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Variation in the volatile constituents of wild and *in vitro* propagated *Tanacetum sinaicum* Del. ex DC through GC-MS chemical fingerprint

Rasha Adel*, Afaf E Abdel-Ghani, Doaa D Abouelenein and Samih I El-Dahmy
Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, 44519 Zagazig, Egypt

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Tanacetum sinaicum (Asteraceae) is a rare perennial herb growing wild in the mountains of Southern Sinai (Egypt). It is a medicinal and endangered plant. So, this work aimed to develop an *in vitro* propagation method for the conservation of this highly threatened plant. Sterile seedlings were used as a source of explants which were cultivated on Murashige and Skoog (M&S) media supplemented with different combinations of growth regulators for callus formation and induction of shoots and roots. M&S media with 1 mg/L kinetin (Kn) showed direct shoot induction. For root induction, excised shoots were transferred to M&S medium supplemented with 1 mg/L naphthalene acetic acid (NAA). Moreover, *n*-hexane extracts of wild and *in vitro* propagated plants were analyzed for their volatile constituents by gas chromatography-mass spectrometry (GC-MS) which resulted in the identification of 38 and 27 constituents, accounting for 87.75 and 75.51 % of their total composition; respectively.

Keywords: GC-MS profile, Micropropagation, n-Hexane extract, *Tanacetum sinaicum*, Tissue culture.
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Introduction

Tanacetum L. is the third largest genus of the tribe Anthemideae in the Asteraceae family. It includes about 160 species distributed in Asia, Europe, northern Africa and North America[1]. In the flora of Egypt, the genus *Tanacetum* is represented by a monotypic species; *Tanacetum sinaicum*[2]. It is a shrubby, fragrant, perennial herb growing wild in rocky mountains of Southern Sinai with discoid, yellow heads having only tubular florets and bipinnatifid woolly leaves[3]. Traditional uses of this plant include treatment of fevers, migraines, stomach ailments, bronchitis and arthritis[4]. Also, many pharmacological studies on the essential oils, extracts, and some chemical constituents of this plant showed that it possesses anti-inflammatory, antimicrobial, antibacterial, pesticide, antiviral and cytotoxic activities[4-10]. This plant is rich in important pharmacologically active secondary metabolites such as terpenoids[4,11-15] and flavonoids[16-17]. Moreover, Previous studies have addressed the chemical composition of the essential oil and the *n*-Hexane extract of *T. sinaicum*[6,7,18].

Rapid urbanization, degradation of plant habitats, ruthless collection of herbs, pollution, and other anthropogenic activities reduced the diversity of medicinal plants in the ecosystem[19]. The development of plant tissue culture technology holds great promise for the conservation and enhancement of valuable medicinal plants[20]. As a result, efforts have been devoted to enhancing the *in vitro* propagation of highly threatened valuable medicinal plants such as *T. sinaicum*.

This study aimed to optimize a protocol for the establishment of *in vitro*, micropropagation, plants in addition to providing a comparison between the volatile constituents of the wild and the *in vitro* propagated plants.

Materials and Methods

Plant material

The aerial parts of *T. sinaicum* Del. ex DC [Synonyms: *Pyrethrum santolinoides* (DC.), *Tanacetum santolinoides* DC., *Chrysanthemum sinaicum* Del.] were collected from rocky mountains of Wadi Elarbaeen, St. Catherine, Sinai Peninsula, Egypt in May 2014. The identity of the plant was confirmed by Prof. Dr Azza El-Hadidy (The herbarium, Department of Botany, Faculty of Science, Cairo University). A voucher specimen (ATS-17) is deposited in the herbarium of the Department of Pharmacognosy, Zagazig University, Egypt.

*Correspondent author
Email: rashaadelelfeqy@gmail.com

Preparation of culture media

To prepare one liter of the Murashige and Skoog (M&S) media (Duchefa, Germany), 600 mL of distilled water, 30 g of sucrose (Adwic, ARE) is added, stirred till dissolved, then 4.4 g of the readymade M&S media were added and stirred till dissolved. The specified amount of phytohormones (Sigma-Aldrich, USA) and other additives were added and then the volume of the media was completed to 950 mL using distilled water. The pH of the media was adjusted at 5.7- 5.8 by a pH meter, using drops of 10% HCl or 1 M NaOH. The prepared media were heated (not boiled) prior to the addition of the purified agar 0.8% (8 g/L) (Bioworld, USA). The media were autoclaved at 394 °K for 15 minutes, then poured into glass jars (50-60 mL per jar), left to cool, then caped with autoclaved plastic caps. The jars were left for 2-3days at room temperature, to ensure the efficiency of the sterilization process[21].

Sterilization and germination of seeds

Fruits containing seeds were sterilized by washing with 70% ethanol for 2 minutes followed by shaking with 5% commercial hypochlorite solution (Clorox®) for 5 minutes. The fruits were rinsed three times with sterile distilled water before culturing on the media. The pH was adjusted at 5.6-5.8 and the jars were kept in the dark at 298 °K. Germination of sterile seeds on 7 different media was evaluated using seed germination percentage and seedling length after 6 weeks of cultivation. Seed germination percentage was calculated using the following formula:

Seed germination percentage =

$$\frac{\text{Number of germinated seeds}}{\text{Total number of seeds cultured}} \times 100$$

Callus induction and maintenance

Induction of callus from in vitro germinated seedlings

Uniformly sized explants (0.5-1 cm) were dissected from the 4-6 weeks old seedlings under sterile conditions. Explants were cultured in jars containing M&S media supplemented with different hormonal combinations; Medium I: 2, 4 dichlorophenoxyacetic acid (2, 4 D) (0.5 mg/L), Medium II: 2, 4 D (1 mg/L), Medium III: 2, 4 D (1mg/L) + Kinetin (Kn) (0.5 mg/L), Medium IV: Kn (1 mg/L), Medium V: Naphthalene acetic acid (NAA) (0.5 mg/L) + Benzyl amino purine (BAP) (1 mg/L), Medium VI: NAA (1 mg/L) + BAP (1 mg/L), Medium VII: NAA (1 mg/L), Medium VIII: BAP (1 mg/L), Medium IX: Indole acetic acid (IAA) (2 mg/L) + Kn (1 mg/L), Medium X: IAA (1 mg/L) + Kn (2 mg/L) and Medium XI: Thidiazuron (TDZ) (0.5 mg/L) + 2, 4 D (1 mg/L) + BAP (0.1 mg/L). All media were supplemented with 3% sucrose 0.8% agar and the cultures were incubated at 298 °K under white fluorescent lamp with light intensity (1000 µ mol m^{-2} s^{-1}), 16/8 hours light/dark periods for 6 weeks, the callus capacity, days for callus initiation and callus dimensions were estimated for each treatment to select the most appropriate media for further investigations. Callus induction percentage (callusing capacity) was calculated using the following formula:

Callus induction percentage =

$$\frac{\text{No of explants produced callus}}{\text{Total No. of explants cultured}} \times 100$$

Effect of some hormonal combinations on callus growth

Media exerted the greatest callusing capacity with high vegetative growth were chosen for further investigations. Growth curves were carried out as subcultures were made by cutting the calli produced in each case to one-gram pieces and transferring them on a fresh medium of the same composition. Fresh weight was measured every 5-7 days for 42 days and the mean values of three readings were plotted against time. Additionally, growth dynamics in callus cultures were calculated as follow:

Growth index (GI) = (Ge – G start)/G start

where Ge is the weight of biomass at the end of generation (final weight); G start is the weight of biomass at zero time (initial weight)[22].

Specific growth rate (µ)= (ln *x* – ln *x*o)/t

where xo is the initial biomass and x is the biomass at time t (21 days)[23].

Doubling time (dt) = ln (2)/µ

dt is the time required for the biomass of a population of cells to double[23].

Somatic embryogenesis

Six weeks old calli with pre-embryogenic masses were selected from those grown on medium V: NAA (0.5 mg/L) + BAP (1 mg/L), and medium XI: TDZ (0.5 mg/L) + 2, 4 D (1 mg/L) + BAP (0.1 mg/L). They were subcultured on the same media as well as M&S hormonal free media with agar (6 g/L) and sucrose (30 g/L). The pH was adjusted to 5.6 - 5.8, the cultures were incubated at 296-298 K under a white fluorescent lamp with a 16 hours photoperiod.

Regeneration of *T. sinaicum* via organogenesis

Direct organogenesis

Explants on **medium IV**: (Kn 1mg/L), showed shoot buds. The achieved shoots were cut and transferred individually to jars containing rooting media which consists of M&S with 30 g/L sucrose, 6 g/L agar and 1 mg/L NAA.

Indirect organogenesis

Both **medium VI**: NAA (1 mg/L) + BAP (1 mg/L) and **medium X**: IAA (1 mg/L) + Kn (2 mg/L) induced calli with numerous shoots. The achieved shoots were cut and transferred individually to jars containing rooting media which consists of MS with 30 g/L sucrose, 6 g/L agar and 1 mg/L NAA.

Acclimatization of the *in vitro* regenerated plants

The *in vitro* regenerated plants (two months old) were washed to be free from media then transferred into pots containing sand: soil (1:1), covered with transparent perforated plastic bags which were removed one week later. Pots were maintained in the greenhouse.

GC-MS analysis

Exactly 10 g of dried wild and micropropagated plants were separately macerated in *n*-hexane, kept on a shaker overnight. After filtration, the hexane was evaporated at a low temperature. The GC-MS analysis was carried out at the Central Agricultural Pesticide Laboratory (CAPL), Cairo, Egypt; on Agilent 6890 gas chromatograph with fused silica capillary column PAS-5 ms (30 mm × 0.25 um film thickness). The carrier gas was Helium with 1 mL/ min. flow rate. The sample injection size was 1 µL. The oven temperature program started at 318 ^{o}K for 2 minutes, then elevated to 553 ^{o}K at the rate of 8 degrees/min and kept isothermal at 553 ^{o}K for 2 minutes. The injector temperature was adjusted at 523 ^{o}K while the detector temperature was at 553 ^{o}K. Mass spectrometry detector was used, scanning from m/z 50 to 500, EI 70 eV. A mixture of standard *n*-alkane (C6–C24) diluted in hexane was injected under the previously mentioned condition to calculate the retention indices (as Kovats indices) of each detected compound. Identification of the compounds was based on the comparison of their retention indices (RI) with those reported by Adams (2007), NIST 08 libraries and literature in addition to, comparing of their EI–mass spectra with the NIST 05 (National Institute of Standards and Technology) and Wiley library spectra. The relative percentages of the individual components were calculated based on GC peak area.

Results and Discussion

T. sinaicum (Asteraceae) is a valuable, rare plant, suffering from the threat of extinction. This study aimed to investigate, for the first time, the tendency of the seeds to germinate *in vitro* and the ability of the explants taken from the growing seedlings to form stable calli that represent the first step of micropropagation. Moreover, the ability of the produced calli to differentiate into organs or to produce somatic embryos was also investigated. The volatile constituent of the hexane extract of both wild and the *in vitro* propagated plants were analyzed by GC-MS to show the differences between their constituents.

***In vitro* germination of seeds**

Germination percentage and seedling length on seven different media were calculated as shown in (Table 1). M&S hormone-free and M&S supplemented with 50 mg/L GA3 media showed the highest germination percentage. No improvement in seed germination with an application of GA3 was evidenced, but a concentration beyond optimum dose caused a reduction in germination percentage. This result was in concurrence with the findings of a previous study[24]. On the other hand, adding phytohormones to the media as 2,4D and Kn decreased germination percentages and suppressed seedling growth.

Table 1 — Germination percentage and seedling length after 6 weeks of cultivation of *T. sinaicum* sterile fruits using different media composition

Media composition	Germination percentage (%)	Seedling length ranges (cm) after 6 weeks
Distilled water	53.33	2.5-4
Murashige and Skoog media (MS) (solid media)	73.33	7-8
Murashige and Skoog media (MS) (liquid media)	46.67	5-5.5
MS with 50 mg/L Gibberellic acid (GA3)	73.33	7.5-8.5
MS with 100 mg/L Gibberellic acid (GA3)	53.00	7.0-8.0
MS with 1 mg/L 2, 4 Dichlorophenoxyacetic acid	53.33	0.7–1.0
MS with 1 mg/L Kinetin	53.33	1.0–1.5

Callus induction and maintenance

Induction of callus from in vitro germinated seedlings

A rough evaluation of the calli growth and their morphological characters after 6 weeks of cultivation on 11 different media were established. Results were recorded in (Table 2), represented in (Fig. 1).

Effect of some hormonal combinations on callus growth

As shown in (Table 3) medium XI followed by medium VI showed the best callus growth rates compared with other media. The graph of medium XI and medium VI (Fig. 1) showed a lag phase of seven days during which, a small increase in fresh weight was observed. The lag phase is considered an energy production period[25]. The lag phase then was followed by an exponential phase, through which the greatest growth rates of the calli were achieved. Callus grown on medium VIII was very healthy and had a good appearance, but exhibited a very slow growth, and represented by a sigmoidal growth curve with lag, exponential, and stationary phases. The adaptive period (lag phase) reached to the 7th day. The exponential phases occurred between the 7th and 28th day. The stationary phase of calli occurred between the 28th and 42nd day where the rate of the cellular division was gradually reduced, then remained constant. Finally, in the stationary phase, nutrients depletion from the culture medium and a reduction of the O_2 amounts inside the cells occurred[26]. Both

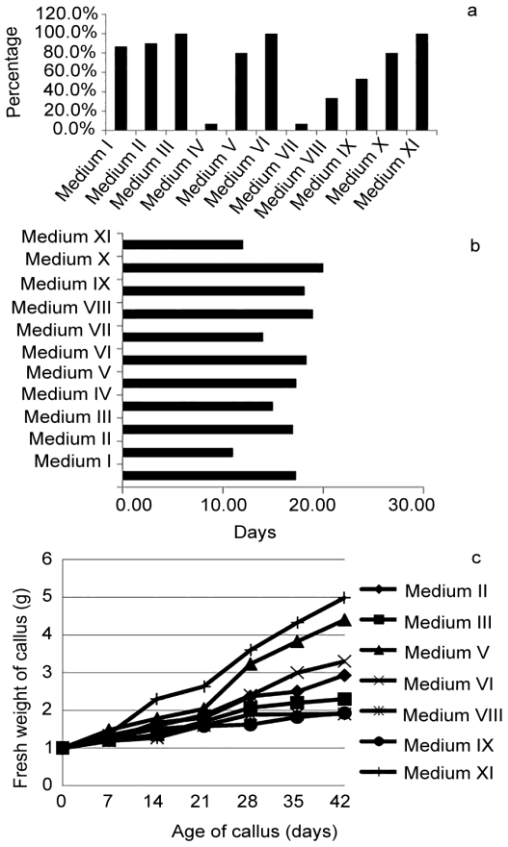

Fig. 1 — Callus induction percentages and days for callus initiation of *T. sinaicum*. Del. a) Callus induction percentage, b) Days for callus initiation, c) Growth curves of calli for 42 days using MS solid media supplemented with different phytohormones.

Table 2 — Days for callus initiation, callus induction percentages (callusing capacity), callus growth and callus morphological characters on eleven culture media after 6 weeks of cultivation. The experiments were repeated 3 times using 15 explants. The results showed the mean and standard error of mean was not included for simplification and did not exceed 10% of the mean

Code	Media composition	Callus growth after 6 weeks	Callus initiation days	Callus induction %	Morphological characters and growth of callus after 6 weeks
Medium I	MS + 2, 4 D (0.5 mg/L)	+	17.30	86.7	Yellowish green; compact
Medium II	MS + 2, 4 D (1 mg/L)	++	11.00	90.0	Buff; compact
Medium III	MS + 2, 4 D (1 mg/L) + Kn (0.5 mg/L)	+++	17.00	100.0	Green to yellow-green
Medium IV	MS + Kn (1 mg/L)	+	15.00	6.7	Yellowish-brown; very small in diameter characterized by direct shoots formation
Medium V	MS + NAA (0.5 mg/L) + BAP (1 mg/L)	+++	17.33	80.0	Green to yellowish green; compact
Medium VI	MS + NAA (1 mg/L) + BAP (1 mg/L)	++++	18.33	100.0	Green; compact with indirect shoot regeneration
Medium VII	MS + NAA (1 mg/L)	+	14.00	6.7	Yellow; very small in diameter
Medium VIII	MS + BAP (1 mg/L)	++	19.00	33.3	Green, very friable
Medium IX	MS + IAA (2 mg/L) + Kn (1 mg/L)	+	18.13	53.3	Pale yellow; very weak growth
Medium X	MS + IAA (1 mg/L) + Kn (2 mg/L)	++	20.00	80.0	Yellow to green; compact with shoot induction
Medium XI	MS + TDZ (0.5 mg/L + 2, 4 D (1 mg/L) + BAP (0.1 mg/L)	++++	12.00	100	Green, compact

+ Very weak growth (2-5 mm in diameter) ++ Moderate growth (5-10 mm in diameter)
+++ Good growth (10-15 mm in diameter) ++++ Very good growth (15-20 mm in diameter).

Table 3 — Growth index (GI), specific growth rate (μ), doubling time (dt) and effect of different hormonal combinations on callus fresh weight (g) using MS solid media supplemented with different phytohormones

Code	Growth index (GI)	Specific growth rate (μ) g/day	Doubling time (dt)	Callus fresh weight (g)					
				7 days	14 days	21 days	28 days	35 days	42 days
Medium II	1.93	0.028	24.76	1.21	1.67	1.80	2.37	2.50	2.93
Medium III	1.30	0.025	27.43	1.21	1.35	1.70	2.07	2.20	2.30
Medium V	3.40	0.034	20.42	1.48	1.78	2.04	3.23	3.83	4.40
Medium VI	2.30	0.030	23.25	1.34	1.59	1.87	2.40	3.00	3.30
Medium VIII	0.90	0.022	30.97	1.19	1.27	1.60	1.89	1.90	1.90
Medium X	0.93	0.022	31.82	1.26	1.51	1.58	1.62	1.82	1.93
Medium XI	3.99	0.046	15.05	1.38	2.30	2.63	3.60	4.33	4.99

medium XI and medium VI showed the highest growth parameters e.g. growth index (GI) and specific growth rate (μ); GI = 3.99 and 3.40 respectively, μ = 0.046 and 0.34 respectively and the lowest doubling time (dt) 15.05 and 20.42 days, respectively as shown in (Table 3) and (Fig. 1). On the other hand, medium VIII showed the lowest growth parameters; GI = 0.9 and μ = 0.022, while dt = 30.97 days.

Somatic embryogenesis

Six weeks old callus grown on medium V and XI were greenish-yellow in colour. The calli at this stage showed pre-embryogenic masses and globular embryos (Fig. 2a).

Callus grown on medium XI gave rise to globular embryos from the primary culture stage. However, the differentiation of embryos was inhibited by a continuous application of these growth regulators. For this reason, media with different growth regulators and also, hormone-free media were used for subsequent subcultures but no embryo differentiation was observed, and browning of callus was detected.

Embryogenic callus maintained on medium V showed somatic embryos as globular (G), heart-shaped (H) and torpedo-shaped (T) (Fig. 2c, d and e). Mature embryos successfully germinated into a cotyledonary embryo, which further developed into cotyledonary leaves of 1 cm height within 7-10 weeks (Fig. 2b). Additionally, well-differentiated vessels and tracheids with lignified and reticulate secondary cell wall patterns and of various shapes and sizes (Fig. 2f) were observed in embryogenic callus grown on medium V.

Regeneration of *T. sinaicum* via organogenesis

Direct organogenesis

Explants on medium IV showed several shoot buds (Fig. 3). Multiple shoots appeared within 20 days which were 3-5 cm in length after 1 month. Shoots were transferred to M&S media supplemented

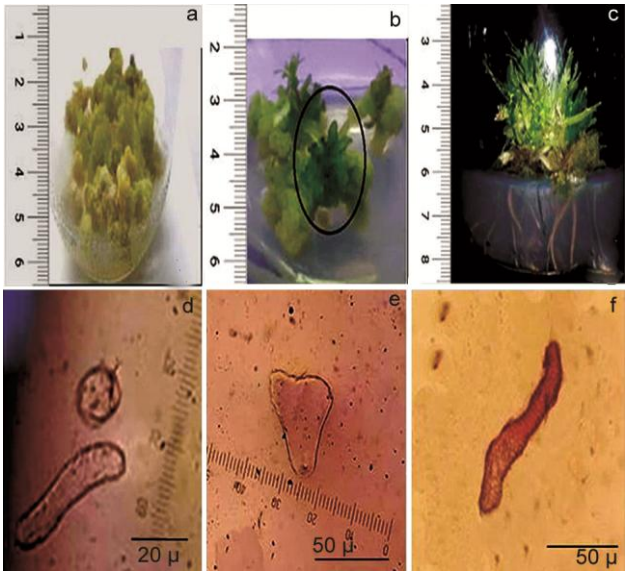

Fig. 2 — Stages of embryogenic callus development of *T. sinaicum. Del*. a) Pockets of embryogenic calli on medium XI, b) Cotyledonary leaves on medium V (0.5 to 1.5 cm height), c) Micropropagated plantlets after 10 weeks on medium V, d) Globular "G" and torpedo shaped "T" embryos, e) Heart shaped "H" embryo, f) Reticulated tracheid with a lignified wall.

with NAA (1 mg/L) where complete regenerated plantlets having well-developed roots, up to 5 cm in length, were obtained. Regeneration of *T. sinaicum* via direct organogenesis hasn't been reported before. However, a previous study has reported an effective and reproducible protocol of *T. cinerariaefolium* micropropagation via direct organogenesis using BAP for direct shoot induction and NAA for rooting[27].

Indirect organogenesis

After 8-10 weeks, a small callus on medium VI showed numerous shoots that were 1-2 cm in length. Additionally, medium X showed multi shooted calli after 4-6 weeks. Rooting of the regenerated shoots did not occur on the shoot induction medium. medium VII was used for rooting of the developed shoots forming complete plantlets with hard roots (Fig. 4).

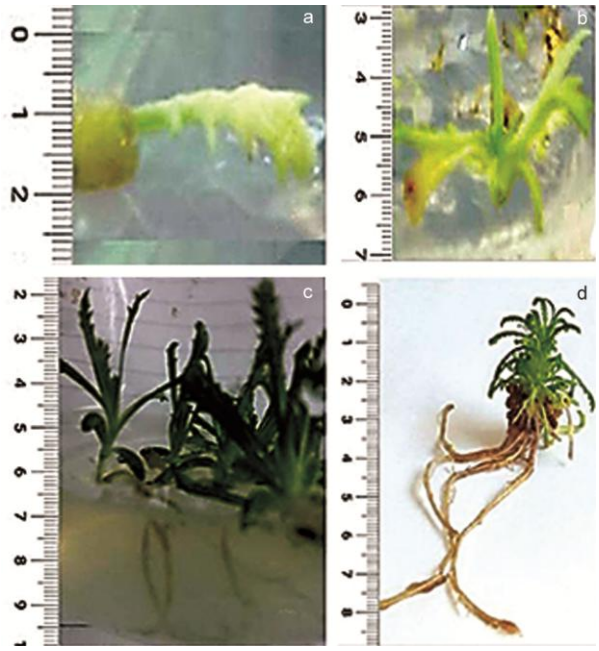

Fig. 3 — Regeneration of *T. sinaicum*. via direct organogenesis. a) Direct adventitious shoot bud induction from cultured leave explants after 2 weeks on medium IV (Kn 1 mg/l), b) Single regenerated shoot from direct shoot bud from leaf explants, c) Root induction, d) 2 months old plantlet.

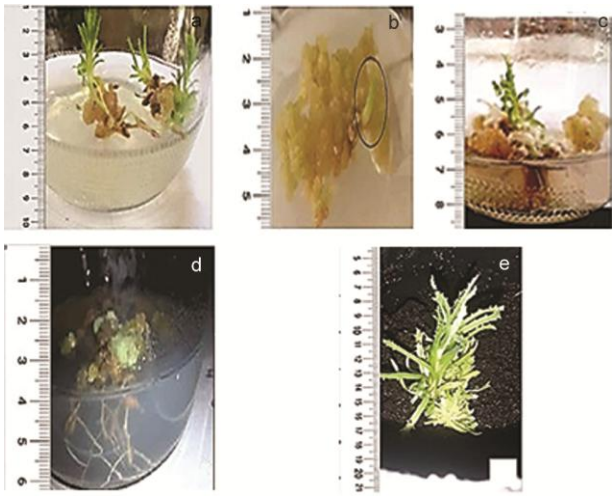

Fig. 4 — Regeneration of *T.sinaicum.* via indirect organogenesis. a) shoot regeneration from callus after 4 weeks on medium X, b) shoot regeneration from callus after 8 weeks on medium VI, formed numerous shoots, c) Rooting of a regenerated shoot on M&S media supplemented with NAA 1 mg/l, d) indirect root induction was observed on medium IX, e) Transplantation of micropropagated plants to soil.

Acclimatization of the *in vitro* regenerated plants

Regenerated plantlets were maintained in the greenhouse after transplantation. However, plants could survive for 2 weeks in the soil before they started to wilt (Fig. 4e).

Characterization of volatile constituents using GC-MS analysis

The volatile constituents of both wild and *in vitro* propagated plants were extracted by *n*-hexane and analyzed by GC-MS under the same conditions. The chemical composition of them was shown in (Table 4). Drastic quantitative and qualitative differences were observed in relation to the constituents of the volatiles. Analyses of the GC-MS chromatograms resulted in the identification of 38 compounds in the *n*-hexane extract of the wild plant, accounting for 87.75% of the total composition; while only 27 compounds were identified in the hexane extract of the *in vitro* plant, accounting for 75.51% of the total composition. The hexane extracts of the wild and *in vitro* plants were dominated by oxygenated monoterpenes with percentages of 39.23% and 38.36 %; respectively**.** The major components of the wild plant were *trans*-thujone (13.75%), *trans*-2, 7-dimethyl-4,6-octadien-2-ol (9.02%), *cis*-thujone (8.18%), pellitorine (6.12%) and artemisia ketone (4.61%), 4,10 (14)-muuroladien-8-β-ol (3.35%), *β-eudesmol* (2.82%) and germacrene-D (2.56%). While, the major components of the *in vitro* propagated plant extract were 4α-Hydroxyachipendol (16.14%), 3-(3-butenyl)-cyclohexanone (10.86%), *cis*-thujone (5.95%), 5-ethyl-2E-undecen-4-one (5.76%), 5-methyl-1,4-Hexadiene (5.28%) and pellitorine (4.73%), spathulenol (3.22%), *β*-eudesmol (2.91%) and massoia lactone (2.43%). The chemical profile of the hexane extract of the *in vitro* regenerated plant is quite different from that of the wild plant. Many constituents such as yomogi alcohol, 1, 8-cineole, artemisia ketone, p-cymen-7-ol, *β*-cubebene, germacrene-D and 4,10 (14)-muuroladien-8-β-ol were absent in the GC chromatogram of the *in vitro* regenerated plant. Also, results showed that both wild and *in vitro* propagated plants contained different concentrations of α-thujone, β-thujone, 4.*β*.-hydroxyachipendol, 4.*α*-hydroxyachipendol, 1,5-epoxysalvial-(14)-ene, spathulenol, salvial-4-(14)-en-1-one, methyl jasmonate, *β*-eudesmol, methyl palmitate, pellitorine, palmitic acid, phytol (Fig. 4). Despite these differences, the *in vitro* plants could produce the major constituent of the wild plant, the *trans*-thujone (Fig. 5).

Yomogi alcohol, Massoia lactone, spathulenol, *Trans*-thujone, *cis*-thujone, artemisia ketone, *β*-*Eudesmol*, Valerenol, Pellitorine, germacrene-D and phytol were previously reported in the essential oil of *T. sinaicum*[6,28].

Table 4 — Chemical composition of the n-hexane extract of wild and *in vitro* regenerated *T. sinaicum*

	Component[a]	RI[b]	MWT	ID[c]	%[d]	
					Wild plant	*In vitro* plant
1	5-methyl-1,4-Hexadiene	991	96	MS	--	5.28
2	Yomogi alcohol	997	139	RI,MS	0.88	--
3	3-Aminopyrazole	1018	83	MS	--	0.73
4	1,8 cineole (cajeputol) = Eucalyptol	1034	154	RI,MS	0.85	--
5	*trans*-2,7-Dimethyl-4,6-octadien-2-ol	1048	154	MS	9.02	--
6	Artemisia ketone	1063	152	RI,MS	4.61	--
7	5-Methyl-3-hexen-2-one	1067	112	MS	--	0.73
8	5-Ethyl-2E-undecen-4-one	1074	196	MS	--	5.76
9	*α*-Thujone = *cis*- thujone = 3-thujanone	1107	152	RI,MS	8.18	5.95
10	*β*-Thujone = *trans*- thujone	1116	152	RI,MS	13.57	1.52
11	4.*β*.- Hydroxyachipendol	1214	155	MS	0.91	1.46
12	4.*α*.- Hydroxyachipendol	1229	155	MS	0.18	16.14
13	3-(3-Butenyl)cyclohexanone	1255	152	MS	--	10.86
14	p-cymen-7-ol (cuminol)	1280	150	RI,MS	0.08	--
15	Carvacrol= p-Cymen-2-ol	1292	150	RI,MS	0.40	--
16	*β*-Bourbonene	1379	204	RI,MS	2.60	--
17	*β*-cubebene	1381	204	RI,MS	0.98	--
18	4-Methylcyclohexene	1398	96	RI,MS	--	1.46
19	Massoia lactone (5-Hydroxy-2-decenoic acid lactone)	1433	168	RI,MS	--	2.43
20	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1 methylethyl)	1470	204	RI,MS	0.25	--
21	2,6-di (t-butyl)-4-hydroxy-4-methyl-2,5 cyclohexadien-1-one	1473	236	RI,MS	--	0.85
22	Germacrene-D	1484	204	RI,MS	2.65	--
23	1,5 -epoxysalvial –(14)-ene	1550	220	RI,MS	0.47	0.60
24	spathulenol	1598	220	RI,MS	1.78	3.22
25	Salvial -4-(14)-en-1-one	1600	220	RI,MS	0.25	0.61
26	Alloaromadendrine epoxide	1649	220	RI,MS	0.52	--
27	Methyl jasmonate	1654	224	RI,MS	0.28	0.97
28	*β*-Eudesmol	1668	222	RI,MS	2.82	2.91
29	4,10 (14)-Muuroladien -8-β ol	1676	220	MS	3.35	--
30	alloaromadendrene oxide-(1)	1678	220	RI,MS	0.59	--
31	3-hydroxyanthranilic acid (2-Amino-3-hydroxybenzoic acid)	1717	153	MS	0.34	--
32	Valerenol	1737	220	RI,MS	--	0.85
33	Hexahydrofarnesyl acetone (HHA)	1819	268	RI,MS	--	1.35
34	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	1835	278	RI,MS	0.96	--
35	Phthalic Acid, Isobutyl Octyl Ester	1860	334	RI,MS	--	0.85
36	n- Nonadecane	1878	240	RI,MS	0.44	--
37	Methyl palmitate	1901	270	RI,MS	1.19	1.15
38	Pellitorine (N-Isobutyl-2,4-decadienamide)	1919	223	RI,MS	6.12	4.73
39	Palmitic acid	1937	256	RI,MS	1.38	1.09
40	Nonadecanol	1993	284	RI,MS	0.89	--
41	Phytol	2008	296	RI,MS	1.22	1.22
42	Methyl linoleate	2014	294	RI,MS	--	0.49
43	Cycloeicosane	2054	280	MS	0.56	--
44	Oleic acid	2124	282	RI,MS	11.55	
45	Tetracosanol (lignocerol)	--	336	MS	0.61	--

(*Contd*.)

Table 4 — Chemical composition of the n-hexane extract of wild and *in vitro* regenerated *T. sinaicum* (*Contd.*)

	Component[a]	RI[b]	MWT	ID[c]	%[d]	
					Wild plant	*In vitro* plant
46	Tetracosane	--	338	MS	3.40	--
47	n - Docosane	--	31	MS	2.37	--
48	Behenic alcohol= 1-Docosanol	--	326	MS	--	0.97
49	Bis (2-ethylhexyl)phthalate	--	390	MS	--	1.33
50	Vitamin E= alpha-Tocopherol	--	430	MS	0.18	--
51	γ -Sitosterol	--	414	MS	0.47	--
52	Triacontyl acetate	--	462	MS	1.13	--
	Monoterpene hydrocarbons (%)				--	--
	Oxygen containing monoterpenes (%)				38.68	38.36
	Sesquiterpene hydrocarbons (%)				6.48	--
	Oxygen containing sesquiterpenes (%)				9.78	9.04
	Others (%)				32.81	28.11
	Total identified compounds (%)				87.75	75.51

[a]: Compounds are listed in order of their elution from a HP-5MS column.
[b]: RI, linear retention indices on HP-5MS column, experimentally determined using homologous series of n- alkanes.
[c]: Identification methods: MS, based on comparison with W9N11.L and NIST11.L MS databases; RI, based on comparison of calculated RI with those reported in ADAMS and NIST libraries.
[d]: Relative percentages.
MWT: Compound molecular weight.

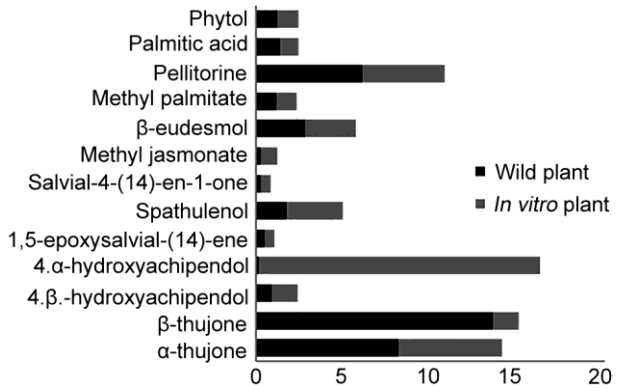

Fig. 5 — Schematic diagram representing common constituents identified by GC-MS in the *n*-hexane extract of wild and *in vitro* regenerated plants.

Conclusion

An *in vitro* propagation method was developed for the conservation and enhancement of *T. sinaicum*. Sterile seeds were germinated over a solid M&S medium with 30 g/L sucrose while adding phytohormones suppressed germination and growth of the seeds. Sterile seedlings were used as a source of explants on M&S media supplemented with different concentrations and combinations of growth regulators for callus growth. Media contains 0.5 mg/L TDZ, 1 mg/L 2, 4 D and 0.1 mg/L BAP showed the best vegetative callus growth but without acquiring any ability to stimulate embryogenesis or organogenesis. Media with 0.5 mg/L NAA and 1 mg/L BAP produced embryogenic calli from which plantlets were developed. On the other hand, media with 1 mg/L Kn had the ability to stimulate shoot formation directly from explants, while media with 1 mg/L NAA and 1 mg/L BAP promoted shoot formation from the callus. For root induction, excised shoots were transferred to media with 1 mg/L NAA. Hexane extracts of wild and *in vitro* propagated plants were analyzed by gas chromatography-mass spectrometry (GC-MS) which resulted in the identification of 38 and 27 constituents; respectively, with wide qualitative and quantitative variations between their constituents. Optimization of the conditions for callus induction and micropropagation of this plant will help in its conservation and could be a starting point for further genetic transformation procedures for the production of valuable biologically active compounds as sesquiterpene lactones.

Conflict of interest

The authors declare no conflict of interest.

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