**MEDIUM-TERM CONSERVATION OF THYME SHOOT CULTURES BY *IN* *VITRO* SLOW GROWTH STORAGE**

**Elif Aylin Ozudogru\*, Ergun Kaya**

Gebze Institute of Technology, Faculty of Science, Department of Molecular Biology and Genetics, Istanbul caddesi, N° 101, 41400, Gebze, Kocaeli, Turkey

\*Fax: 0090 262 605 25 02, \*E-mail: [elifaylinozudogru@yahoo.it](mailto:elifaylinozudogru@yahoo.it), [elif@gyte.edu.tr](mailto:elif@gyte.edu.tr)

**Abstract**

Thyme (*Thymus vulgaris* L.) is an important aromatic and medicinal plant of the Lamiaceae family. It is native to Mediterranean region, but is cultivated worldwide both as a landscape plant, and as a natural source of fresh and dried herbs and essential oils. Thyme essential oils are used extensively for medicinal, culinary and industrial purposes. This makes thyme a unique natural source of products, highly appreciated by the society. However, this growing demand and, as a consequence, uncontrolled harvesting of natural plants lead to the disturbance or even loss of natural thyme populations. Besides, natural populations are usually heterogeneous, and this affects the homogeneity of oil yield and its chemical composition. Today, advanced biotechnological applications provide a unique alternative for propagation and conservation of such valuable germplasm. For instance, *in vitro* clonal shoot lines of selected thyme chemotypes can be established by plant tissue culture methods and maintained *in vitro* for several years or even more by using slow growth storage (‘medium-term conservation’) and cryopreservation (‘long-term conservation’) techniques. The present study is focused on development of efficient medium-term conservation approach for *in vitro* shoot cultures of *T. vulgaris* L. When shoots were transferred to glass jars, on semi-solid MS medium supplemented with 1 mg l-1 kinetin and 0.3 mg l-1 GA3, and stored at 4°C in darkness, maximum storage period was 12 months. Indeed, shoot tips coming from this material showed a good regrowth potential which allowed a fast re-stablishment of shoot cultures in post-conservation. Medium/tissue browning and shoot etiolation were observed, especially starting from 6 months of cold storage. However, shoot etiolation never compromised the regrowth of excised shoot tips when they were moved to standard culture conditions.

**Key words** cold storage, *in vitro* conservation, medium-term conservation, *Thymus vulgaris* L.

**Running title:** Medium-term conservation of thyme

**INTRODUCTION**

Thyme (*Thymus vulgaris* L.), also known as ‘common thyme’, is a small (10-30 cm tall), aromatic and medicinal sub-shrub of the Mediterranean region, belonging to Lamiaceae family (Loziene et al. 2007). The typical aroma of the plant comes from essential oil glands in the leaves, calyces and corallas. Thyme blooms from March to July (in the Northern Hemisphere) dainty pinkish flowers, and thanks to that beautiful colour which covers the land during the summer months, as well to its low-growing stature, it is widely cultivated in gardens as landscape plants (Morales 2002, Lawrence and Tucker 2002).

Other benefits of thyme in animal and human welfare are mainly related to its essential oils (usually referred as thyme oil), used extensively in many industrial (mainly food, fragrance and cosmetic) and pharmaceutical / medical applications (Leung and Foster 1996). Thyme oil, of which main components are thymol and carvacrol, is known to have a high antimicrobial (biostatic and/or biocidal against a wide spectrum of bacteria and fungi), carminative and expectorant activity (de Bouchberg et al. 1976, Leung and Foster 1996, Reddy et al. 1998, Ferhout et al. 1999, Chao et al. 2000, Horne et al. 2001). Thyme is also used widely, especially in Mediterranean region, either as fresh or dried herbs, to add flavour to foods (Lawrence and Tucker 2002). Hence, thyme and its products can be considered as a unique natural source for medicine and industries. This is also reflected by the increasing demands of the society to such natural products (ESTACOM 2000). However, natural thyme populations are far from being adequate to meet those demands (Rey and Sáez 2002). In addition, natural populations are usually heterogeneous, composed of plants of different chemotypes, which also affects the homogeneity of oil yield and its chemical composition (Echeverrigaray et al. 2001). Possible solution to this great variability which thyme exhibits, as well as to the loss of its natural populations in nature, can be the establishment and conservation *in vitro* of shoot lines from selected thyme populations and their exploitation for industrial purposes. Today, thanks to the advanced biotechnological applications, such clonal lines can be established from a single selected explant by using plant tissue culture approaches (especially ‘micropropagation’). One disadvantage of plant tissue culture can be the risk of spontaneous changes, which may arise in response to continuous *in vitro* manipulations (i.e., periodic subculturing to media containing growth regulators). In this sense, the ‘slow growth storage’ technique is an ideal tool for an efficient and safe medium-term conservation of valuable shoot lines. The technique enables shoot cultures to be kept for several months (or, for some species, one year or more) under the aseptic conditions of tissue culture. Depending on the species and the method used, periodic subculturing of the material can be markedly diminished during slow growth storage, without affecting viability and regrowth potential of shoot cultures. Thus, risk of spontenous alterations, as well as manual labor, cost and risk of contamination can be reduced (Lambardi and De Carlo 2003).

The present study represents the first attempt to develop an efficient and repetitive medium-term conservation approach for *in vitro* shoot cultures of *T. vulgaris* L. As a major aim, the developed slow growth storage technique will allow to improve the industrial exploitation of thyme, through the possibility of conserving numerous populations, selected for their valuable characteristics in terms of essential oil profiles.

**MATERIALS AND METHODS**

***Plant material and surface disinfection***

Seeds of *T. vulgaris* L., collected in the Cilento park (Campania, Italy) and provided by Florsilva (Bologna, Italy), were disinfected by 70% ethanol (5 min), 10% H2O2 (5 min) and 20% commercial bleach containing 2% active chlorine (15 min), respectively, with consecutive rinses in sterile dH2O after each step. Seeds were then germinated *in vitro* on semi-solid MS medium (Murashige and Skoog 1962) devoid of growth regulators. 15 day-old seedlings served as a plant source for shoot tip explants.

***Establishment of in vitro shoot cultures***

*In vitro* shoot cultures were initially established in 9-cm Petri dishes by transferring 1-1.5 cm-long shoot tips on MS medium, supplemented with 1 mg l-1 kinetin (Kin) and 0.3 mg l-1 gibberellic acid (GA3) (i.e., regeneration medium, Ozudogru et al., 2011). All the media used in the study were supplemented with 30 g l-1 sucrose and gelled with 3 g l-1 gelrite. The pH was adjusted to 5.8 with 1N NaOH or HCl prior to the inclusion of gelrite and autoclaved for 20 min at 121°C.

Established shoot cultures were maintained in glass jars (98.5 mm height, with a capacity of 170 ml, Sigma® V0633), at 23±2°C, under a 16 h photoperiod, provided by cool daylight fluorescent lamps at 36 µmol m-2 s-1 (i.e., standard culture conditions) by periodically subculturing (at 4-week intervals) to semi-solid MS medium of the same composition.

***Slow growth storage***

Shoot cultures were subcultured to fresh regeneration medium and maintained at standard culture conditions for 14 days, after which they were transferred to cold storage conditions (i.e., 4°C, in darkness). Over 13 months, four randomly-selected glass jars were recovered monthly from cold storage conditions and the shoot cultures evaluated for their characteristics of conservation.

***Re-establishment of in vitro shoot cultures after cold storage***

Shoots showing strong browning and decay in response to cold storage were discarded, while 1-1.5 cm-long shoot tips of healthy shoots (green or etiolated) were excised, plated on Petri dishes, containing fresh regeneration medium, and transferred back to standard culture conditions. Their regrowth potential was evaluated at the end of the first subculture period (i.e., 4 weeks).

***Data collection and statistical analysis***

Two replications of four glass jars, each containing 15 shoots, were used for each storage period (i.e., 1, 2, 3,… 13 months) and each experiment was repeated twice. After each month, the percentage of healthy shoot clusters, (i.e., the clusters showing no sign of decay which could be used for culture re-establishment after shoot tip transfer in standard culture conditions) was determined. Etiolated shoots were considered healthy; however, the level of etiolation was visually evaluated. Tissue and medium browning was also visually evaluated. Following the transfer to standard culture conditions, the regrowth rate of the excised shoot tips (% of shoot tips, able to retain regrowth potential), the average shoot number (no ± SE) and the average shoot length (cm ± SE) were recorded after 4 weeks. Based on these data, Shoot Forming Capacity Index (SFC Index, Lambardi et al. 1993) was calculated as follows:

SFC Index = (average no of shoots per regenerating explant) x (% of regenerating explants)/100

Statistical analysis of percentages was carried out by the *X*2-based Post Hoc Multiple Comparisons test (Marascuilo and McSweeney 1977). Discrete data were subjected to ANOVA, followed by the least significant difference (LSD) test at P≤0.05 to compare means.

**RESULTS AND DISCUSSION**

***Seed disinfection and culture establishment***

The protocol developed previously for peanut seeds (Ozudogru et al. 2005) enabled 100% thyme seed disinfection. Seeds were then germination *in vitro* on semi-solid MS medium, providing 84% germination rate, with all seeds producing 3 shoots on average. The most appropriate regeneration medium was previously determined through the examinations of several medium compositions (Ozudogru et al. 2011). Accordingly, a semi-solid MS medium supplemented with 1 mg l-1 Kin and 0.3 mg l-1 GA3 was selected, providing 97% regeneration rate and 8.6 shoots per explant. The same medium was used as conservation and post-recovery medium in the present study.

***Survival and regrowth potential of shoot tips after slow growth storage***

The maintenance of shoot cultures at low temperature (‘cold storage’), in combination with reduced light intensity or even darkness, is the most common approach to slow growth storage of shoot cultures. Low temperature and light intensity provide many advantages, among which are the reduction of (i) respiration, (ii) water loss and wilting, and (iii) ethylene production (Ozudogru et al. 2010). Temperature usually varies between 0 and 5°C for cold tolerant species, and 15-25°C for the temperate or tropical species (Lambardi and De Carlo 2003). Although there are examples of using different medium compositions or hormone-free medium, cold storage is usually held in the same medium composition as in the proliferation stage. Using this technique, the costly and hand-labour subculture intervals can be decreased to once in months (usually from 3-4 months up to 1-2 years, depending on the species; Ozudogru et al. 2010), and thus the technique is also referred as ‘medium-term storage’ to differentiate it from cryopreservation (‘long-term storage’).

Although some examples exist, application of slow growth storage technique for *ex situ* conservation of Lamiaceae species has been limited. Dube et al. (2011) employed growth retardants, sorbitol and mannitol, to reduce the growth rate of *Coleus forskohlii* Brig. nodal segments, an endangered perennial, aromatic and medicinal plant of the Lamiceae family, and were able to achieve minimal growth 30 days after a culture on MS medium supplemented with 3 M mannitol at 22°C and 16 h photoperiod. These authors, however, did not give any indication in terms of maximum storage time of shoot cultures. Synthetic seeds (containing axillary buds) of *Lavandula angustifolia* L., perennial shrub of the family Lamiaceae, were stored at 4°C in sterile water for up to 6 months, maintaning a germination potential of 52% after 6 months (Leelavathi 2010). Four accessions of *Mentha* spp., aromatic and medicinal plants of Lamiaceae family cultivated for their essential oils, were conserved on hormone-free MS medium at 2°C for 6 months (Islam et al. 2003). For a successful conservation, authors held the cultures at 20°C and 10°C for one and three weeks, respectively, before they were transferred to 2°C. *Mentha arvensis* L., *M. spicata* L., *M. suaveolens* Ehrh. hybrid, and *M. suaveolens* cv. Variegata, were evaluated for survival during storage in MS media containing three concentrations (25%, 50% and 100%) of nitrogen and in four light and temperature regimes (at 4°C and –1°C in darkness, at 4°C with a 12h photoperiod, and at 25°C with a 16h photoperiod; Reed 1999). Author reported that the shoots of all four genotypes stored at 25°C were in excellent condition still after 6 months, and required subculture after 18 months. However, shoot cultures survived longest at 4°C with a 12h photoperiod on 50% nitrogen content. Under this regime, all four genotypes were rated in good condition at 30 months, while declined to poor condition by 36 months.

As slow growth storage protocol for thyme wss still lacking, the present study was designed to develop a novel and effective cold storage protocol for conservation of valuable *in vitro* shoot collections of *Thymus vulgaris* L. The percentage of *in vitro* shoot cultures in healthy condition was maximum (100%) during the first 5 months of storage, and it remained very high also in the following 3 months (being 74% after 8 months of storage; Table 1). Shoot quality tended to decrease slightly but still remained over 50% until 11-month storage period. However, shoot degeneration became drastic in the following 2 months, as percentage of healthy shoots decreased down to 25% after 13 months. Shoots were completely green at the end of the first month, after which they started to gradually etiolate in the following storage period. Etiolation was not so diffused during the first 5 months, was moderate between 6-8 months, and more evident after 9 months. It is worthy of note that in thyme, such as in many other species, shoot etiolation does not compromise shoot-tip regrowth, even when intense. However, from the 11th month of storage, etiolated shoots started to darken and became completely brown at the end of 13 months. Tissue browning was not observed during the first 9 months, it increased gradually only after 10 months. On the contrary, medium browning occurred immediately after the first month of storage, although very low at the beginning, it was still moderate after 5th month and became intense starting from the 9th month of storage (Fig. 1).

In post-storage, the regrowth rate of shoot tips from healthy (green or etiolated) shoots ranged from 92-100% through 11 months of storage, producing multiple shoots (4.4-9.1), with an SFC Index of 4.1-9.1 (Table 2, Fig. 2A). Regrowth rate under standard culture conditions was in the same range (97%), producing 8.6 shoots per explant, with an SFC Index of 8.3. Prolonging the storage period to 12 months resulted with a reduction in regrowth rate, however the mean shoot number and the SFC Index were maintained high (Fig. 2B). It is worthy of note that the mean shoot number and the SFC Index of shoot tips, excised from the shoot clusters after at least 3 months of storage, started to decrease in response to cold storage. Those shoot tips were excised from shoot clusters that did not show clear evidences of decay (e.g., browning, wilting) and, thus, considered healthy (see Table 1). However, probably they contained some shoot tips in which the tissue decay was already started, resulting with a loss in their regrowth potential. Tissue decay was stronger when the storage period was prolonged, especially starting from 9 months of storage; at that time, decayed shoot clusters were evident and, as a consequence, shoot tips were excised only from shoots still vigorous and healthy. This fact was reflected by an increase in mean shoot number and SFC index of shoot tips transferred to standard culture conditions following 9-12 months of storage. The reduction of the SFC Index was very drastic for shoot cultures from 13 months of cold storage (Fig. 2C). Hence, 12 month was considered the maximum storage period for thyme shoot cultures at 4°C and in the dark.

As for shoot length, no variation was observed among the different conservation periods and in comparison to the control samples.

As a conclusion, our results showed that cold storage of *T. vulgaris in vitro* shoot cultures can be effectively prolonged to 12 months without requiring periodic subculturing, and multiplication cycles can easily be re-established by using shoot tips excised from those cultures. Moreover, the study evidences that a careful observation of the stored material and a proper selection of shoots, from which to excised shoot tips, can influence shoot regrowth and a fast re-establishment of shoot cultures in post-conservation.

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**TABLES**

**Table 1.** Survival of *Thymus vulgaris* *in vitro* shoot cultures in response to slow growth storage at 4°C in darkness.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Storage time**  **(months)** | **Healthy shoot clusters1**  **(%)2** | **Tissue**  **browning3** | **Medium browning3** | **Etiolation3** |
| 1 | 100 a | **-** | **-** | **-** |
| 2 | 100 a | **-** | **+** | **+** |
| 3 | 100 a | **-** | **+** | **+** |
| 4 | 100 a | **-** | **+** | **+** |
| 5 | 100 a | **-** | **++** | **+** |
| 6 | 95 a | **-** | **++** | **++** |
| 7 | 84 a | **-** | **++** | **++** |
| 8 | 74 ab | **-** | **++** | **++** |
| 9 | 63 b | **-** | **+++** | **+++** |
| 10 | 66 b | **+** | **+++** | **+++** |
| 11 | 53 b | **++** | **+++** | **++** |
| 12 | 37 c | **+++** | **+++** | **+** |
| 13 | 25 c | **+++** | **+++** | **-** |

(1) ‘Healthy shoot clusters’ refers to green and/or etiolated shoots, not showing any clear sign of decay.

(2) Percentage values statistically analysed by a non-parametric test, the Post Hoc Multiple   
 Comparisons Test (Marascuilo and McSweeney 1977).

(2) Morphological observations indicated as (-), none; (+), low; (++), moderate; (+++), intense.

**Table 2.** Regrowth potential of *Thymus vulgaris* shoot tips 4 weeks after being back to standard culture conditions following slow growth storage at 4°C in darkness.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Storage time**  **(months)** | **Regrowth rate1**  **(%)2** | **Mean shoot number3**  **(no ± SE)** | **Mean shoot length3**  **(cm ± SE)** | **SFC index4** |
| 1 | 100 a | 8.5 ± 0.6 a | 1.2 ± 0.1 a | 8.5 |
| 2 | 100 a | 9.1 ± 0.8 a | 1.3 ± 0.1 a | 9.1 |
| 3 | 92 a | 7.3 ± 0.8 b | 1.6 ± 0.1 a | 6.7 |
| 4 | 93 a | 4.4 ± 0.3 c | 1.0 ± 0.0 a | 4.1 |
| 5 | 100 a | 4.7 ± 0.3 c | 1.0 ± 0.0 a | 4.7 |
| 6 | 95 a | 4.7 ± 0.5 c | 1.6 ± 0.1 a | 4.5 |
| 7 | 97 a | 6.5 ± 0.5 b | 1.1 ± 0.0 a | 6.3 |
| 8 | 92 a | 5.6 ± 0.4 bc | 1.1 ± 0.0 a | 5.2 |
| 9 | 95 a | 7.7 ± 0.4 ab | 1.2 ± 0.0 a | 7.3 |
| 10 | 98 a | 8.4 ± 0.5 a | 1.3 ± 0.0 a | 8.1 |
| 11 | 98 a | 7.4 ± 0.4 b | 1.3 ± 0.0 a | 7.3 |
| 12 | 78 b | 8.8 ± 1.2 a | 1.0 ± 0.0 a | 6.9 |
| 13 | 3 c | 6.0 ± 2.0 bc | 1.2 ± 0.0 a | 0.2 |
|  |  |  |  |  |
| Control5 | 97 | 8.6 ± 0.9 | 1.0 ± 0.0 | 8.3 |

(1) Regrowth rate was calculated based on the total number of shoot tips re-introduced to standard culture conditions.

(2) Percentage values statistically analysed by a non-parametric test, the Post Hoc Multiple   
 Comparisons Test (Marascuilo and McSweeney 1977).

(3) Statistical analysis performed by ANOVA, followed by LSD test at P≤0.05; SE, standard error of means.

(4) SFC index = (mean shoot number per regenerating explant) x (regrowth rate) / 100

(5) *In vitro* plant regeneration from *T. vulgaris* shoot tips at standard culture conditions (i.e.,   
 23±2°C, 16h photoperiod)

**LEGENDS TO FIGURES**

**Figure 1.** *In vitro* shoot cultures of *T. vulgaris* after (A) 1 month (bar, 1.75 cm), (B) 5 months (bar, 1.35 cm), (C) 11 months (bar, 1.20 cm), and (D) 13 months of storage at 4°C, in darkness (bar, 1.25 cm).

**Figure 2.** Regrowth of shoot tips, excised from *in vitro* shoot cultures of *T. vulgaris* L., maintained at cold storage conditions for (A) 11 months (bar, 1.35 cm), (B) 12 months (bar, 1.35 cm), and (C) 13 months (bar, 1.05 cm).