Metabolomics in Foods: Analysis of Secondary Metabolites in Wheat using LC-QTOF/MS

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Abstract

Metabolomics is the study of the specific profile of small molecules and metabolites produced by an organism. Metabolites can act as signals, growth regulators and plant defense agents. In this study, production of secondary metabolites as response to a plant disease called Fusarium Head Blight (FHB), which has been a persistent problem in small grains with worldwide outbreaks was investigated. Wheat samples with distinct genetic background and variation in disease susceptibility were inoculated with Fusarium graminearum, plant tissues were collected and secondary metabolites were extracted. For metabolic profiling of wheat, ultra-performance-liquid-chromatography–quadrupol-time-of-flight (UPLC-QTOF) mass-spectrometry was used. Current experiments resulted in the differentiation of clusters of compounds in response to different treatments. Further analysis of the data will cover the metabolite interactions of the infection process, resulting in information that has not been reported before.

Key words: metabolomics; LC-QTOF/MS; wheat; Fusarium Head Blight

1. Introduction

Wheat (Triticum aestivum) is a very important food grain throughout the world. High levels of relative humidity during cultivation and maturation and insufficient drying during harvesting and storage of grain can lead to fungal activity and mycotoxin production. This strongly reduces the value of food and feed [1]. Many species of Fusarium are globally important pathogens of wheat. Fusarium Head Blight (FHB) is mainly caused by Fusarium graminearum Schawabe (teleomorph: Gibberella zeae (Schweinitz) Petch) and Fusarium culmorum (W.G. Smith) Saccardo [2]. The above mentioned fungal species infect the floral tissues, seedlings; stem bases and roots causing FHB, as well as seedling blight, crown rot and root rot, respectively [3, 4]. Of all the diseases mentioned above, FHB is the one with the greatest significance worldwide, being one of the most destructive wheat pathologies, having both negative economic and health impacts[5]. The disease manifests as premature bleaching of the wheat heads. The resulting low yield and quality loss can be counted in millions of dollars per year in the USA alone [6].

FHB of wheat has become a major problem in the Eastern and Midwestern regions of the United States. Epidemics have caused extensive damage through direct losses in yield, test weight and by price discounting due to the presence of Fusarium-damaged kernels. This is associated with mycotoxins, mainly the trichothecene deoxynivalenol (DON) [7, 8]. The most common pathogen associated with FHB in the Upper Midwest is Fusarium graminearum and its teleomorph
Gibberella zeae [9]. There is known variation for susceptibility to FHB, with examples having high and low susceptibility, but no wheat or barley variety is immune to it [10].

Host resistance and reaction of resistant wheat cultivars to FHB infection is quite complex and are often classified as passive or active. Passive mechanisms may include phenological and morphological traits such as plant height, the presence of awns, spikelet density, and time to flowering [11]. Active mechanisms of FHB resistance have been classified as involving a number of resistance components: 1) resistance to invasion (type I); 2) resistance to spreading (type II); 3) resistance to mycotoxin accumulation (type III); 4) resistance to kernel infection (type IV); and 5) tolerance (type V) [12]. In the case of wheat, type II resistance is considered the most important [13]. While other resistance mechanisms may be ongoing, aside from type II resistance seen on cultivar sources like Sumai 3 and Ning 7840 [14, 15], the genetics of the other types of resistance remain to be discovered.

To fully understand the mechanisms of resistance or susceptibility of FHB, there is a need to determine the molecular pathways and interactions that appear after defense genes respond. The activation and possible interactions of these genes produce molecules that have an effect at some point either in the plant or the pathogen. In this view, metabolic profiling is another strategy for screening the wide array of metabolites or so called “small-molecules” that appear as a response to a stimulus such as a pathogen. Metabolites are highly regulated, integrated and integrating components of the cell, with roles that go from energy and redox control to defense; from structural integrity to signaling. Therefore, studies on these molecules yield direct insight into the points of cellular control of metabolism and many other biological processes [16].

There is a wide diversity of plants and with this also a diverse physicochemical property of many common plant metabolites such as: monoterpenoid volatiles, polar amino acids, or hydrophobic lipids can result in a very complex analytical experiment [17]. Although metabolomic plant analyses aim at the simultaneous detection of all metabolites in plant tissues, no single extraction/separation/detection methodology will satisfy a total profile of the metabolome [17]. Current metabolite profiling methods cover only a fraction of the metabolite complement of the cell. Plants represent a great challenge but also huge opportunities due to the diversity and richness of phytochemicals as well as its ample range of concentration. A range in between 100000–200000 metabolites has been calculated to be present in the plant kingdom [18].

In an attempt to detect, typify and quantify the diversity of compounds, various approaches have been taken: separation techniques like liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE) [19-21] and detection technologies like nuclear magnetic resonance (NMR) and mass spectrometry (MS) are widely used [22]. GC-MS has proven over the years to be a robust and liable tool for the detection of volatile organic compounds [22]. GC-MS is mainly appropriate for compound classes appearing in the primary metabolism (amino acids, fatty acids, carbohydrates and organic acids) Fiehn et al, [23] made possible by derivatization via volatile trimethylsyl (TMS) groups by addition of N-methyl-N-trifluoroacetamide (MSTFA) [24]. LC–MS is more suitable for detecting the overall biochemical diversity of plants. It does not need the prior derivatization of samples to make metabolite groups of interest available for detection. It has been shown to be appropriate for the detection of a wide range of metabolite classes [22, 25]. LC-MS techniques cover the large (semi-polar) group of plant secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, polyamines and derivatives. In addition, LC-MS can detect various
primary metabolites depending on the type of stationary phase use [25]. Notice should be taken that although the number of peaks that may appear may be excessive, they remain as “unknown” until confirmed authentic standards [16].

In the past few years, metabolite profiling studies that discriminate resistance from non-resistant wheat; identification of potential resistance metabolites as biomarkers in potential FHB resistant wheat lines has been performed. These studies relied on GC/MS for processing the samples finding, encountering compounds like fatty acids, aromatic Compounds, p- and m-coumaric acids, myo-inositol and other sugars, malonic acid, amino acids, fatty acids, and aromatics as unique to the plant-pathogen interaction. The use of this technology is prone to detect only relatively low molecular weight compounds [26-29]. Another study uses GC-EI-TOF-MS (Gas Chromatography coupled to Electron Ionization-Time-of-flight mass spectrometry) aimed mainly at polar compounds. They managed to identify several of the resistance related metabolites detected in cultivars which had several FHB resistance QTLs [30].

2. Materials and Method
2.1. Materials: The seeds of hard red spring wheat varieties Glenn, Steele ND and Reeder exhibiting type II resistance to FHB in different degrees, were obtained from North Dakota State University, Department of Plant Sciences. The three varieties were selected based on the North Dakota Hard red Spring Wheat variety trial results for 2011 [31]. Glenn is hard red spring wheat developed at NDSU released by the North Dakota Agricultural Experiment Station (NDAES) in July 2005. It is classified as moderately resistant [32]. Steele ND Steele-ND is hard red spring wheat developed at the NDSU and released by the NDAES in January 2004. It exhibits a moderate level of resistance to FHB [33]. Reeder is hard red spring wheat developed by the North Dakota Agricultural Experiment Station and released by the North Dakota Research Foundation in 1999. It is susceptible to FHB indicated by a 42% of incidence in Greenhouse studies [32].

2.2. Green House Studies: The experiments were designed as randomized complete blocks (RCBD). The experimental scheme is shown in figure 1.

Figure 1. General schematic to determine the type of metabolites related to HRSW plant defense in as a response to *F. graminearum* inoculation.
This consisted of three blocks (replications) with the wheat genotypes within blocks being randomized. For the wheat variety sample set, there were 6 treatments: three cultivars (low, medium and high resistance) combined with two inoculations: water or pathogen. The sample unit for metabolite profiling in both objectives consisted of a pooled sample of 16 spikelets harvested 24 hours after inoculation (hai) or within a time course. Four central spikelets of each of the four spikes of four plants from each cultivar were inoculated with the pathogen. Another batch was mock inoculated with water. The FHB symptoms on pathogen inoculated/mock inoculated wheat spikelets were assessed non-destructively over a period of 21 dai. These results were be treated with ANOVA, using an appropriate statistical package [29].

2.3. UPLC-LC QTOF MS analysis: Data acquisition on metabolic profiles was subjected to further processing using the LC QTOF MS Mass Hunter Qualitative (for alignment and molecular feature extraction) and Mass Hunter Professional (statistical analysis).

3. Results

3.1. Principle component analysis

After the samples were grown, inoculated, collected, extracted and analyzed with UHPLC QTOF/MS, the raw data from the Mass Hunter acquisition software recursive analysis of the data was conducted using Mass Hunter Qualitative (MHQual) and Mass Profiler Professional (MPP) software. The first step of the recursive analysis involved the use of MHQual software to run the molecular feature extraction (MFE) algorithm. Principle component analysis (PCA) was performed and the PCA plot is shown in figure 2.

![Figure 2. Principle component analysis of inoculated and mock wheat variety samples](image)

This showed that the largest source of variation is the difference between Reeder and the other two genotypes. The second largest source of variation is between the varieties Steele-ND and Glenn. For the most part there is very small variation between mock and inoculated samples within the same genotype. Of the 3 genotypes, Glenn shows the most variation between the mock and inoculated treatments. Steele-ND shows less variation between mock and inoculated samples and there is even less variation between the mock and inoculated samples of Reeder.
3.2. Comparison of compounds found in genotypes

The number of features (compounds) in the samples was determined and a Venn diagram (Figure 3) showed the number of compounds found. The total number of compounds found in wheat samples inoculated with F. graminearum was 532, 636 and 565 for Glenn, Reeder and Steele-ND, respectively.

![Venn diagram of compounds found in wheat samples inoculated with F. graminearum](image)

Figure 3. Venn diagram of compounds found in wheat samples inoculated with *F. graminearum*

There were 421 compounds that were found in common for all 3 wheat varieties. There were some compounds that were found to be unique to specific varieties. Reeder had the most compounds (133) that were unique to that variety. The number of compounds which were found to be unique to Steele-ND (47) and Glenn (34) were much lower.

An example of the volcano plots of the wheat samples is shown in Figure 4. This volcano plot given is an example and many more volcano plots were produced for the purpose of statistical analysis and comparison of the samples. The volcano plots show a log plot of the p-value and the fold change of the metabolites. The p-value was set to 0.05 and the fold change was set to two. The higher on the plot a metabolite is, the more significant is the metabolite and the farther from the center the metabolite is, the higher the fold change. The compounds which are significantly (P<0.05) different by at least a 2 fold change are represented by the red squares in the volcano plots. The parameters for creating the volcano plots in MPP can be selected by the user so that different levels of significance or fold changes may be chosen. This allows for narrowing or widening of the determination of which compounds are of interest. Also, the compounds found to be significant by the volcano plot (or other statistical analysis used in MPP) can be compiled into a list for further analysis or for compound identification.

There are many more compounds which are found to be significantly (P<0.05) different by at least a 2 fold change between the inoculated Reeder and mock Reeder samples than were found between treatments in the other 2 varieties. For Steele-ND and Glenn many compounds were found to be significantly (P<0.05) different between treatments but by less than a 2 fold change. The data analysis using MPP was able to determine significant (P<0.05) differences among the wheat varieties and sample treatments (inoculated or mock).
When all inoculated samples were compared to all mock samples, regardless of variety, 20 of 835 compounds were found to be significantly different (P<0.05) by at least a 2.0 fold change. The comparison of the varieties regardless of treatment showed that there were 82 of 796 compounds significantly (P<0.05) different by at least a 2 fold change between Glenn and Reeder, 185 of 674 compounds were significantly (P<0.05) different by at least a 2 fold change between Glenn vs Steele-ND and 77 of 803 compounds were significantly (P<0.05) different by at least a 2 fold change between Reeder and Steele-ND. The analysis also was able to determine that there were 20 of 594 compounds significantly (P<0.05) higher by at least a 2 fold change in inoculated Glenn vs mock Glenn. Among inoculated Reeder vs mock Reeder samples, 114 of 704 compounds were found to be significantly (P<0.05) higher/lower by at least a 2 fold change. For Steele-ND, inoculation with *F. graminearum* resulted in 20 of 618 compounds that were significantly (P<0.05) higher by at least a 2 fold change. Comparison of Glenn inoculated vs Reeder inoculated showed 76 of 733 compounds which were significantly (P<0.05) different by at least a 2 fold change. It was also found that, 19 of 642 compounds were significantly (P<0.05) different by at least a 2 fold change in Glenn inoculated vs Steele-ND inoculated. Differences were also found in Reeder inoculated vs Steele-ND inoculated, such that 69 of 739 compounds were significantly (P<0.05) different by at least a 2 fold change. The compounds determined to be significantly (P<0.05) different in samples which were inoculated with *F. graminearum* may be produced by the wheat or by the pathogen after inoculation. However, further work needs to be done to identify these compounds and to determine their source.

### 4. Discussion

The results of these experiments show some interesting trends and relationships between the wheat varieties used and their level of susceptibility to FHB infection. The PCA plot (Figure 2) shows clear groupings among the samples. The samples of the same genotype are grouped together, while each of the genotypes show variation between each other. This means that there is less variation, within a single genotype, created by inoculation with FHB than the variation
between genotypes. It is interesting that the largest source of variation is the difference between Reeder and the other two varieties since Reeder has high susceptibility to FHB infection, which may be driving the variation between Reeder and the other 2 varieties. Although the PCA shows small amount of variation between mock and inoculated samples, these variations may still be highly significant. The genotype Glenn shows the most variation between the mock and inoculated treatments. There is a clear trend associated with the level of susceptibility of the genotypes to FHB and the amount of variation seen between the mock and inoculated treatments. The amount of variation between mock and inoculated treatments increases with the level of FHB susceptibility. This is of high interest since the genotypes with lower levels of susceptibility may be producing compounds which could be related to their host/pathogen response. Work by Hamzehzarghani et al. [27], has also shown differences in level of susceptibility among wheat lines are able to be categorized based on differences in their metabolic profiles.

Further analysis of the metabolic profiles of these 3 HRS wheat genotypes was done to determine the amount and significance of the differences between genotypes and between the mock and inoculation treatments. The Venn diagram (Figure 3) clearly showed that Reeder has more compounds which are unique to that genotype than the other two genotypes. This data along with the numbers of significantly (P<0.05) different compounds shown in the volcano plots (Figure 4) shown for each variety corroborate the information shown in the PCA plot (Figure 2). Although there is more work to be done with the data from this study, the results of the PCA, Venn diagram and volcano plots (statistical analysis) all support each other strongly. Other research has also shown differences in the metabolites produced by wheat during host/pathogen interaction that were related to the level of susceptibility to FHB [26, 27]. Overall, the numbers of compounds to be found statistically difference between mock and inoculation treatments, as well as between genotypes were highly variable. The relationship between the host and pathogen and the effects on metabolite production is highly complex [27].

5. Conclusions

Overall, metabolic profiling of 3 HRS genotypes varying in FHB susceptibility has been partially completed. Initial analysis has shown differences in compounds between inoculated and mock samples for all 3 genotypes. Significant variation was seen in the compounds detected between each of the 3 genotypes with varying FHB susceptibility. The results of each of the three data visualization and analysis techniques strongly support each other. There is also a clear relationship between the level of FHB susceptibility and the amount of variation seen between the mock and inoculated treatments for the HRS wheat genotypes. There is a considerable amount of research which remains to be done regarding metabolomic profiling of wheat, and response to FHB infection. Continued analysis of data wheat genotypes will need to be done. Also it will be important to work on identification of unique compounds, using various databases, in relation to FHB susceptibility of HRS wheat. Finally, pathway analysis using identified compounds from the database searches may be completed.

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References


