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In vitro propagation and genetic stability assessment of *Plectranthus amboinicus* and *Thymus × citriodorus*

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Abstract
This study aimed to investigate the genetic stability and uniformity of *Plectranthus amboinicus* (Lour.) and *Thymus × citriodorus* (Pers.) Schreb. propagated in vitro for 16 months using Start Codon Targeted (SCoT) molecular markers. Additionally, several growth parameters were assessed, including the number of shoots per explant, shoot length, in vitro rooting percentage, and root and shoot length of rooted plantlets. *T. citriodorus* exhibited a higher number of shoots per explant (29.8 ± 1.26) compared to *P. amboinicus* (15.6 ± 1.22). However, the in vitro rooting percentage was higher in *P. amboinicus* (96.67%) than in *T. citriodorus* (71.67%). Rooted shoots were acclimatized in perlite for 30 days, achieving a 100% survival rate for both species. No polymorphism was detected, confirming the high clonal fidelity of the regenerated plants.

Keywords: aromatic plants, micropropagation, Start Codon Targeted markers,

Introduction

Medicinal and aromatic plants play a crucial role in traditional and modern medicine, as well as in the food and cosmetic industries, due to their bioactive compounds and essential oils [21,23,17]. *Plectranthus amboinicus* (Lour.) Spreng (syn. *Coleus amboinicus*) is widely recognized for its therapeutic potential in treating cough, fever, skin and musculoskeletal disorders, as well as for its antimicrobial, anti-inflammatory, and respiratory benefits [24,7]. Meanwhile, *Thymus × citriodorus* (Pers.) Schreb is valued for its strong antioxidant and antimicrobial properties [18,19,28].

Micropropagation is often used in horticulture for mass plant production and has been applied to a wide range of aromatic plants [3].

Although there is a growing interest in the medicinal properties of *P. amboinicus*, in its special phytochemical profile and health benefits, specific studies focusing on in vitro propagation techniques are relatively limited. For example, [1] examined the effects of plant growth regulators (BAP, NAA and KIN) on shoot induction and proliferation in vitro, demonstrating a high rate of shoot induction. Sari et al. [26] conducted research that explored micropropagation strategies involving diploid and tetraploid variations. In addition, studies by Soliman [27] and Arumugam et al. [2] discussed the establishment of in vitro cultures and rooting techniques, emphasizing the importance of media composition and hormonal influences.

Somaclonal variation is a phenomenon that can arise in plant tissue cultures. [22] showed that the type and concentration of growth regulators, propagation methods, and both the number and duration of subcultures are key factors influencing the frequency of somaclonal variation. This highlights how culture conditions can influence genetic variability, necessitating careful management of in vitro protocols. Long-term tissue culture can induce genetic variability due to disorganized cell division and prolonged exposure to high concentrations of plant growth regulators [14]. These variations underscore the need for rigorous genetic assessments in in vitro propagation. Such evaluations are crucial for the commercial use of micropropagated plants in agriculture, ensuring the consistency and reliability of propagated stock.

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This study aimed to evaluate the genetic stability and uniformity of *Plectranthus amboinicus* and *Thymus* × *citriodorus* propagated in vitro for 15 subcultures using Start Codon Targeted (SCoT) molecular markers.

Material and Method

In Vitro Culture

Two species of aromatic plants were used for the present study: *Plectranthus amboinicus* (Lour.) Spreng and *Thymus* × *citriodorus* (Pers.) Schreb.

The explants used in this experiment consisted of 1.5-2 cm mini-cuttings that came from 14-month-old in vitro cultures. During this period, the cultures were sub-passed once a month on Murashige and Skoog (MS) [16] culture medium supplemented with different plant growth regulators (PGRs). The culture medium for shoot proliferation was Driver and Kuniyaki Walnut (DKW) [11] supplemented with 0.5 mg/L 6-benzyladenine (BA), 30 g/L sugar and solidified with 0.5 % (w/v) Plant agar. The pH of the medium was adjusted to 5.8 before adding the agar. The medium was introduced into 720 mL jars, each with a diameter of 9 cm and a height of 13.5 cm, with a metal lid equipped with a ventilation filter. 100 ml of culture medium were distributed in each container, then they were autoclaved at 120 °C and 1 atm for 20 minutes. Five explants were inoculated in each jar. After inoculation, the culture dishes were incubated in the growth chamber in a controlled environment (22 ± 1 °C, 32.4 $mmol \cdot m^{-2} \cdot s^{-1}$, 16 h photoperiod).

After one month of DKW +0.5 mg/L BA medium growth (Fig. 1a, b, c and Fig 2 a, b, c), the number of shoots/explant and shoot length were measured. In addition, at *P. amboinicus* the number of rooted shoots on the multiplication medium was determined.

For both species, in vitro rooting was tested on DKW medium without PGRs (3 jars × 20 seedlings/jar) (Fig 1. d, e and Fig. 2 d, e). After one month of growth on DKW medium without PGRs, shoot length, root length, and rooting percentage were measured. Subsequently, in vitro rooted plants were acclimatized in perlite and the acclimatization percentage was calculated after one month (Fig, 1F, Fig 2f).

All components were purchased from Duchefa BiochemieBV (Haarlem, The Netherlands).

Genetic Fidelity Assessment of In Vitro Raised Plants Using SCoT Markers

To confirm that the raised plants were genetically true to type compared to their mother plants, a genetic fidelity assessment using SCoT molecular markers was performed. Genomic DNA was isolated from fresh leaves weighing 2 mg. The ex-vitro mother plants and eleven randomly selected in vitro regenerated plants from each analyzed species were used.

We extracted total genomic DNA using a Quick-DNA Plant/Seed Miniprep kit from ZymoResearch, USA, following the supplier's protocol. Six SCoT primers were employed to amplify DNA from each analyzed species of medicinal plants and to confirm the genetic uniformity of in vitro raised plants with their mother plants. The six primers used generated detectable fragments in all analyzed samples. To ensure the reproducibility of results, all PCR reactions were repeated twice. PCR was conducted using a thermocycler system (SuperCycler Trinity by Kyratec, Australia) in a 15 µL PCR mixture containing 3 µL gDNA, 5.6 µL nuclease-free H_2O for the PCR reactions, 2.5 µL GoTaq Flexi Green buffer, 2.5 µL $MgCl_2$, 0.25 µL dNTP mix (Promega, USA), 1 µL SCoT primer (GeneriBiotech, Czechia), and 0.15 µL GoTaq polymerase (Promega, USA). The PCR amplification process started at 94°C for 4 minutes, followed by 35 cycles of 94°C for 60 seconds of denaturation, 90 seconds of annealing at 50-54 °C (touchdown option), 120 seconds of extension at 72°C, and a final extension at 72°C for 7 minutes. Separation of PCR products was performed by electrophoresis on 1.6% agarose gels (Promega, USA) stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotech, South Korea) in 1X TAE (Tris-acetate-EDTA buffer), at 80V and 88 mA for 2.5-3 hours. The electrophoretic band profiles were visualized using the UVP Biospectrum AC Imaging System (UVP BioImaging Systems, Germany).

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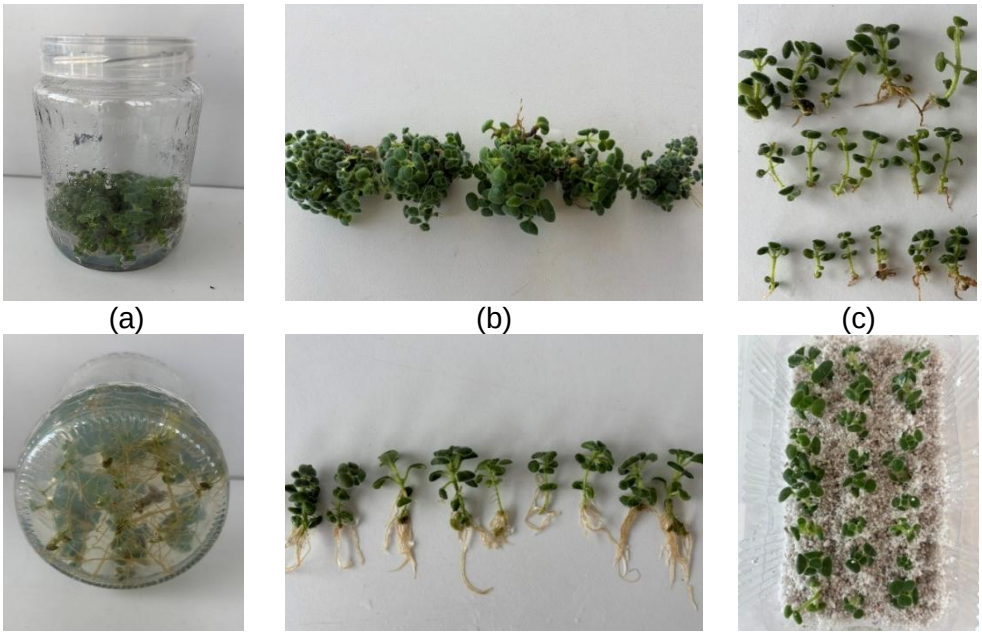
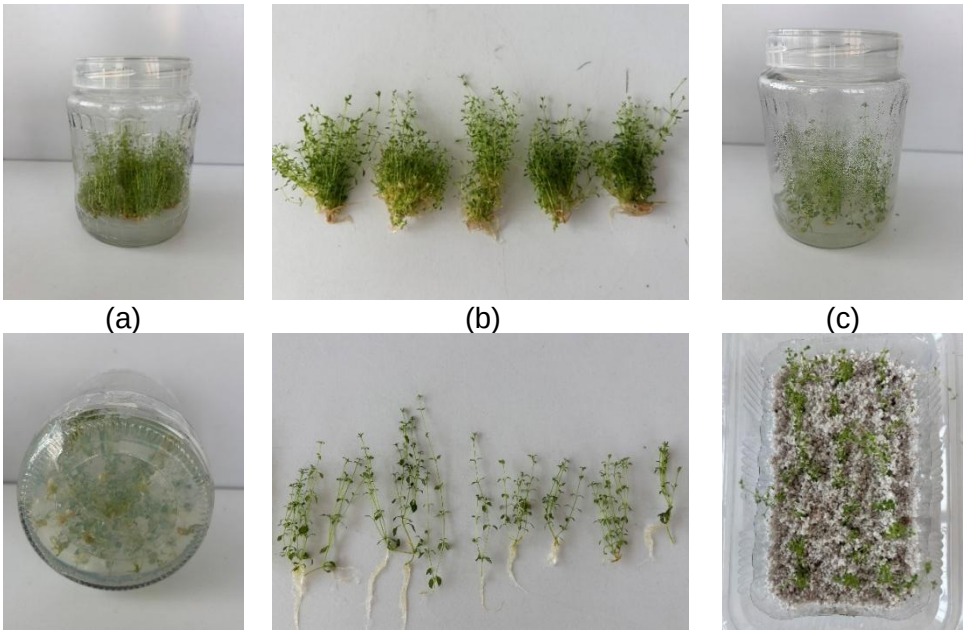


(a) (b) (c)

(d) (e) (f)

Figure 1. In vitro propagation of *P. amboinicus*: (a–c) Multiplication stage on the medium DKW + 0.5 mg/L BA; (d-e) in vitro rooted plantlets on hormone-free DKW; (f) Acclimatization stage.



(a) (b) (c)

(d) (e) (f)

Figure 2. In vitro propagation of *T. citriodorus*: (a–b) Multiplication stage on the medium DKW+0.5 mg/L BA; (c-e) in vitro rooted plantlets on hormone-free DKW; (f) Acclimatization stage.

Data Analysis

Statistical analysis was performed using OriginPro (2021, OriginLab, Northampton, MA, USA). For comparison of means, a one-way ANOVA was applied to evaluate the effect of treatments on shoot and root growth parameters. The significance of differences between the means was determined with Tukey's test at the significance level of $p < 0.05$. All values are expressed as mean ± standard error (SE).

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To assess genetic uniformity between in vitro raised plants and the mother plants, SCoT gel images were analyzed using TotalLab software. The number and size range (base pairs) of amplified bands were recorded, noting that band intensity was not considered in scoring.

Results and Discussion

In Vitro Culture

The effectiveness of a commercial micropropagation protocol depends on the proliferation rate and the number of microshoots obtained from an explant, the accidental induction of roots, and subsequent ex vitro acclimatization of plants [8]. Also, an important step in plant micropropagation is the choice of a culture medium that allows good development and growth of the proliferated shoots [5]. The evaluation of the genetic uniformity of in vitro grown plants is crucial for mass propagation and various molecular techniques have been developed to verify the genetic uniformity and stability of micropropagated plants [10,4,25].

On DKW culture medium supplemented with 0.5 mg/L BA, the number of shoots per explant was 29.8 ± 1.26 in *T. citriodorus* and 15.6 ± 1.22 in *P. amboinicus* (Fig. 3a). In comparison, Tevfik & Yegorova [28] reported a maximum of 8.6 shoots per explant in *T. citriodorus* cultured in vitro on MS medium containing 1.0 mg/L BAP or 1.0 mg/L GA_3. Similarly, for *P. amboinicus* grown in vitro on White basal medium supplemented with various concentrations of BA or Kin (0.125, 0.250, 0.50, 1.0, and 2.0 mg/L), a much lower number of shoots per explant was reported, with the highest number reaching 4.04 shoots per explant at 0.5 mg/L BA [27].

Regarding shoot length, *T. citriodorus* produced longer shoots than *P. amboinicus*, measuring 2.99 ± 0.07 cm compared to 2.33 ± 0.05 cm (Fig. 3b). Similar to shoot proliferation, DKW medium had a positive effect on shoot elongation compared to White medium, where the maximum reported shoot length for *P. amboinicus* was 2.10 cm [27]. For *T. citriodorus* cultured in vitro on MS medium supplemented with 1 mg/L BA, the shoot length was 0.4 ± 0.1 cm, with the highest recorded shoot length being 1.3 ± 0.1 cm in the presence of 1 mg/L Kin + 1 mg/L GA3 [27].

Notably, on the DKW culture medium supplemented with 0.5 mg/L BA, *P. amboinicus* shoots rooted at a rate of 88%, suggesting that the in vitro rooting stage could be omitted for this species.

After subculturing on the DKW medium without PGRs, rooted shoots were obtained, with an average shoot length of 4.75 ± 0.29 cm for *T. citriodorus* and 3.14 ± 0.13 cm for *P. amboinicus* (Fig. 3c). *P. amboinicus* had an average root length of 2.97 ± 0.36 cm, whereas T. citriodorus developed shorter roots (1.36 ± 0.27 cm) (Fig. 3d).

The rooting percentages on DKW medium without PGRs were 96.67% for *P. amboinicus* and 71.67% for *T. citriodorus* (Fig. 3e). The acclimatization of in vitro-rooted shoots was 100% for both species after ex vitro transfer to perlite (Fig. 1f, Fig. 2f, and Fig. 3f). Soliman [27] reported an in vitro rooting percentage of 75% for *P. amboinicus* on MS medium without PGRs, whereas supplementation with different concentrations of IBA increased the rooting percentage to 100%.

Our results suggest that the DKW medium may contribute to the observed differences in growth between the two species, likely due to its distinct composition. Compared to MS medium, DKW has a similar ammonium-to-nitrate ratio but contains less total nitrogen and significantly higher levels of sulfur, calcium, and copper [20].

Genetic Fidelity Assessment

Previous research has demonstrated that evaluating the genetic identity between mother plants and their in vitro regenerated offspring is essential for the large-scale production of medicinal planting material using tissue culture methods [13,6,15]. In our study, the DNA bands produced by amplification with SCoT markers were monomorphic across the micropropagated plantlets and the mother plants (Figure 4), confirming the true-to-type and genetically stable nature of the acclimated plantlets of *Plectranthus amboinicus* and *Thymus* × *citriodorus* after 16 months of in vitro culture.

The six SCoT primers used to analyze the genetic identity between mother plants and each medicinal species' acclimatized in vitro-grown plants produced visible and reproducible DNA-PCR amplified bands. Table 1 presents the number and size range (bp) of the total PCR-amplified bands generated using SCoT primers in the analyzed P*lectranthus amboinicus* and *Thymus* × *citriodorus* plants.

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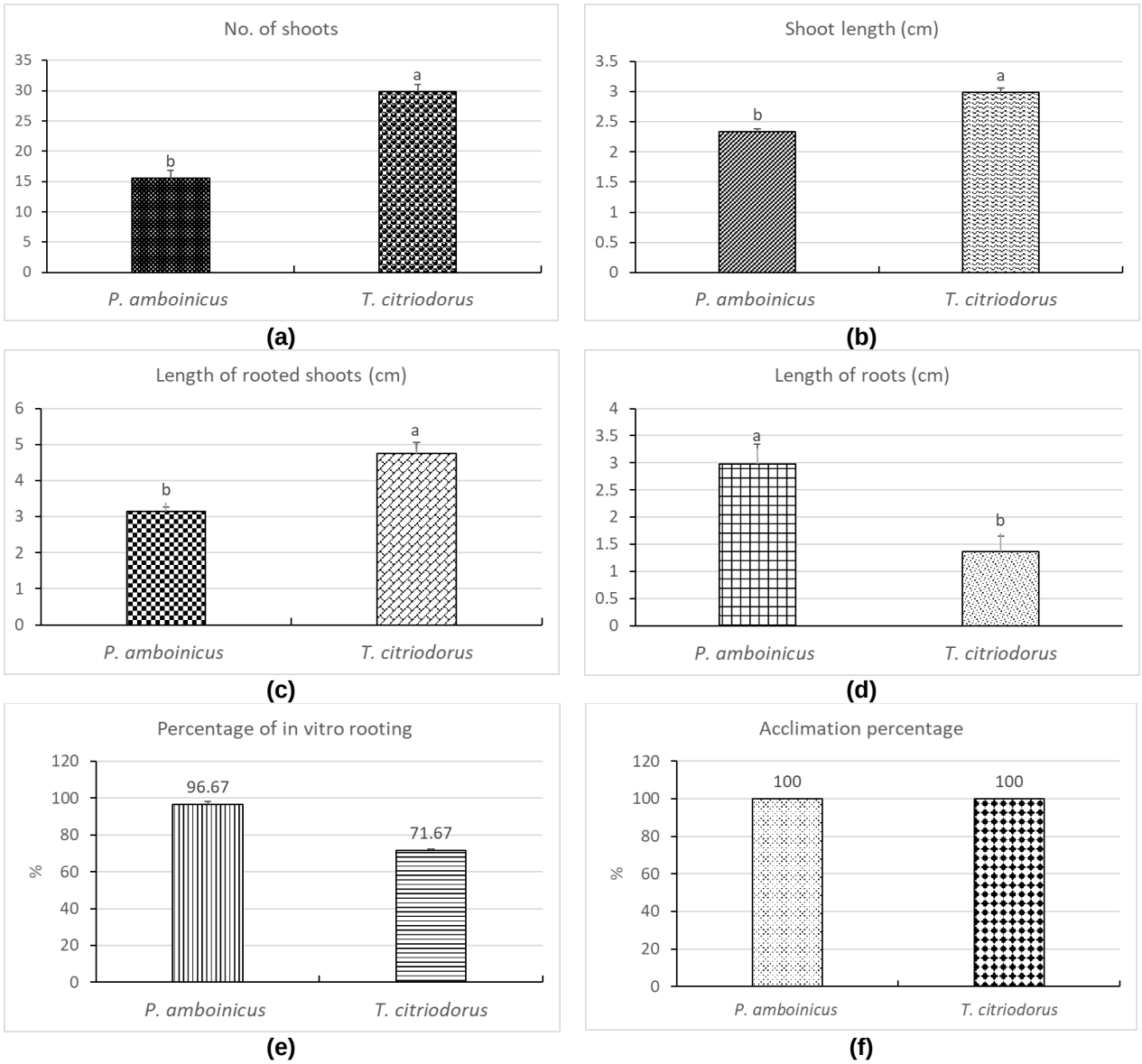

Figure 3. In vitro propagation and acclimatization of *P. amboinicus and T. citriodorus*: (a) - Number of shoots obtained/explant on the medium DKW + 0.5 mg/L BA; (b) - The length of the shoots obtained on the medium DKW + 0.5 mg/L BA; (c) - Length of shoots obtained in the in vitro rooting stage on DKW medium without PGRs; (d) - Length of roots obtained in the in vitro rooting stage on DKW medium without PGRs; (e) - Percentage of in vitro rooting on DKW medium without PGRs; (f) - Percentage of perlite acclimation of the in vitro rooted shoots.

As shown in Table 1, the number of scorable monomorphic bands ranged from 7 (SCoT 1) to 10 (SCoT 25) for *Plectranthus amboinicus* and from 6 (SCoT 1; SCoT 10) to 9 (SCoT 3) for *Thymus* × *citriodorus*. The primer SCoT 3 produced the highest number of monomorphic PCR-amplified bands for both species analyzed. The fewest PCR-amplified bands were generated by primers SCoT 1 and SCoT 10. For *Plectranthus amboinicus*, an average of 8.16 PCR bands per SCoT primer was amplified, while for *Thymus x citriodorus*, the average was 7.16 PCR bands per SCoT primer (Table 1). Similar genetic fidelity studies of in vitro regenerants using SCoT molecular markers have been reported in medicinal plant species such as *Annona reticulata* L. [13], *Solanum khasianum* Clarke [9], and *Solena amplexicaulis* (Lam.) Gandhi [12]. The results of these studies suggest that the SCoT markers system is a valuable tool for assessing genetic fidelity and can be used to test the in vitro micropropagated plant material. To the best of our knowledge, this is the first study to assess the clonal fidelity of micropropagated *P. amboinicus* and *T. citriodorus* using SCoT markers.

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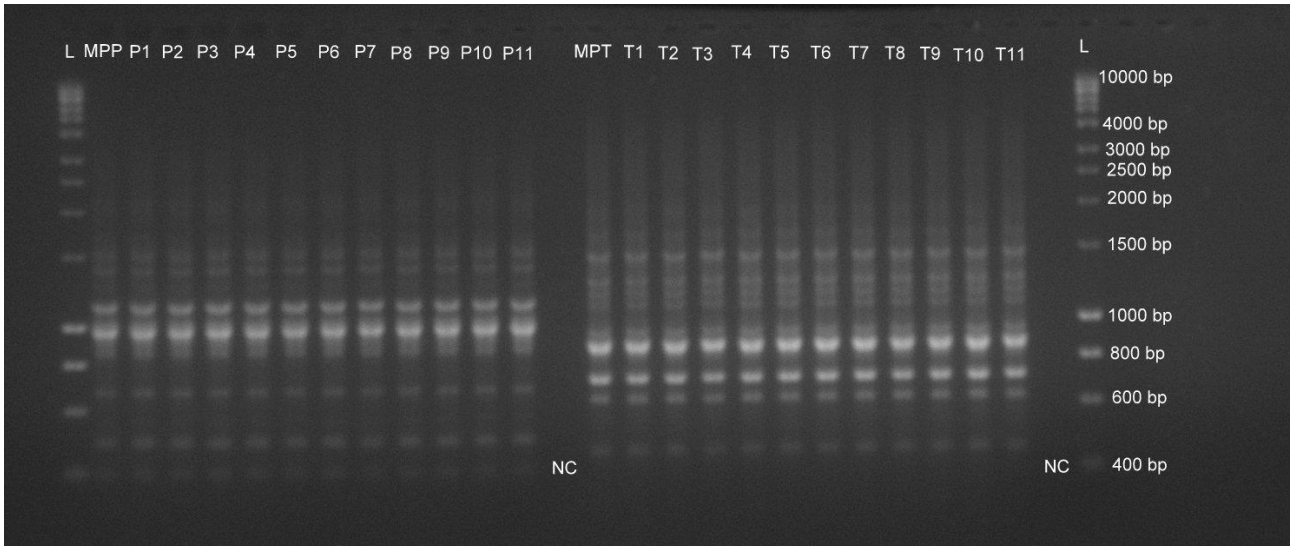

Figure 4. The genetic profile generated with the SCoT 3 primer for the mother plants of *Plectranthus amboinicus* (MPP) and *Thymus* × *citriodorus* (MPT) and there in vitro raised plants (P1-P11; T1-T11) illustrates the genetic identity from each medicinal plant species L – molecular marker, 1 kb Ladder (Fermentas, Leon-Rot, Germany); NC-negative control.

Table 1. Number and size range of monomorphic amplified SCoT bands detected in the medicinal plant species *Plectranthus amboinicus* and *Thymus* × *citriodorus*.

Primer name	Primer sequence 5'-3'	No. of monomorphic bands		Size range of bands (bp)	
		Plectranthus amboinicus	*Thymus* × *citriodorus*	*Plectranthus amboinicus*	*Thymus* × *citriodorus*
SCoT 1	CAACAATGGCTACCACCA	7	6	1000-3500	900-3000
SCoT 3	CAACAATGGCTACCACCG	10	9	400-2500	450-2000
SCoT 4	CAACAATGGCTACCACCT	8	7	500-2000	400-1500
SCoT 10	CAACAATGGCTACCAGCC	7	6	500-2000	450-2000
SCoT 11	AAGCAATGGCTACCACCA	9	8	400-2500	400-2000
SCoT 25	ACCATGGCTACCACCGGG	8	7	400-2500	300-3000
Total		49	43		

Conclusions

Our results demonstrate that DKW culture medium can be successfully used in both the multiplication and in vitro rooting phases for the two aromatic plant species, *P. amboinicus* and *T. citriodorus*. Additionally, BA at a concentration of 0.5 mg/L effectively induces the proliferation of a high number of shoots in both species. The SCoT markers system is a valuable tool for assessing genetic fidelity and can be used to test the in vitro micropropagated plants of *P. amboinicus* and *T. citriodorus*.

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