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STEROID PRODUCTION BY CALLUS AND CELL SUSPENSION CULTURES OF *SOLANUM AVICULARE*

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Solanum aviculare Forst is known for its production of pharmaceutically valuable steroids. The USSR, some eastern European countries, as well as the People's Republic of China, India, New Zealand, Egypt, and Australia produce a proportion of their steroidal raw material, solasodine, from *Solanum laciniatum* and its close relative *S. aviculare*. There is an increasing demand for new sources of steroids because they are used for the synthesis of steroidal drugs required in many areas of medicine (1,2).

Much progress has been made in plant and cell suspension culture techniques, making these approaches to the biosynthesis of useful chemicals feasible. These techniques offer a series of advantages over the use of field-grown plants, although they may fail to produce the desired chemicals in significant amounts (3,4).

A variety of steroids has been detected in callus culures of several *Solanum* species, including *S. laciniatum* (5,6). Studies on the production of diosgenin and solasodine in callus and cell suspension cultures of *S. aviculare* were done by Khanna *et al.* (7,8) who detected their presence, but there is no report on a variety of other steroids in cell suspension cultures of *Solanum* species.

The purpose of this study is to examine the production of various sterols and diosgenin in callus and cell suspension cultures of *S. aviculare*.

EXPERIMENTAL

CALLUS CULTURES.—Surface-sterilized seeds (9) of *S. aviculare*[3] were germinated at 25° in the dark on a Murashige and Skoog medium (10) modified by using 0.4 mg/liter of thiamine HCl, 0.8% agar without glycine, pyridoxine HCl, and nicotinic acid. The pH of the medium was adjusted to 6 with 1 N NaOH. To induce callus formation, the roots and hypocotyls were transferred to the above-mentioned medium, supplemented with 1 ppm of 2,4-D, and incubated at 25° in the dark. The procedure used was similar to that of Hosada and Yatazawa (6). Callus tissues were subcultured every 4 weeks to fresh medium. After six subculturings, callus tissues were harvested and stored in the freezer for subsequent chemical analysis. Prior to extraction, the tissues were thawed at room temperature.

CELL SUSPENSION CULTURES.—Cell suspension cultures composed of free cells and small cell aggregates were developed from pieces of friable callus tissues and were grown in screw-cap Erlenmeyer flasks containing Murashige and Skoog liquid medium (10) supplemented with 2,4-D (1 ppm). The cultures were maintained at 25° under dim room light and agitated on a rotatory shaker at 175 rpm. Cell suspension cultures were subcultured every 8 days in fresh medium. The procedure followed was similar to the one used by Misawa *et al.* (11). After 12 subculturings, cell suspension cultures were harvested, suction filtered, and the cell material was stored in the freezer for chemical analysis. Prior to the extraction, the cell material was thawed at room temperature.

The growth of cell suspension cultures was determined by measuring the dry cell weight (DCW) in mg/ml samples (12).

EXTRACTION PROCEDURE.—Harvested callus tissues (1 kg) and cell material (1 kg) were separately homogenized with Me_2CO and left standing for 24 h. The homogenates were filtered; the filtered materials were extracted again with Me_2CO and left standing for 48 h. The extracted materials were separated by filtration. Both filtrates were combined and acidified with 10% HOAc to a pH of 3.5. The Me_2CO of the filtrates was removed on a rotatory evaporator under reduced pressure, and the concentrates were extracted with CH_2Cl_2. The organic phases were dried ($MgSO_4$), and the solutions were evapo-

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rated on the rotary-evaporator to dryness (6). Standard diosgenin (0.5 g) was added to part of the harvested callus tissues (500 g) and extracted in an identical manner to that described above. The extracts were analyzed under the conditions described below.

FORMATION OF TRIMETHYLSILYLETHERS.—The extracts were dissolved in CH_2Cl_2, and part of the solutions were converted to trimethylsilylethers. Hexamethyldisilazane was added to the extracts and to standard solutions of steroids in a proportion of 5:1 mmol. The mixtures were stirred for 24 h under a dry atmosphere and then hydrolyzed with distilled H_2O (0°). The solutions were extracted with CH_2Cl_2, and the organic phases were dried ($MgSO_4$). The solutions were concentrated on a rotary evaporator for further analysis.

THIN LAYER CHROMATOGRAPHY.—Each extract, along with the standard solutions of squalene,[4] cholesterol,[4] stigmasterol,[4] diosgenin,[5] lanosterol,[6] and β-sitosterol,[6] was applied to activated silica gel 60/kieselguhr, coated, glass plates (0.25 mm) (E. Merck Reagents). The plates were developed in hexane-EtOAc (3:1) and sprayed with a mixture of H_2SO_4 and H_2O (1:1). After heating for 10 min at 110°, colored spots appeared.

GAS CHROMATOGRAPHY-MASS SPECTROMETRIC ANALYSIS.—Samples of derivatized extracts and derivatized standards (diosgenin, lanosterol, cholesterol, β-sitosterol, and stigmasterol) were analyzed separately on a Hewlett Packard 5995A GC/MS/Computer system, equipped with a capillary injector, electron impact ionization chamber (70 eV), quadrupole analyzer, electron multiplier detector, and a 15m, 0.20mm intern. diam., SE-54, quartz, capillary column. Squalene was analyzed using a 2 ft. ¼ in., 3% OV-101, Chromosorb W, glass column. The injector and transfer line temperatures were set at 280°, and the column temperature was programmed from 120° to 320° at a rate of 2°/min. The mass spectrum of each peak was recorded for comparison with the mass spectra of derivatized standards.

COINJECTION OF DERIVATIZED STIGMASTEROL WITH DERIVATIZED CALLUS EXTRACT.—A sample of the derivatized callus extract was analyzed using the same gas chromatographic conditions described previously, and the hydrogen flame ionization detector signal was recorded. Another sample of the derivatized callus extract was coinjected with stigmasterol-trimethylsilylether under the same conditions. The two chromatograms generated were compared.

RESULTS AND DISCUSSION

Friable callus tissues of *S. aviculare* grew actively during 4 weeks of incubation; thereafter, callus tissues gradually darkened and eventually died. The cell suspension cultures composed of free cells and cell aggregates grew rapidly, attaining maximum growth at the ninth day (Figure 1). These results were consistent with those typically obtained in cell and tissue culture studies (6-8,11).

The present study revealed that both callus and cell suspension cultures of *S. aviculare* produced several kinds of steroids as shown by tlc and gc/ms analysis. Squalene, lanosterol, and stigmasterol were detected by tlc and cholesterol; campesterol,[7] β-sitosterol, cycloartenol,[7] lanosterol, and squalene were identified by gc/ms in both callus and cell extracts. Stigmasterol was confirmed to be overlapped with cycloartenol by coinjection with a stigmasterol-trimethylsilylether standard. Other unidentified compounds were present in the regions where the steroids were detected, when callus and cell extracts were analyzed by tlc and gc/ms. The two analytical techniques, tlc and gc/ms, were found to complement each other in the characterization of plant steroids.

Diosgenin was not detected by tlc nor gc/ms in either callus or cell suspension cultures. Had diosgenin been produced by callus and cell cultures in appreciable amounts, it would have been detected because its presence was confirmed by tlc and gc/ms in the callus extract where diosgenin had been previously added.

Diosgenin and solasodine were reported in callus and cell suspension cultures of *S. aviculare* by Khanna *et al.* (7,8), but the presence of other steroids was not examined. A variety of steroids

[4]Aldrich Chemical Co., Milwaukee, WI.

[5]Sigma Chemical Co., St. Louis, MO.

[6]ICN Pharmaceutical Inc., Plainview, NY.

[7]Compared with spectra obtained from E. Stenhagen, S. Abrahamsson, and F.W. McLafferty, "Registry of Mass Spectral Data," New York: Wiley, 1974.

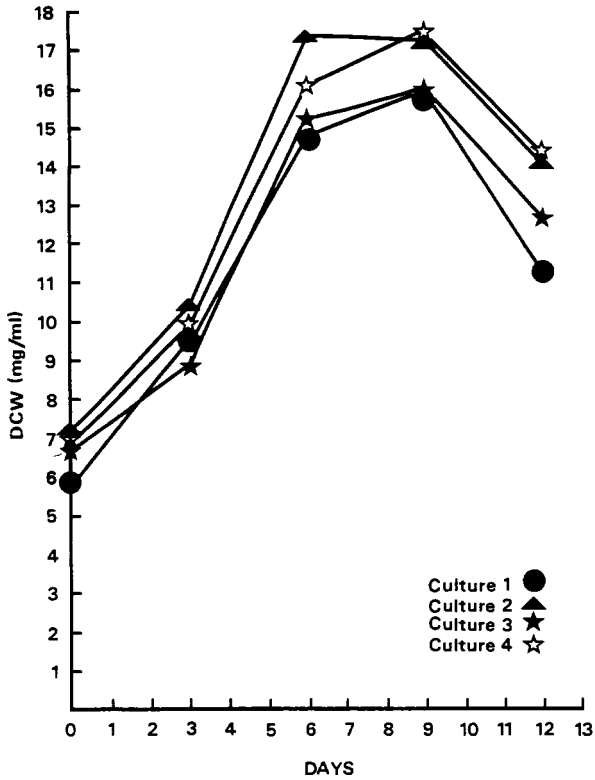

FIGURE 1. Growth of *Solanum aviculare* cell suspension cultures expressed as dry cell weight (DCW) in mg/ml.

has been reported in callus cultures of several *Solanum* species. In callus cultures of *S. elaeagnifolium* and *S. xanthocarpum*, diosgenin, lanosterol, stigmasterol, and β-sitosterol were detected (5). In work done with *S. laciniatum* callus cultures, Hosada and Yatazawa (6) detected the presence of squalene, cholesterol, stigmasterol, β-sitosterol, lanosterol, diosgenin, and solasodine.

The most notable result obtained in this study was that callus and cell suspension cultures of *S. aviculare* produced the same steroids. The absence of diosgenin in callus and cell suspension cultures in the present study could have been due to a series of factors and should be investigated in future work. One of these factors could have been the explant source. The explant source was selected arbitrarily, and there exists the possibility that the explants chosen for the present work were not capable of synthesizing diosgenin in appreciable quantities. Another possible explanation for not detecting diosgenin could be the fact that, in this study, the cultures were not grown under light of sufficient intensity. Thus, if light promotes diosgenin production, its low intensity or absence might have caused the failure of the plant to produce diosgenin. There exists evidence to support this possibility (13).

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LITERATURE CITED

1. Ed. by J.G. Hawkes, N.R. Lester, and A.D. Skelding, "The Biology and

Taxonomy of Solanaceae," London: Academic Press, Inc., 1979, pp. 203-207.
2. E.I. Korneva, L.A. Pikova, and L.F. Matveenko, *Pharm. Chem. J.*, **13**, 516 (1979).
3. Ed. by J. Reinert and Y.P.S. Bajaj, "Plant, Cell, Tissue and Organ Culture," New York: Springer-Verlag, 1977, pp. 649-702.
4. "Plant Tissue Culture as a Source of Biochemicals," Ed. by E.J. Staba, Boca Raton, FL: CRC Press, Inc., 1980, pp. 115-122.
5. P. Khanna, S.K. Mannot, and A.K. Rathore, *Indian J. Exp. Biol.*, **16**, 616 (1978).
6. N. Hosada and M. Yatazawa, *Agric. Biol. Chem.*, **43**, 821 (1979).
7. P. Khanna, A. Uddin, G.L. Sharma, S.K. Manot, and A.K. Rathore, *Indian J. Exp. Biology*, **14**, 694 (1976).
8. P. Khanna, G.L. Sharma, A.K. Rathore, and S.K. Manot, *Indian J. Exp. Biol.*, **15**, 1025 (1977).
9. H. Sweet and W. Bolton, *J. Bot.*, **66**, 692 (1979).
10. T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
11. M. Misawa, M. Hayashi, and H. Tanaka, *Biotechnol. Bioeng.*, **17**, 1335 (1975).
12. "Plant Tissue and Cell Culture," 2nd ed. Ed. by H.E. Street, Berkeley: University of California Press, 1977, pp. 307-385.
13. A. Light, Environmental factors. In, "Plant Tissue Culture as a Source of Biochemicals," Ed. by E.J. Staba, Boca Raton, FL: CRC Press, Inc., 1980, pp. 123-141.

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