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Sphingolipids of plant pathogenic fungi

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Abstract: Glycosphingolipids in filamentous fungi are significant components of the plasma membrane and are vital for different cellular processes, such as growth, morphological transition or signal transduction. Fungal growth inhibitors targeting glycosylinositolphosphoceramide (GIPCs) biosynthesis or antifungal compounds binding to GIPCs present in membranes could present a safe way of preventing fungal growth on crops since GIPCs are not present in mammalian cells. Mass spectrometry-based shotgun lipidomics was used to analyze sphingolipids of 11 fungal strains isolated from plant material. Molecular species with inositol ceramides containing zero to five carbohydrates were identified. Differences in the amount of individual molecular species were influenced by the taxonomic affiliation. All tested strains exhibited a relatively high content (more than 40 mol.%) of GIPCs with three and more saccharides attached to the polar head. It could be assumed that the sphingolipid profiles of the tested plant pathogens would be an adaptation mechanism to antifungal plant defensins.

Keywords: defensin; filamentous fungi; glycosylinositolphosphoceramides; high resolution tandem electrospray mass spectrometry; *Vitis vinifera*

Sphingolipids are abundant components of eukaryotic membranes and are essential compounds in e.g. heat stress response or signalling. Some sphingolipids play an important role in fungal pathogenesis, such as inositolphosphoryl ceramides. These molecules are composed of a very long chain hydroxylated fatty acid condensated to a C18-phytosphingosine and may function as a lipid anchor for molecules such as glycans to form glycosylinositolphosphoceramides (GIPCs) (Fontaine 2017). GIPCs are widely distributed among fungi and plants but are not present in mammalian cells. The expression of GIPCs can be correlated to different growth and morphological stages or pathogenicity. Complex comparative studies on GIPCs production in filamentous fungi are rather rare but the studies performed suggest their large diversity (Buré et al. 2014).

In the past years, it was proved that fungal sphingolipids play an important part in their pathogenicity and could be a potential target in inhibiting fungal growth. Fungicide use is an important part of pest and disease management in agricultural production, e.g. vegetables, grapevine and orchards. However, the regular use of fungicides can potentially lead to environmental pollution (Wightwick et al. 2010) and may be potentially harmful to humans (Hrelia et al. 1996). Since GIPCs are not present in mammalian cells, targeting these acidic lipids could present a safer, more selective and environmentally friendlier way of fighting fungal diseases.

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Fungal GIPCs were found to be important for the survival of many fungal species. These filamentous fungi were susceptible to inhibitors of sphingolipid biosynthesis (Toledo et al. 2010). In a study by Utesch et al. (2018), it was shown that GIPCs, specifically their sugar and phosphate groups, play a crucial role in the attachment of antifungal AFP proteins to fungal membranes. Furthermore, it was found that the antifungal compound chitosan may bind to GIPCs present in filamentous fungi (Zakrzewska et al. 2007). An altered sphingolipid profile is also linked to resistance to plant defensins (Ferket et al. 2003).

Studying the differences in sphingolipid content of different fungal strains and their variety is a significant step in a better understanding of their function and plant-pathogen interactions. In this study mass spectrometry-based shotgun lipidomics was applied to the analysis of sphingolipids present in filamentous fungi newly isolated from *Vitis vinifera* as well as in strains from collections.

MATERIAL AND METHODS

Isolation and identification. The fungal strains *Botrytis cinerea* DMB 4246, DBM 4111 and DBM 4281 were provided by Collection of Yeasts and Industrial Microorganisms, Department of Biochemistry and Microbiology (DBM), University of Chemistry and Technology Prague (UCT). The fungal strains *Aspergillus flavus* CCF 3432, *Alternaria arborescens* CCF 2670 and *Alternaria alternata* CCF 2671 were provided by Culture Collection of Fungi (CCF), Department of Botany, Faculty of Science, Charles University. The fungal strains *Penicilium expansum* IR1, *Fusarium* cf. *graminearum* KH1, *Aureobasidium pullulans* KH2, *Colletotrichum godetiae* KH3 and *Sordaria* cf. *superba* KH4 are isolates from *V. vinifera* grapes and leaves. An overview of the cultivated fungal strains is given in Table. 1. Fungi isolated from infected *V. vinifera* leaves and grapes were placed on a Petri dish with YPD agar medium. After 7 days of incubation at 25 °C, the hyphal tips of each morphologically different mycelium were isolated and purified. The identification process together with the studied sequences of the isolates are given in Table S1 [see electronic supplementary material (ESM)].

Cultivation. The strains were maintained on yeast extract peptone dextrose (YPD) Petri dishes at 4 °C and re-cultured monthly. Fungal cultures on YPD agar slants (7 days old) were used as inoculum for the pre-cultures. The pre-cultures of the fungal strains were cultivated in 100 mL of YPD medium in 500 mL Erlenmeyer flasks at 25 °C on a rotary shaker (Eppendorf, Germany) at 150 rpm for 24 hours. For lipid production, 100 mL volume of YPD medium in 500 mL Erlenmeyer flasks was inoculated with 10 mL of pre-culture and incubated on a rotary shaker at 150 rpm at 25 °C in triplicate parallels. The cultivations were carried out from 6 to 7 days (until early stationary phase) depending on the given strain. After cultivation, the cells were centrifuged (9 000 g, 10 min) and washed twice. Biomass was frozen at –75 °C and lyophilized.

Extraction. The mycelium was disrupted by liquid nitrogen and glass beads, as previously described

Table 1. List of tested fungal strain and their origin

Fungal strain	Class	Culture collection	Origin
A. flavus CCF 3432	Eurotiomycetes	CCF, Charles University	*Zea mays*
P. expansum IR1		isolate	*Vitis vinifera* grapes
B. cinerea DMB 4111	Leotiomycetes	DBM, UCT Prague	*Fragaria ananassa*
B. cinerea DMB 4281		DBM, UCT Prague	*Daucus carota* subsp. *sativus*
B. cinerea DMB 4246		DBM, UCT Prague	air
F. cf. *graminearum* KH1	Sordariomycetes	isolate	*Vitis vinifera* leaves
C. godetiae KH3		isolate	*Vitis vinifera* leaves
S. cf. *superba* KH4		isolate	*Vitis vinifera* leaves
A. pullulans KH2	Dothideomycetes	isolate	*Vitis vinifera* leaves
A. arborescens CCF 2670		CCF, Charles University	*Solanum tuberosum*
A. alternata CCF 2671		CCF, Charles University	*Hordeum vulgare*

CCF – Culture Collection of Fungi; DBM – Department of Biochemistry and Microbiology; UCT – University of Chemistry and Technology Prague

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(Řezanka et al. 2018). Briefly, the cell suspension (10 mL, approximately 15% of dry mass) was ground with glass beads under liquid nitrogen. After thawing, the crushed mycelium was extracted in a blender (Waring, USA) (5 min) by 3 × 5 mL of isopropanol-hexane-water (55 : 25 : 20; v/v/v, upper phase) and 3 × 5 mL of dichloromethane-methanol (1 : 1; v/v lower phase). All six extracts were combined and the lipid solution was further analyzed by negative and positive electrospray ionization mass spectrometry (ESI-MS).

Shot-gun MS. The LTQ-Orbitrap Velos (Thermo Scientific, USA) mass spectrometer equipped with a heated electrospray interface (HESI) was operated in positive and negative ionization mode. The MS scan range was performed in the Fourier transform (FT) cell recording a window between 100–1 000 m/z. The mass resolution was set to 105 000, and the ion spray voltage was set at 3.5 kV (in positive ionization mode) and –2.5 kV (in negative ionization mode). The same parameters were used in both ionization modes, see below. Nitrogen was used as a nebulizer gas set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for collision-induced dissociation (CID) experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The MS data were acquired using the further following parameters: vaporizer temperature, 250 °C; capillary temperature, 230 °C; capillary voltage, 50 V; tube lens voltage, 170 V. The calibration of the MS spectrometer was done with the use of Pierce LTQ Orbitrap negative ion calibration solution. The internal lock mass was used in mass spectra acquisition, i.e. 255.2330 m/z $[M-H]^-$ palmitic acid in negative ESI. The mass accuracy was better than 1.5 ppm.

Statistics. To perform multivariate statistical analyses, we used the program CANOCO 5 (version 5.0). Since the gradient lengths in the data were short, we performed a principal component analysis (PCA) to visualize the variability within the dataset.

RESULTS

The identification of the extract was performed by shot-gun analysis in high-resolution negative ESI. The ceramide derivatives profile of the fungal strain *A. flavus* CCF 3432 is shown in Figure 1. The following facts are apparent from these data. First, inositol ceramides having zero to five carbohydrates are present. In terms of molar percentages, the most abundant inositol ceramides contained zero carbohydrates. With a growing number of carbohydrates present in the molecule, the molar percentage was decreasing (Table 2), however, the more highly glycosylated GIPCs (four and five carbohydrates) accounted for approximately 25 mol.% of the total inositol ceramides in the extract.

Each GIPCs is always a mixture of more than 10 molecular species, with non-hydroxy- and hydroxy-FA, and homologues of bases (phytosphingosine, sphinganine, sphingenine). In total, more than 2 000 molecular species were identified. It should be noted, however, that many of the listed $[M-H]^-$ ions exist as a mixture of at least two molecular species, which was further confirmed by tandem mass spectrometry, according of the paper Buré et al. (2014).

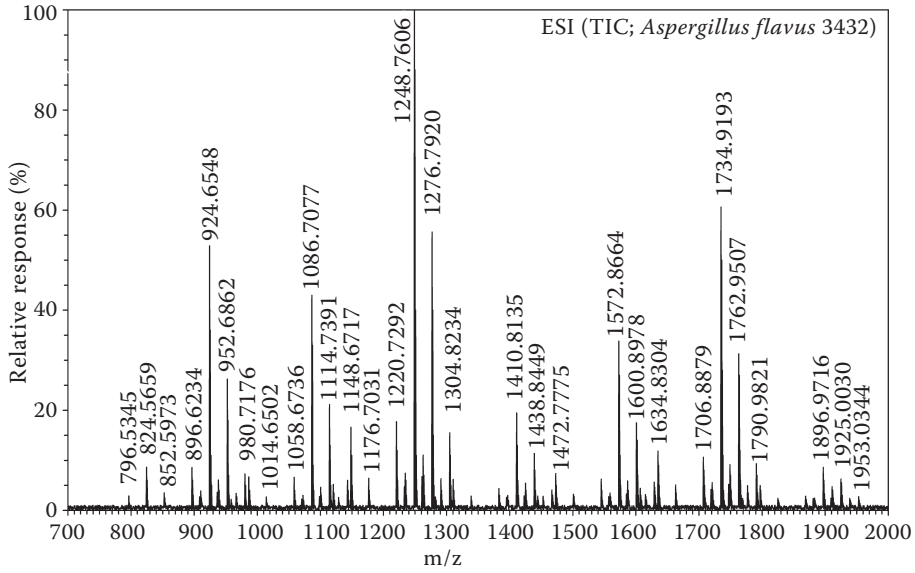

Figure 1. ESI-mass spectrum of sphingolipids from *Aspergillus flavus* CCF 3432 in negative ion mode

TIC – total ion chromatogram

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Tale. 2 The sum of the mol.% of individual molecular species based on the number of carbohydrate bound to the polar head of inositol phosphorylceramides

Fungal strain	Inos + P	SD	Inos + P/Hex	SD	Inos + P/Hex/Hex	SD	Inos + P/Hex/Hex/ Hex	SD	Inos + P/Hex/Hex/ Hex/Hex	SD
A. flavus CCF 3432	23.0	± 0.3	19.4	± 0.2	16.9	± 0.2	15.0	± 0.2	13.8	±0.2	11.9	± 0.1
P. expansum IR1	23.0	± 0.3	19.4	± 0.3	16.9	± 0.2	15.0	± 0.2	13.9	± 0.2	11.8	± 0.2
B. cinerea DMB 4111	23.1	± 0.3	19.4	± 0.3	16.9	± 0.2	15.0	± 0.2	13.9	± 0.2	11.8	± 0.2
B. cinerea DMB 4281	23.0	± 0.3	19.4	± 0.3	16.9	± 0.2	15.0	± 0.2	13.9	± 0.2	11.8	± 0.2
B. cinerea DMB 4246	23.1	± 0.3	19.4	± 0.2	16.9	± 0.2	15.0	± 0.2	13.9	± 0.2	11.8	± 0.2
F. cf. *graminearum* KH1	23.0	± 0.2	19.4	± 0.2	16.9	± 0.1	15.0	± 0.1	13.8	± 0.1	12.0	± 0.1
C. godetiae KH3	23.1	± 0.3	19.4	± 0.3	16.9	± 0.2	15.0	± 0.2	13.9	± 0.2	11.8	± 0.2
S. cf. *superba* KH4	23.0	± 0.3	19.4	± 0.3	16.9	± 0.2	15.0	± 0.2	13.9	± 0.2	11.8	± 0.2
A. pullulans KH2	23.0	± 0.2	19.4	± 0.2	16.9	± 0.1	15.0	± 0.1	13.8	± 0.1	11.9	± 0.1
A. arborescens CCF 2670	23.0	± 0.2	19.4	± 0.2	16.9	± 0.1	15.0	± 0.1	13.8	± 0.1	11.9	± 0.1
A. alternata CCF 2671	23.0	± 0.2	19.4	± 0.2	16.9	± 0.1	15.0	± 0.1	13.8	± 0.1	11.9	± 0.1

SD – standard deviation; P – phospate; Hex – hexose; Inos – inositol; the values are given in %

For example, the molecular species having the chemical formula $C_{54}H_{105}NO_{18}P$ and mass 1 086.7072 Da (calculated 1086.7075 Da, difference 0.3 ppm) contain both Inos+P/Hex/t18:0/2-OH-24:0 and also Inos+P/Hex/t20:0/2-OH-22:0. The key ions defining the structure of Inos+P/Hex/t18:0/2-OH-24:0 molecular species are ions at m/z 421.0753 and at m/z 403.0647 (determines the polar head structure) and ions at m/z 744.5913 (determines the ceramide part of the molecule) and a pair of ions at m/z 383.3531 (structure of fatty acid) and ion at m/z 378.2415 (long-chain sphingoid base). Further molecular species were identified similarly.

Principal component analysis (PCA) was based on the intensity of individual ions obtained from the MS/MS spectra. The proportion of molecular species extracted from biomass of the tested fungi was driven more by the taxonomic affiliation of the strains than by the isolation origin of the given strains, as seen in Figure 2. The only exception was the fungal strain *Fusarium* cf. *graminearum* KH1 (class Sordariomycetes, order Hypocreales), whose sphingolipid profile was more similar to class Dothideomycetes strains rather than to representatives of its own class,

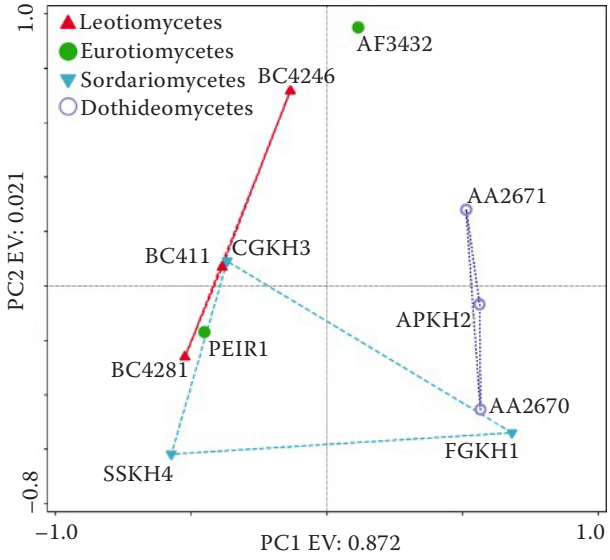

Figure 2. Principal component analysis (PCA) of GIPCs species content in the fungal strains (mol.%)

The variability explained by the first two canonical axes; AF3432 – *A. flavus* CCF 3432; PEIR1 – *P. expansum* IR1; BC4111 – *B. cinerea* DMB 4111; BC4281 – *B. cinerea* DMB 4281; BC4246 – *B. cinerea* DMB 4246; FGKH1 – *F.* cf. *graminearum* KH; CGKH3 – *C. godetiae* KH3; SSKH4 – *S.* cf. *superba* KH4; APKH2 – *A. pullulans* KH2; AA2670 – *A. arborescens* CCF 2670; AA2671 – *A. alternata* CCF 2671; EV – explained variance; GIPCs – glycosylinositolphosphoceramides

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namely *Colletotrichum godetiae* KH3 (order Glomerellales), *S.* cf. *superba* KH4 (order Sordariales). However, differences in the content of individual molecular species among different fungal strains were only in amounts of 0.0–0.4 mol.% of total extracted sphingolipid species (Table 2S).

DISCUSSION

In the past years, there were several studies dealing with molecular species of sphingolipids in filamentous fungi associated with human mycoses (Toledo et al. 2007), however, studies on their content in fungi associated with plant diseases are rather rare. The lack of knowledge about the diversity of fungal GIPCs may be partly attributed to their physicochemical properties. The solubility of GIPCs in usual organic solvents is rather limited due to their large hydrophilic polar head which posed a complication for their isolation and characterization (Buré et al. 2014).

Filamentous fungi pose a great loss for crop production with estimated 10% reduction in crop yields worldwide (Meyer et al. 2016). Inositol ceramides, essential parts of the fungal plasma membranes are targeted with antifungal compounds such as aureobasidin A (Takesako et al. 1993), khafrefungin, rustimicin (Mandala et al. 1998), pleofungin (Yano et al. 2007) or haplofungin (Ohnuki et al. 2009). We proved that the differences in the content of individual molecular species of the tested strains were only in maximal amounts of 0.4 mol.% of total extracted sphingolipid species and the growth of fungal pathogens could thus be inhibited with a broad-spectrum fungicide.

The number of monosaccharide units linked to inositol may be rather large in GIPCs structures in both plants and fungi. Up to 14 carbohydrates were found in tobacco leaves (Kaul & Lester 1975) and a maximum of 20 carbohydrates in *Candida albicans* (Trinel et al. 2002). However, the case is different with filamentous fungi. The analysis of GIPCs from several *Aspergillus fumigatus* strains, opportunistic fungal pathogens of mammalian hosts, showed that they contained a maximum of three to four saccharides attached to inositol (Toledo et al. 2007). In the present study, all phytopathogenic fungal strains produced GIPCs with up to five carbohydrates (Table 2) as seen from the ceramide derivatives profile of *A. flavus* CCF 3432 (Figure 1). A higher degree of glycosylation may play a significant role in the fungal resistance against plant defensins (e.g. antimicrobial peptides produced by different plant species). Ferket et al. (2003) found that highly glycosylated GIPCs (3 and more monosaccharides) were present in defensin resistant mutants in comparison to the wild stain of *Neurospora crassa*.

CONCLUSION

This paper is, to our knowledge, the first comparative study of sphingolipids in plant pathogenic fungi. This work suggests that the taxonomic affiliation of the analyzed strains had a significant impact on the proportion of the extracted sphingolipid species but the differences in the amount of individual molecular species were rather small. Infected crops could thus be treated with broad-spectrum fungicides targeted at inositol phosphorylceramides. The presence of highly glycosylated GIPCs could indicate a defense mechanism of the 11 tested fungal strains to plant defensins.

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