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(RESEARCH ARTICLE)

In vitro propagation of *Satureja thymbra* L. (Lamiaceae): A valuable aromatic-medicinal native plant of the Mediterranean region

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Abstract

Satureja thymbra L. or savory is a highly threatened aromatic-medicinal endemic Mediterranean plant. Micropropagation can play a role in the protection of the natural ecosystem. The disinfection protocol involved the immersion of shoot-tip explants in 70% alcohol (1 min) and 3% NaOCl (15 min) giving 61.18% success. The MS medium supplemented with 30 g/l sucrose and 7 g/l Plant Agar was optimum for the initial establishment stage (10-day culture, 92.31% proliferation) and afterwards supplemented extra with 0.5 mg/l BA for multiple shoot induction (5 weeks) (100% proliferation, 5.5 shoots 14.24 mm long). In rooting stage, 1 mg/l IBA gave the highest rooting (58.62%), 0.5 mg/l NAA the maximum root number (12.13) and 0.1 mg/l IAA the largest root length (34.35 mm) (MS medium + 20 g/l sucrose + 6.5 g/l Plant Agar, 7 weeks) (Experiment No. 1). There was a progressive increase in rooting and shoot proliferation increasing the number of subcultures, being highest (100% rooting, 6 roots 70 mm long, 38.1% shoot multiplication, 2 shoots 30 mm long) in the 4th subculture/ 4.5 weeks each in WPM medium (Experiment No. 2). Therefore, best rooting results (50-100%) were accomplished in 3 different composition media: (1 and 2) WPM medium + 20 g/l sucrose + 6 g/l Plant Agar (1st and 4th subculture) and (3) MS medium + 30 g/l sucrose + 1 mg/l IBA + 7 g/l Plant Agar. The *ex vitro* survival of rooted microshoots after 4 weeks in the greenhouse mist (1 peat moss: 1 perlite) was 81.82%.

Keywords: *Ex situ* conservation; Mediterranean endemic; Micropropagation; Plant growth regulators; *Satureja thymbra*; Tissue culture media

1. Introduction

The aromatic plants are usually collected on their natural stands and used as spices in traditional food and cosmetics, in phytotherapy and aromatherapy. This practice harms the natural habitat, so the development of efficient protocols for rapid clonal propagation and the conservation of germplasm of selected species typical of these islands are badly needed. *In vitro* culture of aromatic and medicinal plants is a useful technique to produce rapidly and in small spaces a large amount of plant material, avoiding the damage and the extinction of natural field grown plants [1]. Moreover, micropropagation represents a valid alternative for rapid clonal propagation and an improvement of the production and marketing of the selected plants. The micropropagation protocols are based on regeneration of plants starting from different organs [2]. The *in vitro* culture technique can be also useful for the production of active compounds naturally present in aromatic plants and in particular enhancing the levels of some metabolites [3].

In vitro conservation has proven to be an alternative tool for the rapid and efficient multiplication of rare and endangered medicinal and aromatic plant genotypes and in the present context, it can provide a continuous supply of

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plant material from threatened germplasm species [4, 5, 6]. To date, there has been an intensified effort to propagate and conserve medicinal plant species by *in vitro* culture techniques from various explant sources [7, 8].

Satureja is an important genus of the Lamiaceae family, which includes 30 species, primarily distributed in the Mediterranean basin [9]. *Satureja* species have economic and medicinal importance because of their high essential oil content. With their pleasant fragrance, they are widely used as herbal teas and spices [10]. Essential oils obtained from the leaves and flowers of different *Satureja* species are commonly used in various industrial applications as flavoring material, perfume and medicine [11].

S. thymbra is an erect much branched, aromatic shrub, 30-50 cm high; old branches covered with flaking, fissured, fuscous-brown bark, young shoots rather indistinctly tetragonal, gland-dotted, pubescent with short down curved white hairs; leaves sessile, generally subtending condensed growth with clustered, reduced leaves, inflorescence an elongate, terminal verticillaster consisting of 3-6 (or more) equally spaced, dense, compact whorls, bracts lanceolate, acuminate about 5 mm long and 2 mm wide; flowers sessile or sub-sessile; calyx narrowly campanulate, thinly hispidulose, pubescent and densely glands dotted externally, glabrous internally, stamens down curved, strongly didynamous, exserted; anthers reniform [12]. This shrub is very common on rocky limestone gully, in garigue; flowering nearly the whole year [12, 13]. *S. thymbra* L. is used as a condiment and in traditional medicine, as herbal tea, owing to its stimulating carminative, antirheumatic, antispasmodic and antibacterial effects to treat various ailments such as nausea, indigestion, cramps, muscle pains, diarrhea and infection diseases [14, 15]. Several reports on the essential oils (EOs) composition of *S. thymbra* from different geographic origin (Greece, Crete, etc.) in the Mediterranean area, state that this species is also rich in carvacrol or thymol or a mixture of both, with ρ-cymene and gamma-terpinene as the other major compounds [16, 17].

S. thymbra L. (Lamiaceae) or savory is a native Mediterranean chamaephyte found in xeric phrygana and grasslands, limestone rocks and stony slopes. It is a bee-keeping and aromatic plant used as a powerful aphrodisiac herb with anti-microbial action, as a delicious flavor and natural preservative with its essential oil being exploited by the beverage industry. It is found in North-East and East-Central Greece, Ionian and Aegean islands, Cyclades, Peloponnese, Crete, Karpathos, South Pindos and Sterea Ellada [18, 19].

Regarding the *in vitro* propagation of related taxa, Arrebola et al. [20] reported shoot multiplication of *S. obavata* from axillary buds. In a very recent study, indirect organogenesis from callus was developed using hypocotyls explants for *S. hortensis* and *S. avoromanica* [21] however, this report is also insufficient to meet the need in time. The present article establishes a platform to achieve high direct organogenesis of *S. thymbra* through shoot multiplication and rooting by testing different basal culture media (MS and WPM), cytokinin BA, different auxin types (IBA, NAA, IAA) and auxin concentrations individually and in combinations as well as the number of successive subcultures in the same basal culture medium.

This species is continuously harvested from its natural habitats. Problems related with its excessive utilization, overgrazing, disruption of the habitat and harvesting before seed set resulted in rare distribution of this valuable medicinal and aromatic plant to use it as cure as well as for industrial purposes. In vitro propagation is very important for germplasm conservation, produces an abundant number of clonal propagules and improves drug-yielding capacity of the plant. However, there is no report on conventional cultivation or *in vitro* propagation of this taxon. Therefore, the main goal of the present study was to develop a holistic approach for *in vitro* multiplication and conservation of *S. thymbra* L. as a potential medicinal plant to obviate the dependence on the natural population for the supply of raw materials. In addition, individual and interactive effects of different auxin and cytokinins in the course of direct regeneration were studied.

2. Material and methods

2.1. Disinfection and initial establishment of plant material

Initially, on 10th March 2017, n= 170 very juvenile shoot tip explants 1.5 mm long were washed with tap water for 5 min. Then surface sterilization was performed by soaking explants in 70 % ethanol solution for 1 min and immediate immersion in 3% sodium hypochloride (NaOCl) solution for 15 min. After that, the explants were washed thrice with sterile double-distilled water and aseptically implanted on the culture media. The MS [20] culture medium was used as the basal nutrient medium of the initial establishment. To provide carbon source, 30 gl^{-1} sucrose was used. Before the addition of plant agar (7 g/l) and autoclaving, the pH of prepared medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl. Afterwards, the medium was sterilized in an autoclave at 121 ^{o}C for 20 min. The explants were placed in 25 mm (width) x 100 mm (height) borosilicate glass test tubes with a flat base (one explant per tube). The cultures were then

incubated at 22±1 °C under a 16-h photoperiod at an irradiance intensity of 40 μmolm^{-2}s^{-1}. The percentage of disinfected and infected (fungi, bacteria) explants, the percentage of explants with multiple shoot production and rooting percentage were recorded at the end of the 10-day culture period.

2.2. Shoot proliferation stage

After 6 weeks from disinfection, initial establishment and culture of shoot tip explants in MS medium free from plant growth regulators (PGRs), the proliferated shoots were divided and transferred into the MS medium supplemented with 30 g/l sucrose, 0.5 mg/l benzyladenine (BA) and solidified with 7 g/l Plant Agar (pH=5.8). The medium was sterilized in an autoclave at 121 ^{o}C for 20 min. The explants were placed in 25 mm (width) x 100 mm (height) borosilicate glass test tubes with a flat base (one explant per tube). The cultures were incubated at 22±1 °C under a 16-h photoperiod at an irradiance intensity of 40 μmolm^{-2}s^{-1}. After 5 weeks of culture, shoot number/ explant, mean shoot length (mm), shoot multiplication and rooting percentages (%) were measured.

2.3. Rooting stage

In the first experiment, the effect of 3 different auxins; indole-3-butyric acid (IBA), α-naphthalene acetic acid (α-NAA) and indole-3-acetic acid (IAA) exogenously applied either individually or in combination on rooting ability of shoot-tip explants was studied. IBA was used alone at 3 concentrations (0.25, 0.5 and 1 mg/l) and 0.25 mg/l IBA with the other 2 auxins (NAA, IAA) in 2 different concentrations (0.25 and 0.5 mg/l) (i.e. 0.25 IBA + 0.25 NAA, 0.25 IBA + 0.25 IAA, 0.25 IBA + 0.5 NAA, 0.25 IBA + 0.5 IAA). NAA was used at 4 concentrations (0.1, 0.25, 0.5 and 1 mg/l) alone and 0.25 mg/l NAA in combination with 2 IAA concentrations (0.25 and 0.5 mg/l) (i.e. 0.25 NAA + 0.25 IAA, 0.25 NAA + 0.5 IAA). IAA was used at 4 concentrations (0.1, 0.25, 0.5 and 1 mg/l) alone, 0.25 and 0.5 mg/l IAA with either 0.25 mg/l IBA or 0.25 mg/l NAA (i.e. 0.25 IAA + 0.25 IBA, 0.25 IAA + 0.25 NAA, 0.5 IAA + 0.25 IBA, 0.5 IAA + 0.25 NAA). The basal culture medium used was the MS supplemented with 20 g/l sucrose and 6.5 g/l Plant Agar (pH=5.8). Afterwards, the medium was sterilized in an autoclave at 121 ^{o}C for 20 min. The explants were placed in 25 mm (width) x 100 mm (height) borosilicate glass test tubes with a flat base (one explant per tube). The cultures were then incubated at 22±1 °C under a 16-h photoperiod at an irradiance intensity of 40 μmolm^{-2}s^{-1}. After 7 weeks of culture, root number/ rooted explant, root length (mm), rooting percentage (%), callusing percentage (%), shoot number/ explant, shoot length (mm), shoot multiplication and necrosis percentages (%) were measured.

Due to the fact that the highest rooting percentage obtained from the first experiment with MS basal culture medium was not adequate (50%), a second experiment with a different basal culture medium; Woody Plant Medium (WPM) (Lloyd and McCown1981) [23] was designed. In the second experiment, the number of subcultures (4 successive cultures) of shoot-tip explants in WPM medium of the same composition each time was studied. The WPM medium was supplemented with 20 g/l sucrose as a carbon source and solidified with 6 g/l Plant Agar as a gelling agent (pH=5.8) and was PGRs-free. Afterwards, the medium was sterilized in an autoclave at 121 ^{o}C for 20 min. The cultures were then incubated at 22±1 °C under a 16-h photoperiod at an irradiance intensity of 40 μmolm^{-2}s^{-1}. After 4.5 weeks of culture, root number/ rooted explant, root length (mm), rooting percentage (%), shoot number/ explant, shoot length (mm) and shoot multiplication percentage (%) were measured.

2.4. *Ex vitro* acclimatization of rooted microshoots

On late-spring (31/05/2017), to eliminate all traces of sucrose and culture medium, well rooted plantlets n=22 in total, derived only from the first experiment with MS basal medium fortified with auxins, were washed thoroughly with sterile water, transplanted into plastic pots containing peat moss (Terrahum): perlite in a ratio of 1:1, and then placed in the internal mist system of an unheated greenhouse for maintaining humidity and gradual hardening to ex vitro conditions. After a 4-week period of maintenance in the mist system (28/06/2017), the survival rate was recorded and acclimatized plantlets were transplanted into 0.33Lt (8x8x7 cm) larger volume pots containing a more enriched in organic matter peat moss (TS2, Classmann): perlite (Perflor): soil mixture in a 2: ½: ½ v/v ratio, respectfully. Afterwards, well irrigated plants using a sprinkling system were transferred to the greenhouse bench and kept under the nursery conditions for 2 more months for further growth. On early-autumn (1/9/2017), the final survival rate was recorded and acclimatized plantlets were transplanted into larger volume pots, 2.5Lt containing a peat moss (TS2): perlite: soil mixture (2:1:1 v/v ratio) and transferred outside greenhouse in the external nature environment under sprinkling irrigation system and shading net conditions.

2.5. Statistical analysis

The first experiment (MS basal medium supplemented with auxins) 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 ± standard error (S.E.) in order significant differences among the treatments

to be established. The main effect of auxin type, auxin concentration and their interaction was performed according to 2-way ANOVA and General Linear Model. The first experiment consisted of 17 treatments with 29 explants (replications) per treatment. The second experiment (WPM basal medium, 4 successive subcultures) consisted of 4 treatments with 4 replicates (Magenta vessels/Baby food jars of 200 ml volume)/ treatment and each replicate with 10 replications/ treatment, giving 40 explants in total/ treatment.

3. Results

After a period of 10 days, 61.18% disinfection success was achieved i.e. 104 out of the 170 in total explants were fungi- and bacteria-free. Accordingly, the 38.82% of explants was infected (11.18% by fungi and 27.06% by bacteria). The shoot multiplication percentage was high, 92.31% (96 out of 104 explants were proliferated) and only the 4.81% of explants was rooted (5 out of 104) (Figure 1a-1i).

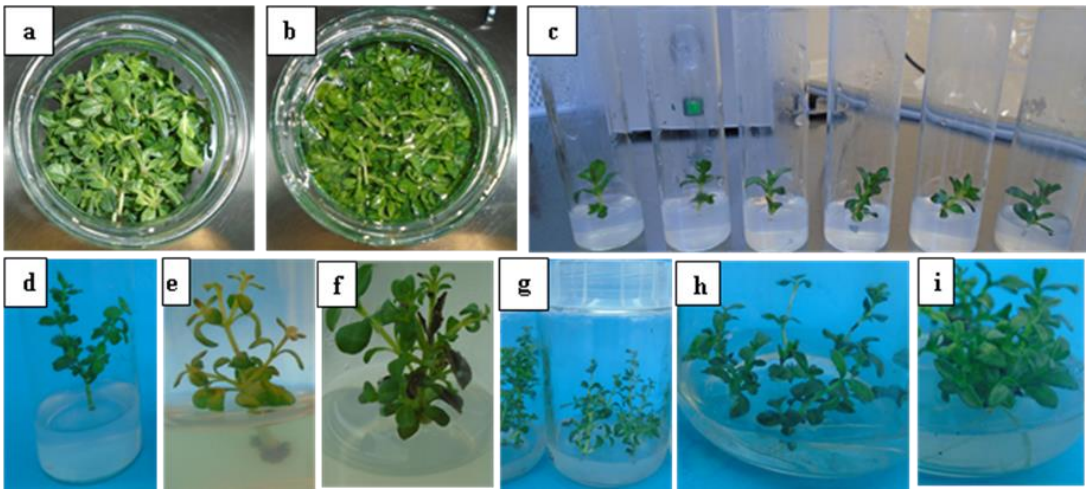

Figure 1 Disinfection process, initial establishment *in vitro*, shoot proliferation and rooting of *Satureja thymbra* L. explants cultured in MS medium supplemented with 30 g/l sucrose and 7 g/l Plant Agar (pH=5.8): (a) explants prior to decontamination, (b) explants after their disinfection, (c) initial transfer of disinfected explants in test tubes, (d, e, f) multiple shoot production after 2, 4 and 6 weeks of culture, respectfully, (g) vegetative growth of explants in larger volume vessels after 4-weeks, and (h, i) shoot proliferation and rooting after another 4 weeks

After 5 weeks in the shoot proliferation medium containing BA, 5.5 shoots/ explant, 14.24 mm long and 100% shoot multiplication percentage were obtained. No root formation was observed (Figure 2a-2d)

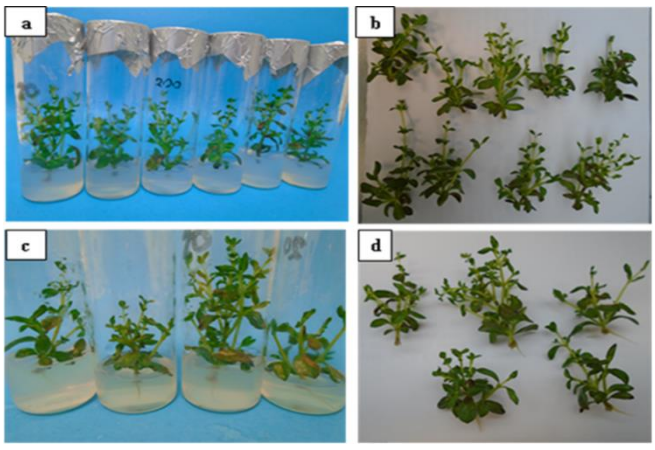

Figure 2 Shoot proliferation and rooting *in vitro* of *Satureja thymbra* L. explants cultured in MS medium supplemented with 30 g/l sucrose, 0.5 mg/l BA and 7 g/l Plant Agar (pH=5.8): (a, b) explants with only multiple shoot formation (5 weeks), (c, d) explants with a simultaneous shoot proliferation and rooting (5 weeks).

In rooting stage, specifically in the first experiment with auxins and MS as the basal nutrient culture medium, rooting percentage 100% was obtained in the presence of 1 mg/l IBA to the culture medium after a 7-week period. Root number was maximum (12.13 roots/ rooted explant) with 0.5 mg/l NAA and root length greater (34.35 mm) with 0.1 mg/l IAA. The individual application of all 3 auxins gave higher rooting percentages (20-58.62%) than the combined effect of 2 each time auxin types (12.5-50%). Taking simultaneously into consideration all macroscopic rooting attributes, IBA proved to be the most effective auxin type and 1 mg/l the optimum auxin concentration for the root formation stage (58.62% rooting, 6.35 roots/ rooted explant, 13.43 mm root length) (Table 1, Figure 3a-3q).

Table 1 Effect of auxin type (IBA, NAA, IAA) and auxin concentration (auxins applied individually and in different combinations) on root number/ rooted explant, root length (mm), rooting and callusing percentages (%) of *Satureja thymbra* L. after 7 weeks of culture in MS medium supplemented with 20 g/l sucrose and 6.5 g/l Plant Agar (pH: 5.8). The main effects of Auxin type (A), Auxin Concentration (B) and their interaction (A)*(B) were determined according to General Linear Model (2-way ANOVA).

Treatments (mg/l)	Root number/ rooted explant	Root length (mm)	Rooting (%)	Callusing (%)
0.25 IBA	5.80 ± 0.38efg	9.69 ± 0.39 a	31.23 d	15.38 d
0.5 IBA	4.67 ± 0.23cde	13.17 ± 0.14 bc	20.00 b	33.33 h
1 IBA	6.35 ± 1.49 fg	13.43 ± 1.04 bc	58.62 j	17.24 e
0.1 NAA	2.00 ± 0.05 a	9.83 ± 0.27 a	35.71 ef	15.38 d
0.25 NAA	4.43 ± 0.42 bcde	8.51 ± 0.30 a	36.84 f	36.84 i
0.5 NAA	12.13 ± 1.02i	13.30 ± 0.29 bc	41.06 g	57.89 k
1 NAA	10.00 ± 0.54 h	10.06 ± 0.01 a	33.33 de	41.67 j
0.1 IAA	3.60 ± 0.27abcd	34.35 ± 1.72 e	41.67 g	8.33 c
0.25 IAA	3.00 ± 0.15abc	12.20 ± 0.55 b	31.58 d	0 a
0.5 IAA	4.00 ± 0.23bcd	19.86 ± 0.92 d	37.50 f	0 a
1 IAA	4.00 ± 0.21bcd	14.81 ± 0.63 c	53.33 i	6.67 b
0.25 IBA + 0.25 NAA	4.00 ± 0.24bcd	12.30 ± 0.31 b	43.75 g	31.25 f
0.25 IBA + 0.25 IAA	4.88 ± 0.38def	18.39 ± 0.49 d	50.00 h	6.25 b
0.25 NAA + 0.25 IAA	6.75 ± 0.58 g	13.24 ± 0.08 bc	18.75 b	31.25 f
0.25 IBA + 0.5 NAA	2.75 ± 0.13 ab	9.38 ± 0.15 a	25.00 c	6.25 b
0.25 IBA + 0.5 IAA	2.00 ± 0.05 a	20.00 ± 0.66 d	25.00 c	8.33 c
0.25 NAA + 0.5 IAA	7.00 ± 0.10 g	8.39 ± 0.03 a	12.50 a	0 a
P-values (2-way ANOVA)				
Auxin type (A)	0.000***	0.000***	0.000***	0.000***
Auxin Concentration (B)	0.000***	0.000***	0.000***	0.000***
(A) * (B)	0.000***	0.000***	0.000***	0.000***

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

The fortification of the culture medium with 1 mg/l IBA, 0.1 mg/l NAA, and 0.25 mg/l IBA + 0.25 mg/l IAA exhibited 1.53-1.94 shoots per explant with non-statistically significant differences among them. Explants treated with 0.25 mg/l IAA gave shoots 36.05 mm in length. The addition of 0.1 mg/l NAA in the medium led to 50% shoot multiplication percentage (Table 2). Auxins, applied alone and in combinations resulted in shoot proliferation (0-50%), however, this percentage was not considered satisfactory for this stage. IAA proved to be the most appropriate auxin type and 0.25 mg/l the ideal auxin concentration only for the elongation of the produced multiple shoots (phase II of shoot proliferation stage following the initial shoot induction one) (Table 2, Figure 3a-3q).

Table 2 Effect of auxin type (IBA, NAA, IAA) and auxin concentration (auxins applied individually and in different combinations) on shoot number/ explant, shoot length (mm), shoot multiplication and necrosis percentages (%) of *Satureja thymbra* L. after 7 weeks of culture in MS medium supplemented with 20 g/l sucrose and 6.5 g/l Plant Agar (pH: 5.8). The main effects of Auxin type (A), Auxin Concentration (B) and their interaction (A)*(B) were determined according to General Linear Model (2-way ANOVA).

Treatments (mg/l)	**Shoot number / explant**	**Shoot length (mm)**	**Shoot multiplication (%)**	**Necrosis (%)**
0.25 IBA	1.46 ± 0.12bcde	24.17 ± 1.31def	30.54 gh	7.69 c
0.5 IBA	1.53 ± 0.13 cde	22.06 ± 0.97bcde	21.34 de	6.67 bc
1 IBA	1.72 ± 0.20 def	19.90 ± 1.43bcd	34.48 h	0 a
0.1 NAA	1.79 ± 0.11 ef	22.30 ± 1.02bcde	50 i	0 a
0.25 NAA	1.00 ± 0.00 a	26.32 ± 1.51efg	0 a	0 a
0.5 NAA	1.47 ± 0.19bcde	28.79 ± 2.56 g	12.9 c	0 a
1 NAA	1.42 ± 0.09abcde	23.82 ± 1.20cdef	25 ef	0 a
0.1 IAA	1.25 ± 0.07abc	28.40 ± 1.51 g	16.67 cd	0 a
0.25 IAA	1.05 ± 0.03 ab	36.05 ± 1.66 h	2.63 ab	0 a
0.5 IAA	1.13 ± 0.07abc	20.52 ± 1.26bcd	6.25 b	0 a
1 IAA	1.40 ± 0.10abcde	27.89 ± 1.80fg	26.67 fg	0 a
0.25 IBA + 0.25 NAA	1.38 ± 0.10abcde	23.33 ± 1.18cde	25 ef	25 e
0.25 IBA + 0.25 IAA	1.94 ± 0.35 f	23.36 ± 0.90cde	18.75 d	6.25 b
0.25 NAA + 0.25 IAA	1.31 ± 0.10abcd	19.42 ± 0.89bc	18.75 d	12.5 d
0.25 IBA + 0.5 NAA	1.13 ± 0.05abc	18.59 ± 0.99 ab	12.5 c	25 e
0.25 IBA + 0.5 IAA	1.25 ± 0.07abc	19.86 ± 0.81bcd	16.67 cd	0 a
0.25 NAA + 0.5 IAA	1.19 ± 0.07abc	15.21 ± 0.58 a	12.5 c	62.5 f
P-values (2-way ANOVA)				
Auxin type (A)	0.033*	0.001***	0.000***	0.000***
Auxin Concentration (B)	0.010**	0.000***	0.000***	0.000***
(A)* (B)	0.000***	0.000***	0.000***	0.000***

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

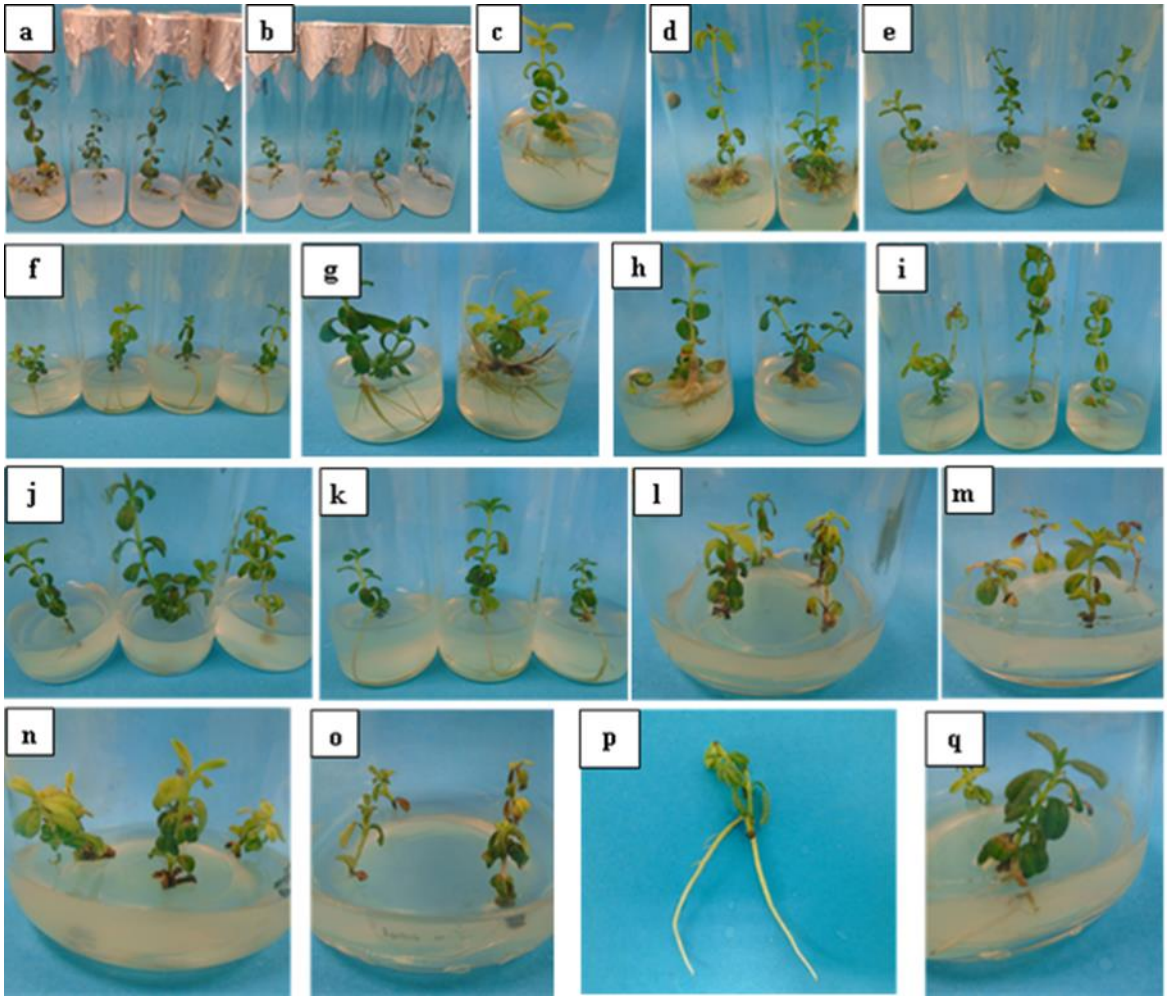

Figure 3 Effect of auxin type (IBA, NAA, IAA) and auxin concentration (mg/l) on *in vitro* rooting and/ or shoot proliferation of *Satureja thymbra* L. shoot-tip explants after 7 weeks of culture in MS medium supplemented with 20 g/l sucrose and 6.5 g/l Plant Agar (pH=5.8): (a) 0.25 IBA, (b) 0.5 IBA, (c) 1 IBA, (d) 0.1 NAA, (e) 0.25 NAA, (f) 0.5 NAA, (g) 1 NAA, (h) 0.1 IAA, (i) 0.25 IAA, (j) 0.5 IAA, (k) 1 IAA, (l) 0.25 IBA + 0.25 NAA, (m) 0.25 IBA + 0.25 IAA, (n) 0.25 NAA + 0.25 IAA, (o) 0.25 IBA + 0.5 NAA, (p) 0.25 IBA + 0.25 IAA, and (q) 0.25 NAA + 0.25 IAA.

In rooting stage, particularly in the second experiment, WPM was the basal nutrient culture medium used. There was a progressive increase in all macroscopic rooting and shoot proliferation attributes (root number, root length, rooting percentage, shoot number, shoot length, shoot multiplication percentage) simultaneously with the increase in the number of successive cultures (1st, 2nd, 3rd and 4thsubculture). From the 1st to the 4th culture (4.5 weeks / each subculture), shoot number was raised from 1.28 to 2 (x 1.6 times increase), shoot length was greater by 1.25 cm (from 17.54 mm to 30 mm, x 1.71 times increase) and shoot multiplication percentage was augmented from 14.71 to 38.1% (x 2.59 times increase). With respect to rooting parameters, root number was raised from 3.5 to 6 (x 1.72 times increase), root length was enhanced by 5.19 cm (from 18.09 to 70 mm, x 3.86 times increase) and rooting percentage was doubled (from 50 to 100%). All rooting and shoot proliferation attributes were highest (100% rooting with 6 roots/ rooted explant 70 mm long and 38.1% shoot multiplication with 2 shoots/ explant 30 mm in length) in the end of the 4th culture of explants (after 4 successive sub-cultures of 4.5 weeks each) in the WPM medium (Table 3, Figure 4a-4d).

Table 3 Effect of the number of cultures (4 successive sub-cultures) on root number/ rooted explant, root length (mm), rooting percentage (%), shoot number/ explant, shoot length (mm) and shoot multiplication percentage (%) of *Satureja thymbra* L. after a 4.5-week period/ each culture in WPM medium supplemented with 20 g/l sucrose and 6 g/l Plant Agar (pH: 5.8).

Number of culture	Root number / rooted explant	Root length (mm)	Rooting (%)	Shoot number / explant	Shoot length (mm)	Shoot multiplication (%)
1st	3.50 a	18.09 a	50 a	1.28 a	17.54 a	14.71 a
2nd	5.00 ab	60.00 b	66.66 b	1.50b	22.50b	20.00 ab
3rd	5.40 ab	65.00c	73.53 b	1.75 c	25.00 c	22.22 ab
4th	6.00 b	70.00 d	100 c	2.00 d	30.00 d	38.10 b
P-values	0.017*	0.000***	0.000***	0.000***	0.000***	0.000***

Means denoted by the same letter in each 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

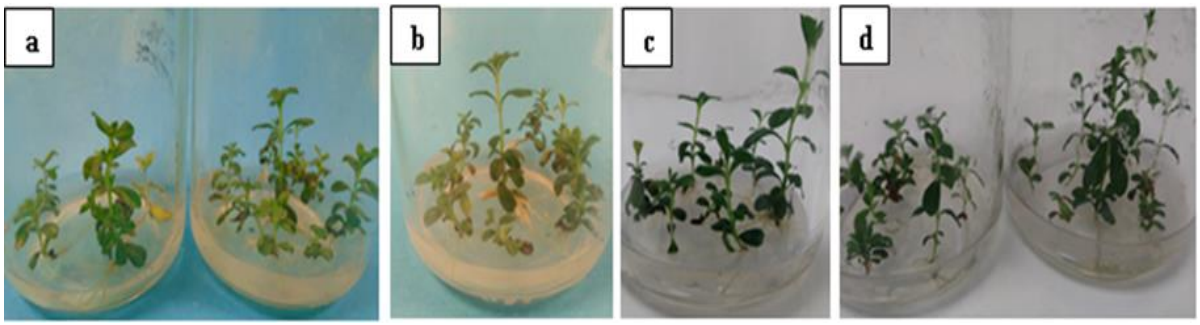

Figure 4 Effect of the number of cultures (4 successive sub-cultures) on *in vitro* rooting and shoot proliferation of *Satureja thymbra* L. shoot-tip explants after a 4.5-week period/ each culture in WPM medium supplemented with 20 g/l sucrose and 6 g/l Plant Agar (pH=5.8): (a) 1st culture (4.5 weeks), (b) 2nd culture (9 weeks), (c) 3rd culture (13.5 weeks), and (d) 4th culture (18 weeks)

After 4 weeks in the mist system inside greenhouse (31/5/2017 - 28/6/2017), the survival rate of rooted microshoots was high, 81.82%, i.e. 18 out of the 22 plantlets in total were successfully acclimatized. After a 2-month period, on early-autumn (28/6/2017 - 1/9/2017), the survival rate of fully acclimatized and hardened plantlets outside greenhouse in the nursery was 100%, i.e. all the 18 plants succeeded to survive to external *ex vitro* conditions. The process of gradual acclimatization and hardening of *S. thymbra* rooted microshoots after their transition from the *in vitro* environment to *ex vitro* conditions outside greenhouse lasted 3 months during the summer period (31/5/2017 – 1/9/2017) (Figure 5a-5f).

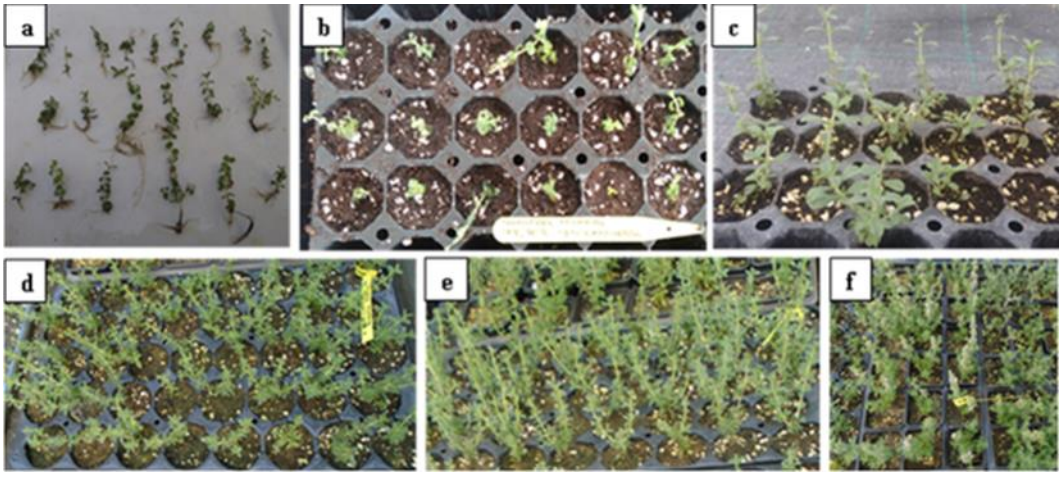

Figure 5 *Ex vitro* acclimatization and adaptation of the *in vitro* rooted *Satureja thymbra* L. plantlets to the greenhouse: (a) rooted microshoots washed thoroughly with sterile water outside the *in vitro* environment, (b) one day after planting in trays containing peat (Terrahum): perlite (1:1 v/v) and transfer in a nylon table bench tunnel with adjustable relative humidity, (c) vegetative growth of acclimatized plantlets (4 weeks in the mist), (d, e) 4 and 8 weeks, accordingly from transplantation in 0.33Lt pots [peat moss (TS2): perlite: sand (2: ½ : ½ v/v)], and (f) vegetative growth and flowering of fully acclimatized plants in 2.5 Lt pots [peat moss (TS2): perlite: sand (2:1:1 v/v)] outside greenhouse.

4. Discussion

In this study, *S. thymbra* explants exhibited best proliferation results (100% multiplication percentage, 5.5 shoots 14.24 mm long) after 5 weeks of culture in the MS basal medium supplemented with 0.5 mg/l BA. Our results substantiate with previous studies, where BA (0.5-1.5 mg/l) was the best PGR for the stimulation of multiple shoot formation, e.g. *Satureja obovata* Lag., [20], *Satureja horetnsis* [21] and *Satureja abyssinica* (Benth.) Briq. [24, 25]. The mechanism for multiple shoot formation can be due to suppression of apical dominance, thus enhancing auxiliary and adventitious shoot formation which is a general role of cytokinins [26]. Superiority of BA for shoot induction has been reported to be due to the ability of plant tissue to metabolize natural hormones more readily than artificial growth regulators or due to the ability of BA to induce production of natural hormones such as zeatin in the tissue and thus work through natural hormone system [27].

In the current study employing *S. thymbra* microshoots, no symptons of hyperhydricity were observed in the proliferation MS medium containing BA. In another *Satureja* species (*S. abyssinica*), however, vitrified cultures were induced in the highest BA concentration [24]. Different basal culture media than MS and different cytokinin types than BA have been reported to promote better the production of multiple shoots during the proliferation stage. According to Ramak et al. [28], the LS culture medium [29] fortified with 1 mg/l BA plus 0.5 mg/l IBA proved to be more appropriate for shoot proliferation of *Satureja khuzistanica* Jamzad nodal explants. Furthermore, in *S. abyssinica*, the best culture initiation (100%) was attained in MS medium in the presence of 1.5 mg/l BAP, maximum shoot number was obtained with 1.25 mg/l kinetin and the greatest shoot height was achieved with 0.5 mg/l BAP [24].

Root establishment and their development highly depend on exogenous auxin [30]. Our findings from the first rooting experiment with MS basal medium and auxins (IBA, NAA, IAA) applied either alone in different concentrations or in combinations revealed that the highest frequency of root formation (58.62%), root number (6.35 roots/ rooted microcutting) and root length (13.43 mm) were seen in the presence of 1 mg/l IBA. Similar results to ours were performed for *S. abyssinica* [24] and *S. punctata* [30], where IBA was the favorable auxin type for root initiation than NAA and IAA.

In the present study with *S. thymbra*, IAA proved to be the most appropriate auxin type and 0.25 mg/l the ideal auxin concentration for the shoot elongation phase following initial shoot induction. In addition, *S. thymbra* explants cultured on MS medium with 0.25 mg/l IBA + 0.25 mg/l IAA gave more shoots (1.94 shoots/ explant). Ours findings are partly in agreement with those presented for *S. abyssinica*, where the combined effect of 0.2 mg/l NAA with 0.5 mg/l IAA gave optimum proliferation results in terms of shoot number and shoot length [24]. In the studied species (rooting experiment No.1), auxins, applied alone and in combinations resulted in non-satisfactory shoot proliferation percentages, being maximum, 50% with 0.1 mg/l NAA, whereas NAA at higher concentrations (0.25-1 mg/l) had an

inhibitory effect. On the contrary, Teshome and Soromessa [24] have reported the negative impact of NAA on shoot proliferation in *S. abyssinica.* The same authors reported that this could be due to the presence of high levels of endogenous NAA and addition of exogenous NAA in the medium raised the total level of auxin, thus leading to increased apical dominancy by reducing shoot proliferation and promoting root induction [24]. In *S. thymbra* under study, on the other hand, the endogenous NAA levels might be low, therefore the exogenous application of a low NAA concentration (0.1 mg/l) did not raise the total auxin level to such a degree so as to decrease apical dominance by promoting multiple shoot induction and diminishing root formation.

In the second rooting experiment with WPM as the basal culture medium, there was a progressive increase in all macroscopic rooting and shoot proliferation attributes simultaneously with the increase in the number of successive cultures (1st, 2nd, 3rd and 4th subculture). In *Satureja khuzistanica* Jamzad, the best condition for rooting was the LS medium supplemented with 0.5 mg/l of IBA [28]. In particular, 100% rooting with 6 roots/ rooted explant 70 mm long and 38.1% shoot multiplication with 2 shoots/ explant 30 mm in length were obtained in the end of the 4th culture for juvenile *S. thymbra* shoot-tip explants (after 4 successive cultures/ 4.5 weeks each). According to Arrebola et al. [20] in *S. obovata* Lag., the rooting of juvenile shoots was accomplished in vivo, while adult shoots were rooted *in vitro* after 3 days of exposure to 1 mg/l IBA followed by subsequent transfer to auxin-free medium. In the present study, *S. thymbra* microshoots showed substantially higher rooting potential to WPM medium PGRs-free (rooting experiment No. 2) than to MS medium with auxins (rooting experiment No. 1). In specific, there was a 2-fold increase in rooting percentage from 0-58.62% when explants were cultured in the MS medium to 50-100% in WPM as well as in root length from 8.51-34.35 mm (MS) to 18.09-70 mm (WPM), accordingly. In *S. abyssinica* (Benth.) Briq., maximum root number (8.00) and rooting frequency (90%) were achieved on half-strength MS medium supplemented with 0.5 mg/l IBA whereas the longest roots (1.07 cm) were produced with 1.4 mg/l IBA [25].

In relation to *S. thymbra* under study, the survival rate of rooted microshoots was 81.82%, after a 4-week period of maintenance in the mist of the greenhouse and 100% after 2 months outside greenhouse in the nursery. Successful *ex vitro* acclimatization and high survival rates (88-96%) have also been achieved for plantlets of other *Satureja* species including *S. abyssinica* [24, 25]. According to Arrebola et al. [20], acclimatization of juvenile *S. obovata* Lag. shoots was accomplished *in vivo* and more than 95% survival of adult rooted plants was obtained during the acclimatization phase. The *ex vitro* acclimatization and hardening of *S. thymbra* plantlets was successfully completed within 3 months during summer.

5. Conclusion

Overharvesting of medicinal plants has led to significant reduction of these species threatening their genetic diversity. Micropropagation can represent a valid alternative to produce rapidly large amounts of plant material. Other advantages of this technique are the protection of the natural ecosystem avoiding the damage of endemic plants. The present study contributes to the conservation and improvement of *S. thymbra* using MS medium supplemented with optimum concentration of BA (0.5 mg/l) for shoot proliferation (5 weeks), 0.25 mg/l IAA for shoot elongation (7 weeks) and 1 mg/l IBA for root induction (7 weeks). The use of WPM basal medium without PGRs for the enhancement of rooting potential (4.5 weeks) is also proposed. In the course of *S. thymbra* domestication process, obtained plants can be further grown in the glasshouse or field to produce seeds which then may be dispersed to establish new populations in the habitat nature of *S. thymbra*. Although, further study also is needed to evaluate *S. thymbra* active bio-compounds and phytochemicals of *ex situ* and *in vitro* grown populations.

Compliance with ethical standards

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Disclosure of conflict of interest

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

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