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SHORT NOTE

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Detoxification of ferulic acid by ectomycorrhizal fungi

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Abstract The ectomycorrhizal fungi *Laccaria amethystina* and *Lactarius deterrimus* grown in liquid culture were used to study the fate of added ferulic acid. *Laccaria amethystina* degraded ferulic acid to the major metabolite vanillic acid. The intermediate vanillin was not detected. *Lactarius deterrimus* showed a completely different detoxification pattern. Two dimers and one trimer of ferulic acid could be identified as polymerization products of this fungus. A bioassay of the possible biological activities of ferulic acid and vanillic acid on these fungi revealed that vanillic acid was less toxic than ferulic acid for *Laccaria amethystina* but that both phenolic acids were toxic for *Lactarius deterrimus*. The results are discussed with respect to ectomycorrhizal fungal growth in the organic layer of forest soils and between living root cells of ectomycorrhizas.

Keywords Ectomycorrhizal fungi · Ferulic acid · *Laccaria amethystina* · *Lactarius deterrimus* · Phenolics

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Introduction

Ectomycorrhizal fungi proliferate in the organic layer of soils in temperate forests where they gain organic and mineral nutrients. However, this layer is also rich in phenolic compounds derived from plant litter (Bending and Read 1997). In particular, soluble, low molecular weight phenolics, such as phenolic acids, are fungitoxic to a variety of fungi (Christie 1965; Haars et al. 1981; Wacker et al. 1990). There is virtually no information available concerning toxicity of known phenolic compounds to ectomycorrhizal fungi (Münzenberger 1991).

Ferulic acid is a widely distributed hydroxycinnamic acid in nature (Rosazza et al. 1995). It belongs to the phenolic derivatives that are related to lignin metabolism. Bacterial and fungal oxidative enzymes such as laccases are able to transform this phenolic acid (Faure et al. 1996; Gramss et al. 1998; Günther et al. 1998). Fungal phenol-oxidizing enzymes of ectomycorrhizas can contribute to humification and detoxification processes in the soil (Günther et al. 1998). Both the abundance of phenolic acids and polyphenols (such as tannin) increase during degradation of litter and during the humification process (Gallet and Lebreton 1995).

Ferulic acid and its dehydrodimers are important in strengthening cell walls in living plant tissue (Fry 1982; Micard et al. 1997; Fry et al. 2000). Ferulic acid is ester-linked to polysaccharides where it may occur in monomeric or dimeric form. Wall flexibility and digestibility are restricted because of these phenolic bridges and plants are protected against invasion by pathogenic fungi (Friend 1981; Graf 1992). However, wall flexibility is necessary in ectomycorrhizas where the fungus grows between the cortical cells to form the Hartig net. Induced defence responses of ectomycorrhizal fungi can lead to an accumulation of phenolic compounds thereby limiting Hartig net formation (Feugey et al. 1999). Mature mycorrhizas of *Laccaria amethystina-Picea abies* contain much lower concentrations of cell wall-bound ferulic acid than non-mycorrhizal fine roots (Münzenberger et al. 1990, 1995). As yet, it is not clear if biosynthesis of

ferulic acid is suppressed in the root tissue or if ferulic acid is detoxified by extracellular enzymes of the ectomycorrhizal fungus itself. Another fascinating possibility is that both mechanisms, suppression of plant defence genes and detoxification by fungal enzymes, work in unison.

For this investigation we have chosen *Laccaria amethystina* (Bolt. ex Hooker) Murr. and *Lactarius deterrimus* Gröger, two ectomycorrhizal fungi, as both are highly sensitive to ferulic acid (Münzenberger 1991). Further, *Lactarius* species are known to produce extracellular polyphenol oxidase (Giltrap 1982; Hutchison 1990; Gramss et al. 1998), an enzyme that is of great importance as it is able to oxidize and polymerize phenolics involved in host defence, rendering them non-toxic (Bending and Read 1997). Both ectomycorrhizal fungi were grown in liquid culture containing ferulic acid as substrate. Newly formed phenolic compounds were analysed by high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) and by nuclear magnetic resonance (NMR) spectroscopy. A bioassay was performed to clarify the toxicity of the phenolic compounds.

Materials and methods

Fungal culture

Fifteen Erlenmeyer flasks were filled with 150 ml modified Melin Norkrans (MMN) nutrient solution containing 0.025 g NaCl, 1.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.05 g $CaCl_2$, 0.15 g $MgSO_4.7H_2O$, 0.05 g $FeCl_3$, 1 ml thiamine hydrochloride and 10 g glucose (Mason 1980) and were autoclaved (20 min, 120°C). Twenty discs each (5 mm diameter) of vigorously growing mycelium of *Laccaria amethystina* TÜ 977 and of *Lactarius deterrimus* TÜ 1384 on agar plates were added to five Erlenmeyer flasks, respectively. The fungal discs were crushed for 5 s using an Ultra-Turrax (Janke and Kunkel, IKA Labortechnik, Staufen). Five Erlenmeyer flasks were left non-inoculated as a control. The flasks were sealed with cotton stoppers and incubated on a rotary shaker (100 r.p.m.) at room temperature. After 1 month, 1 ml of a ferulic acid solution (Serva, Heidelberg) was added to each flask to give a final concentration of 15.4 µM. As biotransformation of ferulic acid was faster for *Lactarius deterrimus*, samples (1 ml) were taken hourly and *Laccaria amethystina* samples were taken daily.

HPLC analysis

The content of ferulic acid and its transformation products were estimated by HPLC analysis of 100 µl of the aqueous culture supernatant for both fungi. HPLC was performed on a Hewlett Packard (Bad Homburg) HPLC apparatus 1050 M equipped with a quarternary pump system, a diode array detector 1040 M series I, and an HP ChemStation. The separation was achieved with a LiChroCart 125–4 RP-18 endcapped (5-µm) column (Merck, Darmstadt). The initial solvent composition was 10% methanol – 90% phosphoric acid (0.1%), reaching 60% methanol and 40% phosphoric acid after 14 min at a flow rate of 1 ml min^{-1}. The amount of substrate and products was calculated from their absorbance at 220 nm in comparison with that of the corresponding standard compounds, ferulic acid (Serva) and vanillic acid (Aldrich, Steinheim).

Extraction of polymeric compounds

Aqueous supernatants of flasks containing *Lactarius deterrimus*, ferulic acid and metabolites were adjusted with 1 N NaOH to pH 7 after an incubation time of 30 min. The supernatant was shaken twice with ethyl acetate (1:1, v/v) to extract the polymeric metabolites into the organic phase. This was dried with Na_2SO_4 and evaporated in vacuo. The residues were redissolved in 1 ml ethyl acetate, taken to dryness with nitrogen and used for structural studies. Similarly the aqueous phase of the same flasks was acidified to pH 2 with 1 N H_2SO_4 and the phenolic acids were extracted as above.

Mass spectrometry

For both fungi analyses of low molecular weight phenolic acids were carried out by gas chromatography-mass spectrometry (GC-MS) after extraction of the aqueous supernatants with ethyl acetate at pH 7.0 and pH 2.0, as described above. Evaporated extracts were dissolved in methanol and analysed directly after derivatization with diazomethane (De Boer and Backer 1956) using GC-MS (GC 8000 and a mass spectrometer MD 800; Fisons Instruments, Mainz). Separation was performed on a 30-m DB5-ms (0.25-mm-diameter) column (J&W Scientific, Folsom, Calif.), with a temperature program of 80–300°C at 10°C min^{-1}. Identification of substances was carried out by comparison of GC retention times and mass spectra with those of authentic ferulic acid (Serva) and vanillic acid (Aldrich).

The positive and negative ion electrospray (ESI) mass spectra of the three oligomeric compounds (1–3, see Fig. 2) were recorded on a Finnigan TSQ 7000 mass spectrometer (San José, USA) operating at an electrospray voltage of 4.5 kV for positive ion and 3.5 kV for negative ion spectra with a heated capillary inlet system (220°C), nitrogen as sheath gas and coupled with a Micro-Tech Ultra-Plus MicroLC system (column: RP18, 5 μm, 1×100 mm; SepServ, Berlin).

NMR spectroscopy

1D (^1H and ^{13}C) and 2D [H-H correlation spectroscopy (COSY), heteronuclear multi-bond connectivity (HMBC)] NMR spectra were recorded at 300°K on either Bruker DPX 300 or ARX 400 NMR spectrometers locked to the major deuterium signal of the solvent, CD_3OD.

Bioassay

MMN agar containing 0.025 g NaCl, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.05 g $CaCl_2$, 0.15 g $MgSO_4{\cdot}7H_2O$, 0.05 g $FeCl_3$, 1 ml thiamine hydrochloride, 10 ml trace elements after Fortin and Piché (1979), 5 g malt and 10 g glucose per litre was prepared and 210 ml were placed in 500 ml Erlenmeyer flasks to which 4 g agar (Merck) was added. After autoclaving, ferulic acid (*trans*-ferulic acid 98%; Serva) or vanillic acid (Aldrich) were dissolved in ethanol (70%) and added to the agar to give final concentrations of 1 mM, 0.5 mM and 0.05 mM. Pure ethanol was added to the agar for control. Petri dishes were each filled with 20 ml agar to give ten replicates per concentration. Both fungi were grown on the agar under dark conditions. After 6 weeks, areas of hyphal growth were measured using an image analysis system (Olympus CUE 3).

Results and discussion

The two ectomycorrhizal fungi showed different strategies to detoxify ferulic acid. *Laccaria amethystina* was capable of oxidizing *trans*-ferulic acid to vanillic acid as the major product within several days (Fig. 1a), indicating an attack at the unsaturated side chain of the aromatic parent compound. Shortening of the propenoyl side-chain has been observed under the biological influence of other fungi and bacteria (Rosazza et al. 1995 and citations therein). For instance, the white rot fungi *Polyporus versicolor* and *Heterobasidion annosum* degrade ferulic acid initially to vanillin and further oxidize this compound to vanillic acid (Ishikawa et al. 1963). In our case, the intermediary metabolite, vanillin, was not detected during the biotransformation. This may indicate that the biotransformation step to vanillic acid was very fast. The study is the first to provide evidence that an ectomycorrhizal fungus (*Laccaria amethystina*) uses this degradation pathway.

A second product detected by HPLC analysis was identified as *cis*-ferulic acid (Fig. 1a). However, this compound was also formed in the control flasks and, obviously, arose from conversion of *trans*-ferulic acid to the *cis*-isomer through exposure of the cultures to light over the incubation period of several days. Accumulation of *cis*-ferulic acid was also reported by Rahouti et al. (1989) for cultures of *Paecilomyces variotii*. Unlike *Laccaria amethystina*, *P. variotii* transformed the *cis*-isomer at the same rate as *trans*-ferulic acid via 4-vinylguaiacol and vanillin to vanillic acid.

Lactarius deterrimus used an alternative strategy to detoxify ferulic acid. Ferulic acid disappeared quickly within a few hours from the medium without any production of vinylguaiacol or vanillic acid (Fig. 1b). This fungus polymerized ferulic acid to several oligomeric compounds whose appearance was apparent through an intensive browning of the culture medium after several hours. Three major oligomeric compounds (1–3, Fig. 2) were identified and fully characterized as two dimeric and a trimeric oligomer of ferulic acid by the combined use of MS and NMR spectroscopy. The ESI-MS data indicated the number of ferulic acid derived units in the molecule, while the NMR data provided rigorous evidence for the identity of the fragments present in the molecules (1D ^{1}H and ^{13}C, and 2D COSY data) and their linkages from long-range correlations between ^{1}H and ^{13}C of different fragments in the structure (2D HMBC data). Finally, the structures and NMR data were compared with those in the literature and are identical to three compounds formed during the initial stages of ferulic acid polymerization by lignin peroxidase (Ward et al. 2001). Micard et al. (1997) described the asymmetrical dimer, compound 2, from sugar-beet pulp. However, Ward et al. (2001) were the first to identify trimeric structures that were derived from lignin peroxidase-catalysed oxidation of ferulic acid. Their results indicated that dehydrodimers and trimers are further oxidized by lignin peroxidase to yield a polymeric product of ferulic acid. We could show for the first time that an ectomycorrhizal fungus can catalyse at least the initial steps of polymerization of ferulic acid thus releasing polyphenolic oligomers.

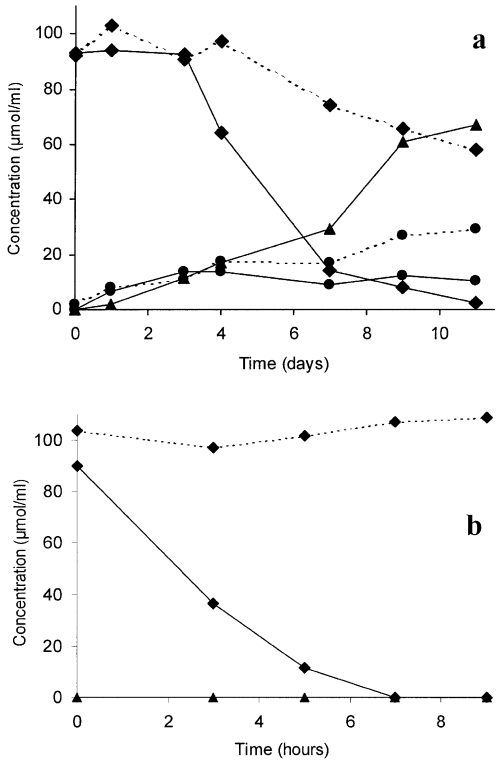

Fig. 1 **a** Decrease in ferulic acid and formation of products by *Laccaria amethystina* (——) and in controls without cells (- - - -), *n*=2. **b** Decrease in ferulic acid in cultures with *Lactarius deterrimus* (——) and in controls without cells (–), *n*=2. –◆– *Trans*-ferulic acid, –▲– vanillic acid, –●– *cis*- ferulic acid

The oxidation of ferulic acid to protocatechuic acid or another oxidative attack on the aromatic ring, which are common biodegradative pathways of microorganisms (Rosazza et al. 1995), were not documented in our study for the ectomycorrhizal fungi *Laccaria amethystina* and *Lactarius deterrimus*, and consequently both ectomycorrhizal fungi seem to be unable to cleave the aromatic ring system. Even if most ectomycorrhizal fungi show only a low ability to degrade phenols compared to wood-decomposing fungi (Bending and Read 1997), the present results show that at least some ectomycorrhizal fungi are very effective in detoxification of phenols through biotransformation to smaller molecules or to polymerization products. The bioassay revealed that vanillic acid, formed by *Laccaria amethystina*, did not inhibit growth of this fungus as compared to ferulic acid (>50% decrease), whereas *Lactarius deterrimus* was inhibited to the same degree by both phenolic acids (~50%). Obviously, the latter has become specialized in forming non-toxic polymerization products. The lowest concentration of both phenolic acids (0.05 mM) stimulated fungal growth up to twofold. Stimulation by phenols at very low concentrations was also found by Lagrange et al. (2001) for growth of the ectomycorrhizal fungus *Pisolithus* sp.

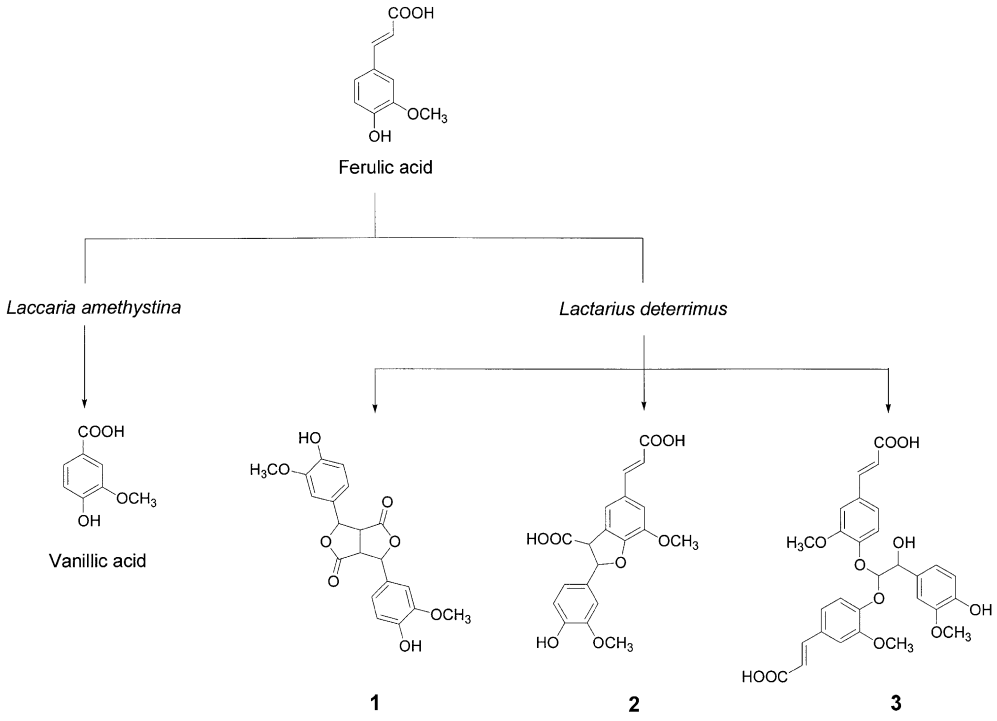

Fig. 2 Compounds formed during the detoxification of ferulic acid by *Laccaria amethystina* and *Lactarius deterrimus*

The present results show that both fungi should be able to participate in the metabolism of organic substances in forest soils such as lignin as well as its degradation products. Ferulic acid is a prominent example of these intermediary phenolic compounds. However, both fungi use different pathways to transform ferulic acid. This might suggest a different function of both fungi in the ecosystem. However, this remains to be demonstrated. Furthermore, we propose that ferulic acid might be degraded inside the root thus preventing cell wall stiffening. How this process might involve Hartig net formation should be a subject for future work.

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