Effect of cytokinins on in vitro multiplication, volatiles composition and rosmarinic acid content of *Thymus leucotrichus* Hal. shoots

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Abstract

An efficient in vitro multiplication protocol was designed to *Thymus leucotrichus*, a subshrub and perennial herb growing naturally in the Northwest of Turkey. Of all basal media studied, Murashige and Skoog medium was found to be superior to the others, providing higher shoot formation and the maximum shoot length. Varying concentrations of cytokinins, i.e., 6-benzyladenine, thidiazuron, 2-isopentenyladenine and kinetin were supplemented in the nutrient media to observe their effects on shoot development and biomass. Rosmarinic acid content and volatile compositions of both naturally growing plants and in vitro multiplied plantlets were also evaluated. 6-benzyladenine (1.0 mg/L) and kinetin (0.5 mg/L) were found to be optimum for shoot number and shoot elongation, respectively. Thidiazuron (1.0 mg/L) was superior for biomass production. Rosmarinic acid content of in vitro multiplied plants was found to be higher than that of wild plants, reaching a maximum with 0.5 mg/L 2-isopentenyladenine, which yielded 10.15 mg/g dry weight. The highest thymol content was obtained with 1.0 mg/L kinetin (55.82%), while thidiazuron (0.1 mg/L) increased carvacrol production (12.53%). Overall, Murashige and Skoog medium supplemented with 1.0 mg/L kinetin was determined to be the most favorable medium studied.

Keywords Biomass · Cytokinins · Volatiles composition · Multiplication · Rosmarinic acid · *Thymus leucotrichus*

Introduction

The genus *Thymus* (Lamiaceae) comprises over 300 ever-green species of herbaceous perennials and subshrubs native to Southern Europe and Asia (Könemann 1999). This genus is represented by 38 taxa, 20 of which are endemic flora of Turkey (Davis 1988). They have been used since antiquity as food and livestock feeds, aromatic flavors and fragrances, beverage additives, fuels and illumination materials, natural

remedies for combating parasites and diseases via soaps and perfumes, leather tanners and clothing dyes. Among them, *Thymus leucotrichus*, called “dağ kekiği” in Turkey, is a subshrub, perennial herb. It is found primarily on rocky slopes in highlands throughout the Eastern Black Sea Mountains at altitudes ranging from 1500 to 2800 m. Fresh, aboveground plant materials are consumed as spices and herbal teas by indigenous people. These species are also known as moonlight thyme or Juniper thyme in continental Europe. The use of *T. leucotrichus* species in Anatolian traditional medicine is well documented elsewhere, reporting its therapeutic uses, such as reducing high cholesterol, fighting cold and flu and relieving abdominal pains (Polat et al. 2015; Ozdemir and Alpınar 2015). Only one report was available in the literature on the in vitro antimicrobial activity of the extracts of *T. leucotrichus* (Sariboga and Korkmaz 2009). However, no information on rosmarinic acid (RA) content or volatiles composition of *T. leucotrichus* is currently available.

In addition to ex situ conservation, alternative approaches including in vitro techniques are useful for multiplication and evaluation of economically important constituents of aromatic plants (e.g., phenolics and essential oils), including *Thymus* species that are important sources of secondary

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metabolites. RA, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is valuable phenolic acid and is a focus of research to increase yield from biomass via different methods (Döring et al. 2014). In vitro production of RA was first achieved in cell suspension cultures of *Coleus blumei* (Razzaque and Ellis 1977). Since then, in vitro RA production from other sources has been reported (Santos-Gomes et al. 2003; Tepe and Sokmen 2007; Costa et al. 2012, 2013; Kikowska et al. 2014; Srivastava et al. 2016).

Plant tissue culture techniques offer an alternative route for in vitro propagation or multiplication, avoiding collection from their natural environment. No literature is currently available about multiplication, RA content and volatile composition of both natural and in vitro multiplied shoots of *T. leucotrichus*. Therefore, this work was designed (i) to optimize in vitro multiplication of *T. leucotrichus* using basal media supplemented with or without cytokinins, (ii) to assess biomass yield and hyperhydricity of each culture line grown in the presence of growth regulators, (iii) to compare the effects of cytokinins on production of RA and volatiles composition.

To the best of our knowledge, no comprehensive report is currently available on in vitro multiplication of *T. leucotrichus* and its RA content and volatiles production. Therefore, we sought to identify the optimal cytokinin for in vitro multiplication and yield of *T. leucotrichus*. We also extracted in vitro multiplied shoots and naturally growing plants and quantified their RA content using high-performance liquid chromatography with diode array detection (HPLC–DAD). Volatiles of all samples were also quantified by solid-phase microextraction (SPME) and gas chromatography/mass spectrometry (GC–MS).

Materials and methods

Plant material

Herbal parts of *T. leucotrichus* var. *leucotrichus* were randomly collected from natural populations in Toplukonak village, Sebinkarahisar-Giresun Turkey (40° 24' 173" N, 38° 34' 945" E; 1915 m) during its flowering season (mid-July, 2014). For multiplication studies, seeds were collected from the same locality towards the end of August, 2014.

Surface sterilization and culture conditions

The seeds of *T. leucotrichus* were surface sterilized with 50% commercial bleach for 15 min using a sonicator and then rinsed with sterile distilled water three times (5 min each). Seeds were then aseptically cultured on 30 mL nutrient media in 98.5 × 59 mm glass containers for germination. To determine the most suitable basal medium, nodal

segments of germinated seedlings were cut into segments 10 mm in length and placed on regulator-free basal media, including Murashige and Skoog (MS) (Murashige and Skoog 1962), Gamborg (B5) (Gamborg et al. 1968), Shenk and Hildebrandt (SH) (Shenk and Hildebrandt 1972) and Linsmaier and Skoog (LS) (Linsmaier and Skoog 1965). The medium pH was adjusted to 5.5–5.6 with 1 N KOH or 1 N HCl before autoclaving at 121 °C, 118 kPa for 20 min. All cultures were incubated at 24 ± 1 °C under white fluorescent lights for a 16 h light photoperiod with a photosynthetic photon flux density of 50 µmol/m² s for 4 weeks.

Effect of cytokinins on multiplication/proliferation

For shoot proliferation trials, microshoots obtained from hormone-free MS medium were cut to 10 mm and placed in glass containers (98.5 × 59 mm) with 40 mL of basal medium with or without cytokinins. As both zeatin and zeatin riboside were not found to be effective plant growth regulators (PGR) in preliminary experiments, the effects of 6-benzyladenine (BA), thidiazuron (TDZ), 2-isopentenyladenine (2iP) and kinetin (KIN) were individually evaluated for shoot proliferation at four different concentrations (0.1/0.5/1.0/2.0 mg/L). Results were compared to regulator-free medium. Shoots were kept subcultured by maintaining under the conditions given above. After the third subculturing, the shoots were harvested and individually evaluated for RA content and volatiles composition.

Determination of rosmarinic acid content

Extraction

For BA, roots formed were excised and removed from the plantlets. Both the aerial parts of intact plants and shoots of in vitro regenerated plantlets were air-dried in the shade and then extracted. For extraction, each seedling sample (0.2 mg) was macerated in 10 mL hexane and 10 mL dichloromethane for 10 min. Extraction solvents were filtered and residues were kept for further processing. Defatted and terpene free residues were extracted in methanol for 10 min followed by filtering with 0.45 µm polytetrafluoroethylene membrane filters.

HPLC conditions

HPLC analyses were carried out with a Shimadzu system composing of an LC-20AT pump, a DGU-20A5 online degasser, an SIL-20A autosampler, a CTO-10ASVP column oven and an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). The separation was performed at 40 °C on a Zorbax Eclipse XDB-C₈ analytical column (150 mm × 4.6 mm, 5 µm). The mobile phase was composed

of A (20 mM phosphate buffer adjusted to pH 2.5) and B (acetonitrile). The applied gradient program was as follows: 0 min, 5% B; 11 min, 8% B; 15 min, 20% B; 25 min, 80% B. A 10 min column re-equilibration time was employed between injections. The sample injection volume was 20 μ L and flow rate was 1.5 mL/min. Detection and quantification of RA was performed at 280 nm.

Analysis of volatile compounds

Headspace solid-phase microextraction

For SPME sampling, puree from each group was individually prepared by blending 100 g of plant for 3 min without water in a Waring blender. The plant puree (10 g) was placed into 10-mL headspace vials, closed with septa. The SPME headspace volatiles were collected using a Supelco 57348 2 cm, 50/30 μ m DVB/Carboxen/PDMS Stable-Flex fiber for 30 min. The fiber was preconditioned at 250 $^{\circ}$ C for 10 min. 30 min was chosen as it yielded the highest concentration of aroma compounds without a qualitative change in composition. After sampling, the SPME device was placed into the injector of the GC throughout the GC analysis time of 62 min using a RTX-5M column.

Identification of components by GC–MS

A Shimadzu QP2010 ultra GC–MS was used for all analyses. The GC–MS was fitted with a PAL AOC-5000 plus auto sampler, a Shimadzu 2010 plus FID and was controlled by Shimadzu Class-5000 Chromatography Workstation software. Gas chromatographic conditions were as followed: the GC oven was fitted with a RTX-5M 30-m capillary column (0.25 mm I.D., 0.25 mm film thickness, Restek, USA) with helium carrier gas at a flow rate of 1.0 mL/min, ramped from 60 $^{\circ}$ C (2 min) to 240 $^{\circ}$ C (5 min) at 5 $^{\circ}$ C/min. The temperatures of the injector and detector were 25 and 230 $^{\circ}$ C, respectively. The ionization voltage was 70 eV. Volatile compounds were identified by comparing the retention indices and experimental mass spectra with the literature data and mass spectral libraries of Wiley and NIST (Adams 2004; Yayli et al. 2005; National Institute of Standards and

Technology 2008). After analysis on a Restek RTX-5M column, quantification was performed using integrated peak areas.

Statistical analysis

Data in tables are given as mean $\pm$ standard errors (SE). For analysis of growth parameters (Tables 1 and 2), data were obtained from ten replicates and experiments were performed three times. For RA analysis (Table 3), replicates of each group were individually pooled and analyses were performed three times. For Table 4, replicates of each group were individually pooled, well mixed and analyzed accordingly. Collected data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's post hoc test. A two-sided *P* value of 0.05 was used to determine statistical significance. All statistical analyses were performed using Microsoft Excel 2010.

Results

In vitro shoot formation

To determine the optimal basal medium for multiplication, explants were transferred to MS, B5, LS and SH media free of growth regulators. At the end of 4 weeks, all microshoots were examined for shoot formation capacities and shoot lengths. In both cases, MS basal medium was found to be superior to the others, providing 56.66% shoot formation as well as giving the maximum shoot length (12.70 ± 0.49 mm) (Table 1).

After determining the optimal basal medium, the effects of cytokinins, as individual growth regulator supplements, were examined by measuring multiple shoot formation and elongation, leaf formation and biomass (based on both fresh and dry weight). To this end, microshoots (10 mm in length) were transferred to the media and supplemented with BA, KIN, 2iP and TDZ, at 0.1, 0.5, 1.0 and 2.0 mg/L. The effects of each cytokinin are presented in Fig. 1. Table 2 and Fig. 1 demonstrate the superiority of BA, which produced the highest mean shoot number (3.30 on average) as well as KIN (1.0 and 0.5 mg/L),

Table 1 Effect of basal media on regeneration rate and shoot elongation of *T. leucotrichus* multiplied shoots

	MS	B5	LS	SH
Regeneration (%)	56.66 ± 6.67^a	43.33 ± 5.77^b	36.66 ± 3.33^{bc}	28.88 ± 3.85^c
Shoot elongation (mm)	12.70 ± 0.49^a	8.19 ± 0.37^b	6.44 ± 0.27^c	4.88 ± 0.19^d
Rooting	ND	ND	ND	ND

Data indicate mean $\pm$ SE. Values followed by the same letter within the same line are not significantly different at *P* < 0.05. Data were recorded after 4 weeks of culture

ND not detected

Table 2 Growth parameters of the shoots in the presence of various cytokinins

PGRs (mg/L)	Mean shoot number	Mean shoot elongation (mm)	Node numbers	Fresh weight (mg)	Dry weight (mg)	Rooting (mm)
BA						
0.1	2.77 ± 0.67 ^{bc}	26.52 ± 1.94 ^a	4.07 ± 0.69 ^a	113.10 ± 15.00 ^{ab}	19.30 ± 4.90 ^a	7.80 ± 0.60 ^a
0.5	2.47 ± 0.97 ^c	19.30 ± 1.61 ^c	3.07 ± 0.69 ^b	103.40 ± 20.00 ^b	11.20 ± 1.80 ^d	10.54 ± 0.94 ^b
1.0	3.30 ± 0.59 ^a	19.48 ± 1.84 ^c	2.97 ± 0.57 ^b	115.00 ± 24.00 ^a	17.50 ± 3.10 ^{bc}	13.40 ± 1.00 ^c
2.0	2.43 ± 0.54 ^c	14.43 ± 1.34 ^d	2.50 ± 0.57 ^c	108.90 ± 20.00 ^{ab}	15.90 ± 3.00 ^c	16.80 ± 1.25 ^d
2iP						
0.1	2.73 ± 0.52 ^a	20.08 ± 1.95 ^d	4.40 ± 0.67 ^b	64.20 ± 7.80 ^d	11.20 ± 0.40 ^d	0.00
0.5	2.33 ± 0.66 ^b	21.67 ± 1.96 ^c	3.63 ± 0.72 ^c	53.70 ± 8.80 ^e	10.70 ± 1.80 ^c	0.00
1.0	1.47 ± 0.51 ^c	24.83 ± 2.70 ^b	5.70 ± 0.88 ^a	108.20 ± 1.80 ^b	18.40 ± 1.90 ^b	0.00
2.0	1.50 ± 0.68 ^c	26.97 ± 2.51 ^a	5.97 ± 0.72 ^a	127.40 ± 1.90 ^a	22.70 ± 3.80 ^a	0.00
KIN						
0.1	2.83 ± 0.59 ^a	40.16 ± 3.81 ^a	5.33 ± 0.88 ^b	204.50 ± 29.80 ^c	26.90 ± 3.00 ^c	0.00
0.5	2.67 ± 0.61 ^{ab}	40.88 ± 3.53 ^a	5.73 ± 0.78 ^a	245.10 ± 24.50 ^a	39.20 ± 3.40 ^a	0.00
1.0	2.47 ± 0.61 ^c	29.38 ± 2.55 ^c	3.97 ± 0.56 ^d	178.30 ± 18.20 ^d	25.40 ± 2.40 ^d	0.00
2.0	2.63 ± 0.73 ^{bc}	36.00 ± 2.89 ^{ab}	4.77 ± 0.68 ^c	234.80 ± 24.40 ^b	30.90 ± 3.20 ^b	0.00
TDZ						
0.1	2.37 ± 0.72 ^b	23.95 ± 2.15 ^a	3.30 ± 0.65 ^a	285.70 ± 19.90 ^d	38.30 ± 2.60 ^d	0.00
0.5	2.07 ± 0.37 ^c	19.78 ± 1.74 ^d	2.20 ± 0.41 ^b	505.20 ± 46.50 ^c	43.70 ± 3.30 ^c	0.00
1.0	2.00 ± 0.26 ^c	21.75 ± 1.86 ^b	2.03 ± 0.18 ^b	697.00 ± 59.70 ^a	61.30 ± 6.80 ^a	0.00
2.0	1.97 ± 0.18 ^c	12.51 ± 1.54 ^e	2.03 ± 0.18 ^b	570.40 ± 67.50 ^b	50.10 ± 4.60 ^b	0.00
Without PGRs	2.93 ± 0.69 ^{ab}	20.51 ± 1.78 ^c	3.47 ± 0.51 ^a	90.60 ± 6.40 ^e	17.80 ± 2.80 ^e	0.00

Data indicate mean ± SE. Values followed by the same letter within the same column are not significantly different at $P < 0.05$. Data recorded after 4 weeks of culture

Table 3 Rosmarinic acid content of multiplied shoots grown on MS medium supplemented with different PGRs at different concentrations and naturally growing plants (mg per gram dry weight)

Cytokinins (mg/L)	BA	2iP	KIN	TDZ
0.1	5.97 ± 0.09 ^c	9.43 ± 0.09 ^a	4.23 ± 0.09 ^d	9.25 ± 0.11 ^a
0.5	5.66 ± 0.10 ^c	10.15 ± 0.10 ^a	1.37 ± 0.09 ^f	6.49 ± 0.09 ^b
1.0	3.64 ± 0.08 ^e	7.55 ± 0.11 ^b	6.35 ± 0.10 ^{bc}	2.95 ± 0.04 ^e
2.0	3.29 ± 0.11 ^e	6.29 ± 0.06 ^{bc}	4.74 ± 0.11 ^d	4.55 ± 0.05 ^d
Without PGR				6.85 ± 0.09 ^b
Naturally growing				6.77 ± 0.09 ^b

Data indicate mean ± SE. Values followed by the same letter are not significantly different at $P < 0.05$. Data were taken after 4 weeks of third subculture

which produced the highest shoot elongation (40.16 ± 3.81 and 40.88 ± 3.53 mm, respectively). In addition, TDZ (1.0 mg/L) was superior for both fresh (697.0 ± 59.7 mg) and dry weight (61.3 ± 6.8 mg) biomass production. However, a sharp decrease was apparent with TDZ due to hyperhydricity in cultured shoots. This effect was also visible during shoot development, with decreasing shoot formation during subsequent subculturing procedures (Fig. 1d). Therefore, despite its high biomass yield, TDZ application at high concentrations was not chosen. Only BA applications led to root formation. In particular, root elongation increased with BA concentration (Table 2 and Fig. 1).

Rosmarinic acid content

Rosmarinic acid was determined in extracts of dried shoots of *T. leucotrichus*. HPLC chromatograms of RA standard and the extract of shoots grown on MS medium containing 2iP (1.0 mg/L) are shown in Fig. 2. RA content of both natural sources and sources multiplied in cytokinin-free medium was around 6.80 mg/g, dry weight biomass. Cytokinin supplements had an interesting effect on RA production (Table 3). For instance, MS containing 2iP resulted in higher RA production at 0.1 and 0.5 mg/L, but not at higher 2iP concentrations. This level of production is comparable

Table 4 Volatile compositions of naturally grown and multiplied plantlets

	RI***	Natural	Without PGR	BA			KIN			2IP			TDZ		
				0.1**	0.5**	1.0**	2.0**	0.1**	0.5**	1.0**	2.0**	0.1**	0.5**	1.0**	2.0**
α -Pinene	939	1.02	–	–	–	2.32	–	–	2.26	–	–	–	–	–	–
3-Octanone	984	2.41	–	–	2.54	–	2.42	–	–	–	4.91	–	1.60	1.32	–
Myrcene	991	4.33	–	4.32	4.26	4.53	4.61	2.53	5.38	3.54	1.38	3.92	1.90	1.87	6.79
α -Terpinene	1017	1.78	–	–	–	–	8.53	–	–	1.35	–	13.58	0.78	0.82	–
Limonene	1024	3.34	–	–	–	–	–	–	–	–	–	–	–	–	–
<i>p</i> -Cymene	1025	20.85	–	11.29	9.31	10.75	6.72	7.13	8.82	6.09	9.09	1.12	6.39	7.38	14.01
Eucalyptol (1,8-Cineole)	1026	1.55	–	–	–	–	–	–	–	–	–	–	–	–	–
γ -Terpinene	1060	8.52	–	5.38	7.31	7.00	6.09	13.26	9.43	4.92	5.95	6.40	2.15	2.21	5.70
Thymol methyl ether	1235	–	–	3.04	–	–	–	5.86	–	1.82	2.66	–	–	–	4.40
Thymoquinone	1252	–	6.24	9.36	4.31	9.22	14.87	4.60	3.72	3.22	6.74	13.30	9.29	10.54	2.22
Thymol	1290	36.86	37.91	37.16	49.19	27.50	28.88	9.45	35.07	55.82	36.60	27.79	50.19	55.54	7.96
Carvacrol	1299	3.31	7.60	1.23	2.15	11.04	7.12	–	2.51	2.15	9.32	7.29	12.53	4.76	–
β -caryophyllene	1419	2.11	0.46	3.84	2.82	4.93	1.37	9.59	3.99	2.71	4.40	4.52	2.43	3.09	5.98
Germacrene- <i>d</i>	1485	–	–	1.42	1.61	1.55	–	6.78	3.05	–	–	1.41	–	12.07	14.09
Bicyclogermacrene	1500	–	–	–	–	–	–	11.94	–	–	–	–	–	15.24	21.08
β -Bisabolene	1506	0.47	1.62	8.61	4.17	6.38	7.03	21.46	8.50	6.60	–	4.52	4.43	4.65	21.08
Minor and unidentified		13.51	46.17	14.35	12.33	14.78	12.36	7.40	17.27	13.13	18.95	15.97	9.09	8.64	–
Monoterpenes		39.84	–	20.99	20.88	24.60	25.95	22.92	25.89	15.90	16.42	25.20	11.20	12.28	26.50
Monoterpenoids		41.72	51.75	50.79	55.65	47.76	50.87	19.91	41.30	63.01	55.32	48.38	72.01	70.84	14.58
Sesquiterpenes		2.58	2.08	13.87	8.60	12.86	8.40	49.77	15.54	9.31	4.40	10.45	6.86	7.74	62.23
Others		2.41	–	–	2.54	–	2.42	–	–	–	4.91	–	1.60	1.32	–

Given as percentage in 1.0 μ L sampling

** Concentration (mg/L)

*** Retention index (RI) values are calculated from retention times relative to that of *n*-alkanes on the RTX-5M column

Fig. 1 4-week-old plantlets after third subculture. All plantlets were grown on MS basal medium supplemented with BA (a), 2iP (b), KIN (c) and TDZ (d). PGR concentrations are numbered as (1) 0.1 mg/L

(2) 0.5 mg/L (3) 1.0 mg/L (4) 2.0 mg/L. (Bars a: 14 mm, b: 20 mm, c: 29 mm and d: 12 mm)

Fig. 2 HPLC chromatograms of (A) RA standard and (B) extract of shoots grown on MS medium supplemented with 2iP (1.0 mg/L)

with productions obtained from natural samples as well as shoots from cytokinin-free medium. In contrast, BA supplement had a negative effect on RA production, with increasing concentrations reducing RA production to values far

below naturally growing plants as well as shoots grown on cytokinin-free medium (Table 3). Finally, the lowest concentration of TDZ (0.1 mg/L) was suitable for high RA production (9.25 mg/g).

Volatiles composition

Samples were randomly taken from well-mixed and dried plant materials. Total ion chromatograms for volatile compounds of shoots of *T. leucotrichus* grown on MS medium containing KIN (1.0 mg/L), TDZ (1.0 mg/L), 2iP (1.0 mg/L) and BA (1.0 mg/L) are presented in Fig. 3a–d. GC–MS of volatile compounds showed 52 components, 16 of which are listed in Table 4, representing about 80% percent of all volatile compounds analyzed.

For field grown (intact) plants, about 94% of volatile compounds were identified. Thymol (36.86%), *p*-cymene (20.85%) and γ -terpinene (8.52%) were the main components, while myrcene (4.33%), limonene (3.34%) and carvacrol (3.31%) were minor components.

In most cases, thymol was found to be the main volatile component of the shoots multiplied in vitro. Among in vitro shoots, the highest thymol content was from cultured in media with 1.0 mg/L KIN (55.82%) and 0.5 mg/L TDZ (55.54%), while the lowest content was from TDZ (1.0 mg/L). Thymol production was also high with 0.5 mg/L BA and 0.1 mg/L TDZ. The medium with 0.1 mg/L TDZ (12.53%) produced higher percentages of carvacrol than the others. In general, carvacrol production was higher in multiplied shoots than in intact plants. Limonene and eucalyptol were not determined in shoots grown in vitro. Total sesquiterpene amount, on the other hand, was the highest with 2.0 mg/mL TDZ (62.23%), with β -bisabolene and bicylogermacrene predominating (21.08% each) (Table 4). However, plants grown in media with KIN (2.0 mg/L) had the lowest total sesquiterpene yield (4.4%).

Discussion

Since in vitro multiplication of *T. leucotrichus* has not been reported previously, experiments started with evaluation of basal media for regeneration (proliferation) rate and shoot elongation. In the absence of growth regulators, nodal segments of selected seedlings were cultured in four basal media. MS basal medium was found to be superior to others, including B5, LS and SH, in terms of both tested parameters as well as shoot health. The efficiency of MS was expected based on earlier experiment using tissue cultures of many *Thymus* species (Furmanowa and Olszowska 1992). Since then many researchers have studied MS basal medium (Ozudogru et al. 2011; Coelho et al. 2012; Bakhtiar et al. 2014; Marco-Medina and Casas 2015).

Cytokinins are important PGRs and critical components of most plant cell culture media. After determining that full strength MS gave the best results, the second step was to evaluate each cytokinin alone. The highest mean number of shoots was obtained with BA, showing a gradual increase with higher applications and reaching a maximum at 1.0 mg/L before decreasing at higher concentrations. These results are in agreement with previous reports emphasizing that shoot proliferation and shoot number are superior with BA compared to the other cytokinins (Sáez et al. 1994; Coelho et al. 2012). However, root formation data was available and root elongation became more apparent with increasing BA concentrations. This formation might be because cytokinins are plant hormones which influence a wide range of plant developmental processes, including inhibition of rooting at higher concentrations. However, cytokinins are commonly applied in plant multiplication to stimulate adventitious root formation (Van Staden and Harty 1988). Spontaneous root formation was also consistent with other

Fig. 3 Total ion chromatograms of volatile compounds of shoots grown on MS medium containing (A) KIN (1.0 mg/L), (B) TDZ (1.0 mg/L), (C) 2iP (1.0 mg/L) and (D) BA (1.0 mg/L)

in vitro propagated *Thymus* species where BA was used as the sole PGR, for instance *T. piperella* (Sáez et al. 1994) and *T. moroderi* (Marco-Medina and Casas 2015).

Mean shoot elongation was best with KIN at low concentrations (0.1 and 0.5 mg/L), while node numbers were best with both KIN (0.5 mg/L) and 2iP. These findings are also in accordance with the results reported elsewhere (Ozudogru et al. 2011; Coelho et al. 2012; Bakhtiar et al. 2014).

The last parameter evaluated is particularly important for biomass yield for in vitro production of valuable metabolites. According to data given in Table 2, TDZ at 1.0 and 2.0 mg/L was optimal for biomass yield. However, at these concentrations, a morphological and physiological syndrome called hyperhydricity occurred, creating a handicap for subculturing. Hyperhydricity is a serious problem that arises during the in vitro production of plants that causes stress due to dramatic changes in the ethylene concentration, producing a glassy appearance, high water content, short internodes and lignification. A number of factors can contribute to hyperhydricity, such as water availability, micronutrient content and hormonal imbalance in the media (Hazarika 2006). Higher cytokinin concentrations were reported to cause hyperhydricity for several multiplied *Thymus* species (Ozudogru et al. 2011; Coelho et al. 2012; Hassannejad et al. 2012). However, in our study, only higher TDZ supplement caused hyperhydricity. A new technique has been recently introduced to prevent hyperhydricity involving incorporating colloidal silver nanoparticles (AgNPs) into MS basal medium (Bernard et al. 2015).

Data obtained in our study showed that RA content of *T. leucotrichus* was lower than in species described above. As seen in Table 3, cytokinin supplement may solely lead to RA overproduction which is prevalent in 2iP (0.1 mg/L) and TDZ (0.1 mg/L) cases. However, auxin supply and precursor or elicitor applications may be necessary for RA production at higher levels. For instance, this was observed in callus and/or cell suspension cultures of *Salvia officinalis* (Santos-Gomes et al. 2003) and *Satureja hortensis* (Tepe and Sokmen 2007), shoot cultures of *Thymus lotocephalus* (Costa et al. 2012) and *Lavandula viridis* (Costa et al. 2013), micropropagated plantlets of *Eryngium maritimum* (Kikowska et al. 2014) and hairy root cultures of *Ocimum basilicum* (Srivastava et al. 2016).

The presence of cytokinin increased the production of thymol, reaching its highest level with KIN (1.0 mg/L), followed by TDZ (0.5 and 0.1 mg/L), 2iP (1.0 mg/L) and finally BA (0.5 mg/L). These results reveal that thymol production in *T. leucotrichus* plantlets depends on the concentration of any particular cytokinin supplement. Increasing TDZ concentration caused a sharp reduction in thymol content, implying a negative correlation between hyperhydricity and thymol production. On the other hand, increases in sesquiterpenes are apparently related to hyperhydricity

and weak growth conditions. In particular, shoots grown in media supplemented with TDZ (1.0 and 2.0 mg/L) gave the lowest thymol content (5.91 and 7.96%, respectively), while total sesquiterpene content was increased (49.19 and 62.23%, respectively) (Table 4). Except for the cases mentioned above, the cytokinin sensitivity does not occur for *T. leucotrichus*, as reported previously for *Thymus moroderi* (Marco-Medina and Casas 2015) who found that the monoterpene and sesquiterpene fractions were higher in multiplied plants than in intact plants (Table 4b).

In a previous report on *Thymus vulgaris* plantlets, cytokinins caused a decrease in *p*-cymene and γ -terpinene and an increase in thymol (Affonso et al. 2009). However, a similar correlation was not observed for *T. leucotrichus* here. Significant differences between intact and clonally multiplied shoots emphasize that the nature as well as the growth regulator concentration influence the production of volatile compounds. No information is available in the literature regarding volatile compound production by field grown *T. leucotrichus*. Therefore, results discussed here are novel in this respect.

Conclusion

No information was available about RA content and volatiles composition of intact *T. leucotrichus* var. *leucotrichus* plants, as well as in vitro multiplication protocol. Here we have devised a preliminary protocol for the in vitro multiplication of this plant species using cytokinins alone.

2iP was a moderately effective regulator for multiplication of *T. leucotrichus*. MS basal medium supplemented with KIN (1.0 mg/L) was optimal for healthy and vigorous shoots as well as reasonable biomass yield, with a single growth regulator. Both RA content and volatiles composition of naturally growing plants and in vitro multiplied shoots were analyzed for these growth conditions.

It is always important to assess the vigor, growth profile and biomass with in vitro production of any particular secondary metabolite. On the whole, MS basal medium supplemented with 1.0 mg/L KIN was superior to the other applications, as RA production was acceptable and a high biomass obtained, with limited hydricity. This study is the first comprehensive effort to measure volatiles composition of both naturally growing plants and in vitro multiplied shoots.

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Compliance with ethical standards

Conflict of interest Authors declare that there is no conflict of interest.

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