

Effect of arbuscular mycorrhizal inoculation on micropropagated *Tetraclinis articulata* growth and survival

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(Received 30 July 1996; accepted 23 September 1996)

Summary — Micropropagated *Tetraclinis articulata* plants were inoculated at acclimatization stage with two types of inoculum of *Glomus fasciculatum*. Substrate consisted of a sterilized mixture of soil, sand and peat. No major differences were observed between the two types of inoculum to the extent of plant root colonization as it affects plant survival. In both cases, mycorrhizal colonization was not very high (37%) but did produce stimulation of plant growth. The arbuscular mycorrhizal inoculation was also associated with an improvement in the survival of plants during the weaning stage. Thus, it is appropriate to introduce inoculation with arbuscular mycorrhizal fungi into the micropropagation protocol of *T. articulata*. It may not only result in better growth during weaning but also perhaps in better adaptation to field xeric conditions.

Cartagena cypress / micropropagation / mycorrhizal dependence / *Glomus* sp / transplant stress

Résumé — Effet de la mycorhization arbusculaire sur la croissance et la survie de *Tetraclinis articulata* micro-propagé. Des plantes de *Tetraclinis articulata* ont été inoculées au moment de l'endomycorhization avec deux types d'inoculum de *Glomus fasciculatum*. Un mélange stérile de sol/sable/tourbe a été utilisé comme substrat. Aucune différence majeure a été observée entre les deux inoculums au niveau de la colonisation des racines et la survie des plantes. Dans les deux cas, la mycorhization n'a pas été très élevée (37 %) mais elle a produit une stimulation de la croissance des plantes et amélioré leur survie pendant le sevrage. Ainsi, il est judicieux d'introduire des champignons mycorhizogènes à arbuscules dans le protocole de micropropagation de *Tetraclinis articulata*. Cela peut induire non seulement une meilleure croissance pendant le sevrage mais peut-être aussi une meilleure adaptation aux conditions xériques du champ.

Tetraclinis articulata* / micropropagation / dépendance mycorhizale / *Glomus fasciculatum

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INTRODUCTION

Due to the widespread use of Cartagena cypress (*Tetraclinis articulata* [Vahl] Masters) in revegetation programmes in Spain, it has recently been propagated in vitro by both axillary and adventitious bud production (Morte et al, 1992; Morte and Honrubia, 1996). However, although it is known that *T articulata* is normally infected by arbuscular mycorrhizal (AM) fungi, and growth enhancements can be obtained by inoculation with AM fungi (Díaz and Honrubia, 1993), the substrates used for the in vitro stages and potting mixtures usually lack AM propagules. Inoculation of these plantlets is thus likely to enhance growth (Gianinazzi et al, 1990). Surface sterilized spores are able to infect some woody plants in vitro (Pons et al, 1983; Ravolanirina et al, 1987) but in vitro inoculation with AM fungi is a lengthy and difficult practice, requiring isolation and sterilization of fungal spores (Ravolanirina et al, 1987; Schubert et al, 1987, 1990). Furthermore, after transplanting, roots grown in vitro are usually replaced (Conner and Thomas, 1981), and as a consequence most of the mycorrhizal roots would be lost at this stage.

In contrast, post vitro inoculation at the time of transplanting the axenically propagated rooted plant to the potting media appears feasible (Ravolanirina et al, 1989; Schubert et al, 1990), and there are now numerous examples of micropropagated plants which have been inoculated at the weaning stage (Adholeya and Cheema, 1990; Chavez and Ferrera-Cerrato, 1990; Azcón-Aguilar et al, 1992; Salamanca et al, 1992; Schubert et al, 1992; Vidal et al, 1992; Lovato et al, 1994; Sbrana et al, 1994). Moreover, in all of these plants, mycorrhization improved plant growth and mineral nutrient status and, in some of them, mycorrhizal inoculation has been demonstrated to increase resistance to transplant stress.

In this study, micropropagated *T articulata* plantlets were inoculated at the acclimatization stage and the influence of mycorrhizal inoculation on growth and development of in vitro propagated plantlets was determined.

MATERIALS AND METHODS

Plant material

T articulata plants were micropropagated from shoot tips of 1-year-old seedling and cotyledon explants fol-

lowing the procedure described by Morte and Honrubia (1996) and Morte et al (1992).

At inoculation stage, each in vitro rooted plantlet had a shoot length of about 4 or 5 cm, and an unramified root, ranging from 7 to 10 cm. Acclimatization was performed by maintaining plantlets at 100% air humidity with a transparent plastic sheet cover for 1 week and then progressively opening the cover, which was finally removed after 2 weeks. Inoculation was carried out at the beginning of the weaning stage.

Fungal inoculum

Glomus fasciculatum (Thaxter) Gerdemann and Trappe emend Walker and Koske was selected as the mycorrhizal fungus because it has been demonstrated to be effective for *T articulata* seedlings (Díaz and Honrubia, 1993). This fungus was provided by the Zaidín Experimental Station (Granada, Spain) and multiplied in pot cultures of *Medicago sativa* L.

Two types of inoculum were used: i) A mixture of soil, mycorrhizal root fragments and spores from the pot cultures. Each replicate received 5 g of this inoculum, placed close to the roots. ii) Washed mycorrhizal roots, of the same pot cultures, with the external mycelium and spores attached, but free from the soil particles. Inoculum (3 g, fresh weight) was cut into 1 cm fragments and applied to each plantlet close to the root system.

Filtered leachates of both types of inoculum were applied to untreated plantlets to compensate for the free-living microbiota associated with the mycorrhizal inoculum. Natural, untreated soil was filtered through filter paper in order to obtain natural microflora without AM propagules; 5 mL of these natural soil sievings were added to the pots.

Growth substrate

The growth substrate consisted of a mixture of soil, sand and peat (5:4:1, v/v) sterilized by autoclaving at 100 °C, 103 KPa, for 1 h, three times on alternative days. The soil consisted of 257.5 g kg⁻¹ total CO₃⁻², 1.20 g kg⁻¹ total N, 4.67 g kg⁻¹ organic C, 8.04 g kg⁻¹ organic matter, 3.87 C/N relation, 1 mg kg⁻¹ available P and 7.79 pH water.

Determinations

Plants were inoculated in plastic pots (400 mL). Plantlets were grown in the greenhouse under natural light/dark conditions and watered when appropriate. The experiments were repeated twice and ten plantlets were used per treatment. Duncan's statistical test (Duncan, 1955) was applied to the results.

Survival of inoculated and control plantlets was observed 4, 8 and 12 weeks following inoculation. The height of shoots and length of roots were recorded. AM fungal colonization of roots was assessed following clearing and staining according to Phillips and Hayman (1970), with minor modifications (Díaz and Honrubia, 1993). Percentage root colonization was then estimated according to the grid-line intersect method (Giovannetti and Mosse, 1980) under a stereoscopic microscope (Olympus SZH, Tokyo, Japan).

RESULTS AND DISCUSSION

No major differences were observed between the two types of inoculum used in colonization on the effects on plant survival (table I). In both cases, colonization by AM fungi was relatively low after 12 weeks: at 36.4% for plantlets inoculated with the mixture of soil, mycorrhizal roots and spores and at 37% for plantlets inoculated with fresh roots (table I). Moreover, these levels were very similar to those previously obtained with *T. articulata* seedlings inoculated with the same fungal strain after a similar period (Díaz and Honrubia, 1993). A relatively low level of root colonization by AM fungi thus seems to be characteristic of this species in the experimental conditions applied. Mycorrhizal roots contained numerous arbuscules and vesicles characteristic of AM fungi.

However, despite this relatively low colonization a stimulation of plant growth occurred but differences in growth were only evident after 12 weeks (fig 1). This could be due to the fact that roots produced in vitro were not colonized and it took this time for new roots to be formed and become colonized by the AM fungus. Mycorrhizal fungi are known to colonize only young, secondary roots before suberization (Barea et al,

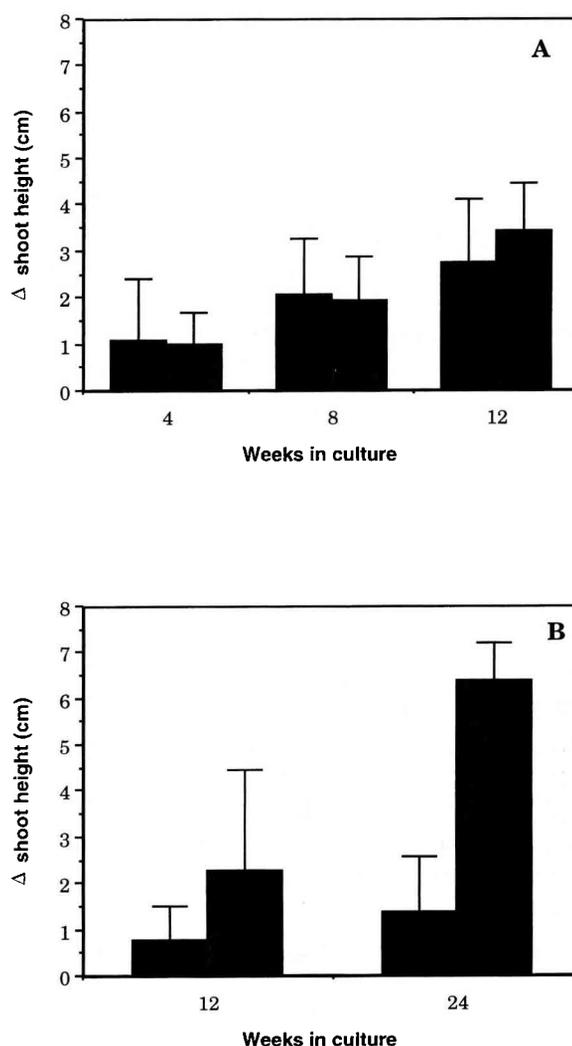


Fig 1. Effect of inoculation with *Glomus fasciculatum* on shoot height of micropropagated plantlets of *Tetraclinis articulata* (black bars = control plants; lighter bars = inoculated plants). **A)** Inoculation with a mixture of soil, mycorrhizal root fragments and spores. **B)** Inoculation with washed mycorrhizal roots. Vertical lines in bars represent standard deviation of means. Δ = increase.

Table I. Effect of inoculation with a mixture of soil, mycorrhizal root fragments and spores (inoculum 1) and washed mycorrhizal roots with external mycelium and spores attached (inoculum 2) of *Glomus fasciculatum* on mycorrhizal colonization and survival of micropropagated *Tetraclinis articulata* plantlets.

Inoculum	Treatment	% mycorrhization 12 wk	% survival		
			4 wk	8 wk	12 wk
1	Control	0 ^a	80 ^a	50 ^a	40 ^a
	Mycorrhization	36.4 ^b	100 ^a	70 ^b	60 ^b
2	Control	0 ^a	66 ^b	33 ^a	33 ^a
	Mycorrhization	37 ^b	100 ^a	50 ^b	50 ^b

^{ab} Values in columns followed by the same letter do not differ significantly at $P < 0.05$ as determined by Duncan's test.

1993). Therefore, because most of the *T articulata* plantlets had an unramified root when they were inoculated, and these susceptible young secondary roots only produced during post vitro development, inoculation during the in vitro stage is unlikely to be useful. The slight reduction in the growth of shoots of inoculated plantlets immediately after inoculation could be due to the fact that, in some cases, plants start their growth during the initial stages of mycorrhizal colonization, only to renew it later (Harley and Smith, 1983).

These results confirm data reported previously (Hayman and Tavares, 1985; Aziz and Habte, 1990; Díaz and Honrubia, 1993) where it was shown that even with low percentages of colonization, AM fungi could significantly improve plant growth. Our results showed that AM inoculation can have similar positive effects on micropropagated *T articulata* plantlets, as previously observed on *T articulata* seedlings (Díaz and Honrubia, 1993). We have not investigated here the mechanism which allows such growth enhancement, but it is likely that the well-proven ability of AM fungi to increase plant nutrient uptake from the soil (Smith and Gianinazzi-Pearson, 1988) may be at least partly responsible. The effects of mycorrhizal inoculation on plant growth may also be due to improved rhizogenesis of the otherwise poorly rooted transplants, as has been suggested with other plants (Heslin and Douglas, 1986; Schubert et al, 1990; Azcón-Aguilar et al, 1992; Vidal et al, 1992). Furthermore, AM fungi are known to improve root formation, possibly by affects on hormone production (Barea and Azcón-Aguilar, 1982) and this could be an important factor.

This could also be the reason why inoculation improved survival of plantlets during the weaning stage (table I). This improvement was observed from 4 weeks on. This positive effect of AM fungi to help plantlets resist environmental stress at transplanting has also been observed by other authors (Barea et al, 1993; Vidal et al, 1992). This difference might be due to the previously mentioned improvement of rhizogenesis, which could be induced by the fungus. Mycorrhizal inoculation has also been reported to increase the production of lateral roots in micropropagated plants (Schellenbaum et al, 1991; Berta et al, 1995). Thus, a morphogenetic effect of the AM fungi on the root system of micropropagated plantlets of *T articulata* cannot be excluded.

In conclusion, inoculation with AM fungi is appropriate to introduce into the micropropagation protocol because it not only improves plantlet

growth, but also survival was superior to control plantlets, which may allow a better posterior adaptation to field xeric conditions.

ACKNOWLEDGMENTS

M Asunción Morte acknowledges the receipt of a grant from Spanish Ministerio de Educación y Ciencia.

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