Chapter 6
Metabolomics and Secondary Metabolite Profiling of Filamentous Fungi

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Introduction

All fungi are heterotrophic organisms, and thus they depend on organic carbon. According to their nutritional needs, fungi can be found in intimate association with major natural carbon sources, i.e., plants, animals, and microbes. Therefore, it is not surprising that this huge group of eukaryotic organisms with an estimated number of 1.5 million species does occupy a myriad of ecological niches. Based on their life and nutritional styles, fungi can roughly be divided into saprotrophs, living on dead organic material (carbon recycling in the natural environment); biotrophs, which use nutrients from the living host-(cells); and necrotrophs, which first kill and then feed on dead host tissue [1].

The various ecological niches populated by filamentous fungi and different lifestyles are reflected by their capability to produce a great number of so-called secondary metabolites. The differentiation between basic and secondary metabolism had originally been suggested by Albrecht Koessel in 1891 [2]. Although a matter of debate since then, the classification in primary (nowadays also called central) and secondary metabolism/metabolites is still used today. According to the current concept, primary metabolism refers to the basic anabolic and catabolic processes required for respiration, nutrient assimilation, and growth, thus primary metabolites mainly comprise sugars, amino acids, fatty acids, and nucleosides, which form the

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building blocks of polysaccharides, proteins, lipids, and nucleic acids. Primary metabolites are needed for the basic functioning of life and hence are produced by every cell of a particular organism. Secondary metabolites are defined as all metabolites other than primary. Thus, fungal secondary metabolites are not essential for the regular growth and function in pure culture, for example, but their formation by the fungus is often restricted to specific parts of the life cycle, a specific physiological/developmental state or certain environmental conditions [3, 4]. Moreover, fungal secondary metabolites generally occur as families of related compounds that are only produced by a limited number of species or even strains [3, 5, 6]. Since fungal secondary metabolites show highly diverse and frequently complicated chemical structures (mainly polyketides, terpenoids, and nonribosomal peptides as well as mixed structures thereof [3]), their analytical determination, isolation, and detailed structure elucidation have remained a great technical challenge.

For a long time, the screening for secondary metabolites has almost exclusively been motivated by the search for novel bioactive ingredients of drugs, while the study of their biological function has largely been neglected [5]. Consequently, the role of most of these compounds remains largely unclear although it is generally acknowledged that fungi employ these secondary metabolites for intra- and interspecies communication and various interactions with their competitors and hosts.

Mainly driven by significant technical developments in analytical instrumentation and computing power as well as novel biological insights, a change in paradigm from reductionist to holistic approaches for the study of filamentous fungi can be observed currently. This development is reflected by the emergence of metabolomics as the latest of the so called -omics disciplines.

Metabolomics of Fungi—An Overview

The General Concept of Metabolomics

Similar to all biological low-molecular-weight molecules, fungal metabolites can be regarded as intermediates and end products of physiological regulatory processes. Their presence can be viewed as the ultimate response of a cell to genetic and environmental variations [7]. In analogy to genomics and other "-omics" disciplines, the total complement of all metabolites present in an organism like, for example, a filamentous fungus, is called the metabolome. The corresponding scientific discipline trying to determine the entirety of all low-molecular-weight metabolites is called metabolomics [8]. Thus, by definition, novel "holistic approaches" such as metabolomics aim at the comprehension of whole biological systems—looking at the biochemical changes taking place in living cells during metabolism. The general goal of most metabolomics studies is to generate a snapshot of the metabolic state of a biological sample and to characterize the changes in the abundances of the measured metabolites arising from natural fluctuations or external, experimental biotic or abiotic perturbations [9]. Due to this generic analytical concept, many different biological systems including filamentous fungi have been studied in various

metabolomics experiments with the aim to investigate various types of scientific questions such as the use of metabolite profiles for chemotaxonomy, biomarker and drug discovery, food safety, or host–fungus interactions (see later in this chapter).

Independent of the scientific question to be studied, most state-of-the-art metabolomics studies share a common workflow (see Fig. 6.1). Typically, a metabolomics study starts with a clear definition of the research question and design of the

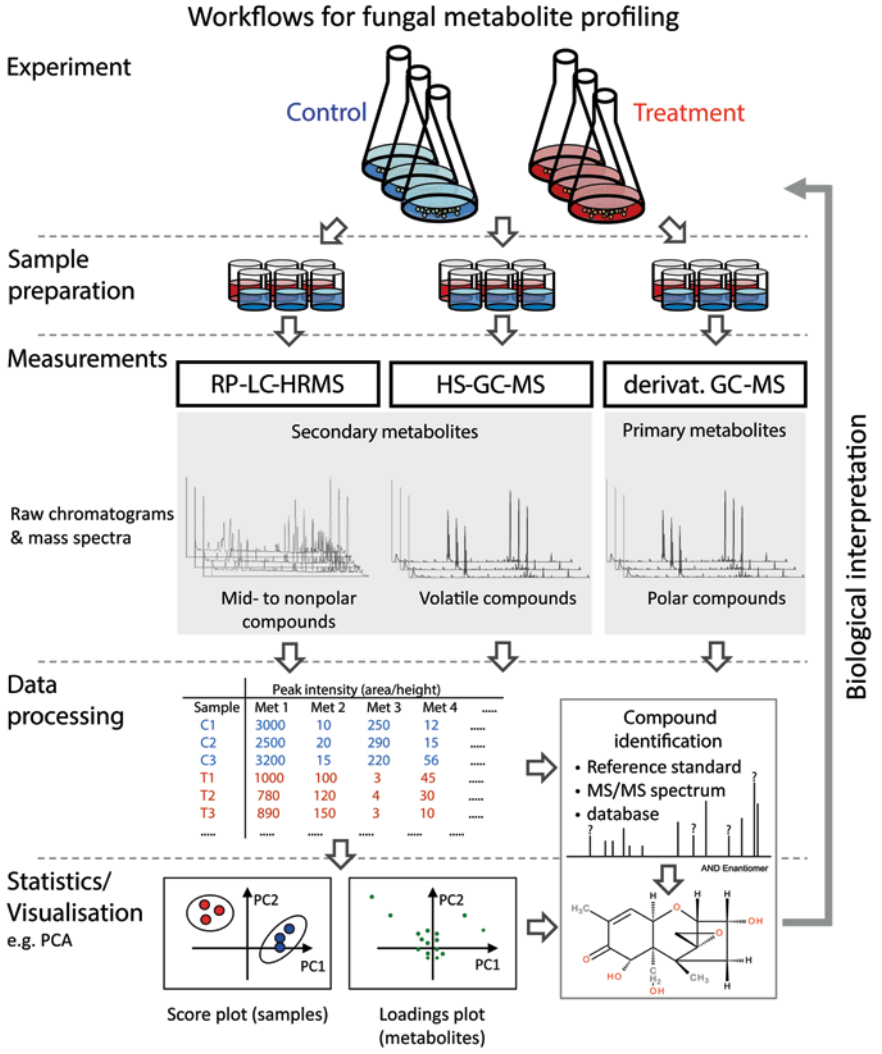


Fig. 6.1 Schematic overview of workflows used for metabolite profiling of filamentous fungi. For secondary metabolites, either reversed phase-liquid chromatography-high-resolution mass spectrometry (RP-LC-HRMS) for mid and nonpolar compounds or headspace-gas chromatography-mass spectrometry (HS-GC-MS) for volatile compounds are employed. Primary metabolites are analyzed after derivatization by GC-MS instrumentation

biological experiment. Separate experimental sample groups representing different experimental conditions (e.g., “control versus treatment” or “wild type versus mutant”) are cultured in parallel and, after defined time periods, samples are taken according to the experimental scheme and stored until further analysis. While the sampling step requires immediate quenching of all metabolic processes without alteration of the metabolic state, the storage conditions shall also conserve metabolite levels without changing the biochemical composition of the biological samples. Subsequently, the samples have to be extracted and prepared for comprehensive analysis, typically by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) enabling the detection of a great number of metabolites simultaneously [10, 11].

Sample measurements with modern analytical instrumentation result in huge amounts of raw data that cannot be evaluated manually anymore. Instead, stepwise treatment of raw data with different methods and automated software tools is required and often comprises the bottleneck of metabolomics workflows. Data handling can be roughly divided in data processing and statistical analysis and has been summarized in several reviews (e.g., [12–14]). Data-processing steps include numerous tasks such as noise and background filtering, feature extraction, spectrum deconvolution (i.e., grouping of peaks that originate from the same metabolite), alignment of chromatograms, and internal standardization. Data processing finally results in the so-called data matrix, a simple-structured table that contains all samples and the abundances of the detected analytical features and is used for further data analysis. Once the data matrix has been prepared, data can be plotted and uni- and multivariate statistics can be carried out with the aim to further reduce data complexity and visualize metabolites, significantly differing between sample groups. The ultimate goal of every metabolomics study is to link the differentially expressed metabolites to the experimental factor, which had been varied to generate the different sample states. Thus, reliable annotation/identification of the detected metabolites is essential for a meaningful biological interpretation of the analytical results.

Analytical Approaches in Metabolomics

The metabolome of any fungus under investigation is highly complex and consists of a multitude of different primary and secondary metabolites. Due to the diverse chemical structures of the metabolites and their occurrence at a wide dynamic range from pico- to millimolar levels, global metabolome analysis cannot be achieved by a single analytical platform. Traditional analytical methods with a low number of predefined target analytes, for which authentic reference standards are available and thus allow absolute quantification, are not feasible in the field of metabolomics. Instead complementary system-wide approaches are applied with the aim to cover as many biochemical compounds as possible (see Fig. 6.1). They usually allow for comparative quantification of (partly) identified metabolites in different biological samples. The different analytical approaches used in metabolomics have been summarized and categorized in numerous well-received general (e.g., [15–17]) as well as microbe-focused review articles (e.g., [18–22]).

Metabolite profiling is one of the strategies applied in metabolomics and can be defined empirically as the (semi)quantitative analysis of a set of metabolites or derivative products (identified or unknown) of a sample, using a particular two-dimensional analytical technique. Most current state-of-the-art metabolomics studies use liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) for metabolite profiling. LC-MS and GC-MS combine high sensitivity and detector linearity over 3–4 orders of magnitude with excellent selectivity (physical metabolite separation by chromatography and separation of co-eluting analytes according to *m/z* ratio of intact ionized molecule- or metabolite-specific fragment ions) and thus allow to determine hundreds of metabolites in a single analytical run. Other techniques such as liquid chromatography-ultraviolet (LC-UV) are used complementary for metabolite profiling (e.g., [23, 24]).

In addition, direct infusion MS, nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy are applied for *metabolite fingerprinting* (sometimes also called as metabolic fingerprinting) without prior metabolite separation. Fingerprinting techniques are mainly used for rapid sample classification without identification of individual metabolites. Chromatography-mass spectrometry based profiling methods result in a three-dimensional data structure (retention time, *m/z* value, and intensity). In contrast, fingerprinting approaches such as direct infusion MS lack the chromatographic separation step and therefore lead to two-dimensional data (*m/z* value and intensity) per sample (e.g., [15, 18]).

It should be noted that the term metabolite fingerprinting has also been used to describe the use of analytical methodologies (e.g., metabolite profiling) for the measurement of intracellular metabolites of a biological system in order to distinguish from the analysis of extracellular metabolites, for which the term metabolic footprinting had been introduced [18, 25, 26]. For the sake of convenience in this chapter, the terms metabolite profiling and metabolite fingerprinting will be used according to Fiehn [15], Goodacre et al. [16], and Dettmer [17].

Independent of the employed analytical technique, two complementary approaches are widely used for metabolite profiling: targeted and untargeted analyses. In the targeted approach, a set of predefined known substances is monitored, which usually allows absolute quantification and definite identification when limited to metabolites available as authentic reference standards. In contrast, untargeted profiling methods try to find analytical features of all detectable compounds and therefore show the potential of probing the entire metabolic space, including substances that are currently unknown (or at least unidentified) at the time of measurement. Thus, untargeted approaches are suitable to detect changes in unexpected parts of the metabolome and frequently lead to new scientific hypotheses.

Annotation and Identification of Metabolites

As already mentioned, meaningful biological conclusions can only been drawn from an (untargeted) metabolomics experiment if the detected and significantly differing analytical features can be reliably assigned to biological molecules. To annotate a compound's chemical structure, NMR is typically the technique of choice. However,

NMR is less sensitive compared to MS. Thus, for detailed chemical structure determination of non-predicted substances in biological samples by NMR techniques, the compounds of interest frequently need to be concentrated and isolated before the actual NMR measurements can be performed. Moreover, when analyzing complex mixtures, especially signals of less-abundant compounds can easily overlap with other signals producing complex spectra that are difficult to interpret [27]. Complementary to this, with LC-HRMS—the most frequently used technique for untargeted metabolomics experiments—compound annotation usually starts with the prediction of molecular formulas by matching accurately measured masses against chemical substance databases. In this respect, well-known databases are ChEBI (http://www.ebi.ac.uk/chebi/) [28], PubChem (http://www.ncbi.nlm.nih.gov/pccompound) [29], or AntiBase 2012 [30], with the latter currently containing more than 40,000 microbial metabolites with 15,220 entries assigned to the "Source [F] = fungus." While knowledge of the molecular formula can lead to putative compound annotation, the elucidation of chemical structures is further complicated by the fact that many structural isomers correspond to a single molecular formula. Consequently, according to the current state of the art, definitive substance identification by LC-HRMS can only be achieved by comparing two or more orthogonal properties such as retention time, accurate mass, or LC-MS/MS spectra with those obtained from an authentic reference standard under identical measurement conditions [31, 32]. The fact that many putatively identified compounds are not available as authentic standards poses a major limitation of current untargeted LC-HRMS-based metabolomics approaches. Another issue is lack of comprehensive LC-MS/MS spectrum databases.

Compared to LC-MS/MS, putative metabolite identification by GC-MS is generally more straightforward. Efficient software tools for chromatographic feature detection, deconvolution (i.e., "purification") of mass spectra, and calculation of retention indices are available and can freely be downloaded from the Internet (e.g., AMDIS, http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis; Metabolite Detector, http://md.tu-bs.de/ [33, 34]). Compared to liquid chromatography tandem mass spectrometry (LC-MS/MS) , compound and structure annotation is largely facilitated by the use of standardized ionization conditions in the electron ionization (EI) source of the MS instrument (typically, 70 eV is used across different instruments and laboratories), which leads to reproducible and information-rich (i.e., fragment ion) mass spectra for a certain compound. Comprehensive MS libraries—such as, for example, the current Wiley Registry 10th Edition/NIST 2012 EI Mass Spectral Library [35] with 870,000 mass spectra—are available for similarity-based spectrum matching. Annotation of the detected compounds is usually achieved by comparison of experimental retention indices and mass spectra with those of library entries. Similar to LC-MS/MS approaches, definitive confirmation of the identity relies on the measurement of authentic standards in parallel [36–38].

This chapter will focus on the two techniques most frequently used in metabolomics experiments dealing with filamentous fungi: (1) GC-MS, which allows the study of both volatiles and -after sample derivatization- polar small metabolites; and (2) LC-MS, which is generally applied in reversed phase mode for the study of mid- to non-polar secondary metabolites.

Analysis of the Fungal Exo- and Endometabolome

Quenching and Sample Preparation for Exo- and Endometabolome Analysis of Nonvolatile Metabolites

The metabolite complement of a filamentous fungus consists of two components: the entirety of intracellular and extracellular metabolites, which have been named endo- and exo-metabolome respectively [19, 39]. Most of the endometabolome consists of primary metabolites that reflect the internal status and "regular" metabolic functioning of the fungus under investigation. Due to fast metabolite turnover rates of intracellular metabolites—in *Escherichia coli*, for example, isocitrate has been estimated to turn over 2.7 times per second [21]—instantaneous quenching of metabolic processes is crucial when the primary metabolome is studied. Moreover, metabolite leakage from quenched cells/fungal mycelium into the medium is of great concern and consequently has been addressed by many researchers. These studies generally revealed that fungi are less prone to leak intracellular metabolites into the medium compared to prokaryotic microbes. Among various quenching methods, the cold (−40 °C) methanol/water (60/40, v/v) method, liquid nitrogen treatment, or rapid filtration and immediate subsequent cooling with liquid nitrogen proved to be best suited for the rapid arrest for metabolism in filamentous fungi. For a detailed discussion of this topic, the reader may refer to review articles of Mashego et al. [21] and Xu et al. [22].

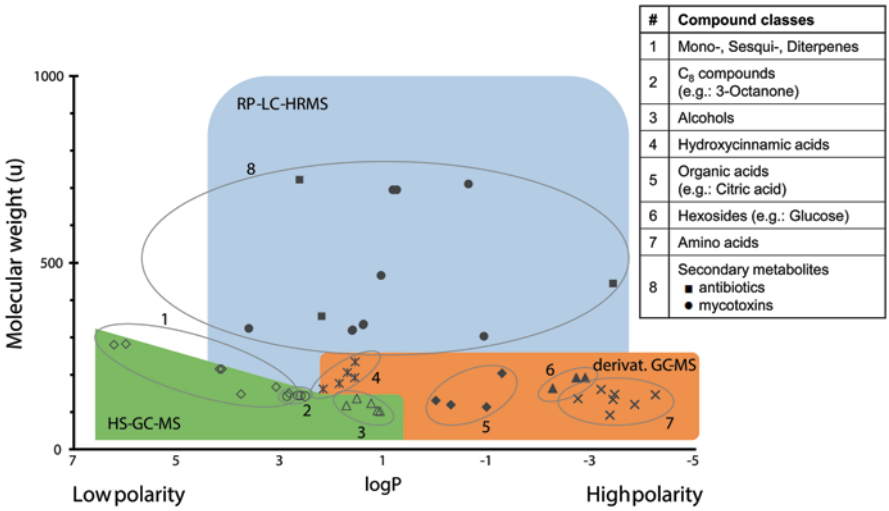
In contrast to the endometabolome, the exometabolome largely represents the secondary metabolites present under certain conditions at the timepoint of investigation. These metabolites can be regarded as being secreted by the organism to interact with its environment. Thus, compared to primary metabolites, secondary metabolites are more promising targets for investigating genus or species-specific traits as well as the interaction between different organisms. They may act as signaling molecules, attracting or repelling other organisms in fungus–fungus or fungus–insect interactions, induce metabolic responses in host organisms, inhibit or kill competitors such as bacteria and other fungi (antibiotics) or mediate susceptibility in host plants (small molecule effectors, typically toxins) [19]. Moreover, nutrition-related compounds such as organic acids and metal chelators (e.g., siderophores) may be produced and secreted into the extra-hyphal space to solubilize, bind, and assimilate inorganic nutrients (e.g., iron in the case of siderophores) . The secreted secondary metabolites do frequently reflect metabolic end points and are therefore less critical with respect to quenching of metabolism than primary metabolites. However, some secondary metabolites are chemically modified by a variety of enzymes (e.g., oxidases, methyltransferases, deacetylases) associated with cell walls or cellular organelles. It also has been argued that rapid quenching, which is necessary to measure intracellular metabolites, can be avoided since the metabolite turnover time is decreased by the dilution of secreted compounds into the larger extracellular culture volume [18].

For being able to distinguish between intracellular and extracellular metabolites, the mycelium and culture supernatant have to be separated prior to sample analysis. This can be achieved by centrifugation or filtration at low temperatures and subsequent washing of the pellet. With solid growth media, fungi can be cultured on cellophane membrane, for example, which enables the separation of the mycelium from the medium (e.g., [40]) before further analysis. Alternatively, it has been described that the mycelium can be carefully scraped from the culture plate [41]. The washed mycelium and/or solid medium can then be extracted, e.g., by (mixtures of) organic solvents, perchloric acid, or potassium hydroxide (reviewed in [21, 22, 25]). Liquid culture filtrates/centrifugates can be further concentrated or directly subjected to the measurement of secondary metabolites. Alternatively, if no separation of extracellular and intracellular metabolites is desired, the whole fungal culture can be extracted directly after quenching.

The Use of GC-MS and LC-MS for the Analysis of Fungal Culture Samples

With GC-MS and LC-MS/MS-based metabolite profiling techniques, which are most commonly used to study fungal exo- and endo-metabolomes, the simultaneous measurement of a group of related metabolites can be realized by single analytical methods. Typically, the simultaneously detected metabolites share certain physical/chemical properties or belong to related metabolic pathways. Using GC-MS under standard conditions (i.e., column temperature between ca. 30 and ≤350 °C) without chemical derivatization, for example, analytes with boiling points between approximately 40 and 450°C can be transported through the GC column by the mobile phase. Thus, metabolites that share the property of being volatile to semi-volatile, according to the World Health Organization (WHO) definition [42], can be determined by GC-MS in a single analytical run. Filamentous fungi have been described to produce a great variety of volatile metabolites belonging to saturated hydrocarbons, alcohols, aldehydes, ketones, lactones, linear esters, ethers, phenols, and terpenoids. Thus, a substantial part of their respective metabolomes can be covered by GC-MS analysis of the volatile organic compounds (VOCs) in liquid culture extracts or by directly probing the headspace above fungal samples (Figs. 6.1 and 6.2 [43]). For a detailed overview of VOC analysis in biological samples, the reader is asked to refer to published review articles [44–47].

Besides VOCs, also polar, non-volatile metabolite can be determined by GC-MS after chemical derivatization. In the area of metabolomics, this approach is still regarded as the gold standard for the comparative quantification of primary metabolites [48]. Derivatization is required to make a certain metabolite less polar, thus more volatile in order to enable proper separation on a GC column. Silylation and alkylation are the two major derivatization procedures that have been described for GC-MS-based profiling of primary metabolites, such as sugars, alcohols, amino acids, non-amino organic acids, and biogenic amines (Fig. 6.2) [43, 49]. A two-step derivatization protocol employing methoximation (to stabilize



#	Compound classes
1	Mono-, Sesqui-, Diterpenes
2	C_8 compounds (e.g.: 3-Octanone)
3	Alcohols
4	Hydroxycinnamic acids
5	Organic acids (e.g.: Citric acid)
6	Hexosides (e.g.: Glucose)
7	Amino acids
8	Secondary metabolites ■ antibiotics ● mycotoxins

Fig. 6.2 Coverage of compound classes by commonly used GC-MS and LC-(HR)MS approaches in metabolomics experiments (based on Halket et al. 2005, modified [43]). Molecular weight of selected fungal metabolites is plotted against the predicted logP value (obtained from http://www.chemicalize.org/; accessed July 2014)

aldehyde and ketogroups) followed by silylation (most frequently using N-methyl-N-(trimethylsilyl) trifluoroacetamide [MSTFA]) has been used in microbial metabolomics [50, 51]. Smart et al. presented an alternative protocol that makes use of methyl chloroformate as a derivatization reagent [29]. The methyl chloroformate alkylation reaction converts primary and secondary amino groups into carbamate derivatives, whereas carboxylate groups are derivatized to form methyl esters, both of which can be analyzed by GC-MS. Although being less universal (as sugars, sugar alcohols, or amino sugars cannot be derivatized), the authors claimed that methyl chloroformate derivatization yields more stable derivatives and is less prone to matrix effects compared to silylation [49]. For the sake of completeness, it shall be noted that complementary to the GC-MS-based procedures, profiling of primary metabolites has also been achieved successfully by hydrophilic interaction chromatography (HILIC) MS [52, 53].

With respect to non-volatile fungal secondary metabolites, however, reversed phase (RP) LC-MS can certainly be regarded as the profiling technique of choice, which will be further discussed in this chapter. This can elegantly be exemplified with recently developed multi-mycotoxin methods, which involve RP stationary phases coupled to either electrospray ionization (ESI), triple quadrupole tandem mass spectrometers (TQMS), or ESI high resolution (HR)MS instrumentation. With the former technique, more than 300 mycotoxins and other fungal secondary metabolites can be measured (semi)quantitatively with a single analytical method [54]. The target analytes cover a broad range of chemical structures, typically produced across a number of different fungal genera and species. Complementary to such a targeted, low-resolution tandem MS approach, LC-ESI-HRMS has successfully

been used for untargeted profiling of fungal metabolites in the full scan mode. LC-ESI-HRMS-based methods are less suited for accurate quantification, however, they show the great advantage of allowing to inspect the presence of fungal metabolites by retrospective data analysis [23, 55, 56]. LC-ESI-HRMS-based untargeted metabolite profiling currently enables the most comprehensive, unbiased coverage of the secondary metabolome. Data processing and metabolite annotation are still considerably hampered by the fact, however, that the majority of the generated data points actually originate from noise and background ions, which should not be attributed to the metabolites contained in the samples under investigation. As a consequence, with the commonly used state-of-the-art LC-ESI-HRMS-based workflows, it is not possible to reliably describe the metabolic composition of individual biological samples, but with untargeted measurements data interpretation is rather restricted to analytical features that are significantly differing between control and treated samples. Novel stable isotope labeling (SIL)-assisted workflows have recently been developed (e.g., [11, 38, 57, 58]), which are excellently suited to circumvent major limitations associated to current LC-ESI-HRMS approaches (see next section).

Applications of Metabolomics Tools to Study Filamentous Fungi

The concept of metabolomics is clearly generic. Metabolomics is not only applicable to any type of biological system but can also be used for the study of various types of (biological) research questions. This young discipline has considerably matured over the last decade and has gained great popularity. In view of the steadily increasing use of metabolomics and metabolite profiling, a comprehensive description of its applications to filamentous fungi is far beyond the scope of this chapter. Instead, a few selected areas of fungal metabolomics and metabolite profiling shall be briefly presented.

Secondary Metabolite Profiling for Chemotaxonomical Classification The concept of metabolite profiling had already been well established and used successfully for medical and diagnostic purposes, long before the term metabolome was first introduced by Oliver et al. in 1998 [26, 59]. Already in 1983, Frisvad and colleagues reported about the “classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites” by thin layer chromatography (TLC) [60]. After 25-years, in 2008, the same author broadly defined the term chemotaxonomy as the use of chemical diversity for taxonomic classification of biological organisms [61].

Nowadays, chemotaxonomical classification approaches are used complementary to DNA-based techniques and are mainly based on primary and more frequently secondary metabolite profiles generated by GC-MS and LC-MS. However, it has to be stated that a limitation of metabolome-based taxonomy is that the expression of genes relevant for taxonomic purposes may not occur because of a variety of environment-dependent regulatory switches (regulatory genes) that are highly variable.

Basically, all fungi under investigation have to be evaluated in parallel in a standardized manner for the ease of comparability. Therefore, the respective fungal strains are cultivated under similar environmental conditions such as temperature, light cycle, and humidity on the same nutrition medium for a certain time period [61] before they are harvested and analyzed. Whereas initially, classification was based on the separation of mycotoxins TLC and HPLC followed by UV/visible (VIS) detection [60, 62], today, MS-based techniques are the methods of choice. These techniques enable a more comprehensive untargeted profiling of the complex metabolite mixtures produced by filamentous fungi. As mentioned, for each sample a metabolite profile is established using either GC-MS [63, 64] or LC-HRMS [65–67]. Both approaches in combination with chemometric analysis allow the distinct separation of fungal cultures into taxonomic classes. In order to evaluate the chemotaxonomic classification, molecular approaches based on the sequence analysis of ribosomal DNA internal transcribed spacers (ITS) for molecular phylogeny have been employed [63, 64, 67]. In the study of Kang and coworkers, which may serve as an example here, a total of 33 strains belonging to seven species of the saprophytic fungi *Trichoderma* were classified using an LC-HRMS and a GC-MS approach in parallel [63]. Metabolite-based chemotaxonomy was established based on principal component analysis (PCA) of LC-HRMS data and revealed most varying metabolite profiles in 16 days old cultures. However, comparison of GC-MS derived data with ITS-based classification showed less correlation compared to LC-HRMS-based metabolite profiles. In the study of Kang and co-workers, the LC-HRMS-derived secondary metabolite profiles seemed to be more reliable for chemotaxonomical classification than the GC-MS-based endometabolome profiles [63]. Despite the undisputable dominance of DNA-based methods for taxonomical classification, chemotaxonomical methods based on secondary metabolites still constitute a valuable complementary tool for the classification of filamentous fungi today.

Use of Metabolite Profiling for the Screening and Production of Fungal Natural Products

Ever since the “discovery” of the penicillin antibiotics, the diversity and bioactive potential of fungal natural products have inspired the pharmaceutical industry to search for lead structures of active drug ingredients [5]. It is obvious that modern MS-based profiling and metabolomics workflows, which are principally suited to screen for hundreds to thousands of natural products simultaneously, can be of great benefit for comprehensive exploration of the pool of putatively beneficial secondary metabolites. All approaches for the discovery of novel bioactive substances have in common that they rely on the existence of an observable phenotype or chemical property, such as biological activity, color, or a known mass, which can be tracked through successive rounds of detection and isolation [68]. Besides drug discovery, for the identification of fungal secondary metabolites, there is also the demand for the screening of active compounds for biological control agents or the discovery of currently unknown fungal metabolites putatively playing key roles in host–fungus interaction.

Many of these fungal compounds produced by fungi belong to one of the three major structural classes of nonribosomal peptides (NRPS), polyketides (PKS), and terpenoids [69–71]. The genes for biosynthesis of secondary metabolites are mostly organized in clusters, and interestingly for many species, the number of these gene clusters exceeds the number of known secondary metabolites produced by the respective fungal strains under laboratory conditions [72, 73]. It has been hypothesized that secondary metabolite formation has evolved for interaction with antagonists and fungal hosts and that therefore some of the metabolites are not produced under standard (axenic) culture conditions, which do not reflect the organism's native habitat.

Since the screening of natural products is often performed under laboratory conditions, the biosynthetic potential of filamentous fungi to produce interesting secondary metabolites has not been fully exploited yet. Various efforts are made to activate the production of these potentially valuable and bioactive metabolites and hence facilitate natural product discovery [73]. The nutrient composition of the cultivation medium can be varied to alter the secondary metabolite profile [74, 75]. In general, the production of secondary metabolite has been described to be higher if the fungi are grown on solid surface substrates. Also the cultivation on mineral-clay pellets coated with a semisolid agar substrate has been found to enhance the chemical diversity of the producing fungi [19]. Another methodology becoming increasingly popular for the activation of silent gene clusters is epigenetic remodeling. To this end, fungi have been treated with DNA methyltransferase and histone deacetylase inhibitors or other small molecule effectors (e.g., 5-azacytidine) with the aim to activate otherwise non-expressed gene clusters encoding secondary metabolite production [76]. Recent studies led to the discovery of a novel, not yet identified metabolite [77] or at least give a hint to the derepression of genes that are involved in the biosynthesis of secondary metabolites [78]. Epigenetic remodeling can be regarded as a promising tool to access biosynthetic pathways for novel, so far unknown secondary metabolites of fungi in the future.

Excessive exploration of the large diversity of fungal strains for the production of natural products, bear the risk of "rediscovering" already known secondary metabolites. Thus, a major task in natural products discovery is the development of efficient dereplication strategies for the identification of already known secondary metabolites during culture screening to avoid subsequent cost-intensive and time-consuming isolation and identification procedures [23, 79–81]. To this end, metabolic profiling using LC-HRMS, often in combination with UV/VIS detection, has evolved as a key technique in screening approaches. Thus, rapid and efficient comprehensive initial analysis of most of the secondary metabolites produced under certain laboratory conditions is of great help as has been recently reviewed by Breitling et al. [82]. As already discussed, current dereplication strategies emphasize a combination of different levels of identification based on authentic reference standards, tentatively identified compounds by LC-MS/MS spectra, UV/VIS spectra, and species-specific metabolite subsets from selected compound databases (e.g., Antibase 2012 [30]). Fungi of interest are cultivated under well-defined conditions and extracts are subsequently measured using LC-HRMS. The accurately measured

m/z value as well as LC-MS/MS and UV/VIS spectra of analytical features of interest are subsequently searched against available databases.

There are, however, still major challenges to be addressed, such as wrongly annotated metabolite ions due to the lack of suitable qualifier and/or fragment ions from literature data. The results described in current research studies demonstrate that still major efforts have to be undertaken to strengthen the reliability of metabolite profiling and metabolomics results, especially when it comes to structure elucidation of new compounds in natural product discovery.

Use of Metabolomics for the Study of Biological Interactions of Fungi

Although the number of metabolomics studies of filamentous fungi is steadily increasing, applications of this technology to investigate host–fungus interactions are still sparse. The intimate association between the fungus under investigation and its interaction partner (e.g., an antagonistic microbe or a plant) coupled with a general commonality of metabolites complicates the separation of the respective metabolomes [83, 84]. Whereas in fungus–fungus [85, 86] or fungus–bacteria interactions both partners can be assumed to contribute significantly to the overall biomass of the investigated samples, this is usually not the case in fungus–plant interactions. In both mutualistic as well as pathogenic plant–microbe interactions, there are several ways to minimize/circumvent the problem of properly dissecting the metabolomes:

1. The systemic response in the host plant can be studied by choosing a sampling site, distant from the physical interaction zone [87, 88].
2. The effect of a pure fungal protein- or fungal small molecule effector can be used to challenge the metabolism of the plant (or vice versa) [89, 90].
3. Depending on the study design, in many fungus–plant interactions it can be assumed that the biomass of the filamentous fungus is so low that its contribution to the sampled plant metabolome can be neglected (e.g., [91]). However, this should be verified by the measurement of fungus-specific metabolites such as mycotoxins or cholesterol [83].
4. An approach employing a plant cell—microbe coculture can be chosen as presented by Allwood and colleagues where plant cells and bacterial pathogen cells are separated by differential filtration after a defined time period of cocultivation [92]. The independent analysis of plant and bacterial cells allowed the metabolite changes within each interacting partner to be assessed [92]. This approach might be adapted for the study of fungus–plant interactions as well.

In recent years, several interesting plant metabolomics studies have provided novel fundamental insight into both mutualistic as well as host–fungus interactions. It has been shown that the combined application of different "-omics" disciplines such as proteomics and metabolomics (e.g., [89]) or metabolomics and transcriptomics (e.g., [91]) provides a more holistic and complementary view on the biochemical response of the organisms under investigation than either of the approaches alone.

Thereby, the combination of the complementary "-omic data" can greatly facilitate a meaningful biological interpretation of the generated study. Voll et al. used a combined transcriptomics and metabolomics approach to investigate the response of primary metabolism of barley leaves upon inoculation with three different biotrophic fungal pathosystems (*Blumeria graminis*, *Ustilago maydis*, and the hemibiotrophic pathogen *Colletrichum graminicola*) with the aim to study if common metabolic response motifs can be revealed for the three pathosystems [91]. Most interestingly, the authors found that common motifs in the response of cereal primary carbon and nitrogen metabolism to the different fungal pathogens were not based on similar transcriptional reprogramming [91]. In the study of Vincent et al. which investigated the effect of the effector protein SnToxA of the necrotrophic pathogen *Stagonospora nodorum* on wheat, the authors reported detailed conclusions on the molecular mechanisms by which necrotrophic pathogens appear to induce oxidative stress and cell death by disruption of photosynthesis and subsequent energy depletion in the effected host cells [89]. Vincent and coworkers emphasized the complementary nature of proteomics and metabolomics, which enabled them to draw general conclusions on how necrotrophs do most probably exploit host cell death mechanisms to promote its own growth and cause disease [89].

Elucidation of Low-Molecular-Weight Gene Products by Metabolite Profiling

Based on recent gene annotations, the number of nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS), and terpene synthase (TPS) gene clusters encoding the enzymes for biosynthesis of secondary metabolites was estimated for different fungal species such as for *Fusarium graminearum* PH1 (NRPS: 20; PKS: 15; TPS: 17) [93] or *Trichoderma virens* (NRPS: 28; PKS: 18; TPS:4) [94]. To understand the function of specific gene clusters and their integration into metabolic pathways, commonly deletion mutants are generated by gene disruption. To this end, a gene cluster of interest is selected for deletion and the resulting phenotype is compared to the corresponding intact wild-type strain when cultivated under similar conditions.

Metabolic profiling approaches have emerged as powerful tools to study the effect of gene deletion on secondary metabolite formation and to draw conclusions on the functionality of the deleted gene cluster. This shall be exemplified with the following few studies. Metabolites associated with genes putatively encoding specific biosynthetic enzymes such the terpene cyclase *vir4* in *T. virens* [95] or VeA, a general regulator of the secondary metabolism in *Aspergillus fumigatus* [96], were successfully studied by GC-MS profiling of volatile compounds in the headspace of living fungal cultures. The authors of the latter study demonstrated that volatiles generated by a VeA disruption mutant are part of the complex regulatory machinery that mediates the effects of VeA on asexual conidiation and sclerotia formation [96]. In a further study, PKS gene products of *Aspergillus flavus* were successfully identified [97]. Comparative metabolomics, using ultra high performance liquid

chromatography (UHPLC) coupled to high resolution Orbitrap MS was used to detect metabolites differentially expressed in the *A. flavus* wild type and Δ(Delta) pks27 mutant strains [96, 97]. Four metabolites were identified that were only present in the wild-type cultures. These included asparasone A (358 Da), an anthraquinone pigment, and two related anthraquinones. The mentioned studies elegantly demonstrate that comparative metabolomics can provide novel insight to functional annotation of fungal secondary metabolite gene clusters either by identification of distinct (novel) metabolites, the production of which is encoded by the genes under investigation or complex metabolic shifts resulting from deletion of other secondary metabolism-associated genes.

Recent Developments for Improved Fungal Metabolomics

Despite considerable progress of metabolomics and its many successful applications to complex biological systems, there are still major challenges. In untargeted LC-HRMS-based approaches, the reliable annotation of truly sample-derived metabolites, their putative identification, accurate comparative metabolite quantification, and proper workflow validation can be considered substantial technical problems.

Stable isotope labeling-assisted techniques make use of the labeling of specific isotopic patterns obtained from the measurement of mixtures of labeled and native biological samples or metabolites. They show great potential to provide improved tools for untargeted GC-MS and LC-MS metabolomics [98, 99]. Heavy stable isotopes of carbon (^{13}C), nitrogen (^{15}N), or sulphur (^{34}S) can be used to enrich tracer metabolites or whole biological samples for metabolomics experiments. It should be noted that hydrogen (^{2}H) and oxygen (^{18}O) can also be used but are less suitable since they can be readily exchanged between individual metabolites or with non-labeled solvent molecules.

Most recent SIL-assisted applications involve the *in vivo* ^{13}C labeling of filamentous fungi for the untargeted metabolome annotation by LC-HRMS [11, 100]. For this purpose, fungi were grown in parallel on substrates containing either a native or labeled carbon source. Bueschl et al. cultured *F. graminearum* on U-^{13}C glucose [98], while Cano et al. used U-^{13}C (/^{15}N)-labeled wheat grains as culture substrate for *Aspergillus fumigatus* [100]. All fungal metabolites produced on these media had incorporated the ^{13}C (/^{15}N) label and thus when mixed with native samples were easily recognized by the corresponding characteristic isotopic patterns in the data. Signals originating from contaminants, solvent clusters, or artifacts were efficiently eliminated. While Bueschl et al. detected around 90 true metabolites originating from *Fusarium* [98], Cano et al. were able to find 21 secondary *Aspergillus* metabolites in the tested samples [100]. Moreover, the methodology offers several advantages over conventional approaches, such as the reduction of sum formula ambiguities by providing the exact number for atoms of the element used for labeling per metabolite ion or the correction of matrix effects by full metabolome internal standardization.

In a recent study, a mixture of the native and U-^{13}C-labeled *Fusarium* mycotoxin deoxynivalenol has been used as a tracer for the untargeted profiling and automated evaluation of its metabolization in flowering wheat ears [101]. The authors were able to find a total of nine wheat-derived toxin derivatives, among them are several novel glutathione-related deoxynivalenol conjugates.

Moreover, fungal metabolomics would benefit from improved temporal and spatial resolution of metabolite analysis, since fungal secondary metabolism is known to be both highly dynamic with respect to cultivation duration as well as age/developmental stage of individual hyphae. Compartmentation by hyphal septa further complicates data interpretation and meaningful biological conclusions.

The latest developments of ambient ionization as well as matrix-assisted laser desorption ionization (MALDI) MS now enable temporally and spatially resolved imaging of metabolite production by living fungal cultures [85, 102–104]. To mention just two of these studies, Moree and colleagues applied MALDI imaging MS directly to agar cultures with the aim to elucidate the bacterial metabolites produced by an antifungal *B. amyloliquefaciens* strain in a side-by-side interaction with an *A. fumigatus* and an *Aspergillus niger* strain [104]. The authors were able to demonstrate that the antifungal activity was mediated by lipopeptides of the iturin family. With such an assay, spatial distribution and relative MS detector intensities can be used to evaluate whether metabolites are secreted by the organisms into the media, confined to the colony, induced, or consumed by neighboring organisms [104].

Another interesting study was conducted by Hu and coworkers employing native and U-^{13}C-labeled glucose as a substrate to study the gradual incorporation and translocation of ^{13}C carbon in fungal hyphae [103]. To this end, the fungus *Neurospora crassa* was grown on glass slides and mass spectra were subsequently recorded after sample preparation using MALDI coupled to MS. Although only highly abundant intracellular metabolites could be mapped by this approach, lateral resolution was high enough to monitor cytoplasmic relocation of ^{13}C isotoplogs in the fungal hyphae.

Conclusion

It can be stated that the latest ambient MS imaging tools will enable a major step forward toward spatially and temporally resolved fungal metabolomics and will provide fascinating new insight into the metabolism of living fungal cultures. Furthermore, novel SIL-assisted approaches and data processing tools show great potential for improved untargeted metabolomics studies of filamentous fungi in the future.

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