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Mycorrhizal associations between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*

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Abstract. We set out to establish root cultures of a host plant with the aim of obtaining dual cultures of *Tuber melanosporum* mycorrhiza on transformed roots. Seedlings of *Cistus incanus* germinated under sterile conditions from seeds collected in the wild were treated with *Agrobacterium rhizogenes*. Nine hairy roots collected from different seedlings were cultured individually by repeated subculturing. The hairy root clones differed in growth rates and in morphology (branching frequency and distance between side roots). Root growth in a liquid medium exhibited a lag phase of about 2 weeks and an exponential phase lasting about 12 days before the start of the stationary phase. Hairy roots could be kept alive on medium M, a special solid minimal medium (low in Fe^{2+} , BO_4^{3-} , Ca^{2+} , Cu^{2+} and Zn^{2+} , very low in PO_4^{3-} and MoO_4^{2-} and lacking NH_4^+ and Co^{2+}), for more than 7 months. *T. melanosporum* could be grown on the same medium for long periods only by subculturing the fungus with the roots. A mycorrhizal association developed between the roots and the *T. melanosporum* mycelium within 3 months. The association consisted of elongated roots with a mantle and a Hartig net surrounding two to three layers of cortical cells. Swollen, club-like root tips were discernible 5 months after inoculation. The mycorrhized roots could be subcultured and propagated on medium M and maintain the mycorrhizal association.

Keywords. *Cistus* - Transformed roots - *Tuber melanosporum* - Dual culture

Introduction

For inoculation purposes, specific isolated cultures of ectomycorrhizal fungi are preferable to spores, particularly because they facilitate the selection of better-performing, mycorrhization-efficient fungal genotypes through the application of the same principles that govern the selection of crop plants with improved performance. However, the ability of isolated mycelia to mycorrhize their host plant is reduced after continuous subculturing on artificial media, rendering long-term use of a specific mycelial culture impractical. This ability may be restored by passage through a mycorrhizal stage (Thompson et al. 1993; Roth Bejerano and Kagan-Zur, unpublished). As the aim of our research was to work with isolated mycelia of *Tuber melanosporum* Vitt., because of the advantage mentioned above, we undertook to establish a transformed root culture of one of its host plants and subsequently to obtain a *T. melanosporum*-mycorrhized root culture. Such a dual culture could be useful with respect to a basic study of *T. melanosporum* mycorrhizal associations, as their interaction and intensity have yet to be elucidated. *Cistus incanus* L. was chosen as the host, since it is one of the naturally occurring plant symbionts of this fungus (Giovannetti and Fontana 1982) and because it grows wild in Israel (Feinbrun-Dotan and Danin 1991).

Cultures of *Agrobacterium rhizogenes*-transformed roots have been used for a number of years to facilitate the cultivation of vesicular arbuscular mycorrhizal fungi, which do not grow readily on artificial media (Becard and Piche 1989; Douds 1997). Such cultures provide a convenient way of obtaining reliable sterile inoculation material (spores or mycorrhized roots) for research (Diop et al. 1992; St. Arnaud et al. 1995; Villegas et al. 1996; Douds 1997; Wan et al. 1997). In the case of isolated ectomycorrhizal fungi, which grow readily on artificial media (Harley and Smith 1983), the cultivation of mycorrhizal roots has not been considered essential for the successful cultivation or study of these fungi. A report on ectomycorrhiza-like structures obtained on spruce callus in culture (Sirrenberg et al. 1995) appears to confirm that the cultivation of ectomycorrhiza on artificial media is indeed feasible. We report here the successful establishment of a *T. melanosporum*-mycorrhized *cistus* hairy root culture.

Materials and methods

Plant and fungal material and in vitro cultures

Cistus incanus seeds collected from the wild were abraded with no. 0 sand paper, immersed in concentrated sulfuric acid for 20 min, rinsed thoroughly, immersed in 1% NaOCl for 6 min, and rinsed again with sterile water. The seeds were then left to soak in the water of the last wash for 2-3 h before sowing. Woody plant medium (WPM) (Lloyd and McCown 1981), containing 0.5 mg l⁻¹ indole-3-butyric acid (IBA) and 0.02% activated charcoal, was used for seed germination and the initial cultivation of seedlings for subsequent preparation of hairy root cultures. Seedlings were allowed to grow for 3 weeks before inoculation with *Agrobacterium rhizogenes*.

A. rhizogenes (strain 15834) was routinely grown in liquid YM medium (Petit and Tempe 1978) for 48 h at 25°C on a shaker rotating at 100 rpm. At the end of 3 weeks of growth, de-rooted *C. incanus* seedlings were inoculated at the epicotyl with *A. rhizogenes*. Thin, delicate hairy roots emanating from the inoculation points appeared 8-11 days after inoculation in about 60% of the inoculated seedlings, and these were transferred individually to N5 medium [MS medium (Murashige and Skoog 1962) with 20% ammonium nitrate and potassium nitrate] containing 500 mg l⁻¹ of ampicillin. After at least one passage on this cleansing medium, nine clones of hairy roots, generally from different seedlings, were

established and transferred regularly on N5 medium without ampicillin. Hairy root cultures were grown on solidified N5 medium in stationary tubes or petri dishes, or in 5 ml of liquid N5 in 50-ml Erlenmeyer flasks agitated at 100 rpm. in order to establish root cultures and subsequent mycorrhization with *Tuber melanosporum*.

T. melanosporum mycelium strain 1015 (kindly donated by Dr. G. Chevalier, INRA, Clermont-Ferrand, France) was subcultured routinely on Fontana agar medium (Bonfante and Fontana 1973) at either 20°C or 25°C (different growth chambers). For mycorrhization, a cube of Fontana agar containing the *Tuber* mycelium was placed onto hairy roots growing on medium M in a glass test tube (Balaji et al. 1995), or the cube was placed alongside the hairy root, both having started at the same time in petri dishes.

The N5, WPM, M, and Fontana media were adjusted to pH 5.8, 5.7, 5.5, and 6.0, respectively, before autoclaving. All media were gelled with 8% Difco Bacto agar and autoclaved at 121°C for 20 min. In some experiments, medium N5 and medium M were gelled with 0.2% gelrite (Phytigel, Sigma).

Test tubes containing seedlings were maintained in a growth chamber at 25°C under a 16-h (light) photoperiod (cool-white, 30 $\mu\text{E m}^{-2} \text{s}^{-1}$). Root cultures were kept in the dark at either 25°C or 20°C in petri dishes or test tubes or agitated on a shaker under conditions of dim light (3 $\mu\text{E m}^{-2} \text{s}^{-1}$).

In vitro fungal growth and colonization evaluation

The growth of *T. melanosporum* in dishes was evaluated by marking the circumference of the mycelium under a binocular microscope starting 10 days after inoculation. The area of the mycelium was then determined with NIH image analysis software. Fungal colonization of the roots was assessed by staining with 0.01% trypan blue in lactic acid, as described by Kagan-Zur et al. (1994).

Results

Characterization of the different *Cistus* hairy root clones

Medium N5, liquid or solidified, was chosen for characterizing the growth habit of the different clones. Some hairy root clones, such as clones L2A and W13, grew slowly, while others, such as clones W10-3 and W51, accumulated 4-5 times more biomass than the slow-growing clones (Table 1). Percentage dry matter ranged between 13% and 14% and was similar among clones, with one exception that had 10% dry matter (data not shown). There was no correlation between absolute growth (fresh weight) and percentage dry matter. For the hairy root cultures in tubes, the roots initially grew horizontally towards the tube wall and then began to grow in circles, finally filling the space above the medium with the main root mass of their growth (around 1 cm high) (Fig. 1). Some root tips eventually grew in a positive geotropic manner, and some even penetrated into the gel, but these had not reached the bottom by the end of 3 months. The growth of clones L2A, W52, and W10-3 was also evaluated in agitated flasks. While roots accumulated much more biomass under agitation in a liquid medium than on stationary solid medium, the relative biomass accumulation in the flasks was the same as that in the tubes: W10-3 > W52 > L2A (Table 2). In general, upon being transferred from solid medium to liquid, agitated medium, the roots first broadened for about 2 weeks and only then developed numerous new root tips. The growth curve of clone W10-3 grown in liquid N5 medium exhibited a lag phase of 12 days, an exponential phase, and a stationary phase that commenced about 30 days after culture initiation (Fig. 2). In this culture, the original root explant changed in color from white to either yellow or dark brown and stained the medium yellow. The roots did not grow when 3% sucrose was replaced with either 1% or 3% glucose.

Table 1. Growth of different *Cistus incanus* hairy root clones after 5 weeks in stationary tubes. Cultures were initiated from 0.6 mg of root apical segments (2 cm long). Values are the means of six replicates \pm standard error (*fw* fresh weight)

| Clone | Fresh weight (mg/tube) | Increase in fresh weight ^a |
|------------|------------------------|---------------------------------------|
| L2A | 18.6 \pm 4.7 | 30 |
| W13 | 28.7 \pm 5.8 | 47 |
| W11 | 32.6 \pm 9.0 | 55 |
| W3 | 42.2 \pm 17.0 | 69 |
| W10-2 | 53.5 \pm 7.2 | 93 |
| W52 | 59.0 \pm 10.8 | 101 |
| W22 | 64.7 \pm 9.5 | 110 |
| W10-3 | 73.2 \pm 8.8 | 121 |
| W51 | 97.5 \pm 34.0 | 162 |
| LSD (0.05) | 35.2 | 58.1 |

^a Increase = (final fw-initial fw)/initial fw

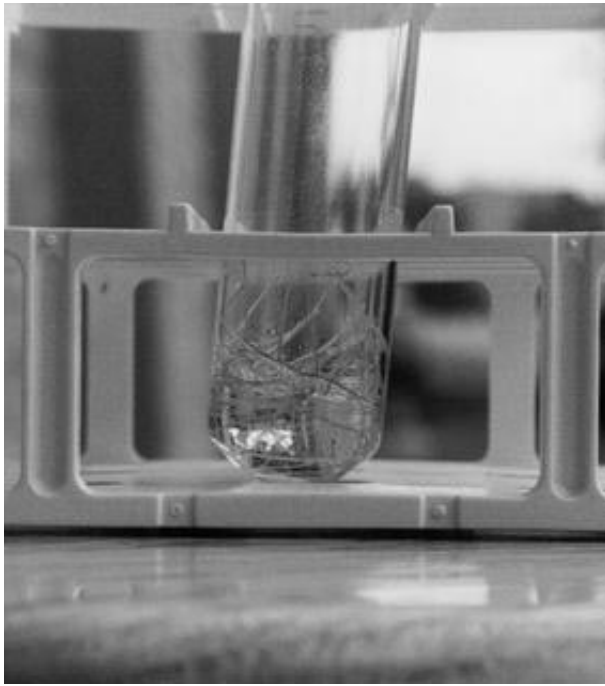


Fig. 1. Transformed *Cistus* root culture. Note lack of geotropism: roots grow into, on, or away from the solid medium

Table 2. Growth of different *C. incanus* hairy root clones after 4 weeks in flasks on a shaker (100 rpm). Values are means of four replicates \pm standard deviation (*fw* fresh weight)

| Clone | Initial fresh weight (mg) | Final fresh weight (mg/flask) | Increase in fresh weight ^a |
|------------|---------------------------|-------------------------------|---------------------------------------|
| L2A | 5 | 64.0 \pm 11.4 | 11.8 |
| W52 | 10 | 134.5 \pm 39.6 | 12.5 |
| W10-3 | 16 | 561.5 \pm 111.1 | 34.1 |
| LSD (0.05) | | 204.8 | 14.4 |

^a Increase = (final fw-initial fw)/initial fw

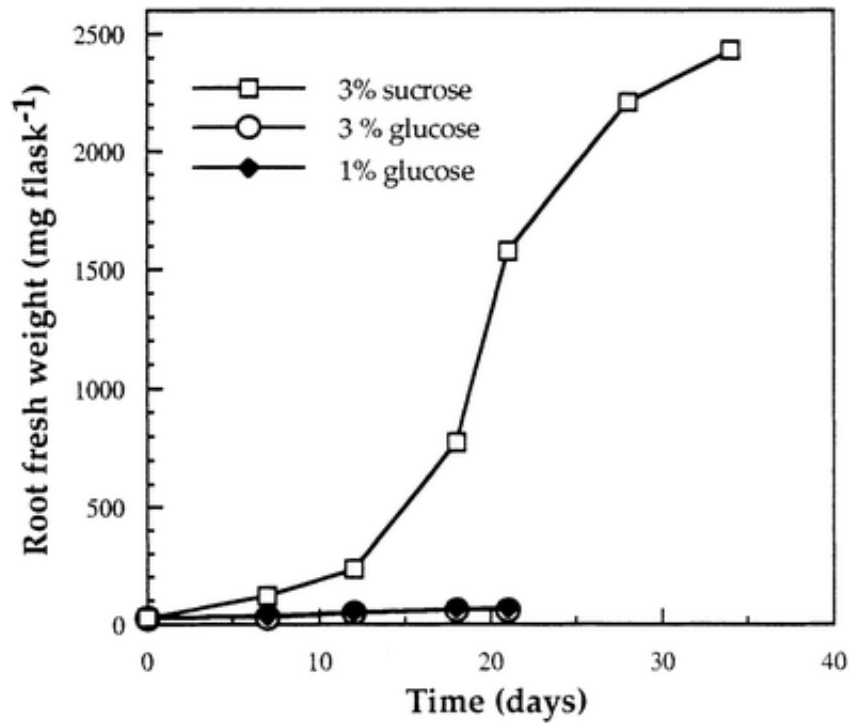


Fig. 2. Growth curve of hairy root clone W10-3 of *Cistus incanus* grown in liquid N5 medium on a shaker (100 rpm) with 3% sucrose or 1% or 3% glucose for 34 days. Each flask was inoculated with three tips, each 10 mm in length. Values are the means of three flasks

The growth habit differed among the hairy root clones, as was clearly evident when those roots grown in flasks were compared (Fig. 3). The variation between clones was expressed in terms of the frequency of side branches and the distances between them, with some clones exhibiting almost no branching. None of the clones in the petri dishes grew in circles as had been observed in tubes.

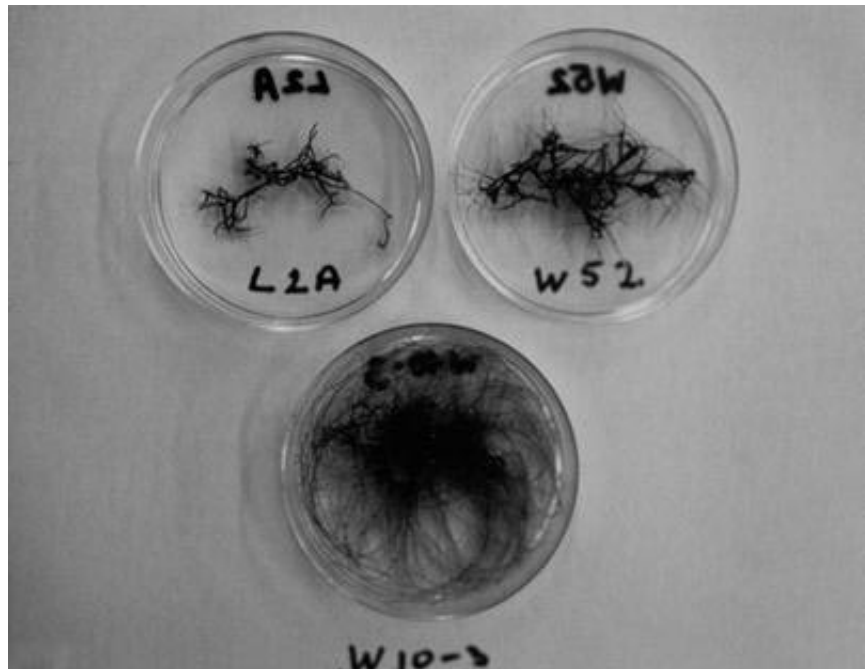


Fig. 3. Hairy root clones of *C. incanus* grown in liquid N5 medium for 4 weeks. Roots were placed in a petri dish for photography

Hairy roots of *Cistus* remained viable for many months when grown on solidified N5 or M medium: new root tips appeared upon transfer, even after long periods between transfers. A piece of a thick root (1 mm in diameter) was able to develop side roots after 200 days. Regrowth from and on medium M was evident after 285 days.

Mycorrhization experiments

Medium M was developed by Balaji et al. (1995) for the mycorrhization of hairy roots of *Pisum* with *Gigaspora margarita*. This medium differs from N5 medium in several of its constituents: it is enriched in Mg^{2+} , lower in Fe^{2+} , BO_4^{3-} , Ca^{2+} , Cu^{2+} , and Zn^{2+} , much lower in PO_4^{3-} and MoO_4^{2-} , and lacking NH_4^+ and Co^{2+} . Medium M contains only 1% sucrose. *Cistus* hairy roots grew on medium M, and while *T. melanosporum* mycelium grew well when first transferred to medium M, after a subsequent transfer to the same medium less mycelium developed. A third transfer to medium Fontana resulted in no growth at all, indicating death of the fungus.

Growth relationships between the fungus and hairy roots in dual culture were studied on medium M in petri dishes. The two partners were indeed able to grow together. Roots as young as 10-25 days and as old as 217 days developed new side roots and induced *T. melanosporum* to develop new hyphae next to them, finally enwrapping them. Mycelium developed faster on medium M when cultured alongside young roots (total new area ranged between 12 and 19 $mm^2 day^{-1}$) than alongside old roots (total new area: 5 $mm^2 day^{-1}$). Root segments covered with hyphae could be transferred to fresh medium M to stimulate proliferation of both the roots and the fungus. For example, a fungus-containing agar piece (medium M) placed next to roots of clone W10-3 produced an area of mycelium at a rate of 12 $mm^2 day^{-1}$ (measured after 32 days), and upon the transfer of a hyphae-enveloped root segment (about 3 months later), mycelium developed at a rate of 4 $mm^2 day^{-1}$ (mean of four replicates).

The first successful symbiotic association took place with roots of the slow-growing clone L2A, which had been growing for 3 months on medium M in glass test tubes. A cube of a Fontana medium containing *T. melanosporum* was placed onto the roots without touching the underlying layer of medium M. Hyphae emanating from these roots could be seen on the sides of the test tubes (data not shown). Three months after inoculation, mycorrhizal associations were detected (Fig. 4) in elongated roots. The hyphae formed a thin mantle and penetrated two to three layers of cells, producing a Hartig net. Swollen, club-like root tips were discernible 5 months after inoculation (Fig. 5). Six months after inoculation, hairy roots were subcultured on two petri dishes containing medium M. Three weeks later, hyphae could be observed emerging from the roots. A Hartig net persisted for 32 months following inoculation in the same roots after three subcultures 6, 7, and 14 months after inoculation. As noted above, the transfer of fungus lacking roots did not result in growth of the fungus.

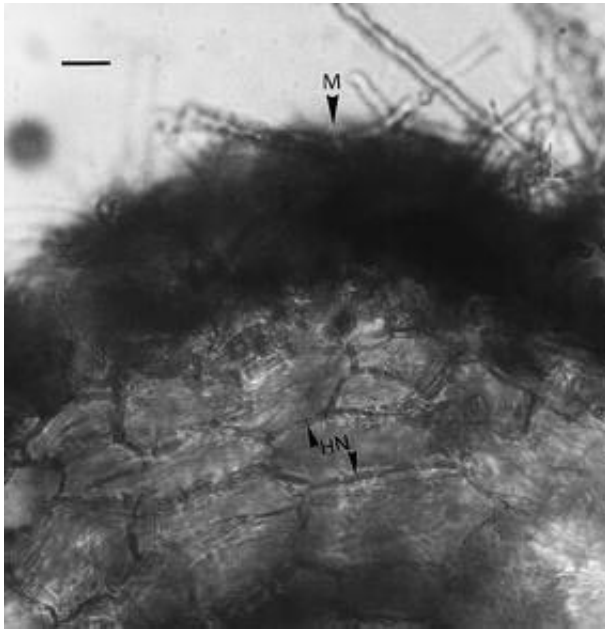


Fig. 4. A cross section of a mycorrhized transformed *Cistus* root revealing a Hartig net. Hyphae appear dark due to trypan blue staining. Note fungal cell septa. *M* mantle, *HN* Hartig net. *Bar*: 14 μ m

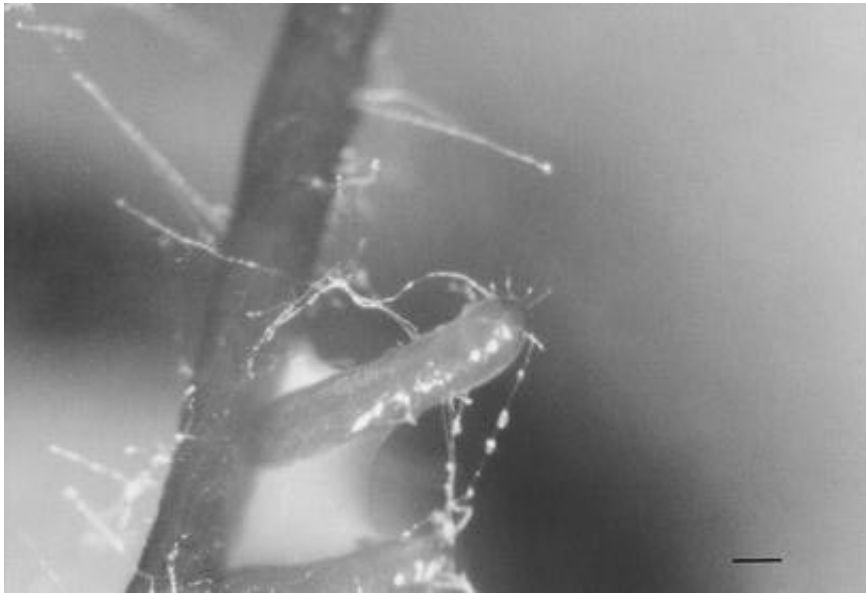


Fig. 5. A club-like mycorrhized root tip with protruding hyphae (spikes). *Bar:* 0.4 mm

Discussion

Among the hairy roots of several species grown in our laboratory, *C. incanus*-transformed roots exhibited unique growth patterns when grown on both solid and liquid media. The circular growth pattern around the test tube walls, resulting in a thick layer of roots, is unique to this species, since hairy roots of other plant species (*Beta vulgaris*, *Symphytum officinale*, and *Alkanna linifolia*) have been observed in our laboratory to grow vertically up the walls. In addition, transformed roots of the latter three species grown in test tubes reached the bottom of the tube within 2 weeks of growth, while the *Cistus* hairy roots, though eventually penetrating the medium, did not reach the bottom of the tube even after 3 months. A reduced geotropism (Otani et al. 1993) is unique to hairy roots. In liquid medium as well, the roots of *Cistus* displayed a growth pattern that differed from that of the other hairy roots mentioned above: instead of first elongating at the root tips, as observed the other hairy roots growing in test tubes, the roots of *Cistus* first thickened for about 2 weeks before forming new side roots. These observations pertained to all of the *C. incanus* clones.

The most interesting difference between *C. incanus* and other hairy root species was apparent in the longevity of the cultures. The life span of *B. vulgaris* hairy roots, for example, is not much more than 2 weeks between transfers in either liquid or on solid medium (unpublished data). Longevity may thus turn out to be a very useful trait that can be exploited for the establishment of ectomycorrhizal associations in vitro. For cultures that display a good longevity, there is no need for frequent subculturing, thus enabling an undisturbed period of mycorrhiza development. Endomycorrhizal associations are quick to form (a matter of 2-4 days; Alexander et al. 1988), while ectomycorrhizal associations are rather slow to form, taking about 4 weeks (Tonkin et al. 1989) to 3 months (Thompson et al. 1993), the latter being the usual period found in practice. There is no information concerning the ability of mycorrhiza to develop when continually disturbed (as would be the case with subculturing). The longevity of *C. incanus* therefore provides the conditions for the full mycorrhizal-forming term to be reached before subculturing is required.

The mycorrhization period of 3 months usually culminates in the formation of club-like swollen, short root tips. However, in our cultured roots, these root tips appeared only after 5 months of culturing. We are not sure whether this means a slower rate of mycorrhizal development *in vitro* because of the lack of an ingredient, a clonal phenomenon, or coincidence. This finding is at present under investigation.

Our *Cistus* clones varied greatly in terms of growth habit and growth rate. The cause of this phenomenon is not clear at present: it may stem either from different integration sites of the Ri T-DNA or from the number of T-DNA copies integrated into the cell. In addition, there may be a difference in the ability of a clone to form mycorrhizae. Research is thus being conducted to answer these questions. Since *T. melanosporum* is a slow-growing fungus (fills up a regular 9-cm-diameter petri dish in 2-3 months), we chose to start our experiments with the slowest growing clone, L2A.

Fusconi (1982) reported that *T. melanosporum* mycorrhization of *C. incanus* roots can follow one of two paths: (1) the penetration of differentiated cortical cells at the more basal part of the root, resulting in the formation of a Hartig net, with a rather thin mantle covering only the distal part of the mycorrhized root, or (2) hyphal penetration of dead root-tip cells or immediate mycorrhization of a rootlet at the point of emergence, resulting in the formation of a mantle, followed by the development of a mantle-attached Hartig net. Under our conditions, it seems that the process took the first path, with a secondary mycorrhization occurring at the emergence of a lateral root.

Roots could grow on the minimal M medium, but *T. melanosporum* showed only residual growth on this medium, probably as a result of the lack of some essential ingredient(s) in the medium. However, when in a mycorrhizal state, the fungus could survive for a long period on this minimal medium (6 months) and was able to regrow out of a mycorrhized root segment upon transfer onto medium M.

In summary, we have demonstrated that *T. melanosporum* and hairy roots of *C. incanus* can grow together on medium M (Balaji et al. 1995) and form typical ectomycorrhizal associations. The culture may be maintained, multiplied, and used as a mass inoculum for the mycorrhization of plants *in vitro* or *ex vitro*.

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