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ANTHRAQUINONE FORMATION IN CALLUS CULTURES OF *CASSIA PODOCARPA*

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ABSTRACT.—Callus tissues from seedlings of *Cassia podocarpa* were grown on Murashige and Skoog agar medium. For pigment production and tissue growth, the optimal concentration of 2,4-D was 0.6 mg/liter medium in the presence of kinetin (0.4 mg/liter). An analytical investigation of callus tissues demonstrated the presence of a number of hydroxyanthraquinones, including rhein and chrysophanol. These were the major compounds. The total yield of anthraquinone compounds in callus cultures (1.36%) was lower than in the plant leaves (1.65%), but visual selection of a high-yielding cell line from callus resulted in an increasing accumulation of 1.3 to 1.8% of the dry wt.

Plant tissue cultures are regarded as a potential source for the production of natural products. For more than two decades, callus and suspension cultures from medicinal plants have been initiated to establish suitable conditions for the possible production of pharmaceutically important products in vitro. This also holds true for plants producing hydroxyanthraquinone derivatives of medicinal value. Formation of hydroxyanthraquinones has, for example, been demonstrated in cell cultures of *Cassia angustifolia* Vahl (1), *Cassia senna* L. (2), *Cassia tora* (3), *Rheum palmatum* (4), and *Rhamnus frangula* (5). These plants are important commercial sources of anthraquinone laxatives. The hydroxyanthraquinones are synthesized in these plants via the acetate malonate pathway. Often the yields in cultures of plants synthesizing anthraquinones via this biosynthetic route are low (4,6). In contrast, cell cultures of plant species synthesizing anthraquinones via the *O*-succinyl benzoic acid pathway belong to a group of the highest producing cell cultures (7).

Cassia podocarpa Guill. & Perr. (Leguminosae) occurs in forest regions of West Africa as a common shrub up to five meters tall. *C. podocarpa* is closely related to recognized "senna," and the leaves and fruits of this plant are mentioned as purgatives (8). This plant has been considered as a possible substitute for *C. senna* (9). Rai and Obayemi (10) have analyzed the anthraquinone composition of the leaves of *C. podocarpa*. This paper describes the detection of anthraquinones in callus cultures of *C. podocarpa* and the effect of plant growth regulators on growth and production. A visual screening for high yielding cell lines is also described.

EXPERIMENTAL

CULTURE METHODS.—Callus cultures were derived from the seedlings of *C. podocarpa* which had been placed on Murashige & Skoog agar medium supplemented with 2,4-D (0.6 mg/liter), kinetin (0.35 mg/liter), and sucrose (35 g/liter). The cultures were kept under fluorescent light of 2000 lux at a temperature of 28°. Calli were cultured for 12 months (subcultured every 6 weeks before being analyzed). To examine the influence of plant growth regulators on growth and pigment production, pieces of callus tissue weighing 0.5 g were transferred to 100-ml flasks, each containing 25 ml agar medium containing 2,4-D (0.3, 0.4, 0.5, 0.6, and 0.7 mg/liter) and kinetin (0.0, 0.2, and 0.4 mg/liter) (Table 1). For every treatment five flasks were sacrificed to estimate fresh wt. The five fresh weights were then combined and dried in an oven at 60° for 24 h prior to the estimation of anthraquinones. The media on which the tissues had grown were also examined for anthraquinones. Growth indices (GI) of these tissues were calculated for each flask:

$$GI = \frac{\text{(Final wet wt} - \text{Initial wet wt)}}{\text{Initial wet wt.}}$$

QUALITATIVE ANALYSIS.—The anthraquinone glycosides and their aglycones in callus tissue ex-

TABLE 1. The Effect of 2,4-D and Kinetin on Growth and Anthraquinone Formation in Callus Cultures of *Cassia podocarpa*.

Concentration of 2,4-D (mg/liter)	Concentration of Kinetin (mg/liter)	Growth Index (GI) ±SE	Total Yield of Anthraquinones (% dry wt)
0.30	0.00	2.00 ± 0.04	0.27
	0.20	3.40 ± 0.02	0.35
	0.40	3.60 ± 0.01	0.39
0.40	0.00	3.80 ± 0.01	0.29
	0.20	4.40 ± 0.02	0.41
	0.40	5.00 ± 0.03	0.80
0.50	0.00	4.20 ± 0.08	0.33
	0.20	5.80 ± 0.03	0.47
	0.40	9.00 ± 0.15	0.83
0.60	0.00	6.40 ± 0.01	0.35
	0.20	6.20 ± 0.05	0.73
	0.40	10.00 ± 0.18	1.31
0.70	0.00	4.60 ± 0.05	0.34
	0.20	5.80 ± 0.03	0.72
	0.40	8.60 ± 0.11	1.00

tracts were separated by tlc on Si gel employing seven different solvent mixtures (11, 12). Separation of the anthraquinones was also achieved by hplc utilizing ion pairing and a reversed-phase H_2O/MeOH gradient elution system (13). Conditions of separation were initially MeOH-H_2O (60:40) with the addition of 1.75 ml/l PIC A (Waters) extending to MeOH-H_2O (86:14) linear gradient, employing Waters 6000A and Waters 6000 pumps and Waters 660 solvent programmer on a column: Apex 5 mm ODS, 30 cm × 4 mm i.d.

QUANTITATIVE ANALYSIS.—The estimation of free and combined anthraquinones (*O*-glycosides) was based on a colorimetric method described in an earlier paper (2). The amount of some individual anthraquinones was also estimated by a thin-layer densitometric method (14).

HIGH-YIELDING CELL LINE.—Callus tissues used were the same as those used for the earlier experiment. The calli of *C. podocarpa* were soft and friable, and the tissue color varied from gray to light brown. The gray and brown tissues of *C. podocarpa* callus cultures were, during subculture, manually separated in different flasks on fresh medium. It was possible to separate only 90% of all the brown tissues and gray tissues from each other. The separation of gray and brown tissues was repeated thrice for each flask at subsequent culture stages, at 6-week intervals.

RESULTS

COMPOSITION OF ANTHRAQUINONES.—Nine compounds (chrysophanol, emodin, rhein, chrysophanol anthrone, chrysophanol dianthrone, emodin anthrone, rhein anthrone, chrysophanol monoglucoside, and rhein monoglucoside) were detected in callus tissue extracts of *C. podocarpa*. Indentification of chrysophanol, emodin, and rhein was also confirmed.

By colorimetric procedure, the free anthraquinones and the combined anthraquinones were estimated to be 0.8% and 0.5% of the dry callus material, respectively.

The concentration of some individual anthraquinones determined by thin-layer densitometric method is shown in Table 2. Rhein and chrysophanol, present both in free and glycosidic forms, represented 65% of the total anthraquinone yield.

THE EFFECT OF AUXIN AND KINETIN.—The results of the experiment on the influence of growth regulators showed that there are considerable differences not only in growth but also in pigment production among callus cultures, depending upon the concentrations of 2,4-D and kinetin in the medium (Table 1). In the absence of kinetin,

TABLE 2. Concentration of Anthraquinone Compounds in Callus Cultures of *Cassia podocarpa* Estimated By Thin-layer Densitometry.[a]

	Aglycones % w/w	*O*-glycosides % w/w
Chrysophanol	0.14	0.22
Emodin	0.15	—[b]
Rhein	0.22	0.26
Total	0.51	0.48

[a]Expressed as the equivalent of alizarin used as an internal standard; each value represents a mean of three determinations.
[b]Not present.

the tissue growth was stimulated by increasing the concentration of 2,4-D to a level of 0.6 mg/liter medium. The addition of kinetin (0.4 mg/liter) to the medium significantly improved the growth when the concentration of 2,4-D was 0.5–0.6 mg/liter. Callus tissues showed no organ formation at any hormonal level tested. As for anthraquinone production, the optimal concentration of 2,4-D was 0.6 mg/liter medium in the presence of kinetin (0.4 mg/liter) which also had the highest growth rate (Table 1).

HIGH YIELDING CELL LINE.—The original callus of *C. podocarpa* was composed of approximately 60% brown cells and 40% gray cells. Little change in qualitative and quantitative spectra with respect to anthraquinone constituents was observed in gray cells and brown cells examined 6 weeks after first and second subculture stages. The chemical analyses carried out 6 weeks after the third transfer also revealed that there was no change in qualitative spectrum, but the total accumulation of anthraquinones had increased in the brown cell line by nearly 40% compared to the original callus (Table 3). The brown cell line thus obtained, after three successive selective transfers, was coded R101. During further subculture of this line after 6, 12, and 18 weeks, the anthraquinone level remained virtually unchanged. The media on which the tissue had grown contained no anthraquinones. It indicates that although the media were colored (faint yellow), anthraquinones are retained in the tissue and not released into the medium.

TABLE 3. Anthraquinone Content of Cell Line R101 and Original Callus of *Cassia podocarpa*.[a]

	Original cells	Brown cell line R101	Gray cells
Free anthraquinones	0.80 ± 0.003	1.00 ± 0.016	0.80 ± 0.003
Bound anthraquinones	0.50 ± 0.003	0.80 ± 0.002	0.40 ± 0.005
Total content	1.30 ± 0.004	1.80 ± 0.016	1.20 ± 0.005

[a]% w/w in dry wt, mean of 10 determinations, ± standard error.

DISCUSSION

An earlier experience (2,6) has shown that 2,4-D is the best choice as auxin and that a combination of 2,4-D and kinetin is essential for growth as well as pigment accumulation in cultures of *Cassia* species. Therefore, it was decided to study the effects of 2,4-D

and kinetin on *C. podocarpa* cultures for determining the optimum levels of 2,4-D and kinetin for both tissue growth and anthraquinone production. The optimum levels for this cell line were 2,4-D (0.6 mg/liter) and kinetin (0.4 mg/liter). Unlike many other plant cell cultures in which growth and secondary metabolism are antagonistic (15), there is a close parallel between the effects of growth regulators (2,4-D and kinetin) on growth and anthraquinone production in *C. podocarpa* callus cultures (Table 1). This seems to be the first case in which the specific yields of hydroxyanthraquinones increase with growth. The inhibition effect of 2,4-D on callus tissue growth at 0.7 mg/liter is similar to that found by Tabata *et al.* (3), although they were using 2,4-D at 1 mg/liter.

The anthraquinone values obtained from the tissue cultures of *C. podocarpa* are high compared to previously reported data for cultures of anthraquinone producing similar species (2). The total yield of anthraquinone compounds in original *C. podocarpa* cultures was lower (1.3% w/w) than that of the plant leaves (1.6% w/w) (10). The visual selection method for brown pigmented tissues yielded a slightly higher anthraquinone content (1.8% w/w) than that present in the plant. The yield increase obtained upon selection, although significant, is not large. It seems possible that the biosynthetic capabilities of callus tissue could be improved progressively by selective subcultures. It may be noted that though rhein dianthrone glycosides (sennosides) occur in whole plants of *C. podocarpa*, they were not detected in callus cultures; this parallels the occurrence of these in plants and its absence in callus cultures of *C. senna* (2) and *R. palmatum* (4). However, Friedrich and Baier (1) were able to recover small quantities of sennosides from callus cultures of *C. angustifolia*.

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