

SHORT COMMUNICATION

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Identification of a mycorrhizal fungus in *Epipogium roseum* (Orchidaceae) from morphological characteristics of basidiomata

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Abstract A mycorrhizal fungus, ME1-1, isolated from an achlorophyllous orchid, *Epipogium roseum*, was identified as *Coprinellus disseminatus* (\equiv *Coprinus disseminatus*) based on characteristics of basidiomata that were artificially induced. Spawn of ME1-1 cultivated on a medium that consisted of sawdust and wheat, sandwiched between two mats of volcanic soils, which was incubated at $20.0^\circ \pm 0.5^\circ\text{C}$ in $80.0\% \pm 0.5\%$ relative humidity in the dark. The basidiomata were formed on the soil after 2 months. Morphological and anatomical characteristics of the basidioma mostly accorded with those of *Coprinellus disseminatus*. We therefore concluded that *C. disseminatus* is one of the mycobionts of *E. roseum*.

Key words Achlorophyllous orchid · Basidiomata formation · *Coprinellus disseminatus* · *Coprinus disseminatus* · Orchid mycorrhizal fungi

Molecular analyses have been recently applied for the identification of orchid mycorrhizal fungi to reveal diverse symbionts in the symbioses (Dearnaley 2007). However, the identification is usually limited to the genus level. Meanwhile, some orchid mycorrhizal fungi have been identified

at the species level based on characteristics of basidioma, such as *Marasmius coniatus* var. *didymoplexis* Berk. et Br. in *Didymoplexis pallens* Griff (Burgeff 1932), *Armillaria jezoensis* J.Y. Cha & Igarashi in *Galeola septentrionalis* Rchb. f. (Cha and Igarashi 1996), *Erythromyces crocicreas* (Berk. & Broome) Hjortstam & Ryvar den in *Erythrorchis ochobiensis* (Hayata) Garay (\equiv *Galeola altissima* (Bl.) Reichenbach f.) (Umata 1995), *Mycena orchidicola* L. Fan & S.X. Guo in *Cymbidium sinense* Willd. (Fan et al. 1996), etc. However, it is difficult to induce basidioma formation under artificial conditions for many orchid mycorrhizal fungi.

Epipogium roseum (D. Don) Lindl. is an achlorophyllous orchid species that is distributed from Japan to tropical region (Tsuyama 1967). For this orchid, Yamato et al. (2005) identified 13 fungal isolates to be in *Coprinus* and *Psathyrella* in Coprinaceae by a phylogenetic analysis based on the DNA sequences of the internal transcribed spacer (ITS) region in rDNA. One of the isolates, ME1-1, was applied for a symbiotic cultivation of this orchid, in which induction of a whole life cycle from seed germination to flowering was achieved (Yagame et al. 2007). The isolate ME1-1 was found to be close to *Coprinellus disseminatus* (Pers.: Fr.) J.E. Lange (\equiv *Coprinus disseminatus* (Pers.: Fr.) S.F. Gray) in the phylogenetic analysis by Yamato et al. (2005). In the present study, basidiomata of the isolate ME1-1, induced by an artificial condition, were examined for the further identification of this fungus.

The isolate ME1-1 was inoculated into a medium that consisted of a mixture of broad-leaved tree sawdust and wheat bran [17:10 (w/w)] with 55% moisture content after autoclaving at 120°C for 60 min. The culture was incubated at $25.0^\circ \pm 0.5^\circ\text{C}$ in the dark for 4 weeks to make a spawn of ME1-1. A plastic container (55.0 \times 35.0 \times 11.5 cm), sterilized by spraying with 70% ethanol, was applied to make a mushroom bed as follows. At the bottom of the container, 1500 ml autoclaved Hyuga soil (a volcanic soil, 15–20 mm in diameter) was first placed to avoid the overlying moisture in the mushroom bed. Then, 600 ml spawn was placed on the Hyuga soil mat, and finally 2000 ml autoclaved akadama soil (a volcanic soil, 3–5 mm in diameter) was placed on the

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Fig. 1. *Coprinellus disseminatus* (isolate ME1-1; Yamato et al. 2005). Basidiomata obtained from an achlorophyllous orchid, *Epipogium roseum*. Arrows show conspicuous yellow-brown ozonium and dark brown rhizomorphs. Bar 1 cm



Fig. 2. *Coprinellus disseminatus* (isolate ME1-1; Yamato et al. 2005). **A** A young basidioma with dark brown veil (*v*) on the pileus and rhizomorphs (*r*) at the base of stipe. **B** Thick-walled brown hyphae of the veil (*v*) in **A**. Bars **A** 1 mm; **B** 20 μ m

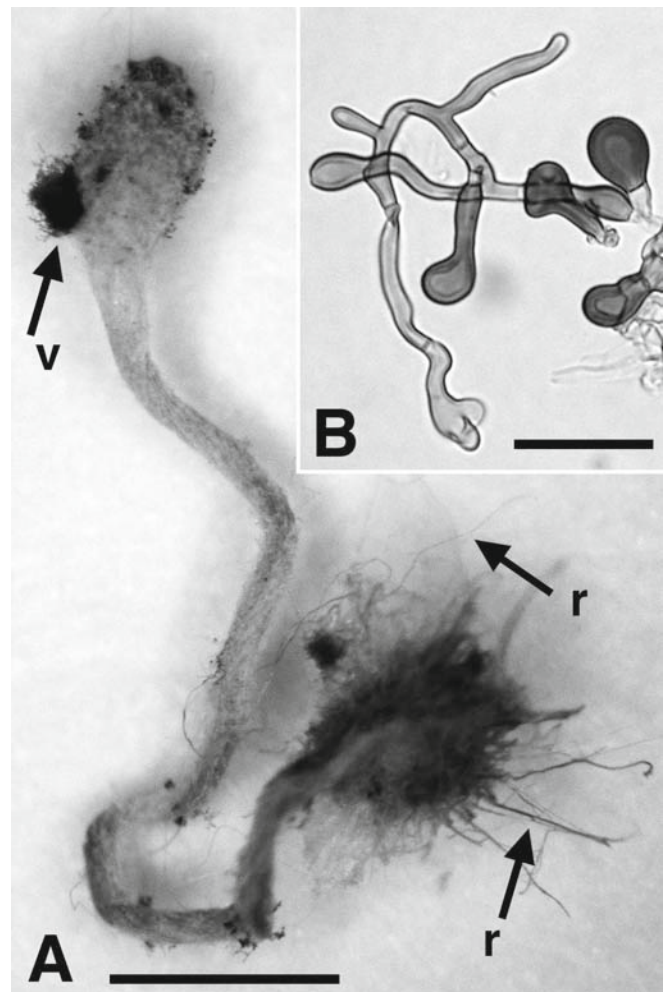
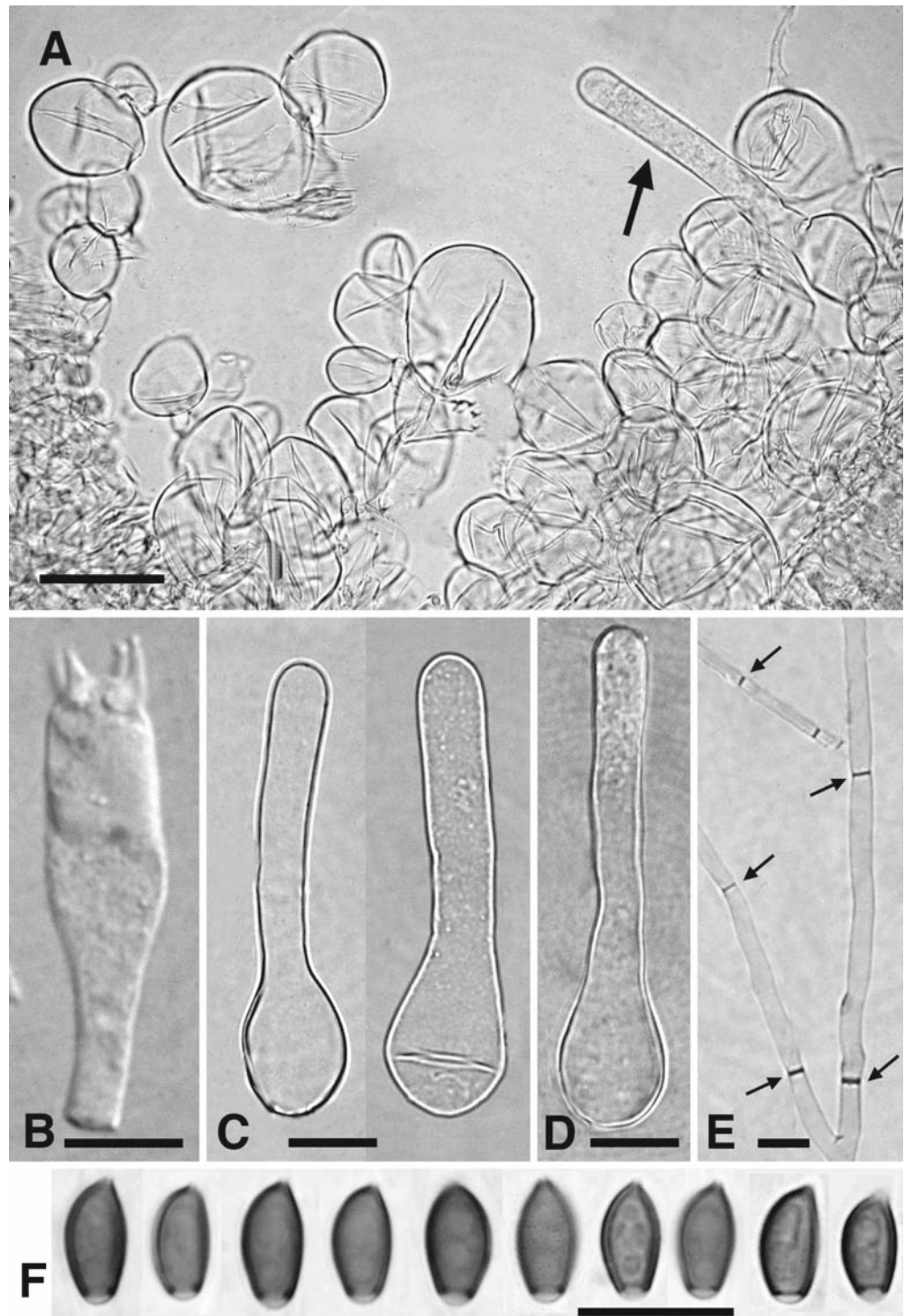


Fig. 3. *Coprinellus disseminatus* (isolate ME1-1; Yamato et al. 2005). **A** Globose pileal veil elements and a pileocystidium (arrow). **B** Basidium with four sterigmata on the top. **C** Pileocystidia. **D** Caulocystidium. **E** Secondary hyphae with single septa (arrows), stained by cotton blue. **F** Basidiospores. Bars **A** 50 μm ; **B** 5 μm ; **C, D** 25 μm ; **E** 20 μm ; **F** 10 μm



spawn mat. The pH and moisture content of the akadama soil after autoclaving were 5.5% and 80%, respectively. The container was placed at $20.0 \pm 0.5^\circ\text{C}$ in $\sim 80.0\%$ of relative humidity in the dark for 2 months to induce basidioma formation. After 2 months, a number of basidiomata were formed (Fig. 1) on the akadama soil and were collected using forceps. The basidiomata were dried at 60°C for 24 h to make a specimen for identification. Anatomic observations and measurements were made on the specimen

mounted in 28% aqueous ammonia. To check the clamp connection in hyphae, ME1-1 was cultured on a water agar plate for 1 week. The newly developed fungal hyphae were isolated from the water agar plate and were stained with 3% cotton blue for 30 s. The hyphae were then observed by a microscope Optiphot VFX-IIA (Nikon, Tokyo, Japan). From the investigated morphological and anatomic characteristics, ME1-1 was identified to be *C. disseminatus*, as described next. The examined specimens, FB-32701 (Her-

barium acronym, CBM), FB-32646 (CBM), and FB-36771 (CBM) were deposited in the Natural History Museum and Institute, Chiba (CBM).

Coprinellus disseminatus (Pers.: Fr.) J.E. Lange, Dansk. Bot. Ark. 9(6): 93, 1938.

≡ *Agaricus disseminatus* Pers., Syn. Meth. Fungi: 403, 1 801.

≡ *Coprinus disseminatus* (Pers.: Fr.) S.F. Gray, Nat. Arr. Br. Pl.1: 634, 1821.

Figs. 1, 2A,B, 3A–F

Pileus ellipsoid to ovoid when young, then hemispherical or obtusely conical to convex (Figs. 1, 2A), 3.5–6.0 (4.6 ± 0.3, mean ± SD, $n = 10$) mm broad, 1.6–2.7 mm high (2.4 ± 0.4), radially sulcated, at first white, later becoming pale brown to ochre with the paler margin, at first covered with fibrillose veil remnants consisted of hyaline, globose elements, about 25–30 µm in diameter (Fig. 3A) and dark brown hyphae (Fig. 2A,B), the surface pruinose because of presence of pileocystidia. Flesh very thin, fragile, white, taste mild, odorless. Lamellae adnate to almost free, not crowded (number of lamellae reaching stipe = 20–30), broad (0.5–0.8 mm wide), white then grayish, finally blackish, not deliquescing. Stipe 15–40 (26.2 ± 1.9) mm long, 0.5–0.8 (0.6 ± less than 0.05) mm wide, cylindrical, equal or more or less tapering upward, sometimes with a clavate base, not rooting, hollow, fragile, surface white to grayish-white, pubescent at the base.

Basidiospores black in mass, dark red brown under the microscope, ovoid to ellipsoid, smooth (Fig. 3F), 6.8–8.3 (7.6 ± 0.4; mean ± SD, $n = 20$) µm long, 3.7–4.3 (4.0 ± 0.2) µm broad in face view, 3.6–4.1 (3.9 ± 0.2) µm in side view, with a central germ pore 1.3–1.8 µm wide (1.7 ± 0.2,) (Fig. 3F). Basidia 14.1–17.2 (14.2 ± 0.7; mean ± SD, $n = 10$) µm long with sterigmata, 4.0–6.1 (5.2 ± 0.4) µm wide, 4-spored (Fig. 3B). Pileocystidia 105–130 × 28–31 (121.4 ± 3.0 × 29.2 ± 0.7) µm, lageniform with cylindrical long neck and rounded apex, thin-walled, hyaline (Fig. 3C). Caulocystidia 100–130 × 24–26 (112.1 ± 4.3 × 25.2 ± 0.6) µm, lageniform with cylindrical long neck and rounded apex, thin-walled, hyaline (Fig. 3D). Cheilocystidia and pleurocystidia absent. No clamp connections were observed in the hyphae on a medium (Fig. 3E).

Conspicuous yellow-brown ozonium mixed with dark brown rhizomorphs (Fig. 2A-r) grew on the soil (Fig. 1). The dark brown rhizomorph was same as that growing on the protocorms of *E. roseum* cocultured with this fungus (Yagame et al. 2007). Under the saprobic condition, this fungus forms numerous gregarious basidiomata (Fig. 1).

Coprinellus disseminatus, a well-known *Coprinus*-type species, is easily recognized by its setulose scurfy fragile cap and gregarious occurrence. This fungus generally forms numerous basidiomata, sometimes in large groups on stumps, buried wood, or roots in forest, parks, gardens, on paths, etc. This fungus is a cosmopolitan species distributed from the Northern Hemisphere to South Africa (Breitenbach and Kränzlin 1995; Uljé 2005). This fungus has distinct morphological characteristics as follows (Orton and Watling 1979; Breitenbach and Kränzlin 1995; Uljé 2005): i.e., con-

spicuous large pileocystidia, caulocystidia with long cylindrical neck, absence of cheilo- and pleurocystidia, subglobose to oval velar elements on the pileus and catenate or hyphoid thick-walled brown velar elements, 4-spored basidium, and smooth central pored basidiospore. *Coprinellus disseminatus* is segregated from other *Coprinus*-type groups by these morphological characteristics. The induced basidiomata of isolate ME1-1 had all these characteristics (Figs. 1, 2, 3), indicating that isolate ME1-1 was *C. disseminatus*.

Some characteristics of ME1-1 were found to be different from those reported in *C. disseminatus*: i.e., smaller pileus, 3.5–6.0 mm in diameter [up to 15 (20) mm, Uljé 2005; 5–12 mm, Breitenbach and Kränzlin 1995; 10–15 mm, Hongo and Imazeki 1987], smaller basidiospores 6.8–8.3 µm long × 3.7–4.3 µm wide in face view (6.5–9.5 µm × 4.0–6.0 µm, Uljé 2005; 7.2–9.7 × 4.1–5.4 µm, Breitenbach and Kränzlin 1995; 8.5–10 × 4–5 µm, Hongo and Imazeki 1987), and shorter pileocystidia, 105–130 µm long (50–200 µm long, Uljé 2005; up to 200 µm long, Breitenbach and Kränzlin 1995; up to 150 µm long, Hongo and Imazeki 1987). Although formation of cheilocystidia has been reported in this species (Orton and Watling 1979; Hongo and Imazeki 1987), it was not detected in ME1-1. However, Breitenbach and Kränzlin (1995) and Uljé (2005) stated that *C. disseminatus* have no cheilocystidia. Actually, Uljé and Bas (1991) interpreted the cheilocystidia to be “escaped” pileocystidia occurring on the lamella edges. In addition, the isolate ME1-1 formed conspicuous long dark orange brown rhizomorphs at the base of the stipe and on substrata (Figs. 1, 2A), and prominent ozonium (Fig. 1), which have not yet been known in *C. disseminatus*. We considered that these differences would be caused by the genetic diversity within the species. Ko et al. (2001) hypothesized that *C. disseminatus* might consist of some cryptic species from a phylogenetic divergence based on the nuclear ITS sequence. Further studies are therefore required for the reappraisal of the species *C. disseminatus*.

In orchid mycorrhizal fungi in Agaricales, there are very few studies of basidiomata formation under artificial conditions. Terashita and Chuman (1987) succeeded in basidioma formation for *Armillaria tabescens* (Scop.) Emel isolated from achlorophyllous orchid *Galeola septentrionalis* Rehb. f. To our knowledge, this is the second study of basidioma formation of orchid mycorrhizal fungi in Agaricales.

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