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FULL PAPER

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Pezicula cinnamomea from cherry tree: pathogenicity tests and photomorphogenesis in culture

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Abstract *Pezicula cinnamomea* (DC.: Fr.) Sacc. was frequently isolated from dead twigs of flowering cherry (*Prunus* × *yedoensis* Matsum.) in Aomori Prefecture, Japan. Inoculations of flowering cherry tree twigs with the fungus resulted in longitudinal browning of heartwood tissues 3–4 cm long after 7 months, showing no apparent symptoms on the bark. The fungus were reisolated at high rates from the affected tissues. Apothecia were formed on cut affected twigs when they were placed in a moist Petri dish and kept under near-UV (Black Light Blue; BL-B) light irradiation. On potato sucrose agar (PSA) plates, the fungus failed to form apothecia in the dark, while many apothecia were formed under BL-B and, to a lesser extent, under white light. In nature, the light, especially near-UV, might be an important factor in apothecium formation. Under near-UV from BL-B conidiomata with conidia formed after 5–6 days and mature apothecia after about 14 days. The developmental processes of the apothecium were histologically studied under light and electron microscopes. *Pezicula cinnamomea*, which is homothallic and fruits easily in culture, may be very useful for life cycle studies and the elucidation of ecological role of this group of fungi.

Key words Endophytic fungi · Near-ultraviolet irradiation · *Pezicula cinnamomea* · Photomorphogenesis · *Prunus* × *yedoensis*

Introduction

The genus *Pezicula* Tul. & C. Tul. (Ascomycetes, Helotiales, Dermateaceae) forms brightly colored flesh apothecia on the bark of various woody plants. Most *Pezicula* species produce in acervuli *Cryptosporiopsis* Bubák & Kabát-type macroconidia, which are typically ellipsoid with rounded apex and truncate base, although some *Cryptosporiopsis* species have not yet been shown to have a *Pezicula* teleomorph (Verkley 1999). Twenty-six species are accepted in *Pezicula*, of which 20 are known to have a *Cryptosporiopsis* anamorph (Verkley 1999). Species of the genus have been predominantly collected in temperate and boreal forests of the Northern Hemisphere (Verkley 1999). There are also a few species that have been collected in the Southern Hemisphere (Zhuang and Korf 1988; Sankaran et al. 1995). *Pezicula* species are generally known as plant pathogenic fungi that primarily cause dieback disease of trees. Recently it has become known that *Pezicula* species have an endophytic nature, playing an important role in "natural pruning," with decay and shedding of dead branches, thus helping to produce a clean bole (Kowalski and Kehr 1996). Although species in the genus generally colonize woody twigs, some occur on stalks of herbaceous plants (Zhong et al. 2001), or on the leaf (Sankaran et al. 1995), bud (Gené et al. 1990), and root of trees (Kowalski and Bartnik 1995; Kowalski et al. 1998).

In Japan, only three species of *Pezicula* are known, i.e., *P. acericola* (Peck) Peck ex Sacc. & Berl., *P. corticola* (C.A. Jørg.) Nannf., and *P. livida* (Berk. & Br.) Rehm (=*P. cinnamomea*, *fide* Verkley) (Kobayashi et al. 1992). A *Cryptosporiopsis* sp. causes twig rot of the cherry tree (Koiwa and Yamada 1996). Recently, *Cryptosporiopsis shigaensis* Matsush. was newly described from unknown plant residue on the forest floor (Matsushima 2001), and *Cryptosporiopsis corticola* (Edgerton) Nannf., the anamorph of *P. corticola*, is reported to cause bull's-eye rot of Japanese pear (Nitta et al. 2002).

In the course of etiological studies of twig blight of flowering cherry *Prunus* × *yedoensis* Matsum., the most popular flowering cherry cultivars in Japan, many *Cryptosporiopsis* isolates, which probably comprise three species, were isolated with tissue isolation. They included, together with two unidentified species, one species that very quickly formed the *Pezicula* state in culture under near-UV

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irradiation. We studied morphology and pathogenicity of the fungus for identification and further performed a series of cultural experiments to investigate the effect of Black Light Blue (BL-B) irradiation on conidiomata and apothecia formation on culture media.

Materials and methods

Isolation procedure and the isolate used

Dead twigs (1–1.5 cm diameter) of flowering cherry trees were collected for isolation in June 1998 from the campus of Hirosaki University. Discolored inner bark and xylem tissues were selected for isolation purposes. The tissue samples were surface-sterilized by dipping in 80% ethanol for 1 min, then in 1% sodium hypochlorite (Antiformin; Wako Chemical, Osaka, Japan) for 2 min. After rinsing with sterilized water and drying on a sterilized filter paper, the tissue samples were cut into segments approximately 2–3 mm thick. Four segments each were placed in a plate containing potato sucrose agar (PSA; potato 200 g, sucrose 20 g, agar powder 20 g, distilled water 1000 ml), and five replicates were made. The plates were first incubated at 20°C in darkness for 7 days, when transferred to 20°C under continuous BL-B (FL8BL-B; National, Osaka, Japan) irradiation. *Pezicula* no. 3115, a single ascospore isolate, was used throughout the experiment.

Cultural studies

Cultures were grown on PSA to investigate colony characteristics. The plates were incubated at 20°C under total dark or continuous BL-B irradiation. Colony colors were classified using the *Methuen Handbook of Colour* (Kornerup and Wanscher 1978).

Inoculation experiments to prove pathogenicity of *P. cinnamomea*

Twigs of 1-year-old potted trees (2 m high; stems 1.5–2 cm diameter, twigs 0.5–1 cm diameter) of *P.* × *yodoensis* were inoculated with conidia or ascospores suspensions (3×10^4/ml) of *P. cinnamomea* no. 3115. The conidia and ascospores were obtained from PSA plate cultures grown under continuous BL-B irradiation at 20°C for 2 weeks. In each of two or three twigs (0.5–1 cm diameter), a small hole (1.5–2 mm diameter) was drilled; spore suspensions (0.1 ml) were put into the hole with a syringe, and the twig was wrapped with plastic tape. Inoculations were done in April 2000 and the potted trees were placed in the field until November, when reisolation was made of the inoculated twigs as previously described. Part of the inoculated twigs was cut off from the tree and put in moist Petri dishes; the dishes were incubated at 20°C under continuous BL-B irradiation to observe apothecial formation on the affected tissues.

Photomorphogenesis of *P. cinnamomea* in culture

Light quality for production of conidia and apothecia

Mycelial disks 6 mm in diameter from the advancing margin of the fungal colonies were placed on PSA plates as inocula. The inoculated plates were first incubated for 5 days at 20°C in the dark, then were continuously irradiated with various fluorescent lights, i.e., white (FL20S·D; National), BL-B (FL20S·BL-B; National), blue (FL20B-F; Mitsubishi, Tokyo, Japan), green (FL20G-F; Mitsubishi), yellow (FL20Y-F; Mitsubishi), and red (FL20R-F; Mitsubishi), all lamps set 15 cm above the plates for 1 month, during which time daily observations were made for development of conidiomata and apothecia.

Developmental processes of conidiomata and apothecia

Observation with scanning electron microscopy (SEM). Agar squares bearing various stages of development of conidiomata and apothecia were put into 2.5% glutaraldehyde, aspirated gently for 30 min to remove the air trapped in the agar, and fixed for 24 h at 5°C, followed with three washes with 0.1 M potassium phosphate buffer at pH 6.6 for 30 min. The materials were then dehydrated with an ethanol series using 50%, 70%, 80%, 90%, and 95% ethanol at room temperature, 30 min at each step, and then with 100% ethanol twice, 20 min each time. After dehydration, the ethanol in the sample was substituted with isoamyl acetate, critical point dried using liquid CO_2 in a JCPD-5 Critical Point Drying Apparatus, attached to an aluminum stub with double-stick tape, and coated with carbon and then gold on a rotary stage in a JFC-1100 Ion Sputterer. The specimens were examined with a SEM (JSM-5300). Terminology in Van Brummelen (1967) is followed in describing some phases of apothecium development.

Observation with light microscopy (LM). Agar pieces with conidiomata and apothecia taken from the PSA plate culture were killed and fixed in FAAII (100 ml 50% ethanol, 6.5 ml formalin, 2.5 ml acetic acid), soaked in tap water for 1–2 days to remove the fixative, then dehydrated with an ethanol series using 50%, 75%, and 95% ethanol at room temperature, 1–2 days at each step, and then 100% ethanol twice, 1–2 days each time. The ethanol was substituted with *n*-butyl alcohol and the samples were then embedded in paraffin and sectioned with a microtome (HM 400R; Microme, Walldorf, Germany). The cellophane tape method (Saho 1974) was used for sectioning (20 μm) the material. The sections on a slide glass were dyed with a double staining method using safranin and fast green FCF (Nishiyama 1961) and mounted in Canada balsam for observation.

Results

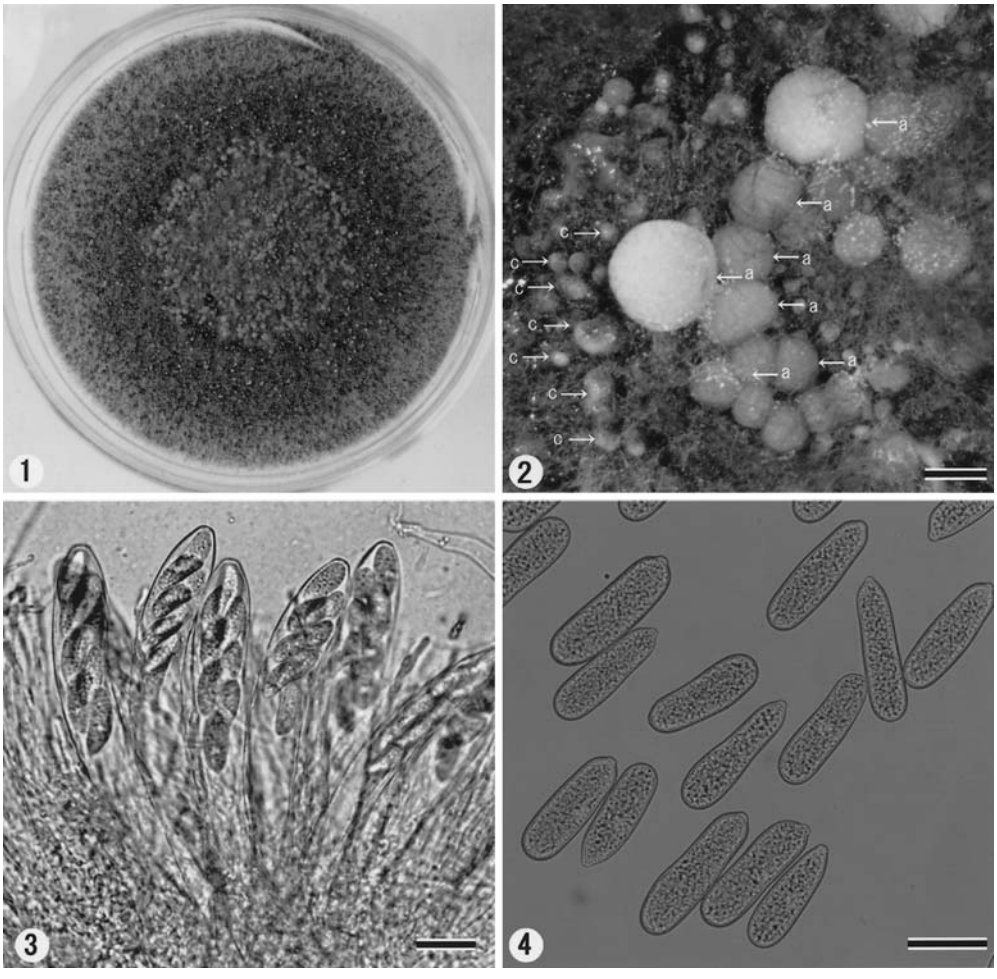
Isolation of *P. cinnamomea* from dead twigs of cherry

In isolation experiments, colonies of various species of fungi were formed on agar plates in several days of incubation, such as species of *Phomopsis* (Sacc.) Bubák, *Dothiorella* Sacc., *Phoma* Sacc., and *Pestalotiopsis* Steyaert, which almost entirely covered the tissue segments. Several days after incubation, however, yellowish-brown colonies with a rather slow rate of growth appeared as sectors from the tissue segments. When such plates were placed under BL-B irradiation at 20°C, conidiomata of *Cryptosporiopsis* and later apothecia of *Pezicula* were formed. The *Pezicula* was identified as *P. cinnamomea*, based on cultural and morphological characteristics, as follows.

Colonies on PSA: margin even, glabrous; aerial mycelium weakly to moderately developed far behind the margin, felty to floccose, whitish; submerged mycelium initially Light Yellow (4A6), becoming Olive Yellow (3D7) from the center with floccose to lanose aerial mycelium. Under BL-B irradiation, colonies initially Grayish Green (29E7), becoming Dark Green (29F6) to Greenish Gray (29F2) gradually; aerial mycelium sparse; fruiting structures, conidiomata and/or apothecia, numerous after 7–14 days, mostly distributed in the center of the colonies (Figs. 1, 2).

Apothecia mostly developing after the anamorph, usually after 2 weeks under near-UV, solitary, or two or three on a basal stroma on the agar surface, sessile or subsessile, disks 0.5–1.5 mm diameter, 0.6–0.7 mm high; hymenium slightly convex, orange-yellow when fresh, yellowish-brown when old; asci (Fig. 3) clavate to cylindrical-clavate, short-stalked, 8-spored, 100–190 × 17.5–22.5 μm; Ascospores ellipsoid to oblong-ellipsoid (L/W 3–3.4), hyaline, continuous, becoming 1- to 4-celled at maturity, straight or slightly curved, irregularly biseriate, 22.5–27.5 × 6.25–7.5 μm, 25.2 × 7.4 μm on average ($n = 100$); paraphyses hyaline, filiform, simple, swollen to 5–6 μm wide at the tip.

Conidiomata eustromatic, mostly superficial on the agar, scattered or gregarious, spherical to irregularly pulvinate, 0.3–1 mm diameter; conidiogenous cells cylindrical, hyaline, smooth, 12.5–30 × 3–7.5 μm, conidiogenesis enteroblastic; macroconidia (Fig. 4) elongated ellipsoid to oblong, straight or slightly curved, rounded at both ends, continuous, thin walled, and hyaline when liberated; (37.5–)40–47.5 × 12.5–15 μm, later 1–3(–5)-septate, then muriform; microconidia filiform, straight, rounded at the apex, 5–10 × 2–2.5 μm.



Figs. 1–4. *Pezicula cinnamomea*, no. 3115. **1** Colony with apothecia and conidial mass on potato sucrose agar (PSA) after 2 weeks incubation under BL-B (Black Light Blue; near-UV) irradiation. **2** Close-up of apothecia (*a*) and conidial mass (*c*). **3** Asci with ascospores. **4** Conidia. *Bars* **2** 1 mm; **3,4** 25 μm

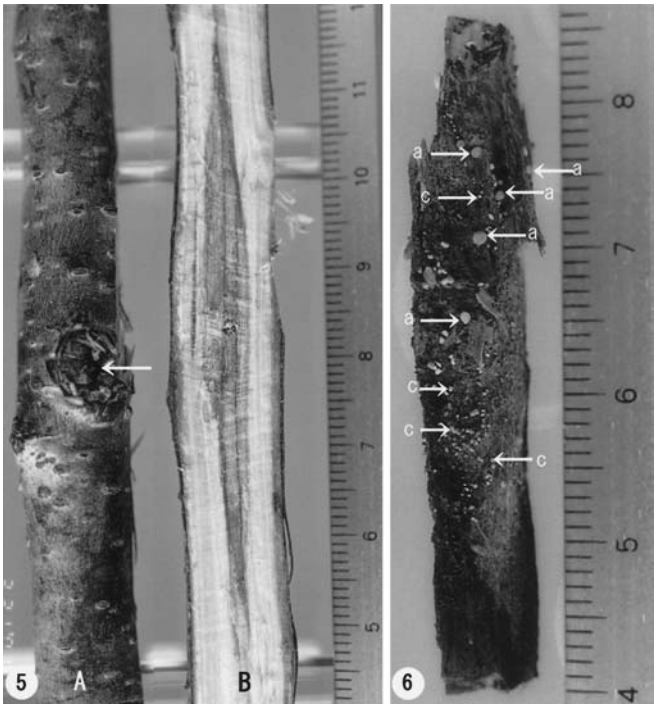


Fig. 5. Symptoms of inoculated twig of flowering cherry tree showing the inoculation site (**A** *arrow*) and browned tissue of heartwood, as seen with the cut halves about 7 months after inoculation (**B**)
Fig. 6. *Pezicula cinnamomea* fructifications on artificially inoculated twig of flowering cherry tree, showing apothecia (*a*) and conidial masses (*c*) produced on the twig in a moist chamber in 1 month under BL-B irradiation

Pezicula sepium (Desm.) Dennis has been isolated from rosaceous plants, particularly species of *Prunus*, affected with bark canker (Verkley 1999). *P. cinnamomea* is different from *P. sepium* in that it is homothallic and the L/W ratio of ascospores is greater (L/W 3–3.4), compared with *P. sepium* [L/W 2–2.2(–2.7)].

Pathogenicity of *P. cinnamomea*

Inoculated twigs showed no apparent symptoms except for slight swelling near the inoculation site even after 7 months (Fig. 5A). However, when the twigs were cut into halves, browning of heartwood was seen, 3–4 cm long in both upper and lower directions from the point of inoculation (Fig. 5B). From the browned heartwood, *P. cinnamomea* could easily be reisolated. Control twigs showed no such browning in heartwood, giving rise to no *Pezicula* in isolation culture. Some affected twigs were cut from the tree and put in a Petri dish with moist filter paper, and the dish was placed under BL-B irradiation for fungal fructification. In 1 month, conidiomata with *Cryptosporiopsis* conidia and a few apothecia of *Pezicula* were formed on the twigs (Fig. 6). The apothecia were apparently smaller (0.6–0.9 mm diameter), than those on the PSA plate (0.5–1.5 mm diameter), but the morphologies of ascus and ascospore were similar in both apothecia.

Photomorphogenesis in culture

Light quality for production of conidiomata and apothecia

Under dark conditions, the colony was brown with no conidiomata nor apothecia even after 1 month of incubation. Under near-UV from BL-B, in contrast, conidiomata with conidia formed after 5–6 days and then mature apothecia after about 14 days. White light was also effective in conidiomata and apothecia formation, but to a lesser extent. Under blue light, small hyphal masses were formed that bore neither conidia nor ascospores. Green, yellow, and red light had no effect on conidiomata or apothecia formation.

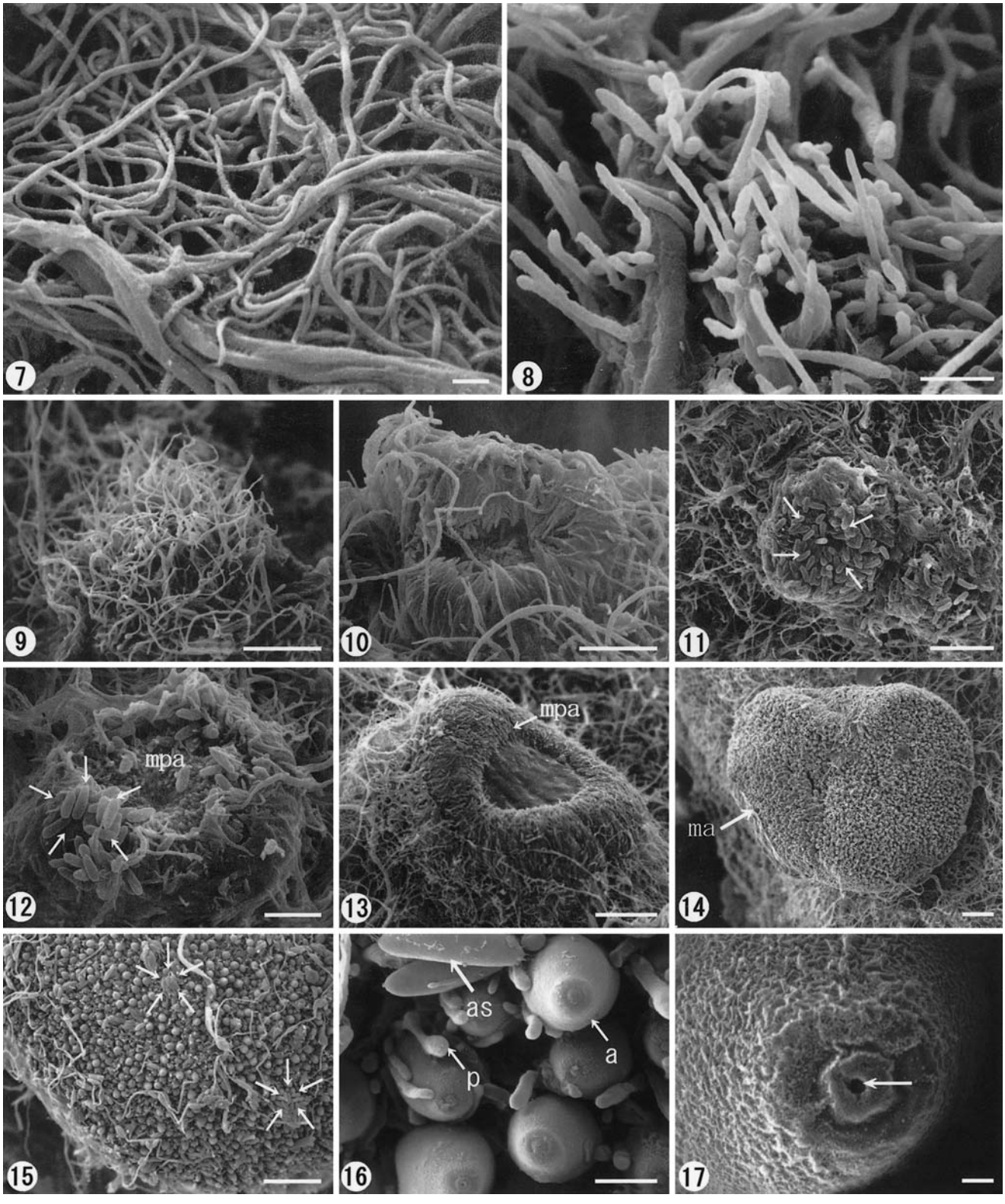
Morphological development of the fungus

Observations with SEM. Development of apothecia was initiated by the formation of rising slender hyphae (2.5–3.5 μm wide) (Fig. 8), which were not seen with the colony in the dark (Fig. 7). The slender hyphae gathered into groups here and there and were interwoven to form spherical masses (Fig. 9). Hyphal density in the mass gradually increased as it developed and finally matured into conidiomata (Fig. 10). Conidia were released from conidiomata in 5–6 days after being transferred to BL-B irradiation (Fig. 11). In 2–4 days more under BL-B irradiation, a mesohymenial-phase apothecium was protruded from the bottom of conidioma (Fig. 12). The apothecium matured as they were pushing up the conidioma in about 2 weeks under BL-B irradiation (Fig. 13). When matured, congested asci and interspersed paraphyses are seen (Figs. 14, 15), the tip of the paraphyses being slightly enlarged (Fig. 16); the ascus apices were conical and of inoperculum type, with a small pore to discharge the ascospores (Fig. 17).

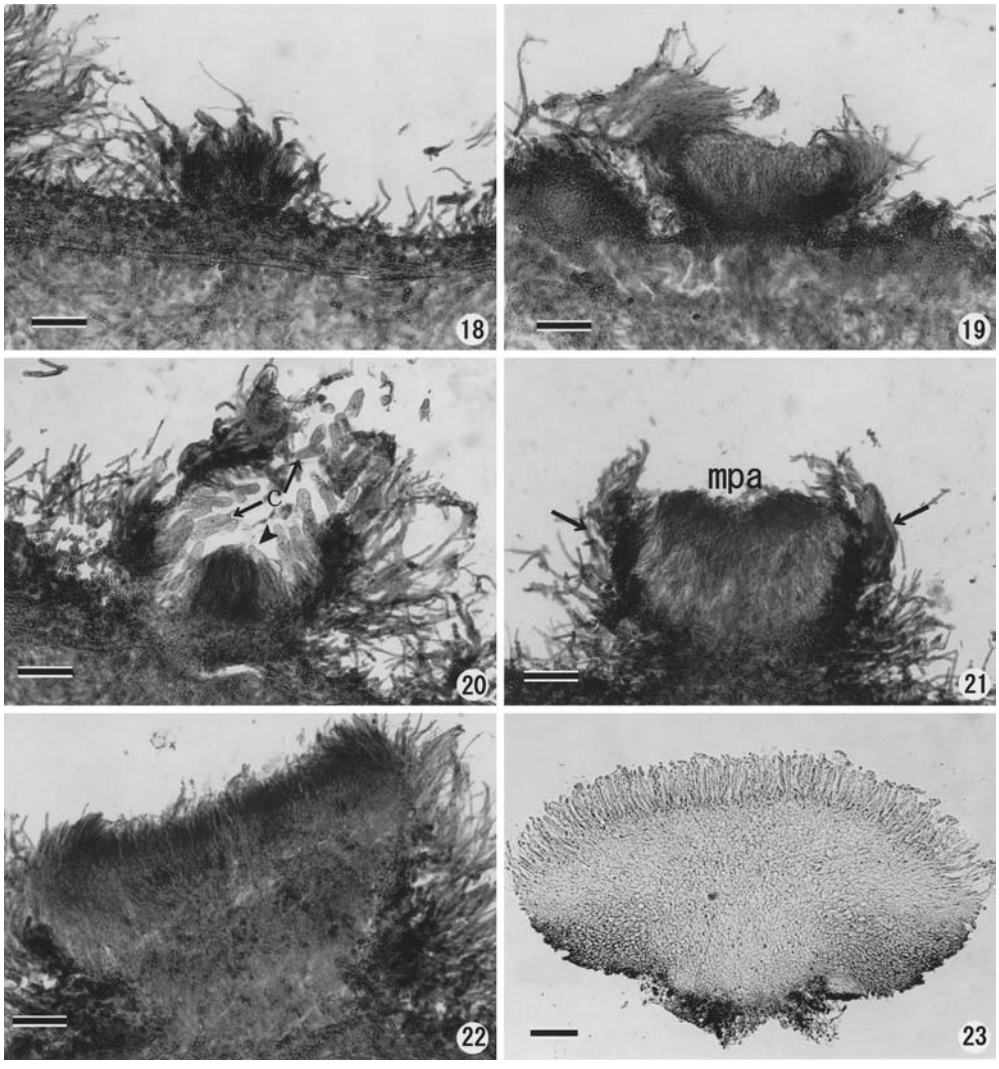
Observations with LM. The hyphal mass formed under BL-B irradiation was of densely interwoven hyphae (Fig. 18), which grew into small immature conidiomata (0.1–0.2 mm diameter) in 2 or 3 days more under BL-B irradiation (Fig. 19). They contained smaller (20–35 × 10–13 μm) and paler immature conidia as well as microconidia (5–10 × 2–2.5 μm). In 5–6 more days, mature conidia [(37.5–)40–47.5 × 12.5–15 μm] were released in masses, following the formation of clear water droplets, from the conidiomata. Sections of conidiomata in this growth stage showed the prohymenial-phase apothecium at the bottom (Fig. 20). The disk of apothecia gradually expanded as it grew from the conidiomata (Figs. 21, 22). In 2 weeks under BL-B irradiation, abundant mature apothecia (Fig. 23) were formed, mostly near the center of the colony.

Discussion

Until recently, *Pezicula cinnamomea* had been considered a broad-leaved tree parasite, distinct from *P. livida* and *P. eucrita* (P. Karst.) P. Karst., the latter being treated as syn-



Figs. 7–17. Scanning electron microscopic observations on conidiomata and apothecia in *Pezicula cinnamomea* grown on PSA at 20°C under BL-B for all figures except for 7. **7** Undifferential creeping hyphae on the surface of the colony in the dark. **8** Slender hyphae arising from the colony surface under BL-B irradiation. **9** Spherical hyphal mass stage. **10** Small immature conidiomata stage. **11** Mature conidiomata with conidial mass (*arrows*). **12** Mesohymenial-phase apothecium (*mpa*) developed from the interior of the conidiomata pushing up the conidial mass (*arrows*). **13** Mesohymenial-phase apothecium (*mpa*), about 10 days after being kept under BL-B irradiation. **14** Mature apothecium (*ma*), about 14 days after being kept under BL-B irradiation. **15** Mature apothecium, slightly enlarged, showing protruding asci and discharged ascospore (*arrows*). **16** Asci (*a*), discharged ascospore (*as*), and paraphyses (*p*). **17** Apex of the ascus, with a small apical pore (*arrow*). *Bars* **7,8** 10 μm; **9,10** 50 μm; **11** 100 μm; **12** 50 μm; **13, 14,15** 100 μm; **16** 10 μm; **17** 1 μm



Figs. 18–23. Light microscopic observations on conidiomata and apothecia formation in *Pezicula cinnamomea* grown on PSA at 20°C under BL-B irradiation. **18** Spherical hyphal mass stage. **19** Small immature conidiomata stage. **20** Mature conidioma with conidia (*c*), with prohymenial-phase apothecium (*arrowhead*) at its bottom. **21** Mesohymenial-phase apothecium (*mpa*) formed inside the conidioma (*arrows*), in about 7 days. **22** Mesohymenial-phase apothecium, about 10 days after being kept under BL-B. **23** A mature apothecium, in about 14 days. *Bars* **18–22** 50 μm; **23** 100 μm

onymous with *P. livida* (Johansen 1949). Verkley (1999), however, recognized that *P. livida* was synonymous with *P. cinnamomea* but separated *P. eucrita* as a distinct species. In Verkley's monograph on *Pezicula* (1999), *P. cinnamomea* had a wide host range, with 27 species of broad-leaved trees and 9 species of conifers. In Japan, Saho and Takahashi (1968) reported for the first time *P. livida* as twig dieback fungi on *Pinus strobus* L., *P. sylvestris* L., *Larix dahurica* Turcz. var. *koreana* Nakai, *L. decidua* Mill., and *L. gmelini* Ledeb. × *L. leptolepis* Gordon, in Hokkaido. Later, 6 species of conifers, *Chamaecyparis obtusa* Endl., *C. pisifera* Endl., *Cryptomeria japonica* D. Don, *Larix leptolepis* Gordon, *Pinus griffithii* McCl., and *Pseudotsuga menziesii* (Mirb.) Franco. were added as hosts of *P. livida* (Takahashi 1979; Kaneko et al. 1985; Kobayashi et al. 1990). On the other hand, only 2 species of broad-leaved trees, *Alnus glutinosa* (L.) Gaertn. and *Fagus crenata* Blume, have been reported in Japan as hosts of *Pezicula livida* and *Pezicula* sp., respectively (Takahashi 1979; Verkley 1999). Therefore, *Prunus* × *yedoensis* is a new hosts for *P. cinnamomea* in this country. In Japan, most recognized hosts for *P. cinnamomea* are conifers rather than species of broad-leaved trees. This finding is in contrast to listed hosts in Verkley's monograph (1999), in which broad-leaved trees are more predominant than conifers, and might reflect the more active investigations by forest pathologists in Hokkaido, northern Japan, than elsewhere. Further investigations for *P. cinnamomea* on broad-leaved trees are needed to elucidate the host range of *P. cinnamomea* in this country.

In Europe and North America, *P. cinnamomea* is a common inhabitant on oak twigs, associating with bark diseases, which is more frequent on sites with a high or fluctuating groundwater table and on poor, very dry sites, especially if accompanied by industrial emissions (Kehr 1991, 1992). Some previous inoculations proved that *P. cinnamomea* produces no symptoms or is a weak parasite (Johansen 1949; Kehr 1991; Kaneko et al. 1996). Our study showed that colonization of *P. cinnamomea* progressed without any observed symptoms. In recent years. *P. cinnamomea* has been known to have an endophytic nature on various tree species (Kowalski and Kehr 1992; Schulz et al. 1999;

Verkley 1999). Under adverse environmental conditions the mechanism of plant defense may be lost and then endophytic fungi could become pathogenic (Kehr 1991, 1992; Kehr and Wulf 1993; Schulz et al. 2002). The factors that concern such change in host–fungus relationships, from symbiosis to parasitism, must be determined with various tree populations in view of plant health.

Light exerts various effects on fungi, such as phototropism, reproduction, morphogenesis, and spore germination. The stimulatory effect of UV on reproduction was first reported by Stevens (1928). He discovered that exposing colonies of *Glomerella cingulata* (Stonem.) Spauld. & Schrenk to quartz-mercury lamp light stimulated the formation of perithecia. Reviews of photoresponses of fungi show that fungi are most sensitive to light of short wavelength, i.e., ultraviolet, near-UV, and blue wavelengths (Carlile 1970; Tan 1978). Regarding Ascomycetes, although the photoresponses of only a few species have been studied, there is some evidence suggesting the possibility of two distinct groups: (1) genera in which ascocarp formation is stimulated only by ultraviolet wavelengths, and (2) genera that respond to both blue and near-UV wavelengths (Leach 1971). In discomycetes, *Sclerotinia triforiorum* Erik. produces mature apothecia under near-UV irradiation (300–390 nm) (Honda and Yunoki 1975), and *Ascophanus carneus* (Pers.) Boud. produces apothecia in abundance when exposed to wavelengths of light 430–450 nm (Leach 1971). Our experiment showed that BL-B irradiation (320–420 nm) is very effective in fructification of *P. cinnamomea*, a result almost the same as Honda's report (1975). Conidia of *P. cinnamomea* were discharged from conidiomata in 3–5 days, followed by the production of many apothecia within 2 weeks after BL-B irradiation. The discomycete *Anthracobia muelleri* (Berk.) Rifai also produced apothecia in 2 weeks after white light irradiation. *Ascodesmis sphaerospora* Obrist, however, produced apothecia only 4 days after white light irradiation (Roxon and Batra 1973). The period of apothecial formation may be determined by various factors, e.g., mycelial growth rate, size of mature apothecia, temperature, kind of media, and light intensity. *P. cinnamomea* produced conidiomata and apothecia on affected twigs as well under near-UV irradiation. This result suggests that light is one of the important factors for apothecia formation in nature.

The capability of *P. cinnamomea* to produce both conidiomata and apothecia on culture media was quite stable throughout the experiment, with little change by subculturing during at least 5 years in our experiment. Therefore, *P. cinnamomea* would be very useful for morphological study in *Pezicula* species. The properties of easily forming apothecia in culture enabled us to observe in detail the process of apothecia formation, from arising slender hyphae to maturation of apothecia, by both SEM and LM. We elucidated that the prohymenial-phase apothecium was initiated from the bottom of conidiomata (basal stroma); this observation agreed well with Kehr (1992), who reported that a basal stroma is present on host twigs that produced not only the anamorph but also the teleomorph of the fungus.

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