Journal of Chromatography A, 1292 (2013) 219–228

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Detection of metabolite induction in fungal co-cultures on solid media by high-throughput differential ultra-high pressure liquid chromatography–time-of-flight mass spectrometry fingerprinting

Samuel Bertrand[a,1], Olivier Schumpp[b,*,1], Nadine Bohni[a], Alban Bujard[a], Antonio Azzollini[a], Michel Monod[c], Katia Gindro[b], Jean-Luc Wolfender[a]

[a] School of Pharmaceutical Sciences, EPGL, University of Geneva, University of Lausanne, Quai Ernest-Ansermet 30, CH-1211 Geneva 4, Switzerland
[b] Swiss Federal Research Station Agroscope Changins-Wädenswil, Route de Duillier, P.O. Box 1012, CH-1260 Nyon, Switzerland
[c] Department of Dermatology and Venereology, Laboratory of Mycology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

ARTICLE INFO

Article history:
Available online 30 January 2013

Keywords:
Fungal interactions
Fusarium
Solid media
Co-culture profiling
Metabolomics
UHPLC–TOF-MS

ABSTRACT

Access to new biological sources is a key element of natural product research. A particularly large number of biologically active molecules have been found to originate from microorganisms. Very recently, the use of fungal co-culture to activate the silent genes involved in metabolite biosynthesis was found to be a successful method for the induction of new compounds. However, the detection and identification of the induced metabolites in the confrontation zone where fungi interact remain very challenging. To tackle this issue, a high-throughput UHPLC–TOF-MS-based metabolomic approach has been developed for the screening of fungal co-cultures in solid media at the petri dish level. The metabolites that were overexpressed because of fungal interactions were highlighted by comparing the LC–MS data obtained from the co-cultures and their corresponding mono-cultures. This comparison was achieved by subjecting automatically generated peak lists to statistical treatments. This strategy has been applied to more than 600 co-culture experiments that mainly involved fungal strains from the *Fusarium* genera, although experiments were also completed with a selection of several other filamentous fungi. This strategy was found to provide satisfactory repeatability and was used to detect the biomarkers of fungal induction in a large panel of filamentous fungi. This study demonstrates that co-culture results in consistent induction of potentially new metabolites.

1. Introduction

Natural products (NPs) obtained from microorganisms are a historical source of lead compounds [1], but the attractiveness of such NPs is diminished because of the difficulties involved in working with complex mixtures [2] and because of the continual rediscovery of the same bioactive chemical structures in pharmacological screens despite the existence of dereplication processes [3]. Such continual rediscovery is inconsistent with the tremendous diversity of the cryptic biosynthetic pathways dedicated to the production of secondary metabolites, which have been revealed by genome sequencing programmes [4–6]. Recently, a variety of strategies have been proposed for the activation of the production of these compounds. One such strategy is the co-cultivation of two microorganisms in a single environment, which is referred to as interspecies crosstalk [5,7–9].

Co-culturing exploits the fact that fungi have evolved highly specialised capacities to occupy environments, such as soil, rhizospheres, plants, mucosal membranes and guts, which also support dense populations of other microorganisms [10]. Interactions between microorganisms lead to the activation of complex regulation mechanisms, which results in the biosynthesis of highly diverse NPs [8], such as pheromones, defence molecules and metabolites that are involved in symbiotic associations [11]. Investigations into co-culture experiments with fungi have been limited, but co-culture has a high potential for the generation of enhanced chemical diversity, an advantage that has been demonstrated in both liquid [9,12–20] and solid co-cultures [11,21–24]. For example, emericellamide A and B were induced during a liquid co-culture of the marine-derived fungus *Emericella* sp. with the marine actinomycete *Salinispora arenicola* [14]. Such stress-induced molecules exhibit specific noteworthy bioactivities, and some of them have been found to have antimicrobial [12,14,17,20,21], anticancer [13,18] and phytotoxic activities [21].

* Corresponding author at: Groupe Mycologie, Station de recherche Agroscope Changins-Wädenswil ACW, Route de Duiller 50, CP 1012, 1260 Nyon, Switzerland. Tel.: +41 22 363 43 71; fax: +41 22 362 13 25.
E-mail addresses: olivier.schump@acw.admin.ch, olivier.schumpp@acw.admin.ch (O. Schumpp).
[1] SB and OS contributed equally to this work.

0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.01.098

For practical reasons, the large-scale production of microbial metabolites is usually accomplished in liquid reactors as they are amenable to easy control of temperature, pressure and atmospheric composition [25,26]. However, solid-media cultures are more similar to the environmental conditions in which fungi evolved. Furthermore, these cultures have been found to yield a substantially higher number of metabolites from diverse microorganisms [27] and references therein. In addition, solid media cultures facilitate visualisation of the interaction zone between the fungi [21,23], which allows for growth rate estimation. This also allows the excision of well-defined parts of the agar media for further analysis of specific zones of the confrontation, an advantage for metabolite localisation studies. Recent developments in scaling up microbial cultures [28] have opened the door to the study of microbial interactions on solid media for the purpose of drug discovery.

To monitor stress-related metabolome modifications in microorganisms, hyphenated analytical methods, such as liquid or gas chromatography coupled to mass spectrometry (LC–MS and GC–MS), are well suited because they can separate metabolites from crude extracts and provide spectroscopic information for full or partial chemical identification [3]. Only a few profiling studies of fungal interactions have been performed to delineate interspecies crosstalk [11,21,23,24]. GC–MS was applied to study the up- and down-regulation of metabolites in co-culture involving *Stereum* and *Coprinus* species. This analytical technique enabled molecule identification through database searches based on fragmentation patterns and retention indexes [23]. Several LC–MS studies have applied direct comparisons of mono-cultures with their corresponding co-cultures [14,18,21]. LC–MS can analyse extracts from microorganisms with minimal sample preparation. Compared to GC–MS, however, the LC–MS dereplication process often generates putative peak annotations only. Nevertheless, the localisation of induced compounds in LC–MS chromatograms enables targeted micro-isolation of such compounds and subsequent *de novo* identification by NMR [29]. This method also has the potential to assess the bioactivity of a given up-regulated metabolite. Recently, direct MALDI-imaging mass spectrometry experiments have revealed the production of high molecular weight (MW) metabolites at the interaction zone between confronting bacteria [30,31].

For metabolite profiling of complex matrices, ultra-high-pressure liquid chromatography coupled to electrospray ionisation and time-of-flight mass spectrometry (UHPLC–TOF-MS) is the latest cutting-edge technology [32]. UHPLC–TOF-MS combines high LC and MS resolutions, high throughput and good reproducibility of the LC–MS data sets, an advantage that is important for data comparison in metabolomics studies [33,34]. This analytical platform has demonstrated its utility for high-throughput fingerprinting of a large set (>15,000) of microbial extracts [35].

In this work, UHPLC–TOF-MS fingerprinting was used to screen a large number of co-culture experiments for any chemical induction of low MW metabolites occurring in fungal co-cultures grown on solid media. Multivariate statistical treatments were applied to the UHPLC–TOF-MS profiles to highlight compounds that were specifically induced because of the interactions between colonies of filamentous fungi. The results of this screening indicate that a large majority of fungi produce new substances when confronted with other fungi on solid media. The significance of these findings in the context of natural product research is discussed below.

2. Experimental

2.1. Biological material

The strains of fungi used in this study were clinical, soil or plant-derived isolates. *Fusarium* strains isolated from the difficult-to-treat onychomycosis were progressively collected at the Centre Hospitalier Cantonal Vaudois (CHUV, Lausanne Switzerland) and stored in the database of Agroscope ACW in vials containing diluted potato dextrose broth (PDB) solution (1:4) at 4 °C (Table S1 and http://mycoscope.bcis.ch/). Other fungal species from the collection that were representative of contrasting ecological niches were also added to the list. Soil isolates utilised in this study included *Aspergillus clavatus*, which is known to produce toxins and bioactive compounds [36], *Cladosporium* sp. and *Hohenbuehelia reniformis*. *Bionectria ochroleuca* and *Eutypa lata* were also included as representatives of plant endophytes with the capability to degrade wood. Both of these fungi are able to form dark-brown substances upon interaction with other fungi (unpublished data and [21]). Two non-*Fusarium* species that cohabit with *Fusarium* in human nails [37] and that were isolated from onychomycosis were also included in this study. The first of these was an *Acremonium* strain known for its capacity to produce uncommon chlorinated metabolic compounds [38], and the second was a dermatophytic *Trichophyton rubrum* strain.

2.2. Chemicals

Extractions were performed with methanol (HPLC grade), isopropanol (HPLC grade), *t*-butanol (PA grade), dichloromethane (HPLC grade), and nano-pure water (Millipore). UHPLC–TOF-MS analyses were performed using ULC/MS-grade acetonitrile and a mixture of water and formic acid (FA) from Biosolve (Valkenswaard, The Netherlands).

2.3. Culture and co-culture conditions

For mono-cultures, a 5 mm agar plug of a fungal pre-culture was inoculated in the centre of a 9 cm petri dish containing 30 mL of potato dextrose agar media (PDA, Difco, BD & Co., Le pont de Claix, France). The petri dishes were incubated at 21 °C.

Similarly, co-culture experiments were inoculated with two 5 mm agar plugs of the appropriate fungal strains on opposite sides of a petri dish containing agar media (PDA), and the petri dishes were incubated at 21 °C.

2.4. Extraction procedure

The confrontation zones of fungal co-cultures were excised with a razor blade as 1 cm × 1 cm pieces of agar and then freeze-dried. The dry material was transferred into an extraction vessel with 20 mL of solvent per 300 mg of dry material. The extraction properties of the following solvents were compared: methanol, isopropanol, *t*-butanol, dichloromethane/methanol/water (64:36:8) and dichloromethane/ethyl acetate/methanol (2:3:1) with or without 1% (v/v) formic acid. The solvent mixtures were freshly prepared, and unless otherwise noted, extractions were performed in a water-bath sonicator (Ultrasonic Cleaver 5200, Branson Ultrasonics Corporation) at room temperature for 20 min. The sonicated samples were filtered through glass cotton. Finally, the extracts were dried under vacuum using a centrifugal evaporator (Genevac HT-4, SP scientific, Ipswich, Suffolk, UK). Uninoculated plates and plates inoculated with one fungal strain (*i.e.*, mono-cultures as opposed to co-cultures) were used as blanks and controls, respectively.

An accelerated solvent extraction (ASE, Dionex, Olten, Switzerland) method was also tested, and the results obtained using two different solvent systems (dichloromethane/methanol/water (64:36:8) and methanol) were compared to those of the sonication procedure.

2.5. Sample preparation

During method development sample enrichment by classical solid phase extraction (SPE) filtration was achieved using Sep Pak Vac SPE C_{18} cartridge (1 cc, 100 mg, Waters, Milford, MA, USA) using methanol/water 85:15 (v/v) as eluent [21].

To facilitate screening a large number of samples, the SPE procedure was simplified as follows. The dry extracts were dissolved in a 4:1 methanol/water mixture at a concentration of 2 mg/mL. A 1 mL aliquot of this solution was transferred into a 1.5 mL microtube containing 200 mg of reversed-phase C_{18} material (Silica gel ZEOprep 60 C_{18}, Zeochem AG, Uetikon, Switzerland) to remove the most apolar compounds, as these compounds would be incompatible with reversed-phase chromatography. The tubes were gently shaken (Rotation Mischer 3300, Vaudaux-Eppendorf, Basel, Switzerland) for 2 min and then centrifuged (Mikroliter Zentrifugen, Hettich) for 5 min. The supernatants were filtered through 0.45 μm filters and diluted 2× with methanol. When the same solvent was used, this procedure was found to produce similar results to those of the classical SPE filtration procedure but was easier to implement for the preparation of a large number of samples.

2.6. UHPLC–TOF-MS analysis

UHPLC–TOF-MS analyses were performed on a Micromass-LCT Premier Time-of-Flight mass spectrometer from Waters (Milford, MA, USA), which had an electrospray (ESI) interface coupled to an Acquity UPLC system (Waters, Baden-Daettwil, Switzerland). In separate runs, detection was achieved in both the positive (PI) and negative ion (NI) modes. The *m*/*z* range was set to be 100–1000 in centroid mode with a scan time of 0.25 s and an inter-scan delay of 0.01 s. The ESI conditions in PI and NI modes were as follows: capillary voltage of 2800 V, cone voltage of 40 V, source temperature of 120 °C, desolvation temperature of 250 °C, cone-gas flow of 20 L/h, and desolvation-gas flow 600 L/h. For internal calibration, a 5 μg/mL solution of leucine-enkephalin from Sigma–Aldrich (Steinheim, Germany) was infused through the lock-mass probe at a flow rate of 5 μL/min using a second Shimadzu LC-10ADvp LC pump (Duisburg, Germany).

UHPLC–TOF-MS profiles were collected with either a 150 mm × 2.1 mm i.d., 1.7 μm Acquity BEH C_{18} UPLC column (Waters, Baden-Daettwil, Switzerland) or an Acquity BEH C4 UPLC column (Waters, Baden-Daettwil, Switzerland) in gradient mode at a flow rate of 0.46 mL/min with the following solvent system: (A) 0.1 vol% FA in water; (B) 0.1 vol% FA in acetonitrile. Analysis began with an isocratic step of 5% B for 3.60 min, which was followed by a gradient from 5% to 95% B over 34.50 min. The column was then washed for 6.93 min with 95% B, reconditioned with 5% B for 0.87 min and finally equilibrated with 5% B for 9.50 min. The temperature was maintained at 40 °C, and the injection volume was 2 μL.

UHPLC–TOF-MS fingerprints were recorded with a 50 mm × 1 mm i.d., 1.7 μm Acquity BEH C_{18} UPLC column (Waters, Baden-Daettwil, Switzerland) in gradient mode at a flow rate of 0.3 mL/min with the solvent system described above. The protocol used to establish the fingerprints included a direct geometric transfer of the metabolite profiling procedure calculated using a dedicated Excel spread sheet, 'HPLC calculator' [39,40]. The gradient was increased from 5% to 95% B in 4 min. The column was then washed for 0.8 min with 95% B, reconditioned with 5% B for 0.1 min and finally equilibrated with 5% B for 1.1 min. The temperature was maintained at 40 °C, and the injection volume was 1 μL. Analyses were performed randomly and included quality control and blank samples after every 10 sample runs. The sample list was randomly generated using an Excel macro (Supplementary material).

2.7. Peak picking and data analysis

Native MassLynx data (Waters, Baden-Daettwil, Switzerland) were converted into nCDF (common data format) data using DataBridge software (Waters, Baden-Daettwil, Switzerland). Automatic feature detection was performed between 0.5 and 4.5 min with MZmine2 software [41] using parameters selected according to the TOF-MS detector. Peaks with a width of at least 0.03 s and an intensity greater than either 20 counts (NI) or 50 counts (PI) were selected with a 5 ppm *m*/*z* tolerance and deconvoluted. Deisotope filtering was applied using the "isotopic peaks grouper" module with tolerance parameters adjusted to 0.03 s and 5 ppm. Feature alignment and gap filling were achieved with an *m*/*z* tolerance of 15 ppm and a retention-time (RT) tolerance of 0.3 min. The features detected from blank samples and uninoculated agar samples were removed from the generated matrix. The full procedure for feature detection is presented in Table S2. The exported feature lists were compared using Microsoft Excel, and multivariate analyses were performed using Unscrumbler X software (CAMO Software AS, Norway). Principal component analysis (PCA) and partial least-squares regression (PLS-DA) applied after unit variance (UV) scaling was used for sample discrimination as well as the generation of biplots and the list of features (loadings).

The detection threshold for selection of the induced peaks was set to 1% of the mean peak area of the 10 most intense features in the dataset. Features that were retained were considered significantly induced and were filtered again according to increases in peak areas. The filtering results were verified for the main biomarkers in the corresponding raw UHPLC–TOF-MS data.

3. Results and discussion

Solid media was preferred over liquid cultures for the screening of induction phenomena in fungal co-cultures for the following reasons. (i) From a fungal ecology perspective, solid media better mimics the natural growth conditions of filamentous fungi in an aerobic environment and enables better development because it is a solid support. (ii) From a practical perspective, solid media enables visualisation of the development of fungal colonies over the experimental time and allows excision of a part of the agar media for further analysis of the location of fungal confrontations.

Mono-cultures of filamentous fungi were produced *via* inoculation of an agar pre-culture plug in the middle of a petri dish, while co-culture plates were inoculated on opposite sides of a single petri dish of the type used for mono-cultures. The co-culture plates were incubated for several weeks. Colony growth leading to interaction with the competing colony was monitored with the unaided eye. Chemical analyses were focused on the confrontation zone, and the results were compared to those obtained from mono-cultures.

The method of metabolic induction through fungal co-culture was evaluated by culturing all of the *Fusarium* strains included in the collection of clinical isolates with representatives of a fungal collection of phytopathogenic and environmentally derived fungal strains (http://mycoscope.bcis.ch/). As described below, more than 600 co-culture experiments were screened.

To highlight the metabolite modifications that are caused by fungal interactions, an LC–MS fingerprinting method was adapted to the analysis of agar solid media. The development of this adapted method included the optimisation of an extraction procedure to detect the largest possible number of metabolites over a wide range of polarities. The repeatability of the fingerprinting method, as well as the metabolite profiles of biological replicates, was verified to

ensure that satisfactory statistical results were obtained even when screening a large number of samples.

3.1. UHPLC–TOF-MS metabolite profiling of fungal cultures in solid media

To profile the largest possible number of fungal metabolites, a repeatable and generic extraction procedure was devised. Because different fungal strains may produce highly divergent metabolite patterns, preliminary analyses were used to select those fungal strains that produced metabolites with a large range of polarities. This representative strain set included two *Fusarium solani* strains, one *A. clavatus* and one *Fusarium oxysporum* strain (See Fig. S1 for the UHPLC–TOF-MS fingerprint of each). All of the strains were grown in standard solid-media conditions and were mixed to form homogenous materials. This pooled solid media was used to optimise the extraction procedure as well as the high resolution metabolite profiling and rapid fingerprinting LC–MS methods.

3.1.1. Extraction of fungal metabolites from solid media

To ensure good extraction repeatability, the solid culture media from the petri dishes was frozen and lyophilised prior to extraction, a step taken to exclude interference from the water content. The metabolic activity and growth speed of some fungal strains from the collection cultivated on agar plates varied significantly according to species. These behavioural differences strongly impact the water content of solid cultures, a result that was observed in the first days after inoculation (data not shown).

Accelerated solvent extraction (ASE) and ultra-sound assisted extraction (UAE) were evaluated as potential extraction methods for this spongy material. The solvent systems used in these evaluations consisted of either methanol or a dichloromethane/methanol/water mixture. No major differences in yield or in chemical composition of the extracts were observed between these two methods (data not shown). Therefore, UAE was preferred for the screening of a large number of samples because it was relatively easy to implement. Such a type of extraction technology was reported to give good extraction yields when applied to solid media [42].

The 4 selected fungal cultures mentioned above were lyophilised, homogenised and subjected to UAE for 20 min with a 70× excess of solvent when compared to the amount used for the dried media (v/m ratio). This high ratio of solvent was necessary to fully submerge the lyophilised material. Repeated extractions revealed that approximately 90% of the extractable material was obtained during the first extraction step. To maintain the simplicity of this procedure and cope with the large number of screened samples, one extraction step was considered to be sufficient. Within this procedure, the metabolite extraction capacities of 6 different solvents or solvent systems comprising a wide range of polarities, while still remaining compatible with reversed-phase HPLC, were compared.

To evaluate the metabolite diversity in the solid culture media extracts, a generic UHPLC–TOF-MS profiling method was applied. The profiling was performed using an extended linear acetonitrile/water gradient on a 150 mm UHPLC column to ensure high peak capacity (P = 408 as calculated by HPLC calculator 3.0 [39,40]) and thus baseline separation of most of the metabolites present. The detection was achieved in both positive (PI) and negative ionisation (NI) modes, which allowed monitoring of the largest possible number of metabolites. A classical SPE sample preparation was performed to remove very lipophilic constituents (see below).

The UHPLC–TOF-MS profiles obtained in NI mode for all 6 extraction solvent systems used are presented in Fig. 1. In all cases, this analytical procedure yielded well-resolved UHPLC–TOF-MS base peak intensity (BPI) chromatograms. When compared

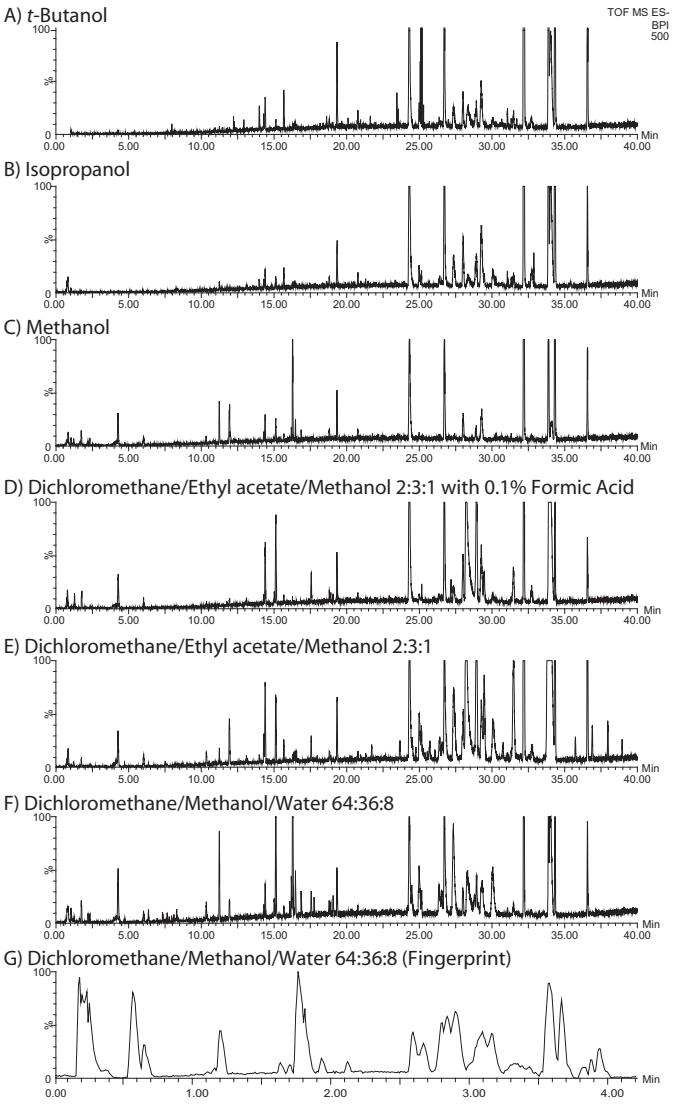

Fig. 1. (A–F) High-resolution NI UHPLC–TOF-MS metabolite profiles (BPI traces) from extracts obtained using 6 different solvent systems (the solvents used are indicated in the figures) from culture media pooled from 4 representative fungal strains (*i.e.*, 2 *Fusarium solani*, 1 *Fusarium oxysporum* and 1 *Aspergillus clavatus*). UHPLC conditions: Acquity C_{18} 150 mm × 2.1 mm i.d., 1.7 μm column, 460 μL/min, 40 °C, 2.6% slope gradient. (G) Fast NI UHPLC–TOF-MS fingerprint obtained after gradient transfer using the DMW extract (F). UHPLC conditions: Acquity 50 mm × 1.0 mm i.d., 1.7 μm column, 300 μL/min, 40 °C, 22.5% slope gradient. This high-throughput method was used to generate the ion maps for the screening of all the involved strains.

to the isopropanol and *t*-butanol extracts, the methanol extract was found to contain a larger number of polar compounds (RT from 0 to 20 min). By contrast, the complement of less-polar metabolites (RT from 20 to 35 min) in the methanol extract was found to be less diverse than those of the isopropanol and *t*-butanol extracts. To optimise the extraction of most metabolites over a large range of polarities, solvent mixtures were also investigated. Three previously reported solvent mixtures were assessed: dichloromethane/methanol/water (64:36:8) (DMW) [21] and dichloromethane/ethyl acetate/methanol (2:3:1) (DEM) (both with and without 1% (v/v) of FA) [42]. The addition of FA into the DEM solvent system led to reduced chemical diversity, as assessed by the drastic decrease in the number of detected peaks.

In the less-polar region of the chromatogram (RT from 20 to 35 min), both the DMW and DEM extracts showed a higher number of peaks than the isopropanol and butanol extracts. Moreover, the

chemical diversity in the apolar region was even more pronounced when DEM was used. In the polar region (RT from 0 to 20 min), DEM performed similarly to methanol.

When compared to all of the previously tested extraction solvent systems, DMW was found to yield a substantially higher number of peaks in the polar region of the chromatogram. Because this solvent system was already found to be appropriate for the extraction of non-polar constituents, it was considered to have a relatively broad extraction capacity and was the extraction solvent system chosen for the rest of this study.

Considering the large number of samples to be analysed, a SPE method prior to UHPLC–TOF-MS analysis was established to prevent sample carryover and maximise the lifespan of the column. Thus, the highly lipophilic compounds were removed from the extracts using C_{18} SPE [21,43]. To assess the number of metabolites lost during the SPE step, the extracts were profiled using a C_4 column that had a lower retention of lipophilic constituents. A comparison of the crude extract profiles with those of the SPE-purified extracts recorded using the C_4 column revealed that, for DMW extracts, there was no significant difference in the detected lipophilic constituents (RT over 26 min). It was found, however, that the SPE procedure eliminated the most lipophilic constituents from the butanol extract (Fig. S2).

Because the SPE protocol reduced the sample weight by approximately 30% without affecting the reversed-phase profiles of DMW extracts, it was systematically applied to all of the screened samples in an effort to preserve experimental reproducibility. To facilitate screening a large number of samples (see below), the SPE procedure was simplified by mixing the samples with C_{18} particles prior to filtration. This modified procedure was found to produce results similar to those of direct SPE filtration.

In conclusion, extraction by UAE with DMW as the solvent system followed by SPE was found to be a highly suitable sample preparation method for generic reversed-phase profiling of fungal metabolites.

3.1.2. Development of the UHPLC fingerprinting methods

To chemically profile a large number of fungal confrontations as well as their respective pure strains (more than 400 samples in this study), a high-throughput UHPLC–TOF-MS fingerprinting method was developed that was based on the high-resolution profiling analysis used to optimise the metabolite extraction. Development of this rapid fingerprinting method was necessary because the high-resolution LC–MS profiling procedure required more than 55 min to complete and was not compatible with the screening of a large number of samples.

To decrease the analysis time, the column length was reduced from 150 mm to 50 mm. This column geometry was found to yield higher peak capacities with gradient times of less than 10 min [44,45]. When a 50 mm column with a diameter of 2.1 mm was used, the optimal flow rate required to reach 90% of the maximum pressure allowed by the system (1000 bar, the optimum condition for high peak capacity) [46] was approximately 1.2 mL/min. This flow rate was not suitable for electrospray ionisation (ESI) [47], and rather than split the flow rate, a reduced-diameter column (1.0 mm) was selected. The optimal flow rate for this column was 0.3 mL/min. This flow rate was suitable for the ESI source of the TOF-MS detector and considerably reduced the solvent consumption. The selectivity of both the profiling and fingerprinting methods were indistinguishable because of the application of a gradient transfer method [39,40]. By altering the column geometry and gradient conditions, the overall analysis time was reduced from 55 min to 6 min (Fig. 1). However, while a tenfold increase in throughput was obtained, these changes resulted in a twofold decrease in theoretical peak capacity (from 408 to 190) [40]. Nevertheless, this analytical procedure was found to be compatible

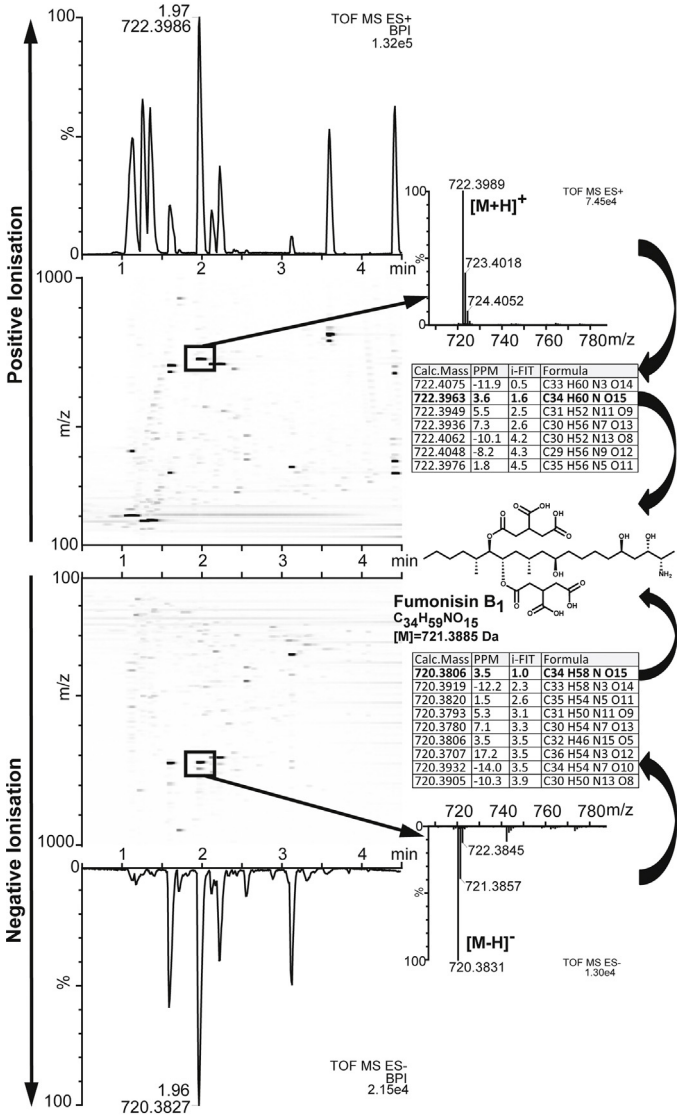

Fig. 2. PI and NI 2D ion maps obtained from the UHPLC–TOF-MS fingerprints of the DMW extract of *Fusarium oxysporum* (Sin106) (see conditions in Fig. 1G). Peak annotations based on the high mass and high spectral accuracy of this technique are used for the assignment of molecular formulae. The figure shows satisfactory resolution of most of the features in the ion maps. For example, the TOF-MS data and literature search provided the identification of fumonisin B_1 or isofumonisin B_1 as the best putative structures associated to the 2 features with masses of 722.3989 (PI) and 720.3831 Da (NI).

with the management of a reasonably timely analysis of a large series of samples without much loss of efficiency in the LC separation. Fig. 1F and G compares the UHPLC–TOF-MS BPI profile and fingerprint obtained from the same DMW extract. Although the fingerprint required a shorter analysis time, the selectivity of this method was similar to that of the profile. This result was confirmed by comparing the extracted ion traces (XICs) of the main LC peaks. The decrease in peak capacity was not detrimental to the detection of features ($m/z \times$ RT) because the orthogonal TOF-MS detection increased metabolite separation and the BPI profiling method did not reveal the presence of many isomeric structures. The UHPLC–TOF-MS fingerprints were thus represented in the form of 2D ion maps, in which the metabolites were resolved in both the LC and MS dimensions. An example of such an ion map is included in Fig. 2, which presents the PI and NI UHPLC–TOF-MS fingerprints of a *Fusarium* pure strain extract with their corresponding BPIs. As shown, the PI and NI fingerprints were very complementary, and

all of the features were well separated despite the use of fast LC separation (Fig. 2).

TOF-MS not only can contribute an additional separation dimension to all of the spectral features but also can allow the user to deduce molecular formulae from its high-mass-accuracy data, a process referred to as dereplication [48]. For example, one of the main peaks of Fig. 2 was detected in both the PI and NI modes at an RT of 1.97 min for m/z values of 722.3989 Da and 720.3885 Da, respectively. The m/z difference of 2 Da between the two ionisation modes was the basis for the identification of the $[M+H]^+$ and $[M-H]^-$ adducts. The high mass-accuracy of the TOF-MS data (<5 ppm) permitted 3 molecular formulae to be deduced in both the NI and PI modes. Moreover, the comparison of theoretical and observed isotopic patterns (low normalised *i*-fit value) as well as the application of heuristic filtering [49] allowed for the determination of an unambiguous molecular formula ($C_{34}H_{59}NO_{15}$) [50]. A search for this molecular formula in natural product databases [51,52] matched with two possible isomers – fumonisin B_1 and isofumonisin B_1 – which are produced by *Fusarium* sp. [53,54]. These results indicate that the screening procedure developed in this work was able to detect known fungal metabolites efficiently and that high-resolution TOF-MS data could be reliably used for the putative identification of features induced by fungal confrontation and the estimation of their novelty.

3.1.3. Repeatability of extraction and UHPLC–TOF-MS fingerprinting analysis for a large set of microbial extracts

To assess the reproducibility of the fungal growth conditions, sample preparation methods and LC–MS analyses, a representative set of 5 strain mono-cultures and 6 co-cultures were selected. The 5 fungal strains used included two *F. solani*, one *T. rubrum*, one *Cladosporium* sp. and one *A. clavatus* strain. 5 co-culture experiments, which involved both of the *F. solani* strains cultured with the 3 others fungal strain, were analysed in parallel to verify the consistency of the metabolite profiles when fungal interactions were present. 9 biological replicates of each sample were grown independently and submitted to UHPLC–TOF-MS fingerprinting.

9 replicates of the uninoculated agar plates were subjected to the same treatment and served as control samples so that the agar background detected in the LC–MS analyses could be excluded. A quality-control (QC) sample, which consisted of an equal mixture of all of the previously described samples (108 in total), was prepared to assess the stability of the system over the time required for analysis of the complete sample set.

The samples were analysed in random order. The blank samples (injection solvent only) and quality QC were analysed every 12 samples. In addition, 6 analyses of both blank samples and QC samples were performed before and after the analysis of the complete set of fungal samples. To facilitate the set-up of an appropriate sample list, an Excel Macro was developed (Supplementary material). Both the PI and NI fingerprints were analysed independently to ensure high MS acquisition frequency and detection stability. Altogether, more than 300 injections were performed sequentially. This procedure was adapted from an established metabolomic protocol that has been applied to the analysis of body fluids [34].

All of the LC–MS fingerprints were automatically processed using open-source peak-picking software [41,55] to generate lists of features (RT, m/z value, and peak area) for the entire set of samples (the fully detailed procedure is explained in Table S2). The system stability over the time required to obtain the whole dataset was evaluated *via* a principal component analysis (PCA, unit variance scaling) involving all the samples with a special focus on the QC samples. The relatively tight clustering of QC samples (data not shown) suggested that the fingerprints were suitable for further detailed analysis of fungal metabolite variations. In addition, the XICs of selected characteristic ions of the QC samples revealed a very good match amongst the RT data (approximately 1% coefficient variation) for more than 25 analytical replicates that were representative of the whole sample list. Blank samples were found to contain very few contaminants, and their patterns were very similar over the whole dataset, indicating no detectable carryover effects.

A PCA analysis of all of the pure strains also revealed satisfactory clustering of the biological replicates (Fig. S3). Within a given pure strain, more than 80% of all the PI- and NI-detected peaks exhibited biological variation of less than 30% (coefficient variation of the peak areas of given peaks for all replicates). When a variation of less than 50% was considered, the detection was 95%. Considering that the growth of fungal strains (growth speed, colony morphology) may be prone to significant variations over subsequent cultures, these results were determined to be very satisfactory (Tables S3 and S4). For the 9 biological replicates of the 6 different co-cultures, the same repeatability was observed among replicates (Tables S3 and S4). This indicates that the metabolite fingerprinting method developed for fungi grown on solid media generates acceptable datasets that can be used for further metabolomic analyses.

3.1.4. Search for metabolite induction in fungal co-culture by data mining

Because agar plates are limited in space and contain restricted amounts of nutrients, the co-cultivation of fungal strains forces competitive interactions [56]. A co-cultivation experimental set-up was used here to stimulate the production of secondary compounds by interacting fungi that would not be released by mono-cultures grown in identical conditions. To evaluate the induction of novel compounds by the 6 representative fungal co-cultures described above, UHPLC–TOF-MS fingerprints of the fungal mono-cultures and their corresponding co-cultures were compared.

As shown in Fig. 3, a visual comparison of the fingerprints displayed as 2D ion maps ($RT \times m/z$) of 2 mono-cultures and their corresponding co-culture was sufficient to detect the most obviously induced metabolites among several hundreds of spectral features. For example, when *F. solani* was confronted with *Cladosporium* sp., 2 intense features (highlighted by a box frame on the UHPLC–TOF-MS ion map in NI and PI mode) were only visible after confrontation and were not observed in spectra obtained from the corresponding mono-cultures. Comparison of the selected ion trace XICs of these features further confirmed that they were not present in detectable quantities in the mono-cultures but were clearly present in the interaction experiment. Observation of this induction phenomenon was consistent over the 9 replicates.

To screen for all of the induced fungal metabolites and detect minor induced compounds as well, a statistical approach was established. Multivariate analysis [57] was applied to 9 replicates of each of the 5 mono-cultures and 6 co-cultures, a total of 99 samples. For a given confrontation, all of the detected features with intensities greater than an appropriate threshold were selected by an automated peak-picking procedure (see Section 2). This procedure was applied to the 9 replicates of the co-culture and mono-cultures of each pair of fungi (27 samples).

As a first step, an unsupervised PCA (unit variance scaling) was performed on these 27 samples for each interaction experiment. For a given interaction (for example, *F. solani* and *Cladosporium* sp.), a well-resolved clustering of the 27 samples in 3 groups was obtained (Fig. 4A). This behaviour was observed for most of the interaction experiments. However, in some cases, PCA analysis resulted in poorly resolved clustering of the co-culture samples apart from their corresponding mono-cultures (Figs. S4 and S5). The well-resolved sample clustering obtained showed that the co-culture fingerprints did not overlap with the 2 corresponding mono-culture clusters. This indicated that the dataset contained

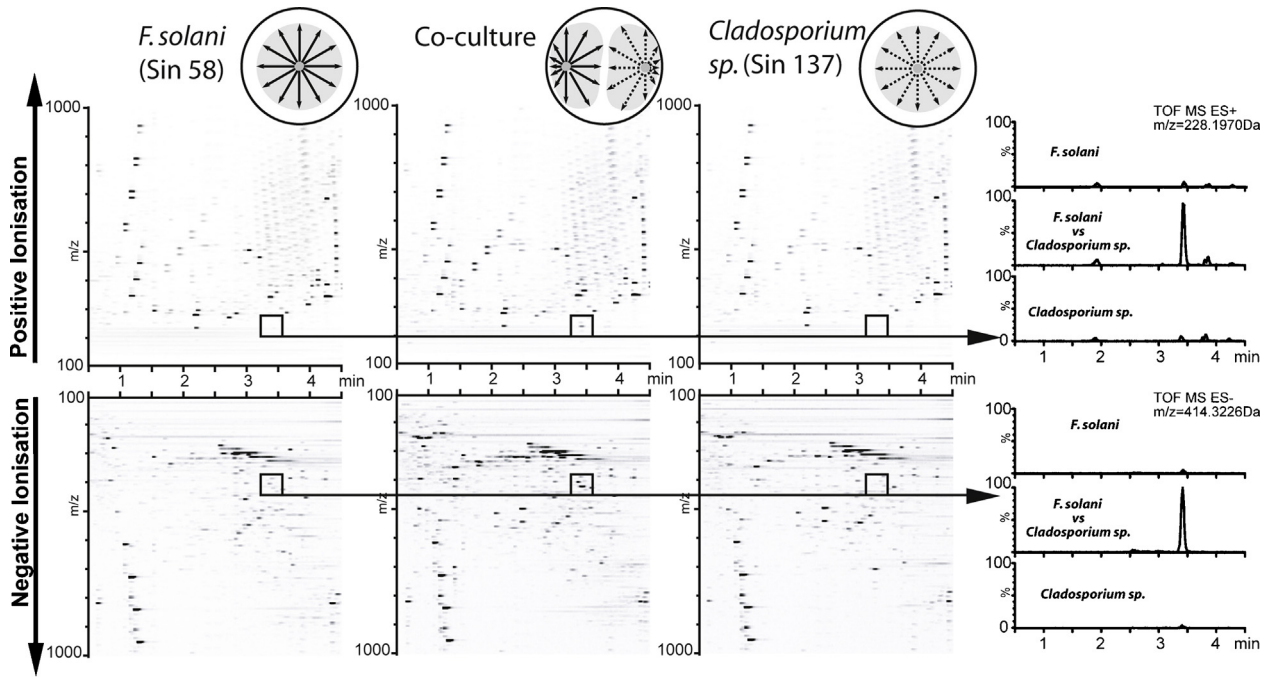

Fig. 3. General strategy to study metabolite induction by fungal co-cultivation on solid media in petri dishes. In this example, *Fusarium solani* (Sin58) was co-cultivated with *Cladosporium* sp. (Sin137). The PI and NI 2D ion map fingerprints are shown. The round insets represent a schematic presentation of the petri dishes of the two mono-cultures with the co-culture in the middle. Visual inspection of the 2D ion maps already enabled the observation of some of the features induced by co-culturing. The XICs of 2 features (228.1970 Da at 3.40 min and 414.3226 Da at 3.42 min) were detected in the PI and NI modes, respectively.

information that discriminated co-cultures from mono-cultures and that, in addition, some features that were only associated with the co-cultures were likely to be present. This was confirmed by further analysis, as features only associated with the co-cultures were found within the dataset of induced compounds (1 co-culture *vs.* 2 mono-cultures, see below). This indicates that fungal interactions modulate the biosynthetic pathways that produce secondary metabolites [9].

As a second step, supervised analysis of the data *via* partial least-square regression (PLS) was used to detect overexpressed

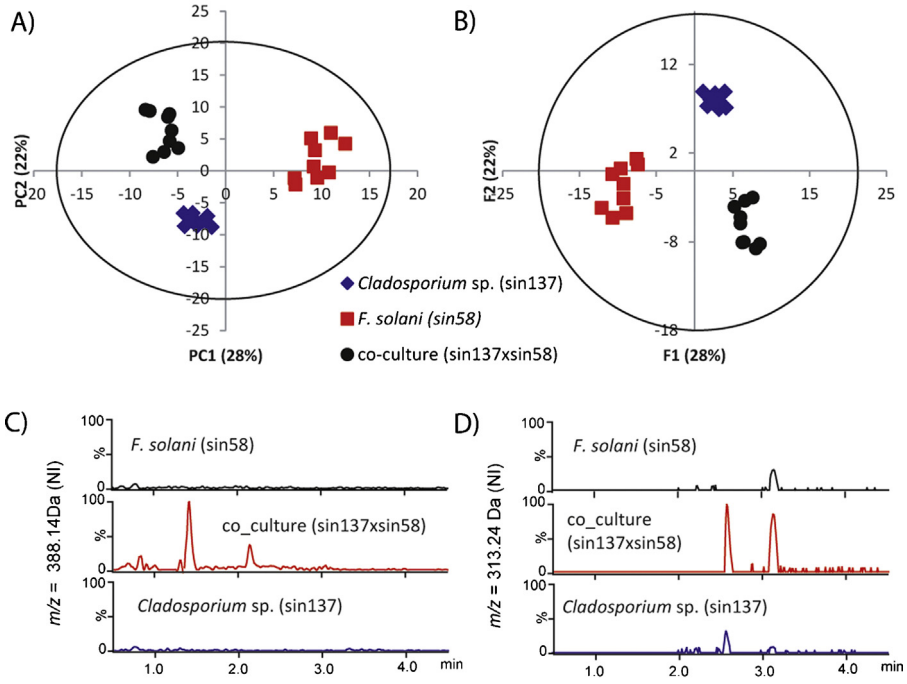

Fig. 4. Unsupervised (PCA) and supervised (PLS) multivariate data analysis (UV scaling) of the fingerprint data included in Fig. 3 (interaction of *F. solani* Sin58 with *Cladosporium* sp. Sin137; 9 replicates of mono- and co-cultures). The PCA (A) showed a clear separation between the 3 groups representing the two mono-cultures and their co-culture. The PLS (B) generated a significant model ($R^2(Y) = 0.97$; $Q^2(Y) = 0.94$). The loadings associated with the PLS, and their further biplot localisation, enabled the detection of metabolites that were significantly induced in all of the replicates. Two examples (C and D) of the XICs of such features that are not directly visible on the ion maps are presented. (C) Example of *de novo* metabolite induction. (D) Up-regulation in the confrontation zone of metabolites present in the corresponding mono-cultures.

compounds in the co-cultures (Fig. 4B). The loadings associated with the PLS separation of the co-cultures and mono-cultures were selected to determine the induced features. A biplot of the loadings and the score plot provided a good means for the ranking of the features responsible for discriminating between the co-cultures and the two corresponding mono-cultures on the PLS plot. For all of these selected features, the induction rate was estimated by comparing the mean peak area of given co-culture biomarkers to those of each mono-culture. Based on this treatment, the most significant features (those with an induction rate higher than threefold) were selected. Finally, the XICs for significantly induced features were plotted to validate these results.

An example of the XICs that correspond to the induced features is given in Fig. 4C and D. In these figures, two types of induction patterns are recorded: (i) *de novo* biosynthesis in which there was no detection in mono-cultures of a compound that was clearly present in co-cultures (Fig. 4C); and (ii) up-regulation in co-cultures of compounds that were already present in mono-cultures, which is expressed as fold changes induction (Fig. 4D).

In the 6 fungal interactions studied using this method, a few tens of features were detected in the PI and/or NI modes (Table S5). When considering the fungal combinations, however, important variations were observed in the number of induced features, a value that ranged from 0 to 29. For example, the interaction between *F. solani* (Sin58) and *Cladosporium* sp. (Sin137) yielded 11 and 29 features in the NI and PI modes, respectively (Table S5).

These results further supported the previously reported existence of chemical induction in fungal co-cultures [5,7,8].

3.1.5. Estimation of chemical novelty generated by fungal interactions

To evaluate whether the induced features described novel NPs, the high mass-accuracy of TOF-MS detection was used to verify matches between the obtained high-accuracy MW and those recorded in the Dictionary of Natural Product (DNP) database [51]. This rapid procedure was preferred over determination of the molecular formula of each induced feature for the purpose of dereplication, as the latter method, described for the peak annotation of constitutive metabolites in Fig. 2, is a more laborious process.

The determination of MWs was performed with a relatively large mass-accuracy tolerance (<15 ppm) to improve the chances of finding a matching candidate. The main suspected adducts (−H and +HCOO in NI and +H and +Na in PI) [58] were taken into account for these calculations. Rough evaluation of chemical novelty was assessed based on the existence of these MW values in the DNP.

Interestingly, a majority of the peaks (around 80%) from mono-culture analysis matched at least one structure recorded in the DNP. This hit number was close to zero when the induced features were searched (Table S5). Such an approach must be used with care, but it can be used for the rapid preliminary estimation of the chemical novelty of the induced compounds detected in filamentous fungi co-cultures.

3.2. Screening for chemical diversity in fungal interactions

The results obtained with the strategy described above, which was based on a limited set of fungal co-cultures, showed that fungal interactions on solid media yield chemical novelty. To further evaluate the potential for chemical induction in a larger variety of fungal interactions, 657 co-culture experiments were examined.

3.2.1. Morphological patterns of fungal interactions on solid media

This series of co-culture experiments was performed on agar-containing petri dishes. The front zone of each interaction was observed over a period of 8 weeks. From these observations, 4 groups that displayed distinct morphological patterns of interaction (interaction types) were defined (Fig. 5).

In the first type of interaction – referred to as *distance-inhibition* type – fungal growth stopped at a distance from the competing fungal colony. This pattern strongly suggested the release of an antifungal substance(s) into the medium that worked to inhibit the growth of both fungi. In the second type of interaction, which was only observed in two cases, the fungal colonies grew large enough to contact one another and produced a dark precipitate similar to the so-called "*zone lines*" observed in wood-decaying fungi confrontation fronts [21]. In this type of interaction, the production of new substances is a confirmed observation of the dark precipitate itself. The third group of interactions included those in which the fungi grew large enough to contact one another but produced no obvious evidence of substance release. This type of interaction was referred to as the *contact inhibition* type. In the last group, interactions resulted in the complete invasion of one colony by the other. This type of interaction was referred to as *overgrowth*.

For the majority of co-culture experiments, the types of interactions observed evolved over time, which led to the observation of intermediate behaviours. Thus, for example, some interactions yielded colonies that would initially stop their development at a distance for a few weeks until one fungal colony would resume its expansion and produce an overgrowth-type interaction. In other cases, colonies would develop up to mycelium contact while the colony's morphology would change substantially in the vicinity of the interaction front.

To verify whether the types of morphological interactions could be related to the number of induced metabolites at the interaction zones, only co-cultures displaying one of the 4 well-defined interaction types (Fig. 5) were kept for LC–MS fingerprint screening.

For this reason, only 138 co-culture experiments (21% of the all interactions) and the mono-cultures of the 83 fungal strains involved in these confrontation experiments were analysed. These selected interactions include 31 distance-inhibition types, 1 zone-line type, 45 contact-inhibition types and 61 overgrowth types (Tables S1 and S6). In this part of the work, and in view of the large number of interactions to be examined, all samples were analysed without replicates to gain rough evaluations of whether a given interaction type could be related to a higher induction of small secondary metabolites.

3.2.2. Chemical induction and types of fungal interactions

To record the fingerprints of all of the 138 co-cultures and optimise the concentrations of those substances specifically released by fungal interactions, the front lines of the co-cultures were excised and processed as described above. By observing fungal interactions on solid media, access to the exact zones of fungal interactions was enabled.

The UHPLC–TOF-MS fingerprints of these co-cultures were analysed in both NI and PI modes and were compared to their corresponding mono-cultures. For this dataset, only the number of *de novo* induced metabolites in the interaction zones was considered. Thus, only the features of sufficient intensity that were detected in the interaction zones but not in the corresponding mono-cultures were taken into consideration. Based on the criteria selected, the number of features induced in each sample (a few tens) varied strongly according to the fungal combinations.

A mean value for the number of induced compounds detected in both PI and NI modes was calculated for each type of interaction (Fig. 5). For all of the interaction types, the number of low-molecular-weight compounds induced *de novo* was not significantly different. Thus, even co-cultures that led to visible antimicrobial activity (distance inhibition) or to the visible formation of new products in the form of dark precipitates were not found to have produced more induced features. This result demonstrates

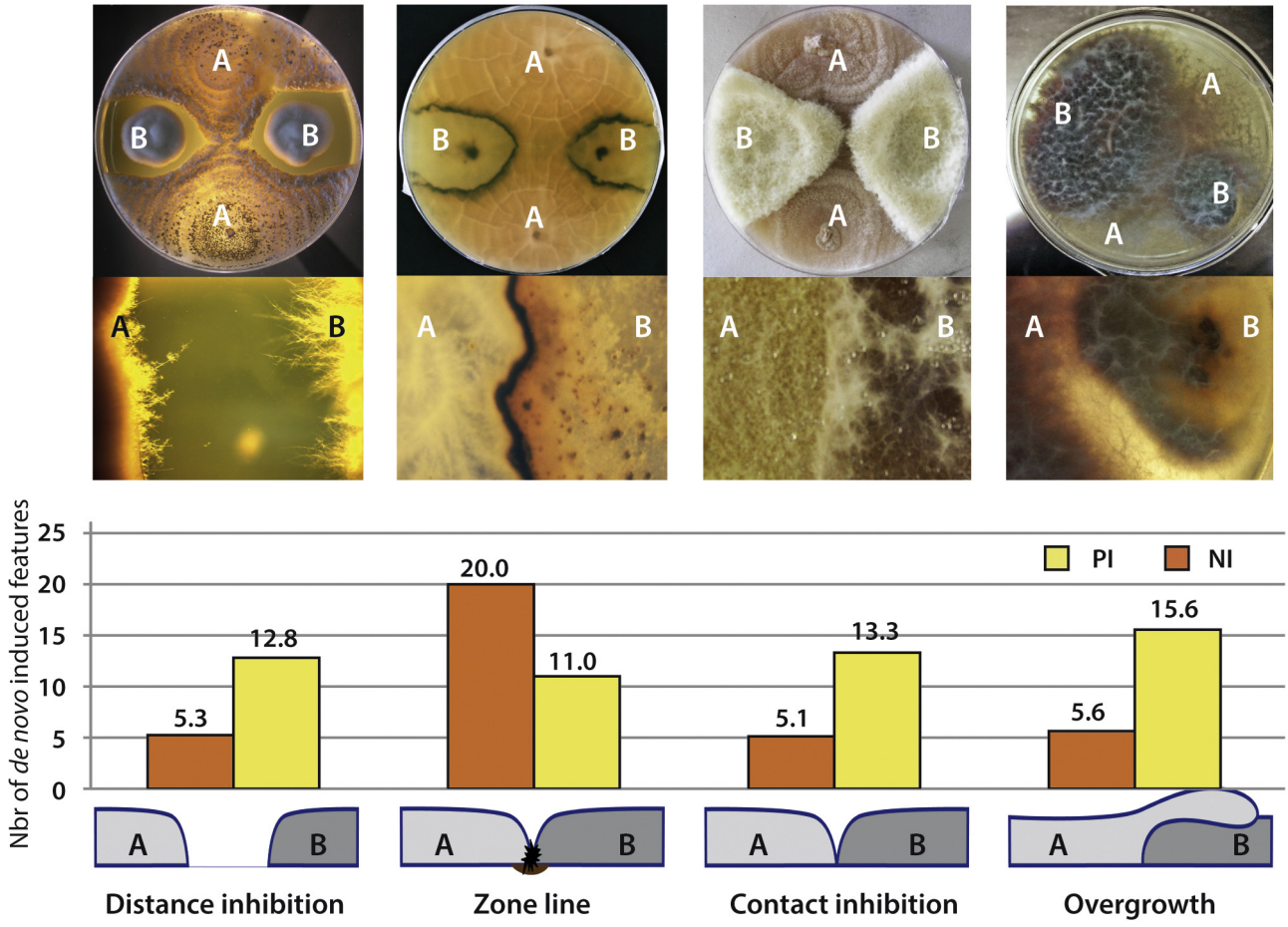

Fig. 5. Colony morphologies of fungal co-cultures. 4 "Interaction types" (*distance-inhibition*, *zone-line*, *contact-inhibition* and *overgrowth*) were defined from observations of 657 co-culture experiments performed on solid agar plates. Mean values of the numbers of induced compounds detected by NI or PI UHPLC–TOF-MS are displayed below the corresponding interaction types. Only well-filtered *de novo* induced features were considered. A schematic of the interaction side-view is provided at the bottom of the figure.

that there is no correlation between the morphological aspects of the interacting colonies and the production of new low MW metabolites detectable with the method used.

Conversely, these results also demonstrate that the large majority of fungi grown on agar plates reacted to the presence of another microorganism by producing substances that were not produced in mono-cultures.

4. Conclusions

The strategy that was developed in this work regarding co-culture experiments of filamentous fungi grown on solid media and subsequent LC–MS fingerprinting has revealed induction phenomena in these microorganisms that may be linked to the activation of cryptic biosynthetic pathways.

The protocol was optimised for co-culturing of fungi on solid media and was found to efficiently generate samples in which metabolite-induction phenomena could be easily observed. Solid-media co-cultures were found to provide a good means of easily following the morphological development of fungal colonies and excise zones in which fungal confrontations occur. The study of more than 600 fungal strains subjected to such co-culturing revealed 4 main morphological types of fungal interactions.

To profile the low-molecular-weight metabolites produced by this large collection of fungi and their co-cultures, a high-throughput UHPLC–TOF-MS method was devised. The sample preparation process developed for the analysis of a large series of samples achieved broad extraction of fungal metabolites from solid agar media with no major interferences from the culture media and no problematic carryover effects. UHPLC fingerprinting conditions were generic and were optimised using high-resolution metabolite profiling. This enabled the acquisition of 10 fungal fingerprints per hour. The high mass and spectral accuracy of the TOF-MS detector was used for dereplication of the main fungal metabolites and for the rough estimation of the novelty of features induced by fungal interactions.

The method was found to be repeatable and generated acceptable datasets that were used for further metabolomic analysis. Furthermore, the advantage of using a profiling method based on UHPLC for this type of study is that this technique is amenable to the possibility of scaling up the separation for the isolation of biomarkers of interest for full identification by NMR and for further characterisation of their bioactivity profiles [21].

The application of this method to a large collection of co-cultures grown on solid media demonstrated that most fungal interactions resulted in the production of compounds that were not produced by the corresponding fungal mono-cultures grown under identical conditions. No correlation between the morphologies of the interacting colonies – defined as the interaction type – and the number of induced compounds could be made.

The screening results did indicate, however, that the compounds specifically produced at the fungal interaction zones in co-cultures seem to correspond to novel chemical compounds that are scarcely represented in natural products databases. These findings indicate

that fungal interaction may be a new and promising source of lead compounds.

Finally, as these low MW compounds seem to be released as a response to microbial interactions, it can be speculated that they may possess antimicrobial activities, a speculation that has been previously reported [12,14,17,20,21]. In this respect, continuing work is under way to measure the differential bioactivity profiles [59] of a large number of co-cultures and their respective mono-cultures. The metabolomic methods presented here will be applied in combination with the bioactivity results to detect the induced compounds responsible for such antimicrobial activities.

Acknowledgements

Roland Todd and Philippe Eugster are acknowledged for their contributions to agar extraction and UHPLC–TOF-MS analysis, respectively. Nadia Marcon of HES-SO (Sion, Switzerland) is thanked for her help with preliminary tests of the Automated Solvent Extraction System. This work was supported by the Swiss National Science Foundation Sinergia Grant CRSII3_127187, which was awarded to J.-L. W., M. M. and K. G.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.01.098.

References

[1] D.J. Newman, G.M. Cragg, J. Nat. Prod. 75 (2012) 311.
[2] F.E. Koehn, G.T. Carter, Nat. Rev. Drug Discov. 4 (2005) 206.
[3] J.L. Wolfender, G. Marti, E.F. Queiroz, Curr. Org. Chem. 14 (2010) 1808.
[4] M. Nett, H. Ikeda, B.S. Moore, Nat. Prod. Rep. 26 (2009) 1362.
[5] J.M. Winter, S. Behnken, Curr. Opin. Chem. Biol. 15 (2011) 22.
[6] E.K. Schmitt, C.M. Moore, P. Krastel, F. Petersen, Curr. Opin. Chem. Biol. 15 (2011) 497.
[7] Y.-M. Chiang, S.-L. Chang, B.R. Oakley, C.C.C. Wang, Curr. Opin. Chem. Biol. 15 (2011) 137.
[8] K. Scherlach, C. Hertweck, Org. Biomol. Chem. 7 (2009) 1753.
[9] V. Schroeckh, K. Scherlach, H.-W. Nützmann, E. Shelest, W. Schmidt-Heck, J. Schuemann, K. Martin, C. Hertweck, A.A. Brakhage, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 14558.
[10] D.Y. Kobayashi, J.A. Crouch, Ann. Rev. Phytopathol. 47 (2009) 63.
[11] A.E. Rodriguez Estrada, A. Hegeman, G. May, Fungal Genet. Biol. 48 (2011) 874.
[12] M. Cueto, P.R. Jensen, C. Kauffman, W. Fenical, E. Lobkovsky, J. Clardy, J. Nat. Prod. 64 (2001) 1444.
[13] K.M. Zuck, S. Shipley, D.J. Newman, J. Nat. Prod. 74 (2011) 1653.
[14] D.-C. Oh, C.A. Kauffman, P.R. Jensen, W. Fenical, J. Nat. Prod. 70 (2007) 515.
[15] S. Angell, B.J. Bench, H. Williams, C.M.H. Watanabe, Chem. Biol. 13 (2006) 1349.
[16] K. Nonaka, T. Abe, M. Iwatsuki, M. Mori, T. Yamamoto, K. Shiomi, S. Omura, R. Masuma, J. Antibiot. 64 (2011) 769.
[17] F. Zhu, G. Chen, X. Chen, M. Huang, X. Wan, Chem. Nat. Compd. 47 (2011) 1.
[18] D.-C. Oh, P.R. Jensen, C.A. Kauffman, W. Fenical, Bioorg. Med. Chem. 13 (2005) 5267.
[19] W. Jonkers, A.E. Rodriguez Estrada, K. Lee, A. Breakspear, G. May, H.C. Kistler, Appl. Environ. Microbiol. 78 (2012) 3656.
[20] C. Li, J. Zhang, C. Shao, W. Ding, Z. She, Y. Lin, Chem. Nat. Compd. 47 (2011) 382.
[21] G. Glauser, K. Gindro, J. Fringeli, J.-P. De Joffrey, S. Rudaz, J.-L. Wolfender, J. Agric. Food Chem. 57 (2009) 1127.
[22] F. Mela, K. Fritsche, W. de Boer, J.A. van Veen, L.H. de Graaff, M. van den Berg, J.H.J. Leveau, ISME J. 5 (2011) 1494.
[23] D. Peiris, W. Dunn, M. Brown, D. Kell, I. Roy, J. Hedger, Metabolomics 4 (2008) 52.
[24] J. Zhou, Q. Ma, H. Yi, L. Wang, H. Song, Y.-J. Yuan, Appl. Environ. Microbiol. 77 (2011) 7023.
[25] R.J. Seviour, B. McNeil, M.L. Fazenda, L.M. Harvey, Crit. Rev. Biotechnol. 31 (2011) 170.
[26] R. Bareither, D. Pollard, Biotechnol. Prog. 27 (2011) 2.
[27] J.A. Zahn, R.E. Higgs, M.D. Hilton, Appl. Environ. Microbiol. 67 (2001) 377.
[28] E. Adelin, N. Slimani, S. Cortial, I. Schmitz-Alfonso, J. Ouazzani, J. Ind. Microbiol. Biotechnol. 38 (2011) 299.
[29] G. Glauser, D. Guillarme, E. Grata, J. Boccard, A. Thiocone, P.-A. Carrupt, J.-L. Veuthey, S. Rudaz, J.-L. Wolfender, J. Chromatogr. A 1180 (2008) 90.
[30] V.V. Phelan, W.-T. Liu, K. Pogliano, P.C. Dorrestein, Nat. Chem. Biol. 8 (2012) 26.
[31] J. Watrous, P. Roach, T. Alexandrov, B.S. Heath, J.Y. Yang, R.D. Kersten, M. van der Voort, K. Pogliano, H. Gross, J.M. Raaijmakers, B.S. Moore, J. Laskin, N. Bandeira, P.C. Dorrestein, Proc. Natl. Acad. Sci. U.S.A. 109 (2012) E1743.
[32] P.J. Eugster, D. Guillarme, S. Rudaz, J.L. Veuthey, P.A. Carrupt, J.L. Wolfender, J. AOAC Int. 94 (2011) 51.
[33] E. Grata, D. Guillarme, G. Glauser, J. Boccard, P.-A. Carrupt, J.-L. Veuthey, S. Rudaz, J.-L. Wolfender, J. Chromatogr. A 1216 (2009) 5660.
[34] E.J. Want, I.D. Wilson, H. Gika, G. Theodoridis, R.S. Plumb, J. Shockcor, E. Holmes, J.K. Nicholson, Nat. Protoc. 5 (2010) 1005.
[35] T. Ito, T. Odake, H. Katoh, Y. Yamaguchi, M. Aoki, J. Nat. Prod. 74 (2011) 983.
[36] J. Varga, M. Due, J.C. Frisvad, R.A. Samson, Stud. Mycol. 59 (2007) 89.
[37] J. Verrier, M. Pronina, C. Peter, O. Bontems, M. Fratti, K. Salamin, S. Schürch, K. Gindro, J.-L. Wolfender, K. Harshman, M. Monod, J. Clin. Microbiol. 50 (2012) 553.
[38] P. Zhang, B. Bao, H.T. Dang, J. Hong, H.J. Lee, E.S. Yoo, K.S. Bae, J.H. Jung, J. Nat. Prod. 72 (2009) 270.
[39] D. Guillarme, D.T.T. Nguyen, S. Rudaz, J.L. Veuthey, Eur. J. Pharm. Biopharm. 68 (2008) 430.
[40] D. Guillarme, D.T.T. Nguyen, S. Rudaz, J.-L. Veuthey, HPLC calculator 3.0, http://www.unige.ch/sciences/pharm/fanal/lcap/telechargement.htm, consulted 11.07.12.
[41] T. Pluskal, S. Castillo, A. Villar-Briones, M. Oresic, BMC Bioinformatics 11 (2010) 395.
[42] S. Jørn, J. Chromatogr. A 760 (1997) 264.
[43] E. Grata, J. Boccard, D. Guillarme, G. Glauser, P.-A. Carrupt, E.E. Farmer, J.-L. Wolfender, S. Rudaz, J. Chromatogr. B 871 (2008) 261.
[44] P. Petersson, A. Frank, J. Heaton, M.R. Euerby, J. Sep. Sci. 31 (2008) 2346.
[45] D. Guillarme, E. Grata, G. Glauser, J.-L. Wolfender, J.-L. Veuthey, S. Rudaz, J. Chromatogr. A 1216 (2009) 3232.
[46] P.J. Eugster, D. Biass, D. Guillarme, P. Favreau, R. Stöcklin, J.-L. Wolfender, J. Chromatogr. A 1259 (2012) 187.
[47] J. Schappler, R. Nicoli, D. Nguyen, S. Rudaz, J.-L. Veuthey, D. Guillarme, Talanta 78 (2009) 377.
[48] D. Guillarme, J. Schappler, S. Rudaz, J.-L. Veuthey, Trends Anal. Chem. 29 (2010) 15.
[49] T. Kind, O. Fiehn, BMC Bioinformatics 8 (2007) 105.
[50] T. Kind, O. Fiehn, BMC Bioinformatics 7 (2006) 234.
[51] Dictionary of Natural Products on DVD (20:1), CRC Press, Taylor & Francis Group, 2001.
[52] K.F. Nielsen, J. Smedsgaard, J. Chromatogr. A 1002 (2003) 111.
[53] S. Naiker, B. Odhav, Mycoses 47 (2004) 50.
[54] S.E. MacKenzie, M.E. Savard, B.A. Blackwell, J.D. Miller, J.W. ApSimon, J. Nat. Prod. 61 (1998) 367.
[55] G. Gürdeniz, M. Kristensen, T. Skov, L.O. Dragsted, Metabolites 2 (2012) 77.
[56] L. Boddy, FEMS Microbiol. Ecol. 31 (2000) 185.
[57] J. Boccard, J.-L. Veuthey, S. Rudaz, J. Sep. Sci. 33 (2010) 290.
[58] K.F. Nielsen, M. Månsson, C. Rank, J.C. Frisvad, T.O. Larsen, J. Nat. Prod. 74 (2011) 2338.
[59] O. Schumpp, N. Bruderhofer, M. Monod, J.L. Wolfender, K. Gindro, Mycoses 55 (2012) 507.