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# *In vitro* growth of *Tagetes patula* L. hairy roots, production of thiophenes and its mosquito larvicidal activity

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Hairy root culture of *Tagetes patula* was studied for thiophene production. Growth parameters (on fresh and dry wt basis) were measured for biomass production. The analysis of thiophene was carried out by the methods of Flame Ionization Detection (FID) and mass spectral analysis (GC-MS). The separation profile of the thiophenes indicated the presence of several structurally different thiophenes, predominantly α-terthienyl, which was confirmed by FID and GC-MS analysis. The maximum accumulation of biomass (0.27g. dry wt/ culture) was recorded on 12$^{th}$ day and thiophene content (0.064%) was recorded as maximum on 9$^{th}$ day. The thiophene produced in hairy roots of *T. patula* showed larvicidal effect against mosquito larvae.

**Key words**: *Tagetes patula,* root culture, thiophene, larvicidal activity

## Introduction

Plant cell cultures have been studied for the production of various high-value compounds of importance in pharmaceutical, food and chemical industries[1-4]. Hairy root culture is a source to produce root-derived compounds[5,6]. Several production processes of various compounds are being scaled up in bioreactors for large-scale production[7]. The measurement of biomass in the submerged cultivation of cells in liquid medium is an important parameter for monitoring the growth process. Various methods (cell number, fresh wt, dry wt, packed cell and settled cell volume), which are reliable but often pose problems in large-scale cultivation systems, are being used[8, 9].

Marigold (*Tagetes* spp.) is a source of thiophenes, which are the group of heterocyclic sulphurous compounds with strong biocidal activity. Thiophene derivatives[10] are widely distributed in *T. patula, T. erecta* and *T. minuta*. α-Terthienyl, the foremost abundant thiophene, present in all tissues of *T. patula*, was found in low concentration in some callus tissues[11,12]. Thiophene accumulation in hairy root cultures of *T. patula* have been reported and studies on elicitation using fungal elicitors were also conducted[13,14].

This communication reports the growth and thiophene production in hairy root cultures of *T. patula* Linn. The qualitative profile of thiophenes was measured by Flame Ionization Detector (FID) and mass spectral analysis (GC-MS). The bioassay of the hairy root extract is also recorded on mosquito larvae.

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## Materials and Methods

### Initiation of Hairy Roots

The hairy roots (500 mg fresh wt) of *T. patula* were cultured in medium[15] containing 3% sucrose and 1.0% agar in 40 ml liquid medium kept in 150 ml Erlenmeyer flasks on a rotary shaker (90 rpm) at 25°C under dark.

### Measurement of Hairy Root Biomass

Hairy roots were harvested at 3 days intervals. Fresh weight was determined after washing the roots with demineralized water to remove the medium salts, and blotting the excess water on filter paper. The hairy roots were dried in hot air oven at 60°C to calculate the dry weight.

### Extraction of Thiophenes

A known weight of dried material was extracted with hexane for 12 hrs at 20°C in the dark. The crude extracts were filtered and washed with hexane. The filtrates were evaporated to dryness under a current of nitrogen and the residues dissolved in hexane. The purified extracts were stored at 4°C in the dark[16]. Analysis of biomass and thiophene content were carried out every 3 days up to 15 days by High Performance Liquid Chromatography (HPLC) method[17]. This extract and the standard thiophene extract were dissolved in hexane and were used for further confirmation with FID and GC-MS analysis and bioassay studies directly.

#### Gas Liquid Chromatography Methods (GLC)

α-Terthienyl was one of the derivatives of the thiophenes, analyzed by GLC using FID detector and also by GC-MS.

#### *(i) FID*

Thiophenes were separated by GLC using Shimadzu-GC-15A with CR-4 recorder, using FID with open tubular column, SE-30 (5%, length of the column, 3 m; i.d 0.5 mm and column temperature (150-240°C) increasing at 2°C/min. The carrier gas was nitrogen with a flow rate of 30 ml/min. Detector and injection temperatures were 280°C and 250°C, respectively. The retention time of the individual peaks in standard sample and hairy root cultures were compared.

#### *(ii) GC-MS*

To identify the constituents of thiophenes and its concentration, GC-MS analysis was undertaken using GLC (SPD-1), column (SE-30); and column temperature (150-240°C) with an increasing rate of 2°C/min. The carrier gas was helium with flow rate of 1 ml/min. Detector and injection temperatures were 280°C and 250°C respectively. The retention time and molecular weight of the individual peaks in standard sample and hairy root cultures were compared.

#### Mosquito Larvae Bioassay of Thiophenes

The mortality properties of hairy root extracted thiophene were studied in comparison with those of standard thiophenes from the mosquito larvae (*Culex quinquefasciatus*). The hexane extract was evaporated to dryness and re-dissolved in acetone. The authentic thiophenes were also dissolved in acetone. Ten mosquito larvae (1-2 day old) were used for each replicate in a medium of 9.9 ml water (in 30 ml flat bottom culture tubes). An aliquot of thiophenes or extract from dry hairy root extract was taken in 0.1 ml acetone only. Bioassay was adopted as reported earlier[18, 19].

## Results and Discussion

#### Growth of Hairy Roots and Thiophene Production

The maximum accumulation of biomass (0.27g dry wt/40 ml) was recorded on 12th day culture, which is 5.36 times higher in the biomass density over initial day of the culture of 500 mg fresh wt/40 ml culture. Whereas accumulation of thiophene progressively increased and reached maximum (0.06%) on 9th day, but later on the content declined (Fig.1).

#### Analysis of Thiophenes

Liquid chromatographic separation profile of hexane extract of the dried hairy roots indicated the presence of several structurally different thiophenes, α-terthienyl being predominant. The results obtained from HPLC, FID and GC-MS investigations clearly indicated the comparative profile of the thiophenes derived from hairy root cultures and the authentic samples (Figs 2, 3 and 4) with respect to their retention time and molecular weight.

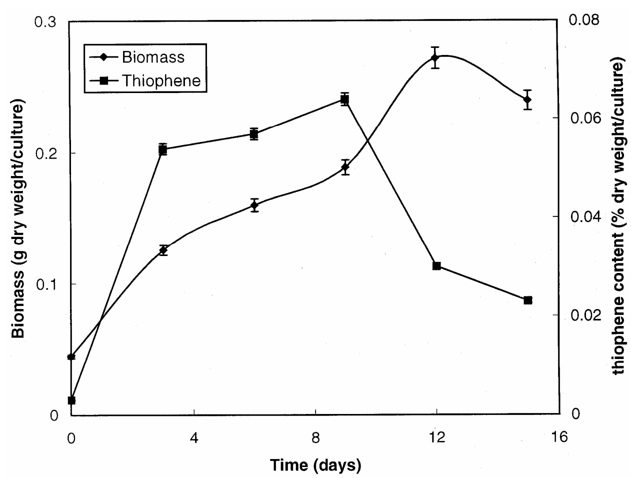


Fig. 1.—Growth and thiophene content in hairy root cultures of *Tagetes patula*

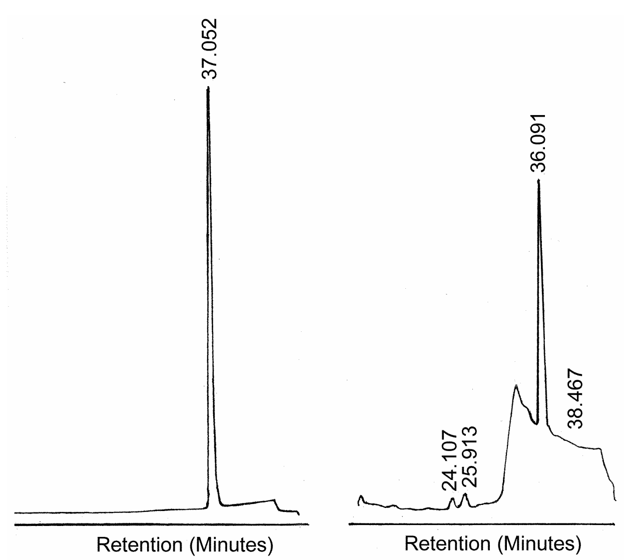


Fig. 2a—FID-profile of authentic α-terthienyl; 2b—FID-profile of hairy root culture extract of *T. patula*

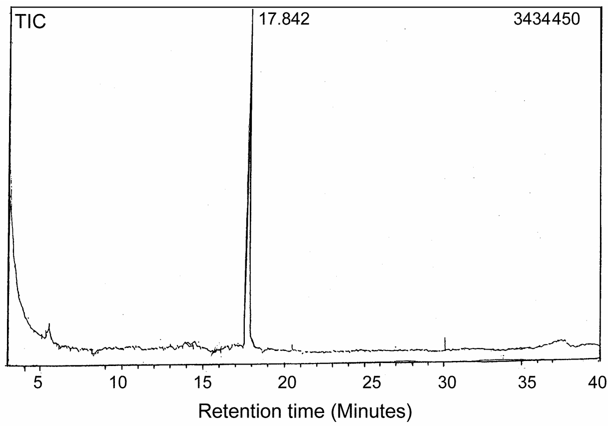


Fig 3a—GC-MS profile of authentic α -terthienyl

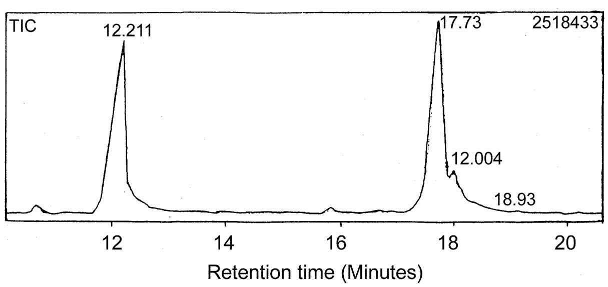


Fig 3b—GC-MS profile of hairy root culture extracts of *T. patula*

**Insecticidal Activity of Hairy Root Extract**

Of phototoxic thiophene insecticides, α-terthienyl has excellent property against mosquito larvae of the genera *Culex, Anopheles* and *Aedes,* as demonstrated in field trials[20] in Canada and Africa. In addition, α-terthienyl is rapidly photo-degraded in the environment, with the half-life[21] of approx 4 hrs and shows virtually no cross-resistance to other pesticides, such as malathion, because of its novel mode of action[22]. Tricyclic thiophenes show great promise as insecticides for disease vector control and as chromotherapeutic agents; quantitative structure-activity relationship analysis has provided an improved understanding of the significance of the contributions made by photochemical properties of the thiophenes to their photo-toxicity[23].

The LC's 50 of 41 tricyclic thiophenes to *Anopheles atropalpus* larvae indicated that 30 of these compounds showed good larvicidal activity with LC's 50 less than 1 ppm or less[24]. The hexane extract of hairy root showed 50% mortality at 0.06 ppm as compared to mosquito larvae which showed 55% mortality at the same level. This result clearly showed that the extract exhibits effective mosquito larvicidal activity as comparable to the standard thiophenes.

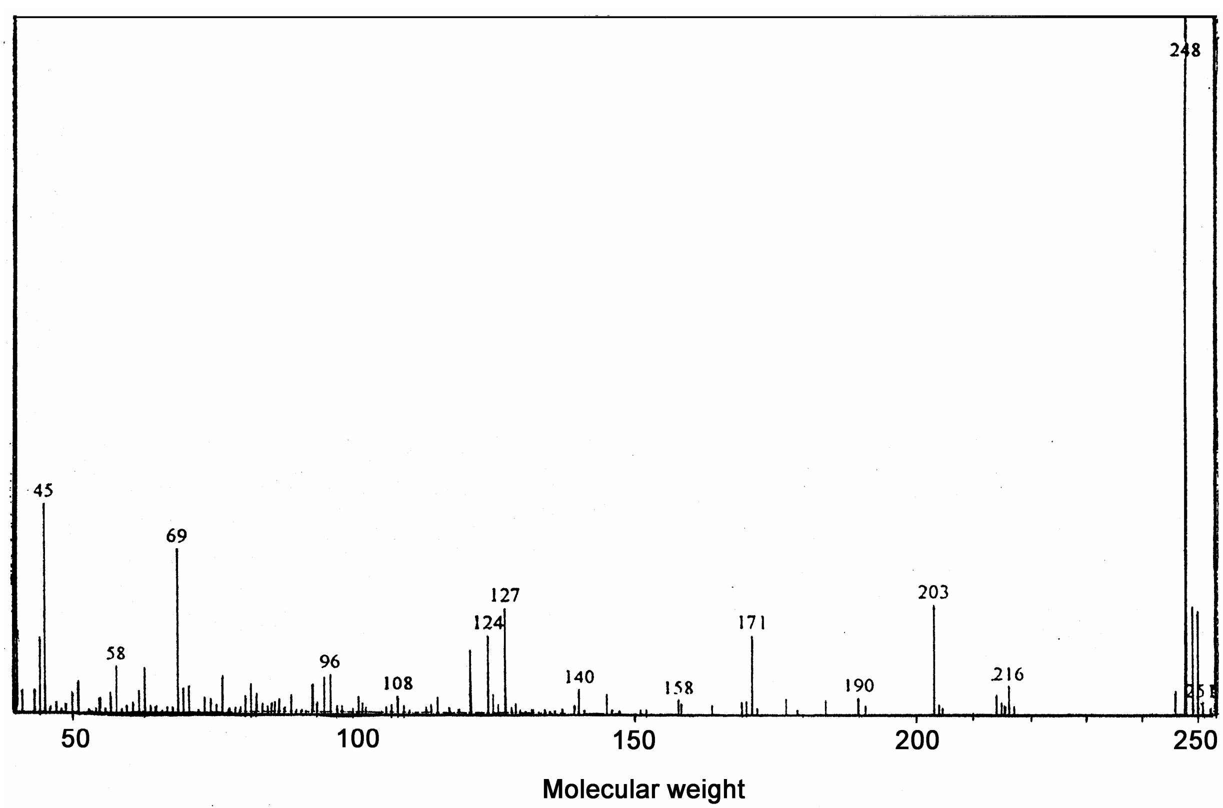


Fig 4a—GC-MS profile of authentic α-terthienyl

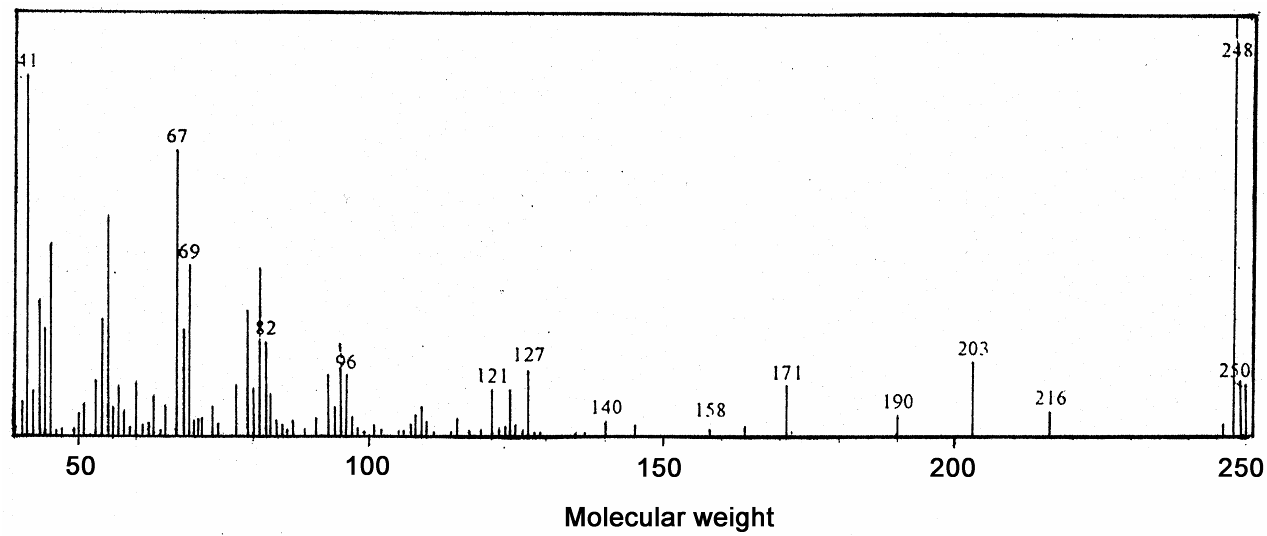


Fig 4b—GC-MS profile of hairy root culture extracts of *T. patula*

## Conclusion

Hairy root cultures of *T. patula* produce thiophenes (0.06% on 9th day of culture) identified by HPLC, FID and GC-MS analysis. The hairy root extract showed mosquito larvicidal activity compared to standard thiophenes.


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