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Hydroxamate siderophores of the ectomycorrhizal fungi *Suillus granulatus* and *S. luteus*

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Abstract Despite indications that *S. granulatus* and *S. luteus* release iron-chelating compounds, the exact spectrum of ferric hydroxamates synthesized by these two *Suillus* species remained unclear. Hence the aim of this study was to identify all of the main siderophores produced by these two ectomycorrhizal fungal species under pure culture conditions. By means of HPLC and LC–MS analyses we show that *S. granulatus* releases cyclic and linear fusigen, ferrichrome, coprogen and triacetylfusarinine C into the nutrient medium, while *S. luteus* culture filtrates contain cyclic and linear fusigen, ferricrocin and coprogen. All of the different siderophores were identified on basis of reference compounds and their specific MS spectra which were recorded on a high resolution MS in positive electrospray ionisation mode. Initial HPLC separations were performed on a C-18 stationary phase, using an acidic eluent (0.1% formic acid in water and acetonitrile) in gradient mode. The potential of these two ectomycorrhizal fungal species to produce siderophores representing three different groups of hydroxamates is discussed in relation to its ecological significance.

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Introduction

Almost all microorganisms synthesize siderophores for mobilization of iron which is abundant in the biosphere, albeit not bioavailable in sufficient quantities in an aerobic environment. A range of different mycorrhizal fungi including those forming ectomycorrhizae were shown to release hydroxamate-type siderophores (Haselwandter and Winkelmann 2007). Nevertheless, the spectrum of fungi screened so far for siderophore release is rather limited especially with regard to ectomycorrhizal fungi (Haselwandter 2008). Within ectomycorrhizal Ascomycota *Cenococcum geophilum* was shown to release ferricrocin as principal siderophore although smaller amounts of the fusarinine monomer, ferrichrome, fusigen and coprogen might also be released (Haselwandter and Winkelmann 2002). The extramatrical mycelium of the basidiomycete *Hebeloma crustuliniforme*, forming ectomycorrhizae with *Pinus sylvestris* seedlings, appears to release also some ferricrocin and even smaller amounts of ferrichrome (van Hees et al. 2006). Without precise description of experimental details including culture conditions Moberg et al.

(2003) reported on the detection of ferricrocin and ferrichrome in cultures of *Suillus variegatus*.

Carpophores of *Suillus* species can be found frequently in conifer dominated ecosystems. With regard to host plants suilloid basidiomycetes display species specificity which leads to a coincidence in distribution of *Suillus* and conifer species in the northern hemisphere (Dahlberg and Finlay 1999). Within the genus *Suillus* the highest species richness correlates with that of the coniferous plant family of the Pinaceae (Molina et al. 1992). Thus, about 90% of the *Suillus* species appears to have a narrow host range. While *S. granulatus* is even restricted to the genus *Pinus* as host plant, *S. luteus* seems to associate with a broader range of genera within the Pinaceae.

The ectomycorrhizal genus *Suillus* and *S. granulatus* in particular are of special interest as Watteau and Berthelin (1994) have claimed that siderophores rather than organic acids like oxalate are the causative agents with respect to fungal weathering processes. Using the *Microbacterium flavescens* JG-9 based bioassay and the chrome azurol S assay Leyval et al. (1992) have shown that *S. granulatus* mycelium produces siderophores under pure culture conditions. Furthermore such a mycelium led to the release of iron from the iron oxyhydroxide goethite. By the use of the chrome azurol S assay Machuca et al. (2006) detected the presence of iron-chelating compounds in solid and liquid nutrient media on which *S. luteus* was grown. On the other hand, the spectrum of siderophores synthesized by both ectomycorrhizal fungal species, *S. granulatus* and *S. luteus*, has hitherto been unknown. Hence the aim of this study was a qualitative analysis of the pattern of siderophores released by these ectomycorrhizal fungi into the nutrient medium when grown under iron-limited conditions.

Materials and methods

Culture conditions

The following strains of fungal isolates were selected for this study: (i) *Suillus granulatus* (L. ex Fr.) O. Kuntze, strain DOM, obtained from INRA-Nancy, F-54280 Champenoux; and (ii) *Suillus luteus* (L. ex Fr.) S.F. Gray, strain CBS-no. 568.96, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

The fungal isolates were sub-cultured in 500 ml of low iron (10 μM) medium (LIM-1 containing 1 g/l of each, L-proline and L-ornithine·HCl; Szaniszlo et al. 1981) at 25°C and 120 rev/min for 10 days. Mycelium of these sub-cultures was then used to inoculate iron free nutrient medium. For the iron free nutrient medium the low iron medium (LIM-1) was adjusted to pH 6.0 and deferrated using Chelex^{R} 100 (Bio-Rad) according to Haselwandter and Winkelmann (2009). The fungal cultures were again incubated at 25°C and 120 rev/min for 21 days (*S. granulatus*) or 39 days (*S. luteus*), respectively.

Extraction and isolation of siderophores

After incubation periods as indicated above the cultures were supplemented with a surplus of $FeSO_4 \cdot 7H_2O$ overnight at 4°C prior to sterile filtration (0.2 μm, cellulose acetate filter) and adjusting the pH to 7.0 with 5 M NaOH. The culture filtrate was loaded onto an Amberlite^{R} XAD-16 column, and the XAD material washed with two volumes of distilled water. Subsequently the siderophores were desorbed with methanol, the concentrated eluate taken up in distilled water, and freeze dried for storage at 4°C.

Analysis of ferric hydroxamates by HPLC and LC–MS

For HPLC analyses the freeze dried samples were dissolved in 5 ml distilled water and filtered through a 0.2 μm Sartorius Minisart^{R} RC 4 filter (Göttingen, Germany). The siderophores were then separated on an Agilent 1200 RRLC system (Waldbronn, Germany), equipped with online degasser (G1322A), binary pump (G1312B), autosampler (G1329B), column compartment (G1316B) and diode array detector (G1315C). Optimum separations were achieved on RP material (Grace Smart RP18, 4.6 × 150 mm, 5 μm; Grace, Deerfield, IL, USA) in gradient elution mode using water (A) and acetonitrile (B), both containing 0.1% formic acid (v/v), as mobile phase. The applied gradient was as follows: from 94A/6B in 10 min to 85A/15B, in 15 min to 10A/90B, and held at this composition for 2 min. Flow rate, column temperature and detection wavelength were set to 0.75 ml/min, 20°C and 435 nm, respectively. The injected sample volume was 5 μl, and after each run

the system was re-equilibrated for 10 min. All solvents used for analytical purposes were of HPLC grade and purchased from Merck (Darmstadt, Germany).

A mixture of standard compounds was used for monitoring the release of siderophores into the nutrient medium during the incubation period. Peak identity was confirmed in LC–MS experiments and by comparison with reference compounds. For LC–MS studies the HPLC instrument was coupled to a micrOTOF-Q II mass spectrometer from Bruker (Bremen, Germany), using the same chromatographic conditions as described above. The LC eluent was introduced to the MS instrument with a solvent split ratio of 3:1, and optimum ionization of the compounds of interest was observed in positive ESI mode. Nebulizer (nitrogen), dry gas (nitrogen) and dry temperature were adjusted to 40 psi, 8 l/min and 200°C; endplate offset and capillary voltage were set to −500 V and 4.5 kV, respectively. Separations were monitored in EIC (extracted ion chromatogram) mode.

Results and discussion

Individual siderophores produced by the two *Suillus* species were identified by HPLC and LC–MS. By comparison of the retention times and UV-spectra of reference compounds with those of respective signals cyclic fusigen ($R_t = 6.1$ min), ferricrocin ($R_t = 10.5$ min), ferrichrome ($R_t = 11.1$ min) and coprogen ($R_t = 14.8$ min) could be unambiguously assigned in the sample solutions at 435 nm. These results were further confirmed in LC–MS analyses, using a time-of flight mass spectrometer (micrOTOF-Q II) for detection. All above mentioned siderophores were clearly identified in positive ESI mode with parent ions at *m/z* values corresponding to $[M+H]^+$. Figures 1 and 2 show typical chromatograms of *S. granulatus* and *S. luteus* samples monitored at 435 nm and in EIC mode, the latter only depicting molecular masses typical for individual compounds. By LC–MS it also was possible to tentatively identify two additional ferric hydroxamates. Both *Suillus* species showed a major signal with a retention time of 6.9 min and an *m/z* value of 798.3. The fragmentation pattern of this compound was similar to that of cyclic fusigen (both show major signals at *m/z* values of 510.2 and 556.2). This and the fact that the molecular masses of both compounds differ by 18 dalton (corresponding to an additional OH group and a proton) can be taken as proof that the later compound eluting at $R_t = 6.9$ min is the linear form of fusigen. Additionally, the presence of triacetylfusarinine C in *S. granulatus* was confirmed. Even not clearly visible at 435 nm (possibly due to its low concentration in the sample) its *m/z* value of 906.7 and elution behaviour were in agreement with published data corresponding to triacetylfusarinine C (Konetschny-Rapp et al. 1988).

The spectra of siderophores released by *S. granulatus* and *S. luteus* are comparatively similar. Both fungal species produce hydroxamates belonging to the fusigen, ferrichrome and coprogen family, respectively. This is perhaps not too surprising as both species are not too distantly related as shown by phylogenetic analyses of internal transcribed spacer (ITS) sequences carried out by Kretzer et al. (1996). In case of *S. granulatus* this study included specimen from Sweden, USA (Michigan) and Nepal, while the specimen of *S. luteus* originated from Germany and USA (Michigan).

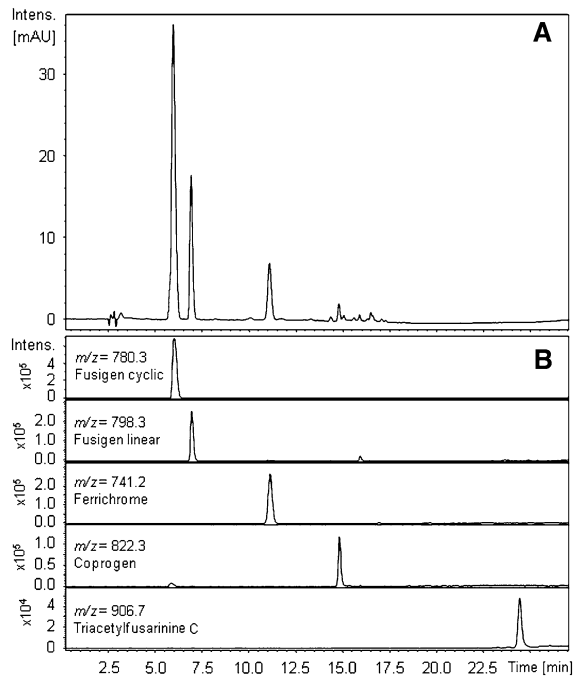

Fig. 1 LC–MS analysis of ferric hydroxamates isolated from filtrates of *S. granulatus* cultures grown in iron free nutrient medium. **a** HPLC separation monitored at 435 nm; **b** extracted ion chromatograms (EIC) for individual compounds

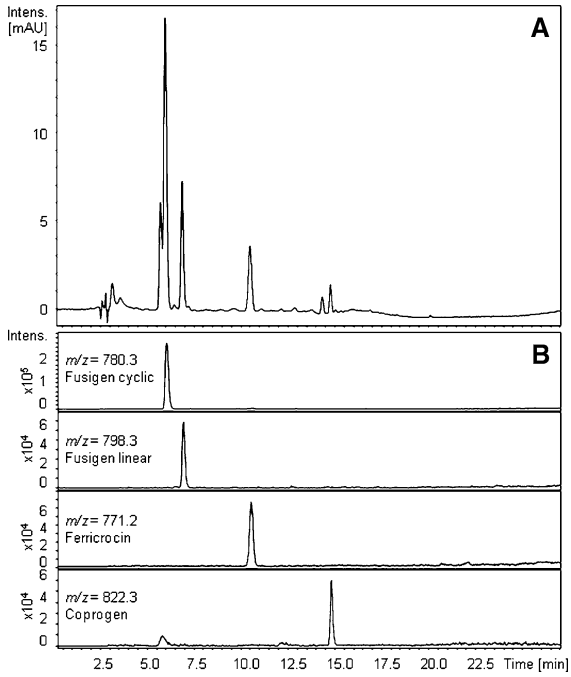

Fig. 2 LC–MS analysis of ferric hydroxamates isolated from filtrates of *S. luteus* cultures grown in iron free nutrient medium. **a** HPLC separation monitored at 435 nm; **b** extracted ion chromatograms (EIC) for individual compounds

The potential to synthesize siderophores representing different families of hydroxamates is most likely to achieve great ecological significance. The three families of siderophores mentioned above differ with regard to physico-chemical properties including solubility and hence mobility in the soil solution (Winkelmann 2007). The different hydroxamate families cover a wide range of lipophilicity. In addition the different electrical charges of the respective siderophores are bound to have an effect on the mobility of the various compounds in soil. Ferrichrome A, for example, has negative charges under neutral conditions which must be expected to affect the adsorption to positively charged soil constituents or via multivalent cations to negatively charged clays or soil organic matter.

Furthermore the fusarinines, ferrichromes and coprogens differ with regard to the characteristic bonds within the molecule, i.e. three ester bonds in fusarinines, six peptide bonds in ferrichromes, and one ester plus two peptide bonds in the coprogens. Thus, the various siderophores can be anticipated to differ with regard to their susceptibility to degradation and 'misuse' by soil microorganisms, e.g. as nitrogen or carbon sources. A pseudomonad, isolated from soil and tentatively designed *Pseudomonas* FC1, is capable of degrading ferrichrome, ferrichrome A and coprogen (Warren and Neilands 1964). *Azospirillum irakense* and *Mesorhizobium loti* were shown to catabolise desferrioxamine B and other linear and cyclic desferrioxamines (Winkelmann et al. 1999; Pierwola et al. 2004). In all three cases the degradation of the siderophores is concomitant with growth of the soil isolates and ascribed to cellular enzymes like proteinases or peptidases. On the other hand proteases, esterases and other hydrolases can be commonly found as extracellular enzymes in various soil types (Nannipieri et al. 2002). To what extent such extracellular enzymes can contribute to degradation of siderophores remains to be ascertained. In any case the ferrated siderophores appear to be more resistant against microbial degradation than the iron free forms. However, when an organism releases instead of a single compound a suite of different siderophores it can be assumed that this enhances the chances that at least one of the siderophores released will fulfil its inherent function and achieve its goal, i.e. iron mobilization for the producing organism.

Iron oxides or hydroxides in the form of hematite and goethite are the most important iron sources found in soil (Blume et al. 2002). Siderophores were shown to be present in soil solutions deriving from various soil types (Powell et al. 1980; Essen et al. 2006). In the soil environment siderophores may function as ligand-based weathering agents leading to iron dissolution (Hoffland et al. 2004). Basic to this process is the formation of siderophore-Fe(III) complexes causing mobilization of iron and hence underlying fungal weathering processes. It was reported that dissolution of iron from such sources can be mediated by relatively low concentrations of oxalate together with small amounts of siderophores (Reichard et al. 2007). However, an entirely siderophore based mechanism for Fe(III)-mineral weathering processes and iron dissolution was demonstrated by Buss et al. (2007). Due to the frequent intimate contact between ectomycorrhizal mycelium and rock material the potential siderophore release by fungal hyphae as shown in this study may well lead to immediate iron dissolution. Following a suggestion made by Courty et al. (2010) a great challenge for the future would be the use of DNA microarrays for an in situ study of the expression of

siderophore biosynthesis genes when fungal mycelium, e.g. of *S. granulatus* and *S. luteus*, is overgrowing goethite or hematite particles.

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