GLC and GLC-MS Analysis of Thiophene Derivatives in Plants and in *in vitro* Cultures of *Tagetes patula* L. (*Asteraceae*)

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The occurrence of thiophenic compounds in diverse plant organs and in *in vitro* root-, callus- and cell suspension cultures of *Tagetes patula* cv. Carmen was investigated using capillary GLC and GLC-MS. The separation of thiophenes by capillary GLC and the group specific MS fragmentation with the typical sulfur isotope peaks allowed the unequivocal assignment of individual thiophenes in complex mixtures, even when occurring in traces and in the presence of different geometrical isomers. The extracts of *Tagetes patula* cv. Carmen contained the following 8 thiophene compounds: 5-(3-buten-1-ynyl)-2,2′-bithienyl (BBT), 5′-methyl-5-(3-buten-1-ynyl)-2,2′-bithienyl (MeBBT), 5-(1-pentynyl)-2,2′-bithienyl (PBT), 5-(4-hydroxy-1-butynyl)-2,2′-bithienyl (BBTOH), 2,2′,5,2″-terthienyl (α-T), 5-(4-acetoxy-1-butynyl)-2,2′-bithienyl (BBTOAc), 5-methylaceto-5′-(3-buten-1-ynyl)-2,2′-bithienyl ($AcOCH_2BBT$), and 5-(3,4-diacetoxy-1-butynyl)-2,2′-bithienyl ($BBT(OAc)_2$). The most complex thiophene profile, including the less common PBT was detected in aerial parts of freshly harvested plant material. Under *in vitro* conditions only the root cultures, but not callus or cell suspension cultures produced substantial amounts of irregular thiophenes confirming that roots are the main site of thiophene biosynthesis.

Introduction

The natural thiophenes, which usually contain 12 carbon atoms, are characteristic secondary metabolites of the genus *Tagetes* (*Asteraceae*). These thiophenes are built up from one to three thiophene rings that are coupled in α-position and carry one to six carbon atom long alkine chains on the remaining free ortho-positions (Bohlmann *et al.*, 1973; Kagan, 1991) (Table I).

Natural thiophenes are biologically active compounds, whose activity is enhanced by irradiation with long wavelength ultraviolet light (UV-A, 320–400 nm) (Camm *et al.*, 1975; Bakker *et al.*, 1979; Chan *et al.*, 1979). Thiophenes exhibit substantial antiviral, antibacterial, antifungal, nematocidal, and insecticidal properties under irradiation whereas their toxicity is much reduced in darkness. In presence of UV light these sulfur-containing polyacetylenes react as type II photosensitisers producing singlet oxygen that causes a wide spectrum of microbial and animal toxicity. The nematocidal activities of thiophenes against plant endoparasitic nematodes takes place in the dark ruling out any light activation. The observed activity might be caused by enzymatic activation of thiophenes in the root of the host plant, caused by the endoparasitic nematode attack (Gommers, 1982; Hudson *et al.*, 1991a). Due to their photodynamic character, thiophenes are of agricultural and medicinal interest as potential medicinal agents or biorational pesticides (Hudson and Towers, 1991b).

Tagetes species are known as a rich source of natural thiophenes. At least a few thiophenes have been detected in every species (Sorensen, 1977; Bohlmann and Zdero, 1985; Kagan, 1991). Because several thiophenes are present in *Tagetes patula*, this species has been commonly used in phytochemical investigations. Basic knowledge on the biosynthesis and occurrence of these secondary metabolites has been elaborated by Bohlmann *et al.* (1973), Bohlmann and Zdero, (1985), Bohl-

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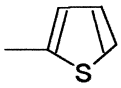
Table I. The most common thiophenes of the genus *Tagetes*.

R_1	Structure R_2	Name	Acronym	No
H–	–C≡C–CH=CH_2	5-(3-buten-1-ynyl)-2,2'-bithienyl	BBT	1
CH_3–	–C≡C–CH=CH_2	5'-methyl-5-(3-buten-1-ynyl)-2,2'-bithienyl	MeBBT	2
H–	–C≡C–CH=CH–CH_3	5-(1-pentynyl)-2,2'-bithienyl	PBT	3
H–	–C≡C–CH_2–CH_2–OH	5-(4-hydroxy-1-butynyl)-2,2'-bithienyl	BBTOH	4
H–		2,2',5',2''-terthienyl, (α-terthiophene,α-terthienyl)	α-T	5
H–	–C≡C–CH_2–CH_2–O–CO–CH_3	5-(4-acetoxy-1-butynyl)-2,2'-bithienyl	BBTOAc	6
CH_3– O–CO–CH_2–	–C≡C–CH=CH_2	5-methylaceto-5'-(3-buten-1-ynil)-2,2'-bithienyl	$AcOCH_2BBT$	7
H–	–C≡C–CH(O–CO–CH_3)– –CH_2–O–CO–CH_3	5-(3,4-diacetoxy-1-butynyl)-2,2'-bithienyl	$BBT(OAc)_2$	8

mann, (1988). They characterised 4 thiophenes as major components, BBT, BBTOH, BBTOAc and α-T, which occur in different concentrations in every part of *Tagetes* plants. The main site of thiophene accumulation is, however, the root (Downum and Towers, 1983; Hogstad *et al.*, 1984; Ketel, 1987).

Production of secondary plant metabolites in *in vitro* cultures is of substantial biotechnological and biochemical interest. Although cell suspension and callus cultures are well suited for mass cultivation, they often produce secondary metabolites only in small amounts or not at all (Wink, 1993). However, organ cultures, such as normal or transformed hairy root cultures often produce the same or similar secondary compounds as the *intact* plants. Usually root cultures are well suited as a model system for biochemical studies and production experiments supposed that the roots are the site of biosynthesis (Walton *et al*, 1999; Wildi and Wink, 2001)

Callus and cell suspension cultures of *Tagetes patula* have already been investigated for 20 and root cultures for 10 years. Using HPLC only the 4 main components, 5-(3-buten-1-ynyl)-2,2′-bithienyl (BBT), 5-(4-hydroxy-1-butynyl)-2,2′-bithienyl (BBTOH), 2,2′,5,2″-terthienyl (α-T), and 5-(4-acetoxy-1-butynyl)-2,2′-bithienyl (BBTOAc) (Table I) could be regularly detected, and seldomly other compounds like 5′-methyl-5-(3-buten-1-ynyl)-2,2′-bithienyl (MeBBT), 5-(3,4-dihydroxy-1-butynyl)-2,2′-bithienyl $BBT(OH)_2$, 5-(3,4-diacetoxy-1-butynyl)-2,2′-bithienyl $(BBT(OAc)_2$, or 5-methyl-2,2′,5′,2″-terthienyl (Me-α-T) (Breteler, 1993; Flores *et al.,* 1988; Hogstad *et al.,* 1984; Sütfeld, 1987). Also other thiophenes of unknown structures have been recorded (Ketel, 1987).

GLC and GLC-MS are suitable methods for the analysis of complex mixtures of thiophenes which contain several stereoisomers or differ only in the number of unsaturated bonds and methyl-groups. More than 25 thiophenes have been explored in *Tagetes* plants using this approach (Bichi *et al.,* 1992; Caniato *et al.,* 1990; Hogstad *et al.,* 1984; Héthelyi *et al.,* 1989; Sütfeld, 1987). Most of the recently discovered new thiophenes are mainly minor components, which can only be identified through coupled techniques such as GLC-MS.

The *in vitro* cultures provide a particularly difficult matrix for phytochemical investigations. This fact probably explains the limited number publications reporting results from GLC-MS analysis of thiophenes in *in vitro* cultures. Although several researchers developed particular purification protocols, the minor thiophenic compounds which are known in plants have usually not been detected in cell and organ cultures (Groneman *et al.,* 1984; Hogstad *et al.,* 1984).

Our work addressed the question, which thiophenes are detectable in intact plants and in *in vitro* cultures of different organisation levels, such as callus cultures, cell suspension and root cultures. Our GLC and GLC-MS analysis was aimed at lowering the detection limit for thiophenes in diverse plant materials, such as intact plants and *in vitro* cultured samples, and at solving the identification problem for thiophenes for which commercial standards are not available.

Experimental

Plant material

Seeds (achenes) of *Tagetes patula* (L.) cv. Carmen came from a commercial source. Plants were grown in the greenhouse of the Institute of Pharmaceutical Biology (University Heidelberg) between May and August under normal day/night regime.

Initiation of sterile in vitro cultures

The achenes were surface sterilised with 70% (v/v) MeOH and 10% (v/v) commercial hypochlorite solution, then rinsed twice with sterile distilled water, and placed on hormon-free Nitsch medium (Nitsch and Nitsch, 1969). The seeds were germinated in darkness at 24 °C. After one week, 1 cm long roots of the seedlings were excised and transferred for the callus or root culture induction (for more details see Margl, 2002).

Cultivation of callus and cell suspension cultures

The surface of the isolated sterile young roots was excised with a scalpel and transferred to solid MS medium (Murashige and Skoog, 1962) supplemented with 8 g/l agarose, 100 mg/l myo-inositol, indoleacetic acid, naphthylacetic-acid, kinetin, 6-benzaminopurine, 30 g/l sucrose, adjusted to pH 5.5 before autoclaving. After 3–4 weeks the primary calli who had been found, were exised and grown under identical condition with 2 week transferring period in unchanged circumstances at 24 °C in light (650 lux).

For initiation of cell suspension growing (ca. 2 g weight) greenish calli were transferred to Erlenmeyer flasks containing the same medium without agarose. The homogenisation of the suspension culture was achieved by shear forces in 10 ml medium in 100 ml Erlenmeyer flasks on the rotary shaker (120 rpm). After 7 days further 10 ml medium was added to the dense grown culture. At the end the stepwise in bigger flasks transferred cultures were kept in 300 ml Erlenmeyer flasks with 100 ml medium.

Cultivation of root cultures

The excised roots of the seedlings were transferred to 10 ml liquid MS medium (Murashige and Skoog, 1962) (in 50 ml Erlenmeyer flasks), supplemented with 100 mg/l myo-inositol, 100 mg/l NZ-amine, 30 g/l sucrose, adjusted to pH 6 before autoclaving. After 10 days of culture further 10 ml fresh medium was added to the fast growing roots, and after 3 weeks they were transferred into bigger flasks. Thereafter, they were transferred every 14 days ensuring that their thiophene production was constant for 3 years. Root cultures were kept on rotary shakers (120 rpm) at 24 °C in light (650 lux).

Steam distillation

Washed roots (30 g) and shoots (50 g) of freshly harvested plants were homogenized in dest. water with a blender. Thiophenes were obtained by steam distillation.

Solvent extraction and purification of thiophenes

Alternatively, thiophenes were extracted from fresh whole roots (6 g), root cultures (5 g) calli (6 g), and cell suspension cultures (16 g) according to the modified method of Croes *et. al.* (1989) with 70% MeOH (10 ml per g plant material). The crude extract was purified twice by partitioning between methanol and a 1:1 (v/v) mixture of hex-

ane/tert-butyl-methylether. The solvent of the apolar phase was collected and evaporated in two steps. First its volume was reduced in a rotavapor. The remaining extract of ca. 0.5 ml was dissolved in 2 ml hexane, which was then evaporated under N_2-atmosphere. The residue was dissolved in 300 µl Et_2O. The second purification step was carried out using micro-scale column chromatography. A Pasteur-pipette of 2 ml served as a column, which was filled with 0.8 g dry silica gel 60 (Macherey & Nagel). The column-material was conditioned with 10 ml Et_2O. The applied extracts were eluted with 2 ml Et_2O into brown bottles. The purified extracts were concentrated under N_2-atmosphere.

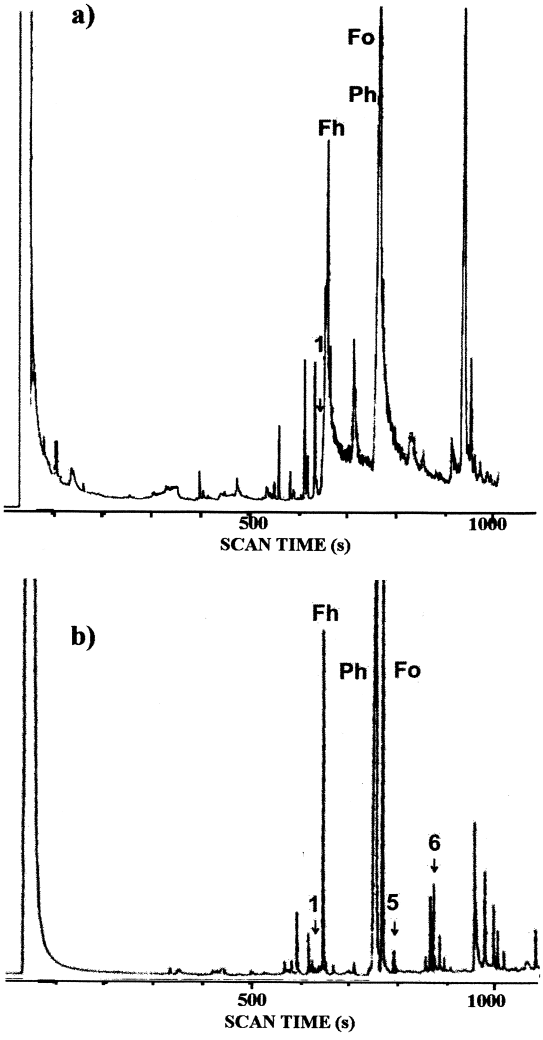


Fig. 1. GLC Chromatogram of prepurified a) and purified b) callus extracts of *Tagetes patula* L. cv. Carmen. 1: BBT, 5: α-T, 6: BBTOAc, Fh: C16 fatty acid methyl ester, Fo: C18 fatty acid methyl ester, Ph: phytol.

In the second purification step the strong apolar thiophenes could be selectively eluted from the crude extracts. Through this procedure the samples were freed from solid particles and from more polar compounds such as lipids and pigments. (The cultures grown in light contain chlorophyll and a functional photosynthetic system (Flores *et al.*, 1988). The effectiveness of this process can be seen in the GLC profiles of crude and purified callus extracts (Fig. 1). The crude extracts are too complex for GLC analysis as too many compounds are present which co-elute with potential thiophenes. In the purified sample traces of BBT, BBTOAc and α-T could be identified.

All extraction steps were carried out under dim light to avoid photochemical reactions.

Gas-liquid chromatography

The temperature-programmed retention indices (I_{PTGC}) were calculated according van den Dool and Kratz (1963). The purified extracts were diluted in hexane. 1 µl hexanic solution of 23 mg/ml n-hexadecane was added as an internal standard at 4 °C to each gram of plant material of the diluted extract source. Thiophenes were quantified by comparing the peak areas of the n-hexadecane and thiophenes (response factor of 0.2440). The analysis was carried out with a Carlo Erba Mega 5360 Gas Chromatograph equipped with fused silica capillary columns 15 m × 0.25 mm i.d.); coated with PERMABOND OV-1 DF (Macherey and Nagel). GLC-conditions: injector 250° C; temp. programme T_0 = 120 °C, t_0 = 2 min, rate of temperature increase = 10 °C/min T_1 = 300 °C, t_1 = 5; pressure = 1 atm; split ratio 1:20; injection volume 2 µl; detector FID. Data evaluation was carried out using a Hewlett Packard 3393 Integrator.

Gas-liquid chromatography-mass spectrometry

The GLC separation was carried out as described above. The only differences were a 30 m capillary column, the heating rate was reduced to 6 °C/min and the detector was a Finnigan MAT 4500 quadrupole mass spectrometer. Mass spectra were recorded at 45 eV.

Results and Discussion

Identification of thiophenes

Sulfur-containing compounds can easily be identified by mass spectrometry because of their typical isotope pattern; the natural abundance of the ^{34}S- isotope is 4.16%. Therefore, sulfur-containing substances show a characteristic corresponding $[M+2]^+$ signal. Fragmentation patterns of isolated pure compounds or published reference spectra (Bichi *et al.,* 1992; Hogstad *et al.*, 1984) were used for the identification of individual thiophenes. 8 thiophenes (BBT, PBT, BBTOH, BBTOAc, $BBT(OAc)_2$, MeBBT, $ACOCH_2BBT$, and α-T) culd be unequivocally identified. Thereafter the temperature programmed retention indices (I_{PTGC}) were determined by GC-FID system. (Table II).

Thiophenes in different plant parts

The thiophene pattern is characteristic for particular plant organs (Héthelyi *et al.*, 1988; Tosi *et al.*, 1988) (Fig. 2 and Table III). Roots which represent the site of thiophene biosynthesis, contain the highest diversity and contents of thiophene. The main component was BBT, followed by BBTOAc and α-T. BBTOH was detectable only in traces in *Tagetes patula* L. cv. Carmen, besides 4 minor components (PBT, $BBT(OAc)_2$, MeBBT, and $AcOCH_2BBT$). Whereas PBT was the main component in the aerial parts of the plant, i.e., in shoots and flowers, BBT occurred only in traces. Trimeric thiophenes, such as α-T, were most abundant in flowers, indicating that either these compounds are made in the roots and are transported to the flowers where they preferentially accumulate or alternatively dimeric thiophenes are transported to the flowers and modified here to the trimeric substances. Although, PBT has been

Table II. Retention indices (RI) by GLC and mass spectral characteristics of thiophenes in *Tagetes patula*.

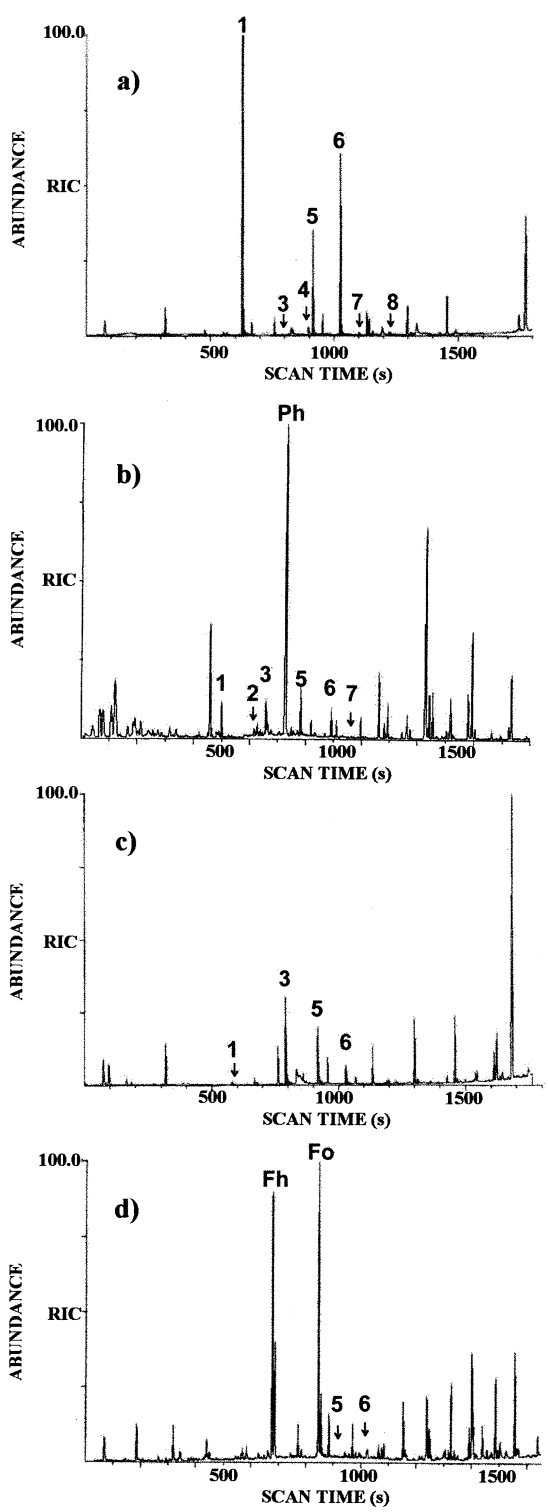
Acronym*	I_{PTGC}	M^+	Fragment ions (relative abundance [%])				
BBT	1892	216	216 (100)	170 (17)	217 (13)	93 (10)	218 (8)
MeBBT	1992	230	230 (100)	83 (37)	229 (34)	231 (17)	102 (17)
PBT	2043	230	230 (100)	229 (23)	231 (18)	232 (10)	197 (9)
BBTOH	2152	234	203 (100)	234 (76)	204 (14)	171 (21)	235 (11)
α-T	2171	248	248 (100)	249 (16)	250 (14)	204 (9)	127 (7)
BBTOAc	2292	276	216 (100)	43 (21)	217 (15)	203 (14)	276 (13)
$AcOCH_2BBT$	2373	288	229 (100)	288 (63)	230 (17)	43 (15)	289 (13)
$BBT(OAc)_2$	2523	334	232 (100)	274 (65)	43 (38)	219 (34)	334 (28)

* see Table I.

Table III. Occurrence of thiophenes in *Tagetes patula* L. cv. *Carmen* plants (total thiophenes = 100%).

Plant material	BBT	MeBBT	PBT	BBTOH	α-T	BBTOAc	$AcOCH_2$ BBT	$BBT(OAc)_2$
Roots SE	63.5%	n. d.	tr.	tr.	8.5%	24.7%	tr.	tr.
Roots SD	93.2%	n. d.	n. d.	n. d.	5.2%	1.6%	n. d.	n. d.
Shoots SE	tr.	n. d.	13.7%	n. d.	n. d.	86.2%	n. d.	n. d.
Shoots SD	7.9%	tr.	46.4%	n. d.	31.8%	12.0%	tr.	n. d.
Flowers SE	3.6%	n. d.	50.0%	n. d.	34.8%	11.6%	n. d.	n. d.
Achenes SE	n. d.	n. d.	n. d.	n. d.	tr.	tr.	n. d.	n. d.

SE: solvent extract, SD: steam distillate, tr.: traces, n.d.: not detected.



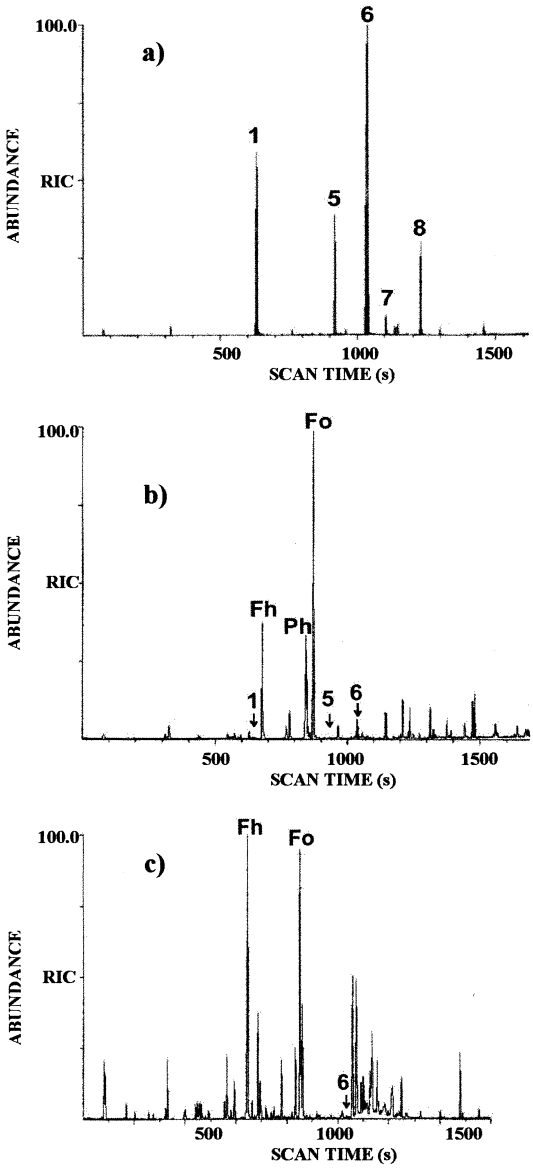


Fig. 3. GLC-MS of extracts from *in vitro* cultures of *Tagetes patula* cv. Carmen.
a) Root culture, b) Callus culture, c) Cell suspension culture. 1: BBT, 5: α-T, 6: BBTOAc, 7: $AcOCH_2BBT$, 8: $BBT(OAc)_2$, Fh: C16 fatty acid methyl ester, Fo: C18 fatty acid methyl ester, Ph: phytol.

Fig. 2. GLC-MS of extracts from *T. patula* L. cv. Carmen organs.
a) Roots, b) Shoots, c) Flowers, d) Achenes.
1: BBT, 2: MeBBT 3: PBT, 4: BBTOH, 5: α-T, 6: BBTOAc, 7: $AcOCH_2BBT$, 8: $BBT(OAc)_2$, Fh: C16 fatty acid methyl ester, Fo: C18 fatty acid methyl ester, Ph: phytol.

Table IV. Occurrence of thiophenes in *in vitro* cultures of *Tagetes patula* L. cv. *Carmen* (total thiophenes = 100%).

Plant material	BBT	BBTOH	α-T	BBTOAc	$AcOCH_2$ BBT	$BBT(OAc)_2$
Root culture SE	23.7%	tr.	11.7%	58.9%	tr.	3.8%
Medium of root culture SE	tr.	n. d.	n. d.	n. d.	n. d.	n. d.
Callus culture SE	20.1%	n. d.	tr.	79.8%	tr.	n. d.
Cell suspension culture SE	n. d.	n. d.	n. d.	tr.	n. d.	n. d.

SE: solvent extract, tr.: traces, n.d.: not detected.

Table V. Thiophene contents in differentiated and undifferentiated plant materials of *Tagetes patula* cv. Carmen.

Plant material	BBT [μg/g FW]	α-T [μg/g FW]	BBTOAc [μg/g FW]	Total [μg/g FW]
Roots of intact plants SE	425.03	21.11	15.18	461.32
Root culture SE	296.92	100.04	392.78	789.74
Callus culture SE	0.01	n.d.	0.01	0.02

SE: solvent extract, n.d.: not detected.

known from flowers since 1984 (Hogstad *et al.*, 1984), the occurrence of this component in the aerial parts had not yet been reported. Achenes are almost free of thiophenes; α-T and BBTOAc were detected only in traces.

Steam distillates of roots and shoots showed a changed thiophene profile as compared to the solvent extracts, in which the less volatile compounds are discriminated or occur only in smaller amounts.

Thiophenes of in-vitro cultures

The thiophene pattern of *in vitro* cultures was more simple than that of the differentiated plants (Fig. 3 and Table IV). Root cultures exhibited the most diverse thiophene profile with altogether 6 thiophenes. As compared to roots of intact plants the ratio of BBT and BBTOAc was reversed, and the content of $BBT(OAc)_2$ was higher. Whereas BBTOH showed a similar abundance in both roots and in root cultures, $AcOCH_2BBT$ was only found in traces. In the medium of the root cultures only BBT was to detected in traces indicating that thiophenes are not released into the culture medium.

Calli showed a similar thiophene pattern as the main components of the root cultures but their content was lower by several orders of magnitude. In cell suspension cultures only traces of BBTOAc could be detected. The main signals seen in the chromatograms of callus and cell suspension cultures are fatty acid methyl esters.

Quantitative analysis of thiophenes

Quantification of thiophenes was carried out by GLC. The main components of the extracts, i.e., BBT, BBTOAc, and α-T, were calculated on a fresh weight basis. The total thiophene contents of *in vitro* cultured root clones were about 40% higher than those of roots *in planta*. Thiophene contents of callus and cell suspension cultures was three orders of magnitudes lower than those of roots and root cultures (Table V).

Conclusion

The GLC analysis revealed a more detailed thiophene pattern than HPLC analyses. The main difference concerns the substantial occurrence of the irregular thiophene PBT instead of BBT in the aerial parts of *Tagetes* plants. Our studies show that root cultures produce several unusual thiophenes as minor components.

Roots are unambiguously the main site of thiophene accumulation in *Tagetes*, from where they are apparently transported to the aerial parts and the flowers where further biotransformations can take place. Among different *in vitro* cultures only root cultures are able to produce thiophene in the same magnitude as roots in *planta* which would well agree with the root being the main site of thiophene biosynthesis.

Genes which encode enzymes of secondary metabolite biosynthesis are apparently regulated in a cell- and tissue-specific fashion in many systems. In undifferentiated cultures or in untypical tissues the corresponding genes are usually switched off. Only upon tissue differentiation a correct regulation of the genes encoding enzymes for biosynthesis or potential transporters is given. If a synthesis takes place in the roots, normal or transformed root cultures represent excellent *in vitro* systems to study the biochemistry of synthesis and accumulation or the biotechnological production of such compounds.

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