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Mass Spectrometry Imaging of Secondary Metabolites Directly on Fungal Cultures

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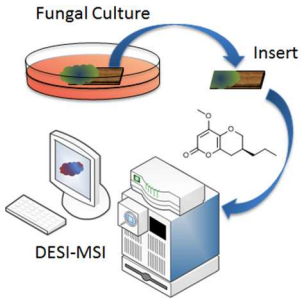
Overcoming the challenges of analysing fungal cultures directly using desorption electrospray ionization mass spectrometry imaging (DESI-MSI).



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Desorption electrospray ionization mass spectrometry (DESI-MS) is an ambient ionization technique that enables imaging experiments directly on fungal cultures. Much information can be gained by examining an organism directly from culture, rather than though an extraction process, as the regional and temporal distribution of bioactive compounds can give a better understanding of interactions in the environment. However, this technique has been underutilized towards the direct analysis of fungal cultures. A major challenge of fungal culture analysis with DESI-MS is the requirement of a firm, flat surface for effective ionization. The media upon which fungi grow can be easily deformed by the pressures from the solvent spray and gas, and the fungal topography is naturally uneven, often containing mycelium and spores that move freely. Furthermore, DESI-MS imaging can only analyse the surface of a sample, thus internal compounds remain undetected. This project first sought to overcome these issues, and then, apply the newly developed methodology to explore the chemical interactions between two distinct fungi. To test the methodology, a fungus that produces antifungal metabolites was grown against a mycotoxin producing fungus, both of the phylum Ascomycota. By comparing the spatial and temporal distribution of secondary metabolites between both isolated cultures and co-cultures, the chemical exchanges that took place were visualized.

Introduction

Desorption electrospray ionization (DESI) is a mass spectrometry (MS) technique that allows for the detection of compounds under ambient conditions.[1] Charged droplets are sprayed on the sample, inducing the compounds to be desorbed from the surface, such that they can be vacuumed into the MS for analysis.[2] By ionizing in a minimally destructive manner, the sample can not only remain in the culture environment, but it can also be reanalysed repeatedly. DESI-MS can be used to image a surface and create a 2D map of the detected compounds. These maps can be superimposed on photos of the initial sample, so as to visualize, determine the location, and estimate the relative concentration of key compounds.[3]

The popular techniques used for the MS analysis and imaging in microbiology include matrix assisted laser desorption ionization (MALDI), laser ablation electrospray ionization (LAESI), DESI and nanoDESI.[4] Both MALDI [5] and LAESI[9] incorporate lasers to assist in the ionization process, thereby ablating the samples during analysis, preventing repetitive analysis over time. Since DESI and nanoDESI[6] rely on the solvent system for ionization, they are minimally abrasive, permitting the repetitive analysis of the same sample. This can be applicable when selected-ion monitoring (SIM), MS/MS, and/or ionization optimization are desired post the initial run, as well as, when instrument limitations prevent simultaneous ionization in both positive and negative modes.

Despite these benefits, DESI is not without challenges. First, high spatial resolutions, such as those observed with MALDI and nanoDESI, are difficult to achieve with DESI.[4] Also, there are many physical parameters that must be in place to effectively ionize a compound. The height, distance, and angle of the spray emitter relative to the sample must be optimized and remain precise and consistent throughout a scan. Therefore, an ideal sample should be flat, as to not alter these measurements, and firm, as to not be deformed by the spray and gas pressure coming from the source.[7] Additionally, the solvent system must be optimized to allow for sufficient desorption. Finally, the charge applied and flow rate of the droplets are also important considerations for optimizing a DESI-MS experiment.

Due to its versatility, DESI-MS has generated great interest in several fields, including the high-throughput screening of pharmaceuticals,[8] environmental analysis,[9] food safety testing,[10] forensics,[11] and biological analyses.[12] In the realm of natural products, DESI-MS is becoming a prominent technique for the detection of compounds directly on the surface.[13] Recent studies have demonstrated MS imaging of secondary metabolites on terrestrial[14, 15] and marine[16] organisms through direct surface analysis. Additionally, Dorrestein and colleagues have been leaders[17] for the direct cultural analysis of microorganisms. However, their research has focused primarily on metabolic exchanges in bacteria[18] often using nanoDESI.

Although microorganisms, such as bacteria, have been imaged using DESI and nanoDESI, members of the fungal kingdom have only undergone limited investigation.[19] Taxa that belong in the phylum Ascomycota are the most speciose[20] and are among the most

prolific producers of bioactive natural products.[21] From an ecological standpoint, fungal communities are also diverse, even in a mircoscale setting,[22] resulting in many fungal interactions. Inhibition zones, colour alteration of mycelia, and sporulation are examples of the physical changes that can occur when fungi grow together,[23] but the chemical interactions that take place are poorly understood. Therefore, DESI-MS presents an opportunity for profiling fungal cultures *in situ* to examine spatial and temporal distribution patterns of secondary metabolites.

The imaging of fungi with DESI-MS raises several issues that are not encountered with plants or bacteria.[24] For instance, plant tissues, such as leaves, stems and seeds, are typically firm and more defined than a fungal culture.[13, 25] Fungal cultures also differ from bacteria, since fungal surfaces often contain topography due to the presence of aerial hyphae, and can, therefore, be dynamic due to the movement of the mycelium and spores. Additionally, organisms grown on media are disadvantageous, since the media can be affected by the gas and spray pressures from the DESI source, which can form divots in the media, altering the distances and angles that are required for consistent and efficient ionization.[2] With bacterial cultures, which grow relatively flat, nanoDESI is often utilized, since it is a less abrasive technique than DESI, thus minimizing deformation of the medium's surface. However, the typical surfaces observed with fungal cultures can be more detrimental to nanoDESI than to DESI. Not only can nanoDESI be hampered by physical issues (i.e. topography), since the fused capillaries are fragile and susceptible to breaking if not carefully monitored, but also, viscous materials (i.e. guttates[19]) can lead to clogging of the secondary capillary.[26] In short, fungal cultures present many challenges for DESI-MS that may not be an issue when analysing natural products from other kingdoms of life.

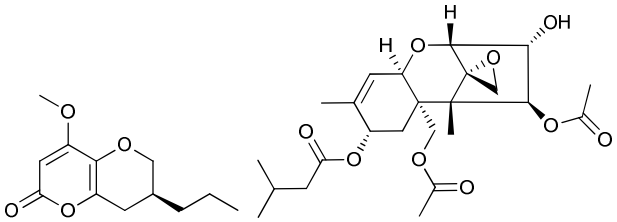
A key advantage to direct culture ionization is the ability to perform mass spectrometry imaging (MSI) experiments. MSI could become an important tool for mycologists interested in the spatial and temporal distribution of secondary metabolites of fungal cultures. Furthermore, visualizing the chemical distribution of metabolites in mixed or co-cultures may provide an understanding of the interactions and chemical communications between organisms. Direct fungal cultures have been imaged using DESI-MS on a limited scale,[19] and most examples of direct culture analysis involve bacterial samples using nanoDESI.[7] Therefore, the methodology developed in this study opens up new avenues for studying the chemistry of fungal interactions *in situ*.

Results and discussion

Development of desorption electrospray ionization methodology for direct fungal analysis

A freshwater fungal strain, coded G100, was identified via ITS sequencing[27] as *Clohesyomyces aquaticus* (Pleosporales, Dothideomycetes, Ascomycota). It was selected for the initial DESI-MS studies due to the biosynthesis of phomopsinone A (**1**), a previously reported antifungal compound.[28] Another culture, coded G3, was also selected because of its observed cytotoxic activity; it displayed 100% cytotoxicity at 10 and 100 ppm in the brine shrimp lethality test.[29] G3, an endophytic fungal strain, was isolated from surface sterilized seeds of *Asimina triloba* (L.) Dunal (Annonaceae) and identified via ITS as *Fusarium* sp. (Hypocreales, Sordariomycetes, Ascomycota). This fungal strain biosynthesized the mycotoxin, T-2 toxin (**2**)[30] (Fig. 1), which has been reported to cause alimentary toxic aleukia and Kashin-Beck's disease. ITS data from both fungal strains were deposited in GenBank (G100: KM589855, G3: KM589854). Compounds **1** and **2** were isolated and identified via natural product protocols common to this research group,[19] including full characterization by NMR and MS (Figs. S1 and S2).

We hypothesized that these two fungi would make an interesting test case, because we could image the interaction of an antifungal compound producing fungus (G100) and a mycotoxin producing fungus (G3). However, such experiments were predicated upon the development of an effective DESI-MS methodology. The initial stages of DESI-MS required optimizing the conditions for these two fungal secondary metabolites. Pure aliquots of **1** and **2** were spotted on Teflon coated slides and sprayed with the DESI emitter. Several solvent systems were tested, including various ratios of CH_3CN:H_2O, CH_3CN:CH_3OH, CH_3CN:CH_3OH:H_2O, and CH_3OH:H_2O (all with 0.1% formic acid). While all permitted ionization at some level, the system comprised of CH_3OH:H_2O (70:30) led to the greatest intensities of the targeted peaks. Furthermore, adjustments to the tube lens voltage had great effects on the classes of compounds that were ionized, coinciding with previous reports suggesting that small molecules ionized best at 100V, while peptides were best at 250V.[11]



1 **2**

Fig. 1 Structures of phomopsinone A (**1**) and T-2 toxin (**2**).

When the 70:30 CH_3OH:H_2O (0.1% formic acid) system was tested initially on a culture of G100 in a Petri dish, compound **1** was detected. However, the signals disappeared rapidly, as the gas pressure from the DESI source moulded a divot on both the fungus and the surrounding agar. It became clear that agar did not provide the firm, flat surface necessary for optimal ionization in the 2D mapping experiments. Since agar is pliable, it was hypothesized that the incorporation of an insert would provide for a surface that was less malleable by the DESI spray and gas pressure (Table 1). Moreover, since fungi play a role in cycling carbon in the environment, cellulosic inserts were explored, so as to provide a nutrient-rich substrate for the test fungus that could be removed easily for analysis. [31]

The first two trials did not yield positive results. Initially, G100 was grown on a cheesecloth insert, so that it could be removed from the agar for DESI-MS analysis. The cheesecloth's porous nature allowed the fungus to grow on top while still gaining additional nutrients from the agar. Unfortunately, the fungus decomposed the cheesecloth, leaving shreds with only trace amounts of fungus remaining on it. Next, filter paper (Ahlstrom Chromatography Blotting Paper Grade 222) was attempted, as its thickness would prevent complete deterioration while still being easily removed from the agar. While that was largely the case, a second problem developed. The sample did not ionize well when imaged by DESI-MS. It was hypothesized that during the analysis, the filter paper absorbed some of the solvent, thereby preventing desorption of secondary metabolites.

Based on those initial failures, cardboard was examined as an insert, since it provided a firm growth substrate that was less absorbent than filter paper. Fortunately, compound **1** was easily ionized off of the surface of G100 grown on cardboard, and the DESI source did not deform the surface. As DESI rastered across the cardboard (Fig. 2), **1** was detected readily, thereby permitting

Table 1 Methods tested for direct imaging of fungal cultures from a Petri dish. Cellulosic inserts were used, since they would provide a nutritious surface for fungal growth.[31] The key goals were both to minimize sample preparation and to limit alterations of the fungal culture by the DESI source.

Insert Type	Hypothesis	Results
None	Analysis of unaltered fungal cultures directly from Petri dish	Pressure from spray and gas deformed the agar, causing an uneven surface that was not ideal for DESI-MS imaging
Cheesecloth	Allows fungal culture to interact with agar medium but could be removed for DESI-MS imaging	Fungal culture deteriorated the cheesecloth
Filter Paper	Thicker insert would minimize deterioration and would allow the fungal culture to be easily removed from the medium	Solvent did not desorb ions effectively off of this absorbent surface
Cardboard	A less absorbent insert could increase ion desorption and provide a firm, flat surface for ionization	Compounds ionized effectively and the fungal culture grew relatively flat on the firm cardboard
Balsa Wood	Wood inserts better simulate the natural environment of the fungus	Fungal cultures grew inconsistently, often uneven, thus decreasing the chances that a culture could be analysed via DESI
Insert in liquid media	Inserts inoculated in liquid media would better simulate a more natural habitat	Culture surfaces were uneven and medium on insert created a malleable surface

relative quantification, i.e. the higher the peak (Fig. 2B), the higher the relative abundance of **1** (Fig. 2C). Moreover, even in the development stage of the methodology, an unanticipated benefit of the DESI-MS emerged; the biosynthesis of **1** was minimal and inconsistent until three weeks of growth. This temporal observation factored into the experimental design of the co-culture experiments.

Balsa wood was also explored as an insert, since some of the test fungi were isolated from submerged woody debris in freshwater habitats.[32] It was hypothesized that balsa wood could provide a more natural substrate to test the spatial distribution of secondary metabolites from aquatic fungi. Unfortunately, the fungal growth patterns on balsa wood were inconsistent. Sometimes they grew raised and bulbous and other times they were transparently thin (Fig. S3). The fungal culture often exhibited a higher preference for agar than remaining on the insert, an issue that was not observed with cardboard.

Another approach to mimic a more natural environment included incubating G100 on the cardboard or balsa wood submerged in liquid media for 7-10 days, followed by placing onto the agar (Fig. S4). Since G100 was isolated from submerged wood in an aquatic habitat, it was hypothesized that this method would better simulate a more natural environment. However, this method resulted in growth that was uneven (Fig. S4). Additionally, a malleable agar layer between the fungal culture and the insert formed, thus negating the firmness provided by the insert (Fig. S4). The submerged inoculation also prevented making correlations between location and age. Younger areas of the culture were indeterminable, and therefore, they could not be paired with the biosynthesis or timing of specific compounds when performing the imaging experiments.

Application of mass spectrometry imaging to fungal cultures.

Since cardboard provided the best surface for DESI-MS of fungal cultures, both G100 and G3 were subjected to this methodology, and 2D mapping experiments were applied in an attempt to observe and quantify the production of compounds **1** and **2**, respectively. For the fungal culture of G100, there were two points of inoculation on a single piece of cardboard, as indicated by the yellow dot (Fig. 3A). Compound **1** was observed at the point of inoculation and increased in concentration towards the edge of its growth, but not at the immediate edge. This radial distribution of **1** in cultures of G100 on cardboard was observed from both inoculation points.

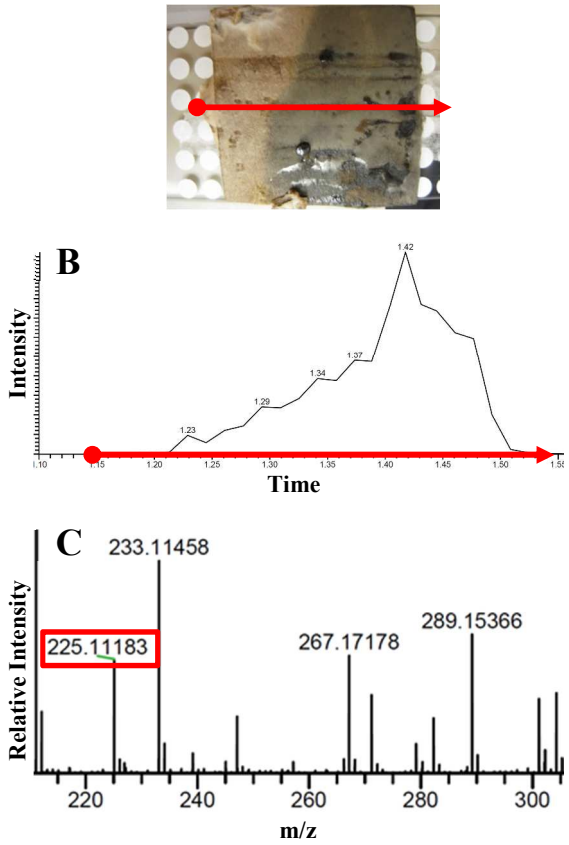


Fig. 2 Analysis of **1** in fungal culture G100. (A) Culture grown on cardboard with visible scan lines from DESI. (B) Extracted-ion chromatogram (XIC) of **1** showing the intensities of secondary metabolites during a single scan across the sample. (C) At 1.42 minutes, the accuracy for $[M+H]^+$ of **1** between the observed and calculated was 1.3 ppm (225.1118 observed vs 225.1121 calculated for $[C_{12}H_{16}O_4+H]^+$.

Conversely, when the identical experiment was performed to monitor **2** on the surface of G3, little to zero **2** was observed (Fig. 3B). To further analyse G3 and determine why compound **2** wasn't detected, a cross-section of G3 with agar was analysed. As is characteristic of *Fusarium* sp., the G3 culture had white aerial hyphae (surface) with a mycelial mat[33] (inner body) colour that was pink to purple.[34] Upon DESI-MS analysis of the cross-section, compound **2** was detected, but only in the areas where the culture was pink/purple in colour (Fig. 4). Therefore, it was determined that **2** was located in the body of the fungal culture, and not on the surface.

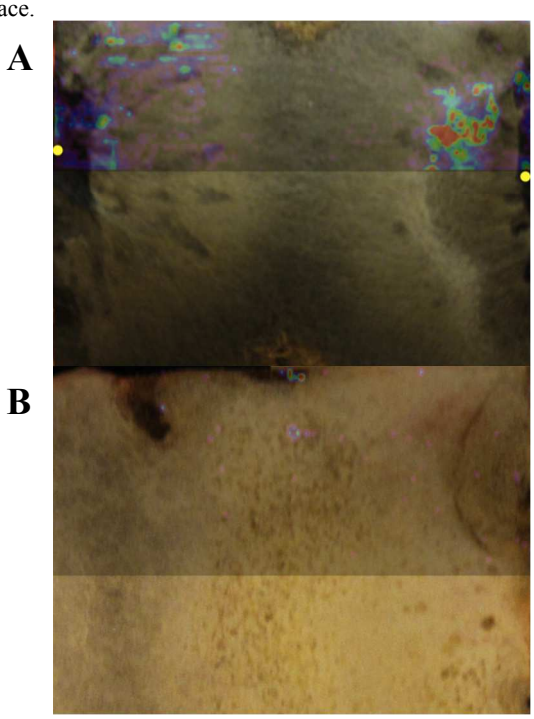


Fig. 3 (A) G100 and (B) G3 grown on cardboard inserts with the DESI-MS image overlaid on the upper half of the photo for phomopsinone A (**1**) and T-2 toxin (**2**), respectively. The DESI-MS illustrates the detection of **1** on the surface of G100 (A), while **2** was not observed on the surface of G3 (B). The yellow circles (A) indicate the points of inoculation. The darker red the spot, the higher the relative concentration of **1**.

These studies demonstrated that external compounds were readily desorbed by DESI but internal compounds were not. Thus, a different method of preparation was required to achieve a firm, flat surface to facilitate imaging of internal compounds (Fig. 5, bottom). Hole-punches were made into the cultures, effectively removing circular cross-sections of fungus on agar. By flash freezing and vacuum drying the removed pieces of culture, the internal

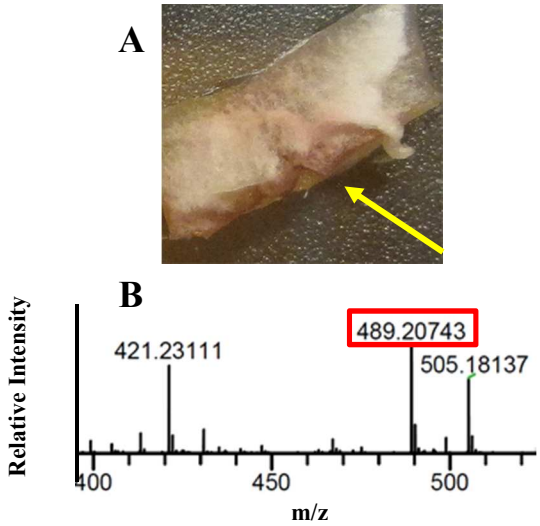


Fig. 4 Analysis of **2** in a fungal culture of G3 grown on agar and cross-sectioned, showing the characteristic pink body colour of *Fusarium* sp. (A). Upon DESI-MS analysis of the cross-section, the Na-adduct of the molecular ion of **2** was observed only in the pink area as indicated by the yellow arrow (B). The accuracy between the observed and calculated for $[C_{24}H_{34}O_9+Na]^+$ was 4.3 ppm (489.2074 observed vs 489.2095 calculated).

compounds were exposed as a ring around the edges. The crude analogy of squishing and flattening a "jelly donut" can be used to visually explain the process. The middle of the excised piece would constitute the surface of the "donut", while the outside edges would contain the "jelly" extruded from the inside. Thus, the compounds on the fungal surface remained intact, while the metabolites on the inside were extruded to the edges as the culture dried under vacuum.

Cross sectioning of tissue samples (i.e. brain, lung, spinal cord, etc.) to facilitate DESI-MS imaging of internal compounds has been reported, particularly in combination with a cryotome. Unfortunately, fungal cultures are often too thin for cryotome sectioning. Thus, freezing and desiccating represented a rapid, effective procedure to obtain a similar outcome with delicate fungal cultures.

This procedure was applied to cultures of G100 and G3 (Figs. 5 and 6). Once again, compound **1** was observed on the surface of the fungal disc of G100. When analysing G3, compound **2** was detected, but only on the outer, pink/purple edges. This supported the hypothesis that the mycotoxin was located inside the body of the fungus, rather than on the surface. While mycotoxins have been well studied in literature,[30] their location within the body of a fungal culture has not been reported.

To evaluate the applications of this technique and to visualize

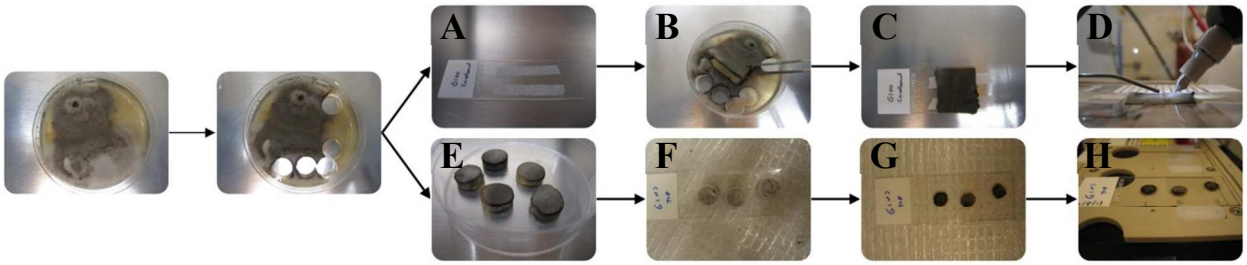


Fig. 5 The general sample preparation steps for DESI-MS analysis of fungal cultures. The top figures show the prepared slide (A), the removal of the insert (B), its application to the slide (C), and the analysis of the fungus via DESI-MS (D). The bottom figures show the hole-punches (E) that are then frozen (F) and vacuum desiccated (G) prior to DESI-MS analysis (H).

secondary metabolite biosynthesis in co-culture, G100 was grown against G3 and then imaged. This experiment tested the antifungal activity of G100, as monitored by DESI-MS for the biosynthesis of **1**, in the context of growth against the mycotoxin producing fungus (G3). For the initial tests, G3 and G100 were inoculated at the same time. On one Petri dish they were inoculated on agar, and on another Petri dish they were inoculated with G3 on the agar and G100 on cardboard. This would allow for surface analysis of **1** on G100 using the cardboard technique and the analysis of **2** from G3 using the freeze/dry procedure. Unfortunately, due to the rapid growth of G3, it quickly overtook the entire plate (Fig. S5); this is a common challenge with cultures of *Fusarium* sp.[34]

As noted earlier, the biosynthesis of **1** was not observed at two weeks or earlier. Therefore, the experiment was repeated with a time delay between inoculations. This time, G100 was inoculated three weeks prior to the inoculation of G3 (Fig. 7), and then both cultures were grown for two weeks before DESI-MS analysis. G100 visibly prevented the growth of G3 in both Petri dishes (with and without cardboard).

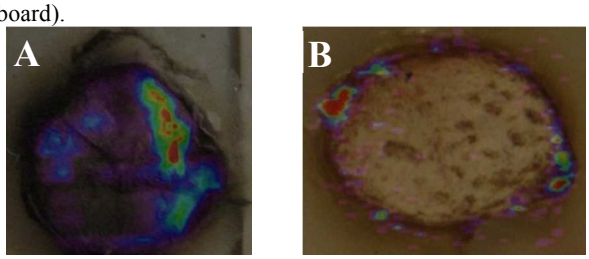


Fig. 6 DESI-MS imaging of **1** in G100 (A) and **2** in G3 (B) after the flash freeze and vacuum dry procedure was applied. The DESI-MS image showed **1** on the surface of G100, while **2** was only observed inside the culture of G3, evident by detection on the edges. The darker red the spot, the higher the relative concentration. However, intensities between images do not relate in concentration.

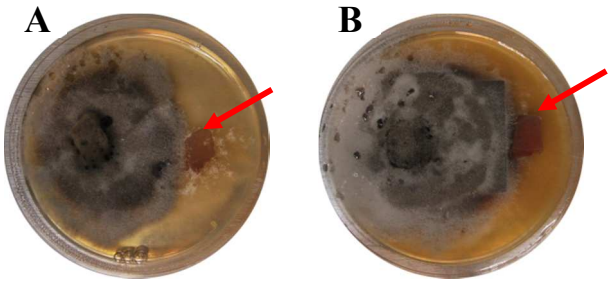


Fig. 7 Two week old co-cultures of G100 on agar (A) or cardboard (B) grown against G3 on agar. G100 (grey mycelium) was inoculated 21 days prior to G3 and inhibited the growth of G3. The reddish rectangle (noted by arrow) is from the initial inoculation of G3.

The cardboard insert of G100 was removed and imaged (Fig. 8) showing the presence of **1** in similar fashion to when it was grown by itself. However, **2** was unable to be detected in the surrounding areas of where G3 was inoculated. Further analysis via the "jelly donut" method was not performed due to the absence of any visual G3 growth. Unfortunately, further exploration into the chemical interactions between these two fungi was hindered due to the attenuation of phomopsinone A (**1**) production. As is often the case with fungi, successive subculturing often leads to decreased biosynthesis of certain metabolites.[35]

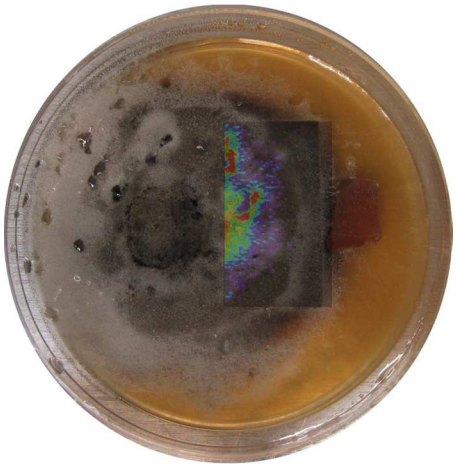


Fig. 8 G100 (left) on cardboard against G3 (right) on agar with the MSI of **1** superimposed.

Conclusions

In conclusion, two methods were developed for the mass spectrometry imaging of fungal cultures. The cardboard insert was an effective and inexpensive way to prepare the fungal culture for imaging of compounds on the surface. Using this, we observed the biosynthesis of **1** at approximately three weeks. This was supported by the observed antifungal properties of G100 in co-culture experiments. Moreover, a second technique was developed to image compounds within the body of a fungal culture. By excising and then freezing and drying a hole-punch from the culture, the mycotoxin (**2**) was observed for the first time in the body of a culture. Studies are ongoing to expand these techniques to other co-culture experiments with a goal of imaging *in situ* the chemical signals between fungal cultures.

Materials and Methods

General experimental procedures

For DESI-MS, a Prosolia OS-3201 DESI source was used with a Thermo LTQ Orbitrap XL system with the following settings: positive mode ionization; capillary temperature, 200 ºC; source voltage, 3.00 kV; capillary voltage, 46 V; tube lens, 100 V; scan time, 150 ms; flow rate, 1.20 µL/min; nitrogen gas pressure, 180 psi; spray angle, 55; inlet height, 1-2 mm. The solvent was CH_3OH:H_2O (70:30) with 0.1% formic acid, and the mass range was set to optimally pass ions from m/z 150-700. A spatial resolution range of 200-300 µm was used for the imaging experiments. Thermo Xcalibur 2.1 software was used to run the queues for mass spectrometry imaging, as well as, to examine the raw data. The raw files were converted to image files via Thermo FireFly 2.1. The image files were opened with BioMAP 3 software and used to create the MS images. Adobe Photoshop CS6 version 13.0 x32 was used to superimpose the MS images over the pictures of the fungal cultures.

DESI inoculation methodology

Fungal cultures were inoculated initially on potato dextrose nutrient agar (PDA, Difco) in 50 × 9 mm sterile Petri dishes to provide stock cultures for subsequent experiments. After 7–10 d of growth, the test fungus was grown on an autoclaved insert. Inserts used included cheesecloth, filter paper, cardboard, and balsa wood, all of which were sterilized via autoclaving. Two methods for inoculating an insert were used. First, a small piece of agar with the fungus was 2-

point inoculated on a small segment of sterile insert (40 mm × 20 mm) placed on a newly prepared Petri dish with PDA media. In a second method, the fungus was grown in liquid media containing 2% soy peptone, 2% dextrose, and 1% yeast extract (YESD media) with a segment of sterile insert and incubated for (7–10 d) at 22 °C with agitation (i.e. shaking at 100 rpms). After 7–10 d, the segment of insert with fungal colony growing on its surface was aseptically transferred onto a Petri dish with PDA media; only cardboard and balsa wood were tested via the second method. Of the explored options for DESI-MS analysis, the first method, using non-corrugated cardboard (40 mm × 20 mm × 1 mm, Staples, item # 521401; Fig. S6) yielded the best surface.

Sample preparation for imaging experiments

The samples grown on inserts (i.e. cardboard and wood) required very little additional preparation (Fig. 5, top). Double-sided tape was placed on a microscope slide. While working in a laminar flow hood, the cardboard/wood was removed from the Petri dish using forceps and placed onto the taped area with minimal pressure, so as to not compromise the surface. In some cases, the inoculant was too elevated relative to the fungal growth, and therefore, was carefully removed using forceps to provide a more even, flat surface. The microscope slides were then placed directly onto the DESI platform for analysis. This method was ideal for analysing metabolites on the surface of the cultures

To analyse the internal metabolites, fungal cultures were grown directly on media (Fig. 5, bottom). Circular sections were made using a 5 mm hole-puncher and placed into a separate, empty plastic Petri dish. Liquid nitrogen was then poured into the Petri dish to flash freeze the sections. Using forceps, the pieces were removed and placed on an unaltered microscope slide. The slide was then placed under vacuum in a desiccator and left overnight. The resulting samples were shrivelled, relatively flat, and stuck to the slide. Finally, the slide was then removed from the desiccator and placed onto the DESI platform for analysis.

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Notes and references

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Electronic Supplementary Information (ESI) available: ^{1}H and ^{13}C NMR data for compounds **1** and **2**, photographs of some of the challenges that were observed with the inserts, and photographs of the cardboard insert. See DOI: 10.1039/b000000x/

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