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THE EFFECT OF PHYTOHORMONES AND SOME ADDITIVES ON TISSUE CULTURE ESTABLISHMENT AND *IN VITRO* PRODUCTION OF ALKALOIDS FROM *ARCANGELISIA FLAVA* MERR.

ผลของฮอร์โมนพืชและสารบางชนิดต่อการสร้างเนื้อเยื่อและการผลิตแอลคาลอยด์ของขมิ้นเครือในหลอดทดลอง

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ABSTRACT

The callus of Arcangelisia flava *Merr. was cultured* in vitro *by tissue culture technique to produce alkaloids: It was found that revised tobacco (RT) medium plus the combination of 2 mg/l naphthalene acetic acid (NAA), 2 mg/l indole-3-butyric acid (IBA) and 1 mg/l 6-furfurylaminopurine (kinetin) was suitable for culturing callus of* A. flava. *This culture could produce berberine similar to nature. The production of berberine was increased in culture grown on medium plus either coconut milk or casein hydrolysate. However, the production of berberine was decreased in culture grown on medium plus citric acid. The production of berberine was high in callus culture grown on coconut milk containing medium. The addition of manipulating agents such as tyrosine, manganese sulfate and aluminium*

sulfate in A. flava *callus culture increased the berberine contents. Nevertheless, the callus could not grow on colchicine containing medium.* A. flava in vitro *culture produced highest berberine when either aluminium sulfate or tyrosine was added. The intensity of yellow color was the indication of amount of alkaloid production. The repeated selection of yellow callus produced high berberine contents.*

บทคัดย่อ

จากการทดลองเลี้ยงเนื้อเยื่อของขมิ้นเครือพบว่า อาหารสูตรอาร์ที (revised tobacco medium) ที่มีส่วนผสมของฮอร์โมนแนฟทาลีนอะซีติกแอซิด 2 มก./ล. อินโดลบิวไทริกแอซิด 2 มก./ล. และไคเนติน 1 มก./ล. เป็นสูตรอาหารที่เหมาะสมต่อการเจริญเติบโตของเนื้อเยื่อขมิ้นเครือ และเนื้อเยื่อขมิ้นเครือนี้สามารถผลิตเบอเบอรีนได้ ปริมาณของเบอเบอรีนจากเนื้อเยื่อขมิ้นเครือจะสูงขึ้นเมื่อเติมเคซีอีนไฮโดรไลเซต โดยเฉพาะอย่างยิ่งเมื่อเติมน้ำมะพร้าวในสูตรอาหาร ปริมาณของเบอเบอรีนจะลดลงเมื่อเติมกรดซิตริก การเติมไทโรซีน แมงกานีสซัลเฟต และอะลูมิเนียมซัลเฟตในสูตรอาหารที่มีน้ำมะพร้าวจะเพิ่มปริมาณของเบอเบอรีน อย่างไรก็ตาม เนื้อเยื่อขมิ้นเครือไม่สามารถเจริญเติบโตได้ในสูตรอาหารที่มีโคลชิซีน ความเข้มของสีเหลืองของเนื้อเยื่อที่เลี้ยง แสดงถึงปริมาณของแอลคาลอยด์ที่ผลิต และการคัดเลือกเนื้อเยื่อขมิ้นเครือที่มีสีเหลืองซ้ำหลาย ๆ ครั้งจะทำให้ได้เบอเบอรีนในปริมาณสูง

INTRODUCTION

For the economical progressive of developing countries such as Thailand, drug is one of the essential basic factor that should be seriously considered and urged to manufacture locally. As a result it would reduce a commercial inbalance from importing drugs. This management would also prevent drug deficiency in critical periods. The berberine alkaloid is one of the potential drugs distributed in some genera of plants including *Arcangelisia flava* Merr., which is a Thai native medicinal plant found wildly grown in southeastern and southern parts of Thailand. It seems to contain a reliable high amount of berberine in its roots and stems. So *A. flava* may become an interesting source of berberine for modern drug production. However, the berberine mostly accumulates in *A. flava* roots and it takes approximately 5 years to produce a proper high alkaloid containing roots. Thus, the application of tissue culture techniques for culturing of *A. flava* in an artificial media was studied to shorten the period of growth and to increase the percentage of berberine by *in vitro* culture. In addition, this investigation would elucidate some basic knowledges of biosynthetic pathway of certain alkaloids in *A. flava* in culture condition.

MATERIALS AND METHODS

Plant material

Various parts of *Arcangelisia flava* Merr. obtained from Chanthaburi Medicinal Plants Garden, Medical Science Department, Ministry of Health, Changwat Chanthaburi and grown at Faculty of Pharmacy, Mahidol University, Bangkok were used to initiate tissue cultures.

Commercial *Arcangelisia flava* or Khamin khruea was bought from traditional drug stores for berberine analysis. *In vitro* culture grown on each medium was harvested and classified. Each available culture was collected and prepared for analysis.

Medium preparation

Media for *in vitro* culture initiation: Revised tobacco (RT) medium was used to initiate callus cultures. These media were supplemented with various concentrations of growth regulators as follows:

$N_2I_2K_1$ = 2 mg/l NAA* + 2 mg/l IBA** + 1 mg/l kinetin***
$N_2I_2B_2$ = 2 mg/l NAA + 2 mg/l IBA + 2 mg/l BAP ****
$N_5I_5B_5$ = 5 mg/l NAA + 5 mg/l IBA + 5 mg/l BAP
$D_1I_1B_1$ = 1 mg/l 2, 4-D***** + 1 mg/l IBA + 1 mg/l BAP
$D_1I_1K_1$ = 1 mg/l 2, 4-D + 1 mg/l IBA + 1 mg/l kinetin
D_1I_1 = 1 mg/l 2, 4-D + 1 mg/l IBA

Media for *in vitro* culture maintenance: The optimum concentration of growth regulators supplemented in the media of *A. flava* was selected. The additives used were; 100 ml/l coconut milk, 20 mg/l casein hydrolysate and 2 g/l citric acid. For the media supplemented with coconut milk, inositol was reduced to 250 mg/l. The medium without additive was used as control.

Media for *in vitro* culture manipulation: The suitable media formulation for maintenance which consisted of optimum concentration of growth regulators and an additive were selected. These were supplemented with 400 μm aluminium sulfate, 0.1% tyrosine and 89.2 mg/l magnesium ion as magnesium sulfate separately. For the medium supplemented with 0.025% colchicine, it was prepared without an additive. The medium without manipulating agent was used as control.

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* naphthalene acetic acid
** idole-3-butyric acid
*** 6-furfurylaminopurine
**** 6-benzyl-aminopurine
***** 2, 4-dichlorophenoxy acetic acid

Establishment of *in vitro* cultures of *A. flava*

Method of culture initiation: Healthy-looking stems, petioles with basal of leaf and leaves were surface sterilized. The green aseptic plant parts were aseptically transferred to revised tobacco (RT) medium. They were incubated in dark cabinet at 27 ± 1 °C to establish the callus cultures. The established callus cultures were then maintained by subculturing to fresh medium every 4 – 5 weeks to observe survival abilities.

Method of rearing and maintaining of *in vitro* cultures: All cultures were reared and maintained by aseptically subculturing onto prepared media every 4 – 5 weeks. The weight of each inoculated yellow healthy-looking callus was approximately 200 mg. The subculturing callus was incubated in dark condition at 27 ± 1 °C.

Method of rearing and manipulating of *in vitro* cultures: Each generation of all cultures was reared and maintained by aseptically subculturing onto prepared media every 3.5 – 4.5 weeks for 3 generations. The first generation of callus grown on media supplemented with colchicine was aseptically transferred to the media without colchicine for the next generation. The weight of inoculated yellow healthy-looking callus was approximately 200 mg. The subculturing callus cultures were incubated in dark condition at 27 ± 1 °C.

Harvesting

The *A. flava* cultures were subcultured to several generations within the period of 12 months until more uniform and healthy-looking callus was obtained. The 4 or 5 week old calli and media of the selected generation were harvested and dried overnight in an oven at 60°C and 80°C respectively, until dry. The dried tissues were stored in the dessicator at 27 ± 1 °C for analysis. The callus grown on medium plus manipulating agents was cultured only for 3 generations. Each generation was harvested, dried and stored as the method described above.

Growth and percentage of survival

The growth rate of the callus cultures was measured within 10 weeks period or until the callus completely died. The measurement of growth rate was based on fresh weight measurement by weighing 15 bottles of each callus every week. The growth rate was calculated as follow:

$$\text{growth rate} = \frac{\text{average final fresh weight}}{\text{average initial fresh weight}}$$

The percentage of survival was measured by visual examination and estimation of the amount of healthy-looking callus remained on the medium each week.

Water content

The water content was measured by weighing 10 bottles of uniform callus, and drying the tissue in a hot air oven at 60°C until dry. The dried weight was measured and the water content, calculated.

Phytochemical detection

The fresh callus was tested with alkaloidal precipitating reagent to detect alkaloids. The dry callus was tested with Fehling's solution to detect free sugar and glycosidie sugar, and with Liebermann-Berchard test to detect steroid.

Analysis of berberine

Optimization of mobile phase: Berberine, palmatine, columbamine, jatrorrhizine, *A. flava* intact plant and *A. flava* callus were chromatographed on silica gel 60G plate and developed with various mobile phases. The spots were visualised by Dragendorff's spraying reagent and UV light to select the optimum developing system.

Selection of optimum concentration of berberine: Various concentrations of berberine hydrochloride were chromatographed on precoated silica gel 60G plate and developed with selected mobiled phase. They were then scanned by using thin layer chromatography (TLC) scanner.

Comparison between 2 methods of berberine extraction: The root powder of *A. flava* was extracted with methanol by maceration and soxhlet extractor.

Assay of berberine in various parts of *A. flava* and commercial Khamin khruea : The berberine content in roots, stems and leaves of *A. flava* collected from Ban-ang forest, Changwat Chanthaburi, and three samples of commercial Khamin khruea bought from three traditional drug stores were determined by high performance thin layer chromatography (HPTLC). Each sample powder was exhaustively extracted by a soxhlet extractor. The extract was evaporated under reduced pressure at 60°C and adjusted to optimum volume. Each extract was then chromatographed on precoated silica gel 60G plate, developed with methanol : water:conc NH_3 (8:1:1) as mobile phase and analysed by TLC scanner.

The leave extract was also chromatographed on precoated silica gel 60G plate with concentration zone. It is then developed with ethylacetate : isopropanol : conc NH_3 (9:8:3) and analysed by TLC scanner by fluorescence reflection mode at an excitation wavelength of 254 nm.

Assay of berberine in *A. flava in vitro* culture: The methanol extracts of *A. flava* tissue cultures were prepared and analysed by using ethylacetate : isopropanol : conc NH_3 (9:8:3) as the mobile phase. The spots were determined with a TLC scanner by fluorescence reflection mode at an excitation wavelength of 254 nm.

Statistical analysis : Results are given as mean (g% w/w) and %CV*, unless otherwise indicated. Analysis of the difference between two means was performed by using student's t-test with the level of significance $P < 0.01$. Significant analysis of more than two experimental groups was performed by using ANOVA and LSD with the level of significance $P < 0.01$, unless other level indicated.

RESULTS

Effectiveness of growth regulators and additives on *in vitro* cultures of *Acangelisia flava* Merr.

Determination of the suitable combination of growth regulators: Among 6 combinations of growth regulators added to revised tobacco media, all combinations yielded tissue cultures with cell proliferation could normally be detected within 2 – 3 weeks after inoculation. The callus initiated on each growth regulator combination and the percentage of initiation were varied (Table 1 and Figure 1). The combinations of $N_2I_2K_1$, D_1I_1 and $D_1I_1K_1$ yielded healthy-looking callus. Though, the callus was continuously subcultured on the same fresh medium every 4 – 5 weeks, only the callus grown on the first combination survived more than 20 months while the two latter calli survived for 2 months. The other combinations of growth regulators yielded unhealthy-looking calli, inspite of continuous subculturing the calli survived within 2 – 3 months. The callus cultured on medium containing the combinations of $N_2I_2K_1$ grew slowly and yielded pale yellow callus.

To establish callus culture from various part of *A. flava*, the stems and petioles with basal of leaf were easily initiated and maintained (Table 1 and Figure 1). The callus obtained from different parts of plant showed some visually differences. As the stem grown on the combination of $N_2I_2K_1$ yielded true callus, the petioles with basal of leaf and leaves yielded compact callus and root-bearing nodules. All parts of *A. flava* grown on the combination of D_1I_1 and $D_1I_1K_1$ yielded true calli.

Determination of additives for maintainance: The combination of $N_2I_2K_1$ was found to be a suitable growth regulator for *A. flava* establishment and maintenance, thus this medium was used for further study. The medium was added with some organic supplements such as coconut milk, citric acid, and casein hydrolysate to enhance growth and secondary products. At first, the starting callus grew slowly and yielded numerous brown calli, and the media eventually turned to brown and black. The dark and unhealthy-looking callus may be due to the toxic effect of phenolic compounds accumulated in the media. After continuous subculturing for a year, the callus culture gradually turned to healthy-looking, produced yellow color and grew fast. The callus grown on medium plus coconut milk was easier

*CV, percentage of coefficient of variation = $\frac{\text{standard deviation}}{\text{mean}} \times 100$

maintained and more healthy-looking than other calli. By visual detection, all calli grown on medium plus organic supplements grew fast and were yellow. However, the yellow callus on medium supplemented with citric acid turned to pale yellow and later white within 5 generations.

The cells grown on all media were unlignified parenchymas with thin walls. Starch grains and yellow droplets were found in all calli. These starch grains gave black blue color when contacted with iodine water. The vessels were scatterly found in only the medium plus coconut milk. When Dragendorff's reagent was added, the cells turned orange. It was thus indicated that this callus should contain the alkaloid. In addition, the callus grown on medium either with or without additives, and root-bearing callus grown on $N_2I_2K_1$ were fluorescent.

The callus was harvested and classified as healthy-looking, unhealthy-looking and brownish calli. Healthy-looking callus was the viable, buff-colored friably loosen mass cells. Its colors were yellow, pale yellow and white yellow. Brownish callus was the overgrowth callus which died before harvesting. Unhealthy-looking callus was clumping and greenish color which was different from healthy-looking one. Each available callus was collected and prepared for analysis.

Determination of supplements for manipulation : The callus on the medium plus coconut milk and either aluminium sulfate or manganese sulfate grew fast and had yellow color. The color was intense at the first generation and gradually pale after the second and third generations. Some unhealthy-looking calli grew together with healthy-looking calli at all generations on medium plus aluminium sulfate. Some unhealthy-looking calli were observed at the second and third generations on medium plus manganese sulfate which was significantly grown faster than the control.

On medium plus coconut milk and tyrosine, the callus grew quite slowly and gave pale yellow color at first generation. Then it turned to intensely yellow and half of it produced numerous root-bearing nodules at the second generation. This root-bearing callus was disappeared at the third generation and the callus became paler.

In order to select high alkaloid productivity cells, colchicine was added to medium. It was found that the callus could not grow on this media. Most of them were dead while some of them turned brown. The calli were subcultured onto fresh medium without colchicine to obtain survival cells, but they were all dead.

Growth rate and percentage of survival

The growth rate was determined weekly by the increasing weight of the callus in the period of 10 weeks. The maximum growth was reached within 6 – 8 weeks (Figure 2). The percentage of survival was maximum within 5 – 6 weeks. Generally, the optimum percentage of survival corresponded to the period of maximum growth. However, at this period the callus was weak and had a light brown color. Thus, subculturing was made during the 4^{th} or 5^{th} week.

Among the calli grown on medium plus different organic supplements, the growth rates were similar. Surprisingly, the growth rates of callus on medium without organic supplement were higher than those tissues in medium with organic supplement. However, the coefficient of variation of harvested callus weight per week was varied significantly and in some cases they were up to 50%.

Percentage of water content in callus

Percentage of water content of *A. flava* callus grown for 4 weeks on solid medium supplemented with $N_2I_2K_1$ was approximately 90 – 92% (Table 2). The additive either coconut milk, citric acid or casein hydrolysate had no effect on the percentage of water content in these calli.

Phytochemical detection in *in vitro* cultures of *A. flava*

Microchemical testing of alkaloids: The callus extracted in salt form gave positive results with all alkaloidal reagents. It could therefore be concluded that the callus cultures of *A. flava* contained alkaloids. The minimum of berberine detection by microcrystallization method was 41.6 μg/ml. It was shown that the alkaloid content was closed to the concentration of 83.2 μg/ml of standard berberine solution. So, it is calculated that the callus contained berberine approximately 0.04% by fresh weight.

Sugar detection: The hot water extracted portion gave intense positive result, as shown by the large amount of red brick precipitation. Thus, it indicated that the *A. flava* callus contained free sugar. The extracted portion obtained from the acidic hydrolysis of the marc also gave red brick precipitation, but to a lesser extent. Thus, the *A. flava* callus may not contain glycosidic sugar and the red brick precipitation may be due to the incomplete extraction of sugar from the first step.

Steroid detection : The purple color was produced when concentrated sulfuric acid was mixed gently with acetic anhydride and the callus extract. The color eventually turned to black. It was thus indicated the absence of unsaturated sterols or triterpene presented when the extract of *A. flava* callus was detected at this concentration.

Assay of berberine from *A. flava* by TLC and HPTLC methods

Optimization of mobile phase: From all mobile phase systems tested, methanol : water : conc NH_3 (8:1:1) was the most suitable mobile phase. Thus, this mobile phase was used throughout the experiment. This system could separate standard alkaloids e.g. berberine, palmatine, columbamine and jatrorrhizine and could also separate the compounds from various parts of *A. flava*. However, this mobile phase could not clearly separate compounds of *A. flava* extract in methanol. When various mobile phase systems were tried, it was found that methanol : isopropanol : conc NH_3 (8:1:1) and ethylacetate : isopropanol : conc NH_3 (8:5:5) seemed to be good mobile phase systems. The ratio of solvents in each system was thus varied and it was found that ethylacetate : isopropanol : conc NH_3 (9:8:3)

was the best mobile phase.

Selection of optimum concentration: A nonlinear relation between berberine concentration and response determined as peak area, was observed over a concentration range of 200 – 4,400 μg/ml (Figure 3). However, at low berberine concentration, there was a linear response over the concentration range of 60 – 400 μg/ml either in fluorescence or absorbance measurement (Figure 4). Thus, this concentration range was selected for the determination of berberine in *A. flava*. Though, the standard berberine and berberine in *A. flava* was separated as streaks, the berberine in *A. flava* callus was not separated as such over the concentration range of 160 – 500 μg/ml. This might be due to other ingredients presented in the sample. This berberine could be separated as a streak at the concentration not more than 120 μg/ml.

Comparison between 2 methods of berberine extraction: Chromatograms of standard alkaloids shown in Figure 5 indicated that berberine was clearly separated from columbamine, jatrorrhizine and palmatine. The retention time of columbamine, jatrorrhizine, palmatine and berberine were 0.85, 0.85, 0.07 and 0.11, respectively. Thus, methanol : water : conc NH_3 (8:1:1) was chosen as an appropriate mobile phase. Regarding to berberine content, Table 3 showed no difference between these two methods at 0.01 level of significance. The TLC chromatogram visuallized by UV light and Dragendorff's spraying reagent showed that there was no difference in alkaloid composition. Time taken for complete extraction were 6 days and 3 – 5 h for maceration and soxhlet extraction, respectively. Therefore, the latter method was selected for extraction purpose.

Percentage of berberine in various parts of *A. flava* and commercial Khamin khruea: The content of berberine was highest in roots at the concentration of 4. 3229%. Its content was sharply reduced to 0.1720% in stems. However, it was found that berberine was not present in leaves (Figure 6). When berberine was added to the leaves, it was well resolved from other leaf constituents because silica gel 60G plate with concentration zone was used as stationary phase and ethylacetate : isopropanol : conc NH_3 (9:8:3) was used as mobile phase.

The content of berberine in commercial Khamin khruea was shown in Table 4. The percentage of berberine in these commercial Khamin khruea were more than ten times of that in *A. flava* stems. However, they were about half of that in its roots. Compare among different sources, it was seen that the percentage of berberine in Khamin khruea bought from Po-ti-pra-did traditional drug store was not different from that from "Song-vait" traditional drug store at 0.01 level of significance. The percentage of berberine in Khamin khruea bought from "Chao-krom-per" traditional drug store was less than that from other two stores at 0.01 level of significance.

Percentage of berberine in *A. flava in vitro* culture: Figure 7 showed chromatograms of standard alkaloids when ethylacetate : isopropanol : conc NH_3 (9:8:3) was used as a new mobile phase. The retention time of columbamine, jatrorrhizine, palmatine and berberine were 15, 14, 21, 27 respectively. Thus, it was seen that berberine was clearly separated from columbamine, jatrorrhizine and palmatine. In addition, the berberine in

A. flava culture was well resolved from other constituents.

1. Percentage of berberine in *in vitro* cultures grown on medium with additive

Callus grown on medium without additive contained 0.3720 – 0.6900% of berberine on dry weight basis (Table 5). The callus grown on this medium generation 19 yielded maximum percentage of berberine (0.6900%) which might be due to continuous selection of yellow callus. There was no difference in berberine content among yellowish healthy-looking callus generations 11 – 13, unhealthy-looking callus generations 10, 12, 15 and brownish callus generation 13 at 0.01 level of significance. In addition, brownish callus contained the same amount of berberine as healthy-looking callus at 0.01 level of significance. However, it was different only at 0.05 level of significance. Thus, the callus might be collected as fresh viable callus or dead callus. However, the viable callus was preferred because it could continually grow and be collected.

Callus grown on medium plus coconut milk contained 0.4875 – 0.8758% of berberine on dried weight basis (Table 6). The addition of coconut milk was found to increase the production of berberine. As the callus was repeatedly subcultured and selected, the content of berberine was increased. When the callus was not selected in generations 12 and 13 the content of berberine was decreased. There was also no difference between brownish callus and yellowish healthy-looking callus grown on medium with coconut milk generation 8 at 0.01 level of significance.

Callus grown on medium plus citric acid contained 0.1740 – 0.3746% of berberine on dry weight basis (Table 7). The percentage of berberine in healthy-looking callus grown on medium plus citric acid of the $3^{rd} - 4^{th}$ generations was not different from yellowish healthy-looking callus grown on medium without additive in the $11^{th} - 13^{th}$ generations which was served as control at 0.05 level of significance. Though the yellowish healthy-looking callus was repeatedly selected due to high berberine content, and repeatedly subcultured onto medium plus citric acid, the content of berberine was gradually decreased.

Callus grown on medium plus casein hydrolysate contained 0.4844 – 0.5744% of berberine on dry weight basis (Table 8). The production of berberine in this callus was increased when compared with the callus grown on medium without additive of the $11^{th} - 13^{th}$ generations at 0.01 level of significance.

2. Percentage of berberine in *in vitro* cultures grown on medium plus manipulating agents

The content of berberine in callus grown on medium plus aluminium sulfate in the 1^{st}, 2^{nd} and 3^{rd} generations was increased when compared with callus grown on medium without a manipulating agent of the 10^{th} generation which was used as control at 0.01 level of significance (Table 9). The percentage of berberine in this callus was maximum in the 2^{nd} generation and was decreased in the 3^{rd} generation. The maximum content of berberine in callus grown on this medium (1.0574%) was superior to the maximum content of berberine in callus grown on medium without manipulating agent (0.8758%) at 0.01 level of significance.

The content of berberine in callus grown on medium plus manganese sulfate was increased in generation 1 at 0.01 level of significance. However, berberine was decreased in calli generations 2 and 3.

The callus grown on medium plus tyrosine in the 1st generation yielded lower berberine than callus grown on medium without manipulating agent at 0.01 level of significance. However, the content of berberine was increased in the 2nd and 3rd generation calli and the calli with root-bearing nodules. These calli contained berberine more than control at 0.05 level of significance. The callus with root-bearing nodules grown on medium plus tyrosine for two subculturings yielded maximum percentage of berberine. However, there was no difference between the callus grown on this medium for 3 subculturings which was subcultured either from true callus or callus with root-bearing nodules. Both the callus with root-bearing nodule grown on medium plus tyrosine for two subculturings and the callus grown on medium plus aluminium sulfate for two subculturings yielded the same maximum amount of berberine at 0.01 level of significance.

When the callus was subcultured onto medium plus colchicine, its berberine content was sharply reduced. It was noticed from chromatograms in Figure 8 that many small peaks appeared only in this callus. It was suggested that berberine might be degraded or the biosynthetic pathway of berberine might be changed so the content of berberine was decreased.

DISCUSSION

It is generally accepted that revised tobacco medium was suitable for growing a wide range of medicinal plant cultures[3], therefore it was selected for the experiment. The combination of growth regulators as 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin ($N_2I_2K_1$) had been found to be optimum for *Arcangelisia flava* Merr. *in vitro* culture establishment and maintainance.

In order to enhance growth and secondary product yield organic supplements such as coconut milk, citric acid and casein hydrolysate were supplied in media for culturing *A. flava* callus. By visuallization, coconut milk was the best organic supplement for enhancing growth. The callus grown on medium plus coconut milk produced higher berberine than that on medium without organic supplement. After repeatedly selection of yellow callus, the content of berberine was gradually increased. The content of berberine was as high as 0.8758% in callus grown on medium plus coconut milk at the 11th generation. Addition of citric acid decreased berberine content in *in vitro* culture. As the callus was repeatedly subcultured onto medium plus citric acid, the berberine content was gradually decreased. The medium plus casein hydrolysate showed promotional effect on berberine production, but its effect was not as much as that on medium plus coconut milk.

Actually at first the circumstances of culturing medium shown, the medium turned to brown and then black brown due to phenolic compounds produced and excreted to accumulate

into the medium by *A. flava* culture. Though, these compounds were beneficial as the precursor of berberine, they were toxic to callus. Thus, the callus grew slowly and turned brown. However, after several subculturing, the culture became yellow and looked healthy which might be due to its selection of resistant cell.

Actually, the percentage of survival was corresponded to the period of maximum growth. However, the *A. flava* culture was weak in that period. This may be due to the injury of the culture caused by the toxic compounds in the medium and the old age of the cells.

Growth could not be an indicator of alkaloid production, since culture had poorer growth rate produced higher alkaloids. For example, the culture of *A. flava* grown on medium supplemented with either coconut milk or casein hydrolysate showed lower growth rate than that of the control, but yielded higher content of berberine. In general, the conditions which promote secondary metabolism in tissue cultures are often suggests as the best way to produce natural products: the first stage allows fast growth and biomass accumulation, then the conditions are changed to support slow or no growth and to favour secondary metabolites.[1]

Fortunately, berberine is yellow, thus the callus containing high berberine could be selected by physical method through the intensity of yellow color. Besides berberine was fluorescent, high yielding cell lines could also be visuallized by its fluorescence nature. However, the first selection method was simpler than the latter selection. By comparison with standard berberine solution using microcrystallization method, the content of alkaloid in tissue culture could be preliminarily determined.

The evaluation of berberine contents in media was made. It was found that the medium without additive of the callus generation 16 contained 0.0379% of berberine with 23.22% of CV (n = 2)*. The medium plus coconut milk of generation 7 contained 0.0519% of berberine with 8.61% of CV (n = 3). The medium plus citric acid of generation 5 contained 0.0231% of berberine with 37.88% of CV (n = 3). The large variation might be due to inhomogenity of samples. The medium was kept as dry form by drying in a hot air over to prevent microbial contamination. However, the dry media lost their gelling effect in solvents such as water, 50% methanol and pure methanol even though they were agitated for 2 h. In addition, the dried media could not be triturated into powder form. It was shown that the content of berberine in media was about one tenth of that in the callus. Thus, it was suggested that the callus might excrete berberine into the medium.

The berberine content obtained from tissue culture of *A. flava* initiated from stem was more than that from basal of leaves and stems of intact plant. However, the berberine content obtained from *in vitro* culture of *A. flava* was less than that from roots of intact plant. It was found that the highest yield of berberine obtained from tissue culture was 1.0574% on dry weight basis which was about half of berberine obtained from commercial

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* n = the number of samples

A. flava. Though the content of berberine was less than that from nature, the application of tissue culture techniques could shorten the period of growth. It would be most economically, if promotion of the berberine production was increased. The result of this experiment should provide the fundamental data for further investigation of *in vitro* berberine production.

CONCLUSION AND RECOMMENDATION

This experiment was the demonstration of *in vitro* cultivation of *Arcangelisia flava* Merr. It showed that revised tobacco medium plus the combination of 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin ($N_2I_2K_1$) was suitable for culturing *A. flava* culture. It demonstrated that *A. flava* culture grown on medium supplemented with coconut milk and casein hydrolysate produced higher berberine than that grown on medium without supplement, but the callus culture grown on medium supplemented with citric acid produced lower berberine than that grown on medium without supplement. The intensity of yellow color was the indication of the content of alkaloid production. The repeatedly selection of yellow callus produced high berberine content. The medium containing tyrosine which is a precursor of berberine caused higher berberine production in callus generations 2 and 3 than control, but decreased the growth rate. The effect of aluminium sulfate, a stressing substance, stimulated the berberine production and increased yield in all callus, but decreased the growth rate resembling the effect of tyrosine. The culture supplemented with manganese sulfate, a mineral salt, increased the production of berberine in the 1^{st} and 3^{rd} generation and also increased the growth rate. The addition of colchicine (0.025%) seriously injured the cells and the culture eventually died.

From HPTLC results, it was interesting to find that major product was berberine. The highest yields of berberine content were obtained when the calli were subcultured on media plus either 400 μM aluminium sulfate or 0.1% tyrosine. In these conditions it was found that the callus contained 1.0574% of berberine derived from callus grown on aluminium containing medium for two subculturings and 1.0468% of berberine obtained from callus grown on tyrosine containing medium. However, among callus grown on medium plus 0.1% tyrosine, only the callus with root-bearing nodules produced highest yields of berberine content.

It was shown that the berberine contents obtained from callus cultures which initiated from a stem were more than those obtained from leaves and stems of the intact plant. However, the berberine content obtained from callus cultures were less than that obtained from the roots of intact plant. Hence, the method for promotion berberine in *A. flava* callus cultures should be further studied to obtain the highest yield of berberine. The factors concerned were light, temperature, aeration, culture vessel agitation and composition of the cultured medium. As it was reported that light decreased berberine production while better aeration increased berberine production. Thus, it was interesting to study the effect of composition of the cultured medium, the most influencial factor, on berberine production.

The experiment on aluminium stressing should be further investigated. The concentration of aluminium sulfate should be increased to provide available aluminium to stress callus. At higher pH values, aluminium ions precipitate as $Al(OH)_3$ and are no longer toxic.[2] Thus, the aluminium might be chelated with EDTA so as to maintain its availability at a higher pH (6.0) which would permit the gelling of the agar. If *A. flava* can grow on liquid medium, the pH of aluminium containing medium should be adjusted to 4.

As it was so far realized that the application of tissue culture techniques will become more beneficial, possibly the plant can grow on liquid medium which was the most economical and suitable way in growing callus cultures for industrial scale. Therefore, the alternative combinations of growth regulator should be investigated to obtain friable tissue culture and organ culture, such as root that can maintain as suspension cultures.

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Table 1. The effect of phytohormonal combinations on establishment of *in vitro* culture from various parts of *Arcangelisia flava*

Hormone	Part used	Amount	Initiation (%)	Callus growth	Detection within (day)
D_1I_1	stems	39	100.00	+6.0	15–25
	petioles	18	61.11	+2.0	16–18
	leaves	37	16.22	+0.5	28
$D_1I_1K_1$	stems	10	90.00	+1.0	11–28
	petioles	9	100.00	+0.1	17–20
	leaves	11	45.45	+0.5	17–75
$D_1I_1B_1$	stems	15	53.33	+0.5	17–25
	petioles	11	36.36	+0.1	9–17
	leaves	19	15.79	+0.1	48
$N_2I_2K_1$	stems	13	76.92	+4.0	14–36
	petioles	14	100.00	+2.0	14–26
	leaves	13	69.23	+1.0	27–57
$N_2I_2B_2$	stems	21	52.38	+1.0	17–19
	petioles	15	13.33	+0.1	19–49
	leaves	13	7.69	+0.1	17
$N_5I_5B_5$	stems	14	78.57	+1.0	17–19
	petioles	8	12.50	0	—
	leaves	19	10.53	+0.5	75

Table 2. Percentage of water content in *A. flava* tissue culture grown for 4 weeks on solid RT medium supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin in dark condition

Additives	Genera-tion	No. of bottles	I			II			Water content	
			Total fresh weight (g)	Total dry weight (g)	Water content (%)	Total fresh weight (g)	Total dry weight (g)	Water content (%)	%Average	%CV
none	18	10	13.1950	1.1956	90.94	15.6963	1.3104	91.65	91.29	0.55
10% coconut milk*	11	10	20.0529	1.7009	91.52	18.1357	1.4586	91.96	91.74	0.34
20 mg/l citric acid	11	10	10.3086	0.9812	90.48	7.1942	0.7158	90.05	90.26	0.34
20 mg/l casein hydrolysate**	12	10	12.2313	1.1358	90.71	13.9248	1.2219	91.22	90.96	0.40

* inositol was reduced to 250 mg/l

** tissue culture was grown for 5 weeks on solid RT medium plus casein hydrolysate

Table 3. Comparison between 2 methods of berberine extraction

Extraction method	Berberine content*	
	% Mean	% CV
Maceration (n = 3)	4.3229	1.25
Soxhlet extraction (n = 3)	4.2850	1.54

* not different at 0.01 level of significance

Table 4. Berberine content in commercial Khramin khruea bought from three traditional drug stores, analysed by HPTLC

Drug stores	Berberine content*	
	% Mean	% CV
Chao-krom-per (n = 3)	2.1807	5.03
Po-ti-pra-did (n = 3)	2.5515	5.32
Song-vait (n = 3)	2.5577	5.30

* not different at 0.01 level of significance

Table 5. Berberine content of *Arcangelisia flava in vitro* culture grown on solid RT medium, supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin

Callus	Generation	n	Berberine content	
			%Mean	%CV
Yellowish healthy-looking callus	11 – 13	3	0.4011	3.64
Unhealthy-looking callus	10,12,15	2	0.3720	2.85
Brownish callus	13	2	0.3991	1.12
Healthy-looking callus	15	3	0.4504	1.24
Brownish callus	15	3	0.4794	6.43
All callus	19	3	0.6900	3.59

Table 6. Berberine content of *Arcangelisia flava in vitro* culture grown on solid RT Medium, supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin plus 10% coconut milk

Callus	Generation	n	Berberine content	
			% Mean	% CV
Yellowish healthy-looking callus	3 – 7	3	0.4875	1.76
Unhealthy-looking callus	5 – 8	3	0.5272	6.50
Pale yellowish healthy-looking callus	7	4	0.5555	5.33
Yellowish healthy-looking callus	8	3	0.6451	3.60
Brownish healthy-looking callus	8	3	0.6133	8.74
Yellowish healthy-looking callus	9	3	0.7454	4.94
All callus	10	2	0.6372	1.89
All callus	11	3	0.8758	2.65
All callus	12	3	0.5739	2.93
All callus	13	3	0.6677	1.09

Table 7. Berberine content of *Arcangelisia flava in vitro* culture grown on solid RT medium, supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin plus 2 g/l citric acid

Callus	Generation	n	Berberine content	
			% Average	% CV
Healthy-looking callus	3 – 4	3	0.3746	2.69
Unhealthy-looking callus	4	3	0.1740	3.10
All callus	5	4	0.2655	2.88
All callus	6	3	0.2480	6.07

Table 8. Berberine content of *Arcangelisia flava in vitro* culture grown on solid RT medium, supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin plus 20 mg/l casein hydrolysate

Callus	Generation	n	Berberine content	
			% Mean	% CV
All callus	10	3	0.5744	1.50
All callus	11	3	0.4844	4.91

Table 9. Effect of various manipulating substances on berberine production in *Arcangelisia flava in vitro* culture grown on solid RT medium, supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin plus 10% coconut milk

Manipulating substances	Generation	n	Berberine content	
			% Mean	% CV
Control	–	2	0.6372	1.89
400 μM Aluminium sulfate	1	3	0.9978	2.40
	2	3	1.0574	2.30
	3	3	0.9205	2.78
89.2 mg/l Manganese sulfate	1	4	0.7625	6.73
	2	4	0.6100	7.50
	3	4	0.6804	5.75
0.1% Tyrosine	1	3	0.5405	2.53
	2*	3	0.6943	1.14
	2**	2	1.0468	4.98
	3*	3	0.7537	0.78
	3**	3	0.7719	7.90
0.025% Colchicine	2	3	0.3089	2.75

* represents true callus

** represents callus with root-bearing nodules

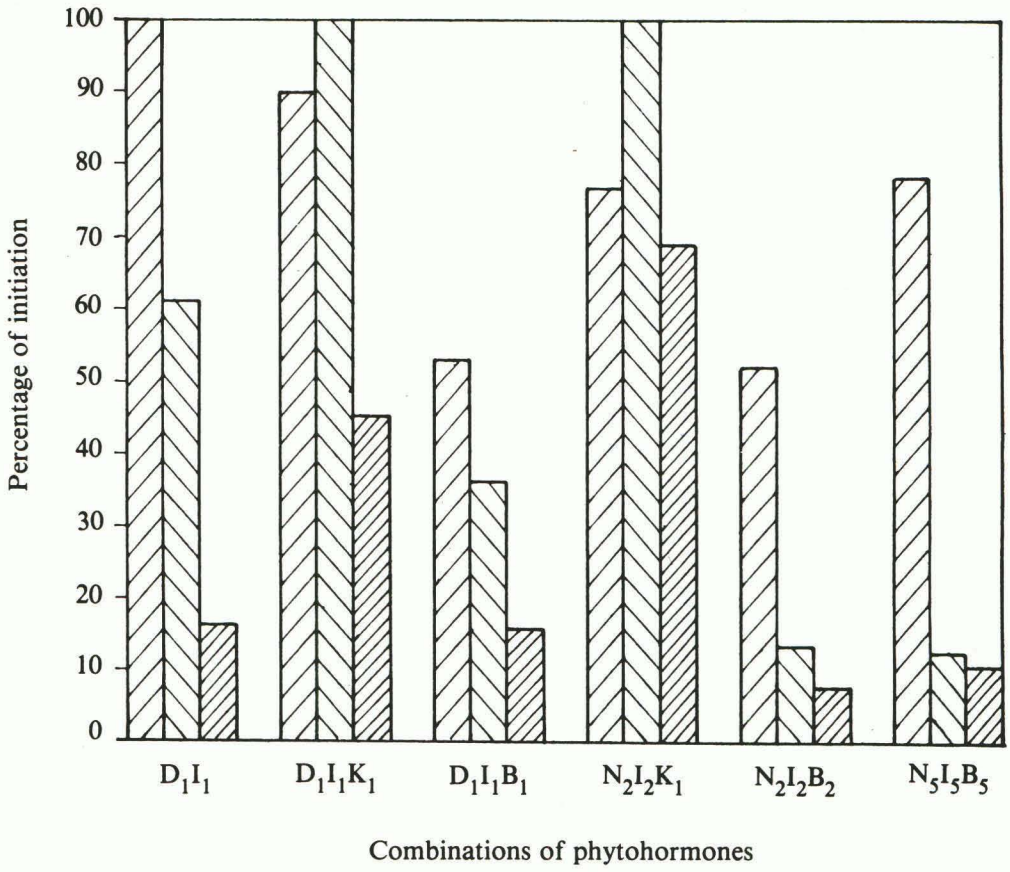

Fig. 1 The establishment of tissue culture from various parts of *Arcangelisia flava* on solid RT medium plus different combinations of phytohormones

: Tissue culture initiated from stems

: Tissue culture initiated from petioles with basal of leaf

: Tissue culture initiated from leaves

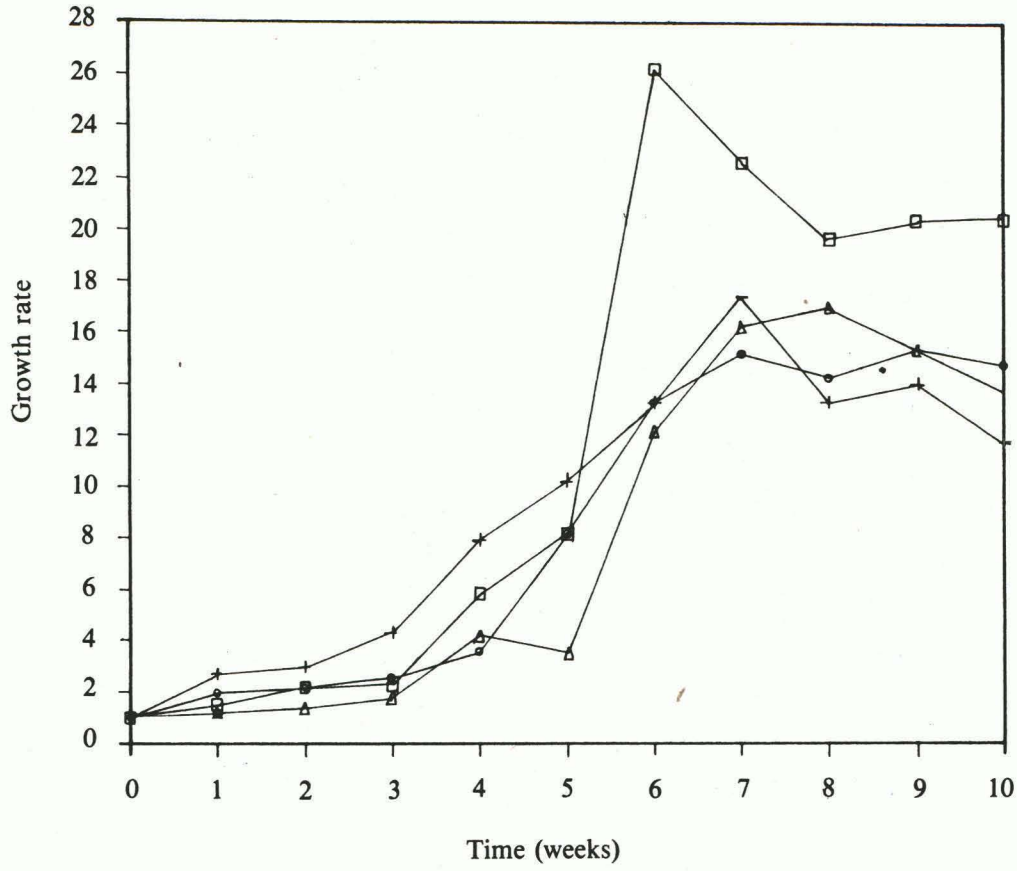

Fig. 2 Growth relatives of *Arcangelisia flava* tissue culture grown on solid RT medium plus various additives, supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin ($N_2I_2K_1$)

□ : Callus grown on RT medium

\+ : Callus grown on RT medium plus 10% coconut milk

○ : Callus grown on RT medium plus 2 g/l citric acid

△ : Callus grown on RT medium plus 20 mg/l casein hydrolysate

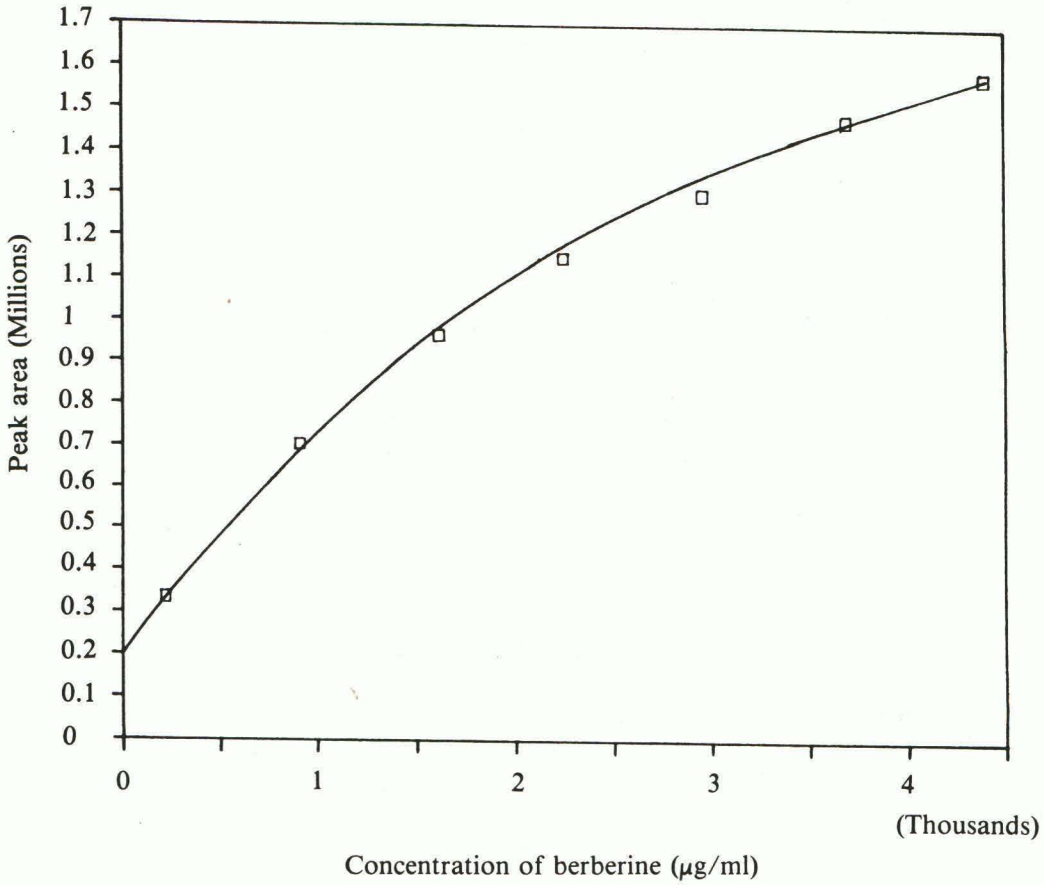

Fig. 3 Nonlinear response of berberine chloride in concentration range of 200 – 4,400 μg/ml, determined by fluorescence measurement

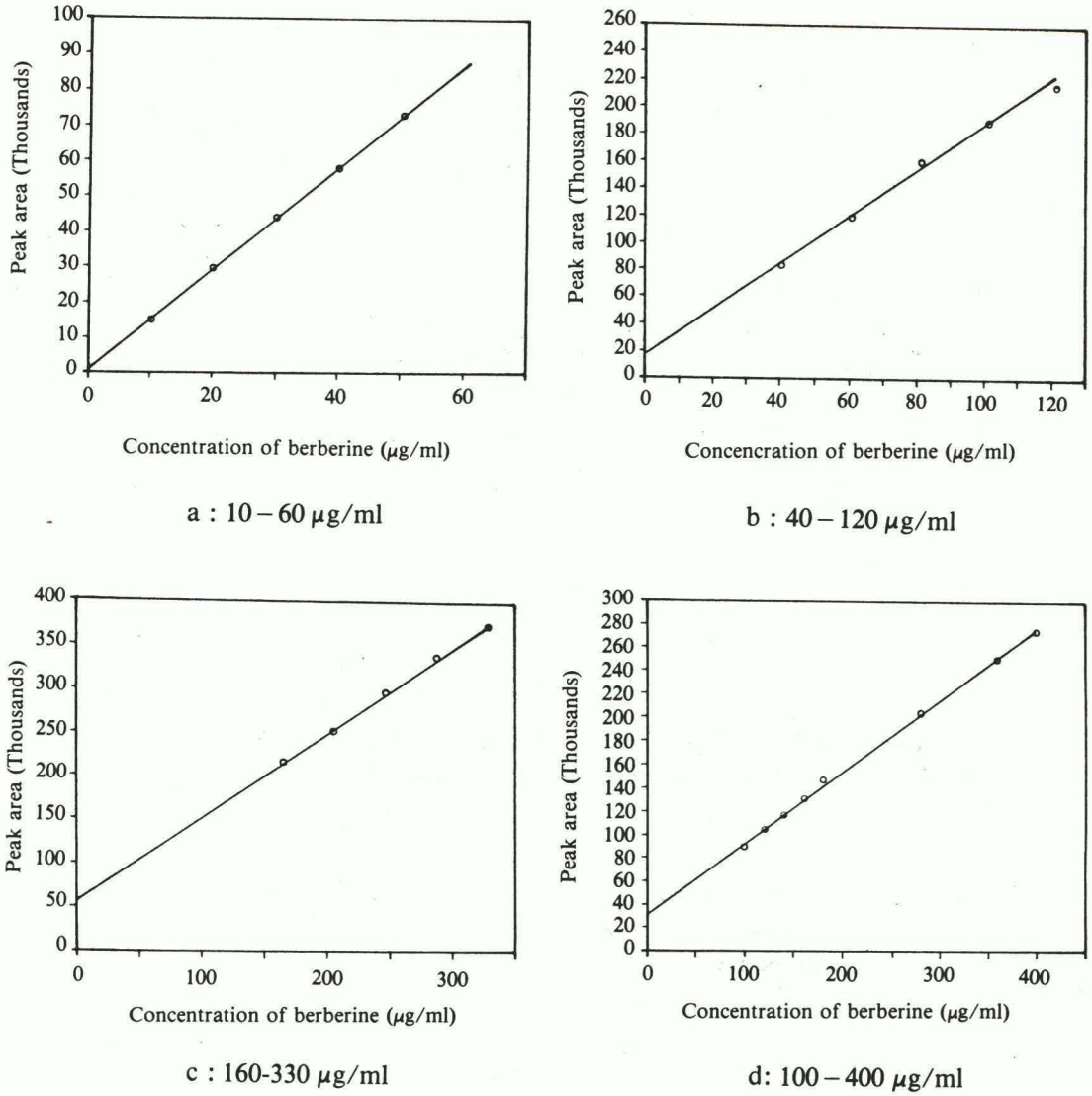

Fig. 4 Calibration curve of berberine chloride in different concentrations, determined by absorbance measurement

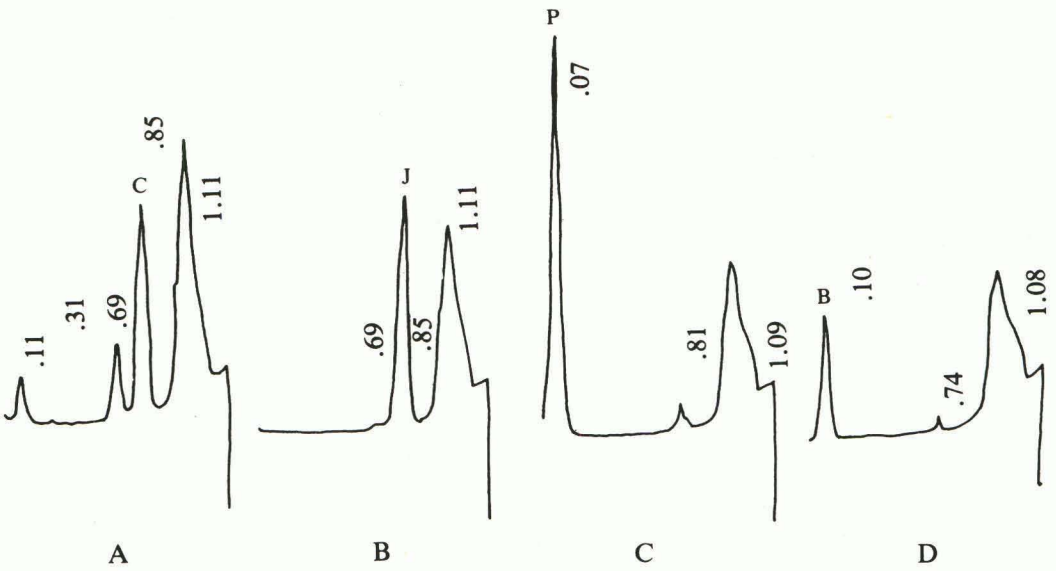

Fig. 5 Chromatograms of standard alkaloids in methanol developed with methanol : water : conc NH_3 (8:1:1) and determined by TLC scanner at absorbtion wavelength 254 nm

A : Columbamine
B : Jatrorrhizine
C : Palmatine
D : Berberine

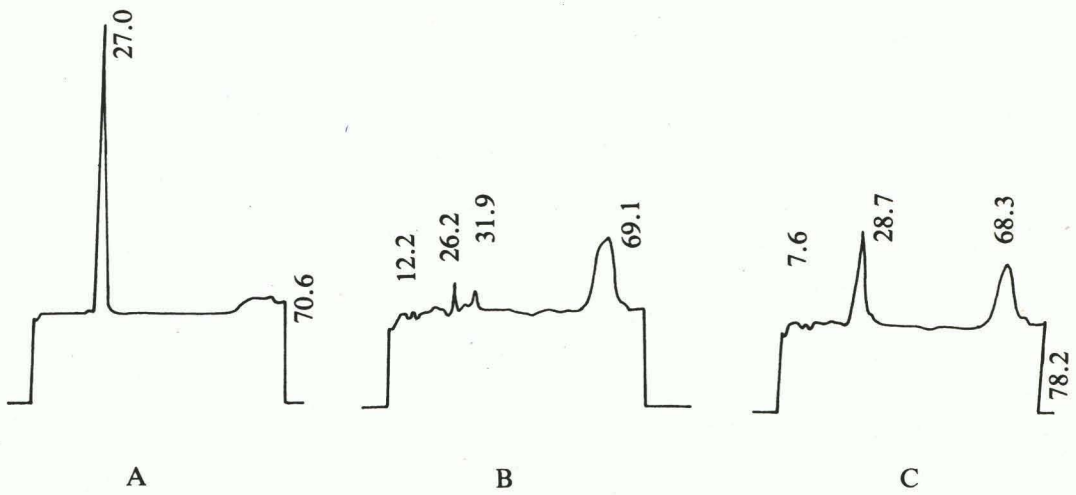

Fig. 6 Chromatograms of methanol extract of leaves and berberine, chromatographed on silica gel 60G plate with concentration zone and developed with ethylacetate : isopropanol : conc NH_3 (9:8:3) and determined by TLC scanner at excitation wavelength of 254 nm

A : Berberine

B : Methanol extract of leaves

C : Methanol extract of leaves + berberine

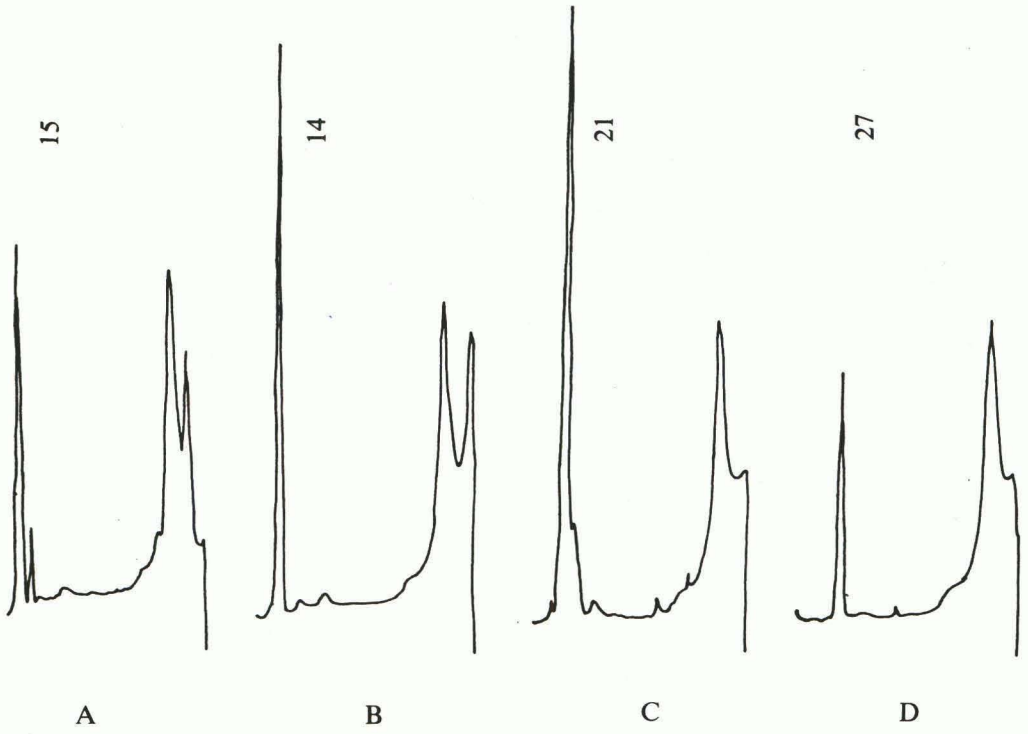

Fig. 7 Chromatograms of standard alkaloids in methanol, developed with ethylacetate : isopropanol : conc NH_3 (9:8:3) and determined by TLC scanner using excitation wavelength at 254 nm

A : Columbamine

B : Jatrorrhizine

C : Palmatine

D : Berberine

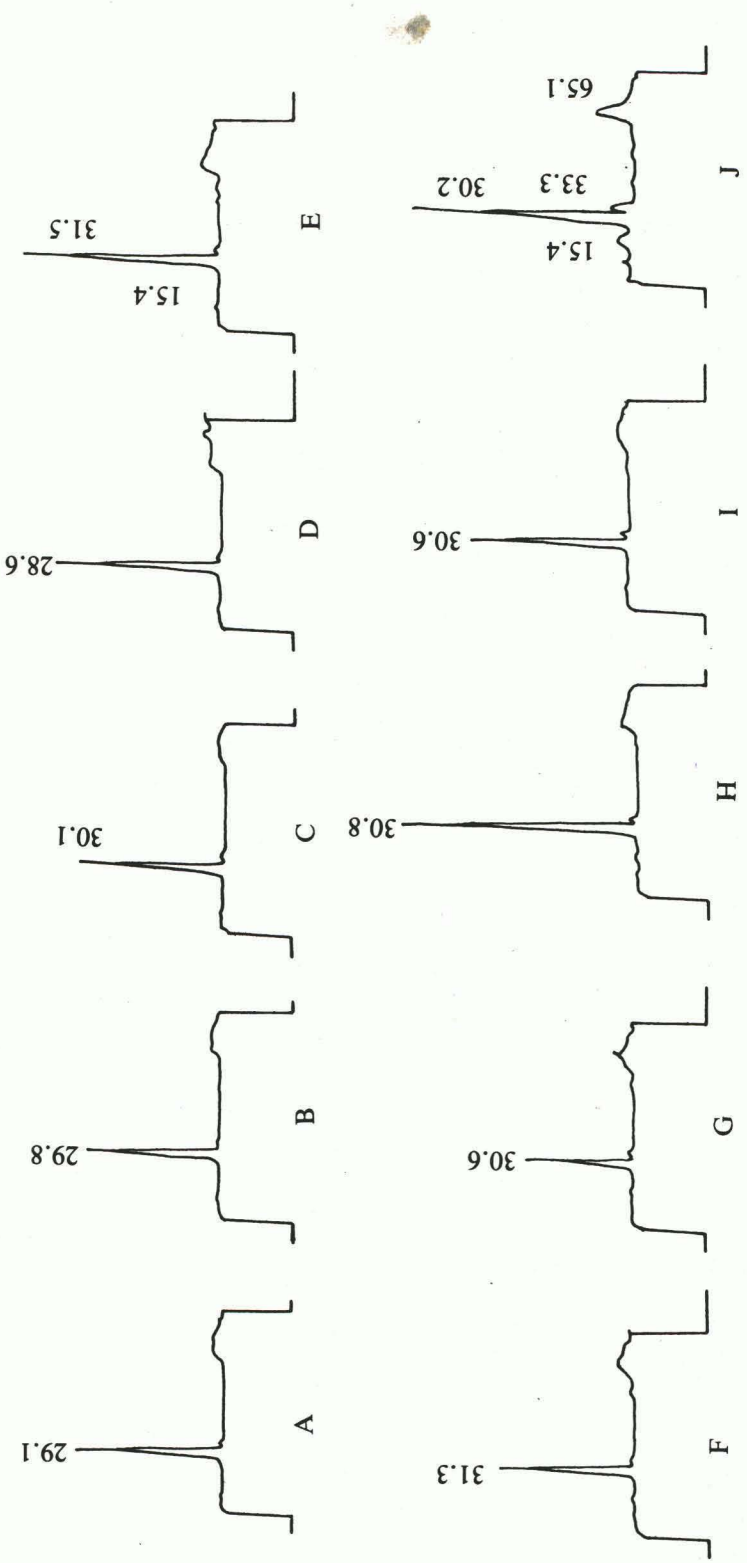

Fig. 8 Chromatograms of *Arcangelisia flava* callus grown on solid RT medium, supplemented with 2 mg/l NAA, 2 mg/l IBA and 1 mg/l kinetin ($N_2I_2K_1$) plus 10% coconut milk and various manipulating agents

A,B,C : Callus grown on medium plus 89.2 mg/l manganese sulfate generation 1,2,3 respectively

D,E,F : Callus grown on medium plus 400 μM aluminium sulfate generation 1,2,3 respectively

G,H,I, : Callus grown on medium plus 0.1% tyrosine generation 1,2,3 respectively

J : Callus grown on medium plus 0.025% colchicine, without 10% coconut milk