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Analytical Methods

Untargeted metabolomics based on ultra-high-performance liquid chromatography–high-resolution mass spectrometry merged with chemometrics: A new predictable tool for an early detection of mycotoxins

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ABSTRACT

In order to explore the early detection of mycotoxins in wheat three standardized approaches (*Fusarium* disease severity, PCR assays for *Fusarium* spp. identification and mycotoxin quantification) and a novel untargeted metabolomics strategy were jointly assessed. In the first phase of this research, standardized approaches were able to quantify mycotoxins and identify *Fusarium* spp. Then, an UHPLC-QTOF metabolic fingerprinting method was developed to investigate plant-pathogen cross-talk. At the same time, chemometrics analysis demonstrated to be a powerful tool in order to distinguish low and strong infection levels. Combining these results, the cross-talk plant pathogen related to the early detection of mycotoxins was discovered. As a rapid response to fungal infection an overexpression of phosphatidic acids was discovered. By contrast, when the infection became stronger an increase of oxylipins and diacylglycerols was revealed.

1. Introduction

Cereals represent one of the most important commodities providing basic nutrients to human diet, since they are rich sources of carbohydrates, proteins, fats, minerals and vitamins. Among them, the average global annual production of wheat was estimated by FAO as 663 million tones (period 2004–2014) (FAO, 2016). In fact, wheat is a crop of many talents; wheat and wheat-based products are used in several sectors, such as food, feed, biofuel, cosmetics and bio-based plastics (Shewry, 2009). Nevertheless, the main sector is the food industry, where wheat is generally ground into flour and is used, among many others, for bread, pasta, and biscuits. Next to wheat flour, the milling process of the grains also produces bran, which is used as food and animal feed ingredients.

The need of specific characters in terms of nutritional and technological properties has increased the breeding pressure towards similar, high quality varieties. Unfortunately, this has led to an increase of susceptibility towards pathogenic diseases due to colonization by various toxicogenic fungi (i.e. *Fusarium* spp.), and subsequent production of secondary metabolites, called mycotoxins (Kumar, Basu, & Rajendran, 2008). *Fusarium* Head Blight (FHB) is the most common fungal disease in small grains occurring worldwide, caused mainly by *F. graminearum* and *F. culmorum* infection (Bottalico & Perrone, 2002; Müllenborn, Steiner, Ludwig, & Oerke, 2008). It is seen most commonly on spring and winter wheat, durum and barley. FHB can cause significant yield losses, quality reductions and accumulation of *Fusarium* mycotoxins, mainly those from the group of trichothecenes, enniatins, and zearalenones. In addition to production of mycotoxins as compounds causing various acute and chronic adverse health effects,

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the *Fusarium* pathogens also usually influence the qualitative and quantitative aspects of the crop yield (Richard, 2007). For this reason, legislated and modified mycotoxins are routinely monitored in cereal grains (McCormick et al., 2015; Nathanail & Syvähuoko et al., 2015; Rubert et al., 2012).

In order to reduce the crop loss, together with the costs of managing noncompliant batches has prompt the search for chemical markers able to identify possible contamination at the earliest stage, and to univocally characterize resistant varieties and infection. In this context, a question of potential early detection of this fungal pathogen on the wheat crop has been arisen.

The early detection of toxigenic fungi directly on cereals can be useful to put an end to the intake of these contaminated materials into the food and feed chain. Initially, these toxigenic fungi have been traditionally identified by microbiological and immunological methods or polymerase chain reaction (PCR) (Huet et al., 2010; Mishra, Fox, & Culham, 2003). Besides classical microbiological and/or PCR-base methods, innovative spectral techniques (i.e. imaging analysis, near-infrared, Raman) have been proposed for the early detection of colonizing fungi (Berardo et al., 2005; Del Fiore et al., 2010). Since fungal growth is not strictly related to mycotoxin accumulation, and to the pattern of occurring mycotoxins, these techniques – although very simple and effective – cannot provide an univocal response on mycotoxin occurrence. On the other side, the identification of specific chemical markers, mainly linked to the plant-pathogen cross-talk, could drive the selection of resistant wheat varieties, and thus support breeding programs. In this frame, metabolomics may represent the golden tool for understanding the biological pathways involved in mechanisms of plant resistance (Cajka et al., 2014; Rubert, Zachariasova, & Hajslova, 2015).

The plant-pathogen cross-talk leading to FHB and mycotoxin accumulation has been significantly studied over the last decade, but the scientific community is still far from a comprehensive scenario, in consideration of the complexity of genetic and environmental factors affecting this interaction (Cajka et al., 2014; Gauthier, Atanasova-Penichon, Chéreau, & Richard-Forget, 2015; Nathanail & Varga et al., 2015; Warth et al., 2015). Recently, Cajka et al. (2014) have developed an analytical procedure optimizing a solid liquid extraction procedure using methanol/water (50:50, v/v) in order to isolate polar/medium-polar barley metabolites followed by ultra high performance liquid chromatography quadrupole-time-of-flight (UHPLC-QTOF). In this research, positive ionization data highlighted a superior discrimination power. In this way, control barley and *Fusarium* infected barley samples were successfully distinguished. In fact, plant stress-related metabolites such as jasmonic acid (JA) or dihydro-7-hydroxymyoporone showed up higher concentrations and correlated positively with increasing concentrations of deoxynivalenol (DON) and its modified forms. Focusing on wheat, a profiling metabolomics strategy has been performed using a stable isotopic labelling approach in order to understand the metabolic fate of HT-2 toxin and T-2 toxin in wheat (*Triticum aestivum L.*) (Nathanail & Varga et al., 2015). The authors demonstrated that the exposure of wheat to either HT-2 toxin or T-2 toxin primarily activates metabolic reactions involving hydroxylation, (de)acetylation, and various conjugations. Furthermore, kinetic data revealed that detoxification progressed rapidly, resulting in the almost complete degradation of the toxins, within 1 week, after a single exposure. In parallel, DON accumulation and *Fusarium* infection in cereals have been recently reviewed by Gauthier et al. (2015) in order to interpret chemical defenses. In this review, the authors have clearly described that when mycotoxins were accumulated the major chemical defenses of the plant cell were related to carbohydrates and amino acid metabolism. These evidences have been recently confirmed by Warth et al. (2015) based on a GC–MS based metabolomics workflow. In this research, DON treatment modified both the primary carbohydrate metabolism and the primary nitrogen metabolism of the plant, and amino acid levels were significantly increased.

Studies aimed at depicting the resistance/susceptibility of grains towards FHB are usually based on artificial grain inoculation in collection fields, in order to decrease natural variability and highlight significant effects. Giorni et al. (2015) reported, on the contrary, the identification of lipid markers of infection in maize naturally infected by *F. verticillioides* under open field conditions. Although the experimental plan involved only few maize varieties in a large number of replicates, the increased variability due to open field conditions affected positively the robustness of the statistical model (Giorni et al., 2015).

The main aim of this research work was to develop a novel metabolomics strategy exploitable for the early recognition of *Fusarium* disease, based on the detection of infection-related metabolites. For this purpose, a set of eighty-six naturally contaminated wheat samples was available. For the proper metabolomics data interpretation, determination of *Fusarium* disease severity was visually determined and *Fusarium* spp. were identified by PCR assays. Subsequently, targeted mycotoxins were quantified by a validated analytical method. In the second phase, an untargeted metabolomics strategy was optimized. First, several extraction solvents and mixtures of them were studied in order to extract the bulk of information, and then an UHPLC-QTOF method was developed to separate and detect metabolites isolated. Afterwards, advanced chemometric tools were used for wheat samples clustering, and metabolic pathways elucidation.

2. Material and methods

2.1. Chemicals and reagents

Polytetrafluoroethylene (PTFE) 50 mL centrifugation cuvettes were obtained from Merci (Praha, Czech Republic). HPLC grade methanol, ethanol, dichloromethane, 2-propanol and hexane were purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were supplied by Sigma–Aldrich (St. Luis, MO, USA). Water was purified by Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Plant material

Altogether, 86 naturally contaminated winter wheat samples (harvest 2012) from the Czech Republic were analyzed within this study. All the samples were collected by the Central Institute for Supervising and Testing in Agriculture as a part of long-term study focused on FHB symptoms assessment and determination of mycotoxins (Chrpová et al., 2016). Regarding the sampling strategy, 25 randomly selected wheat ears from different places of each field were collected and further analyzed.

2.3. Standardized approaches

2.3.1. Visual determination of Fusarium disease severity

The extent of *Fusarium* disease severity was realized at the Crop Research Institute (Prague, Czech Republic). These experiments were visually determined using a 10-point scale (0–9; 0 – no symptoms up to 9 – severe symptoms) introduced by Schaller and Qualset (1980). Description of each level of *Fusarium* disease severity is described Table 1.

2.3.2. DNA extraction and PCR assays for species identification

For the purpose of *Fusarium* species identification, PCR assays were used, as it was recently described by Chrpová et al. (2016).