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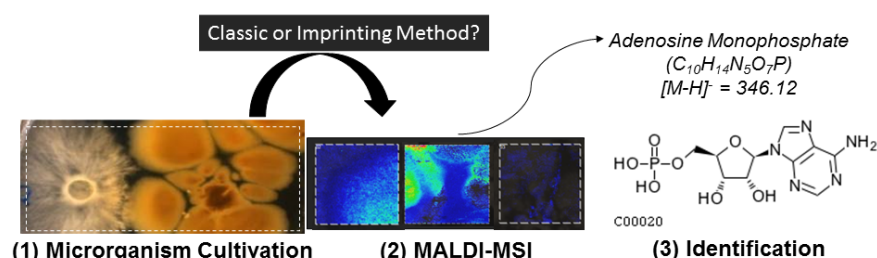
Mass Spectrometry Imaging for Fungal Interaction Analysis: Classic *versus* Imprinting Methods

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Classic or Imprinting Method?

Adenosine Monophosphate
$(C_{10}H_{14}N_5O_7P)$
$[M-H]^- = 346.12$

C00020

(1) Microrganism Cultivation   (2) MALDI-MSI   (3) Identification

Fungi can produce many bioactive metabolites, which are enhanced when challenged in co-culture competition. For a better evaluation of these metabolites, Mass Spectrometry Imaging (MSI) can be used to provide complementary information about the metabolite spatial localization. However, some adaptations are required on available methodologies in MSI for applications in microorganisms, particularly on sample preparation, due to the characteristics of each type of cell that has to be analyzed. The imprinting method has been shown to be a robust method when applied to sample preparation, but to our knowledge it has never been tested for microbial MALDI-MSI. Herein we applied both Classic and Imprinting MALDI-MSI to compare and analyze metabolites produced by *Aspergillus terreus* (ATCC® 20542™) and *Pleurotus pulmonarius* fungi. For the classic method, the fungi were inoculated for 8 days with PDA medium in a MALDI glass slide. For the imprinting method, fungi were also inoculated for 8 days in a MALDI glass slide and then transferred to a filter paper by manual pressure using a homemade apparatus. Samples were then dehydrated and submitted to HCCA matrix application by sublimation. The chemical images were acquired by MALDI-MSI, and the metabolites were identified by UHPLC-ESI-MS/MS. Twelve ions were detected by MALDI-MSI, using classic (*m/z* 210.54, 276.99, 307.45, 321.04, 329.70, 346.12, 351.12, 462.41 and 484.02) and imprinting (*m/z* 313.64, 379.66 and 404.36) methods. Some ions presented a higher intensity in the interaction zone between fungi areas, especially the ions *m/z* 329.70, 351.12 and 484.02. These ions may be related to metabolites involved in communication between microorganisms, because these fungi formed a mutualistic interaction. All ions were investigated by UHPLC-ESI-MS/MS, and two were identified: adenosine monophosphate ($C_{10}H_{14}N_5O_7P$, m/z 346.12, $[M-H]^-$) visualized in the Classic Method, and rubrophen ($C_{22}H_{20}O_6$, m/z 379.66, $[M-H]^-$) visualized in the Imprinting Method. The metabolites from microorganisms are rarely reported in MS/MS databases, which explains the difficulty in the identification of these compounds. Our MSI analysis using the classic method provided a higher number of detected ions. However, both classic and imprinting methods resulted in a complementary information,

leading to the detection of ions that were not previously observed on the classic approach. Despite of the challenges encountered on the sample preparation and metabolite identification, using both classic and imprinting MALDI-MSI for bioprospection of fungi metabolites is a promising approach on the analytical field of mass spectrometry which can be later used in biotechnological applications.

Keywords: MALDI-MSI, *Aspergillus terreus*, *Pleurotus pulmonarius*, metabolomics, biotechnology

INTRODUCTION

Microorganisms naturally grow in complex environments, full of inter- and intra-species interactions. These interactions stimulate the production of metabolites and can have positive or negative effects in the environment. The positive effect, also called mutualistic or neutralist, assist, for example, in communication with other microorganisms. On the other hand, the negative effect, also called antagonistic, can promote the production of antibiotics and organic acids, for example, as a defense mechanism.[1] Both cases are interesting for the discovery of new metabolites or more potent analogues destined for industrial applications.[2] Different types of chemical compounds can be obtained through microbial interaction, such as antibiotics,[3] enzymes,[4] and hormones[5].

Filamentous fungi are known to produce many metabolites when they interact with microorganisms from another kingdom (*Aspergillus fumigatus* and *Streptomyces peucetius*), another genus (*Penicillium pinophilum* and *Trichoderma harzianum*) or species *(Fusarium tricinctum* and *Fusarium begoniae*).[3] These interactions are carried out through co-culture or pairing experiments (enriched agar culture), which consists on the cultivation of one or more microorganisms in the same environment.[1] This competitive environment often challenges microorganisms by stimulating the production of new metabolites.[2] Furthermore, the morphological visualization of the fungi-fungi interaction is classified into four types, namely: inhibition by contact, inhibition by distance, confrontation zone and overlap.[6] Despite the ease of classifying these types of interactions through visible morphological changes (colors, mycelial mass shapes, among others), it is necessary to use more robust analytical tools for a more reliable chemical elucidation of the compounds produced, to complement the information through the spatial visualization which can be vital in order to determine the biological role of a metabolite or group of metabolites.

In this sense, mass spectrometry imaging (MSI) can be a powerful analytical tool. MSI relates the ions detected in the sample (mass-charge ratio, *m/z*) point-by-point, expressing the result through a chemical image, where it has the distribution of ions/metabolites present in the sample.[7] This technique is widely used in the medical field for the analysis of tissue and tumor cells and has recently been expanded to applications in the biotechnology area, analyzing bacteria and fungi.[8-11]

The classic method for analyzing microorganisms by MALDI-MSI involves the cultivation of the microorganism, followed by the removal of the culture medium containing the metabolites produced by them, insertion of this part in the MALDI glass, dehydration of the sample, application of the matrix, acquisition, and interpretation of data.[9,10] However, some adaptations are required on available methodologies in MSI for applications in microorganisms, particularly on sample preparation, to ensure a homogeneous surface for success of MALDI-MSI analysis.[9] The imprinting method has been shown to be a robust method when applied to preparation of samples (plants and microorganisms) analyzed by DESI-MSI and in mammalian tissue sections by MALDI-MSI.[8,12,13] This method allows the sample surface to be as homogeneous as possible, which can facilitate the MALDI ionization process, which requires regular surfaces for the technique to be successful. Furthermore, this surface regularization by imprinting can allow MS (mass spectrometry) ionization methods to access internal parts of the analyzed samples.[8,12,13] But, to our knowledge, the imprinting method has never been tested for MALDI-MSI for microorganisms.

Thus, the objective of this study was to evaluate the interactions between the fungi *Aspergillus terreus* (ATCC® 20542™) and *Pleurotus pulmonarius* (BRM 055674) through mass spectrometry imaging (MSI), comparing the classic and imprinting methods to fungal analysis, and adding information about the visible morphological.

MATERIALS AND METHODS

The fungi used in the work were *Aspergillus terreus* (ATCC® 20542™) and *Pleurotus pulmonarius* (BRM 055674 – belonging to the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries, CMMAABio). Co-cultivation was carried out in petri dishes containing MALDI-MSI (indium tin oxide coating glass slides, Bruker Daltonics) slides and PDA (potato-dextrose agar) culture medium. The fungi were then kept for eight days at 28 °C in a B.O.D. incubator, and then the samples were prepared for analysis by MALDI-MSI.

The sample preparation consisted of removing the MALDI-MSI slides from the petri dishes, containing PDA medium and the cultivated fungi, being later submitted to a vacuum desiccator, until complete dehydration, and the application of the HCCA matrix (α-cyano acid-4-hydroxycinnamic) by sublimation. The slides containing the samples were then inserted into the MTP Slide Adapter II plate (Bruker Daltonics) and chemical images were acquired by MALDI-MSI (UltrafleXtreme, Bruker Daltonics).

MSI data were obtained using the flexControl software version 4.0 (Bruker Daltonics), in negative ionization mode, mass range between *m/z* 500-1500, with laser in ultra-mode (120 µm aperture) and optimized for 90% intensity, gain of detector 10x (2550 V), 1000 shots, 2000 Hz, lateral width (rather width) of 120 µm and with partial random walk through the samples at the time of acquisition. The image data were obtained and processed using the flexImaging software, version 4.0 (Bruker Daltonics, Germany), assigning a gradient color scale, a 1 Da mass filter and RMS (Root Mean Square) normalization.

Extraction of metabolites was performed with methanol and chemical analysis was performed by UHPLC-ESI-MS/MS for high resolution identification (Prominence LC30AD, Shimadzu and Maxis 4G, Bruker Daltonics). The fragmentation patterns obtained were compared with the MetFrag database.[14] The sequential described steps of the methods are resumed in Figure 1.
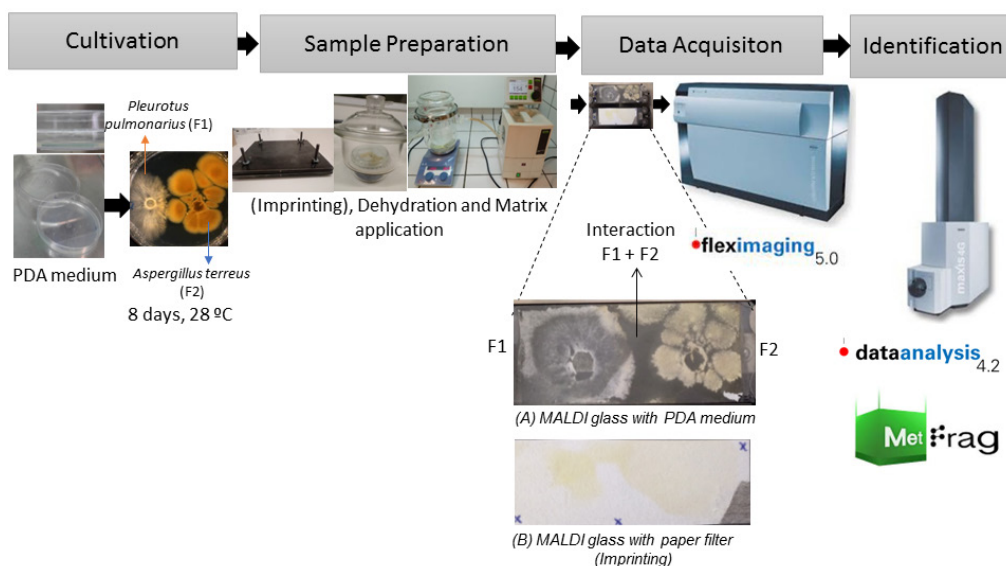

Figure 1. Scheme of the experimental procedure followed for MALDI-MSI analysis of the fungi *P. pulmonarius* (BRM 055674) and *A. terreus* (ATCC® 20542™) using (A) classic and (B) imprinting methods to sample preparation.

RESULTS AND DISCUSSION

Initially, three regions of the same slide were analyzed: co-culture interaction between the two fungi, *P. pulmonarius* and *A. terreus* individually mycelium sides (no interaction zone). Also, in the same slide, it was analyzed a PDA medium without mycelium for a blank analysis. This ensured that all regions were subjected to the same sample preparation (dehydration and matrix application) and MALDI-MSI chemical imaging.

Nine ions were detected by classic method of MALDI-MSI in the co-culture zone, namely: *m/z* 210.54, 276.99, 307.45, 321.04, 329.70, 346.12, 351.12, 462.41 and 484.02 (Figure 02). It is also noted that in the sample blank there were no chemical images related to those nine ions, which indicates that the production of these ions was induced by the co-culture of the two fungi.
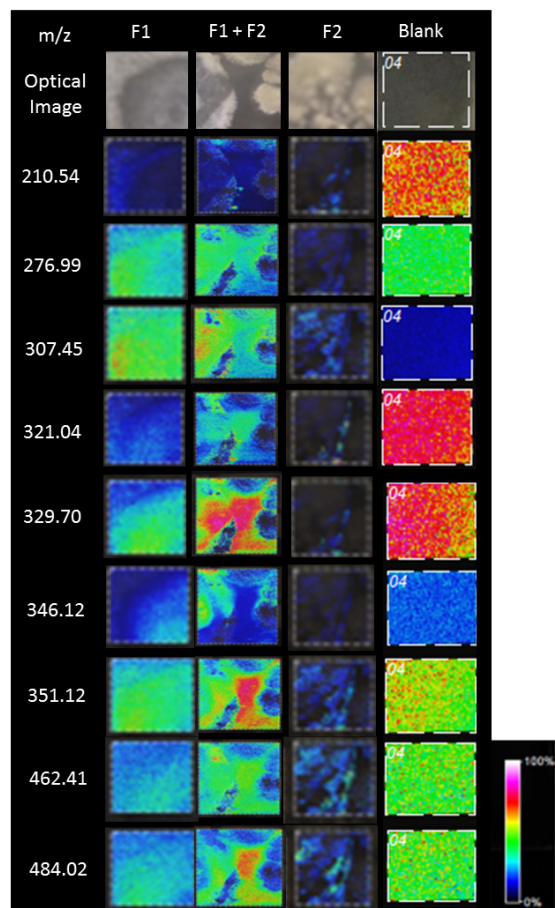

Figure 2. MALDI(-)-MSI applying Classic Method to analysis of metabolites produced by the fungi *Pleurotus pulmonarius* (F1) and *Aspergillus terreus* (F2) individually and in the co-culture zone (F1+F2, interaction), in PDA medium with HCCA matrix applied by sublimation (blank).

The *m/z* ions 329.70, 351.12 and 484.02 were more abundant and had a clear chemical image, in relation to the other ions detected (Figure 2). They are probably related to metabolites involved in the interaction between the microorganisms studied. There are four types of morphological interactions defined according to number of induced metabolites at interaction zones and morphological changes by the naked eye. These interactions are distance-inhibition, zone-line, contact-inhibition, and overgrowth. The first two types of interaction (distance-inhibition, and zone-line) can be subdivided as a negative effect because the growth of one microorganism clearly hinders the development of the other microorganism, with a separation of colonies by looking at the naked eye. While the other two interactions (contact-inhibition, and overgrowth) are classified as a positive effect, because despite being in the same medium, and consuming the same nutrient and space, this competition seems to be beneficial, not distancing the colonies and not leading them to produce defense metabolites.[6]

Analyzing the visible morphological interaction between *Aspergillus terreus* and *Pleurotus pulmonarius* fungi (Figure 3), there is a zone of direct contact between them, evidencing the interaction of the contact inhibition type. This shows that, although there may be competition for nutrients and space in the environment, fungi can interact beneficially (positive effect), without a zone-line or distance-inhibition, as occurs in interactions with a negative effect.
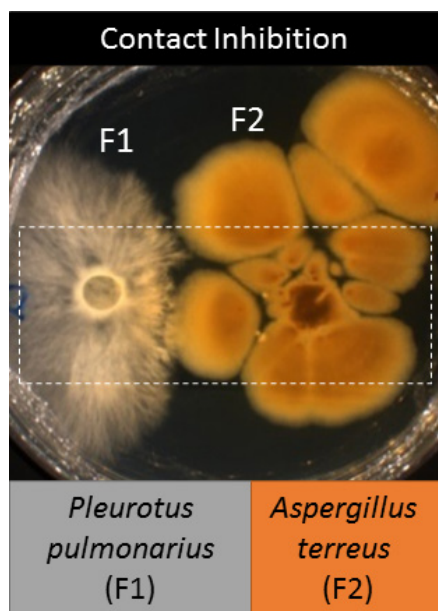
Figure 3. Interaction of *Pleurotus pulmonarius* (F1) and *Aspergillus terreus* (F2) analyzing visible morphological. The rectangle indicates the region of the MALDI slide used for the acquisition of the chemical imaging.

Using the imprinting method, three ions were detected by MALDI-MSI: *m/z* 313.64, 379.66 and 404.36 (Figure 4). In this case, no ions were identified by UHPLC-MS/MS. Despite not detecting as many ions as the classic method (which detected nine ions), it was noted that the use of imprinting, extracting the metabolites from the culture medium onto the filter paper, allowed a visible smoother surface during sample preparation.
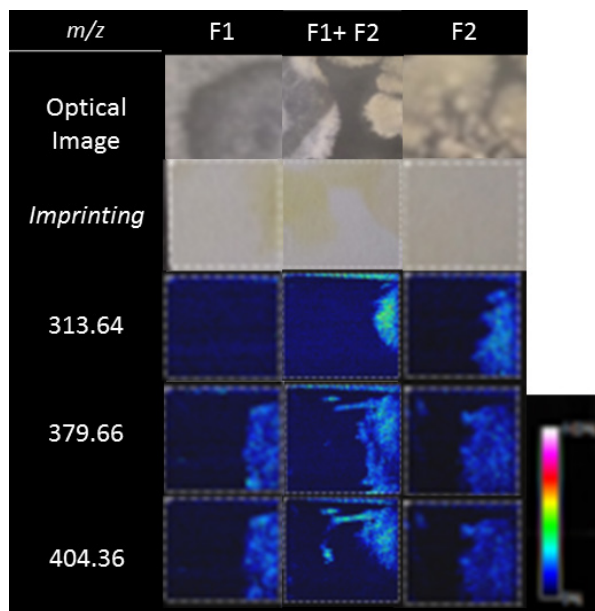

Figure 4. MALDI(-)-MSI applying Imprinting Method to analysis of metabolites produced by the fungi *Pleurotus pulmonarius* (F1) and *Aspergillus terreus* (F2) individually and in the co-culture zone (F1+F2, interaction) in paper filter (imprinting, in third line).

The ions detected in the interaction zone differ in the two approaches probably because of each methods specificity. The classic method allows direct analysis in PDA medium, while imprinting method allows indirect analysis in paper filter, but it can access intern parts. So, it was expected to detect different ions in each approach used. Thus, the two approaches can be used in combination for more external and internal analysis of the sample using the classic and imprinting methods, respectively.

After UHPLC-ESI-MS/MS analysis, the ion *m/z* 346.12, visualized in the Classic Method (Figure 2), was identified in deprotonated form [M-H]⁻ as adenosine monophosphate ($C_{10}H_{14}N_5O_7P$), which corresponds to a primary metabolite related to the develop of the microorganism. The ion *m/z* 379.66, visualized in the Imprinting Method (Figure 4), was identified, and assigned as rubrophen ($C_{22}H_{20}O_6$), also in the deprotonated form [M-H]⁻. This metabolite corresponds to an organic aromatic compound. Identified compounds structures by UHPLC-MS/MS visualized in the MALDI-MSI experiments, and application of these metabolites are shown in Figure 5.
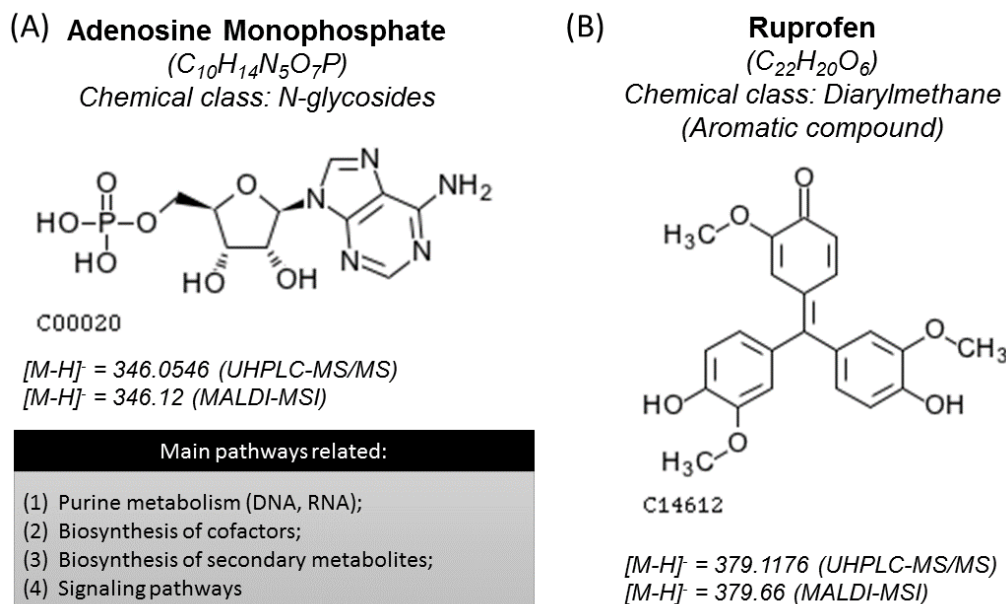

(A) Adenosine Monophosphate
($C_{10}H_{14}N_5O_7P$)
Chemical class: N-glycosides

C00020

[M-H]⁻ = 346.0546 (UHPLC-MS/MS)
[M-H]⁻ = 346.12 (MALDI-MSI)

Main pathways related:
(1) Purine metabolism (DNA, RNA);
(2) Biosynthesis of cofactors;
(3) Biosynthesis of secondary metabolites;
(4) Signaling pathways

(B) Ruprofen
($C_{22}H_{20}O_6$)
Chemical class: Diarylmethane
(Aromatic compound)

C14612

[M-H]⁻ = 379.1176 (UHPLC-MS/MS)
[M-H]⁻ = 379.66 (MALDI-MSI)

Figure 5. Identified compounds' structures by UHPLC-MS/MS, based on MetFrag[14] and KEGG[15] Databases, visualized in the (A) Classic, and (B) Imprinting Methods.

Other methods of metabolite extraction and analysis by UHPLC-MS/MS can be explored in future experiments, to identify a greater number of compounds. It is also important to highlight that those microbial metabolites are produced under specific cultivation conditions and are rarely reported in MS/MS databases, which explains the difficulty in identifying these compounds when searching traditional databases. Some research shows efforts to create platforms that gather information from several databases of microbial metabolites in The Natural Products Atlas (www.npatlas.org) to accelerate and facilitate the process of molecular identification and structural elucidation.[16] However, the participation of the scientific community is widely required to feed these data into the platform, or even the creation of their own database to drive the identification of microbial compounds and their subsequent application.

MSI results confirm and reinforce the importance of the method development in the analysis of microorganisms due to the high resolution of the images obtained. Therefore, our work showed that this field of microorganism metabolomics is highly promising due to the result presented, where several ions were detected with different spatial distributions, which can be better investigated based on their biological function.

CONCLUSIONS
The information on the ions detected by MALDI-MSI in the interaction zone of the studied fungi corroborates the information visualized in the interaction during growth. Both classic and imprinting methods used to MALDI-MSI provided complementary information about fungi interactions, detecting different

ions. The traditional method had a higher number of detected ions when compared to the imprinting protocol (nine and three, respectively). Moreover, the efforts toward entering data into UHPLC-ESI-MS/MS microorganism databases are important to easily identify compounds of fungi origin, especially when exploring internal metabolites analyzed by MS imaging, which is now possible with the use of imprinting, as described in this study. Despite the challenges encountered in identifying metabolites, the use of MALDI-MSI for chemical visualization of metabolites from fungal interactions is a promising approach for biotechnological applications. It is also noteworthy that the work is innovative due to few studies in the field of chemical imaging by mass spectrometry of fungi.

Conflicts of interest
There are no conflicts to declare.

Acknowledgements

REFERENCES
(1) Martínez-Buitrago, P. A.; Ramos, F. A.; Castellanos, L. Binary co-culture selection from marine-derived microorganisms for differential production of specialized metabolites. *Quim. Nova* **2019**, *42*, 713-719. https://doi.org/10.21577/0100-4042.20170388

(2) Netzker, T.; Fischer, J.; Weber, J.; Mattern, D. J.; König, C. C.; Valiante, V.; Schroeckh, V.; Brakhage, A. A. Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Front. Microb.* **2015**, *6*, 299. https://doi.org/10.3389/fmicb.2015.00299

(3) Qian, X.; Chen, L.; Sui, Y.; Chen, C.; Zhang, W.; Zhou, J.; Dong, W.; Jiang, M.; Xin, F.; Ochsenreither, K. Biotechnological potential and applications of microbial consortia. *Biotec. Adv. 2020*, *40*, 107500. https://doi.org/10.1016/j.biotechadv.2019.107500

(4) Sperandio, G. B.; Ferreira Filho, E. X. Fungal co-cultures in the lignocellulosic biorefinery context: a review. *Int. Biod. & Biodeg*. **2019**, *142*, 109-123. https://doi.org/10.1016/j.ibiod.2019.05.014

(5) Bertrand, S.; Bohni, N.; Schnee, S.; Schumpp, O.; Gindro, K.; Wolfender, J-L. Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. *Biotec. Adv*. **2014**, *32* (6), 1180-1204. https://doi.org/10.1016/j.biotechadv.2014.03.001

(6) Bertrand, S.; Schumpp, O.; Bohni, N.; Bujard, A.; Azzollini, A.; Monod, M.; Gindro, K.; Wolfender, J-L. 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. *J. Chromatogr. A* **2013**, *1292*, 219-228. https://doi.org/10.1016/j.chroma.2013.01.098

(7) Dunham, S. J.; Ellis, J. F.; Li, B.; Sweedler, J. V. Mass spectrometry imaging of complex microbial communities. *Acc. Chem. Res*. **2017**, *50* (1), 96-104. https://doi.org/10.1021/acs.accounts.6b00503

(8) Caprioli, R. M.; Farmer, T. B.; Gile, J. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal. Chem*. **1997**, *69* (23), 4751-4760. https://doi.org/10.1021/ac970888i

(9) Yang, J. Y.; Phelan, V. V.; Simkovsky, R.; Watrous, J. D.; Traial, R. M.; Fleming, T. C.; Wenter, R.; Moore, B. S.; Golden, S. S.; Pogliano, K.; Dorrestein, P. C. Primer on agar-based microbial imaging mass spectrometry. *J. Bacter*. **2012**, *194* (22), 6023-6028. https://doi.org/10.1128/JB.00823-12

(10) Araújo, F. D. D. S.; Araújo, W. L.; Eberlin, M. N. Potential of *Burkholderia seminalis* TC3. 4.2 R3 as biocontrol agent against *Fusarium oxysporum* evaluated by mass spectrometry imaging. *J. Am. Soc. Mass Spectrom*. **2017**, *28* (5), 901-907. https://doi.org/10.1007/s13361-017-1610-6

(11) Liu, R.; Khan, R. A. A.; Yue, Q.; Jiao, Y.; Yang, Y.; Li, Y.; Xie, B. Discovery of a new antifungal lipopeptaibol from *Purpureocillium lilacinum* using MALDI-TOF-IMS. *Biochem. Biophys. Res. Com*. **2020**, *527* (3), 689-695. https://doi.org/10.1016/j.bbrc.2020.05.021

(12) Thunig, J.; Hansen, S. H.; Janfelt, C. Analysis of secondary plant metabolites by indirect desorption electrospray ionization imaging mass spectrometry. *Anal. Chem.*, **2011**, *83* (9), 3256-3259. https://doi.org/10.1021/ac2004967

(13) Müller, T.; Oradu, S.; Ifa, D. R.; Cooks, R. G.; Kräutler, B. Direct plant tissue analysis and imprint imaging by desorption electrospray ionization mass spectrometry. *Anal. Chem.* 2**011**, *83* (14), 5754-5761. https://doi.org/10.1021/ac201123t

(14) Ruttkies, C.; Schymanski, E. L.; Wolf, S.; Hollender, J.; Neumann, S. MetFrag relaunched: incorporating strategies beyond in silico fragmentation. *J. Cheminf.* **2016**, *8* (1), 1-16. https://doi.org/10.1186/s13321-016-0115-9

(15) Kanehisa, M.; Araki, M.; Goto, S.; Hattori, M.; Hirakawa, M.; Itoh, M.; Katayama, T.; Kawashima, S.; Okuda, S.; Tokimatsu, T.; Yamanishi, Y. KEGG for linking genomes to life and the environment. *Nucleic Acids Res*. **2007**, *36* (1), D480-D484. https://doi.org/10.1093/nar/gkm882

(16) Van Santen, J. A.; Jacob, G.; Singh, A. L.; Aniebok, V.; Balunas, M. J.; Bunsko, D.; ... Linington, R. G. The natural products atlas: an open access knowledge base for microbial natural products discovery. *ACS Central Sci.*, **2019**, *5* (11), 1824-1833. https://doi.org/10.1021/acscentsci.9b00806