Phytochemistry, Vol. 24, No. 4, pp. 719–722, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

THIOPHENE PRODUCTION BY CROWN GALLS AND CALLUS TISSUES OF *TAGETES PATULA*

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(*Revised received* 6 *September* 1984)

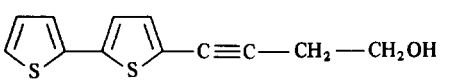
Key Word Index—*Tagetes patula*; compositae; crown gall; tissue culture; thiophene; polyacetylene.

Abstract—Thiophene concentrations were measured in crown gall tissues produced by *Agrobacterium tumefaciens* infections (strains A208, A277) of *Tagetes patula* plants and compared with those of normal and transformed callus tissues. The results showed that it is not possible to predict the amounts of secondary metabolites produced as a result of transfers of genetic material from infected plants to crown galls and then to transformed callus tissues. There appears to be an accumulation of intermediates in some of the biosynthetic routes.

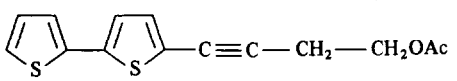
INTRODUCTION

Relatively little information has been published comparing secondary metabolite production by crown gall tissues, or by transformed callus tissues derived from them, with that of normal, healthy plants. Most of the literature deals with alkaloid production although crown gall tissues of *Matricaria chamomilla* were shown to produce polyacetylenes characteristic of the normal plants but in reduced yield [1]. Most of the publications [2, 3] to date contain results which show a reduced yield of secondary metabolite production by crown gall tissues or transformed callus tissues.

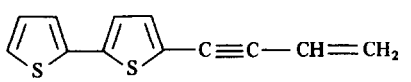
Tagetes patula contains four biosynthetically related thiophenes: 2,2′:5′,2″-terthienyl (α-T), 5-(4-hydroxy-1-butenyl 1-2,2′-bithienyl (BBTOH), 5-(4-acetoxy-1-butenyl)-2,2′-bithienyl (BBTOAc), and 5-(3-buten-1-enyl)-2,2′-bithienyl (BBT). The concentrations of these compounds vary between roots, stems and flowers, with BBT the major thiophene in roots and the acetate the most abundant thiophene in stem and leaf tissue [4]. α-Terthienyl, the fourth most abundant thiophene in *T. patula*, was present in all plant tissues examined but at low concentrations. It was present in some callus tissues but not detected in others and we have not included the results of its estimations here.



BBTOH



BBTOAc



BBT

We have measured the thiophene contents of crown gall tumours produced as a result of infection of healthy plants by *Agrobacterium tumefaciens* strains A208 and A277. Further, the thiophene contents of normal and transformed callus cultures derived from healthy and crown gall tumour tissue, respectively, have also been measured. We wished to determine if a relationship existed between the thiophene compositions of the various systems.

RESULTS AND DISCUSSION

Treatment of normal *Tagetes patula* plants with either strain A208 or strain A277 of *Agrobacterium tumefaciens* induced crown gall formation in the infected plants. Thiophene synthesis occurred in crown gall tissues and all four thiophenes were produced (Table 1); α-terthienyl has not been reported but was present in small amounts. The total yields of BBTOH, BBTOAc, and BBT from gall tissues were greater in total than from normal plant parts although the thiophene pattern did not correspond to that in any of the plant parts (Table 1). For instance, galls synthesized relatively large amounts of both BBTOAc and BBT while BBTOAc represented 70% of the thiophene contents of the stem and BBT 80% of those from the roots. The total thiophene yields from whole plants are 10 mg/g fresh weight.

The thiophene yields from galls produced by infection with strain A208 were greater than those resulting from infections with strain A277 (Table 1). As well, different thiophene patterns were evident: the BBT:BBTOAc ratio was 1:1 in galls from A208 infection while that from A277 infection was 2:1 (Table 1). It was the BBTOAc concentrations that were most affected by the different infections. The alcohol (BBTOH) did not show the wide variations of the BBTOAc yields although the BBTOH yields were greater in either set of galls than from the roots or stems of uninfected plants. Transformed callus tissues showed the

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Table 1. Thiophene recoveries from *Tagetes patula** galls, stems from plants infected by *Agrobacterium tumefaciens* ≠208, ≠277 (August harvest)

Plant	BBTOH	BBTOAc	BBT
		≠208	
Plant 1 (galls)	181±107	3700±356	3860±1230
Plant 2	390±127	5420±1200	4680±2990
Plant 3	197±49	3000±1210	2100±816
		≠277	
Plant 1	153±22	999±394	2860±1378
Plant 2	89±22	900±243	1760±510
Plant 3	240±83	1150±800	1490±776
		Normal plant	
Stem	20	3193±1358	Trace
Roots	30	104	6250+500

*Yields in nmol/g tissue fr. wt.

Table 2. Thiophene recoveries from *Tagetes patula** galls, stems from plants infected by *Agrobacterium tumefaciens* (November harvest)

Plant	BBTOH	BBTOAc	BBT
		≠208	
Plant 1 (galls)	58.8±27	662±93	625±92
Plant 2	76±22	449±214	398±280
Plant 3	90±9.5	352±132	1850±1390
Stem (uninfected)		3193±1358	
		≠277	
Plant 1 (galls)	44.0±24.5	2540±722	878±350
Plant 2	161±10.0	363±110	553±86
Plant 3	72±17	258±55	167±24
Stem (uninfected)		1015±60	
Roots (uninfected)		100	3100

*nmol/g tissue fr. wt.

same irregularities in yields of thiophenes as well as their distribution patterns. The high standard deviations (Tables 1 and 2) reflect these irregularities.

There were substantial differences in overall yields of thiophene from galls harvested in August compared with those harvested in November (Tables 1 and 2). This may result from seasonal variations and different growth rates. The yields of thiophenes from the transformed callus tissues also reflected this seasonal difference (Tables 3 and 4).

Both normal and transformed callus cultures, derived from normal and crown gall tissues, respectively, produced BBT, BBTOAc and BBTOH. As with the galls, the yields of thiophenes from callus tissues derived from plants grown in the summer were greater than from those grown later in the year (Tables 3 and 4). Thiophene yields from transformed callus cultures produced through strain A208 infections were much greater than from tissues produced by A277 infections. Yields from the latter

Table 3. Thiophene contents of *Tagetes patula* normal and transformed callus tissues (August harvest)

Callus (transformed)	BBTOH	BBTOAc	BBT	Nopaline*	Octopine*
A208 1.	76.0	—	258	+	−
A208 2.	290	238	660	+	−
A208 3.	130	94	230	+	−
A208 15.	212	250	300	+	−
A208 16.	243	100	130	+	−
A208 17.	3.1	200	48.0	+	−
A208 19.	16	26.0	142	+	−
A277 8.	5.0	2.0	5.0	−	−
A277 9.	5.0	—	10.0	−	−
A277 10.	2.1	—	—	−	−
A277 23.	—	6.2	50.0	−	−
Galls (A208)	630			+	−
Galls (A277)	2500			−	+
Stem 7	3100	300	1320	−	−
Callus (normal)					
Normal 1.	18.0	55.0	49.0	−	−
Normal 2.	73.0	170	122	−	−
Normal 3.	15.4	22.0	44.0	−	−
Normal 8.	64.0	73.0	25.0	−	−
Normal 9.	1.8	1.8	9.0	−	−

*Determined by the method of ref. [4]. Yields in nmol/g fr. wt.

Table 4. Thiophene recoveries from normal and transformed callus tissues of *Tagetes patula* (December harvest)

Run	BBTOH	BBTOAc	BBT
10a normal	1.7	—	1.4
10b normal	3.1	3.1	1.0
12 normal	0.7	—	1.1
14 normal	—	—	2.0
20 transformed	0.2	—	0.4
22 transformed	0.4	—	1.0
27 transformed	0.7	—	2.5
29 transformed	—	—	0.3

Yields in nmol/g fr. wt.

system were less than those from normal callus tissues (Table 3). Based on BBT recoveries from normal root tissues, the yields of thiophenes from transformed callus tissues (A208) were 2–10% (August run) of those from gall tissues on a fresh weight basis.

The most abundant thiophene (56%) in stem tissue was BBTOAc while that in root tissue was BBT (88%). These two represent the most abundant thiophenes in crown gall tissue although the BBT:BBTOAc ratio in galls (A208) was about 1:1 while that resulting from A277 infection was about 2:1. Such a pattern is not present in thiophene recoveries from either normal or transformed callus cultures (Table 3). The comparatively high yields of BBTOAc from gall tissue produced by infection with strain A208 has a parallel in the *Bidens pilosa* systems [6], heptan-(2-en-4,6-diyn-7-phenyl)-1-ol acetate was recovered from galls in yields greater than from the surrounding tissues. Similarly, the alcohol (BBTOH in *T. patula*) was recovered in yields greater than in any of the plant parts. An explanation for the different concentrations of thiophenes when compared to normal healthy plants may be that in a tumour where growth is less organized than in a plant, some delocalization of substrates and enzymes may result in an accumulation of some of the intermediates in a synthetic route. Such an accumulation in galls and callus tissues is not unusual; polyacetylene intermediates accumulated in callus cultures of *Centaurea ruthenica* [7] in small amounts, while none of the polyacetylene end products of the intact plant were detectable. Although the biosynthetic relationships of the thiophenes in *Tagetes patula* have not been completely described, it seems logical to expect BBT and α-T to be end products of the synthetic routes. The isolation of an esterase [8] which catalyses the conversion of BBTOAc to BBT supports this suggestion. Thus, the irregularities of thiophene productions in both galls and callus tissues (as shown by the high standard deviations, Tables 1 and 2) may simply be a manifestation of relatively disorganized patterns of growth.

Repeated subculturing of transformed callus tissues resulted in a slow decline in thiophene yields to a point where, after six subculturings, the thiophene yields were reduced ten-fold. This result is also different from our experience with subculturing of transformed tissues of *Bidens alba* where, although low, the yields of polyacetylenes are maintained [6]. This shows the unpredictability of secondary metabolite yields by transformed callus tissues. Normal callus tissues of *Tagetes patula* also produce thiophenes but in yields about one-fifth of those transformed tissues (A208).

Transformed callus tissues derived from infections of *T. patula* plants with *A. tumefaciens* produced nopaline (A208) or octopine (A277). It indicates that the bacterial plasmid, or part of it, has been transferred to the plant genomes. However, the wide differences in thiophene yields resulting from the A208 or A277 infections indicates that there is no direct relationship between secondary metabolite production and plasmid transfer. The synthesis of thiophenes by normal callus tissues also supports this conclusion.

It is clear from these experiments that it is not possible to predict the amounts of secondary metabolites resulting from a series of transfers of genetic material from the infected plants to galls and then to transformed callus cultures. There is a large yield variability as well as the accumulation of intermediates of synthetic routes. However, this accumulation of intermediates may provide the opportunity to direct synthesis in some other direction.

CULTURE METHODS

Organisms. Tagetes seed designated "Marigold-French Dwarf Double, Sparky Mixed" was obtained from Buckerfields Ltd., Box 7000, Vancouver, B.C. *A. tumefaciens* strains A208 and A277 were obtained from Dr. Milton Gordon, University of Washington. Strain A208 induces tumours producing nopaline, strain A277 tumours producing octopine.

Methods. Tagetes seeds were planted in soil and grown in a glasshouse. When plants were *ca* 14 cm high, the strains were inoculated in the stems by sterile syringe, 6 and 12 cm from the soil, with a 48 hr culture of *A. tumefaciens* grown in YEB medium [8]. Tumours were harvested 6 weeks after inoculation and sterilized externally by soaking in 70% EtOH for 1 min followed by 10 min in a 10% commercial bleach soln (Javex) with 1 g/l. of detergent (Sparkleen) and then rinsed 4 times in sterile distilled H_2O. Tumours were then cut into slices 1–2 mm thick and grown in 15 mm × 90 mm plastic Petri dishes containing MS medium (Flow Laboratories) to which vitamins and 3% sucrose were added and pH adjusted to 5.7 prior to autoclaving. Filter-sterilized (Millipore) carbenicillin (Ayerst Laboratories) was added at a level of 0.5 g/l. to the autoclaved medium. After 6 weeks cultures which showed no signs of contamination were transferred to the same medium without carbenicillin. Tissues were incubated in dark at 25° and harvested at the time indicated.

Thiophene quantitation. Appropriate samples (0.5–2.5 g) of root, stem, crown gall or callus tissues were crushed to a pulp in 5 ml MeOH contained in a 30 ml test tube. Subsequently, an equal vol. of H_2O was added and the mixture was extracted by vigorous shaking with 2 vol. of petrol (l.b.). 2 extractions were made and, after pooling, the extracts were evaporated to dryness *in vacuo*. The resulting oil was dissolved in 0.2 ml MeOH and stored in the dark for HPLC analysis.

Quantitative determination of the thiophene contents of the plant extracts were made on a Varian Model 5000 liquid chromatograph equipped with a Variscan 634 spectrophotometer and a Spectra Physics model 4100 integrator. The *A* 330 nm was used to detect the compounds eluted from a Varian MCH-10 octadecylsilane reverse phase column (4 × 30 mm). The thiophenes were separated by the solvent system acetonitrile:H_2O (18:7) with the column operating at room temp. The flow rate was 1 ml/min. The three compounds BBT, BBTOH and BBTOAc were quantitated by a comparison of their areas from the chromatograms with those of a stock soln of BBT.

A calibration curve of peak areas vs. nmol BBT was prepared and the results obtained from the analyses of the plant extracts were compared directly with it. BBT was used as the standard in each case; the differences in molar extinction coefficients for BBT, BBTOH, and BBTOAc was 1–2%. Thus, BBT provided a satisfactory standard for quantitation of the three thiophenes. Both normal and transformed callus tissues were treated as described above for extraction and determination of their amounts of thiophenes.

Acknowledgement—This work was supported by the National Research Council contract 24SU-3194-3-0002.

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