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PRODUCTION OF ROTENOIDS BY HETEROTROPHIC AND PHOTOMIXOTROPHIC CELL CULTURES OF *TEPHROSIA VOGELII*

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Key Word Index—*Tephrosia vogelii*; Papilionaceae; heterotrophic; photomixotrophic cell culture; rotenoids; HPLC.

Abstract—Rotenoid compounds provided from the phenylpropanoid pathway are largely accumulated in the leaves of *Tephrosia vogelii*. Heterotrophic and photomixotrophic cell suspension cultures of this tropical plant have been obtained. Both cell lines are able to produce rotenoids, but a specific production is observed in each cell culture. The photomixotrophic cell line accumulated rotenone and deguelin in *Tephrosia* leaves and the heterotrophic cell line produced essentially deguelin and tephrosin.

INTRODUCTION

Rotenone and its derivatives with insecticidal properties are essentially extracted from *Derris*, a tropical plant originally from South America. These compounds are also accumulated in the leaves of *Tephrosia vogelii* Hook f. Up to now, this tropical plant, which is obtained from West Africa, has been traditionally used for its ichtyotoxic, insecticidal and food parasiticidal activities. In fact, a great variety of rotenoids are accumulated in the leaves of this plant where the total rotenoid content can reach 4% of the dry matter. Rotenone and deguelin are the major compounds present [1].

The rotenoids (**1**–**4**) are composed of an isoflavone nucleus C_6-C_3-C_6 (ring ACB) with an isoprene moiety attached at C-8 of ring A. These compounds are classified in the isoflavonoid family, the end product of the phenylpropanoid pathway [2]. The precursor for all phenylpropanoids is L-phenylalanine provided from the shikimate pathway. Phenylalanine is converted into 4-coumaroyl CoA by the concerted action of phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL). PAL, which catalyses the conversion of L-phenylalanine to *trans*-cinnamic acid is subjected to a highly complicated pattern of control comprising endogenous as well as exogenous factors [3, 4]. One of these exogenous factors is light which dramatically increases the enzyme activity after a lag period of a few hours [5]. The subcellular localization of the biosynthetic pathway of the rotenoids is not well known. However, some steps can take place in the chloroplast; phenylpropanoids and/or flavonoids and the enzymes of the phenolic metabolism [3, 6, 7] are located in this organelle of many plants.

In this paper, we studied rotenoid production in chlorophyll-containing and heterotrophic cell lines of *T. vogelii*. Both callus and suspension cultures were first characterized with regard to fresh and dry weight and chlorophyll accumulation.

RESULTS AND DISCUSSION

Heterotrophic and photomixotrophic callus cultures were both obtained from *T. vogelii* hypocotyls. Growth kinetics were established after 15 months of subculture. The fresh and dry weight curves of heterotrophic callus cultures demonstrated a continuous growth throughout the cell culture cycle (Fig. 1A). In chlorophyll-containing cultures intensive growth was only observed between day 10 and day 25 (Fig. 1B). Growth increases were similar in both cultures ($\times 10$ (expressed in dry wt) and 11, respectively, for heterotrophic and photomixotrophic cells). The estimation of the chlorophyll content of green callus cultures (Fig. 1C) demonstrated a loss of chlorophyll material during the first 10 days of culture and the stationary phase of growth; synthesis occurred only during the intense growth phase.

Identical growth patterns were observed when the culture of both cell lines was performed in liquid medium. The phase of growth started from the first day of subculture in the heterotrophic cell suspensions (Fig. 2A). For the photomixotrophic culture (Fig. 2B), the growth phase took place after two days of lag phase. The growth rates, expressed in dry weight, are relatively similar, the dry weight being 2.5-fold higher at the end of both cultures. In the chlorophyll-containing cell suspensions, the lag phase was accompanied by a decrease in the chlorophyll content as observed for the callus cultures

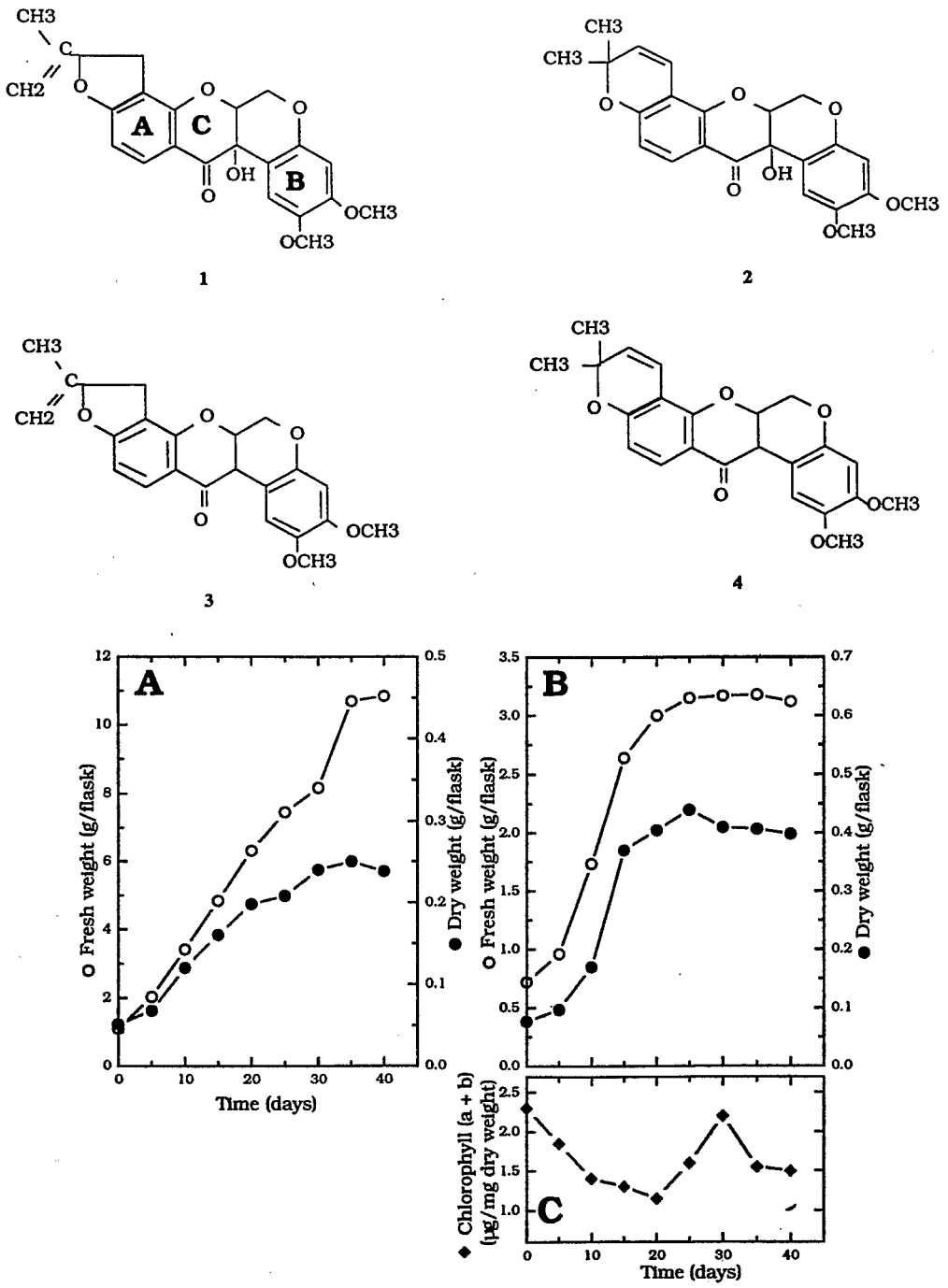

Fig. 1. Growth kinetics of heterotrophic (A) or chlorophyll-containing (B) callus of *T. vogelii* and evolution of chlorophyll content (C).

(Fig. 2C). A synthesis of the chlorophyll occurred only during the intense growth phase and its final level (expressed in $\mu g\, mg^{-1}$ dry wt) raised the value observed at the subculturing. During the transfer to the fresh medium, cells were in the presence of a higher concentration of sucrose which inhibited the chlorophyllogenese [8].

Analysis of the rotenoid content of leaf extracts of *T. vogelii* showed that 1–4 are the main rotenoids produced (Fig. 3). The amount of the rotenoid produced in the *in vitro* material was determined by HPLC analysis with reference to standard solutions of rotenolone (1), tephrosin (2), rotenone (3) and deguelin (4) (Fig. 3).

In the heterotrophic callus cultures, it seemed that 4 was the principal rotenoid produced (Fig. 4A). Compound 1 was not detected and 2 and 3 were poorly synthesized (always $<25\, \mu g\, g^{-1}$ dry wt). The chlorophyll-containing callus cultures produced the same roten-

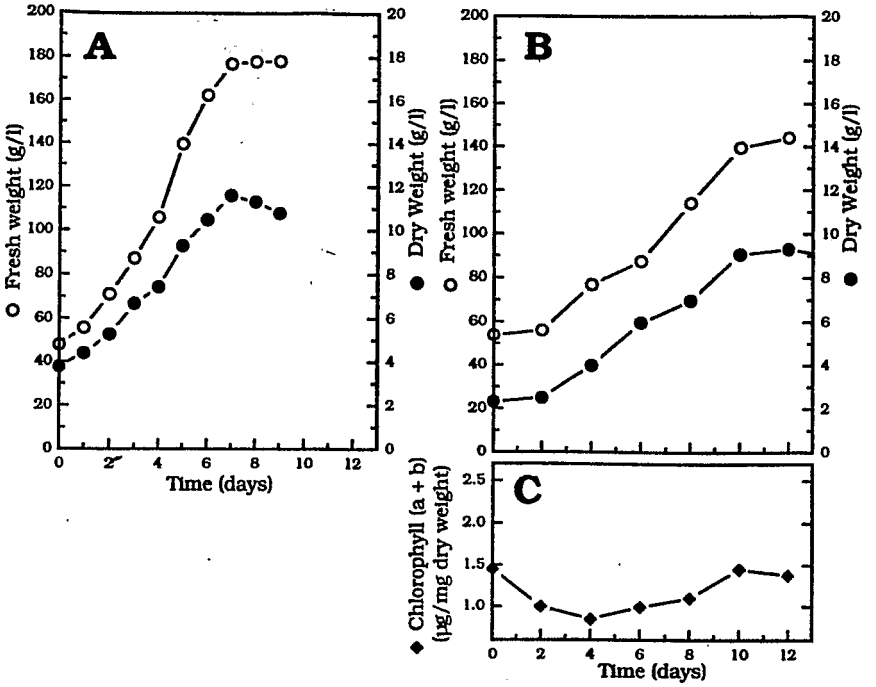

Fig. 2. Growth kinetics of heterotrophic (A) or photomixotrophic (B) cell suspension cultures of *T. vogelii* and evolution of chlorophyll content (C).

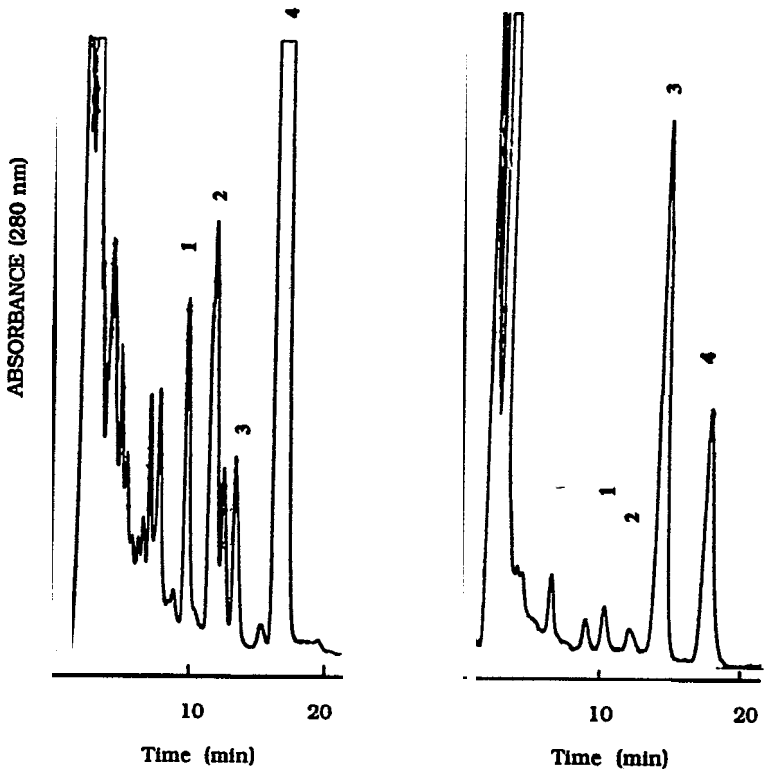

Fig. 3. HPLC analysis of leaf extract (left chromatogram) and callus cultures (right chromatogram) from *T. vogelii*. Each peak number corresponds to its compound number.

oids present in the leaves (Fig. 3). However, **3** appeared to be the main rotenoid throughout the culture cycle (Fig. 4B). Its production rate was approximately four–six-fold higher than that of the other rotenoid compounds. Its highest concentration was observed after 20 days of culture (570 $\mu g\, g^{-1}$ dry wt). Compounds **1** and **2** were produced in lower amounts, their concentration being always lower than 50 $\mu g\, g^{-1}$ dry wt.

In the heterotrophic cell suspension cultures (Fig. 5A), **4** was the major rotenoid produced as in the heterotrophic callus cultures. However, **2** was also found in large amounts, its concentration being equivalent to **4**. Here also, **1** was not detected, although **1** was the major rotenoid produced by the photomixotrophic cell suspension culture (Fig. 5B). Compound **3** was also actively synthesized by these cells. For both cell lines grown in liquid medium, rotenoid synthesis was increased during the growth stage with maximum concentrations of the compounds appearing at the end of the phase of growth. The rotenoid content was also determined in the culture

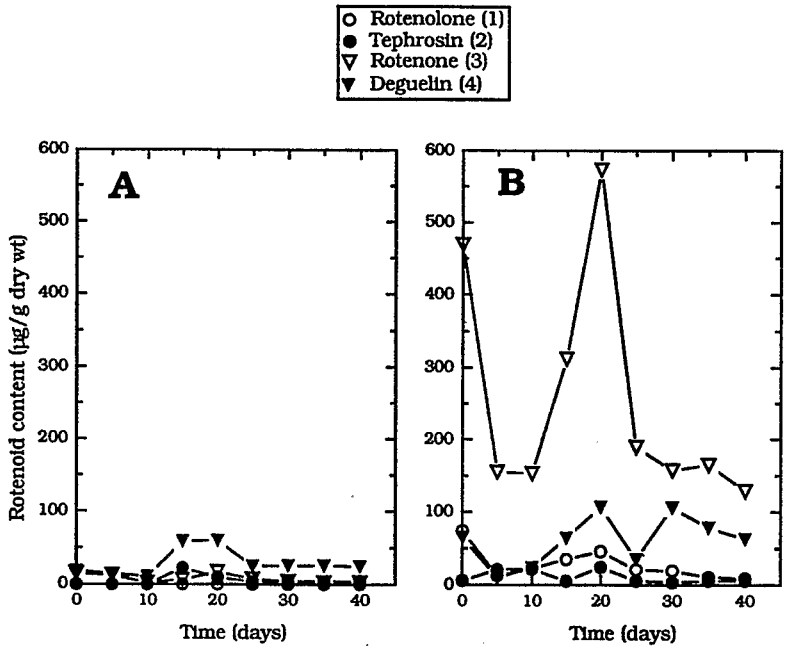

Fig. 4. Rotenoid production in heterotrophic (A) and chlorophyll-containing callus (B) cultures.

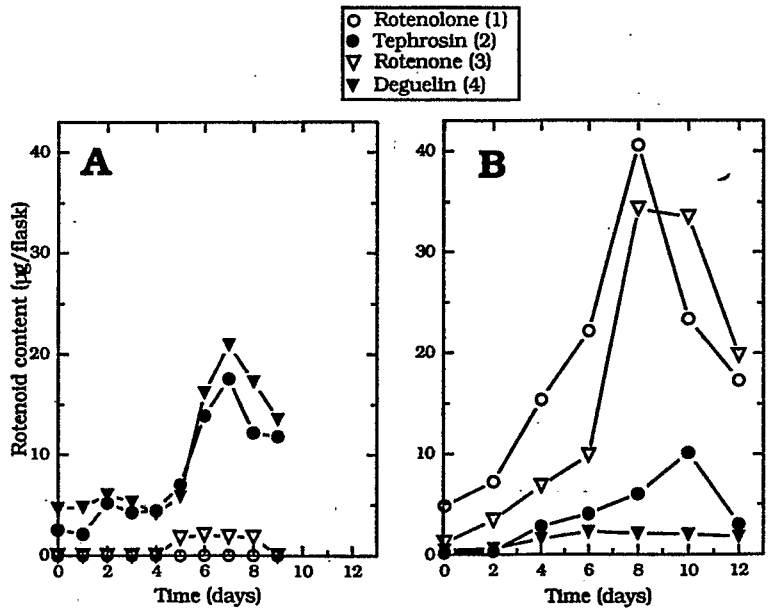

Fig. 5. Rotenoid production in heterotrophic (A) and photomixotrophic (B) cell suspension cultures.

medium of the two cell lines (data not shown). The rotenoids were poorly released and only **4** was found in large amounts. Finally, the modification of some culture conditions (absence of light and different concentration of sucrose) during callus culture allowed the two cell lines to be differentiated by their ability to synthesize chlorophyll. The rotenoid production was markedly different in these two cell lines. Moreover, the rotenoid profile of the chlorophyll-containing cultures was compared to the leaf production. Although *T. vogelii* leaves accumulated **3**, **4** and their derivatives, heterotrophic cell cultures were able to produce only **4** and **2**, while photomixotrophic cell cultures accumulated preferentially compounds like **3**. It seems that the biosynthesis of **3** and **1** is stimulated by photomixotrophic conditions. It is an open question whether light or photosynthesis plays a role in directing the biosynthetic pathway towards **3** or **1**, rather than towards **2** and **4**. Up to now we know that the common precursor to **4** and **3** is rot-2′-enonic acid [9, 10]. Two different enzymes are responsible for the cyclization process which produces **3** and **4**. Compound **1** is an oxidation product of natural rotenone (**3**) while **2** is derived from **4**. It is possible that the relative importance of the second pathway may vary with culture conditions. There is another possibility that the lack of accumulation of **2** and **4** in chlorophyll-containing cells could be attributed to the catabolism of the products or inactivation of some enzymes participating in this metabolic pathway. Detailed studies on the enzymes and intermediate compounds of rotenoid biosynthesis and their localization are needed to elucidate the site, as well as the control mechanism, of the rotenoid biosynthesis in *T. vogelii* cells.

In this study, the establishment of the cell culture system allowed the demonstration that the photomixotrophic condition of the culture (light essentially) promotes the production of rotenoids which are of economical interest. The present investigation also provides another example where the spectra of secondary constituents of photomixotrophic and heterotrophic cell cultures are significantly different [11, 12]. Therefore, depending on the type of compound required, a specific form of cell culture (heterotrophic or photomixotrophic) could be selected for product accumulation and relevant biochemical investigations.

EXPERIMENTAL

Establishment of the cell lines. Chlorophyll-containing callus cultures were initiated from hypocotyl explants of *T. vogelii* seedlings, grown on a modified MS [13] medium [14], supplemented with 5.37 μM 1-naphthalene acetic acid, 4.4 μM benzylaminopurine, sucrose 3% (w/v) and 0.6% (w/v) agar with the pH adjusted to 5.7. These cultures were maintained at 28° under diffuse light (18 hr light–6 hr dark, 100 μmol m^{-2} sec^{-1}). Heterotrophic cultures were obtained by transferring part of the chlorophyll-containing culture to the dark (28°). For each cell line, subculturing was carried out every 3 weeks.

After 8 months of callus subculture, cell suspension cultures were initiated from each callus cell line. Cultures were performed in 300-ml Erlenmeyer flasks containing 80 ml of liquid medium (modified MS medium supplemented with 1.5% (w/v) or 2% (w/v) sucrose for photomixotrophic and heterotrophic cultures, respectively). The photomixotrophic cell line had to be filtered during the first subculture in order to remove cell clumps. Cell suspensions were routinely subcultured (every 1 or 2 weeks, respectively for heterotrophic and photomixotrophic cells) and maintained at 28° on a gyratory shaker (80 rpm) under diffuse light (18 hr light–6 hr dark, 100 μmol m^{-2} sec^{-1}) or in the dark.

Estimation of growth. Cells and the medium were sepd by filtration under partial vacuum through a glass filter. Growth was estimated by measuring the total fresh weight and the dry weight (a 500-mg aliquot of fresh cells was dried at 60° for 48 hr). Data reported are the average of the values from triplicated experiments.

Determination of chlorophyll (a+b) content. Cultured cells collected on Miracloth by filtration (1 g fr. wt) were extracted with Me_2CO (4 ml) in the dark and at 4°. After centrifugation, the supernatant was adjusted to a final vol. of 15 ml with Me_2CO–H_2O (4:1). The quantitative estimation of chlorophyll was by spectrophotometrically using the equation of Lichtenthaler and Wellburn [15].

Extraction of rotenoids. Freeze-dried cells were extracted twice with MeOH–H_2O (9:1) and oxalic acid (1 mg ml^{-1}) [16] at 30° and the extract was evapd to dryness (at 30°). The remaining residue was dissolved in CH_2Cl_2 and partitioned (3×) against 0.25 M NaCl. The CH_2Cl_2 phase was reduced to dryness under vacuum and the final extract taken up in 1 ml of Me_2CO–H_2O (1:1). After centrifugation (27000 *g*, 15 min) the supernatant was decanted and stored at −20°.

Rotenoid analysis. The major rotenoids (**1**–**4**) were characterized qualitatively by TLC (silica gel 60 F 254 plates, Merck) with $CHCl_3$–Me_2CO–HOAc (169:3:1) and quantitatively by HPLC on a Beckman 332, using a Lichrospher 100 CH 8/II column R.P., 25×0.4 cm (5 μm), at a flow rate of 0.8 ml min^{-1}, the mobile phase MeOH–H_2O (65:35), detection at 280 nm.

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