Phytochemistry, Vol. 28, No. 11, pp. 3003–3007, 1989.
Printed in Great Britain.

0031–9422/89 $3.00+0.00

TWO UNEXPECTED COUMARIN DERIVATIVES FROM TISSUE CULTURES OF COMPOSITAE SPECIES

DEREK V. BANTHORPE and GEOFFREY D. BROWN*

Department of Chemistry, University College London, 20 Gordon Street, London WC1H OAJ, U.K.

(*Received in revised form* 24 *April* 1989)

Key Word Index—*Tanacetum parthenium*, *T. vulgare*, *Artemisia vulgaris*; Compositae; tissue culture; isofraxidin; scopoletin; coumarin derivatives; chemotaxonomy.

Abstract—Tissue cultures of *Tanacetum parthenium*, *T. vulgare* and *Artemisia vulgaris* all produced significant amounts of scopoletin and isofraxidin together with stigmasterol and sitosterol under a wide variety of conditions; but none of the lines accumulated detectable quantities of those components, e.g. terpenoids and spiroketal enol ethers, characteristic of the parent plant. The two coumarins predominant in culture were either absent from or were very minor components of the parent plants. Cultures derived from several members of families other than the Compositae did not accumulate the coumarins. The possible significance of these observations is discussed.

INTRODUCTION

Extracts of the flowerheads of *Tanacetum parthenium* (L.) Schultz Bip. [syn. *Chrysanthemum parthenium* (L.) Bernh.; feverfew] are renowned in folk medicine to be efficacious in the treatment of migraine and related ailments, and the active principle may be the sesquiterpene lactone parthenolide [1, 2]. Consequently, we set out to establish culture lines of this species that could be manipulated to produce this target compound perhaps not seriously contaminated by the plethora of other metabolites [3], produced in the floral organs. Concurrently, we established callus lines of two other members of the *Anthemidae* tribe of the Compositae, i.e. *Tanacetum vulgare* L. [syn. *Chrysanthemum vulgare* (L.) Bernh.; tansy] and *Artemisia vulgaris* L. (mugwort) for comparison purposes. The last two species are known to produce monoterpenes with a variety of skeletal types [4–7] and callus lines of both have been established [8–11] and secondary metabolism in those from *T. vulgare* has been investigated [8, 11]. However, *T. parthenium* has not been grown previously in culture. In the event, our cultures did not produce the products characteristic of their parents: lines from all these species yielded unexpected coumarin derivatives and this pattern of products persisted in a variety of cultural conditions.

RESULTS AND DISCUSSION

The main components of the oil extracted from foliage of the three investigated species are listed in Table 1 (extractions were made from flowering plants of all species between late June and early July). GC-MS analyses were carried out and those components that could not be matched with data banks were isolated by column chromatography and/or HPLC and characterized by ^1H and ^{13}C NMR spectroscopy and MS. Each species also produced a variety of lower terpenoids and polyacetylenes as minor components and our analyses are broadly in agreement with those of the previous workers.

Table 1. Components of the essential oil of foliage of Compositae species ($10^2 \times$ % wt/fresh wt) analysed by GC-MS

	Species†		
Compound (type)*	*T. p*	*T. v*	*A. v*
Camphor (C_{10})	8.5	—	—
Thujone (C_{10})	—	6.3	—
Verbenone (C_{10})	—	—	2.9
trans-Chrysanthemyl alcohol (C_{10})	4.1	—	—
Germacrene-D (C_{15})	2.5	3.6	0.5
Caryophyllene (C_{15})	tr.	tr	0.7
Parthenolide (C_{15})	2.9	2.7	—
cis-Spiroketal enol ether (**2**)	1.7	—‡	—
Stigmasterol (C_{30})	0.6	0.2	0.2
Sitosterol (C_{30})	1.3	0.5	0.4

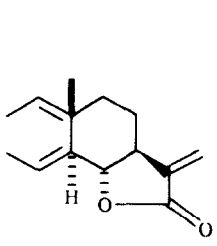
*C_{10}, C_{15} and C_{30} indicate mono-, sesqui-terpenoids and phytosterols respectively. No entry in table indicates that $<10^{-6}$%, if any are present. Tr. indicates trace quantities ca. 10^{-4}%.

†*Tanacetum parthenium*, *Tanacetum vulgare* and *Artemisia vulgaris* respectively.

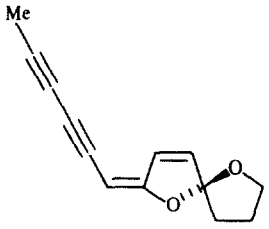
‡Reported detected in roots [12].

Flowerheads of *T. parthenium* were also investigated and yielded an oil (*ca* 1.7% wt/fresh wt) with mostly the same components as in leaves, but with the interesting addition of dehydrosaussurea lactone (**1**) and the pyran analogue of **2**. Root oil from this species (*ca* 0.5% wt/fresh wt) mainly contained **2** and its *trans* isomer (35.7×10^{-2} and 4.2×10^{-2} % respectively) together with the

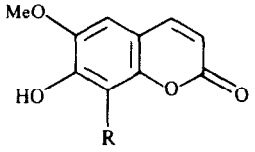
*Author to whom correspondence should be addressed. Present address: Department of Physics, Nottingham University, University Park, Nottingham, NG7 2RD U.K.



1



2



3 R = H
4 R = OMe

pyran compound (5.2×10^{-2}%) and D-friedoolean-14-en-3β-ol (6.0×10^{-2} %).

Callus lines of all three plants species could be readily established on a variety of media and regimes as pale friable material that appeared uniform in morphology and biochemical characteristics over two years and many passages (see Experimental). GC-MS analysis of extracts revealed that under all conditions the terpenoids that were so abundant in the parent plants could not be detected ($< 10^{-6}$% wt/fresh wt; if any); although perhaps as befitting structural components of membranes, the two phytosterols found in the plant extract were accumulated. The lack of monoterpenes in callus of *T. vulgare* is particularly disquieting as a variety of such components had previously been reported from callus established and maintained under very similar conditions [8]. Such irreproducibility may indicate the importance of the physiological condition of the explant in giving rise to callus composed of particular sub-populations with different biochemical properties [11]. However, extracts from a variety of lines of callus of all three species did contain very significant quantities of scopoletin (**3**; 6-methoxy,7-hydroxycoumarin) and isofraxidin (**4**; 6,8-dimethoxy,7-hydroxycoumarin), and these coumarins accounted for virtually all the extracted oil from callus of *T. parthenium*. A representative set of assays is given in Table 2.

As a consequence of these results, the oils from foliage of the parents (and in one case root) were re-examined for the presence of the coumarins which had been found as components of the cultures. Because of the low levels and broad elution profiles in GC (due to the phenolic group), these compounds had not been located in the GC-MS analyses but they could readily be detected by HPLC and their accumulations are recorded in Table 2. The comparisons are on a fresh wet basis and as the callus typically contained more water (wt/wt) than foliage, a dry weight comparison would give a more meaningful index of the synthetic capabilities of the callus.

Isofraxidin occurs in the Compositae but is relatively rare [13, 14]. In contrast, scopoletin and its derivatives are fairly widespread [15] within the family. Coumarins are often found in cell cultures, and root (i.e. organ) cultures of various species contain scopoletin [16–18]. To the best of our knowledge, isofraxidin has not been reported previously in culture but scopoletin is known to occur in tobacco tissue cultures [19].

The accumulation of these novel products was significantly effected by the composition of the medium and the culture conditions, although qualitatively the pattern of secondary metabolism remained unaltered. Thus, an increase in the concentration of sugar in the medium led to sometimes dramatic increases in levels of both products (Table 3) which could not be correlated with either growth rates or sterol content of the extractable oils. For *T. parthenium*, the nature of the sugar appeared immaterial but its concentration was important.

Similarly, the concentration of the auxin in the medium could dramatically influence the production of both coumarins, although the type of phytohormone (e.g. 2,4-D, NAA or 4-CPAA) appeared to be much less influential. This is well illustrated by the levels of accumulation of isofraxidin in *T. parthenium* callus grown under natural and controlled lighting (see Fig. 1). The effect of changing from subdued natural illumination to an artificial 12 hr-diurnal pattern of illumination was to reduce the levels of accumulation of the coumarins. The data in Fig. 2, from a parallel experiment to that of Fig. 1, show that the concentration of NAA in the medium influences accumulation of isofraxidin in a comparable manner to that of 2,4-D.

Fine cell suspensions were not systematically evaluated for their biosynthetic capabilities, but those of *T. vulgare* accumulated the coumarins at levels similar to those of

Table 2. Components of extracts of callus and parent plants ($10^3 \times$ % wt/fresh wt) by HPLC

	Field grown*†				Callus‡		
Compound	*T. p* Foliage	Root	*T. v* Foliage	*A. v* Foliage	*T. p*	*T. v*	*A. v*
Isofraxidin	—	4.6	—	0.7	42.2	1.9	3.3
Scopoletin	—	—	—	4.3	—	0.6	1.8
Stigmasterol	6.0	—	2.1	2.3	0.5	0.4	0.9
Sitosterol	13.0	—	5.4	4.0	0.7	1.1	1.0

T. p etc. as in Table 1.

†— indicates $< 10^{-6}$% if any present.

‡Assays on two-year-old material (18–20 passages). Variations were up to ±20% between different subcultures.

Table 3. Accumulation of isofraxidin and scopoletin in response to sugar in the medium ($10^3 \times$ % wt/fresh wt)

Species	Compound	Concentration of sucrose in medium		
		0.2 g/l	2 g/l	8 g/l
T. parthenium	isofraxidin*	0.9	33.3	53.6
T. vulgare	isofraxidin	0.2	0.8	7.7
	scopoletin	0.01	0.2	1.4
A. vulgaris	isofraxidin	0.6	1.4	18.8
	scopoletin	1.1	1.0	2.9

*Callus of *T. parthenium* did not accumulate any scopoletin. Use of either glucose or fructose (2 g/l) in the medium in place of sucrose led to accumulations of 35.3×10^{-3}% (w/w) and 36.9×10^{-3}% (w/w) respectively.

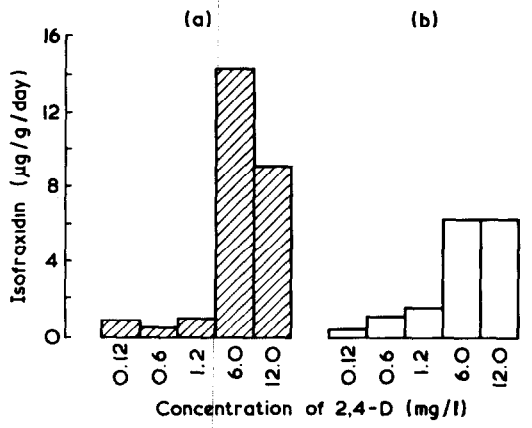


Fig. 1. Variation of accumulation of isofraxidin with concentration of 2,4-D in the medium. (a) subdued natural illumination; max. intensity *ca* 400 lux. (b) diurnal variation: 12 hr at *ca* 2000 lux; 12 hr. darkness.

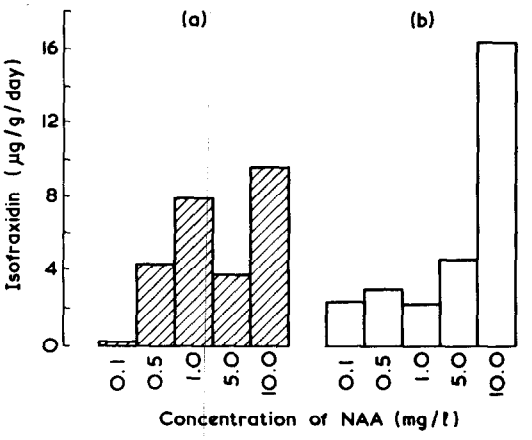


Fig. 2. Variation of accumulation of isofraxidin with concentration of NAA in the medium. (a) and (b) as in Fig. 1.

the parent callus. Thus a typical line yielded (on entry to the stationary phase) 2.3×10^{-3}% (all wt/fresh wt) isofraxidin and 0.9×10^{-3}% scopoletin, compared with levels of 2.0×10^{-3}% and 0.6×10^{-3}% for tissue of the same subculture maintained on a solid agar medium. Again no detectable quantities of terpenoids were present. Others have repeatedly found that suspension cultures rarely match callus tissue in biosynthetic capabilities for secondary metabolism, and one reason could be that the generally rapid growth (typically around a half the passage time of callus) enables fast growing sub-lines that channel material into primary metabolism to predominate [11]. In this context it may be significant that the passage time of our suspensions was long, *ca* 11 weeks, in comparison with a period of four to six weeks for the callus. The reason for this slow growth is not understood. If the accumulations of the coumarins is, however, a facet of undifferentiated metabolism—as is suggested later—it may not be surprising that the two types of tissue behave similarly.

The failure of all three species to accumulate in culture those secondary metabolites characteristic of the parent plant is a situation oft-reported in the literature. However, the accumulation of metabolites which are either absent or present in very low levels in the parents is much less frequent [20] and merits attention. Thus why should isofraxidin—a minor component of the root of field-grown *T. parthenium* and not present in foliage or flowerheads—be virtually the sole metabolite produced in culture, and produced in an ingrained manner under a variety of culture conditions?

One possibility is that scopoletin and isofraxidin occur in these three members of the Compositae as phytoalexin or inhibitin-type compounds. Their incidence in culture might then be due to a failure of the normal repression mechanisms. This function was suggested by our discovery that both coumarins are highly toxic (at levels of less than 1 μg) towards *Cladosporium herbarum*, a common plant pathogen, in simple plating assays (see Experimental). In addition, the occurrence of isofraxidin uniquely in the roots of *T. parthenium* correlated with a persistent infection in this tissue which resulted in explants becoming infected within a few days in culture: in contrast explants of stem and leaf could readily be cultured, using the same rigid sterilization procedures, with negligible infections. Again, the increase in accumulation of the coumarins triggered by increase of sugar in the culture medium could reflect the known elicitation of phytoalexins in other species by sucrose [21]. Indeed, scopoletin has been identified as a phytoalexin in several species [22, 23] as has the β-glucoside of isofraxidin [24]. Unfortunately, we could not induce isofraxidin formation in foliage of *T. parthenium* using spores of either *C. herbarum* or *Botrytis cinerea*, nor was accumulation of coumarins in callus activated by the inclusion of the known elicitor cellulase [25] in the medium (up to 10 μg/ml). Thus we can present no direct evidence for the phytoalexin-nature of these compounds.

Another possibility is that the accumulation of shikimate metabolites is magnified in culture by enhanced levels of phenylalanine ammonia lyase (PAL). The levels of this crucial enzyme are known to be enhanced at high sugar levels in the medium, but our observed effects of auxins and light intensity are not consistent with implication of this enzyme [26].

A third possibility is that the production of simple coumarins is a characteristic of undifferentiated tissue and is a relic of the evolution of secondary metabolism. It may be significant that coumarins predominate in all three species of the Compositae investigated, whereas screening of available culture lines of *Anethum graveolens*

L. and *Carum carvi* L. (Umbelliferae), *Mentha spicata* L. and *Mentha longifolia* L. (Labiatae) and *Pelargonium graveolens* Ait. (Geraniaceae) indicated no detectable traces of these metabolites. Thus production of the coumarins could be a simple chemotaxonomic marker for the *Compositae* (or perhaps for the sub-tribe *Anthemidae*). If this view is valid, screening of cultures of members of different families might reveal other such markers. Because of the likely simplicity of secondary metabolism of most undifferentiated cultures such screening would be easy and cheap (via TLC) in contrast to attempts to locate markers by analysis of the plethora of products produced in the parent tissues.

EXPERIMENTAL

Sources. Tanacetum parthenium (L.) Schultz Bip. [syn. *Chrysanthemum parthenium* (L) Bernh.] was obtained from a private garden in Oxford and was taxonomically verified by Dr Braithwaite (Nottingham University). Voucher specimens have been deposited at the herbarium of the Nottingham Botany Department. *Tanacetum vulgare* L. [syn. *Chrysanthemum vulgare* (L.) Bernh.] and *Artemisia vulgaris* L. were obtained and taxonomically verified by the curator of the herbarium at the Royal Botanic Gardens, Kew, and voucher specimens are held there. (The specimens of *T. vulgare* belonged to a previously characterized chemical taxon [4, 5]).

Culture methods. Portions of stem and leaf were sterilized (2% aq. $Ca(OCl)_2$; 5–15 min) rinsed with sterile H_2O (×3) and explanted onto Murashige and Skoog's medium (Flow Labs, Irvine, Ayrshire, U.K.) containing coconut milk (10% v/v), sucrose (20 g/l) and either 2,4-D (6 mg/l) or a mixture of NAA (0.5 mg/l) and kinetin (0.1 mg/l) made up in Oxoid No. 3 agar (1.2% w/v) adjusted to pH 5.8 before autoclaving. The coconut milk was omitted after the first subculture. For *A. vulgaris* some lines were maintained on 4-CPAA (1 mg/l) as the auxin component. Subcultures were generally performed at 4–6 week intervals (6 to 8 weeks when 4-CPAA was used) when the callus was maintained under subdued natural illumination (*ca* 400 lux max.) at 25°. Callus of *T. parthenium* was also kept on a diurnal cycle of 12 hr (2 000 lux; 30°), followed by darkness at 15°. Fine cell suspension cultures of *T. vulgare* were initiated from callus culture on the above liquid medium shaken at 100 rpm under subdued natural illumination (max. *ca* 400 lux) at 25°. Subculture was made on entry to the stationary phase (1×10^6 cells/ml) at *ca* 11 week intervals.

Extraction and analysis of plant material. Parent tissue or callus (*ca* 100 g) was washed, dried, frozen in liquid N_2 and pulverized. The resulting powder was extracted (Soxhlet) with Et_2O (200 ml), the extract dried ($MgSO_4$) and solvent removed in a stream of N_2 at 0°. Solns of the residue (*ca* 20% v/v) in Me_2CO were analysed by capillary GC-MS (0.1 μl injection) using a BP1 FSOT column (12 m × 0.22 mm i.d.) linked to a mass spectrometer (at 70 e.v. i.p.) coupled to a Finnegan-Incos data system. The splitter ratio on the injector was 1:60. The GC column was programmed from 60° to 320°, at a gradient of 8°/min. and the tandem mass spectrometer made 2.5 scans/sec. from 30–800 a.m.u. Data banks for GC-MS at Bush Boake Allen & Co. Ltd., London, U.K., and the National Bureau of Standards, Washington, U.S.A. were scanned to identify the components of the chromatograms. Other signals were detected corresponding to di-isooctyl adipate and (in smaller amounts) di-isooctyl, di-*n*-butyl and di-isobutyl phthalates. All have been claimed as *bona fide* natural products [27] but all are undoubtedly plasticisers leached out of the Soxhlet thimbles [cf. refs 28 and 29]. Such artefacts were most noticeable when callus tissue (with low levels of secondary metabolites) were analysed.

Isofraxidin and scopoletin could be separated from crude callus extract by CC on silica gel-H with EtOAc–$CHCl_3$ (1:3) as eluent: the R_f values on TLC with the same system were 0.21 and 0.23 respectively. The resulting mixture of the two coumarins could be fully resolved by prep.-scale HPLC on a 5 μM silica column: (10 cm × 4.5 mm) + (25 cm × 7 mm) with EtOAc–petrol (60 80°)–AcOH (10:9:1) as the mobile phase at 3 ml/min and with UV detection at 254 nm. Under these conditions the elution times were 14.5 and 13.3 min respectively.

Isofraxidin. Mp 149–150° (lit. 148–149°) [30]. ^{1}H NMR: 200 MHz; 10% wt/v in $CDCl_3$; δ7.61 (*d*, *J* = 9.5 Hz, 1H), 6.67 (*s*, 1H), 6.28 (*d*, *J* = 9.5 Hz, 1H), 6.10 (*s*, 1H), 4.10 (*s*, 3H), 3.95 (*s*, 3H). The signal at δ6.10 corresponded to the −OH proton and its shift was dependent on the purity of the sample and concentration. Irradiation of the signal at δ3.95, in an NOE expt led to an increase in the intensity at δ6.67 of 8.4%. The converse irradiation produced an enhancement of 3.4% for the signal at δ3.95 and 3.7%, for that at δ7.61. Solvent shifts recorded in C_6D_6 further helped to establish the order of the substituents around the aromatic ring (on the assumption that the solvent interacted with the coumarin skeleton assymetrically; the closest position of approach being in the vicinity of C-4) thus: δ6.59, 5.85, 5.92, 3.66, 3.15. MS *m/z* (rel. int.): 222.0514 $[M]^+$ (100: $C_{11}H_{10}O_5$ requires 222.0528); 207 (27), 194 (15), 179 (21), 151 (12), 123 (20), 95 (15), 79 (17). ^{13}C NMR, IR and UV spectra (the latter recorded in EtOH or EtOH–NaOH (2% v/v)) all were superimposable on spectra of an authentic sample.

Scopoletin. ^{1}H NMR (10% $CDCl_3$ wt/v): δ7.61 (*d*, *J* = 9.5 Hz, 1H), 6.93 (*s*, 1H), 6.86 (*s*, 1H), 6.28 (*d*, *J* = 9.5 Hz, 1H), 6.12 (*s* 1H), 3.96 (*s*, 3H). δ6.12 corresponded to the −OH proton. An NOE expt established that δ6.93 corresponded to H-5, as irradiation of the singlet at δ3.96 led to a signal enhancement at the former of 8.1%. MS *m/z* (rel. int.): 192.0411 $[M]^+$ (100; $C_{10}H_8O_4$ requires 192.0422), 177 (70), 164 (26), 149 (65), 121 (26), 79 (26), 69 (75), 51 (33). Again, ^{13}C NMR, IR and UV spectra superimposed on those of an authentic sample.

A sample of the extract from the foliage of *T. parthenium* chromatographed on a column of silica gel-H with EtOAc–$CHCl_3$ (1:3) gave fractions corresponding to parthenolide and the *cis*-spiroketal enol-ether (I) (contaminated by its *trans* isomer) which had R_f values of 0.45 and 0.61 in the corresponding TLC system. These compounds were then purified by prep. HPLC on 5 μm silica with EtOAc–petrol (60–80°) (1:3) as the mobile phase.

Parthenolide. Mp 95–100° (ex hexane). ^{1}H NMR (10% wt/v $CDCl_3$): δ6.35 (*d*, *J* = 3.6 Hz, 1H), 5.63 (*d*, *J* = 3.3 Hz, 1H), 5.23 (*d*, *J* = 10 Hz, 1H), 3.87 (*t*, *J* = 8.6 Hz, 1H), 2.79 (*d*, *J* = 8.8 Hz, 1H), 2.09–2.44 (*m*, 9H), 1.72 (*s*, 3H), 1.67 (*m*, 2H), 1.31 (*s*, 3H); MS *m/z* (rel. int.): 248 $[M]^+$ (0.3), 233 (0.45), 230 (0.5), 190 (14), 53 (49), 43 (100).

cis-*Spiroketal enol-ether* (**2**). ^{1}H NMR (10% wt/v $CDCl_3$): δ6.70 (*d*, *J* = 5.7 Hz, 1H), 6.21 (*dd*, *J* = 5.7, 1.7 Hz, 1H), 4.94 (*s*, 1H), 4.19 (*ddd*, *J* = 8.5, 5.8, 3.5 Hz, 1H), 4.00 (*ddd*, *J* = 15.1, 8.5, 2.0 Hz, 1H), 2.23–2.00 (*m*, 4H), 1.99 (*d*, *J* = 1.1 Hz 3H); MS *m/z* (rel. int.): 200 $[M]^+$ (100), 199 (25), 185 (15), 170 (30), 157 (25), 115 (40).

trans-*Spiroketal enol ether.* ^{1}H NMR (10% v/v $CDCl_3$): δ6.24 (*d*, *J* = 5.6 Hz, 1H), 6.16 (*dd*, *J* = 5.6, 0.8 Hz, 1H), 4.60 (*s*, *br*, 1H), 4.24 (*ddd*, *J* = 8.5, 8.5, 3.5 Hz, 1H), 4.00 (*ddd*, *J* = 15.4, 8.5, 1.7, 1H), 2.32–2.00 (*m*, 4H), 1.99 (*d*, *J* = 1.2 Hz, 3H). The *trans* stereochemistry was established by irradiation of the singlet at δ4.60 when the intensity at δ6.24 was increased by 8.5% in an NOE experiment.

Fungitoxicity tests. Crude extracts from callus tissue (1 μg)

were chromatographed by TLC on silica gel-H in four different solvent systems (PhMe; EtOAc–$CHCl_3$, 1:3; Me_2CO–$CHCl_3$, 1:2; MeOH–$CHCl_3$, 3:17). The plates were allowed to dry, sprayed with a soln of *C. herbarum* in Czapadek Dox medium and then incubated for 5 days inverted over H_2O in a plastic bag. Fungitoxic compounds appeared as lighter areas on a dark background of fungal hyphae and their identity could be determined by a comparison of the R_f values with those of pure compounds run in the same solvents.

Attempts to induce phytoalexins in the foliage of *T. parthenium* was carried out by the following method. Drops of a suspension of either *C. herbarum* or *B. cinerea* in Czapadek–Dox medium (1.5×10^7 spores/ml; 6 ml in total) were placed on the leaves of a medium-sized (20 cm tall) healthy plant of *T. parthenium*. A control was established, using a second plant covered in a sterile solution of Czapadek–Dox medium. Both plants were maintained under maximum humidity at 25° under a 12/12 hr light-/dark regime for four days (signs of infection became apparent within two days) after which they were extracted. Although there were some differences between the two extracts, there was no *de novo* accumulation of isofraxidin.

Acknowledgements—We thank the SERC for a studentship to GDB; Mr Ian Marr (BBA Ltd. UK) for much help with GC-analyses and Mr S. Corker (UCL) for the HPLC separations. We also thank Dr R. Strange (Botany Dept. UCL) for help with the fungitoxicity and elicitation experiments, and Professors Bohlmann (Berlin) and S. Nishibe (Hokkaido) for authentic samples of isofraxidin and scopoletin.

REFERENCES

1. Berry, M. I. (1984) *Pharm. J.* 611.
2. Johnson, E. S., Kadam, N. P., Hylands, D. M. and Hylands, P. J. (1985) *Br. Med. J.* **291**, 569.
3. Bohlmann, F. and Zdero, C. (1982) *Phytochemistry* **21**, 2543.
4. Tétényi, P., Kaposi, P. and Héthelyi, E. (1975) *Phytochemistry* **14**, 1539
5. Ekundayo, O. (1979) *Z. Pflanzenphysiol.* **92**, 215.
6. Banthorpe, D. V., Baxendale, D., Gatford, C. and Williams, S. R. (1971) *Planta Med.* **20**, 147.
7. Nano, G. M., Bicchi, C., Frattini, C. and Gallino, M. (1976) *Planta Med.* **30**, 211.
8. Banthorpe, D. V. and Wirz-Justice, A. (1972) *J. Chem. Soc. Perkin I*, 1769.
9. Okamura, M., Hayashi, T. and Miyazaki, S. (1984) *Plant Cell Physiol.* **25**, 281.
10. H. Deno *et al.* (1978) *Japanese Patent* 78, 142, 591.
11. Banthorpe, D. V., Branch, S. A., Njar, V. C. O., Osborne, M. G. and Watson, D. G. (1986) *Phytochemistry* **25**, 629.
12. Bohlmann, F., Herbst, P., Arndt, C., Schönowsky, H. and Gleinig, H. (1961) *Chem. Ber.* **94**, 3193.
13. Bell, E. A. and Charlwood, B. V. (1980) in *Secondary Plant Products* Vol. 8, p. 334. Springer, Berlin.
14. Greger, H. (1982) in *World Crops, Production, Utilization* Vol. 7, p. 153. *Aromatic Plants* (Margaris, N., Koedam, A. and Voukou, D. eds). Martinus Nijhoff, The Hague.
15. Goodwin, T. W. and Mercer, E. I. (1983) *Introduction to Plant Biochemistry* 2nd Edn p. 535. Pergamon, Oxford.
16. Steck, W., Bailey, B., Shlyuk, J. P. and Gamborg, O. L. (1971) *Phytochemistry* **10**, 191.
17. von Brocke, W., Reinhard, E., Nicholson, G. and König, W. A. (1971) *Z. Naturforsch.* **26B**, 1252.
18. von Kartnig, Th., Moeckel, H. and Maunz, B. (1975) *Planta Med.* **27**, 1.
19. Okazaki, M., Hino, F., Kominami, K. and Miura, Y. (1982) *Agric. Biol. Chem.* **46**, 609.
20. Banthorpe, D. V., Bilyard, H. J. and Brown, G. D. (1989) *Phytochemistry* (in press).
21. Cooksey, C. J., Garratt, P. J., Dahiya, J. S. and Strange, R. N. (1983) *Science* **220**, 1398.
22. Mors, B. W. and Ribeiro, O. (1957) *J. Org. Chem.* **22**, 978.
23. Greseman, A., Biehl, B. and Lieterei, R. (1986) *J. Phytopathol.* **117**, 373.
24. Malmberg, A. G. and Theander, O. (1980) *Phytochemistry* **19**, 1739.
25. Brooks, C. J. W., Watson, D. G. and Freer, I. M. (1986) *Phytochemistry* **25**, 1089.
26. Camm, E. L. and Towers, G. H. N. (1973) *Phytochemistry* **12**, 961.
27. Gusakova, S. D. and Umarov, A. U. (1975) *Chem. Nat. Prod.* **11**, 527.
28. Cerbulis, J. and Byler, D. M. (1986) *J. Agric. Food Chem.* **34**, 198.
29. Krell, H.-W. and Sanderman, H. Jr. (1986) *J. Agric. Food Chem.* **34**, 194.
30. Späth, A. and Jerzmanowska-Sienkiewiczowa, Z. (1937) *Ber.* 1019.