American Journal of Plant Sciences, 2019, 10, 1482-1502
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Effect of Macronutrients, Cytokinins and Auxins, on *in Vitro* Organogenesis of *Thymus vulgaris* L.

Zineb Nejjar El Ansari[1*], Amina El Mihyaoui[1], Ibtissam Boussaoudi[1], Rajae Benkaddour[1], Ouafaa Hamdoun[1], Houda Tahiri[2], Alain Badoc[3], Aicha El Oualkadi[2], Ahmed Lamarti[1]

[1]Laboratory of Plant Biotechnology, Biology Department, Faculty of Sciences, Abdelmalek Essaadi University, Tetouan, Morocco
[2]Genetic Improvement of Plants Laboratory /National Institute of Agronomic Research (INRA), Tangier, Morocco
[3]Axe MIB (Molécules d'Intérêt Biologique), Unité de Recherche (Enologie EA 4577, USC 1366 INRA), UFR des Sciences Pharmaceutiques, Université de Bordeaux, ISVV (Institut des Sciences de la Vigne et du Vin), Bordeaux, France
Email: *zinebnejjarelansari@hotmail.com

How to cite this paper: El Ansari, Z.N., El Mihyaoui, A., Boussaoudi, I., Benkaddour, R., Hamdoun, O., Tahiri, H., Badoc, A., El Oualkadi, A. and Lamarti, A. (2019) Effect of Macronutrients, Cytokinins and Auxins, on *in Vitro* Organogenesis of *Thymus vulgaris* L. *American Journal of Plant Sciences*, **10**, 1482-1502.
https://doi.org/10.4236/ajps.2019.109105

Received: August 3, 2019
Accepted: September 6, 2019
Published: September 9, 2019

Abstract

The present study reports an efficient protocol for *in vitro* propagation of *Thymus vulgaris* L., an aromatic and medicinal plant in Morocco. Initially, we performed *in vitro* multiplication of *Thymus vulgaris* explants existing in the laboratory and obtained from micropropagation by shoot tip culture. Afterwards, we have evaluated the effect of six macronutrients. After that, seven cytokinins (Kin, BAP, 2iP, DPU, Adenine, Zeatine and TDZ) in three different concentrations (0.46, 0.93, 2.32 µM) have been evaluated to optimize cultures multiplication and elongation. Moreover, the effect of three auxins (IAA, IBA and NAA) at 0.57 µM, combined to 4 cytokinins (Kin, BAP, DPU and Ad.) at 0.46 µM, on shoot rooting has been studied. Thereby, MS medium has been proved the most favorable for plantlets growing. Also, we found that the addition of certain cytokinins, specifically 0.46 Kin, 0.46 and 0.93 BAP, 0.46 2iP, 0.46 DPU, 0.46 Ad. and 0.46 Zeat., ensures better multiplication and growth of vitroplants. In addition, multiplication and rooting of cultures were well optimized after addition 0.46 Kin + 0.57 IAA or NAA, 0.46 DPU + 0.57 IBA and 0.46 Ad. + 0.57 IBA combinations to the culture medium. Lastly, plantlets with roots were successfully acclimatized to ex-vitro conditions and these latter served as a source to establish *in vitro* culture again.

Keywords

Thymus vulgaris L., Macronutrients, Cytokinins, Auxins, *In Vitro* Propagation

1. Introduction

The genus *Thymus* is very common in the Mediterranean region, where certain

species form a particular type of dense vegetation not exceeding 50 cm high, well adapted to the hot summers [1]. Generally, this plant presents gynodioecy, which means that it produces two types of individuals, some with female flowers without stamens and others with hermaphrodite flowers [2].

Plants of the genus *Thymus* have been the subject of numerous chemical and pharmacological studies and their main characteristic is the production of essential oil, accumulated at the level of pelted glandular trichomes [3]. In particular, *Thymus vulgaris*, is a perennial shrub present in the Mediterranean region with at least six different chemotypes [4]; the major compounds reported by the studies carried out on the essential oil of this species are phenolic monoterpenes, namely thymol and carvacrol, in addition to ρ-cymene, γ-terpinene, oxygenated monoterpenes such as borneol and linalool, and sesquiterpenes represented mainly by β-caryophyllene [5] [6] [7] [8] [9]. Also, *Thymus vulgaris* contains tannins, saponins, flavonoids, phenolic acids, steroids and tripterpenes [10] [11] [12].

Actually, the great morphological and chemical variability of *Thymus vulgaris* makes it possible to select plants with the most interesting characteristics for producers. The development of tissue culture methods can contribute to the production of secondary metabolites in several ways. One of them is the rapid multiplication of selected plants by a process to obtain plants genetically identical to the donor plant. Another method could be the production of secondary metabolites by culturing cells and tissues from selected plant explants. With regard to the great variability of thyme, micropropagation could contribute to the production of useful plants for the selection of new varieties, thus providing large quantities of valuable plant material [13] [14] [15]. Thereby, different micropropagation methods of *Thymus vulgaris* were presented, most commonly by axillary buds and using shoot tips or nodal segments to establish cultures [16] [17] [18] [19].

In this way, the present study describes detailed research on the micropropagation of *Thymus vulgaris*, extinct species in Morocco, by axillary buds, shoot tips and nodal segments, through the evaluation and comparison of the effect of different compositions of the culture media.

2. Material and Methods

2.1. Plant Material

We worked on preexisting *Thymus vulgaris* L. plantlets in the Laboratory of Plant Biotechnology of the Faculty of Sciences of Tetouan, obtained by micropropagation from shoot tips and nodal segments.

Cultures were multiplied from nodal segments (5 - 6 mm) on a medium solidified with 0.7% bacterial agar, containing Shah and Dalal [20] macronutrients, Murashige and Skoog [21] micronutrients and vitamins, 100 mg/l myo-inositol, 3% sucrose and 0.46 µM Kin. Plantlets were transplanted into the same medium until sufficient numbers were available to establish the experiments.

2.2. Effect of Macronutrients

Six solutions of macronutrients differing in nitrogen content (NO_3^- and NH_4^+) and in potassium, all added with Murashige and Skoog micronutrients and vitamins were tested: MS (Murashige and Skoog) [21], B_5 (Gamborg) [22], SH (Schenk and Hildebrandt) [23], SD (Shah and Dalal) [20], modified MS (MS_m) according to Badoc [24] and $N_{30}K$ (Margara) [25]. The mineral solution chosen is used for all the following experiments.

2.3. Effect of Cytokinins

Seven cytokinines: Kin (Kinetin), BAP (6-Benzylaminopurine), 2iP (2-isopentenyladenine), DPU (1,3-diphenylurea), Ad. (Adenine), Zeat (Zeatin), and TDZ (Thidiazuron) were evaluated on *Thymus vulgaris* plantlets growth. Three concentrations were tested: 0.46, 0.93 and 2.32 µM/l, plus a control medium containing no growth regulator.

2.4. Effect of Auxins Combined to Cytokinins

Four cytokinins (Kin, BAP, DPU and Ad.) at 0.46 µM were tested alone or combined to three auxins: IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid) and NAA (1-Naphthaleneacetic acid) at 0.57 µM, plus a control medium containing no growth regulator.

2.5. Acclimatization Phase

After removal from the culture media, 30 rooted plantlets were gently washed to remove the rest of the agar medium from roots, and then acclimatized in 250 ml plastic pots, containing a mixture of sterilized peat and vermiculite (2:1, v/v). Each pot was covered by a transparent plastic cup, incubated under specific conditions (photoperiod: 18/6 h, humidity: 90% - 100%, temperature: 24˚C ± 1˚C) and watered, if necessary, with distilled water. After three weeks, the humidity was gradually reduced until the cups were completely eliminated at the end of the fourth week. Regular irrigation was performed during the first two weeks, at intervals of two days from the fifteenth to the twentieth day and as needed until transplantation into larger pots.

2.6. Re-Initiation of *in Vitro* Culture of *Thymus vulgaris* from Acclimatized Plants

Twigs were cut from the acclimatized plants of *Thymus vulgaris*, thoroughly washed with tap water, then surface sterilized under a laminar flow hood according to five methods:

Method 1

- Rinsing with 10% $CaClO_2$ with 4 to 5 drops of Tween 80 for 20 min;
- Rinsing with 10% Mercryl with 4 to 5 drops of Tween 80 for 10 min;
- Rinsing three times with distilled water for 5 min.

Method 2

- Rinsing with ethanol 70˚ for 30 s;
- Rinsing with 10% $CaClO_2$ with 4 to 5 drops of Tween 80 for 30 min;
- Rinsing with 0.1% $HgCl_2$ with 4 to 5 drops of Tween 80 for 5 min;
- Rinsing three times with distilled water for 5 min.

Method 3

- Rinsing with ethanol 70˚ for 30 s;
- Rinsing with 10% $CaClO_2$ with 4 to 5 drops of Tween 80 for 30 min;
- Rinsing with 10% Mercryl with 4 to 5 drops of Tween 80 for 5 min;
- Rinsing three times with distilled water for 5 min.

Method 4

- Rinsing with 10% $CaClO_2$ with 4 to 5 drops of Tween 80 for 30 min;
- Rinsing with 0.1% $HgCl_2$ with 4 to 5 drops of Tween 80 for 5 min;
- Rinsing three times with distilled water for 5 min.

Method 5

- Rinsing with 10% $CaClO_2$ with 4 to 5 drops of Tween 80 for 30 min;
- Rinsing with 10% Mercryl with 4 to 5 drops of Tween 80 for10 min;
- Rinsing three times with distilled water for 5 min.

The sterilized twigs were divided into 2 - 3 cm segments with at least two axillary buds, and these segments were used as explants. For re-initiation of the *in vitro* culture, the explants were placed in glass test tubes (18 × 180 mm), one per tube, containing 15 ml of MS culture medium.

2.7. Culture Conditions

The culture media were supplemented with 3% sucrose and 0.7% E-type bacteriological agar. The pH of the media was adjusted to 5.6 - 5.8 using sodium hydroxide (NaOH). Sterilization of the culture medium was carried out at 121˚C for 20 min. The *in vitro* culture was performed under aseptic conditions in a horizontal laminar flow hood. The vitroplants were incubated in a culture room (photoperiod: 18/6 h with 4000 lux light density, temperature: 24˚C ± 1˚C).

2.8. Evaluation of Plantlets Growth

After one month of growth, the following parameters were evaluated:

- Regeneration rate (%);
- Mean plantlets length (cm);
- Mean number of buds per plantlet;
- Mean number of shoots per plantlet;
- Percentage of rooting (%);
- Mean number of roots per plantlet;
- Mean roots length (cm);
- Dry weight of the aerial part (mg);
- Dry weight of roots (mg).

Dry weight measurements were carried out after placing test samples of shoots and roots in the oven set at 45˚C ± 1˚C, until a constant weight was obtained.

2.9. Statistical Analysis

All measurements were run in triplicates (n = 3); 24 samples were used for each replicate and the values were averaged and given along with standard error (±SE). Analyses were performed with Statistica 6, averages were compared by Duncan test and values beyond $p \leq 0.05$ were considered to be significant.

3. Results

3.1. Effect of Macronutrients on Plantlets Growth

There is not much difference between the six macronutrients in regeneration, rooting and hyperhydricity (Table 1). However, SH macronutrients ensure the best regeneration rate (97.2%), followed by MS and B_5 (95.8%). In addition, MS_m macronutrients show the best rooting rate (58.1%), followed by SH (53.4%) and SD (43.1%). Plantlets showing hyperhydricity appeared in the case of SH (2.8%) and $N_{30}K$ (4.2%).

In addition, buds and shoots multiplication is approximately similar for the six macronutrients, with a maximum number of buds in SH (34.1), followed by B_5 (32.8). The best number of shoots is noted in the case of B_5 (3.4), followed by MS_m (3.1). Elongation of the plantlets is better in the case of MS (2.4 cm) and B_5 (2.2), while the minimum length is observed in the case of MS_m (1.8). For the dry weight of shoots, higher values are noted in the case of MS, B_5 (9 mg) and SH (14) media (Table 2, Figure 1).

Furthermore, root multiplication is optimal in the case of MSm and $N_{30}K$ (6.5 and 6.2, respectively), followed by SH and B_5 (5.8 and 5.6, respectively). For their elongation, there is no big difference between B_5, SH, SD, MS_m and $N_{30}K$, with values ranging from 1.16 (modified MS) to 1.35 cm (SH and SD), except for the MS medium showing a lower length (0.93 cm). In addition, the upper root dry weight is recorded for SH medium (2 mg) and the other macronutrients have the same value (1 mg) (Table 2, Figure 1).

Although SH medium has the best rate of regeneration, hyperhydricity affects 2.8% of the explants, as well as N30K with a rate of 4.2%. MSm and SD macronutrients ensure a good development of the aerial and root parts but show a

Table 1. Regeneration, rooting and hyperhydricity rate of *Thymus vulgaris* plantlets grown on six different macronutrients.

Macronutrients	Regeneration rate (%)	Rooting rate (%)	Hyperhydricity rate (%)
MS	95.83 ± 2.40 a	38.93 ± 1.43 b	0.00 ± 0.00 b
B_5	95.83 ± 2.40 a	42.00 ± 2.50 ab	0.00 ± 0.00 b
SH	97.23 ± 2.80 a	53.40 ± 1.40 a	2.80 ± 0.80 ab
SD	91.67 ± 8.33 a	43.07 ± 2.03 ab	0.00 ± 0.00 b
MS_m	91.67 ± 4.82 a	58.07 ± 1.84 a	0.00 ± 0.00 b
$N_{30}K$	94.43 ± 5.57 a	42.50 ± 3.80 ab	4.17 ± 1.16 a

The data represent Mean ± SE of replicates (n = 3). Values in the same rows carrying different letters are significantly different between treatments and compared by Duncan's multiple range tests at $p \leq 0.05$.

Table 2. Effect of six macronutrients on shoots and roots growth of *Thymus vulgaris* plantlets.

Macronutrients	Mean number of buds	Mean number of shoots	Shoots length	Shoots dry weight (mg)	Mean number of roots	Roots length	Roots dry weight (mg)
MS	31.91 ± 1.33 a	3.06 ± 0.16 a	2.40 ± 0.13 a	9.00 ± 1.00 ab	2.52 ± 0.26 b	0.93 ± 0.08 b	1.00 ± 0.20 b
B_5	32.75 ± 1.49 a	3.35 ± 0.29 a	2.21 ± 0.13 ab	9.00 ± 1.00 ab	5.63 ± 1.12 a	1.22 ± 0.09 a	1.00 ± 0.30 b
SH	34.14 ± 1.44 a	2.93 ± 0.19 a	1.92 ± 0.08 bc	14.00 ± 1.00 a	5.79 ± 0.83 a	1.35 ± 0.05 a	2.00 ± 0.40 a
SD	30.52 ± 1.54 a	2.99 ± 0.17 a	2.05 ± 0.09 bc	6.00 ± 1.00 b	4.79 ± 0.68 ab	1.35 ± 0.06 a	1.00 ± 0.20 b
MS_m	31.70 ± 1.75 a	3.12 ± 0.22 a	1.83 ± 0.08 c	8.00 ± 1.00 ab	6.45 ± 1.07 a	1.16 ± 0.08 a	1.00 ± 0.20 b
$N_{30}K$	30.71 ± 1.60 a	3.10 ± 0.22 a	2.11 ± 0.10 abc	8.00 ± 1.00 ab	6.24 ± 1.20 a	1.29 ± 0.09 a	1.00 ± 0.20 b

The data represent Mean ± SE of replicates (n = 3). Values in the same rows carrying different letters are significantly different between treatments and compared by Duncan's multiple range tests at $p \leq 0.05$.

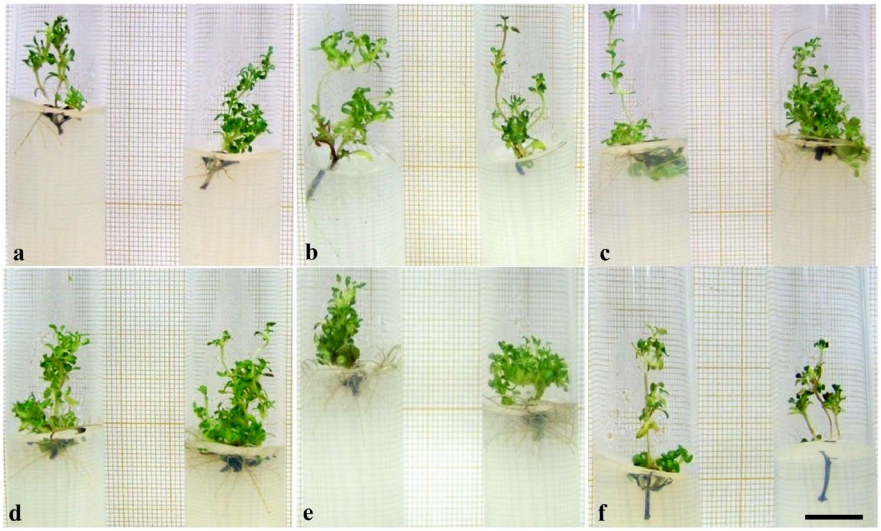

Figure 1. Effect of six macronutrients on shoot and root growth of *Thymus vulgaris* plantlets (**a**—MS; **b**—B_5; **c**—SH; **d**—SD; **e**—MS_m; **f**—$N_{30}K$).

lower rate of regeneration (91.7%). Good results for the multiplication and the elongation of the cultures, as well as for the rooting are recorded in the case of the B5 medium, however, a blackening of the aerial part of the vitroplants is noticed. Indeed, we will opt for MS macronutrients for the rest of our experiments, since they ensure a good regeneration of the aerial part, especially in terms of elongation, with complete absence of hyperhydricity. Rooting will be improved later.

3.2. Effect of Cytokinins on Plantlets Growth

Integration of cytokinins into MS culture medium resulted on several changes, both in shoots and roots.

Thus, the maximum elongation of plantlets is observed for 0.46 Kin (4.1 cm), followed by 2.32 Zeat. (3.8), 0.46 TDZ (3.8) and 0.46 Zeat. (3.7). On the other hand, the minimum elongation (2.3 to 2.5 cm) is recorded in the case of 0.93 BAP, 0.46 Ad., 0.93 Zeat. and TDZ at 0.93 and 2.32 µM. Also, bud multiplication

is maximum for 0.93 2iP (50.8); 0.93 TDZ (34.0) and 0.46 Ad. (31.9), while the minimum number is noted for 0.93 Ad. (18.3). In addition, a maximum number of shoots is regenerated in the case of 0.93 2iP (7.3) and 0.46 Zeat. (6.2), followed by 0.46 2iP (5.8), 0.93 TDZ (4.8), 0.46 Ad. and TDZ (4.1), unlike 0.93 Kin and Ad. (1.7 and 1.4 respectively) generating the lowest number of shoots. Moreover, the maximum dry weight of shoots is noticed for 0.93 TDZ (21.2 mg), followed by 0.93 2iP (10.9 mg) and the minimums for 0.46 and 0.93 Kin (3.1 and 3.2 mg, respectively), 0.46 BAP (2.9 mg), 0.93 DPU (2.6 mg), 0.93 Ad. (3.0 mg) and 0.93 Zeat. (3.4 mg) (Table 3, Figure 2).

Also, the highest number of roots grew for plantlets regenerated on MS medium supplemented with 0.93 2iP (10.1), followed by 2.32 Ad. and 0.93 Zeat. (8.3 and 7.0, respectively), whereas the minimum number is noted in the case of 0.93 and 2.32 TDZ (1.0). Furthermore, root length is optimal at 2.32 BAP (2.7 cm),

Table 3. Effect of seven cytokinins at three concentrations on shoots and roots growth of *Thymus vulgaris* plantlets.

Cytokinins (μM)		Mean number of buds	Mean number of shoots	Shoots length (cm)	Shoots dry weight (mg)	Mean number of roots	Roots length (cm)	Roots dry weight (mg)
Control		30.69 ± 2.73 cd	2.78 ± 0.47 def	3.35 ± 0.21 bcd	6.40 ± 1.10 bc	7.78 ± 0.78 ab	2.07 ± 0.16 ab	2.60 ± 0.50 ab
Kin	0.46	25.25 ± 2.54 def	3.58 ± 0.45 cd	4.12 ± 0.17 a	3.10 ± 0.90 c	3.06 ± 0.47 cd	0.97 ± 0.13 cd	0.30 ± 0.10 bc
	0.93	20.33 ± 1.71 ef	1.70 ± 0.24 f	2.73 ± 0.13 defg	3.20 ± 0.20 c	3.33 ± 0.74 cd	1.95 ± 0.22 ab	0.70 ± 0.10 bc
	2.32	23.05 ± 1.68 def	2.17 ± 0.30 def	2.95 ± 0.26 cdef	6.00 ± 1.20 bc	3.85 ± 1.16 cd	1.77 ± 0.22 abc	1.90 ± 0.30 abc
BAP	0.46	24.33 ± 1.94 def	3.16 ± 0.37 de	3.30 ± 0.28 bcd	2.90 ± 0.50 c	0.00 ± 0.00 e	0.00 ± 0.00 e	0.00 ± 0.00 c
	0.93	27.75 ± 1.77 cd	3.41 ± 0.37 cde	2.47 ± 0.12 fg	9.40 ± 2.20 bc	1.75 ± 0.47 cd	1.70 ± 0.36 abc	0.90 ± 0.20 bc
	2.32	20.44 ± 1.58 ef	2.05 ± 0.27 def	2.61 ± 0.17 efg	6.80 ± 1.20 bc	1.00 ± 0.00 d	2.65 ± 0.15 a	2.00 ± 0.20 abc
2iP	0.46	43.41 ± 2.14 b	5.79 ± 0.38 b	2.60 ± 0.16 efg	6.00 ± 1.00 bc	4.68 ± 0.51 c	0.70 ± 0.06 d	3.70 ± 1.70 a
	0.93	50.76 ± 1.00 a	7.33 ± 1.05 a	3.28 ± 0.21 bcde	10.90 ± 0.41 b	10.05 ± 1.17 a	2.17 ± 0.17 a	1.40 ± 0.50 abc
	2.32	23.52 ± 1.93 def	2.19 ± 0.27 def	3.40 ± 0.25 bcd	3.90 ± 0.60 bc	2.60 ± 0.67 cd	1.22 ± 0.21 bcd	1.60 ± 0.60 abc
DPU	0.46	26.83 ± 2.94 cde	3.70 ± 0.62 c	3.56 ± 0.26 abc	4.30 ± 1.60 bc	5.78 ± 0.49 b	0.47 ± 0.05 d	0.60 ± 0.20 bc
	0.93	19.70 ± 1.94 ef	2.08 ± 0.37 def	2.84 ± 0.14 defg	2.60 ± 0.70 c	4.00 ± 0.51 cd	0.43 ± 0.05 d	0.80 ± 0.20 bc
	2.32	27.70 ± 2.33 cd	2.15 ± 0.26 def	2.94 ± 0.27 cdef	6.60 ± 1.60 bc	6.40 ± 0.56 b	1.77 ± 0.12 abc	1.40 ± 0.80 abc
Ad.	0.46	31.87 ± 1.95 c	4.08 ± 0.53 c	2.39 ± 0.11 fg	3.60 ± 1.10 bc	5.68 ± 0.50 b	0.68 ± 0.08 d	0.60 ± 0.30 bc
	0.93	18.33 ± 1.46 f	1.41 ± 0.13 f	2.62 ± 0.15 efg	3.00 ± 0.50 c	6.52 ± 0.56 b	0.91 ± 0.14 cd	0.90 ± 0.60 bc
	2.32	26.72 ± 1.81 cde	1.88 ± 0.27 ef	2.87 ± 0.18 defg	4.70 ± 0.40 bc	8.33 ± 0.47 ab	1.79 ± 0.11 abc	1.30 ± 0.30 abc
Zeat.	0.46	39.25 ± 2.55 b	6.20 ± 0.76 a	3.71 ± 0.25 ab	5.90 ± 1.20 bc	5.25 ± 0.55 bc	0.71 ± 0.12 d	0.90 ± 0.70 bc
	0.93	19.82 ± 1.41 ef	1.87 ± 0.21 ef	2.46 ± 0.13 fg	3.40 ± 0.40 c	7.00 ± 0.00 ab	2.50 ± 0.00 a	0.70 ± 0.10 bc
	2.32	31.56 ± 2.07 c	2.69 ± 0.36 def	3.76 ± 0.32 ab	8.50 ± 0.70 bc	3.92 ± 0.73 cd	2.33 ± 0.35 a	2.00 ± 0.40 abc
TDZ	0.46	29.25 ± 2.71 cd	4.08 ± 0.60 c	3.75 ± 0.20 ab	4.70 ± 0.60 bc	3.53 ± 0.41 cd	0.63 ± 0.09 d	2.20 ± 0.40 abc
	0.93	34.00 ± 1.93 c	4.77 ± 0.47 bc	2.50 ± 0.16 fg	21.2 ± 0.90 a	1.00 ± 0.00 d	1.20 ± 0.00 bcd	1.80 ± 0.10 abc
	2.32	23.60 ± 1.47 def	2.25 ± 0.33 def	2.25 ± 0.11 g	7.80 ± 1.20 bc	1.00 ± 0.00 d	2.00 ± 0.00 ab	2.10 ± 0.70 abc

The data represent Mean ± SE of replicates (n = 3). Values in the same rows carrying different letters are significantly different between treatments and compared by Duncan's multiple range tests at $p \leq 0.05$.

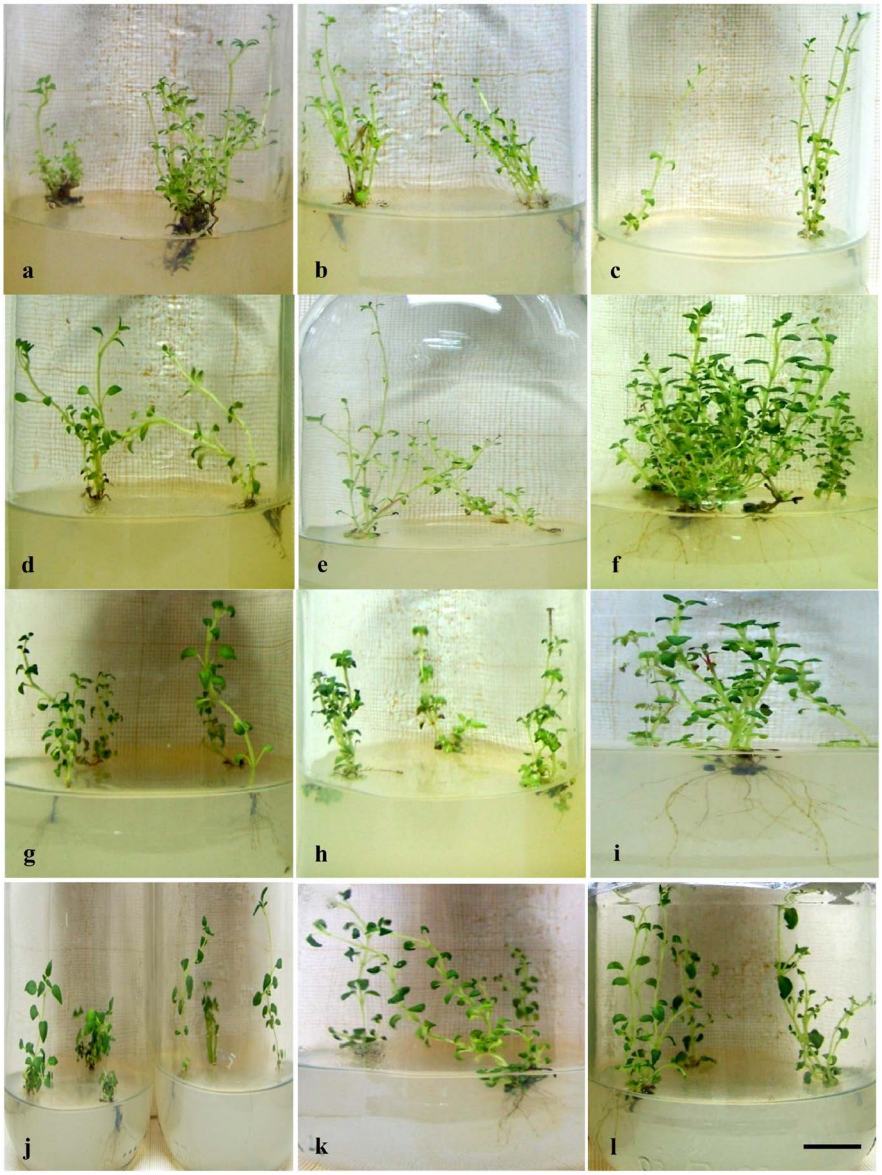

Figure 2. Effect of seven cytokinins at three concentrations on shoot and root growth of *Thymus vulgaris* plantlets (**a**—0.46 µM 2iP; **b**—0.46 µM Ad.; **c**—0.46 µM Kin; **d**—0.46 µM TDZ; **e**—0.46 µM Zeat.; **f**—0.93 µM 2iP; **g**—0.93 µM Ad.; **h**—0.93 µM BAP; **i**—0.93 µM Kin; **j**—2.32 µM Ad.; **k**—2.32 µM DPU; **l**—2.32 µM Zeat.).

0.93 2iP (2.2), 0.93 and 2.32 Zeat. (2.5 and 2.3, respectively), followed by 2.32 TDZ and 0.93 Kin (2.0). Contrariwise, minimum lengths are observed for 0.46 and 0.93 DPU (0.5 and 0.4 cm, respectively). Otherwise, the highest root dry weight is observed for 0.46 2iP (3.7 mg), followed by the control medium (2.6) and the minimum dry weights are noted for 0.46 Kin (0.3 mg), 0.46 DPU and 0.46 Ad. (0.6) (Table 3, Figure 2).

3.3. Effect of Combinations of Cytokinins and Auxins on Plantlets Growth

The combination of cytokinins and auxins affected remarkably the parameters

evaluated for shoots and roots growth.

Thus, we noted an increase in the number of buds, especially in the case of 0.46 Kin + 0.57 IAA (55.7), 0.46 BAP + 0.57 IBA (41.0), 0.46 DPU + 0.57 IBA (45.6) and 0.46 Ad + 0.57 IBA (49.1). Similarly, the number of shoots increased notably for 0.46 Kin + 0.57 IAA (8.3), 0.46 BAP combined with 0.57 IBA or NAA (5.1 and 5.9, respectively), 0.46 DPU + 0.57 IBA (5.6) and 0.46 Ad + 0.57 IBA (5.3). Also, shoots showed higher dry weights after integration of phytohormonal combinations in the culture medium, mainly in the case of 0.46 Kin + 0.57 NAA (32.10 mg) and 0.46 BAP + 0.57 NAA (40.9 mg). However, shoots length decreased for most phytohormonal combinations, except in the case of Ad., for which the combination with auxins did not significantly affect this parameter (Table 4, Figure 3).

Moreover, roots showed positive changes for the majority of phytohormonal combinations and the largest increase in their number is noted in the case of 0.46 Kin + 0.57 IAA (21.2), 0.46 DPU + 0.57 IAA or NAA (16.0 and 11.2, respectively) and 0.46 Ad + 0.57 IBA (11.7). In addition, the highest roots lengths (2.0 to 2.3 cm) are noted in the control medium, 0.46 Kin + 0.57 IAA or NAA, 0.46 DPU + 0.57 IBA or NAA and 0.46 Ad. + 0.57 IBA or NAA. Also, the best values of dry weight are observed in the case of 0.46 Kin + 0.57 IAA or NAA (6.7 and 22.5 mg, respectively) and 0.46 DPU + 0.57 NAA (7.8 mg) (Table 4, Figure 3).

Table 4. Effect of combinations of cytokinins and auxins on shoots and roots growth of *Thymus vulgaris* plantlets.

Cytokinins + Auxins (0.46 µM + 0.57 µM)	Mean number of buds	Mean number of shoots	Shoots length (cm)	Shoots dry weight (mg)	Mean number of roots	Roots length (cm)	Roots dry weight (mg)
Kin + IAA	55.67 ± 1.41 a	8.33 ± 1.14 a	3.24 ± 0.26 bc	14.30 ± 2.00 cd	21.17 ± 2.45 a	2.08 ± 0.14 ab	6.70 ± 0.00 bc
Kin + IBA	32.17 ± 1.70 def	3.50 ± 0.91 cd	2.50 ± 0.13 d	4.90 ± 1.00 fg	6.82 ± 0.96 ef	1.91 ± 0.46 bcd	1.80 ± 0.40 ef
Kin + NAA	41.82 ± 1.93 bc	4.82 ± 1.17 bc	2.61 ± 0.26 cd	32.10 ± 0.30 b	9.20 ± 1.71 cde	2.14 ± 0.32 ab	22.50 ± 1.40 a
BAP + IAA	30.15 ± 1.76 def	3.31 ± 0.54 cd	2.57 ± 0.14 cd	10.10 ± 0.60 def	2.63 ± 0.57 gh	1.49 ± 0.21 cde	3.10 ± 0.30 def
BAP + IBA	41.00 ± 0.72 bcd	5.08 ± 0.71 bc	2.18 ± 0.15 d	10.50 ± 0.60 def	4.56 ± 1.11 fg	1.60 ± 0.21 bcd	3.60 ± 1.30 cde
BAP + NAA	37.80 ± 1.29 cde	5.90 ± 0.74 b	2.11 ± 0.10 d	40.90 ± 2.30 a	3.40 ± 0.65 g	1.45 ± 0.15 de	6.10 ± 0.20 bcd
DPU + IAA	36.33 ± 1.90 def	3.83 ± 0.74 bcd	2.78 ± 0.17 cd	11.90 ± 0.60 cde	16.00 ± 1.31 b	1.75 ± 0.11 bcd	4.30 ± 2.00 cde
DPU + IBA	45.64 ± 1.66 abc	5.55 ± 0.82 b	2.55 ± 0.12 cd	5.10 ± 0.30 fg	9.64 ± 1.27 cde	2.17 ± 0.21 ab	1.60 ± 0.50 ef
DPU + NAA	33.46 ± 1.60 def	3.09 ± 0.51 cd	2.24 ± 0.11 d	17.00 ± 2.40 c	11.18 ± 2.08 cd	2.04 ± 0.23 abc	7.80 ± 1.60 b
Ad. + IAA	34.33 ± 0.65 def	3.75 ± 0.64 bcd	2.62 ± 0.19 cd	3.30 ± 1.20 g	9.92 ± 1.28 cde	1.17 ± 0.16 ef	2.20 ± 1.30 ef
Ad. + IBA	49.17 ± 1.02 ab	5.25 ± 0.45 b	2.44 ± 0.10 d	8.10 ± 0.20 efg	11.67 ± 1.15 c	2.33 ± 0.19 a	2.40 ± 0.60 ef
Ad. + NAA	29.09 ± 1.97 def	2.36 ± 0.53 d	2.34 ± 0.13 d	7.70 ± 0.60 efg	9.64 ± 0.82 cde	2.27 ± 0.28 a	3.50 ± 0.10 def
Kin	25.25 ± 2.55 f	3.58 ± 0.45 cd	4.12 ± 0.18 a	3.10 ± 0.90 g	3.06 ± 0.47 gh	0.98 ± 0.13 efg	0.30 ± 0.10 f
BAP	24.33 ± 1.94 f	3.17 ± 0.37 cd	3.31 ± 0.29 b	2.90 ± 0.50 g	0.00 ± 0.00 h	0.00 ± 0.00 h	0.00 ± 0.00 g
DPU	26.83 ± 2.94 ef	3.71 ± 0.62 bcd	3.57 ± 0.27 ab	4.30 ± 1.60 g	5.78 ± 0.49 fg	0.47 ± 0.06 gh	0.60 ± 0.20 ef
Ad.	31.88 ± 1.95 def	4.08 ± 0.53 bcd	2.40 ± 0.12 d	3.60 ± 1.10 g	5.68 ± 0.51 fg	0.68 ± 0.08 fg	0.60 ± 0.30 ef
Control	30.69 ± 1.73 def	2.78 ± 0.47 cd	3.35 ± 0.21 b	6.40 ± 1.10 efg	7.78 ± 0.78 def	2.07 ± 0.16 ab	2.60 ± 0.50 def

The data represent Mean ± SE of replicates (n = 3). Values in the same rows carrying different letters are significantly different between treatments and compared by Duncan's multiple range tests at $p \leq 0.05$.

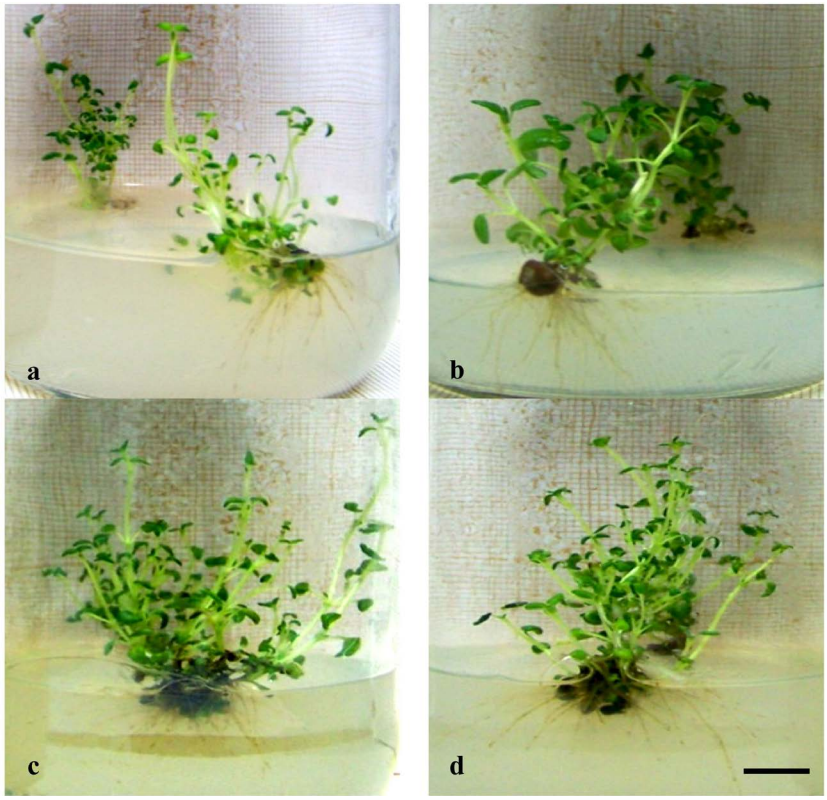

Figure 3. Effect of combinations of cytokinins and auxins on shoot and root growth of *Thymus vulgaris* plantlets (**a**—0.46 µM Ad. + 0.57 µM IBA; **b**—0.46 µM DPU + 0.57 µM IBA; **c**—0.46 µM Kin + 0.57 µM IAA; **d**—0.46 µM BAP + 0.57 µM IBA).

3.4. Acclimatization Phase

The thirty explants developing roots responded well to the acclimatization protocol. One month after the start of acclimatization, 90% of plantlets appeared to be in good health. Three months later, they were transplanted into larger pots. After one year, the acclimatized plants were indistinguishable from the wild plants and 20% developed flowers during the 2nd year, between April and June (**Figure 4**).

3.5. Re-Initiation of *in Vitro* Culture of *Thymus vulgaris* from Acclimatized Plants

Surface sterilization of twigs of acclimatized *Thymus vulgaris* plants proved to be extremely difficult (**Table 5**).

Thus, the first method comprising 10% $CaClO_2$ soaking for 20 min followed by 10% Mercryl for 10 min and rinsing with sterile distilled water three times for 5 min, resulted in high bacterial and fungal contamination rates (50.05% and 46.82%, respectively), in addition to 100% mortality of the tested samples.

The second method, during which we soaked twigs for 30 s in ethanol 70˚, then in $CaClO_2$ 10% for 30 min, after $HgCl_2$ 0.1% for 5 min and finally rinsing with sterile distilled water 3 times for 5 min, resulted in only 20.83% fungal contamination, but 72.92% mortality and just 27.08% of healthy survival.

Figure 4. Acclimatization of *Thymus vulgaris* plantlets (**a** and **b**—Acclimatization after 4 weeks; **c** and **d**—after 8 weeks; **e**—after one year; **f**—inflorescences of *Thymus vulgaris* acclimatized plants).

Table 5. Comparison of sterilization methods of shoot segments from the acclimatized *Thymus vulgaris* plants.

	Bacterial contamination rate (%)	Fungal contamination rate (%)	Death rate (%)	Survival rate (%)
Method 1	50.05 ± 1.56 b	46.82 ± 1.66 a	100.00 ± 0.00 a	0.00 ± 0.00 c
Method 2	0.00 ± 0.00 c	20.833 ± 1.17 b	72.92 ± 2.08 b	27.08 ± 2.08 b
Method 3	97.92 ± 2.08 a	6.25 ± 2.08 cd	100.00 ± 0.00 a	0.00 ± 0.00 c
Method 4	0.00 ± 0.00 c	4.17 ± 0.00 d	35.42 ± 2.08 c	60.42 ± 2.08 a
Method 5	91.67 ± 1.17 a	16.67 ± 2.08 bc	95.83 ± 1.66 a	2.08 ± 0.00 c

The data represent Mean ± SE of replicates (n = 3). Values in the same rows carrying different letters are significantly different between treatments and compared by Duncan's multiple range tests at $p \leq 0.05$.

For the third method where we used ethanol 70˚ for 30 s, then 10% $CaClO_2$ for 30 min, followed by 10% Mercryl for 5 min and finally three rinses with sterile distilled water for 5 min, no proliferation of shoots was noted, in addition to 97.92% of bacterial contamination and 6.25% of fungal contamination.

Similarly, the fifth method including 10% $CaClO_2$ soaking for 30 min, 10% Mercryl for 10 min and 3 rinses with sterile distilled water for 5 min, resulted on 91.67% of bacterial contamination, 16.67% of fungal contamination and 95.83% of mortality, whereas just 2.08% of the samples tested healthy proliferated.

On the other hand, the fourth method, for which we used $CaClO_2$ for 30 min, then 0.1% $HgCl_2$ for 5 min and finally three rinses with sterile distilled water for 5 min, proved to be the most effective, with a total absence of bacterial contamination, 4.17% of fungal contamination, 35.42% of mortality and 60.42% of the explants tested healthy survived.

Healthy plantlets were multiplied by subculture on MS + 0.46 Kin medium. The obtained vitroplants have the morphological criteria mentioned in Table 6 and Figure 5.

4. Discussion

Results obtained concerning the effect of macronutrients on the micropropagation of *Thymus vulgaris* allowed us to choose MS macronutrients for the rest of our experiments, since they ensure a good regeneration of the aerial part, especially in terms of elongation, with complete absence of hyperhydricity.

Table 6. Morphological characteristics of vitroplants obtained after sterilization of shoot segments from the acclimatized *Thymus vulgaris* plants and their multiplication on MS + 0.46 Kin medium.

Plantlets mean length (cm)	3.16 ± 0.21
Mean number of buds	15.82 ± 1.47
Mean number of shoots	2.09 ± 0.23
Mean number of roots	5.75 ± 1.28
Roots mean length (cm)	1.16 ± 0.14

Figure 5. Vitroplants obtained after sterilization of shoot segments from the acclimatized *Thymus vulgaris* plants and their multiplication on MS + 0.46 Kin medium.

Indeed, MS macronutrients have been the most used in the micropropagation protocols established for *Thymus vulgaris* [16] [17] [18] [19] [26] and for species of the genus *Thymus* in general [27]-[36]. However, less concentrated media were opted in some studies, namely 1/2 MS for *Thymus satureioides* Coss. [37] and *Thymus longicaulis* C. Presl [17], DKW medium (1984) [38] for *Thymus membranaceus* Boiss. [39], modified MS according to Gavazzi *et al.* [40] for *Thymus mastichina* (L.) L. [41] and CMS [42] for *Thymus piperella* L. [43]. Furmanowa and Olszowska [15], the first to establish micropropagation of *Thymus vulgaris*, used Nitsch and Nitsch (1969) [44] medium.

Moreover, our results are in concordance with Abdallah *et al.* [45], who obtained the best number of shoots, leaves and leaves/shoots for *Origanum syriacum* on MS medium, compared to NN and B_5, as well as with Özkum [46], who found that MS generates the highest number of parameters, such as the number of shoots and the number of leaves/shoots for *Origanum minutiflorum*, compared to B_5. Also, Nordine *et al.* [47] found that MS medium is the best for survival, multiplication and elongation of *Thymus hyemalis* Lange shoots, compared to 1/2 MS, B_5 and White (1963) [48]. In addition, Arikat *et al.* [49] found that B_5 medium produced shortest shoots compared to MS for *Salvia fruticosa*. However, Yavuz [50] found that culturing *Sideritis stricta* on B_5 results in longer shoots than those grown on MS.

Concerning the effect of cytokinins on the multiplication and elongation of cultures, Kin, BAP, DPU, Zeat. and TDZ at 0.46 µM have the best lengths on the one hand and on the other hand, 2iP, Ad. and Zeat. offer the best multiplication of buds. Also, 2iP, Ad., Zeat. and TDZ in the same concentration show an optimal multiplication of shoots. In addition, Kin, 2iP, Ad. and DPU ensure better root regeneration. Again, root development is absent in the case of BAP and weak in the case of Zeat. and TDZ.

Actually, knowing the effect of types and concentrations of cytokinins is indispensable for cultures multiplication, as reported for several species of the genus *Thymus*. Thus, Nordine and El Meskaoui [30] mentioned that regeneration rate of vitroplants of *Thymus bleicherianus* Pomel was not affected by the addition of low concentrations of cytokinins, whereas the addition of higher concentrations of BAP (8.88 µM) or Kin (9.6 µM) significantly reduced regeneration rate. Also, shoot multiplication was favored by the addition of 4.44 µM BAP, unlike Kin which has no effect. In addition, stressed appearance of plantlets was reported for *Thymus piperella* L. and *T. vulgaris*, characterized by smaller leaves and shorter internodes than *in vivo*, on CMS basal medium and after addition of increasing concentrations of BAP (6.6 to 8.9 µM). In this way, plantlets were subcultured onto the basal medium supplemented with only 2.2 µM BAP [43] [51]. Furthermore, Fraternale *et al.* [41] noted a maximum elongation of *Thymus mastichina* shoots after addition of 0.1 mg/l BAP to MS medium. Moreover, high concentration of Kin (9.3 µM) resulted in minimal survival of *Thymus satureioides* Coss. explants with callus formation at their base and the maximum number of shoots was obtained after addition of 2.22 µM BAP, while their num-

ber decreases with the increase of this concentration to 8.88 µM. Also, Kin proved to be less effective than BAP for shoot multiplication [37]. Besides, the addition of Kin at 4.6 or 6.9 µM ensured complete regeneration of *Thymus hyemalis* plantlets, whereas this rate decreased with higher concentrations (9.3 µM) for explants regenerated from apex. Similarly, the addition of BAP at concentrations higher than 2.2 µM resulted in a decrease in the number of shoots [47]. As well, Bakhtiar *et al.* [29] reported that Kin and TDZ are less effective for the micropropagation of *Thymus persicus* (Ronniger ex Rech.f.) Jalas. Cytokinins types and concentrations effect were established during the micropropagation of several other *Lamiaceae* too [45] [52] [53] [54].

The combination of the four cytokinins (Kin, BAP, DPU and Ad.) at 0.46 µM and the three auxins (IAA, IBA and NAA) at 0.57 µM did not always contribute to the optimization of *in vitro* growth of *Thymus vulgaris* plantlets, though, we obtained interesting results for the majority of evaluated parameters. Thus, Kin, in combination with auxins, did not always ensure better elongation of the number of buds and the number of shoots indeed increased after integration of 0.57 IAA and 0.57 NAA in MS + 0.46 Kin medium. Also, the number of roots and their length generally improved after addition of auxins. In addition, the combination between BAP and auxins is marked mainly by the development of roots and by a better multiplication of buds. As well, apart from shoots length, the combination of 0.46 DPU + 0.57 IBA has the best values for all morphological evaluated criteria. Generally, the combination of Ad. with auxins resulted in better development of rooting part and the best buds and shoots multiplication is observed after addition of 0.57 IBA to MS + 0.46 Ad.

Actually, the choice of the best cytokinin/auxin balance ensures not only a better development of roots, but a better proliferation of all parts of the plant. Thus, a better multiplication of *Thymus caespititius* Brot. was obtained by combining 1 mg/l BAP with 0.25 mg/l IBA [33], as well as 8.9 µM BAP with 2.7 µM NAA for *Thymus persicus* [29], as for Sáez *et al.* [43], the best multiplication of *Thymus piperella* vitroplants was carried out on the CMS medium supplemented with 6.66 µM BAP and 2.85 µM IAA. For *Sideritis stricta*, B_5 medium supplemented with 4.44 µM BAP + 0.54 µM NAA or 8.88 µM BAP + 2.68 µM NAA, was determined to be the most effective medium for shoot formation, while the best rooting was obtained on B_5 medium supplemented with 22.15 µM IBA [50]. Nordine and El Meskaoui [30] reported that the combination of cytokinins and auxins did not significantly improve the micropropagation of *Thymus bleicherianus* and carried out the rooting phase on growth regulators-free MS medium, as did Karalija and Parić [16] for *Thymus vulgaris*. Also, rooting of several *Lamiaceae* was completed on MS or 1/2 MS medium free of growth regulators [54] [55] [56] [57]. In other studies of *Thymus* and other *Lamiaceae* species, it was noted that the combination of IBA with BAP or TDZ inhibits rooting [28] [43] [58] [51]. Macro-Medina and Casas [28] obtained the best rooting of *Thymus moroderi* Pau ex Martínez on a double-phase medium consisting of a solid MS medium without growth regulators and a solution composed of 1/2

Knop macronutrients, 5% sucrose and 0.3% or 0.6% activated charcoal. In addition, rooting of other species of *Thymus* was carried out on media supplemented only with auxins at different concentrations [17] [29] [31] as well as other *Lamiaceae* [49] [59] [60] [61].

The micropropagation protocol established in this study showed that biomass production of *Thymus vulgaris* plantlets differs depending on the medium and growth regulators used. Thus, we found that shoots growing on MS, B5 and SH media hold higher dry weights. Also, the integration of certain cytokinins at specific concentrations resulted in an increase of the dry weight, both for shoots (0.93 TDZ and 0.93 2iP) and roots (0.46 2iP). In addition, combinations between the four cytokinins and the three auxins resulted in an increase in biomass, except for 0.46 Kin + 0.57 IBA, 0.46 DPU + 0.57 IBA and 0.46 Ad + 0.57 IAA.

Indeed, indicators of biomass growth have been used in some micropropagation studies on *Thymus* and other *Lamiaceae* species. Thus, the fresh weight of *Thymus caespititius* vitroplants increased by 7.7% - 8.2% on MS medium supplemented with 1.77 µM BAP in combination with 0.49 µM IBA [33]. In addition, an increase in water content and a loss of dry weight were observed on *Thymus daenensis* Celak. plantlets treated with 4.4 µM BAP on MS medium; these cultures developed morphological characteristics of hyperhydricity, with translucency, very short shoots, rigid and abnormal leaf appearance [34]. Also, Fraternale *et al.* [41] reported a maximum biomass increase of *Thymus mastichina* plantlets, estimated through their fresh and dry weight, on an MS medium supplemented with 0.44 µM BAP, alone or in combination with 10 or 20 µg/l of triacontanol. Likewise, it was found that the fresh and dry mass production of the *Thymus vulgaris* vitroplants is maximum on MS medium supplemented with 5 µM BAP, whereas the increase of the concentration of the medium in IAA, Kin or Zeat. causes a decrease in biomass production [18]. Pourebad *et al.* [62] found that increasing MS medium content in TDZ resulted in a decrease in the fresh weight of *Lallemantia iberica* shoots. Besides, the maximum increase in the fresh weight of *Lavandula dentata* vitroplants was noted on MS medium supplemented with 0.44 µM BAP [63]. Moreover, dry weight of *Ocimum basilicum* shoots grown *in vitro* on MS medium is higher than that of those grown on B_5 and WPM, too, this parameter increased after addition of growth regulators, especially in the case of 2.22 µM BAP combined to 11.42 µM IAA, 9.84 µM IBA or 10.70 µM NAA [52].

Moreover, the difficulty of establishing *in vitro* culture from acclimatized plants did not show up only in the high levels of contamination obtained, but also in ensuring a high rate of survival uncontaminated plantlets. This remark was also reported in the case of other *Thymus* species, namely *Thymus moroderi* [28] with a survival rate not exceeding 28.6%, *Thymus bleicherianus* with 36% [30], *Thymus caespititius* with 8% [33] and *Thymus longicaulis* with 29.4% [17], as well as other *Lamiaceae* such as *Lavandula viridis* [64], *Salvia pratensis* and *S. nemorosa* [65] with about 30%. Actually, explants from field grown plants contain many contaminants that affect the survival and growth of vitroplants, bacte-

ria in most cases. These contaminants are usually introduced with initial explants and are resistant to surface sterilization protocols. Furthermore, contamination may reappear after a long culture period if it is endogenous [66].

Despite these difficulties, we noticed that plantlets that survived following established sterilization protocols have a high growth capacity and allow us to obtain a large number of vitroplants after several subcultures.

5. Conclusions

The present study is a complete and effective protocol for micropropagation of *Thymus vulgaris*, extinct species in Morocco. First, the study of the effect of macronutrients allowed us to conclude that, as with most Lamiaceae, MS macronutrients are the most suitable for *in vitro* vegetative propagation of this species. Then, we found that the addition of certain cytokinins, even at low concentrations, to the culture medium, ensures better multiplication and growth of vitroplants, namely 0.46 Kin, 0.46 and 0.93 BAP, 0.46 2iP, 0.46 DPU, 0.46 Ad. and 0.46 Zeat. Then, we optimized the multiplication and rooting of cultures by establishing combinations between Kin, BAP, DPU and Ad. at 0.46 µM with auxins IAA, IBA and NAA at 0.57 µM. Thus, we obtained interesting results for shoot multiplication and rooting for the combinations 0.46 Kin + 0.57 IAA or NAA, 0.46 DPU + 0.57 IBA and 0.46 Ad. + 0.57 IBA.

Finally, the successful acclimatization and obtaining of plants similar to wild plants, plus the possibility to initiate again *in vitro* culture from acclimatized plants, ensure that the present protocol is an interesting tool for large-scale multiplication of *Thymus vulgaris* plantlets and for the selection and preservation of interesting genotypes.

Acknowledgements

This work is carried out as part of the project in the priority areas of scientific research and technological development (PPR2/2016/35), financed by the Ministry of National Education, Vocational Training, Higher Education and Scientific Research, as well as the National Center for Scientific and Technical Research (CNRST, Morocco).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Morales, R. (2002) The History, Botany and Taxonomy of the Genus *Thymus*. In: Stahl-Biskup, E. and Sáez, F., Eds., *Thyme*: *The Genus Thymus*, Taylor and Francis, Inc., London, 1-43.

[2] Rey, C. (1990) Selection of Thyme (*Thymus vulgaris* L.) for Extreme Areas. *Herba-Hungarica*, **29**, 30-33.

[3] Yamaura, T., Tanaka, S. and Tabata, M. (1989) Light-Dependent Formation of Glandular Trichomes and Monoterpenes in Thyme Seedlings. *Phytochemistry*, **28**, 741-744. https://doi.org/10.1016/0031-9422(89)80106-2

[4] Russo, M., Suraci, F., Postorino, S., Serra, D., Roccotelli, A. and Agosteo, G.E. (2013) Essential Oil Chemical Composition and Antifungal Effects on *Sclerotium cepivorum* of *Thymus capitatus* Wild Populations from Calabria, Southern Italy. *Revista Brasileira de Farmacognosia*, **23**, 239-248. https://doi.org/10.1590/S0102-695X2013005000017

[5] Gedikoğlu, A., Sökmen, M. and Çivit, A. (2019) Evaluation of *Thymus vulgaris* and *Thymbra spicata* Essential Oils and Plant Extracts for Chemical Composition, Antioxidant, and Antimicrobial Properties. *Food Science and Nutrition*, **7**, 1704-1719. https://doi.org/10.1002/fsn3.1007

[6] Mancini, E., Senatore, F., Del Monte, D., De Martino, L., Grulova, D., Scognamiglio, M., Snoussi, M. and De Feo, V. (2015) Studies on Chemical Composition, Antimicrobial and Antioxidant Activities of Five *Thymus vulgaris* L. Essential Oils. *Molecules*, **20**, 12016-12028. https://doi.org/10.3390/molecules200712016

[7] Borugă, O., Jianu, C., Mişcă, C., Goleţ, I., Gruia, A.T. and Horhat, F.G. (2014) *Thymus vulgaris* Essential Oil: Chemical Composition and Antimicrobial Activity. *Journal of Medicine and Life*, **7**, 56-60.

[8] Imelouane, B., Amhamdi, H., Wathelet, J.P., Ankit, M., Khedid, K. and El Bachiri, A. (2009) Chemical Composition and Antimicrobial Activity of Essential Oil of Thyme (*Thymus vulgaris*) from Eastern Morocco. *International Journal of Agriculture and Biology*, **11**, 205-208.

[9] Özcan, M. and Chalchat, J.C. (2004) Aroma Profile of *Thymus vulgaris* L. Growing Wild in Turkey. *Bulgarian Journal of Plant Physiology*, **30**, 68-73.

[10] Asha, D. and Lizzy, M. (2017) Chemical Profiling of *Thymus vulgaris* L. Using HPTLC. *Journal of Pharmacognosy and Phytochemistry*, **6**, 1017-1023.

[11] Fayad, N.K., Al-Obaidi, O.H.S., Al-Noor, T.H. and Ezzat, M.O. (2013) Water And Alcohol Extraction Of Thyme Plant (*Thymus vulgaris*) and Activity Study Against Bacteria, Tumors And Used as Anti-Oxidant in Margarine Manufacture. *Innovative Systems Design and Engineering*, **4**, 41-51.

[12] Damianova, S., Tasheva, S., Stoyanova, A. and Damianov, D. (2008) Investigation of Extracts from Thyme (*Thymus vulgaris* L.) for Application in Cosmetics. *Journal of Essential Oil Bearing Plants*, **11**, 443-450. https://doi.org/10.1080/0972060X.2008.10643652

[13] Chaturvedi, H.C., Jain, M. and Kidwai, N.R. (2007) Cloning of Medicinal Plants through Tissue Culture—A Review. *Indian Journal of Experimental Biology*, **45**, 937-948.

[14] Rout, G.R., Samantaray, S. and Das, P. (2000) *In Vitro* Manipulation and Propagation of Medicinal Plants. *Biotechnology Advances*, **18**, 91-120. https://doi.org/10.1016/S0734-9750(99)00026-9

[15] Furmanowa, M. and Olszowska, O. (1992) Micropropagation of Thyme (*Thymus vulgaris* L.). In: Bajaj, Y.P.S., Ed., *High Tech and Micropropagation III*, Springer-Verlag, Inc., Heidelberg, 230-243. https://doi.org/10.1007/978-3-662-07770-2_14

[16] Karalija, E. and Parić, A. (2011) The Effect of BA and IBA on the Secondary Metabolite Production by Shoot Culture of *Thymus vulgaris* L. *Biologica Nyssana*, **2**, 29-35.

[17] Ozudogru, E.A., Kaya, E., Kirdok, E. and Issever-Ozturk, S. (2011) *In Vitro* Propa-

gation from Young and Mature Explants of Thyme (*Thymus vulgaris* and *T. longicaulis*) resulting in Genetically Stable Shoots. *In Vitro Cellular and Developmental Biology-Plant*, **47**, 309-320. https://doi.org/10.1007/s11627-011-9347-6

[18] Affonso, V.R., Bizzo, R.H., Lage, C.L.S. and Sato, A. (2009) Influence of Growth Regulators in Biomass Production and Volatile Profile of *in Vitro* Plantlets of *Thymus vulgaris* L. *Journal of Agricultural and Food Chemistry*, **57**, 6392-6395. https://doi.org/10.1021/jf900816c

[19] Tisserat, B., Vaughn, S.F. and Silman, R. (2002) Influence of Modified Oxygen and Carbon Dioxide Atmospheres on Mint and Thyme Plant Growth, Morphogenesis and Secondary Metabolism *in Vitro*. *Plant Cell Reports*, **20**, 912-916. https://doi.org/10.1007/s00299-001-0428-6

[20] Shah, R.R. and Dalal, K.C. (1980) *In Vitro* Multiplication of *Glycyrrhiza*. *Current Science India*, **49**, 69-71.

[21] Murashige, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiologia Plantarum*, **15**, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[22] Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient Requirements of Suspension Cultures of Soybean Root Cells. *Experimental Cell Research*, **50**, 151-158. https://doi.org/10.1016/0014-4827(68)90403-5

[23] Schenk, R. and Hildebrandt, A. (1972) Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Cultures. *Canadian Journal of Botany*, **50**, 199-204. https://doi.org/10.1139/b72-026

[24] Badoc, A. (1982) Contribution à l'Etude des Phénomènesd'Organogenèseet de Callogenèse de Tissus de Fenouil Vulgaire (*Foeniculum vulgare* subsp. *capillaceum* var. vulgare (mill.) thellung) Cultivés *in Vitro*; Analyse des Constituants des HuilesEssentielles des Explants. Diplômed'Etudes Approfondies, Université de Lille, Flandres Artois, 74 p.

[25] Margara, J. (1978) Mise au point d'unegamme de milieuxminéraux pour les conditions de la culture *in Vitro*. *Comptes Rendus de l'Académied'Agriculture de France*, **64**, 654-661.

[26] Dolatabadi, H.K., Goltapeh, E.M., Moieni, A., Jaimand, K., Sardrood, B.P. and Varma, A. (2011) Effect of *Piriformospora indica* and *Sebacina vermifera* on Plant Growth and Essential Oil Yield in *Thymus vulgaris in Vitro* and *in Vivo* Experiments. Symbiosis, **53**, 29-35. https://doi.org/10.1007/s13199-010-0104-0

[27] Bernard, F., NavabMoghadam, N. and Mirzajani, F. (2015) The Effect of Colloidal Silver Nanoparticles on the Level of Lignification and Hyperhydricity Syndrome in *Thymus daenensis Vitro* Shoots: A Possible Involvement of Bonded Polyamines. *In Vitro Cellular & Developmental Biology-Plant*, **51**, 546-553. https://doi.org/10.1007/s11627-015-9700-2

[28] Marco-Medina, A. and Casas, J.L. (2015) *In Vitro* Multiplication and Essential Oil Composition of *Thymus moroderi* Pau ex Martinez, an Endemic Spanish Plant. *Plant Cell, Tissue and Organ Culture*, **120**, 99-108. https://doi.org/10.1007/s11240-014-0583-4

[29] Bakhtiar, Z., Mirjalili, M.H., Sonboli, A., MoridiFarimani, M. and Ayyari, M. (2014) *In Vitro* Propagation, Genetic and Phytochemical Assessment of *Thymus persicus*-a Medicinally Important Source of Pentacyclic Triterpenoids. *Biologia*, **69**, 594-603. https://doi.org/10.2478/s11756-014-0346-z

[30] Nordine, A. and El Meskaoui, A. (2014) Rapid *in Vitro* Regeneration and Clonal Multiplication of *Thymus bleicherianus* Pomel, a Rare and Threatened Medicinal

and Aromatic Plant in Morocco. *Medicinal and Aromatic Plants*, **3**, 145.

[31] Nordine, A., Hmamouchi, M. and El Meskaoui, A. (2014) *In Vitro* Clonal Propagation through Direct Shoot Organogenesis of *Thymus broussonetii*—A Vulnerable Aromatic and Medicinal Plant Species. *International Journal of Pharmaceutical Research and Bio-Science*, **3**, 425-439.

[32] Mirshekar, A., Honarvar, M., Mohammadi, F. and Alizadeh, A. (2014) Optimization of Tissue Culture of *Thymus daenensis* Celak. *American-Eurasian Journal of Agricultural & Environmental Sciences*, **14**, 949-953.

[33] Mendes, M.D., Figueiredo, A.C., Oliveira, M.M. and Trindade, H. (2013) Essential Oil Production in Shoot Cultures versus Field-Grown Plants of *Thymus caespititius*. *Plant Cell, Tissue and Organ Culture*, **113**, 341-351. https://doi.org/10.1007/s11240-012-0276-9

[34] Hassannejad, S., Bernard, F., Mirzajani, F. and Gholami, M. (2012) SA Improvement of Hyperhydricity Reversion in *Thymus daenensis* Shoots Culture May Be Associated with Polyamines Changes. *Plant Physiology and Biochemistry*, **51**, 40-46. https://doi.org/10.1016/j.plaphy.2011.10.006

[35] Coelho, N., Gonçalves, S., González-Benito, M.E. and Romano, A. (2012) Establishment of an *in Vitro* Propagation Protocol for *Thymus lotocephalus*, a Rare Aromatic Species of the Algarve (Portugal). *Plant Growth Regulation*, **66**, 69-74. https://doi.org/10.1007/s10725-011-9630-x

[36] Costa, P., Gonçalves, S., Valentão, P., Andrade, P.B., Coelho, N. and Romano, A. (2012) *Thymus lotocephalus* Wild Plants and *in Vitro* Cultures Produce Different Profiles of Phenolic Compounds with Antioxidant Activity. *Food Chemistry*, **135**, 1253-1260.

[37] Nordine, A., TlemcaniChendid, R. and El Meskaoui, A. (2013) Micropropagation of *Thymus satureioides* Coss. an Endangered Medicinal Plant of Morocco. *Journal of Agricultural Technology*, **9**, 487-501.

[38] Driver, J.A. and Kuniyuki, A.H. (1984) *In Vitro* Propagation of Paradox Walnut. *HortScience*, **19**, 507-509.

[39] Pérez-Tortosa, V., Lopez-Orenes, A., Martinez-Pérez, A., Ferrer, M.A. and Calderón, A.A. (2012) Antioxidant Activity and Rosmarinic Acid Changes in Salicylic Acid-Treated *Thymus membranaceus* Shoots. *Food Chemistry*, **130**, 362-369. https://doi.org/10.1016/j.foodchem.2011.07.051

[40] Gavazzi, A., Tonelli, G., Todesco, G., Arreghini, E., Raffaldi, F., Barbuzzi, G. and Biasini, M.G. (1987) Somaclonal Variation versus Chemically Induced Mutagenesis in Tomato (*Lycopersicon esculentum* L.). *Theoritical and Applied Genetics*, **74**, 733-738. https://doi.org/10.1007/BF00247550

[41] Fraternale, D., Giamperi, L., Ricci, D., Rocchi, M.B.L., Guidi, L., Epifano, F. and Marcotullio, M.C. (2003) The Effect of Triacontanol on Micropropagation and on Secretory System of *Thymus mastichina*. *Plant Cell, Tissue and Organ Culture*, **74**, 87-97. https://doi.org/10.1023/A:1023321024040

[42] Collet, G.F. (1985) EnracinementAméliorélors de la Production *in Vitro* de Rosiers. *Revue Suisse de Viticulture Arboriculture Horticulture*, **17**, 259-263.

[43] Sáez, F., Sánchez, P. and Piqueras, A. (1994) Micropropagation of *Thymus piperella*. *Plant Cell, Tissue and Organ Culture*, **39**, 269-272. https://doi.org/10.1007/BF00035981

[44] Nitsch, J.P. and Nitsch, C. (1969) Haploid Plants from Pollen Grains. *Science*, **163**, 85-87. https://doi.org/10.1126/science.163.3862.85

[45] Abdallah, S.A.S., Yakoup, M.Y.A. and Abdalla, M.Y.H. (2017) Micropropagation of Oregano (*Origanum syriacum* L.) through Tissue Culture Technique. *Mansoura Journal of Plant Production*, **8**, 635-639. https://doi.org/10.21608/jpp.2017.40497

[46] Özkum, D., Kürkçüoğlu, M., Başer, K.H.C. and Tipirdamaz, R. (2010) Essential Oils from Wild and Micropropagated Plants of *Origanum minutiflorum* O. Schwarz et Davis. *Journal of Essential Oil Research*, **22**, 135-137. https://doi.org/10.1080/10412905.2010.9700284

[47] Nordine, A, Bousta, D., El Khanchoufi, A. and El Meskaoui, A. (2013b). An Efficient and Rapid *in Vitro* Propagation System of *Thymus hyemalis* Lange, a Wild Medicinal and Aromatic Plant of Mediterranean Region. *International Journal of Pharma, Bioscience and Technology*, **1**, 118-129.

[48] White, P.R. (1963) The Cultivation of Animal and Plant Cells. Ronald Press, New York, 228 p.

[49] Arikat, N.A., Jawad, F.M., Karam, N.S. and Shibli, R.A. (2004) Micropropagation and Accumulation of Essential Oils in Wild Sage (*Salvia fruticosa* Mill.). *Scientia Horticulturae*, **100**, 193-202. https://doi.org/10.1016/j.scienta.2003.07.006

[50] Yavuz, D.Ö. (2016) Optimization of Regeneration Conditions and *in Vitro* Propagation of *Sideritis Stricta* Boiss & Heldr. *International Journal of Biological Macromolecules*, **90**, 59-62. https://doi.org/10.1016/j.ijbiomac.2015.10.064

[51] Lê, C.L. (1989) Microbouturage *in Vitro* du Thym (*Thymus vulgaris* L.). *Revue Suisse Viticulture Arboriculture Horticulture*, **21**, 355-358.

[52] Monfort, L.E.F., Bertolucci, S.K.V., Lima, A.F., de Carvalho, A.A., Mohammed, A., Blank, A.F. and Pinto, J.E.B.P. (2018) Effects of Plant Growth Regulators, Different Culture Media and Strength MS on Production of Volatile Fraction Composition in Shoot Cultures of *Ocimum basilicum*. *Industrial Crops & Products*, **116**, 231-239. https://doi.org/10.1016/j.indcrop.2018.02.075

[53] Morone-Fortunato, I. and Avato, P. (2008) Plant Development and Synthesis of Essential Oils in Micropropagated and *Mycorrhiza* Inoculated Plants of *Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart. *Plant Cell, Tissue and Organ Culture*, **93**, 139-149. https://doi.org/10.1007/s11240-008-9353-5

[54] Sunandakumari, C., Martin, K.P., Chithra, M., Sini, S. and Madhusoodanan, P.V. (2004) Rapid Axillary Bud Proliferation and *ex Vitro* Rooting of Herbal Spice *Mentha piperita* L. *Indian Journal of Biotechnology*, **3**, 108-112.

[55] Grzegorczyk, I., Bilichowski, I., Mikiciuk-Olasik, E. and Wysokińska, H. (2005) *In Vitro* Cultures of *Salvia officinalis* L. as a Source of Antioxidant Compounds. *Acta Societatis Botanicorum Poloniae*, **74**, 17-21. https://doi.org/10.5586/asbp.2005.003

[56] Dode, L.B., Bobrowski, V.L., Braga, E.J.B., Seixas, F.K. and Schuch, M.W. (2003) *In Vitro* Propagation of *Ocimum basilicum* L. (Lamiaceae). Acta Scientiarum. *Biological Sciences*, **25**, 435-437. https://doi.org/10.4025/actascibiolsci.v25i2.2034

[57] Stojakowska, A., Malarz, J. and Kohlmünzer, S. (1999) Micropropagation of *Scutellaria baicalensis* Georgi. *Acta Societatis Botanicorum Poloniae*, **68**, 103-107. https://doi.org/10.5586/asbp.1999.015

[58] Cuenca, S. and Amo-Marco, J.B. (2000) *In Vitro* Propagation of Two Spanish Endemic Species of *Salvia* through Bud Proliferation. *In Vitro Cellular & Developmental Biology-Plant*, **36**, 225-229. https://doi.org/10.1007/s11627-000-0042-2

[59] Ramak, P., Sharifi, M., Osaloo, S.K., Ebrahimzadeh, H. and Behmanesh, M. (2011) Studies on Seed Germination and *in Vitro* Shoot Multiplication of *Satureja khuzistanica* Jamzad, an Important Medicinal Plant. *African Journal of Biotechnology*, **10**, 19407-19414. https://doi.org/10.5897/AJB11.1311

[60] Saha, S., Dey, T. and Ghosh, P. (2010) Micropropagation of *Ocimum kilimandscharicum* Guerke (Labiatae). *Acta Biologica Cracoviensia Series Botanica*, **52**, 50-58. https://doi.org/10.2478/v10182-010-0023-7

[61] Nobre, J. (1996) *In Vitro* Cloning and Micropropagation of *Lavandula Stoechas* from Field-Grown Plants. *Plant Cell, Tissue and Organ Culture*, **46**, 151-155. https://doi.org/10.1007/BF00034849

[62] Pourebad, N., Motafakkerazad, R., Kosari-Nasab, M., Akhtar, N.F. and Movafeghi, A. (2015) The Influence of TDZ Concentrations on *in Vitro* Growth and Production of Secondary Metabolites by the Shoot and Callus Culture of *Lallemantiaiberica*. *Plant Cell, Tissue and Organ Culture*, **122**, 331-339. https://doi.org/10.1007/s11240-015-0769-4

[63] Sudriá, C., Piñol, M.T., Palazón, J., Cusidó, R.M., Vila, R., Morales, C., Bonfill, M. and Cañigueral, S. (1999) Influence of Plant Growth Regulators on the Growth and Essential Oil Content of Cultured *Lavandula dentata* Plantlets. *Plant Cell, Tissue and Organ Culture*, **58**, 177-184. https://doi.org/10.1023/A:1006377003962

[64] Dias, M.C., Almeida, R. and Romano, A. (2002) Rapid Clonal Multiplication of *Lavandula viridis* L'Hér through *in Vitro* Axillary Proliferation. *Plant Cell, Tissue and Organ Culture*, **68**, 99-102. https://doi.org/10.1023/A:1012963021126

[65] Ruffoni, B., Savona, M., Capponi, A., Campagna, G. and Cervelli, C. (2009) Micropropagation of *Salvia pratensis* L. and *Salvia nemorosa* L. Accessions Selected for Ornamental Characters. *Acta Horticulturae*, **812**, 201-203. https://doi.org/10.17660/ActaHortic.2009.812.24

[66] Thakur, R. and Sood, A. (2006) An Efficient Method for Explant Sterilization for Reduced Contamination. *Plant Cell, Tissue and Organ Culture*, **84**, 369-371. https://doi.org/10.1007/s11240-005-9034-6