Mycorrhiza (1994) 5:89–97

ORIGINAL PAPER

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Formation and growth of the ectomycorrhiza of *Cantharellus cibarius*

Abstract New data on the physiology of *Cantharellus cibarius* mycorrhiza formation has resulted in a new aseptic routine method for in vitro formation. The advances are short formation time, healthy plants and reliable colonization. A high glucose demand and a good gas exchange with additional carbon dioxide are important factors in the mycorrhiza formation. Mycorrhiza was observed after 8 weeks, but strong colonization occurred after 10–12 weeks, when mycorrhiza was established to the depth of 5 cm. A *C. cibarius* strain connected to *Picea abies* in nature successfully colonized *Pinus sylvestris* in vitro, but not *Betula pendula*. Mycorrhizal plants have been successfully transferred to unsterile environments in greenhouses. The mycorrhizae continued to colonize new roots and the unsterile peat soil for 10 months. However, *C. cibarius* mycorrhiza is highly sensitive to flooding. With PCR and RFLP, fruit bodies, isolated mycelia and artificially formed mycorrhizae have been compared to prove that *C. cibarius* was used. Climatic changes did not induce primordia formation, but factors behind fruit body formation are discussed.

Key words Mushroom · Polymerase chain reaction Mycorrhiza

Introduction

The research on the physiology of the edible ectomycorrhizal basidiomycete *Cantharellus cibarius* Fr. has not been intensive due to difficulties in obtaining pure mycelium. The fruit bodies of *C. cibarius* and some related species are heavily infected by *Pseudomonas fluorescens* Migula and other bacteria and molds (Danell et al. 1993), and this, together with the slow growth, has been the main reason for the lack of success in isolating sterile mycelium from fruit bodies (Danielson 1984; Froidevaux 1975; Itävaara and Willberg 1988; Modess 1941; Schouten and Waandrager 1979). Fries (1979) did the first successful germination of spores, since repeated by others (Danell and Fries 1990; Itävaara and Willberg 1988; Straatsma et al. 1985). Straatsma et al. (1985) conducted the first isolation of mycelium from fruit bodies, but their technique has been successfully used only by Moore et al. (1989) and Danell and Fries (1990). Moore et al. (1989) synthesized mycorrhizae with *Betula pubescens* Ehrh. and *Pinus sylvestris* L. with Hartig nets one or two cell layers deep. Danell and Fries (1990) synthesized complete mycorrhizae with *Picea abies* (L.: Karst) with fully developed Hartig nets throughout the cortex, limited by the endodermis only. The axenic methods for mycorrhiza formation so far are based on Para film-sealed vessels with plant, fungus and nutrient solution in an inert substrate. However, the methods have not allowed the plant-fungus system to develop beyond the stage of formation of mycorrhiza. The shoots were only a maximum 5 cm in length after 5 months, and root branching was poor. The reasons might be poor gas exchange over the sealing film of the tubes (Danell and Fries 1990), limited space, uncontrolled nutrient concentrations and no withdrawal of excretory products. The same technique but in a greater volume (i.e. in larger vessel) resulted in better mycorrhiza formation and root branching (Danell, unpublished). However, a well-aerated axenic but nonmycorrhizal system was used by McLaughlin (1970) to study fruit body formation in *Chalciporus rubinellus* Singer (Peck.) (as *Boletus rubinellus* Peck.).

Many other methods for mycorrhiza formation have been published since the pioneer work of Melin (1922, 1925). Several of the methods are unsterile and almost none of them allow further development after the formation of mycorrhiza (e.g. Fortin et al. 1980; Kähr and

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Arveby 1986; Molina and Palmer 1982; Unestam and Stenström 1989).

The purpose of this study was to learn more about essential factors behind the mycorrhiza formation of *C. cibarius* and to create a new aseptic system for routine formation. With a technique which allows growth of the double organism beyond the mycorrhiza formation, it would be possible to study the physiology of slow-growing mycorrhizae, e.g. *C. cibarius,* and to do experiments with fruit body formation. Species identification was based on the methods PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism).

Materials and methods

The mycorrhiza formation system

The system is based on the technique of Jentschke et al. (1991). However, almost every part is modified, so the new culture unit system (CUS) will be briefly described.

A single growth unit (Fig. 1) is made of a specially adapted glass beaker of 2 l volume with a broader beaker of the same volume as a lid. The lid is tightened with silicone tubing (outer diameter 5 mm). In the bottom of the beaker is a ceramic lysimeter (Staatliche Porzellanmanufaktur, Berlin, Germany) which is connected via silicone tubing (3 mm wall, 3 mm inner diameter) to a 1-l collection bottle. The bottle is connected to a pipeline with a vacuum pump, which drains the growth unit using a vacuum of −0.8 bar. The drainage bottle is changed every 6 days.

The lysimeter is covered with acid-washed (6M HCl for 2 weeks) quartz sand with an average diameter of 0.9 mm. On top of the sand a 100-ml beaker is placed upside down with a cylinder of filter paper on the outside. The beaker is also covered with sand, yielding a total if 1 l sand as an inert substrate. The autoclaved nutrient solution (see Table 1) is prepared in a 5-l bottle. Heat-sensitive compounds, e.g. glucose, are added to the autoclaved solution after being sterile filtrated (Millipore 0.2 μm). Aliprene tubing (Alitea AB, Stockholm, Sweden), which withstands mechanical stress better than silicone, connects the bottle with a peristaltic pump with 20 inlets. The tubing is attached to the inlet of the growth unit. The pump is automatically activated every 90 min by a microprocessor timer, and 10 ml nutrient solution is added each time. A steel connector on the tubing, placed before the pump, makes it possible to change autoclaved bottles of nutrient solutions, since the end of the steel connector is flamed before connection with the new tubing is made. An air pump with triple filters supplies a growth unit with 1 l air/min via Aliprene tubing. Additional CO_2 is added and mixed with air to a final concentration of 0.2–0.4%. Outlet from the growth units is passive through a glass wool filter.

The whole growth unit is placed in a Fi-totron 600H growth cabinet (Fisons, Loughborough, England) which controls light intensity, photoperiod and temperature. Contaminations are discovered by inoculating substrate on modified Fries medium (MFM) agar (Straatsma and Van Griensven 1986) after harvest.

Table 1 Standard mineral solution used for mycorrhiza synthesis of *C. cibarius* (340 μl stock solution/1000 ml, pH 5.0)

N	17	mg	(60% NH_4, 40% NO_3 w/w; 1:2 mol/mol)
K	11	mg	
P	2.6	mg	
Ca	1.0	mg	
Mg	1.0	mg	
S	1.5	mg	
Fe	126	μg	
Mn	68	μg	
B	34	μg	
Cu	5.1	μg	
Zn	5.1	μg	
Cl	5.1	μg	
Mo	1.2	μg	
Na	0.54	μg	

Host plants

Seeds of *Picea abies* and *Pinus sylvestris* from the south of Sweden and seeds of *Betula pendula* Roth. from Finland were used. The seeds were sterilized for 30 s in 70% ethanol and then for 20 min in H_2O_2. After washing in sterile demineralized water and drying on a sterile filter paper, the seeds were transferred to agar dishes with MFM. The seedling was used as soon as the cotyledons separated from each other. Under a sterile hood six to eight seedlings were transferred to each autoclaved and cooled CUS unit. Immediately after this transfer, 10 ml of a suspension of hyphae was added to every individual root (see below). The growth unit was then sealed and connected to the nutrient, air and drainage pumps in the climate cabinets.

An experiment to show the broad host range of *C. cibarius* was done by inoculating 60 *B. pendula* seedlings and 36 *Pinus sylvestris* seedlings with strain SNGT2 isolated from a fruit body connected to *Picea abies.* Standard incubation and nutrient procedures were used as described below. The degree of mycorrhizal formation was studied after 2 months.

Mycelia

Twenty *C. cibarius* strains were isolated from tissues and spores as described in Danell and Fries (1990). Also one strain of *Lactarius rufus* Scop. ex Fr. was used. Pieces of MFM agar with mycelium (4–8 weeks old) were axenically transferred to 5-cm petri dishes with sterile filtered (Millipore 0.2 μm) liquid MFM. Radial growth rate was 0.5 mm/day. After incubation for 4–6 weeks (20° C, darkness), mycelia from 4–5 petri dishes were fragmented in 100-ml Erlenmeyer flasks containing 50 ml fresh MFM and glass beads. The suspension was filtered (pore size 1 mm) and an additional 150 ml MFM was added to the suspension. The hyphal suspension was introduced to two CUS units as described above. The remaining suspension in the flask was incubated at 20° C in darkness to check vitality of mycelium and occurrence of infections.

Mycelia were also introduced as pieces of agar in four CUS units. Two units were treated according to the standard (see below), and to the other two units added standard nutrient solution without glucose. However, no growth was observed.

Established mycorrhiza was sectioned according to Danell and Fries (1990) to confirm the presence of intercellular Hartig net, which constitutes evidence for mycorrhiza (Harley and Smith 1983). Mycorrhizae were also transferred to MFM agar for reisolation.

Nutrient medium

The nutrient medium is based on Ingestad (1979), with the same proportions between minerals. The complete formulae of stock solutions A and B are given in Nylund and Wallander (1989). However, only 1700 μl stock solution per 5-l bottle (pH 5.0) was used as standard of concentration (instead of 5000 μl). Occasionally concentrations of 1000–2500 μl/5 l were used. Final concentrations are given in Table 1. Experiments with addition of glucose or sucrose were also carried out, using concentrations of 0–0.5%. The standard concentration of glucose during other experiments was 0.2%.

Fruit body-associated bacteria

Garbaye et al. (1990) have shown that a weak *Laccaria* mycelium successfully forms mycorrhiza if mycorrhiza helper bacteria are present. These supply the mycelium with organic acids. I tested the hypothesis that *Pseudomonas fluorescens,* present in all *C. cibarius* fruit bodies (Danell et al. 1993), could replace the addition of glucose. Some 4×10^8 bacteria (four different strains) were added to the hyphal suspension when inoculating CUS units. Eight such units with six spruce plants each were used, with the departure from the standard procedure that no glucose was added. As controls, two units without bacteria, two units without bacteria but with glucose and two units with both bacteria and glucose were used. Incubation was 10 weeks. After harvest, substrate was inoculated on MFM agar to study bacterial viability.

Gas

According to Straatsma and Bruinsma (1986), *C. cibarius* is able to assimilate CO_2. Therefore CO_2 was used in order to study effects on mycorrhiza formation. Two levels were compared. At the low level no CO_2 was added (the CO_2 level of the ambient air in the incubation room was 0.05–0.06%), while the high concentration was set at 0.2%, since higher concentrations hardly increase vegetative growth (Straatsma et al. 1986) but can cause closing of leaf stomata. The standard concentration of CO_2 during other experiments was set at 0.2%. CO_2 was mixed with the air stream before entering the pipeline, and levels were controlled with a flow meter. Measurements of CO_2 were performed with an ADC infra-red CO_2 analyzer. Relative humidity was 49% ±9%.

Light

Each climate cabinet had 12 fluorescent tubes (40-W Thorn warm white). Light intensity was maximal (140 μmol/m^{2}s) after 2 weeks of increasing intensity (50 μmol/m^{2}s 1st week, 110 μmol/m^{2}s 2nd week). Four additional 40-W tungsten red light bulbs per climate cabinet were used to enhance the red spectrum for better photosynthesis.

The effect of a change in photoperiod from 24 h (2 months) to 8 h (1–2 months) was studied. Standard concentrations, *C. cibarius* SNGT2 and *Picea abies* were used.

In the first three series 54 CUS units were covered with aluminum foil up to the substrate level, a precaution that was later abandoned. Standard photoperiod in other experiments was 21 h.

Outplanting of mycorrhizae

From October 1992 pine seedlings with *C. cibarius* mycorrhiza were transferred from the CUS to greenhouses and a forest nursery. The purpose was to study survival of plant and fungus, and fungal ability to colonize soil and other roots. Ten plants (3 months old) with mycorrhiza were separated from each other and placed in clay pots. The filter paper from the growth unit was not removed. Twelve other mycorrhizal plants were placed in a plastic frame with 64 individual compartments (4.5 × 4.5 × 6.5 cm). The substrate that was used was a peat/quartz sand mixture, 25%/75% v/v. Water was added automatically once a day on the felt on which the pots were placed. Through capillary forces and via the roots the water entered the pots. A quantity of 20 ml Ingestad nutrient solution (three times stronger than the standard concentration, Table 1) was added once a week. Temperature was 18° C (day) and 12° C (night), photoperiod 12 h. Humidity was kept at maximum 80%.

Another set of plants (3 months old) was directly transferred from growth units to plastic pots as a clump of six plants. The purpose was to avoid the root and rhizomorph damage caused by separating plants from the glass beaker used for nutrient distribution in the unit. Substrate was peat/quartz sand 50%/50% (v/v). Five pots (30 plants) were placed in another greenhouse. This greenhouse was contaminated with other ectomycorrhizal fungi, e.g. *Laccaria* and *Thelephora,* so the competitiveness of *C. cibarius* was studied. Temperature was 20° C, photoperiod 18 h. Water was added from above when the topsoil became dry. Ingestad nutrient solution (three times stronger than for the growth units) was added once a week.

Six 4-month-old mycorrhizal pine plants in a growth unit had the temperature altered from 20° C to 12° C for 24 h, then 10° C for 24 h and finally 7° C for 3 days. After this treatment the plants were transferred to a pot as described above, and placed in a forest nursery tent, keeping the temperature at approximately 0° C. The purpose was to study resistance to cold and the ability of the mycorrhiza to stand competition from other mycorrhizal fungi.

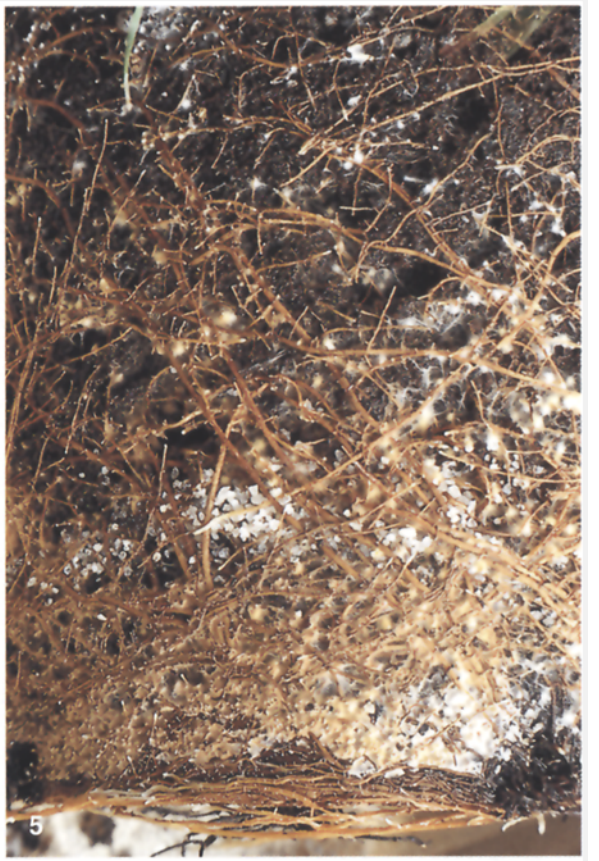
Molecular identification

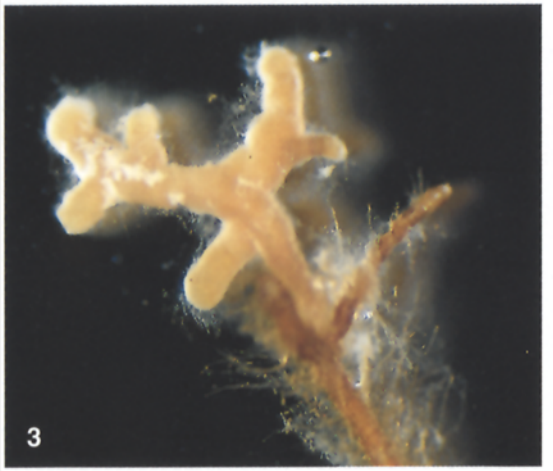
There is always a risk of getting contaminating fungi into culture instead of *C. cibarius.* Thus it is important to compare fruit body mycelium with culture mycelium and mycorrhiza. It would also be valuable to distinguish mycorrhiza of *C. cibarius* from other ectomycorrhizal species when studying the competitiveness of the artificially formed mycorrhiza after transfer to a contaminated greenhouse. In addition to morphological studies, molecular biology methods provide important means of comparing fruit body material, root tips in the soil and axenic material. Straatsma et al. (1985) used dot-blot DNA hybridization to compare vegetative and fruit body mycelium. However, the method is laborious and not applicable for mycorrhizal material where the amount of DNA is very low. I compared ribosomal DNA (rDNA) using PCR followed by RFLP as described below. The internal transcribed spacer (ITS) region of rDNA was chosen as a target for the PCR, since interspecies variations have been recorded by, for example, Gardes et al. (1991).

DNA from frozen fruit bodies and axenic vegetative mycelium (*C. cibarius* and *C. pallens* Pilat), as well as axenic and greenhouse *C. cibarius* mycorrhizae, were used. The DNA extraction procedure is based on that described by Henrion et al. (1994) with the following modifications: The sample was directly crushed in 600 μl CTAB buffer without using liquid nitrogen. Incubation was carried out with 0.1 mg proteinase K (Sigma) at 65° C for 2 h. RNAse was not used after chloroform/phenol, chloroform treatment. Incubation with isopropanol was conducted overnight at −20° C. After pelleting, the DNA was washed with 70% ethanol twice. After drying the pellet for 15 min at 60° C and then for 45 min at room temperature, the DNA was suspended in 50 μl 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and stored at 4° C. Amplification of the entire ITS rDNA region with basidiomycete primers ITS1 and ITS4 (Bioprobe Systems, Montreuil-sous-Bois, France) was done according to Henrion et al. (1992). The number of amplification cycles of the DNA thermal cycler (Perkin Elmer Cetus, Norwalk, USA) was 35. The purity and character of the amplified DNAs were studied after electrophoresis on 1% agarose gel. 10 μl Amplified DNA was fragmented by restriction enzymes (2 U) *Mbo*I, *Hinf*I and *Hae*III (Promega Corp., Madison, USA) for 3 h at 37° C. The fragments were separated by electrophoresis on 1% ME-agarose +2% Nu-Sieve agarose gel (FMC, Rockland, USA) for 3 h at 93 V. DNA was stained in Ethidium bromide (10 mg/l) for 40 min and destained in water for at least 30 min. UV light was used to detect the fragments.

Results

With the standard CUS, mycorrhiza formation in *C. cibarius* and *Picea abies* took 8 weeks, compared to the earlier periods of 4.5 months (Moore et al. 1989) and 5 months (Danell and Fries 1990). Complete mycorrhiza





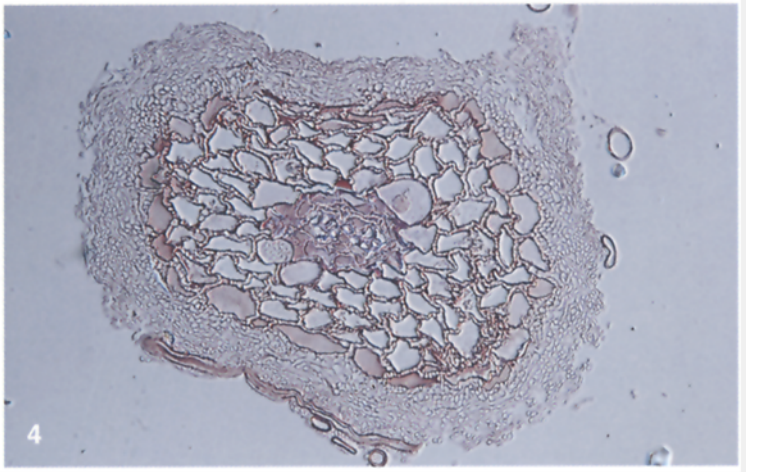


Fig. 1 Photograph of the culture unit system (CUS)

Fig. 2 Yellow mycelium of *C. cibarius after* 8 weeks

Fig. 3 *C. cibarius* mycorrhiza with *Picea abies*

Fig. 4 A section of a *C. cibarius* mycorrhiza with *Picea abies*

Fig. 5 Pot culture of *C. cibarius* and *Pinus sylvestris*

formation of the top 5(–7) cm was established after 10–12 weeks. The yellow mycellum was very dense, aggregating sand particles and creating white airpockets (Fig. 2). Numerous white rhizomorphs occurred. Glucose or sucrose was required to obtain mycelial development and mycorrhiza formation during the first 8 weeks. Once a strong mycelium was established, the addition of carbohydrates was no longer necessary. Glucose addition resulted in colonization a few weeks earlier than with sucrose. Concentrations higher than 0.2% did not give a faster development. *Lactarius rufus* did not need extra carbohydrates, and formed a total mycorrhiza down to the bottom of the CUS (15 cm) in 6 weeks. Within this period, *Lactarius rufus* sometimes spontaneously formed great numbers of primordia, which was also observed by Jentschke et al. (1991).

Contaminations affected less than 5% of the *Lactarius rufus* CUS units. In most cases the contaminations did not affect the growth of plant and fungus. Contaminations in nutrient and drainage bottles were almost negligible. In the *C. cibarius* units where glucose was added, contamination frequency did not increase. However, plants and mycelium were sometimes negatively affected by contamination (often a yeast). In that case, the xenic organism often spread via the tubings to the nutrient bottle. Drainage bottles were almost 100% infected due to frequent changes and carbohydrate content. The contaminating organism could not enter the CUS from the drainage bottle, due to the ceramic lysimeter which served as a mechanical barrier. Sometimes a drainage tubing became plugged by contaminating colonies, which flooded the CUS. Addition of 10 ppm benomyl (Benlate, Dupont) to the nutrient solution decreased contaminations, but was not performed routinely since the influence of benomyl on basidiomycetes is not well known and the contamination problem was considered not severe.

Fruit body-associated bacteria

No growth of mycelium occurred without glucose even in the presence of bacteria. Post-harvest study of the bacterial populations in the CUS units showed complete colonization of the substrate, which was revealed after growth on MFM agar. Mycorrhiza formation occurred normally in the presence of bacteria if glucose was added. *C. cibarius* seemed indifferent to the bacteria.

Gas effects

Without addition of extra CO_2 the *Cantharellus* mycelium developed slowly, and no mycorrhiza was formed after 3 months. The continuous gas flow resulted, however, in healthy and still aseptic pine and spruce plants, reaching 18 cm in 10 weeks. Additional CO_2 was therefore vital for mycorrhiza formation. The additional CO_2 concentration fluctuated a lot, though, mainly due to slow gas exchange of the incubation room where the air pump was operating. Additional CO_2 was thus at least 0.2% and occasionally as high as 0.4%.

Host specificity

Of the 20 strains tested, all formed mycorrhiza with *Picea abies* or *Pinus sylvestris.* One strain originated from a spore collection. However, the density of mycelium and number of mycorrhizal short roots varied greatly between strains. The *C. cibarius* strain from *Picea abies* colonized all units with *Pinus sylvestris* plants, and 20 of 36 plants formed mycorrhiza. However none of the 60 *Betula pendula* plants formed mycorrhiza, and mycelial growth was slow.

Light

No mycelial response to changes in photoperiod was observed.

Outplanting of mycorrhizal seedlings

In total, only two plants died when transferred to pots in the greenhouse. The mycorrhiza survived for at least 10 months and continued to grow and colonize new roots. Frequent mycorrhizae and rhizomorphs were observed along the walls of the pots. Mycorrhiza was found to the bottom of the pot, 12 cm from the soil surface. Mycorrhizae sometimes turned whitish after a few weeks in the greenhouse, a reversible pigment change known from vegetative mycelium (Danell and Fries 1990). The plastic frames were too small for each individual plant. They became either too wet or too dry. The automatic watering from below caused some roots to turn black, something that did not affect roots or mycorrhizae in the topsoil. The mycorrhizae that were prepared for transfer to 0° C survived frosty nights in April.

Mycorrhiza

Since this was the first time artificially formed *C. cibarius* mycorrhizae had been studied on healthy plants in vitro and in pot culture, a morphological study was made, joining those by Moore et al. (1989) and Danell and Fries (1990). *C. cibarius* and *Picea abies* formed a mycorrhiza which at first was unramified and later irregularly pinnate. The mantle surface appeared smooth under the naked eye, but was found to be woolly under a dissecting microscope (Fig. 3). The mantle was yellow but occasionally turned whitish. The mantle remained 45 (± 3) μm thick in the pot cultures. It has been described as plectenchymatous by the previous authors,

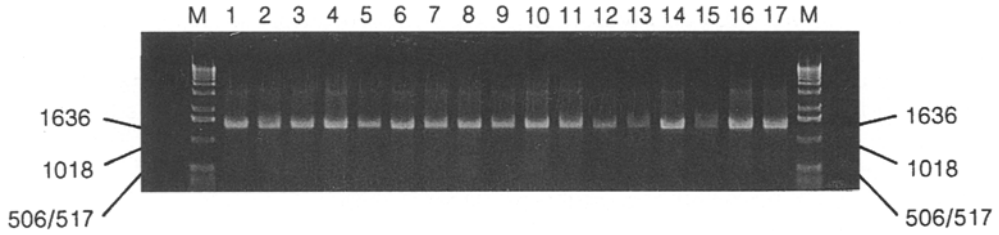


Fig. 6 Gel electrophoresis of amplified ITS of different *C. cibarius/C. pallens* strains. *M* Fragment size marker (5615SA/SB, BRL): vegetative *C. cibarius* mycelia from tissues: *lane 1* SNET1, *lane 2* SNCT1, *lane 3* SNGT2, *lane 4* SVAT7, *lane 5* NTGT4, *lane 6* NTGT1; vegetative *C. cibarius* mycelia from spores: *lane 7* NTGS1, *lane 8* GöHa; fruit bodies of *C. cibarius: lane 9 Pinus sylvestris* biotope, long. 18°23′ E, lat. 60°31′ N, *lane 10 Betula pubescens* ssp. *tortuosa* biotope, 13°42′ E, 64°36′ N, *lane 11 Betula pendula* biotope 14°05′ E, 58°05′ N; fruit body of *C. pallens: lane 12* LVT9; vegetative *C. pallens* mycelia from tissues: *lane 13* LVT3, *lane 14* LVT15, *lane 15* LVT16, *lane 16* LVT17

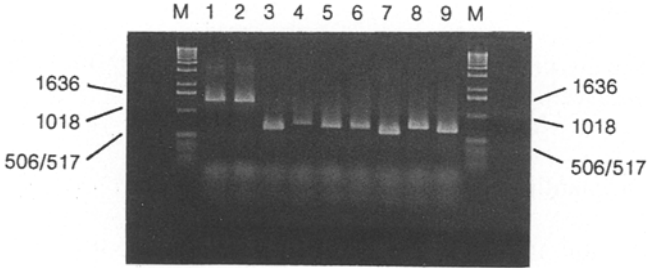


Fig. 7 Gel electrophoresis of amplified ITS of different basidiomycetes. *M* Fragment size marker (see Fig. 1); *lane 1 C. cibarius, lane 2 C. pallens, lane 3 C. tubaeformis* Bull. ex Fr., *lane 4 C. lutescens* Fr., *lane 5 C. melanoxeros* Desm., *lane 6 Gomphus clavatus* SF Gray, *lane 7 Hydnum repandum* (L. ex Fr.) Fr., *lane 8 Sarcodon imbricatus* (L. ex Fr.) Karst., *lane 9 Suillus variegatus* (Sow. ex Fr.) O. Kuntze

but the inner mantle is pseudoparenchymatous (Fig. 4). Fully developed Hartig nets colonized the entire cortex. *C. cibarius* and *Pinus sylvestris* formed a dichotomous mycorrhiza. The yellow surface was sometimes strongly woolly in pot cultures (Fig. 5). Other characteristics were equivalent to those with *Picea abies.*

Molecular identification

The molecular identification proved that *C. cibarius* had been isolated and successfully formed mycorrhiza. The size of the ITS from rDNA of vegetative mycelium corresponded with *C. cibarius* fruit body ITS (Fig. 6). The number of nucleotides of *C. cibarius* and *C. pallens* was much higher (1400 bp) than for other basidiomycetes and members of Cantharellaceae tested (600–800 bp) (Fig. 7). After cutting with the three restriction enzymes, *C. cibarius* and *C. pallens* were found to produce identical RFLP patterns (Fig. 8). The patterns of fruit bodies are similar to the patterns of vegetative mycelia. In Fig. 9, a sterile *C. cibarius* mycorrhiza is compared with mycorrhizae from greenhouse plants originally inoculated with *C. cibarius.* Alien mycorrhizae were clearly distinguished from the *C. cibarius* patterns (data not shown). The minimum number of mycorrhizal root tips that could be used for successful PCR and RFLP was 1 or 2 (DNA extract diluted 1:5 before PCR).

Discussion

Mycorrhizal establishment

The *C. cibarius* demand for additional glucose for growth and mycorrhiza formation was probably due to the artificial environment. So-called early-stage ectomycorrhizal fungi such as *Laccaria, Thelephora* and *Hebeloma* often form fruit bodies soon after mycorrhiza formation in vitro, in forest nurseries or in young plantations (Debaud and Gay 1987; Godbout and Fortin 1990). In these cases the host plant can be very small

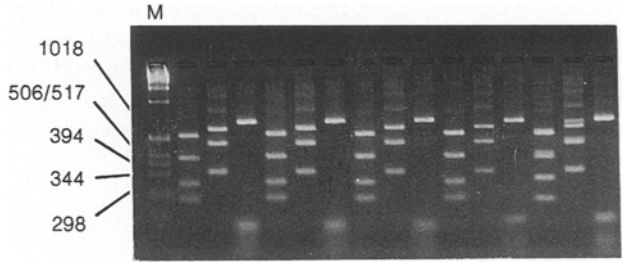


Fig. 8 RFLP analysis of the amplified ITS from *C. cibarius* and *C. pallens,* fruit bodies and axenic mycelia (no difference between strains). *M* Fragment size marker (see Fig. 1). Each strain occupies a set of three lanes: *left lane* restriction enzyme *Mbo*I, *middle lane Hinf*I, *right lane Hae*III

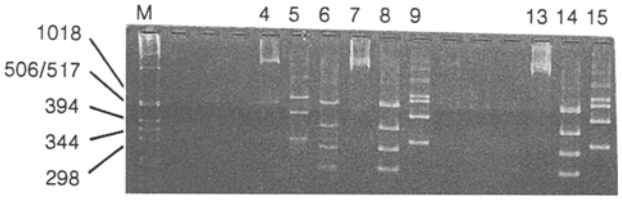


Fig. 9 RFLP analysis of the amplified ITS from *C. cibarius* mycorrhizae. *M* Fragment size marker (see Fig. 1); *lanes 4–6* ITS of axenic mycorrhiza: *lane 4* uncut ITS, *lane 5* restriction enzyme *Hinf*I, *lane 6 Mbo*I; *lanes 7–9* ITS of pot mycorrhiza: *lane 7* uncut ITS, *lane 8 Mbo*I, *lane 9 Hinf*I; *lanes 13–15* ITS of pot mycorrhiza: *lane 13* uncut ITS, *lane 14 Mbo*I, *lane 15 Hinf*I

and it is sometimes killed by the fungus. Successional studies have shown that they are replaced after a few years by late-stage fungi (Last and Fleming 1985; Dighton and Mason 1985). It is important to emphasize that this succession concerns seedlings without contact with older trees. Normally, late-stage fungi such as *C. cibarius* and *Lactarius pubescens* Fr. colonize new roots by vegetative growth from a strong mycelium that is already established (Fleming 1984; Romell 1938). In my study, the mycelium was added in the form of suspended hyphal fragments. In order to recover, glucose was needed. Dighton and Mason (1985) also demonstrated the higher demand for carbohydrates for agar growth of ectomycorrhizal late stage fungi. However, the ability of *C. cibarius* to form permanent mycorrhiza with young seedlings in the CUS shows that the term "late-stage fungus" is not adequate.

Considering the high demand for simple carbohydrates, it is difficult to understand how a young *C. cibarius* spore mycelium in nature can survive until mycorrhiza formation. This study implies that *P. fluorescens* does not act as one of the helper bacteria described by Garbaye et al. (1990, 1992). However, the seasonal production of spores of, for example, a single fruit body of *C. tubaeformis* has been estimated at 1×10^8 (Kälin and Ayer 1983). If such a clone produces many fruit bodies during a few decades, that might be enough to overcome the extremely low possibility of mycorrhiza formation from minute propagules (in laboratory conditions negligible). The overproduction of spores might have another purpose. Fries (1981) suggested that *Leccinum* species produce spores mainly to create mycelia with new genetic combinations that could be fused with the original mycelium (homing reaction). In 1987 Fries concluded that ectomycorrhizal basidiospores are insufficient for long-distance transport.

Gas effects

It is not surprising that *C. cibarius* is adapted to high CO_2 levels. The normal variation in CO_2 levels at 20 cm depth in pine forest soils is 0.5–2% (Magnusson 1992). Straatsma et al. (1986) showed that the presence of a tomato root could be replaced by 0.5% CO_2 in order to stimulate strong mycelial growth of *C. cibarius*. However, my field studies and experimental observations indicate that *C. cibarius* does not survive waterlogging. A similar phenomenon was described by Stenström (1991), who showed that early-stage fungi such as *Hebeloma* and *Laccaria* are not sensitive to waterlogging, while late-stage fungi such as *Suillus* species are. According to Magnusson (1992) waterlogging in forest soils in spring time might result in a 12% oxygen concentration and a 4% CO_2 concentration. This low oxygen concentration might be one reason for *C. cibarius* to be hydrophobic and thus create air pockets, as described by Unestam (1991). It might also be one reason why *C. cibarius* is not found in biotopes with bad water drainage. The occurrence of *C. cibarius* in the top 5 cm in the CUS units is similar to natural conditions, in which mycorrhizae are situated where minerals are released from decaying organic material. However, *Lactarius rufus* grew rapidly to the depth of 15 cm, so perhaps *C. cibarius* is restricted to the top 5 cm also because of higher oxygen demand. In the unsterile pot cultures where *C. cibarius* mycelium was found at 12 cm depth, gas exchange was facilitated by holes in the bottom side. Rainfall is not a problem if the soil permits rapid drainage. In 5-cm petri dishes where the original mycelium is submerged in liquid MFM only a few millimeters at the most, the growth of *C. cibarius* is normal.

Host specificity

Trappe (1962) concludes that *C. cibarius* has a broad host range. However, the unsuccessful colonization of *Betula pendula* with a *C. cibarius* strain from *Picea abies* gives a hint that there might be physiological varieties adapted to different groups of host plants. Strain variations in partner specificity are common among fungi or plants compatible on a species-to-species level (Last et al. 1984). Moore et al. (1989) succeeded in obtaining mycorrhiza with both *Betula pubescens* and *Pinus sylvestris* using isolates from *Quercus robur* L. However, the mycelium on *Pinus sylvestris* did not colonize the entire cortex. RFLP patterns of ITS of *C. cibarius* strains from pine and birch forests did not reveal any differences.

Fruit body formation

Only compact knots of hyphae without visible stipes were observed in *C. cibarius* (fruit body index 0.5 according to Aschan-Åberg 1958; McLaughlin 1970). No change in morphology was observed due to photoperiod and temperature changes. The distinct formation of pointed primordia of *Lactarius rufus* (fruit body index 1) appeared rapidly without any changes of the system. In the field, fruit bodies do not seem to require specific events to trigger formation. Field studies (Danell, unpublished) show that *C. cibarius* and, for example, *Leccinum scabrum* (Fr.) SF Gray and *Paxillus involutus* Batsch ex Fr. may form fruit bodies in early June. *C. cibarius* has also been found in winter and spring. However, in southern Sweden fruit bodies of *C. cibarius* usually begin to appear in the middle of July and are found until late October when fruit bodies are frozen. These field observations and the experiments in the CUS indicate that the photoperiod change after midsummer, which leads to changes in hormone balance in the host plant and a flush of carbohydrates to the roots (Wardlaw 1990), does not trigger fruit body formation. However, Godbout and Fortin (1992) observed in-

creased fruit body production in *Laccaria bicolor* (Maire) Orton when photoperiod was shortened. Temperature cannot be a trigger due to the long possible period of morphogenesis. Godbout and Fortin (1990) showed that *Lactaria bicolor* did not form fruit bodies when temperature was lowered. Probably, the mycelium begins to store nutrients as soon as photosynthesis starts and temperature is above 0° C. When enough nutrients are stored, great numbers of primordia are formed along the roots, as for *Lactarius rufus* in CUS units. Mature primordia will not develop into fruit bodies until rain comes. Agerer (1985) claims that the level of fruit body production is mainly determined by the weekly mean temperature of the first half-year, on condition that there is no drought. This has recently been found to apply to *C. cibarius* as well (Norvell 1992). Fruit bodies appear early if a hot May is followed by a rainy June, as in Uppsala in 1993, when *C. cibarius* fruit bodies appeared before midsummer. Continuous fruit body production is then limited only by drought or frost. An extreme example is the constant fruit body production of *Suillus luteus* (L. ex Fr.) SF Gray in the pine plantation on the mountain Cotopaxi in Ecuador (Hedger 1986). At this site, the temperature is always 8–16° C during the daytime, and fruit bodies of all stages are found around the year. Different ectomycorrhizal species form fruit bodies at different times, which probably reflects the efficiency in accumulating nutrients.

In the case of *C. cibarius*, the CUS revealed its ability to colonize seedlings. Several plants were transferred from aseptic CUS units to the greenhouse. PCR and RFLP showed that *C. cibarius* survived on the hosts for at least 10 months, which implies that *C. cibarius* is not exclusively adapted to older trees and therefore might form fruit bodies when the biomass is big enough. Future research should be focused on optimal greenhouse conditions, and competition between different ectomycorrhizal species can be further evaluated with PCR and RFLP. Genetic and biochemical research on the morphogenesis can be carried out thoroughly only when all the stages of the life cycle of a few strains are under control, something that may be achieved in the future. Commercial growth of *C. cibarius* might be possible through outplanting of colonized seedlings, like the management of the black truffle *Tuber melanosporum* Vitt. (Chevalier and Frochot 1981).

Acknowledgements Financial support came from the Swedish Natural Science Research Council, the Swedish Council for Forestry and Agricultural Research, the Anders Wall Foundations and the Hildur and Sven Wingquist Fund. I want to thank Professor Fries and Professor Eriksson at my department for valuable discussions. I am also grateful to Professor Unestam and his colleagues at the Department of Forest Mycology and Pathology, Swedish University for Agricultural Science and Dr. Martin and his colleagues at the Centre de Recherches Forestieres de Nancy for teaching me PCR, and to Dr. Jentschke of the Forstbotanisches Institut, Göttingen for demonstrating his equipment.

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