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PRODUCTION OF ARTEMISININ IN TISSUE CULTURES OF *ARTEMISIA ANNUA*[1]

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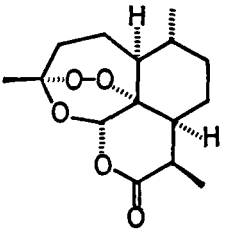
Artemisinin (**1**) ("qinghaosu," QHS) is a potent antimalarial produced by the Chinese medicinal herb "qinghao" (*Artemisia annua* L., Compositae). This compound, which is present mainly in the leaves, varies in concentration from 0.01 to 0.5% (based on dry weight) (1) in Chinese varieties of the plant. In plants collected in the United States, where it is not native but was introduced from Asia, the maximum concentration is about 0.06% (2). Potency of artemisinin and some of its derivatives, notably sodium artesunate (3) (Na salt of the hemisuccinyl ester of 2H-artemisinin) in treatment of malaria, is equal to or better than that of chloroquine substitutes currently used. The herb has been in use in Chinese indigenous medicine for centuries. Also, no side effects, common to many synthetic antimalarials, have been observed during the past 6 years of clinical trials of its active principle, artemisinin. Thus, artemisinin appears to have an excellent safety record. At present only in the People's Republic of China, where this plant grows abundantly, is artemisinin available in large enough amounts for extensive clinical trials and treatment. In spite of two syntheses of this compound (4,5) reported so far, its complex structure makes economical synthesis in the near future unlikely.

The present study was undertaken to determine the feasibility of employing tissue culture techniques to obtain artemisinin at costs affordable by the people of countries where malaria is endemic.



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We have been able to induce callus, root, shoot, or whole plant regeneration using explants from stems, leaves, and flower buds. The method would thus assure a constant supply of the plant material for investigative purposes. Preliminary results with various culture systems indicated the presence of artemisinin in roots and rooted plantlets derived from leaf explants or callus cultures therefrom. Callus and unrooted shoots did not produce the compound in amounts detectable in our highly sensitive assay system.

Initial results indicated also that artemisinin is present in the culture fluid from liquid suspension cultures of callus cells. This latter observation indicates the potential of this methodology for large-scale production of this drug.

[1]This work was presented at the International Congress on Natural Products held at the University of North Carolina, Chapel Hill, North Carolina, July 7-12, 1985.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURE.—Wet plant materials, roots, callus, plantlets, and shoots, were extracted with MeCN (50×v/w) using a tissue grinder. The solution was concentrated to approximately 1/25th its volume and filtered. Culture liquids (2 ml) were extracted with hexane, hexane was removed, and the residue was taken up in 2 ml of MeCN. Samples prepared this way were analyzed for the presence of artemisinin by hplc (Varian 5000, C_{18} column) using an electrochemical (EC) detector (LC4B, Bioanalytical Systems) (6). Before each experiment was conducted, the EC detector used with the hplc was calibrated with authentic artemisinin and an independent reference compound benzoylperoxide. The probability that another EC detectable compound is present in the extracts, which has the same relative retention time (vs. benzoylperoxide) as artemisinin, (QHS) is extremely unlikely.

PLANT MATERIAL.—*A. annua* plants, collected from Brooklyn, New York, and the Washington, DC, area, were potted and maintained in the tropical greenhouse (23°-28°) at the New York Botanical Garden. Specimens are on deposit at the Brooklyn Botanical Garden, Brooklyn, New York.

STERILIZATION AND CULTURE CONDITIONS.—The explants, i.e., leaves, flower buds, and stems, were severed and washed under tap H_2O for 15 min and then were sterilized for 15 min with NaOCl solution containing a detergent [10 ml Chlorox and a few drops of a 1% solution of Triton-X 100, diluted to 100 ml with double-distilled (dd) H_2O] and finally washed three times with sterile dd H_2O. Procedures from this point onwards were conducted under sterile conditions. Explants were trimmed to 4-8 mm segments and cultured in Murashige-Skoog (MS) medium (7) or B5 medium (8). All experiments were repeated at least three times. Cultures were maintained at 27° and at light regimens ranging from 1,500 to 2,500 lux at a 16-8 h light-dark cycle.

MEDIA.—Media used in the experiments were either Murashige-Skoog or B5 inorganic salts modified by supplementing with the following nutrients (mg/liter): thiamine HCl (1.0), inositol (100), nicotinic acid (0.5), pyridoxine HCl (0.5), and sucrose (20,000). These media will be referred to as modified MS and B5 later on in the text. The auxins, 1-naphthalene acetic acid (NAA) and indolebutyric acid (IBA) and the cytokinins, benzylaminopurine (BAP) and kinetin (Kn), were used at different concentrations as described for specific experiments. The pH of the media was adjusted to 5.7 and 5.5, respectively, using dilute KOH solution or dilute HCl as needed, prior to the addition of 0.8% of Bacto-agar (DIFCO). The media (10-12 ml) were dispensed into vials and were autoclaved at 15 psi (123°) for 15 min.

ORGANOGENESIS.—Culture of young leaf segments on either modified MS or B5 medium with IBA or NAA (0.05-2.0 mg/liter) resulted in the development of roots in 6-14 days, mostly from proximal end of the midrib. The roots when extracted showed the presence of artemisinin (QHS), whereas the agar medium did not show any detectable amount of the compound.

There was callus development in 21-35 days on both sides of the leaf when the explant was cultured on modified MS or B5 medium containing NAA (0.05 mg/liter) and BAP (0.1-0.2 mg/liter). The callus subsequently developed chlorophyll, and in about 60 days distinct shoots were evident. These shoots rooted if left on the above medium for a period longer than ca. 3 months or if subcultured in modified MS medium without any growth hormones. Regenerated plantlets were potted in the greenhouse, and their survival rate was 98%.

CALLUS AND CELL SUSPENSION CULTURES.—When leaf explants were cultured on the modified MS or B5 media under the 16-8 h light-dark cycle with low concentrations of NAA (0.02-0.05 mg/liter) and BAP (0.1-0.5 mg/liter), callus was formed in 14-21 days. But they showed tendency to form shoot initials. If the cultures were grown in the dark, callus developed slowly (28-45 days) and later turned brown. Browning was more pronounced when Kn was substituted for BAP. The callus was hard and nonfriable whether grown under light or in the dark.

Callus clumps removed from 60-day-old cultures started from leaf explants were used to initiate suspension cultures. The medium (2 ml/tube) used in these experiments was either modified MS or B5 containing 1.0 mg/liter of NAA. The cultures were incubated in 25×100 mm vials in a rotary apparatus at ca. 4 rpm and were harvested after 2 and 3 weeks after inoculation. Subculturing was not attempted because the large culture clumps, which were nonfriable, made it impractical.

RESULTS

Results are summarized in Figures 1 and 2. Even though these preliminary studies showed that the suspension cultures produce artemisinin in the medium, but not in the callus cells, i.e., the compound leaches into the medium, the amounts obtained were low (ca. 8 μg/ml). The dry weight of the callus was not determined since it produced no ar-

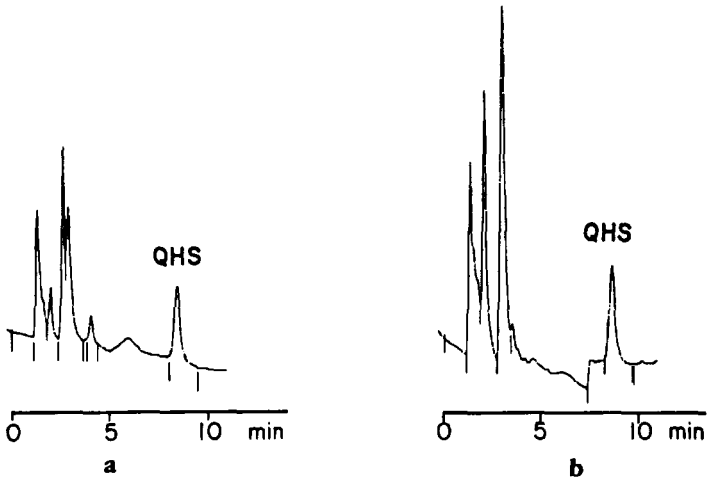

FIGURE 1. Artemisinin production in (a) roots and (b) plantlets regenerated from leaf explants. Rp-hplc; column, C_{18}, 30 cm×3 mm id; sample volume 1 μl; eluent, NH_4OAc buffer (pH 6.9); flow rate, 1 ml/min at 1,800 psi; EC detector.

temisinin. While these microscale experiments do not predict the cost-effectiveness of this method, they clearly indicate that it is feasible to produce artemisinin in batch or continuous suspension cultures. We are currently manipulating the media and other culture conditions to optimize the production of artemisinin. We are also trying to obtain more friable callus and to achieve better separation to promote their rate of growth in liquid medium.

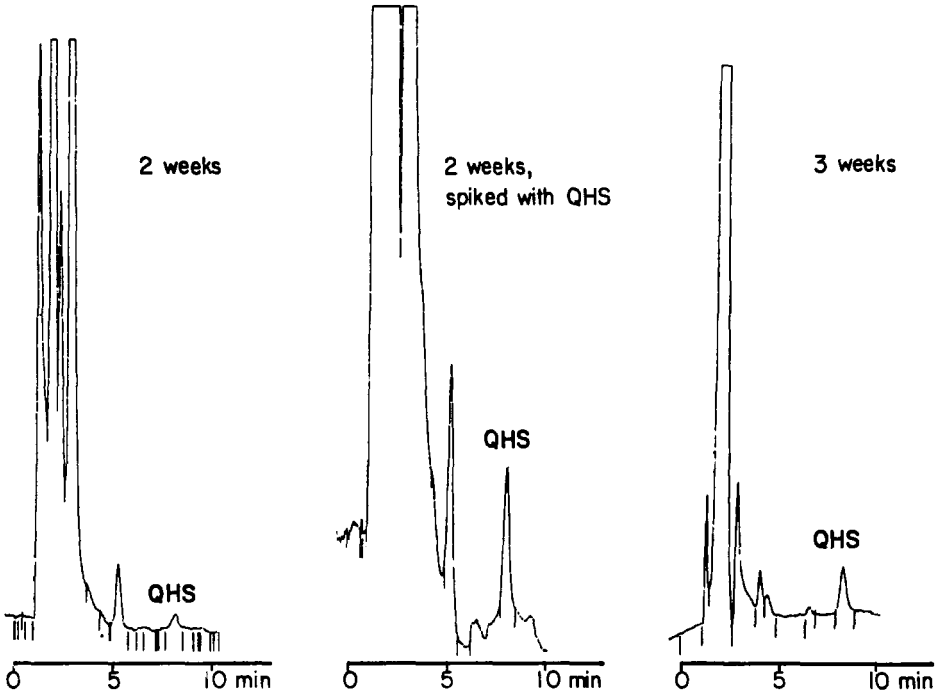

FIGURE 2. Detection of artemisinin in the culture liquids of *Artemisia annua* cell suspension cultures by hplc. Column, C_{18}, 30 cm×3 mm id; sample volume 1 μl; eluent, NH_4OAc buffer (pH 6.9); flow rate, 1 ml/min at 1,800 psi; EC detector.

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