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Treatment and Management of Fusarium Disease in Wheat

Edited by
Valentina Španić and Hrvoje Šarčević

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Contents

About the Editors

Valentina Španić

In the last 17 years, Dr. Valentina Španić has been engaged in wheat breeding and genetics research, combining classical breeding with modern technologies. Today, she is a co-breeder of 22 winter wheat varieties at the Agricultural Institute Osijek (Croatia). The focus of her work is also biotic and abiotic stresses such as Fusarium head blight resistance, and more recently, she spent several months at the University of Minnesota, USA, and the University of Sydney, Australia, working on rusts. Through the KWS-Borlaug stipendium in Germany, she worked on disease resistance, hybrid wheat production, and the evaluation of genetic diversity with SNP markers for a few months. At the Agricultural Institute Osijek, she established Fusarium inoculum production and is trying to combine stress resistance with research on plant physiology. Further, in the last few years, she started to work on drought resistance in wheat. She has been principal investigator of five national/international projects, and as a collaborator, she has participated in four projects. Currently, she is a leader of two projects and a collaborator on one COST action. She is the author of one university book published in 2016, while in 2021, she won the state prize for science in Croatia.

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Dr. Hrvoje Šarčević is a full professor at the University of Zagreb, Faculty of Agriculture, Croatia, where he teaches the undergraduate course "Genetics" and the graduate course "Population genetics in plant breeding" and co-teaches several graduate and postgraduate courses in advanced plant breeding. His research interests focus on the genetics and breeding of wheat, soybean and maize, with emphasis on breeding for biotic and abiotic stresses and grain quality. Since 2007, Dr. Šarčević has been the head of the working group Cereals and Maize of the Croatian Gene Bank and has been involved in the collection and study of maize landraces. He has authored or co-authored more than 50 refereed journal articles and numerous papers in conference proceedings, and has co-authored three book chapters. He supervised six PhDs and more than 40 M.Sc. and B.Sc. theses. He trained his research and teaching skills at North Carolina State University (USA), Nordic Gene Bank (Sweden), NIAB (UK), University of Hohenheim (Germany), and the University of Natural Resources and Life Sciences (Austria). He has participated in several national/international projects as a research leader or collaborator. He is currently a member of the Center of Excellence for Biodiversity and Molecular Plant Breeding, Zagreb, Croatia, and a collaborator on an international project.

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Editorial

Evaluation of Effective System for Tracing FHB Resistance in Wheat: An Editorial Commentary

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A Special Issue of *Agronomy* titled "Treatment and Management of Fusarium Disease in Wheat" published five articles addressing the resistance of winter wheat varieties/lines to Fusarium head blight (FHB). Various approaches were used in these studies, including investigating the effects of different artificial inoculation methods on FHB symptom evaluations, determining the levels of mycotoxins/metabolites produced by *Fusarium* spp., and studying the influence of FHB on protease activity, technological and rheological quality [1–5]. Furthermore, *Fusarium* infection affects plant development and triggers different morphological, physiological, biochemical and molecular changes. In this context, two articles published in this Special Issue investigated the response of different wheat varieties to *Fusarium* infection in terms of their photosynthetic efficiency, and observed chemical and physiological parameters that might be related to the activation of the defense mechanism against FHB [6,7]. One article was focused on spring wheat lines' susceptibility/resistance to crown and root rot caused by *Fusarium culmorum* and *F. pseudograminearum* [8], and one article reported the antifungal activity of *Tamarix gallica* bark extract against *F. acuminatum*, *F. culmorum*, *F. equiseti* and *F. graminearum* associated with FHB [9]. Finally, this Special Issue includes a review on the role of secondary metabolites and antioxidants in wheat defense against FHB [10]. Undoubtedly, all the articles published in this Special Issue highlight FHB as one of the most damaging wheat diseases, leading to a reduction in grain yield and quality [1–10]. Almost all articles mention that FHB is caused by several *Fusarium* species—mainly *F. graminearum*, *F. culmorum* and *F. avenaceum*—and that the predominance of species within the FHB complex is determined by meteorological and agronomical factors [1–6]. However, in one study, another *Fusarium* species was used as a source of inoculum, namely, *F. equisety*, of which can be found in subtropical and warm temperate regions [7].

Besides grain yield, FHB affects the grain protein content by destroying starch granules, storage proteins and cell walls, and consequently decreases the quality of dough. It is also associated with mycotoxin contamination and is a significant threat to animal and human health [2,3,10]. The two articles and the review paper on *Fusarium* mycotoxins/metabolites in this Special Issue mention the main consequences of the consumption of contaminated food (alimentary hemorrhage, vomiting, dermatitis, gastroenteritis, nausea, anorexia, growth retardation, endocrine damage, immunosuppression and reproductive toxicity) depending on *Fusarium* spp. and the mycotoxins/metabolites produced. Current climate change scenarios predict an increase in the number of epidemics caused by this disease, and many different disease control strategies are currently being investigated. Weather conditions at the local level can influence the outbreak of new pests and pathogens due to the rapid emergence of races, their epidemic infection, and the ability to break down host resistance, which also refers to FHB [5]. In this Special Issue, the authors of articles reported that the selection for FHB resistance in high disease pressure environments is more easily achieved by using different methods of artificial inoculation with

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Fusarium spp. [1]. They also indicated that maize debris on the soil surface could serve as a good source of inoculation where wheat is planted afterwards. Still, in the same research, FHB severity was significantly lower in natural infections compared to two methods of artificial inoculation used (spray method and infected maize stalks).

Wheat plants are most vulnerable to FHB infection during flowering to the early dough stage [3]. Optimal temperatures for FHB infection are between 10 and 30 °C, while humidity is a critical factor for the success of pathogen infection [4,5]. FHB symptoms are located on wheat spikes within the spikelets, and consequently the grain, and sometimes the peduncle. A few days after infection, healthy spikes will stay green, while diseased spikelets will start bleaching and the infection will gradually spread through the spike [5]. Sometimes, at high humidity, even pink-to-orange masses of spores may become visible. There are different types of FHB resistance in wheat reported: (1) resistance to initial infection; (2) resistance to spreading within the spike; (3) resistance to the accumulation of mycotoxins; (4) resistance to grain infection; and (5) grain yield tolerance [3,5,6,10]. Plants also can possess passive resistance to FHB encompassing plant morphology and development (earlier flowering, taller plants, spike compactness, degree of anther extrusion, and the presence or absence of awns) [10]. Other ways that plants can participate in the natural defense against FHB are via the production of secondary metabolites including phenolic acids, anthocyanins and flavonoids, alkylresorcinols, benzoxazinoids, volatile organic compounds, phytohormones, carotenoids, etc. [10]. However, it is difficult to find FHB-resistant sources as host resistance is conditioned by numerous low-effect quantitative trait loci (QTL) that are strongly affected by environmental conditions and genetic bases [4]. Multi-mycotoxins produced by toxic *Fusarium* spp. are also significantly influenced by genotypes and the environment [2]. Thus, to combat FHB and minimize the accumulation of *Fusarium* mycotoxins, integrated management is needed by combining resistant wheat varieties, good agronomical practice, the application of fungicides [2,5], and the use of biological agents [4,10]. To minimize the application of fungicides, especially in the context of the Green deal proposed by the EU commission to reduce the use and risk of chemical pesticides, an article in this Special Issue covered this topic by characterizing the phytochemicals found in *T. gallica* bark extract and evaluating its antifungal activity for the control of *Fusarium* spp. [9]. In the same article, two fungicides were tested, and it was observed that the effectiveness of the fungicides against *Fusarium* spp. was substantially lower than that of the *T. gallica* bark extract. Further, the in vitro mycelial growth of *Fusarium* spp. was inhibited by the extract and four phytochemicals (1-(2,4,6-trihydroxyphenyl)-2-pentanone, sinapinaldehyde, trans-squalene and syringaldehyde) [9].

The testing of different wheat lines and varieties in the growth chamber, greenhouse and field conditions revealed new material sources for improving wheat resistance to *Fusarium* fungi that can cause root and crown rot [8]. However, new technology-based approaches (e.g., QTL and GWAS studies) should be implied. Due to the complex quantitative nature and difficult selection for FHB resistance, marker-assisted selection and analyses at physiological and cellular levels could be useful. Therefore, several techniques are used to study the effects of *Fusarium* infestation and their interactions with host plants. One of these is the measurement of photosynthesis, a process that provides the material basis and energy supply for multiple physiological metabolic processes in plants and can be disrupted by biological stress caused by pathogens. The findings of Katanic et al. [6] indicate that the difference in the degree of photosynthetic changes, particularly the analysis of L-band appearance, in the early stages of FHB infection in spikes and leaves could be an indicator of infection. A positive L-band was detected in flag leaves before any visible FHB symptoms, while a negative L-band occurred in spikes, thus indicating an increase in the energetic connectivity in infected spikes between the PSII photosynthetic units. However, location was shown to be a more important factor than genotypes in modulating the response of wheat to FHB [6]. Furthermore, the expression of different genes in defense mechanisms is triggered when physical stress is converted into a biochemical response [7]. The overexpression of some genes, such as pathogenesis-related (*PR-1*), thaumatin-like protein

(*TLP*), chitinase and β-1,3-glucanase, may have created FHB resistance in wheat material by activating a defense mechanism and enhancing the production of different biochemicals. To date, many PR proteins are known, which are classified into 17 families based on their protein sequence similarities, enzymatic activities and biological functions [10].

As mentioned above, grain yield losses and a reduction in technological and rheological parameters are usually observed in winter wheat as a result of *Fusarium* infection. In this Special Issue, an article reported that *Fusarium* inoculation decreased the duration of dough resistance and increased dough softening, but winter wheat varieties were affected to different degrees depending on their FHB resistance/susceptibility [5]. Therefore, dough strength was much more reduced in FHB-susceptible varieties due to alterations in traits measured by extensographs showing a decrease in the average energy value and resistance to extension. In the group of technological quality traits, the sedimentation value and gluten index were primarily affected. The strength of this research lies in the detection of technological and rheological quality changes due to *Fusarium* infections. Another article dealing with wheat quality losses due to FHB showed that extensograph values were strongly affected by FHB, indicating a lower resistance to stretching, extensibility and total stretching energy, thus suggesting that dough functionality and volume loss can be attributed to exogenous fungal proteases [4]. This is related to the reactivation of *Fusarium* proteases during the dough-making process, resulting in negative effects on the rheological properties of dough. Both protein and wet gluten content were significantly influenced by genotype, environment and their interaction. Furthermore, elastic properties of the dough were under gluten influence, whereas a lower degree of softening was reported in more FHB-resistant varieties [4]. The importance of gluten is due to the fact that it is a protein responsible for the baking properties of wheat flour, with gliadins and glutenins being the main protein fractions present in gluten. Overall, both articles related to technological and rheological quality observed that the differences in wheat quality between FHB-resistant and -susceptible varieties are due to genetic and environmental factors (year and location) [4,5].

Fusarium infections also degrade grain quality by increasing the proportion of shriveled grains, and most importantly, by accumulating mycotoxins, that pose a health risk to humans and animals after the consumption of diseased grains or end-use products. The results in this Special Issue highlight the influence of environmental variations on *Fusarium* mycotoxin production where FHB initial resistance (Type I resistance) had a higher impact on the accumulation of mycotoxins than general resistance [3]. Deoxynivalenol (DON) was one of the most abundant mycotoxins [2,3]. In the FHB-inoculated treatment, the DON concentration in the FHB-susceptible variety at two locations was 22,800 μg kg^{-1} and 25,500 μg kg^{-1}, respectively, thus exceeding the permitted level of 200–1750 μg kg^{-1} for DON [3]. Also, DON co-occurred with culmorin and hydroxyculmorins, with a potential role in *Fusarium* virulence. Another article in this Special Issue reported the production of various mycotoxins/metabolites during a three-year study [2]. Twenty-eight *Fusarium* mycotoxins/metabolites were detected and were highly correlated to each other. Special attention should be paid to emerging mycotoxins such as moniliformin (MON), beauvericin (BEA) and enniatins (ENNs), which contribute substantially to the overall contamination of wheat grains. The greatest concentration in the three investigated years was observed for DON (found in 100% of the wheat samples) with mean values of 3245, 5380 and 6743 μg kg^{-1} in 2014, 2015 and 2016, respectively. Herein, in possible epidemic conditions provoked by artificial inoculations, the maximum limits of DON were exceeded. Both articles published in this Special Issue emphasize the need to set limits for modified, masked and emerging forms of mycotoxins as they represent potential health risks for animals and humans [2,3]. As *Fusarium* species *F. graminearum* and *F. culmorum* were used for artificial inoculations in both studies, a number of mycotoxins were expected to be produced, including zearalenone (ZEN), MON, BEA, ENNs and trichothecenes such as DON, nivalenol (NIV), 3- and 15-acetyl-DON (3-AcDON, 15-AcDON), HT-2 and T-2 toxin.

The important fact is that toxin-producing abilities correlate positively with the level of a pathogen's aggressiveness [10].

The articles in the Special Issue on FHB in wheat have provided a wealth of information on the genetic, molecular and physiological mechanisms of resistance to FHB infection. Some gaps in knowledge about FHB were fulfilled, and we hope that these articles provided new ideas for strategies to control this complex plant disease. The search for additional sources of phytochemicals against FHB should be continued to avoid the use of excessive amounts of fungicides. Wheat breeders should keep developing and expanding the range of FHB resistance in wheat material available to market, especially with regard to cost effectiveness and environmental safety.

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References

1. Šarčević, H.; Bukan, M.; Lovrić, A.; Maričević, M. Evaluation of Inoculation Methods for Determination of Winter Wheat Resistance to Fusarium Head Blight. *Agronomy* **2023**, *13*, 1175. [CrossRef]
2. Spanic, V.; Maricevic, M.; Ikic, I.; Sulyok, M.; Sarcevic, H. Three-Year Survey of Fusarium Multi-Metabolites/Mycotoxins Contamination in Wheat Samples in Potentially Epidemic FHB Conditions. *Agronomy* **2023**, *13*, 805. [CrossRef]
3. Sunic, K.; Kovac, T.; Loncaric, A.; Babic, J.; Sulyok, M.; Krska, R.; Drezner, G.; Spanic, V. *Fusarium* Secondary Metabolite Content in Naturally Produced and Artificially Provoked FHB Pressure in Winter Wheat. *Agronomy* **2021**, *11*, 2239. [CrossRef]
4. Peršić, V.; Božinović, I.; Varnica, I.; Babić, J.; Španić, V. Impact of Fusarium Head Blight on Wheat Flour Quality: Examination of Protease Activity, Technological Quality and Rheological Properties. *Agronomy* **2023**, *13*, 662. [CrossRef]
5. Spanic, V.; Dvojkovic, K.; Babic, J.; Drezner, G.; Zdunic, Z. Fusarium Head Blight Infestation in Relation to Winter Wheat End-Use Quality—A Three-Year Study. *Agronomy* **2021**, *11*, 1648. [CrossRef]
6. Katanić, Z.; Mlinarić, S.; Katanić, N.; Ćosić, J.; Španić, V. Photosynthetic Efficiency in Flag Leaves and Ears of Winter Wheat during Fusarium Head Blight Infection. *Agronomy* **2021**, *11*, 2415. [CrossRef]
7. Manghwar, H.; Hussain, A.; Ali, Q.; Saleem, M.H.; Abualreesh, M.H.; Alatawi, A.; Ali, S.; Munis, M.F.H. Disease Severity, Resistance Analysis, and Expression Profiling of Pathogenesis-Related Protein Genes after the Inoculation of *Fusarium equiseti* in Wheat. *Agronomy* **2021**, *11*, 2124. [CrossRef]
8. Özdemir, F. Host Susceptibility of CIMMYT's International Spring Wheat Lines to Crown and Root Rot Caused by *Fusarium culmorum* and *F. pseudograminearum*. *Agronomy* **2022**, *12*, 3038. [CrossRef]
9. Sánchez-Hernández, E.; González-García, V.; Correa-Guimarães, A.; Casanova-Gascón, J.; Martín-Gil, J.; Martín-Ramos, P. Phytochemical Profile and Activity against *Fusarium* Species of *Tamarix gallica* Bark Aqueous Ammonia Extract. *Agronomy* **2023**, *13*, 496. [CrossRef]
10. Chrpová, J.; Orsák, M.; Martinek, P.; Lachman, J.; Trávníčková, M. Potential Role and Involvement of Antioxidants and Other Secondary Metabolites of Wheat in the Infection Process and Resistance to *Fusarium* spp. *Agronomy* **2021**, *11*, 2235. [CrossRef]

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Article

Evaluation of Inoculation Methods for Determination of Winter Wheat Resistance to Fusarium Head Blight

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Abstract: One of the most severe winter wheat (*Triticum aestivum* L.) diseases is Fusarium head blight (FHB). It is believed that selection for resistance to FHB is better in high disease pressure environments, for which various methods of artificial inoculation are used. The standard spray method of artificial inoculation is believed to be technically demanding and labour intensive. Therefore, scattering Fusarium-infected maize stalks onto trial plots after wheat emergence is suggested as a suitable alternative. The aim of this study was to compare the mean values and heritability of the visual rating index (VRI) and the percentage of Fusarium-damaged kernels (FDK) between the two abovementioned methods of artificial inoculation and natural infection, and to determine the phenotypic correlations between the three methods for the studied traits. The achieved levels of VRI and FDK were comparable for the two methods of artificial inoculation and considerably lower under natural conditions. Heritability for VRI ranged over four years from 0.68 to 0.91 for the spray method, from 0.73 to 0.95 for the infected maize stalks, and from 0.26 to 0.65 for natural infection, whereas for FDK it ranged from 0.56 to 0.85, 0.38 to 0.83, and 0.11 to 0.44 for the three inoculation methods, respectively. The strong positive correlation between the two applied methods of artificial inoculation for studied traits suggests that scattering infected maize stalks could serve as a reliable supplement for the technically and labor-intensive spray method of artificial inoculation.

Keywords: winter wheat; Fusarium head blight resistance; artificial inoculation; maize stalks; heritability

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1. Introduction

Fusarium head blight (FHB), caused by *Fusarium graminearum* (teleomorph *Gibberella zeae* (Schw.) Petch) and other *Fusarium* species, is currently one of the most devastating wheat diseases in the world. Symptoms of FHB infection typically include premature bleaching of the entire spike or just a few spikelets, pinkish-red mycelium and spores on infected spikelets, inhibited grain formation, and the development of shriveled, light-weighted, and discolored grain (from white to pink) as a result of mycelial outgrowth from *Fusarium*-colonized grain [1]. Yield reductions due to formation of shriveled grain, reductions in baking and seed quality, and mycotoxin contamination are major threats posed by FHB [2–6]. The frequency of FHB epidemics has increased in recent years in most of the world's major wheat-growing regions [7]. Among different species causing FHB, *F. graminearum* is considered to be the most important globally, due to its widespread occurrence and aggressiveness [8]. It is ranked fourth among plant fungal pathogens based on its scientific and economic importance [9]. The major causal agents of FHB in Europe are *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* [1]. However, the prevalence of certain species varies from country to country and depends on meteorological conditions as well

as agricultural practices, such as crop rotation, previous crop, tillage, and the susceptibility of cultivars [1,10–13].

Central to FHB infection and development are the abundance and aggressiveness of the inoculum during the vulnerable plant growth stage, which essentially spans several days around anthesis, environmental conditions during this critical period, and the susceptibility or resistance status of the plant [14]. Fungicide applications have been shown to be only partially effective in controlling FHB [4,15–17], and are only effective within a narrow time frame [18]. Therefore, host resistance has long been considered the most practical and effective means to control the disease [14,17,19]. However, breeding wheat for durable resistance to FHB has been hindered by a lack of effective resistance genes and by the complexity of resistance in identified sources. FHB resistance is a quantitative trait controlled by multiple genes with either major or minor effects and is significantly influenced by genotype-environment interactions [4,14,19,20]. The combined effect of multiple genes interacting in a complex manner in resistant germplasm and their often poor agronomic and quality performance complicates the utilization of this germplasm [17,21]. According to Mesterhazy et al. [5], despite a 100-year tradition of FHB resistance research and breeding, most cultivated varieties are susceptible or highly susceptible to the disease.

The development of resistant cultivars requires the identification of both resistant sources and resistant lines within breeding populations. Although no source of complete resistance is known and current sources provide only partial resistance, most breeders have found genetic variability for FHB resistance in their existing germplasm. The level of resistance in the adapted germplasm pool will increase if programs actively screen for FHB resistance [22]. Practical breeders are trying to improve FHB resistance by recombining different resistance sources and types while selecting for resistance and desirable agronomic performance. Their ultimate goal is to develop productive cultivars with low disease symptoms and low mycotoxin contamination despite high infection pressures [3].

Resistance testing is best performed under uniform and moderate to high disease pressure, which is typically achieved through artificial inoculation [14]. Common inoculation methods include point inoculation, conidia spray, and the grain-spawn method [22–24]. These methods, while effective in quantifying resistance, are time and labor intensive. As a result, programs often receive data on resistance in breeding material at only one location per year. In addition, some material, often less advanced, may remain untested due to limited resources for FHB resistance screening [23]. Alternatively, sowing in fields with maize stubble on the soil surface or sowing trials in natural hot spots for *Fusarium* infection have been used to provoke infection [3,25]. However, screening for host resistance through natural infection is difficult because disease incidence and severity are inconsistent due to changes in environmental conditions that are difficult to control, such as temperature and precipitation [23,26]. Epidemics of Fusarium head blight have long been considered to originate from an inoculum associated with non-decomposed residues from the previous crop, particularly of small-grain cereals and maize, which provide a site for abundant sporulation in the next growing season [27,28]. Field observations have shown that the severity of FHB and deoxynivalenol (DON) contamination of wheat grains is positively correlated with increasing quantities of maize residues [28]. However, there are limited studies reporting the effects of crop residues as an inoculum source in FHB resistance testing [25,29]. Xue et al. [29] investigated the influence of inoculum sources (conidial suspension, infested barley and maize kernels, or infested wheat debris) on FHB development and DON content. Their results showed that inoculation with infested debris as a natural source of inoculum caused fewer FHB symptoms and lower DON contamination of grains compared to the conidial suspension or infested kernels. In the study by Mesterhazy et al. [25], the spray inoculation and polyethylene bag cover method showed better differentiation among wheat genotypes in terms of visual FHB rating, Fusarium-damaged kernels (FDK), and DON content in grain than method of spray inoculation and mist irrigation or the maize stalks method supported by mist irrigation. Regarding visual FHB ratings, all three methods

showed similar ranking of genotypes, while the results for FDK and DON content were less consistent among methods.

In the Bc Institute's winter wheat breeding program, screening of elite breeding lines for FHB resistance using the spray method of inoculation with *Fusarium* isolates serves as efficient criterion for final selection for registration purposes [30–32]. In the present study, an alternative inoculation method for screening FHB resistance in wheat, known as deployment of *Fusarium*-infected maize stalks, was compared with the standard spray method and natural infection. The objectives of the study were (1) to compare three inoculum sources in terms of their efficacy in inducing FHB symptoms, (2) to determine the relationship between FHB scores within the methods, and (3) to estimate the heritability of investigated traits.

2. Materials and Methods

2.1. Description of Field Experiments for Evaluation of Fusarium Head Blight Resistance

Field experiments including 25 winter wheat genotypes were conducted over four growing seasons (2011–2014) at the Bc Institute's winter wheat breeding nursery in Botinec (Zagreb), Croatia. Each year, a different set of the 18 most promising elite breeding lines and seven controls with varying levels of FHB resistance were evaluated in separate experiments conducted using two methods of artificial inoculation with *Fusarium graminearum* and natural *Fusarium* infection. Among the controls, the cultivars Renan, Roazon, Poncheau, Žitarka, and Lucija were repeated throughout all years of the study, while the control cultivars Apache, Soissons, and resistant breeding lines (D48X42X6)2 and K9_21_AB.14 were included in some years. The soil type at the experimental location was loamy clay. In all four years, sowing was done in late October. The experiments were set up as randomized complete blocks with four replicates. The experimental plots consisted of three 1 m long rows with 25 cm of in-between row spacing. The sowing density was 80 seeds per row. Each year, standard agronomic practices for intensive winter wheat production were used. In the autumn before ploughing, nitrogen (N), phosphorus, and potassium (7:20:30) in the amount of 300 kg ha^{-1} and UREA (46% N) at a rate of 150 kg ha^{-1} were added to the soil. Calcium ammonium nitrate (CAN, 27% N) was applied as a top-dressing at the beginning of wheat tillering (185 kg ha^{-1}) and in the phase of intensive stem elongation, when the first or second node was detectable on the main stem in most genotypes (110 kg ha^{-1}). For weed control, 0.8 g L^{-1} of Pinoxaden (50 g L^{-1}) and Fluroxypyr (360 g L^{-1}) were applied when the majority of genotypes developed first node on the main stem. Fungal diseases were treated with Chlorothalonil (400 g L^{-1}) and Azoxystrobin (80 g L^{-1}) at a rate of 2.5 L ha^{-1} at the time of the emergence of flag leaves in most genotypes.

2.2. Inoculum Production and Inoculation Procedure

The first method of inoculation was the spray method, for which the *Fusarium* inoculum was developed using the "bubble breeding" method proposed by Mesterházy in 1977 [33]. Each year, new isolates were prepared and used for artificial inoculation by the spray method. The fungus *F. graminearum* was isolated from infected wheat grains from the previous year, which came from wheat genotypes with a high FDK score. Different strains of *Fusarium* spp. were isolated from 36 infected wheat grains on PDA medium. The 12 best isolates were selected visually, and based on the appearance of spores under the microscope, their membership to the species *F. graminearum* was confirmed according to the identification keys of Nelson et al. [34]. The aggressiveness test according to Mesterházy [35] was performed on these 12 isolates (Figure S1). The four most aggressive isolates were selected, and liquid inoculum was prepared from them for the spray method immediately before starting artificial inoculation. The concentrations of the inoculum were adjusted to 500,000 spores per ml using a hemocytometer. Equal volumes of liquid inoculum from four isolates were mixed. The liquid inoculum was stored at 4 °C during the inoculation period. The first inoculation of each plot was performed when 50% of the plants were at anthesis. The second inoculation followed two days later. The wheat spikes

were sprayed using a backpack-carried manual sprayer early in the morning, and 40 mL of inoculum was applied to each plot in both sprays. The second method of inoculation was conducted using infected maize stalks collected in an infected maize field from a maize inbred line susceptible to *F. graminearum*. The stalks were cut into 20–30 cm long pieces and scattered on the soil surface (15–20 pieces per experimental plot) in late autumn when the first two leaves emerged on plants (Figure 1). The third type of infection occurred under natural conditions, i.e., no artificial inoculation was performed. In this study, no mist irrigation was applied for either artificial inoculation or natural infection.

Figure 1. Maize stalk residue deployed in the field at the location of Botinec in 2011.

2.3. Fusarium Head Blight Evaluation

The percentage of visually infected spikelets, referred to as the visual rating index (VRI), was estimated on a sample of approximately 100 spikes according to a linear scale from 0 to 100% (Table S1). Disease symptoms were assessed 21 and 25 days after spray inoculation of each genotype and finally expressed as the mean of the two readings. The percentage of Fusarium-damaged kernels (FDK) was determined on ten randomly selected spikes taken from each experimental plot after harvest. The spikes were threshed by hand and the Fusarium-damaged and normal kernels were counted. Only the pinkish white-colored grains along with the slightly infected whitish powdered kernels were considered as Fusarium-damaged, while the normally colored but shriveled kernels were not considered [25].

2.4. Statistical Analysis

An analysis of variance (ANOVA) was performed across inoculation methods for each year as well as for each year × inoculation method combination for two FHB ratings (VRI and FDK) using the PROC GLM of SAS/STAT [36]. The components of variance for the second ANOVA were obtained by equating the observed mean squares from the ANOVA to their expectations and solving for the desired variance components. Genotypic variance (σ^2_G) was calculated as (genotype mean square—error mean square)/r, where r is the number of replicates, and the error variance (σ^2_ε) is equal to the error mean square. Heritability on a plot mean basis was estimated using the equation: $h^2 = \sigma^2_G/(\sigma^2_G + \sigma^2_\varepsilon)$. Spearman's rank correlation coefficients between inoculation methods for two FHB ratings (VRI and FDK) as well as between VRI and FDK within each of the three inoculation methods were calculated using a PROC CORR of the SAS/STAT [36].

3. Results

3.1. Analysis of Variance and Heritability

An analysis of variance across 25 wheat genotypes and three inoculation methods (Table 1) revealed a significant effect of inoculation method (IM) and genotype (G), and a significant IM × G interaction for visual rating index (VRI) and Fusarium-damaged kernels (FDK) in all four years. For the VRI, IM and G had similar effects in 2011, 2012 and 2013, explaining 34 to 39% and 32 to 38% of the total sum of squares (SS) respectively, while the IM × G interaction accounted for 18 to 24% of the total SS. In 2014, variation in VRI was predominantly explained by G (66% of SS), while IM and the IM × G interaction accounted for only 15 and 10% of the total SS, respectively. The variation of FDK in all years was explained primarily by IM, which contributed between 36 and 56% of the total SS, whereas G and IM × G interactions contributed from 20 to 26% and 14 to 19%, respectively. Considering the two methods of artificial inoculation (spray and maize stalk), the ANOVA showed a significant effect of all sources of variation for VRI and FDK, except for IM × G for VRI in 2011 and for FDK in 2014 (Table 1). However, the effect of G, which explained 69 to 88% of SS for VRI and 51 to 70% of SS for FDK, was much larger than in an analysis of all three inoculation methods. Consequently, the contribution of IM for both traits and the IM × G interaction for FDK was of a much smaller magnitude.

Table 1. Analysis of variance for the visual rating index and Fusarium-damaged kernels in four years across 25 genotypes and three inoculation methods (**A**) and two inoculation methods (**B**).

	A. Three Inoculation Methods							**B. Two Inoculation Methods** [a]						
Year	**Inoculation Method (IM)**		**Genotype (G)**		**IM × G**		**Error**	**Inoculation Method (IM)**		**Genotype (G)**		**IM × G**		**Error**
Df	**2**		**24**		**48**		**216**	**1**		**24**		**24**		**144**
	SS%	**Sig**	**SS%**	**Sig**	**SS%**	**Sig**	**SS%**	**SS%**	**Sig**	**SS%**	**Sig**	**SS%**	**Sig**	**SS%**
							Visual rating index (VRI)							
2011	36	**	32	**	18	**	14	1	**	72	**	4	ns	22
2012	34	**	35	**	24	**	7	10	**	69	**	12	**	9
2013	39	**	38	**	18	**	5	1	**	88	**	3	**	8
2014	15	**	66	**	10	**	9	1	**	86	**	6	**	7
							Fusarium-damaged kernels (FDK)							
2011	56	**	23	**	14	**	7	2	**	70	**	12	**	15
2012	56	**	20	**	19	**	5	13	**	57	**	20	**	10
2013	47	**	22	**	19	**	12	17	**	51	**	13	**	19
2014	36	**	26	**	14	**	24	1	*	57	**	5	ns	37

* and ** F test significant (Sig) at $p < 0.05$ and $p < 0.01$, respectively; SS% percent of the total sum of squares; [a] only spray and maize stalk inoculation were included in the ANOVA.

Heritability estimates for the two FHB scores under different inoculation methods for each respective set of 25 genotypes evaluated in 2011, 2012, 2013, and 2014 are shown in Table 2. For VRI, heritability over four years varied from 0.68 to 0.91 for the spray method, and from 0.73 to 0.95 for the infected maize stalk method. The estimated heritability in 2011 and 2013 was similar between the two methods. In 2012, it was higher for the spray method, and in 2014, it was higher for infected maize stalks. The heritabilities for VRI under natural infection were much lower compared to the heritabilities of the two methods of artificial inoculation, ranging from 0.26 to 0.65. For FDK, the heritability varied from 0.56 to 0.85 for the spray method, from 0.38 to 0.83 for the infected maize stalks, and from 0.11 to 0.44 for the natural infection. Heritability estimates in 2011 and 2012 were comparable between VRI and FDK for the three methods, and in 2013 and 2014 they were considerably lower for FDK, especially under the maize stalk inoculation and natural infection.

Table 2. Heritability estimates for the visual rating index (VRI) and the percentage of Fusarium-damaged kernels (FDK) in 25 wheat genotypes evaluated under three inoculation methods (spray, maize stalk and natural) in 2011, 2012, 2013, and 2014.

Year	Inoculation Method			Inoculation Method		
	Spray	Maize Stalk	Natural	Spray	Maize Stalk	Natural
	Visual Rating Index (VRI)			Fusarium-Damaged Kernels (FDK)		
2011	0.68	0.73	0.26	0.85	0.65	0.29
2012	0.91	0.77	0.46	0.85	0.83	0.44
2013	0.90	0.89	0.65	0.80	0.38	0.11
2014	0.83	0.95	0.63	0.56	0.43	0.12

3.2. FHB Scores under Different Inoculation Methods

The visual rating index (VRI) varied significantly among genotypes for all three inoculation methods in all four growing seasons (Figure 2, Table S2). The highest mean VRI values were obtained using spray inoculation (S) in all years, except in 2014 when inoculation with maize stalk (MS) resulted in a significantly higher VRI value. Under natural conditions of infection (N), VRI values were, as expected, the lowest. Despite significant differences between mean VRI values for spray and maize stalk inoculation, VRI values of the two methods were comparable in 2011, 2013, and 2014, with respective mean values of 11.7 and 9.8%, 25.05 and 21.7%, and 27.0 and 31.7%. In the same years, the range of VRI scores was also similar for the two methods of artificial inoculation. The higher mean VRI in 2014 for the maize stalk inoculation method compared to the spray inoculation method primarily resulted from the higher VRI values observed in less resistant genotypes (Figure 2D). In the same year, VRIs under natural conditions was at least ten times higher than in the three previous growing seasons (12.4% in 2014, 1.5% in 2013, 1.1% in 2012, and 0.3% in 2011). Mean FDK values in all four years were significantly higher for the spray method compared to maize stalk method (Figure 3, Table S3). The difference between the mean FDK scores of the spray inoculation and maize stalk inoculation methods, as well as the difference between their ranges was most pronounced in 2013, with means of 21.5 and 11.4% and ranges from 3.8 to 55.3% and 1.9 to 22.1%, respectively. In 2011, 2012, and 2014, the spray and maize stalk inoculations produced comparable FDKs with mean values of 16.9 and 14.6%, 16.9 and 11.9%, and 21.0 and 18.0%. Under natural conditions (N), FDK values were considerably lower than for the two methods of artificial inoculation.

3.3. Correlations

The correlations between inoculation methods for visual rating index (VRI) and Fusarium-damaged kernels (FDK) in the four years of the study are shown in Table 3. The VRIs of the two methods of artificial inoculation (spray and maize stalk) were strongly positively correlated with coefficients of correlation ranging from 0.88 in 2012 to 0.94 in 2013. On the other hand, the correlation between VRI under natural infection and VRI under two types of artificial inoculation was moderately positive in 2011 and 2012, and strong and positive in 2013 and 2014. In 2012, for VRI, the correlation between natural infection and spray inoculation was slightly higher than between natural infection and maize stalk inoculation (0.72 vs. 0.57), whereas in 2014 the corresponding correlation coefficients were 0.83 and 0.93.

The FDK values for the two methods of artificial inoculation were strongly positively correlated, with the correlation coefficients ranging from 0.60 to 0.87. In 2011, the correlation of FDK scores between natural infection and maize stalk inoculation was slightly higher than the corresponding correlation between natural infection and spray inoculation (0.74 vs. 0.54), the correlations of FDK scores were similar in 2012, and the opposite was true in 2014 (0.49 vs. 0.62). In 2013, the correlations of FDK scores between natural infection and both methods of artificial inoculation were not significant.

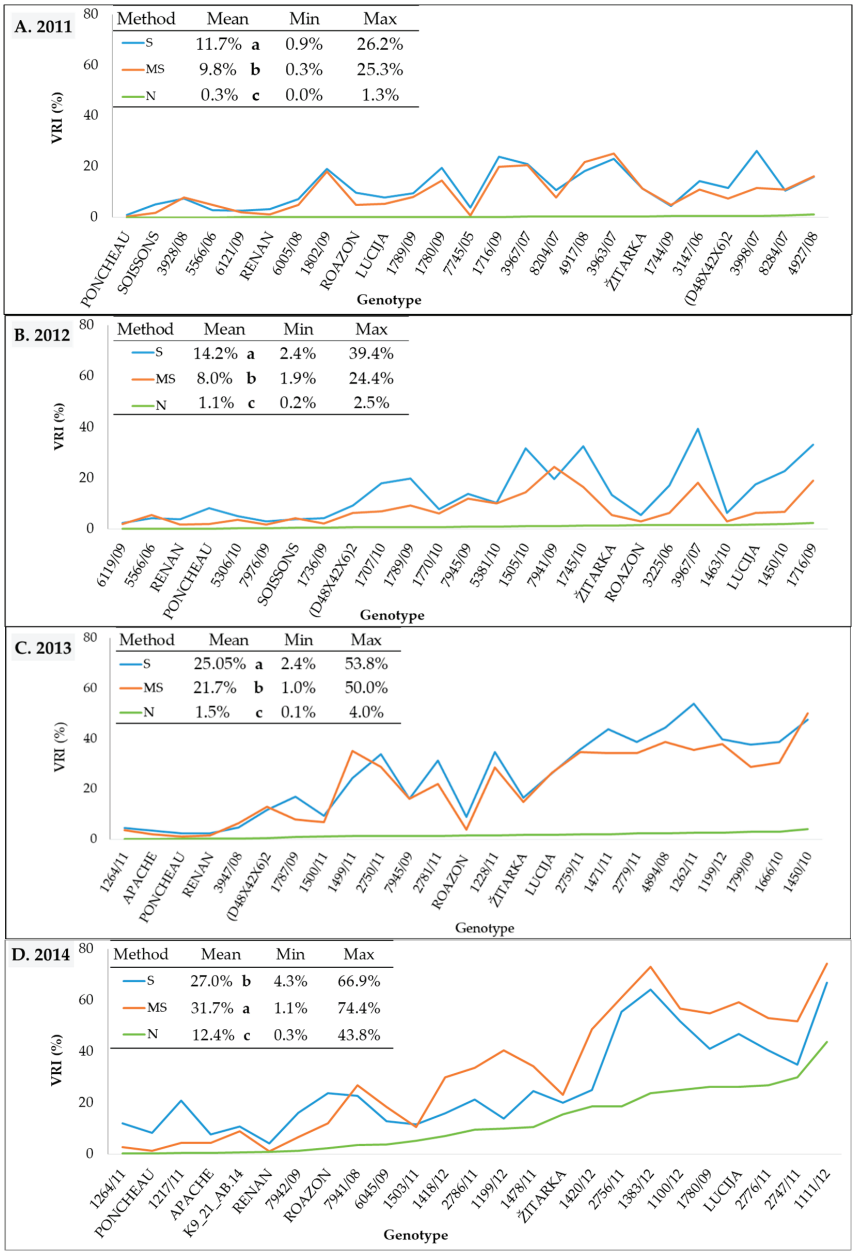

Figure 2. Visual rating index (VRI) in 25 wheat genotypes under spray inoculation (S), maize stalk inoculation (MS), and natural infection (N) in the (**A**) 2011, (**B**) 2012, (**C**) 2013, and (**D**) 2014 growing seasons. Means of the inoculation methods followed by the different letter are significantly different according to LSD test at $p < 0.05$.

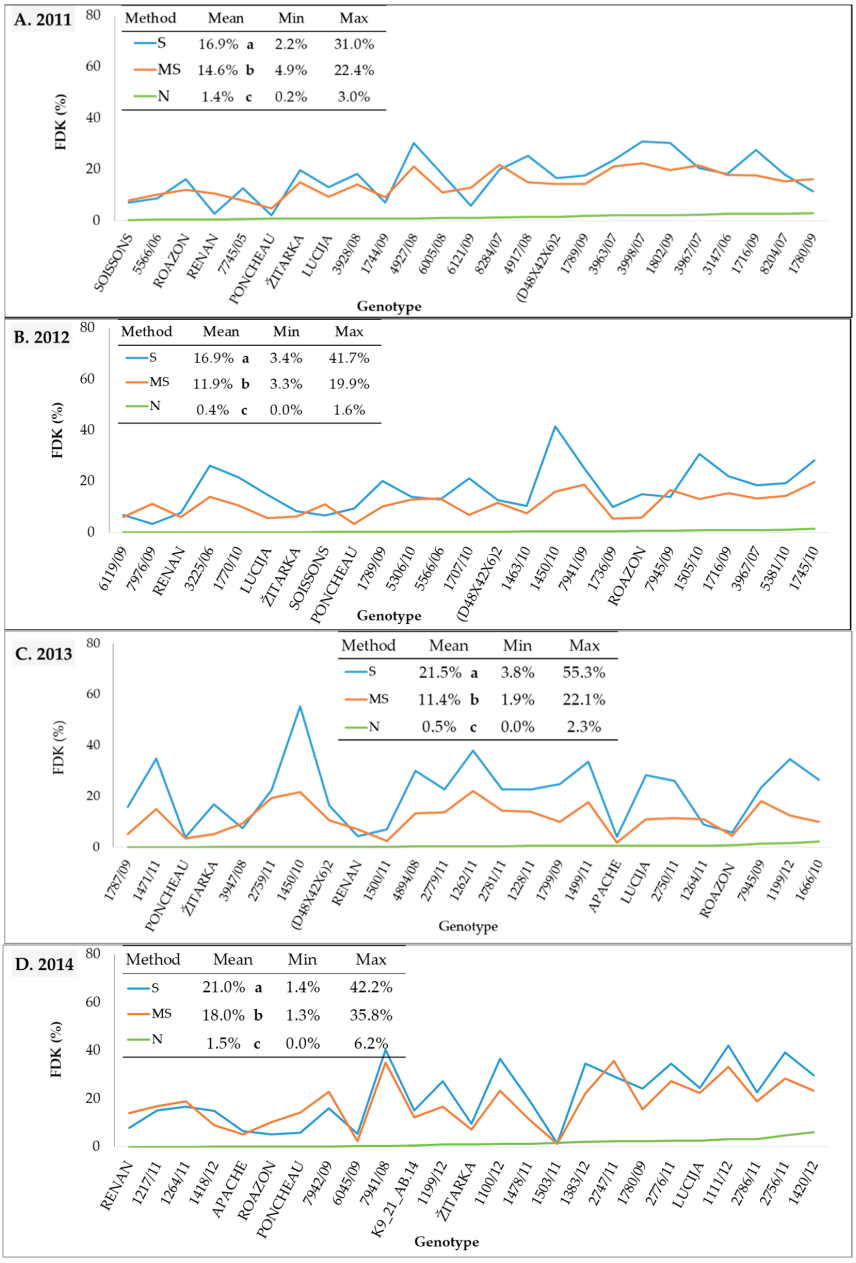

Figure 3. Fusarium-damaged kernels (FDK) in 25 wheat genotypes under spray inoculation (S), maize stalk inoculation (MS), and natural infection (N) in the (**A**) 2011, (**B**) 2012, (**C**) 2013, and (**D**) 2014 growing seasons. Means of the inoculation methods followed by the different letter are significantly different according to LSD test at $p < 0.05$.

Table 3. Spearman's rank correlation coefficients between inoculation methods for visual rating index and Fusarium-damaged kernels in 2011, 2012, 2013, and 2014.

		Visual Rating Index				Fusarium-Damaged Kernels			
Year	**Inoculation Method**	**Spray**		**Maize Stalk**		**Spray**		**Maize Stalk**	
2011	Maize stalk	0.92	**			0.84	**		
	Natural	0.60	**	0.55	**	0.54	**	0.74	**
2012	Maize stalk	0.88	**			0.60	**		
	Natural	0.72	**	0.57	**	0.53	**	0.52	**
2013	Maize stalk	0.94	**			0.77	**		
	Natural	0.88	**	0.82	**	0.19	ns	0.06	ns
2014	Maize stalk	0.90	**			0.87	**		
	Natural	0.83	**	0.93	**	0.62	**	0.49	*

* and ** correlation coefficient significant at $p < 0.05$ and $p < 0.01$, respectively.

The correlations between VRI and FDK were moderate to strong for the two artificial inoculation methods in the four years of study, with coefficients ranging from 0.77 to 0.89 and 0.55 to 0.86 for spray inoculation and maize stalk inoculation, respectively (Table 4). The correlation coefficients were similar for the two methods of artificial inoculation in 2011, 2012, and 2013, while the correlation coefficient was slightly stronger for the spray method in 2014. In natural infection compared to the two artificial inoculation methods, the correlation between VRI and FDK was generally weaker, except in 2014, when it was at the level observed for spray inoculation.

Table 4. Spearman's rank correlation coefficients between visual rating index (VRI) and Fusarium-damaged kernels (FDK) under spray inoculation, maize stalk inoculation, and natural infection in 2011, 2012, 2013, and 2014.

	Inoculation Method					
Year	**Spray**		**Maize Stalk**		**Natural**	
	VRI Versus FDK					
2011	0.83	**	0.86	**	0.43	*
2012	0.81	**	0.72	**	0.46	*
2013	0.89	**	0.77	**	0.16	ns
2014	0.77	**	0.55	**	0.82	**

* and ** correlation coefficient significant at $p < 0.05$ and $p < 0.01$, respectively.

4. Discussion

For routine screening of FHB resistance in large breeding populations, the faster, cheaper, and more reliable inoculation method is preferable [2]. In the present study, an alternative inoculation method for screening FHB resistance in wheat, known as deployment of *Fusarium*-infected maize stalks, was evaluated over four consecutive years. The standard spray method and natural infection were used as controls. As expected, considerably more FHB symptoms in terms of both VRI and FDK were observed under the two artificial inoculation methods compared to natural infection. Although mean VRI and FDK values were generally significantly higher for spray than for maize stalk inoculation, the two inoculation methods resulted in comparable mean values and ranges for both FHB-related traits in three of four study years (Figures 1 and 2). Similar to our study, Mesterhazy et al. [25] compared two variants of spray inoculation with inoculation using maize stalks and reported slightly higher mean FHB scores for the maize stalk method than for the standard spray method, similar mean FDK scores for the two spray methods, and considerably lower deoxynivalenol (DON) grain content for maize stalk method. In their study, the spray method with bags resulted in higher levels of symptoms for all three studied traits compared to the standard spray method and maize stalk method. The authors pointed out that differentiation of genotypes in resistance was more secure at

higher levels of FHB symptoms. In this sense, the similar levels of FHB symptoms for VRI and FDK observed for standard spray inoculation and maize stalk inoculation in the present study indicate the suitability of both methods for screening FHB resistance. An analysis of variance in the present study showed that the inoculation method, genotype, and the inoculation method × genotype interaction had a significant effect on VRI and FDK. Mesterhazy et al. [25] also reported a significant inoculation method × genotype interaction for the FHB score and the FDK and DON content in grain, although the interaction effect was significantly weaker than the main effect of genotype for all of the traits studied. In the present study, the contribution of the inoculation method × genotype interaction effect to the total phenotypic variability for VRI was also much smaller compared to the main genotype effect (Table 1). On the other hand, the contributions of genotype and inoculation method × genotype interaction to the total phenotypic variability for FDK were similar. However, when considering only spray and maize stalk inoculations, the effect of genotype for both VRI and FDK was much larger than the effect of the method × genotype interaction. This suggests that the observed magnitude of the inoculation method × genotype interaction in the present study was mainly due to the method of natural infection. Engle et al. [37] also found a significant effect of the interaction between the genotype and inoculation technique for FHB severity in an experiment in which four methods of artificial inoculation of ears with *F. graminearum* were applied to seven wheat genotypes. Similarly, Miedaner et al. [2] found a significant inoculation × genotype interaction for visual FHB symptoms when comparing point and spray inoculation in wheat and concluded that the genotype-specific response to a particular method may be the result of different contributions of individual components (such as type I and II resistance) to the overall FHB resistance. The available literature shows that not only FHB resistance itself, but also individual FHB resistance components are quantitatively inherited and are often under the control of resistance component-specific QTLs [3,14,20,38–41].

The magnitude of the method × genotype interaction effects over the four years of the present study was reflected in the strength of phenotypic correlations found between the inoculation methods studied. The VRI scores of the two methods of artificial inoculation (spray and maize stalk) were strongly positively correlated with correlation coefficients ranging from 0.88 to 0.94, whereas the correlations of FDK scores were somewhat lower, ranging from 0.60 to 0.87. The correlations of the two methods of artificial inoculation and natural infection were strong and positive for VRI in 2013 and 2014, and were moderate and positive in 2011 and 2012. For FDK, the respective correlations over four years of the study were weak to moderate. In agreement with our results, Mesterhazy et al. [25] also found a higher positive correlation between the standard spray method and the maize stalk method for the FHB score than for FDK (0.73 vs. 0.63, respectively). Miedaner et al. [2] reported lower phenotypic correlation coefficients between spray and point inoculation methods for the percentage of infected spikelets (0.40) and the relative spike weight (0.52). A wide range in the strength of correlations between different inoculation methods for FHB traits observed in the present and previous studies is reflected in the agreement or disagreement in the ranking of genotypes, and suggests that certain genotypes have a specific type of resistance, while others combine different levels of multiple types of resistance. Therefore, the temporal and/or spatial combination of different inoculation methods could provide complementary information on genotype resistance to FHB. However, environmental factors such as temperature, precipitation, and relative humidity cannot be ruled out, as they may affect the development of disease symptoms to different degrees for different inoculation methods.

Another important aspect in evaluating the reliability of an inoculation method is the correlation between the various FHB-associated traits and their heritability for a given method. From a practical point of view, visual evaluation of FHB symptoms on spikes is less laborious and time-consuming than evaluation of Fusarium-damaged kernels (FDK) and is preferred by breeders. In the present study, the correlation coefficients between VRI and FDK were moderately to strongly positive for the two methods of artificial in-

oculation, ranging from 0.77 to 0.89 for spray inoculation and 0.55 to 0.86 for maize stalk inoculation, whereas they were much lower for natural infection in three of four study years. Consistent with our results, correlations between FHB severity and FDK in the study of Kubo et al. [42] ranged from 0.78 to 0.81 over a three-year field experiment including 31 wheat cultivars inoculated with *F. graminearum*. Similar values of correlation coefficients between the two FHB ratings were reported by Mesterhazy [43], who examined the responses of 19 wheat genotypes to seven isolates of *F. graminearum* and *F. culmorum* (0.74), and Goral et al. [44], who evaluated 27 wheat lines inoculated with *F. culmorum* (0.78). On the other hand, He et al. [39] found a much lower correlation between FHB symptoms on spikes and FDK in a RIL population of wheat inoculated with *F. graminearum*, ranging from 0.29 to 0.30 over three years. In addition to the two visual assessments of FHB symptoms, the content of DON is commonly used as an indicator of mycotoxin contamination of grains, but measurement of DON is expensive and impractical for routine breeding practices. Therefore, the extent of correlations between visual FHB ratings and DON content is critical to know for the use of visual FHB ratings as indirect criteria in selecting for low DON content. In several studies, FDK has been shown to be a better predictor of grain DON contamination than visual symptoms on spikes [5,20,25,39,43,45], making FDK a preferred indirect trait when selecting for low DON content.

In the present study, heritability for VRI over four years ranged from 0.68 to 0.91 for the spray method and from 0.73 to 0.95 for the infected maize stalks, whereas it was much lower for natural infection, ranging from 0.26 to 0.65. On the other hand, heritability estimates for FDK compared to VRI were slightly lower for spray inoculation (0.56 to 0.85), moderately lower for maize stalk inoculation (0.38 to 0.83), and much lower for natural infection (0.11 to 0.44). Miedaner et al. [2] reported that the heritability estimates were higher for spray inoculation than for point inoculation, as measured by the percentage of infected spikelets (0.81 vs. 0.77) and relative head weight (0.77 vs. 0.52). These findings are consistent with our study because we found that the differences between methods were greater for the heritability of FHB symptoms estimated on kernels than for the FHB symptoms associated with spikes. Zhang et al. [20] found similar ranges of heritability for FHB symptoms on spikes, FDK, and DON content, ranging from 0.55 to 0.85 in different environments. Moderate to high heritability of FHB resistance components was also reported in some other studies [39,46,47], suggesting that genetic variation plays a major role in the phenotypic variation of FHB-related traits.

5. Conclusions

The inoculation method using the infected maize stalks resulted in fewer FHB symptoms than the spray inoculation method, but from a practical point of view, the differentiation of genotypes according to their resistance was successful, especially for the more practical and quicker visual evaluation. This inoculation method does not require laboratory equipment or inoculum production. Resistance trials can be conducted at locations which are more distant from the laboratory, and flowering time recordings are not required. In this context, maize stalks can also be used as an alternative source of inoculum in early generations of selection when a large number of genotypes with a wide range of flowering times are evaluated. This method also simulates frequent production practice, especially on small family husbandries, where winter wheat is grown after maize and where large amounts of maize debris on the soil surface serves as a good source of inoculation. FHB severity under natural conditions was significantly lower than the FHB severity observed under the two methods of artificial inoculation analysed in the present study, and was largely dependent on the environmental conditions. Nevertheless, the resistance levels determined under natural conditions served as a good control for the resistance levels determined by the two methods of artificial inoculation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13041175/s1, Table S1: Scale for the assessment of visual rating index (% of diseased spikelets per plot); Table S2: Visual rating index (VRI, %) for 25 wheat genotypes under three inoculation methods (spray, maize stalk and natural) in 2011, 2012, 2013, and 2014; Table S3: Fusarium-damaged kernels (FDK, %) for 25 wheat genotypes under three inoculation methods (spray, maize stalk and natural) in 2011, 2012, 2013, and 2014; Figure S1. Aggressiveness test with 12 different isolates of *F. graminearum*.

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References

1. Birr, T.; Hasler, M.; Verreet, J.A.; Klink, H. Composition and predominance of Fusarium species causing Fusarium head blight in winter wheat grain depending on cultivar susceptibility and meteorological factors. *Microorganisms* **2020**, *8*, 617. [CrossRef] [PubMed]
2. Miedaner, T.; Moldovan, M.; Ittu, M. Comparison of spray and point inoculation to assess resistance to Fusarium head blight in a multienvironment wheat trial. *Phytopathology* **2003**, *93*, 1068–1072. [CrossRef] [PubMed]
3. Buerstmayr, H.; Ban, T.; Anderson, J.A. QTL mapping and marker assisted selection for Fusarium head blight resistance in wheat: A review. *Plant Breed.* **2009**, *128*, 1–26. [CrossRef]
4. McMullen, M.; Bergstrom, G.; De Wolf, E.; Dill-Macky, R.; Hershman, D.; Shaner, G.; Van Sanford, D. A Unified effort to fight an enemy of wheat and barley: Fusarium head blight. *Plant Dis.* **2012**, *96*, 1712–1728. [CrossRef]
5. Mesterházy, A.; Varga, M.; György, G.; Lehoczki-Krsjak, S.; Tóth, B. The role of adapted and non-adapted resistance sources in breeding resistance of winter wheat to Fusarium head blight and deoxynivalenol contamination. *World Mycotoxin J.* **2018**, *11*, 539–557. [CrossRef]
6. Martínez, M.; Ramírez Albuguerque, L.; Arata, F.A.; Biganzoli, F.; Fernández Pinto, V.; Stenglein, S.A. Effects of Fusarium graminearumand Fusarium poae on disease parameters, grain quality and mycotoxins contamination in bread wheat (Part I). *J. Sci. Food Agric.* **2020**, *100*, 863–873. [CrossRef]
7. Steiner, B.; Buerstmayr, M.; Michel, S.; Schweiger, W.; Lemmens, M.; Buerstmayr, H. Breeding strategies and advances in line selection for Fusarium head blight resistance in wheat. *Trop. Plant Pathol.* **2017**, *42*, 165–174. [CrossRef]
8. Beccari, G.; Arellano, C.; Covarelli, L.; Tini, F.; Sulyok, M.; Cowger, C. Effect of wheat infection timing on Fusarium head blight causal agents and secondary metabolites in grain. *Int. J. Food Microbiol.* **2019**, *290*, 214–225. [CrossRef]
9. Dean, R.; Van Kan, J.A.; Pretorius, Z.A.; Hammond-Kosack, K.E.; Di Pietro, A.; Spanu, P.D.; Rudd, J.J.; Dickman, M.; Kahmann, R.; Ellis, J. The top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **2012**, *13*, 414–430. [CrossRef]
10. Waalwijk, C.; Kastelein, P.; Vries, I.; Kerény, Z.; van der Lee, T.; Hesselnik, T.; Köhl, J.; Kema, G. Major changes in *Fusarium* spp. in wheat in the Netherlands. *Eur. J. Plant Pathol.* **2003**, *109*, 743–754. [CrossRef]
11. Xu, X.M.; Parry, D.W.; Nicholson, P.; Thomsett, M.A.; Simpson, D.; Edwards, S.G.; Cooke, B.M.; Doohan, F.M.; Brennan, J.M.; Moretti, A.; et al. Predominance and association of pathogenic fungi causing Fusarium ear blightin wheat in four European countries. *Eur. J. Plant Pathol.* **2005**, *112*, 143–154. [CrossRef]
12. Cosic, J.; Jurkovic, D.; Vrandecic, K.; Simic, B. Pathogenicity of Fusarium species to wheat and barley ears. *Cereal Res. Commun.* **2007**, *35*, 529–532. [CrossRef]
13. Spanic, V.; Lemmens, M.; Drezner, G. Morphological and molecular identification of Fusarium species associated with head blight on wheat in east Croatia. *Eur. J. Plant Pathol.* **2010**, *128*, 511–516. [CrossRef]
14. Buerstmayr, M.; Steiner, B.; Buerstmayr, H. Breeding for Fusarium head blight resistance in wheat-progress and challenges. *Plant Breed.* **2020**, *139*, 429–454. [CrossRef]
15. Mesterházy, A.; Toth, B.; Varga, M.; Bartok, T.; Szabo-Hever, A.; Farady, L.; Lehoczki-Krsjak, S. Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of Fusarium head blight and deoxynivalenol. *Toxins* **2011**, *3*, 1453–1483. [CrossRef]
16. Willyerd, K.T.; Li, C.; Madden, L.V.; Bradley, C.A.; Bergstrom, G.C.; Sweets, L.E.; Paul, P.A. Efficacy and stability of integrating fungicide and cultivar resistance to manage Fusarium head blight and deoxynivalenol in wheat. *Plant Dis.* **2012**, *96*, 957–967. [CrossRef]

17. Gilbert, J.; Haber, S. Overview of some recent research developments in Fusarium head blight of wheat. *Can. J. Plant Pathol.* **2013**, *35*, 149–174. [CrossRef]
18. Paul, P.A.; McMullen, M.P.; Hershman, D.E.; Madden, L.V. Meta-analysis of the effects of triazole-based fungicides on wheat yield and test weight as influenced by Fusarium head blight intensity. *Phytopathology* **2010**, *100*, 160–171. [CrossRef]
19. Bai, G.; Shaner, G. Scab of wheat: Prospects for control. *Plant Dis.* **1994**, *78*, 760–766. [CrossRef]
20. Zhang, W.; Boyle, K.; Brûlé-Babel, A.L.; Fedak, G.; Gao, P.; Robleh Djama, Z.; Polley, B.; Cuthbert, R.D.; Randhawa, H.S.; Jiang, F.; et al. Genetic characterization of multiple components contributing to Fusarium head blight resistance of FL62R1, a Canadian bread wheat developed using systemic breeding. *Front. Plant Sci.* **2020**, *11*, 580833. [CrossRef]
21. Miedaner, T.; Korzun, V. Marker-assisted selection for disease resistance in wheat and barley breeding. *Phytopathology* **2012**, *102*, 560–566. [CrossRef] [PubMed]
22. Rudd, J.C.; Horsley, R.D.; McKendry, A.L.; Elias, E.M. Host plant resistance genes for Fusarium head blight: Sources, mechanisms and utility in conventional breeding systems. *Crop Sci.* **2001**, *41*, 620–627. [CrossRef]
23. Thomson, C.J. Evaluation of An Inoculation Method and Quantitative Trait Loci for Fusarium Head Blight Resistance in Wheat. Master's Thesis, University of Illinois at Urbana-Champaign, Champaign, IL, USA, 2010.
24. Imathiu, M.; Edwards, S.G.; Ray, R.V.; Back, M. Artificial inoculum and inoculation techniques commonly used in the investigation of Fusarium head blight in cereals. *Acta Phytopathol. Hung.* **2014**, *49*, 129–139. [CrossRef]
25. Mesterházy, A.; Lehoczki-Krsjak, S.; Varga, M.; Szabo-Hever, A.; Tóth, B.; Lemmens, M. Breeding for FHB resistance via Fusarium damaged kernels and deoxynivalenol accumulation as well as inculation methods in winter wheat. *Agric. Sci.* **2015**, *6*, 970–1002. [CrossRef]
26. Mesterházy, A.; Bartók, T.; Lamper, C.S. Influence of cultivar resistance, epidemic severity, and Fusarium species on the efficacy of fungicide control of Fusarium head blight in wheat and deoxynivalent (DON) contamination of grain. *Plant Dis.* **2003**, *87*, 1107–1115. [CrossRef]
27. Maiorano, A.; Blandino, M.; Reyneri, A.; Vanara, F. Effects of maize residues on the Fusarium spp. infection and deoxynivalenol (DON) contamination of wheat grain. *Crop. Prot.* **2008**, *27*, 182–188. [CrossRef]
28. Blandino, M.; Pilati, A.; Reyneri, A.; Scudellari, D. Effect of maize crop residue density on Fusarium head blight and on deoxynivalenol contamination of common wheat grains. *Cereal Res. Commun.* **2010**, *38*, 550–559. [CrossRef]
29. Xue, A.G.; Butler, G.; Voldeng, H.D.; Fedak, G.; Savard, M.E. Comparison of the influence of inoculum sources on the development of Fusarium head blight and the deoxynivalenol content in spring wheat in a disease nursery. *Can. J. Plant Pathol.* **2006**, *28*, 152–159. [CrossRef]
30. Tomasović, S.; Palaveršić, B.; Ikić, I. Study of wheat lines resistance to Fusarium head blight. *Cereal Res. Commun.* **2005**, *33*, 583–588. [CrossRef]
31. Tomasovic, S.; Palaversic, B.; Mlinar, R.; Ikic, I.; Sarcevic, H. Breeding winter wheat for yield and Fusarium head blight resistance in the Zagreb Bc Institute. *Cereal Res. Commun.* **2008**, *36* (Suppl. B), 179–180.
32. Tomasović, S.; Palaveršić, B.; Ikić, I.; Mlinar, R.; Šarčević, H.; Jukić, K.; Ivanušić, T. Latest results in breeding winter wheat for resistance to Fusarium head blight in the Zagreb Bc Institute. *Plant Breed. Seed Sci.* **2011**, *64*, 141–145. [CrossRef]
33. Mesterházy, Á. The effect of inoculation method on the expression of symptoms caused by Fusarium graminearum Schwabe on wheat in seedling stage. In *Symposium on Current Topics in Plant Pathology*; Kiadó, A., Ed.; Academic Publishing House of the Hungarian Academy of Sciences: Budapest, Hungary, 1975; issued 1977; pp. 223–232.
34. Nelson, P.E.; Toussoun, T.A.; Marasas, W.F.O. Section Discolor. In *Fusarium Species: An Illustrated Manual for Identification*; Pennsylvania State University Press: University Park, PA, USA, 1983; pp. 107–123.
35. Mesterházy, Á. A laboratory method to predict pathogenicity of Fusarium graminearum in field and resistance of wheat to scab. *Acta Phytopathol. Hung.* **1984**, *19*, 205–218.
36. SAS Institute Inc. *SAS/STAT 9.4 Users Guide*; SAS Inc.: Cary, NC, USA, 2013.
37. Engle, J.S.; Madden, L.V.; Lipps, P.E. Evaluation of inoculation methods to determine resistance reactions of wheat to *Fusarium graminearum*. *Plant Dis.* **2003**, *87*, 1530–1535. [CrossRef] [PubMed]
38. Buerstmayr, H.; Lemmens, M.; Hartl, L.; Doldi, L.; Steiner, B.; Stierschneider, M.; Ruckenbauer, P. Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. I. Resistance to fungal spread (Type II resistance). *Theor. Appl. Genet.* **2002**, *104*, 84–91. [CrossRef]
39. He, X.; Dreisigacker, S.; Singh, R.P.; Singh, P.K. Genetics for low correlation between Fusarium head blight disease and deoxynivalenol (DON) content in a bread wheat mapping population. *Theor. Appl. Genet.* **2019**, *132*, 2401–2411. [CrossRef]
40. Mesterházy, A. Updating the breeding philosophy of wheat to Fusarium head blight (FHB): Resistance Components. *Plants* **2020**, *9*, 1702. [CrossRef]
41. Goddard, R.; Steed, A.; Scheeren, P.L.; Maciel, J.L.N.; Caierão, E.; Torres, G.A.M.; Consoli, L.; Santana, F.M.; Fernandes, J.M.C.; Simmonds, J.; et al. Identification of Fusarium head blight resistance loci in two Brazilian wheat mapping populations. *PLoS ONE* **2021**, *16*, e0248184. [CrossRef]
42. Kubo, K.; Kawada, N.; Nakajima, T.; Kazuyuhi, H.; Fujita, M. Field evaluation of resistance to kernel infection and mycotoxin accumulation caused by Fusarium head blight in western Japanese wheat (*Triticum aestivum* L.) cultivars. *Euphytica* **2014**, *200*, 81–93. [CrossRef]

43. Mesterházy, A.; Bartók, T.; Mirocha, C.M.; Komoróczy, R. Nature of resistance of wheat to Fusarium head blight and deoxynivalenol contamination and their consequences for breeding. *Plant Breed.* **1999**, *118*, 97–110. [CrossRef]
44. Goral, T.; Wisniewska, H.; Ochodzki, P.; Nielsen, L.K.; Walentyn-Goral, D.; Stepien, L. Relationship between Fusarium head blight, kernel damage, concentration of *Fusarium biomass*, and *Fusarium toxins* in grain of winter wheat inoculated with *Fusarium culmorum*. *Toxins* **2018**, *11*, 2. [CrossRef]
45. Bai, G.; Plattner, R.; Desjardins, A.; Kolb, F.; McIntosh, R.A. Resistance to Fusarium head blight and deoxynivalenol accumulation in wheat. *Plant Breed.* **2001**, *120*, 1–6. [CrossRef]
46. Buerstmayr, H.; Steiner, B.; Lemmens, M.; Ruckenbauer, P. Resistance to Fusarium head blight in winter wheat: Heritability and trait associations. *Crop Sci.* **2000**, *40*, 1012–1018. [CrossRef]
47. Lu, Q.; Lillemo, M.; Skinnes, H.; He, X.; Shi, J.; Ji, F.; Dong, Y.; Bjørnstad, Å. Anther extrusion and plant height are associated with Type I resistance to Fusarium head blight in bread wheat line 'Shanghai-3/Catbird'. *Theor. Appl. Genet.* **2013**, *126*, 317–334. [CrossRef] [PubMed]

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Three-Year Survey of *Fusarium* Multi-Metabolites/Mycotoxins Contamination in Wheat Samples in Potentially Epidemic FHB Conditions

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Abstract: *Fusarium* head blight (FHB) is a fungal disease of cereals including wheat, which results in significant economic losses and reductions in grain quality. Additionally, the presence of *Fusarium* spp. results in productions of mycotoxins/metabolites, some of which are toxic in low concentrations. The liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was applied to 216 wheat samples from field conditions diseased with FHB. Data obtained show that out of 28 metabolites detected, deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), enniatin B (ENN B), enniatin B1 (ENN B1), culmorin, 15-hydroxyculmorin, and aurofusarin were the most prevalent mycotoxins/metabolites over three years (2014–2016). In 2014–2016, 100, 100 and 96% of the samples were contaminated with zearalenone (ZEN). Of the masked mycotoxins, D3G occurred at a high incidence level of 100% in all three investigated years. Among emerging mycotoxins, moniliformin (MON), beauvericin (BEA) and enniatins (ENNs) showed high occurrences ranging from 27 and 100% during three investigated years. Co-occurrence of *Fusarium* mycotoxins/metabolites was high and almost all were highly correlated to each other but their possible synergistic, additive, or antagonistic effects of toxicity, should be taken into consideration. Our results demonstrated that modified and emerging mycotoxins/metabolites contributed substantially to the overall contamination of wheat grains. To avoid disparagement, it is necessary to analyse these forms in future mycotoxin monitoring programs and to set their maximum levels.

Keywords: crop season; emerging mycotoxins; *Fusarium*; LC-MS/MS; mycotoxins; wheat

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1. Introduction

Fusarium spp. occur regularly each year in cereal crops over the globe, and additional concerns have created new insight into the extremely negative effects of mycotoxins on human and animal health. *Fusarium* head blight (FHB), mainly caused by *Fusarium graminearum* and *F. culmorum*, can significantly reduce grain yield and quality of wheat, and produce mycotoxins that affect food safety [1]. Nowadays, the term "food safety" is increasingly mentioned and hence mycotoxins are increasingly attracting attention, thus encouraging plant biologists and breeders to work on solutions to find resistant wheat genotypes to this widespread disease. Screening and identifying FHB resistant genes in wheat germplasm for development of resistant wheat varieties is the most effective way to manage FHB [2]. But however, agronomic practices and fungicides reduce the risk of damage to some extent. The best fungicide applications, considering the timing and dose of application, can partially reduce FHB symptoms [3]. Furthermore, creation of resistant varieties is hampered as FHB resistance has been classified into multiple types [4]. Type I

resistance is attributed to reduction of initial infection, type II resistance prevents spread of infection within the spike and type III confers resistance to mycotoxin accumulation. Type II resistance has been widely used in breeding programs due to its effective performance in reducing the impact of FHB on grain production. There is also evidence that FHB resistant wheat genotypes accumulate far fewer mycotoxins than susceptible ones [5]. FHB continues to threaten susceptible wheat varieties where environmental conditions such as high humidity and temperature persist during flowering. Warm and humid environmental conditions ideally propagate the pathogen which may result in severe disease outbreaks with substantial crop losses [6]. Typical FHB symptoms include water soaked lesions on glumes, followed by discoloration that spreads from the point of infection to the adjacent spikelets. As infection progresses, symptoms of wilting and blight spread over the entire spike, indicating premature senescence of infected spikes [7]. As previously mentioned, *Fusarium* mycotoxins in wheat are the main secondary metabolites that occur at levels of potential concern for human and animal health [8]. Deoxynivalenol (DON), zearalenone (ZEN), nivalenol (NIV), fumonisins (FB), T-2, and HT-2 toxins are the most important *Fusarium* mycotoxins occurring on a worldwide basis in cereal grains [9]. All of the above mentioned mycotoxins belong to the trichothecene's groups A and B, with the exception of FB and ZEN, which are listed as the most toxic [10]. Furthermore, as food and feed contaminants, they may cause alimentary hemorrhage and vomiting, while direct contact causes dermatitis [11]. In addition, the acute symptoms of trichothecenes detriments are gastroenteritis, nausea, anorexia, growth retardation, endocrine damage and immunosuppression [12]. The main biological effect of the non-steroidal estrogenic mycotoxin ZEN and its metabolites (especially α-zearalenol) is reproductive toxicity [13]. Limits for some mycotoxins have been recommended and specified in unprocessed cereals, milling products, and cereal end-use products: 200–1750 μg kg^{-1} for DON, 20–400 μg kg^{-1} for ZEN, 200–4000 μg kg^{-1} for the sum of B1 + B2 fumonisins (FB1 + FB2 combined) [14], and 15–1000 μg kg^{-1} for the sum of HT-2 and T-2 toxins [15]. European Commission has not yet given any legislative for NIV, but the European Food Safety Authority set a tolerated daily intake (TDI) of up to 1.2 μg kg^{-1} body weight per day [16].

The situation with the occurrence of mycotoxins becomes even more dangerous by the presence of modified *Fusarium* mycotoxins and so-called emerging mycotoxins [17]. *Fusarium* mycotoxins can be altered in their chemical structure, with unexpectedly high toxicity in the digestive tract of humans/animals although the metabolic fate is still not very well studied [18]. They may escape detection methods as they are chemically different from parental mycotoxins and there are currently no regulations for these newly developed metabolites/mycotoxins [19,20]. Previous reports have demonstrated the conversion of DON into DON-3-glucoside (D3G) as well as modifications of ZEN to glucoside or sulphate form [21,22]. Moreover, it was reported about glucoside forms of NIV in wheat products [23]. D3G is formed through the glycosylation of DON as the response to the detoxification process, but the greatest danger is hidden in the fact that D3G has been found in wheat lines with low FHB susceptibility [24]. Although D3G was found in wheat grains at concentrations that reached or exceeded the maximal permitted levels for DON, the toxicity of D3G remains still unknown [25]. On the other hand, the total amount of conjugated forms of ZEN exceeded the concentration of the parental mycotoxin [17], so these forms of ZEN should not be underestimated as conjugated ZEN derivatives can be efficiently hydrolysed. It is also interesting to note that NIV usually co-occurs with DON in wheat grains, where its modified form detected was nivalenol-glucoside (NIV-3G) [26].

Unlike some *Fusarium* mycotoxins, such as DON, T-2, HT-2, FB, and ZEN, whose presence in food and feed has been regulated by authorities, no limits have been set for emerging mycotoxins, such as enniatins (ENNs) [27]. Special attention should be paid to ENN B, as its potential toxicity may be enhanced by co-occurrence with other mycotoxins [28]. The role of other emerging mycotoxins, such as beauvericin (BEA), fusaproliferin (FUP) and moniliformin (MON), is not very well understood up today [29]. It has been reported that BEA is inducer of reactive oxygen species (ROS) leading to cell apoptosis but also it has very

efficient effects in the anticancer, antimicrobial, and insecticidal activities [30]. Although an anti-inflammatory activity of FUP was found [31], there is evidence of FUP phytotoxicity causing structural changes in chloroplasts of plants [32]. In the study of Bertuzzi et al. [33], it was reported that MON reduces the activity of glutathione peroxidase and glutathione reductase, thus increasing oxidative stress in plants. Overall, multi-mycotoxin contamination is very common because mycotoxins have additive/synergistic interactions that pose an additional risk to food safety [8]. This is supported by the fact that co-occurrence of mycotoxins in cereals has been previously reported [34,35]. In addition to problem in food safety, *Fusarium* spp. and their co-occurrence have a detrimental effect on the processing and rheological quality of wheat. FHB infection and its mycotoxins may reduce wheat milling performance, with a strong negative effect on end-use quality [36]. Also, FHB epidemic reduces processing quality in susceptible wheat varieties; primarily, sedimentation value and gluten index, and hence, had negative impact on rheological properties [37]. Of even greater concern, however, is the fact that the technological process can produce conjugated forms of mycotoxins in wheat end-use products [38]. Moreover, microorganisms used in fermentation and malting processes may transform mycotoxins into conjugated forms or even increase some parental forms of mycotoxins [1]. Nevertheless, according to some studies increased temperatures or fermentation can reduce the concentration of some *Fusarium* mycotoxins [39,40]. Nevertheless, the masked forms have significant toxicity due to their conversion by plant's metabolism or technological process.

The objectives of the present study were to investigate the occurrence of *Fusarium* mycotoxins/metabolites and their modified forms in wheat grain under potentially epidemic FHB conditions in three consecutive years and to determine the correlations between the levels of different mycotoxins in contaminated wheat samples.

2. Materials and Methods

2.1. Wheat Material and Field Conditions

Eight winter wheat varieties/lines ranging in FHB resistance and 28 of their progeny were included in the experiment (Supplementary Figure S1). These 36 genotypes were planted in October in three consecutive growing seasons (2013/2014, 2014/2015 and 2015/2016) at location Botinec, Croatia (45°45′11.49″ N; 15°56′4.98″ E) following randomized complete block design with two replicates. Each experimental plot was consisted of two rows with a length of 1 m and a row spacing of 0.25 m. The previous crop in all three growing seasons was rapeseed (*Brassica napus* subsp. *napus*). Fertilization was adapted to intensive wheat production. Before sowing, nitrogen (N), phosphorus and potassium (7:20:30), and UREA (carbonyl diamide with 46% N) in the amount of 300 kg ha^{-1} and 150 kg ha^{-1} were added in the soil. Calcium ammonium nitrate (CAN with 27% N) was added at the beginning of vegetation (185 kg ha^{-1}) and in the phase of intensive growth (110 kg ha^{-1}) (GS 31–33). For weed protection, 0.8 g L^{-1} of Axial 50 EC (pinoxaden 50 g L^{-1}) and Starane 250 (fluroxypyr 360 g L^{-1}) were applied (GS 21–23). Foliar protection was performed using the fungicide Amistar Opti (chlorothalonil 480 g L^{-1} plus azoxystrobin 80 g L^{-1}) at a rate of 2.5 L ha^{-1} (GS 37–38). To prevent insect's influence plants were treated with the insecticide Karate Zeon (lambda cyhalothrin 50 g L^{-1}) at a rate of 0.15 L ha^{-1} (GS 59–60).

Weather data were obtained from the Croatian Meteorological and Hydrological Service. At location Botinec weather data were different in May and June for three consecutive years with precipitation of 88 mm in May and 171 mm in June in 2014, 151 mm in May and 60 mm in June in 2015 and 101 mm in May and 133 mm in June in 2016 (Figure 1). Regarding temperature, the monthly mean values for May were 23, 23 and 24 °C in 2014–2016, respectively, while the monthly mean values for June were 28, 26 and 28 °C in the same years (Figure 2).

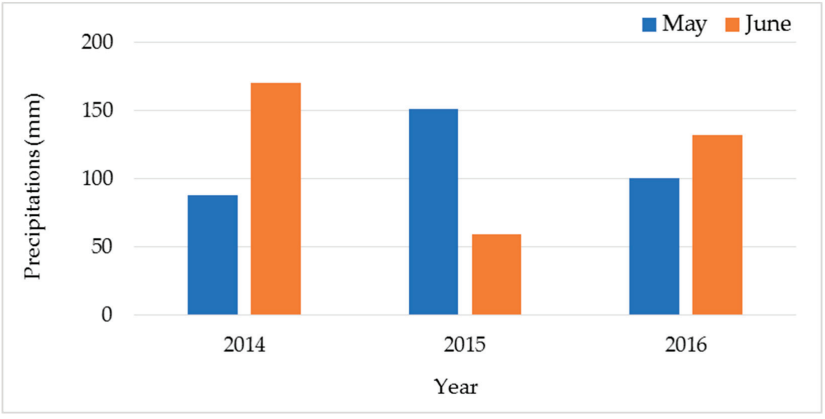

Figure 1. Rainfall in mm in May and June during three consecutive years.

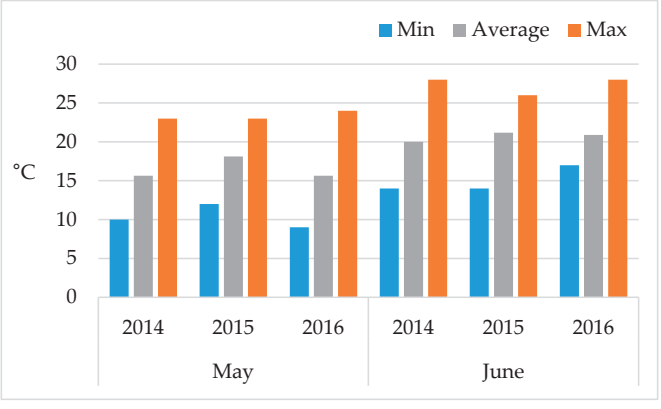

Figure 2. Mean daily temperatures in May and June during three consecutive years.

2.2. Inoculum Production and Inoculation

The four isolates of *F. graminearum* were used for artificial inoculation of plants and were previously collected from wheat in Croatian fields. For inoculum production the bubble-breeding method was used using the medium *Vigna radiata* [L.] R. Wilczek [41]. The concentration of inoculum was set up to 500,000 spores mL^{-1}. Each wheat genotype in the field experiment was inoculated separately at the flowering stage (GS 65–69) in the early morning, and the inoculation procedure was repeated two days later using the back-pack sprayer. Grain samples (total $n = 216$) of wheat genotypes ($n = 36$) in two replicates in three years of investigation were harvested when grain moisture was below 13%.

2.3. Disease Assessment

In each plot, the percentage of visually infected spikelets was estimated 18, 22, 26, and 30 days after the first inoculation. The area under the disease progress curve (AUDPC) was calculated for each plot using the following equation:

$$AUDPC = \Sigma \{[(yi + yi - 1)/2] \times (xi - xi - 1)\}$$

where Σ is the sum over four observations, yi is the score of visually infected spikelets on the ith day, and xi is the day of the ith observation. At harvest maturity (about 13% grain moisture), 10 randomly selected ears were taken from each plot, manually threshed

and the number of *Fusarium* damaged kernels (FDK) was determined and expressed as a percentage of the total number of kernels in the sample.

2.4. Mycotoxin Analysis

Determination of *Fusarium* mycotoxins/metabolites in 216 wheat samples was performed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) at the Department of Agrobiotechnology (IFA-Tulln), Institute of Bioanalytics and Agro-Metabolomics, University of Natural Resources and Life Sciences Vienna (BOKU), Austria, according to the same method previously described in the study by Sunic et al. [6]. Sample values below the LOD (<LOD) were replaced by a constant value of LOD/2 before statistical analysis of the data.

2.5. Statistical Analysis

Combined analysis of variance (ANOVA) was performed across three years and 36 genotypes using the GLM procedure in the statistical program SAS/STAT (SAS Institute Inc., Cary, NC, USA) [42]. Pearson correlation r values were determined using GraphPad Prism 9.4.1 [43].

3. Results

For three consecutive years, Fusarium head blight (FHB) severity was estimated by the area under the disease progress curve (AUDPC) and Fusarium damaged kernels (FDK). In addition, 28 *Fusarium* metabolites/mycotoxins were detected in 216 analysed wheat samples (36 genotypes × 3 years × 2 replicates). Furthermore, these metabolites/mycotoxins were classified into four different groups (trichothecenes and their derivatives, zearelenone and its derivatives, emerging mycotoxins and other *Fusarium* metabolites (Table 1, Figure 3). Analysis of variance revealed a significant effect of year for FDK as well as for all mycotoxins except HT-2 glucoside, moniliformin and enniatin B3, whereas genotype was significant for AUDPC for general resistance, FDK and all mycotoxins except T-2 toxin, HT-2 glucoside and equisetin (Table 1). On the other hand, the genotype × environment interaction was significant for FDK and only nine of the 28 mycotoxins analysed.

Table 1. Analysis of variance for area under the disease progress curve (AUDPC) for general resistance, *Fusarium* damaged kernels (FDK), and content of 28 mycotoxins in grain of 36 wheat genotypes grown in three vegetation years; F values and their significances (F Sign.) are shown.

		Year (Y)		Genotype (G)		G × Y	
Trait		**F**	**F Sign.**	**F**	**F Sign.**	**F**	**F Sign.**
AUDPC for general resistance		1.5	ns	24.8	**	1.0	ns
Fusarium damaged kernels		53.6	**	17.2	**	1.7	**
Trichothecenes and their derivatives	Deoxynivalenol	66.0	**	16.8	**	1.1	ns
	DON-3-glucoside	96.3	**	13.5	**	1.7	**
	3-acetyldeoxynivalenol	75.5	**	6.3	**	0.9	ns
	15-acetyldeoxynivalenol	72.9	**	7.2	**	1.5	*
	Nivalenol	29.7	**	13.4	**	1.2	ns
	T-2 toxin	10.9	**	1.4	ns	0.9	ns
	HT-2 toxin	9.6	**	3.0	**	0.9	ns
	HT-2 glucoside	1.9	ns	1.3	ns	0.8	ns
Zearelenoneand its derivatives	Zearalenone	14.0	**	10.9	**	1.0	ns
	Zearalenone-sulphate	51.3	**	11.1	**	2.7	**
	α-zearalenol	6.3	**	2.5	**	0.8	ns
	β-zearalenol	4.4	*	2.8	**	0.6	ns

Table 1. *Cont.*

		Year (Y)		Genotype (G)		G × Y	
	Trait	**F**	**F Sign.**	**F**	**F Sign.**	**F**	**F Sign.**
Emerging mycotoxins	Moniliformin	1.0	ns	2.9	**	0.6	ns
	Beauvericin	81.3	**	8.7	**	2.4	**
	Enniatin A	17.6	**	4.9	**	1.6	*
	Enniatin A1	18.9	**	5.4	**	1.8	**
	Enniatin B	7.8	**	4.7	**	1.1	ns
	Enniatin B1	17.4	**	6.0	**	1.6	*
	Enniatin B2	5.3	**	4.1	**	0.9	ns
	Enniatin B3	2.6	ns	2.8	**	0.9	ns
Other *Fusarium* metabolites	Culmorin	134.5	**	15.6	**	4.9	**
	5-hydroxyculmorin	11.9	**	4.1	**	0.7	ns
	15-hydroxyculmorin	12.5	**	4.0	**	0.6	ns
	15-hydroxyculmoron	20.2	**	3.5	**	0.7	ns
	Aurofusarin	154.6	**	16.2	**	5.9	**
	Apicidin	3.0	**	1.8	*	0.7	ns
	Chrysogin	10.8	**	6.3	**	0.8	ns
	Equisetin	3.3	*	1.1	ns	0.9	ns

* and **, F significant at $p < 0.05$ and $p < 0.01$, respectively; ns—F not significant.

Figure 3. Percentage of detected mycotoxins by class in 72 analysed wheat samples (36 genotypes x 2 replicates) in 2014, 2015 and 2016 growing seasons. The Microsoft Excel Spreadsheet Software was used to create figure.

3.1. Fusarium Head Blight Assessment

Mean disease severity, measured by area under the disease progress curve (AUDPC) for general resistance and Fusarium damaged kernels (FDK) of 36 wheat genotypes varied over a wide range in all three years of the study (Supplementary Table S1). Values of AUDPC for wheat genotypes ranged from 11 to 710, 4 to 860, and 7 to 810 in 2014, 2015, and 2016, respectively, with the corresponding mean values across genotypes of 255, 284, and 276. FDK values also varied widely among genotypes, ranging from 0.6 to 44.8%, 0.3 to 64.3% and 1.3 to 56.1% in 2014, 2015, and 2016, respectively, with mean FDK values across all genotypes of 11.3% in 2014 and approximately twice as high in 2015 and 2016 (21.1% and 22.2%, respectively).

3.2. Trithothecenes and Their Derivatives

Among all trichothecenes detected in analysed wheat samples the greatest concentration in all three investigated years was observed for DON (found in 100% of the wheat samples) with mean values of 3244.7, 5379.8 and 6742.8 μg kg^{-1} in 2014, 2015 and 2016, respectively (Figure 3, Supplementary Table S2). Considering the means of genotypes over three years, 34/36 of means exceeded the maximum permitted level of DON by legislation (1250.0 μg kg^{-1}), with 12,374.6 μg kg^{-1} being the maximal level found. All wheat samples were contaminated with D3G while the means of each genotype in three years varied between 70.4 and 733.9 μg kg^{-1} (Figure 3, Supplementary Table S2). Similar to concentration of DON, the mean concentration of D3G was higher in the last two years of investigation (2014–297.6 μg kg^{-1}, 2015–375.9 μg kg^{-1}, 2016–565.9 μg kg^{-1}). 3-acetyl-deoxynivalenol (3-ADON) was detected in 85, 100 and 96% of wheat samples, in 2014, 2015 and 2016, respectively, with a minimum of only 4.8 μg kg^{-1}, and a maximum of 144.2 μg kg^{-1}. Nevertheless, mean values of 3-ADON in a particular year did not exceed 90.0 μg kg^{-1} (Figure 3, Supplementary Table S2).

Percentage of positive samples with 15-acetyl-deoxynivalenol (15-ADON) were 50, 86 and 92% in 2014, 2015 and 2016, respectively (Figure 3), with the highest observed concentration of 380.7 μg kg^{-1}, but with the highest mean levels in last two years of investigation. Percentage of positive samples contaminated with NIV were 79, 94 and 94% in the three consecutive years with a minimum level of 5.0 μg kg^{-1} and a maximum level of 220.2 μg kg^{-1}, and the highest mean concentration of 66.5 μg kg^{-1} observed in 2016 (Supplementary Table S2).

T-2, HT-2 and HT-2 glucoside toxins had a lesser occurrence because only 13, 8 and 42% of samples contained T-2 toxin, 28, 15 and 51% of samples contained HT-2 toxin, and 7, 3 and 13% of samples contained HT-2 glucoside toxin, in 2014, 2015, and 2016 crop season, respectively. The lowest concentration of 0.4 μg kg^{-1} was detected for T-2 toxin, while the highest concentration of 40.2 μg kg^{-1} was found for HT-2 toxin (Figure 3, Supplementary Table S2).

3.3. Zearelenone, Zearelenone-Sulphate, α- and β-Zearalenol

Zearalenone (ZEN) was present in 100, 100 and 96% of the samples, while ZEN-sulphate was present in 97, 97 and 74% samples, respectively, during the three crop seasons, with mean values of genotypes of 49.3, 49.4 and 23.6 μg kg^{-1} in 2014, 2015 and 2016, respectively (Figure 3, Supplementary Table S3). The minimum mean levels of ZEN and ZEN-sulphate in investigated wheat genotypes were 1.7 and 2.6 μg kg^{-1}, while the maximum mean values were 194.9 and 797.9 μg kg^{-1} in three investigated years. α-zearalenol was present in 44, 47 and 8% of samples, and β-zearalenol in 36, 47 and 25% of samples in 2014, 2015 and 2016, respectively, with the lowest mean value of genotypes detected in 2016 (Figure 3, Supplementary Table S3). The highest mean values of α- and β-zearalenol for genotypes were 13.4 and 10.1 μg kg^{-1}, respectively.

3.4. Moniliformin, Beauvericin and Enniatins

The rates of contamination with MON were 63, 47 and 53% in set of 216 samples, while BEA was observed in 100% of samples during three crop seasons (Figure 3, Supplementary Table S4). The highest mean concentration of MON was 65.2 μg kg^{-1}, while BEA did not exceed 8.0 μg kg^{-1}. ENN A was present in samples in a rate of 99, 79 and 86% during three years. However, all samples were contaminated with ENN A1, ENN B and ENN B1 in a rate of 100%, except in 2016 where ENN A1 was present in 99% samples (Figure 3). The rates of contamination with ENN B2 were 97, 78 and 86%, respectively, while ENN B3 was present in 63, 38 and 26% of samples during three years, respectively (Figure 3). The highest mean value of 242.6 μg kg^{-1} in genotypes was observed for ENN B1, and further 128.3 μg kg^{-1} for ENN B and 120.2 μg kg^{-1} for ENN A1. The rank order of mean values of these mycotoxins during investigated years was as follow: 67.7 > 26.3 > 22.5 μg kg^{-1} for ENN B1, 35.4 > 19.9 > 14.5 μg kg^{-1} for ENN B, 33.3 > 10.0 > 10.3 μg kg^{-1} for ENN A1, in 2014, 2015 and 2016, respectively (Supplementary Table S4).

3.5. Other Fusarium Metabolites

All wheat samples (n = 216) were contaminated with culmorin, 15-hydroxyculmorin, aurofusarin and chrysogin in a rate of 100%, except in 2016 where chrysogin contamination was 99% (Figure 3). Mean concentration in three investigated years of culmorin ranged between 295.5 and 3157.2 μg kg^{-1}; and further 369.4 and 9011.7 μg kg^{-1}, 106.5 and 17,138.0 μg kg^{-1}, 11.3 and 234.9 μg kg^{-1} for 15-hydroxyculmorin, aurofusarin and chrysogin, respectively. In 2014 culmorin had the mean value of 1257.1 μg kg^{-1}, while in 2015 and 2016 mean values increased to 1570.3 and 2451.8 μg kg^{-1} (Supplementary Table S5). 15-hydroxyculmorin and chrysogin showed the highest mean concentration in 2015 (4347.1 μg kg^{-1} and 125.8 μg kg^{-1}), while aurofusarin had the highest concentration of 8214.4 μg kg^{-1} in 2016. 5-hydroxyculmorin was present in 2016 with a rate of 94%, while 15-hydroxyculmoron was present in 2014 and 2016 with the rates of 96 and 82% in 216 samples. The maximum mean values of 5-hydroxyculmorin and 15-hydroxyculmoron were 5550.0 and 1204.1 μg kg^{-1}. Apicidin was observed in 57, 51 and 47% of samples, while equisetin was found in much lower number of samples (3, 36 and 8%) in the three investigated years (Figure 3). The highest mean values of apicidin and equisetin in genotypes were 30.6 and 1.7 μg kg^{-1} (Supplementary Table S5).

3.6. Co-Occurrence and Correlation of Fusarium Mycotoxins/Metabolites

Very high co-occurrence of mycotoxins has been observed in all investigated wheat samples (Supplementary Tables S2–S5). Further, correlation analysis based on 36 genotypic means over two replicates and three years were performed to show relationships among *Fusarium* severity traits (AUDPC for general resistance and FDK) and *Fusarium* mycotoxins/metabolites (Figure 4, Supplementary Table S6). A strong positive correlation (0.93) was found between AUDPC for general resistance and FDK. Significant positive correlations were also observed between the two measures of FHB severity and the levels of all mycotoxins except equisetin, and were in most cases higher for FDK than for AUDPC for general resistance. Both AUDPC for general resistance and FDK showed the strongest correlations (r > 0.90) with DON and its derivatives, zearalenone, and four other *Fusarium* metabolites. Correlations between 28 metabolites/mycotoxins ranged from moderate to very strong in most cases, except in the case of equisetin, the only *Fusarium* metabolite without any significant correlation with other metabolites/mycotoxins. The most distinguished positive correlations were observed between DON and its derivatives, as well as between DON and its derivatives with aurofusarin and culmorin and its derivatives, NIV, T-2 and HT-2 toxins with BEA and ENNs, while chrysogin showed the strongest positive correlations with DON, ZEN and culmorin and their derivatives.

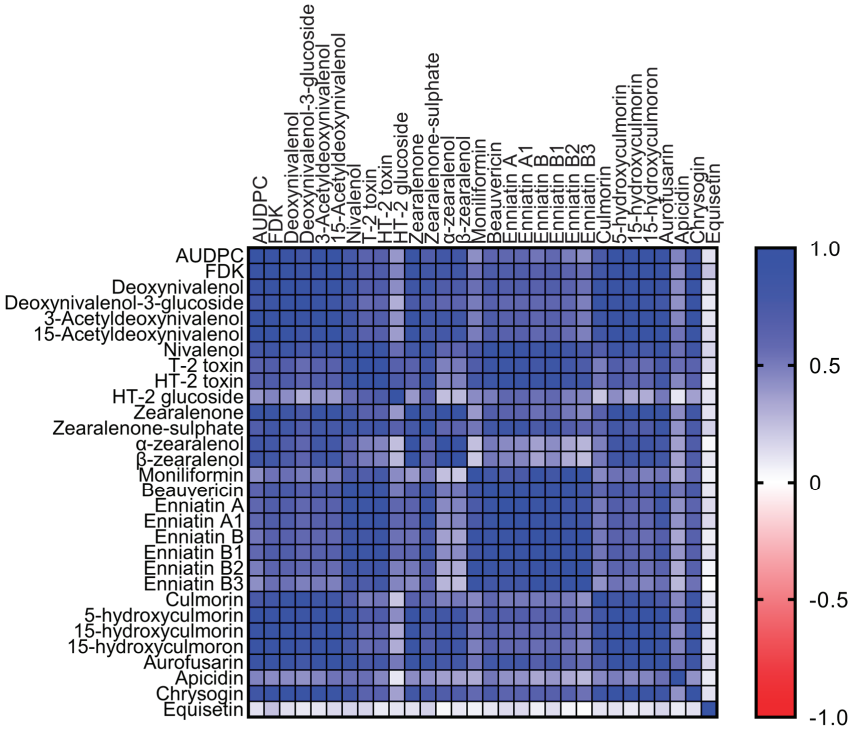

Figure 4. Heatmap presenting Pearson correlation matrix of area under the disease progress curve (AUDPC), *Fusarium* damaged kernels (FDK) and 28 *Fusarium* mycotoxins/metabolites in 36 wheat genotypes. Pearson correlation r values were determined using GraphPad Prism 9.4.1, San Diego, CA, USA. Colors are added for better visualization. The colors span from dark blue to dark red, where dark blue denotes a r value of 1, and dark red indicates a r value of −1.

4. Discussion

Fusarium spp. are fungi that can produce mycotoxins, that pose a potential danger for humans and animals as they can be found in a great variety of food and feed products [44]. The severity of FHB during a given crop season depends on precipitation during wheat flowering, whereas increased levels of *Fusarium* mycotoxins are often observed in seasons with frequent rainfall and high humidity. In the current research the influence of the genotype and year was significant for all *Fusarium* metabolites/mycotoxins, except the influence of genotype for T-2 toxin, HT-2 glucoside, ENN B3 and apicidin, and except the influence of year for HT-2 glucoside, MON and equisetin. Only nine metabolites had significant G × Y interaction. A significant year effect on the concentration of most mycotoxins/metabolites in the current study could be due to observed differences in the precipitation and temperatures, as prevalent factors that have an important effect on *Fusarium* infection, as it has been already observed in previous studies [1,45]. Stanciu et al. [13] suggested that precipitation levels influence fungi and mycotoxin development to a greater extent, compared to temperatures. Previous research showed that FHB susceptible wheat varieties are characterized by a much greater accumulation of DON than the resistant varieties [1]. Further, similarly to results of the current study, previously it was reported that DON significantly correlated with other investigated mycotoxins, and therefore it can be concluded that DON content can be used in the selection of FHB resistant genotypes resulting in lower total toxicity [6,37]. Furthermore, in the current research the level of FHB severity and FDK correlated well with mycotoxins present in the grain, but there was no significant correlation observed for equisetin. It was evident that more FHB infected grains

(with increased % of FDK) were shrivelled and discoloured and thus associated with higher mycotoxin concentrations.

4.1. Trichothecenes and Their Modified Forms

The group with trichothecenes is comprised from large family of structurally related mycotoxins produced by various *Fusarium* species [46]. DON, NIV, 3-ADON, 15-ADON, and fusarenon-X are included in type-B trichothecenes, while type-A group is comprised of T-2 and HT-2 toxin, diacetoxyscirpenol and neosolaniol [47] most of which were detected in wheat samples in the current study. Mycotoxins belonging to type-B trichothecenes group are resistant to milling, processing and heating which results in entrance of these mycotoxins in the food [48]. In the current research, acetylated fungal derivatives 3-ADON and 15-ADON as well as the derived D3G were detected in high occurrence in the most of investigated wheat samples. Similar results were obtained in previous research of Spanic et al. [4]. In the current research, the mixture of four aggressive *F. graminearum* species was used for inoculation, whereas previously it was reported that *F. graminearum* was found to produce three forms of DON (namely DON, 3-ADON and 15-ADON) [49]. Also, this is supported by the fact that DON and its derivatives were significantly positively correlated.

One of the most important type-B trichothecene is DON, being the most prevalent contaminant of cereals and end-use products [50]. In the current research, DON and its derivative forms were one of the most abundant in trichothecenes' group. Similarly, in the study of Nathanail et al. [51] DON and its glucosylated form were found in 93 and 81% of the samples. Concentrations of DON were unusually high in 2015 and 2016, due to increased precipitation in May, thus exceeding the maximum legislative limits for unprocessed wheat grains placed on the market for first-stage processing. Mean concentrations of D3G were not as high as for DON, but still this masked mycotoxin is representing huge concern. On the opposite to the current research, it was reported that D3G concentrations even exceeded those of DON [52]. During some processing, such as malting, DON was successfully converted into D3G [53]. Further problem with D3G is his hydrolysis during mammalian digestion that will return D3G in toxic precursor DON [52]. Thus, the increase in toxicity may occur directly or indirectly by transformation to the parental form of mycotoxin during digestion in the gastrointestinal system [18]. Ovando-Martínez et al. [24] reported that at higher DON concentration, a decrease in the D3G content occurs. On the opposite, in correlation matrix of the current research, D3G and DON were significantly positively correlated. This discrepancy could arise from different sets of genotypes as well as different environmental conditions in the two studies, and in the case of D3G production could be partly explained by the significant genotype × environment interaction observed in the current study. Metabolites such as 3-ADON and D3G were characterised by significantly lower toxicity then DON [54]. On the other hand, 15-ADON is the only derivative of DON whose toxicity is comparable with DON [55]. The levels of 3-ADON and 15-ADON were increased in wheat samples during last two years of investigation (2015 and 2016). In these two years the amount of precipitation during flowering period in May was higher compared to 2014, probably resulting in increased level of 3-ADON and 15-ADON. So, it can be concluded that the DON and its derivatives, dependent on the growing conditions in a particular season, were also significantly affected by the wheat genotype, but only 15-ADON and D3G showed significant G x Y interaction. 15-ADON and D3G should be taken into account in terms of food safety because D3G as "masked" mycotoxin can be converted into DON while 15-ADON is more toxic than DON. Although, another trichothecene B, NIV, is not usually produced in high concentrations, oxidative stress and toxicity induced by NIV contamination are higher than that described for DON [56]. NIV is also very dangerous mycotoxin because it can have damaging effects on mammals through immunotoxicity and hemotoxicity [57]. Further, these authors found similar concentration of NIV in cereals and their products (107.2 $\mu g\ kg^{-1}$), as can be seen in the current research. It was expected to find NIV in samples in increased occurrence in all

samples, because it is an accompanying mycotoxin of DON, as isolates of *Fusarium* spp. that produce DON, also produce NIV [49].

In the current research the occurrence of type-A trichothecenes T-2 and HT-2 toxin and its derivative was lower, compared to other trichothecenes and their modified forms. Similarly, it was concluded for T-2 and HT-2 toxin for maize kernels where they were significantly less common, compared to other toxins produced by *Fusarium* spp. [58]. T-2 toxin can be metabolized into HT-2 toxin and thus the toxicity of T-2 might partly be attributed to HT-2 [59]. Somewhat increased occurrence of T-2 and HT-2 toxin was found in the last year of investigation that could be influenced by more or less equal distribution of rainfall in May and June, before and after flowering. This is in accordance with the results of Hjelkrem et al. [60] who reported that HT-2 + T-2 contamination in oats was influenced by weather conditions both pre- and post-flowering.

4.2. Zearalenone and Its Derivatives

The main characteristic of ZEN is its estrogenic activity causing reproductive disorders in both humans and animals [61]. ZEN was identified in 100% of wheat samples during the first two years of our investigation; whereas its presence was significantly affected by genotype and year. Similar concentrations of ZEN as in the current research were observed by Tan et al. [62] who found that ZEN in maize kernel samples ranged from <LOD to 163.58 μg kg^{-1}. In the current research, the maximum concentration of that toxin in some genotypes exceeded permitted levels while ZEN-sulphate maximum level was 4-fold higher then ZEN's. This could be very hazardous, as for example, ZEN-14-sulphate was produced by *F. graminearum* but yet with unknown toxic effects [63]. Anyway, the sum of ZEN and its modified forms should be taken into account in the health risk management. This is supported by the observation of Gonzalez Pereyra et al. [64] were the presence of highly oestrogenic metabolites, like α-zearalenol and the masked ZEN-4-sulphate, increased the overall toxicity of ZEN contaminated silage. However, glucosylated masked forms of ZEN were not detected in the current research. Therefore, in investigated genotypes they do not represent dangerous, but in other cases they can be very unsafe as they are unstable in the digestive tract of mammals and could formed the main form ZEN [65]. In contrast to DON, ZEN had the highest mean value across genotypes in 2014, and was significantly affected by year but not by G × Y interaction. In opposite to our results, Vogelgsang et al. [66] demonstrated that year had a highly significant effect on both the DON contamination rate and the average content, however no effect of year was observed for ZEN or NIV. Potential risk is also hiding in the fact that ZEN gets synthetized during the malting [53] but also its thermostability is potential danger [67]. Previously, Abid-Essefi et al. [68] reported that toxic effects seem to be relieved by the metabolism of ZEN into α-zearalenol and β-zearalenol. In the current study, the rates and levels of contamination with reduced forms of ZEN, α- and β-zearalenol toxins were low, thus not contributing to toxicity of ZEN.

4.3. Emerging Mycotoxins

In recent years, special attention has been dedicated to so-called "emerging mycotoxins". The huge problem is that there is currently no legislation that would regulate the content of compounds from the group of emerging mycotoxins. Beside MON, BEA, ENNs and FUP, more *Fusarium* metabolites with toxicity falls in the category of emerging mycotoxins. In the current research, MON, BEA and ENNs compounds were found in all investigated wheat samples in all three years. MON was represented with more than 60% in 2014 and somewhat to lesser extent in last two years of investigation, while BEA, ENN B and ENN B1 were present in all investigated years in 100% samples. MON is a mycotoxin that can disrupt the Krebs cycle and cause adenosine triphosphate (ATP) deficiency, causing muscle weakness, heart and respiratory failure in animals [58]. Hietaniemi et al. [69] reported a mean level of 190 μg kg^{-1} and a maximum level of 850 μg kg^{-1} for MON in cereals from Finland. In our study, maximum levels of MON were much lower, where in three years, average value among genotypes did not exceed 66 μg kg^{-1}. Observed differences

among studies could be the consequence of different *Fusarium* spp. present in inoculum, whereas *F. proliferatum* was reported as main producer of MON [70]. BEA is mycotoxin structurally similar to the ENNs, but differs in the nature of the N-methylamino acid, and induces programmed cell death [71]. This toxin is also involved in antimicrobial and antibiotic activities [72]. The same authors described ENNs with cytotoxic activities and genotoxicity, while on the other side exhibiting antifungal and antimicrobial activities. Both, ENNs and BEA, have cytotoxic effects as a result of the induction of oxidative stress [73,74]. In the current research, ENN B1, ENN B and ENN A1 were found in relatively higher concentrations as compared to other "emerging" mycotoxins. Similar results were reported by Reisinger et al. [75] who found that ENN B and ENN B1 were the most abundant with median concentrations of 7 and 6 μg kg^{-1}, respectively, and maximum concentrations of 429 and 555 μg kg^{-1}, respectively. Maximum reported concentrations for BEA in grains and in cereal-based food were 6400.0 and 844.0 μg kg^{-1}, respectively [76]. This was much higher than it was found in the current study, where maximum mean value of BEA in three years was 8.0 μg kg^{-1}. This concentration is much closer to median concentration of 9 μg kg^{-1} found in maize silages [75]. As can be seen from previous studies, all these emerging mycotoxins pose a certain danger for human and animals, and that is why the investigations of their content and occurrence in wheat must not be neglected. Emerging mycotoxins of *Fusarium* spp. with their increased concentrations in possible epidemic conditions should be of concern to official food control authorities and should be incorporated in future legislation.

4.4. Other Fusarium Metabolites

In last few years' metabolite culmorin was also assigned in the group of "emerging mycotoxins" that usually comes with trichothecene mycotoxins thus influencing their toxicity [77]. In the current study mean highest concentrations for culmorin exceeded 2.5 fold the maximal permitted level for DON (250.0 μg kg^{-1}). For 5-hydroxyculmorin and hydroxyculmorin the permitted level was exceeded 4.4 and 7.3 fold, respectively, while for 15-hydroxyculmoron maximal concentration was at the permitted level for DON. Uhlig et al. [78] observed that in natural conditions the concentration of culmorin was about 3-fold higher than concentration of DON. It was previously reported that mixtures of culmorin with DON, 3-ADON, 15-ADON, or NX-3, but not with NIV, inhibited growth of wheat roots in a synergistic manner [79]. It is important to note that culmorin and DON are likely characterised by synergistic toxicity [80]. Culmorin and its derivatives were highly represented in wheat samples in each investigated year in the current study. Results of Streit et al. [81] showed a high occurrence of other *Fusarium* metabolites in natural conditions, where 63, 63, 13, and 7% of feed and feed raw materials (n = 83) was positive for culmorin, 15-hydroxyculmorin, 5-hydroxyculmorin, and 15-hydroxyculmorone. In the study of Spanic et al. [4] aurofusarin was detected in the range of 735.0 to 63,098.0 μg kg^{-1} when significantly fewer samples were investigated than in the current research. In the present study maximum value of aurofusarin was 3.7 fold lower, compared to research of Spanic et al. [4], probably due to different wheat genotypes or *Fusarium* isolates used in these two studies. Recently, it has been reported on the possible induction of oxidative stress by aurofusarin [82]. Sunic et al. [6] reported about connection between production of major *Fusarium* mycotoxins and pigments. However, the available literature data concerning aurofusarin is limited, and further research is needed for better understanding of occurrence and levels of aurofusarin in wheat samples. Chrysogin is *Fusarium* metabolite previously reported in the concentration up to 1320 μg kg^{-1} [6] in contrast to the current study where occurrence was high, but concentrations were 5.6 fold lower. Compared to the concentration of aurofusarin, apicidin had lower mean concentrations in wheat samples in the present study and no significant differences in apicidin concentration among 36 wheat genotypes were found. Although apicidin was detected in low concentration in the current study, we need to be careful with this metabolite as previously Khoshal et al. [83] ranked apicidin as the most toxic as can be seen from their ranking of metabolites acording to order of

toxicity: apicidin > enniatin A1 > DON > beauvericin > enniatin B > enniatin B1 > emodin > aurofusarin. Equisetin was the only metabolite investigated in the present study showing no correlations with other mycotoxins/metabolites and with very low concentrations. Similarly, low occurrence of equisetin was previously found by Spanic et al. [25].

5. Conclusions

The co-occurrence of several mycotoxins/metabolites under potentially FHB epidemic conditions in individual samples confirms the importance of using credible analytical methods for monitoring of *Fusarium* mycotoxins/metabolites in wheat. This is especially important in food risk assessment as most of them are showing synergic or additive effects, and as we observed they are significantly positively correlated. The amount of mycotoxins in wheat grains can be decreased by utilization of FHB resistant genotypes. Also, our results underline the potential of *F. graminearum* to produce multi-mycotoxins simultaneously under the influence of various factors related to the genotype or the environment. In conclusion, both the crop season and genotype significantly affected the levels of mycotoxins/metabolites in wheat grain in response to *Fusarium* infection. Significant differences in the contamination pattern were observed among years for all mycotoxins, except for HT-2 glucoside, MON and equisetin. The differing levels of mycotoxins in three investigated years may be a result of different precipitation patterns among years. In 2016 an equal distribution of precipitations across May and June increased the occurrence of trichothecenes, and decreased the occurrence of ZEN and its derivatives. Special attention needs to be given to masked and emerging mycotoxins, as in the current study they incidence was high in all three investigated years. Mycotoxin content in wheat should be monitored continuously, as the annual levels may vary depending on rainfall and temperature changes, wheat variety type etc.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030805/s1, Figure S1: Mean values of AUDPC for general resistance and Fusarium damaged kernels (FDK); Table S1: Area under the disease progress curve (AUDPC) for general resistance and Fusarium damaged kernels (FDK) of 25 wheat genotypes in 2014, 2015 and 2016; Table S2: Mean values of deoxynivalenol and its derivatives, nivalenol, T-2 and HT-2 toxin and its derivative in three years; Table S3: Mean values of zearelenone, zearelenone-sulphate, α- and β-zearalenol in three years; Table S4: Mean values of moniliformin, beauvericin and enniatins in three years; Table S5: Mean values of other Fusarium metabolites in three years; Table S6: Pearson's correlation coefficients between AUDPC for general resistance, Fusarium damaged kernels (FDK) and concentrations of mycotoxins/metabolites in wheat samples.

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References

1. Spanic, V.; Marcek, T.; Abicic, I.; Sarkanj, B. Effects of Fusarium head blight on wheat grain and malt infected by *Fusarium culmorum*. *Toxins* **2018**, *10*, 17. [CrossRef]
2. Zhang, W.; Boyle, K.; Brûlé-Babel, A.L.; Fedak, G.; Gao, P.; Robleh Djama, Z.; Polley, B.; Cuthbert, R.D.; Randhawa, H.S.; Jiang, F.; et al. Genetic characterization of multiple components contributing to Fusarium Head Blight Resistance of FL62R1, a canadian bread wheat developed using systemic breeding. *Front. Plant Sci.* **2020**, *26*, 11–580833. [CrossRef] [PubMed]

3. Mesterhazy, A.; Toth, B.; Varga, M.; Bartok, T.; Szabo-Hever, A.; Farady, L.; Lehoczki-Krsjak, S. Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of Fusarium head blight and deoxynivalenol. *Toxins* **2011**, *3*, 1453–1483. [CrossRef] [PubMed]
4. Spanic, V.; Viljevac Vuletic, M.; Horvat, D.; Sarkanj, B.; Drezner, G.; Zdunic, Z. Changes in antioxidant system during grain development of wheat (*Triticum aestivum* L.) and relationship with protein composition under FHB stress. *Pathogens* **2020**, *9*, 17. [CrossRef]
5. Wilde, F.; Miedaner, T. Selection for Fusarium head blight resistance in early generations reduces the deoxynivalenol (DON) content in grain of winter and spring wheat. *Plant Breed* **2006**, *125*, 96–98. [CrossRef]
6. Sunic, K.; Kovac, T.; Loncaric, A.; Babic, J.; Sulyok, M.; Krska, R.; Drezner, G.; Spanic, V. *Fusarium* secondary metabolite content in naturally produced and artificially provoked FHB pressure in winter wheat. *Agronomy* **2021**, *11*, 2239. [CrossRef]
7. Bushnell, W.R.; Hazen, B.E.; Pritsch, C. Histology and physiology of Fusarium head blight. In *Fusarium Head Blight of Wheat and Barley*, 1st ed.; Leonard, K.J., Bushnell, W.R., Eds.; The American Phytopathological Society: St. Paul, MN, USA, 2003; Chapter 3; pp. 44–83.
8. Rodrigues, I.; Naehrer, K. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. *Toxins* **2012**, *4*, 663–675. [CrossRef]
9. Cheli, F.; Pinotti, L.; Rossi, L.; Dell'Orto, V. Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. *LWT Food Sci. Technol.* **2013**, *54*, 307–314. [CrossRef]
10. Shank, R.A.; Foroud, N.A.; Hazendonk, P.; Eudes, F.; Blackwell, B.A. Current and future experimental strategies for structural analysis of trichothecene mycotoxins—A prospectus. *Toxins* **2011**, *3*, 1518–1553. [CrossRef]
11. Bennett, J.W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, *16*, 497–516. [CrossRef]
12. Lemos, A.C.; de Borba, V.S.; Badiale-Furlong, E. The impact of wheat-based food processing on the level of trichothecenes and their modified forms. *Trends Food Sci. Technol.* **2021**, *111*, 89–99. [CrossRef]
13. Stanciu, O.; Juan, C.; Berrada, H.; Miere, D.; Loghin, F.; Mañes, J. Study on trichothecene and zearalenone presence in Romanian wheat relative to weather conditions. *Toxins* **2019**, *11*, 163. [CrossRef]
14. Commission Regulation (EC) No 1881/2006 of 19 December 2006 on the Setting Maximum Levels for Certain Contaminants in Foodstuffs. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32006R1881 (accessed on 28 January 2023).
15. Commission Recommendation (ER) No 2013/165/EU of 27 March 2013 on the Presence of T-2 and HT-2 Toxin in Cereals and Cereal Products. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32013H0165 (accessed on 28 January 2023).
16. EFSA. Panel on Contaminants in the Food Chain. Scientific Opinion on risks for animal and public health related to the presence of nivalenol in food and feed. *EFSA J* **2013**, *11*, 3262. [CrossRef]
17. Berthiller, F.; Crews, C.; Dall'Asta, C.; De Saeger, S.; Haesaert, G.; Karlovsky, P.; Oswald, I.P.; Seefelder, W.; Speijers, G.; Strokaet, J. Masked mycotoxins: A review. *Mol. Nutr. Food Res.* **2013**, *57*, 165–186. [CrossRef] [PubMed]
18. Ekwomadu, T.I.; Akinola, S.A.; Mwanza, M. *Fusarium* mycotoxins, their metabolites (free, emerging, and masked), food safety concerns, and health impacts. *Int. J. Environ. Res. Public Health* **2021**, *18*, 11741. [CrossRef]
19. Broekaert, N.; Devreese, M.; de Baere, S.; de Backer, P.; Croubels, S. Modified *Fusarium* mycotoxins unmasked: From occurrence in cereals to animal and human excretion. *Food Chem. Toxicol.* **2015**, *80*, 17–31. [CrossRef]
20. Iqbal, S.Z. Mycotoxins in food, recent development in food analysis and future challenges: A review. *Curr. Opin. Food Sci.* **2021**, *42*, 237–247. [CrossRef]
21. Berthiller, F.; Dall'Asta, C.; Schuhmacher, R.; Lemmens, M.; Adam, G.; Krska, R. Masked mycotoxins: Determination of a deoxynivalenolglucoside in artificially and naturally contaminated wheat by liquid chromatography—Tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 3421–3425. [CrossRef]
22. Borzekowski, A.; Drewitz, T.; Keller, J.; Pfeifer, D.; Kunte, H.J.; Koch, M.; Rohn, S.; Maul, R. Biosynthesis and characterization of zearalenone-14-sulfate, zearalenone-14-glucoside and zearalenone-16-glucoside Using Common Fungal Strains. *Toxins* **2018**, *10*, 104. [CrossRef]
23. Lee, S.Y.; Woo, S.Y.; Tian, F.; Song, J.; Michlmayr, H.; Kim, J.B.; Chun, H.S. Occurrence of deoxynivalenol, nivalenol, and their glucosides in Korean market foods and estimation of their population exposure through food consumption. *Toxins* **2020**, *12*, 89. [CrossRef]
24. Ovando-Martínez, M.; Ozsisli, B.; Anderson, J.; Whitney, K.; Ohm, J.B.; Simsek, S. Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in hard red spring wheat inoculated with *Fusarium Graminearum*. *Toxins* **2013**, *5*, 2522–2532. [CrossRef] [PubMed]
25. Spanic, V.; Katanic, Z.; Sulyok, M.; Krska, R.; Puskas, K.; Vida, G.; Drezner, G.; Sarkanj, B. Multiple fungal metabolites including mycotoxin formation in naturally infected and Fusarium inoculated wheat samples. *Microorganisms* **2020**, *8*, 578. [CrossRef]
26. Nakagawa, H.; Ohmichi, K.; Sakamoto, S.; Sago, Y.; Kushiro, M.; Nagashima, H.; Yoshida, M.; Nakajima, T. Detection of a new Fusarium masked mycotoxin in wheat grain by high-resolution LC–Orbitrap™ MS. *Food Addit. Contam. A* **2011**, *28*, 1447–1456. [CrossRef] [PubMed]
27. Prosperini, A.; Berrada, H.; Ruiz, M.J.; Caloni, F.; Coccini, T.; Spicer, L.J.; Perego, M.C.; Lafranconi, A. A Review of the mycotoxin enniatin B. *Front. Public Health* **2017**, *5*, 304. [CrossRef] [PubMed]

28. Fernandez-Blanco, C.; Font, G.; Ruiz, M.J. Interaction effects of enniatin B, deoxinivalenol and alternariol in Caco-2 cells. *Toxicol. Lett.* **2016**, *241*, 38–48. [CrossRef]
29. Nazari, F.; Sulyok, M.; Kobarfard, F.; Yazdanpanah, H.; Krska, R. Evaluation of emerging *Fusarium* mycotoxins beauvericin, enniatins, fusaproliferin and moniliformin in domestic rice in Iran. *Iran J. Pharm. Res.* **2015**, *14*, e125357. [CrossRef]
30. Wu, Q.; Patocka, J.; Nepovimova, E.; Kuca, K. A Review on the synthesis and bioactivity aspects of beauvericin, a Fusarium Mycotoxin. *Front. Pharmacol.* **2018**, *9*, 1338. [CrossRef]
31. Kuang, Q.-X.; Lei, L.-R.; Li, Q.-Z.; Peng, W.; Wang, Y.-M.; Dai, Y.-F.; Wang, D.; Gu, Y.-C.; Deng, Y.; Guo, D.-L. Investigation of the anti-inflammatory activity of fusaproliferin analogues guided by transcriptome analysis. *Front. Pharmacol.* **2022**, *13*, 881182. [CrossRef]
32. Ceranic, A.; Svoboda, T.; Berthiller, F.; Sulyok, M.; Samson, J.M.; Güldener, U.; Schuhmacher, R.; Adam, G. Identification and functional characterization of the gene cluster responsible for fusaproliferin biosynthesis in *Fusarium proliferatum*. *Toxins* **2021**, *13*, 468. [CrossRef]
33. Bertuzzi, T.; Giorni, P.; Rastelli, S.; Vaccino, P.; Lanzanova, C.; Locatelli, S. Co-Occurrence of moniliformin and regulated *Fusarium* toxins in maize and wheat grown in Italy. *Molecules* **2020**, *25*, 2440. [CrossRef]
34. Bouafifssa, Y.; Manyes, L.; Rahouti, M.; Mañes, J.; Berrada, H.; Zinedine, A.; Fernández-Franzón, M. Multi-occurrence of twenty mycotoxins in pasta and a risk assessment in the Moroccan population. *Toxins* **2018**, *10*, 432. [CrossRef] [PubMed]
35. Mahdjoubi, C.K.; Arroyo-Manzanares, N.; Hamini-Kadar, N.; García-Campaña, A.M.; Mebrouk, K.; Gámiz-Gracia, L. Multi-mycotoxin occurrence and exposure assessment approach in foodstuffs from Algeria. *Toxins* **2020**, *12*, 194. [CrossRef] [PubMed]
36. Siuda, R.; Grabowski, A.; Lenc, L.; Ralcewicz, M.; Spychaj-Fabisiak, E. Influence of the degree of fusariosis on technological traits of wheat grain. *Int. J. Food Sci. Technol.* **2010**, *45*, 2596–2604. [CrossRef]
37. Spanic, V.; Dvojkovic, K.; Babic, J.; Drezner, G.; Zdunic, Z. Fusarium head blight infestation in relation to winter wheat end-use quality—A three-year study. *Agronomy* **2021**, *11*, 1648. [CrossRef]
38. Malachova, A.; Dzuman, Z.; Veprikova, Z.; Vaclavikova, M.; Zachariasova, M.; Hajslova, J. Deoxynivalenol, deoxynivalenol-3-glucoside, and enniatins: The major mycotoxins found in cereal-based products on the Czech market. *J. Agric. Food Chem.* **2011**, *59*, 12990–12997. [CrossRef] [PubMed]
39. Ryu, D.; Hanna, M.A.; Bullerman, L.B. Stability of zearalenone during extrusion of corn grits. *J. Food Prot.* **1999**, *62*, 1482–1484. [CrossRef] [PubMed]
40. Desjardins, A.E.; Manandhar, G.; Plattner, R.D.; Maragos, C.M.; Shrestha, K.; McCormick, S.P. Occurrence of *Fusarium* species and mycotoxins in Nepalese maize and wheat and the effect of traditional processing methods on mycotoxin levels. *J. Agric. Food Chem.* **2000**, *48*, 1377–1383. [CrossRef]
41. Spanic, V.; Lemmens, M.; Drezner, G. Variability of components of fusarium head blight resistance among wheat genotypes. *Cereal. Res. Commun.* **2013**, *41*, 420–430. [CrossRef]
42. SAS Institute Inc. *SAS/STAT 9.2 Users Guide*; SAS Inc.: Cary, NC, USA, 2009.
43. Motulsky, H.J. *Analyzing Data with GraphPad Prism*; GraphPad Software Inc.: San Diego, CA, USA, 1999.
44. Awuchi, C.G.; Ondari, E.N.; Ogbonna, C.U.; Upadhyay, A.K.; Baran, K.; Okpala, C.O.R.; Korzeniowska, M.; Guiné, R.P.F. Mycotoxins affecting animals, foods, humans, and plants: Types, occurrence, toxicities, action mechanisms, prevention, and detoxification strategies—A Revisit. *Foods* **2021**, *10*, 1279. [CrossRef]
45. Wegulo, S. Factors influencing deoxinivalenol accumulation in small grain cereals. *Toxins* **2012**, *4*, 1157–1180. [CrossRef]
46. Alizadeh, A.; Braber, S.; Akbari, P.; Kraneveld, A.; Garssen, J.; Fink-Gremmels, J. Deoxynivalenol and its modified forms: Are there major differences? *Toxins* **2016**, *8*, 334. [CrossRef] [PubMed]
47. Toxicology and Occurrence of Nivalenol, Fusarenon X, Diacetoxyscirpenol, Neosolaniol and 3- and 15-Acetyldeoxynivalenol: A Review of Six Trichothecenes. RIVM Report 388802024/2002. Available online: https://www.rivm.nl/bibliotheek/rapporten/388802024.pdf (accessed on 15 January 2023).
48. Sugita-Konishi, Y.; Park, B.J.; Kobayashi-Hattori, K.; Tanaka, T.; Chonan, T.; Yoshikawa, K.; Kumagai, S. Effect of cooking process on the deoxynivalenol content and its subsequent cytotoxicity in wheat products. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1764–1768. [CrossRef]
49. Castañares, E.; Albuquerque, D.R.; Dinolfo, M.I.; Pinto, V.F.; Patriarca, A.; Stenglein, S.A. Trichothecene genotypes and production profiles of *Fusarium graminearum* isolates obtained from barley cultivated in Argentina. *Int. J. Food Microbiol.* **2014**, *179*, 57–63. [CrossRef]
50. Turner, P.C. Deoxynivalenol and nivalenol occurrence and exposure assessment. *World Mycotoxin J.* **2010**, *3*, 315–321. [CrossRef]
51. Nathanail, A.V.; Syvähuoko, J.; Malachová, A.; Jestoi, M.; Varga, E.; Michlmayr, H.; Adam, G.; Sieviläinen, E.; Berthiller, F.; Peltonen, K. Simultaneous determination of major type A and B trichothecenes, zearalenone and certain modified metabolites in Finnish cereal grains with a novel liquid chromatography-tandem mass spectrometric method. *Anal. Bioanal. Chem.* **2015**, *407*, 4745–4755. [CrossRef]
52. Berthiller, F.; Krska, R.; Domig, K.J.; Kneifel, W.; Juge, N.; Schuhmacher, R.; Adam, G. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicol. Lett.* **2011**, *206*, 264–267. [CrossRef] [PubMed]
53. Spanic, V.; Zdunic, Z.; Drezner, G.; Sarkanj, B. The pressure of *Fusarium* disease and its relation with mycotoxins in the wheat grain and malt. *Toxins* **2019**, *11*, 198. [CrossRef]

54. He, Y.; Yin, X.; Dong, J.; Yang, Q.; Wu, Y.; Gong, Z. Transcriptome analysis of Caco-2 cells upon the exposure of mycotoxin deoxynivalenol and its acetylated derivatives. *Toxins* **2021**, *13*, 167. [CrossRef] [PubMed]
55. Springler, A.; Hessenberger, S.; Reisinger, N.; Kern, C.; Nagl, V.; Schatzmayr, G.; Mayer, E. Deoxynivalenol and its metabolite deepoxy-deoxynivalenol: Multi-parameter analysis for the evaluation of cytotoxicity and cellular effects. *Mycotoxin. Res.* **2017**, *33*, 25–37. [CrossRef] [PubMed]
56. Nagashima, H. Deoxynivalenol and nivalenol toxicities in cultured cells: A review of comparative studies. *Food Saf.* **2018**, *6*, 51–57. [CrossRef]
57. Zingales, V.; Fernández-Franzón, M.; Ruiz, M.-J. Occurrence, mitigation and in vitro cytotoxicity of nivalenol, a type B trichothecene mycotoxin–Updates from the last decade (2010–2020). *Food Chem. Toxicol.* **2021**, *152*, 112182. [CrossRef] [PubMed]
58. Bryła, M.; Pierzgalski, A.; Zapaśnik, A.; Uwineza, P.A.; Ksieniewicz-Woźniak, E.; Modrzewska, M.; Waśkiewicz, A. Recent research on *Fusarium* mycotoxins in maize-A Review. *Foods* **2022**, *11*, 3465. [CrossRef]
59. EFSA. Panel on Contaminants in the Food Chain. Appropriateness to set a group health based guidance value for T2 and HT2 toxin and its modified forms. *EFSA J.* **2017**, *15*, e04655. [CrossRef]
60. Hjelkrem, A.G.R.; Aamot, H.U.; Brodal, G.; Strand, E.C.; Torp, T.; Edwards, S.G.; Dill-Macky, R.; Hofgaard, I.S. HT-2 and T-2 toxins in Norwegian oat grains related to weather conditions at different growth stages. *Eur. J. Plant Pathol.* **2018**, *151*, 501–514. [CrossRef]
61. Poor, M.; Kunsagi-Mate, S.; Sali, N.; Koszegi, T.; Szente, L.; Peles-Lemli, B. Interactions of zearalenone with native and chemically modified cyclodextrins and their potential utilization. *J. Photochem. Photobiol. B* **2015**, *151*, 63–68. [CrossRef]
62. Tan, H.; Zhou, H.; Guo, T.; Zhang, Y.; Ma, L. Zein-bound zearalenone: A hidden mycotoxin found in maize and maize-products. *Food Control* **2021**, *124*, 107903. [CrossRef]
63. Keller, J.; Borzekowski, A.; Haase, H.; Menzel, R.; Rueß, L.; Koch, M. Toxicity assay for citrinin, zearalenone and zearalenone-14-sulfate using the nematode Caenorhabditis elegans as Model Organism. *Toxins* **2018**, *10*, 284. [CrossRef] [PubMed]
64. Gonzalez Pereyra, M.L.; Sulyok, M.; Baralla, V.; Dalcero, A.M.; Krska, R.; Chulze, S.N.; Cavaglieri, L.R. Evaluation of zearalenone, α-zearalenol, β-zearalenol, zearalenone 4-sulfate and β-zearalenol 4-glucoside levels during the ensiling process. *World Mycotoxin J.* **2014**, *7*, 291–295. [CrossRef]
65. Shkhaliyeva, I.; Teker, T.; Albayrak, G. Masked mycotoxins of deoxynivalenol and zearalenone—Unpredicted toxicity. *Biomed. J. Sci. Tech. Res.* **2020**, *29*, 22288–22293. [CrossRef]
66. Vogelgsang, S.; Musa, T.; Bänziger, I.; Kägi, A.; Bucheli, T.D.; Wettstein, F.E.; Pasquali, M.; Forrer, H.R. *Fusarium* mycotoxins in Swiss wheat: A survey of growers' samples between 2007 and 2014 shows strong year and minor geographic effects. *Toxins* **2017**, *9*, 246. [CrossRef]
67. Ben Salah-Abbès, J.; Belgacem, H.; Ezzdini, K.; Abdel-Wahhab, M.A.; Abbès, S. Zearalenone nephrotoxicity: DNA fragmentation, apoptotic gene expression and oxidative stress protected by *Lactobacillus plantarum* MON03. *Toxicon* **2020**, *175*, 28–35. [CrossRef]
68. Abid-Essefi, S.C.; Bouaziz, E.; El Golli-Bennour, Z.; Ouanes, H.B. Comparative study of toxic effects of zearalenone and its two major metabolites α-zearalenol and β-zearalenol on cultured human Caco-2 cells. *J. Biochem. Mol. Toxicol.* **2009**, *23*, 233–243. [CrossRef]
69. Hietaniemi, V.; Ramo, S.; Yli-Mattila, T.; Jestoi, M.; Peltonen, S.; Kartio, M.; Sievilainen, E.; Koivisto, T.; Parikka, P. Updated survey of *Fusarium* species and toxins in Finnish cereal grains. *Food Addit. Contam. A* **2016**, *33*, 831–848. [CrossRef]
70. Waśkiewicz, A.; Łukasz, S. Mycotoxins biosynthesized by plant-derived *Fusarium* isolates. *Arh. Hig. Rada Toksikol.* **2013**, *63*, 437–446. [CrossRef]
71. Logrieco, A.; Moretti, A.; Castella, G.; Kostecki, M.; Golinski, P.; Ritieni, A.; Chelkowski, J. Beauvericin production by *Fusarium* species. *Appl. Environ. Microbiol.* **1998**, *64*, 3084–3088. [CrossRef] [PubMed]
72. Křížová, L.; Dadáková, K.; Dvořáčková, M.; Kašparovský, T. Feedborne mycotoxins beauvericin and enniatins and livestock animals. *Toxins* **2021**, *13*, 32. [CrossRef] [PubMed]
73. Agahi, F.; Álvarez-Ortega, N.; Font, G.; Juan-García, A.; Juan, C. Oxidative stress, glutathione, and gene expression as key indicators in SH-SY5Y cells exposed to zearalenone metabolites and beauvericin. *Toxicol. Lett.* **2020**, *334*, 44–52. [CrossRef] [PubMed]
74. Cimbalo, A.; Alonso-Garrido, M.; Font, G.; Frangiamone, M.; Manyes, L. Transcriptional changes after enniatins A, A1, B and B1 ingestion in rat stomach, liver, kidney and lower intestine. *Foods* **2021**, *10*, 1630. [CrossRef]
75. Reisinger, N.; Schürer-Waldheim, S.; Mayer, E.; Debevere, S.; Antonissen, G.; Sulyok, M.; Nagl, V. Mycotoxin occurrence in maize silage—A neglected risk for bovine gut health? *Toxins* **2019**, *11*, 577. [CrossRef]
76. EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA J.* **2014**, *12*, 3802. [CrossRef]
77. Weber, J.; Vaclavikova, M.; Wiesenberger, G.; Haider, M.; Hametner, C.; Fröhlich, J.; Berthiller, F.; Adam, G.; Mikula, H.; Fruhmann, P. Chemical synthesis of culmorin metabolites and their biologic role in culmorin and acetyl-culmorin treated wheat cells. *Org. Biomol. Chem.* **2018**, *16*, 2043–2048. [CrossRef] [PubMed]
78. Uhlig, S.; Eriksen, G.S.; Hofgaard, I.S.; Krska, R.; Beltrán, E.; Sulyok, M. Faces of a changing climate: Semi-quantitative multi-mycotoxin analysis of grain grown in exceptional climatic conditions in Norway. *Toxins* **2013**, *5*, 1682–1697. [CrossRef]
79. Wipfler, R.; McCormick, S.P.; Proctor, R.; Teresi, J.; Hao, G.; Ward, T.; Alexander, N.; Vaughan, M.M. Synergistic phytotoxic effects of culmorin and trichothecene mycotoxins. *Toxins* **2019**, *11*, 555. [CrossRef] [PubMed]

80. Woelflingseder, L.; Warth, B.; Vierheilig, I.; Schwartz-Zimmermann, H.; Hametner, C.; Nagl, V.; Novak, B.; Sarkanj, B.; Berthiller, F.; Adam, G. The Fusarium metabolite culmorin suppresses the in vitro glucuronidation of deoxynivalenol. *Arch. Toxicol.* **2019**, *93*, 1729–1743. [CrossRef] [PubMed]
81. Streit, E.; Schwab, C.; Sulyok, M.; Naehrer, K.; Krska, R.; Schatzmayr, G. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins* **2013**, *5*, 504–523. [CrossRef] [PubMed]
82. Jarolim, K.; Wolters, K.; Woelflingseder, L.; Pahlke, G.; Beisl, J.; Puntscher, H.; Braun, D.; Sulyok, M.; Warth, B.; Marko, D. The secondary *Fusarium* metabolite aurofusarin induces oxidative stress, cytotoxicity and genotoxicity in human colon cells. *Toxicol. Lett.* **2018**, *284*, 170–183. [CrossRef]
83. Khoshal, A.K.; Novak, B.; Martin, P.G.P.; Jenkins, T.; Neves, M.; Schatzmayr, G.; Oswald, I.P.; Pinton, P. Co-occurrence of DON and emerging mycotoxins in worldwide finished pig feed and their combined toxicity in intestinal cells. *Toxins* **2019**, *11*, 727. [CrossRef]

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Fusarium Secondary Metabolite Content in Naturally Produced and Artificially Provoked FHB Pressure in Winter Wheat

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Abstract: Fusarium head blight (FHB) is an important disease of wheat and production of mycotoxins makes it a major threat in most wheat-producing areas worldwide. This study aimed to identify the impact of epidemic FHB conditions (usage of artificial *Fusarium* inoculation) on mycotoxin levels in unprocessed wheat. *Fusarium* levels were monitored at two locations in two treatments (natural infection and inoculation with *Fusarium graminearum and F. culmorum*) where 13 mycotoxins were evaluated by LC/MS-MS in six winter wheat varieties. Due to favorable conditions for infection with *Fusarium* fungi during the flowering period at location Tovarnik, wheat varieties had higher disease severity and increased mycotoxin accumulation, compared to Osijek. The most abundant mycotoxins in treatment with inoculation with *Fusarium* fungi were deoxynivalenol (DON), culmorin (CUL) and hydroxyculmorins. In treatment with natural infection, DON did not exceed maximum limits set by EU. Varieties with lower initial resistance accumulated DON even in naturally infected samples at Tovarnik. These results highlighted the impact of environment variation in the production of *Fusarium* mycotoxins where FHB initial resistance had a higher impact on the accumulation of mycotoxins than general resistance. Furthermore, wheat samples with higher DON concentration also contained elevated levels of CUL and hydroxyculmorins, showing that CUL can have a possible role in *Fusarium* virulence. The FHB evaluations provide important information about the genetic resistance of wheat varieties, as well as risk assessment considering mycotoxin accumulation in epidemic conditions.

Keywords: *Fusarium culmorum*; *Fusarium graminearum*; mycotoxins; wheat; LC/MS-MS

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1. Introduction

Wheat is one of the major staple and one of the "big three" cereal crops with an annual worldwide production of over 600 million tons [1]. During the period of anthesis, the plant is the most susceptible to diseases that affect wheat heads and one of the main concerns is Fusarium head blight (FHB) caused by fungi of the genus *Fusarium*. The disease can result in direct and indirect economic losses thus causing reduced grain yield and quality, as well as production of mycotoxins [2].

A wide range of factors play different roles in the growth, survival and dissemination of the fungus and thus can influence disease severity and mycotoxin production [3]. Primarily, the aggressiveness of *Fusarium* species and accumulation of mycotoxins is determined by wheat genetic variation [4]. The presence and incidence of different *Fusarium*

species in the crop affect the mycotoxin profile and contamination of the grain [5]. *Fusarium graminearum* and *F. culmorum* are the most dominant and prevalent species causing FHB worldwide, as well as in Croatia [6], where they can be highly aggressive and can produce abundant mycotoxins. Several other species like *F. poae* and *F. avenaceum*, which are also frequently found in winter wheat in East Croatia, are less pathogenic [6,7]. However, less aggressive species are still of great concern since their development with other more aggressive pathogens can also lead to the accumulation of mycotoxins, even if the symptoms of the disease are not expressed to such an extent [2]. Another factor influencing mycotoxin content are optimal climatic conditions for disease development, namely temperature and water availability, which depend on the local and regional environment. Most studies reported that an environment with frequent rainfall and warm temperatures favours the disease development and thus the production of mycotoxins [2,8,9]. Previously it was reported that the influence of climatic conditions on the accumulation of mycotoxins most probably is an indirect factor, influencing primarily fungal growth [3]. However, disease development depends on the substrate, *Fusarium* species present and individual metabolite since every species of FHB complex have different environmental demands. Moderate temperatures and lower water availability favour the production of type A trichothecenes by *F. sporotrichioides*, while warmer humid conditions induce the production of type B trichothecenes by *F. culmorum* and *F. graminearum* [3]. Other studies also reported that the wheat grain contamination with mycotoxins was highly correlated with relative humidity and sum of precipitation in short period around anthesis, namely five day pre-anthesis period, while correlation coefficients for post-anthesis periods showed lower significance [10]. Strong correlation between precipitation during the period of anthesis and infection by the *Fusarium* fungi occurred in some researches [11]. Generally, mycotoxin production by *F. graminearum* and *F. culmorum* seems to be stimulated by narrower weather conditions than that for growth [12].

There are different types of resistance to FHB in wheat: type I (resistance to initial infection), type II (resistance to disease spread), type III (re-sistance to kernel infection), type IV (tolerance) and type V (resistance to mycotoxins) [13]. Mycotoxins produced by *Fusarium* species pose a threat to both, animal and human health [14]. One of the most abundant mycotoxins and therefore most frequently contaminating grains and food products is deoxynivalenol (DON) [8]. Together with its acetylated forms, 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), deoxynivalenol-3-glucoside (D3G), as well as nivalenol (NIV), belong to trichothecenes group B. DON is also known as vomitoxin considering its emetic effect [15]. Watching from the aspect of the cell DON is a potent inhibitor of protein synthesis and some of the mechanisms occurring in response to high DON concentrations involve inflammatory processes, diarrhea, lack of appetite as well as necrosis of certain tissues [14,16]. Nivalenol (NIV) is the mycotoxin mainly produced by *F. graminearum*, *F. culmorum* and *F. poae* [17]. Although there is still scarce evidence of its effect on humans [18], some studies have found that NIV interferes with the synthesis of nucleic acids, induces programmed cell death in vitro and causes immuno- and hematotoxicity in animals. Zearalenone (ZEN) is non-steroidal estrogenic mycotoxin that may cause hyperestrogenism and infertility and even low concentrations can influence the hormonal balance in mammals [14,19]. Main *Fusarium* species known to produce ZEN are *F. graminearum*, *F. culmorum*, *F. crookwellense*, *F. semitectum* and *F. equiseti* [20]. Only a few mycotoxins are regulated and monitored by European Union (EU), while many of them are indicated as "emerging" [21]. In addition to these better-known compounds, other *Fusarium* metabolites require more investigation. Culmorin (CUL), a metabolite that gains a lot of scientific attention, is a tricyclic sesquiterpene diol. According to studies, it does not affect insects or animals [22]. However, recent findings indicated that interactions of CUL and DON increase phytotoxicity levels, namely by CUL inhibiting glycosylation of DON into less toxic DON 3-O-glucose [15,22,23]. Another mycotoxin that acquires more scientific investigation since it is highly mutagenic in in vitro bioassays is fusarin C. It was first isolated from *Fusarium moniliforme* [24]. However, the exact role of fusarin C on

human and animal health has not yet been described [19]. Butenolide (BUT) is a mycotoxin produced by *Fusarium graminearum* and other *Fusarium* species [19]. It is associated with cattle mycotoxicosis called "fescue foot" which can result in edema and gangrenous loss of extremities [19]. Some studies have reported its involvement in Kashin-Beck disease and Keshan disease in China [25]. Despite a fact that aurofusarin is a pigment with antibiotic effect, it was found that aurofusarin can also induce oxidative stress and cytotoxicity in human colon cells [26]. Chrysogin so far has no report on its effect on human and animal health in the scientific literature [5].

Only a small portion of the mycotoxins studied are regulated and for the majority to date, no regulations exist. Therefore, it is necessary to obtain more data on these mycotoxins. Considering the findings of harmful mycotoxin effects on human and animal health, the aim of this study was to estimate the effect of epidemic FHB conditions on mycotoxin levels in artificially inoculated winter wheat, as well as in naturally infected samples.

2. Materials and Methods

2.1. Plant Material and Field Trials

The study was conducted in vegetative season 2019/2020 at two locations, Osijek (45°32′ N, 18°44′ E) and Tovarnik (45°10′ N, 19°09′ E), Croatia. The soil types in these two regions are different with eutric cambisol present at Osijek and black soil chernozem at Tovarnik. During the period of flowering, the average precipitation was 1.7 mm at Osijek (Supplementary Table S1, Figure S1) and 2.3 mm at Tovarnik (Supplementary Table S2, Figure S2), and the average temperature was 15.3 °C at Osijek (Supplementary Table S3, Figure S1) and 15.6 °C at Tovarnik (Supplementary Table S4, Figure S2). The experimental plot area was 7.56 m^2, where treatments (naturally infected and artificially inoculated) were replicated in two plots. In each treatment, same winter wheat varieties (El Nino, Galloper, Tika Taka, Vulkan, Kraljica and Golubica) originated from Agricultural institute Osijek, were used. The seed was treated with Vitavax 200 FF (thiram + carboxin) at a rate of 200 g Vitavax for 100 kg of seeds in order to control seed-borne diseases. Fungicides were excluded in both treatments and in the both investigated environments. Weed control was conducted with a herbicide at wheat tillering (GS 31). Insecticides were sprayed in the spring of the growing season. Fertilization was in proportions N:P:K 130:100:120 kg ha^{-1}. The grains were taken by harvesting the whole plot with a Wintersteiger cereal plot combine-harvester.

2.2. Inoculum Preparation and Inoculation Procedure

The *Fusarium* species used in this experiment were the two most prevalent causal agents of Fusarium head blight: *Fusarium graminearum* (PIO 31), isolated from the winter wheat collected in East Croatia, and *F. culmorum* (IFA 104) obtained from IFA-Tulln, Austria. Conidial inoculum of *Fusarium* spp. were produced by a mixture of wheat and oat grains (3:1 by volume). Conidial concentrations of both fungi were determined using a hemocytometer (Bürker-Türk, Hecht Assistent) and were set to 10×10^4 mL^{-1}. The 100 mL of inoculum was sprayed with sprayers on an area of m^2 at the flowering stage. One treatment was grown according to standard agronomical practice with no usage of fungicide and without misting treatment, while another treatment was subjected to two inoculation events using a tractor-back (Osijek) and hand sprayer (Tovarnik) with *Fusarium* spp. at the time of flowering (Zadok's scale 65) [27] (Figure 1a). Misting was provided by spraying with a tractor back-sprayer on several occasions.

(**a**) (**b**)

Figure 1. Inoculation spraying at Tovarnik (**a**) and the first visible symptoms of the wheat heads seen as bleaching of the spikeletes (**b**).

2.3. Fusarium General Resistance and Type I Resistance

The percentage of bleached spikelets (Figure 1b) per plot and initial infection were estimated on days 10, 14, 18, 22, and 26 after inoculation according to a linear scale (0–100%). Based on the percentages, the area under the disease progress curve (AUDPC) for general resistance and type I FHB resistance of wheat varieties was calculated according to formula:

$$AUDPC = \sum_{i=1}^{n} \left\{ \left[\frac{Yi + Yi - 1}{2} \right] * (Xi - Xi - 1) \right\}$$

where *Yi* is percentage of visibly infected spikelets (*Yi*/100) at the *i*th observation, *Xi* is day of the *i*th observation and *n* is total number of observations.

2.4. Mycotoxin Analysis

Determination of mycotoxin was performed by LC-MS/MS [28]: 5 g of wheat (previously ground by IKA M20, IKA, Staufen, Germany) were extracted using 20 mL extraction solvent (acetonitrile-water-acetic acid, 79:20:1, *v*/*v*/*v*) followed by a 1 + 1 dilution using acetonitrile-water-acetic acid, (20:79:1, *v*/*v*/*v*) and direct injection of 5 µL diluted extract. LC-MS/MS screening of target fungal metabolites was performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIon Spray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 4 × 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, USA).

Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid that exhibit only one fragment ion), which yielded 4.0 identification points according to commission decision [29]. In addition, the liquid chromatography retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.03 min and 30% rel., respectively. Quantification was performed via external calibration using serial dilutions of a multi-analyte stock solution. Results were corrected for apparent recoveries obtained for wheat [28] (Supplementary Tables S5–S9). The accuracy of the method is verified on a continuous basis by regular participation in proficiency testing scheme organized by BIPEA (Gennevilliers, France).

2.5. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) using an appropriate model by Statistica version 12.0 (Statsoft Inc., Tulsa, OK, USA). To estimate disease progress, the AUDPC was used to combine multiple observations from five data points (different dates) into a single value. For correlation analyses, Spearman's coefficient was applied, shown in the supplementary file.

3. Results

3.1. Fusarium General Resistance and Type I Resistance

The FHB symptoms varied among locations, where at Tovarnik there was higher area under the disease progress curve (AUDPC) for general resistance on average for three varieties (El Nino, Galloper and Tika Taka), compared to Osijek. The highest Type I and general resistance at Osijek had Galloper, as well as general resistance at Tovarnik. The lower AUDPC for initial resistance (higher Type I resistance) had Vulkan and Kraljica at Tovarnik. The highest AUDPC for Type I resistance was recorded for El Nino (AUDPC 421) at Tovarnik, followed by Golubica at Osijek (AUDPC 222) (Table 1).

Table 1. Area under the disease progress curve (AUDPC) for general resistance and Type I resistance (initial infection) to Fusarium head blight (FHB) at locations Osijek and Tovarnik and their standard deviations (SD).

Variety	AUDPC for General Resistance Osijek ± SD	AUDPC for General Resistance Tovarnik ± SD	AUDPC for Type I Resistance Osijek ± SD	AUDPC for Type I Resistance Tovarnik ± SD
El Nino	137.3 ± 10.75	212.5 ± 142.5	244 ± 40	421 ± 212.5
Galloper	1.3 ± 0	17.5 ± 9.5	33.1 ± 16.63	87.4 ± 25.85
Tika Taka	42.7 ± 11.35	69.8 ± 7.25	215.3 ± 74.7	137.6 ± 41.95
Vulkan	35.8 ± 6.25	33.8 ± 5.25	119.9 ± 11.35	50.6 ± 4.1
Kraljica	71.5 ± 23.5	18.3 ± 1.75	216.5 ± 80.5	80.1 ± 10.4
Golubica	103.8 ± 22.75	93 ± 7	222.3 ± 29.2	111.3 ± 8.3

3.2. Mycotoxin Analysis

3.2.1. Deoxynivalenol, Deoxynivalenol-3-glucoside and 3-Acetyldeoxynivalenol

The concentrations of deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G) and 3ADON were elevated in *Fusarium* infected samples, compared to naturally infected samples of all winter wheat varieties tested. DON was one of the most abundant mycotoxins produced in artificially inoculated treatment at Osijek and Tovarnik. D3G and 3ADON were also found in all *Fusarium* infected samples at both locations. The levels of DON measured in six artificially inoculated samples at Osijek were 5410 μg kg^{-1} in Galloper, 6370 μg kg^{-1} in Kraljica, 6740 μg kg^{-1} in Vulkan, 17,700 μg kg^{-1} in Tika Taka, 18,300 μg kg^{-1} in El Nino and 22,800 μg kg^{-1} in Golubica (Figure 2a) and at Tovarnik 13,200 μg kg^{-1} in Vulkan, 13,400 μg kg^{-1} in Galloper, 19,800 μg kg^{-1} in Kraljica, 21,000 μg kg^{-1} in Tika Taka, 21,100 μg kg^{-1} in El Nino and 25,500 μg kg^{-1} in Golubica (Figure 2b). The highest concentration of DON in naturally infected samples at Tovarnik was recorded in El Nino variety (620 μg kg^{-1}) and the lowest in Kraljica (19 μg kg^{-1}).

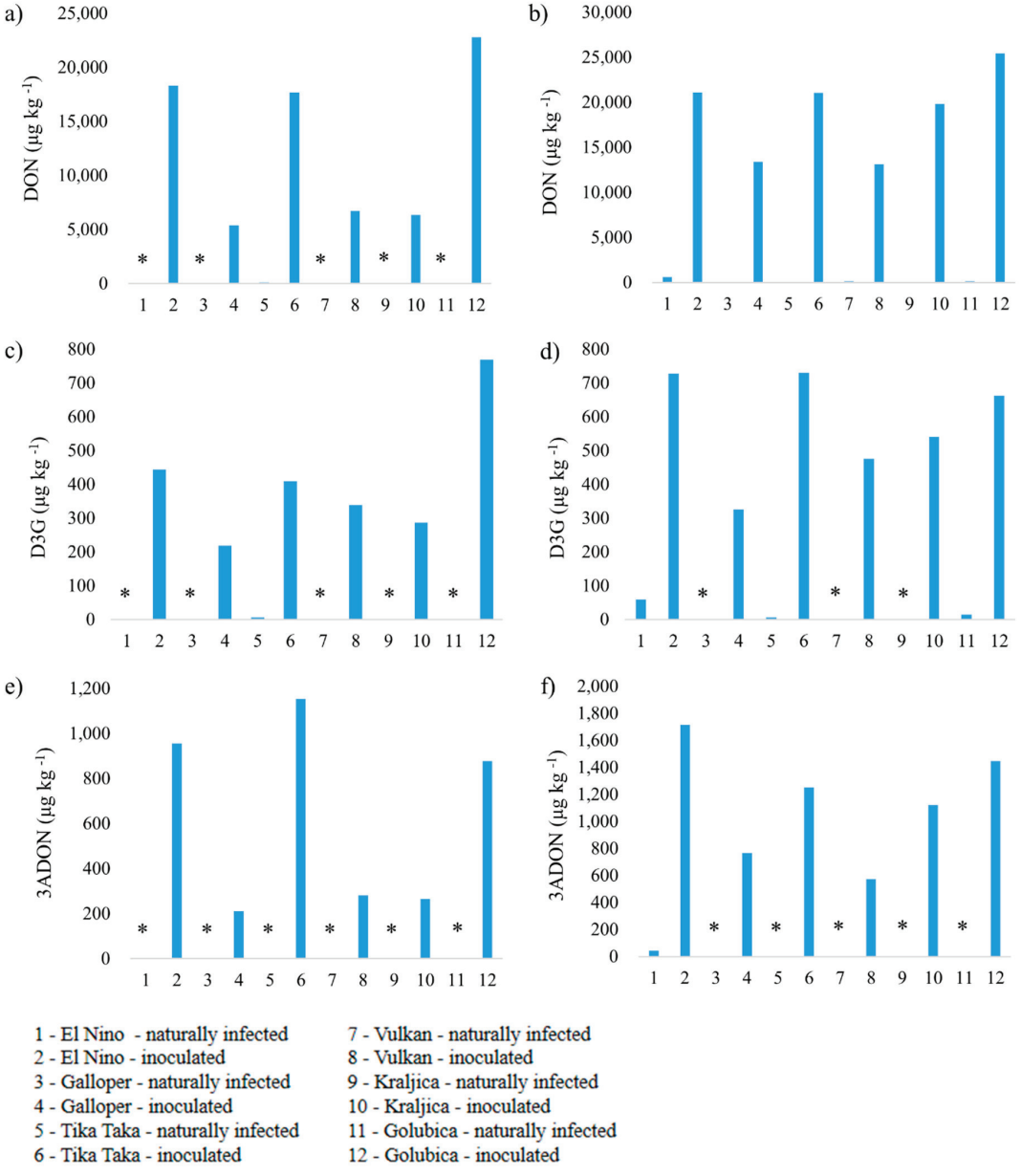

Figure 2. Concentrations of deoxynivalenol (DON) (**a**,**b**), deoxynivalenol-3-glucoside (D3G) (**c**,**d**) and 3-acetylde- oxynivalenol (3ADON) (**e**,**f**) in artificially inoculated and naturally infected samples at Osijek (**a**,**c**,**e**) and Tovarnik (**b**,**d**,**f**). The asterisk (*) indicates that measured values are below LOD values.

Concentrations of D3G and 3ADON were lower than the concentrations of DON. D3G ranged from 219 µg kg^{-1} to 770 µg kg^{-1} at Osijek (Figure 2c) and from 326 µg kg^{-1} to 731 µg kg^{-1} at Tovarnik (Figure 2d), while 3ADON at Osijek ranged from 212 µg kg^{-1} to 1150 µg kg^{-1} (Figure 2e) and at Tovarnik from 572 µg kg^{-1} to 1720 µg kg^{-1} (Figure 2f).

3.2.2. Nivalenol and Zearalenone

Concentrations of nivalenol (NIV) and zearalenone (ZEN) were lower than the rest of the *Fusarium* metabolites studied at both locations. At Osijek, NIV was found only in artificially inoculated samples of most FHB susceptible varieties (El Nino, Tika Taka and Golubica) where the levels were 29 μg kg^{-1}, 31 μg kg^{-1} and 37 μg kg^{-1}, respectively (Figure 3a). At Tovarnik, NIV was found in all artificially inoculated samples and the concentrations ranged from 24 μg kg^{-1} in Galloper to 105 μg kg^{-1} in Golubica with an overall mean of 44 μg kg^{-1} (Figure 3b). In naturally infected samples it was detected only in Golubica (70 μg kg^{-1}).

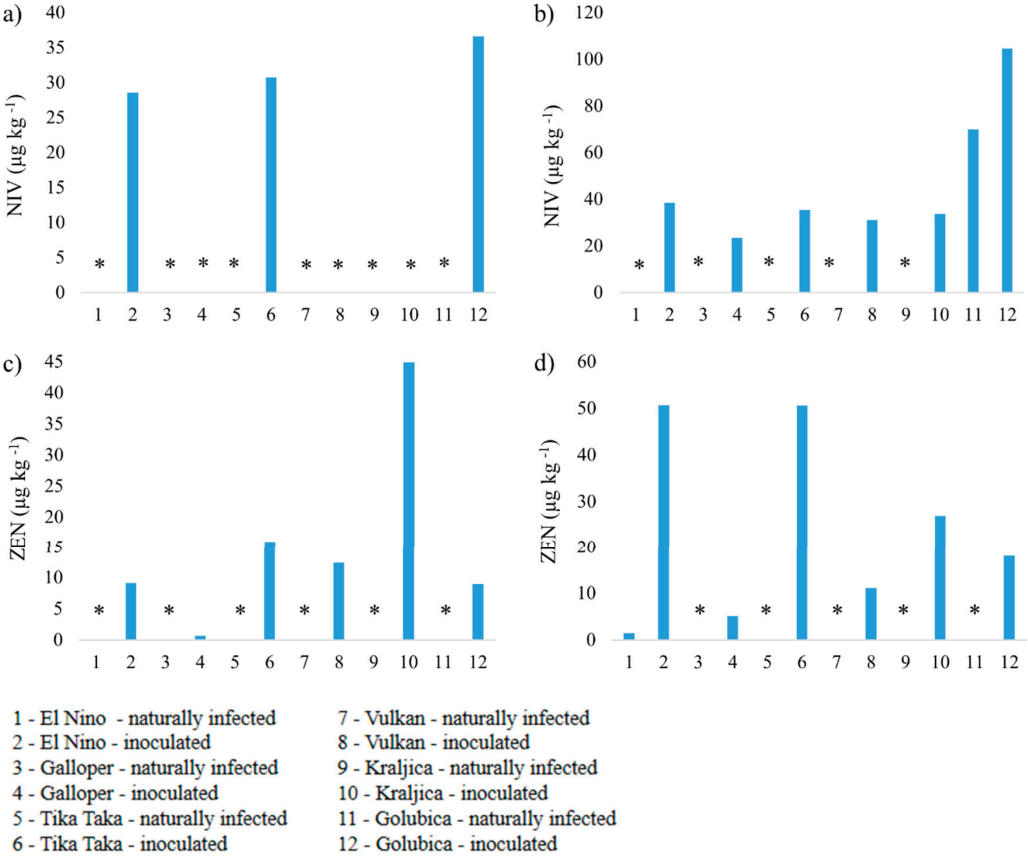

Figure 3. Concentrations of nivalenol (NIV) (**a**,**b**) and zearalenone (ZEN) (**c**,**d**) in artificially inoculated and naturally infected samples at Osijek (**a**,**c**) and Tovarnik (**b**,**d**). The asterisk (*) indicates that measured values are below LOD values.

All artificially inoculated samples at both locations were contaminated with ZEN. None of the naturally infected samples contained ZEN except the El Nino variety at Tovarnik and its concentration was 1 μg kg^{-1}. The levels of ZEN measured at artificially inoculated six varieties at Osijek were 1 μg kg^{-1} in Galloper, 9 μg kg^{-1} in Golubica, 9 μg kg^{-1} in El Nino, 12 μg kg^{-1} in Vulkan, 16 μg kg^{-1} in Tika Taka and 45 μg kg^{-1} in Kraljica with an average level of 15 μg kg^{-1} (Figure 3c) and at Tovarnik 5 μg kg^{-1} in Galloper, 11 μg kg^{-1} in Vulkan, 18 μg kg^{-1} in Golubica, 27 μg kg^{-1} in Kraljica, 51 μg kg^{-1} in El Nino and 51 μg kg^{-1} in Tika Taka (Figure 3d) with an average level of 27 μg kg^{-1}.

3.2.3. Culmorin, 15-Hydroxyculmorin, 15-Hydroxyculmoron and 5-Hydroxyculmorin

El Nino, Tika Taka and Golubica accumulated culmorin (CUL) and its derivatives in much higher concentrations than other varieties. At Osijek, concentrations of CUL were elevated in the artificially inoculated compared to naturally infected samples. The highest amount of CUL was recorded in an artificially inoculated Golubica variety (29,100 μg kg^{-1}). Other concentrations ranged from 7810 μg kg^{-1} in Kraljica to 13,100 μg kg^{-1} in Vulkan. In naturally infected samples CUL was found only in Tika Taka variety at concentration of 220 μg kg^{-1} (Figure 4a). At Tovarnik, concentrations of CUL were elevated even in naturally infected samples. The highest concentration was found in El Nino variety (1000 μg kg^{-1}) and the lowest was recorded in Tika Taka variety (62 μg kg^{-1}), while in Kraljica it was not found. In artificially inoculated samples concentrations ranged from 6100 μg kg^{-1} in Galloper to 14,300 μg kg^{-1} in Golubica with an overall mean of 11,400 μg kg^{-1} (Figure 4b).

Concentrations of CUL derivatives were also elevated at both locations in *Fusarium* infected samples compared to naturally-infected. In artificially inoculated samples at Osijek 15-hydroxyculmorin ranged from 8130 μg kg^{-1} to 28,800 μg kg^{-1} (Figure 4c) and at Tovarnik from 15,600 μg kg^{-1} to 27,000 μg kg^{-1} (Figure 4d). 15-hydroxyculmoron was in range from 570 μg kg^{-1} to 4610 μg kg^{-1} (Figure 4e) and from 1200 μg kg^{-1} to 2990 μg kg^{-1} (Figure 4f) for Osijek and Tovarnik, respectively. Regarding 5-hydroxyculmorin at Osijek, it ranged from 5100 μg kg^{-1} to 24,400 μg kg^{-1} (Figure 4g) and at Tovarnik from 14,000 μg kg^{-1} to 30,000 μg kg^{-1} (Figure 4h). No CUL derivatives were found in naturally infected samples at Osijek except for Tika Taka variety which accumulated 15-hydroxyculmorin at concentration of 89 μg kg^{-1}. At Tovarnik, CUL derivatives were detected not only in artificially inoculated but also in naturally infected samples, as for DON and its derivatives. In naturally infected samples the highest concentrations recorded were 81 μg kg^{-1}, 637 μg kg^{-1} and 1230 μg kg^{-1} in El Nino variety for 15-hydroxyculmoron, 5-hydroxyculmorin and 15-hydroxyculmorin, respectively.

3.2.4. Aurofusarin, Butenolide, Chrysogin and Fusarin C

For other *Fusarium* metabolites, namely aurofusarin, butenolide, chrysogin and fusarin C increases were also recorded in *Fusarium* infected samples compared to naturally infected samples at Osijek and Tovarnik. At Osijek, aurofusarin was recorded in the range from 1870 μg kg^{-1} to 12,511 μg kg^{-1} (Figure 5a). At Tovarnik, aurofusarin levels in artificially inoculated samples were increased, compared to Osijek and the highest concentration was 67,600 μg kg^{-1} in El Nino variety. In addition, aurofusarin was detected only in El Nino even in naturally infected samples (739 μg kg^{-1}) (Figure 5b). Butenolide levels at Osijek ranged from 167 μg kg^{-1} to 1120 μg kg^{-1} (Figure 5c) and at Tovarnik from 188 μg kg^{-1} to 654 μg kg^{-1} (Figure 5d). At Osijek, chrysogin was recorded in range from 346 μg kg^{-1} to 1320 μg kg^{-1} (Figure 5e) and at Tovarnik from 528 μg kg^{-1} to 1170 μg kg^{-1} (Figure 5f). The level of fusarin C at Osijek ranged from 665 μg kg^{-1} to 2660 μg kg^{-1} (Figure 5g) while at Tovarnik it was elevated compared to Osijek and it ranged from 2610 μg kg^{-1} to 6720 μg kg^{-1} (Figure 5h). Butenolide, chrysogin and fusarin C found in artificially inoculated samples were in lower concentrations compared to aurofusarin. Regarding naturally infected samples, only chrysogin was detected in negligible concentrations.

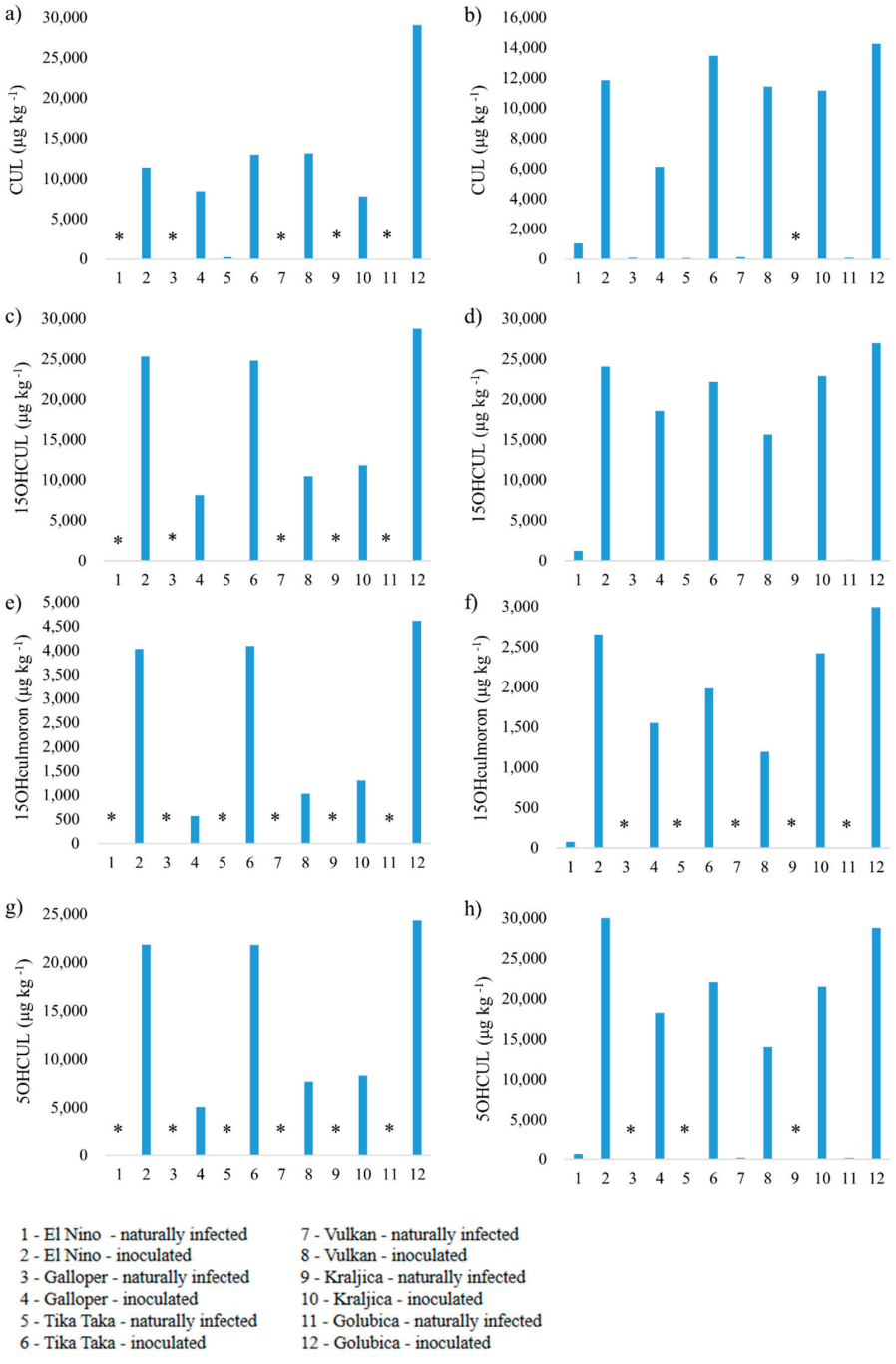

Figure 4. Concentrations of culmorin (CUL) (**a**,**b**), 15–hydroxyculmorin (15OHCUL) (**c**,**d**), 15–hydroxyculmoron (15OHculmoron) (**e**,**f**) and 5-hydroxyculmorin (5OHCUL) (**g**,**h**) in artificially inoculated and naturally infected samples at Osijek (**a**,**c**,**e**,**g**) and Tovarnik (**b**,**d**,**f**,**h**). The asterisk (*) indicates that measured values are below LOD values.

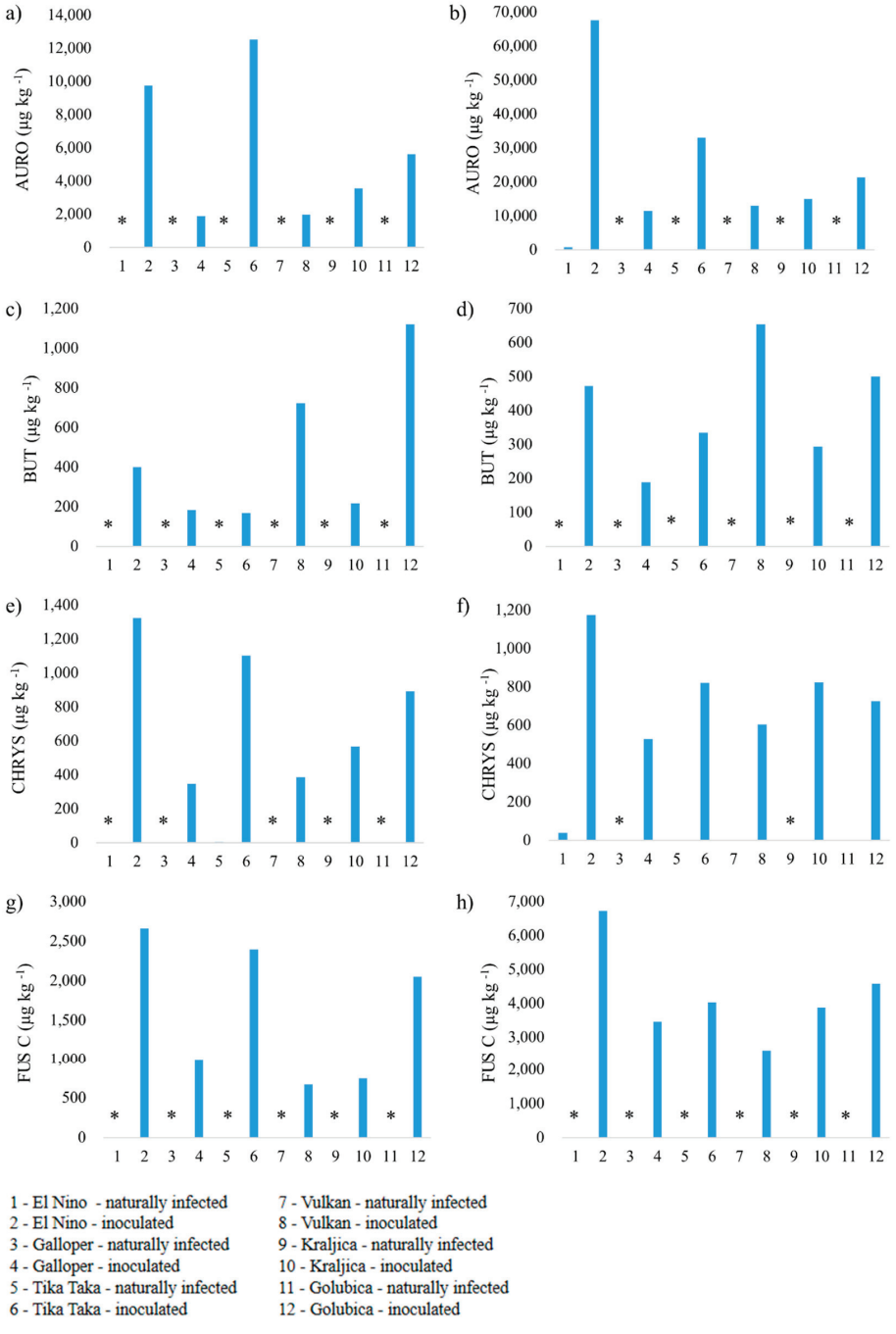

Figure 5. Concentrations of aurofusarin (AURO) (**a**,**b**), butenolide (BUT) (**c**,**d**), chrysogin (CHRYS) (**e**,**f**) and fusarin C (FUS C) (**g**,**h**) in artificially inoculated and naturally infected samples at Osijek (**a**,**c**,**e**,**g**) and Tovarnik (**b**,**d**,**f**,**h**). The asterisk (*) indicates that measured values are below LOD values.

3.3. ANOVA and Correlation Analysis

Analysis of variance (ANOVA) revealed significant differences in 13 investigated mycotoxins among two treatments ($p < 0.001$, $p < 0.01$). Non-significant difference was found between varieties and locations for the most mycotoxins, except for 3-acetyldeoxynivalenol (3ADON), nivalenol (NIV) and fusarin C between locations. Moreover, significant differences were recorded between varieties for NIV ($p < 0.01$) (Table 2).

Correlation analysis showed no statistically significant relationship between the amount of FHB symptoms (Type I and general resistance) and DON contamination although correlation was moderately positive at both locations, Osijek and Tovarnik (r = 0.77, $p < 0.05$) (Supplementary Tables S10 and S11). At Osijek, NIV, hydroxyculmorins and 15-hydroxyculmoron occurred concomitantly with DON (r = 0.88, $p < 0.05$; r = 0.94, $p < 0.01$; r = 0.88, $p < 0.05$ and r = 0.94, $p < 0.01$, respectively) (Supplementary Table S10), while at Tovarnik DON was in high positive correlation with enumerated mycotoxins including 3-ADON (r = 0.94, $p < 0.01$; r = 0.94, $p < 0.01$; r = 0.94, $p < 0.01$; r = 0.94, $p < 0.01$; r = 0.94, $p < 0.01$; r = 0.94, $p < 0.01$, respectively) (Supplementary Table S11). There was also high positive relationship between Type I resistance, general resistance and level of aurofusarin at Tovarnik (r = 0.83, $p < 0.05$ and r = 0.89, $p < 0.05$, respectively) (Supplementary Table S11).

Table 2. Analysis of variance (ANOVA) for 13 analyzed *Fusarium* metabolites.

Source of Variation	Df	F-Value												
		DON	D3G	3ADON	NIV	ZEN	CUL	15-OHCUL	15-OH Culmoron	5-OHCUL	AURO	BUT	CHRYS	FUS C
Location	1	3.43 ns	3.52 ns	4.93 *	8.98 **	1.46 ns	0.51 ns	1.1 ns	0.42 ns	3.68 ns	4.71 ns	0.17 ns	0.01 ns	9.16 **
Treatment	1	87.93 ***	105.19 ***	54.8 ***	13.3 **	18.07 ***	63.33 ***	124.29 ***	43.92 ***	84.31 ***	11.25 ***	33.95 ***	98.56 ***	44.54 ***
Variety	5	2.14 ns	2.03 ns	1.89 ns	4.62 **	1.26 ns	1.65 ns	2.13 ns	1.85 ns	2.07 ns	1.12 ns	1.97 ns	2.66 ns	1.03 ns

***, **, * = significant at $p < 0.001$, 0.01, and 0.05, respectively; ns = not significant ($p > 0.05$); Df-Degrees of freedom, DON–deoxynivalenol, D3G–deoxynivalenol-3-glucoside, 3ADON–3-acetyldeoxynivalenol, NIV-nivalenol, ZEN–zearalenone, CUL–culmorin, 15OHCUL–15-hydroxyculmorin, 15OHculmoron–15-hydroxyculmoron, 5OHCUL–5-hydroxyculmorin, AURO-aurofusarin, BUT–butenolide, CHRYS–chrysogin, FUS C–fusarin C.

4. Discussion

The study has focused on the effect of epidemic FHB conditions on the production of a range of metabolites originated either from the naturally contaminating mycobiota, or from inoculation by *Fusarium graminearum* and *F. culmorum* isolates. Furthermore, this study extended the investigation beyond the well-known mycotoxins to a range of fungal secondary metabolites accumulated in wheat grains. According to previous findings, fungal secondary metabolites commonly found in wheat grains are deoxynivalenol (DON), a type B trichothecene, and zearalenone (ZEN), while predominant species producing these mycotoxins in Europe are found to be *F. graminearum*, *F. culmorum* and *F. avenaceum* [30]. Given the fact that *F. graminearum* widely occurs in Europe, it is also predominantly found in Croatia [31]. However, many *Fusarium* metabolites are far less investigated than DON and ZEN [32] and therefore are not subject to legislation and regular monitoring. Both emerging mycotoxins and modified forms represent a new issue for food contamination [33].

The mycotoxigenic fungi produce several secondary metabolites at the time [34]. Therefore, this study reports the occurrence of 13 *Fusarium* metabolites and their concentrations in the wheat grains of six artificially inoculated as well as in naturally infected (field grown) winter wheat varieties (El Nino, Galloper, Tika Taka, Vulkan, Kraljica and Golubica). The combined use of resistant wheat varieties, fungicides, and specific management practices can reduce part of the Fusarium head blight (FHB) losses [35]. Therefore, the impact of fungicides in the current research was omitted, as well as the influence of management practice, as field experiments were done according to standard agronomical procedures. Considering that precipitation levels between two locations at which the experiment was held differed with Tovarnik having a higher precipitation rate and higher temperatures, levels of *Fusarium* metabolites studied were higher at Tovarnik, compared to Osijek. This is in accordance with previous reports where warm and humid conditions at and shortly after anthesis favour FHB [36]. Humidity determines the intensity of the disease, while precipitation determines inoculum levels [37]. In addition, wheat varieties in treatment with inoculation with *Fusarium* fungi were evaluated for Type I (initial) and general FHB resistance in the field conditions prior to harvest by calculating AUDPC.

4.1. Deoxynivalenol, Deoxynivalenol-3-glucoside and 3-Acetyldeoxynivalenol

Taking into account only proved *Fusarium* mycotoxins and not all metabolites studied, the current research was in accordance with previous studies which reported that DON is the most abundant mycotoxin in wheat grains [38,39]. In this study, varieties with higher initial susceptibility (El Nino, Tika Taka and Golubica) accumulated DON even in naturally infected samples. The average level of DON in naturally infected winter wheat plants (control samples) at location Tovarnik did not exceed the legal limit set by EU (1 250 μg kg^{-1}) for unprocessed cereals [40] while at Osijek in naturally infected samples it was not found. The same results were previously reported where in randomly selected wheat samples from natural infection in Croatia, DON levels were below this threshold [41]. However, in the current research FHB artificially inoculated plants exceeded maximum levels for DON contamination 10-fold at Osijek and 15-fold at Tovarnik. As DON significantly correlated with few investigated mycotoxins, it can be concluded that DON content can be used in the selection for FHB resistance and could potentially participate in lowering total toxicity.

Deoxynivalenol-3-glucoside (D3G) is one of the main DON metabolites known as "modified mycotoxin" [42]. After ingestion it can be hydrolysed to DON [43,44]. D3G was present in *Fusarium* infected samples at both locations, while in naturally infected plants it was observed in susceptible varieties El Nino, Tika Taka and Golubica only at Tovarnik. Concentrations observed in the current research were similar to previous studies reporting the occurrence of DON and D3G in durum wheat in Italy [45]. Previously it was concluded that D3G usually comes in lower concentrations, compared to concentrations of DON [46].

3-acetyldeoxynivalenol (3ADON) was observed in *Fusarium* infected samples at Osijek as well as at Tovarnik. At Tovarnik it was also found in naturally infected samples in

susceptible El Nino variety. In some researches, 3ADON was among the most abundant mycotoxins [47,48], which was not the case in the current research.

Correlations between DON and 3ADON were highly significant at Tovarnik which is in accordance with the previous research [49] where DON highly correlated with 3ADON in barley samples. Also, this correlation was expected in the current research as DON-producing strains with the 3-acetylated precursor are common in Europe [50]. The more pronounced FHB symptoms the higher correlations between those mycotoxins occurred, where higher 3ADON production may be associated with elevated DON content. It was concluded that comparatively higher levels of gene expression may contribute to the higher levels of DON produced by 3ADON strains in infected grains [51]. Although there was not any significant correlation between DON and general and Type I resistance, it can be assumed that high level of DON occurred in varieties with different level of symptoms, as it was evidenced in previous studies [52] reporting that the occurrence of high humidity post-anthesis produced late infections, with a high level of DON, but low level of FHB symptoms.

4.2. Nivalenol and Zearalenone

The obtained results showed that nivalenol (NIV) at Osijek was represented only in varieties with high initial susceptibility (El Nino, Tika Taka and Golubica) in artificially inoculated samples. At Tovarnik, it was found in all *Fusarium* infected plants while in naturally infected samples it was observed only in susceptible variety Golubica. This is in accordance with previous studies [53] where NIV was found only in one naturally infected wheat sample. This was expected for *Fusarium* infected samples as both *F. graminearum* and *F. culmorum* produce NIV at all tested temperatures between 10 and 30 °C [54]. In the previous research the highest concentration of NIV in the organic cereal samples was 106 μg kg^{-1} [55], while the concentration of NIV in the current study was lower in the *Fusarium* infected as well as in the naturally infected samples.

Zearalenone (ZEN) concentrations were the lowest of all *Fusarium* metabolites studied at both locations and did not exceed risk threshold levels of 100 μg kg^{-1} for unprocessed cereals other than maize [40] in artificially inoculated nor in naturally infected samples. Our findings are in accordance with previous studies which showed that concentrations of ZEN found in wheat mostly do not exceed 50 μg kg^{-1} [32,56]. As expected, at Osijek ZEN was found only in inoculated samples while at Tovarnik in naturally infected plants it was observed only in susceptible variety El Nino in negligible concentration. Although there were not any significant correlations between DON and ZEN, it was previously concluded that ZEN is often co-produced with DON by *Fusarium* spp. such as *F. graminearum* [57]. For example, in another study where large European screening for *Fusarium* mycotoxins was obtained, ZEN was found in 32% of approximately 5000 samples of cereal grains and products tested [58].

4.3. Culmorin, 15-Hydroxyculmorin, 15-Hydroxyculmoron and 5-Hydroxyculmorin

In the novel time, there are numerous evidence for culmorin (CUL) being an "emerging mycotoxin". It is confirmed that CUL can inhibit the reaction of glucuronidation and thus increase the toxicity of DON [15,22]. Wheat samples with higher DON concentration contained elevated levels of CUL which implies that CUL can have a possible role in *Fusarium* virulence. Previously, it was reported about interactions between CUL and DON and its toxicity in growing pigs [59]. Furthermore, synergism between CUL and trichothecenes in plants occurred [60]. In *Fusarium* infected samples varieties with higher initial susceptibility (El Nino, Tika Taka and Golubica) accumulated CUL in much higher concentrations than other varieties. In Norway there was a concentration of 100 μg kg^{-1} detected in wheat [61].

Except for CUL, in the analysed samples also occurred 15-hydroxyculmorin, 15-hydroxyculmoron and 5-hydroxyculmorin. Our study was partially in accordance with other researches which showed that in samples artificially inoculated with *F. culmorum*

15-hydroxyculmorin was the most abundant derivative of CUL and 5-hydroxyculmorin second abundant [23] as in the current research at Osijek, while at Tovarnik this was opposite with the most abundant 5-hydroxyculmorin and second 15-hydroxyculmorin. Moreover, in the above-mentioned study in naturally infected samples, CUL was the major metabolite, while in the current research in naturally infected samples CUL and its derivatives were found only at Tovarnik where the major metabolite was 15-hydroxyculmorin. The current study is in accordance with research which reporting that in naturally infected samples of wheat in Croatia concentration of CUL and 15-hydroxyculmorin is at a similar range as the concentration of DON thus implying that DON is correlated with CUL and hydroxyculmorins [62]. In addition to that at Tovarnik, CUL was in a positive significant correlation with AUDPC for general resistance thus implying that CUL role in *Fusarium* virulence is more pronounced in increased FHB pressure.

4.4. Aurofusarin, Butenolide, Chrysogin and Fusarin C

Fusarium metabolites aurofusarin, butenolide, chrysogin and fusarin C observed in this study are recently discovered and therefore far less investigated than others [39]. Under increased FHB pressure, aurofusarin was observed in all varieties at both, Osijek and Tovarnik, while in naturally infected samples it was observed only in Tovarnik in susceptible variety El Nino. In naturally infected samples of wheat aurofusarin was found in wheat in levels up to 4200 μg kg^{-1} [63]. At Osijek, the average level of aurofusarin was in accordance with previous study [64], while at Tovarnik it was observed in much higher concentrations implying that there is a certain connection between the production of major *Fusarium* mycotoxins and pigments. There was an even higher concentration of aurofusarin detected up to 140,000 μg kg^{-1} in Italian samples of durum wheat [65] than in the current research at Tovarnik. Although previous studies report aurofusarin and rubrofusarin accompanied by one another [66], in the current research rubrofusarin was not observed. According to previous studies there is the genetic and biosynthetic origin of aurofusarin and both DON and ZEN [67].

Determined levels of butenolide and chrysogin were lower than those previously reported [64]. However, results are in accordance with the research where fusarin C and chrysogine concentrations were higher in the wheat with *F. graminearum* treatments in contrast to the naturally stored wheat [68]. Higher concentration of fusarin C (average level 40,042 mg kg^{-1}), chrysogin (average level 39 mg kg^{-1}) and butenolide (7300 mg kg^{-1}) were obtained in durum wheat in the fields with natural infection [65], while aurofusarin (average level 76,875 mg kg^{-1}) was at a similar level as in the present study. Butenolide and fusarin C were expected to be detected in the current research because both *F. graminearum* and *F. culmorum* have the ability to produce them [17]. Furthermore, current research revealed high positive correlation between chrysogin and aurofusarin, which was expected, as chrysogin is also pigment produced by *Fusarium* sp. [69].

5. Conclusions

This study performed at two different locations indicated that winter wheat samples with higher deoxynivalenol (DON) concentration contained elevated levels of culmorin (CUL) and hydroxyculmorins, showing that CUL can have a possible role in *Fusarium* virulence, which became more pronounced in elevated infection with *Fusarium* fungi. Since DON significantly correlated with few investigated metabolites, it can be assumed that DON can participate in lowering total toxicity. Furthermore, according to elevated aurofusarin levels, it is also assumed that there is a certain connection between the production of major *Fusarium* mycotoxins and pigments under increased FHB pressure. As the impact of some fungal secondary metabolites on food and feed safety, i.e., human and animal health is still unclear, it is of great importance to investigate their toxicity, as well as consequently regulate maximal allowed concentration for specific food and feed. Furthermore, possible synergistic effects between certain metabolites need to be investigated more closely as they could interact together thus giving total toxicity. An inevitable practical conclusion of

this manuscript is also information about the genetic resistance of winter wheat varieties investigated which will be useful for future risk assessment, considering FHB pressure and consequently mycotoxin production.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112239/s1, Figure S1: Climate diagram for May 2020 at Osijek. Figure S2: Climate diagram for May 2020 at Tovarnik. Table S1: Daily precipitation (mm) during May, June and July at Osijek in 2020. Table S2: Daily precipitation (mm) during May, June and July at Tovarnik in 2020. Table S3: Daily temperatures (°C) during May, June and July at Osijek in 2020. Table S4: Daily temperatures (°C) during May, June and July at Tovarnik in 2020. Table S5: Apparent recovery values, LOD values and LOQ values for 13 Fusarium secondary metabolites analyzed. Table S6: Amounts of Fusarium secondary metabolites in naturally infected samples at Osijek. Table S7: Amounts of Fusarium secondary metabolites in samples inoculated with Fusarium fungi at Osijek. Table S8: Amounts of Fusarium secondary metabolites in naturally infected samples at Tovarnik. Table S9: Amounts of Fusarium secondary metabolites in samples inoculated with Fusarium fungi at Tovarnik. Table S10: Correlation analysis between metabolite accumulation and the area under the disease progress curve (AUDPC) for general and Type I (initial) resistance at Osijek. Table S11: Correlation analysis between metabolite accumulation and the area under the disease progress curve (AUDPC) for general and Type I (initial) resistance at Tovarnik.

Author Contributions: K.S. wrote the manuscript and interpreted the results. T.K., A.L., J.B. and G.D. interpreted the results, done statistical analysis and revised the manuscript. M.S. and R.K. performed mycotoxin research and revised the manuscript. V.S. conceived the study, performed the field experiments, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Shewry, P.R. Wheat. *J. Exp. Bot.* **2009**, *60*, 1537–1553. [CrossRef]
2. Osborne, L.E.; Stein, J.M. Epidemiology of Fusarium head blight on small-grain cereals. *Int. J. Food Microbiol.* **2007**, *119*, 103–108. [CrossRef] [PubMed]
3. Popovski, S.; Celar, F.A. The impact of environmental factors on the infection of cereals with Fusarium species and mycotoxin production—A review. *Acta Agric. Slov.* **2013**, *101*, 105–116. [CrossRef]
4. Spanic, V.; Marcek, T.; Abicic, I.; Sarkanj, B. Effects of Fusarium Head Blight on Wheat Grain and Malt Infected by *Fusarium culmorum*. *Toxins* **2018**, *10*, 17. [CrossRef] [PubMed]
5. Beccari, G.; Arellano, C.; Covarelli, L.; Tini, F.; Sulyok, M.; Cowger, C. Effect of wheat infection timing on Fusarium head blight causal agents and secondary metabolites in grain. *Int. J. Food Microbiol.* **2019**, *290*, 214–225. [CrossRef]
6. Spanic, V.; Lemmens, M.; Drezner, G. Morphological and molecular identification of Fusarium species associated with head blight on wheat in East Croatia. *Eur. J. Plant. Pathol.* **2010**, *128*, 511–516. [CrossRef]
7. Desjardins, A.E. *Fusarium Mycotoxins: Chemistry, Genetics, and Biology*, 1st ed.; American Phytopathological Society (APS Press): St. Paul, MN, USA, 2006.
8. Mielniczuk, E.; Skwaryło-Bednarz, B. Fusarium Head Blight, Mycotoxins and Strategies for Their Reduction. *Agronomy* **2020**, *10*, 509. [CrossRef]
9. Xu, X.M.; Nicholson, P.; Thomsett, M.A.; Simpson, D.; Cooke, B.M.; Doohan, F.M.; Brennan, J.; Monaghan, S.; Moretti, A.; Mule, G.; et al. Relationship Between the Fungal Complex Causing Fusarium Head Blight of Wheat and Environmental Conditions. *Phytopathology* **2008**, *98*, 69–78. [CrossRef] [PubMed]
10. Czaban, J.; Wróblewska, B.; Sułek, A.; Mikos-Szymańska, M.; Boguszewska, E.; Podolska, G.; Nieróbca, A. Colonisation of winter wheat grain by *Fusarium* spp. and mycotoxin content as dependent on a wheat variety, crop rotation, a crop management system and weather conditions. *Food Addit. Contam. Part A* **2015**, *32*, 874–910. [CrossRef]
11. Bernhoft, A.; Torp, M.; Clasen, P.-E.; Løes, A.-K.; Kristoffersen, A.B. Influence of agronomic and climatic factors on Fusarium infestation and mycotoxin contamination of cereals in Norway. *Food Addit. Contam. Part A* **2012**, *29*, 1129–1140. [CrossRef]
12. Hope, R.; Aldred, D.; Magan, N. Comparison of environmental profiles for growth and deoxynivalenol production by *Fusarium culmorum* and *F. graminearum* on wheat grain. *Lett. Appl. Microbiol.* **2005**, *40*, 295–300. [CrossRef] [PubMed]
13. Mesterhazy, A. Types and components of resistance to Fusarium head blight of wheat. *Plant. Breed* **1995**, *114*, 377–386. [CrossRef]

14. Cuomo, C.A.; Güldener, U.; Xu, J.-R.; Trail, F.; Turgeon, B.G.; Di Pietro, A.; Walton, J.D.; Ma, L.-J.; Baker, S.; Rep, M.; et al. The *Fusarium graminearum* Genome Reveals a Link Between Localized Polymorphism and Pathogen Specialization. *Science* **2007**, *317*, 1400–1402. [CrossRef]
15. Woelflingseder, L.; Warth, B.; Vierheilig, I.; Schwartz-Zimmermann, H.; Hametner, C.; Nagl, V.; Novak, B.; Šarkanj, B.; Berthiller, F.; Adam, G.; et al. The Fusarium metabolite culmorin suppresses the in vitro glucuronidation of deoxynivalenol. *Arch. Toxicol.* **2019**, *93*, 1729–1743. [CrossRef]
16. Pestka, J.J. Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. *Arch. Toxicol.* **2010**, *84*, 663–679. [CrossRef]
17. Pasquali, M.; Giraud, F.; Brochot, C.; Cocco, E.; Hoffmann, L.; Bohn, T. Genetic Fusarium chemotyping as a useful tool for predicting nivalenol contamination in winter wheat. *Int. J. Food Microbiol.* **2010**, *137*, 246–253. [CrossRef]
18. Bretz, M.; Knecht, A.; Göckler, S.; Humpf, H.-U. Structural elucidation and analysis of thermal degradation products of the *Fusarium mycotoxin* nivalenol. *Mol. Nutr. Food Res.* **2005**, *49*, 309–316. [CrossRef] [PubMed]
19. Desjardins, A.; Proctor, R. Molecular biology of *Fusarium* mycotoxins. *Int. J. Food Microbiol.* **2007**, *119*, 47–50. [CrossRef] [PubMed]
20. Rai, A.; Das, M.; Tripathi, A. Occurrence and toxicity of a *Fusarium* mycotoxin, zearalenone. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2710–2729. [CrossRef]
21. Kovač, T.; Borišev, I.; Kovač, M.; Lončarić, A.; Kenjerić, F.Č.; Djordjevic, A.; Strelec, I.; Ezekiel, C.N.; Sulyok, M.; Krska, R.; et al. Impact of fullerol C60(OH)24 nanoparticles on the production of emerging toxins by *Aspergillus flavus*. *Sci. Rep.* **2020**, *10*, 725–810. [CrossRef]
22. Wipfler, R.; McCormick, S.P.; Proctor, R.H.; Teresi, J.M.; Hao, G.; Ward, T.J.; Alexander, N.J.; Vaughan, M.M. Ward Synergistic Phytotoxic Effects of Culmorin and Trichothecene Mycotoxins. *Toxins* **2019**, *11*, 555. [CrossRef] [PubMed]
23. Ghebremeskel, M.; Langseth, W. The occurrence of culmorin and hydroxy-culmorins in cereals. *Mycopathology* **2001**, *152*, 103–108. [CrossRef]
24. Farber, J.; Scott, P.; Fusarin, C. *Fusarium*, 1st ed.; Chełkowski, J., Ed.; Elsevier BV: Amsterdam, The Netherlands, 1989; Volume 2, pp. 41–52.
25. Woelflingseder, L.; Adam, G.; Marko, D. Suppression of Trichothecene-Mediated Immune Response by the Fusarium Secondary Metabolite Butenolide in Human Colon Epithelial Cells. *Front. Nutr.* **2020**, *7*, 127. [CrossRef]
26. Jarolim, K.; Wolters, K.; Woelflingseder, L.; Pahlke, G.; Beisl, J.; Puntscher, H.; Braun, D.; Sulyok, M.; Warth, B.; Marko, D. The secondary Fusarium metabolite aurofusarin induces oxidative stress, cytotoxicity and genotoxicity in human colon cells. *Toxicol. Lett.* **2018**, *284*, 170–183. [CrossRef]
27. Zadoks, J.C.; Chang, T.T.; Konzak, C.F. A decimal code for the growth stages of cereals. *Weed Res.* **1974**, *14*, 415–421. [CrossRef]
28. Sulyok, M.; Stadler, D.; Steiner, D.; Krska, R. Validation of an LC-MS/MS-based dilute-and-shoot approach for the quantification of > 500 mycotoxins and other secondary metabolites in food crops: Challenges and solutions. *Anal. Bioanal. Chem.* **2020**, *412*, 2607–2620. [CrossRef] [PubMed]
29. European Commission. COMMISSION DECISION of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of result. *Off. J. Eur. Union L.* **2002**, *221*, 8–36.
30. Bottalico, A.; Perrone, G. Toxigenic Fusarium species and mycotoxins associated with head blight in small-grain cereals in Europe. In *Mycotoxins in Plant Disease*, 1st ed.; Logrieco, A., Bailey, J.A., Corazza, L., Cooke, B.M., Eds.; Springer: Dordrecht, The Netherlands, 2002; pp. 611–624.
31. Spanic, V.; Lemmens, M.; Drezner, G.; Mesterházy, Á. Variability in components of fusarium head blight resistance among wheat genotypes. *Cereal Res. Commun.* **2013**, *41*, 420–430. [CrossRef]
32. Stanciu, O.; Banc, R.; Cozma, A.; Filip, L.; Miere, D.; Mañes, J.; Loghin, F. Occurence of *Fusarium* Mycotoxins in Wheat from Europe—A Review. *Acta Univ. Cibiniensis. Ser. E Food Technol.* **2015**, *19*, 35–60. [CrossRef]
33. Mortensen, A.; Granby, K.; Eriksen, F.D.; Cederberg, T.L.; Friis-Wandall, S.; Simonsen, Y.; Broesbøl-Jensen, B.; Bonnichsen, R. Levels and risk assessment of chemical contaminants in byproducts for animal feed in Denmark. *J. Environ. Sci. Health Part B* **2014**, *49*, 797–810. [CrossRef]
34. Streit, E.; Schatzmayr, G.; Tassis, P.; Tzika, E.; Marin, D.; Taranu, I.; Tabuc, C.; Nicolau, A.; Aprodu, I.; Puel, O.; et al. Current Situation of Mycotoxin Contamination and Co-occurrence in Animal Feed—Focus on Europe. *Toxins* **2012**, *4*, 788–809. [CrossRef]
35. Hollingsworth, C.R.; Motteberg, C.D.; Wiersma, J.V.; Atkinson, L.M. Agronomic and Economic Responses of Spring Wheat to Management of Fusarium Head Blight. *Plant. Dis.* **2008**, *92*, 1339–1348. [CrossRef]
36. Gautam, P.; Dill-Macky, R. Fusarium head blight development and trichothecene accumulation in point inoculated Fusarium infected wheat heads. *World Mycotoxin J.* **2012**, *5*, 45–55. [CrossRef]
37. Francl, L. Development of Fusarium head blight in relation to environment and inoculum. In *Proceedings National Fusarium Head Blight Forum*; Michigan State University, University Printing: East Lansing, MI, USA, 1998; pp. 26–27.
38. Van der Fels-Klerx, H.; de Rijk, T.; Booij, C.; Goedhart, P.W.; Boers, E.; Zhao, C.; Waalwijk, C.; Mol, H.G.J.; van der Lee, T. Occurrence of Fusarium Head Blight species and *Fusarium* mycotoxins in winter wheat in the Netherlands in 2009. *Food Addit. Contam. Part A* **2012**, *29*, 1716–1726. [CrossRef] [PubMed]
39. Stanciu, O.; Juan, C.; Miere, D.; Loghin, F.; Mañes, J. Occurrence and co-occurrence of *Fusarium* mycotoxins in wheat grains and wheat flour from Romania. *Food Control.* **2017**, *73*, 147–155. [CrossRef]

40. European Commission (EC). Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union L.* **2006**, *364*, 5–24.
41. Pleadin, J.; Vahčić, N.; Perši, N.; Ševelj, D.; Markov, K.; Frece, J. *Fusarium* mycotoxins' occurrence in cereals harvested from Croatian fields. *Food Control.* **2013**, *32*, 49–54. [CrossRef]
42. Kovač, M.; Šubarić, D.; Bulaić, M.; Kovač, T.; Šarkanj, B. Yesterday masked, today modified; what do mycotoxins bring next? *Arch. Ind. Hyg. Toxicol.* **2018**, *69*, 196–214. [CrossRef]
43. Berthiller, F.; Krska, R.; Domig, K.J.; Kneifel, W.; Juge, N.; Schuhmacher, R.; Adam, G. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicol. Lett.* **2011**, *206*, 264–267. [CrossRef] [PubMed]
44. Bryła, M.; Ksieniewicz-Woźniak, E.; Waśkiewicz, A.; Szymczyk, K.; Jędrzejczak, R. Natural Occurrence of Nivalenol, Deoxynivalenol, and Deoxynivalenol-3-Glucoside in Polish Winter Wheat. *Toxins* **2018**, *10*, 81. [CrossRef] [PubMed]
45. Dall'Asta, C.; Dall'Erta, A.; Mantovani, P.; Massi, A.; Galaverna, G. Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin J.* **2013**, *6*, 83–91. [CrossRef]
46. Lemmens, M.; Steiner, B.; Sulyok, M.; Nicholson, P.; Mesterhazy, A.; Buerstmayr, H. Masked mycotoxins: Does breeding for enhanced Fusarium head blight resistance result in more deoxynivalenol-3-glucoside in new wheat varieties? *World Mycotoxin J.* **2016**, *9*, 741–754. [CrossRef]
47. Miedaner, T.; Reinbrecht, C.; Lauber, U.; Schollenberger, M.; Geiger, H.H. Effects of genotype and genotype-environment interaction on deoxynivalenol accumulation and resistance to Fusarium head blight in rye, triticale, and wheat. *Plant. Breed.* **2001**, *120*, 97–105. [CrossRef]
48. Zhao, J.; Cheng, T.; Xu, W.; Han, X.; Zhang, J.; Zhang, H.; Wang, C.; Fanning, S.; Li, F. Natural co-occurrence of multi-mycotoxins in unprocessed wheat grains from China. *Food Control.* **2021**, *130*, 108321. [CrossRef]
49. Tucker, J.R.; Badea, A.; Blagden, R.; Pleskach, K.; Tittlemier, S.A.; Fernando, W.G.D. Deoxynivalenol-3-Glucoside Content Is Highly Associated with Deoxynivalenol Levels in Two-Row Barley Genotypes of Importance to Canadian Barley Breeding Programs. *Toxins* **2019**, *11*, 319. [CrossRef]
50. Miller, J.D.; Greenhalgh, R.; Wang, Y.; Lu, M. Trichothecene Chemotypes of Three Fusarium Species. *Mycologia* **1991**, *83*, 121–130. [CrossRef]
51. Amarasinghe, C.C.; Fernando, W.G.D. Comparative Analysis of Deoxynivalenol Biosynthesis Related Gene Expression among Different Chemotypes of *Fusarium graminearum* in Spring Wheat. *Front. Microbiol.* **2016**, *7*, 1229. [CrossRef]
52. Cowger, C.; Arrellano, C. Plump Kernels with High Deoxynivalenol Linked to Late *Gibberella zeae* Infection and Marginal Disease Conditions in Winter Wheat. *Phytopathology* **2010**, *100*, 719–728. [CrossRef]
53. Spanic, V.; Zdunic, Z.; Drezner, G.; Sarkanj, B. The Pressure of Fusarium Disease and Its Relation with Mycotoxins in the Wheat Grain and Malt. *Toxins* **2019**, *11*, 198. [CrossRef]
54. Brennan, J.M.; Fagan, B.; van Maanen, A.; Cooke, B.M.; Doohan, F.M. Studies on in vitro Growth and Pathogenicity of European Fusarium fungi. *Eur. J. Plant. Pathol.* **2003**, *109*, 577–587. [CrossRef]
55. Juan, C.; Ritieni, A.; Mañes, J. Occurrence of *Fusarium* mycotoxins in Italian cereal and cereal products from organic farming. *Food Chem.* **2013**, *141*, 1747–1755. [CrossRef] [PubMed]
56. Klarić, M.; Cvetnić, Z.; Pepeljnjak, S.; Kosalec, I. Co-occurrence of Aflatoxins, Ochratoxin A, Fumonisins, and Zearalenone in Cereals and Feed, Determined by Competitive Direct Enzyme-Linked Immunosorbent Assay and Thin-Layer Chromatography. *Arch. Ind. Hyg. Toxicol.* **2009**, *60*, 427–434. [CrossRef] [PubMed]
57. Jestoi, M. EmergingFusarium-Mycotoxins Fusaproliferin, Beauvericin, Enniatins, and Moniliformin—A Review. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 21–49. [CrossRef] [PubMed]
58. Gareis, M.; Zimmermann, C.; Schothorst, R.; Paulsch, W.; Vidnes, A.; Bergsten, C.; Paulsen, B.; Brera, C.; Miraglia, M.; Grossi, S.; et al. Collection of Occurrence Data of Fusarium toxins in Food and Assessment of Dietary Intake by the Population of EU Member States. 2001. Available online: http://europa.eu.int/comm/food/fs/scoop/task3210.pdf (accessed on 20 May 2021).
59. Rotter, R.G.; Trenholm, H.L.; Prelusky, D.B.; Hartin, K.E.; Thompson, B.K.; Miller, J.D. A preliminary examination of potential interactions between deoxynivalenol (DON) and other selected Fusarium metabolites in growing pigs. *Can. J. Anim. Sci.* **1992**, *72*, 107–116. [CrossRef]
60. Weber, J.; Vaclavikova, M.; Wiesenberger, G.; Haider, M.; Hametner, C.; Fröhlich, J.; Berthiller, F.; Adam, G.; Mikula, H.; Fruhmann, P. Chemical synthesis of culmorin metabolites and their biologic role in culmorin and acetyl-culmorin treated wheat cells. *Org. Biomol. Chem.* **2018**, *16*, 2043–2048. [CrossRef]
61. Uhlig, S.; Eriksen, G.S.; Hofgaard, I.S.; Krska, R.; Beltrán, E.; Sulyok, M. Faces of a Changing Climate: Semi-Quantitative Multi-Mycotoxin Analysis of Grain Grown in Exceptional Climatic Conditions in Norway. *Toxins* **2013**, *5*, 1682–1697. [CrossRef] [PubMed]
62. Kifer, D.; Sulyok, M.; Jakšić, D.; Krska, R.; Klarić, M. Šegvić Fungi and their metabolites in grain from individual households in Croatia. *Food Addit. Contam. Part. B* **2021**, *14*, 98–109. [CrossRef]
63. Kotik, A.N.; Trufanova, V.A. Detection of naphtoquinone fusariotoxin aurofusarin in wheat. *Mikol. Fitopatol.* **1998**, *32*, 58–61.
64. Spanic, V.; Katanic, Z.; Sulyok, M.; Krska, R.; Puskas, K.; Vida, G.; Drezner, G.; Šarkanj, B. Multiple Fungal Metabolites Including Mycotoxins in Naturally Infected and Fusarium-Inoculated Wheat Samples. *Microorganisms* **2020**, *8*, 578. [CrossRef]

65. Beccari, G.; Colasante, V.; Tini, F.; Senatore, M.; Prodi, A.; Sulyok, M.; Covarelli, L. Causal agents of Fusarium head blight of durum wheat (*Triticum durum* Desf.) in central Italy and their in vitro biosynthesis of secondary metabolites. *Food Microbiol.* **2018**, *70*, 17–27. [CrossRef]
66. Frandsen, R.J.; Schütt, C.; Lund, B.W.; Staerk, D.; Nielsen, J.; Olsson, S.; Giese, H. Two Novel Classes of Enzymes Are Required for the Biosynthesis of Aurofusarin in *Fusarium graminearum*. *J. Biol. Chem.* **2011**, *286*, 10419–10428. [CrossRef]
67. Malz, S.; Grell, M.N.; Thrane, C.; Maier, F.J.; Rosager, P.; Felk, A.; Albertsen, K.S.; Salomon, S.; Bohn, L.; Schäfer, W.; et al. Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the Fusarium graminearum species complex. *Fungal Genet. Biol.* **2005**, *42*, 420–433. [CrossRef] [PubMed]
68. Garcia-Cela, E.; Kiaitsi, E.; Medina, A.; Sulyok, M.; Krska, R.; Magan, N. Interacting Environmental Stress Factors Affects Targeted Metabolomic Profiles in Stored Natural Wheat and That Inoculated with *F. graminearum*. *Toxins* **2018**, *10*, 56. [CrossRef] [PubMed]
69. Wollenberg, R.D.; Saei, W.; Westphal, K.R.; Klitgaard, C.S.; Nielsen, K.L.; Lysøe, E.; Gardiner, D.M.; Wimmer, R.; Sondergaard, T.E.; Sørensen, J.L. Chrysogine Biosynthesis Is Mediated by a Two-Module Nonribosomal Peptide Synthetase. *J. Nat. Prod.* **2017**, *80*, 2131–2135. [CrossRef] [PubMed]

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Impact of Fusarium Head Blight on Wheat Flour Quality: Examination of Protease Activity, Technological Quality and Rheological Properties

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Abstract: Wheat infections caused by *Fusarium* represent a global agricultural problem that reduces grain yield and negatively impacts wheat's technological and rheological quality. Although fungal proteases or an increase in endogenous proteases due to *Fusarium* infection could negatively influence wheat storage proteins and dough performance, little research has been performed on either of these topics. The primary objective of this study was to identify the effect of *Fusarium* infection on protease activity in 25 wheat cultivars grown in two distinct locations in eastern Croatia. Apart from proteolytic activity, this paper describes the impact of Fusarium head blight (FHB) infection on the technological quality parameters of wheat flour and the dough's rheological properties. The first treatment consisted of naturally grown, healthy wheat without fungicides, while the second treatment utilized wheat varieties subjected to intense FHB infection. Protein and wet gluten content in wheat grain and flour of uninfected cultivars were heavily influenced by testing location, soil type, and quality. *Fusarium* infection increased the activity of nonspecific proteases by 43% in flour samples from Osijek and 125% in flour samples from Tovarnik. Estimates of effect size showed that FHB infection had twice as big an effect on protease activity in Tovarnik as in Osijek, and a similar trend was found for dough softening. Moreover, the infection significantly impacted wheat cultivars' extensograph values, indicating a lower resistance to stretching, extensibility, and total stretching energy in infected flour samples, indicating that dough functionality and volume loss can be attributed to exogenous fungal proteases. Still, the magnitude of the effect varied depending on the growth location and the cultivar's traits. Multivariate data analysis identified three clusters of wheat cultivars, each with varying degrees of the *Fusarium* infection's effects. Some cultivars displayed consistent protease activity and flour quality across sites. In contrast, others showed variability in their responses due to environmental conditions. To conclude, genetic resistance could provide adequate control of FHB, guaranteeing the successful protection of wheat quality. However, the possibility of confounding factors influencing genetic and cultivation conditions must be considered, and further research is needed to understand their interaction.

Keywords: nonspecific protease; wheat flour; genotype variation; environmental factors; rheological properties; FHB infection

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1. Introduction

Wheat's advantage as a crop species is mainly reflected in the quality and characteristics of the dough formed from its flour. Wheat is the most important grain in the human diet, and wheat flour is a source of essential dietary components. As a result, cultivating wheat free of numerous pollutants, such as mycotoxins, which can threaten human health, is of great interest [1]. Fusarium head blight (FHB) is one of wheat's most economically devastating diseases and a global problem, causing a considerable loss of yield and grain

quality due to reduced protein and starch content [2,3]. This disease's primary causative agent is *Fusarium graminearum*, which forms the FGSC (*F. graminearum* species complex) with at least 16 phylogenetically different species [4,5]. Still, many species of the genus *Fusarium* are also considered causative, e.g., *F. culmorum*, *F. poae,* and *F. avenaceum*, and several species may be present simultaneously, interacting with each other and leading to infection and mycotoxin production [6].

The most susceptible period for *Fusarium* infection of wheat is the flowering stage, with temperatures ranging between 20 and 25 °C and a moisture content of 95%, whereas the earliest symptoms of the disease occur about ten days after flowering [7,8]. However, in warm and wet conditions, the first symptoms appear even earlier [9]. The sheer intensity of infection is affected by the causative agent's pathogenicity, the cultivar's susceptibility, and the timing of infection during the growing season [2,6,10,11]. Control strategies that can reduce the occurrence of FHB include various agrochemical techniques, including selective pre-crop planting, crop rotation, tillage, fungicide and biological control applications, fertilization, and the creation of FHB-resistant cultivars [1,12–14]. Although agrochemical measures are helpful, they are only partially efficient in preventing FHB. Integrated control of FHB spread and avoidance of mycotoxins buildup in grains [15,16] relies heavily on developing resistant cultivars, which is the most cost-effective and long-term approach. Disease resistance is linked to the plant's hypersensitive reaction, which occurs at the site of pathogen penetration and results in the premature death of spikes or blight [17]. According to Schroeder and Christensen [18], there are two major types of FHB resistance. Type I resistance refers to the initial infection, whereas Type II resistance refers to the plant's ability to reduce the spread of disease symptoms through a spike. Moreover, wheat plants have evolved different defense responses to fight off the invasion by *Fusarium* spp., involving physiological and molecular mechanisms triggered by pathogen attack [8,19]. This is supported by the fact that, until today, more than 500 quantitative trait loci (QTL) for FHB resistance have been discovered [20]. However, breeding wheat for FHB resistance is difficult due to quantitative inheritance and complicated mechanisms in wheat–pathogen interaction, and it requires a thorough understanding of the physiological and molecular mechanisms of defense responses in wheat plants to *Fusarium* spp.

Although there are a large number of studies on the pathogenicity and epidemiology of FHB, resistance mechanisms, mycotoxins, and measures to combat infection, the literature about the impact of *Fusarium* infection on the quality of wheat flour and its end products is not so abundant. Some studies have been based primarily on food safety and the possibility of avoiding wheat grain contamination with mycotoxins [12,21,22]. Nevertheless, there has been a noticeable increase in the number of studies dealing with the impact of FHB on the effectiveness of wheat milling, grain quality, flour properties, and quality of end-use products [23–27]. Even though the microbial load is mainly found on the grain's surface, the dry milling process can redistribute contamination and deteriorate wheat flour quality [28]. For example, it was determined that *Fusarium* infection affects the product's technological quality, including its sedimentation value and gluten index, and thus negatively impacts the dough's rheological properties, such as stability, resistance to extension, and energy value [29].

Proteolytic cleavage of peptide bonds, as one of the essential protein modification mechanisms during the seed maturation and germination process, is greatly affected by fungal infections of cereals [30,31]. Increased protease activity was found in barley and wheat grains infected with *Fusarium* [31–33]. Moreover, when present excessively in flour, proteases alter the gluten network, thus impacting the dough's quality, allowing considerable gas retention, and affecting bread's quality and texture [34]. *Fusarium* proteases remain idle in the harvested grain but can be reactivated during the dough-making process, negatively affecting the rheological properties of dough [33,35]. It is well known that the dough's physical properties and baking depend not only on the gluten amount but also on its strength, whereas higher protease activity reduces gluten strength by partially breaking down its polymeric form. Excessive elasticity results in insufficient dough rising because

the gas pressure required to stretch the dough is too high, while extreme stretching without sufficient strength leads to the cracking of air bubbles, forming large cavities during baking. Since *Fusarium* protease remains active through the dough-making and kneading phases, the extension of these processes will likely result in a significant loss of dough strength and bread shape [33]. Recent studies aimed to characterize the proteases synthesized by *Fusarium* species and find proteins that could inhibit these enzymes, minimizing changes in the dough's quality [36–39].

The advantage of wheat as a crop species is not only in its high level of adaptation to different climatic conditions and in maintaining a high grain yield in these conditions but is also largely reflected in the quality and characteristics of the dough produced from its flour. These properties are derived from its storage proteins. Although the wheat protein content is relatively low compared to other grains, the role of storage proteins in gluten formation makes wheat one of the most consumed grains. Thus, the content and concentration of wheat proteins become some of wheat's leading commercial value indicators, making the research of FHB's impact on protease activity significant. Therefore, this research primarily aimed to determine the influence of *Fusarium* infection on the protease activity of 25 winter wheat varieties sown in two distinct locations in Eastern Croatia. In addition to proteolytic activity, technological quality properties (grain protein content, sedimentation value, gluten index, and falling number) and rheological characteristics (water absorption, farinograph quality number, dough stability, degree of the dough softening, dough resistance to extension, extensibility, and extension energy) of the wheat flour and dough were also examined. We were curious to see how the wheat testing location affects the wheat's ability to resist *Fusarium* infection and how the protease activity of infected and uninfected wheat flour samples from two distinct cultivation areas reflects the dough quality for baking. Furthermore, does the genotypic variability of wheat varieties significantly influence protease activity and other wheat grain and flour properties, or do environmental factors prevail?

2. Materials and Methods

2.1. Wheat Samples, Field Experiments, FHB Inoculations, and Disease Assessment

Twenty-five winter wheat cultivars (Table 1) were sown in a completely randomized block design in the 2019–2020 crop season at two locations in eastern Croatia (Osijek, OS, at 45°32′ N, 18°44′ E, and Tovarnik, TOV, at 45°10′ N, 19°9′ E). During the wheat growing season, Osijek had an average air temperature of 11.1 °C and a total precipitation of 408.6 mm. In Tovarnik, the average air temperature was 11.7 °C, and the total rainfall was 448.3 mm (data from the Croatian Meteorological and Hydrological Service). At both locations, standard agro-technical measures were applied, but the application of fungicides was omitted. The experiment consisted of two replicates for each cultivar of uninfected control samples (treatment 1) and two replicates of samples artificially infected with *Fusarium* species that cause FHB (treatment 2). Inoculum for artificial inoculation was produced by mixing two of the most common *Fusarium* species (1:1), *F. graminearum* and *F. culmorum*, and producing spores on autoclaved wheat and oat grains as described in previous research [40]. For inoculation, the conidium concentration was set to 10^5 mL^{-1} and sprayed on the whole plots of 7.56 m^2 when >50% of plants per plot were in the full flowering stage (Zadoks scale of 65, [41]). After inoculation, plants were sprayed with water to initiate infection, thus maintaining increased moisture for 24 h.

Infection symptom assessment was performed on the 10th, 14th, 18th, 22nd, and 26th day after inoculation. Initial resistance (Type I resistance) was scored on a random sample of 30 wheat heads and represented a percentage of diseased ears per plot, while general resistance was evaluated on a linear scale as a percentage of diseased spikelets on the entire

plot. Further, the area under the disease progress curve (AUDPC) for initial and general resistance (AUDPC-In and AUDPC-Gen) was calculated as follows:

$$\text{AUDPC} = \sum_{i=1}^{n} \left[\left\{ \frac{Y_i + Y_{(i-1)}}{2} \right\} \times \left(x_{(i+1)} - x_i \right) \right] \quad (1)$$

where Y_i is disease severity on the ith date; x_i is ith day; n is the number of days on which FHB infection was recorded.

Table 1. Code names of tested winter wheat cultivars with different resistance levels to FHB used in tables and figures. Letters indicate cultivars' resistance to *Fusarium* infection: R—resistant, MR—moderately resistant, MS—moderately susceptible, S—Susceptible.

Code	Winter Wheat Cultivars	Code	Winter Wheat Cultivars
1	Osk.107/15 MS/S	14	Imported * W2 S
2	Osk. 51/15 R	15	Imported * W3 R
3	Osk. 54/15 MS/MR	16	Osk. 108/04 S
4	Osk. 84/15 MR/R	17	Imported * W4 R
5	Osk. 251/02 MR/R	18	Osk. 106/03 S
6	Osk.111/08 MR/R	19	Osk. 44/11 MS/MR
7	Osk. 70/14 S	20	Osk.116/09 MR
8	Osk. 78/14 S	21	Osk. 4.40/7–82 S
9	Osk.138/14 MR/R	22	Osk. 381/06 MR
10	Osk. 52/13 R	23	Osk. 114/08 MR
11	Osk. 89/05 S/MS	24	Osk. 10287 S
12	Imported * W1 R	25	Osk 120/06 MS/MR
13	Osk.7733 S/MS		

* imported cultivars—data can be provided by the Agricultural Institute Osijek, Department for breeding and genetics of small cereal crops (Osijek, Croatia).

Final harvesting occurred when grain moisture fell below 14% in July 2020.

2.2. Specific Properties of Grain and Flour

2.2.1. Technological Properties

The protein content (PC, %) was measured in grain samples on Infratec 1241 (Foss Tecator). The white flour of each sample was produced by laboratory-scale milling using a Quadrumat mill (Brabender OHG, Duisburg, Germany). The proportion of wet gluten (WG, %) was determined according to the ICC Standard No. 155 [42] by a Perten Glutomatic® 2000 System (PerkinElmer Inc., Waltham, MA, USA), and the sedimentation value (SV, mL) was determined according to the standard method HRN EN ISO 5529 [43]. Sedimentation value indicates the ability to swell gluten proteins in lactic acid, depends on the quantity and quality of wheat proteins, and is an indicator of protein quality.

2.2.2. Amylolytic Activity

Since a certain amount of α-amylase activity is necessary for final product quality, it was estimated as a falling number (FN, s) using the Hagberg–Perten falling number system according to the standard method HRN EN ISO 3093 [44]. The FN test examines the effect of α-amylase on gelatinized starch granules in flour which are gradually broken down (cleaved) by amylase action. The test temperature maximizes enzymatic activity in the flour/water mixture. The FN refers to the time in seconds needed to stir and allow the viscometer stirrer to fall a measured distance through a hot slurry or gel of wheat flour and has an inverse relationship with the activity of α-amylase. Therefore, when the enzymatic activity is high, the starch is rapidly broken down, and the device descends fast through the relatively liquid paste. The FN is low when the viscous fluid opposes the flow with some resistance. If, on the other hand, the enzyme activity is low, the device takes longer to

cover the distance of its fall. This signifies that the falling number is high. So, the larger the number, the lower the activity of $\alpha-$amylase, and vice versa [44].

2.2.3. Proteolytic Activity

For the evaluation of proteolytic activity in wheat flour samples, a modified standard test for determining the activity of nonspecific proteases with phosphoprotein casein as a substrate was used [45]. This quality control procedure is based on the protease digestion of casein to form peptides soluble in trichloroacetic acid (TCA). These peptides contain the amino acids tyrosine and tryptophan residues that react with the Folin-Ciocalteu (FC) reagent to form a blue-colored chromophore, which is then quantified by a UV-VIS spectrophotometer (Analytik Jena, Specord 40).

The standard protocol was modified for micro volumes and adapted to the tested samples. The optimal incubation and reaction times, as well as temperature, were determined before testing. To extract the enzyme, flour samples were suspended in 10 mM CH_3COONa and 5 mM $(CH_3COO)_2Ca$ buffer (pH 7.5, 37 °C) in a ratio of 1:5 (*w/v*) and pre-incubated for 12 h at 37 °C. After centrifugation (15 min, 10,000× *g*, 20 °C), to 50 µL of enzyme extract, 20 µL of 0.5% casein in a 50 mM potassium phosphate buffer (pH 7.5, 37 °C) was added and incubated for 10 min at 37 °C. The reaction was stopped with 110 mM trichloroacetic acid (TCA, 20 µL), re-incubated for 30 min at 37 °C, and centrifuged for 5 min at 20 °C (10,000× *g*). The supernatant (250 µL) was then mixed with 25 µL 500 mM Na_2CO_3 and 125 µL 0.5 N FC reagent and incubated for 30 min at 37 °C, after which samples with developed blue chromophores were transferred to cuvettes and absorbance was measured at 660 nm.

Absorbance values generated by protease activity were compared with a standard curve obtained by reacting known quantities of tyrosine (L-Tyrosine, Sigma-Aldrich) with the FC reagent to compare changes in absorbance with the amount of tyrosine in µmol. Nonspecific protease activity was expressed as µmol of tyrosine equivalents released from casein min/mL and recalculated per gram of tested flour. All measurements were replicated three times.

2.3. Rheological Properties of Dough

In addition to the main parameters of the technological properties of wheat flour, we also determined the rheological properties of wheat flour dough with a farinograph [46] and an extensograph [47] using 50 and 300 g of flour samples, respectively. The obtained results provide information on dough behavior during kneading and the properties of gluten during dough formation. The determination of the flour quality by farinograph is based on registering changes in the physical characteristics of the dough during a specific stirring time. Using a farinograph, we determined: (1) the ability of flour to absorb water (WA, %), which indicates the proportion of water to be added to the flour to knead the dough of optimal consistency and is expressed in farinographic units (FU), where the optimal consistency is about 500 FU; (2) the farinograph quality number (FQN) as the length from the water point to the point of 30 FU below the center line of the largest consistency along the time axis; (3) dough stability (DS, min), which indicates the time from the maximum achieved optimal consistency to its decrease by 10 FU; and the (4) degree of the dough softening (DoS, FU) as the difference between the maximum resistance to mixing (i.e., the optimal consistency of the dough) and the middle of the curve at the end of mixing (12 min later).

Using extensograph, the dough resistance to extension (RtE, EU), extensibility (Ext, mm), and extension energy (E, cm^2) were determined. Dough resistance to extension represents the necessary force for stretching the dough to a certain length. The resistance to extension is a curve obtained after 50 mm of stretching the dough and is expressed in extensographic units (EU). The dough extensibility (Ext) refers to the length of stretched dough from the extension beginning to the moment of cracking, while the extension energy (E) refers to the amount of energy consumed by dough extension and is obtained by calculating the area below the formed curve (cm^2) on the extensograph.

2.4. Statistical Analysis

Statistical analyses and data visualizations were performed in Excel [48] and XLSTAT 2022.2.1.1304 [49]. For the comparison of mean values of all wheat flour samples from both locations (n = 50 for control and n = 50 for infected samples) and for the assessment of the impact of FHB infection and testing location, as well as their interaction on measured parameters, a two-way analysis of variance (two-way ANOVA) and the Tukey HSD as a post hoc test were used. Before testing, the Shapiro–Wilk test was used to check if the data followed normality. Levene's test was used to check the assumption of equal variances, and their homogeneity was graphically verified (q-q plot, residuals). When the premises were not rejected, variance analysis was performed. However, when the data were shown not to be normally distributed and followed different distributions, the Kruskal–Wallis test, the non-parametric version of ANOVA, with multiple pairwise comparisons using the Conover–Iman procedure, was used. Statistical analyses were performed with untransformed data, which is why the effects of higher-order interaction were limited because of the low number of replications available for every parameter. However, percentages were replaced with proportions. All tests were performed at the $p < 0.05$ level of significance.

The impact of location or FHB infection on a particular measured outcome was estimated as the effect size. To better observe the differences between the FHB-infected and control groups and then among individual cultivars, the difference between the mean value of the infected and the mean value of the control group for each tested location and/or cultivar was marked as the mean difference. Since the mean difference does not consider the standard deviation within the groups, the Hedges effect size [50,51], a quantitative measure of the strength of an effect based on the overall standard deviation, was calculated by standardizing the mean difference between two groups $(\overline{x_1} - \overline{x_2})$ by the pooled, weighted standard deviation $\left(SD_{pooled}\right)$ of the sampled population $(n_1 + n_2)$ and Ellis's unbiased form of effect size was used because this article focuses on data from small independent samples:

$$d = (\overline{x_1} - \overline{x_2})/SD_{pooled} \quad (2)$$

$$SD_{pooled} = \sqrt{((n_1 - 1)SD_1^2 + (n_2 - 1)SD_2^2)/(n_1 + n_2 - 2)} \quad (3)$$

$$corrected\ (Hedges\ d) \cong d[1 - (3/(4(n_1 + n_2) - 9)] \quad (4)$$

A higher effect size means more impact on the measured parameter. On the other hand, negative effect size values refer to the reduction of a particular parameter compared to the control group, and the higher its absolute value is, the more significant the impact is. The statistical significance (calculated by the Welch t-test or Steel test) of the differences between the infected and control groups in most parameters coincided with a high positive or negative effect of infection on the measured outcome. Estimates of effect size and its 95% confidence intervals were graphically presented by high-low-close stock graphs. This way, a better and more informative representation of the proven values was shown.

Statistical data processing also involved applying multivariate analysis to determine the structure of the obtained results. Principal component analysis (PCA) was used to reduce the data set's dimensionality while preserving the variables' characteristics that account for most of the variance. Linear combination factors of initial variables reveal potential causes of correlation of the obtained results in this manner. PCA was performed on normalized data to avoid the excessive influence of one variable on the main components and was based on a Spearman correlation matrix due to previously described distributions of extensograph data. Hierarchical agglomerative clustering was then performed on principal components by grouping with the Ward method of quadrated Euclidean distances so that the degree of similarity within the group is maximized while minimizing the similarity between the groups.

3. Results

3.1. Technological Quality of Wheat Samples

By examining the impact of the interaction of FHB infection and the wheat testing location on the protein content (PC), the proportion of wet gluten content (WG), and the sedimentation value (SV), a two-way ANOVA showed a significant effect of both testing location and FHB infection ($F_{3,96}$ = 21.9 for PC, 32.87 for WG, and 24.5 for SV, $p < 0.0001$). However, the model for PC, WG, and SV explained only 41, 51, and 43% of the dataset variability (Figure 1a,c,e). The most influential was the interaction of location and treatment. The testing location influenced the FHB infection's effect on PC, WG, and SV differently. For example, the analysis of differences in PC proportion between uninfected (control) and FHB-infected samples cultivated at Osijek revealed a statistically significant and relevant estimate of the FHB infection effect that caused an average increase in PC by 18%. In contrast, FHB infection did not affect the proportion of PC in wheat grains grown at Tovarnik (Figure 1a,b).

Similar to the protein content proportion, WG also increased in FHB-infected samples of wheat cultivars grown at Osijek. Like PC, both FHB-infected and uninfected flour samples of wheat cultivars grown at Tovarnik had significantly greater WG values than those produced at Osijek. But, contrary to Osijek, the FHB infection of wheat at Tovarnik showed no significant influence on the WG proportion (Figure 1c,d). The sedimentation value reacted differently from PC and WG (Figure 1e,f). On average, FHB infection did not cause significant changes in the SV of wheat cultivars grown at Osijek. However, the change in SV ranged from a 20% increase to a 17% decrease, depending on the cultivar. At the same time, in flour samples of wheat cultivars grown at Tovarnik, an average 24% decrease in SV was recorded. Furthermore, depending on the cultivar, the reduction in SV ranged from 0% to 40%. Thus, the most influential variable for SV was the FHB treatment, followed hierarchically by the FHB treatment's interaction with testing location. The analysis of differences between uninfected and infected samples confirmed both a statistically significant and relevant effect of FHB infection on the SV of flour samples from Tovarnik (Figure 1f).

The amylolytic activity was estimated as a Hagberg falling number (FN), which, as stated before, indirectly measures the amount of α-amylase presence, and the proteolytic activity was calculated as the activity of nonspecific proteases (PA). Neither location nor FHB infection influenced FN (two-way ANOVA, $F_{3,96}$ = 1.75, $p = 0.16$), as shown in Figure 2a,c. However, both FHB infection and wheat growing location significantly affected the proteolytic activity of flour samples, with 80% of the variability in the proteolytic activity explained by the two-way ANOVA model ($F_{3,96}$ = 134.9, $p < 0.0001$). FHB infection caused an increase in the activity of nonspecific proteases by an average of 43% in flour samples of wheat cultivars grown at Osijek. At the same time, in flour samples of wheat cultivars grown at Tovarnik, an average of a 125 % increase in protease activity was determined. The estimates of effect size confirmed twice as strong an effect of FHB infection on increasing protease activity of the flour samples from Tovarnik compared to Osijek. This disparity in the effect size and the mean differences between uninfected and FHB-infected samples was due to a large range of increased protease activity in both Tovarnik (from 81% to 235 % increase) and Osijek samples (from 3% to 139 % increase), as shown in Figure 2b,c.

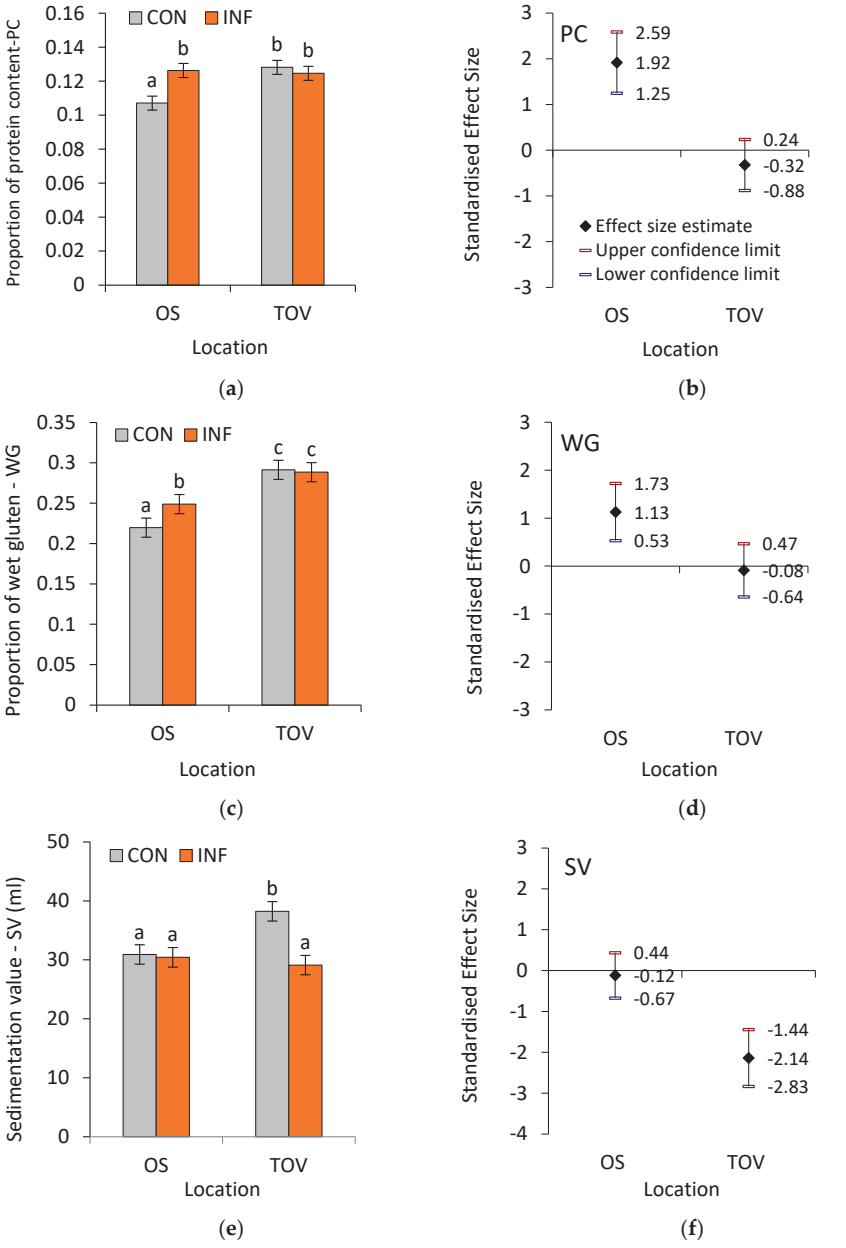


Figure 1. Average values and 95% confidence interval of (**a**) protein content (PC) proportion in wheat grains, (**c**) proportion of wet gluten content (WG), (**e**) sedimentation value (SV, mL) in control (uninfected, CON) (n = 25) and infected (INF) (n = 25) flour samples of winter wheat cultivars grown at Osijek (OS) and Tovarnik (TOV) locations. An estimate of standardized effect size with a 95% confidence interval of *Fusarium* infection on PC (**b**), WG (**d**), and SV (**f**) of winter wheat cultivars grown at OS and TOV. For all variables with the same letter, the difference in means is not statistically significant (at $p < 0.05$, Tukey HSD).

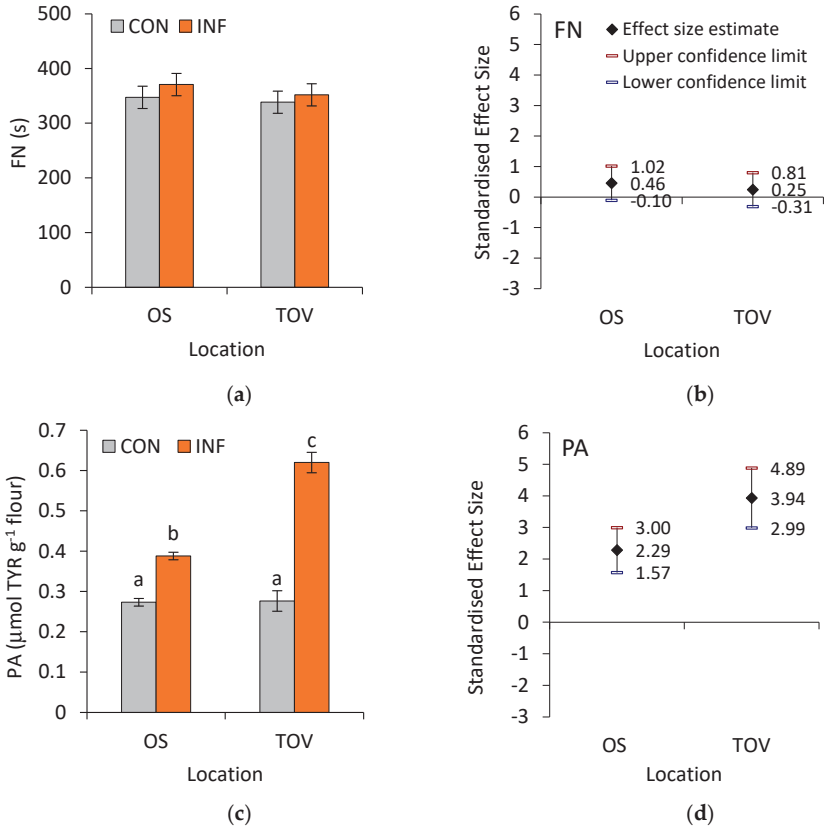
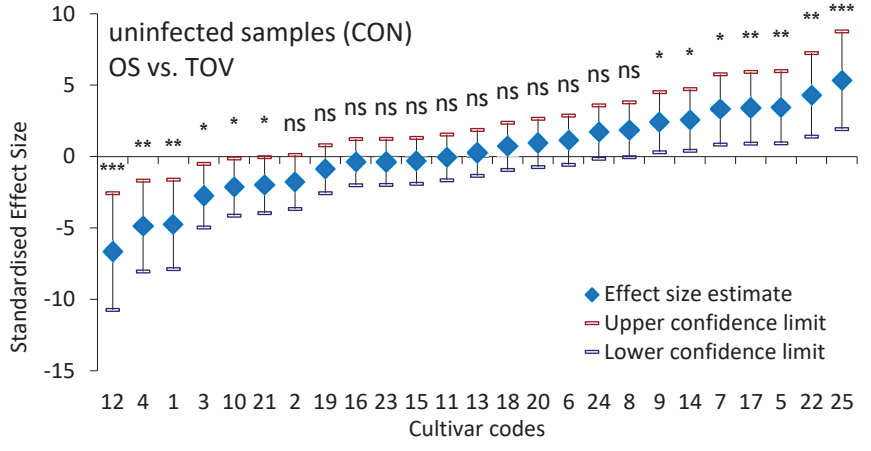


Figure 2. Average values and 95% confidence interval of (**a**) falling number (FN, s) and (**c**) proteolytic activity (PA, µmol TYR g^{-1} flour) in control (uninfected, CON) (n = 25) and infected (INF) (n = 25) flour samples of winter wheat cultivars grown at Osijek (OS) and Tovarnik (TOV) locations. An estimate of standardized effect size with a 95% confidence interval of *Fusarium* infection on FN (**b**) and PA (**d**) of winter wheat cultivars grown at Osijek (OS) and Tovarnik (TOV). For all variables with the same letter, the difference in means is not statistically significant (at $p < 0.05$, Tukey HSD).

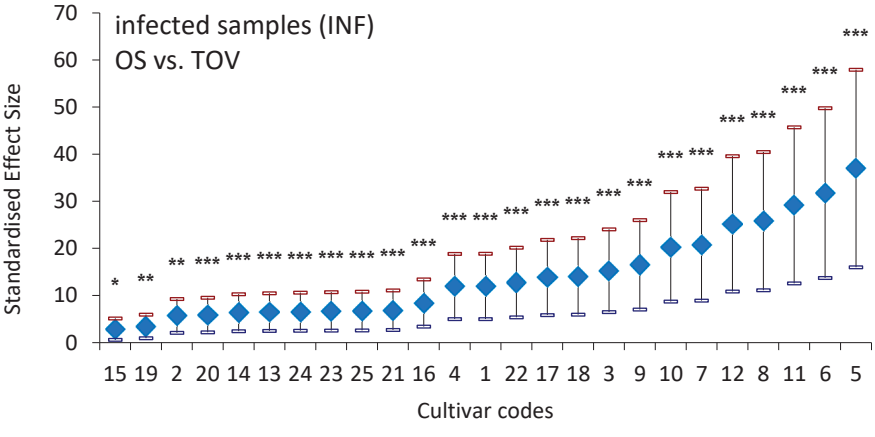
3.2. Proteolytic Activity of Wheat Samples

Although there were no differences in the activities of nonspecific proteases (PA) between uninfected wheat flour samples from Osijek and Tovarnik, we included genotypic variability in the model due to the high range of PA over cultivars. According to this new model (two-way ANOVA, $F_{49,100} = 15.43$, $p < 0.0001$, $R^2 = 88\%$), the most influential variable affecting differences in protease activity was actually genotype (based on Type III sum of squares SS = 0.052, F = 19.43, $p < 0.0001$), hierarchically followed by its interaction with the location (Type III SS = 0.032, F = 11.99, $p < 0.0001$). The analysis of differences between uninfected control samples of both locations identified three groups of samples (Figure 3a): the first group consisted of cultivars with significantly lower protease activity when grown at Tovarnik, compared to Osijek (codes 12, 4, 1, 3, 10, 21), a second group included cultivars with no significant differences in the activity of proteases no matter where they were grown at (codes 2, 19, 16, 23, 15, 11, 13, 18, 20, 6, 24 and 8), and a third group consisted of cultivars in which protease activity was significantly higher when they were grown at Tovarnik as opposed to Osijek (codes 9, 14, 7, 17, 5, 22, and 25). The same was done for infected samples. However, extracting groups of cultivars was not possible because the effect of the

infection was much higher in Tovarnik than in Osijek, and the effect size of the differences between cultivars ranged from 2.76 (cultivar 15) to a maximum of 25.8 (cultivar 5), all of which fall into the category of "very high effect" (Figure 3b). Considering these very large effects depend on the context and known sources of variability, some coherence exists as the relations between individual cultivars were mirrored in the infected samples, suggesting that genetics and environment influenced PA.



(**a**)



(**b**)

Figure 3. Estimates of the standardized effect size with a 95% confidence interval of the *Fusarium* infection impact on proteolytic activity (PA, µmol TYR g^{-1} flour) of 25 winter wheat cultivars grown at (**a**) OS—Osijek and (**b**) TOV—Tovarnik locations. Signification codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05, ns–non significant.

3.3. Rheological Quality of Wheat Samples

Further analysis included the farinograph and extensograph rheological properties of wheat flour samples from all cultivars grown at both locations. The ability of uninfected wheat flour to absorb water ranged from 50.5% (cultivar 20) to 61.3% (cultivar 1) at Osijek and from 52.2% (cultivar 20) to 62.7% (cultivar 1) at Tovarnik. The FHB infection did not influence the water absorption of tested wheat flour from either location (Table 2,

Figure 4). The same was determined for dough stability (DS). However, the range from minimal to maximal values of DS was relatively high (1.3 and 1.5 min in uninfected samples from Osijek and Tovarnik, 1.9 and 3.1 min in infected samples from Osijek and Tovarnik), suggesting an influence of cultivar variability.

Table 2. Average values and standard error of rheological properties obtained using Farinograph for uninfected (control, CON) and FHB-infected (INF) flour samples of winter wheat cultivars grown at Osijek (OS) and Tovarnik (TOV) locations: water absorption—WA (%); dough stability—DS (min); degree of dough softening—DoS (FU); Fischer F statistics, p values, and regression coefficients (R^2) of two-way variance analysis model (ANOVA) for differences between locations, treatments, and their interaction. The letters are used to group mean values according to the analysis of the differences between categories, where values indicated by the same letter do not differ significantly (Tukey-HSD test at the significance level of $p < 0.05$).

	Location OS		**Location TOV**	
	CON (n = 25)	**INF (n = 25)**	**CON (n = 25)**	**INF (n = 25)**
WA (%)	ANOVA ($F_{3, 96} = 3.02$, $p = 0.03$, $R^2 = 0.09$)			
	56.28 ± 0.49 ab	56.18 ± 0.47 a	57.91 ± 0.48 b	56.74 ± 0.37 ab
DS (min)	ANOVA ($F_{3, 96} = 0.69$, $p = 0.56$, $R^2 = 0.02$)			
	0.46 ± 0.07 a	0.46 ± 0.09 a	0.63 ± 0.09 a	0.57 ± 0.13 a
DoS (FU)	ANOVA ($F_{3, 96} = 46.99$, $p < 0.0001$, $R^2 = 0.59$)			
	83.88 ± 4.54 b	107.96 ± 5.42 c	56.08 ± 4.81 a	150.04 ± 7.51 d
FQN	ANOVA ($F_{3, 96} = 8.71$, $p < 0.0001$, $R^2 = 0.214$)			
	42.68 ± 6.8 a	42.92 ± 4.9 a	78.92 ± 8.4 b	41.40 ± 3.6 a

The degree of dough softening (DoS) behaved slightly differently than other rheological properties determined by farinograph. It differed significantly between cultivars, locations, and treatments (Table 2). The FHB infection caused a significant increase in DoS, on average, of more than 400% in Tovarnik samples, as opposed to Osijek, where the rise in DoS caused by the infection was around 40%. Therefore, FHB infection had the most significant impact on the variability of DoS, where the estimated effect size in samples from Tovarnik was actually three times, and not ten times, greater than the infection effect on samples from Osijek (Figure 4c). The extensive range in the DoS of uninfected samples at both Osijek (31 to 135 FU) and Tovarnik (3 to 98 FU) also suggested significant genotypic variability. The farinograph quality number (FQN), a combined value of dough development time, stability, and mixing tolerance index, expressing flour quality as a single value, showed that uninfected wheat flour samples from Tovarnik were more robust (stronger), having the highest average values and thus indicating better gluten quality. Furthermore, a significant decrease in FQN was determined in FHB-infected flour samples from Tovarnik, while in Osijek, FHB infection had no influence on the average values of the flour sample's FQN (Table 2).

Since indices of dough processing characteristics measured on the extensograph were not normally distributed and had different distributions in uninfected and FHB-infected samples at both locations, the non-parametric Kruskal–Wallis ANOVA was used to show differences among the medians of the tested parameters (location, treatment). The results showed significant differences among tested samples for energy—E in cm^2 (Kruskal–Wallis $\chi^2 = 42.9$, c = 7.815, $p < 0.0001$), resistance to extension—RtE in EU (Kruskal–Wallis $\chi^2 = 55.5$, c = 7.815, $p < 0.0001$) and extensibility—Ext in mm (Kruskal–Wallis $\chi^2 = 11.9$, c = 7.815, $p = 0.007$). Multiple pairwise comparisons using the Conover–Iman procedure

showed no difference in E, RtE, and Ext between control samples grown at Osijek and Tovarnik locations (Figure 5a,c,e). However, FHB infection resulted in a significant decrease in the extensograph rheological quality of wheat flour in terms of E, RtE, and Ext at both locations. The effect of location on the differences was substantial, and the effect size for the FHB infection was slightly larger at Tovarnik for E and RtE but not for Ext (Figure 5b,d,e).

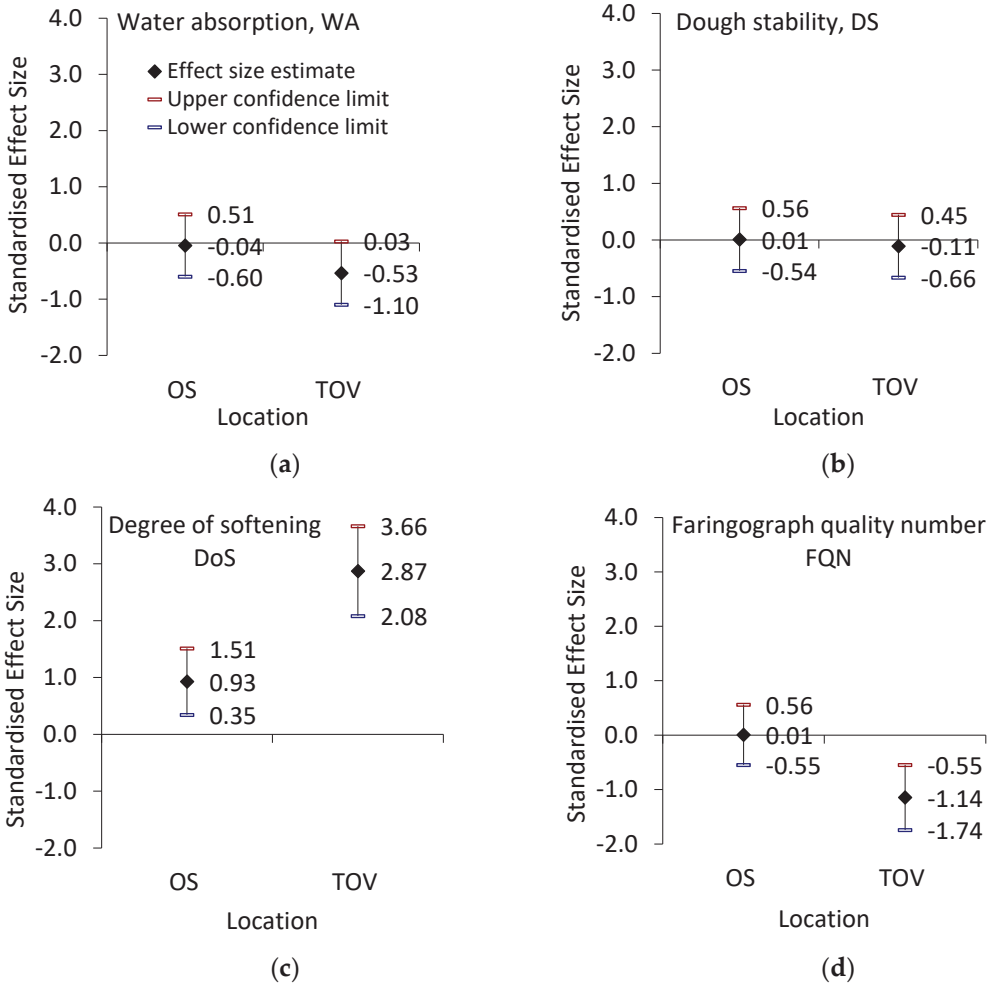


Figure 4. Estimates of standardized effect size with a 95% confidence interval of *Fusarium* infection impact on farinograph rheological properties: water absorption (**a**), dough stability (**b**), degree of dough softening (**c**), and farinograph quality number (**d**) of winter wheat cultivars grown at Osijek (OS) and Tovarnik (TOV) locations.

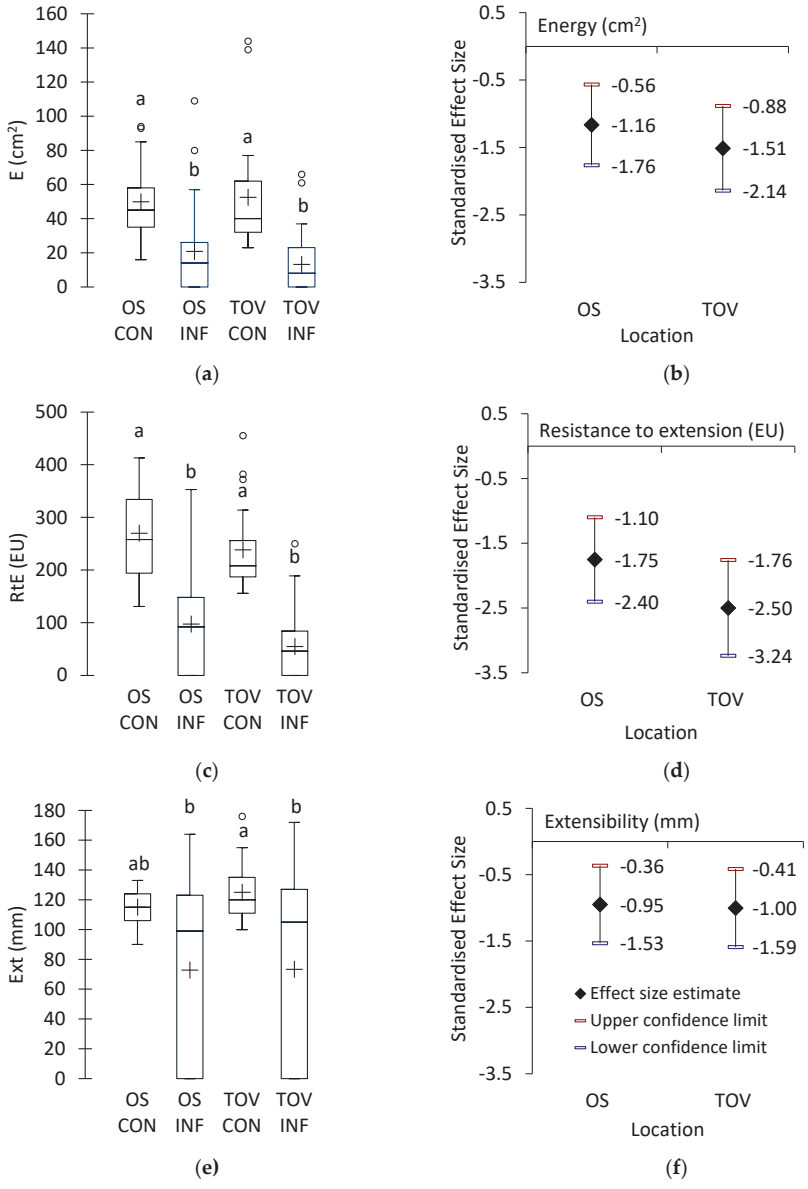


Figure 5. Boxplot plots of extensographic dough properties for investigated control (uninfected, CON) (n = 25) and infected (INF) (n = 25) winter wheat cultivars grown at different locations (OS, TOV): (**a**) energy (E, cm^2), (**c**) resistance to extension (RtE, EU) and (**e**) extensibility (Ext, mm), and estimates of standardized effect size with a 95% confidence interval of *Fusarium* infection impact on the same properties: E (**b**), RtE (**d**), and Ext (**f**). The box represents the middle 50% of observed values; the bottom of the box is the 25th percentile and the top of the box is the 75th percentile of the data; the line in the middle of the box is the median (50th percentile) and the plus sign (+) is the mean value; the whiskers extend to the lowest and greatest non-outliers' value, and circles are used to represent outliers. Boxplots marked with the same letter are not significantly different according to multiple pairwise comparisons using the Conover-Iman procedure/two-tailed test at a $p < 0.05$ significance level.

3.4. Multivariate Analysis

The existence of outliers and an extensive interquartile range for extensograph indices in infected samples from both locations also revealed a strong influence of genotype variability on the grains' and flour's technological quality due to FHB infection. Therefore, a multivariate data analysis based on projection methods was used to observe and uncover the relationships between observations and variables, as well as trends, clusters, and outliers in the data. First, we tested the factorability of 14 variables that measure the impact of *Fusarium* infection on wheat cultivars' technological quality and rheological properties. The Spearman correlation matrix revealed that all variables, except FN, were correlated by at least one other variable (Supplementary Material Table S1). Therefore, FN was removed from further analysis. The Keiser–Meyer–Olkin value of 0.743 confirmed sample adequacy, and Bartlett's test of sphericity confirmed variable suitability for structure detection, allowing factor analysis with the 13 remaining variables. Principal component analysis (PCA) without any rotations was used to group samples of winter wheat cultivars, visualize proximities, and condense the original variables into coherent factors. The first three factors had eigenvalues greater than one and explained 37.2%, 24.9%, and 9.5% of the variance in the dataset, respectively. However, the leveling of eigenvalues in the scree plot suggested a solution with two factors carrying 62.1% of the initial information (Supplementary Material Table S2 and Figure 1). Due to the complexity of variables loaded on both PCs with a loading factor greater than 0.4, we ran exploratory factor analysis with the varimax and oblimin solutions. A correlation of 0.13 between the two factors suggested choosing the varimax solution. Following rotation, PC1 was loaded with eight variables, each explaining grain and flour-specific properties (PC, SV, WG, PA) and farinograph rheological dough properties (WA, DS, DoS, FQN). Figure 6a shows six variables loaded on PC2 that explain the extensograph rheological dough properties (E, RtE, and Ext) and the level of initial and general resistance to *Fusarium* infection (AUDPC). Observations were then projected onto the main components of PCA, and three groups of samples were generated using agglomerative hierarchical clustering (AHC), as shown in Figure 6b,c. Figure 6c depicts a dendrogram obtained using Ward's method of minimizing variance, from which an acceptable solution with three clusters is visible: the C1 Cluster (green), which included some samples from Osijek (codes 1, 2, 4, 5, 9, 10, 12, 15, 17, 22 OS) and a few samples from Tovarnik (codes 2, 4, 12, 15 TOV); the C2 Cluster (red), which included the remaining samples from Osijek; and the C3 cluster (blue), which included the majority of samples from Tovarnik.

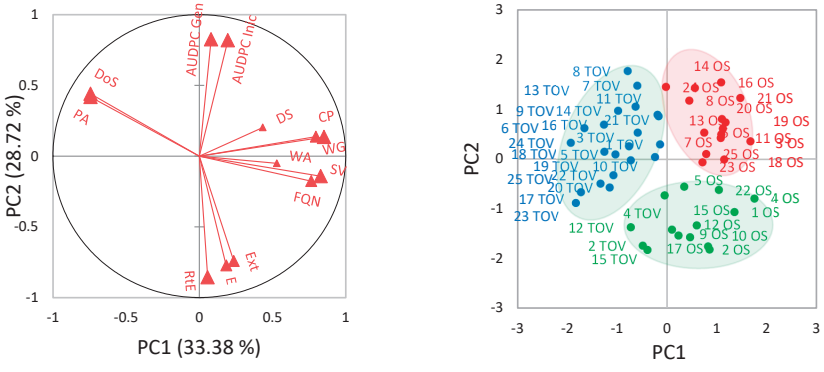
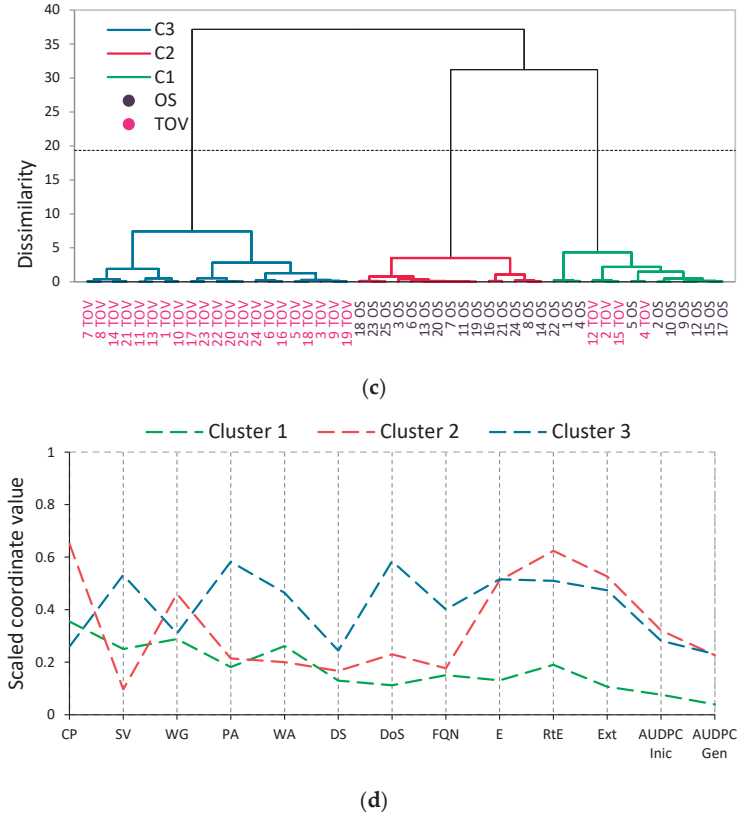


Figure 6. *Cont.*



(**c**)

(**d**)

Figure 6. Principal component analysis of measured quality variables derived as the mean difference between infected and control (uninfected) samples of 25 winter wheat cultivars cultivated at two locations (Osijek-OS and Tovarnik-TOV). (**a**) Correlations between measured variables and the two principal components; (**b**) Projections of cultivars on the PCA coordinate plot; "green points" represent Cluster 1 (C1), "red points" represent Cluster 2 (C2), "blue points" represent Cluster 3 (C3) displayed and sorted by (**c**) Agglomerative hierarchical cluster analysis; (**d**) Parallel coordinate plot of differences representing mean rescaled value (0,1) of distances for validating each cluster. (CP—crude protein, SV—sedimentation value, WG—wet gluten, PA—proteolytic activity, WA—water absorption, DS—dough stability, DoS—degree of dough softening, FQN—farinograph quality number, E—energy, RtE—resistance to extension, Ext—extensibility, AUDPC Inic—initial and AUDPC Gen—general resistance).

Further analysis included a parallel coordinate plot of differences with mean rescaled distance values from 0 to 1 to validate and describe each cluster (Figure 6d). As can be seen, a sharp increase in the distance measure value indicates a lower association between the clusters. The results suggested that cultivars of the first cluster (green) show the slightest changes in grain and flour technological and specific properties due to FHB infection. They also had the highest resistance to FHB infection (low AUDPC values) and the smallest changes in dough properties. Cluster 2 (red) cultivars grown in Osijek showed the greatest changes in protein and wet gluten content, as well as in extensograph dough processing characteristics, but the smallest changes in protein quality, protease activity, and farinograph rheological properties due to FHB infection. Finally, the third cluster (blue), which includes the majority of cultivars grown at Tovarnik, exhibits low changes in protein content, wet gluten content, and dough stability but significant changes

in protein quality, protease activity, and farinograph dough properties as a result of FHB infection. Both red and blue clusters mainly showed lower resistance to FHB infection, but there was no significant difference between them in AUDPC values for initial and general resistance values.

4. Discussion

In plant breeding, multi-environment trials identify superior genotypes with desirable traits. This task, however, is difficult due to the prevalence of genotype-environment interaction (GEI) [52]. Understanding how genotype, environment, and GEI affect wheat grain quality is crucial because it reduces the need for quality-based selection [53]. The significance of assessment, quantification, and the degree to which elements like the environment and GEI are accountable for phenotypic variation in different quality parameters have been reported previously [54]. We used two treatments on the different types of wheat we tested at both of our experimental locations. The first treatment consisted of naturally grown wheat without the use of fungicides, while the second treatment utilized wheat varieties that had been subjected to intense FHB. The purpose of the experiment was to accurately select wheat varieties with superior traits by evaluating and quantifying the effects of genotype, environment, and GEI on wheat grain and flour quality. Predictably, the results demonstrated that the response of 25 winter wheat cultivars to FHB infection was significantly influenced by genotype variability and by the location of their growth (Osijek vs. Tovarnik).

4.1. Influence of Fusarium Infection and Nonspecific Proteolytic Activity on Technological Quality of Wheat Cultivars Grown at Two Locations

Protein and wet gluten content in wheat grain and flour of uninfected (control) cultivars were considerably influenced by testing location, with greater values in grains grown in Tovarnik. On the one hand, this may result from climatic conditions, such as temperature and precipitation during the grain-filling period. On the other hand, it can be a consequence of soil type and quality. Additionally, it is acknowledged that grains' protein content corresponds strongly with flour's protein content and quality [55,56]. Despite the fact that Tovarnik received slightly more rain than Osijek, particularly during the critical grain-filling stage [29], and that standard agro-technical measures were applied at both locations, we presume that the higher protein content in the control samples from Tovarnik is more likely due to the type and quality of the soil. The soil at Tovarnik is a humus-rich, dark Chernozem high in phosphorus and ammonium compounds. In comparison, the soil in Osijek is Eutric Cambisol, a slightly acidic to neutral soil with lower humus and phosphorus concentrations and a moderately higher clay content [29]. This implies that Tovarnik soil was able to provide more nutrients required for wheat growth, resulting in higher protein content. Furthermore, multiple authors [57–62] stated that nitrogen-rich soil increases protein content and influences protein composition in wheat grains. It has also been suggested that environmental factors, rather than inherited traits, have a greater impact on protein content [63].

Another quality trait reported to be highly influenced by genotype, environment, and GEI is wet gluten content [64]. Wheat flour samples from these two locations showed an intriguing correlation between FHB infection and the amount of protein and wet gluten content. Among all cultivars, wheat samples from Osijek had higher protein and gluten concentrations after being infected with FHB. Infected wheat grown in Tovarnik didn't appear to lose any protein or the proportion of wet gluten. However, sedimentation values decreased in the Tovarnik samples while remaining unchanged in the Osijek samples. Previous research primarily indicated a decrease in the quality of *Fusarium* spp.-infected wheat flour, particularly in terms of protein content and total glutenin, a composition of gluten [24,31,65]. Furthermore, it has been shown that fungal protease enzymes secreted at a later stage of infection break down gluten, resulting in a deterioration of wheat flour quality and its industrial properties [66]. Protease activity was observed to be more stable when

$scCO_2$, which is utilized in the food technology industry, was introduced as compared to other enzymes [67]. Moreover, the intensity of infection influences inter-genotypic variability in specific quality measures of flour [68–70]. For instance, Ortega et al. [71] discovered that the greater severity and virulence of infection resulted in a more significant decrease in protein content. This is consistent with Wang et al. [33], who discovered that a more potent infection with *F. culmorum* resulted in a more significant glutenin reduction. Another example of the relationship between disease and protein content was explained by Eggert et al. [72], who investigated the effects of naturally occurring and artificially generated diseases and found that artificial infection leads to significantly lower protein values.

On the other hand, the not-so-strong influence of infection was determined in the work of Gärtner et al. [23]. Fungal proteins can contribute to the overall protein content, according to Boyacioğlu and Hettiarachchy [65]. However, Eggert et al. [72] found that only 0.3% of the total protein in infected wheat was of fungal origin. We suggest that the average increase in protein content and lack of change in protein quality found in Osijek samples may be due to the activation of defense-related proteins by establishing systematic resistance in plants. This is further supported by the higher frequency of wheat samples from Osijek having lower AUDPC values during the reproductive period, which is consistent with our findings that more cultivars displayed better FHB resistance in Osijek than in Tovarnik. Moreover, the increase in protein content in infected samples may be attributable to a shift in the starch-to-protein ratio in favor of protein content [73] due to the fungal consumption of carbohydrates [33]. Even if the total amount of protein remained relatively consistent in most cultivars grown at Tovarnik, the data demonstrated that the protein quality was altered by the FHB infection. As previously indicated, increased precipitation and moisture during wheat growth could have made cultivars susceptible to FHB disease. Therefore, a slightly higher infection intensity caused an enzymatic breakdown of proteins by pathogens, which can explain the decline in wheat's protein quality (due to increased swelling) and is consistent with the findings of Gärtner et al. [23]. This suggests that FHB infection during grain-filling reduces the availability of high-quality proteins in the grain, lowering protein quality. Protein content was shown to be somewhat affected by protease activity in severely infected wheat samples, which may have implications for dough qualities, according to another study [74]. However, because FHB infection does not always influence grain starch and protein content equally [25], the rationale may lie in the early buildup of stored proteins during the grain-filling stage.

4.2. Influence of Fusarium Infection and Nonspecific Proteolytic Activity on Rheological Quality of Wheat Cultivars Grown at Two Locations

Functional properties of dough are usually evaluated with mixograph, alveograph, and farinograph analysis [75]. The current research used a farinograph to determine water absorption, stability, and the quality number of the dough, as well as the degree of dough softening. Among the tested wheat flour samples in this study, there are no statistically significant differences in water absorption or stability of the dough, although a significant difference exists for the degree of dough softening due to FHB infection. The ability to absorb water is one of the most important indications of flour quality since it directly influences the quality and yield of the final product, and it is essential in order to establish flour strength and estimate bakery product prices [76]. Martin et al. [77] observed that infection and changes in environmental conditions had a minor impact on water absorption but a significant effect on the dough stability and the degree of softening. Regarding the water absorption capacity, Okuda et al. [78] note that the average values for the water absorption capacity of wheat flour vary from 50 to 70%, similar to the results found in this research. Some studies have demonstrated a slight increase in water absorption with an increase in the severity of *Fusarium* spp. infection [33] due to a larger fraction of damaged starch granules in infected wheat. Protein-rich cultivars have been found to have a greater

capacity to absorb water [79]. However, this investigation confirms our prior findings that protein-rich cultivars show decreased water absorption following FHB infection [29].

The FHB infection showed the most significant influence on the degree of dough softening variability, where the estimated effect of the infection on the samples from Tovarnik was three times larger than the infection effect on the Osijek wheat samples. It was discovered that FHB infection enhanced the degree of dough softening for all wheat cultivars, which is in line with some previous research [77]. Because it interferes with grain maturation and the digestion of starch and protein in food, FHB disease lowers the quality of wheat end products [80]. The degree of softening and the stability of the dough reflect the highly elastic properties of the dough that gluten is responsible for, and a low degree of softening indicates that the gluten proteins were intact [81]. In fact, the good dough stability and lesser degree of softening imply that such dough is also suited for more extensive mechanical processing. This investigation showed a substantial positive association between the activity of proteases and the degree of softening of the dough, which is in line with previous research [76] that suggested that dough softening might be used as an indicator of proteolytic degradation of proteins. Furthermore, exogenous proteases originating from *Fusarium* spp. can remain dormant in stored grains but might be reactivated during dough preparation, thus influencing dough characteristics and the baking process [35]. As for the extensograph values, it was determined that only FHB infection significantly affected changes in the resistance to stretching, extensibility, and total stretching energy. Significantly lower values were discovered in samples of infected flour from both locations, with the effect of infection being slightly more pronounced in samples from the Tovarnik location.

The presence of outliers and an extensive interquartile range for extensograph indices in infected samples from both locations also revealed a strong influence of genotype variability on the grains' and flour's technological quality. Due to the inability to replicate technological and rheological properties, it was not possible to confirm the significance of the influence of genotype on these characteristics; therefore, the strength of the effect of genotype as a variable was determined for the activity of nonspecific proteases, considering the influence of location and intensity of FHB infection. Even though no significant differences were determined in the average activity of specific proteases between control wheat flour samples from Osijek and Tovarnik, the difference analysis indicated that some cultivars displayed consistent protease activity across sites. In contrast, others showed variability in their response due to environmental conditions. Therefore, the inclusion of genotypic variability in the model revealed that genotype significantly influences differences in protease activity.

4.3. Multivariate Analysis of Quality Parameters among 25 Winter Wheat Varieties at Two Locations under Two Treatments

A multivariate data analysis based on projection methods uncovered the relationships between observations and variables. The results showed that dough formation depends on several factors, including the initial and general resistance to *Fusarium* infection. Nightingale et al. [31] attributed the loss of dough functionality and volume to exogenous fungal proteases. Because *Fusarium* proteases remain active throughout all stages of dough processing, longer resting processes result in a greater loss of dough strength and bread shape [26]. Further analysis showed that some of the differences between cultivars were due to genetic factors and some to environmental ones. For example, the cultivars in the first cluster (green) grown at both locations provided evidence of a high degree of resistance to FHB infection. However, although cultivars from clusters 2 and 3 had no significant differences in resistance to FHB, they showed distinct responses to the infection, influencing protein quality, protease activity, and dough properties. Thus, it appears that the different geographical regions in which these cultivars were grown affected the wheat response to FHB infection. According to Scala et al. [82], soil management practices and local (micro)environmental conditions may be held responsible for the FHB outbreak.

Therefore, it is suggested that the quality of grain and flour products is determined by a combination of genotype, environment, and resistance to FHB. Previous research has shown that environmental conditions, including humidity and temperature, as well as cultivar susceptibility and cultivation methods, all play a role in the likelihood of a crop being infected by *Fusarium* spp. [2,83]. Surma et al. [84] performed a multivariate analysis and found that genotype and treatment significantly influenced all measured traits. It should be emphasized that most investigations relied on artificial inoculation to acquire *Fusarium*-infected wheat and that it is anticipated that these samples will produce very high mycotoxin levels. However, specific resistant genotypes may still have some defense against FHB. Thus, we continue to study these strains. Together, these three factors highlight the critical nature of preventing *Fusarium* infection in the wheat crop and flour milling industry.

However, we must consider the possibility that genetic and environmental factors were influenced by confounding factors, such as the variability of the types used in the tests. Here, multivariate analysis showed that FHB had minimal effect on both technological and rheological variables in the first cluster of wheat cultivars, but this cluster contained only a small number of cultivars displaying low susceptibility to FHB. As a result, we demonstrated that genetic resistance could provide adequate control of FHB, guaranteeing the successful protection of wheat quality, similar to the results of Wegulo et al. [13].

5. Conclusions

It can be concluded that genotype variability strongly influenced the technological quality and rheological properties of wheat cultivars infected with Fusarium head blight (FHB), while testing location had a significant effect on protein content and quality. Three distinct clusters of samples were generated, each exhibiting unique changes in their properties due to FHB infection and displaying varying levels of resistance to the disease. Genotype was the most influential variable affecting differences in protease activity, followed by its interaction with the location. Therefore, we can conclude that conditions at testing locations substantially determine the direction of the FHB infection effect, whether it is an increase, a decline, or an impact without a quantifiable influence. Further research is needed to investigate the specific mechanisms behind the interaction of environmental (location conditions) and genetic factors in determining particular cultivar responses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030662/s1. Table S1. Spearman correlation matrix of technical and rheological properties for uninfected and infected winter wheat cultivars (n = 50) grown at two locations (OS and TOV). The display option used is blue-red and presents a negative correlation with cold colors (blue for correlations close to -1) and positive correlations with warm colors (red for correlations close to 1). Values in bold differ from 0 with a significance level $\alpha = 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Table S2. Eigenvalues and the proportion of explained variation by the principal components. Figure S1. The scree plot for the eigenvalues of factors arranged in descending order of magnitude with explained cumulative variability. Table S3. Correlations between variables and factors in principal component analysis before and after varimax rotation. Table S4. PCA of genotypes characteristics factor scores and contribution to the main components after Varimax rotation. Values in bold correspond for each observation to the component for which the squared cosine is the largest (data not shown) to avoid interpretation errors due to projection effects (for example, when the squared cosines associated with the axes used on a chart are low, the position of the observation in question cannot be interpreted). Yellow to green color scale is used to visualize contribution (green high, yellow low). Figure S2. Violin plot of initial and general resistance to *Fusarium* infection (AUDPC-In and AUDPC-Gen) of tested winter wheat cultivars grown at two distinct locations (OS—Osijek and TOV—Tovarnik). Box plots represent the interquartile range with a mean (+) and median ($-$), whiskers show 1.5 $\times$ IQR, and dots outliers, and the shape of the violin display frequencies of values. The broader distribution illustrates a higher frequency of data points at those values.

Author Contributions: Conceptualization, V.Š. and V.P.; methodology, V.Š., J.B., I.V. and V.P.; software, V.Š. and V.P.; validation, V.Š. and V.P.; formal analysis I.B.; investigation, V.Š., I.B., J.B., I.V. and V.P.; resources, V.Š.; data curation, V.Š. and V.P.; writing—original draft preparation, V.P.; writing—review and editing, V.Š. and V.P.; visualization, V.P.; supervision, V.Š.; project administration, V.Š.; funding acquisition, V.Š. All authors have read and agreed to the published version of the manuscript.

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References

1. Leslie, J.F.; Moretti, A.; Mesterházy, Á.; Ameye, M.; Audenaert, K.; Singh, P.K.; Richard-Forget, F.; Chulze, S.N.; Ponte, E.M.; del Chala, A.; et al. Key Global Actions for Mycotoxin Management in Wheat and Other Small Grains. *Toxins* **2021**, *13*, 725. [CrossRef]
2. Alconada, T.M.; Moure, M.C.; Ortega, L.M. Fusarium Infection in Wheat, Aggressiveness and Changes in Grain Quality: A Review. *Vegetos* **2019**, *32*, 441–449. [CrossRef]
3. Bellesi, F.J.; Arata, A.F.; Martínez, M.; Arrigoni, A.C.; Stenglein, S.A.; Dinolfo, M.I. Degradation of Gluten Proteins by *Fusarium* Species and Their Impact on the Grain Quality of Bread Wheat. *J. Stored Prod. Res.* **2019**, *83*, 1–8. [CrossRef]
4. O'Donnell, K.; Kistler, H.C.; Tacke, B.K.; Casper, H.H. Gene Genealogies Reveal Global Phylogeographic Structure and Reproductive Isolation among Lineages of *Fusarium graminearum*, the Fungus Causing Wheat Scab. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7905–7910. [CrossRef]
5. Goswami, R.S.; Kistler, H.C. Heading for Disaster: *Fusarium Graminearum* on Cereal Crops. *Mol. Plant Pathol.* **2004**, *5*, 515–525. [CrossRef] [PubMed]
6. Xu, X.-M.; Nicholson, P.; Thomsett, M.A.; Simpson, D.; Cooke, B.M.; Doohan, F.M.; Brennan, J.; Monaghan, S.; Moretti, A.; Mule, G.; et al. Relationship between the Fungal Complex Causing Fusarium Head Blight of Wheat and Environmental Conditions. *Phytopathology* **2008**, *98*, 69–78. [CrossRef]
7. Marček, T.; Vuletić, M.V.; Bakula, I.; Alivojvodić, S.; Španić, V. Time-Course Experiment of *Fusarium* Infestation of Wheat Genotypes with the Emphasis on the Physiological Response. *Croat. J. Food Sci. Technol.* **2018**, *10*, 58–63. [CrossRef]
8. Španić, V.; Zdunić, Z.; Drezner, G.; Vuletić, M. Differences in Physiological Traits at the Initial Stage of Fusarium Head Blight Infection in Wheat. *Biol. Plant* **2020**, *64*, 174–181. [CrossRef]
9. Martínez, M.; Biganzoli, F.; Arata, A.; Dinolfo, M.I.; Rojas, D.; Cristos, D.; Stenglein, S. Warm Nights Increase Fusarium Head Blight Negative Impact on Barley and Wheat Grains. *Agric. For. Meteorol.* **2022**, *318*, 108909. [CrossRef]
10. Siou, D.; Gélisse, S.; Laval, V.; Repinçay, C.; Canalès, R.; Suffert, F.; Lannou, C. Effect of Wheat Spike Infection Timing on Fusarium Head Blight Development and Mycotoxin Accumulation. *Plant Pathol.* **2014**, *63*, 390–399. [CrossRef]
11. del Ponte, E.M.; Fernandes, J.M.C.; Bergstrom, G.C. Influence of Growth Stage on Fusarium Head Blight and Deoxynivalenol Production in Wheat. *J. Phytopathol.* **2007**, *155*, 577–581. [CrossRef]
12. Schaafsma, A.W.; Tamburic-Ilincic, L.; Hooker, D.C. Effect of Previous Crop, Tillage, Field Size, Adjacent Crop, and Sampling Direction on Airborne Propagules of *Gibberella Zeae/Fusarium Graminearum*, Fusarium Head Blight Severity, and Deoxynivalenol Accumulation in Winter Wheat. *Can. J. Plant Pathol.* **2005**, *27*, 217–224. [CrossRef]
13. Wegulo, S.N.; Baenziger, P.S.; Hernandez Nopsa, J.; Bockus, W.W.; Hallen-Adams, H. Management of Fusarium Head Blight of Wheat and Barley. *Crop Protection* **2015**, *73*, 100–107. [CrossRef]
14. Champeil, A.; Fourbet, J.F.; Doré, T.; Rossignol, L. Influence of Cropping System on Fusarium Head Blight and Mycotoxin Levels in Winter Wheat. *Crop Protection* **2004**, *23*, 531–537. [CrossRef]
15. Kollers, S.; Rodemann, B.; Ling, J.; Korzun, V.; Ebmeyer, E.; Argillier, O.; Hinze, M.; Plieske, J.; Kulosa, D.; Ganal, M.W.; et al. Whole Genome Association Mapping of Fusarium Head Blight Resistance in European Winter Wheat (*Triticum Aestivum* L.). *PLoS ONE* **2013**, *8*, e0057500. [CrossRef]
16. Zhu, Z.; Hao, Y.; Mergoum, M.; Bai, G.; Humphreys, G.; Cloutier, S.; Xia, X.; He, Z. Breeding Wheat for Resistance to Fusarium Head Blight in the Global North: China, USA, and Canada. *Crop J.* **2019**, *7*, 730–738. [CrossRef]
17. Wu, F.; Zhou, Y.; Shen, Y.; Sun, Z.; Li, L.; Li, T. Linking Multi-Omics to Wheat Resistance Types to Fusarium Head Blight to Reveal the Underlying Mechanisms. *Int. J. Mol. Sci.* **2022**, *23*, 2280. [CrossRef]
18. Schroeder, H.W.; Christensen, J.J.; Christensen, J.D.; Platz-Christensen, J.; Schroeder, H.W. Factors Affecting Resistance of Wheat to Scab Caused by *Gibberella zeae*. *Phytopathology* **1963**, *53*, 831–838.
19. Steiner, B.; Lemmens, M.; Griesser, M.; Scholz, U.; Schondelmaier, J.; Buerstmayr, H. Molecular Mapping of Resistance to Fusarium Head Blight in the Spring Wheat Cultivar Frontana. *Theor. Appl. Genet.* **2004**, *109*, 215–224. [CrossRef]

20. Buerstmayr, M.; Wagner, C.; Nosenko, T.; Omony, J.; Steiner, B.; Nussbaumer, T.; Mayer, K.F.X.; Buerstmayr, H. Fusarium Head Blight Resistance in European Winter Wheat: Insights from Genome-Wide Transcriptome Analysis. *BMC Genom.* **2021**, *22*, 470. [CrossRef]
21. Drakopoulos, D.; Kägi, A.; Six, J.; Zorn, A.; Wettstein, F.E.; Bucheli, T.D.; Forrer, H.-R.; Vogelgsang, S. The Agronomic and Economic Viability of Innovative Cropping Systems to Reduce Fusarium Head Blight and Related Mycotoxins in Wheat. *Agric. Syst.* **2021**, *192*, 103198. [CrossRef]
22. Magan, N.; Aldred, D. Post-Harvest Control Strategies: Minimizing Mycotoxins in the Food Chain. *Int. J. Food Microbiol.* **2007**, *119*, 131–139. [CrossRef] [PubMed]
23. Gärtner, B.H.; Munich, M.; Kleijer, G.; Mascher, F. Characterisation of Kernel Resistance against Fusarium Infection in Spring Wheat by Baking Quality and Mycotoxin Assessments. *Eur. J. Plant Pathol.* **2007**, *120*, 61–68. [CrossRef]
24. Kreuzberger, M.; Limsuwan, S.; Eggert, K.; Karlovsky, P.; Pawelzik, E.; Eggert, M. Impact of *Fusarium* Spp. Infection of Bread Wheat (*Triticum Aestivum* L.) on Composition and Quality of Flour in Association with EU Maximum Level for Deoxynivalenol. *J. Appl. Bot. Food Qual.* **2015**, *88*, 177–185. [CrossRef]
25. Matthäus, K.; Dänicke, S.; Vahjen, W.; Simon, O.; Wang, J.; Valenta, H.; Meyer, K.; Strumpf, A.; Ziesenib, H.; Flachowsky, G. Progression of Mycotoxin and Nutrient Concentrations in Wheat after Inoculation with *Fusarium Culmorum*. *Arch. Anim. Nutr.* **2004**, *58*, 19–35. [CrossRef] [PubMed]
26. Bacala, R.; Fu, B.X.; Cordova, K.; Hatcher, D.W. Wheat Fusarium Protease Specificity and Effect on Dough Properties. *Foods* **2021**, *10*, 1585. [CrossRef]
27. Horvat, D.; Španić, V.; Dvojković, K.; Šimić, G.; Magdić, D.; Nevistić, A. The Influence of *Fusarium* Infection on Wheat (*Triticum Aestivum* L.) Proteins Distribution and Baking Quality. *Cereal Res. Commun.* **2015**, *43*, 61–71. [CrossRef]
28. Berghofer, L.K.; Hocking, A.D.; Miskelly, D.; Jansson, E. Microbiology of Wheat and Flour Milling in Australia. *Int. J. Food Microbiol.* **2003**, *85*, 137–149. [CrossRef]
29. Španić, V.; Dvojković, K.; Babić, J.; Drezner, G.; Zdunić, Z. Fusarium Head Blight Infestation in Relation to Winter Wheat End-Use Quality—A Three-Year Study. *Agronomy* **2021**, *11*, 1648. [CrossRef]
30. Diaz-Mendoza, M.; Diaz, I.; Martinez, M. Insights on the Proteases Involved in Barley and Wheat Grain Germination. *Int. J. Mol. Sci.* **2019**, *20*, 2087. [CrossRef]
31. Nightingale, M.J.; Marchylo, B.A.; Clear, R.M.; Dexter, J.E.; Preston, K.R. Fusarium Head Blight: Effect of Fungal Proteases on Wheat Storage Proteins. *Cereal Chem. J.* **1999**, *76*, 150–158. [CrossRef]
32. Eggert, K.; Rawel, H.M.; Pawelzik, E. In Vitro Degradation of Wheat Gluten Fractions by *Fusarium Graminearum* Proteases. *Eur. Food Res. Technol.* **2011**, *233*, 697–705. [CrossRef]
33. Wang, J.; Wieser, H.; Pawelzik, E.; Weinert, J.; Keutgen, A.J.; Wolf, G.A. Impact of the Fungal Protease Produced by *Fusarium Culmorum* on the Protein Quality and Breadmaking Properties of Winter Wheat. *Eur. Food Res. Technol.* **2005**, *220*, 552–559. [CrossRef]
34. van Oort, M. Enzymes in Bread Making. In *Enzymes in Food Technology*; Wiley-Blackwell: Oxford, UK, 2009; pp. 103–143.
35. Koga, S.; Rieder, A.; Ballance, S.; Uhlen, A.K.; Veiseth-Kent, E. Gluten-Degrading Proteases in Wheat Infected by *Fusarium Graminearum*—Protease Identification and Effects on Gluten and Dough Properties. *J. Agric. Food Chem.* **2019**, *67*, 11025–11034. [CrossRef] [PubMed]
36. Pekkarinen, A.I.; Jones, B.L. Purification and Identification of Barley (*Hordeum Vulgare* L.) Proteins That Inhibit the Alkaline Serine Proteinases of *Fusarium Culmorum*. *J. Agric. Food Chem.* **2003**, *51*, 1710–1717. [CrossRef] [PubMed]
37. Xu, L.; Wang, H.; Zhang, C.; Wang, J.; Chen, A.; Chen, Y.; Ma, Z. System-Wide Characterization of Subtilases Reveals That Subtilisin-like Protease FgPrb1 of *Fusarium Graminearum* Regulates Fungal Development and Virulence. *Fungal Genet. Biol.* **2020**, *144*, 103449. [CrossRef]
38. Elagamey, E.; Abdellatef, M.A.E.; Arafat, M.Y. Proteomic Insights of Chitosan Mediated Inhibition of *Fusarium Oxysporum* f. Sp. Cucumerinum. *J. Proteomics* **2022**, *260*, 104560. [CrossRef]
39. Pekkarinen, A.I.; Longstaff, C.; Jones, B.L. Kinetics of the Inhibition of *Fusarium* Serine Proteinases by Barley (*Hordeum Vulgare* L.) Inhibitors. *J. Agric. Food Chem.* **2007**, *55*, 2736–2742. [CrossRef]
40. Snijders, C.H.A.; van Eeuwijk, F.A. Genotype x Strain Interactions for Resistance to Fusarium Head Blight Caused by *Fusarium Culmorum* in Winter Wheat. *Theor. Appl. Genet.* **1991**, *81*, 239–244. [CrossRef]
41. Zadoks, J.C.; Chang, T.T.; Konzak, C.F. A Decimal Code for the Growth Stages of Cereals. *Weed Res.* **1974**, *14*, 415–421. [CrossRef]
42. *ICC Standard No. 155*; Determination of Wet Gluten Quantity and Quality (Gluten Index Ac. to Perten) of Whole Wheat Meal and Wheat Flour (Triticum Aestivum). International Association for Cereal Science and Technology: Vienna, Austria, 1994.
43. *HRN EN ISO 5529*; Wheat—Determination of the Sedimentation Index—Zeleny Test (ISO 5529:2007). ISO: Geneva, Switzerland, 2010.
44. *HR EN ISO 3093*; Wheat, Rye and Their Flours, Durum Wheat and Durum Wheat Semolina—Determination of the Falling Number According to Hagberg-Perten. ISO: Geneva, Switzerland, 2009.
45. Cupp-Enyard, C. Sigma's Nonspecific Protease Activity Assay—Casein as a Substrate. *J. Vis. Exp.* **2008**, *17*, e899. [CrossRef]
46. *HRN EN ISO 5530-1*; Wheat Flour—Physical Characteristics of Doughs—Part 1: Determination of Water Absorption and Rheological Properties Using a Farinograph. ISO: Geneva, Switzerland, 2015.

47. *HRN EN ISO 5530-2*; Wheat Flour—Physical Characteristics of Doughs—Part 2: Determination of Rheological Properties Using an Extensograph. ISO: Geneva, Switzerland, 2015.
48. Microsoft Corporation Microsoft Excel 2019. Available online: https://office.microsoft.com/excel (accessed on 17 January 2023).
49. Addinsoft XLSTAT Statistical and Data Analysis Solution 2022. Available online: https://www.xlstat.com (accessed on 17 January 2023).
50. Hedges, L.V.; Olkin, I. Estimation of a Single Effect Size: Parametric and Nonparametric Methods. In *Statistical Methods for Meta-Analysis*; Elsevier: Amsterdam, The Netherlands, 1985; pp. 75–106.
51. Ellis, P.D. *The Essential Guide to Effect Sizes: Statistical Power, Meta-Analysis, and the Interpretation of Research Results*; Cambridge University Press: Cambridge, UK, 2010.
52. Eltaher, S.; Baenziger, P.S.; Belamkar, V.; Emara, H.A.; Nower, A.A.; Salem, K.F.M.; Alqudah, A.M.; Sallam, A. GWAS Revealed Effect of Genotype × Environment Interactions for Grain Yield of Nebraska Winter Wheat. *BMC Genom.* **2021**, *22*, 2. [CrossRef]
53. Surma, M.; Adamski, T.; Banaszak, Z.; Kaczmarek, Z.; Kuczyńska, A.; Majcher, M.; Ługowska, B.; Obuchowski, W.; Salmanowicz, B.; Krystkowiak, K. Effect of Genotype, Environment and Their Interaction on Quality Parameters of Wheat Breeding Lines of Diverse Grain Hardness. *Plant Prod. Sci.* **2012**, *15*, 192–203. [CrossRef]
54. Nehe, A.; Akin, B.; Sanal, T.; Evlice, A.K.; Ünsal, R.; Dinçer, N.; Demir, L.; Geren, H.; Sevim, I.; Orhan, Ş.; et al. Genotype x Environment Interaction and Genetic Gain for Grain Yield and Grain Quality Traits in Turkish Spring Wheat Released between 1964 and 2010. *PLoS ONE* **2019**, *14*, e0219432. [CrossRef]
55. Williams, R.M.; O'Brien, L.; Eagles, H.A.; Solah, V.A.; Jayasena, V. The Influences of Genotype, Environment, and Genotypeenvironment Interaction on Wheat Quality. *Aust. J. Agric. Res.* **2008**, *59*, 95–111. [CrossRef]
56. Abedi, T.; Alemzadeh, A.; Kazemeini, S.A. Wheat Yield and Grain Protein Response to Nitrogen Amount and Timing. *Aust. J. Crop Sci.* **2011**, *5*, 330.
57. Wang, R.; Wang, H.; Jiang, G.; Yin, H.; Che, Z. Effects of Nitrogen Application Strategy on Nitrogen Enzyme Activities and Protein Content in Spring Wheat Grain. *Agriculture* **2022**, *12*, 1891. [CrossRef]
58. Ghimire, D.; Das, S.; Mueller, N.D.; Creech, C.F.; Santra, D.; Baenziger, P.S.; Easterly, A.C.; Maust, B.; Maharjan, B. Effects of Cultivars and Nitrogen Management on Wheat Grain Yield and Protein. *Agron. J.* **2021**, *113*, 4348–4368. [CrossRef]
59. Stupar, V.; Paunović, A.; Madić, M.; Knežević, D.; Đurović, D. Influence of Genotype, Nitrogen Fertilisation, and Weather Conditions on Yield Variability and Grain Quality in Spring Malting Barley. *J. Cent. Eur. Agric.* **2021**, *22*, 86–95. [CrossRef]
60. Xue, C.; Matros, A.; Mock, H.-P.; Mühling, K.-H. Protein Composition and Baking Quality of Wheat Flour as Affected by Split Nitrogen Application. *Front. Plant Sci.* **2019**, *10*, 642. [CrossRef] [PubMed]
61. Xue, C.; Schulte auf'm Erley, G.; Rücker, S.; Koehler, P.; Obenauf, U.; Mühling, K.H. Late Nitrogen Application Increased Protein Concentration but Not Baking Quality of Wheat. *J. Plant Nutr. Soil Sci.* **2016**, *179*, 591–601. [CrossRef]
62. Xue, C.; Erley, G.S.A.; Rossmann, A.; Schuster, R.; Koehler, P.; Mühling, K.H. Split Nitrogen Application Improves Wheat Baking Quality by Influencing Protein Composition Rather than Concentration. *Front. Plant Sci.* **2016**, *7*, 738. [CrossRef] [PubMed]
63. Malik, A.H.; Kuktaite, R.; Johansson, E. Combined Effect of Genetic and Environmental Factors on the Accumulation of Proteins in the Wheat Grain and Their Relationship to Breadmaking Quality. *J. Cereal Sci.* **2013**, *57*, 170–174. [CrossRef]
64. Zecevic, V.; Knezevic, D.; Boskovic, J.; Madic, M. Effect of Genotype and Environment on Wheat Quality. *Genetika* **2009**, *41*, 247–253. [CrossRef]
65. Boyacioğlu, D.; Hettiarachchy, N.S. Changes in Some Biochemical Components of Wheat Grain That Was Infected with *Fusarium Graminearum*. *J. Cereal Sci.* **1995**, *21*, 57–62. [CrossRef]
66. Barneix, A.J. Physiology and Biochemistry of Source-Regulated Protein Accumulation in the Wheat Grain. *J. Plant Physiol.* **2007**, *164*, 581–590. [CrossRef]
67. Leitgeb, M.; Knez, Ž.; Hojnik Podrepšek, G. Effect of Green Food Processing Technology on the Enzyme Activity in Spelt Flour. *Foods* **2022**, *11*, 3832. [CrossRef]
68. Tóth, B.; Mesterházy, Á.; Horváth, Z.; Bartók, T.; Varga, M.; Varga, J. Genetic Variability of Central European Isolates of the *Fusarium Graminearum* Species Complex. *Eur. J. Plant Pathol.* **2005**, *113*, 35–45. [CrossRef]
69. Akinsanmi, O.A.; Backhouse, D.; Simpfendorfer, S.; Chakraborty, S. Mycelial Compatibility Reactions of Australian *Fusarium Graminearum* and F. *Pseudograminearum* Isolates Compared with AFLP Groupings. *Plant Pathol.* **2008**, *57*, 251–261. [CrossRef]
70. Malbrán, I.; Mourelos, C.A.; Girotti, J.R.; Aulicino, M.B.; Balatti, P.A.; Lori, G.A. Aggressiveness Variation of *Fusarium Graminearum* Isolates from Argentina Following Point Inoculation of Field Grown Wheat Spikes. *Crop Prot.* **2012**, *42*, 234–243. [CrossRef]
71. Ortega, L.M.; Moure, M.C.; González, E.M.; Alconada, T.M. Wheat Storage Proteins: Changes on the Glutenins after Wheat Infection with Different Isolates of *Fusarium Graminearum*. *Int. Microbiol.* **2019**, *22*, 289–296. [CrossRef] [PubMed]
72. Eggert, K.; Wieser, H.; Pawelzik, E. The Influence of Fusarium Infection and Growing Location on the Quantitative Protein Composition of (Part I) Emmer (*Triticum Dicoccum*). *Eur. Food Res. Technol.* **2010**, *230*, 837–847. [CrossRef]
73. Pawelzik, E.; Permady, H.H.; Weinert, J.; Wolf, G.A. Effect of Fusarium Contamination on Selected Quality Criteria of Wheat. *Getreide Mehl. Brot.* **1998**, *52*, 264–266.
74. Juodeikiene, G.; Basinskiene, L.; Vidmantiene, D.; Bartkiene, E.; Bakutis, B.; Baliukoniene, V. Acoustic Sensing of Deoxynivalenol in Co-Occurrence with Zearalenone and T-2/HT-2 Toxin in Winter Wheat Cultivar Sirvinta from Lithuania. *World Mycotoxin J.* **2011**, *4*, 395–404. [CrossRef]

75. Khatkar, B.S.; Bell, A.E.; Schofield, J.D. A Comparative Study of the Inter-Relationships Between Mixograph Parameters and Bread-Making Qualities of Wheat Flours and Glutens. *J. Sci. Food Agric.* **1996**, *72*, 71–85. [CrossRef]
76. Hadnađev, T.D.; Torbica, A.; Hadnađev, M. Rheological Properties of Wheat Flour Substitutes/Alternative Crops Assessed by Mixolab. *Procedia Food Sci.* **2011**, *1*, 328–334. [CrossRef]
77. Martin, C.; Schöneberg, T.; Vogelsgang, S.; Vincenti, J.; Bertossa, M.; Mauch-Mani, B.; Mascher, F. Factors of Wheat Grain Resistance to Fusarium Head Blight. *Phytopathol. Mediterr.* **2017**, *56*, 154–166. [CrossRef]
78. Okuda, R.; Tabara, A.; Okusu, H.; Seguchi, M. Measurement of Water Absorption in Wheat Flour by Mixograph Test. *Food Sci. Technol. Res.* **2016**, *22*, 841–846. [CrossRef]
79. Zaidul, I.; Karim, A.A.; Manan, D.; Ariffin, A.; Norulaini, N.N.; Omar, A.M. A Farinograph Study on the Viscoelastic Properties of Sago/Wheat Flour Dough Systems. *J. Sci. Food Agric.* **2004**, *84*, 616–622. [CrossRef]
80. Martínez, M.; Ramírez Albuquerque, L.; Arata, A.F.; Biganzoli, F.; Pinto, V.F.; Stenglein, S.A. Effects of *Fusarium Graminearum* and *Fusarium Poae* on Disease Parameters, Grain Quality and Mycotoxins Contamination in Bread Wheat (Part I). *J. Sci. Food Agric.* **2020**, *100*, 863–873. [CrossRef]
81. Aydoğan, S.; Şahin, M.; Akçacık, A.; Sümerya, A.; Seyfi, H.; Bahri, T.; Uluslararası, D.; Araştırma, T.; Müdürlüğü, E. Relationships between Farinograph Parameters and Bread Volume, Physicochemical Traits in Bread Wheat Flours. *J. Bahri Dagdas. Crop Res.* **2015**, *3*, 14–18.
82. Scala, V.; Aureli, G.; Cesarano, G.; Incerti, G.; Fanelli, C.; Scala, F.; Reverberi, M.; Bonanomi, G. Climate, Soil Management, and Cultivar Affect Fusarium Head Blight Incidence and Deoxynivalenol Accumulation in Durum Wheat of Southern Italy. *Front. Microbiol.* **2016**, *7*, 1014. [CrossRef] [PubMed]
83. Havrlentová, M.; Šliková, S.; Gregusová, V.; Kovácsová, B.; Lančaričová, A.; Nemeček, P.; Hendrichová, J.; Hozlár, P. The Influence of Artificial Fusarium Infection on Oat Grain Quality. *Microorganisms* **2021**, *9*, 2108. [CrossRef] [PubMed]
84. Surma, M.; Adamski, T.; Wiśniewska, H.; Kaczmarek, Z.; Mejza, I.; Mejza, S.; Kuczyńska, A.; Krystkowiak, K.; Mikołajczak, K.; Ogrodowicz, P. Uni- and Multivariate Approaches to Evaluating the Susceptibility of Wheat Hybrids to Fusarium Head Blight. *Czech J. Genet. Plant Breed.* **2016**, *52*, 132–138. [CrossRef]

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Fusarium Head Blight Infestation in Relation to Winter Wheat End-Use Quality—A Three-Year Study

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Abstract: Fusarium head blight (FHB) is one of the major threats for wheat production worldwide. It reduces yield, quality, and feeding value of wheat grains. In addition, mycotoxins produced by *Fusarium* pathogens can have a negative effect on livestock and human health. The aim of this study was to assess changes in technological quality traits and end-use quality of winter wheat varieties after artificial inoculation with *Fusarium* spp. over three years. Differences in dough development duration and extensibility were measured as the means of relative reductions due to different environments and varieties' characteristics. Differences in dough softening during kneading were determined as the means of relative increases due to FHB inoculation. In addition, dough had reduced strength, was stickier, and therefore was more difficult to handle, due to a decrease of the average energy value and resistance to extension in FHB-inoculated wheat, compared to naturally infected plants. Dough development time, stability, and resistance usually varied in a similar way, with FHB-resistant varieties showing a good response to FHB inoculation and maintaining good quality. Increasing the level of *Fusarium* spp. contamination in more FHB-susceptible wheat varieties worsened their technological quality, primarily, the sedimentation value and the gluten index, and hence had a negative effect on the rheological properties.

Keywords: extensograph; farinograph; Fusarium; technological quality; wheat

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1. Introduction

Wheat production is endangered by the fungal pathogens from *Fusarium* spp., which cause Fusarium head blight (FHB). This disease not only causes grain yield losses, but also decreases wheat quality and causes the presence of mycotoxins in the grains [1–3], which are potentially harmful to human and animal health. It can destroy starch granules, storage proteins, and grain cell wall and subsequently affect the quality of dough [4]. Consequently, FHB infection results in the reduction of end-use quality [5]. The negative influence of FHB on wheat flour properties and its products was previously reported [6]. Furthermore, FHB resistance is mainly categorized into two types: Type 1 (resistance to initial infection) and Type 2 (resistance to spread within the head) [7], although several other forms of resistance have been proposed. It was suggested that the rheological properties under FHB infection pressure influence grain resistance to the disease [8]. The most important method for FHB control and the reduction of mycotoxin concentration is the development of FHB-resistant wheat varieties [9]. Besides that, the use of specific cultural practices, fungicides, and biological control can help reduce FHB infection [10].

Climatic conditions, especially during wheat anthesis, can affect *Fusarium* species [11]. *Fusarium graminearum* and *F. culmorum* are the most prevalent species causing FHB [12,13], but their prevalence may change throughout the year [14]. Infection by *Fusarium* spp. can

occur between 10 and 30 °C [15], and therefore, the availability of moisture is a predominant factor for the success of pathogen infection [16]. Under favorable weather conditions with high relative humidity and optimal temperatures during flowering, *Fusarium* infection will start in a wheat spikelet and then will expand within the whole head, causing the characteristic symptoms of bleaching [17].

It is essential to understand the impact of *Fusarium* infestation not only on grain properties but also on health, due to the large consumption of wheat products worldwide [18]. Wheat flour, imparting viscoelastic properties to dough, is used in a diverse range of end-use products including breads, cakes, noodles, crackers, cookies, and pasta [19]. In fact, gluten proteins are able to form a network in the dough, where carbon dioxide is blocked [20]. The final baking quality, defined by the rheological parameters of dough, is influenced by wheat variety background and environmental conditions [21]. The quality of wheat in relation to its end products is particularly determined by its protein content. Research on the impact of *Fusarium* infestation on wheat quality is scarce, compared to that on FHB resistance mechanisms. Besides, the majority of studies are focused on technological grain quality [22,23], and only few on rheological dough properties [24]. The reason of that could be the fact that the current method to evaluate end-use quality are time-consuming and costly, compared to those used to measure technological quality traits, which are considered fast and inexpensive. However, it is important to notice that the quality of the final product is evaluated on the basis of the rheological properties of the wheat dough, including dough elasticity, viscosity, and extensibility. The current study is focused on the detection of technological and rheological quality changes in winter wheat varieties under natural infection and *Fusarium* artificial inoculation, using standard fariongraph and extensograph tests for technological quality determination and rheological evaluation.

2. Materials and Methods

2.1. Field Experiments

Twenty-five winter wheat varieties (Table 1) were studied in field experiments in the vegetative seasons 2014/2015, 2015/2016, and 2016/2017 at Osijek (45°27′ N, 18°48′ E) in Croatia. The annual precipitation during the growing seasons 2014/2015, 2015/2016, and 2016/2017 were 513, 706, and 482 mm, and the average annual temperatures were 11.3, 11.0, and 10.0 °C, respectively (Figure 1a–c). During the heading stage, the highest rainfall was recorded in 2017 (48.8 mm), followed by 2016 (45 mm). The lowest amount of rainfall around the heading stage was recorded in 2015 (12.1 mm) with the highest average temperature (19.2 °C), compared to 2016 and 2017 (12.0 and 15.3 °C, respectively) (Figure 2a–c). As a control of seed-borne diseases, Vitavax 200 FF (thiram + carboxin) was used at a rate of 200 mL 100 kg^{-1}. During the vegetative season, insecticides and herbicides were applied as needed for weeds and aphid protection of the field experiments. Fertilization with standard amounts of NPK fertilizers differed during the study (120–140/80–100/120–130 kg ha^{-1}). Wheat varieties were sown in 7.56 m^2 plots in two replications per treatment (two treatments in total) with a Hege Seedmatic machine in October of each year of study. One sample was left under natural conditions (without use of fungicides) and another was subjected to *Fusarium* artificial inoculations when 50% of the wheat plants inside each plot were at the flowering stage (Zadok's scale 65) [25]. A *Fusarium* inoculum was applied with a tractor-back sprayer, and afterwards the plots were irrigated twice with water to maintain humidity in the 24 h after the inoculations. The FHB inoculations were repeated two days later. Disease severity (general FHB resistance) and incidence (Type I resistance) were recorded on days 10, 14, 18, 22, and 26 after the last inoculation. The percentage of bleached spikelets (disease intensity) per plot was estimated according to a linear scale (0–100%), while disease incidence was calculated as the percentage of diseased ears after assessing a random sample of 30 heads. The area under the disease progress curve (AUDPC) was calculated [26] and used for further statistical analysis.

Calculation of AUDPC:

$$AUDPC = \sum_{i=1}^{n} \left\{ \left[\frac{Yi + Yi - 1}{2} \right] * (Xi - Xi - 1) \right\}$$

Yi—percentage of visibly infected spikelets ($Yi/100$) at the ith observation

Xi–day of the ith observation, n—total number of observations

Wheat plots were harvested by a Wintersteiger cereal plot combine-harvester in the beginning of July, at a grain moisture content of 14.5–16.0%.

Table 1. Origin, year of release, and susceptibility to *Fusarium* of 25 investigated winter wheat varieties.

Varieties	Origin [1]	Year of Release	Susceptibility [2] to *Fusarium*
GOLUBICA	HR, AIO	1997	S
SUPER ZITARKA	HR, AIO	1997	S
BASTIDE	FRA	2003	S
FELIX	HR, AIO	2007	S
BC ANICA	HR, BC	2010	S
LUCIJA	HR, AIO	2001	MS
SRPANJKA	HR, AIO	1989	MS
RENATA	HR, AIO	2006	MS
KATARINA	HR, AIO	2006	MS
SANA	HR, BC	1983	MR
BEZOSTAYA	Former USSR	1955	MR
ALKA	HR, AIO	2003	MR
ZITARKA	HR, AIO	1985	MR
ANTONIJA	HR, AIO	2011	MR
FLAMURA 85	ROM	1989	MR
KRALJICA	HR, AIO	2010	MR
DROPIA	ROM	2006	MR
OLIMPIJA	HR, AIO	2009	R
VULKAN	HR, AIO	2009	R
DIVANA	HR, JS	1995	R
GRAINDOR	FRA	2006	R
APACHE	FRA	1998	R
U1	HR, AIO	1936	R
RENAN	FRA	1991	R
SIRBAN PROLIFIC	HU	1905	R

[1] AIO, Agricultural Institute Osijek, JS-Jost sjeme, BC, BC Institute; [2] S, susceptible, MS, moderately susceptible, MR, moderately resistant, R, resistant.

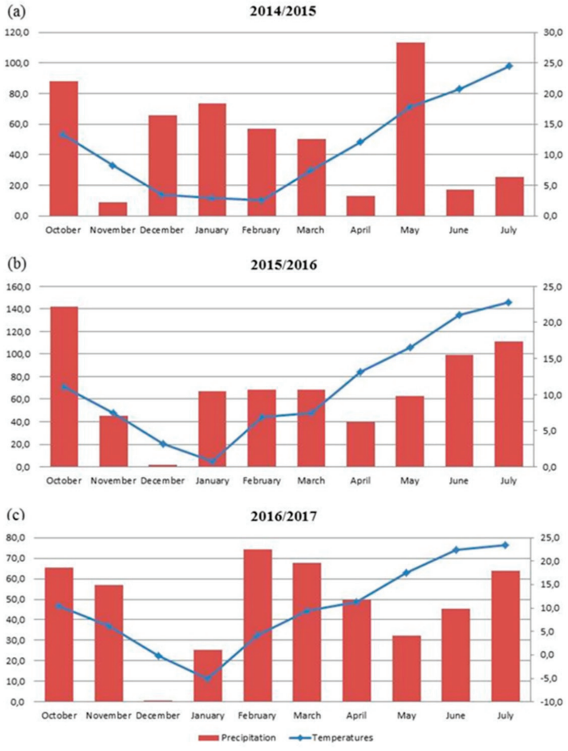

Figure 1. Climate diagrams for the vegetation seasons 2014/2015 (**a**), 2015/2016 (**b**), and 2016/2017 (**c**) in Osijek, Croatia.

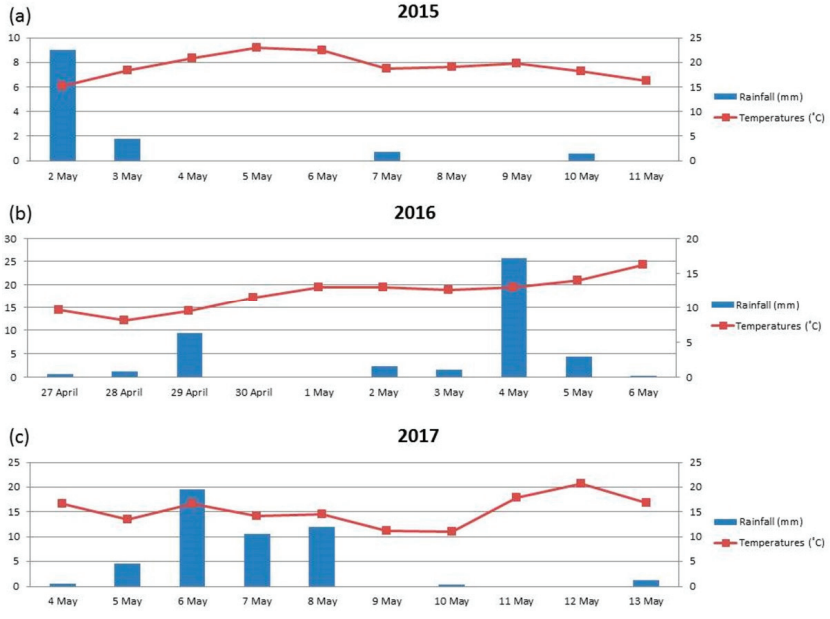

Figure 2. Climate diagrams for temperatures and rainfall around the heading stage in 2015 (**a**), 2016 (**b**), and 2017 (**c**) in Osijek, Croatia.

2.2. Fusarium Isolates and Production of the Inoculum

The conidia of two *Fusarium* isolates (*Fusarium graminearum*, PIO 31 and *F. culmorum*, IFA 104) were stored in permanent cultures at +4 °C before the study. The strains were cultured on a synthetic low-nutrient (SNA) medium consisting of water containing KH_2PO_4, KNO_3, $MgSO_4$*$7H_2O$, glucose, sucrose, and agar [27]; the medium was kept for one week in the dark at room temperature. For a mass production of the conidia of each isolate in the proportion 1:1., two discs (5 mm diam.) from a well-grown colony were transferred to the mixture of wheat and oat (3:1), previously soaked in water overnight, and autoclaved [28]. Conidial concentrations were set to 10×10^4 mL^{-1} by a hemocytometer. The *Fusarium* inoculum (100 mL) was sprayed on an area of m^2 per plot.

2.3. Milling, Grain Technological Properties, and Dough Properties

Wheat grain samples were conditioned to 14% moisture content and milled using Quadrumat Senior break (C.W. Brabender Inc., South Hackensack, NJ, USA). Protein content was measured by Infratec 1241, Foss Tecator. Wet gluten content and the gluten index were obtained by the ICC method No. 155 [29]. Zeleny sedimentation volume and falling number were measured by the ICC method No. 116/1 [30] and the ICC method No. 107/1 [31]. Dough properties were evaluated using 50 g of flour with a Farinograph (Brabender, Duisburg, Germany) according to HRN ISO 5530-1:1999 [32] and 300 g of flour with an Extensograph (Brabender, Duisburg, Germany) according to HRN ISO 5530-2:1999 [33]. Relative differences of technological and dough quality parameters (relative technological or dough traits in %) were determined for *Fusarium*-treated samples relative to naturally infected samples.

2.4. Statistical Analysis

The data distribution was evaluated by the Shapiro–Wilk W-test, but since the data of six parameters did not show a normal distribution, the comparison between treatments (naturally infected and inoculated) was performed by the Mann–Whitney U test for those traits. Analysis of variance (ANOVA) using the main-effects model and relative differences for grain technological, farinograph, and extensograph properties between FHB-inoculated and naturally infected samples were analyzed for statistically significant differences by the Fisher's least significant difference (LSD) test ($\alpha = 0.05$) by Statistica version 12.0 (Statsoft Inc., Tulsa, OK, USA).

3. Results

3.1. Fusarium Head Blight (FHB) Severity and Incidence

Twenty-five winter wheat varieties were evaluated for general FHB and Type I resistance in FHB-inoculated plots in three-year field experiments. The symptoms of FHB disease appeared after 7–8 days from inoculation (dpi), and disease resistance was recorded for the first time at 10 dpi and again every 4 days till 26 dpi. FHB symptoms were not found in non-inoculated plots (naturally infected plots); therefore, in the plots with natural infection, disease scoring was not performed. FHB symptoms were more severe in the last vegetative season 2016/2017, than in 2014/2015 and 2015/2016 (Figure 3a,b).

Overall, the variety Golubica showed significantly greater disease scores for general resistance (higher FHB susceptibility). (Figure 3a). FHB symptoms were observed at high severity (average of 25 wheat varieties, 430 AUDPC) in the last vegetative season (2016/2017), compared to previous years, except for U1, Sirban Prolific, and Renan, which showed a mean severity of 5.5, 5.8, and 8.0 AUDPC, respectively. Sirban Prolific in 2014/2015 and 2015/2016, along with U1 in 2015/2016, presented no FHB symptoms. AUDPC for general FHB resistance in 25 wheat varieties inoculated with *Fusarium* spp. averaged 28.2, 42.2, and 165.3 in 2014/2015, 2015/2016, and 2016/2017, respectively.

Sirban Prolific, Renan, and U1 had significantly different Type I resistance (less pronounced relative differences between FHB-inoculated and naturally infected samples). (Figure 3b). For the fraction of plants showing initial disease symptoms, AUDPC ranged

from 0 (Sirban Prolific) to 75.0 (Golubica) in 2014/2015, from 0 (Sirban Prolific) to 191.6 (Felix) in 2015/2016, and from 18.3 (U1) to 647.5 (Golubica) in 2016/2017.

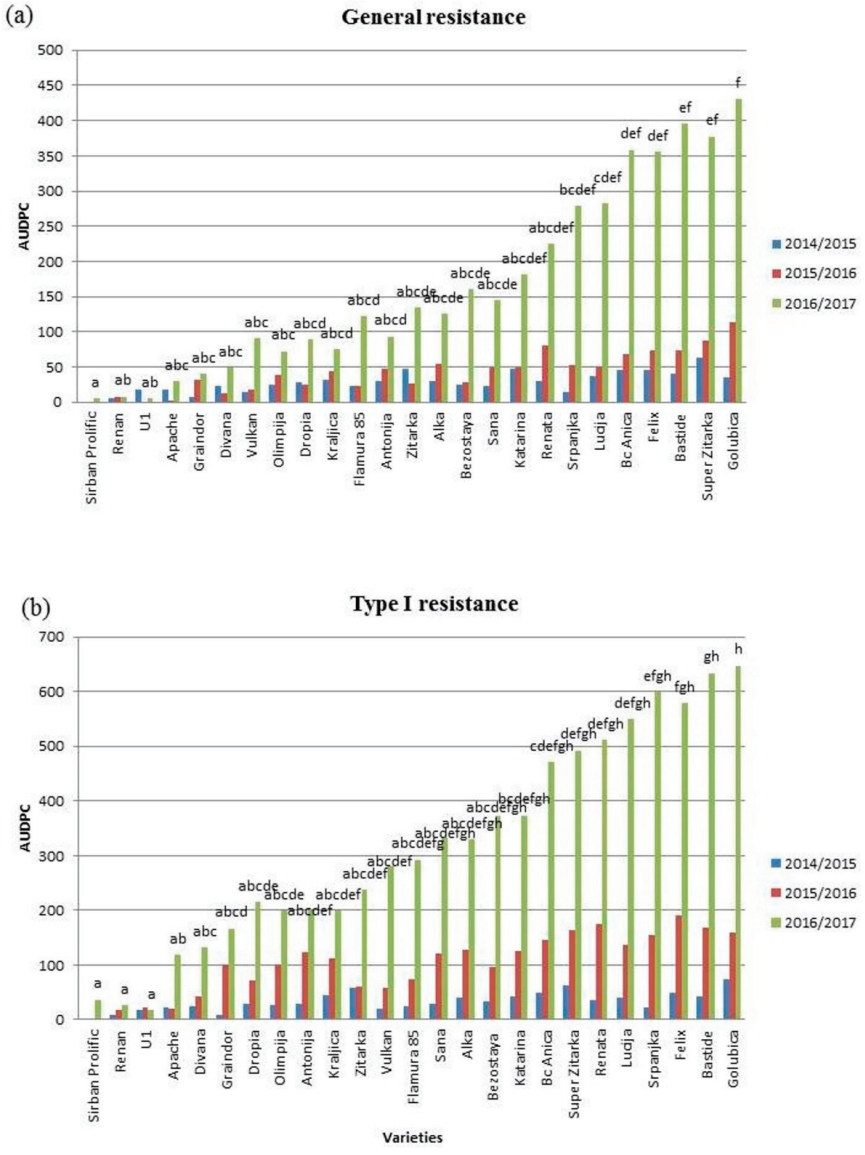

Figure 3. AUDPC for general FHB resistance (**a**) and Type I resistance (**b**) in the three examined years for 25 winter wheat varieties. Different lower-case letters represent significantly different values ($p < 0.05$) for each wheat variety in the three years in average.

3.2. Impact of Fusarium Infections on Grain Technological and Rheological Parameters

The analysis of variance for five grain technological and eight dough rheological properties revealed that the mean squares (MS) for 25 winter wheat varieties and two treatments (natural disease infection and artificial inoculation) in the three studied years were highly significant for sedimentation value, gluten index, dough stability, resistance

and degree of softening, energy value, and resistance to extension ($p < 0.001$) (Table 2). In general, the year showed the largest effect, followed by variety, on protein and wet gluten content, water absorption, dough development, and extensibility. The treatment had the strongest effect, compared to variety and year, on sedimentation value, gluten index, degree of softening, energy value, and resistance to extension.

Table 2. Analysis of variance for five grain technological and eight dough rheological properties in the three years of investigation.

		MS												
Source of Variation	Df	PC	SV	WG	GI	FN	WA	D	S	R	SOF	E	RES	EXT
Variety (V)	24	9.27 ***	94.7 ***	45.3 ***	278 ***	4863 ***	26.3 ***	8,565 ***	2.0549 ***	15.249 ***	2508 ***	2267 ***	16088 ***	1317 ***
Treatment (T)	1	0.14	1115.2 ***	3,1	2875 ***	501	0,5	4,002	3,0246 *	14,291 **	42538 ***	38785,0 ***	409248 ***	604
Year (Y)	2	13.08 ***	1015.6 ***	70.8 ***	1046 ***	34602 ***	164,5 ***	63,713 ***	8.8346 ***	119,587 ***	21600 ***	13066,7 ***	80694 ***	8403 ***
Error	122	0.32	14,8	4,9	59	885	1,1	1,047	0,7357	2,136	623	338.1	2265	370

***, **, * = significant at $p < 0.001$, 0.01, and 0.05, respectively; Df, degrees of freedom, MS, mean square. PC, protein content, SV, sedimentation value, WG, wet gluten content, GI, gluten index, FN, falling number, WA, water absorption, D, dough development, S, dough stability, R, dough resistance, SOF, degree of softening, E, energy value, RES, resistance to extension, EXT, extensibility.

3.2.1. Technological Quality Parameters

We did not find any significant relative difference in protein content between the two treatments in all wheat varieties (Figure 4a). Renata in 2014/2015 and Divana in 2015/2016 had higher relative differences of protein content (>10%) after *Fusarium* inoculation, compared to naturally infected samples. On average, Felix and Bc Anica showed the highest increase of protein content due to *Fusarium* inoculation (3.4%).

The varieties Bc Anica, Lucija, Bezostaya, Bastide, Super Zitarka, and Golubica presented significant relative differences of sedimentation values between the two treatments, compared to the varieties Renan, Sirban Prolific, and Graindor (Figure 4b). On average, the sedimentation value was decreased in FHB-inoculated samples in the three years of study. After FHB treatment, Bezostaya showed a decrease in the sedimentation value up to 40% in 2015/2016, followed by Dropia (37%), Bastide, and Super Zitarka (29%).

The varieties Srpanjka and Sirban Prolific had significant relative differences of wet gluten content, compared to Zitarka and Kraljica (Figure 4c). In 2016/2017, in most wheat varieties, wet gluten content was increased in FHB-inoculated samples, compared to naturally infected plots (except for U1 and Sirban Prolific). The differences between FHB-inoculated and naturally infected samples with respect to wet gluten content were about 5.0, 5.2, and −9.4% in 2014/2015, 2015/2016, and 2016/2017, respectively. Felix and Srpanjka had the highest relative losses in 2014/2015 (20 and 18%), while Super Zitarka in the same year showed increased wet gluten content in FHB-inoculated samples (5%). The variety Golubica showed the highest relative decrease of gluten index in FHB-inoculated samples, compared to naturally infected ones, with the highest relative loss in 2016/2017 (44.4%) (Figure 4d). Furthermore, the highest relative losses, on average, occurred in 2016/2017.

The varieties Renan and Flamura 85 reported significant relative differences for falling number, compared to Bezostaya, Felix, and U1 (Figure 4e). Bezostaya had a higher falling number, up to 32.2%, after FHB inoculation, compared to naturally infected plants in 2016/2017, Felix up to 24.5% in 2015/2016, and Flamura 85 up to 26.7% in 2014/2015.

3.2.2. Farinograph Parameters

The varieties Bezostaya, Sirban Prolific, Antonija, Bastide, Divana, Apache, and Olimpija showed significant relative differences between the two treatments for water absorption, compared to Kraljica, Bc Anica, and Sana (Figure 5a). The highest relative loss was recorded for Super Zitarka in 2016/2017 (4.1%).

The relative differences of dough development between the two treatments in Renan were significant with respect to other wheat varieties, except for Lucija, Flamura 85, Felix, and Katarina (Figure 5b). The variety Antonija, with the highest relative differences, had significant relative differences compared to Renan, Lucija, Flamura 85, and Felix.

The varieties Divana and Renan showed higher dough stability after FHB inoculation, compared to naturally infected plants, in 2016/2017, while lower dough stability was obtained after FHB inoculation in 2014/2015 and 2015/2016 (Figure 5c). Apache, with the highest relative losses after FHB treatment (64, 92, and 67% in 2014/2015, 2015/2016, and 2016/2017, respectively), was significantly different from Renan, Divana, and Katarina.

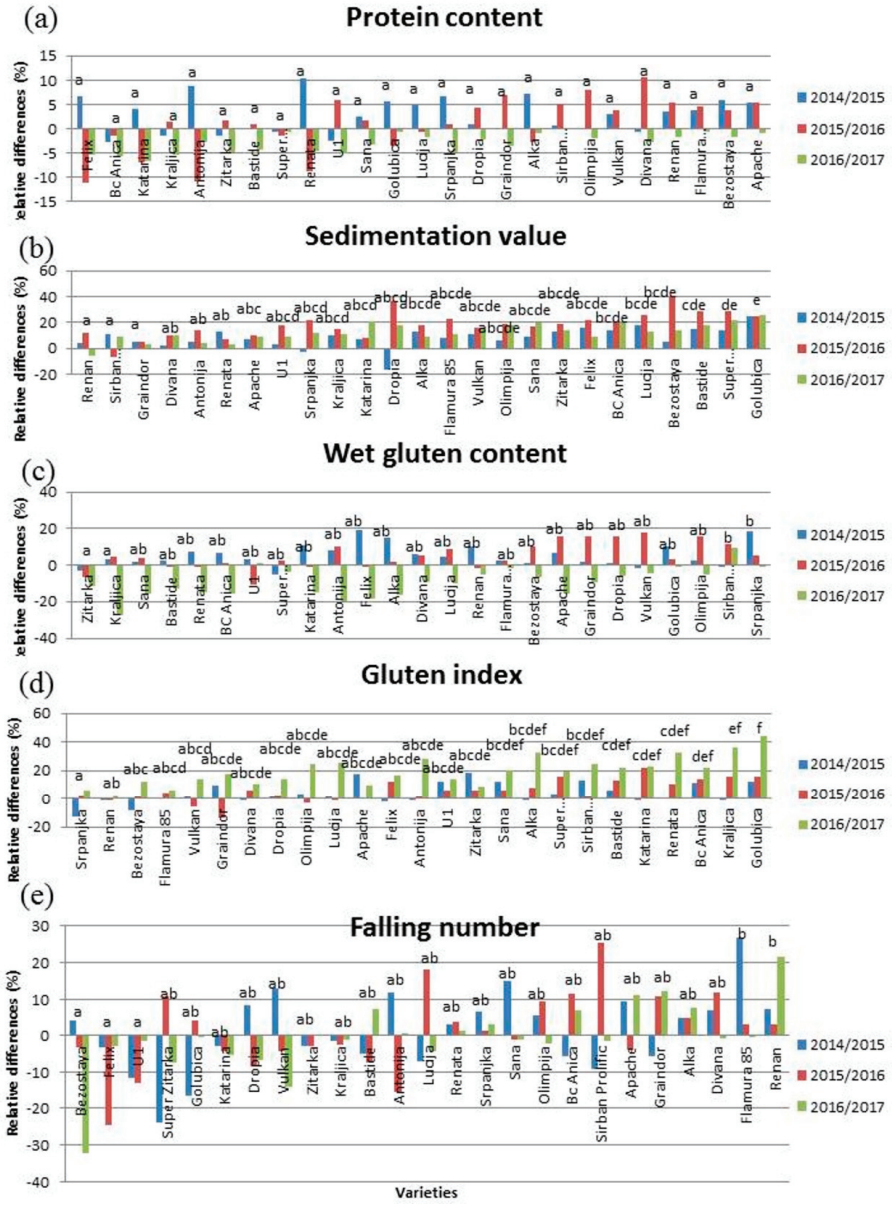

Figure 4. Relative differences in technological quality parameters (protein content (**a**), sedimentation value (**b**), wet gluten content (**c**), gluten index (**d**), and falling number (**e**)) between FHB-inoculated and naturally infected plants in the three years of the study (2014/2015, 2015/2016, and 2016/2017) for 25 winter wheat varieties. Different lower-case letters represent significantly different values ($p < 0.05$) for each wheat variety in the three years, on average.

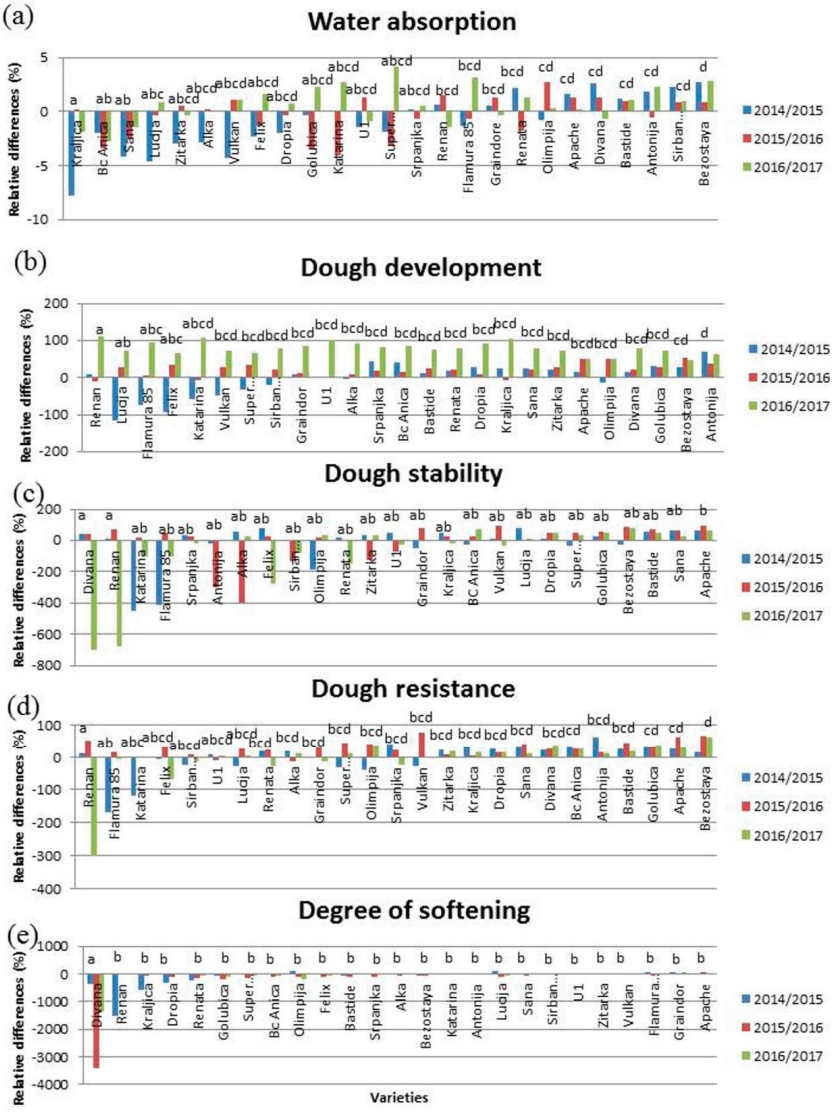

Figure 5. Relative differences of farinograph parameters (water absorption (**a**), dough development (**b**), stability (**c**), resistance (**d**), and degree of softening (**e**)) between FHB-inoculated and naturally infected samples in the three years of study (2014/2015, 2015/2016, and 2016/2017) for 25 winter wheat varieties. Different lower-case letters represent significantly different values ($p < 0.05$) for each wheat variety in the three years, on average.

The variety Renan showed the same pattern of behavior for dough resistance as for dough stability in the three years of study. Bezostaya, Apache, and Golubica showed significant relative differences, compared to Renan and Flamura 85 (Figure 5d).

The variety Divana had the highest increase in the degree of softening in the three examined years for FHB-inoculated plants, compared to naturally infected ones and was significantly different from all other varieties (Figure 5e). The degree of softening increased after FHB inoculation by 2.5 and 8.9-fold for the variety Divana in 2015/2016, compared to 2016/2017 and 2014/2015.

3.2.3. Extensograph Parameters

The varieties Sirban Prolific, Renan, Apache, Vulkan, Divana, Graindor, U1, Olimpija, and Flamura 85 were similar as regards the relative differences of energy value between FHB-inoculated and naturally infected plants (Figure 6a). The highest loss was recorded in 2015/2016 (97.3%) in Alka, followed by Golubica in 2014/2015 (85.3%) and Bc Anica in 2016/2017 (79.6%).

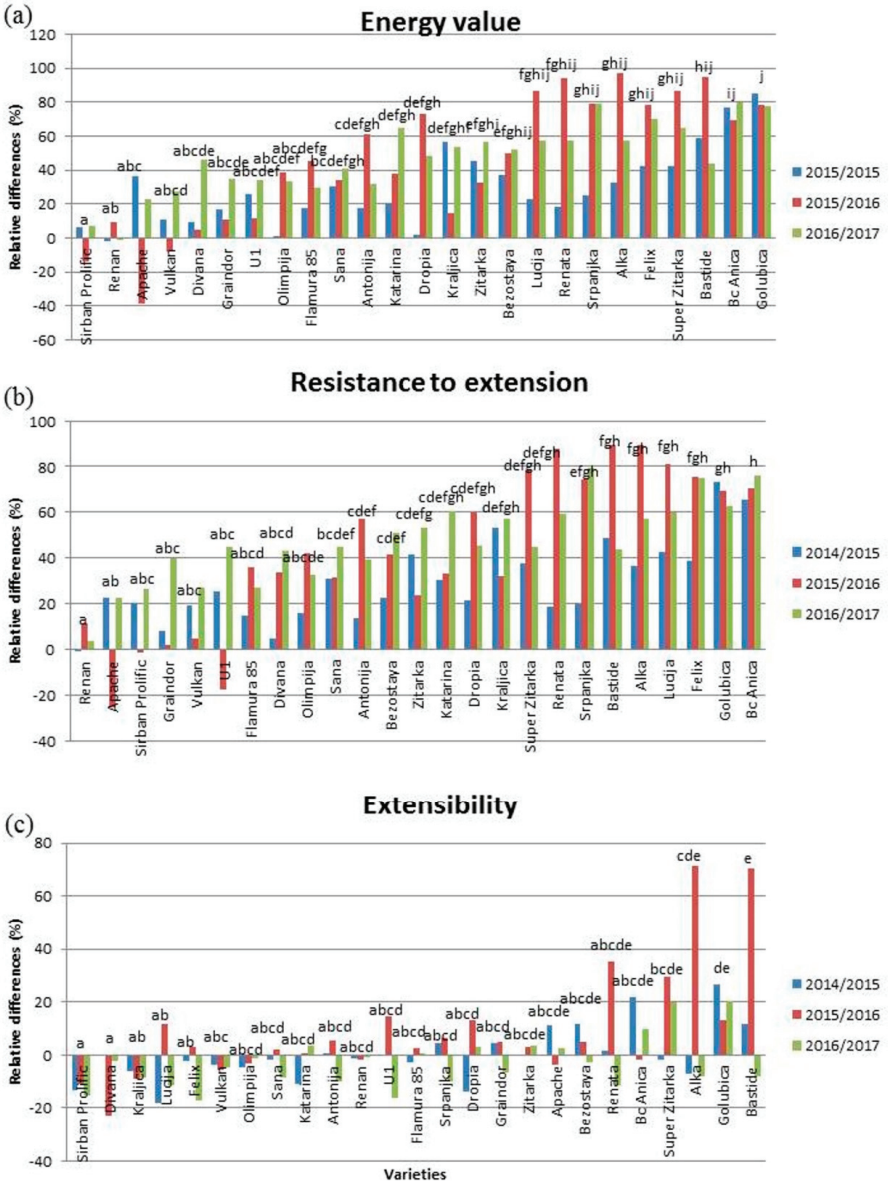

Figure 6. Relative differences of extensograph parameters (energy (**a**), resistance to extension (**b**), and extensibility (**c**) between FHB-inoculated and naturally infected samples in the three years of study (2014/2015, 2015/2016, and 2016/2017) for 25 winter wheat varieties. Different lower-case letters represent significantly different values ($p < 0.05$) for each wheat variety in three years in average.

The variety Renan had relative differences of resistance to extension between two treatments at the same significant level as Apache, Sirban Prolific, Graindor, Vulkan, U1, Flamura 85, Divana, and Olimpija (Figure 6b). Bc Anica had losses up to 65.7, 70.5, and 75.9%, Golubica 73.4, 69.4, and 62.9%, and Felix 38.9, 75.4, and 75.0% in 2014/2015, 2015/2016, and 2016/2017, respectively.

The varieties Bastide, Golubica, Alka, and Super Zitarka were significantly different for extensibility, compared to Sirban Prolific and Divana, in relation to FHB-inoculated and naturally infected plants (Figure 6c). ANOVA did not show significant differences between the two treatments for extensibility.

4. Discussion

Fusarium head blight (FHB) infection requires wet or moist conditions before and during anthesis as well as during the early grain development stages [34]. In the current study, winter wheat varieties were differentiated on the basis FHB infestation, which was expected, as modern and older winter wheat varieties with different genetic background in the field experiments were included. The weather conditions during the three vegetative seasons of 2014/2015, 2015/2016, and 2016/2017 varied widely, which may explain the differences between wheat varieties in different years for FHB general or Type I resistance. In 2014/2015, the average monthly precipitation in May was at least 1.5 times as high as in the same month in 2015/2016 and 2016/2017, but it is important to notice that they occurred at the latest after flowering, compared to 2015/2016 and 2016/2017. The average monthly temperatures were the highest in June in 2016/2017, thus providing the most favorable temperature ranges for FHB infection in that year. Furthermore, in 2014/2015, there precipitation was low during June, compared to 2015/2016 and 2016/2017. In 2016/2017, the average precipitation in pre-anthesis was higher in April than in 2014/2015 and 2015/2016. Lower temperatures in 2015/2016 and a lower amount of precipitation in April and June in 2014/2015 prevented an epidemic, as occurred in 2016/2017 when fungal infestation was extensive due to the weather conditions, since the AUDPC for FHB general resistance was 3.9- and 5.9-fold higher in 2016/2017, compared to 2015/2016 and 2014/2015, respectively. In general, precipitation during anthesis is particularly favorable to wheat infestation by *Fusarium* spp. [35], as it could be observed in 2017 in the current study. It was previously concluded that winter wheat kernel infection by *Fusarium* spp. depended primarily on weather conditions and then on wheat variety [36]. An FHB outbreak can occur due to environmental conditions at a local level [37].

In addition, most of the total variance in all quality traits was partially determined by the year and the wheat variety. The effects of the environment were very important when breeding wheat for end-use quality [38]. We found that 48% of the total variation of deoxynivalenol contamination as a consequence of FHB infection depended on the year [39]. In the current study, a most prominent influence of the year was observed in all parameters when compared to the effect of wheat variety [40], which was also observed for farinograph properties. Treatment had the strongest significant effect, compared to variety and year, on sedimentation value, gluten index, degree of softening, energy value, and resistance to extension. The rheological properties of dough were not affected to the same extent for all winter wheat varieties. The relative differences of technological and rheological traits caused by FHB were calculated to reflect the different impact of FHB in different wheat varieties.

4.1. Technological Quality Properties of Wheat Grain under FHB Pressure

Fusarium infection did not have a significant effect on protein content, as seen by ANOVA analysis, whereas protein content was significantly influenced by variety and year. This was previously reported by other researchers [41,42]. In the current study, in some years, protein content was higher in FHB-inoculated samples, compared to naturally infected plants, especially in 2016/2017 when the strongest FHB infestation occurred, compared to 2014/2015 and 2015/2016. Furthermore, FHB-susceptible wheat varieties

showed increased protein content in FHB-inoculated samples. Similar findings were after *Fusarium* spp. contamination [43]. This could be due to the consumption of carbohydrates by *Fusarium* pathogens [42]. In contrast, it was found that total protein content was lower in *Fusarium*-damaged grains [44], while some researchers revealed only a slight decrease of it [18]. In the current study, a decrease occurred in most cases in FHB-inoculated plants, compared to naturally infected plants, most probably as a consequence of the enzymatic degradation of proteins by FHB [45]. According to some researchers, 1% more or less proteins increases or decreases, respectively, the baking volume by about 25 mL for 100 g of flour [21].

The sedimentation value is a measure of protein swelling in an acid or sodium dodecyl sulfate (SDS) solution, indicating protein quality. The small variation between winter wheat varieties suggests a stronger influence of year and treatment on the sedimentation value. On average, the sedimentation value showed a tendency to decrease in FHB-inoculated plants, compared to naturally infected plants. Only in the two most FHB-resistant varieties, Renan and Sirban Prolific, the sedimentation value tended to increase under increased FHB pressure in some years; this was also observed for Srpanjka and Dropia in 2014/2015. On the other hand, in more than half of the varieties, on average, the decrease in the sedimentation value was >10%, which resulted in negative effects on quality. This is in accordance with research showing a decrease of the sedimentation value with increased FHB infection [18]. A higher sedimentation value means a higher gas retention, affecting dough stability and baking volume. Our results indicated that, although the total amount of protein remained quite stable in most wheat varieties, FHB infection could alter protein quality, as a slight degradation of proteins might lead to their increased swelling.

Gluten proteins determine wheat processing quality, and glutenins and gliadins play the most important role in the viscoelastic properties of dough [46]. According to ANOVA, the treatment did not significantly influence wet gluten content. Similar results were obtained previously, indicating that wet gluten content was not significantly influenced by *Fusarium* infection [47]. However, according to some studies, wet gluten content was reduced in artificially *Fusarium*-inoculated samples [43]. The results of the current study showed that wet gluten content was significantly influenced by the year and the variety. In 2016/2017, on average, the lowest values of wet gluten content occurred, compared to previous years examined in this study. In spite of the lowest values of wet gluten content in 2016/2017, increased wet gluten content in FHB-inoculated plants occurred compared to naturally infected plants. Previously, an increase of wet gluten content in FHB-damaged grains was reported [48]. In 2016/2017, the highest FHB severity and incidence. on average, were recorded. This brought us to the conclusion that more heavily FHB-infected wheat plants will increase their wet gluten content, together with protein content.

The gluten index, as ab indication of gluten strength, shows whether the gluten quality is weak (<30%), normal (30–80%), or strong (80%) [49]. The gluten index was the parameter most strongly influenced by the treatment, as the most FHB-susceptible wheat variety showed the greatest decreases of gluten index after FHB inoculation, compared to naturally infected plants. Furthermore, the greatest decreases of gluten index occurred in 2016/2017, the year with the highest FHB severity and incidence.

The falling number is a measure of α-amylase activity in the grain, indicating sprout damage. This parameter was not significantly influenced by the treatment in the current study. In most cases, the falling number showed decreased values in FHB-inoculated plants, which is in accordance with other studies, where it was expected that α-amylase degraded starch [50]. In 2016/2017, when the most extensive FHB infection occurred, the falling number was less than 310 s on average, which indicated low enzyme activity, with negative consequences for baking products due to low raising and small volume of the dough. In few cases, an increase in FHB-inoculated plants occurred, as previously reported by some studies, and FHB-infected grains could mature earlier, thus causing negative consequences for wheat quality [45].

4.2. Farinograph Analysis of Dough in FHB-Inoculated and Naturally Infected Plants

Dough resistance was estimated by the farinograph test, whereby the behavior of dough against mixing at a specified constant speed with specified water addition could be observed. The viscous and elastic properties of the dough could be measured when gluten was mixed with water [51]. In the current study, all farinograph parameters were significantly influenced by year and variety. Dough stability, resistance, and degree of softening significantly affected by the treatment.

The water absorption of flour is an indicator for dough and bread yield [52]. Flour with good bread-making properties has higher absorption, takes longer to mix, and is more tolerant to over-mixing than poor-quality bread flour [53]. Therefore, a higher water absorption of flour leads to a higher dough yield. In the current study, we did not find any significant effect of the treatment on water absorption, as previously reported [18]. However, some studies showed at least a slight increase of water absorption with increased *Fusarium* infection [42], which might have resulted from a higher proportion of damaged starch granules in FHB-infected plants. The FHB-susceptible variety Super Zitarka had the highest decrease of water absorption after FHB inoculation in 2016/2017, compared to naturally infected plants. The varieties with higher protein content absorbed a higher amount of water [54], but according to the current study, the varieties with higher protein content showed a decreased water absorption after FHB inoculation.

Dough development time is a measure of gluten strength and increases as protein content increases [55]. Stronger flours with a higher protein content have a longer development time than weaker flours. The low-quality variety Antonija showed the greatest decrease of dough development under FHB pressure. In general, greater relative losses in dough development after FHB inoculation were obtained in 2016/2017, when increased FHB infestation occurred, compared to previous years.

In the current study, dough stability, dough resistance, and degree of softening were significantly influenced by treatment. *Fusarium* inoculations exerted strong effects on dough stability duration and dough softening during kneading [8]. Dough stability is a measurement of how well flour resists to overmixing. Strong flours are usually more stable than weak ones. Previously, positive correlations between dough development and stability were reported [56]. Therefore, Divana and Renan had increased dough stability after FHB inoculation, on average, during the three years of the study, while for 12 varieties, a decrease occurred [8].

The variety Renan showed the same pattern of dough resistance as that found for stability in the three studied years. Previously, it was found that FHB-susceptible wheat varieties were negatively affected by FHB as regards water absorption, dough softening, and dough resistance, while FHB-resistant varieties were not affected [18]. In the current study, few FHB-susceptible varieties showed low relative differences between the two treatments.

The low degree of softening indicated that gluten proteins were intact [55]. The greatest impact of *Fusarium* inoculations on dough softening was measured for the variety Divana that showed the greatest increase in samples from FHB-inoculated plots, compared with naturally infected plants. Divana, an enhanced-quality wheat, was classified as an FHB-resistant variety and showed an increased degree of softening after FHB inoculation, which can be explained by the low degree of softening after natural infection, indicating a great discrepancy between treatments. For all wheat varieties through the three examined years, FHB inoculation increased the degree of softening, which is in accordance with previous research [8].

4.3. Extensograph Analysis of Dough in FHB-Inoculated and Naturally Infected Plants

An extensograph was used to determine energy value, resistance, and extension ability of the dough obtained from different winter wheat varieties after the two treatments. By this analysis, the viscoelastic behavior of the dough was measured [57]. High resistance to extension with increased energy and long extensibility results in dough with good bread-making quality [58].

The energy value showed dough's resistance to processing and the degree of dough stretching. The higher this value, the greater the gas-holding capacity and fermentation tolerance of the dough. It was generally found that FHB-susceptible wheat varieties displayed greater decreases in energy value after FHB inoculation. The energy value should be higher than 80 cm^2 for the gas-holding capacity and fermentation tolerance of the dough to be high [59]. Only in naturally infected samples from 2014/2015, the energy value was higher than 80 cm^2, suggesting that the year with the least FHB symptoms gave the best energy results.

The ability of wheat to be processed into different products and the baking properties of flour were determined by measuring the resistance to extension and extensibility. Bread volume increases when the dough is highly resistant to extension [60]. A similar pattern of behavior as for the energy value was obtained for FHB-susceptible wheat varieties, that showed the greatest decreases in resistance to extension after FHB inoculation.

It was reported that the extensibility value increased with the protein content [61]. A similar pattern of behavior as for the energy values was obtained for FHB-susceptible wheat varieties, that showed the greatest decreases in extensibility after FHB inoculation. A decrease in the resistance to extensibility explained the difficulties in bread making [62].

The measured technological and rheological parameters confirmed that extensive *Fusarium* spp. infection worsened both sedimentation value and gluten index, with consequential effects on dough stability, resistance, and degree of softening, thus exerting a negative impact on energy values and resistance to extension. The impact of FHB inoculation on dough stability and resistance was weaker than that of the year. Previously, it was concluded that FHB inoculation significantly worsened standard technological quality parameters and rheological parameters [63]. Moreover, in the case of a very strong FHB pressure, induced by artificial inoculation, it is possible to presume that also the content of *Fusarium* mycotoxins would be high [64]. The greater the wheat grain resistance, the more reduced was the impact on dough properties [8]. Overall, *Fusarium* inoculation decreased the duration of dough stability as well as dough resistance and increased dough softening, and wheat varieties were affected differently dependently on FHB resistance/susceptibility. The most informative traits to determine quality loss as a result of FHB infection were the rheological traits such as the extensograph parameters (e.g.,135 min Energy) and farinograph dough stability [65]. In addition, dough had reduced strength, was stickier, and therefore was more difficult to handle, as energy value and resistance to extension were lower after FHB inoculation. Dough resistance and proofing time recorded with a maturograph increased in samples with a higher DON content as a consequence of greater FHB severity, whereas proofing stability, also measured with a maturograph, decreased [24]. Furthermore, the farinograph and extensograph curves showed that the presence of *Fusarium*-damaged grains decreased dough consistency and resistance to extension [44].

5. Conclusions

The effect of *Fusarium* spp. infection was visible in both sedimentation value and gluten index of wheat grains and consequently significantly influenced the rheological properties of dough, including dough stability, resistance, degree of softening, energy value, and resistance to extension. Favorable climatic conditions had the highest impact on *Fusarium* infestation in the vegetative season 2016/2017, thus causing the highest losses in technological and rheological traits. Overall, on average, *Fusarium* inoculation, compared to natural infection, decreased the duration of dough resistance and increased dough softening, and winter wheat varieties were affected differently. Fungal protease activity can destroy the gluten network, therefore reducing tolerance during dough mixing, as observed for severely infected *Fusarium* samples. It can be concluded that none of the modern wheat variety with better quality is completely resistant to the spread of *Fusarium* spp. Increasing FHB incidence and severity evidently worsened the technological quality, and its negative effects on the rheological properties of the flour were obvious in end-use quality. These results imply that the endosperm storage proteins of highly and moderately

FHB-susceptible winter wheat varieties included in this study might contain valuable genes associated with high quality, which could be transferred to bread wheat in an attempt to improve flour baking quality; however, the simultaneous incorporation of genes for FHB resistance is necessary. In contrast, some FHB-resistant varieties do not result in good end-use quality. Besides the technological properties that are well known to undergo FHB pressure, the detection of changes in end-use quality is also an important step to identify modifications that can pose new safety risks. Accordingly, the newly released wheat varieties must pose a low health risk to secure food safety standards with satisfactory end-use quality.

Author Contributions: V.S. conceived and designed the experiments; V.S., K.D., and G.D. performed the field experiments and analyzed the data; J.B. statistical analysis; V.S. wrote the paper; K.D., J.B., G.D., and Z.Z. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Snijders, C.H.A. Fusarium head blight and mycotoxin contamination of wheat, a review. *Neth. J. Plant Pathol.* **1990**, *96*, 187–198. [CrossRef]
2. Bottalico, A.; Perrone, G. Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. *Eur. J. Plant Pathol.* **2002**, *108*, 611–624. [CrossRef]
3. McMullen, M.P.; Bergstrom, G.C.; De Wolf, E.; Dill-Macky, R.; Hershman, D.E.; Shaner, G.; Van Sanford, D.A. A unified effort to fight an enemy of wheat and barley: Fusarium head blight. *Plant Dis.* **2012**, *96*, 1712–1728. [CrossRef] [PubMed]
4. Dexter, J.E.; Marchylo, B.A.; Clear, R.M.; Clarke, J.M. Effect of Fusarium head blight on semolina milling and pasta-making quality of durum wheat. *Cereal Chem.* **1997**, *74*, 519–525. [CrossRef]
5. Mauler-Machnik, A.; Suty, A. New findings on the epidemiology, importance and control of Fusarium ear blight on wheat. *Cereal Res. Commun.* **1997**, *25*, 705–709. [CrossRef]
6. Dexter, J.E.; Nowicki, T.W. Safety Assurance and Quality Assurance Issues Associated with Fusarium Head Blight in Wheat. In *Fusarium Head Blight of Wheat and Barley*; Leonard, K.J., Bushnell, W.R., Eds.; APS Press: St. Paul, MN, USA, 2003; pp. 420–460.
7. Schroeder, H.W.; Christensen, J.J. Factors affecting resistance of wheat to scab caused by *Giberella zeae*. *Phytopathology* **1963**, *53*, 831–838.
8. Martin, C.; Schöneberg, T.; Vogelgsang, S.; Vincenti, J.; Bertossa, M.; Mauch-Mani, B.; Mascher, F. Factors of wheat grain resistance to Fusarium head blight. *Phytopathol. Mediterr.* **2017**, *56*, 154–166. [CrossRef]
9. Buerstmayr, H.; Ban, T.; Anderson, J.A. QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat: A review. *Plant Breed* **2009**, *128*, 1–26. [CrossRef]
10. Shah, L.; Ali, A.; Yahya, M.; Zhu, Y.; Wang, S.; Si, H.; Rahman, H.; Ma, C. Integrated control of fusarium head blight and deoxynivalenol mycotoxin in wheat. *Plant Pathol.* **2018**, *67*, 532–548. [CrossRef]
11. Kelly, A.C.; Clear, R.M.; O'Donnell, K.; McCormick, S.; Turkington, T.K.; Tekauz, A.; Gilbert, J.; Corby Kistler, H.; Busman, M.; Ward, T.J. Diversity of Fusarium head blight populations and trichothecene toxin types reveals regional differences in pathogen composition and temporal dynamics. *Fungal. Genet. Biol.* **2015**, *82*, 22–31. [CrossRef]
12. Edwards, S.G. Fusarium mycotoxin content of UK organic and conventional wheat. *Food Addit. Contam.* **2009**, *26*, 496–506. [CrossRef]
13. Spanic, V.; Lemmens, M.; Drezner, G. Morphological and molecular identification of Fusarium species associated with head blight on wheat in East Croatia. *Eur. J. Plant Pathol.* **2010**, *128*, 511–516. [CrossRef]
14. Covarelli, L.; Beccari, G.; Prodi, A.; Generotti, S.; Etruschi, F.; Juan, C.; Ferrer, E.; Mañes, J. *Fusarium* species, chemotype characterisation and trichothecene contamination of durum and soft wheat in an area of central Italy. *J. Sci. Food Agric.* **2015**, *95*, 541–551. [CrossRef] [PubMed]

15. Brennan, J.M.; Egan, D.; Doohan, F.M. Effect of temperature on head blight of wheat caused by *Fusarium culmorum* and *F. graminearum*. *Plant Pathol.* **2005**, *54*, 156–160. [CrossRef]
16. Lacey, J.; Bateman, G.L.; Mirocha, C.J. Effects of infection time and moisture on development of ear blight and deoxynivalenol production by *Fusarium* spp. in wheat. *Ann. Appl. Biol.* **1999**, *134*, 277–283. [CrossRef]
17. Kang, Z.; Buchenauer, H. Cytology and ultrastructure of the infection of wheat spikes by *Fusarium culmorum*. *Mycol. Res.* **2000**, *104*, 1083–1093. [CrossRef]
18. Gartner, B.H.; Munich, M.; Kleijer, G.; Mascher, F. Characterisation of kernel resistance against Fusarium infection in spring wheat by baking quality and mycotoxin assessments. *Eur. J. Plant Pathol.* **2008**, *120*, 61–68. [CrossRef]
19. Khan, H. Genetic improvement for end use quality in wheat. In *Quality Breeding in Field Crops*; Qureshi, A., Dar, Z., Wani, S., Eds.; Springer: Cham, Switzerland, 2019; pp. 239–253.
20. Shewry, P.R. Wheat. *J. Exp. Bot.* **2009**, *60*, 1537–1553. [CrossRef]
21. Seling, S. Die Bedeutung des Proteingehaltes von Backweizen aus Sicht der Wissenschaft. *Getreidetechnologie* **2010**, *64*, 103–110.
22. Matthaus, K.; Danicke, S.; Vahjen, W.; Simon, O.; Wang, J.; Valenta, H.; Meyer, K.; Strumpf, A.; Ziesenib, H.; Flachowsky, G. Progression of mycotoxin and nutrient concentrations in wheat after inoculation with *Fusarium culmorum*. *Arch. Anim. Nutr.* **2004**, *58*, 19–35. [CrossRef]
23. Wang, J.H.; Pawelzik, E.; Weinert, J.; Zhao, Q.; Wolf, G.A. Factors influencing falling number in winter wheat. *Eur.Food Res.Technol.* **2008**, *226*, 1365–1371. [CrossRef]
24. Lancova, K.; Hajslova, J.; Kostelanska, M.; Kohoutkova, J.; Nedelnik, J.; Moravcova, H.; Vanova, M. Fate of trichothecene mycotoxins during the processing: Milling and baking. *Food Addit. Contam.* **2008**, *25*, 650–659. [CrossRef] [PubMed]
25. Zadoks, J.C.; Chang, T.T.; Konzac, F.C. A decimal code for the growth stages of cereals. *Weed Res.* **1974**, *14*, 415–421. [CrossRef]
26. Buerstmayr, H.; Steiner, B.; Lemmens, M.; Ruckenbauer, P. Resistance to Fusarium head blight in winter wheat: Heritability and trait associations. *Crop Sci.* **2000**, *40*, 1012–1018. [CrossRef]
27. Gerlach, W.; Nirenberg, H. The genus *Fusarium*: A pictorial atlas. *Mitteil. Biol.* **1982**, *209*, 1–406. [CrossRef]
28. Lemmens, M.; Haim, K.; Lew, H.; Ruckenbauer, P. The effect of nitrogen fertilization on Fusarium head blight development and deoxynivalenol contamination in wheat. *Phytopathology* **2004**, *152*, 1–8. [CrossRef]
29. International Association for Cereal Science and Technology. *Determination of Wet Gluten Quantity and Quality (Gluten Index ac. to Perten) of Whole Wheat Meal and Wheat Flour (Triticum aestivum)*; ICC Standard No. 155; International Association for Cereal Science and Technology: Vienna, Austria, 1994.
30. International Association for Cereal Science and Technology. *Determination of Sedimentation Value (ac. to Zeleny) as an Approximate Measure of Baking Quality*; ICC Standard No. 116/1; International Association for Cereal Science and Technology: Vienna, Austria, 1994.
31. International Association for Cereal Science and Technology. *Determination of the 'Falling Number' according to Hagberg-Perten as a Measure of the Degree of Alpha-Amylase Activity in Grain and Flour*; ICC Standard No. 107/1; International Association for Cereal Science and Technology: Vienna, Austria, 1995.
32. International Association for Cereal Science and Technology. *Method for Using the Brabender Farinograph*; ICC Standard No. 115/1; International Association for Cereal Science and Technology: Vienna, Austria, 1992.
33. International Association for Cereal Science and Technology. *Method for Using the Brabender Extensograph*; ICC Standard No. 114/1; International Association for Cereal Science and Technology: Vienna, Austria, 1992.
34. De Wolf, E.D.; Madden, L.V.; Lipps, P.E. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. *Phytopathology* **2003**, *93*, 428–435. [CrossRef]
35. Fedak, G.; Cao, W.; Xue, A.; Savard, M.; Clarke, J.; Somers, D.J. Wheat production in stressed environments. In *Development in Plant Breeding*; Buck, H.T., Nisi, J.E., Salomón, N., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 91–95.
36. Czaban, J.; Wróblewska, B.; Sułek, A.; Mikos, M.; Boguszewska, E.; Podolska, G.; Nieróbca, A. Colonisation of winter wheat grain by *Fusarium* spp. and mycotoxin content as dependent on a wheat variety, crop rotation, a crop management system and weather conditions. *Food Addit. Contam.* **2015**, *32*, 874–910. [CrossRef] [PubMed]
37. Scala, V.; Aureli, G.; Cesarano, G.; Incerti, G.; Fanelli, C.; Scala, F.; Reverberi, M.; Bonanomi, G. Climate, Soil Management, and Cultivar Affect Fusarium Head Blight Incidence and Deoxynivalenol Accumulation in Durum Wheat of Southern Italy. *Front. Microbiol.* **2016**, *7*, 1014. [CrossRef]
38. Kaya, Y.; Akcura, M. Effects of genotype and environment on grain yield and quality traits in bread wheat (*T. aestivum* L.). *Food Sci. Technol.* **2014**, *34*, 386–393. [CrossRef]
39. Schaafsma, A.W.; Tamburic-Ilinic, L.; Miller, J.D.; Hooker, D.C. Agronomic considerations for reducing deoxynivalenol in wheat grain. *Can. J. Plant Pathol.* **2001**, *23*, 279–285. [CrossRef]
40. Grausgruber, H.; Oberforster, M.; Werteker, M.; Ruckenbauer, P.; Vollmann, J. Stability of quality traits in Austrian-grown winter wheats. *Field Crops Res.* **2000**, *66*, 257–267. [CrossRef]
41. Prange, A.; Birzele, B.; Kramer, J.; Meier, A.; Modrow, H.; Kohler, P. Fusarium inoculated wheat: Deoxynivalenol contents and baking quality in relation to infection time. *Food Control* **2005**, *16*, 739–745. [CrossRef]
42. Wang, J.H.; Wieser, H.; Pawelzik, E.; Weinert, J.; Keutgen, A.J.; Wolf, G.A. Impact of the fungal protease produced by *Fusarium culmorum* on the protein quality and breadmaking properties of winter wheat. *Eur. Food Res. Technol.* **2005**, *220*, 552–559. [CrossRef]

43. Boyacioğlu, D.; Hettiarachchy, N.S. Changes in some biochemical components of wheat grain that was infected with *Fusarium graminearum*. *J. Cereal Sci.* **1995**, *21*, 57–62. [CrossRef]
44. Nightingale, M.J.; Marchylo, B.A.; Clear, R.M.; Dexter, J.E.; Preston, K.R. Fusarium Head Blight: Effect of Fungal Proteases on Wheat Storage Proteins. *Cereal Chem.* **1999**, *76*, 50–158. [CrossRef]
45. Wang, J.H.; Pawelzik, E.; Weinert, J.; Wolf, G.A. Impact of *Fusarium culmorum* on the polysaccharides of wheat flour. *J. Agric. Food Chem.* **2005**, *53*, 5818–5823. [CrossRef] [PubMed]
46. Sluková, M.; Levková, J.; Michalcova, A.; Skřivan, P.; Skřivan, P. Effect of the dough mixing process on the quality of wheat and buckwheat proteins. *Czech J. Food Sci.* **2017**, *35*, 522–531. [CrossRef]
47. Polišenská, I.; Vaculová, K.; Jirsa, O.; Sedláčková, I.; Frydrych, J. The effect of *Fusarium culmorum* on yield and grain characteristics of winter wheat cultivars. *Zemdirb. Agric.* **2020**, *107*, 113–122. [CrossRef]
48. Siuda, R.; Grabowski, A.; Leszek, L.; Ralcewicz, M.; Spychaj-Fabisiak, E. Influence of the degree of fusariosis on technological traits of wheat grain. *Int. J. Food Sci. Technol.* **2010**, *45*, 2596–2604. [CrossRef]
49. Cubadda, R.; Carcea, M.; Pasqui, L.A. Suitability of the gluten index method for assessing gluten strength in durum wheat and semolina. *Cereal Foods World* **1992**, *37*, 866–869.
50. Hareland, G.A. Effects of Pearling on Falling Number and α-Amylase Activity of Preharvest Sprouted Spring Wheat. *Cereal Chem.* **2003**, *80*, 232–237. [CrossRef]
51. Wieser, H. Chemistry of gluten proteins. *Food Microbiol.* **2007**, *24*, 115–119. [CrossRef] [PubMed]
52. Webb, C.; Owens, G.W. *Milling and flour quality In Bread Making: Improving Quality*; Cauvain, S.P., Ed.; Woodhead Publishing Limited: Cambridge, UK, 2003; pp. 200–218.
53. William, A.A. Wheat and flour testing. In *Wheat Flour*; William, A.A., Ed.; American Association of Cereal Chemists: St. Paul, MN, USA, 2001.
54. Zaidul, I.S.M.; Karim, A.A.; Manan, D.M.A.; Ariffin, A.; Norulaini, N.A.N.; Omar, A.K.M. A farinograph study on the viscoelastic properties of sago/wheat flour dough. *J. Sci. Food Agric.* **2004**, *84*, 616–622. [CrossRef]
55. Aydoğan, S.; Şahin, M.; Göçmen Akçacık, A.; Hamzaoğlu, S.; Taner, S. Relationships between farinograph parameters and bread volume, physicochemical traits in bread wheat flours. *J. Bahri Dagdas Crop. Res.* **2015**, *3*, 14–18.
56. Singh, N.; Kaur, A.; Katyal, M.; Bhinder, S.; Ahlawat, A.K.; Singh, A.M. Diversity in quality traits amongst Indian wheat varieties II: Paste, dough and muffin making properties. *Food Chem.* **2015**, *197*, 316–324. [CrossRef]
57. Rosell, C.M.; Rajan, J.A.; Benedito, D.E.; Barber, C. Influence of hydrocolloids on dough rheology and bread quality. *Food Hydrocoll.* **2001**, *15*, 75–81. [CrossRef]
58. Shelton, D. *Wheat and Flour Testing Methods: A Guide to Understanding Wheat and Flour Quality*, 2nd ed.; Kansas State University: Manhattan, KS, USA, 2008.
59. Elgün, A.; Türker, S.; Bilgiçli, N. *Tahıl ve Ürünlerinde Analitik Kalite Kontrolü*; Selçuk University Ziraat Fakültesi Gıda Mühendisliği Bölümü: Konya, Turkey, 2001.
60. Hruskova, M.; Smejda, P. Wheat flour dough alveograph characteristics predicted by NIR systems 6500. *Czech J. Food Sci.* **2003**, *21*, 28–33. [CrossRef]
61. Salazar-Garcıa, M.G.; Torres, P.I.; Reyes-Moreno, C.; Ramirez-Wong, B. Extensional flow studies on wheat flour dough swith different protein content. *J. Text Stu.* **2003**, *34*, 449–464. [CrossRef]
62. Maforimbo, E.; Skurray, G.; Uthayakumaran, S.; Wrigley, C.W. Improved functional properties for soy–wheat doughs due to modification of the size distribution of polymeric proteins. *J. Cereal Sci.* **2006**, *43*, 223–229. [CrossRef]
63. Capouchová, I.; Papoušková, L.; Konvalina, P.; Vepříková, Z.; Dvořáček, V.; Zrcková, M.; Janovská, D.; Škeříková, A.; Pazderů, K. Effect of *Fusarium* spp. Contamination on Baking Quality of Wheat. In *Wheat Improvement, Management and Utilization*; Wanyera, R., Owuoche, J., Eds.; IntechOpen: London, UK, 2017. [CrossRef]
64. Smith, K.P.; Evans, C.K.; Dill-Macky, R.; Gustus, C.; Xie, W.; Dong, Y. Host genetic effect on deoxynivalenol accumulation in Fusarium head blight of barley. *Phytopathology* **2004**, *94*, 766–771. [CrossRef] [PubMed]
65. Ács, K.; Lehoczki-Krsjak, S.; Varga, M.; Kótai, C.; Ács, E.; Salgó, A.; Mesterházy, Á. Reduction of deoxynivalenol (DON) contamination by improved fungicide use in wheat. Part 3. Reduction of Fusarium head blight and influence on quality traits in cultivars with different resistance levels. *Eur. J. Plant Pathol.* **2018**, *151*, 21–38. [CrossRef]

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Photosynthetic Efficiency in Flag Leaves and Ears of Winter Wheat during Fusarium Head Blight Infection

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Abstract: Fusarium head blight (FHB) is one of the most serious fungal diseases of wheat (*Triticum aestivum* L.). It causes major reduction of grain yield and quality, while the safety of wheat products is at risk due to mycotoxin contaminations. To contribute to a better understanding of mechanisms governing more efficient defense strategies against FHB, an evaluation of photosynthetic efficiency was performed during different phases of infection, i.e., before visual symptoms occur, at the onset and after the development of disease symptoms. Six different winter wheat varieties were artificially inoculated with the most significant causal agents of FHB (*Fusarium graminearum* and *F. culmorum*) at two different locations. Photosynthetic efficiency was assessed in flag leaves and ears of inoculated and untreated (control) plants based on measurements of chlorophyll *a* fluorescence rise kinetics and the calculation of JIP-test parameters. Obtained results indicate that the response of wheat to *Fusarium* infection includes changes in photosynthetic efficiency which can encompass alternating reductions and increases in photosynthetic performance during the course of the infection in both flag leaves and ears. FHB-induced photosynthetic adjustments were shown to be somewhat variety-specific, but location was shown to be a more significant factor in modulating the response of wheat to *Fusarium* infection. Changes in chlorophyll *a* fluorescence rise kinetics could be detected prior to visible symptoms of the disease. Therefore, this method could be applied for the early detection of *Fusarium* infection, particularly the analysis of L-band appearance, which showed a similar response in all inoculated plants, regardless of variety or location.

Keywords: chlorophyll *a* fluorescence; *Fusarium* spp.; OJIP kinetics; photosynthesis

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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the world's most important cereal crops. Global demand for wheat is growing but achieving yield and quality increases is challenging [1]. Along with the decreasing availability of suitable agricultural land, climate change, and unfavorable abiotic conditions, wheat production is significantly affected by pathogenic fungi [2]. Fusarium head blight (FHB), which is among the most serious wheat diseases, is caused by several *Fusarium* spp., such as *F. graminearum*, *F. culmorum*, and *F. avenaceum*, and other related fungi [3]. Infection with *Fusarium* fungi significantly reduces grain yield and causes poor wheat grain quality [4–6]. In addition, wheat grains and products become contaminated with mycotoxins produced by FHB pathogens, which are harmful for humans and animals [7–9].

Appropriate crop management practice and selected chemical treatments may lower losses associated with FHB. Along with this, the selection of more FHB resistant wheat genotypes, which are able to retain adequate grain yield and quality and low mycotoxin contamination when exposed to FHB, represents a long-term sustainable strategy of wheat

production. Furthermore, fungicide treatment was shown to be more effective at reducing FHB severity and decreasing the accumulation of the main *Fusarium* mycotoxin deoxynivalenol (DON) in moderately resistant, compared to highly FHB susceptible varieties [10]. However, selecting for such genotypes is hampered by the complexity of FHB resistance traits [11,12]. The mechanisms governing more efficient defense against FHB are not fully uncovered [13]. The resistance reaction of wheat to *Fusarium* infection includes the following components: Type I, resistance to initial infection; Type II, resistance to spread of symptoms [14]; Type III, resistance to toxin accumulation [15]; Type IV, resistance to kernel infection [16,17]; Type V, yield tolerance [16,17].

In general, pathogen attack can induce significant changes in secondary metabolic processes associated with plant defense, but it can also impair primary metabolism, such as photosynthesis, assimilate partitioning, and source–sink regulation [18]. During *Fusarium* infection, various changes at the physiological and molecular level can be detected. These changes affect wheat ears where infection is located, but other parts of the infected plant such as leaves can also be affected [13,19,20]. FHB was shown to be associated with changes in oxidative stress levels and modifications in antioxidative response as well as callose deposition, which are considered to be related to FHB resistance [13,20–23]. It was also implicated that photosynthesis is interconnected with plant immune defense against *Fusarium* species [24]. More significant reduction of net photosynthesis and stomatal conductance of flag leaves was detected in a resistant, compared to susceptible wheat sister line, while the opposite effect was observed for yield components [19]. A possible role of photosynthesis in response to FHB was also indicated by changes of leaf and ear transcriptome [13], while Ajigboye et al. [25] pointed to changes in photosystem II (PSII) photochemistry in detached glumes caused by different *Fusarium* species. However, in some studies, a correlation between leaf photosynthetic efficiency and *Fusarium* infection was not indicated [26].

In C3 cereals, such as wheat, the flag leaf is considered to be the main photosynthetic tissue, but the ear is also photosynthetically active and can contribute to final grain yield, especially under unfavorable growth conditions [27]. The evaluation of photosynthetic efficiency of flag leaves and ears of wheat in this study was based on the measurement of chlorophyll *a* fluorescence rise kinetics (OJIP kinetics) combined with a multiparametric analysis of the recorded fluorescence transients (JIP-test). This is an efficient and non-invasive approach to obtain insights into the functioning of photosynthetic machinery [28–30]. It has been intensively used for the investigation of abiotic stress response in various plant species and the adaptation of plants to different growth conditions [31–37]. Chlorophyll *a* fluorescence (ChlF) measurements were also used for the evaluation of the plant's response to pathogen attack and disease severity [25,38–41]. The aim of this study was to provide a better understanding of the response of the photosynthetic apparatus of different winter wheat varieties to *Fusarium* infection and to explore the possible role of FHB-induced changes in photosynthetic efficiency in overall disease outcome. Since the impact of FHB as well as defense strategies can vary significantly between different wheat varieties and due to specific growth conditions and/or agricultural practices [42,43], the evaluation of dynamic of FHB-induced changes in photosynthetic efficiency was performed at two different locations.

2. Materials and Methods

2.1. Plant Material

The experiment was conducted on six winter wheat (*Triticum aestivum* L.) varieties from Agricultural Institute Osijek: Golubica, El Nino, Galloper, Tika Taka, Vulkan, and Kraljica. Golubica is a winter variety with excellent quality, belonging to A1 quality group, with more than 14% protein content. However, it is subject to underproduction due to its high susceptibility to FHB. El Nino is a winter variety with plant height of 89 cm. It has tolerance to low temperatures and mild drought. Furthermore, it has medium quality properties with average 1000 kernel weight of 41 g. Galloper is a medium early variety

with plant height of 92 cm. Galloper has an average 1000 kernel weight is 45 cm with medium quality properties (B1 quality group). Plant regulators are recommended for good efficiency of genetic potential. It has good resistance to most widespread wheat diseases. Tika Taka is a new high yielding variety belonging to A2-B1 quality group, the same quality group as Vulkan. It has good lodging resistance with plant height of 82 cm and 1000 kernel weight of 42 g. Vulkan is a winter variety with owns, with high genetic potential. It has a large number of productive tillers per unit area but is taller than Kraljica with plant height of 87 cm. Kraljica is a high yielding winter wheat variety which belongs to A2 quality group. It is also the most widespread variety in Croatia. Kraljica has an average 1000 kernel weight of 40 g. It demonstrates tolerance to low temperatures and good resistance to lodging.

2.2. Field Experiments

The experiment was conducted during the vegetative season 2019/2020 at two experimental locations: Osijek (45°27′ N, 18°48′ E) and Tovarnik (45°10′ N, 19°09′ E). Those two locations differ in soil type and climatic condition [44]. At Osijek, the soil is eutric cambisol (pHKCl–6.25, humus–2.00–2.20%), while Tovarnik has black soil chernozem (pHKCl–7.42, humus–2.75–3.00%). At both locations, a completely randomized block design was applied. Wheat was sown during October 2019 in 7.56 m^2 plots. The agrotechnical practice used was standard for commercial wheat production, except for fungicide application which was omitted in this experiment. The experiment included two different treatments (*Fusarium* inoculation and untreated control) where one treatment consisted of two replications (two plots). Each wheat variety was planted in four plots at each location and same experimental design was used at both locations (Osijek and Tovarnik). Plant density was 3000–3500 plants per 7.56 m^2.

In order to assess the effects of FHB on photosynthesis, artificial inoculation on selected wheat varieties was performed at the flowering stage (Zadok's stage 65) [45] at two replicated plots with *Fusarium* inoculum composed of 1:1 *F. graminearum* stain PIO 31 (obtained from the wheat in East Croatia) and *F. culmorum* strain IFA 104 (obtained from IFA, Tulln, Austria). Plants from untreated plots were used as a control in the experiment. Inoculum preparation and inoculation were performed as previously described [44]. Disease severity (general resistance) was estimated based on the percentage of bleached spikelets per plot according to a linear scale (0–100%) at 10, 14, 18, 22, and 26 days after inoculation. All these values were used to calculate the area under the disease progress curve (AUDPC) for FHB severity [46] and additionally values for days when ChlF was recorded (10 and 18 dpi) are presented. Disease incidence (Type I resistance) was calculated as the percentage of diseased ears after assessing a random sample of 30 heads at 10, 14, 18, 22, and 26 days after inoculation. All these values were used to calculate the area under the disease progress curve (AUDPC) for Type I resistance [46]. Additionally, values for days when ChlF was recorded (10 and 18 dpi) are presented. Agronomical and quality traits for all varieties included in this study have been assessed at both Osijek and Tovarnik in season 2019/2020 [44]. The grain yield was measured by harvesting the whole area of each plot followed by correction to 14% moisture (on a wet basis) and expressed as dt ha^{-1}.

2.3. Measurement and Analysis of Fast Cholophyll a Fluorescence

The chlorophyll *a* fluorescence (ChlF) measurements on flag leaves and ears of artificially inoculated and untreated wheat were performed at three measurement points: (1) prior to the development of symptoms at three days post-inoculation (dpi) (Zadok's stage 65 [45]); (2) at the onset of first visible disease symptoms at 10 dpi (Zadok's stage 70 [45]); and (3) after the development of disease symptoms at 18 dpi (Zadok's stage 75 [45]).

The OJIP fluorescence transients were measured with a Handy-PEA fluorimeter (Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn, Norfolk, England). At both locations (Osijek and Tovarnik), for each of the six varieties, 20 plants (10 from each plot) from inoculated plots and 20 plants (10 from each plot) from untreated plots were analyzed

by performing measurements on flag leaves and ears. After dark adaptation for 30 min, ChlF transients were induced by red light (peak at 650 nm) of 3000 μmol photons m^{-2} s^{-1} and recorded for 1 s with 12 bit resolution. The JIP-test was applied to analyze and compare ChlF transients [28,30]. JIP-test parameters included in this study are presented in Table 1. Additionally, transients were normalized as relative variable fluorescence (W_{OP}, W_{OJ}, W_{OK}) and presented as difference kinetics ($\Delta W = W_{inoculated} - W_{untreated}$) between plants inoculated with *Fusarium* fungi and untreated control plants. The difference kinetics ΔW_{OP} was used to reveal major changes occurring in the O–J and O–I phases after *Fusarium* inoculation. The difference kinetics ΔW_{OJ} is used to reveal K band (at 300 μs) which, when positive, is considered to reflect an inactivation of the oxygen evolving complex and/or an increase of the functional PSII antenna size. The difference kinetics ΔW_{OK} is used to reveal the L band (at 150 μs), which is positive when energetic connectivity (grouping) between the PSII photosynthetic units is lower in inoculated plants, compared to untreated control plants, and negative when energetic connectivity is higher in inoculated plants, compared to untreated control plants [28,30].

Table 1. List of JIP-test parameter used in the study including their calculations and descriptions [28–30]. Parameters written in bold are presented in the study and other parameters included in the table are required for their calculation.

Data extracted from the recorded ChlF transient	
Minimal fluorescence intensity at 20 ms—O step	F_0
Maximal fluorescence intensity—P step	F_m
Fluorescence intensity at 300 μs	F_{300}
Fluorescence intensity at 2 ms—I step	F_I
Fluorescence intensity at 30 ms—J step	F_J
Fluorescence intensity at any time t	Ft
Time (in ms) to reach maximal fluorescence F_M	t_{FM}
Total complementary area between fluorescence induction curve and F = FM	Area
Fluorescence parameters derived from the extracted data	
Maximal variable fluorescence	$F_V = F_m - F_0$
Relative variable fluorescence at 2 ms (J step)	$V_J = (F_J - F_0)/(F_m - F_0)$
Relative variable fluorescence at 30 ms (I step)	$V_I = (F_I - F_0)/(F_m - F_0)$
Relative variable fluorescence normalized to the amplitude at any time t	$W_{OP} = (Ft - F_0)/(F_m - F_0)$
Relative variable fluorescence normalized to the amplitude of the O–J phase (K-band)	$W_{OJ} = (F_t - F_0)/(F_J - F_0)$
Relative variable fluorescence normalized to the amplitude of the O–K phase (L-band)	$W_{OK} = (F_t - F_0)/(F_K - F_0)$
Approximated initial slope (in ms^{-1}) of the fluorescence transient V = f(t)	$M_0 = (dV/dt)_0 = 4 \times (F_{300} - F_0)/(F_M - F_0)$
Quantum yields and efficiencies	
Maximum quantum yield for primary photochemistry	$TR_0/ABS \equiv \varphi_{P0} = [1 - (F_0/F_M)] = F_v/F_M$
Quantum yield for electron transport (ET)	$ET_0/ABS \equiv \varphi_{E0} = (1 - F_0/F_M) \times (1 - V_J)$
Efficiency/probability for electron transport (ET), i.e., efficiency/probability that an electron moves further than Q_A^-	$ET_0/TR_0 \equiv \psi_{E0} = (1 - VJ)$
Efficiency/probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the photosystem I (PSI acceptor) side (RE)	$RE_0/ET_0 \equiv \delta_{R0} = (1 - V_I)/(1 - V_J)$

Table 1. *Cont.*

Quantum yields and efficiencies	
Probability that PSII chlorophyll (Chl) molecule functions as RC	$\gamma_{RC} = Chl_{RC}/Chl_{total} = RC/(ABS + RC)$
Q_A-reducing RCs per PSII antenna Chl (reciprocal of ABS/RC)	$RC/ABS = \gamma_{RC}/(1 - \gamma_{RC}) = \varphi_{P0}\,(V_J/M_0)$
Specific energy fluxes per active (Q_A- reducing) photosystem II (PSII) reaction center (RC)	
Absorption flux (of antenna Chls) per active RC	$ABS/RC = M_0 \times (1/V_J) \times (1/\varphi_{P0})$
Trapping flux (leading to Q_A reduction) per active RC	$TR_0/RC = M_0 \times (1/V_J)$
Electron transport flux (further than Q_A^-) per active RC	$ET_0/RC = M_0 \times (1/V_J) \times \psi_{E0}$
Dissipation flux per active RC	$DI_0/RC = (ABS/RC) - (TR_0/RC)$
Performance indexes	
Performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors	$PI_{ABS} = [\gamma_{RC}/(1 - \gamma_{RC})] \times [\varphi_{P0}/(1 - \varphi_{P0})] \times [\psi_{E0}/(1 - \psi_{E0})]$
Performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors	$PI_{TOTAL} = PI_{ABS} \times (\delta_{R0}/1-\delta_{R0})$

2.4. Statistical Analysis

Statistical data analysis included factorial analysis of variance followed by post-hoc Tukey's HSD (honestly significant difference) test in order to determine statistically significant differences among artificially inoculated and untreated plants of each variety separately. For statistical analysis, Statistica 13.4.0.14 software (TIBCO Software Inc., Palo Alto, CA, USA) was used. Differences were considered significant at $p < 0.05$.

3. Results

3.1. FHB Severity, Incidence and Effect on Grain Yield

FHB severity and incidence were assessed for the first time at 10 days post inoculation (dpi) and assessment continued every four days until 26 dpi in order to calculate AUDPC for general FHB and AUDPC for Type I resistance (Table 2). Symptoms of FHB were not visible at 3 dpi for any of tested varieties. However, FHB symptoms became visible at all *Fusarium* inoculated plots during the experiment, indicating the success of applied artificial inoculation. Moreover, symptoms of FHB were not visible on wheat untreated with *Fusarium* spp. during the experiment at both Osijek and Tovarnik. FHB severity varied between 0 and 2.5 at 10 dpi and between 0 and 22.5 at 18 dpi (Table 2). AUDPC for FHB severity was the highest for El Nino, followed by Golubica at both locations, and it was lowest for Galloper, compared to other varieties at Osijek. Meanwhile, at Tovarnik, Galloper and Kraljica had the same AUDPC for FHB severity. FHB incidence at 10 dpi varied between 0 and 11.5 and between 3 and 23.5 at 18 dpi (Table 2). AUDPC for Type I resistance at Osijek was the highest for El Nino, but it was also quite high for Golubica, Tika Taka, and Kraljica varieties, while it was lower for Galloper and Vulkan. At Tovarnik, differences in AUDPC for Type I resistance between varieties were more pronounced with El Nino showing a much higher value compared to other varieties, and the lowest value was obtained for Vulkan followed by Kraljica and Galloper.

Table 2. Fusarium head blight (FHB) severity at 10 days post inoculation (dpi) and 18 dpi, AUDPC for general FHB (calculated from the percentage of bleached spikelets per plot at 10, 14, 18, 22 and 26 dpi), FHB incidence at 10 dpi and 18 dpi, AUDPC for Type I resistance (calculated from the percentage of diseased ears after assessing a random sample of 30 heads at 10, 14, 18, 22 and 26 days after inoculation) and grain yield (GY) decrease for six winter wheat varieties at two different locations (Osijek and Tovarnik).

Variety	FHB Severity at 10 dpi	FHB Severity at 18 dpi	AUDPC for FHB Severity	FHB Incidence at 10 dpi	FHB Incidence at 18 dpi	AUDPC for Type I Resistance	GY Decrease (%)
Osijek							
Golubica	1.5	7.5	104	4.85	22	222	43
El Nino	0.5	9	137	3	23.5	244	39
Galloper	0	0	1	1.5	3	33	8
Tika Taka	0.5	3	43	3.5	18.5	215	25
Vulkan	1	2	36	4.85	8.35	120	16
Kraljica	2	4.5	72	6.85	18.5	217	9
Tovarnik							
Golubica	0	3	93	0	4.85	111	59
El Nino	2.5	22.5	213	11.5	42	421	54
Galloper	0	1	18	0	4.85	87	13
Tika Taka	0	2	70	0	11.85	138	34
Vulkan	0	1.5	34	0	3	51	29
Kraljica	0	0.5	18	1.5	3	80	14

Fusarium inoculation caused a decrease in the grain yield of all tested varieties at both locations. The grain yield decrease was lowest for Galloper, compared to other varieties at both locations, while Golubica and El Nino showed more pronaunced grain yield reduction. All varieties showed lower grain yield at Tovarnik compared to Osijek and stronger yield reduction due to *Fusarium* infection.

3.2. Phytosynthetic Parameters in Flag Leaves

Location, variety, and treatment significantly affected all three quantum yields (TR_0/ABS, ET_0/ABS, ET_0/TR_0), while measurement time point significantly influenced only the maximum quantum yield for primary photochemistry (TR_0/ABS) and probability that an electron moves further than Q_A^- (ET_0/TR_0), while it did not affect quantum yield for electron transport (ET_0/TR_0) (Table 3). Specific energy fluxes per active (Q_A-reducing) photosystem II (PSII) reaction center (RC), including absorption, trapping, electron transport, and dissipation flux (ABS/RC, TR_0/RC, ET_0/RC, DI_0/RC), were affected by all tested factors, as was the performance index for energy conservation from exciton to the reduction of PSI end acceptors (PI_{TOTAL}). The performance index for energy conservation from exciton to the reduction of intersystem electron acceptors (PI_{ABS}) was affected by location, variety and measurement time point, while the efficiency with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor (RE_0/ET_0) was affected by variety, treatment, and measurement time point. Interactions of tested factors are further presented in Table 3. The interaction of all four tested factors significantly affected ET_0/ABS, ET_0/TR_0, both performance indexes (PI_{ABS} and PI_{TOTAL}) and RE_0/ET_0, but not specific energy fluxes per active RC.

Table 3. Mean squares from the analysis of variance for selected JIP-test parameters measured in flag leaves of six wheat varieties at two different locations (Osijek and Tovarnik) and three measurement points (3, 10 and 18 dpi). Treatment refers to artificially inoculated plants and untreated control plants. Description of used JIP-test parameters is presented in Table 1.

		MS									
Source of Variation	**df**	**TR_0/ABS**	**ET_0/ABS**	**ET_0/TR_0**	**ABS/RC**	**TR_0/RC**	**ET_0/RC**	**DI_0/RC**	**PI_{ABS}**	**RE_0/ET_0**	**PI_{TOTAL}**
Location (L)	1	0.0164 *	0.0844 *	0.0695 *	0.2011 *	0.4948 *	0.8105 *	0.0650 *	15.371 *	0.0016 ns	11.838 *
Variety (V)	5	0.0008 *	0.0100 *	0.0116 *	0.4886 *	0.2943 *	0.0417 *	0.0283 *	6.5287 *	0.0675 *	7.4672 *
Treatment (T)	1	0.0014 *	0.0120 *	0.0257 *	1.1816 *	0.6566 *	0.6015 *	0.0765 *	0.1362 ns	0.0163 *	2.2237 *
Measurement point (M)	2	0.0010 *	0.0013 ns	0.0043 *	2.5874 *	1.5735 *	0.6717 *	0.1263 *	3.5233 *	0.3737 *	52.807 *
L × V	5	0.0003 ns	0.0009 ns	0.0009 ns	0.0406 ns	0.0226 ns	0.0108 ns	0.0042 ns	0.4499 ns	0.0121 *	1.5749 *
L × T	1	0.0008 *	0.0286 *	0.0512 *	1.1390 *	0.6550 *	0.8227 *	0.0665 *	0.6890 ns	0.0014 ns	0.2751 ns
V × T	5	0.0002 ns	0.0014 ns	0.0014 ns	0.2623 *	0.1551 *	0.0378 *	0.0149 *	1.3500 *	0.0015 ns	1.5473 *
L × M	2	0.0005 *	0.0243 *	0.0399 *	1.3478 *	0.9452 *	0.7080 *	0.0401 *	2.1542 *	0.0398 *	0.3673 ns
V × M	10	0.0004 *	0.0021 *	0.0020 *	0.1023 *	0.0587 *	0.0206 *	0.0081 *	1.1179 *	0.0028 *	0.5757 *
T × M	2	0.0023 *	0.0007 ns	0.0000 ns	0.8547 *	0.4265 *	0.1553 *	0.0748 *	4.6259 *	0.0105 *	1.2659 *
L × V × T	5	0.0001 ns	0.0018 *	0.0022 *	0.0632 ns	0.0415 *	0.0112 ns	0.0028 ns	0.8940 *	0.0044 *	0.3705 ns
L × V × M	10	0.0003 ns	0.0021 *	0.0024 *	0.0865 *	0.0448 *	0.0169 *	0.0077 *	0.9259 *	0.0015 ns	0.3350 ns
L × T × M	2	0.0009 *	0.0107 *	0.0159 *	0.1900 *	0.0870 *	0.1127 *	0.0199 *	3.4890 *	0.0046 *	4.1613 *
V × T × M	10	0.0001 ns	0.0002 ns	0.0003 ns	0.0283 ns	0.0164 ns	0.0036 ns	0.0024 ns	0.2175 ns	0.0018 ns	0.3449 ns
L × V × T × M	10	0.0002 ns	0.0015 *	0.0018 *	0.0339 ns	0.0199 ns	0.0092 ns	0.0033 ns	0.6381 *	0.0033 *	0.5676 *

ns—not significant; *—statisticaly significant at $p < 0.05$; df—degrees of freedom; MS—mean sum of squares.

The chlorophyll *a* fluorescence (ChlF) transients of the dark-adapted flag leaves at three measurement points had the expected OJIP shape for all untreated control plants as well as for FHB inoculated plants of all six winter wheat varieties at both experimental locations (Osijek and Tovarnik), demonstrating that all measured flag leaves were photosynthetically active (data not shown). Differences in average variable fast fluorescence transients between O and P steps in flag leaves are presented as difference kinetics ΔW_{OP} (Figure 1). It revealed differences between tested varieties in the changes occurring in the O–J and O–I phase as well as considerable impact of the environment on ChlF, especially before and at the onset of symptoms development. Negative peaks at J-step can be seen at 3 dpi in all varieties (Figure 1a–f) at Osijek, but this was not observed at location Tovarnik where *Fusarium* infection had opposite effect at J-step (Figure 1g–l). Negative peaks at J-step were also visible for variety El Nino (Figure 1b), Galloper (Figure 1c), Vulkan (Figure 1e), and Kraljica (Figure 1f) at 10 dpi at Osijek, but only for variety Golubica at location Tovarnik (Figure 1g). The effect of FHB at both locations at 18 dpi was similar at J-step for varieties Golubica (Figure 1a,g), Galloper (Figure 1c,i), Tika Taka (Figure 1d,j), and Vulkan (Figure 1e,k) and opposite for variety El Nino (Figure 1b,h) and Kraljica (Figure 1f,l). At location Osijek,

negative amplitude at I-step was observed for Golubica (Figure 1a), Tika Taka (Figure 1d), and Vulkan (Figure 1e) at 3 dpi and only for Tika Taka (Figure 1d) at 10 dpi, while the effect of FHB at I-step was relatively small at 18 dpi compared to other measurement points for all varieties. Moreover, the impact of FHB on amplitude of I-step at location Tovarnik was mostly not very pronounced at 3 dpi (Figure 1g–l).

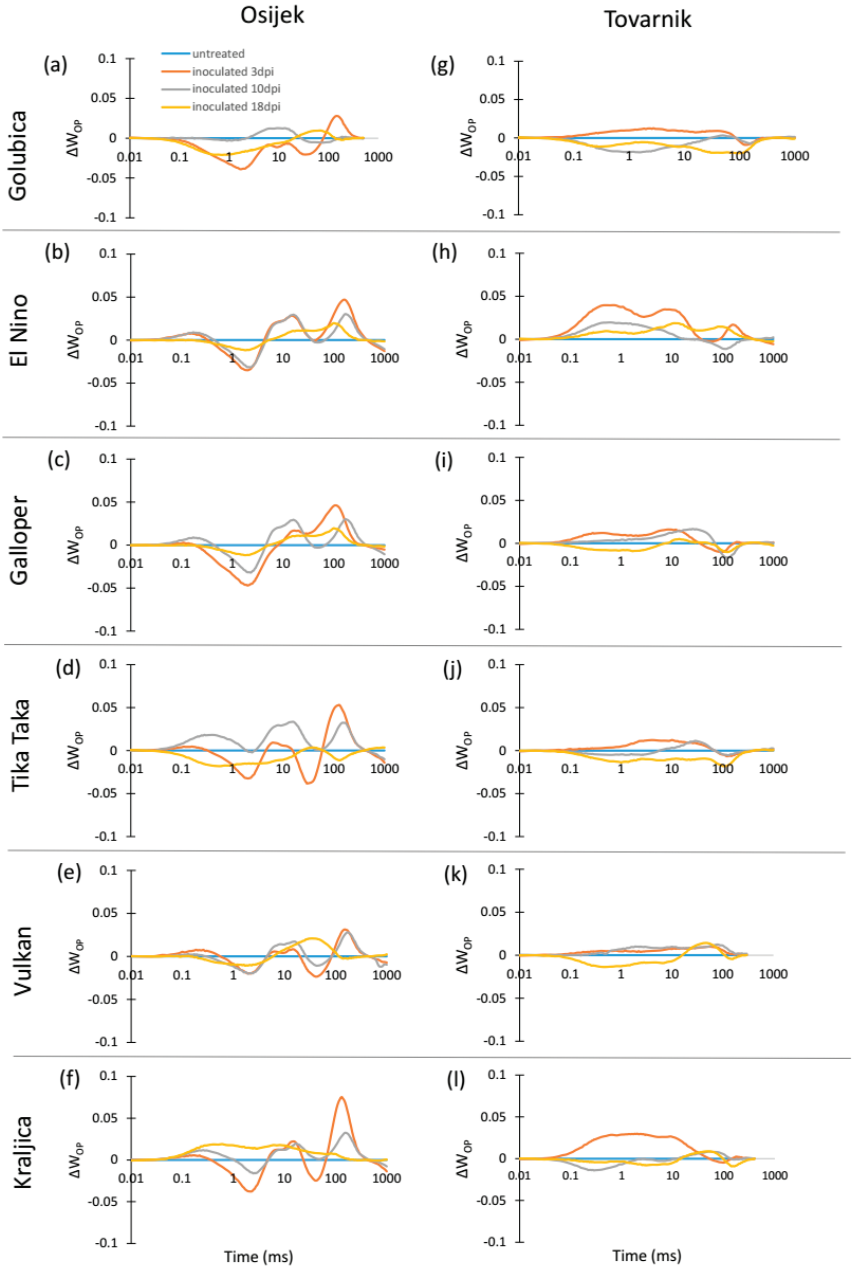

Figure 1. Changes in the shape of the chlorophyll *a* fluorescence transient curves in flag leaves of winter wheat variety Golubica (**a**,**g**), El Nino (**b**,**h**), Galloper (**c**,**i**), Tika Taka (**d**,**j**), Vulkan (**e**,**k**) and Kraljica (**f**,**l**) measured at 3 (orange), 10 (gray)

and 18 (yellow) days post-inoculation (dpi) at locations Osijek and Tovarnik. Each curve represents average kinetics of 20 replicates and time (ms) refers to time after onset of illumination during chlorophyll *a* fluorescence measurement. Average fluorescence data were normalized between O–P steps and plotted as difference kinetics ΔW_{OP}. Average values measured in corresponding untreated control plants were used as referent value for each variety, measurement point and location.

The occurrence of K-bands and L-bands in flag leaves after FHB inoculation is presented at Figure 2. For variety Golubica, only small differences in K-band are induced by FHB at both locations (Figure 2a), while El Nino showed a positive K-band, especially in earlier phases of infection (Figure 1b). Galloper had a less pronounced positive K-band than El Nino, which at location Tovarnik was visible only at 3 dpi (Figure 2c). Variety Tika Taka at location Osijek had a positive K-band at 3 dpi and 10 dpi and negative K-band at 18 dpi, while the effect of FHB on K-band of this variety at location Tovarnik was very small (Figure 2d). The effect of FHB on K-band of variety Vulkan was also less pronounced at Tovarnik compared to Osijek (Figure 2e). Variety Kraljica showed a positive K-band at all three measurements at Osijek with the highest peak observed at 3 dpi, while at Tovarnik it had a positive K-band only at 3 dpi, and a negative K-band was visible at 10 dpi in Kraljica (Figure 2f). Compared to the variable effect of *Fusarium* infection on the occurrence and amplitude of K-band, all plants infected by *Fusarium* at both locations showed similar patterns in L-band occurrence (Figure 2g–l). Overall, the L-band was mostly positive for all inoculated plants during different phases of infection, but the most considerable effect of FHB is the induction of a high positive L-band at 3 dpi for all six varieties.

Changes in JIP-test parameters in flag leaves after inoculation with *F. graminearum* + *F. culmorum* spores for each variety separately at location Osijek are presented in Figure 3a–f. *Fusarium* treatment caused some changes in flag leaves of treated plants, compared to untreated control plants before (3 dpi) and at the onset of symptoms development (10 dpi) at location Osijek. However, at 18 dpi, the difference between treated and untreated plants at this location was not detected in flag leaves for any of the tested varieties. For variety El Nino at 3 dpi, TR_0/ABS was slightly decreased (Figure 3b), but for all other varieties at all measuring time points at location Osijek *Fusarium* infection did not affect TR_0/ABS (Figure 3a,c–f). Furthermore, *Fusarium* treatment at location Osijek did not induced changes in PI_{ABS}. Performance index PI_{TOTAL} was also generally unchanged in treated compared to control plants, except for the decreased value of PI_{TOTAL} for variety El Nino at 3 dpi (Figure 3b) and variety Tika Taka at 10 dpi (Figure 3d). Variety Tika Taka showed statistically significant increase of all four specific energy fluxes per active RC (ABS/RC, TR_0/RC, ET_0/RC, DI_0/RC) at 3 and 10 dpi (Figure 3d) and similar was observed for El Nino, except that change of DI_0/RC in flag leaves of El Nino was not statistically significant. Additionally, ABS/RC and TR_0/RC were also affected in variety Vulkan (Figure 3e) at 3 dpi (Figure 3b,e) and in variety Kraljica at 3 and 10 dpi (Figure 3f).

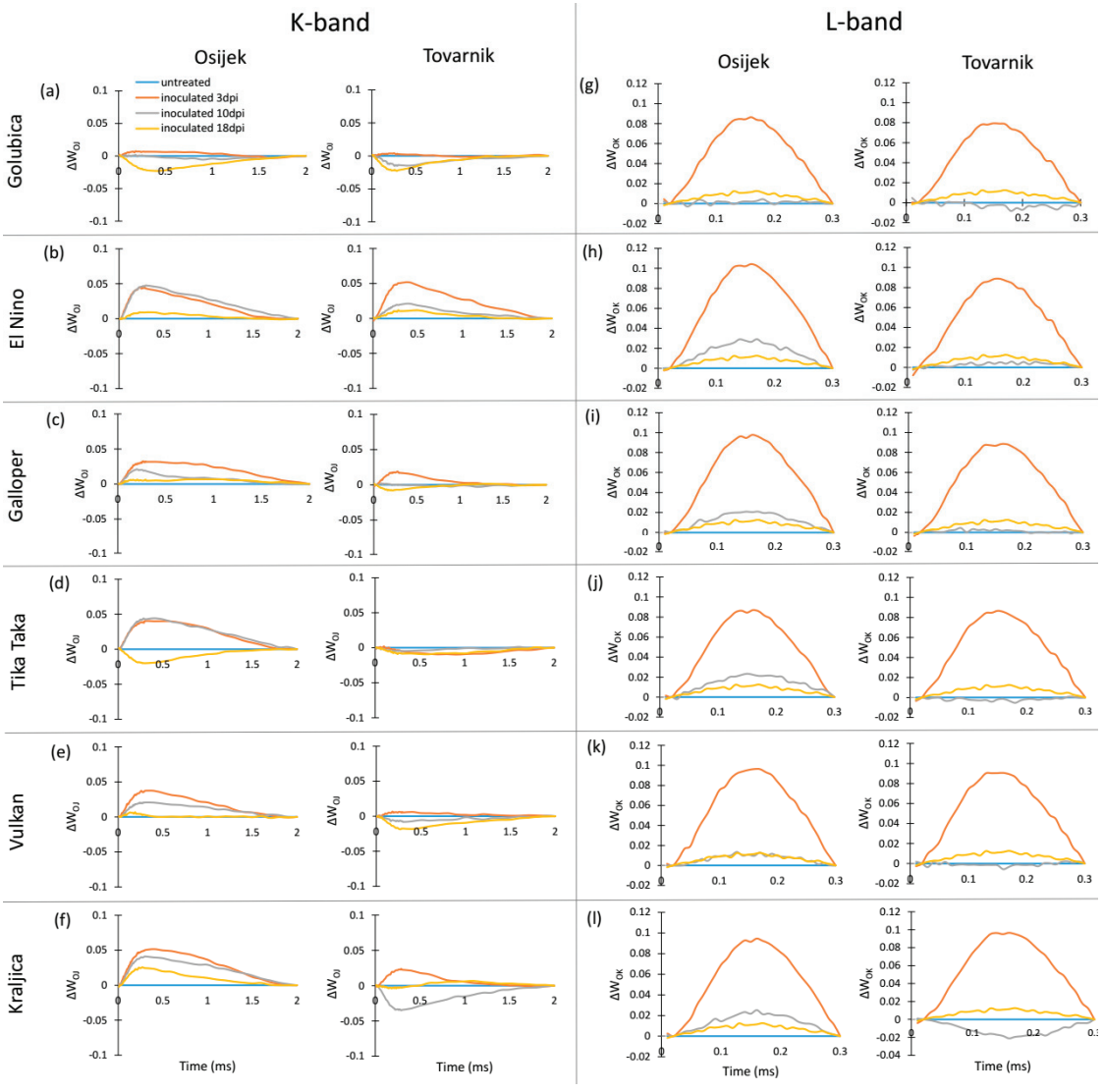

Figure 2. Changes in the shape of the chlorophyll *a* fluorescence transient curves in flag leaves of winter wheat variety Golubica (**a**,**g**), El Nino (**b**,**h**), Galloper (**c**,**i**), Tika Taka (**d**,**j**), Vulkan (**e**,**k**) and Kraljica (**f**,**l**) measured at 3 (orange), 10 (gray) and 18 (yellow) days post-inoculation (dpi) at locations Osijek and Tovarnik. Each curve represents average kinetics of 20 replicates and time (ms) refers to time after onset of illumination during chlorophyll *a* fluorescence measurement. Average fluorescence data were normalized between O–J steps (K-band) and between O-K steps (L-band) and plotted as difference kinetics ΔW_{OJ} (**a**–**f**) and ΔW_{OK} (**g**–**l**). Average values measured in corresponding untreated control plants were used as referent value for each variety, measurement point and location.

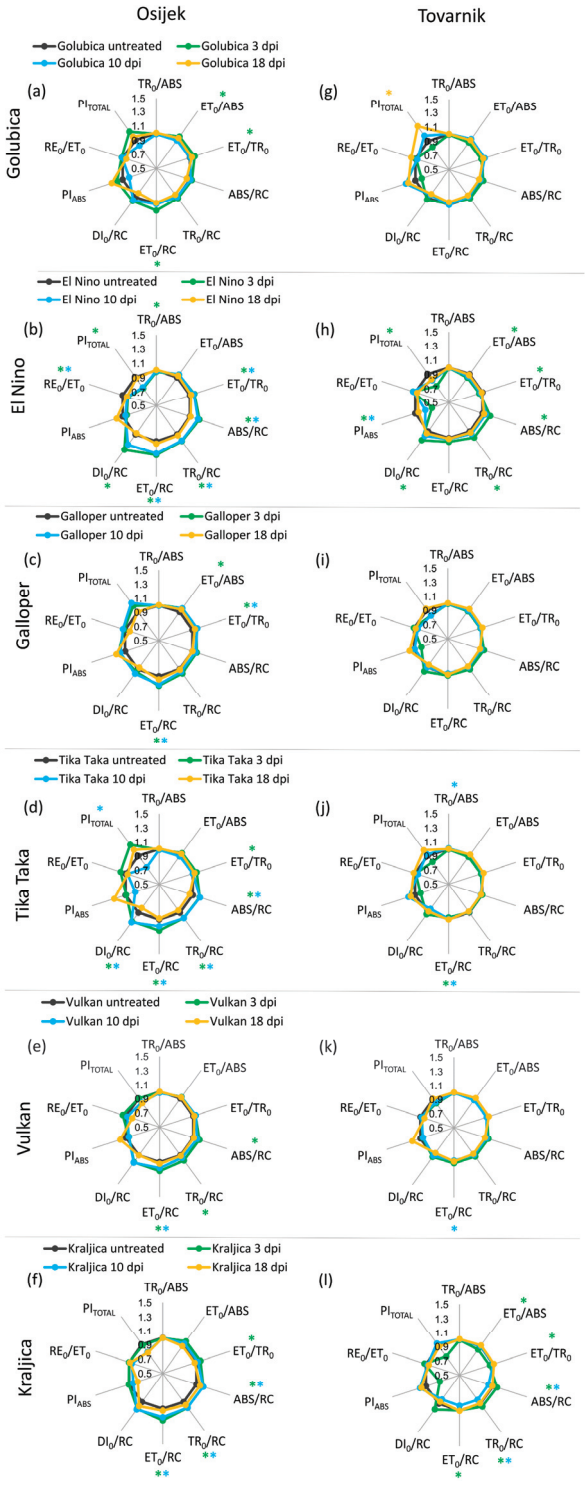

Figure 3. Spider plots of selected JIP-test parameters in flag leaves of winter wheat variety Golubica

(**a**,**g**), El Nino (**b**,**h**), Galloper (**c**,**i**), Tika Taka (**d**,**j**), Vulkan (**e**,**k**) and Kraljica (**f**,**l**) measured at 3 (green), 10 (blue) and 18 (yellow) days post-inoculation (dpi) at locations Osijek and Tovarnik. Values for inoculated plants are shown as difference compared to the untreated plants at the same time point for two locations separately. Statistically significant differences (ANOVA, Tukey's HSD) in measured parameters in inoculated plants compared to corresponding untreated plants are indicated by asterix (*). Descriptions of used JIP-test parameters are presented in Table 1.

Changes in JIP-test parameters in flag leaves after inoculation with *F. graminearum* + *F. culmorum* for each variety separately at location Tovarnik are presented in Figure 3g–l. At location Tovarnik, similar to what was observed at location Osijek, *Fusarium* infection generally had a greater effect on measured JIP-test parameters in earlier phases of infection. However, for variety Galloper, none of the analyzed parameters in flag leaves at this location were affected by FHB (Figure 3i), and the only difference between treated plants and the untreated control of variety Golubica at location Tovarnik was seen in a statistically significant increase in PI_{TOTAL} at 18 dpi (Figure 3g). Changes in variety El Nino were observed at 3 dpi when ET_0/ABS, ET_0/TR_0, PI_{ABS}, and PI_{TOTAL} were decreased and ABS/RC, TR_0/RC, and DI_0/RC were increased (Figure 3h). Additionally, El Nino showed a decrease of PI_{ABS} at 10 dpi (Figure 3h). Values of TR_0/ABS were mostly not affected by FHB at location Tovarnik, similarly to what was observed at location Osijek. Only slight but statistically significant increase of this parameter was detected at 10 dpi in variety Tika Taka (Figure 3j). Furthermore, ET_0/RC was affected at 3 and 10 dpi for this variety (Figure 3j). Variety Vulkan showed only small decrease of ET_0/RC at 10 dpi (Figure 3k), while variety Kraljica had decreased values ET_0/ABS and ET_0/TR_0 at 3 dpi, and increased values of ABS/RC, TR_0/RC, and ET_0/RC at 3 dpi as well as increased values of ABS/RC and TR_0/RC and 10 dpi in treated, compared to corresponding inoculated plants at location Tovarnik (Figure 3l).

3.3. Phytosynthetic Parameters in Ears

Variety, treatment, and measurement time point significantly affected all analysed JIP-test parameters, while location affected all of them, except RE_0/ET_0 (Table 4). Moreover, various interactions of tested parameters mostly had a significant effect on analysed parameters, and interactions of all four tested factors were significant for all JIP-test parameters in wheat ears (Table 4).

The chlorophyll *a* fluorescence (ChlF) transients of the dark-adapted ears at three measurement points had the expected OJIP shape for all untreated control plants as well as for FHB inoculated plants of all six winter wheat varieties at both experimental locations (Osijek and Tovarnik), demonstrating that all measured ears were photosynthetically active (data not shown). Differences in average variable fast fluorescence transients between O and P steps in ears are presented as difference kinetics ΔW_{OP} (Figure 4). Similarly, as in flag leaves, changes occurring in the O–J and O–I phase between inoculated and untreated plants were considerably affected by location. Generally, a strong J-peak and I-peak were not visible for the majority of varieties regardless of measurement time, although more considerable differences were observed between inoculated and untreated plants of variety El Nino (Figure 4h), Vulkan (Figure 4k), and Kraljica (Figure 4l) at location Tovarnik.

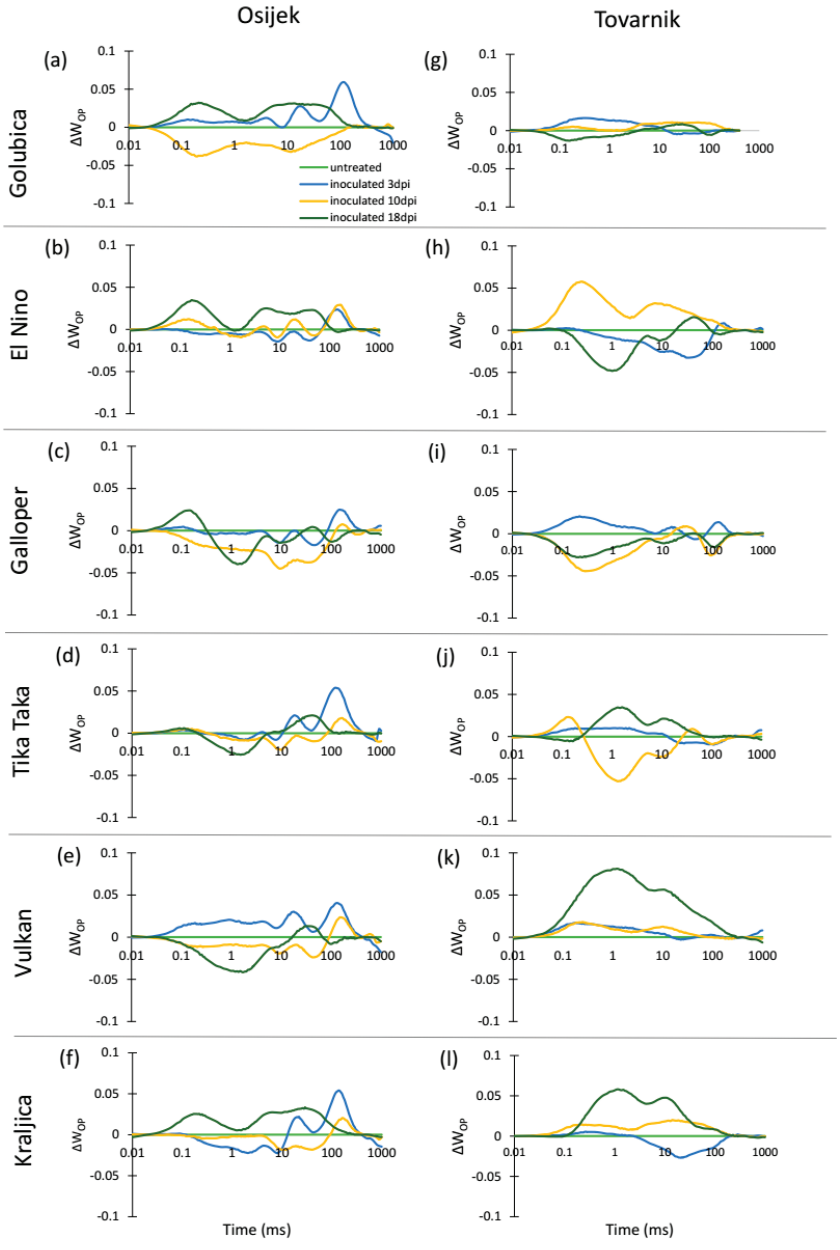

Figure 4. Changes in the shape of the chlorophyll *a* fluorescence transient curves in ears of winter wheat variety Golubica (**a**,**g**), El Nino (**b**,**h**), Galloper (**c**,**i**), Tika Taka (**d**,**j**), Vulkan (**e**,**k**) and Kraljica (**f**,**l**) measured at 3 (blue), 10 (yellow) and 18 (green) days post-inoculation (dpi) at locations Osijek and Tovarnik. Each curve represents average kinetics of 20 replicates and time (ms) refers to time after onset of illumination during chlorophyll *a* fluorescence measurement. Average fluorescence data were normalized between O–P steps and plotted as difference kinetics ΔW_{OP}. Average values measured in corresponding untreated control plants were used as referent value for each variety, measurement point and location.

Table 4. Mean squares from the analysis of variance for selected JIP-test parameters measured in ears of six wheat varieties at two different locations (Osijek and Tovarnik) and three measurement points (3, 10 and 18 days post-inoculation). Treatment refers to artificially inoculated plants and untreated control plants. Descriptions of used JIP-test parameters are presented in Table 1.

		MS									
Source of Variation	**df**	**TR_0/ABS**	**ET_0/ABS**	**ET_0/TR_0**	**ABS/RC**	**TR_0/RC**	**ET_0/RC**	**DI_0/RC**	**PI_{ABS}**	**RE_0/ET_0**	**PI_{TOTAL}**
Location (L)	1	0.1965 *	0.0047 *	0.1776 *	11.822 *	1.6843 *	3.0867 *	4.5816 *	10.468 *	0.0003 ns	14.352 *
Variety (V)	5	0.0101 *	0.0523 *	0.0491 *	1.8584 *	0.6258 *	0.0928 *	0.3545 *	11.416 *	0.0894 *	8.8653 *
Treatment (T)	1	0.0375 *	0.0107 *	0.0006 ns	3.9920 *	0.8694 *	0.3737 *	1.1355 *	10.060 *	0.0367 *	12.178 *
Measurement point (M)	2	0.0217 *	1.0873 *	1.9154 *	4.4020 *	1.3606 *	14.373 *	0.8684 *	89.485 *	0.0273 *	99.214 *
L × V	5	0.0046 *	0.0045 *	0.0033 *	0.4963 *	0.1504 *	0.0404 ns	0.1309 *	1.5515 *	0.0061 *	3.2458 *
L × T	1	0.0134 *	0.0014 ns	0.0204 *	0.2670 ns	0.0000 ns	0.1007 *	0.2613 *	0.0584 ns	0.0040 ns	0.1454 ns
V × T	5	0.0021 *	0.0036 *	0.0092 *	0.3110 *	0.1135 ns	0.0825 *	0.0627 *	0.6261 *	0.0218 *	6.2946 *
L × M	2	0.0028 *	0.2210 *	0.3933 *	0.3258 *	0.3597 *	1.5361 *	0.0008 ns	22.316 *	0.0954 *	38.717 *
V × M	10	0.0024 *	0.0093 *	0.0104 *	0.4299 *	0.2365 *	0.1653 *	0.0604 *	0.8278 *	0.0235 *	4.4150 *
T × M	2	0.0060 *	0.0091 *	0.0065 *	0.1906 ns	0.0117 ns	0.0203 ns	0.1246 *	2.8933 *	0.0367 *	2.1364 *
L × V × T	5	0.0026 *	0.0053 *	0.0060 *	0.2133 ns	0.1064 ns	0.0394 ns	0.0572 *	0.6986 *	0.0089 *	4.0147 *
L × V × M	10	0.0022 *	0.0039 *	0.0077 *	0.2696 *	0.1467 *	0.0460 *	0.0489 *	0.6680 *	0.0115 *	2.8763 *
L × T × M	2	0.0009 ns	0.0073 *	0.0069 *	1.1995 *	0.9113 *	0.5624 *	0.0199 ns	0.0269 ns	0.0609 *	0.9325 ns
V × T × M	10	0.0023 *	0.0036 *	0.0030 *	0.4347 *	0.1987 *	0.0663 *	0.0678 *	1.3925 *	0.0229 *	3.1128 *
L × V × T × M	10	0.0023 *	0.0058 *	0.0079 *	0.3705 *	0.1362 *	0.0561 *	0.0738 *	1.0498 *	0.0157 *	3.7578 *

ns—not significant; *—statisticaly significant at $p < 0.05$; df—degrees of freedom; MS—mean sum of squares.

The occurrence of K-bands and L-bands in ears after inoculation with *Fusarium* fungi is presented at Figure 5. At 3 dpi, the occurrence of the small but positive K-band was generally observed and at this measurement point a more pronounced positive K-band was only seen for Galloper at Tovarnik (Figure 5c). A high positive K-band at 10 dpi was specific for El Nino (Figure 5b) and Tika Taka (Figure 5d) at Tovarnik, while a substantial negative K-band at 10 dpi was observed for Golubica (Figure 5a) at Osijek. At 18 dpi, all varieties at location Osijek showed a positive K-band, except Vulkan (Figure 5e) which did not show considerable changes in K-band occurrence in inoculated, compared to control plants. In contrast, Golubica (Figure 5a), Galloper (Figure 5c), Tika Taka (Figure 5d), and Kraljica (Figure 5f) had a negative K-band at 18 dpi at Tovarnik. Although some differences between varieties and locations in occurrence of L-band were visible, the response of L-band for all varieties before the development of symptoms at 3 dpi was similar, including a visible negative L-band.

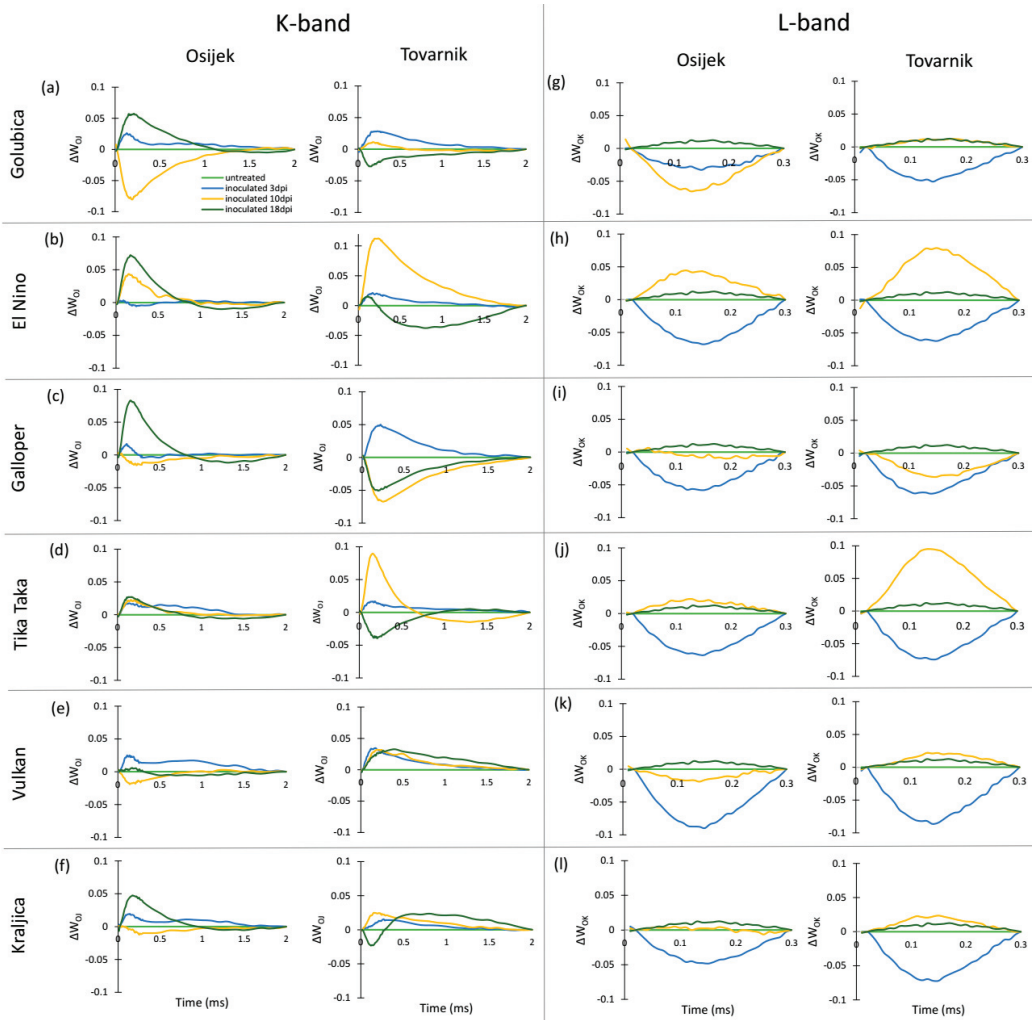

Figure 5. Changes in the shape of the chlorophyll *a* fluorescence transient curves in flag leaves of winter wheat variety Golubica (**a**,**g**), El Nino (**b**,**h**), Galloper (**c**,**i**), Tika Taka (**d**,**j**), Vulkan (**e**,**k**) and Kraljica (**f**,**l**) measured at 3 (blue), 10 (yellow) and 18 (green) days post-inoculation (dpi) at locations Osijek and Tovarnik. Each curve represents average kinetics of 20 replicates and time (ms) refers to time after onset of illumination during chlorophyll *a* fluorescence measurement. Average fluorescence data were normalized between O–J steps (K-band) and between O–K steps (L-band) and plotted as difference kinetics ΔW_{OJ} (**a**–**f**) and ΔW_{OK} (**g**–**l**). Average values measured in corresponding untreated control plants were used as referent value for each variety, measurement point and location.

Changes in JIP-test parameters in ears after inoculation with *F. graminearum* + *F. culmorum* for each variety separately at location Osijek are presented in Figure 6a–f. In comparison to leaves where changes were observed only in earlier measurement points, *Fusarium* treatment caused the same changes before as well as after the development symptoms in ears of treated plants compared to untreated control plants. The exception here was variety Tika Taka which showed differences due to FHB only at 3 dpi, including a decrease of TR_0/ABS and performance indexes (PI_{ABS} and PI_{TOTAL}), and an increase of DI_0/RC (Figure 6d). Golubica showed a statistically significant decrease of performance indices (PI_{ABS} and PI_{TOTAL}) in ears of treated plants, compared to untreated control plants at 3 dpi and 10 dpi. Additionally, *Fusarium* infection decreased ABS/RC, TR_0/RC, and

ET_0/RC in variety Gloubica at 10 dpi, while at 18 dpi inoculated plants had increased ABS/RC and DI_0/RC compared to the corresponding control at location Osijek (Figure 6a). For variety El Nino at 3 dpi and 10 dpi, TR_0/ABS was decreased and a decrease in ET_0/ABS was observed at 3 dpi in ears of treated compared to untreated plants. Performance index PI_{ABS} was decreased due to FHB in variety El Nino at 3 dpi and 10 dpi, while PI_{TOTAL} and RE_0/ET_0 were significantly decreased at 18 dpi. Furthermore, ABS/RC and TR_0/RC were increased at 18 dpi and DI_0/RC was lower at all three measuring time points in ears of infected compared to control plants of variety El Nino at location Osijek (Figure 6b). Both performance indexes (PI_{ABS} and PI_{TOTAL}) as well as TR_0/ABS and DI_0/RC remained unchanged during the experiment for variety Galloper, while ET_0/ABS increased at 18 dpi when ABS/RC, TR_0/RC, and ET_0/RC flux were also increased in ears of inoculated compared to untreated control plants of this variety and ET_0/TR_0 increased at 10 dpi and at 18 dpi. The only effect of FHB on JIP-test parameters in ears for variety Vulkan at location Osijek included a statistically significant increase of ET_0/ABS and PI_{ABS} at 18 dpi (Figure 6e), while for variety Kraljica a decrease of TR_0/ABS and PI_{ABS} along with an increase of DI_0/RC was detected at 10 dpi and at 18 dpi. RE_0/ET_0 was increased in ears of inoculated compared to untreated control plants (Figure 6f).

Changes in JIP-test parameters in ears after inoculation with *F. graminearum* + *F. culmorum* for each variety separately at location Tovarnik are presented in Figure 6g–l. Similar to what was observed at location Osijek, certain differences between measured JIP-test parameters were detected at each measurement time point (3, 10, and 18 dpi). However, the pattern of changes in measured JIP-test parameters during the course of infection was different to what was observed at location Osijek. Variety Golubica showed a slight but statistically significant decrease of TR_0/ABS at 3 dpi in inoculated, compared to untreated plants as well as a decrease of PI_{ABS} and increase of DI_0/RC (Figure 6g). At 3 dpi, only an increase in RE_0/ET_0 and no other changes in ears were induced in variety El Nino by FHB. However, at 10 dpi, inoculated plants of variety El Nino had significant increased values of all specific energy fluxes per active RC (ABS/RC, TR_0/RC, ET_0/RC, DI_0/RC) as well as decreased values of both performance indices (PI_{ABS} and PI_{TOTAL}) and values of RE_0/ET_0, compared to corresponding control plants, while an increase of ET0/TR0 and decrease of RE_0/ET_0 were detected at 18 dpi (Figure 6h). Although at Osijek variety Galloper showed FHB-induced changes at 18 dpi, at location Tovarnik differences between inoculated and untreated plants of variety Galloper were observed only at 3 and 10 dpi. These differences included a very significant increase in PI_{TOTAL} accompanied by an increase of RE_0/ET_0 at 3 dpi. Furthermore, at 3 dpi, inoculated plant of variety Galloper had decreased TR_0/ABS and PI_{ABS} as well as increased ABS/RC, ET_0/RC, and DI_0/RC. At 10 dpi, *Fusarium* infection induced a decrease in ABS/RC and DI_0/RC along with an increase in performance index PI_{ABS} in inoculated compared to untreated plants of variety Galloper (Figure 6i). For variety Tika Taka, statistically significant variations between inoculated and untreated plant were detected only at 3 dpi at Osijek, while at Tovarnik no differences were observed before the development of symptoms. At the onset of symptom development, Tika Taka showed increased ET_0/ABS, ET_0/TR_0, and ET_0/RC and decreased value of RE_0/ET_0 in ears of inoculated, compared to untreated plants. Additionally, ET_0/ABS, ET_0/TR_0, and ET_0/RC were decreased at 18 dpi in FHB inoculated plants of variety Tika Taka (Figure 6j). Changes in measured JIP-test parameters in ears of variety Vulkan at Tovarnik occurred only at 18 dpi, as was observed at Osijek. However, changes in Tovarnik were more pronounced and negative compared with Osijek since the decrease of ET_0/ABS, ET_0/TR_0, and ET_0/RC as well as both performance indexes PI_{ABS} and PI_{TOTAL} along with an increase in DI_0/RC were observed at 18 dpi at location Tovarnik in inoculated, compared to untreated plants of variety Vulkan (Figure 5k). The most pronounced effect of FHB in ears of variety Kraljica at Tovarnik was a major decrease in PI_{TOTAL} along with a decrease in RE_0/ET_0 at 3 dpi when an increase in TR_0/ABS was also observed in inoculated, compared to untreated plants. Furthermore, variety Kraljica showed decreased values of ET_0/ABS, ET_0/TR_0, and PI_{ABS} in infected compared to untreated plants at 18 dpi (Figure 6l).

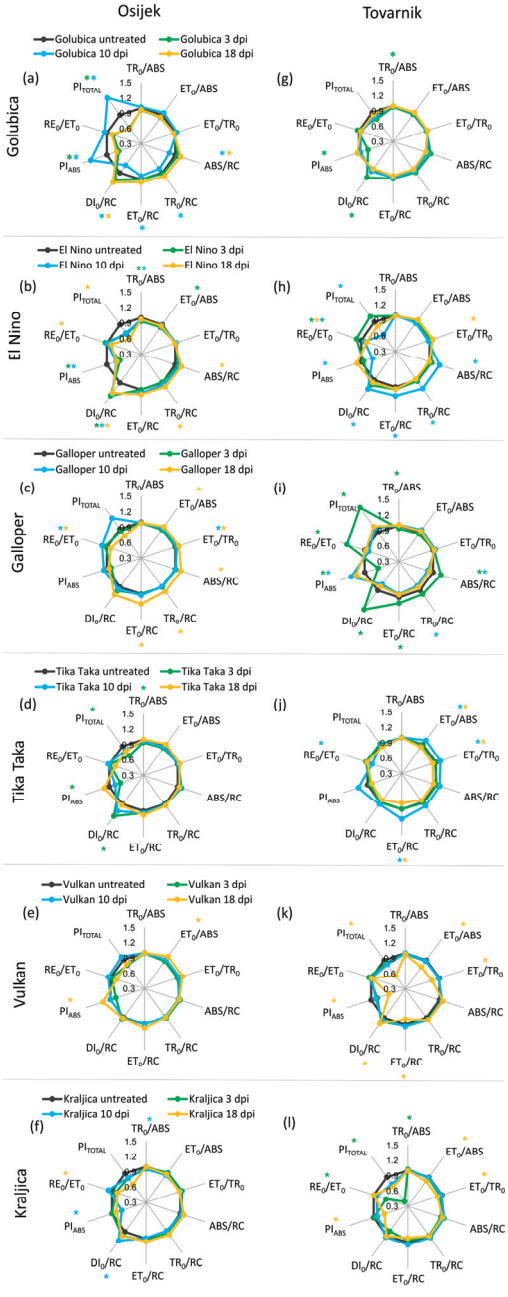

Figure 6. Spider plots of selected JIP-test parameters in ears of winter wheat variety Golubica (**a**,**g**), El Nino (**b**,**h**), Galloper (**c**,**i**), Tika Taka (**d**,**j**), Vulkan (**e**,**k**) and Kraljica (**f**,**l**) measured at 3 (green), 10 (blue) and 18 (yellow) days post-inoculation (dpi) at location Osijek and Tovarnik. Values for inoculated plants are shown as difference compared to the untreated plants at the same time point for two locations separately. Statistically significant differences (ANOVA, Tukey's HSD) in measured parameters in inoculated plants compared to corresponding untreated plants are indicated by asterix (*) for each measurement point. Descriptions of used JIP-test parameters are presented in Table 1.

4. Discussion

Due to a major reduction of yield, quality, and safety, FHB epidemics significantly threaten wheat production worldwide [3,5,47]. Various morphological and developmental wheat characteristics and specific physiological mechanisms affect the development of FHB [11]. Moreover, during field growth, plants are generally exposed to several unfavorable factors and a specific combination of factors exists at each growth season and at each location. During the quite long growth period from sowing to harvest, winter wheat is exposed to different combinations of abiotic factors which can modify its response and final outcome of *Fusarium* infection.

In this study, the effect of *Fusarium* infection on photosynthesis was assessed by measuring ChlF in flag leaves and ears of six winter wheat varieties with different susceptibility to *Fusarium* infection [44]. According to FHB severity at 10 and 18 dpi and AUDPC for FHB severity as well as FHB incidence at 10 and 18 dpi and AUDPC for Type I resistance, Golubica and El Nino varieties were highly susceptible at both locations, while Galloper showed the highest level of FHB resistance among tested varieties, together with Kraljica at Tovarnik. The characterization of varieties from this study showed the expected response of yield and yield components under FHB stress which were much more reduced for susceptible varieties than for resistant varieties [44]. Moreover, yield reduction was stronger at location Tovarnik compared to Osijek. Analysis of OJIP transients and comparison of values of JIP-test parameters pointed to the general functionality of PSII in both flag leaves and ears of wheat plants infected with *Fusarium* fungi. However, variations in several JIP-test parameters during the course of infection were observed when inoculated plants were compared to untreated control plants. It is particularly important that some of these alterations in photosynthetic apparatus functioning were measurable before disease symptoms were visible (at the 3 dpi). Although this method is not applicable as a reliable diagnostic method, the obtained results support the assumption that routine monitoring of ChlF changes around anthesis, when *Fusarium* infection is expected to occur, might be useful for the early detection of infection [25]. The applicability of JIP-test analysis to detect plant stress before visible symptoms of the disease was suggested for other plant pathogens as well [38]. According to the results of this study, the occurrence of L-band might be the most applicable for the purpose of early FHB detection since this parameter showed a similar response in all inoculated plants regardless of variety or environmental conditions. A positive L-band was detected in flag leaves of inoculated plants at 3 dpi before any visible symptom of infection could be observed, at the same time as a negative L-band occurred in inoculated ears. This indicates that an early response of wheat to *Fusarium* infection includes lowering of the energetic connectivity (grouping) between the PSII photosynthetic units in flag leaves and increase of energetic connectivity in infected ears.

Various host and pathogen related factors affect epidemics of FHB, e.g., physiological state and genetic make-up of the host and adaptation and virulence of the pathogen. Local and regional environment factors also contribute to the dynamic of *Fusarium* infections [3]. The impact of FHB as well as defense strategies can vary significantly between different wheat varieties and due to specific growth conditions and/or agricultural practices [42,43]. Therefore, the severity and impact of the disease may differ for particular wheat variety if it is exposed to different environmental conditions. Results of this study indicate that changes in the photosynthetic efficiency due to *Fusarium* infection vary depending on environmental conditions. Environment, i.e., location, was shown to significantly modify the response of both flag leaves and ears to FHB stress. Experimental locations included in this study (Osijek and Tovarnik) are located in the continental part of Croatia and are about 60 km apart but have different soil types and slightly different climatic conditions. These differences between Osijek and Tovarnik were sufficient to induce variability in the response to FHB considering agronomical and quality traits [44] as well as JIP-test parameters, as observed in this study. This points to a possibility that modification in photosynthetic efficiency is interconnected with the response of wheat to *Fusarium* infection as previously indicated [24,25].

Despite indicating variations in the functioning of photosynthetic apparatus, obtained results did not indicate a heavy impairment of PSII or electron transport in wheat infected with *Fusarium*. Maximum quantum yield for primary photochemistry (TR_0/ABS) in flag leaves was mostly unchanged during the course of this experiment, which is in accordance with previously conducted field measurements [26]. One exception here is the minor decrease of TR_0/ABS in inoculated, compared to untreated plants of variety El Nino at location Osijek at 3 dpi and minor increase of this parameter in inoculated compared to untreated plants of variety Tika Taka at location Tovarnik at 10 dpi. In this study, slight changes in TR_0/ABS in ears due to FHB were observed for some varieties in earlier phases of infection, but the maximum quantum yield of primary photochemistry in ears was mostly unchanged during the course of infection, as in flag leaves. Previously, a decrease of maximum quantum yield of primary PSII photochemistry as a response to *Fusarium avenaceum* and *F. culmorum* in detected glumes was reported [25]. Two photosynthetic performance indexes (PI_{ABS} and PI_{TOTAL}) were calculated and compared as a more reliable indicator of photosynthetic efficiency than TR_0/ABS. Compared to previous studies [26], the result of this study showed that values of PI_{ABS} and PI_{TOTAL} occasionally responded to *Fusarium* infection, mostly in earlier phases of infection, but also at 18 dpi.

Some previous studies indicated different impacts of FHB on photosynthetic activity of susceptible and resistant wheat varieties and assumed the involvement of photosynthesis in the disease response [24]. Some authors showed that a decrease of net photosynthetic rate (Pn) and stomatal conductance (Gs) is more pronounced in the flag leaves of resistant variety compared to its susceptible sister line [19]. However, decreases in measured photosynthetic parameters yield components were less affected by *Fusarium* infection in the resistant line compared to the susceptible one. The downregulation of photosynthesis is considered to contribute to higher FHB resistance, which includes more significant physiologic modifications, but results in only modest yield loss [19]. On the contrary, some research associated increasing photosynthetic efficiency with higher resistance to FHB [48]. They observed decreasing photosynthetic efficiency in susceptible cultivar Rebelde compared to resistant cultivar Sumai3 after inoculation and suggested that cross-talk between genes regulating stomatal closure and opening are important in the development of FHB resistance. Results of our study also support the variety-specific response of photosynthetic apparatus in flag leaves as well as in ears to *Fusarium* infection. However, ChlF measurements of six varieties in this study does not seems to reflect their disease susceptibility. In this study, susceptible variety El Nino had 39% and 54% decreases in yield at Osijek and Tovarnik, respectively. This variety showed pronounced changes in analyzed JIP-test parameters due to *Fusarium* infection, indicating a levering of photosynthetic efficiency and inefficient use of absorbed energy in both flag leaves and ears. On the other hand, variety Golubica, which was also susceptible to FHB and showed 53% and 59% decreases in yield, did not show such a decrease in photosynthetic efficiency in flag leaves nor in ears at location Tovarnik, where an even higher yield decrease is recorded compared to location Osijek. The response of leaves of resistant variety Galloper was similar to those of variety Golubica at location Osijek and no changes in flag leaves of this variety was observed at location Tovarnik. However, response of ears of this variety was more pronounced at location Tovarnik compared to location Osijek. Variety Tika Taka and Vulkan showed similar yield decreases at both locations, but the photosynthetic efficiency of variety Tika Taka was slightly more affected by FHB compared to variety Vulkan. For variety Kraljica, FHB slightly more affected photosynthetic efficiency at location Tovarnik, where a higher yield loss for this variety was observed compared to location Osijek. Altogether, results of this study and previous studies [19,48] might be interpreted as an indicator that the modification of photosynthetic efficiency occurs as a response of some varieties to *Fusarium* infection, while for some varieties other defense strategies might be more important. Moreover, it should be noted that measurements were performed using different methodological approaches in each of these studies, including the difference in growth conditions and methods for photosynthetic evaluation, and therefore they should be compared with caution.

Photosynthetic adjustments of wheat infected by *Fusarium* fungi were shown to be dynamic, including alternating down-regulation and up-regulation during the course of infection. Observed variability in photosynthetic efficiency among six tested wheat varieties might result from a specific genotype-associated response on complex interactions of all factors acting in a particular growth environment along with FHB. It is possible that some of the parameters which were different in flag leaves and ears between control and treated plants are significant in terms of the defense and ultimate response of the variety as previously suggested [19,24], especially since those changes mainly occur before or at the beginning of the development of symptoms. However, field conditions make it difficult to distinguish such an association between specific PSII functioning and better response to infection.

5. Conclusions

Variety-specific alternations in photosynthetic efficiency in flag leaves mostly occurred only in earlier phases, while some changes in the functioning of photosynthetic apparatus in ears were also detected after the development of symptoms. Changes in PSII photochemistry and electron transport in FHB-inoculated, compared to untreated control plants, differed between two experimental locations (Osijek and Tovarnik) as well as overall disease outcome and grain yield. ChlF measurement, as a fast, non-invasive, and field-adjusted technique could be adopted to efficiently supplement other methodological approaches for the early detection of FHB epidemics since changes in ChlF could be detected prior to visible symptoms of the disease. The L-band, which showed similar responses in all inoculated plants regardless of variety or location, might be particularly useful for the purpose of early FHB detection. However, results of this study indicate the limited applicability of OJIP kinetics in field conditions as an indicator of the degree of FHB resistance. In future, experiments in controlled conditions, such as a greenhouse, and the evaluation of early changes in ChlF (starting from few hours post-inoculation) should be tested in order to determine when the response of the photosynthetic apparatus to *Fusarium* infection begins and whether these early changes are uniform between varieties or if they could indicate the level of FHB-resistance.

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References

1. Hawkesford, M.J.; Araus, J.L.; Park, R.; Calderini, D.; Miralles, D.; Shen, T.; Zhang, J.; Parry, M.A.J. Prospects of doubling global wheat yields. *Food Energy Secur.* **2013**, *2*, 34–48. [CrossRef]
2. Figueroa, M.; Hammond-Kosack, K.E.; Solomon, P.S. A review of wheat diseases—A field perspective. *Mol. Plant Pathol.* **2018**, *19*, 1523–1536. [CrossRef]
3. Osborne, L.E.; Stein, J.M. Epidemiology of Fusarium head blight on small-grain cereals. *Int. J. Food Microbiol.* **2007**, *119*, 103–108. [CrossRef] [PubMed]
4. Parry, D.W.; Jenkinson, P.; Mcleod, L. Fusarium ear blight (scab) in small grain cereals—A review. *Plant Pathol.* **1995**, *44*, 207–238. [CrossRef]

5. Dweba, C.C.; Figlan, S.; Shimelis, H.A.; Motaung, T.E.; Sydenham, S.; Mwadzingeni, L.; Tsilo, T.J. Fusarium head blight of wheat: Pathogenesis and control strategies. *Crop. Prot.* **2017**, *91*, 114–122. [CrossRef]
6. Spanic, V.; Horvat, D.; Drezner, G.; Zdunic, Z. Changes in protein composition in the grain and malt after Fusarium infection dependently of wheat resistance. *Pathogens* **2019**, *8*, 112. [CrossRef] [PubMed]
7. da Rocha, M.E.B.; da Freire, F.C.O.; Erlan Feitosa Maia, F.; Izabel Florindo Guedes, M.; Rondina, D. Mycotoxins and their effects on human and animal health. *Food Control* **2014**, *36*, 159–165. [CrossRef]
8. Škrbić, B.; Malachova, A.; Živančev, J.; Veprikova, Z.; Hajšlová, J. Fusarium mycotoxins in wheat samples harvested in Serbia: A preliminary survey. *Food Control* **2011**, *22*, 1261–1267. [CrossRef]
9. Spanic, V.; Katanic, Z.; Sulyok, M.; Krska, R.; Puskas, K.; Vida, G.; Drezner, G.; Šarkanj, B. Multiple fungal metabolites including mycotoxins in naturally infected and Fusarium-inoculated wheat samples. *Microorganisms* **2020**, *8*, 578. [CrossRef]
10. Mesterházy, Á.; Tóth, B.; Varga, M.; Bartók, T.; Szabó-Hevér, Á.; Farády, L.; Lehoczki-Krsjak, S. Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of Fusarium head blight and deoxynivalenol. *Toxins* **2011**, *3*, 1453. [CrossRef]
11. Bai, G.; Su, Z.; Cai, J. Wheat resistance to Fusarium head blight. *Can. J. Plant Pathol.* **2018**, *40*, 336–346. [CrossRef]
12. Zhang, W.; Boyle, K.; Brule-Babel, A.; Fedak, G.; Gao, P.; Djama, Z.R.; Polley, B.; Cuthbert, R.; Randhawa, H.; Graf, R.; et al. Evaluation of genomic prediction for Fusarium head blight resistance with a multi-parental population. *Biology* **2021**, *10*, 756. [CrossRef]
13. Li, X.; Zhong, S.; Chen, W.; Fatima, S.A.; Huang, Q.; Li, Q.; Tan, F.; Luo, P. Transcriptome analysis identifies a 140 Kb region of chromosome 3B containing genes specific to Fusarium head blight resistance in wheat. *Int. J. Mol. Sci.* **2018**, *19*, 852. [CrossRef] [PubMed]
14. Schroeder, H.W.; Christensen, J.J.; Christensen, J.D.; Platz-Christensen, J.; Schroeder, H.W. Factors affecting resistance of wheat to scab caused by Gibberella zeae. *Phytopathology* **1963**, *53*, 831–838.
15. David Miller, J. Degradation of deoxynivalenol by suspension cultures of the Fusarium head blight resistant wheat cultivar frontana. *Can. J. Plant Pathol.* **1986**, *8*, 147–150. [CrossRef]
16. Mesterhazy, A. Types and components of resistance to Fusarium head blight of wheat. *Plant Breed.* **1995**, *114*, 377–386. [CrossRef]
17. Mesterházy, Á.; Bartók, T.; Mirocha, C.G.; Komoroczy, R. Nature of wheat resistance to Fusarium head blight and the role of deoxynivalenol for breeding. *Plant Breed.* **1999**, *118*, 97–110. [CrossRef]
18. Berger, S.; Sinha, A.K.; Roitsch, T. Plant physiology meets phytopathology: Plant primary metabolism and plant-pathogen interactions. *J. Exp. Bot.* **2007**, *58*, 4019–4026. [CrossRef] [PubMed]
19. Yang, S.; Li, X.; Chen, W.; Liu, T.; Zhong, S.; Ma, L.; Zhang, M.; Zhang, H.; Yu, D.; Luo, P. Wheat resistance to Fusarium head blight is associated with changes in photosynthetic parameters. *Plant Dis.* **2016**, *100*, 847–852. [CrossRef]
20. Spanic, V.; Vuletic, M.V.; Horvat, D.; Sarkanj, B.; Drezner, G.; Zdunic, Z. Changes in antioxidant system during grain development of wheat (Triticum aestivum l.) and relationship with protein composition under FHB stress. *Pathogens* **2020**, *9*, 17. [CrossRef]
21. Walter, S.; Nicholson, P.; Doohan, F.M. Action and reaction of host and pathogen during Fusarium head blight disease. *New Phytol.* **2010**, *185*, 54–66. [CrossRef] [PubMed]
22. Bai, G.; Shaner, G. Management and resistance in wheat and barley to Fusarium head blight. *Annu. Rev. Phytopathol.* **2004**, *42*, 135–161. [CrossRef] [PubMed]
23. Khaledi, N.; Taheri, P.; Falahati-Rastegar, M. Evaluation of resistance and the role of some defense responses in wheat cultivars to Fusarium head blight. *J. Plant Prot. Res.* **2017**, *57*, 396–408. [CrossRef]
24. Yang, H.; Luo, P. Changes in photosynthesis could provide important insight into the interaction between wheat and fungal pathogens. *Int. J. Mol. Sci.* **2021**, *22*, 8865. [CrossRef]
25. Ajigboye, O.O.; Bousquet, L.; Murchie, E.H.; Ray, R.V. Chlorophyll fluorescence parameters allow the rapid detection and differentiation of plant responses in three different wheat pathosystems. *Funct. Plant Biol.* **2016**, *43*, 356–369. [CrossRef]
26. Spanic, V.; Vuletic, M.V.; Drezner, G.; Zdunic, Z.; Horvat, D. Performance indices in wheat chlorophyll a fluorescence and protein quality influenced by FHB. *Pathogens* **2017**, *6*, 59. [CrossRef] [PubMed]
27. Tambussi, E.A.; Maydup, M.L.; Carrión, C.A.; Guiamet, J.J.; Araus, J.L. Ear photosynthesis in C3 cereals and its contribution to grain yield: Methodologies, controversies, and perspectives. *J. Exp. Bot.* **2021**, *72*, 3956–3970. [CrossRef]
28. Yusuf, M.A.; Kumar, D.; Rajwanshi, R.; Strasser, R.J.; Tsimilli-Michael, M.; Govindjee; Sarin, N.B. Overexpression of γ-tocopherol methyl transferase gene in transgenic Brassica juncea plants alleviates abiotic stress: Physiological and chlorophyll a fluorescence measurements. *Biochim. Et Biophys. Acta Bioenerg.* **2010**, *1797*, 1428–1438. [CrossRef]
29. Strasser, R.J.; Srivastava, A.; Tsimilli-Michael, M. The fluorescence transient as a tool to characterize and screen photosynthetic samples. In *Probing Photosynthesis: Mechanisms, Regulation and Adaptation*; Yunus, M., Pathre, U., Mohanty, P., Eds.; Taylor & Francis: London, UK, 2000; pp. 445–483.
30. Strasser, R.J.; Tsimilli-Michael, M.; Srivastava, A. Analysis of the chlorophyll a fluorescence transient. In *Chlorophyll A Fluorescence*; Springer: Dordrecht, The Netherlands, 2004; pp. 321–362. [CrossRef]
31. Begović, L.; Galić, V.; Abičić, I.; Lončarić, Z.; Lalić, A.; Mlinarić, S. Implications of intra-seasonal climate variations on chlorophyll a fluorescence and biomass in winter barley breeding program. *Photosynthetica* **2020**, *58*, 995–1008. [CrossRef]

32. Begović, L.; Mlinarić, S.; Antunović Dunić, J.; Katanić, Z.; Lončarić, Z.; Lepeduš, H.; Cesar, V. Response of lemna minor L. to short-term cobalt exposure: The effect on photosynthetic electron transport chain and induction of oxidative damage. *Aquat. Toxicol.* **2016**, *175*, 117–126. [CrossRef]
33. Mlinarić, S.; Begović, L.; Tripić, N.; Piškor, A.; Cesar, V. Evaluation of light-dependent photosynthetic reactions in reynoutria japonica houtt. leaves grown at different light conditions. *Front. Plant Sci.* **2021**, *12*, 1–13. [CrossRef]
34. Pavlović, I.; Mlinarić, S.; Tarkowská, D.; Oklestkova, J.; Novák, O.; Lepeduš, H.; Bok, V.V.; Brkanac, S.R.; Strnad, M.; Salopek-Sondi, B. Early brassica crops responses to salinity stress: A comparative analysis between chinese cabbage, white cabbage, and kale. *Front. Plant Sci.* **2019**, *10*, 1–16. [CrossRef] [PubMed]
35. Rapacz, M.; Sasal, M.; Kalaji, H.M.; Kościelniak, J. Is the OJIP test a reliable indicator of winter hardiness and freezing tolerance of common wheat and triticale under variable winter environments? *PLoS ONE* **2015**, *10*, 1–18. [CrossRef]
36. Kalaji, H.M.; Jajoo, A.; Oukarroum, A.; Brestic, M.; Zivcak, M.; Samborska, I.A.; Cetner, M.D.; Łukasik, I.; Goltsev, V.; Ladle, R.J. Chlorophyll a fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. *Acta Physiol. Plant.* **2016**, *38*, 1–11. [CrossRef]
37. Pollastrini, M.; Desotgiu, R.; Camin, F.; Ziller, L.; Gerosa, G.; Marzuoli, R.; Bussotti, F. Severe drought events increase the sensitivity to ozone on poplar clones. *Environ. Exp. Bot.* **2014**, *100*, 94–104. [CrossRef]
38. Christen, D.; Schönmann, S.; Jermini, M.; Strasser, R.J.; Défago, G. Characterization and early detection of grapevine (vitis vinifera) stress responses to esca disease by in situ chlorophyll fluorescence and comparison with drought stress. *Environ. Exp. Bot.* **2007**, *60*, 504–514. [CrossRef]
39. Baghbani, F.; Lotfi, R.; Moharramnejad, S.; Bandehagh, A.; Roostaei, M.; Rastogi, A.; Kalaji, H.M. Impact of Fusarium verticillioides on chlorophyll fluorescence parameters of two maize lines. *Eur. J. Plant Pathol.* **2019**, *154*, 337–346. [CrossRef]
40. Zhori, A.; Meco, M.; Brandl, H.; Bachofen, R. In situ chlorophyll fluorescence kinetics as a tool to quantify effects on photosynthesis in Euphorbia cyparissias by a parasitic infection of the rust fungus Uromyces pisi. *BMC Res. Notes* **2015**, *8*, 1–7. [CrossRef] [PubMed]
41. Warzecha, T.; Skrzypek, E.; Adamski, T.; Surma, M.; Kaczmarek, Z.; Sutkowska, A. Chlorophyll a fluorescence parameters of hulled and hull-less barley (Hordeum vulgare l.) DH lines inoculated with Fusarium culmorum. *Plant Pathol. J.* **2019**, *35*, 112–124. [CrossRef]
42. Matić, M.; Vuković, R.; Vrandečić, K.; Čamagajevac, I.Š.; Ćosić, J.; Vuković, A.; Sabljić, K.; Sabo, N.; Dvojković, K.; Novoselović, D. Oxidative status and antioxidative response to Fusarium attack and different nitrogen levels in winter wheat varieties. *Plants* **2021**, *10*, 611. [CrossRef]
43. Vaughan, M.; Backhouse, D.; del Ponte, E.M. Climate change impacts on the ecology of Fusarium graminearum species complex and susceptibility of wheat to Fusarium head blight: A review. *World Mycotoxin J.* **2016**, *9*, 685–700. [CrossRef]
44. Spanic, V.; Cosic, J.; Zdunic, Z.; Drezner, G. Characterization of agronomical and quality traits of winter wheat (Triticum Aestivum L.) for Fusarium head blight pressure in different environments. *Agronomy* **2021**, *11*, 213. [CrossRef]
45. Zadoks, J.C.; Chang, T.T.; Konzak, C.F. A decimal code for the growth stages of cereals. *Weed Res.* **1974**, *14*, 415–421. [CrossRef]
46. Sunic, K.; Kovac, T.; Loncaric, A.; Babic, J.; Sulyok, M.; Krska, R.; Drezner, G.; Spanic, V. Fusarium secondary metabolite content in naturally produced and artificially provoked FHB pressure in winter wheat. *Agronomy* **2021**, *11*, 2239. [CrossRef]
47. Torres, A.M.; Palacios, S.A.; Yerkovich, N.; Palazzini, J.M.; Battilani, P.; Leslie, J.F.; Logrieco, A.F.; Chulze, S.N. Fusarium head blight and mycotoxins in wheat: Prevention and control strategies across the food chain. *World Mycotoxin J.* **2019**, *12*, 333–355. [CrossRef]
48. Francesconi, S.; Balestra, G.M. The Modulation of stomatal conductance and photosynthetic parameters is involved in Fusarium head blight resistance in wheat. *PLoS ONE* **2020**, *15*, 1–21. [CrossRef]

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Disease Severity, Resistance Analysis, and Expression Profiling of Pathogenesis-Related Protein Genes after the Inoculation of *Fusarium equiseti* in Wheat

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Abstract: Wheat (*Triticum aestivum*) is an important cereal crop, grown throughout the temperate and in some tropical and sub-tropical zones, at higher elevations. Several biotic and abiotic factors influence the production of wheat. In the present study, two wheat varieties have been subjected to disease severity and resistance analysis against *Fusarium equiseti*. Disease severity analysis revealed Shafaq-2006 to be more resistant than Sahar-2006. Both varieties were further subjected to the expression analysis of six important defense-related genes by RT-PCR and quantitative real-time PCR. This analysis revealed that PR-1, TLP, Chitinase, and β-1,3-glucanase genes were highly expressed in Shafaq-2006 and possibly play a significant role in its defense mechanism. In addition, biochemical and physiochemical parameters were also studied to further explore the difference between resistant and susceptible varieties. With total proline and protein contents, sugar and chlorophyll contents also increased significantly in resistant variety. Likewise, higher relative water content, total plant length, and the high root–shoot ratio was observed in resistant plants, compared to susceptible wheat plants. These increases in chemical and physiological parameters might be related to the activation of the defense mechanism due to the higher expression of PR genes in the resistant wheat variety. These genes can further be employed for cloning into wheat and other transgenic crops to develop resistance against *F. equiseti*.

Keywords: plant defense mechanism; plant disease; pathogenicity; *Triticum aesitivum*; Shafaq-2006; Sahar-2006

1. Introduction

Wheat (*Triticum aestivum*) is regarded one of the major cereal crops that is being challenged by various biotic and abiotic stresses which influence and disturb metabolic processes in plants. Plants can be affected by many abiotic factors, including excessive temperature, nutrient deficiency, ultraviolet radiations, drought, pollution, and lack of oxygen as well as biotic agents, such as bacteria, fungi, viruses, and insects [1,2]. Various

fungal species have been known to cause severe diseases in plants. As a predominant soil saprophyte, *F. equiseti* is associated with fruit rots as well as dead and dying plant tissues. It also acts as an important pathogenic agent on various agricultural plants [3]. It is a typically soil-borne pathogen that frequently exists in subtropical and warm temperate regions [4]. *F. equiseti* is known to cause different diseases in many plants, such as root rot in winter, wheat and stem rot in maize [5], and Fusarium head blight in barley and wheat [6,7]. In a previous report, *F. equiseti* isolates obtained from saline sea-bed soil samples exhibited pathogenicity during seedling pre-emergence [8]. To overcome the influences of these unusual pathogens or environmental stresses, plants activate different defense systems [9].

Plants have established several constitutive and inducible defense mechanisms, including the expression of different genes in response to pest/pathogen infection. Hypersensitive reaction (HR) is the most effective response, which induces cell necrosis quickly around the infection site. This response is related to an integrated and coordinated set of metabolic changes involved in the inhibition of further pathogen entrance [10]. Biotic stresses affect the growth and development of plants and in response, they change the expression of different genes in their defense [11]. Plants trigger various signaling pathways soon after recognizing external changes and convert physical stress into a biochemical response. As a result, they stimulate the expression of stress-response genes [12].

Under stress, several proteins are accumulated in plants that are known as pathogenesis-related (PR) proteins. PR proteins are studied to be coded by the host plant specifically under biotic stresses [13]. These proteins are not only accumulated in the infected leaves, but are also produced systemically, associated with the development of systemic acquired resistance (SAR). It has been found that PR-proteins induce in many species of various plant families [14], which suggests their general role in the adaptation to different biotic stresses. Many studies show that the PR-proteins are accumulated more in resistant plants as compared to susceptible plants [15]. Artificial constitutive over-expression of PR-proteins has been proved to be very useful in inducing stress resistance [9].

Several PR-1 proteins are identified in Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), wheat, maize (*Zea mays*), tomato (*Solanum lycopersicum*), and pepper (*Capsicum annuum*) [16]. Among PR-proteins, PR-1 is the most abundant, which is accumulated in about 1–2% of the total protein contents in a leaf [17]. PR-1 gene expression serves as a molecular marker that indicates a defense response against pathogens [17]. Thionins are usually small (5 kDa) cysteine-rich PR proteins initially isolated from cereals. They have widespread in vitro antibacterial and antifungal activity [18]. Thionin accumulation in the cell wall of resistant wheat spikes indicates its role in plant defense [19]. Thaumatin-like proteins (TLPs) in plants are associated with developmental processes and defense against phytopathogens and elicitors [20]. Due to the antifungal property of TLPs, they have been efficiently used in genetic engineering for producing disease-resistant plants [21]. Chitinases are accumulated in plants in response to fungal infection as well as other abiotic and biotic stressors [22]. Studies have reported that the expression of Chitinase increases against phyto-pathogens and its induction is stronger in resistant varieties of wheat [23], sugar beet (*Beta vulgaris*) [24] and tomato [25]. The Chitinase gene has been reported to be induced in response to fungal infection in maize and wheat [26]. Plant β-1,3-glucanases are members of the PR-2 family of pathogenesis-related proteins, which play an important function in plant defensive responses against pathogen infection [22]. Plant β-1,3-glucanases have been recognized as one of the important components of defense mechanism against phytopathogens [27]. They are strongly induced when plants respond to infection or wounding by fungal, viral, or bacterial pathogens [28]. Another group of PR proteins is the plant defensins, which have been identified in different plant families, including the *Solanaceae, Brassicaceae,* and *Fabaceae*. These proteins are either constitutively expressed in reproductive or storage organs or induced in the result of injury or pathogen attack, as part of a systemic defense response [29]. The antifungal activity of defensins has been studied extensively, while only a few plant defensins have been known to prevent the growth of bacteria [30].

Thirty different wheat varieties have been cultivated in Pakistan; of these, 22 have been considered as high-yielding and eight as low-yielding [31]. Shafaq-2006 is currently the major cultivated wheat variety that exhibits resistance against aphids [32]. It has also been reported as a high-yielding variety with a durable resistance mechanism against yellow rusts [32]. Sahar-2006 is susceptible in its reactions against different inoculum [33]. Under natural field conditions, this variety behaves as moderately resistant to abiotic stresses [34]. This experiment has been designed to study the behavior of important pathogenesis-related genes in two different wheat varieties (Shafaq-2006 and Sahar-2006). These genes can further be used for cloning into wheat and other transgenic crops to create resistance to *F. equiseti*.

2. Materials and Methods

2.1. Fungus Culture and Inoculum Preparation

The identified fungus strain *Fusarium equiseti* was obtained from the National Agriculture Research Center (NARC), Islamabad, Pakistan. *F. equiseti* was grown on Sabouraud Dextrose Agar (SDA) medium for seven days at 25 °C in an incubator (Figure 1). Pure culture of this fungus was obtained by pouring 5 mL of sterilized distilled water and scraping the agar surface with the help of a spatula to isolate fungal spores in petri dishes. Subsequently, the spore suspension was filtered using a muslin cloth, and the spore number was counted and adjusted to 10^7 mL^{-1} by hemocytometer. This culture was further used for the inoculation.

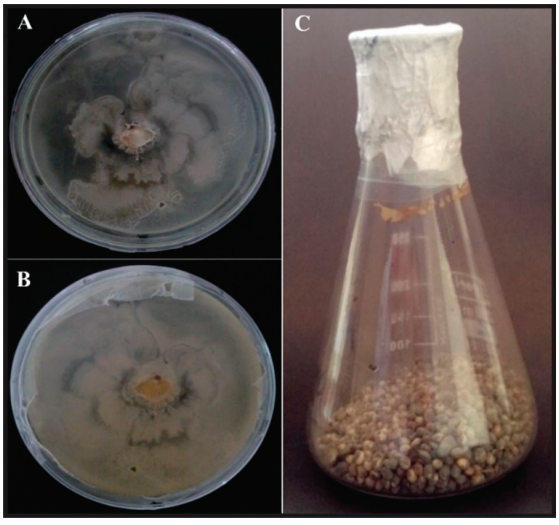

Figure 1. Growth of *Fusarium equiseti* on SDA at 25 °C and inoculum preparation. (**A**) Plate from front side, (**B**) Plate from reverse side, and (**C**) Inoculation preparation of fungus.

2.2. Seed Sterilization and Soil Inoculation

The seeds of two wheat varieties viz. Shafaq-2006 and Sahar-2006 were collected from the National Agriculture Research Center (NARC), Islamabad. Seeds of both wheat varieties were soaked in 75% ethanol for 3 min for surface sterilization and washed thoroughly with distilled water, three times. The sterilized seeds (eight seeds per pot) were sown in plastic pots for 21 days in a growth chamber at 25 °C, 80% relative humidity and 14 h photoperiod. *F. equiseti* was used for the standard systemic inoculation method [35]. Briefly, sorghum seeds were sterilized in 70% ethanol, rinsed thrice with distilled water, and soaked overnight in distilled water. The next day, sorghum seeds were dried on filter papers and autoclaved at 121 °C for 21 min. Autoclaved sorghum seeds were inoculated by mixing 1.15 g of *F. equiseti* spore suspension (filtered using a muslin cloth) in a flask

and kept in an incubator at 25 °C for 15 days. The flask was shaken daily to mix the fungus evenly with the sorghum seeds. Two grams of inoculated sorghum seeds were mixed in one kg of autoclaved soil. Each one kg fungal inoculated soil was used for four pots and seeds of each wheat variety were sown in respective pots. In addition, the seeds of both wheat varieties were also grown in non-inoculated soil (autoclaved soil without fungal inoculation), which served as control. The plants were irrigated on a daily bases to maintain soil humidity. The experiment involved three replicates for each treatment, including control. Harvesting was done 3 weeks after sowing.

2.3. Disease Severity Analysis

Lesions were counted at the time of harvesting by using a standard protocol [36]. All the plants were observed carefully to calculate disease severity in percentage.

2.4. Biochemical Parameters

Different biochemical parameters were measured to see biochemical changes in resistant and susceptible wheat varieties. Leaves of both varieties were used to measure sugar contents, following the protocol of Dubois et al. [37], with slight modifications [38]. The methods of Bates et al. [39] and Lowry et al. [40] were followed to determine proline and protein contents, respectively. The method of Arnon [41] was used to determine chlorophyll contents.

2.5. Physiological Parameters

Different physiological parameters were also studied to analyze the effects of *F. equiseti* on resistant and susceptible wheat varieties. A measuring tape was used to measure the length of roots and shoots of both varieties during harvesting, and their fresh weight was recorded. Fresh samples were kept in an oven at 70 °C for 24 h to calculate their dry weight.

Leaf relative water contents (RWCs) of plants were measured by using the standard method of Whetherley [42]. For this purpose, the fresh leaf weight of the samples was measured and placed in distilled water. After 24 h, the weight of fully turgid leaves was recorded, and leaves were kept in an oven at 70 °C. The dry weight of these samples was determined after 72 h to calculate leaf RWCs.

2.6. RNA Extraction, Quantification, and cDNA Synthesis

Total RNA was extracted from leaves of three-week-old plants using a Thermo scientific® Gene JET plant RNA purification kit. By using Nanodrop, the quantification of RNA samples was performed and stored at –80 °C for further use. Total RNA was subjected to cDNA synthesis by using a Thermo scientific® cDNA synthesis kit and stored at –80 °C.

2.7. Primer Designing, RT-PCR, and qRT-PCR Analysis

Primers were designed to amplify six PR-family genes (Table 1) using Primer3 Input (http://bioinfo.ut.ee/primer3-0.4.0/, accessed on 20 December 2019).

Table 1. Primer sequences used for RT-PCR and wheat genes they are targeting.

Gene	Accession Number	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Product Size (bp)
PR-1	HM489878.1	GCCAGCTACTACTCTCTCCG	AGGTATCCCATGCACGACTC	175
Thionins	AY253444.1	AAGCACTTCTGGATTTCGCC	CATCCTGTTCATCGCTGCAG	168
TLP	KJ764822.1	TTCCTCCTCCTGGCTGTTTT	ATATCCTCCCGGCTTTGGTG	175
Chitinase 2	AB029935.1	ACGGCGATATGGTTCTGGAT	TAGCGCTTGTAGAACCCGAT	209
β-1,3-glucanase	DQ090946.1	CTACAGGTCCAAGGGCATCA	GCGGCGATGTACTTGATGTT	210
Defensin	KJ551546.1	TGTCCAATAAGAACTGCGCG	TGGTTCCATGGGCTAGCTAG	161
Actin	GQ339780.1	GAGAAGCTCGCATATGTGGC	TCCAGCAGCTTCCATACCA	180

RT-PCR was performed to check primer specificity for the amplification of six selected genes, such as PR-1 (HM489878.1), Thionins (AY253444.1), Chitinase 2 (AB029935.1), β-1,3-glucanase (DQ090946.1), Thaumatin like protein (TLP) (KJ764822.1), and Defensin

(KJ551546.1) from both varieties. The actin gene (GQ339780.1) was used as a reference control. Total cDNA was used as a template for the RT-PCR.

RT-PCR was performed in a 25-µL reaction mixture comprising of 16 µL water, 2.50 µL 10× PCR buffer, 1.50 µL dNTPs, 1.50 µL $MgCl_2$, 1 µL template, 0.50 µL Taq polymerase, and 1 µL from each forward and reverse primers (Thermo scientific®). The following thermal profile was set up for the reactions: 94 °C for 5 min, followed by 25 cycles of 94 °C for 40 s, 49 °C for 1 min, and 72 °C for 1 min, and the final extension at 72 °C for 5 min. 1.50% agarose gel was used to run the PCR product.

The qRT-PCR was carried out by using Applied Biosystems 7300 Real-Time PCR System. A volume of 3 µL first strand cDNAs and SYBR Green PCR Master Mix (Thermo scientific®) was used to perform thermal cycling with following conditions: initial denaturation at 95 °C for 1 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 49 °C for 15 s, and extension at 72 °C for 45 s. Relative quantitation was calculated and normalized to the housekeeping Actin gene.

2.8. Statistical Data Analysis

The analysis of collected data was carried out by using the Statistics 8.1 software. Statistical analyses were performed using two-way ANOVA followed by the Tukey test. The results are presented as mean ± SDa. The differences among groups were considered statistically significant at a p-value < 0.05.

3. Results

3.1. Disease Severity Profiling

Both varieties showed symptoms on leaves after 15–20 days of germination in *F. equiseti* inoculated soil. No symptoms appeared on control plants. These results confirm the successful systemic inoculation of fungus. Shafaq-2006 showed less disease symptoms than Sahar-2006. In addition, comparing to control, the less number of plants were observed under fungal treatments, where most of the plants of susceptible variety (Sahar-2006) died as compared to resistant variety (Shafaq-2006) (Figure 2). For Shafaq-2006, 2% diseased plants were observed, whereas 18.80% diseased plants were found for Sahar-2006, as shown in Table 2.

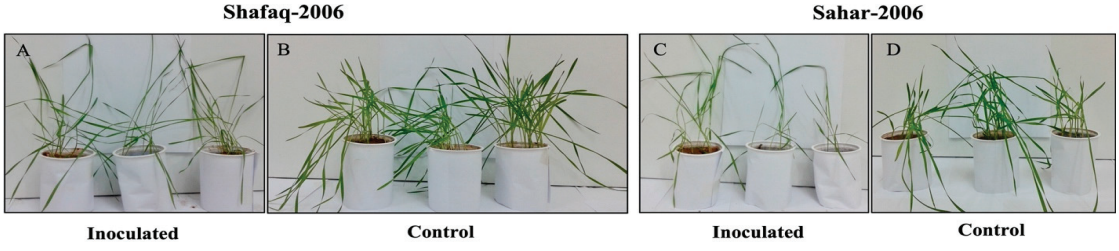

Figure 2. Disease severity analysis of the resistant (Shafaq-2006), and susceptible (Sahar-2006) wheat varieties. (**A**) Shafaq-2006 (Inoculated), (**B**) Shafaq-2006 (Control), (**C**) Sahar-2006 (Inoculated), and (**D**) Sahar-2006 (Control).

Table 2. Mean number of leaf spots and diseased plants' percentage.

Variety	Treatment	Mean Number of Spots per Leaf	Diseased Plants (%)
Shafaq-2006	Control	0	0
	Inoculated	4	2
Sahar-2006	Control	0	0
	Inoculated	16.60	18.80

3.2. Biochemical Parameters

Different biochemical parameters were measured in order to analyze the effects of *F. equiseti* on susceptible and resistant wheat varieties. Total proline and protein contents significantly increased by 44.66% and 68.07%, respectively, in the resistant variety (Shafaq-2006) as compared to the susceptible variety (m) under *F. equiseti* inoculation. The amount of total proline contents was the same in control plants of both varieties (Figure 3A), but the total proline contents of inoculated Sahar-2006 decreased 29.28%, compared to control. While a significant difference was observed in protein contents of both varieties in control plants. High protein contents (32.58%) were observed in control plants of Shafaq-2006 than Sahar-2006 (Figure 3B). The analysis of sugar contents showed a significant difference between the inoculated and control wheat varieties. Where, a significant increase in the sugar contents of Shafaq-2006 was recorded, compared to control. Similarly, a significant difference was observed in the sugar contents of both inoculated varieties, where Shafaq-2006 exhibited 51.10% higher sugar contents, compared to Sahar-2006. Additionally, the sugar content of Shafaq-2006 increased by 35.50% compared to that of Sahar-2006 under control conditions (Figure 3C). However, the sugar content of Sahar-2006 significantly decreased by 18.58% under fungal inoculation, compared to control.

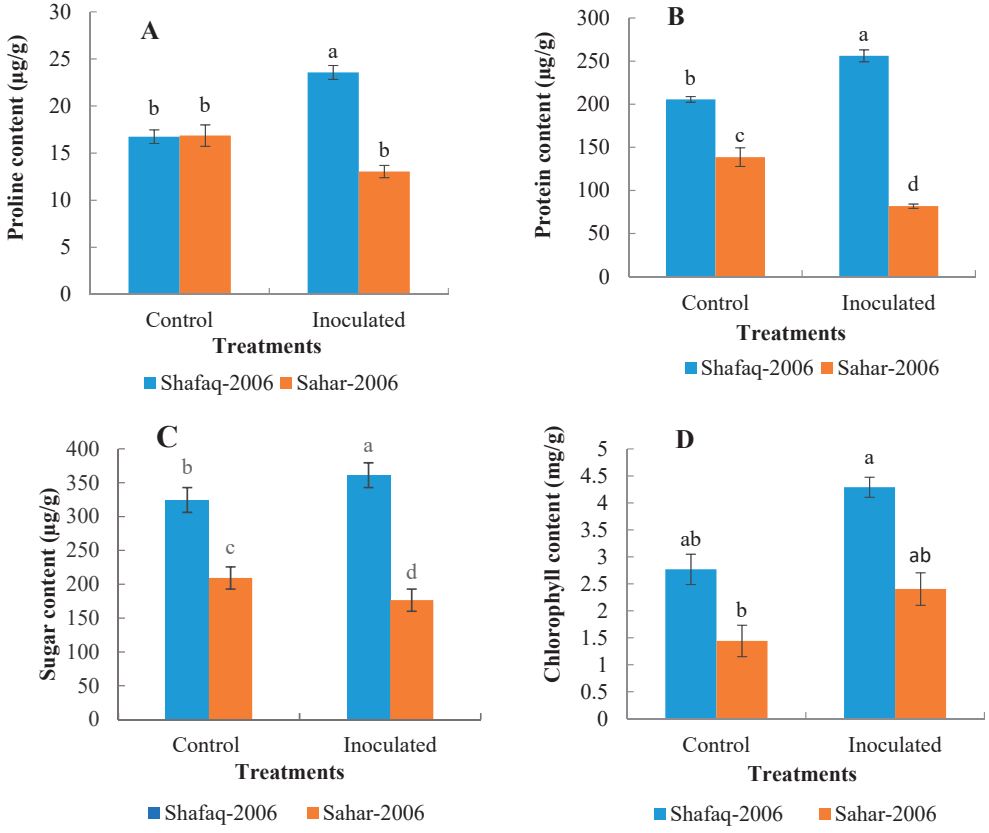

Figure 3. Measurement of the biochemical parameters, such as (**A**) Proline contents, (**B**) Protein content, (**C**) Sugar content, and (**D**) Chlorophyll content after the systemic inoculation of *Fusarium equiseti* in the Shafaq-2006 and Sahar-2006. All the means sharing common letter(s) are not significantly different at $p < 0.05$ and vertical bars represent the standard error of means (n = 3), while all the means with different letter(s) are significantly different at $p < 0.05$.

Although there was not a significant difference in total chlorophyll contents of both varieties, the chlorophyll contents of Shafaq-2006 increased by 43.99% to that of Sahar-2006,

which exhibited a considerable reduction in chlorophyll contents under fungal inoculation. Higher chlorophyll contents were observed in the control plants of resistant variety than susceptible one (Figure 3D). However, the inoculated Sahar-2006 exhibited 40.02% higher total chlorophyll contents as compared to control.

3.3. Physiological Parameters

Different physiological parameters were also measured to assess the effect of *F. equiseti*. The RWC of Shafaq-2006 was observed to be significantly higher under inoculation as well as control conditions, compared to Sahar-2006. In control, the higher RWCs (36.15%) were observed in Shafaq-2006, compared to Sahar-2006. However, an increase in RWC was noted under inoculated treatment, where Shafaq-2006 exhibited 55.40% increase in RWC, compared to Sahar-2006 (Figure 4A). However, there was no significant difference in RWC of Sahar-2006 under inoculation and control conditions. In the case of root–shoot ratio, both varieties did not show a significant difference between their inoculated and control plants. The root–shoot ratio of inoculated Sahar-2006 rather decreased than control. However, a little increase in the root–shoot ratio of inoculated Shafaq-2006 was recorded, compared to control. However, the results exhibited a significant difference in inoculated treatment, where in, the root–shoot ratio of Shafaq-2006 increased by 58.11% in comparison with Sahar-2006 (Figure 4B). In addition, plant length was also analyzed, which clearly indicated the susceptibility and resistance of Sahar-2006 and Shafaq-2006 to *F. equiseti*, respectively. Because the plant length of inoculated Shafaq-2006 was significantly increased by 20.68%, compared to control, it increased by 55.21% to that of Sahar-2006 under *F. equiseti* inoculation treatment. However, Shafaq-2006 exhibited a higher plant length (15.94%), compared to Sahar-2006 in control (Figure 4C). Moreover, the plant length of inoculated Sahar-2006 also decreased by 48.86% compared to that of control.

3.4. Expression Analysis by RT-PCR and qRT-PCR

RT-PCR was used in order to check the bands' intensity of six genes, including PR-1, Thionins, Thaumatin-like protein (TLP), Chitinase 2, β-1,3-glucanase, and Defensin in inoculated Shafaq-2006 wheat variety. PR-1 and TLP genes were observed to be more expressed in inoculated Shafaq-2006 by observing higher bands' intensity. Moreover, the bands' intensity of β-1,3 glucanase and Chitinase 2 was observed to be higher (Figure 5). These results indicate a possible role of these genes in resistance against *F. equiseti* stress in Shafaq-2006. Low bands' intensity of Thionins and Defensins genes were observed, which shows that these genes do not play any considerable role against *F. equiseti* stress in Shafaq-2006.

The expression analysis of six genes, including PR-1, Thionins, Thaumatin-like protein (TLP), Chitinase 2, β-1,3-glucanase, and Defensin was observed by qRT-PCR. The qRT-PCR analysis showed the higher expression of PR-1 in inoculated Shafaq-2006. The expression levels of TLP, β-1,3 glucanase, and Chitinase were also observed to be higher in the inoculated Shafaq-2006 (Figure 6). It indicates that these genes are involved in resistance against *F. equiseti* in Shafaq-2006. The expression level of Defensins and Thionins was observed to be lower in Shafaq-2006 as compared to other genes, which shows that these genes do not play any considerable role in resistance against *F. equiseti* stress in inoculated Shafaq-2006. In the case of Sahar-2006, the results of the qRT-PCR analysis showed minute expression of these genes under stress, thus suggesting that these genes do not play a role in conferring resistance against *F. equiseti* stress.

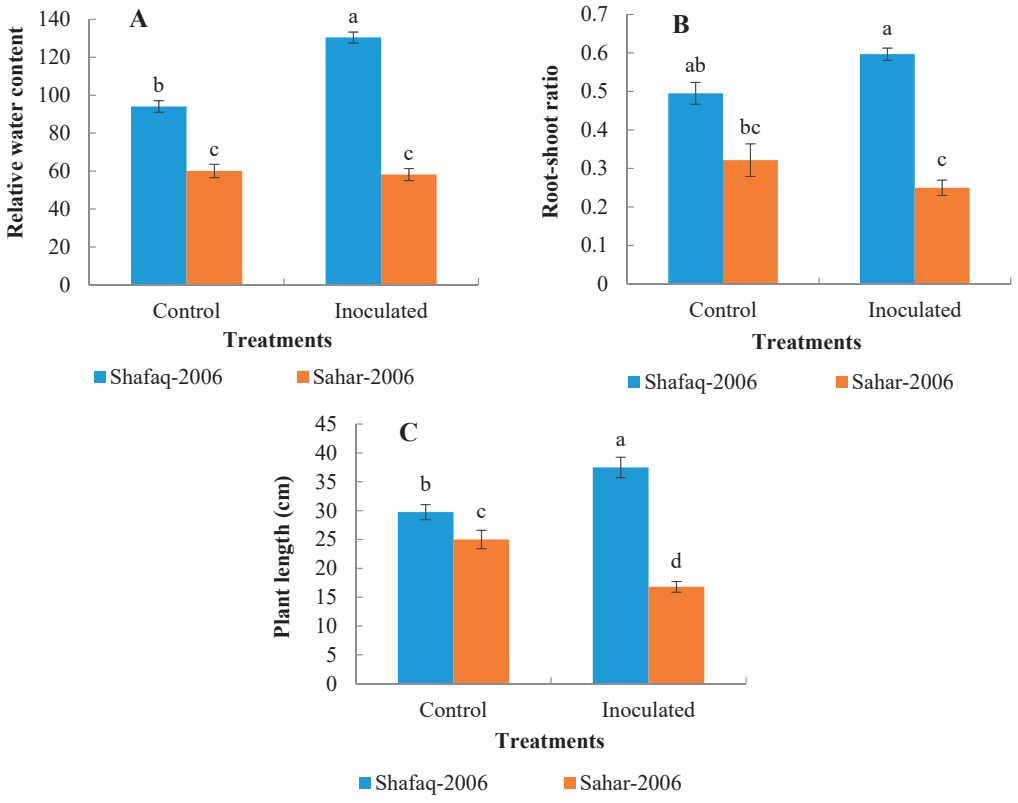

Figure 4. Measurement of the physiological parameters, such as (**A**) RWCs, (**B**) Root–shoot ratio, and (**C**) Plant length after the systemic inoculation of *Fusarium equiseti* in the Shafaq-2006 and Sahar-2006. All the means sharing common letter(s) are not significantly different at $p < 0.05$ and vertical bars represent the standard error of means (n = 3), while all the means with different letter(s) are significantly different at $p < 0.05$.

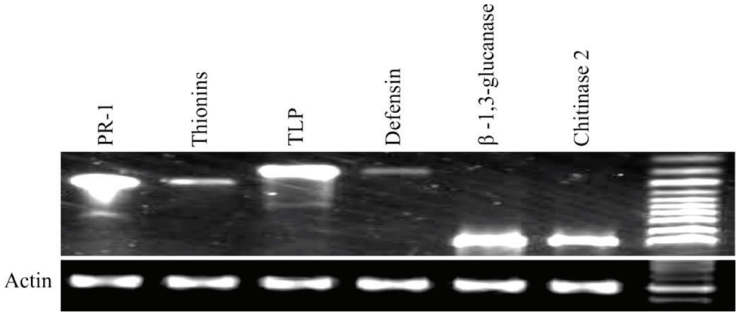

Figure 5. RT-PCR analysis of six PR-family genes in the Shafaq-2006 after the inoculation of *Fusarium equiseti*. Actin was used as a control.

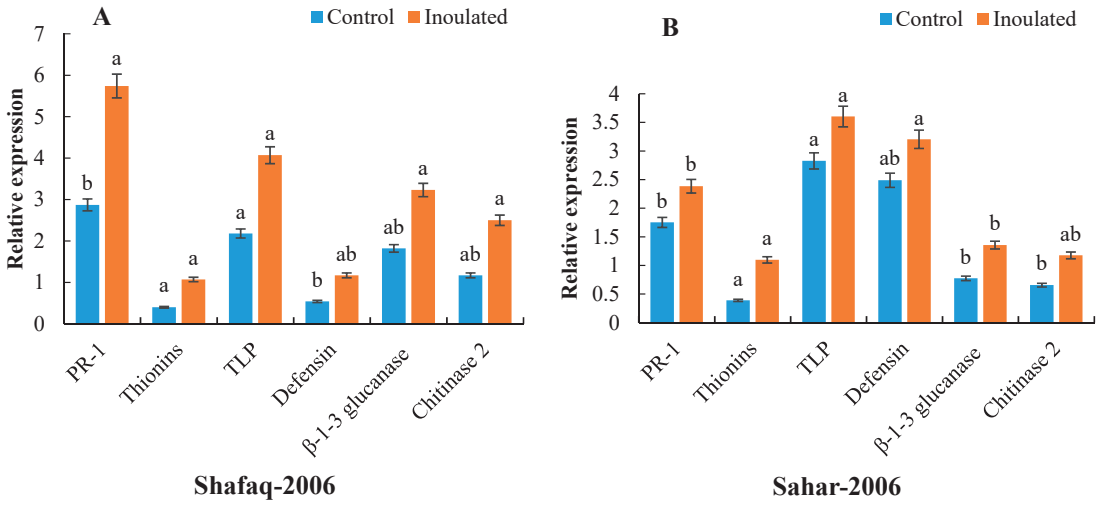

Figure 6. Relative expression of pathogenesis-related genes by qRT-PCR in inoculated and control wheat varieties. (**A**) Relative expression of pathogenesis-related genes in Shafaq-2006. (**B**) Relative expression of pathogenesis-related genes in Sahar-2006. All the means sharing common letter(s) are not significantly different at $p < 0.05$ and vertical bars represent standard error of means (n = 3), while all the means with different letter(s) are significantly different at $p < 0.05$.

4. Discussion

This experiment was conducted to examine the disease severity and expression of defense-related genes in wheat. Disease severity analysis proved Shafaq-2006 to be resistant, while Sahar-2006 was found susceptible to *F. equiseti*. Although Shafaq-2006 has not been reported to be fungus resistant before, previous studies have described it to be aphid resistant [32]. Sahar-2006 has been described to be susceptible in its reactions against the pressure of inoculum, especially with root inoculation methods [33].

In this experiment, we have observed higher proline contents in the inoculated resistant variety and a reduction in proline contents were observed in the inoculated susceptible variety. According to Claussen [43], a certain stress level is required for proline accumulation. Under various stress conditions, for instance, high salinity, drought, and biotic stress, proline accumulates in high concentration [44]. Studies have shown that proline contents in wheat were increased under water stress [45,46]. As a compatible solute, proline is involved in osmotic adjustment and mainly accumulates in most of the plants in stress condition [47]. Protein contents were observed to be increased in inoculated resistant variety, while a decrease in protein contents was observed in susceptible variety after infection. The analysis of the present results revealed that the protein contents in Shafaq-2006 were increased to that of Sahar-2006 in control as well as stress condition. Under *F. equiseti* stress (inoculation), the protein contents of Sahar-2006 were highly decreased; in contrast, protein contents of Shafaq-2006 significantly increased by 68.07% more than Sahar-2006. Thus, it has been reported that the increase in total protein contents plays an important role in plant defense [48]. Moreover, the higher sugar contents were recorded for the resistant wheat variety both in inoculated and control conditions. Similarly, Mohammadkhani and Heidari [49] reported the higher sugar and proline contents of wheat under stress. Sugar also plays a primary role in the plant defense mechanism against pathogens [50]. The sugar level is correlated with disease reaction in many plants and the high sugar level is considered responsible for the resistance to disease [50]. Generally, some pathogenic infections bring change in photosynthetic rate and respiratory pathway that cause fluctuation in sugar content [51]. Moreover, the increase in total chlorophyll contents was observed in inoculated Shafaq-2006, while Sahar-2006 exhibited a considerable reduction in total chlorophyll contents as compared to Shafaq-2006 after *F. equiseti* infection. The results

of the present study are in accordance with Manghwar et al. [52], who reported higher total chlorophyll contents in resistant wheat variety than the susceptible one after fungal infection. Interestingly, the total chlorophyll contents of Sahar-2006 also increased under fungal stress, compared to control plants. An increase in chlorophyll content might be due to the presence of a large number of bundle sheath chloroplast in the leaves of inoculated plants [52,53]. The reduction in chlorophyll contents in stress condition has been regarded as a typical symptom of oxidative stress that might be the result of chlorophyll degradation and pigment photo-oxidation [54]. In general, the resistant wheat variety (Shafaq-2006) exhibited higher biochemical parameters in the *F. equiseti* inoculated soil. These results are supported by many studies, which report that the increase in biochemical parameters under stress might be associated with a plant defense mechanism against pathogens [52,55,56].

An increase in RWCs was observed in Shafaq-2006, while low RWCs were observed in Sahar-2006 after *F. equiseti* inoculation. The increase in RWCs of the resistant wheat variety might be related to its higher protein production and total chlorophyll contents, which can promote the photosynthetic capacity by enhancing the gas exchange ability and water status under stress [57,58]. An increase in the total length of the plant was observed in the inoculated resistant variety. In the inoculated susceptible variety, the total length was significantly reduced. This decrease might be due to the fact that the water stress reduces the rate of plant height and leaf appearance [59]. Additionally, a higher root–shoot ratio (58.11%) of plants was observed in the inoculated resistant variety compared to the susceptible variety. Similarly, the root–shoot ratio of sorghum enhanced under water stress [60], whereas the root–shoot ratio of the inoculated susceptible variety was reduced. The reduction in the root length is an obvious plant response to fungal infection [61]. The studies have reported that the stress conditions at the seeding stage decrease the weight of endosperm and retard the growth of radicle, coleoptile, root, and shoot [62]. In brief, by analyzing the results of biochemical and physiological parameters, it could be concluded that Shafaq-2006 is resistant while Sahar-2006 is susceptible against *F. equiseti*. Furthermore, to assess the defense mechanism of resistant wheat variety and the expression of defense-related genes, both the inoculated and control wheat varieties were subjected to RT-PCR and qRT-PCR.

The high bands' intensity of PR-1, TLP, chitinase 2, and β-1,3-glucanase genes were observed by RT-PCR in the inoculated Shafaq-2006 wheat variety. These results indicate a possible role of these genes in the resistance against *F. equiseti* stress in Shafaq-2006. Low band intensities of Thionins and Defensins genes were observed. The high expression of PR-1, TLP, chitinase 2, and β-1,3-glucanase genes was shown during the qRT-PCR in Shafaq-2006 variety, while thionins and defensins did not show any expression. The expression of these genes was inconsiderable in the inoculated and control Sahar-2006 varieties. This might be the reason why Sahar-2006 could not overcome the infection caused by *F. equiseti* after the inoculation. As compared to Shafaq-2006, the effect of infection on Sahar-2006 was prominent, higher concentration of proline, reduced chlorophyll, sugar, protein, and water contents in addition to the overall reduction in growth rate showed acute effects of *F. equiseti.* RT-PCR and qRT-PCR results have shown that PR-1, TLP, Chitinase, and β-1,3-glucanase genes might be involved in playing major roles in inducing disease resistance in inoculated resistant plants. It has also been shown by the results that thionins and defensins do not play any considerable role in disease resistance of Shafaq-2006 against *F. equiseti*. Higher levels of these genes' expression may have reduced the infection against *F. equiseti*. Several PR proteins, including PR-1, 2, 3, 4, and 5 have been reported to inhibit the growth of fungi. PR-1 inhibits the growth of *Pseudomonas syringae* bacterium and *Cercospora nicotianae* fungus as well as oomycetes, including *Peronospora tabacina* and *Phytophthora parasitica* [17]. PR-1 proteins are involved in the thickening of the cell wall and may provide resistance against the pathogen spread in the apoplast [63]. In plants, the overexpression of TLP genes has been found to provide enhanced tolerance against fungal pathogens [64,65]. Thaumatin-like proteins (TLPs) are not normally expressed constitutively in healthy plants, but are induced exclusively in response to a

pathogenic attack or wounding [66]. The Chitinase gene is thought to play a dual role in fungal growth inhibition by digesting the cell wall and releasing pathogen-borne elicitors that are involved in inducing further defense reactions in the host. Overexpressions of β-1,3-glucanases and Chitinases are well-known examples of protection conferred by the transgenic expression of plant antifungal genes [65,67]. Chitinases expressed at high levels appear to be effective against plant-pathogenic fungi [68]. Chitinase genes are upregulated by *F. graminearum* during the early stages of barley and wheat spikes' infection [69]. Plant β-1,3-glucanases have been proposed as important components of plant defense mechanisms against pathogens [27]. Plant β-1,3-glucanases are thought to be involved in playing a key role in plant defense responses to pathogen infection [22].

5. Conclusions

It is concluded that Shafaq-2006 is a more resistant variety than Sahar-2006 against *F. equiseti*. All the PR-family genes do not play an equal role in the defense mechanism of Shafaq-2006. The higher expression of PR-1, TLP, Chitinase, and β-1,3-glucanase genes in Shafaq-2006 predicts their possible role in the defense mechanism of this variety against *F. equiseti*. These overexpressed genes might have created disease resistance by activating a defense mechanism and improving the production of different biochemicals such as protein, proline, chlorophyll, and sugar contents and also physiological parameters, such as RWC, total length of the plant, root and shoot length, and fresh and dry weight of root and shoot. Further research needs to be done to overexpress these genes by genetic engineering in Shafaq-2006 in order to increase its immunity against *F. equiseti* and other fungi.

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References

1. Panhwar, Q.A.; Ali, A.; Depar, N.; Shah, J.A. Screening of plant growth promoting rhizobacteria for sustainable wheat (*Triticum aestivum* L.) crop production. *Pak. J. Bot.* **2020**, *52*, 345–353. [CrossRef]
2. Kong, H.; Zhang, Z.; Qin, J.; Akram, N.A. Interactive effects of abscisic acid (ABA) and drought stress on the physiological responses of winter wheat (*Triticum aestivum* L.). *Pak. J. Bot.* **2021**, *53*, 1545–1551. [CrossRef]
3. Naz, S.; Perveen, S. Response of wheat (*Triticum aestivum* L. var. galaxy-2013) to pre-sowing seed treatment with thiourea under drought stress. *Pak. J. Bot.* **2021**, *53*, 1209–1217. [CrossRef]
4. Messiaen, C.; Cassini, R. Recherches sur les Fusarioses. IV. La systématique des Fusarium. *Ann. Epiphyt.* **1968**, *19*, 387–454.
5. Booth, C. The genus fusarium. *Genus Fusarium*, 1971; 237p. [CrossRef]
6. Shaner, G. Epidemiology of Fusarium head blight of small grain cereals in North America. *Fusarium Head Blight Wheat Barley* **2003**, 84–119.
7. Gale, L. Population biology of Fusarium species causing head blight of grain crops. *Fusarium Head Blight Wheat Barley* **2003**, 120–143.
8. Palmero, D.; Iglesias, C.; De Cara, M.; Lomas, T.; Santos, M.; Tello, J. Species of Fusarium isolated from river and sea water of southeastern Spain and pathogenicity on four plant species. *Plant Dis.* **2009**, *93*, 377–385. [CrossRef] [PubMed]

9. Munis, M.F.H.; Tu, L.; Deng, F.; Tan, J.; Xu, L.; Xu, S.; Long, L.; Zhang, X. A thaumatin-like protein gene involved in cotton fiber secondary cell wall development enhances resistance against Verticillium dahliae and other stresses in transgenic tobacco. *Biochem. Biophys. Res. Commun.* **2010**, *393*, 38–44. [CrossRef]
10. Van Loon, L. Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Plant Pathol.* **1997**, *103*, 753–765. [CrossRef]
11. Xu, L.; Zhu, L.; Tu, L.; Guo, X.; Long, L.; Sun, L.; Gao, W.; Zhang, X. Differential gene expression in cotton defence response to *Verticillium dahliae* by SSH. *J. Phytopathol.* **2011**, *159*, 606–615. [CrossRef]
12. Veronese, P.; Ruiz, M.T.; Coca, M.A.; Hernandez-Lopez, A.; Lee, H.; Ibeas, J.I.; Damsz, B.; Pardo, J.M.; Hasegawa, P.M.; Bressan, R.A. In defense against pathogens. Both plant sentinels and foot soldiers need to know the enemy. *Plant Physiol.* **2003**, *131*, 1580–1590. [CrossRef] [PubMed]
13. Mohamed, I.A.; Shalby, N.; MA El-Badri, A.; Saleem, M.H.; Khan, M.N.; Nawaz, M.A.; Qin, M.; Agami, R.A.; Kuai, J.; Wang, B. Stomata and xylem vessels traits improved by melatonin application contribute to enhancing salt tolerance and fatty acid composition of *Brassica napus* L. plants. *Agronomy* **2020**, *10*, 1186. [CrossRef]
14. Hameed, A.; Akram, N.A.; Saleem, M.H.; Ashraf, M.; Ahmed, S.; Ali, S.; Abdullah Alsahli, A.; Alyemeni, M.N. Seed treatment with α-tocopherol regulates growth and key physio-biochemical attributes in carrot (*Daucus carota* L.) plants under water limited regimes. *Agronomy* **2021**, *11*, 469. [CrossRef]
15. Nawaz, M.; Wang, X.; Saleem, M.H.; Khan, M.H.U.; Afzal, J.; Fiaz, S.; Ali, S.; Ishaq, H.; Khan, A.H.; Rehman, N.; et al. Deciphering plantago ovata forsk leaf extract mediated distinct germination, growth and physio-biochemical improvements under water stress in maize (*Zea mays* L.) at early growth stage. *Agronomy* **2021**, *11*, 1404. [CrossRef]
16. Borad, V.; Sriram, S. Pathogenesis-related proteins for the plant protection. *Asian J Exp Sci* **2008**, *22*, 189–196.
17. Sudisha, J.; Sharathchandra, R.; Amruthesh, K.; Kumar, A.; Shetty, H.S. Pathogenesis related proteins in plant defense response. In *Plant Defence: Biological Control*; Springer: Berlin/Heidelberg, Germany, 2012; pp. 379–403.
18. Bohlmann, H.; Clausen, S.; Behnke, S.; Giese, H.; Hiller, C.; Reimann-Philipp, U.; Schrader, G.; Barkholt, V.; Apel, K. Leaf-specific thionins of barley—A novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. *EMBO J.* **1988**, *7*, 1559–1565. [CrossRef]
19. Kang, Z.; Buchenauer, H. Immunocytochemical localization of cell wall-bound thionins and hydroxyproline-rich glycoproteins in Fusarium culmorum-infected wheat spikes. *J. Phytopathol.* **2003**, *151*, 120–129. [CrossRef]
20. Bryngelsson, T.; Green, B. Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. *Physiol. Mol. Plant Pathol.* **1989**, *35*, 45–52. [CrossRef]
21. Pierpoint, W.; Jackson, P.; Evans, R.M. The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). *Physiol. Mol. Plant Pathol.* **1990**, *36*, 325–338. [CrossRef]
22. Ebrahim, S.; Usha, K.; Singh, B. Pathogenesis related (PR) proteins in plant defense mechanism. *Sci. Against Microb. Pathog.* **2011**, *2*, 1043–1054.
23. Anguelova-Merhar, V.; VanDer Westhuizen, A.; Pretorius, Z. β-1, 3-glucanase and chitinase activities and the resistance response of wheat to leaf rust. *J. Phytopathol.* **2001**, *149*, 381–384. [CrossRef]
24. Nielsen, K.K.; Mikkelsen, J.D.; Kragh, K.M.; Bojsen, K. An acidic class III chitinase in sugar beet: Induction by Cercospora beticola, characterization, and expression in transgenic tobacco plants. *Mol. Plant Microb. Interact.* **1993**, *6*, 495. [CrossRef]
25. Lawrence, C.B.; Singh, N.P.; Qiu, J.; Gardner, R.G.; Tuzun, S. Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria solani* and may function as an elicitor release mechanism. *Physiol. Mol. Plant Pathol.* **2000**, *57*, 211–220. [CrossRef]
26. Caruso, C.; Chilosi, G.; Caporale, C.; Leonardi, L.; Bertini, L.; Magro, P.; Buonocore, V. Induction of pathogenesis-related proteins in germinating wheat seeds infected with Fusarium culmorum. *Plant Sci.* **1999**, *140*, 87–97. [CrossRef]
27. Mauch, F.; Staehelin, L.A. Functional Implications of the Subcellular Localization of Ethylene-Induced Chitinase and [beta]-1, 3-Glucanase in Bean Leaves. *Plant Cell* **1989**, *1*, 447–457. [CrossRef]
28. Kombrink, E.; Somssich, I. Pathogenesis-related proteins and plant defense. In *Plant Relationships*; Springer: Berlin/Heidelberg, Germany, 1997; pp. 107–128.
29. Broekaert, W.F.; Cammue, B.P.; De Bolle, M.F.; Thevissen, K.; De Samblanx, G.W.; Osborn, R.W.; Nielson, K. Antimicrobial peptides from plants. *Crit. Rev. Plant Sci.* **1997**, *16*, 297–323. [CrossRef]
30. De Oliveira Carvalho, A.; Moreira Gomes, V. Plant defensins and defensin-like peptides-biological activities and biotechnological applications. *Curr. Pharm. Design* **2011**, *17*, 4270–4293. [CrossRef] [PubMed]
31. Atiq-ur-Rehman, R.; Shahzad, A.; Muhammad, F.; Muhammad, Z.; Shamim, I.; Yasmin, A. Status of foliar diseases of wheat in Punjab, Pakistan. *Mycopath* **2011**, *9*, 39–42.
32. Wains, M.S.; Ali, M.A.; Hussain, M.; Anwar, J.; Zulkiffal, M.; Sabir, W. Aphid dynamics in relation to meteorological factors and various management practices in bread wheat. *J. Plant Protect. Res.* **2010**, *50*, 385–392. [CrossRef]
33. Beniwal, M.; Chawla, P.; Singh, R. Comparison of different methods of inoculation and evaluation of wheat material against *Neovossia indica* (Mitra) Mundkur. *Crop Res. (Hisar)* **2001**, *21*, 105–108.
34. Ullah, H.Z.; Haque, M.; Rauf, C.; Akhtar, L.; Munir, M. Comparative virulence in isolates of *Tilletia indica* and host resistance against Karnal bunt of wheat. *J. Animal Plant Sci.* **2012**, *22*, 467–472.

35. Ibrahim, T.; Bano, A.; Chaudhary, H.J.; Imran, M.; Mehmood, Z.; Hassan, S.W.; Muhammad, N.; Naqvi, S.A.H.; Munis, M.F.H. Evaluation of different inoculation methods for the induction of spot blotch caused by *Helminthosporium sativum* in wheat. *Philipp. J. Crop Sci.* **2014**, *39*, 27–33.
36. Ahmed, B.; Sharif, F.; Sarhan, A. Effect of certain micronutrients on Fusarium wilt of tomato. *J. Agric. Water Res. Plant Protec.* **1987**, *6*, 13–28.
37. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.T.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
38. Johnson, R.R.; Balwani, T.L.; Johnson, L.; McClure, K.; Dehority, B. Corn plant maturity. II. Effect on in vitro cellulose digestibility and soluble carbohydrate content. *J. Animal Sci.* **1966**, *25*, 617–623. [CrossRef]
39. Bates, L.S.; Waldren, R.P.; Teare, I. Rapid determination of free proline for water-stress studies. *Plant Soil* **1973**, *39*, 205–207. [CrossRef]
40. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]
41. Arnon, D.I. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* **1949**, *24*, 1. [CrossRef]
42. Wheatherley, P. Studies in the water relations of cotton plants. I. The field measurement of water deficit in leaves. *New Phytol.* **1950**, *49*, 81–87. [CrossRef]
43. Claussen, W. Proline as a measure of stress in tomato plants. *Plant Sci.* **2005**, *168*, 241–248. [CrossRef]
44. Mattioli, R.; Costantino, P.; Trovato, M. Proline accumulation in plants: Not only stress. *Plant Signal. Behav.* **2009**, *4*, 1016–1018. [CrossRef] [PubMed]
45. Vendruscolo, E.C.G.; Schuster, I.; Pileggi, M.; Scapim, C.A.; Molinari, H.B.C.; Marur, C.J.; Vieira, L.G.E. Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. *J. Plant Physiol.* **2007**, *164*, 1367–1376. [CrossRef]
46. Qayyum, A.; Razzaq, A.; Ahmad, M.; Jenks, M.A. Water stress causes differential effects on germination indices, total soluble sugar and proline content in wheat (*Triticum aestivum* L.) genotypes. *Afr. J. Biotechnol.* **2011**, *10*, 14038–14045.
47. Amirjani, M.R.; Mahdiyeh, M. Antioxidative and biochemical responses of wheat to drought stress. *J. Agric. Biol. Sci.* **2013**, *8*, 291–301.
48. Arjunan, G.; Vidhyasekaran, P.; Kandaswamy, T.K. Changes in amino acids and amides content in jowar leaves infected by *Helminthosporium turcicum*. *Curr. Sci. (India)* **1976**, *45*, 229–230.
49. Mohammadkhani, N.; Heidari, R. Water Stress Induced by Polyethylene Glycol 6000 and Sodium. *Pak. J. Biol. Sci.* **2008**, *2*, 92–97.
50. Nagaveni, T. *Resistance Factors for Leaf Blight of Barley Caused by Helminthosporium Sativum Pam, King and Bakke*; University of Agricultural Sciences, Bangalore: Bengaluru, India, 2005.
51. Klement, Z.; Goodman, R. The hypersensitive reaction to infection by bacterial plant pathogens. *Annu. Rev. Phytopathol.* **1967**, *5*, 17–44. [CrossRef]
52. Manghwar, H.; Hussain, A.; Ullah, A.; Gul, S.; Shaban, M.; Khan, A.H.; Ali, M.; Sani, S.G.A.S.; Chaudhary, H.J.; Munis, M.F.H. Expression analysis of defense related genes in wheat and maize against *Bipolaris sorokiniana*. *Physiol. Mol. Plant Pathol.* **2018**, *103*, 36–46. [CrossRef]
53. Krishna, K.; Bagyaraj, D. Growth and nutrient uptake of peanut inoculated with the mycorrhizal fungus Glomus fasciculatum compared with non-inoculated ones. *Plant Soil* **1984**, *77*, 405–408. [CrossRef]
54. Anjum, S.A.; Xie, X.-Y.; Wang, L.-C.; Saleem, M.F.; Man, C.; Lei, W. Morphological, physiological and biochemical responses of plants to drought stress. *Afr. J. Agric. Res.* **2011**, *6*, 2026–2032.
55. Devi, M.C.; Reddy, M. Growth response of groundnut to VAM fungus and *Rhizobium* inoculation. *Plant Pathol. Bull.* **2001**, *10*, 71–78.
56. Rajasekaran, S.; Nagarajan, S. Effect of dual inoculation (AM Fungi and Rhizobium) on chlorophyll content of *Vigna unguiculata* (L.) Walp. Var. Pusa 151. *Mycorrhiza News* **2005**, *17*, 10–11.
57. Aroca, R.; Ruiz-Lozano, J.M.; Zamarreño, Á.M.; Paz, J.A.; García-Mina, J.M.; Pozo, M.J.; López-Ráez, J.A. Arbuscular mycorrhizal symbiosis influences strigolactone production under salinity and alleviates salt stress in lettuce plants. *J. Plant Physiol.* **2013**, *170*, 47–55. [CrossRef]
58. Wang, J.; Fu, Z.; Ren, Q.; Zhu, L.; Lin, J.; Zhang, J.; Cheng, X.; Ma, J.; Yue, J. Effects of arbuscular mycorrhizal fungi on growth, photosynthesis, and nutrient uptake of *Zelkova serrata* (Thunb.) Makino seedlings under salt stress. *Forests* **2019**, *10*, 186. [CrossRef]
59. Niakan, M.; Darvishkhezri, M.; Iranbakhsh, A.; Barzegar, A. Changes of sorghum growth in response to drought and allelopathy stresses. *Annals Biol. Res.* **2013**, *4*, 18–22.
60. Younis, M.; El-Shahaby, O.; Abo-Hamed, S.; Ibrahim, A. Effects of water stress on growth, pigments and $^{14}CO_2$ assimilation in three sorghum cultivars. *J. Agron. Crop Sci.* **2000**, *185*, 73–82. [CrossRef]
61. Al-Abdalall, A.H.A. Assessment of yield loss caused by root rots in wheat and barley. *J. Food Agric. Environ.* **2010**, *8*, 638–641.
62. Jafar, M.S.; Nourmohammadi, G.; Maleki, A. Effect of water deficit on seedling, plantlets and compatible solutes of forage Sorghum cv. Speedfeed. In Proceedings of the 4th International Crop Science Congress, Brisbane, Austrialia, 26 September–1 October 2004.
63. Eyal, Y.; Sagee, O.; Fluhr, R. Dark-induced accumulation of a basic pathogenesis-related (PR-1) transcript and a light requirement for its induction by ethylene. *Plant Mol. Biol.* **1992**, *19*, 589–599. [CrossRef]

64. Velazhahan, R.; Muthukrishnan, S. Transgenic tobacco plants constitutively overexpressing a rice thaumatin-like protein (PR-5) show enhanced resistance to Alternaria alternata. *Biol. Plant.* **2003**, *47*, 347–354. [CrossRef]
65. Datta, K.; Velazhahan, R.; Oliva, N.; Ona, I.; Mew, T.; Khush, G.; Muthukrishnan, S.; Datta, S. Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to Rhizoctonia solani causing sheath blight disease. *Theor. Appl. Genet.* **1999**, *98*, 1138–1145. [CrossRef]
66. Monteiro, S.; Barakat, M.; Piçarra-Pereira, M.A.; Teixeira, A.R.; Ferreira, R.B. Osmotin and thaumatin from grape: A putative general defense mechanism against pathogenic fungi. *Phytopathology* **2003**, *93*, 1505–1512. [CrossRef]
67. Brogue, K.; Chet, I.; Holliday, M.; Cressman, R.; Biddle, P.; Knowlton, S.; Mauvais, C.J.; Broglie, R. Transgenic plants with enhanced resistance to the fungal pathogen Rhizoctonia solani. *Science* **1991**, *254*, 1194–1197. [CrossRef] [PubMed]
68. Okay, S.; ÖZCENGİZ, G. Molecular cloning, characterization, and homologous expression of an endochitinase gene from Bacillus thuringiensis serovar morrisoni. *Turk. J. Biol.* **2011**, *35*, 1–7.
69. Tripathi, A.; Aggarwal, R.; Yadav, A. Differential expression analysis of defense-related genes responsive to *Tilletia indica* infection in wheat. *Turk. J. Biol.* **2013**, *37*, 606–613. [CrossRef]

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Host Susceptibility of CIMMYT's International Spring Wheat Lines to Crown and Root Rot Caused by *Fusarium culmorum* and *F. pseudograminearum*

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Abstract: The destructive soilborne *Fusarium* species is one of the most serious challenges facing agriculture. Mycotoxins produced by *Fusarium* spp. can induce both acute and chronic toxic effects on humans and animals. Massive investments have been made in the last few decades to develop an appropriate management strategy to control *Fusarium* species in cereals, particularly in wheat, using genetic resistance and other practices, with varied outcomes. The purpose of this research was to find new sources of resistance to both *Fusarium culmorum* and *F. pseudograminearum*, which are wheat's most destructive pathogens in seedlings and adult plants stages. In this study, 26 lines were selected and promoted from a total of 200 spring wheat germplasm received from CIMMYT Mexico plus 6 local check lines. The 32 lines were screened for their resistance reactions to both *Fusarium* species under different environmental conditions. The discriminant factorial analysis indicated that 7, 12, and 5 were resistant lines against *F. culmorum* under field, greenhouse, and growth room conditions, respectively. Four lines, L12, L19, L21, and L26, were found to be jointly resistant at the adult and seedling stages in the field and greenhouse. On the other hand, only moderately resistant lines were found for *F. pseudograminearum* but not completely resistant, which was limited to growth room conditions. Interestingly, five lines (L10, L13, L17, L25, and L28) have shown resistant properties to both *Fusarium* species. To further evaluate the yield performance of the best-selected 26 lines plus 6 check lines, field trials were conducted under ± *F. culmorum* inoculum. The highest yield values were obtained from three check lines, as well as the L26, which showed consistency in its reaction to *F. culmorum* under both field and greenhouse conditions, and produced a high yield (5342 kg/ha). Based on the result obtained, L26 showed a high potential to improve wheat yield and resistance to *F. culmorum*-caused root and crown rot; therefore, it should be used in wheat crossing programs. Having *Fusarium*-resistant varieties will ultimately reduce crown rot symptoms and increase grain quality by reducing mycotoxin levels.

Keywords: *Fusarium* crown and root rot; *Triticum*; resistance screening

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1. Introduction

Wheat (*Triticum aestivum* L. and *T. durum* L.) is a major staple food crop that provides sustenance to around 40% of the world's population [1]. It offers 55% of the carbohydrates and 20% of the dietary calories consumed on a global scale [1,2]. In 2020, a total of 760.9 million tons (MT) of grain were globally produced over an area of 219 million hectares (Mha) (FAOSTAT 2022). Turkey is the tenth-largest wheat producer, with an average annual production of 20 MT produced over 7 Mha [3].

By 2050, the world's population is predicted to reach 9–10 billion people; as a result, grain output will need to increase by 50% by 2030 to meet the growing demand [4]. Wheat consumption is rising as the world's population grows, but wheat production has been dropping in recent years due to abiotic and biotic factors [5]. For instance, approximately 90% of wheat production is in rainy areas or where semi-supplemental irrigation is applied.

The majority of wheat-producing areas face drought challenges, especially at the post-anthesis stages [6]. At the same time, political instability, drought, other weather extremes, and persistent pest and disease pressure have exacerbated volatility in wheat yields, exports, and prices [7]. Above the yield damage that microorganisms cause, they also affect wheat crop quality [1]. *Fusarium* species cause severe and chronic diseases in cereals in many parts of the world. Disease symptoms known as foot rot (FFR), crown rot (FCR), and head blight (FHB) are caused primarily by *Fusarium culmorum*, *F. pseudograminearum* (formerly *F. graminearum* group 1), and *F. graminearum* (formerly *F. graminearum* group 2) are of high economic importance in wheat crops globally [8]. These three species have been reported to be associated with crown rot in wheat and cause significant yield damage in West Asia (Azerbaijan and Kazakhstan), North Africa (Egypt, Tunisia, and Morocco), the USA, Canada, Australia, and Turkey [8–12]. The same species have been reported for FHB epidemics in Asia, Canada, China, Europe, and South America [13].

Studies have demonstrated that the isolates of FCR and FHB pathogens can cause both diseases under appropriate climatic conditions. FCR occurs especially under hot and drought conditions [14] in rainfed and wheat monoculture systems, whereas FHB occurs in wet and warm environments. The pathogens cause necrosis and dry root, crown, and basal stem, known as FCR, whereas the same species infect floral tissue and cause blighting of grains, known as FHB.

The devastating and widespread disease FHB results in yield and quality loss in most wheat-growing regions [15]. Apart from reductions in grain yield and seed quality, the major risk due to FHB is the contamination of the crop with toxic fungal secondary metabolites known as mycotoxins [16,17]. They pose a chronic health risk; prolonged exposure through diet has been linked to cancer, diseases of the kidney and liver, and suppression of the immune system. In addition, mycotoxins can be present in livestock feed, reducing productivity in meat and dairy production. When these toxins find their way from feed into milk or meat (carry-over), they become a food safety hazard in these products as well [18]. Mycotoxins may be produced by *Fusarium* species during various growth stages in the field as well as during the storage of grains and other products.

Many cereal-infecting *Fusarium* species produce trichothecenes, including deoxynivalenol (DON) and nivalenol (NIV), and other mycotoxins in plant tissue that are harmful to animal and human health. Deoxynivalenol is the most frequent mycotoxin reaching the highest concentration levels also under the conditions of Central Europe [16]. DON accumulation in the grain is an acute problem as it is toxic to humans and livestock [18]. DON concentration varies with *Fusarium* species, weather conditions, and the plant organ affected. As FHB pathogens, *F. culmorum* and *F. graminearum* produce more DON in grains than *F. pseudograminearum,* but as FCR pathogens, *F. graminearum* and *F. pseudograminearum* produce similar amounts of DON in straw. When DON is produced in the infected stem base tissue, the water-soluble metabolite can be translocated into other plant parts. FCR infection by all three species, *F. culmorum*, *F. graminearum*, and *F. pseudograminearum* can lead to DON contamination of grains [10].

Crown and root rot, caused primarily by *Fusarium* species, are severe soilborne diseases that limit productivity in dryland wheat production areas across the world's major wheat-producing countries, as well as in Turkey [1,10–12,19,20]. Rotted seeds, seedlings, roots, crowns, and basal stems are among the cereal damage caused by these *Fusarium* species [21]. The etiology of *Fusarium* crown and root rot (FCRR) is complex, with multiple species frequently isolated from infected plants [22–24]. *Fusarium acuminatum*, *F. algeriense*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. hostae*, *F. graminearum*, *F. oxysporum*, *F. pseudograminearum*, *F. redolens*, etc. are the most common species which have been reported causing FCRR [10,23,25–28]. Infections by *Fusarium* species may exist independently but might tend to co-exist in the same locations and even in the same plants [23].

Several studies on *Fusarium* species causing FCRR revealed that *F. culmorum* and *F. pseudograminearum* are the most common and destructive species [22,25,29,30]. Both species share a number of physiological, genetic, and pathological similarities [31,32] as

well as the prevalence of both pathogens increased by hotter temperatures in dryland areas. However, *F. culmorum* prefers cooler temperatures than *F. pseudograminearum* [14,33].

Yield losses of up to 35% have been recorded from crown rot in the Pacific Northwest (PNW) of America, 25–58% in Australia [8,9,34], and up to 49% in Tunisia [35]. In Turkey, losses in winter wheat reached up to 43% [36] and 54% in durum wheat in the Central Anatolian Plateau (CAP) [37]. Aktaş et al. (1999) reported a disease intensity of 36.2% in winter cereals as a result of root and crown rots. Production losses have been estimated at $13 million in PNW, and the potential loss due to the reduced grain yield and quality in Australia is $80 million [24,35,38].

Several disease management strategies are already in use to reduce the burden of *Fusarium* species. Crop debris or contaminated plant stubble harbors the inocula [39]; therefore, managing stubble can have a significant impact on disease control [40]. Crop rotation is another agronomic strategy for reducing FCRR growth. In Turkey, the winter wheat–summer fallow rotation is the most common cropping system; however, wheat rotation with legumes is practiced in some areas. A further agronomic approach that can reduce disease incidence is the proper control of weed grasses that harbor *Fusarium* species. Seed treatment with fungicides or the application of fungicides to stem bases does not appear to provide adequate protection from *Fusarium* infections [41,42], particularly in winter crops, where *Fusarium* infections are more noticeable in the later stages when drought is present at maturity. However, more recently, successful use of fungicides against FCRR caused by *F. culmorum* has been reported, either as a seed dressing or as a foliar spray (treated twice at Zadoks development stages 31 and 45 with fluquinconazole, tebuconazole, or epoxiconazole with carbendazim) [43–45]. As can be evident, agronomic and chemical interventions aimed at reducing FCRR incidence are not always compatible with economic and practical concerns.

After all the above mentioned, increasing the genetic resistance of wheat cultivars against FCRR diseases is a prime priority. Controlling FCRR is difficult due to the scarcity of commercial cultivars resistant to all *Fusarium* species., No complete resistance exists even against a single pathogen, and the term "resistance" in this sense only refers to "partial resistance", which is a measurement of disease symptom development and/or fungal biomass. However, despite having a high fungal load, it is likely that some grain genotypes are tolerant and thus can maintain their yield potential or show reduced symptom development when infected. In the beginning, the accurate identification of the causative agents is critical for resistant cultivar breeding studies. In inbreeding programs and field studies, where diverse genotypes are examined for their reaction to a given pathogen, a mixture of isolates from the same species is typically utilized. The use of different field ranking procedures by different workers makes finding glasshouse inoculation procedures that correlate with field rankings more difficult [46]. It is also likely that different inoculation procedures used to detect partial resistance mechanisms operate differently at different stages of development [47].

The International Wheat and Maize Improvement Centre (CIMMYT) in Turkey receives approximately 1000 accessions of wheat each year from the CIMMYT Mexico spring wheat program and the International Winter Wheat Program (IWWIP, www.iwwip.org (accessed on 1 September 2021)). Accessions received are screened against various pathogens, including cereal nematodes and different *Fusarium* species in various geographical regions throughout Turkey, growth rooms, and greenhouses, to have single germplasm resistant to multiple soilborne diseases. The germplasm with multiple resistance is then distributed to international collaborators to employ in their breeding programs. This is a critical strategy to ensure that a chosen resistant wheat line can target as many distinct species/isolates as possible.

Therefore, the objectives of this study were to (i) screen spring wheat germplasm provided by CIMMYT with good quality characteristics against *F. culmorum* and *F. pseudograminearum* under controlled growth room conditions, (ii) validate the resistance situation of the best-selected lines to *F. culmorum* under the greenhouse and field conditions (iii) study yield perfor-

mances of the best-selected lines under *F. culmorum*-infested field. The best resistant/tolerant line will then be recommended to be used in international breeding programs.

2. Materials and Methods

2.1. Germplasm Selection

Out of 200 spring wheat germplasm obtained from CIMMYT Mexico, a set of 26 lines with good quality characteristics were chosen based on their resistance performance to be further validated for their resistance potential to *F. culmorum* (isolate FC14) and *F. pseudograminearum* (isolate FPG03). Three standard moderate resistant check lines: 249, Altay, and Yelken, as well as three susceptible check lines, Kızıltan, Gerek, and Kutluk, were included as controls due to their known reactions (Table 1). This set of 26 lines plus 6 checks (total 32), was tested for *F. culmorum* resistance in three different field conditions in Eskişehir, Yozgat, and Konya, as well as in Eskişehir's greenhouse and growth room facilities. The meteorological conditions for each region are displayed according to the Turkish State Meteorological Service (TSMS) in Table 1. To screen for *F. pseudograminearum* resistance, only growth room facilities were used. Furthermore, the same set was tested for yield performance in Konya under plus/minus *F. culmorum* in field conditions (Table 2).

Table 1. Meteorological characteristics of each region during the 2017–2018 growing season.

Locality	Average Annual Temperature	Annual Precipitation	Humidity
Eskişehir	12.7 °C	200–390 mm	67.1%
Yozgat	13.3 °C	450–570 mm	54.6%
Konya	23.6 °C	124–300 mm	57.9%

Table 2. The list of the spring wheat germplasm used in the field studies and the six check lines.

Ent	CNAME	CID
1	PRL/2*PASTOR//WAXWING*2/KRONSTAD F2004/4/PBW343*2/KUKUNA//KRONSTAD F2004/3/PBW343*2/KUKUNA	546,349
2	DANPHE/2*BAJ #1	546,357
3	PICAFLOR #1/5/FRET2/KUKUNA//FRET2/3/YANAC/4/FRET2/KIRITATI	553,138
4	DANPHE/3/PBW343*2/KUKUNA//PBW343*2/KUKUNA	553,204
5	FRANCOLIN #1/BAJ #1	553,377
6	SOKOLL/3/PASTOR//HXL7573/2*BAU*2/4/PASTOR//MILAN/KAUZ/3/BAV92	554,318
7	TUKURU//BAV92/RAYON/6/NG8201/KAUZ/4/SHA7//PRL/VEE#6/3/FASAN/5/MILAN/KAUZ*2/7/KINGBIRD #1	559,533
8	WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1*2/6/WBLL1*2/4/YACO/PBW65/3/KAUZ*2/TRAP//KAUZ/5/KACHU #1	559,752
9	PBW343*2/KUKUNA//PBW343*2/KUKUNA/3/2*BAJ #1	559,939
10	PAURAQ/4/HUW234+LR34/PRINIA//PBW343*2/KUKUNA/3/ROLF07	545,670
11	ATTILA*2/PBW65/5/PRL/2*PASTOR/4/CHOIX/STAR/3/HE1/3*CNO79//2*SERI/6/PFUNYE #1	546,353
12	WHEAR/KUKUNA/3/C80.1/3*BATAVIA//2*WBLL1/8/VEE#8//JUP/BJY/3/F3.71/TRM/4/BCN/5/KAUZ/6/MILAN/KAUZ/7/SKAUZ/PARUS//PARUS/9/KACHU	546,418
13	KACHU*2/BECARD	546,469
14	BABAX/LR42//BABAX/3/ER2000/4/2*MUNAL	549,129
15	ROBIN,KEN	448,396
16	BABAX/LR42//BABAX*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ*2/5/MUNAL #1	546,537
17	MERCATO/4/FRAME//MILAN/KAUZ/3/PASTOR/5/WHEAR/SOKOLL	548,932
18	KENYA SUNBIRD/2*KACHU	541,193
19	SOKOLL/3/PASTOR//HXL7573/2*BAU/4/NAVJ07	549,534

Table 2. *Cont.*

Ent	CNAME	CID
20	SOKOLL/3/PASTOR//HXL7573/2*BAU/4/HUW234+LR34/ PRINIA//PBW343*2/KUKUNA/3/ROLF07	549,549
21	SOKOLL/3/PASTOR//HXL7573/2*BAU/4/MASSIV/PPR47.89C	549,913
22	W15.92/4/PASTOR//HXL7573/2*BAU/3/WBLL1/5/SOKOLL/3/ PASTOR//HXL7573/2*BAU	552,587
23	SOKOLL/3/PASTOR//HXL7573/2*BAU/4/GLADIUS	552,597
24	W15.92/4/PASTOR//HXL7573/2*BAU/3/WBLL1*2/5/WHEAR/ SOKOLL	554,181
25	TRCH/SRTU//KACHU*2/3/PVN	559,568
26	SOKOLL/3/PASTOR//HXL7573/2*BAU/4/2*PASTOR//HXL7573/2*BAU/3/SOKOLL/WBLL1	560,562
27	249 (Check-CR-Mr)	
28	Altay (Check-CR-Mr)	
29	Yelken (Check-CR-Mr)	
30	Kızıltan (Check-CR-S)	
31	Gerek (Check-CR-S)	
32	Kutluk (Check-CR-S)	

Ent: Entity; CNAME: Cross Name; CID: Cross Identification Number; * means crosses

2.2. Inoculum Preparation

Monosporic isolates of *F. culmorum* and *F. pseudograminearum* were plated on the Spezieller Nährstoffarmer Agar (SNA) medium (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 \times 7H_2O$, 0.5 g KCl, 0.2 dextrose, 0.2 sucrose, 20 g agar, distilled water to 1 L) and cultured for 10 days at 23 °C ± 1 °C with a 12 h photoperiod. Oven bags (35 cm × 48 cm), quarter filled with wheat bran, were humidified and sealed with cotton. Separate wheat brans were allocated for each inoculum. The bags were autoclaved at 121 °C for 20 min for 3 successive days. The spore suspension was prepared by adding sterilized distilled water to each Petri dish containing *Fusarium* cultures. Autoclaved wheat bran bags were allowed to cool before being inoculated with the spore suspension under sterilized conditions. Inoculated wheat bran was mixed by shaking the bags and incubated at 23 °C ± 1 °C for 2–3 weeks at a 12 h photoperiod or until the fungus sufficiently colonized the bran. Finally, the fungus-infested wheat bran was allowed to dry at room temperature. The fungus-colonized bran was used as a source of inoculum in experiments conducted in the growth room, greenhouse, and field.

2.3. Growth Room Experiment

Infested wheat bran was suspended in distilled water before being filtered through two layers of cheesecloth. Before use, the spore concentration was adjusted to 10^6 conidia mL^{-1} of water, and methylcellulose (0.1% v/v) was added to the conidial suspension. To achieve sufficient plantlets with a similar phenological stage, ten wheat seeds were placed on moist blotting paper in sterilized Petri dishes for germination at 22 °C for 2–3 days. Each pre-germinated seed was sown in a separate plastic tube (2.5 cm in diameter × 16 cm in height) filled with potting mix and covered in the same substrate. A sterile potting mix of sand, soil, and organic manure (50:40:10; $v/v/v$) was used for growth room and greenhouse trials. One week after sowing, the stem base of each seedling (0.5–1 cm above the soil level, including the coleoptile) was inoculated with 1 mL (1×10^6 conidia mL^{-1}) of the abovementioned spore suspension. Following incubation, the seedlings were kept in a growth room for 42 days (early tillering, Zadoks growth stage 14), with a day/night photoperiod of 16/18 h at a temperature of 23 °C ± 1 °C and relative humidity of 60/80% (±5%). A randomized complete block design with five replications (1 plant per replicate) was used, and the experiment was repeated twice.

2.4. Greenhouse

Each tube was sown with two seeds of each wheat accession, and received 0.5 g of fungal colonized wheat bran (as inoculum source). To facilitate root growth, these tubes

were set on a platform of sand in a greenhouse characterized by a range of temperatures from 16 °C to 35 °C with relative humidity (RH) of 25–90%. During the growing seasons, the experiments were watered as needed. Plants were exposed to water stress at maturity stages to promote disease development. A randomized complete block design was used to set up the experiment, which had six replications (two plants per replicate).

2.5. Field Conditions

During the 2017/18 growing season (October to June), plant materials and check cultivars were planted in field conditions at the ILCI Çiçekdağı Agricultural Enterprise (ICAE) in Yozgat (Latitude 39.63806; Longitude 34.46722), at the Transitional Zone Agricultural Research Institute (TZARI) in Eskişehir (Latitude 39.76670; Longitude 30.40518), and the Bahri Dağdaş International Agricultural Research Institute (BDIARI) in Konya (Latitude 37.85789; Longitude 32.556), Turkey. Each 5 g seed sample of entries was sown in a one-meter row and inoculated with 2 g of fungus-colonized wheat bran of *F. culmorum*. Experiments with three replications were set up using a randomized complete block design. Disease symptoms were scored by picking up 15 individual plants from each row.

2.6. Disease Assessment and Data Analysis

Plants were harvested, and stems were collected at the end of the growing season. Seedling resistance was tested on plants that were grown in a growth room (Zadoks growth stage 14). At the end of the maturity stage, plants grown in greenhouses and fields were examined, and adult plant resistance was tested. Plants were scored on a numeric scale of 1–5 for the typical symptoms of browning percentage on the crown (by observing the disease on the crown) and the main stem (by measuring the disease symptoms on the stem). The scale was modified from Wildermuth et al. [40] (1: 1–9% as resistant, 2: 10–29% moderately resistant, 3: 30–69% moderately susceptible, 4: 70–89% susceptible, and 5: 90–99% highly susceptible). Plants grown under field conditions were also scored for whitehead symptoms at the ripening stage using the same 1–5 scale.

2.7. Yield Performance

The selected spring wheat germplasm was evaluated for yield performance under field conditions in Konya, both with and without *F. culmorum* inoculation. Each entry was replicated three times and planted in a 6 m^2 plot of six rows in a randomized complete block design with plus or minus artificial inoculum. For inoculated plots, a 140 g seed sample from each entry was inoculated with 5 g of fungus-colonized wheat bran, as described in the 'Field conditions' section. Grain yield was weighted and recorded in kg per ha per plot for all plots.

2.8. Statistical Analysis

The analysis of variance was used to analyze all of the data (ANOVA). Protected least significant difference at $p < 0.001$ was used to detect significant differences between studied lines using SPSS statistical software V 17.0 (SPSS Inc., Chicago, IL, USA). A linear discriminant analysis (AFD) was performed using R 3.4.3 software to distinguish the mainline groups based on their disease index. Linear regression analyses were also performed to uncover relationships between each line's plant height and grain weight. All other analyses were carried out with the XLSTAT software (2016.02.28451) (Addinsoft Inc, New York, NY, USA).

3. Results

The selected 26 spring wheat lines were tested against the crown rot disease caused by *F. culmorum* and precisely evaluated via the disease index for each condition (Figure 1), and the discriminant factorial analysis (AFD) revealed four groups of lines: Group 1, Group 2, Group 3, and Group 4, comprising resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S) lines, respectively (Figure 2A). In all field conditions, eight lines, L1, L9, L16, L22, L29, L30, L31, and L32, had an index of > 3.1, indicating a significantly higher severity (Figure 1A) and corresponding to S Group 4 (Figure 2A). MS

Group 3 comprised three lines: L3, L7, and L14, with disease indices of 2.8, 2.9, and 2.8, respectively. Fourteen lines (L2, L4, L5, L6, L8, L10, L13, L15, L17, L20, L23, L24, L25, and L28) had mean indices of between 2.4 and 2.7 and belonged to MR Group 2. Seven lines, L11, L12, L18, L19, L21, L26, and L27, had the lowest severity between indices of 2.2 and 2.3 and belonged to R Group 1.

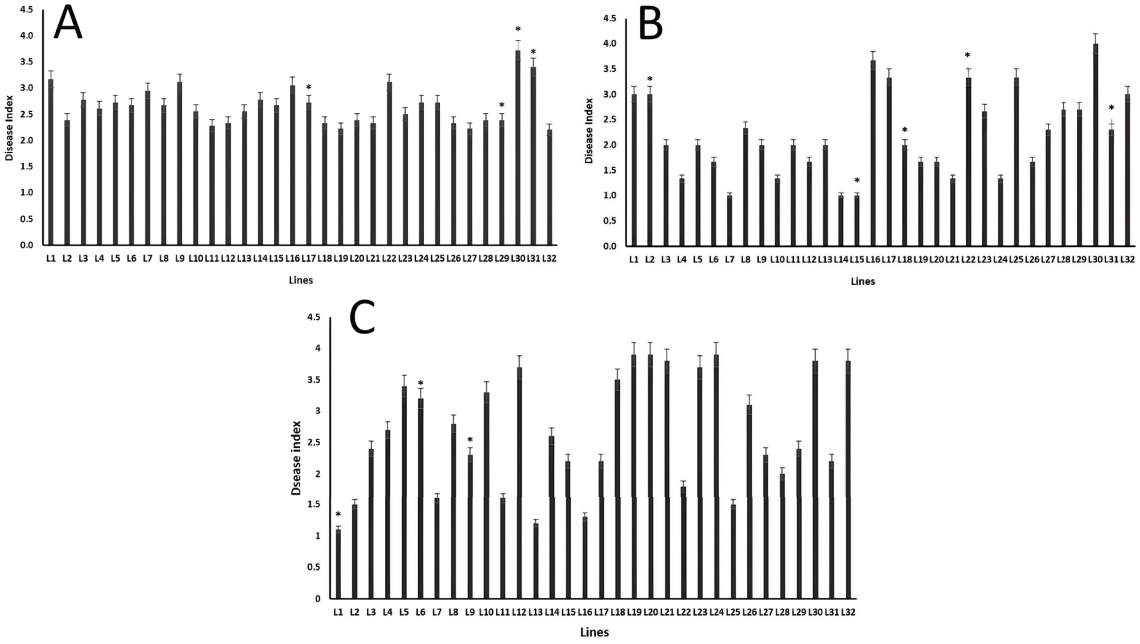


Figure 1. Crown rot disease index of 32 spring wheat lines (including 6 check lines) to *Fusarium culmorum*. (**A**) Disease index in field conditions of three locations (Eskişehir, Yozgat, and Konya). (**B**) Greenhouse conditions in Eskişehir. (**C**) Growth room conditions in Eskişehir. Asterisks (*) represent homogeneous groups based on the protected least significant difference test for each variable at $p < 0.001$. Error lines on bars represent the standard error (n = 6).

In Eskişehir greenhouse conditions (Figure 2B), the same number of groups were observed. The *F. culmorum* disease index ranged from 1 (L7, L14, and L15) to 4 (L30) (Figure 1B). Line 16, with an index of 3.7, exhibited the highest severity and was included in the S Group 4 together with the susceptible check line 30. Nine lines: L1, L2, L17, L22, L23, L25, L28, L31, and L32, also exhibited high disease severity with indices between 2.7 and 3.3 and were included in MS Group 3, while nine other lines: L3, L5, L8, L9, L11, L13, L18, L27, and L29, belonged to MR Group 2 and had significantly lower values between 2.0 and 2.3. The lowest severity indices between 1.0 and 1.7 were found in 12 lines: L4, L6, L7, L10, L12, L14, L15, L19, L20, L21, L24, and L26, which were included in R Group 1.

Within the analyzed lines, *F. culmorum*-induced disease index values were increased significantly in growth chamber settings (Figure 1C), resulting in five discrete resistance categories (Figure 2C). Aside from the other conditions, the fifth group, Group 5, included three highly susceptible (HS) lines, L19, L20, and L24, each with an index value of 3.9. Six lines, L5, L10, L12, L18, L21, and L23, as well as check lines L30 and L32, had a significantly high severity that exceeded the mean index value of 3.3 and were included in S Group 4. Five lines, L4, L6, L8, L14, and L26, had lower indices ranging from 2.6 to 3.2 and were assigned to MS Group 3. MR Group 4 included seven lines (L3, L7, L9, L11, L15, L17, and L22) as well as four check lines (L27, L28, L29, and L31). The lowest disease index values,

ranging from 1.1 to 1.5 were observed in five lines, L1, L2, L13, L16, and L25, all of which were part of the R Group 1.

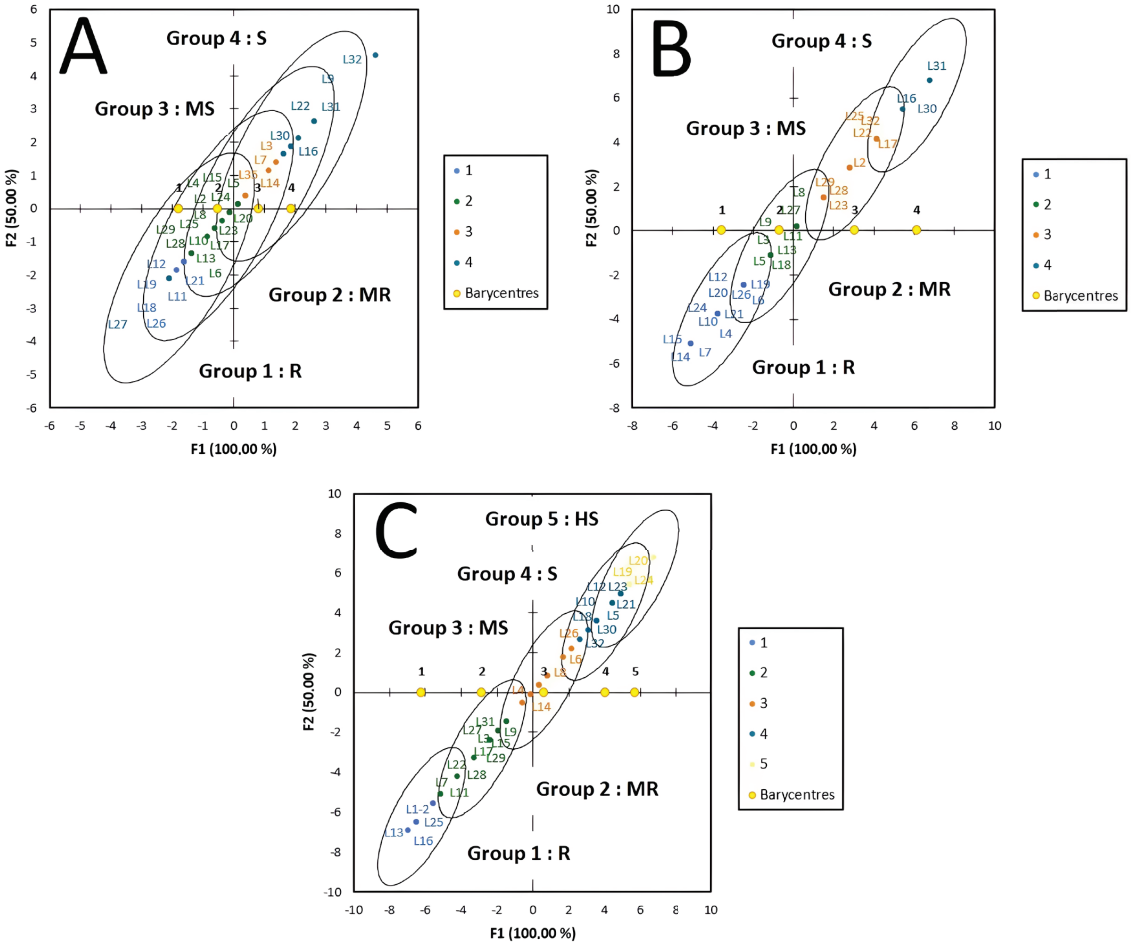
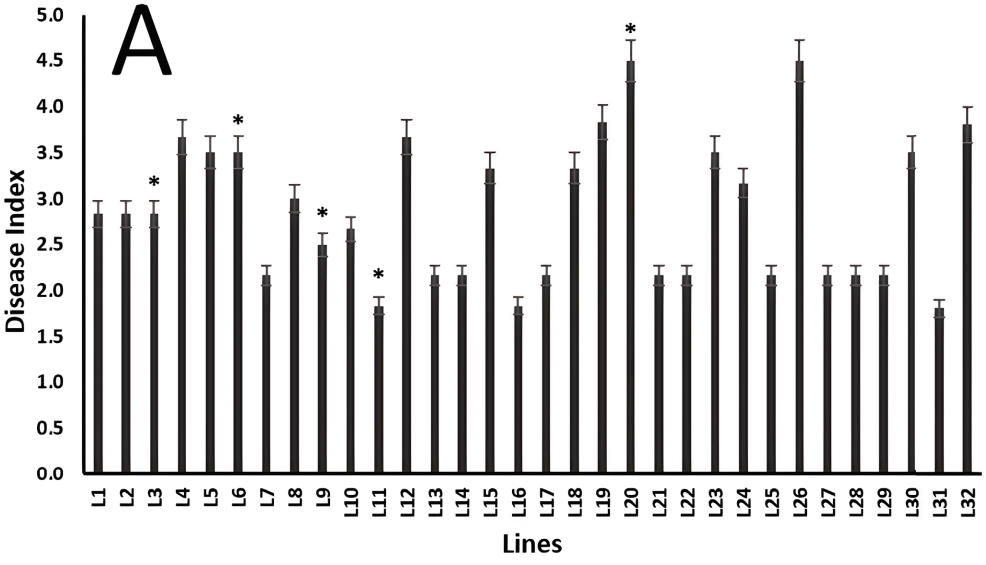


Figure 2. Discriminant factorial analysis (AFD) showing the population structure for a set of 32 lines from CIMMYT's spring wheat nursery based on their resistance reaction against *Fusarium culmorum*. (**A**) Field conditions of three locations (Eskişehir, Yozgat, and Konya). (**B**) Greenhouse conditions in Eskişehir. (**C**) Growth room conditions in Eskişehir. Numbers represent resistance reaction: 1: resistant; 2: moderately resistant; 3: moderately susceptible; 4: susceptible; 5: highly susceptible. Yellow points represent the barycenter's defining groups.

There were significant differences ($p < 0.001$) across the evaluated 32 lines in terms of the crown rot disease index for *F. pseudograminearum* (Figure 3A). The AFD analysis revealed that there are four distinct groups of lines based on their resistance reaction to *F. pseudograminearum* (Figure 3B). Among them, 12 lines were shown to have a high disease index that exceeded 3, and just 2(L20 and L26) in HS Group 5 were able to attain the maximum 4.5 and 5 index values, respectively. The remaining eight lines: L4, L5, L6, L12, L15, L18, L19, and L23, and two check lines (L30 and L32), were in the S Group 4. Among the 15 moderately resistant lines (MR) in the first group, L11 and L16 gave the lowest index value (1.8). L1, L2, L3, L8, and L24 were moderately susceptible (MS) lines in Group 3. L7,

L9, L10, L11, L13, L14, L16, L17, L21, L22, L25, L27, and L28, as well as 2 check lines (L29 and L31), were other members of MR Group 2.



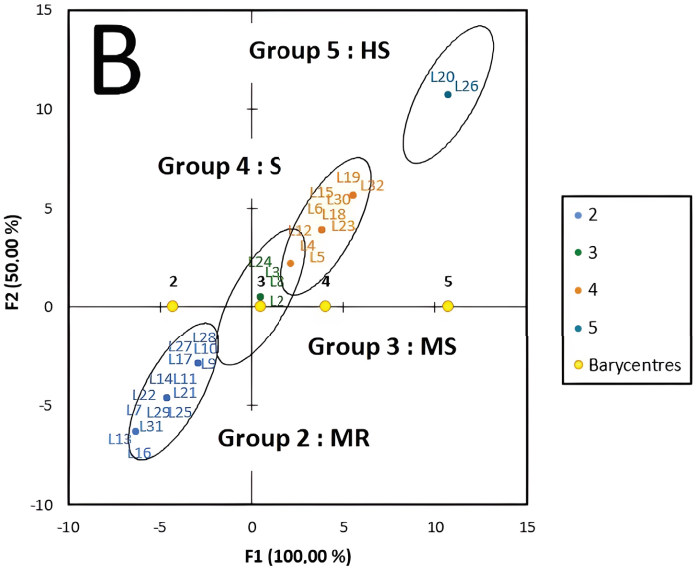


Figure 3. (**A**) Crown rot disease index of 32 spring wheat lines (including six check lines) of *Fusarium pseudograminearum* in Eskişehir. Asterisks (*) represent homogeneous groups based on the protected least significant difference test for each variable at $p < 0.001$. Error lines on bars represent the standard error (n = 6). (**B**) Discriminant Factorial Analysis (AFD) showed the population structure for a set of 32 lines from CIMMYT's spring wheat nursery based on their resistance reaction against *F. pseudograminearum* in growth room conditions. Numbers represent resistance reaction: 2: moderately resistant; 3: moderately susceptible; 4: susceptible; 5: highly susceptible. Yellow points represent the barycenter's defining groups.

The grain yield of the 32 lines was evaluated in big plots of 6 m^2 with or without *F. culmorum* inoculum. It is indicated that fungal inoculation showed various grain yield responses (Figure 4). L1, L2, L3, L4, L5, L11, L12, L19, L20, L21, L23, L26, L27, L28, L29, and L30, for example, were negatively affected by *Fusarium* inoculation and yielded less, demonstrating these lines' susceptibility. However, 14 lines, namely L7, L8, L9, L10, L13, L14, L15, L16, L17, L18, L22, L24, L25, and L31, increased their yielding potential, whereas two lines, L6 and L32, were unaffected by inoculation, indicating resistance to *F. culmorum*. Those lines might have a tolerant reaction as well. Indeed, the inoculation appeared to increase rather than decrease the productivity of these lines. Except for check lines, L26 had the highest yield (5342 kg/ha), while L1 had the lowest (2623 kg/ha). The yield values of three reference lines (L30, L31, and L32) were exceptionally high. The other three, L27, L28, and L29, had a moderate yield associated with pathogenic fungal damage.

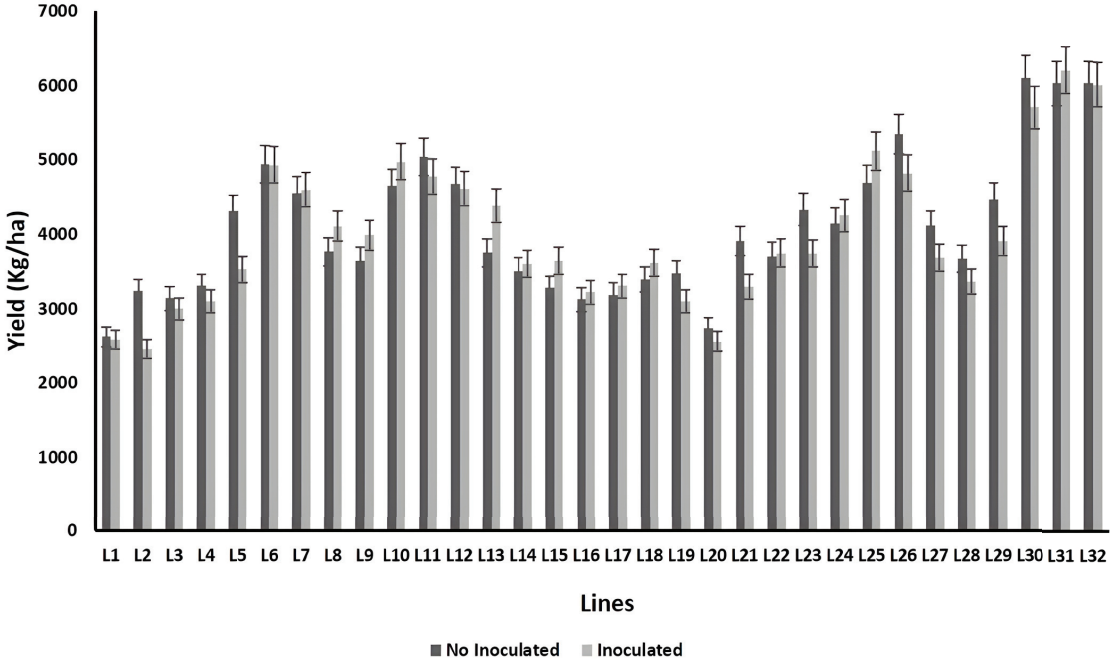


Figure 4. Grain yield of 32 CIMMYT's spring wheat lines, including 6 check lines, inoculated and noninoculated with *Fusarium culmorum*, in Konya, Turkey.

4. Discussion

Fusarium root and crown rot of wheat are most commonly caused by the infection of a diverse species of *Fusarium* genus, including *F. pseudograminearum*, *F. culmorum*, *F. avenaceum*, and *F. graminearum*, which rots small-grain cereal seeds, seedlings, roots, crowns, basal stems, and heads. On wheat plants, these fungi cause browning and decay by infecting the coleoptile, leaf sheath, and stem base of seedlings.

The most effective technique to manage crown and root rot infections is through wheat breeding. Wheat plants generally lack genetic resistance against these pathogens, and these diseases are more frequently caused by multiple pathogens in the field; thus, the goal is to find genetic resistance to these pathogens in wheat germplasm that offer resistance to as many pathogens as possible. However, few wheat germplasms have moderate levels of resistance to *F. culmorum* or *F. pseudograminearum*. Because most wheat varieties are susceptible to these pathogens, identifying new resistance sources and crossing them with

high-yielding cultivars is essential. Therefore, for effective wheat breeding applications, large-scale screenings of resistant wheat germplasm are still required.

The primary goal of this study was to assess the resistance in 32 wheat germplasm to *F. culmorum* or *F. pseudograminearum* at the adult plant stage under field conditions in the provinces of Yozgat, Eskişehir, and Konya in Turkey, as well as at the seedling stage in greenhouse and growth chamber conditions in Eskişehir. Because investigating resistant or tolerant lines necessitates a thorough understanding of the pathogenicity spectrum and pathotypes of the target species, highly virulent and aggressive isolates of both species were used in all experiments. Approximately 65.6% of the lines (21 lines) were resistant (7 lines) to moderately resistant (14 lines) against FCRR caused by *F. culmorum* at the adult plant stage. Seedling resistance screening in the greenhouse identified two, nine, nine, and 12 lines as S, MS, MR, and R against *F. culmorum*, respectively. In the field and greenhouse, four lines (L12, L19, L21, and L26) were found to be jointly R, and three lines (L5, L8, and L13) were jointly MR at the adult and seedling stages, respectively. At the seedling stage, six other lines that were MR at the adult plant stage (L4, L6, L10, L15, L20, and L24) were R in the greenhouse. In that manner, Özdemir et al. [48] have screened many wheat varieties from Turkey, Australia, and the Pacific Northwest of the USA for their resistance to *F. culmorum* and *F. pseudograminearum* using the Real-Time PCR method. Three types of lines were detected, Resistant-Tolerant, Resistant-Intolerant, and Susceptible-Intolerant. Good correlations between the results of specific resistant lines under different conditions suggest that these lines could be useful for pyramiding genetic variants for long-term resistance. Controlled (growth room), greenhouse, and field-based experiments sometimes produced diverse results among lines, which is understandable. However, it is assumed that controlled conditions are more suitable and reliable for disease screening due to their reduced variation error. For instance, *F. culmorum*-induced disease index values increased significantly in growth chamber settings within the analyzed lines, indicating that *F. culmorum* found optimal conditions to emerge on the studied spring wheat lines. Furthermore, some studies [49–51] have found that earlier inoculations result in more severe disease infection, which can lead to higher disease levels, and that the early response to FCRR is not always related to adult plant responses. Three check lines (L30, L31, and L32) and L26 had the highest yield values. *Fusarium* inoculation had a negative effect on 16 lines, causing them to yield less, demonstrating their susceptibility. However, inoculation did not affect 16 lines, indicating resistance/tolerance to *F. culmorum*. Indeed, the inoculation appeared to boost rather than reduce the productivity of these lines. This has to do with tolerance attributes of spring wheat lines related to the decent yielding proprieties despite the pathogen's inoculum. In the case of *F. pseudograminearum*, screening was limited to growth room conditions, and no R lines were identified, but 15 MR (four of which were checked) lines were observed. On the other hand, only five lines (L10, L13, L17, L25, and L28) have shown resistant status to both *Fusarium* species which is an important perspective for breeding.

Genetic wheat resistance against *F. pseudograminearum* has been highlighted in many studies. However, no completely resistant (R) lines currently exist that support our findings in this context [52]. A few lines of bread wheat were reported to offer partial resistance to *F. pseudograminearum* under field conditions [53]. The mechanism of this resistance is related to the colonization and growth characteristics of *F. pseudograminearum* in wheat seedlings. Therefore, partially resistant cultivars were shown to have slow mycelium growth (in both plant's xylem and phloem) compared to the susceptible ones [54]. Interestingly, the resistance pattern against *Fusarium* pathogens could exhibit eventual variations in the field conditions due to the associated meteorological factors. For instance, Birr et al. [55] emphasized a positive correlation between climatic variables (e.g., precipitation and relative humidity) and *F. graminearum*'s abundance. In addition, mycotoxin concentrations fluctuated across wheat grains due to the high moisture factors.

Deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), nivalenol (NIV), and zearalenone (ZEA) toxins are some of the most important mycotoxins found in harvested

wheat grains all of which are produced by *Fusarium* species. Earlier studies showed that disease severity caused by *Fusarium* species and mycotoxin contamination in harvested wheat grain were remarkably correlated [56]. It is considered that the determination of resistant wheat varieties against *Fusarium* species will also help to reduce the contamination level of the mycotoxins in harvested wheat grains. This part of the research will be given a high priority by the International Soil Borne Pathogens Platform in Turkey to evaluate R vs. S to accumulate mycotoxins in wheat plants at different growing stages.

A major impediment to FCRR resistance breeding is the lack of research and understanding of the genetic basis of resistance [57]. Furthermore, there is a scarcity of dependable, reproducible, and high-throughput phenotyping procedures for screening large numbers of genotypes, leading to the discovery of novel sources of FCRR resistance and the underlying genetic factors [52,58]. In Turkey, the main *Fusarium* species involved in wheat fields is *F. culmorum* and, since research collaborations are made with other *Fusarium*-infested countries (mainly with *F. pseudograminearum*), lines with multiple resistance traits to both species are extremely needed for breeding programs.

In conclusion, the present study has revealed new sources of resistance to *F. culmorum* and *F. pseudograminearum* derived from experiments in the growth chamber, greenhouse, and field conditions. New R or MR germplasm resources found in the present research may therefore hold a lot of promise for improving wheat resistance to these fungi that cause root and crown rot, as there is currently no durable resistant cultivar for both species and they can further be exploited in breeding programs for the development of disease-resistant commercial cultivars. Internationally, it is recommended to perform a rank test of wheat lines based on their resistance to *Fusarium* spp. This test could involve many scientists from main *Fusarium*-infested countries, and it could be an innovative way to produce innovative management solutions to this disease. However, screening procedures must be improved in the sense that all resistance/tolerance aspects will be well covered from breeding perspectives. For instance, using new technologies-based approaches (e.g., QTL and GWAS studies) could be extremely doable to enforce resistance discovery in wheat cultivars. Additionally, it could be useful to adopt these conceptional genetics to promote high grain yield despite *Fusarium* infestations through investing in tolerance traits.

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References

1. Bowden, R.L.; Hunger, R.M.; Morrill, W.L.; Murray, T.D.; Smiley, R.W. *Compendium of Wheat Diseases and Pests*; Bockus, W.W., Ed.; APS Press: Saint Paul, MN, USA, 2010; Volume 3. [CrossRef]
2. Breiman, A.; Graur, D. Wheat evolution. *Isr. J. Plant Sci.* **1995**, *43*, 85–98. [CrossRef]
3. FAOSTAT. Food and Agriculture Organization Statistical Database. Available online: http://www.fao.org/faostat/en/#data/QC (accessed on 20 February 2022).
4. Alexandratos, N.; Bruinsma, J. World Agriculture towards 2030/2050: The 2012 Revision. Available online: https://ageconsearch.umn.edu/record/288998/ (accessed on 18 November 2021).

5. Adhikari, S.; Kumari, J.; Jacob, S.R.; Prasad, P.; Gangwar, O.P.; Lata, C.; Kumar, S. Landraces-potential treasure for sustainable wheat improvement. *Genet. Resour. Crop Evol.* **2022**, *69*, 499–523. [CrossRef]
6. Braun, H.J.; Săulescu, N.N. Breeding winter and facultative wheat. *Cent. Asia* **2002**, *9*, 5.
7. Bentley, A.; Donovan, J.; Sonder, K.; Voss, R.; Rutsaert, P.; Poole, N.; Kamoun, S.; Saunders, D.G.O.; Hodson, D.; Hughes, D.P.; et al. Another Food Crisis? The Ukraine Conflict, Global Wheat Supply and Food Security (Version V1). *Zenodo* 2022. Available online: https://zenodo.org/record/6380085#.Y3y1zH1ByUk (accessed on 15 July 2021).
8. Chakraborty, S.; Liu, C.J.; Mitter, V.; Scott, J.B.; Akinsanmi, O.A.; Ali, S.; Dill-Macky, R.; Nicol, J.; Backhouse, D.; Simpfendorfer, S. Pathogen population structure and epidemiology are keys to wheat crown rot and Fusarium Head Blight management. *Australas. Plant Pathol.* **2006**, *35*, 643–655. [CrossRef]
9. Smiley, R.W.; Gourlie, J.A.; Easley, S.A. Pathogenicity of fungi associated with wheat crown rot complex in Oregon and Washington. *Plant Dis.* **2005**, *89*, 949–957. [CrossRef] [PubMed]
10. Tunali, B.; Nicol, J.M.; Hodson, D.; Uckun, Z.; Buyuk, O.; Erdurmus, E.; Hekimhan, H.; Aktas, H.; Aydın Akbudak, M.; Bagci, S.A. Root and crown rot fungi associated with spring, facultative, and winter wheat in Türkiye. *Plant Dis.* **2008**, *98*, 1299–1306. [CrossRef]
11. Özer, G.; Paulitz, T.C.; Imren, M.; Alkan, M.; Muminjanov, H.; Dababat, A.A. Identity and pathogenicity of fungi associated with crown and root rot of dryland winter wheat in Azerbaijan. *Plant Dis.* **2020**, *104*, 2149–2157. [CrossRef] [PubMed]
12. Bozoğlu, T.; Derviş, S.; Imren, M.; Amer, M.; Özdemir, F.; Paulitz, T.C.; Morgounov, A.; Dababat, A.A.; Özer, G. Fungal pathogens associated with crown and root rot of wheat in central, eastern, and southeastern Kazakhstan. *J. Fungi* **2022**, *8*, 417. [CrossRef] [PubMed]
13. Goswami, R.S.; Kistler, H.C. Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* **2004**, *5*, 515–525. [CrossRef]
14. Cook, R.J.; Veseth, R.J. *Wheat Health Management*; American Phytopathological Society: St. Paul, MN, USA, 1991. [CrossRef]
15. Buerstmayr, H.; Ban, T.; Anderson, J.A. QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat: A review. *Plant Breed.* **2009**, *128*, 1–26. [CrossRef]
16. Chrpova, J.; Sip, V.; Matejova, E.; Sykorova, S. Progression of deoxynivalenol concentrations in spikes and kernels of winter wheat cultivars after inoculation with *Fusarium culmorum*. *Czech. J. Genet. Plant Breed.* **2006**, *42*, 137–141. [CrossRef]
17. Bhat, R.V.; Vasanthi, S. Mycotoxin food safety risk in developing countries. In *Food Safety in Food Security and Food Trade*; (No. 569-2016-39053); International Food Policy Research Institute: Washington, DC, USA, 2003.
18. Sneller, C.; Guttieri, M.; Paul, P.; Costa, J.; Jackwood, R. Variation for resistance to kernel infection and toxin accumulation in winter wheat infected with *F. graminearum*. *Phytopathology* **2012**, *102*, 306–314. [CrossRef] [PubMed]
19. Burgess, L.W.; Backhouse, D.; Summerell, B.A.; Swan, L.J. Crown rot of wheat. In *Fusarium—Paul E. Nelson Memorial Symposium*; Summerell, J.F., Leslie, D., Backhouse, W.L., Bryden, L.W.B., Eds.; American Phytopathological Society Press: St. Paul. MN, USA, 2001; pp. 271–295. [CrossRef]
20. Gonzalez, M.S.; Trevathan, L.E. Identity and pathogenicity of fungi associated with root and crown rot of soft red winter wheat grown on the upper coastal plain land resource area of Mississippi. *J. Phytopatol.* **2000**, *148*, 77–85. [CrossRef]
21. Paulitz, T.C.; Smiley, R.W.; Cook, R.J. Insights into the prevalence and management of soilborne cereal pathogens under direct seeding in the Pacific Northwest, USA. *Can. J. Plant Pathol.* **2002**, *24*, 416–428. [CrossRef]
22. Backhouse, D.; Abubakar, A.A.; Burgess, L.W.; Dennisc, J.I.; Hollaway, G.J.; Wildermuth, G.B.; Henry, F.J. Survey of *Fusarium* species associated with crown rot of wheat and barley in eastern Australia. *Australas. Plant Pathol.* **2004**, *33*, 255–261. [CrossRef]
23. Smiley, R.W.; Patterson, L.M. Pathogenic fungi associated with Fusarium foot rot of winter wheat in the semiarid Pacific Northwest. *Plant Dis.* **1996**, *80*, 944–949. [CrossRef]
24. Akinsanmi, O.A.; Mitter, V.; Simpfendorfer, S.; Backhouse, D.; Chakraborty, S. Identity and pathogenicity of *Fusarium* spp. isolated from wheat fields in Queensland and northern New South Wales. *Aust. J. Agric. Res.* **2004**, *55*, 97–107. [CrossRef]
25. Bozoğlu, T.; Özer, G.; Imren, M.; Paulitz, T.C.; Dababat, A.A. First report of crown rot caused by *Fusarium redolens* on wheat in Kazakhstan. *Plant Dis.* **2021**, *105*, 3302. [CrossRef]
26. Özer, G.; İmren, M.; Bayraktar, H.; Paulitz, T.C.; Muminjanov, H.; Dababat, A.A. First report of *Fusarium hostae* causing crown rot on wheat in Azerbaijan. *Plant Dis.* **2019**, *103*, 3278. [CrossRef]
27. Özer, G.; Erper, I.; İmren, M.; Bozoglu, T.; Ozdemir, F.; Dababat, A.A. First report of crown rot caused by *Fusarium algeriense* on wheat in Kyrgyzstan. *Plant Dis.* **2022**. [CrossRef]
28. Gebremariam Shikur, E.; Sharma-Poudyal, D.; Paulitz, T.C.; Erginbas-Orakci, G.; Karakaya, A.; Dababat, A.A. Identity and pathogenicity of Fusarium species associated with crown rot on wheat (*Triticum* spp.) in Türkiye. *Eur. J. Plant Pathol.* **2018**, *150*, 387–399. [CrossRef]
29. Xu, F.; Yang, G.; Wang, J.; Song, Y.; Liu, L.; Zhao, K.; Li, Y.; Han, Z. Spatial distribution of root and crown rot fungi associated with winter wheat in the North China Plain and its relationship with climate variables. *Front. Microbiol.* **2018**, *9*, 1054. [CrossRef]
30. Mishra, P.K.; Tewari, J.P.; Clear, R.M.; Turkington, T.K. Genetic diversity and recombination within populations of *Fusarium pseudograminearum* from western Canada. *Int. Microbiol.* **2006**, *9*, 65–68. [CrossRef] [PubMed]
31. Pettitt, T.; Xu, X.; Parry, D. Association of Fusarium species in the wheat stem root complex. *Eur. J. Plant Pathol.* **2003**, *109*, 769–774. [CrossRef]

32. Dyer, A.T.; Johnston, R.H.; Hogg, A.C.; Johnston, J.A. Comparison of pathogenicity of the *Fusarium* crown rot (FCR) complex (*F. culmorum*, *F. pseudograminearum* and *F. graminearum*) on hard red spring and durum wheat. *Eur. J. Plant Pathol.* **2009**, *125*, 387–395. [CrossRef]
33. Alkan, M.; Göre, M.E.; Bayraktar, H.; Özer, G. Genetic variation of *Fusarium* spp. isolates associated with root and crown rot of winter wheat using retrotransposon-based iPBS assays. *Int. J. Agric. Wildlife Sci.* **2014**, *5*, 250–259. [CrossRef]
34. Chakraborty, S.; Obanor, F.; Westecott, R.; Abeywickrama, K. Wheat crown rot pathogens *Fusarium graminearum* and *F. pseudograminearum* lack specialization. *Phytopathology* **2010**, *100*, 1057–1065. [CrossRef]
35. Chekali, S.; Gargouri, S.; Paulitz, T.; Nicol, J.M.; Rezgui, M.; Nasraoui, B. Effects of *Fusarium culmorum* and water stress on durum wheat in Tunisia. *Crop Prot.* **2011**, *30*, 718–725. [CrossRef]
36. Hekimhan, H.; Bagci, S.A.; Nicol, J.; Arisoy, R.Z.; Taner, S. Dryland root rot: A major threat to winter cereal production under sub-optimal growing conditions. In Proceedings of the 4th International Crop Science Congress, Brisbane, Australia, 27 September–1 October 2004.
37. Bagci, S.A.; Hekimhan, H.; Mergoum, M.; Aktas, H.; Taner, S.; Tulukcu, E.; Ekiz, H. Effects of Foot and Root Rot Pathogens on Yields of Some Cereal Genotypes and Determination of Resistance Sources. In Proceedings of the 4th Field Crops Congress, Tekirdağ, Turkey, 17–21 September 2001; pp. 115–120.
38. Aktas, H.; Kinaci, E.; Yildirim, A.F.; Sayin, L.; Kural, A. Determination of the effects of root and foot rot pathogens on yield components in cereals which are problems in Konya province and solutions. In Proceedings of the Cereal Symposium, Konya, Türkiye, 8–11 June 1999; Ekiz, H., Ed.; Ministry of Agriculture and Rural Affairs, Bahri Dağdaș International Winter Cereals Research Center: Konya, Turkey, 1999; pp. 392–403.
39. Swan, L.J.; Backhouse, D.; Burgess, L.W. Surface soil moisture and stubble management practice effects on the progress of infection of wheat by *Fusarium pseudograminearum*. *Aust. J. Exp. Agric.* **2000**, *40*, 693–698. [CrossRef]
40. Wildermuth, G.B.; Thomas, G.A.; Radford, B.J.; McNamara, R.B.; Kelly, A. Crown rot and common root rot in wheat grown under different tillage and stubble treatments in southern Queensland, Australia. *Soil Tillage Res.* **1997**, *44*, 211–224. [CrossRef]
41. Evans, M.L.; Hollaway, G.J.; Dennis, J.I.; Correll, R.; Wallwork, H. Crop sequence as a tool for managing populations of *Fusarium pseudograminearum* and *F. culmorum* in south-eastern Australia. *Australas. Plant Pathol.* **2010**, *39*, 376–382. [CrossRef]
42. Simpfendorfer, S. Seed-Borne *Fusarium* Threatens Crown Rot Control Strategies—Tamworth 2011. Northern Grains Region Trial Results Autumn 2013 Research & Extension—Independent Research for Industry Editors: Loretta Serafin, Steven Simpfendorfer, Matthew Gardner and Guy McMullen NSW Department of Primary Industries. 2013, pp. 137–139. Available online: https://eprints.usq.edu.au/25153/1/Moore_etal_PV.pdf (accessed on 16 November 2017).
43. Akgül, D.S.; Erkilic, A. Effect of wheat cultivars, fertilizers, and fungicides on Fusarium foot rot disease of wheat. *Turk. J. Agric. For.* **2016**, *40*, 101–108. [CrossRef]
44. Moya-Elizondo, E.A.; Rew, L.J.; Jacobsen, B.J.; Hogg, A.C.; Dyer, A.T. Distribution and prevalence of Fusarium crown rot and common root rot pathogens of wheat in Montana. *Plant Dis.* **2011**, *95*, 1099–1108. [CrossRef] [PubMed]
45. Pariyar, S.R.; Dababat, A.A.; Nicol, J.M.; Erginbas-Orakci, G.; Goll, M.B.; Watrin, C.; Sikora, R. Fungicide seed treatment and host resistance for the management of wheat crown rot caused by *Fusarium culmorum*. *Basic Res. J. Agric. Sci. Rev.* **2014**, *3*, 116–121.
46. Sohail, Q.; Erginbas-Orakci, G.; Ozdemir, F.; Jighly, A.; Dreisigacker, S.; Bektas, H.; Dababat, A.A. Genome-Wide Association Study of Root-Lesion Nematodes Pratylenchus Species and Crown Rot *Fusarium culmorum* in Bread Wheat. *Life* **2022**, *12*, 372. [CrossRef]
47. Kazan, K.; Gardiner, D.M. Fusarium crown rot caused by *Fusarium pseudograminearum* in cereal crops: Recent progress and future prospects. *Mol. Plant Pathol.* **2018**, *19*, 1547–1562. [CrossRef]
48. Ozdemir, F.; Koc, N.K.; Paulitz, T.; Nicol, J.M.; Schroeder, K.L.; Poole, G. Determination of fusarium crown rot resistance in wheat to *Fusarium culmorum* and *Fusarium pseudogramineaum* using real time PCR. *Crop Prot.* **2020**, *135*, 105204. [CrossRef]
49. Mitter, V.; Zhang, M.C.; Liu, C.J.; Ghosh, R.; Ghosh, M.; Chakraborty, S.A. high-throughput glasshouse bioassay to detect crown rot resistance in wheat germplasm. *Plant Pathol.* **2006**, *55*, 433–441. [CrossRef]
50. Li, X.; Liu, C.; Chakraborty, S.; Manners, J.M.; Kazan, K. A simple method for the assessment of crown rot disease severity in wheat seedlings inoculated with *Fusarium pseudograminearum*. *J Phytopathol.* **2008**, *156*, 751–754. [CrossRef]
51. Yang, X.M.; J Yang, X.; Ma, J.; Li, H.; Ma, H.; Yao, J.; Liu, C. Different genes can be responsible for crown rot resistance at different developmental stages of wheat and barley. *Eur. J. Plant Pathol.* **2010**, *128*, 495–502. [CrossRef]
52. Liu, C.; Ogbonnaya, F.C. Resistance to Fusarium crown rot in wheat and barley: A review. *Plant Breed.* **2015**, *134*, 365–372. [CrossRef]
53. Bovill, W.D.; Horne, M.; Herde, D.; Davis, M.; Wildermuth, G.B.; Sutherland, M.W. Pyramiding QTL increases seedling resistance to crown rot (*Fusarium pseudograminearum*) of wheat (*Triticum aestivum*). *Theor. Appl. Genet.* **2010**, *1211*, 127–136. [CrossRef] [PubMed]
54. Knight, N.L.; Sutherland, M.W. Histopathological assessment of wheat seedling tissues infected by *Fusarium pseudograminearum*. *Plant Pathol.* **2013**, *623*, 679–687. [CrossRef]
55. Birr, T.; Hasler, M.; Verreet, J.A.; Klink, H. Composition and predominance of Fusarium species causing Fusarium head blight in winter wheat grain depending on cultivar susceptibility and meteorological factors. *Microorganisms* **2020**, *8*, 617. [CrossRef]
56. Spanic, V.; Zdunic, Z.; Drezner, G.; Sarkanj, B. The pressure of Fusarium disease and its relation with mycotoxins in the wheat grain and malt. *Toxins* **2019**, *11*, 198. [CrossRef]

57. Collard, B.C.Y.; Grams, R.A.; Bovill, W.D.; Percy, C.D.; Jolley, R.; Lehmensiek, A.; Sutherland, M.W. Development of molecular markers for crown rot resistance in wheat: Mapping of QTLs for seedling resistance in a '2-49′×'Janz'population. *Plant Breed.* **2005**, *124*, 532–537. [CrossRef]
58. Zadoks, J.C.; Chang, T.T.; Konzak, C.F. A decimal code for the growth stages of cereals. *Weed Res.* **1974**, *14*, 415–421. [CrossRef]

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Phytochemical Profile and Activity against *Fusarium* Species of *Tamarix gallica* Bark Aqueous Ammonia Extract

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Abstract: French tamarisk, *Tamarix gallica* L. (family *Tamaricaceae*) is a deciduous tree that, like other halophytes, grows in a wide variety of saline habitats thanks to its powerful phenolics-based antioxidant system. Given that antioxidant properties are usually linked to the presence of compounds with antifungal properties, in the work presented herein the antimicrobial activity of *T. gallica* bark extract was investigated against four phytopathogenic species of genus *Fusarium*. According to the results of gas chromatography–mass spectroscopy, the phytochemical profile of the aqueous ammonia extract included 1-(2,4,6-trihydroxyphenyl)-2-pentanone; 3,5-dimethoxy-4-hydroxycinnam aldehyde; *trans*-squalene; 4-hydroxy-3,5-dimethoxy-benzaldehyde; dihydro-3- methylene-2,5-furandione; 1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone; and 4-hydroxy-3,5-dimethoxy-benzoic acid as main constituents. Concerning in vitro antifungal activity, EC_{90} effective concentrations in the 335–928 $\mu g \cdot mL^{-1}$ range were obtained against *F. acuminatum*, *F. culmorum*, *F. equiseti*, and *F. graminearum*, remarkably lower than those of two conventional fungicides (viz. mancozeb and fosetyl-Al). The antifungal activity of the extract was tested further in wheat and maize grain protection bioassays, confirming that the treatment effectively controlled *F. graminearum* at a concentration of 375 $\mu g \cdot mL^{-1}$. Given this promising activity, *T. gallica* bark extracts may be susceptible to valorization as a natural and sustainable biorational for *Fusarium* spp. control.

Keywords: antifungal; *Fusarium* spp.; FHB; GC−MS; halophyte; *Tamarix gallica*

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1. Introduction

Halophytes can complete their life cycles in highly salinized habitats without having significant detrimental effects on their growth or development. However, they account for, approximately, just 1% of all terrestrial plants, and the majority of them have neither ornamental nor economic value, which restricts their growth and use. It is therefore essential to look for (and make use of) beneficial halophytes in the development of moderately and severely salinized areas, which are vulnerable to desertification and ecological fragility owing to their lack of cover vegetation [1].

More than 60 species of halophytic plants are included in the genus *Tamarix* (family *Tamaricaceae*), popularly known as 'tamarisk' and 'salt cedar', which can be cultivated practically everywhere in the globe, improving the environment while also bringing economic advantages [2] (except in humid environments, in which they behave as invasive plants, impeding the development of other native species). Native to hot and arid areas, tamarisk species may also be found in temperate climates [3]. These plants are distinguished by having needle-shaped leaves covered with salt secreted by salt glands, which play a key

function in ionic balance regulation and in osmotic and turgor pressure maintenance under high salinity [4]. Studies on various *Tamarix* species have revealed a number of phytochemicals, the most significant of which are polyphenolic substances such as tannins, phenolic acids, and flavonoids, which are related to their main pharmacological properties, summarized in the review by Bahramsoltani et al. [5].

Tamarix gallica L. is a deciduous halophyte tree or shrub with a long lifespan, native to coastal and arid environments. It can withstand a variety of environmental stresses, including salt, high temperatures, and drought whilst growing up to 4 m. Its hermaphrodite flowers are small, five-petaled, white to pink, and flower throughout the spring and summer; they grow in long, drooping, narrow clusters that are up to three inches long. Seeds are small and black, with a sessile tuft of hygroscopic unicellular hairs attached to one end. *Tamarix gallica* has a smooth, reddish-brown bark that becomes furrowed and ridged with age [6].

As noted above, halophytes have a powerful antioxidant system based on certain phenolic compounds, terpenoids (carotenoids and essential oils), and vitamins, which are crucial to plants' normal growth, development, and defense against damage and infection [7]. In addition, these compounds have a wide spectrum of medicinal properties, such as anti-inflammatory, anti-allergic, antithrombotic, cardioprotective, and vasodilator effects, hepatoprotective and chemopreventive properties, and promising behavior as antioxidant and antimicrobial agents [8].

In the case of *T. gallica*, a total phenol content of 334.19 $\pm$ 8.47 mg GAE/g DW (and a flavonoids content of 159.73 $\pm$ 6.28 mg CE/g DW) was reported for a leaf methanolic extract [9], higher than that obtained for a methanolic extract of shoots (with a total phenol content of 200 mg GAE/g DW) [10]. The flower phenolic fingerprint of *T. gallica* includes seven phenolic acids (chlorogenic, *trans*-cinnamic, *p*-coumaric, gallic, sinapic, syringic, and vanillic acid) and six flavonoids (amentoflavone, apigenin, (+)-catechin, flavone, isoquercetin, and quercetin). As for the leaves, in addition to the phenolics identified in flowers, rosmarinic and ferulic acids were identified by Ksouri et al. [7]. Aside from these chemicals, Boulaaba et al. [11] reported the presence of the flavonoid kaempferol in flower extracts, and the existence of six compounds in the leaf extract, including quercetin 3-*O*-glucuronide. In turn, Said et al. [10] identified the phenolics naringin and caffeic acid in the leaf extract. The above phytochemicals, it has been suggested, account for the antibacterial activity of *T. gallica* against *Micrococcus luteus* (Schroeter) Cohn and its antifungal activity (especially against *Candida glabrata* (H.W. Anderson) S.A. Mey. and Yarrow and *Candida albicans* (C.P. Robin) Berkhout) [11].

Concerning the opportunities for the valorization of *T. gallica* extracts, their application as biorationals for crop protection may be particularly interesting. Among staple food crops, wheat and maize are especially important in terms of their contribution to food security [12]. However, cereal production is threatened by climate change and plant disease epidemics [13]. For instance, Fusarium head blight (FHB) severely reduces grain production quality and quantity in cereal crops including wheat, maize, and barley [14]. More than sixteen species, including *Fusarium graminearum* Schwabe (the major FHB pathogen), *Fusarium culmorum* (Wm.G. Sm.) Sacc., *Fusarium pseudograminearum* O'Donnell and T. Aoki, *Fusarium avenaceum* (Fr.) Sacc., *Fusarium equiseti* (Corda) Sacc., and *Fusarium poae* (Peck) Wollenw., are part of the FHB species complex. All produce mycotoxins, low molecular weight toxic secondary metabolites of high thermal stability and bioaccumulation capacity, which are potentially harmful to both human and animal health [15].

Although unpredictable, *Fusarium* outbreaks have increased in frequency in northern and central Europe as *F. graminearum* has invaded areas formerly dominated by the presence of *F. culmorum* [16]. Fungicide applications are regarded as a crucial and often utilized method for managing FHB. Factors such as the active molecule applied, timing, manner, rate of administration, cereal variety, and the presence of *Fusarium* species and pathogenic races affect the efficacy of the treatments and mycotoxin reduction [17]. Triazoles (i.e., tebuconazole, metconazole, and prothioconazole), carbendazim, strobilurins (i.e., azoxystrobin),

and their combinations are frequently used to control FHB [18]. In particular, azoxystrobin alone should be avoided, given that it may enhance the production of the deoxynivalenol toxin [19]. Alternatives to synthetic fungicides are being sought to reduce the accumulation of pesticide residues in food and the environment.

In this context, with the aim of searching for alternatives to the application of fungicides, taking into consideration Article 14 of Directive 2009/128/EC, this work covers the use of gas chromatography–mass spectrometry (GC−MS) to characterize the phytochemicals found in *T. gallica* bark aqueous ammonia extract, as well as the evaluation of its antifungal activity for the control of *Fusarium* spp. The effectiveness of this extract was first tested in vitro against *F. acuminatum*, *F. culmorum*, *F. equiseti*, and *F. graminearum*, and further tested for grain protection at storage against *F. graminearum*. The reported findings may be useful for the sustainable postharvest protection of wheat and maize grains, promoting their storability and food safety.

2. Material and Methods

2.1. Reagents

Ammonium hydroxide solution (CAS No. 1336-21-6, 50% *v/v* aq. soln.) was supplied by Alfa Aesar (Ward Hill, MA, USA). Acetic acid (CAS No. 64-19-7, purum, 80% in H_2O); squalene (CAS No. 111-02-4, analytical standard); 1-(2,4,6-trihydroxyphenyl)-2-pentanone (CAS No. 443678-79-3); syringaldehyde (CAS No. 134-96-3); sinapinaldehyde (CAS No. 4206-58-0); and Tween® 20 (CAS No. 9005-64-5) were purchased from Sigma Aldrich Química S.A. (Madrid, Spain). Becton, Dickinson, and Company (Franklin Lakes, NJ, USA) supplied the potato dextrose broth (PDB) and potato dextrose agar (PDA).

The Plant Health and Certification Service of the Government of Aragon provided the commercial fungicides used for comparison purposes, namely Vondozeb® (mancozeb 75%; reg. no. 18632; UPL Iberia) and Fesil® (fosetyl-Al 80%, reg. no. 18795; Bayer).

2.2. Fungal Isolates

Fungal isolates of *F. acuminatum* (42/63/2022) and *F. graminearum* (CRD 002/99) were supplied by the Regional Diagnostic Center of Aldearrubia (Junta de Castilla y León); *F. equiseti* (MYC-1403) was obtained from the Centre for Agri-Food Research and Technology of Aragon (CITA); and *F. culmorum* (CECT 20493) was acquired from the Spanish Type Culture Collection (CECT; Valencia, Spain).

2.3. Plant Material and Extraction Procedure

To attain the dissolution of polyphenols and other bioactive compounds of interest contained in *T. gallica* bark, an aqueous ammonia extraction medium was chosen, given its ability to remove acetyl groups from xylan polymers, reduce cellulose crystallinity, selectively breakdown and remove lignin from substrates, and increase porosity while releasing low amounts of sugar degradation compounds. Aqueous ammonia pretreatment is also affordable, non-corrosive, non-polluting, safe to use, and recyclable [20]. This choice is supported by other recent work involving bark extracts [21–23].

The extract was prepared from a composite sample of the bark of ten specimens of *T. gallica* from the *Paseo de San Pedro,* in Llanes (Asturias, Spain; 43°25′30.9″ N 4°45′31.2″ W), collected in May 2021 (Figure 1). The bark samples were thoroughly mixed, dried, and ground into a fine powder to facilitate the extraction process. The preparation of the bark extract followed the procedure previously reported in reference [22]. The bark powder sample (previously digested in aqueous ammonia solution for 2 h) was sonicated for 10 min, with a 2 min pause after every 2.5 min of sonication, using a model UIP1000hdT probe-type ultrasonicator from Hielscher Ultrasonics (Teltow, Germany). The sample was then allowed to stand for 24 h, and acetic acid was used to bring the pH to neutral. Finally, the solution was centrifuged for 15 min at 9000 rpm, and the supernatant was filtered using Whatman No. 1 paper.

Figure 1. (**a**) Tamarisk of the *Paseo de San Pedro*, in Llanes (Asturias, northern Spain), (**b**) trunk of a *T. gallica* specimen, (**c**) detail of *T. gallica* bark.

2.4. Extract Characterization

The infrared vibrational spectrum was recorded using a Nicolet iS50 Fourier-transform infrared spectrometer from Thermo Scientific (Waltham, MA, USA) with an in-built diamond attenuated total reflection (ATR) system. The spectrum was acquired with a resolution of 1 cm^{-1} spanning the 400–4000 cm^{-1} range, using the interferograms produced by co-adding 64 scans.

The aqueous ammonia extract of *T. gallica* bark was studied by gas chromatography–mass spectrometry (GC–MS) at the University of Alicante's Research Support Services (STI), with an Agilent Technologies (Santa Clara, CA, USA) model 7890A gas chromatograph connected to a model 5975C quadrupole mass spectrometer. The operating conditions were as follows: 280 °C injector temperature; splitless mode; 1 µL injection volume; 60 °C initial temperature for 2 min, followed by a ramp of 10 °C per min up to a final temperature of 300 °C, kept for 15 min. An Agilent Technologies HP-5MS UI chromatographic column (30 m in length, 0.250 mm diameter, and 0.25 µm film) was used for the separation of the compounds. The mass spectrometer settings were as follows: 230 °C electron impact source temperature; 150 °C quadrupole temperature; 70 eV ionization energy. For calibration, test mixture 2 for apolar capillary columns according to Grob (Supelco 86501) and PFTBA tuning standards supplied by Sigma Aldrich Química S.A. (Madrid, Spain) were utilized. For chemical identification, mass spectra and retention times were compared to those of reference compounds and the National Institute of Standards and Technology database.

2.5. In Vitro Antifungal Activity Evaluation

The antimicrobial activity of the treatments was evaluated using the agar dilution method (or 'poisoned food method'), in accordance with EUCAST standard antifungal susceptibility testing protocols [24]. To obtain concentrations in the 62.5−1500 $\mu g \cdot mL^{-1}$ range, aliquots of stock solution were mixed into a pouring PDA medium. Mycelial disks (Ø = 5 mm) from the margins of 1-week-old PDA cultures of the *Fusarium* spp. tested were transferred to PDA plates prepared with the aforementioned concentrations (three plates per treatment and concentration, with two duplicates). Incubation was conducted at 25 °C in the dark for one week. As a control, pure PDA media was used. Growth inhibition was calculated as $((d_c - d_t)/d_c) \times 100$, where d_c and d_t represent the mean diameters of the control and treated colonies, respectively. Determination of EC_{50} and EC_{90} values (50% and 90% maximal effective concentration, respectively) was carried out using PROBIT analysis in IBM SPSS Statistics v.25 (IBM, Armonk, NY, USA).

2.6. Preparation of Conidial Suspension of F. graminearum

A conidial suspension of *F. graminearum* was produced using the approach reported by Buzón-Durán et al. [25], with slight changes. Conidia of *F. graminearum* were harvested from 1-week-old PDB cultures (200 mL broth maintained in the dark at 25 °C and 140 rpm in an orbital stirrer incubator). To eliminate hyphal fragments, the suspension was filtered through two layers of sterile muslin. A hemocytometer (Weber Scientific International Ltd.,

Teddington, Middlesex, UK) was used for spore concentration determination, and the final concentration was adjusted to 1×10^6 spores (conidia)·mL^{-1}.

2.7. Stored Wheat and Maize Grain Protection Assays

The effect of *T. gallica* bark extract on the protection of stored wheat and maize grains against *F. graminearum* was determined according to Perczak et al. [26], with slight modifications. Soft winter wheat variety cv. 'Rimbaud' grains (Agrusa; Mollerussa, Lérida, Spain) and maize cv. 'P0937′ grains (DuPont Pioneer; Johnston, IA, USA), supplied by Piensos y Cereales Isabelio Sánchez-García (El Tejado de Béjar, Salamanca, Spain), were used in the experiments. Grains were surface sterilized by immersion in sodium hypochlorite 3% for 2 min and then rinsed with sterile milli-Q water three times, before being dried at room temperature in a laminar flow hood on sterile absorbent paper. Grain treatments (50 g of wheat or maize grains per treatment) were conducted by immersion in 100 mL of *T. gallica* extract (at a concentration equivalent to the MIC obtained in the in vitro experiments, adding 0.2% Tween® 20) at room temperature, under agitation, for 15 min. In the positive and negative controls, distilled water with 0.2% Tween® 20 was used. After drying for 30 min, at room temperature in a laminar flow hood, the grains were inoculated with the conidial suspension (prepared as described in the previous subsection). The samples were then incubated in a dark chamber at 25 °C for 28 days. Each treatment was repeated three times.

2.8. In Vitro Germination Assays

The effect of *T. gallica* bark extract on the germination of wheat and maize grains was assessed according to International Seed Testing Association (ISTA) standards [27]. The procedure was similar to the one indicated for the stored grain protection assays, using 20 maize grains and 50 wheat grains per treatment and replicate. Each treatment was repeated three times, and, for each treatment, three replicates of wheat or maize grains were placed in glass plates, using the between-paper method, and maintained under constant humid conditions. Germination was evaluated after four and six days for wheat and maize, respectively, with grains deemed germinated if they produced a well-developed seedling.

2.9. Statistics

Provided that the homogeneity and homoscedasticity requirements were met, according to the Shapiro–Wilk and Levene tests, the results of the in vitro mycelium growth inhibition experiments were analyzed using a one-way analysis of variance, followed by Tukey test for the post hoc comparison of means at $p < 0.05$.

3. Results

3.1. Bark Vibrational Characterization

Table 1 provides a summary of the primary infrared absorption bands found in the bark of *T. gallica*, which are consistent with the presence of functional groups such as polyphenols, alkaloids, organic acid esters, and other phytoconstituents. The main bands of the leaf vibrational spectrum [28] are also indicated for comparison purposes.

3.2. Bark Extract Constituents

Among the twenty-five compounds identified in the aqueous ammonia extract by GC−MS (Table 2), the nine most abundant (percentages > 3.5%) were: 1-(2,4,6-trihydroxyphenyl)-2-pentanone (11.8%); sinapinaldehyde or 3,5-dimethoxy-4-hydroxycinnamaldehyde (10%); *trans*-squalene or supraene (9.9%); syringaldehyde or 4-hydroxy-3,5-dimethoxy-benzaldehyde (8.1%); dihydro-3-methylene-2,5-furandione (7.5%); 1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone (7.2%); 4-hydroxy-3,5-dimethoxy-benzoic acid (6.6%); 2,6-dimethoxy-phenol (4%); and hexadecanoic acid, methyl ester (3.7%). Figure 2 depicts the chemical structures of the main phytochemicals found in *T. gallica* bark extract.

Table 1. Main bands in the infrared spectra of *T. gallica* bark and leaves.

Bark	Leaves [28]	Assignment
	3393	OH stretching; hydrogen bonds
3358		OH group in phenolic compounds
2917	2925	$–CH_2$ asymmetric stretching of alkyls (cutine, wax, pectin)
2850	2861	$–CH_2$ symmetric stretching (cutine and wax); CH_2–(C6)– bending (cellulose)
	1732	C=O stretching of alkyl ester
1628	1652	C=O stretching (hemicellulose, bonded ketones, . . .); C=C stretching
1594		C=C stretching
1504	1519	Aromatic skeletal. Typical of carotenoids
1460	1442	Symmetric aromatic ring stretching vibration (C=C ring); C–H deformation; O–CH_3 stretching
1421		C–H deformation
1328		CH in-plane bending in cellulose I and cellulose II
1223	1261	Amide III; C–C–O asymmetric stretching acetylated glucomannan; C–O and OH of COOH; in-plane rocking vibration signal of the $–CH_2–$ group
1153	1153	C–O–C asymmetric stretching in cellulose I and cellulose II; C–C in-plane (β-carotene)
1123		H–C–O bond bending
1030	1052	C–O stretching; O–H out plane bending

Table 2. Major phytochemical compounds identified in the aqueous ammonia extract of *T. gallica* bark by GC−MS.

RT (min)	Area (%)	Assignment	Qual
5.0425	7.5026	2,5-furandione, dihydro-3-methylene-	91
9.1734	2.9246	Benzofuran, 2,3-dihydro-	68
11.0490	3.9897	Phenol, 2,6-dimethoxy-	96
11.7078	1.1151	Vanillin	96
12.2064	2.1478	Ethanone, 1-(3-hydroxyphenyl)-	90
13.7495	2.4736	4-methyl-2,5-dimethoxybenzaldehyde	72
14.7764	1.8309	2,3,4,5-tetramethylbenzoic acid	30
14.8595	8.1339	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	96
15.0909	2.7329	4-methoxymethyl-6-methyl-1H-pyrazolo [3,4-b]pyridin-3-ylamine	52
15.3105	1.6158	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	89
15.5005	0.9374	Methyl tetradecanoate	96
15.6845	7.2470	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	92
16.0762	2.9054	Benzoic acid, 4-hydroxy-3,5-dimethoxy-, hydrazide	95
16.4917	3.7293	Benzoic acid, 4-hydroxy-3,5-dimethoxy-	98
16.6460	2.9655	Aspidinol	59
16.6994	1.6692	2-fluorenamine	46
17.3938	1.3234	9-hexadecenoic acid, methyl ester, (Z)-	95
17.5897	3.6982	Hexadecanoic acid, methyl ester (or methyl palmitate)	98
17.9102	2.2142	n-hexadecanoic acid	99
18.0705	1.4350	Benzeneacetic acid, .alpha.-phenyl-, methyl ester	72
18.2188	10.0460	3,5-dimethoxy-4-hydroxycinnamaldehyde	98
18.2960	11.8101	2-pentanone, 1-(2,4,6-trihydroxyphenyl)	53
19.2694	1.9702	11-octadecenoic acid, methyl ester	99
19.4949	1.7364	Methyl stearate	99
25.0919	9.9556	Supraene	98

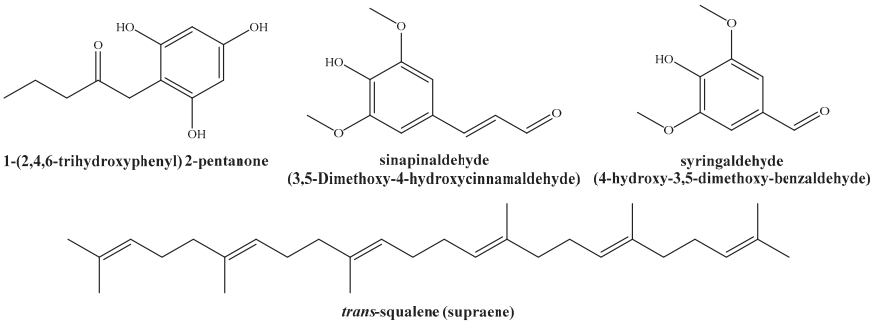

Figure 2. Main phytochemicals identified in the aqueous ammonia extract of *T. gallica* bark.

If the aforementioned compounds are grouped into categories, the extract of *T. gallica* bark consists of phenolic compounds (50%), triterpenes (12%), flavonoids (10%), alkaloids (10%), and fatty acid methyl esters (5%).

3.3. Extract Antifungal Activity

3.3.1. In Vitro Activity

The results of in vitro anti-Fusarium activity tests of *T. gallica* bark extract and its main phytochemical constituents are depicted in Figure 3 and Figures S1–S4. *Tamarix gallica* bark aqueous ammonia extract suppressed *Fusarium* spp. growth at concentrations ranging from 375 to 1000 $\mu g \cdot mL^{-1}$, depending on the *Fusarium* species, and showed the highest efficacy against *F. graminearum* (MIC = 375 $\mu g \cdot mL^{-1}$). Regarding its four main phytoconstituents, 1-(2,4,6-trihydroxyphenyl)-2-pentanone featured the highest antifungal activity, with inhibition values in the 250–375 $\mu g \cdot mL^{-1}$ range, better than those obtained for sinapinaldehyde (in the 500–750 $\mu g \cdot mL^{-1}$ range) and for *trans*-squalene and syringaldehyde (ranging from 375 to 750 $\mu g \cdot mL^{-1}$). To facilitate the comparison of their efficacies, effective concentration values are summarized in Table 3.

Table 3. Effective concentrations (expressed in $\mu g \cdot mL^{-1}$) against *F. acuminatum*, *F. culmorum*, *F. equiseti*, and *F. graminearum* of *T. gallica* bark aqueous ammonia extract and four of its main constituents.

Treatment	Effective Concentration	*F. acuminatum*	*F. culmorum*	*F. equiseti*	*F. graminearum*
T. gallica bark extract	EC_{50}	568.8	272.8	440.2	238.3
	EC_{90}	928.0	825.6	698.3	334.8
1-(2,4,6-trihydroxyphenyl)-2-pentanone	EC_{50}	147.9	81.5	114.3	95.8
	EC_{90}	236.2	213.4	238.2	190.7
Sinapinaldehyde	EC_{50}	257.1	117.0	209.4	169.9
	EC_{90}	555.2	367.7	530.2	299.5
Trans-squalene	EC_{50}	242.5	179.6	153.7	114.3
	EC_{90}	507.3	380.2	393.5	258.0
Syringaldehyde	EC_{50}	322.7	176.8	144.1	124.6
	EC_{90}	601.4	374.8	316.1	246.6

For comparison purposes, two conventional synthetic fungicides were also tested against the aforementioned four *Fusarium* taxa. Results are summarized in Table 4. At the recommended dose (i.e., 1500 $\mu g \cdot mL^{-1}$), dithiocarbamate (mancozeb) resulted in complete suppression of the mycelial growth of *F. acuminatum*, but it required ten times the recommended dose (15,000 $\mu g \cdot mL^{-1}$) to completely inhibit *F. culmorum*, *F. equiseti*, and *F. graminearum*. The organophosphorus fungicide (fosetyl-Al) completely inhibited the growth of *F. culmorum* and *F. graminearum* at the recommended dose (i.e., 2000 $\mu g \cdot mL^{-1}$), but required a higher concentration (i.e., 20,000 $\mu g \cdot mL^{-1}$) to achieve complete inhibition of *F. acuminatum*. It is worth noting that, at the latter concentration, only 64.4% of the

mycelial growth of *F. equiseti* was inhibited, thus indicating that a concentration higher than 20,000 μg·mL^{-1} would be required for complete inhibition.

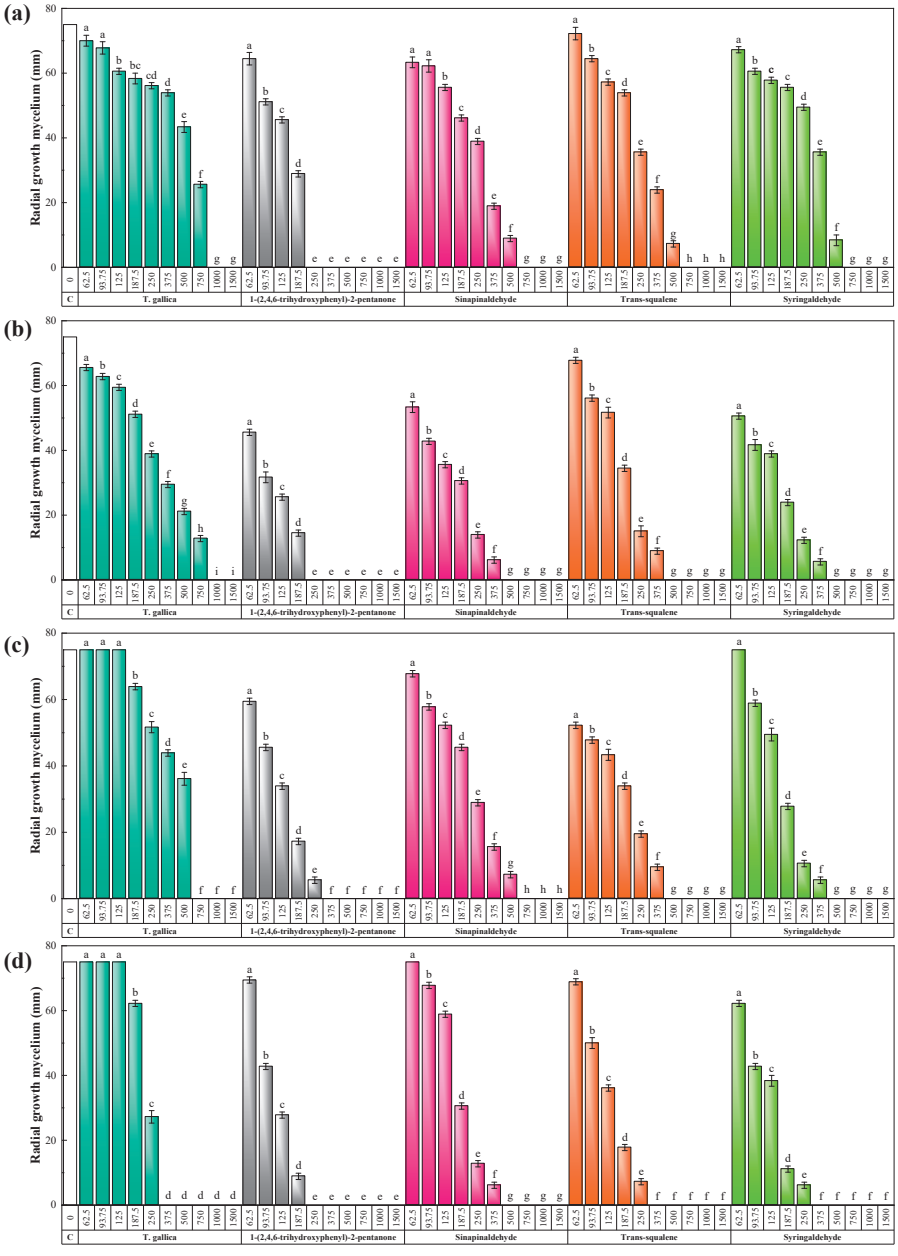

Figure 3. Inhibition of the radial growth of the mycelium of (**a**) *F. acuminatum*, (**b**) *F. culmorum*, (**c**) *F. equiseti*, and (**d**) *F. graminearum* in the in vitro tests performed in PDA medium incorporating different concentrations (in the 62.5–1500 μg·mL^{-1} range) of *T. gallica* bark extract or of its main phytochemical constituents (viz., 1-(2,4,6-trihydroxyphenyl)-2-pentanone, sinapinaldehyde, *trans*-squalene, and syringaldehyde). The efficacies of concentrations labeled with the same letters are not statistically different at $p < 0.05$. Error bars represent standard deviations.

Table 4. Radial growth of the mycelium of *F. acuminatum*, *F. culmorum*, *F. equiseti*, and *F. graminearum* in the in vitro assays performed on a PDA medium with different concentrations of two commercial synthetic fungicides, namely a tenth of the recommended dose (Rd/10), the recommended dose (Rd), and ten times the recommended dose (Rd × 10).

Commercial Fungicide	Pathogen	Radial Growth of Mycelium (mm)				Inhibition (%)		
		Control (PDA)	Rd/10	Rd *	Rd × 10	Rd/10	Rd *	Rd × 10
Mancozeb	*F. acuminatum*	75	65	0	0	13.3	100	100
	F. culmorum	75	75	5	0	0	93.3	100
	F. equiseti	75	70	25	0	6.7	66.7	100
	F. graminearum	75	75	5	0	0	93.3	100
Fosetyl-Al	*F. acuminatum*	75	66.7	35	0	11.1	53.3	100
	F. culmorum	75	75	0	0	0	100	100
	F. equiseti	75	75	60	26.7	0	20	64.4
	F. graminearum	75	33.3	0	0	55.6	100	100

* Rd = 1.5 mg·mL^{-1} of mancozeb (2 g·L^{-1} for Vondozeb®, mancozeb 75%) and 2 mg·mL^{-1} of fosetyl-Al (2.5 g·L^{-1} for Fesil®, fosetyl-Al 80%). All mycelial growth values (in mm) are average values (n = 3).

3.3.2. Protection of Wheat and Maize Grains

To assess the effectiveness of the *T. gallica* bark extract for the postharvest protection of wheat and maize grains, promoting their storability and food safety, ex situ tests were conducted against *F. graminearum*. After 28 days of incubation, in wheat and maize grain samples artificially infected with this pathogen, no mycelial development was observed in the grains treated with *T. gallica* bark extract, while the positive control grains (inoculated and treated only with distilled water) showed clear fungal colonization (Figure 4). Therefore, the treatment showed a clear protective effect on both wheat and maize stored grains exposed to *F. graminearum* at a concentration of 375 µg·mL^{-1} (i.e., the MIC value determined in the in vitro tests).

Figure 4. Effect of the application of *T. gallica* bark extract on the growth of *F. graminearum*: (**a**) negative control wheat grains, (**b**) positive control wheat grains, (**c**) wheat grains inoculated with *F. graminearum* and treated with *T. gallica* bark extract at a dose of 375 µg·mL^{-1}, (**d**) negative control maize grains, (**e**) positive control maize grains, (**f**) maize grains inoculated with *F. graminearum* and treated with *T. gallica* bark extract at a dose of 375 µg·mL^{-1}. Only one replicate per treatment is shown.

3.4. Germination Assays

Regarding germination tests (Figure 5), no significant differences were observed between the negative control (grains treated with distilled water; not shown), with a 99−100% germination rate, and the grains treated with *T. gallica* bark extract at 375 µg·mL^{-1}, with germination percentages of 98 and 96% for wheat and maize grains, respectively. This finding suggests that the application of *T. gallica* bark extract would not be phytotoxic to

wheat and maize grains. The germination percentage of the positive control (i.e., artificially inoculated grains with no treatment) was notably lower, with germination rates of 78 and 88% for wheat and maize, respectively, but it clearly improved in the case of inoculated and treated grains (89 and 95.5% germination rate, respectively).

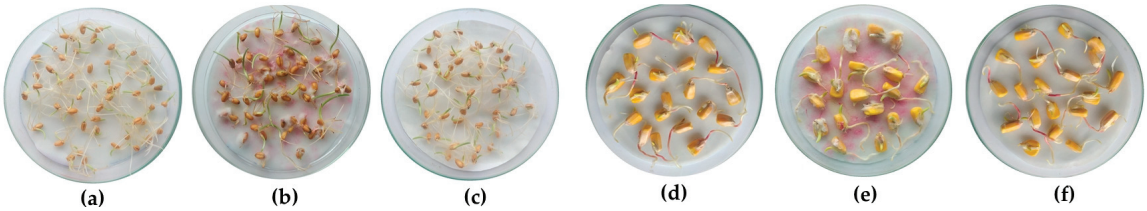

Figure 5. Germination tests: (**a**) wheat grains treated with *T. gallica* bark extract at a dose of 375 μg·mL^{-1}; (**b**) positive control wheat grains (inoculated with *F. graminearum* and treated with distilled water); (**c**) wheat grains inoculated with *F. graminearum* and treated with *T. gallica* bark extract at a dose of 375 μg·mL^{-1}; (**d**) maize grains treated with *T. gallica* bark extract at a dose of 375 μg·mL^{-1}; (**e**) positive control maize grains (inoculated with *F. graminearum* and treated with distilled water); and (**f**) maize grains inoculated with *F. graminearum* and treated with *T. gallica* bark extract at a dose of 375 μg·mL^{-1}. Only one replicate per treatment is shown.

4. Discussion

4.1. On the Phytochemical Composition and Mode of Action

The high phenolics content is in agreement with that reported in flowers by Boulaaba et al. [29] (135.3 mg GAE/g DW) and would explain the high antioxidant activity observed by Nisar et al. [30] and by Lefahal et al. [31].

Concerning the antifungal mechanism of the main compound categories identified in the extract (viz. phenolic, flavonoids, and organic acids), according to a recent study on *Tamarix aphylla* (L.) Karst. extracts by Al-Otibi et al. [32], their activity should be ascribed to their ability to induce hyper acidification via proton donation at the plasma membrane interface and intracellular cytosolic acidification, disrupting ATP synthesis [33]. Makarewicz et al. [34] hypothesized that the hydrophobic phenolic compounds initially bind to the plasma membrane, cell wall, and lipopolysaccharide–water interface of the cell without penetration. Their stacking on the plasma membrane would affect membrane fluidity, resulting in destabilization and partial disruption, which would allow the phenolic compounds to enter the cytosol. Their toxicity mechanism against microorganisms would also include enzyme inhibition and nonspecific interactions with proteins. On the other hand, the flavonoid antifungal activity has been attributed to their ability to complex with extracellular and soluble proteins and cell walls [35].

In a more detailed analysis, the activity of the extract should be referred to the most representative phytochemicals (or to synergies between some of them), as discussed below.

1-(2,4,6-trihydroxyphenyl)-2-pentanone is a phenolic compound previously reported, for instance, in *Elaphoglossum spathulatum* (Bory) T. Moore methanol extract [36], in *Polygala javana* DC ethanolic extract [37], in pyroligneous acid obtained from slow pyrolysis from palm kernel shell [38], in wood extractives of *Populus tomentosa* Carrière [39], and in *Aquilaria malaccensis* Lam. ethanolic extract [40]. The latter was shown to have antibacterial activity against *Acinetobacter baumannii* Bouvet and Grimont 1986 and *Klebsiella pneumoniae* (Schroeter 1886) Trevisan 1887.

3,5-dimethoxy-4-hydroxycinnamaldehyde (or sinapinaldehyde) is a low molecular weight phenolic acid intermediate in the formation of lignin. Sinapinaldehyde has previously been found, in lower proportions than those reported for the aqueous ammonia extract of *T. gallica* bark, in the aerial parts of the halophyte *Cladium mariscus* L. (Pohl.) [41], in the leaves of *Strelitzia nicolai* Regel and Koch [42], and in raw materials such as in the wood of *Populus lasiocarpa* Oliv. and *P. tomentosa* (0.35 and 0.34%, respectively) [39], in the

heartwood of *Fraxinus excelsior* L. and *Fraxinus americana* L. [43], in the fibers of *Senra incana* Cav. and *Cocos nucifer*a L., and in the seeds of *Coix lacryma-jobi* L. [44]. Shreaz et al. [45] examined several cinnamaldehydes, including sinapaldehyde, finding that it was an effective anticandidal agent against several azole-sensitive and azole-resistant clinical isolates, with MIC values in the 100−200 $\mu g \cdot mL^{-1}$ range. Its antifungal activity was related to the inhibition of plasma membrane-ATPase (PM-ATPase), the lowering of intracellular pH, and the depletion of NADPH, together with damage caused to membranes and cell walls. Its limited toxicity together with its broad spectrum of activity suggested that sinapaldehyde could be developed as an antifungal.

Squalene is a lipophilic triterpene, a natural precursor of ergosterol, crucial in the plasmatic membrane of fungi [46]. It has previously been identified in *Acalypha indica* L., *Ammannia baccifera* L., *Abrus precatorius* L., *Abutilon indicum* L., *Cuscuta reflexa* Roxb. [47], *Cucurbita maxima* Duchesne [48], *Jasminum grandiflorum* L. [49], and *Leucas aspera* (Willd.) Link [50], and—more recently—by our research group in the bark of *Quercus ilex* subsp. *ballota* (Desf.) Samp., with a content of 13% [21], slightly higher than that obtained in the bark of *T. gallica* (9.9%). It has been demonstrated that squalene has antifungal properties against *Candida* spp. [51]. Intracellular accumulation of squalene is known to disrupt fungal cell membranes, possibly via the formation of squalene vesicles that weaken fungal cells by removing critical membrane lipid components [52]. Terbinafine and other antifungal drugs' mode of action is based on inhibiting squalene peroxidase, resulting in squalene accumulation [53]. Currently, research on squalene monooxygenase and epoxidase enzymes is a promising area for the development of new antifungal drugs [54,55]. Reports on the antifungal action of *trans*-squalene for other supraene-rich natural products have been summarized in [21].

Syringaldehyde or 3,5-dimethoxy-4-hydroxybenzaldehyde is a phenolic aldehyde found in a wide range of plants, according to the comprehensive summary by Wu et al. [56]. It possesses significant broad-spectrum antimicrobial activity, being highly effective against bacteria such as *Bacillus subtilis* (Ehrenberg) Cohn, *K. pneumonia*, *Staphylococcus aureus* Rosenbach, and *Pseudomonas aeruginosa* (Schroeter) Migula, and against the formation of *Aspergillus* spp. biofilms [57,58].

Given the activity demonstrated in the in vitro tests by the four phytochemicals discussed above (Figure 3), and taking into consideration that their antimicrobial activity is further supported by the findings of other research groups, the antifungal activity of the extract should be mainly ascribed to these compounds. Nonetheless, contributions from other constituents present in the extract in lower amounts and the existence of synergistic behaviors cannot be ruled out.

4.2. Antimicrobial Activity Comparison

4.2.1. Comparison with Other *Tamarix gallica* Extracts

The high content of polyphenols (including quercetin, kaempferol, coumarin, and rhamnocitin, among others) reported for other *T. gallica* organs, primarily flowers, would be responsible for their biological capacity against multidrug-resistant clinical infections (*S. aureus, Micrococcus luteus, Escherichia coli* (Migula) Castellani and Chalmers, *Pseudomonas* spp., *Klebsiella* spp., *Enterococcus faecalis* (Andrewes and Horder 1906) Schleifer and Kilpper-Balz 1984, *Bacillus* spp., *Listeria monocytogenes* (Murray et al.) Pirie, and *Candida* spp.) as shown in Table S1. However, it is worth noting that the concentrations assayed in [7,11], ranging from 100 to 300 $mg \cdot mL^{-1}$, were two to three orders of magnitude higher than those assayed herein and that complete inhibition was not attained in most cases.

4.2.2. Comparison with other *Tamaricaceae* Family Bark Extracts

A literature survey for other species of the *Tamaricaceae* family with established antimicrobial activity was conducted to compare the results.

The antimicrobial activity of *T. aphylla* bark is the one that has received the most attention in the literature. Bibi et al. [59] studied its antifungal activity against *Aspergillus flavus*

Link, *Aspergillus fumigatus* Fresen., *Aspergillus niger* Tiegh., *Fusarium oxysporum* Schltdl., *Penicillium notatum* Westling, and *Saccharomyces cerevisiae* Desm. Extracts in different solvents were assayed (viz. methanol, ethanol, chloroform, distilled water, and acetone), finding that the chloroform extract was the most effective, inhibiting the growth of *F. oxysporum* by 97.68%, *A. flavus* by 88.48%, *A. fumigatus* by 91.46 %, and *P. notatum* by 87.46% at a concentration of 2000 $\mu g \cdot mL^{-1}$. Iqbal et al. [60] investigated the efficacy of a fixed oil against bacteria and fungi. Its maximum effectiveness was obtained against *B. subtilis* (MIC = 125 $\mu g \cdot mL^{-1}$), *C. glabrata* (MIC = 400 $\mu g \cdot mL^{-1}$), and *E. coli* (MIC = 500 $\mu g \cdot mL^{-1}$); additionally, it showed moderate activity against *C. albicans*, *S. aureus*, *Shigella flexneri* Castellani and Chalmers, and *Trichphyton longifusus* (Flórián and Galgoczy) Ajello, with MIC values in the 1000−2000 $\mu g \cdot mL^{-1}$ range. Finally, it showed low efficacy against *Salmonella typhi* (Schroeter) Warren and Scott. (MIC = 3000 $\mu g \cdot mL^{-1}$) and *Fusarium solani* (Mart.) Sacc. (MIC = 4000 $\mu g \cdot mL^{-1}$); there was no inhibitory effect against *P. aeruginosa*, *Microsporum canis* E. Bodin ex Guég., or *A. flavus*. Its antimicrobial activity was related to the presence of capric acid and lauric acid in high amounts [61].

On the other hand, Ren et al. [62] evaluated a *Tamarix ramosissima* Ledeb. bark ethanolic extract against some foodborne pathogens, finding a moderate-low bactericidal effect against *S. aureus*, *L. monocytogenes*, *Bacillus cereus* Frankland and Frankland, and *Shigella flexneri* Castellani and Chalmers, with MIC values of 5000 $\mu g \cdot mL^{-1}$; and a lower activity against *E. coli* (MIC = 10,000 $\mu g \cdot mL^{-1}$), *P. aeruginosa* and *S. typhi* (MIC > 10,000 $\mu g \cdot mL^{-1}$). However, it showed no activity against the four fungi tested: *Penicillium expansum* Link, *A. niger*, *Acremonium strictum* (Gams) Summerbell, and *Penicillium citrinum* Thom. Mikaeili et al. [63] assessed an aqueous decoction of *T. ramosissima* bark against *Trichophyton verrucosum* Bodin and *Epidermophyton floccosum* (Harz) Langeron and Miloch., reporting inhibition zone values of 18.3 and 23.3 mm, respectively, at a concentration of 500,000 $\mu g \cdot mL^{-1}$.

Although comparisons of the activities reported above for other tamarisk species extracts with those reported in this work for *T. gallica* should be taken with care (given that the activity is solvent- and fungal isolate-dependent), if inhibitory values against *Fusarium* spp. are analyzed, it may be inferred that *T. aphylla* would have lower effectiveness than *T. gallica* (with inhibition values higher than 2000 $\mu g \cdot mL^{-1}$ against *F. oxysporum* and *F. solani*, vs. 375−1000 $\mu g \cdot mL^{-1}$ for *T. gallica* against *F. acuminatum*, *F. culmorum*, *F. equiseti*, and *F. graminearum*).

4.2.3. Comparison with Conventional Fungicides

Several fungicides, including those in the benzimidazole group (carbendazim, benomyl), azoles (hexaconazole, prochloraz, propiconazole, tebuconazole, and triadimenol), and dithiocarbamates (mancozeb) are useful for the control of FHB. The basic technique for managing FHB involves the use of azoles, which block the ergosterol production pathway and decrease mycotoxin concentration and FHB symptoms [64]. Strobirulins (azoxystrobin), on the other hand, limit FHB by blocking electron transport in the mitochondrial respiratory chain, reducing aerobic energy production and inhibiting fungal growth [65]. None of them, however, has led to total FHB control [14]. The severity of the disease, the crop's level of natural resistance, and the spraying method play a significant role in the effectiveness.

In this work, two conventional fungicides were tested against the four *Fusarium* isolates for reference purposes. As shown in Table 4, their effectiveness was substantially lower than that of the *T. gallica* bark extract (Table 3): while full inhibition was attained for the natural product at concentrations in the 375−1000 $\mu g \cdot mL^{-1}$ range, doses of 1500 and 15,000 $\mu g \cdot mL^{-1}$ were needed in the case of mancozeb, and fosetyl-Al concentrations in the 2000−20,000 $\mu g \cdot mL^{-1}$ range were required to control three of the *Fusarium* taxa (provided that complete inhibition of *F. acuminatum* was not reached even at the highest dose of this last chemical).

4.3. Limitations of the Study

According to Tokarev et al. [66], who tested four fungicides in vitro (viz. pyraclostrobin, thiram, fludioxonil, and a combination of imazalil+metalaxyl+tebuconazole)

against ten strains of *Fusarium* spp., the sensitivity of *F. acuminatum*, *F. graminearum*, *F. semitectum*, *F. culmorum*, *F. sporotrichioides*, and *F. equiseti* strains to fungicides was higher than that of strains belonging to *F. oxysporum*, *F. solani*, *F. verticillioides*, and *F. proliferatum*. Hence, further tests on the effectiveness of the bark extract against these later taxa would be needed before moving to field trials.

Another important point would be related to the presence of mycotoxins in the treated grains. There is growing evidence that fungicides may not be as effective at reducing the generation of toxins because, in some circumstances, they may act as stressors that trigger the biosynthesis of toxins. Certain *Fusarium* species can produce mycotoxins when exposed to sublethal levels of some fungicides: for instance, application of sublethal doses of tebuconazole induced fumonisin expression in *Fusarium verticillioides* (Sacc.) Nirenberg and *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach and Nirenberg [67], and trichothecenes in *Fusarium langsethiae* Torp and Nirenberg, as did low doses of prochloraz [68]. However, in *F. graminearum* the application of low concentrations of tebuconazole did not lead to a significant increase in trichothecenes, whereas the application of propiconazole did [69]. Additional research is needed to determine the influence of *T. gallica* bark extracts at different doses on mycotoxin production.

5. Conclusions

Gas chromatography–mass spectroscopy characterization of *Tamarix gallica* bark aqueous ammonia extract allowed for the identification of 1-(2,4,6-trihydroxyphenyl)-2-pentanone; 3,5-dimethoxy-4-hydroxycinnam aldehyde; *trans*-squalene; 4-hydroxy-3,5-dimethoxy- benzaldehyde; dihydro-3-methylene-2,5-furandione; and 1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone as the main constituents. In vitro mycelial growth inhibition tests showed that the extract and the aforementioned four phytochemicals displayed high activity against four *Fusarium* taxa responsible for the so-called Fusarium head blight (FHB) in cereals, resulting in complete inhibition at concentrations ranging from 375 to 1000 $\mu g \cdot mL^{-1}$ in the case of the extract, and in the 250–750 $\mu g \cdot mL^{-1}$ range for its constituents. These inhibitory concentration values were lower than those required when using mancozeb and fosetyl-Al synthetic fungicides, tested for comparison purposes. Further ex situ bioassays on wheat and maize grains artificially infected with *F. graminearum* confirmed the effectiveness of the bark extract against this pathogen, attaining full protection of wheat and maize grains at a concentration equal to the MIC determined in the in vitro tests (375 $\mu g \cdot mL^{-1}$), with no symptoms of phytotoxicity based on germination tests. These findings support the potential of this halophyte as a valuable source of natural bioactive compounds and pave the way for the valorization of its bark to obtain high added-value products, such as biorationals for cereal protection against FHB.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13020496/s1, Figure S1: Growth inhibition of *F. acuminatum* for *T. gallica* bark, 1-(2,4,6-trihydroxyphenyl)-2-pentanone, sinapinaldehyde, *trans*-squalene, and syringaldehyde. Figure S2: Growth inhibition of *F. culmorum* for *T. gallica* bark, 1-(2,4,6-trihydroxyphenyl)-2-pentanone, sinapinaldehyde, *trans*-squalene, and syringaldehyde. Figure S3: Growth inhibition of *F. equiseti* for *T. gallica* bark, 1-(2,4,6-trihydroxyphenyl)-2-pentanone, sinapinaldehyde, *trans*-squalene, and syringaldehyde. Figure S4: Growth inhibition of *F. graminearum* for *T. gallica* bark, 1-(2,4,6-trihydroxyphenyl)-2-pentanone, sinapinaldehyde, *trans*-squalene, and syringaldehyde. Table S1. Antimicrobial activity of *T. gallica* leaf and flower extracts reported in the literature.

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References

1. Duan, Q.; Zhu, Z.; Wang, B.; Chen, M. Recent progress on the salt tolerance mechanisms and application of tamarisk. *Int. J. Mol. Sci.* **2022**, *23*, 3325. [CrossRef]
2. Han, Z.; Yin, W.; Zhang, J.; Niu, S.; Ren, L. Active anti-erosion protection strategy in tamarisk (*Tamarix aphylla*). *Sci. Rep.* **2013**, *3*, 3429. [CrossRef]
3. Zhang, D.; Yin, L.; Pan, B. Biological and ecological characteristics of *Tamarix* L. and its effect on the ecological environment. *Sci. China Ser. D Earth Sci.* **2002**, *45*, 18–22. [CrossRef]
4. Dawalibi, V.; Monteverdi, M.; Moscatello, S.; Battistelli, A.; Valentini, R. Effect of salt and drought on growth, physiological and biochemical responses of two *Tamarix* species. *Iforest Biogeosci. For.* **2015**, *8*, 772–779. [CrossRef]
5. Bahramsoltani, R.; Kalkhorani, M.; Abbas Zaidi, S.M.; Farzaei, M.H.; Rahimi, R. The genus *Tamarix*: Traditional uses, phytochemistry, and pharmacology. *J. Ethnopharmacol.* **2020**, *246*, 112245. [CrossRef]
6. Elamin, M. A review of biological and pharmacological activities from the aerial part of tamarisk. *Int. J. Pharm. Res. Allied Sci.* **2016**, *5*, 22–36.
7. Ksouri, R.; Falleh, H.; Megdiche, W.; Trabelsi, N.; Mhamdi, B.; Chaieb, K.; Bakrouf, A.; Magné, C.; Abdelly, C. Antioxidant and antimicrobial activities of the edible medicinal halophyte *Tamarix gallica* L. and related polyphenolic constituents. *Food Chem. Toxicol.* **2009**, *47*, 2083–2091. [CrossRef]
8. Lee, J.M.; Yim, M.-J.; Lee, D.-S.; Lee, M.S.; Park, Y.G.; Jeon, J.H.; Choi, G. Comparison of biological activities of Korean halophytes. *Nat. Prod. Sci.* **2018**, *24*, 247–252. [CrossRef]
9. Ferreira, M.J.; Pinto, D.C.G.A.; Cunha, Â.; Silva, H. Halophytes as medicinal plants against human infectious diseases. *Appl. Sci.* **2022**, *12*, 7493. [CrossRef]
10. Said, S.; Noureddine, G.; Eddine, L.S.; Abdelmadjid, G.; Djamel, B.; Tliba, A. Phenolic content, HPLC analysis and antioxidant activity extract from *Tamarix gallica* and *Tamarix articulata* growing in Southeast of Algeria. *Res. J. Pharm. Technol.* **2018**, *11*, 3826–3832. [CrossRef]
11. Boulaaba, M.; Snoussi, M.; Saada, M.; Mkadmini, K.; Smaoui, A.; Abdelly, C.; Ksouri, R. Antimicrobial activities and phytochemical analysis of *Tamarix gallica* extracts. *Ind. Crops Prod.* **2015**, *76*, 1114–1122. [CrossRef]
12. Grote, U.; Fasse, A.; Nguyen, T.T.; Erenstein, O. Food security and the dynamics of wheat and maize value chains in Africa and Asia. *Front. Sustain. Food Syst.* **2021**, *4*, 617009. [CrossRef]
13. Gurung, S.; Hansen, J.M.; Bonman, J.M.; Gironella, A.I.N.; Adhikari, T.B. Multiple disease resistance to four leaf spot diseases in winter wheat accessions from the USDA National Small Grains Collection. *Crop Sci.* **2012**, *52*, 1640–1650. [CrossRef]
14. Dweba, C.C.; Figlan, S.; Shimelis, H.A.; Motaung, T.E.; Sydenham, S.; Mwadzingeni, L.; Tsilo, T.J. Fusarium head blight of wheat: Pathogenesis and control strategies. *Crop Prot.* **2017**, *91*, 114–122. [CrossRef]
15. Pitt, J.I.; Taniwaki, M.H.; Cole, M.B. Mycotoxin production in major crops as influenced by growing, harvesting, storage and processing, with emphasis on the achievement of Food Safety Objectives. *Food Control* **2013**, *32*, 205–215. [CrossRef]
16. Moretti, A.; Pascale, M.; Logrieco, A.F. Mycotoxin risks under a climate change scenario in Europe. *Trends Food Sci. Technol.* **2019**, *84*, 38–40. [CrossRef]
17. Leslie, J.F.; Moretti, A.; Mesterházy, Á.; Ameye, M.; Audenaert, K.; Singh, P.K.; Richard-Forget, F.; Chulze, S.N.; Ponte, E.M.D.; Chala, A.; et al. Key global actions for mycotoxin management in wheat and other small grains. *Toxins* **2021**, *13*, 725. [CrossRef]

18. Feksa, H.R.; Do Couto, H.T.Z.; Garozi, R.; De Almeida, J.L.; Gardiano, C.G.; Tessmann, D.J. Pre- and postinfection application of strobilurin-triazole premixes and single fungicides for control of fusarium head blight and deoxynivalenol mycotoxin in wheat. *Crop Prot.* **2019**, *117*, 128–134. [CrossRef]
19. Chen, H.; Wu, Q.; Zhang, G.; Wu, J.; Zhu, F.; Yang, H.; Zhuang, Y. Carbendazim-resistance of *Gibberella zeae* associated with fusarium head blight and its management in Jiangsu Province, China. *Crop Prot.* **2019**, *124*, 104866. [CrossRef]
20. Zhang, H.; Wu, J. Statistical optimization of aqueous ammonia pretreatment and enzymatic hydrolysis of corn cob powder for enhancing sugars production. *Biochem. Eng. J.* **2021**, *174*, 108106. [CrossRef]
21. Sánchez-Hernández, E.; Balduque-Gil, J.; Barriuso-Vargas, J.J.; Casanova-Gascón, J.; González-García, V.; Cuchí-Oterino, J.A.; Lorenzo-Vidal, B.; Martín-Gil, J.; Martín-Ramos, P. Holm Oak (Quercus ilex subsp. ballota (Desf.) Samp.) Bark Aqueous Ammonia Extract for the Control of Invasive Forest Pathogens. *Int. J. Mol. Sci.* **2022**, *23*, 11882. [CrossRef]
22. Sánchez-Hernández, E.; Martín-Ramos, P.; Martín-Gil, J.; Santiago-Aliste, A.; Hernández-Navarro, S.; Oliveira, R.; González-García, V. Bark extract of *Uncaria tomentosa* L. for the control of strawberry phytopathogens. *Horticulturae* **2022**, *8*, 672. [CrossRef]
23. Sánchez-Hernández, E.; González-García, V.; Casanova-Gascón, J.; Barriuso-Vargas, J.J.; Balduque-Gil, J.; Lorenzo-Vidal, B.; Martín-Gil, J.; Martín-Ramos, P. Valorization of *Quercus suber* L. bark as a source of phytochemicals with antimicrobial activity against apple tree diseases. *Plants* **2022**, *11*, 3415. [CrossRef]
24. Arendrup, M.C.; Cuenca-Estrella, M.; Lass-Flörl, C.; Hope, W.; EUCAST-AFST. EUCAST technical note on the EUCAST definitive document EDef 7.2: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). *Clin. Microbiol. Infect.* **2012**, *18*, E246–E247. [CrossRef]
25. Buzón-Durán, L.; Martín-Gil, J.; Marcos-Robles, J.L.; Fombellida-Villafruela, Á.; Pérez-Lebeña, E.; Martín-Ramos, P. Antifungal activity of chitosan oligomers–amino acid conjugate complexes against *Fusarium culmorum* in spelt (*Triticum spelta* L.). *Agronomy* **2020**, *10*, 1427. [CrossRef]
26. Perczak, A.; Gwiazdowska, D.; Gwiazdowski, R.; Juś, K.; Marchwińska, K.; Waśkiewicz, A. The inhibitory potential of selected essential oils on *Fusarium* spp. growth and mycotoxins biosynthesis in maize seeds. *Pathogens* **2020**, *9*, 23. [CrossRef]
27. Olesen, M.; Duijn, B.v.; Boelt, B. Introduction of New Methods: Spectral Imaging. *Seed Test. Int.* **2014**, *147*, 10–13.
28. Nasrollahzadeh, M.; Sajadi, S.M.; Maham, M. *Tamarix gallica* leaf extract mediated novel route for green synthesis of CuO nanoparticles and their application for N-arylation of nitrogen-containing heterocycles under ligand-free conditions. *RSC Adv.* **2015**, *5*, 40628–40635. [CrossRef]
29. Boulaaba, M.; Tsolmon, S.; Ksouri, R.; Han, J.; Kawada, K.; Smaoui, A.; Abdelly, C.; Isoda, H. Anticancer effect of *Tamarix gallica* extracts on human colon cancer cells involves Erk1/2 and p38 action on G2/M cell cycle arrest. *Cytotechnology* **2013**, *65*, 927–936. [CrossRef]
30. Nisar, J.; Ali Shah, S.M.; Ayaz, S.; Akram, M.; Rashid, A.; Mustafa, I.; Nisar, Z. In vitro comparative evaluation of *Tamarix gallica* extracts for antioxidant and antidiabetic activity. *Exp. Biol. Med.* **2022**, 15353702221139208. [CrossRef]
31. Lefahal, M.; Makhloufi, E.-H.; Trifa, W.; Ayad, R.; El Hattab, M.; Benahmed, M.; Keskin, M.; Akkal, S. The cosmetic potential of the medicinal halophyte *Tamarix gallica* L. (Tamaricaceae) growing in the eastern part of Algeria: Photoprotective and antioxidant activities. *Comb. Chem. High Throughput Screen.* **2021**, *24*, 1671–1678. [CrossRef] [PubMed]
32. Al-Otibi, F.; Moria, G.A.; Alharbi, R.I.; Yassin, M.T.; Al-Askar, A.A. The antifungal properties of *Tamarix aphylla* extract against some plant pathogenic fungi. *Microorganisms* **2023**, *11*, 127. [CrossRef] [PubMed]
33. Takó, M.; Kerekes, E.B.; Zambrano, C.; Kotogán, A.; Papp, T.; Krisch, J.; Vágvölgyi, C. Plant phenolics and phenolic-enriched extracts as antimicrobial agents against food-contaminating microorganisms. *Antioxidants* **2020**, *9*, 165. [CrossRef] [PubMed]
34. Makarewicz, M.; Drożdż, I.; Tarko, T.; Duda-Chodak, A. The interactions between polyphenols and microorganisms, especially gut microbiota. *Antioxidants* **2021**, *10*, 188. [CrossRef]
35. Othman, L.; Sleiman, A.; Abdel-Massih, R.M. Antimicrobial activity of polyphenols and alkaloids in Middle Eastern plants. *Front. Microbiol.* **2019**, *10*, 911. [CrossRef]
36. Socolsky, C.; Salvatore, A.; Asakawa, Y.; Bardón, A. Bioactive new bitter-tasting p-hydroxystyrene glycoside and other constituents from the fern *Elaphoglossum spathulatum*. *Arkivoc* **2003**, *10*, 347–355. [CrossRef]
37. Alagammal, M.; Tresina, P.; Mohan, V. GC-MS determination of bioactive components of *Polygala javana* DC. *Int. J. Curr. Pharm. Res.* **2012**, *4*, 42–44.
38. Rabiu, Z.; Hamzah, M.A.A.M.; Hasham, R.; Zakaria, Z.A. Characterization and antiinflammatory properties of fractionated pyroligneous acid from palm kernel shell. *Environ. Sci. Pollut. Res.* **2021**, *28*, 40535–40543. [CrossRef]
39. Peng, W.; Li, D.; Zhang, M.; Ge, S.; Mo, B.; Li, S.; Ohkoshi, M. Characteristics of antibacterial molecular activities in poplar wood extractives. *Saudi J. Biol. Sci.* **2017**, *24*, 399–404. [CrossRef]
40. Kamal, K.M.; Hashim, Y.Z.H.Y.; Sani, M.S.A.; Rahim, N.A.; Maifiah, M.H.M.; Jihadi, N.I.M. Combination of polymyxin B and *Aquilaria malaccensis* extract enhanced the killing and inhibited the growth of *Acinetobacter baumannii* and *Klebsiella pneumoniae*. *Malays. J. Microbiol.* **2021**, *18*, 27–36. [CrossRef]
41. Rodrigues, M.J.; Custódio, L.; Mecha, D.; Zengin, G.; Cziáky, Z.; Sotkó, G.; Pereira, C.G. Nutritional and phyto-therapeutic value of the halophyte *Cladium mariscus* L. (Pohl.): A special focus on seeds. *Plants* **2022**, *11*, 2910. [CrossRef] [PubMed]

42. Ghareeb, M.; Saad, A.; Ahmed, W.; Refahy, L.; Nasr, S. HPLC-DAD-ESI-MS/MS characterization of bioactive secondary metabolites from *Strelitzia nicolai* leaf extracts and their antioxidant and anticancer activities in vitro. *Pharmacogn. Res.* **2018**, *10*, 368–378. [CrossRef]
43. Sanz, M.; de Simón, B.F.; Cadahía, E.; Esteruelas, E.; Muñoz, A.M.; Hernández, T.; Estrella, I.; Pinto, E. LC-DAD/ESI-MS/MS study of phenolic compounds in ash (*Fraxinus excelsior* L. and *F. americana* L.) heartwood. Effect of toasting intensity at cooperage. *J. Mass Spectrom.* **2012**, *47*, 905–918. [CrossRef] [PubMed]
44. Rencoret, J.; Ralph, J.; Marques, G.; Gutiérrez, A.; Martínez, Á.T.; del Río, J.C. Structural characterization of lignin isolated from coconut (*Cocos nucifera*) coir fibers. *J. Agric. Food. Chem.* **2013**, *61*, 2434–2445. [CrossRef]
45. Shreaz, S.; Bhatia, R.; Khan, N.; Muralidhar, S.; Manzoor, N.; Khan, L.A. Influences of cinnamic aldehydes on H^+ extrusion activity and ultrastructure of *Candida*. *J. Med. Microbiol.* **2013**, *62*, 232–240. [CrossRef]
46. Zare, B.; Sepehrizadeh, Z.; Faramarzi, M.A.; Soltany-Rezaee-Rad, M.; Rezaie, S.; Shahverdi, A.R. Antifungal activity of biogenic tellurium nanoparticles against *Candida albicans* and its effects on squalene monooxygenase gene expression. *Biotechnol. Appl. Biochem.* **2014**, *61*, 395–400. [CrossRef]
47. Suman, T.Y.; Elumalai, D.; Kaleena, P.K.; Rajasree, S.R.R. GC–MS analysis of bioactive components and synthesis of silver nanoparticle using *Ammannia baccifera* aerial extract and its larvicidal activity against malaria and filariasis vectors. *Ind. Crops Prod.* **2013**, *47*, 239–245. [CrossRef]
48. Stevenson, D.G.; Eller, F.J.; Wang, L.; Jane, J.-L.; Wang, T.; Inglett, G.E. Oil and tocopherol content and composition of pumpkin seed oil in 12 cultivars. *J. Agric. Food. Chem.* **2007**, *55*, 4005–4013. [CrossRef]
49. Wei, F.H.; Chen, F.L.; Tan, X.M. Gas chromatographic-mass spectrometric analysis of essential oil of *Jasminum officinale* L var *grandiflorum* flower. *Trop. J. Pharm. Res.* **2015**, *14*, 149–152. [CrossRef]
50. Anandan, A.; Eswaran, R.; Doss, A.; Sangeetha, G.; Anand, S. Chemical compounds investigation of *Lucas aspera* leaves-a potential folklore medicinal plant. *Asian J. Pharm. Clin. Res.* **2012**, *5*, 86–88.
51. Masuda, A.; Akiyama, S.-I.; Kuwano, M.; Ikekawa, N. Potentiation of antifungal effect of amphotericin B by squalene, an intermediate for sterol biosynthesis. *J. Antibiot.* **1982**, *35*, 230–234. [CrossRef] [PubMed]
52. Ryder, N.S. Terbinafine: Mode of action and properties of the squalene epoxidase inhibition. *Br. J. Dermatol.* **1992**, *126*, 2–7. [CrossRef] [PubMed]
53. Elewski, B.E. Mechanisms of action of systemic antifungal agents. *J. Am. Acad. Dermatol.* **1993**, *28*, S28–S34. [CrossRef]
54. Belter, A.; Skupinska, M.; Giel-Pietraszuk, M.; Grabarkiewicz, T.; Rychlewski, L.; Barciszewski, J. Squalene monooxygenase–a target for hypercholesterolemic therapy. *Biol. Chem.* **2011**, *392*, 1053–1075. [CrossRef]
55. Nowosielski, M.; Hoffmann, M.; Wyrwicz, L.S.; Stepniak, P.; Plewczynski, D.M.; Lazniewski, M.; Ginalski, K.; Rychlewski, L. Detailed mechanism of squalene epoxidase inhibition by terbinafine. *J. Chem. Inf. Model.* **2011**, *51*, 455–462. [CrossRef]
56. Wu, J.; Fu, Y.-S.; Lin, K.; Huang, X.; Chen, Y.-j.; Lai, D.; Kang, N.; Huang, L.; Weng, C.-F. A narrative review: The pharmaceutical evolution of phenolic syringaldehyde. *Biomed. Pharmacother.* **2022**, *153*, 113339. [CrossRef] [PubMed]
57. Fillat, A.; Gallardo, O.; Vidal, T.; Pastor, F.; Díaz, P.; Roncero, M. Enzymatic grafting of natural phenols to flax fibres: Development of antimicrobial properties. *Carbohydr. Polym.* **2012**, *87*, 146–152. [CrossRef]
58. Musthafa, S.A.; Dabdoub, W.; Sadiq, M.; Munuswamy-Ramanujam, G. Syringaldehyde isolated from *Abutilon indicum* Linn. leaves exhibits broad spectrum anti-microbial activity. *Mater. Today Proc.* **2022**, *50*, 335–339. [CrossRef]
59. Bibi, S.; Afzal, M.; Aziz, N.; Din, B.; Khan, M.; Khan, A.; Komal, H. Antifungal activity of *Tamarix aphylla* (L.) Karst. stem-bark extract against some pathogenic fungi. *Am. Eurasian J. Agric. Environ. Sci.* **2015**, *15*, 541–545.
60. Iqbal, A.; Begum, N.; Rabbi, F.; Akhtar, N.; Khan, W.M.; Shah, Z. In-vitro antimicrobial, antioxidant and enzyme inhibitory activities of fixed oil extracted from stem bark of *Tamarix aphylla*. *Pharm. Chem. J.* **2022**, *56*, 1116–1122. [CrossRef]
61. Bergsson, G.; Arnfinnsson, J.; Steingrímsson, O.l.; Thormar, H. In vitro killing of *Candida albicans* by fatty acids and monoglycerides. *Antimicrob. Agents Chemother.* **2001**, *45*, 3209–3212. [CrossRef]
62. Ren, X.; Bao, Y.; Zhu, Y.; Liu, S.; Peng, Z.; Zhang, Y.; Zhou, G. Isorhamnetin, hispidulin, and cirsimaritin identified in *Tamarix ramosissima* barks from southern Xinjiang and their antioxidant and antimicrobial activities. *Molecules* **2019**, *24*, 390. [CrossRef]
63. Mikaeili, A.; Karimi, I.; Modarresi, M.; Shahbazi, A.; Jalilian, N. Antimycotic activity of aqueous extract of *Tamarix ramosissima* Ledeb. bark on in vitro and in vivo guinea pig model of dermatophytosis. *Int. J. Life Sci. Pharma Res.* **2018**, *8*, 8–15.
64. Paul, P.A.; McMullen, M.P.; Hershman, D.E.; Madden, L.V. Meta-analysis of the effects of triazole-based fungicides on wheat yield and test weight as influenced by Fusarium head blight intensity. *Phytopathology* **2010**, *100*, 160–171. [CrossRef] [PubMed]
65. Cendoya, E.; Nichea, M.J.; Monge, M.d.P.; Zachetti, V.G.L.; Chiacchiera, S.M.; Ramirez, M.L. Effect of fungicides commonly used for Fusarium head blight management on growth and fumonisin production by *Fusarium proliferatum*. *Rev. Argent. Microbiol.* **2021**, *53*, 64–74. [CrossRef] [PubMed]
66. Tokarev, Y.; Orina, A.; Gavrilova, O.; Gagkaeva, T.; Glupov, V. The effect of fungicides on growth of *Fusarium* fungi in vitro. *BIO Web Conf.* **2020**, *18*, 00022. [CrossRef]
67. Marín, P.; de Ory, A.; Cruz, A.; Magan, N.; González-Jaén, M.T. Potential effects of environmental conditions on the efficiency of the antifungal tebuconazole controlling *Fusarium verticillioides* and *Fusarium proliferatum* growth rate and fumonisin biosynthesis. *Int. J. Food Microbiol.* **2013**, *165*, 251–258. [CrossRef] [PubMed]

68. Mateo, E.M.; Valle-Algarra, F.M.; Mateo, R.; Jiménez, M.; Magan, N. Effect of fenpropimorph, prochloraz and tebuconazole on growth and production of T-2 and HT-2 toxins by *Fusarium langsethiae* in oat-based medium. *Int. J. Food Microbiol.* **2011**, *151*, 289–298. [CrossRef]
69. Kulik, T.; Lojko, M.; Jestoi, M.; Perkowski, J. Sublethal concentrations of azoles induce tri transcript levels and trichothecene production in *Fusarium graminearum*. *FEMS Microbiol. Lett.* **2012**, *335*, 58–67. [CrossRef]

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Review

Potential Role and Involvement of Antioxidants and Other Secondary Metabolites of Wheat in the Infection Process and Resistance to *Fusarium* spp.

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Abstract: This article provides a summary of current knowledge about wheat metabolites that may affect resistance against Fusarium head blight (FHB). The mechanisms of resistance, the roles of secondary metabolites in wheat defense, and future directions for breeding are assessed. The soluble phenols play an important role in redox regulation in plant tissues and can act as antimicrobial compounds. The color of cereal hulls and grains is caused by such natural pigments as anthocyanins in the aleurone, endosperm, and pericarp layers of the grain. Phenolic acids, alkylresorcinols, and phytohormones actively participate in the defense system, whereas carotenoids show various effects against *Fusarium* species that are positively correlated with the levels of their mycotoxins. Pathogen infestation of vegetative tissues induces volatile organic compounds production, which can provide defensive functions to infested wheat. The efficient use of native resistance in the wheat gene pool, introgression of resistant alleles, and implementation of modern genotypic strategies to increase levels of native secondary metabolites with antifungal properties can enhance the FHB resistance of new varieties. Expanding the breeding interest in the use of forms with different grain color and plant organs can be a potential benefit for the creation of lines with increased resistance to various stresses.

Keywords: wheat; breeding; plant defense; Fusarium head blight; genetics and resistance; efficient wheat metabolites and antioxidants

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1. Introduction

Fusarium head blight (FHB) and Gibberella ear rot, mainly caused by *Fusarium graminearum* Schwabe and *Fusarium culmorum* (W.G. Smith) Sacc., are two of the most devastating diseases of small grain cereals and corn [1]. These fungi substantially reduce grain yield and affect grain quality. Mycotoxin contamination of human food and animal feed has become a more important aspect than direct yield losses in affecting the economics of small grain production [2–4]. Many mycotoxins are produced in culture, but the most important are trichothecenes (which include deoxynivalenol [DON], also known as vomitoxin, nivalenol [NIV], HT-2 and T-2 toxins), zearalenone (ZEA), and fumonisins [5]. Consumption of grains containing trichothecenes may cause intestinal irritation in mammals, feed refusal in livestock, vomiting, skin dermatitis, and immunological problems [6]. *Fusarium culmorum*, *F. graminearum*, and *F. pseudograminearum* (O'Donnell and T. Aoki; group I) (=*Gibberella coronicola*) are the most devastating fungal pathogens on small grain cereals [7]. *F. graminearum* sensu lato is today the most frequently isolated causal agent of FHB worldwide [8,9]. The *F. graminearum* species complex (FGSC) includes 16 phylogenetic species: *F. graminearum*, *F. asiaticum* (O'Donnell et al.), *F. austroamericanum* (T. Aoki et al.), *F. brasilicum* (T. Aoki et al.), *F. cortaderiae* (O'Donnell et al.), *F. meridionale* (T. Aoki et al.), *F. boothii* (O'Donnell et al.), *F. mesoamericanum* (T. Aoki et al.), *F. acaciae-mearnsii* (O'Donnell et al.), *F. pseudograminearum*, *F.*

gerlachii (T. Aoki et al.), *F. vorosii* (B. Tóth et al.), *F. aethiopicum* (O'Donnell et al.), *F. nepalense* (T. Aoki et al.), *F. louisianense* (Gale et al.), and *F. ussurianum* (T. Aoki et al.) [10].

Among the several means of fighting this disease includes the use of fungicides, cultural practices, resistant cultivars, and biological agents [11]. As true of most plant diseases, host resistance is recommended as the most effective and economical method of management [12,13]. Fully resistant cultivars are not available to date, but some cultivars have usable levels of partial resistance that limit yield loss and mycotoxins accumulation [14].

This article discusses the main secondary metabolites and antioxidants of wheat that can contribute to resistance in wheat species and cultivars. Although they are contained in relatively small amounts in cereals, they can work together to enhance antioxidant efficiency and resistance to *Fusarium*.

Current information is presented regarding the contents and effects of these metabolites and antioxidants in relation to wheat species' and genotypes' resistance to FGSC.

2. Infection Process

Plants are constantly subjected to biological pressures that could compromise their development. Plant and pathogen interests are opposed and antagonistic, thus generating an evolutionary dynamic between the two. This is stimulated by the confrontation between the plant's resistance and the ability of the pathogen to generate infection of the plant. Significant progress has been made in recent years toward better understanding the processes involved in FHB infection, and especially in the case of *F. graminearum* [15–17]. *F. graminearum's* infection process includes a biotrophic phase, occurring within six hours post inoculation (hpi). The pathogen then shifts to a necrotrophic phase between 24 and 72 hpi via production of trichothecenes and cell wall-degrading enzymes [18]. *Fusarium* spp. are able to penetrate and invade a host with the help of secreted cell wall-degrading enzymes, thus enabling the pathogen to infect, penetrate, and grow through the wheat tissue. Among cell wall-degrading enzymes are important pectinases, xylanases, cellulases, feruloyl esterases, proteases, endo-peptidases, and lipases [19]. The glycogen synthase kinase gene (*FGK3*) in *F. graminearum* is known to be an important virulence factor for this pathogen [20].

The cell wall-degrading enzymes produced by *F. culmorum* and *F. graminearum* facilitate rapid colonization of wheat spikes [21]. Lipases are important for phytotoxicity of *F. graminearum* [22]. *F. verticillioides* lactamases constitute another group of enzymes in wheat, rye, and corn get part in the resistance process of fungi to antimicrobial environment [23]. Important for these enzymes to be active and function is the presence of encoding genes, such as the lactamase encoding gene *FVEG_08291* in *F. verticillioides* [23] that imparts resistance against lactams with benzoxazinoid rings produced by wheat, corn, and rye [24]. It is noteworthy that *Fusarium* spp. possess more than 40 lactamase encoding genes [23].

Infection with *Fusarium* species can result in the contamination of cereals with health-threatening mycotoxins. These are mainly type A and type B trichothecenes, such as T-2 and HT-2, or nivalenol (NIV) and deoxynivalenol (DON). *Fusarium* mycotoxins include also other toxic secondary metabolites, such as fusaproliferin, moniliformin, and enniatins [25]. Another minor *Fusarium* mycotoxin on wheat is beauvericin, which, in addition to its toxic activity in higher animals, possesses insecticidal, antifungal, and antibacterial activity [25]. Mycotoxins play an important role in the infection process. It has been found that toxin-producing ability correlates positively with the level of a pathogen's aggressiveness [26]. DON kills the host cells by disrupting the cell membrane, thus causing cellular electrolyte leakage and an increase in cytoplasmic Ca^{2+} ions that leads to imbalance in cellular homeostasis [27,28]. Increased production of such mycotoxins as DON and the emerging mycotoxin culmorin (CUL) having synergistic toxic effects resulting in increased pathogen aggressiveness and enhanced host colonization [29]. Lu and Edwards [30] revealed small, secreted cysteine-rich proteins as a common source of *F. graminearum*–wheat interaction effectors involved in triggering resistance or susceptibility between wheat and *Fusarium*. In a recent study by Fabre et al. [31] examining the aggressiveness of three

F. graminearum strains, the findings show that contrasts were based not upon the existence of strain-specific molecules, but rather upon the ability of a strain to accumulate sufficient effector protein abundance. Protein abundance variance was mostly driven by the strain genetics and part was also influenced by the host cultivar; however, strains by cultivar interactions were marginally detected, depicting that strain-specific protein accumulations did not depend on the host cultivar.

3. Plant Defense

3.1. Mechanisms of Resistance

Cultivar resistance is an important factor that may significantly affect infection of plants, and cultivated genotypes may have different mechanisms of resistance. Wheat's resistance against FHB includes many resistance mechanisms [32]. In the case of *Fusarium* infection, this includes the following components: type I, resistance to initial infection; type II, resistance to spread of symptoms [33]. After types I and II resistance, there also exists a type III resistance to toxin accumulation [34,35]. Mesterházy [36,37] distinguished the following components (types) of head blight resistance: I. resistance to invasion; II. resistance to spreading; III. resistance to kernel infection; IV. Tolerance; V. resistance to toxin accumulation; VI. resistance to late blighting; and VII. resistance to head death above infection site. All these types of resistance are interdependent, but they are presumably based upon different mechanisms and inherited independently.

There are two types of plant protection against infection, active and passive.

3.1.1. Active Resistance

The interaction of *F. graminearum* with small grain cereals has been studied in various cellular, molecular, and biochemical areas. Plant defenses are based on both physical barriers, such as the cell wall and its modifications, as well as chemical defense mechanisms that are induced in response to external stimuli [38–40].

After recognizing the pathogen, the host plants' basal defense responses lead to activation of several resistance mechanisms. These include production of reactive oxygen species (ROS), enzymatic and non-enzymatic antioxidants [41], cell wall reinforcement associated with phenylpropanoid metabolism [32], and callose deposition [42]. ROS accumulation and removal are controlled in plant–pathogen interactions by enzymatic and non-enzymatic antioxidants. Such enzymatic antioxidants as peroxidase (POX) and catalase (CAT) are involved in scavenging H_2O_2, whereas superoxide dismutase is a scavenger of O_2^- and changes this molecule to H_2O_2 in living cells [43]. The soluble phenols play a significant function in redox regulation in plants and can have an effect as antimicrobial compounds. In addition to ROS, there are several types of reactive nitrogen species (RNS), including nitric oxide (NO). In particular, this signaling molecule might be involved in defense reactions mediated by ROS, such as production of phytoalexins and polyamines, transcription activation, or cell wall reinforcement [43]. Recently, Khaledi et al. [44] found that NO production increased in ears and seedlings of wheat varieties after inoculation with *F. graminearum*, and a greater increase was characteristic of the more resistant variety compared to the susceptible one. Therefore, NO might be involved in wheat defense responses to the pathogenic *Fusarium* species and the relationship between ROS and RNS should be investigated in more detail.

ROS accumulation and programmed cell death as its consequence would be helpful defense strategies leading to reduced progress of the hemibiotrophic *F. graminearum* in the host tissues and increased resistance at the early time points after inoculation, when this pathogen is in its biotrophic phase [43]. Wheat plants' secondary metabolites can play an important active role in their resistance against *Fusarium* spp. A wide range of secondary metabolites with both antioxidant and pro-oxidant properties (depending upon their concentrations), such as phenolic compounds, carotenoids, and linoleic acid-derived hydroperoxides, are synthesized and act as modulators of mycotoxin biosynthesis [26,45].

In addition to the induction of phenolics and phytoalexins, active plant defense also involves expression of pathogenesis-related (PR) proteins. When stimulated by various pathogens or conditions that mimic the effects of pathogen infection, the host is thought to inhibit growth, multiplication, and/or spread of the invading pathogen by synthesizing PR proteins [40,46]. PR proteins are presently grouped into 17 families based upon their protein sequence similarities, enzymatic activities, and biological functions [47,48].

Carotenoid and tocopherol effects on FHB and trichothecene accumulation are less investigated [49]. Boba et al. [50] were able in their study to decrease carotene content through the suppression of a lycopene β-cyclase gene. The suppression of this gene in transgenic flax then led to an increase in tocopherols, squalene, gibberellic acid, and menthol. An increase in *Fusarium* resistance was driven by these changes in the transgenics.

Table 1 reports determined contents of endogenous wheat phytochemicals and metabolites potentially involved in protection against oxidative stress caused by *Fusarium* spp. in wheat. Anthocyanins in wheat are based upon six aglycons–anthocyanidins, which differ only in their glycosylation patterns in attached sugar moieties and/or their esterification with phenolic acids [51]. This may be due to the antioxidant activity of anthocyanins, which is known to increase plant resistance [52].

Table 1. Contents of bioactive antioxidant natural metabolites and phytochemicals in wheat (mg kg^{-1} dry matter [DM]).

Compound	Content	Reference
Total 5-n-alkylresorcinols	761 bread wheat, 743 spelt, 654 durum, 697 emmer, 737 einkorn 300–943 common wheat, 194–687 durum wheat, 545–654 einkorn wheat, 531–784 emmer wheat, 490–741 spelt wheat	[53,54]
5-n-Heptadecylresorcinol (C17:0)	32–34 common wheat 1.2 (*T. turgidum* ssp. *dicoccum*) 26.0 (*T. turgidum* ssp. *turgidum*)	[55,56]
5-n-Nonadecylresorcinol (C19:0)	250–272 common wheat 20.4 (*T. turgidum* ssp. *dicoccum*) 187.9 (*T. aestivum*)	[55,56]
5-n-Heneicosylresorcinol (C21:0)	368–474 common wheat 196.5 (*T. turgidum* ssp. *dicoccum*) 653.1 (*T. aestivum*) 164.4 (*T. turgidum* var. *durum*) 65.4 (*T. aestivum*)	[55,56]
5-n-Tricosylresorcinol (C23:0)	84–108 common wheat	[55]
5-n-Pentacosylresorcinol (C25:0)	26–33 common wheat	[55]
Total anthocyanins	210 Pp grain; 430 Pp bran 21–157 Ba, R, 78 Pp	[51,57]
Cyanidin-3-glucoside	Ba 3.07, Pp 10.34, R 4.02	
Cyanidin-3-rutinoside	8.42 Ba, Pp	
Delphinidin-3-glucoside	13.68 Ba	
Delphinidin-3-rutinoside	33.44 Ba	
Malvidin-3-glucoside	12.04 Ba, 0.48 Pp, 0.22 R	
Peonidin-3-arabinoside	2.22 Ba, Pp	
Peonidin-3-glucoside	0.88 Pp	
Peonidin-3-galactoside	1.94 Ba, 0.58 Pp, 0.33 R	
DIMBOA-glucoside	18 common wheat	[58]
Total carotenoids	1.63–4.19 einkorn, 4.73–13.64 emmer, 2.69–8.38 durum, 1.62–2.98 spelt, 1.40–4.90 bread wheat 5.47 mg β-carotene kg^{-1} DM (*T. turgidum* var. *durum*) 3.3 < 1.4–6.6 > wheat grains 3.2 < 1.6–4.7 > white wheat grains 3.1 < 1.4–4.1 > red wheat grains 6.0 < 4.7–6.6 > black wheat grains	[59–61]

Table 1. *Cont.*

Compound	Content	Reference
α-Carotene	7.3–13.4 *T. monococcum*	[62]
β-Carotene	0.116 spring wheat, 0.195 einkorn	
Zeaxanthin	0.144 spring wheat, 0.351 einkorn, 0.138 emmer wheat	
Lutein	1.096 spring wheat, 5.246 einkorn, 0.761 emmer wheat	
Total phenolics	1499; 1545.7 mg FAE kg^{-1} DM 559.1, 506.5–659.8 mg GAE kg^{-1} 1265.7 < 837.0–2233.7 > wheat grains 1231.7 < 837.0–1759.0 > white grains 1401.8 < 1105.8–1850.9 > red grains 1546.4 < 1122.8–2233.7 > black grains	[60,61,63]
Total flavonoids	270.0, 236.2–319.3 mg RE kg^{-1} DM 252 < 147–397 > winter wheat grains 241 < 147–351 > white grains 290 < 218–389 > red grains 361 < 321–397 > black grains	[61,63]
Apigenin	2.512 control, 104.565 inoculated with *F. culmorum*	[64]
Kaempferol	6.009 control, 124.739 inoculated with *F. culmorum*	
Luteolin	7.117 control, 458.404 inoculated with *F. culmorum*	
Naringenin	7.115 control, 127.787 inoculated with *F. culmorum*	
Quercetin	6.958 control, 512.934 inoculated with *F. culmorum*	
Rutin	13.764 control, 332.44 inoculated with *F. culmorum*	
Vitexin	6.481 control, 148.256 inoculated with *F. culmorum*	
Total phenolic acids	987.3; 4061.4 mg kg^{-1} DM	[60]
Salicylic acid	< 0.3–0.8 > free salicylic acid in leaves	[65]
Protocatechuic acid	< 6.8–13.3 > bran; 9.2	[66]
Ferulic acid	270–1446; 3000 bran 194.18 grain at 10 days post-anthesis flower tissues (S) 69.9; (MR) 99.0; (R) 101; developing grains 10 days post-anthesis (S) 97.1, (MR) 122.3, (R) 126.2, 130.1–233 developing grains	[67,68]
4-Hydroxybenzoic acid	87.3 control, 87.3 infected	[67]
Gallic acid	< 1–37 >; control 57, infected 77,3	
Vanillic acid	< 30–70 >; control 26.7, infected 37.0	
Syringic acid	< 1–62 >; control 30.7, infected 23	
t-Cinnamic acid	< 3–83 >; control 127.0, infected 343.3	
p-Coumaric acid	< 1–63 >; bran 90; control 45.7, infected 44.0	
Caffeic acid	< 2–90 >; bran 38; control 40, infected 46.7	
Sinapic acid	< 2–2017 >; bran 200; control 136.0, infected 360.0	
Chlorogenic acid	< 10–69 >; control 38.0, infected 39.0	
Abscisic acid	Increase from 86 to 154 ng g^{-1} DW after inoculation with *F. graminearum*	[69]
Indol-3-acetic acid	Increase from 83 to 26 328 ng g^{-1} DW after inoculation with *F. graminearum*	
Jasmonic acid	Increase from 29 to 410 ng g^{-1} FW after inoculation with *F. graminearum*	
(-)-β-Caryophyllene	Increase from 9 to 104 ng $sample^{-1}$ after inoculation with *F. graminearum*	[70]
β-Linalool	Increase from 12 to 405 ng $sample^{-1}$ after inoculation with *F. graminearum*	

Table 1. *Cont.*

Compound	Content	Reference
(-)-Thujopsene	Increase from 0.005 to 0.018 ratio unit after inoculation with *F. culmorum*	[71]
Trichodiene	Increase from 0.009 to 0.027 ratio unit after inoculation with *F. culmorum*	
(-)-β-Chamigrene	Increase from 0.003 to 0.012 ratio unit after inoculation with *F. culmorum*	
(Z)-hex-3-enal	Increase from 14 to 139 ng sample^{-1} after inoculation with *F. graminearum*	[70]
(E)-hex-2-enal	Increase from 1 to 709 ng sample^{-1} after inoculation with *F. graminearum*	
(E)-hex-2-en-1-ol	Increase from 9 to 881 ng sample^{-1} after inoculation with *F. graminearum*	
(Z)-hex-3-en-1-yl acetate	Increase from 22 to 218 ng sample^{-1} after inoculation with *F. graminearum*	
Hex-1-en-1-yl acetate	Increase from 3 to 477 ng sample^{-1} after inoculation with *F. graminearum*	

Pp: purple pericarp; Ba: blue alerone, R: red grain colour; FAE: ferulic acid equivalent; GAE: gallic acid equivalent; RE: retinol equivalent; MR: moderately resistant; R: resistant; S: susceptible; DW: dry weight; DM: dry matter; FW: fresh weigh.

3.1.2. Passive Resistance

Plant morphology can play an important role during infection and provide a passive way of resistance or susceptibility to FHB [72–74]. In general, Steiner et al. [72] and Jones et al. [73] confirm that earlier flowering varieties and taller plant height show greater resistance compared to later flowering and shorter varieties [75–77]. Positive correlations between spike compactness and FHB severity have recently been reported from a study by Giancaspro et al. [76]. Other traits associated with FHB include heading time, degree of anther extrusion, and presence or absence of awns. The extent of anther retention after flowering and FHB severity were shown to be positively correlated with the semi-dwarfing allele *Rht-D1b* [78].

4. Secondary Metabolites

Many naturally occurring secondary metabolites in plants are involved in resistance mechanisms against FHB. The majority of these are phenolic compounds with antioxidant properties. Significantly contained in wheat are phenolic acids (in free, conjugated, and bound forms) [79], flavonoids [80,81], alkylresorcinols [82], benzoxazinoids [83], phytohormones [84], and volatile organic compounds [85].

4.1. Phenolic Compounds/Antioxidants

Constituting a broad spectrum of genetic plant defense mechanisms against pathogens, the accumulation of phenolic compounds has been shown to inhibit in vitro growth and reproduction across a wide array of fungal genera and can help in reducing *Fusarium* trichothecene mycotoxin accumulation in cereal grains [86]. Phenolic compounds are secondary metabolites produced by the phenylpropanoid pathway and are synthesized by plants from the amino acid phenylalanine [87]. Plant biosynthesis produces various phenols that are commonly grouped as phenolic acids and flavonoids.

4.1.1. Phenolic Acids

Phenolic acids are predominant in cereal grain extracts and are derivatives of either cinnamic acid or benzoic acid (Figure 1). In wheat, they include (in descending quantity) ferulic, sinapic, 4-hydroxybenzoic, vanillic, and caffeic acids [67]. Their contents in common wheat are substantially greater as compared with durum wheat (Table 1). This corresponds

to the facts that durum wheat (*Triticum turgidum* sp. *durum*) is notable for its extreme susceptibility to FHB and that sources of FHB resistance are rare in the gene pool of the tetraploid wheat [77]. Indeed, Stuper-Szablewska and Perkowski [67] found in durum wheat only ferulic, *p*-coumaric, and syringic acids, whereas common wheat contained in addition gallic, 4-hydroxybenzoic, vanillic, chlorogenic, caffeic, and sinapic acids. Phenolic acids can be ranked as follows in ascending order of toxicity toward *F. graminearum*: chlorogenic acid < *p*-hydroxybenzoic acid < caffeic acid < syringic acid < *p*-coumaric acid < ferulic acid [80]. Martin [68] found a weak but significant effect of ferulic acid (FA) on resistance against *Fusarium* according to FA levels in grains but suggests that FA levels in grains are generally low (Table 1). Across all genotypes, however, the FA content increased significantly from 97.1 mg kg^{-1} in flowering tissues to 120.4 mg kg^{-1} 10 days after anthesis. The effectiveness of phenolic acids against *Fusarium* spp. could be related to their antioxidant activity, which Verma et al. [66] measured in six wheat cultivars. In their study, high antioxidant activity as determined by ABTS test (using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was proven for *trans*-ferulic acid, syringic acid, sinapic acid, and caffeic acid, all of which were obtained by acid hydrolysis. Among the alkaline hydrolyzed phenolic acids, higher antioxidant activity was shown for ferulic acid, *p*-coumaric acid, sinapic acid, and vanillic acid, respectively. Generally, the total content of all phenolic acids may be an important factor in their protective effect against *Fusarium* spp.

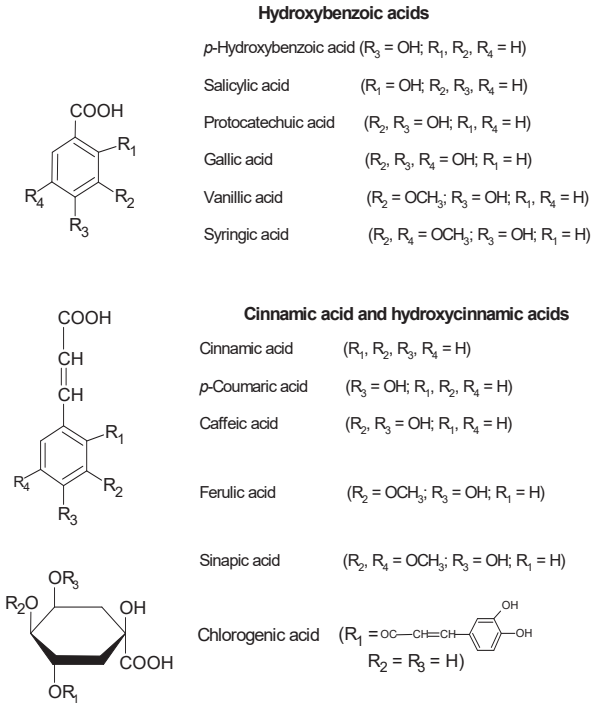


Figure 1. Structures of phenolic acids in wheat.

Phenolic acids found in cereals exist in both soluble (free) and insoluble (cell wall-bound) forms [88]. For free phenolic acids, 80% ethanol [66,89], methanol/water (80:20, *v*/*v*) [88,90], or methanol-water (70:30, *v*/*v*) acidified with 0.1% formic acid is generally used [91]. In addition, different ratios methanol/water are used (7:3, 1:1, *v*/*v*) [56,92], but also 95% ethanol or dimethyl sulfoxide has been used [93]. Bound or conjugated phenolic acids should be released by alkaline (with 2 M NaOH) and acidic hydrolysis (with 6 M HCl) [56,66,88]. Martini et al. [60] used, for extraction of phenolic acids, ethanol/water

(80:20, *v*/*v*) and ethyl acetate, alkaline hydrolysis after initial ethanol/water extraction with 2 m sodium hydroxide, and successive extraction with ethyl acetate.

The major part of phenolic compounds is in the germ and bran tissues of grains. Moreover, phenolic acids, and most predominantly ferulic and *p*-coumaric acids, have an important role in limiting polysaccharide degradation by exogenous enzymes, where they act as a cross-link between polysaccharides and between polysaccharides and lignin [89]. Phenolic compounds in plants are involved in the pathogen and plant interaction. In wheat (winter and spring cultivars), significantly greater amounts of free phenolic compounds were detected in the glumes, lemmas, and paleas of the spring cultivars prior to and at all sampling times after inoculation compared to the winter wheat cultivars [94]. The spring cultivars show resistance against initial infection by the fungus [94]. Inasmuch as *p*-coumaric acid increases significantly in the glumes, lemmas, and paleas of the spring cultivars, it appears that phenolic compounds play a role in cultivars' resistance to *F. culmorum*. In cereals, cell wall-bound ferulic acid (FA), along with its dehydrodimers, as well as free chlorogenic acid and its hydrolyzed product caffeic acid, could be key components of resistance to toxigenic *Fusarium* species [95]. In a later study by Schöneberg et al. [93], FA had a significant influence on the growth of *Fusarium* species (*F. poae*, *F. graminearum*, and *F. langsethiae*) in comparison with the control treatment ($p < 0.001$). In their study, the black oat varieties Gailette and Zorro differed significantly and had as much as two times higher levels of FA compared with the yellow or white varieties Canyon and Husky. Inoculation with *F. langsethiae* caused reduction relative to the controls (by as much as 96–100% for FA and 97–100% for *p*-hydroxybenzoic acid). The reduction was complete after *F. langsethiae* inoculation in the case of vanillic acid across all examined varieties. Despite the overall reduction, the black oat varieties had significantly higher FA levels in comparison with white and yellow-hulled varieties. FA had a significant effect on the growth of all *Fusarium* species. In the case of *F. graminearum*, increasing FA concentration significantly decreased fungal growth relative to the control treatment, the reduction ranging from 3% (at 100 μg FA g^{-1}, $p > 0.05$) to 64% (at 1000 FA μg g^{-1}, $p < 0.001$) and to 88% (at 5000 μg FA g^{-1}, $p < 0.001$) [93]. The same trend in reduction was observed for *F. langsethiae*. In contrast to FA, however, *F. poae* and *F. graminearum* exhibited an increase in growth when exposed to *p*-hydroxybenzoic acid and vanillic acid, whereas *F. langsethiae* showed mostly a decrease in growth. FA and *p*-hydroxybenzoic acid showed no significant effect on any mycotoxin, whereas quercetin had a minor but significant decreasing effect on neosolaniol and diacetoxyscirpenol. Giordano et al. [96] observed a significant negative correlation between DON contamination in corn (*Zea mays* L.) at harvest maturity and free phenolic acids and total antioxidant activity at the beginning of kernel development, whereas no significant correlation was observed with fumonisin contamination. Ferulic, *p*-coumaric, and caffeic acids were the main cell wall-bound phenolic acids during kernel development, whereas chlorogenic acid was the main free phenolic acid. In a study of fungal biotransformation of chlorogenic acid with *F. graminearum*, Gauthier et al. [95] demonstrated that *F. graminearum* possesses the ability to degrade chlorogenic acid into caffeic, hydroxychlorogenic, and protocatechuic acids, as well as caffeic acid into protocatechuic and hydroxycaffeic acids. Some of these metabolic products can contribute to the inhibitory efficiency of chlorogenic acid, thereby corroborating the contribution of chlorogenic acid to the chemical defense that cereals employ to counteract *F. graminearum* and its production of mycotoxins. Among the phenolic acids, derivatives of cinnamic acid, such as caffeic, ferulic, and *p*-coumaric acids, are most recognized as contributors to FHB resistance [91,95]. Cinnamic acid derivatives also have strong antioxidant properties, which constitute an important primary factor for the ability of phenolic acids to modulate mycotoxin production [95]. Similarly, in corn, the most efficient resistance factors were shown to be pericarp propanoids, mainly *trans*-ferulic acid, *cis*-ferulic acid, *p*-coumaric acid, and diferulates [97].

Another phenolic acid, salicylic acid, plays a significant role in plant immunity as a signaling molecule in eliciting resistance and increases its activity during the early phase of infection by *F. graminearum* [98,99]. In a recent study, Rocheleau et al. [99] reported that

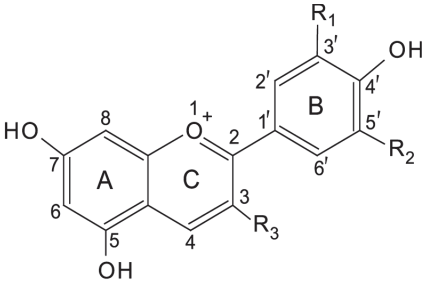
F. graminearum could utilize salicylic acid as a sole source of carbon to grow. Salicylate 1-monooxygenase and catechol 1,2-dioxygenase are two of the first key enzyme steps for salicylate degradation via catechol in the β-oxoadipate pathway. There also exists, however, a nonoxidative decarboxylation pathway of salicylic acid conversion to catechol via 2,3-dihydroxybenzoic acid [99].

Deoxynivalenol (DON) accumulation is enhanced by peroxide stress [92], and the inhibition of its production by phenolic acids is consistent with their ability to scavenge reactive oxygen radicals [91]. Nevertheless, there exist differences between strains of *F. graminearum* and *F. culmorum* carrying the DON chemotype, where enhancement of deoxynivalenol and acetyldeoxynivalenol production was recorded, and those strains carrying the nivalenol chemotype, wherein the same treatment yielded a 2.4- to 7-fold decrease in nivalenol and fusarenone accumulation [100,101]. There should be further investigation of interactions between different phenolic compounds that frequently co-occur in cereal grains [93].

4.1.2. Anthocyanins and Flavonoids

Flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, coumarins, stilbenes, and lignans are the main flavonoids and have various functions, including pigmentation and resistance to pathogens in plants [102,103]. The abundance and composition of these compounds in cereal grains contribute to either constitutive or induced synthesis and are highly variable depending upon the species, cultivar, and environmental conditions (Figure 2). Many phenolic compounds are bound to the cell wall, indicating that they are parts of the preformed general defense system against potential pathogens [104]. The specific biochemical pathways and mechanisms of their antifungal activity are not yet fully understood, however. Flavonoids comprise another group of compounds with antioxidant activity and have been identified from many plants. Some of them suppress trichothecene biosynthesis [49,52,93]. Anthocyanins have a protective role under conditions of extreme temperature, drought, and/or salinity, as they prevent lipid oxidation and protect the plasma membrane from damage [105]. Because infection of plants by various pathogens is accompanied by oxidative stress, the presence of anthocyanins in a plant may have a positive effect on the resistance to abiotic stress [106].

Anthocyanins in a plant are extracted with acidified methanol with HCl-methanol/hydrochlorid acid (85:15, *v*/*v*) [68,107], flavonoid aglycones [90] with 96% ethanol [9], for flavonoid glycosides methanol and acetonitrile are eluents, followed by alkaline hydrolysis (water/2 M NaOH, 1:4, *v*/*v*) [9,64] and acid hydrolysis (6 M HCl) and diethyl ether. Additionally, dimethyl sulfoxide (DMSO, 0.5–100 μM) can be used [102]. Several anthocyanins were identified by NMR spectroscopy and mass spectrometry after sequential extraction of blue bread wheat 'UC66049' with solvents of various polarities and multiple chromatographic fractionations [108].



Aglycone	R_1	R_2	R_3
Delphinidin	OH	OH	OH
Cyanidin	OH	H	OH
Petunidin	OCH_3	OH	OH
Peonidin	OCH_3	H	OH
Malvidin	OCH_3	OCH_3	OH
Pelargonidin	H	H	OH

Figure 2. Structure of anthocyanins identified in wheat grains.

Antifungal activity of grain-endogenous flavonoids has recently been reported in barley and wheat cultivars resistant to FHB [109]. The dihydroquercetin-accumulating barley mutant ant 18–159 inhibits hyphal penetration by F. *graminearum* and *F. culmorum* into the grain testa. This resistance has been attributed to the mutant's excessive production of dihydroquercetin due to a specific mutation in the gene encoding for dihydroflavonol reductase [93,110]. Furthermore, wheat varieties with dark and purple-colored grains have been shown to contain large amounts of FA and vanillic acid [93,111]. It is nevertheless likely that the fungus metabolized an extensive amount of the endogenous phenolic compounds, thereby leading to the observed differences between control and inoculated treatments. This was reported also by Bilska et al. [9]. In their study, trichothecene accumulation by *F. culmorum* and *F. graminearum* was significantly reduced by quercetin, kaempferol, luteolin, apigenin, and naringenin, although this effect was dependent upon the fungal strain and flavonoid levels (Figure 3). Nevertheless, correlation between antiradical and antioxidant properties of flavonoids and their effect on *Fusaria* was established. Antioxidant properties of flavonoids such as quercetin can interfere with mycotoxin production, but FA is considered the most potent phenolic acid with antifungal activity against *Fusarium* species [80]. It is clear that the antioxidant properties of phenolic acids and flavonoids apply here. In fact, the most consistent overall inhibition of mycelial growth was observed with FA treatments. Nevertheless, impacts of naringenin, apigenin, kaempferol, and quercetin at concentrations 400 mg kg^{-1} and 800 mg kg^{-1} in inhibiting mycotoxin accumulation were also reported [9]. The flavanone naringenin with a single bond between C2 and C3 was more efficient than apigenin having a double bond between these two carbon atoms. In a study by Bollina and Kushalappa [112], naringenin at LD_{50} concentration 1.58 mM and quercetin at LD_{50} concentration 2.95 mM totally inhibited biosynthesis of DON and 3-acetyldeoxynivalenol in barley, as similarly did *p*-coumaric, sinapic, ferulic, and caffeic acids, respectively, at LD_{50} concentrations 1.15, 1.74, 1.76, and 2.50 mM. Levels of apigenin, kaempferol, luteolin, naringenin, quercetin, rutin, and vitexin were significantly increased in winter wheat after inoculation with *Fusarium culmorum* [64] (Table 1). Gunnaiah and Kushalappa [113] found that in resistant wheat cultivars, accumulation of such phenylpropanoids as syringyl-rich monolignols and their glucosides reduced pathogen colonization and increased wheat cell wall thickening. They considered phenylpropanoid pathway genes responsible for

flavonoid biosynthesis to have enhanced host resistance mechanisms and reduced pathogen growth due to the antifungal and antioxidant properties of biosynthesized flavonoids and lignols [113] (Figure 3).

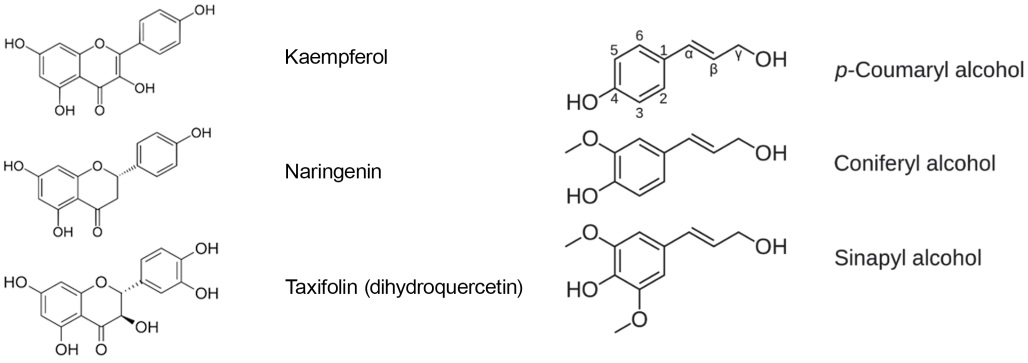


Figure 3. Structure of (**a**) aglycones of flavonoids and (**b**) monolignols determined in wheat grains.

Lignans have been discovered in different parts of plants, including seeds [114]. These are vascular plant secondary metabolites, which are attributed for a wide range of physiological functions and beneficial properties [115]. Pathogen attack may accelerate the rate of lignin and lignans synthesis and deposition, which results in an inhibition of pathogen growth and its confinement [116].

4.1.3. Alkylresorcinols

Alkylresorcinols (AR), also known as resorcinolic lipids, are phenolic lipids composed of long aliphatic chains and resorcinol-type phenolic rings (Figure 4). Alkylresorcinols are relatively rare in nature, with the main known sources being wheat, rye, barley, and triticale (i.e., cereal grasses). Alkylresorcinols are present in large amounts in the bran layer (e.g., pericarp, testa, and aleurone layers) of wheat and rye (0.1–0.3% of dry weight) [117]. Alkylresorcinols can also be found in rice, though not in the edible parts of the rice plant [118]. They are present in the endosperm (the part of cereal grain used to make white flour) only in exceptionally low amounts, which means that alkylresorcinols can be used as biomarkers for people who eat foods containing wholegrain wheat and rye rather than cereal products based upon white flour [54,119]. Similarly, in a study by Ziegler et al. [53] bread wheat (761 ± 92 mg g^{-1} DM) and spelt (743 ± 57 mg g^{-1}) belonging to the hexaploid species showed higher AR levels than did the tetraploid durum (654 ± 48 mg g^{-1}, $p < 0.05$), while the levels found in the diploid einkorn (737 ± 91 mg g^{-1}) and the tetraploid emmer (697 ± 94 mg g^{-1}) did not differ significantly from those in the other species.

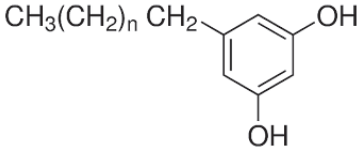


Figure 4. Structure of 5-n-Alkylresorcinols identified in wheat grains: 5-n-heptadecylresorcinol (C17:0), 5-n-nonadecylresorcinol (C19:0), 5-n-heneicosylresorcinol (C21:0), 5-n-tricosylresorcinol (C23:0), 5-pentacosylresorcinol (C25:0).

Alkylresorcinols are extracted by acetone, methanol, or mixture methanol/methyl-*tert.* butyl ether (MeOH/MTBE, 1:1, *v*/*v*), ethyl acetate or n-hexane [53]. Landberg [54] used diethyl ether and methanol, while Suzuki et al. [118] used 10% MeOH/$CHCl_3$.

Righetti et al. [56] have suggested involvement of the lipophilic phenolic fraction in mycotoxin accumulation in wheat. The contamination, expressed as the sum of DON and deoxynivalenol 3-glucoside, was found to be significantly lower in common wheat and spelt than in emmer, durum wheat, and einkorn, while following the trend hexaploid < tetraploid < diploid species. The mycotoxins content negatively correlated with the total 5-n-alkylresorcinols, and the AR21:0/AR23:0 ratio (AR21:0 is 5-n-heneicosylresorcinol, where the saturated hydrocarbon chain attached to position 5 of resorcinol consists of 21 carbon atoms; AR23:0 is 5-n-tricosylresorcinol having 23 carbon atoms and no double bonds, Figure 4) was recently reported by Righetti et al. [56] as an indicator of antifungal activity. Their results suggest that only the lipophilic phenolic fraction in wheat exerts an inhibitory effect on mycotoxin accumulation [56].

4.2. Benzoxazinoids

The principal phytoanticipin in wheat and corn is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, Figure 5). For the extraction of benzoxazinoids, 70% methanol coupled with accelerated extraction system is generally used, as it is described by Kowalska and Kowalczyk [120]. DIMBOA accumulation is regulated by jasmonic acid in both the aboveground parts of wheat and the roots [121]. A recent study found that jasmonic acid signaling and DON detoxification have relevance for seedling resistance and that seedling development and root growth are jasmonic acid-controlled processes [122]. The results of this study confirm that development-specific determinants of resistance against *Fusarium* are more significant than are the organ-specific determinants, and suggest roots to be an important organ in studies of *Fusarium*–wheat interactions.

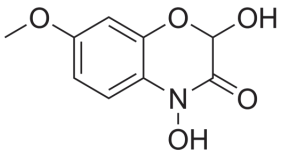


Figure 5. Structure of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) detected in wheat grains.

4.3. Volatile Organic Compounds

Pathogen infestation of vegetative tissues can induce volatile organic compounds (VOCs) production, which can in turn provide defensive functions to both injured and uninjured plants. In wheat, oats, and barley, the blend of VOCs induced after *Fusarium* spp. infestation was dominated by (Z)-hex-3-enal, (E)-hex-2-enal, (E)-hex-2-en-1-ol, (Z)-hex-3-enyl acetate, 1-hex-1-enyl acetate, β-linalool, and β -caryophyllene [70] (Figure 6). Buśko et al. [71] recently reported findings about VOCs contained in the grain of winter wheat varieties under controlled conditions after inoculation with *F. culmorum*. Among hydrocarbons, alcohols, aldehydes, ketones, aromatics, terpenes, and other components, terpenes were of particular importance [71] (Figure 6). Interestingly, the terpenes produced in wheat grains were further changed by *F. culmorum* into other compounds that were more toxic. Significantly large quantities of terpenes were observed in wheat grains inoculated with *F. culmorum* compared to uninoculated control samples. Trichodiene, thujopsene, and β-chamigrene were dominant in inoculated samples, while α-pinene, indane, and 3-carene dominated in control samples.

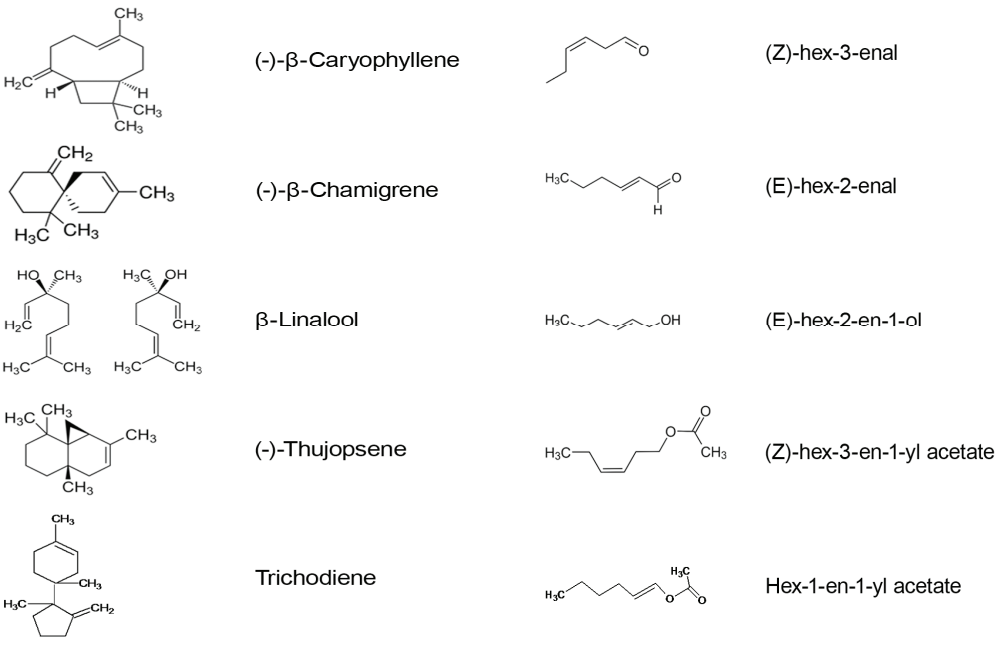


Figure 6. Structure of (**a**) terpenes and (**b**) volatile organic compounds determined in wheat grains.

Nazareth et al. [123] used the volatile gaseous fumigant allyl isothiocyanate that is not contained in wheat (an antimicrobial organosulfur compound that can be obtained from mustard *Brassica* spp.) to restrict the production of beauvericin and enniatin produced by *Fusarium poae* in wheat flour. Synthesis of beauvericin was more inhibited than was that of enniatin.

4.4. Phytohormones

When studying the influence of phytohormones on the defense response of wheat against *F. graminearum* infection, Qi et al. [69] determined that infection of heads with *F. graminearum* induced accumulation of salicylic acid, jasmonic acid, abscisic acid, and indole acetic acid [69] (Figure 7). Small et al. [124] and Trapp et al. [125] reported the extraction of these phytohormones can be provided with methanol, ethyl acetate, mixture acetone-ethanol-water (1:1:2, *v/v/v*) or 100% cold methanol, dichlormethane, or isopropanol, respectively.

Jasmonic acid treatment reduced *F. graminearum* growth and FHB symptoms even as an increase in FHB was observed with abscisic acid [69]. After the application of some elicitors, including methyl jasmonate, on *Fusarium verticillioides* in corn, however, Small et al. [124] determined that these were not effective for reducing Fusarium ear rot and fumonisin contamination.

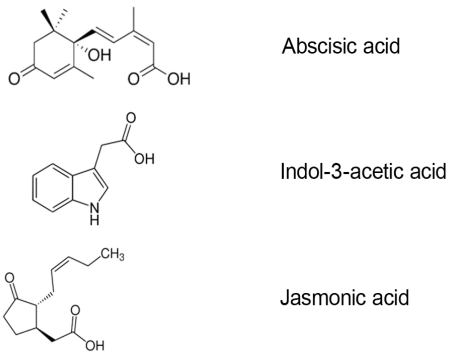


Figure 7. Structure of phytohormones determined in wheat grains.

4.5. Carotenoids

The carotenoids are yellow pigments with antioxidant and photoprotective properties that belong to the terpenes, and their basic structure consists of eight isoprene units. Two classes of carotenoids can be distinguished: (1) carotenes are pure hydrocarbons, and (2) xanthophylls are derivatives containing one or more oxygen atoms. A wide range of carotenoids may be present in wheat grain, including lutein, β-carotene, β-cryptoxanthin, zeaxanthin, antheraxanthin, taraxanthin (lutein 5,6-epoxide), triticoxanthin, and flavoxanthin [51] (Figure 8). Carotenoids are efficiently extracted with mixture ethanol/acetone/hexane (1:1:2, $v/v/v$) [126]. In another study, extraction with hexane/ethyl acetate mixture (9:1, v/v) after incubation with KOH, 95% ethanol, NaCl (10 g L^{-1}) and pyrogallol (60 g L^{-1} ethanol) has been applied [127]. Lutein is the most abundant carotenoid, followed by zeaxanthin, antheraxanthin, α-carotene, and β-carotene, while β-cryptoxanthin is a minor component or it occurs at non-detectable levels [51,126].

Bread wheat (*Triticum aestivum* L.) is poor in carotenoids, but durum wheat (*Triticum durum* Desf.) and other related species, such as einkorn (*T. monococcum* L.), showed higher carotenoid content and thereby potential as donors of useful traits [128]. Carotenoid content and color are influenced by intrinsic genotypic characteristics [51,129], and to a lesser extent by environmental conditions [62]. Delgado et al. [127] observed durum wheat cultivars to show greater lutein content than did common wheat cultivars. In their study, durum wheat was more susceptible to mycotoxin contamination than was common wheat. Positive correlations between the levels of lutein and mycotoxins in durum wheat cultivars were detected for the following mycotoxins: DON and its derivative DON-3-glucoside, moniliformin, as well as culmorin and its derivatives [127]. Martini et al. [60] observed stronger impact of genetic factors on the content of yellow (carotenoid) components and total antioxidant capacity in durum wheat, while content of total polyphenolics and phenolic acids was mostly affected by environmental conditions.

In wheat genotypes with higher levels of FHB resistance, the individual metabolites involved with particular efficiency in protection processes are represented in various proportions and they all contribute to final resistance. The degree of a particular genotype's resistance will depend upon its genetic and enzymatic equipment for biosynthesis of the active metabolites and corresponds to the content of these compounds in the grain. Therefore, the representation of compounds with resistance activity in each genotype should be comprehensively determined and assessed.

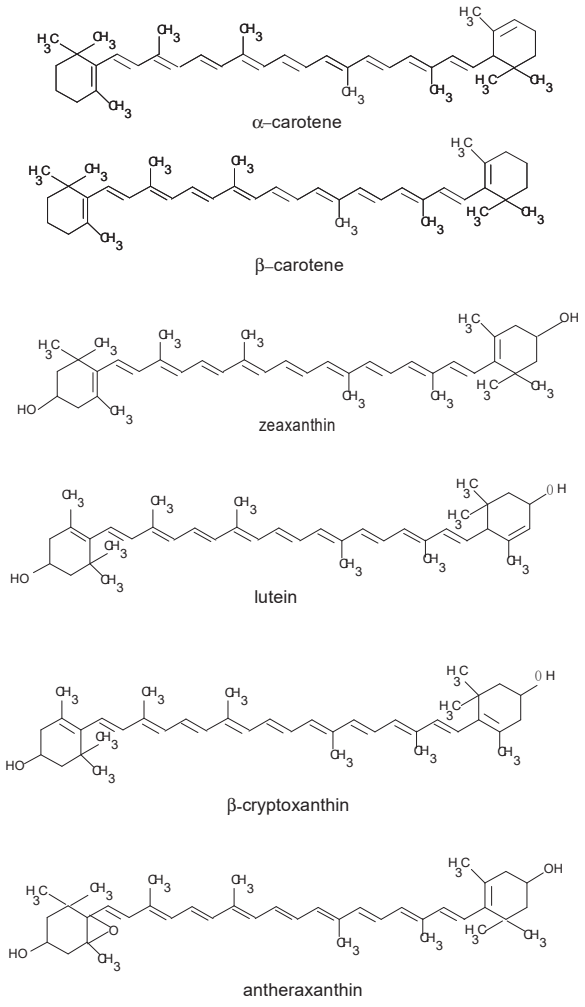


Figure 8. Structures of carotenoids determined in wheat grains.

5. Pigmentation of Grains and Fhb Resistance

The host deploys distinctive resistance mechanisms in different organs of wheat against *F. graminearum* [130,131]. Grain color and its relationship to resistance is the aspect most intensively studied. There exists various coloration of the grain in wheat due to levels of biologically active pigments possessing antioxidant capacity [51]. Descriptions of grain color in wheat are generally qualitative in nature: white, yellow, red, blue, or purple. Red pigmentation is associated with deposition of proanthocyanidins in the testa, whereas both blue and purple pigmentations derive from the accumulation of anthocyanins in the aleurone and the pericarp [132,133].

Anthocyanins can be synthesized in wheat genotypes in grains and in various organs, and they can be involved in the plant response to oxidative stress. Anthocyanins can be contained in the pericarp (purple coloration) or aleurone (blue coloration). When they are contained in both tissues, the color of the grains turns to a dark brown–black shade [134]. Hexaploid wheat varieties are being bred today with increased carotenoid content in the grain, and especially with higher lutein content. The highest carotenoid contents have been observed in yellow- and purple-grained genotypes [126].

In addition to the yellow grain color shades occurring due to carotenoids content, wheat varieties also exist that contain anthocyanins in the aleurone or pericarp layers causing blue and purple grain coloring [135].

6. Use of Antioxidants in Breeding for FHB Resistance

The best strategy for controlling FHB is to breed new varieties with reduced susceptibility. Colored-grain wheats are a potentially great alimentary source of other health-enhancing compounds, such as anthocyanins [51,136], tocols [137], and phenolic acids [88]. Expanding the breeding interest in the use of wheat forms with different grain color and plant organs can be a potential benefit for the creation of lines with increased resistance to various stresses. Spanić et al. [138] have demonstrated that differences in the antioxidant response of wheat varieties can be a valuable marker for the selection of FHB resistance. Rapid activation of the antioxidant system appears to be important in overcoming FHB, but the timing and type of antioxidant enzymes expressed are important. A large amount of variability in enzyme activity and H_2O_2 content exists within wheat varieties [138]. Measurements of ROS levels and the scavenging activities of antioxidant contents may be very useful for breeding programs to screen and select FHB-resistant varieties [41]. An example of a variety in which secondary metabolites have been implicated in FHB resistance is the most commonly used source of breeding material for FHB resistance, the cultivar Sumai 3 [113]. Gunnaiah and Kushalappa [113] report that several resistance-related metabolites produced in Sumai-3 can explain several mechanisms of resistance. Resistance in the Sumai-3 cultivar to FHB is mainly due to phenylpropanoid and flavonoid metabolites. Antimicrobial compounds and cell wall thickening by hydroxycinnamic acid amides in Sumai-3 also resist FHB [113].

Results obtained by Etzerodt et al. [49] could form a basis for choosing wheat cultivars using metabolite profiling as a marker for selecting wheat cultivars with improved resistance against FHB and accumulation of trichothecene toxins in wheat heads. They found that several phenolic acids, lutein, and β-carotene affected DON accumulation, but the effect varied for the two studied wheat types (spring versus winter wheat).

Furthermore, positive experiences in examining crops other than wheat have also been reported. In corn silk and kernels, induced expression of 3-deoxyanthocyanidins and flavan-4-ols imparted resistance to *F. verticillioides* and *F. graminearum* [40]. Expression data revealed that flavonoid pathway *P1* and *P2* genes were active during the early stages of silk development and *PR-4* and *PR-5* genes showed developmental and fungus-induced expression [40]. Anthocyanins can contribute to antioxidant properties of the polyphenolic complex, but they are unstable and can be easily degraded. Thus, their effects on fungi are not yet entirely clear. In their study of *F. graminearum* effect on wheat genotypes, Martin et al. [139] found no relationship with anthocyanin levels, which were affected more by environmental conditions. Meanwhile, Bernardi et al. [140] determined the red corn cultivar Rostrato Rosso having the greatest accumulation of anthocyanins to be highly resistant to the penetration and diffusion of *F. verticillioides.* Similarly, in a study by Lorenz-Kukuła et al. [141], expression of specific genes in flax increased resistance against *Fusarium* and resulted in a significant increase in the levels of anthocyanins and flavonoids and in their antioxidant capacity. In addition, metabolomic analysis of a red cotton mutant (S156) resistant to *Verticillium dahliae* showed enrichment of flavonoids and anthocyanins and upregulated expression of flavonoid biosynthesis genes [142].

On the other hand, it is known that there also exists varieties with colored grains that show susceptibility to FHB. An example is the variety Skorpion with blue grain. Although the anthocyanins which it contains are considered to offer health benefits due to their antioxidant effects, the variety shows susceptibility to FHB [90]. Thus, grain color alone cannot be taken as a marker to detect resistance. Moreover, for varieties with colored grain, it is always necessary to evaluate varietal resistance in trials through artificial infection.

7. Conclusions and Future Directions

In conclusion, many compounds like hydrophilic and lipophilic antioxidants (phenolic compounds such as phenolic acids, anthocyanins, flavonoids, and lipophilic carotenoids), alkylresorcinols, volatile organic compounds, phytohormones, and benzoxazinoids can be involved in protective mechanisms against FHB, the most common disease affecting wheat. However, these protective compounds affect *Fusarium* strains in varying degrees depending upon their antioxidant activity and different biochemical and cellular mechanisms. Complete resistance to *F. graminearum* is not detected in any host plant, and selection of FHB-resistant genotypes remains challenging. Therefore, considerable effort is needed to gain more in-depth insight into the genetics of the pathogen populations and to find novel and effective resistance markers in various hosts, as well as to identify the major components of cereals' defense against the pathogen. It follows that more detailed studies are required to obtain a better understanding of the protective effects and modes of activity of these metabolites. Indirect selection for an antioxidant response associated with FHB resistance can be performed and the antioxidative mechanism plays a significant role against *Fusarium* biotic stress in wheat and other cereals. The efficient use of native resistance in the wheat gene pool, introgression of resistant alleles, and implementation of modern genotypic strategies to increase levels of native secondary metabolites with antifungal properties can enhance FHB resistance of new wheat varieties. A complex of secondary metabolites composed of individual antioxidants and compounds having antifungal efficiency, with their various contents and possible synergistic effects, can determine the resistance of wheat genotypes. In short, more detailed studies are warranted concerning new wheat genotypes, their native resistance metabolites, and the effects of these upon FHB.

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References

1. Mielniczuk, E.; Skwaryło-Bednarz, B. Fusarium Head Blight, Mycotoxins and Strategies for Their Reduction. *Agronomy* **2020**, *20*, 509. [CrossRef]
2. Zain, M.E. Impact of Mycotoxins on Humans and Animals. *J. Saudi Chem. Soc.* **2011**, *15*, 129–144. [CrossRef]
3. Pleadin, J. Mycotoxins in Grains and Feed—Contamination and Toxic Effect in Animals. *Biotechnol. Anim. Husb.* **2015**, *31*, 441–456. [CrossRef]
4. Chrpová, J.; Šíp, V.; Štočková, L.; Milec, Z.; Bobková, L. Resistance of Winter Wheat Varieties Registered in the Czech Republic to Fusarium Head Blight in Relation to the Presence of Specific Rht alleles. *Czech J. Genet. Plant Breed.* **2010**, *46*, 122–134. [CrossRef]
5. Edwards, S. Investigation of *Fusarium* Mycotoxins in UK Wheat Production-Update. In *Society of Feed Technologists, Proceedings of the 2nd International Symposium on Fusarium Head Blight, Orlando, FL, USA, 11–15 December 2004*; Society of Feed Technologists, Michigan State University: East Lansing, MI, USA, 2005; pp. 398–400.
6. Krska, R.; Baumgartner, S.; Josephs, R. The State-of-the-Art in the Analysis of Type-A and -B Trichothecene Mycotoxins in Cereals. *Fresenius J. Anal. Chem.* **2001**, *371*, 285–299. [CrossRef]

7. Spanu, F.; Scherm, B.; Camboni, I.; Pani, G.; Oufensou, S.; Macciotta, N.; Pasquali, M.; Migheli, Q. FcRav2, a Gene with a ROGDI Domain Involved in Fusarium Head Blight and Crown Rot on Durum Wheat Caused by *Fusarium culmorum*. *Mol. Plant Pathol.* **2018**, *9*, 677–688. [CrossRef] [PubMed]
8. Bilska, K.I.; Jurczak, S.; Kulik, T.; Ropelewska, E.; Olszewski, J.; Zelechowski, M.; Zapotoczny, P. Species Composition and Trichothecene Genotype Profiling of *Fusarium* Field Isolates Recovered from Wheat in Poland. *Toxins* **2018**, *10*, 325. [CrossRef] [PubMed]
9. Bilska, K.; Stuper-Szablewska, K.; Kulik, T.; Buśko, M.; Załuski, D.; Jurczak, S.; Perkowski, J. Changes in Phenylpropanoid and Trichothecene Production by *Fusarium culmorum* and *F. graminearum* Sensu Stricto via Exposure to Flavonoids. *Toxins* **2018**, *10*, 110. [CrossRef]
10. Aoki, T.; Ward, T.J.; Kistlet, H.C.; O'Donnell, K. Systematics, Phylogeny and Trichothecene Mycotoxin Potential of Fusarium Head Blight Cereal Pathogens. *JSM Mycotoxins* **2012**, *62*, 91–102. [CrossRef]
11. Wagacha, J.M.; Muthomi, J.W. *Fusarium culmorum*: Infection Process, Mechanisms of Mycotoxin Production and Their Role in Pathogenesis in Wheat. *J. Crop Prot.* **2007**, *26*, 877–885. [CrossRef]
12. D'Mello, J.P.; Placinta, C.M.; Macdonald, A.M. *Fusarium* Mycotoxins: A Review of Global Implications for Animal Health, Welfare and Productivity. *Anim. Feed Sci. Technol.* **1999**, *80*, 183–205. [CrossRef]
13. Buerstmayr, H.; Stierschneider, M.; Steiner, B.; Lemmens, M.; Griesser, M.; Nevo, E.; Fahima, T. Variation for Resistance to Head Blight Caused by *Fusarium graminearum* in Wild Emmer (*Triticum dicoccoides*) Originating from Israel. *Euphytica* **2003**, *130*, 17–23. [CrossRef]
14. Pereyra, S.A.; Dill-Macky, R.; Sims, A.L. Survival and Inoculum Production of *Gibberella zeae* in Wheat Residue. *Plant Dis.* **2004**, *88*, 724–730. [CrossRef] [PubMed]
15. Liu, X.; Wang, J.; Xu, J.; Shi, J. FgIlv5 is Required for Branched-Chain Amino Acid Biosynthesis and Full Virulence in *Fusarium graminearum*. *Microbiology* **2014**, *160*, 692–702. [CrossRef]
16. Sperschneider, J.; Gardiner, D.M.; Thatcher, L.F.; Lyons, R.; Singh, K.B.; Manners, J.M.; Taylor, J.M. Genome-Wide Analysis in Three *Fusarium* Pathogens Identifies Rapidly Evolving Chromosomes and Genes Associated with Pathogenicity. *Genome Biol. Evol.* **2015**, *7*, 1613–1627. [CrossRef]
17. Dweba, C.C.; Figlan, S.; Shimelis, H.A.; Motaung, T.E.; Sydenham, S.; Mwadzingeni, L.; Tsilo, T.J. Fusarium Head Blight of Wheat: Pathogenesis and Control Strategies. *Crop Prot.* **2017**, *91*, 114–122. [CrossRef]
18. Khaledi, N.; Taheri, P.; Falahati-Rastegar, M. Identification, Virulence Factors Characterization, Pathogenicity and Aggressiveness Analysis of *Fusarium* spp., Causing Wheat Head Blight in Iran. *Eur. J. Plant Pathol.* **2017**, *147*, 897–918. [CrossRef]
19. Kikot, G.E.; Hours, R.A.; Alconada, T.M. Contribution of Cell Wall Degrading Enzymes to Pathogenesis of *Fusarium graminearum*: A Review. *J. Basic Microbiol.* **2009**, *49*, 231–241. [CrossRef] [PubMed]
20. Qin, J.; Wang, G.; Jiang, C.; Xu, J.R.; Wang, C. 1Fgk3 Glycogen Synthase Kinase is Important for Development, Pathogenesis, and Stress Responses in *Fusarium graminearum*. *Sci. Rep.* **2015**, *5*, 8504. [CrossRef]
21. Wanyoike, W.M.; Kang, Z.; Buchenauer, H. Importance of Cell Wall Degrading Enzymes Produced by *Fusarium graminearum* During Infection of Wheat Head. *Eur. J. Plant Pathol.* **2002**, *108*, 803–810.
22. Feng, J.; Liu, G.; Selvaraj, G.; Hughes, G.R.; Wei, Y. A secreted Lipase Encoded by LIP1 is Necessary for Efficient Use of Saturated Triglyceride Lipids in *Fusarium graminearum*. *Microbiology* **2005**, *151*, 3911–3921. [CrossRef]
23. Gao, S.; Glenn, A.E.; Blacutt, A.A.; Gold, S.E. Fungal Lactamases: Their Occurrence and Function. *Front. Microbiol.* **2017**, *8*, E1775. [CrossRef]
24. Glenn, A.E.; Davis, C.B.; Gao, M.; Gold, S.E.; Mitchell, T.R.; Proctor, R.H.; Stewart, J.E.; Snook, M.E. Two Horizontally Transferred Xenobiotic Resistance Gene Cluster Associated with Detoxification of Benzoxazolinones by *Fusarium* Species. *PLoS ONE* **2016**, *11*, e0147486. [CrossRef]
25. Luz, C.; Saladino, F.; Luciano, F.; Mañes, J.; Meca, G. Occurrence, Toxicity, Bioaccessibility and Mitigation Strategies of Beauvericin, a Minor *Fusarium* Mycotoxin. *Food Chem. Toxicol.* **2017**, *107*, 430–439. [CrossRef]
26. Kosová, K.; Chrpová, J.; Šíp, V. Cereal Resistance to Fusarium Head Blight and Possibilities of Its Improvement through Breeding. *Czech J. Genet. Plant Breed.* **2009**, *45*, 87–105. [CrossRef]
27. Bushnell, W.; Perkins-Veazie, P.; Russo, V.; Collins, J.; Seeland, T. Effects of Deoxynivalenol on Content of Chloroplast Pigments in Barley Leaf Tissues. *Phytopathology* **2010**, *100*, 33–41. [CrossRef] [PubMed]
28. Cossette, F.; Miller, J.D. Phytotoxic Effect of Deoxynivalenol and Gibberella Ear Rot Resistance of Corn. *Nat. Toxins* **1995**, *3*, 383–388. [CrossRef]
29. Wipfler, R.; Mc Cormick, S.P.; Proctor, R.H.; Teresi, J.M.; Hao, G.; Ward, T.J.; Alexander, N.J.; Vaughan, M.M. Synergistic Phytotoxic Effects of Culmorin and Trichothecene Mycotoxins. *Toxins* **2019**, *11*, 555. [CrossRef] [PubMed]
30. Lu, S.; Edwards, M.C. Genome Wide Analysis of Small Secreted Cysteine-Rich Proteins Identifies Candidate Effector Proteins Potentially Involved in *Fusarium graminearum*—Wheat Interactions. *Phytopathology* **2016**, *106*, 166–176. [CrossRef] [PubMed]
31. Fabre, F.; Bormann, J.; Urbach, S.; Roche, S.; Langin, T.; Bonhommer, L. Unbalanced Roles of Fungal Aggressiveness and Host Cultivars in the Establishment of the Fusarium Head Blight in Bread Wheat. *Front. Microbiol.* **2019**, *10*, 2857. [CrossRef] [PubMed]
32. Ravensdale, M.; Rocheleau, H.; Wang, L.; Nasmith, C.; Ouellet, T.; Subramaniam, R. Components of Priming Induced Resistance to Fusarium Head Blight in Wheat Revealed by Two Distinct Mutants of *Fusarium graminearum*. *Mol. Plant Pathol.* **2014**, *15*, 948–956. [CrossRef]

33. Schroeder, H.W.; Christensen, J.J. Factors Affecting Resistance of Wheat to Scab by Gibberella zeae. *Phytopathology* **1963**, *53*, 831–838.
34. Miller, J.D.; Arnison, P.G. Degradation of Deoxynivalenol by Suspension Cultures of the Fusarium Head Blight Resistant Wheat Cultivar Frontana. *Can. J. Plant Pathol.* **1986**, *8*, 147–150. [CrossRef]
35. Spanic, V.; Marcek, T.; Abicic, I.; Sarkanj, B. Effects of *Fusarium* Head Blight on Wheat Grain and Malt Infected by *Fusarium culmorum*. *Toxins* **2018**, *10*, 17. [CrossRef] [PubMed]
36. Mesterházy, Á. Types and Components of Resistance to Fusarium Head Blight of Wheat. *Plant Breed.* **1995**, *114*, 377–386. [CrossRef]
37. Mesterházy, Á. Theory and Practice of the Breeding for Fusarium Head Blight Resistance in Wheat. *J. Appl. Gen.* **2002**, *43A*, 289–302.
38. Malinovsky, F.G.; Fangel, J.U.; Willats, G.T. The Role of the Cell Wall in Plant Immunity. *Front. Plant Sci.* **2014**, *5*, 178. [CrossRef] [PubMed]
39. Miedes, E.; Vanholme, R.; Boerjan, W.; Molina, A. The Role of Secondary Cell Wall in Plant Resistance to Pathogens. *Front. Plant Sci.* **2014**, *5*, 358. [CrossRef]
40. Sekhon, R.S.; Kuldau, G.; Mansfield, M.; Chopra, S. Characterization of *Fusarium*-Induced Expression of Flavonoids and PR Genes in Maize. *Physiol. Mol. Plant Pathol.* **2006**, *69*, 109–117. [CrossRef]
41. Khaledi, N.; Taheri, P.; Falahati-Rastegar, M. Reactive Oxygen Species and Antioxidant System Responses in Wheat Cultivars during Interaction with *Fusarium* Species. *Austr. Plant Pathol.* **2016**, *45*, 653–670. [CrossRef]
42. Ellinger, D.; Voigt, C.A. Callose Biosynthesis in Arabidopsis with a Focus on Pathogen Response: What We Have Learned Within the Last Decade. *Ann. Bot.* **2014**, *114*, 1349–1358. [CrossRef] [PubMed]
43. Taheri, P. Cereal Diseases Caused by *Fusarium graminearum*: From Biology of the Pathogen to Oxidative Burst-Related Host Defense Responses. *Eur. J. Plant Pathol.* **2018**, *152*, 1–20. [CrossRef]
44. Khaledi, N.; Taheri, P.; Falahati-Rastegar, M. Evaluation of Resistance and the Role of Some Defense Responses in Wheat Cultivars to Fusarium Head Blight. *J. Plant Protect. Res.* **2018**, *57*, 205–217. [CrossRef]
45. Boutigny, A.L.; Richard-Forget, F.; Barreau, C. Natural Mechanisms for Cereal Resistance to the Accumulation of *Fusarium* Trichothecenes. *Eur. J. Plant Pathol.* **2008**, *121*, 411–423. [CrossRef]
46. Jain, D.; Khurana, J.P. Role of Pathogenesis-Related (PR) Proteins in Plant Defense Mechanism. In *Molecular Aspects of Plant-Pathogen Interaction*; Singh, A., Singh, I., Eds.; Springer: Singapore, 2018; pp. 265–281.
47. Ali, S.; Ganai, B.A.; Kamili, A.N.; Bhat, A.A.; Mir, Z.A.; Bhat, J.A.; Tyagi, A.; Islam, S.J.; Mushtaq, M.; Yadav, P.; et al. Pathogenesis-Related Proteins and Peptides as Promising Tools for Engineering Plants with Multiple Stress Tolerance. *Microbiol. Res.* **2018**, *212–213*, 29–37. [CrossRef] [PubMed]
48. Sels, J.; Mathys, J.; De Coninck, B.M.; Cammue, B.P.; De Bolle, M.F. Plant Pathogenesis-Related (PR) Proteins: A Focus on PR Peptides. *Plant Physiol. Biochem.* **2008**, *46*, 941–950. [CrossRef]
49. Etzerodt, T.; Gislum, R.; Laursen, B.B.; Heinrichson, K.; Gregersen, P.L.; Jørgensen, L.N.; Fomsgaard, I.S. Correlation of Deoxynivalenol Accumulation in *Fusarium*-Infected Winter and Spring Wheat Cultivars with Secondary Metabolites at Different Growth Stages. *J. Agric. Food Chem.* **2016**, *64*, 4545–4555. [CrossRef] [PubMed]
50. Boba, A.; Kulma, A.; Kostyn, K.; Starzycki, M.; Starzycka, E.; Szopa, J. The Influence of Carotenoid Biosynthesis Modification on the *Fusarium culmorum* and *Fusarium oxysporum* Resistance in Flax. *Physiol. Mol. Plant Pathol.* **2011**, *76*, 39–47. [CrossRef]
51. Lachman, J.; Martinek, P.; Kotíková, Z.; Orsák, M.; Šulc, M. Genetics and Chemistry of Pigments in Wheat Grain—A Review. *J. Cereal Sci.* **2017**, *74*, 145–154. [CrossRef]
52. Pani, G.; Scherm, B.; Azara, E.; Balmas, V.; Jahanshiri, Z.; Carta, P.; Fabbri, D.; Dettori, M.A.; Fadda, A.; Dessi, A.; et al. Natural and Natural-Like Phenolic Inhibitors of Type B Trichothecene In Vitro Production by the Wheat (*Triticum* sp.) Pathogen *Fusarium culmorum*. *J. Agric. Food Chem.* **2014**, *62*, 4969–4978. [CrossRef]
53. Ziegler, J.U.; Steingass, C.B.; Longin, C.F.; Würschum, T.; Carle, R.; Schweiggert, R.M. Alkylresorcinol Composition Allows the Differentiation of *Triticum* spp. Having Different Degrees of Ploidy. *J. Cereal Sci.* **2015**, *65*, 244–251. [CrossRef]
54. Landberg, R. *Alkylresorcinols as Biomarkers of Whole Grain Wheat and Rye Intake*; Acta Universitatis Agriculturae Sueciae: Uppsala, Sweden, 2009.
55. Bordiga, M.; Locatelli, M.; Travaglia, F.; Arlorio, M.; Reyneri, A.; Blandino, M.; Coisson, J.D. Alkylresorcinol Content in Whole Grains and Pearled Fractions of Wheat and Barley. *J. Cereal Sci.* **2016**, *70*, 38–46. [CrossRef]
56. Righetti, L.; Cirlini, M.; Folloni, S.; Ranieri, R.; Galaverna, G.; Bertuzzi, T.; Dall'Asta, C.; Battilani, P.; Giorni, P. 5-n-Alkylresorcinols but Not Hydroxycinnamic Acids Are Directly Related to a Lower Accumulation of Deoxynivalenol and Its Glucoside in *Triticum* spp. Genotypes with Different Ploidity Levels. *J. Cereal Sci.* **2019**, *85*, 214–220. [CrossRef]
57. Abdel-Aal, E.S.; Hucl, P.; Rabalski, I. Compositional and Antioxidant Properties of Anthocyanin-Rich Products Prepared from Purple Wheat. *Food Chem.* **2018**, *254*, 13–19. [CrossRef] [PubMed]
58. Etzerodt, T.; Maeda, K.; Nakajima, Y.; Laursen, B.; Fomsgaard, I.S.; Kimura, M. 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) Inhibits Trichothecene Production by *Fusarium graminearum* through Suppression of Tri6 Expression. *Int. J. Food Microbiol.* **2015**, *214*, 123–128. [CrossRef]
59. Shewry, P.R.; Hey, S. Do "Ancient" Wheat Species Differ from Modern Bread Wheat in Their Contents of Bioactive Components? *J. Cereal Sci.* **2015**, *65*, 236–243. [CrossRef]

60. Martini, D.; Taddei, F.; Ciccoritti, R.; Pasquini, M.; Nicoletti, I.; Corradini, D.; D´Egidio, M.G. Variation of Total Antioxidant Activity and of Phenolic Acid, Total Phenolics and Yellow Coloured Pigments in Durum Wheat (*Triticum turgidum* L. var. durum) as a Function of Genotype, Crop Year and Growing Area. *J. Cereal Sci.* **2015**, *65*, 175–185.
61. Ma, D.Y.; Sun, D.X.; Zuo, Y.; Wang, C.Y.; Zhu, Y.J.; Guo, T.C. Diversity of Antioxidant Content and Its Relationship to Grain Color and Morphological Characteristics in Winter Wheat Grains. *J. Integr. Agric.* **2014**, *13*, 1258–1267. [CrossRef]
62. Lachman, J.; Hejtmánková, K.; Kotíková, Z. Tocols and Carotenoids of Einkorn, Emmer and Spring Wheat Varieties: Selection for Breeding and Production. *J. Cereal Sci.* **2013**, *57*, 207–214. [CrossRef]
63. Li, Y.G.; Ma, D.Y.; Sun, D.X.; Wang, C.Y.; Zhang, J.; Xie, Y.X.; Guo, T.C. Total Phenolic, Flavonoid Content, and Antioxidant Activity of Flour, Noodles, and Steamed Bread Made from Different Colored Wheat Grains by Three Milling Methods. *J. Crop Prod.* **2015**, *3*, 328–334. [CrossRef]
64. Buśko, M.; Gòral, T.; Ostrowska, A.; Matysiak, A.; Walentyn-Gòral, D.; Perkowski, J. The Effect of Fusarium Fungicide Application on Concentrations of Flavonoids (Apigenin, Kaempferol, Luteolin, Naringenin, Quercetin, Rutin, Vitexin) in Winter Wheat Cultivars. *Am. J. Sci.* **2014**, *5*, 3727–3736. [CrossRef]
65. Kovács, V.; Gondor, O.K.; Szalai, G.; Darkó, É.; Majláth, I.; Janda, T.; Pál, M. Synthesis and Role of Salicylic Acid in Wheat Varieties with Different Levels of Cadmium Tolerance. *J. Hazard. Mater.* **2014**, *280*, 12–19. [CrossRef]
66. Verma, B.; Hucl, P.; Chibbar, R.N. Phenolic Acid Composition and Antioxidant Capacity of Acid and Alkali Hydrolysed Bran Fractions. *Food Chem.* **2009**, *116*, 947–954. [CrossRef]
67. Stuper-Szablewska, K.; Perkowski, J. Phenolic Acids in Cereals Grain: Occurrence, Metabolism and Role in Living Organisms. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 664–675. [CrossRef] [PubMed]
68. Martin, C. Resistance Elements of Small Grain Cereals against Fusarium Head Blight and Contribution of Health Promoting Compounds. Ph.D. Thesis, University of Neuchâtel, Neuchâtel, Switzerland, 2017.
69. Qi, P.F.; Balcerzak, M.; Rocheleau, H.; Leung, W.; Wei, Y.M.; Zheng, Y.L.; Ouellet, T. Jasmonic Acid and Abscisic Acid Play Important Roles in Host-Pathogen Interaction between *Fusarium graminearum* and Wheat during the Early Stages of Fusarium Head Blight. *Physiol. Mol. Plant Pathol.* **2016**, *93*, 39–48. [CrossRef]
70. Piesik, D.; Pańka, D.; Delaney, K.J.; Skoczek, A.; Lamparski, R.; Weaver, D.K. Cereal Crop Volatile Organic Compound Induction After Mechanical Injury, Beetle Herbivory (*Oulema* spp.), or Fungal Infection (*Fusarium* spp.). *J. Plant Physiol.* **2011**, *168*, 878–886. [CrossRef]
71. Buśko, M.; Gòral, T.; Boczkowska, M.; Perkowski, J. Relationships between Volatile Organic Compounds with Emphasis on Terpene Compounds and Genetic Matrix in Inoculated and Non-inoculated Winter Wheat Cultivars. *Chem. Ecol.* **2019**, *35*, 971–986. [CrossRef]
72. Steiner, B.; Buerstmayr, M.; Michel, S.; Schweiger, W.; Lemmens, M.; Buerstmayr, H. Breeding Strategies and Advances in Line Selection for Fusarium Head Blight Resistance in Wheat. *Trop. Plant Pathol.* **2017**, *42*, 165–174. [CrossRef]
73. Jones, S.; Farooqi, A.; Foulkes, J.; Sparkes, D.L.; Linforth, R.; Ray, R.V. Canopy and Ear Traits Associated with Avoidance of Fusarium Head Blight in Wheat. *Front. Plant Sci.* **2018**, *9*, 1021. [CrossRef] [PubMed]
74. Tessmann, E.W.; Van Sanford, D.A. Associations Between Morphological and FHB Traits in a Soft Red Winter Wheat Population. *Euphytica* **2019**, *215*, 189. [CrossRef]
75. McCartney, C.A.; Brûlé-Babel, A.L.; Fedak, G.; Martin, R.A.; McCallum, B.D.; Gilbert, J.; Hiebert, C.W.; Pozniak, C.V. Fusarium Head Blight Resistance QTL in the Spring Wheat Cross Kenyon/86ISMN 2137. *Front. Microbiol.* **2016**, *7*, 1542. [CrossRef]
76. Giancaspro, A.; Giove, S.L.; Zito, D.; Blanco, A.; Gadaleta, A. Mapping QTLs for Fusarium Head Blight Resistance in an Interspecific Wheat Population. *Front. Plant Sci.* **2016**, *7*, 1381. [CrossRef]
77. Haile, J.K.; N'Diaye, A.; Walkowiak, S.; Nilsen, K.T.; Clarke, J.M.; Kutcher, H.R.; Steiner, B.; Buerstmayr, H.; Pozniak, C.J. Fusarium Head Blight in Durum Wheat: Recent Status, Breeding Directions and Future Research Prospects. *Phytopathology* **2019**, *109*, 1664–1675. [CrossRef]
78. Buerstmayr, M.; Buerstmayr, H. The Semidwarfing Alleles *Rht D1b* and *Rht B1b* Show Marked Differences in Their Associations with Anther-Retention in Wheat Heads and with Fusarium Head Blight Susceptibility. *Phytopathology* **2016**, *106*, 1544–1552. [CrossRef] [PubMed]
79. Giordano, D.; Beta, T.; Vanara, F.; Blandino, M. Influence of Agricultural Management on Phytochemicals of Colored Corn Genotypes (*Zea mays* L.). Part 1: Nitrogen Fertilization. *J. Agric. Food Chem.* **2018**, *66*, 4300–4308. [CrossRef] [PubMed]
80. Atanasova-Penichon, V.; Barreau, C.; Richard-Forget, F. Antioxidant Secondary Metabolites in Cereals: Potential Involvement in Resistance to *Fusarium* and Mycotoxin Accumulation. *Front. Microbiol.* **2016**, *7*, 566. [CrossRef]
81. Žilič, S. Phenolic Compounds of Wheat: Their Content, Antioxidant Capacity and Bioaccessibility. *MOJ Food Process. Technol.* **2016**, *2*, 85–89. [CrossRef]
82. Tian, S.; Sun, Y.; Chen, Z.; Zhao, R. Bioavailability and Bioactivity of Alkylresorcinols from Different Cereal Products. *J. Food Qual.* **2020**, *2020*, 5781356. [CrossRef]
83. Niculaes, C.; Abramov, A.; Hannemann, L.; Frey, M. Plant Protection by Benzoazinoids—Recent Insights into Biosynthesis and Function. *Agronomy* **2018**, *8*, 143. [CrossRef]
84. Matsuura, T.; Mori, I.C.; Himi, E.E.; Hirayama, T. Plant Hormone Profiling in Developing Seeds of Common Wheat (*Triticum aestivum* L.). *Breed. Sci.* **2019**, *69*, 601–610. [CrossRef]

85. Wenda-Piesik, A.; Piesik, D.; Ligor, T.; Buszewski, B. Volatile Organic Compounds (VOCs) from Cereal Plants Infested with Crown Rot: Their Identity and Their Capacity for Inducing Production of VOCs in Uninfested Plants. *Int. J. Pest Manag.* **2010**, *4*, 377–383. [CrossRef]
86. Nakabayashi, R.; Saito, K. Integrated Metabolomics for Abiotic Stress Responses in Plants. *Curr. Opin. Plant Biol.* **2015**, *24*, 10–16. [CrossRef] [PubMed]
87. Santos-Sánchez, N.F.; Salas-Coronado, R.; Hernández-Carlos, B.; Villanueva-Cañongo, C. Shikimic Acid Pathway in Biosynthesis of Phenolic Compounds. In *Plant Physiological Aspects of Phenolic Compounds*; Soto-Hernández, S., García-Mateos, R., Palma-Tenango, M., Eds.; IntechOpen: London, UK, 2019; Chapter 3; pp. 35–50.
88. Paznocht, L.; Kotíková, Z.; Burešová, B.; Lachman, J.; Martinek, P. Phenolic Acids in Kernels of Different Coloured-Grain Wheat Genotypes. *Plant Soil Environ.* **2020**, *66*, 57–64. [CrossRef]
89. Chateigner-Boutin, A.L.; Lapierre, C.; Alvarado, C.; Yoshinaga, A.; Barron, C.; Bouchet, B.; Bakan, B.; Saulnier, L.; Devaux, M.F.; Girousse, C.; et al. Ferulate and Lignin Cross-Links Increase in Cell Walls of Wheat Grain Outer Layers during Late Development. *Plant Sci.* **2018**, *276*, 199–207. [CrossRef]
90. Martinek, P.; Škorpík, M.; Chrpová, J.; Fučík, P.; Schweiger, J. Development of the New Winter Wheat Variety Skorpion with Blue Grain. *Czech J. Genet. Plant Breed.* **2013**, *49*, 90–94. [CrossRef]
91. Gauthier, L.; Atanasova-Penichon, V.; Chéreau, S.; Richard-Forget, F. Metabolomics to Decipher the Chemical Defense of Cereals against *Fusarium graminearum* and Deoxynivalenol Accumulation. *Int. J. Mol. Sci.* **2015**, *6*, 24839–24872. [CrossRef]
92. Ponts, N.; Pinson-Gadais, L.; Barreau, C.; Richard-Forget, F.; Quellet, T. Exogenous H_2O_2 and Catalase Treatments Interfere with *Tri* Genes Expression in Liquid Cultures of *Fusarium graminearum*. *FEBS Lett.* **2007**, *581*, 443–447. [CrossRef] [PubMed]
93. Schöneberg, T.; Kibler, K.; Sulyok, M.; Musa, T.; Bucheli, T.D.; Mascher, F.; Bertossa, M.; Foegele, R.T.; Vogelsang, S. Can Plant Phenolic Compounds Reduce *Fusarium* Growth and Mycotoxin Production in Cereals? *Food Addit. Contam. Part A* **2018**, *35*, 2455–2470. [CrossRef]
94. Siranidou, E.; Kang, Z.; Buchenauer, H. Studies on Symptom Development, Phenolic Compounds and Morphological Defense Responses in Wheat Cultivars Differing in Resistance to Fusarium Head Blight. *J. Phytopathol.* **2003**, *150*, 200–208. [CrossRef]
95. Gauthier, L.; Bonnin-Verdal, M.N.; Marchegay, G.; Pinson-Gadais, L.; Ducos, C.; Richard-Forget, F.; Atanasova-Penichon, V. Fungal Biotransformation of Chlorogenic and Caffeic Acids by *Fusarium graminearum*: New Insights in the Contribution of Phenolic Acids to Resistance to Deoxynivalenol Accumulation in Cereals. *Int. J. Food Microbiol.* **2016**, *221*, 61–68. [CrossRef]
96. Giordano, D.; Beta, T.; Reyneri, A.; Blandino, M. Changes in the Phenolic Acid Content and Antioxidant Activity during Kernel Development in Corn (*Zea mays* L.) and Relationship with Mycotoxin Contamination. *Cereal Chem.* **2017**, *94*, 315–324. [CrossRef]
97. Sampietro, D.A.; Fauguel, C.M.; Vattuone, M.A.; Presello, D.A.; Catalán, C.A. Phenylpropanoids from Maize Pericarp: Resistance Factors to Kernel Infection and Fumonisin Accumulation by *Fusarium verticillioides*. *Eur. J. Plant Pathol.* **2013**, *135*, 105–113. [CrossRef]
98. Erayman, M.; Turktas, M.; Akdogan, G.; Gurkok, T.; Inal, B.; Ishakoglu, E.; Ilhan, E.; Unver, T. Transcriptome Analysis of Wheat Inoculated with *Fusarium graminearum*. *Front. Plant Sci.* **2015**, *6*, 867. [CrossRef] [PubMed]
99. Rocheleau, H.; Al-Harthi, R.; Quellet, T. Degradation of Salicylic Acid by *Fusarium graminearum*. *Fungal Biol.* **2019**, *123*, 77–86. [CrossRef] [PubMed]
100. Ponts, N.; Couedelo, L.; Pinson-Gadais, L.; Verdal-Bonnin, M.N.; Barreau, C.; Richard-Forget, F. *Fusarium* Response to by H_2O_2 is Trichothecene Chemotype-Dependent. *FEMS Microbiol. Lett.* **2009**, *293*, 255–262. [CrossRef]
101. Ferrigo, D.; Raiola, A.; Bogialli, S.; Bortolini, C.; Tapparo, A.; Causin, R. In Vitro Production of Fumonisins by *Fusarium verticillioides* under Oxidative Stress Induced by H_2O_2. *J. Agric. Food Chem.* **2015**, *63*, 4879–4885. [CrossRef] [PubMed]
102. Steinkellner, S.; Mammerler, R. Effect of Flavonoids on the Development of *Fusarium oxysporum* f. sp. *lycopersici*. *J. Plant Interact.* **2007**, *2*, 17–23. [CrossRef]
103. Jimenez-Garcia, S.N.; Garcia-Mier, L.; Garcia-Trejo, J.F.; Ramirez-Gomez, X.S.; Guevara-Gonzalez, R.G.; Feregrino-Perez, A.A. Chapter 3. *Fusarium* Mycotoxins and Metabolites that Modulate Their Production. In *Fusarium Plant Diseases, Pathogen Diversity, Resistance and Molecular Markers*; Askun, T., Ed.; IntechOpen: London, UK, 2018; pp. 23–40.
104. Stuper-Szablewska, K.; Kurasiak-Popowska, D.; Nawracała, J.; Perkowski, J. Quantitative Profile of Phenolic Acids and Antioxidant Activity of Wheat Grain Exposed to Stress. *Eur. Food Res. Technol.* **2019**, *245*, 1595–1603. [CrossRef]
105. Pervaiz, T.; Songtao, J.; Faghihi, H.; Halder, M.S.; Fang, J. Naturally Occurring Anthocyanin, Structure, Functions and Biosynthetic Pathway in Fruit Plants. *J. Plant. Physiol. Biochem.* **2017**, *5*, 187. [CrossRef]
106. Shoeva, O.Y.; Gordeeva, E.I.; Arbuzova, V.S.; Khlestkina, E.K. Anthocyanins Participate in Protection of Wheat Seedlings from Osmotic Stress. *Cereal Res. Commun.* **2017**, *45*, 47–56. [CrossRef]
107. Eliášová, M.; Kotíková, Z.; Lachman, J.; Orsák, M.; Martinek, P. Influence of Baking on Anthocyanin Content in Coloured-Grain Wheat Bread. *Plant Soil Environ.* **2020**, *66*, 381–383. [CrossRef]
108. Tyl, C.E.; Bunzel, M. Antioxidant Activity-Guided Fractionation of Blue Wheat (UC66049 *Triticum aestivum* L.). *J. Agric. Food Chem.* **2012**, *60*, 731–739. [CrossRef] [PubMed]
109. Isah, T. Stress and Defense Responses in Plant Secondary Metabolites Production. *Biol. Res.* **2019**, *52*, E39. [CrossRef] [PubMed]
110. Wang, X.; Olsen, O.; Knudsen, S. Expression of Dihydroflavonol Reductase Gene in an Anthocyanin-Free Barley Mutant. *Hereditas* **1993**, *119*, 67–75. [CrossRef]

111. Masisi, K.; Beta, T.; Moghadasian, M.H. Antioxidant Properties of Diverse Cereal Grains: A Review on In Vitro and In Vivo studies. *Food Chem.* **2016**, *196*, 90–97. [CrossRef] [PubMed]
112. Bollina, V.; Kushalappa, A.C. In Vitro Inhibition of Trichothecene Biosynthesis in *Fusarium graminearum* by Resistance-Related Endogenous Metabolites Identified in Barley. *Mycology* **2011**, *2*, 291–296.
113. Gunnaiah, R.; Kushalappa, A.C. Metabolomics Deciphers the Host Resistance Mechanisms in Wheat Cultivar Sumai-3, against Trichothecene Producing and Non-Producing Isolates of *Fusarium graminearum*. *Plant Physiol. Biochem.* **2014**, *83*, 40–50. [CrossRef]
114. Durazzo, A.; Zaccaria, M.; Polito, A.; Maiani, G.; Carcea, M. Lignan Content in Cereals, Buckwheat and Derived Foods. *Foods* **2013**, *2*, 53–63. [CrossRef] [PubMed]
115. Peterson, J.; Dwyer, J.; Adlercreutz, H.; Scalbert, A.; Jacques, P.; McCullough, M.L. Dietary Lignans: Physiology and Potential for Cardiovascular Disease risk reduction. *Nutr. Rev.* **2010**, *68*, 571–603. [CrossRef] [PubMed]
116. Bagniewska-Zadworna, A.; Barakat, A.; Łakomy, P.; Smoliński, D.J.; Zadworny, M. Lignin and Lignans in Plant Defence: Insight from Expression Profiling of Cinnamyl Alcohol Dehydrogenase Genes during Development and Following Fungal Infection in *Populus*. *Plant Sci. J.* **2014**, *229*, 111–121. [CrossRef]
117. Mattila, P.; Pihlava, J.M.; Hellstrom, J. Contents of Phenolic Acids, Alkyl- and Alkenylresorcinols, and Aventhramides in Commercial Grain Products. *J. Agric. Food Chem.* **2005**, *53*, 8290–8295. [CrossRef] [PubMed]
118. Suzuki, Y.; Kurano, M.; Esumi, Y.; Yamaguchi, I.; Yoshiharu, D. Biosynthesis of 5-alkylresorcinol in Rice: Icorporation of Putative Fatty Acid Unit in the 5-alkylresorcinol Carbon Chain. *Bioorg. Chem.* **2003**, *31*, 437–452. [CrossRef]
119. Ross, A.B.; Kamal-Eldin, A.; Åman, P. Dietary Alkylresorcinols: Absorption, Bioactivities, and Possible Use as Biomarkers of Whole-Grain Wheat- and Rye-Rich Foods. *Nutr. Rev.* **2004**, *3*, 81–95. [CrossRef] [PubMed]
120. Kowalska, I.; Kowalczyk, M. Determination of benzoxazinoids in Spring and Winter Varieties Using Ultra-Performance Liquid Chromatography Coupled with Mass Spectrometry. *Acta Chromatogr.* **2019**, *31*, 179–182. [CrossRef]
121. Meyer, J.; Murray, S.L.; Berger, D.K. Signals that Stop the Rot: Regulation of Secondary Metabolite Defences in Cereals. *Physiol. Mol. Plant Pathol.* **2016**, *94*, 156–166. [CrossRef]
122. Wang, Q.; Shao, B.; Shaikh, F.I.; Friedt, W.; Gottwald, S. Wheat Resistances to *Fusarium* Root Rot and Head Blight Are Both Associated with Deoxynivalenol- and Jasmonate-Related Gene Expression. *Phytopathology* **2018**, *108*, 602–616. [CrossRef]
123. Nazareth, T.M.; Bordin, K.; Manyes, L.; Meca, G.; Mañes, J.; Luciano, F.B. Gaseous Allyl Isothiocyanate to Inhibit the Production of Aflatoxins, Beauvericin and Enniatins by *Aspergillus parasiticus* and *Fusarium poae* in Wheat Flour. *Food Control* **2016**, *62*, 317–321. [CrossRef]
124. Small, I.M.; Flett, B.C.; Marasas, W.F.; McLeod, A.; Stander, M.A.; Viljoen, A. Resistance in Maize Inbred Lines to *Fusarium verticillioides* and Fumonisin Accumulation in South Africa. *Plant Dis.* **2012**, *96*, 881–888. [CrossRef]
125. Trapp, M.A.; De Souza, G.D.; Rodrigues-Filho, E.; Boland, W.; Mithöfer, A. Validated Method for Quantification in Plants. *Front. Plant Sci.* **2014**, *5*, 417.
126. Paznocht, L.; Kotíková, Z.; Šulc, M.; Lachman, J.; Orsák, M.; Eliášová, M.; Martinek, P. Free and Esterified Carotenoids in Pigmented Wheat, Tritordeum and Barley Grains. *Food Chem.* **2018**, *240*, 670–678. [CrossRef] [PubMed]
127. Delgado, R.M.; Sulyok, M.; Jirsa, O.; Spitzer, T.; Krska, R.; Polišenská, I. Relationship between Lutein and Mycotoxin Content in Durum Wheat. *Food Addit. Contam. Part A* **2014**, *31*, 1274–1283. [CrossRef] [PubMed]
128. Hidalgo, A.; Fongaro, L.; Brandolini, A. Colour Screening of Whole Meal Flours and Discrimination of Seven *Triticum* Subspecies. *J. Cereal Sci.* **2017**, *77*, 9–16. [CrossRef]
129. Colasuonno, P.; Lozito, M.L.; Marcotuli, I.; Nigro, D.; Giancaspro, A.; Mangini, G.; De Vita, P.; Mastrangelo, A.M.; Pecchini, N.; Houston, K.; et al. The Carotenoid Biosynthetic and Catabolic Genes and Their Association with Yellow Pigments. *BMC Genet.* **2017**, *18*, 122. [CrossRef]
130. Golkari, S.; Gilbert, J.; Prashar, S.; Procunier, J.D. Microarray Analysis of *Fusarium graminearum*-Induced Wheat Genes: Identification of Organ-Specific and Differentially Expressed Genes. *Plant Biotechnol. J.* **2007**, *5*, 38–49. [CrossRef]
131. Gunnaiah, R.; Kushalappa, A.C.; Duggavathi, R.; Fox, S.; Somers, D.J. Integrated Metabolo-Proteomic Approach to Decipher the Mechanisms by Which Wheat QTL (Fhb1) Contributes to Resistance against *Fusarium graminearum*. *PLoS ONE* **2012**, *7*, e40695. [CrossRef]
132. Zykin, P.A.; Andreeva, E.A.; Lakholay, A.N.; Tsvetkova, N.V.; Voloylokov, A.V. Anthocyanin Composition and Content in Rye Plants with Different Grain Color. *Molecules* **2018**, *23*, 948. [CrossRef] [PubMed]
133. Gordeeva, E.I.; Shoeva, O.Y.; Khlestkina, E.K. Marker-Assisted Development of Bread Wheat Near-Isogenic Lines Carrying Various Combinations of Purple Pericarp (Pp) Alleles. *Euphytica* **2015**, *203*, 469–476. [CrossRef]
134. Syed Jaafar, S.N.; Baron, J.; Siebenhandl-Ehn, S.; Rosenau, T.; Böhmdorfer, S.; Grausgruber, H. Increased Anthocyanin Content in Purple Pericarp x Blue Aleurone Wheat Crosses. *Plant Breed.* **2013**, *132*, 48–58. [CrossRef]
135. Francavilla, A.; Joye, I. Anthocyanins in Whole Grain Cereals and Their Potential Effect on Health. *Nutrients* **2020**, *12*, 2922. [CrossRef]
136. Paznocht, L.; Burešová, B.; Kotíková, Z.; Martinek, P. Carotenoid Content of Extruded and Puffed Products Made of Colored-Grain Wheats. *Food Chem.* **2021**, *340*, 127951. [CrossRef]
137. Lachman, J.; Hejtmánková, A.; Orsák, M.; Popov, M.; Martinek, P. Tocotrienols and Tocopherols in Colored-Grain Wheat, Tritordeum and Barley. *Food Chem.* **2018**, *240*, 725–735. [CrossRef] [PubMed]

138. Spanić, V.; Viljevać Vuletić, M.; Abicić, I.; Marček, T. Early Response of Wheat Antioxidant System with Special Reference to Fusarium Head Blight Stress. *Plant Physiol. Biochem.* **2017**, *115*, 34–43. [CrossRef] [PubMed]
139. Martin, C.; Schöneberg, T.; Vogelsang, S.; Vincenti, J.; Bertossa, M.; Mauch-Mani, B.; Masher, F. Factors of Wheat Grain Resistance to *Fusarium* Head Blight. *Phytopathol. Mediterr.* **2017**, *56*, 154–166.
140. Bernardi, J.; Stagnati, L.; Lucini, L.; Rocchetti, G.; Lanubil, A.; Cortellini, C.; De Poli, G.; Busconi, M.; Marocco, A. Phenolic Profile and Susceptibility to *Fusarium* Infection of Pigmented Maize Cultivars. *Front. Plant Sci.* **2018**, *9*, 1189. [CrossRef]
141. Lorenz-Kukuła, K.; Wróbel-Kwiatkowska, M.; Starzycki, M.; Szopa, J. Engineering Flax with Increased Flavonoid Content and thus *Fusarium* Resistance. *Physiol. Mol. Plant Pathol.* **2007**, *70*, 38–48. [CrossRef]
142. Long, L.; Liu, J.; Gao, Y.; Xu, F.C.; Zhao, J.R.; Li, B.; Gao, W. Flavonoid Accumulation in Spontaneous Cotton Mutant Results in Red Coloration and Enhanced Disease Resistance. *Plant Physiol. Biochem.* **2019**, *143*, 40–49. [CrossRef] [PubMed]

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