

# Growth and Secondary Product Formation of *in vitro* Cultures from the Liverwort *Reboulia hemisphaerica*

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*Reboulia hemisphaerica* has been cultivated on Gamborg B5 medium with various sugars. Differentiated cultures grew best on agar medium with the addition of 0.5% sucrose and a light regime of 18 h light/6 h dark. Callus was induced either by a combination of phytohormones (2 mg/l  $\alpha$ -naphthylacetic acid + 1 mg/l kinetin) or by 4% glucose. The differentiated cultures on agar produced sesquiterpenes in a tenfold increased amount compared to undifferentiated suspension cultures. Cultures kept in dark produced no sesquiterpenes, but 5-hydroxy-7,8,4'-trimethoxyflavone was present whereas apigenin-7,4'-dimethylether was the only flavonoid in cultures cultivated in the light.

## Introduction

Liverworts are a rich source of different secondary products, especially of terpenoids [1]. The amount of plant material available for chemical analysis is often limited. *In vitro* cultures offer the possibility of obtaining sufficient amounts [2]. *Reboulia hemisphaerica* (L.) Raddi, Grimaldiaceae, is a thalloid liverwort containing dark brown oil bodies dispersed in the thalli. A number of sesquiterpene hydrocarbons as well as aristolone have been previously described for the plant [3–5]. Recently, we described the identification of (+)-gymnomitr-8(12)-en-9 $\alpha$ -ol and (R)-(-)-8,11-dihydro- $\alpha$ -cuparenone from material collected in southern France [6]. Although *R. hemisphaerica* was among the first liverworts to be aseptically cultivated *in vitro* [7], nothing is known about secondary product formation in the cultures.

## Materials and Methods

Aseptic cultures of *R. hemisphaerica* were provided by the Institute of Botany, Czecho-Slovak Academy of Sciences, Culture Collection of Autotrophic Organisms, Trebon, C.S.F.R. The cultures had been isolated in 1941 and kept at 15 °C and 8 h illumination per day on agar medium with 1 M  $\text{NH}_4\text{NO}_3$ , 0.1 M  $\text{MgSO}_4$ , 1 M  $\text{CaCl}_2$  and 0.01 M

$\text{FeCl}_2$ . The cultures used were derived from the cultures obtained from Trebon. Basal media for the cultivation was either B5 [8] or MS [9]. Trace elements were those of MSK-2 [10]. Surface cultures were with the addition of 9 g/l Difco Bacto-Agar. The media were adjusted to pH 6.0 before autoclaving. Suspension cultures were shaken on a reciprocal shaker at 120 rpm. The standardized growth conditions were continuous fluorescent white light of about 2500 lx at  $22 \pm 1$  °C. The cultures were kept in 200 ml Erlenmeyer containing 50 ml liquid or solidified medium. Inoculum size was 2 g fresh weight. The influence of sugars on growth and morphology was tested with the following sugars: sucrose 2%, 1% and 0.5%; glucose 0.5%; lactose 0.5%, and galactose 0.5%.

Callus cultures were on MS-medium either with 2 mg/l  $\alpha$ -naphthylacetic acid (NAA) and 1 mg/l kinetin (KIN) or with 4% glucose. Glucose was determined with an enzyme kit "sucrose/glucose" from Boehringer (Mannheim). Nitrate, ammonium and phosphate were determined with the respective test system "spectroquant" from Merck (Darmstadt). A 30 l fermenter was run for 30 days in the dark with an inoculum of about 5 g by Diversa Company Hamburg. 120 g dried plant material grown under continuous light were extracted with  $\text{Et}_2\text{O}$  at room temperature for isolation and structure elucidation of  $\beta$ -hydroxy-gymnomitr-9-one (100 mg) and other terpenoids [11] and apigenin-7,4'-dimethylether (80 mg). 133 g dry weight of dark grown cultures were extracted with  $\text{CH}_2\text{Cl}_2$  at room temperature for the isolation of 5-hydroxy-

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7,8,4'-trimethoxyflavone (80 mg). The flavonoids were isolated by a combination of column chromatography on silicagel with petrolether/EtOAc gradients and on Sephadex LH-20 with  $\text{CHCl}_3/\text{MeOH}$  (1:1). They were characterized by their MS-, UV-, IR-, and  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR-spectra.

**5-Hydroxy-7,4'-dimethoxyflavone (6):** UV ( $\lambda_{\text{max}}^{\text{EtOH}}$  nm: 268, 294 (sh) 327);  $\delta_{\text{H}}$  12.78 (s, OH), 7.81 (2H, d,  $J=9$  Hz, H-2', 6') and 7.00 (2H, d,  $J=9$  Hz, H-3', 6'), 6.55 (s, H-3), 6.45 and 6.33 (2H, both s,  $J=9.2$  Hz, H-8, 6).

**5-Hydroxy-7,8,4'-trimethoxyflavone (7):** UV ( $\lambda_{\text{max}}^{\text{EtOH}}$  nm: 274, 301, 320 (sh);  $\delta_{\text{H}}$  12.60 (s, OH), 7.88 (2H, d,  $J=9.2$  Hz, H-2', 6') and 7.02 (2H, d,  $J=9.2$  Hz, H-3', 5'), 6.56 and 6.41 (2H, both s), 3.93, 3.92 and 3.88 (3  $\times$  3 H, s).

The main sesquiterpene, (+)-gymnomitr-8(12)-en-9 $\alpha$ -ol, was quantified through gaschromatography (GC) with methylpalmitate as internal standard. GC equipment and conditions: Vega 6000, Carlo Erba (Milano, Italy), DB1 fused silica column, 30  $\times$  0.25 mm, J. and W. Scientific; Helium; injector at 250  $^{\circ}\text{C}$ ; FID at 230  $^{\circ}\text{C}$ ; temperature program: 110  $^{\circ}\text{C}$  – 3 min, 110–150  $^{\circ}\text{C}$ , 2  $^{\circ}\text{C}/\text{min}$ ; 150–220  $^{\circ}\text{C}$ , 5  $^{\circ}\text{C}/\text{min}$ ; 220  $^{\circ}\text{C}$  – 10 min.

## Results and Discussion

**Growth and morphology of the cultures:** *R. hemisphaerica* grew as differentiated plants on Gamborg B5 media. The shape of the thalli varied under the influence of different kinds and concentrations of added sugars. The broadest thallus (20 mm) was achieved with 2% sucrose. When 1% and 0.5% sucrose, 0.5% glucose or 0.5% lactose were added, the thalli reached only 10 mm and only 5 mm with 0.5% galactose. Rhizoids were numerous on medium with sucrose and glucose, their number was however reduced on media with lactose or galactose. The number of oil cells was highest (20–30  $\text{mm}^2$ ) when 1% sucrose and lower (10–20  $\text{mm}^2$ ) when any other sugar was added. Growth under standard conditions showed the typical sigmoid pattern (Fig. 1).

Maximal growth under standard conditions in continuous light with various sugars was achieved with 0.5% glucose (Fig. 2). Sucrose concentrations above 0.5% did not lead to a further increase in fresh weight. Variation of the light regime, 18 h light/6 h dark or 12 h light/12 h dark, resulted in a better growth (compared to continuous light) (Fig. 3).

Callus induction was achieved either on MS-medium with the addition of 3% sucrose, 2 mg/l NAA and 1 mg/l KIN, or on the same basal medium with 4% glucose. The morphology of the two different calli was similar. It consisted of dark green lumps with spherical or oval cells of 30 to 50  $\mu\text{m}$  diameter. Fresh weight increase was higher in the phytohormone-induced callus (Fig. 4) than

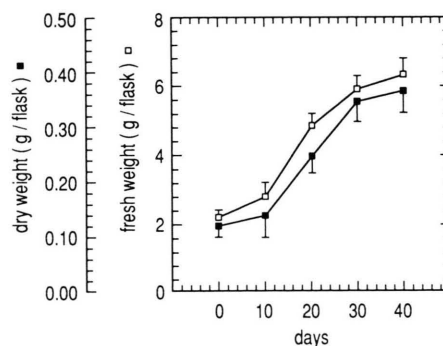
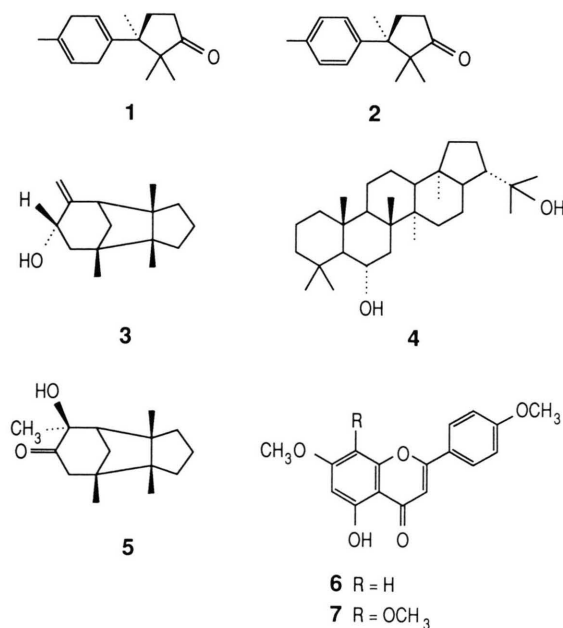


Fig. 1. Fresh (□) and dry (■) weight increase of *R. hemisphaerica* cultures on B5-medium with 0.5% sucrose under continuous light of 2500 lx at 22  $^{\circ}\text{C}$  (mean of 4 independent series of cultivation).

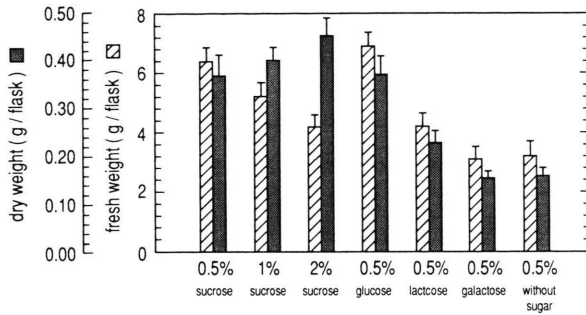


Fig. 2. Fresh (▨) and dry (■) weight of *R. hemisphaerica* cultures on B5-medium with various sugars under continuous light of 2500 lx at 22 °C after 40 days of cultivation. Inoculum 2 g fresh weight corresponding to 0.12 g dry weight (mean of 4 independent series of cultivation).

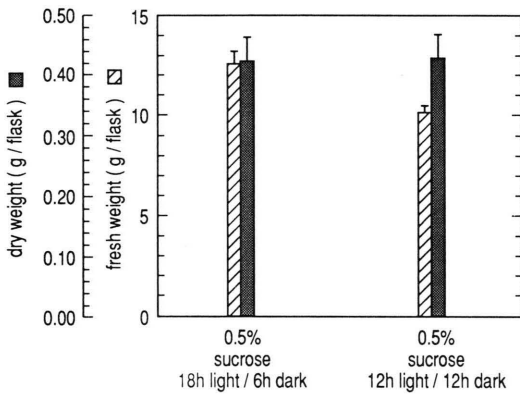


Fig. 3. Fresh (▨) and dry (■) weight of *R. hemisphaerica* cultures on B5-medium, 0.5% sucrose with a light regime of 18 h light/6 h dark and 12 h light/12 h dark, respectively, after 40 days of cultivation (mean of 4 independent series of cultivation).

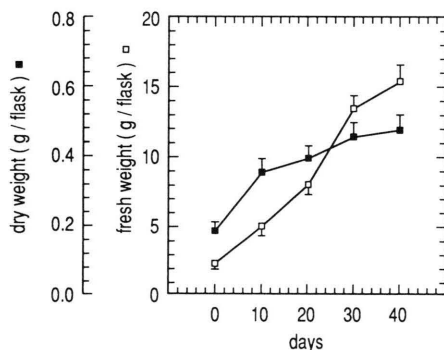


Fig. 4. Fresh (□) and dry (■) weight increase of callus cultures of *R. hemisphaerica* on MS-medium with the addition of 3% sucrose, 2 mg/l NAA and 1 mg/l KIN, under continuous light of 2500 lx at 22 °C (mean of 4 independent series of cultivation).

in the callus induced with 4% glucose (Fig. 5). However, dry weight increase was higher in the glucose generated calli. Both cultures differentiated to plantlets when retransferred to standard B5 media with 0.5% sucrose.

Starting from callus cultures on agar with 4% glucose, we established suspension cultures. They grew in undifferentiated form on B5 medium with 2% glucose added. The growth characteristics under continuous light are outlined in Fig. 6. Dry weight increase stops at day fourteen when glucose, ammonium, nitrate, and phosphate in medium are almost exhausted (Fig. 6, 7). From the two nitrogen sources, ammonium is preferentially taken up in the beginning whereas nitrate is used in

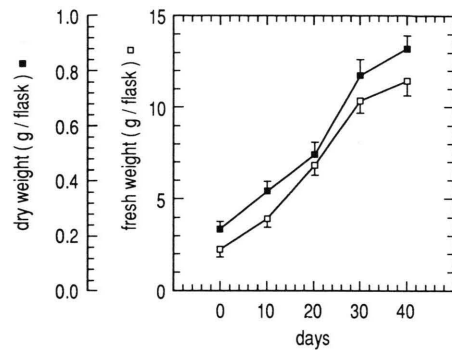


Fig. 5. Fresh (□) and dry (■) weight increase of callus cultures of *R. hemisphaerica* on MS-medium with the addition of 4% glucose, without phytohormones, under continuous light of 2500 lx at 22 °C (mean of 4 independent series of cultivation).

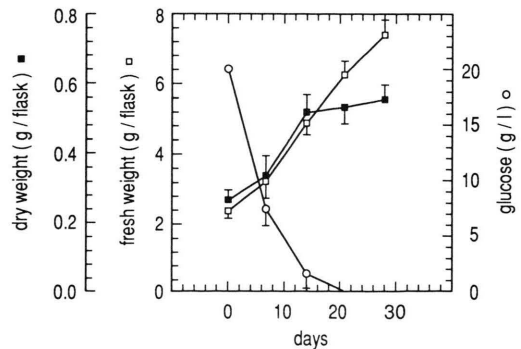


Fig. 6. Fresh (□) and dry (■) weight increase and glucose concentration (○) of *R. hemisphaerica* suspension cultures in B5-medium with 2% glucose under continuous light of 2500 lx at 22 °C (mean of 4 independent series of cultivation).

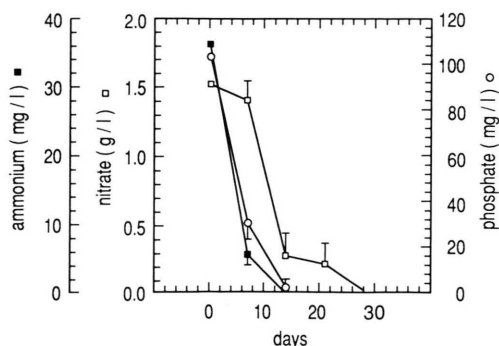


Fig. 7. Nitrate (□), ammonium (■) and phosphate (○) concentration of *R. hemisphaerica* suspension cultures in B5-medium with 2% glucose under continuous light of 2500 lx at 22 °C (mean of 4 independent series of cultivation).

substantial amounts only from day seven when ammonium ions are almost consumed. Unlike suspension cultures of higher plants only a few mosses and liverworts could be cultivated in the dark [12]. Cultures of *Marchantia polymorpha*, *Jungermannia subulata*, *Calypogeia granulata* and *Heteroscyphus bescherellei* did not grow in the dark even if a sufficient amount of sugar was present in the medium [12–14]. In contrast, *Barbula unguiculata* [15] and *Marchantia paleacea* [16] continued to grow and synthesize chlorophyll in complete darkness. Recently, Takio *et al.* [17] reported the photosynthetic ability of dark-grown *R. hemisphaerica*. Our suspension cultures from *R. hemisphaerica* cultivated in the dark showed similar growth characteristics as in the light (Fig. 8). However, glucose was con-

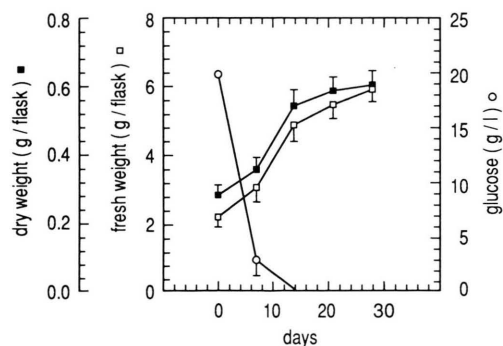


Fig. 8. Fresh (□) and dry (■) weight increase and glucose concentration (○) of *R. hemisphaerica* suspension cultures in B5-medium with 2% glucose cultivated in the dark at 22 °C (mean of 4 independent series of cultivation).

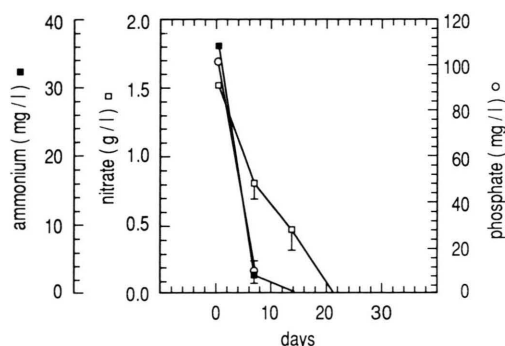


Fig. 9. Nitrate (□), ammonium (■) and phosphate (○) concentration of *R. hemisphaerica* suspension cultures in B5-medium with 2% glucose cultivated in the dark at 22 °C (mean of 4 independent series of cultivation).

sumed more rapidly and dry weight paralleled fresh weight increase. Ammonium and nitrate were equally consumed from the beginning (Fig. 9). A 30 liter fermenter was run for 30 days with a 20% inoculum. The fresh weight increase was 7 g/l in 30 days which was low compared to 12 g/l and to 17 g/l within 7 days for shaken cultures in 1000 ml and 2000 ml Erlenmeyer flasks used for upscaling.

#### Production of secondary compounds

Recently, from material collected in southern France we isolated the sesquiterpenes 8,12-dihydro- $\alpha$ -cuparenone (1),  $\alpha$ -cuparenone (2), (+)-gymnomitr-8(12)-en-9 $\alpha$ -ol (3) and  $\alpha$ -zeorin (4) [6]. (3) was the main compound with 130 mg from 100 g of dry material. (3) was also the main compound for cultivated differentiated cultures with 700 mg/100 g. From the culture material we further isolated 83 mg/100 g of a new gymnomitran-derivative which was determined to be 8 $\beta$ -hydroxy-gymnomitran-9-one (5) [11]. (5) could not be detected in field-collected material and is not yet described in the literature. However, as we worked with different material, such material which was collected in southern France and cultivated material isolated more than forty years ago from a place near Hamburg, it remains unclear if the formation of the product is due to a different environment or to genetic variation. In the case of flavonoids detected in the culture different structures were obtained from light and dark grown cultures. From cultures

cultivated under continuous light, we isolated 67 mg/100 g of 5-hydroxy-7,4'-dimethoxyflavone (= apigenin-7,4'-dimethylether) (**6**). From the dark grown fermenter cultures we isolated 60 mg/100 g 5-hydroxy-7,8,4'-trimethoxyflavone (**7**). (**6**) could not be detected in dark grown cultures. (**7**) could not be detected in light grown cultures. The main sesquiterpene (**3**) was quantified by gas chromatography for differentiated light grown agar cultures and for undifferentiated light grown suspen-

sion cultures. Its amounts were 700 mg/100 g in the agar cultures and 60 mg/100 g in the suspension cultures.

It is interesting to note that undifferentiated cultures contained less than one tenth of the main sesquiterpenes compared to the differentiated cultures. This is in agreement with similar results from *Fossombronina pusilla* [18]. However, Takeda and Katoh [14] reported an even higher yield in undifferentiated cells than in intact plants.

- [1] Y. Asakawa, Chemical constituents of the Hepaticae, in: Fortschr. Chem. Org. Naturstoffe (W. Herz, H. Grisebach, and G. W. Kirby, eds.), Vol. **42**, p. 1, Springer Verlag, Wien, New York 1982.
- [2] G. Wurzel and H. Becker, Z. Naturforsch. **45c**, 13 (1990).
- [3] Y. Asakawa, R. Matsuda, M. Toyota, C. Suire, T. Takemoto, and S. Huneck. J. Hattori Bot. Lab. **50**, 107 (1981).
- [4] Y. Asakawa and R. Matsuda, Phytochemistry **21**, 2143 (1982).
- [5] H. Beyer, Terpene aus freilebenden Lebermoosen und deren Sterilkulturen. Dissertation, Universität Heidelberg 1988.
- [6] R. M. S. C. Morais, L. J. Harrison, and H. Becker, J. Chem. Research (S) **1988**, 380.
- [7] A. Allsopp, Nature **179**, 681 (1957).
- [8] O. L. Gamborg, R. A. Miller, and K. Ojima, Exp. Cell Res. **50**, 151 (1968).
- [9] T. Murashige, F. Skoog, Physiol. Plant. **15**, 473 (1962).
- [10] K. Katoh, M. Ishikawa, K. Miyake, Y. Ohta, Y. Hirose, and T. Iwamura, Physiol. Plant. **49**, 241 (1980).
- [11] R. M. S. C. Morais, L. J. Harrison, and H. Becker, Phytochemistry (1990) in press.
- [12] S. Takami and S. Takio, J. Plant Physiol. **130**, 267 (1987).
- [13] K. Katoh, Physiol. Plant. **57**, 67 (1983).
- [14] R. Takeda and K. Katoh, Planta **151**, 525 (1981).
- [15] S. Takio, M. Kajita, S. Takami, and S. Hino, J. Hattori Bot. Lab. **60**, 407 (1986).
- [16] S. Takio, S. Takami, and S. Hino, J. Plant Physiol. **132**, 195–198 (1988).
- [17] S. Takio, C. Akita, V. W. Ngumi, and Takami, Plant Cell Reports **8**, 575 (1990).
- [18] M. Sauerwein and H. Becker, Planta Medica **56**, 364 (1990).