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Techniques for controlled synthesis of the Douglas-fir – *Laccaria laccata* ectomycorrhizal symbiosis

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Summary — *Laccaria laccata* (Scop ex Fr) Cke is an ectomycorrhizal basidiomycete which is very efficient for the controlled mycorrhization of Douglas-fir (*Pseudotsuga taxifolia* Poir Britt). Studying the biology of this symbiosis led to the development of a number of experimental techniques for aseptic and non-aseptic synthesis. This paper describes seed treatments, fungal inoculum preparation, substrates, nutrient solutions, aseptic experimental systems (test tubes and Petri dishes) and non-aseptic systems (pot experiments in the glasshouse) and bare-root nursery techniques. The specificity of each technique is discussed according to the experimental purpose.

ectomycorrhizas / aseptic synthesis / non-aseptic synthesis / *Pseudotsuga taxifolia* / *Laccaria laccata*

Résumé — Techniques d'étude de la symbiose ectomycorrhizienne entre le douglas et *Laccaria laccata*. *Laccaria laccata* (Scop ex Fr) Cke est un champignon basidiomycète ectomycorrhizien très efficace pour la mycorrhization contrôlée du douglas (*Pseudotsuga taxifolia* Poir Britt). L'étude de la biologie de cette symbiose a conduit à la mise au point d'un certain nombre de techniques expérimentales pour réaliser sa synthèse en conditions aseptiques ou non aseptiques. Cette note décrit le traitement des graines, la préparation de l'inoculum fongique, les solutions nutritives, les substrats, les systèmes expérimentaux aseptiques (tubes à essais [fig 1] et boîtes de Petri [fig 2]) et non aseptiques (expériences en pots en serre [figs 3, 4, 5, 6] et techniques de pépinière à racines nues). Les techniques aseptiques in vitro permettent d'étudier l'effet de divers facteurs expérimentaux sur la dynamique de l'infection ectomycorrhizienne, mais pas l'effet de la mycorrhization sur la croissance de la plante. Cet effet se manifeste en serre et en pépinière. Certains des dispositifs proposés pour les expériences en serre permettent l'observation directe et non destructive du système racinaire.

ectomycorrhizes / synthèses axéniques / synthèses non-axéniques / *Pseudotsuga taxifolia* / *Laccaria laccata*

* Present address: As for J Garbaye (2)

INTRODUCTION

Douglas-fir is presently the dominant forest tree species used for reforestation in France. Field experiments have shown that the ectomycorrhizal fungus *Laccaria laccata*, when inoculated to planting stocks in the nursery, stimulates the early growth of outplanted Douglas fir (Molina, 1980; Le Tacon *et al*, 1983, 1985, 1988; Mortier *et al*, 1988).

As practical applications of these results are developing, different aspects of the association are being investigated by INRA in order to improve performance and to select more efficient fungal strains. The physiology of the symbiosis is studied in aseptic *in vitro* systems, and experiments in glasshouse and nursery conditions are carried out in order to study how mycorrhizal establishment is affected by environmental factors and compare the behavior of inoculated and non-inoculated seedlings submitted to different treatments, in conditions close to practice.

The aim of this note is to help readers working on similar symbiotic systems choose the technic the best adapted to their own experimental purpose.

PRODUCTION OF FUNGAL INOCULUM

Maintenance of the fungal strain

The ectomycorrhizal basidiomycete *Laccaria laccata* (Scop ex Fr) Cke isolate S-238 from USDA (Corvallis, OR) is maintained in Petri dishes (6 cm diameter) on modified Pachlewski agar medium (Pachlewski and Pachlewska, 1974). The composition is for 1 liter as follows: diammonium tartrate: 0.5 g; KH_2PO_4 : 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g; maltose: 5.0 g; glucose: 20 g; thiamine: 1 μg ; Fe EDTA: 0.6

mg; Mo: 0.03 mg; B: 0.13 mg; Mn: 0.5 mg; Cu: 0.06 mg; Zn: 0.23 mg; Agar: 20 g. Micronutrients (Fe, Mo, B, Mn, Cu and Zn) are applied together as 0.1 ml of a concentrated commercial solution: Kanieltra (CO-FAZ, BP 198-08, Paris, France). Cultures are kept at 25 °C in a dark incubation chamber. When growing, the mycelium develops a bright lilac color, which reaches maximal intensity after 1 month. Cultures are transferred into fresh medium after 2 months, when the colonies have a diameter of about 4 cm.

Liquid inoculum

L. laccata is grown in 1-liter Erlenmeyer flasks stoppered with cotton wool and containing 500 ml of liquid modified Pachlewski medium. The flasks are inoculated with 8 agar disks (6 mm diameter) cut from the margin of a culture on modified Pachlewski agar medium. After 2 weeks, the mycelium develops a lilac color which permits detection of contaminants. This typical colour does not develop as well in other media such as Melin (Melin, 1936), malt extract or brewery wort. Flasks are kept in the dark at 25 °C on an orbital shaker for 1 month. The mycelium is then washed in tap water in order to remove residual nutrients, homogenized in a Waring blender for ≈ 10 s and resuspended in distilled water. This kind of fungal inoculum is quantified by measuring the fungal dry weight per ml or by counting living propagules (determination of colony forming units by spreading 1 ml of suspension on a 6-cm Petri dish with nutrient agar). The viability of the suspension does not decrease before 4 weeks at 4 °C.

Vermiculite-peat inoculum (adapted from Marx and Bryan, 1975)

Glass jars (1.6 l) containing 1.3 l expanded vermiculite-sphagnum peat mixture (4:5-

1:5, v:v, pH = 5.5) are autoclaved (120 °C, 20 min). Another ratio of vermiculite–peat can be used (2:3–1:3). The peat can release some substances which are toxic for fungal growth. For this reason, the first ratio mixture is preferred. Then the mixture is moistened to field capacity with 600 ml modified liquid Pachlewski medium. The jars are stoppered with lids with a 1-cm diameter hole. This hole is fitted with a 4-cm long tube filled with cotton wool. The jars are then autoclaved a second time (120 °C for 20 min). After cooling, 8 mycelial plugs are laid on top of the substrate. Mycelium grows down into the substrate, which is completely colonized after 6 weeks at 25 °C. For faster growth, jars can be filled with a smaller quantity of substrate and shaken after mycelia have colonized a few centimeters: in this manner, mycelium is evenly distributed throughout the substrate and incubation time is shortened. This inoculum can be stored at 4 °C for up to 6 months.

Alginate beads inoculum

The process of including fungal mycelium in polymeric gels (especially calcium alginate) has been previously described (Domergues *et al*, 1979; Le Tacon *et al*, 1983, 1985). The inoculum prepared in this manner is more efficient than the classical vermiculite–peat inoculum (Mortier *et al*, 1988) because the mycelium is protected in the gel from physical stresses (*eg* water stress) and from competitor microorganisms. With this technique, it is possible to accurately control the weight of mycelium or the number of living propagules contained in the inoculum. Different attempts have been made to measure the quantity of mycelium in the vermiculite–peat inoculum: ergosterol assay (Martin *et al*, 1990); chitin assay (Vignon *et al*, 1986), but none

of them gave reliable results (Mortier *et al*, 1988) because of the peat which interferes with colorimetric measurements.

A mycelial suspension, obtained as previously described, is mixed (1:1, v:v) with distilled water containing 20 g l⁻¹ sodium alginate and 50 g l⁻¹ autoclaved dry powdered sphagnum peat. When aseptic inoculum is needed, the alginate solution and the peat should be autoclaved separately. The final solution is pumped through a pipe with 2-mm holes. The drops fall into a 100 g l⁻¹ CaCl₂ solution and form beads of reticulated calcium alginate gel (Maupein *et al*, 1987). The beads are kept in CaCl₂ for 24 h at room temperature in order to ensure complete reticulation. They are then washed with tap water to remove NaCl and CaCl₂ and stored in air-tight containers at 4 °C in order to prevent drying. This type of inoculum can be kept up to 9 months in these conditions. The beads are prepared with 1–2 g mycelium (dry weight) per l of final solution (Mortier *et al*, 1988).

ASEPTIC MYCORRHIZAL SYNTHESIS

As for all the techniques described below, the seeds of Douglas-fir (*Pseudotsuga taxifolia* (Poir) Britt (syn *P douglasii* (Lindl) Carr, syn *P menziesii* (Mirb) Franco, syn *P mucronata* (Raf) Sudw) are from provenance zone 412 (Snoqualmie Falls, Washington State, USA). They are supplied by Vilmorin (La Méritré, 49250 Beaufort-en-Vallée, France).

All the aseptic experimental systems presented here are designed for studying the dynamics of symbiosis establishment between the plant and the fungus. However, due to the limited volume of the vessel containing the roots, they are not suitable for the expression of a growth effect on the plant.

Root exudates provide the carbon needed for the fungal growth (Harley and Smith, 1983). The release of these substances is linked to photosynthesis (Hacs-kalylo, 1973). Therefore, in order to obtain normal photosynthesis, the aerial part of the plant is kept under non-axenic conditions outside the tube or the Petri dish and the roots are kept inside the culture vessel under axenic conditions. If the aerial part of the plant were kept inside, parameters affecting gas exchange (temperature, CO₂, humidity) would be altered.

Cultures are set in a climate-controlled growth chamber with 23 °C day, 17 °C night, 16 h photoperiod with 240 µE.m⁻².s⁻¹ (Mazda MAIH 400 lamps), 80% relative humidity.

The seeds are surface-sterilized in 30% H₂O₂ for 90 min, washed for 4 h in sterile water, and plated on glucose (1 g.l⁻¹) agar in order to detect contamination. Contaminated seeds are discarded and germinants are used when taproots are 1–2 cm long.

Test-tube system (fig 1)

The 2 components of the system (fungus, plant) are aseptically confronted in glass test-tubes (3 x 15 cm) filled with autoclaved (120 °C, 20 min) peat-vermiculite (1:1, v:v) moistened to field capacity with modified Shemakanova mineral nutrient solution (Shemakanova, 1962): MgSO₄, 7H₂O: 150 mg; (NH₄)₂HPO₄: 125 mg; (NH₄)₂SO₄: 125 mg; CaCl₂, 2H₂O: 50 mg; KCl: 108 mg; Kanieltra: 0.1 ml; distilled water: 1 liter). Fungal inoculation can be achieved with peat-vermiculite inoculum (either mixed throughout the substrate (1:10, v:v) or laid on top of the tube (1–2 cm), alginate beads (5 beads laid on top of the tube) or mycelium suspension (injected with a syringe or deposited with a pipette). The tubes are covered with alumin-

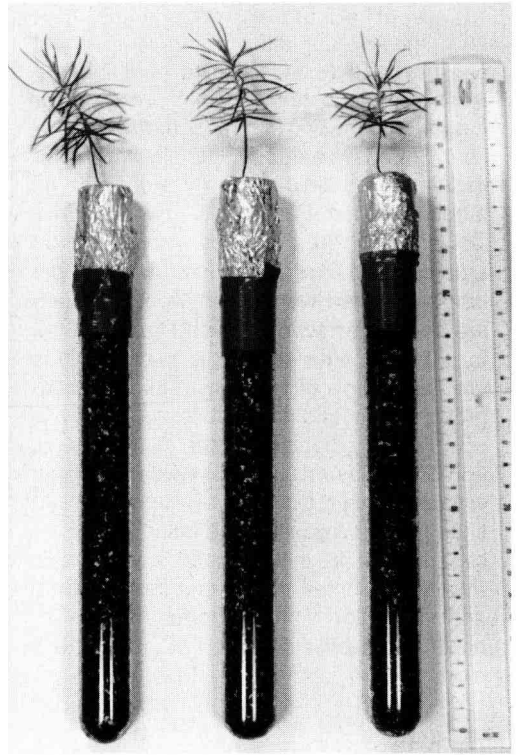


Fig 1. Four-week-old Douglas fir seedlings in test-tubes with vermiculite-peat substrate. The substrate and the root system are kept aseptic by a putty seal.

ium foil and the rootlet of one aseptically germinated seed is introduced through a hole in the foil and sealed with autoclaved coachwork putty (Terosta 2, Teroson SA, Asnières, France). The roots are maintained in axenic conditions, while the aerial part of the plant develops outside the tube.

After 1 month of culture, the plant is removed from the tube and roots are observed with a stereomicroscope. Each seedling bears ≈ 100 short roots, 30–100% of them being mycorrhizal with *Laccaria laccata*, depending on variable factors.

Petri dish systems (fig 2)

Circular (diameter = 12 cm) or square (12 x 12 cm) Petri dishes can be used. They are filled to the lid with substrate: autoclaved soil, autoclaved silica sand (0.5–1.2 mm) washed with 6 N HCl and rinsed with tap water or vermiculite–peat mixture (1:1, v:v). Soil is moistened to field capacity with distilled water and the 2 other substrates with modified Shemakanova nutrient solution.

The rootlets or 2 or 3 aseptically germinated seeds are introduced through holes 2–3 cm apart in the side wall of the dish and sealed with autoclaved coachwork putty. The lid is sealed with plastic adhesive tape. The dishes are set upside down at a 45° angle in the grown chamber, so that

roots grow down against the lid. As in test tubes, mycorrhiza can be obtained after 1 month of culture, with the same number of short roots per plant and the same mycorrhizal infection rate.

Compared with the test tubes, this Petri dish system enables observation of the roots through the lid with a stereomicroscope (monitoring root and fungal growth, counting mycorrhizas, etc). Dishes can also be opened under sterile atmosphere for root sampling or addition of various inocula or chemicals.

**NON-ASEPTIC SYNTHESIS
IN THE GLASSHOUSE**

Before sowing, Douglas-fir seeds are either pretreated in moist sphagnum peat for

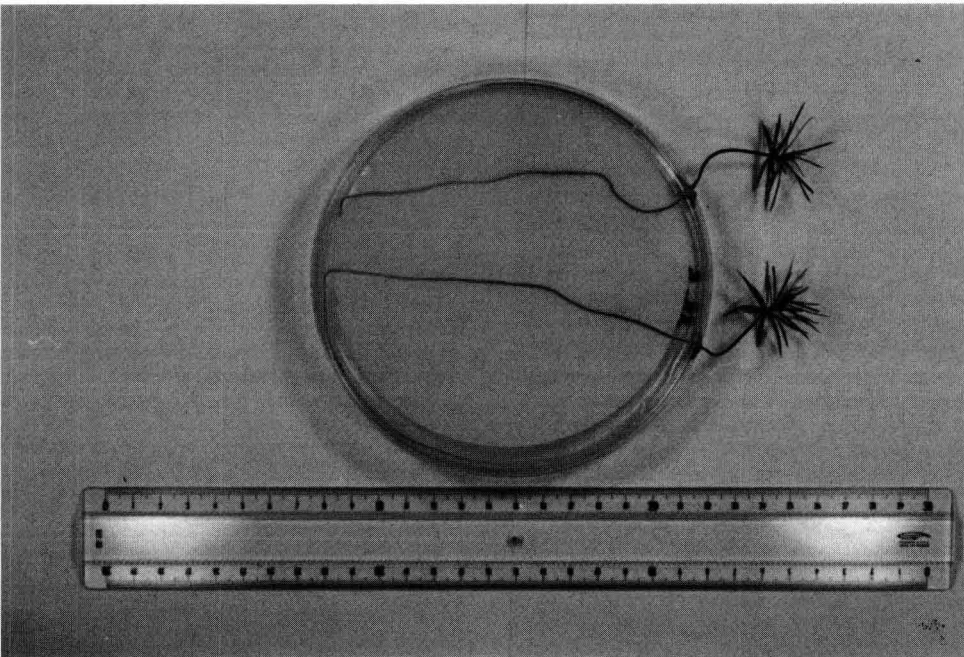


Fig 2. Two-week-old Douglas-fir seedlings in a Petri dish (substrate removed to show roots). The content of the dish and the root system are kept aseptic by a putty seal.

8 weeks at 4 °C or surface sterilized in 30% H₂O₂ for 90 min, washed for 4 h in sterile water and kept overnight in water at 4 °C. The germination rate is better with the first technique but the risks of damping off due to *Rhizoctonia* spp, *Fusarium* spp or other pathogens is higher. The method using H₂O₂ eliminates the pathogens and suppresses seed dormancy.

The seedlings can be grown on soil (disinfected or not by steam or methyl bromide fumigation) or non-disinfected vermiculite-peat mixture (1:1, v:v). Several kinds of container can be used for growing Douglas fir seedlings in the glasshouse, depending on the aim of the experiment.

Hiko containers (fig 3)

The black high-density cast polyethene Hiko containers are manufactured in Swe-

den. They are trays containing 24 or 40 cells of 150 or 93 cm³, respectively. They are easy to fill and occupy the room in the glasshouse very efficiently. However, holes at the bottom are wide and flowing substrates such as sandy soils have to be maintained by peat or glass-wool plugs. Another drawback is that Hiko containers cannot be opened. One seedling is grown in each cell.

Transparent boxes (fig 4)

These are 20 x 7.5 x 2.2 cm clear polystyrene boxes (Ref LH 275.22, Établissements Caubère, Paris, France). One extremity is cut open and three 1-cm holes are bored in the other end in order to ensure drainage. Boxes are wrapped with black polythene film to prevent green algae proliferation, and maintained inclined at

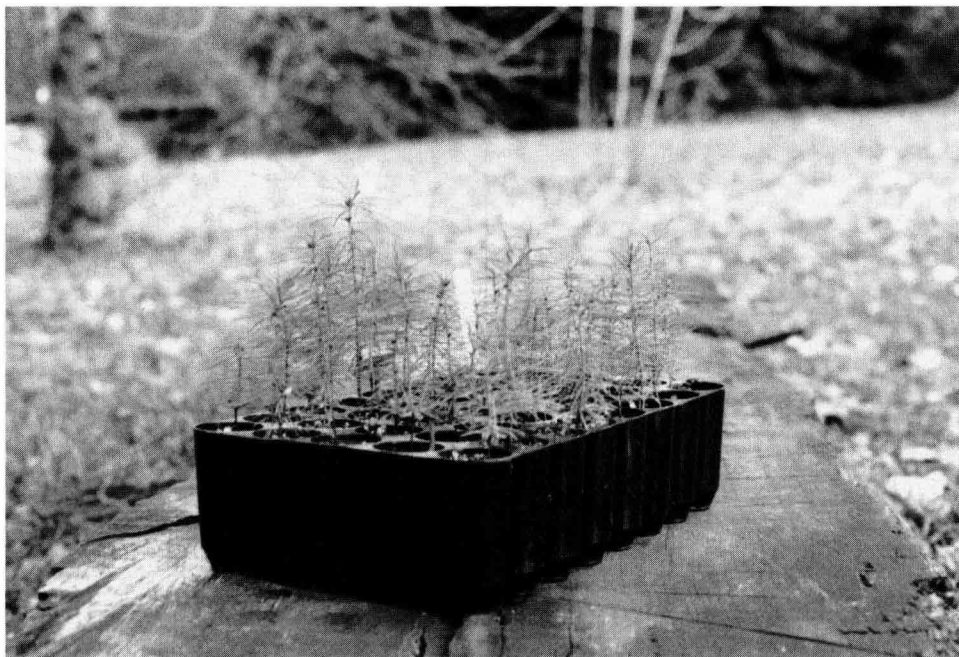


Fig 3. Six-month-old Douglas-fir seedlings in a set of Hiko containers (40 cells, 93 ml each).

45° in order to force root growth against the lower wall. Two or 3 seedlings are grown per box. This type of container presents the same advantages as the Petri dish system previously described for aseptic cultures: non-destructive observation of roots, sampling and various manipulations.

Roottrainers (fig 5)

These thermoformed PVC containers (Spencer-Lemaire Industries Ltd, Edmonton, Alberta, Canada) are like books forming cells when closed. The "books" are packed in trays. Different capacities are

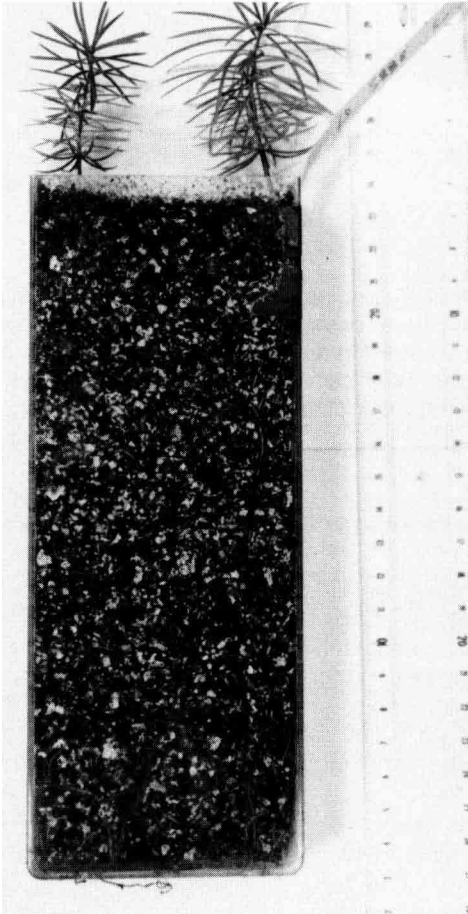


Fig 4. Six-month-old Douglas fir seedlings in a transparent box with vermiculite-peat substrate.

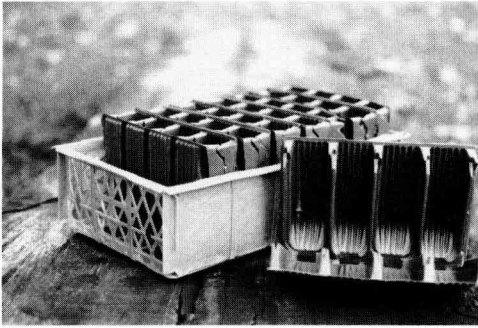


Fig 5. "Roottrainer" containers. The tray contains 32 cells, 115 ml each. The "book" in the foreground is open.

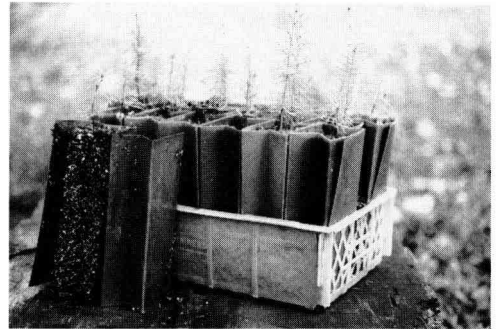


Fig 6. Six-month-old Douglas-fir seedlings in "M" containers. The container in the foreground is open and shows the vermiculite-peat clump.

available: 115, 175, 250 and 1 300 ml per cell. Root and mycorrhiza development can be monitored any time by opening the "books". Roottrainers present the same drawback as Hiko containers when filled with flowing substrates.

"M" containers (fig 6) (Riedacker, 1978)

The "M" containers (Thermoflan, Molières-Cavaillac, Le Vigan, France) are made of 2 folded PVC parts, fitted into each other, which can be separated any time for non-destructive root observations. They are completely opened at the bottom and can only be filled with pure peat without plugging. Their capacity is 400 ml.

Whatever the container type, 2 or 3 seeds are sown per cell in order to ensure at least 1 germinating seed. When seedlings are 5 weeks old, they are thinned to 1 per individual container. When soil is used as a substrate, seedlings are watered daily with deionized water. When the

vermiculite-peat substrate is used, the following nutrient solution is applied in excess twice a week in addition to daily watering with deionized water: for 1 liter, KNO_3 : 80 mg; $\text{Ca}(\text{NO}_3)_2$, $4\text{H}_2\text{O}$: 19 mg; NaH_2PO_4 , H_2O : 9 mg; MgSO_4 , $7\text{H}_2\text{O}$: 74 mg; Kanieltra: 10 μl . The composition of this solution has been experimentally determined in order to provide optimal mycorrhizal establishment. Concentrations of macroelements in mg l^{-1} are: P: 1.8; N: 33.5; K: 31.0; Ca: 32.0; Mg: 7.2.

Fungal inoculation can be performed either by mixing vermiculite-peat or alginite inoculum throughout the substrate before filling the containers or by opening them when roots are well developed and spreading any of the 3 previously described inoculum types on the root system.

In all these glasshouse experiments, mycorrhizal infection begins 8 weeks after sowing. After one growing season (5–6 months), mycorrhizal rate can be close to 100% for seedlings \approx 10–12 cm tall in the smallest containers.

LARGE SCALE SYNTHESIS IN BARE-ROOT NURSERY CONDITIONS

The seeds are pretreated in moist sphagnum peat for 8 weeks at 4 °C before sowing. The nursery soil, freshly tilled and at 10 °C minimum, is fumigated in spring with cold methyl bromide (75 g per m², soil covered with clear polythene film for 4 days). The film is removed 3 weeks before inoculating and sowing. Toxicity is eliminated during this time. This fumigation destroys all the microorganisms which can compete with the inoculated fungus (Le Tacon *et al*, 1983).

The nursery beds are divided into 0.5-m² plots separated from each other by 50 cm uninoculated and unsown zones. The fungal inoculum is broadcast and incorporated into the 10 cm topsoil at the dose of 2 l per m² for peat-vermiculite inoculum (in this case, it is impossible to determine the quantity of mycelium) or 1 liter per m² for alginate beads (2 g mycelium (dry weight) per m²).

The culture is managed using routine nursery practices except that fertilization is suppressed or considerably reduced, and that systemic fungicides are banned.

Under these conditions, mycorrhizal rate with *Laccaria laccata* ranges from 60–80% at the end of summer, with seedlings 5–20 cm in height depending on the nursery. Under these nursery conditions, the effect of *Laccaria laccata* inoculation is dramatic: the seedling height is doubled if compared with an uninoculated control (Le Tacon *et al*, 1988).

In this case of all non-aseptic synthesis (glasshouse or nursery), seedlings uninoculated with *L. laccata* are mycorrhizal with *Thelephora terrestris*, a contaminant ectomycorrhizal basidiomycete which is very common as airborne spores in all temperate regions and adapted to these culture

conditions. Therefore, it is generally impossible to produce non-mycorrhizal control seedlings.

Experiments using the techniques presented here can be found in Le Tacon *et al* (1983, 1985, 1987, 1988), Le Tacon and Bouchard (1986), Mortier *et al* (1988), Garbaye *et al* (1990) and Duponnois and Garbaye (1991).

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