Cell suspension culture of *Amsonia tabernaemontana* Walter: growth, organogenesis and alkaloid production

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Abstract

The paper discusses the growth of cell suspension cultures of *Amsonia tabernaemontana* Walter established from callus of hypocotyl origin. The cell number and growth increment were determined. Cellular aggregates developed well in the Wood and Braun (WB) medium with 1 mg/l NAA and 0.5 mg/l kinetin (growth increment 712.4). When the aggregates were cultured on WB media without NAA and kinetin or with 0.02 mg/l kinetin and 3 mg/l IAA, roots developed on the aggregates. Examination of the roots and cell suspensions indicates that the roots are richer in alkaloids than the callus and cell suspensions.

INTRODUCTION

Amsonia tabernaemontana Walter (Apocynaceae) is a North American perennial, cultivated in Poland in botanical gardens. The Apocynaceae family is rich in alkaloid-containing species, some of the members such as *Catharanthus* G. Don and *Rauwolfia* L. are well known for their medicinal properties.

Many alkaloids have been isolated from the roots, leaves, flowers and seeds of *Amsonia tabernaemontana* Walter. Tabersonine, the main seed alkaloid is a precursor of vindoline which is a part of vinblastine and vincristine, the dimeric indole alkaloids isolated from *Catharanthus roseus* (L.) G. Don and used as an antineoplastic agent (Kohlmeier 1980).

So far, little work has been done on the production of secondary metabolites in tissue culture of the *Amsonia* genus. The first studies of *Amsonia tabernaemontana* Walter in tissue culture were undertaken by

Furmanowa and Rapczewska (1973, 1977) and Rapczewska (1978). They dealt with callus cultures and alkaloid contents.

The aim of the present study was to establish cell suspension cultures of the same species and to determine the conditions under which alkaloid can be formed.

MATERIAL AND METHODS

The methods used in the culture of the callus and the conditions under which these cultures can be induced were described by Furmanowa and Rapczewska (1977).

CELL SUSPENSION CULTURE

The suspension culture of *Amsonia tabernaemontana* Walter was initiated from the 2nd to 6th passage of the callus of hypocotyl origin by transferring 2 to 3 g of the callus to 60 ml liquid medium contained in

Table 1

Media used for cell suspension culture of *Amsonia tabernaemontana* Walter

Medium	Number of callus passage used for suspension	Kinetin mg/l	NAA mg/l	2,4 D mg/l	IAA mg/l	Alkaloid precursor	Growth increment (fresh weight, g)	Microscopical observation
MS	2-3	0.1	—	2.0	—	—	282.5	cells in aggregates
„	2-3	0.1	—	1.0	—	—	305.6	„
„	2-3	0.1	—	0.5	—	—	261.8	„
„	2-3	0.1	—	—	1.0	—	154.1	„
„	5-6	0.1	—	—	1.0	—	106.9	„
WB	2-3	0.5	1.0	—	—	—	712.4	„
„	5-6	0.5	1.0	—	—	—	405.3	„
„	5-6	0.1	1.0	—	—	—	314.0	„
„	5-6	0.5	1.0	—	—	geraniol	462.8	„
„	5-6	0.5	1.0	—	—	50.0	408.1	„
„	2-3	0.5	1.0	—	—	geraniol	781.5	„
„	2-3	0.5	1.0	—	—	50.0	286.5	„
„	2-3	0.5	1.0	—	—	tryptamine	310.0	„
„	2-3	0.5	1.0	—	—	20.0	250.0	„
„	2-3	—	—	—	—	tryptophan	250.0	„
„	2-3	—	—	—	—	20.0	250.0	centres of meristematic activity in aggregates

a 250-ml Erlenmeyer flask. The variously modified media of Wood and Braun (WB) (1961) and Murashige and Skoog (MS) (1962) were used. Some of them are shown in Table 1. To stimulate the production of alkaloids, precursors such as tryptamine, tryptophan and geraniol were added. The flasks were incubated on a horizontal rotary shaker at 120 r.p.m. at 250° C under continuous fluorescent light (2000 lux). The suspension culture was subcultured every 4 weeks by transfer of 10 ml suspension with the use of a pipetting unit fitted with a cannula of 3 mm bore. After that period the culture was in a stationary phase.

The cell number per 1 ml was determined by the techniques of Henshaw et al. (1966).

Growth increment was calculated by the method described by Staba (1969). The following formula was used:

$$\frac{100 (\text{final weight} - \text{initial weight})}{\text{initial weight}}$$

The aggregates were fixed in acetic ethanol (glacial acetic acid: absolute ethanol — 1:3), dehydrated, clarified through an ethanol-xylol series and embedded in paraffin. Microtome sections were then stained in 1% ethanol solution of safranin (Konar et al. 1972).

EXCISED ROOT CULTURES

Four to five days old seedlings were transferred to a 250-ml Erlenmeyer flask containing 50 ml Street and McGregor (1952) liquid medium, supplemented with 200 mg/l casein hydrolysate. After two weeks 20-mm root tips were excised and cultured in this medium. The roots were subcultured after 4 weeks incubation at 24° C. During that time the main axis produced slowly growing laterals. The continuous growth of roots was maintained for two years. During that time only 20-mm root tips were cut off and transferred to fresh culture medium. The remaining parts of the roots were used for chemical investigations.

EXAMINATION OF CULTURES FOR PRESENCE OF ALKALOIDS

The alkaloid content was investigated in: the cell suspension after 4 weeks of growth, liquid culture medium, roots initiated from cell aggregates, roots of seedling origin, roots from intact plants (Warsaw University Botanical Garden), seeds and seedlings. Alkaloid extraction was done by the method described by Furmanowa and Ołędzka (1980). For thin-layer chromatography glass plates (200×200 mm) were covered with silica gel G (Merck) and preactivated at 105° C for 1 h.

The alkaloids reserpine, ajmalicine, tetrahydroalstonine, yohimbine, tabersonine, eburnamonine, vincadiforimine, vincadine, quebrachamine, dehydroaspidospermidine were used as reference compounds. All of them are tertiary bases. The samples were applied with a 10 μ l pipette in a 0.1% solution. The following solvents were chosen for the chromatograms:

1. n-butanol:glacial acetic acid:distilled water (4:1:5, v/v; 4:1:1, v/v; 6:1:3, v/v),
2. ethyl acetate:absolute ethanol (3:1, v/v),
3. methanol.

The distance between the solvent front and the point of sample application was approximately 100 mm. The alkaloids were chromatographed as the hydrochloride salts. A modified Dragendorff's reagent (Linskens 1959) and ceric ammonium sulphate (CAS) reagent were used for detection of the alkaloids.

The CAS reagent is very useful for chromogenic differentiation of certain *Catharanthus* alkaloids. It was prepared according to Farnsworth et al. (1964), who described eight chromogenic classes, when using the reagent, according to the colour reaction of the *Catharanthus* alkaloids. They are: Class I — red, Class II — orange, Class III — yellow, Class IV — green, Class V — blue, Class VI — gray, Class VII — purple and Class VIII — no chromogenic reaction. Since one class was not detected with the CAS reagent each plate in our study was sprayed with modified Dragendorff's reagent twenty four hours after the initial CAS treatment. Previous application of the CAS reagent did not interfere with the application of Dragendorff's reagent. According to Farnsworth et al. (1964), chromogenic reactions were observed immediately, after five minutes, fifteen minutes, one hour and twenty four hours.

Cochromatography was also applied. The TLC analyses allowed qualitative determination of each alkaloid extract (Table 2).

RESULTS AND DISCUSSION

GROWTH OF CELLS IN SUSPENSION AND ITS ORGANOGENESIS

The batch suspension cultures of *Amsonia tabernaemontana* consisting of cells and cell aggregates were maintained for three years (twenty five passages). The studies, showed that the growth was dependent on the composition of the medium, the age of the callus from which the suspension culture was initiated and the age of the suspension culture (number of passages). WB medium was better for suspension culture than MS medium. The growth increment on WB medium was approximately twice higher than on MS medium.

The experiments with callus indicated that the callus from the 2nd and 3rd passage was best for initiation of suspension culture. A suspension initiated from older callus passages, i.e. the 5th, 6th to 10th developed slower than normally and contained very few aggregates.

The suspension which was maintained for 2 further passages on the WB medium with 0.5 mg/l kinetin and 1 mg/l NAA was used to establish experimental cultures in various media. These suspensions were at first finely dispersed but after several passages they contained many pale yellow aggregates. The growth increment counted after three weeks of incubation showed that the best growth of suspension was achieved in WB medium with 1 mg/l NAA, 0.5 mg/l kinetin and 50 mg/l geraniol (growth increment 781.5). When the total cell number in suspension culture was plotted against the incubation time a lag phase, a growth phase and a stationary phase could be distinguished in the sequence.

The lag phase refers to the initial period of a batch culture when no cell division is apparent. The stationary phase is the terminal phase of the batch culture growth cycle where no synthesis of biomass or increase in cell number is apparent. The growth phase consists of a phase of acceleration (exponential), a linear phase when the maximum rate is observed and a progressive deceleration when the growth rate subsides rapidly (Street 1973).

With geraniol the lag phase is short (2 to 3 days), the linear phase is between the 4th to 9th day, but the progressive deceleration phase is prolonged to the 16th day of culture. After this time the cells remain in the stationary phase (Fig. 1).

The microscopical observations of cell suspension during one passage (21 days) indicated certain changes in the cell shapes. The cells from

Fig. 1. Growth curve for the suspension culture of *Amsonia tabernaemontana* Walter in the WB medium with kinetin (0.5 mg/l), NAA (1 mg/l), geraniol (100 mg/l and 50 mg/l)

the stationary phase used to initiate the culture are elongated and single rather than grouped in aggregates. They have not much cytoplasm (Fig. 2). A marked increase of cytoplasm is typical of the cells at the end of the lag phase. The cells in the growth phase are small and spherical and collected in aggregates (Fig. 3).

When the aggregates were transferred to a WB medium without NAA and kinetin some of the aggregates formed roots (Fig. 4). Sections through the cell aggregates show centres of meristematic activity from which roots arise (Fig. 5, 6). After 6 weeks of incubation the roots were 2 cm long. Root-bearing aggregates were observed only in suspension culture in their 2nd to 6th passages.

If a callus subculture was delayed, the resulting suspensions were more highly aggregated and showed a higher level of organogenesis than when initiated from the callus earlier in the incubation period (the incubation period of the callus was 4 weeks).

The results presented in Table 1 indicate that the growth increment of the cell suspension culture was also dependent upon the age of the suspension culture. The best results were obtained in the 2nd and 3rd passages of the suspension culture. In the subsequent passages the growth increment was decreased (Table 1).

CHROMATOGRAPHIC INVESTIGATIONS

The phytochemical analyses carried out by TLC indicated, that the suspension culture with the highest growth increment contained no alkaloids. The results obtained with the suspension culture line are in accordance with our first experiments which concerned alkaloid production in the callus of *Amsonia tabernaemontana* (Furmanowa and Rapczewska 1977). The best growth of the callus was achieved in WB medium with geraniol in a concentration of 50 mg/l, but alkaloids were absent there.

In the present study the best growth of the suspension culture was observed in the WB medium with 0.5 mg/l kinetin, 1 mg/l NAA and 50 mg/l geraniol but alkaloids were not found there. In this case our observations agree with the results of Deus (1978) who showed that addition of 50 mg/l geraniol to a suspension culture of *Catharanthus roseus* increased the cell yield, but at the same time the serpentine production was reduced by 50%.

Only one spot ($R_f = 0.55$) was given by the cell suspension growing in:

1. WB medium with 0.5 mg/l kinetin and 1 mg/l NAA,
2. WB medium with 20 mg/l tryptamine,
3. WB medium with 20 mg/l tryptophan,
4. WB medium without NAA and kinetin.

Fig. 2. Stationary phase cells used for initiating a new passage of culture, $\times 300$

Fig. 3. Growth phase cells showing aggregates, $\times 300$

Fig. 4. Root-bearing aggregates from suspension cultures in the WB medium with 1 mg/l NAA and 0.5 mg/l kinetin after 25-day incubation (natural size)

Fig. 5. Cross-section through a cell aggregate showing the centre of meristematic activity from which roots arise, $\times 100$

Fig. 6. Root primordium developed from the centre of meristematic activity (longitudinal section), $\times 100$

Table 2

Distribution of alkaloids in *Amsonia tabernaemontana* Walter cultured *in vitro* and *in vivo*

Plant material	Rf value and colour reaction of examined alkaloids (n-butanol: glacial acetic acid: water — 6: 1: 3)																			
	0.60 Res.	0.57 Ajm.	0.56 Thalst	0.55	0.54 Yoh.	0.53 Tab.	0.52 Eb.	0.51	0.51	0.50	0.49 Vinf.	0.48 Vinc.	0.47	0.46	0.45 Queb.	0.43	0.39	0.38	0.37	0.35 Dhasp.
	Yell.	Yell.	Yell.		Yell.	Blue					Blue	Pink			Pink with grey centre					Red
Roots of aggregate origin in liquid medium	—	—	—	+	—	—	—	—	—	+	blue	—	—	—	—	+	blue	—	—	—
Roots of seedling origin in liquid medium	—	—	—	+	—	—	—	—	++ blue with yell. centre	—	—	—	—	—	—	+	blue	—	—	—
Roots from intact plant	—	—	—	—	—	—	—	+	—	—	—	—	+	—	—	+	—	+	—	+
Seedlings	—	—	—	—	—	++ blue	—	—	—	++	—	—	—	—	—	+	—	—	++ blue	—
Seeds	—	—	—	—	—	++ blue	—	—	—	—	—	—	—	++	—	—	—	—	+	—

Explanations: Res. — Reserpine; Ajm. — Ajmalicine; Thalst. — Tetrahydroalstonine; Yoh. — Yohimbine; Tab. — Tabersonine; Eb. — Eburnamonine; Vinf. — Vincadiformine; Vinc. — Vincadine; Queb. — Quebrachamine; Dhasp. — Dehydroaspidospermidine

+ — small spots; ++ — bigger spots with intensive colour reaction. Observation of colour reaction after one hour

More alkaloids were detected in the roots which arose from aggregates (WB medium without NAA and kinetin). In extracts from those roots (after Dragendorff's and CAS treatments) 4 spots of alkaloids were present: one of them was yellow (Class III), one blue and one pink-blue-violet (Class V) and one without chromogenic reaction (Class VIII). One of the alkaloids which belongs to Class V was blue and did not change its colour ($R_f = 0.50$), while the other one was at first pink, after one hour blue and after 24 hours violet ($R_f = 0.39$). Essentially, our studies demonstrated that alkaloid production is not only a feature of the roots derived from seedlings, but also of the roots initiated from suspension culture poor in alkaloids. Similar results were described by Raj Bhandary et al. (1969) and West and Mika (1957) who studied alkaloid production in *Atropa belladonna* tissue culture.

In the present study thin-layer chromatograms of extracts of the roots of aggregate origin suggested that the detected alkaloids belonged to three chromogenic classes. These classes are interesting from the pharmaceutical point of view, since Class III includes among others, reserpine, Class V leurocristine (vincristine) and Class VIII serpentine and alstonine, all of them important therapeutic agents (Farnsworth et al. 1964). None of the detected spots gave R_f values corresponding to the reference alkaloids used in the investigations (Table 2).

In roots derived from intact plants 6 spots were detected on the chromatograms, that is two spots more than in those of aggregate origin. They belong to three chromogenic classes: Class I — one pink spot changes to red after one hour, Class III — two yellow spots and Class VIII three spots without chromogenic reaction. One spot with R_f value = 0.35, colour reactions after CAS (pink changes to red) and colour (grey and green) corresponding to hydroaspidospermidine.

The thin-layer chromatogram of extracts of seedlings was not identical with TLC obtained from seeds. Both these chromatograms differed from those of extracts of roots of various origin mentioned above.

Very typical of extracts from seedlings and seeds was the spot with R_f value = 0.53 (blue after CAS). That largest spot was identified as tabersonine (R_f value, colour reaction after CAS — blue and UV colour — blue and violet). To the same Class V belongs the alkaloid with $R_f = 0.37$ which was also detected in seed and seedling extracts. Cochromatography confirms the presence of both tabersonine (in seeds and seedlings) and dehydroaspidospermidine (in roots from intact plants).

Two chromatograms showed the greatest similarity: the one from roots of aggregate origin and the one from roots of seedling origin. On these two chromatograms 4 spots were detected. Three of them had the same R_f values: 0.55, 0.43, 0.39. The R_f values of the fourth spot were very similar: 0.51 and 0.50 (Table 2). They differed in colours:

one was blue with yellow centre ($R_f = 0.51$) and the other one was only blue and did not change its colour after 24 hours ($R_f = 0.50$). It seems interesting that only one spot ($R_f = 0.43$, yellow after CAS) was found to be common for the roots of aggregate origin, roots of seedling origin, roots from an intact plant and seedlings. That alkaloid was absent in the suspension culture and in seeds.

The liquid medium used for suspension culture did not contain alkaloids.

Tryptamine and tryptophan added to the cell suspension culture decreased the growth increment but they had no influence on alkaloid formation. The suspension culture grew slowly and no differentiation was observed in the aggregates. The undifferentiated cultures are in many cases much less active in alkaloid production than the intact plants. Our studies confirm this observation. Only the roots of aggregate origin gave 4 spots on the chromatogram, cell aggregates gave 1 spot, but when the growth was very vigorous they did not give a positive reaction with CAS or Dragendorff's reagent on the chromatograms. In our earlier studies (Furmanowa and Rapczewska 1977) reactive substances corresponding in R_f value to tabersonine and vincadiforine were extracted from the differentiated callus.

The present results indicate that, in tissue culture the roots derived from aggregates in the suspension culture and those of seedling origin growing in liquid medium are the best source of alkaloids. They have, however, fewer alkaloids than the excised roots from intact plants. Out of 4 spots obtained from the extracts of roots of aggregate origin and out of 6 spots obtained from the extracts of roots of intact plants only one showed the same R_f value = 0.43 (yellow after CAS reagent).

Our observations suggest that the conditions leading to the synthesis of the alkaloids of *Amsonia tabernaemontana* Walter in tissue culture are mainly achieved in association with the organization of the roots.

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Amsonia tabernaemontana Walter w kulturze zawieszinowej — wzrost, organogeneza i tworzenie się alkaloidów

Streszczenie

Zbadano wzrost hodowli zawieszinowej *Amsonia tabernaemontana* Walter w różnych pożywkach. Agregaty komórkowe najlepiej rozwijały się w pożywkach Wooda i Brauna (WB) z 1 mg/l NAA i 0,5 mg/l kinetyny (wskaźnik wzrostu 712,4).

W hodowli prowadzonej w pożywce WB pozbawionej NAA i kinetyny albo w pożywce WB z 0,02 mg/l kinetyny i 3 mg/l IAA z agregatów komórkowych wyrosły korzenie. Badania fitochemiczne wskazują, że korzenie wyrosłe z agregatów komórkowych dają na chromatogramie 4 plamy alkaloidów ($R_f = 0,55; 0,50; 0,43; 0,39$), komórki z hodowli zawieszinowej — 1 plamę ($R_f = 0,55$) a pożywki, w których kultury były prowadzone nie zawierają alkaloidów. Korzenie wyrosłe w gruncie są najbogatsze w alkaloidy (6 plam na chromatogramie). Najcenniejsze pod względem zawartości alkaloidów są, w kulturze *in vitro*, korzenie wyrosłe z agregatów komórkowych.