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New report of the mycorrhizal association between *Pisolithus tinctorius* (Sclerodermataceae, Basidiomycota) and *Quercus coccifera* (Fagaceae, Angiospermae)

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Abstract – In this paper we described for the first time the mycorrhiza between the fungus *Pisolithus tinctorius* and the kermes oak *Quercus coccifera,* obtained by inoculation. The synthesis was performed under nursery conditions using vegetative (40 mL/plant) or sporal (10^8 spores/plant) inoculum of *P. tinctorius* basidiomata collected from two different locations and putative hosts, and containers filled with a sterile mixture of sphagnum peat/ black peat/perlite+vermiculite. The mycorrhizal association is described and illustrated in detail and compared with other known *P. tinctorius* mycorrhiza. Mycorrhizae were monopodial-pinnate to pyramidal pinnate, cream to brown colour, mantle surface loosly woven, extramatrical mycelium abundant, rhizomorophs scarce and well differentiated, sclerotia frequent, subglobose to lemon-shaped, mantle plectenchymatous with three layers. The conditions of inoculation are also discussed. A beneficial effect of the mycorrhizal symbiosis on plant growth attributes is suggested.

mycorrhiza / sclerotia / rhizomorph / inoculum / *Pisolithus* / kermes oak

INTRODUCTION

Quercus coccifera L., the kermes oak, is a widely distributed shrub in the western Mediterranean basin, from Morocco to Greece. It grows under altitudes of 1000 m and is well adapted to the high temperatures and water stress conditions that characterize the Mediterranean climate. Therefore, it is used in the reforestation of disturbed lands in this area.

Quercus is considered to be an ectomycorrhizal genus and several studies describe the occurrence of ectomycorrhizae in a wide variety of species. Some have been reported to form mycorrhizae with the fungus *Pisolithus tinctorius* (Pers.) Coker & Couch, such as *Quercus alba* L. (Walker & McLaughlin, 1991), *Q. gambelli* × *Q. turbinata* (Walker, 1990), *Q. ilex* L. (de Roman & de Miguel, 2005), *Q. ilex* subsp. *ballota* (Desf.) Samp. (Domenech *et al.,* 2004), *Quercus myrsinaefolia* Blume (Tam & Griffiths, 1993), *Q. palustris* Münchh. (Maronek *et al.,* 1981), *Q. pyrenaica* Wild. (Duñabeitia *et al.,* 2004), the tropical *Q. serrata* Thunb. and *Q. acutissima* Carruth (Oh *et al.,* 1995), *Q. rubra* L. (Crunkilton *et al.,* 1992), *Q. suber* L. (Díez *et al.,* 2000) and *Q. virginiana* Mill. (Davies *et al.,* 1990). However, very little information is available about *Q. coccifera*, mainly in relation

to the effect of inoculation in field experiments in semi-arid ecosystems (Maestre *et al.,* 2002, Caravaca *et al.,* 2005), whereas descriptions of mycorrhizae remain unreported. Morphological descriptions of mycorrhiza are essential to understand new plant-fungi associations and therefore constitute a useful tool to identify the mycorrhizae obtained in nursery or field studies.

The objective of this study was to establish the mycorrhizal synthesis between *Q. coccifera* and *Pisolithus tinctorius* under nursery conditions in an attempt to determine the most effective conditions for the production of mycorrhizal seedlings, and to characterize the synthesized mycorrhizae for subsequent studies.

MATERIALS AND METHODS

Plant material: Acorns of *Q. coccifera* were collected from a plantation at the Campus of the University of Murcia, Murcia, Spain. They were soaked in water for 24 h and the floating seeds were rejected. Seeds were pre-germinated at room temperature in peat and transferred to the container (one per container) at the radicle emergence (15-30 days). A high level of germination was reached (94%). *Q. coccifera* seedlings were cultivated in 350 cm^3 plastic, removable containers using as potting substrate a mixture of sphagnum peat/black peat/ perlite+vermiculite (2:1:1 v/v) autoclaved twice (1h, 120ºC) at 24 h interval.

Fungal inoculation: P. tinctorius basidomata were collected from two locations: a mixed forest of *Pinus halepensis* Miller-*Q. rotundifolia* Lam. at Moratalla, Murcia, Spain, and a plantation of *Q. coccifera* at the Campus of the University of Murcia, Murcia, Spain. Reference basidiomata were deposited at the Laboratory of Mycology-Mycorrhizas collection of the University of Murcia, Spain (UM E005-09).

Two types of inocula were used: (1) Mycelia were isolated by explants from basidiomata tissue on modified Melin-Norkrans medium (MMN) (Marx, 1969) and transferred on fresh medium every 3-4 months. The strains used were: 42 AM (from the mixed forest) and 01 IN (from the *Q. coccifera* plantation). Mycelial inoculum was prepared using 500 mL flasks filled with a sterilized (120ºC, 20 min) mixture of peat and vermiculite (1:4, v/v), moistened with liquid MMN medium (400 mL/L substrate) and sterilized again (120ºC, 20 min). Flasks were then inoculated with 20-30 plugs/L of mycelium growing on MMN agar plates and incubated at 25ºC in the dark for 4-8 weeks. (2) Sporal inoculum was prepared according to Brundrett *et al.,* (1996). Basidiomata were crushed and sieved through a 250-500 µm mesh sieves and stored in dry conditions at room temperature until their use. At the moment of inoculation, spores were suspended in sterile tap water with a few drops of Tween 20 as surfactant.

Seedlings were inoculated six month after germination. Mycelial inoculum was applied at the dose of 40 mL/plant onto the root surface by opening the container. Sporal inoculum was applied by watering 3 times at one-month interval at the dose of 0.1 g/plant which corresponded to 10^8 spores/plant. Not inoculated plants remained as controls.

Plants were grown outdoors under natural climatic and day/night conditions at the University of Murcia, Spain. The climate is dry Mediterranean, with an average annual rainfall of 300 mm, mostly concentrated in the autumn,

and a mean annual temperature of 17.8 ºC. They were watered with mini-diffusers when needed. Neither fertilization, nor fungicide and pesticide treatments were applied during the experiment. 224 replicates were used for each treatment.

Mycorrhizal assessment: Four months after inoculation all the plants were assessed for the presence of mycorrhizae. The percentage of mycorrhizal seedlings was calculated as the ratio between seedlings that became mycorrhizal and total inoculated seedlings, for each treatment. Data were analyzed by contingency tables with the software package SPSS 10.0 for Windows.

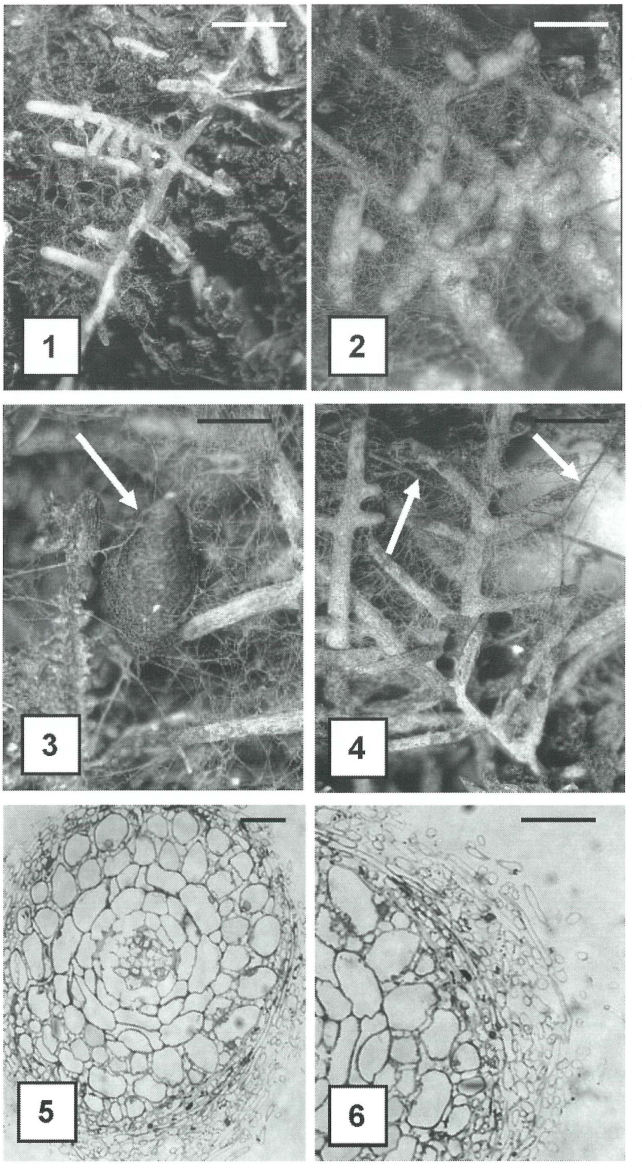
Description of mycorrhizae: Fresh mycorrhizae were described and photographed using an Olympus® SZH zoom stereo-microscope. Selected root pieces were fixed in FEA (13 mL formol-200 mL 50 % ethanol-5 mL acetic acid), washed in distilled water and put into an ultrasonic cleaner (Fungilab S.A.) to detach substrate debris from the surfaces. Then, they were washed three times in cold phosphate buffer 0.2 M pH 7.2, included in 1 % OsO_4 for 2.30 h and maintained for 2 h in 4.8 % uranyl acetate. Serial ethanol dehydration and Spurr resin embedding were carried out according to Roland & Vian (1991). Semi-thin (1.0 µm) sections were sliced with a Reichert-Jung® Ultracut microtome, using glass knives. Sections were stained with 1% toluidine blue in 1% acetic acid, and then observed and photographed using a light Olympus® BH-2 microscope. Mycorrhizae were described following the criteria of Agerer (1987-2002). Measurements are given as minimum-maximum intervals on a basis of 30 samples. Voucher samples of the fixed mycorrhizae were deposited at the Laboratory of Mycology-Mycorrizas of the University of Murcia (UM E005-09).

Plant growth measurements: Ten months after sowing, non destructive plant growth parameters were measured. Height, basal stem diameter and number of branches were measured on the seedlings. Data were analyzed by ANOVA with the software package SPSS 10.0 for Windows and significant differences among treatments were determined using the least significant difference (LSD) test at $p < 0.05$.

RESULTS

Mycorrhizae description

Mycorrhizae monopodial-pinnate to pyramidal pinnate in the basal part, variable in size, (4)-10-25-(50) mm long and 0.3-0.6 mm in diameter of the main axis. Unramified ends rather straight, sometimes sinuous, 1.0-2.5 mm in length and 0.2-0.4 mm in diameter, with rounded, cylindric apex of cream colour when young and dark cream to brown colour when old. Mantle surface loosely woven with scarce emanating hyphae not specifically distributed. **Rhizomorphs** scarce, well differentiated, pale brown, formed by aggregation of mantle hyphae, 100-150 µm in diameter, roundish, connected to mycorrhizae on mantle surface with tightly interwoven hyphae. **Extramatrical mycelium** abundant. **Sclerotia** frequent, dark brown, subglobose to lemon-shaped, outer layer plectenchymatous and center pseudoparenchymatous, 0.8-1.8 mm length and 1-1.4 mm diameter, connected to mycelial strands. (Figs. 1-4). **Mantle** in cross-section 25-40 µm thick, with three different layers distinguishable, all of them plectenchymatous. Outer layer with hyphae, 2-5 µm width. Middle layer of loosely arranged hyphae, 2-5 µm



Figs. 1-4. Mycorrhiza between *Pisolithus tinctorius* and *Quercus coccifera*. **1-2.** Monopodial-pinnate to pyramidal-pinnate mycorrhizal system with extramatrical mycelium. **3.** Sclerotia connected to mycelia strands. **4.** Rhizomorphs connected to the mycorrhiza. **5-6.** Cross-section showing the mantle and Hartig net. Bars: 1 = 0.8 mm, 2, 3, 4 = 1 mm, 5, 6 = 20 µm.

width and inner layer of densely arranged hyphae 2-6 µm width. **Hartig net** extending two to three layers of cortical cells, with irregular hyphae (1.5)-2-(4) µm in diameter (Figs. 5-6).

Mycorrhizal formation

Successful mycorrhizal synthesis was obtained with all the inoculation treatments. The two strains assessed produced similar results in terms of infectivity. However, the number of mycorrhizal seedlings obtained varied depending on the type of inoculum applied. Vegetative inoculum on peat/ vermiculite substrate was more effective than sporal inoculum for the two fungal strains used (Table 1).

Seedlings growth

Mycorrhizal seedlings were taller and had a larger stem diameter than the uninoculated ones, but the number of leaves was similar in all the treatments. The height of the seedlings inoculated with mycelial inoculum of the strain 42 AM of *P. tinctorius* and the stem diameter of the seedlings inoculated with sporal inoculum were significantly greater than those of the control plants (Table 2).

Table 1. Percentage of mycorrhizal seedlings of *Quercus coccifera* seedlings inoculated with two types of inoculum of *Pisolithus tinctorius.* Data are obtained from 224 replicates. Values followed by a different letter are significantly different according to contingency tables.

Fungal origin	*Type of inoculum*	*Mycorrhizal seedlings (%)*
P. halepensis-Q. rotundifolia mixed forest	Mycelial- strain 42 AM	76.6 b
	Sporal	27.1 a
Q. coccifera plantation	Mycelial-strain 01 IN	73.8 b
	Sporal	23.3 a

Table 2. Plant growth parameters of nonmycorrhizal and mycorrhizal *Quercus coccifera* seedlings inoculated with two types of inoculum of *Pisolithus tinctorius.* Mean ± standard error (n = 50). Values followed by a different letter are significantly different according to LSD test ($p < 0.05$).

Inoculation treatment	*Height (cm)*	*Leaves (number)*	*Stem diameter (mm)*
P. tinctorius-mixed forest			
Mycelial-42 AM	15.8 ± 5.1 b	28.5 ± 6.9 a	3.16 ± 0.5 ab
Sporal	14.2 ± 2.6 ab	29.9 ± 11.2 a	3.31 ± 0.53 b
P. tinctorius-plantation			
Mycelial-01 IN	13.7 ± 3.0 ab	28.3 ± 13 a	2.99 ± 0.13 ab
Sporal	13.2 ± 3.6 ab	27.9 ± 9 a	3.28 ± 0.79 b
Uninoculated control	11.7 ± 2.8 a	27.3 ± 7.5 a	2.85 ± 0.64 a

DISCUSSION

P. tinctorius is a well-known fungus that has been widely used for the inoculation of forest plants (Marx *et al.,* 1984). Most studies have focused on field or nursery responses, but do not describe mycorrhiza features. The mycorrhizal association with *Q. coccifera* is described for the first time in this study, which indicates the host compatibility of kermes oak with *P. tinctorius*.

The features of synthesized mycorrhizae were similar for both the inoculated fungal strains and consistent with the description of *P. tinctorius* mycorrhizae provided for *Picea abies* (L.) H. Karst. (Weiss, 1992; Agerer & Rambold, 2004-2008), *Betula alleghaniensis* Britton (Massicotte *et al.,* 1990), *Eucalyptus* (Rose *et al.,* 1981) or *Quercus ilex* L. (de Miguel & de Roman, 2005). Colour differed from the characteristic golden-yellow colour (Torres & Honrubia, 1994; Oh *et al.* 1995; Díez *et al.,* 2000; Moyersoen & Beever, 2004; de Miguel & de Roman, 2005), but was the same as that observed in the mycorrhizae obtained between *Pinus halepensis* and a *P. tinctorius* isolated from the location of this study (Díaz *et al.,* 2004). It is noteworthy that the cuticle of these basidiomata is white when young but brown to brown-violet when mature, and that the gleba is ochre to brown but not yellow (unpublished data). Similarities between the basidioma and mantle are frequent, so the differences observed in the colour of the mantle may be due to variations in the origin of the fungal strains for one same fungal species. Molecular studies would be convenient to define the differences between *P. tinctorius* populations.

The mantle of *Q. coccifera-P. tinctorius* is plechtenchymatous and multilayered, which agrees with Agerer & Rambold (2004-2008), de Miguel & de Roman (2005), Díez *et al.* (2000), but differs from the uniform, prosenchymatous mantle described for *Pinus massoniana* Lamb. and *P. elliottii* Engel. by Tam (1994).

The formation of sclerotia is an important character that has not much been documented for *P. tinctorius*. Sclerotia are resistance structures related to ecological adaptations to dry conditions. In this sense, the abundance of sclerotia observed may be due to the fungi and plant species used, which came from semiarid conditions, and also to the experimental conditions of this study. Likewise, the capacity to form sclerotia confers an elevated tolerance to water stress conditions, which is characteristic of the Mediterranean climate. Further studies are needed to elucidate the contribution of these structures to physiological plant responses to stress.

Rhizomorphs appeared scarce but well differentiated and match the long-distance exploration rhizomorph-type described by Agerer (2001). The amount and differentiation of rhizomorphs and the extramatrical mycelium as a transport structure are also factors of ecological importance that account for nutrient uptake and play a key role in plant growth and performance (Roussau, 1994). The rhizomorphs found in the *Q.coccifera-P.tinctorius* association might perform a relevant function in soil system exploration in the Mediterranean ecosystems where these symbionts are common.

The two fungal strains used were able to form mycorrhizae no matter what their origin and putative plant host, thus demonstrating the lack of specificity of *P. tinctorius* under the experimental conditions of this study. The differences observed in terms of infectivity were due to the type of inoculum used. Although spores obtained from basidiomata and stored for a period of time have been proposed for inoculation given their ability to survive storage (Brundrett *et al.,*

1996), vegetative inoculum is usually highly effective with *P. tinctorius* (Díaz *et al.*, 2010). Indeed, vegetative inoculum enables the use of fungal strains that have been specifically selected for criteria like effectiveness, plant growth or field performance.

Mycorrhizal seedlings exhibited increased root branching when compared with non-mycorrhizal ones, which may provide advantages in field conditions after outplanting. The non-destructive plant growth parameters measured are insufficient to drow definitive conclusions in terms of effectiveness. Nevertheless, certain enhance plant growth was observed in mycorrhizal seedlings, suggesting a beneficial effect of the mycorrhizal symbiosis on plant growth attributes.

The production of *Q. coccifera* seedlings mycorrhized with *P. tinctorius* is feasible under nursery conditions. The synthesized mycorrhiza may be recognized by morphological and anatomical criteria, which could prove a useful tool for practical purposes. Mycorrhizal inoculation induces positive plant growth responses in seedlings and this may have ecological implications for field performance.

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