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Short Communication

Endomycorrhiza and Pseudo-Ectomycorrhiza Produced *in vitro* by Two Species of *Tuber* on Transformed *Cistus incanus* Roots

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Abstract

The ability of mycelial strains of *Tuber borchii* and *T. melanosporum* to produce either ecto- or endomycorrhiza with two clones of transformed roots of *Cistus incanus* was studied *in vitro* in relation to nitrogen and phosphate concentrations in the culture medium. While differing in their response to the *Cistus* clones at particular P and N concentrations, both *Tuber* species displayed the ability to form endomycorrhiza and pseudo-ectomycorrhiza without Hartig net; however, typical ectomycorrhiza with mantle and Hartig net was not obtained. These results support the notion that *Tuber* can produce different types of mycorrhiza depending on external conditions.

ABBREVIATIONS

VAM: Vesicular-Arbuscular mycorrhizas; ECM: Ectomycorrhiza; EM: Endomycorrhiza; EEM: Ectendomycorrhiza; IAA: Indole 3-Acetic Acid; MMN: Melin And Norkrans Medium; TR: *Cistus incanus* Transformed Roots; Ps-ECM: Pseudo-Ectomycorrhiza

INTRODUCTION

The notion that the majority of land plants are completely dependent on symbiotic microorganisms for growth and survival is now well established, highlighting the importance of soil microbes as regulators of plant diversity and abundance [1]. Among the various symbiotic associations discovered so far, the one linking roots and fungi – the mycorrhiza – is very widespread and is considered to be the determining factor in the evolution of land plants [2,3].

Several types of mycorrhizas have been described, and different systems of mycorrhizal classification have been proposed based on their anatomy [4,5]. Increasingly, however, results of *in vitro* experiments [6,7] and field and greenhouse observations [8], coupled with the new picture of the phenomenon in nature revealed by molecular identification tools [9], support the idea that there are basically just two classes of mycorrhiza: 1) those produced by the aseptate mycelia of species belonging to the Glomerulomycota and Mucoromycotina [10], usually referred to as vesicular-arbuscularmycorrhizas (VAM) in view of the structures formed inside the host cells – although some *Endogonaceae* can also produce ectomycorrhizas [11]; 2) mycorrhizas produced by the septate mycelia of septomycetes, that is, members of the Ascomycota and Basidiomycota.

Ectomycorrhizas (ECMs) are particularly widespread among the septomycetes and have been extensively studied for their role in forest ecosystems. They were discovered in the late nineteenth century (1885) by B. Frank and were the first form of mycorrhizal symbiosis to be described. In an ectomycorrhiza the fungal hyphae completely cover the root tip, forming a mantle (mycoclena) from which they penetrate the apoplastic spaces of the cortical layers of cell wall surfaces [12].

The genus *Tuber* (Ascomycota, Pezizales), from which our earliest knowledge about mycorrhizas was derived [13], has long been considered a typical ectomycorrhizal fungus. However, molecular tools [9] have revealed that certain fungal species hitherto considered to be exclusively ectomycorrhizal (including *Tuber* species) may produce septateendomycorrhizas (orchid

mycorrhiza, OM) with the achlorophyllic orchid *Neottia*. This has been repeatedly confirmed with other species of orchids, including chlorophyllic species [14], and with other plants such as Ericaceae[15].

The possibility of obtaining two or even three different types of mycorrhiza *in vitro* –ectomycorrhiza (ECM), endomycorrhiza (EM), and ectendomycorrhiza (EEM) – was already known from studies conducted with *Terfezia* spp, (desert truffles) which form both ectomycorrhizas characterized by a Hartig net [6,16]and septateendomycorrhizas with hyphae penetrating into cortical host cells, in both cases without a hyphal mantle [6,17]. As the former are observed under rich nutritional conditions while the latter occur on media poor in phosphorus [6], it would appear that the nutritional status of the host plant determines which type of mycorrhiza will develop. The notion that the phosphate status of a plant affects mycorrhizal synthesis is well established [18]. It seems that P deficient plant roots exude a greater range and volume of substances to their environment, probably due to increased permeability of the plasma membranes [19]. It has lately been shown that P deficient *Arabidopsis* plants are characterized by an increased sensitivity to auxin [20]. Auxins have long been known to be implicated in ectomycorrhizal formation. More especially, they have been shown to play a role in the morphological changes that roots undergo during mycorrhiza formation [21]. It has been demonstrated that a fungal mutant overproducing indole 3-acetic acid (IAA) forms three to five times more mycorrhiza than a wild-type mycelium [22]. In some instances, such a mutant has been reported to form ectendomycorrhiza characterized by intracellular penetration and Hartig net [23]. External auxin level has also been shown to play a role in determining the type of mycorrhiza produced [7].

Less studied is the effect of nitrogen concentrations on the determination of mycorrhizal types: Haug et al. [24] working on mycorrhizas of *Pisolithus tinctorius* in *Picea abies* roots showed that high concentrations of either nitrate or ammonia resulted in a diminished Hartig net and in hyphal penetration into cortical cells. Brunner and Scheidegger [25] studying colonization of the same plant species by *Hebeloma crustuliniforme* reported thatNH_3concentrations in excess of 30 mM caused a similar shift in mycorrhizal morphology, namely from the known wild type ectomycorrhiza to an atypical endomycorrhiza. Lately, Flores-Monterroso et al. [26] demonstrated that either excess of NH_3 or its deficiency, altered gene expression and ectomycorrhiza stability in maritime pine roots.

In the study reported below, we examined the types of mycorrhizal association formed *in vitro* by *Tuber borchii* Vittad. and *Tuber melanosporum* Vittad. With two transformed roots clone of *Cistus incanus* L. in relation to diverse external nitrogen and, to some extent, phosphate concentrations, using an *in vitro* culture system. *In vitro* culture systems offer a controlled environment in which the mechanisms involved in the morphogenesis of mycorrhizas may conveniently be explored, as demonstrated by Ventura et al. [27] and Zaretsky et al. [7].

MATERIALS AND METHODS

Tuber cultures

Tuber melanosporum strain 1015 and *Tuber borchii* strain ATCC96540 mycelia were sub cultured routinely on modified Melin and Norkrans (MMN) medium at 25°C for 40 days.

Cistus incanus transformed roots

For mycorrhization, we employed two clones of *Cistus incanus* transformed roots (TR) previously used by [7], namely M2 and W51. These were subcultured for 60 days at 25°C on N5 medium (MS medium with 20% of the amount of nitrates), solidified with 0.2% Phytagel adjusted to pH 5.8 before autoclaving and supplemented with 500 mg l^{-1} ampicillin.

Mycorrhization

M medium was used to obtain mycorrhizal synthesis based on the results of Wenkart et al. [32] and Zaretsky et al. [7]. TRs were transferred to Falcon 50 ml tubes containing 30 ml of medium for all experiments.

Transformed roots were allowed to grow for 3 weeks before inoculation. A cube of MMN agar containing the *Tuber* mycelium was then added to each TR containing tube. The co-cultures were kept in the dark at 25°C for 2-4 months prior to examination.

Experimental design

The following formulations of M medium were tested:

- original minimal M medium (4.8 mg/ l KH_2PO_4 and 80 mg/l KNO_3)
- M medium modified with respect to phosphorus concentration: low P (0.96 or 0.48 mg/l KH_2PO_4)
- M medium modified with respect to nitrogen concentration: low N (20.2, 80, or 101.1 mg/l KNO_3), and high N (404 or 606 mg/l KNO_3).

Both basic and modified M media were gelled with 0.7% Plant Agar (Sigma) adjusted to pH 5.5 before autoclaving.

Each test was performed with at least five samples.

Microscopy

Fungal mycorrhizal colonization of the roots after 2-4 months was assessed by staining with cotton blue (0.1% methyl blue in Amman lactophenol). Prior to staining, each sample was cleared by heating in 10% KOH for 15-30 min in a warm bath (60°C). Next, it was then washed in 0.1 N HCl and bidistilled water, placed in cotton blue, and heated to boiling, then rinsed in lactophenol and observed in the same liquid, or embedded in the resin Tissue-Tek OCT (Sakura) and then deep frozen at -20ºC.

Sections of 12 µm were obtained using a cryostat and observed in lactophenol with a Leitz DMRB microscope.

RESULTS

The results of the mycorrhization experiments are presented in (Table 1). They may be summarized as follows:

Effect of varying the N concentration from 20 to 606 mg/l

P 4.8, N 20.2: the two truffle species react in the same way, each forming endomycorrhiza (Figure 1a) with clone M2 and pseudo-ectomycorrhiza (Figure 1b,c) with clone W51, i.e. both exhibit differential responses to the TR clones.

Table 1: Types of mycorrhiza formed by *Tuber melanosporum* and *Tuber borchii* with transformed roots (TR) of two clones of *Cistus incanus* (M2 and W51) under various concentrations of nitrogen (N) and phosphate (P). ENDO - septate endomycorrhiza; Ps-ECM - pseudo-ectomycorrhiza; none -no growth of mycelium and/or mycorrhizal symbiosis.

Medium (mg/l)		TR	Mycorrhizal type	
P	N		*T. melanosporum*	*T. borchii*
4.8	20.2	M2	ENDO	ENDO
		W51	Ps-ECM	Ps-ECM
4.8	80	M2	ENDO	ENDO
		W51	ENDO	ENDO
4.8	101.1	M2	None	Ps-ECM
		W51	None	Ps-ECM
4.8	404	M2	None	ENDO
		W51	None	ENDO
4.8	606	M2	None	ENDO
		W51	None	ENDO
0.96	80	M2	ENDO	Ps-ECM
		W51	Ps-ECM	Ps-ECM
0.48	80	M2	ENDO	Ps-ECM
		W51	Ps-ECM	Ps-ECM

Abbreviations: TR: Transformed Roots; ENDO: septate endomycorrhiza; Ps-ECM: pseudo-ectomycorrhiza; None: no growth of mycelium and/or mycorrhizal symbiosis

P 4.8, N 80 (original M medium): the two truffle species produce endomycorrhiza with both TR clones, i.e. they react in the same way to both clones.

P 4.8, N ranging from 101 to 606: *T. melanosporum* does not enter into mycorrhizal relations at all while *T. borchii* does, with endomycorrhiza predominating. The differences observed are between fungal species.

Effect of varying the P concentration from 4.8 to 0.48 or 0.96 mg/l

At P 0.48 or 0.96, N 80, *T. melanosporum* reacts differently to each of the root clones, forming endomycorrhiza with M2 but Ps-ECM with W51. *T. borchii* consistently forms Ps-ECM mycorrhiza with both root clones. In this situation, differences were found both between fungal species and between root clones.

DISCUSSION

Our main finding is that both fungal species proved capable of forming septate endomycorrhiza on both *Cistus incanus* transformed root clones, given appropriate conditions (Table 1, Figure 1a). On the other hand, in none of the experiments performed in this study was a true ectomycorrhiza formed by either fungal species on either clone. This is surprising in view of the finding that, under appropriate conditions, *Terfezia* spp did form such associations *in vitro* with clone W51 [7], and so did *T. melanosporum* with clone L2A; we note that the latter clone is no longer available. The reasons for the discrepancy are unknown at the moment, though it seems reasonable to suppose that they reflect differences between the clones. To be certain of our result, the roots in this study were most scrupulously analyzed. However, underdeveloped ectomycorrhizal relations have been described before. Malajczuk et al. [29] described in detail what they termed 'superficial ectomycorrhiza' in which fungal hyphae of two different species never penetrated the cortex of the *Eucalyptus* species studied. Brundrett [4] analyzed ectomycorrhizal forms and also reported epidermal or superficial forms. We believe that the term 'pseudo-ectomycorrhiza' best describes our findings, as no true penetration of hyphae into apoplastic spaces between root cells was observed.

Clone M2 differs from Clone W51 mainly in its greater sensitivity to external auxins [7]. The only instances where mycorrhizal type differed between the two clones resulted in

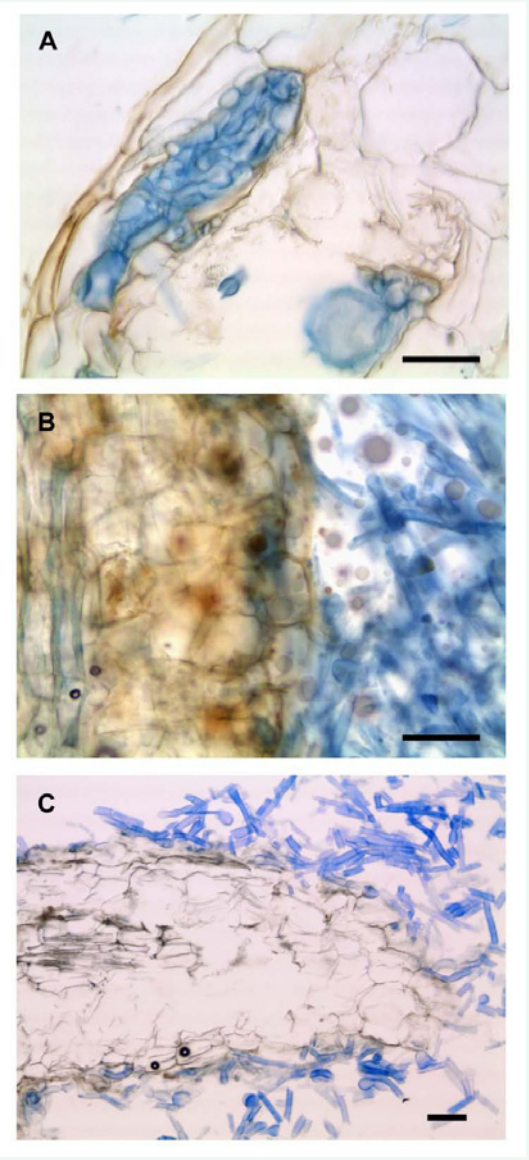

Figure 1 Types of mycorrhiza produced in vitro by *Tuber* strains cultivated with transformed roots of *Cistus incanus*: (A) – Septate endomycorrhiza; (B) – Squash of a pseudo-ectomycorrhiza; (C) – Thin section of a pseudo-ectomycorrhiza lacking any visible true Hartig net. Bar 50 µm.

the formation of endomycorrhiza with clone M2, suggesting that under these two controlled conditions (4.8 mg/ml P and 20.2 mg/ml N, as well as 0.48 P and 80 N) the differential response of the two clones may have stemmed from differences in auxin excretion by the two fungal species. As auxin secretion levels were not measured in this research, this must be seen as an assumption at this time.

Analysis of the different responses of the two fungal species leads us to conclude that *T. melanosporum* is more sensitive to high nitrogen concentration than *T. borchii*. Indeed, *T. melanosporum* failed to form any kind of association with either root clone when N concentration reached 101 mg/ml or higher, while *T. borchii* did produce mycorrhiza at these levels, whether ENDO or Ps-ECM.

CONCLUSION

Above we described conditions that support *in vitro* formation and maintenance of endomycorrhizal associations of two *Tuber* species with transformed roots of *Cistus incanus*. Indeed, the most interesting conclusion to be drawn from this study seems to be that, under suitable conditions, any ectomycorrhizal fungus may be capable of forming an underdeveloped type of septate endomycorrhiza [7,8,13,16,22,30].

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REFERENCES

1. van der Heijden MG, Bardgett RD, van Straalen NM. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett. 2008; 11: 296-310.
2. Pirozynski KA, Malloch DW. The origin of land plants: a matter of mycotrophism. Biosystems. 1975; 6: 153-164.
3. Simon L, Bousquet J, Lévesque RC, Lalonde M. Origin and diversification of endomycorrhizal fungi and co-incidence with vascular land plants. Nature.1993; 363: 67-69.
4. Brundrett M. Diversity and classification of mycorrhizal associations. Biol Rev Camb Philos Soc. 2004; 79: 473-495.
5. Imhof S. Arbuscular, ecto-related, orchid mycorrhizas-three independent structural lineages towards mycoheterotrophy: implications for classification? Mycorrhiza. 2009; 19:357-363.
6. Fortas Z, Chevalier G. Effect does conditions de culture sur la mycorhization de l Helianthemum guttatum par troisespèces de terfez des genres *Terfezia* et *Tirmania* d'Algérie. Can J Bot.1992; 70:2453-2460.
7. Zaretsky M, Kagan-Zur V, Mills D, Roth-Bejerano N. Analysis of mycorrhizal associations formed by Cistus *incanus* transformed root clones with *Terfezia boudieri* isolates. Plant Cell Rep. 2006; 25: 62-70.
8. Gutiérrez A, Morte A, Honrubia M. Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire. Mycorrhiza. 2003; 13: 299-307.
9. Selosse MA, Faccio A, Scappaticci G, Bonfante P. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla*,(Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb Ecol. 2004; 47: 416-426.
10. Bidartondo MI, Read DJ, Trappe JM, Merckx V, Ligrone R, Duckett JG,. The dawn of symbiosis between plants and fungi. Biol Lett. 2011; 7: 574-577.
11. Warcup JH. Taxonomy, culture, and mycorrhizal associations of some zygosporic Endogonaceae. Mycol Res. 1990; 94: 173-8.
12. Peterson RL, Massicotte HB, Melville LH. Mycorrhizas: Anatomy and Cell Biology. CABI Publ., Wallingford, Oxon, UK. 2004.
13. Pacioni G, Comandini O. Tuber. In Cairney JWG, Chambers SM (eds) Ectomycorrhizal fungi: key genera in profile. Springer Verlag, Berlin. 1999; pp 163-186.
14. Pandey M, Sharma J, Taylor DL, Yadon VL. A narrowly endemic photosynthetic orchid is non-specific in its mycorrhizal associations. Mol Ecol. 2013; 22: 2341-2354.
15. Krpata, D, Mühlmann, O, Kuhnert R, Ladurner H, Göbl F, Peintner U. High diversity of ectomycorrhizal fungi associated with *Arctostaphylos uva-ursi* in subalpine and alpine zones: Potential inoculum for afforestation. Forest Ecol Manag. 2007; 250: 167-175.
16. Roth-Bejerano N, Livne D, Kagan-Zur V. *Helianthemum-Terfezia* relations in different media. New Phytol. 1990; 114:235-238.
17. Awameh MS. The response of *Helianthemum salicifolium* and *H. ledifolium* to infection by the desert truffle *Terfezia boudieri*. Mushroom Sci. 1981; 11:843-853.
18. Harley JL, Smith SE. Mycorrhizal symbiosis. Academic Press, Inc., London. 1983.
19. Graham JH, Leonard RT, Menge JA. Membrane-mediated decrease in root exudation responsible for phorphorus inhibition of vesicular-arbuscular mycorrhiza formation. Plant Physiol. 1981; 68: 548-552.
20. López-Bucio J, Hernández-Abreu E, Sánchez-Calderón L, Nieto-Jacobo MF, Simpson J, Herrera-Estrella L. Phosphate availability alters architecture and causes changes in hormone sensitivity in the *Arabidopsis* root system. Plant Physiol. 2002; 129:244-256.
21. Barker SJ, Tagu D. The Roles of Auxins and Cytokinins in Mycorrhizal Symbioses. J Plant Growth Regul. 2000; 19: 144-154.
22. Gay G, Normand L, Marmeisse R, Sotta B, Debaud JC. Auxin overproducer mutants of Hebelomacylindrosporum Romagnesi have increased mycorrhizal activity. New Phytol. 1994; 128: 645-657.
23. Gea L, Normand L, Vian B, Gay G. Structural aspects of ectomycorrhiza of *Pinus pinaster* (Ait.) Sol. formed by an IAA-overproducer mutant of *Hebeloma cylindrosporum* Romagnesi. New Phytol. 1994; 128: 659-670.
24. Haug S, Ritter T, Kottke I, Oberwinkler F. Steigerung der Leistungsfahigkeit von Forstpflanzen (*Fagus silvatica* L. und *Quercus robur* L.) durch kontrollierte Mykorrhizierung. Allg Forst Jagdztg. 1992; 163:72-79.
25. Brunner I, Scheidegger C. Effects of high nitrogen concentrations on ectomycorrhizal structure and growth of seedlings of Picea abies (L.) Karst. New Phytol. 1994; 129:83-95.
26. Flores-Monterroso A1, Canales J, de la Torre F, Ávila C, Cánovas FM. Identification of genes differentially expressed in ectomycorrhizal roots during the Pinus pinaster-Laccaria bicolor interaction. Planta. 2013; 237: 1637-1650.
27. Ventura Y, Mills D, Kagan-Zur V, Roth-Bejerano N, Bustan A. Mycorrhized Ri-transformed roots facilitate *in vitro* inoculation of *Cistus incanus* with *Tuber melanosporum*. Plant Cell Tiss Org. 2006; 85: 53-61.

28. Wenkart S, Roth-Bejerano N, Mills D, Kagan-Zur V. Mycorrhizal associations between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. Plant Cell Rep. 2001; 20: 369-373.

29. Malajczuk N, Dell B, Bougher NL. Ectomycorrhiza formation in *Eucalyptus*. III. Superficial ectomycorrhizas initiated by *Hysterangium* and *Cortinarius* species. New Phytol. 1987; 105: 421-428.

30. Roth-Bejerano N, Navarro-Ródenas A, Gutierrez A. Types of mycorrhizal association. In Kagan-Zur V, Roth-Bejerano N, Sitrit Y, Morte A (eds). Desert truffles. Soil Biology, Vol. 38. Springer. 2014; pp 69-80.

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