Growth and Terpenoid Production of an Axenic Culture from the Liverwort *Ricciocarpos natans*

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Ricciocarpos natans grows best on Gamborg B5 medium supplemented with 2% sucrose. Six other mineral media were less effective on the growth. During a culture period, pH of the media changed depending on the ammonium and nitrate content of the respective media. In media of a molar ratio of ammonium to nitrate of 1:1 or 1:1.9, pH first dropped from 6.0 to about 3.5 and then rose again. In media where nitrate is the only nitrogen source or where there is a ratio of ammonium to nitrate of 1:12.5, pH rose during the first two weeks and after this dropped. Differences in the light intensity (2000 and 6000 lx) and light regime (14 h light/10 h dark) had no effects on the growth. The cultures produced a variety of terpenoids. The same pattern of compounds was seen in field-collected material as well as in cultivated material. The content of the terpenoids decreased on nitrogen and phosphorous-deficient media.

Introduction

Bryophytes have been investigated rather late – compared with other plant groups – for their chemical compositions [1]. These investigations showed bryophytes containing a large variety of secondary metabolites hitherto unknown from other plant sources. Of the three classes of bryophytes – hornworts, mosses, and liverworts – only the latter contain oil bodies [2] which are intracellular structures containing various mono-, sesqui-, and diterpenoids [3] some of which exert interesting biological activities [4].

Plant material available for chemical research which must be followed by pharmacological screening is often very limited. Axenic cultures offer the possibility of obtaining sufficient amounts. However, unlike in higher plants, there are only a few studies of optimal growth and terpenoid production in liverworts [5, 6].

Ricciocarpos natans (L.) Corda, Ricciaceae, grows floating on the surface of stagnant waters in temperate regions of both the northern and southern hemisphere. If the marshy pool dries out it keeps growing on the moist soil. In Germany it is dispersed and shows the phenomenon of changing its habitat [7]. Within three years the authors found it only once in its natural habitat.

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Ricciocarpos natans is the only species containing oil bodies within the family of the Ricciaceae. Its chemistry is still unknown. Lorenzen *et al.* [8] reported this liverwort to be growing quite well in aerated liquid cultures. Therefore, a study of its *in vitro* growth and terpenoid production seemed promising.

Materials and Methods

About 100 g fresh weight of *Ricciocarpos natans* were collected from a marshy pool northwest of Karlsruhe, F.R.G., in August 1986 and were extracted with CH_2Cl_2. Aseptic cultures of *Ricciocarpos natans* were provided by the Institute of Botany, Czecho-Slovak Academy of Sciences, Culture Collection of Autotrophic Organisms, Trebon, C.S.S.R. The cultures had been isolated in 1947 and had been kept at 15 °C and 8 h illumination per day on agar medium with 1 M NH_4NO_3, 0.1 M KH_2PO_4, 0.1 M $MgSO_4$, 1 M $CaCl_2$, and 0.01 M $FeCl_2$. The cultures used in the study were derived from the cultures obtained from Trebon. They were maintained in our laboratory on Gamborg B5 medium [9] under the conditions described below and were subcultured every five weeks. The cultures resembled very much the aquatic form of field material.

All experiments were run with an inoculum of about 1.5 g fresh weight in 200 ml Erlenmeyer flasks containing 70 ml of liquid media. The following media with their respective macronutrient

composition were tested: Lorenzen *et al.* [8], Benecke [10], Knop b [10], Knudsen [11], MSK-2 [12], White [13], and Gamborg B5 [9]. Trace elements were those described by Murashige and Skoog [14]. 10 g/l of sucrose were added to the media for standard cultures and pH adjusted to 6.0 before autoclaving. The cultures were kept under continuous illumination of about 2000 lx at 22 ± 1 °C. Each time, five flasks were harvested, fresh and dry weight determined, and the pH value of the remaining solution was measured.

1050 g of fresh plant material were extracted with CH_2Cl_2 for isolation and structure elucidation of terpenoids [15]. The content of the main constituents (Fig. 1), (−)-limonene (**1**), cuprenolide (**2**), ricciocarpin A (**4**), and phytol (**7**) was determined by gas chromatography and methyl-10-undecenoate (Merck, Darmstadt) as internal standard. Four flasks were measured individually, the measuring was repeated twice for each sample, thus a given value represents the mean of eight determinations. The measurement data were evaluated statistically by means of the U-test according to Mann and Whitney [16].

GC conditions: Varian aerograph 2700 (Varian, Bremen); 2 m steel column ⅛″, 5% OV 101 on Chromosorb WAW 80–100 mesh; carrier gas: N_2, 1.7 bar; temperature program: 60–280 °C, 12 °C/min; Integrator 308 (Dialog GmbH, Düsseldorf); retention times and response factors isolated compound/standard: (−)-limonene 4.5 min (0.76), standard 10.2 min, cuprenolide 15.7 min (1.22), ricciocarpin A 16.6 min (1.18), phytol 17.7 min (1.10).

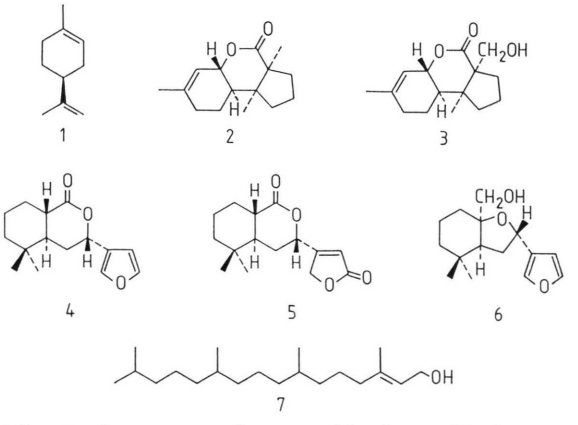

Fig. 1. Structures of terpenoids from *Ricciocarpos natans*.

Preparation for quantitative determination: To 1.5 g of fresh plant material 1 ml of a solution of the standard (5 mg/100 ml) in CH_2Cl_2 and 15 ml CH_2Cl_2 were added and the mixture homogenized for 30 sec at 24,000 rpm by an Ultra-Turrax 18/2 (Janke & Kunkel, Staufen). Further 5 ml CH_2Cl_2 were added and the suspension was once more homogenized. After 5 min the solution was filtered and the filtrate chromatographed on a silica gel column. The column (0.5 × 12 cm) was run with CH_2Cl_2/EtOAc (95:5) until the chlorophyll eluted. The eluent was concentrated *in vacuo* to 0.2 ml and the residue submitted to GC.

Results and Discussion

Growth and morphology on different media and under different light regimes

Ricciocarpos natans grew as differentiated plants on any of the different mineral media. Growth curves (Fig. 2 and 3) show the typical sigmoid pattern. The duration of the lag phase, the rise within the growth phase, and the beginning of the stationary phase differed for the various media.

Differences were also observed in the appearances of the cultures reaching the stationary phase. Plants on media of Benecke, Knop b, Lorenzen, and White turned from dark-green to light-green and subsequently their thalli turned brown starting from the basal part. The ventral scales changed to violet on Lorenzen's and White's medium as early as six days after the start of the culture and on Benecke's and Knop's medium ten days after the start of the culture. Plants on B5-, MSK-2-, and Knudsen's medium remained dark-green for even as long as 35 days. Ventral scales started to turn violet at the end of the culture period only. The violet colouring agent is bound to the cell wall as can be seen by plasmolyse experiments. Primarily investigations have shown this pigment being different from anthocyanins and sphagnorubins, membranochromes found in *Sphagnum* species [17]. Its nature has not yet been determined.

Growth expressed by fresh weight was best on B5 medium (Fig. 2 and 4) reaching a maximum of 18.5 g/flask after a five weeks-cultured period. On the other media growth was from 5.5 g/flask to

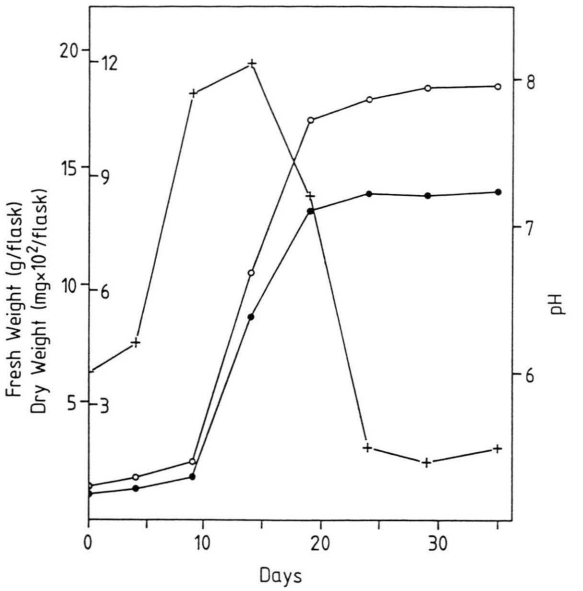

Fig. 2. Growth of an axenic culture of *Ricciocarpos natans* and changes in the pH of the medium. Gamborg B5 medium, 1% sucrose, ○ = fresh weight (g/flask); ● = dry weight (mg/flask); + = pH of the medium.

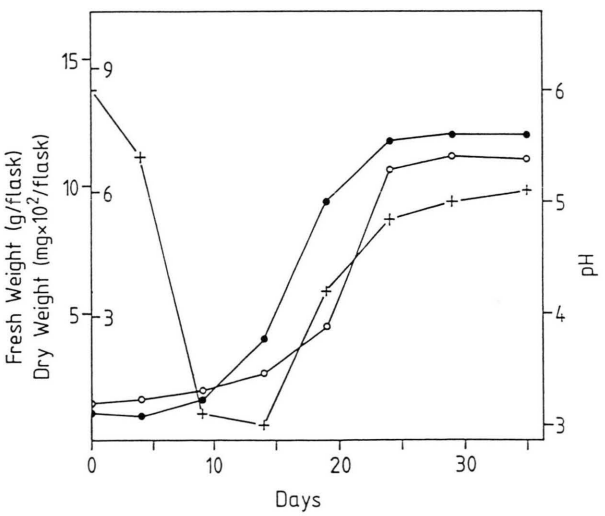

Fig. 3. Growth of an axenic culture of *Ricciocarpos natans* and changes in the pH of the medium. Knudsen's medium, 1% sucrose. ○ = fresh weight (g/flask); ● = dry weight (mg/flask); + = pH of the medium.

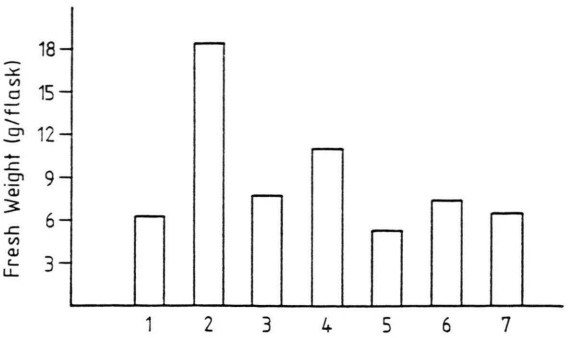

Fig. 4. Growth of an axenic culture of *Ricciocarpos natans* on different mineral media. Fresh weight (g/flask) after a 35 days' culture period. 1 = Benecke's medium; 2 = Gamborg B5 medium; 3 = Knop b medium; 4 = Knudsen's medium; 5 = Lorenzen's medium; 6 = MSK-2 medium; 7 = White's medium.

11.0 g/flask (Fig. 4). There was no correlation between the absolute amount of the mineral salts in the media and the growth of the cultures.

The differences in dry weight were less pronounced since the percentage of dry weight on B5 medium is only 4.6% compared to 6.7% on Knudsen's and to values of 9.2% to 10.0% on the other media. Reducing the amount of nitrate or phosphate in B5 medium to 1/10 of their original concentration fresh weight decreased, as expected, to 45% and 75%, respectively, but dry weight on the medium with reduced nitrate only decreased to 80%. Thus the percentage of dry weight on B5 medium with reduced nitrate is just in the range of the other media.

Changes of pH-values in the media during one passage showed two different patterns. In B5 (Fig. 2), Knop's, Lorenzen's, and White's medium pH increased from 6.0 to a value above 7.5 during the first two weeks to drop to about 5.5 at the end of the culture period. In the other three media, Benecke, Knudsen (Fig. 3), and MSK-2, pH dropped during the first ten to fourteen days to a value between 3.0 and 3.5, then increased to reach a final value of 5.1 for Knudsen and MSK-2 or 4.2 for Benecke.

The changes in pH for MSK-2 medium have already been described for the liverworts *Marchantia polymorpha* [12, 18] and *Jungermannia subulata* [19]. pH-changes reflect the utilization of nitrate and ammonium by the cultures. Ammonium is first taken up and pH drops simultaneously. Ni-

trate is only utilized when ammonium is exhausted causing a rise of pH in the medium. In Benecke and Knudsen the molar ratio of ammonium to nitrate is about 1:1 and in MSK-2 1:1.9. In B5 medium the molar ratio of ammonium to nitrate is 1:12.5 and in the other three media (Knop b, Lorenzen, and White) with an initial rise in pH nitrate is the only nitrogen source. When nitrate in B5 medium was reduced to 1/10 so that the molar ratio of ammonium to nitrate changed to 1:1.2, pH during a passage dropped first, as expected, to 3.2 and then increased to 5.2.

Liverworts can be grown photoautotrophically on mineral media without any addition of a carbon source [10]. As early as 1962, Machlis [20] showed that the growth of the *in vitro* cultured liverwort *Sphaerocarpos donnellii* can be multiplied by adding sugar to the medium. For other liverworts it has been demonstrated that glucose and sucrose as carbon sources have an optimum in stimulating growth at certain concentrations and lead to callus induction at concentrations from 2% to 4% [5, 21, 22]. According to a review by Ohta and Hirose [23] and by Ono *et al.* [22] callus cultures have been established for 25 liverwort species. Ono *et al.* [22] also report callus induction for *Ricciocarpos natans* with 2% glucose.

We tested B5 medium without any carbon source and with sucrose and glucose at concentrations of 0.5%, 1%, 2%, and 4% for their effects on growth and for morphological effects in cultures of *Ricciocarpos natans*. Growth without any carbon source was very poor (Fig. 5). Stationary phase was already reached after two weeks and increase of fresh weight was only 2.5-fold. Sucrose and glucose addition stimulated growth to a high extent. Optima for both sugars were 2% with the maximum of 13-fold fresh weight increase for sucrose. Glucose was slightly less effective. A concentration of 4% sugar does not lead to callus induction, the thalli, however, show significant morphological changes. Their size was reduced and the surface was curved to the ventral side; the number of rhizoids was increased. The rhizoids as well as the ventral scales at 4% sugar were shorter than those of plants grown at lower sugar concentrations.

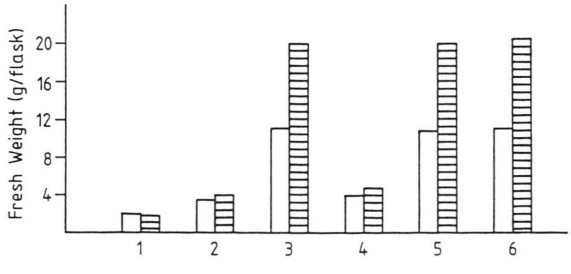

Fig. 5. Effects of light intensity and light regime on the growth of an axenic culture of *Ricciocarpos natans*. Fresh weight (g/flask) after a 14 days' (left) and a 24 days' (right) culture period on Gamborg B5 medium. 1 = dark, 2% sucrose; 2 = 24 h light, 2000 lx, no sucrose; 3 = 24 h light, 2000 lx, 2% sucrose; 4 = 24 h light, 6000 lx, no sucrose; 5 = 24 h light, 6000 lx, 2% sucrose; 6 = 14 h light/10 h dark, 6000 lx, 2% sucrose.

Lorenzen *et al.* [8] have observed a better growth at higher light intensities in a 2% CO_2 atmosphere. We tested *Ricciocarpos natans* in the dark, with 24 h continuous illumination at 2000 lx and 6000 lx, and at 6000 lx and 14 h light and 10 h dark. Two experiments at 2000 lx and 6000 lx were run without sucrose. The other experiments were run with B5 medium containing 2% sucrose. The results (Fig. 5) show that the tested light intensities and photoperiod have no influence on growth of *Ricciocarpos natans*. However, the culture did not grow in the dark, and plants kept in the dark showed a decrease in chlorophyll content and started to turn brown from the basal part.

Production of terpenoids

The axenic culture of *Ricciocarpos natans* enabled the production of sufficient amounts of plant material for chemical analysis. 1050 g of fresh weight were extracted and the following terpenoids isolated and their structures elucidated by spectroscopical methods [15]: (−)-limonene (**1**), cuprenolide (**2**), cuprenolidol (**3**), ricciocarpin A (**4**), ricciocarpin B (**5**), ricciofuranol (**6**), and phytol (**7**) (Fig. 1). Ricciocarpin A (**4**) was also isolated from field-collected material. A comparison by thin-layer chromatography showed a good resemblance of the two sources with ricciocarpin A (**4**) as the main constituent in both field-collected and -cultivated material.

Of the isolated compounds, (−)-limonene (**1**), cuprenolide (**2**), ricciocarpin A (**4**), and phytol (**7**) were quantified by gas chromatography in dependence of culture conditions during one culture period. The content of the four terpenoids, calcu-

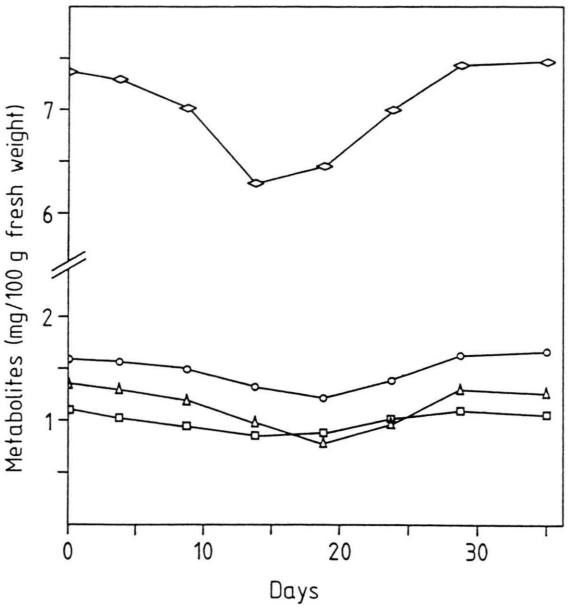

Fig. 6. Production kinetics of an axenic culture of *Ricciocarpos natans* grown on Gamborg B5 medium. Content (mg/100 g fresh weight) of ○ = (−)-limonene, □ = cuprenolide, ◇ = ricciocarpin A, and △ = phytol.

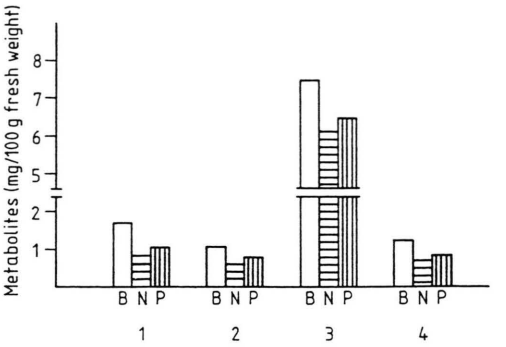

Fig. 7. Effects of nutrient deficiencies on the terpenoid production of an axenic culture of *Ricciocarpos natans*. Content (mg/100 g fresh weight) of 1 = (−)-limonene, 2 = cuprenolide, 3 = ricciocarpin A, and 4 = phytol after a 35 days' culture period. B = Gamborg B5 medium; N = Gamborg B5 medium, nitrate-reduced to 1/10; P = Gamborg B5 medium, phosphate-reduced to 1/10.

lated per fresh weight, decreased during the first two weeks (Fig. 6). Cuprenolide (**2**) and ricciocarpin A (**4**) show a slight increase between the second and the third week while limonene (**1**) and phytol (**7**) still decrease. An increase of all four terpenoids takes place after three weeks after which time the cultures reach the stationary phase. Both the decrease and the increase were shown to be significant (error probability $\alpha < 0.001$ for decrease and increase of limonene (**1**), ricciocarpin A (**4**), and phytol (**7**) and for increase of cuprenolide (**2**); $\alpha = 0.012$ for decrease of cuprenolide (**2**)).

Plant tissue cultures often produce higher amounts of secondary metabolites on media with nitrogen or phosphorus deficiencies [24, 25]. For *Ricciocarpos natans* no such effect could be observed when nitrate or phosphate were reduced to 1/10 of their usual concentrations. The content of the four terpenoids was even lower on the deficient media (Fig. 7). Light intensity (2000 lx *versus* 6000 lx continuous illumination) had no effect on terpenoid production. Light regime, 14 h light/10 h dark, at 6000 lx caused a reduction of about 10% for limonene (**1**) and ricciocarpin A (**4**) and about 20% for cuprenolide (**2**) and phytol (**7**). As described in the previous chapter this light regime had no influence on fresh weight.

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