In vitro mycorrhization of micropropagated *Helianthemum almeriense* plantlets with *Terfezia claveryi* (desert truffle)

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The mycorrhizal synthesis of *Terfezia claveryi* with micropropagated plantlets of *Helianthemum almeriense* was carried out *in vitro* on modified Modified Melin-Norkrans (MMN) agar medium with pH 8.0. The mycorrhization rate was about 80% after 12 weeks. *T. claveryi* formed ectendomycorrhizas without hyphal mantle. The effect of the fungus on *in vitro* rooting was also studied. *T. claveryi* did not enhance *in vitro* rooting of microcuttings of *H. almeriense*. *In vitro* survival of the plantlets was the limiting step and mycorrhizal inoculation appeared to improve the survival rate of rooted plantlets. The effect of the mineral composition and pH of the medium on survival are discussed.

Key words: micropropagated Cistaceae plantlets, *in vitro* synthesis

Introduction

Helianthemum almeriense Pau belongs to the Cistaceae and is one of the most abundant shrubs of the semi-arid areas in Spain. This species, which is of great interest for reafforestation, establishes ecto-endomycorrhizae with Ascomycetes such us *Terfezia* sp. and *Balsamia* sp. (desert truffles).

Terfezia claveryi is a hypogeous fungus found in marl-gypsum soils under *H. almeriense* and in marl calcareous and sandy soils under *H. almeriense* and *H. lavandulifolium* in the region of Murcia (HONRUBIA et al. 1992) . This fungus, called "turma" in Spanish semi-arid zones, is well adapted to the xeric conditions of the Southern Mediterranean area, where it is prized and is of marked gastronomic and economic importance.

We report here the first *in vitro* mycorrhization of micropropagated *H. almeriense* plantlets with *Terfezia claveryi* .

Material and methods

Plant material

H. almeriense plants were micropropagated following the procedure described by MORTE and HONRUBIA (1992). After 4 weeks, plantlets for experiments were collected from the rooting medium of MURASHIGE and SKOOG (1962) with salt strength diluted to 1:4, without auxins.

At inoculation, each rooted plantlet had a shoot length of about 3 cm, and four to five nodal segments with two leaves each. The average number of roots per plantlet was three. They were un-

Fig. 1. *In vitro* system for inoculating micropropagated *H. almeriense* plantlets with *T. claveryi*, on a modified MMN medium. (Photo: M.A. Morte)

ramified, with lengths ranging from 1 to 1.5 cm. These plantlets were called R+ to differentiate them from unrooted explants with the same shoot characteristics called R–.

Cultivation conditions throughout the process of micropropagation were 25 ± 2° C, 40 $\mu mol.m^{-2}.s^{-1}$ Growlux fluorescent light and 16-h photoperiod. These culture conditions were maintained for the mycorrhizal synthesis experiments, too.

Fungal material

Isolates of *T.claveryi* were obtained from fruitbody tissues. The best growth medium was the Modified Melin-Norkrans (MMN) agar medium (MARX 1969) (in $mg.l^{-1}$; ClCa 50; ClNa 25; $PO_4H(NH_4)_2$ 250; $MgSO_4.7H_2O$ 150; Cl_3Fe 1%; thiamine-HCl 100) with glucose 10 $g.l^{-1}$, malt 3 $g.l^{-1}$ and solidified with 15 $g.l^{-1}$ of agar and pH 8.0.

Experiments

Two kinds of substrate were used for the *in vitro* inoculation of *H. almeriense* with *T. claveryi*:

1) A sterilized mixture of vermiculite: peat (2:1, v/v) watered with MMN liquid medium with 2.5 $g.l^{-1}$ of glucose and pH 8.0. Five pieces of agar, 0.25 cm^2 in surface, with mycelium of *T. claveryi* grown on a modified MMN (MMN nutrients less malt and $PO_4H(NH_4)_2$) liquid medium with 2.5 $g.l^{-1}$ of sucrose were used as inoculum. The rooted plantlets obtained on the MS/4 medium were cultivated with the mycelium and 100 ml of substrate in Erlenmeyer flasks of 250 ml . The experiments were repeated three times with 20 inoculated and 20 control plantlets each time.

2) Modified MMN medium with 8 $g.l^{-1}$ of agar and 2.5 $g.l^{-1}$ of glucose, pH 8.0. Pieces of agar, 0.25 cm^2 in surface, with mycelium of *T. claveryi* grown on MMN medium (15 $g.l^{-1}$ of agar and pH 8.0) were used as inoculum. To facilitate the inoculation process and to see the effect of this fungus on rooting, rooted plantlets (R+) and unrooted microcuttings (R–) were inoculated with two pieces of agar with mycelium per tube. The tubes had a diameter of 2.5 cm and were 20 cm long. 25 ml of medium per tube was used (Fig. 1). The experiments were repeated twice,

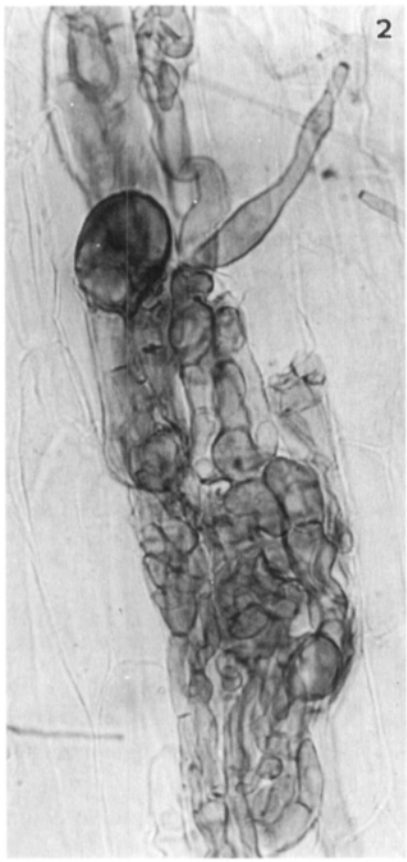

Fig. 2. Longitudinal section of infected root; the hyphae form coils that fill the host cells, showing a bead shape. 40 x. (Photo: M.A. Morte)

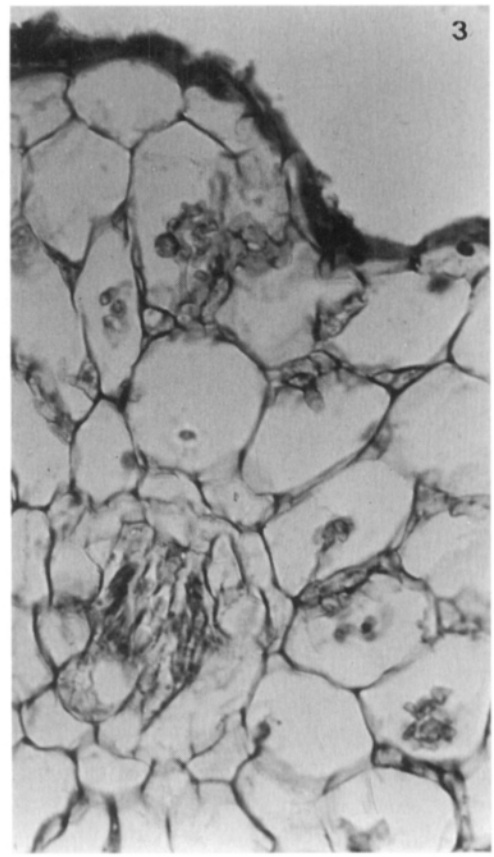

Fig. 3. Cross-section showing absence of hyphal mantle, and inter- and intracellular infections affecting only the cortical cells. 40 x. (Photo: M.A. Morte)

with 15 plantlets or explants being used for each treatment each time.

Fungal colonization was assessed on cleared and stained root samples (PHILLIPS and HAYMAN 1970). The percentage of root infection was estimated according to the grid-line intersect method (GIOVANNETTI and MOSSE 1980).

Results and discussion

Experiment 1

The mycelium grew and had colonized the whole substratum after just four weeks. Plantlets elongation was clearly visible after the same period of time. However, no mycorrhization was observed 2, 4 and 6 months after inoculation. The survival rate of the plantlets after 24 weeks *in vitro* was almost the same (about 80%) for both the control and inoculated plantlets.

We consider it probable that plantlets did not form mycorrhizas because the substrate used was very rich in nutrients.

Experiment 2

Rooting:

In comparison with plantlets rooted on MS/4 medium (MORTE and HONRUBIA 1992), the rooting rate of explants within the first 4 weeks of cul-

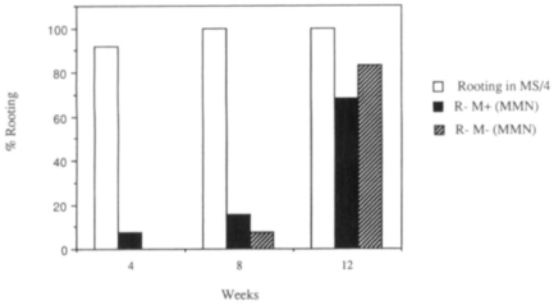

Fig. 4. Rooting percentage of *H. almeriense* microcuttings grown on MS/4 and MMN media and inoculated with *T. claveryi*.

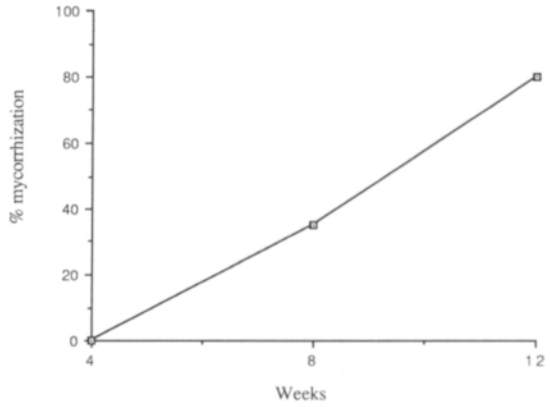

Fig. 5. *In vitro* mycorrhization percentages of R+ M+ plantlets.

ture on MMN was low (Fig. 4) . *In vitro* rooting of explant is a complex process that can be influenced by many factors (PIERIK 1987). One of the factors causing the low rooting rate in our experiments may have been the carbon source. It has been firmly established that sugar is necessary for adventitious root formation (PIERIK 1987). The kinds of carbohydrates and their concentrations also influence rooting (MONCOUSIN et al. 1992, UOSUKAINEN 1992, CABONI et al. 1992). Generally, sucrose and glucose, both at low concentrations (10–20 $g.l^{-1}$), are the most effective carbon sources for rooting (CHALUPA 1977, CHENG and VOQUI 1977, TIMMIS and RITCHIE 1984). Our experiments suggest that the kind of sugar (glucose) and the low concentration (2.5 $g.l^{-1}$) used were not conducive to rooting. Also mineral nutrients may have affected rooting, since the MMN-modified medium we used contains few mineral nutrients, and they are at lower concentrations than in the MS medium.

In this specific, and probably not optimal, nutrient context, the presence of the mycorrhizal fungus *T. claveryi* did not improve rooting of the explants (Fig. 4). IAA release of *T. claveryi* was not investigated but, if present IAA production under our experimental conditions but if any, this did not enhance *in vitro* rooting of the *H. almeriense* cuttings.

Mycorrhization:

The mycelium grew from the surface of the inoculum into the substrate within 4 weeks. This mycelial growth into the agar may have been due either to the fact that *Terfezia* is a hypogeous fungus or to the different agar concentrations (8 $g.l^{-1}$ in the tube and 15 $g.l^{-1}$ for the pieces of inoculum). It allowed the fungus to colonize the root system very well.

Until the 4th week, no mycorrhization was observed, only some infection points were visible. After 8 weeks, the mycorrhization rate was 35% and it increased to 80% after 12 weeks (Fig. 5). The newly produced roots of R– M+ microcuttings were very short (0.1–0.3 cm), with an average of five roots/explant, and did not form mycorrhizas.

Using non-micropropagated plants of *Helianthemum guttatum* and a substrate of perlite watered with a nutrient solution, FORTAS and CHEVALIER (1992) obtained mycorrhizas of *Terfezia* under axenic conditions. ROTH-BEJERANO et al. (1990) also obtained mycorrhizas with seedlings of *Helianthemum sessiliflorum* and *Terfezia leonis* in a half-strength Hoagland's solution with agar and activated charcoal.

The morphology of the mycorrhizas formed in our experiments was similar to that of the ectendomycorrhizas described by FORTAS and CHEVALIER (1992). Both the extramatricial and intercel-

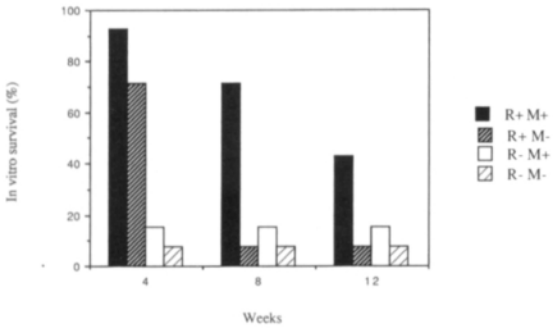

Fig. 6. *In vitro* survival percentages of mycorrhizal (R+M+) (R–M+) and non mycorrhizal (R+M–) (R–M–) plantlets or microcuttings grown on an MMN modified medium.

lular hyphae were moniliform or beadlike. The intracelullar hyphae formed coils which filled the whole lumen (Fig. 2).

Hyphal mantle was absent from some sections of the mycorrhizas (Fig. 3). Only some hyphae growing along the surface of the roots were observed. The infection was inter- and intracellular, affecting the outer layers of cortical cells. The epidermis, endodermis and central cylinder were uninfected. Similar features characterized the mycorrhizas formed under gnotoxenic conditions by seedlings of *H. almeriense* inoculated with sporal suspension of *T. claveryi* in an autoclaved soil (Cano et al. 1991).

Inoculation of *T. claveryi* improved *in vitro* survival of rooted plantlets (Fig. 6). However, this positive effect did not prevent apical necrosis and leaf fall at 6 weeks. This indicates that the MMN medium is not adequate. In comparison with MS medium, MMN is a nutrient-poor medium. Moreover, its high pH (8) is known to inhibit plant development *in vitro* (Pierik 1987).

In conclusion, the results of mycorrhizal synthesis have shown that it is possible to obtain *in vitro* mycorrhizal symbiosis between micropropagated plantlets of *H. almeriense* and *T. claveryi* on a modified MMN medium. Our further research will concentrate on developing a modified MS medium in order to obtain better plant growth and mycorrhization *in vitro,* and on studies of *post vitro* development of mycorrhizal *H. almeriense* plantlets.

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SELOSTUS

Terfezia claveryi -sienen *in vitro* siirrostus mikrolisättyihin *Helianthemum almeriense* -taimiin

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Terfezia claveryi-sienen ja mikrolisättyjen *Helianthemum almeriense*-mikrotaimien välistä mykorritsasymbioosia tutkittiin *in vitro*- olosuhteissa muunnetulla Melin-Norkrans (MMN) agaralustalla, jonka pH oli 8,0. Kahdentoista viikon jälkeen taimien mykorritsaprosentti oli 80. *T. claveryi* muodosti isäntäkasvin kanssa tupettoman ektendomykorritsan.

Sienen vaikutusta tutkittiin myös *in vitro* -juurrutuksessa. *T. calveryi* ei parantanut *H. almeriense* mikropistokkaiden *in vitro* juurtumista. Mikropistokkaiden eloonjääminen *in vitro* -olosuhteissa muodostui rajoittavaksi tekijäksi. Mykorritsasiirrostus näytti parantavan juurtuneiden mikrotaimien eloonjäämisprosenttia. Tutkimuksessa pohdittiin myös agaralustan ravinnekoostumuksen ja pH-luvun vaikutusta eloonjäämiseen.