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Secondary chemistry and DNA-analyses of the Australian lichen *Heterodea muelleri* (Hampe) Nyl. and culture of the symbionts

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Abstract
The study aimed to figure out suitable culture conditions and nutrient requirements for the biosynthesis of secondary metabolites in the cultured mycobiont of *Heterodea muelleri*. The production of biologically active substances like usnic acid and diffractaic acid in the cultured mycobionts was of high interest. The collected thalli contained diffractaic acid as the major substance in all samples, barbatic acid as a minor compound and 4-O-demethyldiffractaic acid in traces. The cultured *Heterodea*-mycobionts produced secondary metabolites only under stress-conditions, e.g. shortwave UV-light and low temperatures. Both stress parameters initiated the production of the same substances as in the voucher specimens, but in case of barbatic acid in much higher concentrations. Moreover, the stressed mycobionts produced 4-O-demethylbarbatic acid in minor quantity. All secondary compounds were analysed by HPLC, TLC and an additional mycrocrystallization test. DNA-analyses confirmed the identity of the aposymbiotically grown mycobionts with the original lichen samples. In a further test series, the photobiont of *H. muelleri* was isolated and cultured. DNA-analyses and microscopical investigations demonstrated that *Trebouxia jamesii* is probably the algal symbiont of *H. muelleri*.

Keywords: *Heterodea*, lichen culture, diffractaic acid, barbatic acid, UV stress, temperature stress

1. Introduction

Recent culture experiments (e.g. Adler et al., 2003) clearly showed that 'standardized culture conditions' inhibited the formation of secondary compounds in mycobiont cultures. Instead of the typical lichen substances, fatty acids and triglycerides were formed. By studying published results (e.g. Yamamoto et al., 1987; Hamada, 1989; Yoshimura et al., 1994a,b; Yamamoto et al., 1995; Hamada et al., 1996) and by further experimentation it became obvious that various internal and external factors were involved. A complex interrelationship of environmental factors, specific nutrient requirements and a considerable morphogenetic capacity of mycobionts were found to influence the induction of secondary pathways, and the production of specific lichen substances in culture (Stocker-Wörgötter, 2001a,b, 2002).

DNA-analyses are very important routines for examining the identity of cultured mycobionts, since lichenicolous and parasitic fungi may grow within and on the lichens, which may confuse molecular identification. Our studies showed that higher amounts of DNA could be extracted for sequencing from cultures than from the tested voucher specimens (Stocker-Wörgötter, 2001a).

The Australian lichen flora has already been intensively studied morphologically and taxonomically (e.g. Elix, 1994; Filson, 1994; McCarthy, 2003); our investigation intends to be a first experimental approach studying the chemical profile of selected cultured mycobionts, e.g. *Heterodea muelleri*.

The Heterodeaceae is a monogeneric family with two species in Australia and New Zealand. *H. muelleri* occurs in all States of Australia, the Northern island of New Zealand and also in New Caledonia. The lichen grows loosely fixed to the ground among grasses and litter, occasionally on damp logs or tree bases in forests (Filson, 1978).

Heterodea muelleri (Parmeliaceae, Fig. 1) is a foliose lichen growing on soil and can be recognized by its eye-catching greenish color. The thalli produce the depsides diffractaic acid and barbatic acid as secondary compounds, which were identified by two different methods (HPLC, TLC). Filson (1992) reported that there should be also the dibenzofurane usnic acid in the cortex, which could not be detected in our study. The aim of the study was to figure out suitable culture conditions and nutrient requirements for the biosynthesis of secondary metabolites in the cultured mycobiont of *Heterodea muelleri*. The production of biologically active substances in the cultured mycobionts is of high interest, as they are a source of potential anti-tuberculosis agents (Ingólfsdóttir et al., 1998).

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2. Material and Methods

Voucher specimen

The three voucher specimens were collected in SE-Australia, on the hills of Canberra (Mt. Ainslie, Mt. Majura and Black Mountain) in the Australian Capital Territory. Two lichen collections were sterile, whereas that from Mt. Ainslie had apothecia.

Culture methods

'Tissue' (fragment) culture method (Yamamoto-method; Yamamoto, 1990, modified)

Small pieces of the lichen thalli were washed in bidistilled water containing a drop of Tween 80 and afterwards homogenized in sterile water using a mortar and a pestle. Then the suspension was filtered through two sieves (with 500 and 150 µm mesh size). Lichen-fragments of 150–300 µm size were transferred into tubes (containing various media e.g. MS, S2% and LBM+S) using bamboo sticks. This method was used both for myco- and photobionts. After 2–3 months incubation in darkness, small fungal or algal colonies had formed. The mycobionts were gently homogenized to obtain subcultures on different media (e.g. MS, M-Y, LBM, LBM+S, PDA, S2%). The algae were subcultured in Petri dishes containing M-Y medium.

Single spore isolation (Ahmadjian, 1973)

Apothecia were cut off the thallus and transferred to 10 ml of water in a beaker (for 10 minutes). After removal of the water film using a filter paper, the apothecia were fixed to the top cover of a petri dish with petrolium jelly. Spores were discharged downwards in a petri dish containing sterile S2% medium. Within two days, spores were discharged to the surface of the agar plate.

Afterwards, the cover containing apothecia was removed to avoid further contaminations. Germinated single spores were transferred to tubes containing S2% medium. Mycelia that developed from these spores (incubation of 4–6 months) were gently homogenized to obtain subcultures on different test media (e.g. MS, M-Y, LBM, LBM+S, PDA, S2%).

Culture media

LBM [Lilly & Barnett medium (Lilly and Barnett, 1951)]. LBM+S [Lilly & Barnett medium with soil extract (Lilly and Barnett, 1951)]. The soil extract was prepared by the method of Esser (1976). MS [Murashige & Skoog medium (Stocker-Wörgötter, 2001b)]. M-Y [Malt-Yeast medium (Yamamoto, 1990)]. PDA [Potato-Dextrose-Agar (Sigma P-2182)]. S2% [Sabouraud-2%-Glucose-Agar (Fluka 84086)].

Culture conditions

The cultures were maintained in a culture chamber under a changing 12h/27°C light and 12h/24°C dark regime and a light intensity of 50–100 µE $m^{-2}s^{-1}$.

Stress parameters

Well-developed subcultures (spore isolates from Mt. Ainslie samples) of *Heterodea muelleri*, grown on Sabouraud 2% medium for 4 months, were subjected to a drop of temperature (14h/20°C light and a 10h/10°C dark cycle and a light intensity of 50–100 µE $m^{-2}s^{-1}$) for 6 weeks. A control was kept under the original standard conditions (12h/27°C light and a 12h/24°C dark cycle and a light intensity of 50–100 µE $m^{-2}s^{-1}$). Methanol-extracts of the freeze-dried cultures were analysed by HPLC.

Subcultures of the same lichen, but grown on Murashige & Skoog medium, were exposed to shortwave UV-light (254 nm) for one hour each day at room temperature for a period of two weeks. The cultures were maintained under the standard conditions (as described above) the rest of the time. A control was kept as a reference. Methanol-extracts of the freeze-dried mycobionts were analysed by HPLC.

Chemical analyses and preparation of the specimens

Extraction and preparation of the thallus samples

5–6 thallus pieces (c. 3–5 mm in diam.) were transferred to glass tubes and extracted with methanol for 4 hours. The extracts were then transferred to HPLC vials and an aliquot of 20 µl from every sample was injected.

Extraction and preparation of the cultured mycobionts

Circular plugs (c. 1 cm in diam.) were cut out from every agar plate overgrown by the mycobiont, and the origin of each subculture was recorded. To prevent contamination, the plugs for chemical testing were prepared in a hood under sterile conditions. The samples were vacuum freeze-dried at –42°C for 24 hours, using an ice condenser connected to a vacuum pump. The dried discs were transferred to glass tubes and extracted with methanol for 4 hours. Such extracts were then transferred to HPLC vials and an aliquot of 20 µl from every sample was injected.

HPLC analyses

Natural compounds were characterized by high-performance liquid chromatography (HPLC) with retention index values (RI) calculated from benzoic acid and solorinic acid controls (Elix, 1996; Elix and Wardlaw, 2000; Feige et al., 1993). For HPLC a Merck Hitachi Spectra System and a Beckman 5C18 column, 250 by 4.6 mm and spectrometric detectors operating at 254 nm with a flow rate of 1 ml/min were used. Two solvent systems, A and B were used: 1% aqueous ortho-phosphoric acid and methanol in the ratio 7:3 (A) and methanol (B). The run started with 100% A, and was raised to 58% B within 15 min, then to 100% B within a further 15 min, followed by isocratic elution in 100% B for a further 10 min. The HPLC was coupled to photodiode array detectors for ultraviolet spectroscopic comparisons. By this means the ultraviolet spectra observed for the various components eluting in the HPLC chromatogram were recorded and computer-matched against a library of ultraviolet spectra recorded for authentic metabolites under identical conditions.

TLC

Standardized method for TLC of lichen substances (Culberson and Kristinsson, 1970; Culberson and Ammann, 1979; Culberson and Johnson, 1982) with atranorin and norstictic acid as references.

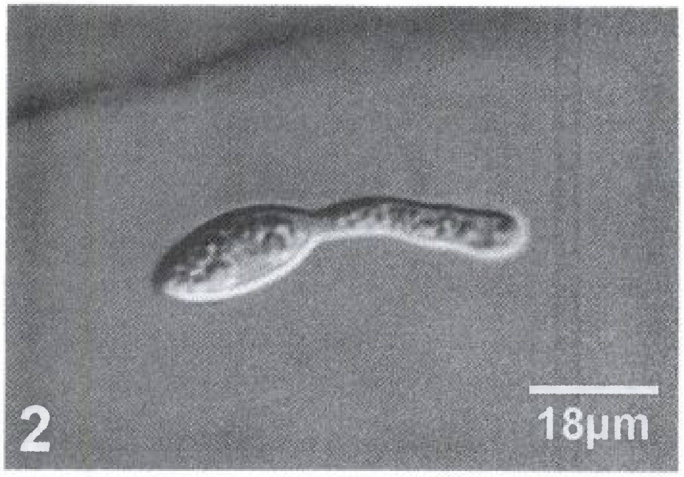
DNA analyses

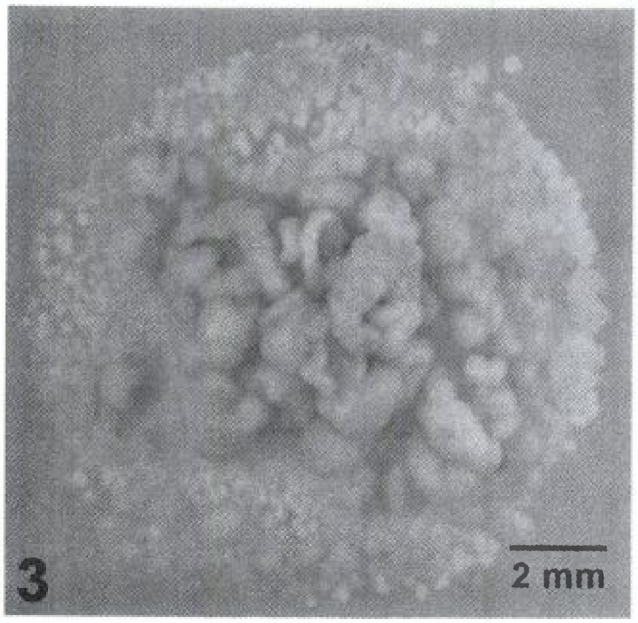
DNA-extraction

Total DNA was extracted from cultured mycobionts and for the comparisons from the voucher specimens (Armaleo and Clerc, 1995).

DNA-amplification and purification

The ITS-regions, the 5.8 region and the flanking parts of the small and large subunits (SSU 18S and LSU 28S) of the rDNA were amplified using a GeneAmp PCR System thermal cycler. Primers for the PCR were ITS1F (Gardes and Bruns, 1993), ITS2 and ITS4 (White et al., 1990) designed for the lichen fungus. The PCR mix contained 1.25 units of Dynazyme Taq polymerase (Finnzymes), 0.2 mM of each of the four dNTPs, 0.5 µM of each primer and





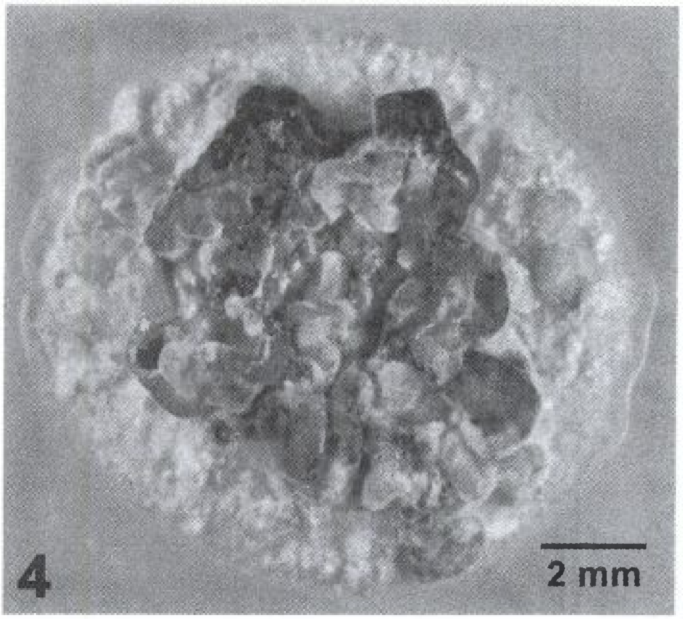


Figure 1. *Heterodea muelleri* thallus.

Figure 2. Germinated spore of *Heterodea muelleri.*

Figure 3. Cultured mycobiont (*Heterodea muelleri*) on Sabouraud 2% medium, forming clumpy colonies without substances.

Figure 4. Cultured mycobiont (*Heterodea muelleri*) on Murashige & Skoog medium, producing an unknown brown pigment.

10–50 ng genomic DNA. The PCR products were cleaned by using a Quiaquick PCR product purification kit. Sequences were run on a ABI 310 automated sequencer. For the alignments of the sequences, a Pile-up Programme was used and the adjustments were done manually. For the photobionts, special *Trebouxia* primers (ITS1T and ITS4T, Kroken and Taylor, 2000) were used.

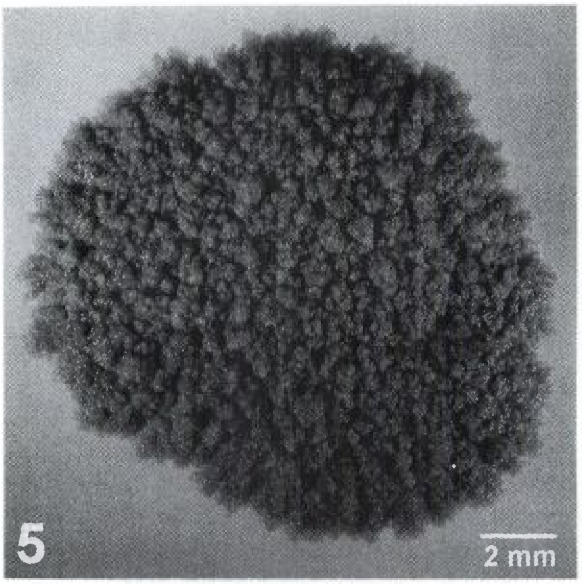
Microcrystallization test

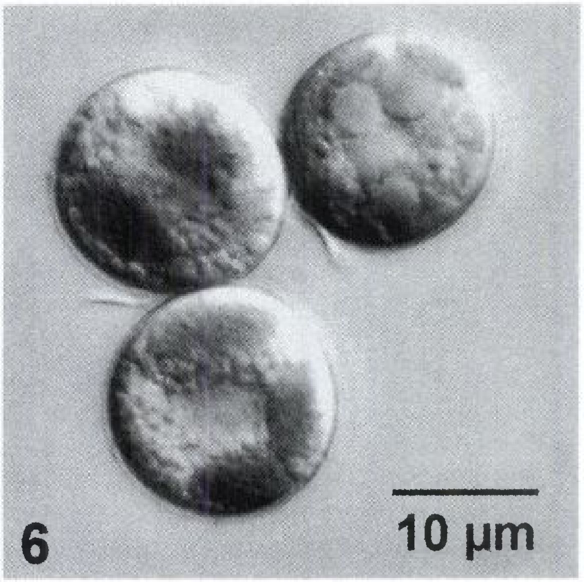
The lichen substances were extracted from the thalli with acetone, and afterwards recrystallized with glycerine-acetic acid (Asahina, 1940; Huneck and Yoshimura, 1996).

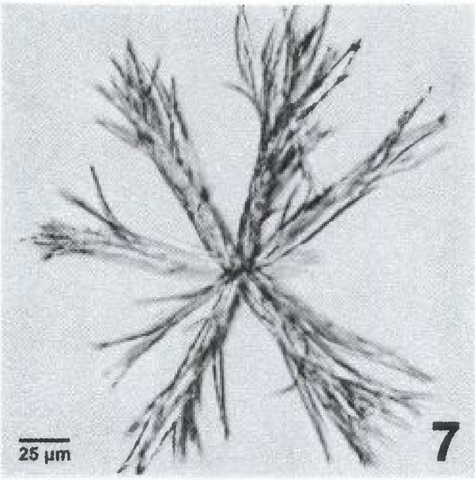
3. Results

The HPLC analyses of *Heterodea muelleri* demonstrated that all thallus samples contained diffractaic acid as a major substance and barbatic acid as a satellite compound (Figs. 9, 11 and 13). 4-O-demethyldiffractaic acid was found as a trace in all samples. TLC and the microcrystallization test confirmed diffractaic and barbatic acid to be present in the lichen thalli (Figs. 7, 8).

Comparing the isolations of the three voucher specimens, the mycobiont of Mt. Anslie exhibited the highest growth rates, both on S2%-medium and MS-medium.







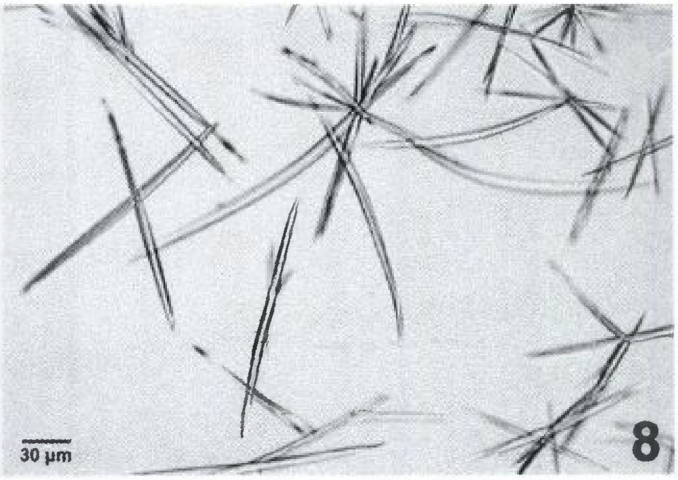
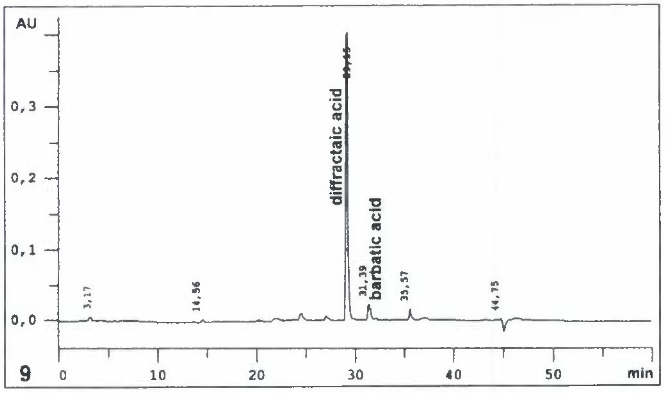


Figure 5. Cultured photobiont (*Heterodea muelleri*) on malt yeast agar.
Figure 6. *Trebouxia* sp. (*jamesii*), isolated from *Heterodea muelleri*.
Figure 7. Diffractaic acid from *Heterodea muelleri* thallus, recrystallized with glycerine-acetic acid.
Figure 8. Barbatic acid from *Heterodea muelleri* thallus, recrystallized with glycerine-acetic acid.



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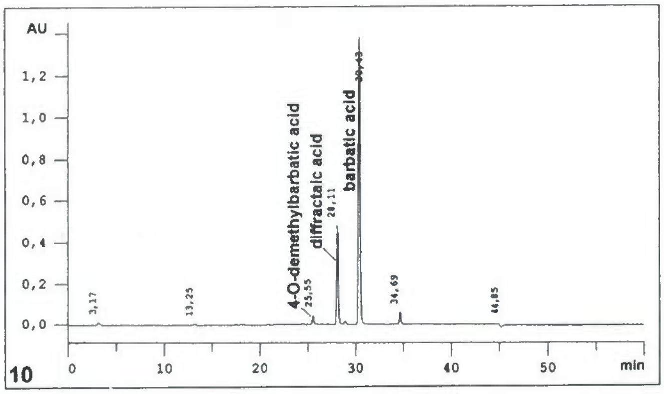

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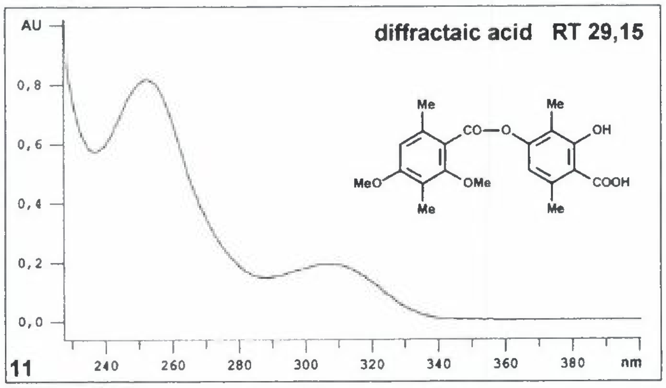

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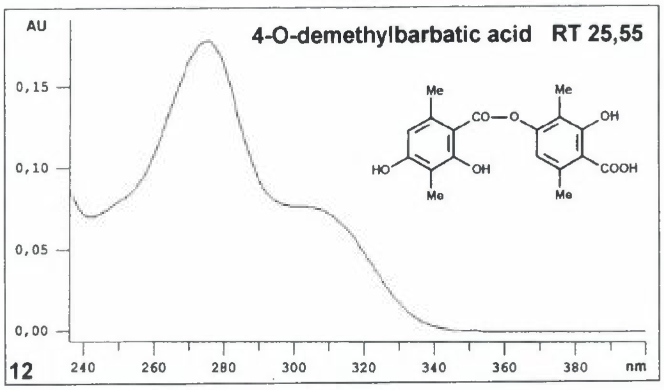

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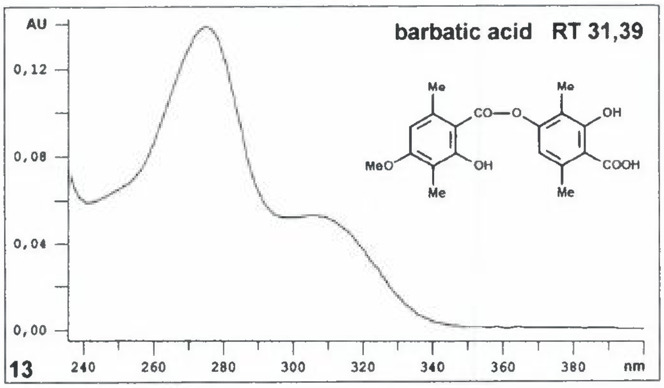

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Figure 9. HPLC Chromatogram of the thallus of *Heterodea muelleri*.
Figure 10. HPLC Chromatogram of the cultured mycobiont of *Heterodea muelleri* maintained under temperature stress.
Figure 11. UV spectrum of diffractaic acid from the thallus of *Heterodea muelleri*.
Figure 12. UV spectrum of 4-O-demethylbarbatic acid from the cultured mycobiont of *Heterodea muelleri* maintained under temperature stress.
Figure 13. UV spectrum of barbatic acid from the thallus of *Heterodea muelleri*.

On the other tested nutrient media (LBM, LBM+S, M-Y and PDA), the mycobionts showed either poor development or contaminations. When grown on MS-medium, the *Heterodea muelleri* fungus produced an unknown brown pigment which could not be identified by HPLC.

The mycobiont (Black Mountain sample) grew very slowly on every test medium which excluded further experiments. All culture efforts of the Mt. Majura mycobiont failed because of contaminations. However, three single spore isolations of the Mt. Ainslie thallus successfully germinated and formed a mycelium that could be subcultured (Figs. 2, 3). The spore-derived subcultures showed the same medium preferences (e.g. S2% and MS medium) as the fragment cultures, also producing an unknown brown pigment on MS-medium (Fig. 4).

The cultures maintained under a changing 12h/27°C light and 12h/24°C dark cycle (the standard conditions in our investigation) did not produce any secondary compounds.

Surprisingly, the mycobiont cultures under a colder temperature than the standard condition produced barbatic acid as the major substance (Fig. 10). Diffractaic, 4-O-demethylbarbatic and 4-O-demethyldiffractaic acids were formed as satellite and minor compounds.

The subcultures, exposed to shortwave UV-light, formed the same depsides as the voucher specimens, except 4-O-demethylbarbatic acid (Fig. 12), which additionally was probably produced as a precursor.

The data-sets of the fungal DNA-analyses confirmed that the true mycobiont had been cultured. The natural lichen thalli samples gave only weak bands of DNA whereas the cultures provided broad bands that could easily be sequenced.

DNA-sequences of the thalli and the cultured photobionts (Figs. 5, 6), using *Trebouxia* primers, proofed the identity of the thallus algae and the symbiotic partner. Comparisons with the gene-databank of NCBI pointed out that *Trebouxia jamesii* can be considered as the photobiont of *Heterodea muelleri.*

4. Discussion

Our investigation has shown that mycobionts inoculated from thallus fragments show considerably higher growth rates than those grown from single spore isolates.

A couple of months were needed to grow mycelia of a spore-derived mycobiont, whereas fragment cultures produce sufficient biomass to be subcultured after an incubation time of 2 months. However, equal size inoculates (grown from spore or 'tissue') of *Heterodea muelleri* show a similar growth rate when subcultured. A clear advantage of single spore isolates is that they always lack contaminations with lichenicolous fungi or epibiontic bacteria. For clean DNA templates, single spore isolates are requested.

Sabouraud 2% medium and Murashige & Skoog medium were obviously preferred by the *Heterodea* mycobiont, but further media compositions have to be screened in future experiments. The tissue culture method is well fitted to gain a high quantity of fungal material. Moreover, this method is recommended for secondary metabolism experiments that aim to test bioactive substances (Yamamoto, 1991).

Stress (change of ecological parameters) was found to be necessary for the production of the typical polyketides in mycobiont cultures, as it was shown in several former studies (e.g. Yamamoto et al., 1987; Hamada, 1989; Yoshimura et al., 1994a,b; Yamamoto et al., 1995; Hamada et al., 1996; Stocker-Wörgötter and Elix, 2002).

Filson (1992) reported that *Heterodea muelleri* in the natural environment produces usnic acid in the cortex and diffractaic acid in the medulla.

The *Heterodea muelleri* samples of our study contained the depside diffractaic acid as major substance and another depside, barbatic acid, as a satellite compound. Moreover, 4-O-demethyldiffractaic acid was found in very small quantities. The UV-treatment of the mycobiont caused the mycelia to produce the same chemistry as the voucher specimens plus an additional precursor of barbatic acid. Why exposure to shortwave UV can induce the biosynthesis of related lichen substances is still an open question.

A further interesting result was that cultures grown under a lower temperature regime produced considerable higher quantities of diffractaic and barbatic acids. Especially, the increase of barbatic acid in the cultured mycobiont under low-temperature stress was remarkable. A connection between low temperature treatment and increased production of secondary compounds was reported by Stocker-Wörgötter (2001a) for *Umbilicaria mammulata.*

More test series with mycobionts of *Heterodea muelleri* and other lichens are requested to clarify the complex biochemical processes occurring in lichens.

Diffractaic acid is well known to be a biologically active compound (e.g. Müller, 2001). Huneck (1999) reported a growth-inhibitory effect of diffractaic acid, barbatic acid and 4-O-demethylbarbatic acid on lettuce seedlings, performed by Nishitoba et al. (1987).

DNA-analyses and microscopical observations showed that the photobiont of Heterodea muelleri belongs to the genus *Trebouxia.* Identification by morphological characters only is difficult, but in accordance to the findings of Friedl (1989), *Trebouxia jamesii* is considered to be the algal partner of the investigated lichen.

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