Phytochemistry, 1981, Vol. 20, pp. 353–355. © Pergamon Press Ltd. Printed in England. 0031-9422/81/0201-0353 $02.00/0

GROWTH AND FUROQUINOLINE ALKALOID PRODUCTION IN CULTURED CELLS OF *CHOISYA TERNATA*

M. SEJOURNE,* C. VIEL,*† J. BRUNETON‡, M. RIDEAU* and J. C. CHENIEUX*

*Faculté de Pharmacie, Tours, France; ‡Faculté de Pharmacie, Angers, France; †Faculté de Pharmacie, Châtenay-Malabry, France

(*Received* 18 *April* 1980)

Key Word Index—*Choisya ternata*; Rutaceae; furoquinolines; plant cell cultures; alkaloid production time-course.

INTRODUCTION

The Mexican shrub *Choisya ternata* Kunth (Rutaceae) contains numerous furoquinoline alkaloids [1–5] and we reported previously on the isolation from leaves of two dihydrofuroquinoline alkaloids, namely balfourodinium with plant growth-inhibiting properties, and platydesminium [6].

The present investigation was undertaken to compare the alkaloid composition and contents of cell suspension cultures with those of the original plant, and to study the alkaloid production time-course during the growth phases.

RESULTS AND DISCUSSION

Choisya ternata can be grown successfully on Murashige and Tucker medium [7]. Callus tissues are greenish, friable and unorganized. Previous studies with different concentrations of growth hormones (auxins, cytokinins, adenine) failed to induce organogenesis and these data suggested that the callus lacked potential for organ induction. Suspension cultures can easily be obtained from callus cell and grow in the light in 1-l. shaken Erlenmeyer flasks or in 2- or 7.5-l. fermentors as small aggregates (up to 500 μm) and isolated cells.

Table 1. Alkaloid content* of *Choisya* leaves and cell cultures

Alkaloid	Leaves	Cell cultures
Balfourodinium	0.15	Trace
Platydesminium	0.05	0.1
Skimmianine	0.11	Trace
Choisyine	0.07	—
Evoxine	0.05	—
Kokusaginine	0.03	0.009

* Values are expressed in % dry wt.

The alkaloid patterns (Table 1) have been compared in *Choisya* leaves (the richest organ) and in cell suspensions. From *Choisya* leaves, the tertiary furoquinoline alkaloids already known in this species: skimmianine (0.11% dry wt), evoxine (0.05%), choisyine (0.07%) and kokusaginine (0.03%) were isolated as well as the two dihydrofuroquinoline salts (−)(*S*)-balfourodinium Cl^- (0.15%) and (−)(*S*)-platydesminium ClO_4^- (0.05%).

Comparatively, the TLC pattern of the ether-soluble tertiary alkaloid fraction of cell suspension was simpler than that of the original plant and gave three alkaloidal spots *A*, *B*, *C*. After chromatographic separation on alumina column, *B* was identified by NMR spectra as kokusaginine, the minor alkaloid in the leaves. *C*, isolated in very low amounts, has been tentatively identified by TLC as being skimmianine, the main furoquinoline alkaloid in *Choisya* leaves. The total amount of furoquinoline alkaloids in suspension cultures was much lower than that of the original plant (0.01% vs 0.25%).

The ether-insoluble quaternary alkaloid fraction of cell suspension, after separation on silica gel column and identification by UV, NMR and MS, gave platydesminium, and only traces of balfourodinium, the main dihydrofuroquinoline alkaloid of the leaves and stems. Thus, *in vitro* cells apparently show a decrease in specific enzymes for hydroxylation and methylation. Platydesminium is produced in approximately the same amount (0.1% dry wt) as in the original plant (0.05%). Similar alkaloid pattern has been obtained in suspension cultures growing on D-medium or in callus tissues, and the same alkaloids have been isolated.

The quantitative analysis of platydesminium salt in suspension cultures was carried out by a fluorometric procedure [9] at various times during a 6 week period of growth. Figure 1 shows a typical time course of growth and the production of this alkaloid in well established *Choisya ternata* suspensions growing in shaken flasks. The fresh wt and dry wt growth curves are sigmoidal. During the second half of the log phase, protein content reached a peak after that of the RNA content. The lag phase was accompanied by a decrease of the platydesminium content of the cells. Platydesminium was then synthesized and the production occurred maximally in the late stationary phase.

In our assays, production growth pattern of *Choisya ternata* may be thus classified in Gaden's 'second type' [12] but with particular decrease of the alkaloid content during the first stage of growth. However this last point is not unknown and has been found by Tabata with alkaloids produced in a *Scopolia japonica* callus [13] or with nicotine produced by tobacco cells [14]. Whether this is due to metabolism of platydesminium during the lag phase or to excretion into the medium is being investigated.

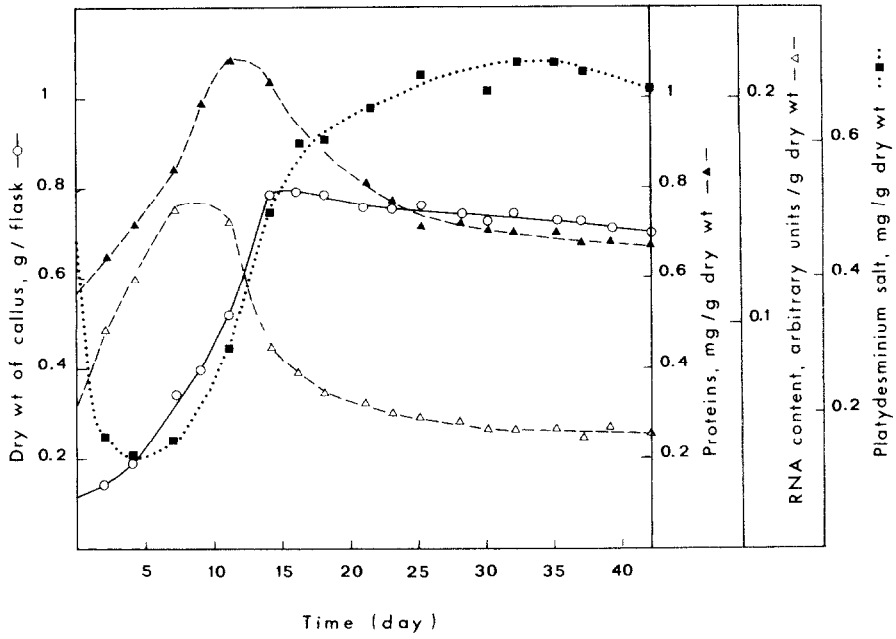

Fig. 1. Time course of *Choisya ternata* cell culture and total nucleic acids, proteins and platydesminium salt production over 40 days (D-medium).

EXPERIMENTAL

The callus culture stock originally derived from the young stem of *Choisya ternata* Kunth has been maintained on the Murashige and Tucker medium [7] supplemented with 1 mg/l. NAA and 0.1 mg/l. K (N-medium) at 26 ± 1°, under 1000 lx, for over 7 yr by successive transfers carried out with 3 week intervals. Suspension cultures, established by inoculating callus cells in a liquid medium of the same composition (except that NAA is substituted by 0.01 mg/l. 2,4-D and that K is omitted: D-medium), were maintained by serial culture every week for 6 months before studies of growth characteristics were made. They were grown in the light (2000 lx) at 26 ± 1° in 1-l. cotton-stoppered flasks containing 200 ml medium on a rotary shaker (100 rpm).

Cell culture alkaloid analysis. Suspension cultures were grown in aerated 2-l. or 7.5-l. fermentors (Biolafitte) containing respectively 1 or 5 l. N-medium. The fresh cells (9.6 kg) were freeze-dried (980 g) and the total alkaloids extracted by MeOH lixivation. MeOH extracts were filtered through neutral Al_2O_3 (200 g; Prolabo); solvent was distilled off and the residue was taken up in 1 l. distilled water and extracted with 500 ml Et_2O (×3). The ether-insoluble fraction was evapd and the residue dissolved in 250 ml · 10^{-3} M bromothymol blue solution buffered at pH 6 and the alkaloid–dye complexes were repeatedly extracted by CH_2Cl_2. The organic layer was chromatographed over a Si gel G column (150 g; Merck). Elution by EtOAc–HCOOH–H_2O (10:1:1) gave two alkaloids which were crystallized as perchlorates from MeOH–$HClO_4$, and identified as platydesminium and balfourodinium by TLC, UV, NMR and mass spectra compared with those of bases previously isolated from *Choisya* leaves [6].

The ether-soluble fraction was chromatographed over Al_2O_3. $CHCl_3$–EtOAc (4:1) eluted 3 alkaloids. *A*—UV (HCl 0.1 N) nm: 243, 303, 314, 341. *B*—UV (HCl 0.1 N) nm: 244, 250, 287, 330. *C*—UV (HCl 0.1 N) nm: 250, 320, 345. The main alkaloid *B* was identified as kokusaginine by means of TLC and NMR spectra. *C* migrates at the same R_f as skimmianine in numerous solvent systems on Al_2O_3: toluene–EtOAc–HCOOH (5:4:1) and Si gel G: $CHCl_3$–EtOAc (4:1), C_6H_6–Me_2CO (4:1).

Choisya *leaves' alkaloid analysis.* Freeze-dried, powdered *Choisya* leaves (2500 g fresh wt) were extracted with MeOH. (−)(*S*)-Balfourodinium and (−)(*S*)-platydesminium were isolated as described previously [6]. Tertiary alkaloid fraction was chromatographed over Al_2O_3 column. A hexane–EtOAc gradient gave 4 alkaloids in the following sequence: an unidentified alkaloid, kokusaginine (47 mg), skimmianine (305 mg) and choisyine (225 mg). Then MeOH eluted evoxine (100 mg). These well-known furoquinoline bases were identified by NMR spectra.

Growth characteristics of Choisya *cell suspensions and platydesminium production time-course.* 250 ml shake flask experiments were conducted under the same conditions as described above for maintenance of stock cultures, during 40 days, in the light. The flasks contained 50 ml D-medium. Cell density was 4.5×10^4 per ml at the start of the culture. Every day or every other day, 2 flasks were taken off, and growth of the cultures was recorded by fresh wt, dry wt and packed vol. determinations, by measurement of cell density and by decrease of the medium conductivity as described elsewhere [8].

Platydesminium was extracted by MeOH from 50 mg freeze-dried cells, and evaluated by a spectrofluorometric method [9].

Lipid-free, cold-acid insoluble residues were prepared from 50 mg freeze-dried cells. Nucleic acids and proteins were extracted from the residues by the Bryant and Rees procedure [10]. Total nucleic acids (i.e. essentially RNA) [11] were measured spectrophotometrically at 260–290 nm. Proteins were estimated by the Lowry method using bovine serum albumin (Sigma) as standard.

REFERENCES

1. Frolova, V. I. and Kuzovkov, A. D. (1963) *Zh. Obshch. Khim.* **33,** 121.
2. Johns, S. R., Lamberton, J. A. and Sioumis, A. A. (1967) *Aust. J. Chem.* **20,** 1975.
3. Dreyer, D. L., Pickering, M. V. and Conan, P. (1972) *Phytochemistry* **11,** 705.
4. Grundon, M. F., Harrison, D. M. and Spyropoulos, C. G. (1974) *J. Chem. Soc. Perkin Trans.* I, 2181.
5. Tea, S. H. (1977) Thèse Doct. Pharmacie, Toulouse.
6. Rideau, M., Verchère, C., Hibon, P., Chénieux, J. C., Maupas, Ph. and Viel, C. (1979) *Phytochemistry* **18,** 155.
7. Murashige, T. and Tucker, D. P. H. (1969) *Proc. 1st Int. Citrus, Symp.* Vol. 3, p. 1155. Chapman & Hall, London.
8. Séjourné, M., Rideau, M. and Chénieux, J. C. (1980) *Bull. Soc. Bot. Fr.* (in preparation).
9. Montagu-Bourin, M., Rideau, M. and Chénieux, J. C. (1981) *Planta Med.* **38,** 50.
10. Bryant, J. A. and Rees, T. (1971) *Phytochemistry* **10,** 1191.
11. Fraser, R. S. S. and Loening, U. E. (1974) *J. Exp. Botany* **25,** 847.
12. Gaden, E. L. (1955) *Chem. Ind.* 154.
13. Tabata, M., Yamamoto, H. and Hiraoka, N. (1970) *Coll. Int. CNRS* No. 193, 389.
14. Tabata, N., Yamamoto, H., Hiraoka, N., Marumoto, Y. and Konoshima, M. (1971) *Phytochemistry* **10,** 723.