An experimental approach to study multi-species interactions implicated in Fusarium-Head Blight complex assembly in wheat.

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Miguel Ángel Corrales Gutiérrez[1], Harleen Kaur[1], Francesca Desiderio[2], Dimitar Kostadinov Douchkov[3], Jiasui Zhan[1], Heriberto Vélëz[1,4,*], Salim Bourras[5*].

Authors affiliation

[1]. Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences. Almas Allé 5, 75007 Uppsala, Sweden.

[2]. Council for Agricultural Research and Economics (CREA) - Research Centre for Genomics and Bioinformatics, Fiorenzuola d'Arda, Italy

[3]. Leibniz-Institute of Plant Genetics and Crop Plant Research, 06466-Gatersleben, Germany.

[4]. Current address: College of Science, Mathematics and Technology. Wenzhou-Kean University. 88 Daxue Rd, Ouhai, Wenzhou, Zhejiang Province 325060, China.

[5]. Department of Plant Biology, Swedish University of Agricultural Sciences. Almas Allé 5, 75007 Uppsala, Sweden.

Authors for correspondence

Dr. Heriberto Vélëz

hvelez@kean.edu

Prof. Dr. Salim Bourras

salim.bourras@slu.se

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Diagnostic of head blight and leaf blotch diseases of wheat in Sweden using a mycobiome isolation approach.

Harleen Kaur, Elisa Vilvert, Miguel Ángel Corrales Gutiérrez, Jiasui Zhan, Dimitar Douchkov, Francesca Desiderio, Heriberto Vélëz, Salim Bourras.

Abstract

Fusarium Head Blight (FHB) is a major disease of cereal crops present everywhere where wheat is produced. A complex of several, distinctly different, species from the genus *Fusarium* are responsible for causing the disease, with important taxa being *F. graminearum*, *F. culmorum*, and *F. avenaceum*. FHB is an adult stage, late season disease affecting wheat at anthesis. However, recent discoveries are now challenging the basic assumption that anthers and spikes are the only ecological niches relevant for resistance to FHB. Most prominently, it has been established that pathogens typically occurring earlier in the season, such as *Zymoseptoria tritici*, induce strong systemic changes in the plant immune system, that expand way beyond their primary ecological niche. Furthermore, taxa implicated in FHB can be isolated from virtually all wheat tissues, including typical blotch symptoms on wheat leaves. Strikingly, such important biological observations about the complex lifestyle of FHB causing taxa are overlooked as potential targets in resistance breeding. In this work we present a simplified approach allowing the dissection of the intricate lifestyles of the FHB causing *Fusarium* taxa. In doing so, we provide an experimental proof-of-concept that systemic signals from *Z. tritici* can affect the basic response of wheat to *F. graminearum*. We further demonstrate that differences in *Fusarium* species composition affect the outcome of the interaction in a genotype-specific manner. We argue that efforts to study hidden layers of the biology of FHB associated taxa are crucial to developing new resistance breeding strategies, based on a better understanding of the genetic drivers of the disease.

Introduction

Fusarium Head Blight (FHB) is a wheat disease caused by a multispecies complex of up to 17 distinctly different *Fusarium* and non-*Fusarium* taxa (Karlsson *et al*., 2021). In nature, *Fusarium* ssp. are ubiquitous colonizers of monocot grasses that can be found in all tissues from roots to seeds. This is consistent with the piling evidence suggesting that pathogenic *Fusarium* taxa have evolved from endophytic *Fusarium* species, including those implicated in FHB (Hill *et al*., 2022; Ma *et al*., 2013). Despite this evolutionary trajectory, the *Fusarium* genomes have no clear signatures of lifestyle transition, even though such events are frequent in *Fusarium* fungi (Hill *et al*., 2022). This is strikingly contrasting with other ubiquitous colonizers such as Hypocreales fungi, where comparative genomic studies have revealed clear, lifestyle specific, genetic signature underlying the ability of these fungi to inhabit different hosts and environments (Wu & Cox, 2021). This suggests that lifestyle requirements in *Fusarium* fungi are very plastic, which is indeed the case with those associated with FHB. For instance, *F. graminearum* can survive as a saprophyte on wheat debris, while the most common pathogenic taxa implicated in FHB including *F. graminearum*, *F. culmorum*, and *F. avenaceum* can be found as latent endophyte inhabiting wheat stems, leaves, and roots (Kaur & Vilvert, *et al*., 2024; Lidia *et al*., 2021). Furthermore, it was also shown that FHB associated taxa can cause blotch-like symptoms on wheat leaves thus demonstrating that lifestyles shifts can occur in tissues where *Fusarium* spp. are present as silent endophytic colonizers (Kaur & Vilvert, *et al*., 2024).

Altogether, these data indicate that FHB causing taxa are present in the wheat host, very early in the season, throughout development, prior to anthesis. This is major because the etiology of FHB is often assimilated to the biology of *F. graminearum* with a two-step process is initiated at anthesis by for instance airborne spores landing in the anthers, which further spread through the rachis. Several studies have addressed the relative predominance of the different taxa implicated in FHB in relation to years and/or location. In Australia, several FHB epidemics in the 1980s, 1990s, and 2000s were incited by *F. pseudograminearum* (Burgess *et al*., 1987; Obanor *et al*., 2013; Obanor & Chakraborty, 2014). This is striking, because *F. pseudograminearum* is mostly associated with a disease called Fusarium Crown Rot, which affects a distinctly different wheat tissue at a much earlier developmental stage (Kazan & Gardiner, 2018). Thus, there can be large variations in complex composition, including several cases where *F. graminearum* was not the most prevalent species (Doohan *et al*., 2003; Lukanowski *et al*., 2008; Parry *et al*., 1995; Waalwijk *et al*., 2003; Xu *et al*., 2005). For

instance, a field study evaluated the percentage of FHB infected wheat ears colonised by *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, and *M. nivale* in seven location throughout east and west Flanders in Belgium (Audenaert *et al*., 2009). The authors found large variation in complex composition across wheat genotypes and environments, with several cases where *F. poae* was the most predominant species causing FHB (Audenaert *et al*., 2009). In Italy, a large population study showed large shifts in FHB complex composition where *F. graminearum* was replaced by *F. poae* as the main taxon, and even supplanted by *M. Nivale* and *F. verticillioides* (Valverde-Bogantes *et al*., 2020). Finally in Kenya, a field survey where the incidence and severity of FHB were assessed at the hard dough stage showed that *F. avenaceum*, *F. poae* and *F. tricinctum* were the most abundant taxa. In one case. *F. graminearum* was the lowest in abundance of all isolated taxa (Maina *et al*., 2016) .

This again demonstrates the ecological plasticity of FHB associated taxa, further highlighting the importance of lifestyle transition as a potential driver of FHB, and strengthening the argument that resistance to this disease cannot be reduced to *F. graminearum*. It also challenges to genetic assumptions restricting the definition of resistance to FHB to resistance to initial infection (Type I), resistance to the spread of the fungus through the rachis (Type II), resistance to toxin accumulation or the ability to degrade the mycotoxins (Type III), resistance to kernel infection (Type IV), and finally tolerance to yield loss (Type V). Strikingly, all types are restricted to flower/spike as the only tissue relevant to resistance, and all except 'Type IV' have a strong developmental stage-dependent, structural component. Furthermore, 'Type IV' is strongly challenged by recurrent and repeated observations that associations between mycotoxin production and FHB severity are inconsistent, which is a reasonable expectation considering piling evidence that *Fusarium* mycotoxins as antimicrobial compounds primarily directed against other microbes (i.e. not the host) (Sweany *et al*., 2022). Furthermore, *Fusarium* mycotoxin production in wheat is possibly a reminiscence of ancient association with grasses (i.e. the wild ancestors of cereals) where such compounds provide protection against herbivores (Gerling *et al*., 2023). Indeed, wild grasses serve as reservoir for *Fusarium* infections and mycotoxin contamination of wheat, which pinpoints the importance of understanding the endophytic lifestyle and its plasticity among members of the FHB species complex (Gerling *et al*., 2023).

Thus, the very genetic models serving as a basis for resistance breeding fail at accounting for such complexity, and the interplay between different members of the FHB complex. Similarly,

such models also overlook important findings highlighting the importance of pathogen-induced systemic signals to wheat immunity (Seybold *et al*., 2020). It has been shown that infection with the foliar wheat pathogen *Zymospetoria tritici* induces systemic and long-lasting immunosuppression in the host (Seybold *et al*., 2020). Such effect was observed in distant organs and tissues that were not infected by *Z. tritici*, thus suggesting that early-stage infection of wheat with foliar pathogens can alter the response to disease occurring later in the season with the most prominent example being FHB. Another striking observation challenging the resistance 'Type' nomenclature, is that none of the 556 QTL identified in wheat as conferring resistance to FHB correspond to a major locus with dominant effect (Steiner *et al*., 2017; Venske *et al*., 2019). Furthermore, not a single immune receptor has been shown to control any form of resistance to FHB (Steiner *et al*., 2017; Venske *et al*., 2019). This is striking considering that the exceptional ability of *Fusarium* spp. to colonize a variety of tissues as silent apoplectic endophytes, which requires initial and permanent suppression of pattern recognition receptors (PRRs) (Saijo *et al*., 2018; Stotz *et al*., 2014). Indeed, the *Fusarium* genomes encode effector proteins, which is a class of fungal factor commonly associated with the suppression of plant immunity in fungal pathogens and non-pathogenic fungal endophytes (Collinge *et al*., 2022; Redkar *et al*., 2022; Rovenich *et al*., 2014). Furthermore, prominent members of the FHB complex such as *F. graminearum*, and *F. culmorum* behave as typical hemibiotrophs in leaf infection assays, with an initial latent, asymptomatic biotrophic phase followed by necrosis induction (Diamond & Cooke, 1999; Kaur & Vilvert, *et al*., 2024).

Thus, the basic expectation is that the wheat immune response to FHB should involve some form of receptor-ligand interaction implicating well defined host immune receptors. However, the ability to genetically explore such hypothesis is largely restricted by the phenotyping strategies used to score FHB, which are largely aligned to the pre-defined resistance types I to V. Most prominently, interactions between FHB causing taxa and the host are almost exclusively assessed from anthesis onwards, with a large focus on scoring blight symptoms on the spikes and mycotoxin accumulation in the grain (Alisaac & Mahlein, 2023; Khan *et al*., 2020; Mesterhazy, 2020). Still, several alternative phenotyping strategies have been developed to allow large phenotypic screens of more complex traits that cannot be implemented in the field. These include detached leaf assays (Diamond & Cooke, 1999), seedling resistance assays (Mesterhazy, 1987), seed germination assay (Browne & Cooke, 2005), and coleoptile infection assay (Wu *et al*., 2005). Strikingly, while none of these strategies is targeting anthesis, all have proven useful for the identification of resistance traits against FHB in wheat and other cereals.

156 Most prominently, and of direct relevance to this work, assays based on artificial infections of
157 detached leaf assay has been particularly used due to the high correlation between pathogen
158 latent period and quantitative disease resistance in the field (Diamond & Cooke, 1999; Niks &
159 Skinnes, 1998).
160
161 In this work we present a phenotyping strategy designed to investigate the host genetic factors
162 affecting the endophytic lifestyle of *Fusarium* taxa associated with FHB and their ability to
163 associate in a complex. We developed a fully featured phenotyping strategy based on highly
164 controlled detached leaves infections, combined with machine-learning aided image analysis
165 allowing accurate quantification of disease symptoms. In doing so, we also produce an
166 experimental proof-of-concept for the potential role of systemic signals and the host genetic
167 background in controlling lifestyle transition and complex assembly, thus providing a working
168 system for mining potentially novel FHB resistance traits.
169
170 **Methods**
171 **Plant growth conditions.**
172 An overview of the phenotyping pipeline is provided in **Figure 1**. In this study, we used the
173 reference hexaploid wheat cultivars Chinese Spring, Fielder, and Bob-white; the breeding lines
174 Agadir, Artico, and Victo which are *Triticum aestivum* spp. *aestivum*; Latino, a *T. turgidum* spp.
175 *durum* cultivar, MG5323, a *T. dicoccum* accession, and Zardak, an old cultivar of *T. turgidum*
176 spp. *turanicum*. We also used 30 commercial spring wheat cultivars commonly grown in
177 Sweden provided by the Swedish Agricultural Cooperative Lantmännen Lantbruk (Sweden).
178 All genotypes were germinated in pots with sterilized substrate at 20-21 °C, RH of 70% and
179 with light intensity of 300 µmol and 18 h light/ 6 h dark photoperiod. Plants for inoculation
180 with *Z. tritici* were maintained in a closed infection chamber inside a growth chamber with the
181 same above conditions, and supplemented with additional LED light sources enriching the light
182 spectrum in the 380 and 700 nM wavelength, respectively (**Supplementary Figure 1**).
183
184 **Preparation of *Zymoseptoria tritici* inoculum**
185 *Z. tritici* isolates ST99CH_3B8 (abbreviated as 3B8), ST99CH_3C4 (3C4), ST99CH_3C7
186 (3C7), ST99CH_3D5 (3D5), ST99CH_3D7 (3D7), ST99CH_3F2 (3F2), ST99CH_3G6 (3G6)
187 were isolated in Switzerland in 1999 (courtesy of Prof. Bruce McDonald, Federal Institute of
188 Technology, ETH Zürich, Switzerland). *Z. tritici* isolates were incubated in yeast extract-malt
189 extract-starch agar medium (YMS 4 g/L yeast extract, 4 g/L malt extract, 4 g/L sucrose, 16 g/L

190 agar), in darkness at 20 °C for 11 days until the day of the infection. Spores were collected
191 from the plates by scraping the cells in sterile conditions and resuspend in TWEEN 0.01%. The
192 solution was filtered with a cloth with pore diameter of 22-25 µm. Spore concentration was
193 determined with a hemocytometer and adjusted in all isolates to $1x10^6$ spores/ml. Before air
194 gun infection, all isolates were mixed at equal volume and concentration.
195
196 **Preparation of *Fusarium* inoculum**
197 *F. avenaceum*, *F. culmorum* and *F. graminearum* were collected from Swedish fields in summer
198 of 2021 and characterized morphologically for species determination, and genotyped by Kaur
199 & Vilvert *et al*. (2024) using universal ITS primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-
200 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and species specific primers for *F.*
201 *graminearum* marker GOFW (5'-ACCTCTGTTGTTCTTCCAGACGG-3') GORV (5'-
202 CTGGTCAGTATTAACCGTGTGTG-3'), and *F. culmorum* with marker Fc03 (5'-
203 TTCTTGCTAGGGTTGAGGATG-3') Fc02 (5'-GACCTTGACTTTGAGCTTCTTG-3')
204 (Astrid Bauer & Seigner, 2015; De Biazio *et al*., 2008). For co-infections with the three
205 *Fusarium* species, isolates were grown for two weeks at 25 °C on oatmeal agar (OMA: 3 g/L
206 organic oatmeal flower and 16 g/L agar) and sporulation was induced by exposure to natural
207 light for 3 days. For co-infections with *Z. tritici*, all *Fusarium* species were incubated in muesli
208 agar (MA: 25 g/L organic muesli: oatmeal, wheat spikes, barley and rye flakes, sunflower
209 seeds, and sunflower lecithin; 2 g/L malt extract and 12 g/L agar), and incubated at 25 °C for
210 30 days. After the incubation period, spores were collected by scratching from the plate and
211 suspended in TWEEN 0.01%. The final spore concentration was adjusted to 250 spores/µL and
212 aliquoted into vials at -20 °C until infection day.
213
214 **Coinfection assays**
215 For coinfections with different *Fusarium* species, 6 cm long leaf segments were cut from 15-
216 days-old wheat seedlings. Leaf segments were punctured and inoculated with 15 µL of a spore
217 solution at the desired concentration. For co-infections with different *Fusarium* species, an
218 equal volume and concentration of spores (10 spores/µL) were mixed into a 15 µL drop and
219 inoculated as described above. As a control, a group of leaf segments were inoculated with
220 TWEEN 0.01%. Leaf segments were placed in Benzimidazole Agar Medium (BAM: 5% water
221 agar supplemented with 0.03 mg/L benzimidazole). Custom frames made of polylactic acid
222 (PLA) were designed and 3D printed on a Flashforge Guider 2s machine (Flashforge). These
223 were used to keep the leaf surface in contact with BAM medium, preventing desiccation and

curling which happens when incubation time extends over 4-5 days. Pictures were taken at 8 dpi using a DSLR camera. Phenotyping of the commercial cultivars, lab standards, and breeding lines was performed using shorter 4 cm length leaf segments, with a spore concentration of 250 spores/µL and an incubation time of 5 days without PLA frames.

For *F. graminearum – Z. tritici* coinfections, 9-days-old wheat seedlings were placed in closed chamber made of two stacked clear PLA containers. The *Z. tritici* inoculum consisted of a mix at an equal ratio of the seven isolates described above, sprayed with a spray gun over the seedlings using 0.33 ml per seedling. As a control, a group of plants were inoculated with TWEEN 0.01%. After inoculation, the climate in the infection chamber was maintained at high humidity (over 70% of RH) and in darkness for 24 hours. Then plants were placed back in a day-night cycle as described above, and kept at high RH by spraying distilled water, using a low-pressure spray gun. Then, 6 cm length leaf segments were cut from *Z. tritici*-infected and mock-inoculated plants at 3 days post infection (dpi). Secondary infections with *F. graminearum* were performed as described above, using a 15 µL drop with a *F. graminearum* spore concentration of 250 sp/µl. Leaf segments were kept on BAM plates and maintained in darkness with high RH for 24 hours. Then, plates were moved back to a day/night cycle at high RH, incubated for 4 days (MG5323) or 5 days (Fielder), then photographed.

Machine learning-aided image analysis

Each leaf segment from the original picture was extracted individually using ImageJ. To quantify disease severity, Jupyter Notebooks were used to run code from the PlantCV and OpenCV packages (Gehan *et al*., 2017). Images showing the juxtaposition of different masks were obtained using the Jupyter Notebooks terminal interface. Bash commands were used to simultaneously analyze multiple samples from one directory with the PlantCV based script, which creates a JSON file as output for further analysis. The implementation of PlantCV allows binary image transformation from original RGB pictures, mask creation, and pixel classification in different predefined classes using a multiclass Naïve Bayes (NB) algorithm.

To define our region of interest (ROI) and reduce the error rate of the algorithm, the picture area inside the PLA frames holding the leaves was extracted. Each leaf segment was extracted as individual image using PlantCV based script (Gehan *et al*., 2017). Pixels were manually captured with ImageJ, and their RGB parameters (red, green, blue) were saved in a tab delimited file where we define each class of pixel. To avoid class imbalance, we collected the

same number of pixels for each class (necrosis, chlorosis, healthy tissue, and background). The training set consisted of over 1000 different pixels from each class. Once the RGB values of each class were defined in a training set, a Naïve Bayes (NB) algorithm (Gehan *et al*., 2017) was used to develop a probability function for each pre-defined class. After the training step, a probability function was used by the NB algorithm to classify each pixel, and the data was exported to JSON file containing the number of counted pixels in each predefined class. Data was transformed to a tabular file and uploaded to the R statistical environment for further analysis.

Statistical analysis

Data was initially tested for normality and homocedasticity, then a linear model with fixed effects was designed after performing an aligned-rank transformation with the values of necrosis or chlorosis (y) as response variable and cultivar (x_1), zymoseptoria (x_2) and its interaction (x_3) as explanatory variables with their corresponding coefficients ($\beta_1, \beta_2, \beta_3$). The constant μ represents the intercept.

$$y = \mu + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3$$

To test statistical differences, we performed an aligned-rank-transformation-two-way-ANOVA (robust two-way-ANOVA) and a *post hoc* test with Tukey adjustment. A Principal Component Analysis (PCA) was carried out with the average value of each cultivar and *Fusarium* species combination (each combination has at least 5 biological replicates). Coordinates of each sample in the PCA were extracted to calculate the distance matrix which was used as input for cluster analysis using a Hierarchical Agglomerative Nesting (AGNES) algorithm. All statistical analyses were performed with R (version 4.2.2) in the Rstudio environment (R Core Team, 2022) and the packages: readxl, dplyr, tidyr, stringr, reshape2, ggplot2, multcompView, forcats, ARTool, rcompanion, factoextra, and cluster (Graves *et al*., 2023; Kassambara & Mundt, 2020; Kay *et al*., 2021; Maechler *et al*., 2022; Mangiafico, 2023; Wickham, *et al*., 2023; Wickham, *et al*., 2023; Wickham, 2007, 2016, 2022, 2023; Wickham & Bryan, 2023).

Results

Phenotypic analysis of single *vs.* mixed *Fusarium* species infection of wheat leaves

We selected the bread wheat cultivar Fielder for this assay based on the consistent and clear phenotypes we obtained in our preliminary tests and our previous work (Kaur and Vilvert, *et al*. 2024). We adapted a leaf segment infection strategy based on previous work demonstrating

291 high correlation between the phenotypes observed in leaf segment assays and quantitative
292 disease resistance to FHB in the field (Kumar et al., 2011; Perochon & Doohan, 2016). Leaf
293 segments were inoculated with *F. avenaceum*, *F. culmorum*, *F. graminearum* and their
294 combinations by drop inoculation (see Methods). At 8 dpi, variability in disease severity among
295 the different pathogens and their combinations was assessed by eye (**Figure 2**). Here, we
296 reasoned that if interactions between *Fusarium* species are a major component of how these
297 species act in a complex, then such assay should lead to clear differential phenotypes i.e.
298 observable by a trained person. Indeed, *F. graminearum* showed the most severe symptoms,
299 triggering necrosis in large areas of the leaf segment. By contrast, the combination of *F.*
300 *culmorum* and *F. graminearum* caused much weaker symptoms as compared to single
301 infections with *F. graminearum*. These results suggest that *F. graminearum* is the most
302 aggressive of the three *Fusarium* species tested, and that there are interactions among *F.*
303 *avenaceum*, *F. culmorum* and *F. graminearum* that could change the plant response and or
304 directly affect complex assembly. We conclude that the detached leaf assay is sensitive to
305 *Fusarium* species composition and can reveal quantitative difference in disease severity
306 resulting from pure *vs*. mixed species inocula.
307
308 **Precision phenotyping using Image-based quantification of disease severity**
309 In another series of experiments, we aimed at developing an image-based precision
310 phenotyping strategy to distinguish and quantify two important components of the observed
311 symptoms, namely: necrosis and chlorosis. We reasoned that these two types of tissue
312 alterations denote different physiological processes taking place during the progression of
313 disease, and their prevalence may vary between genotypes thus pinpointing to distinctly
314 different host factors involved in the response to *Fusarium* sp. We analyzed the pictures using
315 a NB algorithm trained with a set of RGB values from different pixels classified as 'necrosis',
316 'chlorosis', 'healthy tissue' and 'background' (see Methods). We used a test set of 64 leaf
317 segment pictures to test this approach. We were able to clearly separate each type of tissue, and
318 create so called 'masks' (see Methods) allowing the visualization and the quantification of the
319 infected area undergoing necrosis *vs*. chlorosis (**Figure 3**). The results of the quantification of
320 each leaf segment grouped by treatment are illustrated in **Figure 4**. They also could validate
321 that the script correctly identified the infection with *F. graminearum* as the infection which
322 triggers the most severe response (the highest amount of necrotic tissue). The levels of necrosis
323 observed in the control group are close to 0, indicating a high performance of the classifier
324 algorithm. We could also corroborate and quantify the cases where mixed infection resulted in

325 less necrosis than infection with a pure culture of *F. graminearum*. Altogether, we conclude
326 that our image-based, machine learning aided phenotyping pipelines can correctly and
327 precisely quantify disease symptoms and reveal the smallest differences in the wheat response
328 to single *vs*. mixed *Fusarium* inocula.
329
330 **Comparison of disease severity values scored visually *vs.* using machine-learning**
331 We then compared the disease scoring obtained from a trained person with the results obtained
332 with machine-learning (ML) aided image analysis. Here, we aimed at identifying the added
333 value of accurate segmentation of the phenotype into necrosis and chlorosis (i.e. expressed as
334 a percentage), which we argued are very difficult to separate from the composite disease
335 phenotype scored by eye (expressed as a scale from 1 to 5). We used an image library previously
336 generated by us (Kaur and Vilvert., *et al*, 2024). It consisted of images of detached leaf infection
337 assays with *F. avenaceum*, *F. culmorum*, *F. graminearum* and their combinations in a panel of
338 different wheat lines. Due to the large number of putative comparisons (39 lines and 7 different
339 treatments) and considering the complexity of the data, we decided to perform an exploratory
340 cluster analysis with the coordinates of each sample obtained in a PCA and compare the relative
341 position of each cultivar.
342
343 We found that the most coherent clustering was obtained with the percentage of necrosis scored
344 by ML based on a Naïve Bayes algorithm (**Figure 5, Supplementary Tables 2, 3, 4, 5**). This
345 approximation aggregates the samples in the breeding lines (blue), lab standards (pink) and
346 commercial lines (green, red and yellow). Moreover, the clusters obtained with the values of
347 necrosis percentage are similar to the groups obtained with visual scores. In both cases there
348 are two clear groups of commercial lines close to the lab standard and the breeding lines (blue
349 and yellow in manual scoring and orange and yellow in ML scoring), and a third commercial
350 line group which is less similar to the lab standards (green, ML scoring), which is split in two
351 closely related groups in the manual scoring (green and pink). In contrast, with manual scoring,
352 the parental lines are spread between the cluster of lab standards (Latino, Zardak and MG5323,
353 tetraploid durum wheat) and one of the clusters of commercial lines (Artico, Victo and Agadir,
354 hexaploid wheat). However, beyond these differences, the relative position of each line in
355 comparison with the others remains relatively similar. For example, the lab standard Fielder is
356 close to Chinese Spring, Bob-White, Eleven, Amulett, and Rogue in both cases, with manual
357 scoring and with necrosis calculated with Naïve Bayes algorithm. In summary, we found that
358 the ML-based scoring allowed better defined clusters differentiating the different genotypes

359 used in this study. These results further indicate that the ML strategy surpassed the visual
360 scoring in terms of (i) tissue segmentation accuracy, (ii) sensitivity to small differences, and
361 (iii) precise quantification of different tissue types, all of which are of paramount importance
362 for clustering genotypes based on similar disease responses.
363
364 We then performed a robust ANOVA analysis (**Supplementary Table 6**), to detect statistically
365 significant differences between different groups of cultivars vs. treatments. The results of the
366 ANOVA indicate that observed differences (i.e. as in the clustering) between the groups of
367 cultivars, *Fusarium* combinations and the combination of both, are statistically significant ($p <$
368 0.01). This further demonstrates that our strategy can detect single *Fusarium* species and
369 *Fusarium* complex-specific responses in a cultivar-dependent manner, which is an important
370 validation of our strategy as a potential tool for mapping host factors underlying FHB complex
371 assembly in wheat.
372
373 **Phenotypic analysis of the effect of primary infection with *Z. tritici* on *F. graminearum***
374 In a recent study by us we could demonstrate that *Fusarium* taxa commonly implicated in FHB
375 largely co-exist with wheat leaves showing blotch symptoms typically caused by *Z. tritici* in
376 Sweden (Kaur and Vilvert, *et al*, 2024). We further isolated several isolates of *F. graminearum*
377 and *F. culmorum* from typical blotch-like necrotic lesions, and demonstrated using our leaf
378 segment assays that they can indeed cause blotch-like symptoms that can be easily confound
379 with *Z. tritici*, or *Pyrenophora-tritici repentis* (Kaur and Vilvert, *et al*, 2024). We thus aimed at
380 developing a phenotyping strategy that would allow us to explore these intriguing interactions
381 between *Z. tritici* and *Fusarium* spp.
382
383 We used cultivar Fielder, a universal susceptible control for *Z. tritici* which has also been the
384 basis for our leaf segment infection assays with *F. graminearum*. We used a mix at equal ratio
385 of seven *Z. tritici* isolates representing genotypes with a broad and non-overlapping spectrum
386 of virulence originating from an extensively characterized population developed at ETH Zurich
387 (Zhan *et al*., 2003, 2005). Considering the high genetic diversity of *Z. tritici* populations
388 commonly present in a single field, we reasoned that our assays would gain in biological
389 relevance may we integrate a higher-level of inoculum complexity. We also used *F.*
390 *graminearum*, as it was the *Fusarium* specie which resulted in the most severe symptoms in
391 our assay.
392

393 First, we checked the sensitivity of the image analysis strategy to detect dose-dependent effects
394 where *F. graminearum* inoculations were done at 10, 100 and 250 spores/µl on material that
395 was initially infected with *Z. tritici* (see Methods, **Figure 6A**). The NB algorithm recognized
396 higher levels of necrosis and chlorosis in leaf segments infected at a concentration of 10
397 spores/µl in comparison with the negative controls, although the differences are not consistent
398 enough to be statistically significant (**Figure 6B**). Leaf segments inoculated with a
399 concentration of 100 and 250 spores/µl showed similar levels of necrosis and chlorosis,
400 although higher concentrations tend to give more severe disease symptoms (**Figure 6B, 6C**).
401 Most importantly, we also detected a reduction of the necrosis and chlorosis caused by *F.*
402 *graminearum* on the leaf segments pre-infected with *Z. tritici* (**Figure 6B**). It is noteworthy
403 that, considering the timing of the co-infections, the observed interactions are taking place
404 during the biotrophic phase of the hemibiotrophic lifestyle of *Z. tritici*. Therefore, these results
405 suggest there is possible antagonism from *Z. tritici*, during its biotrophic stage, to suppress the
406 necrotic lifestyle of *F. graminearum*, which can be detected by our strategy.
407
408 To further explore the capacity of our pipeline to reveal such phenotypes, we expanded our
409 analysis to one tetraploid wheat accession (MG5323) which has been consistently more
410 susceptible to *F. graminearum* leaf infections than Fielder in our assays. We wanted to assess
411 if an inhibitory effect from *Z. tritici* would still be visible provided with higher virulence from
412 *F. graminearum* on the host. Here, comparisons between MG5323 and Fielder come handy as
413 both genotypes are susceptible to *Z. tritici*, thus the outcome of interactions should be mainly
414 influenced by the virulence level of *F. graminearum*.
415
416 The results from the image analysis show that our pipeline can still detect an inhibitory effect
417 of *Z. tritici* on *F. graminaerum* (**Figure 7A**), however, this effect is weaker in MG5323 where
418 *F. graminearum* is more virulent (**Figure 7B**). We also observed that such an effect seems to
419 preferentially act on necrosis rather than chlorosis in Fielder, where inhibition allows more
420 refined phenotypic readout. On the contrary, the effect on chlorosis was more prominent in
421 MG5323 where we were able to detect a statistically significant reduction in presence of *Z.*
422 *tritici*. Together, these results suggest that *Z. tritici* can modify the host response to *F.*
423 *graminearum* which seems to be conserved across different wheat species. We propose that
424 such mechanisms are likely to alter the endophytic lifestyle of the *Fusarium* species thus
425 playing a possible role in shaping *Fusarium* populations in the field.
426

427 **Discussion**

428 In this study we presented an original experimental set up allowing the study of disease
429 interactions in wheat with a focus on FHB. Detached leaf assays are highly suitable for large
430 scale screens due to the relatively short time from seed to inoculation, and the low requirements
431 of space. In fact, the full procedure can be performed in 17 days thus facilitating the screening
432 of large populations. Several procedures have been developed to phenotype resistance to FHB
433 ranging from spikelet infection in the lab and in the field (Chhabra *et al*., 2024; Giancaspro *et*
434 *al*., 2016), *in vitro* detached head inoculation (Huang *et al*., 2020), seedling assays (Soresi *et*
435 *al*., 2015), coleoptile infection assays (Wu *et al*., 2005), and detached leaf assay (Perochon &
436 Doohan, 2016). Even though detached leaf assays are commonly used, precision phenomics
437 strategies to quantify the resulting phenotypes have not been exploited, with a few attempts
438 restricted to the measurement of lesion diameter (Perochon & Doohan, 2016; Roesler *et al*.,
439 2022). A major feature of our strategy is the possibility to simultaneously decompose the
440 symptoms into two types of alterations (necrosis and chlorosis) and precisely (i.e. to the pixel)
441 measure their relative areas. Furthermore, we demonstrate that there are measurable differences
442 in the ability of FHB associated taxa to cause either of these symptoms on different genotypes
443 (alone or in combination). Thus, in this work, we took full advantage of such reductionist
444 approaches to explore the complexity of multi-pathogen species interactions within the FHB
445 complex, and between FHB and other wheat pathogens.
446
447 The first interesting finding of this work was the inherent variation in the capacity of different
448 FHB associated *Fusarium* species to induce chlorosis or necrosis. While the mechanisms
449 underlying the expression of these tissue alterations are conserved among plants, their
450 physiological basis is very different. Chlorosis is typically a nutrient deficiency phenotype
451 commonly induced when micronutrients such as iron (Fe) and zinc (Zn) are depleted from the
452 cells (Nogiya *et al*., 2016). Chlorosis is not to be confused with 'senescence' which is a highly
453 regulated developmental process in plants which may also result from nutrient deficiency (i.e.
454 starvation) but is largely controlled by genetic programming (Noodén *et al*., 1997). Necrosis is
455 a typical marker for biotic stress, triggered by the plant immune system in response to
456 pathogens (Minina *et al*., 2005). In this context, our results indicated that FHB associated
457 species have different abilities to differentially alter two highly conserved tissue damage-
458 associated mechanisms reminiscent of blight symptoms. This could indicate a form of
459 functional specialization among species of the FHB complex.
460

461 Furthermore, we showed that the ability to cause these symptoms and their relative prevalence
462 is genotype-specific, and species combination-dependent. Such genotype-specific variation in
463 the hemibiotrophic lifestyle was also observed in other pathogenic *Fusarium* species such as
464 *F. temperatum*, and *F. oxysporum* (Hill *et al*., 2022; Robles-Barrios *et al*., 2022; Srivastava *et*
465 *al*., 2024). We therefore speculate that such variation may reflect dynamic changes in the
466 hemibiotrophic lifestyle of *Fusarium* sp. Here, a classical progression would correspond to an
467 early endophytic colonization without visible symptoms, followed by the induction of chlorosis
468 as nutrients are gradually depleted by the fungus, then a shift to a necrotrophic lifestyle where
469 the pathogen switches to feeding on dead tissues. Thus, what our assay could be revealing a
470 possibly new types of resistance to FHB based on the suppression of deleterious tissue
471 alterations by chlorosis and necrosis.
472
473 Another interesting finding from this study was the observation that co-existence of different
474 *Fusarium* species can have a measurable negative impact on the performance of the complex
475 to cause lesions on the leaves. In fact, we found a statistically significant difference between
476 single *F. graminearum* infections and inoculations with *F. culmorum* and *F. graminearum*. This
477 is counter intuitive considering that disease complexes such as FHB are largely considered as
478 based on a form of synergism between microbial pathogens (Lamichhane & Venturi, 2015).
479 This is for instance represented in our assays by the *F. avenaceum* and *F. graminearum*
480 combination, which tends to have a higher disease severity than *F. avenaceum* single infection.
481 Similarly, evidence shows that co-infection with *F. aglaonematis sp. nov.* and *F. elaeidis*
482 triggers more severe disease symptoms in *Aglaonema modestum* as compared with single
483 infections (Zhang *et al*., 2022). This further highlight the difficulty to predict multispecies-
484 complex dynamics, which are proposed to result from evolutionary competition and pathogen
485 niche specialization (Abdullah *et al*., 2017).
486
487 Altogether these observations may indicate that the ability of taxa from the FHB complex to
488 cooperate *vs*. compete may have a fitness cost which could be partially determined by the host.
489 Such cost could for instance be associated with tradeoffs between the maintenance of virulence
490 factors important to infect the host vs. antimicrobial factors important for competition with
491 other taxa in the complex. Numerous studies showing that filamentous plant pathogens
492 significantly impact the host microbiome, and recent discoveries suggesting that pathogen
493 genomes encode large arrays of so-called effectors which are primarily targeted to outcompete
494 other microbes (Flores-Nunez & Stukenbrock, 2024; Snelders et al., 2022). While at the same

time, it was also shown that the maintenance of such large repertoire of virulence factors can also have an impact on pathogen fitness (Montarry et al., 2010). These observations are highly relevant for the cases where we saw possible signs of competition over plant immunity and plant resources in two cases where *F. graminearum* was combined with another *Fusarium* species or *Z. tritici*. This is also consistent with previous studies demonstrating that both *F. greaminearum* and *Z. tritici* have the capacity to manipulate the plant microbiome during infection, including the identification of specific effector proteins with antifungal activity in from *Z. tritici* (Kettles et al., 2018; Rojas et al., 2020). In fact, competition between *Fusarium* species as a strategy for biological control has been discussed and tested with some success (Aimé *et al*., 2013; Alabouvette *et al*., 2009).

We therefore conclude that the ability to reveal differences in the plant immune response to single vs. species infection is an important step forward in understanding the factors controlling FHB complex assembly, which further demonstrates the future potential of our experimental pipeline to be used a genetic tool for resistance screening.

Data availability

All data is available within the manuscript or in the supplementary material. Additional information will be made available upon request to the corresponding author.

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527 **Supplementary Material**

528

529 *Supplementary figures*

530 **Supplementary Figure 1.** Infection chamber used for *Z. tritici* inoculations of wheat
531 seedlings.

532

533 *Supplementary tables*

534 **Supplementary Table 1.** Disease severity score index.

535 **Supplementary Table 2.** Cluster composition based on manual scoring of disease severity
536 from Figure 5A.

537 **Supplementary Table 3.** Cluster composition based on naïve bayes scoring of 'necrosis'
538 from Figure 5B.

539 **Supplementary Table 4.** Cluster composition based on naïve bayes scoring of 'chlorosis'
540 from Figure 5C.

541 **Supplementary Table 5.** Cluster composition based on naïve bayes scoring of 'necrosis +
542 chlorosis' from Figure 5D.

543 **Supplementary Table 6.** ANOVA performed with disease symptom quantification obtained
544 with our machine learning based set up.

545

546 *Supplementary files*

547 **Supplementary File 1.** G-code for the 3D printed frames shown in figure 1. The frames are
548 used to keep the leaf segments in contact with the agar medium.

549 **Supplementary File 2.** Jupyter Notebook containing the scripts to analyze pictures with the
550 naïve bayes algorithm.

551 **Supplementary File 3.** Numerical data underlying the statistical analysis of figures 2, 5, 6
552 and 7.

553 **Supplementary File 4.** Html file with R analysis and necessary scripts to reproduce all the
554 analysis showed in the figures.

555 *Data availability*

556 **GitHub DOI.** 10.5281/zenodo.10697381

557

558

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821 **Figures**

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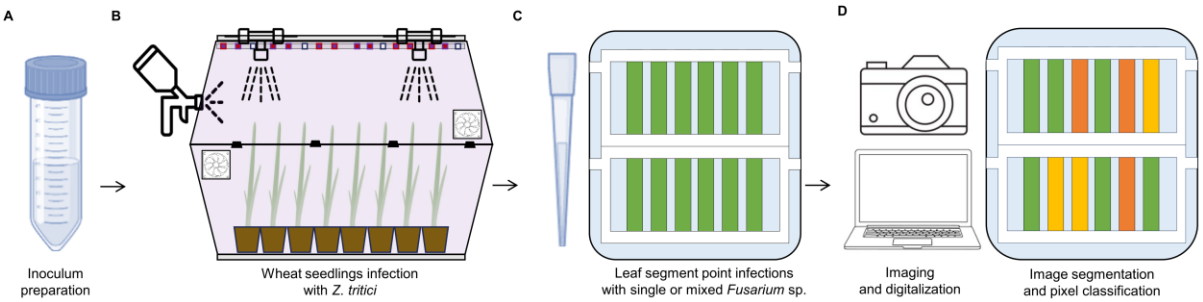

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826 **Figure 1**. Overview of the phenotyping pipeline.

827 A 11-days-old *Z. tritici* (an hemibiotroph) culture was scratched from YMS plates, resuspended in
828 TWEEN 0.01% (A) and sprayed in a closed environment (named as The Hive) where plants have kept
829 until the appropriate stage (B). After 3 days, leaf fragments are harvested, mounted on agar plates
830 and infected with a spore suspension of *Fusarium* spp. (a necrotroph) (C). After 4-7 dpi of incubation,
831 pictures of the disease symptoms were taken with a Nikon camera. The pictures were analyzed with
832 bash code throughout python scrIpts and the results were analyzed with R (D).

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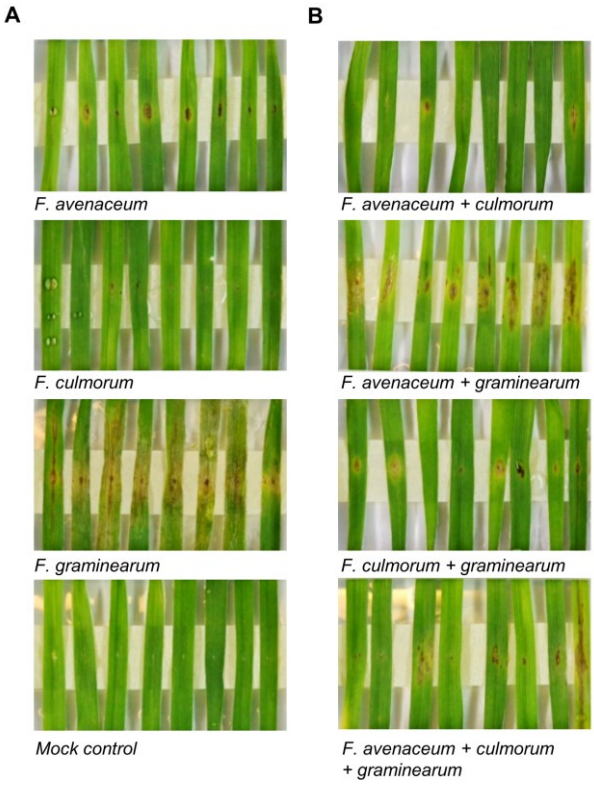

Figure 2. Phenotyping of Fusarium coinfections.

The cultivar Fielder was inoculated with *F. avenaceum*, *F. culmorum* and *F. graminearum* isolates from Sweden. Pictures were taken at 8 dpi. We perform two independent experiments with 8 biological replicates per treatment. We show representative pictures of one of the experiments.

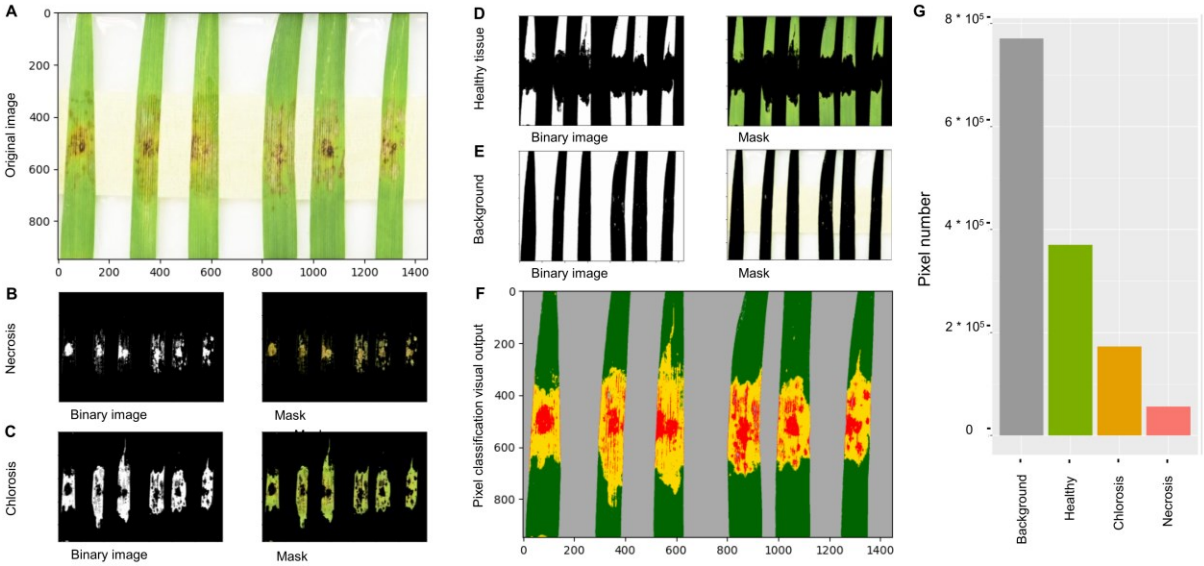

Figure 3. Steps for image analysis throughout quantification of Fusarium symptoms. A Naïve Bayes algorithm use a training set of pixels collected with the free software ImageJ to develops a probability function with the aim of classify all picture pixels in each of the predefined classes (necrosis, chlorosis, healthy tissue, or background). The result can be visualized in a binary image that is used to create a mask. All masks can be overlapped in a final image where is counted the number of pixels in each class, allowing the quantification of the disease symptoms.

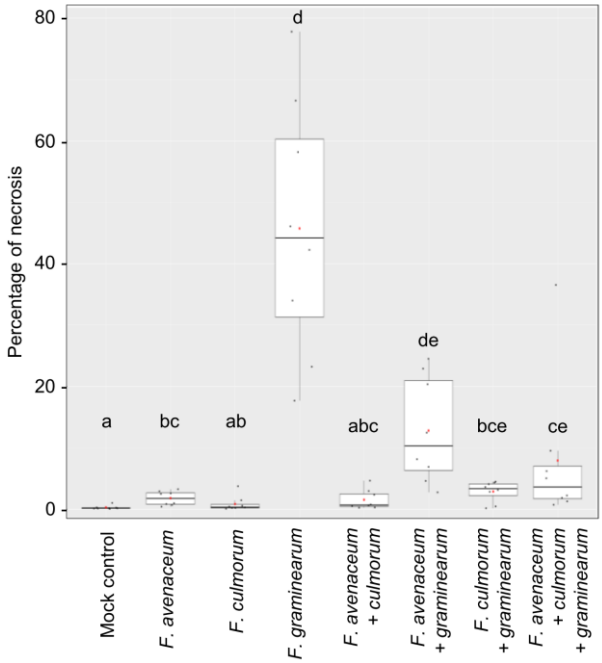

Figure 4. Symptom disease severity quantification through Naïve Bayes algorithm.
Pictures from Figure 2 were analyzed by the set up shown in Figure 3. The number of pixels classified as “Necrosis” was divided by the number of pixels calculated in the different groups of leaf pixels (“necrosis”, “chlorosis” and “healthy tissue”). The letters a-e refer to two groups of treatments that present differences statistically significative (two-way robust ANOVA: $p < 0.05$)

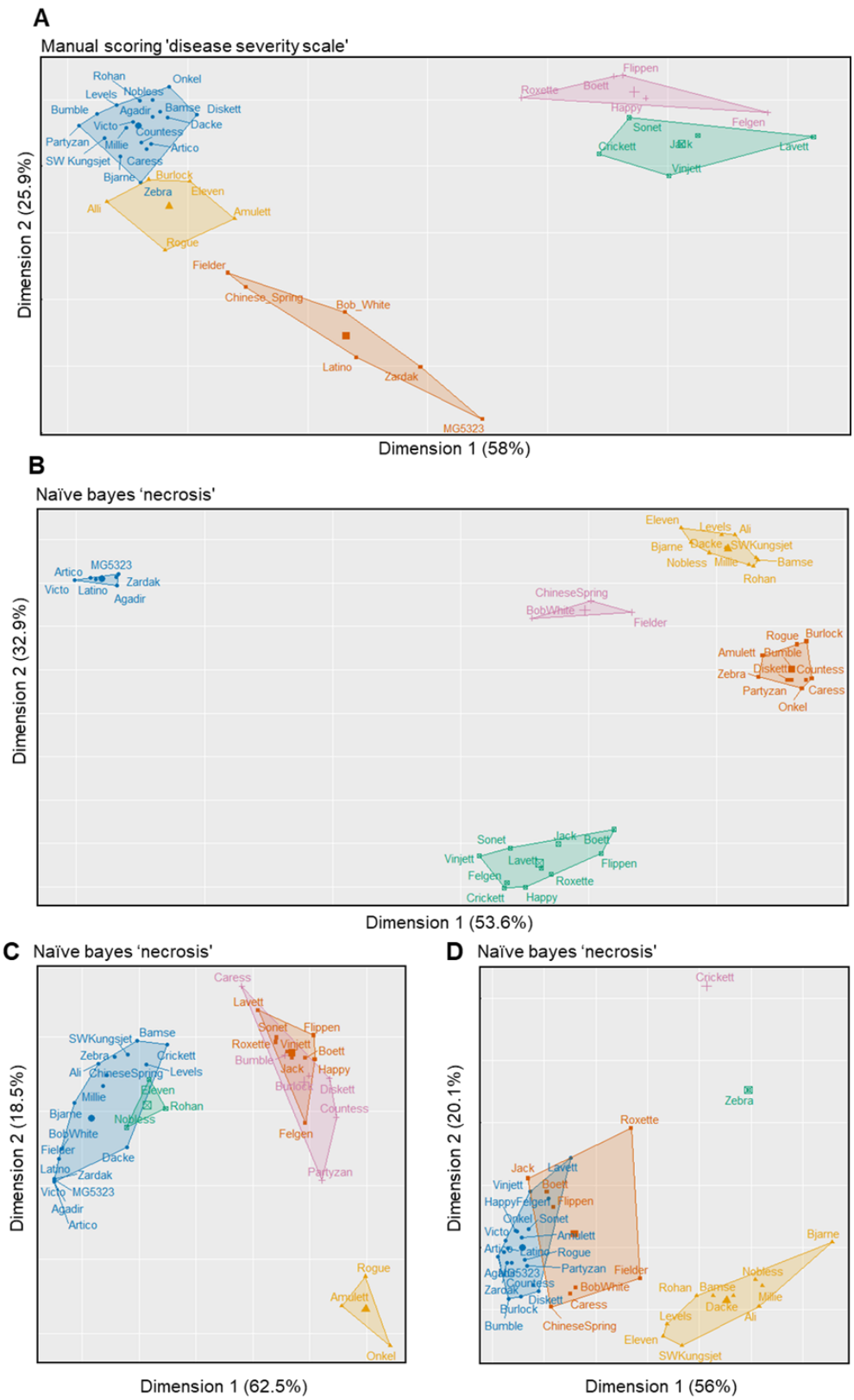

Figure 5. Cluster analysis of wheat cultivars infected with *F. avenaceum*, *F. culmorum*, *F. graminearum* alone or in combination.

The Agglomerative nesting (AGNES) algorithm was applied to the distance matrix of the mean value of the PCA coordinates of each sample to develop the dendrogram. The defined number of clusters is 5 (k = 5).

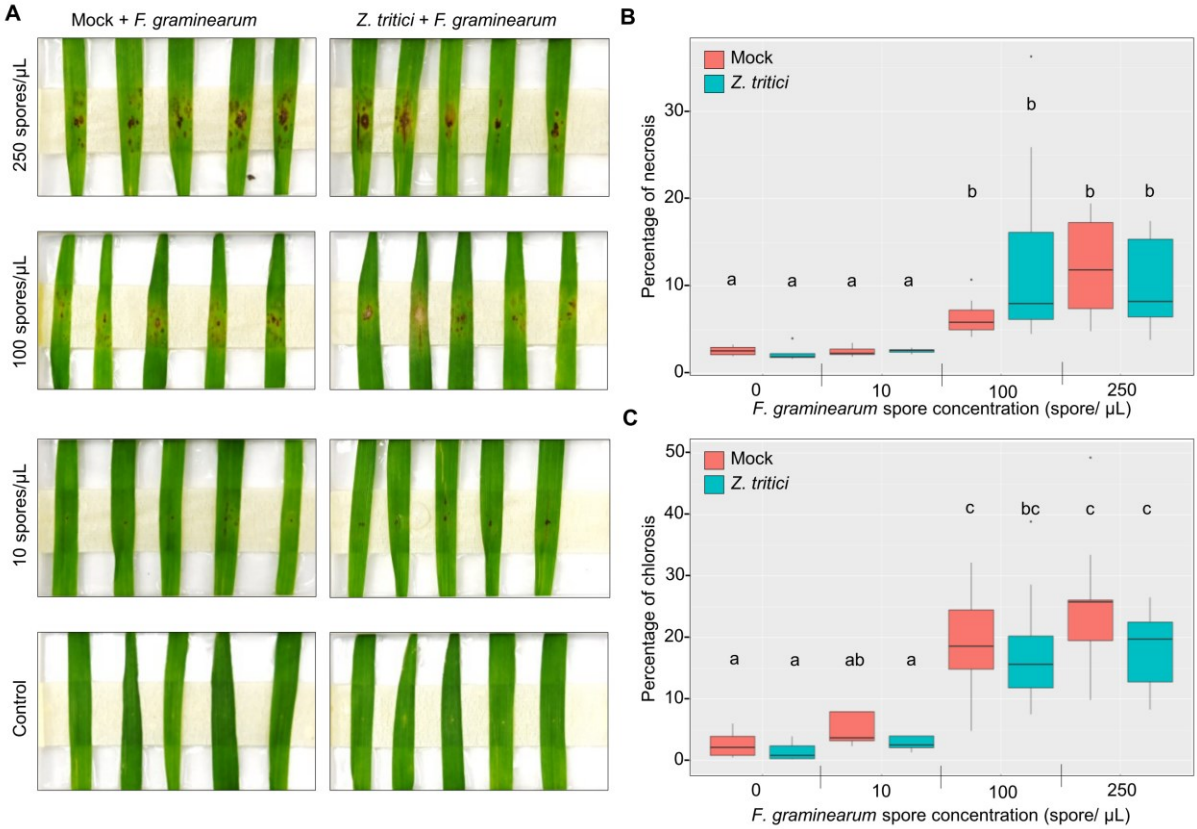

Figure 6. Quantification of the disease symptoms of *F. graminearum* at different spore concentrations in coinfections assays with *Z. tritici*.

(A) Pictures from one experiment with 5 biological replicates were analyzed with our setup at 5 dpi. The symptom quantification was obtained dividing the number of pixels counted in the class necrosis (B) or chlorosis (C) between the sum of the number of pixels in necrosis, chlorosis and healthy tissue classes. The pink color indicates mock treatment and blue color indicates plants coinfected with Z. tritici. The letters a-b refer to two groups of treatments that present differences statistically significative (two-way robust ANOVA: $p < 0.05$)

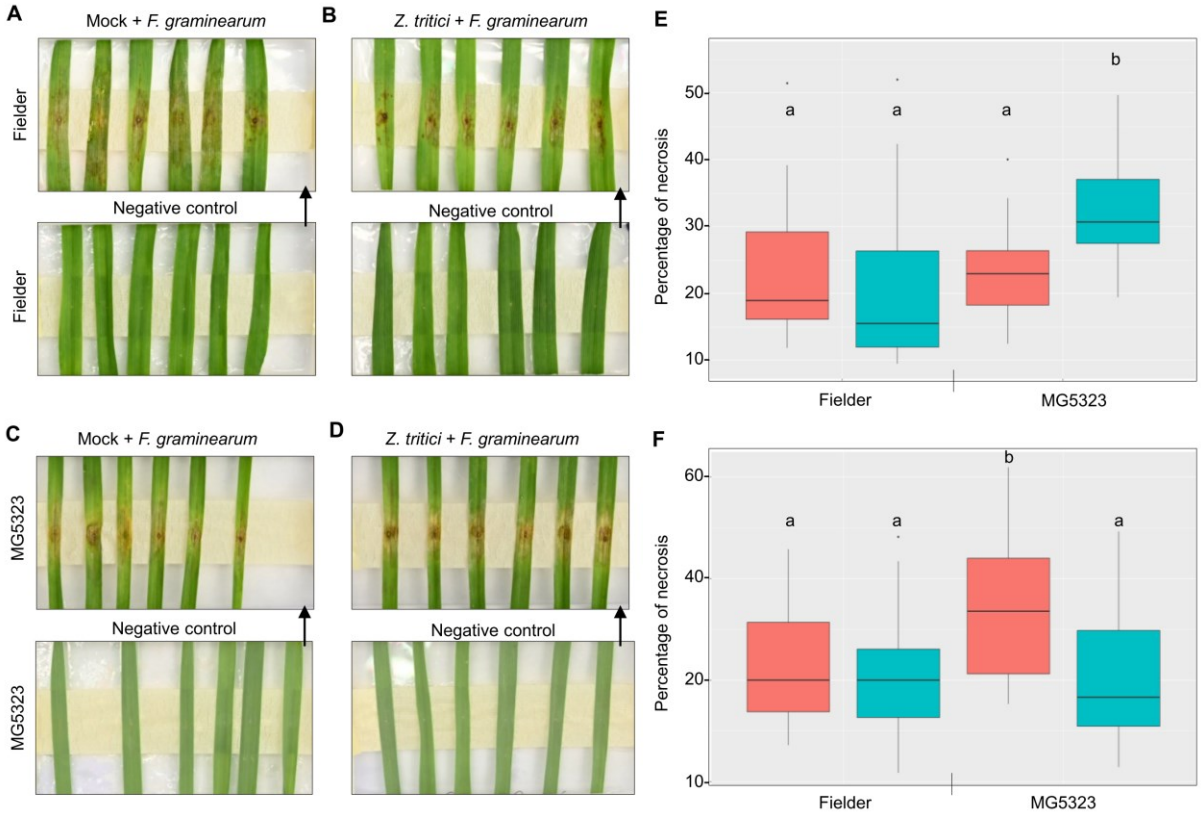

Figure 7. Quantification of the disease symptoms of *F. graminearum* in coinfections assays with *Z. tritici*.

(A) Pictures from two experiments with 6 biological replicates were analyzed with our setup. We show characteristic pictures of one experiment. The symptom quantification was obtained dividing the number of pixels counted in the class necrosis (B) or chlorosis (C) between the sum of the number of pixels in necrosis, chlorosis, and healthy tissue classes. The pink color indicates mock treatment and blue color indicates plants coinfected with *Z. tritici*. The letters a-b refer to two groups of treatments that present differences statistically significative (two-way robust ANOVA: $p < 0.05$)