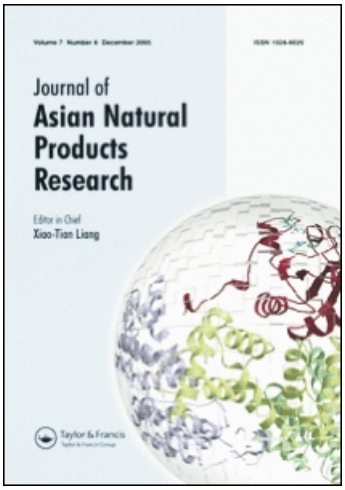

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CALLUS CULTURES OF *ANNONA SQUAMOSA* FOR THE PRODUCTION OF ANNONACEOUS ACETOGENINS

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Callus cultures of *Annona squamosa* were induced using different explants including petals, seed contents (megagametophyte and embryo) and fruits (mesocarp). Growth of the calli induced from the explants was found to be influenced by the type, concentration and ratio of auxin vs. cytokinin. The content of squamocin (67.8 $\mu g\, g^{-1}$ dry weight) in calli cultured on Gamborg B-5 medium containing 5.0 $mg\, l^{-1}$ naphthalene acetic acid and 4.0 $mg\, l^{-1}$ zeatin was nearly seven times higher than that in intact fruits.

Keywords: *Annona squamosa*; Callus culture; Acetogenins; Squamocin

INTRODUCTION

Annona squamosa (Annonaceae), also known as the custard apple, is a kind of world fruit tree that has been naturalized throughout the tropics [1]. In China, it is used in folk medicine as an insecticide and a parasiticide. It was reported that its roots could be used to treat acute dysentery, depression and spinal marrow diseases, and its leaves used for prolapse of the anus, sores and swelling [2]. Since the discovery of uvaricin, the first annonaceous acetogenin [3], up to now approximately 320 this type of natural polyketides have been isolated from the Annonaceae [4]. The interest shown for this class of compounds is justified by their highly potent and diverse bioactivities, including promising cytotoxicities and *in vivo* antitumor effects (activities of some compounds *in vitro* tests are 40–300 times higher than taxol) [4–6]. These results prompted us to look for a source of annonaceous acetogenins. Here, we report on the first establishment of callus cultures of this species and the study of their secondary metabolites.

We have induced callus cultures using explants of *Annona squamosa* in completely defined culture media and analyzed the ability of the cultures to produce acetogenins. Squamocin (**1**) (Figs. 1 and 2), a major acetogenin in *Annona squamosa* [7] (The standard

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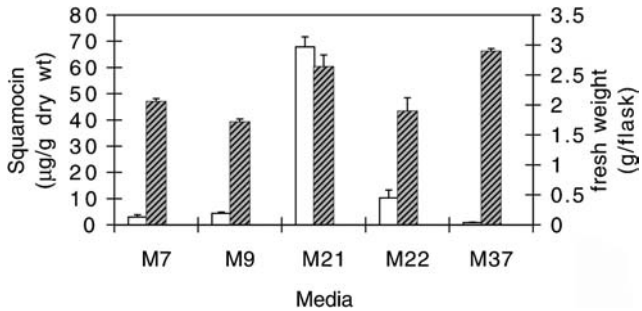

FIGURE 1 Growth (fresh weight, diagonally striped area) and squamocin yield (blank area) of calli. Media are containing different auxin and cytokinin as described in Table I. Data are mean ± SE from the independent replicates.

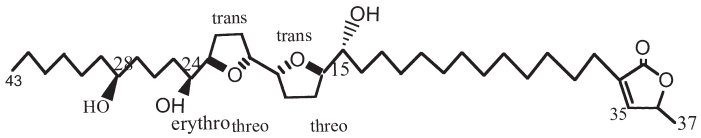

FIGURE 2 Structure of squamocin.

sample was isolated from the seeds by the authors and its structure was determined by IR, ^{1}HNMR, ^{13}CNMR) was detected in calli induced from various explants.

RESULTS AND DISCUSSION

We have attempted to induce the callus by the use of petals, seeds and fruits cultured on GB5 medium supplemented with auxin (NAA: Naphthalene acetic acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid) and cytokinin (KT: kinetin, 6-BA: 6-Benzyl amino acid). The best source of explant tested for callus induction was seed contents of 50 mm-diameter fruits. It failed to induce calli from petals and mesocarps of 20 mm-diameter fruits. Calli started to develop on the cut area not immersed in the culture media after approximately 25 days in culture. The calli were friable and light yellow in color. Subculture was necessary every 15–20 days of culture, otherwise the calli became dark after approximately 25 days and eventually died. The culture medium containing auxin and cytokinin for induction was shown to be a favorable medium for growth.

The optimal conditions for callus growth and the production of **1** were also investigated. Different ratios and concentrations of auxin (NAA, 2,4-D) and cytokinin (ZT: Zeatin, KT, 6-BA) do affect significantly the growth and the content of squamocin in calli (Fig. 1). Increasing in fresh weight of calli after 16 days of culture in GB5 (Gamborg B-5 medium) was estimated to be from two to six times as the initial fresh weight of calli (data not shown). It was shown that the medium containing NAA is more advantageous for callus growth and accumulation of compound **1** than that containing 2,4-D. A higher concentration of NAA ($10\,mg\,l^{-1}$) increased the growth of callus, but $5.0\,mg\,l^{-1}$ of NAA were found to be more efficient for the production of **1**. The yield of **1** is low on medium containing $2.0\,mg\,l^{-1}$ of 2,4-D, though the growth rate is the highest. The combination of NAA with 6-BA is better than that of NAA with ZT or KT.

TABLE I Callus induction rate from various explants (%)

	P	*M20*	*M50*	*S20*	*S50*
F1	0	0	0	0	18.2
F2	0	0	0	0	14.3
F3	0	0	64.5	7.7	5.0
F4	0	0	81.0	40.0	87.1

P: Petals; M20: Mesocarp of 20 mm-diametre fruits; M50: Mesocarp of 50 mm-diametre fruits; S20: Seeds of 20 mm-diametre fruits; S50: Seeds of 50 mm-diametre fruits; F1: B_5 medium containing 2.0 mg l^- 2,4-D; F2: B_5 medium containing 2.0 mg l^- NAA; F3: B_5 medium containing 2.0 mg l^- NAA and 1.0 mg l^- KT; F4: B_5 medium containing 2.0 mg l^- 2,4-D and 1.0 mg l^- 6-BA.

Several approaches could be considered to increase the yield of secondary metabolites in plant tissue cultures, such as optimization of culture conditions, precursor feedings, selection of high yielding cell lines and adding elicitors treatment. The present study demonstrated the possibility of obtaining callus from *Annona squamosa* L. The data indicated that the yield of squamocin in cultures could be enhanced by manipulating the plant growth regulators and the highest yield reached 67.8 $\mu g\, g^{-1}$ dry weight, which is about seven times higher as that in the fruit of intact plant (10.1 $\mu g\, g^{-1}$ dry weight) (Fig. 1). The system described can be served as basis for future development of commercial-scale production of annonaceous acetogenins.

EXPERIMENTAL SECTION

In Vitro Culture

In vitro callus cultures were induced from petals, seeds and fruits of *Annona squamosa* L., collected in May and July 1997 in Kunming (the capital of Yunnan Province in P. R. China, the voucher specimen was deposited in Department of Natural Medicines, Peking University). Callus cultures were initiated on solid GB5 medium containing four combinations of auxin (2,4-D, NAA) and cytokinin (6-BA, KT) [8]. Supplementation of medium with 0.5% (w/v) insoluble Polyvinylpyrrolidone (PVP) to minimize oxidation of phenolics which were produced and released by the explants. Cultures were grown at 25 ± 1°C in the dark. Small pieces of callus were transferred onto GB5 solid medium containing different concentrations of auxin and cytokinin as described in Table I. At the end of exponential growth phase, the callus was routinely subcultured with 2 weeks' intervals.

Extraction and HPLC Analysis

Two grams of dry weight (corresponding to 22.2 g in fresh weight) of ground lyophilized callus biomass with 2 weeks of age were extracted with chloroform using Soxhlet apparatus at 60°C water bath for 3 h. The chloroform extract was injected into HPLC column. The HPLC analysis was performed with a TSP System equipped with a UV detector. Elution was carried out with MeCN/H_2O (80:20) in a reversed-phase column Prodigy ODS (250 × 4.6 mm, 5 μm partical size) at a flow rate of 1 ml min^{-1}, and the detection wavelength was set at 220 nm.

Quantitation of Squamocin (1)

The content of **1** in the calli obtained from various culture conditions was determined by HPLC. The retention time for **1** was 16.2 min in above mentioned conditions. The results were from at least three replicate determinations. For the determination of **1**, 20 μl was

injected into HPLC column. The content of **1** in each sample was calculated by reference to the standard sample, based on the peak-area ratios.

Acknowledgements

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