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Isolation and regeneration of protoplasts from the ectomycorrhizal ascomycete *Cenococcum geophilum* Fr.

Abstract Protoplasts of the ectomycorrhizal ascomycete *Cenococcum geophilum* were isolated from mycelium grown in liquid medium. The method was optimized with regard to culture conditions, preincubation, lytic enzyme system, pH value of the incubation medium, osmotic buffer and incubation temperature for *C. geophilum* strains SIV and 1448. The yields were $1–3 \cdot 10^8$ and $7 \cdot 10^6$ protoplasts per gram fresh weight for *C. geophilum* SIV and *C. geophilum* 1448, respectively. Protoplasts from *C. geophilum* SIV exhibited plasma membrane integrity close to 100% (fluorescein diacetate staining). At least 50% of the protoplasts contained a nucleus (staining with acridine orange). The regeneration of protoplasts from *C. geophilum* is described for the first time. The regeneration frequency was up to 13%, and, dependent on the conditions of culture (liquid medium, agarose, agar), four types of regeneration patterns could be distinguished. Regenerated protoplasts of *C. geophilum* were capable of forming mycorrhizas with spruce (*Picea abies*) seedlings.

Key words *Cenococcum graniforme* · Mycorrhiza Protoplast isolation · Protoplast regeneration

Introduction

Ectomycorrhizas – symbioses between plants and fungi – are characterized by the formation of a Hartig net in the cortical layer of the root. In this region the two symbionts stay in close contact and an exchange of metabolites takes place (Kottke and Oberwinkler 1986). There is general agreement that the fungus is provided with organic carbon by the plant, whereas the plant receives inorganic nutrients from the fungus (Harley and Smith 1983). However, our knowledge about the exchange of metabolites between the symbionts and the chemical nature of the substances is rather limited. Such questions can be well investigated by using protoplasts, which are an important experimental tool for physiological and biochemical studies as well as for genetic manipulation (Fowke and Gamborg 1980; Maheshwari et al. 1986; Peberdy 1989; Hashiba 1991). However, few reports about the isolation of protoplasts from ectomycorrhizal fungi exist (Kropp and Fortin 1986; Hébraud and Fèvre 1988; Barrett et al. 1989; Anunciacao et al. 1990; Farquhar and Peterson 1990).

In a recent paper, we described an approach to the isolation of functional protoplasts from an ectomycorrhizal basidiomycete, *Amanita muscaria* (Chen and Hampp 1993a). Transport studies with these protoplasts revealed specific systems for sugar uptake, with a preference for glucose. Basidiomycetes such as *A. muscaria* accumulate trehalose, with glucose being the main substrate (Niederer 1989; Wallenda et al. 1993). Ascomycetes, in contrast, preferentially accumulate mannitol, which is formed from fructose (Niederer 1989; Wingler et al. 1993). If sugar transport across the plasma membrane of fungi is related to the respective pathways of sugar utilization, then ascomycetes should favour uptake of fructose. Such a preference could influence the exchange of metabolites in mycorrhizas. A prerequisite for testing this assumption is the availability of functional protoplasts from an ascomycete.

In this paper we describe a method which is a great improvement of an existing technique (Barrett et al. 1989) for the isolation of protoplasts from the ectomycorrhizal ascomycete *Cenococcum geophilum*, a widespread and rapidly growing fungus, with regard to both yield and integrity.

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Materials and methods

Organisms

Seeds of *Picea abies* (L.) Karst. were obtained from the Staatsklenge Nagold, Germany. *C. geophilum* Fr. strains SIV and 1448 were supplied by Dr. I. Kottke, Department of Botany, University of Tübingen. Both strains were isolated from mycorrhizas of spruce.

Chemicals

Chemicals from the following companies were used: Difco Laboratories, Detroit, Mich., malt extract; FMC BioProducts, Rockland, Calif., SeaPlaque Agarose; Honsha, Tokyo, Cellulase Onozuka RS; Merck, Darmstadt, Germany, casein hydrolysate, agar agar 1614; Boehringer Mannheim, Mannheim, Germany, bovine serum albumin, fraction V; Serva, Heidelberg, Germany, Macerozyme R-10; Sigma, St. Louis, Mo., Lysing Enzymes (*Trichoderma harzianum*).

Media

Culture media were based on modified MMN (Guttenberger and Hampp 1992). The thiamine hydrochloride content was increased to 1 mg/l. In addition, the different media contained the following substances: MMNC, 20 g/l glucose monohydrate, 10 g/l malt extract, 2 g/l casein hydrolysate; $MMNC_{1/10}$, 2 g/l glucose monohydrate, 1 g/l malt extract, 0.2 g/l casein hydrolysate; MMNC-KCl, MMNC with 0.7 M KCl; $MMNC_{1/10}$–KCl, $MMNC_{1/10}$ with 0.7 M KCl; MMNCaG, 20 g/l glucose monohydrate, 2 g/l casein hydrolysate; MMNCaF, 20 g/l fructose, 2 g/l casein hydrolysate; MMNCaS, 20 g/l sucrose, 2 g/l casein hydrolysate. The washing medium used for the isolation of protoplasts consisted of 0.8 M KCl, 5 mM MES, 1 mM $CaCl_2$, 1% (w/v) bovine serum albumin (fraction V), 0.2% (w/v) ascorbate (disodium salt) in distilled water.

Culture conditions

If not stated otherwise, *C. geophilum* SIV and *C. geophilum* 1448 were grown in liquid MMNC medium. Suspension cultures (85 ml) were incubated in 300-ml Erlenmeyer flasks with one indentation at 20° C on a rotatory shaker (80 rpm). Mycelia were subcultured every second or seventh day for strains SIV and 1448, respectively. Flasks containing 60 ml of fresh medium were inoculated with 25 ml of fresh homogenate (Ultra-Turrax, 9500 rpm, 45 s).

Isolation of protoplasts

One-day-old cultures of *C. geophilum* were collected on a 40-μm nylon mesh and rinsed with distilled water. Residual water was removed with a filter paper. Mycelium of *C. geophilum* SIV was suspended in washing medium (25 mg/ml) containing 0.625 mg/ml Lysing Enzymes, 1.25 mg/ml Macerozyme R-10 and 2.5 mg/ml Cellulase Onozuka RS. The pH of the mycelial suspension was adjusted to 5 with 20% phosphoric acid. Mycelium of *C. geophilum* 1448 (25 mg/ml) was incubated in washing medium containing 2.5 mg/ml Lysing Enzymes, 5 mg/ml Macerozyme, 10 mg/ml Cellulase Onozuka RS without further adjustment of the pH (approximately 5.8). Mycelial suspensions were incubated at 19° C for approximately 24 h. Protoplasts were separated from the mycelium by successive filtration through 40- and 20-μm nylon meshes and washed by centrifugation (3 min, 1700 *g*) and resuspension in washing medium. The yield was determined by counting the protoplasts in a Fuchs-Rosenthal haemocytometer.

Regeneration of protoplasts

After centrifugation the protoplasts were resuspended in $MMNC_{1/10}$–KCl using three different protocols:

(a) $MMNC_{1/10}$–KCl, 2.5 ml, containing 0.8% low melting agarose (28–30° C) was mixed with the same volume of protoplast suspension and poured into a sterile 9-cm petri dish.

(b) The protoplast suspension was plated on MMNC-KCl-agar (2% agar agar).

(c) The protoplast suspension was maintained as a liquid culture.

The protoplasts were incubated at 20° C and regeneration was monitored using an inverted microscope.

Characterization of protoplasts

Integrity of protoplasts was shown by staining the cells with fluorescein diacetate at a final concentration of 0.005% (w/v). Nuclei of protoplasts were stained with acridine orange at a final concentration of 0.005% (w/v). Regeneration of the fungal cell wall was followed by staining the cells with Calcofluor White (0.01% w/v). The fluorescence of the different structures was shown by microscopy 15–20 min after staining (fluorescein diacetate and acridine orange: excitation 450–490 nm, emission >515 nm; Calcofluor White: excitation 340–380 nm, emission: 430 nm).
Respiration of protoplasts was measured with a Clark-type oxygen electrode.

Formation of mycorrhizas

Surface-sterilized (25 min, 30% H_2O_2) seeds of *P. abies* were germinated on MMN agar (0.8% agar agar) in petri dishes. The dishes were maintained horizontally for 1 week and vertically for another 2 weeks (20° C; 150 $\mu Em^{-2} s^{-1}$). Mycorrhizas were synthesized according to Kottke et al. (1987) and Guttenberger and Hampp (1992). MMN agar plates (2% agar agar) were covered with charcoal filters and the root system of a seedling was spread on the filter. An aliquot (1 ml) of mycelial suspension (culture age 2 days) was applied to the filter close to the root. Root and mycelium were covered with a sheet of cellophane that had been boiled in water for 15 min. Dental rolls were placed between the cellophane and the lid of the petri dish to fix the cellophane and to absorb condensed water. Petri dishes were incubated as described by Guttenberger and Hampp (1992).

Embedding of mycorrhizas

Mycorrhizas were fixed in Karnowsky reagent [40 g/l p-formaldehyde and 2.5% glutaraldehyde dissolved in 0.2 M N-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), pH 7.2] for 1 week. Tissues were dehydrated in a graded ethanol series, infiltrated with LR White and flat-embedded between two slides. Slices (0.5 μm) were stained with a solution consisting of 0.6 g new fuchsin, 0.6 g crystal violet, 40 g glycerol and 0.6 g sodium tetraborate dissolved in 60 ml distilled water.

Results

Isolation of protoplasts

C. geophilum 1448

Lysing Enzymes (2.5 mg/ml), Macerozyme R-10 (5 mg/ml) and Cellulase Onozuka RS (10 mg/ml) were most

effective for the liberation of protoplasts from *C. geophilum* 1448 in combination. The yield of protoplasts was increased by a preincubation of the mycelium with 1.2 U/ml trypsin added to the culture for 1–1.5 h (27° C). A higher incubation temperature (27° C instead of 20° C) increased the yield from $7.7 \cdot 10^5$ to $1.6 \cdot 10^6$ protoplasts/g fresh weight (FW). When KCl was used as osmoticum the highest yield ($7.2 \cdot 10^6$ protoplasts/g FW) was obtained at a concentration of 0.77 M KCl.

C. geophilum SIV

The typically black or brown mycelium of *C. geophilum* SIV was, when used for the isolation of protoplasts, white in colour due to the culture conditions (homogenization and subcultivation every 2 days).

Reduction of the concentration of the enzyme mixture given above by 50% had little effect on the yield of protoplasts. A further reduction to 25% decreased the yield to about 60% of that obtained with the original enzyme concentration ($1.7 \cdot 10^8$ protoplasts/g FW). Release of protoplasts was observed down to 1% of the initial enzyme concentration.

KCl, sorbitol and $MgSO_4$ were tested as osmotic stabilizers. While intact (confirmed by fluorescein diacetate staining) protoplasts were obtained with KCl and sorbitol, $MgSO_4$ caused the release of a large number of very small protoplasts which did not stain with fluorescein diacetate. The highest yields were observed in the range 0.72 M–0.86 M KCl (1.2–1.5 osmol; $6.5 \cdot 10^7$–$9.1 \cdot 10^7$ protoplasts/g FW) or 0.73 M sorbitol (0.9 osmol; $3 \cdot 10^7$ protoplasts/g FW). When sorbitol was used as osmoticum, the stability of the protoplasts was high in the range 1.1 M (1.4 osmol)–1.4 M (1.8 osmol) (Fig. 1a,b).

The optimal pH was found to be about 5 in three independent experiments. A pH below 4 caused precipitation of protein and consequently cell lysis. During the incubation (24 h), the pH of the mycelial suspension showed a transient change from 5.0 to 5.5 to 4.7. Protoplasts could be isolated at all temperatures investigated (6° C–35° C; Fig. 2), but the yield was rather low at temperatures above 30° C. Incubation temperatures above 25° C resulted sometimes in high yields of protoplasts but also in lysis of protoplasts during centrifugation. High yields were reliably obtained at 20° C.

Within the first 3 days of culture, there was little influence of culture age on the yield of protoplasts; however, the culture conditions proved to be critical. Culturing the mycelia on a rotatory shaker at 80 rpm increased the yield significantly compared with 65 rpm ($7.8 \cdot 10^7$ versus $2.8 \cdot 10^7$ protoplasts/g FW). The yield (and the colour of the mycelium) also varied when the carbon source of the medium was changed (Table 1).

The yield and the diameter of protoplasts increased during incubation (Fig. 3). The release of protoplasts started as early as 15 min after the beginning of the incubation and continued even after 5 days. The yield did not increase continuously but in bursts which occurred after slightly different incubation times in different experiments. By using the method described above, 1–$3 \cdot 10^8$ protoplasts/g FW were reliably obtained.

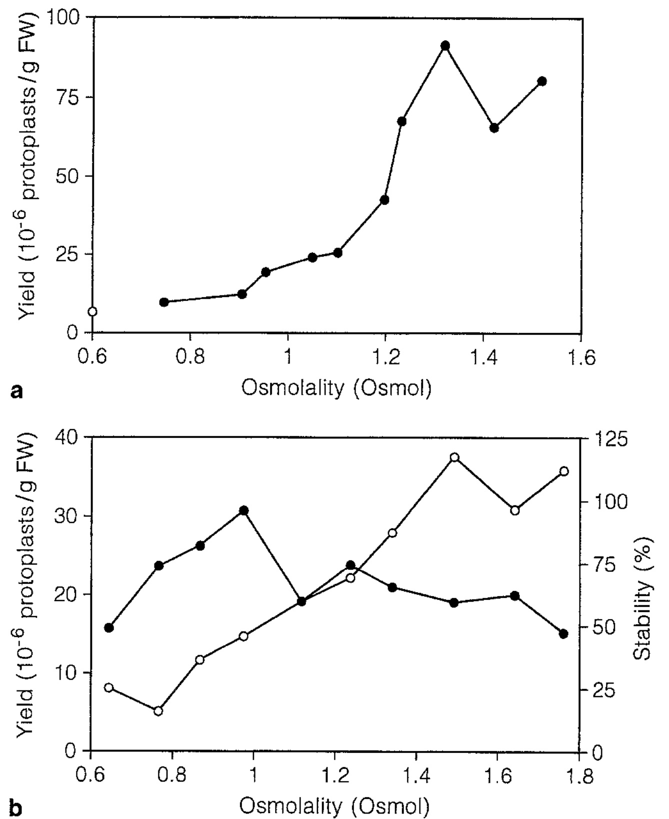


Fig. 1 **a** Influence of the concentration of KCl on the yield of protoplasts (*Cenococcum geophilum* SIV). **b** Influence of the concentration of sorbitol on the yield (●) and stability (O) of protoplasts (*C. geophilum* SIV). The stability was calculated as percentage of protoplasts still present after 4 h of incubation in media containing different concentrations of sorbitol

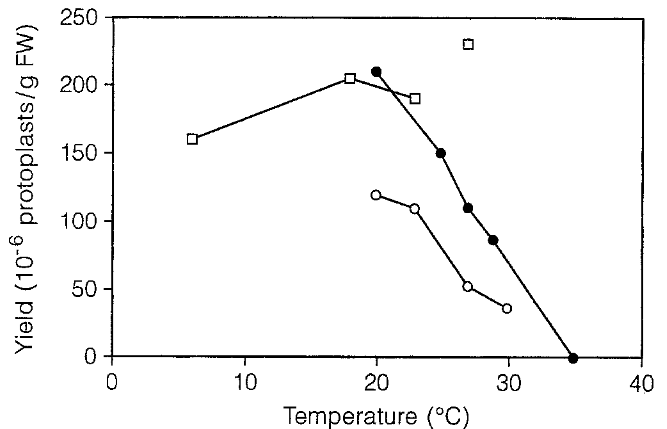


Fig. 2 Influence of the incubation temperature on the yield of protoplasts (*C. geophilum* SIV). Results of three different experiments are shown

Table 1 Colour of the mycelium and yield of protoplasts (P) after 6 h and 48 h of cell wall lysis for *Cenococcum geophilum* SIV cultured on different carbon sources (MMN: modified Melin-Norkrans medium; MMNC: MMN + malt extract + glucose; MMNCaG: MMN + casein hydrolysate + glucose; MMNCaF: MMN + casein hydrolysate + fructose; MMNCaS: MMN + casein hydrolysate + sucrose). The yield obtained from mycelium grown in MMNC medium was defined as 100% (*FW* fresh weight)

Medium	Mycelium	6 h		48 h	
		Yield (10^{-7} P/g FW)	%	Yield (10^{-8} P/g FW)	%
MMNC	White	8.7	100	1.7	100
MMNCaG	Grey	0.5	6	0.5	30
MMNCaF	White-brownish	0.4	5	1.4	82
MMNCaS	White	0.5	5	1.7	101

Properties of protoplasts from *C. geophilum* SIV

Nearly 100% of the protoplasts were stained with fluorescein diacetate (Fig. 4a,b). Approximately 50% of the protoplasts had a nucleus as estimated by staining with acridine orange and some of the protoplasts had more than one nucleus (Fig. 4c). The respiration rate of the protoplasts was 100 to 500 pmol $O_2/(10^6$ protoplasts·min).

Regeneration of protoplasts from *C. geophilum* SIV

Protoplasts plated on MMNC-KCl agar showed a regeneration frequency of 2–3%. The regeneration frequency increased to about 12–15% by immobilization of the protoplasts in 0.4% agarose (three independent experiments), but protoplasts were quite sensitive to mechanical and temperature stress during the immobilization. Protoplasts cultivated in liquid medium agglutinated during the first hours of regeneration. Determination of the regeneration frequency was thus impossible.

Four types of regeneration patterns could be distinguished: in liquid medium or agarose, the protoplasts formed hyphae either immediately (Fig. 4d) or after a period of yeast-like growth (Fig. 4e). The formation of a germ tube-like structure was observed occasionally (Fig. 4f).

On agar plates, protoplasts showed some kind of hypertrophy before a number of hyphae started to grow out simultaneously from the centre of the cell (Fig. 4g,h). Like mycelial cell walls, the cell wall of regenerates could be stained with Calcofluor White (Fig. 4e) indicating the presence of β-1,4/β-1,3 glucans (Nagata and Takebe 1970; Benitez et al. 1976). This fluorescence became visible a few hours after the isolation of protoplasts and later intensified.

Formation of mycorrhizas

All mycelia obtained from cultures of regenerated protoplasts investigated (five cultures) were capable of forming ectomycorrhizas with spruce. The mycorrhizas exhibited the same morphological and histological structures as those formed by mycelium of the original culture. The fungal sheath was thin (Fig. 5a) and consisted of hyphae with dark brown (outer layer) or hyaline (inner layer) cell walls. The Hartig net was well de-

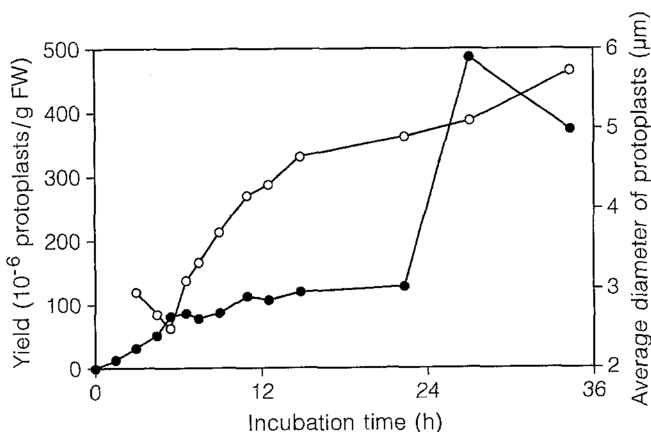
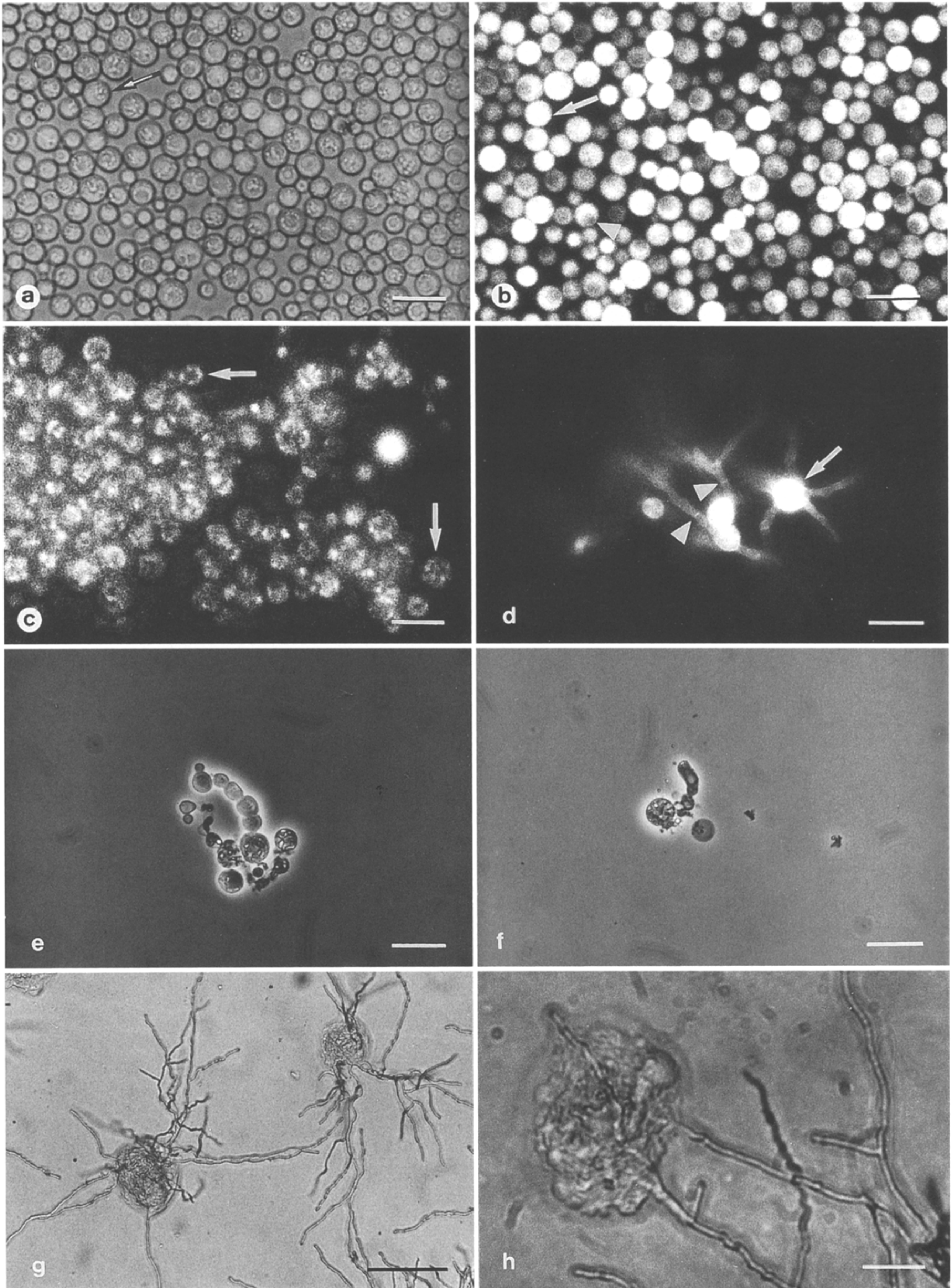


Fig. 3 Influence of the incubation time on the yield (●) and average diameter (○) of protoplasts (*C. geophilum* SIV). Both determinations were carried out in the same experiment. The yield increased strongly after 22 h of incubation

Fig. 4a–h Protoplasts of *C. geophilum* SIV and their regeneration. **a** Representative preparation of protoplasts. **b** Fluorescence after staining with fluorescein diacetate. The photograph is identical to that in **a**; *arrows* mark the same protoplast. Vacuoles are visible as less fluorescing areas of the protoplasts (*arrowhead*). **c** Fluorescence after staining with acridine orange. *Arrows* mark protoplasts containing more than one nucleus. **d** Fluorescence of 4-day-old regenerates stained with Calcofluor White. The protoplasts were cultured in liquid medium. Regeneration started with the development of hyphae. The fluorescence of the regenerating protoplasts (*arrow*) and hyphal septa (*arrowhead*) is much stronger than that of the hyphae, indicating a different cell wall composition. **e** Yeast-like regeneration of protoplasts cultured in liquid medium for 1 day. **f** Formation of a germ tube-like structure by a protoplast cultured in liquid medium for 1 day. **g** Regeneration of protoplasts cultured on agar-plates. The cells are hypertrophed. **h** Regenerating protoplasts cultured on an agar-plate. The regenerating protoplast is well structured. It is clearly visible that hyphae grow out from structures originating from the inner part of the regenerating protoplast. *Bars* **a,b,d–f** 20 µm; **c** 10 µm; **g** 100 µm; **h** 25 µm ▶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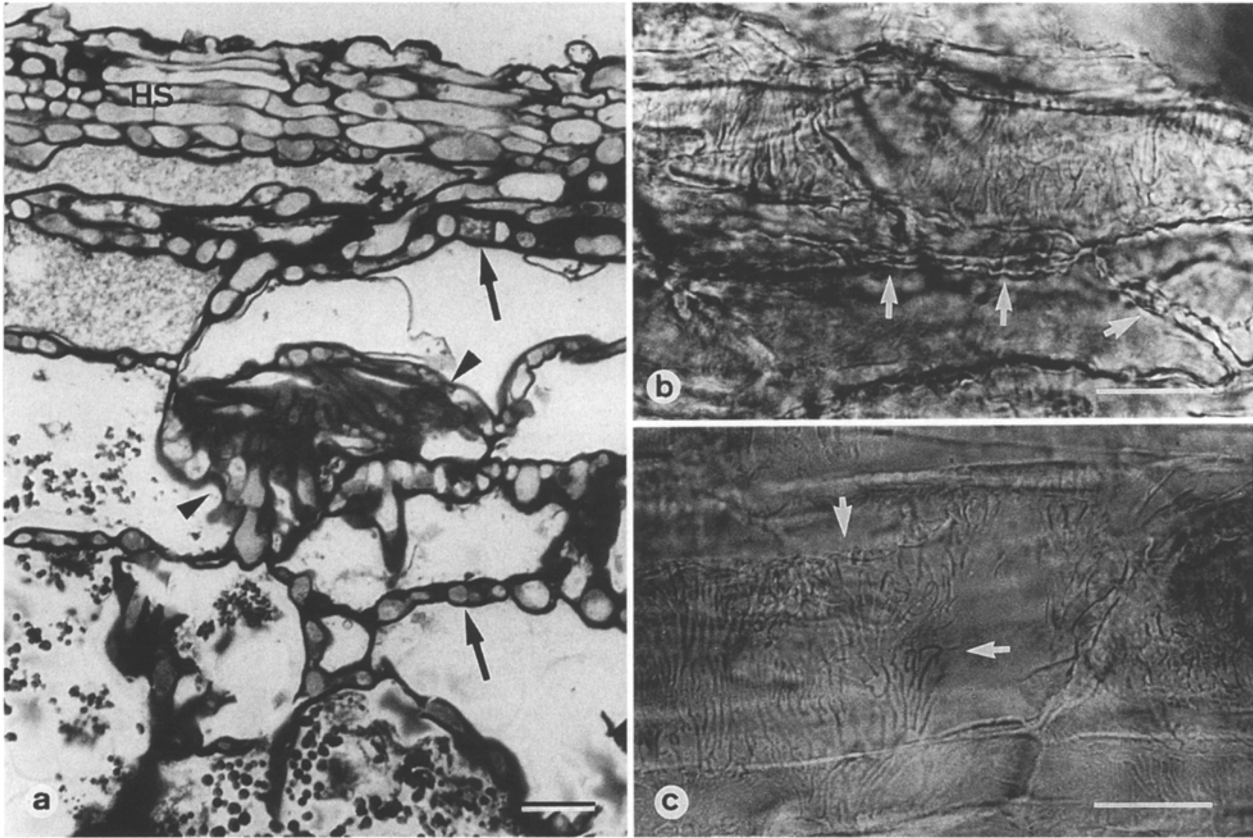

Fig. 5a–c Mycorrhizas formed by mycelium of *C. geophilum* SIV and cultures of regenerated protoplasts. **a** Longitudinal section of a mycorrhiza of the original culture of *C. geophilum* SIV with spruce (*Picea abies*). The hyphal sheath (*HS*) is thin, the Hartig net well developed. In the area of the Hartig net, hyphae between cortical cells (*arrows*) and hyphae forming lobes (*arrowheads*) on the surface of cortical cells can be seen. **b,c** Longitudinal section of a mycorrhiza formed by mycelium of a regenerated protoplast and spruce. Hyphae between cortical cells (*arrows*) in the area of the Hartig net are visible in **b**. Hyphae forming lobes (*arrows*) on the surface of a cortical cell in the area of the Hartig net are visible in **c**. *Bars* **a** 10 μm; **b,c** 20 μm

veloped in all samples and extended over the whole cortical layer of the root (Fig. 5a–c). Hyphae of the Hartig net had hyaline cell walls. Mycorrhizas were greyish or yellow-brownish coloured.

Discussion

Mycelia of the ascomycete *C. geophilum* delivered high yields of intact protoplasts when incubated under appropriate conditions, although there were differences between strains. The yield of protoplasts was ten times higher for white mycelium of *C. geophilum* SIV than for black mycelium of *C. geophilum* 1448. Our data also show that culture conditions exert considerable influence upon protoplast yield. Formation of protoplasts from *C. geophilum* SIV was better after culture in MMNC and MMNCaS than in MMNCaG. Mycelia grown in the former were white (Table 1). The grey colour that developed in mycelia during culture in MMNCaG is presumably due to encrustation of the cell wall with melanins. These may protect the fungal cell wall against the lytic enzymes employed. This assumption is in accordance with results of Bull (1970), who showed that β-1,3-glucanase and chitinase are inhibited by melanins. Taylor et al. (1987) describe a higher resistance of wild strains of *Wangiella dermatitis* to cell wall lytic enzymes than of melanin-deficient mutants. Similarly Jang et al. (1993) observed lower yields of protoplasts when well-pigmented strains of *Cryphonectria parasitica* were used. Protection of the cell wall of *C. geophilum* by melanins is also indicated by the fact that well-pigmented hyphae, in contrast to hyaline ones, were not stained with Calcofluor White (data not shown) or aniline blue (Mikola 1948).

At osmolalities of 1.2 osmol and above, the yield of protoplasts was lower with sorbitol than with KCl (*C. geophilum* SIV), although the protoplasts exhibited a high stability with sorbitol (Fig. 1). This might be due to inhibition of the lytic enzymes by high sorbitol concentrations, as suggested by Sipiczki et al. (1985) for Novozyme 234. Novozyme 234 is an enzyme preparation similar to the Lysing Enzymes used in our experiments.

The optimal parameters found for the formation of protoplasts from *C. geophilum* SIV and *C. geophilum* 1448 differ from those published for *C. geophilum* 155 (Barrett et al. 1989). For the latter strain, the osmolality

was 2.5 osmol rather than 1.4 osmol, and the optimal pH 6.3 rather than 5.0. In addition, protoplasts from *C. geophilum* SIV could be isolated at a lower temperature (20° C versus 31° C) and at lower enzyme concentrations than protoplasts from *C. geophilum* 155. This might be due to differences in the cell wall composition of the three strains. Jang et al. (1993) point out that the optimal conditions for the isolation of protoplasts must be tested for each strain of a species, and for a number of species different methods have been described in the literature (*Agaricus bisporus*: Sonnenberg et al. 1988; Chen and Hampp 1993b. *Hebeloma cylindrosporum*: Hébraud and Fèvre 1988; Barrett et al. 1989. *Laccaria laccata*: Kropp and Fortin 1986; Barrett et al. 1989). However, for *C. geophilum* SIV ten times higher yields were obtained than for *C. geophilum* 155 (1 ml mycelium of *C. geophilum* SIV = 22 mg DW = 130 mg FW = $2.6 \cdot 10^7$ protoplasts; 1 ml mycelium of *C. geophilum* 155 = $2.2 \cdot 10^6$ protoplasts) (Barrett et al. 1989); the incubation time for both strains was 24 h. The low incubation temperature and enzyme concentration used for the isolation of protoplasts from *C. geophilum* SIV might be of special importance for the viability of protoplasts. *C. geophilum* is known to be sensitive to temperatures of 30° C and above (Mikola 1948; Hacskaylo et al. 1965; Hutchison 1991). Novozyme 234 (Roncal et al. 1991), presumably in common with Lysing Enzymes, contains cell lytic activities which can influence the viability of protoplasts if the agent is used at high concentrations e.g. 3 mg/ml for Novozyme (Roncal et al. 1991).

The regeneration of protoplasts from *C. geophilum* is described here for the first time. Protoplasts from *C. geophilum* isolated by Barrett et al. (1989) had no nuclei and failed to regenerate. The regeneration frequency of 13% is high enough for genetic experiments and in the range reported for other ectomycorrhizal fungi (Kropp and Fortin 1986; Hébraud and Fèvre 1988; Barrett et al. 1989; Anunciacao et al. 1990).

Regeneration of protoplasts of *C. geophilum* exhibited four different patterns. These patterns observed in liquid media were similar to those described by Annè et al. (1974) for protoplasts of *Penicillium chrysogenum*. The fourth pattern, found on agar plates with a high agar content (2%), is not known from the literature. It is not clear whether the hypertrophed cells were really hypertrophed or if a mucilaginous material was excreted into the medium. This latter would also explain why the hyphae seemed to grow out from the middle and not from the margin of the cell.

Regenerated protoplasts of *C. geophilum* SIV are able to form ectomycorrhizas with spruce seedlings. Both mycelium obtained from the original isolate and from regenerated protoplasts failed to form a well-developed, black fungal sheath in our experiments. This might be due to the culture conditions. An influence of culture conditions on the structure of the fungal sheath of ectomycorrhizas was described by Kottke and Oberwinkler (1986). Park (1970) described white and yellow-brown ectomycorrhizas of *C. geophilum* with *Tilia americana* and postulated a dependency of the colour of mycorrhizas of *C. geophilum* on the physiological state and age of the mycorrhizas. However, the formation of ectomycorrhizas by regenerated protoplasts strongly indicates that the formation of protoplasts does not affect the physiological properties of *C. geophilum*.

In preliminary experiments, we have shown that protoplasts of *C. geophilum* can be subjected to silicon oil filtration as described for protoplasts from *A. muscaria* (Chen and Hampp 1993a). As a next step, we will try to characterize systems for metabolite transport at the plasma membrane of protoplasts from *C. geophilum*.

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