# Features of morfogenesis of *Thymus x citriodorus* (Pers.) Schreb. and *Thymus marschallianus* Willd. explants at the second stage of clonal micropropagation *in vitro*

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**Abstract.** Many species of the genus *Thymus* are widely known as valuable essential oil and medicinal plants that have antiseptic, anti-inflammatory and analgesic properties. The use of biotechnological methods makes it possible to increase the efficiency of traditional methods of breeding and seed production. Our investigation aimed to study the influence of the different factors on the explants morphogenesis at the 2th stage of *Thymus marschallianus* Willd. and *Thymus* × *citriodorus* (Pers.) Schreb. clonal micropropagation. It was established that the highest morphogenetic potential was possessed by explants of *T. marschallianus*, whose multiplication index reached 19.9, while that of *T.* × *citriodorus* was 10.3. The optimal culture media for *T. marschallianus* (½MS with 0.5 mg/l naphthylacetic acid and 20 g/l glucose) and for *T.* × *citriodorus* (MS hormone free and 20 g/l sucrose) were determined. The efficiency of culturing *T.* × *citriodorus* explants in glass jars (closed with foil) was demonstrated, which allowed increasing the multiplication index up to 2.9–6.2 times compared to using test tubes or flasks as culture vessels. It was found that a longer growing cycle (70 days) in *T.* × *citriodorus* contributed to an increase the multiplication index by 3.6 times, and in *T. marschallianus* – by 6.2 times compared to the standard growing cycle (40 days).

## 1 Introduction

To expand the range of natural products used in the pharmaceutical, food, perfumery and cosmetic industries, it is necessary to introduce new promising species of medicinal and essential oil plants into agricultural production. One of such valuable plants is thyme. Its essential oil has analgesic, antiseptic, anti-inflammatory, antispasmodic and antioxidant effects. Two thyme species, *Thymus vulgaris* L. and *T. serpyllum* L., are actively used in the pharmaceutical industry of Russia and other countries [1]. There is information about the use of *T.* × *citriodorus* plant raw materials in the composition of medicines and in cooking [2-5]. Essential oil of this species is also used in cosmetology, alcoholic beverage

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and perfume industries. It was shown that the essential oil of *T.* × *citriodorus* contains terpinolene (59.0–71.0%) and α-terpineol (20.0–29.6%) [6]. Another study found that the main components of the essential oil of this species are geraniol (54.2–72.5%) and citral (up to 15%), which gives it a pleasant lemon aroma [5]. A number of publications have shown the effectiveness of using *T. marschallianus* plant raw materials as an antimicrobial [7, 8], antitumor [9] and antioxidant [10] agent. Data that the essential oil of *T. marschallianus* contains 42.5% thymol and 13.2% limonene [10], as well as carboxylic [11] and hydroxycinnamic acids [12] are presented.

The solution of many problems of plant selection and seed production is associated with the use of biotechnological methods, in particular, clonal micropropagation *in vitro*, which has many advantages over traditional methods [13-15]. The efficiency of clonal micropropagation is influenced by a number of endogenous and exogenous factors: plant genotype, type and physiological state of the explant, *in vitro* cultivation conditions, composition of the culture medium, and many others. In addition, when culturing under aseptic conditions, researchers note a number of problems: poor survival or high contamination of explants, shoots vitrification, low multiplication index [15-18]. By studying and identifying optimal limiting factors, it is possible to regulate plant morphogenesis processes in artificial systems and create effective biotechnological methods on this basis. Many publications are devoted to the study of thyme *in vitro* micropropagation, most of them concern the optimization of the culture media composition for the main stages of this process [19-21]. However, we have practically never seen similar publications for *T. marschallianus* and *T* × *citriodorus* in the literature. Our investigation aimed to study the influence of the different factors on the explants morphogenesis at the 2nd stages of *Thymus marschallianus* and *T.* × *citriodorus* clonal micropropagation.

## 2 Materials and methods

As plant material we used tissues and organs of essential oil plants: *Thymus marschallianus* Willd. and *Thymus x citriodorus* (Pers.) Schreb. (cv. 'Doone Valley'). The initial donor plants were grown under greenhouse conditions. Preparation of materials, equipment and culture media for work in aseptic conditions, analysis of growth processes were carried out according to recommendations [22, 23]. To sterilize the plant material, we used 70% ethanol (Rosspirtprom, Russia) and 0.3% DezTab solution (Akhlor Donge LTD, China).

For clonal micropropagation, stem segments with one node (5–10 mm) were used as explants. Cultivation was carried out on modifications of the Murashige and Skoog (MS) culture medium with the addition of plant growth regulators in various concentrations: β-indolyl-3-acidic acid (IAA), α-naphthylacetic acid (NAA), kinetin (Kin), 6-benzylaminopurine (BAP), thidiazuron (TDZ), β-gibberellic acid ($GA_3$) (Sigma, USA). The explants were grown in a culture room at a temperature of 24–26 °C, 70% air humidity and illumination of 2–3 klux with a 16-hour photoperiod. For micropropagation, the following were used as culture vessels: test tubes (150×15 mm) closed with cotton-gauze plug or foil, as well as glass jars or flasks (200 ml) closed with foil. 1–2 explants were placed in test tubes with 10 ml of culture medium, and 4–5 explants were placed in jars or flasks with 30 ml of medium. The duration of the growing cycle varied from 30 to 70 days. During the analysis, the number of shoots (pcs. per explant), shoot length (cm), number of nodes on the shoot (pcs.), frequency of hydrated shoots (%), multiplication index and other parameters were determined. When calculating the multiplication index, the number of shoots formed on the explant was multiplied by the average number of nodes on the shoot.

In each experimental variant, at least 20 explants were analyzed in 2–3 replicates. The data were processed statistically using the Microsoft Office software package (Excel 2010). The reliability of differences between variants was calculated using Student's t-test at the

P≤0.05 level of significance. The results obtained are presented in tables as the arithmetic mean with standard error, and in graphs as the arithmetic mean with confidence intervals.

## 3 Results and discussions

It was established that in the studied species during the cultivation of explants, along with the development of axillary shoots, adventitious shoots were formed. Moreover, the efficiency of micropropagation depended on the hormonal composition of the culture medium, genotype, culture vessel and duration of the growing cycle.

When studying the influence of the culture medium composition on the morphometric parameters of *T.* × *citriodorus* microshoots, it was revealed that the maximum number of shoots (8.6 pcs. per explant) was obtained on the MS medium containing 1.0 mg/l BAP or 1.0 mg/l $GA_3$ (Fig. 1). In *T. marschallianus*, an increase in this indicator was noted when using the MS culture medium with the addition of TDZ together with $GA_3$ or IAA. In this experiment, in all MS medium modifications, sucrose (20 g/l) was used as a source of carbohydrates.

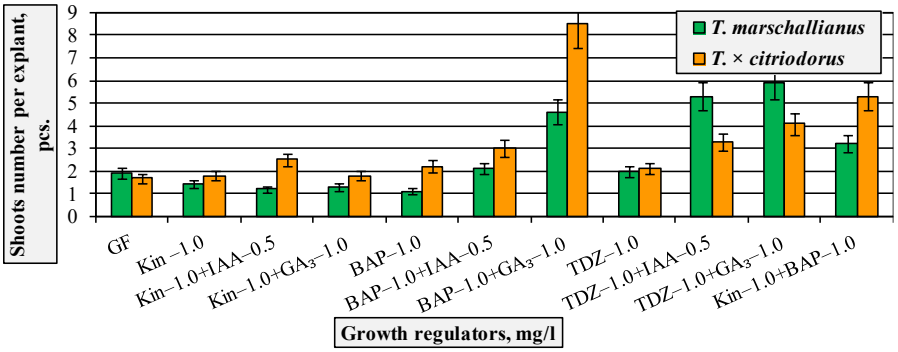


**Fig. 1.** The influence of the culture medium composition and genotype on the shoots number (pcs. per explant) at the second stage of *Thymus* clonal micropropagation.

Analysis of the dependence of shoot length on the growth regulators composition in the culture medium showed that this parameter reached its maximum value (1.6 cm) in *T.* × *citriodorus* when using MS hormone-free medium (GF) or MS with the addition of kinetin and $GA_3$ (Table 1). Addition of BAP or TDZ to the medium contributed to a decrease in the microshoots length by 2.2-4.0 times. In Nordine 's and colleagues research [19] the maximum number of microshoots of *T. broussonetii* was obtained on a medium supplemented with 0.5 mg/l BAP and 0.2 mg/l NAA. However, developed microshoots were short. In our studies, a similar decrease in shoot length was also observed in *T. marschallianus*, but only when cultivated on media with TDZ.

**Table 1.** The influence of the culture medium composition on the explant development at the second stage of *Thymus* clonal micropropagation.

| Growth regulators in MS culture medium, mg/l | *T. marschallianus* | | | *T.* × *citriodorus* | | |
|---|---|---|---|---|---|---|
| | Shoot length, cm | Multiplication index | Frequency of hydrated shoots,% | Shoot length, cm | Multiplication index | Frequency of hydrated shoots,% |
| _ | 0.6±0.1 | 2.1±0.2 | 0 | 1.6±0.2 | 4.6±0.3 | 0 |
| Kin –1.0 | 0.9±0.1 | 1.5±0.1 | 0 | 1.1±0.1 | 2.2±0.2 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Kin–1.0+IAA–0.5 | 0.8±0.1 | 1.6±0.2 | 0 | 0.7±0.2 | 3.8±0.4 | 0 |
| Kin–1.0+ $GA_3$–1.0 | 0.9±0.2 | 2.9±0.3 | 0 | 1.3±0.1 | 2.7±0.3 | 0 |
| BAP–1.0 | 0.8±0.1 | 1.2±0.1 | 0 | 0.4±0.1 | 4.0±0.4 | 21.6±2.4 |
| BAP–1.0+IAA–0.5 | 0.7±0.1 | 3.8±0.4 | 8.1±0.9 | 0.5±0.1 | 4.8±0.4 | 29.8±2.9 |
| BAP–1.0+ $GA_3$–1.0 | 0.9±0.2 | 5.1±0.5 | 19.6±1.8 | 0.5±0.1 | 5.2±0.5 | 78.6±7.9 |
| TDZ–1.0 | 0.4±0.1 | 2.8±0.3 | 0 | 0.6±0.1 | 2.3±0.2 | 0 |
| TDZ–1.0+IAA–0.5 | 0.4±0.1 | 5.8±0.6 | 22.1±1.9 | 0.6±0.1 | 3.4±0.4 | 18.1±1.9 |
| TDZ–1.0+ $GA_3$–1.0 | 0.3±0.1 | 6.5±0.7 | 28.4±2.8 | 0.6±0.1 | 5.7±0.6 | 36.5±3.9 |
| Kin–1.0+BAP–1.0 | 1.0±0.1 | 3.5±0.4 | 0 | 0.6±0.1 | 5.8±0.6 | 68.1±6.9 |

When cultivating explants, the maximum multiplication index (up to 6.5) was noted on media with 1.0 mg/l TDZ or BAP (Table 1). However, the high frequency of vitrified shoots formation (up to 78.6%) indicates the inappropriateness of using these cytokinins for micropropagation of the studied thyme species. Shoot vitrification was also observed in *T. sibthorpii* [20]. At the same time, the maximum morphometric parameters at the stage of introduction *in vitro* were obtained using MS medium with 0.1–2.0 mg/l BAP [21, 24] or this cytokinin together with 0.2 mg/l NAA [19]. Comparing three cytokinins (BAP, kinetin, TDZ), Turkish scientists obtained the best results for *T. leucotrichus* multiple shoots formation using 1.0 mg/l BAP [18]. Similar results were shown by Baktiar et al. for *T. persicus.* In this case, the optimal culture medium at the second stage of micropropagation was MS with 2.0 mg/l BAP and 0.5 mg/l NAA [21]. Nordine Aicha et al. [24] proposed to use ½MS medium with 1.0 mg/l BAP during *T. satireioides* micropropagation. Similar results on the study of the dependence of the length of microshoots on cytokinins in the culture medium were obtained in our studies.

On MS culture media without growth regulators or with the addition of kinetin, the formation of hydrated shoots was not observed, which allows them to be recommended for the 2nd stage of micropropagation. Thus, the highest multiplication index in *T.* × *citriodorus* (4.6) was obtained when cultivating explants on a hormone-free MS medium, and in *T. marschallianus* (2.9) – on a medium with 1.0 mg/l kinetin and 1.0 mg/l $GA_3$. According to the literature data, cultivation of some thyme species at the second stage of clonal micropropagation was effective on a hormone-free MS medium [20, 25, 26].

Since the multiplication index in these experiments for two studied thyme species were low, we analyzed the possibility of using a longer growth cycle (up to 70 days), different types of culture vessels (test tubes, jars, flasks) and plugs to close them.

It was found that in *T. marschallianus*, the number of shoots when cultivated in jars was 1.4–1.6 times higher on a medium with BAP, compared to cultivation in test tubes or flasks (Table 2). On a culture medium with TDZ, this indicator was higher in test tubes and flasks with cotton-gauze plugs. At the same time, in *T.* × *citriodorus*, the number of shoots in different culture vessels did not differ significantly.

**Table 2.** The influence of the culture vessel and culture medium composition on the shoots number (per explant) at the second stage of two thyme species clonal micropropagation (40 days of cultivation).

| Species | Growth regulators in MS culture medium, mg/l | Shoots number, pcs. per explant | | | |
|---|---|---|---|---|---|
| | | test tubes with cotton gauze plug | test tubes with foil | jars with foil | flasks with cotton gauze plug |
| *T.* × *citriodorus* | – | 1.1±0.1 | 1.1±0.1 | 1.6±0.3 | 1.3±0.1 |
| | Kin–1.0 | 1.4±0.1 | 1.5±0.1 | 1.7±0.1 | 1.7±0.1 |

| | | | | | |
|---|---|---|---|---|---|
| | Kin–1.0+$GA_3$–1.0 | 1.5±0.2 | 2.0±0.2 | 1.8±0.1 | 1.8±0.2 |
| | BAP–1.0 | 1.2±0.1 | 1.3±0.1 | 2.0±0.2 | 2.0±0.3 |
| | TDZ–1.0 | 1.7±0.1 | 1.3±0.1 | 2.1±0.2 | 1.8±0.1 |
| *T. marschal-lianus* | – | 2.7±0.3 | 2.7±0.3 | 2.2±0.1 | 2.3±0.2 |
| | Kin–1.0 | 2.1±0.1 | 2.1±0.1 | 2.4±0.1 | 2.5±0.3 |
| | Kin–1.0+$GA_3$–1.0 | 2.2±0.1 | 2.2±0.1 | 2.5±0.1 | 2,3±0.1 |
| | BAP–1.0 | 3.6±0.4 | 3.6±0.4 | 5.6±0.7 | 4.0±0.5 |
| | TDZ–1.0 | 6.6±0.8 | 4.6±0.8 | 5.2±0.8 | 6.1±0.4 |

The multiplication index when culturing *T.* × *citriodorus* explants in jars was 1.6–2.4 times higher compared to culturing in test tubes or flasks for 40 days (Fig. 2A). With a longer growth cycle (70 days) for this species on an optimal medium (MS without growth regulators), this parameter when using jars was 2.9–7.4 times higher compared to other culture vessels (Fig. 2B).

An analysis of the cultivation of *T. marschallianus* explants on media without growth regulators or with BAP showed the advantage of using jars as a cultural vessel. However, on a medium with TDZ, the multiplication index was higher in flasks and test tubes with cotton-gauze plug.

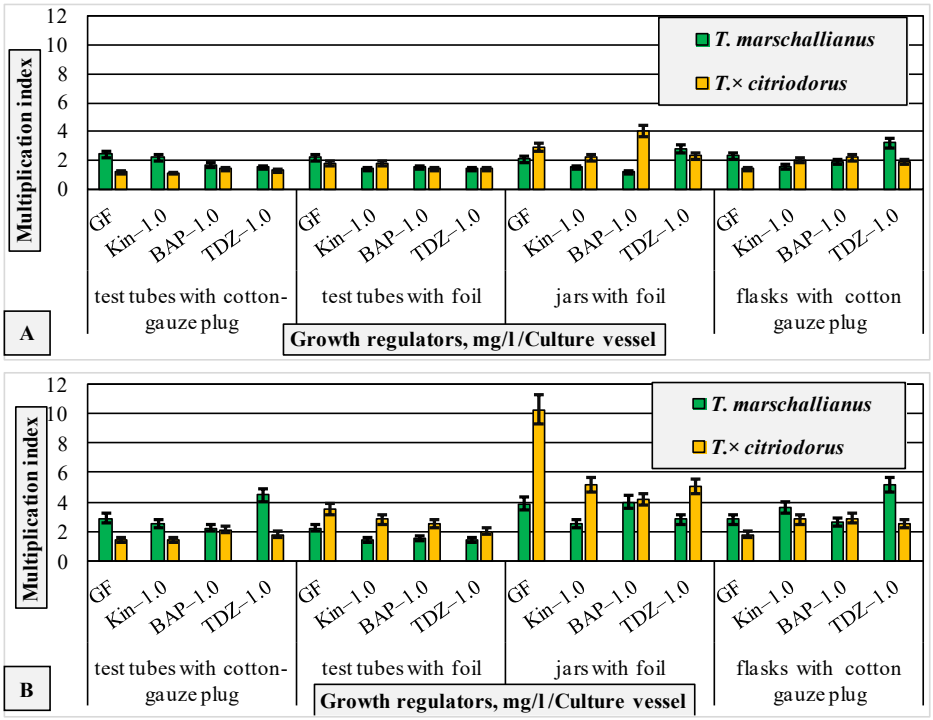


**Fig. 2.** The influence of the culture vessel and culture medium composition on the multiplication index at the second stage of two thyme species clonal micropropagation (A – 40 days of cultivation; B – 70 days of cultivation).

In our previous studies of *T. vulgaris*, it was found that with a standard growing cycle (40 days), the multiplication index did not exceed 4.0–4.1. However, with a longer growing cycle (70 days), this parameter increased to 12.8 [27]. In this regard, the cultivation of *T. marschallianus* and *T.* × *citriodorus* during 30–70 days was analyzed (Fig. 3). It was found that

on a hormone-free MS medium in *T.* × *citriodorus*, the multiplication index increased significantly with increasing duration of cultivation. Cultivation for 70 days contributed to an increase in this parameter by 3.6 times compared to 40 days.

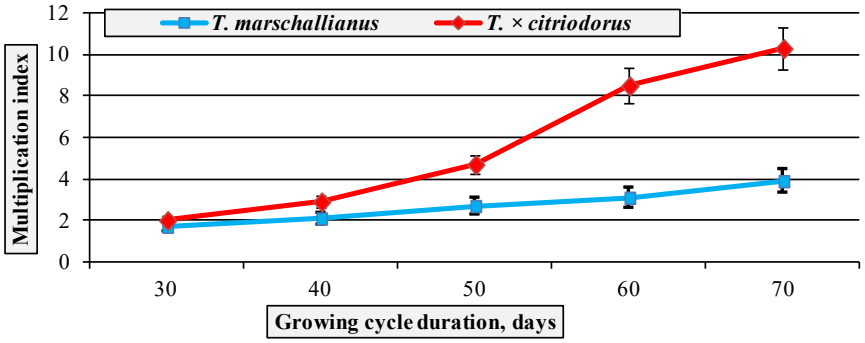


**Fig. 3.** The influence of growing cycle duration on the multiplication index at the second stage of two thyme species clonal micropropagation.

Thus, for *T.* × *citriodorus*, the media compositions and cultivation conditions were determined to ensure effective microcloning with a multiplication index up to 10.3 (with an optimal combination of factors). The morphometric parameters of *T. marschallianus* explant development were low, therefore an additional experiment was conducted to determine for this species the optimal culture medium (Table 3). Analysis of the obtained data showed that a greater number of shoots (6.7–8.3 pcs./explant) were obtained on the ½MS medium with 20 g/l glucose or on the ½MS medium with 0,5 mg/l NAA. On the MS medium with TDZ, this indicator was maximum (9.3 pcs./explant). However, the addition of TDZ to the medium caused a high vitrification rate (52.4%). When sucrose was used instead of glucose, hydrated microshoots were also formed with a frequency of up to 79.3%. The shoot length was greatest (up to 3.5 cm) when explants were cultivated on MS hormon free medium or ½MS medium with the addition of NAA.

**Table 3.** The influence of the culture medium composition on the morphometric parameters of explants at the second stage of *T. marschallianus* clonal micropropagation (70 days of cultivation).

| Composition of culture medium | Shoots number per explant, pcs. | Shoot length, cm | Multiplication index | Frequency of hydrated shoots,% |
|---|---|---|---|---|
| ½MS+0,5 mg/l NAA+20 g/l glucose | 8.3±0.7 | 3.4±0.3 | 19.9±1.7 | 0 |
| ½MS+0,5 mg/l NAA+20 g/l sucrose | 5.5±0.9 | 1.2±0.2 | 6.3±0.5 | 79.3±8.4 |
| ½MS+0,5 mg/l NAA+1 mg/l Kin+20 g/l sucrose | 3.0±0.2 | 0.9±0.1 | 3.7±0.1 | 50.2±2.4 |
| ½ MS+20 g/l glucose | 6.7±0.5 | 2.9±0.3 | 14.3±1.1 | 0 |
| ½MS+0,5 mg/l NAA+1 mg/l Kin+20 g/l glucose | 3.8±0.3 | 1.6±0.1 | 6.1±0.6 | 0 |
| MS+0,5 mg/l NAA+1 mg/l Kin+20 g/l glucose | 2.5±0.2 | 1.9±0.2 | 4.2±0.4 | 0 |
| MS+20 g/l glucose | 3.3±0.3 | 3.5±0.2 | 8.3±0.7 | 0 |
| MS+1 mg/l BAP+20 g/l glucose | 4.2±0.3 | 0.9±0.1 | 4.2±0.3 | 0 |
| MS+1 mg/l TDZ+20 g/l glucose | 9.3±0.9 | 1.3±0.1 | 12.0±1.1 | 52.4±5.4 |
| MS+1 mg/l Kin +20 g/l glucose | 3.5±0.4 | 1.3±0.1 | 4.6±0.4 | 0 |

A study of the influence of the culture medium composition on the multiplication index showed that when replacing sucrose with glucose (medium ½MS + 0.5 mg/l NAA), this parameter in *T. marschallianus* increased by 3.2 times (Table 3). A halving of the macro- and microelements content in the MS medium (MS hormone free and 20 g/l glucose or MS with the addition of 0.5 mg/l NAA, 1 mg/l Kin and 20 g/l glucose) contributed to an increase in the multiplication index by 1.4-1.7 times. During the research, it was revealed that the maximum multiplication index (19.9) was obtained on a ½MC culture medium supplemented with 0.5 mg/l NAA and 20 g/l glucose. Therefore, we recommend using this medium for the 2nd stage of *T. marschallianus* micropropagation.

An analysis of the dependence of the multiplication index on the growing cycle duration in *T. marschallianus* showed that cultivation for 70 days (on an optimal medium) made it possible to increase this index by 6.2 times compared with 30 days of cultivation (Fig. 4).

Thus, for two thyme species, possible ways to increase the morphogenetic potential were studied and the culture media composition and cultivation conditions to ensure effective microcloning with a multiplication index up to 10.3 (*T. × citriodorus*) and 19.9 (*T. marschallianus*) were established.

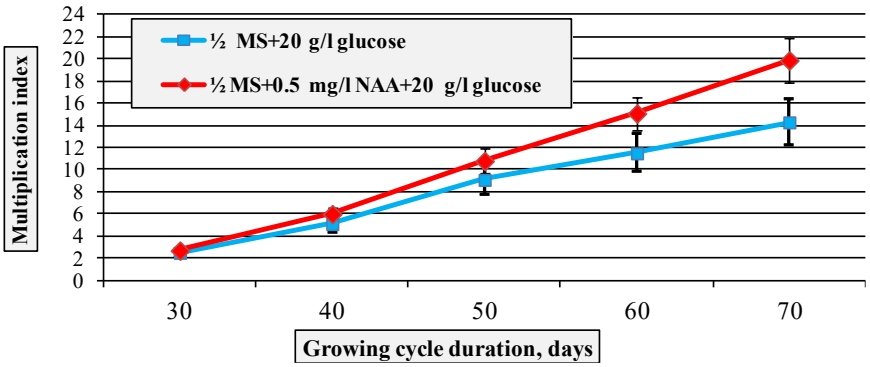


**Fig. 4.** The influence of growing cycle duration on the multiplication index at the second stage of *T. marschallianus* clonal micropropagation.

## 4 Conclusions

In order to determine possible ways to increase the morphogenetic potential of *T. marschallianus* and *T.× citriodorus*, the influence of various factors on explants morphogenesis at the second stage of clonal micropropagation was studied. The optimal culture media for *T. marschallianus* (½MS with 0.5 mg/l NAA and 20 g/l glucose) and for *T. × citriodorus* (MS without growth regulators and 20 g/l sucrose) were identified. When comparing two thyme species, it was shown that *T. marschallianus* explants had a higher morphogenetic potential – its multiplication index reached 19.9, while that of *T. × citriodorus* was 10.3. It was found that cultivating *T. × citriodorus* explants in glass jars (closed with foil) increased the multiplication index up to 2.9–7.4 times compared to the use of test tubes or flasks as a culture vessels. For two thyme species, the feasibility of using a longer growing cycle (70 days) was demonstrated, which made it possible to increase the multiplication index by 3.6-6.2 times compared to the standard growing cycle (40 days). The obtained results are the basis of an *in vitro* propagation technique, which can be used for the accelerated propagation of promising thyme samples with an increased content of secondary

metabolites and other valuable traits, as well as for the mass production of planting material of new *T.* ×*citriodorus* and *T. marschallianus* cultivars.

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