THE DEMI-LICHENIZATION OF *TRAMETES VERSICOLOR* (L.:FRIES) PILAT (POLYPORACEAE): THE TRANSFER OF FIXED $^{14}CO_2$ FROM EPIPHYTIC ALGAE TO *T. VERSICOLOR*.

MICHAEL S. ZAVADA[1], LAURA DIMICHELE[2], AND CHARLES R. TOTH[1]

Abstract - Basidiocarps of *Trametes versicolor* with an epiphytic flora (including *Trebouxia* sp.) collected from north-central Rhode Island were incubated in an atmosphere of ^{14}C labeled CO_2 for 6 hours. Using autoradiography, the location of the fixed $^{14}CO_2$ was determined at 6h, 12h, 24h, 48h, 96h, and 192h. The fixed ^{14}C was recorded in the epiphytic algae after 6h, and at all subsequent times. After 192h the ^{14}C was recorded in the fibrillar matrix associated with the epiphytic algae, and appeared to be present in the fungal hyphae in the top smooth, silky layer of the basidiocarp. The results suggest that the epiphytic algae secrete the fixed carbon in a form that *Trametes versicolor* can potentially assimilate, however, the slow assimilation of the carbon (4–8 days) suggest a casual relationship between *T. versicolor* and its epiphytic algal flora.

INTRODUCTION

The wood decaying *Trametes versicolor* (L.:Fries) (Polyporaceae: Basidiomycetes) is found in conifer and broadleaf forests throughout North America. It was recently found that the upper silky-smooth layer of *Trametes* basiodiocarps are often colonized by a variety of epiphytic organisms (Zavada and Simoes 2001). Among these organisms the lichen forming phycobiont *Trebouxia* and *Stichococcus* were identified (Zavada and Simoes 2001).

A majority of the 13,500 species of lichenized fungi are Ascomycetes. Five families of Basidiomycetes are known to be lichenized. The nature of the fungal-algal association in lichens has been questioned (Ahmadjian and Jacobs 1981, Goward 1999, Sanders 2001). In many general biology texts (e.g., Raven and Johnson 2002) lichens are considered to be a mutualistic or a symbiotic association; however, Ahmadjian and Jacobs (1981) termed the association; a "controlled parasitism." At present over 44 genera of algae and cyanobacteria have been identified in lichens (Ahmadjian 1993). This is indicative of the diversity of organisms which fungi can exploit. In addition to the algae in lichens, bacteria are invariably associated with the lichen thallus. Bacteria are often embedded in an extracellular matrix associated with the algae and

[1]Department of Biology, Providence College, Providence, RI 02918. [2]Biomedical Engineering, University of North Carolina, Chapel Hill, NC 27599. Corresponding author - mzavada@providence.edu.

fungus, and bacteria may play a more active role in the lichen association than previously thought (Ahmadjian 1982, Stracke et al. 2002).

Carbohydrate transfer from the photobiont to the mycobiont in lichens is variable. In the cyanobacterial photobiont the carbohydrate transferred to the mycobiont is glucose (Ahmadjian 1993). Algal symbionts secrete a variety of polyols. Two commonly lichenized algae are often present on *Trametes*, *Stichococcus,* and *Trebouxia* (Zavada and Simoes 2001). In lichenized *Stichococcus*, sorbitol is the primary carbohydrate transferred to the mycobiont, and in *Trebouxia* it is ribitol (Ahmadjian 1993). The rate at which the carbohydrates are released to the mycobiont also vary and can range from minutes, e.g., in *Peltigera polydactyla* (Neck.) Hoffm. (Drew and Smith 1967), to more extended periods of time in other lichens (hours). The rate of transfer may be influenced by the presence of light and water (Ahmadjian 1993, Richardson 1973). The purpose of this study is to determine if $^{14}CO_2$ fixed by the epiphytic algae that occur on the basidiocarps of *Trametes versicolor* is available as a carbon source for *Trametes*.

MATERIALS AND METHODS

From late September to early December 2000 three collections of basidiocarps of *Trametes versicolor* were made from Snake Den State Park, Smithfield, Rhode Island. The basidiocarps were collected from a forest bordering a 1.5 km trail that transects the park. Approximately 15 individual basidiocarps attached to the substrate were brought back to the laboratory from each collection. The samples were placed in airtight, polystyrene desiccation chambers (Fisher Scientific) measuring 23 cm long x 22 cm wide and 17 cm deep (8602 cm^3). Each chamber was equipped with a maximum-minimum temperature thermometer to monitor the daily temperature fluctuations in the chamber. The samples were misted with distilled water daily to insure continual growth. The chambers (control and treatment) were placed in a 28 °C incubator one week prior to the beginning of the experiment. The basidiocarps were maintained on a 12-h light/dark cycle and exposed to approximately 10.75 PAR.

Five hours prior to illuminating the growth chambers on the day of the experiment a 15 ml Eppendorph tube with *Saccharomyces cerevisiae* was placed in the experimental chamber. A strain of *S. cerevisiae* that over expressed ornithine decarboxylase was used to maintain the $^{14}CO_2$ atmosphere around the basidiocarp. This strain was produced from a *yODC* (*SPE1*) genomic clone from University of California (Fonzi and Sypherd 1987) and clones with three *yODC* gene copies (3x strain) were constructed (Sikorski and Hieter 1989). The 3x yODC strain was used for $^{14}CO_2$ generation, but prior to placing the *S. cerevisiae* lysate in the chambers, the cells were washed in H_2O and resuspended in 450 µl of

lysis buffer (0.02% Brij, 25 mM Tris pH 8.0, 0.1 mM EDTA, 2 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 0.1% Triton X-100, 1 mM $MgCl_2$), disrupted by agitation with glass beads (BioSpec Bead-Beater), and 14,000 xg supernatant recovered. $^{14}CO_2$ was generated in the chambers by a yODC enzymatic assay using release of $^{14}CO_2$ from ^{14}C-carboxy-labeled ornithine (Tyagi et al. 1981). A protease inhibitor mix containing phenylmethylsulfonyl fluoride, leupetin, aprotinin, and pepstatin A (Roche Molecular Biochemicals) was included in the lysis buffer (Toth and Coffino 1999). The introduction of the $^{14}CO_2$ producing yeast into a 28 °C chamber produced approximately 500 pm of $^{14}CO_2$ per minute per mg of extract based on the enzymatic activity of the over expressed ornithine decarboxylase. Approximately 250 µg of extract was used for the generation of $^{14}CO_2$ in the experiment. The 5 microcuries of ^{14}C ornithine used for the experiment approximately produced 100 pm of $^{14}CO_2$. The advantage of producing the $^{14}CO_2$ using this method is that over the 5-hour period the $^{14}CO_2$ is accumulated in the chamber at a slow and steady rate ensuring that all of the $^{14}CO_2$ produced remains atmospheric until fixed by the algae.

In the growth chamber was a covered petri dish with $Ba(OH)_2$ soaked sponges, the cover of which was connected to a string to the outside of the chamber. The chamber was sealed and maintained in darkness to generate $^{14}CO_{2\,(g)}$ at 28 °C for 5 h. At the end of 5 hours the incubator lights were turned on (Time 0), and the epiphytic algae were allowed to fix the $^{14}CO_2$ for 6 h. After 6h the petri dish with the $Ba(OH)_2$ sponges was uncovered, and the remaining atmospheric $^{14}CO_2$ was precipitated. The yeast lysate and the petri dish was removed from the chamber, and the chambers were resealed and returned to the incubators. At 6h (the time at which exposure to $^{14}CO_2$ was terminated), 12h, 24h, 48h, 96h, and 192h, 1 cm^2 samples of the basidiocarps were excised and cut into 1–2 mm^2 pieces, and immediately fixed according to the method of Bozzola and Russell (1999) for autoradiography.

Following fixation, dehydration and embedding in Spurr's Low Viscosity Resin, 0.5 to 5 µm thick sections were cut with glass knives on a MTX ultra-microtome and mounted on glass slides. A thin film of Kodak NTB-2 photographic emulsion for autoradiography was then applied by dipping a 7-cm wire loop in the liquified photographic emulsion which was maintained at 40 °C under darkroom conditions, and lit by a Kodak Safelight Filter No. 2. The sections from each of the treatments were incubated from 6–24 weeks in complete darkness at 1 °C. Following incubation, the photographic emulsions were developed, washed, then fixed, and the sections were stained for 4 m in Toludine Blue, dried and mounted with a cover slip using Paramount. The sections were viewed on a Zeiss Axiophot.II, and photographed using Kodak Tech Pan black and white film.

RESULTS

Sections of the upper silky-smooth surface of the basidiocarps show that this region of the basidiocarp is composed of epiphytic algae, bacteria, an extracellular fibrillar matrix, and the loose anastomosing hyphae of the basidiocarp (Fig. 1a,b; also see Zavada and Simoes 2001). Basidiocarps that exhibited a well-developed epiphytic flora (Fig. 1a) were divided into two sets of chambers. One sample of basidiocarps was not exposed to $^{14}CO_2$ (the controls) and a second sample of basidiocarps was exposed to $^{14}CO_2$. The controls showed no diffuse staining (Fig. 1b). Sections of the silky-smooth layer of the treated basidiocarps showed a strong concentration of black grains associated with the epiphytic algae that were collected at 6h,12h, 24h, 48h, and 96h. This indicates the presence of fixed ^{14}C (Fig. 2 arrows, 6h). At 6h, 12h, 24h, 48h, and 96h it appears that the ^{14}C is restricted to the algal component. However, at 192h the black grains became more diffuse in the algal component (Figs. 3–5), as indicated by smaller, discrete black particles, and were distributed not only in the algal component, but also in the extracellular matrix and the fungal hyphae (Figs. 3–5), indicating that portions of the ^{14}C fixed by the algae were now located in the extracelluar matrix and the fungus (Figs. 3–5). These data indicate that the ^{14}C fixed by the algal component was secreted 4–8 days following exposure to the $^{14}CO_2$ and is available for assimilation by the fungus.

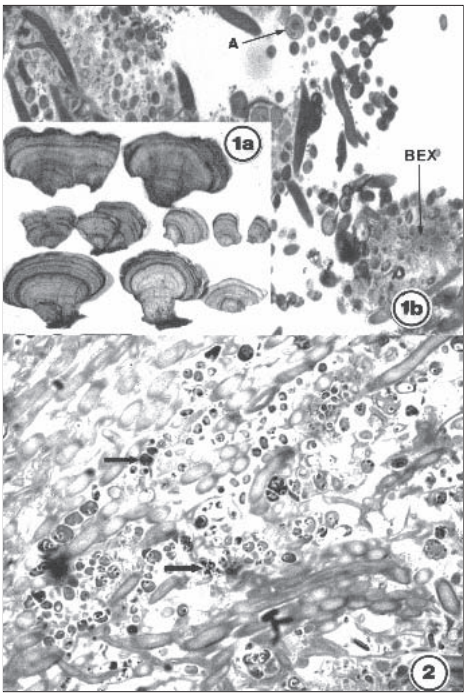
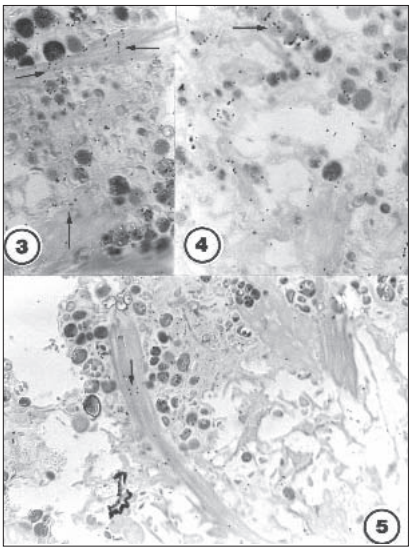


Figure 1a. A representative collection of *T. versicolor* with a well-developed epiphytic flora, X 2. Figure 1b. A microscopic section of the upper smooth-silky layer showing fungal hyphae associated with epiphytic algae (A), and bacteria embedded in an extracellular matrix (BEX). This is a section of one of the control basidiocarps showing the lack of defuse black grains. X 1,000. Figure 2. A microscopic section of the smooth-silky layer in *T. versicolor* showing a concentration of black grains associated with the algal cells (arrows) after 6h, X 1,000.

DISCUSSION

The results of this study indicate that $^{14}CO_2$ fixed by the epiphytic algae on the basidiocarps of *Trametes versicolor* becomes available for assimilation by the fungus 4–8 days following exposure. This study, however, did not determine what the assimilate is, or the quantity available to the fungus. This alternative carbon source may be important to *T. versicolor* for sporocarp formation, and growth and maintenance when carbon derived from the rotting wood has been depleted, or when competition for the primary source of carbon becomes intense due to the invasion of other species of wood decaying fungi (Chapela et al. 1988, Coates and Raynor 1985). The occurrence of epiphytes on the basidiocarps of *T. versicolor* is a common, but not a universal occurrence. This study suggests that *T. versicolor* has the capacity to demilichenize the algal epiphytes. Major differences between this association and the fungal-algal association in lichens are the slow transfer of carbon from the alga to the fungus, the lack of morphological modification of the fungus, the lack of reproductive synchrony or production of unique secondary compounds, and the establishment of a long-term association. The fungal-algal association in lichens has recently been reinterpreted (Goward 1999, Sanders 2001). It has been suggested that the fungi are engaged in algaculture, i.e., the controlled cultivation and manipulation of the associated algae (Goward 1999, Sanders 2001), an association that has no benefit to the phycobiont. In addition, the fungus may stimulate the embedded algae to secrete significant amounts of fixed carbon in the form of polyols, i.e.,70–90% of the fixed carbon may be secreted for fungal use (Fahselt 1994, Smith 1980). Smith (1980)



Figures 3–5. treated microscopic sections of *T. versicolor* at 192h. Figure 3. A DIC microscopic section showing the dense black granules associated with the extracelluar matrix and the fungal hyphae (arrows), X 1,000. Figure 4. A microscopic section showing the dense black granules associated with the extracelluar matrix and the fungal hyphae. Note that a majority of the granules are still associated with the algal component (arrow), X 1,000. Figure 5. A microscopic section showing the dense black granules associated with the fungal hyphae (arrow). Note that a majority of the granules are still associated with the algal component, X 1,000.

found when the phycobiont is isolated from the lichen thallus that the secretion of carbon is reduced to 1–2% of the total fixed carbon (however, see Maruo et al. 1965; also see Fahselt 1994). The fungal-algal relationship in *T. versicolor* may be more casual. The fungal influence over the secretion of usable carbon compounds may be limited; the fixed carbon did become available to *T. versicolor* 4–8 days after the initial fixation of the $^{14}CO_2$, a time that is longer than that observed in fungal-algal associations in lichens.

Another interesting aspect of this study is the identification of *Trebouxia* as one of the epiphytes. Ahmadjian (1967, 1970, 1988) suggests that *Trebouxia* does not occur in the free living state (however, see Bubrick et al. 1984; Nakano 1971a,b; Tschermak-Woess 1978). One function that the more casual fungal-algal associations may play (e.g., in *T. versicolor*) is that the short-lived basidiocarps may act as refugia for the fungal dependent algae until a more permanent association can be established.

Although fungi are thought of as saprotrophic or parasitic, more than one third of the known fungi are involved in mutualistic symbioses (Kendrick 1991). Thus, fungi have the capacity to establish associations with a variety of living organisms. These associations run a gamut from ephemeral associations, coupled with a specific life history phase (as possibly exists in *T. versicolor*), to obligate, intimate, and life long relationships as observed in many lichens, and mycorrhizae. Whether the carbon transferred from the epiphytes to *T. versicolor*, is a significant, supplementary, or inconsequential source, this study demonstrates the capacity for *T. versicolor* to exploit algae as a carbon source.

ACKNOWLEDGEMENTS

We wish to thank the Department of Biology, Providence College for partial support of this project. This project was the partial fulfillment of an undergraduate research project for Laura DiMichele.

LITERATURE CITED

Ahmadjian, V. 1967. A guide to the algae occurring as lichen symbionts: Isolation, culture, cultural physiology, and identification. Phycologia 6:127–160.

Ahmadjian, V. 1970.The lichen symbiosis: Its origin and evolution. Pp. 163–184, *In* T. Dobzansky, M.K. Hecht, and W.C. Steere (Eds.). Evolutionary Biology, Volume 4 , Appleton-Century Crofts, New York, NY.

Ahmadjian, V. 1988. The lichen alga *Trebouxia*: Does it occur free-living? Plant Systematics and Evolution 158:243–247.

Ahmadjian, V. 1993. The Lichen Symbiosis. John Wiley, New York, NY. 250 pp.

Ahmadjian, V. 1982. Algal-fungal symbioses. Pp. 179–233, *In* F.E. Round and D.J. Chapman (Eds.). Progress in Phycological Research, Volume 1, Elseveir Biomedical Press, Amsterdam, Holland.

Ahmadjian, V., and J.B. Jacobs. 1981. Relationship between fungus and alga in the lichen *Cladonia cristatella* Tuck. Nature 289:169–172.

Bozzola, J.J., and L.D. Russell. 1999. Electron Microscopy, Principles and Techniques for Biologists. Jones and Bartlett, Boston, MA. 670 pp.

Bubrick, P., M. Galun, and A. Frensdorff. 1984. Observations on free-living *Trebouxia* De Puymaly and *Pseudotrebouxia* Archibald and evidence that both symbionts from *Xanthoria parietina* (L.) Th. Fr. can be found free-living in nature. New Phytologist 97:455–462.

Chapela, I.H., L. Boddy, and A.D.M. Raynor. 1988. Structure and development of fungal communities in beech logs four and a half years after felling. Microbial Ecology 53:59–70.

Coates, D., and A.D.M. Raynor. 1985. Fungal population and community development in cut beech logs. I. Establishment via the aerial and cut surface. New Phytologist 101:153–171.

Drew, E.A., and D.C. Smith. 1967. Studies in the physiology of lichens. VIII. Movement of glucose from alga to fungus during photosynthesis in the thallus of *Peltigera polydactyla*. New Phytologist 66:389–400.

Fahselt, D. 1994. Carbon metabolism in lichens. Symbiosis 17:127–182.

Fonzi, W.A., and P.S. Sypherd. 1987. The gene and primary structure of ornithine decarboxylase from *Saccharomyces cerevisiae*. Journal of Biological Chemistry 262:10127–10133

Goward, T. 1999. The Lichens of British Columbia. Part 2. Ministry of Forests Research Programs, BC, Canada.

Kendrick, B. 1991. Fungal symbioses and evolutionary innovations. Pp. 249–261, *In* L. Margulis and R. Fester (Eds.). Symbiosis as a Source of Evolutionary Innovation. MIT Press, Cambridge, England.

Maruo, B., T. Hattori, and H. Takahashi. 1965. Excretion of ribitol and sucrose by green algae into the culture medium. Agricultural and Biological Chemistry 29: 1084–1089.

Nakano, T. 1971a. Some aerial and soil algae from the Ishizuchi mountains. Hikobia 6:139–152.

Nakano, T. 1971b. Subaerial algae of Patagonia, South America I. Bulletin of the Biological Society of Hiroshima University, 38:2–12.

Raven, P.H., and G.B. Johnson. 2002. Biology. 6th Ed., McGraw Hill, Boston, MA 1238 pp.

Richardson, D.H.S. 1973. Photosynthesis and carbohydrate movement. Pp. 249–288, *In* V. Ahmadjian and M.E. Hale, (Eds.). The Lichens. Academic Press, New York, NY.

Sanders, W.B. 2001. Lichens: The interface between mycology and plant morphology. Bioscience 51:1025–1035.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122:19–27

Smith, D.C. 1980. Mechanisms of nutrient movement between the lichen symbionts. Pp. 197–227, *In* C.B. Cook, P.W. Pappas, and E.D. Rudolph, (Eds.). Cellular Interactions in Symbiosis and Parasitism. Ohio State University Press, Columbus, OH.

Stracke, S., C. Kistner, S. Yoshida, L. Mulder, S. Sato, T. Kaneko, S. Tabata, N. Sandal, J. Stougaard, K. Szczyglowski, and M. Parniske 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417:959–962.

Toth, C., and P. Coffino. 1999. Regulated degradation of yeast ornithine decarboxylase. Journal of Biological Chemistry 274:25921–25926.

Tschermak-Woess, E. 1978. Myrmecia reticulata as a phycobiont and free-living *Trebouxia*: The problem of *Stenocybe septata*. Lichenologist 10:69–79.

Tyagi, A.K., C.W. Tabor, and H. Tabor. 1981. Ornithine decarboxylase from *Saccharomyces cerevisiae*. Purification, properties and regulation of activity. Journal of Biological Chemistry 256:12156–12163.

Zavada, M.S., and P. Simoes. 2001. The possible demi-lichenization of the basidiocarps of *Trametes versicolor* (L.: Fries) Pilat (Polyporaceae). Northeastern Naturalist 8:101–112.