



In vitro cultivation of tansy (*Tanacetum vulgare* L.): a tool for the production of potent pharmaceutical agents

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Abstract

In this study, tansy (*Tanacetum vulgare* L.) in vitro culture was established from seeds collected from natural populations. The multiplication of plantlets was conducted through shoot tips that exhibited potent apical growth and regeneration capacities on basal medium (BM), without the addition of any plant growth regulators (PGRs). PGRs were also omitted for the establishment and cultivation of tansy root cultures. Both abaxial and adaxial leaf surfaces of in vitro micropropagated plantlets were covered with glandular biseriate trichomes. Histochemical staining showed that glandular secretions were rich in lipid and terpene compounds, confirmed by GC-MS analysis of essential oil (EO). In the total EO, similar portions of oxygenated monoterpenes (38.5% m/m) and oxygenated sesquiterpenes (22.6% m/m) were detected. Chemical profiles of methanol extracts of in vitro cultured tansy shoots and roots varied in quantity and quality from those obtained from wild-growing tansy. HPLC analysis indicated that the methanol extracts of in vitro cultured roots were the richest in 3,5-*O*-dicaffeoylquinic acid (3,5-*O*-DCQA), in which the concentration was 6 times higher (10.220 mg/g DW) than that in the extract obtained from roots of wild-growing tansy (1.684 mg/g DW). This result is noticeable in the manner of industrial production of biologically active 3,5-*O*-DCQA that has been shown to have antioxidant, hepatoprotective, antiviral, antimutagenic, and immunomodulatory activity. Biotechnological interventions on secondary metabolite production taking place in trichomes could further enhance the production of some important tansy metabolites and further investigation will be directed toward the elucidation of the pharmaceutical potential of tansy in vitro obtained metabolites, as mixtures or single moieties.

Keywords Tansy · *In vitro* cultivation · Essential oil · Methanol extracts · Histochemical analysis · Phytochemical analysis

Introduction

The current pharmacological industry mostly relies on synthetic drugs, due to the intensive development of a computational approach, combinatorial chemistry techniques, and

instruments during the second half of the twentieth century (Liu et al. 2017). Despite what was expected, the rate of new chemically synthesized pharmaceuticals reaching the market has continually decreased, with only 24.6% of the 1881 new drugs approved from 1981 to 2019 obtained synthetically (Newman and Cragg 2020). This has forced the pharmaceutical industry recurrently turn to natural products, compounds derived from microorganisms, plants, and animals used in traditional medicine for thousands of years. Among them, secondary metabolites of plants make the most promising pool of potential pharmacological drugs. From the total number of plant species discovered so far, approximately 20% have been screened for biologically active compounds, and about 135 registered drugs have been approved (Newman et al. 2003; David et al. 2015).

Since 1853, when acetylsalicylic acid was first produced from natural salicin isolated from the bark of the white willow (*Salix alba*), the pharmaceutical industry has been oriented to

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the chemical synthesis of products isolated from living organisms. Some of the most famous are paclitaxel (Taxol®) from the bark of *Taxus brevifolia* L. used in the treatment of breast cancer (Cragg 1998), phorbol ester (Prostratin®) isolated from the bark of *Homalanthus nutans* used for the treatment of lymphoblastic cells infected with HIV-1 (Dias et al. 2012), or chloroquine extracted from the bark of *Cinchona officinalis* and used to prevent and treat malaria (Plowe 2005). Chloroquine also showed potent antiviral effects (Savarino et al. 2003) and was promoted for the treatment of patients infected with SARS-CoV-2 during the 2019/2020 pandemic spread of this virus (Wang et al. 2020; Devaux et al. 2020).

In parallel, drug formulations of complex partly purified plant extracts have become equally adopted as “single-molecule” medicines. This approach relies on traditional medicine experiences, where complex mixtures of plant compounds were used for the treatment of different health problems without any knowledge about the contained bioactive compounds. In 2008, the first botanical extract, Veregen®, an enriched extract of polyphenols from green tea (*Camellia sinensis*), was approved in the USA for the treatment of genital warts caused by the human papilloma virus (Scheinfeld 2008). Although there is no doubt that these herbal mixtures provide a synergism of positive effects (Arora and Koul 2014), many of the regulatory approval processes were impeded for various reasons (Chugh et al. 2018). One of the issues is the intrinsic variability of plant material caused by environmental factors (i.e., actual climate, harvesting time, soil composition, altitude, storage conditions) and/or biotic interactions occurring in natural populations before harvest. This can cause significantly altered phytochemical composition of obtained extracts, as well as low yields of targeted products and high abundance and toxicity of some active compounds.

The employment of in vitro cultivation of plants is among the biotechnological solutions favored by researchers. The establishment of in vitro multiplication protocols enables the production of uniform plant material and controlled manipulation with environmental conditions, plant growth regulators, and elicitation strategies that can boost the production and overall yield of phytochemicals. Growing plants under controlled environmental conditions (temperature, light intensity, and duration), on growing media prepared according to strict recipes for plant’s nutritional necessities, showed to be good practice for avoiding the instability in chemical composition that naturally occurs (Robles and Garzino 2000; Demetzos et al. 2002). Strictly aseptic conditions prevent the presence of unexpected compounds synthesized by endophytic fungi and/or bacteria or produced by plants as a result of the interaction with them (David et al. 2015), or harmful residues of pesticides that are often present in plants collected from nature. While intensive wild-crafting and unsustainable harvesting techniques of either whole plants or their particular organs could bring about their persistence and sustainability in

natural habitats and even make plant populations threatened (Cordell 2011), in vitro micropropagation techniques offer a proper alternative for the cultivation of valuable plants and large-scale production of targeted metabolites.

Relying on information coming from traditional medicine, we aimed to investigate the phytochemical properties of *Tanacetum vulgare* L. (tansy, *Asteraceae/Compositae*). This aromatic perennial herb is a native of Europe. Its generic name, *Tanacetum*, comes from the Greek word “*athanatos*” meaning immortality. In ancient times, the unique rejuvenating powers and ability to preserve dead bodies from corruption were ascribed to its long-lasting flowers (Mitich 1992). Since then, tansy has broadly been used in traditional medicines worldwide for the treatment of a variety of medical disorders, such as intestinal worms, rheumatism, colds and fevers, digestive disorders, epileptic seizures, hysteria, gout, kidney problems, and tuberculosis. Many laboratory reports have claimed that essential oils and extracts of tansy display a wide range of biological activities, including antioxidant, antimicrobial, insecticidal, cytotoxic, antiviral, and anti-inflammatory activities (Mordujovich-Buschiazco et al. 1996; Onozato et al. 2009; Rosselli et al. 2012; Gospodinova et al. 2014; Devrnja et al. 2017). A purified complex extract of flavonoids and phenol carbonic acids from tansy flowers, commercially known as Tanacechol®, is registered in Russia as a choleric and spasmolytic agent for chronic cholecystitis and biliary dyskinesia (Karabaeva et al. 2015). Septimeb™, an herbal extract including *T. vulgare* (tansy), *Rosa canina* (dog rose), and *Urtica dioica* (stinging nettle) in addition to selenium, flavonoids, and carotenes, showed positive effects on the reduction of sepsis severity (Pourast et al. 2017). The weaker version of this mixture, named Setarud (IMOD™), was proposed as an immunomodulator in the treatment of HIV-positive patients (Paydary et al. 2012).

In general, this species is insufficiently investigated since it is endowed with an array of valuable phytochemicals. It is well known for its impressive chemical diversity with more than 30 chemotypes identified by dominant terpenoids (Keskitalo et al. 2001; Rohloff et al. 2004; Wolf et al. 2012). Additionally, there are limited data regarding suitable micropropagation techniques for its in vitro cultivation, and only procedures for the regeneration of petiole and leaf explants and the production of protoplast-derived callus have been reported (Keskitalo et al. 1995). Our previous research (Devrnja et al. 2017) highlighted the phytochemicals in essential oil and methanol extracts from wild-growing tansy plants that manifested strong antioxidant and antimicrobial activity. Special attention was given to its anticancer properties and in vitro results showed a high antiproliferative effect in the micrograms range (up to 100 µg/mL) on human cervical adenocarcinoma (HeLa) cells.

This study aimed to investigate the potential of tansy plants to produce valuable chemicals by in vitro culture. The general

aims were to establish both shoot and root cultures of tansy and to analyze the phytochemical composition of obtained essential oil and methanol extracts with histochemical analyses of production sites. Comparing the composition of metabolites mixtures from in vitro grown plants with those obtained from wild-grown parental plants will indicate the sustainability of the proposed thesis that in vitro cultures could provide an efficient tool for the production of innovative pharmaceutical drugs from tansy metabolites in controlled conditions.

Materials and methods

Establishment and maintenance of in vitro shoot and root cultures

Seeds collected from a native population (from the Ada Huja locality, Belgrade, Serbia) were used for in vitro culture establishment. The seeds were washed in tap water containing 2–3 drops of liquid commercial detergent for 30 min and rinsed three times with sterile distilled water. Washed seeds were then surface sterilized using a 30% solution of commercial bleach (4% NaClO) for 30 min, and 15% bleach for 15 min, and subsequently rinsed three times with sterile distilled water for 10 min. Seeds dried on sterile filter paper were aseptically placed in 90-mm Petri dishes (20 seeds per dish) containing 25 mL of basal medium (BM) for germination.

The BM contained MS (Murashige and Skoog 1962) macro and micro mineral salts, 2% sucrose, 100 mg/L myo-inositol, and LS (Linsmaier and Skoog 1965) vitamins, and was solidified with 0.7% (w/v) agar (Torlak, Belgrade, Serbia). The pH of the medium was adjusted to 5.5 before sterilization in an autoclave at 114 °C (80 kPa) for 25 min. The seeds were germinated under controlled conditions of 24 ± 2 °C, 16-h light/8-h dark photoperiod, and under cool white fluorescent light with a photosynthetic photon flux rate of $40 \mu\text{mol}/(\text{m}^2\text{s})$, as measured by an LI-1400 DataLogger equipped with an LI-190SA Quantum sensor, LICOR Biosciences.

When the resulting axenic seedlings fully developed 4–5 leaves and a branched root system, they were used as donor material for shoot multiplication. Micro shoots were subcultured on fresh BM in glass 100-mL Erlenmeyer flasks every 4 weeks, and material for phytochemical analyses was collected continuously. In parallel, roots excised from the plantlets rooted spontaneously on BM were used for the establishment of in vitro root cultures. Roots were transferred into Erlenmeyer flasks (400 mg in each) with 100-mL liquid half-strength BM ($\frac{1}{2}$ BM; with reduced content of MS components) and maintained on a horizontal shaker (90 rpm) under the aforementioned conditions with reduced light ($2 \mu\text{mol}/(\text{m}^2\text{s})$). After 4 weeks in culture, roots were removed from the medium and dried on filter paper to remove any adherent culture medium and air-dried for further phytochemical analyses.

Morpho-anatomical analysis

For the investigation of morpho-anatomical structures that produce and accumulate secondary metabolites, the second and third fresh leaves, isolated from shoots cultured in vitro, were used and subjected to scanning electron (SEM) and light microscopy. For SEM, leaf samples were coated with a thin layer of gold in a BAL-TEC SCD 005 (BAL-TEC GmbH, Schalksmühle, Germany) sputter coater. Both adaxial and abaxial surfaces were examined with a JEOL JSM-6390 LV (JEOL, Tokyo, Japan).

For anatomical investigation by a light microscope, the fresh leaves were fixed with FAA (formalin: acetic acid: 70% ethyl alcohol; 10:5:85) for 24 h, dehydrated in a graded ethanol series, subsequently cleared with xylol, and embedded in paraffin wax at 58 °C. Sections (8–10 μm thick) were stained with hematoxylin and photographed using a Zeiss Axiovert (Carl Zeiss GmbH, Jena, Germany) microscope.

Histochemical analysis

To detect the presence of secondary metabolites in secretory cells of trichomes, the second and third fresh leaves were hand-sectioned and the following histochemical staining methods were used: Sudan Black B, Sudan IV, and Sudan Red 7B/hematoxylin for total lipids (Jensen 1962); Nile Blue A for neutral and acidic lipids (Cain 1947); Nadi reagent for terpenoids (David and Carde 1964); periodic acid-Schiff (PAS) reagent for polysaccharides (Jensen 1962); Ruthenium Red for pectins (Johansen 1940); Wagner (Furr and Mahlberg 1981), Dragendorf (Svendsen and Verpoorte 1983) and Ellram (Furr and Mahlberg 1981) reagents for alkaloids; ferric chloride (FeCl_3) for polyphenols (Gahan 1984); and Toluidine Blue O for lignins and tannins (Baker 1966). Following the methods of specific authors, standard control procedures were carried out simultaneously for all histochemical methods used. Observations were made on a Zeiss Axiovert microscope. The autofluorescence investigation was carried out using the same instrument.

Isolation and phytochemical analysis of essential oil

Essential oils from the aerial parts of in vitro grown plants were obtained by steam distillation for 2 h in a Clevenger-type apparatus. Before distillation, air-dried plants were blended to a fine powder. The oil distillate was separate from traces of water by extraction with *n*-hexane and kept at 4 °C until analysis. The oil yield was expressed as mL per 100 g of plant dry weight.

Gas chromatography-mass spectrometry (GC-MS) analysis of essential oils was performed on an Agilent Technologies 7890 A GC system equipped with 5975 CI Inert MSD and flame ionization detector (FID). The GC system was equipped

with the split-splitless injector and HP-5MS capillary column (30 m × 0.25 mm, 0.25- μ m film thickness). Helium was the carrier gas, and its flow rate was 3 mL/min. The ion source energy was 70 eV and chromatographic conditions were as follows: column temperature was linearly programmed from 60 to 300 °C (at the rate of 3 °C/min) and held isothermally at 300 °C next 10 min; injector temperature was 250 °C; ion source temperature was 230 °C; and quadrupole temperature was 150 °C.

The constituents were identified by comparison of their mass spectra to those from Wiley275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1., National Institute of Standards and Technology- NIST, Standard Reference Data Program, Gaithersburg, MD, USA), compared to those from available literature (Adams 2007), and used as an additional tool to approve MS findings.

Preparation and phytochemical analysis of methanol extracts

Tansy methanol extracts were prepared from both aerial parts and roots of plants grown *in vitro*. To compare chemical profiles of extracted compounds, methanol extracts were also made from native tansy plants collected from the Ada Huja locality, Belgrade, Serbia.

For all extracts, 500 mg of air-dried plant material was blended to a fine powder and extracted with 10 mL of methanol in an ultrasonic bath for 20 min. After sonication, the extraction was continued by maceration of tissue for 48 h in the dark at room temperature. Finally, the extracts were filtered through Whatman filter paper No. 1 to a volumetric flask and filled with methanol up to 10 mL. The extracts were stored at room temperature for further analyses.

High-pressure liquid chromatography (HPLC) of the methanol extracts was carried out on an Agilent 1100 chromatograph with a column compartment equipped with a Zorbax SB-C18 analytical column (150 mm × 4.6 mm, 5 μ m, Agilent Technologies, Waldbronn, Germany) and diode array detector. The mobile phase consisted of a 1% (v/v) solution of orthophosphoric acid in water (A) and acetonitrile (B). The flow rate of the mobile phase was 0.8 mL/min, and the injection volume was 10 μ L. A gradient program was used as follows: 90–75% A, 0–30 min, 75–45% A, 30–40 min, 45–0% A, 40–50 min. The chromatograms were recorded at 280 nm and 360 nm. Identification of 3,5-*O*-dicaffeoylquinic acid and chlorogenic acid was confirmed by the co-injection method using commercial standard samples purchased from Sigma-Aldrich. The content of 3,5-*O*-DCQA and chlorogenic acid was determined by the external standard quantification method.

Results

After 15 days, the percentage of germinated tansy seeds was 97.8% (Fig. 1a, b). Grown on BM, seedlings developed 4–5 leaves and a branched root system in 4 weeks (Fig. 1c). The *in vitro* grown tansy plants displayed a typical tansy phenotype with alternate and pinnately lobed leaf morphology (Fig. 1c). Healthy looking seedlings were used for the establishment of both shoot and root cultures on solid or liquid BM, respectively (Fig. 1d, e). After 4 weeks of cultivation on solid BM, average shoot length was 48.54 ± 0.2 mm, without any lateral branching. The roots cultivated in liquid BM increased

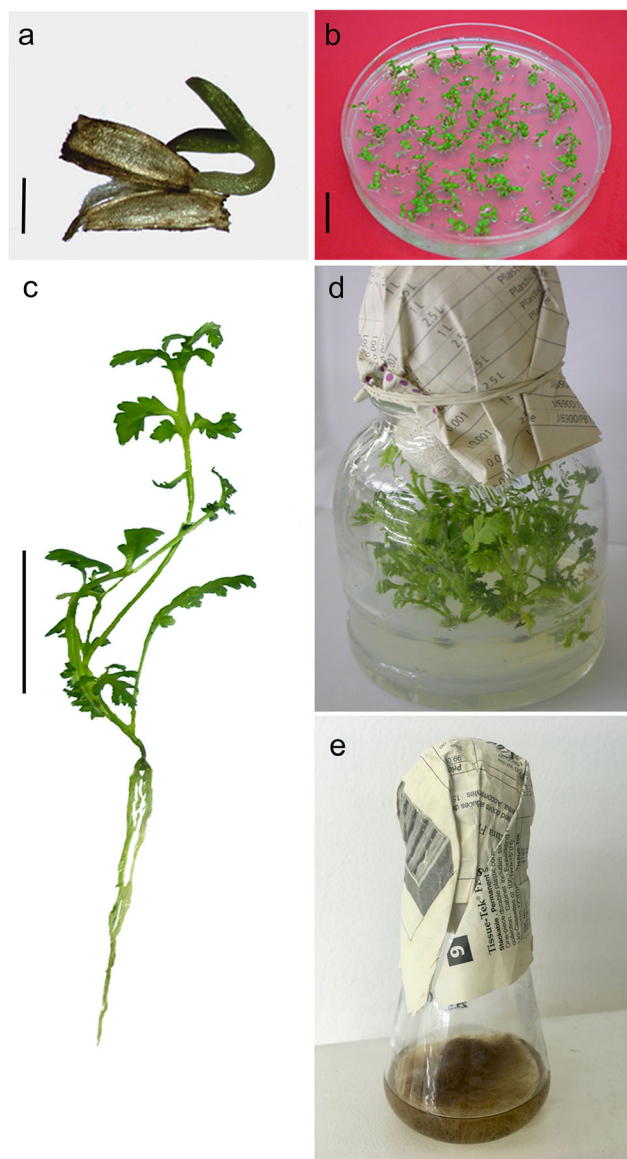


Fig. 1 *In vitro* micropropagation of tansy. **a** Seedlings observed with binocular magnifier; bar = 1 mm; **b** Petri dish with germinated seedlings; bar = 2 cm; **c** Nicely develop tansy seedling with 4–5 leaves and branched root; bar = 2 cm; **d** Tansy shoots grown on solid medium; **e** Erlenmeyer with tansy roots grown in liquid medium

their weight 3 times (from an initial 400 mg to 1.2 ± 0.03 g average). After 7 days of air-drying period, the roots' weight was decreased with average FW/DW index = 6.02 ± 0.05 .

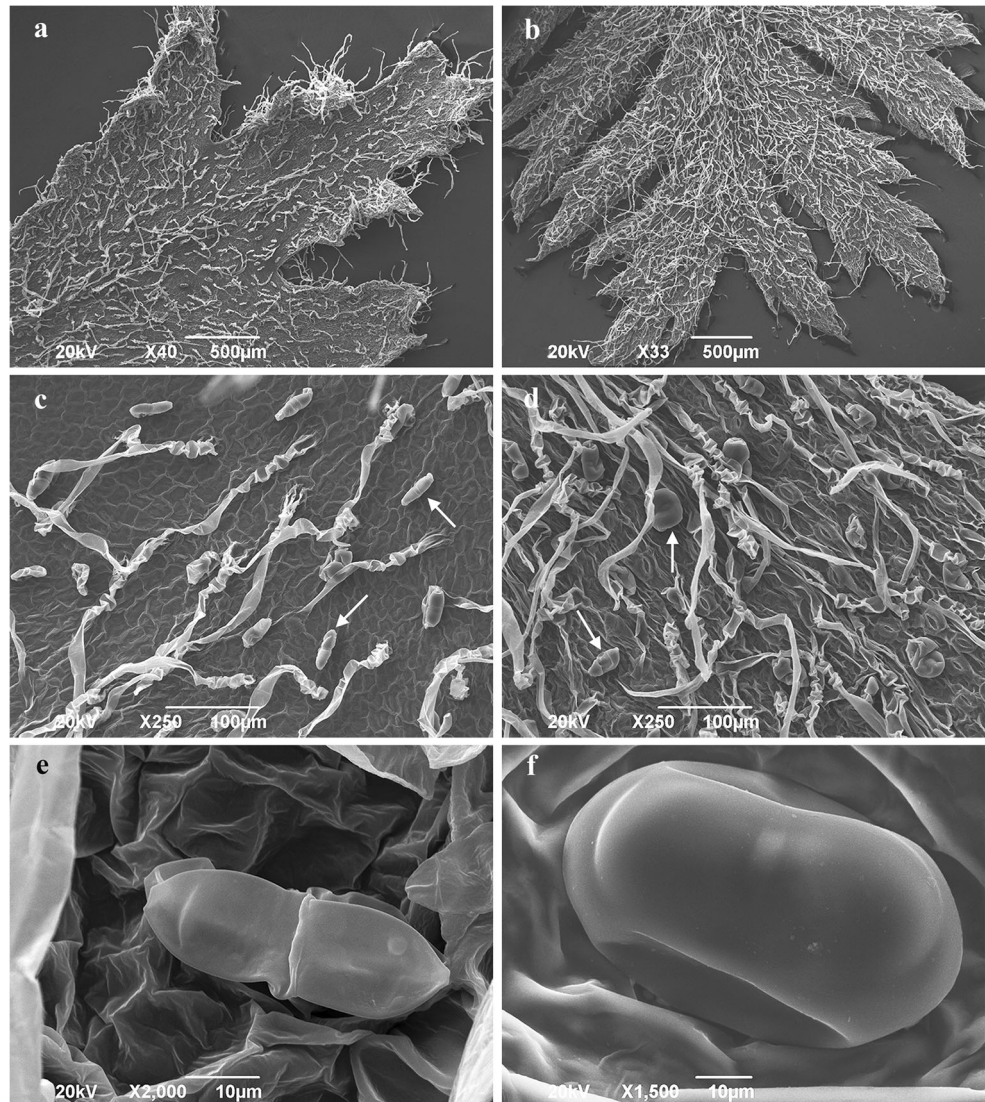
Morpho-anatomical and histochemical characteristics of leaves from tansy grown in vitro

Both adaxial and abaxial surfaces of leaf indumentum consisted of non-glandular and glandular trichomes. Long, uniseriate non-glandular trichomes were more frequent on the abaxial leaf surface (Fig. 2a–d). The biseriata glandular trichomes were observed in tansy grown in vitro (Fig. 2e, f). The surface of immature biseriata glands appears wrinkled, indicative of the close attachment of the cuticle to the secretory upper cell walls (Fig. 2e). During maturation, the gland surface became smoother as the secretory products accumulated within the developing subcuticular space formed by a detachment of the cuticle (Fig. 2f).

The anatomical investigation of leaf cross-sections by light microscopy revealed a isobilateral leaf type with an upper and lower epidermis with stomata cells, palisade, and spongy mesophyll (Fig. 3a, b). The epidermis consisted of a single layer of cells and was covered with a thin cuticle layer. Photosynthetic tissue was represented as 1–2 layers of palisade tissue cells and 2–3 layers of loosely, spongy mesophyll tissue with closed, collateral vascular bundles (Fig. 3a). Biseriate glandular trichomes were in various stages of development and consisted of two basal cells placed in the epidermis, one pair of cells forming a short stalk and three to four pairs of cells forming the secretory head (Fig. 3b). The cuticle layer over the secretory head expanded and formed the subcuticular space.

To detect and localize the lipids, terpenoids, phenols, tannins, polysaccharides, pectins, and alkaloids in glandular trichomes, tansy leaves grown in vitro were treated with specific histochemical staining tests (Table 1).

Fig. 2 Scanning electron micrographs of in vitro grown tansy foliar surface. **a** Adaxial leaf surface; **b** abaxial leaf surface; **c** glandular (arrow) and non-glandular trichomes on the adaxial leaf surface; **d** glandular (arrow) and non-glandular trichomes on the abaxial leaf surface; **e** biseriata glandular trichome at the beginning of the secretory phase; **f** mature biseriata glandular trichome on the adaxial leaf surface in the full secretory phase



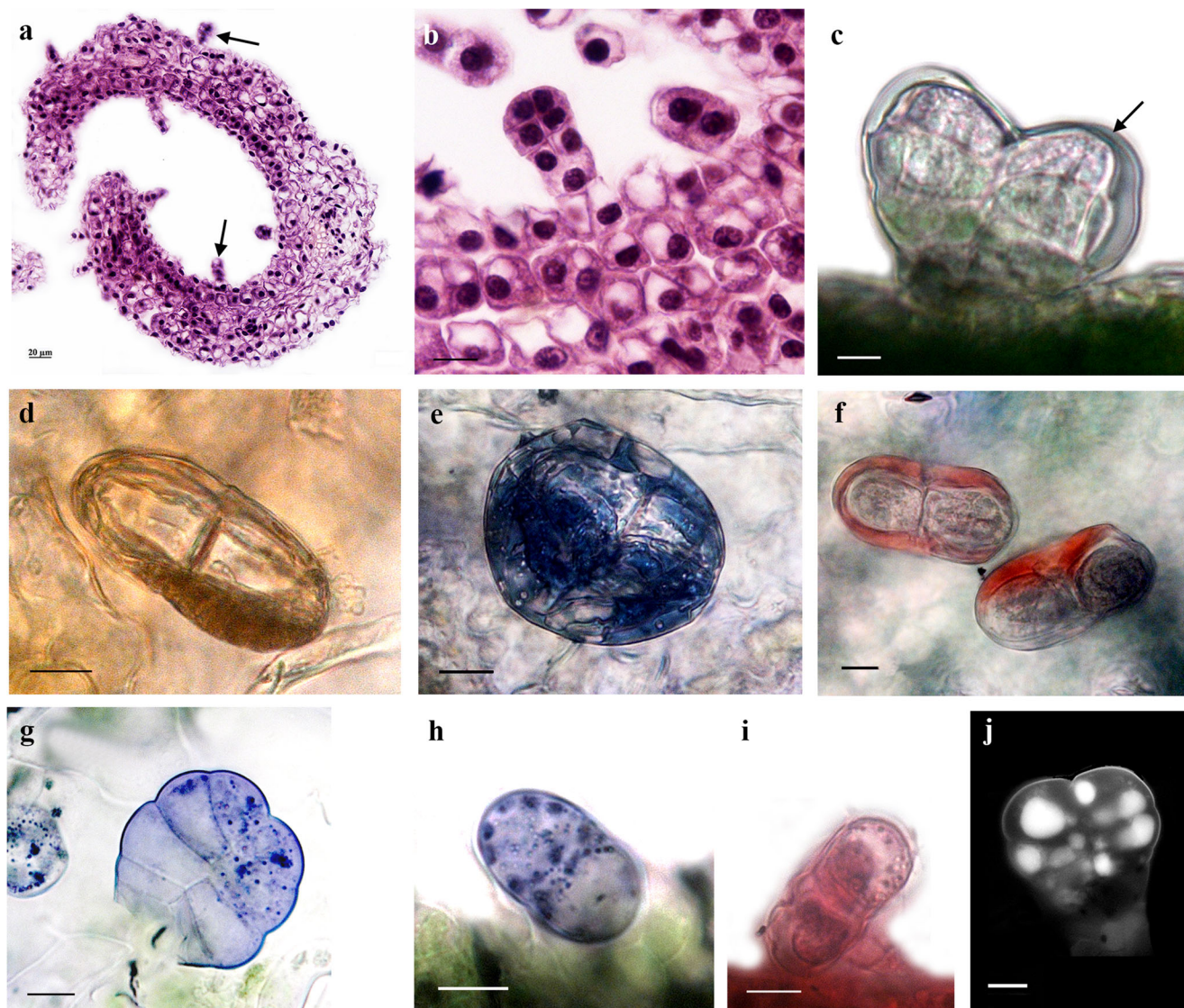


Fig. 3 Structural and histochemical features of leaf glandular trichomes from *in vitro* grown tansy. **a** Cross-section of tansy leaf, note: trichomes (arrow) on adaxial and abaxial leaf surface; **b** young, immature leaf glandular trichomes, note: two basal cells, a short stalk, and secretory head of three pairs of cells; **c** unstained biseriolate trichome with subcuticular space (arrow); **d** orange-brown colored of secretory material in subcuticular

space after stained with Sudan Red 7B/hematoxylin; **e** dark-blue color of lipophilic substance after staining with Sudan black B; **f** neutral lipids/essential oils stained blue with Nil blue A; **g–h** positive reaction with NADI reagent, violet-blue droplets indicate terpene secretion; **i** positive reaction with PAS; **j** UV-autofluorescence micrographs of leaf glandular trichomes. Bar = 10 μ m

On the *in vivo* leaf surface, biseriolate trichomes with secretions in the subcuticular space were observed (Fig. 3c). The histochemical staining showed that the secretion contained lipophilic substances (Fig. 3d, e). In the glandular secretions localized in the subcuticular space, neutral lipids/essential oils were also detected (Fig. 3f). The terpenoids, represented as dark violet droplets, referred to as essential oils, were localized in the secretory cells and subcuticular space (Fig. 3g, h).

Polysaccharides in the secretory cells and subcuticular space were stained pink-red (Fig. 3i). This reaction was positive with insoluble polysaccharides of non-glandular trichomes and epidermal cell polysaccharides. Drops of secretion localized in the

secretory cells of the glandular head emitted intense autofluorescence under UV excitation (Fig. 3j).

Essential oil of *in vitro* plantlets

Results of the GC-MS analysis of the essential oil are presented in Table 2. The essential oil of *in vitro* tansy plantlets was composed of 88 volatile compounds of which 42 were identified, representing 72.3% of the total oil. Only four compounds were made up by more than 5% of the total oil and 14 volatiles were present only in traces (< 0.1%). The monoterpenes were the most abundant compounds (40%), followed

Table 1 Histochemical analyses perform to identify the main metabolites secreted by leaf biseriate glandular trichomes of in vitro grown tansy

Metabolite	Reagent	Reaction	Color
Lipids			
<i>Total</i>	Sudan Black B	+	Orange-brown
	Sudan IV	-	-
	Sudan Red 7B/hematoxylin	+	Blue
<i>Neutral and acidic</i>	Nil Blue A	+	Red
Terpenoids (<i>essential oils and oleoresins</i>)	NADI reagent	+	Violet-blue
Polysaccharides	Periodic acid-Schiff (PAS)	+	Pink-red
Pectins	Ruthenium Red	+	Red
Alkaloids	Wagner reagent	-	-
	Dragendorff reagent	-	-
	Ellram reagent	-	-
Polyphenols	Ferric chloride	-	-
Lignins and tannins	Toluidine Blue O	-	-

by sesquiterpenes (33.4%). All identified compounds belonging to six chemical groups: oxygenated monoterpenes (38.5%), monoterpene hydrocarbons (< 0.7%), oxygenated sesquiterpenes (22.6%), sesquiterpene hydrocarbons (10.6%), aromatic hydrocarbons (0.2%), and aromatic alcohols (< 0.2%). The oxygenated monoterpene trans-thujone (22.7%) and oxygenated sesquiterpene neryl-isovalerate (20.6%) were the dominant compounds in the oil.

Methanol extract compounds

The HPLC chromatograms recorded at 280 nm revealed differences between the chemical compositions of methanol extracts derived from native tansy and from tansy cultured in vitro (Fig. 4). Based on characteristic UV spectra, flavonoids and phenolic acids were detected, while chlorogenic acid (CGA) and 3,5-*O*-dicaffeoylquinic acid (3,5-*O*-DCQA) were identified in all extracts tested. The highest content of CGA and 3,5-*O*-DCQA was recorded in the methanol extract of roots grown in vitro (Fig. 4d). The amount of 3,5-*O*-DCQA was up to 6 times higher in the methanol extract of in vitro roots (Fig. 4d; 10.220 mg/g DW) compared with methanol extract of native tansy roots (Fig. 4c; 1.684 mg/g DW). The methanol extracts of tansy aerial parts from native (Fig. 4a) and in vitro plants (Fig. 4b) were characterized by relatively similar amounts of the acid (2.274 mg/g DW and 3.579 mg/g DW, respectively).

Discussion

Plant micropropagation is proposed to be the most promising system for the efficient production of useful secondary metabolites under controlled cultivation conditions. In vitro culture allows rapid production of genetically identical pathogen- and

contaminant-free plants through clonal propagation, using relatively small amounts of space, supplies, and time. In this study, tansy *in vitro* culture was established from seeds collected from natural populations, whose composition of secondary metabolites in essential oils and methanol extracts were determined in our previous study (Devrnja et al. 2017). Successful multiplication of plantlets was conducted through shoot tips that exhibited potent apical growth and regeneration capacities on BM without the addition of any PGRs. It has been previously reported that exogenously applied PGRs, especially from the cytokinin and auxin groups, may impair genetic stability and lead to somaclonal variation (Rani and Raina 2000; Bairu et al. 2007), which is considered to be undesirable in the production of plant material via in vitro culture, especially if it will be used for secondary metabolite isolation (Bairu et al. 2007; Passinho-Soares et al. 2017). Moreover, for the establishment and propagation of tansy in vitro root cultures, PGR supplementation was also unnecessary, and roots increased their mass 3 times in 4 weeks in PGR-free liquid BM. For roots detached from the spontaneously rooted micropropagated shoots and further in vitro maintenance, auxins were considered a necessary treatment. Roots that were excised and propagated in PGR-free medium, often turned brown and died soon after being removed from the “mother” plant (Stanišić et al. 2019).

Light and SEM microscopy of the leaf surface of in vitro micropropagated tansy plants revealed the presence of glandular biseriate trichomes randomly distributed on both adaxial and abaxial leaf surfaces. These were surrounded by long non-glandular ones, which are known to be typical for all species belonging to the *Asteraceae* family (Ciccarelli et al. 2007). Glandular trichomes were also present on the tansy stems. In many cases, glandular trichomes were abundant on both in vitro gentle leaf surfaces, while in aged ex vitro leaves, these structures were only present on the abaxial surface

Table 2 GC-MS analysis of in vitro grown tansy plantlets

No.	Compound	KI ^e	RRT	%m/m	No.	Compound	KI ^e	RRT	%m/m
1	Tricyclen	919	0.456	tr.	45	Spathulenol	1577	2.651	0.3
2	α -Tujene	921	0.46	tr.	46	Neryl-isovalerate	1585	2.676	20.6
3	α -Pinene	932	0.481	tr.	47	n.i.	1589	2.69	0.3
4	Camphene	946	0.514	0.1	48	Salvial-4(14)-en-1-on	1593	2.706	0.3
5	Sabinene	971	0.571	0.4	49	n.i.	1597	2.718	0.1
6	β -Pinene	974	0.58	tr.	50	n.i.	1603	2.737	0.1
7	Dehydro-1,8 cineole	986	0.614	tr.	51	n.i.	1605	2.746	0.1
8	α -Terpinene	1010	0.686	tr.	52	n.i.	1609	2.755	0.5
9	p-Cimene	1023	0.706	0.2	53	n.i.	1613	2.771	0.2
10	1,8 Cineole	1029	0.725	0.5	54	n.i.	1618	2.788	0.1
11	γ -Terpinene	1057	0.809	0.1	55	n.i.	1830	2.824	0.6
12	Cis-sabinene hydrate	1065	0.834	0.1	56	n.i.	1634	2.839	0.4
13	Terpinolene	1086	0.905	tr.	57	Gossonorol	1638	2.849	0.2
14	n.i.	1099	0.935	0.1	58	n.i.	1655	2.902	0.7
15	Cis-thujone	1106	0.96	0.2	59	n.i.	1658	2.912	0.1
16	Trans-thujone	1117	1	22.7	60	n.i.	1659	2.928	0.2
17	n.i.	1133	1.059	0.1	61	n.i.	1667	2.939	0.1
18	Trans-pinocarveol	1135	1.075	0.1	62	n.i.	1672	2.959	0.2
19	Camphor	1142	1.093	10.1	63	n.i.	1675	2.967	0.1
20	n.i.	1154	1.133	0.1	64	n.i.	1680	2.982	0.1
21	Cis-chrysanthenol	1160	1.157	0.5	65	n.i.	1686	3.004	0.2
22	Borneol	1163	1.167	1	66	n.i.	1689	3.013	0.7
23	Terpinen 4-ol	1175	1.21	0.5	67	n.i.	1714	3.09	0.1
24	n.i.	1183	1.238	tr.	68	n.i.	1716	3.098	tr.
25	α -Terpineol	1088	1.26	0.1	69	n.i.	1719	3.105	tr.
26	n.i.	1108	1.33	0.1	70	n.i.	1721	3.112	tr.
27	Bornyl acetate	1284	1.61	0.3	71	n.i.	1724	3.121	tr.
28	Neryl acetate	1364	1.901	2.1	72	n.i.	1730	3.137	0.3
29	n.i.	1375	1.942	0.1	73	n.i.	1733	3.147	tr.
30	Modheph-2-ene	1379	1.956	0.1	74	n.i.	1751	3.202	0.1
31	α -Isocomene	1385	1.981	0.4	75	n.i.	1753	3.209	0.1
32	β -Isocomene	1405	2.051	0.2	76	n.i.	1768	3.251	4.2
33	Caryophyllene	1419	2.1	0.4	77	n.i.	1776	3.279	0.2
34	α -Humulene	1453	2.221	0.1	78	n.i.	1781	3.299	0.2
35	β -Pharnesen	1457	2.234	1.6	79	n.i.	1791	3.326	0.1
36	Amorpha 4,7-dien	1460	2.245	0.1	80	n.i.	1825	3.423	0.1
37	cis-muurola-4(14)5-diene	1463	2.255	0.1	81	2-Pentadecanone 6,10,14-trimethyl	1845	3.48	0.7
38	n.i.	1476	2.299	tr.	82	n.i.	1901	3.65	0.9
39	Germacrened	1481	2.32	1.9	83	n.i.	1920	3.694	0.4
40	γ -Chimachalen	1484	2.328	1.5	84	Methyl hexanoate	1927	3.713	0.1
41	E- β -Ionon	1487	2.328	0.5	85	n.i.	1953	3.788	0.9
42	Bicyclogermacrene	1497	2.373	0.1	86	n.i.	1974	3.849	1.6
43	γ -Z-Bisabolene	1517	2.441	0.1	87	n.i.	1997	3.921	10
44	β -Sesquiphellandrene	1525	2.469	4.2	88	n.i.	2028	3.989	0.4
Number of detected compounds				88	KI ^e - Kovats index experimentally determined				
Number of identified compounds				42	RRT-relative retention time				
Identified compounds belonging to				(%)	CI-concentration index				
Oxygenated monoterpenes				38.5	% m/m-percentage relative to total EO composition				
Monoterpene hydrocarbons				< 0.7	n.i.-non-identified				
Oxygenated sesquiterpenes				22.6	tr.-present in traces (< 0.1%)				
Sesquiterpene hydrocarbons				10.6					
Aromatic hydrocarbons				0.2					

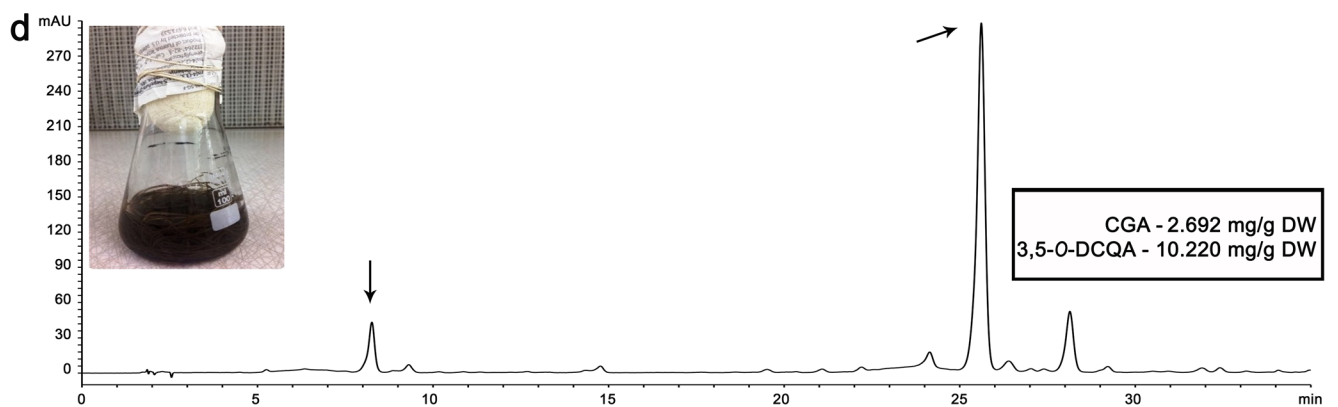
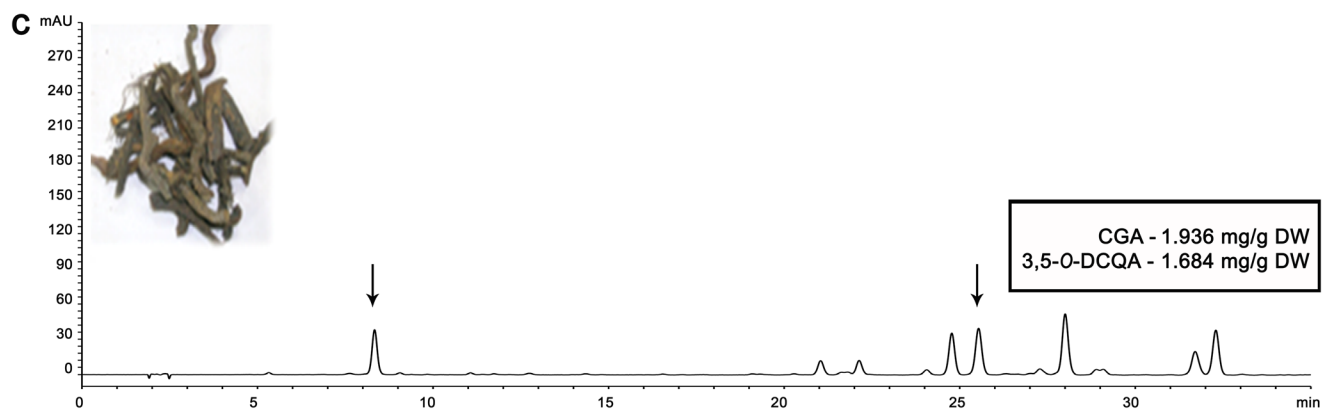
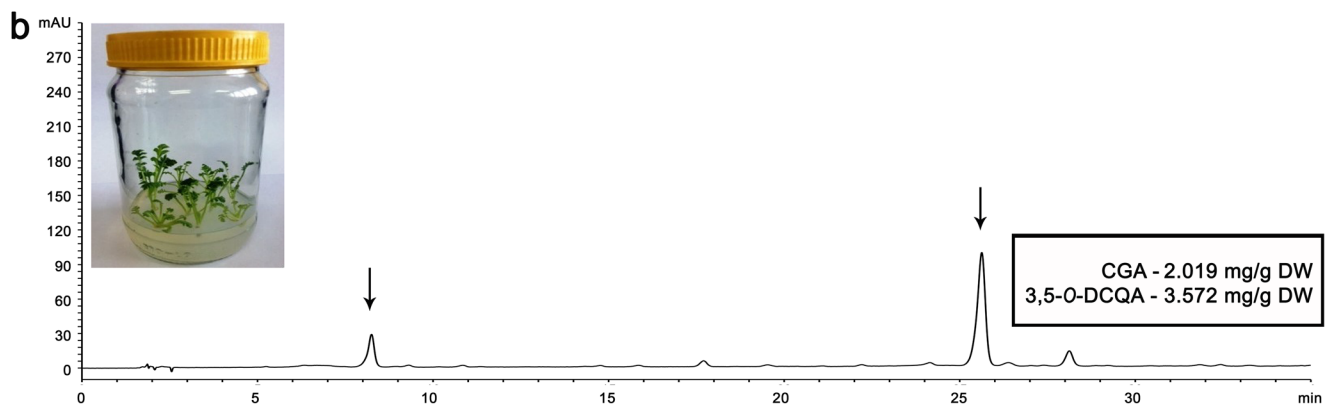
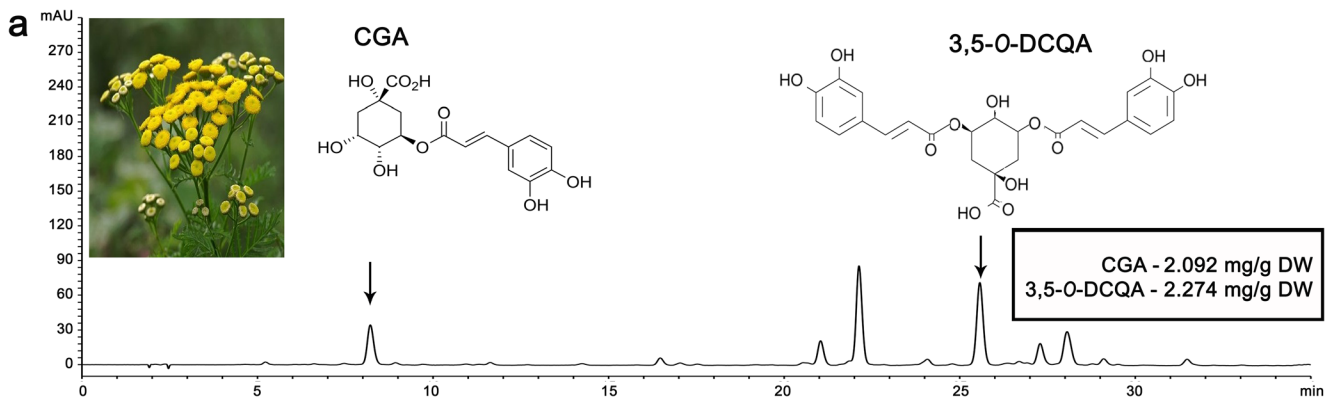
(Bandyopadhyay et al. 2004). Frequent in vitro sub-culturing practices in tansy, which occur every 4 weeks, could be responsible for retarding plantlets in the juvenile stage and prevent trichome distribution related to the maturation process.

The presence of storage glands is intrinsically associated with terpenoid metabolism (Maffei 2010), which was confirmed by histochemical analysis of in vitro tansy leaves. Glandular secretions were rich in lipid and terpene compounds, which was also confirmed by GC-MS analysis of EO. Tansy biseriate trichomes contained a storage subcuticular space in which volatile compounds were retained in their liquid state, like in other representatives of the *Asteraceae* family (Turner et al. 2000). The morphology of these trichomes is adapted for the fast release of volatile defense compounds upon herbivory attack or other mechanical damage. During the first decades of the twenty-first century, special attention was given to the investigation of site-specific regulation of synthesis machinery for plant secondary metabolites (Lange et al. 2011; Tissier 2012a; Sultana et al. 2015). Impressive progress has been made in the identification of promoters driving trichome-specific gene expression, which could be used to control trichome density and, consequently, to improve the productivity of EOs or even some particular constituents (Tissier 2012b). The recorded presence of numerous, highly metabolically active biseriate trichomes on the surface of in vitro grown tansy could be the starting point for the utilization of obtained knowledge.

A review of the available literature found no data referring to phytochemical analyses of EOs from in vitro tansy cultures. Essential oil constituents of native tansy, of which the seeds were taken for the establishment of in vitro cultures presented in this study, were predominantly oxygenated monoterpenes, with *trans*-chysantenyl acetate as a dominant compound (Devrnja et al. 2017). Interestingly, in vitro cultivation significantly affected EO composition that may be favored by the high chemical plasticity reported for tansy (Lawrence 2000). Like in some other reports (Arumugam et al. 2020), in EO obtained from in vitro cultivated plants, a significantly greater number of chemical compounds was detected, as compared to that of wild-grown tansy (88 vs. 65, respectively). In vitro cultivation conditions also enhanced the accumulation of sesquiterpenes with respect to EO from field-grown “mother” plants, with a similar portion of identified monoterpene and sesquiterpene compounds. Although EO chemistry is genotype determined, the plasticity of secondary metabolism allows plants to alter their defenses in response to environmental and/or physiological-stress circumstances (Usano-Alemany et al. 2016; Kirimer et al. 2017). Strikingly, the dominant sesquiterpene from in vitro tansy, neryl-isovalerate, present with 20.6% in total EO, was not detected in native tansy plants. Neryl-isovalerate is a registered food flavoring agent and adjuvant (Joint FAO/WHO Expert Committee 1997; EU Commission 2012). Additionally, the dominant compounds in the EO of

in vitro tansy, *trans*-thujone and camphor, were identified in a much higher portion (22.7% and 10.1%, respectively) than in the oil of native tansy (9.04% and 4.9%, respectively). At this point, the observed differences in EO metabolite production in tansy trichomes affected by cultivation practice are hard to explain. Several authors have reported that these quantity-quality variations induced by in vitro culture conditions could be accounted for by the requirement of some metabolites for a specified environmental factor, such as light or temperature, for highly differentiated in vitro cells/tissues or for cells/tissues in a specified stage of development (Dörmenberg and Knorr 1997; Avato et al. 2005; Batista et al. 2016; Passinho-Soares et al. 2017). Variation in EO composition could also be the consequence of different ontological stages, with in vitro plants being in the juvenile-stage by definition and mature native tansy plants in the flowering stage. The elevated prevalence of camphor in micropropagated plants, compared to field-grown plants, was also observed in *Salvia officinalis* (Avato et al. 2005), which was explained by the correlation of camphor levels with juvenile stages of development of micropropagated plants relying on earlier works by Croteau et al. (1981, 1987), who have shown that the presence of camphor in the oil decreases with the age of the plant. Although the specific growing conditions could alter the chemotype of “mother” plants, in vitro cultivation could offer a supply of constant EO, unrelated to environmental conditions, time of the year, and presence of unwanted biotic interactions. Biotechnological interventions on secondary metabolites production taking place in trichomes could further enhance the production of some important metabolites in tansy EO. Guided by a similar goal, Sultana et al. (2015) used the *Tanacetum cinerariifolium* promoter of chrysanthemol synthase (TcCHS), coding for the first enzyme in the pyrethrum biosynthesis pathway, fused to a GFP (Green Fluorescent Protein) and GUS (β -glucuronidase) reporter genes, to transform *Chrysanthemum morifolium* and *Nicotiana tabacum* plants. The accumulation of pyrethrum was detected only in glandular trichomes of both species.

Quantity and quality variations were reflected also in the chemical profile of methanol extracts of in vitro cultured tansy herbs and roots compared to wild-grown tansy. In both in vitro and native plant material, extracts were rich in phenolics and flavonoids. The strong antioxidant, antimicrobial, and anti-cancer potential of tansy extracts obtained from wild-grown plants were attributed to these compounds (Devrnja et al. 2017). The most striking differences were observed for 3,5-*O*-DCQA, one of the dicaffeoylquinic acid derivatives with two caffeic acid moieties substituted at the 3 and 5 positions of the quinic acid. Results indicated that the methanol extract of in vitro cultured roots was the richest in 3,5-*O*-DCQA content, whose concentration was 6 times higher than that in the extract obtained from roots of wild-growing tansy. This is a noticeable result in the manner of industrial production of biologically active 3,5-*O*-DCQA, which has been shown to have



◀ **Fig. 4** Chromatograms of tansy methanol extracts. Methanol extract of a native tansy herb; **b** in vitro grown tansy herb; **c** native tansy roots; **d** in vitro grown tansy roots. The arrows point to chlorogenic acid (CGA) and 3,5-*O*-dicaffeoylquinic acid (3,5-*O*-DCQA) peaks with given amounts of targeting compounds as mg per g of dry weight (DW) for each type of extracts

antioxidant, hepatoprotective, antimutagenic, and immunomodulatory activity (Peluso et al. 1995; Tatefuji et al. 1996; Basnet et al. 1996; Yoshimoto et al. 2002; Juan-Badaturuge et al. 2009; Kim et al. 2012, 2017). It has also been reported that 3,5-*O*-DCQA improves pancreatic function in type 2 diabetic mice (Yin et al. 2018) and is a potent inhibitor of HIV-1 integrase, an enzyme that is essential for viral replication and subsequent HIV infection of humans (Tamura et al. 2006).

Conclusions

The establishment of an efficient and inexpensive method for *in vitro* multiplication of tansy is a promising starting point for the introduction of high-capacity bioreactors and mass micropropagation of shoots and roots for stable secondary metabolites production. In addition, well-developed protocols open up new directions for research that could affect secondary metabolite production by the manipulation of chemical and physical cultivation conditions, application of precursors and/or elicitors, or genetic transformation that would affect the expression of genes from the biosynthetic pathway of the target compound. The significance of these results is even higher since studies related to the *in vitro* root cultures or leaf secretory structures of *Tanacetum vulgare* are not available to our knowledge. EO and methanol extract obtained from *in vitro* grown tansy qualitatively and quantitatively differed from the previously analyzed wild-grown plants, being the source of new mixtures shown to be rich in biologically active compounds. Further investigation will be directed toward the optimization of *in vitro* cultivation conditions, including elicitation, for obtaining maximum production of tansy secondary metabolites and the elucidation of their pharmaceutical potential, as mixtures or single moieties.

Author statement Nina Devrnja collected samples and data, carried out the experiments, performed data analyses, and prepared the original draft. Dijana Krstić Milošević and Vele Tešević performed chemical analyses of the samples. Dušica Janošević performed histochemical analyses. Branka Vinterhalter contributed in *in vitro* culturing. Jelena Savić contributed in designing the experiment and critically reviewed the manuscript. Dušica Čalić designed the experiment.

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Compliance with ethical standards

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

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