Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.082576

ARTICLE

Metabolic Responses of Wheat Spike Tissue Associated with *Fusarium* Infection in Genotypes Differing in Type II Resistance

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Received: 18 March 2026; Accepted: 18 May 2026; Published: 30 July 2026

ABSTRACT: In this study, metabolomic profiling using gas chromatography–mass spectrometry (GC-MS) revealed clear distinctions between *Fusarium*-infected and control wheat spikes, as shown by the separate clustering of infected samples. Out of 222 detected features, eight were found to be significantly altered in infected spikes compared to controls, highlighting specific metabolic changes associated with the plant's response to Fusarium head blight (FHB) stress. In response to *Fusarium* infection, wheat exhibited significant metabolic reprogramming, with both reductions and accumulations of key metabolites. A decrease in 3-methoxytyramine (28.9–85.3%), octylamine, malic acid, and homonojirimycin suggests their early involvement in defence-related pathways either through rapid turnover, utilization in stress-induced lipid remodelling, energy diversion, or conversion into downstream protective compounds. Conversely, the accumulation of metabolites such as cis-aconitic acid, N-hexanoyl homoserine lactone, ribonic acid, and 1,3-diaminopropane points to disrupted central metabolism, increased microbial signalling activity, oxidative sugar degradation, and heightened polyamine catabolism. Together, these shifts reflect a coordinated host response involving energy redistribution, signalling modulation, and metabolic adjustments aimed at limiting pathogen spread and damage. Collectively, these findings provide insights into the metabolic responses associated with *Fusarium infection* in wheat genotypes that differ in type II resistance. Also, these results characterize metabolic responses to *Fusarium* infection in wheat genotypes differing in Type II resistance, used as a phenotypic reference of disease spread.

KEYWORDS: *Fusarium*; GC-MS; metabolites; type II resistance; winter wheat

1 Introduction

Fusarium head blight (FHB), a dangerous wheat disease, is mainly caused by *Fusarium graminearum* Schwabe and *F. culmorum* [1]. *Fusarium* fungi are not only causing significant grain yield losses, but also contaminate grains with mycotoxins. Mycotoxins are secondary metabolites that are harmful to human and animal health. A positive correlation was observed between the levels of FHB-associated metabolites in wheat grains and the severity of FHB symptoms [2]. This suggests that metabolite accumulation may be closely linked to the timing and progression of infection. The most susceptible stage of wheat plants for infection by FHB is the anthesis stage [3]. Although varietal susceptibility and pathogen aggressiveness affect disease development, climatic conditions during anthesis, particularly temperature and rainfall, play a key role. Cultivation of FHB-resistant genotypes represents the most effective and sustainable

strategy for controlling this disease [4]. However, FHB resistance is quantitatively controlled by the combined effects of many small to medium effect quantitative trait loci (QTL). Five different types of wheat resistance to FHB disease are known. Type I resistance prevents initial infection, Type II resistance refers to resistance to the spread of symptoms, while Type III resistance is defined as resistance to mycotoxin accumulation [5]. Type IV resistance reflects resistance to kernel infection in wheat and Type V refers to grain yield tolerance [6]. Although FHB resistance is commonly classified into five resistance types, these components are often interconnected, and individual wheat genotypes may express multiple resistance mechanisms simultaneously. In the present study, we specifically focused on Type II resistance, which reflects the plant's ability to restrict pathogen spread within the spike. In general, wheat genotypes differ in the type and degree of resistance to FHB; however, none is fully immune because of pathogen adaptability, variability in virulence, and strong environmental influences. Thus, FHB remains a serious threat to grain yield and quality, and ultimately global food security [7].

Compounding this issue, climate change continues to intensify challenges in wheat production by affecting growth conditions and promoting the spread of *Fusarium*, further jeopardizing the stability of global food supply [8]. To address this, there is an urgent need to accelerate wheat breeding through the application of modern biotechnological tools. One such approach, metabolomics, enables researchers to analyse plant metabolic responses in detail, facilitating the development of wheat genotypes with enhanced resilience to environmental stress. Metabolomics enables large scale analysis of metabolites and has become a powerful tool for uncovering how plants reorganize metabolic pathways to cope with stress [9]. Analysing stress-induced changes in metabolic pathways allows researchers to pinpoint key metabolites involved in plant defence responses and adaptation mechanisms [10].

Primary metabolites are essential for growth, development, and sustaining life processes, while secondary metabolites, derived from primary metabolites, play important roles in environmental responses, stress adaptation, and plant defence against biotic stress, including pathogen attacks [11]. Understanding the role of secondary metabolites in plant defence has prompted deeper investigations into the genetic basis of their production. Consequently, the complete genome sequencing of *F. graminearum* has identified an increasing number of functional genes. These genes are involved in the production of secondary metabolites, hyphal differentiation, sexual and asexual reproduction, virulence, and pathogenicity [12].

Previous studies have highlighted the involvement of specific metabolites and signalling pathways in wheat defence responses. For example, nitric oxide (NO) plays a signalling role in wheat defence against Fusarium, influencing downstream metabolic pathways and promoting the accumulation of defence-related compounds such as phenolics, flavonoids, and osmolytes [13,14]. In contrast, increased content of abscisic acid (ABA) have been associated with higher susceptibility to FHB [15], while less susceptible genotype exhibit upregulation of secondary metabolism following infection [16]. Additionally, p-aminobenzoic acid (PABA) has been shown to inhibit fungal growth and reduce FHB development under both *in vitro* and field conditions [17]. Dong et al. [18] demonstrated that the plant-derived metabolites such as phenylalanine and malate significantly suppressed *Fusarium* infection when the genes encoding these biosynthetic enzymes were upregulated in wheat spikes. Using some of the most resistant wheat lines, Dhokane et al. [19] reported a high accumulation of metabolites associated with the phenylpropanoid, lignin, glycerophospholipid, flavonoid, fatty acid, and terpenoid pathways. This finding was further supported by the upregulation of corresponding biosynthetic genes. To further explore how these signalling and hormonal pathways translate into resistance traits, metabolite profiles were examined in the grains of inoculated and control plants. The 25 metabolites that varied between control and *Fusarium* inoculated grains belonged to diverse functional groups, such as amino acids and amines, saturated and unsaturated fatty acids, polyphenols and

their derivatives, nucleotides, terpenoids, benzyl cyanides, small organic (carboxylic) acids, hydroxysteroids, and carbohydrates [20]. Despite extensive metabolomic studies on FHB, metabolomic signatures specifically associated with Type II resistance remain poorly defined, particularly during early stages of infection. We hypothesized that early metabolic responses to *Fusarium* infection differ among wheat genotypes exhibiting contrasting levels of Type II resistance, which is used here as a phenotypic reference of disease spread.

The aim of this study was to characterize metabolomic changes associated with *Fusarium* spp. infection in wheat genotypes differing in Type II resistance. Understanding genotype-specific metabolic responses will contribute to a deeper understanding of the wheat-*Fusarium* pathosystem and support the development of targeted breeding and management strategies for FHB.

2 Materials and Methods

2.1 Experimental Layout

Seeds of six winter wheat (*Triticum aestivum* L.) genotypes (Vulkan, Kraljica, Galloper, Tika Taka, El Nino, and Golubica) originated from Agricultural Institute Osijek (Croatia) were first sown in seedling trays and kept at room temperature to germinate. After five days, the trays containing the seedlings were transferred to a plant growth chamber for six weeks to undergo vernalization. During this period, plants were grown under controlled temperature, photoperiod, and humidity conditions. Following vernalization, the plants were transported into 2.5 L pots filled with soil (pH 5.5–7.0; organic matter 70.0–85.0%; nitrogen 100–200 mg L^{-1}; P_2O_5 100–150 mg L^{-1}; K_2O 200–400 mg L^{-1}) and moved to a greenhouse (Gis Impro d.o.o., Vrbovec, Croatia) four in randomized block design. Each treatment consisted of four biological replicates, with one pot representing one replicate, and containing four plants. These replicates represent independent biological units. The experiment was not independently repeated. Plants were grown under controlled greenhouse conditions with regulated temperature and photoperiod and irrigated as needed during the experiment.

2.2 Fusarium Inoculum Preparation

The *Fusarium* species used in this experiment were *Fusarium graminearum* (PIO 31), isolated from winter wheat collected in eastern Croatia, and *F. culmorum* (IFA 104), obtained from IFA-Tulln, Austria. One isolate per species was used. Aggressiveness was not measured because it is known to vary over time and decrease during subculturing, making single-timepoint measurements potentially unreliable. The aim of the study was to assess overall pathogenic effects rather than isolate-specific aggressiveness. The conidial inoculum was produced using a mixture of wheat and oat grains (3:1 by volume). The grains were soaked overnight in glass jars filled with distilled water. The next day, excess water was drained, and the grains were sterilized. To inoculate the grains with macroconidia, a piece of synthetic nutrient agar (SNA) containing fungal mycelium from each isolate was placed into the respective jars. The jars were then kept at room temperature under diffused daylight and shaken daily for two weeks to ensure proper aeration and drying. After colonization, macroconidia were washed off the grains with sterilized water, and the suspension was diluted. Final conidial concentrations of both fungi were determined using a hemocytometer (Bürker-Türk, Hecht Assistent, Sondheim vor der Rhön, Germany) and adjusted to 5×10^4 conidia mL^{-1}. The use of two inoculation events was intended to ensure uniform infection efficiency and is consistent with standard protocols for FHB assessment. The mixed inocula of *F. graminearum* and *F. culmorum* (50:50) were used to better reflect the natural co-occurrence of multiple *Fusarium* species in the field. This approach has been consistently applied in our previous studies, allowing methodological continuity and comparability of results. Mixed inoculation is widely accepted in resistance testing, as it provides a relevant composite

challenge for disease evaluation [21]. However, this approach does not allow discrimination between species-specific effects, and the results are interpreted as a combined response.

2.3 Inoculation Technique

When flowering stage began, marked by anther extrusion (Zadoks scale 61), plants were inoculated with a mixture of *F. graminearum* and *F. culmorum*. Each treatment included four replicates arranged in a randomized complete block design, with four plants per pot in each replicate. Untreated plants served as controls. A 20 µL aliquot of the prepared inoculum mixture was injected into the middle two spikelets of each plant's spike using an automatic pipette (Eppendorf, Wien, Austria). Each plant underwent two inoculation events, spaced two days apart. To promote infection, misting began one hour after each inoculation and continued for 36 h. During this period, forgers sprayed water every hour for 2 min. Spike tissue was sampled seven days after first inoculations when symptoms started to show up.

2.4 Type II Resistance

Type II resistance to FHB (resistance to disease spread within the spike) was evaluated by counting the number of infected spikelets on the *Fusarium* inoculated spike of a single plant per pot at 19 days post inoculation. Type II resistance represents one component of overall FHB resistance and was used in this study as a phenotypic reference trait reflecting disease spread within the spike. The 19 dpi time point was selected because disease symptoms were stable under our experimental conditions and this time point has been consistently applied in our previous studies. Disease assessment was performed on plants originating from the same biological replicates used for metabolomic analysis.

2.5 Metabolic Profiling

Metabolomic analysis was performed using four biological replicates per genotype and treatment, corresponding to four independently inoculated pots. Each biological replicate consisted of pooled spike tissue collected from four spikes (one spike per plant) within a single pot. Spike tissue sampled 7 days after inoculations was flash-frozen in liquid nitrogen and ground for 2 min in 10 mL tubes with a grinding ball using an automatic cryogenic grinder (Labman, Middlesbrough, UK). Polar metabolites were extracted from frozen spike tissue using a methanol–chloroform–water protocol following established GC-MS workflows for plant metabolite profiling [22–24]. Extracts were derivatized by methoxyamination and trimethylsilylation and analyzed on an Agilent 8890 GC coupled to a LECO Pegasus BT time-of-flight mass spectrometer operated in electron ionization mode at 70 eV. Metabolites were separated on a DB-35MS capillary column under a standard temperature gradient, and retention indices were calibrated using a C8–C30 FAME mixture. Metabolite identification was performed using ChromaTOF and the Golm Metabolome Database, and peak intensities were processed with the TargetSearch package in R [25].

2.6 Statistical Analysis

Determination of differences among genotypes for Type II resistance within FHB treatment was done using one-way analysis of variance (ANOVA), followed by Fisher LSD *post hoc* test ($p < 0.05$). Data processing and multivariate analysis, including partial least squares discriminant analysis (PLS-DA), heatmap analysis, and significant analysis of metabolites (SAM) were determined using MetaboAnalyst version 6.0. Partial least squares discriminant analysis (PLS-DA) was used solely as an exploratory visualisation method to assess overall patterns of group separation. Formal model validation metrics (R^2, Q^2) and permutation testing were not performed therefore, the PLS-DA model was not used for predictive purposes and results were

interpreted with caution. Greater emphasis was placed on univariate statistical analyses and consistently observed metabolite changes.

3 Results

3.1 Type II Resistance in Investigated Genotypes

The most pronounced FHB symptoms on 19-day post inoculation (dpi) were recorded in the genotypes El Nino, Golubica, and Tika Taka. The least severe symptoms were observed in the genotypes Vulkan and Kraljica (Fig. 1).

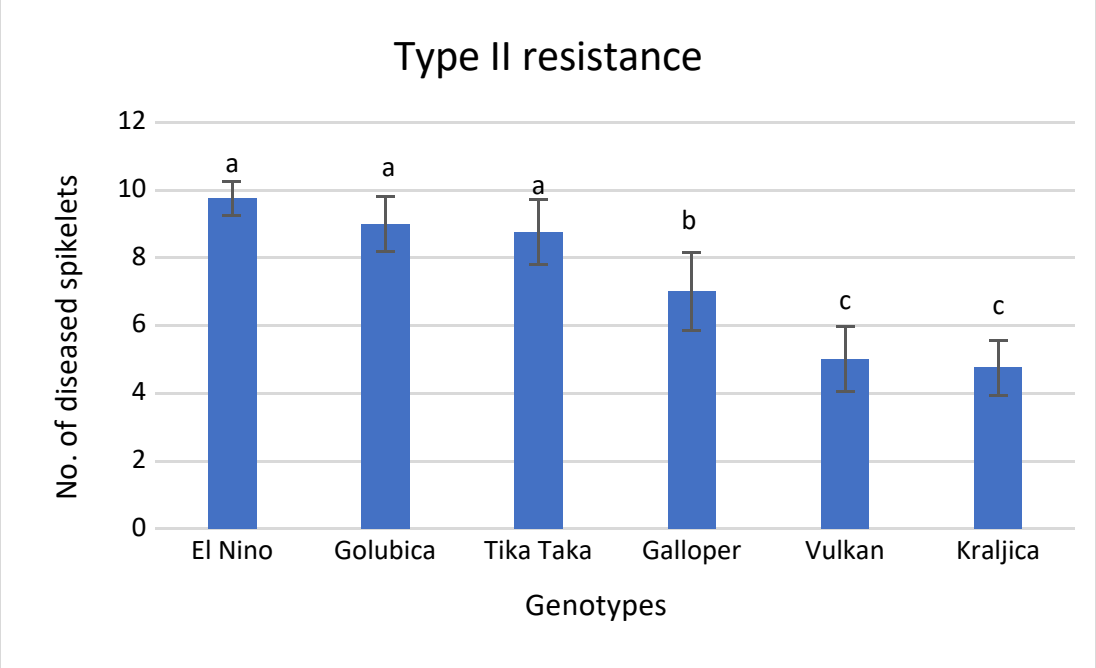

Figure 1: Type II resistance (resistance to disease spread) to Fusarium head blight (FHB) measured at 19 days post inoculation. Bars represent mean values of four independent biological replicates ± SD. Different small letters indicate significant difference between genotypes ($p < 0.05$). Higher value indicate lower FHB resistance.

3.2 Significantly Altered Metabolites between Control and Fusarium-Inoculated Treatments

A total of 222 metabolites were detected. Among all metabolic features analysed, eight metabolites, including organic acids (malic acid, cis-aconitic acid, ribonic acid), amines and polyamines (3-methoxytyramine, octylamine, 1,3-diaminopropane), iminosugar (homonojirimycin) and a signal-ling-related compound (N-hexanoyl homoserine lactone) showed significant changes between the control and *Fusarium* infected spikes ($p < 0.05$). These were selected for further analyses, and the SAM plot (Fig. 2) visualized these significant metabolites.

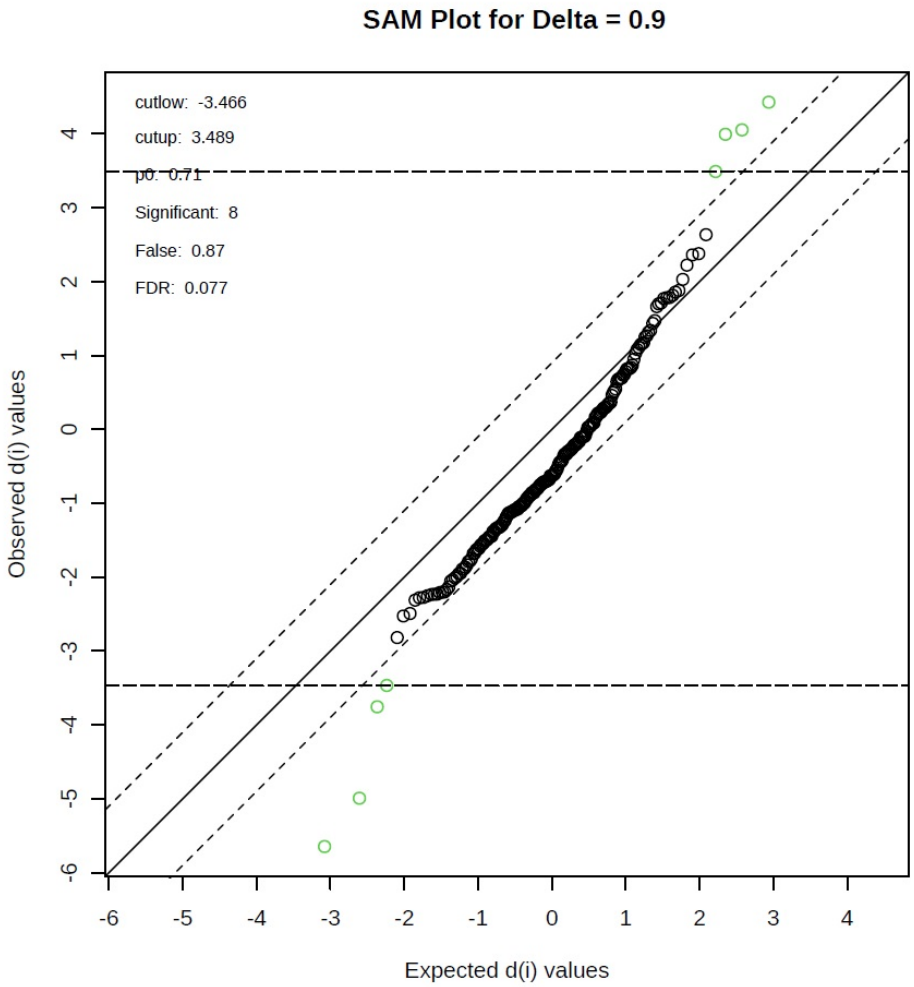

Figure 2: Significance analysis of metabolites (SAM) plot. The green points represent metabolites that are differentially regulated between control and *Fusarium* infected spikes. The solid diagonal line represents "observed = expected". The more the variable deviates from the "observed = expected", the more likely it is to be significant.

3.3 Genotypic Differences in Metabolic Response to Fusarium Infection

Subjecting the significant metabolite data to partial least squares discriminant analysis (PLS-DA) revealed high variability in the contributions of metabolites to *Fusarium* responses (Fig. 3). PC1 explained 27.6%, while PC2 explained 9.4% of the overall variability. All control samples (CS) tended to cluster together, suggesting similar metabolite profiles. Among *Fusarium* inoculations samples (FS), Golubica and El Nino were located in the upper left quadrant, compared to other genotypes under *Fusarium* treatment, indicating genotype-specific differences in their metabolic responses to *Fusarium* infection. Overall, the separation of the two treatment groups along PC1 indicates that the primary source of variation in metabolite composition was treatment-related. The distribution of genotypes along PC2 suggests that genotype-specific differences further influenced the metabolic response. This separation nevertheless indicates that *Fusarium* infection is associated with distinct metabolic shifts between treatments. However, as PLS-DA was not supported by formal validation metrics, these results are presented for exploratory purposes only.

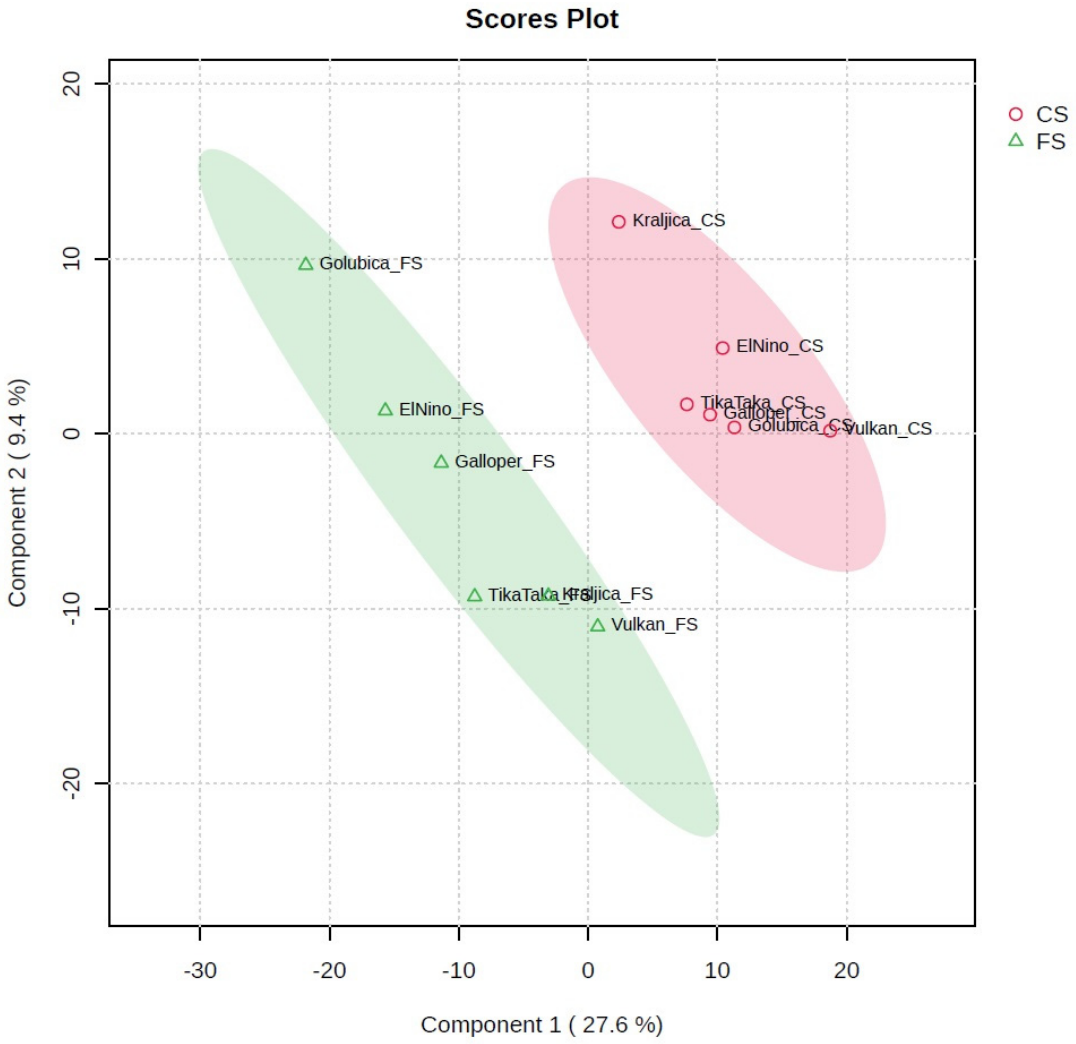

Figure 3: Partial least square discriminant analysis (PLS-DA) in spikes of control (CS) and six *Fusarium* (FS) infected winter wheat genotypes. Control samples and *Fusarium* infected samples did not overlap with each other indicating an altered state of metabolite levels in the *Fusarium* infected wheat spikes. PLS-DA was used as an exploratory visualization method.

Heatmap analysis further resolved the metabolic changes associated with *Fusarium* infection in wheat spikes and showed clear treatment and genotype related patterns (Fig. 4). Heatmap separated the CS and FS groups, confirming the clear metabolic reprogramming already evident before analysis. In all genotypes, except genotype Vulkan, the metabolites octylamine, 3-methoxytyramine, homonojirimycin, and malic acid were consistently reduced under *Fusarium* infection compared to the control samples. In contrast, 1,3-diaminopropane, ribonic acid, N-hexanoyl homoserine lactone, and cis-aconitic acid accumulated more under *Fusarium* infection compared to the control. Genotype specific trends were also recognisable. Vulkan showed a lower overall metabolite abundance in both treatments.

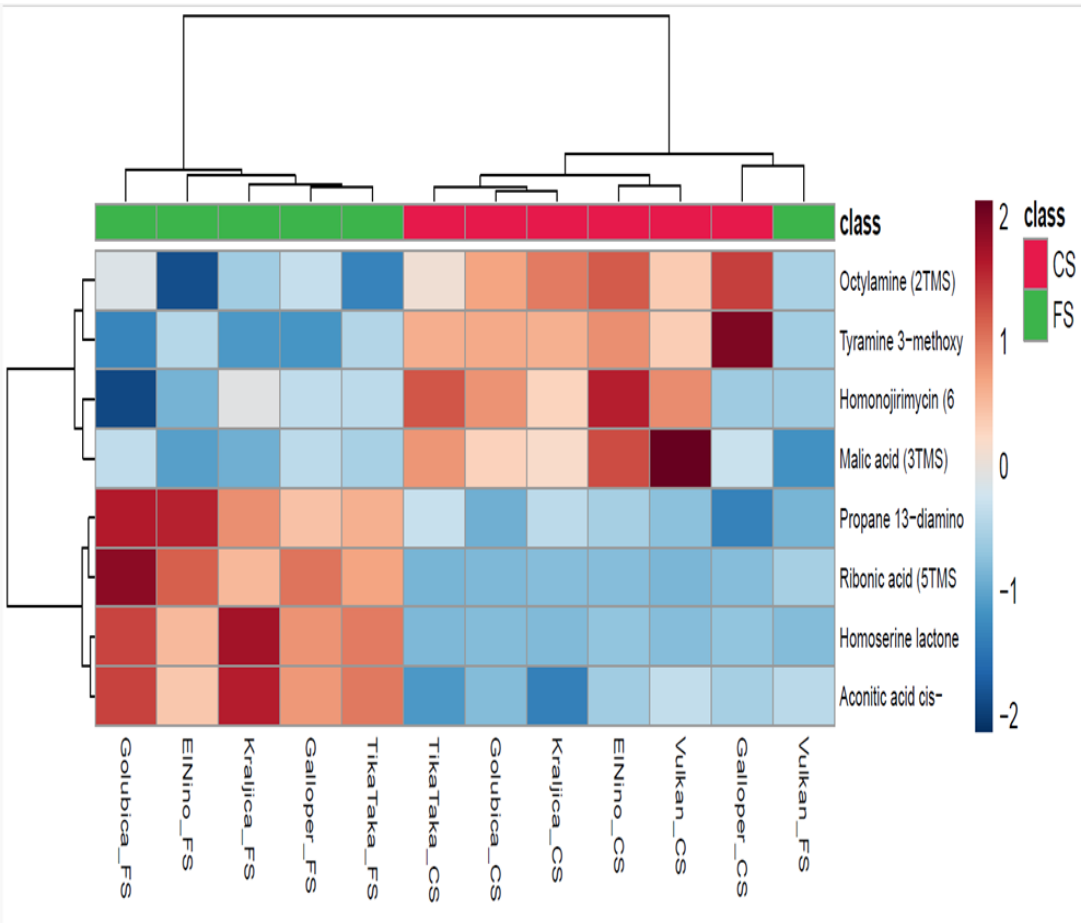

Figure 4: Heatmap analysis in spikes of six winter wheat genotypes under control (El Nino, Golubica, Tika Taka, Galloper, Vulkan, and Kraljica (CS)) and *Fusarium* (FS) infected six winter wheat genotypes. The red colour indicates relative abundance compared to noninoculated control, while blue colour indicates a reduction compared to noninoculated control.

When comparing FHB treatment and controls, 3-methoxytyramine, octylamine, and malic acid generally decreased across genotypes, whereas homonojirimycin showed a genotype dependent response (Fig. 5A–D). The decrease in 3-methoxytyramine in FHB treatment varied between 28.9% and 85.3% compared to the control (Fig. 5A). Octylamine levels decreased in all genotypes, however, the magnitude of this decrease was lower in Kraljica and Vulkan (65.6% and 62.7%, respectively) compared with the greater reductions observed in the remaining four genotypes (Fig. 5B). Increase of malic acid in the FHB stressed spikes of the genotype Kraljica was observed, compared to the other genotypes which showed a decrease of this metabolite (Fig. 5C). In the FHB-stressed spikes, homonojirimycin levels increased in most genotypes, while in four genotypes (El Nino, Golubica, Tika Taka, Vulkan), a higher value was recorded, compared to controls (Fig. 5D).

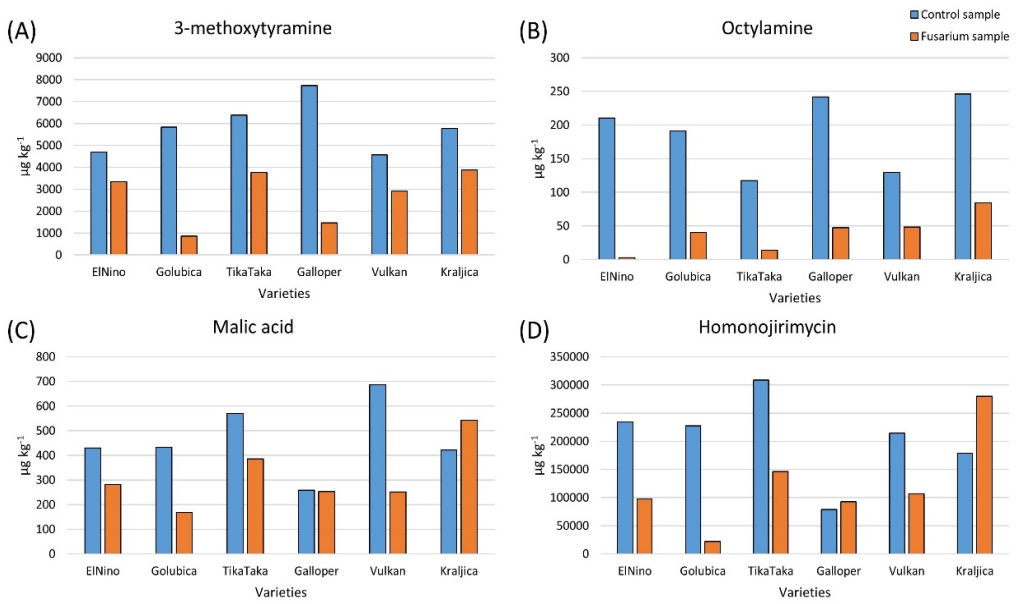

Figure 5: Concentrations of 3-methoxytyramine (**A**), octylamine (**B**), malic acid (**C**), and homonojirimycin (**D**) in control samples and *Fusarium* infected samples (artificially inoculated) of six winter wheat genotypes (El Nino, Golubica, Tika Taka, Galloper, Vulkan, and Kraljica) from four replicates.

Higher value of cis-aconitic acid, N-hexanoyl homoserine lactone, ribonic acid, and 1,3-diaminopropane in FHB treatment (except cis-aconitic acid and 1,3-diaminopropane for Vulkan) was recorded. The most prominent increase was observed in the genotype Kraljica which showed an increase in cis-aconitic acid and N-hexanoyl homoserine lactone by 100% and 99.4%, respectively, compared to the controls (Fig. 6A–D). These metabolite changes suggest involvement of pathways related to energy metabolism, signalling, and defence responses under *Fusarium* infection.

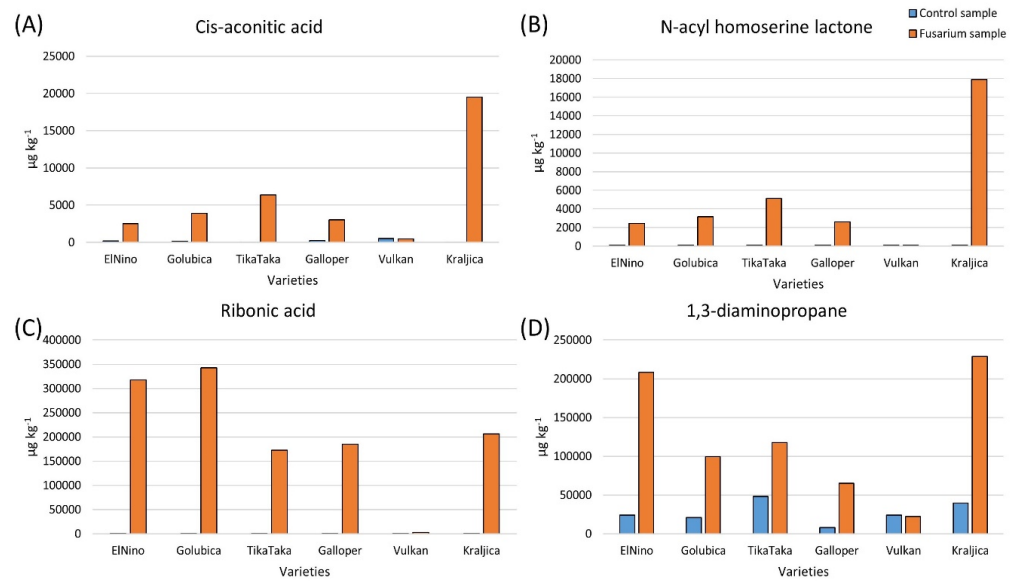

Figure 6: Concentrations of cis-aconitic acid (**A**), N-hexanoyl homoserine lactone (**B**), ribonic acid (**C**), and 1,3-diaminopropane (**D**) in control samples and *Fusarium* infected samples (artificially inoculated) of six winter wheat genotypes (El Nino, Golubica, Tika Taka, Galloper, Vulkan, and Kraljica) from four replicates.

4 Discussion

In recent years, metabolomics has been increasingly employed to investigate wheat responses to biotic stress [11]. Metabolic profiling has revealed key insights into wheat's defence responses to pathogen invasion, supporting the discovery of defence-related metabolites, genetic traits, and biological control strategies [17]. Among these defence-related compounds, secondary metabolites, including terpenoids, flavonoids, alkaloids, and phenolics, play a crucial role to plant resistance by serving as a first line of defence against pathogens [10]. Further, the study of Nussbaumer et al. [26] revealed that rearrangements in primary metabolism and translational machinery, along with distinct changes in glutamate metabolism, particularly in wheat lines carrying the *Qfhs.ifa-5A* gene. This may enhance fungal resistance, potentially due to the activity of two amino acid permeases located within the QTL confidence interval. Given the importance of metabolic responses in plant-pathogen interactions, investigating genotype-dependent metabolic variation can offer valuable insights into resistance mechanisms. In this study, to observe the differences in metabolites, GC-MS based metabolic profiling were conducted on six winter wheat genotypes. While the experiment was not independently repeated, consistent trends were observed across all treatments, suggesting that the effects are not random. Nevertheless, the lack of experimental repetition represents a limitation. Therefore, the results should be interpreted as exploratory. The combined use of the two species was intended to mimic natural infection conditions; however, this design does not allow conclusions regarding species-specific pathogenicity. The approach has been used in our previous work and is supported by the literature, which shows that mixed inocula provide a realistic challenge for assessing resistance while reflecting interactions between co-occurring *Fusarium* species [21]. This methodological choice ensures that results are relevant to field situations where multiple species are commonly present. In addition, *Fusarium* aggressiveness was not directly assessed, and equal conidium concentrations may not reflect equal aggressiveness. Future studies incorporating experimental replication and direct aggressiveness measurements are warranted. These limitations may affect the generalizability of the findings and should be considered when interpreting the results. The findings should therefore be interpreted as exploratory. Furthermore, the use of PLS-DA without formal validation represents a methodological limitation, and the observed group separation should be regarded as indicative rather than confirmatory.

4.1 Metabolic Distinctions across Genotypes and Treatments

Plant metabolic responses to biotic stress are often genotype-dependent, with resistant and susceptible genotypes exhibiting distinct biochemical profiles [27]. These genotype-specific metabolic patterns reflect underlying genetic control and suggest that FHB resistance may be mediated by constitutive or inducible metabolic traits. This study showed that separation of samples by PC1 reflects that *Fusarium* infection is the main source of variation in metabolite composition. In contrast, dispersion of genotypes along PC2 indicates that genotype-specific metabolic traits further modulate the biochemical response. The study of Warth et al. [28] also showed that PCA clearly separated deoxynivalenol treated and control samples along PC1 across multiple genotypes, showing that the treatment has a dominant effect on metabolite profiles; genotype differences also were apparent but less relevant. It should be noted that the metabolomic profiles observed in this study represent the integrated response of wheat spikes to combined *Fusarium* infection. In addition to pathogen-specific interactions, early metabolic changes may also reflect general stress responses associated with inoculation, including wound-induced signalling and defence priming. The use of a mixed inoculum and the inoculation procedure may therefore contribute to broad stress-related metabolic adjustments, particularly at early time points. These effects are considered part of the host's

defence activation under controlled *Fusarium* infection conditions and do not detract from the comparative interpretation of genotype-specific responses.

Also, *Fusarium* treatment induced changes in metabolite levels (e.g., jasmonic acid, phenylpropanoid pathway) which can separate infected vs. uninfected genotypes in defence metabolite response (genotype effect) [17]. When infected by *F. graminearum*, FHB-resistant and susceptible wheat plants exhibited distinct metabolic responses. The resistant plants, in particular, activating shikimate-mediated secondary metabolism to inhibit fungal growth and reduce mycotoxin production [29]. In Sumai 3, a well-known *Fusarium*-resistant wheat genotype, metabolomic analysis that enhanced activity in specific pathways is associated with restricted pathogen progression [30]. Phytohormones are considered important regulators of plant immune responses and have been shown to modulate the severity of FHB in wheat [15]. In contrast, the susceptible genotype exhibited no clear separation due to FHB infection in the spikes, which might indicate either a more localized response to FHB or a different underlying resistance mechanism [16]. It is important to note that resistance to FHB is a complex trait comprising multiple components. Type I resistance refers to resistance to primary infection and is typically evaluated under field conditions, while type II resistance refers to resistance to fungal spread within the spike and is typically assessed by point inoculation of a single floret under controlled conditions. Both components contribute to total resistance, and are often evaluated in combination to better understand disease response [31].

In the study of Tan et al. [17], the heatmap clearly separated control and *Fusarium*-stressed groups, supporting earlier findings of substantial metabolic shifts induced by pathogen stress. In this study, in all genotypes except Vulkan, metabolites such as 3-methoxytyramine, octylamine, malic acid, and homonojirimycin were consistently reduced under *Fusarium* infection compared to controls. These decreases may reflect the disruption of key metabolic pathways involved in stress adaptation, such as amino acid metabolism, polyamine biosynthesis, and the TCA cycle [32]. In contrast, other metabolites including cis-aconitic acid, N-hexanoyl homoserine lactone, ribonic acid, and 1,3-diaminopropane showed consistent accumulation across most genotypes following infection. This may suggest their involvement in pathogen-induced metabolic responses or defence signalling. Notably, genotype-specific trends were also observed. Vulkan, previously identified as a highly FHB-resistant genotype [15], exhibited a generally lower overall metabolite abundance under infected conditions. This pattern may reflect a resistance strategy based on metabolic suppression or homeostatic stability, which has been proposed as a mechanism to limit pathogen access to host resources and reduce oxidative damage [33]. Such a strategy contrasts with more metabolically dynamic responses seen in the other resistant genotype Kraljica, which displayed higher increases in several metabolites under FHB stress. These findings emphasize the diversity of resistance mechanisms among wheat genotypes, ranging from metabolic activation to metabolic restraint. This highlights the potential for metabolite profiling to uncover key biomarkers of disease resistance.

Although disease progression was monitored at multiple time points, an area under the disease progress curve (AUDPC)-based analysis was not included in this study. The experimental design focused on relating early metabolic responses at 7 dpi to a defined late-stage disease outcome at 19 dpi, rather than modelling disease progression over time. Metabolomic profiling at 7 dpi was designed to capture early metabolic reprogramming, whereas disease severity assessed at 19 dpi reflects a stabilized late-stage phenotype. This approach was chosen to link early metabolic reprogramming to the final extent of disease spread within the spike. PLS-DA was used as an exploratory visualization tool to illustrate metabolic differences between treatments and genotypes. As formal model validation metrics (R^2, Q^2) and permutation testing were not applied, PLS-DA results should be interpreted cautiously and in conjunction with univariate statistical analyses.

4.2 Significant Metabolic Decrease Induced by Fusarium Inoculation

Fusarium infection in wheat can also lead to the suppression of key metabolic processes. This metabolic shift may compromise plant growth and development, particularly in susceptible genotypes, which may show a more pronounced decline in these essential functions during FHB infection. The compound 3-methoxytyramine, also known as 3-methoxy-4-hydroxyphenethylamine, is a metabolite derived from the aromatic amino acid tyrosine and is present in different species of plants [34]. The enzymatic decarboxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in plants serves as a physiological process bridging primary and secondary metabolism. This process facilitates the production of defensive compounds in response to attacks by insects, herbivores, and pathogens [35]. For instance, in *Citrus* species, glucosylated forms of tyramine and its N-methylated derivatives have been identified, suggesting a role in plant defence against biotic stress [36]. Additionally, Zhou et al. [37] reported that plant metabolites, including phenolic compounds, contribute to antimicrobial activity and reinforcement of cell walls. This further supports the hypothesis that 3-methoxytyramine may function similarly in wheat's defence response. The observed decrease of 3-methoxytyramine in *Fusarium*-inoculated wheat samples compared to controls may reflect its rapid turnover or conversion into downstream defence-related compounds. This suggests its early involvement in the plant's metabolic response to pathogen attack.

Plants produce various amines (like putrescine, spermidine, and other biogenic amines) involved in growth and stress responses. While octylamine itself is not typically found among naturally occurring plant amines its relevance can be considered due to its link to fatty acid or lipid metabolism in plants [38]. Since octylamine is linked to fatty acid metabolism (precursor or breakdown product), the decrease of octylamine in FHB-infected wheat samples may indicate its utilization or breakdown during defence-related lipid remodelling or stress-induced metabolic shifts triggered by *Fusarium* infection. Interestingly, Kraljica and Vulkan, the genotypes previously characterized as the most resistant to FHB, exhibited smaller reductions in octylamine, compared to both their controls and the other more susceptible genotypes. This milder decline may reflect a more stable metabolic response to FHB stress, possibly linked to resistance mechanisms.

Malic acid undergoes reversible oxidative decarboxylation catalysed by NADP-malic enzyme, a key enzyme involved in multiple metabolic processes in plants [39]. The presence of malic acid in wheat grains suggests its involvement in fundamental metabolic processes within the plant [40]. Additionally, malic acid and its metabolism can be involved in plant responses to stress, including pathogen attack. Its levels might fluctuate under conditions like fungal infection [41]. The decrease of malic acid in *Fusarium*-infected wheat samples may reflect its consumption in energy-demanding defence responses or a shift in central metabolism. This occurs as the plant reallocates resources from primary metabolism toward the production of defence-related compounds. An increase in malic acid was observed only in the FHB-stressed spikes of Kraljica. Given malic acid's role in stress tolerance and energy metabolism, this pronounced accumulation in Kraljica may contribute to its enhanced defence capacity.

Homonojirimycin, a member of the iminosugar class of compounds, is primarily reported from microbial sources, particularly certain bacteria and fungi, rather than directly from plants [42]. In the case of plant-associated fungi such as *Fusarium*, either the fungus itself or its microbial endophytes may produce homonojirimycin or influence its accumulation in plant tissues. Many fungi and bacteria are known producers of iminosugars that affect host metabolism [43]. In this study, the decrease of homonojirimycin in *Fusarium*-infected wheat samples may suggest its involvement in early defence responses or its utilization in metabolic pathways related to pathogen resistance. This could lead to reduced accumulation as the infection progresses. The relatively moderate accumulation in the resistant genotype Kraljica could indicate a more efficient or regulated response to infection.

4.3 Significant Metabolic Increase Induced by Fusarium Inoculation

During *Fusarium* infection, several metabolites showed increased abundance in wheat spike tissue. These metabolic shifts may reflect host responses associated with *Fusarium* infection and defence-related metabolic reprogramming. Cis-aconitic acid is an intermediate in the tricarboxylic acid (TCA) cycle (also known as the Krebs cycle), formed by the dehydration of citric acid and converted to isocitric acid by the enzyme aconitase [44]. Aconitase activity can be inhibited or impaired by oxidative stress generated during the plant's defence response. This is because the enzyme contains iron-sulphur clusters that are highly sensitive to reactive oxygen species (ROS). This observation aligns with findings from a study by Yuan et al. [45] who indicated that *F. graminearum* infection leads to higher changes in the wheat transcriptome, affecting various metabolic pathways, including those related to energy metabolism. These alterations suggest that the pathogen induces metabolic reprogramming in the host plant, which could involve changes in TCA cycle intermediates. This accumulation may indicate altered regulation of the TCA cycle under *Fusarium* infection, aligning with previous findings that *F. graminearum* infection alters energy metabolism and related pathways in wheat. These interpretations remain speculative and require experimental validation to determine the origin and functional relevance of homoserine lactone (HSL) accumulation in infected wheat spikes.

Fusarium oxysporum is known to encode lactonase enzymes [46], which hydrolyse cyclic ester substrates such as DL-pantoyl lactone. Given the structural similarity between pantoyl lactone and N-acyl homoserine lactones, this suggests a potential mechanism by which *Fusarium* species may degrade quorum-sensing signals like N-hexanoyl homoserine lactone (C6-HSL). This remains to be experimentally verified. While the study of Alramadhan et al. [47] focuses on *Bacillus thuringiensis*, the presence of lactonase activity in a closely related bacterium suggests a broader potential. *Fusarium* species, which occupy similar ecological niches and engage in comparable interactions, may also produce lactonases capable of degrading C6-HSL. The increase of C6-HSL in *Fusarium*-infected wheat samples may reflect the accumulation of microbial quorum-sensing signals, possibly produced by co-inhabiting bacteria or even the pathogen itself. Although *F. oxysporum* is known to encode lactonase enzymes that can degrade lactone-based signals like C6-HSL, the observed increase may indicate either a suppression or inefficiency of lactonase activity under infection conditions. Alternatively, it could result from an overproduction of HSLs by associated microbial communities as part of a complex plant microbe interaction. Interpretations involving microbial signalling or quorum-sensing–related metabolites are speculative and require experimental validation.

Fusarium spp. or co-colonizing microbes may consume sugar acids, including ribonic acid, as carbon sources [48]. The increase of ribonic acid in *Fusarium*-infected wheat samples may reflect enhanced oxidative degradation of sugars, such as ribose. This likely occurs as part of the plant's stress response or metabolic reprogramming under infection. The accumulation could also result from cell wall breakdown or altered pentose phosphate pathway activity, both of which are commonly triggered during pathogen attack [49].

1,3-diaminopropane is a catabolic product of spermidine and spermine via polyamine oxidases (PAOs). The activity of these enzymes is often upregulated during plant stress and pathogen infection, contributing to defence-related H_2O_2 production [50]. The increase of 1,3-diaminopropane in FHB-infected wheat samples may be associated with enhanced catabolism of polyamines spermidine and spermine via upregulated PAO activity during pathogen attack. This process not only generates 1,3-diaminopropane but also produces hydrogen peroxide (H_2O_2). H_2O_2 functions as a signalling molecule to activate defence responses and strengthen the plant's resistance to *Fusarium* infection.

The marked increases in specific metabolites, particularly in Kraljica, may reflect genotype specific metabolic response patterns associated with *Fusarium* infection. The absence of similar increases in Vulkan, another resistant genotype, suggests that different resistance mechanisms or metabolic strategies may be involved. Some of the observed metabolic changes may represent general stress responses induced by inoculation and pathogen challenge rather than resistance mechanisms per se. Also, comparing these early responses with later symptom development could provide further insight into the physiological effects of the QTLs, as their influence may vary among traits (disease index, FDK, DON accumulation). This represents an important avenue for future research to assess agreement or divergence between early molecular markers and later phenotypic outcomes. Nevertheless, the observed variation between early metabolomic changes and disease symptoms suggests that FHB resistance should be studied as part of a wider, integrated system. Comprehensive analyses incorporating disease symptoms, grain contamination, and metabolomic profiling are needed to fully understand the physiological mechanisms of resistance and to increase the practical utility of molecular data.

5 Conclusions

This study highlights the complex metabolic reprogramming that occurs in wheat spike tissues during *Fusarium* infection. The observed decrease in metabolites such as 3-methoxytyramine, octylamine, malic acid, and homonojirimycin may be associated with early stress related responses or their utilization in infection induced metabolic pathways. In contrast, the accumulation of compounds such as cis-aconitic acid, N-hexanoyl homoserine lactone, ribonic acid, and 1,3-diaminopropane may indicate alterations in central metabolism, oxidative balance, polyamine turnover, and microbe-associated signalling under *Fusarium* challenge. Together, these metabolic shifts highlight coordinated metabolic adjustments related to *Fusarium* infection. In addition, observed metabolic differences among genotypes suggest genetic variation in infection associated metabolic responses. However, given the experimental design and associated limitations, these findings should be interpreted as exploratory. Further studies are needed to elucidate the specific mechanisms underlying these metabolic changes and to explore their roles in wheat resistance. Future research could extend metabolomic approaches to other types of FHB, such as resistance to initial infection (Type I) or resistance to mycotoxin accumulation (Type III), and should include independent biological replication to confirm the robustness and general applicability of the findings.

Acknowledgement: Not applicable.

Funding Statement: Financial support by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben by visiting programme for scientists from transitions countries is gratefully acknowledged.

Author Contributions: Conceptualization: Valentina Spanic and Jurica Duvnjak; methodology: Valentina Spanic, Jurica Duvnjak and John C. D'Auria; formal analysis: Jurica Duvnjak, Katarina Sunic Budimir, Daniel Kujundzic and John C. D'Auria; investigation: Valentina Spanic, Jurica Duvnjak, Katarina Sunic Budimir, Daniel Kujundzic and John C. D'Auria; resources: Valentina Spanic and John C. D'Auria; writing—original draft preparation: Jurica Duvnjak; writing—review and editing: Valentina Spanic, Katarina Sunic Budimir, Daniel Kujundzic and John C. D'Auria; visualization: Jurica Duvnjak; project administration: Valentina Spanic and John C. D'Auria. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings are available on request from the corresponding author.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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