Mycorrhization of vitroplants raised from somatic embryos of cork oak (Quercus suber L.)

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Abstract

The technique described herein allows in vitro ectomycorrhizal synthesis in Quercus suber vitroplants raised from somatic embryos with Pisolithus tinctorius and Scleroderma polyrhizum strains. Only strains of this species coming from fruit bodies collected in Quercus suber stands (strain QS241 and strain QS247) formed ectomycorrhizas, and hence these species seem to exhibit host adaptation. The in vitro mycorrhization facilitated the development of secondary roots and the ex vitro weaning of cork oak vitroplants. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The cork oak (Quercus suber L.) is one of the most important trees of the Mediterranean forest and has economic value based on the production of cork. This is a natural product with multipurpose applications used mainly in floor coverings and wine bottling. The increasing demand for cork and the low natural regeneration of this species justify intensive planting with improved material. Current tree improvement strategies in Quercus place great emphasis on breeding and cloning (Savill and Kanowski, 1993; Cuenca et al., 1999). One of our main goals is to develop the micropropagation of cork oak based on somatic embryogenesis.

Somatic embryogenesis is envisaged as one useful tool in cloning elite genotypes of forest trees (Hogberg et al., 1998; Taber et al., 1998; Lelu et al., 1999; Radojevic et al., 1999; Svobodova et al., 1999). It is considered as a relatively stable way of micropropagation and hence prevents additive and non-additive genetic variation. Somatic embryos are similar to their zygotic counterparts, showing a vascular connection between root and shoot: therefore, they can reduce the risk of intraclonal variation displayed by rooted cuttings due to differences on efficiency among cuttings regarding vascular connection between shoot and adventitious roots. Somatic embryogenesis also offers high rates of multiplication based on the process of recurrent embryogenesis (Merkle, 1995).

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Somatic embryogenic lines were obtained from leaves of mature trees. The somatic embryos can be obtained by a secondary embryogenesis which allows a high level of multiplication. We also achieved moderate percentages of germination of somatic embryos but regenerated plants transferred to substrate stopped growing and died (Fernández-Guijarro et al., 1995; Puigderrajols et al., 1996; Celestino et al., 1998).

In vitro mycorrhization of micropropagated plants can be used to increase survival and growth during ex vitro weaning (Nowak, 1998). For instance, in the case of fruit trees, the inoculation with arbuscular fungi facilitated vitroplants adaptation to ex vitro conditions (Sbrana et al., 1994). It has also been reported that in vitro ectomycorrhization can improve microcutting rooting (Normand et al., 1996) and enables vitroplants to acclimate more readily (Martins et al., 1996). The in vitro mycorrhization of micropropagated plants, e.g. Helianthemum spp. (Morte et al., 1994) and Cistus spp. (Díez and Manjón, 1996), has been obtained only in very few Mediterranean species.

2. Material and methods

Cork oak vitroplants were obtained from the somatic embryogenic line M10 by secondary embryogenesis as described by Fernández-Guijarro et al. (1995). This embryogenic line was obtained from a leaf of an elite mature tree (Toribio et al., 1998), and it is preserved at the germoplasm collection of the IMIA Institute, Alcalá de Henares, Spain. In vitro culture was carried out in closed Magenta™ vessels (Sigma™, 95 x 67 mm², height x width, 60 ml medium) at 25 ± 1 °C and 16 h photoperiod (mixed cool-white and grolux fluorescent lamps, 50 μmol m⁻² s⁻¹).

Maturation of embryos was achieved by nutrient depletion in ½ G medium (Gamborg, 1966) for 30 days, which synchronizes shoot and root embryo development, followed by a storage at 4 °C for 30 days to switch embryos from embryogenic pathway to germination (Fernández-Guijarro et al., 1995).

One embryo per vessel was germinated in Magenta™ vessels with 30 ml liquid SH medium (Schenk and Hildebrandt, 1972) and perlite filling up to 4 cm height of the vessels. Two months later, the SH medium was taken out with the aid of a pipette, the perlite was washed twice with sterile water before adding 30 ml of liquid BAF medium (Moser, 1960) at one-half of the phosphate concentration (Fig. 1). Then, each vessel was inoculated with a 0.7 x 0.7 cm² piece of a fungal colony of a mycorrhizal fungus grown on solid BAF medium. Several strains available in our laboratory were used, mainly strains of P. tinctorius (Pers.) Coker & Couch and Scleroderma (S. meridionale Demoulin & Malcz. and S. polyrhizum G.F. Gmel.). These strains were obtained from fruit bodies collected: (a) in a Pinus pinaster Aiton stand (Segovia, Spain) (S. meridionalis strain PP10); (b) in a Eucalyptus camaldulensis Dehnh. plantation (Miravete, Cáceres, Spain) (P. tinctorius strain EC204); (c) in a Q. suber stand in Arcacena Mountains (Huelva, Spain) (P. tinctorius strain QS241, S. polyrhizum G.F. Gmel. strain QS247). The strains are deposited at the Departamento de Biología Vegetal of Alcalá University, and fruit body samples at Alcalá Herbarium (AH). One month after inoculation, morphological (dissecting microscope) and anatomical (light microscope) examinations were carried out in order to confirm the mycorrhizal formation. The ectomycorrhizas were described according to Agerer (1987–1993).

Vitroplants were acclimatized in sand/peat/crushed pine bark (v:v:v) at a gradually reduced relative humidity by removing a plastic cover progressively during a period of 4 weeks.
3. Results

Plants raised from somatic embryos inoculated with strains coming from a Pinus, Cistus or Eucalyptus vegetation did not form mycorrhizas. In some cases, the mycelium of these strains did not grow. In other cases they grew covering completely the plantlets which finally died. In contrast, ca. 85% of vitroplants inoculated with P. tinctorius QS241 and ca. 90% of those inoculated with S. polyrhizum G.F. Gmel. QS247 formed ectomycorrhizas. One month after inoculation, inoculation percentages of inoculated vitroplants ranged from 50–75% to 60–70% of the tips of the secondary roots, respectively. Whereas mycorrhizated plants displayed a very ramified root system, the non-inoculated plants lacked secondary roots.

Both strains coming from fruit bodies collected in Q. suber stands (strain QS241 and strain QS247) formed ectomycorrhizas. The perlite substrate was full of mycelium growth associated to the roots.

The P. tinctorius–Q. suber mycorrhizas were golden-yellow. Although they were young, their ramification system was monopodial-pinnate. There were young rhizomorphs which were yellow and not differentiated yet. Mycelium grew mainly associated to the root system. Despite the mantle being sometimes young and thin, it was often well-developed. The mantle was plectenchymatic. The outer layer of the mantle was formed by yellow running hyphae, some of them with crystals on their surface. The inner layer was formed by compact and sometimes parallel-running hyphae; this structure was the same at the young parts of the mantle. At the well-developed parts of the mantle, it was possible to observe the structure of the Hartig net.

The S. polyrhizum–Q. suber mycorrhizas were brown with abundant mycelium giving them a white colour. Their ramification system was monopodial-pinnate. Mycelium grows on the substrate. There were rhizomorphs which were from hyaline to light-yellow in light microscope. The rhizomorphs were highly differentiated with centrally arranged thicker hyphae. Mantle was often well-developed, though it was sometimes very young and thin. At both parts, the mantle was plectenchymatic. Its outer layer showed a ring-like structure according to the terminology of Agerer (1995). The inner layer was plectenchymatic too with more or less the same structure. The Hartig net could be observed under the well-developed parts of the mantle.

Whereas non-inoculated vitroplants died during acclimatization, ca. 80% of mycorrhizated vitroplants survived; thus, mycorrhization seems to facilitate the weaning process of vitroplants raised from somatic embryos.

4. Discussion and conclusion

We observed that strains collected under other species in vitro did not establish mycorrhizas with Q. suber. For instance, P. tinctorius strains associated to Eucalyptus failed to form mycorrhizas with Q. suber plantlets raised from somatic embryos. These findings led us to undertake the isolation of strains from basidiocarps collected in cork oak stands. We inoculated vitroplants raised from somatic embryos with strains collected associated with Q. suber woods. These strains belonged to P. tinctorius and S. polyrhizum taxa and were collected in a cork oak stand in Aracena Mountains (southern Spain). In vitro these strains formed mycorrhizas with plants raised from somatic embryos. Therefore, it seems to be a different ability of the strains of Pisolithus to form mycorrhizas with cork oak, depending on the host from which they were isolated. This could indicate that a host specificity exists among the different strains of Pisolithus as molecular ecology studies had also previously indicated (Anta et al., 1998).

Regarding in vitro mycorrhizal establishment, some authors use an agar medium to synthesize in vitro mycorrhizas (Malajczuk and Hartney, 1986). Others use perlite or perlite–peat. In our case, synthesis was carried out in a hydroponic medium using perlite as substrate. We did not obtain synthesis in agar medium. This indicates that in our case hydroponic medium is more suitable than agar-based mediums.

The in vitro synthesized mycorrhizas showed the typical ectomycorrhizal morphology and anatomy. They had Hartig net and well-developed mantle. The morphology and anatomy of these mycorrhizas are in agreement with the mycorrhizas previously described in other wood species.

In the case of the mycorrhizas of P. tinctorius the morphological and anatomical features are the same as in the nursery-obtained mycorrhizas of Q. suber.
seeding (Anta et al., unpublished). This description is also congruent with the previous descriptions in other hosts such as *Picea abies* (Weiss, 1990, 1991), *Betula alleghaniensis* (Massicotte et al., 1990) or eucalyptus (Rose et al., 1981). This description is in agreement with the description of the in vitro synthesised mycorrhizas of *P. tinctorius* with eucalyptus (Tonkin et al., 1989).

According to the literature, there is no description of the mycorrhiza formed by *S. polyrhizum* in any host species. The synthesized mycorrhizas showed the main anatomical characteristics of the mycorrhizas of the genus *Scleroderma*, such as the characteristic structure of the mantle and rhizomorphs described in the case of *S. citrinum* (Pers.:Pers.), *S. bovista* and *S. verrucosum* (Waller and Agerer, 1993; Agerer, 1995). This is the first time that the morphology of the ectomycorrhiza of *S. polyrhizum* has been studied.

Boutekrabt et al. (1990) and Boutekrabt and Pargeny (1991) investigated the ultrastructure of the in vitro synthesized mycorrhizas of *Tuber melanosporum* Vitt. with microcuttings of *Q. robur* L. and *Q. pubescens* Willd. These authors concluded that there were no differences between the mycorrhizas formed on microcuttings and seedlings. We can suppose that in the case of plants of cork oak raised from somatic embryos the mycorrhizas are similar to the mycorrhizas of seedlings. To confirm this fact we compared the in vitro synthesized mycorrhizas with those obtained ex vitro by spore inoculation of cork oak seedlings. In the case of *P. tinctorius* both mycorrhizas showed similar anatomy and morphology.

Although in the literature there are works on in vitro mycorrhization (Burgess et al., 1994; Wiemken, 1995; Sudhakara and Satyanarayana, 1998a,b), it must be remarked that few of them used vitroplants raised from somatic embryos. When micropropagated plants have been used, most cases involved microcuttings (Strullu et al., 1986; Tonkin et al., 1989) rather than plants raised from somatic embryos. Sasa and Krogstrup (1991) achieved ex vitro formation of mycorrhizas in *P. sitchensis* plantlets raised from somatic embryos. Piola et al. (1995) observed that in vitro ectomycorrhization improved the root development of *Larix* somatic embryos. Moreover, the benefit of the “biotization” of micropropagated plants with microbial inoculants is well known (Nowak, 1998). In our case, in vitro mycorrhization increased the formation of secondary roots and the survival after acclimatization of vitroplants raised from somatic embryos. Thus, this approach is likely to leave behind the bottleneck so far encountered by *Q. suber* micropropagation by somatic embryogenesis.

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References


