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SHORT NOTE

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Ectomycorrhizal associations with *Cedrus atlantica* (Endl) Manetti ex Carrière. I. Mycorrhizal synthesis with *Tricholoma tridentinum* Singer var. *cedretorum* Bon

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Abstract The roots of *Cedrus atlantica* grown in a cedar forest soil under gnotoxenic conditions formed a mycorrhizal association with *Tricholoma tridentinum* Singer var. *cedretorum* Bon when this fungus was inoculated into the soil as a mycelial form. This association was not observed when plants were grown on an artificial substrate or when mycelium was immobilized in a calcium alginate gel. The influence of host receptiveness, fungal survival and cultural conditions on the mycorrhizal infection of *C. atlantica* is discussed.

Key words Ectomycorrhizal association · *Cedrus atlantica* · *Tricholoma tridentinum* · Mycorrhizal synthesis · Receptiveness

Introduction

The controlled mycorrhization of *Cedrus atlantica* (Endl.) Manetti ex Carrière (Atlas cedar) has been little studied despite the importance of this forest species for afforestation in Mediterranean countries. Since young cedars artificially inoculated with mycorrhizal fungi show more vigor (Nezzar-Hocine et al. 1996a), their survival at outplanting may thus be increased. This could result in a significant improvement of afforestation in regions currently experiencing problems of regeneration (Le Poutre 1961; Hocine et al. 1994).

According to previous surveys carried out in a natural Algerian forest, fungi belonging to the genus *Tricholoma* are the most representative of the mycorrhizal mycoflora in native Atlas and French cedar forest (Nezzar-Hocine et al. 1996b, 1998). *Tricholoma tridentinum* Sing var. *cedretorum* Bon is among the rare fungi specific to *C. atlantica*.

In France, inoculation of Atlas cedar has been attempted from pure cultures of *Cortinarius herculeus* Malç., *Suillus collinitus* Fr., *Tricholoma tridentinum* Sing., *Tricholoma terreum* (Sch.: Fr.) Kumm. and *Tuber albidum* Pico (Argillier et al. 1994). Only inoculations with spores of *Tuber albidum* (=*T. borchii* Vitt) (Mousain et al. 1987; Argillier et al. 1994), *Tuber melanosporum* Vitt., and *Tuber uncinatum* Chatin (Chevalier unpublished data) resulted in mycorrhizal associations. However, Ruehle et al. (1981) in the USA reported mycorrhizal synthesis of *Pisolithus arrhizus* (Scop.) S. Rauschert (=*P. tinctorius*) and *Thelephora terrestris* Fr.: Fr. with *C. atlantica*, although the level of mycorrhization was extremely low. In Morocco, Abourouh (1983, 1994) failed to obtain cedar mycorrhizas by inoculation with *Thelephora terrestris* and *Rhizopogon* sp. and very few mycorrhizas developed following inoculation with mycelial cultures of *Pisolithus tinctorius*.

Successful mycorrhization of Atlas cedar has also been reported with *Laccaria laccata* (Scop.: Fr) Cooke (Nezzar-Hocine et al. 1996a) and *Hebeloma crustuliniforme* (Bull.) Quél., *H. cylindrosporum* Romagn., *Tuber albidum*, *Cenococcum graniforme* (Sow.) Ferd. & Wing. (Nezzar-Hocine 1998). Controlled mycorrhizal infection with *Tricholoma tridentinum* var. *cedretorum* has never been reported, although in 1941 Modess

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(Norkrans 1949) successfully inoculated pine and spruce with *Tricholoma pessundatum* (Fr.) Quél. which is morphologically very close to *Tricholoma tridentinum*. This paper is the first report of the successful controlled mycorrhization of *C. atlantica* with *Tricholoma tridentinum* var. *cedretorum*.

Materials and methods

Plant material

Seeds of Atlas cedar were collected in a cedar forest in Lubéron (France) in 1986 by the Robin Nursery (F-05500, Saint Laurent du Cros, France). The seeds were moistened and placed in polyethylene bags at 4 °C for 30–40 days. Then seeds were sown in containers of two types: R430 (Robin Nursery) (430 ml parallepipedic) and Hillson Rootrainers (Spencer-LeMaire Ind. Ltd. Edmonton, Alberta, Canada 175-ml.) Both containers can be opened periodically to examine the roots. Containers were filled with cedar forest soil or a peat-vermiculite mixture (1:1, v:v), autoclaved at 120 °C for 30 min and autoclaved again after 24 h. The soil was collected in Ceyrat (Puy-de-Dôme, France) from the A1 horizon of a 50 to 60-year-old cedar plantation and sieved through a 5-mm screen. Details of the physical and chemical properties of the soil are summarized in Table 1. The seedlings were grown under gnotoxenic conditions in a greenhouse (20 °C mean temperature, natural light, 50% RH, spring time) or in a growth chamber (18/22 °C, 16-h day, light intensity 110 $\mu E\ m^{-2}\ s^{-1}$, 65–80% RH). Growing substrates were fertilized with slow-release fertilizer (Osmocote 14-8-8) at 3 kg/m^3. Modified (P/5) Morizet nutrient solution (Morizet and Mingeau 1976) was distributed weekly over all seedlings older than two months. Seedlings in soil were watered 2 or 3 times a week with tap water (pH 6.9–7.0). Seedlings in the artificial substrate were alternately given water or nutrient solution.

Inoculations were performed either by placing the solid inoculum (perlite or peat moss-vermiculite or peat-perlite) onto 8-week-old containerized root systems (50 ml for R430 containers, 20 ml for Spencer- LeMaire) or by mixing alginate beads with the substrate at planting (25 ml/l).

Inoculum preparation

Two isolates of *Tricholoma tridentinum* were used: T. tri.1 isolated by Mousain et al. in 1987 and T. tri 2 isolated by Chevalier in 1989. Two types of inoculum were used: (1) A solid inoculum was prepared in three different forms by growing mycelium in 600-ml flasks containing 400 ml of perlite or peat moss-vermiculite mix (1:9, v:v) or peat-perlite (1:4, v:v) culture medium moistened with Pachlewski-Oddoux (PO) liquid medium (Rapior and Andary 1987) and autoclaved for 1 h at 120 °C. The flasks were inoculated with squares (1 cm^2) of agar medium cut and removed from the edge of a 2-month-old culture on PO medium. Inoculum was used after 9 weeks growth in the dark at 20 °C. (2) An alginate bead inoculum was prepared by growing mycelium for 8 weeks in liquid culture on PO medium in 175-ml flasks. Entrapment of mycelium in alginate was performed according to Mauperin et al. (1987). The viability of the inoculum was checked immediately after bead production and before inoculation by placing three replicates of 10 beads randomly sampled on PO medium in Petri dishes.

Experimental design

Ten or 16 single seedling replicates were used for each treatment as described in Table 2.

Mycorrhizal assessment

Two months after inoculation and onwards, the containers were opened to assess mycorrhizal development; morphological characteristics of mycorrhizas were observed under a stereoscopic microscope (non-destructive method). Selected root pieces were fixed in formaldehyde 70%: ethanol: acetic acid (5:90:5 v:v:v). To examine anatomical characteristics microscopically sections were cut by hand with a razor blade, stained with lactophenol-cotton blue, and placed in a drop of Amman's solution on a slide. Seed-

Table 1 Physical and chemical properties of cedar forest soil (Ceyrat, Puy de Dôme) receptive to *Tricholoma tridentinum*

Texture (% of fine soil)

Clay	Loam		Sand	
	Fine	Rough	Fine	Rough
(<2 μm)	(2–20 μm)	(20–50 μm)	(50–300 μm)	(200–2000 μm)
16.8	15.1	6.2	9.2	52.4

Chemical characteristics (mg/g)

pH (H_2O)	OM	C (Anne)	N (Kjeldahl)	C/N	P_2O_5 (Dyer) (assimilable)	K (ammonium acetate) (exchangable)	Ca (ammonium acetate) (exchangable)	$CaCO_3$ Total	Mg (ammonium acetate) (exchangable)
5	45.9	26.7	1.83	14.59	0.254	0.188	1.67	2	0.3

Table 2 Experimental design: number of single seedling replicates for each treatment

	Greenhouse		Growth chamber
Inoculum form	Solid inoculum		Alginate beads
Mode of inoculation	Onto root system		Mixed with substrate
Container	Robin	Spencer L.	Spencer L.
Growth substrate			
Soil	10	10	16
Artificial substrate	10	10	16

ling shoot height was recorded 13 months after inoculation. Data were analysed by ANOVA (SAS Institute 1989) with two factors and their interactions. Multiple comparisons of treatment means were carried out using least significant difference (*t*–test).

Results

Ectomycorrhiza formation

All inoculation attempts with T. tri1 and those with T. tri 2 on artificial substrates failed. Three months after inoculation, mycorrhizas were formed only on seedlings grown in cedar forest soil inoculated with the isolate T. tri 2. The mycorrhizas showed a poorly developed mantle and very few rhizomorphs. The first true mycorrhizas were observed only 7 months after inoculation (Fig. 1). They were white in colour, typical of *Tricholoma* as described by Agerer (1987–1995) from field samples. Rapid mycorrhizal establishment was only successful when solid inoculum of T. tri 2 was placed onto the root systems of plants grown in cedar forest soil in R430 containers, independent of the inoculum form used. More than 70% of the plants were mycorrhizal and the infection rate was high (60% of short roots). Inoculation with alginate beads failed. Mycorrhizal formation occurred from natural contaminants in various substrates, initially with *Thelephora terrestris* Fr.:Fr. and later in the season with *Hebeloma mesophaeum* (Pers.) Quél.

Table 3 Analysis of variance of seedling height

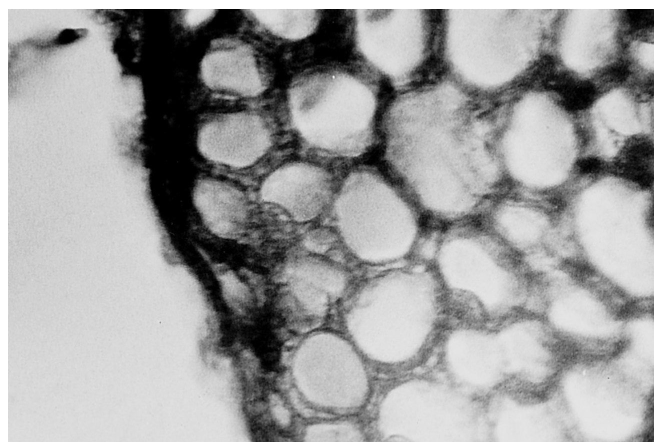
	DF	Mean square	F value	Probability
Strain	2	130.32	8.67	0.0005
Substrate	1	23.43	1.56	0.2171
Strain*substrate	2	5.41	0.36	0.6992

Table 4 Mean height of seedlings 7 months after inoculation with *Tricholoma tridentinum*. Values with the same letter are not significantly different at $P=0.05$

Strain	Mean height (cm)
T. tridentinum (T. tri2)	27.15 A
T. tridentinum (T. tri1)[a]	23.97 B
Control	22.10 B

[a] non – mycorrhizal plants

a



b

Fig. 1 **a** Typical ectomycorrhiza with white rhizomorphs emanating from the fungal mantle (X 57.5) developed in a cedar forest soil 7 months after inoculation with a mycelial inoculum of isolate *Tricholoma tridentinum* T. tri2. **b** Transverse section of an ectomycorrhiza 3 months after inoculation with a mycelial inoculum of isolate T. tri2. The mantle is plectenchymatous and the Hartig net is well developed (×100) (cedar forest soil, solid inoculum)

Plant growth

Mycorrhizal plants were taller than control plants regardless of growth substrate (Tables 3, 4). Only fungal strain had a significant influence on seedling height, i.e. there was no effect of substrate type nor any interaction between fungal strain and substrate. Shoot growth of plants mycorrhizal with T. tri2 was significantly higher than plants inoculated with T. tri.1 or non-inoculated control seedlings.

Discussion

Mycorrhizal synthesis between *Tricholoma tridentinum* var. *cedretorum* and *Cedrus atlantica* has never been reported previously. Mycorrhizas obtained with strain T. tri2 in the gnotoxenic conditions of our study were morphologically similar to those observed by Nezzar-Hocine (1998) in field conditions.

Failure of mycorrhizal development by strain T. tri1 or by strain T. tri2 in some conditions may be related to factors such as plant receptiveness, fungal persistence or culture conditions. Development of the cedar root system is characterized by the late appearance of feeder roots receptive to mycorrhizal infection. Delay in fine-

root formation can be increased by growth factors such as culture substrate, container form and fertilization rate (Nezzar-Hocine 1998). A slow development of the root system limits mycorrhization since fewer fine roots are available during the period when the fungal inoculum is viable in the substrate.

Failure of mycorrhizal infection may result from variations in fungal persistence, either the death of mycelium or its inability to infect fine roots. Since inoculum preparation and inoculation of both strains were performed at the same time and under similar conditions, the lack of mycorrhizal development by strain T. tri1 may well be due to its inherent inability to colonize Atlas cedar. This result agrees with previous work carried out with the same strain (Argillier et al. 1994). The better effectiveness of mycelium grown on solid inoculum, whatever the form, rather than alginate beads may be related to the inoculation method rather than to mycelium survival. Indeed mycelium of both strains regrew well from alginate beads on culture medium. In previous experiments, inoculation of cedar with alginate-entrapped mycelium of *Laccaria laccata* and *Hebeloma crustuliniforme* proved very successful (Nezzar-Hocine et al. 1996a; Nezzar-Hocine 1998).

The formation of ectomycorrhizas is also influenced by environmental factors and soil properties (Slankis 1974). Mycorrhizal associations were only observed on plants grown in cedar forest soil. Results in this paper demonstrate the higher receptiveness of natural soil than artificial substrates, as previously shown by Dupré et al. (1982) for *Tuber melanosporum* development in natural truffle soil. This particular soil allowed earlier development of more branched root systems than the artificial substrate and thus optimized subsequent mycorrhizal infection. The physico-chemical properties of cedar forest soil may favour mycorrhization with T. tri2 by better fulfilling the ecological requirements of the fungus. Receptiveness is greatly influenced by the physical, chemical and biological characteristics of soil (Mosse et al. 1981; Harley 1984; Perrin et al. 1996). Perrin et al. (1988) reported high variability in the receptiveness to *Laccaria laccata* of a wide range of forest soils.

Tricholoma–like ectomycorrhizas were first observed on 6-month-old seedlings, although the fungus is classified among the medium- or late-stage species (Mason et al. 1983; Fleming et al. 1986). The development of mycorrhizas on young seedlings can be better explained in terms of inoculum availability in relation to plant receptiveness (Newton 1992).

The controlled mycorrhization of cedar with mycelial cultures is more difficult than with other conifer species such as *Pinus*, *Picea* and *Pseudotsuga* (Strullu 1991), as a consequence of specific characteristics of early development of the host-plant root system. Nevertheless, mycorrhiza formation can be obtained under certain circumstances, even with fungi such as *Tricholoma*, which do not easily form mycorrhizal associations subsequent to artificial inoculation.

References

Abourouh M (1983) Essai de mycorhization en pépinière de *Cedrus atlantica* Manetti (cèdre de l'Atlas). Ann Rech For Maroc 23:93–328

Abourouh M (1994) Les ectomycorhizes du cèdre de l'Atlas: état des connaissances et perspectives. Ann Rech For Maroc 27:337–348

Agerer R (1987–1995) Colour atlas of ectomycorrhizae, 1st–9th edn. Einhorn, Schwäbisch Gmünd

Argillier C, Falconnet G, Mousain D, Guehl JM (1994) Production technique of Atlas cedar outside the ground. Ann Rech For Maroc 27:487–498

Dupré C, Chevalier G, Morizet J, Leblevenec L (1982) Influence de l'azote et du phosphore sur la mycorhization de *Quercus pubescens* Willd. par *Tuber melanosporum* Vitt. en conditions contrôlées. In: Gianinazzi S, Gianinazzi-Pearson V, Trouvelot A (eds) Mycorrhizae, an integral part of plants: biology and perspectives for their use. Les colloques de l'INRA, No. 13, INRA, Paris, pp 147–161

Fleming LV, Deacon JW, Last FT (1986) Ectomycorrhizal succession in a Scottish birch wood. In: Gianinazzi-Peason V, Gianinazzi S (eds) Physiological and genetical aspects of mycorrhizae. INRA, Paris, pp 259–263

Harley JL (1984) Mycorrhiza and soil ecology. Biol Rev 2:127–158

Hocine H, Belarbi R, Perrin R, Chevalier G (1994) Possibilités de mycorhization de *Cedrus atlantica* Manetti. Ann Rech For Maroc 27:350–361

Le Poutre B (1961) Recherches sur les conditions édaphiques de regénération des cédraies Ann Rech For Maroc 6:1–21

Mason PA, Wilson J, Last FT, Walker C (1983) The concept of succession in relation to the spread of sheathing mycorrhizal fungi on inoculated tree seedlings growing in unsterile soils. Plant Soil 71:247–256

Mauperin C, Mortier F, Garbaye J, Le Tacon F, Carr G (1987) Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. Can J Bot 65:2326–2329

Morizet J, Mingeau M (1976) Influence des facteurs du milieu sur l'absorption hydrique (étude effectuée sur tomate décapitée en exsudation). I. Facteurs nutritionnels. Ann Agron 27:183–205

Mosse B, Stribley DP, Le Tacon F (1981) Ecology of mycorrhizae and mycorrhizal fungi. In: Alexander M (ed) Advances in microbial ecology 4. Plenum, New York, pp 137–210

Mousain D, Falconnet G, Gruez J, Chevalier G, Tillard P, Bousquet N, Plassard C, Cleyet-Maret JC (1987) Controlled ectomycorrhizal development of Mediterranean forest seedlings in the nursery. First results and prospects. In: Sylvia DM, Hung LL, Graham JH (eds) Proceedings of the 7th North American Conference on Mycorrhizae, 3–8 May 1987, University of Florida, Gainsville, p 129

Newton AC (1992) Towards a functional classification of ectomycorrhizal fungi. Mycorrhiza 2:75–79

Nezzar-Hocine H (1998) Associations mycorhiziennes naturelles de *Cedrus atlantica* dans le massif du Djurdjura (Algérie) et mycorhization contrôlée. PhD thesis, Université Blaise Pascal, Clermont-Ferrand II, France

Nezzar-Hocine H, Halli-Hargas R, Chevalier G, Perrin R (1996a) Mycorrhization of *Cedrus atlantica* with *Laccaria laccata* under controlled conditions. In: Azcon-Aguilar C, Barea JM (eds) Mycorrhizas in integrated systems from genes to plant development. European Commission, EUR 16 728, Luxembourg, pp 565–568

Nezzar-Hocine H, Bouteville RJ, Halli-Hargas R, Chevalier G (1996b) La macroflore fongique de *Cedrus atlantica* (Endl.) Manetti ex Carrière. I. Inventaire des espèces d'une cédraie du massif du Djurdjura (Algérie) et connaissances actuelles sur les champignons des cédraies. Crypt Mycol 17:85–103

Nezzar-Hocine H, Bouteville RJ, Halli-Hargas R, Chevalier G (1998) La macroflore fongique de *Cedrus atlantica* (Endl.) Manetti ex Carrière. II. Les champignons ectomycorhiziens d'une cédraie du massif du Djurdjura (Algérie). Crypt Mycol (in press)

Norkrans B (1949) Some mycorrhiza-forming *Tricholoma* species. Sven Bot Tidskr 43:485–490

Perrin R, Duvert P, Plenchette C (1988) Substrate receptiveness to mycorrhizal association: concepts, methods and applications. Acta Hortic 221:223–228

Perrin R, Parlade X, Pera J (1996) Receptiveness of forest soils to ectomycorrhizal association. I. Concept and method as applied to *Laccaria bicolor* (Maire) Orton, *Pinus pinaster* Art and *Pseudotsuga menziesii* (Mirb.) Franco symbiosis. Mycorrhiza 6:469–476

Rapior S, Andary C (1987) *Cortinarius* section *orellani*: isolation and culture of *Cortinarius orellanus*. Trans Br Mycol Soc 9:41–44

Ruehle JL, Marx DH, Abourouh M (1981) Development of *Pisolithus tinctorius* and *Thelephora terrestris* ectomycorrhizae on seedlings of coniferous trees important to Morocco. Ann Rech For Maroc 21:281–296

SAS Institute (1989) SAS / STAT © User's Guide, Version 6, 4th edn, vol 2. SAS, Cary N.C.

Slankis V (1974) Soil factors influencing formation of mycorrhizae. Annu Rev Phytopathol 12:437–457

Strullu DG (1991) Les mycorhizes des arbres et plantes cultivées. Lavoisier, Paris