BRYONOLIC ACID PRODUCTION IN HAIRY ROOTS OF *TRICHOSANTHES KIRILOWII* MAX. VAR. *JAPONICA* KITAM. TRANSFORMED WITH *AGROBACTERIUM RHIZOGENES* AND ITS CYTOTOXIC ACTIVITY

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The hairy roots of *Trichosanthes kirilowii* MAX. var. *japonica* KITAM. were induced by *Agrobacterium rhizogenes* (ATCC 15834) on sterile shoots. The axenic culture of hairy roots proliferated 30 to 60-fold based on the initial fresh weight after three weeks of culture in Murashige & Skoog liquid media. Bryonolic acid as the main triterpenoid was isolated in a high yield, together with chondrillasterol from the hairy roots of this plant. Bryonolic acid showed strong inhibition of the growth of B-16 melanoma cells.

KEYWORDS *Trichosanthes kirilowii* var. *japonica* ; hairy root; bryonolic acid; *Agrobacterium rhizogenes*; B-16 melanoma cell

Trichosanthin, a protein from the root tubers of a Chinese medicinal herb *Trichosanthes kirilowii* MAX. was found to be effective in inducing abortion in several mammalian models[1-3] and in humans.[4] It has been used for abortion and for therapy of choriocarcinoma and hydatidform moles in China. Recently we have reported the isolation and characterization of new abortifacient proteins, Karasurin A (K-A) and B, from root tubers of other species *Trichosanthes kirilowii* MAX. var. *japonica* KITAM. (the Japanese name is Kikarasuuri).[5] Production of proteins by tissue cultures, such as the root culture and the hairy root of *T. kirilowii* MAX. var. *japonica* KITAM., has not been reported yet. *Agrobacterium rhizogenese* causes the formation of transformed hairy roots by introducing T-DNA of the Ri plasmid into the genomic DNA of the host plant cells. The hairy roots have the advantages of rapid growth and an occasional higher production of secondary metabolites.

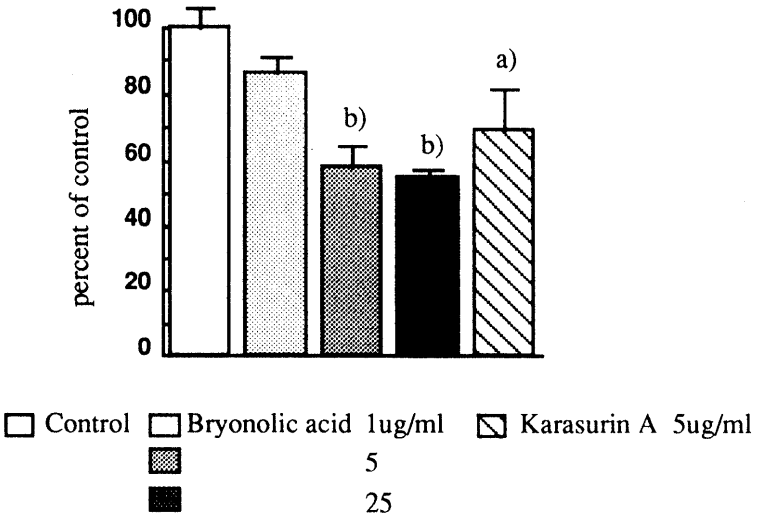
Trichosanthes kirilowii MAX. var. *japonica* KITAM. was efficiently transformed by direct inoculation of *A. rhizogenes* (ATCC 15834)[6] on sterile shoots. After several subcultures on hormone-free Murashige and Skoog (MS) solid medium[7] containing 0.5 g/l Claforan (Hoechst Japan Ltd.) to remove the bacteria, the axenic hairy roots were maintained on the MS agar medium without antibiotics. Opines (mannopine and agropine) of the hairy roots were detected using high voltage paper electrophoresis.[8] The hairy roots were cultured in large scale in MS liquid medium at 100 rpm on a rotatory shaker at 25 ° C, in the dark for three weeks before harvest.

This paper describes production and isolation of relatively low molecular weight compounds. Production of proteins by the hairy root cultures will be discussed elsewhere. The chloroform extract (372 mg) of the dried hairy roots (11.9 g) was purified by silica gel column chromatography to give two compounds. Their structures were elucidated to be chondrillasterol (**1**)[9] and bryonolic

acid (**2**)[10,11] by the ^{1}H-NMR, ^{13}C-NMR and other spectral data. The isolation yield (dry weight) of **1** was up to 0.11%, and the yield of **2** was 1.35%. The yield of **2** is higher than the content in mother plants (less than 0.02%, analyzed from the tuber roots of the mother plants) and also callus culture (0.02%).[11c] Bryonolic acid was first isolated from the roots of *Bryonia dioica* JACQ.,[10a] and later from those of *T. kirilowii* MAX. var. *japonica* KITAM.[11a] and also its cell culture.[11c] Tabata *et al.* reported the isolation of bryonolic acid from the cultured cells of *Luffa cylindrica* (Cucurbitaceae) and the anti-allergic activity of this compound against both homologous passive cutaneous anaphylaxis and delayed hypersensitivity in rodents.[12] They found that cultured cells are capable of producing a large amount of bryonolic acid (ca. 3% of dry wt.), which may be considered a promising natural product for the treatment of a delayed-type allergy for their unique properties.

We assayed the effect of bryonolic acid on the growth of B-16 melanoma cells (kindly supplied by Japanese Cancer Research Resources Bank) *in vitro* according to the MTT method.[13] As shown in Fig. 1, bryonolic acid significantly inhibited the cell growth above 5μg/ml.[14]

In summary, hairy root cultures of T. *kirilowii* MAX. var. *japonica* KITAM. produced bryonolic acid in more than 50-fold higher yield than callus cultures or tuber roots of the plant. Therefore, the hairy roots might potentially be a suitable source for large-scale production of bryonolic acid, which exhibit cytotoxic as well as anti-allergic activities, because of their high growth rate and productivity.



Each bar and vertical line represent the mean and S.E. of 6-8 experiments in each experimental group.
a) $p < 0.05$, b) $p < 0.01$ vs. control.

Fig. 1. Effects of Bryonolic Acid on Growth of B-16 Cell

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1: ^{13}C-NMR data were consistent with the reference data reported by T. Akihisa *et al.*,
1-OAc: Acetylation of compound **1** with acetic anhydride and pyridine gave **1-OAc**, ^{13}C-NMR ($CDCl_3$): δ 28.40(C-16), 20.94(C-21), 12.46(C-29) (ref. 24β-epimer: 28.39(C-16), 20.93(C-21), 12.45(C-29), 24α-epimer=spinasterol, 28.49(C-16), 21.10(C-21), 12.25(C-29)). The 24-ethyl-7,21-dien-sterol isolated from the hairy roots of *T. kirilowii* was proved by ^{13}C-NMR to be a single 24β-epimer.

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2: ^{13}C-NMR (d_5-Py) δ: 16.5, 18.0, 19.6, 20.1, 21.1, 22.4, 25.5, 28.0, 28.6, 28.7, 30.5, 30.7, 31.1, 31.3, 31.5, 33.3, 35.1, 35.5, 37.5, 37.7, 37.8, 39.4, 40.6, 42.1, 45,2, 51.0, 78.0, 134.1, 134.6, 181.2

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14) About 2 x 10^3 cells in 0.1 ml of culture medium (Earle's MEM supplemented with 10% fetal calf serum) were inoculated into 96-well culture plates. After 24 h of incubation in a humidified 5% CO_2 atmosphere at 37° C, bryonolic acid in culture medium was added to each cell at various concentrations (25μg, 5 μg,1μg/ ml). Cells were incubated for 48 h. The inhibition of cell growth with bryonolic acid was assayed by measurement of dehydrogenase activity using MTT(Sigma).

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