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Changes in the fungus-specific, soluble-carbohydrate pool during rapid and synchronous ectomycorrhiza formation of *Picea abies* with *Pisolithus tinctorius*

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Abstract. A simple and convenient culture system has been developed for the analysis of ectomycorrhiza formation under controlled conditions. Rapid and synchronous mycorrhiza synthesis was observed when thin and even layers of *Pisolithus tinctorius* (Pers.) hyphae were brought at once into contact with the entire root system of 3-month-old *Picea abies* (L. Karst) plants. Suitable fungal layers were grown on cardboard with limiting glucose supply in the medium to maximize radial growth. The glucose was almost consumed by the time the fungus had spread over the whole cardboard and was ready for inoculation of the roots. At this stage, the fungus contained trehalose and arabitol as the main soluble carbohydrates. A few hours after the assembly of the culture system, contacts between roots and aerial hyphae were observed and a sheath was formed 3 days later, suggesting very rapid ectomycorrhiza formation under these conditions. The pool of soluble carbohydrates of the inoculum, i.e. the extramatrical mycelium, declined after inoculation of the roots and was almost zero after 2 weeks. The supply of carbon by the plant was then sufficient for the fungus to expand the soluble pool efficiently in both the mycorrhizas and the extramatrical mycelium. The kinetics of the carbohydrate pool and the observed differentiation of the short roots to mycorrhizas imply that in our culture system fully functional symbiosis was established no later than 14 days after the plants were inoculated with the fungus.

Key words: *Picea abies* - *Pisolithus tinctorius* - Mycorrhiza formation - Trehalose - Polyols

Introduction

Several methods have been described for the axenic culture of ectomycorrhizas. Initially, flask cultures with various media were used to confirm mycorrhizal associations (Marx and Zak 1965). Subsequently, ectomycorrhizas were successfully produced in growth pouches and tubes (Fortin and Piche 1980; Nylund and Unestam 1982; Yang and Wilcox 1984). However, the lack of aseptic conditions in the first case and slow mycorrhiza formation due to plug inoculation in both methods were major drawbacks. Culture-dish techniques introduced by Grellier et al. (1984) proved to be superior since they allowed for more appropriate climatic conditions for the shoot in terms of humidity and light intensity. Moreover, axenic conditions were maintained in the root compartment and the formation of mycorrhizas could be monitored continuously. With one exception, namely mycorrhiza formation on primary roots of eucalyptus seedlings (Horan et al. 1988), the fungus under study was still introduced as a plug at one or several points of the root system (Wong and Fortin 1989), or it was forced to grow in patches through charcoal paper, eventually spreading along the roots from the points of contact (Kottke et al. 1987). Thus, various developmental stages of mycorrhiza were always present on the root system.

For our current studies on the response of *Picea abies* to ectomycorrhiza formation, it was essential that the mycorrhizas developed rapidly and concurrently at all possible sites on the root system. Moreover, accurate determinations of enzyme activities and metabolite concentrations are feasible only if sufficient quantities of mycorrhizas at an identical developmental stage can be obtained. The culture-dish technique described here fulfils the above criteria and allowed the direct observation of the timing of mycorrhiza development and the related events. This was probed by analysing the kinetics of the fungus-specific, soluble-carbohydrate pool.

Materials and methods

Treatment of the seedlings

Spruce seeds (*P. abies* L., Karst) were kindly provided by the Eidgenössische Forschungsanstalt für Wald, Schnee und Landschaft,

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Birmensdorf, Switzerland. The seeds were surface-sterilized in 30% (v/v) aqueous H_2O_2 for 15 min at room temperature and washed with sterile distilled water with changes at 5, 15, and 30 min, and after 3–4 h. Germination was carried out at 27° C in the dark on 1.5% agar with modified MMN medium (Marx 1969) containing less glucose (2 g/l) and malt extract (0.6 g/l) than in the original recipe. After approximately 20 days, three germinated seeds with radicules 2–4 cm in length were transplanted aseptically into a wide-neck Erlenmeyer flask containing a mixture of vermiculite (Vermica AG, Bözen, Switzerland), fertilized peat (Flora guard TKS 2, 300–450 mg N/l, 200–350 mg P_2O_5/l, 350–600 mg K_2O/l) and distilled water (1:1:2, v/v/v), which had been sterilized twice at 120° C for 40 min with an interval of 3 days. The flasks with the seedlings were stoppered with cotton and incubated in a phytotron with a day/night cycle of 16 h 240 $\mu E\, m^{-2}\, s^{-1}$ at 20° C and 8 h dark at 16° C. Light was provided by Sylvania cool white lamps (F 96T12/CW/VHO) and measured by a Li-cor 1600 radiometer. Under these conditions, the seedlings were ready for ectomycorrhiza synthesis after 3 months.

Cultivation of the fungal inoculum

The culture of *Pisolithus tinctorius* (Pers.) Cokeret et Couch, strain Lelly/Marx 298, was kindly provided by Dr. I. Kottke and grown at 27° C on 1.5% agar-containing MMN medium. Plugs of inoculum from this culture were transferred to agar plates with nine different glucose and malt extract concentrations. Culture diameter was measured when the fungus reached the edge of the Petri dish on one of the various media. The fungus was grown also on various supports placed on MMN agar and culture diameter measured as above. In this way the optimal culture conditions for rapid and synchronous ectomycorrhiza formation were determined.

Experimental conditions for mycorrhiza synthesis

A 3-month-old axenic plant with several first-order laterals was collected from one of the Erlenmeyer flasks and carefully freed of vermiculite and peat with forceps. The roots were placed into a Petri dish with the wall cut in one place to allow the shoot to protrude, and the whole root system covered by a cardboard overgrown with the fungus. A wad of cotton served to store nutrients (30 ml MMN medium without glucose and malt extract) and maintain ideal moisture conditions, and it also pressed the cardboard gently against the root system. There were thus no problems with either water logging or drought (Kottke et al. 1987). Finally, the culture dish was sealed along the edge with parafilm. The entire procedure was carried out rapidly in a laminar-flow bench to maintain axenic conditions inside the culture dish.

Determination of soluble carbohydrates

Clusters of mycorrhizal short roots were collected and freeze dried. The samples were then ground to powder in a Retsch mill (Germany). The solute was extracted from the cardboard support after sampling of the hyphal mat and was concentrated in a Speedvac (Zivy, Switzerland). Aliquots of the powdered mycorrhizas, the cardboard extract and the entire mycelial sample were extracted with 80% (v/v) aqueous methanol at 65° C. The soluble carbohydrates were freed from acids, derivatized, and analysed by gas chromatography (Shimadzu 14A) according to Frehner et al. (1984).

Results

Cultivation of the fungus

Radial growth of the fungus on MMN agar increased when glucose and malt extract were lowered step by step from 20 g/l to 200 mg/l and 3 g/l to 30 mg/l, respectively. At still lower glucose and malt extract concentrations, the growth rate gradually decreased (Fig. 1).

On agar with the usual MMN medium, *P. tinctorius* grew slowly and formed concentric rings with either

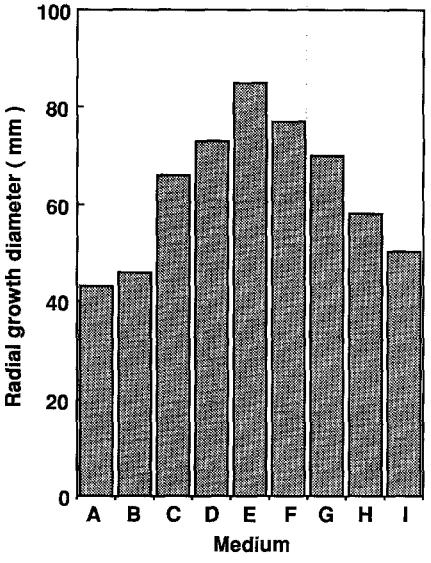

Fig. 1. Growth of *Pisolithus tinctorius* on 1.5% agar with MMN medium and various concentrations of glucose and malt extract, respectively. *A* 20 g/l, 3 g/l; *B* 7 g/l, 1 g/l; *C* 2 g/l, 0.3 g/l; *D* 700 mg/l, 0.1 g/l; *E* 200 mg/l, 30 mg/l; *F* 70 mg/l, 10 mg/l; *G* 20 mg/l, 3 mg/l; *H* 7 mg/l, 1 mg/l; *I* 1 mg/l, 0 mg/l

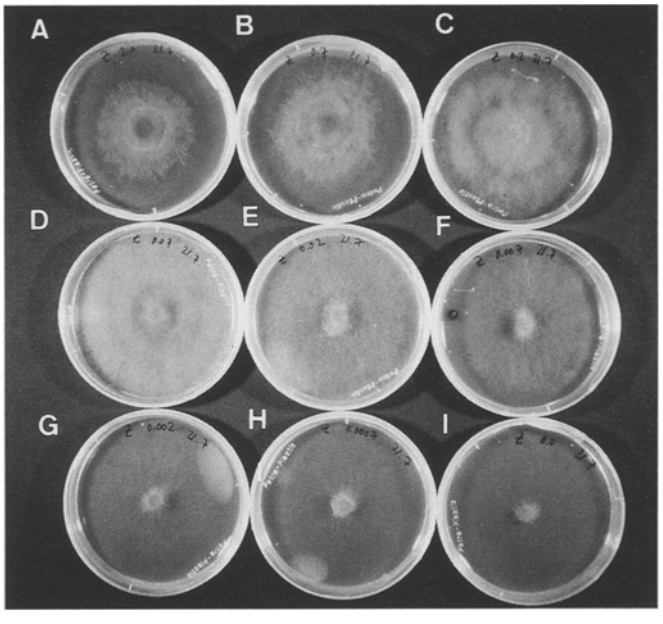

Fig. 2. Mycelial mats of *P. tinctorius* grown with media *A–I* given in Fig. 1

abundant and few aerial hyphae (Fig. 2). These cultures were unsuitable as inocula for mycorrhiza formation, since only part of the fungus made good contact with the root system. Lowering the glucose and malt extract concentrations in the medium resulted not only in more rapid radial growth but also in progressively smoother fungal layers. A thin and homogeneous mat was obtained at the optimal concentration for fast radial growth, namely with 200 mg/l glucose and 30 mg/l malt extract.

Growth differed markedly on various supports (Fig. 3). Of all the materials tested, ordinary filter paper was clearly the worst. Better results were obtained with activated charcoal paper, dialysis membrane, cellulose nitrate and glass fibre filters, but the growth rates were not nearly as good as on cardboard, for which the fungus had an obvious preference. It should be noted that in the presence of a solid support, the sugar concentrations in the medium had to be adjusted to 2 g/l for glucose and 0.3 g/l for malt extract to achieve fungal layers of the quality shown in Fig. 2E. Under these conditions, it took about 14 days for the hyphae to grow from the plug of inoculum to the edge of the Petri dish, at which point the cardboard was placed into the culture dish to inoculate the *P. abies* roots.

The extract of a cardboard support which had been left on modified MMN agar for several hours contained about 2.4 mg/ml glucose (day −14, Fig. 4). When the cardboard was completely overgrown by the fungus, the glucose content was decreased to 0.3 mg/ml (day 0); it vanished completely after an additional 7 days of cultivation (day 7). We should point out that the residual glucose was diluted further from 0.3 mg/ml to less than 0.1 mg/ml when the cardboard was placed into the culture dish since the mineral solution supplied with the cotton wad did not contain any sugar. Thus the external glucose supply was effectively depleted when the fungus came into contact with the host root system. The fructose and maltose present in modified MMN medium are also potential carbon/energy sources, but the results in Fig. 4 demonstrate that these sugars were hardly used by the fungus.

Under optimal growth conditions for mycorrhiza synthesis, *P. tinctorius* hyphae showed high contents of trehalose and arabitol at the time of harvest, i.e. when the hyphae had reached the edge of the culture dish after 14 days (Fig. 5). This internal carbohydrate pool was gradually consumed when the fungus was left further on the glucose-depleted medium at 27° C. After 7 days, trehalose was reduced by 90% and arabitol by 30%. About 35% of the initial arabitol was still present after 90 days of cultivation, and by that time constituted the bulk of the soluble carbohydrates (16 µg/mg dry wt.). Mycorrhiza formation was still possible with fungal mats which had grown for a total of 21 days, but the best results with regard to rate and homogeneity of mycorrhiza synthesis were obtained with 14-day-old inocula which had just grown over the entire support. The data suggest that without help from the host plant, *P. tinctorius* could subsist on glucose-depleted medium for several months, slowly consuming its internal sugar reserves.

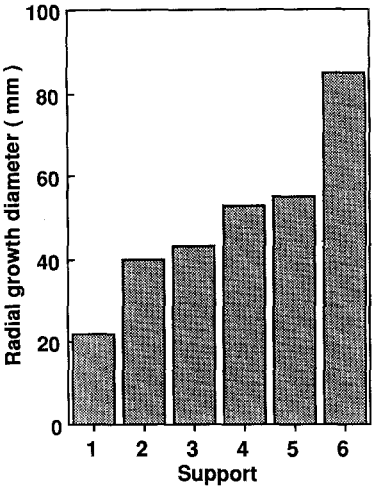

Fig. 3. Radial growth of *P. tinctorius* on various supports placed on 1.5% agar with MMN medium and 2 g/l glucose and 0.3 g/l malt extract. *1* Filter paper, Schleicher and Schuell LS 14; *2* carbon filter paper, Machery and Nagel MN 728; *3* dialysis membrane, Spectra/Por 4; *4* membranes of cellulose nitrate, Millipore HA 0.45 µm; *5* glass fibre paper, Whatman GF; *6* cardboard, Schleicher and Schuell, 293

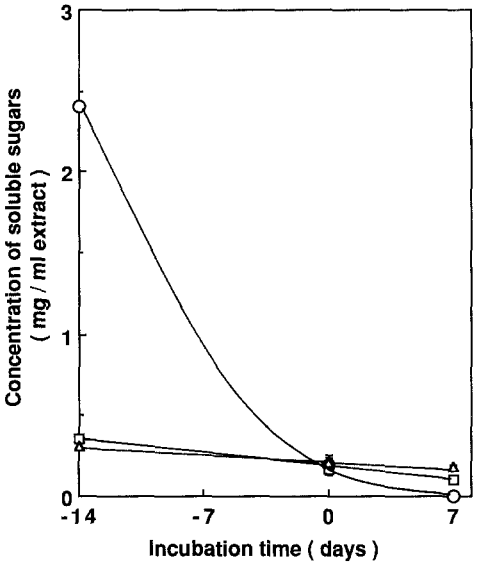

Fig. 4. Soluble sugars extracted from the cardboard before inoculation of *P. tinctorius* (day −14), when the fungus had reached the edge of the Petri dish (day 0), and 7 days later (day 7). Glucose (○), fructose (□), maltose (△)

Ectomycorrhiza development

The formation of mycorrhizas could be observed through the bottom of the culture dish. Pictures of a single short root were taken on consecutive days following inoculation (Fig. 6). Initially, some aerial hyphae were in contact with the roots (Fig. 6A). On the 3rd day, the network of hyphae around the short roots was considerably denser, and the rootlet, although growing, did not form any root hairs (Fig. 6B). Ten days after inoculation, the rootlet had doubled in length and was cov-

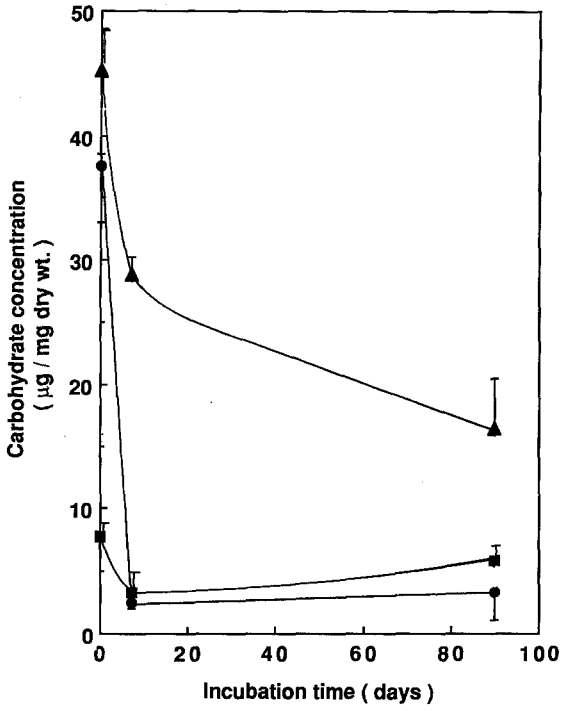

Fig. 5. Soluble carbohydrates of *P. tinctorius* mycelium. Samples were taken 14 days after inoculation, i.e. when the fungus had reached the edge of the Petri dish (day 0), and 7 and 90 days later. Arabitol (▲), mannitol (■), trehalose (●)

ered with a rough, brown mantle (Fig. 6C). On the 28th day, the fungus had completely enveloped the root (Fig. 6D). The mycorrhiza appeared to grow intermittently, with bulges and constrictions representing phases of vigorous and slow growth, respectively. As intended by the experimental design, mycorrhiza formation proceeded concurrently on all short roots and in much the same way as on the one shown in Fig. 6. Strands originating at and spreading from rootlets served as morphological markers and strongly suggested a mutual exchange of substances (Fig. 6E). In the overview of a typical section of the root system (Fig. 6F), the single short root displayed in the previous panels is emphasized by an arrow.

Changes in the fungus-specific carbohydrates during rapid and synchronous mycorrhiza formation

The fungus-specific, soluble carbohydrates were analysed in samples of the mycorrhizas and in the extramatrical mycelium (Figs. 7, 8). Whilst trehalose and mannitol were already found in the developing mycorrhizas 4 days after inoculation, the arabitol content was at the limit of detection (Fig. 7). The trehalose content began to increase rapidly 14 days after inoculation and the arabitol content several days later. Mannitol remained roughly at the level reached after 14 days.

Initially, the fungal inoculum had a high content of arabitol and trehalose but these pools decreased to zero 14 days after inoculation (Fig. 8). During this period, the fungus grew rapidly and formed a thick mantle around the developing short roots (Fig. 6). Since the medium was practically free of glucose and other carbon sources at the time of inoculation, the observed fungal growth could only be sustained by internal carbon reserves (e.g. glycogen) or by carbon provided by the plant. The contents of trehalose and arabitol began to increase rapidly after 14 days and 21 days, respectively. During this period, the fungal sheath was still growing in thickness, but the carbon supply was apparently so high that the pool of soluble carbohydrates expanded to include the whole extramatrical mycelium. Mannitol, which was hardly detectable initially, started to increase from the 10th day, i.e. before trehalose and arabitol, but remained at a comparatively low level up to day 30.

As a control, fungal mats were cultivated without plants but otherwise under similar conditions as for ectomycorrhiza synthesis (Fig. 9). The soluble carbohydrate pool of the mycelium decreased as in the presence of the plant, but in contrast to the symbiotic system the soluble pool was not replenished after 14 days.

Discussion

The long-term goal of our studies is to understand better the physiological and biochemical events which control the formation of ectomycorrhizas. This goal can be realized only with an experimental system that allows rapid and concurrent mycorrhiza synthesis on all parts of a host root system; otherwise events related to the development of the various root tissues may obscure the processes of the symbiosis between fungus and plant. The experimental procedure presented here was designed to this specification.

To check whether transfer and inoculation of the roots was possible with very little disturbance and physiological response, the plant-stress indicator ethylene was measured (Wiemken et al. 1990). Ethylene production by the roots did not increase after the transplantation of the plantlets from the Erlenmeyer flasks to Petri dishes. Thus, handling of the plantlets was possible without wounding. It was also important that the various tissues could be harvested quantitatively and undamaged. During the experimental period, neither the roots nor the fungus penetrated the cardboard support, although they did penetrate substrates such as charcoal and filter paper. The fungal mats could be lifted off in one piece, and the roots did not break when removed from the support. Sequential sampling of mycorrhizas, extramatrical mycelium, and of root and fungal exudates in the cardboard is possible using the culture system described here.

Fungal soluble carbohydrates have received considerable attention for some time (for a review, see Lewis 1989). The fungus-specific sugar trehalose, which structurally and functionally resembles the plant disaccharide sucrose, was found to be an excellent stress protectant, especially in yeast (Wiemken 1990). The rate of trehalose synthesis from glucose supplied in the medium was

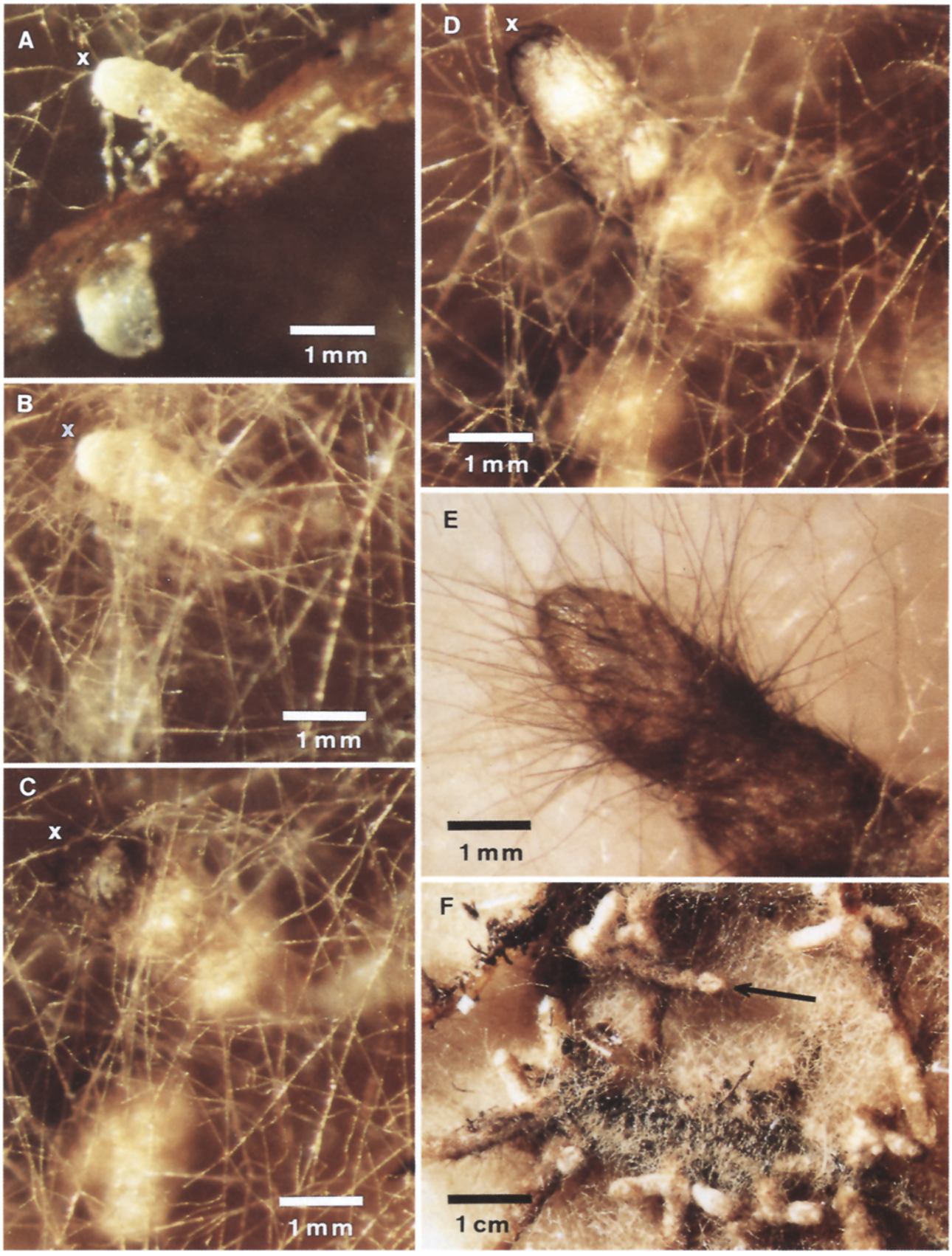

Fig. 6A–F. Mycorrhiza formation of one single side root and a typical picture of a single and a group of mycorrhizas. After **A** 1 day; **B** 3 days; **C** 10 days; **D** 28 days; **E** typical mycorrhiza with spreading strands of hyphae, 28 days; **F** part of the root system crowded with mycorrhizas, no bare roots, 28 days

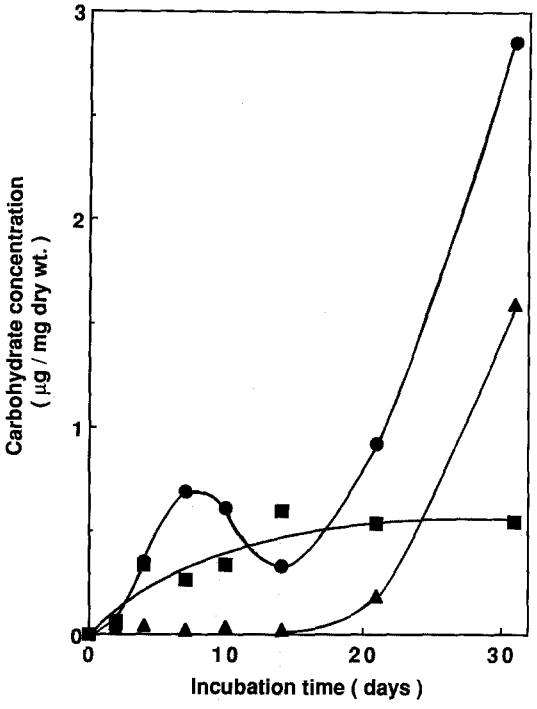

Fig. 7. Changes in the fungus-specific, soluble carbohydrate pool during the development of mycorrhizas. Arabitol (▲), mannitol (■), trehalose (●)

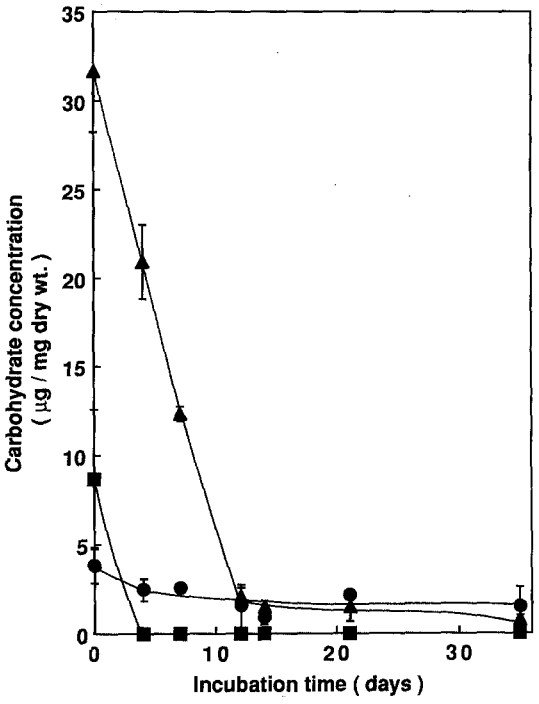

Fig. 9. Changes in the soluble carbohydrate pool of the mycelium of *P. tinctorius* kept without either a plant or C source in the medium in the Petri dish culture system. Arabitol (▲), mannitol (■), trehalose (●)

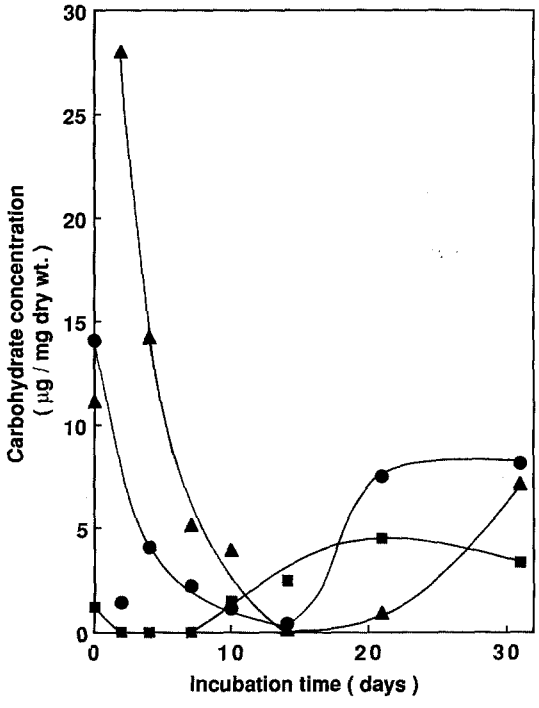

Fig. 8. Changes in the soluble carbohydrate pool of the extramatrical mycelium during the development of mycorrhizas. Arabitol (▲), mannitol (■), trehalose (●)

taken as measure of vitality for excised ectomycorrhizas of spruce (Niederer et al. 1989). In mycorrhizas, the plant sugars are transformed into fungal carbohydrates and thus become unavailable to the plant (Lewis and Harley 1965). During the development of the mycorrhiza, the pattern of fungus-specific, soluble carbohydrates changed characteristically (Figs. 7, 8). The entire soluble pool of the fungal inoculum was consumed during the intensive growth phase, i.e. the mantle formation, which started as soon as the fungus and the root were brought together (Fig. 7). Since plant sugars were not yet available, the entire fungal mat of the inoculum probably became the source of carbohydrates for the hyphae which were in touch with the short roots and were the main sink. At first, the plant was probably, the source of carbohydrates only for the mycorrhiza-forming part of the fungus (increase after 4 days) and then later for the whole fungal mat (increase after 14 days). With mycorrhiza formation starting at one or several points, we would expect an almost constant level of soluble carbohydrates in the extramatrical mycelium from the beginning until mycorrhizas had become established over the whole root system.

The 1 : 1 ratio of mannitol and trehalose found 4 days after inoculation may suggest that glucose and fructose from plant sucrose was already the carbon source of the fungus, since excised mycorrhizas supplied with glucose or fructose formed mainly trehalose and mannitol, respectively (Lewis and Harley 1965). However, *P. tinctorius* is not able to grow on fructose as sole C source (data not shown) and another explanation for the pres-

ence of mannitol at the beginning of mycorrhiza formation is required. It is known that organic N sources favour an enhanced mannitol and a reduced trehalose content (Holligan and Jennings 1972). The hyphae in contact with the short roots might be fed with organic N compounds of plant origin (exudate) and not via extramatrical mycelium. Note that on the 4th day no mannitol was present in the extramatrical mycelium.

As the symbiosis was progressively established (after 21 days), trehalose was the main carbohydrate in mycorrhizas. According to Martin et al. (1988), it is probable that the fungus maintained a small mannitol pool and formed trehalose via a mannitol cycle from the surplus of carbon delivered by the plant. Trehalose could then serve for carbohydrate transport, intermittent storage or stress protectant.

To conclude, we suggest that the carbon supply by the plant in our system started around the 4th day for hyphae which were in contact with the roots, and was well established after 2 weeks when the whole fungal mat was able to enlarge its soluble carbohydrate pool during a period of intense growth.

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