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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%.