Mycoscience 40: 367–371, 1999

Short Communication

Germination and growth of *Erythrorchis ochobiensis* (Orchidaceae) accelerated by monokaryons and dikaryons of *Lenzites betulinus* and *Trametes hirsuta*

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Accepted for publication 28 May 1999

Four sib-monokaryons and two reconstituted dikaryons of two basidiomycetes, *Lenzites betulinus* and *Trametes hirsuta*, accelerated the seed germination of *Erythrorchis ochobiensis*, an achlorophyllous orchid. All isolates of *L. betulinus* and three isolates of *T. hirsuta* induced the development of plants from germinated seeds. Although three monokaryotic isolates of *T. hirsuta* failed to induce the development of plants, the reconstituted dikaryons induced the development.

Key Words——achlorophyllous orchid; dikaryon; monokaryon; orchid mycorrhiza; seed germination.

About 90% of basidiomycetous fungi are known to be heterothallic and to be tetra- or bipolar (Whitehouse, 1949a, 1949b). In the tetrapolar species, four different types of monokaryons and two different types of dikaryons are produced from a dikaryotic parent as a result of sexual production. These genetically different types may interact differently in their mycorrhizal association with a host plant. The variability in ectomycorrhizal formation among monokaryons and reconstituted dikaryons has been investigated by coculture, through which considerable information has been obtained (e.g. Debaud et al., 1995; Kropp and Anderson, 1994; Rosado et al., 1994). The ability of monokaryons to form ectomycorrhizas varies with the species of fungus (Debaud et al., 1988; Kropp et al., 1987; Kropp and Fortin, 1988; Wong et al., 1989). In orchid mycorrhizas, the symbiotic mycorrhizal association varies not only with the species of fungus but also among isolates of the same species (e.g. Alexander and Hadley, 1983; Masuhara and Katsuya, 1991; Umata, 1997, 1998). However, no information has yet been reported from cocultures using monokaryons and reconstituted dikaryons. In this investigation, cocultures were carried out in vitro between *Erythrorchis ochobiensis* (Hayata) Garay, an achlorophyllous orchid, and sib-monokaryons and reconstituted dikaryons of *Lenzites betulinus* (L.: Fr.) Fr. and *Trametes hirsuta* (Wulfden: Fr.) Pilát. The purposes of this investigation were, (i) to determine whether monokaryons show symbiotic association in the orchid mycorrhiza, and (ii) to assess inter- and intraspecific variation in the mycorrhizal symbiosis among isolates.

About 50 capsules of the orchid were collected at Kutinoerabujima Isl., Kagoshima Prefecture, and seeds from the capsules were mixed well and refrigerated after air-drying.

Basidiomes of *L. betulinus* and *T. hirsuta*, both of which are tetra-polar (Nobles, 1965), were collected on the same dead wood of *Prunus serrulata* var. *spontanea* Makino at Takakuma Experimental Forest of Kagoshima University Forests. Neither polypore has been observed to colonize roots of *E. ochobiensis* in the field or been examined for its symbiotic potential with the orchid. Basidiospores obtained aseptically from a single basidiome were inoculated on a plate of malt extract agar medium (MEA) and incubated at 25°C. After 4 d of incubation, about 15–20 germinated spores of each fungus were transferred to fresh MEA plates and compatibility tests were carried out. Di- and monokaryons were distinguished by the presence or absence of clamp connections. From the results of compatibility tests, four sib-monokaryons and two reconstituted dikaryons of each fungus were selected. They were four monokaryotic isolates and two dikaryons, L1×L17 and L8×L13, of *L. betulinus*; and four monokaryotic isolates and two dikaryons, T4×T16 and T5×T10, of *T. hirsuta* (Table 1).

The isolates were cocultured with the orchid seeds of *E. ochobiensis* for 4 mo at 30°C in darkness on the modified medium of Mori et al. (1969) using sawdust of *Quercus acutissima* Carr. or *Lithocarpus edulis* (Makino) Nakai as detailed previously (Umata, 1997, 1998).

To assess the symbiotic ability of the isolates, seedling development and peloton formation were observed under the microscope. Two types of seed-coat of *E. ochobiensis* were distinguishable: the outer seed-coat is brown, hard, and shell-like; and the inner seed-coat is transparent and wing-like. In this study, symbiotic association was judged by the appearance of pelotons in the cells of the enlarged embryo, protocorm, and root of

Table 1. Compatibility tests of sib-monokaryons of *Lenzites betulinus* and *Trametes hirsuta*.

Lenzites betulinus

Isolate	L1	L8	L13	L17
L1	—	×	×	○
L8	×	—	○	×
L13	×	○	—	×
L17	○	×	×	—

Trametes hirsuta

Isolate	T4	T5	T10	T16
T4	—	×	×	○
T5	×	—	○	×
T10	×	○	—	×
T16	○	×	×	—

Note: ○, ×, and — show clamp connections formed, no clamp connections formed, and untested, respectively.

the orchid. Seed germination and successive development were recorded as the six stages described previously (Umata, 1998) with slight revision, as follows. Stage 0: No sign of rupture of outer seed-coat. Stage 1: Rupture of outer seed-coat: outer seed-coat ruptures at one end, enlarged embryo is visible, and fungus colonizes inside the base of embryo and forms several pelotons. Stage 2: Protocorm formation: inner seed-coat ruptures and embryo enlarges and becomes protocorm, pelotons increase in number. Stage 3: Scaly leaf and main root formation on protocorm: pelotons are abundant. Stage 4: Lateral root formation on protocorm. Stage 5: Plant formation: organs develop and scaly leaves and lateral roots are formed successively. The numbers of seeds, protocorms, or plants in each stage were counted after 4 mo of incubation. Table 2 shows the numbers and percentages of seeds in each stage.

Cocultures with *Lenzites betulinus* Seeds of *E. ochobiensis* germinated and developed into plants (stage 5) in the presence of all six isolates, as shown in Table 2 and Figs. 1–3, while the rupture of outer seed-coats was not observed in the absence of isolates. The highest overall germination rate (75.2%) was obtained with isolate L1, and the lowest (59.3%) with isolate L1 × L17. Pelotons were observed in the cells of enlarged embryos, protocorms and both main and lateral roots. In all cocultures, the majority of germinated seeds reached stage 3, while considerably lower percentages reached higher stages. When cocultured with dikaryotic isolates L1 × L17 and L8 × L1, 2.0 and 1.3% of inoculated seeds developed to stage 5, respectively, lower proportions than those cocultured with monokaryons, as shown in Table 2.

Cocultures with *Trametes hirsuta* Seeds germinated and developed into protocorms in the presence of all six isolates (Table 2), while the rupture of outer seed-coats was again not detected in the absence of isolates. The highest overall germination rate (64.7%) was obtained with isolate T4, and the lowest (28.0%) with isolate T10. Three isolates, namely, monokaryon T16 and the two dikaryons induced plant formation, while other three monokaryons, isolates T4, T5, and T10, failed to induce the full development, as shown in Table 2 and Figs. 4–9. Pelotons were observed in the cells of enlarged embryos, protocorms, and both main and lateral roots of the plants. Soft rot involving browning, withering, and softening of plant tissues, was observed in the protocorms of stages 2 and 3 associated with isolates T4 (Fig. 4) and T5. The highest proportion of germinated seeds was found in stage 1 for all isolates except T4 (stage 2), and much lower proportions were found in the later stages. The proportions of seeds which reached stage 5 with the two dikaryotic isolates differed considerably: 15.7% with isolate T4 × T16 and 1.9% with T5 × T10.

Of the monokaryons isolated from a single basidiome of *Laccaria laccata* (Scop.: Fr.) Berk. & Br., some were unable to form ectomycorrhizas while others readily formed a mantle and Hartig net on *Pinus banksiana* Lamb. (Kropp, et al., 1987; Wong et al., 1989). On the other hand, no variability of mycorrhization were detected among monokaryons of *Hebeloma cylindosporum* Romagnési and *Pinus pinaster* (Ait.) Sol. (Debaud et al., 1988). These results suggested that mycorrhization by monokaryons depends on the species of fungus. In the present investigation, all four monokaryons of *L. betulinus* induced the development of germinated seeds into plants, while in the case of *T. hirsuta*, only one of four monokaryons induced plant formation. These results show that interspecific variability in symbiotic potential of monokaryons is present in orchid mycorrhizas as well as in ectomycorrhizas.

Although one constituent monokaryon formed ecto-

Figs. 1–3. From seed to protocorm and lateral root formation of *Erythrorchis ochobiensis* cocultured with *Lenzites betulinus*.
1. Seed (A) with perfect wing-like inner seed-coat (IC) and shell-like outer seed-coat (OC) showing no rupture (stage 0), seed (B) with ruptured outer seed-coat and enlarged embryo (E) (stage 1), and protocorm (C) with ruptured inner and outer seed-coat (stage 2). The inner seed-coat is much larger than the outer seed-coat which covers only embryo. The inner seed-coat in the center was broken when the seed was removed from a mass of germinated seeds. 2. Plant with scaly leaves (SL), main root (MR) and distinct epidermal hairs (EH) (stage 3). 3. Plant with scaly leaves (SL), lateral root (LR), and main root (MR) (stage 4). Bars = 1 mm.

Figs. 4–9. Growth of *E. ochobiensis* cocultured with four sib-monokaryons and two reconstituted dikaryons of *Trametes hirsuta*, showing the highest stage induced by each isolate after incubation for 4 mo at 30°C in darkness.
4. Plants at growth stages 3–4 induced by monokaryotic isolate T4. Soft rotted protocorms (SRP) were often observed. 5–6. Plants at stage 5 induced by monokaryotic isolate T16 (Fig. 5) and dikaryotic isolate T4 × T16 (Fig. 6). Lateral roots show negative gravitropism. 7. Plants at stage 4 induced by monokaryotic isolate T5. 8. Plants at stage 3 induced by monokaryotic isolate T10. 9. Plants at stage 5 induced by dikaryotic isolate T5 × T10. Plants are very few compared with those induced by isolate T4 × T16 (Fig. 6). Bars = 1 cm.

mycorrhizas while another one did not, their reconstituted dikaryon formed mycorrhizas vigorously (Kropp et al., 1987; Kropp and Fortin, 1988). In another case, the dikaryon reconstituted from two monokaryons that both formed no ectomycorrhizas, formed ectomycorrhizas in 2 mo (Ducamp et al., 1986). Kropp and Anderson (1994) reasoned that some species of fungi require information in addition to that carried in a haploid genome to be capable of ectomycorrhiza formation and a functional symbiosis. In *T. hirsuta*, 1.9% of seeds reached stage 5 in association with the T5 × T10 dikaryon, while its constituent monokaryons induced development only to stage 3 or 4 (imperfect symbiotic potential). On the other hand, 15.7% of seeds developed fully with the T4

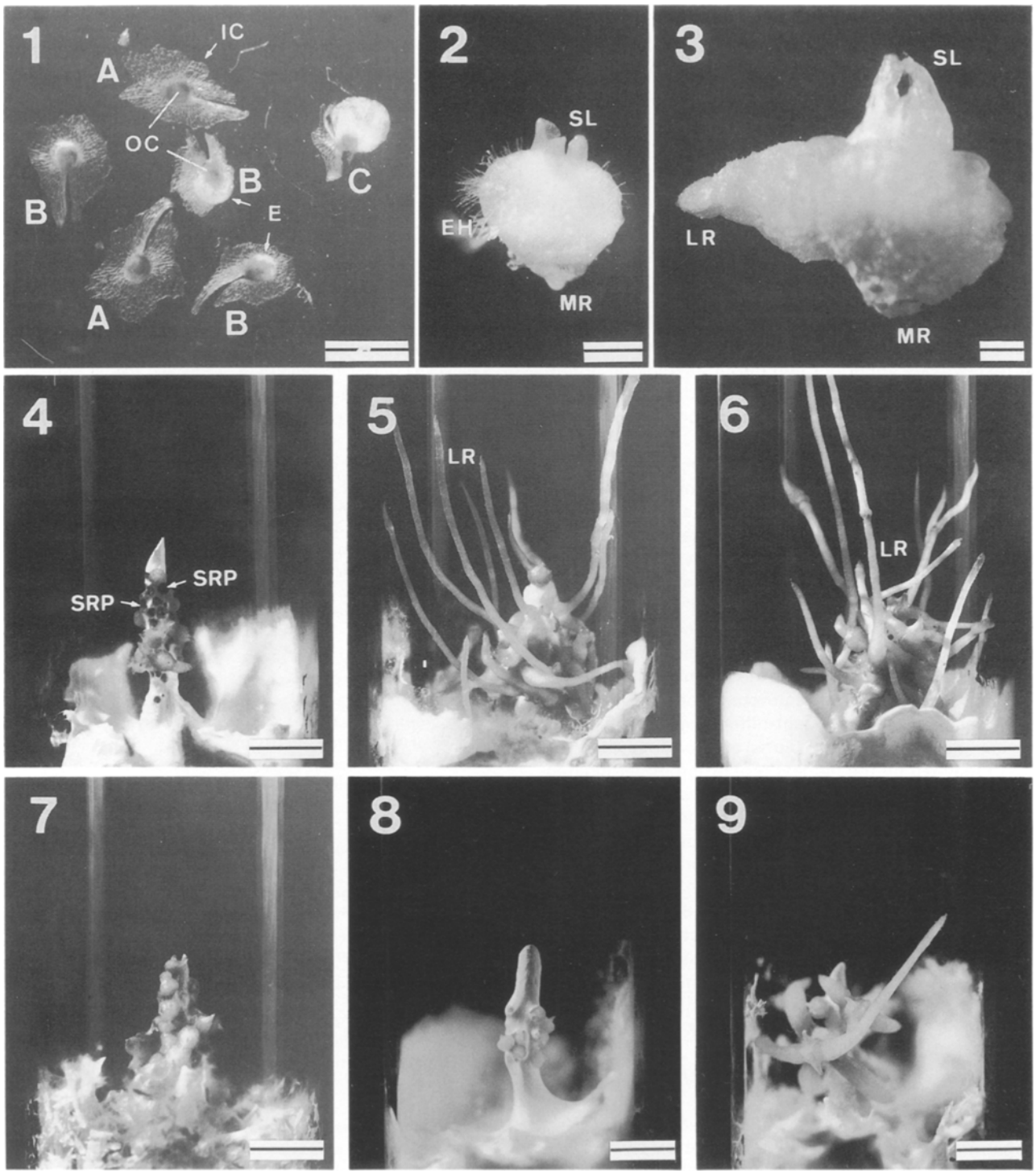

Table 2. Growth of *Erythrorchis ochobiensis* induced by 4 sib-monokaryons and 2 reconstituted dikaryons of *Lenzites betulinus* and *Trametes hirsuta* after incubation at 30°C for 4 mo in darkness.

Fungus and isolate	Inoculated seeds	Number and proportion (%) of seeds						Overall number and proportion (%) of germinated seeds (stages 1 to 5)
		Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	
Lenzites betulinus								
L1	2144	532 (24.8)	90 (4.2)	52 (2.4)	1284 (59.9)	96 (4.5)	90 (4.2)	1612 (75.2)
L17	2312	872 (37.8)	212 (9.2)	156 (6.7)	876 (37.9)	88 (3.8)	108 (4.7)	1440 (62.3)
L1×L17	2131	867 (40.7)	237 (11.1)	160 (7.5)	750 (35.2)	75 (3.5)	42 (2.0)	1263 (59.3)
L8	2478	676 (27.3)	70 (2.8)	236 (9.5)	1336 (53.9)	110 (4.4)	50 (2.0)	1802 (72.7)
L13	2259	630 (27.9)	27 (1.2)	63 (2.8)	993 (44.0)	213 (9.4)	333 (14.7)	1629 (72.1)
L8×L13	1869	756 (40.4)	189 (10.1)	204 (10.9)	684 (36.6)	12 (0.6)	24 (1.3)	1113 (59.6)
Trametes hirsuta								
T4	1866	658 (35.3)	483 (25.9)	592 (31.7)	129 (6.9)	4 (0.2)	0 (0.0)	1204 (64.7)
T16	1540	660 (42.9)	524 (34.0)	72 (4.7)	188 (12.2)	40 (2.6)	56 (3.6)	880 (57.1)
T4×T16	1932	1016 (52.6)	364 (18.8)	120 (6.2)	84 (4.3)	44 (2.3)	304 (15.7)	916 (47.4)
T5	1806	919 (50.9)	556 (30.8)	94 (5.2)	215 (11.9)	22 (1.2)	0 (0.0)	887 (49.1)
T10	1968	1416 (72.0)	444 (22.6)	36 (1.8)	72 (3.7)	0 (0.0)	0 (0.0)	552 (28.0)
T5×T10	1720	904 (52.6)	656 (38.1)	64 (3.7)	56 (3.3)	8 (0.5)	32 (1.9)	816 (47.4)

×T16 dikaryon, of which the constituent monokaryons had respectively imperfect and perfect symbiotic potentials. These results indicate that dikaryotization enhanced the ability of *T. hirsuta* to show perfect symbiotic potential with the orchid.

Previous studies showed that homokaryotic mycelia formed typical ectomycorrhizas with similar microscopic and ultrastructural morphology to the ectomycorrhizas formed by the parental dikaryon. The ultrastructural localization of acid phosphatase activities was also comparable in homokaryotic and dikaryotic mycorrhizas (Debaud et al., 1988). In the present investigation, the mycorrhizas formed by constituent monokaryons in both fungi were observed to be morphologically similar to those formed by the respective dikaryons at the macroscopical level, though ultrastructural observation was not carried out. A significant difference was observed between the two fungi in terms of the stage of plant growth reached by the highest proportion of germinated seeds, stage 3 in the case of *L. betulinus* and stage 1 in the case of *T. hirsuta*. An intraspecific variation was also observed; for example, the dikaryon of *T. hirsuta*, T4×T16, showed apparently high proportion of plants at stage 5, compared with those of T5×T10, while the dikaryons of *L. betulinus*, L1×L17 and L8×L13, showed almost equal proportion of plants at stage 5. These results confirmed that the symbiotic association varied not only with fungal species but also among isolates of the same species, as previously stated (e.g. Alexander and Hadley, 1983; Masuhara and Katsuya, 1991; Umata, 1997, 1998).

Soft rot was often observed on *E. ochobiensis* protocorms colonized by *T. hirsuta*, which is suggested that the fungus was not only symbiotic but also parasitic in some in vitro conditions, as reported previously in other orchid species (Hadley, 1970; Beyrle et al., 1995).

Acknowledgements——I wish to thank Drs. T. Etoh and K. Arai, Kagoshima University, and Dr. T. Terashita, former Professor at Kagoshima University, for offering helpful suggestions.

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