

Article

# Exploring Fusarium head blight resistance in a winter triticale germplasm collection

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**Abstract:** Fusarium Head Blight (FHB) is a destructive disease affecting the grain yield and quality of wheat, barley, rye and triticale. Developing varieties with genetic resistance is integral to successfully managing FHB. However, significant knowledge gap exists in the genetic diversity present in triticale for FHB resistance. This information is critical for breeding new varieties of triticale as its production continues to increase. In the present study, a set of 298 winter triticale accessions from a worldwide collection were screened for their type-2 FHB resistance in an artificially inoculated misted nursery with high levels of inoculum density. Most of the triticale accessions were susceptible to FHB, and only 8% of accessions showed resistance in the field nursery screening. The resistant accessions identified in the nursery screening were selected and further screened for three years in greenhouse conditions. Seven accessions were found to show robust FHB resistance over the three years of greenhouse testing. Thirteen accessions showed significantly lower levels of Deoxynivalenol accumulation when compared to the susceptible triticale control. The accessions identified in the study will be useful in triticale and wheat breeding programs for enhancing FHB resistance and reducing DON accumulation.

**Keywords:** Fusarium head blight; deoxynivalenol; triticale; genetic resistance; disease evaluation

## 1. Introduction

Triticale ( $\times$ Triticosecale Wittmack) is a man-made cereal crop developed by hybridizing wheat (*Triticum* spp.) and rye (*Secale cereale* L.). It combines the superior grain quality and high yield potential of wheat with resistance to abiotic and biotic factors of rye [1], [2]. European countries have spearheaded the development and breeding of Triticale to adapt it to diverse environments and soil conditions [3]. With more than 92% of world's Triticale being grown in Europe; Poland, Germany, France, and Belarus are the top producer countries of Triticale (FAOSTAT, 2019). The first improved commercial Triticale cultivar was released in Hungary in 1968 [5]. In North America, Triticale breeding started at the University of Manitoba in 1954, and the first variety Rosner, was released in 1969 [6]. In the USA, breeding efforts for Triticale have just started towards the end of the last century [7]. In 2012, ~0.45 million acres was under triticale cultivation in the USA, whereas in 2015 this acreage increased to ~0.77 million acres. Further, in 2020, ~1.19 million acres of land in the USA was under triticale cultivation (<https://www.fsa.usda.gov/news-room/efoia/electronic-reading-room/frequently-requested-information/crop-acreage-data/index>). This increased cultivation demonstrates the constantly increasing production and popularity of triticale in the USA. Triticale is being used as a superior forage crop because of its high biomass yield, high protein content, high digestibility coefficient and good amino acid profile [7]. Triticale has also gained popularity as a cover crop because of its high nitrogen use efficiency and biotic and abiotic

stress tolerance, providing it an advantage over nutrient poor soils over traditional cereal crops [5], [7], [8].

Fusarium head blight (FHB), caused by *Fusarium graminearum* in the USA, is a major disease of wheat and barley [9]. In addition to causing direct yield losses worth millions of dollars annually, FHB also contaminates grain with associated mycotoxins such as deoxynivalenol (DON) and nivalenol [9], [10]. These mycotoxins are potent protein-synthesis inhibitors, and symptoms associated with their intake in humans include headache, fever, emesis, diarrhea, and loss of appetite [11]–[13]. In animals, it leads to reduced feeding, poor growth, lower egg production, reduced carcass quality, poor fertility and hatchability of eggs and immunosuppression [12], [14]. Genetic resistance is one of the major strategies of managing FHB and DON accumulation in wheat and barley [9], [15].

Genetic resistance against FHB is quantitative in nature, and more than 550 QTL with varying effects on FHB severity and DON content have been reported in wheat [16], [17]. Several wheat varieties with at least some level of effective resistance are available in all the wheat growing regions of the world [16], [17]. Triticale, being a synthetic crop developed by combining wheat and rye, is expected to be susceptible to FHB and DON accumulation [18]–[21]. Veitch et al. (2008) analyzed 7 winter type and 5 spring type triticale varieties in multi-year and multi-site tests, and found them to have higher FHB susceptibility and DON content accumulation as compared to the wheat checks. Similarly, Góral et al. [19] analyzed 32 winter triticale and 34 winter wheat accessions and found that FHB severity and Fusarium damaged kernel percentages were lower for triticale, whereas DON content was higher in triticale than wheat. However, all these experiments have screened a relatively small number of local lines, not providing a clear information on the frequency of high level of FHB genetic resistance among larger more diverse collections of triticale.

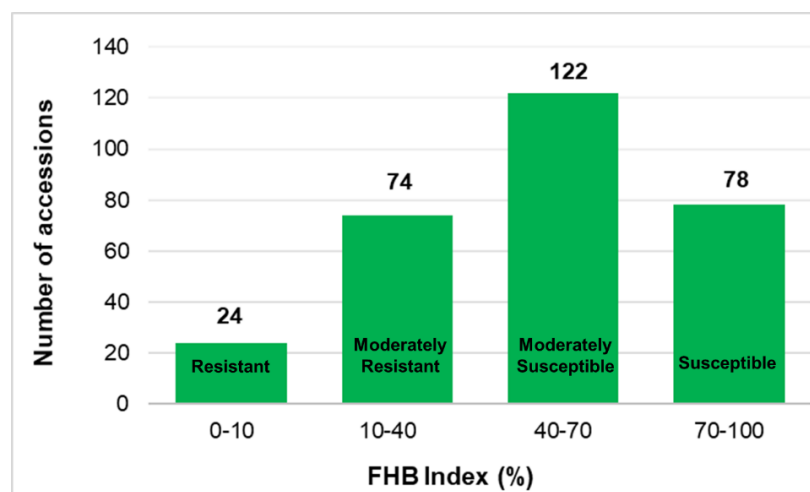
In the present study, we screened type-2 FHB resistance of a large set of diverse winter-type triticale collection comprising two hundred and ninety-eight accessions, under high FHB pressure misted nursery field conditions. Field studies for FHB evaluation involve considerable Genotype\*Environment interaction, making it difficult to analyze the effectiveness of genetic resistance [21], [22]. Therefore, the lines showing high level of type-2 resistance selected from the field screening were tested in greenhouse conditions for two years. Subsequently, a small set of highly resistant triticale accessions were tested in greenhouse a third time for type-2 FHB resistance and DON content accumulation, with the goal of identifying robust sources of genetic resistance for FHB that can be used in triticale breeding. In addition, if stable, these sources could also be used for wheat and barley improvement.

## 2. Results

### 2.1. Evaluation of FHB index, plant height and flowering times

A wide range of diversity for FHB indices was observed among the 298 winter-type accessions screened in Fusarium-inoculated corn spawn misted nursery field conditions. Disease Index among the accessions varied from 3% to 100%, showing: a) maintenance of very high-disease pressure conditions in the nursery, and b) wide range of diversity present in the triticale panel. Figure 1 shows the frequency distribution of accessions according to FHB indices. The accessions were divided into four groups: resistant (0-10% FHB index), moderately resistant (10-40% FHB index), moderately susceptible (40-70% FHB index) and susceptible (70-100% FHB index). With 24 accessions classified in group-1, the resistant group was the smallest among all the four groups. Groups 2, 3, and 4 contained 74, 122, and 78 accessions, respectively. The 24 lines from group-1 having lowest FHB index in field testing in 2017 were selected for further greenhouse testing in 2018 and 2019. Susceptible accession UMDTE\_197, having a disease index of 100% was used as a susceptible check in all the greenhouse experiments. Plant height of the 298 accessions showed a wide distribution ranging from 54 cm to 174 cm, whereas flowering times ranged from 187 days to 220 days after planting (Supplementary Table 1). The height of the selected 24 accessions ranged from 75 cm to 142 cm, whereas flowering times of the selected set varied

from 187 days to 210 days after flowering, indicating absence of association of FHB index with plant height or flowering times.



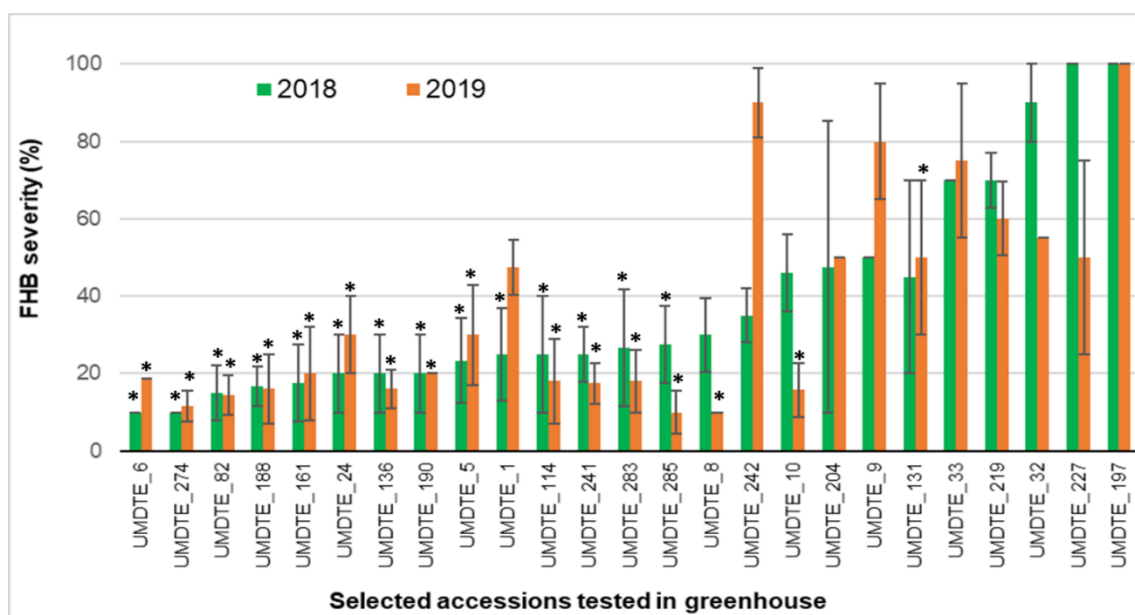
**Figure 1.** Frequency distribution of the 298 accessions into four groups according to their FHB index in field testing.

## 2.2. Greenhouse evaluation of FHB severity in selected resistant lines in 2018 and 2019

In greenhouse conditions, the selected resistant accessions showed a wide range of FHB severity in 2018 and 2019 (Figure 2). A two-way ANOVA with interaction revealed significant genotype effect at  $p < 0.001$  whereas no significant Genotype\*Year interaction was observed (Table 1). Susceptible check Triticale accession UMDTE\_197 showed disease severity of 100% in both years. In 2018, fourteen accessions (UMDTE\_6, UMDTE\_274, UMDTE\_82, UMDTE\_161, UMDTE\_188, UMDTE\_24, UMDTE\_190, UMDTE\_136, UMDTE\_114, UMDTE\_5, UMDTE\_283, UMDTE\_1, UMDTE\_241, and UMDTE\_285) showed significantly lower FHB severity as compared to the susceptible check UMDTE\_197. In 2019, seventeen accessions showed resistance, out of which fourteen were common with 2018. Additionally, three accessions UMDTE\_8, UMDTE\_10 and UMDTE\_131 showed significantly higher resistance than control. In 2018 also these three accessions had numerically lower mean FHB severity (albeit statistically not different at  $\alpha = 0.01$ ) than that of control UMDTE\_197. DON content accumulation was not measured in either 2018 or 2019. A final set of 17 accessions along with susceptible control UMDTE\_197 showing low FHB severity in both the years was selected for a final evaluation of FHB severity and DON content accumulation in 2020 in greenhouse.

**Table 1.** Analysis of variance of FHB severity recorded in 2018 and 2019 greenhouse testing of selected 24 accessions.

Response: FHB_Severity(%)_trans ( Type III tests)				
	Sum Sq	Df	F value	Pr(>F)
(Intercept)	12.000	1	205.7322	<2.2e-16 ***
Genotype	5.1211	24	3.6582	9.884e-07 ***
Year	0.0000	1	0.0000	1.0000
Genotype: Year	1.7799	24	1.2715	0.1969
Residuals	7.4660	128		



**Figure 2.** FHB severity of selected 24 accessions and susceptible control UMDTE\_197 tested in the greenhouse in 2018 and 2019. Error bars represent standard deviation. ‘\*’ indicate significant difference from control at  $\alpha=0.01$ .

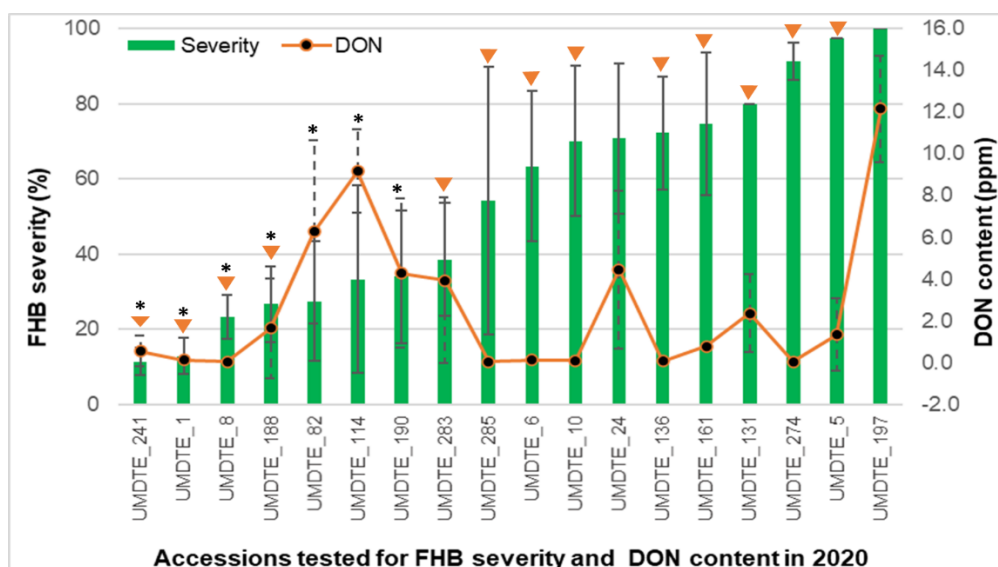
### 2.3. Greenhouse evaluation of FHB severity and DON content of final selected set in 2020

A one-way ANOVA for FHB severity of the selected 17 accessions along with susceptible check UMDTE\_197, tested under greenhouse conditions, revealed significant genotypic effect at  $p < 0.001$  (Table 2). Seven accessions (UMDTE\_241, UMDTE\_1, UMDTE\_8, UMDTE\_188, UMDTE\_82, UMDTE\_114, UMDTE\_190) showed significantly lower mean FHB severity as compared to the susceptible check, which showed a 100% average FHB severity (Figure 3). Other accessions had numerically lower, but statistically similar mean FHB severity to control.

**Table 2:** Analysis of variance of FHB severity recorded for the selected 17 accessions along with susceptible check in 2020 greenhouse testing.

Response: FHB_Severity(%)_trans ( Type III tests)‡				
	Sum Sq	Df	F value	Pr(>F)
(Intercept)	12.000	1	136.5720	<2.2e-16 ***
Genotype	12.137	17	7.6739	1.235e-13 ***
Residuals	12.389	141		

‡Data log transformed for the analysis.



**Figure 3.** FHB severity and DON content of the final set of selected 17 accessions and susceptible control UMDTE\_197 tested in the greenhouse in 2020. “\*” indicate significant difference from control for FHB severity at  $\alpha=0.01$ . Orange triangles indicate significant lower DON content from control at  $\alpha=0.01$ . Solid black error bars indicate standard deviation in FHB severity, dashed error bars indicate standard deviation in DON content.

ANOVA for DON content in the 2020 greenhouse test showed significant genotypic effect at  $p<0.001$  (Table 3). With the average DON content of 12.2 ppm, susceptible check UMDTE\_197 was found to have highest DON accumulation among all the tested accessions. Thirteen accessions (UMDTE\_285: 0 ppm, UMDTE\_8: 0 ppm, UMDTE\_274: 0.1 ppm, UMDTE\_136: 0.1 ppm, UMDTE\_10: 0.1 ppm, UMDTE\_1: 0.1 ppm, UMDTE\_6: 0.1 ppm, UMDTE\_241: 0.5 ppm, UMDTE\_161: 0.8 ppm, UMDTE\_5: 1.3 ppm, UMDTE\_188: 1.6 ppm, UMDTE\_131: 2.4 ppm, and UMDTE\_283: 3.9 ppm) were found to have significantly lower DON content than the control (Figure 3). It is important to note that accessions UMDTE\_82, UMDTE\_114, and UMDTE\_190 had low average FHB severity, but had statistically similar mean DON content to the control, indicating different regulation of these two parameters of FHB resistance.

**Table 3.** Analysis of variance of DON content recorded for the selected 17 accessions along with susceptible check in 2020 greenhouse testing.

Response: DON_trans ( Type III tests)‡				
	Sum Sq	Df	F value	Pr(>F)
(Intercept)	36.206	1	67.9290	8.307e-10***
Row_no	51.023	17	5.6312	6.753e-06 ***
Residuals	19.188	36		

‡Data square-root transformed for the analysis.

### 3. Discussion

Genetic resistance is one of the most important strategies of FHB management and in wheat over 500 QTL with major or minor effect on the trait have been reported and many have been utilized in breeding [16], [17], [21]. Triticale is a synthetic crop and is

gaining popularity as a livestock feed crop and a cover crop [7]. However, limited information is available on genetic FHB resistance in Triticale [20], [23]. In this study, we systematically screened a large collection of Triticale accessions for identification and confirmation of accessions that have a high level of FHB resistance and low DON content accumulation. In the field testing in year-1 of the study, 298 accessions were screened in a corn-kernel inoculated FHB misted nursery. High FHB pressure was maintained in the nursery as indicated by highest FHB index shown by at least 26% of the tested lines (Figure 1). A relatively small number of lines (8%) showed high level of FHB resistance in the field screening, whereas the majority of the accessions (67%) were moderately to highly susceptible. Since field evaluation of FHB response of the plants is subject to genotype\*environment interaction, the lines selected for their low FHB index from the field were subsequently tested for three more seasons in greenhouse condition with point inoculation. The final number of lines having consistently very low FHB severity was narrowed down to 7 after further rounds of testing. The consistent sifting of the lines removed several accessions with every round of testing, which might have shown misleading phenotypes on account of the disease escape. As a consequence, the final selected resistant lines represent only 2.3% of the original set. This very small percentage of lines with robust resistance is not surprising, considering that most of the resistant sources in wheat have been reported from Asian sources [15], [16], but most of the Triticale lines are of European origin. Even in wheat, a previously reported analysis of 34,571 wheat landraces and other germplasm from China and Japan identified 1,765 lines showing high levels of FHB resistance [24], which accounted for 5.1% of the lines. The small set of lines identified in the present study having low FHB severity and DON content constitute useful sources of FHB resistance for use not only in Triticale breeding programs, but also for wheat improvement.

Plant height has been reported to be associated with FHB severity, with shorter genotypes developing more severe disease [25]–[28]. This might be specifically applicable for field-based screening, as chances of splash dispersal of *Fusarium* spores to the spikes are higher at levels closer to the soil [28], [29]. In the present work, FHB severity was not found to be correlated to plant height ( $r=0.03$ ). In the final set of 7 resistant accessions obtained after three rounds of greenhouse testing displayed a wide range of plant height (75 cm – 142 cm), with three accessions having average heights of less than 100 cm, further confirming the lack of association between plant height and FHB severity. Although Miedaner and Voss reported increased FHB severity in reduced height mutants of a set of near isogenic lines for reduced height genes in wheat field testing, they concluded that the contribution of reduced height to FHB severity can be counteracted by a more resistant genetic background [27]. This supports that genetic resistance is independent of plant height and is consistent with our observations.

Correlations between heading and flowering times with FHB severity have previously been reported in wheat, rye and triticale [25], [26], [30], [31]. Early flowering lines are considered predisposed to having higher FHB intensity [25]. However, FHB severity and heading time were not found to be correlated in our study ( $r=0.03$ ). The heading times of the final seven resistant lines in our study varied considerably with four of the lines having the shortest heading times. This discrepancy might be related to the fact most of these previous studies were conducted in field where environmental factors may favor earlier development of disease. All of these traits are pleiotropic and multigenic and association among them becomes complicated in field conditions. In our final set of lines, which were confirmed multiple times in controlled environmental conditions, we did not find any particular association between FHB severity and heading times.

It is important to note that the DON content of the finally selected triticale accessions did not show a correlation with the FHB severity ( $r=0.1$ ). Only 5 of the thirteen low DON content lines also had significantly lower FHB severity in the final 2020 set compared to the susceptible control (Figure 3). Three accessions having low FHB severity displayed DON content similar to the susceptible check, whereas 9 accessions with low DON content had FHB severity statistically similar to the control. Miedaner et al. also reported poor correlation between DON content and FHB severity in a doubled haploid population of

146 individuals of triticale and, concluded that prediction of DON content from FHB severity was not possible [32]. Such poor correlation has also been reported for wheat [33], [34]. Paul et al. (2005) performed a meta-analysis of 163 studies in wheat reporting FHB visual symptoms and DON content and found a low level of correlation between FHB incidence and DON content. In fact, the correlation between FHB severity and DON content in triticale has been reported to be even lower than in wheat [32], [35]. All these studies, including the current study indicate that there are different genetic controls or mechanisms of FHB severity and DON accumulation that appear to be independent of each other. This indicates that breeding efforts must focus on both the traits separately. The lines rigorously phenotyped and identified in the present work that have low FHB severity and DON content provide sources of genetic resistance for breeding improved triticale and wheat varieties.

#### 4. Materials and Methods

##### 4.1. Plant material and Experimental Design for Field Testing

Experiments were conducted at the Beltsville Research Farm Facility of the University of Maryland (-76.833195 longitude, 39.011599 latitude) during the planting season of 2017-2018. A set of 298 diverse winter-type Triticale accessions was obtained from the National Small Grains Collection (NSGC) Repository, Aberdeen, Idaho, USA. Fifty seeds of each accession were planted as 4 feet long single rows. Plant height and flowering times were recorded for each line. All experiments were conducted on no-till plots with corn-stubble from previous growing cycles to ensure high inoculum load for infection of the plants, as crop residues are known to enhance FHB in wheat [36].

##### 4.1.1. Fungal Inoculation for Field testing

Three *F. graminearum* isolates collected from Maryland (one each from Clarksville, Beltsville and Wye farm locations in the state) were used for generating corn-spawn inoculum for field. Corn kernels inoculated with Fungal plugs from 50% glycerol stocks were cultured on Potato Dextrose Agar (PDA) plates for each isolate and grown at room temperature. Maize kernels (13-16 lbs) were rinsed with water and autoclaved twice for 30 min with each autoclave cycle. One-week after starting fungal cultures each corn tray was inoculated with cultures from the 3 PDA plates using 150 ml of autoclaved water containing 0.2 g streptomycin sulfate to prevent bacterial contamination. Inoculated trays were covered with aluminum foil and incubated at room temperature for 2.5 weeks. Fungal growth was monitored at weekly intervals by observing pink pigmentation and white mycelial growth. After 2.5 weeks the inoculum was transferred to autoclaved burlap sacks to half of their capacity and then dried for 1 week at 35° C [37]. At the tillering stage the corn-spawn inoculum was manually spread in the field at an application rate of 40 g inoculum per square meter. High humidity was maintained at the inoculated site by artificially by daily misting the field overnight for 5 minutes every hour from 9 pm – 6 am until the latest flowering line reached anthesis, after which the misting was stopped.

##### 4.1.2. Field FHB index data

Disease incidence and severity were evaluated as indicators of FHB spread. Readings were taken 25 days after anthesis for each line. Twenty spikes per row were randomly selected for phenotyping. Disease incidence (DI) was calculated by visually assessing the percentage of spikes infected in a whole head row. Disease severity (DS) was calculated by taking average of number of diseased spikelets per spike. Diseased spikelets looked pre-maturely bleached whereas healthy spikes were still green. Disease Index was calculated as a product of DI and DS divided by 100. Lines were classified into different FHB response groups based on their Disease Index.

On the basis of Disease Index scores, a subset of 24 lines was selected for further testing for type-2 resistance in the greenhouse in year 2018 and 2019. Disease index up to 10% was chosen as the criterion for selection.

#### 4.2. Greenhouse testing

##### 4.2.1. Greenhouse planting

Seeds of the selected 24 accessions from field testing were planted in the greenhouse in 2018 and 2019. In 2020 a smaller subset of 18 lines were planted to analyze FHB severity and DON accumulation. In each year, three plants per accession were grown in individual 6-inch pots (1 plant/ pot) and vernalized for 6 weeks at 4 °C. Following vernalization plants were transferred to a greenhouse with a day temperature of 23-25 °C, a night temperature of 16-18 °C, and 16 hour of light and 8 hour of darkness.

##### 4.2.2. Fungal Inoculum preparation

*F. graminearum* isolate GZ3639, known for its strong virulence (Desjardins et al., 1997; Rawat et al., 2016), was used for all the greenhouse experiments. For macroconidia production, 2 plugs of Potato Dextrose Agar mycelial culture of the fungus were inoculated in Mung bean broth, which was shaken at 200 rpm at 28°C for 7-10 days. Macroconidia were counted on a hemocytometer and inoculum was prepared by diluting the culture to a concentration of  $1 \times 10^5$  spores/ml using sterile water.

##### 4.2.3. Inoculation strategy and FHB severity measurement

Inoculation was performed at pre-anthesis stage on spikes, which was about 2 days prior to anthers emerging out of the spikes. The tenth and eleventh spikelets (counted from the base of the spikes) were marked with a black sharpie pen, and 10 µl macroconidial inoculum was injected between the lemma and palea of the florets (one floret/spikelet), avoiding injury to any other part of the florets. Spikes were covered with moisture saturated zip lock bags for 72 hours to provide high humidity for optimal fungal growth. For each genotype, 8-10 spikes were tested in each experiment. FHB severity was calculated by dividing the number of bleached spikelets downward from the point of inoculation by 10 and multiplying by 100.

##### 4.2.4. DON content measurement

DON content of seeds from the 18 lines selected for 2020 testing was measured by GC/MS following Mirocha et al. (1998). Seeds from infected spikes from all the three plants of each accession were manually threshed, bulked and divided in three technical replicates. Briefly, 1 g of ground samples were extracted with 12 mL of acetonitrile/water (84/16, v/v) in 15 mL centrifuge tubes. Each sample was placed on a shaker for 24 hrs, and then 4 ml of the extract was passed through a column packed with C18 and aluminum oxide (1/3, w/w). Two milliliter of the filtrate was evaporated to dryness under nitrogen at room temperature, and 70 µl of Trimethylsilyl (TMS) reagent (TMSI/TMCS, 100/1) was added to the vial, rotating the vial so that the reagent makes contact with residue on the sides of the vial. The vial was placed on a shaker for 10 min, and then 700 µL of isooctane containing 0.5 µg/mL mirex was added and shaken gently. HPLC water (700 µl) was added to quench the reaction and the vial was vortexed so that the milky isooctane layer becomes transparent. The upper layer was transferred into a GC vial for GC/MS analysis (Shimadzu GCMS-QP2020, Shimadzu Corporation, Kyoto, Japan) and readings were recorded.

#### 4.3. Statistical Analysis

Data was analyzed in R (vR x64 3.6.3) and R studio using packages lme4, car, and ggplot2. All experiments were conducted in a Completely Randomized Design (CRD). Each spike was considered as an individual replicate. Data was analyzed for normality and homogeneity of variance assumptions beforehand. Genotype and year were both

considered as fixed effects. A two-way ANOVA (Type-3) with interaction was performed for 2018 and 2019 FHB severity data, whereas one-way ANOVA (Type-3) was performed for 2020 FHB severity data and 2020 DON content data. Log<sub>10</sub> transformation was done for FHB severity data that was not normalized. Square root transformation was done for DON content data. Pair-wise comparisons between susceptible check (UMDTE\_197) and other selected genotypes were done with post-hoc test on Least Significant Difference at  $\alpha=0.01$ .

**Supplementary Materials:** Supplementary Table 1 with Accession numbers, flowering times and height data of winter Triticale used in the study has been provided online.

**Author Contributions:** Conceptualization, N.R. and V.T.; methodology, S.W.; Statistical analysis, B.C.; DON content measurement, Y.D.; Triticale accessions, X.M.; Funding acquisition, N.R., V.T., G.C.; writing—original draft preparation, N.R., S.W. All authors have read and agreed to the published version of the manuscript.

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