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IN VITRO CLONAL PROPAGATION THROUGH DIRECT SHOOT ORGANOGENESIS OF *THYMUS BROUSSONETII* – A VULNERABLE AROMATIC AND MEDICINAL PLANT SPECIES

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Abstract: This paper reports, for the first time, a rapid and reliable micropropagation protocol for high-frequency shoot regeneration and plant establishment of *Thymus broussonetii,* a vulnerable and highly valuable aromatic and medicinal plant. This plant species is threatened by over collection due to its socioeconomic importance. Hence, an attempt has been made for *in vitro* cultivation of this plant for large scale multiplication as well as conservation. Several treatments such as cytokinin types and concentrations, cytokinin/auxin ratio, gelling agents, and explant density were tested on the shoot bud induction. Effects of various concentrations of auxins were also examined on the micropropagated shoots during rooting stage. Full-strength Murashige and Skoog (MS) medium supplemented with 6- benzylaminopurine (4.4 µM) and naphthalene acetic acid (1 µM) promoted significantly shoot bud induction while affecting shoot length. The use of 3 g/l of gellan gum (Phytagel™) promoted shoot bud induction and growth. For micropropagated shoot rooting, hormone-free MS medium exhibited the best results. The micropropagated plantlets were successfully hardened and 94% of them were found healthy after transplantation to *ex vitro* conditions. The micropropagated plants established in soil showed normal flowering did not show any morphological abnormality during one year of growth.

Keywords: Explant Density, Gelling Agent, Kinetin, Micropropagation, Naphthalene Acetic Acid, *Thymus Broussonetii.*

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INTRODUCTION

Thyme species (genus *Thymus*, family Lamiaceae, subfamily Nepetoidae) are well known aromatic perennial herbs used extensively throughout the Mediterranean region [1]. Several of them are used in traditional phytotherapy in the form of tisane or powder as an antimicrobial, antispasmodic, anti-inflammatory, antioxidant, and antifungal agent [2-7]. These biological activities are related to the presence of phenolic compounds in the extracts such as thymol and/or carvacrol and other compounds [8-10]. In the Moroccan flora, *Thymus broussonetii* Boiss. is an endemic species [11] which present a great economic importance [12]. Indeed, the leaves, flowering parts and stems barks of this highly valuable species were used as powder, decoction or infusion form to treat digestive disorder, diarrhea, fever, coughs, rheum and numerous infected areas of the body. It is also used as a tonifying agent [12,13]. Recently, the antiparasitic effect (against *Toxoplasma gondii* cysts) of *Thymus broussonetii* essential oil was found [9]. It is reported that extracts of *T. broussonetii* possess not only anti-inflammatory effects [14], but also stimulatory effects on the immune system in addition to protection against stress by a neurotropic and antinociceptive activity [15]. This is due to the active compounds that possess its extracts and/or essential oils such as carvacrol and thymol. The accumulation levels of the active compounds in such MAPs however are very low, and therefore a large amount of plant material is needed.

In fact, the excessive pressure on wild plant populations caused by wild harvesting combined with land conversion has led diminished and scattered distribution of these species. The harvest of this species occurs mainly during the flowering period before seed formation. This causes a decrease in regeneration rate and progressive degradation of wild populations [16]. In addition, the biosynthesis of secondary metabolites, although controlled genetically, is affected strongly by environmental conditions [17]. Abiotic environmental factors as well as biotic effects were proven to influence both essential oil and polyphenol production, leading to a chemical variability [18]. Therefore, it is impossible to find a chemically homogeneous and standardized raw material of *Thymus* species in the natural habitat. This imposes many disadvantages such as the heterogeneity of plant material, the difficulty of predicting supplies for industry and lack of control. In addition, the natural products market requires an efficient system for production of plant tissues free from seasonal and somatic variations, infection by bacteria and fungi, and environmental pollution that can affect the economic value of harvested tissues [19]. Faced with this situation, the propagation of this herbal plant in commercial scale is ideal to reduce some of these problems and to ensure the sustainable supply of *T. broussonetii* raw material. Plant cell tissue and organ culture technologies have been expected to be efficient and useful tools for the breeding (selection) of high-quality MAPs and for the preservation of endangered species [20]. Micropropagation is one of the techniques that can be applied for propagation of *T.*

broussonetii in large scale. It is capable of producing large numbers of genetically similar, disease free *T. broussonetii* plants in a short period of time and limited space. This technique was applied for many *Thymus* species such as *Thymus vulgaris* [21-23], *Thymus piperella* [24], *Thymbra spicata* L. var. spicata L. [25], *Thymus lotocephalus* [26], *Thymus satureioides* [27], *Thymus hyemalis* [28,29] and *Thymus bleicherianus* [30], To avoid natural population depletion and heterogeneity of raw material, it becomes imperative to develop a protocol for *in vitro* propagation of this vulnerable plant species. It would of great utility for *ex situ* conservation, genotypes selection and clonal production.

2. Materials and Methods

2.1. Seeds germination and *in vitro* culture establishment

The plant species was identified by a botanist and a voucher specimen (INP. 264) was deposited at the herbarium of the National Institute of Medicinal and Aromatic Plants; Taounate. University of Sidi Mohamed Ben Abdellah Fez Morocco. Mature seeds of wild *Thymus broussonetii* were collected from the Khenifra area, Middle Atlas of Morocco, between July and August (2009). Seeds were separated from the inflorescence cleaned and dry stored at 4°C until the beginning of the experiments in the February 2010. To surface sterilized, seeds were soaked in 70% ethanol (v/v) for 3 minutes, followed by 10 minutes in 10% (v/v) commercially prepared bleach (6% active ingredient sodium hypochlorite) and a drop of surfactant, Tween 20. Afterwards, they were rinsed thoroughly with sterile distilled water and dried on sterile filter paper. Seeds were germinated in Petri dishes (9 cm of diameter) containing 25 ml of Murashige and Skoog (MS) medium [31] devoid of Plant growth regulators (PGRs) and solidified with 3 g/l of gellan gum (Phytagel™). Healthy nodal segments and shoot tips (1-1.5 cm long) were aseptically excised from *in vitro* seedlings and cultured in glass flasks (175 ml) containing 30 ml of MS media. Subcultures of one genotype were performed every 3 weeks until a sufficient stock was available for subsequent experiments.

2.2. Shoot proliferation

In order to establish a reliable and rapid micropropagated system, three experiments were carried using nodal segment (1 cm long) with a pair of axillary buds derived from uniform shoots of one genotype. MS medium were solidified using 3 g/l of gellan gum (Phytagel™). The results of each experiment were evaluated after 3 weeks of culture period.

2.2.1. Experiment 1: Effect of plant growth regulators on shoot proliferation

This experiment aimed to investigate the effect of PGRs on multiplication rate of *T. broussonetii*. MS medium was supplemented with two cytokinins at different concentrations [6-benzylaminopurine (BAP) (2.2; 4.4; 6.6 or 8.8 µM) and kinetin (KIN) (1.8; 4.6; 6.9 or 9.3 µM)]

and auxin / cytokinin combinations (0.5 or 1 µM α-naphthalene acetic acid (NAA) with 4.4 µM BAP or 6.9 µM KIN). Proliferation frequency and averages of number of shoots per explant and shoot length were recorded.

2.2.2. Experiment 2: Effect of gelling agent on shoot proliferation

The aim of this experiment is to evaluate the effect of gelling agent type on proliferation shoots. Three types of gelling agent [3 g/l gellan gum (Phytagel™), 8 g/l bacteriological agar and 8 g/l difco-bacto agar] were tested using the best MS proliferation medium resulting from experiment 1. Average number of shoots per explant and Fresh weight (FW) were recorded.

2.2.3. Experiment 3: Effect of explant density on shoot proliferation

To evaluate the effect of explant density on shoots proliferation, the numbers 2, 4, 6, 8, 10 and 12 explants per flask with 6 replicates were examined by recording the average number shoot and FW of shoots. The MS proliferation medium was solidified, in this case, with 3 g/l of phytagel.

2.3. Shoot rooting

For root induction, shoots (about 2.5 cm long) were transferred to MS medium solidified with 3 g/l of phytagel, and supplemented with Indole 3 acetic acid (IAA) (2.8, 3.6, 7.3 or 10.9 µM), or indole 3 butyric acid (IBA) (1, 2.5, 5 or 7.4 µM) or NAA (0.5, 1, 1.5 or 2 µM). The effects of these treatments were compared to free PGR MS medium on the root induction of micropropagated and they were recorded as a rooting ratio (%) and root number after 4 weeks of the culture period.

2.4. Environment conditions

In all the experiments reported, all media were supplemented with 30 g/l sucrose, and the pH was adjusted to 5.8 before autoclaved at 121°C and 100 KPa for 15 minutes. All *in vitro* culture were incubated in a growth chamber at 23 ± 2°C, with illumination provided by cool white florescent lamps at 60 µmol m^{-2} $s^{-1}$with a 16-h light photoperiod.

2.5. Acclimatization

Rooted shoots were removed from rooting medium and washed from the phytagel, they were transferred to 250 ml plastic pots containing a mixture of peat and vermiculite (2:1 *v/v*) covered with clear plastic cups. The humidity was maintained between 70 and 80% in the first 3 days and it was decreased gradually thereafter by the gradual removal of plastic. At the end of the second week of acclimatization, the cups were removed completely and the plants were

irrigated daily for the first 3 days, in 2-day intervals during 7 to 14 days, and in 4-days or more (as needed) until transplantation to the soil.

2.6. Statistical analysis

The experimental treatments were completely randomized. Each experiment was consisted by 6 replicates with 5 explants (excepted density experiment) per replicate. Statistical analysis of data was carried out by means of the software "SPSS for Windows". The homogeneity was carried out by leven's test and significant differences between means were determined using Duncan's New Multiple Range Test (P = 0.05).

3. Results and discussion

3.1. Seeds germination and *in vitro* culture establishment

The protocol used for decontamination has been effective, and 96% of *in vitro* seedlings did not manifest symptoms of bacterial and fungal contamination. The use of seeds for the establishment of primary cultures can prevent most of the decontamination problems that are often associated with explant establishment (Figure 1a). This method was successfully used in recent micropropagation studies on T. *satureioides* [27] and *T. hyemalis* [28]. Healthy nodal segments were aseptically excised from *in vitro* seedlings and cultured in glass flasks (175 ml) containing 30 ml of MS media. Subcultures of one genotype were performed every 3 weeks until a sufficient stock was available for subsequent experiments aimed to optimize proliferation and rooting stages.

3.2. Shoot proliferation

3.2.1. Effect of PGRs on shoot proliferation

Good knowledge of the effect of plant growth regulators is an important factor in the establishment of plant tissue culture. It is well recognized that the cytokinin/auxin ratio reported to overcome apical dominance, induce high number of shoot buds and release lateral bud from dormancy [32]. Therefore, the effects of two cytokinins, BAP and KIN in the presence or absence of NAA, on regeneration rate, shoot number, and shoot length of *T. broussounetii* were examined. Compared to control, the results showed that there were no significant differences between all treatments used on the regeneration rate excepting when medium was supplemented with 8.8 µM of BAP; which affect significantly the regeneration rate. Regarding the effect on the shoot number, the ratio of BAP (4.4 µM) and NAA (1 µM), gave better shoot number (7.33 ± 0.35) compared to control (Table 1). Thus, the results of this experiment indicate that the MS medium when supplemented with BAP (4.4 µM) and NAA (1 µM) promoted significantly shoot bud induction and proliferation as reported also in *T. vulgaris* [23],

while affect shoot length compared to control. No difference was noted between cytokinins (BAP and KIN) tested in the regeneration rate, induction and shoot length *in T. broussounetii*. However, in a recent studies the supply of 2,22 µM BAP in *T. satureioides* [27] and 1,8 µM KIN in *T. hyemalis* [28] promoted significantly shoot bud induction similarly as reported in a previous studies of *T. piperella* [24] and *T. mastichina* [33]. Hence, the supply of cytokinin alone or high cytokinin / auxin ratio were more suitable for shoot bud induction and regeneration of micropropagated plantlets in *Thymus* species. In this study the multiple micropropagated shoots obtained on a various PGR treatments failed to elongate on the same medium while the control (free PGR MS medium) remains the most appropriate for the micropropagated shoot elongation with an average around 4 cm after three weeks of *in vitro* culture establishment.

3.2.2. Effect of gelling agent on shoot proliferation

The effect of the type of gelling agent on the shoot number and growth of micropropagated shoots of *T. broussonetii* is shown in table 2. The use of gellan gum (Phytagel™) in the MS medium instead of Agar (Difco bacto agar and Bacteriological agar) had a very strong positive effect on the shoot bud induction and growth (Table 2). The gellan gum (Phytagel™) promoted shoot bud induction than Agar, thus more shoot bud was induced (8.63 $\pm$ 0.26) and more growth expressed as FW (1.93 $\pm$ 0.11). The difference between the effects of the type of gelling agent on the induction and shoot growth could be attributed to the mineral composition [34-36]. It known that agar is thought to contain agropectins with its sulphate groups and some other organic impurities such as phenolic compounds that might have inhibitory or toxic effects on the cell division and explant growth [34,37]. Therefore, the choice of the gelling agent is of considerable importance for the optimization of micropropagation as reported [35,38]. Hence, the use of gellan gum (Phytagel™) is more suitable for *in vitro* establishment and growth of micropropagated shoots of *T. broussonetii.*

3.2.3. Effect of explant density

In this experiment, the shoot number and fresh weight were influenced by the explant density (Table 3). The highest number of shoots (9.19 $\pm$ 0.23) per explants with a best FW was obtained when the density used was 6 explants/flask (Figure 1b). In addition, the micropropagated shoots produced at this density were healthy and morphologically superior compared to other densities. When the density increased to 8, 10 and 12 explants per flask, the average number of regenerated shoots was decreased and the regenerated shoots having thin stem and small leaves. When the density increased, tissue browning has been observed from the second weeks of culture period. The effects of explant density in plant tissue culture are well known and documented [39,40]. This effect was thought to be due to secondary compounds diffusing from explants into culture medium and also to the depletion and competition of nutrients.

3.3. Rooting and acclimatization

To evaluate the effects of auxin types and concentrations on the rooting stage, shoots were excised from proliferation medium and transferred into rooting MS medium during 4 weeks of culture period. The results showed that free PGR MS medium, and MS medium supplemented by IAA (2.8 µM) or by NAA (2 µM) were appropriate for rooting stage while IBA affected shoot rooting. Hence, practically the free PGR MS medium is the most suitable for rooting the micropropagated shoots of *T. broussonetii*. It promoted a 100% rooting rate with an average of 4.75 ± 1.13 roots per micropropagated shoot (Figure 1c, Table 4). The use free PGR MS medium as rooting medium was reported also in *T. vulgaris* [23] and in recent study in *T. satureioides* [27]. Furthermore, comparing the rooting effects of different auxins used in this study, the results showed that IAA and NAA were better than IBA. In *T. hyemalis* the rooting stage was achieved when medium culture was supplemented by IBA [28] while in *T. mastichine*, auxins cited above promoted shoot rooting [33]. Hence, the differences in rooting response into thyme species could be attributed to the genotype sensitivity or the endogenous level of cytokinin/auxin ratio among others factors [41,42].

Besides, acclimatization of rooted micropropagated shoots is as a major phase for successful establishment of micropropagated plantlets [43]. After acclimatization stage, 94% of the *in vitro* derived plants were found healthy in *ex vivo* conditions. After one year in the field, the micropropagated plants did not show any morphological abnormality (Figure 1d and e).

In conclusion, this study describes a reliable and highly efficient protocol for rapid *in vitro* clonal propagation of *T. broussonetii*. *In vitro* establishment and shoot multiplication of this vulnerable MAP can be done on the full-strength MS medium supplemented with BAP (4.4 µM) NAA (1 µM), 3 g/l of phytagel, and with density of 6 explants. For the elongation and rooting stages, free PGR MS medium remains the most appropriate. Hence, this *in vitro* clonal protocol could provide a promising alternative tool for large-scale propagation that increases the number of plantlets for cultivation, genotypes selection, and aid preservation of natural of *T. broussonetii* populations, since data on its micropropagation are not available.

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Table 1 Effect of PGRs on regeneration (%), shoot number and shoot length of *T. broussonetii* micropropagated plants

PGRs (µM)	Regeneration (%)	Shoot number	Shoot length (cm)
Control	100 [a]	4.91 ± 0.19 [bcde]	4.05 ± 0.31 [a]
BAP			
2.2	81.25 [a]	3.44 ± 0.66 [def]	1.02 ± 0.17 [bcde]
4.4	91.67 [a]	6.25 ± 0.38 [ab]	0.73 ± 0.06 [def]
6.6	91.67 [a]	5.25 ± 0.76 [bc]	0.96 ± 0.04 [bcde]
8.8	41.67 [b]	1.75 ± 0.75 [f]	0.37 ± 0.13 [f]
KIN			
1.8	91.67 [a]	3.25 ± 0.25 [def]	1.23 ± 0.15 [bcd]
4.6	100 [a]	3.08 ± 0.36 [def]	0.95 ± 0.17 [bcde]
6.9	100 [a]	4.25 ± 0.92 [cde]	0.84 ± 0.04 [cdef]
9.6	81.25 [a]	2.48 ± 0.72 [ef]	0.59 ± 0.13 [ef]
BAP+NAA			
4.4+0.5	100 [a]	4.69 ± 0.53 [bcd]	1.46 ± 0.33 [ab]
4.4+1	93.75 [a]	7.33 ± 0.35 [a]	1.53 ± 0.11 [ab]
KIN+NAA			
6.9+0.5	100 [a]	5.37 ± 0.32 [bc]	1.49 ± 0.26 [ab]
6.9+1	93.75 [a]	2.76 ± 0.40 [ef]	1.17 ± 0.28 [abc]

Data indicate mean ± SE. Values followed by the same letter within the same column are not significantly different at $P < 0.05$. Data recorded after 3 weeks of culture.

Table 2 Effect of gelling agent on shoot number and fresh weight (FW) of *T. broussonetii* micropropagated plants.

Gelling agent	Shoot number	FW (g)
Bacteriological agar (8 g/l)	3.94 ± 0.19^{b}	0.90 ± 0.22^{c}
Phytagel (3 g/l)	8.63 ± 0.26^{a}	1.93 ± 0.11^{a}
Difco bacto agar (8 g/l)	4.38 ± 0.53^{b}	1.45 ± 0.12^{a}

Data indicate mean ± SE. Values followed by the same letter within the same column are not significantly different at $P < 0.05$. Data recorded after 3 weeks of culture.

Table 3 Effect of explant density on number and fresh weight of shoots per explant of *T. broussonetii* micropropagated plants

Number of explant	Number of shoot/explant	Fresh weigth/flask
2	5.38 ± 0.43^{c}	1.16 ± 0.01^{ab}
4	8.22 ± 0.28^{ab}	1.24 ± 0.04^{ab}
6	9.19 ± 0.23^{a}	1.4 ± 0.02^{a}
8	7.03 ± 0.61^{b}	0.76 ± 0.03^{b}
10	5.54 ± 0.44^{c}	0.72 ± 0.01^{c}
12	5.69 ± 0.37^{c}	0.64 ± 0.02^{c}

Data indicate mean ± SE. Values followed by the same letter within the same column are not significantly different at $P < 0.05$. Data recorded after 3 weeks of culture.

Table 4 Effect of type and concentration of auxins on rooting (%) and root number of *T. broussonetii* micropropagated plants

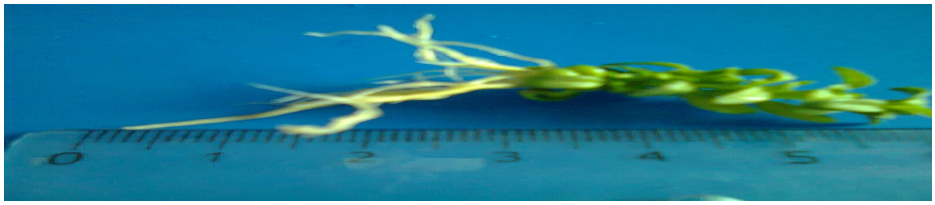
Auxins (µM)	Rooting (%)	Root number
Control	100 [a]	4.75 ± 1.13 [ab]
IAA		
2.8	91.67 [a]	6.42 ± 0.85 [a]
3.6	66.67 [bc]	2.67 ± 0.88 [bdc]
7.3	50.00 [cde]	1.50 ± 0.25 [cd]
10.9	33.33 [efg]	1.11 ± 0.95 [d]
IBA		
1	25 [fg]	1.60 ± 0.52 [cd]
2.5	60 [cd]	2.92 ± 01.23 [bcd]
5	50 [cde]	1.39 ± 0.69 [cd]
7.4	41.67 [def]	1.80 ± 0.48 [cd]
NAA		
0.5	16.67 [g]	0.18 ± 0.13 [d]
1	25.00 [fg]	0.70± 0.58 [d]
1.5	58.33 [cd]	2.25 ± 0.43 [bcd]
2	83.33 [ab]	4.00 ± 1.44 [abc]

Data indicate mean ± SE. Values followed by the same letter within the same column are not significantly different at $P < 0.05$. Data recorded after 4 weeks of culture.

Figure legends

Figure 1. Micropropagation of *Thymus broussonetii* (a) Germinated seeds on PGR-free MS medium (b) 6 explants cultured on MS medium supplemented with 4.4 µM of BAP and 1 µM of KIN and solidified with 3 g/l phytagel (c) Rooted plants on MS medium with 2.8 µM of IAA (d) Acclimatized plants in large pots after 3 months (e) Micropropagated plants after one year of transfer into soil.

(a) **(b)**



(c)

(d) **(e)**

Figure 1

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