Yoshiaki KON*, Hiroyuki KASHIWADANI** & Syo KUROKAWA***:

Induction of lichen thalli of *Usnea confusa* Asah. ssp. *kitamiensis* (Asah.) Asah. in vitro

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ツブコナサルオガセにおける培養組織からの地衣体の再生

(Pl. I)

Successful artificial reestablishment of lichens from isolated fungi and algae has been reported for *Endocarpon* (Ahmadjian et al. 1970), *Cladonia* (Ahmadjian et al. 1980) and *Xanthoria* (Burbrick & Galun 1986). On the other hand, successful tissue culture of lichens was reported by Yamamoto et al. (1985) for *Usnea rubescens* and *Ramalina yasudae*. However, induction of lichen thalli from cultured tissue as well as morphological features of induced lichen thalli have never been reported for these lichens. The direct induction of lichens from natural lichen tissues will play an important role for the studies of lichen physiology and may open a new dimension in culture studies of soredia and isidia. Recently, we obtained lichen fibrils induced from actively growing tissues of *Usnea confusa* Asah. ssp. *kitamiensis* (Asah.) Asah. These fibrils showed morphological features similar to those of *Usnea confusa* ssp. *kitamiensis* in nature.

In the present paper, we will describe laboratory conditions for the induction of fibrils from cultured tissues of *Usnea confusa* ssp. *kitamiensis* and developmental stages of induced fibrils. This paper also compares induced fibrils and natural thalli of the subspecies with respect to morphology and chemistry.

Material and method A specimen of *Usnea confusa* ssp. *kitamiensis* was collected at Tanzawa, Kanagawa Pref. in May 1987. Growing tissues were obtained by the method reported by Yamamoto et al. (1985), except the light condition. The method is schematically shown in Fig. 1. Minute segments (about 150–300 μm thick) were cultured onto the 5 ml slant media (malt-yeast extract medium) in test tubes (15 mm×85 mm) under the

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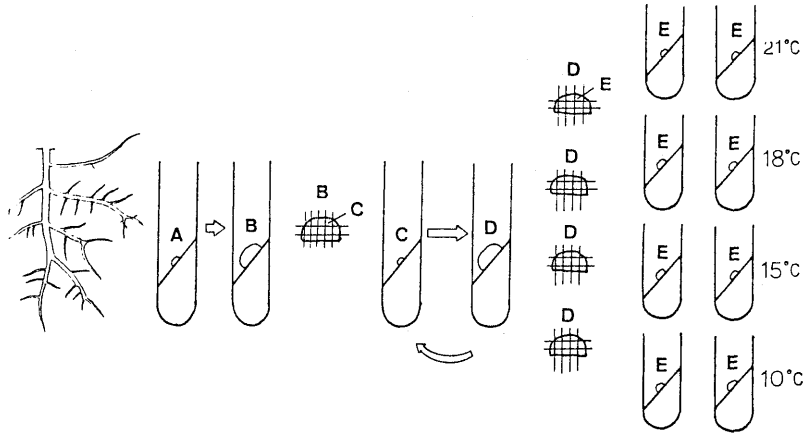

Fig. 1. Method for obtaining cultured lichen tissues and their transplantation. A, a minute segment of the thallus. B and D, cultured lichen tissues. C and E, part of cultured lichen tissues.

continuous illumination of fluorescent lamp (1000 lux) at 20°C. Cultured lichen tissues composed of hyphae and algae were recognizable with the naked eyes about one month after incubation. About two months after incubation, most actively growing tissues were selected and were cut into smaller masses of tissue with a scalpel. Each of these masses was transplanted onto fresh medium and was cultured under the same condition as mentioned above. The following transplantations were repeated ten times every 40 days after incubation, following the same procedure. The most actively growing tissues thus obtained were used for the present study.

The growing tissues obtained after repeating transplantation were cut again into small masses of tissue. Then these masses were transplanted onto fresh medium in each of 80 test tubes. Each set of ten test tubes was incubated under a 12/12 hr light-dark cycle about 1000 lux at different temperatures, 21°C, 18°C, 15°C and 10°C, respectively. In addition, each set of ten test tubes was incubated in dark at four different temperatures as mentioned above.

For light microscopic observation, lichen thalli were mounted in GAW (glycerin+alcohol+water=1 : 1 : 1) solution or lactophenol cotton blue solution. Lichen thalli were prepared for scanning electron microscopy by the method described by Ahmadjian & Jacobs (1985). Samples of thalli were placed in

small cuvetts filled with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 and fixed for 30 minutes at 0°C. Samples were rinced twice in 0.1 M phosphate buffer for 30 minutes and then fixed for 30 minutes at 5°C in 1% OsO_4 in 0.1 M phosphate buffer. The specimens were dehydrated through an ascending alcohol series of 50%, 70%, 90%, 95%, 100% and absolute alcohol for 20 minutes respectively and dried in a desiccator with silica gel. Chemical analyses were carried out by the standardized thin-layer chromatographic method (Culberson 1972).

Results and discussion Lichen fibrils similar to natural lichen thalli of *Usnea confusa* ssp. *kitamiensis* were formed in number of test tubes kept in incubators. The frequency of fibril development varied in different part of the culture. Some areas of a culture had many fibrils (Fig. 2F and Pl. I D), while other areas had only scattered, individual fibrils. Lichen fibril formation rates (percentages of number of test tubes, in which fibrils, or at least a fibril, were formed, to ten test tubes tested) varied in accordance with the temperatures. On 50 days after incubation, they were 0% at 21°C, 90% at 18°C, 20% at 15°C and 0% at 10°C, and 150 days after incubation 0% at 21°C, 100% at 18°C, 70% at 15°C, and 10% at 10°C. It is noteworthy that fibril formation was not observed at 21°C and well-developed fibrils were observed at 18°C. At the temperatures lower than 18°C, it is noticeable that fibril formation rates become lower and it takes longer time to form lichen fibrils. In dark condition, fibril formation was observed only at 18°C and 15°C and the rates were as low as 10-20%.

As mentioned above, the optimum condition to form lichen fibrils is 18°C in light. Thus, various stages of fibril formation were observed under this condition. About one month after incubation, fibrils were observed as small whitish cylindrical projections (about 30-40 μm high) on the surface of the growing tissues and visible only under stereo-microscope (20×). After about two months, these projections elongated upright to form green fibrils about 1 mm long and 70 μm in diameter. They grew up 2-3 mm in length in 3-4 months after incubation. However, fibrils longer than 3 mm were not formed on any of slant media used for the present experiment. These fibrils, in addition, were streight and unbranched.

When the development of lichen fibrils was observed by SEM, the mycobiont and phycobiont were loosely attached each other (Fig. 2A) in initial stages of

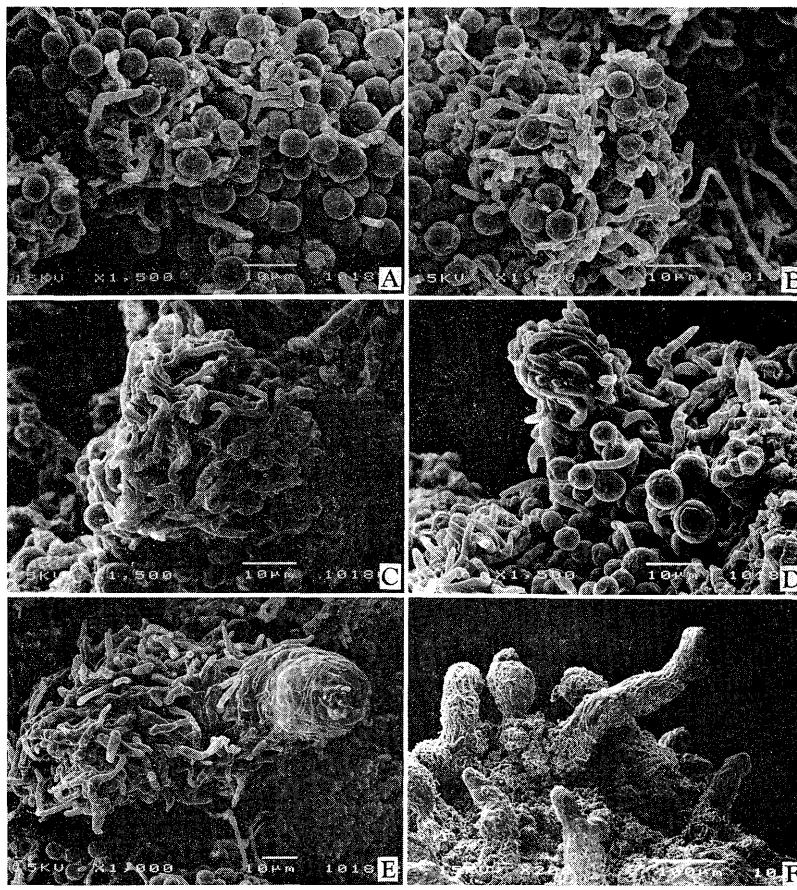

Fig. 2. Developmental stages of the fibrils of *Usnea confusa* ssp. *kitamiensis* observed by SEM. A, initial stage of development of fibril. Mycobiont hyphae and phycobiont cells are loosely attached each other. B, early stages of development of fibril. Mycobiont hyphae and phycobiont cells are increasing in number, forming soredia like cluster. C, early stage of development of fibril. Mycobiont hyphae envelope soredial cluster. D, juvenile fibril. The surface is covered with a network of hyphae. E, elongated fibril. The surface is coated with mucilaginous substance near the tip of fibril. F, short fibrils formed on the cultured lichen tissue.

formation of fibrils. Although they grew together, mycobiont cells were very rare or almost absent in the periphery of the colony, where phycobiont cells were dominant. Mycobiont and phycobiont cells increased in number and mycobiont cells usually enveloped the surface of the phycobiont cells. In this stage,

lichen fibrils could be observed as small projections (Fig. 2B). These small projections resembled the soredial clusters reported by Ahmadjian & Jacobs (1982) in basic stage of the development in the reestablishment of lichens. Then these fibrils grew upward (Fig. 2C) and the surface was covered with a network of hyphae of the mycobiont (Fig. 2D). In later stages, fibrils were coated with mucilaginous substance near the tips, even though the substance seemed to be almost lacking near the base (Fig. 2E and F).

The inner structure was observed in fibrils cultured for three months. The fibril was 1000 μm long and 100 μm in diameter (Pl. IA). The cortex was thin (about 5 μm thick) and was composed of 1-2 layers of thin-walled hyphae running parallel to the surface. These hyphal cells were 7.5-10 μm long and about 2.5 μm in diameter. The medulla was 110-150 μm thick and was composed of large thin-walled hyphae (10-16$\times$6-10 μm). Although gonidia were variable in number of cells in different part of fibrils, they scattered throughout the medulla (Pl. I B). However, gonidia were totally lacking near the apex of fibril (Pl. I B). The axis was 40-58 μm in diameter and was composed of more or less conglutinated longitudially running hyphae (about 1.8 μm in diameter). It is noteworthy that the axis extended downward into the growing tissue supporting the fibril, especially when cultured in dark condition (Pl. I C).

These morphological features of cultured fibrils are basically similar to those of thalli of *Usnea confusa* ssp. *kitamiensis* in nature. However, the following minor differences are noted. In natural thalli of the present subspecies, the cortex is composed of thick-walled, firmly conglutinated and radially running hyphae (Asahina 1956) and is coated by outer amorphous layer (Asahina 1956). In regenerated fibrils, in contrast, the cortex was composed of thin-walled, loosely conglutinated and longitudinally running hyphae and was covered with a very thin mucilaginous substances as reported by Ahmadjian & Jacobs (1985). While gonidia are located or restricted to just below the cortex in the medulla in the natural thalli, they are scattered throughout the medulla and a separated medullary zone was not apparent in regenerated fibrils. Medullary hyphae of regenerated fibrils, in addition, are much more rounded (6-10$\times$10-16 μm) than hyphae of separated medulla of the natural thalli. However, similar rounded hyphae are also found in hyphae located among or near gonidial cells in the medulla of natural thalli. These minor differences were also observed in early stages of fibril formation of resynthesized thalli of *Usnea strigosa* (Ahmadjian

& Jacobs 1982). Therefore, these features can be considered as normal morphological characters in early stages of the development of fibrils of species of the genus *Usnea*, even though early stages of fibril formation in nature is not known well at present.

Secondary products of regenerated fibrils were tested by the TLC method. Usnic acid and norstictic acid, which are also produced in this subspecies in nature, were demonstrated on the chromatograms. Although these substances were demonstrated in all fibrils tested, the concentration of them was considerably variable. It should be noted here that these substances were also demonstrated in growing tissues cultured in test tubes.

On the other hand, Ahmadjian & Jacobs reported deposits of numerous crystals on the cortical surface or even on the surface of phycobiont cells of resynthesized thalli of *Usnea strigosa*. However, they were not observed in any stages of regenerated fibrils of the present subspecies.

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地衣体の一部から無菌的に得られた組織を 20°C で継代培養することにより活発に増殖する組織を得た。この増殖組織を 18°C，明所に移すことによって，組織表面から多数の突起が形成され，これらの突起はさらに生長して，後には皮層，髄層，および軸の分化を示し，基本的には自然界のツブコナサルオガセと同じ内部構造を持つ地衣体が形成される。この再生地衣体の発生過程ならびに生産される化学物質について報告した。

再生地衣体の発生過程では，次の四段階が観察された。1) 菌糸と藻細胞のゆるやかな接触，2) 藻細胞の表面を菌糸がゆるく被ったソレデイア（粉芽）状小突起の形成，3) 表面が菌糸で被われた突起の長軸方向への伸長，4) 先端部分が粘液性物質で被われた突起の形成である。再生地衣体と天然の地衣体とを比較すると，次のような微妙な相違が認められる。すなわち，天然のツブコナサルオガセは肥厚した細胞壁をもつ菌糸からなる皮層を持ち，外側は不定形の薄い膜で被われているのに対して，再生地衣体の皮層の菌糸では，細胞壁の肥厚はみられず，皮層の外側は薄い粘液性物質で被われている；髄層の菌糸の細胞は，自然界のものでは皮層直下のものを除いて円筒形をしているが，再生地衣体の場合は球状に近い，また，再生地衣体の軸の菌糸においても皮層の場合とおなじく，細胞壁の肥厚はみられないなどである。これらの違いは，培養条件下の特殊な環境によってひきおこされるものであるか，あるいは，自然界でも地衣体形成のごく初期の段階では同様な過程を示すものであるかは目下不明である。本亜種にはウスニン酸，ノルスチクチン酸が含まれるが，同じ化学物質は再生地衣体でも検出された。また，この両物質は低濃度ながら再生地衣体を支持する培養組織からも検出された。

Explanation of plate I

Pl. I. Regenerated fibrils of *Usnea confusa* ssp. *kitamiensis*. A, a fibril regenerated under light condition (×110), showing differentiation of cortex, medulla, gonidial layer, and axis. B, a fibril regenerated under light condition (×440). The cortex is very thin. C, a fibril regenerated under dark condition (×110), showing axis extending downward into the center of cultured lichen tissue. D, numerous fibrils formed on the surface of cultured tissue (×1.8).