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In vitro production of thiophenes using hairy root cultures of *Tagetes erecta* (L.)

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Marigold (*Tagetes* spp.) is a source of thiophenes, which are a group of heterocyclic sulfurous compounds possessing strong biocidal activity, thus making *Tagetes* plants very useful as natural source of agents for controlling pathogens such as nematodes, insects, fungi and bacteria. Hairy root cultures of *Tagetes erecta* L. were developed using *Agrobacterium rhizogenes* mediated transformation. The hairy root clones showed great variations in growth pattern and total thiophene content (0.31 to 0.96 mg/gfw). Four types of thiophenes that accumulated in root cultures of *Tagetes* were butenenylbithiophene (BBT), hydroxybutenenylbithiophene (BBTOH), acetoxybutenenylbithiophene (BBTOAc) and α-terthienyl (α-T). Total thiophene contents in these isolated rhizoclones were found to be four fold higher than that of wild type hairy root cultures. The developed method of producing hairy cultures of *T. erecta* can be used for producing thiophenes at large scale.

Key words: *Agrobacterium rhizogenes,* marigold, rhizoclones, biocidal.

INTRODUCTION

The biocidal properties attributed to marigold (*Tagetes* species), particularly the nematicidal property, are related to the presence of thiophenes (Chan et al., 1975; Gommers and Geerligs, 1973). Thiophenes are sulphur-heterocyclic compounds found in many plant species (Abegaz, 1991; Hudson et al., 1986) and are well represented in the *Tagetes* species (Bohlmann et al., 1973; Downum and Towers, 1983). Thiophenes act as toxins that are activated by sunlight or UV radiation (300 to 400 nm), killing pathogens such as nematodes, insects, fungi and bacteria (Champagne et al., 1984). Nematodes causes an estimated $100 billion annually in worldwide crop damage making it agriculture largest unmet pest control need (Luc et al., 1990). Nematode

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Abbreviations: BBT, Butenenylbithiophene; **BBTOH,** hydroxybutenenylbithiophene; **BBTOAc,** acetoxybutenenylbithiophene; **α-T,** α-terthienyl; **EPA,** environmental protection agency; **MeBr,** methyl bromide.

control has traditionally depended on highly toxic contact and fumigant pesticides which have now been restricted or eliminated in the United States by the Environmental Protection Agency (EPA). Similar restrictions have occurred in other countries. Chemicals such as organophosphate and carbamate contact nematicides, fenamiphos, carbofuran, aldicarb and fumigants like metam-sodium and 1,3-dichloropropene has been further restricted. Similarly, methyl bromide (MeBr) production and import ended in the U.S.A in 2005 under the Montreal Protocol (Gareau and Dupuis, 2009). Use of the remaining stockpiles requires annual critical use exemptions from the international protocol. Therefore, new classes of nematicidal compounds are constantly being sought, but there are currently no promising materials close to commercial developments. Avermectins, a microbial origin and powerful anthelmintics have been developed for veterinary use. Its efficacy against plant parasitic nematodes is well established, however, it cannot be used successfully as soil treatments because the compound is complex. It is estimated that the current market for nematicides is between $700 million and $1 billion each year worldwide (Global Nematicide Market, 2012).

Hairy root cultures have been proven to be an efficient means for producing secondary metabolites that are normally biosynthesized in roots of differentiated plants and difficult to be synthesized chemically (Hu and Du, 2006). Thiophene accumulation in Plants/calli of different species of *Tagetes* have been recorded by various groups like Croes et al. (1989), Kyo et al. (1990), Menelaou et al. (1991), Mukundan and Hjortso (1991a, b) etc. Rajasekaran et al. (2004) also reported hairy root culture of *Tagetes patula* for production of thiophene. The thiophene produced in hairy roots of *T. patula* showed larvicidal effect against mosquito larvae. Thus, it was evident that organization is a prerequisite for production of thiophene, and hence it was concluded that root cultures would be a suitable alternative to calli/cell suspension cultures. Therefore, this experiment was carried out to establish the hairy root culture of *Tagetes erecta* L. by *Agrobacterium rhizogenes* mediated transformation for *in vitro* production of thiophenes.

MATERIALS AND METHODS

Plant material

Seeds of *T. erecta* were acquired from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. They were surface sterilized with 0.1% mercuric chloride for 90 s, and washed three times with autoclaved distilled water for 10 min each. The disinfected seeds were germinated on media containing MS salts (Murashige and Skoog, 1962) and 30 gL^{-1}sucrose solidified with 0.8% agar. The cultures were incubated at 25±2°C under cool-white fluorescent lamp intensity of 40 µm m^{-2} s^{-1} with a 16 h light/8 h dark cycle (Trypsteen, 1991). The young leaves obtained from *in vitro* grown *T. erecta* plants were used as explants source.

Induction of hairy roots by *A. rhizogenes*

The wild type A4 strain of *A. rhizogenes* harboring an agropine-type pRi A4 was grown in liquid YMB media up to $O.D_{600}$ = 0.9-1.0 (Birot et al., 1987; Hooykass et al., 1977). Transformation with *A. rhizogenes* and hairy root induction was done following a procedure previously reported by Pal et al. (2013). The young leaves were infected by pricking with needle, and loaded with suspension culture of *A. rhizogenes.* The inoculated leaf sections were placed on MS basal medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose and grown in dark. The control leaves were also kept after pricking with sterile needle. Roots produced at the infected sites were excised and placed on semi solid MS medium containing cefotaxime (250 mg L^{-1}). The putative hairy roots were made bacteria free by regular sub-culturing on fresh medium containing cefotaxime. The bacteria free hairy roots (100 mg) were transferred on to liquid ½ MS medium with 20 g L^{-1} sucrose, and incubated with agitation (60 rpm) at 25°C in the dark. The cultures were maintained for 45 days, and the root samples were collected for thiophene estimation at an interval of 5 days from the 15th day onwards (that is, 15, 20, 25, 30, 35, 40 and 45 days). Non-transformed root cultures were also initiated and maintained on same medium by excising roots from aseptically grown non-transformed *T. erecta* plantlets (Figures 1 and 2).

PCR analysis

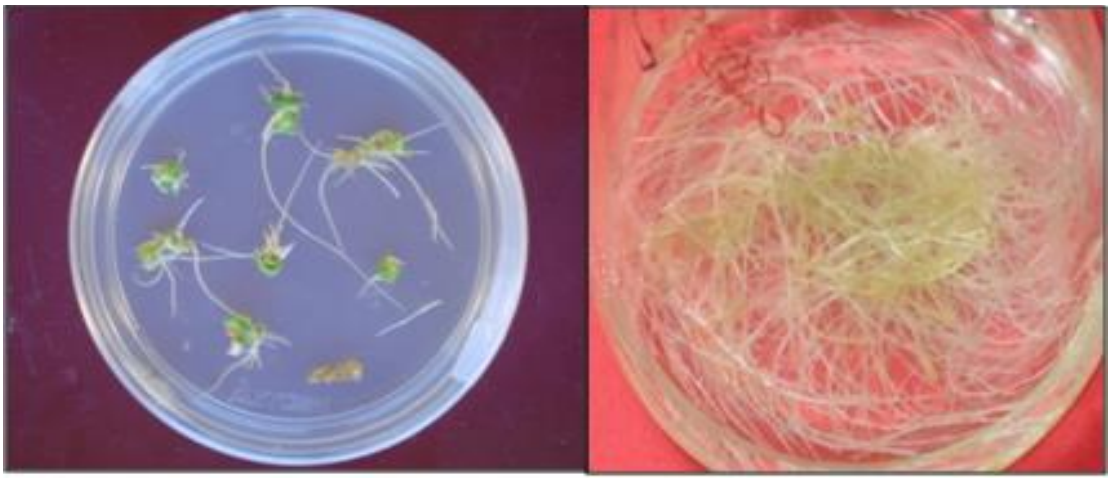
Genomic DNA was extracted from root tissue using CTAB method (Murray and Thompson, 1980), and used as template in polymerase chain reaction. The reaction was carried out using *rol C* (540 bp) gene specific primers (Bulgakov et al., 2005). Each 25 µl reaction mixture contained 1X PCR buffer, 3.5 mM $MgCl_2$, 25 p mol of each forward (5'-ATGGCTGAAGACGACCTGTT-3') and reverse (5'-TTAGCCGATTGAAAACTTGCAC-3') primers with 0.2 mM dNTPs and 1U of Taq DNA polymerase (Bangalore Genei Private Ltd. Bangalore, India), and ~10 ng of genomic DNA was added as template in respective reactions. The reaction with no template served as negative control. Amplification cycle included initial denaturation for 4 min at 94°C, followed by 30 cycles of 45 s denaturation at 94°C, annealing for 60 s at 55°C, extension at 72°C for 120 s and 10 min final extension at 72°C in a programmable peltier thermal cycler (PTC-200, M J Research, USA).

Measurement of biomass

Sampling for biomass measurement was done from the transformed as well as non-transformed root cultures at different intervals (that is, 15th days of culture initiation and thereafter at 5 days interval up to 45 days). In this ways a total of seven samples were drawn (15, 20, 25, 30, 35, 40, and 45 days culture). The fresh weight was determined after washing the roots with deionized water so as to remove the medium salts, and blotting the excess water on filter paper. The experiment was laid out in randomized complete block design and had three replications. The data of fresh weight of transformed as well as non-transformed roots at different time intervals was analyzed using the software SPSS ver. 20.0 and the same is presented as mean ± standard deviation in Figure 3.

Extraction and estimation of thiophenes

The different root samples from different time intervals were dried at 40°C in a hot-air oven. Thiophene extraction and estimation from such dried root samples were carried out according to Rajasekaran et al. (1999). A known weight of dried material was extracted with hexane for 12 h at 20°C in the dark. The crude extracts were



a b

Figure 1. a) Hairy root induction from leaf discs infected with *A. rhizogenes.* **b)** Growth of isolated rhizoclone in liquid MS media.

filtered and washed with hexane. The filtrates were evaporated to dryness and residues dissolved in hexane. The purified extracts were stored at 4°C in the dark. An aliquot of hexane extract of hairy root cultures or standard of thiophenes sample was injected to HPLC column (LC-10A HPLC, SHIMADZU JAPAN), C18 (3.9 × 300 mm) (Bondapack) and run at isocratic condition using solvent mixture of hexane-dioxan (95:5), with flow rate of 2.5 $ml.min^{-1}$ and pump pressure of 125 $kg.cm^{-1}$. Detector (Photodiode array detector) was set at 330 nm. Quantification was done by comparison of peak area with those of authentic standards, that is, 5-(3-buten-1-ynyl)-2,2-bithienyl (BBT), 5-(4-hydroxy-1-butynyl)-2,2- bithienyl (BBTOH) 5-(4-acetoxy-1-butynyl)-2,2-bithienyl (BBTOAc), and α-T (2,20:50,20- terthienyl). The experiment was conducted with three biological replicates and the data is represented as means of the standard deviation.

RESULTS

Induction and culture of transformed hairy roots

After one week of incubation on MS basal medium, roots emerged directly from the sites of infection on leaf discs (Figure 1a). The frequency of root initiation was nearly 80% from leaf explants inoculated with *A. rhizogenes*. A total of 11 hairy root lines were isolated and fast growing hairy root lines were selected for further analysis. The developed hairy roots showed rapid growth with extensive lateral branching (Figure 1b). The cultures adapted well to liquid medium. Great morphological variations were evident among the isolated rhizoclones (Figure 3 and 4). Inoculation was done with 100 mg fresh root sample of both the transformed as well as non-transformed lines. Growth of the root cultures was in exponential phase till 15 days. From 20 days onwards, cultures were in log phase. Accumulation of biomass showed a specific growth rate reaching maximum until 45 days (Figure 3).

PCR detection and opine assay for confirmation of transgenic hairy roots

Genomic DNA isolated from putative transgenic roots yielded expected size of 540 bp PCR amplification with *rol C* gene specific primers. Non template control (ntc) and wild type roots (W) did not produce any amplification (Figure 5). Thus, there was stable integration and expression of *rol* genes in transformed hairy roots.

Thiophene accumulation

Thiophene production was observed in all the rhizoclones, that is, Tr1- 4 and wild type, and NTr hairy roots. Chromatographic separation of hexane extracts showed at least four peaks representing all four structurally different thiophenes, that is, BBT, BBTOH, BBTOAc, and a-T (Figures 1 and 2). In all cases, thiophene content increased rapidly and reached to peak level in 15 day cultures, and thereafter declined gradually till 45 days (Figure 6). Thiophene accumulation pattern showed a growth associated correlation, with a maximum thiophene content on the 15^{th} day which were in exponential phase. The decline of thiophene content was found in cultures in log phase. However, total thiophene content in some of the transformed clones was nearly 4 times higher than that of wild type roots (Figure 6). The maximum thiophene content was found in line Tr-2 while the minimum thiophene content was found in line Tr-4 as compared to the non-transformed (NTr) control line.

DISCUSSION

Thiophenes found in Marigold plants make them

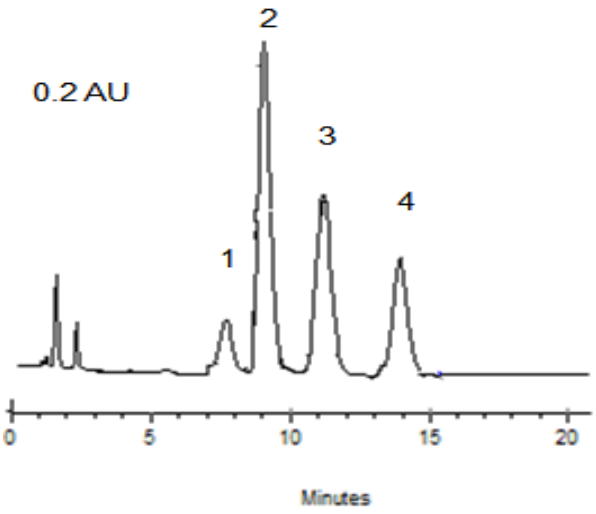


Figure 2. HPLC profile of *T. erecta* rhizoclone extracts. Peaks numbered as 1-4 corresponds to various thiophenes, that is, BBT, BBTOH, BBTOAc, and a-T respectively.

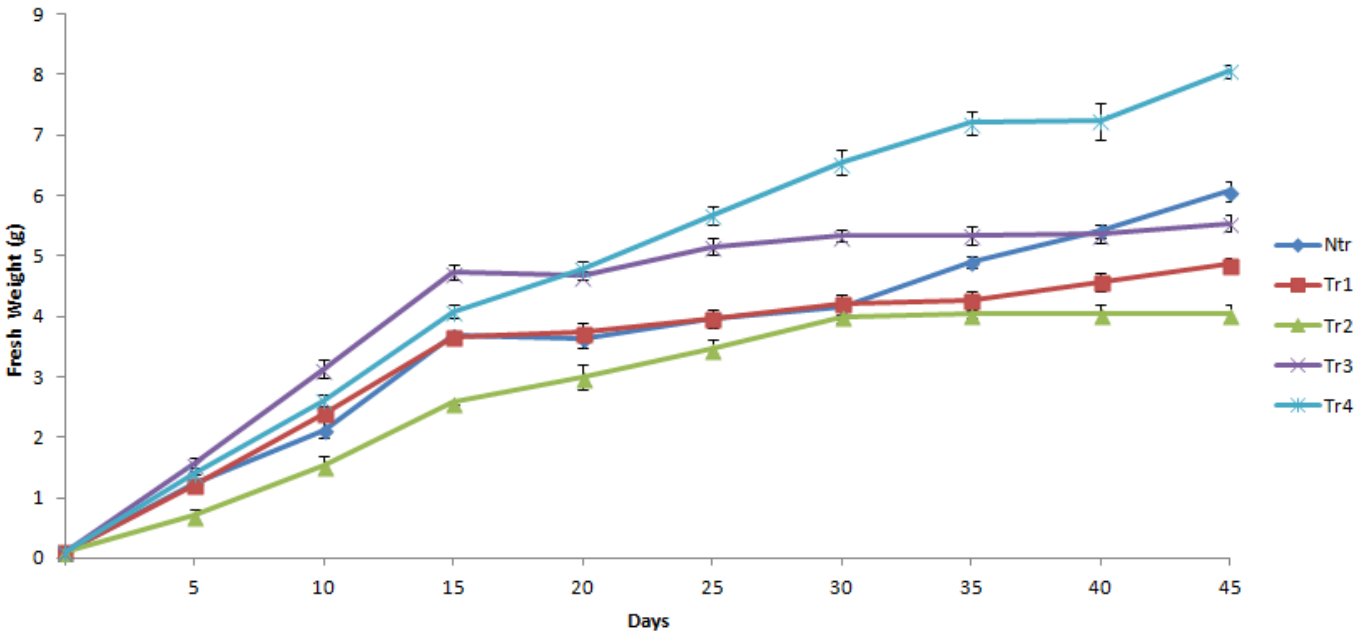


Figure 3. Growth pattern of hairy root clones of *T. erecta.* Ntr is nontransgenic /wild type root cultures; Tr1- Tr4 are transgenic hairy root clones.

attractive potential candidates for the development of plant based biopesticides. In the present work, we have attempted to establish the method of *in vitro* production of thiophenes by hairy root cultures of *T. erecta* plant. The PCR analysis (Figure 5) indicated the presence of *rol C* gene, a part of the *Agrobacterium* pRi T_L–DNA and T_R–DNA, in the host plant genome (Chilton et al., 1982; White et al., 1982). The fast growth and easy maintenance of hairy root cultures are advantageous to be used as continuous sources for the production of valuable secondary metabolites. Therefore, prove economical for commercial production purposes. All four structurally different thiophenes detected by us in *T. erecta* (Figures 1 and 2) were earlier reported in other *Tagetes* species, that is, *Tagetes laxa*, *Tagetes terniflora*, *Tagetes minuta* and *Tagetes campanulata*. (Rodriguez

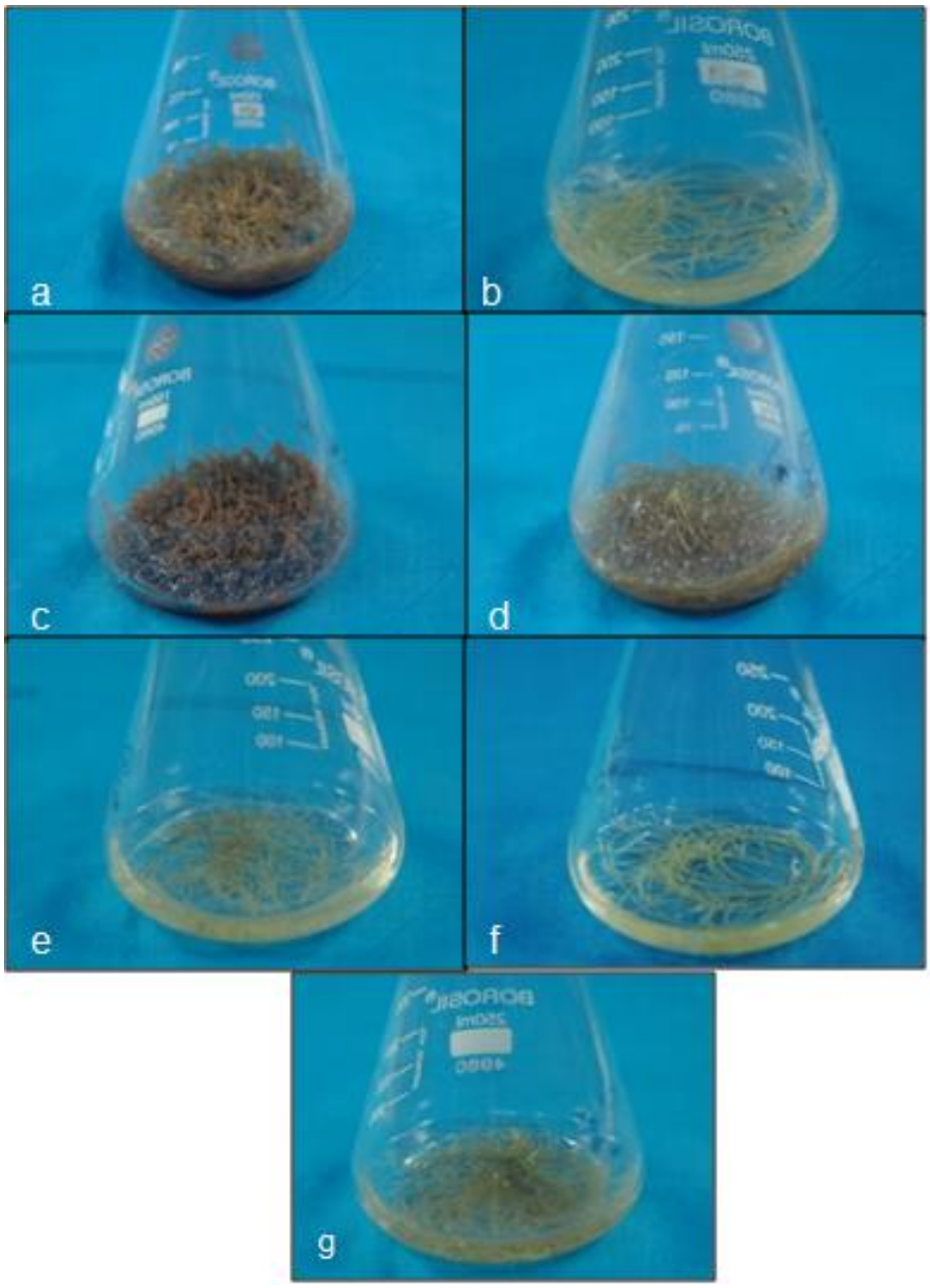


Figure 4. Various rhizoclones of *T. erecta*, that is, a-g, showing morphological variations.

Talou et al., 1994), but it differed from *Tagetes filifolia* where BBT (OAc) 2 type of thiophene was reported (Massera et al., 1998). In our studies marked differences in growth rate and root morphology were observed in root cultures (Figure 3 and 4). These morphological differences could be because of variation of auxin levels. Since *Agrobacterium* strains carry genes encoding auxins synthesis, this might be linked to variation in auxin levels which results in morphological variations (Croes et al., 1989; Arroo et al., 1995). *Agrobacterium rol* and *aux* genes are involved in rhizogenesis through the modification of plant cell growth and developmental regulation and these genes are located in the TR-DNA and TL-DNA regions of the Ri (root-inducing) plasmid of *A. rhizogenes* agropine strains. Some of the genes are also involved in auxin biosynthesis and/or auxin sensitivity, that cause differences in hairy root growth and morphology when compared to the non-transformed roots (Meyer et al., 2000; Christey, 2001). Very often noteworthy phenotypic and growth variations have been observed among hairy root clones which are derived from independent transformation events. In most cases, the

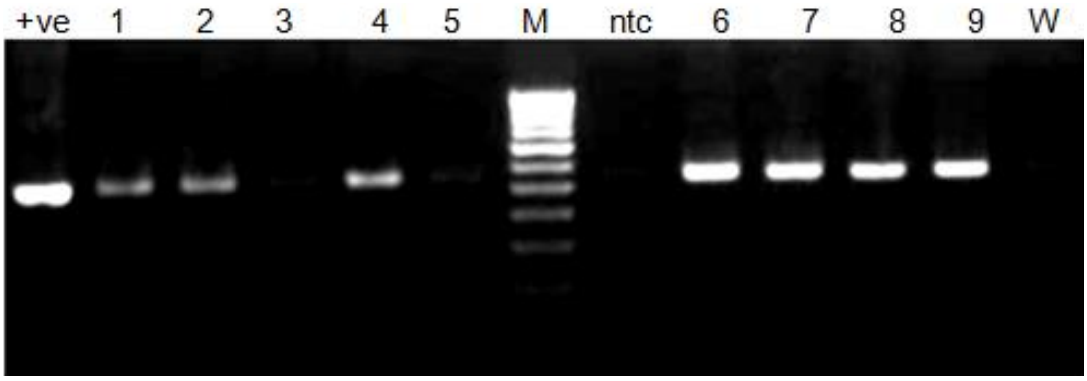


Figure 5. PCR detection of *rol C* gene from transformed roots. *+ve* is the plasmid DNA, lanes 1 to 5 and 6 to 9 are the rhizoclones, ntc is the no template control; *W* is the wild type hairy roots and *M* is the DNA marker.

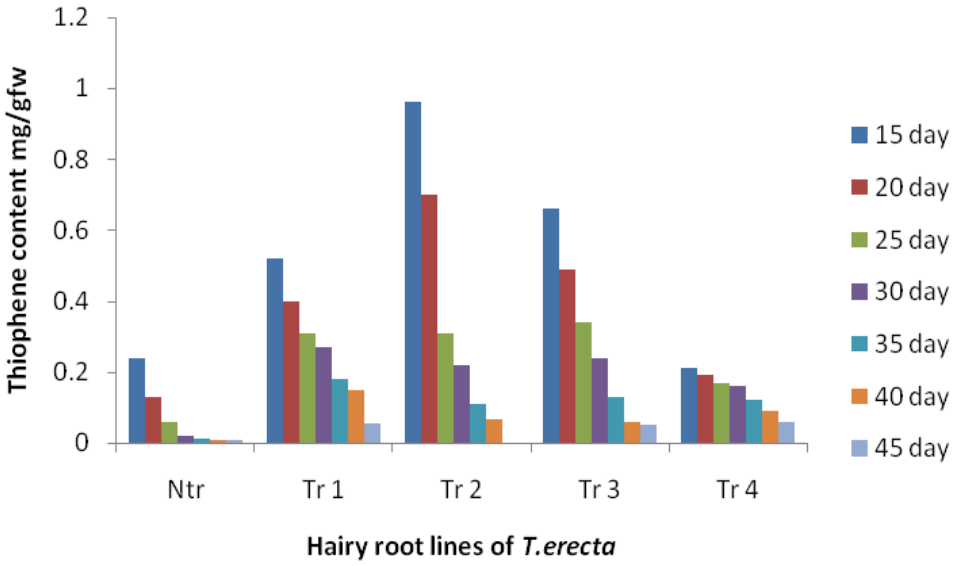


Figure 6. Accumulation of thiophene contents in *T. erecta* root cultures at various time intervals.

variability that generally affects the branching intensity, the root diameter and growth rate has been visually described. Differences in the integration of genes from the TL-DNA and TR-DNA regions of *A. rhizogenes* in the host genome are considered to be the major cause of phenotype variations (Ambros et al., 1986; Mano et al., 1986, 1989; Jouanin et al., 1987). In our results the hairy root cultures showed profuse lateral branching (Figures 3 and 4). Auxins in the presence of sucrose are known to stimulate lateral branching (Reed et al., 1998). Abundant lateral branching has very often been mentioned as one of the most typical traits within the altered phenotype of hairy roots (Tepfer, 1984; Spano et al., 1988; Guivarch et al., 1999). Our results shows that growth rate of hairy root cultures was in exponential phase till 15 days; similar results were also obtained by Mukundan and Hjortso (1991a) in *T. patula* where a well-defined exponential growth phase was identified during the initial growth phase (Bajaj, 2001). Although, the qualitative growth dynamics of the transformed *Tagetes* roots is similar to that of microbial culture or cell cultures, a quantitative description of the root growth kinetics cannot rely uncritically on models that have been developed primarily for microbial cultures. Hairy root growth data are not always so simple that single kinetic parameter, such as a specific growth rate can adequately describe them. Accumulation of biomass showed a specific growth rate reaching maximum till 45 days and thus became stationary which might be due to the depletion of limiting nutrients (Asha and Nutan, 2004). The highest level of

thiophene accumulation was found in all rhizoclones was 0.96 mg/g (Figure 6). The variations observed in thiophene accumulation in different clones could also be ascribed to plant tissue reaction to auxins. Because T-DNA integration takes place at random site into the host plant genome, the resulting rhizoclones often show different accumulation patterns of secondary metabolites (Mano et al., 1989; Rodriguez Talou et al., 1994). On the basis of the present results, 15 day hairy root cultures appear to be the best stage for the *in vitro* production of thiophenes. Further improvements might be helpful for commercial production purpose, such as screening large number of rhizoclones, optimization of various strains of *A. rhizogenus* and various media composition since the chemical synthesis of thiophenes is not economically possible (Ketel, 1987) and absence of any commercially available nematicidal compounds makes this study very useful.

Conflict of Interests

The authors have not declared any conflict of interest.

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