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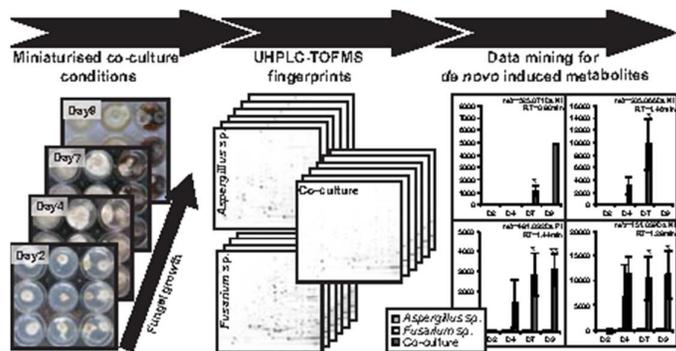
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A multi-well approach was developed for time series studies of *de novo* metabolite-induction by fungal co-culture using untargeted metabolomics.

ARTICLE

Multi-well Fungal Co-Culture for *de novo* Metabolite-Induction in Time Series Studies based on Untargeted Metabolomics

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2014,
Accepted 00th January 2014

DOI: 10.1039/x0xx00000x

www.rsc.org/

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The induction of fungal metabolites by fungal co-cultures grown on solid media was explored using multi-well co-cultures in 2-cm diameter Petri dishes. Fungi were grown in 12-well plates to easily and rapidly obtain the large number of replicates necessary for employing metabolomic approaches. Fungal culture using such a format accelerated the production of metabolites by several weeks compared with using the large-format 9-cm Petri dishes. This strategy was applied to a co-culture of a *Fusarium* and an *Aspergillus* strain. The metabolite composition of the cultures was assessed using ultra-high pressure liquid chromatography coupled to electrospray ionisation and time-of-flight mass spectrometry, followed by automated data mining. The *de novo* production of metabolites was dramatically increased by nutrient reduction. A time-series study of the induction of the fungal metabolites of interest over nine days revealed that they exhibited various induction patterns. The concentrations of most of the *de novo* induced metabolites increased over time. However, interesting patterns were observed, such as with the presence of some compounds only at certain time points. This result indicates the complexity and dynamic nature of fungal metabolism. The large-scale production of the compounds of interest was verified by co-culture in 15-cm Petri dishes; most of the induced metabolites of interest (16/18) were found to be produced as effectively as on a small scale, although not in the same time frames. Large-scale production is a practical solution for the future production, identification and biological evaluation of these metabolites.

1. Introduction

Microorganisms are a historical source of natural bioactive lead compounds;^{1, 2} however, the attractiveness of such natural products (NPs) is reduced due to the continual rediscovery of the same bioactive chemicals in pharmacological screens despite the existence of dereplication processes.³ Various approaches to increase NP chemodiversity, have been recently developed, such as non-targeted metabolic engineering, epigenetic modification or elicitation and the production of unnatural-natural products.^{2, 4-7} Among such approaches, the activation of cryptic biosynthetic pathways through microorganisms co-culture (growing two microorganisms in a single confined environment) has gained attention because co-culture has led to the production of new or rare secondary metabolites under standard laboratory conditions.^{4-6, 8-11} Genomic sequencing has revealed a considerable diversity of secondary metabolite encoding biosynthetic genes, and such approaches may permit the elucidation of potential metabolite diversity of a given microorganism¹² and reduce the continual rediscovery of NPs.

Moreover, most stress-induced molecules exhibit interesting biological activities,¹¹ such as antimicrobial,¹³⁻²³ anticancer,^{20, 24, 25} antiprotozoal²⁶ and phytotoxic activities.¹⁵ These molecules are thus worth studying using drug-discovery approaches.

To study the induction of metabolite biosynthesis in fungal co-cultures, sensitive high-resolution (HR) techniques, mainly based on mass spectrometry, are required. Metabolite profiling based on ultra-high pressure liquid chromatography coupled to electrospray ionisation and time-of-flight mass spectrometry (UHPLC-TOFMS) provides a rapid and efficient overview of the metabolome of complex matrices, such as microbial extracts.^{27, 28} This approach was successfully applied to fungal co-cultures and allowed an efficient comparison of the metabolomic profiles of pure-strain cultures ("pure-strain cultures" will be referred to as "pure cultures" in the manuscript) and their corresponding co-culture metabolomic profiles for the identification of *de novo* induced compounds (secondary metabolites present in the co-culture but not in the pure cultures).^{15, 29, 30}

Various studies have explored the induction of fungal metabolites in fungal co-cultures grown on solid media in 9-cm Petri dishes using untargeted liquid chromatography-mass spectrometry (LC-MS) metabolomics.^{15, 29, 30} In these studies, numerous co-cultures were screened, and various phenomena were observed:

- 1) The growth rate of different fungi may vary significantly.³¹ Therefore, many fungi cannot easily be grown with others. It might be necessary to inoculate one of the two fungi days before inoculating the second one.³²
- 2) The duration of growth has a huge effect on the metabolites present.³³ In addition, the period of growth required to obtain confronting fungal colonies on 9-cm Petri dishes may be weeks or even months. This period can be even longer when larger dishes are used.
- 3) The use of 9-cm diameter Petri dishes leads to a low concentration of material such that the quantity of the purified compounds may not be sufficient for comprehensive identification of the induced metabolites.³⁰

In the present paper, an approach based on small-scale co-culture experiments in 2-cm diameter Petri dishes is presented. This approach partially overcomes the problems mentioned above and provides a rational way to highlight *de novo* induced metabolites and study the evolution of their accumulation over time. To develop this protocol, two model fungi, *Aspergillus clavatus* (Sin141) and *Fusarium* sp. (PS54743), were selected for use because of their respective differential growth rates in 9-cm Petri dishes. In addition, large-scale production of the *de novo* induced metabolites was evaluated using 15-cm Petri dishes to assess the feasibility of up-scaling the method for future isolation and structural elucidation of these metabolites.

2. Results and Discussion

To address the issues related to sampling in a metabolomics study, a down-scaled 2-cm Petri dish procedure was developed for the study of the *de novo* induction of secondary metabolites by fungal co-culture. Surprisingly, cultivation in this small-scale system strongly stimulated fungal growth. The surface of the well was covered within less than a week using most of the tested fungal species, whereas reaching this level of coverage takes several weeks in standard 9-cm Petri dishes). The rare exceptions of species with slow growth characteristics required no more than two weeks to fully cover the well's surface. Thus, compared to the traditionally utilised 9-cm Petri dish co-cultures, a larger number of experiments can be performed in a shorter period.

For practicality, 12-well plates were used instead of independent 2-cm Petri dishes because they correspond to twelve 2-cm Petri dishes. The use of this format enabled the rapid and efficient generation of a large number of replicates for each pure and co-culture. One 12-well plate (Fig. S1A) allowed three replicates for each pure culture, the co-culture and the non-inoculated agar (blanks); one metabolomics study required at least two plates.

Volume optimisation of the culture medium

The volume of potato dextrose agar (PDA) medium added to the Petri dish was optimised to allow the rapid induction of *de novo* secondary metabolite. The four following volumes were tested: 1, 2, 3 and 4 mL, in wells with a capacity of approximately 5 mL. The two model fungi (*A. clavatus* and *Fusarium* sp.) used in this study were grown in triplicate for three days. Subsequently, the content of each well was lyophilised and extracted by sonication using a monophasic dichloromethane–methanol–water solvent mixture. In our previous studies, this solvent system was found to be optimal for the extraction of a broad range of fungal metabolites.²⁹ The extracts were dried under vacuum and then subjected to reversed-phase solid-phase extraction (SPE) to remove highly nonpolar compounds and reduce their carryover effects in LC-MS. Finally, the enriched extracts were solubilised for further analysis. Compared to when using standard Petri dishes, extracting these smaller culture volumes significantly accelerated the sample-preparation procedure and substantially simplified the manipulation of a large number of cultures.

The samples were finally analysed according to a previously established protocol, using UHPLC-TOFMS in both positive (PI) and negative ionisation (NI) modes.²⁹ This procedure takes advantage of the high-resolution and rapid separation capabilities of UHPLC as well as the HR detection offered by TOFMS.³⁴ The UHPLC-TOFMS fingerprints generated 2D ion maps (retention time (RT) x m/z), in which all of the features associated with the fungal metabolites were resolved in both the LC and MS dimensions with high repeatability.^{29, 35} All of the features (RT x m/z x peak area) detected in the fingerprints of each replicate were deconvoluted using an automated peak-picking procedure that was optimised for the retrieval of only the relevant chromatographic ion traces.³⁶ A low signal-to-noise threshold was set to maximise the retention of relevant information.

The *de novo* induction of metabolites was systematically evaluated for each set of samples based on the volume of culture medium used (Fig. 1). Ions corresponding to the same compound but detected using both ionisation modes (NI and PI) were counted only once. In addition, the unusual formation of adducts related to co-eluting compounds was also verified as such phenomenon may be sometimes observed in ESI, and may lead to features highlighted as *de novo* induced features during the data analysis process which would then be false positive. Fig. 1 shows that after three days of growth, more metabolites were detected when the smaller volumes of culture medium (1 and 2 mL) were used. The number of induced metabolites was consistent with the previously reported results obtained using 9-cm Petri dishes.^{29, 30} In contrast, fewer metabolites were induced in co-culture experiments performed using the larger volumes of culture medium (3 and 4 mL). However, metabolite intensity on the base peak chromatogram was similar between the different extracts. This indicated that metabolite production was improved when larger culture media volume was used as metabolite/sugar ratio is unchanged. The differences of *de novo* induction observed suggested that the reduction of nutrients increased the

competition between the two microorganisms and led to an increased number of *de novo* induced metabolites. Similar results were previously reported for pure cultures of *Streptomyces*, in which antibiotic production was triggered by poor nutrient conditions.³⁷

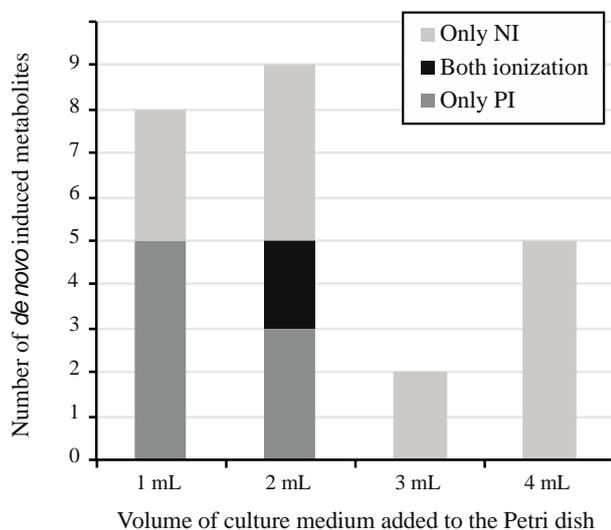


Fig. 1. Effect of the volume of culture medium added to a 2-cm Petri well on the number of *de novo* induced metabolites by fungal co-culture after three days of growth, based on the UHPLC-TOFMS fingerprints obtained using both ionisation modes.

The smallest colonies were observed when the fungi were grown using 1 mL of culture medium (Fig. S2). However, no clear difference in the colony size was observed in cultures grown using 2, 3 or 4 mL of culture medium. In addition, the standard deviation for the extract mass obtained was more than six times lower for 1 or 2 mL of culture medium compared with 3 or 4 mL (Fig. S3), indicating less variability under the former than under the latter conditions. Consequently, a volume of 2 mL was selected for use in the 2-cm diameter wells to ensure the release of largest number of metabolites with the maximal growth rate and satisfactory repeatability.

Dynamics of *de novo* compound induction in the fungal co-culture

Using the optimised condition (2-ml cultures), the induction of compounds was assessed at four different time points (two, four, seven and nine days, which will be referred to as D2, D4, D7, D9) to determine the optimal growth period for significant *de novo* induction in the 12-well plates. For that purpose, six replicates of the co-culture experiment were used (six pure cultures of *A. clavatus* and *Fusarium sp.* and six co-cultures) (Fig. S1 shows the 12-well plate format and Fig. S4 shows the colony morphologies).

After growth, the content of each well was lyophilised and extracted by sonication using a monophasic dichloromethane–methanol–water solvent mixture. The weight of each extracts was compared for each time point. From D2 to D7, the extract mass decreased and then, it remained constant between D7 and D9 (Fig. 2A). Proton NMR (¹H-NMR) profiling of the co-culture

extracts revealed that the main constituent was glucose (anomeric protons at δ_{H} 5.2 and 4.5 ppm), which is the main source of carbon in the culture medium (Fig. 2B). However, the presence of sugar drastically decreased from D0 (day zero) to D7 and then, only slightly reduced between D7 and D9 (Fig. S5). This decrease is logically related to the sugar consumption required for fungal growth. This result further comforted the choice for cultures to reduce volumes (2 mL) to lower the sugar quantity extracted from the medium. In term of extract composition, smaller culture volumes favour a better secondary metabolite to sugar ratio. Large quantity of sugar from the growth medium would significantly impede secondary metabolites analysis.

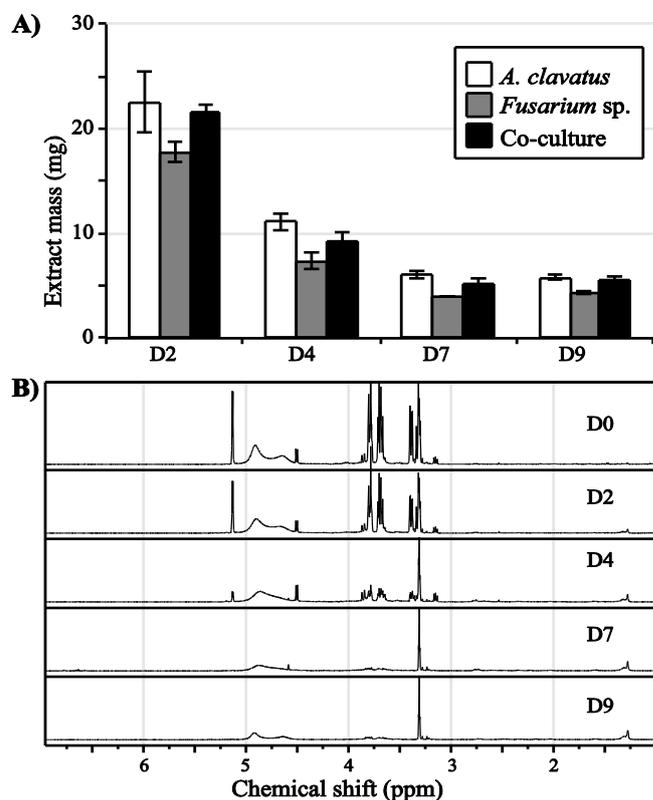


Fig. 2. Amount of extract obtained after different periods of fungal growth (A). The trend is consistent with the decrease in the glucose content demonstrated in the ¹H-NMR spectra (B) of the co-culture extracts (the full content of one well dissolved in 600 μL of CD_3OD ; the spectra were normalised based on the residual CD_3OH signal at 3.31 ppm).

To evaluate the *de novo* induction of metabolites during fungal growth, the fungal extracts were enriched by SPE and analysed using UHPLC-TOFMS for untargeted metabolite profiling. Generic linear RP LC gradients and both PI and NI electrospray ionisation (ESI) modes were used to perform a comprehensive survey of the metabolite composition of the extracts (Fig. S6).²⁹ Comparison of the total metabolites detected in all of the profiles revealed that only a few metabolites were present in the co-culture at the early time points (up to D4), whereas the number of metabolites was greater at later time points (after D4). This trend

was inversely related to the sugar content, as determined using NMR (Figs. 2B and Fig. S6).

The *de novo* induced features were counted at each time point by an automatic search for ions that were detected in the co-culture but were not detected in either of the pure strain cultures (Fig. 3, Table S1).³⁰ As during the optimisation of the culture medium volume used for fungal growth, the unusual formation of adducts related to co-eluting compounds was also verified. At D2, the level of *de novo* induction was very low: only one metabolite was highlighted in profiles showing only few other secondary metabolites (Fig. S6). At D4, more induced metabolites were observed, and at D7 and D9, the number of *de novo* induced metabolites detected had further increased. The number of secondary metabolites that were revealed was in the range of that previously reported for *de novo* induction after several weeks of culture in 9-cm Petri dishes.²⁹

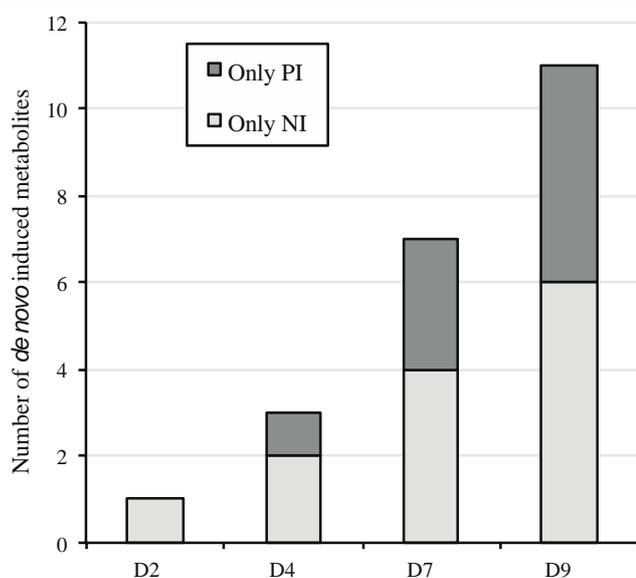


Fig. 3. Number of *de novo* induced metabolites present at different points of fungal growth, as assessed using either PI or NI TOFMS detection.

Altogether, 18 features were highlighted to be induced in the co-culture at a minimum of one time point. To study the trend of production of each of these metabolites, their level at each time point was monitored.

Among the 18 features, nine were not detected at any time point in the pure cultures. These features were considered as *de novo* induced *in stricto sensu*. Therefore, the strain producing these metabolites could not be determined; in the remainder of the manuscript, only these features will be described as *de novo* induced. The other nine highlighted features were detected in at least one of the pure cultures at a minimum of one time point. This result indicated that their production was up-regulated due to co-culture; and these particular features will be discussed later

in the manuscript. Consequently, the fungi which was producing these particular features was defined.

As expected, many of the *de novo* induced features accumulated in the co-cultures over time (Fig. 4). This was the case for seven of the nine highlighted features. Features PI481.032@1.44 (the notation corresponds to an ion detected using the PI mode that had an m/z of 481.032 Da at a RT of 1.44 min), NI205.087@1.44 and PI265.178@2.55 were produced from D4 to D9. Features NI427.103@1.44, NI525.071@0.80 and NI445.114@1.03 accumulating from D7, and feature NI581.131@1.15 was produced only from D9.

Interestingly, not all the *de novo* induced features simply accumulated as a function of the period of growth. Two features, NI387.038@0.77 and NI305.066@1.46, were produced at D4 and D7 and could not be detected at D9. This result indicates the complexity and dynamic nature of fungal metabolism.

Dynamics of metabolite up-regulation in fungal co-cultures

In addition to the previously discussed *de novo* induced features, other ions were highlighted (Fig. 5). These ions correspond to features that were induced at one time point and that were produced at some stage of the experimental period by either *Fusarium* sp. or *A. clavatus*. This allowed identifying which of the two fungi had produced the metabolite. Four of these metabolites were produced by *Fusarium* sp. and five were detected in the pure culture of *A. clavatus* (Fig. 5).

These results revealed other induction patterns resulting from fungal co-culture. One particular pattern was the up-regulation of the production of a given metabolite by fungal co-culture. Feature PI585.391@3.15 was produced by *A. clavatus* under standard pure culture conditions, however, its production was clearly up-regulated during co-culture. The production of two other features (PI289.069@1.46 and PI286.070@1.85) by *Fusarium* sp. was highly up-regulated under co-culture conditions at D4. At the later time points (D7 and D9), these two features were not detected in the pure culture samples but their production was still evident in the co-culture sample. Therefore, they represent two examples of features that were highly up-regulated and that were induced over a longer time span in the co-culture compared to in the pure culture. Another feature displayed similar characteristics. NI151.039@1.28 was present in the co-culture from D4 to D9 and was detected in similar quantities only at D4 in the pure culture of *Fusarium* sp. Two other features that were detected in the pure culture of *A. clavatus* followed a similar pattern. PI194.153@0.77 and PI444.162@2.55 were both present in the pure cultures at D4 and D7 and in contrast, were detected in the co-culture extract only at D9. Interestingly, whereas this feature was produced only at D9 in the co-culture, it was produced at a comparatively lower concentration at D4 and D7 in the pure culture of *A. clavatus*.

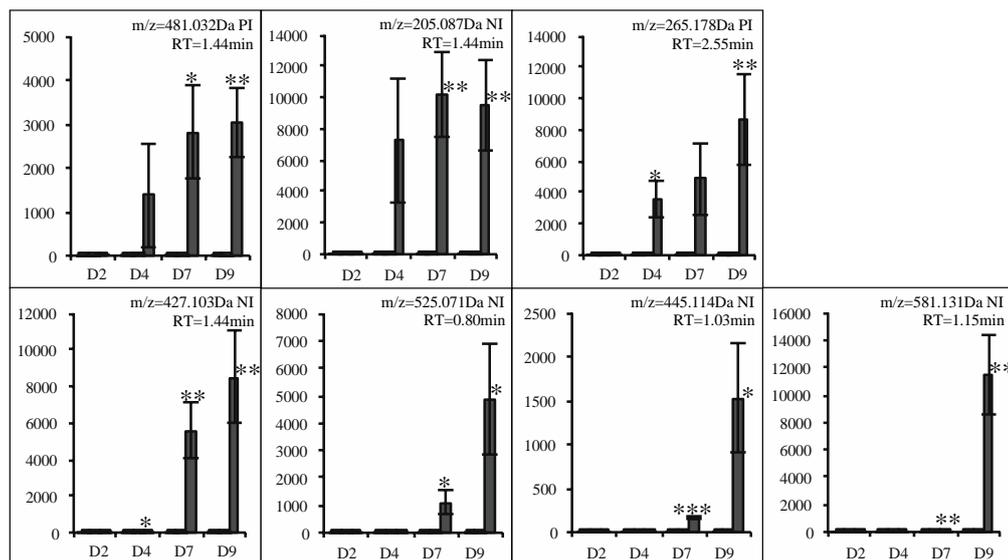
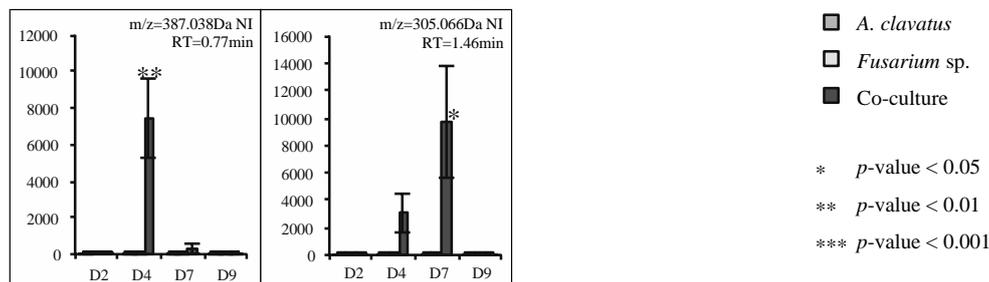
De novo induced features accumulating with time**De novo induced features with unusual induction pattern**

Fig. 4. *De novo* induction patterns (peak area values of extracted ion chromatograms in the ordinate axis) of some of the metabolites that were detected in the fungal co-cultures. The metabolites were sorted according to their induction pattern during the co-culture. The *p*-values were calculated by comparing the peak areas of the metabolites in the co-culture with those of the corresponding metabolites with the higher value in the two pure cultures.

Another induction pattern, the early production of specific metabolites in response to co-culture, was displayed by some of the features of interest. NI175.060@0.60, for example, was not detected in the pure culture of *Fusarium* sp. before D9 and was relatively up-regulated at D7 in the co-culture. The induced expression of this feature had begun by D4 because it was detected at a very low concentration at that point. Similarly, *A. clavatus* began to produce NI588.353@3.18 at D4 in the co-culture and later, at D7, in the pure culture. In addition, feature NI476.277@2.69 was detected already at D2 in the co-culture and was not observed in the pure cultures of *A. clavatus*. However, its concentration at D4 to D9 in the co-culture was reduced compared with that attained in the pure culture.

These differences in the induction pattern evolution over time appear to be metabolite-dependant, highlighting the complexity of metabolomic studies of microbial interactions that require integration of these dynamic issues. Using the strategy developed in this study, it has been shown that the dynamic of the induction of given metabolites observed as features were reproducible (N=6). However, key biochemical events may occur at different points in time and the induced features may be present only transiently. Significant variations in the metabolite

levels within a short time frame may explain why fungal metabolomic studies focusing on metabolite induction are known to be prone to variations and difficulties in terms of reproducibility.³⁸

Dereplication of the selected metabolites

The identification of the metabolites described above was beyond the scope of the current study; however, preliminary information was obtained through dereplication analysis based on the obtained HR-MS spectra.^{29, 30} Molecular formulae were deduced based on exact mass accuracy, isotopic pattern matching³⁹, heuristic filtering⁴⁰ and searches of the fungal metabolites included in the Dictionary of Natural Products.⁴¹ Multiple commonly detected adducts were considered to identify the features.²⁷ When multiple results were found, a cross search based on the genus of the studied fungi (*Fusarium* and *Aspergillus* species) was performed. Only compounds that could be associated with fungi of the same genus or family were considered as putatively identified. For compounds produced by a pure strain with up-regulated expression in the co-culture, only the strain of origin was considered for the dereplication analysis. This strategy allowed drastically reducing the number of putative

identifications (Fig. S7) to only a few possibilities for each considered feature. Finally, five compounds were putatively identified and four features were not identifiable due to a large number of possible compounds (Table S1). Nine peaks were putatively unannotated.

In the co-culture, gibepyrone F (NI151.039@1.28)⁴² and bostrycoidin (PI286.070@1.85)⁴³ were produced by *Fusarium* sp. Fumiquinazoline C or D (PI444.162@2.55)⁴⁴ was produced by *A. clavatus*. Two other *de novo* induced compounds were putatively identified as mitorubrin (NI427.103@1.44)⁴⁵ and brefeldin A (PI265.178@2.55);⁴⁶ based on phylogenetic information, these two compounds were produced by *A. clavatus*. It is important to note that all the putatively assigned metabolites possess antimicrobial activities.^{42-44, 47, 48}

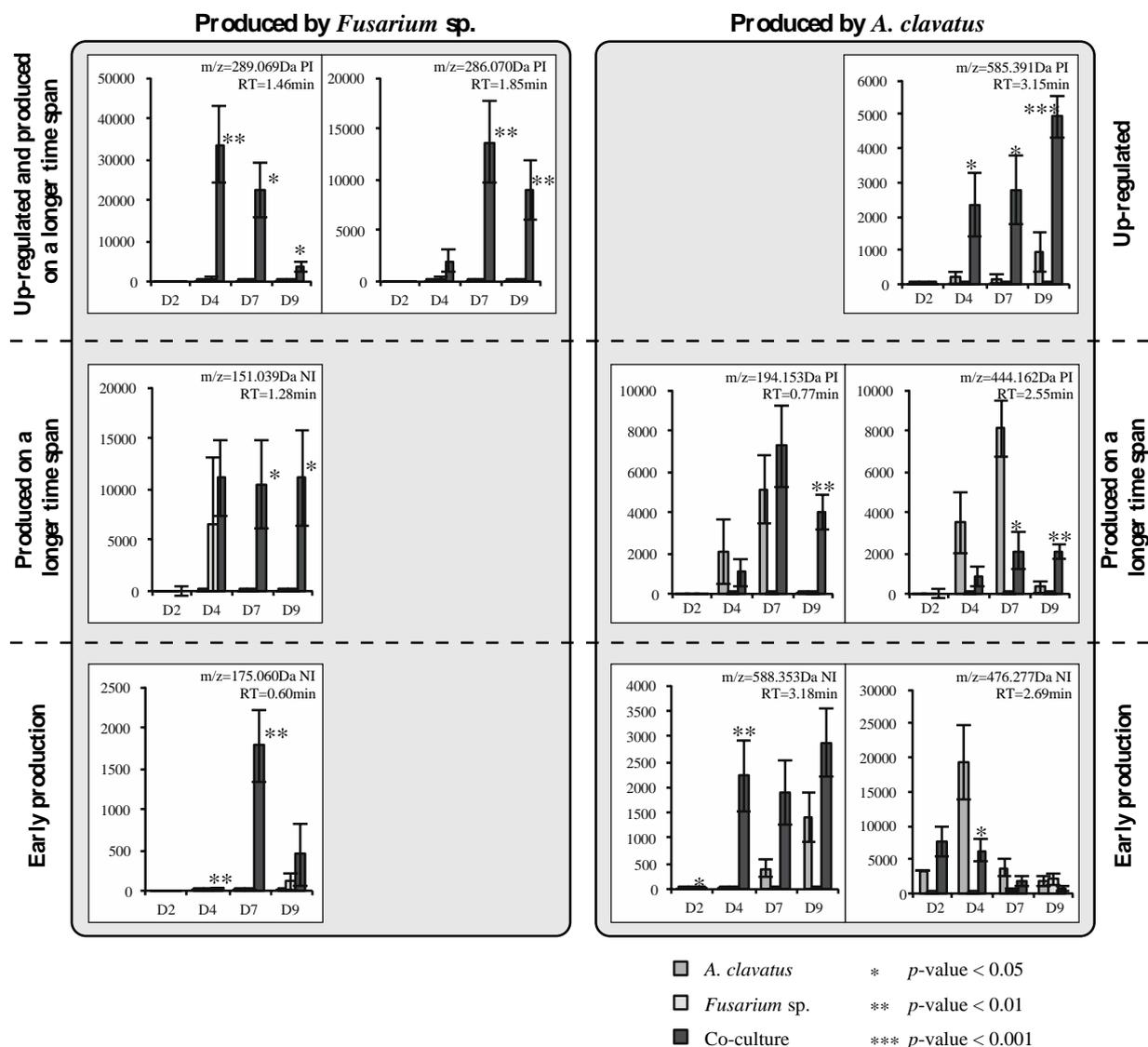


Fig. 5. Induction patterns (peak area values of extracted ion chromatograms in the ordinate axis) of some of the metabolites during fungal growth. The metabolites were sorted according to their induction patterns in the co-culture. The p -values were calculated by comparing the peak areas of the metabolites in the co-culture with those of the corresponding metabolites with the higher value in two the pure culture.

ARTICLE

Feasibility of up-scaled production for targeted isolation of *de novo* induced metabolites

Whereas several selected metabolites produced by the pure strains were dereplicated, the large majority of the *de novo* induced metabolites of interest in the present study could not be identified. This result emphasises the high potential of fungal co-cultures to generate novel NPs, but at the same time, renders the identification of such metabolites very challenging. Thus, a comprehensive *de novo* structural identification of such fungal metabolites and characterisation of their biological activities can be attained only through their targeted purification from the crude co-culture extract.³⁰ Because the multi-well system described here yielded an average of only 5 mg of crude extract per well (and the extracts contain large amounts of sugar), production at a larger scale is necessary to obtain at least microgram amounts of pure constituents for further characterisation. The co-culture condition for scale-up were investigated using large Petri dishes (15 cm) containing 60 times more growth medium and 14 times more growth space. UHPLC-TOFMS metabolite profiles of such a culture were obtained and compared to those obtained using the 12-well plate method.

At such a large scale, the growth rate of both fungi species was different from that at the small scale; *A. clavatus* grew slowly compared with *Fusarium* sp. Consequently, it was decided to inoculate *Fusarium* sp. in the middle of the Petri dish and to induce competition with four inocula of the second fungus, which were placed equidistant at the edges of the Petri dish (Fig. S1B). This technique permitted replicating the species' confrontation at different locations, which was expected to increase the concentration of the induced metabolites. After 24 days, the plate was almost completely covered by the fungi and the area covered by each fungus was similar (Fig. S8); therefore, the growth was stopped. The culture medium was extracted using the same protocol as for the 12-well plates with adaptation of the volumes. Approximately 350 mg of large-scale extract was obtained from one large Petri dishes. After extract enrichment, PI and NI mode UHPLC-TOFMS analysis was conducted, the features that were the focus of the small scale study were searched by extraction of their specific ion chromatograms.

From the 18 metabolites of interest, 16 were detected in the extract of the large-scale co-culture. Only NI305.066@1.46 and NI476.277@2.69 were not observed in the large-scale UHPLC-TOFMS profiles. The induction patterns of these two features in the 12-well plates showed that their concentrations reached a maximum at D7 and D2, respectively, before decreasing during subsequent fungal growth. Their production may have followed

a similar pattern in the large-scale co-cultures, which would explain their absence in the extract.

These results indicated that scaling-up the co-culture might allow the large-scale production of the induced metabolites of interest for further purification (three large Petri dishes producing 1 g of crude extract) and the identification of potentially novel fungal metabolites.

3. Conclusions

A multi-well procedure was devised to study fungal co-cultures based on 12-well plates containing 2 ml of growth medium using two model fungi. This strategy allowed obtaining rapidly a large number of replicates even with fungi having different growth rates (data not shown). Access to a sufficient number of replicates at different growth times provided a good way to confirm *de novo* metabolite induction phenomena with good confidence. The study of the induction of selected fungal metabolites in time series in such conditions showed the complexity of fungal interactions and highlighted how metabolomics experiments can tackle such issues. In the studied model co-culture, 18 metabolites were found to be *de novo* induced at some stage of the co-culture. The five features that could be putatively identified by dereplication correspond to compounds that have been reported to possess antimicrobial activities. This indicates that induction of cryptic pathways through co-culture is able to generate new NPs with interesting bioactivities, most likely related to defence.

The proposed strategy is applicable to different types of microorganisms and can be employed to study naturally occurring microbial interactions.⁴⁹ This may therefore provide valuable data for the interaction mechanisms triggered by small molecules.

The method was reliably up-scalable to a large-scale 15-cm Petri dish format. Large amounts of crude extract can be obtained from cultures grown in multiple large Petri dishes (15 cm in diameter).⁵⁰ This aspect is crucial for isolation of the metabolites of interest and their full structural and biological characterisation. This result suggested that even larger metabolite production could be accomplished using semi-industrial agar-supported solid-state fermenters.⁵¹ Notably, the generic 12-well plate strategy is also compatible with HPLC microfractionation coupled to biological testing for the early-stage identification of antifungal compounds⁵² because the amount of material generated for the metabolomics study (50 mg) are sufficient for the identification of induced metabolites with a high bioactivity potential at a very early stage. This strategy will therefore be applied in future co-culture studies for the rapid identification of antimicrobial compounds.

4. Materials and methods

Chemicals

The extractions were performed using methanol (HPLC grade, Fisher Scientific), dichloromethane (HPLC grade, VWR International), and nano-pure water (Millipore). The UHPLC-TOFMS analyses were performed using ULC/MS-grade acetonitrile and a mixture of water and formic acid (FA) purchased from Biosolve. PDA (Difco) was used as the culture medium.

Biological Materials

Two fungi of very different origins were selected as models for this study. *Aspergillus* patient *clavatus* (Sin141) was isolated from soil. In contrast, the *Fusarium* sp. (PS54743) utilised was recovered from a blood sample of an immuno-compromised patient who was diagnosed with invasive fusariosis at the Geneva University Hospital. The two fungal strains were stored in the Agroscope ACW bank in vials containing a diluted potato dextrose broth solution (1:4) at 4 °C (<http://mycoscope.bcis.ch/>).

Multi-well Culture and Co-Culture Conditions

For optimisation, 1, 2, 3 or 4 mL of PDA was placed in the wells of 12-well plates (wells with a 2-cm internal diameter); 2 mL of PDA was selected for further experiments.

The two pure cultures, Sin141 and PS54743, were inoculated by placing 2-mm agar plugs of fungal pre-cultures in the centre of position A1-3 and B1-3, respectively, of a 12-well plate (Figure S1A). Similarly, the co-cultures were prepared by inoculation two 2-mm agar plugs of pre-cultures of the two different fungal strains on the opposite sides of well C1-3. Wells A4, B4 and C4 were not inoculated and were used for the blank samples. The cultures were incubated at 21 °C for 3 days for studies of the effect of the volume of culture medium and for 2, 4, 7 and 9 days for studies the evolution of *de novo* metabolite induction.

Large-Scale Co-Culture Conditions

Large (15-cm) Petri dishes were prepared in triplicate using 120 mL of PDA. This volume corresponded to a 60-fold increase in the amount of growth medium and a 14-fold increase in the growth surface compared with those of the 2-cm wells. The plates were inoculated by placing one 5-mm agar plug of a pre-culture of *Fusarium* sp. in the centre. In addition, four 5-mm agar plugs of a pre-culture of *A. clavatus* were placed equidistant at the edges of the Petri dish (Figure S5B). The Petri dishes were incubated at 21 °C for 24 days.

Extraction Procedure for the Small-Scale Samples

Extraction was performed according to previously described procedures²⁹, with adaptations to account for the reduced volume of the material to be extracted. Briefly, each fungal pure culture, fungal co-culture or non-inoculated agar sample was transferred to a glass tube and freeze-dried using a centrifugal evaporator (Genevac HT-4, SP Scientific). The extraction solvent mixture, dichloromethane–methanol–water 64:36:8 (v/v), was added

(20 mL per 30 mg of dry material). In the case of the selected 2 mL volume of PDA used in the well of the 12-well plates, 4 mL of the extraction solvent mixture was used. The extractions were performed by sonication directly in the tubes in a water-bath sonicator (Ultrasonic Cleaver 5200, Branson Ultrasonics Corporation) at room temperature for 20 minutes. The sonicated samples were filtered through glass cotton and the extracts were dried under vacuum using a centrifugal evaporator. Each extract was weighted.

Extraction Procedure for Large-Scale Co-Culture Samples

The large-scale co-culture samples were cut into 1 x 1 cm agar pieces using a razor blade and were freeze-dried (Freeze Dryer Alpha 2-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH). The dry material was extracted three times by sonication with 750 mL of dichloromethane–methanol–water (64:36:8 (v/v)) in a water-bath sonicator at room temperature for 20 min. The sonicated samples were filtered through glass wool. The three extracts were pooled and were dried under vacuum. Approximately 350 mg of extract was obtained from one 15-cm Petri dish.

NMR Profiling of Fungal Extracts

The extract was entirely dissolved in 600 µL of CD₃OD and analysed using a Varian Unity Inova 500 MHz NMR instrument (Agilent Technologies) at 25 °C. ¹H-NMR analysis was conducted using 64 transients at 302 °K (29 °C). The spectra obtained were analysed using MestReNova software (version 8.0, Mestrelab Research S. L.), and the chemical shifts were referenced to the residual protonated solvent signal (CD₃OD, 3.31 ppm).

UHPLC-TOFMS Analysis

SPE was conducted to remove the most lipophilic compounds, which would ensure good repeatability of reversed-phase chromatography over a long series. Sample enrichment by classical SPE filtration was achieved using Sep Pak Vac SPE C18 cartridges (1 cc, 100 mg, Waters).¹⁵ Briefly, 1 mg of extract was dissolved in 1 mL of nano-pure water and eluted through the C18 cartridge. The extract was then eluted using methanol–water 85:15 (v/v) and the solution was adjusted to 1 mL.

UHPLC-TOFMS analyses were performed using a Waters Micromass-LCT Premier Time-of-Flight mass spectrometer that had an ESI interface coupled to an Acquity UPLC system (Waters). In separate runs, detection was achieved using both the PI and NI mode. The *m/z* range was set to 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 the 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 (Sigma-Aldrich) was infused through the lock-mass probe at a flow rate of 5 µL/min using a second Shimadzu LC-10ADvp LC pump.

UHPLC-TOFMS fingerprints were recorded with a 50 mm × 1 mm i.d., 1.7 μm Acquity BEH C18 UPLC column (Waters) in gradient mode at a flow rate of 0.3 mL/min with the following solvent system: (A) 0.1% v/v formic acid (FA) in water; (B) 0.1% v/v FA in acetonitrile. 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 to 5% B in 0.1 min and finally equilibrated with 5% B in 1.1 min. The temperature was maintained at 40 °C, and the injection volume was 1 μL of a 1 mg/mL solution based on the extract content prior to SPE. The analyses were performed randomly and included quality control and blank samples after every 10 sample runs. The sample list was randomly generated using a dedicated Excel macro.²⁹

Peak Picking and Data Analysis

Native MassLynx data (Waters) were converted into netCDF (common data format) using DataBridge software (Waters). Automatic feature detection was performed between 0.4 and 4.5 min with MZmine 2 software³⁶ using parameters selected according to the TOFMS detector. Peaks with a width of at least 0.03 s and an intensity greater than 30 counts (both NI and PI) were picked with a 5 ppm *m/z* tolerance and the generated peak lists were 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 a *m/z* tolerance of 15 ppm and a RT tolerance of 0.2 min. The features detected from blank samples and non-inoculated agar samples were removed from the generated matrix. The full procedure for feature detection is presented in Table S2.

To select *de novo* induced features, the 'detected' status (peak height over a certain threshold) of every feature was used. The features uniquely 'detected' in the co-culture replicates were selected (at least 5 times out of 6). A simple Excel script was employed to explore the large data set generated through MS detection.³⁰ Selected features were validated by exploration of the raw data using MassLynx. A particular attention was paid to adduct formed by two co-eluting compounds by searching manually for two molecular ions which may explain the formation of the selected features in the MS spectrum.

The statistical significance of the highlighted features was calculated using student's test to compare each pure culture and the co-culture data point. Only the *p*-value that corresponded to the comparison of the co-culture with the pure culture having the higher mean peak area for that specific feature was considered.

Dereplication of the Selected Metabolites

The dereplication process was based on the high mass accuracy molecular weights of ions detected using the TOFMS system. Only the [M-H]⁻, [M+HCO₂]⁻, [M+H]⁺ and [M+Na]⁺ adducts were considered²⁷. The molecular formulae of the selected ions were determined based on the exact mass accuracy, isotopic pattern matching and heuristic filtering.^{39, 40} Finally, the molecular weight was searched in the Dictionary of Natural Products⁴¹ for positive matches among the chosen metabolites

that were produced by *Fusarium* and *Aspergillus* sp. or by phylogenetically related fungi (based on the Catalogue of Life, Species2000, <http://www.catalogueoflife.org/>).

Acknowledgements

We are grateful to Philippe J. Eugster for his contribution to the UHPLC-TOFMS analysis. This study was supported by the Swiss National Science Foundation Sinergia Grant CRSII3_127187, which was awarded to J.-L.W., M.M., and K.G.

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Electronic Supplementary Information (ESI) available:

Organisation and inoculation of the Petri dishes. Images of the fungal colonies in pure cultures and co-cultures (effect of the volume of culture medium and of growth time). Mass of the fungal co-culture extract obtained from a 2-cm diameter Petri dish (effect of the amount of culture medium over time). Amount of glucose in the extract relative to the growth period. UHPLC-TOFMS profiles of the co-culture extracts according to different growth periods. Effect of biological-resource limitation on the number of positive dereplications. Images of the large-scale co-culture. List of *de novo* induced features with their corresponding putative identification. Precise peak-picking procedure using MZmine 2. See DOI: 10.1039/b000000x/

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