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Micropropagation of *Thymus vulgaris* L., an Important Medicinal and Aromatic Plant

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Abstract The aim of this work was to elaborate an *in vitro* propagation protocol for the *Thymus vulgaris* species using seeds as a source of explants. Four weeks after inoculation, the percentage of germinated seeds was 81% in the case of cultures mentained in the light and 50% in the case of cultured mentained in the dark. The highest multiplication rate (5.3 microshoots/explant) and the highest average length of shoots (6.5 cm) were obtained on the nutrient medium without phytohormones. MS culture medium [18] with mineral salts reduced by half, supplemented with 2 mg/l IBA has been shown to be most effective for *in vitro* rooting of *T. vulgaris* microshoots. The rooted vitroplants have successfully accommodated to *ex vitro* conditions, achieving an acclimatized rate of 96%. The results obtained throughout the *in vitro* regeneration phases confirm that the micropropagation technique is an efficient method of multiplication for the *T. vulgaris* species.

Key words

thyme, *in vitro* culture, growth regulators, multiplication, rooting

The genus *Thymus* L. belongs to the *Lamiaceae* family and consists of over 400 species of herbaceous annuals and perennial plants that are extensively used, for medicinal and nonmedicinal purposes. These plants are widely distributed throughout the Old World [17, 26].
The dried leaves and flowering tops are employed as flavouring agents for food and beverages, and as sources of essential oil for the pharmaceutical and cosmetic industries. Thyme is an important medicinal plant, and essential oils extracted from the plant, commonly known as thyme oils, have a wide range of therapeutic applications and properties including antirheumatic, antiseptic, antibacterial, carminative, diuretic and expectorant [4, 5, 9, 16, 19, 30].
Advances in biotechnological approaches provide a set of techniques that contribute to solving problems of extinction or genetic erosion in particular of plants.
Alternatives for fast multiplication, like micropropagation, offers independence from climatic factors, elimination of geographical and political boundaries, shorter production cycles and novel products not found in nature. In recent years, there have been numerous publications on micropropagation of various species of the *Thymus* genus: *T. bleicherianus* [23], *T. caespititius* [15], *T. daenensis* [2, 10], *T. hyemalis* [21], *T. satureioides* [22], *T. longicaulis* [25], *T. lotocephalus* [3], *T. mastichina* [6, 14], *T. membranaceus* [27], *T. moroderi* [13], *T. persicus* [1, 31], *T. piperella* [28], *T. marschallianus* Willd. and *T. serphyllum* [29].
Even though *T. vulgaris* is the most common species of this genus and is grown in large areas, scientific literature on the possibilities of propagation of this species in *in vitro* cultures is not extensive and the results presented therein are often divergent. Therefore, this study attempts to identify the most efficient method of thyme propagation in *in vitro* plant cultures.

Material and Method

Culture initiation
The biological material used for the initiation of *in vitro* cultures consisted of *T. vulgaris* seeds. The seeds were first washed with tap water and were sterilized in 6% calcium hypochlorite for 10 minutes, after which were performed three rinses with sterile distilled water to remove traces of sterilizing agent. After sterilization, the seeds were inoculated on the MS culture medium [18] without growth regulators.
At this stage of the experiment, as well as at subsequent stages, the medium was supplemented with 40 g/l glucose (as carbon source), 32 g/l NaFeEDTA (as iron source) and 7 g/l agar (for solidification of culture media). The culture media were sterilized by autoclaving at 120°C for 20 minutes. Before autoclaving, the pH of the medium was adjusted to 5.6-5.8.
The inoculation of seed was carried out under aseptic conditions at the hood with sterile laminar air flow.
Cultures were transferred to the growth room where controlled conditions of temperature (22-24°C), photoperiod (16 hours) and light intensity (3000 lx) were ensured. At the initiation stage, half of the cultures were subjected to the aforementioned conditions, and the other half was cultured in the dark.

After four weeks, the percentage of germinating seeds was determined.

Multiplication phase

In order to culture proliferation, the apexes and nodal fragments resulting from the partitioning of the microshoots regenerated in the initiation cultures phase were inoculated on the MS culture medium supplemented with different types of cytokinins (BAP - benzylaminopurine, 2iP - 2 isopentyladenine, KIN - kinetin) in concentrations of 1, 2 and 5 mg/l. As a control variant, the MS medium without growth regulators was used. Subcultivation on fresh medium was performed at an interval of four weeks.

The following morphological characteristics of the analysed plants were determined: number of shoots and shoots length.

Rooting phase

Microshoots regenerated on the multiplication nutrient media were detached from the culture, individualized and cultivated on rooting medium represented by a variant of the MS basal medium with the mineral salts reduced to half in which the type of auxin (NAA - naphthalenacetic acid, IAA - indolylacetic acid, IBA - indolylbutyric acid) and its concentration (0.5; 1; 2 mg/l) was varied. As a control variant, the ½MS medium without growth regulators was used.

After four weeks of culturing was calculated rooting rate as a ratio between the number of shoots at which the rhizogenesis process took place and the total number of shoots transferred to the rooting culture medium.

Acclimatization phase

In vitro rooted microshoots were transferred to pills of peat type Jiffy and peat, perlite and manure mixture in a ratio of 2:1:1 for acclimatization to *ex vitro* conditions. The percentage of acclimatized plants was calculated as the ratio of the number of viable acclimatized plants and the total number of plants transferred *ex vitro*.

Results and Discussions

In vitro propagation technique generally involves four distinct stages: initiation of culture, multiplication, rooting of microshoots and acclimatization.

"In vitro" culture initiation

The cultures initiation is a basic technological sequence in the *in vitro* multiplication of different plant species. One of the essential conditions on which depends the success of initiation and maintenance of a cell culture is that of ensuring asepsis. The method of the biological material sterilization varies depending on the origin of the material, the physiological state and the type of organ.

In our research for *in vitro* culture initiation they were used seeds as explants. The observations made revealed that the inclusion of *T. vulgaris* species in this culture system does not pose particular problems, the use of calcium hypochlorite (6% solution for 10 minutes) for the seeds sterilization proved to be efficient. Also, at this stage the morphogenetic potential of the *T. vulgaris* explants was evaluated, by monitoring the percentage of germinated seeds. The use of MS basal medium without growth hormones favored seed germination and the production of neoplantlets which were then used to test the morphogenetic reaction of *T. vulgaris* explants on different hormonal formulations of the MS medium (Figure 1)..

Fig. 1 Plantlets regenerated from seed germination on MS medium without growth regulators, after 4 weeks from initiation of *in vitro* culture

The seeds began to germinate three days after inoculation on the culture medium, and after one week from the beginning of the experiment the percentage of germinated seeds grown in the light was 58% compared to 43% in the case of seeds grown in the dark. After four weeks, in the case of the cultures mentained in the light the percentage of germinated seeds was 81% compared to 50% in the case of cultures mentained in the dark (Figure 2).

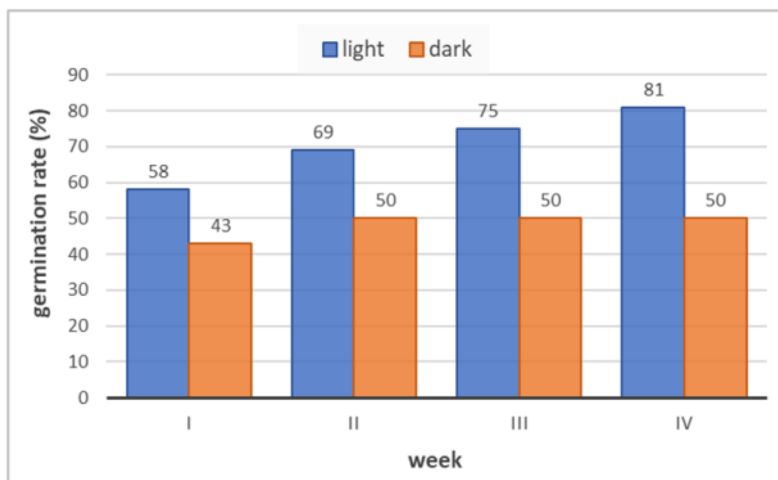

Fig. 2. The percentage of *in vitro* germinated seeds to light and to dark

For most species of the *Thymus* genus, decontamination of seeds is carried out by soaking in NaOCl solution at a concentration of 1% as for *T. hyemalis* [21] to 5% as for *T. persicus* [1] and *T. lotocephalus* [3], or in 0.1% $HgCl_2$ solution as for *T. mastichina* [6]. Other agents used for decontamination of *T. vulgaris* seeds were 5% calcium hypochlorite [8], 20% NaOCl solution [11], or 10% H_2O_2 solution [25].

Effect of cytokinin type and concentration on the "in vitro" proliferation of shoots

The propagation phase spans several subcultures with a duration of approximately four weeks. The number of subcultures must be limited (varies by species), any prolongation of this phase requiring further verification of the genetic stability of the material.

In this phase, the main purpose is to obtain a many vitroplants as possible while maintaining the genetic stability of the material. The increase of the multiplication rate can be obtained by stimulating the development of the axillary buds, by stimulating the elongation of the shoots or by both methods simultaneously. This is usually done by increasing the amount of cytokinins in the medium or by a hormonal balance inclined in favor of cytokinins.

Research on propagation of *in vitro* thyme cultures was initiated by Olszowska and Furmanowa [7, 24]. The aforementioned authors multiplied the plants on modified NN medium [20] with addition of 10 $mgdm^{-3}$ adenine sulphate (AS) and 0.5 $mgdm^{-3}$ IBA.

In our experiments, the research was carried out on three of the most important cytokinins, namely BAP, 2iP and KIN.

However, the control variant without growth regulators led to the best morphogenetic response (Figure 3). The results are consistent with those reported by other authors, in the case of thyme supplementation of the nutrient medium with cytokinins resulting in the decrease of the microshoots length [12, 23].

Fig. 3. Biological material multiplied *in vitro* on MS medium without growth regulators

Supplementation of the culture medium with cytokinins had an inhibitory effect on the growth of microshoots. In the case of all the cytokinins used, the induction of the regenerative processes was reduced with the increase of their concentration (Table 1).

Table 1. The influence of cytokinin type and concentration on the number of shoots/explants and the shoots length at *T. vulgaris* species (values are the mean of 3 repetitions)

Cytokinin	Concentration (mg/l)	No. of shoots/explant	Shoots length (cm)
Control	0	5.3	6.5
BAP	1	3.4	4.3
	2	1.8	2.6
	5	1.2	2.0
2iP	1	2.7	3.4
	2	2.5	2.1
	5	1.3	1.9
KIN	1	4.0	5.6
	2	2.4	2.8
	5	1.1	2.6

The results showed that between the three cytokines used, the most effective in the shoots regeneration has been shown to be kinetin. The highest values regarding the multiplication rate (4 microshoots/explant) and the shoots length (5.6 cm) were obtained on the MS medium supplemented with 1 mg/l KIN. Increasing the concentration of kinetin resulted in a weaker morphogenetic response (multiplication rate 1.1 microshoots/explant and average shoots length 2.6 cm in the case of KIN in the concentration of 5 mg/l).

The results of our own research are in contradiction with those obtained by Kulpa et al. [12] in which KIN at a concentration of 0.5 $mgdm^{-3}$ resulted in development of plants of the lowest mass and smallest number of side shoots.

In their extensive research on the effect of cytokinins (6-benzyladenine, kinetin, and thidiazuron) on the multiplication of *T. vulgaris* and *T. longicaulis*, Ozudogru et al. [25] found semi-solid MS medium supplemented with 1 $mgdm^{-3}$ kinetin and 0.3 $mgdm^{-3}$ GA_3 to be the most efficient medium at the multiplication stage. Also, Nordine et al. [21] claimed that the best medium to be used at this stage of the experiment was the medium supplemented with 0.5 $mgdm^{-3}$ KIN.

In our experiments, the replacement of KIN with BAP had inhibitory effect on the shoots regeneration, the induction of regenerative processes being reduced as the cytokinin concentration in the nutrient medium increased. Thus, in the case of using the concentration of 1 mg/l BAP, a multiplication rate of 3.4 microshoots/explant was obtained and an average shoots length of 4.3 cm, higher than those obtained on the medium supplemented with 5 mg/l BAP (propagation rate 1.2 microshoots/explant and average shoots length 2 cm).

Variants of the nutrient media supplemented with 2iP allowed the induction of regenerative processes, but to a lesser extent. The highest values in terms of the multiplication rate (2.7 microshoots/explant) and the shoots length (3.4 cm) were obtained at the concentration of 1 mg/l 2iP, these values decreasing with increasing cytokinin concentration (multiplication rate 1.3 microshoots/explant and average shoots length 1.9 cm in case of 2iP in concentration of 5 mg/l).

The results of our own research are in contradiction with those obtained by Kulpa et al. [12] who indicate that the cytokinin which had the most positive impact on plant development at the multiplication stage was 2iP (medium supplemented with 5 $mgdm^{-3}$).

After approximately 30 days, the shoots obtained were transferred to fresh medium that supported the regenerative processes. From the qualitative point of view, the biological material resulting from the regeneration of explants had a normal morphology, without vitrification aspects, necrosis or callus differentiation.

Effect of auxin type and concentration on the "in vitro" rooting of shoots

It is known that, in *in vitro* cultures, the auxins are responsible for stimulating root development and cell elongation. However, high concentrations of auxin added to the nutrient media result in the development of callus and inhibition of root system development.

In the present study, nine variants of rooting medium were tested in which the type of auxin (NAA, IAA, IBA) and its concentration (0.5; 1; 2 mg/l) were varied. As a control variant, the ½MS medium without growth regulators was used. The effect of the type and concentration of auxin on the *in vitro* rooting ability of *T. vulgaris* microshoots was evaluated four weeks after the initiation of the experiment.

The highest rates of rooting were obtained when the culture medium was supplemented with IBA, followed by NAA and IAA. For all auxins used, the rooting rate increased with increasing their concentration.

The highest value of the rooting rate (100%) was obtained on the ½MS medium without growth hormones. However, in comparison with the variant supplemented with 2 mg/l IBA (in which microshoots were rooted in a proportion of 97%), on the medium without auxin, a smaller number of roots was obtained, an important parameter for the success of the next stage, the acclimatization. Therefore, the ½MS medium

supplemented with 2 mg/l IBA is recommended for *in vitro* rooting of *T. vulgaris* microshoots (Table 2 and Figure 4).

Table 2. Effect of auxin type and concentration on the *in vitro* rooting ability of *T. vulgaris* microshoots (values are the mean of 3 repetitions)

Auxin	Concentration (mg/l)	Rooting rate (%)
Control	0	100
IBA	0.5	89
	1	91
	2	97
NAA	0.5	78
	1	83
	2	87
IAA	0.5	65
	1	69
	2	72

Fig. 4. Rooting of *T. vulgaris* microshoots on ½MS medium supplemented with 2 mg/l IBA

The results obtained in the course of our own research are consistent with those reported by Kulpa et al. [12], in the case of thyme shoots rooted on MS medium without auxins produced the shortest plants with the smallest number of roots. They were found that the tallest plants with the greatest mass and the longest and most developed root systems were cultured on the medium supplemented with IBA at a concentration of 1 mgdm^{-3}. According to Nordine and El Meskaoui [23], Nordine et al. [21] and Coelho et al. [3], the most suitable medium to be used for rooting *Thymus* sp. is MS medium without plant growth regulators or medium supplemented with IBA at a concentration of 0.5 or 1.5 mgdm^{-3}.

Ozudogru et al. [25] claimed that MS medium supplemented with 0.01 mgdm^{-3} NAA is the most efficient for rooting thyme shoots. Results of the research conducted by Coehlo et al. [3] proved that the best medium used at the rooting stage is a combination of MS medium with 1 mgdm^{-3} NAA or 0.5 mgdm^{-3} IAA. In contradiction, in the research made by Kulpa et al. [12] it was found that NAA added to the medium at a concentration from 0.2 to 2 mgdm^{-3} results in development of less tall plants with roots shorter than those found in plants multiplied on the control medium.

Influence of the nutrient substrate on the acclimatization to "ex vitro" conditions of the T. vulgaris vitroplants

Acclimatization is the final stage, but absolutely necessary, which is found in all types of plant micropropagation. Regardless of the *in vitro* culture method adopted, its success depends on the ability to transfer the plants *ex vitro*, to an acceptable economic level. This involves adapting the vitroplants to the new environmental conditions such as: lower relative humidity, higher light intensity, temperature fluctuations and stress caused by different diseases.

The experiments performed in this study led to the conclusion that the nutrient substrate has an important role on the success of the acclimatization of *T. vulgaris* vitroplants, the best results being obtained when were used pills of peat type Jiffy (96% acclimatized plants). When using the mixture of peat, perlite and manure in proportion of 2:1:1, the percentage of acclimatized plants was lower (79%), this substrate ensuring a good fortification of the acclimatized plants.

After the period of acclimatization and fortification in pots, the plants were transplanted in the greenhouse to continue their growth and development. The plants obtained by *in vitro* propagation have retained the morphological characteristics of the donor plants (Figure 5 a and b).

Fig. 5. *T. vulgaris* plant fortified in pot (a) and soil (b)

We conclude that the results obtained throughout the *in vitro* regeneration phases certify that for the *T. vulgaris* species, the micropropagation technique represents an advantageous alternative to the classic methods of propagation, which allows the rapid obtaining of high quality seedlings.

Conclusions

Our research shows that the shoot multiplication of *T. vulgaris* is depended upon the treatment with growth regulators that is used. The new regenerated plants of thyme have been appeared normal and no morphological variation was shown. The *in vitro* culture system successfully established for *T. vulgaris* offers a viable tool for preservation, multiplication and sustainable production of this very valuable medicinal species. This protocol can ensure a stable supply of this commercial crop in limited time and space, irrespective of seasonal variations and thus meet the global demand for its essential oil. Regenerated plants could also serve as potential sources for the extraction of active compounds for pharmaceutical purposes.

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