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MICROPROPAGATION OF *THYMUS* x *CITRIODORUS* AND GENETIC UNIFORMITY ASSESSMENT OF *IN VITRO* REGENERATED PLANTS

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Abstract

Thymus x citriodorus (Pers.) Schreb. (lemon thyme) is a perennial aromatic plant valued for its culinary, medicinal, and ornamental properties. This study aimed to establish an efficient micropropagation protocol for lemon thyme and to evaluate the genetic fidelity of in vitro regenerated plants using RAPD molecular markers. The highest shoot multiplication rate (13.77±2.51 shoots/explant) was obtained on Murashige and Skoog (MS) medium containing 3 mg/L BAP. The longest shoots (3.76±0.46 cm) were generated in the presence of 0.5 mg/L 2iP and 5 mg/L GA_3. Rooting was most efficient (100% rooting rate) on ½MS medium supplemented with IBA (0.5 or 1 mg/L) and in the presence of IBA (0.5 or 1 mg/L) in combination with GA_3 (3 mg/L). Acclimatized plants showed a 100% survival rate. Molecular analyses revealed monomorphic banding patterns in RAPD profiles, confirming the genetic fidelity of micropropagated plants.

Key words: *lemon thyme, micropropagation, plant growth regulators, genetic fidelity, RAPD markers.*

INTRODUCTION

Thymus x citriodorus (Pers.) Schreb (lemon thyme) is a perennial species of the *Lamiaceae* family, well known for its strong lemon-like aroma and high content of essential oils, which possess antimicrobial, antifungal, and antioxidant properties (Oliveira et al., 2022; Oliveira et al., 2023; Tevfik and Yegorova, 2024). This plant is widely used in the food, cosmetic, and pharmaceutical industries, as well as in landscaping, being a popular ornamental and melliferous species (Zheljazkov et al., 2008).

Conventional propagation methods, such as stem cuttings or clump division, are limited by the species' slow growth rate and low ability to multiply. Moreover, propagation by seeds is often inefficient due to the low viability and high genetic variability of regenerated plants (Kintzios, 2000). In this context, *in vitro* culture techniques offer a promising alternative for the rapid and large-scale production of healthy and genetically uniform plant material (George et al., 2008). Micropropagation is a valuable method for preserving and multiplying elite genotypes; however, it can also induce somaclonal variations due to physiological stress under *in vitro* conditions (Jain, 2001). Therefore, assessing the genetic stability of *in vitro* regenerated plants is essential for their subsequent use in commercial production or research (Kudikala et al., 2020; Biswas et al., 2022; Mudoi et al., 2023). DNA molecular markers, such as RAPD, ISSR, or SSR, have proven effective in detecting genetic variation and validating clonal fidelity (Rani and Raina, 2000; Bairu et al., 2011).

This study aimed to develop an efficient micropropagation protocol for *T. x citriodorus* and to evaluate the genetic uniformity of regenerated plants using RAPD molecular markers. The results will contribute to optimizing *in vitro* propagation strategies and ensuring the genetic quality of plant material intended for commercial and therapeutic use.

MATERIALS AND METHODS

In vitro propagation

The *in vitro* propagation technique involves four essential stages: culture initiation, shoot multiplication, shoot rooting, and acclimatization of regenerated plantlets to *ex vitro* conditions.

In vitro culture initiation stage

The explants used for initiating *in vitro* cultures consisted of apexes and uninodal fragments taken from shoots harvested from *T. x citriodorus* mother plants selected from a phytosanitary point of view and for the authenticity of the variety.

The shoots were defoliated, fragmented, and first washed with tap water, after which they were sterilized with calcium hypochlorite ($CaCl_2O_2$) in different concentrations: 6%, 8%, and 10%. The sterilization time was 10 minutes. Subsequently, three rinses with sterile distilled water were performed to remove traces of sterilizing agent.

After disinfection of the plant material, the explants were sampled and inoculated under aseptic conditions on MS culture medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs).

At this stage of the experiment, as well as in subsequent stages, the culture medium was supplemented with 40 g/L glucose, 32 mg/L NaFeEDTA, and 7 g/L agar.

The culture media were sterilized by autoclaving at 120°C for 20 min. Before autoclaving, the pH of the medium was adjusted to 5.6-5.8 with 1N KOH or 1N HCl.

The cultures were maintained in the growth chamber under controlled conditions of temperature (22-24ºC), photoperiod (16 hours light/8 hours dark), and light intensity (2500 lx).

Four weeks after culture initiation, the following parameters were calculated: the percentage of infected explants (explants contaminated with fungal and/or bacterial infections), the percentage of necrotic explants (uncontaminated but non-viable explants, burned by the disinfectant agent), and the percentage of initiation (viable explants that regenerated shoots).

The experiments were organized in 3 repetitions. For each experimental variant and each repetition, 10 explants were used.

In vitro multiplication stage

The apexes and nodal fragments resulting from the division of regenerated shoots during the *in vitro* culture initiation phase were inoculated on MS medium supplemented with different types, combinations, and concentrations of PGRs. MS medium without PGRs was used as control medium (Table 1).

Table 1. Experimental variants - *in vitro* multiplication of *T. x citriodorus* shoots

Variant	PGRs (mg/L)				
	BAP	KIN	2iP	IAA	GA_3
V0	-	-	-	-	-
V1	0.5	-	-	-	-
V2	3	-	-	-	-
V3	5	-	-	-	-
V4	-	0.5	-	-	-
V5	-	3	-	-	-
V6	-	5	-	-	-
V7	-	-	0.5	-	-
V8	-	-	3	-	-
V9	-	-	5	-	-
V10	0.5	-	-	1	-
V11	3	-	-	1	-
V12	5	-	-	1	-
V13	-	0.5	-	1	-
V14	-	3	-	1	-
V15	-	5	-	1	-
V16	-	-	0.5	1	-
V17	-	-	3	1	-
V18	-	-	5	1	-
V19	-	-	-	-	0.5
V20	-	-	-	-	3
V21	-	-	-	-	5
V22	0.5	-	-	-	0.5
V23	3	-	-	-	0.5
V24	5	-	-	-	0.5
V25	0.5	-	-	-	3
V26	3	-	-	-	3
V27	5	-	-	-	3
V28	0.5	-	-	-	5
V29	3	-	-	-	5
V30	5	-	-	-	5
V31	-	0.5	-	-	0.5
V32	-	3	-	-	0.5
V33	-	5	-	-	0.5
V34	-	0.5	-	-	3
V35	-	3	-	-	3
V36	-	5	-	-	3
V37	-	0.5	-	-	5
V38	-	3	-	-	5
V39	-	5	-	-	5
V40	-	-	0.5	-	0.5
V41	-	-	3	-	0.5
V42	-	-	5	-	0.5
V43	-	-	0.5	-	3
V44	-	-	3	-	3
V45	-	-	5	-	3
V46	-	-	0.5	-	5
V47	-	-	3	-	5
V48	-	-	5	-	5

BAP - 6-benzylaminopurine; KIN - kinetin; 2iP - 2-isopentyl adenine; IAA - indole-3-acetic acid; GA_3 - gibberellic acid.

Subculturing of biological material on fresh medium was performed every four weeks.
Four weeks after subcultivation, the number of shoots per explant and shoot length were evaluated.
The experiments were organized in 3 repetitions. For each experimental variant and each repetition, 10 explants were used.
To evaluate the *in vitro* regenerative potential of the studied species in successive subcultures, the regenerated biological material was cultured on MS medium supplemented with 40 g/L glucose, 32 mg/L NaFeEDTA, 7 g/L agar, and 3 mg/L BAP. Three successive subcultures were performed every four weeks. The parameters evaluated after each of the three subcultures were the number of shoots per explant and shoot length.

In vitro rooting stage
Shoots regenerated on the propagation medium were individualized and cultivated on several variants of rooting medium. ½MS medium without PGRs was used as control medium (Table 2).

Table 2. Experimental variants - *in vitro* rooting of *T. x citriodorus* shoots

Variant	PGRs (mg/L)			
	IBA	NAA	IAA	GA_3
V0	-	-	-	-
V1	0.5	-	-	-
V2	1	-	-	-
V3	-	0.5	-	-
V4	-	1	-	-
V5	-	-	0.5	-
V6	-	-	1	-
V7	0.5	-	-	3
V8	1	-	-	3
V9	-	0.5	-	3
V10	-	1	-	3
V11	-	-	0.5	3
V12	-	-	1	3

IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; IAA - indole-3-acetic acid; GA_3 - gibberellic acid.

Four weeks after the initiation of the experiment, the rooting rate was calculated.
The experiments were organized in 3 repetitions. For each experimental variant and each repetition, 10 shoots were used.

Acclimatization stage
In vitro rooted shoots were carefully removed from the culture vessels, and their root system was rinsed thoroughly under running tap water to remove residual agar medium and reduce the risk of contamination with phytopathogenic microorganisms. They were subsequently transplanted into a substrate composed of peat, manure, and perlite (in a ratio of 2:1:1) and Jiffy peat pellets.
The plants were placed under glass jars or covered with plastic bags to create a high-humidity environment and prevent dehydration. Two to three weeks after transfer to *ex vitro* conditions, when the plants began to grow, the humidity was gradually reduced by uncovering the cultures, allowing the plants to adapt to natural environmental conditions. The appearance of the first *ex vitro* leaves signaled the end of the acclimatization phase.
Four weeks after the start of the experiment, the acclimatization rate was calculated.
The experiments were organized in 3 repetitions. For each experimental variant and each repetition, 30 plantlets were used.
The acclimatized plants were then transplanted into pots in a peat-based mixture for further fortification and development.

Genetic uniformity assessment of *in vitro* regenerated plants
The genetic fidelity of *in vitro* propagated *T. x citriodorus* plants was assessed using Random Amplified Polymorphic DNA (RAPD) markers. The biological material for genetic analyses consisted of young leaves of ten *T. x citriodorus* plants: the mother plant, four *in vitro* plants, and five acclimatized plants.

Genomic DNA extraction
Genomic DNA was extracted using the Qiagen DNeasy Plant Mini kit, following the manufacturer's protocol with minor adjustments.

DNA quality assessment
The concentration and purity of extracted DNA were determined spectrophotometrically using a BioPhotometer plus (Eppendorf, Germany).

RAPD amplification
For the RAPD analysis, a total of sixteen primers from the OPA and OPB series (OPA-09 to OPA-20 and OPB-01 to OPB-04) were selected based on their proven ability to generate polymorphic and reproducible banding patterns (Kim et al., 2020).
PCR reactions were performed in a final volume of 25 µL, containing 5 µL of 5×

FirePol Master Mix Ready to Load (Solis BioDyne, Estonia), 3 µL of genomic DNA (~10 ng), 2 µL of primer, and ultrapure water. Amplifications were performed in a Techne TC-512 Thermal Cycler under the following conditions: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 35°C for 40 s, and extension at 72°C for 60 s, with a final extension of 5 min at 72°C.

Electrophoretic analysis

Amplified DNA fragments were separated by electrophoresis in 1.2% agarose gels prepared in 1× TAE buffer and stained with ethidium bromide. Visualization was carried out under UV illumination using the Gene Flash Syngene Bio Imaging system.

Data analysis

Statistical interpretation of data was performed using SPSS 10 for Windows program. The differences between the mean values of the analyzed morphological characteristics of *in vitro* plants grown on nutrient media with different hormonal formulas compared to the control were analyzed with One-way ANOVA – LSD, being considered significant at $P<0.05$. Values shown are means ± standard deviation (SD).

RESULTS AND DISCUSSIONS

***In vitro* propagation**

To develop an efficient *in vitro* propagation protocol for *T. x citriodorus*, the optimal conditions were identified for each regeneration stage.

In vitro culture initiation stage

One of the essential conditions on which the success of initiating a cell culture depends is the selection of the appropriate sterilization method for disinfecting the explants.

In this experiment, the inclusion of *T. x citriodorus* species in the *in vitro* culture system did not raise any particular problems, the use of $CaCl_2O_2$ for sterilizing the explants proving effective.

Between 66.67% and 83.33% of the explants regenerated shoots. The highest initiation percentage was obtained at the lowest concentration of the sterilizing agent, namely 6%. As the concentration of the disinfectant agent increased, a decrease in the initiation rate was observed, with the lowest values being obtained at a concentration of 10% $CaCl_2O_2$ (Figure 1).

The percentage of infected explants after the first four weeks of culture ranged between 6.67% and 13.33%. The lowest contamination rate was recorded when using 10% $CaCl_2O_2$, but in this case, the percentage of necrotic explants that did not regenerate shoots was higher compared to that recorded when using 6% or 8% $CaCl_2O_2$ (Figure 1).

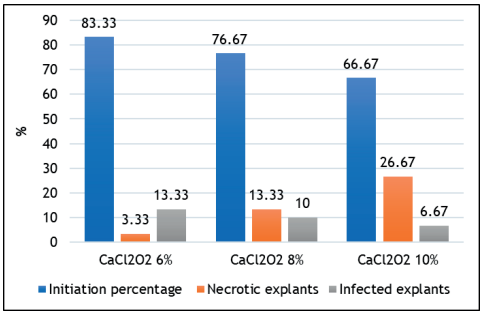

Figure 1. Influence of sterilization method on the *in vitro* regenerative potential of *T. x citriodorus* explants at the culture initiation stage. Values shown are means of 3 repetitions.

Considering that when $CaCl_2O_2$ at a concentration of 6% was used as a disinfectant, the explants had the best regenerative potential, we can conclude that this sterilization method was the most effective.

The use of MS basal medium without PGRs favored the production of plantlets that were subsequently used to test the morphogenetic response of *T. x citriodorus* explants on nutrient media with different hormonal formulas (Figures 6 a and 6 b).

In contrast to our results, a low survival rate of initial explants has been reported in other *Thymus* species, such as *T. mastichina* (Mendes and Romano, 1999), *T. caespititius* (Mendes et al., 2013), and *T. moroderi* (Marco-Medina and Casas, 2015).

In vitro multiplication stage

The highest proliferation rate in *in vitro* culture usually requires the search for an appropriate phytohormonal balance.

In our study, supplementing the nutrient

medium with PGRs induced an increase compared to the control medium (without PGRs) in the number of shoots per explant, in most variants the increases being significant. The highest values (13.77±2.51 shoots/explant) were obtained on MS medium supplemented with 3 mg/L BAP, followed by culture medium supplemented with 5 mg/L BAP and 5 mg/L GA_3 (10.53±0.85 shoots/explant). The lowest multiplication rate values were recorded in the control variant lacking PGRs (1.13±0.06 shoots/explant) (Table 3). In comparison, the study of the influence of culture medium composition on the morphometric parameters of *T.* × *citriodorus* microshoots, conducted by Tevfik and Yegorova (2024), revealed that the maximum number of shoots (8.6 shoots/explant) was obtained on MS medium containing 1.0 mg/L BAP and 1.0 mg/L GA_3. Clapa et al. (2025) obtained 29.8±1.26 shoots per explant in *T. x citriodorus* on Driver and Kuniyaki Walnut (DKW) culture medium (Driver and Kuniyuki, 1984) supplemented with 0.5 mg/L BA. In *T. marschallianus*, an increase in this parameter was observed when MS culture medium supplemented with 1.0 mg/L TDZ and 1.0 mg/L GA_3 or 0.5 mg/L IAA was used (Tevfik and Yegorova, 2024). *T. caespititius* was optimally multiplied in the presence of 1 mg/L BA and 0.25 mg/L IBA (Mendes et al., 2013). The results obtained by Kulpa et al. (2018) indicated that the cytokinin that had the most positive impact on shoot development of *T. vulgaris* L. at the multiplication stage was 2iP (at a concentration of 5 mg/L). In the case of the species *T. vulgaris* and *T. longicaulis*, the optimized proliferation medium consisted of semi-solid MS medium supplemented with 1 mg/L kinetin and 0.3 mg/L GA_3 (Ozudogru et al., 2011). In *T. lotocephalus*, BA gave the best proliferation results, but also high percentages of hyperhydric shoots (Coelho et al., 2012). In research conducted by Nordine and El Meskaoui (Nordine and El Meskaoui, 2014) on *T. broussonetii*, the maximum number of shoots was obtained on a medium supplemented with 0.5 mg/L BAP and 0.2 mg/L NAA, but the developed shoots were short.

Regarding shoot length, in our study, the shortest shoots were recorded on the culture medium supplemented with 5 mg/L BAP (0.79±0.07 cm), and the longest shoots were generated in the presence of 0.5 mg/L 2iP and 5 mg/L GA_3, respectively 3.87±0.66 cm and 3 mg/L GA_3 with an average of 3.76±0.46 cm (Table 3).

Table 3. Influence of type, concentration, and combination of PGRs on the number of shoots/explant and shoot length in the species *T. x citriodorus*. Values shown are means ± SD

Variant	No. of shoots/ explant	Shoot length (cm)
V0	1.13±0.06	1.68±0.04
V1	4.53±1.12*	1.28±0.09*
V2	13.77±2.51*	1.18±0.06*
V3	3.17±0.64*	0.79±0.07*
V4	4.03±0.35*	1.11±0.02*
V5	2.87±0.86*	0.90±0.03*
V6	3.93±0.35*	0.92±0.07*
V7	1.57±0.21	1.33±0.22*
V8	3.03±0.55*	1.05±0.03*
V9	2.47±0.15*	0.91±0.08*
V10	5.07±0.23*	0.93±0.07*
V11	4.90±0.87*	0.81±0.08*
V12	5.67±0.87*	0.82±0.03*
V13	2.60±0.20*	1.20±0.09*
V14	2.90±0.20*	0.84±0.07*
V15	3.03±0.55*	0.80±0.05*
V16	1.73±0.25	1.50±0.23
V17	5.00±0.70*	1.18±0.03*
V18	4.67±0.96*	0.93±0.09*
V19	1.13±0.12	2.47±0.53*
V20	1.23±0.15	3.76±0.46*
V21	1.70±0.46	3.20±0.29*
V22	4.17±0.57*	1.20±0.13*
V23	4.37±0.25*	1.08±0.04*
V24	4.27±0.21*	1.10±0.04*
V25	4.40±0.26*	1.55±0.16
V26	4.17±0.15*	1.35±0.13*
V27	4.70±0.35*	1.14±0.03*
V28	5.00±1.22*	1.35±0.12*
V29	5.23±0.61*	1.25±0.09*
V30	10.53±0.85*	1.29±0.03*
V31	2.53±0.61*	3.53±0.46*
V32	2.93±0.23*	1.14±0.03*
V33	2.87±0.15*	1.09±0.09*
V34	3.50±0.17*	2.10±0.02*
V35	3.40±0.44*	1.43±0.20
V36	3.80±0.36*	1.42±0.16
V37	3.63±0.06*	2.60±0.10*
V38	3.43±0.45*	1.72±0.16
V39	3.87±0.64*	1.67±0.09
V40	2.23±0.25*	2.32±0.22*
V41	3.03±0.49*	1.40±0.19
V42	2.83±0.31*	1.34±0.11*
V43	1.63±0.12	2.99±0.22*
V44	3.17±0.21*	1.98±0.26
V45	2.33±0.21*	1.96±0.08
V46	1.77±0.15	3.87±0.66*
V47	2.50±0.40*	2.36±0.32*
V48	2.57±0.40*	2.18±0.11*

*significance of the difference at $p < 0.05$

Research conducted by Tevfik and Yegorova (2024) on the dependence of *T.* × *citriodorus* shoot length on the type, concentration, and combination of PGRs in the culture medium showed that this parameter reached the highest values when MS hormone-free medium (1.6±0.2 cm) or MS medium supplemented with 1.0 mg/L Kin and 1.0 mg/L GA_3 (1.3±0.1 cm) was used. Clapa et al. (2025) obtained shoots with a length of 2.99±0.07 cm in *T. x citriodorus* on DKW culture medium supplemented with 0.5 mg/L BA. In *T. marschallianus*, the longest shoots were observed on medium supplemented with 1.0 mg/L Kin and 1.0 mg/L BAP (1.0±0.1 cm) (Tevfik and Yegorova, 2024). According to Nordine and El Meskaoui (Nordine and El Meskaoui, 2014), multiplication of thyme on media supplemented with cytokinins resulted in a decrease in shoot length.

In our study, on culture media supplemented with 0.5 mg/L 2iP and 1 mg/L IAA (V16), 0.5 mg/L GA_3 (V19), 3 mg/L GA_3 (V20), and 5 mg/L GA_3 (V21), regenerated shoots also formed roots. The rhizogenesis process also occurred in the control variant lacking PGRs (V0).

The *in vitro* regenerative potential of the studied species remained approximately constant throughout the three subcultures performed. There were no statistically significant differences between the three subcultures in terms of the number of shoots/explant (Figure 2) and shoot length (Figure 3).

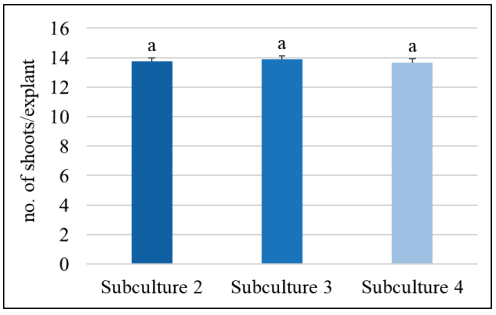

Figure 2. Influence of the number of subcultures on the number of shoots/explant in *T. x citriodorus*. The mean values are accompanied by error bars indicating standard deviations. Letters indicate the significance of differences between subcultures according to the LSD test ($P < 0.05$)

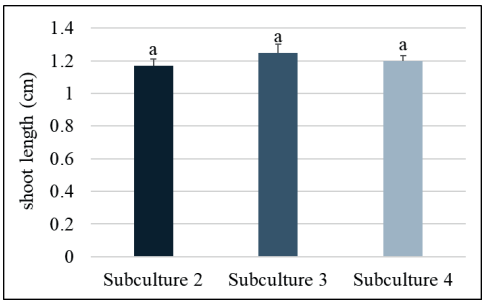

Figure 3. Influence of the number of subcultures on shoot length in *T. x citriodorus*. The mean values are accompanied by error bars indicating standard deviations. Letters indicate the significance of differences between subcultures according to the LSD test ($P < 0.05$)

From a qualitative point of view, the biological material regenerated *in vitro* had normal morphology, without aspects of vitrification, necrosis, or callus differentiation (Figures 6 c and 6 d).

***In vitro* rooting stage**

For effective *in vitro* propagation protocols, shoots must produce roots in large proportions. In the absence of roots, the survival rate of acclimatized plants is reduced.

It is known that, in *in vitro* cultures, auxins are responsible for stimulating root development and cell elongation. However, high concentrations of auxins added to nutrient media result in callus development and inhibition of root system development (Yankovskaya et al., 2011).

Thus, to select the optimal composition of the nutrient medium for the rooting of *T. x citriodorus* shoots, several medium variants were tested in the present study, in which the type of auxin (IBA, NAA, IAA) and its concentration (0.5 and 1 mg/L) were varied. The combination of auxin with gibberellic acid was also tested.

The highest values of rooting rate (100%) were obtained when the culture medium was supplemented with IBA (0.5 or 1 mg/L). A rooting rate of 100% was also obtained in the presence of IBA (0.5 or 1 mg/L) in combination with GA_3 (3 mg/L) (Figure 4).

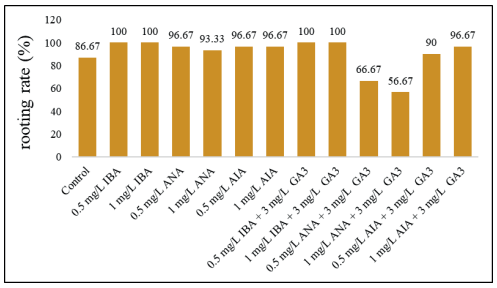

Figure 4. Influence of type, concentration, and combination of PGRs on the *in vitro* rooting capacity of *T. x citriodorus* shoots. Values shown are means of 3 repetitions

Similar results were reported by Kulpa et al. (2018) for *T. vulgaris*, the most developed root system being obtained on medium supplemented with 1 mg/L IBA. In contrast, Ozudogru et al. (2011) reported that the most effective medium for *in vitro* rooting of thyme shoots was MS medium supplemented with 0.01 mg/L NAA. Research conducted by Coelho et al. (2012) demonstrated that the best results in the rooting stage were obtained on ¼MS medium without auxins or supplemented with 0.5 mg/L IAA. The rooting percentage obtained by Clapa et al. (2025) for *T. x citriodorus* on DKW medium without PGRs was 71.67%. According to Nordine and El Meskaoui (2014) and Nordine et al. (2013), the most suitable medium for rooting of *Thymus* sp. shoots was MS medium without PGRs.

Acclimatization stage

The acclimatization is the final stage of microclonal propagation. For efficient micropropagation protocols, it is essential that *in vitro* regenerated plants successfully acclimate to *ex vitro* conditions.

In our experiments conducted on *T. x citriodorus*, an acclimatization rate of 100% was recorded in both nutrient substrates used: Jiffy peat pellets and a mixture of peat, manure, and perlite (in a ratio of 2:1:1) (Figure 5).

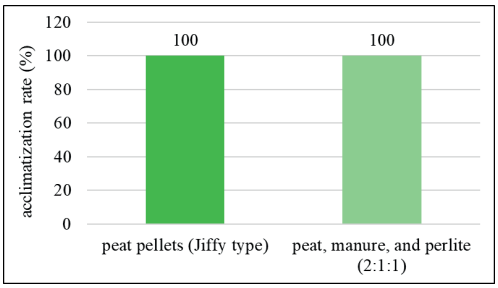

Figure 5. Influence of nutrient substrate on the acclimatization capacity of *in vitro* rooted plants to *ex vitro* conditions. Values shown are means of 3 repetitions

Similar results were obtained by Clapa et al. (2025) when acclimatizing *in vitro* rooted shoots of *T. x citriodorus* after *ex vitro* transfer to perlite.

Successful acclimatization of *in vitro* regenerated plantlets to *ex vitro* conditions was also achieved in other thyme species: *T. vulgaris* (a survival rate of 100%) (Ozudogru et al., 2011), *T. bleicherianus* (a survival rate of 85%) (Nordine and El Meskaoui, 2014), *T. hyemalis* (a survival rate of 90%) (Nordine et al., 2013), and *T. lotocephalus* (a survival rate of 93.33%) (Coelho et al., 2012).

Figure 6 shows aspects of the main stages of *in vitro* culture of *T. x citriodorus*.

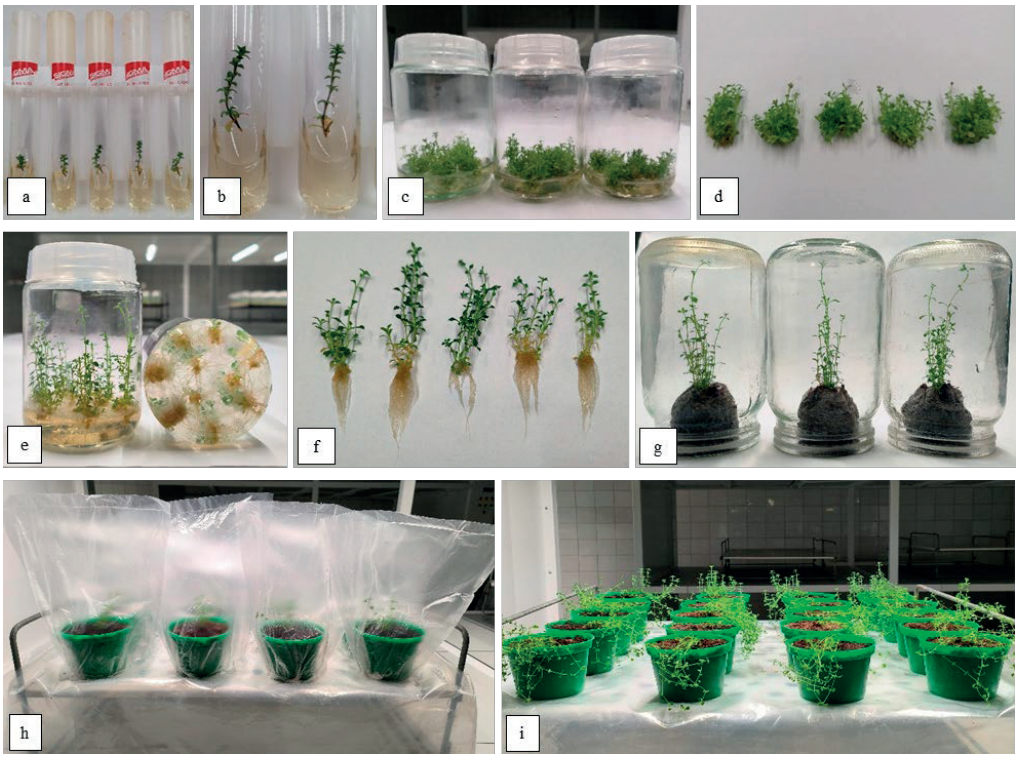

Figure 6. Aspects of *in vitro* culture of *T. x citriodorus*: a, b - explants in the initiation phase on MS medium without PGRs; c, d - shoot propagation on MS medium supplemented with 3 mg/L BAP; e, f - *in vitro* rooted shoots; g - acclimatization of plantlets in Jiffy peat pellets; h - acclimatization of plantlets in a mixture of peat, manure, and perlite (in a ratio of 2:1:1); i - acclimatized plants fortified in pots

Genetic uniformity assessment of *in vitro* regenerated plants

The type and concentration of PGRs, propagation methods, number and duration of subcultures are the main factors influencing the induction of somaclonal variation (Pastelín Solano et al., 2019). According to Kushnir and Sarnatska (2005), in *in vitro* propagation, the number of subcultures should be limited to 10-15. Any extension of these requires additional checks on the genetic stability of the material, as *in vitro* plant tissue culture for a long period of time can induce genetic variability due to prolonged exposure to high concentrations of PGRs (Li et al., 2019). Various molecular techniques have been developed to verify the genetic uniformity of micropropagated plants (Clapa and Hârţa, 2022; Badhepuri et al., 2024; Saptari et al., 2024; Clapa et al., 2025).

In the present study, RAPD analysis was employed to assess the genetic fidelity of *in vitro* propagated *T. x citriodorus* plants.

DNA from the mother plant, *in vitro* propagated plantlets, and acclimatized plants was analyzed for the presence/absence of bands. Scoring of RAPD bands in a binary matrix (1 = present, 0 = absent) revealed 100% similarity among all samples (Bakhtiar et al., 2014).

The concentration and purity of extracted DNA were determined spectrophotometrically. The absorbance ratios (A260/A280) ranged from 1.8 to 2.0, indicating high-purity DNA, free of protein contaminants. The yields were consistent across all samples, and the extracted DNA was found to be of sufficient integrity and concentration, being suitable for subsequent PCR amplification.

Amplification with RAPD primers produced clear and reproducible banding patterns in all ten plants examined. These results are consistent with those reported in other studies conducted on the species *T. persicus* (Bakhtiar et al., 2014) and *T. quinquecostatus* (Kim et al., 2020). Representative profiles are shown in Figure 7.

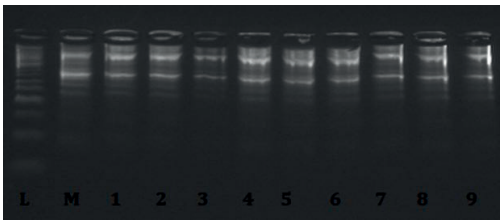

Figure 7. Representative RAPD electrophoretic profiles obtained with primer OPA-09. Lanes 1-10: DNA from mother plant, *in vitro* propagated plantlets (four stages), and acclimatized plants (five individuals). L: DNA ladder (100 bp)

For all primers used, the electrophoretic patterns were similar in the donor plant, four *in vitro* propagated shoots at different stages, and five acclimatized plants. All primers generated a comparable number of bands, ranging from 3 to 8 per primer, with fragment sizes between 200 and 2000 bp. No polymorphic bands were detected in any of the analyzed samples, indicating high genetic stability of the *in vitro* propagated *T. x citriodorus* material. These results confirm that *in vitro* culture did not induce detectable somaclonal variations. Similar results have been reported in other studies conducted on the species *T. persicus* (Bakhtiar et al., 2014) and *T. quinquecostatus* (Kim et al., 2020).

The use of RAPD markers has proven effective for monitoring genetic uniformity, providing a rapid and reliable tool for quality control of tissue culture-derived plants. The total number of bands, their sizes, and reproducibility underline the robustness of the RAPD method for detecting potential variations.

CONCLUSIONS

The use of 6% $CaCl_2O_2$ for the disinfection of explants and the MS medium without PGRs for their inoculation proved effective in inducing *in vitro* regenerative processes in the species *T. x citriodorus*. The highest shoot multiplication rate was obtained on MS medium containing 3 mg/L BAP. The longest shoots were generated in the presence of 0.5 mg/L 2iP and 5 mg/L GA_3. The most effective culture medium for *in vitro* shoot rooting proved to be ½MS medium supplemented with IBA (0.5 or 1 mg/L) or with IBA (0.5 or 1 mg/L) in combination with GA_3 (3 mg/L). *In vitro* regenerated plants were efficiently acclimatized to *ex vitro* conditions. Molecular analyses confirmed the genetic fidelity of micropropagated plants. These results validate the applied micropropagation protocol as a reliable strategy for clonal propagation and germplasm conservation of *T. x citriodorus*.

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