Chromatographic study of marmesin and visnagin occurrence in *Ammi visnaga* Lam. suspension tissue cultures

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Abstract

Chromatographic examination of tissue from suspension cultures of *A. visnaga* proved their ability to biosynthesis of furanochromone-visnagin and furanocoumarin-marmesin. The occurrence of these two compounds depends on the composition of medium which also influences culture growth and embryogenesis, after subculture for at least 1 year.

INTRODUCTION

Fruits of the mediterranean plant *Ammi visnaga* Lam. (*Umbelliferae*) contain furochromons (Späth, Gruber, 1938, 1941; Hörhammer, Wagner, 1962; Fantl; Salem 1930; Smith, Hosansky, Bywater, 1956). The most interesting of them are khellin and visnagin, especially the first one which exhibits a spasmolytic action on the muscles of urinary ducts, intestines and bronchi (Uhlenbrock, Mulli, 1957; Samaan, 1931; Fahmy, El Keiy, 1931). It is considered an effective drug in the therapy of cardiac asthma (Uhlenbrock, Mulli, Schmidt 1953 a, b; Best, Coe, 1950).

Coumarins of the seselin group which have been identified in this plant (Karawya, Sina, Nour, 1969; Karawya, Nour, Sina, 1971; Hörhammer, Wagner, 1962; Smith, Hosansky, Bywater, 1956, Bencze, Schmid, 1954), among them visnadin, samidin and dihydrosamidin show a more effective action on heart than khellin and papaverine hydrochloride.

The content of these coumarins in *A. visnaga* has stimulated interest for "in vitro" tissue culture of this species in which biosynthesis of

visnagin has been established (Kaul, Staba 1965, 1967; Chen, Stohs, Staba, 1969).

Ammi visnaga cultivated in our experimental garden of medicinal plants showed a high khellin and visnagin content (Supniewska, 1954, 1957). Therefore tissue culture of this plant was undertaken with the aim to study some metabolite biosynthesis in cells cultivated *in vitro*.

MATERIAL AND METHODS

Fruits of *Ammi visnaga* were sterilised with 2% calcium hypochlorite for 30 min and then aseptically germinated on Petri dishes on moistened filter paper. Germination in field conditions is very long. In our experiment attempts were made to shorten the germination period by using variable temperature i.e. 4–6°C and/or 26–30°C at intervals of 24 and 48 hrs, respectively. The seeds sprouted in 4–28 days.

These germinating seeds were aseptically transferred into test tubes according to Heller (1949) on the diluted (1:1) Knop's nutrient medium. They were kept in an isolated compartment of the greenhouse under natural light conditions at ca 25°C (day) and 15°C (night). After 3–4 leaves had developed, the explants of roots, stems and leaves were taken and inoculated on solid media in test tubes.

The following nutrient media were used: 1) Murashige's medium modified by Lin and Staba (1961), enriched with 0.5 mg/l of kinetin and 6 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D), 2) Wood's medium (Harris, Nylund, Carew 1964) with addition of 0.5 mg/l of kinetin and 2 mg/l of naphthaleneacetic acid (NAA).

Callus tissues were obtained from all organs. The best developed were those from roots and stem basis explants of seedlings immediately placed on agar solid media. After some passages on Wood's medium, development of numerous foliated shoots in some test tubes (ca 15%) was observed (Plate Ia).

After 4–5 subcultures (in the course of ca 4 months) tissue cultures were propagated in Petri dishes on U-shaped glass tubes covered with filter paper partly immersed into liquid medium, the same as that used in the solid media.

Observation during 1 year as well as chromatographic examinations of extracts obtained from the cultivated tissues were the basis for the proper choice of nutrient medium for suspension cultures.

The well developed callus tissues were used as starting material for establishing these cultures.

a) Aerated cultures were run in a glass apparatus (300 ml) with ste-

rile air flow through a sintered glass filter. The apparatus was made in our laboratory for cultivation of the small tissue amounts (Plate Ib).

The callus tissues were inoculated on 200 ml of Wood's medium with diminished NAA content (0.1 mg/l). The weight of inoculum was 12—18 g and/or 7—9 g. The cultures were kept at 26—28°C in a thermostat equipped with a "White" 40 W fluorescent lamp 5—61 giving 800—1000 lux light. The cultures were grown for 17 or 31 days. They were then filtered off and washed repeatedly with distilled water, dried at 55—60°C and ground in an electric mill.

b) Agitated suspension cultures were obtained by inoculating 12—18 g of callus on 250 ml of medium in 1-l Erlenmayer flasks. Two kinds of media were used: 1) Wood's medium (see above) and 2) Linsmaier-Skoog's medium with addition of 0.5 mg/l 2,4-D instead of indoleacetic acid (IAA) and 0.2 mg/l of kinetin. The Erlenmayer flasks were shaken at 75 strokes/min and kept in the thermostat under the same conditions as the aerated cultures. After 33 and 72 days the suspended biomass was filtered off and treated like the material obtained by the aeration method.

Extracts from the material obtained by cultivation were prepared by following methods:

a) after Karawya, Nour, Sina (1971): 2.5 g of powdered raw material was placed on 5 g acid alumina in a Soxhlet apparatus and extracted exhaustively with chloroform for 8hr until the absence of coumarins and chromones was confirmed. For this purpose thinlayer chromatography (TLC) was used. The fluorescence of the developed chromatograms was analysed under UV light. The concentrated chloroform extract (5 ml) was placed on the column (Ø1.5 cm) filled with ca 22 g acid alumina (activity II according to Brockmann) and eluted with ca 100 ml of chloroform-methanol (95:5) mixture under UV light. The two first chlorophyll-containing fractions were discarded and the next nine fractions were collected according to their colours.

b) after Balbaa, Zaki, Abdel-Wahab (1968). The powdered material (10 g) was exhaustively refluxed with ethanol. The concentrated crude extract was placed on the top of the column and eluted as above.

c) after Adamska, Kaczmarek (1974): 10 g of the callus material was percolated with chloroform (200 ml) on a column (Ø 1.6 cm) filled with 22 g of acid alumina (activity II according to Brockmann). Five fractions of 40 ml were collected. Then a mixture of chloroform-methanol (90:10) was used as developing solvent system (Karawya, Nour, Sina, 1971; Karawya, Sina, Nour, 1969).

All extracts were tested by thinlayer chromatography (TLC) on Silica gel G Merck, 0.25-mm layers and 17-cm distance.

The following developing systems were used: I) Chloroform-methanol 98:2 v/v (Karawya, Nour, Sina 1971); II) benzene-acetone

90:10 v/v (Stahl 1967); III) ethyl acetate-toluene-water 50:75:50 v/v/v (Balbaa, Hilal, Haggag 1973).

The amounts of chromatographed extracts from callus tissues and fruits were the same. Both were obtained in the same way. They were comparatively chromatographed to evaluate their chemical composition. Thus, standard substances were chromatographed i.e.: khellin and visnagin (author's own standard, Supniewska 1957), khellol glycoside and visnagin (Karawya Cairo), umbelliferone (Fluka A. G.), marmesin (Kohlmünzer Cracow). These substances were also spotted together with the extract investigated as a single spot (single spot chromatography).

The developed chromatograms were observed under UV light before and after treatment with a) 10% potassium hydroxide in methanol (Hörhammer, Wagner 1962), b) 15% antimonium trichloride c) Dragendorff's reagent (Hörhammer, Wagner 1962; Stahl, Schorn, 1961).

RESULTS

Preliminary observations of stationary callus tissue cultures on agar nutrient media or liquid one in Petri dishes showed that Wood's medium was the most suitable and gave the best growth rates. Callus tissues cultured on this medium were vigorous and green, while those growing on modified by Lin and Staba Murashige's medium were weaker and gradually turned brown. Therefore Murashige's medium (as above) was omitted and instead of it Linsmayer-Skoog's medium which has a higher level of mineral salts was introduced for suspension cultures.

In aerated cultures on Wood's medium a great increase of green biomass in the form of cell aggregates in 2-week-old cultures was visible. They were attached to the fermentor walls above the medium level and more loosely suspended in the liquid (Plate Ib).

In Table 1 the average results of growth rates of 17- and 31-day-old aerated cultures are presented. It was found that most suitable was the use of over 15 g of inoculum, giving better daily increases of the biomass and higher growth rates. When smaller amounts of callus were used (under 10 g/200 ml of medium), the growth rates were distinctly lower and even prolongation of the cultures to 31 days gave no better results.

A different result was obtained in experiments performed with agitated cultures (Table 2). In Linsmaier-Skoog's medium only a slight increase of growth was observed, after 20 days a limited increase occurred and after 33 days the growth efficiency index was 3.70. The prolonged cultures (72 days) showed a very limited increases of

PLATE I

a — Plant grown up from callus on agar medium; *b* — Set for aerated suspension culture; *c* — Agitated culture showing embryogenesis in callus tissue; *d* — The same enlarged with inceptions of bundles; *e* — Embryo from agitated culture

growth (efficiency index 3.8), even if inoculated with a high amount of callus (15-17 g).

The enhanced growth in agitated cultures on Wood's medium was better (Table 2), but not as good as in aerated cultures. The growth efficiency index after 33 days in these conditions was 5.80. In three

Table 1

Aerated suspension tissue cultures

in 200 ml of Woods medium supplemented with 0.1 mg/l NAA and 0.5 mg/l kinetin

Weight of tissue, g		Period of growth days	Daily increase, g	Growth efficiency
initial	final			
16.57±1.00	78.71±1.41	17	3.56±0.088	17.81±1.41
8.95±1.32	60.31±4.69	31	1.65±0.016	8.30±0.62

Weights are mean-values calculated from 10 cultures.

The increment of tissue was measured in growth-efficiency units according to the formula: $\frac{W_f - W_i}{t \cdot l}$, W_f = final weight, W_i = initial weight, t = period of growth in days, l = liters of medium.

flasks a mass of compact uniformly suspended clusters with white striplings were observed. Microscopical examination showed advanced organogenesis. Initials of vessels as well as formed embryos with quite long roots were visible (Plate Ic-e).

Table 2

Agitated suspension tissue cultures

Medium	Weight of tissue, g		Period of growth, days	Daily increase, g	Growth efficiency
	initial	final			
A) Linsmaier-Skoog	13.7±1.00	38.0±2.26	33	0.75±0.07	3.70±0.37
	16.2±0.63	72.5±7.80	72	0.76±0.10	3.80±0.57
B) Wood	17.1±1.06	55.5±6.78	33	1.16±0.21	5.80±1.02

Weights are mean-values calculated from: A) 10 cultures, B) 8 cultures. Growth efficiency was measured as in Table 1.

In phytochemical investigations the most useful method was exhaustive extraction method according to Karawya et al. (1971). Fig. 1 shows chromatograms of extracts obtained by this method in three different solvent systems. After discarding the first chlorophyll fractions 1 and 2, following fractions were chromatographed parallelly with analogous fractions obtained from fruits and authentic substances. Fractions 8 and 9 contained only traces of the compounds under investigation.

The presence of visnagin in fractions 5-7 was established by comparative TLC with authentic substance on the basis of:

a) fluorescence in UV light (yellow-greenish changing to yellow-orange after spraying with SbCl_3 or KOH), b) R_f values (0.54, 0.14, 0.28 — in solvent systems I, II, III, respectively) confirmed by cochromatography.

Fig. 1. TLC chromatograms of chloroform extracts developed in 3 solvent systems I — all developed spots under UV light, II and III — only the spots of searched compounds;

A — extract from fruits of *A. visnaga*, B — from tissue cultures, C — standards

b — blue, lb — light-blue, br — brown, bry — brown-yellow y — yellow, ly, light-yellow, yg — yellow-green, v — violet

1 — khellol-glycoside, 2 — umbelliferone, 3 — marmesin, 4 — visnagin, 5 — khellin, 6 — visnadin

Furochromone khellin was not found contrary as in fruit extracts in which it was detected in fractions 4-7.

A spot with R_f values 0.45, 0.14, 0.35 in I, II and III systems with violet fluorescence in UV light was visible in fractions 3-6. After spraying with SbCl_3 it was not changed but after treatment it becomes turquoise-greenish. This chromatographic mobility and reactions as well as cochromatography with furocoumarin-marmesin showed the identity of both substances.

A further spot had R_f values of 0.78, 0.83 and 0.77 in I, II and III systems with light blue fluorescence before and after spraying with SbCl_3 , which became stronger after KOH treatment what is cha-

racteristic for some coumarins of the seselin group (Hörhammer, Wagner, 1962; Karawya, Sina, Nour, 1969; Karawya, Nour, Sina, 1971). However, this spot was not ideotical with that of visnadin (the only substance of this type which was available as a standard), in view of its different chromatographic behavior: lower R_f values, violet fluorescence in UV light and separate spots in single-spot chromatography.

DISCUSSION

It is well known that great changes concerning metabolite biosynthesis occur in tissue cultured *in vitro* as compared with intact plants. Our experiments concern mainly suspension cultures. In these cultures biosynthesis of visnagin depended on the general medium composition. Visnagin was absent in slowly growing callus tissues on Linsmaier-Skoog's medium. The same was true for marmesin biosynthesis. It can be assumed that these cultures growing for a few weeks only were not able to accomodate to the changed nutrient conditions, i.e. higher mineral salts content, auxin and sugar concentration changes.

In tissues growing of Wood's medium the presence of visnagin and marmesin was established, especially in those with apparent embryogenesis. Nevertheless, the chromatographic spots obtained from extracts of these callus tissues were less pronounced, than those obtained from fruit extracts. It indicates that the content of these compounds in tissue cultures was decreased in comparison with fruits.

Uhlenbrock, Mulli, Schmidt (1953 a, b) claimed hypothetically that khellin can be regarded as a catalyser of biochemical processes because its maximal content was found in gynaeceum and unripe fruits. This fact may be attributed to the role of kinetin in organogenesis. The author's own studies have confirmed the highest concentration of khellin in the above mentioned organs (Supniewska, 1957). However, tissue cultures in which the biosynthesis of primary metabolites predominates are not able to produce greater amounts of this compound.

Coumarins are natural plant constituents and have been often produced by tissue cultures *in vitro*, e.g. scopoletin is produced by tissues of tobacco (Fritig, Hirth, Ourisson, 1970), psoralen, bergapten, xanthotoxin, umbelliferone are biosynthetized by *Ruta graveolens* tissue cultures (Reinhard, Corduan, Volk, 1968).

It has been proved that umbelliferone and 7-demethylsuberosin are the precursors of linear furocoumarins of psolaren type and marmesin is a further intermediate in this biosynthesis (Austin, Brown, 1973; Dall'Acqua, Innocenti, Caporale, 1975). In our cultures no umbelliferone has been found (Fig. 1, B). It is a normal component of

Ammi visnaga fruits (Crowden, Harborne, Heywood, 1969), and it was also confirmed in fruits by our chromatographic analyses (Fig. 1A). This would suggest that *in vitro* this compound is quickly converted to marmesin.

Reinhard, Corduan and Volk (1968) reported that coumarin biosynthesis *in vitro* can be achieved by addition of auxin such as 2,4-D or NAA. Our results have not confirmed this observation. It may be assumed that in our experiments lower concentrations of auxins were used, below their stimulating effect on coumarin biosynthesis (NAA 10^{-7} g/ml and 2,4-D 5×10^{-7} g/ml, while the mentioned authors used 10^{-6} M/l). However, differences in the action of the substances were observed: in tissues grown on Wood's medium (with NAA content) visnagin and marmesin have been found, especially when embryogenesis occurred, whereas in cultures on Linsmaier-Skoog's medium (containing 2,4-D) they were absent in spite of a 5-fold auxin dosis.

It seems that the biosynthesis of these metabolites *in vitro* depends not only on concentration but also on the type of auxin used as well as on the general medium composition and tissue organisation.

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*Chromatograficzne badania występowania marmezyny i wisnaginy
w hodowlach zawiesinowych Ammi visnaga Lam.*

Streszczenie

Otrzymano i prowadzono hodowle zawiesinowe *A. visnaga* na pożywkach Wooda i Linsmaiera-Skooga w dwojaki sposób, przez wytrząsanie lub przez areację sterylным powietrzem. Lepszy przyrost masy tkankowej wykazywały hodowle na pożywce Wooda, osiągając najwyższe wartości w warunkach hodowli aerowanej.

Odsączoną od pożywki, wysuszoną tkankę ekstrahowano chloroformem, a następnie oczyszczano na kolumnie z kwaśnego tlenku glinu. Zebrane frakcje badano chromatograficznie (TLC) w 3 układach rozwijających.

W żadnej z prowadzonych hodowli nie stwierdzono furanochromonu-keliny, która prawdopodobnie wiąże się z powstawaniem słupków a później owoców.

W tkankach z pożywki Wooda znaleziono furanochromon — wisnaginę i furanokumarynę — marmezynę, przy czym większe ich ilości wystąpiły w hodowlach wykazujących daleko posuniętą organogenezę. W tkankach z pożywki Linsmaiera-Skooga, prowadzonych w krótszym okresie czasu (poniżej 1 roku), nie wykryto wisnaginy ani marmezyny, co wskazuje na zależność biosyntezy tych związków od składu pożywki i okresu adaptacji na niej.