FORMATION OF GOSSYPOL BY *GOSSYPIUM HIRSUTUM* L. CELL SUSPENSION CULTURES

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ABSTRACT.—Callus and cell suspension cultures of *Gossypium hirsutum* L. Paymaster 303, Tamcot Sp37 and Acala SJ5 in Linsmaier-Skoog medium containing 10^{-6}M 2,4-dichlorophenoxy acetic acid and 10^{-6}M naphthalene acetic acid were initiated which produced hemigossypol and gossypol. Authenticity of the isolated gossypol was substantiated by nmr, mass spectroscopy and thin layer chromatography and compared with standard gossypol. In addition [5-^{14}C] DL-mevalonate, when added to the growth medium, was readily incorporated into gossypol, as verified by repeated thin layer chromatography and co-crystallization to constant specific activity. Growth kinetics and gossypol production showed that rapidly growing cultures (4–5 days required to double their cell mass) produced significantly less gossypol (0.02% of dry weight) compared to slow growing cultures (doubling time 11 days) which produced 0.11 g gossypol per 60 g dried cell mass per liter of suspension culture.

Both callus (1–4) and suspension cultures (5–7) of *Gossypium* species have been obtained by a number of laboratories. In general, these studies were undertaken to potentially apply these *in vitro* systems to genetic and crop improvement. However, in none of these reports has the formation of *in vivo* occurring pigments such as gossypol been reported. In the search for a more convenient system for catalyzing the biosynthetic reactions leading to gossypol, we have initiated callus and suspension cultures of *Gossypium* sp. which are capable of synthesizing gossypol and related pigments.

EXPERIMENTAL

CULTURE CONDITIONS.—Seeds of *Gossypium hirsutum* Tamcot Sp37, Paymaster 303 and Acala SJ5 were delinted with concentrated sulfuric acid, washed thoroughly with water, sterilized for 20 min. in a 5.25% hypochlorite solution containing 2 drops of Tween 20/100 ml, washed with sterile water and then germinated in sterile Petri dishes containing 0.6% agar. Root, stem, and cotyledon tissue was dissected and transferred to a solid medium containing 8 g/liter agar (Bacto-Agar), inorganic salt formulation of Linsmaier and Skoog (8), 30 g sucrose/liter, 10^{-6}M 2,4-dichlorophenoxyacetic acid (2,4-D) and 10^{-6}M naphthalene acetic acid (NAA). The pH of the medium was adjusted to 6.0 with NaOH and the solution was then sterilized by autoclaving for 20 min. at 18 lbs. and 125°. Callus tissue was maintained at 30° in the dark and was transferred every 6 weeks to fresh medium.

Suspension cultures were initiated from callus tissue in the same medium as described above (hereafter referred to as LS medium) except that the agar was omitted. Cell suspension cultures were maintained at 30° in the dark while being agitated on a gyrotory shaker (120 rpm). The cultures were transferred to new LS medium every 10 days or every 25 days. To measure growth kinetics and gossypol production as related to growth, a ten day old 200 ml culture was filtered and 2 cm^3 (0.7 ± 0.05 g wet weight) of loosely packed cells were each inoculated into thirty 125 ml Erlenmeyer flasks, containing 25 ml of LS medium. Twenty-four hrs after inoculation and every second day thereafter two cultures were harvested and analyzed for gossypol and hemigossypol as well as for increases in weight.

ISOLATION OF GOSSYPOL.—Cells were harvested from individual suspension cultures by filtration with suction. An aliquot of the residual cell mass was dried at 60° for 72 hrs. to determine dry weight. The remaining cells were extracted 2 times with methanol (10 ml each extraction for a 25 ml culture) saturated with sodium bisulfite. The combined methanol extracts were evaporated to dryness under vacuum, at $<50°$ and in the dark. The residue was dissolved in a small amount of ethylacetate and an aliquot analyzed by thin layer chromatography (tlc). The following tlc systems were used to separate gossypol and related pigments: Silica gel G (Brinkmann Instruments, Inc.), hexane-ether-formic acid (65:35:1); Poly-

amide 11 F-254 (Brinkman Instruments, Inc.), benzene-chloroform-methanol-acetic acid (130:60:10:2). The gossypol was visualized by spraying with a solution of equal parts of 5% phloroglucinol in 95% ethanol and concentrated HCl (10); the two spots corresponding to gossypol (Rf 0.61) and hemigossypol (Rf 0.17) were each placed in a separate test tube and extracted with 95% ethanol. The intensity of the color of the extract was measured at 550 nm (9, 10).

After the medium was saturated with NaCl, gossypol and related pigments in the medium (filtrate above) were extracted with three equal volumes of ethylacetate. After concentration, the amounts of gossypol and hemigossypol were determined by tlc as described above.

The authenticity of the gossypol isolated from the cell suspension cultures was substantiated by nmr (^{1}H-nmr, 100 MHz, $CDCl_3$, TMS, δ 1.54 (d), 2.13, 3.82 (m), 5.78, 6.40, 7.76, 10.98, 14.68) and mass spectroscopy (80 ev, *m/e* 518, 2%, M^+; *m/e* 500, 72%, M^+-H_2O; *m/e* 482, 100%, M^+-2H_2O; *m/e* 485, 9%, $M^+-H_2O-CH_3$; *m/e* 467, 44%, $M^+-2H_2O-CH_3$) and compared with literature spectra (11, 12).

Formation of gossypol and hemigossypol was further tested by the incorporation of [5-^{14}C] DL-mevalonate. Three two-day-old cell suspension cultures (each containing 1 cm^3 loosely packed cells in 8 ml LS medium) were each fed 4.16 μCi of [5-^{14}C] DL-mevalonate (11.8 mCi/m mole). After shaking for 72 hrs. at 30° in the dark, the cells were harvested and the gossypol was isolated and purified from the pooled incubations as described above. The percent of incorporation of [5-^{14}C] DL-mevalonate into gossypol was obtained by tlc analysis of the extract. In another experiment standard, nonradioactive gossypol was added to partially purified gossypol which had been synthesized from [2-^{14}C] DL-mevalonate. The mixture was recrystallized from an ether-hexane mixture to constant specific radioactivity.

RESULTS AND DISCUSSION

Initially, a number of media reported previously by others (1–7) were tried and found to promote callus tissue growth (results not shown). However, under these conditions none of the callus tissue produced gossypol. After a number of experiments with different inorganic salt solutions (media described in 1–7), hormones (2,4-D, NAA, indole acetic acid, 6-benzyl adenine, and kinetin at various concentrations), and carbon sources (sucrose, glucose and fructose at various concentrations), a medium was selected which consisted of Linsmaier-Skoog mineral salts (8), sucrose as the carbon source and 2,4-D and NAA as hormones. This medium promoted the growth of callus tissue as well as the production of phloroglucinol positive (9) pigments in callus tissue which had a brown coloration (fig. 1). The callus tissue which appeared white in coloration (fig. 1) grew much more rapidly but did not contain pholoroglucinol positive pigments. Callus tissue formed after 6–10 days from all plant tissue that had been transferred. No visual distinction (color or appearance) or growth characteristics of the callus tissue could be associated with the origin of tissue (roots, stem or cotyledons). The transfer of completely dark callus tissue always resulted in the formation of some white callus tissue (fig. 1, far left). Similarly, transfer of all white callus tissue resulted in the proliferation of mostly white tissue, however dark callus tissue always appeared as well (fig. 1, second from left.).

FIG. 1. Callus tissue of *Gossypium hirsutum* L. cultivar Acala SJ5 (two cultures on the left) and Paymaster 303 (two cultures on the right).

Cell suspension cultures were readily obtained in LS medium from white or dark callus tissue. However, during the first 5 transfers the color of these suspension cultures appeared to be intermediate to dark in color (yellow-brown). Successive transfer of two identical cell suspension cultures, one every 10 days and one every 25 days, resulted in the selection of a completely white culture (10-day transfer), which grew rapidly, with a doubling time of 3–4 days, and in the selection of a very dark culture (25-day transfer), which grew more slowly, with a doubling time of 9–12 days. The presence of phloroglucinol positive pigments coincided directly with the color of the cultures. As such, a culture with a dark coloration contained 310 mg of gossypol equivalents (for definition see Bell (9)) in the cell mass per liter of culture after 12 days in culture. However, a culture which appeared completely white did not contain any pholoroglucinol positive compounds.

If the above transfer schedule was maintained, the dark cultures were stable. Although the color of the cultures, and therefore the pigment concentration, did vary slightly from transfer to transfer, no completely white cell suspension cultures were obtained (a total of 18 transfers). Some of the white cultures (45 total transfers) sometimes turned dark from one transfer to the next but acquired the original white color after 4–6 transfers.

The authenticity of the presence of gossypol was established by comparing the nmr and mass spectra of the isolated compound with the data in the literature (11, 12) and with spectra of authentic compounds. In all experiments the migration of gossypol and hemigossypol on tlc plates was compared with standards.

The biosynthetic formation of gossypol was substantiated through the incorporation of [5-^{14}C] DL-mevalonate into gossypol. An incorporation of 0.8 to 1.1% (table 1) of the C^{14} into gossypol, based on the assumption that only one isomer of DL-mevalonate was utilized, compares favorably with an incorporation of 3–5% in cell-free systems (13), in view of the observation that plant cells do

TABLE 1. Incorporation of mevalonate into gossypol.

Gossypium hirsutum sp.	Incorporation[a] %
Paymaster 303	0.8
Acala SJ5	1.0
Tamcot Sp37	1.1

[a]Incorporation was calculated from the amount of radioactivity from one isomer of [5-^{14}C] DL-mevalonate associated with gossypol extracted after 3 days from the cell mass of a suspension culture (1 cm^3 of cells inoculated into 8 ml of LS medium) and purified through two tlc systems.

not readily take up mevalonate (14). That the radioactivity was associated with gossypol is supported by little loss of radioactivity upon tlc in two solvent systems and by crystallization which resulted in constant specific radioactivity after three recrystallizations (table 2).

The growth pattern and production characteristics of the two *G. hirsutum* varieties, Paymaster 303 and Tamcot Sp37, are shown in figs. 2 and 3. The cells used to carry out the experiment in fig. 2 were obtained from a Paymaster

TABLE 2. Co-crystallization of gossypol from *G. hirsutum* cultures with authentic material.

Crystallization	dpm/mg
1st	7619
2nd	3336
3rd	3368
4th	3320

Gossypol was isolated (one tlc system) from *G. hirsutum* Tamcot Sp37 cell suspension cultures fed [5-^{14}C] DL-mevalonate. After addition of authentic gossypol recrystallization from hexane-ether was continued until constant specific radioactivity.

303 culture whose color was yellow-brown. These cultures (referred to as "medium-white") grew rapidly, doubled their dry weight in about 4–5 days and showed a characteristic log phase. Gossypol production paralleled growth up to day 10 and then decreased (fig. 2). The amount of gossypol produced reached about 27 mg/liter culture. In contrast, a similar experiment (fig. 3) initiated with Tamcot Sp37, a dark culture, produced a maximum of 110 mg/liter culture of gossypol at day 16. However, growth in these cultures was much slower (fig. 3) than Paymaster 303 (fig. 2). Tamcot Sp37 never entered a true log phase of growth and doubled in weight after approximately 11 days (fig. 3). The amount of hemigossypol in the cells of both cultures (figs. 2 and 3) was less than the lower limit detectable by tlc and the phloroglucinol method.

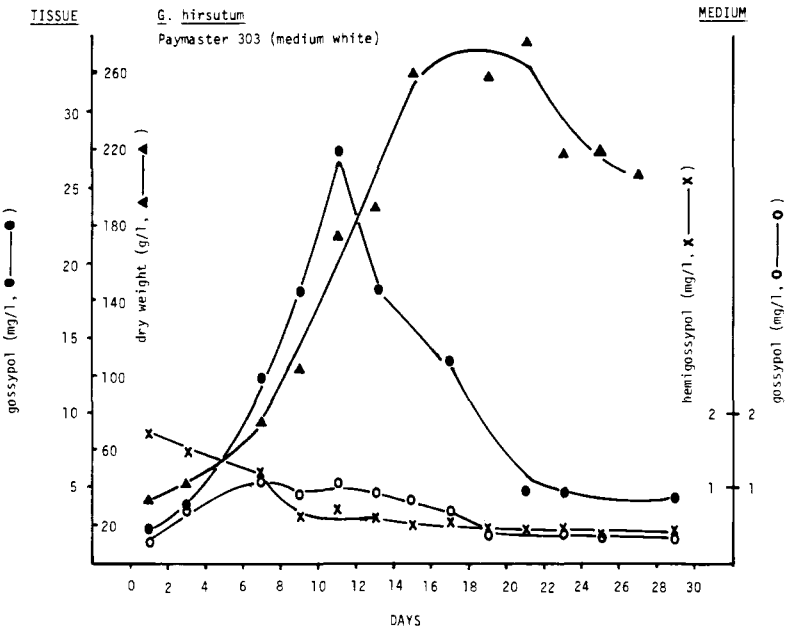

FIG. 2. Growth kinetics and gossypol production by *Gossypium hirsutum* L. cultivar Paymaster 303 grown at 30° in the dark in 25 ml shake cultures. Each point represents the average of two determinations.

The medium of both cultures contained small amounts of gossypol and hemigossypol (figs. 2 and 3). In both cases the hemigossypol concentration appeared to be highest during the first 6–7 days and then decreased. Gossypol concentration reached a maximum at about day 11 in the medium of either Paymaster 303 (fig. 2) or Tamcot Sp37 (fig. 3) but was much lower than the concentration in the cell mass. The initial increase of hemigossypol followed by an increase in gossypol concentration with the concomitant decrease in hemigossypol would support the proposal that hemigossypol is a precursor of gossypol (15). In both Paymaster 303 (fig. 2) and Tamcot Sp37 (fig. 3) cultures, the dry weight/liter decreased after day 20, suggesting cell lysis. However, little if any hemigossypol or gossypol appeared in the medium. These findings indicated that the earlier appearance of hemigossypol and gossypol in the medium could be due to an excretion process.

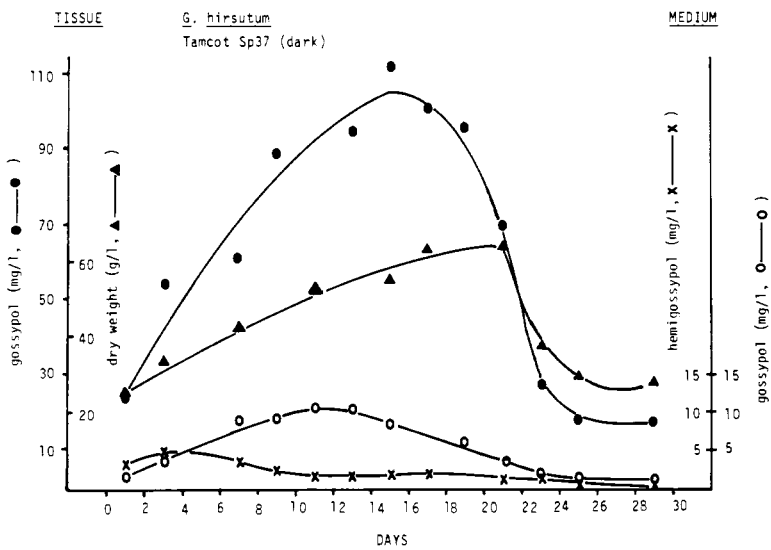

FIG. 3. Growth kinetics and gossypol production by *Gossypium hirsutum* L. cultivar Tamcot Sp37 grown at 30° in the dark in 25 ml shake cultures. Each point represents the average of two determinations.

The experiments in figs. 2 and 3 suggested that terpenoid aldehyde pigment production and growth are inversely related. A rapidly growing culture of *G. hirsutum* tended to be white in appearance and produced little or no gossypol or related pigments, whereas a dark culture produced significant amounts of gossypol and related pigments but grew very slowly. Perhaps this inverse relationship of growth and pigment production has its origin in the proposed phytoalexin properties of gossypol and related compounds (9).

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