Indole Alkaloid Production in Callus Cultures of *Uncaria rhynchophylla* (Miq.) Miquel

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The effects of growth hormones and nutrients on growth and alkaloid production in callus cultures of *Uncaria rhynchophylla* (Miq.) Miquel (Rubiaceae) were investigated. Gamborg B5 (B5) medium supplemented with indoleacetic acid (10^{-4} M) and 6-benzylaminopurine (3×10^{-5} M) was optimized both for the growth (1.8 g fresh weight/5 weeks) and alkaloid production (1.73 mg/g dry weight/5 weeks). The influence of sucrose concentration was examined, and 2% sucrose was found to be the most appropriate for the growth and alkaloid production. The effect of the composition of nitrogen sources was examined. Maximal growth and alkaloid production were obtained when 6 mM ammonium chloride and 25 mM potassium nitrate were added to the B5 medium. Hirsuteine, hirsutine, 3α-dihydrocadambine and ursolic acid were isolated from the callus. The 3α-dihydrocadambine concentration of callus was *ca.* 50-fold higher than that of the hooks and stems of *U. rhynchophylla*.

Key words *Uncaria rhynchophylla*; callus culture; alkaloid production; hirsuteine; hirsutine

In Chinese (Kampo) medicine, the crude drug "Choto-ko" has long been used as a spasmolytic, an analgetic, a sedative, and for the treatment of headache in hypertension, dizziness, cerebral arteriosclerosis and convulsion.[1] The principle of the drug is mainly in the hooks and stems of *Uncaria rhynchophylla* (Miq.) Miquel (Japanese origin) or *U. sinensis* (Oliv.) Havil (Chinese origin). Many alkaloids have been isolated from the hooks and stems of *U. rhynchophylla*, *e.g.* rhynchophylline, isorhynchopylline, hirsuteine, hirsutine, dihydrocorynantheine, corynantheine, corynoxeine, isocorynoxeine, akuammigine, geissochizine methyl ether.[2–4]

In spite of many phytochemical studies, there has been no report on the tissue culture of this plant and its alkaloid production. For the purpose of producing effective alkaloids from this plant, callus was induced, and the culture conditions which were required for producing the maximum amount of alkaloid were determined. Finally, three major alkaloids and a triterpene were isolated from the callus.

Experimental

General Methods The melting point was determined on a Yanagimoto micromelting point apparatus and was uncorrected. Optical rotation was on a Union Giken PM-101 polarimeter. The IR spectrum was recorded with a Shimadzu IR-408 spectrometer and the UV spectrum was recorded with a Nihon Bunko Kogyo UVIDEC-460 double-beam spectrometer. The ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-FX 100 spectrometer in a C_5D_5N and $CDCl_3$ solution using tetramethylsilane (TMS) as an internal standard.

Thin-layer chromatography (TLC) was performed on pre-coated Kieselgel 60 F_{254} (Merck) and detection was achieved by spraying 10% H_2SO_4 followed by heating and treatment with Dragen-dorff reagent. Column chromatography was carried out on Kieselgel 60 (Art. 7734, Merck), Kieselgel 60H (Art. 7736, Merck), Diaion HP-20 (Mitsubishi Chem. Ind. Co., Ltd., Tokyo, Japan) and Licroprep RP-18 (25—40 μm).

Plant Material and Cell Culture The leaves, cut from *U. rhynchopyhlla* plants cultivated at the Medicinal Plant Garden of Hiroshima University, were surface-sterilized with 70% ethanol for 10 s followed by treatment with 8% chlorinated lime and were then rinsed twice with sterilized water. They were cut into 5 mm squares and then placed on Murashige and Skoog (MS)[5] and Gamborg B5 (B5)[6] and White (W)[7] agar-gelled medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and Kinetin (Kin). The callus was maintained in the same medium by subculturing every 5—6 weeks. Cultures were maintained at 25±1 °C in light for 16 h and 8 h of darkness.

For experiments on the effects of growth hormones and nutritional factors, fresh callus (0.2 g) were inoculated on the changed medium (4 replicates) and harvested at 5-week intervals for 15 weeks. The growth hormones used were 2,4-D, indoleacetic acid (IAA), indolebutylic acid (IBA), NAA, BAP and Kin.

For kinetic studies under static conditions, *ca.* 1.2—1.3 g of fresh callus was inoculated and the tissue was harvested at 7—10 d intervals over a 55-d-long culture passage, and the substance was then chemically analyzed for its alkaloid composition.

Alkaloids Analysis Samples of lyophilized callus were extracted with CH_3CN–H_2O–AcOH (50 : 100 : 1) overnight. After filtration, the alkaloid extract was analyzed by TLC and high performance liquid chromatography (HPLC). The known alkaloids were identified by comparison with authentic samples.

TLC was carried out on silica gel plates using acetone–MeOH (20 : 1) and AcOEt–*n*-hexane (1 : 1) as solvents. The detection reagent used was a Dragen-dorff reagent.

HPLC was carried out on TSKgel ODS-120T (250 mm × 4.6 mm i.d., 5 μm) at a flow rate of 0.75 ml/min at 40 °C. The mobile phase was CH_3CN–H_2O–AcOH (50 : 100 : 1). The effluent was detected at 254 nm.

Quantitative Analysis of Alkaloids For quantitative analysis of alkaloids, the callus of 4 flasks were harvested and the fresh and the dry weights of each sample were determined. In all cases, 50 mg of lyophilized callus were extracted with 2 ml of CH_3CN–H_2O–AcOH (50 : 100 : 1) as described above, and were analyzed by HPLC. The system was calibrated with standard hirsuteine and hirsutine. The calibration plots were obtained from the peak areas of hirsuteine and hirsutine.

Extraction and Isolation of Constituents in Callus Lyophilized callus (80 g), which had been cultured in a B5 solid medium (10^{-4} M IAA–3×10^{-5} M BAP), was extracted with benzene and then that residue was extracted with MeOH. The benzene extractives (1.1 g) were chromatographed on a silica gel column using $CHCl_3$, $CHCl_3$–MeOH and $CHCl_3$–MeOH–H_2O. The fraction eluted with $CHCl_3$–MeOH (50 : 1) gave a colorless powder **1** (63 mg, 0.079%). The fraction eluted with $CHCl_3$–MeOH (25 : 1) was purified by preparative TLC, and was chromatographed on a silica gel column using acetone–MeOH. The fraction eluted with acetone–MeOH (20 : 1) gave an amorphous powder **4** (13 mg, 0.016%).

The MeOH extractives (31 g) were chromatographed on a Diaion HP-20 column using 15, 30, 50, 80% aqueous MeOH, MeOH, $CHCl_3$. The combined fraction eluted with 80% aqueous MeOH, MeOH and

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$CHCl_3$ was chromatographed on a silica gel column using $CHCl_3$–acetone–MeOH. The fraction eluted with $CHCl_3$–acetone–MeOH (15:2:0.1) was chromatographed on a silica gel column using AcOEt–EtOH–H_2O. The fraction eluted with AcOEt–EtOH–H_2O (11:2:0.8) gave an amorphous powder **3** (18 mg, 0.023%). The rest was chromatographed on a silica gel column using $CHCl_3$–MeOH–H_2O, and AcOEt–EtOH–H_2O, respectively. Further purification by Lichroprep RP-18 gave a pale yellow powder **2** (60 mg, 0.075%).

Compound **1** was obtained as a colorless powder, mp 258—260 °C [lit.[8] mp 278—280 °C], $[\alpha]_D^{20} = +68°$ (c=0.27, MeOH) [lit.[8] $[\alpha]_D = +76.8°$]. The ^{13}C-NMR spectrum (C_5D_5N) of **1** (Table 1) showed a carboxylic acid carbon signal [δ 179.7 (C-28)], a hydroxyl carbon signal [δ 78.0 (C-3)], two olefinic carbon signals [δ 125.6 (C-12) and 139.2 (C-13)], seven methyl carbon signals [δ 15.7, 16.5, 17.4, 17.5, 21.4, 23.9, 28.8] and 20 other carbon signals. This suggested **1** to be oleanolic acid or ursolic acid. Comparing it with the authentic sample,[9] compound **1** was deduced to be ursolic acid.

Compound **2** was obtained as a pale yellow powder, mp 179—184 °C (dec.), $[\alpha]_D^{21} = -103°$ (c=0.67, MeOH) [lit.[4] $[\alpha]_D = -91°$]. UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 226 (4.44), 274 (3.66), 282 (3.68), 290 (3.59). IR γ_{max}^{KBr} cm^{-1}: 3300 (NH, OH), 1680 (C=O), 1630 (C=C). The ^{1}H-NMR spectrum (CD_3OD) of **2** showed a methyl ester group [δ 3.79 (3H, s)], a hydroxyl methyne proton [δ 4.29 (1H, br t, J=6 Hz)], an anomeric proton [δ 5.56 (1H, d, J=9 Hz)], an aromatic proton [δ 6.9—7.4 (4H, m)] and an olefinic proton [δ 7.54 (1H, s)]. These data suggested the presence of an indole. On the other hand, the ^{13}C-NMR spectrum (C_5D_5N) of **2** showed signals due to a glucose moiety [δ 101.3 (glc-1), 74.5 (glc-2), 78.7 (glc-3), 71.5 (glc-4), 78.4 (glc-5), 62.7 (glc-6)]. Comparing with authentic sample,[4] compound **2** was deduced to be 3α-dihydrocadambine.

Compound **3** was obtained as an amorphous powder, $[\alpha]_D^{20} = +46°$ (c=1.2, $CDCl_3$) [lit.[2] $[\alpha]_D^{30} = +66.5°$]. UV $\lambda_{max}^{CHCl_3}$ nm ($\log\varepsilon$): 224 (4.17), 283 (3.76), 292 (3.68). IR $\gamma_{max}^{CHCl_3}$ cm^{-1}: 3450 (NH), 1700 (C=O), 1645 (C=C). The ^{1}H-NMR spectrum ($CDCl_3$) of **3** showed a primary methyl group [δ 0.78 (3H, m)], a methoxy group [δ 3.67 (3H, s)], a methyl ester group [δ 3.75 (3H, s), a C-3β proton [δ 4.63 (1H, m)] and a NH group [δ 8.75 (1H, br s)]. The ^{13}C-NMR spectrum ($CDCl_3$) is shown in Table 1. Analysis of the TLC and HPLC of **3** was in good agreement with that of hirsutine. In comparison with the authentic sample,[2] compound **3** was deduced to be hirsutine.

Compound **4** was obtained as an amorphous powder, $[\alpha]_D^{20} = +60°$ (c=0.83, $CDCl_3$) [lit.[2] $[\alpha]_D = +68.5°$]. UV $\lambda_{max}^{CHCl_3}$ nm ($\log\varepsilon$): 224 (4.15), 282 (3.73), 292 (3.65). IR $\gamma_{max}^{CHCl_3}$ cm^{-1}: 3450 (NH), 1690 (C=O), 1640 (C=C), 930 (–CH=CH_2). The ^{1}H-NMR spectrum ($CDCl_3$) showed a methoxy group [δ 3.64 (3H, s)], a methyl ester group [δ 3.71 (3H, s)], a vinyl group [δ 5.28 (1H, m) and 4.95 (2H, m)], a C-3β proton [δ 4.50 (1H, m)] and a NH group [δ 8.20 (1H, br s)]. The ^{13}C-NMR ($CDCl_3$) is shown in Table 1. Analysis of TLC and HPLC of **4** was in good agreement with that of hirsuteine. In comparison with the authentic sample,[2] compound **4** was deduced to be hirsuteine.

Results and Discussion

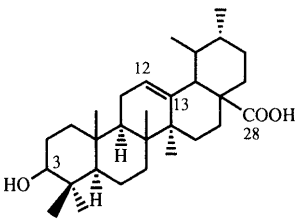
Callus Induction and Alkaloid Production Callus initiation from the leaves of *U. rhynchophylla* was assessed on MS solid medium supplemented with various concentrations and combinations of 2,4-D, Kin, NAA and BAP in light for 16 h or in the dark. No callus was induced in the light for 16 h. Callus was only induced in MS medium supplemented with 10^{-4} M NAA–10^{-6} M Kin (MN4K6), 10^{-4} M NAA–10^{-5} M Kin (MN4K5) and 10^{-4} M NAA–10^{-4} M Kin (MN4K4) in the dark, and was subcultured on the same medium in the dark at 5-6 week intervals for over one year.

TLC analysis of CH_3CN–H_2O–AcOH (50:100:1) extracts of these callus showed that some alkaloids were produced in MN4K4 callus. HPLC analysis of the CH_3CN–H_2O–AcOH (50:100:1) extract of MN4K4 callus showed that hirsuteine and hirsutine were produced as major alkaloids and corynoxeine, isocorynoxeine, rhynchophylline and isorhynchophylline were produced as minor alkaloids. However, corynantheine, dihydrocorynantheine and geissoschizine methyl ether were not detected. So, the amounts of hirsuteine and hirsutine were measured as indicators of alkaloid production.

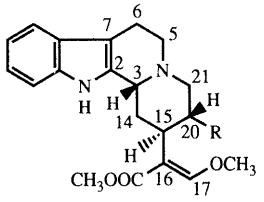
Table 1. ^{13}C-NMR Spectral Data of **1**—**4**

Carbon	**1**[a]	**2**[a]	**3**[b]	**4**[b]
1	39.1			
2	28.0	136.1	131.2	132.7
3	78.0	63.1	54.6	54.1
4	39.3			
5	55.8	55.2	51.1	51.2
6	18.8	22.8	16.7	17.0
7	33.6	108.3	106.9	107.8
8	40.0	127.8	127.4	127.8
9	48.0	118.4	117.9	117.9
10	37.3	119.2	119.5	119.3
11	23.6	121.4	121.7	121.3
12	125.6	111.6	111.5	111.2
13	139.2	137.6	136.3	136.0
14	42.5	36.7	31.1	31.1
15	28.8	33.0	34.2	34.0
16	24.9	110.2	111.0	111.6
17	48.0	152.5	160.1	159.6
18	53.5	58.6	11.1	115.4
19	39.3	65.8	24.1	139.3
20	39.3	44.1	38.0	42.9
21	31.1	97.0	50.4	51.2
22	37.3	167.1	168.7	168.7
23	28.8			
24	16.5			
25	15.7			
26	17.4			
27	23.9			
28	179.7			
29	17.5			
30	21.4			
COOMe		50.9	51.3	51.2
OMe			61.6	61.4
glc-1		101.3		
-2		74.5		
-3		78.7		
-4		71.5		
-5		78.4		
-6		62.7		

a) In C_5D_5N. *b*) In $CDCl_3$.



ursolic acid (**1**)



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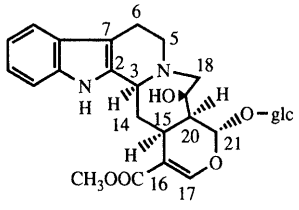
hirsutine (**3**) : R = -CH_2CH_3
hirsuteine (**4**) : R = -CH_2=CH_2



3 α -dihydrocadambine (**2**)

Chart 1

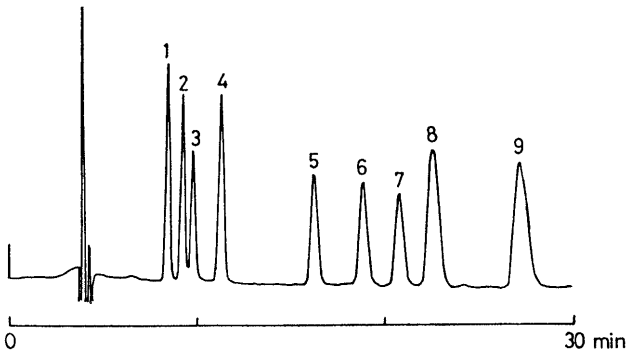


Fig. 1. Chromatogram of HPLC of Standard Alakloids of *Uncaria rhynchophylla*

1, isocorynoxeine; 2, isorhynchophylline; 3, corynoxeine; 4, rhynchophylline; 5, corynantheine; 6, dihydrocorynantheine; 7, geissoschizine methyl ester; 8, hirsuteine; 9, hirsutine.

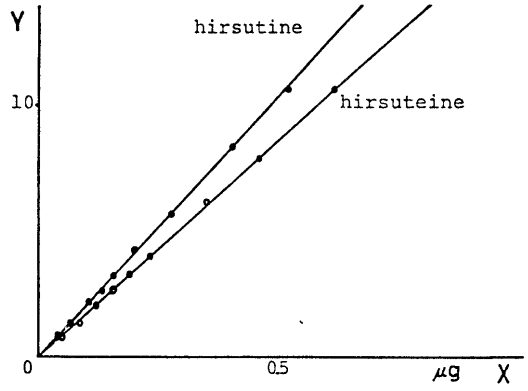


Fig. 2. Calibration Curve of Hirsuteine and Hirsutine

$Y=17.40X-0.02$ ($r=0.9999$), $Y'=20.98X'-0.07$ ($r=0.9999$) X, hirsuteine (X', hirsutine) concentration; Y, hirsuteine (Y', hirsutine) peak area.

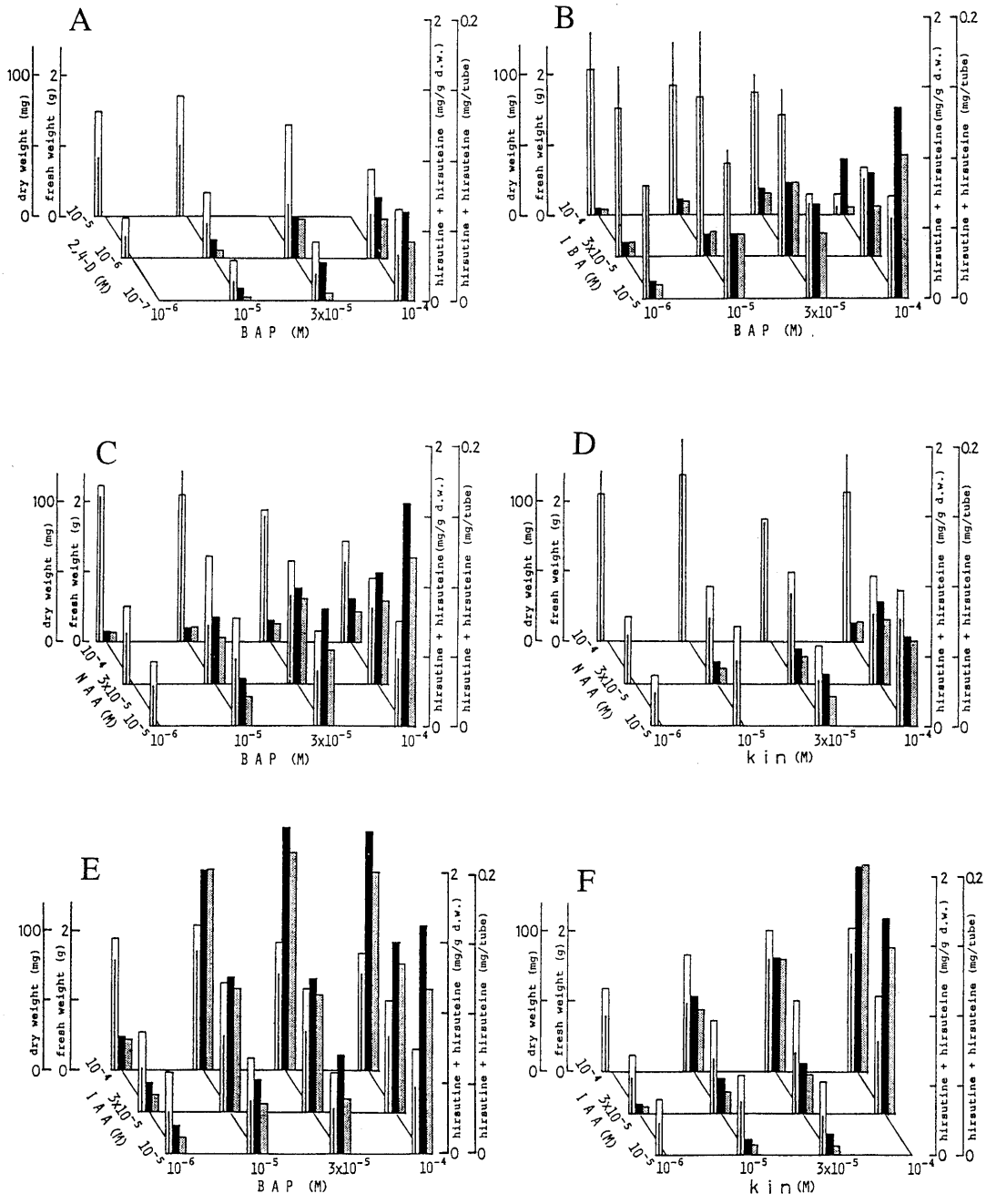


Fig. 3. Effect of Growth Homones on Growth and Hirsuteine–Hirsutine Production in Callus Cultures of *Uncaria rhynchophylla*

Combination of 2,4-D and BAP (A), IBA-BAP (B), NAA-BAP (C), NAA-Kin (D), IAA-BAP (E), IAA-Kin (F) in Gamborg B5 medium. —, fresh weight (g); □, dry weight (mg); ■, hirsuteine–hirsutine content (mg/g dry weight.); ▨, hirsuteine–hirsutine content (mg/tube).

HPLC proved to be successful for the separation of authentic samples (Fig. 1). The calibration curve was quite linear (Fig. 2).

The growth of MN4K4 callus was very slow (*ca.* 3 times for 6 weeks). When this callus was transferred to B5 solid medium supplemented with 10^{-4} M NAA–10^{-4} M Kin (GN4K4), the growth was faster than that of MN4K4 callus. In addition, GN4K4 callus produced alkaloids.

Effect of Growth Hormone on Callus Growth and Alkaloid Production The growth and alkaloid concentrations were examined in callus cultured in B5 medium supplemented with various concentrations and combinations of IAA, IBA, 2,4-D, NAA, BAP and Kin (Fig.3), for 15 weeks at 5 week intervals. The combination of 2,4-D–BAP (condition A) was unsuitable both for callus growth and alkaloid production. The combination of IBA–BAP (condition B) was suitable for callus growth. In particular, B5 medium supplemented with 3×10^{-5} M IBA–10^{-5} M BAP was suitable for callus growth (3.2 g fresh weight/5 weeks). However, this combination was unsuitable for alkaloid production. The effect of a combination of NAA–BAP (condition C) and NAA–Kin (condition D) gave similar results to that of IBA–BAP. The combinations of IAA–BAP (condition E) and IAA–Kin (condition F) were suitable both for callus growth and alkaloid production. It was found that the callus growth (1.8 g fresh weight/5 weeks) and alkaloid production (1.73 mg/g dry weight/5 weeks, 0.156 mg/tube/5 weeks) were optimal when the callus was cultured in B5 medium supplemented with 10^{-4} M IAA–3×10^{-5} M BAP.

Influence of the Culture Media on Callus Growth and Alkaloid Production The callus growth and alkaloid production were examined in callus cultured in three media, supplemented with 10^{-4} M IAA–3×10^{-5} M BAP (condition A) and 3×10^{-5} M IBA–10^{-5} M BAP (condition B) (Fig. 4). MS medium improved the callus growth, but

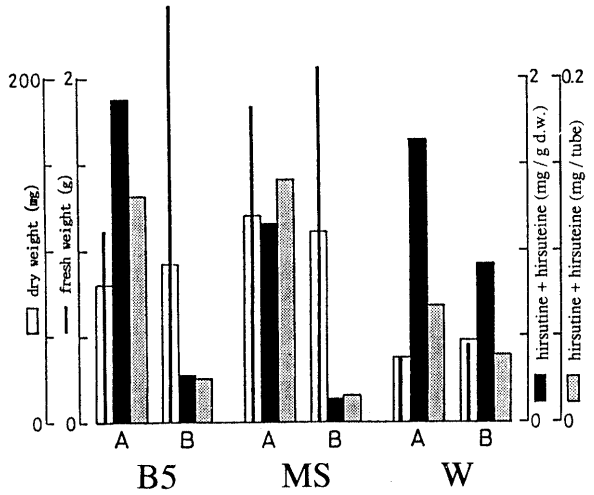


Fig. 4. Effect of Basal Medium on Growth and Hirsuteine–Hirsutine Production in Callus Cultures of *Uncaria rhynchophylla*

A, 10^{-4} M IAA and 3×10^{-5} M BAP; B, 3×10^{-5} M IBA and 10^{-5} M BAP B5, Gamborg B5; MS, Murashige–Skoog; W, white.

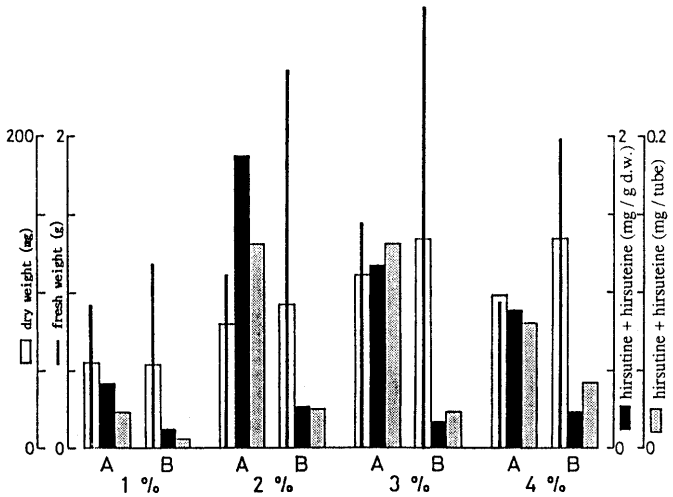


Fig. 5. Effect of Sucrose Concentration on Growth and Hirsuteine–Hirsutine Production in Callus Cultures of *Uncaria rhynchophylla* in Gamborg B5 Medium

A, 10^{-4} M IAA and 3×10^{-5} M BAP; B, 3×10^{-5} M IBA and 10^{-5} M BAP.

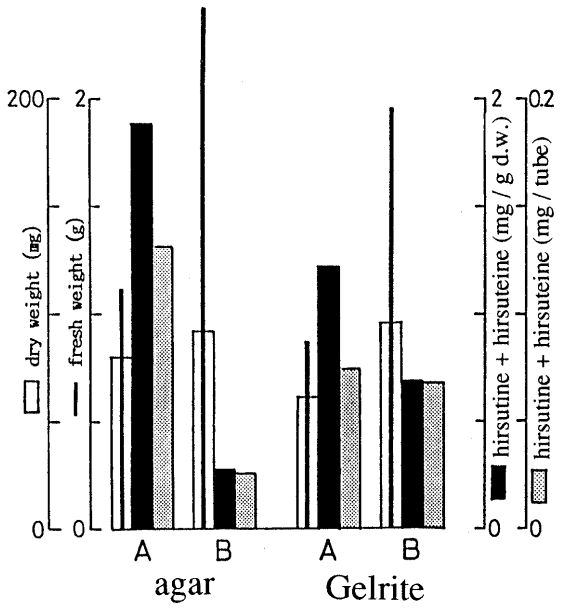


Fig. 6. Effect of Gelling Agents on Growth and Hirsuteine–Hirsutine Production in Callus Cultures of *Uncaria rhynchophylla* in Gamborg B5 Medium

A, 10^{-4} M IAA and 3×10^{-5} M BAP; B, 3×10^{-5} M IBA and 10^{-5} M BAP.

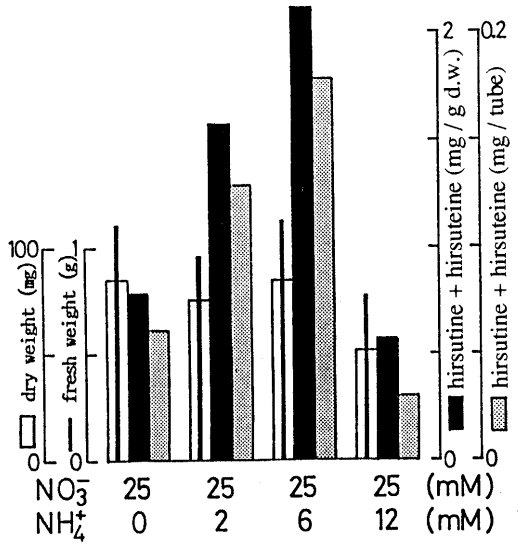


Fig. 7. Effect of Nitrogen Sources on Growth and Hirsuteine–Hirsutine Production in Callus Cultures of *Uncaria rhynchophylla* in Gamborg B5 Medium

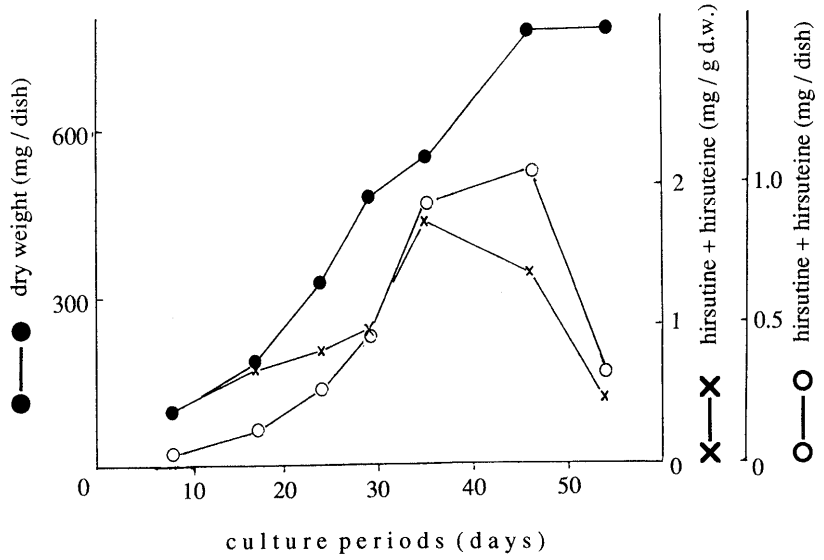


Fig. 8. Kinetics of Growth and Hirsuteine–Hirsutine Production in Callus Cultures of *Uncaria rhynchophylla*

did not significantly affect alkaloid production. White medium improved alkaloid production, but the callus did not grow. These results determined that B5 medium was suitable both for the callus growth and alkaloid production.

Effect of Sucrose Concentration on Callus Growth and Alkaloid Production The callus growth and alkaloid production in B5 medium supplemented with 1%, 2%, 3% and 4% sucrose are showed in Fig. 5. Maximal growth was obtained with 3% sucrose. However, maximal alkaloid production was obtained with 2% sucrose. These same callus supplemented with 1% and 4% sucrose did not improve either growth or alkaloid production.

Effect of Gelling Agents on Callus Growth and Alkaloid Production Callus growth and alkaloid production were examined in the B5 medium solidified with agar (0.7%) and Gelrite (gellan gum 0.2%) (Fig. 6). For condition A, callus growth and alkaloid production in agar-solidified medium were significantly higher than that with Gelrite. In contrast, alkaloid production in the Gelrite-solidified medium under condition B was significantly higher than that of agar. It was found that the effect of gelling agents depended on the supplemented growth hormones.

Effect of Nitrogen Sources on Callus Growth and Alkaloid Production It has been showed that both the type and amount of nitrogen sources in the basal medium affect the biosynthesis of various compounds in the plant cell culture. *Lithospermum* suspension cells could only accumulate shikonin derivatives with NO_3^- as the sole nitrogen source,[10] and produced rosmarinic acid and lithospermic acid instead of shikonin when cultured in LS medium, which contains about 20 mM NH_4^+ in addition to 40 mM NO_3^-.[11]

The effect of the composition of nitrogen sources on callus cultures was examined by varying the rate of NH_4^+ and NO_3^- without changing the NO_3^- concentration (Fig. 7). Ammonium chloride and potassium nitrate were used as sources of NH_4^+ and NO_3^-, respectively. Callus growth was not affected by 0—6 mM NH_4^+ concentrations. In contrast, alkaloid production was increased gradually with increasing NH_4^+ concentration. Both callus growth and alkaloid production decreased with 12 mM NH_4^+ concentration.

Maximal growth and alkaloid production was obtained when 6 mM ammonium chloride and 25 mM potassium nitrate were added to the B5 basal medium.

Kinetics of Growth and Alkaloid Production in Callus The growth of callus, which was cultured in B5 medium supplemented with 10^{-4} M IAA–3×10^{-5} M BAP, was investigated over a period of 50 d (Fig. 8). The callus showed about an 8-fold increase in dry weight for 50 d of culture, and this growth rate remained suitable through the subcultures. However, hirsuteine and hirsutine concentrations were at a maximum for 35—40 d and then decreased. The total amounts of hirsuteine and hirsutine formed reached a maximum of 1.9 mg/g dry weight at 35 d.

Conclusion

Callus was induced from the leaf of *U. rhynchophylla* on MS agar-solid medium supplemented with 10^{-4} M NAA–10^{-6} M Kin, 10^{-4} M NAA–10^{-5} M Kin and 10^{-4} M NAA–10^{-4} M Kin in the dark. The callus growth of *U. rhynchophylla* and its alkaloid production was optimal when callus was cultured in B5 agar-solid medium supplemented with 10^{-4} M IAA–3×10^{-5} M BAP, 2% sucrose and 6 mM ammonium chloride and 25 mM potassium nitrate. As major compounds, hirsuteine, hirsutine, 3α-dihydrocadambine and ursolic acid were isolated from the callus, which was cultured in B5 agar-solid medium supplemented with 10^{-4} M IAA–3×10^{-5} M BAP. Interestingly, the 3α-dihydrocadambine concentration of the callus was *ca.* 50-fold higher than that of the hooks and stems of *U. rhynchophylla*. Study of other compounds isolated from the callus are in progress.

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