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Development of *Peltigera praetextata* lichen thalli in Culture

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Abstract

We obtained well-developed dorsiventral lichen thalli from cultured fragments of the natural thallus of the cyanolichen, *Peltigera praetextata*. The thallus obtained was composed of both dorsal and ventral cortices, a *Nostoc* photobiont layer, and a medulla. The dorsal cortex was composed of paraplectenchymatous hyphal tissue, but the ventral cortex was an undifferentiated plectenchymatous tissue. Different developmental stages of the cyanolichen thalli were observed under controlled laboratory conditions. Changes in the shape of *Nostoc* colonies were observed from the free-living filamentous stage to the symbiotic cell aggregates. The development of the cyanolichen starting from excised thallus segments appears to be very similar to that from soredia, even though *P. praetextata* does not produce soredia. Cultured lichen thalli (subcultured for 1 year) were compared with natural thalli. Although veins, rhizines, and epicortex were not observed in cultured thalli, most morphological features were very similar to the natural thalli.

Keywords: lichen culture *in vitro*, cyanolichens, *Peltigera praetextata*

1. Introduction

The culture of lichens under laboratory conditions is difficult. However, the artificial synthesis of lichens under laboratory conditions has increased in

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recent years as reviewed by Ahmadjian (1989, 1990), i.e. Ahmadjian, 1966; Bertsch and Butin, 1967; Ahmadjian and Heikkilã, 1970; Marton and Galun, 1976; Ahmadjian and Jacobs, 1982, 1985; Bubrick and Galun, 1986; Stocker-Wörgötter and Türk, 1987). Development of soredia was followed by Bitter (1904), Tobler (1911, 1942–1943), Bachmann (1927), Margot and De Sloover (1984), James and Henssen (1975), Schuster et al. (1985), Stocker-Wörgötter and Türk (1988), and Ott (1987a,b, 1988). The direct initiation of lichens from natural thalli was recently achieved by Yamamoto et al. (1985, 1987). Small fibrils were successfully cultured from several *Usnea* species (Yamamoto, 1988, 1990; Kon et al., 1990). Ahmadjian (1989) mixed cultured of *Peltigera canina* bionts on agar. Although spore germination and early biont interactions did occur on agar, he did not achieve synthesis on mica. In this paper, we describe the different stages of development of the cyanolichen, *P. praetextata*, thalli under controlled laboratory conditions.

2. Materials and Methods

Peltigera praetextata (Flörke) Vain. grows on rocky and nutrient-poor soils as well as siliceous rocks between mosses. The specimens for culture were collected in Takao, Kyoto, Central Honshu, Japan during the summer of 1987. Part of the collection was preserved as herbarium specimens no. 8704.

Lichen cultures were started a few days after collection. Segments of thalli (150–300 μm in diameter) were excised as described previously (Yamamoto et al., 1985) and cultured on three different culture media: Modified Deter Medium (MDM) (Watanabe, 1960); Malt-Yeast Extract Medium (M-Y) (Ahmadjian, 1967); and 2% water agar (Nacalai tesque, Inc.; Code 010–28) at different pH values (pH=7.0, 8.0, and 9.0) at 15°C in light (13–14 $\mu E/m^2 \cdot sec$) with daily cycle (16 hr light/8 hr drak). Lichens were subcultured for more than two years, at one year intervals.

Cross-sections of cultured lichen thalli were obtained by means of a freeze-microtome. Sections of thalli were stained with lactophenol cotton-blue. *Nostoc* filaments were stained with Giemsa's solution or methyl green. The development of lichen thalli was observed using both binocular stereo and transmission light microscopes and recorded by macro- and microphotography.

3. Results and Discussion

More than 80% of the test tube cultures were contaminated when inoculated with *Peltigera praetextata*. The contamination rate was higher than in our

previous work (Yamamoto et al., 1985), using lichens with green algae (1–50%); however, terricolous lichen cultures usually show high contamination rates (Yoshimura et al., 1987).

Neither *Nostoc* nor the mycobiont of *Peltigera praetextata* did grow on the M-Y medium. Incomplete synthesis of *Nostoc* with the mycobiont was obtained from 2% of inoculated test tubes on the MDM medium. Among the three different media tested, only the one without organic nutrients (water-agar medium) provided successful cultures up to the juvenile thallus of *P. praetextata*, which was obtained from approx. 6% of inoculated test tubes. Among water-agar media of different pH values, the neutral culture medium seemed to support better growth of juvenile *P. praetextata* thalli than those of basic media. In previous studies (Yamamoto et al., 1985; Yoshimura and Kurokawa, 1989), most lichens with green algae could be cultured by using the M-Y medium; however, *Nostoc* by itself would not grow on M-Y medium. Dorsiventral lichen thalli were obtained only on 2% water agar medium. All the further observations described and photographed were carried out from the cultures on 2% water-agar medium.

After inoculation, thallus segments of *P. praetextata* showed no change for two-to-six weeks, nearly the same as that of soredia development (Stocker-Wörgötter and Türk, 1988). After this relatively long lag phase the first fungal growth occurred. In contrast to lichens with green algae, i.e. *Usnea* spp., *Cladonia* spp., *Ramalina* spp. (Yamamoto, 1988), the fungal growth from the thallus segments was not clear because of the covering, coherent slimy material derived from the *Nostoc* cells and the original segments of thalli. The production of *Nostoc* colonies from the original segments was more obvious. We have not yet been successful in separating and culturing the mycobiont of *Peltigera praetextata*. The *Nostoc* photobiont can be separated from the mycobiont, and cultured but usually with some bacterial contaminants.

4. *Nostoc* differentiation

Nostoc colonies often grew out at three to eight weeks after inoculation from the excised thallus segments. They consisted of filaments enveloped in a gelatinous matrix often forming loops. Their cells were: spherical, ellipsoid, or barrel-shaped, 2,5-(3–3.5)–4 μ long, 2.5–4 μ wide; heterocysts were nearly the same size as other trichome cells; a filament was sheathed, 4.5 μ wide and up to 250 μ long. The colonies normally consisted of single rows of spherical and ellipsoidal cells with individual gelatinous matrix. Older filaments, however, were usually composed of multi-rowed cells. Finally, the filaments became globose as irregular aggregated cell-colonies in a gelatinous matrix (Fig. 1).

The cells in globose aggregates were somewhat larger (4–5 μm) than normal free-living cells of trichomes.

During lichenization, algal cells increased in size after envelopment by hyphae as in *Heppia echinulata* (Marton and Galun, 1976) and in *Dermatocarpon miniatum* (Ahmadjian and Heikkilä, 1970). In *P. praetextata*, *Nostoc* cells also increased in size during lichenization. *Nostoc* cells in globose cell aggregates were somewhat larger than those in normal trichomes, even before being enveloped by mycobiont hyphae (Fig. 1). The transformation of *Nostoc* colonies may be controlled by a soluble substance, because it was observed without direct contact of mycobiont hyphae. The size of the algal cells probably is a result of retardation of division frequency (Ahmadjian and Heikkilä, 1970).

5. Initiation of New Thalli

Hyaline mycobiont hyphae often developed radially from the coherent slimy layer of *Nostoc* aggregates. According to our observation, globose *Nostoc* colonies composed of rather large swollen cells were enveloped by mycobiont hyphae, and seemed to be the initial bodies of lichen thalli. The uni-layered hyphal envelopes of globose *Nostoc* aggregates were seen only in very early stages of development (Fig. 2). The relative abundance of hyphal cells to *Nostoc* was low at the early stages of thallus primordia (Fig. 2), but as differentiation of the lichen continued the hyphal biomass increased (Fig. 3). The

Figure 1. Formation of globose *Nostoc* aggregates from filamentous multiseriate *Nostoc* colonies. Scale = 10 μm.

Figure 2. *Nostoc* colonies surrounded by hyphae on agar-medium. Scale = 10 μm.

Figure 3. Cross-section of the thallus primordia attached to the agar medium showing rather few *Nostoc* cells with undifferentiated hyphal tissues. Scale = 10 μm.

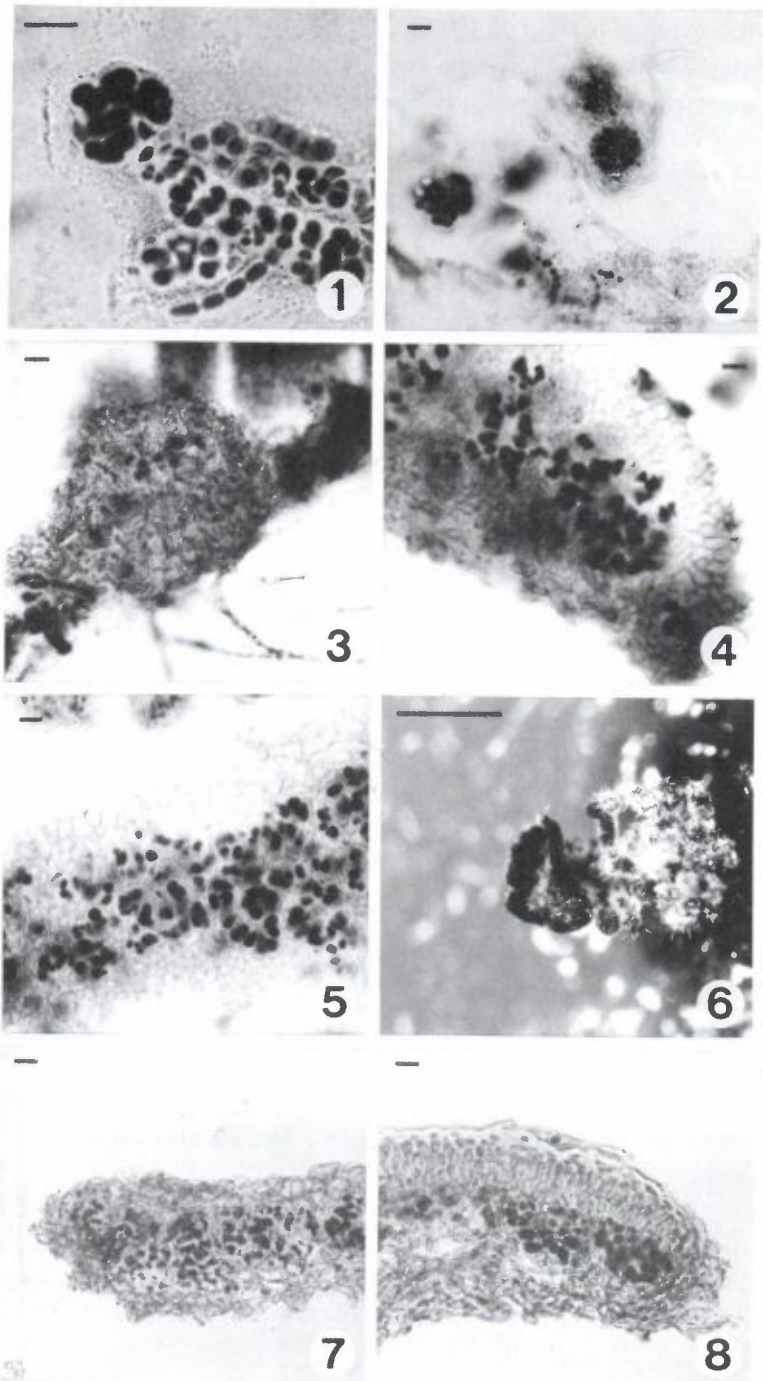
Figure 4. Cross-section of juvenile lichen thallus on agar medium showing developed dorsal cortex and poorly differentiated ventral cortex. Scale = 10 μm.

Figure 5. Cross-section of a basal portion of cultured young lichen thallus attached to an agar-substratum, showing differentiated lichen tissues; paraplectenchymatous dorsal cortex, medulla with *Nostoc* cells, and poorly differentiated ventral sides. Scale = 10 μm.

Figure 6. Young cultured lichen thalli of *Peltigera praetextata* with white thallus primordia on agar-medium. Scale = 10 μm.

Figure 7. Cross-section of an apical portion of a cultured lichen thallus, showing paraplectenchymatous dorsal cortex, a *Nostoc* photobiont layer, narrow medulla, and very poorly differentiated ventral cortex. Scale = 10 μm.

Figure 8. Cross-section of an apical portion of a natural lichen thallus for comparison, showing the presence of well developed paraplectenchymatous dorsal cortex with epicortex, a clear *Nostoc* photobiontlayer, and wide medulla, and the absence of the ventral cortex. Scale = 10 μm.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young thalli were dark blue or blackish, and composed of differentiated hyphal tissues with *Nostoc* cells (Fig. 4). Ott (1988) observed a single-layered cortex of closely-attached pseudoparenchymatous cells in the development of *P. venosa* (a synonym of *P. didactyla*). We observed a multi-layered cortex typical of the juvenile thallus of *P. praetextata*. The multi-layered cortex is composed of closely-attached pseudo-parenchymatous cells with thin walls (Fig. 4), and is very similar to that of the thalloid exciple of *Leptogium* apothecia. Dorsiventral thallus primordia often formed three months after the formation of globose thallus primordia.

The ventral side of the lichen thallus became embedded in the agar-media by means of long filamentous hyphae (Fig. 3). The primordia gradually became transformed into dorsiventral lichen thalli (Fig. 4). The thallus was composed of both dorsal and ventral cortices and a central medulla with *Nostoc* cells (Figs. 4–5). The dorsal cortex was composed of a paraplectenchymatous hyphal tissue composed of two or three layers of cells, in which the cell lumina were large (about 10 μ in diameter) and the cell walls rather thin (Fig. 5). The ventral cortex was composed of a rather undifferentiated plectenchymatous hyphal tissue of irregularly arranged, round cells. The medulla was composed of loosely interwoven hyphae among the *Nostoc* cells.

6. Comparison of Cultured Thalli With Natural Thalli

Although cultured thalli (Fig. 6) were very small (up to 3 mm in width), their lobe-shape, color, and anatomy were very similar to those of developed natural thalli (ca. 20 cm in width). The cross-section of the apical portion of a cultured thallus (Fig. 7) was compared with that of a natural thallus (Fig. 8). The dorsal cortex of both cultured and natural thalli was composed of well differentiated paraplectenchymatous hyphal tissues; however, the epicortex was totally absent in the cultured thalli, but usually present in natural thalli. In cultured thalli, algal cells were distributed throughout the medulla especially in the apical portion of thallus lobes and gradually restricted to the dorsal portion of the medulla toward the basal portion of thallus lobes. In natural thalli, algal cells are scattered in the medulla only at the apical portion of the lobes; however, they are soon restricted below the dorsal cortex (heteromerous). Long medullary hyphae without algal cells, comprise the medulla of natural thalli, while the medulla of our cultured colonies was composed of long hyphae and contained *Nostoc* cells. Veins and rhizines have not been formed in cultured lichen thalli, probably because we obtained only juvenile stages of thallus formation, although they are always present at the ventral side of the natural

thalli of *P. praetextata*. Epicortex formation may require more severe (dry) environmental conditions than provided by the culture conditions.

The stages in development of *P. praetextata* thalli *in vitro* seem to be very similar to those in the natural condition as Ott (1988) reported on *P. venosa*. Formation of new thalli of *P. praetextata* followed the same manner previously reported by Marton and Galun (1976) in *Heppia echinulata* on a different medium; dissociation of the components, then reversion to their free-living form, and finally a complete resynthesis. These processes were observed as continuous under the same culture conditions.

The development of a cyanolichen directly from segments of lichen thalli seems to be very similar to that from soredia (Stocker-Wörgötter and Türk, 1988). *Nostoc* cells initially form filaments, but then form aggregate globose colonies like those seem in *Peltigera* thalli. Similar structures to the 'arachnoidal stages' described by Schuster et al. (1985) were observed in white thallus primordia on agar and along margins of cultured thalli (Fig. 6). *P. praetextata*, however, does not produce soredia, only isidia. Some juvenile, white thallus primordia in our culture were similar to the "arachnoidal stages" seen in the development of soredia in cultures of *P. didactyla* (Stocker-Wörgötter and Türk, 1988). The formation of 'arachnoidal stages' in a non-sorediate species suggests that they are typical early stages of thallus development and not just sorediate growth. The extensively covering hyphal outgrowth from the thallus primordia is the result of the moist culture conditions (Fig. 6), but each fungal outgrowth is limited probably because of a lack of nutrients. Arachnoidal stages may characterize development under dry conditions, because arachnoidal stages or globose thallus primordia were more easily formed when subcultured on mica (unpublished data).

Lichen cultures using our technique may be applicable in fundamental morphogenetic studies of various fields of lichenology, not only for nutritional research (Yamamoto et al., 1985, 1987; Yamamoto, 1988, 1990). Some other attempts for ecological studies have already been made: the ecology of *Cladonia vulcani* was studied using cell aggregates in different pH media (Yoshimura et al., 1987); lichen growth at different temperatures was studied by using cell aggregates of various lichens (Yamamoto, 1988, 1990; Yoshimura et al., 1989, 1990). The present study suggests wider experimental uses of cultured bionts in the future.

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