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Zoosporangium development, zoospore release and culture properties of *Halophytophthora mycoparasitica*

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***Halophytophthora mycoparasitica*, a fungal parasite with unique zoosporangial ornamentation, was isolated from submerged fallen leaves of mangrove collected in the South-west Islands, Japan, since the original description from Malaysia. Isolates were examined for the development of zoosporangia and the process of the zoospore release. Scanning electron microscope observation revealed that the denticulate ornamentation of the surface of the zoosporangium is composed of many sets of assembling spines which are densely covered with mucilage. The mucilage is exuded from the spines and spreads over the zoosporangium as the zoosporangium develops. The mucilage works to gather spines into groups, which are angled towards the center of each assembly. Details were observed on the process from dehiscence plug development, through its ejection, to zoospore release. Mycoparasitic behavior was also observed in a mixed culture with a host fungus. Culture studies showed the species adapts well in its growth and asexual reproduction to brackish and sea water in a mangrove environment.**

Key Words——*Halophytophthora mycoparasitica*; mangrove; mycoparasite; zoosporangium ornamentation.

Halophytophthora mycoparasitica (Fell & Master) Ho & Jong was originally described as *Phytophthora mycoparasitica* by Fell and Master (1975) from submerged decomposing leaf of *Rhizophora* sp. collected from mangrove in Malaysia. The species was transferred to the genus *Halophytophthora* Ho & Jong along with other marine *Phytophthora* species by Ho and Jong (1990). *Halophytophthora* species are saprophytic oomycetes inhabiting marine and brackish waters, especially mangrove regions, and play an important role in decomposition of submerged fallen mangrove leaves (Newell et al., 1987; Nakagiri et al., 1996; Newell, 1996). Thirteen species and two varieties have been hitherto described in the genus, mainly from submerged mangrove leaves. Among halophytophthoras, *H. mycoparasitica* may be a rare species, because it has not been reported since the original description in 1975. It is characterized by its spiny zoosporangium, thick-walled zoosporangial wall near the base of the dehiscence tube, conical evanescent dehiscence plug, and mycoparasitism on *Pestalotiopsis* sp. and other fungal hyphae (Fell and Master, 1975). All the cultures of *H. mycoparasitica*, including one (ATCC 28292) derived from the holotype, have been lost, probably owing to the difficulty in maintaining such mycoparasitic strains in pure culture. Thus, at present no culture is available from any culture collection.

During the study of mangrove fungi in the South-west Islands, Japan, we obtained several isolates of *H. mycoparasitica* from submerged fallen leaves of *Bruguiera gymnorrhyza* Lamrk. The characteristics of our isolates corresponded closely with those described by Fell and Master (1975). Having observed the developmental process of zoosporangia and dehiscence plugs under a light and a scanning electron microscope (SEM), we report here details of the process of ejection of the dehiscence plug and subsequent zoospore release, and also the development of the spiny ornamentation on the surface of the zoosporangium. The surface structure was originally described as being denticulate, or consisting of clusters of spines (Fell and Master, 1975), but later Stamps et al. (1990) suggested it was made of clusters of bacteria attaching to the surface of the zoosporangium rather than spines, judging from the figures in the original description. Our study reveals the nature and development of the ornamentation. We also conducted culture studies with or without the host fungus, *Pestalotiopsis* sp., to clarify the fungal parasitic feature of *H. mycoparasitica*. Growth and reproduction at different temperatures and salinities were examined to know how the species adapts its growth and asexual reproduction to the mangrove environment. Efficient methods for cultivating and preserving the strains in pure culture were also investigated.

Materials and Methods

Isolation Submerged fallen leaves of various mangrove trees were collected from the Maira River, Iriomote Is., Okinawa Pref., Japan on 25 Oct. 1996. Disks of 8 mm diam were cut with a cork borer from the leaves and washed five times in a sterilized artificial sea water (Jamarin S; Jamarin Laboratory, Osaka) (salinity 15 ppt) by shaking in a test tube. After draining on a sterilized

filter paper, the disks were placed on plates of half-strength corn meal seawater agar (1/2 CMSWA) (a half strength of corn meal agar (Nissui, Tokyo) with compensatory agar was dissolved in 15 ppt seawater). The plates were incubated at 15°C for 2 wk. From several disks of orange colored fallen leaves of *B. gymnorrhiza*, *H. mycoparasitica* appeared together with hyphae of *Pestalotiopsis* sp. Agar blocks containing hyphae were transferred to plates of vegetable juice seawater agar (VJSWA-20: 10% (v/v) vegetable juice, 0.3% (w/v) calcium carbonate, 2% agar in 20 ppt salinity sea water). Isolation of *H. mycoparasitica* often resulted in mixed cultures with *Pestalotiopsis* sp. To purify the cultures, single zoosporangia of *H. mycoparasitica* were transferred to new plates. Six strains of pure cultures (AN-1571–AN-1576) and two strains of mixed cultures (AN-1577 and AN-1578) were obtained. A culture of the host, *Pestalotiopsis* sp. (AN-1614), was also obtained by isolating conidia.

Incubation Pure cultures of *H. mycoparasitica* grew so poorly on 1/2CMSWA that VJSWA-20 was employed to incubate them. However, the pure culture on VJSWA-20 still showed slower growth than the mixed culture with *Pestalotiopsis* sp. To find a medium to improve the growth of the pure culture, *Pestalotiopsis*-extract medium (PEM) was prepared as follows: a strain of *Pestalotiopsis* sp. (AN-1614) was incubated on a slant agar medium of VJSWA-20 in a test tube for 3 d at room temperature (20–25°C) until its hyphae covered the medium, then the medium was autoclaved and poured into a Petri dish.

Preservation For stable and prolonged maintenance of the cultures, we investigated efficient methods for freezing preservation. Agar blocks containing hyphae were frozen at a constant cooling rate (1°C/min) and stored in a vapor of liquid nitrogen.

Observation of development of zoosporangia Zoosporangia were produced abundantly when agar blocks of VJSWA or PEM containing hyphae were submerged in 20 ppt salinity sea water for ca. 12 h. The developmental process of the zoosporangium and the dehiscence plug at the apex of the dehiscence tube and the following zoospore release was observed under a phase contrast microscope. Fine structures of the surface of the zoosporangia and the apex of the dehiscence tube were examined under SEM. To prepare the SEM specimens, the submerged agar blocks (ca. $5 \times 5 \times 2$ mm) with zoosporangia were fixed in 1% OsO_4 (in 10 ppt salinity sea water) for 2 h at room temperature, then dehydrated in an ethanol series and substituted with isoamyl acetate. After critical point drying and coating with platinum-palladium, the specimens were observed with a JSM 5400 (JEOL) operated at 15 kV.

Tests for growth and reproduction Effects of salinity and temperature on the hyphal growth and zoosporangium formation of two strains (AN-1573 and AN-1574) were examined as reported previously (Nakagiri, 1993; Nakagiri et al., 1994) with some modifications. VJSWA-20 was employed for the growth tests at different temperatures (10, 15, 20, 25, 30, and 37°C) and VJSWA with different salinities (0, 10, 20, 30, 40, and 50 ppt) were used for the salinity tests at 25°C. To obtain inocula, the two strains were subcultured on VJSWA-30 at 25°C for 9 d. Agar blocks (2 mm square) containing mycelium were used as inocula. To test zoosporangium formation, the isolates were subcultured on PEM at 25°C for 7 d, then agar blocks containing hyphae were transferred into sterilized sea water of different salinities (0, 10, 20, 30, and 40 ppt) or temperatures (10, 15, 20, 25, 30, and 37°C). The salinity tests were performed at 25°C and the temperature tests were done in 30 ppt salinity sea water. The abundance of produced zoosporangia was examined under a microscope after overnight submersion.

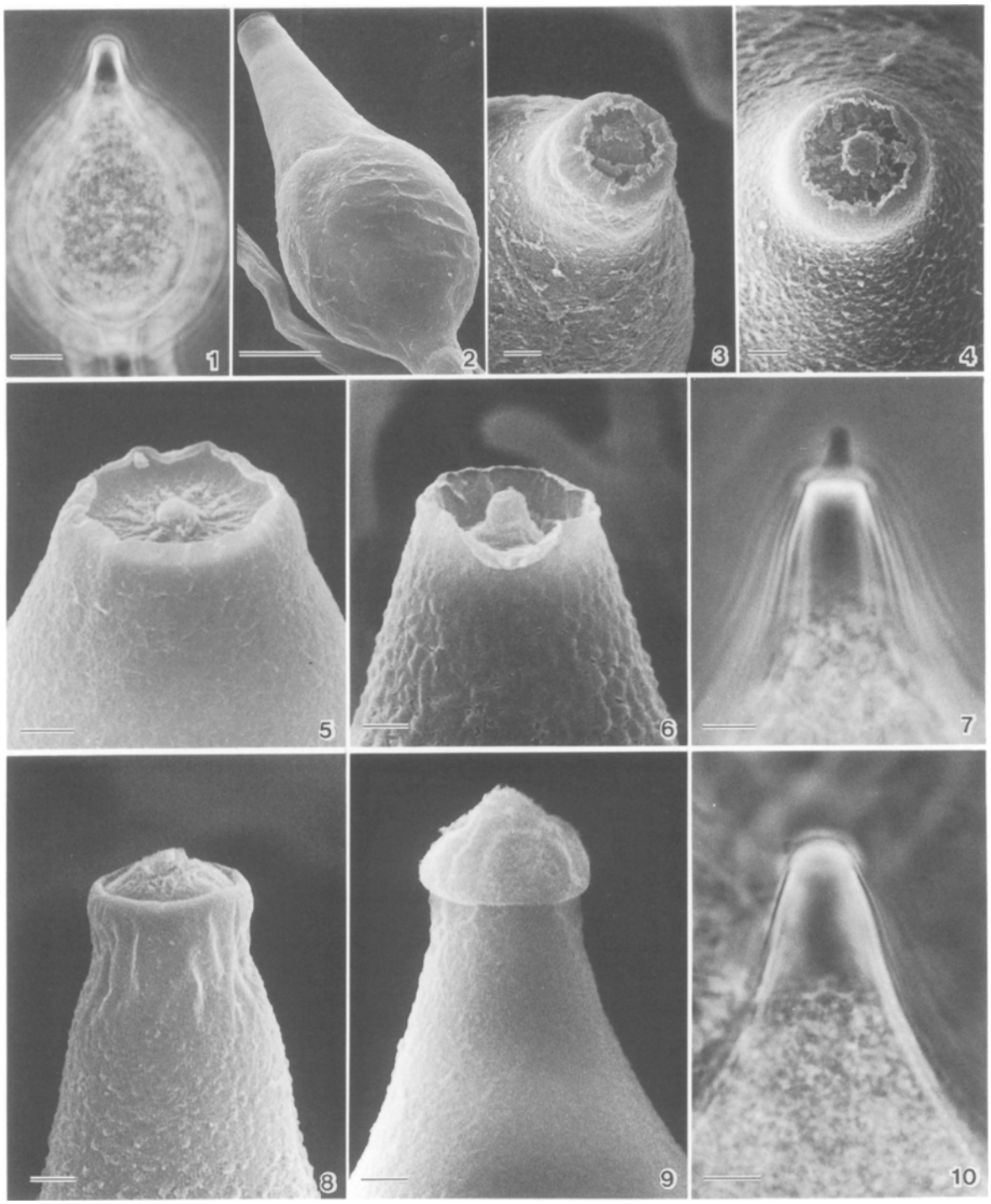
Observation of parasitism Interaction between *H. mycoparasitica* (parasite) and *Pestalotiopsis* sp. (host) was investigated by incubating them on a 1/2CMSWA plate, on which inocula of both species were placed separately. The point of contact of the two colonies was examined under a phase contrast light microscope.

Results and Discussion

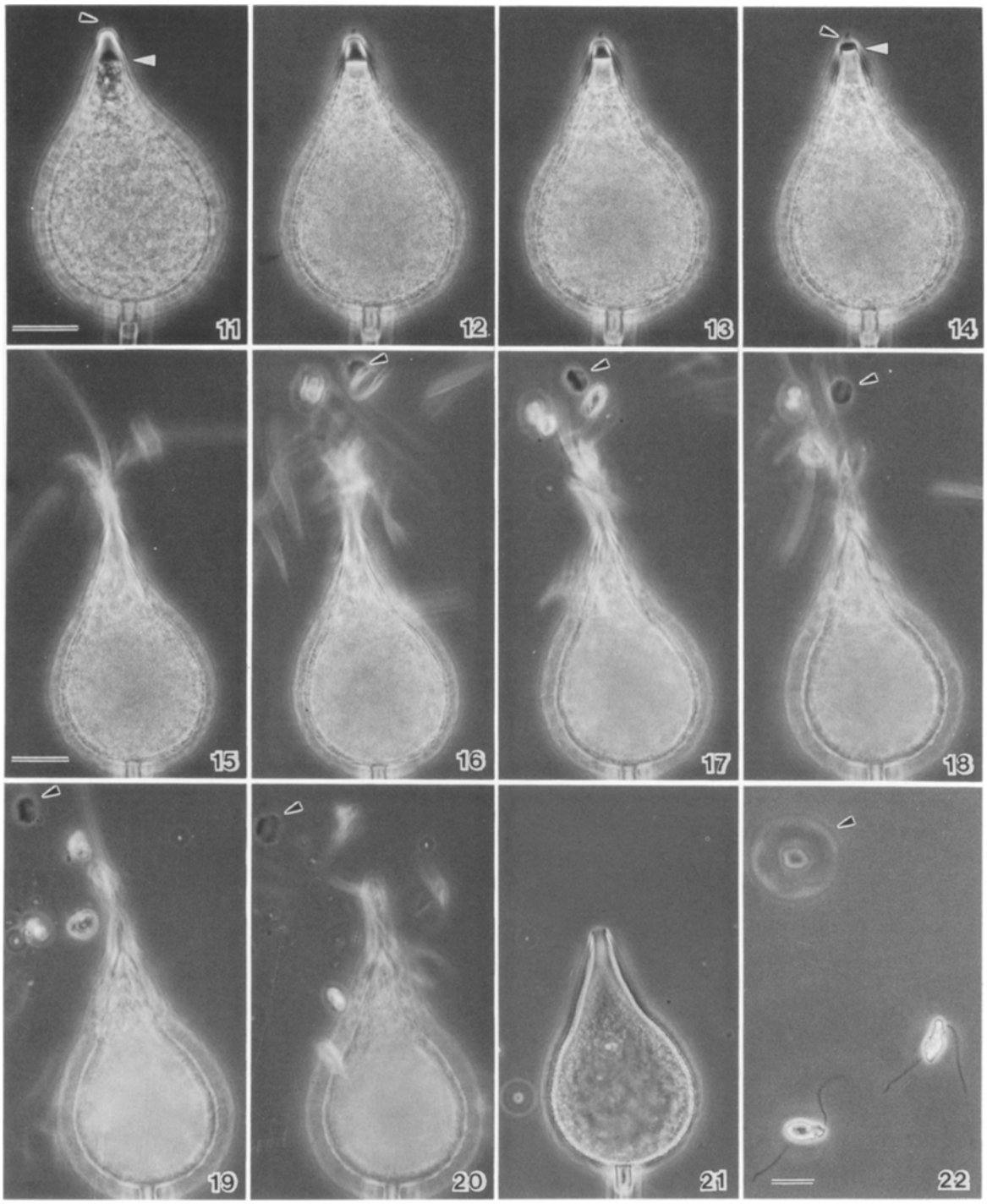
Development of zoosporangia and dehiscence plug At the early stage of development, zoosporangia have a smooth surface and form a short conical dehiscence tube with a rounded apex (Figs. 1, 2). When the transparent plug material begins to deposit inside the apex of the dehiscence tube, the surface wall of the apex is perforated in the center and the wall turns back toward the periphery to enlarge the pore (Fig. 3). Within the pore, the apical part of the dehiscence plug is exposed and a nipple-like protuberance arises at the center (Figs. 4, 5). The protuberance of the plug apex elongates in time (Fig. 6). The plug of this stage appears "mucronate" (Fell and Master, 1975) with a membranaceous protuberance when observed under a light microscope (Fig. 7). Then the plug apex begins to rise beyond the end of the dehiscence tube (Figs. 8, 10). The plug develops a rounded distal end with a papilla (Fig. 9) and a concave proximal end (Figs. 10, 11). At this stage, the cytoplasm in the zoosporangia differentiates to zoospores.

Process of zoospore release Approaching the zoospore release, the proximal end of the plug is pressed up toward the apex and the volume of the plug is decreased by compression (Figs. 11–14). Subsequently, the plug is ejected from the dehiscence tube and zoospores swim away one by one through the tube (Figs. 15–21). No vesicle is formed at the end of the tube. The ejected plug becomes hemispherical cushion-shaped with an apical protuberance, having a circular depression at the central part of the proximal end (Figs. 16–20, 22), which may look like a disk with a central thinner part and a peripheral thick ring when observed upright under phase contrast (Figs. 18, 22). The plug swells and evanesces in 1–2 min after ejection.

Morphology and development of the apex of the dehiscence tube and the dehiscence plug of *H. mycoparasitica* resemble those of *H. masteri* Nakagiri & Newell (see Figs. 22–31 in Nakagiri et al., 1994).



Figs. 1–10. *Halophytophthora mycoparasitica*.
Phase-contrast microscope (1, 7, 10) and SEM (2–6, 8, 9) micrographs. 1, 2. Young zoosporangium with a conical dehiscence tube. Note tiny protuberances emerging on the surface. 3–6. Departmental process of the apex of the dehiscence tube. Along with enlargement of the apical pore of the dehiscence tube, the distal end of the dehiscence plug becomes exposed and a nipple-like protuberance arises at the center. 7. Mucronate apex of the dehiscence tube observed under LM. 8–10. The distal end of the dehiscence plug rising beyond the end of the tube. The plug has a rounded distal end with a papilla and a concave proximal end. Bars: 1=10 μm; 2, 7, 10=5 μm; 3–6, 8, 9=1 μm.



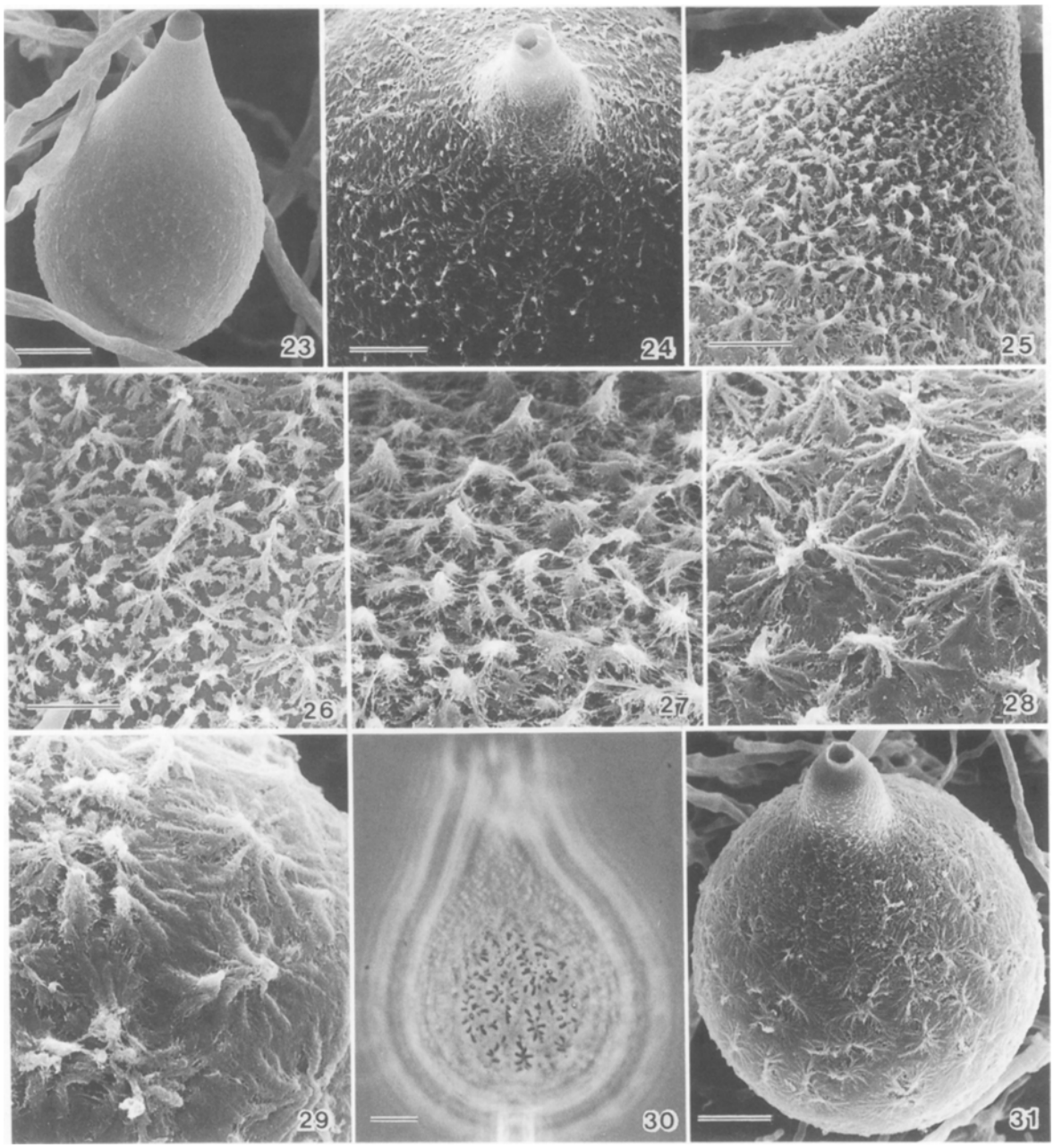
Figs. 11–22. *Halophytophthora mycoparasitica.*
Phase-contrast micrographs of zoospore release from a zoosporangium. 11–14. Before ejection of the plug, the proximal end of the dehiscence plug (white arrowhead) rises up toward the apex, so that the plug material becomes compressed. The plug has a protuberance (black arrowhead) at the distal end. 15–21. Zoospore release. Following the ejection of the plug, zoospores swim away one by one through the dehiscence tube. Note the hemispherical, cushion-shaped plug (arrowheads). 22. Biflagellate zoospores and an ejected and swollen, disk-shaped plug (arrowhead). Bars: 11 (=12–14), 15 (=16–21)=20 μm; 22=10 μm.

However, the latter species differs in that the ejected plug elongates up to 40 μm and persists for more than 3 h after ejection, and a vesicle is formed at the opening of the dehiscence tube (Nakagiri et al., 1994).

Development of spiny ornamentation of zoosporangia

As zoosporangium development proceeds, spiny orna-

mentation begins to develop on the surface. Our observation using SEM revealed the following developmental process of the ornamentation. The young zoosporangium has small protuberances distributed sparsely on the surface except at the dehiscence tube (Fig. 23). As the zoosporangium develops, the protuberances elongate into spines and a mucilaginous material deposits on the surface of the zoosporangium (Figs. 24, 25). The mucilage is deposited only on the zoosporangia, not on hyphae and zoosporangiophores, and less on the dehiscence tube. The mucilage is exuded from the spines (protuberances) and spreads to the surface of the



Figs. 23–31. *Halophytophthora mycoparasitica*.
SEM (23–29, 31) and phase-contrast (30) microphraphs of zoosporangia. 23. Young zoosporangium with small protuberance on the surface. 24, 25. The protuberances elongate into spines, from which mucilaginous material is exuded. 26, 27. The mucilage spreads from the spines to the surface of the zoosporangium. Note the neighbor spines are connected with the mucilage. 28, 29. Spines surrounded densely by the mucilage. They gather into groups by joining the spines nearby with the mucilage. 30. Phase-contrast micrograph focused on the surface of the zoosporangium. Note the spines clustering irregularly or into groups. 31. SEM of a mature zoosporangium covered with spines and mucilage. Spines are clustered into groups. Compare with Fig. 30. Bars: 23, 24, 30, 31 = 10 μm; 25, 26 (= 27–29) = 5 μm.

zoosporangium (Figs. 26, 27). Continuous deposition of mucilage on the spine during its growth results in a tapering stick-like structure (up to 5 μm long) (Fig. 28) composed of a spine at the core and a dense covering of mucilage. The mucilage works to join nearby sticks (spines) together, so that they gather in groups, probably around a central higher spine in each assembly (Figs. 28, 29). This developmental process of the ornamentation may explain how "the spines are clustered in small, irregular groups with the spines angled towards the center of the group" (Fell and Master, 1975). The sizes (1–5 μm long) and distribution pattern of the sticks (spines) observed under SEM (Fig. 31) correspond to those seen under the light microscope (Fig. 30; see also Fig. 17 in Fell and Master (1975)). Stamps et al. (1990) claimed of this ornamentation that "the published figures suggest that the sporangia may have borne clusters of bacteria rather than spines." Our observations refute this suggestion.

Among halophytophthoras, spiny ornamentation of the surface of zoosporangia is known only in *H. mycoparasitica* and two varieties of *H. spinosa* (Fell & Master) Ho & Jong. None of *Phytophthora* species possesses such ornamented zoosporangia (Waterhouse, 1970; Stamps et al., 1990). Exudation of mucilage over the zoosporangium is also a unique characteristic of *H. mycoparasitica*. Mature zoosporangia are covered with the spiny ornamentation and thick mucilage except around the dehiscence tube (Fig. 31). To investigate a possible function of the mucilage, we tried to observe some interaction between the zoosporangia and mangrove-isolates of bacteria and *Labyrinthula* sp., which often parasitize and disrupt the hyphae of halophytophthoras. Their cultures were introduced onto agar plates on which zoosporangia had been formed. But no evident defensive effect of the mucilage against the bacteria and *Labyrinthula* sp. was observed. Further studies to clarify the biological or ecological function of the mucilage and spines, if any, are necessary.

Growth and reproduction Growth of pure cultures of *H. mycoparasitica* on VJSWA-15 was moderate, with colony diam reaching 8–12 mm after 4 d incubation at 25°C, while on PEM the growth rate was doubled, with colony diam reaching 20–22 mm under the same culture conditions. Zoosporangium formation was observed on the surface of the media (more abundantly on PEM) after 5–10 d incubation at 25°C. When submerged in seawater for ca. 12 h, the mycelia on both media produced zoosporangia abundantly.

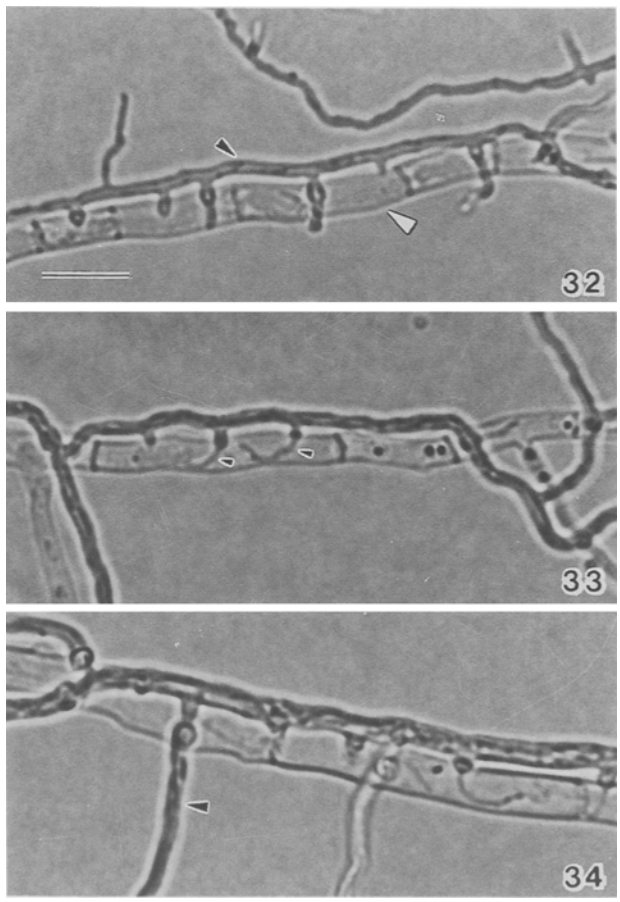
Hyphal growth on VJSWA-20 was good at 20–30°C and optimal at 25°C (Fig. 35). No growth was observed at 10 and 37°C. As to salinity, good growth was observed at above 10 ppt and the optimum was at 40 ppt (Fig. 36). Growth was strongly depressed in fresh water (0 ppt).

Asexual reproduction was observed in sea water (30 ppt) at 15–30°C and optimally at 25°C (Table 1). At 30°C, most of the zoosporangia showed thin and aborted forms which failed to form zoospores. At 25°C, zoosporangia formation occurred well in the sea water above 10 ppt salinity, especially at 30 ppt (Table 2). Fresh water (0 ppt) strongly depressed the asexual reproduction.

These cultural properties indicate that *H. mycoparasitica* is a facultative mycoparasitic oomycote adapting well to brackish and seawater environments.

Parasitism In the facing cultures of *H. mycoparasitica* and *Pestalotiopsis* sp. on 1/2CMSWA, the hyphae of the former twined around those of the latter at the contact points of the two colonies. Short and simple branches of hypha of *H. mycoparasitica* (parasite) made contact with hypha of *Pestalotiopsis* sp. (host) and inserted a very thin hypha (intracellular haustorium?) into the host hypha (Figs. 32, 33). From the invading hypha (haustorium), a hypha of the parasite sometimes protrudes and grows out of the host hypha (Fig. 34).

Our isolates of *H. mycoparasitica* were observed parasitizing hyphae of *Pestalotiopsis* sp. on the isolation medium plates as shown by Fell and Master (1975), who



Figs. 32–34. *Halophytophthora mycoparasitica* parasitizing hyphae of *Pestalotiopsis* sp.
32, 33. Hypha of *H. mycoparasitica* (black arrowhead) inserting haustoria (small black arrowheads) into *Pestalotiopsis* sp. hypha (white arrowhead). 34. A hypha originated from the haustorium (arrowhead) grows out of the host hypha. Bar: 32 (=33, 34)=10 μm.

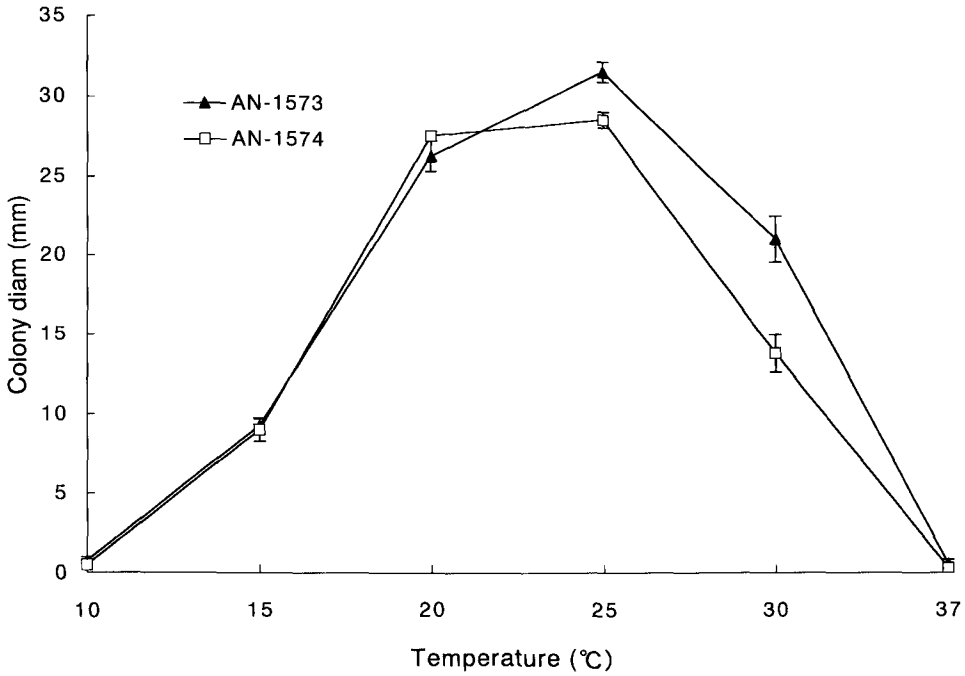


Fig. 35. Hyphal growth of *Halophytophthora mycoparasitica* (AN-1573 and AN-1574) at different temperatures. Colony diam (mean value and s.e. of duplicate tests) after incubation on VJSWA-20 plates for 9 d is plotted.

Table 1. Zoosporangium formation of two strains of *H. mycoparasitica* induced by submersion in sea water (30 ppt) of different temperatures.[a)]

Strain	10	15	20	25	30	37(°C)
AN-1573	−[b)]	+	+++	++++	(++)[c)]	−
AN-1574	−	++	+++	++++	(+++)[c)]	−

a) Agar blocks containing mycelia were submerged in sea water for 1 d.

b) Relative abundance of produced zoosporangia is shown as −, +, ++, +++, and ++++.

c) Most zoosporangia produced were aborted.

Table 2. Zoosporangium formation of two strains of *H. mycoparasitica* induced by submersion in sea water (25°C) of different salinities.[a)]

Strain	0	10	20	30	40 (ppt)
AN-1573	±[b)]	+	+++	++++	++
AN-1574	−	+	++	++++	+++

a) Agar blocks containing mycelia were submerged in sea water for 1 d.

b) Relative abundance of produced zoosporangia is shown as −, ±, +, ++, +++, and ++++.

also found *Penicillium* sp. could be a host. In the process of mangrove leaf decomposition, *Pestalotiopsis* sp. is one of the prevalent endophytic fungi which invades living and senescent leaves on the tree (Nakagiri et al., 1989). After leaf fall into brackish water, *H. mycoparasititca* will invade the fallen leaf by its zoospores and parasitize the hyphae of the pre-existing host. This parasitism may serve for delivering nutrients derived from the leaves and formerly colonizing fungi to a later colonizer without much loss of nutrients in the aquatic environment after leaf fall. It is known that fallen mangrove leaves colonized by oomycotes and fungi are transformed through nitrogen immobilization into a nutritious food for intertidal animals (Fell and Master, 1980). The mycoparasitc phenomenon may be an important mechanism to transfer nutrients of leaf and colonizing fungi to the leaf-eating consumers in the mangrove ecosystem (Nakagiri et al., 1989, 1996).

Preservation of the isolates Two pure cultures (AN-1573 and AN-1574) of *H. mycoparasitica* were subcultured on PEM, and the other four pure (AN-1571, AN-1572, AN-1575 and AN-1576) and two mixed cultures (AN-1577 and AN-1578) with *Pestalotiopsis* sp. were subcultured on VJSWA-20. All the strains were successfully preserved by freezing with 10% (w/v) glycerin (a cryo-protectant) at a constant cooling rate (1°C/min) and storing in a vapour phase of liquid nitrogen (at ca. −170°C) (for details of the procedure, see Nishii and Nakagiri, 1991). The frozen cultures of the two pure strains (AN-1573 and AN-1574) were deposited in the culture collection of the Institute for Fermentation, Osaka as IFO 32966 and IFO 32967, respectively.

Our isolation of *H. mycoparasitica* is probably the second since the original finding from Malaysia in 1975 and the first from the South-west Islands, Japan, where we have been regularly investigating the mycoflora on mangrove fallen leaves for the past decade. Thus, this

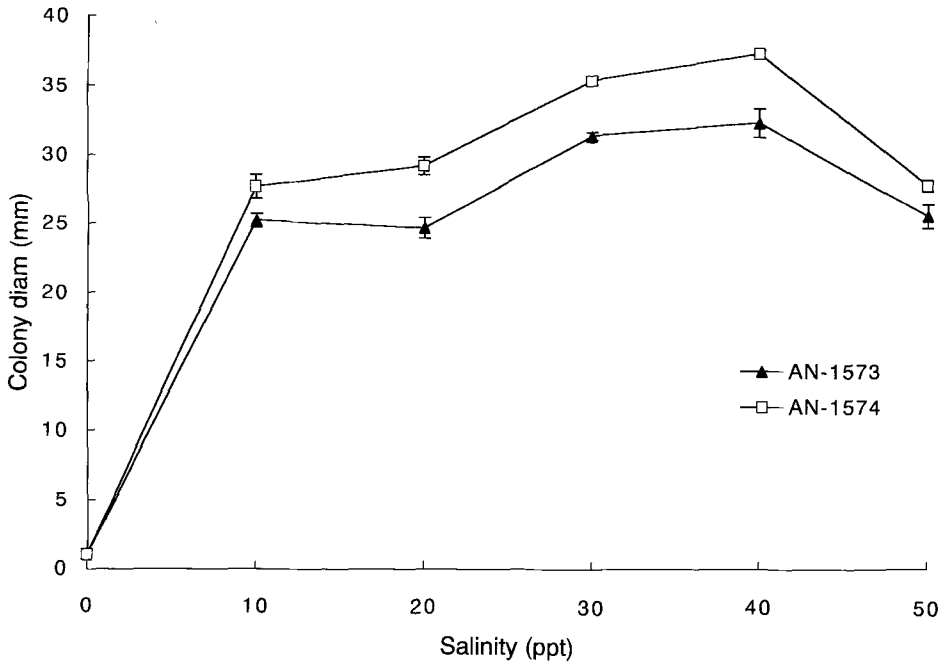


Fig. 36. Hyphal growth of *Halophytophthora mycoparasitica* (AN-1573 and AN-1574) at different salinities. Colony diam (mean value and s.e. of duplicate tests) after incubation on VJSWA plates with different salinities at 25°C for 10 d is plotted.

may be a rare species compared to other halophytophthoras, such as *H. vesicula* (Anastasiou & Churchiland) Ho & Jong and *H. spinosa* var. *lobata*, etc. However, it is possible that our isolation method affects the frequency of isolation. In this study, we incubated leaf disks on 1/2CMSWA at 15°C for 2 wk, instead of our usual conditions (culturing on CMSWA or VJSWA at 25°C for 3–5 d). Incubation of the leaf disks on the low nutrient medium and at low temperature might enable a mycoparasitic species to occur with its host, probably because such incubation brought a proper balance of the activities of the host and the parasite, and also retarded the growth of other prevalent, fast-growing fungi which may usually grow over the slow-growing parasitic species. Studies to develop a better method for detecting the mycoparasitic species are necessary to understand properly their geographical distribution.

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