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ORIGINAL ARTICLE

In vitro propagation, DNA content and essential oil composition of *Teucrium scorodonia* L. ssp. *scorodonia*

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Abstract *Teucrium scorodonia* ssp. *scorodonia* is a valuable medicinal plant. It is rare in Poland, therefore availability of raw material is limited and an effective method of producing in vitro-derived plants for pharmaceutical reasons would be desirable. However, the protocol for micropropagation of this species has not been developed yet. Here, its regeneration ability on media supplemented with different growth regulators was evaluated using shoot tip and nodal segment explants. Murashige and Skoog (MS; Physiol Plant 15:473–497, 1962. doi:10.1111/j.1399-3054.1962.tb08052.x) medium supplemented with indole-3-acetic acid (IAA) (0.1 mg L^{-1}) and 6-benzyladenine (BA) (2 mg L^{-1}) was the best for multiple shoot induction, resulting on average in about 7 and 8 axillary shoots per shoot tip and nodal explant, respectively, within 5 weeks. Flow cytometric analysis revealed that most of the in vitro-developed shoots of *T. scorodonia* possessed similar nuclear DNA content as seedlings (about 2.1 pg/2C). In vitro rooting of shoots was achieved at 100 % efficiency on MS medium without growth regulators or containing auxin. The addition of auxin, IAA, indole-3-butyric acid (IBA) or α-naphthaleneacetic acid (NAA) led to an increase in the number of roots. Rooted plantlets were transferred into ex vitro conditions and further grown in the field, where they matured and flowered. The essential oils from aerial parts of the in vitro-originated plants and their seed-derived counterpart were isolated and analyzed by gas chromatography-mass spectrometry (GC–MS). In the oils, 84 components were identified. Although the essential oils from the micropropagated plants and the seed-derived plants were qualitatively similar, quantitative differences were found.

Keywords Micropropagation · Cytokinins · Flow cytometry · Genome size · Essential oils · *Teucrium scorodonia*

Introduction

The genus *Teucrium* includes over 300 species spread all over the world, but most of them are found in the Mediterranean basin. Many species of *Teucrium* possess different biological properties, such as antibacterial, antifungal, anti-inflammatory, antioxidant, antipyretic, diuretic and hypoglycemic activities, and they are used in traditional medicine (Ulubelen et al. 2000; Kovacevic et al. 2001; Maccioni et al. 2007; Küçük et al. 2008; Djabou et al. 2013). The aromatic plants are also used in the production of flavoured wines and beers, bitters and liqueurs (Djabou et al. 2012; Maccioni et al. 2007).

In the Polish flora, the genus *Teucrium* is represented by five species (Mirek et al. 2002); among which, *T. scorodonia* ssp. *scorodonia* (herein referred to as *T. scorodonia*) has been identified. In Poland, *T. scorodonia* is a

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rare plant and it has been found only in Western Pomerania and Lower Silesia. It is a perennial shrub, about 30–50 cm high, characterized by greenish-yellow flowers with a corolla about twice as long as the calyx, which has short hairs and few or no glands (Tutin et al. 1972).

The leaves and flowers of *T. scorodonia* are known for their astringent, antiseptic, expectorant and digestion-improving properties (Maccioni et al. 2007). In some regions, the plant is used as a hop substitute for flavoring beer. The characteristics of *T. scorodonia* are mainly associated with the presence of essential oils that are rich in sesquiterpene hydrocarbons (Maccioni et al. 2007). Particularly important components of this group of metabolites are β-caryophyllene and germacrene D. β-caryophyllene is known to be a selective agonist of cannabinoid receptor type-2 (CB_2). The receptors may be targeted in the treatment of depression and anxiety (Guimarães-Santos et al. 2012; Bahi et al. 2014). Moreover, caryophyllene inhibits the growth of *Trypanosoma cruzi* and *Leishmania brasiliensis* parasites (Leite et al. 2013). Germacrene D demonstrates antifungal, antimicrobial and insecticidal properties (Picaud et al. 2006; Noge and Becerra 2009; Zarai et al. 2011). Apart from the essential oil, other secondary metabolites such as diterpenoids, caffeic acid, ursolic acid, and flavonoids were identified in *T. scorodonia* (Marco et al. 1982, 1983; Rodriguez-Hanh et al. 1994; Valant-Vetschera et al. 2003).

Micropropagation is a common method for the rapid and large-scale production of many medicinal plant species, including some *Teucrium* species, such as *T. polium* (Al-Qudah et al. 2011), *T. fruticans* (Frabetti et al. 2009), and *T. stocksianum* (Bouhouche and Ksiksi 2007). However, to the best of our knowledge, in vitro propagation of *T. scorodonia* has not been reported before. The aim of the present research was to develop an effective protocol for micropropagation of this species.

The study examines the morphogenic responses of shoot tips and nodal explants of *T. scorodonia* to various cytokinins: 6-benzyladenine (BA), zeatin (ZEA), 6-furfurylaminopurine (kinetin, KIN), and thidiazuron (TDZ); as well as the effect of various auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthaleneacetic acid (NAA), on the rooting of the obtained shoots. Although production of shoots directly from meristematic structures ensures high genetic stability, culture conditions, such as the presence of exogenous growth regulators, can lead to somaclonal variation (Bairu et al. 2011; Matsuda et al. 2014; Lebedev and Shestibratov 2016). Somaclonal variation can affect the quality and quantity of secondary metabolites produced by micropropagated plants (Oksman-Caldentey 2014; Skała et al. 2015). Therefore, flow cytometry was used to compare the nuclear DNA content in *T. scorodonia* seedlings and shoots multiplied under in vitro conditions. Moreover, gas chromatography coupled with mass spectrometry (GC–MS) was employed to study the composition of hydrodistilled essential oils from aerial parts of *T. scorodonia* micropropagated plants. The results were compared with the respective plants derived from seeds.

Materials and methods

Shoot culture establishment

Teucrium scorodonia seeds obtained from the Botanical Garden of Lodz (Poland) were surface sterilized in 2 % sodium hypochlorite solution for 10 min, washed three times with sterile distilled water for 15 min each, then placed on MS agar (0.7 % w/v SIGMA, Aldrich) medium containing 3 % sucrose, gibberellic acid (GA_3) (1 mg L^{-1}) and kinetin (KIN) (0.2 mg L^{-1}) for germination. Shoot tips (0.5 cm in length) from 35-day-old seedlings were excised and transferred onto MS agar medium supplemented with 0.1 mg L^{-1} IAA and 0.2 mg L^{-1} BA. The cultures were subcultured every 42 days. They were grown at 26 ± 2 °C in a 16 h photoperiod. Light at intensity of 40 µmol m^{-2} s^{-1} was provided by cool white fluorescent lamps (POLAMP, Poland).

Effect of cytokinins on shoot multiplication

Two types of explants: shoots tips (0.5 cm in length) and nodal segments (0.5 cm in length, having two axillary buds) from in vitro cultured shoots (obtained as described above) were transferred onto MS agar medium supplemented with 0.1 mg L^{-1} IAA and different concentrations of BA, ZEA, KIN (0.2, 0.5, 1, 2 mg L^{-1}) or TDZ (0.02, 0.05, 0.1, 0.2 mg L^{-1}). Two control media were applied: (1) MS medium without growth regulator, and (2) MS medium supplemented with auxin IAA only (0.1 mg L^{-1}). After 35 days the number of shoots per explant, shoot length, and frequency of hyperhydricity were determined.

Root development, plantlet acclimatization, and plant cultivation

After 35 days, excised axillary shoots derived from shoot tips and nodal segments were transferred onto MS agar medium, either without plant growth regulators or supplemented with auxin (IAA, IBA or NAA) at concentration of 0.1 or 0.5 mg L^{-1}. The shoots were rooted within 42 days, when the number of roots per shoot was recorded.

The in vitro-derived rooted shoots (42 days old) were transplanted into pots (10 cm in diameter) containing a

sterilized mixture of soil, sand and peat (4:3:3 v/v/v) and placed in the greenhouse (26 ± 2 °C, natural light). To ensure high humidity, the plants were covered with glass beakers, which were removed after 14 days of acclimatization. Sixty days after transplanting the plantlets to the pots, the survival rate was assessed and after another successive 30 days, the plants were moved to the field. The ex vitro plants were propagated from seeds originated from the same source as those used for shoot culture initiation. They were grown in the greenhouse for 90 days and then were moved to the field. All the plants were grown in the field (compost substrate, sunny stand) at the Department of Pharmacognosy Medicinal Plant Garden, Medical University of Lodz, Poland, where they exhibited normal development and reached the flowering stage in the second year of cultivation. The aerial parts were collected at the flowering stage and dried at 25 °C. The plants were positively identified as *T. scorodonia* spp. *scorodonia* according to Flora Europaea (Tutin et al. 1972). Voucher specimens (N° 5 and 6) were deposited in the Department of Biology and Pharmaceutical Botany, Medical University of Lodz.

Genome size estimation

Flow cytometry was used to check the stability of genome size in in vitro-derived shoots of *T. scorodonia*. The leaves of 35-day-old seedlings, and of shoots multiplied on MS media with BA, KIN or TDZ were used for estimation of nuclear DNA content. *Vicia villosa* 'Minikowska' (2C = 3.32 pg; Dzialuk et al. 2007) served as an internal standard. Samples were prepared as previously described (Thiem and Sliwinska 2003), using Galbraith's buffer (Galbraith et al. 1983), supplemented with propidium iodide (PI; 50 µg mL^{-1}), ribonuclease A (50 µg mL^{-1}), and polyvinylpyrrolidone (PVP-10; 1 % w/v). Nuclear DNA content was estimated by a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer, on five plants from each combination. For each sample, DNA content in 3000-6000 nuclei was measured, using linear amplification. Histograms were evaluated using the FloMax program (Partec GmbH, Münster, Germany). The coefficient of variation (CV) of the G_0/G_1 peak of *T. scorodonia* ranged between 3.5 and 6.4 %. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of *T. scorodonia/V. villosa* on a histogram of fluorescence intensities.

Isolation and analysis of essential oil

For analysis of essential oils, the following plant material was selected: seed-derived plants (SP), two samples of in vitro derived plants obtained from shoot tips cultured on MS medium in the presence of 0.1 mg L^{-1} IAA, and one of the cytokinins: BA (0.2 mg L^{-1}, V_1P) or KIN (0.5 mg L^{-1}, V_2P). All analyzed plants were grown in the field under the same conditions.

The essential oils of dried plant material (50 g) were obtained by hydrodistillation for 3 h, according to European Pharmacopoeia 5.0 (2005), using Clevenger-type apparatus. After assessment of volume, the oils were collected in diethyl ether and dried over anhydrous sodium sulfate. Chemical composition of the essential oils was analyzed using GC–MS on a Trace GC Ultra apparatus (Thermo Electron Corporation, Milan, Italy) with FID and MS DSQ II detector after dilution in diethyl ether (10 µL in 1 mL). Simultaneous GC-FID and MS analyses were performed using a MS-FID splitter (SGE, Analytical Science). Operating conditions were as follows: Apolar capillary column Rtx-1 ms (Restek), 60 m × 0.25 mm i.d., film thickness 0.25 µm; temperature program, 50–310 °C at 2 °C/min; SSL injector temperature 280 °C; FID temperature 300 °C; split ratio 1:20; carrier gas helium at a regular pressure of 300 kPa. Mass spectra were acquired over the mass range 30–400 Da, ionization voltage 70 eV; ion source temperature 200 °C. The identification of the components was based on a comparison of their mass spectra and relative retention indices with data stored in the NIST 98.1, Wiley 8th Ed. and MassFinder 4.1. libraries (Adams 2002). Retention indices (RI, apolar column) were determined with relation to a homologous series of alkanes (C_8–C_{26}) under the same conditions with linear interpolation. Percentages were calculated from FID response without the use of correction factors.

Statistical analysis

Results are presented as the mean ± standard error (SE). The shoot multiplication experiment was replicated three times in three subsequent treatments, through three successive passages. In total, 32–42 shoot tips and nodal explants were used per each cytokinin treatment. The rooting experiment was also performed in three replicates. The total number of 30 shoots derived from shoot tips and 30 shoots derived from nodal explants were raised for each treatment. The results presented in Figs. 1 and 2 were analyzed by the Kruskal–Wallis test to compare the means. The results were regarded as statistically significant at $P \leq 0.05$. The results of DNA content measurements were evaluated using the one-way analysis of variance (ANOVA) and Tukey's test at $P \leq 0.05$. All statistical analyses were performed using STATISTICA v. 12.5 (STATSoft) software.

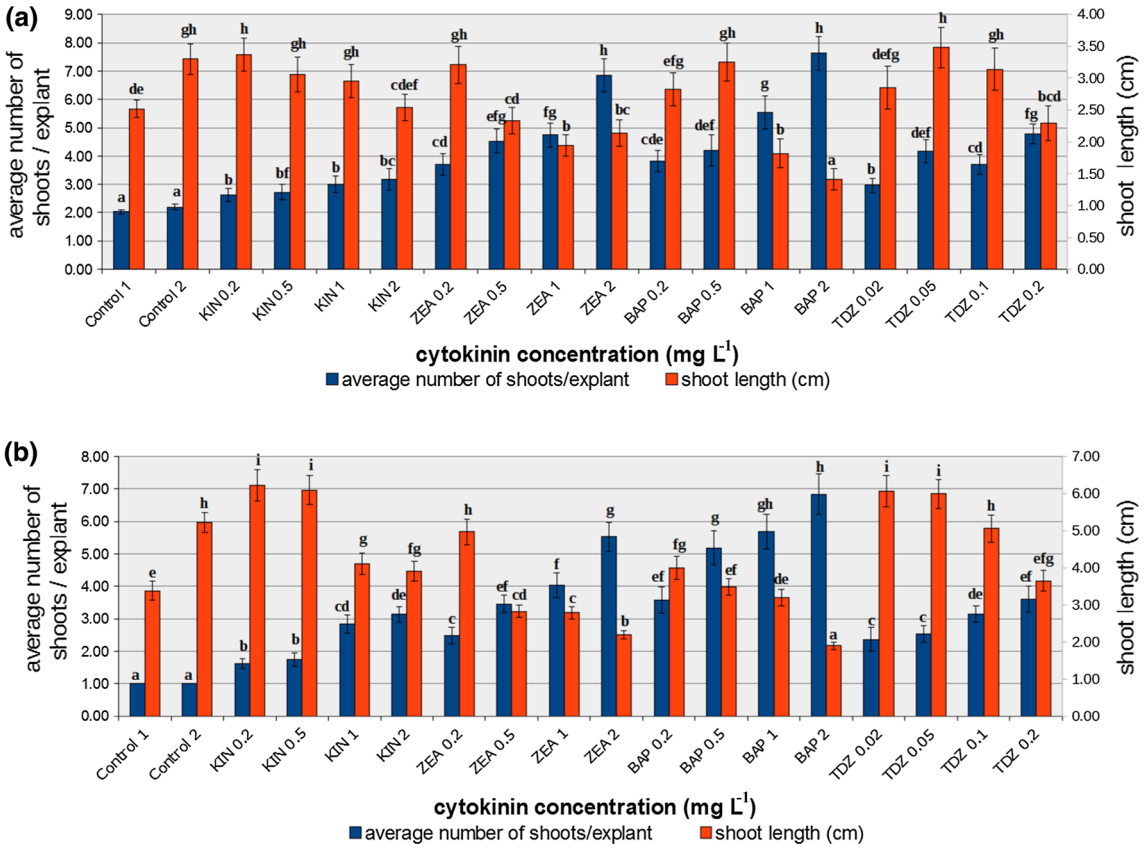

Fig. 1 Effect of different cytokinins on shoot multiplication from **a** nodal explants and **b** shoot tips of *T. scorodonia* after 35 days of culture on MS medium containing IAA (0.1 mg L^{-1}). The *values* represent the means of three replicates. Means followed by the *same letter* at each parameter were not significantly different at $P \leq 0.05$ according to the Kruskal–Wallis test. Control 1—MS medium without growth regulators; Control 2—MS medium supplemented only with IAA (0.1 mg L^{-1})

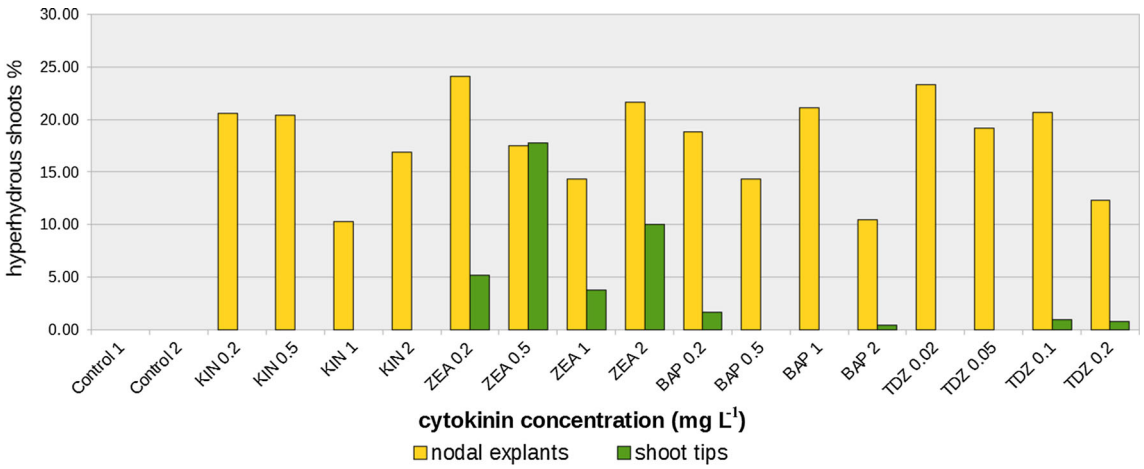

Fig. 2 Hyperhydricity (%) of multiple shoots of *T. scorodonia* regenerated from nodal explants and shoot tips under different cytokinin concentrations (on MS medium with 0.1 mg L^{-1} IAA)

Results and discussion

Micropropagation of *T. scorodonia*

No multiple shoot formation was observed when *T. scorodonia* shoot tip and nodal explants were cultured on MS medium without growth regulators or containing IAA (0.1 mg L^{-1}) alone. One or two axillary shoots were developed from pre-existing buds (Fig. 1a, b). For development of multiple shoots the presence of cytokinin was required. After 5 weeks of culture on MS medium supplemented with 0.1 mg L^{-1} IAA and BA, ZEA, KIN (0.2, 0.5, 1, and 2 mg L^{-1}) or TDZ (0.02, 0.05, 0.1, and 0.2 mg L^{-1}) all the shoot tips and 86–100 % of the nodal segments developed axillary shoots without callus formation. It was observed that, in general, higher numbers of regenerated shoots per explants, and shorter average shoot lengths were observed in media with higher cytokinin concentrations (Fig. 1a, b).

The best multiplication results were recorded when nodal explants were cultured on medium containing 0.1 mg L^{-1} IAA and 2 mg L^{-1} BA or 2 mg L^{-1} ZEA; mean shoot number was about 8 and 7 per explant, respectively (Fig. 1a). The elongation of shoots was significantly better on medium containing 2 mg L^{-1} ZEA (shoot length over 2 cm) than in the presence of 2 mg L^{-1} BA (mean 1.4 cm). However, hyperhydric shoots grown on medium supplemented with ZEA were more frequent than those on medium with BA (Fig. 2). Consequently, the number of good quality shoots (without hyperhydricity) with derived growth potential was lower in the presence of ZEA. Substituting BA with TDZ or KIN resulted in reduction of shoot multiplication. In the presence of TDZ at optimal concentration (0.2 mg L^{-1}), fewer than 5 shoots per nodal explant were produced. The average length of the shoots was 2.3 cm (Fig. 1a), and above 12 % of them showed hyperhydricity (Fig. 2).

The number of shoots obtained in the presence of BA or ZEA in combination with IAA was also higher than in the presence of KIN or TDZ, when shoot tips of *T. scorodonia* were used as explants (Fig. 1b). However, the values for shoot tip cultures were slightly lower compared to those achieved from nodal explants on the media with the same growth regulators (Fig. 1a, b). The highest number of shoots from shoot tips occurred in the presence of 0.1 mg L^{-1} IAA and 2 mg L^{-1} BA (almost 7 shoots/explant) (Figs. 1b, 3a), followed by 2 mg L^{-1} ZEA (5.5 shoots/explant), 0.2 mg L^{-1} TDZ (4 shoots/explant), and 2 mg L^{-1} KIN (3 shoots/explant). On the other hand, shoots cultured with optimal concentration of BA (2 mg L^{-1}) and IAA (0.1 mg L^{-1}) were about two-fold shorter than those grown with 0.2 mg L^{-1} BA (1.9 vs 4 cm), and 15 % shorter (1.9 cm) than those grown with 2 mg L^{-1} ZEA and IAA (2.2 cm). Hyperhydricity of shoots (over 3 %) occurred only when shoot tips were cultured on the media supplemented with ZEA (Fig. 2). It is well known that higher concentrations of cytokinin promote shoot hyperhydricity in many plant species, for example *Salvia przewalskii*, *Aloe polyphylla*, *Laburnum anagyroides*, and *Scutellaria alpina* (Skała et al. 2007; Ivanova and van Staden 2008, 2011; Timofeeva et al. 2014; Grzegorczyk-Karolak et al. 2015). However, as it was observed in our study, the frequency of hyperhydric shoots in *T. scorodonia* culture did not evidently depend on cytokinin concentrations, but occurred more frequently when nodal segments were used as the explants. Also Fraga et al. (2004) reported that the type of explant was significantly related to hyperhydricity in regenerated shoots of *Dianthus gratianopolitanus* cultivar Frosty Fire. The authors observed that in the presence of NAA and BA in MS medium, the shoots proliferated from nodal explants were hyperhydrous in 40 % of cases, whereas no hyperhydrous shoots were observed when shoot tips were used as explants under the same conditions (Fraga et al. 2004). The results described by the authors and these obtained in our study may be explained by differences in the concentration of endogenous cytokinins and their metabolism in various plant tissues, as well as the different water content in the explants used (Pospíšilová et al. 2000; Kevers et al. 2004; Offord and Tyler 2009; Skała et al. 2015; Trifunović et al. 2015).

Based on the results presented in Fig. 1a, b, BA at concentration of 2 mg L^{-1} together with 0.1 mg L^{-1} IAA was considered the best combination for *T. scorodonia* axillary shoot multiplication from both shoot tips and nodal explants. BA at concentration of 6.6 μM (1.5 mg L^{-1}) was also the best cytokinin for shoot formation from nodal segments of *T. fruticans* (Frabetti et al. 2009). However, the shoot multiplication rate (2.8 shoots/explant within 35 days) was considerably lower than the one achieved in our study. The poorest results in respect to shoot multiplication of *T. scorodonia* were obtained in the presence of KIN, despite the fact that this cytokinin appeared to be more effective than BA and TDZ for *T. polium* shoot multiplication (Al-Qudah et al. 2011).

Regardless of the composition of rooting medium (i.e. without growth regulators or with auxin), all the shoots formed roots within 42 days of culture (Fig. 4). This suggests that the endogenous concentration of auxins in the shoots was sufficient for root induction in hormone-free media as it was earlier reported by Piątczak and Wysokińska (2003) in *Centaurium erythraea* shoots. However, in our study the presence of auxin, its type and concentration affected the number of roots formed per shoot of *T.*

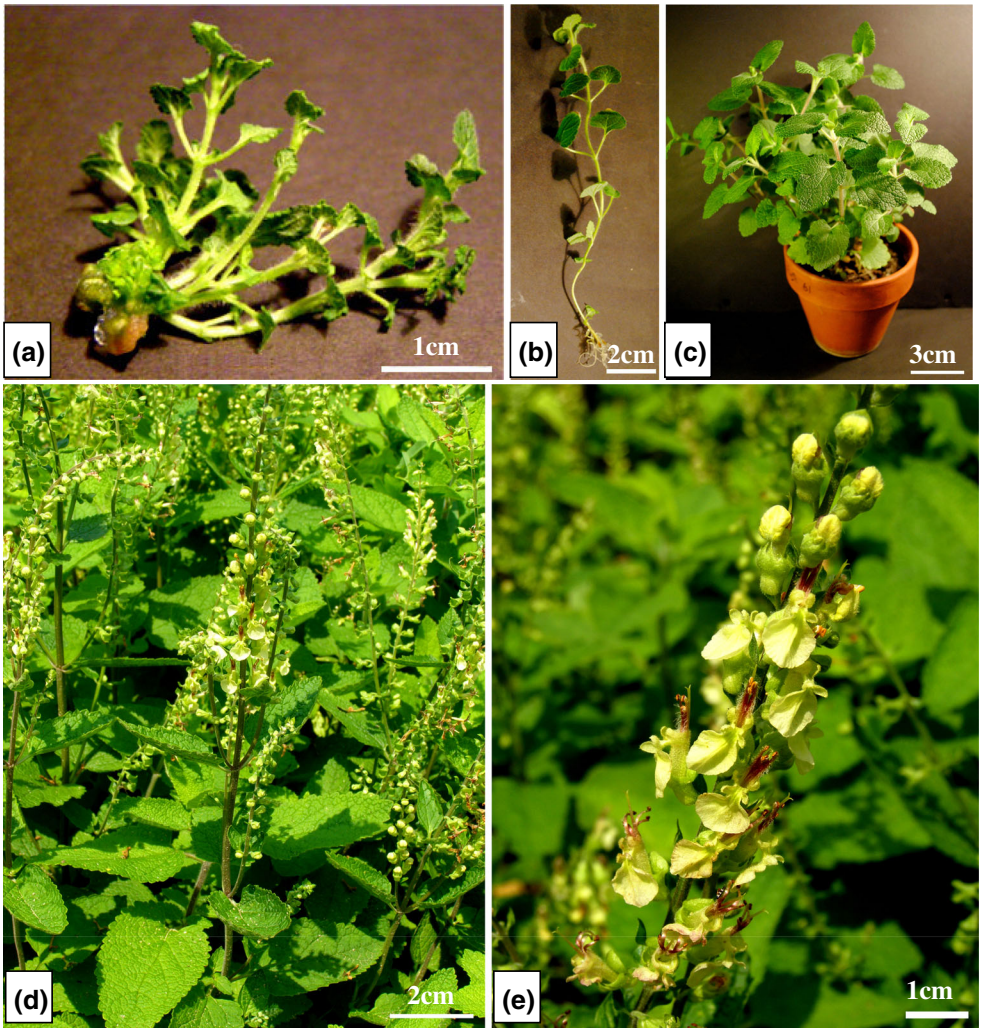

Fig. 3 In vitro micropropagation of *T. scorodonia*: **a** multiple shoot induction from shoot tip on MS medium supplemented with IAA (0.1 mg L^{-1}) and BA (2 mg L^{-1}); **b** rooted shoot on MS with IAA (0.1 mg L^{-1}); **c** plant after 60-day pot growth; **d** in vitro propagated plants grown in the field; **e** inflorescence in vitro-derived plant

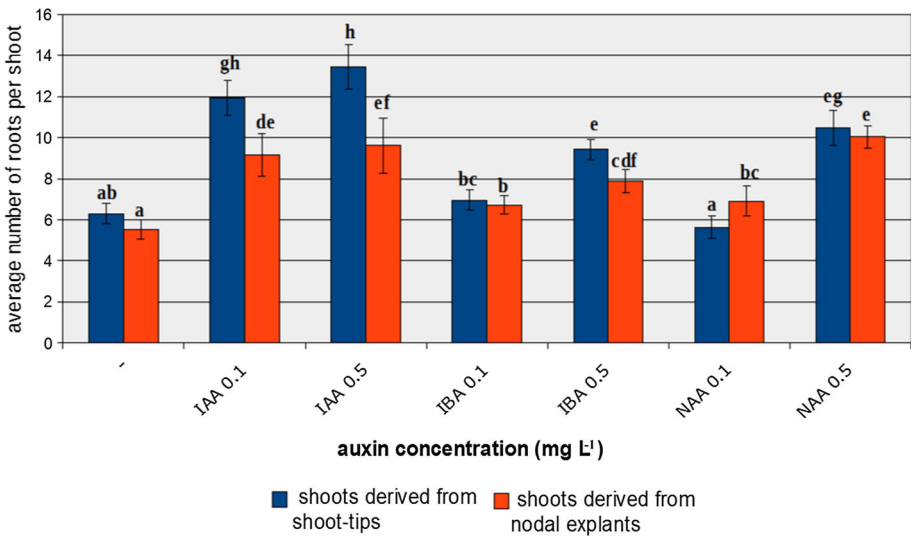

Fig. 4 Effect of auxin on number of roots produced by shoots of *T. scorodonia* after 42 days of culture on MS medium. The *values* represent the means of three replicates. Means followed by the *same letter* were not significantly different at $P \leq 0.05$ according to the Kruskal–Wallis test

scorodonia (Figs. 3b, 4). When the concentration of auxin in a medium increased from 0.1 to 0.5 mg L^{-1}, the number of roots increased slightly in the presence of IAA and significantly in the presence of IBA or NAA. IAA appeared to be the most effective source of auxin for root formation of *T. scorodonia*. The highest number of roots per shoot was obtained when shoots were cultured with 0.5 mg L^{-1} IAA, resulting in about 13 roots from shoot tips and about 10 from nodal explants. For shoots derived from nodal explants, NAA at 0.5 mg L^{-1} was also efficient (about 10 roots per shoot). A study using in vitro-derived shoots of *T. fruticans* (Frabetti et al. 2009) and *T. stocksianum* (Bouhouche and Ksiksi 2007) and where IBA was used for rooting, found that the number of roots increased with increasing auxin concentrations (0.01–2 mg L^{-1}). In contrast, in *T. polium* shoots, no root induction was observed in the presence of IBA or IAA (0.1–2 mg L^{-1}) in the medium, and the best rooting was obtained when 0.8 mg L^{-1} of NAA was added (Al-Qudah et al. 2011). These results suggest that the effect of auxin depends on the species of *Teucrium*. In the present study, about 90 % of *T. scorodonia* plants survived the acclimatization period when they were transferred to the greenhouse (Fig. 3c). The plants then grew to maturity in the field and achieved blooming and fruiting stage in the second year. The micropropagated plants (Fig. 3d,e) were phenotypically indistinguishable from their seed-derived counterparts.

Genome size

Flow cytometric analyses revealed that the nuclear DNA content ranged from 2.11 to 2.17 pg/2C and there was no significant difference between the genome size of plants obtained in vitro and seedlings (Table 1; Fig. 5). Only in the shoots grown on the MS medium supplemented with TDZ the DNA content was significantly higher; however, only by about 3 % (Table 1).

The stability of DNA content has previously been found in in vitro-produced plants among other medicinal species, e.g. *Oenothera paradoxa, Inula verbascifolia, Rubus chamaemorus, Solidago virgaurea, S. graminifolia, Plantago asiatica,* and *Eryngium planum* (Sliwinska and Thiem 2007; Makowczyńska et al. 2008; Thiem et al. 2013). The present results confirmed that the proposed protocol guarantees in vitro production of plants with a stable genome size.

The genome size of *T. scorodonia* reported by Bennett and Smith (1991) was 2.4 pg/2C. The difference between this value and the one established here is probably due to the different method (Feulgen densitometry) used previously.

Composition of essential oils

The hydrodistillation of aerial parts at flowering stage from in vitro and seed-derived plants resulted in an essential oil yield of 0.04 % (v/w) and 0.05 % (v/w), respectively, which was comparable to that of *T. scorodonia* plants growing in Italy (0.03 % w/w) (Maccioni et al. 2007). Previous studies on other *Teucrium* species reported oil yields ranging from 0.05 to 1.5 % (Djabou et al. 2013).

The chemical composition of essential oils with their relative amounts are listed in Table 2. Eighty-four compounds were identified, representing 96.3, 95.9, and 94.8 % of the total oils from V_1P, V_2P, and SP samples, respectively. The chemical profiles of the oils were similar and the variation was mainly due to the concentration of individual components. The oils were characterized by high percentage of sesquiterpene hydrocarbons (72.2 % in V_1P, 75.6 % in V_2P, and 54.4 % in SP) such as: β-caryophyllene, germacrene D, α-humulene. The main component of the fraction was β-caryophyllene (25.4, 26, and 22.3 % in the oils from V_1P, V_2P, and SP samples, respectively). β-Caryophyllene was also the main essential oil component of *T. scorodonia* ssp. *scorodonia* from Italy (25.2 %) (Maccioni et al. 2007) and from 10 locations in Corsica (France) (17.3–25.2 %) (Djabou et al. 2012). Our results reveal a major difference between oils from in vitro- and

Table 1 Nuclear DNA content in leaves of *T. scorodonia* seedlings and in vitro derived shoots (cultured on MS medium with 0.1 mg L^{-1} IAA and cytokinin)

Plant material origin	Cytokinin (concentration in mg L^{-1})	DNA content (pg/2C ± SE)
Seedlings		2.118 ± 0.012a
In vitro-derived shoots		
From shoot tips	0.2 BA	2.109 ± 0.009a
	0.5 BA	2.112 ± 0.017a
	0.5 KIN	2.128 ± 0.008ab
	0.05 TDZ	2.171 ± 0.010b
From nodal explants	0.2 BA	2.109 ± 0.005a
	0.5 BA	2.109 ± 0.008a

Values followed by the same letters within a column are not significantly different ($P \leq 0.05$) according to Tukey's test

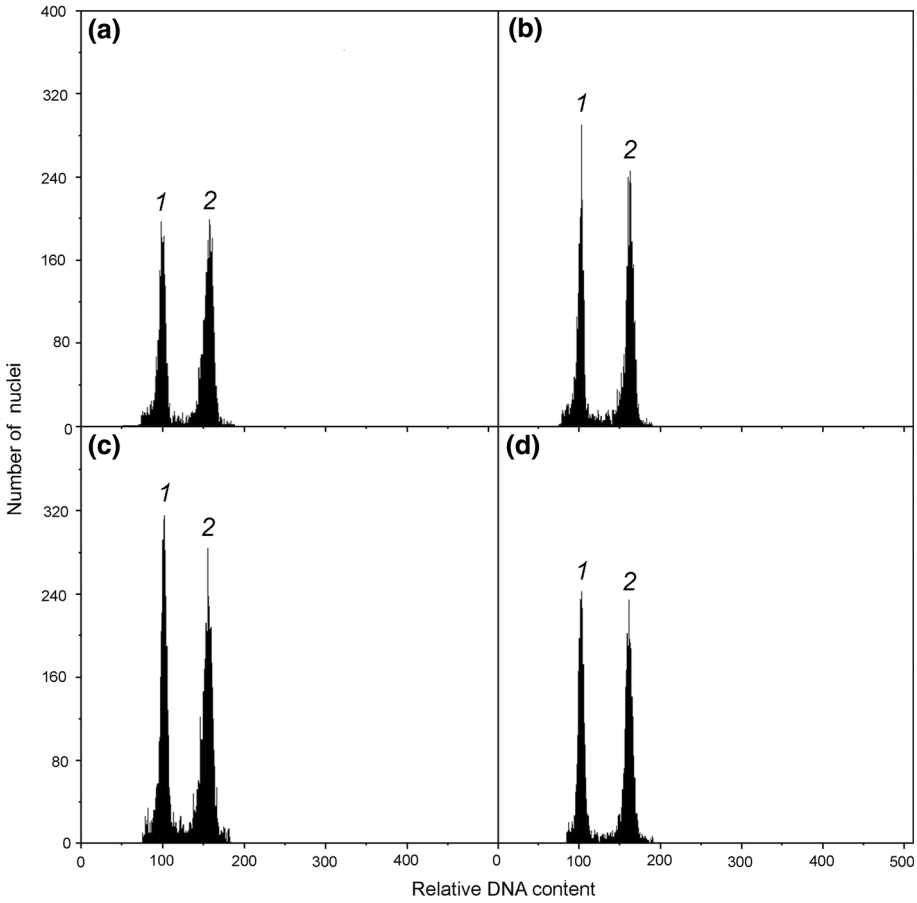

Fig. 5 Flow cytometric histograms of PI fluorescence intensity of the nuclei isolated from leaves of *Vicia villosa* (internal standard) and **a** *Teucrium scorodonia* seedling; **b** shoot derived from shoot tip grown in the presence of 0.5 mg L^{-1} BA and 0.1 mg L^{-1} IAA or **c** 0.05 mg L^{-1} TDZ and 0.1 mg L^{-1} IAA; **d** shoot derived from nodal explant grown in the presence of 0.2 mg L^{-1} BA and 0.1 mg L^{-1} IAA. 1—peak G_1/G_0 of *T. scorodonia*, 2—peak G_1/G_0 of *V. villosa*

seed-derived plants of *T. scorodonia* regarding the content of germacrene D: in the oils of V_1P and V_2P samples the amount of the sesquiterpene (15.4 and 22.4 %) was almost 5 and 7 times higher than that found in the SP sample (3 %). The concentration of this compound was found to be 6.3 % in essential oils isolated from aerial parts of *T. scorodonia* grown in Italy (Maccioni et al. 2007), and 4.6–10.1 % in Corsica (Djabou et al. 2012), which is higher than the seed-derived sample and lower than the in vitro-derived samples found in the present experiment. The third sesquiterpene hydrocarbon, α-humulene, was found to constitute 8.4–8.7 % of the analyzed oils, which is similar to previous findings (Maccioni et al. 2007; Djabou et al. 2012). Other important sesquiterpene hydrocarbons such as α- and β-cubebene, α-copaene, α-gurjunene, δ-cadinene were present in the SP sample in higher amounts than in the in vitro samples, with the exception of (E)-α-bisabolene. Caryophyllene and humulene epoxides were significant components of all essential oils. However, another difference among the essential oils analyzed in the present study was that caryophyllene oxide, the main oxygenated sesquiterpene, was found to be higher in the SP (15.4 %) samples than the VP (6.2 and 3.9 %) samples.

It is worth noting that in all essential oils, nine C13 compounds with the same carbon skeleton were identified as the products of carotenoid degradation: the hydrocarbon α-ionene, the ketones (E)-β-ionone, (E)-β-damascone and (E)-β-damascenone and their derivatives, as well as oxides (theaspiranes and dihydroedulan). The total content of these components was low (2.7–3.0 %). Ionones and damascones have been found to occur in a wide range of essential oils but in small amounts. However, they significantly contributed to the fragrance of the oils (Sell 2010). Some compounds of this group were previously found in *T. scorodonia* essential oils (Djabou et al. 2012) and in essential oils of other *Teucrium* species (De Martino et al. 2012). All oils of *T. scorodonia* analyzed here were poor in monoterpenes both hydrocarbons (1.1–1.9 %) and oxygenated compounds (2.2–2.3 %).

A comparative analysis of essential oils from micropropagated plants and their seed-derived counterparts was previously performed on some other species and numerous differences in yields and oil profiles were recorded. For example, the yield of essential oils isolated from *Salvia sclarea* plants, derived in vitro from shoot tips, was 0.1 %, while plants propagated from seeds produced 0.2 % of

Table 2 Constituents of essential oils from in vivo/in vitro *Teucrium scorodonia* plants

Peak number	Compound	RI	RI_{lit}	SP (%)	V_1P (%)	V_2P (%)
1	α-Pinene	930	934	t	t	0.1
2	Oct-1-en-3-one	956	956	–	–	t
3	Octane-2,3-dione	961	959	t	t	0.1
4	**Oct-1-en-3-ol**	964	966	**4.4**	**3.0**	**4.3**
5	β-Pinene	969	974	t	–	0.1
6	2-Pentylfuran	978	977	0.1	0.1	t
7	Myrcene	982	983	0.2	0.1	0.2
8	Car-3-ene	1004	1006	–	–	0.1
9	Phenylacetaldehyde	1008	1012	–	t	0.1
10	p-Cymene	1011	1015	0.1	0.1	t
11	Limonene	1020	1025	0.6	0.2	0.6
12	(*Z*)-β-Ocimene	1027	1029	0.1	0.3	0.3
13	(*E*)-β-Ocimene	1038	1041	0.1	0.2	0.2
14	γ-Terpinene	1048	1051	0.1	0.1	t
15	*cis*-Linalool oxide (furanoid)	1073	1072	t	t	t
16	Terpinolene	1078	1082	0.3	0.1	0.3
17	**Linalool**	1084	1086	**1.8**	**1.9**	**1.7**
18	Oct-1-en-3-yl acetate	1093	1093	0	0.1	0.2
19	Oct-3-yl acetate	1108	1110	0	0.1	0.2
20	*allo*-Ocimene	1118	1117	t	t	t
21	2-Methylbenzofuran	1147	1149	0.3	0.3	t
22	Terpinen-4-ol	1160	1164	0.2	0.1	0.1
23	Methyl salicylate	1168	1171	t	0.1	0.2
24	α-Terpineol	1171	1176	0.1	t	0.1
25	β-Cyclocitral	1195	1196	0.1	0.2	0.1
26	α-Ionene	1243	1240	0.3	0.4	0.2
27	Dihydroedulan	1288	1290	t	t	t
28	Theaspirane A	1290	1293	t	t	0.1
29	Theaspirane B	1304	1304	t	t	0.1
30	1,1,5,6-Tetramethylindane	1308	1311	0.1	0.1	t
31	Bicycloelemene	1333	1340	0.1	0.4	0.6
32	Neryl acetate	1340	1342	t	t	0.1
33	**α-Cubebene**	1350	1354	**5.3**	**3.0**	**3.0**
34	Geranyl acetate	1358	1362	t	t	0.2
35	(*E*)-β-Damascenone	1360	1363	0.2	0.3	0.3
36	α-Ylangene	1371	1376	0.2	0.5	0.4
37	**α-Copaene**	1375	1379	**2.2**	**1.3**	**1.0**
38	β-Bourbonene	1382	1386	0.8	1.1	0.8
39	**β-Cubebene**	1385	1390	**2.5**	**1.7**	**1.7**
40	(*E*)-β-Damascone	1394	1398	0.1	0.1	0.1
41	7,8-Dihydro-β-damascenone	1397	–*	1.3	1.3	1.1
42	α-Gurjunene	1409	1413	1.3	1.0	1.1
43	**β-Caryophyllene**	1421	1420	**22.3**	**25.4**	**26.0**
44	β-Copaene	1426	1430	0.3	2.1	1.3
45	*trans*-α-Bergamotene	1431	1434	0.1	0.1	0.1
46	Aromadendrene	1440	1443	0.2	0.2	0.3
47	(*E*)-β-Farnesene	1446	1446	0.7	1.5	0.2
48	**α-Humulene**	1452	1455	**8.4**	**8.7**	**8.7**

Table 2 continued

Peak number	Compound	RI	RI_{lit}	SP (%)	V_1P (%)	V_2P (%)
49	α-Cadinene	1457	1459	0.6	1.2	1.2
50	β-Ionone-5,6-epoxide	1460	1460	0.7	t	0.1
51	(*E*)-β-Ionone	1463	1467	0.4	0.8	0.7
52	γ-Muurolene	1472	1474	0.4	0.5	0.3
53	**Germacrene D**	1479	1479	**3.0**	**15.4**	**22.4**
54	(3*Z*,6*E*)-α-Farnesene	1481	1480	0.3	0.8	t
55	4-*epi*-Cubebol	1486	1490	0.7	0.7	0.6
56	Bicyclogermacrene	1491	1494	0.6	1.5	2.0
57	(3*E*,6*E*)-α-Farnesene	1493	1498	0.2	0.6	0.6
58	β-Bisabolene	1499	1503	0.5	0.7	0.5
59	γ-Cadinene	1505	1512	0.5	0.6	0.5
60	*trans*-Calamenene	1508	1514	0.3	0.1	t
61	**δ-Cadinene**	1513	1516	**1.5**	**1.2**	**1.3**
62	Cadina-1,4-diene	1523	1523	0.2	0.2	0.1
63	**(*E*)-α-Bisabolene**	1531	1530	**1.4**	**1.9**	**1.5**
64	β-Caryophyllene oxide	1539	1546	1.4	0.8	0.2
65	β-Calacorene	1545	1541	0.2	0.2	t
66	Germacrene B	1550	1552	0.3	0.3	t
67	**Spathulenol**	1564	1569	**2.2**	0.8	0.7
68	**Caryophyllene oxide**	1570	1573	**15.4**	**6.2**	**3.9**
69	Salvial-4(14)-en-1-one	1577	1584	0.3	0.5	t
70	Humulene epoxide 1	1582	1587	0.4	0.3	0.1
71	**Humulene epoxide 2**	1593	1597	**4.4**	**1.8**	**1.3**
72	Torilenol	1599	1601	0.3	0.2	t
73	1-*epi*-Cubenol	1613	1619	0.6	0.2	0.1
74	Humulene epoxide 3	1618	1624	t	t	t
75	Caryophylla-4(14),8(15)-dien-5α-ol	1620	1626	0.2	t	0.1
76	T-Muurolol	1625	1632	1.7	1.1	0.6
77	T-Cadinol	1627	1633	0.3	0.6	0.1
78	Cubenol	1630	1636	0.2	0.2	0.1
79	α-Cadinol	1637	1642	0.6	0.7	0.6
80	Phenantrene	1747	1744	0.1	t	0.1
81	Farnesylacetone	1888	1895	–	0.2	0.2
82	Phytol	2099	2099	t	–	1.3
83	Pentacosane	2500	2500	–	t	0.1
84	Heptacosane	2700	2700	–	–	0.1
	Total identified			94.8	96.3	95.9
	Sesquiterpene hydrocarbons			54.4	72.2	75.6
	Oxygenated sesquiterpenes			28.7	14.1	8.4
	Monoterpene hydrocarbons			1.5	1.1	1.9
	Oxygenated monoterpenes			2.2	2.2	2.3
	C13 compounds			3.0	2.9	2.7
	Others			5.0	3.8	5.0

The concentrations of the main compounds were signified in bold

RI relative retention index on Rtx-1 ms column, RI_{lit} relative retention index of literature, *SP* essential oil from in vivo plants, V_1P and V_2P essential oils from in vitro plants derived from shoots cultured on MS medium with either 0.1 mg L^{-1} IAA and 0.2 mg L^{-1} BA, or 0.1 mg L^{-1} IAA and 0.5 mg L^{-1} KIN

t = traces (< 0.05 %)

* Unavailable data

essential oils (Kuźma et al. 2009). The same tendency was observed for species of *S. przewalskii* (Skała et al. 2007) and *T. polium* (Al-Qudah et al. 2011). Also qualitative differences in oil composition of in vitro- and in vivo-derived plants of *S. przewalskii* were reported by Skała et al. (2007). The authors implied that those differences could be due to the conditions of in vitro cultures, especially the presence of plant growth regulators. Al-Qudah et al. (2011) reported that the presence of BA and NAA in the culture medium affected β-caryophyllene level in oil isolated from in vitro regenerated plants of *T. polium*. No β-caryophyllene was found in the in vitro sample that was grown on MS medium without plant growth regulators. Affonso et al. (2009) reported that in vitro shoot culture of *Thymus vulgaris* differed in essential oil composition depending on the plant hormones in the media. Amoo et al. (2012) conclude that the effect of growth regulators may be reduced during long-term ex vitro cultivation. This may be the reason why our findings revealed that field-grown micropropagated and seed-derived *T. scorodonia* were similar in respect to essential oil content and composition. Similarly, only small differences were detected in the contents of essential oil components between grown ex vitro and in vitro plants of four *Solidago* species (Kalemba and Thiem 2004).

In conclusion, our study provides the first demonstration of an efficient micropropagation procedure for *T. scorodonia*. Shoot tips and nodal explants were used as good starting material for axillary shoot multiplication. Multiple shoots possessed the same nuclear DNA content as seedlings, except when they were grown on the medium supplemented with TDZ. After three cycles on optimized multiplication medium (35 days each) and three cycles on rooting medium (42 days each) it was possible to obtain about 300 and 400 plants from a single shoot tip and nodal explant, respectively. The present results also show that in vitro-derived plants of *T. scorodonia* can be used as a potential source of essential oils whose main components are β-caryophyllene and germacrene D. The developed procedure appeared to be suitable for sustainable production of plants for pharmaceutical use.

Author contribution This research was accomplished with the collaboration of all authors. JM performed the micropropagation experiments, evaluated results, took photographic documentation, performed statistical analyses, prepared samples for essential oil analysis and wrote the manuscript. ES performed flow cytometric determination of DNA contents, evaluated the results, and participated in writing the manuscript. DK performed GC–MS analyses and wrote part of the manuscript. EP and HW were responsible for verification of the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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