GENOME-WIDE ASSOCIATION STUDIES FOR FUSARIUM HEAD BLIGHT RESISTANCE IN SPRING WHEAT GERMPLASM FOR NORTHERN EUROPE

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Title

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Abstract

Wheat (*Triticum aestivum L.*) is one of the major crops in the world and an important agricultural commodity in Finland with various uses. Fusarium head blight (FHB) is a deadly disease of cereal crops and with the gradual increase in temperature and precipitation, it is becoming alarming to Finnish agriculture. Deoxynivalenol (DON) is a vomitoxin produced by *Fusarium graminearum* species during the FHB infection and is hazardous to health if taken in larger quantities by humans and animals. European Union has legalized the maximum allowed DON content in wheat flour for human consumption at 1.75 ppm. Various types of resistance against FHB are known till date, including tolerance and escape from the disease. Anther extrusion (AE) is a highly heritable trait in wheat and is mechanistically involved in resistance against FHB by preventing the availability of nutrients for the fungus. Other traits such as heading, maturity, and height have shown correlations with FHB incidence and severity in previous studies. Genomic information is crucial to identify markers to accelerate wheat breeding programs against FHB. This experiment was conducted at Boreal Plant Breeding Ltd. Finland using 198 spring wheat breeding lines in a row-and-column design with three replications in an artificially spawn-inoculated *F. graminearum* field. The goal of the project was to evaluate the genetic diversity for various agronomic and FHB-resistance traits and to estimate correlations among them. A genome-wide association study was also performed by using 11,987 SNP markers to investigate any marker-trait association(s) in the spring wheat breeding germplasm. Larger phenotypic variability was observed in both agronomic and FHB-resistance related traits. Many spurious associations were found with general linear models (Naïve and Q model). No marker-trait associations were observed among the traits in mixed linear model (K) after including kinship as a covariate. Cryptic relatedness among breeding lines has shown a significant role during association mapping. An unexpected negative correlation was found between DON and Fusarium severity indicating inaccuracies in phenotyping. A negative phenotypic and genotypic correlation was found between AE and DON. Future studies on the validation of AE as a phenotypic marker against DON accumulation is recommended. Repeating the experiment with the inclusion of more lines with *Fhb1* gene in homozygous state might be helpful in finding reliable associations for FHB-resistance related traits.

Keywords

Genome-wide association, Fusarium head blight, spring wheat, anther extrusion, genotypic correlation, phenotypic correlation

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Table of Contents

Abstract Form 2

1. Introduction 5

2. Literature Review 7

2.1 Wheat 7

2.2 Fusarium head blight 9

2.2.1 *Fusarium graminearum* 9

2.2.2 Life cycle of *F. graminearum* 10

2.2.3 Infection cycle of *F. graminearum* 11

2.2.4 *F. graminearum* and environment factors 12

2.3 Deoxynivalenol (DON) 12

2.4 FHB Resistance in Wheat 13

2.4.1 Types of FHB resistance 13

2.4.2 Genetic associations of FHB resistance(s) 14

2.4.3 Breeding against FHB 14

2.5 Morphological traits of wheat related to FHB resistance 15

2.5.1 Heading, maturity class and FHB 15

2.5.2 Anther Extrusion and FHB 15

2.5.3 Plant Height and FHB 16

2.6 GWAS; A promising approach to study plant traits 16

3. Research Objectives 18

4. Material and Methods 19

4.1 Plant Material and Experimental Design 19

4.2 Selection of suitable markers and Genotyping 20

4.3 Phenotyping and Data collection 20

4.3.1 Days to heading and Maturity groups 21

4.3.2 Plant Height and Anther Extrusion 21

4.3.3 Visual disease score, incidence and severity 21

4.3.4 DON testing 21

4.4 Data Analysis 22

4.4.1 Correlation, Heritability and Descriptive Statistics 23

4.4.2 GWAS Analysis 24

5. Results 26

5.1 Phenotypic Parameters 26

5.2 Genotypic data and Population Structure 28

5.3 Heritability and Genetic Correlations 29

5.4 Genome-wide association study 32
6. Discussion 35
6.1 Phenotypic data 35
6.2 Heritability and Correlations 36
6.3 Population structure and GWAS 37
7. Conclusion 39
8. Acknowledgements 40
9. Bibliography 41
10. Appendices 49
10.1 Appendix 1: PC plot 49
10.2 Appendix 2: Scree Plot 50
10.3 Appendix 2: Manhattan and Q-Q plot for Height 51
10.4 Appendix 3: Manhattan and Q-Q plot for Maturity class 52
10.5 Appendix 4: Manhattan and Q-Q plot for Anther extrusion 53
10.6 Appendix 5: Manhattan and Q-Q plot for Fusarium incidence 54
10.7 Appendix 6: Manhattan and Q-Q plot for Fusarium severity 55
10.8 Appendix 7: Manhattan and Q-Q plot for DON content 56
..... 56

1. Introduction

Fusarium head blight (FHB) is considered as a widely spread problem for global grain production that poses threats from field scale to the dining table. *Fusarium graminearum* Schwabe is one of the common fungus causing blight in small grain cereals such as oats, barley and wheat grown around the globe (Hietaniemi et al., 2016). The fungus produces a naturally occurring harmful mycotoxin called as deoxynivalenol (DON) (Kushiro, 2008). DON is a type B trichothecene, an epoxy-sesquiterpenoid (Cope, 2018) and have ability to cause acute toxicity in human foods leading to diarrhea, vomiting, nausea, headache and fever (Pestka, 2010). *F. graminearum* is an emerging problem for small grains and have been recently reported in many European countries. Spread of this fungus has been observed in Netherlands, UK, Sweden, Norway, Finland and north-west Russia (Hietaniemi et al., 2016). Climatic conditions have a direct influence on the content of mycotoxins accumulated during the growing season. It has been reported that extended humid conditions from flowering till harvesting significantly increases the infection and in turn mycotoxin levels in grains (Brennan et al., 2005; Lacey et al., 1999; van der Burgt et al., 2011).

Wheat is one of the major food crops in Finland whereas contamination with DON is a serious concern to the food and feed security in the area. The EU legislation has strict recommendations for maximum DON concentration in unprocessed cereals (1250 µg/kg) and for bread and breakfast cereals (750 µg/kg) for human consumption (European Food Safety Authority, 2013). Finnish Food Authority has adopted the EU recommendations for maximum daily intake of dangerous mycotoxins including DON (Regulation 2005/856/EC). The changing climatic conditions have directly affected Finnish agriculture on one hand and the market dynamics of wheat grain products on the other. Previously, *F. culmorum* was reported to be the main DON producer in all Scandinavian countries, including Finland (Bottalico and Perrone, 2002) whereas, *F. graminearum* was dominant in warmer climates of central and southern Europe (Logrieco and Moretti, 2008). But during the last decade *F. graminearum* has become more active in most countries of the European continent because the higher temperature and humidity conditions favors its prevalence in the area (Madgwick et al., 2011; Miedaner et al., 2008; Stepien and Chełkowski, 2010).

Apart from climatic conditions, the genetic potential of a cultivar is an important factor allowing various plant-microbe interactions resulting in resistance or susceptibility of the plant to a certain pest (Grażyna Podolska, 2017). The most effective and sustainable way to cope with FHB is to develop resistant cultivars and follow appropriate crop management practices (Ollier et al., 2020). Identification and utilization of resistant wheat genotypes is crucial to mitigate the problem by using tools of molecular plant breeding. It has already been reported that genetic resistance to FHB is non-

race specific and is controlled by multiple loci with effects ranging from low to high (van Eeuwijk et al., 1995). Similarly, the heritability of the trait ranges from medium to high and is dependent of the genetic population under consideration (Bai and Shaner, 1994). Two types of FHB resistance has already been reported in wheat during 1960's which includes today's Type I resistance as the resistance to initial infection, and Type II resistance as the resistance against the spread of infection on wheat spike (Schroeder and Christensen, 1963). It has been also demonstrated that Type II resistance is more stable and is less influenced by environmental factors (Bai and Shaner, 1994). Although FHB resistance in wheat is a complex trait and probably comprises of complex interactions of genetic networks and signaling pathways (Bai and Shaner, 2004).

Anther extrusion (AE) is a quantitative trait that refers to the extrusion of yellow stage anthers coming out of the florets. Higher AE has been reported for exhibiting a negative association with FHB severity, whereas less or partially extruded anthers showed association with increased infection (He et al., 2016; Kubo et al., 2013; Lu et al., 2013). The anthers if retained inside florets serves as nutrients source for *F. graminearum*, whereas if the anthers extrudes out, the colonization of the pathogen becomes more difficult (Xu et al., 2019). Selecting the desirable genotypes based on AE would be a helpful strategy for plant breeders to breed wheat crop against FHB (Strange et al., 1978). Moreover, plant height (PH) also shows a close association with FHB resistance; greater the height, lesser is the probability of infection (Muqaddasi et al., 2017b). Over the past two decades, many quantitative loci (QTL) have been mapped related to FHB resistance in wheat but the populations used for such studies were mostly biparental with relatively smaller sizes (Pais de Arruda et al., 2015).

Genome-wide association studies (GWAS) is a promising strategy for the identification of QTLs related to the trait(s) of interest and exploits the recombination events to a higher mapping resolution (Pais de Arruda et al., 2015). Therefore, there is a need to study spring wheat genotypes adapted to Northern climates for identification of FHB resistant loci and in turn assist the wheat breeding programs in Finland. It is also important to validate AE as a phenotypic marker for FHB resistance to reduce the expensive cost of DON testing and to assist in selection process for the plant breeders.

2. Literature Review

2.1 Wheat

Wheat (*Triticum aestivum L.)* is an annual, self-pollinated hexaploid (AABBDD genome, 2n=6x=42) cereal that has been first cultivated about 9,000 years ago (Shewry, 2009). Wheat is currently one of the most important staple crops worldwide and is contributing to 40% of the nutrient intake of human population (Giraldo et al., 2019). Modern day wheat has been evolved from the hybridization among earliest cultivated species of wheat named as *Triticum turgidum* (AABB genome, 2n=4x=28) and an unrelated wild grass species *Triticum tauschii* (DD genome, 2n=2x=14) (**Fig. 1**). Wheat contributes substantially in global food security as the livelihood of about 80 million farmers rely on wheat crop, especially in developing countries (Giraldo et al., 2019). Wheat is also one of the principal staple crops after rice and with the passage of time, the demand of wheat products is rapidly growing in reference to tremendously escalating population coupled with changes in food preferences and dietary intake of people (Singh et al., 2019).

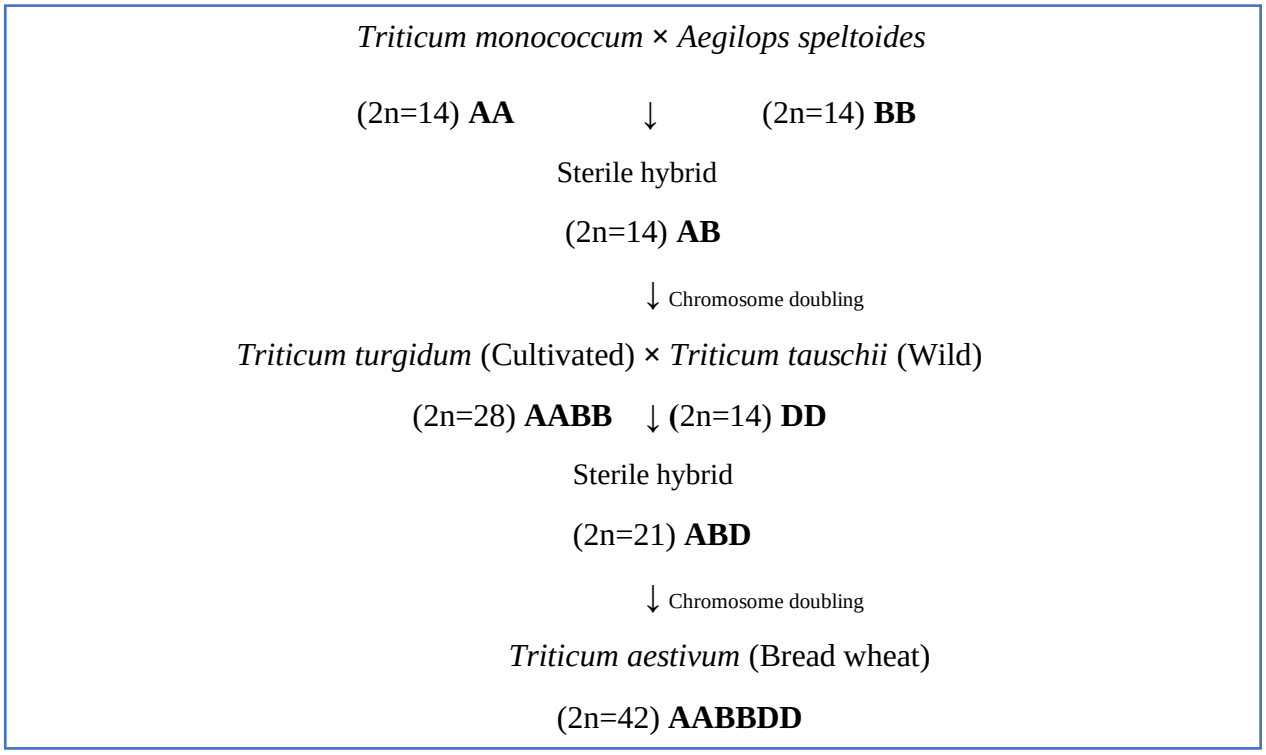

Figure 1 Flowchart diagram representing possible evolutionary hybridization events among various wheat types forming *Triticum aestivum L.* about 9000 years ago.

Morphologically, wheat plant produces multiple leaves, stems/tillers and finally spikes on top of productive tillers which bear grains at maturity. Some of the tillers are unable to produce spikes and eventually grains hence known as unproductive tillers (Wang et al., 2016). In comparison with other

arable crops, wheat roots are fairly deep and can extend as far down as 2m. Whereas, leaves are produced first from the apical meristem during the initial phases of the development and later on, the spike and other respective parts are produced. Time period needed to transition between the vegetative phase and reproductive (flowering phase) strongly influences the heading of the particular wheat variety (Bonnet, 1936). A flag leaf is the last leaf produced on wheat stem, majorly contributes in providing carbohydrates to the developing ear because it has higher photosynthesis rate due to broader leaf area (Pajević et al., 1999). Presence and absence of awns on spikes is variety dependant but is an appreciable structure in wheat varieties cultivated in countries with hot and drought-prone conditions (Duwayri, 1984). Awns also enhances grain size but might reduce grain number (Rebetzke et al., 2016). On the other hand, European wheat has shown a decrease in climate resilience so it has been suggested that awned wheat varieties could be a solution to combat this issue (Kahiluoto et al., 2019).

Wheat grows successfully between the latitudes of 27°S and 40°S and 30°N and 60°N, but it has also demonstrated effective growth beyond these limits. The maximum and minimum growth temperature requirement for wheat cultivation is 34–36°C and 3-4°C, respectively but the mean optimum growing temperature is about 25°C (Mergoum, 2009). In terms of moisture, nearly three-fourth of the wheat cultivated land area receives annual precipitation of almost 375 and 875 mm but wheat has also adapted to a range of moisture conditions and can be grown in areas where annual precipitation ranges from 250 to 1,750 mm (Mergoum, 2009). The harvesting month of wheat varies in various parts of the world, but in the temperate zones of the Northern hemisphere, the maximum volumes are harvested in April and September, while in the southern hemisphere, wheat is mainly harvested in between October and January (Mergoum, 2009).

Currently, wheat is cultivated on about 218 million hectares worldwide, and the land area as well as trade of wheat is more in comparison to any other crop all around the world (Giraldo et al., 2019). Since 1960s, the cultivation of wheat along with other cereals has increased to three-fold and is expected to increase further by the middle of 21st century (Godfray et al., 2010). In Finland, most of the consumer's daily energy needs are fulfilled by cereals, meat and dairy. In year 2019, nearly 1.07 million hectares was cultivated by cereal crops in Finland that were used as animal feed (62 %), as food (16 %), as seed (9 %) and as industrial use (13 %), like brewing bears and distilling other liquors (http://stat.luke.fi/en/cereals-balance-sheet). Out of this, 206,800 hectares were used for wheat cultivation overall among which 166,700 hectares for spring wheat, and 40,000 hectares for cultivating winter wheat (Partala, 2019). In terms of production in year 2019, winter wheat accounted for 222.3 million kg while spring wheat for 697.3 million kg, which brought the total to approximately 919 million kg of annual wheat produce in Finland. Whereas, the average yield per hectare in 2019

for winter wheat was 5620 kg/ha and 4300 kg/ha for spring wheat (Luke, 2019). The concerns regarding the quality of wheat in Finland poses serious challenges to meet the demands of coming days. Consequently, it is imperative to set research goals and policies for productivity maximization in order to encounter the raising demand of wheat and wheat-based products keeping in view the importance of defined quality standards.

2.2 Fusarium head blight

Fusarium head blight (FHB), commonly known as scab, is a potentially devastating and deceptive disease of barley and wheat, mostly prevalent in humid and semi-humid areas of the world (Schroeder and Christensen, 1963). Before 1990s FHB was mainly reported in South America, East Asia and parts of Europe. But after that, FHB threat has also been rising in Canada and USA primarily due to climate change, crop rotations and adoption of conservation agricultural practices (McMullen et al., 2012). In China, severe epidemics of FHB affected more or less 0.7 million hectares of wheat which reduced the yield to more than 1 million tons (Hongxiang MA, 2019; Leonard and Bushnell, 2003). In USA, various intense outbreaks of FHB on barley and wheat from 1991-1997 lead to almost 1.3 billion USD worth losses that badly influenced the overall economy of the States (Bai and Shaner, 2004). Although there are a lot of Fusarium species that can cause FHB but *F. graminearum* is the commonly reported pathogen in various studies done in different parts of the world (Bai and Shaner, 1994; Bíliková and Hudec, 2013).

2.2.1 *Fusarium graminearum*

Until now, isolation of more than seventeen species of Fusarium from naturally infected spikes of wheat and barley have been accomplished (Becher et al., 2013; Dweba et al., 2017; Khan et al., 2020). All of the isolated species have various levels of virulence and can infect wheat and barley spikes on inoculation. Although *F. poae* and *F. culmorum* have been reported to be common in some of the European countries (Bottalico and Perrone, 2002) but *F. graminearum* is one of the most studied pathogen worldwide in cereal crops (Goswami and Kistler, 2004). *F. graminearum* can survive not only on various cereal crops like wheat, rice, soybean, corn and barley but also on the rotten and dead tissues of many other plant species (Mielniczuk and Skwaryło-Bednarz, 2020; Osborne and Stein, 2007). The fungal pathogen can use the crop residues on the soil surface as the major reservoir of nutrients (Shaner, 2003). Macroconidia, hyphal fragments, chlamydospores, and ascospores, all of them can be used as an inoculum by the fungal strains (Bai and Shaner, 1994), but ascospores majorly act as primary inoculum that can initiate epidemics (Shaner, 2003).

2.2.2 Life cycle of *F. graminearum*

The sexual development of *F. graminearum* starts by the formation of binucleate cells hyphae. The fungus cells are homothallic containing genetically identical nuclei and hence there is no requirement for a separate sexual partner for the development of sexual spores (ascospores). The mating behaviour of the fungus is under a strong genetic control (MAT genes) resulting in two mating types (Mat1-1 and Mat1-2) (Yun et al., 2000). In one study, deletion of the mating-type locus was done to eliminate the sexual reproductive stage and it brought prominent disease reduction during field trials determining the importance of the locus in fungal pathogenicity (Desjardins et al., 2004). Generally *F. graminearum'*s binucleate cells use to form further small coiled cells, that later develops into fruiting body initials (Trail and Common, 2000). When the initials are cultured, they quickly develop into perithecia that is flask-shaped and is filled with asci which are sacs that are tubular in shape and contain the ascospores that are formed by meiosis. At the final stage of maturation, the asci extend themselves up to the opening of the perithecium and later the ascospores are discharged into the air (**Fig. 2**) (Trail, 2009). A complete life cycle of *F. graminearum* takes about two weeks inside the laboratory conditions, while the maturation of asci and the release of ascospores usually takes place during the last four days (Bowden and Leslie, 1999; Trail et al., 2002).

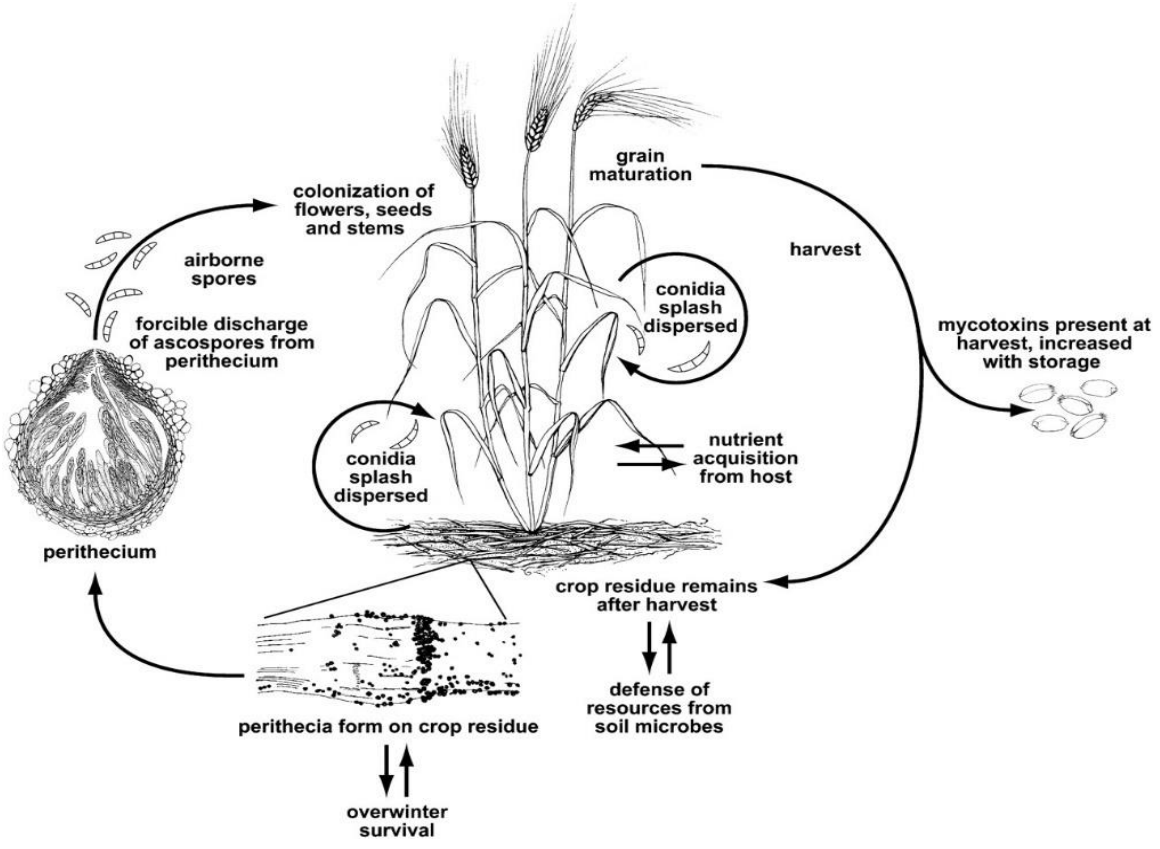

Figure 2 Life cycle of *F. graminearum* in field conditions (Adopted from (Trail, 2009))

2.2.3 Infection cycle of *F. graminearum*

In field conditions, the primary inoculum of FHB is airborne ascospores and it is considered to be a monocyclic disease (Trail, 2009). Infection begins to initiate when pathogenic spores that were released into air from the residues of crop lands come in contact with the growing wheat plant. Just after this novel contact, spores start germinating and enter the plant either through degenerating anther tissues or via natural openings of lemma and palea (Bushnell, 2003). The extruded anthers are promptly infected and then the fungus further penetrates through the developing rachis and floral bracts (Bai and Shaner, 1994; Bushnell, 2003). Soon after the initiation of infection, water soaked, dark brown spots begins to appear on the infected floret's glumes and later the entire floret gets blighted. Further the infection can spread through the entire susceptible wheat spikelet by vascular bundles of rachis and rachilla. There the fungus radially spreads and the necrosis initiates as the pathogen begins to grow intracellularly and starts colonizing the tissues (Bushnell, 2003). If the vascular tissues in the rachis gets clogged, it can lead to premature bleaching of spike head, and the tissues that get bleached might form a band of several florets right in the centre of the spike head (**Fig. 3**) which means that even grains that were not infected directly will also get shrivelled primarily due to a shortage of water and nutrients (Bai, 1995). The severity of blight increases as the pathogen further spreads within the spike, and ultimately the whole spike will get blighted. The infected florets are either unable to produce grains, or the produced grains are not completely filled which significantly influences the grain yield and quality due to the accumulation of mycotoxins produced by fungus (Bushnell, 2003).

Figure 3 Phenotypic appearance of Fusarium head blight infection on Wheat spikes. Different spikes showing various infection intensities in terms of visible bleaching of spikelets

https://fieldcrops.cals.cornell.edu/small-grains/diseases-small-grains/fusarium-head-blight-scab/

2.2.4 *F. graminearum* and environment factors

The presence of favourable weather conditions particularly temperature, moisture and the abundance of primary inoculum during and after the period of anthesis usually influence the severity and spread of Fusarium head blight (Goswami and Kistler, 2004; Markell and Francl, 2003). In Finland, spring cereals have been observed to have more susceptibility for *Fusarium* spp. and hence the accumulation of mycotoxins as well (Yli-Mattila et al., 2008). It has been reported that humidity combined with temperature during flowering stage helps the dissemination of inoculum consequently increases the chances of FHB infection (Kriss et al., 2010). Risk of the FHB infection seems to increase during the anthesis stage for most of the cereal crops (Doohan et al., 2003). In some other studies, it has been reported that DON accumulation also depends upon wind and the intensity of light apart from temperature and humidity (Doohan et al., 2003). Changing climate with possible increase in temperature can be potentially feasible for *Fusarium* spp. to spread in Finland and probably be more damaging to cereal crops in coming days (Hietaniemi et al., 2016).

2.3 Deoxynivalenol (DON)

F. graminearum, the most common cause of FHB, mainly produces deoxynivalenol (DON), a mycotoxin for whom regulations have been set in a lot of organizations and countries to guarantee food safety (Buerstmayr and Buerstmayr, 2015). DON is commonly considered as a vomitoxin, and develops inside FHB infected grains of wheat, barley and oats. In wet and humid conditions, FHB may start to infect grain heads especially when the plant is under flowering or grain filling stage (McMullen et al., 2012). The infection of FHB does not always indicate the presence of DON, but if harvested grains have high level of scabby kernels, it indicates that DON will probably be present (He et al., 2019). The toxicity levels of DON have been established by United States department of Agriculture (USDA), to ensure provision of safe food. Generally, the presence of DON does effect flavour and sensory attributes but its consumption in safe levels do not pose any threat to human health and it also does not contain any carcinogenic properties like that of aflatoxins in corn (Reddy et al., 2010). DON is only believed to be causing serious health situations if ingested in high amounts, some of the symptoms are vomiting, diarrhoea, headache and vertigo (Pestka and Smolinski, 2005). Therefore, the maximum tolerable consumption levels of DON in terms of wheat grains were set at 0.5 to 2 ppm in Canada, USA as well as in some European countries (Leonard and Bushnell, 2003; Osborne and Stein, 2007). On a similar note, European Union (EU) have also adopted certain recommendations on the threshold of food and feed mycotoxins. According to the EU Regulation (EC) No 1881/2006, the maximum level of DON in human food is settled at 1250 µg/kg in case of unprocessed cereals and at 200 µg/kg for food items suitable for infants and young children (European

Food Safety Authority, 2013). Most of the DON is present in the outer seed cover of bran and if FHB infection develops during very initial stages of kernel development, it can reduce yield by lowering down the kernel numbers as well (Sinha and Savard, 1997). Whereas, the infection at slightly later stages leads to chalky white or discoloured, shrunken and scabby kernels, that are also called as tombstones (**Fig. 4**). FHB infection at later stages of plant development may bring no visible damage, but the kernels may still possess high levels of DON (Góral et al., 2018). A Canadian study evaluated the DON content in FHB infected kernels and reported that DON content was 274 ppm in pink coloured kernels, 174 ppm in white tombstones, 2-5 ppm in shrunken kernels and 1-1.2 ppm in normal kernels (Sinha and Savard, 1997). Shrunken kernels are removed prior to milling in order to improve flour quality but this brings significant loss in milling yield (Dexter et al., 1997). In case of oats, de-hulling of the seeds may lead to a reduction in toxin concentrations by 90 % (Schwake-Anduschus et al., 2010). On the other hand, the application of various cleaning methods was found to reduce the mycotoxins levels up to 36% in wheat (Schaarschmidt and Fauhl-Hassek, 2018).

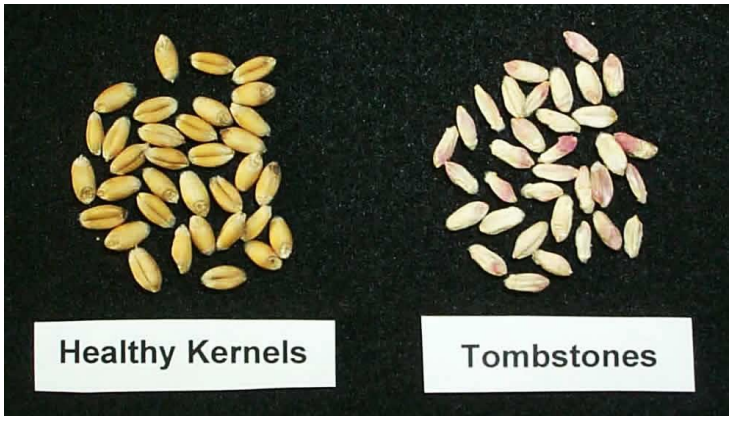

Figure 4 Picture taken from healthy wheat kernels (right) and FHB infected tombstones (left).

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2.4 FHB Resistance in Wheat

FHB resistance is a complex trait and is influenced by multiple factors, ranging from plant genetic constituents (QTLs) that influence intrinsic plant defence mechanisms to plant characteristics that have an indirect impact on decreasing susceptibility to FHB. These active and passive resistance factors are sometimes related to morphological and/or developmental traits of wheat.

2.4.1 Types of FHB resistance

In hexaploid wheat, various kinds of FHB resistances have been recognized till date. The ability to resist the incidence of infection is known as Type I resistance while the ability to prevent the severity or spread of FHB is referred as Type II resistance. Both of these resistances have been reported by

various studies in bread wheat (Burt et al., 2015; Chu et al., 2011; Lemes da Silva et al., 2019), although Type II resistance is regarded as most stable and effective against FHB (Bai and Shaner, 2004) hence was studied in many breeding populations (Buerstmayr et al., 2002; Jiang et al., 2020; Zhang et al., 2018). A plant exhibiting good Type I resistance but week Type II resistance may easily get infected whenever the inoculum is abundant (Bai, 1996). On the other hand, Type III and IV resistance against FHB is usually less studied in comparison to that of Type II resistance. Type III resistance was conceived for stopping infection initiation in kernels and also has referred to different resistance capacities of some lines to degrade DON (David Miller and Arnison, 1986). Whereas, Type IV resistance has been reported for preventing the accumulation of DON (Liu et al., 2009; Löffler et al., 2009). The low level of DON in the infected kernel might be because of three possible causes: (a) The fungus produced less amount of DON, (b) The plant enzymes degraded the DON during development of kernel, or (c) The DON content was higher in spike tissue but it failed to move inside kernels during the developmental process (David Miller and Arnison, 1986).

2.4.2 Genetic associations of FHB resistance(s)

Studies has shown that days to heading (DH) and plant height (PH) are associated with development of Type I resistance (He et al., 2016; Mao et al., 2010). Type I and Type II resistances have also shown association with Type III and Type IV resistance mechanisms. A meta-analysis study showed that various types of FHB resistances were studied by QTL mapping among which only 22 and 25 out of 209 QTLs were associated with Type IV and Type III resistance respectively but none of them were disassociated from Type I or Type II (Liu et al., 2009). Miller et al. (1985) first monitored Type III resistance and reported that the resistant cultivars depicted significant ability to promote degradation and can prevent synthesis of DON (Miller et al., 1985). Later in an in vitro experiment, embryo callus cultures of a resistant cultivar ‘Frontana’ and a susceptible cultivar ‘Casavant’ were compared that resulted in an 18% degradation of DON in resistant cultivar while just 5% decrease in the susceptible one, indicating the activity and variation among genetic mechanisms of Type III resistance is cultivar dependant (David Miller and Arnison, 1986).

2.4.3 Breeding against FHB

Traditional breeding approaches for breeding wheat against FHB are quite difficult, time-consuming and require laborious efforts. Quantitative mode of inheritance and substantial environmental influence makes FHB even more complex trait to breed in a conventional way (Stack, 2003). Over the course of past twenty years, more than 250 QTLs conferring FHB resistance have been reported on different wheat chromosomes (Buerstmayr et al., 2009; Löffler et al., 2009). Majority of these QTLs have been identified in association with Type II resistance and are found important in a

breeding perspective (Liu et al., 2009). *Fhb1* gene has been intensively studied and is believed to be a major player in FHB resistance mechanism against various isolates of Fusarium species (Stack et al., 1997). A Chinese spring wheat Cultivar 'Sumai 3' and its progenitor lines were found to have *Fhb1* gene in fixed state hence was extensively used in breeding wheat against FHB around the globe (Brown-Guedira et al., 2008). Marker-assisted selection (MAS) ensures an effective way of breeding against FHB in more accurate and speedy way (Li et al., 2019). Therefore, the identification of robust markers associated with resistance traits is of prime importance in today's resistance breeding on commercial scale.

2.5 Morphological traits of wheat related to FHB resistance

Phenological and morphological traits like loose spikelet distribution, absence of awns, staggered flowering time and tallness could contribute in the development of Type I resistance (Mesterházy, 1995). Recent advances in scientific studies for breeding against FHB has also shown that anther extrusion, plant height and days to heading are some of the important traits to consider as various resistance mechanisms for FHB (Lu et al., 2013; Mao et al., 2010; Skinnes et al., 2010).

2.5.1 Heading, maturity class and FHB

Days to heading and maturity are important traits in wheat breeding and are useful in terms of yield components (Anwar et al., 2009) and resistance against FHB (Mesterházy, 1995). Studies have shown contrasting results for marker-traits associations among FHB and both days to heading and maturity. One study in U.S.A has shown no significant association between markers and plant maturity (Chu et al., 2011) whereas, another study has shown a negative correlation between heading and FHB resistance (Moreno-Amores et al., 2020). It is important to see how different maturity groups behave in terms of *F. graminearum* infection for breeding lines in Northern Europe. The practical suggestions for such relations could belong to an escape strategy of plant types to avoid FHB infection.

2.5.2 Anther Extrusion and FHB

One of the practically suggested resistance mechanisms against FHB in wheat is via anther extrusion (AE) since it was reported that the initial infection of FHB occurs via anthers (Buerstmayr et al.; Muqaddasi et al., 2017a). AE is generally regarded as a quantitative trait that is influenced by various genes and is majorly studied to enhance the ability of cross-pollination in hybrid wheat (Muqaddasi et al., 2017a; Muqaddasi et al., 2019). The potential of enhanced AE to prevent FHB has been confirmed by an assay of *F. graminearum* green fluorescent protein strain on two wheat cultivars 'Sumai 3' and 'Roblin'. In both of the plant types, anthers developed infection rapidly but in resistant cultivar Sumai 3, the infestation of pathogen was hindered by the closure of xylem and phloem tissues

that were near the infected florets (Miller et al., 2004). It was reported in 1970's that various growth stimulants present inside anthers accelerate the growth of *F. graminearum* (Strange and Smith, 1971). These substances were further identified as betaine, choline and glycine that are present in high concentrations inside anthers (Pearce et al., 1976; Strange et al., 1974). However, these studies were revisited and no prominent influence of betaine and choline on growth of fungus was observed (Engle et al., 2003). It has also been suggested that emasculation and shedding of anthers is an effective tool against FHB (Strange et al., 1978). The partially extruded anthers, or those that are stuck between palea and lemma can enable the penetration of FHB inside the floret cavity but in case of full anther extrusion, FHB pathogen colonized on the anthers would face difficulty in infecting other floret tissues (Skinnes et al., 2010). A strong link between AE and duration of flower opening was also reported in the past (Singh et al., 2007). This might be because of the short opening duration or the fine angle between palea and lemma whereas, the anthers might retain inside the late flowering types (De Vries, 1971). A strong positive correlation between AE and FHB resistance was also observed and published in an extensive report on 60 different European winter wheats (Graham and Browne, 2009).

2.5.3 Plant Height and FHB

Plant height is an important morphological trait for wheat crop and has been under selection for decades. A semi-dwarf wheat cultivar is desirable due to better yield and it minimizes the risk of lodging in field conditions as well (Laing and Fischer, 1977). On the other hand, it has also been reported that FHB Type I resistance is associated with plant height (Mao et al., 2010). A study indicated that a certain resistant QTL was found on chromosome 4D at a location closer to the *Rht-D1* locus controlling plant height (Draeger et al., 2007). The association among these two loci is found to be a result of linkage among genes and the germplasm exhibiting such association can be used for resistance breeding purposes. Various studies on different breeding populations suggested a negative association between FHB resistance and plant height suggesting that the taller lines were more resistant to the disease (Buerstmayr et al., 2002; Buerstmayr et al., 2000; Buerstmayr et al., 2003; Somers et al., 2003). The mechanistic explanation of such a negative association could be an escape strategy of wheat plants against the fungus attack. Genetic associations among various plant traits often enables plant breeders to do indirect and effective selections for traits that are difficult to evaluate or practically expensive to phenotype (Aytaç, 2009).

2.6 GWAS; A promising approach to study plant traits

Recently developed techniques of DNA sequencing made it possible to genetically improve many traits like biotic and abiotic stress tolerance and grain quality in field crops. One of the useful and

successful tools for identification of regions close to candidate genes and related loci is genome-wide association study (GWAS) (Alqudah et al., 2020). GWAS works by investigating the statistical association between a genetic marker and the phenotypic trait which has been scored across distantly related or heterogeneous lines of a diverse collection of individuals (Huang and Han, 2014). The efficiency and robustness of GWAS in exploration of complex phenotypic traits among crops like wheat and barley had already been reported and with the aid of high throughput sequencing technology and currently available large populations, it is expected that GWAS will become more proficient in the identification of causative genes that are responsible for various quantitative traits (Alqudah et al., 2020). The variation of a phenotypic trait may involve few or many loci i.e. the trait might have a complex genetic structure (polygenetic e.g. heading date) or have a simple genetic architecture (e.g. barley spot blotch) (Bykova et al., 2017). The identification of factors to determine the basic genetic architecture of a trait is the main objective of a common GWAS study. On the other hand, Bernardo (2016) has criticized the use of GWAS for finding major but rare QTLs in breeding populations (Bernardo, 2016). Apart from the existence contrasting perspectives, GWAS approach is well-suited to predict the best performing candidates in the breeding population with the use of relatively cheaper and abundant markers (SNPs) instead of designing a methodology to create a cultivar (Bernardo, 2016). The possible utilization of a GWAS study in a plant breeding program is summarized in **Fig. 4**.

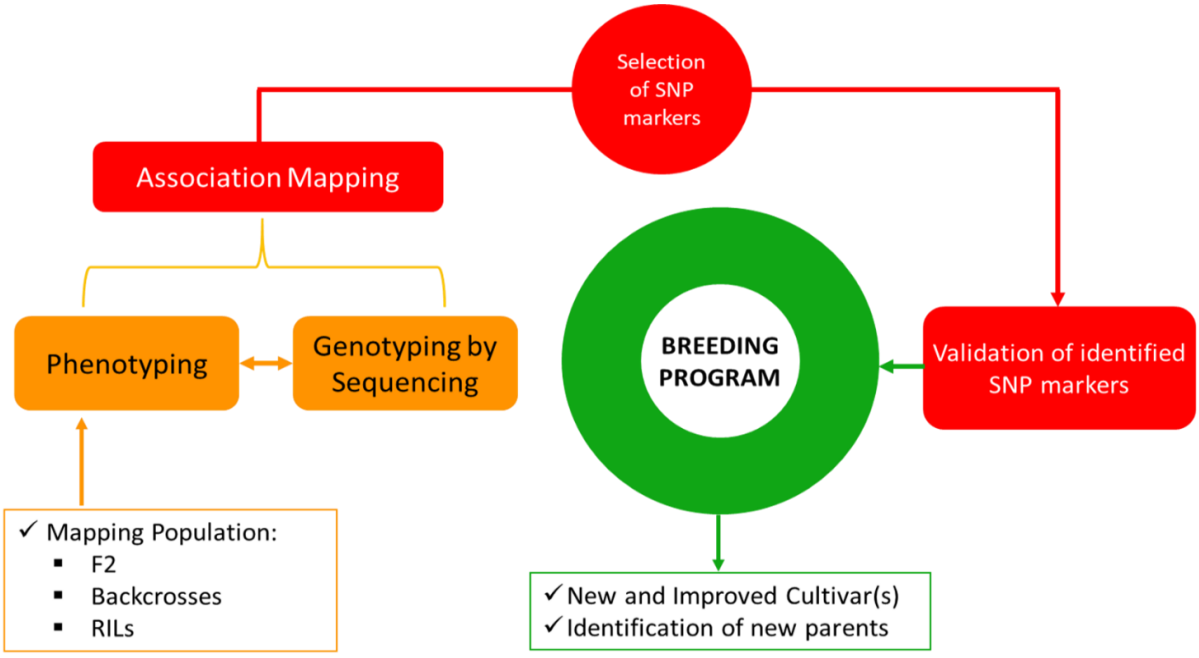

Figure 3 Schematic representation of a genome-wide association mapping in a plant breeding program (Modified from Emre Aksoy)

3. Research Objectives

The objective of the current study are as follows:

1. To evaluate the genetic diversity in the breeding germplasm in terms of agronomic as well as FHB-resistance related traits.
2. To inquire if there is any significant association between SNP markers and studied traits?
3. Does Anther Extrusion have any significant correlation with DON concentration (i.e. FHB)?

4. Material and Methods

4.1 Plant Material and Experimental Design

A training population consisted of 198 spring wheat genotypes were provided by Boreal Plant Breeding Ltd. (Finland) to perform the study. Genetic divergence and breeding importance were held in mind during the process of genotypes selection. The panel consisted of a diverse breeding population of spring wheat containing both commercial cultivars and breeding lines, adopted to the Northern growing conditions. About 75% of the genotypes belong to the main type spring wheat maturity group suitable for growing in Finland, Sweden and Baltic countries. Whereas, the remaining 25% were from very early maturing spring wheat group for northern regions. One line (Genotype ID 48848) with *Fhb1* gene (homozygote) was also included in the experiment.

Experiment was conducted during the growing season 2019 at Jokioinen (60.811163, 23.497720), Finland (**Fig. 5**). A row-column design (Piepho and Williams, 2010) was used with three replications. Sowing was done at 5th June 2019 by using sowing machine (Linjarivikylvokone HEGE 75). Each experimental unit consisted of a single genotype sown in row of 1 m length. Guard rows were sown as borders to equalize disease pressure within rows.

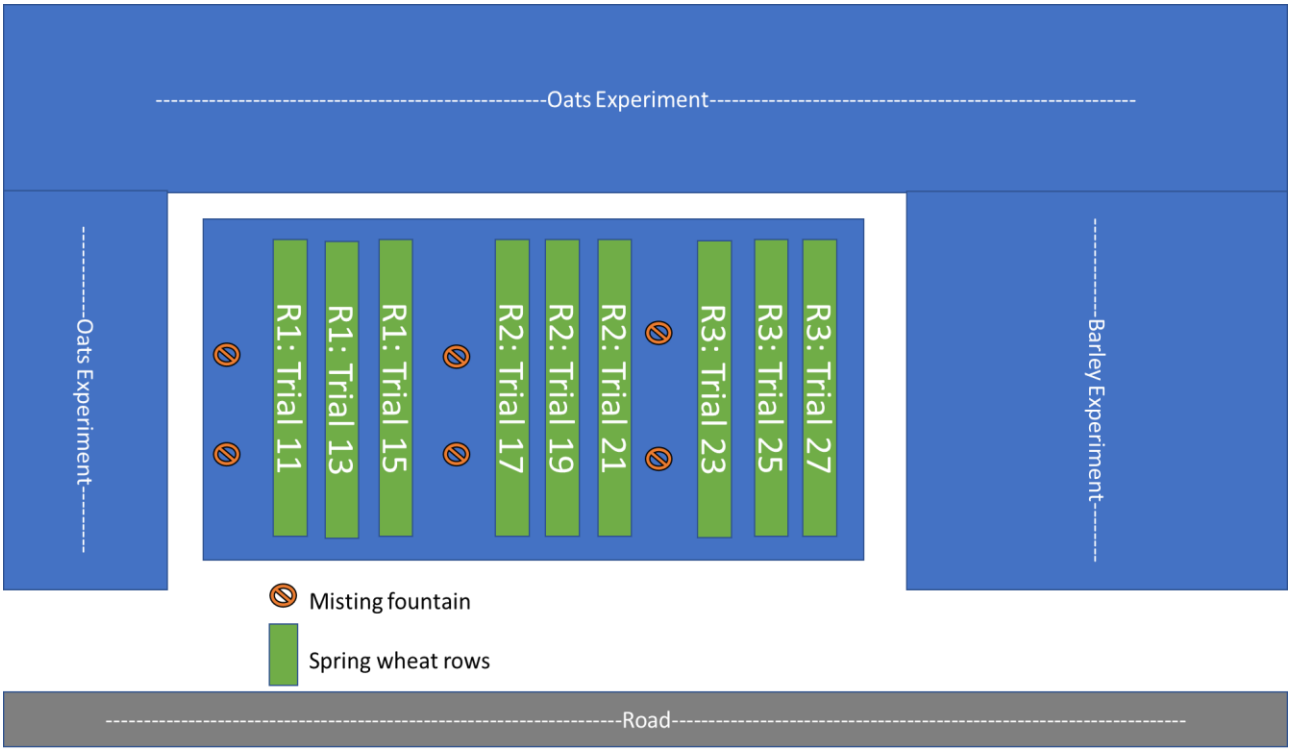

Figure 4 Map of artificially inoculated Spring wheat field (located at Jokioinen, Finland).

The inoculum was initially prepared by following the method of (Tekle et al., 2018) and was composed of a mixture of five *F. graminearum* isolates (12007, 12010, 05011, 05039 and 06249) collected from Finnish fields. Later, a spawn inoculation method was adopted by which heat-killed oat seeds (**Fig. 6**) infected with inoculum were evenly spread between all the rows at latest at Zadoks stage 49 (Zadoks et al., 1974). The experimental field was mist irrigated daily from 7pm to 10pm from inoculation until two weeks after the last flowering was observed. At maturity, the lines were sickle harvested and each individual row was bagged. Lastly, each bag was separately threshed by using Thresher (Kurt Pelz Maschinenbau Postfach 5300). Threshed samples were collected in small bags with respective genotype tags and then later subjected to milling before analysing DON concentration.

4.2 Selection of suitable markers and Genotyping

All breeding lines were genotyped by using a customized, unpublished single nucleotide polymorphism (SNP) chip. In total, 11,987 SNP markers were considered for the study and quality of the markers was taken into account by following criteria:

i. If the missing values at any SNP marker was more than 10% (i.e. SNP call rate < 90%), such marker(s) were excluded.
ii. If the minor allele frequency of a marker was < 5%, such markers were also removed because they are not informative enough in a GWAS analysis.
iii. If the heterozygosity of a marker was >5%, such marker was not included as well.
iv. Apart from that such lines were also removed, if >10% of their markers were found heterozygous and/or have 10% of missing values.

Finally, the missing values were imputed with average values and 11,987 SNP markers were selected. SNPs that couldn't fit in any of the mapped chromosome were assigned to chromosome '22'. Imputations for missing values were done with a mean value by using A.mat function of Ridge Regression and Other Kernels for Genomic Selection (rrBLUP) (Endelman, 2011). All the computations were resolved in R (Team., 2010).

4.3 Phenotyping and Data collection

Data was collected on seven plant parameters for each genotype in the experiment. The parameters and methodology adopted to collect the data is described below:

4.3.1 Days to heading and Maturity groups

Days to heading was considered by counting the number of days from sowing to the visibility of at least 50% of the spikes in each individual row in all three replications. Maturity was scored on a scale of "1-5" in which ‘1’ means earliest and ‘5’ refers the latest maturing genotype group.

4.3.2 Plant Height and Anther Extrusion

Plant height of each genotype was determined by choosing the average looking plant in a row and measuring the length (in cm), starting from base of the plant till the tip of spike with the help of a meter rod. Whereas, for anther extrusion the methodology by Skinnes et al. (2010) was followed (Skinnes et al., 2010). A linear scale from 0-9 was developed with 10% interval between two individual points (**Fig. 7**) and data was collected by visually inspecting randomly collected spikes from the material under study. After the completion of heading and just before flowering, two equal-sized spikes were bagged together to decrease the risk of losing the anthers by wind, water and shedding (**Fig. 8**). A visual inspection of all the lines was done at proper stage by using aforementioned scale and scores were taken. In case of strong shedding effect, scores from the bagged spikes were taken into consideration.

4.3.3 Visual disease score, incidence and severity

Visual disease was observed for 10 spikes per row on a scale of ‘0-5’ after three weeks of spawn inoculation, at Zadoks stage 91 (Zadoks et al., 1974). The scale was as follows: 0=none of the spikelet; 1=one spikelet; 2=two spikelets; 3=3 spikelets or under half of the spikelets; 4=more than half of the spikelets and 5=the whole spike had disease symptoms. Data was recorded in terms of Fusarium severity and Fusarium incidence. Fusarium incidence refers to the percentage of wheat spikes exhibiting disease symptoms in a single row and it corresponds to the resistance against initial infection (Type I resistance). Whereas, Fusarium severity refers to the average score of infection in percentage in a single row and it corresponds to resistance against spread of the fungus from initial point (Type II resistance).

4.3.4 DON testing

A grain sample of 10g was taken from each wheat genotype in the study and was milled with the help of milling machine (Lab Mill 120, Perten Instruments, Hägersten, Sweden). DON concentration was measured by using Enzyme-linked immunosorbent assay (ELISA) kit (R5906 Ridascreen DON 96 test, R-Biopharm, Darmstadt, Germany). A commercial reference sample (TR-D100) was also used to control variability among DON measurements.

Figure 6 A single heat-killed oat seed treated with Inoculum. Black spots on the seed are Fusarium spores growing and onset of disseminating the disease to wheat plants in field

Figure 8 Bagged Spikes in one of the spring wheat rows to prevent shedding from wind and rain

Figure 7 Scale used for visual scoring of Anther Extrusion (AE) in field.

A corresponds to'0'; no AE,

B refers to '5'; medium AE and

C means '9'; complete AE

4.4 Data Analysis

Once the data from field experiment was obtained, spatial corrections were done by using suitable models for each trait. Statistical analysis were done using SPSS Version 25.0 (IBM, 2017). Uncorrected data was compared with data after applying various models such as models considering blocks, columns, rows and column within rows. The most obvious outliers were omitted, and the most suitable model was chosen for each trait respectively (**Table. 1**). The basis of selection of model was Bayesian information criterion (BIC) value. Finally, the best linear unbiased estimates (BLUEs) were computed and subjected for the data analysis. SPSS Version 25.0 (IBM, 2017) was used to do the corrections and to find least significant difference (LSD, α=0.05) and co-efficient of variation (CV %) values for all the traits.

Table 1 Phenotypic traits with respective spatial correction models applied to remove outliers.

TRAIT	Selected Model
Height	block, column, row, column within row
Maturity	block, column, row
Heading	block, column, row
Anthers extrusion	block, column
Fusarium incidence	block, column
Fusarium severity	block, column, row
DON	block, column, row, column within block

4.4.1 Correlation, Heritability and Descriptive Statistics

Descriptive statistics for all the traits was computed in SPSS (Version 35.0) (IBM, 2017) while the heritability estimates and correlation analysis was conducted in Derivative-free approach to Multivariate analysis (DMU) software (Madsen et al., 2014).

Variance components as well as narrow-sense heritability (h^2) for each trait were estimated with DMU software (Madsen et al., 2014) by using single trait model. The formula used to calculate h^2 is as follows:

$$h^2 = \frac{d(\mathbf{G})\sigma_a^2}{d(\mathbf{G})\sigma_a^2 + \sigma_e^2}$$

Where h^2 refers to narrow-sense heritability, $d(\mathbf{G})$ indicates the mean of diagonal element of $\mathbf{G}$ matrix, whereas, σ_a^2 and σ_e^2 refers to additive genetic and residual variances, respectively. Computations were done by using DMU software (Madsen et al., 2014) taking two-trait model into consideration with following structure:

$$\mathbf{y}' = [\mathbf{y}_1' \quad \mathbf{y}_2'], \mathbf{e}' = [\mathbf{e}_1' \quad \mathbf{e}_2']$$

$$\mathbf{b} = \begin{bmatrix} \mathbf{b}_1 \\ \mathbf{b}_2 \end{bmatrix}, \mathbf{X} = \begin{bmatrix} \mathbf{X}_1 & 0 \\ 0 & \mathbf{X}_2 \end{bmatrix}$$

$$\mathbf{a} = \begin{bmatrix} \mathbf{a}_1 \\ \mathbf{a}_2 \end{bmatrix}, \mathbf{Z} = \begin{bmatrix} \mathbf{Z}_1 & 0 \\ 0 & \mathbf{Z}_2 \end{bmatrix}$$

The assumptions regarding distribution for two traits model is as shown:

$$a \sim N(0, \mathbf{G_0} \otimes \mathbf{G})$$

In which, $\mathbf{G}_0$ and $\mathbf{G}$ shows genetic covariance between two traits and additive relationship matrix, respectively,

$$e \sim N(0, \mathbf{R_0} \otimes \mathbf{I}),$$

Where, $\mathbf{R}_0$ and $\mathbf{I}$ are residual matrix and incidence matrix, respectively. Also, $E(y) = Xb$ and $Cov(a, e) = 0$. The sign $\otimes$ indicates the Kronecker product of the two matrices. Symbolizing two traits as i and j, the correlation among i and j was calculated as:

$$\frac{d(\mathbf{G})(\mathbf{G}_0)_{ij}}{\sqrt{d(\mathbf{G})(\mathbf{G}_0)_{ii} d(\mathbf{G})(\mathbf{G}_0)_{jj}}} = \frac{(\mathbf{G}_0)_{ij}}{\sqrt{(\mathbf{G}_0)_{ii}(\mathbf{G}_0)_{jj}}}$$

In which,

$d(\mathbf{G})$ = average of diagonal element of G-matrix
$(\mathbf{G}_0)_{ij}$ = genetic covariance between two traits
$(\mathbf{G}_0)_{ii}$ = genetic variance for trait i
$(\mathbf{G}_0)_{jj}$ = genetic variance for trait j

and computations for phenotypic correlations (Pearson's correlation) were done in MVApp (Julkowska et al., 2019).

4.4.2 GWAS Analysis

Genomic Association and Prediction Integrated Tool (GAPIT) was used to perform GWAS analysis (Lipka et al., 2012). GAPIT is a dynamic open source package in R (Team., 2010) environment and provides state-of-the art mixed model approaches to perform GWAS analysis.

Firstly, Principal Component Analysis (PCA) was performed in R (Team., 2010) by using function 'prcomp()' to determine number of PCs to be considered by visually observing the flattening of scree plot (**Appendix. 1**) so that the amount of variation explained by each PC was considered for further analysis. Moreover, BIC values from GAPIT were also used to set the code ("model.selection = TRUE and PCA.total = 10") before running actual GWAS analysis.

After that the GWAS analysis was performed with four different models to determine the marker-trait association. Following models were used with the respective code settings:

1. **Naïve model:** This model does not consider any population structure and relatedness among lines during association. Statistically, the model can be described as follows:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{e}$$

In which, **y** refers to the BLUEs determined for each trait, **X** shows the design matrix and **b** is the vector that considers the fixed effects that are only the markers in this case while **e** reflects the random

error. The code settings in GAPIT ("group.from = 1, group.to = 1") for Naïve model were so that it disabled the kinship calculation which otherwise is enabled by default.

2. **Q model:** This model includes the population structure as covariate as per number of PCs decided to take into account but does not include any relationship between the lines. In principle, the model is similar to Naïve model i.e $\mathbf{y = Xb + e}$ except that **b** refers to the markers as well as eigenvalues from the PCA.

3. **K model:** This model considers the cryptic relatedness with a kinship (**K**) matrix, while no population structure being taken into account (No PCs but just the markers). In GAPIT, the computation was done by default "VanRanden" method (VanRaden, 2008). Statistically, K model can be explained by following equation:

$$y = Xb + Za + e$$

Where, **y** is the BLUEs for each trait, vector **b**, **a** and **e** show the fixed effects (markers), additive gene effects including **K** matrix and random error, respectively. Whereas, **X** and **Z** describe the design matrices associating fixed and random effects, respectively, with BLUEs of each trait.

4. **QK model:** This model includes both the kinship matrix and chosen number of PCs to determine the association between phenotypic and genotypic data. Statistically, QK models is similar to K model i.e. $\mathbf{y = Xb + Za + e}$ except that vector **b** represents both the eigenvalues and markers.

A detailed set of assumptions for all the above-mentioned models can be found in GAPIT Manual (http://www.zzlab.net/GAPIT/gapit_help_document.pdf). The significance of the association study was set on basis of Bonferroni multiple testing correction as well as by False discovery rate (FDR) adjusted p-value (0.10) (Benjamini and Hochberg, 1995). The final output from the GAPIT contains Microsoft Excel files with numeric data, graphs and images, quantile-quantile (Q-Q) plots and Manhattan Plots.

5. Results

5.1 Phenotypic Parameters

Measurements were taken on two types of traits in artificially inoculated spring wheat genotypes, the agronomic traits (plant height, days to heading, maturity class) and the FHB-resistance related traits (anther extrusion, fusarium incidence, fusarium severity, and DON content). The frequency distribution of all the measured traits can be seen in **Fig. 9**. After the spatial corrections with respectively suitable models, almost all the traits have followed a normal distribution that satisfies the need of further statistical analysis. Plant height, days to maturity and FHB-resistance related parameters have shown almost a perfect normal distribution as compared to days to heading and anther extrusion (**Fig. 9**).

Descriptive statistics for all the measured traits are shown in (**Table. 2**). The mean value for anther extrusion was overall higher i.e. 6.87 with a maximum of 9 in some genotypes. Similarly, Fusarium incidence was also higher on average basis, ranging from a minimum of 17% up to a maximum of 100%. On the other hands, Fusarium severity was found to have a comparatively lower mean value of about 29%, with a minimum and maximum values of 5.6% and 72%, respectively. Whereas, DON content on average was about 18.95 ppm with a minimum of 2.86 ppm and a maximum value of 53.5 ppm, showing that in either case it was exceeding the recommended EU levels (1.25 ppm) for unprocessed wheat.

Coefficient of variation (CV %) for Fusarium severity and DON are among the highest indicating a lot of variation in the germplasm. On the other hand, plant height showed a minimum CV % (**Table. 2**). Even after performing spatial corrections, higher CV values were observed for maturity, and for all the FHB-resistance related traits (CV > 10). Similarly, higher least significant differences (LSD) were also found for FHB-resistance related traits, indicating a lot of variation in the data (**Table. 2**).

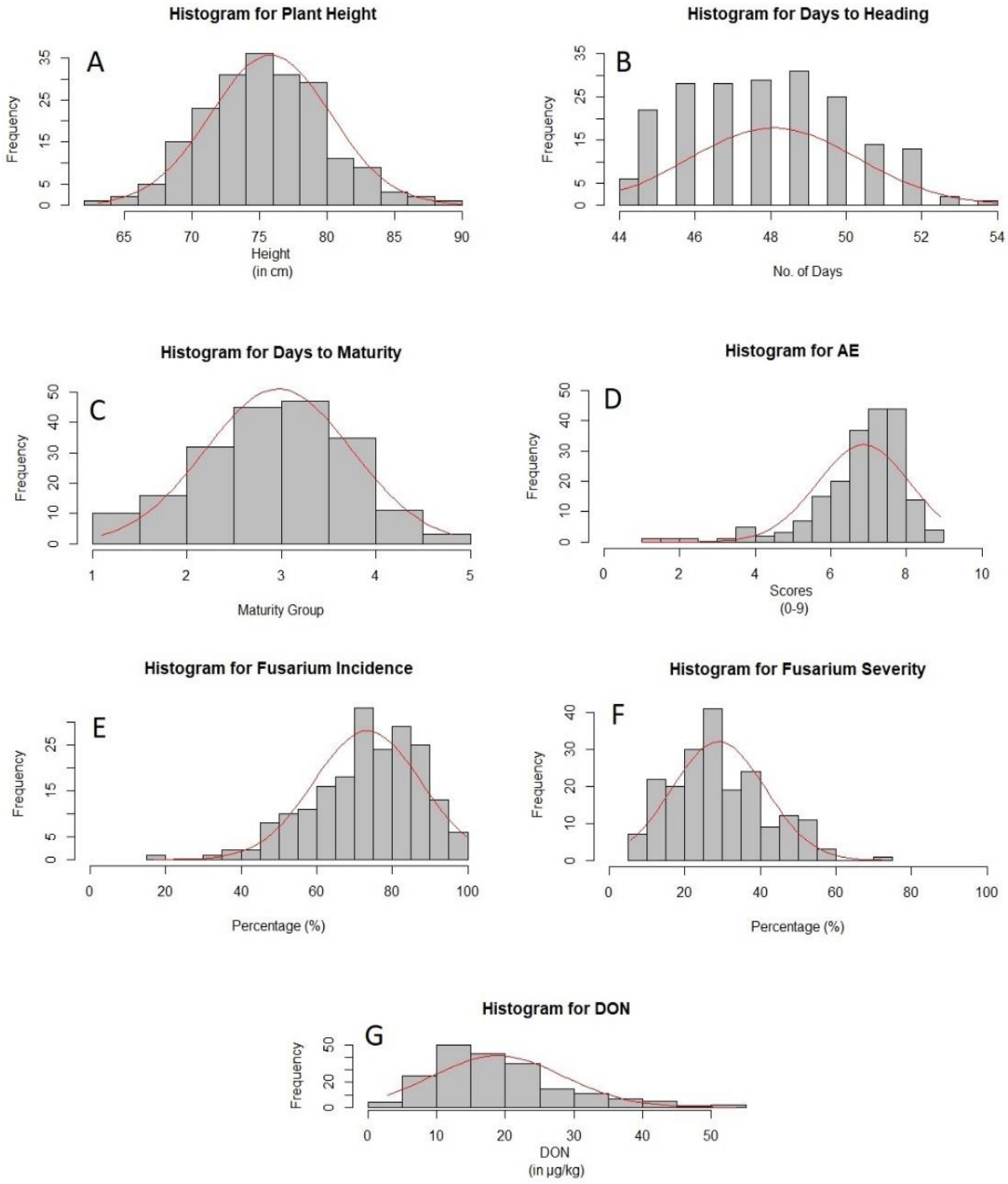

Figure 9 Frequency distribution of all the measured traits with reference to a normal distribution curve (red line)

Table 2 Mean values, range, least significant differences (α = 0.05), and coefficient of variation (%) for all measured traits

TRAIT	Mean	Min.	Max.	LSD (α = 0.05)	CV %
Height (cm)	75.85	63	90	6.34	5.22
Maturity (1-5)	2.96	1	5	0.85	17.86
Heading (no. Of days)	48.06	44	54	2.02	2.63
Anthers extrusion (0-9)	6.87	1	9	1.44	12.92
Fusarium incidence	73.5	17	100	25.67	21.73
Fusarium severity	29.05	5.6	72	21.56	40.95
Don (ppm)	18.95	2.86	53.5	8.87	29.51

5.2 Genotypic data and Population Structure

Scatter plot (**Fig. 10**) for 198 spring wheat lines showed that there is no prominent population structure. A small aggregation near the bottom might be due to the fact that almost 3/4th of the genotypes were from the main type maturity group and were suitable for growing in Northern climatic conditions. Principal component analysis (PCA) was conducted to approximate the population structure (**Fig. 10**). First and second principal components explained 5.9% and 4.9% of the total variation, respectively (**Appendix. 2**).

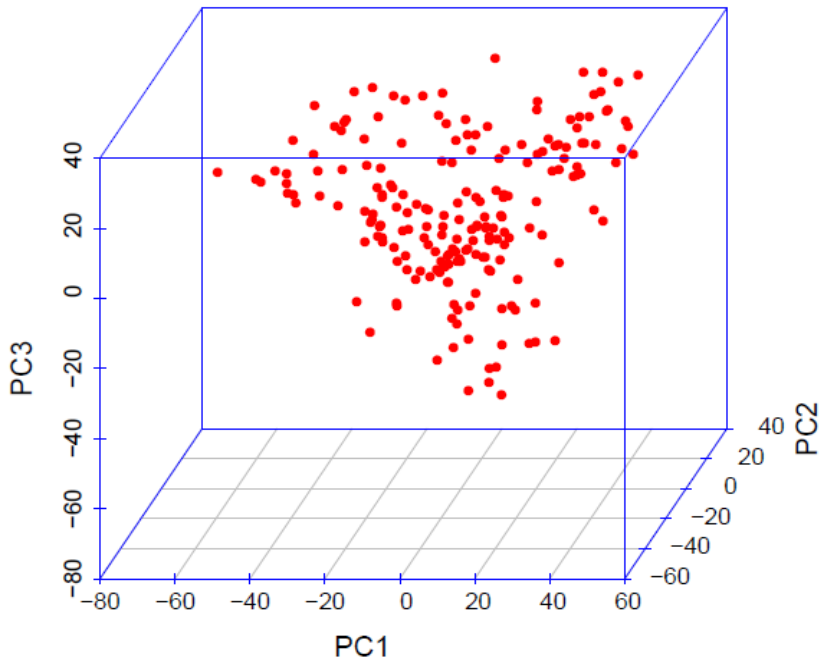

Figure 10 Scatter plot obtained from the genotypic data in three-dimensions

5.3 Heritability and Genetic Correlations

Narrow-sense heritability estimates and respective variance components are shown in **Table. 3**. It can be seen that the agronomic traits for spring wheat showed relatively higher heritability than the FHB-resistance related traits. The parameters such as days to heading, maturity class and anther extrusion exhibited the highest heritability (> 60%) values among all the measured traits. Whereas, Fusarium incidence and DON content showed the lowest heritability estimates of 34% and 39% respectively.

Table 3 Variance components and narrow-sense heritability estimates for all measured traits

TRAIT	σ_a^2	σ_a^2 (SE)	h^2	σ_e^2	σ_e^2 (SE)
Height (cm)	8.18	3.27	0.40	12.09	2.51
Maturity (1-5)	0.34	0.08	0.69	0.15	0.04
Heading (No. of days)	2.89	0.77	0.64	1.60	0.46
Anthers Extrusion (0-9)	1.14	0.32	0.69	0.50	0.18
Fusarium incidence	69.51	31.20	0.34	130.81	25.52
Fusarium severity	63.99	20.97	0.47	70.09	15.00
DON (ppm)	35.21	14.11	0.39	54.51	11.00

Phenotypic correlations were computed for all the pair of traits and can be seen in **Table. 4**. Days to heading had a highly significant positive correlation with maturity class (0.68, α=0.01) and DON content (0.41, α=0.01) whereas, a negative correlation can be observed with anther extrusion and DON content (-0.5, α=0.01). Plant height had a significant positive correlation with anther extrusion (0.15, α=0.05), but a non-significant negative association can be seen with DON content. Maturity class has shown a significant positive phenotypic correlation with DON content (0.24, α=0.05) while a highly significant negative association with fusarium incidence (-0.49, α=0.05) and severity (-0.6, α=0.05). Anther extrusion had shown a positive correlation with fusarium severity (0.26, α=0.05).

Genotypic correlations were computed with two traits mixed model for all the pair wise traits (**Table. 4**). Confidence interval show that the days to heading has a stronger positive correlation with maturity class (0.83) but a negative association can be seen with FHB-severity (-0.76). Similarly, maturity class also showed a strong negative correlation with Fusarium incidence (-0.6) and severity (-0.9). Whereas, confidence interval for plant height indicated weak positive correlation with anther extrusion (0.20 but not with any of the FHB-resistance related traits. The anther extrusion has shown a strong negative genotypic correlation with DON content (-0.8), but a negative genotypic correlation with Fusarium severity (-0.4).

Table 4 Phenotypic correlation coefficient calculated as Pearson correlation (α=0.05, 0,01) (above the diagonal) and genotypic correlation (below the diagonal) for all the pair-wise measured traits, presented with confidence intervals in parenthesis.

TRAIT	DH	PH	MC	AE	Fussev	Fusinc	DON
DH	-	0.13	0.68**	−0.16*	−0.47**	−0.34**	0.41**
PH	0.07 (-0.15, 0.30)	-	0.04	0.15*	0.08	0.04	−0.08
MC	0.83 (0.75, 0.92)	-0.13 (-0.34, 0.07)	-	−0.12	−0.6**	−0.49**	0.24**
AE	0.07 (-0.12, 0.27)	0.29 (0.06, 0.52)	-0.03 (-0.22, 0.14)	-	0.26**	0.05	−0.5**
Fussev	-0.76 (-0.89, -0.62)	0.08 (-0.17, 0.33)	-0.95 (-1.03, -0.88)	0.36 (0.17, 0.55)	-	0.48**	−0.33**
Fusinc	-0.835 (-1.04, -0.62)	-0.16 (-0.46, 0.14)	-0.69 (-0.86, -0.53)	0.10 (-0.14, 0.36)	0.88 (0.74, 1.03)	-	−0.11
DON	0.39 (0.19, 0.59)	-0.03 (-0.31, 0.25)	0.16 (-0.05, 0.38)	-0.80 (-0.92, -0.67)	-0.44 (-0.66-0.22)	0.01 (-0.27, 0.31)	-

DH; days to heading, PH; plant height, MC; maturity class, AE; anther extrusion, Fussev; Fusarium severity, Fusinc; Fusarium incidence. *, ** $P < 0.05, 0.01$, respectively. For genotypic correlations, the confidence interval (values in parenthesis) decides the direction of the correlation and if it includes zero, no conclusion can be made for positive or negative nature of the relationship between the two traits.

The distribution of DON content versus anther extrusion (**Fig. 11**) showed strong, negative, almost linear association between the two traits. Breeding lines with a lower anther extrusion has shown a broader range of DON content in respective lines but overall, more the anthers extruded out, less is the probability of DON accumulation.

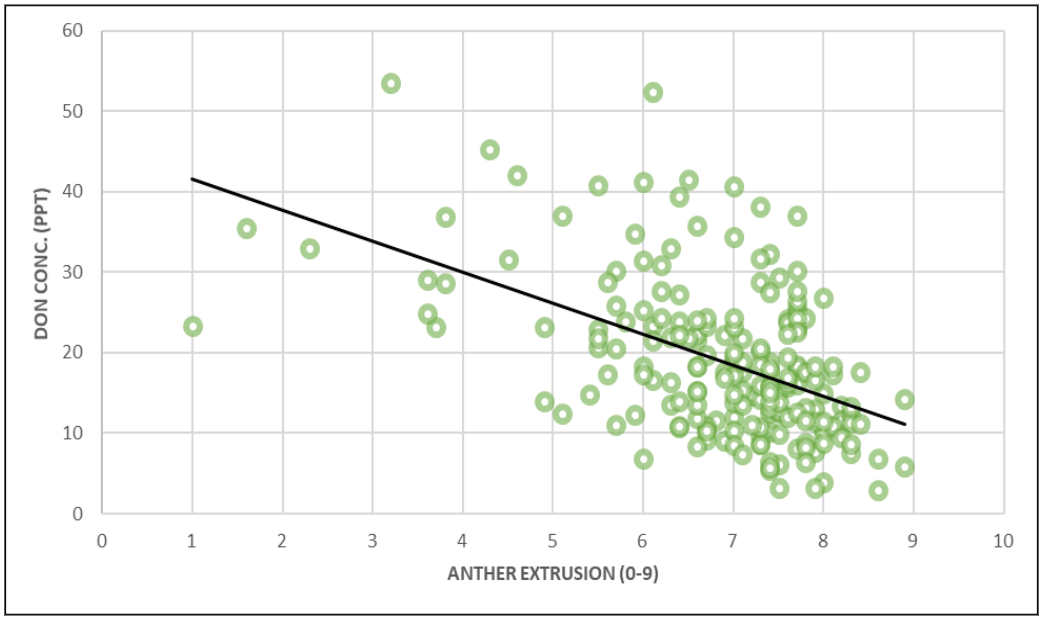

Figure 11 Relationship between DON content and Anther Extrusion for 198 spring wheat lines

The distribution of DON content versus Fusarium severity was found to be moderately negative. A dispersed structure of distribution was observed even after spatial corrections for both the traits (**Fig. 12**). Genotypes with more fusarium severity has shown lesser DON accumulation in the kernels.

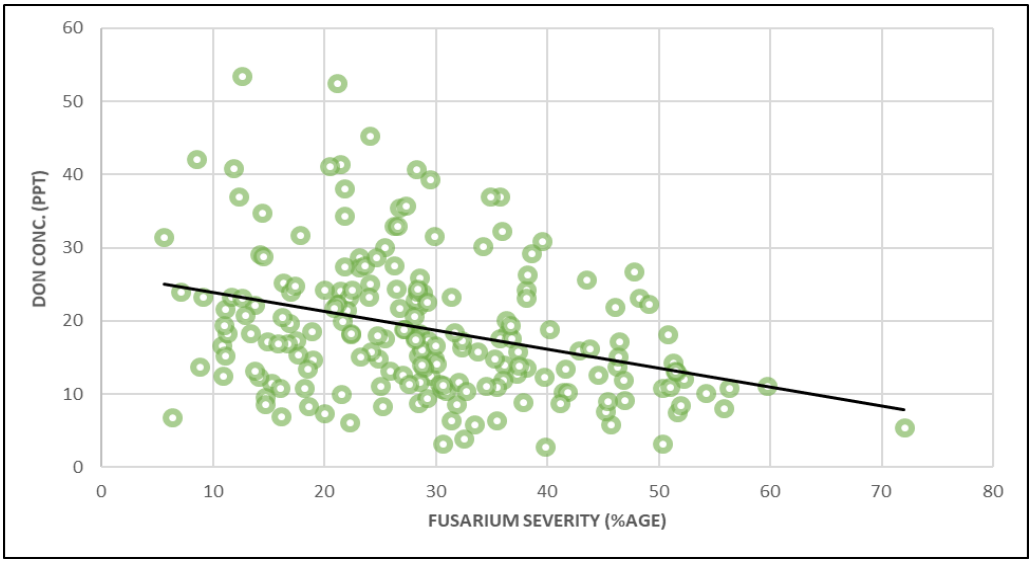

Figure 12 Relationship between DON content (ppm) and Fusarium severity (%) for 198 spring wheat lines

A set of genotypes were taken from the spring wheat panel with successively increasing anther extrusion values and was plotted against respective DON content (**Fig. 13**). It was observed that the more the anther extrusion, lesser is the DON accumulation. However, the *Fhb1* homozygote line (48848) had a comparatively lesser DON content compared to its nearby lines whereas, the anther extrusion score was not among the highest in the field (**Fig. 13**).

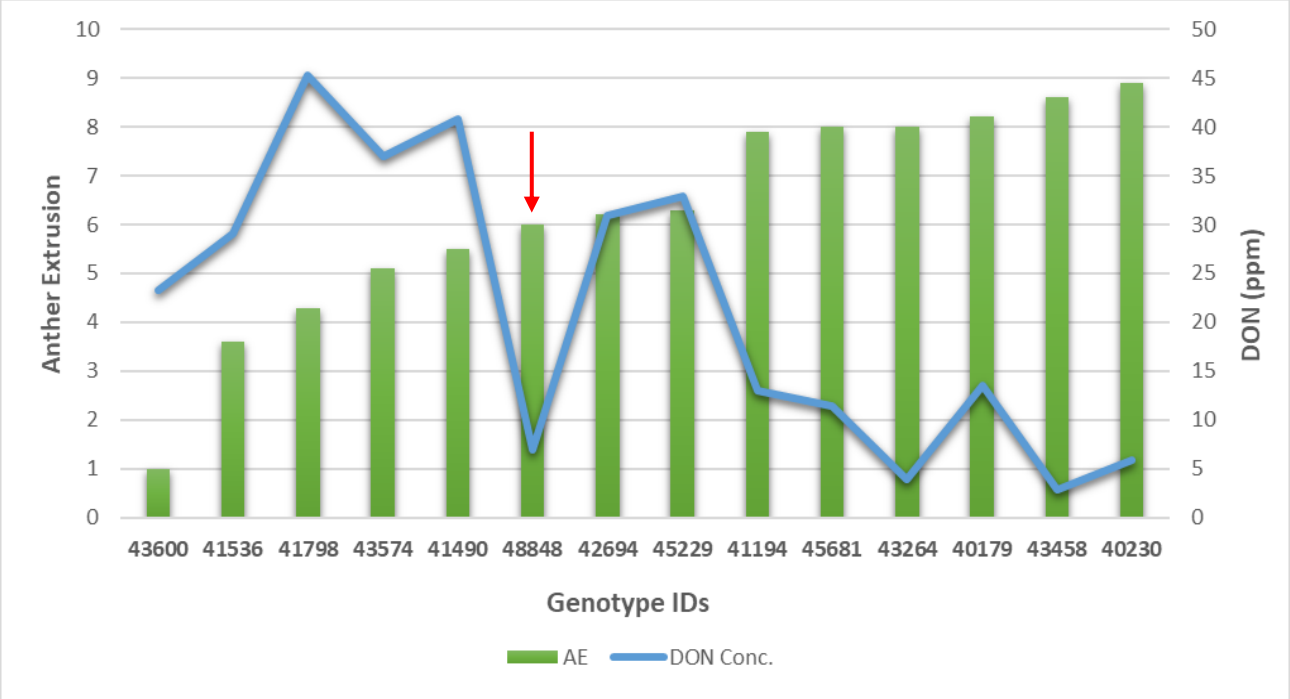

Figure 13 Mean DON (ppt) values for some selected breeding lines from all three anther extrusion groups (low, medium and high). Red arrow indicating line with fixed Fhb1 gene (homozygous)

5.4 Genome-wide association study

The number of PCs to include in the association analysis to correct population structure is determined before executing the analysis. It was found by looking at respective BIC values that **one** PC was suitable as a covariate for traits such as maturity class and Fusarium severity, while no PCs (**zero**) were required to add while performing association analysis for rest of the traits. According to the scree plot (**Appendix. 1**), the variation (%) explained by each PC have shown the flattening of curve after 6th PC, hence 6 PCs were added for each trait where population structure needs to be considered.

Marker-trait associations was computed for all the measured traits by using four models and the significant associations for all four-models are summarized in **Table. 5**. Manhattan plots and QQ-

plots for days to heading are shown in **Fig. 14** and for all remaining traits in (**Appendices 2-7**). A large number of associations were observed for most of the traits (except Fusarium incidence) with Naïve model that does not include population structure or a kinship matrix. Further, when population structure was included in association (Q-model), still significant associations were found for agronomic traits and anther extrusion, but no associations were detected for FHB-resistance related traits. On the contrary, when a kinship matrix was considered in the association analysis alone (K-model), no significant associations were found for any of the measured traits. Similarly, when both the kinship and population structure were included together in the analysis (QK-model), no significant associations were observed. Interestingly, Fusarium incidence showed zero associations with any model used in the analysis.

Table 5 GWAS models applied to each trait during association analysis and respective marker-trait associations for each model. False-discovery-rate (FDR)-adjusted p-values was considered to set significance level ($\alpha = 0.10$).

TRAIT	Naïve model	Q model	K model	QK model
Height	36	49	0	0
Maturity	3355	147	0	0
Heading	2134	50	0	0
Anthers Extrusion	300	185	0	0
Fusarium incidence	0	0	0	0
Fusarium severity	2664	0	0	0
DON	301	0	0	0

Manhattan plots and Q-Q plots for days to heading is provided in **Fig. 14**. The Manhattan plots for first two models i.e. Naïve and Q model showed significant associations even with stringent Bonferroni correction, but the significance was reduced to zero once cryptic relatedness alone and combined with population structure were considered in the latter two models i.e. K model and QK model, respectively. While the Q-Q plots for the Naïve and Q model showed a prominent deviation even from the start, whereas by adding cryptic relatedness, the deviation was diminished in case of both K model. Moreover, in case of QK model where both kinship and population structure were taken into account, the last data points again start deviating from the expected value (**Fig. 14**).

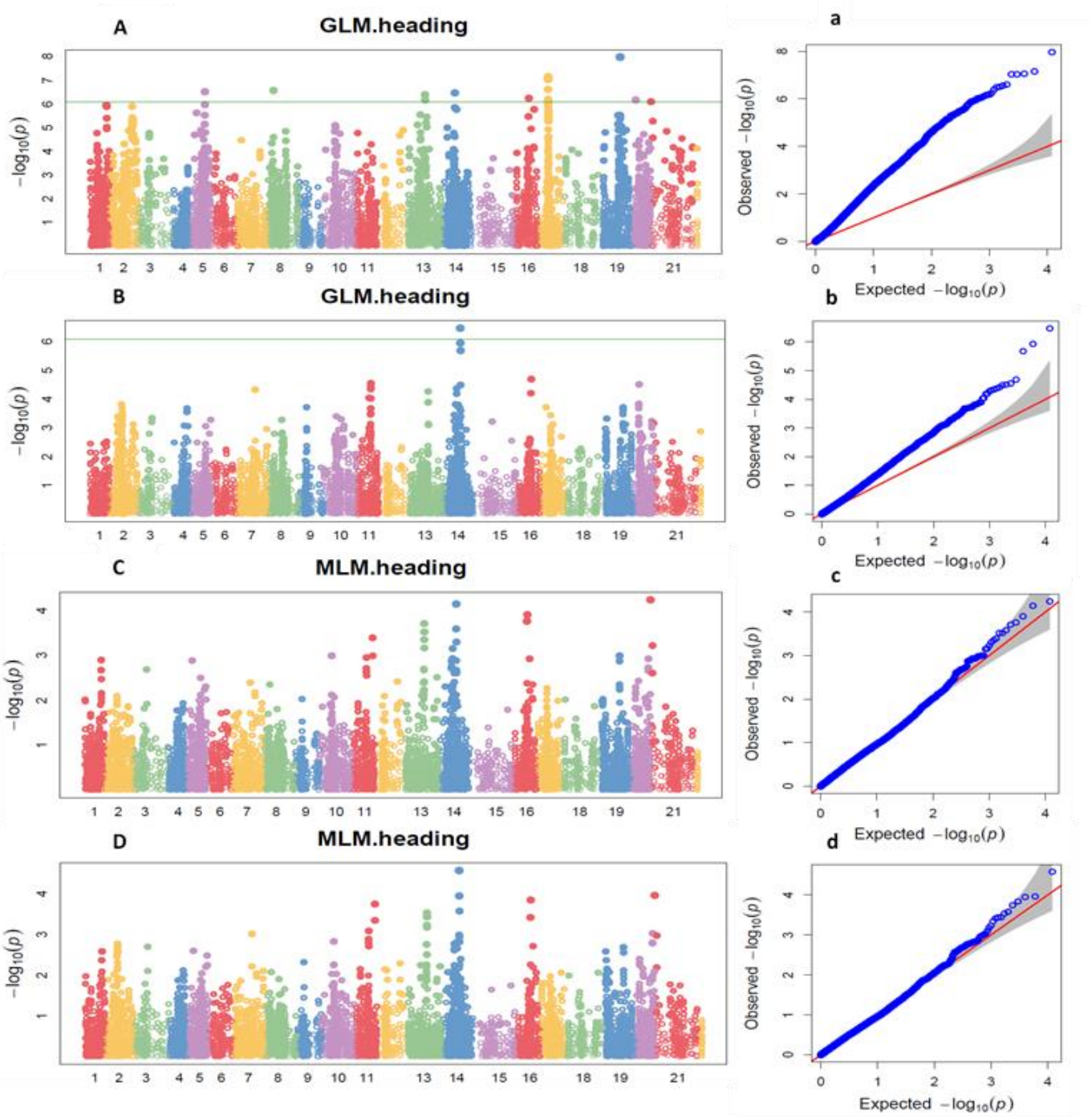

Figure 14 Manhattan Plots and Q-Q plots for days to heading with all four models used in association analysis. **A, a** refers to Manhattan and Q-Q plot for Naïve Model. **B, b** refers to Manhattan and Q-Q plot for Q Model. **C, c** refers to Manhattan and Q-Q plot for K Model. **D, d** refers to the Manhattan and Q-Q plot for QK Model, respectively. Various colours on x-axis of Manhattan plots represent individual wheat chromosome, and the dots represent each SNP marker included in the study, whereas the filling of dot and its vertical position on vertical -log scale shows the strength of association. Horizontal green line on **A** and **B** shows Bonferroni's correction. In the Quantile-Quantile (Q-Q) plots, red line shows the expected trend of no association between SNP and trait. Whereas, grey area shows 95% confidence interval under the assumption of null hypothesis of no marker-trait association and the blue dots above the grey area shows marker trait association.

6. Discussion

Fusarium head blight (FHB) is a deadly disease of cereal crops and affects quality of the produce in addition to losses in yield and germination capacity (Tekle et al., 2013). Wheat is an obvious target for many fungal pest species worldwide but in Finland, *Fusarium graminearum* is getting more and more importance due to the yearly rise in temperature and probable rotation of maize crop with spring wheat, both of which might increase chances of FHB and DON accumulation (Blandino et al., 2010; Landschoot et al., 2013). In one study, DON accumulation has shown a positive correlation with temperature and humidity in a multi-year yield trial on wheat (Landschoot et al., 2012). Till date, DON accumulation in wheat kernels has driven a lot of scientific studies to look for FHB resistance in both spring and winter wheat germplasm worldwide but in Finland, notably less reporting was done in this regard. Practically, DON is an expensive trait to phenotype in terms of both economy and time for plant breeding industry. Today, the utilization of genomic information is vital to speed up resistance breeding programs and finding valid correlations between traits that can allow indirect selections for expensive traits.

6.1 Phenotypic data

A lot of variation was observed for most of the measured traits in the artificially inoculated spring wheat experiment. The CV% values for agronomic traits were relatively less as compared to the FHB-resistance related traits (**Table. 2**). A higher CV% values for Fusarium incidence and severity might be due to cumbersome phenotyping of these traits, especially while dealing with a diverse set of genotypes. Similarly, higher LSD values were observed in our study that showed larger variations in the data. This might be due to the protocols followed for phenotyping FHB-resistance related traits and also non-uniform conditions observed in the field during the whole growing period. It has been reported that the uniformity (microclimate changes) of the field affects phenotyping and can possibly influence results where such factors will not be taken in to account (van der Burgt et al., 2011). In general, a lower CV% for various traits indicates a reliable phenotypic outcome but, in our study, all the traits except heading and height showed a CV% value. This makes our observations in agreement with Haikka et al, 2020 for all the oat traits except DON content, which in our case showed lesser CV% (29.51%). Similarly, a comparatively higher CV% for DON content was found in a study on Oats in Canada (Yan et al., 2010). The mean DON content (18.95 ppm) was found to be more than the EU limits i.e. 1.75 ppm indicating that the spawn-inoculation methods was working well and could be potentially used for such studies where needed. Whereas, a high magnitude of variability observed in our disease related data complements with the situation where disease scoring was done earlier in the season for even late maturing genotypes, probably resulting in a false estimation of

disease incidence and severity. This also make our results less reliable and evokes a need to repeat the experiment and to take measurements on time in relevance to the maturity group of the respective genotypes, so that earlier genotypes get an earlier score and late genotypes get score later in the season accordingly. It is also recommended to be more careful in terms of crop management to increase field uniformity to make phenotyping easy and more reliable.

6.2 Heritability and Correlations

The narrow-sense heritability estimates for maturity class, heading and anther extrusion were higher (> 60%) as compared to the resistance related traits and plant height (< 50%) (**Table. 3**). Anther extrusion has shown a narrow-sense heritability of about 69% indicating that the trait is highly heritable and can be subjected to effective selection in wheat breeding programs. Our results are in disagreement with the results by Atashi- Rung and Lucken (1978) who reported low narrow-sense heritability for anther extrusion (19%). The higher heritability for anther extrusion in our case depicts the influence of major genes controlling the trait in spring wheat population.

In this study, the time taken by genotypes to initiate heading has shown a significantly negative phenotypic (-0.47, -0.34) as well as strong genotypic (-0.8, -0.7) correlation with Fusarium incidence and severity, respectively. This implies that the late heading genotypes might have adopted an escape strategy to get less prone to initial infection and hence less severe symptoms in contrast to the early flowering ones. Although the phenotyping on the late maturing genotypes was also done quite earlier which might have an impact on our interpretations. It also seems like the late flowering genotypes are less susceptible to the infection and its spread as maturity class has shown negative phenotypic (-0.49, -0.6) as well as genotypic correlation (-0.6, -0.9) with both fusarium incidence and severity, respectively. Similar results were reported in various studies on different wheat germplasm in past (Gervais et al., 2003; Paillard et al., 2004; Schmolke et al., 2005). Whereas, DON content has shown an unexpected negative phenotypic (-0.3) and genotypic (-0.4) correlation with Fusarium severity, and is not in line with other studies on this topic (Haikka et al., 2020). This might happen due to early disease scoring of the late maturing genotypes and also with a relatively smaller sample size in non-uniform field conditions in our case. This argument is supported by a positive phenotypic correlation between maturity class and DON content (0.24), implying that the DON accumulation increased with time, hence the disease, indicating that the earlier scores were not reliable. It has been also emphasized in past that the development stages might have an effect on DON accumulation in wheat kernels (Del Ponte et al., 2007). Although, similar positive correlation between DON content and maturity was reported in Oat breeding lines in Finland (Haikka et al., 2020). It has also been reported that FHB infection at later stages of plant development may bring no visible damage, but the kernels

may still possess high levels of DON (Góral et al., 2018). Therefore, a keen understanding is needed to make valuable conclusions regarding DON accumulation for various plant types.

Selecting anther extrusion on routine basis could be effective to enhance the frequency of the trait in spring wheat breeding programs (Atashi-Rang and Lucken, 1978). Anther extrusion is reported as a stable trait in wheat crop with high heritability and breeding can be helpful in further improvement of this trait (Muqaddasi et al., 2017a). It has been also suggested that plant varieties can be developed with higher anther extrusion and relatively lower plant height to combat with FHB (Lu et al., 2013), as the semi-dwarf cultivars are more adopted to today's cultivation system (Graham and Browne, 2009). Our data showed that anther extrusion had a significantly moderate negative phenotypic (-0.5) and strong negative genotypic (-0.8) correlation with DON content. This implies that the genotypes with a higher anther extrusion score may have resisted the DON accumulation conferring resistance against DON accumulation. Our results are in agreement with previous studies where negative correlation was reported between AE and DON accumulation (Skinnes et al., 2010; Skinnes et al., 2008). Our results might be helpful in adopting AE as a phenotypic marker for selecting against DON accumulation in spring wheat breeding programs. Our recommendations are also supported by a study on European wheat germplasm which showed that selections made for FHB resistance on the basis of anther extrusion could be helpful without penalising yield (Graham and Browne, 2009). Hence, the anther extrusion needs to be tested and verified in a broader range of genotypes at multiple locations before including as FHB-marker trait in actual wheat breeding programs in Finland. A path-coefficient analysis of traits associated with AE can also provide a better understanding of the trait behaviour.

6.3 Population structure and GWAS

A great importance has been given to the population structure while performing a GWAS study as it might result in false marker-trait associations (Matthies et al., 2012). Our data showed decreased stratification in this experiment and no obvious population structure was observed for our genotypes. The first and second PC accounted for 5.9% and 4.9% variation respectively (**Appendix. 1**). Our plant material was mainly composed of breeding lines from breeding programs that might result in lower variation compared with other studies on the same subject with oat plants (Haikka et al., 2020).

In this study, the data acquired from artificially inoculated field experiment depicted no significant genome-wide association with FHB-resistance related traits. Precisely, no significant associations were detected for both agronomic and FHB-resistant traits with mixed linear model. Furthermore, Q-Q plots become more closer to the expected values after adding kinship into the model. On the other

hand, Q model after correcting with population structure does not make any significant change in Q-Q plot to follow the expected trend (Naïve Model). Hence, it can be concluded that correcting for population structure alone in our material was not sufficient and might have caused an overestimation of marker-trait association in case of Q-model. But including kinship matrix in the association (K Model), all the false associations were diminished showing that a high magnitude of cryptic relatedness is affecting the analysis. This is logical to understand that there is a possibility of close relatedness among the germplasm as it is composed of breeding lines with possibly shared pedigrees. Interestingly, no marker-trait association was observed in case of Fusarium incidence even with the model that does not consider population structure (Naïve) and relatedness (Q) which otherwise result in false associations. This suggests that the phenotypic data was not reliable and phenotyping for FHB resistance was not done properly. One reason could be that only one genotype with *Fhb1* gene was included, which probably reduced the success of association mapping. This was also emphasized by Rex Bernardo (2016) that an association study carried out with a population with relatively low frequency of resistant alleles seems not to be successful (Bernardo, 2016). Hence, it is recommended that to get more realistic and consistent results, the study should be repeated with the inclusion of more lines with *Fhb1* gene in fixed state.

7. Conclusion

There was a high magnitude of variability present in the tested germplasm for both agronomic as well as FHB-resistance related traits. Days to heading, maturity and anther extrusion were the highly heritable traits compared to plant height and FHB-resistance related traits. No marker-trait associations were found in the current study indicating the possible influence of relatedness among the genotypes and inclusion of only one line with *Fhb1* gene in fixed state in the study. An unexpected negative correlation between DON accumulation and Fusarium severity was found, implying inaccuracies in phenotyping the disease in the artificially inoculated spring wheat field. Whereas, a negative correlation between anther extrusion and DON content was found to be the most interesting result of the study. Apart from repeating the experiment by rectifying identified gaps, it is highly recommended to study anther extrusion in detail with a special reference to breeding spring wheat against Fusarium head blight in Northern Europe.

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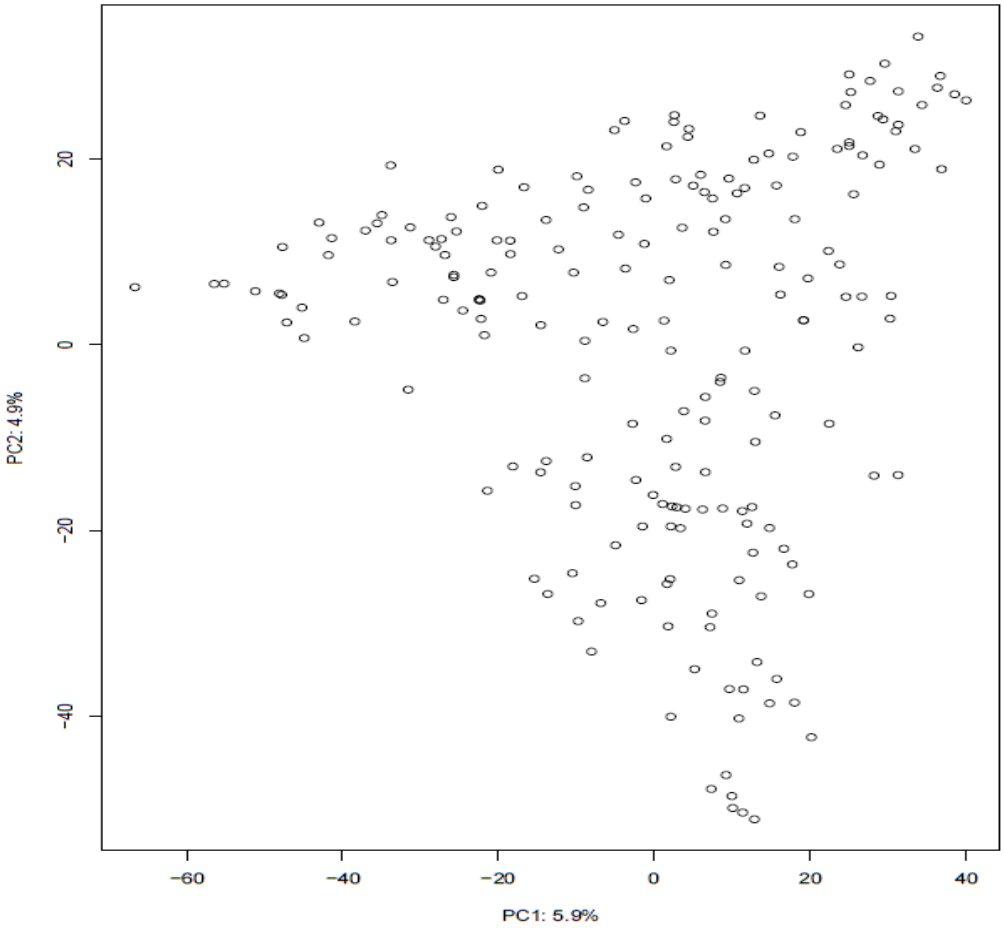
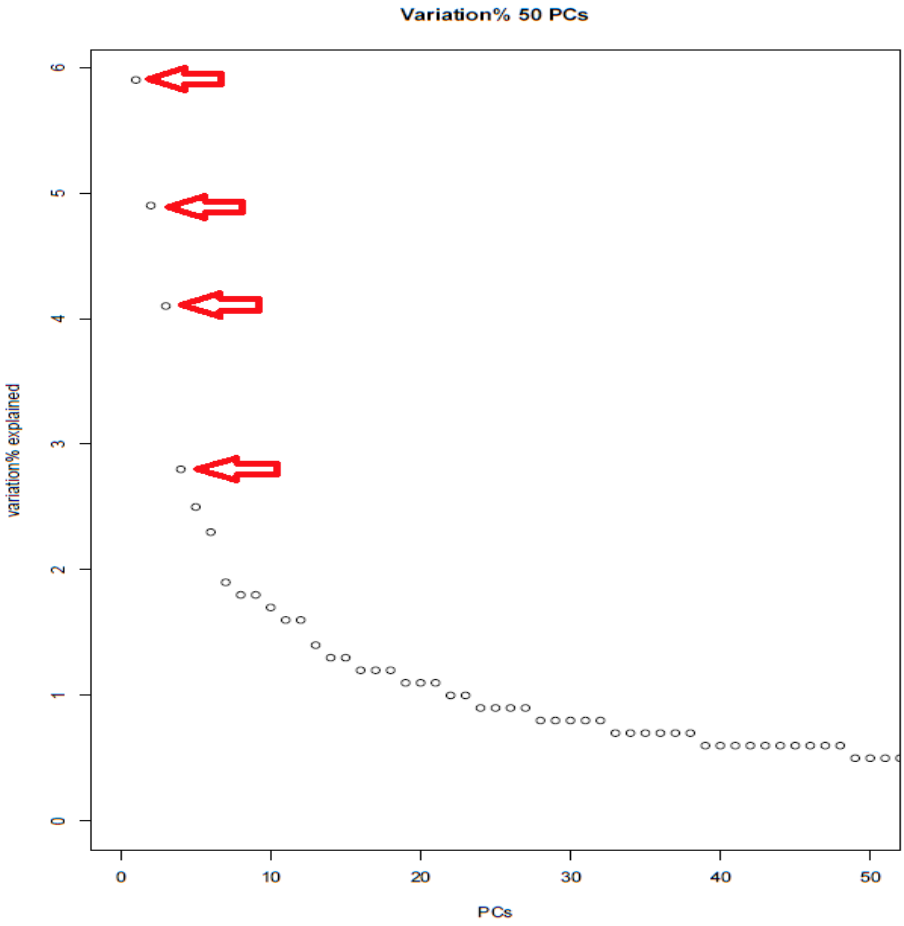
10. Appendices

10.1 Appendix 1: PC plot



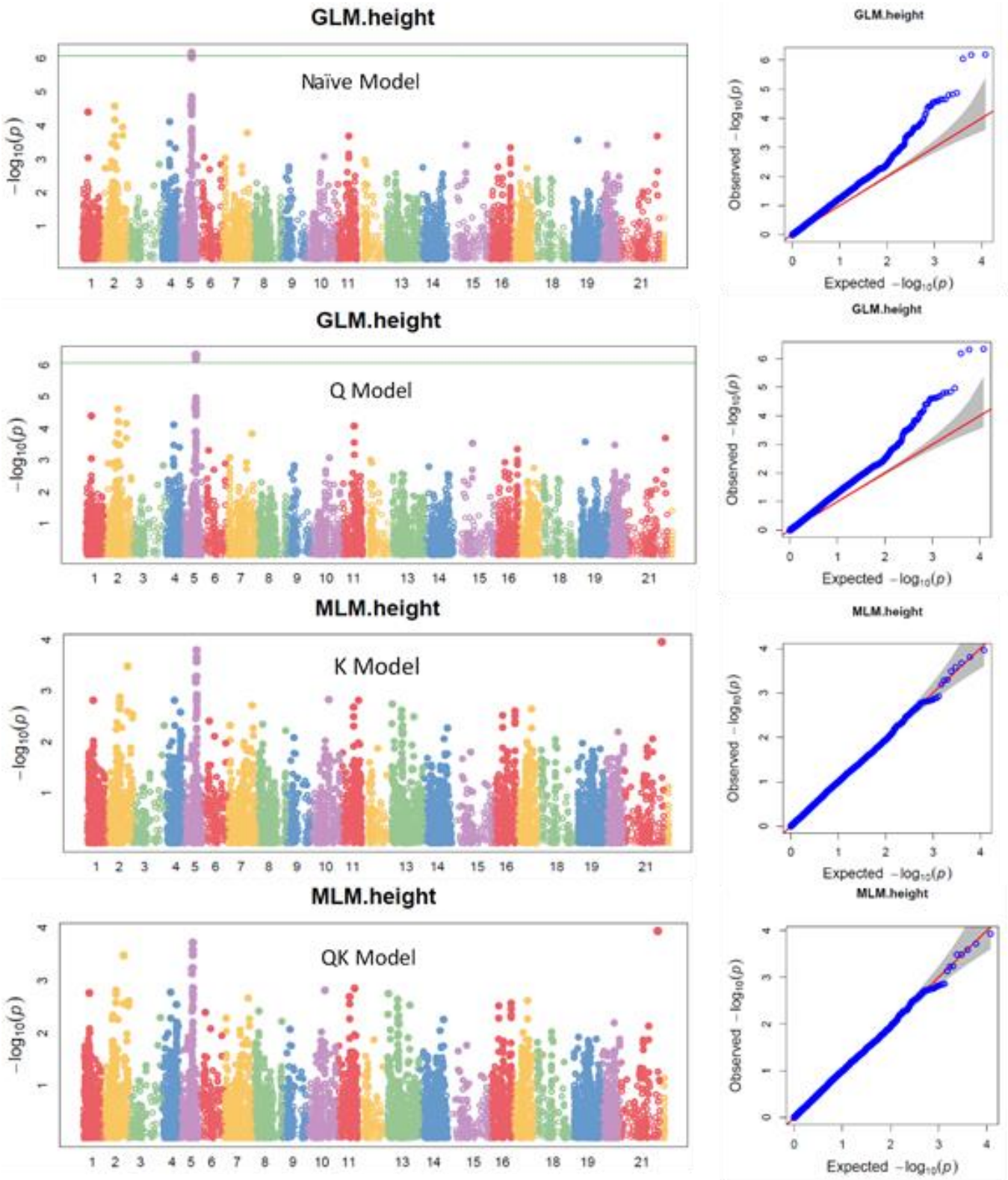
Principal components showing the variation explained by each PC1 and PC2

10.2 Appendix 2: Scree Plot

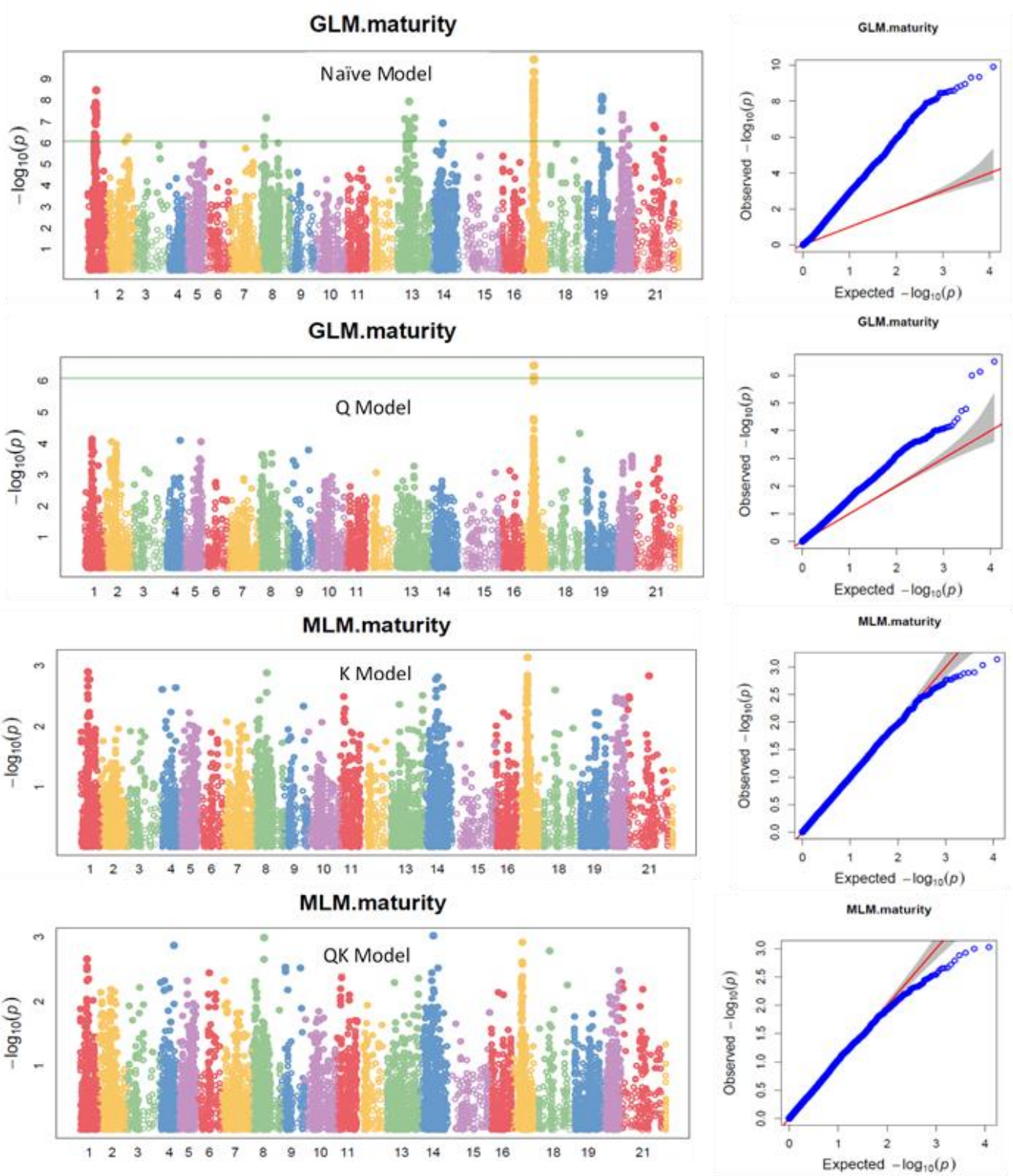


Scree plot representing the variation explained by each PC

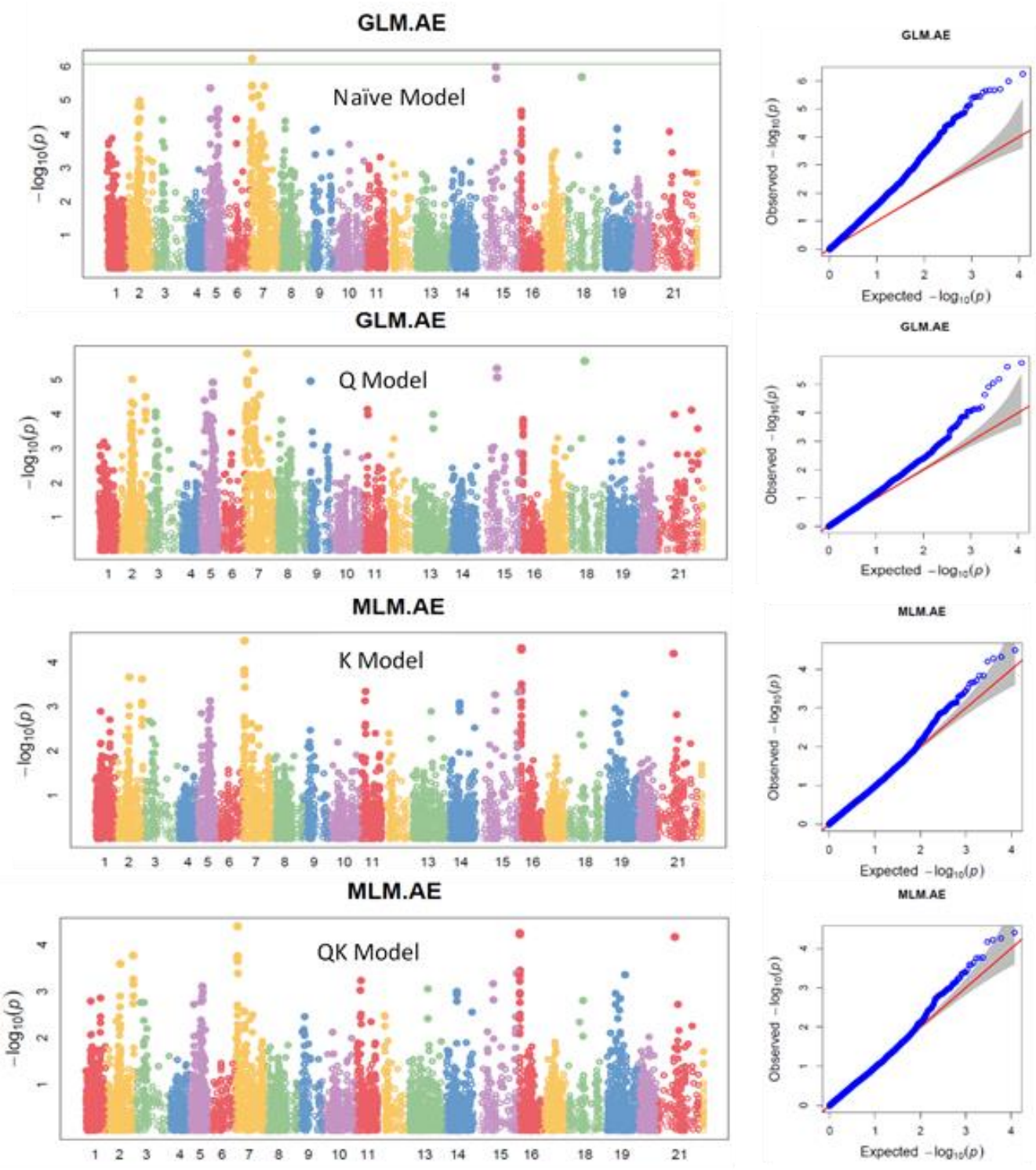
10.3 Appendix 2: Manhattan and Q-Q plot for Height



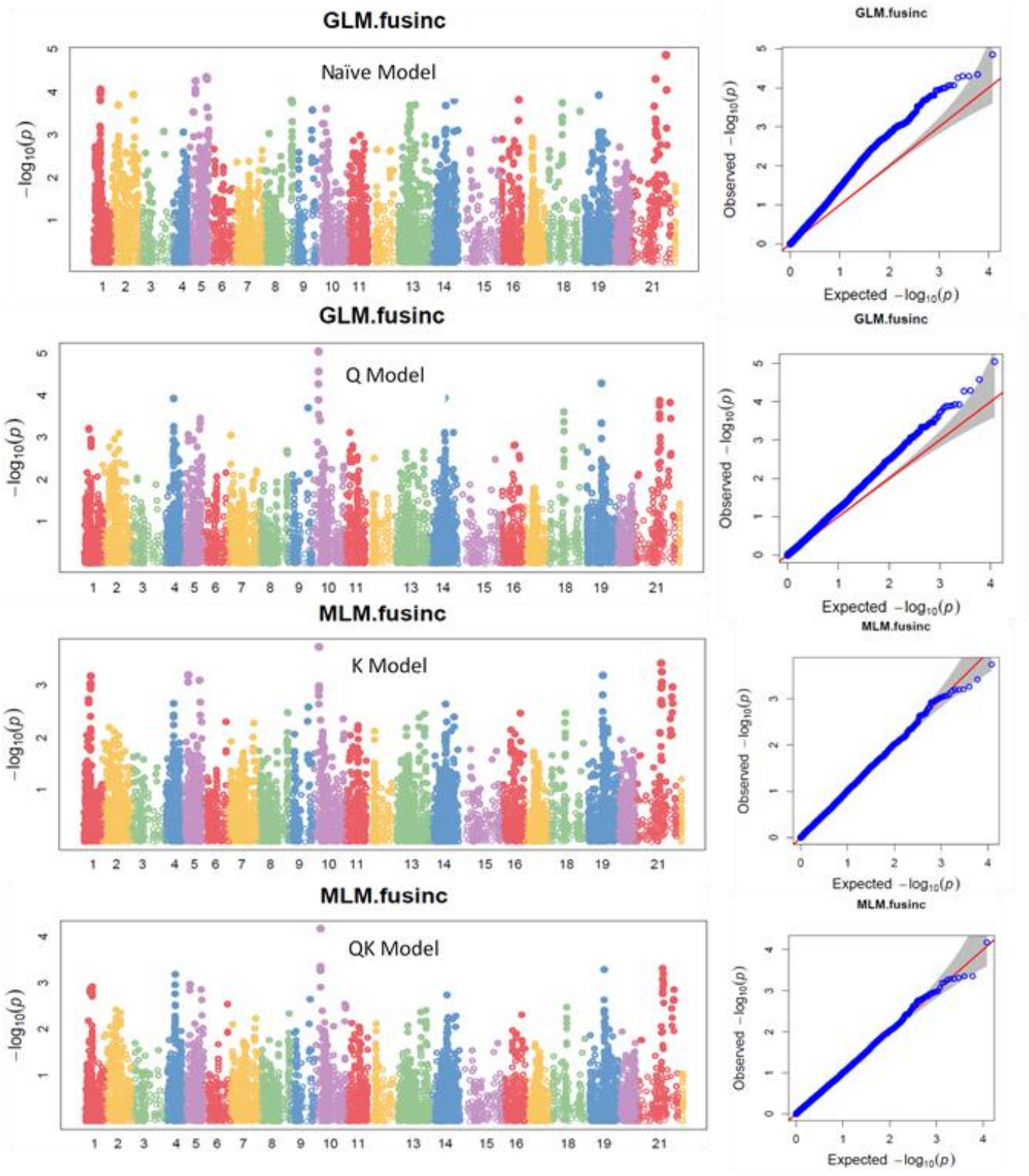
10.4 Appendix 3: Manhattan and Q-Q plot for Maturity class



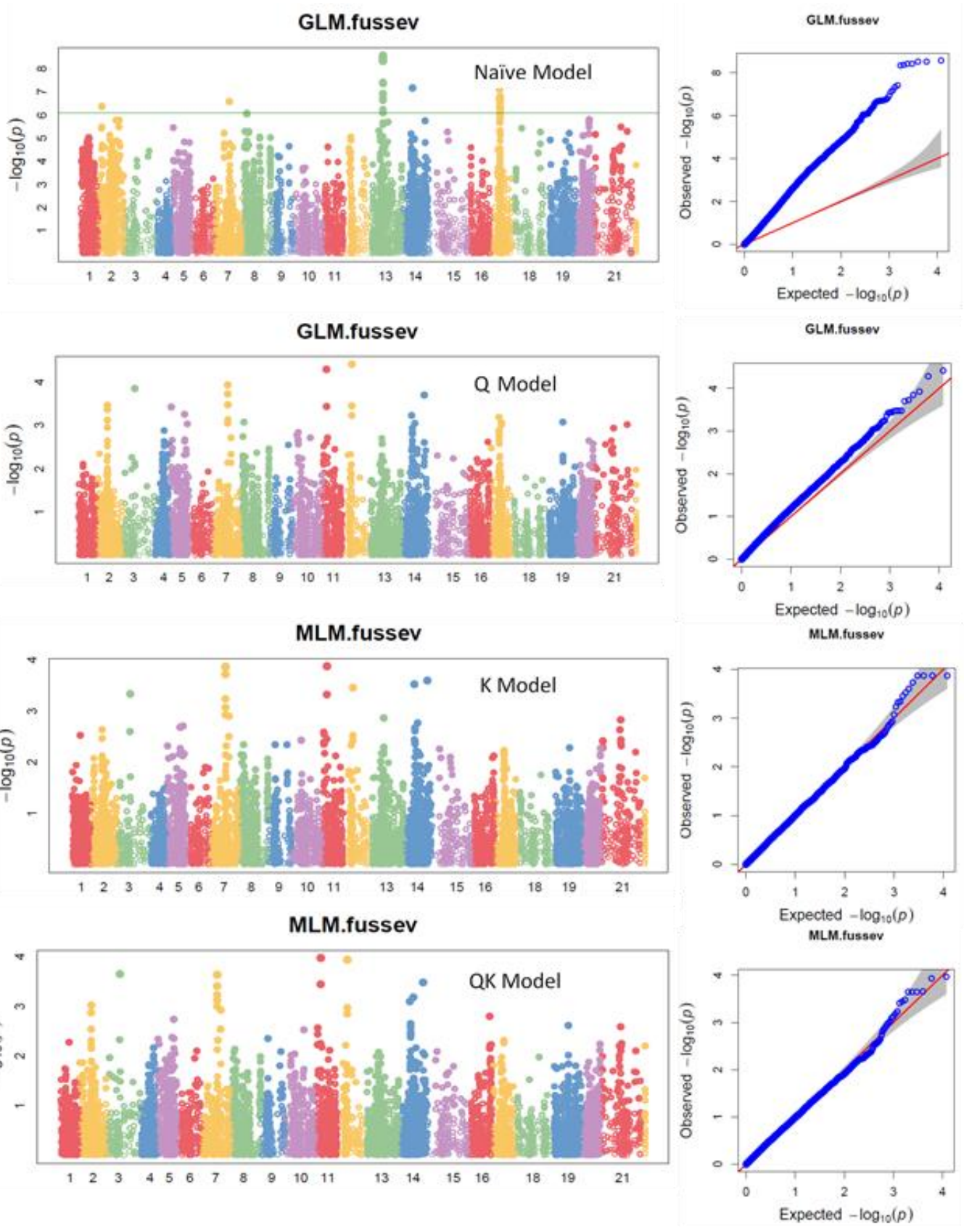
10.5 Appendix 4: Manhattan and Q-Q plot for Anther extrusion



10.6 Appendix 5: Manhattan and Q-Q plot for Fusarium incidence



10.7 Appendix 6: Manhattan and Q-Q plot for Fusarium severity



10.8 Appendix 7: Manhattan and Q-Q plot for DON content