

**Research Article** 

# Rapid *in Vitro* Regeneration and Clonal Multiplication of Thymus Bleicherianus Pomel, a Rare and Threatened Medicinal and Aromatic Plant in Morocco

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**Medicinal & Aromatic Plants** 

#### Abstract

This paper reports, for the first time, an efficient and reliable protocol for *in vitro* clonal propagation of *T*. *bleicherianus*, a rare aromatic and medicinal plant in Morocco. Initially, nodal explants collected from wild plant were used for *in vitro* culture establishment on Murashige and Skoog (MS) basal salts medium without plant growth regulators (PGR). Browning and contaminants were the major obstacles for *in vitro* culture establishment using explant derived from wild plants. To overcome this problem, the use of polyvinylpyrrolidone together with dark treatment decreased browning from 100 to 25%. The aseptic and living explants were then transferred into MS medium and all cultures were placed under light conditions. The cytokinin type and concentration were the most important factors affecting shoot multiplication. Axillary shoot bud proliferation was achieved using plant (6.39 ± 0.21) was obtained on MS medium supplemented with 4.44 µM of 6-benzylaminopurine. Regenerated shoots were rooted on plant growth regulators-free MS medium. Plantlets were successfully acclimatized to *ex vitro* conditions with a survival rate of 85%. All regenerated plants were morphologically identical to the mother plants. The *in vitro* culture system successfully established for *T. bleicherianus* offers a viable tool for mass micropropagation, multiplication and conservation of this very rare and threatened species.

**Keywords:** *In vitro* propagation; BAP-6-benzylaminopurine; Kinetin; NAA; α-naphthalene acetic acid; Medicinal and aromatic plants; PVP- polyvinylpyrrolidone; Plant growth regulators; *Thymus bleicherianus* 

## Introduction

The genus Thymus includes many species of perennial, aromatic herbs and sub-shrubs native to Europe and North Africa. Various types of Thyme are used worldwide as condiments, ornamentals and sources of many important compounds as flavones, rosmarinic acid, triterpenes, carbohydrates and especially essential oil [1-3]. Thyme oil possesses numerous biological activities including insecticidal, antibacterial, antimycotic, antioxidative, and food preservative properties [4-10].

In Moroccan flora, the genus Thymus consists of about 21 species, 13 among them are endemic [11]. *Thymus bleicherianus* Pomel is a very rare and threatened species represented by a very few populations in the Morocco [12]. In spite of this, this plant species is over harvested and widely used in the culinary arts [1] and in traditional medicine as an infusion or powder for the treatment of digestive disorders. Recently, analyses of essential oil of this species showed that is rich in  $\alpha$ -terpinene (42.23%) and thymol (23.95%), which gives it a significant antifungal, antibacterial and antioxidant activity suggesting prospects for its application in food, cosmetics and pharmaceutical industry [13,14].

However, the continuous human pressure, mainly due to local uses, is reducing and destroying populations, making this very rare species extremely threatened. Extinction risk of this species has led us to contribute to the *ex situ* conservation. Plant tissue culture techniques are important biotechnological tools for the *in vitro* propagation of rare and endangered plant species. The multiplication by *in vitro* culture, means micropropagation, is a very important methodology to obtain a great number of high-quality plants in a short period of time [15]. This technique has already been developed and successfully established in many *Thymus* species, such as *T. satureioides* [16], *T. hyemalis* [17,18], *T. vulgaris* [19-21], *T. piperella* [22], *T. mastichina* [23], *T. longicaulis* 

[21] and *T. lotocephalus* [24]. Up until now, there are no reports on the micropropagation of T. *bleicherianus*. Therefore, we try to establish a suitable *in vitro* propagation protocol for conservation, genotype selection and consistent clonal production of this species.

## Materials and Methods

#### Plant material and sterilization of explant

The original plant material was collected from the Meknes region (Moroccan Tafoghalt Forest,). The plant was identified by the botanist in the National Centre for Forestry Research, Rabat, Morocco. The wild plants were transferred and maintained in large pots, then they were transplanted in the garden of National Institute of Medicinal and Aromatic Plants; Taounate. University of Sidi Mohamed Ben Abdellah–Fez–Morocco. Shoot segments of *T. bleicherianus* about 5-6 cm long were harvested from mother plants and were then washed thoroughly under running tap water for about 30- 60 min. Washed material was then soaked in 70% ethanol for 5 min, 0.1% mercuric chloride (w/v) for 5 min. Later, explant immersed for 10 min in 10% (v/v) commercially prepared bleach (6% active ingredient sodium hypochlorite) and a drop of surfactant, Tween 20. Finally, the segments were washed four times with sterile distilled water. The surface-sterilized explants were

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PGR (µM)	Regeneration (%)	Shoot number	Shoots length
Control	100 ª	$3.7\pm0.83~^{\text{bcde}}$	$3.2\pm1.02~^{\text{a}}$
BAP			
2.2	100 ª	$4.0\pm0.5~^{\text{bcd}}$	$1.7\pm0.7~^{\text{ab}}$
4.4	100 ª	$6.4\pm0.2{}^{\text{a}}$	2.0 0.1 ab
6.6	93.7 <sup>ab</sup>	$3.0\pm0.3~^{\text{def}}$	$1.1\pm0.3$ $^{\text{b}}$
8.8	50 <sup>d</sup>	$2.0\pm0.2~^{\rm f}$	$1.0\pm0.3$ $^{\text{b}}$
KIN			
1.8	81.2 <sup>ab</sup>	$2.1\pm0.5~^{\rm f}$	$1.3\pm0.3~^{\text{b}}$
4.6	75 <sup>bc</sup>	$2.3\pm0.4~^{\text{ef}}$	1.1 ± 0.5 <sup>b</sup>
6.9	87.5 <sup>ab</sup>	$4.1\pm0.4~^{\text{bcd}}$	$2.2\pm0.5~^{\text{ab}}$
9.3	56.2 <sup>cd</sup>	$1.5\pm0.2$ $^{\rm f}$	$1.5\pm0.5$ $^{\text{b}}$
BAP+NAA			
4.4+0.5	87.5 <sup>ab</sup>	$5.0\pm0.5~^{\rm bc}$	$2.2\pm0.4~^{\text{ab}}$
4.4+1	93.7 <sup>ab</sup>	$3.0\pm0.8~^{\text{def}}$	$2.2\pm0.4~^{\text{ab}}$
KIN+NAA			
6.9+0.5	87.5 <sup>ab</sup>	$5.0\pm0.6~^{\text{b}}$	$2.3\pm0.3~^{\text{ab}}$
6.9+1	100 ª	$3.0\pm0.2~^{\text{cdef}}$	$2.4\pm0.2~^{\text{ab}}$

 Table 1: Effect of PGR on regeneration (%) and shoots number and length of micropropagated Shoot of *T. beicherianus*.

Values represent the means  $\pm$  SE. Means followed by the same letter in a same column are not significantly different ( $P \le 0.05$ ). Data recorded after 3 weeks of culture period.

trimmed at the cut ends to 1–1.5 cm each having two nodes, prior to inoculation on culture media.

## **Raising Aseptic Culture and Shoot Bud Proliferation**

To investigate the effects of light and Polyvinylpyrrolidone (PVP) on browning and regeneration rate of explants, aseptic explants were cultured in glass flasks (175 ml) containing 30 ml of free Plant growth regulators (PGR) Murashige and Skoog (MS) basal salts medium [25] supplemented with or without PVP (0.5%) and then placed under light or dark conditions. Hence, four randomly treatments were carried out Figure 2. After 3 weeks of the culture period, browning percentage (calculated as the number of explants surrounded by a browning '100/ total number of explants) and regeneration rate were determined.

Sequentially, all explants for each treatment were then transferred and cultivated on the free PGR MS medium without PVP under light conditions to evaluate the subsequent effects of above treatments on the growth of cultures. After three weeks of the culture period, data were recorded as regeneration rate, number and length of shoots.

To investigate the effect of PGR on the proliferation and on the growth of axillary bud shoot, nodal segments (1 cm long) with a pair of axillary buds were placed on MS medium containing different concentrations of cytokinins, 6-benzylaminopurine (BAP - 2.2, 4.4, 6,6 and 8.8  $\mu$ M) or Kinetin (KIN - 1.8, 4.6, 6.9 and 9.3  $\mu$ M) combined or not with two concentrations of auxin,  $\alpha$ -naphthalene acetic acid (NAA - 0.5 or 1  $\mu$ M) Table 1. After 3 weeks of culture period, data were evaluated as regeneration rate, number and length of micropropagated shoots.

# Rooting and hardening of micropropagated shoots

For rooting stage, healthy micropropagated shoots were transferred into PGR-free MS medium. After 4 weeks, *in vitro*-rooted shoots were removed and washed to remove the agar from the roots. The plantlets were then transplanted into plates with wells of 3 cm in diameter filled by peat and vermiculite (2/3:1/3 v/v) mixture and were covered with a plastic for providing the condition of high humidity. When new leaves developed in the micropropagated plants, the plastic cover was removed progressively during 10 days. Then, the plants were transferred to large pots filled with the same mixture and were regularly irrigated by water. After 3 months of growth in pots, the plantlets were transferred to soil in open field.

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## **Environmental conditions**

For all culture media, MS medium was enriched with 3% sucrose (w/v), 0.4 gellan gum (w/v) and pH was adjusted to 5.8 before being autoclaved at 121°C and 100 KPa for 15 min. All the cultures were incubated in a growth chamber at temperature of  $23 \pm 2^{\circ}$ C under darkness or light conditions with illumination provided by cool white florescent lamps at 60 µmol m<sup>-2</sup> s<sup>-1</sup> with a 16-h of light photoperiod.

## **Statistical Analysis**

The experiment was consisted by six replicates with four explants 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 the mean values were calculated and were compared by Duncan's multiple range tests at  $P \le 0.05$ .

# **Results and Discussion**

An *in vitro* propagation protocol, based on axillary bud shoot proliferation, has been developed for *T. bleicherianus*, a rare and endangered medicinal and aromatic plant. The establishment of free contamination cultures was very difficult due to high incidence of contamination (Figure 1A). In this study, sterilization treatments are effective for disinfecting the field grown Thymus explants intended for *in vitro* culture establishment. These treatments yielded aseptic cultures



**Figure 1:** Contamination and browning during *in vitro* culture establishment of *T. bleicherianus*, A) contaminated explant, B) *In vitro* culture on Polyvinylpyrrolidone (PVP)-free Murashige and Skoog (MS) medium incubated under lighting environment, C) *In vitro* culture on MS medium containing PVP incubated under dark condition.

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with an average of 36%. It is well known that explant taken from wild plants contain more pathogens that seriously affect the viability and the growth of shoot. These contaminants are particularly dangerous when they are plant pathogens. In most cases bacteria are the frequent contaminants. They are usually introduced with the explant and may survive surface sterilization of the explant because they are in interior tissues. Endogenous contamination may occur during new buds initiation after a long culture period [26]. Latent contamination is particularly dangerous because it can easily be transferred among cultures. So, aseptic technique is absolutely necessary for the successful establishment and maintenance of plant cell, tissue and organ cultures.

Besides, lethal blackening was another serious problem occurring during *in vitro* culture of explants taken from wild *T. bleicherianus* (Figure 1B). Similarly, this phenomenon has been encountered during *in vitro* establishment of *Thymus vulgaris* [19] and in many others plant species [27].

This process resulted by the exudation of phenolic compounds (secondary metabolites) from excised explants (Figure 1B). Therefore identification of a suitable treatment to minimize phenolic oxidation is an essential prerequisite to successful in vitro culture establishment. The prevention of oxidation browning of in vitro culture by using some antioxidants such as PVP is reported in many works [27,28]. Likewise, dark treatment has been reported to reduces browning and improve bud shoot induction [29-31] by controlling exudation of phenolic compounds into the culture media. Thereby, addition of the PVP into culture medium of T. bleicherianus significantly reduced browning rate from 100% to 52% and from 62.5% to 25% under lighting and dark (Figure 1C) conditions respectively (Figure 2). Otherwise, to evaluate the subsequent effects of above treatments on the growth of cultures, all explants for each treatment were then sequentially transferred and cultivated on the free PGR MS medium without PVP under light conditions. Thus, the results showed that a highest regeneration rate (45%) was obtained in the cultures pretreated with PVP under dark environment compared to other pretreatments (Figure 3 and 4A). Hence, for successfully establishing induction of axillary bud shoots in T. bleicherianus, plant tissue culture initiation must be done on a MS culture medium containing PVP under a dark environment during three weeks of culture period.





**Figure 3:** Subsequent effect of pretreatments dark and PVP on regeneration rate (%) of axillary bud shoots of *T. bleicherianus*. Bar represent SE.



Figure 4: Micropropagation of *T. bleicherianus*, A) Subsequent activation and regeneration of axillary bud shoots after dark and PVP pretreatments, B) *In vitro* rooted Shoots on PGR-free MS medium, C) Acclimatized plantlets.

Besides, in order to promote growth and axillary budding good knowledge of the effect of plant growth regulators is an important factor in the successful establishment and maintenance of plant cell, tissue and organ cultures. Usually, the cytokinin/auxin ratio reported to overcome the apical dominance on shooting, and enhance number of shoot buds, and release lateral bud from dormancy [32]. In this study, the results of the effects of PGR on the *in vitro* proliferation stage in *T. bleicherianus* were summarized in Table 1. There was no difference

of regeneration rate when nodal explants were cultured on PGR-free MS medium or on media containing a lower cytokinin concentrations. Addition of higher concentrations of BAP (8.88  $\mu$ M) or KIN (9.6  $\mu$ M) significantly decreased regeneration rate from 100% to reach 50 and 56.25% respectively. Interestingly, a highest multiplication rate (6.39  $\pm$ 0.21 shoot/explant) was promoted by MS medium supplemented with 4.44 µM of BAP. However, no significant effect on shoot proliferation on the culture medium containing different KIN concentrations tested. Furthermore, addition of auxin to cytokinin into culture medium appeared unnecessary since their combination could not significantly improve shoot multiplication Table 1. It is known that auxin inhibits the activation of axillary buds, and hence shoot multiplication, while cytokinin has the opposite effect. As well, effect of concentration and cytokinin types is critical for axillary budding shoot as reported in many medicinal plants [22,23,33] including some Thymus species [16,17,20,21]. Likewise, the results indicates that shoot elongation was also affected by the type and concentration of PGRs. Indeed, PGRfree MS medium (control) gave a maximum shoot length (3.25 cm), while cytokinin (BAP or KIN) decreased significantly shoot elongation Table 1. However, the combination of auxin-cytokinin led to slightly increase shoots length compared to use of cytokinins only Table 1. It is very interesting to note the high and significant improvements of shoot multiplication increased as the number of subcultures increased (see control in Table 1 and Figure 3). This may be due to adaptation of the explants to in vitro environment.

For rooting stage, healthy micropropagated shoots were harvested from the proliferation medium, and they were cultured into PGR-free MS medium during for 4 weeks. The rooting rate of micropropagated shoots was about 100% as reported in similar studies including *T. vulgaris* [19,21] and other species [18,34] suggesting that the use of auxins for rooting is not essential that this can reduce the time and cost of production. *In vitro* rooted shoots (Figure 4B) were then successfully acclimatized, with a survival rate of 85%, and no morphological variation was observed Figure 4C.

## Conclusion

The *in vitro* culture system successfully established for *T. bleicherianus* offers a viable tool for preservation, multiplication and sustainable production of this very rare and threatened species.

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