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Production of Thiophene from *Tagetus patula*

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Abstract

Thiophene chemistry proved to be very interesting for several industrial branches. The thiophene moiety was found to have larvicidal action on mosquito larvae. Thiophene is present in the leaves of *T. patula*. It was extracted from leaves by solvent extraction and also by tissue culture. The results were analysed by Spectrophotometer, Fourier Transform Infrared Spectroscopy and larvicidal action.

Keywords: Thiophene; Leaves; Tissue culture

Introduction

Plant derived products are gaining importance in the field of biotechnology [1]. Apart from various advantages compared to synthetic products they provide low cost drugs and vaccines. But commercializing of these products is minimal due to lack of good manufacturing practices to field grown plants.

In the past years the plant kingdom has been of great interest as a source of potential of insecticidal products. In addition natural insecticides can provide core structures from which new insecticidal agents can be synthesized. Although the natural products are very effective against many insects, their synthetic preparation is not that efficacious. Plant insecticides still represent only a small fraction of the insecticidal material used very year. However as a consequence of stricter environmental legislation, increased resistance of pests to synthetic pesticides, growing residue awareness among the consumers, mounting industrial research and the development cost of chemical insecticides, there has been shift towards the interest for the use of natural insecticides. Thiophene is a photosensitive natural insecticide which is really effective and has many applications both in industrial and in pharmaceutical products.

The leaf tissue of *T. patula* was used to initiate the callus growth and the medium used was the Murashige and Skoog's with 2-4 dichlorophenoxyacetic acid and kinetin [2]. Maximum thiophene content and maximum biomass accumulation was recorded.

Materials Required

Marigold stem and leaf branches, Distilled water, Methanol, Hexane, 20.20 blade, solvent extractor, Bavistin, 0.1% $HgCl_2$, Hitastin, Glass Jar (250 ml), Soxhlet apparatus, MS medium.

Procedure

Media preparation

MS Medium (Stock): Medium prepared in varying concentration of hormones. Murashige and Skoog's medium was prepared using Macronutrients Stock 1 (Table 1), Macronutrients Stock 2 (Table 2), Macronutrients Stock 3 (Table 3), Micronutrients Stock 4 (Table 4) and Vitamins (Table 5). The medium for tissue culture contained macronutrients, micronutrients and vitamins. The components of MS medium (Stock 1) are ammonium nitrate, potassium nitrate, potassium dihydrogen phosphate and magnesium sulphate. Specific amounts of each were measured and the solution was made upto 1 L using distilled water. Stock 2 contained calcium chloride. Stock 3 contained disodium salt of EDTA and ferrous sulphate hydrate. Stock 4 contained Magnesium sulphate tetrahydrate, zinc sulphate, boric acid, Potassium iodide, disodium molybdate, cuprous sulphate pentahydrate and cobalt chloride. Stock 5 contained glycine vitamins nicotinic acid, pyridoxine HCL and thiamine.

Media preparation: Stock solution from 1-5 were mixed in specific amounts. 20 ml of stock I, 20 ml of stock II, 10 ml of stock III, 10 ml

Components	Weight (g/l)
Ammonium nitrate	82.5
Potassium nitrate	95
Potassium dihydrogen phosphate	8.5
Magnesium sulphate	18.5

Murashige and Skoog's MS medium, Composition (ml/l).

Table 1: Macronutrients (Stock : I).

Component	Weight (g/l)
Calcium chloride	22

Table 2: Macronutrients (Stock: II).

Components	Weight (g/l)
Disodium salt of EDTA	3.7
Ferrous sulphate hydrate	2.8

Table 3: Macronutrients (stock: III 20ml).

Components	Weight (g/l)
Magnesium sulphate tetra hydrate	2.23
Zinc sulphate	0.860
Boric acid	0.620
Potassium iodide	0.083
Disodium molybdate	0.02
Cuprous sulphate penta hydrate	0.002
Cobalt chloride	0.002

Table 4: Micronutrients (stock: IV 10ml).

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Components	Weight (mg/l)
Glycine	200
Nicotinic acid	50
Pyridoxine HCl	50
Thymine HCl	10

Table 5: Vitamins (stock: V 10ml).

	Kinetin	2,4-D
Media 1	0.1 mg	0.2 mg
Media 2	0.2 mg	0.4 mg
Media 3	0.3 mg	0.6 mg
Media 4	0.4 mg	0.075 mg

Table 6: Concentration of hormones.

stock IV, 10 ml of stock V Added 1% myo- inositol, 3% sucrose and different concentration of hormones: kinetin and 2-4 D (Table 6) were added with pH maintained at 5.8. Four medias of varying hormone concentration were prepared for callus culture.

Tissue Culture

Sterilization

The shoots of the Marigold plant were sterilized using disinfectant Bavistin. Shoots were cut at 2 internodes interval. Branches were placed in separate glass jars of 250 ml capacity. Used autoclaved sterile water and kept swirling continuously to remove any remaining dirt. Then drain the water and fill the jar till half with mercuric chloride (0.1%) and water and keep swirling for 5 minutes. Again drain out the liquid mixture and add sterile water and swirled for 5 minutes twice.

Initiation

Switch on the UV for 20 minutes before entering the lab. Surface sterilized the laminar hood (top and table) with ethanol. Sterilized all equipment to be used in the steripot (SHIMAS).The leaves were cut a bit away from the node [3] and put the leaf in various bottles containing full MS medium with same hormonal composition.

Subculture

The calluses obtained in different bottles were collected. Glass jars of 250 ml capacity were filled with about 100 ml of MS medium of varying hormonal concentration. The callus were then inserted a bit into different test media (1,2,3 and 4) with mouth facing the HEPA filter to avoid contamination. This procedure was repeated for two weeks [4] (Figure 1) shows the callus grown in medium, (Figures 2-4) show the callus growth observed on the fourth, eighth and twelfth day.

Solvent extraction

Fresh leaves of Marigold were taken and sterilized with distilled water. 500 ml conical flask was filled with about 150 ml of hexane. The soxhlet apparatus was set and water connection to top and bottom of the condenser was given. Temperature was set at about 65°C. Extraction was continued for about 6 hours. Light pale yellow color extract was obtained in the conical flask (Figure 5). Stored the extract in dark bottles as thiophene is photoactive. After the initial extract was concentrated, it was again redissolved in hexane. The above procedure was repeated for callus of *T. patula*. Fourier Transform Infrared Analysis was done analysis for detection of thiophene.

Results and Discussion

The callus growth was observed in Media 1, 2, 3 and 4 (Figures 6-9). The figures show the size of the callus grown on four Medias. It was observed that the callus biomass was the highest in the media 4 containing 0.4 mg kinetin and 0.075 mg 2-4 D.

Spectrophotometric analysis (Uv)

Peak was observed in the range 200-250 nm for both extract of *T. patula* callus as well as *T. patula* leaves. The peak in the range of 200-250 among other peaks was similar to the standard data for thiophene [5].

Figure 1: Test-tube containing callus.

Figure 2: Callus observed on the fourth day.

Figure 3: Callus observed on the eighth and twelfth day.

Figure 4: Callus observed on the eighth and twelfth day.

Figure 5: Marigold leaf extract with Hexane.

Figure 6: Media 1.

Figure 7: Media 2.

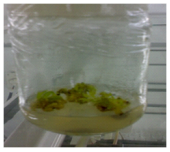

Figure 8: Media 3.

Figure 9: Media 4.

Ft-Ir Spectrometric analysis

The various derivatives of thiophene were observed at different wave numbers [6].

Extract 1

Callus culture of *T. patula* extracted with hexane as solvent was subjected to FT-IR spectroscopy. The result was as obtained in the table 7. The derivatives of hexane were observed at wave numbers in the range of 2969-2965/cm, 2929-2912/cm, 2884-2883/cm, 2861-2849/cm, the compound observed was n-alkalene. At 1466-1468/cm n-alkanes were observed.

Thiophene derivatives were present from 1375-688/cm. At 1375-1360/cm, 1172-1165/cm, 1145-1165/cm, 1070-1040/cm, 1040-990/cm, 984.30/cm, 884.59/cm, 818/cm, 760-650/cm, 730- 720/cm and 688/cm, the derivatives of thiophene: aliphatics sulfonates, pyrazoles, benzene, thiophene, tetra hydropyran, tert-butyl group, primary chloroalkanes and tetra hydro thiophene were present respectively.

Extract 2

Leaves of *T. patula* extracted with hexane as solvent and subjected to FT-IR spectroscopy. The result was as obtained in the table 8. The derivatives of hexane were observed at wave numbers in the range 2969-2965/cm, 2929-2912/cm, 2884-2883/cm, 2861-2849/cm the compound observed was n-alkalene. At 1466-1468/cm n-alkanes was observed.

Thiophene derivatives were present from 1375-688/cm. At 1375-1360/cm, 1282-1275/cm, 1280-1240/cm, 1145-1165/cm, 1070-1040/cm, 1040-990/cm, 1010-990/cm, 984.30/cm, 951.65/cm, 884.59/cm, 818/cm, 760-650/cm and 730-720/cm represent derivatives of thiophene: secondary nitro alkanes, alkyl nitrates, epoxy derivatives, dialkyl sulfones, aliphatic sulfoxides, pyrazoles, Mono meta and 1,3,5 substituted benzene, benzene, methyl sulphonate, thiophene, tetra hydropyran, tert-butyl group and primary chloroalkanes were present respectively.

Larvicidal test

The solvent extract of *T. patula* callus and the leaves were used for

WAVE NUMBER (FREQUENCY,/Cm)	VIBRATION	COMPOUND
2969-2965	Anti symmetric	n-alkalene
2929-2912	Antisymmetric CH_2 stretch	n-alkalene
2884-2883	Symmetric CH_3 stretch	n-alkalene
2861-2849	Symmetric CH_2 stretch	n-alkalene
1466-1468	CH_3 deformation	n-alkanes
1375-1360	Symmetric NO_2 stretch	Secondary nitro alkanes
1282-1275	Symmetric NO_2 stretch	Alkyl nitrates
1280-1240	Ring stretch	Epoxy derivatives
1172-1165	Symmetric SO_2 stretch	Alkyl sulfonates
1145-1125	Symmetric SO_2 stretch	Dialkyl sulfones
1070-1040	S=O stretch(1 or 2 bands)	Aliphatic sulfoxides
1040-990	Ring vibrations	Pyrazoles
984.30	Ring breathing	Benzene
951.65	S bond CH_3 rocking vibration	
884.59	Weak vibration	Thiophene
818	Ring breathing	Tetra hydro pyran
760-650	Symmetrical skeletal stretching	Tert-butyl group
730-720	Ccl stretch PC conformation	Primary chloroalkanes
688	Ring breathing	Tetra hydro thiophene

Table 7: FT-IR analysis of *T. patula* leaf callus.

WAVE NUMBER (FREQUENCY,/Cm)	VIBRATION	COMPOUND
2969-2965	Anti symmetric	n-alkalene
2929-2912	Antisymmetric CH_2 stretch	n-alkalene
2884-2883	Symmetric CH_3 stretch	n-alkalene
2861-2849	Symmetric CH_2 stretch	n-alkalene
1466-1468	CH_3 deformation	n-alkanes
1375-1360	Symmetric NO_2 stretch	Secondary nitro alkanes
1282-1275	Symmetric NO_2 stretch	Alkyl nitrates
1280-1240	Ring stretch	Epoxy derivatives
1145-1125	Symmetric SO_2 strectch	Dialkyl sulfones
1070-1040	S=O stretch(1 or 2 bands)	Aliphatic sulfoxides
1040-990	Ring vibrations	Pyrazoles
1010-990	Triagonal ring breathing	Mono meta and 1,3,5 substituted benzene
984.30	Ring breathing	Benzene
951.65	S bond CH_3 rocking vibration	Methyl Sulphonate
884.59	Weak vibration	Thiophene
818	Ring breathing	Tetra hydro pyran
760-650	Symmetrical skeletal stretching	Tert-butyl group
730-720	CCl stretch PC conformation	Primary chloroalkanes

Table 8: FT-IR analysis of *T. patula* leaves.

the larvicidal test. The mortality rate of mosquito larvae was observed to be 80% for callus and 60% for *T. patula* leaves.

Conclusion

From the above results we came to the conclusion that media 4 containing higher amounts of kinetin and lower amounts of 2-4 D produced callus of large biomass as compared to media 1,2 and 3.The amount of thiophene present in the callus is directly proportional to the biomass. Hence maximum thiophene content was present in the Media 4 calluses observed on the 12th day.

The UV spectrophometric analysis showed the present of thiophene and its derivatives from 200-250 nm in both hexane extract of callus and leaves as compared to standard data.

The results of FT-IR analysis showed that thiophene and their derivative were present in both the leaf calluses of *T. patula* and in the leaves of *T. patula*. From the FT-IR analysis showed the presence of thiophene at 884.59/cm in both the callus and leaves extract.

Higher percentage of larvicidal action was observed in callus compared to that of leaves. The results of FT-IR analysis showed that thiophene and their derivatives were present in both the leaf callus of *T. patula* and in the leaves of *T. patula*. Thiophene acts specifically on superoxide dismutase enzyme present in the gut of mosquito and thereby leading to its death. The larvicidal action was observed to be 80% for leaf calluses and 60% for leaves of *T. patula*. The prominence of thiophene in callus than in the leaves was observed. The larvicidal action would help in repressing diseases such as malaria, dengue, chikungunya caused by mosquito as vector.

Hence thiophene presence was confirmed in callus and leaves by UV spectrophotometer analysis, FT-IR analysis and Larvicidal test.

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