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Micropropagation of the Moroccan Endemic Plant *Thymus broussonetii* Boiss. with Aromatic-Medicinal Value and Conservation Concern

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Abstract

Micropropagation from shoot tips and nodal segments was carried out for the conservation and domestication of spontaneous Moroccan thyme, *Thymus broussonetii* Boiss. subsp. *broussonetii* (endemic threatened). The mineral composition of the culture medium, as well as the succession of different growth regulators, influenced the *in vitro* growth of this species. Sterilized achenes of *T. broussonetii* were able to germinate on an agar medium containing Gautheret macronutrients with a rate of 25% and a degree of contamination of less than 4%. Shoot apices of 15-day seedlings (two cotyledon leaves) were cultivated on SD + 0.46 µM Kin medium and the explants obtained were transplanted every month. Six macronutrients (MS, B_5, SH, SD, MS_m and $N_{30}K$) were tested and $N_{30}K$ was chosen for the following experiments. Seven cytokinins (Kin, BAP, 2iP, DPU, adenine, Zeat and TDZ) at 0.46, 0.93 and 2.32 µM/l were evaluated and the addition of 0.93 µM adenine to $N_{30}K$ medium favored significantly the induction of buds and the elongation of explants. Three polyamines (putrescine, spermidine and spermine) at 2, 5, 10 and 20 µM/l were tested. A better multiplication of buds, shoots and roots was noted for $N_{30}K$ + 10 µM spermine. Cytokinin-auxin combinations led to better root multiplication and an increase in the number of buds and the length of explants, particularly for 0.46 µM Kin + 2.85 µM IAA. Acclimatization was successfully carried out using vitroplants developing a good root system. One month after the start of acclimatization,

97% of *T. broussonetii* plantlets were healthy. Three months later, they were transplanted into larger pots. 100% of the acclimatized plants developed flowers in the 2nd year between June and August. Re-initiation of the *in vitro* culture was carried out from sterilized twig segments collected from the acclimatized plants of *T. broussonetii* with 1 - 2 nodes on the medium $N_{30}K$ + 0.46 µM Kin, and 52.1% of the explants healthily proliferated. Finally, two micropropagation prototypes were developed: shoot tip culture from seedlings obtained after germination of achenes and node culture from acclimatized plants.

Keywords

Thymus broussonetii, Micropropagation, Shoot Tip, Nodes, Macronutrients, Cytokinins, Polyamines, Auxins, Prototypes, Conservation

1. Introduction

Thymus broussonetii Boiss. is a very rare medicinal and aromatic plant which exists in the form of two subspecies: subsp. *hannonis* (Maire) R. Morales endemic to Morocco and subsp. *broussonetii* endemic to Morocco, Algeria and Tunisia [1] [2] [3]. In Morocco, it is found in the High Atlas, in northern and middle Atlantic Morocco [2].

Thymus broussonetii is a small shrub 12 to 40 cm height with flat leaves ciliated at the base with whole edges; flowers are gathered towards the top of shoots in dense oval-cylindrical inflorescences, with floral leaves wider than the leaves, often colored with purple, attenuated and sharp at the end, with cilia on the edges; the calyx, 6 - 7.5 mm long, has 2 lips, the upper not very toothed; the corolla is pink to purple, 2 to 3 times longer than the calyx, with a distinctly prominent narrow tube. The subsp. *hannonis* differs from the type by its smaller size (12 vs. 15 - 40 cm), its shorter leaves (5 - 10 vs. 10 - 18 mm) not subpetiolate (2.5 vs. 1 mm), and floral bracts densely covered by longer hairs and not only on abaxial side [4] [5].

Moreover, the essential oil of *Thymus broussonetii* is mainly rich in thymol, carvacrol, borneol, *ρ*-cymene, *α*-pinene and *γ*-terpinene [6] [7] [8] [9]. These bioactive compounds attribute to this species its biological properties as antimicrobial, [10] [11], anti-inflammatory [12], antinociceptive [13] and antioxidant [7]. The stimulation of the immunizing system and the protection against the stress by a neurotropic activity was attributed to aqueous and ethyl acetate extracts from *T. broussonetii* that increased *in vivo* the number of leucocyte categories studied including polynuclears, total lymphocytes, TCD4+, TCD8+ and NK cells [14]. Also, an antitumor effect of *T. broussonettii* essential oil was proved against the human ovarian adenocarcinoma IGR-OV1 parental cell line OV1/P resistant to chemotherapy [15]. In addition, the essential oil was able to inhibit efflux pump systems of resistant Gram-negative bacteria, such as *Esche-*

richia coli, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella enterica* serotype *Typhimurium* and *Pseudomonas aeruginosa* [16]. However, the levels of accumulation of bioactive compounds are very low and a large quantity of plant material is therefore needed [17].

Actually, the excessive pressure exerted on wild plant populations, by harvesting from their natural habitat, combined with land conversion, has resulted in a reduced and scattered distribution of this species. Its harvest takes place mainly during the flowering period before the formation of seeds. This causes a decrease in the rate of regeneration and a progressive degradation of wild populations [18]. Besides, the biosynthesis of secondary metabolites, although genetically controlled, is strongly affected by environmental conditions [19]. In addition, the market of natural products requires an efficient system of production of plant tissues, free from seasonal and somatic variations, infections by bacteria and fungi, as well as environmental pollution likely to affect the economic value of collected tissues [20]. Indeed, micropropagation is one of the techniques that can be applied for the large-scale propagation of *T. broussonetii*, through the production of a large number of genetically similar and disease-free plants, in a short period of time and in a small space. Nordine *et al.* (2014) [17] have already applied this technique for the same species.

The present study completes the already established micropropagation protocol of this species, through the evaluation of the effect of several macronutrients, cytokinins, polyamines and auxins, in addition to the re-initiation of *in vitro* culture from acclimatized plants.

2. Material and Methods

2.1. Plant Material

Thymus broussonetii Boiss. subsp. *broussonetii* achenes were provided by the National Institute of Agronomic Research (INRA) of Rabat and were used as a source of plant material.

2.2. Seed Germination

2.2.1. Seed Sterilization

Achene surface was sterilized according to the following protocol:

- Immersion in a filtered solution of calcium hypochlorite ($Ca(ClO)_2$) 7% (w/v), containing a few drops of Tween 80 for 15 minutes;
- Rinsing with sterile distilled water for 5 minutes;
- Immersion in mercury chloride ($HgCl_2$) solution 0.1% (w/v) for 2 minutes;
- Three successive rinses with sterile distilled water (5, 10 and 15 minutes).

Achenes were then soaked for 48 h in sterile distilled water in 250 ml conical flasks installed on a rotary shaker (12 rpm, Fisher Scientific).

2.2.2. *In Vitro* Germination

After imbibition, seeds were germinated *in vitro* into glass test tubes (18 × 180

mm), one seed per tube, this latter containing 15 ml of a culture medium composed of Gautheret macronutrients [21] and Murashige and Skoog (MS, 1962) [22] micronutrients, solidified with 0.7% bacteriological agar, previously sterilized at 121˚C. The tubes were placed in a culture room, with a temperature of 24˚C ± 1˚C and 60% of relative humidity. The lighting was supplied 18 hours a day by fluorescent tubes (4000 lux, Philips). Germinated seeds were counted 24 hours after the beginning of the experiment. Germination was considered complete when the radicle pierced the seminal envelopes.

2.2.3. Shoot Tip and Node Culture

The 2-week-old seedlings resulting from *in vitro* germination were used in the following experiments, since their organs (hypocotyls, cotyledons and shoot tip) have developed and their roots were short.

Thus, cultures were induced from nodal segments (5 - 6 mm) with axillary buds obtained from 4-week-old aseptic seedlings, on a medium solidified with 0.7% bacteriological agar, containing Shah and Dalal (SD, 1980) [23] macronutrients, Murashige and Skoog (MS, 1962) [22] micronutrients and vitamins, 100 mg/l myo-inositol, 3% sucrose and 0.46 µM/l kinetin. Seedlings were transplanted in the same medium until enough plantlets were available to establish experiments. The vitroplants were incubated in a culture room (photoperiod: 18/6 h with 4000 lux light intensity, temperature: 24˚C ± 1˚C).

2.3. Effect of Macronutrients

Six solutions of macronutrients differing in nitrogen content (NO_3^- and NH_4^+,) and in potassium, all added with MS micronutrients and vitamins, were tested: MS [22], B_5 [24], SH [25], SD [23], modified MS (MS_m) according to Badoc (1982) [26] and $N_{30}K$ [27]. The mineral solution chosen is used for all the following experiments.

2.4. Effect of Cytokinins

Seven cytokinins (Sigma-Aldrich): Kin (kinetin), BAP (6-benzylaminopurine), 2iP (2-isopentenyladenine), DPU (1,3-diphenylurea), adenine, Zeat (zeatin) and TDZ (thidiazuron) were evaluated on *Thymus broussonetii* plantlets growth. Three concentrations were tested: 0.46, 0.93 and 2.32 µM/l, plus a control medium containing no growth regulator.

2.5. Effect of Polyamines

Three polyamines (putrescine, spermidine and spermine, Sigma-Aldrich) were tested at four concentrations: 2, 5, 10 and 20 µM/l.

2.6. Effect of 0.46 Kin or 0.46 Adenine Combined to Auxins

Kin or adenine at 0.46 µM/l were tested, alone or combined to three auxins (Sigma-Aldrich): IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid) and NAA (1-Naphthaleneacetic acid) at five concentrations: 0.057, 0.3, 0.57, 2.85 or

5.71 µM/l.

2.7. 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 plastic pots (9 × 6 × 8 cm), 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. After complete elimination of the cups, 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 (17 × 13 × 15 cm).

2.8. Re-Initiation of *in Vitro* Culture of *Thymus Broussonetii* from Acclimatized Plants

Twigs were cut from the acclimatized plants of *Thymus broussonetii*, thoroughly washed with tap water, then surface sterilized under a laminar flow hood. Several sterilization methods, differing by the products used for this purpose, were tested. The chosen method is based on soaking twigs first in 10% $Ca(ClO)_2$ (w/v) with 4 to 5 drops of Tween 80 for 30 min, then in 0.1% $HgCl_2$ (w/v) with 4 to 5 drops of Tween 80 for 5 min and finally dipping three times in sterile 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 a medium composed of $N_{30}K$ [27] macronutrients, Murashige and Skoog (1962) [22] micronutrients and vitamins, 100 mg/l myo-inositol, 3% sucrose and 0.46 µM/l Kin. After multiplication, plantlets were transferred to bigger flasks.

2.9. Culture Conditions

The culture media were supplemented with 3% sucrose and 0.7% bacteriological agar. The pH of the media was adjusted to 5.6 - 5.8 using sodium hydroxide (NaOH 1 N). Sterilization of the culture media 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 intensity, temperature: 24˚C ± 1˚C).

2.10. 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;
- Rooting rate (%);
- Mean number of roots per plantlet;
- Hyperhydricity rate (%).

2.11. 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. Achenes Germination

Germination of *Thymus broussonetii* achenes does not begin until 48 hours. The final germination rate is 25% and the degree of contamination does not exceed 4% (Figure 1).

3.2. Effect of Macronutrients

The differences between the six macronutrients in terms of regeneration and rooting are not significant contrarily to hyperhydricity. $N_{30}K$ macronutrients ensure total regeneration of the vitroplants, followed by MS and MS_m (93.1). SD macronutrients show the best rooting rate (71.2%), followed by SH (67.7) and $N_{30}K$ (62.5). Hyperhydricity appears for MS_m (31.2%) and MS (41.6) and is absent for B_5 and SD.

In addition, a better shoots elongation is noticed in the case of MS (3.04 cm) and SD (2.91). The minimum length is observed for SH (2.08). The multiplication of shoots and buds is approximately similar for the six macronutrients, with a maximum number of shoots in the case of $N_{30}K$ (3.6), followed by MS (3.1). The best number of buds is noted in the case of $N_{30}K$ (28.6), followed by B_5 (27.5). Root multiplication is optimal in the case of SD and MS (4.58 and 4.44, respectively), followed by SH and $N_{30}K$ (4.02 and 3.80, respectively).

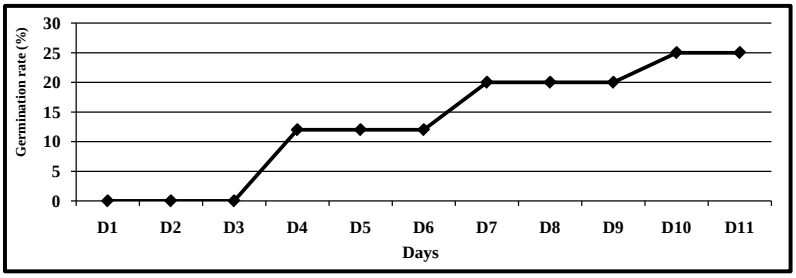

Figure 1. Monitoring curve of *Thymus broussonetii* germination. Culture medium: Gautheret macronutrients + Murashige and Skoog micronutrients + 0.7% bacteriological agar. Temperature: 24˚C ± 1˚C, relative humidity: 60%, photoperiod: 18/6 hours, light intensity: 4000 lux.

In short, B_5 and SD macronutrients have lower regeneration rates and MS and MS_m show very high hyperhydricity rates (41.6% and 31.2%, respectively). Between SH and $N_{30}K$ macronutrients, the second ones were selected, since they ensure total regeneration and better multiplication of buds and shoots, with a low hyperhydricity rate (Table 1, Figure 2 and Figure 7).

3.3. Effect of Cytokinins

The integration of cytokinins into the culture media has produced several changes in the aerial as in the root parts of the vitroplants (Table 2, Figure 3 and Figure 7).

Thus, a total regeneration of the explants is noted for cytokinins 2iP and adenine at all concentrations, at 0.46 µM for DPU, Zeat and TDZ, as well as 0.93 µM DPU and 2.32 µM Zeat. The lowest rates are observed in the case of 2.32 µM Kin (80.6%), as well as BAP (83.3) and TDZ (87.5) at 0.93 µM.

Among the regenerated explants, 100% develop roots in the case of DPU and Kin at 2.32 µM, as well as BAP and adenine at 0.93 µM; 98.6% for 2.32 µM adenine; 97.2% for 0.46 µM adenine and 0.93 µM DPU. Very low rooting rates were noted in the case of 0.93 and 2.32 µM TDZ (11.7 and 4.5, respectively), besides 0.46 µM Zeat (37.5).

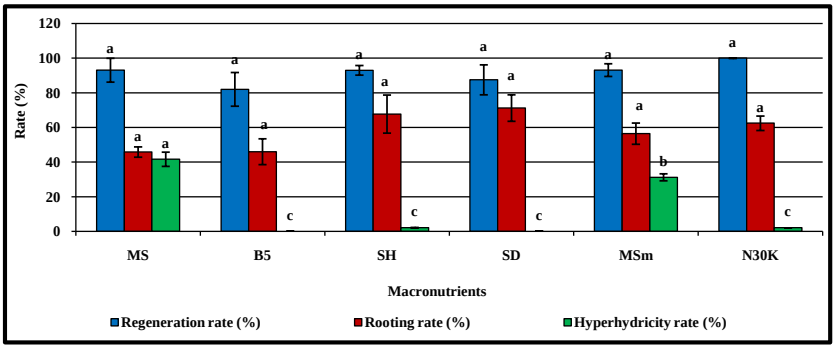

Figure 2. Effect of six macronutrients on the regeneration, rooting and hyperhydricity rates of *Thymus broussonetii* explants.

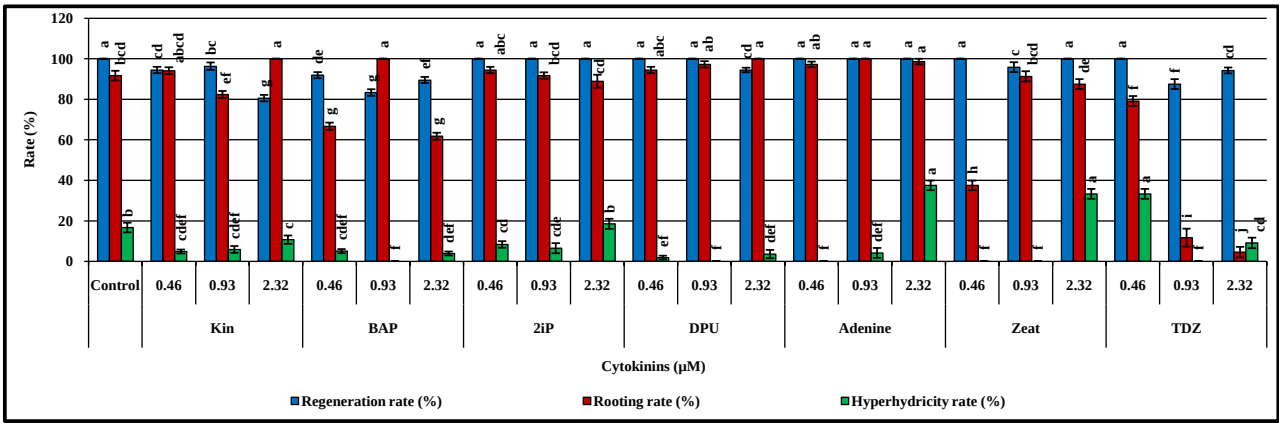

Figure 3. Effect of seven cytokinins at three concentrations on the regeneration, rooting and hyperhydricity rates of *Thymus broussonetii* explants.

Table 1. Effect of six macronutrients on the micropropagation of *Thymus broussonetii*.

Macronutrients	Regeneration rate (%)	Shoots length (cm)	Number of shoots	Number of buds	Rooting rate (%)	Number of roots	Hyperhydricity rate (%)
MS	93.1 ± 6.9 ^{a}	3.04 ± 0.15 ^{a}	3.15 ± 0.22 ^{ab}	27.08 ± 1.01 ^{ab}	45.8 ± 23.0 ^{a}	4.44 ± 0.54 ^{a}	41.6 ± 4.1 ^{a}
B_5	82.0 ± 9.7 ^{a}	2.14 ± 0.12 ^{b}	2.62 ± 0.18 ^{b}	27.50 ± 1.19 ^{a}	46.0 ± 7.4 ^{a}	3.14 ± 0.37 ^{ab}	0.0 ± 0.0 ^{c}
SH	93.0 ± 2.8 ^{a}	2.08 ± 0.08 ^{b}	2.75 ± 0.17 ^{b}	25.82 ± 0.89 ^{abc}	67.7 ± 11.0 ^{a}	4.02 ± 0.59 ^{ab}	2.1 ± 0.0 ^{c}
SD	87.5 ± 8.7 ^{a}	2.91 ± 0.16 ^{a}	2.64 ± 0.15 ^{b}	23.37 ± 0.68 ^{c}	71.2 ± 7.6 ^{a}	4.58 ± 0.50 ^{a}	0.0 ± 0.0 ^{c}
MS_m	93.1 ± 3.7 ^{a}	2.14 ± 0.12 ^{b}	3.06 ± 0.21 ^{ab}	24.96 ± 1.10 ^{bc}	56.4 ± 6.2 ^{a}	2.70 ± 0.35 ^{b}	31.2 ± 2.0 ^{b}
$N_{30}K$	100.0 ± 0.0 ^{a}	2.36 ± 0.11 ^{b}	3.64 ± 0.25 ^{a}	28.64 ± 0.95 ^{a}	62.5 ± 4.2 ^{a}	3.80 ± 0.36 ^{ab}	2.1 ± 0.0 ^{c}

The data represent mean ± SE of replicates (n = 3). Values in a same row carrying different letters are significantly different by Duncan's multiple range test at $p \leq 0.05$.

Table 2. Effect of seven cytokinins at three concentrations on the micropropagation of *Thymus broussonetii*.

Cytokinins (µM)		Regeneration rate (%)	Shoots length (cm)	Number of shoots	Number of buds	Rooting rate (%)	Number of roots	Hyperhydricity rate (%)
Control		100.0 ± 0.0 ^{a}	5.58 ± 0.49 ^{b}	1.96 ± 0.20 ^{abc}	20.67 ± 1.45 ^{bc}	91.7 ± 2.4 ^{bcd}	8.81 ± 1.10 ^{bc}	16.7 ± 2.4 ^{b}
Kin	0.46	94.4 ± 1.6 ^{cd}	2.94 ± 0.25 ^{hij}	1.88 ± 0.14 ^{bc}	19.97 ± 1.01 ^{bc}	94.1 ± 1.7 ^{abcd}	5.61 ± 0.57 ^{cde}	4.9 ± 0.9 ^{cdef}
	0.93	96.3 ± 1.8 ^{bc}	3.29 ± 0.35 ^{fghij}	1.59 ± 0.13 ^{cd}	18.88 ± 0.90 ^{c}	82.3 ± 1.7 ^{ef}	6 .74 ± 0.87 ^{bcd}	5.9 ± 1.7 ^{cdef}
	2.32	80.6 ± 1.6 ^{g}	3.87 ± 0.36 ^{efgh}	2.07 ± 0.14 ^{ab}	21.14 ± 1.34 ^{abc}	100.0 ± 0.0 ^{a}	7.18 ± 0.71 ^{bcd}	10.7 ± 2.1 ^{c}
BAP	0.46	91.9 ± 1.6 ^{de}	2.57 ± 0.13 ^{ij}	2.36 ± 0.19 ^{ab}	21.27 ± 1.17 ^{ab}	66.7 ± 1.7 ^{g}	5.36 ± 0.84 ^{de}	5.0 ± 1.0 ^{cdef}
	0.93	83.3 ± 1.6 ^{g}	2.37 ± 0.26 ^{j}	1.66 ± 0.12 ^{cd}	17.38 ± 1.02 ^{c}	100.0 ± 0.0 ^{a}	4.93 ± 0.57 ^{def}	0.0 ± 0.0 ^{f}
	2.32	89.5 ± 1.5 ^{ef}	2.35 ± 0.11 ^{j}	1.94 ± 0.16 ^{abc}	18.82 ± 0.93 ^{c}	61.8 ± 1.7 ^{g}	4.05 ± 0.42 ^{def}	3.9 ± 0.9 ^{def}
2iP	0.46	100.0 ± 0.0 ^{a}	3.58 ± 0.25 ^{efghi}	1.97 ± 0.15 ^{ab}	19.44 ± 0.94 ^{bc}	94.4 ± 1.6 ^{abc}	7.58 ± 0.69 ^{bcd}	8.3 ± 1.6 ^{cd}
	0.93	100.0 ± 0.0 ^{a}	4.98 ± 0.39 ^{bcd}	1.64 ± 0.20 ^{cd}	21.53 ± 1.00 ^{ab}	91.7 ± 1.6 ^{bcd}	9.39 ± 0.81 ^{bc}	6.5 ± 2.4 ^{cde}
	2.32	100.0 ± 0.0 ^{a}	5.24 ± 0.37 ^{bc}	1.75 ± 0.12 ^{c}	19.83 ± 0.70 ^{bc}	88.9 ± 3.2 ^{cd}	8.03 ± 0.62 ^{bcd}	18.5 ± 2.4 ^{b}
DPU	0.46	100.0 ± 0.0 ^{a}	4.04 ± 0.24 ^{defg}	1.92 ± 0.15 ^{abc}	23.11 ± 1.06 ^{ab}	94.4 ± 1.6 ^{abc}	7.67 ± 0.99 ^{bcd}	1.8 ± 0.9 ^{ef}
	0.93	100.0 ± 0.0 ^{a}	5.65 ± 0.46 ^{b}	1.75 ± 0.14 ^{c}	24.83 ± 1.25 ^{a}	97.2 ± 1.6 ^{ab}	10.03 ± 0.83 ^{ab}	0.0 ± 0.0 ^{f}
	2.32	94.4 ± 1.1 ^{cd}	5.59 ± 0.37 ^{b}	2.07 ± 0.21 ^{ab}	24.50 ± 1.59 ^{a}	100.0 ± 0.0 ^{a}	9.75 ± 0.55 ^{abc}	3.6 ± 2.1 ^{def}
Adenine	0.46	100.0 ± 0.0 ^{a}	4.20 ± 0.24 ^{cdef}	1.96 ± 0.18 ^{abc}	21.96 ± 1.51 ^{ab}	97.2 ± 1.4 ^{ab}	6.04 ± 0.86 ^{bcde}	0.0 ± 0.0 ^{f}
	0.93	100.0 ± 0.0 ^{a}	6.65 ± 0.54 ^{a}	1.71 ± 0.20 ^{c}	22.08 ± 1.20 ^{ab}	100.0 ± 0.0 ^{a}	8.38 ± 0.98 ^{bc}	4.2 ± 2.4 ^{def}
	2.32	100.0 ± 0.0 ^{a}	5.07 ± 0.42 ^{bcd}	2.46 ± 0.23 ^{a}	23.42 ± 1.42 ^{a}	98.6 ± 1.4 ^{a}	13.26 ± 1.13 ^{a}	37.5 ± 2.4 ^{a}
Zeat	0.46	100.0 ± 0.0 ^{a}	3.32 ± 0.16 ^{fghij}	2.00 ± 0.19 ^{ab}	19.63 ± 1.19 ^{bc}	37.5 ± 2.4 ^{h}	2.11 ± 0.39 ^{ef}	0.0 ± 0.0 ^{f}
	0.93	95.8 ± 2.4 ^{c}	4.60 ± 0.31 ^{bcde}	1.39 ± 0.12 ^{cd}	17.91 ± 0.93 ^{c}	91.3 ± 2.5 ^{bcd}	5.00 ± 0.78 ^{def}	0.0 ± 0.0 ^{f}
	2.32	100.0 ± 0.0 ^{a}	4.30 ± 0.36 ^{cdef}	2.25 ± 0.19 ^{ab}	21.92 ± 1.34 ^{ab}	87.5 ± 2.4 ^{de}	7.95 ± 0.89 ^{bcd}	33.3 ± 2.4 ^{a}
TDZ	0.46	100.0 ± 0.0 ^{a}	3.40 ± 0.29 ^{fghij}	2.21 ± 0.22 ^{ab}	18.75 ± 1.18 ^{c}	79.2 ± 2.4 ^{f}	4.50 ± 0.74 ^{def}	33.3 ± 2.4 ^{a}
	0.93	87.5 ± 2.4 ^{f}	2.99 ± 0.28 ^{ghij}	1.15 ± 0.08 ^{d}	15.20 ± 0.69 ^{d}	11.7 ± 4.4 ^{i}	2.25 ± 0.63 ^{ef}	0.0 ± 0.0 ^{f}
	2.32	94.2 ± 1.4 ^{cd}	3.66 ± 0.26 ^{efghi}	1.50 ± 0.11 ^{cd}	13.64 ± 0.58 ^{d}	4.5 ± 2.6 ^{j}	1.00 ± 0.00 ^{f}	9.1 ± 2.6 ^{cd}

The data represent mean ± SE of replicates (n = 3). Values in a same row carrying different letters are significantly different by Duncan's multiple range test at $p \leq 0.05$.

Very high hyperhydricity rates are observed in the case of 2.32 µM adenine (37.5%), 2.32 µM Zeat and 0.46 µM TDZ (33.3). Values ranging from 1.8% to 18.5% are noted in the rest of cases, except for BAP, DPU, Zeat and TDZ at 0.93 µM, in addition to adenine and Zeat at 0.46 µM, where an absence of hyperhydricity is noticed.

The maximum shoots length is observed for 0.93 µM adenine (6.65 cm), followed by 0.93 and 2.32 µM DPU (5.65 and 5.59, respectively) and 2.32 µM 2iP (5.24). A low elongation is recorded in the case of 0.93 µM TDZ (2.99 cm), 0.46 µM Kin (2.94), as well as 0.46, 0.93 and 2.32 µM BAP (2.57, 2.37 and 2.35, respectively).

A maximum number of shoots is regenerated in the case of 2.32 µM adenine (2.46) and 0.46 µM BAP (2.36), followed by 2.32 µM Zeat (2.25), 0.46 µM TDZ (2.21), plus Kin and DPU at 2.32 µM (2.07). The minimum of shoots is observed in the case of 0.93 µM TDZ (1.15).

The multiplication of buds is maximum for 0.93 and 2.32 µM DPU (24.83 and 24.50, respectively), 2.32 µM adenine (23.42) and 0.46 µM DPU (23.11). The minimum number of buds is noted for 2.32 µM TDZ (13.64).

Also, the greatest number of roots grow for explants regenerated on N30K medium supplemented with 2.32 µM adenine (13.26), followed by 0.93, 2.32 µM DPU (10.03 and 9.75, respectively) and 0.93 µM 2iP (9.39). The minimum number of roots is noted in the case of 2.32 µM TDZ (1.00).

In conclusion, 0.93 µM adenine and 0.93 µM DPU alone in N30K medium are the best for the micropropagation of *T. broussonetii*: they ensure total regeneration of the vitroplants, optimal elongation and maximum multiplication of buds. In addition, a good development of the root part is noted, with high rooting rates and high multiplication of roots.

3.4. Effect of Polyamines

The addition of polyamines to the culture media resulted in some modifications in the *in vitro* growth of *T. broussonetii* vitroplants (Table 3, Figure 4 and Figure 7).

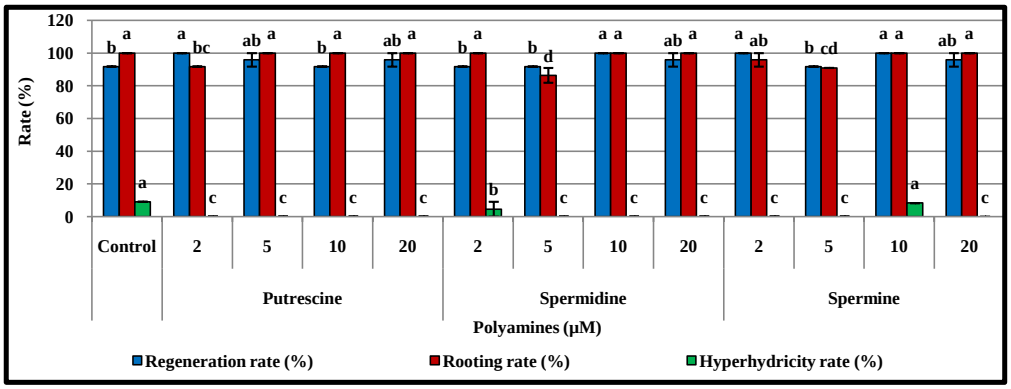

Figure 4. Effect of three polyamines at four concentrations on the regeneration, rooting and hyperhydricity rates of *Thymus broussonetii* explants.

Table 3. Effect of polyamines on the micropropagation of *Thymus broussonetii*.

Polyamines (µM)		Regeneration rate (%)	Shoots length (cm)	Number of shoots	Number of buds	Rooting rate (%)	Number of roots	Hyperhydricity rate (%)
Control		91.7 ± 0.0 ^{b}	4.69 ± 0.42 ^{b}	1.73 ± 0.24 ^{ab}	21.00 ± 1.07 ^{cd}	100.0 ± 0.0 ^{a}	6.45 ± 0.65 ^{ef}	9.1 ± 0.0 ^{a}
Putrescine	2	100.0 ± 0.0 ^{a}	5.82 ± 0.41 ^{a}	1.92 ± 0.23 ^{ab}	23.83 ± 1.58 ^{abc}	91.7 ± 0.0 ^{bc}	7.96 ± 0.74 ^{def}	0.0 ± 0.0 ^{c}
	5	95.8 ± 4.2 ^{ab}	4.05 ± 0.35 ^{bc}	1.96 ± 0.17 ^{ab}	25.78 ± 1.31 ^{ab}	100.0 ± 0.0 ^{a}	8.52 ± 0.80 ^{def}	0.0 ± 0.0 ^{c}
	10	91.7 ± 0.0 ^{b}	4.30 ± 0.29 ^{bc}	1.59 ± 0.16 ^{b}	25.18 ± 1.90 ^{abc}	100.0 ± 0.0 ^{a}	13.86 ± 0.87 ^{a}	0.0 ± 0.0 ^{c}
	20	95.8 ± 4.2 ^{ab}	2.74 ± 0.14 ^{d}	1.68 ± 0.26 ^{ab}	18.48 ± 1.28 ^{d}	100.0 ± 0.0 ^{a}	10.56 ± 0.70 ^{bc}	0.0 ± 0.0 ^{c}
Spermidine	2	91.7 ± 0.0 ^{b}	6.24 ± 0.53 ^{a}	2.27 ± 0.30 ^{a}	25.50 ± 1.31 ^{abc}	100.0 ± 0.0 ^{a}	8.55 ± 0.78 ^{cde}	4.5 ± 4.5 ^{b}
	5	91.7 ± 0.0 ^{b}	3.82 ± 0.32 ^{bc}	1.46 ± 0.14 ^{b}	19.41 ± 1.43 ^{bcd}	86.4 ± 4.5 ^{d}	7.47 ± 0.94 ^{bcd}	0.0 ± 0.0 ^{c}
	10	100.0 ± 0.0 ^{a}	4.76 ± 0.31 ^{b}	1.38 ± 0.12 ^{b}	23.83 ± 1.62 ^{abc}	100.0 ± 0.0 ^{a}	12.21 ± 0.84 ^{ab}	0.0 ± 0.0 ^{c}
	20	95.8 ± 4.2 ^{ab}	3.92 ± 0.21 ^{bc}	2.35 ± 0.27 ^{a}	21.65 ± 1.45 ^{bcd}	100.0 ± 0.0 ^{a}	11.09 ± 0.87 ^{b}	0.0 ± 0.0 ^{c}
Spermine	2	100.0 ± 0.0 ^{a}	5.99 ± 0.42 ^{a}	1.83 ± 0.12 ^{ab}	21.58 ± 1.09 ^{bcd}	95.8 ± 4.2 ^{ab}	5.96 ± 0.59 ^{f}	0.0 ± 0.0 ^{c}
	5	91.7 ± 0.0 ^{b}	3.63 ± 0.33 ^{cd}	2.00 ± 0.22 ^{ab}	22.18 ± 1.26 ^{bcd}	90.9 ± 0.0 ^{cd}	7.35 ± 0.81 ^{ef}	0.0 ± 0.0 ^{c}
	10	100.0 ± 0.0 ^{a}	4.18 ± 0.28 ^{bc}	2.29 ± 0.30 ^{a}	27.42 ± 1.48 ^{a}	100.0 ± 0.0 ^{a}	10.50 ± 0.84 ^{bc}	8.3 ± 0.0 ^{a}
	20	95.8 ± 4.2 ^{ab}	2.82 ± 0.14 ^{d}	2.35 ± 0.22 ^{a}	23.00 ± 1.36 ^{abc}	100.0 ± 0.0 ^{a}	10.65 ± 0.77 ^{bc}	0.0 ± 0.0 ^{c}

The data represent mean ± SE of replicates (n = 3). Values in a same row carrying different letters are significantly different by Duncan's multiple range test at $p \leq 0.05$.

Thus, a total regeneration is noticed in the case of 2 µM putrescine, 10 µM spermidine, 2 and 10 µM spermine. In addition, the rooting rate is 100%, except for the control (91.7%), 2 µM putrescine (91.7), 5 µM spermidine (86.4), 2 and 5 µM spermine (95.8 and 90.9, respectively). Also, a certain number of plants show a translucent appearance in the case of 2 µM putrescine (4.5%) and 10 µM spermine (8.3), but lower than that of the control (9.1).

Compared with shoot length on the control medium (4.69 cm), an improvement is recorded after adding 2 µM putrescine, 2 µM spermine and 10 µM spermidine (5.82; 5.99 and 6.24, respectively). On the other hand, it decreases for the rest, especially after the addition of 20 µM putrescine (2.74) and 20 µM spermine (2.82).

An increase in the number of shoots is noted for 2 and 20 µM spermidine (2.27 and 2.35, respectively), as well as 10 and 20 µM spermine (2.29 and 2.35, respectively), while this number decreases in the case of 10 µM spermidine (1.37).

The number of buds increases for most polyamines, particularly for 5 µM putrescine (25.78), 2 µM spermidine (25.50) and 10 µM spermine (27.42). The lower number of buds is observed in the case of 20 µM putrescine and 5 µM spermidine (18.48 and 19.41, respectively).

Furthermore, vitroplants growing on N30K medium supplemented with polyamines develop in most cases greater number of roots, essentially for 10 µM putrescine and spermidine (13.86 and 12.21, respectively). The lower number of roots is noticed in the case of 2 µM spermidine (5.96).

In conclusion, the addition of 10 µM putrescine influences positively the multiplication of buds and roots, while 2 µM spermidine optimizes elongation as well as buds and shoots multiplication. Besides, 10 and 20 µM spermine contribute to increase the number of buds, shoots and roots.

3.5. Effect of Cytokinins and Auxins Combinations

3.5.1. Effect of Auxins Combined to 0.46 µM Kin

The combination of the three auxins (IAA, IBA and NAA) with 0.46 Kin resulted in some changes in the *in vitro* growth of *T. broussonetii* vitroplants (Table 4, Figure 5 and Figure 7).

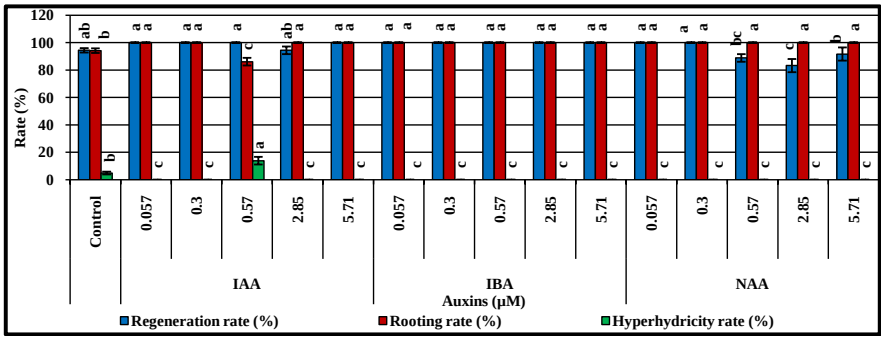

Figure 5. Effect of three auxins at five concentrations, combined to 0.46 µM Kin, on the regeneration, rooting and hyperhydricity rates of *Thymus broussonetii* explants.

Table 4. Effect of 0.46 µM Kin combined to auxins on the micropropagation of *Thymus broussonetii*.

Auxins concentration (µM)		Regeneration rate (%)	Shoots length (cm)	Number of shoots	Number of buds	Rooting rate (%)	Number of roots	Hyperhydricity rate (%)
Control	0	94.4 ± 1.6 ^{ab}	2.94 ± 0.25 ^{b}	1.88 ± 0.14 ^{abc}	19.97 ± 1.01 ^{bc}	94.1 ± 1.7 ^{b}	5.61 ± 0.57 ^{e}	4.9 ± 0.9 ^{b}
IAA	0.057	100.0 ± 0.0 ^{a}	3.02 ± 0.24 ^{b}	1.67 ± 0.26 ^{bc}	19.00 ± 2.05 ^{c}	100.0 ± 0.0 ^{a}	8.67 ± 1.24 ^{de}	0.0 ± 0.0 ^{c}
	0.3	100.0 ± 0.0 ^{a}	2.92 ± 0.26 ^{b}	1.83 ± 0.21 ^{abc}	20.50 ± 2.41 ^{bc}	100.0 ± 0.0 ^{a}	8.50 ± 0.92 ^{de}	0.0 ± 0.0 ^{c}
	0.57	100.0 ± 0.0 ^{a}	2.96 ± 0.27 ^{b}	2.50 ± 0.42 ^{ab}	21.17 ± 1.66 ^{bc}	86.1 ± 2.8 ^{c}	7.70 ± 1.32 ^{de}	13.9 ± 2.8 ^{a}
	2.85	94.4 ± 2.8 ^{ab}	3.03 ± 0.27 ^{b}	1.91 ± 0.21 ^{abc}	21.64 ± 2.36 ^{abc}	100.0 ± 0.0 ^{a}	14.54 ± 1.31 ^{a}	0.0 ± 0.0 ^{c}
	5.71	100.0 ± 0.0 ^{a}	5.37 ± 0.51 ^{a}	2.00 ± 0.17 ^{abc}	26.17 ± 2.25 ^{ab}	100.0 ± 0.0 ^{a}	10.58 ± 0.66 ^{bcd}	0.0 ± 0.0 ^{c}
IBA	0.057	100.0 ± 0.0 ^{a}	3.56 ± 0.34 ^{b}	2.42 ± 0.26 ^{abc}	25.33 ± 1.91 ^{abc}	100.0 ± 0.0 ^{a}	13.08 ± 1.41 ^{abc}	0.0 ± 0.0 ^{c}
	0.3	100.0 ± 0.0 ^{a}	4.99 ± 0.41 ^{a}	2.58 ± 0.40 ^{a}	28.50 ± 2.72 ^{a}	100.0 ± 0.0 ^{a}	12.92 ± 1.20 ^{abc}	0.0 ± 0.0 ^{c}
	0.57	100.0 ± 0.0 ^{a}	3.40 ± 0.42 ^{b}	1.67 ± 0.19 ^{bc}	20.67 ± 0.90 ^{bc}	100.0 ± 0.0 ^{a}	11.42 ± 0.83 ^{abcd}	0.0 ± 0.0 ^{c}
	2.85	100.0 ± 0.0 ^{a}	3.18 ± 0.32 ^{b}	2.00 ± 0.27 ^{abc}	23.67 ± 2.89 ^{abc}	100.0 ± 0.0 ^{a}	13.33 ± 1.72 ^{abc}	0.0 ± 0.0 ^{c}
	5.71	100.0 ± 0.0 ^{a}	3.62 ± 0.37 ^{b}	2.17 ± 0.37 ^{abc}	24.83 ± 2.12 ^{abc}	100.0 ± 0.0 ^{a}	14.00 ± 0.71 ^{ab}	0.0 ± 0.0 ^{c}
NAA	0.057	100.0 ± 0.0 ^{a}	4.81 ± 0.52 ^{a}	1.58 ± 0.26 ^{c}	23.83 ± 2.34 ^{abc}	100.0 ± 0.0 ^{a}	9.92 ± 1.18 ^{cd}	0.0 ± 0.0 ^{c}
	0.3	100.0 ± 0.0 ^{a}	3.54 ± 0.36 ^{b}	1.75 ± 0.22 ^{abc}	26.17 ± 2.56 ^{ab}	100.0 ± 0.0 ^{a}	10.50 ± 1.20 ^{bcd}	0.0 ± 0.0 ^{c}
	0.57	88.9 ± 2.8 ^{bc}	2.52 ± 0.27 ^{b}	1.91 ± 0.21 ^{abc}	22.18 ± 1.84 ^{abc}	100.0 ± 0.0 ^{a}	10.45 ± 1.53 ^{bcd}	0.0 ± 0.0 ^{c}
	2.85	83.3 ± 4.8 ^{c}	3.45 ± 0.49 ^{b}	1.90 ± 0.23 ^{abc}	22.80 ± 2.00 ^{abc}	100.0 ± 0.0 ^{a}	9.10 ± 1.11 ^{de}	0.0 ± 0.0 ^{c}
	5.71	91.7 ± 4.8 ^{b}	2.39 ± 0.26 ^{b}	1.90 ± 0.31 ^{abc}	20.00 ± 2.15 ^{bc}	100.0 ± 0.0 ^{a}	7.70 ± 1.48 ^{de}	0.0 ± 0.0 ^{c}

The data represent mean ± SE of replicates (n = 3). Values in a same row carrying different letters are significantly different by Duncan's multiple range test at $p \leq 0.05$.

Thus, a total regeneration is observed for all phytohormonal combinations, except 2.85 µM IAA (94.4), as well as 0.57, 2.85 and 5.71 µM NAA (88.9, 83.3 and 91.7, respectively). In addition, the rooting rate increases for all combinations between 0.46 µM Kin and auxins (100%), but decreases for 0.57 µM IAA (86.1%). Besides, a total absence of hyperhydricity is noted, except for 0.57 µM IAA (13.9%).

Shoots length increases after integration of auxins in the medium $N_{30}K$ + 0.46 µM Kin, especially in the case of 5.71 µM IAA (5.37 cm); 0.3 µM IBA (4.99) and 0.057 µM NAA (4.81). However, it decreases insignificantly for 0.3 µM IAA (2.92), 0.57 and 5.71 µM NAA (2.52 and 2.39, respectively).

Moreover, the number of shoots increases for certain phytohormonal combinations, namely 0.57 µM IAA (2.50), 0.057 and 0.3 µM IBA (2.42 and 2.58, respectively), but decreases in the case of 0.057 µM NAA (1.58), 0.57 µM IBA (1.67) and 0.057 µM IAA (1.67).

In addition, an increase in the number of buds is noted for almost all the combinations between 0.46 µM Kin and auxins, in particular for 0.3 µM IBA (28.50), 0.3 µM NAA (26.17) and 5.71 µM IAA (26.17). However, this number decreases in the case of 0.057 µM IAA (19.00).

Besides, the number of roots increases significantly after adding the auxins to the medium $N_{30}K$ + 0.46 µM Kin, in particular in the case of 2.85 µM IAA (14.54), 5.71 µM IBA (14.00) and 0.3 µM NAA (10.50).

3.5.2. Effect of Auxins Combined to 0.46 µM Adenine

After adding IAA to $N_{30}K$ + 0.46 µM adenine medium, the regeneration rate decreases from 100% to 86.1% for 0.3 µM IAA and IBA, to 88.9 for 2.85 µM IBA and NAA, while it is maintained at 100% for all the other auxins and concentrations. All of the regenerated explants develop roots after integration of auxins into the culture media, except for 0.3 and 0.57 µM IBA (93.3% and 91.7%, respectively) and 0.57 µM NAA (83.3). In addition, a total absence of hyperhydricity is noted.

Shoots length decreases compared to the control medium (4.20 cm) after the addition of IAA. However, a small increase is noted in 5.71 µM IAA (4.32). Also with IBA, the highest value is mentioned in the case of 0.057 (4.63) and 0.57 µM (5.24). For NAA, shoots length increases only for 0.57 µM (4.90). On the other hand, the lowest values are recorded at 5.71 µM NAA (2.27) and 0.057 µM IAA (2.22).

In comparison with the number of shoots on the control medium (1.96), an important increase is noted for 0.3 µM IBA (2.80), as well as 0.57 µM NAA (2.75). On the other hand, their number decrease with 5.71 µM IBA (1.60) and 0.3 µM NAA (1.18).

The number of buds increases after the addition of IBA and NAA, with maximum values at 0.57 µM IBA (27.33) and 2.85 µM NAA (28.00). However, this number decreases with 5.71 µM IBA and NAA (21.00 and 20.00, respectively) compared to the control (21.96). Even for IAA, a larger number of buds are observed with 2.85 and 5.71 µM (24.00 and 24.83, respectively). However, their

number decreases in the case of 0.057 and 0.3 µM (17.33 and 19.80, respectively).

Moreover, a significant increase is remarked in the number of roots after the addition of auxins, in particular for 2.85 and 5.71 µM IAA (13.92 and 11.50, respectively), 0.057 and 2.85 µM IBA (12.08 and 12.00, respectively), as well as 2.85 µM NAA (12.80) (Table 5, Figure 6 and Figure 7).

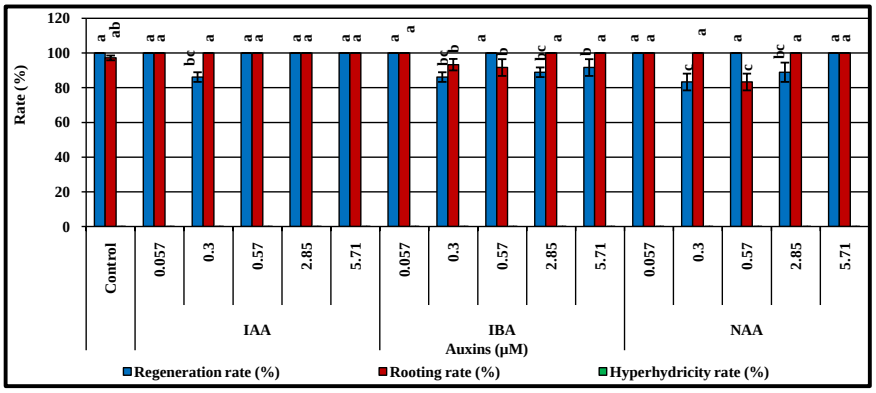

Figure 6. Effect of three auxins at five concentrations, combined to 0.46 µM adenine, on the regeneration, rooting and hyperhydricity rates of *Thymus broussonetii* explants.

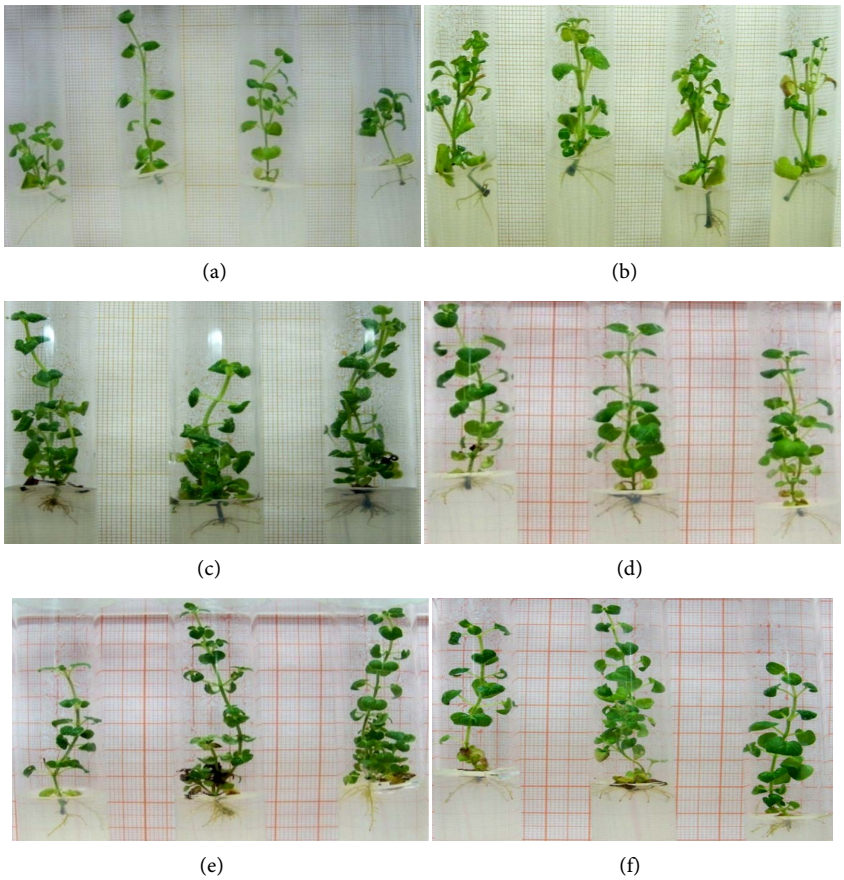

Figure 7. Micropropagation of *Thymus broussonetii* (Explants in $N_{30}K$ medium (a) without growth regulators; (b) +0.93 µM adenine; (c) +10 µM spermine; (d) +0.46 µM Kin + 0.3 µM IBA; (e) +0.46 µM adenine + 5.71 µM IAA; (f) +0.46 µM Kin + 2.85 µM IAA).

Table 5. Effect of 0.46 µM adenine combined to auxins on the micropropagation of *Thymus broussonetii*.

Auxins concentration (µM)		Regeneration rate (%)	Shoots length (cm)	Number of shoots	Number of buds	Rooting rate (%)	Number of roots	Hyperhydricity rate (%)
Control	0	100.0 ± 0.0 ^{a}	4.20 ± 0.24 ^{abcd}	1.96 ± 0.18 ^{abc}	21.96 ± 1.51 ^{abcd}	97.2 ± 1.4 ^{ab}	6.04 ± 0.86 ^{e}	0.0 ± 0.0
	0.057	100.0 ± 0.0 ^{a}	2.23 ± 0.20 ^{g}	1.92 ± 0.26 ^{abc}	17.33 ± 1.29 ^{d}	100.0 ± 0.0 ^{a}	8.17 ± 0.85 ^{cde}	0.0 ± 0.0
	0.3	86.1 ± 2.8 ^{bc}	2.89 ± 0.35 ^{efg}	2.00 ± 0.39 ^{abc}	19.80 ± 1.72 ^{cd}	100.0 ± 0.0 ^{a}	6.20 ± 1.08 ^{e}	0.0 ± 0.0
IAA	0.57	100.0 ± 0.0 ^{a}	2.55 ± 0.20 ^{fg}	2.17 ± 0.27 ^{ab}	21.83 ± 1.47 ^{abcd}	100.0 ± 0.0 ^{a}	9.58 ± 1.14 ^{bcd}	0.0 ± 0.0
	2.85	100.0 ± 0.0 ^{a}	2.47 ± 0.18 ^{g}	2.08 ± 0.19 ^{abc}	24.00 ± 1.92 ^{abcd}	100.0 ± 0.0 ^{a}	13.92 ± 0.98 ^{a}	0.0 ± 0.0
	5.71	100.0 ± 0.0 ^{a}	4.32 ± 0.38 ^{abcd}	1.83 ± 0.11 ^{abc}	24.83 ± 1.24 ^{abc}	100.0 ± 0.0 ^{a}	11.50 ± 1.51 ^{abc}	0.0 ± 0.0
	0.057	100.0 ± 0.0 ^{a}	4.63 ± 0.68 ^{abc}	2.33 ± 0.23 ^{ab}	24.17 ± 2.12 ^{abcd}	100.0 ± 0.0 ^{a}	12.08 ± 0.97 ^{ab}	0.0 ± 0.0
	0.3	86.1 ± 2.8 ^{bc}	3.34 ± 0.67 ^{defg}	2.80 ± 0.47 ^{a}	25.00 ± 1.69 ^{abc}	93.3 ± 3.3 ^{b}	10.00 ± 0.97 ^{bcd}	0.0 ± 0.0
IBA	0.57	100.0 ± 0.0 ^{a}	5.24 ± 0.58 ^{a}	2.08 ± 0.36 ^{abc}	27.33 ± 3.52 ^{ab}	91.7 ± 4.8 ^{b}	11.36 ± 0.93 ^{abc}	0.0 ± 0.0
	2.85	88.9 ± 2.8 ^{bc}	3.84 ± 0.43 ^{bcde}	2.18 ± 0.44 ^{ab}	25.46 ± 3.70 ^{abc}	100.0 ± 0.0 ^{a}	12.00 ± 1.04 ^{ab}	0.0 ± 0.0
	5.71	91.7 ± 4.8 ^{b}	3.74 ± 0.57 ^{bcdef}	1.60 ± 0.31 ^{bc}	21.00 ± 3.20 ^{abcd}	100.0 ± 0.0 ^{a}	10.00 ± 0.93 ^{bcd}	0.0 ± 0.0
	0.057	100.0 ± 0.0 ^{a}	3.84 ± 0.27 ^{bcde}	1.75 ± 0.31 ^{bc}	25.83 ± 1.77 ^{abc}	100.0 ± 0.0 ^{a}	9.42 ± 1.04 ^{bcd}	0.0 ± 0.0
	0.3	83.3 ± 4.8 ^{c}	3.40 ± 0.26 ^{cdefg}	1.18 ± 0.12 ^{c}	24.18 ± 1.13 ^{abcd}	100.0 ± 0.0 ^{a}	10.64 ± 1.11 ^{abcd}	0.0 ± 0.0
NAA	0.57	100.0 ± 0.0 ^{a}	4.90 ± 0.65 ^{ab}	2.75 ± 0.35 ^{a}	26.17 ± 3.02 ^{abc}	83.3 ± 4.8 ^{c}	9.73 ± 0.73 ^{bcd}	0.0 ± 0.0
	2.85	88.9 ± 5.6 ^{bc}	3.47 ± 0.33 ^{cdefg}	2.20 ± 0.39 ^{ab}	28.00 ± 2.39 ^{a}	100.0 ± 0.0 ^{a}	12.80 ± 1.01 ^{ab}	0.0 ± 0.0
	5.71	100.0 ± 0.0 ^{a}	2.28 ± 0.19 ^{g}	1.92 ± 0.23 ^{abc}	20.00 ± 1.67 ^{bcd}	100.0 ± 0.0 ^{a}	7.42 ± 0.73 ^{de}	0.0 ± 0.0

The data represent mean ± SE of replicates (n = 3). Values in a same row carrying different letters are significantly different by Duncan's multiple range test at $p \leq 0.05$.

3.6. Acclimatization Phase

The thirty explants developing roots respond well to the applied acclimatization protocol. One month after the start of acclimatization, 97% of the plantlets appear healthy. Three months later, they are transplanted into larger pots and 100% develop flowers during the 2nd year, between June and August (**Figure 8**).

3.7. Re-Initiation of *in Vitro* Culture of *Thymus broussonetii* from Acclimatized Plants

Surface sterilization of twigs from *T. broussonetii* acclimatized plants proved to be very difficult. Several sterilization methods using different products were tested. The method that consists of soaking first in $Ca(ClO)_2$ for 30 minutes, then in 0.1% $HgCl_2$ for 5 minutes and finally dipping three times in sterile distilled water for 5 minutes, proves to be the most adequate, with a total absence of bacterial contamination, 6.2% of fungal contamination, 37.5% of mortality and 52.1% of the explants tested have proliferated healthily.

The healthy and alive explants are multiplied by subculturing them on $N_{30}K$ + 0.46 µM Kin medium. The vitroplants obtained (**Figure 9**) present the morphological criteria mentioned in **Table 6**.

(a) (b)

(c) (d)

(e) (f)

Figure 8. Acclimatization phase (Acclimatization after (a) 4 weeks; (b) 8 weeks; (c) 6 months; (d) *Thymus broussonetii* inflorescences; (e) and (f) Acclimatization after 1 year).

Figure 9. Vitroplants obtained after sterilization of nodal segments from *Thymus broussonetii* acclimatized plants and their multiplication on $N_{30}K$ + 0.46 µM Kin medium.

Table 6. Morphological characteristics of vitroplants obtained after sterilization of shoot segments from the acclimatized *Thymus broussonetii* plants and their multiplication on $N_{30}K$ + 0.46 µM Kin medium.

Plantlets mean length (cm)	4.26 ± 0.19
Mean number of buds	19.30 ± 0.75
Mean number of shoots	1.92 ± 0.11
Mean number of roots	3.68 ± 0.34

4. Discussion

The results obtained, following the germination of *Thymus broussonetii* achenes, show that the protocol used for decontamination was efficient, since the rate of fungal and bacterial contamination did not exceed 4%. The use of achenes for initiation of *in vitro* culture can avoid most of the decontamination problems, often associated with the nature of the starting plant part used [17]. Achenes sterilization was also established in other studies on the *in vitro* culture of *Thymus* species, namely *T. hyemalis* Lange [28], *T. satureioides* Coss. [29] and *T. lotocephalus* G. López & R. Morales [30], but with different steps.

Concerning the effect of macronutrients, we opted for $N_{30}K$, since they ensured total regeneration of the explants, as well as the best multiplication of buds and shoots, with a low hyperhydricity rate. Also, Nobre (1996) [31] carried out the *in vitro* culture of *Lavandula stoechas* on a medium containing the $N_{30}K$ macronutrients. However, Nordine *et al.* (2014) [17] used MS macronutrients during the *in vitro* culture of *T. broussonetii*, like most of the studies established on species of *Thymus*, such as *T. daenensis* Celak. [32], *T. moroderi* Pau ex Martínez [33], *T. bleicherianus* Pomel [34] and *T. caespititius* Brot. [35]. Other researchers established culture on other less concentrated media, namely *T. membranaceus* Boiss. subsp. *membranaceus* [36] on DKW medium [37] and *T. vulgaris* L. [38] on Nitsch and Nitsch medium [39].

Furthermore, the addition of some cytokinins at determined concentrations to the culture medium contributed to the improvement of the morphology of *T. broussonetii* vitroplants. Thus, we noted an improvement in shoots length in the case of 0.93 µM adenine and 0.93 µM DPU. Also, the number of buds and rooting rate increased after the addition of 0.93 and 2.32 µM adenine, 0.93 µM DPU, as well as 2.32 µM Kin. Besides, the addition of 2.32 µM adenine and 0.93 µM DPU to the culture medium contributed to better root multiplication.

The effect of cytokinins has been tested in other studies on the genus *Thymus*, especially in the multiplication phase. In this way, Bakhtiar *et al.* (2014) [40] reported that *T. persicus* vitroplants cultured on MS medium without cytokinins did not succeed to proliferate, even after 4 weeks, while 100% regenerated in MS medium supplemented with different concentrations of cytokinins. Indeed, a variation in regeneration rate and in the number of shoots, as a function of the type and concentration of cytokinin was observed. On the contrary, *T. moroderi* has been described as a sensitive species to cytokinins, since low concentrations

produced a negative effect on vitroplants growth [33]. Also, higher concentrations of BAP (>2.22 µM) proved to be the most favorable for the multiplication of *T. lotocephalus* shoots [30]. In addition, a total regeneration of plantlets obtained from nodal segments of *T. hyemalis* was ensured after the addition to MS medium of 4.6 or 6.9 µM Kin, but a lower rate was noted in the case of 4.4 or 8.8 µM BAP. However, plantlets obtained from shoot tips showed a lower regeneration capacity in the case of 9.3 µM Kin. Also, BAP concentrations higher than 2.2 µM caused a reduction in the number of shoots obtained from both nodal segments and shoot tips [28]. Abdallah *et al.* (2017) [41] demonstrated that 4.65 µM Kin provided better shoot elongation and multiplication in the case of *Origanum syriacum*. Also, Sunandakumari *et al.* (2004) [42] obtained the best regeneration and the optimal shoot multiplication of *Mentha x piperita* on MS medium supplemented with 2.32 µM Kin.

Moreover, the use of polyamines contributed, in most cases, to the improvement of the morphology of *T. broussonetii* vitroplants. Thus, a better shoot elongation occurred in the case of 2 µM putrescine, 2 µM spermidine and 2 µM spermine. Also, a better multiplication of buds was noted after adding 5 and 10 µM putrescine, 2 µM spermidine and 10 µM spermine. As well, we noticed a maximum shoot multiplication after adding 2 and 20 µM spermidine, as well as 10 and 20 µM spermine. In addition, we found better root multiplication, especially for 10 and 20 µM putrescine, 10 and 20 µM spermidine, as well as 10 and 20 µM spermine.

The first application of exogenous polyamines aimed to increase rooting and to improve the quality of roots in olives. When polyamines were combined with auxins, this promoted early rooting and increased the percentage of final rooting and the number of roots per explant [43] [44]. Also, putrescine was implicated in the induction of roots by increasing the activity of total peroxidases at the base of the explants. It also promoted rapid root growth and increased the frequency of rooting [45]. Moreover, the integration of a combination between the three polyamines (0.2 mM putrescine + 0.2 mM spermidine + 0.05 mM spermine) in MS or DKW medium, supplemented with BAP at several concentrations, stimulated shoot elongation and increased the number of buds of *Corylus avellana* [46]. Nevertheless, Erland and Mahmoud (2014) [47] found that the integration of the same three polyamines at high concentrations (100 and 1000 µM) in WPM medium [48] caused browning of tissues, necrosis and inhibition of the growth of vitroplants of *Lavandula x intermedia* "Roughly", but the vitroplants treated with these polyamines on MS medium were generally healthier. In addition, the number of roots of *Tectona grandis* and their length increased after the use of IBA (2.46, 4.92 or 7.38 µM) in combination with putrescine at 1.815 mM [49].

For most combinations of 0.46 µM Kin and 0.46 µM adenine with auxins, better development of the root part was observed. Thus, the maximum of roots grew in the media added with the combinations 0.46 µM Kin + 2.85 µM IAA and

0.46 µM adenine + 2.85 µM IAA. In addition, the integration of auxins in the culture media optimized the development of the aerial part, especially in terms of bud multiplication. The most notable combinations are 0.46 µM Kin + 0.3 µM IBA and 0.46 µM adenine + 2.85 µM NAA.

Alone or combined with cytokinins, auxins were integrated into the culture media for the induction of roots and to optimize multiplication of *Thymus* vitroplants. Subsequently, Sáez *et al.* (1994) [50], after having experienced the effect of several combinations, concluded that MS medium, modified according to Collet (1985) [51], added with the combination 6.6 µM BAP + 2.8 µM IAA is the best for shoots multiplication of *T. piperella* L. Also, shoot tips of *T. vulgaris* responded better when MS medium was enriched with auxins combined with Kin: NAA generated the best results in terms of regeneration, shoot multiplication and elongation, as well as rooting. The rooting phase was carried out in media supplemented only with auxins and 0.23 µM 2,4-D gave the best results [52]. In addition, the combination 4.4 µM BAP + 1 µM NAA in MS medium favored the induction and proliferation of buds of *T. broussonetii*. The best rooting was obtained in MS medium added with 2.8 µM IAA or 2 µM NAA [17]. Coelho *et al.* (2012) [30] obtained the best rooting in 1/4 MS medium free of growth regulators. Furthermore, Abdallah *et al.* (2017) [41] obtained the best multiplication of *Origanum syriacum* shoots after incorporation into MS medium of the combination 2.32 µM Kin + 0.27 µM NAA. Moreover, the best development of roots was observed for MS + 4.92 µM IBA and 1/2 MS + 5.71 µM IAA. Furthermore, the best regeneration of *Origanum acutidens* vitroplants was obtained on MS medium supplemented with 8 µM BAP + 1.07 µM NAA and the best rooting was carried out on MS + 1.07 µM NAA [53].

During the establishment of the *in vitro* culture from the acclimatized plants, two difficulties were encountered: high contamination rate and low survival of the non-contaminated explants. It was the same case for other species of the genus *Thymus*, for which the culture was carried out *in vitro* after sterilization of shoot tips and nodes, namely *T. moroderi* [33] with a survival rate not exceeding 28.6%, *T. bleicherianus* with a rate of 36% [34], *T. caespititius* with 8% [35] and *T. longicaulis* C. Presl with 29.4% [52], as well as other *Lamiaceae* such as *Lavandula viridis* [54], *Salvia pratensis* and *Salvia nemorosa* [55] with around 30%. Despite these difficulties, we have noticed that explants which have survived following sterilization protocols have a great capacity for *in vitro* growth and allow the proliferation of a large number of vitroplants obtained after several subcultures.

5. Conclusions

The present study represents a complete description, from *in vitro* achenes germination until acclimatization to *ex-vitro* conditions, for the micropropagation of *T. broussonetii*, very rare and endemic species in Morocco.

First, the protocol used to decontaminate achenes before *in vitro* germination

was effective, since the rate of fungal and bacterial contamination did not exceed 4%. Then, the study of the effect of macronutrients resulted in the choice of $N_{30}K$, since they ensure total regeneration of the vitroplants, as well as best multiplication of buds and shoots, with a low hyperhydricity rate.

After multiplication of cultures, the evaluation of the effect of seven cytokinins at three concentrations showed that 0.93 µM adenine, added to $N_{30}K$ medium, favored significantly the induction of buds and the elongation of explants of *T. broussonetii*.

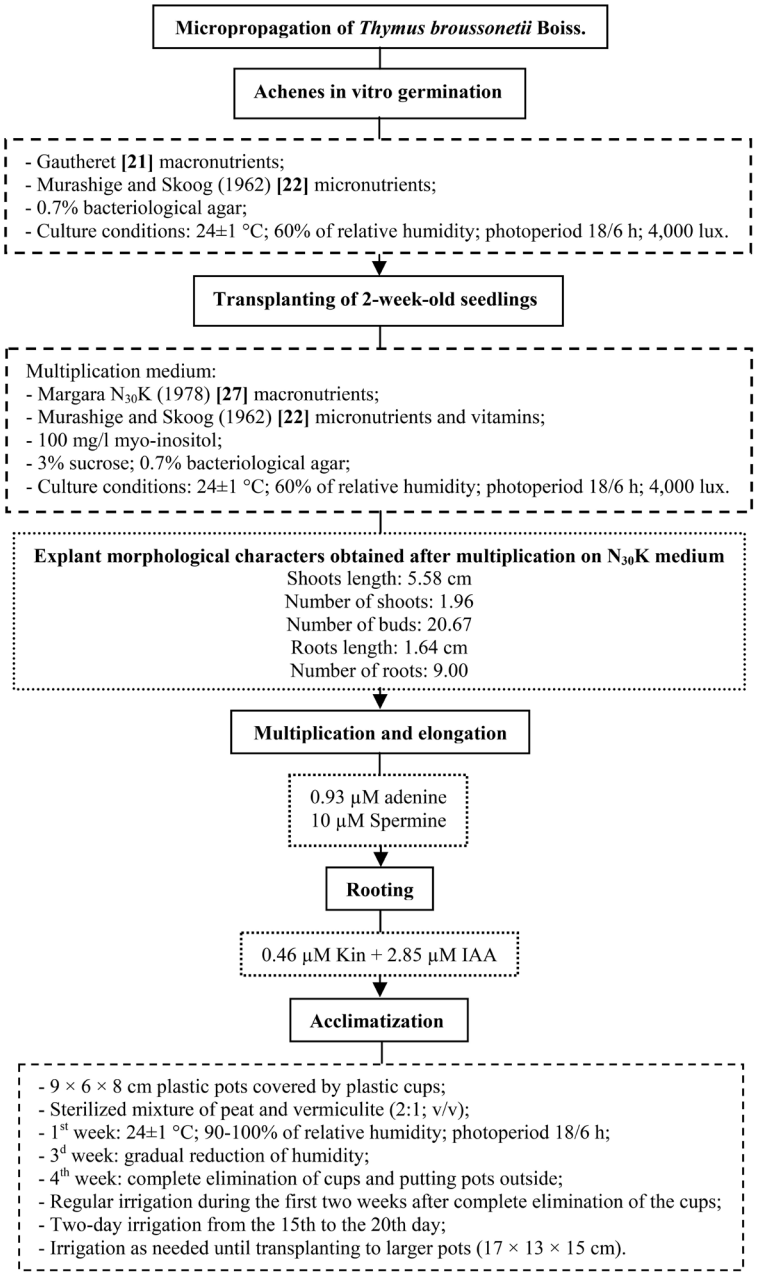

Figure 10. Prototype of the micropropagation of *Thymus broussonetii* subsp. *broussonetii* by shoot tip culture taken from 15-day seedlings at two cotyledon leaves stage obtained from achene germination.

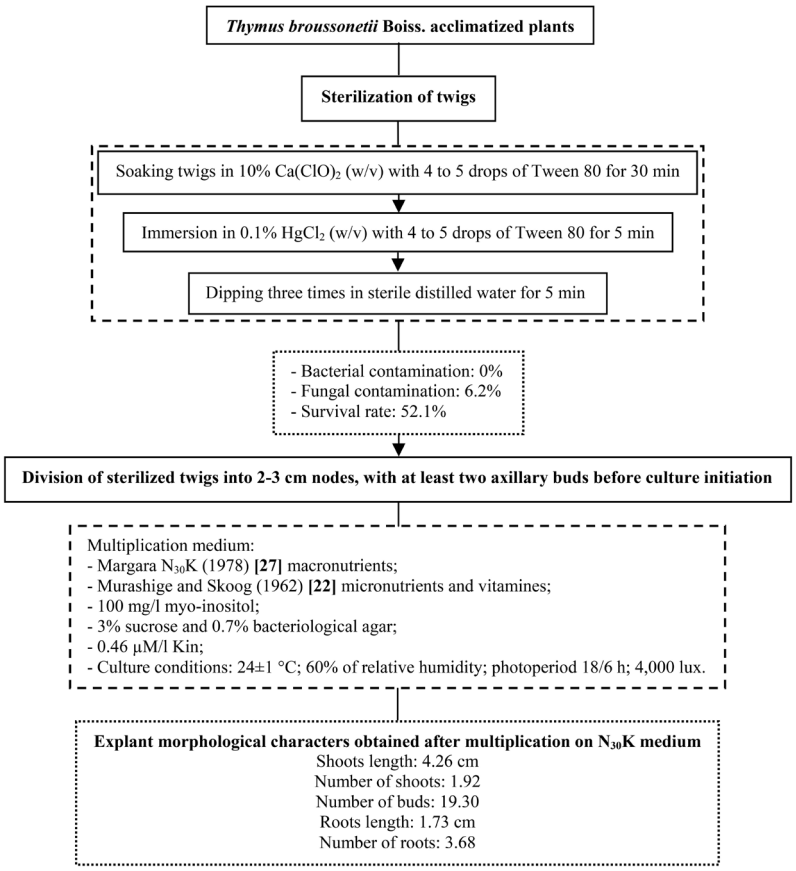

Figure 11. Prototype of vegetative propagation by nodes culture from acclimatized plants of *Thymus broussonetii* Boiss. subsp. *broussonetii*.

In addition, the use of polyamines contributed, in most cases, to the improvement of the morphology of *T. broussonetii* vitroplants, namely a better multiplication of buds, shoots and roots in $N_{30}K$ + 10 µM spermine.

Then, after integration into the culture media of 0.46 µM Kin or 0.46 µM adenine, combined with auxins, better roots multiplication and an increase in the number of buds and the length of *T. broussonetii* explants were noted, particularly for 0.46 µM Kin + 2.85 µM IAA and 0.46 µM adenine + 2.85 µM IAA.

Finally, acclimatization was successfully carried out using vitroplants developing a good root system. One month after the start of acclimatization, 97% of *T. broussonetii* plantlets were healthy and 100% of the acclimatized plants developed flowers in the 2nd year between June and August.

Re-initiation of the *in vitro* culture was carried out from sterilized segments of twigs collected from the acclimatized plants of *T. broussonetii*, with 1 - 2 nodes on the medium $N_{30}K$ + 0.46 µM Kin and 52.1% of the explants healthily proliferated.

In practice, this protocol can ensure a sustainable supply of this important rare species in a limited time and space, regardless of seasonal variations and thus, meet the growing demand for its essential oil. Also, regenerated plants could serve as potential sources for the extraction of bioactive compounds.

At the end of this work, two prototypes were established. The first (Figure 10) concerns shoot tip culture from seedlings obtained after the germination of achenes, and the second (Figure 11) concerns the nodes culture from acclimatized plants.

Conflicts of Interest

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

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