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Anthracene derivatives in tissue culture of *Cassia senna* L.

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Tissue cultures from *Cassia senna* L. have been established. Chrysophanol, physcion, emodin, rhein and aloe emodin have been identified in the oxidative-hydrolysate of the callus. Chrysophanol was the major component. With the exception of physcion all of these compounds were found in the glycosidic as well as the free form. The concentration in the dried callus of the free anthraquinones was 0·8% w/w and of the glycosidic (combined) anthraquinones 0·4% w/w. These results and the absence of sennosides in the cultures emphasize the variation in the anthracene derived constituents of the derived callus and the parent plant.

The use of plant tissue culture for secondary product biosynthesis, particularly in plants of pharmaceutical importance, holds promise for the controlled production of plant constituents. It has been suggested (Petiard & Demarly, 1972) that given appropriate culture conditions the biochemical potentialities of the *in situ* cells would be expected to be genetically inherent in the cultured callus cells. *Cassia angustifolia Vahl* has previously been cultured and the presence of several anthracene derivatives, including a trace of sennosides, reported (Baier & Friedrich, 1970; Friedrich & Baier, 1972; 1973). In view of the existing biochemical similarities between the two species *Cassia senna L.* and *C. angustifolia* we have investigated the constituents produced by the callus cultures of *C. senna.*

MATERIALS, METHODS AND RESULTS

Alexandrian Senna seed was germinated and plants raised in a greenhouse. The material was authenticated as *C. senna* by its morphological and cytomorphological characters including numerical value of its stomatal index.

Induction of callus. Dry seeds of *C. senna* were surface sterilized in 80 vol hydrogen peroxide, rinsed (3× in sterile water), and germinated in darkness at 24 ± 1° on sterile agar, 0·8%. After five days, pieces of cotyledon approximately 6 mm^2 and segments of hypocotyl approximately 10 mm in length were aseptically transferred to Murashige & Skoog's (1962) revised tobacco medium supplemented with 2,4-dichlorophenoxyacetic acid 0·55, kinetin 0·3 mg $litre^{-1}$, sucrose 35 g $litre^{-1}$ to which coconut water 18% v/v was added (Turner, 1971a, b). Callus, showing no gross difference in the cellular constituents or characters, was successfully initiated from cotyledon and hypocotyl. Hypocotyl explants being more readily available, further experimentation was confined to hypocotyl derived callus.

Modifications of the medium with napthalene acetic acid, indol-3yl-acetic acid alone or with kinetin or 2,4,-dichlorophenoxyacetic acid did not improve callus growth.

The medium described sustained satisfactory growth of healthy callus cultures of *C. senna* for two years with subculturing at intervals of 4 to 6 weeks. All cultures

were maintained in continuous warm white light 100 lumen ft² at 28 ± 0·5°. After seven passages callus material in the fresh or freeze dried state was examined for the constituents. Callus showing differentiation and organ regeneration was discarded. A healthy callus of *C. senna* consists of a soft friable mass in which the tissue colour varies from pale green to light brown. Although brown pigments are usually associated with dead or dying callus, tissue so coloured when cultured only gave rise to rapid growth of the same green-brown callus. It appears that this colour pattern is an integral part of the callus character. The general cell form was thin walled isodiametric parenchymatous cells varying from 50 to 540 μm in diameter.

Qualitative examination of callus anthracenes

Methods for the separation of plant anthracene derivatives by thin-layer chromatography have been described (Horhammer, Wagner & Bittner, 1963; Gyanchandani & Nigam, 1963; Danilovic & Stevanovic, 1965; Labadie, 1969). The analysis and identification of anthraquinone glycosides are effected by their conversion into corresponding aglycones. The free aglycones can be separated by an organic solvent. We have used three separate extraction procedures to characterize the anthracene derivatives. The first was designed to give some free oxidized and reduced aglycones, the second, aglycones of the *O*-glycosides and the third, free aglycones as well as the aglycones of the combined forms in the total extract obtained by oxidative-hydrolysis.

Free aglycones

Powdered callus (0·5 g) was extracted to exhaustion with hot benzene (25 ml) and concentrated under reduced pressure at 30–40° to about 2 ml. Samples and standard solutions were applied to thin-layer plates [Kieselgel G (Merck) and Kieselguhr G (Merck) 1:6 (Labadie, 1970)], and developed with light petroleum (40–60°)–ethylformate–formic acid (90:40:1). Dried chromatograms were either sprayed with 5% KOH w/v or with pyridine–methanol (1:1 v/v) and heated at 120° for 20 min. The results are summarized in Table 1.

Table 1. *Thin-layer chromatographic examination of benzene extracts of callus material originating from* C. senna.

Compound	hR_F	Colour response			
		In daylight	In ultra-violet (350nm)	5% KOH (w/v) spray	Pyridine–methanol, (v/v) 1:1 spray +heat
Chrysophanol-anthrone*	80·2	Faint yellow	Light red red	Yellow	Violet
Chrysophanol	75·0	Yellow	Orange	Purple	Yellow-orange
Physcion	61·8	Yellow	Orange	Purple	Yellow-orange
Chrysophanol-dianthrone†	50·1	Yellow	Red	Yellow	Violet
Unknown	36·7	Yellow	Red	Yellow	Violet
,,	20·0	Yellow	Red	Yellow	Violet
,,	2·8	Light orange	Red	Yellow	Yellow-orange

* Prepared by the reduction of chrysophanol with stannous chloride.

† Prepared according to Auterhoff & Scherff (1960).

O-Glycosidic anthracenes

The free anthraquinones in powered callus (0·5 g) were completely extracted with ether (30 ml) and the marc heated with dilute hydrochloric acid (0·1 N) for 20 min. The solution was cooled and the liberated aglycones extracted with ether. This extract and the aqueous phase were examined chromatographically.

After their reduction *in vacuo* at 35°, the ether extract and reference compound solutions were applied to silica gel plates, which were developed in benzene–ethyl formate–formic acid (75:24:1) and located with 5% w/v KOH in methanol. Chrysophanol, emodin, rhein and aloe emodin were present in the extract; physcion was not but it was present with chrysophanol, emodin, rhein and aloe emodin as free aglycones in the initial ether percolate examined by the same chromatographic system. Thin-layer chromatography of the aqueous phase indicated that the sugar molecule of the hydrolysed glycoside is glucose.

Aglycones of the oxidative-hydrolysate

Powdered callus (0·5 g) was added to aqueous $FeCl_3$ (5 ml; 25%) + HCl (10 ml; 4N) and refluxed in a boiling water bath for 30 min. The mixture was extracted with chloroform (15 ml), and the extract evaporated to dryness. The residue was taken up in methanol (2 ml) + water (1 ml). Samples (5 μl) of the solution and of standard solutions of chrysophanol, physcion, emodin, rhein and aloe emodin were applied to thin-layer plates of silica gel G. Each chromatogram was first developed to 7·5 cm with benzene–ethyl formate–formic acid (75:24:1), and, after drying, to 15 cm with light petroleum (40–60°)–ethyl acetate–glacial acetic acid (90:5:5). All the compounds were visible as yellow spots which changed to orange-pink when sprayed with 5% KOH in methanol. In comparison with the reference solutions, spots observed in the callus extract showed hR_F identical to chrysophanol, 71·4; physcion, 66·1; emodin, 40·2; rhein, 33·7; and aloe emodin, 25·9.

About 20–25 mg of each of the compounds detected on the chromatograms was isolated by preparative thin-layer chromatography and its identity further confirmed by melting point, mixed melting point, ultraviolet, visible and infrared spectra.

Compounds isolated are listed in Table 2.

Quantitative estimation of the callus anthracenes

Most of the published methods for estimating free and combined anthraquinones in crude drugs do not allow the compounds to be determined separately (Lemli, 1966; Shah, Quadry & Bhatt, 1972). In the present method the total anthracene derivative content is estimated colorimetrically and the amount of the individual components determined on thin-layer adsorbent material using a 'Vitatron' densitometer.

Colorimetric estimation

Since the anthraquinones have similar molecular weights and molar absorptivities, they were estimated as the equivalent of 1,8-dihydroxyanthraquinone. Maximum extinction for 1,8-dihydroxyanthraquinone in 5% w/v NaOH + 2% ammonia was at 510 nm. A linear relation was obtained between concentration and extinction.

For the estimation of the free anthracenes, 0·1 g of the powdered freeze dried callus was extracted to exhaustion with chloroform (50 ml), the solution evaporated to dryness, the residue taken up with 10 ml of 5% NaOH 2% ammonia solution and the extinction measured at 510 nm.

For the estimation of the glycosidic anthracenes, the marc was heated with water (20 ml) for 20 min to extract the water soluble glycosides and the suspension was cooled and centrifuged. To 10 ml of the supernatant $FeCl_3$ (20 ml; 10%) was added and the mixture refluxed for 20 min, then HCl (2 ml; 10N) was added and refluxing continued for 20 min. The solution was cooled, extracted with ether (3 × 25 ml) and the extracts combined and washed with water (25 ml) to remove excess acid. The volume of this extract was made up to 100 ml with ether. 10 ml of this solution was evaporated and the residue taken up with 10 ml of 5% NaOH 2% ammonia solution and the extinction measured at 510 nm.

The estimated content of free anthraquinones was 0·8% w/w and of combined anthraquinones (present as glycosides) 0·4% giving a total of 1·2% w/w dry weight.

Thin-layer densitometric estimation

The quantitative determination of aglycones in the colorimetric method is subject to error due to the presence of pigments in the extract. Evaluation of individual compounds was carried out using a 'Vitatron TLD 100 Universal Densitometer' employing a 'flying spot' scanning system. The extraction procedures, the adsorbents and developing solvent systems were the same as described for the qualitative processes, 5% KOH in methanol w/v was used as the spray reagent. Scanning of the coloured spots on the thin-layer plate was with transmitted light in a logarithmic mode using a filter 525 nm. 1,2-Dihydroxyanthraquinone shows a linear relation between concentration and integrated peak area over a range of 0·4 to 6 μg and was used as an internal standard. The concentrations of anthraquinones were calculated as 1,2-dihydroxyanthraquinone and expressed as a percentage dry weight of the original callus material.

By the above procedure, it was estimated that chrysophanol was the major anthracene component of the callus while rhein was present only in low concentration. Taking rhein as unity, the quantitative ratio for free oxidized anthraquinones can be

Table 2. *Nature and concentration* of compounds isolated from callus of* C. senna.

Free oxidized aglycones		Free reduced aglycones		*O*-Glycosides	
Compound	% w/w (dry weight)	Compound	% w/w	Compound	% w/w
Chrysophanol	0·450	Chrysophanol-anthrone	0·048	Chrysophanol	0·252
Physcion	0·050			Emodin	0·057
Emodin	0·055	Chyrsophanol-dianthrone		Rhein	0·040
Rhein	0·045		0·032	Aloe emodin	0·051
Aloe amodin	0·055				
Total	0·655		0·080		0·400

* Expressed as the equivalent to 1,2-dihydroxyanthraquinone.

expressed as chrysophanol 1:10, physcion 1:1·1, emodin 1:1·2 and aloe emodin 1:1·2, and for *O*-glycosidic compounds the ratios are chrysophanol 1:6·3, emodin 1:1·4 and aloe emodin 1:1·2. Percentages w/w of all compounds are in Table 2.

DISCUSSION

Comparison of plant tissue cultures with the parts from which they were initiated

have been made on several species, e.g. *Citrus limon* (Bove, Bove & Raveux, 1957); *Solanum tuberosum* (Steward, Thompson & Pollard, 1958); *Agave toumeyane* (Weinstein, Nickell & others, 1959); *Atropa belladonna* (West & Mika, 1957); *Datura stramonium* (Chan & Staba, 1965); *Digitalis purpurea* and *D. lanata* (Staba, 1962). In each report, marked differences were observed in the nature and concentration of the constituents present in the plant part and derived callus, and although some similarities exist, it is generally regarded that plant tissue cultures are biochemically distinct from the parent plant part. Fairbairn & Shreshtha (1967), working with *C. senna* L., showed that chrysophanol and aloe emodin occur in seedlings in the early stages of germination. We found that five-day-old seedlings contained anthracenes, and chromatographic examination revealed a spot identical to chrysophanol, thus at the time of excision of seedlings and their subsequent culture on synthetic medium the tissues contained chrysophanol. This was unusually dominant in future synthesis. Friedrich & Baier (1972) demonstrated that in their callus culture of *C. angustifolia* chrysophanol was also the compound present in the highest concentration and that rhein was present in the lowest percentage. Our results indicate that the callus of *C. senna* performs in a similar manner but differs particularly in the absence of sennosides; it has a concentration of free anthraquinones approximately four times greater than the plant and a concentration of glycosides only one sixth of that found in the mature plant. These results demonstrate not only the expected variations between the constituents of a callus and its parent plant, but also indicate the difference between callus from the two *Cassia* species.

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