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On the life history and host-specificity of *Blastophysa rhizopus* (Codiales, Chaetosiphonaceae), an endophytic green alga from Muroran in laboratory cultures[1)]

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The developmental morphology and life history of the endophytic green alga *Blastophysa rhizopus* REINKE from Muroran were investigated in unialgal and bialgal cultures. The results in unialgal culture correspond with the previous descriptions obtained from North American strains. Bialgal cultures between *B. rhizopus* and various other species of seaweeds as host plants were carried out for the first time. Although *B. rhizopus* has been reported as the endophyte of various plants, it only shows quick and strong penetration into the original host *Grateloupia turuturu* with slow and weak penetration into some other red algae. It does not naturally penetrate the other green, brown and red algae examined, however penetration can be induced by artificial wounding. *B. rhizopus* does not have host-specificity, but seems to have favorable limited hosts as substrates growing in the same season at different habitats. The mode of host tissue penetration is also discussed.

Since the temperature response of laboratory cultures was found to reflect the seasonal variation in nature, this endophyte from Muroran may be considered to share its life with *G. turuturu* as the host.

Key Index Words: bialgal culture; Blastophysa rhizopus; *Chlorophyta; endophyte; host-specificity; life history.*

The endophytic green alga, *Blastophysa rhizopus*, was first described by REINKE (1888) growing in the basal disc of *Dumontia filiformis* and the thallus of *Hildenbrandia* sp.. Since then many workers have reported various hosts for *B. rhizopus: Enteromorpha compressa* (HUBER 1892), *Nemalion schrammi* (BØRGESEN 1911), *Sphacelaria tribuloides, Ruppia maritima* and *Ulva lactuca* (COLLINS and HERVEY 1917), *Hildenbrandia* sp. and *Zostera* sp. (PRINTZ 1926), *Anadyomene stellata* (SCHUSSING 1930), *Neodilsea yendoana, Grateloupia turuturu* and *Schizymenia dubyi* (TOKIDA and MASAKI 1948), *Dumontia incrassata, Eudesme virescens, Punctaria* sp. and *Acrothrix novae-angliae* (SEARS 1966), *Dumontia* sp. (IRVINE *et al.* 1975), *Predaea feldmannii* (SEARLES and LEISTER 1980), *Dudresnaya* sp. and *Liagoropsis schrammi* (BALLANTINE and WYNNE 1986).

Of those investigations, only SEARS (1966) performed culture experiments with *B. rhizopus*. He worked on the developmental morphology, life history and cytology of this alga in both plants from unialgal culture and nature.

There are, however, no culture experiments to distinguish this alga as a true endophyte and not an epiphyte. It appears to have neither a few restricted or specific hosts nor indiscriminate substrates,

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although it seems to be endophytic in a wide range of algal species in the descriptions as mentioned above.

We have carried out the present study to confirm the development, life history and host-specificity in *B. rhizopus* from Muroran in unialgal and bialgal cultures. We describe here in detail the morphogenesis of this endophyte, especially in bialgal cultures and also consider the relationship between the effects of culture conditions and seasonal changes on the occurrence of this alga.

Materials and Methods

Isolation and unialgal culture

Blastophysa rhizopus was collected at Charatsunai, Muroran on Sep. 8 and 22, 1984, Sep. 14, 27, Oct. 14, 22 and Nov. 16, 1985, growing in vegetative and fertile thalli of *Grateloupia turuturu*.

Fertile sporangia of *B. rhizopus* were isolated by pipetting from cross-sectioned *G. turuturu* blades. Vegetative coenocytes were also crude cultured within the pieces of original host tissue, generating fertile sporangia in high temperature conditions (18–22°C) within 1–2 weeks. These fertile sporangia released bi- or quadriflagellate swarmers. Swarmers were pipetted onto glass slides with a few drops of medium and maintained as unialgal cultures in vessels (6.5 cm × 8.0 cm) containing 180–200 m*l* medium.

The culture medium employed was PES (PROVASOLI 1966) and was renewed every month. Culture experiments were conducted in 10 incubators equipped with Cool-White 40 W fluorescent lamps (ca. 12–18 W. m^{-2}) under the following temperature and photoperiod regimes: 5°C, $\underline{14}:\overline{10}$ (no. 1) or $\underline{10}:\overline{14}$ (no. 2); 10°C, $\underline{14}:\overline{10}$ (no. 3) or $\underline{10}:\overline{14}$ (no. 4); 14°C, $\underline{14}:\overline{10}$ (no. 5) or $\underline{10}:\overline{14}$ (no. 6); 18°C, $\underline{14}:\overline{10}$ (no. 7) or $\underline{10}:\overline{14}$ (no. 8); 22°C, $14:\overline{10}$ (no. 9) or $10:\overline{14}$ (no. 10).

Cytology

Chromosome counts were made using unialgal culture plants derived from bi- and quadriflagellate zoospores. These coenocytes were fixed in ethanol: acetic acid (3:1 v/v) and stained with an aceto-iron-haematoxylin-chloral hydrate solution (WITTMANN 1965).

Bialgal culture

For bialgal cultures with *B. rhizopus* cultured from swarmers, tetraspores and carpospores of the original host species *Grateloupia turuturu* were isolated and cultured by the capillary pipette method. Other algal species listed in Table 1 were also used. Most species were cultured from spores released from fertile plants collected at Charatsunai, Muroran by the capillary pipette method. Only one species, *Pachymeniopsis lanceolata*, was collected at Shimoda, Shizuoka and obtained as a unialgal culture from tetraspores.

Electron microscopy

Bialgal cultured plants were prepared for scanning and transmission electron microscopy as follows:

For the SEM, samples were fixed in 1% glutaraldehyde in seawater for 2 hr at 4°C, post-fixed in 1% OsO_4 for 2 hr at 4°C, and then dehydrated in a graded acetone series. They were critical point dried, coated with gold, and viewed with a Hitachi S–510 SEM.

For the TEM, samples were fixed in 2% glutaraldehyde and 1% paraformaldehyde in 0.1M cacodylate buffer (pH 7.2) with 2% NaCl for 2 hr at 4°C; and post-fixed in 2% OsO_4 for 3 hr at 4°C in the same buffer with 2% NaCl; then they were bloc-stained with 2% uranyl acetate, dehydrated in acetone and embedded in SPURR's epoxy resins (SPURR 1969). Sections were cut with a diamond knife on a Porter-Blum MT–1 ultramicrotome and double stained with uranyl acetate and REINOLD's lead citrate solution (REINOLD 1963). They were observed with a Hitachi H–300 elec-

tron microscope.

Results

Field observation

Blastophysa rhizopus grows as an endophyte only in the thallus of *Grateloupia turuturu* in the lower intertidal zone at Charatsunai, Muroran, from August to December, the period of occurrence of the host alga (Figs. 1, 2). It disappears in December with the decaying of *G. turuturu* and is not found from January to July even in or on other algae.

At the beginning of its occurrence, *B. rhizopus* is restricted to the basal portion of the *G. turuturu* thallus. Most other parts of the host thallus are not affected by the endophytic infection (Fig. 2). During maturation period of *G. turuturu, B. rhizopus* extends into various parts of the matured host tissue as a green patch about 1–2 cm in diameter, while the host cells around these green patches bleach and die.

Plant morphology

Endophytic plants grow abundantly in the cortical tissue of *Grateloupia turuturu* (Fig. 3). Cells vary in shape, from spherical to tubular and 20–60 μm in diam. or 60–150 μm in length. Each cell is connected by colorless slender filaments ranging from 5–10 μm in diam.. Cells are multi-nucleate coenocytes and have numerous pyrenoids. Plants growing in host tissue project colorless hairs (3–5 μm in diam.) into the outer surface of the host.

Reproduction and development

Fertile cells are more round than vegetative ones and become sporangia (Fig. 4) which produce about 30–60 swarmers. Swarmers are released one by one through the opening of a colorless tube projected into the outer surface of the host (Fig. 5). They are bi- (Fig. 6) or quadri-flagellate (Fig. 7) asexual zoospores and do not show any sexual behavior. These two kinds of zoospores are not released from the same sporangium, but are produced from different sporangia of one individual plant.

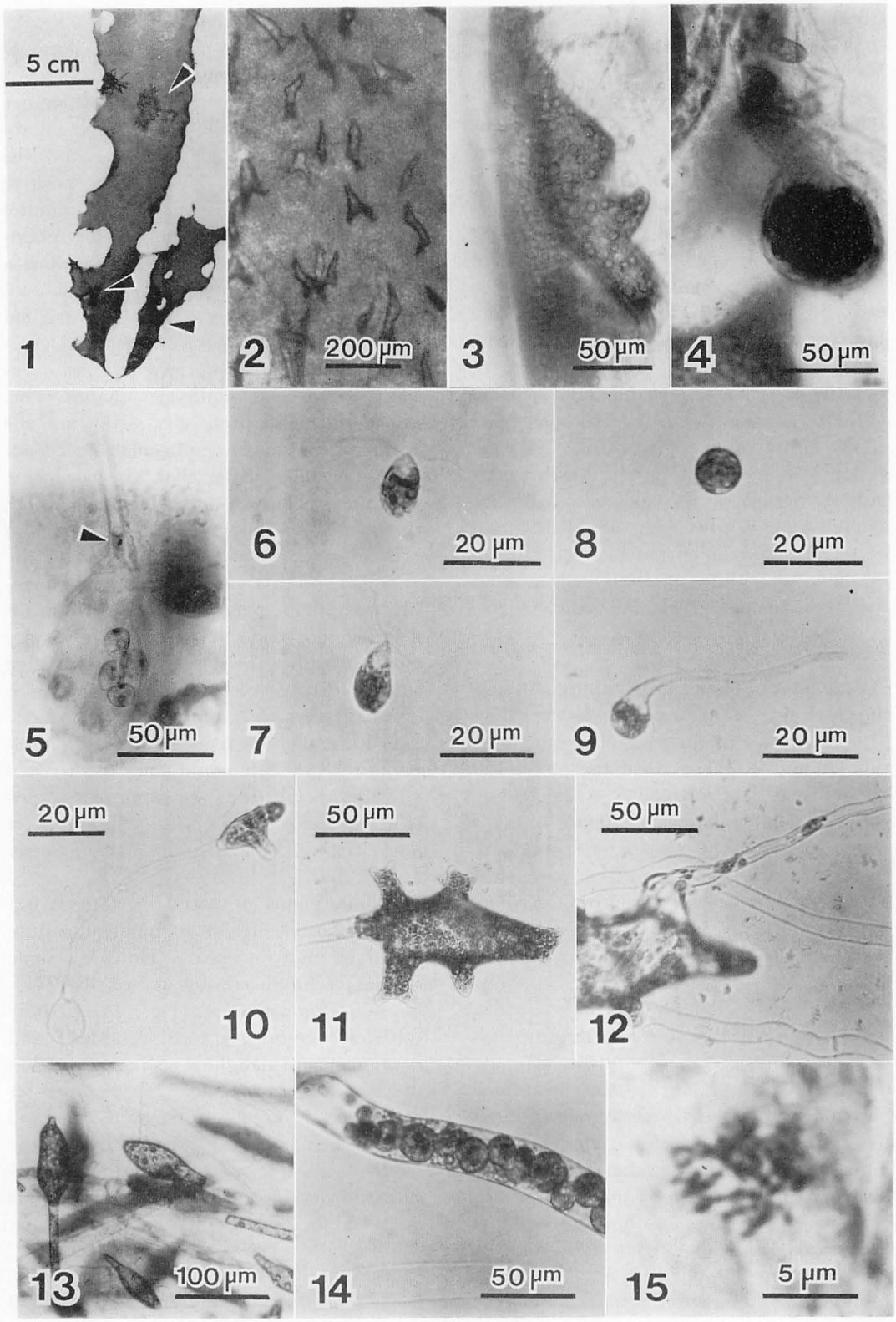
Both kinds of zoospores are pyriform or subspherical shaped measuring 16–18 μm $\times$ 8–10 μm and there is no marked difference in size between them. They have a chloroplast with an orange-colored eye-spot in the posterior of the cell. They show positive phototaxis and after swimming 1–5 minutes settle to the substratum and become spherical (Fig. 8). Settled zoospores produce a germination tube (Fig. 9) into which all the cytoplasm migrate, leaving the original cell empty (Fig. 10). The migrated cytoplasm with a single nucleus enlarges irregularly at the end of the germination tube. Nuclear divisions occur successively and the germling becomes a multi-nucleate coenocyte (Fig. 11). After that, the coenocyte elongates its growth tubes in one or more directions and its cytoplasm migrates into the elongated growth tube little by little (Fig. 12) and enlarges at the distal end forming a daughter coenocyte. Finally it forms a net-work of coenocytes connected by many elongated tubes (Fig. 13). This vegetative tubular extension occurs in all directions, especially toward a source of light.

Culture plants grew well at high temperatures (18–22°C) and long day (14:10 LD), but their growth was suppressed slightly at low temperatures (10–14°C) and short day (10:14 LD), and inhibited completely at 5°C.

Culture plants grew well vegetatively but did not become fertile in unialgal culture for more than one year. However, some of them reproduced vegetatively by cytoplasmic segmentation (Fig. 14). Many spherical protoplasts were produced and developed into daughter coenocytes within the mother coenocyte. Such daughter coenocytes enlarged in size and were all released at one time by the rupture of the mother wall. They settled on the substratum and developed in the same pattern as described for zoospore-development.

Cytology

The chromosome number was about 30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regardless of the flagellum number of zoospore (Fig. 15). Nuclear division occurred synchronously in each coenocyte.

Bialgal cultures

a) *Blastophysa rhizopus* with *Grateloupia turuturu*

A colony of coenocytes brought into contact with a basal disc or blade of *G. turuturu* adhered to the surface of such a host material tightly after a few days (Fig. 16). These coenocytes then began to penetrate into the host material and their endophytic growth occurred in one week-old culture (Fig. 17). When coenocytes were placed apart from a host material in the same culture vessel they produced long tubular filaments which enlarged toward the host and adhered to the host by their tip (Fig. 18). The host cells adjacent to the penetrating coenocyte gradually discolored and bleached completely within 3 months culture (Fig. 19).

An electron microscopic observation showed that the host cells were penetrated via pressure of the endophytic growth of coenocytes. Neighboring host cells were completely dead, but no evidence of any enzymatic digestion of these host cells was seen ultrastructurally (Figs. 20, 21).

b) *B. rhizopus* with other species

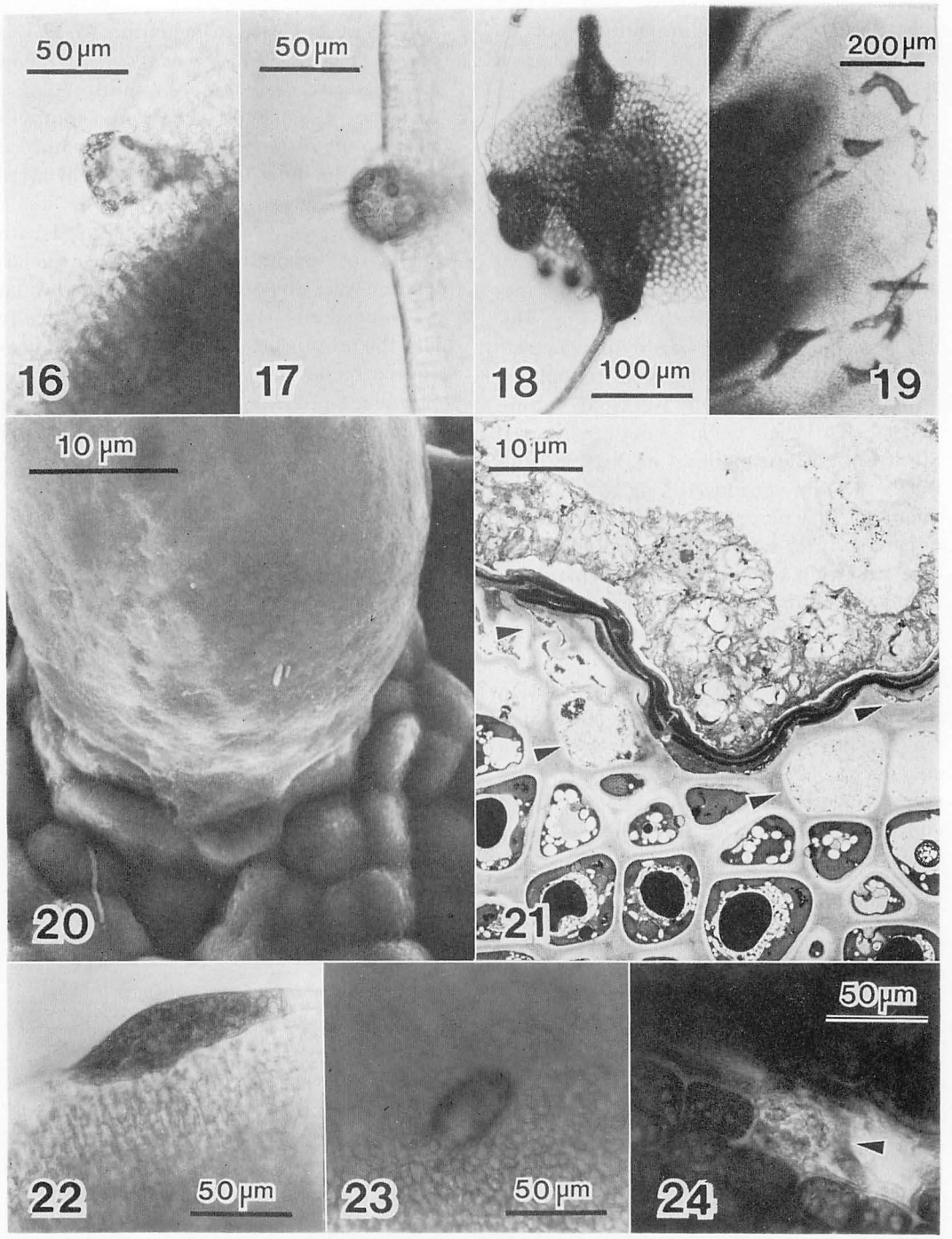
Results of bialgal cultures with other species, including the host algae reported by previous authors, are shown in Table 1. *B. rhizopus* from Muroran did not show any endophytic behavior with a short-term bialgal culture for up to 2 weeks. Coenocytes grown epiphytically on their surface were easily removed similar to those grown on a glass slide. In long-term bialgal cultures (1–3 months) however, they could adhere to and weakly penetrate *Grateloupia filicina* (Fig. 22), *Rhodymenia pertusa, Pachymeniopsis lanceolata* (Fig. 23) and *Neodilsea yendoana*. Coenocytes grew only epiphytically on the surface of other algae examined even in the long-term cultures and were easily removed.

However, when they were cultured with host tissues wounded or abraded artificially, they were able to penetrate through such a wounded site and extend endophytically into the host tissue. Fig. 24 shows that coenocytes penetrated into the wounded site of a blade of *Dictyopteris divaricata* although *B. rhizopus* could not penetrate a healthy blade of this brown alga.

Table 1. Results of bialgal culture of *B. rhizopus* with various algal species as hosts (−: only epiphytic, +: weak penetration, ++: strong penetration).

	short-term cul. (1–2 weeks)	long-term cul. (1–3 months)
Chlorophyta		
Ulva pertusa	−	−
Phaeophyta		
Dictyopteris divaricata	−	−
Laminaria japonica	−	−
Fucus evanescens	−	−
Pelvetia wrightii	−	−
Rhodophyta		
Grateloupia turuturu	+	++
G. filicina	−	+
Neodilsea yendoana	−	+
Pachymeniopsis lanceolata	−	+
Chondrus yendoi	−	−
Gigartina japonica	−	−
Rhodymenia pertusa	−	+
Palmaria palmata	−	−
Ptilota pectinata	−	−

Fig. 1, *Blastophysa rhizopus* as green patches (arrowheads) on *Grateloupia turuturu* (mature carposporophytes). Fig. 2, Surface view of the vegetative host thallus infected by many coenocytes. Fig. 3, Cross section of the host thallus and a vegetative coenocyte of *B. rhizopus*. Fig. 4, Fertile sporangium. Fig. 5, Release of zoospore (arrowhead). Figs. 6–14, Reproduction and development of *B. rhizopus* in unialgal culture: Fig. 6, Biflagellate zoospore. Fig. 7, Quadriflagellate zoospore. Fig. 8, Settled zoospore. Fig. 9, Settled zoospore producing germination tube. Fig. 10, One-day-old germling. Fig. 11, Terminated coenocyte elongating growth tubes in many directions. Fig. 12, Cytoplasmic migration through growth tubes. Fig. 13, Coenocytes net working connected by tubes. Fig. 14, Coenocytes produced by cytoplasmic segmentation. Fig. 15, Chromosomes of *B. rhizopus* counted about 30.



Figs. 16–21. Bialgal culture of *B. rhizopus* with the natural host, *Grateloupia turuturu*: Fig. 16, A coenocyte adhered to a basal disc (in 1 week bialgal culture). Fig. 17, Penetration into a thallus surface (in 1 week). Fig. 18, Adhesion to a young germling. Fig. 19, A basal disc infected by many coenocytes (after 3 months). Fig. 20, SEM photograph of penetration of a coenocyte into a thallus surface (after 1 week). Fig. 21, TEM photograph of a section of boundary region between a coenocyte and host cells. Neighboring host cells are pressed and bleached, but no digestion seems to have occurred (arrowheads). Figs. 22–24, Bialgal culture with other species: Fig. 22, Adhesion to a basal disc of *Grateloupia filicina* (in 3-week-old bialgal culture). Fig. 23, Weak penetration of a thallus of *Pachymeniopsis lanceolata* (after 3 weeks). Fig. 24, Penetration of artificially wounded sites of a thallus of *Dictyopteris divaricata* (arrowhead).

Reproductive maturation

In unialgal culture *Blastophysa rhizopus* only grew vegetatively. However, in bialgal culture with *Grateloupia turuturu*, *B. rhizopus* reached reproductive maturity within 5–6 months. Each coenocyte became round and produced bi- or quadriflagellate zoospores again. These zoospores were released through an opening in the distal end of a tube projected into the outer surface of the host tissue in the same manner as described for field plants. Further development of zoospores occurred as described previously. However, these second generation coenocytes reproduced zoospores in 2–3 month-old culture even in unialgal cultures.

Discussion

The present results of reproduction, development and cytology in unialgal culture of *Blastophysa rhizopus* from Muroran, Hokkaido (Figs. 6–15) agree with those of SEARS (1966), working on the materials from North America (including UTEX no. 1029 BROOKS strain). A coenocyte makes vegetative tube growth and forms a net-work of numerous coenocytes connected to each other with a filamentous colorless tube. Reproduction is either vegetative by cytoplasmic segmentation, or asexual by bi- and quadriflagellate zoospores. Crossing experiments between biflagellate swarmers derived from different individuals were attempted several times, but sexual behavior was not observed, the chromosome number was always about 30 and meiotic figures were not seen in any phase of development. According to SEARS (1966), it is possible that this species is dioecious and the proper mating strains have not been crossed, although sexual fusion between biflagellate zoospores was not observed. Moreover, in plants collected from Nagasaki, Kyushu (MIGITA, personal comm.), both macro- and micro-swarmers are often found. Therefore further crossing experiments between different geographically isolated strains will be needed to prove the absence of sexual reproduction in this species.

As noted in the introduction, other authors have reported various species as host plants for *Blastophysa rhizopus*. Therefore, this endophyte is considered to be apparently indiscriminate regarding substrate species. SEARS (1966) already pointed out that *B. rhizopus* can grow unialgally in various growth media without specific host tissues, and the relationship between this alga and its host tissue is apparently that of a substrate requirement for a coenocyte which lacks a holdfast mechanism. In the present study, however, it was found only in *Grateloupia turuturu* tissue and was not found to grow in other species or host free in the natural habitat of Muroran.

In bialgal cultures between *B. rhizopus* and other species, the results suggest that the plant from Muroran has few hosts or nearly complete host specificity (Table 1). This endophyte showed quick and strong penetration of the original host plant, *Grateloupia turuturu*, but slow and weak penetration of four red algae; *G. filicina*, *Neodilsea yendoana*, *Rhodymenia pertusa* and *Pachymeniopsis lanceolata*. In other species examined as partners in bialgal cultures, *B. rhizopus* coenocytes showed only epiphytic growth even in long-term culture (6 months). However, in bialgal cultures with those partners wounded or abraded artificially, the coenocyte can easily infect its partners at a wound site and grow endophytically in their tissue (Fig. 24). From this, it is understandable that various plants, including species of spermatophytes, have been reported as the host plants by previous authors. It is clear that *B. rhizopus* seems to commonly have limited host plants in each habitat but penetrates indiscriminately in wounded plant. It can grow well vegetatively on the glass slide in unialgal culture and completely repeat a monophasic life cycle without any host plant, although zoosporogenesis in unialgal culture lags behind the one in bialgal cultures by several months. It means that this endophyte does not need a supply of any nutrients from the

host. Therefore, it does not have a host-specificity, but does have favorable limited hosts as substrates. These are coarser algae of soft tissue and belong mainly to the Cryptonemiales or Nemalionales (Rhodophyta) and some of the Chordariales (Phaeophyta). They grow with *Blastophysa* in the same season at each habitat as described by previous authors.

The mode of penetration into the host tissue is important because it gives an understanding of how the endophytic relationship is established. It is well known that endophytic and parasitic algae that require a wound or abraded site on the host material usually penetrate by either enzymatic action or through the pressure effect of the terminal cell of the filamentous germling (including rhizoidal cell). A parasitic red alga, *Harveyella mirabilis* requires wound site on the host *Odonthalia floccosa* for spore penetration and development (GOFF and COLE 1976), while the spores of *Janczewskia* spp. do not require any wound or abraded site on the host plants *Laurencia* spp. for their penetration (FELDMANN and FELDMANN 1958, NONOMURA 1979). According to NONOMURA, working on spore development of *Janczewskia morimotoi*, the rhizoid of this alga elongates, pushing between or directly through the host cells. However, penetration may occur partly as a result of digestive rather than a completely mechanical mode. RAWLENCE (1972) investigated the relationship between an obligate epiphyte *Polysiphonia lanosa* and its specific host *Ascophyllum nodosum* ultrastructurally. The rhizoid of this epiphyte was found to digest its way into the host tissue. According to WHITE and BONEY (1969), working on an endophytic filamentous alga *Acrochaetium endophyticum*, the mode of entry of the filament appears to be due to pressure effects on the surface of the host *Heterosiphonia plumosa*, but there is no evidence of any enzymatic dissolution of the host wall. In *Blastophysa polymorpha*, PRINTZ (1926) described that the germination tube enters between the surface cells of the host tissue and later is restricted to the cortical layer of the host cells. Thus the enlarging coenocytes are pushed out of shape, but generally are not destroyed.

In the present study, ultrastructural observations showed that the coenocytic filament can penetrate any part of *Grateloupia turuturu* without any wound sites through pressure effects of the tip of the filament (Fig. 20). No evidence of enzymatic action for penetration of the host wall was found. However, in a long-term culture (Fig. 19), the host tissue adjacent to endophytic coenocytes became gradually discolored producing white patches. In nature, young host cells adjacent to coenocytes are not killed, but when both the host and endophyte become mature, the host cells around endophytic cells bleach and die.

Although there are no reports that host plants are ever killed by parasitic or endophytic algae, the two red parasites *Harveyella* and *Choreocolax* do cause some degree of minor host disruption (KUGRENS and WEST 1973, GOFF 1976, CALLOW *et al.* 1979). Still, there is no appearance of substantial deleterious effect on the hosts. TOKIDA and MASAKI (1948) reported that *B. rhizopus* is a pathogenic green alga which causes "green spot rotting" on *Neodilsea yendoana*. According to them, however, the endophytic growth of *B. rhizopus* only destroys host tissue mechanically. These wound sites become discolored patches resulting from bacterial activity which does not have deleterious effect on the endophyte. If the endophytic coenocyte has no ability to digest or lyse the host tissue, it is possible that some bacteria may have something to do with penetration of the host. If so, further experiments on the mode of infection process are needed to be carried out in axenic bialgal cultures.

Development of zoospores is influenced by culture conditions, especially temperature. The coenocytes derived from zoospores can grow at temperatures ranging from 10–25°C, remarkably well at 18–22°C, but cease to grow completely at 5°C. This

laboratory temperature response is reflected in the seasonal variation of *Blastophysa rhizopus* growth in nature. The water temperatures at Muroran range from 20–10°C from August to November, during which *B. rhizopus* grows abundantly in the host *Grateloupia turuturu*. This temperature range is suitable for growth and reproductive maturity of this endophyte. In December, however, the host plants decay and water temperature falls to below 5°C. During the winter months (3–5°C), *B. rhizopus* growth is completely suppressed and can not be found in or on any algal species. It may survive in the prostrate discs (remaining holdfasts or new germlings) of host plants as a spherical coenocyte or a few celled colony until June-July when temperature rises to 10–14°C. With the growth of erect blades from the prostrate discs of *G. turuturu* in summer, the endophytes grow rapidly in the host tissue and finally appear as green patches. As mentioned above, the growth in relation to regarding temperature in culture agrees well with the short-term appearance of this species at Muroran. Therefore, *B. rhizopus* from Muroran may be considered to share its life with the host plant *G. turuturu*. Still, there seems to be geographical variation of host plants reflected in the presence of various substrate species in different localities (but with other host plants in different habitats).

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飯間雅文・舘脇正和：室内培養における室蘭産内生緑藻アワミドリ（ミル目ケートシフォン科）の発生・生活史と宿主特異性について

室蘭産内生緑藻アワミドリ *Blastophysa rhizopus* Reinke の発生・生活史が，単藻及び種々の海藻との二藻培養で調べられた。単藻培養の結果，生活史はこれまでの北米産の報告と一致した。アワミドリの宿主海藻との二藻培養は初めて行われ，これまで様々な海藻が宿主として記載されているにもかかわらず，室蘭産アワミドリは天然宿主紅藻ツルツルにのみ速やかな着生と組織への侵入を示し，数種類の紅藻に対してゆっくりとした弱い侵入を示した。他の多くの緑藻，褐藻，紅藻の組織には全く侵入しなかったが，それらの海藻にも人偽的に傷をつけたところその部位に侵入を示した。本種には宿主特異性はないが，地域ごとに着生基質として同時期に生育する宿主藻が限られていることが推察される。また宿主藻組織への侵入方法についても考察された。さらに室蘭産アワミドリの生長の温度に対する反応は，天然での季節的消長を反映していることから，この内生藻は宿主藻ツルツルと一体の生活史を営んでいると考えられる。（051 室蘭市母恋南町1-13 北海道大学理学部附属海藻研究施設）