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Production of Thiophenes in Calli and Suspension Cultures of *Tagetes patula* L. as Influenced by Light/Dark Succession.

By

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With 3 Figures

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Summary

POLI F., TOSI B., DALL'OLIO G. & BRUNI A. 1992. Production of thiophenes in calli and suspension cultures of *Tagetes patula* L. as influenced by light/dark succession. – *Phyton* (Horn, Austria) 31 (2): 257–262, 3 figures. – English with German summary.

HPTLC-densitometry was used to evaluate thiophene accumulation in leaf calli and cell suspension of *Tagetes patula*. Calli obtained from 7–8 weeks old leaves were subcultured in supplemented MS for many months. The secondary calli showed 4 to 6-fold higher fresh weight increment in 2 weeks while the tertiary ones only doubled their weight in the same period. A considerable increase of thiophene production (about 16.5 -fold) was obtained growing calli under successive light/dark periods. Repeated subculturing of callus tissues resulted in a slow decline of the thiophene yield. Under the same conditions, cell suspension cultures did not exhibit an appreciable increment in thiophene content which decreased with subculturing.

Zusammenfassung

POLI F., TOSI B., DALL'OLIO G. & BRUNI A. 1992. Thiophenproduktion beeinflusst durch Licht/Dunkel-Abfolge in Kallus- und Suspensionskulturen von *Tagetes patula* L. – *Phyton* (Horn, Austria) 31 (2): 257–262, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Thiophenakkumulation in Blatt-Kallus- und Zellsuspensionskulturen von *Tagetes patula* wurde mittels HPTLC-Densitometrie bestimmt. Die Kalli, gewonnen von 7–8 Wochen alten Blättern, wurden mehrere Monate in einem erweiterten MS-

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Medium kultiviert. Die sekundären Kalli zeigten ein 4–6fach höheren Frischgewichtszuwachs in 2 Wochen, wogegen die tertiären Kalli ihr Gewicht im selben Zeitraum bloß verdoppelten. Eine bedeutende Zunahme der Thiophen-Produktion (um das 16,5fache) wurde bei Kalli unter wechselnder Licht/Dunkel-Abfolge erzielt. Wiederholtes Übertragen von Kallusgeweben führte zu einer schwachen Abnahme der Thiophenausbeute. Unter den selben Bedingungen zeigten Zellsuspensionskulturen keine nennenswerte Zunahme im Thiophengehalt, welche mit fortgesetzter Kultur abnahm.

Introduction

Natural or artificial thiophenes are secondary compounds that may function as biocides (GOMMERS & al. 1981). They were frequently found in *Tagetes* species (marigolds) where their accumulation changes according to the physiological state of plants and during ontogenesis (SÜTFELD 1982, TOSI & al. 1988). Lately, the studies on thiophene production by *in vitro* cultures were performed with the aim to select criteria for the choice of explants having a high thiophene production (KETEL 1986, 1987). In particular, cell cultures of *Tagetes erecta*, *T. patula* and *T. minuta* were carried out to evaluate thiophene accumulation in connection with the morphogenic potency, the cell specialization, and the release of compounds into the medium (KETEL & BRETELER 1988). However, despite a lot of promising results (see CROES & al. 1989), the optimization of cultures having a high thiophene production is yet far from being achieved. We herewith report a re-examination of the yield of the four most abundant thiophenes [5-(4-hydroxy-1-butenyl)-2,2'-bithienyl, BBTOH; 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl, BBTOAC; 5-(3-buten-1-enyl)-2,2'-bithienyl, BBT; 2,2' : 5',2"-terthienyl, α -T] from calli and cell suspension cultures of *Tagetes patula* cv. "Petit Gold" in different stages of growth. Particular attention was devoted to the influence of light on the thiophene content and to the optimization of high performance thin-layer chromatography (HPTLC) densitometric quantification as a quick procedure for thiophene screening.

Materials and Methods

Plant material and callus production.

Seeds of *Tagetes patula* cv. "Petit Gold", purchased from the market were left to germinate on moistened paper in the dark. The seedlings were then grown in pot with common soil in a phytotrone (Heraeus, VEPHQ 5/1350) for a photoperiod of 16 h (10,000 lux) at 20 ± 1 °C and with $80 \pm 10\%$ of humidity. After 7–8 weeks leaves were surface-sterilized in ethanol (70%) and NaCl (5%) and placed on MS (MURASHIGE & SKOOG 1962) medium supplemented with 2% sucrose, 0.5 mg/1 NAA (naphthaleneacetic acid) and 5 mg/1 BA (6-benzyladenine) and solidified with 0.8% (w/v) agar (Difco). The incubation conditions were as follows: temperature 25 ± 1 °C, photoperiod 16 h, light 5,000 lux (Philips T4D58W/25 lamps). When formed, primary calli were subcultured after 3 weeks transferring a half of callus on the same medium.

Successively, calli were subcultured every 2 weeks. At least 30 calli were used and after weighing processed for thiophene analysis.

For evaluating the influence of the alternation of light and dark in the production of thiophenes, portions of the same callus line were cut and processed as shown in Fig. 3.

Cell suspension cultures

Cell suspension cultures were started from tertiary callus tissue. To subculture, every 3 weeks, 2.5 g of cells were inoculated in 40 ml of fresh MS medium (supplemented as previously specified) in 250 ml Erlenmeyer flasks on a rotary shaker (120 rpm) under 5,000 lux (16 h photoperiod). MS media supplemented in different manner were also tested, but no appreciable enhancement of cell growth and thiophene production was gained.

Thiophene analysis

Extraction of thiophenes was carried out under dimmed room light on calli samples at different stages of growth and on cell suspensions (0.2–2 g, f. w.). The extraction and quali/quantitative analysis of thiophenes were performed following the operating conditions reported in Tosi & al. (1988).

Results and Discussion

Calli obtained from 7–8 weeks old leaves of *Tagetes patula* were subcultured for many months in a MS medium supplemented with NAA and BA. Other media with different hormone combinations did not improve

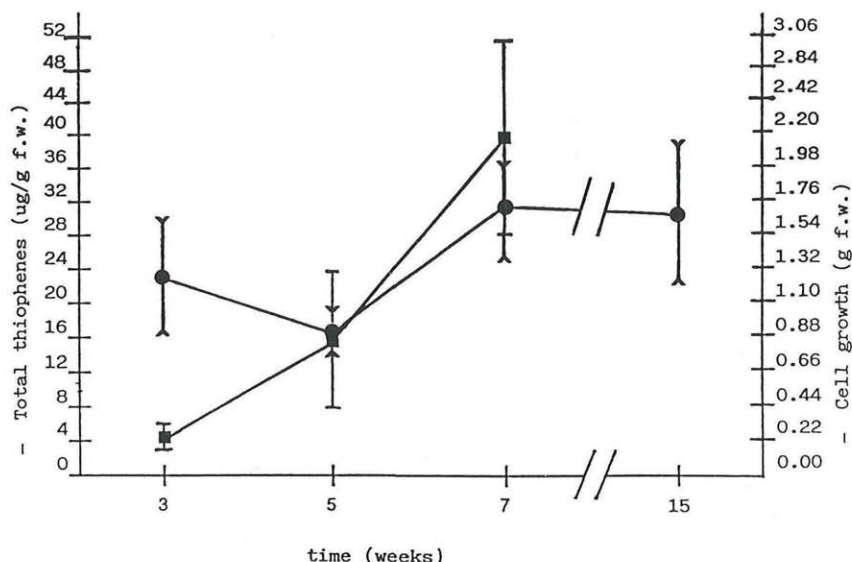


Fig. 1: Total thiophene production (●-●) and timecourse of cell growth (■-■) in *T. patula* calli. The data are the average of 40 determinations.

callus growth and thiophene yield. The fresh weight of secondary callus increased 4 to 6-fold in 2 weeks, whereas that of tertiary calli only doubled in the same period. However, a large variability in the cell growth rate was observed as shown by the high standard deviation (Fig. 1). Calli were cauliflower-like, green-yellowish and friable. No sample of calli was devoid of thiophenes even though a wide variation in their yield was found.

The total thiophene production initially decreased until the 5th week, then increased gaining the maximum on the 7th week. Calli at the 3rd passage showed on the average more chemicals than secondary and primary calli. After 7 subcultures the thiophene content was stationary (Fig. 1). Repeated subculturing of callus tissues resulted in a slow decline in thiophene yield and after 20 subculturings the thiophene amount was reduced 5-10-fold respect to the 3rd subculture.

The contents of the four most abundant thiophenes of the calli were more heterogeneous as shown by the high standard deviation. However, there was a tendency to increase of BBTOH and BBTOAC with subculturing (after 15 weeks the means values are $12.2 \pm 8.8 \mu\text{g/g f. w.}$ and $14.1 \pm 8.6 \mu\text{g/g f. w.}$ respectively), whereas BBT and α -T were present in low concentration and were tending downwards ($3.1 \pm 3.0 \mu\text{g/g f. w.}$ and $0.8 \pm 0.8 \mu\text{g/g f. w.}$ respectively) (Fig. 2).

Our data are in agreement with the pattern reported in literature to the age of the explants (KETEL 1987, NORTON & al. 1985, CROES & al. 1988). It was confirmed that both BBTOH and BBTOAC are the most abundant thiophenes of *Tagetes patula in vitro* cultures.

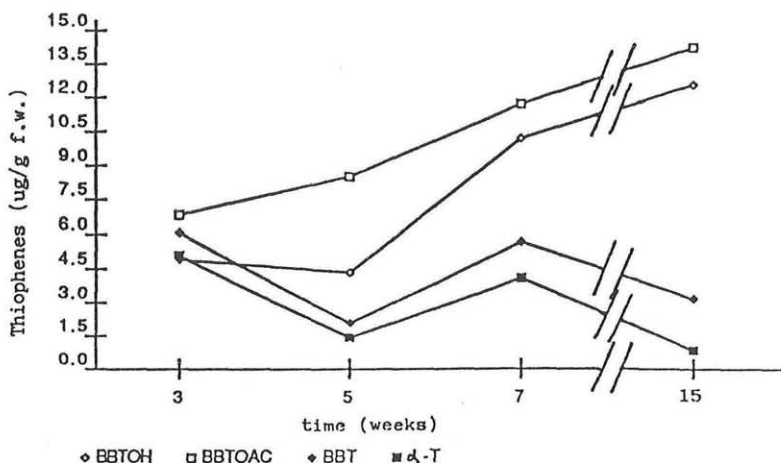


Fig. 2: Time-course of the production of BBTOH, BBTOAC, BBT and α -T in *T. patula* calli. The data are the average of 40 determinations.

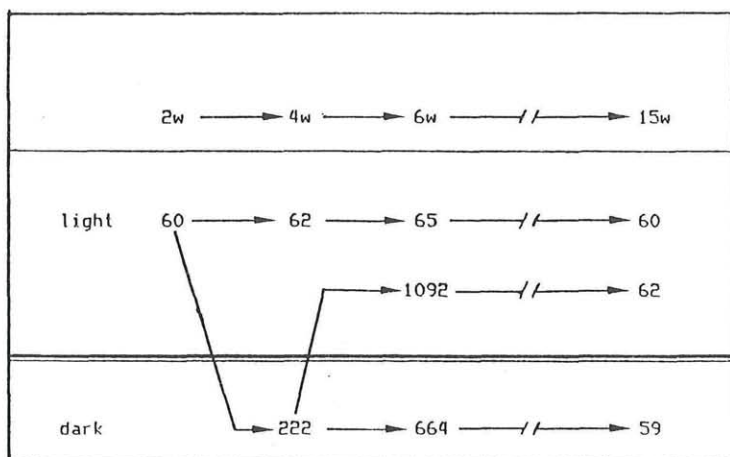


Fig. 3: Effects of light/dark succession on the total thiophene content in calli of *Tagetes patula*. The data are the average of at least four determinations, values expressed in $\mu\text{g/g}$ fresh weight of total thiophenes, w = weeks of subculturing.

Since the change light/dark and *vice versa* can improve the thiophene production in seedlings of *Tagetes patula* (SÜTFELD 1982), we applied cycles of light and dark to callus cultures following the scheme described in Fig. 3.

Collected data indicate that the passages of the callus cells from the light to the dark determined a 4-fold increase of the total thiophene content. The major thiophenes observed in calli were BBTOH and BBTOAC. When callus cells were cultivated again in the light after a dark period, the total thiophene content increased 5-fold. In this case the four tested thiophenes enhanced in quantity. Probably these increases were determined by the better cell growth occurring in these conditions. In the light, in fact, the cell rate was 300% in 2 weeks while in the dark reached only 100%.

Tertiary leaf calli, that were very friable, were used to set up cell suspension cultures. After 4–5 passages the cultures were principally formed by large green cell aggregates (10–20 mm) or by little yellow-brown aggregates (3–5 mm). The total thiophene content ($32.08 \mu\text{g/g}$ f. w.) was similar to the one present in original calli. BBTOH ($14.54 \mu\text{g/g}$ f. w.) and BBTOAC ($15.90 \mu\text{g/g}$ f. w.) were well represented while α -T and BBT were in very low concentration ($0.80 \mu\text{g/g}$ each) and sometimes were not detectable. The release of thiophenes into the medium was not very high (about $1.2 \mu\text{g/ml}$) with a small prevalence of BBTOH, since the thiophene is very soluble in water. In conclusion, the amounts of polar (BBTOH) and non-polar (BBTOAC and BBT) thiophenes in cell aggregates of *Tagetes patula* gradually decreased to more than 90% over 60 to 80 days of subculturing in liquid media, while the succession of light and dark periods did not determine substantial differences.

From a biotechnological point of view, calli are promising starting material for the production of thiophenes. However, as suggested by some authors (see CROES & al. 1988), many efforts are still necessary for evaluating growth conditions, the need of elicitors or of stress conditions to increase the thiophene production. Further analytical experiments are in progress to explore the effects of elicitors together with succession of light/dark periods.

Acknowledgements

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