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# Moss cell cultures as sources of arachidonic and eicosapentaenoic acids

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Lipid classes from tissue cultures of the moss *Leptobryum pyriforme* (Hedw.) Wils. were analyzed. In the total lipid fraction, this species contained 20% arachidonic acid and about 7% eicosapentaenoic acid. The distribution of these fatty acids showed a preference for the phospholipid fraction. In particular, the phosphatidylethanolamine fraction was enriched in arachidonic acid. The arachidonic acid content of *Leptobryum* could be altered by transferring the cultures to different culture conditions. Mosses show high organic mass production in tissue cultures in relatively simple media. The great potential of using mosses as sources for the production of polyunsaturated fatty acids is evident.

*Fatty acid* *Arachidonic acid* *Icosapentaenoic acid* *Phospholipid* *Cell culture* Leptobryum pyriforme

## 1. INTRODUCTION

Mosses are known to produce lipids containing high proportions of fatty acids with 20 carbon atoms, having 4 or 5 double bonds [1–3]. The occurrence of these polyunsaturated fatty acids constitutes an outstanding compositional difference between mosses and higher plants. It is known that these fatty acids are constituents of membranes in animal tissues which are essential for many physiological processes [4]. Arachidonic acid (20:4*n*6) is a biogenetic precursor of the biologically active prostaglandins and leukotrienes [5]. Recently, lipids which structurally resemble prostaglandins could be detected in some moss species [6]. Little effort has been made so far to elucidate the function of 20:4 and 20:5 fatty acids in spore-producing plants. Mosses can reproduce vegetatively with great ease, and show excellent growth under distinct conditions, even in liquid culture. Moss cells are regeneratively totipotent and can produce a new plant [7]. The filamentous moss protonemata of many species contain special cells (tmemen), which facilitate the loosening of the filaments into small units of 3–5 cells under mechanical treatment such as shaking in a culture medium.

Plant cell culture can be very useful for studying the biosynthesis of lipids from exogenous precursors and as sources for the production of various compounds [8]. This paper presents data which demonstrate that mosses are very useful organisms in terms of plant cell culture. They can be easily manipulated, are stable, and show high organic mass production in liquid culture. It should be possible to use them as sources for the isolation of biologically active lipids [9,10].

*Abbreviations:* MGDG, monogalactosyldiacylglycerols; DGDG, digalactosyldiacylglycerols; SQDG, sulphoquinovosyldiacylglycerols; PG, phosphatidylglycerols; PI, phosphatidylinositols; PE, phosphatidylethanolamines; PC, phosphatidylcholines

## 2. EXPERIMENTAL

### 2.1. *Plant material and lipid extraction*

A protonema culture of *Leptobryum pyriforme* (Hedw.) Wils. was obtained from the bryophyte collection of the Ceskoslovenske Akademic Ved.,

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Praha, Czechoslovakia (M 200, 2n strain). Protonemata were grown in a modified Bennecke liquid medium in 300-ml Erlenmeyer flasks at a constant temperature of 20°C, 2000 lux white light (Osram TL 40W/25 fluorescent tubes), in a 16 h light–8 h dark illumination regime. The medium contained 82 $\mu$mol $CaCl_2 \cdot 2H_2O$, 789 $\mu$mol $KNO_3$, 1 mmol $KH_2PO_4$, 46 $\mu$mol $MgSO_4 \cdot 7H_2O$, 7 $\mu$mol $FeCl_3$ and 10 mmol glucose per l; pH 5.8.

The protonemal cells were collected on a nylon net, and treated with boiling isopropanol to avoid lipid degradation by lipases. The suspension was homogenized (50 mg dry wt) with purified quartz sand in a mortar with a pestle. The homogenate was extracted with chloroform:methanol (2:1, v/v) following an established procedure [11]. The crude lipid extract was evaporated to dryness, the residue dissolved in hexane, and stored at −20°C.

### 2.2. *Separation and identification of lipids*

The hexane extract was fractionated by low-pressure column chromatography on silicic acid (column 10 mm diameter and 60 mm long, SIL-LC, 325 mesh, Sigma) [12]. Silicic acid (1.5 g) in hexane was slurried into the column. Samples of about 10 mg lipids dissolved in hexane were applied to the column. The solvents were pumped through at a rate of 60 ml/h.

Separation flow sheet:

| Fraction | ml | Solvents (v/v) | Lipids eluted |
|---|---|---|---|
| 1 | 14 | hexane:diethyl ether (99:1) | carotenes |
| 2 | 15 | hexane:diethyl ether (4:1) | triacylglycerols |
| 3 | 20 | chloroform | chlorophylls |
| 4 | 12 | acetone:chloroform (2:1) | MGDG |
| 5 | 13 | acetone:methanol (29:1) | DGDG |
| 6 | 20 | acetone:methanol (19:1) | SQDG (+DGDG, trace) |
| 7 | 12 | acetone:methanol (2:1) | PG+PI |
| 8 | 9 | methanol | PE |
| 9 | 23 | methanol | PC |

Further fractionation of fraction 7 (PG + PI):

| | | | |
|---|---|---|---|
| 7.1 | 10 | chloroform:methanol (7:1) | PG |
| 7.2 | 6 | chloroform:methanol (4:1) | PG |
| 7.3 | 3 | chloroform:methanol (1:1) | PI (+PG, trace) |
| 7.4 | 8 | chloroform:methanol (1:4) | PI |

The identity and purity of acyl lipids were checked by thin-layer chromatography (TLC) on silica gel G with the following solvents: triacylglycerols – *n*-heptane:diethyl ether:acetic acid (75:25:4, v/v) [13]; glycolipids and phospholipids – chloroform:methanol:acetic acid:water (85:15:10:3, v/v) [14]. Pure reference substances were cochromatographed for identification.

The indicators used were: triacylglycerols – cupric acetate [15]; glycolipids – $\alpha$-naphthol [16]; phospholipids – ninhydrin [17], and molybdenum trioxide/sulphuric acid [18].

### 2.3. *Fatty acid analyses*

Acyl lipids were trans-esterified with 5% $H_2SO_4$ in methanol (v/v) for 4 h at 80°C. Gas-liquid chromatography (GLC) was used to analyze the resulting methyl esters (DANI 3800 GLC equipped with WCOT fused silica capillary column, 50 m × 0.23 mm i.d., flame ionization detector, FID). The stationary phase was CP Sil88 (Chrompack, film thickness 0.20 $\mu$m). Nitrogen was used as carrier gas at a flow rate of 1.1 ml/min (split 1:100). The column temperature was 200°C. Identification and quantitative determination were carried out using standards and GLC-MS coupling [19]. The data shown are mean values of at least 3 determinations.

## 3. RESULTS AND DISCUSSION

Bryophytes are generally not damaged by fungi, insects, and earthworms. Nevertheless, until recently, the phytochemistry of the bryophytes has been neglected (review [9,10]). This may be due, in part, to difficulties in collecting large quantities of pure material of certain species. The methods of cell and tissue culture should reduce this problem.

Liquid culture is an excellent method for growing mosses in large batches [20]. Growth can be manipulated in a wide range, as demonstrated in

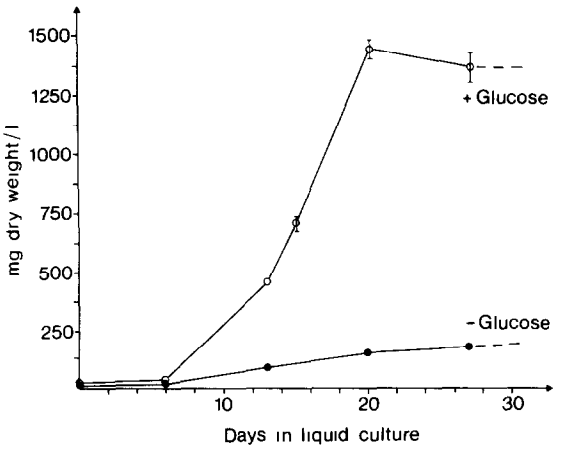


Fig.1. Influence of glucose on the organic mass production of *Leptobryum* in liquid culture. The data represent mean values ± SD of 3 determinations. SD of measurements carried out with the − glucose medium were too small to fit in the graph.

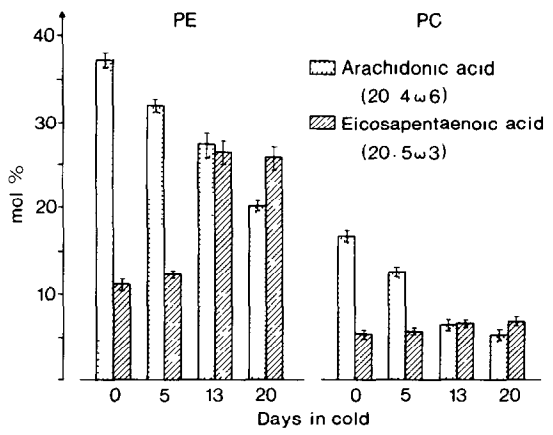


Fig.2. Modulation of the arachidonic acid and eicosapentaenoic acid contents of PE and PC after transferring *Leptobryum* from normal growth condition to a temperature of 1°C.

fig.1. In studying stress physiology and moss development, we usually cultivate the mosses under conditions aimed at remaining below the fastest growth rate. This procedure is preferred to avoid the acceleration of senescence. There are some possibilities to modulate growth, e.g. by increasing the level of mineral components in the medium, or the addition of organic substances, such as glucose (fig.1) or amino acids. Liquid cultures must be shaken in order to avoid stress condition. If not shaken, normal growth and development may be disturbed [21].

Table 1 lists the results of fatty acid analyses of *L. pyriforme*. The high proportions of polyunsaturated fatty acids are evident.

*L. pyriforme* constitutes a typical example of the high stability of mosses propagated in tissue and cell culture. This species has been in culture since 1921 [22], and since 1978 has been used in our group as a standard species in sterile liquid culture. During this time it was transferred to fresh medium every 10 days. The fatty acid pattern of the lipids did not change significantly for at least 3 years. The highest mol percentage of unsaturated fatty acids is found in the phospholipid fractions (table 1). This fraction shows the highest proportion of arachidonic acid (39 mol%) esterified in PE. The triacylglycerols and galactolipids contain only one-third of the levels found in the phospholipids.

A possibility of modulating the arachidonic acid content is demonstrated in fig.2. The protonemata were transferred from normal temperature to cold conditions (1°C) for 14 days. During this time the PE fraction showed a decrease in constituent arachidonic acid accompanied by an increase in eicosapentaenoic acid. This result indicates a close biochemical correlation between both fatty acids. The decrease of arachidonic acid occurred only in the PC fraction. The proportions of eicosapentaenoic acid did not change in the cold.

One may hypothesize that arachidonic acid and other polyunsaturated fatty acids which occur in complex lipids of subcellular membranes may contribute to the survival of moss tissues under extreme ecological conditions [19,23]. Mosses can overcome strong water stress, after years of dryness, and can be reactivated by watering. We have obtained evidence that modulation of lipid metabolism is involved in adaptation to this stress situation. This may also be true for temperature stress, cold acclimatization, for which we presented an example.

Table 1

Fatty acid patterns of lipid classes of the moss *Leptobryum pyriforme*

| | 14:0 | 16:0 | 16:1 | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 ω6 | 18:3 ω3 | 18:4 ω3 | 20:0 | 20:3 ω6 | 20:3 ω3 | 20:4 ω6 | 20:5 ω3 | 24:0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| nmol/mg dry wt | tr | 46.7 | 8.6 | 2.2 | 32.6 | 8.9 | 7.7 | 8.4 | 2.3 | 68.2 | tr | 1.3 | 3.3 | tr | 20.0 | 6.4 | tr |
| mol% | tr | 21.2 | 3.9 | 1.0 | 14.8 | 4.1 | 3.5 | 3.8 | 1.0 | 31.0 | tr | 0.6 | 1.5 | tr | 9.1 | 2.9 | tr |
| Triacylglycerols nmol/mg dry wt | tr | 10.6 | 1.0 | – | 0.8 | 3.6 | 3.8 | 2.4 | 0.4 | 4.7 | tr | 0.4 | 1.2 | 0.4 | 3.6 | 1.2 | tr |
| mol% | tr | 30.5 | 2.9 | – | 2.3 | 10.5 | 11.0 | 6.9 | 1.2 | 13.6 | tr | 1.1 | 3.4 | 0.8 | 10.4 | 3.5 | tr |
| Glycolipids nmol/mg dry wt | tr | 16.9 | 3.0 | 2.2 | 31.7 | 4.1 | 1.3 | 2.4 | 0.7 | 52.2 | tr | 0.7 | 0.6 | tr | 5.0 | 2.3 | – |
| mol% | tr | 13.6 | 2.4 | 1.8 | 25.6 | 3.3 | 1.0 | 1.9 | 0.6 | 42.1 | tr | 0.6 | 0.5 | tr | 4.0 | 1.9 | – |
| Phospholipids nmol/mg dry wt | tr | 19.2 | 4.6 | tr | tr | 1.2 | 2.6 | 3.6 | 1.1 | 11.2 | tr | tr | 1.6 | tr | 11.3 | 2.9 | 0.4 |
| mol% | tr | 31.4 | 7.5 | tr | tr | 2.0 | 4.2 | 5.9 | 1.8 | 18.3 | tr | tr | 2.6 | tr | 18.5 | 4.7 | 0.6 |
| MGDG (mol%) | tr | 3.8 | 0.9 | 2.3 | 37.1 | 3.4 | 0.8 | 1.4 | 0.6 | 41.9 | tr | 0.6 | tr | tr | 5.0 | 1.4 | – |
| DGDG | tr | 16.1 | 0.8 | 1.4 | 16.7 | 3.7 | tr | 1.5 | 0.6 | 50.7 | tr | 0.6 | 0.8 | tr | 2.6 | 3.5 | – |
| SQDG | tr | 41.1 | 9.4 | tr | 1.7 | 2.5 | 3.1 | 4.4 | 0.7 | 31.0 | tr | tr | 0.7 | tr | 3.1 | 1.2 | – |
| PG | tr | 30.8 | 23.9[a] | tr | tr | 1.5 | 4.0 | 4.7 | – | 31.3 | – | tr | tr | – | 1.6 | tr | – |
| PI | tr | 41.1 | 6.2 | tr | – | 3.7 | 11.0 | 6.0 | – | 11.2 | – | 0.8 | 0.6 | 2.0 | 13.3 | 3.1 | – |
| PE | tr | 23.4 | 2.2 | – | – | 2.1 | 1.5 | 3.7 | tr | 6.9 | – | 0.8 | 5.6 | 0.6 | 38.8 | 8.9 | 3.5 |
| PC | tr | 32.1 | 1.0 | – | – | 1.5 | 3.5 | 7.1 | 3.8 | 17.0 | 1.1 | tr | 3.3 | tr | 22.1 | 6.1 | tr |

[a] Probably 16:1ω13t; tr (trace) 0.5 mol%

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## REFERENCES

[1] Schlenk, H. and Gellerman, J.L. (1965) J. Am. Oil Chem. Soc. 42, 504–511.
[2] Wagner, H. and Friedrich, H. (1969) Phytochemistry 8, 1603.
[3] Kohn, G. (1982) Thesis, University of Mainz.
[4] Bergström, S., Carlson, L.G. and Weeks, J.R. (1968) Pharmacol. Rev. 20, 1–48.
[5] Marx, J.L. (1982) Science 215, 1380–1383.
[6] Ichikawa, T., Yamada, K., Namikawa, M., Sakai, K. and Kondo, K. (1983) The World Conference of Bryology, Book of Abstracts C-VI-1, p.44.
[7] Jahnen, W. and Hartmann, E. (1983) in: Protoplasts (Potrykus, I. et al. eds) pp.182–183, Birkhäuser, Basel.
[8] Radwan, S.S. and Mangold, H.K. (1980) in: Advances in Biochemical Engineering, Plant Cell Cultures I, vol.16 (Fiechter, A. ed.) pp.109–133, Springer, Berlin.
[9] Asakawa, Y. (1982) in: Progress in the Chemistry of Organic Natural Products, 42 (Herz, W. et al. eds) pp.1–285, Springer, Wien.

[10] Huneck, S. (1983) in: New Manual of Bryology, vol.1 (Schuster, R.M. ed.) pp.1–116, Hattori Botanical Laboratory, Nichinan.
[11] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
[12] Gellerman, J.L., Anderson, W.H., Richardson, D.G. and Schlenk, H. (1975) Biochim. Biophys. Acta 388, 277–290.
[13] Korte, K. and Casey, M.L. (1982) J. Chromatogr. 232, 47–53.
[14] Nichols, B.W. (1965) Biochim. Biophys. Acta 106, 274–279.
[15] Fewster, M.E., Burns, B.J. and Mead, J.F. (1969) J. Chromatogr. 43, 120–126.
[16] Jacin, H. and Mishkin, A.R. (1965) J. Chromatogr. 18, 170–173.
[17] Patton, A.R. and Chism, P. (1951) Anal. Chem. 23, 1683–1685.
[18] Dittmer, J.C. and Lester, R.L. (1964) J. Lipid Res. 5, 126–127.
[19] Vandekerkhove, O., Euler, R., Kohn, G. and Hartmann, E. (1984) J. Hattori Bot. Lab. 56, 187–193.
[20] Naef, J. and Simon, R. (1978) Saussurea 9, 51–56.
[21] Kofler, L. (1959) Rev. Bryol. Lichenol. 28, 1–202.
[22] Pringsheim, E.G. (1921) Jahrb. Wiss. Bot. 60, 499–530.
[23] Hartmann, E. and Hock, K. (1985) in: The Physiological Properties of Plant Protoplasts (Pilet, P.E. ed.) pp.190–199, Springer, Berlin.