Secondary Metabolism in Tissue Culture of Artemisia annua

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SECONDARY METABOLISM IN TISSUE CULTURE OF *ARTEMISIA ANNUA*

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ABSTRACT.—Undifferentiated callus of *Artemisia annua* produced the coumarin, scopoletin and the triglyceride, glycerol 1,2-di-9-octadecenoate 3-octadecanoate, but failed to accumulate any of the terpenoids found in the parent plant. The biosynthetic capabilities of shoots which had differentiated from callus, however, were comparable to the whole plant.

Artemisia annua L. (Compositae) produces the antimalarial agent artemisinin, which has proven effective in clinical trials (1), especially in the treatment of cerebral malaria (2). In parts of Southeast Asia artemisinin is now the only effective treatment for malaria. Artemisinin represents a considerable synthetic challenge (3–7), and several researchers have chosen to investigate an alternative, potentially more cost-effective strategy of producing artemisinin in vitro (2, 8–11) from *A. annua* tissue cultures, with little success to date. This study attempts to define the full pattern of secondary metabolism in *A. annua* cultures and is intended to establish a "baseline" for future research aimed at producing artemisinin in vitro.

Callus cultures of *A. annua* were obtained from explants of seedlings on media comprised of Murashige and Skoog mineral salts and vitamins (12) supplemented with various hormone mixtures. Callus grown on medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; medium 1) showed no tendency to differentiate and accumulated the triglyceride 1,2-di-9-octadecenoate 3-octadecanoate as the principal metabolite (stigmasterol, sitosterol, and chlorophyll were also found). Callus grown on medium containing both naphthaleneacetic acid and benzyladenine purine (NAA, 0.5 mg/liter and BAP, 0.5 mg/liter; medium 2) differentiated into shoots towards the end of the growth cycle: undifferentiated callus produced scopoletin (13) in addition to the above triglyceride, while differentiated shoots qualitatively resembled extracts of the aerial parts of the whole plant in their ability to accumulate mono- and sesquiterpenes, phytosteroids, and pigments (quantitative similarities were also noted for the less volatile sesquiterpene lactones, see Table 1). While increasing the concentration of BAP in medium 2 (to 2.5 mg/liter) suppressed this tendency to differentiate, and removal of BAP (NAA alone at 2.5 mg/liter) promoted differentiation, neither treatment qualitatively affected this pattern of secondary metabolism in culture.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on a Bruker 400 MHz machine and were used to identify and quantify scopoletin and glycerol 1,2-di-9-octadecenoate 3-octadecanoate. Volatile terpenoids were quantified by gc on a Perkin-Elmer gas chromatograph (FSOT BP1 capillary column 12 m×0.15 mm i.d.) equipped with an integrator and operating a temperature program (60–300°; 8°/min); R_t (min) artemisia ketone 6.01; camphor 7.51; *trans*-β-selinene 14.27; *epi*-deoxyarteannuin B 19.75; arteannuin B 22.00; artemisinin 22.04 and 23.73 [two peaks observed due to thermal decomposition on the column (14)]. Phytosterols and pigments were assayed qualitatively by tlc (Merck PK6F plates) in three different solvent systems (15% EtOAc/hexane, 30% EtOAc/hexane, and 50% EtOAc/hexane); R_f β-carotene 0.60, 0.72, 0.84; stigmasterol and sitosterol 0.22, 0.36, 0.76; chlorophyll a 0.08, 0.29, 0.64; xanthophyll 0, 0.08, 0.27.

INITIATION OF CULTURES.—Tissue cultures

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TABLE 1. Secondary Metabolites in Tissue Culture and Whole Plants of *Artemisia annua* (mg Metabolite/100 g Plant).

Component	Medium 1	Medium 2 Undifferentiated	Medium 2 Differentiated	Whole Plant
Monoterpenes				
Camphor[a]	–	–	0.3	8.0
Artemisia ketone[a]	–	–	0.2	9.1
Sesquiterpenes				
Artemisinin[a]	–	–	3.8	4.5
Arteannuin B[a]	–	–	4.6	9.0
epi-Deoxyarteannuin B[a]	–	–	0.6	5.5
trans-β-Selinene[a]	–	–	0.6	18.5
Higher terpenoids				
trans-β-Carotene[b]	–	–	+	+
Xanthophyll[b]	–	–	+	+
Stigmasterol and sitosterol[b]	+	+	+	+
Miscellaneous				
Triglyceride[c]	199.8	80.2	4.0	7.6
Scopoletin[c]	–	17.0	2.0	3.6
Chlorophyll a[b]	+	+	+	+

–No entry indicates $<10^{-6}$%, if any, present.

[a]Identification and quantification by gc against known amounts of reference compound (camphor obtained commercially from Aldrich; other compounds obtained by prep. hplc of *A. annua* plants and identified by comparison of nmr data against published spectra).

[b]Qualitative tlc assay in 3 different solvent systems against reference compounds (xanthophyll obtained by prep. hplc of *A. annua* plants and identified by comparison of nmr data with published spectra; other compounds obtained commercially from Aldrich).

[c]Identification and quantification by ^{1}H-nmr spectroscopy against known amounts of reference compound (scopoletin obtained commercially from Aldrich; glycerol 1,2-di-9-octadecenoate 3-octadecanoate obtained by prep. hplc of *A. annua* plants and identified from its ^{1}H- and ^{13}C-nmr spectra and by hydrolysis with caustic EtOH to oleic acid, stearic acid (2:1) and glycerol, and by enzymatic digestion (with porcine pancreatic lipase) to stearic acid, oleic acid (1:1) and glycerol-2-oleate).

of *A. annua* were initiated from 20-day-old plantlets (2–3 cm high), grown under fluorescent light under greenhouse conditions (seeds were obtained from and taxonomically verified by Dr. J. Twibel of the NCCPG collection at Cambridge). Explants of the hypocotyl region of the plantlet were surface sterilized in 2% $Ca(OCl)_2$ solution, rinsed three times in sterile H_2O, then transferred to sterile agar media.

MEDIA.—The media for growth comprised Murashige and Skoog mineral and vitamin mixture (12) (Imperial Laboratories; 4.71 g/liter), sucrose (20 g/liter), agar (Aldrich 0.24 g/20 ml), and supplementary plant hormones. Four different kinds of media were prepared: 1. 2,4-D (1 mg/liter); 2. NAA (0.5 mg/liter)+BAP (0.5 mg/liter); 3. BAP (2.5 mg/liter)+NAA (0.5 mg/liter); 4. NAA (2.5 mg/liter).

MAINTENANCE AND EXTRACTION OF CULTURES.—Callus typically appeared within 2–3 weeks when explants were maintained at 24° with a 16 h/8 h light/dark period. Callus could then be subcultured at 6-week intervals, and five subcultures were made before each callus line was extracted. In the extraction procedure, if differentiation was evident, differentiated and undifferentiated materials were first separated by hand, then pulverized to a fine powder under liquid N_2 and extracted in a Soxhlet apparatus (Et_2O). Ethereal extracts were dried and solvent removed under reduced pressure.

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