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Implication of *Fusarium graminearum* primary metabolism in its resistance to benzimidazole fungicides as revealed by ^{1}H NMR metabolomics

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ABSTRACT

Fungal metabolomics is a field of high potential but yet largely unexploited. Focusing on plant-pathogenic fungi, no metabolomics studies exist on their resistance to fungicides, which represents a major issue that the agrochemical and agricultural sectors are facing. Fungal infections cause quantitative, but also qualitative yield losses, especially in the case of mycotoxin-producing species. The aim of the study was to correlate metabolic changes in *Fusarium graminearum* strains' metabolomes with their carbendazim-resistant level and discover corresponding metabolites-biomarkers, with primary focus on its primary metabolism. For this purpose, comparative ^{1}H NMR metabolomics was applied to a wild-type and four carbendazim-resistant *Fusarium graminearum* strains following or not exposure to the fungicide. Results showed an excellent discrimination between the strains based on their carbendazim-resistance following exposure to low concentration of the fungicide (2 mg L^{-1}). Both genotype and fungicide treatments had a major impact on fungal metabolism. Among the signatory metabolites, a positive correlation was discovered between the content of *F. graminearum* strains in amino acids of the aromatic and pyruvate families, L-glutamate, L-proline, L-serine, pyroglutamate, and succinate and their carbendazim-resistance level. In contrary, their content in L-glutamine and L-threonine, had a negative correlation. Many of these metabolites play important roles in fungal physiology and responses to stresses. This work represents a proof-of-concept of the applicability of ^{1}H NMR metabolomics for high-throughput screening of fungal mutations leading to fungicide resistance, and the study of its biochemical basis, focusing on the involvement of primary metabolism. Results could be further exploited in programs of resistance monitoring, genetic engineering, and crop protection for combating fungal resistance to fungicides.

1. Introduction

Fungal metabolomics is a rapidly evolving field of biological sciences, however, in contrast to environmental and human metabolomics [1–6], its potential is yet largely unexploited. To date, metabolomics approaches have been developed for the study of various fungal species, including model species for applications in numerous scientific disciplines, such as, *Aspergillus nidulans* [7,8] and *Saccharomyces cerevisiae* (yeast) [9–12], and arbuscular mycorrhizal fungi (AMF) [13–15].

Focusing on fungal species of agricultural interest, metabolomics have been applied in the study of their metabolism [16–18], their interactions with plants [19–21] and other fungi [22,23], and their taxonomy [9,24–26]. Nonetheless, to our knowledge, no metabolomics studies exist related to fungal resistance to fungicides and its correlation to their metabolism regulation.

Fungal resistance to fungicides is among the main challenges that the agrochemical and agricultural sectors are currently facing, resulting in severe quantitative and qualitative yield losses, and threatening food supply and security [27,28]. Fungal resistance to fungicides is the acquired, stable, heritable reduction in their sensitivity to specific fungitoxic agent(s) [29]. There are many mechanisms by which fungi acquire resistance to fungicides, with the most common ones being the genetic modification of the target site, overexpression of the target gene, detoxification of the fungicide, and the increased efflux of fungicides by the activity of ATP-binding cassette (ABC) efflux transporters [29].

Although fungal primary metabolism is vital for their survival and

Abbreviations: DON, deoxynivalenol; *FG1*, *FG2*, *FG3*, *FG6*, *Fusarium graminearum* isolates; FHB, Fusarium Head Blight disease; HCA, hierarchical cluster analysis; HR, highly resistant; KEGG, Kyoto Encyclopedia of Genes and Genomes; MR, moderate resistant; PLS-DA, partial least squares-discriminant analysis; TSP, 3-(trimethylsilyl)-propionic acid-2,2,3,3-d4-sodium salt; *WT*, wild-type; ZEA, zearalenone

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responses to stresses, in contrast to the study of secondary metabolism [30–32], information on its regulation in response to fungicide treatments is yet largely fragmented. Within this context, as a proof-of-concept, we have undertaken a proton nuclear magnetic resonance (^{1}H NMR) spectroscopy metabolomics study in order to investigate the correlation between fungal genomes, metabolomes, and phenomes, focusing on fungal resistance to fungicides, and how the different fungal resistance levels are correlated with certain patterns of metabolism regulation. As model organism the plant pathogenic fungus *Fusarium graminearum* [telomorph: *Giberella zeae*, Ascomycete] was chosen, and the fungicide carbendazim, which belongs to the benzimidazole fungicides, was selected as the fungicide to be studied. Five *F. graminearum* strains with variable carbendazim-resistance levels to carbendazim, which were obtained in one of our previous studies [33], were selected.

F. graminearum is a very important plant pathogen that causes the Fusarium Head Blight disease (FHB) of small grain cereals, leading to severe yield losses [34]. In addition to quantitative losses, the pathogen causes significant qualitative losses due to the production of toxic metabolites know as mycotoxins, such as trichothecenes [*e.g.*, deoxynivalenol (DON), nivalenol (NIV)] and the oestrogenic zearalenone (ZEA), which pose threat for the human and animal health [35–37]. The latter, is a polyketide which is derived from the condensation of acetate units by polyketide synthases (PKSs) [38].

Although several approaches have been assessed for potential implementation in crop protection against *F. graminearum* [39,40], currently, crop management mainly relies on the use of synthetic fungicides. Benzimidazole fungicides used to be one of the most successful fungicide groups that had been extensively applied for more than three decades in the control of FHB disease [41]. It is an old group of fungicides, introduced in the late '60s, that exert their toxicity through the inhibition of the polymerization of β-tubulin during mitosis [42,43]. However, their efficacy against *F. graminearum* was dramatically decreased due to the selection and prevalence of resistant fungal strains [44]. Here, the fungicide carbendazim was selected for the study of *F. graminearum* resistance, which used to be the most commonly applied benzimidazole fungicide. Based on its mutagenic and reprotoxic activities it was recently banned from use in crop protection, however, it represents an excellent active ingredient for the metabolic study of fungal resistance. Molecular studies have indicated that target site modifications in the homologous β_2-tubulin gene binding are the most likely mechanism for fungal resistance to carbendazim [33,45].

Nonetheless, fungal resistance comes at a price, fitness-related costs; mutations in the β_2-tubulin gene could significantly affect the stability of microtubules and finally could reduce fungal fitness, which is a very important information for crop protection [46,47]. The effect of *F. graminearum* resistance to carbendazim on its fitness and mycotoxin biosynthetic capacity were investigated in a recent study [33], which revealed that the six carbendazim-resistant strains that were studied, exhibited variable mycotoxin-biosynthetic capacity and pathogenicity and reduced sporulation and conidial germination compared to the wild-type strain. The observed resistance was attributed to mutations in β_2-tubulin gene, whereas for two of the mutants, resistance was not attributed to such mutation. Nonetheless, no links between the resistant genotypes, metabolomes and phenotypes were made, and information on the topic is non-existent.

2. Materials and methods

2.1. Chemicals, reagents and inhibitors

Deuterium oxide (D_2O) of 99.9% purity, containing 0.05% v/v 3-(trimethylsilyl)-propionic acid-2,2,3,3-d4-sodium salt (TSP) (Sigma-Aldrich, St. Gallen, Switzerland), was used in ^{1}H NMR analyses. The fungicide carbendazim (99.0%, *v*/v) was kindly provided by Bayer CropScience AG (Monheim am Rhein, Germany). Stock solutions of carbendazim were prepared in HPLC-grade ethanol (Thermo Fisher Scientific GmbH, Darmstadt, Germany) at a concentration of 300 µg mL^{-1}, which were stored at −25 °C until further use.

Table 1
Wild-type *(WT)* and carbendazim-resistant *Fusarium graminearum* strains used in the study.

Strain	Rf[a]	Resistance basis	Phenotype[b]
CBS 110261 (*WT*)	1	Wild-type	Sensitive
FG1	174.6	Unknown	*HR*
FG2	42.09	Unknown	*HR*
FG3	13.53	Mutation at codon 6 of β_2-tubulin ($A \rightarrow N$)	*MR*
FG6	21.24	Mutation at codon 200 of β_2-tubulin ($F \rightarrow Y$)	*MR*

[a] Resistant factor to carbendazim; the ratio of EC_{50} for the individual resistant mutant to the EC_{50} of the *WT*.
[b] HR; highly resistant, MR; moderately resistant.

2.2. Biological material

The isolate CBS 110261 of *F. graminearum* (wild type, *WT*) was obtained from the CBS Fungal Biodiversity Centre (Royal Netherlands Academy of Arts and Sciences, KNAW, Amsterdam, The Netherlands). The *F. graminearum* isolates *FG1*, *FG2*, *FG3*, and *FG6* (Table 1) had been obtained from the parental isolate CBS 110261 by UV mutagenesis as previously described [33].

2.3. Culture conditions and bioassays

Starter cultures of *F. graminearum* strains were grown on potato dextrose agar (PDA, Neogen, Auchincruive, Scotland, UK) in Petri plates (9 cm in diameter), at 25 °C in the dark. The inoculum that was used in the experiments consisted of 5-mm in diameter culture plugs taken from the edges of 6-day old starter cultures using a cork borer. All handling and bioassays were performed under aseptic conditions in a laminar flow cabinet following good laboratory practice (GLP) and standard operating procedures (SOP). The plugs were then placed in the center of sterile cellophane membranes (9-cm in diameter, 500 PUT, UCB, North Augusta, USA) in a Petri plate (9-cm in diameter) containing 25 mL of PDA amended or not (untreated) with 2 mg L^{-1} of carbendazim, which is the *WT*'s sub-lethal concentration [33], or 10 mg L^{-1}. All fungal isolates, including the *WT*, were treated with 2 mg L^{-1} carbendazim, whereas the high concentration of 10 mg L^{-1} was applied only to the *FG1* and *FG2*, which exhibit high-resistance level to the fungicide.

In total, fifteen cultures were used per treatment (biological replications). Ten days after treatments, mycelia were carefully collected from the surface of the cellophane membranes using a spatula. Collected hyphae from five cultures were pooled to provide one pooled sample in plastic falcon tubes (50 mL), which was immediately flash frozen in liquid N_2 for metabolism quenching. In total three pooled samples (obtained from the fifteen biological replications), and a quality control (QC) sample were obtained for each of the treatments. Samples were immediately extracted for ^{1}H NMR metabolomics.

2.4. Sample preparation and extraction for ^{1}H NMR metabolomics

Sample preparation and extraction for ^{1}H NMR metabolomics was performed as previously described [16], with minor modifications. Briefly, the obtained pooled samples were pulverized to a fine powder in a mortar with a pestle using liquid N2 and transferred to falcon tubes (50 mL) and then, they were stored at −80 °C until further processing. For the removal of water, sub-samples (100 mg each) were lyophilized for 24 h. Polar metabolites were extracted by adding D_2O (0.8 mL) to the lyophilized samples in Eppendorf tubes (1.5 mL). Initially,