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Micropropagation of *Thymus satureioides* Coss. an endangered medicinal plant of Morocco

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Abstract The objective of this study was to develop a rapid system for regeneration of an important endemic medicinal plant of Morocco, *Thymus satureioides* Coss. (Labiatae). Initially *in vitro* grown seedling were exposed to full strength Murashige and Skoog, 1962 (MS) medium or reduced to ½ MS or ¼ MS hormone-free. Then, for axillary shoots proliferation, BAP, KIN, NAA and IAA were tested for their ability to multiply *T. satureioides*. Shoots obtained on proliferation medium were exposed to elongation medium containing gibberellic acid (GA_3) (0.5, 1.0, 2.5, or 5.0 µM). The effect of the auxins IAA, IBA and NAA, on the *in vitro* rooting of the shoots was studied. Maximum number of shoots (5.25±0.52) was observed on the ½ MS medium containing 2.22 µM of BAP. Incorporation of 1 µM of GA_3 in ½ MS medium significantly improved the shoot elongation within 3 weeks of culture. For rooting, rhizogenezis was promoted on half strength MS medium hormone-free. Regenerated plants were transferred to dimpled plates filled by peat and vermiculite (2/3:1/3 v/v) mixture. Micropropagated *Thymus satureioides* plants had a 95% survival rate, and showed vigorous and uniform growth.

Key words: *in vitro* culture, Micropropagation, Plant growth regulators, *Thymus satureioides*

Introduction

Medicinal plants play crucial role in the provision of health care in many developing countries (Timmermans, 2003). The food industry is becoming increasingly interested in aromatic herbs, mainly of the Lamiaceae family due to growing consumer demands for healthy natural foods. In addition to the food industry, Lamiaceae herbs are also of high demand in dyeing, fragrances, cosmetics, beverages and smoking industries (Papageorgiou *et al*., 2008).

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Lamiaceae herbs, namely rosemary, oregano, marjoram, sage, basil and thyme, are popular aromatic herbs. The genus *Thymus* L. (Labiatae) consists of about 215 species of herbaceous perennials and sub-shrubs (Nickavar *et al*., 2005).

The Mediterranean region can be described as the centre of the genus (Stahl-Biskup and Sáez, 2002). Various *Thymus* species are commonly used as spices and as traditional medicine remedies. In several studies, different *Thymus* species have been reported as the sources of flavonoids (Miura, 2002; Benkiniouar *et al*., 2007). Some previous studies have confirmed the antimicrobial effect of *T. broussonetii*, *T. zygis* and *T. satureioides* (Lattaoui *et al*., 1993; Lattaoui and Tantaoui-Elaraki, 1994). Van Den Brouck and Lemli (1981) have demonstrated the antispasmodic effect of *T. satureioides*, which is due to its flavonoids. However, the biosynthesis of secondary metabolites, although controlled genetically, is affected strongly by environmental influences (Yanivie and Palevitch, 1982). Abiotic environmental factors (temperature, moisture, soil and climatic conditions, elevation, etc.) as well as biotic effects (human disturbance, herbivores, etc.) were proven to influence both essential oil and polyphenol production of *Thymus* species as well as the chemical composition of coenopopulations in the course of time (Sáez, 2001).

In addition, *Thymus* species were characterized by a great chemical polymorphism (Corticchiato *et al*., 1998; Thompson *et al*., 1998; Stahl-Biskup, 2002). Therefore, it is impossible to find a chemically homogeneous and standardized raw material of *Thymus* species in the natural habitat. Since the harvest of medicinal plants on a mass scale, from their natural habitats is leading to a depletion of plant resources (Sen, 1991). In Morocco, endemism rate of genus *Thymus* is 57%, represented by 13 species (Benabid, 2000). *T. satureioides* is an important endemic plant in Morocco, popularly known as "azukni" it is used as traditional medicine, in form of infuse and decoctions to treat whooping cough, bronchitis and rheumatism (Bellakhadar *et al*., 1991). Its anti-inflammatory properties can be attributed to two triterpenes, i.e. ursolic and oleanolic acids (Ismaili *et al*., 2001, 2002, 2004). It is reported that thymol and/or carvacrol and considerable amounts of γ-terpinene, *p*-cymene, and borneol are the main components of essential oil of *T. satureioides* (Alaoui *et al*., 2012). Nowadays, in Morocco, *Thymus* species are threatened due to their overexploitation, such as *T. satureioides*, which is widely exported as herbs or essential oil. As a result, this species are now endangered, and the conservation of this valuable plant is imperative (Abbad *et al*., 2011). This problem can be overcome by *in vitro* propagation methods often used; the first hand, to produce clones of a plant with a large-scale (Sahoo *et al*., 1997; Vigya *et al*., 2012) and the other hand, to preserve endangered species (Fay, 1992; Benson, 1999).

Despite the potential applications of the *in vitro* culture methods, very few researchers have used them with *Thymus* genus. Micropropagation of *T. lotocephalus* (Coelho *et al.* 2012), *T. vulgaris* (L ê, 1989; Furmanowa and Olszowska, 1992; Ozudogru *et al*., 2011), *T. piperilla* (Sa éz *et al*., 1994), *T. mastichina* (Mendes and Romano, 1999) and micropropagation of garden thyme (*Thymbra spicata* L. var. spicata L.) (Daneshvar-Royandezagh *et al*., 2009) were reported. A bibliographical survey, to date, showed that there is no report on the *in vitro* culture of *T. satureioides*. This paper focuses on a method of micropropagation of this species from seedlings. Firstly, to preserve naturel resources, and secondly, to produce uniforms plants to avoid plant material heterogeneity.

Materials and methods

Seed source

In July 2009 (the period when *Thymus* fruits mature), inflorescences of *Thymus satureioides* Coss. were collected from wild populations from the Azilal region, mid-southwest of Morocco. The plants were identified and authenticated by Prof. Elouwalidi J. Voucher specimens (RAB 78018) were deposited in the herbarium of Scientific Institute, University Mohammed V, Rabat, Morocco. Seeds were separated from the inflorescence, cleaned and dry stored at 4 ℃ temperature (until use for experiment).

Seed germination

Seed were sorted out for uniform size and similar external characteristics, discarding those with obvious alterations or malformations. Seed of *T. satureioides* were germinated either *ex vitro* by sowing about 200 seeds in dimpled plates with wells 3 cm in diameter filled by peat and vermiculite (2/3:1/3 v/v) without any pre-treatment. Or *in vitro* after sterilization according to the following protocol; seeds were incubated in 70% (v/v) alcohol for 3 min, followed by 10 min in a solution of 1% sodium hypochlorite. They were then rinsed thrice with sterile distilled water, and finally dried on sterile filter paper.

After decontamination, seeds were placed on Petri dish (9 cm of diameter) containing 25 ml of MS medium (Murashige and Skoog, 1962) devoid of Plant growth regulators (PGRs). A seed was considered to have germinated at the emergence of the radical (radical > 1 mm) (Bewley and Black, 1994). The germination was recorded for a period of 6 weeks. The germination parameter evaluated was germination rate, expressed as the percentage of seeds

germinated. For *in vitro* germination; four replicates (30 randomly seed each) were used.

In vitro establishment

Shoot tips (0.5-1.0 cm) that did show any contamination, were excised from *in vitro* seedlings. They were then cultured under aseptic conditions on three different formulations of MS macronutrients; full strength MS, ½ MS and ¼ MS without PGRs. This was done in order to determine the most useful *in vitro* basal medium for seedling development. After the third subculture (subculturing at 3-week intervals) the development of seedlings was evaluated considering the morphological criteria such as response (%), shoot number, number of nodes and shoot length (cm). Seven replicates consisting of four explants per flask (Erlenmeyer 250 ml) were used. Well-developed shoots on ½ MS hormone-free were used as starting material in the axillary shoot propagation experiment.

The axillary shoot propagation

Microshoots formed on ½ MS medium (3 weeks old) were subcultured on ½ MS fresh medium hormone-free every 3 weeks until enough stock was available. In order to establish a system which could be utilized for continuous microplant production and multiplication, uniform nodal segments (derived from the same genotype) were used as explants, and various cytokinins at different concentrations; BAP (2.22, 4.44, 6.66 or 8.88 µM) and KIN (1.8, 4.6, 6.9 or 9.3 µM) and cytokinins combined with auxins; NAA (0.5 or 1 µM) and IAA (1.1 or 2.8 µM) were tested for their ability to multiply *in vitro* shoots of *T. satureioides*. Nodal segments (1 cm long) were implanted into half strong MS medium with PGRs. The ½ MS medium hormone-free was included as control. The experiment was conducted with 4 replicates consisting of four explants per flask. After three weeks of culture, the efficacy of each medium variant on shoot proliferation and growth was determined by recording: regeneration (%), mean number of shoots per explant, and shoot length (cm).

Shoot elongation

The result from the proliferation stage, showed that the shoots obtained on proliferation medium (2.22 µM BAP) were relatively short (1.07±0.16). Therefore, an elongation of shoots was required. The explants with shoot clusters from three weeks of culture on proliferation medium, were transferred to same medium, but lacking PGRs and supplemented with varied

concentrations of GA_3 at 0.5, 1.0, 2.5, and 5.0 µM for 3 weeks of culture to allow for the elongation of shoots.

Rooting and acclimatization

Elongated shoots (3-4 cm long) were cultured for rooting on half strength MS medium, supplemented with IAA (2.8, 3.6, 7.3 or 10.9 µM), IBA (1, 2.5, 5 or 7.4 µM) and NAA (0.5, 1, 1.5 or 2 µM) or on half strength MS medium auxin-free included as control. Each treatment was repeated three times and there were four explants in each replicate. Three weeks later, data were recorded by percent of root formation, number and length of roots. Well rooted plantlets were removed from the culture medium, and rinsed with water to remove agar. These were transferred into dimpled plates with wells 3 cm in diameter filled by peat and vermiculite (2/3:1/3 v/v). Dimpled plates were placed into plastic container covered with transparent plastic to avoid evapotranspiration and maintain high humidity. Every two days, the plastic cover was perforated thrice (about 1 cm^2 for each hole). The cover was also removed briefly thrice a day in order to manually spray the plant. Two weeks later, the plastic was removed completely. After 5 weeks, when new leaves developed in the micropropagated plants, the plants were transferred to large pots filled with peat and vermiculite (2/3:1/3 v/v). Finally, after a 10 weeks acclimatization period in the greenhouse, the plants were carefully transferred into soil in open air.

Culture conditions

The culture media for germination, seedling development, axillary shoot propagation, elongation and rooting were supplemented with 3% sucrose and 0.4% gellen gum, pH adjusted to 5.8 before autoclaved at 121℃ and 100 KPa for 15 min. All the cultures were incubated in a growth chamber at 23±2℃, with illumination provided by cool white florescent lamps at 60 µmol m^{-2} s^{-1} with a 16-h light photoperiod.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) to assess treatment differences using the SPSS software. Significant differences between means were determined using Duncan's New Multiple Range Test (P=0.05). Data are presented as means±Standard Error (SE).

Results

Seed germination

In this study, the seed sterilization protocol was successful. Sterile cultures were obtained in a high proportion (95%). Seeds were germinated either *ex vitro* or *in vitro* for a period of 6 weeks. The result showed that *in vitro* germination rate achieved 30.83% and the seedlings showed a normal appearance (Fig. 1a), however germination *ex vitro* was poor and produced a 5.5% germination rate only.

In vitro establishment

Shoot tips (0.5-1 cm long) were transferred to MS, ½ MS and ¼ MS media for seedling development. During the development stage, it was observed that ½ MS was the best medium for seedling development compared to MS and ¼ MS. It had highest significant response percentage (78.57%), number of shoots (2.11±0.18) and number of nodes (3.36±0.19). However, there was no significant difference in the shoot length among the three media used (Table 1). In the ¼ MS medium, the seedlings showed an abnormal appearance and died completely within one month. Based on the above results, ½ MS medium was used to assess the effect of plants growth regulators on the shoot proliferation, elongation and rooting experiments for *T. satureioides*.

Table 1. Effect of MS medium concentration on response (%), number of shoot/explant, number of nodes/explant and average shoot length (cm) of *T. satureioides in vitro* propagated shoots

Media	Response (%)	Number of shoots/explant	Number of nodes/explant	Shoots length (cm)
MS	35.71^{b}	0.93±0.36^{b}	1.50±0.51^{b}	0.72±0.22^{a}
½ MS	78.57^{a}	2.11±0.18^{a}	3.36±0.19^{a}	1.07±0.16^{a}
¼ MS	25.00^{b}	0.48±0.16^{b}	0.64±0.22^{b}	0.50±0.22^{a}

Values represent mean±SE. Values followed by the same letter in each column are not significantly different ($p<0.05$). Results recorded after 3 weeks of culture

The axillary shoot proliferation

In this experiment, nodal segments of *T. satureioides* developed in ½ MS medium were used as explant, and various combinations of two auxins (NAA

and IAA) and two cytokinins (BAP and KIN) were tested (Table 2). In terms of regeneration, all explants showed the ability of regeneration more than 75%, in all tested media, except the explants exposed to cytokinins (BAP or KIN) combined with 2.8 µM of IAA. On this medium, we observed that the most of explants are transformed into callus. It was also observed that the highest KIN concentration (9.3 µM) gave a lower regeneration rate (33.33%) with callus at the base of explant. When the cytokinins were used without auxins, the highest mean number of shoots 5.25±0.52 was obtained with 2.22 µM of BAP (Table 2). This result was significantly ($P<0.05$) higher than these obtained with all concentrations tested. Increasing BAP concentration from 2.22 to 8.88 µM, decrease the average number of shoots per explants to 1.75±0.14. However, when KIN was tested and compared with BAP, it proved to be less effective than BAP. It was observed that the maximum number of shoots was 2.25±0.38 at 6.9 µM KIN. Furthermore, addition of auxins (NAA or IAA) to BAP (2.22 µM) containing medium did not improve multiplication rate. However, when 1 µM NAA was used with 6.9 µM KIN, the multiplication rate (3.51±0.1) was significantly increased compared with 2.25±0.38 obtained in 6.9 µM KIN alone (Table 2). In this experiment, the length of shoots was also evaluated. The highest average shoot length (2.25±1 cm) was observed on MS medium with KIN (6.9 µM) combined with NAA (1 µM) followed by a shoot length of 1.67±0.17 cm on MS medium hormone-free. But these values are not statistically different between them. However, shoots obtained on 2.22 µM BAP containing medium remained relatively short (0.92±0.16 cm).

Table 2. Effect of cytokinin type and concentration, and cytokinin/auxin combinations on the regeneration (%), number of shoots/explant and shoot length (cm) of *T. satureioides in vitro* propagated shoots

PGRs (µM)	Regeneration (%)	Number of shoot/explant	Shoot length (cm)
Control	75.00^{a}	2.33±0.33^{cd}	1.67±0.17^{ab}
BAP			
2.22	100.00^{a}	5.25±0.52^{a}	0.92±0.16^{bcd}
4.44	83.33^{a}	3.25±0.29^{b}	1.08±0.33^{bcd}
6.66	100.00^{a}	2.67±0.42^{bc}	1.00±0.38^{bcd}
8.88	93.33^{a}	1.75±0.14^{cde}	0.58±0.16^{cd}
BAP+Auxins			
2.22+0.5 (NAA)	78.33^{a}	1.00±0.14^{efg}	0.50±0.14^{cd}
2.22+1 (NAA)	86.67^{a}	0.50±0.29^{fg}	0.42±0.22^{cd}
2.22+1.1 (IAA)	86.67^{a}	2.08±0.36^{cd}	0.83±0.08^{cd}
2.22+2.8 (IAA)	16.67^{b}	1.00±0.14^{efg}	0.33±0.08^{cd}
KIN			
1.8	100.00^{a}	1.46±0.11^{def}	1.17±0.08^{bc}
4.6	100.00 ^{a}	1.83±0.30^{cde}	0.67±0.22^{cd}
6.9	100.00^{a}	2.25±0.38^{cd}	1.08±0.17^{bcd}
9.3	33.33^{b}	0.58±0.30^{fg}	0.25±0.14^{d}
KIN+Auxins			
6.9+0.5 (NAA)	83.33^{a}	1.42±0.36^{def}	0.67±0.17^{cd}

6.9+1 (NAA)	100.00[a]	3.51±0.1[b]	2.25±1[a]
6.9+1.1 (IAA)	83.33[a]	1.67±0.17[de]	0.75±0.14[cd]
6.9+2.8 (IAA)	25.00[b]	0.25±0.14[g]	0.29±0.23[d]

Values represent mean±SE. Values followed by the same letter in each column are not significantly different ($p<0.05$). Results recorded after 3 weeks of culture.

Shoot elongation

Since the shoots were relatively short (0.92±0.16 cm) on proliferation medium (BAP 2.22 µM), the goal of this phase of the experiment was to improve the shoots length before rooting and acclimatization stage. The effect of various concentration of GA_3 (0.5, 1.0, 2.5, and 5.0 µM) was evaluated. The results showed that incorporation of 1.0 µM GA_3 to MS media significantly ($p<0.05$) enhanced the shoot length (max 3.69±0.67) (Fig. 1b) followed by the 0.5 µM GA_3 concentration (2.63±0.43 cm) (Table 3).

Table 3. Effect of GA_3 on shoot elongation (cm) of *T. satureioides*

GA_3 (µM)	**Shoots length (cm)**
0	1.31±0.28[c]
0.5	2.63±0.43[ab]
1	3.69±0.67[a]
2.5	1.88±0.07[bc]
5	1.72±0.14[bc]

Values represent mean±SE. Values followed by the same letter in each column are not significantly different ($p<0.05$). Results recorded after 3 weeks of culture

Rooting and acclimatization

Elongated shoots were cultured for rooting on half strength MS medium hormone-free or supplemented with IAA, IBA or NAA at various concentrations. According to the results obtained (Table 4), the highest rooting rate was induced by ½ MS auxin-free (91.66%), followed by NAA (83.33%), IBA (75%) and IAA (58.33%) at 2.0; 2.5 and 7.3 µM respectively. For both IAA and IBA a slight decline in rooting percentage was observed at the highest concentration (10.9 µM IAA and 5 or 7.4 µM IBA). However, the rooting rate increased as the concentration of the NAA increased, reaching a maximum (83.33%) at the highest concentration tested (2 µM). In term of number of root a high average number (2.42±079) was obtained with NAA (2 µM) followed by MS auxin-free (2.08±0.22), but these values were not statistically different.

Length of root was achieved a maximum 1.33±0.17 cm on ½ MS without auxins. It should be noted that both NAA (2 µM) and IAA (more than 7.3 µM) not promoted only rhizogenesis, but also caused callus formation at the basal end of the shoots. Therefore the ½ MS hormone-free medium; proved to be the most effective for root induction directly at the base of the shoot with no

intermediate callus (Fig. 1c). Plantlets rooted on this medium and transferred to *ex vitro* conditions (Fig. 1d&e) through the protocol described above, had a 95% survival rate. The established plants were apparently uniform and did not show any detectable variation after 10 weeks of acclimatization in soil (Fig. 1e).

Table 4. Effects of ½ MS medium supplemented with different concentrations of IAA, IBA and NAA on rooting (%), number of root/shoot and root length of *T. saturioiedes*

Auxins (µM)	Rooting (%)	Number of root/shoot	Root length (cm)
Control	91.66^{a}	2.08 ±0.22^{ab}	1.33 ±0.17^{a}
IAA			
2.8	25.00^{de}	0.50 ±0.38^{de}	0.50 ±0.29^{ab}
3.6	41.67^{cde}	1.00 ±0.38^{bcde}	0.58 ±0.08^{ab}
7.3	58.33^{abcd}	1.50 ±0.25^{abcd}	0.83 ±0.30^{ab}
10.9	41.67^{cde}	1.03 ±0.15^{bcde}	0.37 ±0.19^{ab}
IBA			
1	50.00^{bcd}	1.06 ±0.22^{bcde}	0.42 ±0.11^{ab}
2.5	75.00^{ab}	1.83 ±0.08^{abc}	0.91 ±0.04^{ab}
5	66.67^{abc}	1.08 ±0.08^{bcde}	0.77 ±0.16^{ab}
7.4	58.33^{abcd}	0.83 ±0.17^{cde}	0.50 ±0.00^{ab}
NAA			
0,5	8.33^{e}	0.04 ±0.04^{e}	0.08 ±0.08^{b}
1	25.00^{de}	0.66 ±0.66^{cde}	0.91 ±0.9^{ab}
1.5	50.00^{bcd}	1.00 ±0.25^{bcde}	0.58 ±0.11^{ab}
2	83.33^{ab}	2.42 ±0.79^{a}	0.83 ±0.08^{ab}

Values represent mean ±SE. Values followed by the same letter in each column are not significantly different $p<0.05$. Data was recorded for three weeks of culture.

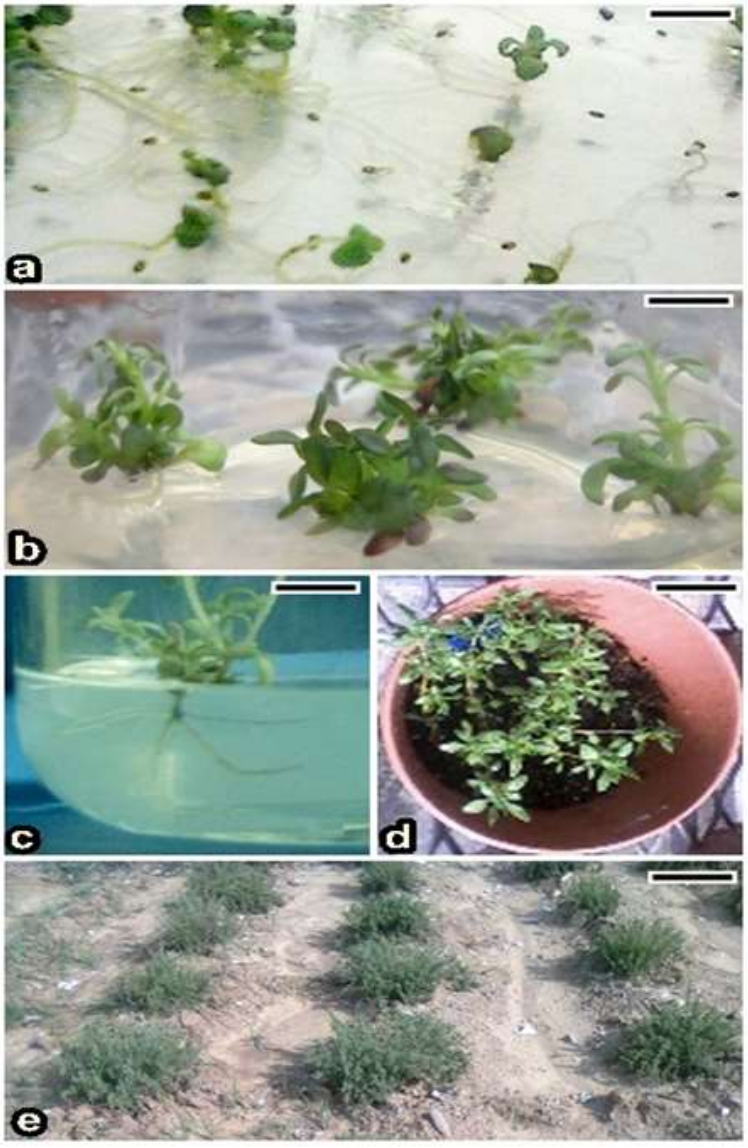

Fig. 1. *In vitro* propagation of *Thymus satureioides*; a) Seedlings after 6 weeks of germination on MS medium (*bar* 1 cm); b) Elongated shoots on ½ MS with 1 µM of GA_3 (*bar* 1.05 cm); c) Rooted plants on ½ MS without hormones (*bar* 1.6 cm); d) Transferred plants into large pots (*bar* 3 cm); e) Field-transferred plant (bar 16.98 cm).

Discussion

The use of seeds as a means of propagation is advantageous; on the one hand, seeds constitute a perfect material to initiate *in vitro* culture, because they produce plants which differ genetically. As a result, the maintenance of a wider

genetic base (Fay, 1992; Benson *et al*., 2000; George and Debergh, 2008; Gonçalves *et al*., 2010). On the other hand, an alternative for obtaining uncontaminated explants is to take explants from seedlings which are aseptically grown from surface sterilized seeds. The hard surface of the seed is less permeable to penetration of harsh surface sterilizing agents, such as hypochlorite, so the acceptable conditions of sterilization used for seeds can be much more stringent than for vegetative tissues (Akin-Idowu *et al*., 2009). Initiation of thyme tissue culture from seedling has been previously well documented (Sáez *et al*., 1994; Daneshvar-Royandezagh *et al*., 2009; Ozudogru *et al*., 2011; Coelho *et al*., 2012). According to some studies conducted previously, *in vitro* germination of most plant seeds is achieved by use of basal salts medium. Murashige and Skoog formulation is the most commonly used medium (Molia, 2000).

For *in vitro* establishment, ½ MS medium was better suited for seedling development compared to MS and ¼ MS. Therefore, intermediate nutrient level is most effective for regeneration response in *T. satureioides*. There are several reports that study direct organogenesis on half MS medium (Nhut *et al*., 2001; Martin 2004; Sarwar *et al*., 2009). Contrary to our result, full strength MS was found the best medium for *in vitro* establishment of *T. lotcephalus*, compared with ½ MS and ¼ MS (Coelho *et al*., 2012). In the present study, we found that BAP is the most suitable cytokinin for *T. satureioides* micropropagation. These results are in agreement with those obtained by some searchers; Sáez *et al*. (1994) working with *T. piperella*, Baba Erdag and Yurekli (2000) working with *T. sipyleus* or Mendes and Romano (1999) and Fraternale *et al.* (2003) working with *T mastichina*. They too confirmed the favorable effect of BA or BAP. On the contrary, in *T. vulgaris* a favorable effect of KIN was found (Furmanowa and Olszowska 1992; Ozudogru *et al*., 2011). But in another work, the use of plant growth regulators has been shown to be disadvantageous for *T. vulgaris* micropropagation; it was reported that the addition of PGRS in the culture medium caused a disturbance in growth; which could be the interaction between endogenous and exogenous hormones (Lê, 1989). In our work, BAP induced a higher multiplication rate of shoot; however the multiple shoots obtained remained shorts. Therefore, it was necessary to develop a suitable medium for shoot elongation. The result showed that incorporation of 1 µM GA_3 to MS media enhanced the shoot elongation of *T. satureioides*. The promotive effect of GA_3 on elongation of shoots generated on BAP-containing medium, has been reported in several other plants species (Demeke and Hughes, 1990; Jordan and Oyanedel, 1992; Purohit and Singhvi, 1998; Sugla *et al*., 2007; Purkayastha *et al*., 2008). GA_3 is considered to stimulate shoot

elongation by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger 1998).

For rooting, GA_3-elongated shoots isolated from elongation stage were cultured in ½MS medium hormone-free, or containing various auxins. The ½MS basal medium hormone-free promoted to be the most effective for root induction. Similarly, a reduction of 25–50% of macronutrients promoted root formation in *T. piperilla* (Stahl-Biskup and Sa éz 2002). The best rooting without hormones was previously reported in *T. vulgaris* (L ê 1989; Ozudogru *et al.* 2011). However, roots development of *T. mastichina* L., was promoted with different auxins; NAA (100%), IBA (100%), and IAA (80%) (Mendes and Romano, 1999). The differences observed in rooting response, between species, may be due to several factors such as the endogenous cytokinin/auxin ratio, the influence of shoot multiplication medium, the sensitivity of tissues to absorb or use the exogenous auxin, and the type of initial explants (mature or juvenile), among others (de Klerk *et al*., 1999; de Klerk, 2002).

Conclusion

This investigation illustrates a protocol for shoot regeneration of *Thymus satureioides*. In summary, (i) the best shoot proliferation was obtained on half MS medium supplemented with 2.22 µM of BAP, (ii) best shoot elongation was obtained with 1 µM of GA_3, (iii) the best root induction was obtained on half MS medium hormone-free. This is an effective protocol for the large scale multiplication of *Thymus satureioides*, then, it highlights a successful and rapid technique that can be used for commercial propagation in order to meet the growing market needs and ex situ conservation of this important medicinal species.

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