural Organization - 'Demeter', Institute of Plant Breeding and Genetic Resources, Laboratory for the Conservation and Evaluation of Native and Floricultural Species, Balkan Botanic Garden of Kroussia, Thermi P.C. 57001, Thessaloniki, P.O. Box 60458, Greece.

Abstract

Despite being protected, the number of plants that belong to *Sideritis* genus has decreased in the last years due to the urbanization of its natural habitat. *Sideritis raeseri* Boiss and Heldr. subsp. *raeseri* known as mountain tea of Parnassus or Velouchi is a valuable genetic resource of the Greek flora because of its special characteristics, so, the development of a micropropagation protocol is crucial to its conservation and use in breeding programs. The objectives of the study were to evaluate the effects of glycine betaine (GB), polyvinylpyrrolidone (PVP-10) and D-mannitol applied in different concentrations in combination with auxins [α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA)] on shoot proliferation and rooting of shoot-tip explants of the studied species. Better shoot proliferation results were obtained in the following two combination treatments: (1) 100 mg/l GB + 0.5 mg/l NAA (5.5 shoots/explant 2.34 cm long, 75% shoot multiplication), and (2) 2.5 g/l D-mannitol + 3 mg/l IBA (2.92 shoot/explant 83.33% shoot multiplication percentage). In relation to rooting, better results were performed only with PVP at 0.5 g/l applied simultaneously with 0.5 mg/l NAA (12 roots/rooted microcutting, 1.47 cm root length, 90% rooting). The *ex vitro* survival percentage of rooted microcuttings derived from all 5 experiments was ranged between 68% and 93%.

Keywords: D-mannitol; Glycine betaine; Greek flora; Micropropagation; Polyvinylpyrrolidone; *Sideritis* spp.

1. Introduction

Sideritis species have been used in folk medicine for their antimicrobial, antiulcerogenic, digestive and anti-inflammatory properties. In the folk medicine of the Balkan countries, *Sideritis raeseri* is used as a herbal tea in the treatment of inflammation, gastrointestinal disorders and coughs, and also as a tonic, whereas extracts are used as a component of dietary supplements for anaemia. Its dried inflorescences are used to prepare a beverage called 'mountain tea' [1]. Although the disease prevention and health promotion effects of *S. raeseri* Boiss & Heldr. are not clearly established in terms of a prospective cohort study, several *in vitro* and *in vivo* studies have suggested that *S. raeseri* Boiss. & Heldr. exhibits hypotensive and vasodilatory actions [2] and exerts vasoprotective and gastroprotective effects [3].

Investigation of seed germination of several *Sideritis* species [4, 5] has revealed a strong correlation between germination and environmental conditions. Using tissue culture techniques to study regeneration ability of three *Sideritis* species, it is reported that germination ratio was very low [6]. In the last decades, *in vitro* culture of plants has become an integral part of advances in plant science research. Plant tissue culture techniques allow for close monitoring and precise manipulation of plant growth and development, indeed, the *in vitro* system offers the advantage that relatively little space is needed to culture plants and this system allows a rigorous control of physical environment and

---

* Corresponding author
E-mail address: vsarrop@gmail.com

nutrient status parameters, which are difficult to regulate with traditional experimental system [7]. Furthermore, any complex organ-organ and plant-environment interaction can be controlled or removed, and the level of stress can be accurately and conveniently controlled [8]. All this together makes that some aspects of plant growth, that were barely understood before the advancement of the science of tissue culture, such as the metabolism and interaction of plant hormones, as well as their physiological effects can be deeply studied [9].

Glycine Betaine (GB) (trimethylglycine or oxyneurine) is a quaternary ammonium compound [10] that is naturally formed during the oxidation of choline. It is considered to be a provitamin. In plant cells, it imparts insulation against drought and salt stress [11]. GB not only preserves the osmotic balance through stabilization of protein structures via a chaperone-like action but also protects the photosynthetic apparatus [12]. Yang and Lu [13] concluded that if endogenous level of GB reaches a threshold level due to either foliar application of high concentrations of GB or by increasing the number of foliar applications of effective concentration, or applied through the rooting medium for a longer duration even at low concentration, it does not act as an osmoregulatory substance, thereby causing phytotoxicity. The use of antioxidant substances such as polyvinylpyrrolidone (PVP-10) is often reported in micropropagation, however it is still very restricted in the rooting of vegetative cuttings [14]. PVP is an adsorbent compound that binds to phenols and prevents oxidation, in addition to adsorbing the products of phenol oxidation [15].

Being most common carbohydrate in the phloem sap of many plants [16] and due to its cheap and easy availability, sucrose is often assumed to be the sugar of choice in cell and tissue culture media [17, 18]. However, it is not always the best carbohydrate to achieve shoot proliferation [19] because a number of carbon sources besides sucrose are also translocated in plants [20]. Some reports [21] showed that not only media with elevated concentrations of carbohydrates, but also media containing additional carbohydrates, such as mannitol, stimulate plant differentiation in cultured cells. Shen et al. [22] suggested that the exogenously applied mannitol can additionally act as free radical-scavenger and reduce oxidative damage of cells caused by hydroxyl radicals.

To our knowledge, no study has been reported on the influence of PVP, GB, mannitol, on micropropagation of *S. raeseri* Boiss and Heldr. subsp. *raeseri.* However, the current study focused on the combined effect of the above mentioned additives with auxins in order to get rapid and satisfied proliferation and adventitious rooting concurrently, and subsequently increase the survival percentage of the acclimatized plants in the greenhouse. The present study was also undertaken to show the relative efficiency of different combinations of plant growth regulators for *in vitro* shoot and root regeneration from shoot-tip explants and to select the best combination for maximal shoot and root initiation and elongation and establishment of complete plantlets.

2. Material and methods

2.1. Plant material and *in vitro* culture conditions

The experimental plant material was shoot tip explants, 2-3 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. The nutrient medium used was the MS [23] supplemented with all the essential macronutrients, micronutrients, vitamins and amino acids in full strength.

Five experiments were conducted. In the first experiment, GB (Sigma-Aldrich) applied exogenously at 5 concentrations (0, 50, 100, 200, 400 mg/l) combined with 0.5 mg/l NAA (Sigma-Aldrich) in MS basal culture medium supplemented with 30 g/l sucrose (Duchefa Biochemie) and 3 g/l Gelrite (Duchefa Biochemie) as a gelling agent. The experiment included 5 treatments with 12 replications (explants)/treatment and 4 shoot tip explants in each Magenta vessel (Baby food jars, autoclavable, reusable, 62.4 mm × 95.8 mm, size: 200 mL), each containing 35 mL of MS medium. Magenta™ B-caps were used for covering the vessels. After 8 weeks of culture, measurements were taken regarding shoot number/explant, root number/rooted microcutting, shoot length (mm), root length (mm) and percentages (%) of shoot multiplication, rooting, callus formation, vitrification and necrosis.

In the second experiment, PVP (Sigma-Aldrich) was added at 7 concentrations (0, 0.5, 1, 2.5, 5, 10, 20 g/l) simultaneously with 1 mg/l IBA (Sigma-Aldrich) in MS basal culture medium supplemented with 30 g/l sucrose and 3 g/l Phytagel (Sigma-Aldrich) as a gelling agent. The experiment included 7 treatments with 20 replications (explants)/treatment and 4 shoot tip explants in each Magenta vessel. After 9 ½ weeks of culture, measurements were taken regarding shoot

number/explant, root number/rooted microcutting, shoot length (mm), root length (mm) and percentages (%) of shoot multiplication, rooting, callus formation, vitrification and necrosis.

In the third experiment, PVP was tested at 5 concentrations (0, 0.5, 1, 2.5, 5 g/l) in conjunction with 0.5 mg/l NAA. The MS culture medium was also supplemented with 30 g/l sucrose and 3 g/l Gelrite. The experimental plant material was transferred into flat-base glass test tubes of 25 x 100 mm containing 10 ml of MS medium and covered with aluminium foil. The experiment included 5 treatments with 10 replications/treatment and one shoot tip explant in each 25 x 100 mm flat-base glass test tube. After 8 weeks of culture, measurements were taken regarding shoot number/explant, root number/rooted microcutting, shoot length (mm), root length (mm) and percentages (%) of shoot multiplication, rooting, callus formation, vitrification and necrosis.

In the fourth experiment, D-mannitol (Sigma-Aldrich) added at 7 concentrations (0, 1, 2.5, 5, 10, 25, 50 g/l) simultaneously with 3 mg/l IBA in MS basal culture medium supplemented with 30 g/l sucrose and 3 g/l Phytagel. The experiment included 7 treatments with 12 replications (explants)/treatment and 4 shoot tip explants in each Magenta vessel. After 5 weeks of culture, measurements were taken regarding shoot number/explant, root number/rooted microcutting, shoot length (mm), root length (mm) and percentages (%) of shoot multiplication, rooting, callus formation, vitrification and necrosis.

In the fifth experiment, D-mannitol was tested at 4 concentrations (0, 1, 2.5, 5 g/l) in conjunction with 0.5 mg/l NAA. The ½ MS culture medium (50% in macro- and micronutrients) was also supplemented with 30 g/l sucrose and 3 g/l Phytagel. The experimental plant material was transferred into flat-base glass test tubes of 25 x 100 mm containing 10 ml of ½ MS medium and covered with aluminium foil. The experiment included 4 treatments with 15 replications/treatment and one shoot tip explant in each 25 x 100 mm flat-base glass test tube. After 8 weeks of culture, measurements were taken regarding shoot number/explant, root number/rooted microcutting, shoot length (mm), root length (mm) and percentages (%) of shoot multiplication, rooting, callus formation, vitrification and necrosis. The pH of the media in all 5 experiments was adjusted to 5.8 before adding the gelling agent and afterwards the medium was sterilized at 121 °C for 20 min. All cultures were maintained in a growth chamber. The chamber was programmed to maintain 16-h light duration (40 µmol/m²/s) supplied by cool white fluorescent lamps and a constant temperature of 22± 2°C.

2.2. *Ex vitro* acclimatization of rooted microcuttings

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 to 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 four days with 65-72% relative humidity and the next three days with 55-62%. After one week the trays with the plants were transferred to one of the benches of the greenhouse (50 ± 5% relative humidity) for 2 more weeks, wherein watered by sprinkling. After this period, the plants were transplanted into pots of 0.33 Lt with enriched white peat moss (TS2, Clammann): perlite (Perflor): sand (2:0.5:0.5 v/v) soil substrate and transferred to the greenhouse bench (45-55% relative humidity) for 2 more weeks, wherein watered by sprinkling. After 8 weeks from transplantation, the adjusted plants were transferred to pots of larger capacity, 2.5 Lt, filled with enriched white peat moss (TS2, Clammann): perlite (Perflor): sand (2:0.5:0.5 v/v) soil substrate and maintained in the greenhouse. After a period of 5 weeks, the plants were transferred outside of the greenhouse nursery to the external environment within 2.5 Lt pots, where their acclimatization and hardening was completed. Finally, after 12 weeks in total from the initial transition of the *in vitro* rooted plantlets to the *ex vitro* environment, their survival percentage was recorded.

2.3. Statistical analysis

The experiment was completely randomized and analyzed with Analysis of Variance (ANOVA) using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, USA) at P≤0.05, according to Duncan's multiple range test ± standard error (S.E.) in order significant differences among the treatments to be established.

3. Results

3.1. Effect of GB on micropropagation of *S. raeseri*

The addition of 100 mg/l GB in the MS culture medium containing 0.5 mg/l NAA was the optimum treatment exhibiting 5.5 shoots/explant and 75% shoot multiplication percentage, causing a 2.5-fold increase in relation to the control (GB-free). The percentage of explants with hyperhydricity and necrotic symptoms was lower, 8.33% and 25% respectively

with 100 mg/l GB. Except 100 mg/l GB, all the other concentrations (50, 200, 400 mg/l) raised shoot length (28.84-30.85 mm) with respect to the control (15.23 mm) (Table 1, Figure 1A-1E).

Table 1 Effect of GB concentration (0-400 mg/l) combined with 0.5 mg/l NAA on shoot number/explant, shoot length, percentages (%) of shoot multiplication, vitrification and necrosis in *S. raeseri*

GB (mg/l)	Shoot number /explant	Shoot length (mm)	Shoot multiplication (%)	Vitrification (%)	Necrosis (%)
Control	1.75 ± 0.39[a]	15.23 ± 1.17[a]	33.33[a]	25[c]	58.33[d]
50	2.08 ± 0.43[a]	28.84 ± 5.20[b]	50[c]	16.67[b]	33.33[b]
100	5.50 ± 1.16[b]	23.38 ± 1.88[ab]	75[d]	8.33[a]	25[a]
200	2.33 ± 0.74[a]	30.85 ± 4.41[b]	41.67[b]	41.57[d]	66.67[e]
400	2.00 ± 0.39[a]	30.60 ± 3.79[b]	50[c]	91.67[e]	41.67[c]
P-values	0.001**	0.016*	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001

Rooting was adversely affected due to GB application. Root number was decreased by 1.5 times and rooting percentage approximately by 20-40%. Root number was maximum (14.86) and rooting (70%) higher in the control treatment. However, root length was raised from 15.16 mm (control) to 22.58 mm (0.7 cm-increase) when explants treated with 100 mg/L GB. Callusing percentage was lower (33.33%) with 400 mg/l GB (Table 2, Figure 1A-1E).

Table 2 Effect of GB concentration (0-400 mg/l) combined with 0.5 mg/l NAA on root number/rooted microcutting, root length, rooting and callusing percentages (%) in *S. raeseri*

GB (mg/l)	Root number	Root length (mm)	Rooting (%)	Callusing (%)
Control	14.86 ± 1.50[b]	15.16 ± 0.49[ab]	70[d]	50[b]
50	9.67 ± 1.62[a]	14.50 ± 0.96[ab]	50[b]	100[e]
100	8.83 ± 1.48[a]	22.58 ± 2.73[c]	50[b]	91.67[d]
200	9.71 ± 0.80[a]	18.46 ± 1.68[bc]	58.22[c]	75[c]
400	9.75 ± 0.75[a]	12.92 ± 0.94[a]	33.22[a]	33.33[a]
P-values)	0.008**	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. **P ≤ 0.01, ***P ≤ 0.001
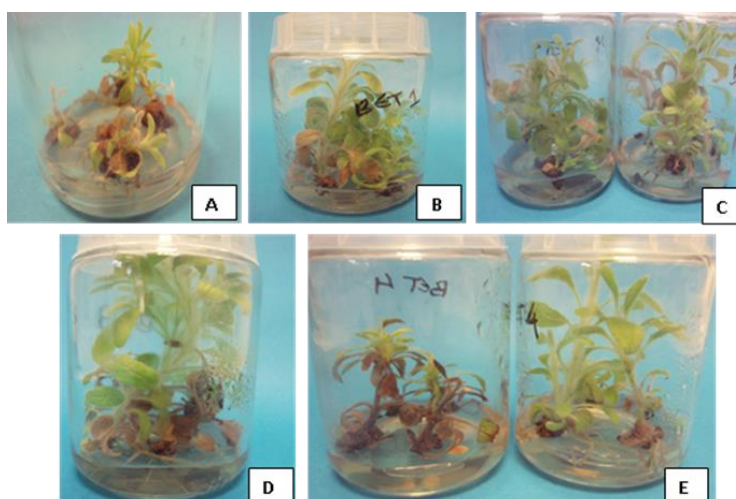

Figure 1 Effect of GB concentration (0-400 g/l) combined with 0.5 mg/l NAA (MS medium) on micropropagation of *S. raeseri*: (A) Control (GB-free), (B) 50 g/l, (C) 100 g/l, (D) 200 g/l and (E) 400 g/l GB

3.2. Effect of PVP + IBA on micropropagation of *S. raeseri*

PVP (1-20 g/l) decreased shoot number/explant from 7.05 (control) to 3.44-5.75. The 2 higher PVP concentrations (10 and 20 g/l) reduced shoot length (10.89-11.71 mm) in relation to the control (14.99 mm). Shoot multiplication percentage was positively affected reaching 100% when explants treated with the 2 lower PVP concentrations (0.5 and 1 g/l), with respect to the control (90%). However, PVP intensified hyperhydricity symptoms raising vitrification percentage from 35% (control) to 50-81.25%. Complete disappearance of explants with necrotic symptoms was observed with 20 g/l PVP (Table 3, Figure 2A-2F).

Table 3 Effect of PVP concentration (0-20 g/l) combined with 1 mg/l IBA on shoot number/ explant, shoot length, percentages (%) of shoot multiplication, vitrification and necrosis in *S. raeseri*

PVP (g/l)	Shoot number /explant	Shoot length (mm)	Shoot multiplication (%)	Vitrification (%)	Necrosis (%)
Control	7.05 ± 0.89[c]	14.99 ± 0.66[b]	90[c]	35[a]	10[b]
0.5	5.75 ± 0.65[bc]	17.78 ± 1.73[b]	100[e]	50[b]	10[b]
1	4.95 ± 0.48[ab]	16.97 ± 1.13[b]	100[e]	55[c]	15[c]
2.5	4.90 ± 0.64[ab]	17.66 ± 1.41[b]	95[d]	60[d]	20[d]
5	4.00 ± 0.32[ab]	15.55 ± 1.15[b]	90[c]	60[d]	25[e]
10	4.75 ± 0.65[ab]	10.89 ± 0.60[a]	85[b]	80[e]	30[f]
20	3.44 ± 0.38[a]	11.71 ± 0.98[a]	75[a]	81.25[e]	0[a]
P-values	0.002**	0.000***	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. $**P \leq 0.01$, $***P \leq 0.001$

The incorporation of 2.5 g/l PVP increased root number (8.38) in comparison to control (6.20). Root length was greater (13.01 and 14.17 mm) with 2.5 and 10 g/l, accordingly. PVP at 1 g/l raised rooting percentage from 50% (control) to 60%, whereas root number (5.25) and root length (9.01 mm) were similar to the control's levels. The highest PVP concentration of 20 g/l completely inhibited rooting. The percentage of explants with callusing was lower (43.75-45%) in the 2 higher PVP concentrations (10 and 20 g/l) (Table 4, Figure 2A-2F).

Table 4 Effect of PVP concentration (0-20 g/l) combined with 1 mg/l IBA on root number/ rooted microcutting, root length, rooting and callusing percentages (%) in *S. raeseri*

PVP (g/l)	Root number	Root length (mm)	Rooting (%)	Callusing (%)
Control	6.20 ± 1.22[c]	9.96 ± 0.67[bc]	50[e]	75[b]
0.5	5.71 ± 0.55[c]	8.77 ± 0.42[b]	35[c]	75[b]
1	5.25 ± 0.66[c]	9.01 ± 0.71[b]	60[f]	85[c]
2.5	8.38 ± 0.55[d]	13.01 ± 0.70[d]	40[d]	80[bc]
5	3.43 ± 0.46[b]	10.90 ± 0.68[c]	35[c]	75[b]
10	3.33 ± 0.15[b]	14.17 ± 0.73[d]	15[b]	45[a]
20	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0[a]	43.75[a]
P-values	0.000***	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. $***P \leq 0.001$
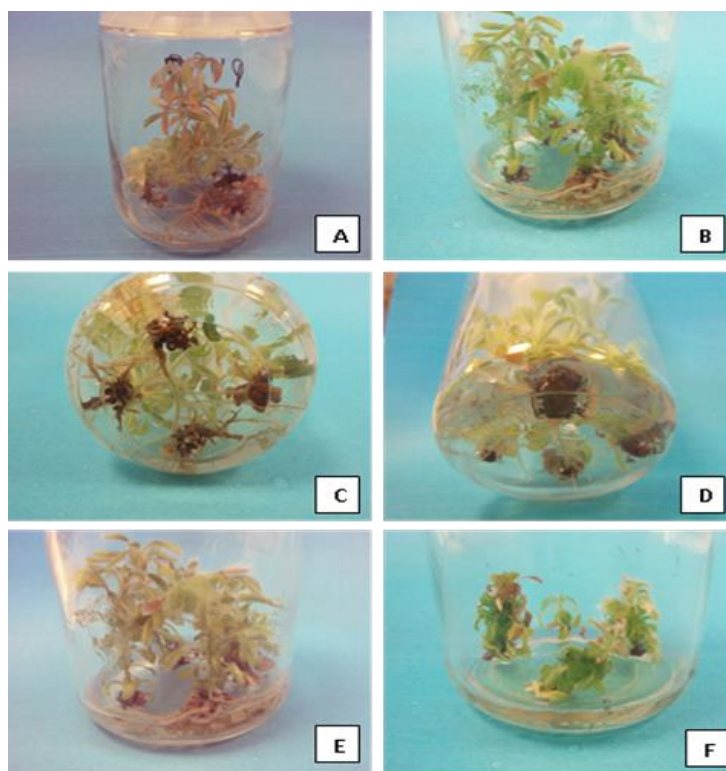
Figure 2 Effect of PVP concentration (0-20 g/l) combined with 1 mg/l IBA (MS medium) on micropropagation of *S. raeseri*: (A) Control (PVP-free), (B) 0.5 g/l, (C) 1 g/l, (D) 2.5 g/l, (E) 5 g/l and (F) 20 g/l PVP

3.3. Effect of PVP + NAA on micropropagation of *S. raeseri*

PVP (1-5 g/l) caused a 2.5 to 4-fold decrease in shoot number (1-1.5 shoot) compared with the control (4.1 shoots). Shoot length was hardly affected by PVP. PVP negatively influenced shoot multiplication, being higher (80%) in the control. The highest PVP concentration of 5 g/l completely inhibited shoot proliferation. No necrotic symptoms were observed in control treatment, however, PVP caused vitrification to 10-20% of explants (Table 5, Figure 3A-3E).

Table 5 Effect of PVP concentration (0-5 g/l) combined with 0.5 mg/l NAA on shoot number, shoot length, shoot multiplication-vitrification-necrosis percentages (%) in *S. raeseri*

| PVP (g/l)	Shoot number /explant	Shoot length (mm)	Shoot multiplication (%)	Vitrification (%)	Necrosis (%)
Control	4.10 ± 0.92[c]	23.73 ± 2.28[ab]	80[d]	10[a]	0[a]
0.5	3.10 ± 0.89[bc]	28.44 ± 4.61[b]	50[c]	10[a]	10[b]
1	1.50 ± 0.31[ab]	19.00 ± 1.91[a]	30[b]	10[a]	20[c]
2.5	1.50 ± 0.27[ab]	18.50 ± 1.75[a]	30[b]	20[b]	20[c]
5	1.00 ± 0.00[a]	16.00 ± 1.00[a]	0[a]	20[b]	20[c]
P-values	0.003**	0.013*	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

PVP did not differentiate root number (8.88-13.5) compared to control (11.71 roots). An increase in root length (17.29 mm) by 0.6 cm with respect to the control (11.08 mm) was observed with 2.5 g/l PVP. Rooting was higher (90%) with 0.5 g/l PVP. PVP led to a 10% decrease in callusing from 100% (control) to 90% (Table 6, Figure 3A-3E).

Table 6 Effect of PVP concentration (0-5 g/l) combined with 0.5 mg/l NAA on root number/ rooted microcutting, root length, rooting and callusing percentages (%) in *S. raeseri*

PVP (g/l)	Root number	Root length (mm)	Rooting (%)	Callusing (%)
Control	11.71 ± 1.92[a]	11.08 ± 0.69[ab]	70[a]	100[b]
0.5	12.00 ± 1.99[a]	14.68 ± 1.16[bc]	90[c]	90[a]
1	13.50 ± 1.61[a]	13.11 ± 0.48[ab]	80[b]	90[a]
2.5	8.88 ± 1.11[a]	17.29 ± 2.39[c]	80[b]	90[a]
5	9.43 ± 1.10[a]	10.60 ± 0.31[a]	70[a]	90[a]
P-values	0.234 ns	0.003**	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. ns $P \geq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$
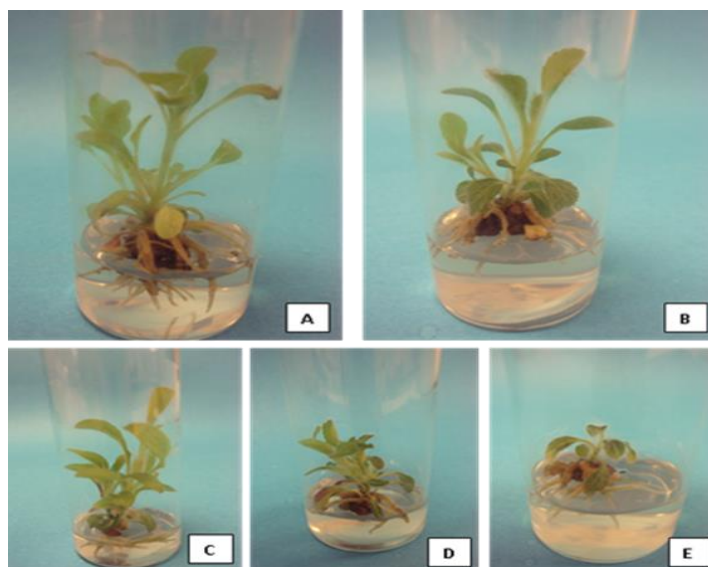
Figure 3 Effect of PVP concentration (0-5 g/l) combined with 0.5 mg/l NAA (MS medium) on micropropagation of *S. raeseri*: (A) Control (PVP-free), (B) 0.5 g/l, (C) 1 g/l, (D) 2.5 g/l and (E) 5 g/l PVP

3.4. Effect of D-mannitol + IBA on micropropagation of *S. raeseri*

A 2.5-3 times increase in shoot number was observed with 2.5 and 5 g/l D-mannitol (2.88-2.92 shoots) compared to control. Shoot length was negatively affected (50% decrease) with intermediate D-mannitol concentrations (2.5-25 g/l). Production of multiple shoots was evident when explants treated with 1-10 g/l D-mannitol. Shoot multiplication percentage was higher (83.33%) with 2.5 g/l D-mannitol. Neither hyperhydricity nor necrosis symptoms observed in the D-mannitol-untreated explants. D-mannitol caused vitrification to the 8.33-16.67 of explants. A positive correlation was observed between D-mannitol concentration (1-50%) and percentage of explants with necrosis (8.33-100%) (Table 7, Figure 4A-4G).

D-mannitol lower concentrations (1-5 g/l) reduced root number from 10 roots/rooted microcutting (control) to 4-6 roots. D-mannitol applied at 2.5 g/l increased root length from 3.34 mm (control) to 4 mm and rooting percentage from 16.67% (control) to 25%. The 3 higher applied D-mannitol concentrations (10, 25, 50 g/l) led to complete inhibition of rooting. Callusing percentage was raised from 8.33% (control) to 33.33-66.67% when 1-10 g/l D-mannitol was used, while no callus initiation was observed with 25 and 50 g/l D-mannitol (Table 8, Figure 4A-4G)

Table 7 Effect of D-mannitol concentration (0-50 g/l) combined with 3 mg/l IBA on shoot number/ explant, shoot length, percentages (%) of shoot multiplication, vitrification and necrosis in *S. raeseri*

D-mannitol (g/l)	Shoot number /explant	Shoot length (mm)	Shoot (%) multiplication	Vitrification (%)	Necrosis (%)
Control	1.00 ± 0.00[a]	30.83 ± 4.07[d]	0[a]	0[a]	0[a]
1	1.92 ± 0.34[a]	23.65 ± 3.58[cd]	41.67[b]	8.33[b]	8.33[b]
2.5	2.92 ± 0.47[b]	14.93 ± 1.91[ab]	83.33[d]	16.67[c]	16.67[c]
5	2.88 ± 0.51[b]	13.02 ± 1.21[a]	50[c]	16.67[c]	25[d]
10	1.75 ± 0.30[a]	18.51 ± 2.02[abc]	41.67[b]	16.67[c]	58.33[e]
25	1.00 ± 0.00[a]	21.67 ± 2.07[bc]	0[a]	16.67[c]	100[f]
50	1.00 ± 0.00[a]	25.42 ± 3.17[cd]	0[a]	16.67[c]	100[f]
P-values	0.000***	0.000***	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. ***$P \leq 0.001$

Table 8 Effect of D-mannitol concentration (0-50 g/l) combined with 3 mg/l IBA on root number/ rooted microcutting, root length, rooting and callusing percentages (%) in *S. raeseri*

D-mannitol (g/l)	Root number	Root length (mm)	Rooting (%)	Callusing (%)
Control	10.00 ± 0.62[d]	3.34 ± 0.01[b]	16.67[c]	8.33[b]
1	6.00 ± 0.00[c]	3.00 ± 0.00[b]	8.33[b]	66.67[f]
2.5	6.00 ± 0.37[c]	4.00 ± 0.12[c]	25[d]	41.67[d]
5	4.00 ± 0.25[b]	3.00 ± 0.12[b]	16.67[c]	62.50[e]
10	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0[a]	33.33[c]
25	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0[a]	0[a]
50	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0[a]	0[a]
P-values	0.000***	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. ***$P \leq 0.001$
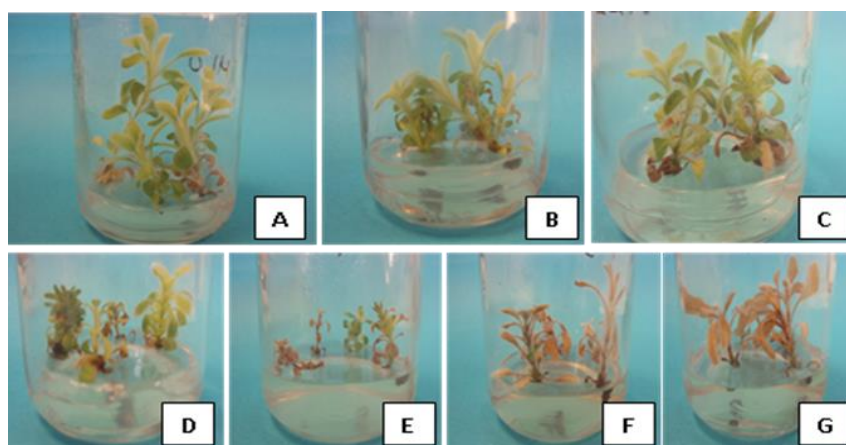
Figure 4 Effect of D-mannitol concentration (0-50 g/l) combined with 3 mg/l IBA (MS medium) on micropropagation of *S. raeseri*: (A) Control (mannitol-free), (B) 1 g/l, (C) 2.5 g/l, (D) 5 g/l, (E) 10 g/l, (F) 25 g/l and (G) 50 g/l mannitol

3.5. Effect of D-mannitol + NAA on micropropagation of *S. raeseri*

D-mannitol (1-5 g/l) combined with NAA did not affect shoot number with respect to the control. Shoot length was greater (18.28 mm) by 0.45 cm compared to control (14.69 mm) with 5 g/l D-mannitol. Shoot multiplication was higher (70%) with 2.5 g/l D-mannitol. The 2 higher D-mannitol concentrations (2.5 and 5 g/l) reduced the vitrification

percentage from 50% (control) to 30-40%. On the other hand, D-mannitol increased the percentage of explants with necrotic symptoms from 10% (control) to 30-70% (Table 9, Figure 5A-5D).

Root number was not differentiated substantially due to D-mannitol application (7.4-10.29 roots/rooted microcutting) compared with the control (9.14 roots). The incorporation of 2.5 g/l D-mannitol into the culture medium led to an increase in root length by 0.3 cm, from 10.95 mm (control) to 13.51 mm. D-mannitol applied at 2.5 g/l gave the same rooting percentage (70%) with respect to the control, whereas lower and higher D-mannitol concentrations had an inhibitory effect. A 10% decrease in callusing percentage from 100% (control) to 90% was recorded when D-mannitol was used (Table 10, Figure 5A-5D).

Table 9 Effect of D-mannitol concentration (0-5 g/l) combined with 0.5 mg/l NAA on shoot number, shoot length, shoot multiplication-vitrification-necrosis percentages (%) in *S. raeseri*

D-mannitol (g/l)	Shoot number /explant	Shoot length (mm)	Shoot (%) multiplication	Vitrification (%)	Necrosis (%)
Control	2.50 ± 0.56[b]	14.69 ± 0.73[ab]	30[b]	50[c]	10[a]
1	1.20 ± 0.13[a]	17.50 ± 1.11[bc]	10[a]	50[c]	50[c]
2.5	3.00 ± 0.39[b]	14.04 ± 1.00[a]	70[d]	40[b]	30[b]
5	2.20 ± 0.39[ab]	18.28 ± 1.21[c]	40[c]	30[c]	70[d]
P-values	0.019*	0.011*	0.000***	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. *P ≤ 0.05, ***P ≤ 0.001

Table 10 Effect of D-mannitol concentration (0-5 g/l) combined with 0.5 mg/l NAA on root number/ rooted microcutting, root length, rooting and callusing percentages (%) in *S. raeseri*

D-mannitol (g/l)	Root number	Root length (mm)	Rooting (%)	Callusing (%)
Control	9.14 ± 1.97[a]	10.95 ± 0.78[a]	70[c]	100[b]
1	7.40 ± 0.50[a]	11.73 ± 0.90[ab]	50[b]	90[a]
2.5	10.29 ± 1.10[a]	13.51 ± 0.66[b]	70[c]	90[a]
5	10.25 ± 1.05[a]	12.25 ± 0.50[ab]	40[a]	90[a]
P-values	0.340 ns	0.099 ns	0.000***	0.000***

Means ± standard error (S.E.) with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at P ≤ 0.05. ns *P* ≥ 0.05, ***P* ≤ 0.001

Figure 5 Effect of D-mannitol concentration combined with 0.5 mg/l NAA (½ MS medium in macro- and micronutrients) on micropropagation of *S. raeseri*: (A) Control (mannitol-free), (B) 1 g/l, (C) 2.5 g/l and (D) 5 g/l Mannitol

3.6. *Ex vitro* acclimatization of *in vitro* rooted plantlets

The survival percentage of *in vitro* rooted plantlets originated from the GB-enriched containing 0.5 mg/l NAA MS culture medium after 12 weeks from their transition to the *ex vitro* environment outside unheated greenhouse was 93%. On the other hand, 73% and 81% survival percentage was recorded when microplants were treated with PVP in combination with 1 mg/l IBA and 0.5 mg/l NAA, respectively. *In vitro* rooted plantlets derived from D-mannitol + 3 mg/l IBA supplemented MS culture media were successfully acclimatized *ex vitro* exhibiting 86% survival percentage. However, the lowest *ex vitro* survival percentage (68%) was exhibited from plantlets rooted *in vitro* in ½ MS culture medium fortified with D-mannitol + 0.5 mg/l NAA (Figure 6A-6C).
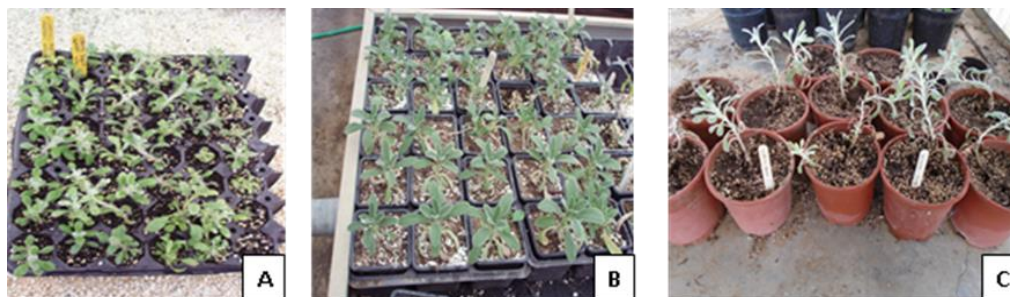

Figure 6 *Ex vitro* acclimatization and adaptation of *in vitro* rooted *S. raeseri* plantlets: (A) internal mist system inside greenhouse conditions, planted to multi-seat discs with peat:perlite (1:1 v/v) soil substrate and watered by sprinkling, (B) transplantation of plants in pots of 0.33 Lt with peat:perlite:sand (2:0.5:0.5 v/v) soil substrate, maintenance in greenhouse bench and watered by sprinkling, (C) transplantation of plants in pots of 2.5 Lt with peat:perlite:sand (2:0.5:0.5 v/v) soil substrate, maintenance outside greenhouse and watered by sprinkling

4. Discussion

4.1. Effect of GB on micropropagation of *S. raeseri*

In *S. raeseri* Boiss & Heldr., the 100 mg/l GB + 0.5 mg/l NAA combination treatment appears suitable for the *in vitro* shoot proliferation stage (5.5 shoots/explant 2.34 cm long and 75% shoot multiplication percentage). Saini et al. [24] reported the stimulatory effect of 125-1,000 mg/l GB on growth, shoot number and shoot multiplication rate in two commercially important UPASI (U-9 and U-10) cultivars of tea (*Camellia sinensis* L. (O.) Kuntze) with the best response achieved with 1,000 mg/l GB. In contrast, sugar beet leaf discs treated with GB did not result in adventitious shoot formation under *in vitro* conditions with respect to other nitrogen supplements in the culture medium [25]. In apple microcuttings, the foliar application of 0.1-0.3 M GB onto newly transplanted plantlets led after 8 weeks to an increase in shoot growth by 30–76 % compared to the non-treated control plants [26].

In the current study employing *S. raeseri* microplants, 100 mg/l GB stimulated root length by 0.75 cm, however initial stage of root induction (root number and rooting percentage) was inhibited due to GB application, irrespective of its concentration (50-400 mg/l). It is evident therefore that GB is not a promising rooting agent for the studied species. Contradictory results were recorded for the wheat cultivars (*Triticumn aestivum L.*) Sakha 94 (sensitive var.) and Sakha 93 (resistant var.), where GB (10 mM) improved growth vigor by increasing plant height and root length [27].

4.2. Effect of PVP on micropropagation of *S. raeseri*

In *S. raeseri* Boiss & Heldr. microplants, 0.5-1 g/l PVP applied with 1 mg/l IBA even though enhanced shoot multiplication percentage is not suitable for shoot proliferation stage because intensified hyperhydricity problem. In addition, shoot proliferation of *S. raeseri* explants was negatively affected due to D-mannitol + 0.5 mg/l NAA, therefore D-mannitol seems inappropriate for this micropropagation stage. Sarropoulou et al. [28] reported that PVP had no significant effect on shoot length in the three cherry rootstocks studied. In addition, Parris [29] found no substantial changes in shoot length of Magnolia cv. Ann cultures by incorporating 1g/l PVP into the MS culture medium.

In the current study employing *S. raeseri* microplants, better rooting results (12 roots 1.47 cm long, 90% rooting) within 8 weeks were obtained by adding 0.5 g/l PVP + 0.5 mg/l NAA to the MS culture medium, thus promoting *in vitro* rooting stage. Similarly, Sarropoulou et al. [28] found that rooting percentage of CAB-6P and Gisela 6 cherry rootstocks was positively influenced when explants treated with 1 g/l PVP supplemented in half strength MS medium, while for the M × M 14 cherry rootstock adding 5 g/l PVP in both full and half strength MS media exhibited 100% rooting. On the

contrary, PVP adversely affected rooting percentage in mini-cuttings of three clones of *Eucalyptus urophylla × Eucalyptus grandis* [30].

In this research study, even though the combination effect 1 g/l PVP + 1 mg/l IBA led to increased rooting percentage, however its use is not considered appropriate at this stage as a result of escalation in vitrified explants. Similarly, in marula tree (*Sclerocarya birrea* subsp. *caffra*), 0.8 g/l PVP in combination with 1 mg/l IBA gave the maximum rooting percentage (68%) [31]. Rooting of cotton (*Gossypium hirsutum* L. cv. SVPR2) plants was promoted due to PVP application [32]. Positive effects of PVP on root length have been reported for oak (*Quercus robur* L.) microcuttings [33], *Betula platyphylla* var. *japonica* [34] and *Taxus mairei* embryos [35]. In the apple cvs Delicious and Starkspur, PVP hardly affected root number or root length and even though decreased rooting percentage in the induction phase it had a stimulatory effect during the initiation and elongation phase [36].

4.3. Effect of D-mannitol on micropropagation of *S. raeseri*

In *S. raeseri* microplants, D-mannitol applied at 2.5 g/l with 3 mg/l IBA had a positive effect on initial shoot proliferation stage taken into consideration shoot number (2.92) and shoot multiplication percentage (83.33%). On the other hand, Moges et al*.* [37] in chrysanthemum and Shibli et al. [38] in bitter almond found that mannnitol caused a reduction in shoot growth compared to sucrose. However, the elongation of produced *S. raeseri* shoots in the present study was not enhanced as a result of D-mannitol + IBA application. For instance, shoot length was optimum (30.83 mm) with 3 mg/l IBA (D-mannitol-free). According to Sarropoulou et al. [39], in CAB-6P cherry rootstock, the supplementation of the MS medium with 5 g/l mannitol positively influenced shoot length. Although, the combination treatment 2.5 g/l D-mannitol + 0.5 mg/l NAA led to increased shoot multiplication percentage (70%) caused at the same time extensive problems of hyperhydricity and necrosis to the explants, thus its use is not recommended for the *in vitro* shoot proliferation stage of the species under study. Leva et al. [40] have reported that mannitol improved the *in vitro* propagation of agamic olive explants, collected from mature trees growing in the field. In olive (*Olea europaea* L.), mannitol gave higher shoot length than sucrose [41].

The fortification of MS culture medium with D-mannitol + IBA adversely affected root number of *S. raeseri* microcuttings, however, 2.5 g/l D-mannitol stimulated root length (4 mm) and raised rooting percentage (25%) but at levels not acceptable in a commercial level. In CAB-6P cherry rootstock, root number and rooting percentage were maximum in half MS medium supplemented with 5 g/l mannitol and 15 g/l sucrose while root length was greatest with 10 g/l mannitol and 30 g/l sucrose in full MS medium (Sarropoulou et al. 2016). In the current study employing *S. raeseri* microplants, root length was also enhanced due to 2.5 g/l D-mannitol + NAA combination treatment. In Gisela 6, another cherry rootstock, 20 g/l mannitol + 15 g/l sucrose in full MS medium exhibited the maximum root number and 10 g/l mannitol + 30 g/l sucrose the greatest root length [39]. Between the 2 combination treatments, better results for *S. raeseri* explants (10.29 roots 1.35 cm long, 70% rooting) were obtained with 2.5 g/l D-mannitol + NAA, thus promoting *in vitro* rooting stage.

5. Conclusion

Advances in biotechnological approaches provide a set of techniques that contribute to solving problems of extinction or genetic erosion in particular of plants. Alternatives for fast multiplication, like "*in vitro* propagation" that enables propagation of plants under controlled environmental conditions, can help in multiplying selected plants after molecular and antifungal studies or subjected to excessive demand by the people. Plants are rich sources of pharmaceutically important compounds; but there is a need to synthesis these compounds within laboratory conditions. Micropropagation is an important technology since many secondary plant metabolites can't be synthesized chemically. Many plant species are undiscovered and their medicinal properties unknown; and even the medicinal remedies past down from generations are being lost. Further research and conservation of all plant species including medicinal plants is needed to preserve nature's natural drugs. Advanced biotechnological methods of culturing plant cells and tissues should provide new means for conserving and rapidly propagating valuable, rare, and endangered medicinal plants.

Compliance with ethical standards

2007-2013 (NSRF 2007-2013) coordinated by the Hellenic Agricultural Organisation "DEMETER" (Institute of Plant Breeding and Genetic Resources/Scientific supervisor: Dr Eleni Maloupa).

Disclosure of conflict of interest

The authors of this article declare that there is no conflict of interest

References

[1]   Romanucci V, Di Fabio G, D'Alonzo D, Guaragna A, Scapagnini G and Zarrelli A. (2017). Traditional uses, chemical composition and biological activities of *Sideritis raeseri* Boiss. & Heldr. Journal of the Science of Food and Agriculture, 97(2), 373-383.

[2]   Kitic D, Brankovic S, Radenkovic M, Savikin K, Zdunic G, Kocic B and Velickovic-Radovanovic R. (2012). Hypotensive, vasorelaxant and cardiodepressant activities of the ethanol extract of *Sideritis raeseri* spp. *raeseri* Boiss. & Heldr. Journal of Physiology and Pharmacology, 63, 531-535.

[3]   Brankovic S, Kitic D, Radenkovic M, Veljkovic S, Jankovic T, Savikin K and Zdunic G. (2011). Spasmolytic activity of the ethanol extract of *Sideritis raeseri* spp. *raeseri* Boiss. & Heldr. on the isolated rat ileum contractions. Journal of Medicinal Food, 14, 495-498.

[4]   Estrelles E, Güemes J, Riera J, Boscai U, Ibars A and Costa M. (2010). Seed germination behavior in *Sideritis* from different Iberian habitats. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 38, 9-13.

[5]   Yankova-Tsvetkova E, Yurukova-Grancharova P and Vitkova A. (2013). Reproductive biology of the Balkan endemic *Sideritis scardica* (Lamiaceae). Botanica Serbica, 37, 83-87.

[6]   Uçar E and Turgut K. (2009). *In vitro* propagation of some mountain tea (Sideritis) species. Ziraat Fakültesi Dergisi Akdeniz Üniversitesi, 22, 51-57.

[7]   Shibli RA, Smith MAL and Spomer LA. (1992). Osmotic adjustment and growth responses of three *Chrysanthemum morifolium* Ramat cultivars to osmotic stress induced *in vitro*. Journal of Plant Nutrition, 15, 1374-1381.

[8]   Stephen RG, Zeng L, Shannon MC and Roberts SR. (2002). Rice is more sensitive to salinity than previously thought. Annual Report of U.S. Department of Agriculture's Research Service, 481.

[9]   Bairu MW and Kane ME. (2011). Physiological and developmental problems encountered by *in vitro* cultured plants. Plant Growth Regulation 63:101-103.

[10] McNeill DS, Nuccio ML and Hanson AD. (1999). Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiology, 120, 945-949.

[11] Hayashi H, Mustardy L, Deshnium P, Ida M and Murata N. (1997). Transformation of *Arabidopsis thaliana* with the codA gene for choline oxidase: accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant Journal, 12, 133-142.

[12] Park EJ, Jeknic Z and Chen THH. (2006). Exogenous application of glycine-betaine increases chilling tolerance in tomato plants. Plant and Cell Physiology, 47(6), 706-714.

[13] Yang X and Lu C. (2006). Effects of exogenous glycinebetaine on growth, CO_2 assimilation, and photosystem II photochemistry of maize plants. Physiologia Plantarum, 127, 593-602.

[14] Fachinello JC, Hoffmann A, Menezes AL and Nachtigal JC. (1993). Effect of PVP and IBA on rooting of strawberry guava (*Psidium cattleyanum* Sabine) on different substrates. Revista Brasileira de Fisiologia Vegetal, São Carlos 5.

[15] Teixeira JB. (2001). Limitations to the process of *in vitro* cultivation of woody species. Brasília: Embrapa - Genetic Resources and Biotechnology, 2001. Available at http://www.redbio.org/portal/encuentros/enc_2001/index.html

[16] Ahmad T, Abbasi NA, Hafiz IA and Ali A. (2007). Comparison of sucrose and sorbitol as main carbon energy source in morphogenesis of peach rootstock GF-677. Pakistan Journal of Botany, 39(4), 1264-1275.

[17] Jain N and Babbar SB. (2003). Effect of carbon source on the shoot proliferation potential of epicotyle explant of *Syzygium cuminii*. Biologia Plantarum, 47(1), 133-136.

[18] Faria RTD, Rodrigues FN, Oliveira LVR and Muller C. (2004). *In vitro Dendrobium nobile* plant growth and rooting in different sucrose concentrations. Horticultura Brasileira, 22(4), 780-783.

[19] Blance G, Ferriere NM, Teisson C, Lardet L and Carron MP. (1999). Effect of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis*. Plant Cell, Tissue and Organ Culture, 59, 103-112.

[20] Moing A, Carbonne F, Rashad MH and Jean-Pierre G. (1992). Carbon fluxes in mature peach leaves. Plant Physiology 100, 1878-1884.

[21] Li SH, Kuoh CHS, Chen YH, Chen HH and Chen WH. (2005). Osmotic sucrose enhancement of single-cell embryogenesis and transformation efficiency in *Oncidium*. Plant Cell, Tissue and Organ Culture, 81, 183-192.

[22] Shen B, Jensen RG and Bohnert HJ. (1997). Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiology 113(4), 1177-1183.

[23] Murashige T and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

[24] Saini U, Kaur D, Chanda S, Bhattacharya A and Ahuja PS. (2012). Application of betaine improves solution uptake and *in vitro* shoot multiplication in tea. Plant Growth Regulation, 67, 65-72.

[25] Tsai CJ and Saunders JW. (1999). Evaluation of sole nitrogen sources for shoot and leaf disc cultures of sugarbeet. Plant Cell, Tissue and Organ Culture 59, 47-56.

[26] Uosukainen M, Rantala S, Manninen A and Vestberg M. (2000). Improvement of microplant establishment through *in vitro* and ex vitro exogenous chemical applications. Acta Horticulturae, 530, 325-332.

[27] Aldesuquy HS, Abo-Hamed SA, Abbas MA and Elhakem AH. (2012). Role of glycine betaine and salicylic acid in improving growth vigour and physiological aspects of droughted wheat cultivars. Journal of Stress Physiology and Biochemistry, 8(1), 149-171.

[28] Sarropoulou V, Dimassi-Theriou K and Therios I. (2015). Medium strength in inorganics and PVP concentration effects on cherry rootstocks *in vitro* rooting. Horticultural Science (Prague), 42(4), 185-192.

[29] Parris JK. (2012). Basal salt composition, cytokinins, and phenolic binding agents influence *in vitro* growth and *ex vitro* establishment of Magnolia 'Ann'. HortScience, 47, 162-1629.

[30] Melo B, Pinto JEBP, Luz JMQ, Peixoto JR and Juliatti FC. (2001). Different antioxidants in oxidation control, germination and seedling development *in vitro* culture of embryos guarirobeira (*Syagrus oleracea* (Mart.) Becc.). Ciência e Agrotecnology 25, 1301-1306.

[31] Mollel MHN and Goyvaerts EMA. (2012). Micropropagation of marula, *Sclerocarya birrea* subsp. *caffra* (Anarcadiaceae) by axillary bud proliferation and random amplified polymorphic DNA (RAPD) analysis of plantlets. African Journal of Biotechnology, 11, 16003-16012.

[32] Ganesan M and Jayabalan N. (2006). Influence of cytokinins, auxins and polyamines on *in vitro* mass multiplication of cotton (*Gossypium hirsutum* L. cv. SVPR2). Indian Journal of Experimental Biology, 44, 506-513.

[33] Malá J, Kálal J, Cvrcková H, Cvikrová M and Eder J. (1999). The effect of reduction of exuded phenolic substances level on rooting of oak microcuttings. Acta Horticulturae, 530, 353-362.

[34] Vaario LM, Otomo Y, Soda R and Ide Y. (1995). Plant regeneration from root tissue and establishment of root culture of Japanese white Birch (*Betula platyphylla* var. japonica). Plant Tissue Culture Letters, 12, 251-258.

[35] Chang SH and Yang JC. (1996). Enhancement of plant formation from embryo cultures of *Taxus mairei* using suitable culture medium and PVP. Botanical Bulletin of the Academia Sinica, 37, 35-40.

[36] Standardi A and Romani F. (1990). Effects of some antioxidants on *in vitro* rooting of apple shoots. HortScience, 25, 1435-1436.

[37] Moges AD, Karam NS and Shibli RA. (2003). Slow growth *in vitro* preservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. Advances in Horticultural Science, 17, 1-8.

[38] Shibli RA, Shatnawi MA, Ajlouni MM, Jaradat A and Adham Y. (1999). Slow growth *in vitro* conservation of bitter almond (*Amygdalus communis* L.). Advances in Horticultural Science, 13, 133-134.

[39] Sarropoulou V, Dimassi-Theriou K and Therios I. (2016). Mineral strength, sucrose level and mannitol concentration effects on cherry rootstocks micropropagation. Agriculture and Forestry, 62(3), 7-25.

[40] Leva AR, Petruccelli R, Muleo R, Goretti R and Bartolini G. (1995). Influence of trophic, regulatory and nutritional conditions on the *in vitro* culture of different olive cultivars. Conference Proceedings: Mediterranean Olive Growing: status and perspectives of cultivation and research. Instituto Experimentale perla Olivicoltura, Rende, Italy, 239-248.

[41] García JL, Troncoso J, Sarmiento R and Troncoso A. (2002). Influence of carbon source and concentration on the *in vitro* development of olive zygotic embryos and explants raised from them. Plant Cell, Tissue and Organ Culture, 69, 95-100.

How to cite this article

Sarropoulou V and Maloupa E. (2019). Effect of glycine betaine, polyvinylpyrrolidone and D-mannitol on micropropagation of *Sideritis raeseri* Boiss and Heldr. subsp. *raeseri*. GSC Biological and Pharmaceutical Sciences, 7(2), 29-42.