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FORMATION OF GERMICHRYSONE BY TISSUE CULTURES OF *CASSIA TOROSA*: INDUCTION OF SECONDARY METABOLISM IN THE LAG PHASE

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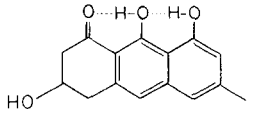
Key Word Index—*Cassia torosa*; Leguminosae; callus; shake culture; induction; secondary metabolism; polyketide; germichrysone.

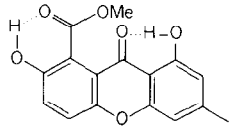
Abstract—A callus culture of *Cassia torosa* which produced germichrysone, an octaketide hydroanthracene, in high yield was established on Murashige–Skoog's medium containing IAA (3 ppm) and benzyladenine (0.1 ppm). In six-week-old callus culture the main pigment was pinselin and the germichrysone content was markedly decreased. This may indicate that pinselin was formed from germichrysone. A shake culture was established in liquid Murashige–Skoogs medium containing IAA and benzyladenine. The course of germichrysone production in relation to growth was investigated with the shake culture and the production of germichrysone was found to possess two maxima. The first maximum was observed in lag phase and the second coincided with active growth. Incorporation experiments with [1-^{14}C] acetate clearly demonstrated that the secondary metabolism was induced in the lag phase.

INTRODUCTION

Changes in the main pigments during germination are a characteristic feature of *Cassia torosa* [1, 2]. The main pigment in the seeds is an anthraquinone glycoside, whereas a hydroanthracene, germichrysone (**1**), is the characteristic pigment of the seedlings [3, 4]. In our previous investigation on pigment production in *C. torosa* tissue culture, a callus culture was shown to produce germichrysone (**1**) in a much higher yield than the seedlings [5]. The present work is a continuation of our work with the tissue cultures of *C. torosa* and is mainly concerned with the effect of varied concentrations of auxins and cytokinins on growth and pigment production as well as with the establishment of a shake culture. The time course production of germichrysone (**1**) in relation to growth rate was investigated with the established shake culture to clarify the kinetics of germichrysone (**1**) production.



1 Germichrysone



2 Pinselin

RESULTS

The callus culture, established as described in the previous paper [5] and subcultured on Murashige–Skoog's medium (MS medium) [6], stayed black so long as it was maintained with 2,4-D and kinetin. When the black callus tissue was transferred on to MS medium containing NAA (1–3 ppm) and kinetin (0.1 ppm) and subcultured several times at transfer intervals of 4–6 weeks, a part of the callus tissue became greenish yellow. When this greenish-yellow callus tissue was maintained on fresh MS medium containing NAA and kinetin and subcultured at appropriate intervals for several months, it gave a callus culture of stable greenish-yellow colour. The effects of auxins and cytokinins on the growth and pigment production by this culture are shown in Table 1. All the media tested supported growth and pigment production. Replacement of NAA by 2,4-D had no significant effect on growth and the colour of callus tissue, but pigment production was slightly suppressed. It was evident that a combination of IAA and benzyladenine was the best among those tested both for growth and pigmentation (Table 1). Zeatin did not show any advantage over benzyladenine. 4-Pyridylurea, a synthetic cytokinin [7], tended to yield white callus tissue (not shown in Table 1). Schenk and Hildebrandt's medium [8], tested as a basal medium, was no better than the MS medium. The callus tissue grown on the MS medium with a high concentration of IAA (3 ppm) was softer and more friable than those grown with a low concentration of IAA or with NAA (1–3 ppm). Since a soft and friable callus tissue was advantageous in an investigation to establish a shake culture, the greenish-yellow callus culture was maintained with IAA (3 ppm) and benzyladenine (0.1 ppm). The callus culture thus established could be maintained by subculturing at transfer intervals of 3 weeks. When the callus culture was incubated for 6 weeks without subculturing, the

Table 1. Effects of auxins and cytokinins on growth and germichrysone formation in a 3-week-old culture of *C. torosa* callus

Medium	Auxin (ppm)		Cytokinin (ppm)		Fr. wt of callus (g)	Germichrysone (mg/g fr. wt)	(mg/l.)
MS*	2,4-D	1	Kinetin	0.1	3.3	0.19	15.8
		1	Kinetin	0.5	3.1	0.18	14.0
		1	Kinetin	1.0	2.7	0.17	11.5
		3	Kinetin	0.1	3.0	0.31	23.5
	NAA	1	Kinetin	0.1	4.7	0.42	48.8
		3	Kinetin	0.1	3.4	1.17	100
	IAA	1	Kinetin	0.1	4.7	0.52	60.0
		3	Kinetin	0.1	8.2	0.48	99.0
		1	Benzyladenine	0.1	7.2	0.96	174
		3	Benzyladenine	0.1	10.4	0.48	125
		3	Zeatin	0.1	5.6	0.55	77.5
SH*		3	Kinetin	0.1	3.7	0.48	45.0
		3	Benzyladenine	0.1	4.2	0.48	50.0

*MS, Murashige–Skoog's medium; SH, Schenk and Hildeblandt's medium.

Table 2. Germichrysone content in 3- and 6-week-old callus cultures of *C. torosa*

Medium	Auxin (ppm)		Cytokinin (ppm)		Culture period (week)	Fr. wt of callus (g)	Germichrysone (mg/l.)	(mg/g fr. wt)
MS*	IAA	3	Kinetin	0.1	3	8.2	95.0	0.47
		3	Kinetin	0.1	6	13.5	15.0	0.04
		3	Benzyladenine	0.1	3	10.4	128	0.48
		3	Benzyladenine	0.1	6	14.9	15.0	0.04

*See Table 1.

content of germichrysone (**1**) decreased sharply as shown in Table 2. Addition of ascorbate significantly prevented the degradation of germichrysone (**1**) in long-term incubation. The main pigment of 6-week-old callus tissue was pinselin (**2**) [5]. These observations suggest that germichrysone (**1**) was converted into pinselin (**2**) in the callus culture under long-term incubation.

A shake culture was established in the MS liquid medium with IAA and benzyladenine from the greenish-yellow callus tissue. A high concentration of IAA (3 ppm) gave a better growth rate than a lower concentration (1–0.3 ppm) and, therefore, the shake culture was maintained in the MS liquid medium containing IAA (3 ppm) and kinetin (0.1 ppm) by subculturing at appropriate intervals for 6 months. The intervals between subculturing were relatively long at the initial stage, *ca* 20 days, but gradually became shorter. The shake culture thus established could be maintained by subculturing at transfer intervals of 10 days. The effects of auxins and cytokinins were examined by using this shake culture. The results demonstrated that a combination of IAA and benzyladenine was the best for growth and pigment production (Table 4). Gamborg's B5 medium, which was reported to be effective in anthraquinone production in *Morinda* tissue culture [9], showed no advantages over the MS medium used in the shake culture.

The pattern of accumulation of germichrysone (**1**) in relation to the growth of the established shake culture is shown in Fig. 1. The maximum growth was attained some 6–8 days after inoculation, however, the production of germichrysone (**1**) showed two

Table 3. Effects of ascorbate on growth and germichrysone production in long-term incubations of *C. torosa* callus culture

Medium	Auxin (ppm)		Cytokinin (ppm)	Culture period (week)	Fr. wt of callus (g)	Germichrysone (mg/l.)	(mg/g fr. wt)
MS*	IAA	3	Kinetin 0.1	3	10.9	76.3	0.28
		6	Kinetin 0.1	6	9.0	34.5	0.15

*See Table 1.

Table 4. Effects of auxins and cytokinins on growth and germichrysone formation in *C. torosa* shake culture

Medium	Auxin (ppm)		Cytokinin (ppm)		Culture period (days)	Fr. wt of cultured tissue (g/flask)	Germichrysone (mg/l.)	Germichrysone (mg/g fr. wt)
MS*	IAA	3	Benzyladenine	0.1	10	8.8	139	1.90
		6	Benzyladenine	0.1		2.2	22.5	1.22
	NAA	3	Benzyladenine	0.1		5.6	30.8	0.66
		3	Kinetin	1.0		7.1	56.7	0.97
B5†	IAA	3	Benzyladenine	0.1		7.2	13.3	0.22
MS*		3	Benzyladenine	0.1	20	9.8	55.0	0.67
		6	Benzyladenine	0.1		2.3	23.3	1.22
	NAA	3	Benzyladenine	0.1		14.6	50.0	0.44
		3	Kinetin	1.0		14.1	58.3	0.50
B5†	IAA	3	Benzyladenine	0.1		11.3	13.3	0.14

*See Table 1.
†B5, Gamborg's B5 medium.

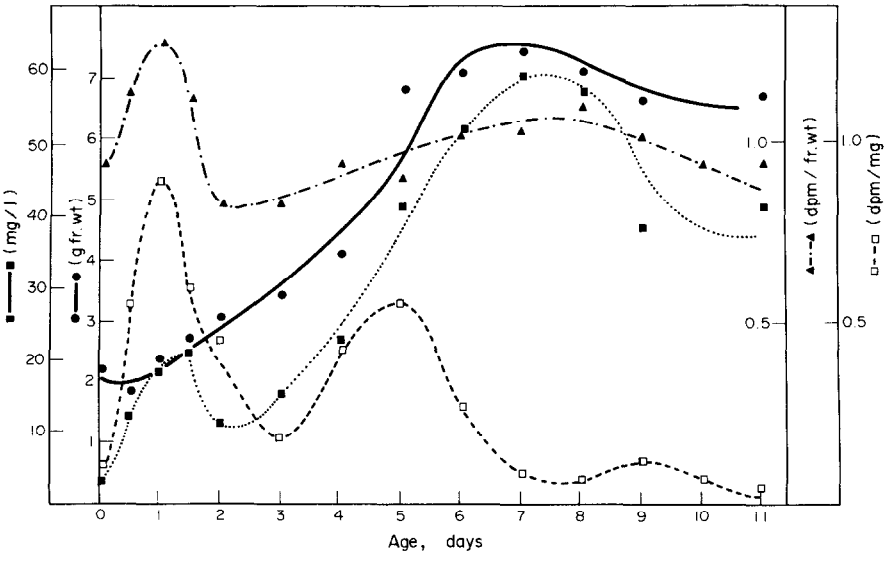


Fig. 1. Changes in germichrysone production (■), fresh weight (●), germichrysone content (▲) and sp. radioactivity of germichrysone labelled with [1-^{14}C]acetate (□) in *C. torosa* shake culture.

maxima. The first maximum was observed during lag phase (0–2 days) and then the second maximum was on the seventh and eighth days. This characteristic feature of pigment production was more evident in the time course change of germichrysone (1) content in the tissue cells. The pigment content reached its first maximum during lag phase, declined (second day) and then gradually rose to the second maximum (seventh and eighth days). The second maximum of germichrysone production coincided with the increase of fresh weight. The curve of specific activity of germichrysone labelled by [1-^{14}C] acetate in short time feeding experiments (1 hr) clearly demonstrated the presence of two maxima in the rate of germichrysone formation. The first maximum was at 24 hr after inoculation and the second maximum on the fourth and fifth days, the latter of which coincided with active growth.

DISCUSSION

The callus tissue of *C. torosa* was composed of small cell aggregates, when it was maintained on the MS medium with IAA and benzyladenine. 2,4-D and NAA gave somewhat hard callus tissue due to the presence of big cell aggregates and to a lower content of water. The effect of 2,4-D on pigment production is in contrast to Zenk's observation that the production of anthraquinones in the tissue culture of *Morinda citrifolia* is completely suppressed by 2,4-D [9]. A similar observation was made by Furuya *et al.*, who found IAA is much more favourable than 2,4-D to the production of anthraquinones in the tissue culture of *Digitalis lanata* [10]. In contrast, Tabata *et al.* have reported that anthraquinone production in the tissue culture of *C. torosa* is maintained nearly at the same level with either 2,4-D or IAA [11]. The different effects of auxins are attributed to the difference of

anthraquinone biosynthesis by the plants [11]. The anthraquinones of *Morinda* and *Digitalis* are derived from 2-succinylbenzoate and mevalonate [12], whereas the anthraquinones of *Cassia* are assumed to be polyketides formed from acetate and malonate [13]. The results of our studies provide another example of the tissue culture producing the polyketide that is not strongly affected by 2,4-D.

The time course of secondary metabolite production reflects the regulation of enzyme activities responsible for the biosynthesis. Kinetic studies of the rate of metabolite formation and cell growth are essential to obtain a basic understanding of secondary metabolism in plant tissue culture [14]. Growth and production patterns of tissue cultures are classified into several types [14]. In the tissue culture of *Lithospermum officinale* var. *erythrorhyzon*, the accumulation of shikonin, a naphthoquinone, occurs after active protein synthesis has ceased [15, 16]. Polyphenol production in the tissue cultures of Paul's Scarlet rose and *Acer pseudoplatanus* shows a similar pattern [7, 8]. In contrast, the production of nicotine, tropane alkaloids and *Morinda* anthraquinones proceeds almost in parallel with cell growth [9, 19, 20]. Diosgenin production belongs to another type. Growth and production curves are diphasic and the production curve lags behind the growth curve [21]. Total phenolic production in suspension culture of *Sylibum marianum* is reported to be biphasic [22]. A high level of total phenolic content at one week after inoculation rapidly decreases to a minimum level in 3–4 weeks and then gradually increases to attain the maximum level in 8–9 weeks, after which the phenolic content again begins to decrease. The production of phenolics takes place after the increase of cell weight has almost ceased.

The time course production of germichrysone in the shake culture of *C. torosa* showed a distinct biphasic pattern (Fig. 1). However, growth and production pattern are not exactly the same as those of *Sylibum marianum*. The initial increase of the rate of germichrysone production is clearly shown in the curve of its content per unit weight of tissue, as well as in the curve of specific activity of germichrysone labelled by [1-^{14}C] acetate in short time feeding experiments.

It is generally recognized that metabolic activities in cultured cells increase immediately after inoculation. For example, enzyme activities relating to carbohydrate metabolism [23] and oxygen consumption increase immediately after inoculation [24, 25]. The activity of phenylalanine ammonia lyase (PAL) is not only induced by light but also by dilution in fresh medium [26]. The specific activity of germichrysone in 1-hr feeding experiments with [1-^{14}C] acetate is highest at 24 hr after inoculation, i.e. *ca* 1.6 times higher than at the second maximum (fourth and fifth days) (Fig. 1). The induction of germichrysone production is presumably caused by the inoculation of tissue, i.e. by dilution in fresh medium. In contrast, the second maximum of germichrysone production occurs in the logarithmic phase. The results so far obtained indicate that the production of germichrysone is induced by two different mechanisms. The first induction is triggered by the dilution of tissue cells in the fresh medium and the second induction is closely associated with active cell growth. This is the first report of the induction of secondary metabolism in the lag phase and the results of further studies will be reported in separate papers.

EXPERIMENTAL

Plant material and culture methods. A callus culture initiated from the seedlings of *C. torosa* and maintained as described in the previous paper was the source of the tissue culture used in this study [5]. All the callus cultures were grown on 40 ml of medium solidified with 0.9% agar (Bacto Agar; DIFCO) in 100-ml flasks in the dark at 25–28°. The Murashige–Skoog's (MS) medium was mainly used as a basal medium and Schenk and Hildebrandt's medium was also tested as a basal medium in callus cultures. In expts to test the influence of auxins and cytokinins, 5–10 flasks were used for each experiment with culture period of 3 weeks. A shake culture was initiated by transferring callus tissue grown on 40 ml of medium into 100 ml of liquid MS medium in 500-ml flasks and grown on a rotary shaker (180 rev/min) in the dark at 25–28°. A 20% inoculum was transferred into fresh medium and subcultured at appropriate intervals. Gamborg's B5 medium was also tested as a basal medium in shake culture experiments. In growth curve expts, 20-ml aliquots were dispensed into 500-ml conical flasks containing 100 ml of medium and these cultures were used for various expts. 4–5 flasks were used to measure fr. wt and pigment production.

Quantitative estimation of pigments. Fresh callus tissue was extracted with Me_2CO under reflux (×3) and the extracts were evapd to dryness. The residue was dissolved in a known vol. (50–100 ml) of Me_2CO and an appropriate vol. of the soln was subjected to TLC on 0.5 N $(COOH)_2$ treated Merk GF_{254} (reactivated) developed with C_6H_6–Me_2CO (4:1). The quantities of pigments were measured with a Shimazu dual-wavelength TLC zig-zag scanner (Model 900) with 402 and 700 nm as sampling and reference wavelengths, respectively. Standard calibration curves were made from solns of known concentration of authentic samples [5].

Incorporation of [1-^{14}C]*acetate.* [1-^{14}C]-Acetate (0.45 μCi; 45–60 mCi/mM) was added to two flasks of shake culture of various ages, which were further incubated for 1 hr. The cultures were harvested and washed with H_2O. The harvested cells were extracted with EtOAc under reflux and the extracts subjected to TLC (system just described). The band of germichrysone was scraped off the developed plate and extracted with EtOAc. The germichrysone was estimated by UV spectrophotometry and the radioactivity was measured in a liquid scintillation counter. Sp. acts. of germichrysone were the mean value of two samples in each expt.

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REFERENCES

1. Takido, M., Nakamura, Y. and Nitta, K. (1960) *Pharm. Bull. Nihon Univ.* **3–4**, 18.
2. Takido, M., Takahashi, S., Masuda, K. and Yasukawa, K. (1977) *Lloydia* **40**, 191.
3. Takahashi, S., Takido, M., Sankawa, U. and Shibata, S. (1976) *Phytochemistry* **15**, 1295.
4. Takahashi, S., Kitanaka, S., Takido, M., Sankawa, U. and Shibata, S. (1978) *Phytochemistry* **16**, 999.
5. Takahashi, S., Takido, M., Yeh, S., Otsuka, H., Noguchi, H., Iitaka, Y. and Sankawa, U. (1981) *Shoyakugakuzasshi* **35**, 22.

6. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
7. Takahashi, S., Shudo, K., Okamoto, T., Yamada, K. and Isogai, Y. (1978) *Phytochemistry* **17**, 1201.
8. Schenk, R. U. and Hildebrandt, A. C. (1972) *Lloydia* **38**, 131.
9. Zenk, M. H., El-Shagi, H. and Shulte, U. (1975) *Planta Med.* **29**, Suppl. 79.
10. Furuya, T. and Kojima, H. (1971) *Phytochemistry* **10**, 1607.
11. Tabata, M., Hiraoka, N., Ikenoue, M., Sano, Y. and Konoshima, M. (1975) *Lloydia* **38**, 131.
12. Dansette, P. and Azerad, R. (1970) *Biochem. Biophys. Res. Commun.* **40**, 1090.
13. Leistner, E. and Zenk, M. K. (1969) *J. Chem. Soc. Chem. Commun.* 20.
14. Tabata, M. (1977) in *Plant Tissue Culture and Its Biotechnological Application* (Barz, W., Reinhard, E. and Zenk, M. H., eds.) p. 3. Springer, Berlin.
15. Tabata, M., Mizukami, H., Hiraoka, N. and Konoshima, M. (1974) *Phytochemistry* **13**, 972.
16. Mizukami, H., Konoshima, M. and Tabata, M. (1977) *Phytochemistry* **16**, 1183.
17. Davies, M. E. (1972) *Planta* **104**, 50.
18. Phillips, R. and Henshaw, G. G. (1977) *J. Exp. Botany* **28**, 785.
19. Furuya, T., Kojima, H. and Syono, K. (1971) *Phytochemistry* **10**, 1597.
20. Chan, W. and Staba, E. J. (1965) *Lloydia* **28**, 55.
21. Kaul, B., Stoh, S. J. and Staba, E. J. (1969) *Lloydia* **32**, 347.
22. Becker, H. and Schrall, R. (1977) *Planta Med.* **31**, 185.
23. Fowler, M. W. (1971) *J. Exp. Botany* **22**, 714.
24. Shimizu, T., Clifton, A., Komamine, A. and Fowler, M. W. (1977) *Physiol. Plant.* **40**, 125.
25. Kanamori, I., Ashihara, H. and Komamine, A. (1979) *Z. Pflanzenphysiol.* **93**, 437.
26. Schroder, J., Betz, B. and Hahlbrock, K. (1978) *Plant Physiol.* **60**, 440.