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FULL PAPER

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Ultrastructure of developing basidiospores in *Rhizopogon roseolus* (= *R. rubescens*)

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Abstract The ultrastructure of developing basidiospores in *Rhizopogon roseolus* is described. When viewed in the fruiting body chamber using scanning electron microscopy, basidiospores appear narrowly ellipsoid and have smooth walls. Eight basidiospores are usually produced on the apex of each sterigma on the basidium. Transmission electron micrographs showed that basidiospores formed by movement of cytoplasm (including the nuclei) via the sterigmata, and then each basidiospore eventually became separated from its sterigma by an electron-lucent septum. The sterigma and basidium subsequently collapsed, resulting in spore release. Freshly released spores retained the sterigmal appendage connected to the collapsed basidium. After spore release, the major ultrastructural changes in the spore concerned the lipid bodies and the spore wall. During maturation, lipid bodies formed and then expanded. Before release, the spore wall was homogeneous and electron-lucent, but after release the spore wall comprised two distinct layers with electron-dense depositions at the inner wall, and the dense depositions formed an electron-dense third layer. The mature spore wall complex comprised at least four distinct layers: the outer electron-lucent thin double layers, the mottled electron-dense third layer, and the electron-lucent fourth layer in which electron-lucent granular substances were dispersed.

Key words Ectomycorrhizal fungi · Lipid body · *Rhizopogon roseolus* · Spore development · Spore wall

Introduction

Rhizopogon roseolus (Corda) Th. M. Fr. (= *R. rubescens* Tul. & C. Tul.), known as "shoro" in Japan, is a hypogeous basidiomycete that is an important ectomycorrhizal symbiont of Pinaceae (Morina and Trappe 1994). The fruiting bodies of this fungus are found in the sandy soils of *Pinus thunbergii* Parl. forest in seashore habitats. *Rhizopogon roseolus* is a popular edible fungus in Japan, but factors such as deforestation, poor forest management, and indiscriminate harvesting have led to recent decreases in production (Nagasawa 2000). To cultivate this fungus, pine seedlings can be aseptically inoculated with mycelium or spores; then, these mycorrhizal seedlings can be planted into a natural environment, and the fungus can be encouraged to grow (Rincón et al. 2001; Yamada et al. 2001; Wang et al. 2002). However, cultivation techniques have not been fully refined because of the low efficiency with which mycorrhizal trees can be obtained and the low levels of fruiting body production.

To successfully cultivate the fungus, inoculation and propagation methods must be developed, and strains with superior traits must be selected or bred. Such traits as vigorous mycelial growth, a tendency to form mycorrhizas, and high levels of fruiting body production are desirable. However, the sexual processes of this fungus are poorly understood, as are the mating systems that might permit new isolates to be obtained. Morphological observations of basidiospore formation, release, and maturation are prerequisites for understanding the sexual processes of the Basidiomycota.

In this article, we provide, using scanning and transmission electron microscopy, details of basidiospore development in the fruiting bodies of *R. roseolus* and discuss the significance of the spore structure with respect to the habitat of the fungus.

Materials and methods

Fruiting bodies of *R. roseolus* were collected from a seashore *P. thunbergii* forest in Tottori Prefecture, Japan. Figure 1 shows cross sections of the fruiting bodies at three developmental stages: white (immature), beige (intermedi-

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ate), and brown (mature) stages. For scanning electron microscopy (SEM), tissue fragments of the fruiting body were cut from gleba and fixed in 2% glutaraldehyde in phosphate buffer (pH 7.2) for 2 h at 4°C. After being rinsed with the buffer, these tissues were postfixed overnight in 2% osmium tetroxide in the buffer at 4°C. The fixed material was dehydrated in an ethanol series, transferred to isoamyl acetate, and critical-point dried in a Hitachi HCP-2 dryer using carbon dioxide. The dried materials were coated with platinum and examined using a Hitachi S-800 field-emission scanning electron microscope at 15 kV. For transmission electron microscopy (TEM), small pieces of gleba were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.2) for 2 h at 4°C. The fixed samples were washed in buffer and postfixed in 2% osmium tetroxide in the same buffer for 2 h at 4°C. They were then dehydrated in an ethanol series, transferred to propylene oxide, and embedded in Spurr resin (Spurr 1969). Infiltration and polymerization of the Spurr resin were carried out according to the procedure modified for preparation of *Alternaria alternata* spores (Park et al. 1990). Thin sections were cut using an LKB ultratome III with a diamond knife, and the sections were stained with 2% uranyl acetate for 3 min, then 3% lead citrate for 1 min. The samples were observed using a JEM-100CX II electron microscope at 80 kV.

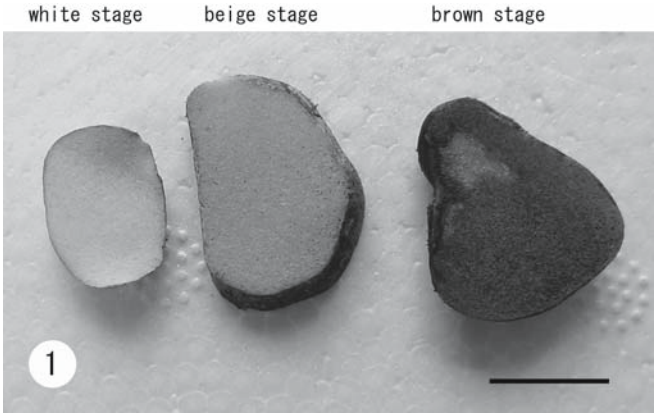
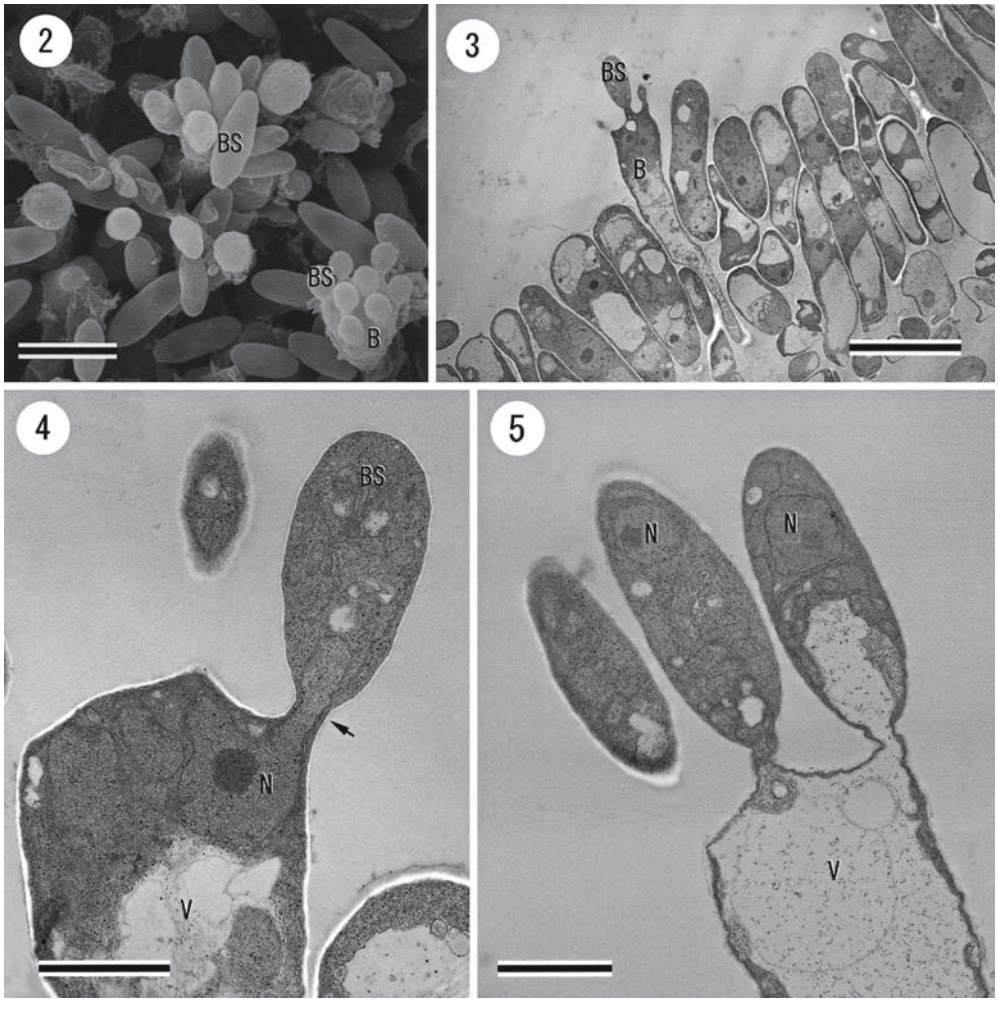


Fig. 1. Cross sections of *Rhizopogon roseolus* fruiting bodies at three different developmental stages: the white (immature), beige (intermediate), and brown (mature) stages. *Bar* 1 cm

Results

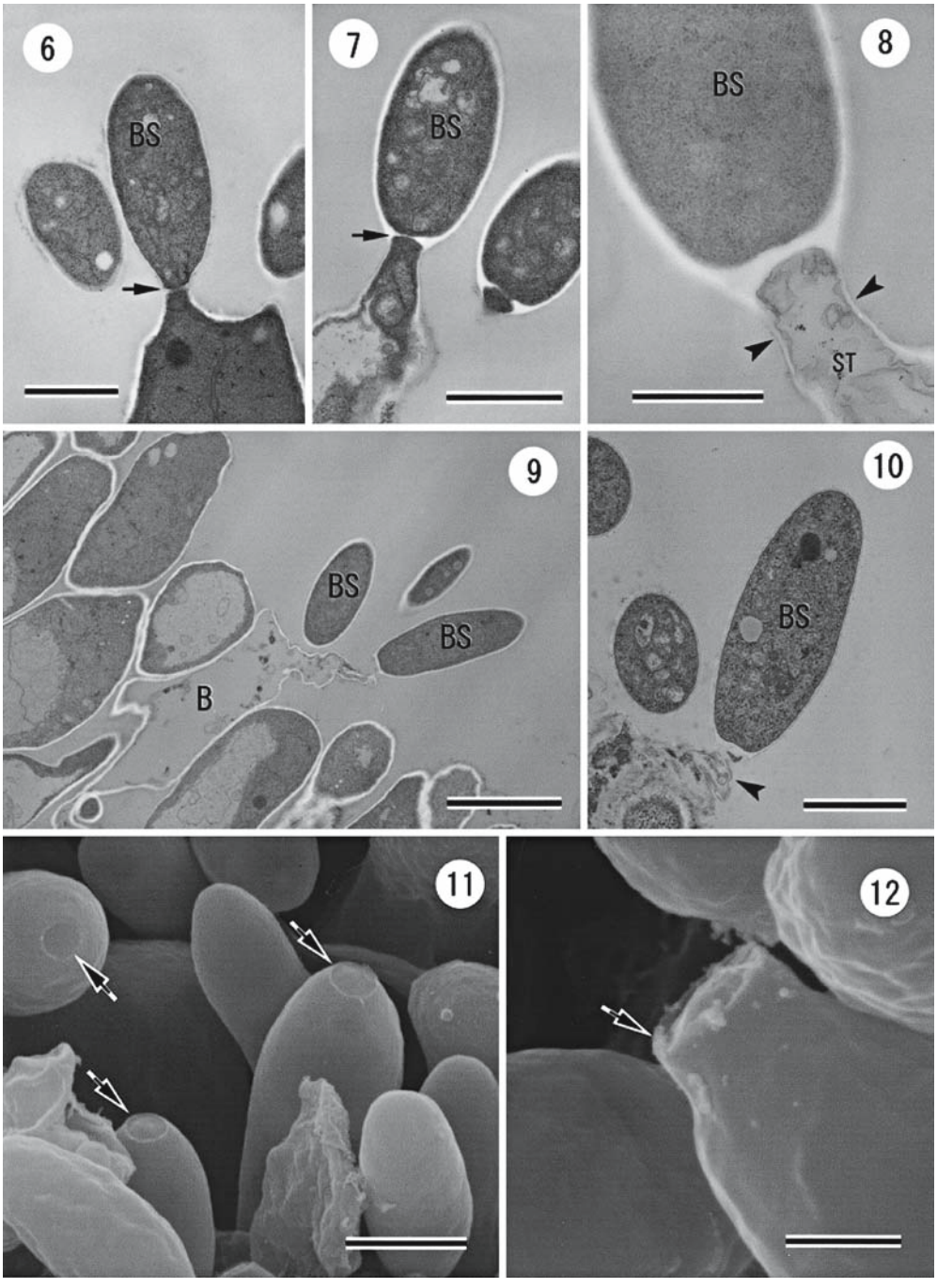
Figures 2–5 show the ultrastructure of the developing basidiospores in the fruiting body chambers of white (immature) stage *R. roseolus*. When viewed within the



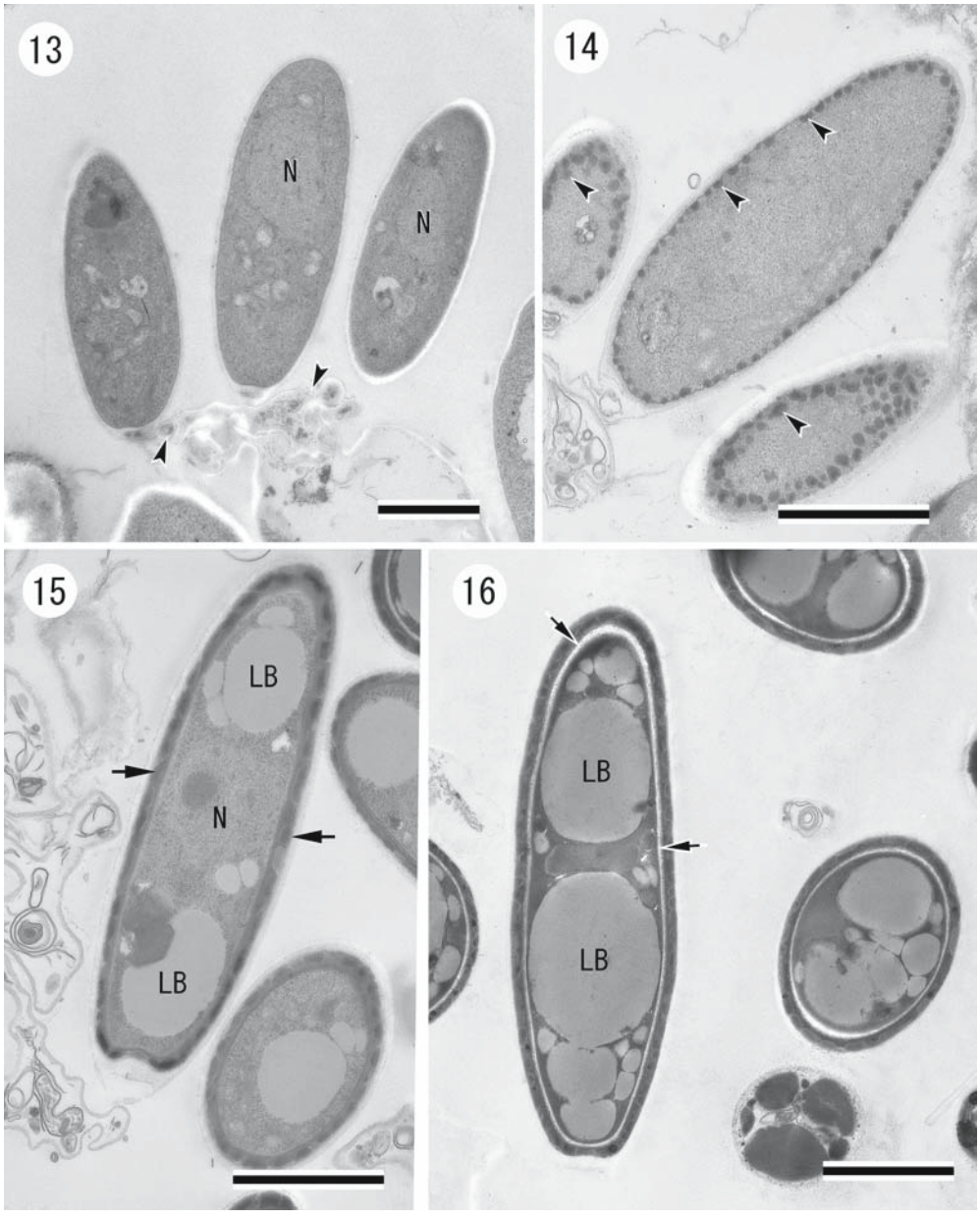
Figs. 2–5. Basidiospore formation in white (immature) stage fruiting body of *Rhizopogon roseolus*. **2** Scanning electron micrograph of basidiospores (*BS*) and basidia (*B*). **3** Transmission electron micrograph of the hymenium of the gleba. A basidium (*B*) bearing a basidiospore (*BS*) can be seen arising from the hymenium. **4** Nucleus caught in sterigma (*arrow*). **5** Basidiospores with nuclei (*N*) and a vacuolated basidium. A portion of a vacuole was occasionally found in the basidiospore. *B*, basidium; *BS*, basidiospore; *N*, nucleus; *V*, vacuole. *Bars* **2** 5 μm; **3** 10 μm; **4, 5** 2 μm

chambers using SEM, the basidiospores appeared narrowly ellipsoid with a smooth wall. Eight basidiospores were usually observed per basidium (Fig. 2). Each basidium arose from the hymenium and bore basidiospores (Fig. 3). In the basidium, a large vacuole was detectable on the side opposite to the basidiospore. A nucleus caught in the sterigma was observed (Fig. 4). A portion of a vacuole was occasionally found in the basidiospore, suggesting that the cytoplasmic movement was driven by vacuole development (Fig. 5). Figures 6–12 show the ultrastructure of the released basidiospores in the chambers of white (immature) stage fruiting bodies. The cytoplasm of basidiospores eventually separated from their sterigmata by an electron-lucent septum (Figs. 6–8). Afterward, the cell wall of the sterigma became thin, and the cytoplasm of the sterigma became disorganized and nearly empty (Fig. 8). In some sections, vacuolated basidia (Fig. 9) and disrupted sterigmal appendages (Fig. 10) were observed in close proximity to basidiospores. Figures 11 and 12 show SEM images of released basidiospores in the fruiting body chamber. Each basidiospore had a subhilar depression on its axial end (Fig. 11). In some cases, the spore retained a part of the sterigmal appendage (Fig. 12). Neither a hilar appendage nor a Buller's drop was observed.

Figures 13–16 show the ultrastructural features of the released basidiospores. Figure 13 shows the basidiospores



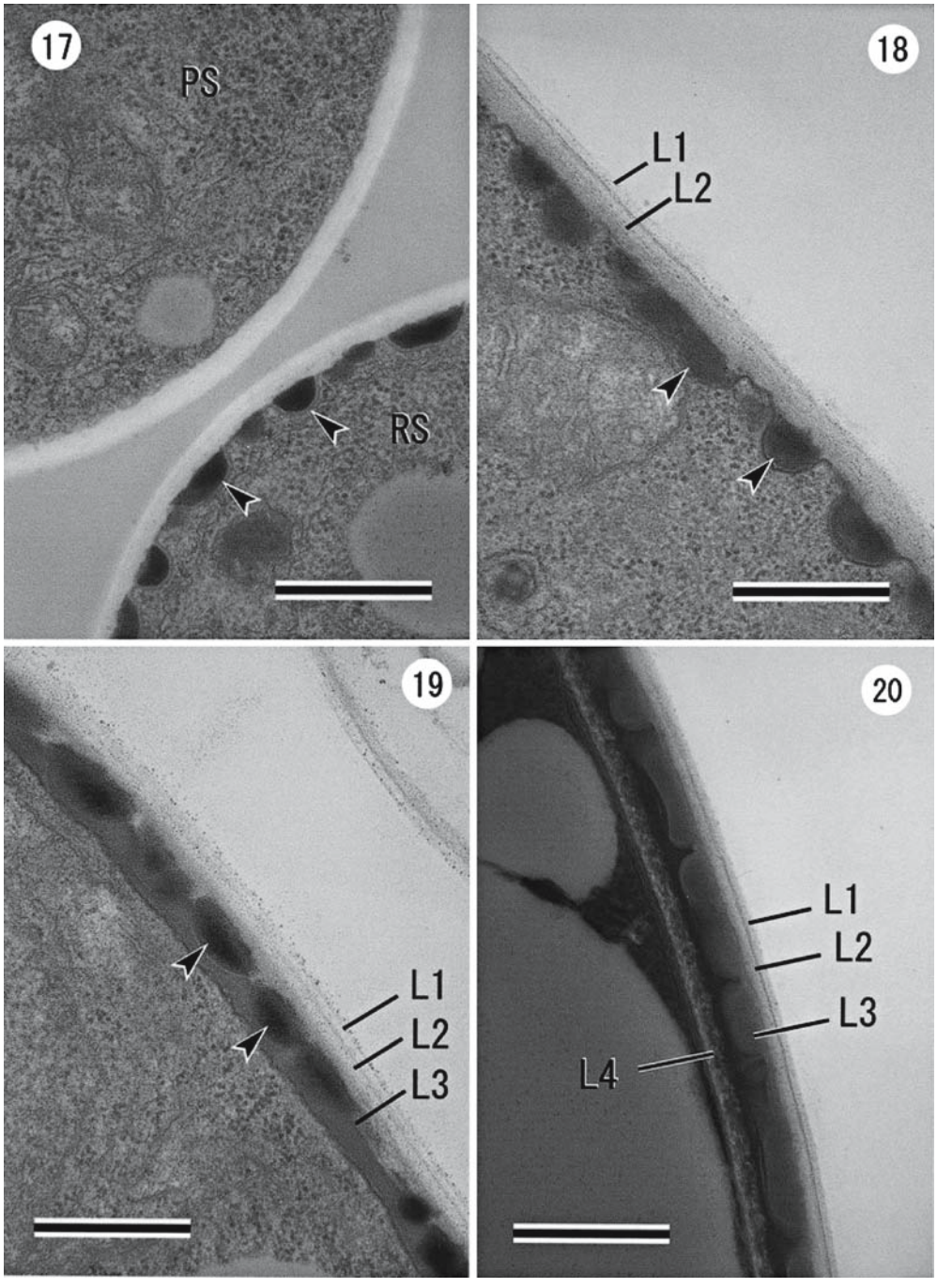
Figs. 6–12. Release of basidiospores in white (immature) stage fruiting body of *Rhizopogon roseolus*. **6, 7** Thin sections of basidiospores (*BS*) and sterigmata showing septum formation at the apex of the sterigmata (*arrows*). **8** Thin section of a basidiospore (*BS*) and sterigma (*ST*) showing the thin cell wall (*arrowheads*) and vacuolated cytoplasmic components of the sterigma. **9** Basidiospores (*BS*) and a vacuolated basidium (*B*). **10** A basidiospore (*BS*) retained a part of the collapsed sterigmal appendage (*arrowhead*). **11, 12** Scanning electron micrographs of released basidiospores in the fruiting body chamber. The *arrows* in **11** indicate subhilar depressions; *arrow* in **12** indicates a portion of a sterigmal appendage. *B*, basidium; *BS*, basidiospore; *ST*, sterigma. *Bars* **6, 7, 10, 11** 2 µm; **8, 12** 1 µm; **9** 5 µm



Figs. 13–16. Basidiospore development in the fruiting bodies of *Rhizopogon roseolus*. **13** Basidiospores of a white (immature) stage fruiting body with their sterigmal appendage (*arrowheads*). **14** Maturing basidiospores in a beige (intermediate) stage fruiting body showing electron-dense depositions (*arrowheads*) at the inner spore walls. **15** Maturing basidiospores in a beige (intermediate) stage fruiting body with electron-dense spore walls (*arrows*) and lipid bodies (*LB*). **16** Basidiospores in a brown (mature) stage fruiting body with electron-lucent layers (*arrows*) at the inner spore walls and expanded lipid bodies (*LB*). *LB*, lipid body; *N*, nuclei. *Bars* 2 μm

of a white (immature) stage fruiting body. Freshly released spores retained their sterigmal appendage (Fig. 13, arrowheads), which remained in contact with the collapsed basidium. Maturing basidiospores in a beige (intermediate) stage fruiting body are shown in Figs. 14 and 15. Electron-dense materials were deposited on the inner spore walls (Fig. 14). Figure 15 shows basidiospores with electron-dense spore walls, which probably comprise abundant dense materials. At this stage, lipid bodies were consistently detectable. In the brown (mature) stage, numerous basidiospores were found in the fruiting body chambers, and their ultrastructural features are shown in Fig. 16. The mature basidiospores were consistently surrounded by electron-lucent layers at the inner spore walls and contained expanded lipid bodies.

Figures 17–20 show ultrastructural details of the spore walls. In the white (immature) stage, spore walls were homogeneous and electron-lucent. Small electron-dense depositions were observed at the inner spore wall in released spores but were never found in prereleased spores (Fig. 17). Thin sections of spore walls from a beige (intermediate) stage fruiting body are shown in Figs. 18 and 19. The spore wall comprised two distinct layers: the outer thin layer (L1) and the inner electron-gray layer (L2), which was lined with electron-dense depositions (Fig. 18). As the spore matured, the gray layer became a thin lucent layer (L2), and an additional electron-dense third layer (L3) developed, probably comprising the dense materials (Fig. 19). At the brown (mature) stage, the spore wall complex comprised at least four distinct layers: the outer electron-lucent thin double layers (L1 and L2), the mottled electron-dense third layer (L3), and the electron-lucent fourth layer (L4) in which electron-lucent granular substances were dispersed (Fig. 20).



Figs. 17–20. Spore wall development in *Rhizopogon roseolus* basidiospores. **17** Thin section of basidiospores in a white (immature) stage fruiting body, with homogeneous and electron-lucent spore walls. Small electron-dense depositions (*arrowheads*) were observed at the inner spore wall in released spores (*RS*), but were never found in prereleased spores (*PS*). **18** Thin section of a basidiospore in a beige (intermediate) stage fruiting body, with a two-layered spore wall (*L1* and *L2*) with electron-dense depositions (*arrowheads*). **19** Thin section of basidiospore in a beige (intermediate) stage fruiting body, with a three-layered spore wall (*L1*, *L2*, and *L3*). Note the presence of electron-dense materials (*arrowheads*) in the dense layer (*L3*). **20** Thin section of a basidiospore in a brown (mature) stage fruiting body, with a four-layered spore wall (*L1*, *L2*, *L3*, and *L4*). Note the dispersed electron-lucent granular substance in the lucent layer (*L4*). *Bars* 0.5 µm

Discussion

Previous SEM observations of basidiospores from *R. roseolus* fruiting bodies collected in Europe showed that the exosporium was uniformly reticulated and covered by a smooth, thin perisporium (Martín and Ferran 1995; Martín 1996). In the present SEM observations, we did not observe a reticulate exosporium covered by perisporium. Furthermore, in the present TEM observations we found that the spore wall comprised several layers, but no reticulate exosporium was observed. However, the mottled electron-dense third layer observed in the matured spore wall may be associated with the reticulate ornamentations found in previous SEM observations (Martín and Ferran 1995; Martín 1996). For *R. roseolus* isolates distributed in Japan, taxonomic suitability has been confirmed based on the morphological characters by Hosford and Trappe (1988). In addition, we previously found that the rDNA internal transcribed spacer (ITS) sequences of Japanese *R. roseolus* isolates have very high levels of homology with *R. roseolus* sequences in the public database (Matsumoto et al. 2006). Therefore, it seems that a little morphological difference in spore surface exists between geographically distant speci-

mens of *R. roseolus*. Further taxonomic study is needed to determine whether the difference is caused by morphological variation within this species.

Most basidiomycetous mushrooms propel their spores away from the basidium (ballistospory). Such spores are known as "ballistospores," referring to the apparently active nature of their discharge (Money 1998). The ultrastructural features underlying basidiospore discharge have been studied in some Hymenomycetes, including *Lentinula edodes*, *Coprinus cinereus*, and *Boletus rubinellus* (Nakai and Ushiyama 1974a,b, 1975; McLaughlin 1977; Yoon and McLaughlin 1984, 1986). Spore discharge in these mushrooms involves specific structure for spore projection: a hilar appendage and a Buller's drop. In the Gasteromycetes, however, the basidiospores exhibit statismospory, with no active release from the basidium but a passive falling off at or near maturity (Miller and Miller 1988). However, ultrastructural details of statismospory have not been fully revealed in Gasteromycetes. The present TEM study revealed that these specific structures for spore projection were absent in *R. roseolus*. We speculate that spore release in this fungus is caused by septum formation at the tip of sterigma and subsequent collapse of the sterigma, and that released spores accumulate and mature in a glebal chamber.

In the present study, we described the ultrastructural changes that maturing basidiospores undergo in *R. roseolus*. After spore release, the major ultrastructural changes in the spore concerned the lipid bodies and the spore wall. During maturation, lipid bodies formed and expanded, and the spore walls became electron dense and multilayered. High lipid content is a feature of most mushroom spores that have been studied ultrastructurally (Voelz and Niederpruem 1964; Manocha 1965; Hyde and Walkinshaw 1966; Stock and Hess 1970; Heintz and Niederpruem 1971; Nakai and Ushiyama 1974b; Greuter and Rast 1975; Arita 1979; Fineran and Fineran 1984; Ruch and Motta 1987; Ruch and North 1988). According to Reisener (1976), differences among fungal species with respect to the lipid content of dormant spores reflect the physiological behavior of the spores, and lipid is the chief storage metabolite and substrate during the germination process. Spores with large amounts of stored lipid are able to germinate without an external supply, whereas spores with little stored lipid rely on an exogenous substrate for germination (Reisener 1976). Thus, the high lipid content of the basidiospores of *R. roseolus* implies that they do not rely on an exogenous substrate for germination.

Basidiospore walls are rich in fine structural features, leading to a myriad of different structures, surface markings, and chemical reactivities. These features are conserved at the species level and are frequently characteristic for genera or even families. Spore wall structure in the Hymenomycetes has been grouped into several categories (Clémençon 1997; Clémençon et al. 2004). Also, it seems that the ultrastructure of the spore wall in Gastromycetes has been poorly investigated, although the diversity of the spore morphology was examined based on light microscopy (Pegler et al. 1995). The present study showed that the *R. roseolus* spore wall was characterized by an electron-dense third layer and an electron-lucent fourth layer. This spore wall structure does not fit into any of the categories proposed in Hymenomycetes (Clémençon 1997; Clémençon et al. 2004) and might be specific in this fungus. Further, the electron-dense third layer was presumably formed by the electron-dense materials observed between the cell wall and plasma membrane during maturation. It seems therefore that deposition of the electron-dense materials in the spore walls is closely associated with the color change of gleba with maturation.

Fruiting bodies of *R. roseolus* are gasteroid, hypogeous in habit, and often found in sand, especially along seashores, in groves of *P. thunbergii* (Hosford and Trappe 1988). Basidiospores of *Rhizopogon* either remain in situ after fruiting body decomposition (Miller et al. 1994) or are dispersed locally by mycophagous small animals (Johnson 1996). It is therefore considered that basidiospores of *R. roseolus* possess some superior characters for survival in such sandy habitants and in the guts of animals. Spore survival depends on resistance to dryness and UV light. Spores with thin and colorless walls survive only a few days, but thick-walled, heavily pigmented spores can survive for month or years (Hess and Weber 1976). Resistance to dryness of pigmented spores kept in a herbarium is known for *Psilocybe* (2 years, Watling 1963; 9 years, Sussman 1968) and *Conocybe* (3 years, Watling 1963). Also, several studies (Kotter and Farentinos 1984; Colgan and Claridge 2002) have revealed that basidiospores of *Rhizopogon* retained viability after passage through the gut of small animals, suggesting stability for the digestion process. For *R. roseolus*, the spore wall may also be physically reinforced by four distinct layers and heavily pigmented by deposition of electron-dense materials. Therefore, it seems that these specific spore wall structures may play a role in physical tolerance against the harsh environmental conditions that might exist in sandy soil, which could lead to severe dehydration and overheating, and also against digestion by mycophagous animals.

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