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In vitro culture and *ex situ* conservation of the endangered and medicinal mountain tea plant species *Sideritis raeseri* Boiss & Heldr., endemic to Greece under the influence of activated charcoal, salicylic acid and α- & β-cyclodextrins

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Abstract
In this study the effects of activated charcoal (AC), salicylic acid (SA) and α- & β-cyclodextrins (α-CD & β-CD) on micropropagation of *Sideritis raeseri* Boiss & Heldr. were investigated. The 2.5 g/l AC + 0.5 mg/l α-napthaleneacetic acid (NAA) treatment is proposed for rooting and shoot proliferation (4.5 shoots/explant, 48.34 mm, 83.33% shoot multiplication, 16.4 roots/rooted microcutting, 23.5 mm root length, 83.33% rooting). SA (25 μM) increased shoot multiplication by 20% and root length by 1.24 cm, however did not promote root induction stage. The 2.5 g/l α-CD + 0.5 mg/l NAA treatment is suitable only for shoot proliferation (5.5 shoots/explant, 91.67% shoot multiplication). For rooting stage, the use of 0.5 g/l α-CD or 1 g/l β-CD + 0.5 mg/l NAA is recommended (12.29-12.33 root number, 91.67-100% rooting). The *ex vitro* survival percentage was 96% for AC-treated explants, 92% in β-CD, 86% in α-CD and 79% in SA-enriched culture media.

Keywords: activated charcoal, cyclodextrins, micropropagation, salicylic acid, *sideritis* SPP

1. Introduction
Sideritis raeseri Boiss. & Heldr. is endemic to the Balkan Peninsula and is reported to grow in Greece, R. Macedonia and Albania. Aerial parts of this plant are widely utilized in Mediterranean folk medicine in the form of a decoction or infusion. *S. raeseri*, known in Greece as "Mountain tea of Velouchi or Parnassus" has been an object of interest for a long time due to its valuable therapeutic properties. Being a native plant from the Mediterranean region, it is used as a herbal tea in the treatment of inflammations (against anti-human immunodeficiency virus replication), gastrointestinal disorders (anti-ulcerogenic, stomachic, carminative), coughs (analgesic), and as a tonic, while the extract is used as a component of dietary supplements for anemia [1].
Alternative and low-cost substances, such as AC, have extensively been used in tissue culture media to reinforce growth and development. The advantageous outcomes of AC on the *in vitro* tissue response may derived from the darkening of the culture medium for manifold root formation [2] or the adsorption of plant growth regulators and other organic compounds [3]. The incorporation of AC into the nutritional substrate brings about a balance between plants growth regulators and other materials included inside the culture medium; it also takes active role in differentiation and modification of the cultivated tissues [4]. At the rooting stage, AC retards the segregation of the auxin incorporated into the medium and pragmatically influences plant development by adsorbing inhibitory substances such as abscisic acid and phenols, stimulating therefore rooting [5]. The usage of AC is not always serviceable in absorbing growth regulators, thiamine and nicotinic acid which are essential for micropropagation. The outcome of this adverse effect results in diminishing availability of the previously reported compounds within the nutritional substrate. Moreover, AC in tissue culture media causes acidification by lowering pH value and managing therefore solid gelly condition [6].
SA has been used to promote *in vitro* regeneration in several plant species [7]. In specific, SA has a promoting impact on shoot and root growth and development of plants [8]. The stimulating role of SA in callus growth, shoot development, rooting and hardening of *in vitro* derived plantlets has been noted in *Ziziphus spina-christi* [9]. The *in vitro* strengthening regeneration in *Hibiscus acetocella* and *H. moschentos* applying SA has been reported by Sakhanokho and Kelly [10]. In a number of woody and herbaceous plant species, SA boosted the rooting of cuttings under *in vivo* conditions when applied simultaneously with auxin [11]. SA inhibited the beneficial effect of IAA on apple stem slices *in vitro* rooting by amplifying the IAA oxidation during the auxin sensitive phase (24-96 h) [12]. Therefore, SA is a substance with hormone-like activity playing a significant role in the regulation of plant growth and development [13].
CDs belong to the category of cyclic carbohydrates containing six or more glucopyranose units linked by a-1,4 bonds. The most common and frequent used representatives are a-CD and b-CD (with six and seven glucose units, accordingly), derived from starch by enzyme conversion. They have a plinth shape, are internally hydrophobic and externally hydrophilic and have the ability of forming complexes with organic compounds, amending the guest molecules solubility [14]. An increment in rooting under *in vivo* conditions was observed by Mura *et al.* [15] when *Olea europea* L. cv. Leccio del Corno

cuttings were treated with indole-3-butyric acid (IBA) and a-CD. According to Salminen *et al.* [16], in strawberry microplants CD caused morphological changes and retarded their growth. On the other hand, in *Cynara scolymus* L. cv. Early French *vitro* culture, supplementing the medium with NAA and CD resulted in augmented rooting [17].

The objectives of this study was to determine whether application of exogenous AC, SA as well as α- or b-CDs to *in vitro* grown meristem shoots could improve micropropagation of *Sideritis raeseri* Boiss & Heldr., Hellenic mountain tea of Velouchi or Parnassus.

2. Materials and Methods

2.1 Plant material and culture conditions

The experimental material was shoot tip explants 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:perline (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 [18] supplemented with all the essential macronutrients, micronutrients, vitamins and amino acids in full strength.

Three experiments were conducted. In the first experiment, AC (Duchefa Biochemie) applied exogenously at 6 concentrations (0, 0.25, 0.5, 1, 2.5, 10 g/l) combined with 0.5 mg/l 6-benzyladene (BA) (Sigma-Aldrich). In the second experiment, SA (Sigma-Aldrich) added at 8 concentrations (0, 1, 2.5, 5, 10, 25, 50, 100 μM) simultaneously with 10.74 μM NAA (Sigma-Aldrich). In the third experiment, both α- and b-CDs (Sigma-Aldrich) were tested at 6 concentrations (0, 0.1, 0.5, 1, 2.5, 5 g L^{-1}) in conjunction with 0.5 mg/l NAA. The MS culture medium was also supplemented for all three experiments with 30 g/l sucrose (Duchefa Biochemie). In the first and second experiment with AC and SA, respectively, 3 g/l Phytagel (Sigma-Aldrich) was used as a gelling agent while for the third experiment with α- and β-CDs, 3 g/l Gelrite (Duchefa Biochemie).

The pH of the media 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 cm long for the SA-experiment, 2-2.5 cm for the CDs-enriched culture medium and 2.5-3 cm for the experiment with AC. The plant material was transferred for the second experiment with SA into flat-base glass test tubes of 25 x 100 mm containing 10 ml of MS medium and covered with aluminium foil, whereas for the first and third experiment with AC and CDs, respectively into Mangenta vessels (Baby food jars, autoclavable, reusable, 62.4 mm × 95.8 mm, size: 200 mL), each containing 35 mL of 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 16-h light duration (40 μmol/m^2/s) supplied by cool white fluorescent lamps and a constant temperature of 22 ± 2 ºC.

The first and third experiment with AC and α- & β-CDs, respectively included 6 treatments with 12 replications (explants)/treatment and 4 shoot tip explants in each Magenta vessel while the second experiment with SA included 8 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 for all 3 experiments, 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.

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 a non-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 0.5 lt and transferred to the nursery outside greenhouse where their acclimatization was completed (5 weeks). After 8 weeks from transplantation, the adjusted plants were transferred to pots of larger capacity, 1.5 mL, 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.

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 $P \leq 0.05$, 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 Experiment 1: Effect of AC on micropropagation of *S. raeseri*

In relation to the control (Figure 1a) containing only 0.5 mg/l NAA, the 0.25-5 g/l AC + 0.5 mg/l NAA combination treatments yielded similar results regarding shoot number/explant (Figure 1b-1f, Table 1). Significant increase in shoot length by 2 cm, from 28.8 to 48.34 mm, occurred when the explants were exposed to 2.5 g/l AC. In addition, AC in the concentration of 2.5 g/l increased shoot multiplication percentage from 66.67% (control) to 83.33%. Both in the case of the control (AC absence) and the presence of 0.25-5 g/l AC in the culture medium no vitrified explants were observed. Symptoms of necrosis/browning were observed to the 8.33-33.33% of the microcuttings only when AC was applied at high concentrations (1-5 g/l) whereas at lower AC concentrations (0.25-0.5 g L^{-1}) there was no trace of necrosis in plant tissues. Therefore, better proliferation results

taking simultaneously into consideration the number of shoots/explant (4.5), the average length of shoots (48.34 mm) and shoot induction rate (83.33%) gave the treatment of the explants with 2.5 g/l AC.

Table 1: Effect of AC concentration (0-5 g/l) combined with 0.5 mg/l NAA on shoot number/explant, shoot length (mm), shoot multiplication and necrosis percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments NAA (mg/l) AC (g/l)	Shoot number/explant	Shoot length (mm)	Shoot multiplication percentage (%)	Necrosis percentage (%)
0.5 NAA + 0 AC	3.33 ± 0.62 bc	28.80 ± 3.62 a	66.67 e	0 a
0.5 NAA + 0.25 AC	2.08 ± 0.56 ab	36.04 ± 7.22 ab	33.33 b	0 a
0.5 NAA + 0.5 AC	2.17 ± 0.37 ab	51.92 ± 9.20 b	58.33 d	0 a
0.5 NAA + 1 AC	1.00 ± 0.00 a	52.08 ± 5.13 b	0 a	25 c
0.5 NAA + 2.5 AC	4.50 ± 0.70 c	48.34 ± 7.69 b	83.33 f	8.33 b
0.5 NAA + 5 AC	2.00 ± 0.39 ab	25.24 ± 3.07 a	50 c	33.33 d
P-values	0.000***	0.007**	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n = 12, ** $P \leq 0.1$,*** $P \leq 0.01$

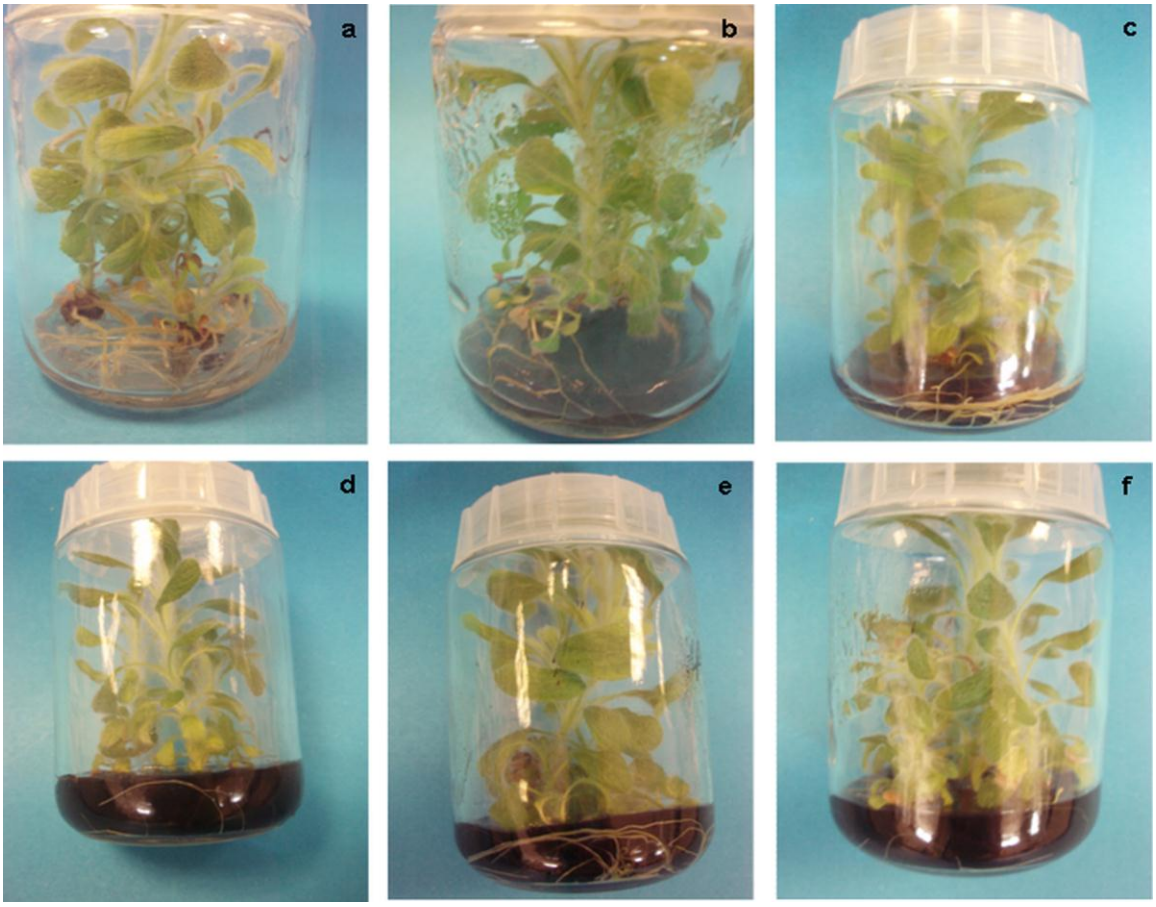

Fig 1: Effect of AC concentration combined with 0.5 mg/l NAA (MS medium) on micropropagation of *Sideritis raeseri* Boiss & Heldr.: (a) Control (AC-free), (b) 0.25 g/l AC, (c) 0.5 g/l AC, (d) 1 g/l AC, (e) 2.5 g/l AC, (f) 5 g/l AC

The control treatment (AC absence) gave 91.67% callogenesis (Table 2, Figure 1a). However, AC (0.25-5 g/l) resulted in complete inhibition of callus induction. Regarding root regeneration, low (0.25-0.5 g/l) and high AC concentrations (5 g/l) negatively affected the number of roots/rooted microcutting. Instead, AC at 0.25-5 g/l significantly enhanced the elongation of roots (Figure 1b-1f) being maximal (28.12 mm) in the presence of 0.5 g/l AC in the culture medium. Small increase in the rooting percentage from 83.33% (control) to 91.67% was exhibited when the explants were treated with 0.5 g/l AC. The maximum absolute value of root number (16.4) was recorded when the microshoots received the influence of 2.5 g/l AC.

Table 2: Effect of AC concentration (0-5 g/l) combined with 0.5 mg/l NAA on root number/rooted microcutting, root length (mm), rooting and callus induction percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments NAA (mg/l) AC (g/l)	Root number/rooted microcutting	Root length (mm)	Rooting percentage (%)	Callus induction percentage (%)
0.5 NAA + 0 AC	16.75 ± 1.77 c	13.32 ± 0.84 a	83.33 b	91.67 b
0.5 NAA + 0.25 AC	4.10 ± 0.63 a	23.81 ± 1.41 bc	41.67 a	0 a
0.5 NAA + 0.5 AC	7.20 ± 0.96 ab	28.12 ± 2.09 c	91.67 c	0 a
0.5 NAA + 1 AC	12.73 ± 2.44 bc	26.09 ± 2.04 bc	83.33 b	0 a
0.5 NAA + 2.5 AC	16.40 ± 3.94 c	23.50 ± 2.56 bc	83.33 b	0 a
0.5 NAA + 5 AC	9.70 ± 1.82 ab	21.58 ± 1.53 b	83.33 b	0 a
P-values	0.000***	0.000***	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n = 12, *** $P \leq 0.01$.

3.2 Experiment 2: Effect of SA on micropropagation of *S. raeseri*

Compared to the control containing only 10.74 μM NAA (Figure 2a), SA (1-100 μM) when combined with 10.74 μM NAA gave similar results in terms of shoot number/explant (1-1.8) and shoot length (14.67-19.1 mm) (Figure 2b-2h, Table 3). When the explants were treated with 25 μM SA exhibited the highest shoot multiplication rate, from 30% (control) to 50% and the maximum shoot number/explant (1.8). No hyperhydricity symptoms were observed at any treatment (0% vitrification). Symptoms of necrosis/browning were observed to the 30-90% of the microcuttings in the presence of 1-100 μM SA including the control treatment (50%).

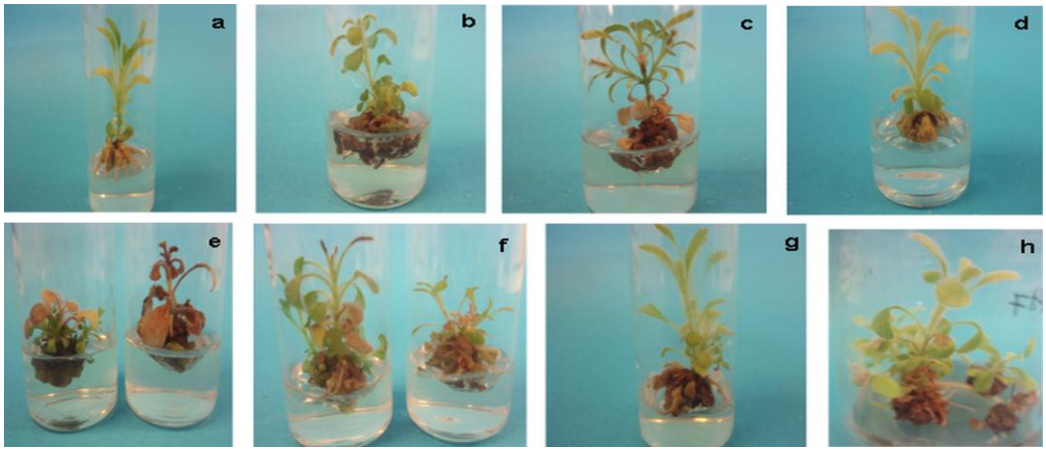

Fig. 2 Effect SA concentration combined with 10.74 μM NAA (MS medium) on micropropagation of *Sideritis raeseri* Boiss & Heldr.: (a) Control (SA-free), (b) 1 μM SA, (c) 2.5 μM SA, (d) 5 μM SA, (e) 10 μM SA, (f) 25 μM SA, (g) 50 μM SA, (h) 100 μM SA

In terms of rhizogenesis, root number/rooted microcutting was substantially augmented from 8.29 (control) to 12 by adding 5 μM SA to the culture medium (Table 4). Treatment of explants with 25 μM SA resulted in the increase of root length. The ability of microshoots to form roots was adversely affected due to exogenous SA (1-100 μM) application as there was a reduction in rooting percentage from 70% (control) to 0-66.67% (Fig. 2a-2h). SA (1-100 μM) led to an increase of callus formation rate, from 80% (control) to 83.33-100%.

Table 3: Effect of SA concentration (0-100 μM) combined with 10.74 μM NAA on shoot number/explant, shoot length (mm), shoot multiplication, vitrification and necrosis percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments (μM) 10.74 NAA + SA	Shoot number/explant	Shoot length (mm)	Shoot multiplication percentage (%)	Necrosis percentage (%)
0	1.40 ± 0.18 ab	15.17 ± 1.86 a	30 d	50 d
1	1.20 ± 0.17 ab	15.67 ± 0.66 a	10 b	80 f
2.5	1.20 ± 0.17 ab	16.00 ± 0.55 a	10 b	90 g
5	1.00 ± 0.00 a	17.22 ± 0.89 a	0 a	55.56 e
10	1.30 ± 0.25 ab	14.88 ± 0.70 a	10 b	90 g
25	1.80 ± 0.24 b	14.67 ± 1.15 a	50 e	30 a
50	1.58 ± 0.31 ab	19.10 ± 1.86 a	25 c	41.67 c
100	1.75 ± 0.33 b	18.58 ± 2.50 a	50 d	33.33 b
P-values	0.156 ns	0.214 ns	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n=10, ns-$P \geq 0.05$, ***$P \leq 0.01$.

Table 4: Effect of SA concentration (0-100 μM) combined with 10.74 μM NAA on root number/rooted microcutting, root length (mm), rooting and callus induction percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments (μM) 10.74 NAA + SA	Root number/rooted microcutting	Root length (mm)	Rooting percentage (%)	Callus induction percentage (%)
0	8.29 ± 1.28 e	6.15 ± 0.22 c	70 f	80 a
1	3.00 ± 0.00 b	5.00 ± 0.00 b	10 b	90 c
2.5	3.33 ± 0.26 bc	5.83 ± 0.18 c	30 c	100 d
5	12.00 ± 1.17 f	5.94 ± 0.14 c	33.33 c	100 d
10	0.00 ± 0.00 a	0.00 ± 0.00 a	0 a	100 d
25	5.83 ± 0.58 d	7.39 ± 0.56 d	60 e	100 d
50	5.25 ± 0.69 cd	5.90 ± 0.42 c	66.67 f	100 d
100	5.00 ± 0.37 bcd	5.96 ± 0.16 c	41.67 d	83.33 b
P-values	0.000***	0.000***	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n = 10, *** $P \leq 0.01$.

3.3 Experiment 3: Effect of α- & β-CDs on micropropagation of *S. raeseri*

Concerning *in vitro* shoot regeneration using α-CD, the maximum shoot number/explant (5.5) and the highest shoot multiplication percentage (91.67%) were recorded in the 5 g/l α-CD + 0.5 mg/l NAA combination treatment, differing substantially from the control (3.17 shoots/explant) (Table 5). With respect to the control where 24.14 mm shoot length was obtained (Figure 3a), α-CD (0.1-5 g/l) did not influence shoot elongation (20.92-28.63 mm) (Figure 3b-3f). Neither hyperhydricity nor necrotic symptoms were observed in the α-CD-untreated explants. However, α-CD (0.1-5 g/l) caused vitrification to the 16.67-50% of the microshoots and total browning in a 33.33-83.33% rate.

Concerning *in vitro* root regeneration, 1 g/l α-CD gave the maximum root number per rooted microcutting (12.33) compared to the control (10 roots) (Table 6). In relation to the control (15.23 mm) (Figure 3a), root length was hardly affected due to α-CD application (12.86-17 mm) (Figure 3b-3f). Rooting percentage (75-100%) was increased when explants treated with 0.1-2.5 g/l α-CD, compared to the α-CD-untreated microshoots (66.67%). α-CD applied at 0.5 g/l resulted in 100% rooting. Slight increase in callus induction frequency, from 91.67% (control) to 100% was exhibited with α-CD (0.1-5 g/l).

Table 5: Effect of α-CD concentration (0-5 g/l) combined with 0.5 mg/l NAA on shoot number/explant, shoot length (mm), shoot multiplication, vitrification and necrosis percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments 0.5 mg/l NAA + α-CD (g/l)	Shoot number/explant	Shoot length (mm)	Shoot (%) multiplication percentage	Vitrification percentage (%)	Necrosis percentage (%)
0	3.17 ± 0.40 ab	24.14 ± 2.07 a	75 c	0 a	0 a
0.1	2.58 ± 0.46 a	28.25 ± 4.31 a	58.33 b	16.67 b	41.67 c
0.5	3.42 ± 0.61 ab	20.92 ± 1.39 a	58.33 b	50 e	41.67 c
1	5.42 ± 1.56 bc	21.32 ± 1.99 a	75 c	41.67 d	33.33 b
2.5	5.50 ± 1.03 c	24.06 ± 3.28 a	91.67 d	33.33 c	33.33 b
5	3.00 ± 0.85 ab	28.63 ± 2.27 a	33.33 a	33.33 c	83.33 d
P-values	0.091 ns	0.211 ns	0.000***	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n = 12, ns-$P \geq 0.05$, ***$P \leq 0.01$

Table 6: Effect of α-CD concentration (0-5 g/l) combined with 0.5 mg/l NAA on root number/rooted microcutting, root length (mm), rooting and callus induction percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments 0.5 mg/l NAA + α-CD (g/l)	Root number/rooted microcutting	Root length (mm)	Rooting percentage (%)	Callus induction percentage (%)
0	10.00 ± 1.37 b	15.23 ± 0.69 ab	66.67 a	91.67 a
0.1	9.00 ± 1.97 ab	12.86 ± 0.73 a	83.33 c	100 b
0.5	10.67 ± 2.31 bc	17.74 ± 1.43 b	100 e	100 b
1	12.33 ± 1.82 c	17.00 ± 0.96 b	91.67 d	100 b
2.5	7.22 ± 0.58 a	13.39 ± 0.94 a	75 b	100 b
5	7.43 ± 0.97 a	13.15 ± 1.09 a	66.67 a	100 b
P-values	0.025*	0.001**	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n=12, *$P \leq 0.5$, **$P \leq 0.1$, ***$P \leq 0.01$

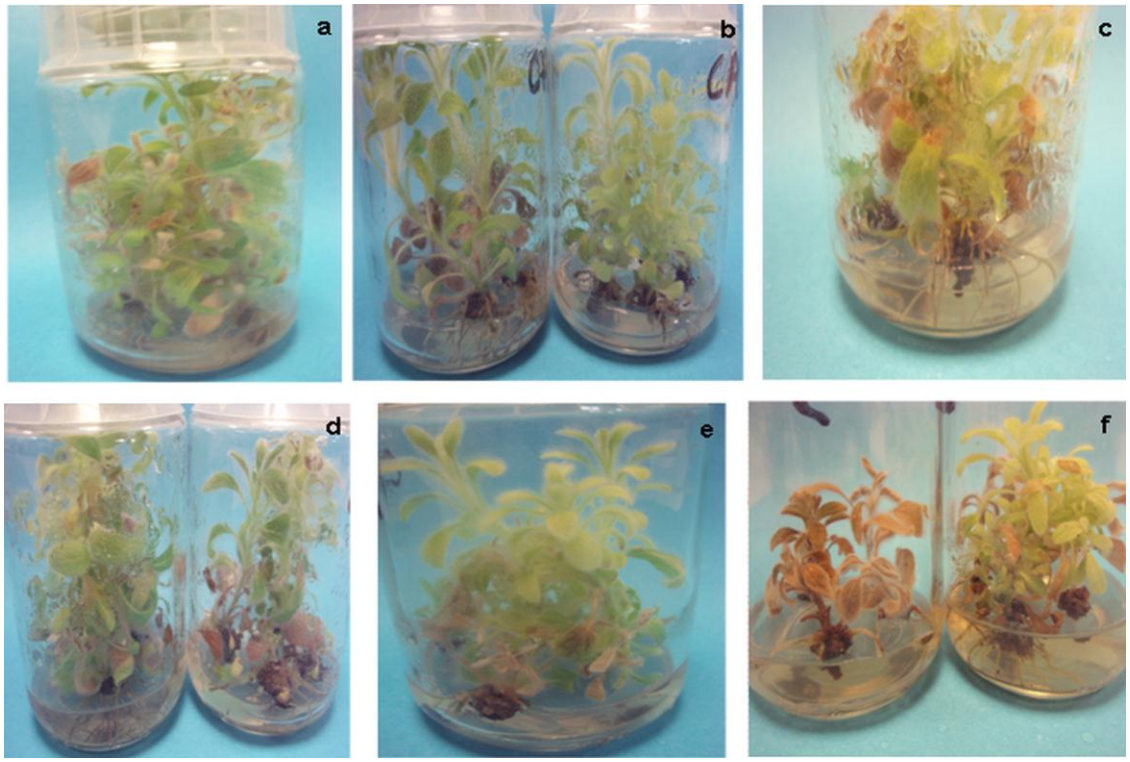

Fig 3: Effect of α-CD concentration combined with 0.5 mg/l NAA (MS medium) on micropropagation of *Sideritis raeseri* Boiss & Heldr.: (a) Control (α-CD-free), (b) 0.1 g/l α-CD, (c) 0.5 g/l α-CD, (d) 1 g/l α-CD, (e) 2.5 g/l α-CD, (f) 5 g/l α-CD

Regarding *in vitro* multiple shoot induction using β-CD, shoot elongation was not differentiated significantly due to β-CD (0.1-5 g/l) application (19.17-31.04 mm), exhibiting similar results to the control (24.14 mm) (Table 7). In relation to the control (Figure 4a), β-CD (0.1-5 g/l) did not promote shoot regeneration in terms of shoot number and shoot multiplication rate (Figure 4b-4f). On the contrary, the highest applied β-CD concentration of 5 g/l caused a 2-fold decrease in shoot number/explant, from 2.88 (control) to 1.42. Shoot proliferation percentage was significantly reduced from 75% (control) to 16.67-66.67% when the explants were treated with 0.1-5 g/l β-CD. Neither hyperhydricity nor necrotic symptoms were observed in the β-CD-untreated explants. However, β-CD (0.1-5 g/l) caused vitrification to the 16.67-41.67% of the microshoots and total browning in a 33.33-100% rate. The inclusion of β-CD at 5 g/l in conjunction with 0.5 mg/l NAA to the MS medium led to 100% necrosis and 41.67% vitrification, the highest percentages among treatments.

Regarding *in vitro* root induction using β-CD, the fortification of medium with 0.5 g/l β-CD gave the maximum root number per rooted microcutting (12.29) compared to the control (10 roots) (Table 8). Root elongation was adversely influenced due to β-CD at 0.5-5 g/l concentrations (9.21-12.06 mm) in relation to the control (15.23 mm). Compared to the β-CD-untreated microshoots where 66.67% rooting was achieved (Figure 4a), augmented rooting rate (75-100%) was exhibited by adding 0.1-2.5 g/l β-CD to the medium (Figure 4b-4e) Rooting of 100% was achieved with the simultaneous application of 1 g/l β-CD and 0.5 mg/l NAA. On the contrary, the highest applied β-CD concentration of 5 g/l caused a 2.5-3-fold decrease in root number, from 10 (control) to 3.5, and in rooting percentage from 66.67% (control) to 25% as well as a reduction in root length almost by 0.55 cm (Figure 4f). Callogenesis was apparent in all β-CD (0.1-5 g/l) treatments including that of control to the 66.67-100% of the explants.

Table 7: Effect of β-CD concentration (0-5 g/l) combined with 0.5 mg/l NAA on shoot number/explant, shoot length (mm), shoot multiplication, vitrification and necrosis percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments 0.5 mg/l NAA + β-CD (g/l)	Shoot number/explant	Shoot length (mm)	Shoot multiplication percentage (%)	Vitrification percentage (%)	Necrosis percentage (%)
0	2.88 ± 0.43 b	24.14 ± 2.07 ab	75 e	0 a	0 a
0.1	1.25 ± 0.14 a	31.04 ± 5.17 b	16.67 a	25 c	50 c
0.5	3.00 ± 0.39 b	19.17 ± 1.62 a	75 e	50 e	33.33 b
1	3.17 ± 0.70 b	22.52 ± 2.69 ab	66.67 d	41.67 d	33.33 b
2.5	2.25 ± 0.50 ab	20.89 ± 2.66 a	33.33 c	16.67 b	35.00 b
5	1.42 ± 0.18 a	23.47 ± 2.83 ab	25 b	41.67 d	100 d
P-values	0.005**	0.123 ns	0.000***	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n = 12, ns - $P \geq 0.05$, ** $P \leq 0.05$, *** $P \leq 0.01$.

Table 8: Effect of β-CD concentration (0-5 g/l) combined with 0.5 mg/l NAA on root number/rooted microcutting, root length (mm), rooting and callus induction percentages (%) in *Sideritis raeseri* Boiss & Heldr.

Treatments 0.5 mg/l NAA + β-CD (g/l)	Root number/rooted microcutting	Root length (mm)	Rooting percentage (%)	Callus induction percentage (%)
0	10.00 ± 1.37 c	15.23 ± 0.69 c	66.67 b	91.67 c
0.1	5.20 ± 0.74 ab	14.24 ± 1.33 c	75 c	91.67 c
0.5	12.29 ± 1.35 d	12.06 ± 0.62 b	75 c	75 b
1	7.90 ± 0.92 bc	9.21 ± 0.56 a	100 d	100 d
2.5	5.56 ± 0.57 ab	9.49 ± 0.77 a	75 c	66.67 a
5	3.50 ± 0.15 a	9.75 ± 0.22 a	25 a	75 b
P-values	0.000***	0.000***	0.000***	0.000***

Means ± S.E. followed by the same letters within each column are not significantly different at $P \geq 0.05$ based on Duncan's multiple range test, n = 12, *** $P \leq 0.01$.

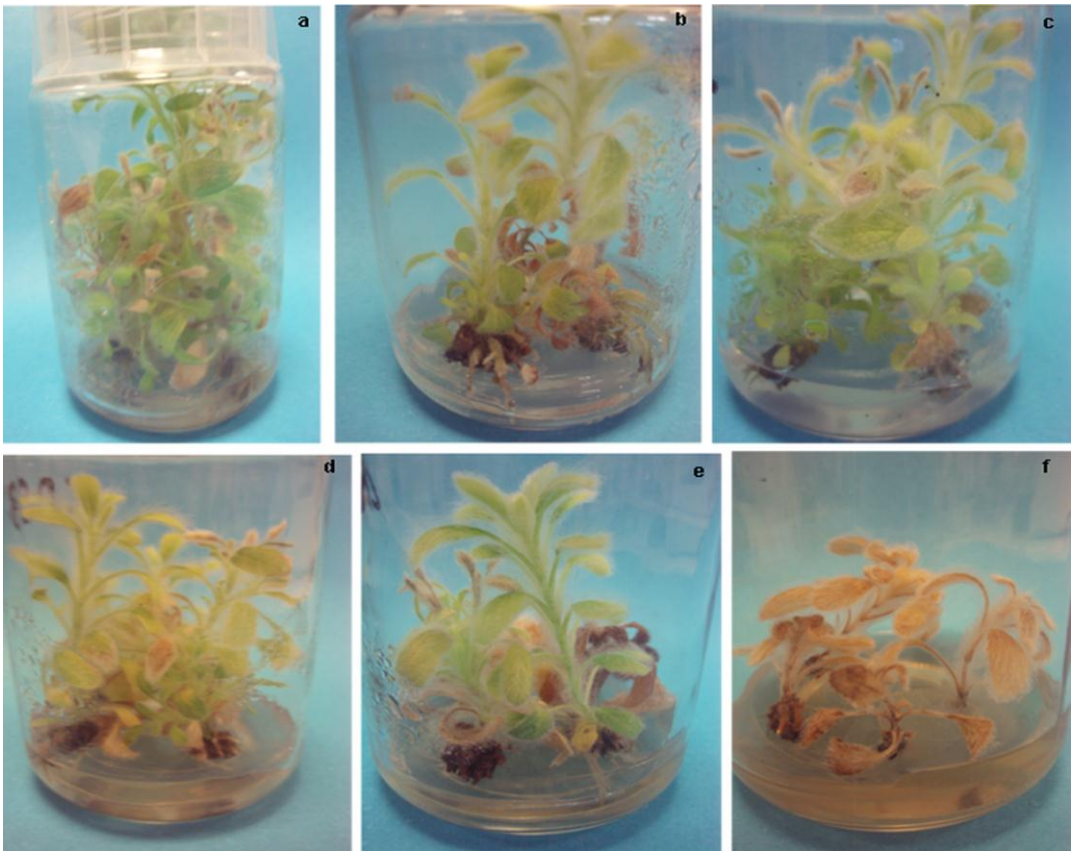

Fig 4: Effect of β-CD concentration combined with 0.5 mg/l NAA (MS medium) on micropropagation of *Sideritis raeseri* Boiss & Heldr.: (a) Control (β-CD-free), (b) 0.1 g/l β-CD, (c) 0.5 g/l β-CD, (d) 1 g/l β-CD, (e) 2.5 g/l β-CD, (f) 5 g/l β-CD

3.4 *Ex vitro* acclimatization of *in vitro* rooted plantlets

The survival percentage of the *in vitro* rooted plantlets originated from the SA-enriched containing 10.74 μM NAA MS culture medium after eight weeks from their transition to the *ex vitro* environment of the unheated greenhouse was 79%. On the other hand, 92% and 86% survival rate was recorded when the microplants were treated with β-CD and α-CD, respectively in combination with 0.5 mg/l NAA. However, the highest *ex vitro* survival percentage (96%) was exhibited from the plantlets that were rooted under *in vitro* conditions in MS culture medium fortified with 0.5 mg/l NAA + 0.25-5 g/l AC (Figure 5).

Fig 5: *Ex vitro* acclimatization and adaptation of *in vitro* rooted *S. raeseri* Boiss & Heldr. plantlets to the greenhouse bench wherein watered by sprinkling.

4. Discussion

Conventional breeding techniques of woody fruit trees are often difficult and slow due to elevated heterozygosity levels and time-consuming successful generation between subsequent intersections [19]. These inconveniences require the development of swift and effective regeneration protocols for micropropagation of elite genotypes.

In the current study employing *S. raeseri*, AC (0.25-2.5 g/l) did not differentiate the number of produced shoots considerably whereas 5 g/l AC had an inhibitory effect. Similarly, AC led to a decrement in the number of branches in the *Cymbidium forrestii* [20]. Shoot elongation of *S. raeseri* explants was significantly enhanced due to AC application at 0.5-2.5 g/l concentrations. In accordance to our findings, Faria *et al.* [21] noted that *Cattleya walkeriana in vitro* plantlets when treated with 2 g/l AC achieved the optimum vegetative growth. Our results are also in line with those presented by Hemphill *et al.* [22], who reported that shoot elongation in cotton (*G. hirsutum*) was stimulated by adding 3 g/l AC to the MS medium. Positive effects of AC on shoot elongation have also been reported in shoot cultures of *Ulmus campestris* [23] and *Picea galuca* [24].

The simultaneous use of 2.5 g/l AC and 0.5 mg/l NAA resulted in an increase in shoot proliferation percentage of *S. raeseri* by 16.67%, pointing out that there is a synergistic action between AC and NAA. On the contrary, reduced shoot formation and increased shoot elongation were observed for microshoots of cashew (*Anacardium occidentale* L.) cultured on media containing AC [25]. In addition, according to Webb *et al.* [26], AC inhibited shoot proliferation of *Pinus strobus* L. The addition of AC to both liquid and semi-solid media is a recognized practice and its influence in growth and development may be attributed mainly to the adsorption of inhibitory substances in the culture medium [27], drastic decrease in the phenolic oxidation or brown exudates accumulation [28], alteration of medium pH to an optimum level for morphogenesis [29] and establishment of a darkened environment in medium and hence simulate soil conditions [30].

Regarding rhizogenesis, a slight promotion of rooted *S. raeseri* microshoots percentage was exhibited with 0.5 g/l AC. According to Poudel *et al.* [31], AC alone or with IBA did not improve rooting frequency of *Vitis ficifolia* var. *ganebu* and its interspecific hybrid but significantly increased root length in both genotypes. In black wattle (*Acacia mearnsii*) micropropagation nodal cuttings, on the other hand, 12.5% of shoots exhibited rooting on medium containing 2 g/l AC while no rooting was observed on AC-free media [32]. In other plant species, 0.5 g/l AC enhanced *in vitro* root induction of microshoots in *Robinia pseudoacacia* [33] and *Morus alba* L. [34]. In addition, 100% rooting was reported for camphor tree (*Cinnamomum camphora)* shoots cultured *in vitro* on WPM medium supplemented with IBA + 2 g/l AC [35]. In the present study with *S. raeseri*, AC concentrations lower or higher than 2.5 g/l adversely affected the number of roots per rooted microcutting. These findings are not consistent with those reported for Carrizo citrange (*Citrus sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.), in which the addition of 12 µM AC to the rooting medium resulted in positive effects on root number [36]. According to Tang *et al.* [37], AC enhanced root development, particularly root branching in walnut plantlets. One of the recent reports on application of AC in plant tissue culture includes *Swertia chirayita* wherein 0.5 g/l AC had been employed along with NAA for rooting [38]. In *Fortunella crassifolia*, maximum rooting was achieved on MS medium supplemented with NAA, kinetin (Kn) and 0.5 g/l AC [39]. BitHua [40] showed that rooting of *Spathiphyllum xiangshui* was best on a ½ MS medium containing 0.5 mg/l IBA + 0.5 g/l AC. These studies suggested that AC could promote rooting by adsorbing rooting inhibitors as it provides a darkened environment at the shoot base simulating soil conditions and allowing accumulation of photosensitive auxins [5].

In *S. raeseri* shoot tip explants, AC (0.25-5 g/l) positively influenced root elongation. The potentiality of AC on root elongation has also been reported by several other workers [41]. The positive effect of AC on root growth had been shown by Sarma and Rogers [42] on their studies on *Juncus effusus* L. According to Nissen and Sutter [43], AC eliminates light and provides a reasonable physical environment for the rhizosphere, preventing undesirable callusing and helping rooting.

AC (0.25-5 g/l) led to complete inhibition of callus induction in the base of *S. raeseri* microshoots. Previous studies reported that the use of AC either alone or in combination with auxin significantly reduced the callus formation of *Simmondsia chinensis* explants [44]. Similarly, black wattle (*Acacia mearnsii*) micropropagation nodal cuttings when cultured on modified MS medium supplemented with 2 g/l AC gave rise to a lower rate of callus induction (27%) than the media which did not contain AC [32].

In *S. raeseri* shoot tips grown *in vitro*, SA (1-100 µM) neither favour nor inhibited shoot proliferation regarding shoot number and shoot multiplication percentage. Different results were obtained in maize (*Zea mays* L.), where 10,000 µM SA slightly increased shoot length of the seedlings under non-salt stressed conditions [45]. Similarly, according to Bideshki *et al.* [46], SA (100 and 500 µM) augmented shoot length of garlic (*Allium sativum* L.) plants. In contrast to our findings for *S. raeseri*, in banana (*Musa acuminata* cv. 'Berangan', AAA) *vitro* culture, 2000 µM SA positively influenced shoot proliferation in terms of shoot number/explant [47]. Our records in the present study are also not in line with those presented in Safed Musli *(Chlorophytum borivilianum* Sant. Et Fernand.) *vitro* shoot cultures, where 5-100 mg/l SA led to a significant decrease in shoot number and shoot length [48].

The *S. raeseri* explants ability to produce multiple shoots was positively stimulated (20% increase) by adding 25 or 100 µM SA to the 10.74 µM NAA MS containing medium. Similarly, the exogenous application of 500 µM SA had a beneficial effect on shoot multiplication in both *Hibiscus* species, *H. moscheutos* (cv 'Luna Red') and *H. acetosella* under non-saline conditions [10]. SA is a hormone-like substance that has been reported to enhance *in vitro* regeneration in several plant species, including *Coffea arabica* [49], *Astragulus adsurgens* [50] and *Avena nuda* [51]. The positive effect of SA on the proliferation of plant tissue cultures could be a reflection of an increase in the number of meristematic cells [10]. SA stimulates the organogenesis and embryogenesis by regulation the cell division, enlargement and/or activating DNA replication

without concomitant nuclear division [50].

In terms of root regeneration, root number was increased by applying 5 μM SA and root length in the presence of 25 μM SA to the medium. Similarly, the application of 4000 mg/l NAA + 200 mg/l SA caused a substantial increase in root number and root length in Henna (*Lawsonia inermis* L.) cuttings [52]. According to Li and Li [53], 400 μM SA treatment of rape (*Brassica napus* L.) seedlings enhanced lateral root formation significantly, in which the number of lateral roots was increased by 47.8% compared with the control. Consistent results to ours were also obtained by Khodary [45] in maize (*Z. mays* L.), where 10,000 μM SA slightly increased root length of the seedlings under non-saline conditions. According to Bideshki *et al.* [46], SA (100 and 500 μM) increased root number of garlic (*Allium sativum* L.) plants. Our findings are also in line with those presented by Sakhanokho and Kelley [10], who found that 500 μM SA were beneficial for root elongation of *H. moscheutos* (cv 'Luna Red') and *H. acetosella* species.

Negative was the effect of SA (1-100 μM) on rooting percentage of *S. raeseri* microshoots. In contrast, root formation of 2 *Hibiscus* species were promoted due to SA exogenous application [10]. On the other hand, SA found to be inhibitory on *in vitro* rooting of stem discs of apple when applied before auxin [54]. This effect was attributed to enhanced oxidation of indole-3-acetic acid (IAA) during the auxin sensitive phase by SA[12]. Different results were recorded for poinsettia (*Euphorbia pulcherrima*) cuttings, where 100-300 mg/l SA increased rooting percentage whereas 100-400 mg/l SA did not differentiate root number in a considerable degree but remarkably inhibited root elongation [55]. SA (1-100 μM) treated *S. raeseri* explants exhibited a 10-20% increase in callogenesis percentage compared to the control. This finding is in disagreement with that reported for poinsettia (*Euphorbia pulcherrima*) cuttings, in which 100-300 mg/l SA reduced callus percentage [55].

Root length of *S. raeseri* microshoots was not affected significantly by fortifying the medium with α-CD (0.1-5 g/l). Different results were exhibited in jojoba microshoots where the combination treatments 0.1 mM α-CD + IBA and 0.03 mM b-CD + IBA had an enhancing effect on root length [56]. Greater root number and more rooted *S. raeseri* explants were obtained with the inclusion of 1 g/l α-CD to the 0.5 mg/l NAA-enriched MS medium. Accordingly, a 2-fold increase in root number and a 3-fold one regarding rooting percentage were recorded when *Cynara scolymus* cv. Early French *vitro* plants were treated with CD and NAA whereas no alteration was evident in devoid of the NAA auxin [57]. In consistency to our results, the addition of 0.1 mM a-CD and IBA to the nutritional substrate gave the highest root number in jojoba (*Simmondsia chinensis* Link Schn.) *vitro* shoot explants [56]. Mura *et al.* [15] justified the augmented rooting of olive tree cuttings to the formation of complexes between IBA and CD leading to increased and better solubility of IBA. In *S. raeseri* explants, 100% rooting was recorded with the presence of 0.5 g/l α-CD in the medium. According to Apóstolo *et al.* [56], 100% rooting from jojoba (*S. chinensis* Link Schn.) *vitro* shoot cultures was obtained by adding 0.03-0.5 mM α-CD to the 0.015 mM IBA containing MS medium. The promoting influence of CDs on rooting of jojoba shoots [56] may be ascribed to the involvement of CDs in the increase of permeability within cell membranes [58].

In general, 0.1-2.5 g/l β-CD exerted positive results regarding rooting percentage of *S. raeseri* and 100% rooting was achieved in the combined effect 1 g/l β-CD + 0.5 mg/l NAA. In jojoba *vitro* culture (*S. chinensis* Link Schn.), 100% rooting was performed under the influence of 0.03-0.5 mM β-CD [56]. Similar results were also recorded in globe artichoke spring cultivar 'Romanesco C3', where substantially higher rooting percentages were observed in the NAA + β-CD (83%) treatments in comparison to the 65% obtained in the presence of NAA alone [59]. In a previous study conducted by Cavallaro *et al.* [60] in early artichoke [*Cynara cardunculus* L. subsp. *scolymus* (L.) Hegi] cv. Violet Margot, the combined effect 2 mg/l NAA + 2 g/l β-CD gave the greatest percentage of rooted plantlets (62%) and adequate root quality. On the contrary, in *S. raeseri*, AC (0.5-5 g/l) led to a decrease in root length, whereas root number was augmented in the 0.5 g/l β-CD + 0.5 mg/l NAA combination treatment. According to Arbaoui *et al.* [61], in kenaf (*Hibiscus cannabinus* L.) rooting was strongly favoured by fortifying the MS medium with 0.25 mg/l IBA + 25 mg/l β-CD, in which 9.22 roots/vitroplant and 1.9 cm root length was recorded. In accordance with our findings in *S. raeseri* shoot tip explants, in *Nolina recurvata* Hemsl., rooting percentage and root number were highest in the 5 μM IBA + 1.76 mM of β-CD (MS medium) combination treatment [62].

5. Conclusions

S. raeseri shoot tip explants treated with 2.5 g/l AC + 0.5 mg/l NAA responded adequately and efficiently by exhibiting the most promising results regarding all shoot proliferation and rooting macroscopic attributes including alleviation of callogenesis, vitrification and necrosis phenomena. The 2.5 g/l α-CD + 0.5 mg/l NAA combination treatment enhanced *in vitro* multiple shoot induction in a considerable degree whereas both 0.5-1 g/l α- & β-CD substantially promoted rooting. However, β-CD did not improve further shoot regeneration. *S. raeseri* microshoots' ability to proliferate was adversely influenced by exogenous β-CD. SA, on the other hand did not stimulate micropropagation of the Hellenic mountain tea of Velouchi or Parnassus. To our knowledge, no reports exist in the literature regarding the effect of CDs on shoot proliferation in plant tissue cultures. There are only some limited published works about the impact of CDs but on the *in vitro* rooting process and in other plant species. Our results indicate the usefulness of both AC and CDs in the micropropagation of *S. raeseri* Boiss & Heldr. and presumably in other species with rooting difficulties.

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