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Pure culture synthesis of ectomycorrhizas with *Cantharellus cibarius*

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SUMMARY

In a pure culture synthesis experiment, ectomycorrhizas were formed between two isolates of *Cantharellus cibarius* Fr. and the host plants *Pinus sylvestris* L. and *Betula pubescens* Ehrh. The mycorrhizal associations are described macroscopically and microscopically. Re-isolated mycelium from the mycorrhizas was found to be identical to the original inoculum. The ectomycorrhizal status of *C. cibarius* with *P. sylvestris* and *B. pubescens* is thus verified.

Key-words: Betula pubescens, Cantharellus cibarius, ectomycorrhizal synthesis, mycorrhiza, *Pinus sylvestris*.

INTRODUCTION

An ectomycorrhizal status for fungi is often the conclusion drawn from field observations concerning the consistent association of fruitbodies with one or more tree species. Direct evidence for this association can be obtained using the pure culture synthesis technique developed by Melin (1936), which requires isolation of the fungus into pure culture. This is problematic for many species, amongst others *Cantharellus cibarius* Fr.

Cantharellus cibarius, commonly known as the chanterelle, is one of the most well-known, edible, wild mushrooms of Europe and North America. It has been described as an ectomycorrhizal fungus following its observed association with a broad range of tree hosts (Mousain 1979). Although several have failed to grow mycelium of this fungus (Modess 1941; Froidevaux 1975; Schouten & Waandrager 1979; Danielson 1984), others have been successful (Doak 1934; Torev & Toreva 1968; Fries 1979; Straatsma *et al.* 1985). Only one report exists, however, on the pure culture synthesis of *C. cibarius* mycorrhizas (Doak 1934), and it lacks a description of the mycorrhizas formed and methods used. More recent attempts have failed (Straatsma *et al.* 1986).

The purpose of the current study was to attempt mycorrhizal synthesis and thus clarify the mycorrhizal status of *C. cibarius*.

MATERIALS AND METHODS

The fungal symbionts studied in this experiment were *C. cibarius* isolates S26 and T6. Isolate S26 is a plurisporous culture obtained in 1987 from a fruitbody collected near *Quercus robur* L. in Zwiggelterveld, Drenthe, The Netherlands, using a modification of the

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method of Fries (1979). Spores suspended in sterile deionized water were plated onto chanterelle–agar medium containing 0·05% activated charcoal powder (w/v).

The chanterelle medium (C-medium) was based on Fries (1978) and included modifications by Straatsma & Van Griensven (1985). It contained 2 g glucose, 2 g fructose, 0·58 g NH_4Cl, 0·59 g succinic acid, 0·2 g KH_2PO_4, 0·1 g $MgSO_4.7H_2O$, 20 mg NaCl, 26 mg $CaCl_2.2H_2O$, 10 mg meso-inositol, 9·5 mg Na-EDTA, 7 mg $FeSO_4.7H_2O$, 0·85 mg $MnSO_4.H_2O$, 0·32 mg H_3BO_3, 1·44 mg $ZnSO_4.7H_2O$, 0·38 mg KI, 12·5 µg $Na_2MoO_4.2H_2O$, 1·25 µg $CuSO_4.5H_2O$, 1·25 µg $CoCl_2.6H_2O$, 100 µg thiamin, 100 µg pyridoxine, 100 µg riboflavin, 25 µg biotin, 100 µg nicotinic acid, 100 µg biotin, 100 µg nicotinic acid, 100 µg *p*-aminobenzoic acid, 100 µg pantothenic acid, and de-ionized water to 1 l. The pH was adjusted to 5·5 with 1 M KOH before filter sterilization through 0·2-µm cellulose nitrate filters.

Fries (1979) has shown that spores of *C. cibarius* require a stimulus to germinate which can be supplied by *Rhodotorula glutinis* (Fres.) Harrison. In this experiment, *R. glutinis* was replaced by a *C. cibarius* isolate from the Horst collection. Petri plates containing the existing isolate on C-agar medium were used as a lid above Petri plates containing spores of S26, and incubated at 20°C. Isolate T6, one of the existing isolates in the Horst collection, was obtained in 1981 from tissue of a chanterelle fruitbody growing near *Q. robur* in Elsloo, Friesland, The Netherlands, as described by Straatsma *et al.* (1985).

Colonies of each isolate, grown for 4–5 weeks at 20°C in liquid C-medium, were used to make hyphal suspension inoculum (Straatsma *et al.* 1986) in filter-sterilized modified Melin–Norkrans (MMN) medium (Marx 1969), retaining the hyphal fraction 150–1000 µm. Sterilized perlite in capped glass culture tubes (145 × 25 mm) was drenched with 7·0 ml of the hyphal suspension to give 0·1–0·4 mg of mycelial dry weight per tube, and incubated for 1–2 weeks at 20°C before addition of the host.

Pinus sylvestris L. and *Betula pubescens* Ehrh. were used as host plants in combination with each isolate. One aseptic seedling (from seed germinated on water agar following surface sterilization in 30% hydrogen peroxide) was transfered to each tube. These were incubated at 20–26°C, under a light intensity of 1500–2000 lux for 12 h day^{-1}. *Q. robur* was not tested as a host plant due to difficulties in obtaining aseptic seedlings.

After the formation of mycorrhizas was observed, the fungal symbiont was re-isolated on C-agar medium from sample mycorrhizas and pieces of perlite for all four host–isolate combinations.

For microscopic observations the mycorrhizas were fixed in formaldehyde–acetic acid–50% alcohol (5:5:90) solution, embedded in paraffin, sectioned to 5 µm thickness, and stained with safranin-fast green.

The colours used to describe the mycorrhizas and re-isolated mycelium are according to Kornerup & Wanscher (1978), and are referred to as Methuen.

RESULTS

Perlite was found to be a satisfactory inert medium carrier, despite a previous report by Straatsma & Van Griensven (1986) that it inhibits growth of *C. cibarius*. The mycelium grew slowly through the perlite–MMN and colonized it within 1 month. At 3 months monopodial mycorrhizas of both isolates were visible on both hosts. At 4·5 months dichotomous mycorrhizas had developed on *P. sylvestris* and racemous mycorrhizas on *B. pubescens*. Seedlings were then removed from the tubes for inspection and it was observed that perlite-grown mycelium had the distinct fruity smell of apricots associated with fruitbodies of *C. cibarius* (Corner 1966).

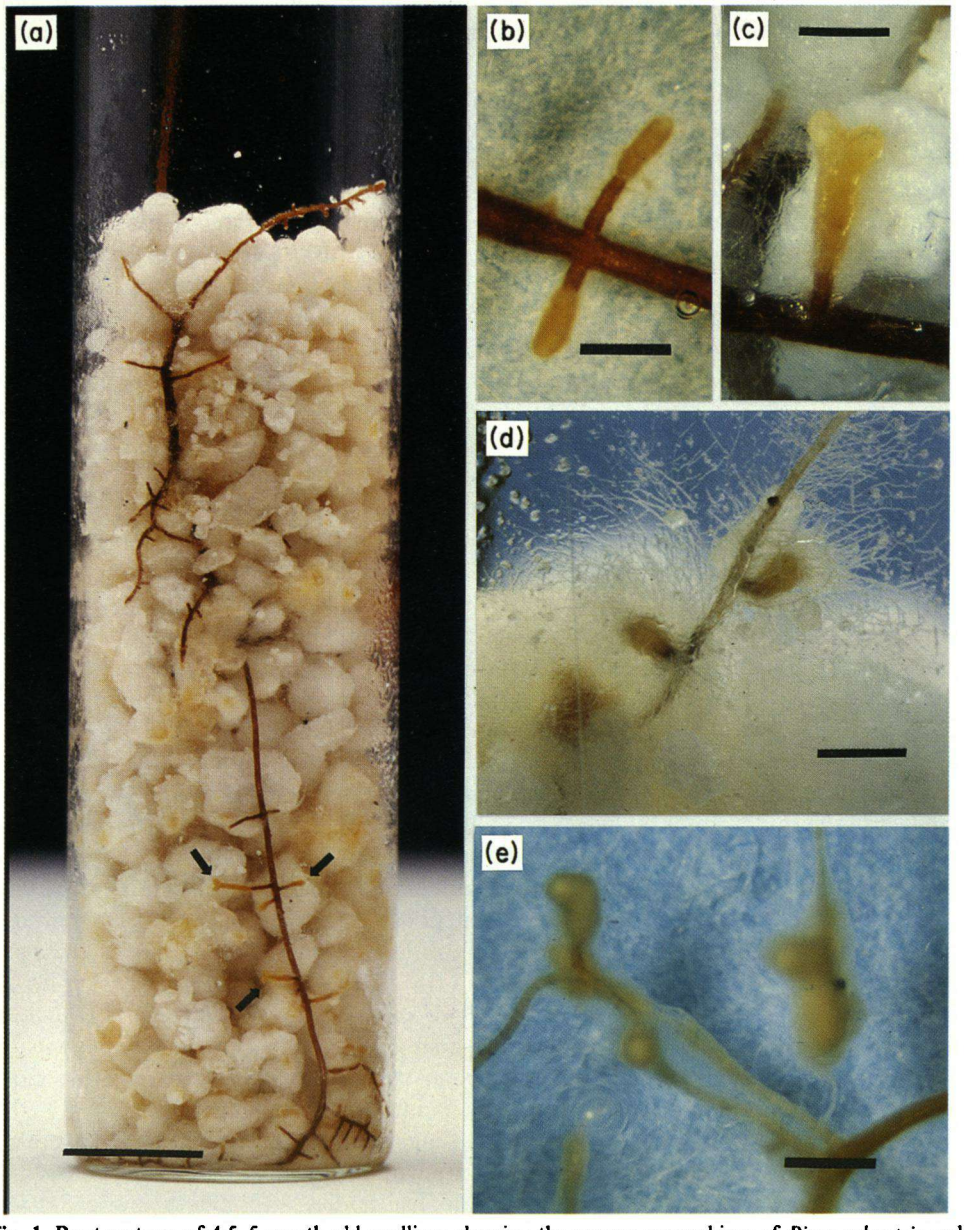

Fig. 1. Root systems of 4·5–5 month old seedlings showing the orange mycorrhizas of *Pinus sylvestris* and *Cantharellus cibarius* T6 in culture tubes (a, see arrows) and in detail (b,c), and of *Betula pubescens* and *C. cibarius* T6 (d,e). Scale bars indicate 10 mm (a) and 1 mm (b–e).

Pinus sylvestris and *C. cibarius* mycorrhizas (Fig. 1) can be described as follows. Dimensions 1–3(–4) mm × (0·3–)0·4–0·5 mm. Distinctly swollen compared to uninfected root tips of diameter 0·2–0·27 mm. Monopodial or dichotomously branched. Colour orange to greyish orange or brownish orange (Methuen 5A7, 5B5–5B8, 5C6), but with extreme tip much paler to almost white. Surface very finely woolly, felty at high

magnification (50 ×), at lower magnification smooth. At places surrounded by mycelial tufts of pale orange hyphae. No rhizomorphs. Mantle prosenchymatic. Fungal mantle thickness variable, 10–25 μm for S26 and 15–35 μm for T6 (Fig. 2). Uniseriate to biseriate with a Hartig net predominantly one cell layer thick (1·7–2·6 μm), occasionally two cell layers thick (4·3–6·0 μm) between the outermost layer of cortical cells (Fig. 2).

Betula pubescens and *C. cibarius* mycorrhizas (Fig. 1) can be described as follows. Dimensions 0·8–1·4(–1·8)mm × (0·2–)0·3–0·58 mm. Distinctly swollen compared to uninfected root tips of diameter 0·1–0·2 mm. Monopodial or racemous. Colour orange to light orange or greyish orange (Methuen 5A4–5A8, 5B6–5B8). Surface very finely woolly, smooth to felty at magnification lower than 50 ×. At places surrounded by mycelial tufts of pale orange hyphae. No rhizomorphs. Mantle prosenchymatic. Fungal mantle thickness variable, 15–40 μm for S26 and 35–85 μm for T6 (Fig. 2). Uniseriate to biseriate with a Hartig net predominantly one cell layer thick (1·7–2·6 μm), occasionally two or three cell layers thick (4·3–8·6 μm) between the outermost layer of cortical cells (Fig. 2).

Mycelium re-isolated from the mycorrhizas on C-agar medium can be described as follows. At 4 weeks, aerial mycelium white to orange white (Methuen 5A2), on the agar reverse side pale orange at the colony edge (Methuen 5A3) to light orange or orange (Methuen 5A4–5A7) in the colony centre; darkening with age. Hyphae 1·3–3·4 μm wide, with thin, smooth, yellow-coloured walls. Clamp connections present. These characteristics are the same as those of the original T6 and S26. No fungal contaminants grew from either mycorrhizas or pieces of perlite.

DISCUSSION

There are no previous descriptions of mycorrhizas formed with *C. cibarius* in pure culture to be used in comparison with these results. Natural *Pseudotsuga menziesii* (Mirb.) Franco and *C. cibarius* mycorrhizas, as described by Froidevaux (1975), have a Hartig net present in the entire cortex, plus abundant and dense attached rhizomorphs. The discrepancies concerning Hartig net development and rhizomorph formation, between these natural mycorrhizas and the mycorrhizas described above, may be caused by the cultural conditions used (Harley & Smith 1983) and the differences in plant host species and age.

Both T6 and S26 are isolates obtained from fruitbodies collected near *Q. robur* hosts. The fact that they form mycorrhizas with two other tree hosts, *P. sylvestris* and *B. pubescens*, suggests that physiological strains adapted to one specific host do not exist in *C. cibarius*. This would support the conclusion by Harley & Smith (1983) that close specificity is not a common characteristic of ectomycorrhizal fungi.

Under natural conditions ectomycorrhizal fungi are dependent upon their host plant for fructification (Romell 1938). In pure culture without the host plant almost none are capable of forming mature, spore-producing fruitbodies although a few can form primordia or immature fruitbodies (Debaud & Gay 1987). Primordia-like structures of *C. cibarius* have been observed in pure culture on perlite–MMN (L.M. Moore, unpublished observations), but no reports exist on the formation of its fruitbodies. The addition of a host plant to *C. cibarius* mycelium, as reported here, is therefore considered an essential step towards the development of its fruitbodies. It is a continuation of the research programme begun in 1980 at the Dutch Mushroom Experimental Station to domesticate this highly prized edible species. This approach is similar to that used at the Bordeaux Mushroom Research Station, France, for the production of mycorrhizal fungi in plantations (Delmas 1978), and which has led to the production of fruitbodies of

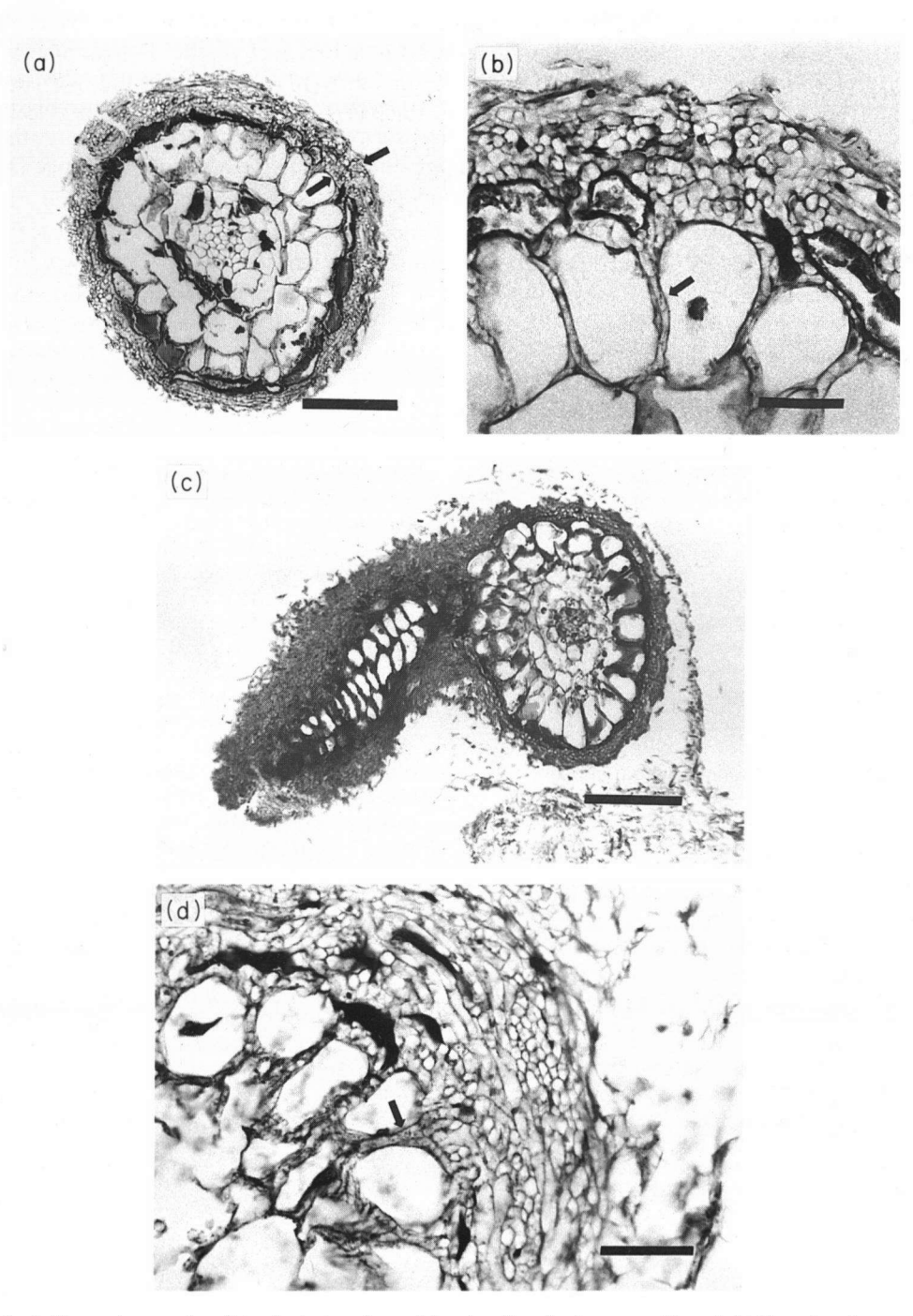

Fig. 2. Photomicrographs of histological sections of *Cantharellus cibarius* mycorrhizas. (a,b) Host plant is *Pinus sylvestris*. The fungal mantle of T6 is indicated (see arrows, a) and the cells of the Hartig net are visible (see arrow, b) between layers of root cortex cells. (c,d) Host plant is *Betula pubescens*. A branched mycorrhiza of S26 is indicated in both cross and tangential (not median) section (c). Hartig net cells of T6 are visible (see arrow, d) between root cortex cells. Scale bars indicate 100 μm (a,c) and 25 μm (b,d).

Tuber melanosporum Vitt., *Suillus granulatus* (Fr.) O. Kuntze and *Lactarius deliciosus* (L. ex Fr.) S.F. Gray in recent years (Olivier & Delmas 1987).

The synthesis of chanterelle mycorrhizas reported here verifies the status of *C. cibarius* as an ectomycorrhizal fungus with the hosts *P. sylvestris* and *B. pubescens*. It also opens new possibilities for fructification attempts and for physiological work to interpret the observed decline of the natural chanterelle population in The Netherlands (Jansen & De Wit 1978; Jansen *et al.* 1985; Jansen & Van Dobben 1987).

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