Cell Wall Pigment Formation of *in vitro* Cultures of the Liverwort *Ricciocarpos natans**

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The liverwort *Ricciocarpos natans* has been cultivated on Gamborg B5 medium with different levels of ammonium, nitrate, phosphate and sucrose. The formation of the cell wall pigments riccionidin A and B was shown to be dependent on the level of these nutrients as well as on the intensity of light. A decrease in nitrogen supply (mainly nitrate) induced the formation of both wall pigments, whereas phosphate and high levels of sucrose inhibited the pigment synthesis. Decreasing the illumination rate led to lower anthocyanidin contents. Dedifferentiated cultures also produced wall pigments, but their amount was much lower compared to differentiated cultures.

Introduction

Numerous species of bryophytes are known to form reddish or violet pigments predominantly attached to the cell wall (Herzfelder, 1921). Up to now the knowledge about the chemistry of these compounds (Bendz *et al.,* 1962, 1963; Vowinkel, 1975; Mentlein and Vowinkel, 1984) and the factors influencing their formation (Rudolph, 1964) is very low. Recently we reported the isolation and structure elucidation of the wall pigments riccionidin A (Fig. 1) and B (a dimeric compound based on riccionidin A, exact structure still unknown) from *R. natans* grown in axenic culture (Kunz *et al.,* 1994). Using *in vitro* cultures of *R. natans* we studied the formation of these compounds during growth and the dependency of their formation on several nutrients and illumination.

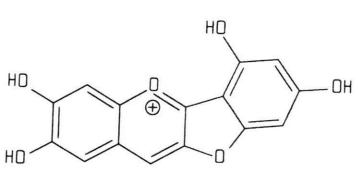

Fig. 1. Riccionidin A.

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Materials and Methods

Aseptic cultures of *R. natans* were derived from cultures obtained from the Institute of Botany, Czecho-Slowak Academy of Sciences, Trebon, formerly C.S.F.R. These cultures had been isolated in 1947 and 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 were grown in 200 ml Erlenmeyer flasks containing 70 ml liquid B5 medium (Gamborg *et al.,* 1968), respectively, with trace elements of MS (Murashige and Skoog, 1962) and 2% sucrose. The flasks were kept under constant illumination (5000 lx; white light, Universalweiß/25, Fa. Osram, Fernheim) at 22 °C. Inoculum size was 2 g fresh weight. Suspension cultures were shaken on a reciprocal shaker at 120 rpm. These cultures were grown on the same basal medium, but containing 4% glucose. Calli were induced on solidified B5 medium with 6% glucose at 500 lx.

For testing the influence of nitrogen supply the content of available nitrogen in the medium was reduced from 27 mmol/l to 4.5 mmol/l. This was achieved by reducing either only the nitrate content (from 25 to 2.5 mmol/l; → nitrate deficiency medium) or both nitrate (from 25 to 4.2 mmol/l) and ammonium content (from 2 to 0.3 mmol/l; → nitrate/ammonium deficiency medium). For testing the influence of phosphate the content of phosphate was reduced to a tenth of

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the original concentration in the medium (from 1 to 0.1 mmol/l). The tests on the influence of sucrose were performed with 4% sucrose (instead of 2%). Tests with reduced illumination were carried out at 2000 lx.

The sucrose content was determined with an enzyme kit "sucrose/glucose" from Boehringer Mannheim (Mannheim). Ammonium and phosphate were determined using "spectroquant" test systems from Merck (Darmstadt). A nitrate selective electrode (Model 93-07, Orion Research Inc., Boston) was used for analysis of nitrate content.

Quantitative analysis of riccionidin A and B was performed as follows: each sample (about 100 mg) of freeze-dried, milled plant material was extracted with 10 ml MeOH–HCl (99.5 + 0.5) utilizing a Branson Sonifier B12 equipped with a titan microtip. The extract was filtered and evaporated to dryness. The residue was redissolved in 1 ml MeOH–H_2O–HCl (80 + 20 + 1) and subjected to solid phase extraction using an Adsorbex RP18 column (Merck, Darmstadt). The eluate was diluted to 2.0 ml using H_2O–HCl (100 + 1) and 100 µl of this solution were subsequently analyzed by HPLC: column, LiChrosorb RP18 7 µm (Merck, Darmstadt), 4×250 mm; eluent, MeOH–H_2O–trifluoroacetic acid (45 + 55 + 1); flow rate 1.0 ml/min; detection, absorption at 490 nm; retention times, riccionidin B 6.25 min, riccionidin A 9.60 min; quantification by peak area, external standard method, integration software, Hyperdata Chromsoft V2.06 (Bischoff, Leonberg). Method validation: linearity (0.2–10.0 µg/ml), 6 concentrations, three runs respectively, r = 0.9999; recovery, 92%; reproducibility, RSD = 1% (six-fold analysis).

Results and Discussion

Under standard conditions (B5 medium) differentiated cultures of *Ricciocarpos natans* showed a growth curve with typical sigmoid pattern (Fig. 2) and similar increase of fresh and dry weight. Stationary phase was reached after about 4 weeks. Beginning after 3 weeks reddish pigmentation was visually detectable. The ventral side and the basal edges of thalli first turned to red. Then the reddish-black colouration further progressed to the growing tip and finally to the middle of thalli. Analysis of extractable pigments showed that the formation of riccionidin A and B had already started a week before visual detection was possible (Fig. 3). After 3 weeks then the content of both pigments strongly rose. The fact that the nutrients tested for have been consumed at the same time (Fig. 4) indicated a link between nutrient supply and pigment formation. Although the reddish-black colour of the thalli became more intense towards the end of the period, the extractable amount of riccionidin A and B significantly decreased after 5 weeks, which may possibly be caused by an increased polymerization or attachment to the cell wall.

For testing the influence of nitrogen supply on growth and pigment formation *R. natans* was grown on a modified nutrient medium containing only a tenth of the original nitrate concentration

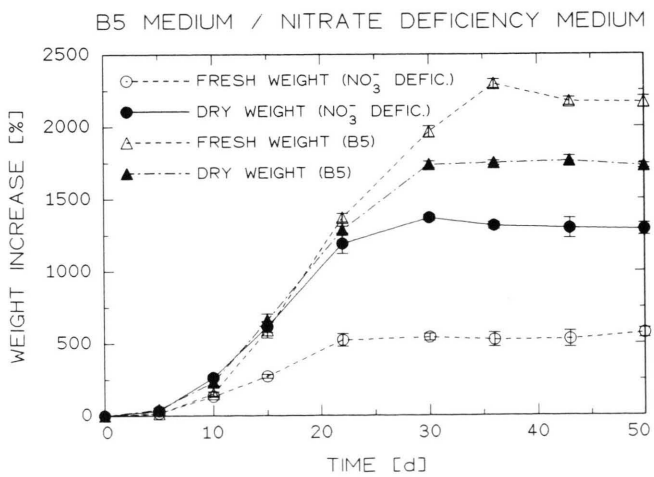

Fig. 2. Fresh and dry weight increase of *R. natans* cultures on B5 medium or nitrate deficiency medium (mean of five flasks, respectively; each bar marks standard deviation).

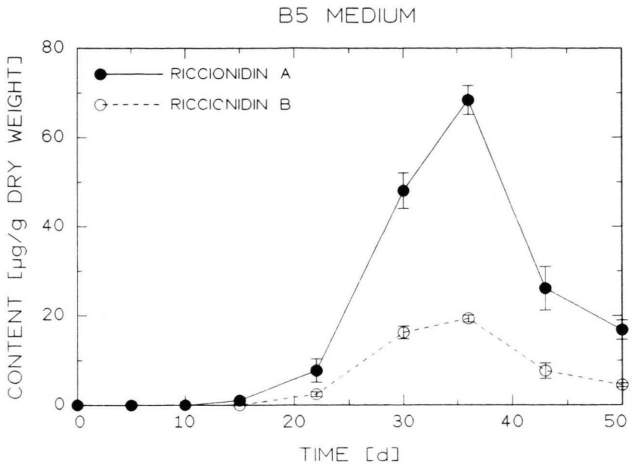

Fig. 3. Content of riccionidin A and B in *R. natans* on B5 medium (mean of four flasks, respectively; each bar marks standard deviation).

of the B5 medium. Again the growth curves were sigmoid. The fresh weight of the thalli however rose much less compared to the growth on B5 medium, whereas the increase of dry weight was equivalent to the standard conditions (Fig. 2). Analysis of pigment content (Fig. 5) showed, that the deficiency of nitrate significantly enhanced the formation of both riccionidin A and B, which has also been reported for the formation of anthocyanins (Yamakawa *et al.*, 1983; Do and Cormier, 1991) and sphagnorubins (Rudolph, 1964). As shown in Fig. 5, both riccionidins have already been formed in detectable amounts after 5 to 10 days, when all the nitrate had been consumed. But higher amounts of both pigments were not accumulated, until also the phosphate had been totally consumed. Presumably the phosphate still present in the medium inhibited the pigment formation, an effect which has also been observed in cell cultures of *Vitis* species (Yamakawa *et al.*, 1983). Similar results for growth and pigment formation were obtained when the ratio of nitrate and ammonium in the N-deficiency medium was changed from 2.5:2 mmol/l (nitrate deficiency) to 4.2:0.3 mmol/l (nitrate/ammonium deficiency). This showed that in opposite to the results from *Vitis* species (Yamakawa *et al.*, 1983) the ammonium to nitrate ratio had no significant influence on the pigment formation.

Growing *R. natans* on a phosphate deficiency medium both riccionidins could also be detected after 5 days. Immediately after consumption of the phosphate the content of both pigments strongly increased to a maximum at the 15th day. After-

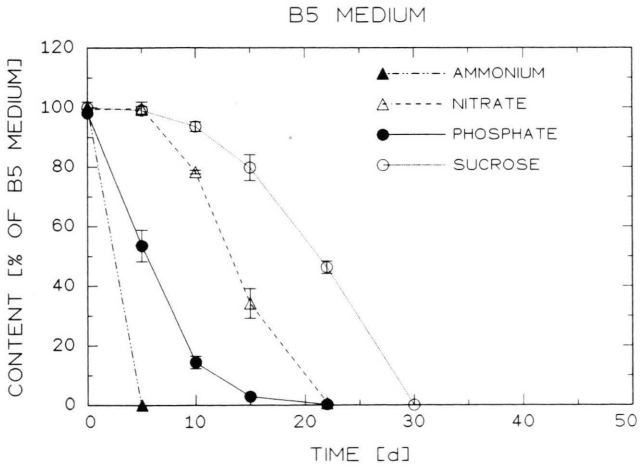

Fig. 4. Content of essential nutrients in the B5 medium during growth of *R. natans* (mean of five flasks, respectively; each bar marks standard deviation).

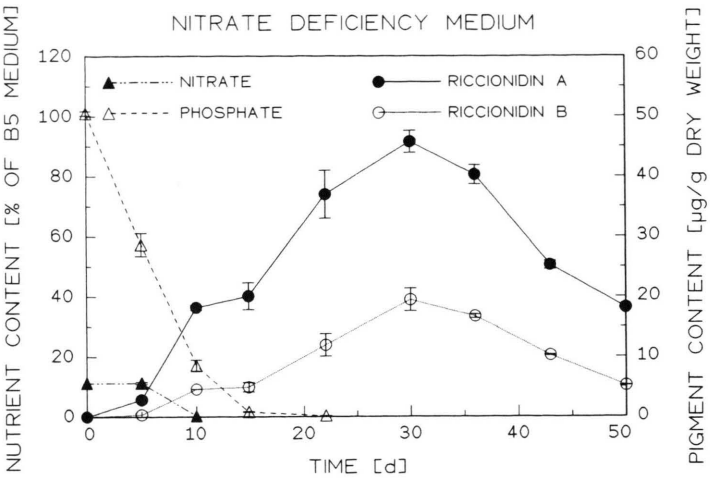

Fig. 5. Content of riccionidin A and B in *R. natans* on nitrate deficiency medium and content of nitrate and phosphate in the medium.

wards the content of the extractable pigments dropped down again, as already observed on the standard medium and the nitrate deficiency medium as well. Furthermore the pigment formation and nutrient content of the medium revealed that – in contrast to the observation for phosphate – high amounts of nitrate did not inhibit the biosynthesis of both riccionidins.

As shown in Fig. 6, a lack of both nutrients caused an increase in pigment content surpassing each of the individual deficiency effects. Moreover the content did not decrease towards the end of the observation period which differed from the observations made for the standard conditions and the other deficiency media.

In order to test the influence of high sugar concentrations on the pigment synthesis, *R. natans* was also grown on a B5 medium containing double the amount of sucrose (4%). Whereas the growth was only slightly affected (a small increase of dry weight compared to standard conditions), the formation of riccionidins was strongly reduced to a tenth compared to the content on the medium with 2% sucrose. On the nitrate/phosphate deficiency medium the concentrations of riccionidins also decreased when the sucrose content was increased from 2 to 4%. Therefore an increase of sugar content in the medium is not appropriate to enhance the formation of riccionidins, which is in contrast to the results from studies on structurally related compounds, *i.e.* anthocyanins (Do and Cormier, 1990, 1991; Yamakawa *et al.*, 1983; Yamamoto *et al.*, 1989) and sphagnorubins (Rudolph, 1964).

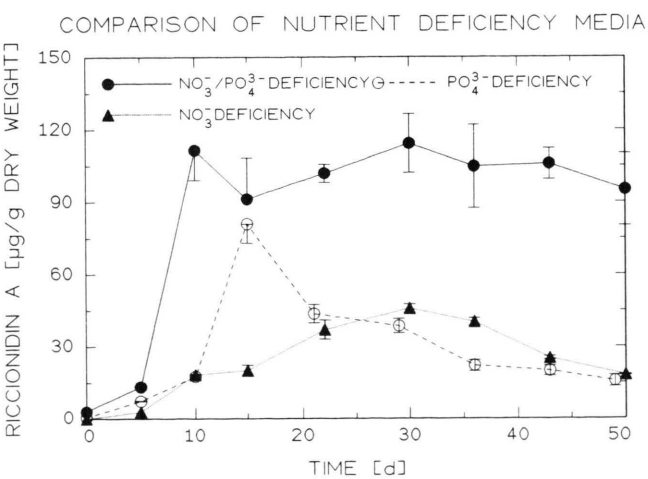

Fig. 6. Comparison of riccionidin A content in *R. natans* grown on nitrate deficiency medium, phosphate deficiency medium or nitrate/phosphate deficiency medium.

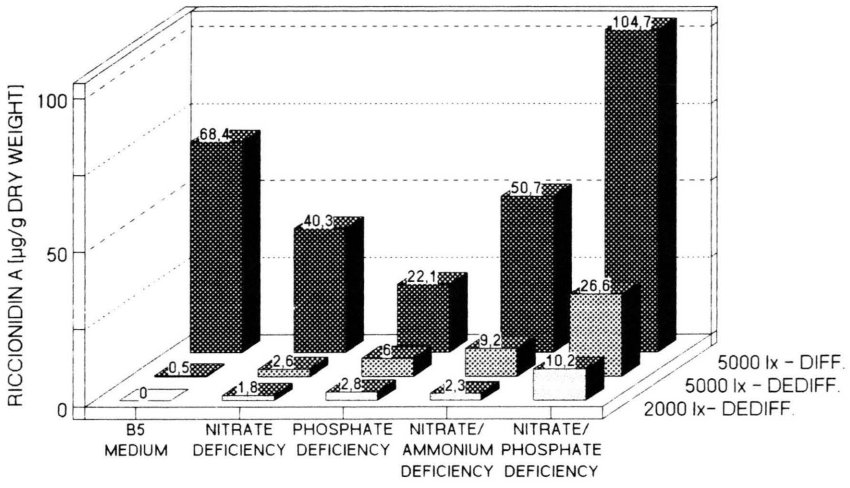

Fig. 7. Comparison of riccionidin A content in cell suspension cultures of *R. natans* grown at 2000 lx or 5000 lx and differentiated cultures grown at 5000 lx, after 35 days on five different media respectively (mean of five flasks).

In addition to testing the nutrient influence, the effect of illumination rate on pigment formation was studied on dedifferentiated cell suspension cultures of *R. natans*. Whereas growth was not affected by the change of illumination rate (not illustrated), the pigment formation decreased on each nutrient medium when a lower illumination rate (2000 lx compared to 5000 lx) had been applied (Fig. 7). This is in accordance with the results obtained for *Vitis* (Yamakawa *et al.*, 1983) and *Sphagnum* (Rudolph, 1964).

In addition to this the comparison of the pigment content between differentiated and dedifferentiated cultures of *R. natans* grown on several media (Fig. 7) revealed in each case a significantly lower riccionidin content of the dedifferentiated cell suspension culture. A similar decrease in secondary metabolites content has previously been reported for *Reboulia hemisphaerica* when the plant is growing in the dedifferentiated form (Morais and Becker, 1991). However, in the case of *R. natans* the lower level of pigments might also be caused by the high content of sugar in the medium necessary to maintain the cell culture in the dedifferentiated form.

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