論　文

Inoculum potential of mycobiont regulates the establishment of mycorrhizal symbiosis with *Erythrorchis altissima*, a fully myco-heterotrophic orchid

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Summary

The achlorophyllous orchid *Erythrorchis altissima* establishes a mycorrhizal association with wood decaying fungi. In the present investigation, we examined the relationship between symbiosis establishment and wood decay. Protocorms of the orchid previously grown symbiotically with two kinds of wood decaying mycobiont, R204 or R374, were cultured on beech cubes (2 cm long sides), of which dry weight varied from 0.62–0.80 g/cm^3. The following results were obtained: (i) the ratio of successful symbiosis establishment (that is, onward growth of protocorm) was positively proportionate to wood volume density, (ii) for achievement of a success rate of 50%, volume density was needed ≥ 0.675 g/cm^3 for R204 and ≥ 0.65 g/cm^3 for R374, respectively, and (iii) the decline in volume density as a result of decay brought about by either mycobiont was positively proportionate to initial volume density and greater under co-culture as compared with mono-culture conditions. These results suggest that (i) there is a positive proportional relationship between symbiosis establishment and wood volume density, (ii) inoculum potential (IP) of mycobiont regulates symbiosis establishment, (iii) continuous wood decay should decrease IP, thereby leading to plant death, and (iv) symbiosis establishment increases the wood decay capacity of the mycobiont.

Keywords: Achlorophyllous orchid, Co-culture, Symbiosis establishment, Wood decay, Wood volume density

1. Introduction

Orchid plants establish symbiotic associations with Basidiomycetous or Ascomycetous fungi to form mycorrhizae, in which the mycobiont enters root cortical cells and forms pelotons, supply the host with water and nutrients, including C-sources (Smith and Read 2008). If the orchid remains achlorophyllous even after the production of aerial shoots (i.e. is fully myco-heterotrophic, MH), the orchid must continue to receive nutrients from its mycobiont in order to sustain growth and development from seed into adulthood (Leake 1994). Fully MH orchids associate with saprophytic or ectomycorrhizal fungi. For example, *Cyrtosia septentrionalis* (syn: *Galeola septentrionalis*), a perennial terrestrial orchid native to temperate East Asia, associates with wood decomposing *Armillaria* species (Hamada 1939; Cha and Igarashi 1995, 1996; Terashita 1985, 1996; Terashita and Chuman 1989) and *Physisporinus* species (Umata et al. 2013), whereas *Cephalanthera austinae* and *Corallorhiza maculata* associate with ectomycorrhizal fungi belonging to the Thelephoraceae and Russulaceae, respectively (Tayler and Bruns 1997).

Erythrorchis altissima (syn. *Galeola altissima*, *E. ochobiensis*), a perennial, vine-like, fully MH orchid, climbs up the trunk of forest trees, particularly that of *Castanopsis sieboldii* subsp. *sieboldii* (Fig. 1A; Umata et al. 1994). The orchid's stem can attain up to ca. 36 m long (Makino and Nemoto 1931) and it produces flowers from May to June and a cluster of fruits from October to December (Fig 1 A, B). The orchid associates with many kinds of wood decaying fungi (Hamada und Nakamura 1963; Umata 1998; Umata et al. 2000; Ogura-Tsujita et al. 2018) and even with monokaryotic

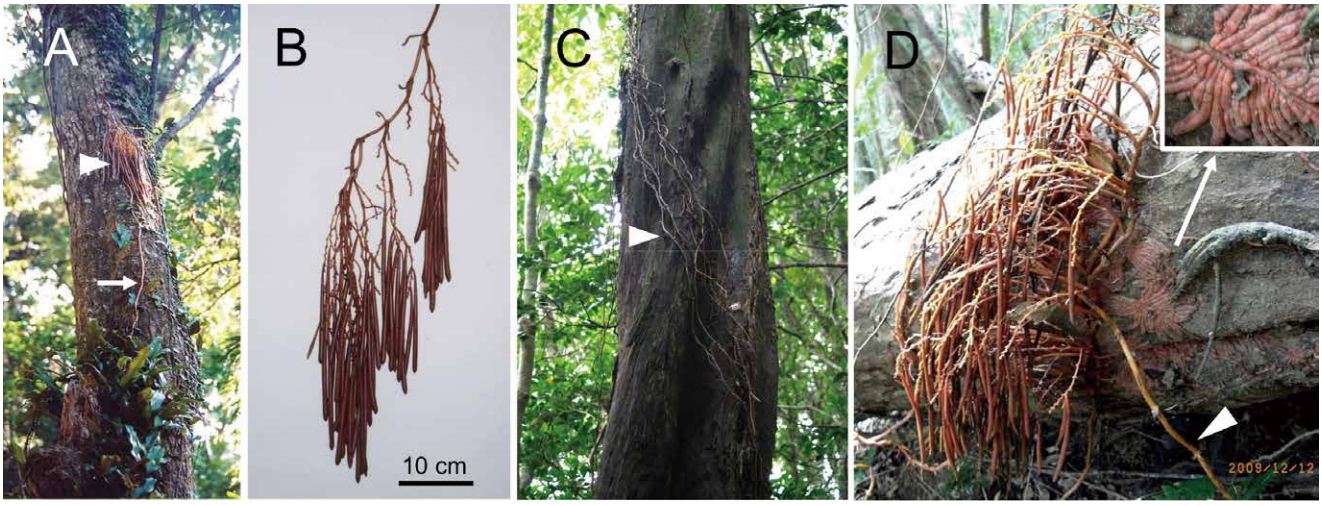

Fig 1. *Erythrorchis altissima* growing in natural forest on Tanegashima Island, Southwest Japan.

A: *Erythrorchis altissima* climbing up an individual of *Castanopsis sieboldii* subsp. *sieboldii* in October 1995. White arrowhead and arrow indicate a cluster of seed capsules and a stem, respectively.

B: Close-up of the seed capsules shown in A.

C: A dead individual of *Erythrorchis altissima* (white arrowhead) on a dead host tree, the bark of which is peeling off in November 2002. The tree collapsed the following year.

D: Another *Erythrorchis altissima* individual with a cluster of seed capsules on a fallen trunk of *Distylium racemosum* in the same forest in December 2009. The inset image shows many mycorrhizal roots adhering to the host trunk and branching out extensively from the stem marked with a white arrowhead in the main image. This structure is termed a root-mat ("Wurzelmatte"). This individual orchid was found dead three years later.

isolates of these fungal species (Umata 1999, 2013). In the course of our field surveys on Tanegashima Island, the northernmost distribution of this orchid in Kagoshima Prefecture, southwestern Japan, we found a dead individual of the orchid on a dead individual of *C. sieboldii* subsp. *sieboldii* (Fig. 1C). Curiously, however, this orchid was inferred to have been alive the previous year, as evidenced by the presence of recently dehisced fruits, and the tree had also been seen alive several years previously (Fig 1A). Because the orchid normally grows on dead wood and forms mycorrhizal roots which branch out extensively, a structure named "Wurzelmatte" (root-mat) (Fig. 1D; Hamada und Nakamura, 1963), we were unable to understand how the orchid died. Recently, Ogura-Tsujita et al. (2021) found that many stems of *E. altissima* on well-decayed wood were dead whereas others on as-yet only partially decayed wood were still alive.

From these events, we infer that symbiosis establishment may play a role in the progression of wood decay and that the progression of wood decay by mycobionts may in turn cause the death of the orchid. In order to explore this hypothesis, we sought to examine the relationship between wood decay and the establishment of symbiosis in *E. altissima* under co-culture conditions.

2. Materials and methods

2-1. Orchid seeds

Mature seeds were obtained from fruits of a single individual of *E. altissima* growing on a fallen trunk of *Distylium racemosum* in a natural broadleaved evergreen forest on Tanegashima Island, Kagoshima Prefecture, Japan, in December 1995 and 2009 (Fig. 1B, D). The seeds were stored in silica gel at 1–5 °C until used in the following experiments.

2-2. Fungal mycobionts

Two species of fungi, Isolates R204 and R374, were used. Both were obtained from the roots of adult *E. altissima* following the methods of Warcup and Talbot (1967). Isolate R204 was reported to induce seed germination and enhance onward growth of this orchid (Umata 1998; Umata et al. 2000) while Isolate R374 has not been investigated for symbiotic ability.

2-3. Preparation of mycorrhizal protocorms

The seeds were firstly sterilized in 75% ethanol for 1 min and then washed in a 10% solution of calcium hypochlorite for 20 min. They were then rinsed three or four times in sterile distilled water and air-dried aseptically for at least 4 hrs. The seeds were then attached to sterilized bamboo needles (4 mm diam × 5 mm long)

A sawdust medium was prepared for the co-culture experiments. The medium was composed of 300 g of air-dried beech sawdust and 150 g of raw rice bran suspended in 1,000 ml of tap water. This mixture was autoclaved for 60 min at 121 °C. Each isolate was inoculated in a 200 ml flask containing about 70 g of the medium and incubated at 25 °C for 3–4 wks. The needles bearing the seeds were planted into the medium in the flasks, which were

Table 1. Number per wood volume density of beech cube (2×2×2 cm) prepared for co-culture with R204 and R374.

	Volume density (g/cm^3)					
Isolate	<0.65	0.65–<0.675	0.675–<0.7	0.7–<0.725	0.725–<0.75	≥0.75
R204	16	11	9	8	6	6
R374	16	8	13	2	5	6

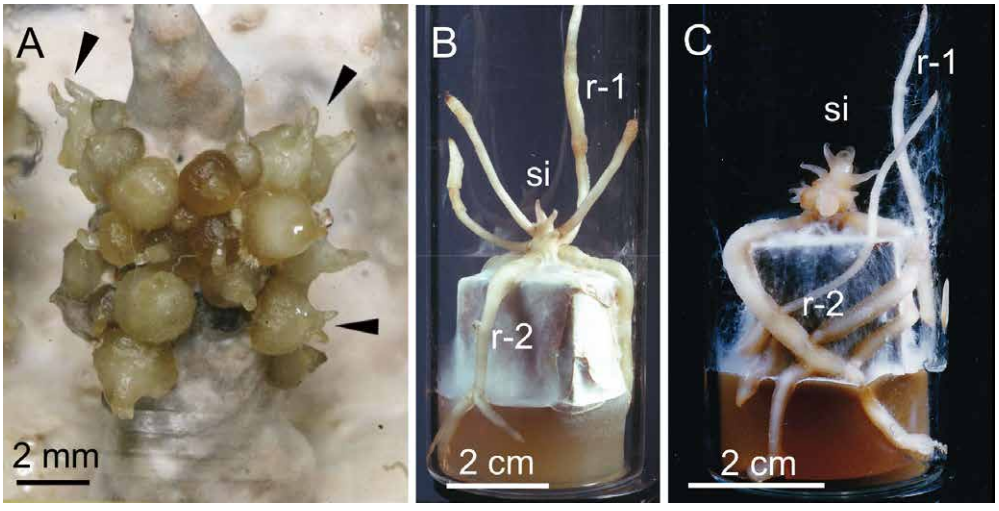

Fig 2. Culture of *Erythrorchis altissima* protocorms grown with mycobionts R204 and R374 on beech wood cubes (2×2×2 cm).

A: Protocorms grown with R374 with apical bud initials at their apex (black arrowheads), after 10 wks incubation at 27.5 °C in the dark.

B, C: Successful onward growth of protocorm into seedling on beech wood cube with mycobiont R204 (B) and R374 (C) after 13 wks incubation at 23 °C in the dark. The seedling have produced a stem initial (si), aerial roots (r-1), and adhering roots (r-2).

then incubated at 27.5 °C in darkness, with protocorms being obtained after 10 wks (Fig. 2A). The dimensions and volume of the protocorms were as follows: 2.5 ± 0.4 mm wide by 2.8 ± 0.6 mm height and 10.2 ± 5.6 mm^3 for isolate R204 (n=16), and 2.7 ± 0.6 mm wide by 3.0 ± 0.7 mm height and 12.6 ± 7.1 mm^3 for isolate R374 (n=16). There was no significant difference in the size and volume of the protocorms obtained from either mycobiont (Student's t-test, $P<0.01$).

2-4. Culture of mycorrhizal protocorms on beech wood

Sterilized 200 ml flasks containing 100 ml of 1% agar medium composed of a modified Mori solution were prepared as detailed previously (Umata 1997, 1998), with the addition of 2% mannitol and 0.5% Difco yeast extract to form the basal medium (BM). R204 and R374 were then inoculated onto this agar medium and the flasks were incubated at 27.5 °C for 2 wks in darkness. In addition, a wood cube (2×2×2 cm) of beech (*Fagus crenata*) was immersed in liquid BM without a C-source for around 16 hrs, autoclaved at 121 °C for 1 hr, and then allowed to cool to room temperature. These wood cubes were then placed onto the agar BM medium overgrown with the mycobiont in the flasks. Finally, one protocorm growing with either R204 or R374 was introduced onto one wood cube and then incubated at 23 °C for 13 wks in darkness. The wood was oven-dried at 105 °C for 48 hrs before examination and number per volume density (g/cm^3) of wood prepared for co-culture with R204 and R374 was shown in Table 1.

2-5. Measurement of volume density decline in beech wood cubes as a result of wood decay

The same methods described in section 2–4 were repeated but a protocorm was not introduced into the flasks. The wood was oven-dried at 105 °C for 48 hrs pre- and post-incubation and weighed for each fungus. The difference in weight loss in the beech cubes as a result of wood decay by either fungus was tested using Student's t-tests.

2-6. Assessment of successful symbiosis establishment

Successful symbiosis establishment was defined as the point when inoculated protocorms exhibited signs of onward growth (Fig. 2B, C), whereas failure was defined as when the protocorm either died or showed no signs of growth after 13 wks.

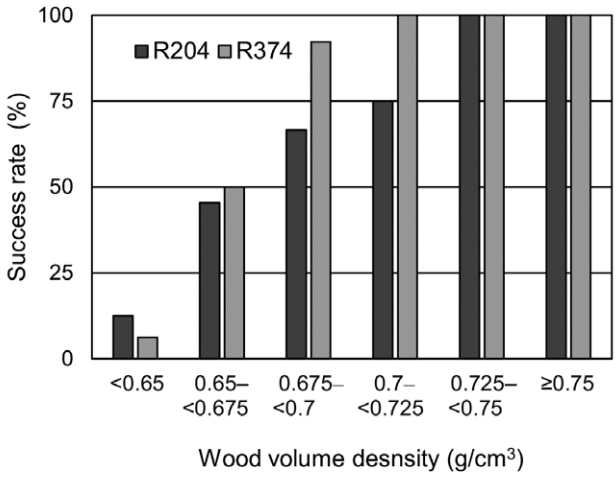

Fig 3. Volume density of beech wood and successful onward growth of protocorms of *Erythrorchis altissima* with Isolate R204 and R374, respectively, after 13 wks incubation at 27.5 °C in the dark.

3. Results

3-1. Wood volume density and symbiosis establishment

Fig. 3 shows the relationship between wood volume density (g/cm^3) and symbiosis establishment with R204 and R374. As volume density increased, the frequency of successful establishment increased for both mycobionts. When the success rate reached 50%, volume density was 0.675–0.7 g/cm^3 for R204 and 0.65–0.675 g/cm^3 for R374, respectively.

3-2. Declines in wood volume density under the mono-culture and co-culture conditions

The declines in volume density achieved by R204 and by R374 was significantly larger under co-culture conditions as compared with mono-culture conditions (Fig. 3).

4. Discussion

4-1. Inoculum potential regulates the symbiosis establishment

The present investigation revealed that there is a positive proportional relationship between successful symbiosis establishment and initial wood volume density in *Erythrorchis altissima*. Further, a minimum threshold volume density is needed for successful establishment, with a volume density of ≥ 0.675 g/cm^3 required for a 50% success rate in the case of R204, and ≥ 0.65 g/cm^3 required in the case of R374. These findings indicate that volume density is a crucial factor regulating the establishment of mycorrhizal symbiosis between *E. altissima* and its mycobionts. Therefore, progression of wood decay, and associated decrease in volume density, is inferred to lead to death of the orchid. We suspect that the death of the orchid observed in the wild on Tanegashima was due to the extensive decay of the *C. sieboldii* subsp. *sieboldii* host tree by wood-decaying mycobionts and a similar cause is implicated for the events described by Tsujita et al. (2021).

Garrett (1960) defined inoculum potential (IP) as the amount of energy required for a fungus to infect its host at the site of infection. Availability of nutrients directly affects pathogen IP. In the present investigation, volume density is a proxy for resource availability for the fungus, and we therefore hypothesize that mycobiont IP regulates the establishment of mycorrhizal symbiosis. Our hypothesis is supported by several other reports, including, for example, the observation that the symbiotic growth of protocorms of *Dactylorhiza purpurella* with *Thanatephorus* (= *Rhizoctonia*) species on agar medium slowed down and eventually ceased, even though the protocorms underwent dramatic onward growth when cellulose was added on the medium (Hadley and Williamson 1971). Further, higher cellulose levels were found to increase the inoculum potential of *Thanatephorus cucumeris* (= *Rhizoctonia solani*), resulting in the increased severity of damping-off in radish (Chung et al. 1988), and successful establishment of dipterocarp seedlings required that soil at the site contained an adequate amount of ectomycorrhizal inoculum prior to out planting (Ingleby et al. 2000). In addition, Sauvageau et al. (2019) reported that an inoculum density of 97 propagules of *Pythium tracheiphilum* per gram of dry soil was required to cause infection on lettuce cultivar Estival, whereas an inoculum density of 46 propagules was sufficient on cultivar Prestige.

4-2. Symbiosis establishment and wood decay

It is generally understood that two kinds of achlorophyllous plants have lost photo-autotrophic ability and that they must therefore obtain nutrients from other organisms. Firstly, parasitic plants invade the aerial parts or underground roots of their host plants to obtain water, carbohydrates and minerals (Yoder 1999; 2001), thereby causing considerable damage. For example, *Aeginetia indica* (Orobanchaceae), a parasite on Gramineae species including *Saccharum officinarum* (sugar cane) and *Miscanthus sinensis*, significantly reduced sugar crop in sugar cane (Kusano 1908), and the obligate root hemiparasite *Striga* species (also Orobanchaceae) decimate agriculture in much of Africa and parts of Asia, attacking major cereal grains and legumes (Ejeta and Gressel, 2007). Secondly, mycotrophic plants such as the orchid *E. altissima* associate with wood-decaying fungi as described here. However, in this case, it remains obscure exactly what impacts the plant exerts on its mycobiont host. In the present investigation, we demonstrated that decline in volume density is significantly greater under co-culture conditions as compared with mono-culture conditions, indicating that symbiosis establishment enhances the wood decay capacity of the mycobiont. This finding is reasonable, because some amount of the wood must be utilized by the orchid as nutrients for growth over and above that used by the mycobiont, suggesting that mycorrhizal symbioses play a crucial role in nutrient cycling in nature.

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共生菌の感染ポテンシャルが全菌従属栄養植物タカツルランの菌根共生の成立を調節する

馬田　英隆・ステファン ウイリアム ゲイル

要　旨

無葉緑植物タカツルランは木材腐朽菌と菌根共生を成立する。本研究では，菌根共生の成立と木材腐朽との関係を調べた。予め２種類の共生菌 R204と R374（いずれも木材腐朽菌）のそれぞれで育てたプロトコームをブナ材（2×2×2 cm）上で培養した。ブナ材の容積密度は0.62 ～ 0.80 g/cm^3 であった。次のような結果が得られた。(i) 共生成立の割合，すなわち，プロトコームが成長を示した割合はブナ材の容積密度（g/cm^3）に正比例した。(ii) 成立割合が50％に必要なブナ材の初期容積密度は R204で0.675 g/cm^{3}以上，R374で0.65 g/cm^{3}以上であった。(iii) 二つの共生菌の腐朽によるブナ材の重量損失量はブナ材の容積密度に正比例し，いずれの共生菌においても二者培養のほうが一者培養よりも多かった。これらの結果から，以下のようなことが考えられる。（１）共生の成立と木材の容積密度との間には正の比例関係がある。（２）共生菌の感染ポテンシャル（IP）は共生の成立を調節する。（３）継続する木材腐朽は IP を低下させ，タカツルランを死に至らしめる。（４）共生は共生菌の木材腐朽能を増加させる。

キーワード：共生培養，共生の成立，無葉緑ラン，木材腐朽，木材容積密度

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