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ORIGINAL PAPER

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Identification of a mycorrhizal fungus in the roots of achlorophyllous *Sciaphila tosaensis* Makino (Triuridaceae)

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Abstract The identity of a mycorrhizal fungus in the roots of achlorophyllous *Sciaphila tosaensis* was investigated by DNA analysis and examination of the morphology of the association. The morphological features of the mycorrhizal fungus, i. e. aseptate hyphal coils, vesicles, arbuscule-like branching, and degenerate coils were similar to those previously reported for other achlorophyllous plants. Spore-like propagules identified as a glomalean fungus were propagated from root pieces of *S. tosaensis* in pot culture using alfalfa as the host trap plant. A PCR product was obtained from colonized root of *S. tosaensis* using the taxon-specific primers, VANS1 and VAGLO. Sequence analysis of the DNA fragment showed it to be almost identical to other *Glomus* species. Although it has been reported many times that the morphology of mycorrhizal fungi in achlorophyllous plants is quite similar to that of arbuscular mycorrhizal fungi, this is the first report of the isolation and identification of such a fungus itself.

Keywords *Sciaphila* · Triuridaceae · Arbuscular mycorrhiza · Achlorophyllous plant · Myco-heterotrophy

Introduction

Sciaphila tosaensis Makino (Triuridaceae) grows in deeply shaded forests in the western part of Japan (Kitamura et al. 1992). The Triuridaceae includes approximately 70 species in seven genera, all of which are achlorophyllous (Maas-van de Kamer 1995) as far as is known.

Although the morphological features of some Triuridaceae species have been described in earlier works (Poulsen 1886, 1890; Johow 1889; Malme 1896; Janse 1896–1897; Fiebrig 1922; Milanez and Meira 1943), reports of the detailed examination of mycorrhizas in Triuridaceae plants are scarce. Recently, fungal morphology, intracellular aseptate hyphal coils and their degeneration to clumps of amorphous fungal material were described in *Triuris hyalina* Miers (Imhof 1998). Among achlorophyllous plants, a similar mycorrhizal morphology has been described in the Polygonaceae (van der Pijl 1934), Gentianaceae (von Knöbel and Weber 1988; Imhof 1997, 1999a; Imhof and Weber 1997) and Burmanniaceae (Groom 1895; van der Pijl 1934; McLennan 1958; Terashita and Kawakami 1991; Imhof 1999b). These mycorrhizas were considered to be arbuscular on the basis of their morphological features (Leake 1994). However, the isolation and identification of such fungi in a chlorophyllous plants have not previously been attempted.

As achlorophyllous plants do not conduct photosynthesis, they are completely dependent upon their colonizing mycorrhizal fungi for carbon compounds as well as other nutrients, i.e. they are myco-heterotrophic. It is well known that Orchidaceae species are also myco-heterotrophic, especially at the protocorm stage. In the latter case, some of the mycorrhizal fungi are closely related to *Rhizoctonia* and the origin of the carbon compounds lies in organic matter (Hadley 1969). In the Monotropaceae, which are also achlorophyllous, association with ectomycorrhizal fungi as monotropoid mycorrhiza has been verified using molecular identification methods (Culling et al. 1996). Evidence for the transfer of carbon compounds from neighboring trees has also been obtained by ^{14}C tracer studies in forests (Björkman 1960).

As arbuscular mycorrhizal fungi (AMF) are themselves obligate biotrophs, their association with roots of achlorophyllous plants implies that carbon compounds originate from chlorophyllous host plants and are transferred to the roots of achlorophyllous plants through hyphal bridges. AMF themselves may have a function in the transfer of carbon between plants (Newman 1988). Thus, it is necessary to investigate the association of AMF with achlorophyllous plants.

Mycorrhizal fungi colonizing roots can be detected directly by polymerase chain reaction (PCR) on DNA

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extracted from the roots. Since taxon-specific primers have been designed for AMF (Simon et al. 1992, 1993), it is now possible to identify the fungi at the genus level. This technique seems to be most promising for identification of mycorrhizal fungi colonizing achlorophyllous plants. In this present study, the mycorrhizal fungus in the roots of achlorophyllous *S. tosaensis* was identified by DNA analysis and examination of the morphology of the association.

Materials and methods

Morphology of the association

In September 1999, five specimens of *S. tosaensis* Makino were collected from the understory of *Camellia japonica* L. in Fukui prefecture, approximately 60 m above sea level. The samples were dug out with other plant roots.

The roots of *S. tosaensis* were cut into 1-cm fragments and those with yellow pigment, indicating possible colonization, were selected. The fraction used for examination of fungal morphology was fixed and preserved in formaldehyde 6.5 ml, acetic acid 2.5 ml, ethanol 45.5 ml, distilled water 45.5 ml, cleared in 10% KOH, stained with 0.1% chlorazol black E and mounted on slides in polyvinyllactoglycerol. The stained root pieces were examined by Nomarski interference-contrast microscopy.

Isolation of mycorrhizal fungi

Fungus in the roots of *S. tosaensis* was isolated and propagated from root samples by pot culture (Brundrett et al. 1996). The root pieces were cleaned thoroughly by ultrasonication and five pieces from each plant were used as inoculum. Akadama-soil and river sand (1:1,v:v) autoclaved at 121°C for 30 min were put into five pots (500 ml per pot) and the inoculum was placed at a depth of approximately 5 cm. Seeds of *Medicago sativa* L. (alfalfa) were sown and the pots were placed in a greenhouse. Peters soluble fertilizer (N-P-K=20-5-30, W.R. Grace and Co., Fogelsville, Pa.) was supplied once a week at a final N concentration of 100 mg l^{-1}. After 4 months, part of the roots was harvested and washed gently to remove soil particles. Associated hyphae and spore-like propagules were observed under dissecting and compound microscopes. Roots were then stained with 0.1% trypan blue and mycorrhizal colonization was examined by Nomarski interference-contrast microscopy.

DNA analysis

DNA was extracted from 1-cm-long root and shoot pieces of *S. tosaensis* using cetyltrimethylammonium bromide, phenol/chloroform extraction and isopropanol precipitation (Gardes and Bruns 1993). DNA amplification was conducted with Takara Taq polymerase (Takara Shuzo, Kyoto). Pre-PCR with universal primers was conducted after direct amplification proved to be unsuccessful. DNA was first amplified with SS38 (Bousquet et al. 1989) and NS21 (Simon et al. 1992) and then 1 µl of the PCR products was re-amplified with the primer VANS1 (Simon et al. 1992) specific for glomalean fungi and one genus-specific primer: VAGLO for *Glomus*, VAACAU for *Acaulospora* and *Enterophospora*, or VAGIGA for *Gigaspora* and *Scutellospora* (Simon et al. 1993). The reaction was carried out in a TaKaRa PCR Thermal Cycler 480 (Takara Shuzo, Kyoto) in the initial step at 94°C for 90 s for denaturation, for the first 14 cycles out of 40 at 94°C for 30 s for denaturation, at 50°C for 55 s for annealing and at 74°C for 45 s for polymerization. In the following 11 cycles, polymerization was prolonged to 2 min, in the remaining 15 cycles to 3 min. The final elongation step was at 74°C for 10 min. The PCR products were directly ligated to the pT7blueT (Takara Shuzo, Kyoto) vector and sequenced.

Results and discussion

Sciaphila tosaensis was found on the deeply shaded forest floor. The roots of the plants coexisted with roots of *C. japonica* that formed a canopy over the sampling sites.

Infection was mostly initiated through root hairs (Fig. 1). This type of infection was also observed in the achlorophyllous plant *Voyria aphylla* (Imhof 1999a). Colonization occurred in the form of cell-to-cell intracellular spread (Fig. 2) as in *Paris*-type arbuscular mycorrhizas (Smith and Smith 1997). Aseptate hyphal coils occupied most of the cortical cells and intercalary vesicles and arbuscule-like hyphal branchings were observed associated with some coils (Fig. 2). The branched hyphae were slightly thicker than coiled hyphae and branching itself was not as developed as arbuscules in chlorophyllous plants. This kind of branching has also been observed in achlorophyllous *Triuris hyalina* Miers (Imhof 1998). Some of the hyphal coils had degenerated to clumps of amorphous fungal material (Fig. 3), as was also reported for achlorophyllous *T. hyalina* Miers (Imhof 1998). Aseptate hyphal coils, vesicles, arbuscule-like branching and degenerate amorphous fungal material are features very similar to those of mycorrhizal colonization reported in the achlorophyllous plants *Voyria*, *Burmannia*, *Psilotum*, and *Lycopodium* (Peterson et al. 1981; Terashita and Kawakami 1991; Schmid and Oberwinkler 1993; Imhof 1998, 1999a, b).

In some samples, a further type of hyphal coil was observed in cells near the central cylinder (Fig. 4). These hyphae were aseptate and developed moniliform structures totally different from AMF. Colonization was infrequent but some of the hyphal coils had also degenerated (Fig. 5). A similar phenomenon of secondary fungal colonization was also reported in achlorophyllous *Burmannia tenella* (Imhof 1999b).

Isolation of mycorrhizal fungi was successful in only one pot out of five inoculated with root pieces of *S. tosaensis*. This may indicate that the viability of the colonizing fungus was reduced in *S. tosaensis* roots even before the degeneration. The diameter of spore-like propagules was 20–30 µm and the fungus was identified as a species of the Glomales on the basis of morphological features (Fig. 6). Arbuscules and vesicles were observed in the roots of colonized alfalfa (Fig. 7). The plants colonized by the fungus grew better than non-inoculated controls (data not shown). These observations imply that the isolated fungus behaves as a normal arbuscular mycorrhizal fungus in chlorophyllous plants.

In the DNA analysis, PCR products of ca. 190 bp were obtained from all the roots of *S. tosaensis* examined using the taxon-specific primers VANS1 and VAGLO. One PCR product (STr-1) was sequenced and compared using the database search programs Gapped BLAST and PSI-BLAST (Altschul et al. 1997). The sequence was almost identical to that of a *Glomus* clone glo2, accession No. U25154, reported by Clapp et al. (1995) (Fig. 8). PCR products of this size were not obtained with the

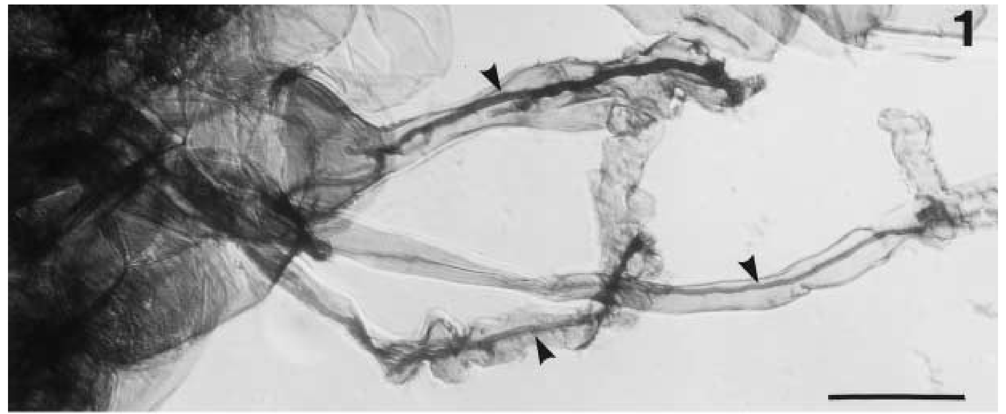


Fig. 1 Root hairs of *Sciaphila tosaensis*. Infection of mycorrhizal fungi was initiated from root hairs. *Arrowheads* indicate hyphae of mycorrhizal fungi. Figures 1,2,3,4 and 5 are micrographs from Nomarski interference-contrast microscopy; *bars* 100 µm

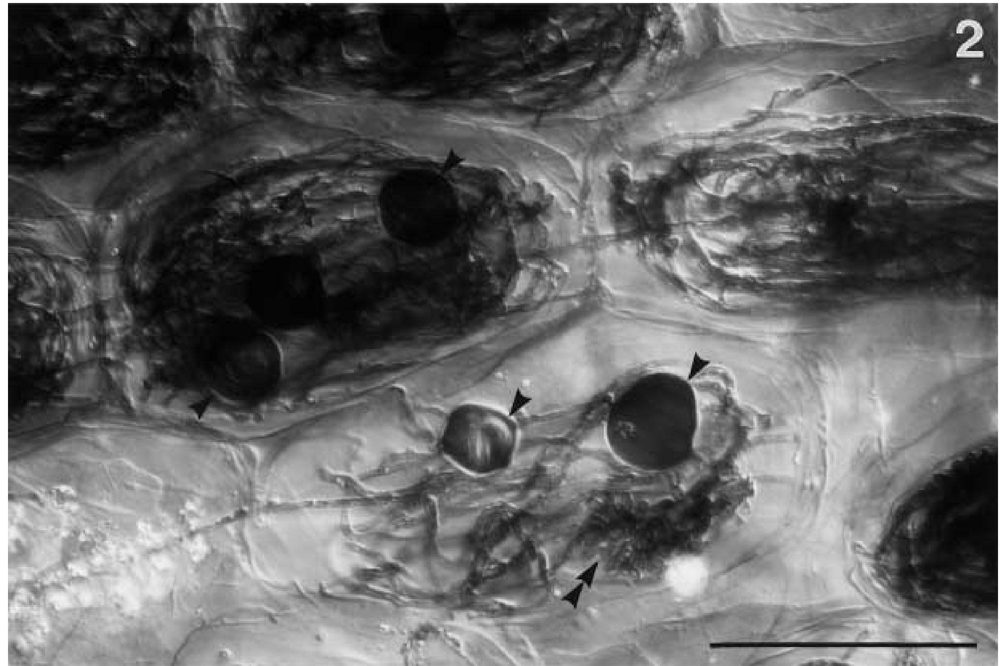


Fig. 2 Aseptate hyphal coils showing vesicle (*arrowheads*) and arbuscule-like branchings (*double arrowhead*)

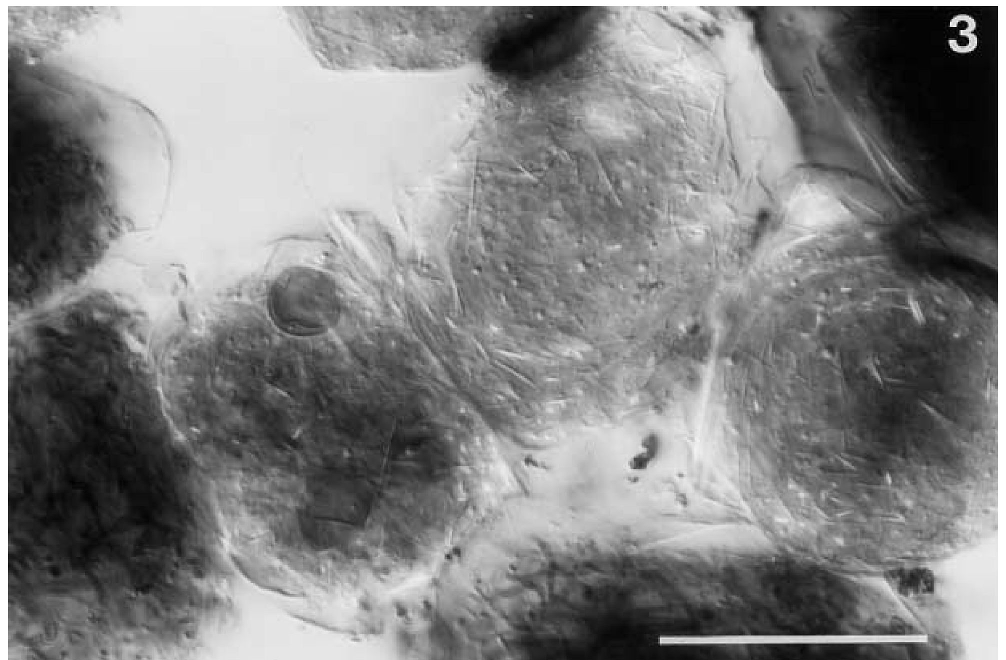
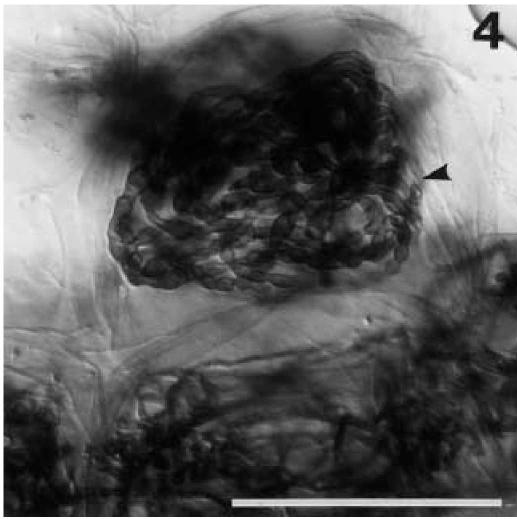


Fig. 3 Hyphal coils degenerated to amorphous fungal material in the cells of *S. tosaensis*



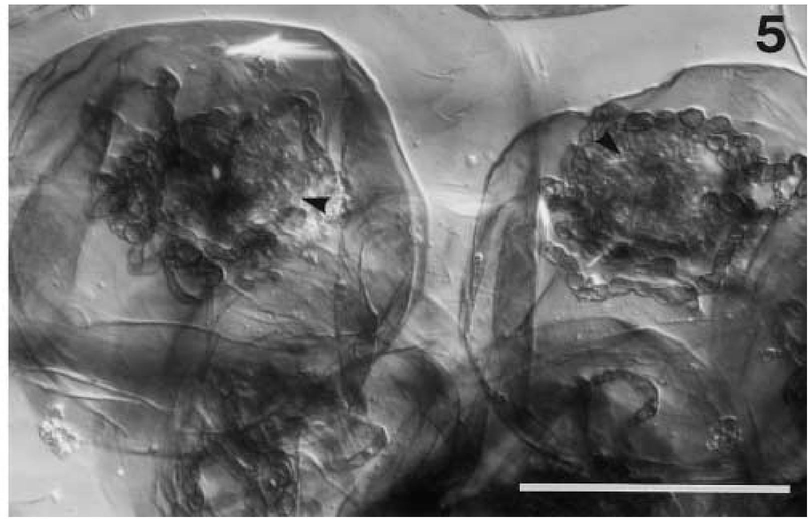


Fig. 4 Hyphal coils of putative saprophytic fungus (*arrowhead*) in the cells of *S. tosaensis*

Fig. 5 Hyphal coils of putative saprophytic fungus degenerated to amorphous fungal material (*arrowheads*) in the cells of *S. tosaensis*

primers VAACAU or VAGIGA or when DNA extracted from the shoot of *S. tosaensis* was used as template. Thus, DNA of a *Glomus* species was clearly present in DNA extracted from *S. tosaensis* root.

It can be concluded from these results that the fungus colonizing the root of *S. tosaensis* is an AMF and a species of *Glomus*. Since there is almost no host specificity between plants and AMF, different plant species can be linked by hyphal bridges (Newman et al. 1994). It is, therefore, possible that the AMF colonizes both *S. tosaensis* and coexisting plants such as *C. japonica* in the field through the formation of hyphal bridges. Since AMF are obligate biotrophs and achlorophyllous plants are not thought to supply carbon compounds to mycorrhizal fungi, these may be generated by associated chlorophyllous plants.

The morphology of the degenerate hyphal coils in *S. tosaensis* is similar to that of orchid mycorrhizas (Burgeff 1959), where some of the mycorrhizal fungi are close to saprophytic *Rhizoctonia*. Orchid mycorrhizal fungi form intracellular hyphal coils supplying carbon compounds to the plants. The hyphal coils degenerate and are lysed in the plant cells in late stages of the interaction (Smith 1967; Hadley 1969; Strullu and Gourret 1974). Although carbon is supplied before fungal degeneration occurs (Hadley and Williamson 1971), lysis is

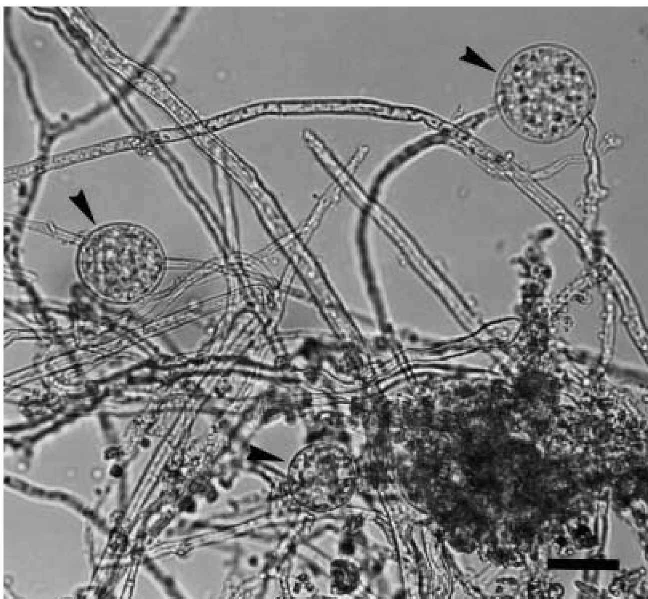


Fig. 6 Spore-like propagules (*arrowheads*) of *Glomus* sp. isolated and propagated from roots of *S. tosaensis* with alfalfa as the host trap plant; *bar* 20 µm

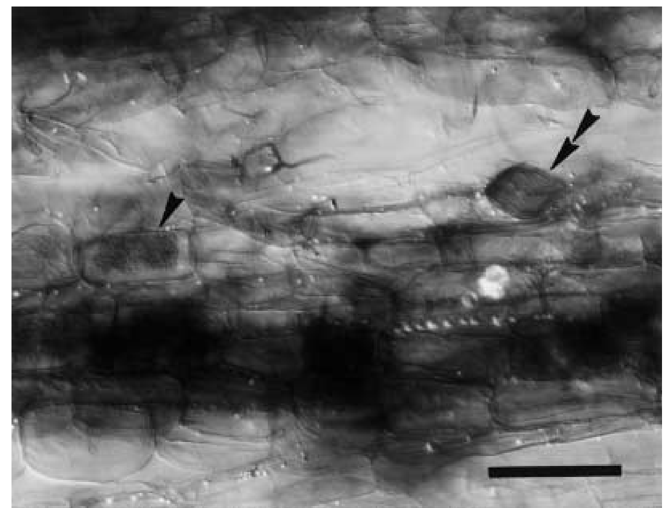


Fig. 7 Morphology of mycorrhizal fungus isolated from roots of *S. tosaensis* in cortical layers of alfalfa. *Arrowhead* and *double arrowhead* indicate arbuscules and vesicles, respectively; *bar* 100 µm

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        1                                                        50
STr-1:  gtctagtata atcgttatac aggTGAAACT GCGAATGGCT CATTAAATCA
        |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
glo2:   gtctagtata atcgttatac aggTGAAACT GCGAATGGCT CATTAAATCA

        51                                                      100
        GTTATAATTT ATTTGATAGT GAAATTACTA CTTGGATAAC CGTGGTAATT
        |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
        GTTATAATTT ATTTGATAGT GAAATTACTA CTTGGATAAC CGTGGTAATT

        101                                                     150
        CTAGAGCTAA TACATGCTAA AATTCCC[T]GA CTTCTGGAAA GGGATGTATT
        |||||||||| |||||||||| |||||||   || |||||||||| ||||||||||
        CTAGAGCTAA TACATGCTAA AATTCCC[C]GA CTTCTGGAAA GGGATGTATT

        151
        TATTAGATAA AAAACCAATa tcgggcaacc gattcccttg
        |||||||||| |||||||||| |||||||||| ||||||||||
        TATTAGATAA AAAACCAATa tcgggcaacc gattcccttg
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Fig. 8 Alignment of DNA sequences amplified using primers VANS1 and VAGLO. Lower and upper case letters indicate the primer sequences and genuine sequences, respectively. Of the 190 bp, the sequences only differ at position 128 (shown in a *box*) (*STr-1* amplified from root of *S. tosaensis*, *glo2 Glomus* sp. clone glo2 ribosomal RNA gene (Clapp et al. 1995)

also assumed to take part in nutrient transfer. The similar phenomenon observed in *S. tosaensis* may indicate that carbon transfer occurs at least at the stage of hyphal degeneration.

Hyphal coils morphologically different from those of arbuscular mycorrhiza were observed in the roots of *S. tosaensis*. The possibility exists that this second putative saprophytic fungus also supplies carbon compounds to the plant.

In order to determine the role of AMF in achlorophyllous plants, it is necessary to establish a tripartite symbiosis of chlorophyllous host plant, achlorophyllous plant and mycorrhizal fungi in pot culture. This could be used to establish whether arbuscular mycorrhizal colonization alone is sufficient for growth of the achlorophyllous plant in the presence of a host plant, or whether a second putative saprophytic fungus is also required. Carbon transfer from the chlorophyllous to the achlorophyllous plant through mycorrhizal hyphae could also be examined using tracer ^{14}C.

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