Mycoscience VOL.65 (2024) 68–78

Mycoscience

An open access journal for general mycology

The Mycological Society of Japan

Full paper

Variable, life stage-dependent mycorrhizal specificity and its developmental consequences in the fully myco-heterotrophic orchid *Cyrtosia septentrionalis*

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ABSTRACT

The degree of specificity between fully myco-heterotrophic (MH) orchids and mycorrhizal fungi is regarded as high, but some species undergo a mycobiont shift as they transition from juvenile to adult plant. We investigated morphological and physiological aspects of the interaction between the fully MH *Cyrtosia septentrionalis* and its four known mycobionts to elucidate developmental consequences of variable, life-stage-dependent specificity. Of five randomly sampled germinated seeds co-cultured with each mycobiont, *Physisporinus* sp. '*TK-10*' colonized all, *Armillaria gallica* and *Desarmillaria tabescens* colonized one, and *A. mellea* subsp. *nipponica* colonized none. Whereas 16.2% of aseptically germinated seeds exhibited onward growth when co-cultured with *TK-10*, just 1.5–2.6% did so with the other species. Even so, *A. gallica* colonized and formed internal rhizomorphs within rhizomes established with *TK-10*, suggesting that this mycobiont can replace and potentially can oust the latter. We infer that the orchid can associate with *Armillaria* and *Desarmillaria* mycobionts throughout its life, but that *TK-10* enhances early growth. However, because *TK-10* has a higher wood-rotting capacity than *A. gallica*, rapid resource exhaustion may cause nutrient supply shortages. We hypothesize that secondary colonization by *Armillaria* or *Desarmillaria* species triggers *TK-10* displacement and that this mycobiont shift sustains orchid growth for longer.

Keywords: *Armillaria*, Fungal nutritional mode, Internal rhizomorph, Mycobiont shift, *Physisporinus*

Article history: Received 1 September 2023, Revised 28 November 2023, Accepted 6 December 2023, Available online 31 March 2024.

1. Introduction

All orchids produce tiny seeds that lack an endosperm and other essential nutrients (Rasmussen, 1995). To overcome this deficiency, they engage in symbiosis with Basidiomycetous or Ascomycetous fungi (mycobionts) to acquire the nutrients necessary for germination (Smith & Read, 2008). In the case of achlorophyllous orchids, this association persists throughout their life cycle. These orchids are thus entirely dependent on the carbon and other nutrients supplied by the mycobionts, making them fully myco-heterotrophic (MH). A higher degree of specificity has been observed in the mycorrhizal relationships of such orchids (Leake, 1994). However, some terrestrial MH orchids establish mycorrhizal relationships with two or more different mycobionts during successive life history stages (Kitahara et al., 2022; Li et al., 2022). For example, whereas the fully MH orchid *Gastrodia elata* Blume requires the assistance of the litter decomposer *Mycena osmundicola* J.E. Lange for seed germination, adult plants associate exclusively with parasitic or pathogenic *Armillaria* species, such as *A. gallica* Marxm. & Romagn., *A. mellea* (Vahl.) P. Kumm., *A. ostoyae* (Romagn.) Herink or *A. sinapia* Bérubé & Dessur. (Cha & Igarashi, 1995a; Kusano, 1911; Xu & Guo, 2000; Xu & Mu, 1990). *Armillaria mellea* has an inhibitory effect on germination in this species, even though it is essential for the development of mycorrhizal protocorms (Park et al., 2012; Xu & Guo, 2000). It is thought that gastrodianin, a lectin produced by the orchid protocorm, regulates this interaction by inhibiting the colonization of *A. mellea* during seed germination and early protocorm development stages (Hu et al., 1999; Wang et al., 2001). However, a mechanistic understanding of how this mycobiont shift is achieved remains wanting.

As for *G. elata*, *Cyrtosia septentrionalis* (Rchb.f.) Garay (syn. *Galeola septentrionalis*) is a fully MH orchid that is thought to switch mycobiont as it transitions through seed germination, protocorm development and subsequent life history phases (Umata et al., 2022). Over the course of their life, plants of this species develop a giant body comprising an extensive root system of 20 or more extended adventitious roots, some of which may reach up to 5 m in length, as well as numerous shorter lateral roots (Hamada, 1939; Umata et al., 2022); some individuals produce more than 30 flower-

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doi: 10.47371/mycosci.2023.12.002

ing shoots, each attaining up to 1.7 m in height (Chen & Cribb, 2009; Hamada, 1939; Umata et al., 2022). As an adult plant, *C. septentrionalis* exhibits high specificity for mycobionts in the parasitic/pathogenic genera *Armillaria* and *Desarmillaria* (Cha & Igarashi, 1995a, 1995b; Hamada, 1939; Terashita, 1985; Terashita & Chuman, 1987, 1989), with the latter being a recent segregate of the former (Koch et al., 2017). Terashita (1985) demonstrated that *A. mellea* subsp. *nipponica* J.Y. Cha & Igarashi can colonize aseptic rhizomes to enhance onward growth to the seedling stage. In contrast, Umata et al. (2013) found that *Physisporinus* sp. '*TK-10*', a wood-rotting polypore isolated from protocorms growing naturally at a soil depth of ca. 15 cm, stimulates seed germination and facilitates the development of rhizomes. It therefore seems reasonable to infer that the orchid switches mycobionts from the *Physisporinus* species to an *Armillaria* or *Desarmillaria* species during early growth. However, in a more recent fine-scale, site-based study, Umata et al. (2022) revealed that, of three naturally occurring adult *C. septentrionalis* plants that emerged in close proximity to one another, two were colonized by *A. gallica* and one with the *Physisporinus* mycobiont. Although colonization by *Armillaria* or *Desarmillaria* species has not been observed in juveniles of *C. septentrionalis* in nature, it is possible that the orchid associates with either the *Physisporinus* mycobiont or *A. gallica* throughout juvenile and adult life history phases, but that the *Physisporinus* mycobiont is replaced by *A. gallica* in some cases.

Prior *in vitro* research has shown that seeds of *C. septentrionalis* can be germinated on a sawdust-based medium at 30 °C in the presence of either of two *Armillaria* mycobionts (viz *A. gallica* and *A. mellea* subsp. *nipponica*) or *Desarmillaria tabescens* (Scop.) R.A.Koch & Aime (formerly *A. tabescens*), even if there is no direct contact between them (Umata et al., 2013). However, this could only be achieved under modified atmospheric conditions comprising a reduced O_2 (13.1–17.0%) and elevated CO_2 (2.7–4.9%) concentration (Nakamura et al., 1975). As these conditions are established by the mycobiont's wood-rotting activity in nature (Umata et al., 2013), Umata et al. (2021) proposed that fungal nutritional mode plays a key role in the germination of the orchid–and thus the outcome of the mycorrhizal relationship. Moreover, in addition to a modified atmosphere, germination initiation (as defined by seed coat rupture) was enhanced by supplementing the growth medium with kinetin, α-naphthalene acetic acid and certain reactants involved in early plant defense against pathogenic attack (Chandra et al., 1996; Ebel & Scheel, 1997; Mika et al., 2004), including K^+, Ca^{2+} and H_2O_2 (Umata et al., 2021). However, because direct contact co-culture experiments using *Armillaria* or *Desarmillaria* species have not yet been conducted, it remains unknown whether either or both of these mycobionts can colonize germinated seeds.

It also remains unclear whether *Armillaria* or *Desarmillaria* mycobionts can enhance the onward growth of germinated seeds and protocorms of the orchid, as well as whether they can associate with rhizomes already growing symbiotically with the *Physisporinus* mycobiont, and potentially even displace it. Previously, we hypothesized that direct physical contact between a growing *C. septentrionalis* root and an *A. gallica* mycelium that enters its rhizosphere might be sufficient to induce such a mycobiont shift (Umata et al., 2022). Given the apparent importance of fungal nutritional mode in the mycorrhizal establishment and possible mycobiont shift, the relative capacity of *Armillaria* or *Desarmillaria* species and the *Physisporinus* mycobiont to decompose wood also deserves further attention. The sheer size of adult plants implies enormous demand for organic nutrients over long periods, and it is plausible that these needs are met by the wood-rotting or parasitic behavior of the mycobiont.

In addition, the physiological and developmental means by which internal mycelial organs are formed requires clarification. Not only do *Armillaria* species produce linear, free-living, mycelial rhizomorphs (Hartig, 1874), but *A. gallica* and *A. mellea* subsp. *nipponica* also form specialized internal rhizomorphs–so-called "intraradicale Rhizomorpha"–within mycorrhizal roots of *C. septentrionalis* (Hamada, 1939; Terashita, 1985; Umata et al., 2022). Hamada (1939) speculated that these organs play a central role in enabling the host to attain such giant sizes. To shed light on this aspect of the MH orchid–mycobiont relationship and address the above hypotheses, we sought to clarify the mechanism by which *Armillaria* or *Desarmillaria* species colonize adult *C. septentrionalis* plants and ascertain the functional significance of internal rhizomorphs.

2. Materials and methods

2.1. Collection and storage of seeds

Mature seed pods of *C. septentrionalis* were collected from a wild population in Isa City, Kagoshima Prefecture, southwest Japan, in Oct 2013. The seeds were removed from the pods and stored in silica gel at 1–5 °C until used in the experiments described below.

2.2. Mycobionts

We examined the mycorrhization of *C. septentrionalis* using its four known mycobionts. *Armillaria gallica* and *Desarmillaria tabescens* were sourced from the National Institute of Technology and Evolution Biological Resource Center in Chiba, Japan (accession numbers NBRC 31621 and NBRC 31620, respectively); *A. mellea* subsp. *nipponica* was isolated from a fruit body as described by Umata et al. (2013); and *Physisporinus* sp. '*TK-10*' was isolated from a naturally growing *C. septentrionalis* protocorm (Umata et al., 2013).

2.3. Surface sterilization of seeds

The seeds were sterilized by immersion in electrolyzed hypochlorous acid water (50 μg/mL HCOL; pH 5.0–6.5 HouRai Medec Co., Kagoshima, Japan) and 0.1% (v/v) Tween-20 for 20–25 min, and then rinsed three times in sterilized distilled water. A mean (±SD) of 68.7 (±28.0) seeds were then loaded onto sterilized 8 mm-diam paper disks (Advantec, Tokyo, Japan), and these 'seed-disks' were transferred to 9 mL vials containing 2.5–3 mL of a 0.8% water-agar (WA) + 100 mM KCl medium and monitored for 2 wk at 23 °C for contamination. Contaminated seed-disks were discarded.

2.4. Mycobiont culture

A sawdust-based medium was prepared as a mycobiont bed following the methods of Umata et al. (2013). This was used to line two types of containers: polycarbonate boxes (100 mm tall, 60 mm wide, 60 mm deep) were used to obtain symbiotically germinated seeds and mycorrhizal rhizomes, and test tubes (150 mm tall, 30 mm diam) were used to co-culture aseptically germinated seeds and protocorms with mycobionts. For the polycarbonate boxes, 80–100 mL of the sawdust-based medium were placed at the bottom and this was overlaid with 80–100 mL of river sand sterilized at 120 °C for 1 h (Fig. 1A); a 7–10 mm diam hole was created at the center of sand for mycobiont inoculation of the medium beneath. For the test tubes, 25 mL of the sawdust-based medium were placed

at the bottom (Fig. 1B). All boxes and tubes were autoclaved for 2 h at 120 °C, and then one of the four mycobionts was inoculated onto the medium. The cultures were incubated for 5–6 wk at 30 °C before being used in the following experiments.

2.5. Classification of germination and onward development

In all subsequent experiments, seed germination and onward development were evaluated according to the following six, readily discernable stages (Umata et al., 2021).

Stage 0: No germination. Seed coat intact, seed unchanged.

Stage 1: Germination initiated. Seed coat ruptured. Because the embryo is not visible through the thick, brown seed coat in *C. septentrionalis*, opening of the seed coat represents the first clear indication of germination.

Stage 2: Full germination. Embryo enlarged and emerging from the ruptured seed coat, diameter equal to or exceeding the width of the seed coat.

Stage 3: Protocorm. Distinct apical bud visible at the apex of the enlarged embryo.

Stage 4: Rhizome. Protocorm elongation and subsequent organ formation apparent.

Stage 5: Seedling. Shoot and roots forming on the rhizome. For this study, we inferred that plantlets had successfully transitioned to an adult once they reached this stage.

2.6. Preparation of aseptically germinated seeds and protocorms

We employed the methods of Umata et al. (2021) to germinate seeds aseptically in jars in the presence of an O_2-absorbing and CO_2-generating agent (AnaeroPack-MicroAero No. A-28, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan), which establishes micro-aerophilic conditions (6–12% O_2, 5–8% CO_2). When incubated at 30 °C in the dark, 55.2 ±7.5 (67.1%) of seeds on each disk germinated (Stage 1) within 8–10 wk. To obtain aseptic protocorms (Stage 3), seeds that had successfully germinated were transferred onto the medium of Nakamura (1982) which had been modified by replacing the vitamin mixture (thiamine, nicotinic acid, and pyridoxine) with 1% Ebios yeast powder (Asahi Food & Health Co., Tokyo, Japan) and the casein hydrolysate with 0.5% lacto albumin (Difco Laboratories, Michigan, USA). Aseptic protocorms were obtained after culturing at 25 °C for 8–10 wk in the dark.

2.7. Confirmation of mycobiont colonization in symbiotically germinated seeds

Once the mycobionts had been successfully established in the sawdust-based medium in the polycarbonate boxes, four sterilized seed-disks were placed onto the bed, the lid was resealed (Fig. 1A) and the box was incubated at 30 °C for a further 8–10 wk in the dark; three replicate boxes were prepared for each of the four mycobionts. After that period, five seed-disks were selected from the 12 seed-disks per mycobiont, and one germinated seed (Stage 1) was randomly sampled from each. The size of sampled germinated seeds was different. These germinated seeds were fixed in FAA (formaldehyde–acetic acid–ethanol), then dehydrated through an ethanol series before being embedded in Technovit 7100 resin (Kulzer, Wertheim, Germany) for sectioning on a microtome. Serial resin sections were cut at a thickness of 4–5 μm and stained with Toluidine blue, then embedded in an Entellan mounting medium (Merck, Darmstadt, Germany). The sectioned samples were examined under a light microscope to confirm mycobiont colonization.

2.8. Co-culture of aseptically germinated seeds and protocorms with an Armillaria or Desarmillaria mycobiont

A single seed-disk bearing either 55.2 ±7.5 aseptically germinated seeds (Stage 1) or seven aseptic protocorms (Stage 3) was inserted onto the mycobiont bed in each tube, and this was covered with a 10 × 10 mm nylon mesh plus a further 7 mL of sawdust medium; a second inoculation of the fungus was then introduced (Fig. 1B). The nylon mesh was used to prevent the plants from being obscured by sawdust, which was important for observation. All tubes were capped with a polypropylene lid then sealed with adhesive tape and incubated at 25 °C for 6 wk or longer in the dark. Ten replicates were prepared for each of the four mycobionts. Germinated seeds that developed beyond the protocorm stage during this period were regarded as having successfully established a mycorrhizal association, as were protocorms that developed beyond the rhizome stage (Stage 4). However, we didn't co-culture of protocorms with *TK-10*, because seeds co-cultured with *TK-10* grew to Stage 4 in the previous investigation (Umata et al., 2013) and aseptic germinated seeds colonized with *TK-10* exhibited the highest proportion of successful onward growth in the present investigation (Table 1).

A longer co-culture was also carried out using the polycarbonate boxes to obtain plants at more advanced growth stages. After establishing mycobiont beds colonized with *A. mellea* subsp. *nipponica*, a 5 × 5 cm piece of non-woven fabric (used instead of nylon mesh) was placed over the bed and four aseptically germinated-seed (Stage 1) disks were placed on top of that. This was followed by overlaying with 50 mL of a sterilized sawdust-vermiculite medium composed of beech-wood sawdust, vermiculite, rice bran and water in a ratio of 5:5:1:2 by volume (i.e., similar to the setup shown in Fig. 1B but without secondary mycobiont inoculation). Three replicates were prepared, and the boxes were then sealed with adhesive tape and incubated at 25 °C for 7 mo in the dark.

2.9. Co-culture of Armillaria gallica and mycorrhizal rhizomes colonized with TK-10

To examine whether *A. gallica* is capable of replacing *TK-10* as

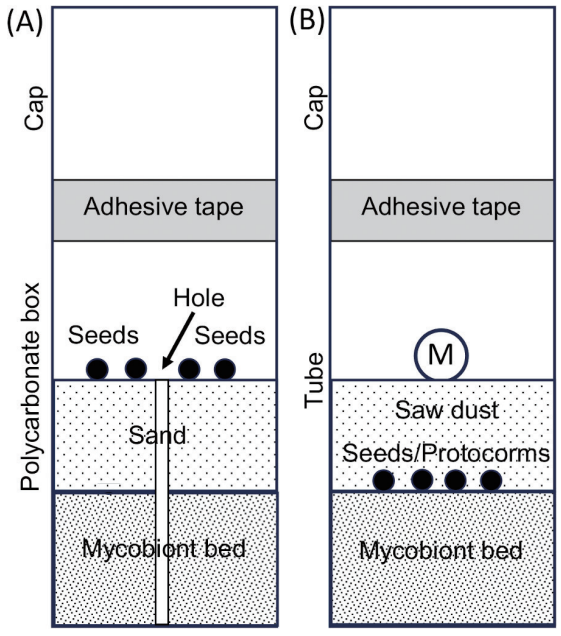

Fig. 1. Schematic illustration of the culture containers used in this study. **A:** Polycarbonate box. **B:** Test tube. M in (B), second inoculation of mycobiont.

Table 1. Mean (±SD) proportion of aseptically germinated seeds and protocorms that successfully exhibited onward growth in culture on a sawdust-based medium in the presence of four mycobionts. Different letters in a column indicate significantly different values ($p < 0.05$, multiple Tukey-Kramer's test).

	Germinated seeds [(1)]		Protocorms [(2)]	
Mycobiont	Protocorms (%)	n	Rhizomes (%)	n
Armillaria gallica	1.5 ±3.3 [a]	10	10.0 ±15.7 [a]	10
A. mellea subsp. *nipponica*	1.5 ±2.8 [a]	10	10.0 ±20.3 [a]	10
Desarmillaria tabescens	2.6 ±4.0 [a]	10	7.1 ±14.6 [a]	10
TK-10	16.2 ±20.2 [b]	10	N/A	N/A

[(1)] One seed-disk was placed in each replicate container, with each seed-disk bearing a mean of 55.2 (±7.5) germinated seeds (Stage 1). Successful mycorrhization led to onward growth to the protocorm (Stage 3) or later stage.
[(2)] Seven protocorms (Stage 3) were placed in each replicate tube. Successful mycorrhization led to onward growth to the rhizome (Stage 4) or later stage.

the sole mycobiont in *C. septentrionalis* roots, we cultured mycorrhizal rhizomes colonized with *TK-10* in the presence of *A. gallica*. Firstly, seeds were symbiotically germinated with *TK-10* in polycarbonate boxes as described in Section 2.7, and these were overlaid with 80 mL of river sand sterilized at 120 °C for 1 h and then incubated at 25 °C for 8–10 wk to induce rhizome formation (Stage 4). Separately, *A. gallica* was cultured on sticks of *Quercus acutissima* Carruth. following the methods of Park et al. (2012). In brief, four or five sticks (4–5 cm long, 8–10 mm diam) and several *Q. acutissima* leaves (cut into 2–3 cm pieces) were submerged in 100 mL of a sterile liquid medium composed of tap water and rice bran in a 4:1 ratio by volume in sterilized containers; *A. gallica* was then inoculated onto the medium. Rhizomorphs formed on the sticks within 4 mo of incubation in the dark at 25 °C. Three sticks colonized with *A. gallica* rhizomorphs were placed beside roots emanating from rhizomes colonized with *TK-10* in the polycarbonate boxes; five replicate boxes were prepared. These were sealed with adhesive tape and incubated at 25 °C for 3 mo or longer in the dark.

2.10. Direct observation of rhizomorph formation

From the detection of internal rhizomorphs in the roots of *C. septentrionalis* in nature (Hamada, 1939; Umata et al., 2022) and that in a root of seedling grown with *A. mellea* subsp. *nipponica in vitro* (Terashita, 1985), we hypothesized that the root epidermis may play an important role for the rhizomorph formation. To elucidate developmental aspects of internal rhizomorph formation within roots of *C. septentrionalis*, seeds co-cultured with either *A. gallica* or *TK-10* were transferred onto 10 mL of a potato dextrose agar (PDA) medium in 9 cm diameter Petri dishes, covered with a 5 × 5 cm sheet of sterilized plastic wrap to serve as substitute for the root epidermis, and incubated at 25 °C for 6 wk in the dark. Observations of surface morphology and color were then made under a stereomicroscope.

2.11. Wood-rotting ability

To compare the relative wood-rotting ability of *TK-10* and *A. gallica*, 1 cm^3 beech (*Fagus crenata* Blume) blocks were oven-dried at 105 °C for 48 h and weighed. These blocks were then immersed in water for 24 h to absorb water. Each of the two mycobionts was cultured in a 200 mL flask containing 50 mL of agar medium comprising 4% glucose, 0.3% peptone, 1.5% Difco yeast extract and 2% agar for 4 wk at 25 °C in the dark. The wooden blocks were sterilized in water at 121 °C for 2 h, placed on the medium with one of the mycobionts, and then incubated at 25 °C for 4 wk in the dark. We set up nine replicates for *TK-10* and 13 for *A. gallica*; a further five blocks were incubated on the same medium lacking either mycobiont as a control. After the incubation period, the blocks were oven-dried again at 105 °C for 48 h and then reweighed.

2.12. Statistical analysis

The proportion of seeds that successfully germinated and protocorms or rhizomes that exhibited onward growth was assessed using a multiple Tukey-Kramer's test. The variance of weight loss among control wooden blocks versus those colonized with either *A. gallica* or *TK-10* was assessed using a Student's t-test

3. Results

3.1. Microscopic examination of symbiotically germinated seeds

Seed germination took place in the presence of all four mycobionts (Fig. 2A–H; Stage 1–2). Each mycobiont except for *A. mellea* subsp. *nipponica* firstly infected a basal cell of the enlarged embryo (Fig. 2C–G) and then spread throughout the growing embryo (Fig. 2H, I; Stage 2–3). However, the level of colonization varied among the four mycobionts: of the five seeds examined under a light microscope following germination with each of them, *A. mellea* subsp. *nipponica* had failed to colonize any of the cells of enlarging embryos (Fig. 2B; Stage 1), *A. gallica* and *D. tabescens* had each colonized one (Fig. 2 C–E; Stage 1–2) and *TK-10* had colonized all five (Fig. 2F–H; Stage 1–2). Only in the case of *TK-10* did some seeds exhibit onward growth to the protocorm stage (Stage 3) and more extensive mycorrhizal spread (Fig. 2I).

3.2. Co-culture of aseptically germinated seeds and protocorms

Aseptically germinated seeds (Fig. 3A; Stage 1) and protocorms (Fig. 3B; Stage 3) also successfully established mycorrhizal growth through co-culture with all four examined mycobionts (Table 1; Fig. 3C–J; Stage 3–5), but the frequency of successful mycorrhizal establishment was very low with both *Armillaria* and *Desarmillaria* mycobionts (Table 1). Whereas *TK-10* successfully colonized 16.2% of the aseptic germinated seeds on average, thereby enabling them to undergo onward growth to the protocorm stage (Stage 3), the proportion achieved by *A. gallica*, *A. mellea* subsp. *nipponica* and *D. tabescens* was significantly lower ($p < 0.05$), at just 1.5–2.6%. In fact, *A. mellea* subsp. *nipponica* induced the development of just two out of ca. 660 germinated seeds into seedlings (Stage 5) during 7 mo of incubation (Fig. 3J, K), with the remainder failing to grow and remaining unchanged relative to their initial condition (Fig. 3J; Stage 1). Even so, internal rhizomorphs were visible as a series of

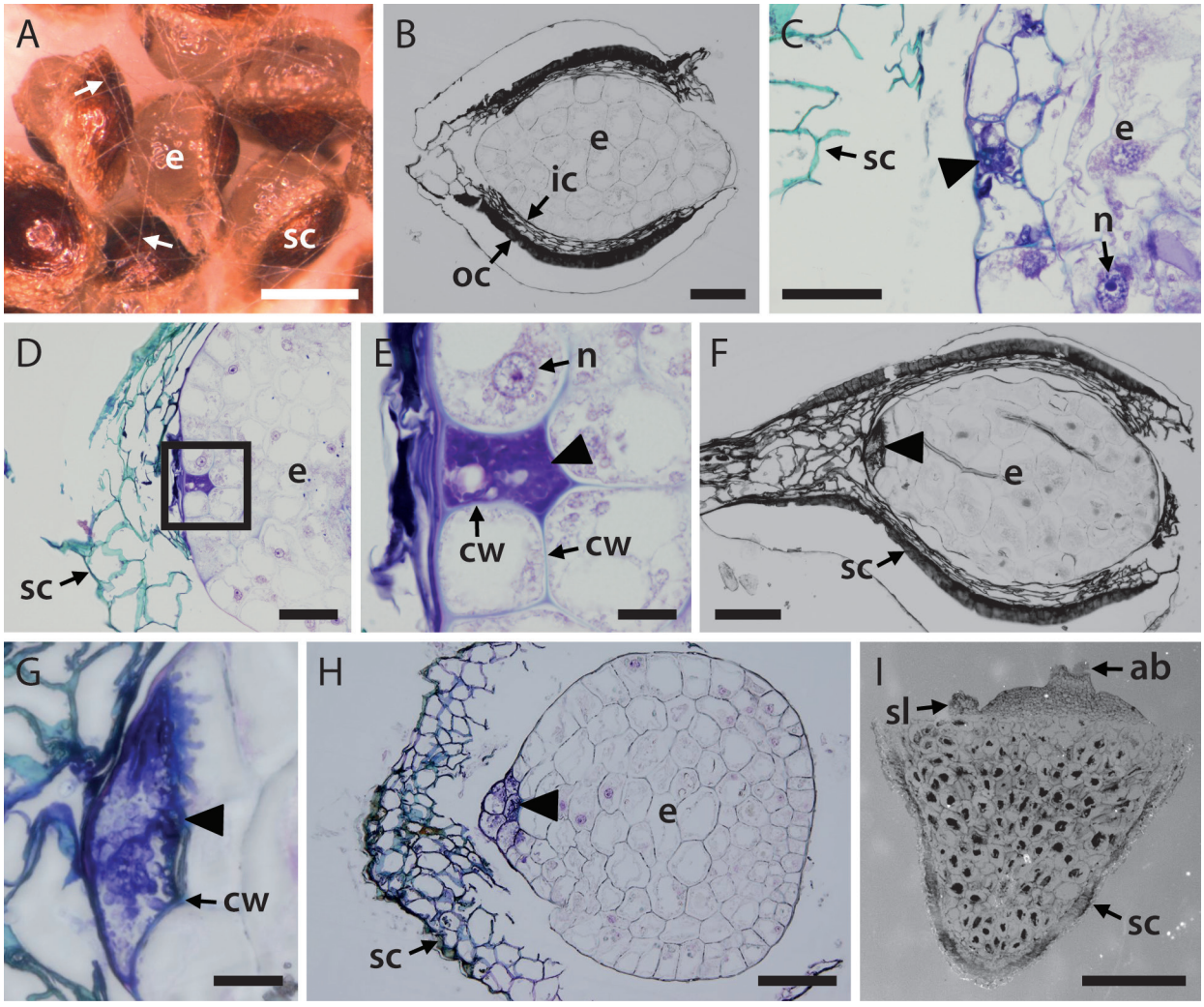

Fig. 2. Co-culture of *Cyrtosia septentrionalis* seeds. **A**: Symbiotically germinated seeds (Stage 1) with an enlarged embryo (e) rupturing the seed coat (sc) following co-culture with *Desarmillaria tabescens*. Arrows indicate individual hyphae of *D. tabescens*. **B**: Longitudinal section thorough a symbiotically germinated seed (Stage 1) with an enlarged embryo (e) following co-culture with *Armillaria mellea* subsp. *nipponica*. The basal region (left-hand-side) is free from fungal infection. The seed coat is composed of an inner (ic) and outer (oc) layer. **C**: Basal region of a Stage 2 embryo (e) infected with *D. tabescens* (arrowhead). The seed coat (sc) and an enlarged nucleus (n) are indicated. **D**: Basal region of a Stage 2 embryo (e) infected with *A. gallica*. The seed coat (sc) is indicated. **E**: Magnification of the square in D, with the convoluted hyphae of *A. gallica* visible inside a single cell (arrowhead). An enlarged nucleus (n) and cell walls (cw) are indicated. **F**: Longitudinal section thorough a symbiotically germinated seed (Stage 1) following co-culture with *TK-10*. An area of colonization (arrowhead) is visible in the basal region of an embryo (e). The seed coat (sc) is indicated. **G**: Magnification of the area of colonization in F, with the convoluted hyphae of *TK-10* (arrowhead) visible inside one or two cells. The cell wall (cw) is indicated. **H**: Longitudinal section thorough a symbiotically germinated seed (Stage 2) following co-culture with *TK-10*. The area of colonization (arrowhead) has spread into several cells of the enlarged embryo (e). The seed coat (sc) is indicated. **I**: Longitudinal section thorough a protocorm (Stage 3) growing symbiotically with *TK-10*. Numerous pelotons (black masses) are visible throughout, except towards the apex. The apical bud initial (ab), a scaly leaf initial (sl) and the seed coat (sc) are indicated. *Bars*: A, I 1 mm; B, F, H 100 μm; C, D 50 μm; E 10 μm; G 20 μm.

whitish striations near the base of emerging roots in the seedlings formed by this mycobiont (Fig. 3J, K). However, 7.1–10.0% of aseptic protocorms were successfully colonized by these three mycobionts, enabling them to develop into the rhizome stage (Fig. 3E–I; Stage 4).

3.3. Co-culture of Armillaria gallica and mycorrhizal rhizomes colonized with TK-10

Armillaria gallica rhizomorphs emerged from the *Quercus acutissima* sticks and directly contacted sterile roots of *C. septentrionalis* emanating from rhizomes (Stage 4) colonized with *TK-10* (Fig. 4A–C). They then formed internal rhizomorphs beneath the root epidermis (Fig. 4D). These organs were highly organized in structure, with a thick outer rind enclosing a central cavity (Fig. 4D).

3.4. Rhizomorph formation within plant and beneath sheet of plastic wrap

Mycobiont colonization occurred primarily towards the base of both swelling embryos and protocorms (Fig. 2C–I; Stages 1–3) and internal rhizomorphs subsequently formed within protocorms (Fig. 3C, D, 5A; Stage 3) and rhizomes (Figs. 3F–H, 5B; Stage 4). *Armillaria* species formed internal rhizomorphs as early as the protocorm stage (Fig. 3C, D; Stage 3) as well as in the basal portion of rhizomes (that is, in the main root) in more developed plants (Fig. 3F–H; Stage 4). Whereas colonized areas appeared brown, with the whitish internal rhizomorphs being visible beneath the

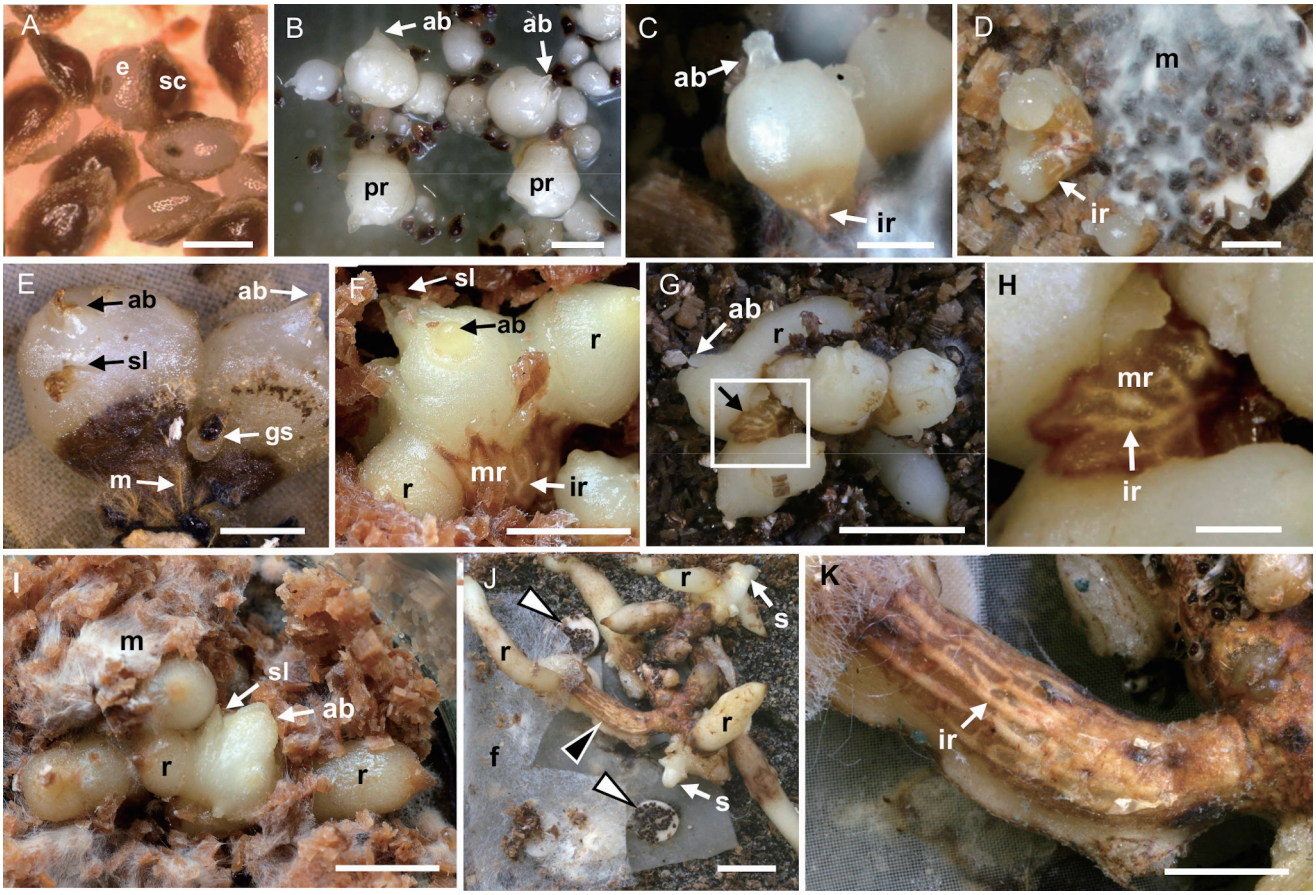

Fig. 3. Co-culture of axenically germinated seeds and protocorms of *Cyrtosia septentrionalis*. **A**: Aseptically germinated seeds at Stage 1 prior to co-culture. **B**: Aseptic protocorms at Stage 3 (pr) with apical buds (ab) indicated. **C**: Successful onward growth of aseptically germinated seeds into protocorms with an apical bud (ab) following co-culture with *A. gallica*. Internal rhizomorphs (ir) are visible at the base of a protocorm. **D**: Successful onward growth of aseptically germinated seeds into protocorms following co-culture with *A. mellea* subsp. *nipponica* (m). Internal rhizomorphs (ir) are visible at the base of a protocorm. However, note that only three seeds show onward growth. **E**: Successful onward growth of aseptically germinated seeds into protocorms following co-culture with *D. tabescens*. Apical bud initials (ab), a scaly leaf initial (sl), a loose aggregation of mycelia (m) and a Stage 2 germinated seed (gs) that has failed to undergo onward growth are indicated. **F**: Successful onward growth of aseptic protocorms into rhizomes at Stage 4 following co-culture with *A. gallica*. An apical bud (ab), scaly leaf (sl), a main root (mr), and adventitious roots (r) are indicated. **G**: Successful onward growth of aseptic protocorms into rhizomes at Stage 4 following co-culture with *A. mellea* subsp. *nipponica*. An apical bud (ab), a main root (black arrow), and an adventitious root (r) are indicated. **H**: Magnification of the area marked with square in G, showing internal rhizomorphs (ir) beneath a main root surface (mr). **I**: Successful onward growth of aseptic protocorms into rhizomes at Stage 4 following co-culture with *D. tabescens* (m) without forming inner rhizomorphs. An apical bud (ab), a scaly leaf initial (sl), and adventitious roots (r) are indicated. **J**: Successful onward growth of aseptically germinated seeds into seedlings at Stage 5 following co-culture with *A. mellea* subsp. *nipponica*. The two marked shoots (s) belong to different individuals. Internal rhizomorphs are visible within a root (black arrowhead). Adventitious roots (r), a shoot (s) and the non-woven fabric (f) used to cover the germinated seeds are indicated. However, most germinated seeds failed to undergo onward growth (white arrows). **K**: Magnification of the area marked with the arrowhead in J showing several internal rhizomorphs (ir) beneath the root surface. *Bars*: A, C, H 1 mm; B, D, E 2 mm; F, G, I, K 5 mm; J 1 cm.

root epidermis, sterile parts remained cream-white (Fig. 3F–H). In contrast, *D. tabescens* only formed loosely aggregated mycelia on the surface of the protocorms (Fig. 3E) and no internal rhizomorphs were visible within the rhizomes (Fig. 3I). *TK-10* formed whitish internal rhizomorphs beneath the epidermis near the base of protocorms and in the roots of developing rhizomes and seedlings (Fig. 5A–D; Stage 3–5), but these were not as organized as those formed by *A. gallica* (Fig. 4D), with no clear differentiation between outer rind and central cavity (Fig. 5D).

Both *A. gallica* and *TK-10* continued growing on PDA in Petri dishes underneath the sheet of plastic wrap, but initially they failed to form linear mycelial organs. Only once their hyphae had grown beyond the plastic wrap and thus become exposed to the air did they firstly begin vigorous production of aerial mycelia and then commence formation of linear organs beneath the center of the sheet (Fig. 5E, F). Whereas the linear organ of *A. gallica* was dark and smooth, that of *TK-10* was white and rough (Fig. 5E, F).

3.5. Wood-rotting ability

Values for weight loss in the *Fagus crenata* blocks attributable to wood rotting by *A. gallica* and *TK-10* are shown in Table 2. These indicate that the wood-rotting ability of *TK-10* was three times greater than that of *A. gallica* in the culture condition. A small degree of weight loss was also documented in the control blocks (0.404 g, ca. 5.9%), which may reflect a reduction in water-soluble extractives.

4. Discussion

4.1. Cyrtosia septentrionalis can associate with Armillaria and Desarmillaria mycobionts through successive life history stages, but Physisporinus mycobiont (TK-10) enhances early growth

The current study corroborates earlier findings by Umata et al.

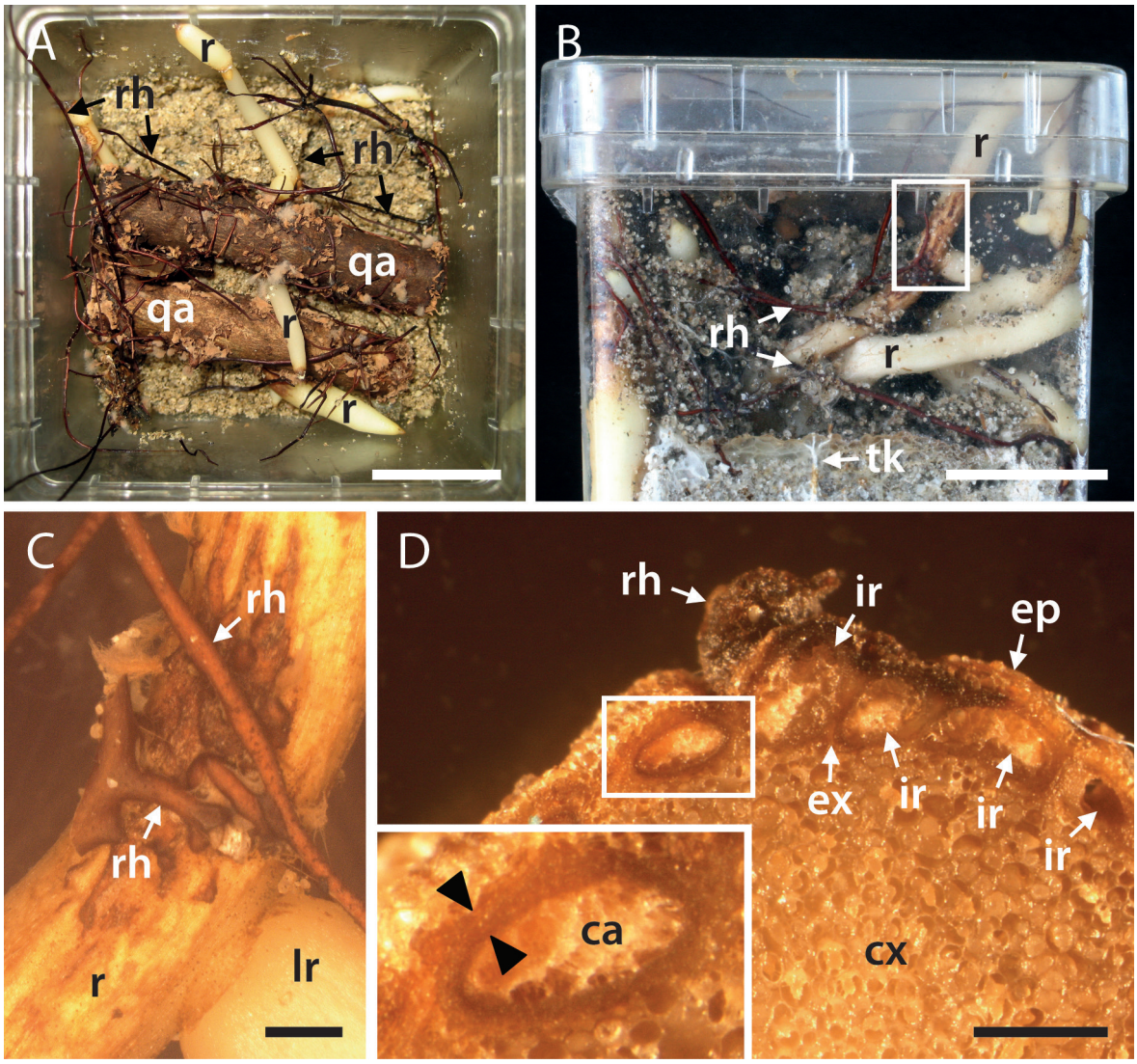

Fig. 4. Rhizomes of *Cyrtosia septentrionalis* previously colonized with *Physisporinus* sp. *TK-10* that have been subsequently co-cultured with *Armillaria gallica*. **A**: Sticks of *Quercus acutissima* (qa) colonized with *A. gallica* placed next to a *C. septentrionalis* rhizome at Stage 4 previously grown with *TK-10*. Roots (r) and rhizomorphs of *A. gallica* (rh) are indicated. **B**: *Armillaria gallica* rhizomorphs (rh) successfully connecting with roots (r) developing on the rhizome grown with *TK-10* (tk). **C**: Magnification of the square in B showing external rhizomorphs of *A. gallica* (rh) connecting with *C. septentrionalis* roots (r). A lateral root (lr) is indicated. **D**: Cross-section of the root in C showing an external rhizomorph (rh) attached to the root and several internal rhizomorphs of *A. gallica* (ir) that have formed between the epidermis (ep) and exodermis (ex). Inset is a magnification of the single internal rhizomorph in the white box showing a thick outer rind (arrowheads) encircling a central cavity (ca). *Bars*: A, B 2 cm; C 2 mm; D 1 mm.

(2013, 2021) that *A. gallica*, *A. mellea* subsp. *nipponica* and *D. tabescens* can all induce seed germination in *C. septentrionalis* without direct colonization. Additionally, our research reveals that all of the tested mycobionts can colonize germinated seeds. Such colonization fosters subsequent growth into rhizomes and seedlings, thereby indicating the potential for the orchid to form enduring mycorrhizal associations through successive life history stages. Collectively, our findings strongly suggest that the three *Armillaria* and *Desarmillaria* species can colonize aseptically germinated seeds, thereby stimulating the growth of a small proportion of them (1.5–2.6%) into protocorms. This interaction contrasts with the mechanism observed in *G. elata*, where a shift in mycobiont is required for developmental progression from juvenile to emergent plant (Park et al., 2012).

Although we were unable to detect successful colonization by *A. mellea* subsp. *nipponica* in the five randomly sampled germinated seeds, this absence may be attributable to the limited sample size. As this mycobiont has previously been shown to be capable of facilitating orchid growth into rhizomes and seedlings (Terashita, 1985), it seems plausible that *A. mellea* subsp. *nipponica* can colonize aseptically germinated seeds and promote early myco-heterotrophic growth.

Nevertheless, the *Physisporinus* mycobiont exhibited greater affinity for the orchid during early plant growth stages, proving capable of colonizing and successfully establishing a symbiotic relationship with all germinated seeds examined. Furthermore, our results demonstrate that this leads to a higher likelihood of successful onward growth, with 16.2% of aseptically germinated seeds progressing into protocorms. We infer that the *Physisporinus* mycobiont enhances more vigorous development during seed germination, protocorm, and rhizome growth stages. In contrast, the notably lower rate of mycorrhizal establishment and the ensuing limited

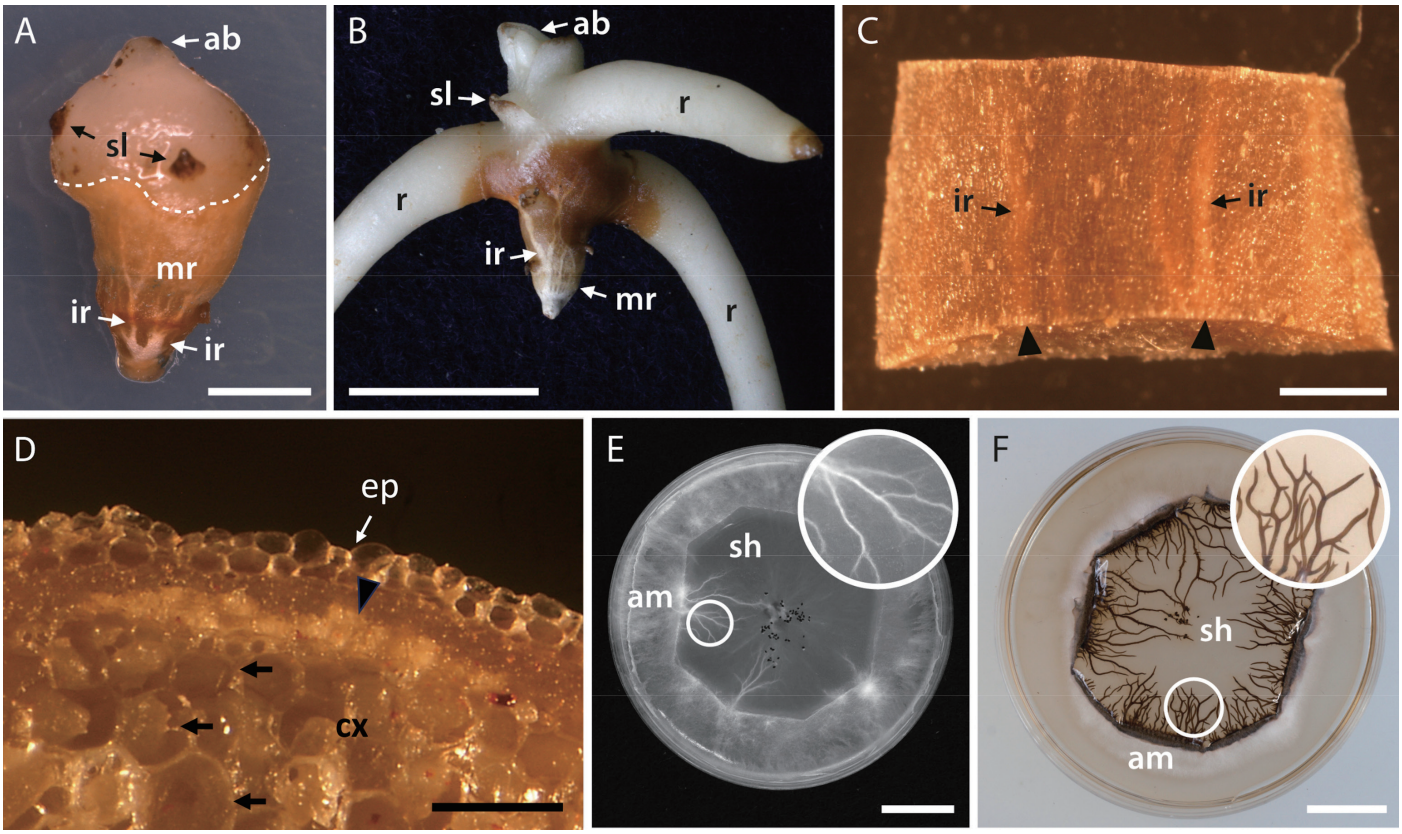

Fig. 5. *Physisporinus* sp. *TK-10* internal rhizomorph formation inside *Cyrtosia septentrionalis*. **A**: Internal rhizomorphs of *TK-10* (ir) beneath the epidermis at the base of a Stage 3 protocorm. An apical bud initial (ab) and two scaly leaf initials (sl) are indicated. The dotted line indicates the boundary between sterile and colonized parts of the main root initial (mr). **B**: Internal rhizomorphs of *TK-10* (ir) beneath the epidermis of a main root (mr) of a seedling at Stage 5. The apical bud (ab), scaly leaves (sl), main root (mr) and adventitious roots (r) are indicated. The brown colored portion clearly demarcates the colonized portion from the sterile white parts. **C**: Surface view of an adventitious root colonized by *TK-10*, showing faint white internal rhizomorphs (ir) beneath the epidermis. The arrowheads indicate the positions of the two rhizomorphs shown in D. **D**: Cross-section of the root shown in C with one flat internal mycelial cord (arrowheads) (right arrowhead in Fig. 5C) visible beneath the epidermis (ep). The organization of this rhizomorph is not as elaborate as that of *A. gallica* shown in Fig. 4D. Cells in the cortex (cx) are filled with round pelotons (arrow). **E**, **F**: Rhizomorphs of *TK-10* (E) and *A. gallica* (F) beneath a sheet of plastic wrap (sh) with aerial mycelia (am) growing vigorously outside the sheet. Inset are magnifications of the smaller circles. *Bars*: A 2 mm; B, E, F 2 cm; C 0.2 mm; D 1 mm.

Table 2. Weight loss in *Fagus crenata* wooden blocks following culture with either *Armillaria gallica* or *Physisporinus* species '*TK-10*' for 4 wk at 23 ºC. Different letters in a row indicate significantly different values ($p = 0.01$, multiple Tukey's test).

	A. gallica	n	*TK-10*	n	Control	n
Weight before (g)[(1)]	6.868 ±0.106	13	6.828 ±0.130	9	6.900 ±0.062	5
Weight loss (g)	0.589 ±0.043 [a]	13	0.934 ±0.150 [b]	9	0.404 ±0.112	5
Actual weight loss (g)[(2)]	0.185 ±0.043 [a]	13	0.530 ±0.150 [b]	9	-	-

[(1)] Initial values did not differ significantly ($p = 0.01$, Student's t-test).
[(2)] The actual weight loss caused by *A. gallica* and *TK-10* was calculated by firstly subtracting 0.404 g (i.e., the mean weight loss observed in the control treatment) from the end weight of each block. Weight loss in the control blocks might be due to removal of water-soluble extractives (Kuriyama, 1967).

progression achieved by *Armillaria* and *Desarmillaria* species indicates a weaker ability to promote growth. However, *Armillaria* and *Desarmillaria* species enhanced more onward growth of the protocorms (7.1–10%) than germinated seeds (1.5–2.6%). Indeed, Terashita (1985) reported that *A. mellea* subsp. *nipponica* enhanced the development of one in six rhizomes into seedlings (i.e., a 16.7% success rate). These finding suggest that there is a close relation between successful symbiosis establishment and plant growth stage. Further research is required to shed light on the mechanisms underlying these differences, considering both variable mycobiont complementarity and the influence of specific plant growth stages on the outcome of the plant–mycobiont interaction.

4.2. An emerging mechanism for Armillaria and Desarmillaria colonization of adult C. septentrionalis plants

Previously, we hypothesized that direct physical contact between a root of *C. septentrionalis* colonized by the *Physisporinus* mycobiont and a mycelium of *A. gallica* in its rhizosphere is sufficient to induce a mycobiont shift (Umata et al., 2022). However, we were unable to specify a mechanistic basis for the interaction between *A. gallica* and other *C. septentrionalis* mycobionts. Here, we reveal that *A. gallica* can displace the *Physisporinus* mycobiont as the dominant mycobiont through secondary colonization of root tissue and subsequent internal rhizomorph formation, although it is probable that, because *A. gallica* colonized a sterile root in the present investigation, both mycobionts persist within the same in-

dividual. In this regard, the mycorrhizal association in *G. elata* is similar: protocorms of this orchid colonized with *Mycena osmundicola* exhibited onward growth to the rhizome stage when placed near pieces of wood infected with a known *Armillaria* mycobiont (Park et al., 2012). In fact, Smith and Read (2008) hypothesized that mass nutrient supply from *A. mellea* is essential for the onward growth of *G. elata* protocorms. This model, which entails a shift to a mass-nutrient supplier that both enables onward growth to successive life history stages and supplants an existing mycobiont, was also invoked in stimulating the development of *Cephalanthera falcata* (Thunb.) Blume, a partial mycoheterotrophic orchid in which seedlings are predominantly colonized by *Thelephoraceae* fungi and adults by members of the *Sebacinales* to induce growth into giant individuals in *situ* (Yamato et al., 2021). Interestingly, the *Sebacinales* fungi examined in that study were inferred to possess a greater capacity for carbon transfer. Conversely, the present study reveals that the *Physisporinus* mycobiont has a significantly greater wood-decaying potential than *A. gallica*, and previous research also has shown it to have a wood-rotting capacity more than double that of *D. tabescens* (Umata et al., 2013). By inference, the *Physisporinus* mycobiont ought therefore to be capable of supplying more carbon to the orchid than either of the other two mycobionts.

However, more recently, Umata and Gale (2023) argued that inoculum potential *sensu* Garrett (1960) regulates the establishment of a mycorrhizal association in *Erythrorchis altissima* (Blume) Blume (syn. *E. ochobiensis*), a perennial, climbing hemi-epiphytic fully MH orchid (Comber, 1990) which associates with wood-rotting fungi (Ogura-Tsujita et al., 2018; Umata et al., 2000). When protocorms of this species that had previously been grown with either of two mycobionts (denoted R204 or R374) on beech blocks of varying density, the ratio of successful mycorrhizal establishment with the mycobionts declined in direct proportion to a decrease in initial wood density (Umata & Gale, 2023). Accordingly, they suggested that continuous wood decay could lead to nutrient supply shortages, thereby bringing about the death of the plant. In a field investigation on *E. altissima*, Ogura-Tsujita et al. (2021) suggested that excessive wood decay leads to the death of this orchid, presumably as a consequence of a mycobiont-mediated nutrient supply shortage. Analogously, we infer that a *C. septentrionalis* plant colonized by *TK-10* is likely to decline and die sooner owing to its mycobiont's greater propensity for wood decay, thereby causing more rapid exhaustion of its carbon source, unless secondarily colonized by a suitable *Armillaria* or *Desarmillaria* partner. Indeed, Umata et al. (2022) proposed that the relatively low frequency at which the *Physisporinus* mycobiont is encountered in adult *C. septentrionalis* plants in the wild is likely to be a result of the mycobiont's high wood-decay rate. In contrast, *Armillaria* species tend to form vast colonies over large areas by forming rhizomorph networks and live for many years (Anderson et al., 1979; Ferguson et al., 2003; Korhonen, 1978). For example, *A. gallica* (syn. *A. bulbosa*) can occupy a habitat of 15 ha, weigh over 10,000 kg and live for more than 1,500 y although its genet is suspected to spread fragmentary (Smith et al., 1992). Whereas *D. tabescens* does not form rhizomorphs in nature (Koch et al., 2017), it may persist for over 4 y in tree roots or butts after colonizing them, and it is believed to extend its mycelia into soil and infect neighboring tree roots or butts, thereby spreading disease more widely (Furukawa et al., 2015). *Cyrtosia septentrionalis* has been observed co-occurring with a large colony of this fungus in nature (Kaneko et al., 1998). From the perspective of *C. septentrionalis*, this would make it an ideal partner for sustained, long-term nutrient supply.

On the other hand, *G. elata* can interact with a broad range of fungi beyond *Mycena* species and this orchid may undergo several mycobiont shifts through successive growth phases until forming a stable interaction with an *Armillaria* species (Chen et al., 2019). This suggests that other kinds of mycobiont can also colonize juvenile plants of *C. septentrionalis* besides the *Physisporinus* mycobiont, although further research is needed to corroborate this.

4.3. Internal rhizomorph formation

Our observation that the *Physisporinus* mycobiont can form internal linear mycelial organs beneath the epidermis of *C. septentrionalis* protocorms, rhizomes and seedlings is the first confirmation of this ability beyond *Armillaria* species (Hamada, 1939; Terashita, 1985; Umata et al., 2022). Crucially, these structures conform to the definition of a rhizomorph as specified by Rayner et al. (1985), whose key criterion was a linear organ with an apically dominant growing tip. In fact, other *Physisporinus* species can form rhizomorphs in nature (Shino et al., 2022). Based on these findings, we re-examined images of *C. septentrionalis* plants colonized by the *Physisporinus* mycobiont in material studied in our previous investigations, and confirmed the presence of these organs in both protocorms (Umata et al., 2013) and roots (Umata et al., 2022).

The linear organ transports nutrients and water toward its growing tip (Gray et al., 1996; Yafetto, 2018). *Armillaria* species are known to form the most morphologically and functionally complex rhizomorphs among the mycelial cord-forming basidiomycetes (Baumgartner et al., 2011) with a distinct cell layer forming an outer rind that functions in apical or lateral growth (Motta, 1969) and a central cavity for gas exchange (Pareek et al., 2006). The present study revealed that *A. gallica* even forms rhizomorphs within protocorms, suggesting that this mycobiont may be active in mass nutrient transfer from a very early stage of plant development. In contrast, *D. tabescens* was only able to form a relatively loose aggregation of mycelia on the surface of the protocorm and did not form the internal rhizomorphs, which could reflect physiological properties of this fungus as described above. The rhizomorph formed by the *Physisporinus* mycobiont was structurally simple in terms of tissue organization, with no clear differentiation between the outer rind and central cavity. This may indicate that the *Physisporinus* mycobiont has a lower capacity for nutrient transfer as compared with *A. gallica*. Even so, both the *Physisporinus* mycobiont and *A. gallica* produced rhizomorphs under plastic wrap, suggesting that these mycobionts are destined to form these organs when contained within the plant epidermis. However, in our experiments, these species only form rhizomorphs once their hyphae had grown beyond the plastic wrap, suggesting that aeration is needed for rhizomorph initiation. Similarly, rhizomorph initiation was not triggered in a submerged inoculum of *A. mellea* until its mycelium had reached the substrate-air interface (Snider, 1959; Worrall et al., 1986). How *C. septentrionalis* detects, selects and exerts an influence over either rhizomorph-forming fungus as an active mycorrhizal partner remains unclear, but the potential size, structure, nutritional mode, and ecology of rhizomorph-forming *Armillaria* species certainly appear to make them the most suitable mycobiont for this giant, structurally complex orchid. This may be one of the key reasons why *Armillaria* species tend to serve as the ultimate mycobiont in this species. On the other hand, further investigation for the non-rhizomorph forming *D. tabescens* will be needed to resolve how this fungus can supply nutrients to its orchid host.

Conclusion

We conclude that both *Armillaria* and *Desarmillaria* mycobionts can associate with *C. septentrionalis* from germinated seed to

adult plant, but that *TK-10* confers higher rates of germination induction and onward growth during rhizome and seedling growth stages. *Armillaria* species (in particular *A. gallica*) are capable of secondarily colonizing plants with an established relationship with *TK-10*, and the latter may be superseded within the plant in the process. However, *TK-10* exhibits a greater capacity for wood decomposition than *A. gallica* and may thus can support higher levels of mass nutrient supply, but this may lead to nutrient supply shortages and, conceivably, early death of this plant. Secondary colonization by a competent *Armillaria* or *Desarmillaria* species appears to be critical in overcoming this disadvantage. Overall, the highly organized, rhizomorph-forming *Armillaria* species appear to represent the most reliable, long-term partner for *C. septentrionalis*. However, further investigation for the non-rhizomorph forming *D. tabescens* will be needed to resolve how this fungus can supply nutrients to the orchid.

Disclosure

The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of Japan.

Acknowledgements

The authors thank Mr. K. Maruno, Mr. S. Chuman and Mr. M. Nishi for their help in collecting *Cyrtosia septentrionalis* fruit capsules, and Mr. T. Yamamoto at the Graduate School of Science, Kyoto University, for technical support in preparing sections of *Cyrtosia* protocorms. This work was partially supported by grant - in - aid from the Japan Society for the Promotion of Science (KS: 15K18470).

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