Adsorbent Filter – A Tool for the Selection of Plant Suspension Culture Cells Producing Secondary Substances

Bernd Knoop and Rolf Beiderbeck

Botanisches Institut der Universität Heidelberg, Im Neuenheimer Feld 360, D-6900 Heidelberg

Z. Naturforsch. **40c**, 297–300 (1985); received November 27, 1984/February 11, 1985

Suspension Culture, Selection of Strains, Adsorbent, Secondary Substances

Cells and cell aggregates from suspension cultures are plated onto selection plates, consisting of:

1. A layer of nutrient medium solidified with agar
2. A filter paper coated on the upper side with activated charcoal (AC) as an adsorbent
3. covered by a sheet of cellophane as cell carrier.

Substances produced migrate through the cellophane and are locally bound to the adsorbent layer. After transfer of the cells together with the cellophane to another dish the adsorbent filter is dried and the substances bound to the AC are eluted to the white side of the filter paper where they can be detected by means of standard methods of TLC and PC.

The substance pattern mirrors the pattern of cell material and allows to discover those cells with deviating substance production.

This method is used to recover cell aggregates of *Nicotiana tabacum* and *Matricaria chamomilla* from a mixture of both cultures.

Many plant species synthetize secondary substances which are used by man as drugs, perfume and aroma base or insecticide. Rapidly growing cell cultures of the same species usually do not produce these typical secondary substances or if they do, only in trace amounts. An economically profitable production via liquid culture is a rare exception [1, 2]. Therefore experimental efforts to increase the secondary substance production have been made in different ways:

1) by empirical design of culture media favorable for substance production [2–4];
2) by the addition of solid or liquid accumulation phases [5, 6];
3) by the isolation of cell mutants or variants with constitutive secondary substance synthesis.

The third procedure requires methods for cell mutation and selection. Whereas methods to produce mutants are published in great numbers [*e.g.* 7] the successful selection of high producing mutants has remained problematic except in the case of coloured compounds like *e.g.* anthocyanins [8].

Usually colorless products can be detected in the medium or cells only after scaling up the cultures, a time and labour consuming process. The radioimmun-assay (RIA) proposed as a general selection method is complicated, expensive and not always suitable [9].

Here we describe a method which makes it possible to select production mutants or variants among great numbers of small cell aggregates (≥ 1 mm^2) by detecting products released from the living cells to the culture medium.

The principle is to accumulate those substances produced by different cell aggregates into a thin layer of an adsorbent. After a suitable time the accumulated secondary substances are eluted from the adsorbent to a white filter paper carrying the adsorbent and detected there by means of UV or staining methods.

Material and Methods

Cell culture

A suspension culture of *Nicotiana tabacum L.* was cultured in MS-medium with 1 mg$*$l^{-1} NAA and 0.03 mg$*$l^{-1} kinetin and transferred weekly [10]. A crown-gall suspension culture of *Matricaria chamomilla L.* was subcultured weekly into hormone-free MS-medium [5].

Adsorbent filter plates

Petri dishes of 9 cm diameter are equipped with
– culture substrate: a basal layer of MS-medium with

Reprint requests to Dr. B. Knoop.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/85/0500–0297 $ 01.30/0

or without hormones (see above) solidified with 0.9% agar.

– an adsorbent filter: filter paper (no. 589 or 602eh from Schleicher & Schüll, Dassel, Germany, diameter 7 or 9 cm), coated on the upper side with activated charcoal (AC; Merck, Darmstadt, Germany) according to one of the following methods:

– – 1.2 g AC suspended in a solution of 100 mg cellulose nitrate (*e.g.* membrane filter material from Sartorius, Göttingen, Germany) in 20 ml acetone;

– – 1 g AC suspended in 4 ml prewarmed agar solution (1.5–2.5%).

About 2 ml of this AC-suspension was pipetted onto the filter paper and distributed uniformly over the surface by means of a glass rod. Then the filter was dried.

Adsorbent filters prepared in one of these ways were autoclaved and placed on the culture substrates.

– cell carrier foil: cellophane (Einmach-Cellophan, Kalle, Wiesbaden, Germany) 20 µm thick and with 7 cm diameter was autoclaved in MS-medium and placed on the adsorbent filter [11]. Air bubbles were removed.

Plating

Cell suspensions were left standing for half an hour and the resulting sediment was partially transferred to adsorbent filter plates and distributed over the cellophane by means of a Drigalsky spatula. Dishes were closed with Parafilm and incubated in near darkness at 27 °C.

Detection of secondary substance production

After the incubation the cell carrier foil and the adsorbent filter below were cut simultaneously at their margins to mark their relative position to each other. The cell carrier foil together with the cell aggregates was then transferred to another dish with MS-medium for further culture.

The adsorbent filter was dried and stretched in a metal frame. While the lower white side of the filter was blown with warm air from a hair dryer the upper side with the AC was sprayed with a mixture of ethanol/ KOH (4+1) several times [6]. This method generates a solvent stream from the upper to the lower side of the adsorbent filter eluting secondary substances from the AC and carrying them to the white back. This procedure is referred to as "development". Secondary substances can be detected on paper by means of their UV-fluorescence (365 nm).

Resolving power

A solution of umbelliferone (100 $mg*l^{-1}$) in water was applied to the AC-layer of an adsorbent filter as dots using a drawing pen (Standardgraph 55S, outer diameter 0.7 mm). The filter was dried and the umbelliferone moved to the white side of the filter as described above.

Results and Discussion

Model experiment:

Immediately after punctiform application of umbelliferone onto the AC-layer of an adsorbent filter no fluorescence could be detected on its back under UV, not even after development with water. This means that umbelliferone is completely adsorbed to the AC. Development with ethanol/KOH however moves the fluorescent dye to the paper and it can be detected by UV (Fig. 1).

A comparison between the diameters of the drawing pen used (0.7 mm) and the fluorescing spots (0.8–1.2 mm) on the white side of the adsorbent filter shows the very limited lateral diffusion of umbelliferone during development. The "headlight-effect" (broadening of the substance-"beam") amounts to about 0.5 mm at maximum and is independent of the spot diameter. As a consequence larger dots are portrayed more precisely. In this

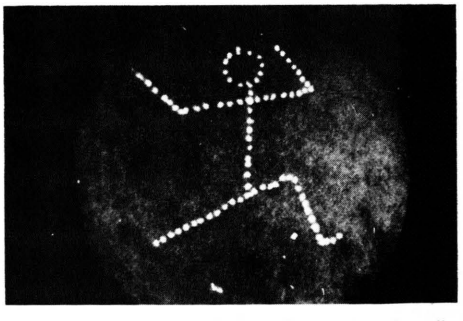

Fig. 1. Model experiment demonstrating fluorescence of umbelliferone after adsorption to and elution from an adsorbent filter.
Photo: filter back under UV 365 nm.

experiment the method resolves two neighboured spots with about 1 mm distance between their centres.

Dilution series allow the following calculation of sensitivity: If 2 µl of an umbelliferone solution are applied to form a spot of 3–4 mm diameter the limit of detection is reached at 0.2 ng of pure substance.

Detection of secondary substances produced by suspension cultures

If cells and cell aggregates of suspension cultures are plated onto the cell carrier foil of adsorbent filter plates these cells serve as sources of secondary substances. Those substances leaving the cells migrate into the AC-layer and are adsorbed as the umbelliferone in the model experiment.

Cells of two different suspension cultures, one a normal culture of *Nicotiana tabacum,* the other a crown-gall culture of *Matricaria chamomilla,* were plated onto adsorbent filter plates either separately or together.

After 2–7 days of incubation the cells were removed and the filters developed with ethanol/ KOH. As a main component *Matricaria chamomilla* produces the yellow fluoresceing coniferyl aldehyde [6]; cells of *Nicotiana tabacum* produce a light blue fluoresceing substance which has been identified as scopoletine by behaviour during purification, thin layer chromatography in four different systems, absorbance and fluorescence spectra [12–14].

If a combination of aggregates of these two cultures is plated for some days the developed filters show yellow and blue spots on their lower side. The pattern of these spots is a precise mirror image of the cell aggregate pattern on the cellophane so that the origin of each spot can be traced back to corresponding cells. This makes it possible to select tobacco cells from those of *Matricaria* and *vice versa* (Fig. 2).

In accordance with the model experiment with umbelliferone (see above) cell aggregates of 1.5 mm diameter produce spots of less than 2.5 mm and 3.5 mm aggregates produce spots of about 4 mm diameter.

With cellophane as a cell carrier aggregates larger than 2 mm diameter tend to dry up within 1 week but this is no serious obstacle since selection is mostly desired on the level of aggregates as small as possible.

The most important advantages of this method are:

- Production of secondary substances by small cell aggregates can be detected without preceding mass propagation of the cultures;
- Total production of each cell aggregate during the incubation period is collected on the adsorbent and detected at the same time (amplification);
- Test plates are identical with culture plates during the test phase;
- Detection methods can be widely varied (fluorescence, colour, specific reagents of paper and thin layer chromatography).

An intelligent choice of adsorbents and procedures of development should adapt this method to many questions of secondary substance production. Such adaptation experiments have been started.

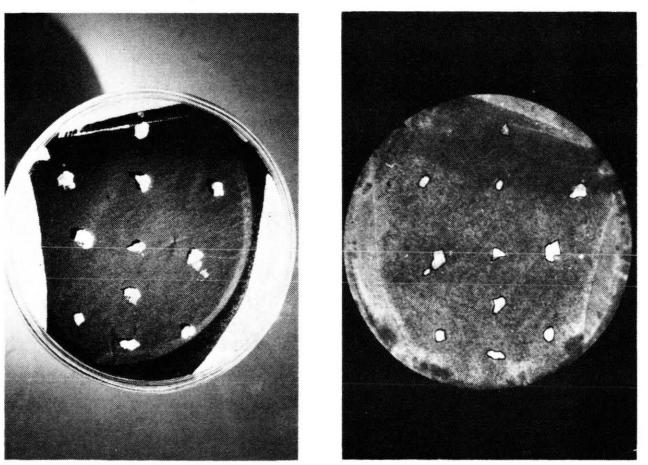

Fig. 2. a. Adsorbent filter plate with cell aggregates from suspension cultures of *Nicotiana tabacum* and *Matricaria chamomilla.*
b. Fluorescence pattern (UV 365 nm) on the white filter back as mirror image of the corresponding pattern of cell aggregates (compare a.).
The black-and-white photo does not discriminate blue and yellow fluorescences.

[1] B. E. Ellis, IAPTC Newsletter **38,** 13 (1982).
[2] Y. Yamada and Y. Fujita, in: Handbook of Plant Cell Culture, **Vol.** 1 (D. A. Evans, W. R. Sharp, P. V. Ammirato, Y. Yamada eds.), 717, New York 1983.
[3] K. H. Knobloch and J. Berlin, Z. Naturforsch. **35C,** 551 (1980).
[4] M. H. Zenk, H. El-Shagi, H. Arens, J. Stöckigt, E. Weiler and B. Deus, in: Plant Tissue Culture and its Biotechnological Application (W. Barz, E. Reinhard, M. H. Zenk eds.), 27, Berlin 1977.
[5] R. Beiderbeck, Z. Pflanzenphysiol. **108**, 27 (1982).
[6] B. Knoop and R. Beiderbeck, Z. Naturforsch. **38C,** 484 (1983).
[7] C. E. Flick, in: Handbook of Plant Cell Culture, **Vol. 1** (D. A. Evans, W. R. Sharp, P. V. Ammirato, Y. Yamada eds.), 393, New York 1983.
[8] W. Alfermann and E. Reinhard, Experientia **27,** 353 (1971).
[9] E. W. Weiler and M. H. Zenk, Phytochemistry **15,** 1537 (1976).
[10] R. Beiderbeck and B. Knopp, Z. Naturforsch. **39C,** 45 (1984).
[11] R. Beiderbeck and R. Hohl, Biochem. Physiol. Pflanzen **175,** 45 (1980).
[12] R. Hegnauer, in: Chemotaxonomie der Pflanzen, Birkhäuser Verlag, Basel 1962–1973.
[13] J. A. Sargent and F. Skoog, Plant Physiol. **35,** 934 (1960).
[14] J. A. Sargent and F. Skoog, Physiol. Plantarum **14,** 504 (1961).