Metabolomics (2008) 4:52–62
DOI 10.1007/s11306-007-0100-4

ORIGINAL ARTICLE

Metabolite profiles of interacting mycelial fronts differ for pairings of the wood decay basidiomycete fungus, *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminatus*

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Received: 12 July 2007 / Accepted: 5 November 2007 / Published online: 4 December 2007

Abstract The paper presents the first proof-of principle study of metabolite profiles of the interacting mycelial fronts of a wood decomposer basidiomycete, *Stereum hirsutum*, paired with two competitor basidiomycetes, *Coprinus disseminatus* and C. *micaceus*, using TLC and GC-TOF-MS profiling. GC-TOF-MS profiles were information rich, with a total of 190 metabolite peaks detected and more than 120 metabolite peaks detected per sample. The metabolite profiles were able to discriminate between the interactions of *S. hirsutum* with the two species of *Coprinu*s. In confrontation with *C. micaceus*, where *S. hirsutum* mycelial fronts always overgrew those of *C. micaceus*, there were down-regulations of metabolites in the interaction zone, compared to monocultures of both *S. hirsutum* and *C. micaceus*. In contrast, in pairings with *C. disseminatus*, whose mycelia overgrew those of *S. hirsutum*, there were some up-regulations compared with monoculture controls, the majority of the metabolites being characteristic of the *S. hirsutum* monoculture profile. These differences indicate that up-regulation of metabolites in the mycelia of *S. hirsutum* may be connected to a defensive role or to stress. The results also show proof of principle for the employment of metabolic profiling for biological discovery studies of metabolites produced by fungi that could be applied to natural product screening programmes.

Keywords Metabolite profiling · Basidiomycete fungi · Mycelial interactions

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1 Introduction

Successions of species of decomposer fungi can be observed on wood and leaf litter in terrestrial ecosystems, usually by the occurrence of fruit bodies of the fungi, but also by temporal changes in the spatial distribution of mycelia within the wood or in the litter on the forest floor (Williams et al. 1981; Boddy and Rayner 1983; Hedger 1985). These temporal changes in fungal communities, perhaps lasting decades in the case of large tree trunks (Magnenot 1952), are variable, and have many possible sequences of species, even on the same resource type (Rayner and Boddy 1988).

However these authors also point out that species of decomposer fungi can be broadly assigned to different temporal stages of the decomposition sequence. *Primary colonisers*, some of which arise from cryptic endophytic infections of the plant tissue, are the first to be detected and may be host specific, and are followed by main *decomposition stage fungi*, arising both from spores and invasive mycelial fronts, cords and rhizomorphs. They are eventually replaced by *late stage colonizers* by which time the resource may be highly decomposed. The positions of different species in this sequence have been interpreted by Boddy (2000) and others as the result of the effect of 'driving variables' on their mycelia. These include

environmental stresses, such as nutrient content, water activity, temperature or gaseous environments, which can be alleviated or increased at different stages of the succession. In addition, colonization will be affected by disturbance, which may increase as the resource becomes more fragmented in the later stages of decomposition. However, direct combative competition between mycelia to defend or occupy resources is likely to be the most important factor in the occupation of resources in early stages of the colonization sequence.

Boddy and Rayner (1983) considered that, in conditions where environmental stress and disturbance are low, usually in the middle stage of successions, the structure of fungal decomposer communities is largely the outcome of these combative interactions between the mycelia, both interspecific and between genets of the same taxon. However, resource retention by early colonisers and invasion of late colonisers may be a mixture of adaptation to stress and disturbance together with combative defense or attack. Such combat includes inhibition, and sometimes stimulation, of mycelia following contact between fronts, but also at a distance. Changes which occur include simple inhibition of extension growth by one or both fungi, but also morphological changes, such as the formation of mycelial barrages along one or both of the advancing fronts (Boddy and Rayner 1983). This implies recognition of the presence of other mycelia and the switching on of a combative response, but how this recognition is mediated is not clear. Reactivity of mycelia at a distance implies the release and recognition of diffusible compounds, both water soluble and volatile, but in some instances contact between hyphae must occur before a response is stimulated, sometimes followed by cell death in one or both of the mycelial fronts (Ikediugwu and Webster 1970). Changes occurring during interactions can include the production of extra-cellular secondary metabolites, notably phenolic and quinonoid compounds (Griffith et al. 1994). A number of such metabolites have been identified during the past decade (Gloer 1995; Shearer 1995; Humphris et al. 2001; Wheatley 2002) in interactions between mycelia of wood decay fungi. More recently Hynes et al. (2007) have shown that production of volatiles was also increased in interactions between the mycelia of the wood decomposer basidiomycetes *Hypholoma fasciculare* and *Resinicium bicolor*. As well as metabolite changes, other studies have shown that there can be an induction of phenoloxidase enzymes and peroxidase enzymes such as laccase and manganese dependent peroxidase, during mycelial interactions (White and Boddy 1992; Gregorio et al. 2006).

Recently Heilmann-Clausen and Boddy (2005) have also pointed out that domains occupied by wood decay fungi show changes which offer a 'passive' defense against other fungi, as against active changes induced by the presence of other mycelia. These passive defenses, which increase stress to other mycelia, include changes in water content, usually by reduction, and also chemical changes, which make the colonised wood stimulatory or inhibitory to mycelia of other fungi and thereby influence the colonisation pattern.

One of the problems of interpretation of the results of the published studies on the metabolites produced during mycelial interactions is the difficulty of detecting significant changes in the many different metabolites present, especially those at low concentration, which could be of interest in understanding the mechanisms of the interactions between mycelia. To explore these ideas more fully we chose to use a metabolomic approach to a study of the combative interactions between the mycelium of a wood decay fungus, *Stereum hirsutum*, with two competitors *Coprinus micaceus and Coprinus disseminatus*. These species were selected as a simplified model of a putative successional sequence of fungi on fallen branches of Pedunculate Oak (*Quercus robur*), observed by ourselves and from the literature (Rayner and Boddy 1988; Heilmann-Clausen and Boddy 2005). *Stereum hirsutum* is an early coloniser, and its internal occupation of sapwood causes white rot decay. Production of basidiocarps on the surface can occur within a year of branch fall and the mycelium can persist for many years. In contrast *Coprinus disseminatus* is a later invasive species, colonising the branches once they have been in contact with the soil for a number of years but with the wood still firm. *C. micaceus* is also a later colonizing species but invades fallen wood after a longer period of decomposition at soil level, usually once it has reached a friable softened consistency, by means of the yellow brown mycelial cords and mycelial fronts moving inside the bark.

In this work we have aimed to study the morphology of the interactions and relate them to the expression of metabolites along the confronting mycelial fronts of *S. hirsutum* and the two species of *Coprinus*. To explore these ideas we grew the fungi in pairings on agar plates, which although criticisable as not being representative models of the natural environment, did offer growth on a solid substrate, and allowed easy extraction of metabolites for profiling.

2 Material and methods

2.1 Isolation of cultures

Fungal mycelia were isolated from basidiocarps collected from woodland in W. Sussex. For *C. disseminatus* and *C. micaceus*, pilei of basidiocarps were dissected way from the stipe and stuck to the inner side of 9 cm Petri dish lid with Vaseline and incubated overnight to deposit a spore print on-to a Petri dish of 3% w/v Malt Extract Agar

(Oxoid, UK) supplemented with 1% streptomycin (Sigma-Aldrich, UK). After 12 h of incubation at room temperature, spores from the print were streaked on-to the medium and lids were replaced with new ones. A similar technique was used for *S. hirsutum*, using a portion of basidiocarp. In both cases Petri dishes were resealed with 'Parafilm' and incubated at 25°C in the dark. Developing mycelia were checked under a microscope for clamp connections to confirm identity of the fungi as basidiomycetes.

Isolates were sub-cultured onto 3%w/v Malt Extract Agar and stock cultures were maintained in water culture in 1.5 ml cryovials (Nalgene Ltd.) with 1 ml sterilised distilled water, at room temperature.

2.2 Interactions on agar plates

The experiments were performed in 9 cm Petri dishes containing 15 ml Potato Dextrose Agar (Sigma-Aldrich, UK). Agar plugs (5 mm) were taken with a sterilized no. 3 cork borer from edges of actively growing cultures (25°C) and were placed 3 cm from the edge of the plate and 3 cm away from the other plug. *S. hirsutum* was separately confronted with *C. disseminatus* and *C. micaceus* and all three organisms were also self-paired. All combinations were carried out in 10 replicates. Plates were incubated at 25°C in the dark. Relative combative abilities of the mycelia were assessed by following changes in the appearance of the mycelia for up to 8 days, noting whether the fronts deadlocked, with growth of both ceasing after contact, or whether the fronts of one species overgrew the mycelium of the other. Once mycelial fronts had contacted each other, growth was allowed to continue for a further 48 h and the fronts were then cut out for metabolite profiling.

2.3 Metabolite extraction

The mycelial fronts were removed from paired cultures by using a sterile scalpel to cut out a strip of agar containing the front and the mycelium 5 mm on either side of it. Control strips cut across the centre of un-inoculated PDA plates were prepared in the same way. These strips were then freeze dried for 24 h. Dried material was then crushed with clean glass rods in a 50 ml plastic sample tube and extracted with 5 ml methanol for 12 h at 4°C then dried in a centrifugal evaporator (Genevac Ltd.) and stored in 7 ml glass vials at −20°C.

2.4 Analysis of metabolites using thin layer chromatography

Dried methanolic extracts from mycelial fronts were re-dissolved in 1 ml methanol:dichloromethane (1:9 v/v) prior to TLC analysis. TLC analyses of extracts from every pairing were carried out in triplicate by loading 10 µl samples on normal phase silica gel aluminium backed TLC plates (Merck) containing 60 F_{254} indicator. Chromatographs were developed in TLC tanks containing 7:3 v/v ethyl acetate; n-hexane mobile phase. The position of solvent front was marked and plates were visualised under UV (254 and 366 nm) prior to spraying with 4% w/v vanillin in sulphuric acid for further development. Extraction and TLC analysis was carried out on three replicate plates for each experimental treatment, plus three PDA control plates.

2.5 GC-TOF-MS

Dried sample extracts from 10 replicates of each pairing and the un-inoculated PDA controls were reconstituted in 700 µl methanol (AR grade, Sigma-Aldrich), spiked with an internal standard solution (1.73 mg/ml succinic d_4 acid, glycine d_5 and malonic d_2 acid) and 150 µl aliquots were lyophilised (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Thermo Life Sciences, Basingstoke, UK). Two-stage chemical derivatisation was performed. Firstly, oximation was performed by heating the samples with O-methylhydroxylamine (50 µl; 20 mg/ml in pyridine; 80 min; 40°C) and then the samples were trimethylsilylated with MSTFA (N-acetyl-N-(trimethylsilyl)-trifluoroacetamide; 50 µl; 80 min; 40°C).

Derivatised samples were analysed by GC-TOF-MS using a Agilent 6890 gas chromatograph (Stockport, UK) coupled to a Leco Pegasus III mass spectrometer (St Joseph, USA), controlled with ChromaTof software v2.15. Sample analysis was randomized and three machine replicates were performed for each sample. Analytical conditions have been previously described (O'Hagan et al. 2005). Three random samples from each of 6 classes were chosen and peak deconvolution performed with Leco ChromaTof software (peak width = 3 s, baseline = 1, smoothing 3). Analyst defined metabolite peaks were added to a database with associated retention index (RI) and mass spectrum, in total 190 metabolite peaks. After chromatographic peak deconvolution of all samples, peak matching to those peaks in the database was performed (RI ± 5, mass spectral match > 700). Data was exported as ASCII files to Microsoft Excel for further data analysis. Normalisation of peaks was performed using the internal standard succinic d_4 acid to calculate the response ratio (peak area-metabolite/peak area-internal standard). Chemical identification of metabolite peaks was performed by mass spectral library searches of three mass spectral libraries (NIST/EPA/NIH02 (http://www.nist.gov/srd/nist1a.htm), MPI-Golm prepared mass spectral/RI library (http://csbdb.mpimp-golm.mpg.de/csbdb/

gmd/msri/gmd_contributions.html) and an authors (WBD) prepared mass spectral/RI library containing over 500 entries). Preliminary identification was confirmed by a mass spectral match > 700 and identification was confirmed by analysis of the authentic metabolite standards (purchased from Sigma-Aldrich (Gillingham, UK) or ACROS Chemicals (Loughborough, UK)) with the same analytical conditions and showing the same RI (±10).

For further data analysis, all data were imported into Matlab® Version 7.1 (http://www.mathworks.com) running under Windows XP on an IBM-compatible PC. Within Matlab®, exploratory analysis using principal components analysis (PCA), (Joliffe 1986) implemented using the NIPALS algorithm (Wold 1966) was performed on the data sets to reduce the dimensionality of the multivariate data and preserve most of the variance. Univariate statistical analysis was performed, using the non-parametric Kruskal–Wallis test (Kruskal and Wallis 1952), to calculate whether there were statistical significant difference for any given peak between the means of the different biological classes. Many peaks were identified as being significantly different between the strains at $\alpha = 0.01$ but no peaks were identified as significant using the highly-conservative Bonferroni Correction (Abdi 2007), α/No. of peaks (0.01/190, 0. 0.000053). This correction is used when testing n independent hypotheses as performing multiple tests of statistical significance on the same data, where 1/1,000 hypotheses tested will appear be significant at the $P = 0.001$ level purely due to chance.

3 Results and discussion

3.1 Observations of morphological changes

Morphological responses began after mycelial contact (5 days) and were fully developed by day 7. Release of orange coloured pigments (possibly phenolics, quinones or aromatic-based metabolites) into the medium had begun by this time beneath the interaction zone of *C. disseminatus* and *S. hirsutum*, (Fig. 1a). *C. disseminatus* began to form a mycelial 'barrage' (Griffith et al. 1994) along the interface at day 5, followed by further overgrowth of the *S. hirsutum* front in the next 48 h, after which time the interaction zone was removed for metabolite analysis (day 7). In plates where growth was allowed to continue *C. disseminatus* had occupied the whole plate by day 10–11.

In contrast, there was no evidence of pigment production in the interface between *S. hirsutum* and *C. micaceus* after the mycelial contact on day 5 and initially a zone of 'mutual inhibition' (Griffith et al. 1994) occurred (no mycelial contact and a clear inhibition zone of c1mm between fronts), but by day 7–8 mycelia of *S. hirsutum* had crossed this zone and started overgrowing the *C. micaceus* mycelial front. This was accompanied formation of dense knots of white aerial mycelium by *C. micaceus* adjacent to the front (Fig. 1b). However these were covered by the *S. hirsutum* front within 24 h and by day 10–11 it had completely overgrown the *C. micaceus* mycelium.

Morphological changes especially pigment production in the interaction zone of *S. hirsutum* and *C. disseminatus* might be associated with extracellular enzymes such as laccase. In unpublished studies we found a significant increase of laccase (ρ-diphenol oxidase, EC 1.10.3.2) activity in the co-culture of *S. hirsutum* and C. *disseminatus*. compared to monocultures, a finding also reported by others (White and Boddy 1992) in a study of interaction of *Hypholoma fasciculare* with other wood decomposer fungi and in a study of interactions of two tropical litter decomposer basidiomycetes by Gregorio et al. (2006). Although the primary role of laccase is considered to be in lignin degradation, these enzymes have been implicated in a number of functions during mycelial interactions. Laccases can act upon phenolic compounds and convert them to free radicals such as reactive quinone compounds (Rayner et al. 1994). These free radicals are involved in polymerization with cell surface components resulting in a change in the hydrophobicity of the hyphal cell walls (Score et al. 1997). This leads to chemical cross linking of adjacent hyphae and formation of hyphal aggregations such as hyphal strands or 'barrages'. Changes in hyphal hydrophobicity could also help to protect the fungus from hydrolytic enzymes of the other organisms. These reactive quinone compounds can also undergo polymerization to result in melanin and melanin-like compounds which can act as physical barriers for cell wall degrading enzymes of other organisms. Therefore the formation of pigments in the interaction zone may indicate the formation of melanin or melanin like compounds.

3.2 Thin layer chromatography (TLC)

Results of TLC of the methanolic extracts of freeze dried interaction zones taken from paired cultures of *S. hirsutum*/*C. disseminatus* and *S. hirsutum*/*C. micaceus* plus monoculture controls are shown in Fig. 2. The interaction zone in the paired cultures of *S. hirsutum* and *C. disseminatus* (Fig. 2a) had a profile which was not a simple summation of monoculture profiles. One aspect was apparent up-regulation of a metabolite present in monoculture of *S. hirsutum*, marked as X in Fig. 2a. There also appeared to be expression of new metabolites possibly not present in either monoculture, for example metabolite Y in Fig. 2a. However, in the interaction zones of *S. hirsutum* and *C. micaceus* (Fig. 2b) there was neither apparent up regulation of

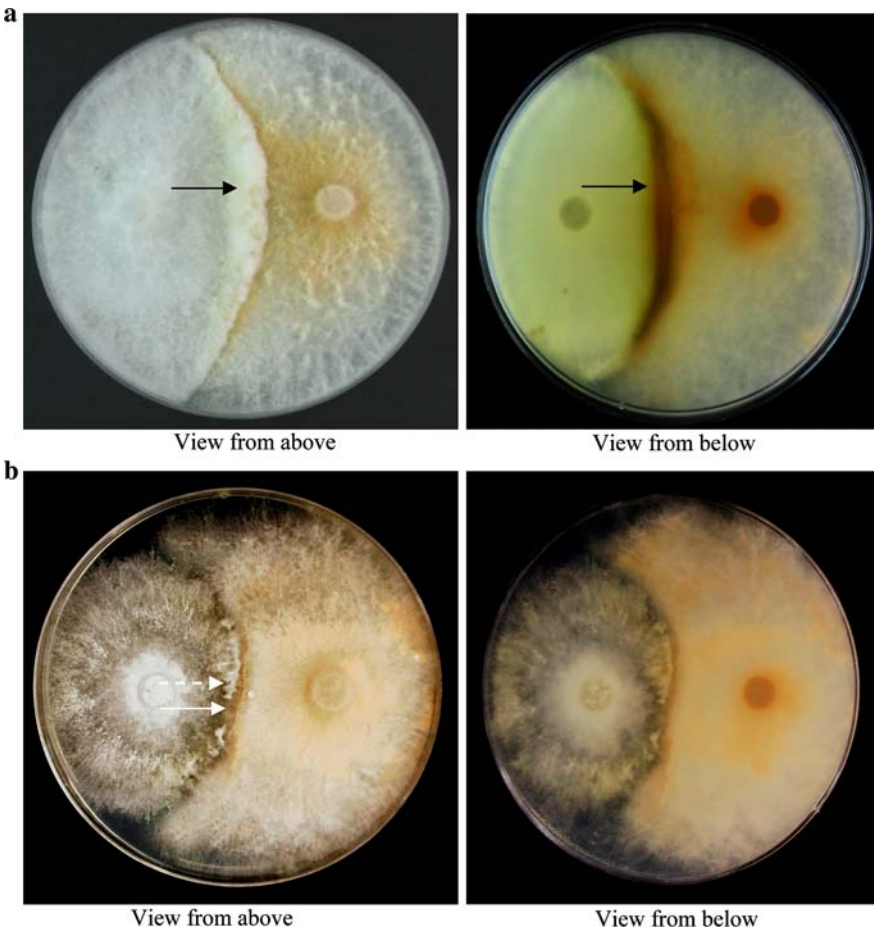


Fig. 1 Mycelia of *S. hirsutum* interacting on PDA with mycelia of *C. disseminatus* and *C. micaceus* , after 7 days growth at 27°C in a 9 cm Petri dish. (**a**) *S. hirsutum* (right) versus *C. disseminatus* (left), view from above: note overgrowth by *C. disseminatus* accompanied by formation of a mycelial barrage (arrowed); view from below: note pigment release into the medium (arrowed) below the barrage. (**b**) *S. hirsutum* (right) versus *C. micaceus* (left), view from above: note beginning of overgrowth of *C. micaceus* front by the *S. hirsutum* mycelial front (arrowed) accompanied by formation of dense aerial mycelium in 'hyphal knots' by *C. micaceus* (dashed arrow); view from below, note absence of pigment release

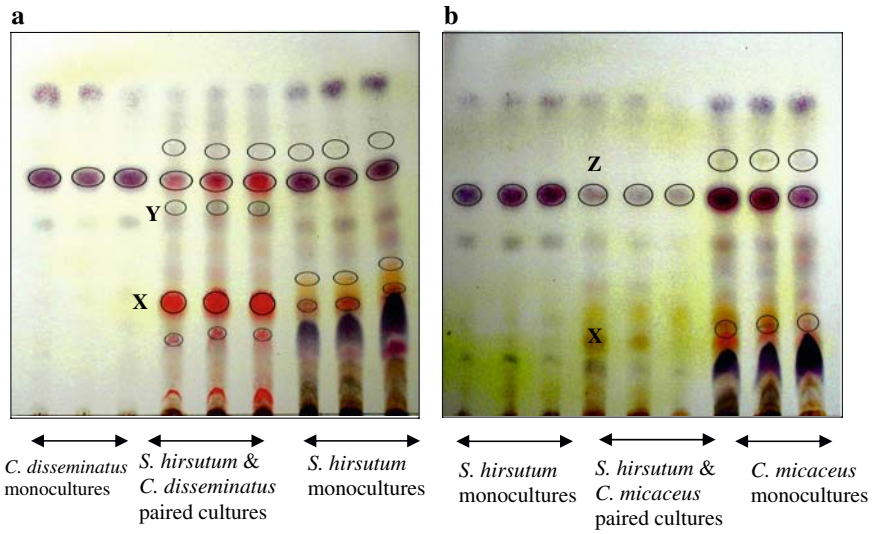


Fig. 2 TLC profiles of methanolic extracts of the interaction zones of the mycelia of *S. hirsutum* paired with mycelia of *C. disseminatus* (**a**) *C. micaceus* (**b**). In (**a**) note the up-regulation in the paired cultures of a *S. hirsutum* metabolite (X) and appearance of new metabolites, e.g. (Y). In (**b**) note the down regulation of *S. hirsutum* metabolites in the paired culture for example X and Z. Circles indicate the positions of metabolites visualized under UV (254 and 366 nm)

metabolites present in the monoculture nor appearance of new metabolites. However there appeared to be an apparent down-regulation of some of the metabolites detected in the *S. hirsutum* monoculture. These included metabolite X, already noted as up-regulated in the paired culture with *C. disseminatus* (Fig. 2a), but not detected in the paired culture with *C. micaceus* (Fig. 2b). Another example is marked as Z in Fig. 2b, a metabolite not detected in the dual culture extracts, but present in the *S. hirsutum* monoculture extract. Interestingly this metabolite was neither up or down regulated in the dual cultures of *S. hirsutum* with *C. disseminatus* (Fig. 2a)

3.3 Gas chromatography-TOF-mass spectrometry

Results obtained by TLC provided a rapid screen of samples, but contained limited biological and metabolic information. To obtain a greater biological insight and have the opportunity to identify metabolites of interest, GC-TOF-MS was used in a metabolic profiling approach. Typical analytical ion chromatograms (m/z 73) of methanolic extracts of interacting mycelial fronts are shown in Figs. 3 and 4. m/z 73 Was chosen as it is a distinct fragment ion of all trimethylsilyl derivatised metabolites and therefore selective for analytes of interest in this study.

190 Unique metabolite peaks were detected in all samples, with an average of 127 peaks detected per sample. 51 Peaks detected in the media-only samples were also detected in fungal extraction samples. Of the 190 peaks, 35 (relating to 27 metabolites) were definitively chemically identified by matching of mass spectrum and retention time to the author's (WBD) mass spectral/retention index library, whereas 15 were provisionally identified by matches by mass spectrum only to three separate mass spectral libraries (Table 1). Of interest was the absence of many common metabolites, including amino acids but the presence of many different low molecular weight organic acids. Also many sugars and sugar alcohols were detected, of which the majority were present in the culture medium, but not all. Table 1 shows identified metabolites, many not routinely detected in other microbial studies undertaken by

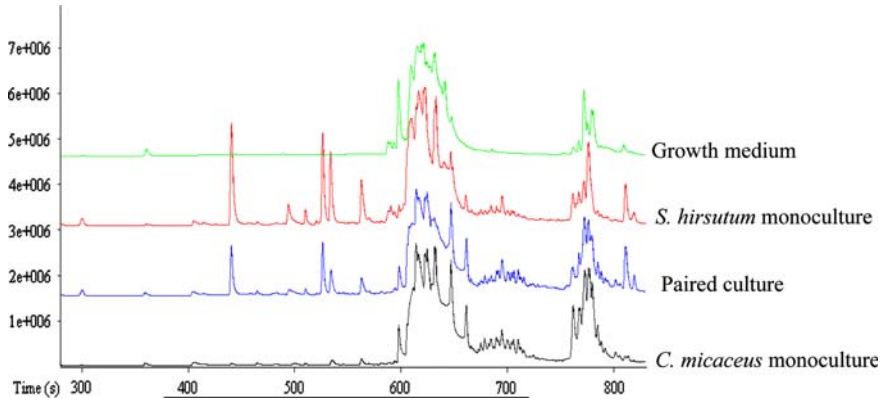


Fig. 3 Single ion chromatograms (m/z 73) for monocultures of *S. hirsutum*, *C. micaceus* and paired cultures of both fungi. A growth medium control is also shown

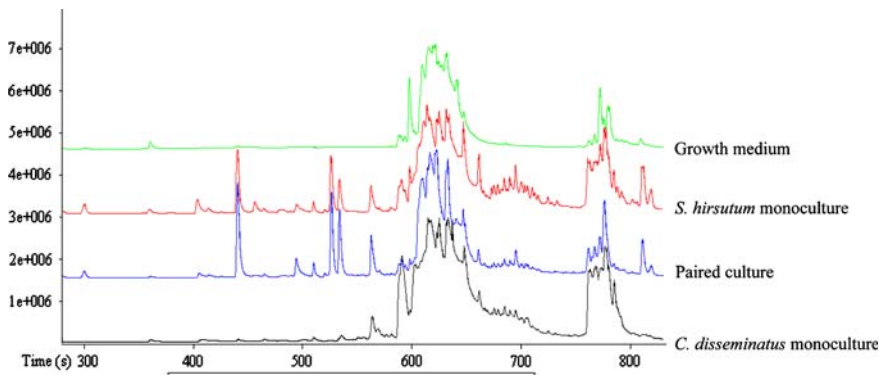


Fig. 4 Single ion chromatograms (m/z 73) for monocultures of *S. hirsutum*, *C. disseminatus* and paired cultures of both fungi. A growth medium control is also shown

Table 1 List of metabolites identified either by combined mass spectral and retention index matching, or by mass spectral matching only

Metabolites definitively identified by matching mass spectrum (match ≥750) and retention index (RI ±10)
2,3 Butanediol*, lactic acid*, pyruvic acid*, glycerol*, 2-hydroxyhexanoic acid, glycine, phosphate, alanine, 2,3-dihydroxybutanoic acid, threonine, succinic acid*, glutamic acid, threitol*, meso-erythritol, citramalic acid, malic acid*, ribose, glucose, 6-deoxyglucose, glucose*, 2-oxoglutaric acid, myo-inositol*, pyridoxine, hexadecanoic acid octadecanoic acid, trehalose, cellobiose*, sucrose*
Metabolites identified by matching mass spectrum only (match ≥750)
3-Methyl,3-hydroxybutanoic acid, 2-methyl,3-hydroxybutanoic acid, 2,3-dihydroxybutanoic acid, 2,4-bihydroxybutanoic acid*, 1-methyl, 3,5-bihydroxybenzene, 3,4,5-trihydroxybutanoic acid, 4-hydroxyphenylethanol, pinitol*, monoamidomalonic acid, 1,2-dihydroxyanthraquinone, 9,12-octadecadienoic acid, 1,2-dihydroxyanthraquinone, myo-inositol-2-phosphate, 5-hydroxy-indole-3-acetate, galactinol, 1-hydroxy,3-methoxy-6-methylanthraquinone
Metabolites marked with * were present in the culture medium

the authors. Although not definitively identified, more than 14 metabolite peaks provided mass spectra that may indicate they are aromatic in nature, either single benzene ring or fused benzene ring metabolites. Two such metabolites are 1-methyl-3,5-dihydroxybenzene and 1,2-dihydroxyanthraquinone, matched by mass spectral searches of the NIST/EPA/NIH02 library. Certain phenolic compounds have been implicated as mediators of laccases (Bourbonnais et al. 1995). (Johannes and Majcherczyk 2000) have shown that two aromatic compounds, 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid effectively mediate laccase production. Therefore those aromatic compounds might have a role as mediators of the increase in laccase production already observed in cultures of *S. hirsutum* when co-cultured with the other two fungi. A similar suggestion was made in their study of laccase induction in paired culture of *Marasmiellus troyanus* and *Marasmius pallescens* by Gregorio et al. (2006).

The high intensity peaks observed at 600–700 s are monosaccharides and related metabolites were observed in both the media and samples, i.e. unconsumed sugars, though novel sugars only detected after metabolic activity were detected from 670 to 730 s. Also of interest are the higher concentrations of disaccharides in samples compared to the media contents showing metabolic activity related to conversion of monosaccharides to disaccharides. These large excesses of sugar metabolites are problematic to the GC instrumentation (and other analytical technologies) and data analysis, and further validation of discriminatory peaks was undertaken to ensure accuracy of results. Future work will address metabolite profiles in interactions on media with low sugar content, for example by the use of cellulose as a carbon source to overcome these difficulties.

Simple inspection of the single ion chromatograms showed that most of the media components had retention times of greater than 600. Between 400 and 600 s there seemed to be metabolites unique to each species. No metabolites were detected that were unique to paired cultures, showing that each species produce metabolites even when not stressed, an interesting biological approach to this chemical warfare between species. In a relatively harsh environment with limited resources each species produces these metabolites not on demand when stressed but in a continuous approach using limited resources. General differences were visible when comparing analytical chromatograms of mycelial fronts of combined cultures relative to monoculture controls, as shown in Figs. 3 and 4. Univariate and multivariate statistical analyses were undertaken to assess these possible differences in more detail.

For statistical analysis, the data was separated into two groups; (1) *S. hirsutum* monoculture/*C. micaceus* monoculture/paired cultures of *S. hirsutum* with *C. micaceus* and (2) *S. hirsutum* monoculture/*C. disseminatus* monoculture/paired cultures of *S. hirsutum* and *C. disseminatus*. Principal Component Analysis was performed to assess for sample outliers before univariate analyses. One sample was observed to be an outlier, observed to be outside clustering of any classes. When this outlier was removed distinct clusters were seen, showing that biological differences are present for each class compared to within class variability (data not shown).

Univariate Kruskal Wallis analyses were performed to assess statistical differences between different monocultures and paired cultures. Only metabolite peaks with $P < 0.001$ were reported as being statistically significant (Tables 2 and 3). For paired cultures of *S. hirsutum* and *C. micaceus* and monoculture controls, 24 metabolite peaks were shown as being significantly different between different cultures (Table 2), being either in single or combined cultures. All these were peaks not present in the media controls. For these peaks no single peak was shown to be up-regulated in paired cultures compared to single cultures, in fact seven peaks were down-regulated (lower concentration) in the paired culture (peak numbers 23, 50, 90, 103, 107, 119, 175). Three examples, 2-methyl-2, 3-dihydroxypropionic acid, pyridoxine and an unidentified metabolite are shown in Fig. 5. Pyridoxine is a cofactor in many enzymes in amino acid metabolism (Tanaka et al. 2005). In addition pyridoxine plays a role in the resistance of the filamentous fungi *Cercospora nicotianae* to its own toxin cercosporin (Bilski et al. 2000). Pyridoxines act as quenchers of singlet molecular oxygen ($^1O_2$) cercosporin. Since there is a down regulation of majority of the metabolites produced by *S. hirsutum*, presumably including toxic compounds, during its interaction with *C. micaceus* there might not be a need to produce pyridoxine in higher quantities.

For paired cultures of *S. hirsutum* and *C. disseminatus* compared to monoculture controls ten peaks not present in the growth medium were shown as significantly different at $P < 0.001$ (Table 3) and of these three were shown to be up-regulated in paired culture (an unidentified metabolite, malic acid and 1,2-dihydroxyanthraquinone, Fig. 6). This indicates increased synthesis by *S. hirsutum* of these metabolites in the paired culture relative to monoculture. The ability to acidify their environment confers a competitive advantage to acid producing fungi by inhibiting the growth of other organisms which cannot grow in low pH environment (Magnuson and Lasure 2004). Therefore the increased production of malic acid in the paired cultures of *C. disseminatus* and *S. hirsutum* might be associated with defensive strategies employed by *S. hirsutum* against *C. disseminatus.* 1,2-dihydroxyanthraquinone, the other up-regulated metabolite in the co-culture of *C. disseminatus*

Table 2 Metabolites shown to be statistically significantly different ($P < 0.001$) for the *S. hirsutum* and *C. micaceus* sample set using Kruskal Wallis analysis

Metabolite peak	*P* value	χ^2	Metabolite identification
9	0.00015	17.6	Unidentified
16	9.36E-05	18.6	Unidentified
20	0.00035	15.9	Unidentified
23[a]	0.00010	18.4	2-Methyl-2,3-dihydroxypropanoic acid
27	5.36E-05	19.7	2,3-Dihydroxybutanoic acid
37	1.07E-05	22.9	Meso-erythritol
50[a]	4.89E-05	19.9	Unidentified
52	0.00033	16.0	Unidentified
62	8.78E-05	18.7	Unidentified
90[a]	0.00026	16.5	Pyridoxine
103[a]	0.00014	17.8	Unidentified
107[a]	0.00063	14.7	Unidentified
108	0.00013	17.9	Unidentified
119[a]	0.00056	15.0	Unidentified
142	4.88E-05	19.9	Myo-inositol-2-phosphate
161	0.00010	18.4	1-Hydroxy-3-methoxy-6-methylanthraquinine
170	4.19E-05	20.2	Unidentified
175[a]	0.00039	15.7	Unidentified
176	4.35E-05	20.1	Unidentified
179	2.45E-05	21.2	Unidentified
182	0.00013	17.9	Unidentified
185	1.07E-05	22.9	Unidentified
187	2.45E-05	21.2	Unidentified
188	1.41E-05	22.3	Unidentified

[a] Peak with lower concentration in paired cultures compared to monoculture of *S. hirsutum*

Table 3 Metabolites which were shown to be statistically significantly different ($P < 0.001$) for the *S. hirsutum* and *C. disseminatus* sample set using Kruskal Wallis analysis

Metabolite peak	*P* value	χ^2	Metabolite identification
3	0.00045	11.15	Unidentified
9[a]	9.52E-05	14.53	Unidentified
37	0.00038	11.54	Meso-erythritol
43[a]	4.47E-08	40.23	Malic acid
73	2.13E-05	18.24	Unidentified
84	0.00096	9.70	Unidentified
85	0.00015	13.46	Unidentified
86[a]	1.04E-12	124.18	1,2-Dihydroxyanthraquinone
104	4.87E-06	22.45	Unidentified
142	3.15E-05	17.22	Myo-inositol-2-phosphate

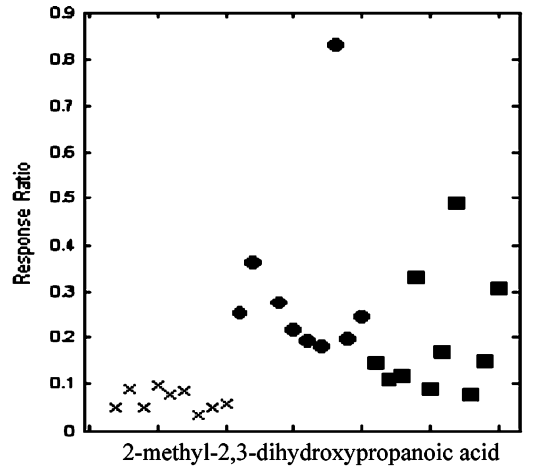
[a] Peak with higher concentrations in paired cultures compared to monoculture of *S. hirsutum*

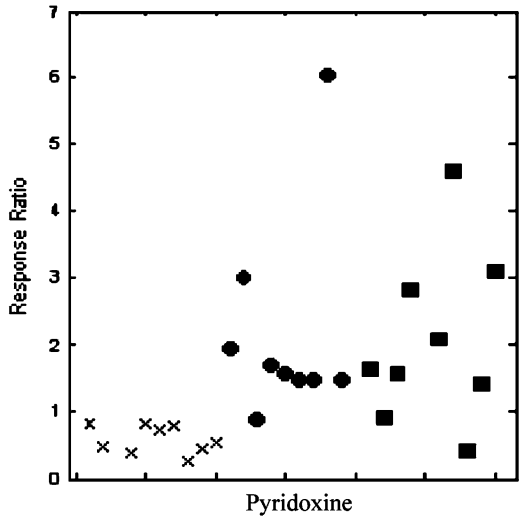
and *S. hirsutum* is a natural pigment commonly present in a number of dicotyledon plant families, especially Rubiaceae (Han et al. 2001), and is commonly used as a dye, and as a antibacterial, antifungal and anti-leukemic agent (Collin 2001). For example, Sung et al. (2004) showed that 1,2-dihydroxyanthraquinone isolated from the seeds of *Cassia obtusifolia* strongly inhibited the growth of *Clostridium perfringens* and *E. coli.*

Most of the metabolites were not present in the mass spectral libraries employed in the study, two of these libraries being composed mainly of primary metabolites. The unidentified metabolites are most likely to be secondary metabolites synthesised by the fungi. These unique metabolites may have interest for future research, including as anti-fungal compounds. Of course, GC based analyses require metabolites to be volatile and thermally stable, in this investigation after chemical derivatisation. Sample preparation will be biased to metabolites not lost during lyophilisation (less volatile metabolites). For example, low MW alcohols and other compounds with boiling points less than 50°C will be lost during lyophilisation. Undoubtedly there are metabolites of higher MW and low volatility that will not be detected by GC-MS and HPLC-MS is a more appropriate analytical technology and will produce complementary results. There should also be production of volatile compounds during fungal interactions, as reported by Hynes et al. (2007) who used LC-MS based method to explore the volatile metabolites produced during interactions between two basidiomycetes fungi *H. fasciculare* and *R. bicolor.* They observed increased production of volatiles during the interaction, including methylbenzoate, benzyl alcohol and γ-muurolene. In the present study 15 of the metabolites detected were also aromatics.

In conclusion, both TLC and GC-TOF-MS profiles indicated that there was an up-regulation of some metabolites in the interaction zone when *S. hirsutum* was overgrown by *C. disseminatus*. In contrast, when *S. hirsutum* mycelia had contacted those of *C. micaceus*, by day 7 there was a down-regulation of metabolites in the interaction zone. The majority of up- or down-regulated metabolites were characteristic of the *S. hirsutum* monoculture profile. This may suggest the existence of a relationship between the synthesis and release of secondary metabolites and the outcome of mycelial interactions. In short, the up-regulation of metabolites by *S. hirsutum* may be connected to a defensive role, or to imposed stress, and not to an aggressive role.

In terms of the ecology of these three wood decomposer fungi, *S. hirsutum* has already been noted as a primary colonizer of Oak and other hardwoods and the two *Coprinus* species occur much later in the decomposition sequence. Mycelia of *S. hirsutum* are likely to confront those of *C. disseminatus* in the intermediate stages of the succession, perhaps after several years, and we have





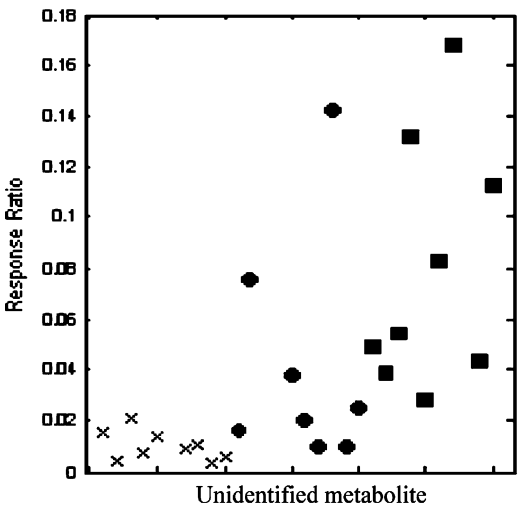
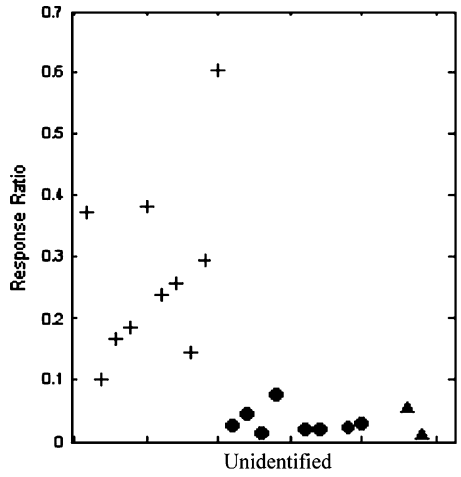
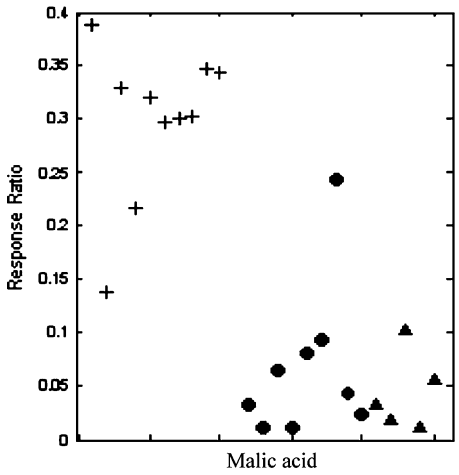


Fig. 5 Plots of metabolite peaks down-regulated in paired cultures (×) when compared to separate monocultures of *S. hirsutum* (●) and *C. micaceus* (■)





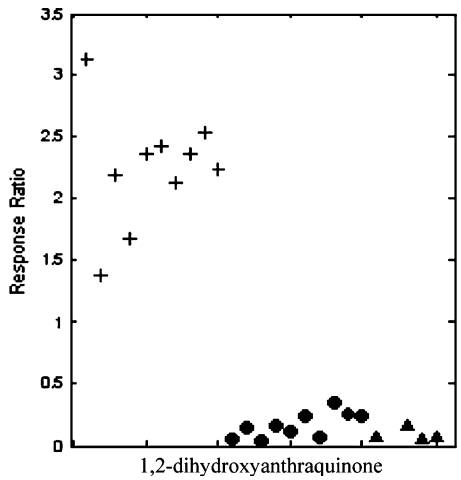


Fig. 6 Plots of metabolite peaks up-regulated in paired cultures (×) when compared to separate monocultures of *S. hirsutum* (●) and *C. disseminatus* (■)

observed *C. disseminatus* firstly occupying the bark and invading into the outer sapwood of branches of Oak after 3–4 years on the ground. In this situation relative combativeness (Rayner and Boddy 1988) is of importance in determining progress of the mycelia into domains occupied by the primary colonizers like *S. hirsutum.* The up-regulation of metabolites we have observed by the

mycelium of *S. hirsutum* in the presence of *C. disseminatus* may indicate a defensive reaction of *S. hirsutum* mycelia against the stress imposed by the invasive mycelia of *C. disseminatus.* In contrast, *C. micaceus* is characteristic of a much later phase of decomposition, often when well rotted wood is almost incorporated into the soil of the woodland, perhaps after 10–15 years of decomposition, and it is likely to succeed by being tolerant of low nutrient status or stressed conditions (Rayner and Boddy 1988) rather than combative replacement of existing mycelia. It often colonises by cord formation over the surface of well-rotted logs on the solid surface. Normally mycelia of *S. hirsutum* and *C. micaceus* are therefore separated both temporally and by different resource requirements. However the more combative primary colonizer, *S. hirsutum*, is likely to overgrow *C. micaceus* in the non-nutrient limited conditions of a nutrient agar plate, although the confrontation in vivo is not likely to occur, since intolerance of nutrient stress would mean *S. hirsutum* would not naturally colonise the same resources as *C. micaceus*.

We believe the metabolomic approach adopted in this proof of principle study of mycelial interactions could provide a useful approach to a broader understanding of fungal colonization successions and in particular raises the question as to whether up-regulation of metabolites and perhaps accompanying induction of phenoloxidase enzymes is a feature of a 'defence' or stress reaction in combative interactions. This can only be answered by a much larger scale study involving groups of taxa characteristic of different stages of a decomposition sequence of fungi in wood or litter. The research also provides a route to isolation of metabolites produced by fungi, especially where metabolites normally produced in small quantities by a fungal species are up-regulated in interacting cultures, of potential interest in natural products discovery programmes.

Acknowledgement This work was financially supported by University of Westminster, Cavendish Scholarship.

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