Characterisation of resistance in wheat to Fusarium Head Blight complex

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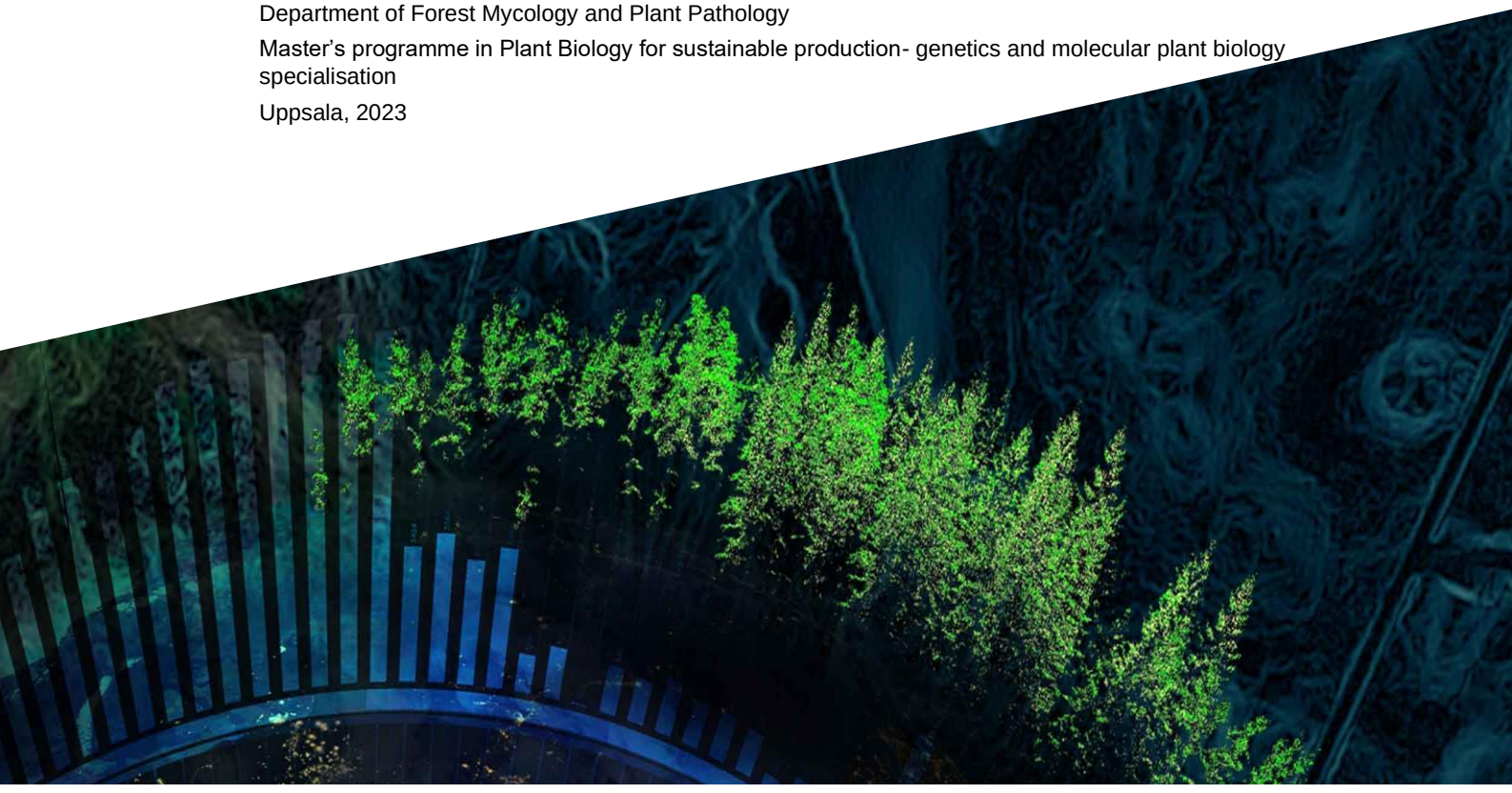

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Abstract

The plant pathobiome is described as a set of microorganisms that interact with each other and with the plant biotic environment to influence disease progress. Recent studies have led to the realisation that a disease in a plant is not always associated with single organisms rather is a result of complex interactions between various taxa, the host, and the environment. In this study, we investigated the pathobiome composition of diseased wheat kernels as well as the role of host genotype in determining the assembly of the Fusarium Head Blight (FHB) disease complex. For this purpose, we isolated *Fusarium* spp. from infected wheat spikes and conducted infection assays on wheat to observe the interaction within the FHB complex, as well as the possible role of the plant genotype in disease progression. The outcome suggested that complex interactions occur within the FHB complex, as well as between the complex and the wheat genotype to cause disease. Hence, the plant genotype has a role in how the communities within the pathobiome interact to cause disease.

Keywords: Wheat, Pathobiome, Fusarium Head Blight (FHB), Phenotyping

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Abbreviations

ANOVA	Analysis of Variance
CMA	Corn Meal Agar
FHB	Fusarium Head Blight Complex
GLM	General Linear Model
MEV	Malt Extract and Vitamins
OMA	Oatmeal Agar
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
PDA	Potato Dextrose Agar
RIL	Recombinant Inbred Line
SLU	Swedish University for Agricultural Sciences
YMS	Yeast Malt Sucrose

1. Introduction

1.1 Pathobiome

Plants are a habitat to diverse and complex microbial communities that play various roles in relation to overall plant growth and development (Brader et al. 2017) . The pathobiome is a concept introduced to describe a set of diverse and ubiquitous prokaryotic, viral, and complex eukaryotic communities that interact with the plant and its biotic environment to cause a disease (Vayssier-Taussat et al. 2014). Studies have established that multiple interactions occur between the pathogen and the plant pathobiome that can influence disease process positively or negatively (Jakuschkin et al. 2016). Hence, one pathogen- one disease approach based on Koch's postulates does not always hold true in case of complex communities of microorganisms involved in disease formation. In cases where a single pathogen is concerned, it is possible that the microorganisms associated with this pathogen are responsible for increasing or decreasing the disease severity and hence, should also be included as a part of disease process (Mannaa & Seo 2021). Therefore, we need to shift the focus to a more realistic and comprehensive approach wherein, we take into consideration the various organism involved, their interaction between each other as well as their interaction with the host plant and its environment to form a disease (Fig. 1). Recently, it has been shown that the plant genotype and its environment play an important role in shaping the microbial communities (Van Overbeek & Van Elsas 2008; Mina et al. 2020; Aira et al. 2010). Therefore, it would be interesting to observe the microbial composition during infection in a plant and the interactions occurring within the pathobiome that influence the disease.

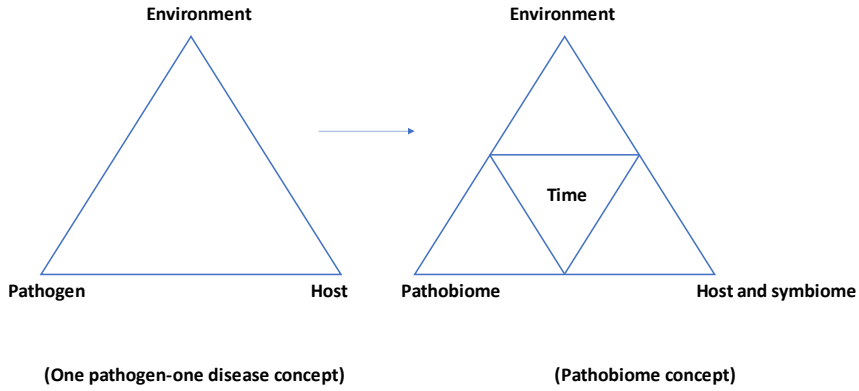

Figure 1. A transition from the disease triangle based one pathogen- one disease hypothesis to a comprehensive approach of disease pyramid which incorporates the concept of pathobiome that interacts dynamically (time) with host plant and its biotic (symbiome) and abiotic environment.

1.2 Fusarium Head Blight of Wheat (FHB) and interactions in the FHB complex

Fusarium Head Blight (FHB) disease of wheat is considered as one of the most devastating diseases causing losses in both quantity of production and the quality of grains produced (Dubin 1997; Khan et al. 2020). FHB is characterised by bleaching of the entire spike (Fig. 2) or part of it depending on where the infection starts. The symptoms also include shrivelled, lightweight, and discoloured grain formation (Birr et al. 2020). FHB is caused by a complex of 17 different *Fusarium* species with major drivers being *Fusarium graminearum* and *F. culmorum* (Doohan et al. 1998). Several studies have been carried out to demonstrate interactions between the various species of the FHB complex. These interactions depend on several factors like timing of infection, host species, timing of establishment at infection sites, environment and plant biomass. (Simpson et al. 2004; Xu et al. 2007; Xu & Nicholson 2009; Tan et al. 2021). Synergistic as well as competitive interactions have been established in the various species of FHB complex. *Fusarium poae* has been shown to hamper the effect of *F. graminearum* if pre-inoculated on wheat (Tan et al. 2021). Similarly, *Microdochium majus* and *M. nivale* show selective advantage on wheat and rye respectively (Simpson et al. 2004). These studies show that these interactions are very complex and cannot be

determined just by ‘one plus one is two’ logic. Presence of such complex interactions in the FHB complex makes it an excellent model to study the interactions of different species in a complex to develop a disease. It is not only important to know the role of interactions in a complex but also the role of genotype of the host to impart resistance or susceptibility to the pathogen complex. Hence, it will be interesting to investigate the role of the plant genotype on host plant resistance/susceptibility with respect to disease development by the FHB complex.

Figure 2. FHB symptom in wheat spike (Picture by H. Vélëz, Hammarbyallén, Uppsala)

1.3 Evolution of Wheat with respect to FHB

Bread wheat (*Triticum aestivum*) originated from crossing of tetraploid wheat, *Triticum turgidum* (AABB) and diploid *Aegilops tauschii* (DD) (Szabo-Hever et al. 2018). The D sub-genome is an important source of resistance genes in bread wheat. Durum wheat (*Triticum durum*, AABB) is particularly susceptibility to FHB possibly due to the absence of the D genome, and breeding for FHB resistance is a major focus in this crop. Domestication of the durum wheat has created a genetic bottleneck leading to a lack of sources of FHB resistance in the primary gene pool (Dweba et al. 2017). Hence, it is very important to find novel sources of resistance in durum wheat against the FHB complex. One of the

potential sources of resistance for durum wheat is the tetraploid wheat, *Triticum dicoccum* which is the wild relative of the now domesticated durum wheat. Breeding programs can take advantage *T. dicoccum* population to identify potential source of resistance via genome wide association studies and these can then be incorporated into resistance breeding programmes to susceptible durum wheat cultivars (Fig. 3) (Haile et al. 2019). Therefore, it would be interesting to assess how parental lines of different recombinant inbred line (RIL) populations being used in breeding of durum wheat for resistance/susceptibility against FHB differ in disease severity to the FHB complex.

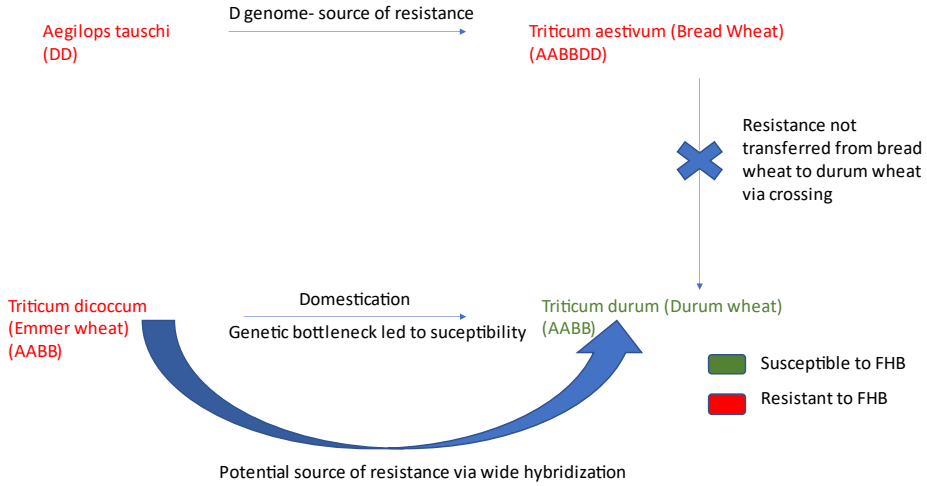

Figure 3. Sources of resistance in Bread wheat and Durum wheat.

1.4 Host resistance to FHB

Active resistance in wheat to FHB is described as a response of genes to pathogen or a complex invasion and infection in the form of a physiological change in the host plant. A total of five types of active resistance to FHB have been described in wheat (Mesterházy 1995; Mesterházy et al. 1999). Type I resistance describes the resistance to invasion by the pathogen which helps in limiting the initial infection by the pathogen. Type II resistance describes the resistance that inhibits the spread of the pathogen within a spike. Type III is for resistance to mycotoxins accumulation by the pathogen/complex, Type IV for resistance to spread of disease to the kernels and Type V is for tolerance. Most of the breeding programmes focus on Type II resistance for evaluating the resistance in wheat to FHB. However, most of the wheat that is being sown to this date has only partial resistance to FHB (Imathiu et al. 2009). Very little effort has been made to use Type I resistance for

evaluation (Bai & Shaner 2004). To assess resistance that prevents disease incidence in the first place, it is important to incorporate Type I resistance for evaluating resistance in wheat to FHB. Several studies have incorporated detached leaf assays for phenotypic assessment of Type I resistance in wheat successfully (Imathiu et al. 2009; Browne & Cooke 2003). Therefore, it is of interest to use detached leaf assays for phenotypic assessment of Type I resistance in infected wheat.

1.5 Objectives

Based on the fact that several factors are responsible for wheat-FHB complex interaction, it is important to study the role of plant genotype in disease development without any contribution of external factors. Our hypothesis was that individual species in the FHB complex interact differently with each other in disease development in the same environment conditions and that the disease severity changes with the plant genotype as a result of interactions between the host genotype and the FHB complex.

In this research project we aimed to investigate:

i. Composition of the fungal communities isolated from infected wheat spikes with particular focus on species of *Fusarium* genus.
ii. Interactions between species of the Fusarium Head Blight complex to influence disease.
iii. Effect of wheat genotype on the observed interactions between different FHB-implicated *Fusarium* species.

Firstly, we assessed the fungal communities isolated from infected wheat spikes collected from various locations in Sweden using culturing methods followed by molecular identification of the fungal isolates. Secondly, we assessed how different species in FHB complex interact to cause disease along with the role of the plant genotype in disease progress by carrying out detached leaf assays. We expected to see variation in disease severity between various *Fusarium* species as well as wheat genotypes.

2. Material and Methods

2.1 Isolation of *Fusarium* complexes from wheat and their purification

Two set of samples were used for isolation of Fusarium complexes from wheat. The first set includes seeds from the wheat cultivars Dala, Rohan, Dacke, Fielder, Bobwhite, Stanley, Dula, Alderon, and Happy, and the Triticale cultivar Trado, harvested from untreated garden plots near Märsta, Sweden (no use of fungicide or insecticide). These seeds were then surface sterilised for one minute in 1% Sodium Hypochlorite, followed by 30 sec in 70% Ethanol, and washed twice for 30 sec each using autoclaved Milli-Q water. Individual seeds were placed in Petri plates containing fungal culture media (i.e., half-strength potato dextrose agar (½PDA); yeast-malt sucrose agar (YMS); oatmeal agar (OMA); and cornmeal agar (CMA)), amended with 50 µg/ml of Kanamycin (Appendix, Supplementary Information Table 1). In addition to this, seeds of above-mentioned cultivars were washed with just autoclaved Milli-Q water for 2 minutes and then plated similarly (i.e., one seed per plate and one plate per medium per cultivar).

The second set includes seeds and glumes from FHB infected spikes collected from seven different wheat fields in Sweden (VSC034 SPK001, VSC035 SPK002, VSC038 SPK 003, VSC039 SPK 004, VSC040 SPK 005, VSC041 SPK 006 and VSC042 SPK 007). The spikes were coded VSC (Växtskyddscentralen) by the plant protection officer who collected the samples from the fields and the SPK code was given by the lab group to distinguish the individual spikes collected from the same field. The seeds and the glumes were then treated similarly as the first set of samples (both sterile and non-sterile treatment).

The plates were then incubated at 20°C for 7 days until colonies were visible. Individual colonies were picked and transferred to new plates and incubated at 20°C. The process was repeated until a single pure colony was obtained (Fig. 4).

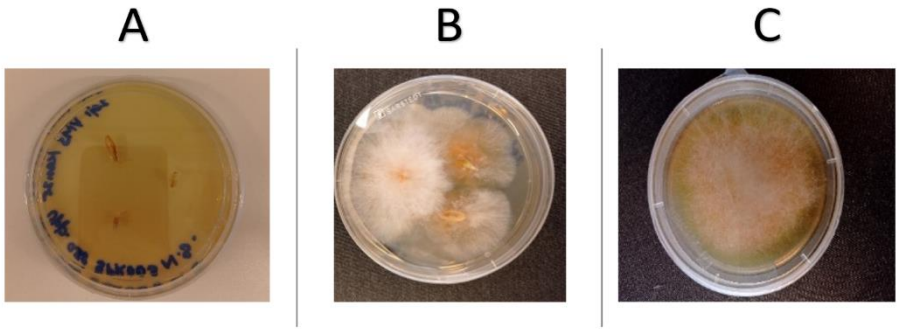

Figure 4. A) Culturing seeds and glume from infected wheat spikes on YMS medium B) Fungal colonies obtained from culturing C) Single pure colony obtained from transferring individual colonies.

2.2 DNA extraction from isolated fungal colonies

A modified cetyltrimethylammonium bromide (CTAB) based method of DNA extraction was used to extract DNA of fungal isolates (Brandfass & Karlovsky 2008). Briefly, fungal-plugs from each isolate were transferred to liquid cultures containing malt extracts and vitamins (folic acid, thiamine chloride and biotin) (MEV) and incubated at 25℃ for two weeks. Fungal hyphae were harvested, homogenised with CTAB buffer (3% CTAB; 150 mM TRIS-HCl; 2.6 M NaCl; 20 mM EDTA), and placed in a water bath at 65℃ for at least 30 min. The samples were centrifuged at 10’000 g for 10 min and the supernatant was transferred to a new Eppendorf tube. An equal volume of chloroform was added and after mixing, the samples were centrifuged as before. Once again, the supernatant was transferred to a new Eppendorf tube and an equal amount of isopropanol was added to precipitate the DNA. After a half an hour incubation at -20℃, the samples were centrifuged as before, and the pellet obtained was washed using 70% ethanol. The samples were centrifuged as before and after drying, the DNA pellet was dissolved in TE buffer.

2.3 Molecular identification of isolated fungal colonies

Identification of the isolated species was carried out by Polymerase Chain Reaction (PCR) of the genomic DNA followed by amplicon sequencing of conserved nuclear locus, internal transcribed spacer (ITS) rDNA. For ITS PCR, primer pair ITS1 and ITS4 (White et al. 1990) (Table 1) were used for amplification (Table 2, 3), followed by PCR clean-up (Table 2) using exonuclease using ThermoFisher Scientific PCR clean up kit (Table 4). The amplicons were subsequently sent for sequencing to Macrogen Europe. Online database BLAST (blastn) was used to identify the species by DNA sequence homology (*Database resources of the National Center for Biotechnology Information,* 2016).

Table 1. Primers used for molecular identification of fungal isolates by ITS sequencing.

Locus	Primer	Orientation	Sequence (5'-3')	Length (bp)
ITS	ITS1	Forward	TCCGTAGGTGAACCTGCGG	19
ITS	ITS4	Reverse	TCCTCCGCTTATTGATATGC	20

Table 2. Reaction mixture for PCR amplification and PCR product clean-up.

PCR reaction	volume	PCR clean-up	volume
Water	5µl	PCR mixture	5µl
2X DreamTaq Master Mix*	10µl	Exonuclease I	0.5µl
ITS1	1µl	FastAmp	1µl
ITS4	1µl		
gDNA	3µl		

Table 3. PCR amplification specifications.

PCR (Total cycles- 30)	One –step Cycle	
	95°C	2 min
	95°C	30 s
	60°C	30 s
	72°C	1 min
	72°C	10 min
	15°C	∞

Table 4. PCR clean-up specifications

PCR clean-up	Cycle	
	37℃	15 min
	85℃	15 min

The fungal species identified were then arranged based on the same source that is the fungal species were isolated from the same spike from which they were isolated, growing on the same media and given the same treatment. This will result in grouping of the fungal species into communities, and we assume that the fungal species belonging to one community form a true interacting complex and that in nature they had been interacting with each other and the host. Hence, individual fungal species from a community can be used for infection assays on wheat. Fungal species isolated from VSCO39 SPK004 showed presence of *Fusarium graminearum, Fusarium culmorum and Fusarium avenaceum* and was verified by PCR amplification using *Fusarium* specific primers (Table 5) (Kuzdraliński et al. 2017). After verification, it was used for carrying out infection assays on the wheat.

Table 5. PCR primers for identifying specific Fusarium species.

Primer	Target DNA sequence	Sequence (5'-3')	Size (bp)	Reference
Fusarium graminearum				
GOFW	gaoA	ACCTCTGTTGTTCTTCCAGACGG	435	(de Biazio et al. 2008)
GORV		CTGGTCAGTATTAACCGTGTGTG		
Fusarium culmorum				
Fc03	SCAR	TTCTTGCTAGGGTTGAGGATG	144	(Astrid Bauer & Seigner 2015)
Fc02		GACCTTGACTTTGAGCTTCTTG		
Fusarium avenaceum				
FAF1	ITS	AACATACCTTAATGTTGCCTCGG	314	(Mishra et al. 2003)
FAR		ATCCCCAACACCAAACCCGAG		

2.4 Plant material and growth conditions

The plant material in this study comprised three different sets of cultivars. The first set included the reference genotypes (Fielder, Bobwhite, and Chinese Spring), the second set includes eight species- SW023 (Accession of *T. dicoccum*); SW001, SW004, SW034 (*cultivars of T. aestivum*); SW020 (cultivar of *T. durum*) and SW036 old cultivar of *T.durum*) obtained from the Council for Agricultural Research and Economics, Research Centre for Genomics and Bioinformatics, Fiorenzuola d'Arda, Italy (CREA – Dr. Francesca Desiderio), and the third set included 30 commercial bread wheat cultivars (Table 9) provided by the Swedish Agricultural Cooperative (Lantmännen Lantbruk, Sweden). The plants were grown in the controlled climate chambers for three to four weeks at 20℃, 70% relative humidity and 300µMOL light intensity, 16 hours light, and 8 hours dark conditions.

2.5 Preparation of Fusarium complex inoculum

The inoculum was composed of isolate HK48 (*F. graminearum*), HK50 (*F. culmorum*) and HK53 (*F. avenaceum*), isolated from sample VSC093 SPK004. The single-spore isolates were cultured on OMA at 20°C for two weeks under constant darkness. The conidia were harvested by flooding the cultures with 10 ml of sterile water and the suspension was then passed through 50 µm nylon mesh to remove mycelium. The inoculum cell density was measured using a haemocytometer and adjusted to a working concentration of 250 conidia/ µl.

2.6 Phenotypic assessment of Fusarium complex virulence in wheat

Type I resistance was assessed in the inoculated wheat leaves according to a protocol developed by the Leibniz institute of Plant Genetics and Crop Plant Research (Dr. Dimitar Douchkov, IPK Gatersleben, personal communication). Three- to four-weeks-old wheat leaves were randomly selected and cut into 4 cm length. A hole was punctured in the middle of leaf segment and inoculated with 15µl of pathogen inoculum. The total inoculum volume and spore concentration was kept constant, also in species combination treatments. The inoculated leaf was placed in water agar and incubated at 20℃, 70% relative humidity and 300µMOL light intensity, 16 hours light, and 8 hours dark conditions for four days (Fig. 5). The necrosis was observed in each leaf segment and a score from 0 to 5 was given according to the area covered with necrosis (Table 6). Seven different combinations of *Fusarium spp.* were used for infection, "*F. graminearum (Fg)"; "F. culmorum (Fc)"; "F. avenaceum (Fa)"; "F. graminearum* and *F. avenaceum (Fga)"; "F. graminearum* and *F. culmorum (Fgc)"; "F. culmorum* and *F. avenaceum (Fca)"* and all the three combined (*Fgca*). In the case of the lab standards, a total 12

replicates were used while in the case of the breeding lines and commercial cultivars, a total 6 replicates were used for carrying out phenotypic assessment. Infection scores were given based on the necrosis chart (Table 6) to each replicate and a heat map was generated to compare the necrosis within the three set of cultivars used (Table 7, 8 and 9).

2.7 Statistical methods

R software version 4.2.0 was used for statistical evaluation and plot generation (R Core Team, 2022). For carrying out analysis, normality and heteroscedasticity of the data was checked by generating a linear model (LM) and a general linear model (GLM) with symptom severity as result of the variables cultivar type and *Fusarium* combination. Even though the initial values of the disease severity (infection scores) were discrete, the data was treated as a continuous variable to quantify numerically a complex qualitative variable. Neither the LM nor the GLM passed the normality and heteroscedasticity tests. However, the density plot showed that the residuals of the LM are distributed in a Gaussian bell (Fig 6). The peaks observed were a result of not having intermediate levels of disease severity. Hence, the data was assumed to be normally distributed and heteroscedastic. PCA plot generation and an analysis of variances (ANOVA) was conducted to determine the effect of cultivar and *Fusarium* species on the disease severity. The analyses were run at a significance of P = 0.01.

Figure 5. Infection assays carried out on wheat leaf segments, before incubation (left) and after (right).

Table 6. Infection score chart.

Phenotype	Infection score
	0- No necrosis around the wounded area
	1- The wounded area surrounded by slight ring of necrosis
	2- Necrosis spread to up to 25% of leaf segment area
	3- Necrosis spread up to 25- 50 % of leaf segment area
	4- Necrosis spread up to 50-75% of the leaf segment area
	5- Necrosis spread more than 75% to entire segment.

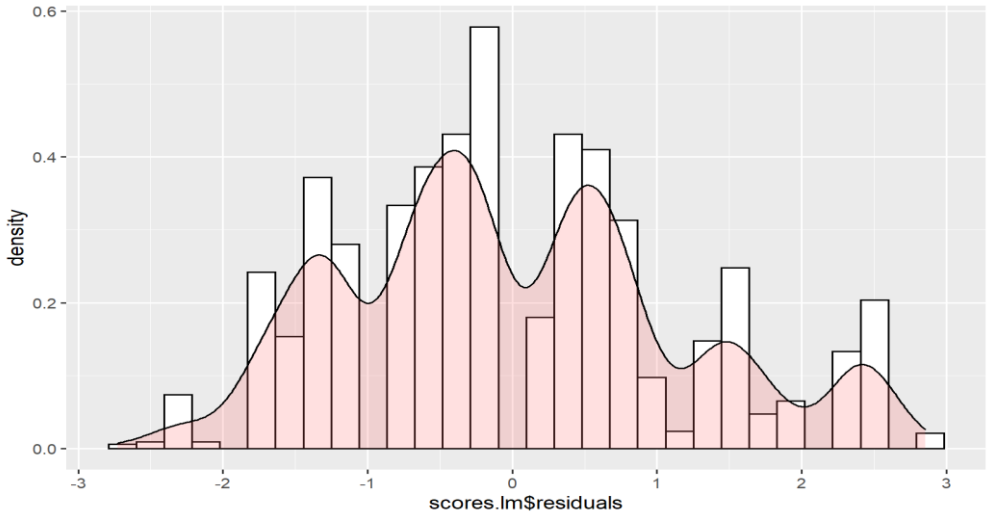

Figure 6. Density plot generated using disease score of all replicates. R software was used to develop linear model. The y axis represents the density of each value and the x axis represent the different residuals. Residuals of our linear model distributed among 6 values, corresponding to the 5 severity scores and 0. The global distribution of residuals is similar to a gaussian bell, corresponding to a normal distribution.

3. Results

3.1 Composition of the isolated fungal communities and selection of Fusarium species for infection

A total of 285 fungal isolates were obtained from culturing glumes and seeds of wheat spikes collected from 7 different locations across Sweden. The isolates amounted to 21 operational taxonomic units (OTUs). After species annotation, the most commonly observed OTUs were found to represent *F. culmorum* (47%) and *F. graminearum* (38%) (Fig. 7). Considering the treatment given before culturing, 35% of the isolates were found in the interior of plant tissue (sterilised glume and seed) and 65% on the surface, respectively (non-sterilised glume and seed) (Fig. 8). Depending on the source of the isolates, 71% were obtained from the glumes and 28% from the seed of the wheat spikes (Fig. 9). Although, the isolates from both glume and seed for sterilised and non-sterilised treatment were predominantly *Fusarium* species, the pathogen abundance was more in the case of non-sterilised treatment (Fig. 8) as expected. In addition to this, a total of 278 isolates were obtained from the seeds harvested from untreated seed plots which remain to be sequenced.

We were able to group the isolated fungal species into communities and selected the ones which came from the same source, i.e., the community was isolated from the same spike, in the same treatment. The selected community was assumed to be a pure interacting complex and further verified using species specific primers. We were able to isolate three such *Fusarium spp.* corresponding to *F. graminearum (isolate HK48)*, *F. culmorum (isolate HK50)* and *F. avenaceum (isolate HK53)* from VSC039 SPK 004 growing on ½PDA medium and obtained from the surface of the tissue. Primers specific to *F. graminearum* were used for isolate HK48, *F. culmorum* specific primers for isolate HK50, and *F. avenaceum* specific primers for isolate HK53 (Fig. 10). The three selected isolates gave clear bands at appropriate amplicon length except for HK 53 which was verified again later (Table 5) and hence, this selected community was further used for infection assays.

While transferring colonies to new media, it was observed that *Fusarium* colonies grew differently in OMA compared to YMS or ½PDA. Instead of mycelial growth, clumps formed on the surface of the media (Fig. 11A). These clumps when viewed under the microscope turned out to be aggregated *Fusarium* macroconidia (Fig. 11B). This meant, *Fusarium* produced conidia even under constant darkness in OMA and hence, we used OMA for obtaining conidia for carrying out the infection assays (Fig. 11C).

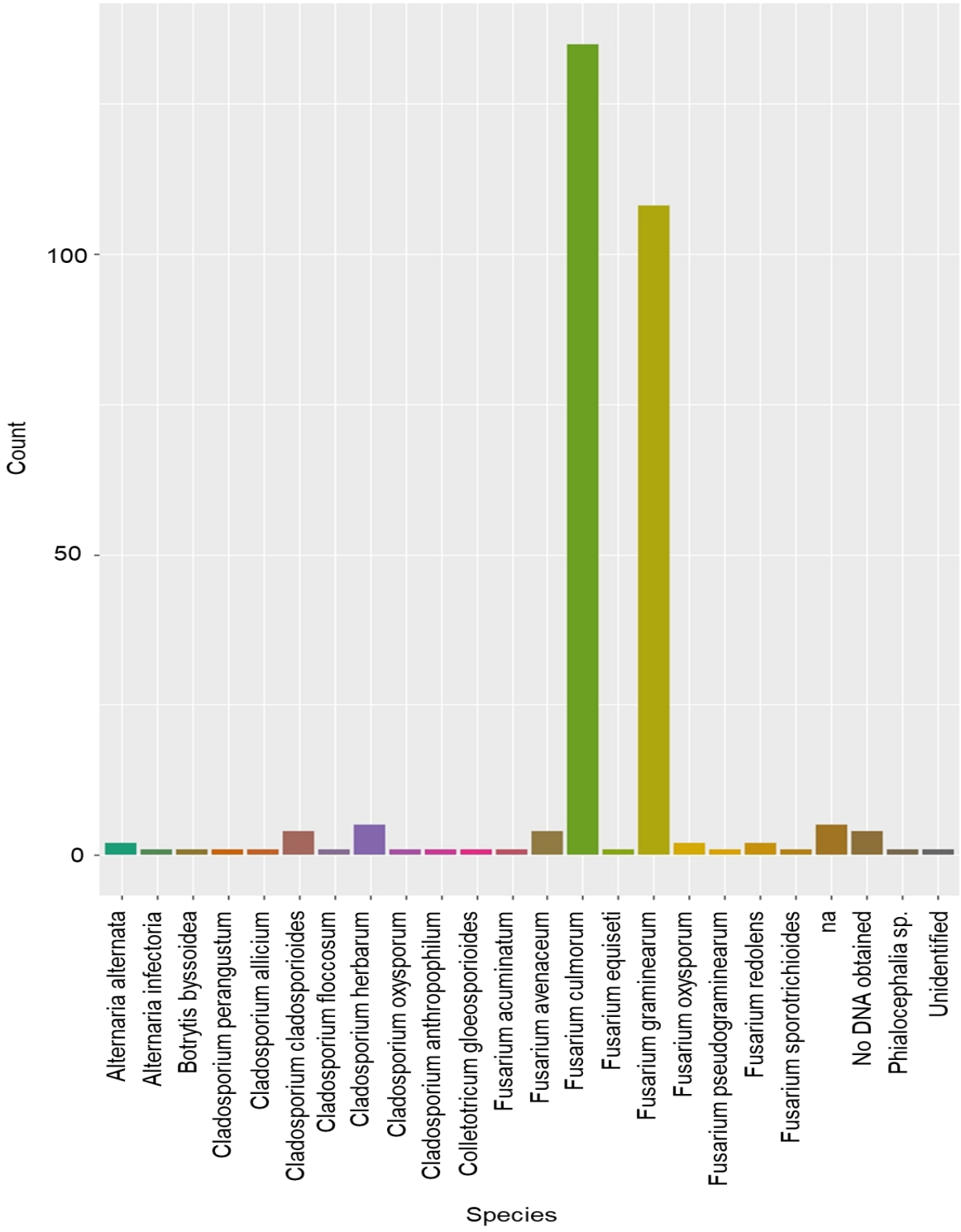

Figure 7. Total species isolated from the infected wheat spikes. Total 285 isolates were obtained from which 21 OTUs were identified. F. culmorum and F. graminearum were the most observed OTU amounting to 47% and 38% of the identified species respectively. Total isolates also included samples from which no DNA was obtained (4 samples), DNA was obtained but remain to be sequenced (unidentified) (1 samples) and sent for sequencing but sequencing results were of not good quality to provide blast results (na) (5 samples).

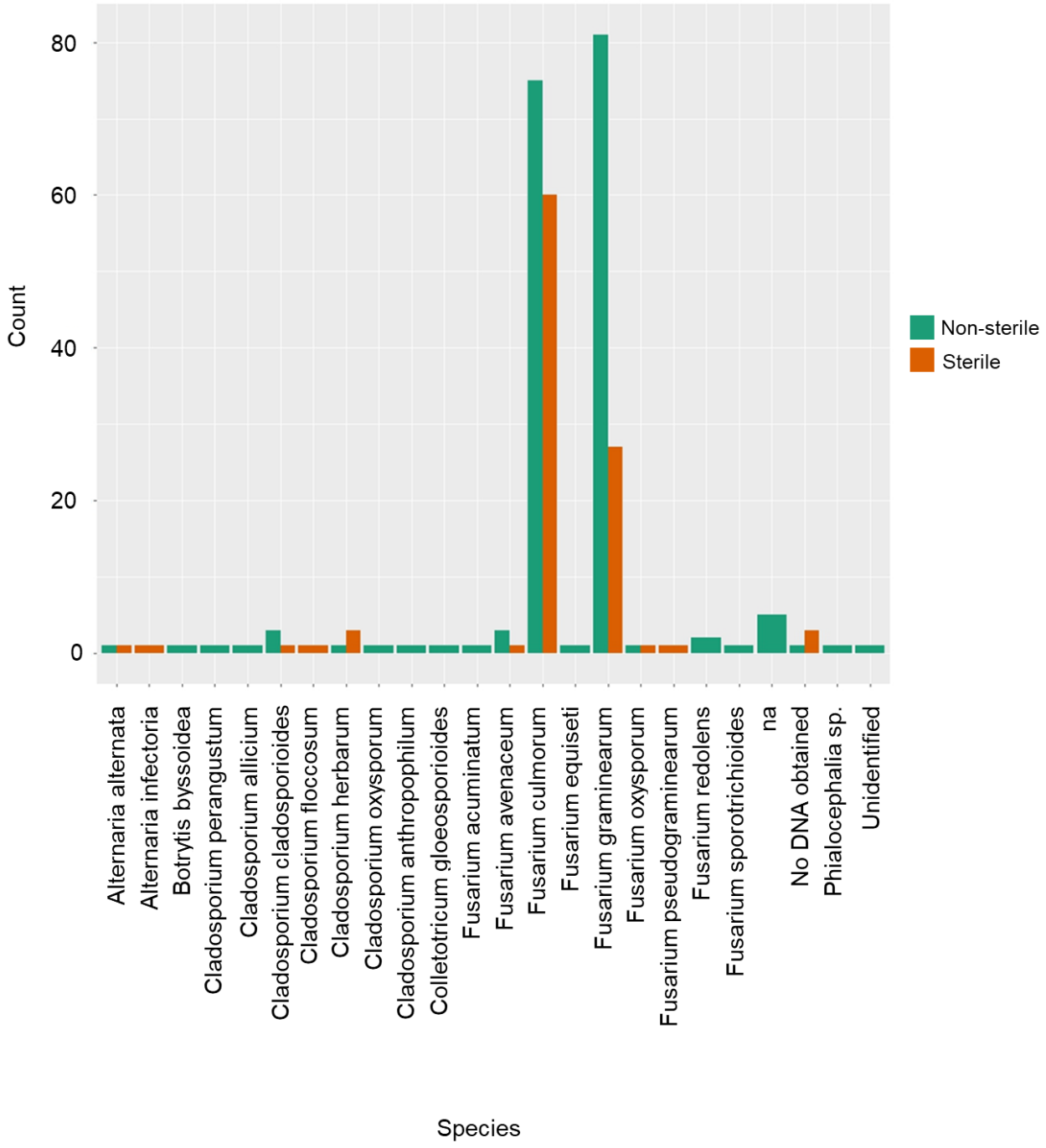

Figure 8. Comparison of total species isolated from the sterile and non-sterile treatment of infected wheat spikes. 35% of the isolates were found in the sterilised glume and seed) and 65% in case of non-sterilised glume and seed. The pathogen abundance is more in the case of non-sterilised treatment (green) compared to sterilised treatment (orange).

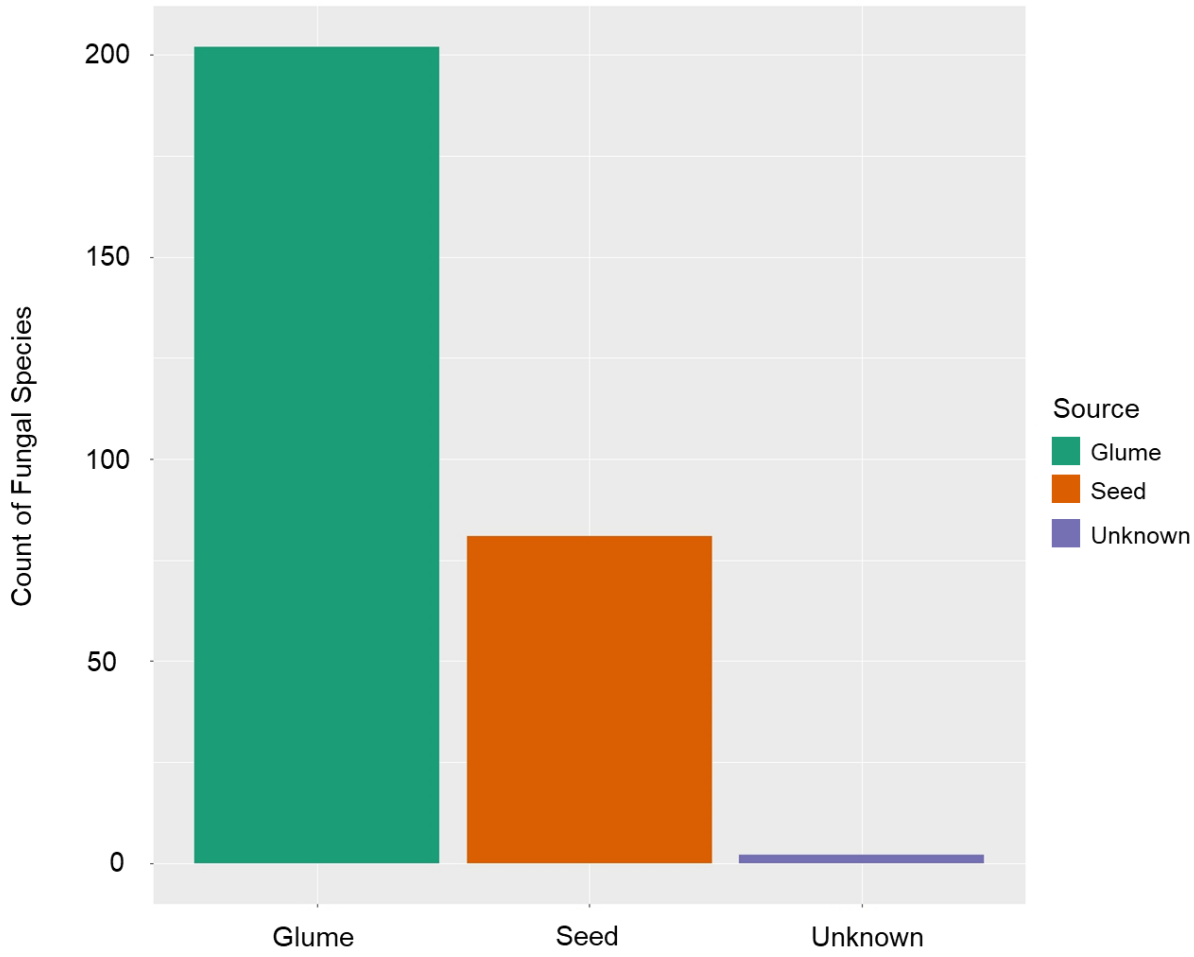

Figure 9. Comparison of total species isolated from the glumes (green) and seeds (orange) of the infected wheat spikes. 71% were obtained from the glumes and 28% from the seed of the wheat spikes and for remaining 1%, the source remains unknown (purple) due to error while labelling.

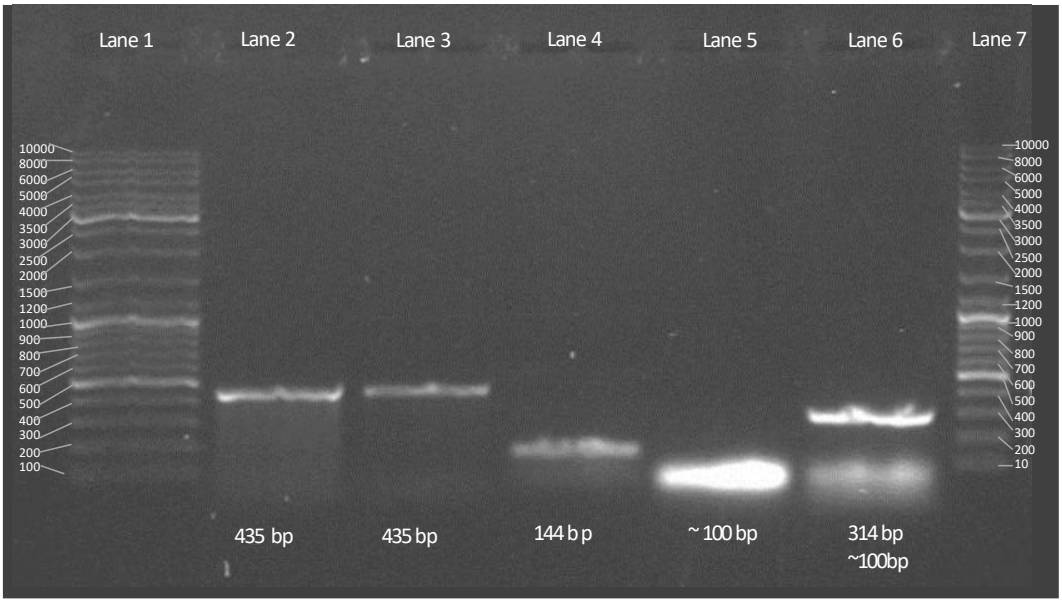

Figure 10. Verification of species identity by Agarose Gel Electrophoresis using Fusarium specific primers- Lane 1 Gene Ruler DNA ladder mix (100bp- 10k bp), Lane 2 (HK47, Fusarium graminearum) Lane 3 (HK48, Fusarium graminearum), Lane 4 (HK50, Fusarium culmorum), Lane 5 (HK53, unsure) (Fusarium avenaceum verified later), Lane 6 (HK54, unsure) (Fusarium avenaceum verified later) and Lane 7 Gene Ruler DNA ladder mix (100bp- 10k bp).

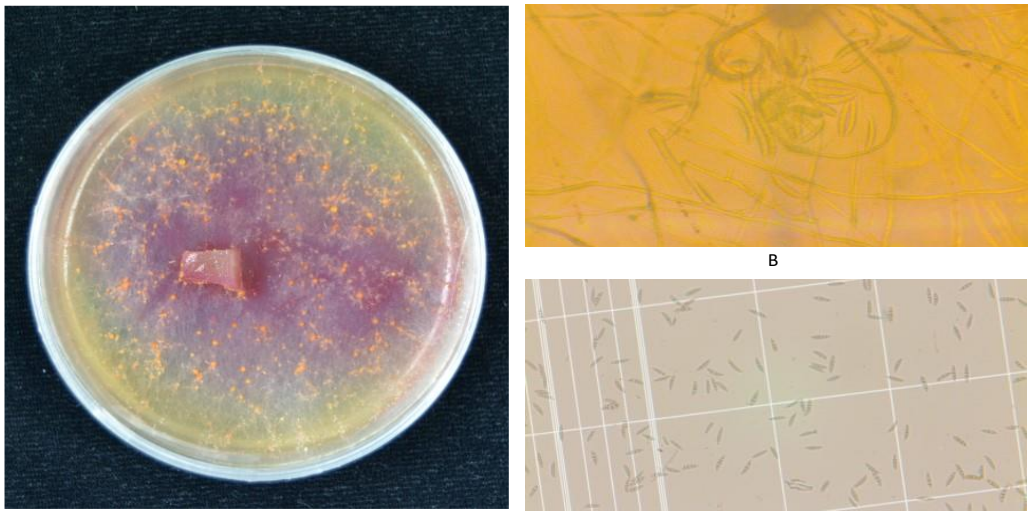

Figure 11. A) Fusarium graminearum growing in OMA media. B) Clumps of Fusarium graminearum conidia observed while growing on plate (20x). C) Fusarium graminearum conidia (10x).

3.2 Species implicated from the FHB complex interact with each other and the host genotype to influence disease progress

We first investigated the role of the plant genotype and pathogen complex interaction in disease formation in reference plant genotypes, Fielder, Bobwhite, and Chinese Spring. We observed that individual *Fusarium* species as well their different combinations cause infection differently in the same genotype. In case of Fielder (Table 7), all the different Fusarium combinations are causing disease differently. This suggests that the *Fusarium spp.* are interacting with each other during co-infection and that in nature, the observed variation in symptoms is possibly the result of fluctuations in the abundance of different *Fusarium* species. In addition to this, the same combination of *Fusarium spp.* is causing disease differently across different reference genotypes. In the case of co-infection with *Fusarium graminearum* and *Fusarium culmorum*, differences were observed between all three reference genotypes, from Bobwhite being more susceptible to the combination to Chinese spring being more resistant. This would suggest that the plant genotype could possibly be influencing the dynamics within the FHB complex.

Table 7. Heat map of infection scores of reference genotypes. Seven different combinations of Fusarium spp. were used for infection, "F. graminearum (Fg)"; "F. culmorum (Fc)"; "F. avenaceum (Fa)"; "F. graminearum and F. avenaceum (Fga)"; "F. graminearum and F. culmorum (Fgc)"; "F. culmorum and F. avenaceum (Fca)" and all the three combined (Fgca). Colour code was given based on a score from 0 to 5 assigned according to the area covered with necrosis, with "Red (5)" highest level of necrosis, "white (2.5)" intermediate level of necrosis to "green (0)" no necrosis.

Plant material	Combinations						
Reference genotypes	*Fg*	*Fc*	*Fa*	*Fga*	*Fgc*	*Fca*	*Fgca*
Fielder	3.5	2.58	3.08	3.8	2.75	1.75	2.8
Bobwhite	3.91	2.41	2.75	3.91	3.3	2.58	2.91
Chinese spring	3.6	2.33	3	3	2.4	2.41	2.75

*Key: 0 | 1 | 2 | 3 | 4 | 5

3.3 Phenotypic assessment of FHB infection in commercial cultivars and breeding lines

We carried out phenotypic assessment of eight breeding lines and 30 commercially grown bread wheat cultivars. Out of the eight breeding lines, two (SW032 and SW038) failed to germinate. SW023 and SW020 (belonging to one cross) infection differed slightly in response to *F. culmorum*, "*F. graminearum* and *F. culmorum*", and "*F. culmorum* and *F. avenaceum*" (Table 8). This suggests that SW023 is more susceptible to *F. culmorum* compared to SW020. Also, SW004 and SW001 (belonging to one cross) show slight variation in response to *F. avenaceum*, "*F. avenaceum* and *F. graminearum*", and all the three combined (Table 8). This may suggest that SW004 is more susceptible to *F. avenaceum* compared to SW001. This means different plant species respond differently to various combinations of *Fusarium sps*. In addition to this, SW036 (an old cultivar of *T. durum*), SW023 (accession of *T. dicoccum*) and SW020 (cultivar of *T. durum*) show high susceptibility to *Fusarium spp.* compared to others which are bread wheat cultivars (Table 8).

In most cases of commercial cultivars (Table 9), *F. graminearum* had the highest infection scores as compared to other species of the complex (individually and/or combination). Also in most cases, "*F. culmorum* and *F. avenaceum*" co-infections caused the least symptoms. Infection by the other combinations varied differently across the genotypes. In some cases, like SW035, SW019, SW016, SW012, SW031, SW030, SW017, SW008, SW021 and SW018, showed increased susceptibility to "*F. graminearum* and *F. culmorum*" and "*F. graminearum* and *F. avenaceum*" was observed. However, they still showed less susceptibility to "*F. culmorum* and *F. avenaceum*" combined. This shows that *F. culmorum* and *F. avenaceum* are antagonistic in their interaction, but both facilitate *F. graminearum* infection separately.

Table 8. Heat map of infection scores of Breeding lines used for the generation of mapping populations at CREA. Seven different combinations of Fusarium spp. were used for infection, "F. graminearum (Fg)"; "F. culmorum (Fc)"; "F. avenaceum (Fa)"; "F. graminearum and F. avenaceum (Fga)"; "F. graminearum and F. culmorum (Fgc)"; "F. culmorum and F. avenaceum (Fca)" and all the three combined (Fgca). Colour code was given based on a score from 0 to 5 assigned according to the area covered with necrosis, with "Red (5)" highest level of necrosis, "white (2.5)" intermediate level of necrosis to "green (0)" no necrosis.

Plant material	Combinations						
	Fg	*Fc*	*Fa*	*Fga*	*Fgc*	*Fca*	*Fgca*
Breeding lines							
SW034	2.5	2.667	2.667	2.33	2.67	1.5	1.5
SW036	4.33	4	2.333	3.5	4.17	2.5	2.67
SW004	3	2.667	2.333	3	2.83	0.83	2
SW001	3	2.667	1.833	2.33	2.5	0.83	1.5
SW023	4	3.833	3.167	3.5	4.5	2.17	3.5
SW020	4.17	3	3.333	3.67	3.67	1.67	3.33

*Key	0	1	2	3	4	5

Table 9. Heat map of infection scores of Commercial cultivars. Seven different combinations of Fusarium spp. were used for infection, "F. graminearum (Fg)"; "F. culmorum (Fc)"; "F. avenaceum (Fa)"; "F. graminearum and F. avenaceum (Fga)"; "F. graminearum and F. culmorum (Fgc)"; "F. culmorum and F. avenaceum (Fca)" and all the three combined (Fgca). Colour code was given based on a score from 0 to 5 assigned according to the area covered with necrosis, with "Red (5)" highest level of necrosis, "white (2.5)" intermediate level of necrosis to "green (0)" no necrosis.

Plant material	Combinations						
	Fg	*Fc*	*Fa*	*Fga*	*Fgc*	*Fca*	*Fgca*
Commercial cultivars							
SW002	4	2.33	2.33	2	2	1.83	2.5
SW007	4	1.83	2.33	2.17	2.17	1.67	1.67
SW025	3.33	1.83	1.83	2.5	2.67	1.17	1.67
SW005	2.83	1.5	1.67	1.5	2	1.5	2.17
SW013	2.83	1.5	2	2.67	2.33	1.83	2.33
SW033	2.83	2.33	1.83	2.33	1.67	1.33	2.67
SW029	2.33	2.17	2.17	1.67	2	1.33	1.5
SW022	3	1.5	2	2	1.83	1.5	2
SW024	3.33	1.83	1.67	1.83	1.83	1.33	2.17
SW015	3.5	2.17	1.5	1.5	2.17	2.17	2.33
SW037	2.67	2.5	3.17	2	2.5	1	2.83
SW014	2.67	1.5	2.67	3	2.5	2	2.83
SW003	3.83	2.83	2.33	1.83	2.83	2.5	3.17
SW028	3.67	3	2.5	2.5	2.33	2.67	2.5
SW027	2.67	2.17	2.5	2	2.33	1.33	2.33
SW009	2.67	2	2.33	2.67	2	1.33	1.83
SW026	2.33	2.17	2.17	2.33	2.5	1.33	2.33
SW006	3	2.67	2.83	2.33	2.17	2.17	3
SW011	2.83	2.5	2.83	1.83	2.17	1	2
SW010	2.67	1.67	3.17	1.83	2.83	1.17	1.67
SW035	2.33	1	1.67	4.33	4.83	2.83	1.67
SW019	1.67	1.17	1.83	4.83	4.83	2.5	1.17
SW016	1.33	1	1.17	5	5	1.83	2
SW012	2.33	1.17	1.83	4.5	4.5	2.5	1.33
SW031	2.17	0.83	1.5	4.83	4.17	1.5	1.5
SW030	2.17	1.17	1.67	3.67	4.17	1.33	1.17
SW017	1.33	0.33	1.5	4.67	3.5	1.67	1.33
SW008	1.5	1.17	1.17	4.33	4	1.67	1.67
SW021	1.33	0	1.33	4.67	4.33	2.67	1
SW018	2	0.33	1.5	4.67	4.33	1.33	1.5

*Key	0	1	2	3	4	5

3.4 Statistical analysis

The data generated with our linear model (LM) did not pass the Lilliefors normality test and the Breush-Pagan heteroscedasticity test. We also treated the data by logarithm and radicals and created a general linear model (GLM), but we did not obtain any model which could pass the normality and heteroscedasticity assumptions. The main reason our model did not pass the test was due to the discrete nature of the disease score values. Since it was biologically reasonable to treat our data as normal (a detached leaf can have a disease severity between two of the disease score used values), we decided to perform a parametric statistical analysis.

Principal Component Analysis (PCA) was first used to analyse the contribution of each variable to the distribution of our data. The PCA plot (Fig. 12) demonstrated the total variance of two major principal components (72.4%) with dimension 1 accounting to 39.1% and dimension 2 accounting to 33.3%. According to the PCA plot, the variable 'cultivar' is the major contributor to explain the observed variability of the disease severity (result of difference in resistance/ susceptibility of the different cultivars). Interestingly, the PCA plot also suggests that the disease score and FHB complex are correlated which can be expected as each specie and species combination trigger different levels of disease severity like *Fgc* and *Fga* elicit more severe response compared to *Fca* in case of commercial cultivars.

The difference among groups were statistically evaluated using a two ways analysis of variance (ANOVA). The ANOVA (Table 10) showed that there were statistical differences between cultivar/Fusarium-species and in the interaction between these two variables. This suggested that there are differences among the groups of different *Fusarium* species and cultivars tested and between each combination of *Fusarium*/cultivar evaluated. These results could indicate that the disease severity depends on the interaction of the pathogen species and on the cultivar genotype.

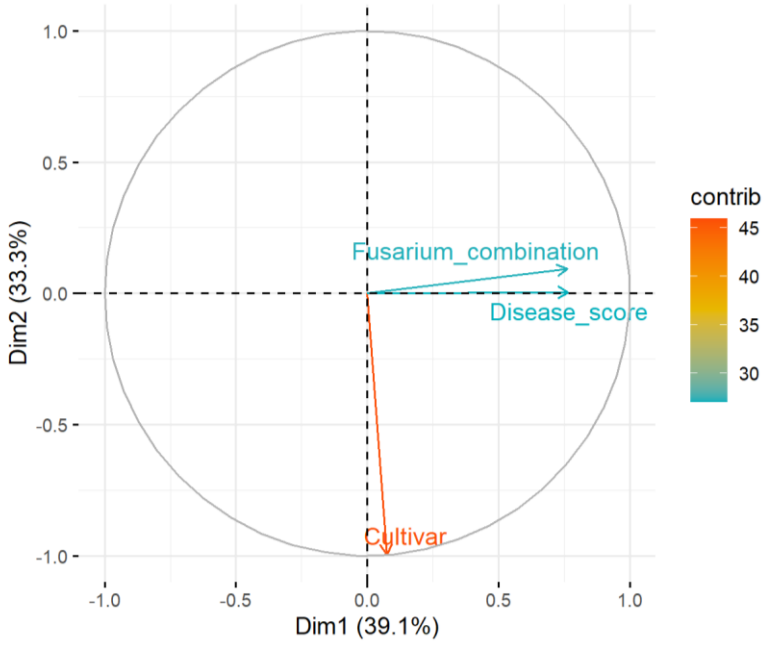

Figure 12. PCA plot with Cultivar genotype and Fusarium species combination as variables. R software was used to develop PCA analysis. The first dimension (Dim1) is mainly composed by the variable cultivar and the second dimension (Dim2) is mainly composed by the Fusarium species (Fusarium combination) and the symptom severity (disease score). The first dimension explains 39.1% and the second dimension explain 33.3% of the observed variability. Contribution of each variable to each dimension is described by the colour key (contrib), red indicating high contribution and blue indicating a low contribution of the variable to the dimension.

*Table 10. ANOVA of the disease score with respect to cultivars (Cultivar), Fusarium species combinations (Fusarium_combination) and both combined (Cultivar:Fusarium_combination). The analysis was performed with R software. We show the degrees of freedom (Df), the sum of squares (Sum Sq), the mean of the squares (Mean Sq), the Fisher-Snedecor statistic (F value), the P-value (Pr(>F)) and the level of significance ("***" for p=0, "**" for p=0.001, "*" for p=0.01, "." for p=0.05, " " for 0.1)*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Cultivar	38	302.7	7.97	13.575	<2e-16	***
Fusarium_combination	6	445.4	74.24	126.506	<2e-16	***
Cultivar:Fusarium_combination	228	808.3	3.55	6.041	<2e-16	***
Residuals	1487	872.6	0.59			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4. Discussion

The main aim of carrying out isolation of fungal communities instead of a total community analysis using sequencing, was to obtain fungal isolates that can be used to experimentally study plant-pathogen (in this case wheat and FHB complex) interactions under laboratory conditions. Therefore, it is understood that our strategy is not aimed at describing the total microbiome. Because isolation by culturing is limited by a strong bias for species that are favoured by the media used, we tried to isolate as many taxa as possible by using four different media. The purpose of grouping the isolated fungal species into communities was to obtain a pure interacting complex. We assumed that if fungal species were isolated from the same spike (we used two glumes and one seed from each spike), under same treatment and even the same media, we believe that these fungal species form a true interacting complex and replicates how they interact in the field conditions.

Detached leaf assays have been successfully used in determining *Fusarium langsethiae* pathogenicity and aggressiveness in oats and wheat (Tan et al. 2021) as well as determining the role of *F. poae* in facilitating the infection by the *F. graminearum* (Imathiu et al. 2009). Researchers at the Liebig Institute of Plant genetics and Crop Plant research (IPK) have shown that it is possible to predict the differences in FHB development due to plant genotype in the field conditions based on leaf segment inoculations in the lab (Dr. Dimitar Douchkov, IPK Gatersleben, personal communication). This is an important information so we can apply a reductionist approach by using leaf segments to assess FHB. Hence, we can assess the exclusive role of plant genotype in phenotypic assessment without any influence from the environment using the detached leaf assays.

Infection assays on the lab standard established that the disease progress occurs at two levels, at one level different *Fusarium* species interact with each other, then, and at a second level, the host genotype influences the host resistance or susceptibility to disease progress by the formed pathogen complex. This shows that a complex dynamic exists between host genotypes and pathogen complexes, and both factors are important to consider while studying disease development. It is interesting to note that there was a general trend in the disease severity by various combination of *Fusarium* species in the lab standards. *Fusarium graminearum* individually resulted in higher disease severity than any other combinations which has been observed in other studies too (Xu et al. 2005, 2007). It might be possible that the other Fusarium species are competitive towards *F. graminearum* and result in decreased severity in terms of necrosis in leaves when inoculated together with *F. graminearum*. It is also possible that *Fusarium graminearum* is very aggressive

and predominates any infection caused by other species in co-inoculations (Velluti et al. 2000).

Fusarium culmorum and *Fusarium avenaceum* combination showed the lowest disease severity of all pathogen combinations. This was also observed in case of parental RILs and commercial bread wheat cultivars. This negative interaction has been observed in pre-penetration studies wherein *F. culmorum* inhibits germ tube formation by *F. avenaceum* (Wagacha et al. 2012). This suggests that *F. culmorum* and *F. avenaceum* are antagonistic to each other. This is interesting because the chosen cultivars are from different breeders and with different breeding histories, with the only common feature being that they can be grown in Sweden. It is interesting to note that in case of commercial wheat cultivars when the host genotype is resistant to "*F. culmorum* and *F. avenaceum"* and if *F. graminearum* is in combination with the two individually, the host genotype tends to respond the same way as to "*F. culmorum* and *F. avenaceum"*. This suggests that the host genotype may recognise certain factors from *F. avenaceum* and *F. culmorum* which might result in more resistance compared to *F. graminearum* (Xu & Nicholson 2009). This emphasizes the fact that both the plant genotype and the pathobiome are important considerations while carrying out resistance studies. This might also explain why when all the species are combined, the resulting infection is less than individual infection by *F. graminearum*. This level of complexity is difficult to assess in field infections, thus highlighting the usefulness of our approach.

It is also interesting to note that most of our fungal species isolated were *Fusarium graminearum* and *Fusarium culmorum*. We were able to isolate only four *Fusarium avenaceum* in total of 285 isolates. This is in accordance with the study shown in Finland (Yli-Mattila et al. 2004) wherein presence of *Fusarium culmorum* was positively correlated with *Fusarium graminearum* but negatively correlated with *Fusarium avenaceum.* It might be possible that this antagonism must be prevalent in the field conditions to affect the prevalence of one species over another.

In case of parental RIL lines, increased susceptibility of durum wheat lines compared to bread wheat show how the conventional breeding programmes need to improve their approach by selecting germplasm sources with more resistance. It was surprising to note that SW023 which is an accession of *T. dicoccum* showed high susceptibility to *Fusarium spp.* since *T. dicoccum* lines are considered to have more resistance comparatively (Haile et al. 2019). Also, the current breeding programmes screen for FHB resistance by visual examination which is inefficient and tedious. Detached leaf assays are compatible with high throughput phenotyping and hence, can be employed by breeders to accelerate crop breeding (Shakoor et al. 2017) thus, showing usefulness of our approach.

5. Conclusion

Fusarium Head Blight disease of wheat is one of the most devastating fungal diseases of wheat with losses affecting yield, seed, food and feed quality (Dweba et al. 2017). In this study, we showed that the disease formation by FHB complex is highly complicated, and the plant genetic makeup is important to implicate the resistance or susceptibility to disease progress. This approach provides a new insight of the host-dependent disease development which could be potentially used in identifying host factors contributing to resistance in breeding of FHB-resistant cultivars. It could also help in identifying probiotic microbiota which could be used to suppress disease development (Mina et al. 2020). For future research purposes, it would be interesting to use an image analysis software to measure the area of necrosis as well as implementing temporal and spatial variation during detached leaf assays to investigate at what level the interaction between the *Fusarium* species exists. Also, measurement of mycotoxin accumulation along with necrosis can be carried out to see if the interaction between species differs based on toxin accumulation by carrying out experiment in the attached leaves. Also, the fungal species isolated from untreated seed plots can be further investigated to check for more diverse fungal communities that may have role in the FHB complex.

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Popular science summary

Fusarium Head Blight (FHB) disease of wheat is one of the most devasting diseases of wheat worldwide and in Sweden. The disease is characterised by bleaching of the spikes and reducing the quality of the grains produced by releasing toxins. This results in high yield losses up to 40% as well as human and animal health risk. FHB has been associated with 17 different *Fusarium* species with main drivers being *Fusarium graminearum* and *Fusarium culmorum*. Several practices are being used to control the disease like application of fungicides and cultural control. However, these practices only help in avoiding spread of the pathogen and we need to find ways to prevent the disease in the first place. One such way is breeding of wheat cultivars with more resistance to the FHB. However, current breeding programmes mainly focus on resistance to *F. graminearum*. It is important to carry out resistance analysis by taking into consideration other *Fusarium* species as well as establish that plant genotype plays a role in resistance or susceptibility to FHB.

In this lab study we investigated the composition of *Fusarium* communities isolated from infected wheat spikes collected from various parts of Sweden. This will help us to assess the resistance in cultivars using *Fusarium* species that are found in Swedish wheat fields. We selected three different isolates, *F. graminearum, F. culmorum* and *F. avenaceum,* to carry out further investigations. Secondly, we observed infection on different wheat cultivars by various combinations of selected isolates to see how different *Fusarium* species interact with each other and the plant genotype to form disease. We observed that disease severity varied with various wheat genotypes as well as different combinations of species of FHB complex. This shows that both the plant genotype as well as FHB complex interact dynamically with each other to influence the disease development. In most cases, *F. culmorum* and *F. avenaceum* act antagonistically to each other and result in less disease severity. In some wheat cultivars, combination of *F. graminearum* and *F. culmorum* as well as *F. graminearum* and *F. avenaceum* caused more disease severity compared to other combinations. This shows that breeding programmes should assess the *Fusarium* species population for the target area and focus on resistance to the combinations that particularly result in high disease severity.

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Appendix

Supplementary Information Table 1. Recipes for the culture media used for isolating Fusarium colonies.

Media	Recipe	Measurements
½ PDA	BD Difco PDA	19.5g
	Agar	4g
	Water	1000ml
YMS	Yeast	4g
	Malt extract	4g
	Sucrose	4g
	Agar	16g
	Water	1000ml
OMA	Oat-Meal	2g
	Agar	16g
	Water	1000ml
CMA	Corn Meal	2g
	Agar	16g
	Water	1000ml

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