Trees (1987) 1: 191–194

Structure and Function
Trees

Short communication

An in vitro method for establishing mycorrhizae on coniferous tree seedlings

I. Kottke, M. Guttenberger, R. Hampp, and F. Oberwinkler

Institut für Biologie I, Universität Tübingen, Auf der Morgenstelle, 7400 Tübingen, Federal Republic of Germany

Summary. A method for in vitro synthesis of mycorrhizae on coniferous tree seedlings is described. Tree seedlings (*Larix decidua* Mill., *Picea abies* (L.) Karst. and *Pinus sylvestris* L.) and fungi (*Amanita muscaria* (L. ex Fr.) Hooker, *Piloderma croceum* Erikss. et Hjorst., *Pisolithus tinctorius* (Pers.) Coker et Couch. and *Suillus grevillei* (Klotzsch) Singer were maintained under sterile conditions in petri dishes. Typical ectomycorrhizae were established within 2–3 weeks after inoculation and within 2 months after germination of seedlings. Eventually a high percentage of mycorrhizal root tips was obtained.

Key words: Basidiomycetes – Coniferous trees – Ectomycorrhiza – In vitro culture – Symbiosis

Introduction

Recently, there has been a resurgence of interest in the cellular processes involved in establishing mycorrhizae on roots of higher plants. Culture in petri dishes under controlled, sterile conditions removes many technical constraints that an investigator would otherwise face. Advantages of the use of such cultures include the ability [1] to monitor continuously the entire root system along with the symbiont, [2] to exclude any adverse effects of contaminating microorganisms, [3] to control parameters such as developmental stage and [4] to excise tissues for study with the minimum system perturbation.

Offprint requests to: I. Kottke

We describe here a new culture method. Under the reported conditions, root growth is typical and symbiosis is established within 2–3 weeks.

Materials and methods

Media. The MMN solutions (medium a and b) used were a modification of those of Marx (1969); thus: [1] the concentrations of ferric chloride, glucose and malt extract were lowered, [2] the concentration of nitrogen was increased, [3] trace elements (Johnson et al. 1957) and casein hydrolysate were added as detailed below. The two solutions were:

(a) $CaCl_2$ 0.05 g, NaCl 0.025 g, KH_2PO_4 0.5 g, $(NH_4)_2HPO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, $FeCl_3 \cdot 6H_2O$ about 1 mg, thiamine hydrochloride 100 µg, glucose monohydrate 5 g, stock solutin of trace elements (see below) 10 ml, per litre.
(b) as (a), but $(NH_4)_2HPO_4$ 0.25 g, glucose monohydrate 10 g, malt extract 5 g, casein hydrolysate 1 g.

MMN-agar contained 2% (w/v) Agar Agar (Merck 1614).
Stock solution of trace elements: KCl 3.728 g, H_3BO_3 1.546 g, $MnSO_4 \cdot H_2O$ 0.845 g, $ZnSO_4 \cdot 7H_2O$ 0.575 g, $CuSO_4 \cdot 5H_2O$ 0.125 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.018 g, per litre.

Plant culture. Seeds of *Picea abies* (L.) Karst., *Pinus sylvestris* L. and *Larix decidua* Mill. were from the Staatsklenge Nagold (FRG). Before germination, seeds were surface sterilized by shaking them gently in hydrogen peroxide (30%) for 25 min at ambient temperature. Germination was on modified MMN-agar (a) in petri dishes. This medium contained sugar, which was not necessary for plant growth. The inclusion of sugar served as an indicator of contamination. Upon needle emergence seedlings were transferred into 500-ml Erlenmeyer flasks that contained 300 ml of a perlite-sphagnum mixture (90/10) moistened with 150 ml of modified MMN-nutrient solution (a). The flasks were sealed with a cotton stopper and kept in a growth chamber for 4 weeks (16 h light, 25 W m^{-2} at the level of the plants: Osram Universal White fluorescent tubes; 8 h dark, 22° C). The most vigorous plants were selected for inoculation.

Fungal culture. Mycelia of *Amanita muscaria* (L. ex Fr.) Hooker and *Suillus grevillei* (Klotzsch) Singer were isolated from fruiting bodies. *Piloderma croceum* Erikss. et Hjorst. strain

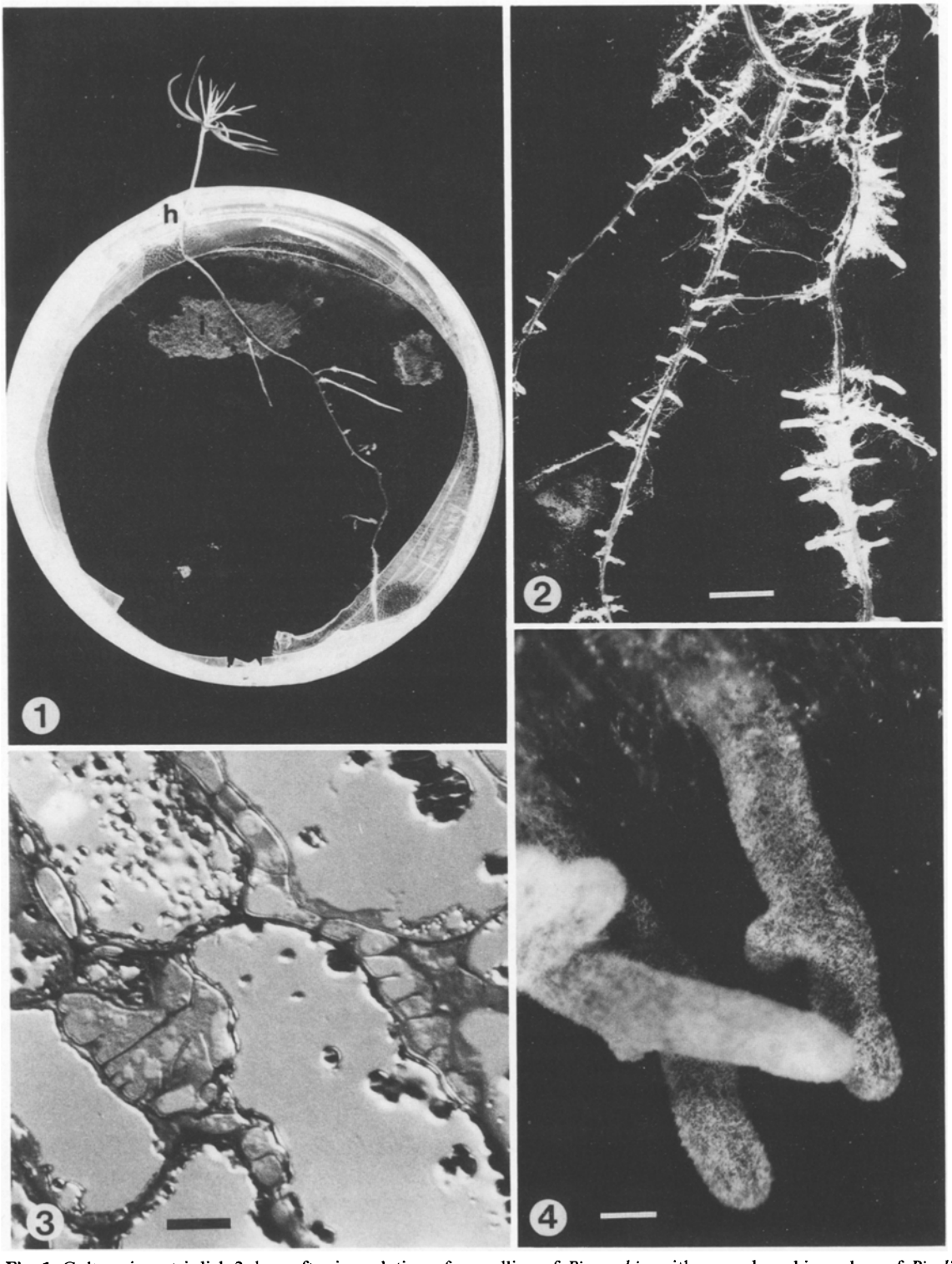

Fig. 1. Culture in petri dish 2 days after inoculation of a seedling of *Picea abies* with paper-based inoculum of *Pisolithus tinctorius*; *i* inoculum on charcoal paper, *h* hole in the petri dish

Fig. 2. Totally mycorrhizal root system of *Pinus sylvestris* 3 weeks after inoculation with *Pisolithus tinctorius*. *Scale mark* is 5 mm

Fig. 3. Normally developed Hartig net structure of *Suillus grevillei-Larix decidua* with fingerlike branched hyphae. *Scale mark* 10 μm

Fig. 4. Mycorrhiza of *Amanita muscaria-Picea abies* with typical mantle structure formed on charcoal paper. *Scale mark* 100 μm

C106 was obtained from the Department of Forest Mycology and Pathology, Uppsala. *Pisolithus tinctorius* (Pers.) Coker et Couch. strain Lelley/Marx 298 was obtained from the Department of Botany, Munich/Weihenstephan, FRG. Mycelia were stored in test tubes on modified MMN-agar (b) at 8° C. Prior to use as inoculate the mycelia were cultivated on MMN-agar (b) for about 3 weeks. Five to ten small plugs of actively growing mycelia were transferred to discs of sterilized, activated charcoal paper (active carbon paper; Macherey-Nagel + Co, Düren, FRG; MN 728; 9 cm in diameter) in petri dishes. Five millilitres of nutrient solution (b) was added. Mycelia covered the paper in 10–14 days; these papers were used to inoculate seedlings.

Inoculation. Inoculated seedlings were grown in a modified 90 mm petri dish (Fig. 1). The sidewall of the bottom half of the petri dish was punctured with a hot scalpel. The roots of a single seedling were inserted through this hole. Empirically, we found that the optimum-sized seedling had three to four main roots and several initials of short roots. The seedling, including the roots, was positioned horizontally. Then the roots were covered with the filter-paper inoculum, which restricted the roots to a single plane between the bottom of the petri dish (lower side) and the filter paper (upper side). Three millilitres of nutrient solution (a) was added. The petri dish was closed and a strip of Parafilm used to seal the dish. Aluminium foil was wrapped around the dish to keep the roots in darkness.

Symbiont culture. Cultures were maintained in a special planting vessel, the bottom of which was covered with moist cotton. As an additional measure to prevent evaporation, the planting vessel was covered with a plastic top. Culture conditions were otherwise as described under "plant culture".

Cultures were monitored twice weekly. Addition of fresh nutrient solution was at least biweekly. Measurement of growth rates of mycorrhizal and non-mycorrhizal roots was carried out by means of a Mop-Videoplan (Zeiss-Kontron). The development of the root system was marked on the bottom of the petri dish.

Results and discussion

This method for in vitro synthesis of mycorrhizae was developed in order to overcome technical difficulties which we experienced in adapting established techniques to our experimental demands. Intending to work on different stages of ontogeny, a technique suitable for continuous measurement of root growth was especially important. Nylund and Unestam (1982) synthesized mycorrhizae on filter paper in glass tubes. However, glass tubes are not convenient for observation and measurement of root growth. With the growth pouches described by Fortin et al. (1983) maintenance of sterile conditions proved difficult. The sandwich technique of Chilvers et al. (1986) does not allow for direct observation of mycorrhizal development. In addition, only small seedlings can be enclosed in petri dishes.

The synthesis of mycorrhizae on filter paper in petri dishes under aseptic conditions seemed promising in dealing with these problems. However, under these conditions seedlings did not thrive; root growth was frequently retarded. This, perhaps, can be ascribed to the swollen apices, which failed to form fungal associations. These pathological symptoms were not observed if granular activated charcoal or peatmoss were included, indicating the presence of a noxious exudate from the fungus or plant. An absorptive medium seemed to be required for undisturbed root growth, which is necessary for establishing symbiosis. Therefore, we replaced the filter paper by activated charcoal paper. The advantage of activated charcoal has also been described by Duclos and Fortin (1983) for the in vitro synthesis of ericoid mycorrhizae.

Mycorrhizae still only occurred in the upper parts of the petri dish, where the roots were not surrounded by a water film. A drastic reduction of the amount of nutrient solution eventually resulted in a high percentage (nearly 100%) of mycorrhizal root tips. Accordingly, low moisture of the substrate is an indispensable prerequisite. A water film surrounding the root inhibited mycorrhiza development (perhaps, because partially anoxic conditions developed). Insufficient moisture had a deleterious effect on both organisms. It was thus critical to maintain optimum moisture conditions.

The advantage of a paper-based inoculum is the expanse of the mycelium. Thus, contact with roots is enhanced. In addition, agar plugs tend to detach from the (vertical) support, unless they are affixed by growing mycelium. As the root system of the seedlings spreads on the surface of the paper, convenient observation of mycorrhization under the stereo microscope was possible. Fungi and tree species that were examined, yielded good results with this technique (Figs. 2–4).

Acknowledgements. The authors are indebted to Professor W. H. Outlaw, Jr., for correcting the English. The skilful technical assistance of O. Ebinger-Gäbele is gratefully appreciated. This work was supported by grants from the Deutsche Forschungsgemeinschaft (R. H.) and the Projekt Europäisches Forschungszentrum, Karlsruhe (I. K.).

References

Chilvers GA, Douglass PA, Lapeyrie FF (1986) A paper-sandwich technique for rapid synthesis of ectomycorrhizas. New Phytol 103: 397–402

Duclos JL, Fortin JA (1983) Effect of glucose and active charcoal on in vitro synthesis of ericoid mycorrhiza with *Vaccinium* spp. New Phytol 94: 95–102

Fortin JA, Piché Y, Godbout C (1983) Methods for synthesizing ectomycorrhizas and their effect on mycorrhizal development. Plant Soil 71: 275–284

Johnson CN, Stout PR, Broyer RC, Carlton AB (1957) Comparative chlorine requirements of different plant species. Plant Soil 8: 337–353

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59: 153–163

Nylund J-E, Unestam T (1982) Structure and physiology of ectomycorrhizae. I. The process of mycorrhiza formation in Norway Spruce in vitro. New Phytol 91: 63–79

Received July 13, 1987