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Associations between anther retention and Fusarium head blight resistance in winter wheat breeding lines and the relationship between the *Fusarium* resistance QTL *Qfhs.ifa-5A* and anther retention

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List of Abbreviations

AcDON	acetyl-deoxynivalenol
ANOVA	analysis of variance
AE	anther extrusion
AR	anther retention
AUDPC	area under disease progress curve
C3	Remus_NIL3
CIMMYT	International Maize and Wheat Improvement Center
Dai	days after inoculation
DH	double haploid
DON	deoxynivalenol
FDK	Fusarium damaged kernels
FHB	Fusarium head blight
FUS	fusarenone
IFA	Department for Agrobiotechnology
NIL	near isogenic line
NIV	nivalenol
PH	plant height
QTL	quantitative trait loci
RIL	recombinant inbred line
ZEA	zearalenone

Abstract

Fusarium head blight (FHB) is a cereal disease of global importance which causes severe economic losses in epidemic years and poses a possible health threat to humans and animals, because of the production of mycotoxins.

Anther extrusion (AE) of wheat is a morphological trait that influences FHB resistance in specific resistance to initial infection, also known as type 1 resistance. In a one year field trial, 403 different wheat cultivars and breeding lines were evaluated for anther retention (AR) and FHB severity after spray inoculation with *Fusarium culmorum*. The wheat lines showed a significant variation for AE and FHB severity and both traits were significantly correlated ($r = 0.50$, $P < 0.001$). Selection for high AE in FHB resistance breeding appears to be a promising approach to enhance type 1 resistance in breeding lines. Since AE is a highly heritable morphological trait, it can be used in breeding programmes in the development of resistant lines.

In addition, the relationship of the major FHB resistance QTL *Qfhs.ifa-5A* and AR was analysed in more detail. Therefore, the susceptible cultivar 'Remus' and its near isogenic line 'Remus NIL3', carrying *Qfhs.ifa-5A*, were evaluated for both traits. 'Remus' exhibited a much higher degree of retained anthers and was more diseased than 'Remus_NIL3'. The association between AE and *Qfhs.ifa-5A,* which confers mainly type 1 resistance, was further examined in a glasshouse experiment. Different pre-treatments, which consisted in removing the anthers or compressing them inside the florets, plus control heads with no pre-treatments, were applied to 'Remus' and 'Remus_NIL3', prior to the inoculation with the fungus. After single head spray inoculation, control heads of 'Remus' were significantly more diseased than 'Remus_NIL3' control plants. When the anthers were removed, no significant difference in FHB severity was detected between the two genotypes 10 days after inoculation. When the anthers were compressed inside the florets, 'Remus' was slightly more diseased than 'Remus_NIL3'. The results indicate that the major resistance QTL *Qfhs.ifa-5A* acts as a passive resistance mechanism controlling anther extrusion and FHB resistance. Furthermore, additional resistance genes may be present in the QTL interval

Key words: Fusarium head blight, anther extrusion, *Qfhs.ifa-5A*, resistence

Zusammenfassung

Ährenfusariose ist eine Getreidekrankheit mit weltweiter Bedeutung, die in epidemischen Jahren schwere finanzielle Verluste verursacht und aufgrund der Bildung von Mykotoxinen eine potentielle gesundheitliche Gefahr für Mensch und Tier darstellt.

Antherenausstoß im Weizen ist ein morphologisches Merkmal welches die Ährenfusarioseresistenz zu Beginn der Infektion, auch Typ 1 Resistenz genannt, beeinflusst. Vierhundertdrei verschiedene Weizensorten und Zuchtlinien wurden in einem einjährigem Feldversuch nach Sprühinokulation mit *Fusarium culmorum* auf Antherenausstoß und Fusariumanfälligkeit untersucht. Zwischen den Weizenlinien wurden signifikante quantitative Unterschiede für Antherenausstoß und Fusariumanfälligkeit festgestellt. Die Korrelation zwischen den beiden Merkmalen war signifikant ($r = 0.50$, $P < 0.001$). Die Selektion auf Antherenausstoß in der Ährenfusarioseresistenzzüchtung scheint eine vielversprechende Methode zu sein um die Typ 1 Resistenz in Zuchtlinien zu verbessern. Da der Antherenausstoß ein hoch vererbbares morphologisches Merkmal ist, kann dieses in Züchtungsprogrammen für die Entwicklung resistenter Linien genutzt werden.

Zusätzlich wurde der Zusammenhang zwischen Antherenausstoß und dem *Qfhs.ifa-5A* Ährenfusarioseresistenz-QTL untersucht. Dazu wurde die anfällig Sorte ‘Remus’ und ihre nah isogene Linie ‘Remus_NIL3’ mit dem *Qfhs.ifa-5A* QTL auf beide Merkmale hin untersucht. ‘Remus’ hatte viel mehr zurückgehaltene Antheren und zeigte mehr Krankheitssymptome als ‘Remus_NIL3’. Der Zusammenhang zwischen Antherenausstoß und *Qfhs.ifa-5A*, das hauptsächlich Typ 1 Resistenz verleiht, wurde in einem Glashausversuch weiter untersucht. ‘Remus’ und ‘Remus_NIL3’ wurden vor der Inokulation mit dem Pilz verschiedenen Vorbehandlungen unterzogen, welche aus dem Entfernen oder Hineinpressen der Antheren, sowie Kontrollähren ohne Behandlung bestanden. Nach Einzelährensprühinokulation zeigten die Kontrollähren der ‘Remus’ Pflanzen mehr Symptome als die Kontrollähren von ‘Remus_NIL3’. Als bei beiden Genotypen die Antheren entfernt wurden konnte kein signifikanter Unterschieden zwischen ‘Remus’ und ‘Remus_NIL3’ festgestellt werden. Als bei beiden Genotypen die Antheren hineingepresst wurden war ‘Remus’ etwas anfälliger als ‘Rems_NIL3’. Die Ergebnisse zeigen, dass der Resistenz-QTL *Qfhs.ifa-5A* als passiver Resistenzmechanismus fungiert, der den Antherenausstoß und Ährenfusarioseresistenz kontrolliert. Außerdem scheint es in dem QTL-Intervall zusätzliche Resistenzgene zu geben.

Schlüsselwörter: Ährenfusariose, Antherenausstoß, *Qfhs.ifa-5A*, Resistenz

1 Research questions

The general objective of this thesis was to investigate the role of anther extrusion in FHB resistance of wheat.

In a field trial, 403 different cultivars and breeding lines, as well as the susceptible cultivar 'Remus' and its near isogenic line 'Remus_NIL3' (C3) carrying the *Fusarium* resistance QTL *Qfhs.ifa-5A*, conferring mainly resistance to initial infection, were evaluated for FHB severity and anther retention. With the field trial following research question shall be answered:

- Is FHB resistance influenced by the retention of anthers?
- Is there variation for FHB resistance and anther retention in the breeding material?
- What is the degree of correlation between trapped and partially extruded anthers and FHB severity? Is there a difference in the association of the traits between the different sets of materials?
- Is there a marked difference for anther retention and FHB susceptibility between 'Remus' and 'C3'?

With a glasshouse experiment, including a susceptible ('Remus') and a resistant genotype ('C3') differing only in the possession of the *Fusarium* resistance QTL *Qfhs.ifa-5A*, following research questions shall be answered:

- Is wheat more susceptible to FHB when all anthers are retained inside the floret?
- Is it possible to increase the level of resistance of a susceptible genotype by removing the anthers?
- Will the resistant genotype turn out to have the same level of resistance as the susceptible genotype when it is manually manipulated in a way that all anthers stay inside the spikelet?
- Is the resistance QTL *Qfhs.ifa-5A* associated with both traits, FHB resistance and anther retention/extrusion?
- Is the resistance QTL *Qfhs.ifa-5A* one gene controlling FHB resistance and anther retention/extrusion?

2 Introduction

2.1 Fusarium head blight

2.1.1 Introduction

Fusarium head blight (FHB) is a cereal disease which causes severe economic losses in epidemic years. Furthermore, it poses a possible health threat to humans and animals due to the production of mycotoxins. It is caused by various species of the genus *Fusarium* and under certain conditions it can also be induced by *Microdochium nivale*. The most prominent species causing FHB are *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae*. *F. graminearum* is reported to occur in continental climates, whereas *F. culmorum* and *F. avenaceum* are more prevalent in cooler European areas with maritime climate (Bottalico and Perrone 2002). FHB can attack all small grain cereals (i.e. wheat, barley, triticale, rye, and oats) during the flowering period. Wheat and barley, however, are the most affected crops. One of the main causal agents of FHB, *F. graminearum,* is on the fourth place of a top 10 fungal plant pathogen list created by the journal *Molecular Plant Pathology*, underlining its scientific and economic importance (Dean et al. 2012).

2.1.2 History and epidemics

FHB, often called scab, was first reported more than hundred years ago in the United States and England (Arthur 1891; Chester 1890; Smith 1884; Parry D. W. et al. 1995). In the beginning of the 20th century, Minnesota already suffered from FHB outbreaks (MacInnes and Fogelman 1923). Some of the major wheat producing countries have to deal with FHB epidemics every few years. In the USA, the upper Midwest states, that is to say Minnesota, North Dakota, South Dakota, Illinois, Indiana, Kentucky, Michigan, Missouri and Ohio, is the region most prone to this fungal disease (Nganje et al. 2004). In Canada, western parts of the country, especially Manitoba, are the most affected ones (Clear and Patrick 2000). Epidemics have also been reported from Australia (Obanor et al. 2013), Argentina (Palazzini et al. 2015) and China, where the provinces along the Yangtze River are the ones most severely affected (Wang 1996). The economic impact of FHB is severe. Nganje et al. (2004) stated that in North Dakota the average FHB losses exceeded 10 % of the value of the wheat crop during the 1998-2000 harvest seasons.

2.1.3 Causal organisms

There is an extensive list of *Fusarium* species associated with FHB. Some of the *Fusarium* species that have been isolated from head blight affected wheat are *F. avenaceum*, *F. culmorum*, *F. poae*, *F. equiseti*, *F. cerealis*, *F. tricinctum*, *F. sporotrichioides*, *F. acuminatum*, *F. subglutinans*, *F. sambucinum*, *F. proliferatum*, *F. crookwellense*, *F. moniliforme* and *F. oxysporum* (Buerstmayr et al. 2012; Wilcoxson et al. 1988). Nevertheless, *F. graminearum* (teleomorph: *Gibberella zeae*) predominates in most parts of the world (Wang 1996; Boutigny et al. 2014; Guenther and Trail 2005). It is found on all continents (Backhouse 2014) and together with *F. culmorum* it is known to be the most aggressive FHB species (Wilcoxson et al. 1988). *Microdochium nivale* (teleomorph: *Monographella nivalis*), a non-mycotoxin-producing fungus causing snow mold, has also been mentioned to cause FHB. However, this only seems to occur under exceptionally cool and wet conditions (Liddell 2003).

2.1.4 Effects on grain yield, grain quality and the contamination by mycotoxins

Fusarium head blight infection impairs grain yield as well as grain quality. Reduced grain set and shrivelled, light-weight *Fusarium* damaged kernels (FDK) result in yield losses, while mycotoxin contamination leads to rejection or downgrading of grain at marketing (McMullen et al. 2012).

Milling reduces the mycotoxin content of wheat, because the outer layers of the kernel, i.e. the pericarp and aleurone tissues, which are removed during milling, are more heavily infected than the endosperm (Bechtel et al. 1985). As a consequence, flour contains less of the most important toxin deoxynivalenol (DON) than whole wheat (Seitz et al. 1985). Studies that analysed milling fractions found that the DON concentration is highest in bran, followed by shorts, and least in flour (Gärtner et al. 2007; Trigo-Stockli et al. 1996). However, it is to note that baking does not destroy DON, and thus, the mycotoxin is often retained in the bread (Scott et al. 1983). Moreover, the fungus digests storage proteins and starch inside the kernel (Bechtel et al. 1985) and produces adverse effects on baking performance, such as reduced dough stability and loaf volume (Dexter et al. 1996; Gärtner et al. 2007).

Fusarium infections have also negative effects for the brewing and malt industry. Aside from rendering barley unsafe for beer brewing due to the contamination with mycotoxins, the fungus is known to produce gushing in beer. The uncontrolled foaming is positively correlated with the amount of hydrophobins, small fungal proteins, produced by *Fusarium* species (Sarlin et al. 2005).

One of the reasons why so much effort has been undertaken to combat FHB is the accumulation of mycotoxins in the grain and its potential health hazard to humans and animals. Several countries have set limits for the two most important mycotoxins produced by *Fusarium* species, DON and zearalenone (ZEA). Each *Fusarium* species has its own mycotoxin profile. *Fusarium graminearum*, is for instance known to produce the trichothecenes DON, acetyl-deoxynivelnol (acDON) and nivalenol (NIV), as well as ZEA and fusarenone (FUS). *Fusarium culmorum* produces DON, NIV and ZEA (Bottalico and Perrone 2002). Deoxynivalenol belongs to the trichothecenes group and is a potent protein synthesis inhibitor (Pestka and Bondy 1994).

Swine are the most sensitive farm animals to *Fusarium* mycotoxins (Prelusky et al. 1994) and manifest reduced body weight, reduced weight gain and reduced feed consumption when fed DON contaminated grain (Friend et al. 1986). ZEA has an estrogenic effect and produces reproductive and fertility problems when fed to animals (Prelusky et al. 1994).

Diseases caused by mycotoxins associated with *Fusarium* species have also been reported in humans. In the Soviet Union, alimentary toxic aleukia, a fatal disease, was caused by the consumption of overwintered cereal grain contaminated with *F. poae* and *F. sporotrichioides* (Yagen and Joffe 1976). In Japan and Korea, red mold disease, provoked by the ingestion of grain contaminated with *F. graminearum*, produced nausea, vomiting, diarrhea, abdominal pain, fever and throat irritation (Beardall and Miller 1994). Fusariotoxicoses have also been reported from China, with 35 outbreaks of intoxication between 1961-1985 (Luo 1988).

In order to minimize health risks for humans and animals, the European Union established maximum levels for the *Fusarium* toxins DON and ZEA. The current maximum level for DON in unprocessed cereals other than durum wheat, oats and maize is 1250 µg/kg. For cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption and dry pasta the maximum level is 750 µg/kg and for bread and breakfast cereals the maximum level is 500 µg/kg. The maximum level for ZEA is 100 µg/kg for unprocessed cereals other than maize. For cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption the maximum level is 75 µg/kg (European Commission 2007). In the USA, the Food and Drug Administration set an advisory limit for DON of 1000 µg/kg in finished wheat products (e.g. flour, bran, germ) for human consumption (FDA 2010).

A scientific cooperation task of the European Commission collected occurrence data of *Fusarium* toxins in food in several European countries and assessed the dietary intake by the population. Fifty-seven percent of the evaluated 11022 cereal samples tested positive for DON, but only 7% had a DON level of 750 µg/kg or higher. The report concluded that the average

level intake for DON does not exceed 46.1% of the tolerable daily intake of 1 µg/kg bodyweight (European Commission 2003).

2.1.5 Epidemiology

Fusarium head blight is a floral disease that affects wheat spikes during, or shortly before/after anthesis (Andersen 1948). Infection is initiated when air-borne ascospores or rain-splash dispersed macroconida land on wheat spikes and penetrate the florets (Bushnell et al. 2003). Infection becomes visible through water-soaked spots, darkened necrotic lesions ('scab') and subsequent premature bleaching of the spikelets. The fungus can spread to adjacent spikelets, thus affecting the entire head. It also affects developing kernels causing mycotoxin contamination. Colonized kernels, sometimes called 'tombstone' kernels, usually have a shrivelled and discoloured appearance and are lighter than healthy grain. Under high humidity a pinkish mycelium may become visible on the spikes and at a later stage of disease development black perithecia can be seen on the plant tissue (Bottalico and Perrone 2002; Osborne and Stein 2007; Goswami and Kistler 2004).

Plant residues serve as a major inoculum source. The pathogen survives saprophytically on infested crop debris and can also colonize weed species (Mourelos et al. 2014; Inch and Gilbert 2003). A study by Pereyra et al. (2004) showed that the ascospore production level of *Gibberella zeae* is higher in wheat and barley residues than in the residues of corn, fescue and gramineous weeds. The fungus could not be recovered from forage legumes and did not produce perithecia on sunflower residues, although it saprophytically colonized it. In China, where wheat-rice rotations are practiced, ascospores are reported to be produced on paddy stubble (Wang 1996).

The fungus is able to produce inoculum on crop residues until they are completely decomposed (Pereyra et al. 2004). Buried residues degrade faster than residues on the soil surface; therefore, non-inversion tillage has an unfavourable effect on the inoculum survival (Leplat et al. 2013). Reduced tillage is considered as one of the factors responsible for the re-emergence of FHB epidemics in the U.S. (Shaner 2003).

FHB outbreaks are not only associated with management practices influencing the amount of inoculum, but also with favourable weather conditions. Rainfall before or during anthesis, together with temperatures between 15 and 30°C, can trigger infection (McMullen et al. 1997). Epidemic years have been found to coincide with rainfall above the monthly average during the time of anthesis (Obanor et al. 2013).

Temperature requirements of *Gibberella zeae* have already been studied more than 50 years ago. Andersen (1948) and MacInnes and Fogelman (1923) have conducted *in vitro* studies

and reported minimum, optimum and maximum temperatures for mycelial growth. The first author found them to be 4°, 28° and 32°C, whereas the second author described 3°, 25 to 27° and 33°C as cardinal growth temperatures on artificial media. MacInnes and Fogelman (1923) observed 100% spore germination after 48 hr at 25°C. A more recent study by Brennan et al. (2003) recorded 25°C as the optimum growth temperature for *F. graminearum*, *F. culmorum* and *F. poae*, and 20°C for *F. avenaceum* and *Microdochium nivale*. This finding adds evidence to the influence of temperature on the distribution patterns of *Fusarium* species.

2.1.6 Study of the infection process and the role of anthers

A study that investigates the infection process was presented by Kang and Buchenauer (2000), who examined the infection by *F. culmorum* with light and electron microscopy. In artificially inoculated plants, spore germination took place within a few hours after inoculation and was observed on the inner surfaces of lemma, glume, palea, stigma and on the upper part of the ovary, as well as on the outer surfaces of lemma, glume and rachis. However, extensive hyphal growth was found only on the inner surfaces of lemma, palea and glume, and on caught anthers and pollen grains on the brush hair of the ovary. Spores that had landed on the outer surfaces generated hypha that grew over the edge to reach the inner surfaces, where host penetration occurred.

The observation that initial infection takes place on the inner parts of the florets is consistent with an earlier study by Pugh et al. (1933) who had noted the same in histological studies of inoculated spikelets. Another study was done by Miller et al. (2004) who investigated the infection by a strain of *F. graminearum* transformed with green fluorescent protein. He observed the colonization of the ovary and subsequent spread into the rachis via vascular tissues and parenchyma. The fungus spread downward the rachis from the point of infection, rather than upward throughout the wheat spike. This observation leads the author to assume that the bleaching of the part of the spike above the point of infection is not due to fungal colonization, but arises from the occlusion of the vascular tissues and the following restriction of nutrient flow.

Kang and Buchenauer (2000), as well as Miller et al. (2004) affirmed the role of trapped anthers in the infection process. In their studies, the growth of hyphal network was more prolific on pollen and anthers, suggesting that trapped anthers could increase initial infection. This agrees with an earlier work by Pugh et al. (1933) who reported an increased percentage of infected kernels in a variety with a higher amount of trapped anthers compared to another variety with fewer retained anthers.

An experiment carried out by Strange and Smith (1971) also showed that anthers promoted infection. They compared the number of infected spikelets from non-emasculated plants and plants from which the anthers had been removed and conducted an *in vitro* growth experiment using anther and pollen extracts as growth stimulants for the fungus. Non-emasculated plants showed higher infection rates, and anther as well as pollen extracts had a higher growth-stimulating-activity on the fungus than extracts from other parts of the plant. A few years later, choline and betaine were identified as the growth stimulating components in wheat anthers (Strange et al. 1974).

Engle et al. (2004) used choline, betaine and anther extracts to study their effect on hyphal growth of two *F. graminearum* strains in nine different wheat genotypes. Choline and betaine slightly enhanced radial growth at concentrations of 0.01, 0.1 and 1.0 µM on water agar. However, no significant effect was found at concentrations of 10, 100 and 1000 µM. Anther extracts also showed to increase hyphal growth on water agar.

2.1.7 Control

To control FHB, different strategies should be combined. Cultural practices, including type of tillage and crop rotation, fungicide application and the use of resistant cultivars, can help to reduce disease severity. *Fusarium* species survive saprophytically on crop debris, which together with weed host plants serve as primary inoculum source. Crop residues should be buried using inversion tillage to reduce primary inoculum. This augments the decomposition rate of the residues and also helps to control weed species (Leplat et al. 2013).

Another way to reduce inoculum in the field is to practice adequate crop rotations. The adverse effect of corn-wheat rotations has already been mentioned in early literature (Koehler 1924) and more recent studies confirm this finding. Dill-Macky and Jones (2000) reported that corn-wheat, as well as wheat-wheat rotations increased FHB incidence and severity compared to soybean-wheat rotations. DON levels in wheat planted after corn were twice as high as in wheat planted after soybean.

The use of fungicides to control FHB can only be recommended at higher yield levels taking into account the cost of application. Paul et al. (2008) conducted a multivariate meta-analysis to determine the effect of triazole fungicides for FHB control in wheat. Overall mean percent control was higher for FHB index (32-52%) than for DON (12-45%), and prothioconazole + tebuconazole were the most effective fungicides in reducing FHB severity. Metconazole was the most effective fungicide in reducing DON. From these results it is evident that applying a fungicide as a single control measure under high disease pressure will not be sufficient to reduce FHB to an acceptable level. Moreover, fungicides are more effective in

resistant varieties than in susceptible varieties (Mesterházy et al. 2003). Also, fungicide application poses a challenge to the producer because of the short window of application, uneven flowering in the field and wet weather conditions during anthesis (McMullen et al. 2012). To help producers evaluate the risk of a disease outbreak and the need for a fungicide application, several forecasting systems that take in account weather variables have been developed. One of them is the 'Fusarium Head Blight Risk Assessment Tool' in the United States (Wegulo et al. 2015).

Biological control agents, like species of yeast and endospore-forming bacteria, have been tested to control FHB and their antagonistic activity against *Fusarium* spp. has been shown (da Luz et al. 2003). However, none of them are available on the market until now (Wegulo et al. 2015).

To improve grain quality at harvest, combine harvest configurations can be changed in order to blow away FDK. They are lighter than healthy grain and will be removed by increasing the fan speed and the shutter opening. This results in lower yield, but also in a reduction of price discounts that are applied due to the presence of FDK and mycotoxins. The grain quality improvement should compensate for the reduced yield in terms of economic benefit (Salgado et al. 2011).

2.1.8 Resistance breeding

Another way to reduce FHB severity is to breed and use resistant cultivars. Resistance to FHB is a quantitative trait and more than 100 quantitative trait loci (QTL) for FHB resistance in hexaploid wheat have been reported so far (Buerstmayr et al. 2009). FHB resistance is not race-specific, nor species-specific, which means that by using one aggressive *Fusarium* isolate in resistance breeding selection can be done to confer resistance to all other FHB species (Mesterházy et al. 2005).

Two major resistant components, named type 1 and type 2 resistance have been proposed by Schroeder and Christensen (1963). Type 1 resistance reduces initial infection and type 2 resistance moderates pathogen spreading in infected tissue. Other active resistance mechanisms are the ability to degrade DON (Miller and Arnison 1986), resistance to kernel infection (Mesterházy et al. 1999) and tolerance, which means the ability to produce higher yields than predicted by FHB resistance (Mesterházy 1995).

Lemmens et al. (2005) found that the detoxification of DON to DON-3-*O*-glucoside co-localizes with the major FHB resistance QTL, *Qfhs.ndsu-3BS*, which is located on chromosome 3B. This QTL, also called *Fhb1,* has been detected in at least 26 QTL mapping studies and confers type 2 resistance (Buerstmayr et al. 2012). The two other most repeatable QTL are *Qfhs.ifa-5A*

on chromosome 5A and *Fhb2* on chromosome 6BS, the former primarily conferring type 1 resistance and the latter type 2 resistance (Buerstmayr et al. 2009).

The most widely used resistance source in breeding programs worldwide is 'Sumai-3'. The variety was released by a Chinese Institute of Agricultural Science in 1974 and derived from a cross of two moderately susceptible cultivars; 'Funo' from Italy and 'Taiwanxiaomai' from China (Liu and Wang 1991). 'Sumai-3', as well as its related line 'Nang 7840', another excellent resistance source, carry both the *Fhb1* QTL, conferring type 2 resistance (Buerstmayr et al. 2012). Other genetic resources used for resistance breeding are Chinese landraces like 'Wangshuibai' (Bai and Shaner 2004). Resistant germplasm is also found in European winter wheats, Japanese spring wheats (e.g. 'Nobeokabozu Komugi') and Brazilian spring wheats (e.g. 'Frontana') (Snijders 1990).

In addition to the active or physiological resistance mechanisms mentioned above, passive resistance mechanisms have been described. These include morphological traits like plant height, the presence or absence of awns, ear compactness (Mesterházy 1995), the width of flower opening (Gilsinger et al. 2005) and the extent of anther extrusion (Skinnes et al. 2010). Mesterházy (1995) found that under natural epidemic conditions dwarf genotypes were more susceptible to FHB infection.

Skinnes et al. (2010) assessed anther extrusion (AE) in a mapping population of 75 double haploid (DH) lines and analysed its relationship to FHB and DON content under field conditions over three years. He reported a highly significant negative correlation between AE and FHB ($r = -0.53$ to -0.69, $P < 0.0001$), as well as DON ($r = -0.39$ to -0.46, $P < 0.0001$). The calculated genotype by year interaction for AE was very low and the trait was highly heritable ($H^2 = 0.91$). This study was the first to report QTL for anther extrusion. The author identified four QTL for AE, which explained 53.6% of the total phenotypic variation. QTL on chromosome 1AL explained 18.3% of the total phenotypic variance, QTL on 5AS explained 15.6%, QTL on 4DL explained 13.3% and QTL on 1B explained 7.4%.

Lu et al. (2013) evaluated a recombinant inbred line (RIL) population from the cross of Shanghai-3/Catbird (moderately resistant, high AE) with the German spring wheat cultivar Naxos (susceptible, low AE) for FHB resistance, anther extrusion and plant height. The observed negative correlation between AE and FHB severity ($r = -0.45$ to -0.64, $P < 0.0001$) was similar to the correlation found by Skinnes et al. (2010). Heritability for AE was high too ($H^2 = 0.80$). Plant height was also negatively correlated with FHB with a correlation coefficient of $r = -0.48$ ($P < 0.0001$) for spawn inoculation and $r = -0.37$ ($P < 0.0001$) for spray inoculation for the FHB mean over two years. The author also detected QTL associated with AE that all coincided with FHB severity. However, they were located in different chromosomal regions

than the QTL found by Skinnes et al. (2010). This indicates that AE is controlled in a quantitative manner.

Buerstmayr and Buerstmayr (2015) found QTL for AE that are different from those reported by Skinnes et al. (2010) and Lu et al. (2013). The authors evaluated a RIL population from the cross between 'Arina' (resistant) and 'Capo' (moderately resistant) and detected QTL for AE on chromosome 4AL, 6BL and 5AS, of which 4AL and 6BL coincided with QTL for FHB severity. They also reported a significant correlation (r = 0.63) between AR and FHB severity.

He et al. (2014) assessed AE, plant height (PH), FHB, DON and FDK of 140 selected lines from the CIMMYT wheat germplasm bank. The Pearson correlation coefficients between AE and FHB index, DON content and FDK were -0.43 ($P < 0.001$), -0.35 ($P < 0.001$) and -0.26 ($P < 0.01$) respectively. Lines with low AE/PH showed a wide range of FHB index, whereas lines with high AE/PH tended to have low FHB scores.

Kubo et al. (2013) used DH lines derived from F_1 plants from crosses between closed-flowering and opened-flowering varieties to study the relationship between FHB and the degree of AE. Closed-flowering lines showed the highest FHB resistance and lines with partially extruded anthers showed higher FHB scores than lines with fully extruded anthers.

The observation that closed-flowering lines are more resistant agrees with an earlier study by Kubo et al. (2010), who tested a RIL population from a cross between a cleistogamous (closed flowering) and a chasmogamous (opened flowering) wheat variety for FHB resistance. However, the improved resistance of closed flowering RILs was not always accompanied by resistance to grain deterioration and mycotoxin accumulation in this study.

The influence of flower opening on FHB was also demonstrated by Gilsinger et al. (2005), who characterized a RIL population for FHB incidence and flower opening width and duration. The author showed that flower opening width was positively correlated with FHB incidence ($r = -0.6$, $p < 0.0001$) and hypothesised that lines with narrow flower opening escaped infection by reducing the time and area in which *Fusarium* conidia can enter the floret. He also remarked that in narrow flower opening lines anthers are more prone to become trapped within the floret.

2.2 Flower morphology and flowering biology of wheat (*Triticum aestivum*)

The flower morphology and the flowering process have been described precisely in early literature (Leighty and Sando 1924; Percival 1921; Obermayer 1916; Vries 1971). The wheat flower is an inflorescence called spike, with its main axis termed rachis. The axis of the spikelets which bears the florets is termed rachilla (Fig. 1). Each spikelet generally consists of two to five florets, of which usually merely two to three will set grain. The upper florets of a spikelet are smaller and less developed, sometimes even imperfect and sterile. The outer bract

of the florets is called lemma, the inner bract is called palea. The entire spikelet is also enclosed by bracts, called glumes. Each floret bears three stamens and a single carpel with a feathery stigma (Fig. 2). The stamens are composed of an elongated part, the filaments, and a terminal, pollen producing part, the anthers. The bi-lobed anthers are green when young and turn yellow when ripe.

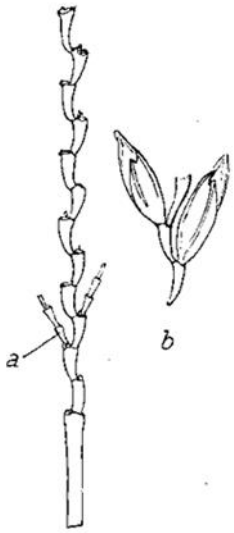

Figure 1: Rachis of an ear. a) rachilla b) portion of rachis with attached spikelets (Percival 1921)

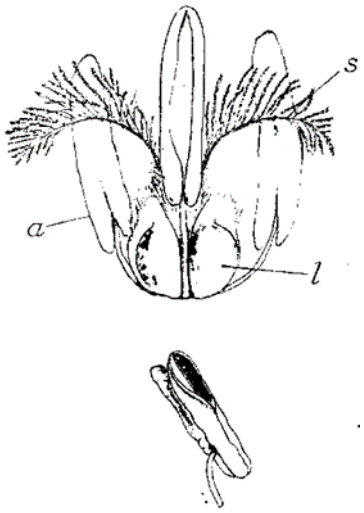

Figure 2: Flower before anthesis with dehiscing anther below. a) anther s) style l) lodicule (Percival 1921).

When the flowering period, called anthesis, starts, the lodicules swell and push lemma and palea apart, until a separation of three to four millimetres. At this time, the filaments elongate from two to three millimetres to a length of seven to ten millimetres and push the anthers outside the florets. Lemma and palea remain open for about 5 to 15 minutes. Cross-pollination may occur during this time, although self-pollination is the rule, because the anthers already release the pollen inside the floret before they are ejected. When the lodicules collapse, lemma and palea close again and the flowering process has ended. The time from the beginning of the opening until the end of the closing takes 8 to 30 minutes or more.

Flowering normally starts at the lower half of the uppermost third of the spike, and within a spikelet, the lower florets usually flower first. The flowering of an entire spike takes three to five days under warm and sunny conditions and the whole plant completes flowering in about eight days.

At the end of flowering, the spent and ejected anthers dangle from the spikelets and remain on the spikelets until they are blown away by the wind. In some florets, however, anthers may

become trapped between lemma and palea when they close again. This can be provoked by a very small angle of separation between lemma and palea or a very short opening duration. In other cases, anthers may be retained inside the floret. The proportion of extruded anthers to trapped or retained anthers depends on the cultivar and external conditions. In some cultivars and under certain weather conditions, i.e. on cloudy or rainy days, closed flowering is possible.

3 Materials and Methods

3.1 Evaluation of breeding lines for FHB resistance and anther retention in the field

3.1.1 Plant material and experimental design

A total number of 403 winter wheat breeding lines were cultivated at the experimental station of IFA-Tulln (latitude 48°20´0´N, longitude 16°3´0´E, altitude 177 m, 600 mm average annual precipitation, 9.5°C average annual temperature) in 2015. This panel consisted of 374 $F_{4:6}$ derived lines and 29 F_7 experimental varieties (PrecW), i.e. potential candidates for official trial testing and registration, as well as the check varieties 'Midas', 'Balitus', 'Ubicus' and 'Bernstein'. The 374 $F_{4:6}$ derived lines split into two sets of which 191 lines (YoungEast) were adapted to the agroecological conditions of Eastern Austria, Hungary, Serbia and Romania, while the other 183 lines (YoungWest) were adapted to Western European conditions. All lines were tested in randomized complete block design with two replications and phenotyped for FHB severity and AR.

The susceptible spring cultivar 'Remus' was planted in 32 replications and its near isogenic line (NIL) 'Remus_NIL3' (C3) that carries the FHB resistance QTL *Qfhs.ifa-5A* in the susceptible 'Remus' background, was planted in 24 replications. *Qfhs.ifa.5A* confers type 1 resistance by lowering the rate of initial infection. The donor of the resistance QTL *Qfhs.ifa.5A* is 'CM-82036' (abbreviation of 'CM-82036-1TP-10Y-OST-10Y-OM-OFC') that originates from the cross 'Sumai#3'/'Thornbird-S' and was developed by a shuttle breeding program between CIMMYT Mexico and South America (Buerstmayr et al. 2002).

'Remus' ('Sappo'/'Mex'//'Famos') was developed by the Bavarian State Institute for Agronomy in Freising, Germany. The 'NIL3' derived from a cross of the susceptible cultivar 'Remus' and the highly resistant line 'CM-82036' followed by five backcrosses with 'Remus'. In the BC_5F_2 generation, 'NIL3' was selected, possessing the resistant 'CM-82036' alleles at *Qfhs.ifa-5A* in a 98.5% Remus background. The presence of *Qfhs.ifa-5A* was confirmed with flanking microsatellite markers *gwm304*, *barc186* and *barc1*. (Schweiger et al. 2013).

Wheat lines were sown on November 5 and on November 27 in plots of two rows and 65 cm length in 2014. The spacing between the rows was 17 cm and the spacing between the plots was 33 cm. The seed rate was 5g/plot and the preceding crop was soybean. Seeds were treated with 'Celest Extra 050 FS'. The plants were fertilized with NPK 17:6:18+7S on October 4 at a rate of 300 kg/ha and with KAS 27% N on May 13 at a rate of 200 kg/ha. The herbicide 'Andiamo Maxx' was applied on April 4 at a rate of 1.5 l/ha and the herbicide

‘Puma Extra’ was applied on May 19 at a rate of 1 l/ha. The insecticide treatment consisted in an application of ‘Biscaya’ at a rate of 0.3 l/ha on May 20.

3.1.2 Inoculation

Artificial inoculation was performed with a motor driven back-pack sprayer in the late afternoon. The *F. culmorum* isolate Fc 91015 was applied at a rate of 25.000 conidia/ml. Inoculum was produced according to the standard operating procedure of the Institute of Biotechnology in Plant Production, IFA-Tulln (see appendix). After spray inoculation, an automated mist-irrigation system provided humidity for 20 hr. It was activated every 20 min for about 10 sec. Inoculation was done every two days from the beginning of anthesis until the last plots were fully flowering. In this way, all plots of replication 1 and 2 were inoculated eight times.

3.1.3 FHB and anther retention assessment

‘Remus’ and ‘C3 were assessed for FHB severity, FHB incidence and anther retention. FHB incidence was measured as the percentage of symptomatic heads within a plot from 40 heads evaluated. FHB severity was measured as the percentage of symptomatic spikelets per plot and was evaluated 10, 14, 18, 22, 26 and 30 dai. Additionally, the area under disease progress curve (AUDPC) was calculated as described by Buerstmayr et al. (2000) as follows:

$$AUDPC = \sum_{i=1}^{n} \left\{ \left[\frac{Y_i + Y_{i+1}}{2} \right] (X_i - X_{i-1}) \right\}$$

The variable Y_i is the percentage of number of infected spikelets per spike on the i^{th} day, X_i is the day of the i^{th} observation, and n is the total number of observations.

The anther retention characteristics of the genotypes were evaluated by randomly choosing five heads per plot and inspecting four florets per head for trapped anthers, giving a maximum score of 20.

Assessment of anther retention takes into account anthers trapped inside the floret, but also anthers trapped between lemma and palea. Anthers trapped inside the floret can be inspected by opening lemma and palea and looking inside. Anthers trapped between lemma and palea are those that only have a small tip protruding from the floret.

FHB scorings were carried out 14, 18, 22 and 26 dai.

3.1.4 Statistical analysis

Statistical analysis was performed with the statistical package R (R development core team, 2015) using the lme4 package for the mixed model analysis. A linear mixed model for anther retention and FHB severity was set up, where replication, replication-by-set interaction and genotype were treated as random effects to derive variance components. The randomization of the design was restricted within each block so that lines belonging to each of three different sets respectively were grown together in a sub-block, thus an additional random effect for sets nested within blocks had to be introduced. Normality and homogeneity of variance of the residuals were verified with a QQ-plot and a residual plot. Heritability within a year was estimated using the formula $h^2 = \sigma_g^2/(\sigma_g^2 + \frac{\sigma_\varepsilon^2}{r})$. Differences among genotypes concerning FHB severity and anther retention were verified with ANOVA.

3.2 Analysis of the relationship between *Qfhs.ifa-5A* and anther retention

3.2.1 Plant Material

Two genotypes were used in the glasshouse experiment; the susceptible spring wheat cultivar ‘Remus’ and the near isogenic line ‘Remus_NIL3’ (C3) that carries the FHB resistance QTL *Qfhs.ifa.5A* in the susceptible Remus background.

3.2.2 Experimental design and growth conditions

Seeds were germinated in March 2015. The seedlings received a cold treatment (5 °C) for one week to improve tillering before transplanting them into pots with 20 cm diameter. Each pot was filled with 4 L of substrate consisting of 500 L heat-sterilized compost, 250 L peat, 10 kg sand and 250 g rock flour. Five plants of the same genotype were put into each pot which made a total number of 88 pots with 440 ‘Remus’ plants and 90 pots with 450 ‘C3’ plants. The pots were allocated in double rows on each site of the glasshouse, alternating double rows with genotype ‘Remus’ and genotype ‘C3’. At the end of tillering, 2 g of mineral fertilizer (COMPO Blaukorn® ENTEC® N/P/K/Mg: 14/7/17/2) were applied per pot. Until heading, sulphur (sulphur evaporator, Nivola®) was administered twice a week during 10 hr at night to prevent mildew. Temperature and duration of illumination were regulated according to the growth stage (Table 1). The seedlings reached the flowering stage after nine to ten weeks.

Table 1: Day and night temperature and duration of illumination in the glasshouse, according to the growth stage of the wheat plants.

Growth stage	Day temperature [°C]	Night temperature [°C]	Illumination [hr]
Planting – end of tillering	12	10	12
End of tillering – mid stem extension	14	10	14
Mid stem extension – start of heading	16	14	14
Start of heading – start of flowering	18	14	14
Start of flowering – end of the experiment	22	18	16

3.2.3 Pre-treatments of wheat spikes prior to inoculation

Post anthesis and before spikes were spray inoculated, four and three different types of pre-treatments were applied to the spikes of the two genotypes in experiment 1 and 2 respectively. Each type of pre-treatment was assigned to a colour code, abbreviated by a letter (Table 2).

Table 2: Pre-treatments applied in glasshouse experiment 1

Pre-treatment	Colour code	Abbreviation
No manipulation	green	G
Middle florets removed	blue	B
Middle florets and anthers removed	yellow	Y
Middle florets removed and anthers compressed inside the florets	red	R

In order to obtain an equal number of spikelets/spike, spikelets were reduced to twelve, by cutting off the least developed basal and apical spikelets. The middle florets of the spikelets were removed in pre-treatment ‘B’, ‘Y’ and ‘R’. This resulted in spikelets with two well developed florets, which can be easily manipulated (Fig. 3).

Figure 3: Wheat spike before manipulation (left), removing middle florets (middle), wheat spike with removed middle florets (right).

In pre-treatment ‘Y’, all anthers were removed with a forceps. In pre-treatment ‘R’, on the other hand, the previously removed anthers were placed inside the florets again. By doing this, anthers could not be extruded anymore and stayed inside the floret. Pre-treatment ‘B’ had only the middle florets removed and pre-treatment ‘G’ was not manipulated, apart from equalling the number of spikelets/spike to twelve. Pre-treatment ‘B’ and ‘G’ constituted the control.

In experiment 2, only three pre-treatments were applied and the middle florets were not removed (Table 3).

Table 3: Pre-treatments applied in glasshouse experiment 2

Pre-treatment	Colour code	Abbreviation
No manipulation	green	G
Anthers removed	yellow	Y
Anthers compressed inside the florets	red	R

Post anthesis, pre-treatments were applied every two days to all wheat spikes flowering on that day and primed spikes were marked with adhesive labels with colour codes. This was done until completing approximately 60 replications per pre-treatment and genotype. The pre-treatments were randomly assigned to the spikes in a pot, in a way that all types of pre-treatments were represented at least once in every pot used.

3.2.4 Inoculation of the primed spikes

The inoculum for artificial inoculation was prepared by diluting 1 ml of *F. graminearum* isolate IFA 65/66 in one litre of filtered water, resulting in a concentration of 20.000 conidia/ml. Tween-20 was added to the prepared inoculum to increase wetting of the wheat spikes. The primed wheat spikes were inoculated one day after the preparation by spraying about 2 ml conidia suspension on the whole head using an atomizer. Subsequently, the colour codes of the inoculated spikes were marked with a number indicating the date of inoculation. Inoculated spikes were covered with plastic bags for 24 hr to assure high humidity for the germination of macroconidia (Fig. 4).

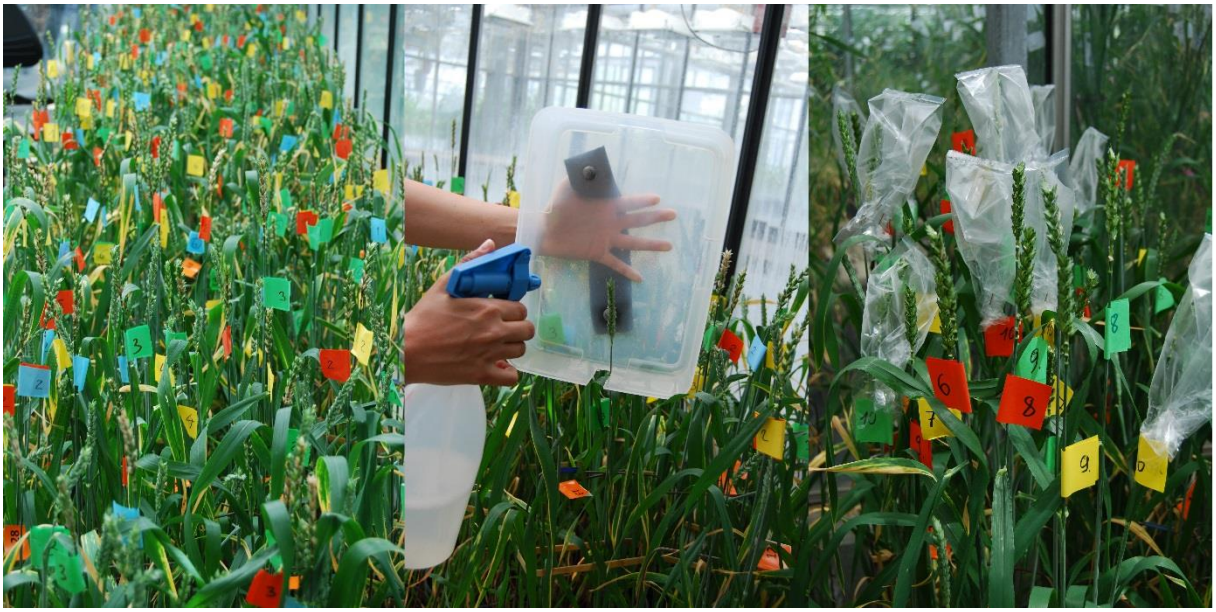

Figure 4: Labelled wheat heads (left), inoculation with an atomizer (middle), inoculated heads covered with polyethylene bags (right).

3.2.5 Disease assessment

Development of FHB on individual spikes was determined at 6, 10, 14, 18 and 22 days after inoculation (dai), abbreviated to S1, S2, S3, S4 and S5 in the following, for disease scoring 1 to 5, and scored on a 0-12 scale. A score of 0 stands for no diseased spikelets, whereas the maximum score of 12 means that all spikelets/spike were diseased. As previously pointed out, the maximum number of spikelets/spike was twelve, given that spikelet number was deliberately reduced to obtain an equal number for all spikes.

3.2.6 Statistical Analysis

Statistical analysis of the greenhouse experiment was also conducted with the statistical package R (R development core team, 2015). Experiment 1, with four pre-treatments and

experiment 2, with three pre-treatments were analysed separately. A linear mixed model was set up with the factors genotype and pre-treatment as fixed effects and the variable ‘head’ as a random effect. The variable ‘head’ represents the individual spikes for every pre-treatment/genotype. To check the assumptions of normality and homogeneity a QQ-plot and a residual plot were created. Analyses of variance (ANOVA) were conducted separately for the parameters S1, S2, S3, S4 and S5.

4 Results

4.1 Evaluation of breeding lines for FHB resistance and anther retention in the field

4.1.1 Winter wheat breeding line panel

A diverse panel of 403 winter wheat breeding lines was planted in two replications, and was assessed for FHB severity and anther retention. ANOVA revealed significant differences ($P < 0.001$) between genotypes for these two traits (Table 4 and Table 5). Heritability-within-a-year was 0.85 for FHB severity and 0.77 for anther retention.

Table 4: Analysis of variance for anther retention of the different genotypes.

Traits	Source	DF	Sum of squares	Mean squares	F value	p value
genotype	genotype	402	15028	37.38	4.7730	< 0.001
residuals	genotype	452	3540.2	7.83		

Table 5: Analysis of variance for FHB severity of the different genotypes.

Traits	Source	DF	Sum of squares	Mean squares	F value	p value
genotype	genotype	402	67952	169.04	8.3828	< 0.001
residuals	genotype	452	9114	20.16		

Most lines manifested a relatively low (6-14%) FHB severity, while some were highly susceptible (Fig. 5).

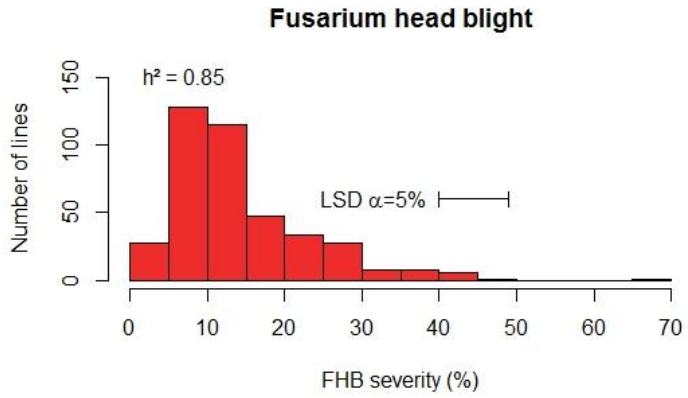

Figure 5: Frequency distribution of Fusarium head blight response, depicted as the mean value for the four FHB ratings for the 403 lines evaluated.

There are very few genotypes with a high anther retention rate, and relatively few with a very low anther retention rate (Fig. 6). The anther retention of most genotypes is at a medium level.

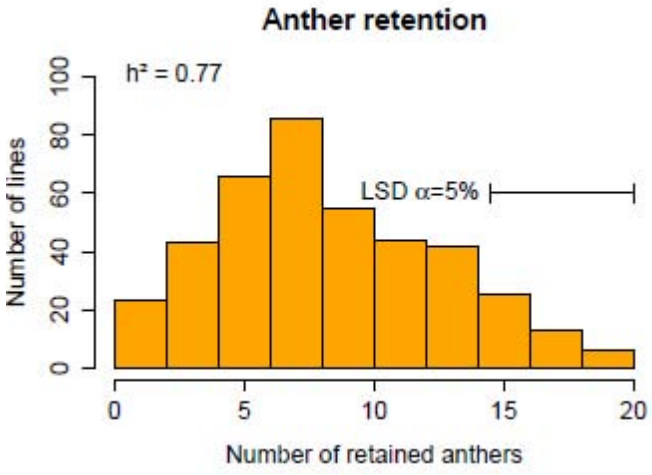

Figure 6: Frequency distribution of retained anthers for the 403 lines evaluated.

FHB severity and AR was low in the 'PrecW' set, while the $F_{4:6}$ lines from the 'YoungEast' set showed a low FHB severity and intermediate AR (Fig. 7). Some lines from the 'YoungWest' set were among the most susceptible ones and the average FHB severity was markedly higher than in the other two sets. Notably all sets showed a considerable range of AR.

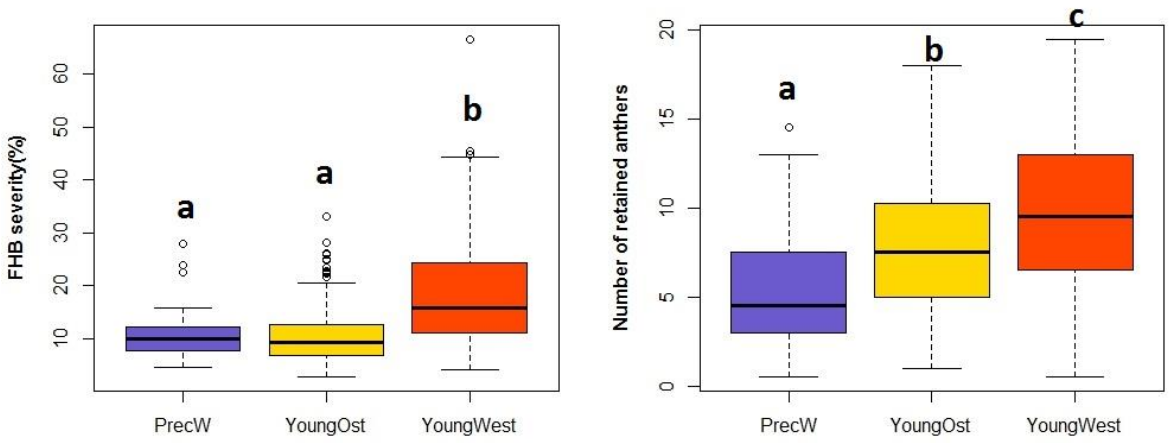

Figure 7: Boxplots for FHB severity (mean value over the four scorings) and anther retention for the three different sets.

FHB severity is positively correlated with AR, with a correlation coefficient of $R^2 = 0.50$ ($P < 0.001$). Genotypes with few retained anthers tend to have low FHB severity. However, with increased level of anther retention the variation in FHB severity increases. Some of the genotypes with high anther retention have high FHB severity, whereas others show low FHB severity.

When the correlation coefficients are calculated separately for each set, differing correlation coefficients can be observed (Table 5). For the set 'PrecW' the correlation between FHB severity and AR is not significant. The correlation is highest for the set 'YoungWest' and lowest for 'YoungOst'.

Table 6: Correlation coefficients among FHB and AR for the different sets.

Set	Correlation coefficient
All sets together	0.50***
PrecW	0.30
YoungEast	0.36***
YoungWest	0.53***

*** significance at $P < 0.001$

4.1.2 'Remus' and its near isogenic line 'C3'

The anther retention characteristics of the two genotypes were assessed under field conditions, as described in the 'Materials & Methods' section. 'Remus' showed significantly more retained or trapped anthers than 'C3' (Fig. 8 and Table 7). Its calculated mean value for anther retention was 11.54, compared to 4.25 for 'C3'.

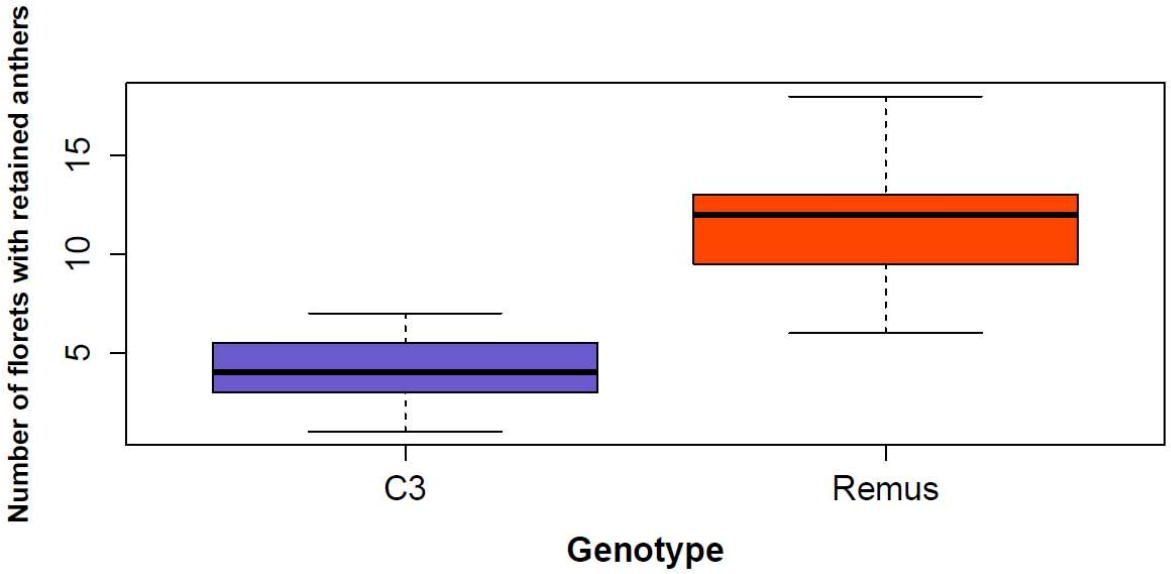

Figure 8: Number of florets with retained anthers (from 5 spikes, 4 florets/spike) for the two genotypes.

Table 7: Analysis of variance for the number of retained anthers of 'Remus' and 'C3'.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	1	729.17	729.17	171.23	< 0.001
residuals	54	229.96	4.26		

The AUDPC for the first five disease scorings was calculated and also this trait showed marked differences between the two genotypes (Fig. 9 and Table 8).

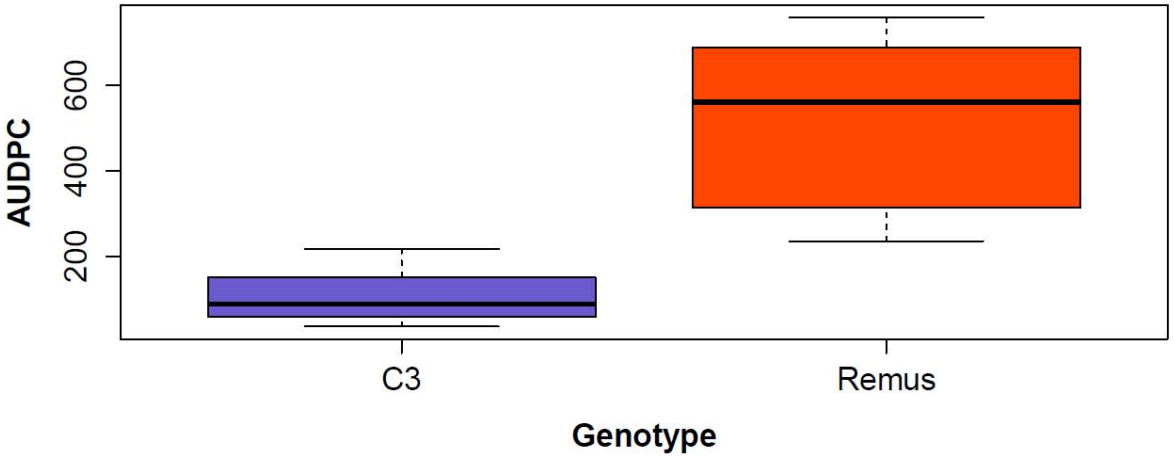

Figure 9: Area under disease progress curve for the first five FHB scorings for the two genotypes.

Table 8: Analysis of variance for the AUDPC of 'Remus' and 'C3'.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	1	2189568	2189568	131.57	< 0.001
residuals	54	898661	16642		

Disease incidence was calculated by counting the number of heads out of 40 that showed disease symptoms. The difference for the disease incidence between the two genotypes, expressed as the percentage of symptomatic heads, gave the same picture as the AUDPC with 'C3' showing a significant lower disease incidence than 'Remus' (Fig. 10 and Table 9).

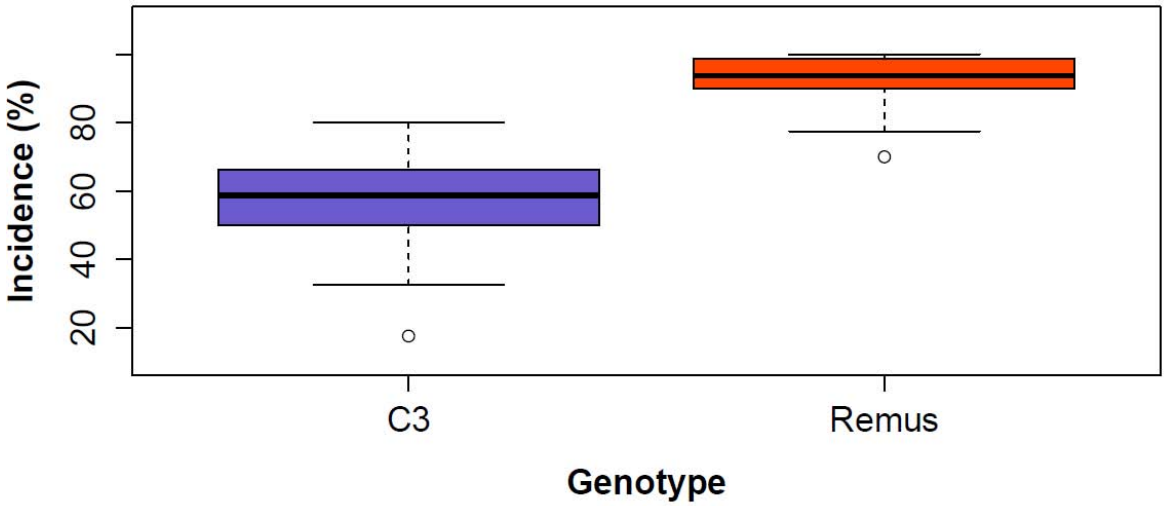

Figure 10: Disease incidence for the two genotypes, expressed as the percentage of symptomatic heads out of 40.

Table 9: Analysis of variance for the FHB incidence of 'Remus' and 'C3'.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	1	16675	16675.2	136.99	< 0.001
residuals	54	6573	121.7		

All in all, 'Remus' and the NIL possessing the FHB resistance QTL *Qfhs.ifa-5A* differ significantly for AR, FHB severity and FHB incidence.

4.2 Analysis of the relationship of *Qfhs.ifa-5A* and anther retention

The susceptible 'Remus' and the moderately resistant NIL of Remus, possessing *Qfhs.ifa-5A,* were cultivated in the glasshouse to investigate the association between AR and the FHB resistance QTL *Qfhs.ifa-5A*. The four types of pre-treatments, which consisted in removing or compressing the anthers inside the florets, plus the controls, were applied to the genotypes and FHB development was scored after spray inoculation. Analysis of variance was performed in order to identify significant differences for FHB resistance between the pre-treatments and genotypes.

4.2.1 Experiment 1

In experiment 1, ANOVA revealed high statistical significance ($P < 0.001$) for the factors genotype and pre-treatment for all five FHB scorings (see the appendix for a detailed ANOVA output). The same significant difference was observed for the genotype by pre-treatment interaction at the time points S1 and S2, but the significance level was considerably lower for S3 ($P < 0.01$), as well as for S4 and S5 ($P < 0.05$).

In table 10 below, means for 'Remus' and 'C3' 10 dai are depicted for each pre-treatment (detailed results for all other disease scorings of experiment 1 are presented in the appendix).

Table 10: Genotype by pre-treatment means for the number of diseased spikelets in experiment 1, for the disease scoring S2, 10 days after inoculation.

	Pre-treatment			
Genotype	B	G	R	Y
C3	$4.3^{A\ a}$	$1.7^{B\ b}$	$3.8^{A\ c}$	$0.9^{B\ d}$
Remus	$7^{A\ e}$	$4.6^{B\ ac}$	$5.4^{B\ f}$	$1.4^{C\ bd}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

The plants without manipulation of pre-treatment 'G' are supposed to show the same disease incidence as the plants from the control 'B', where only the middle floret has been removed. Contrary to expectations, however, pre-treatment 'G' and 'B' differed significantly from each other, for both genotypes. Surprisingly, pre-treatment 'B' resulted in a higher disease incidence.

In experiment 1, the effect of pre-treatment 'R' was already visible (Fig. 11). Wheat spikes with removed anthers were significantly less diseased than wheat spikes with their anthers retained inside the floret.

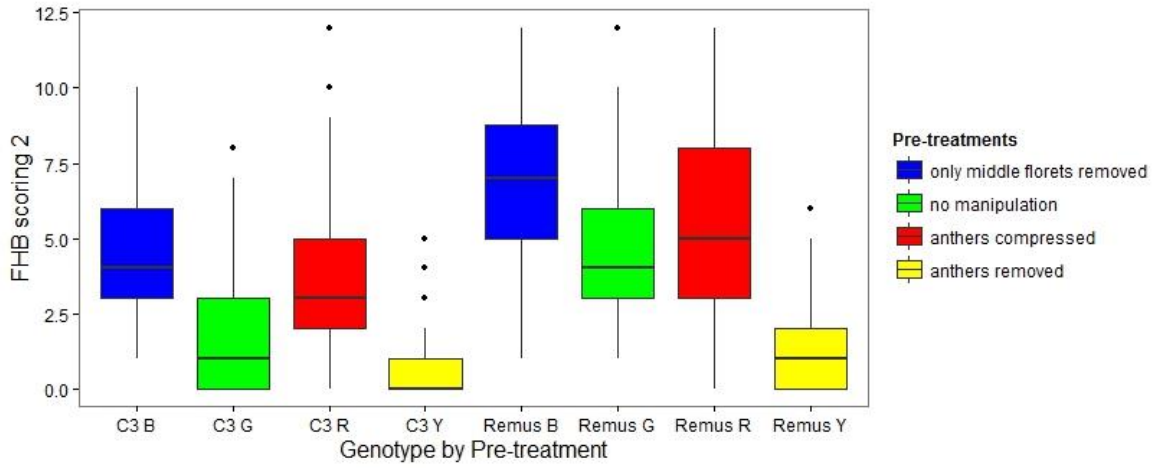

Figure 11: FHB severity in experiment 1, 10 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.

4.2.2 Experiment 2

This study aims to investigate the role of anthers by applying different pre-treatments and the results should not be biased by any effect resulting from the manipulation of the florets. Taking into account the unwanted and statistically proven effect of pre-treatment 'B', a second experiment, named experiment 2, was conducted. As mentioned in the 'Materials & Methods' section, in experiment 2 I desisted from removing the middle florets and only applied three pre-treatments, 'G', 'R' and 'Y'.

The boxplots below (Fig. 12 to Fig. 16) visualize the distribution of the FHB scorings S1 to S5, for the three pre-treatments and the two genotypes.

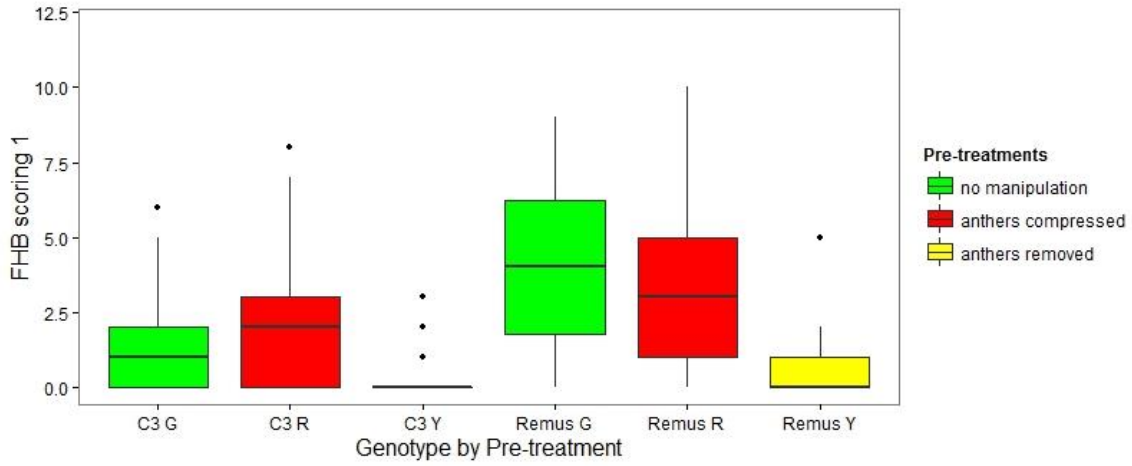

Figure 12: FHB severity in experiment 2, 6 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.

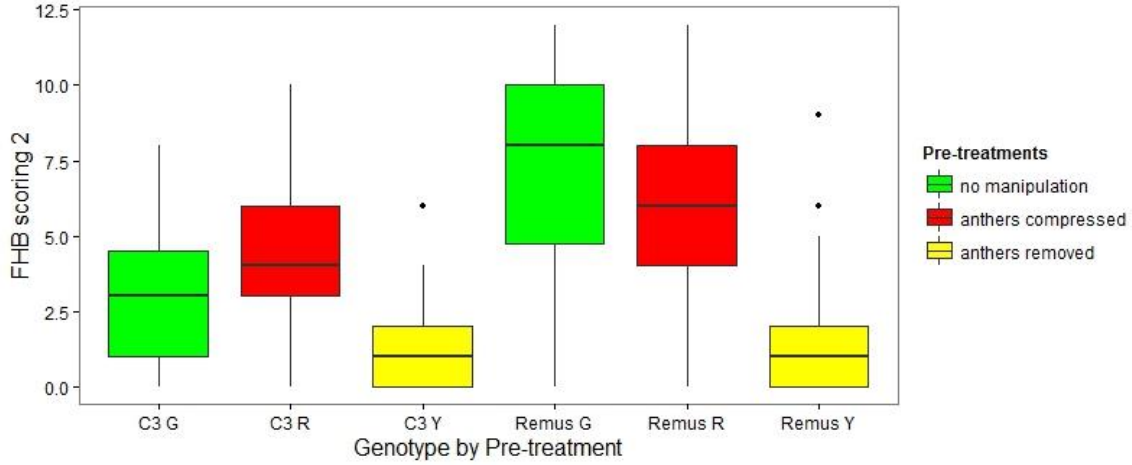

Figure 13: FHB severity in experiment 2, 10 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.

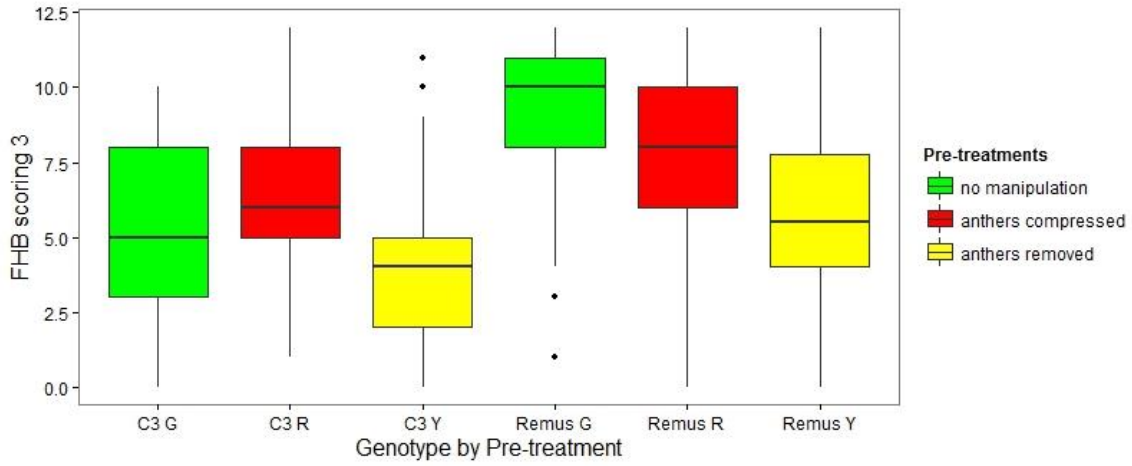

Figure 14: FHB severity in experiment 2, 14 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.

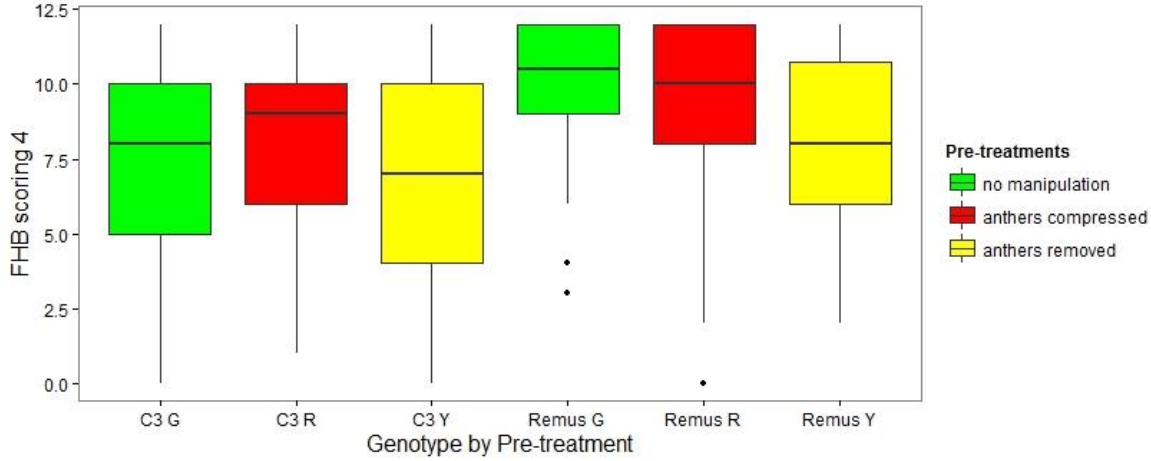

Figure 15: FHB severity in experiment 2, 18 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.

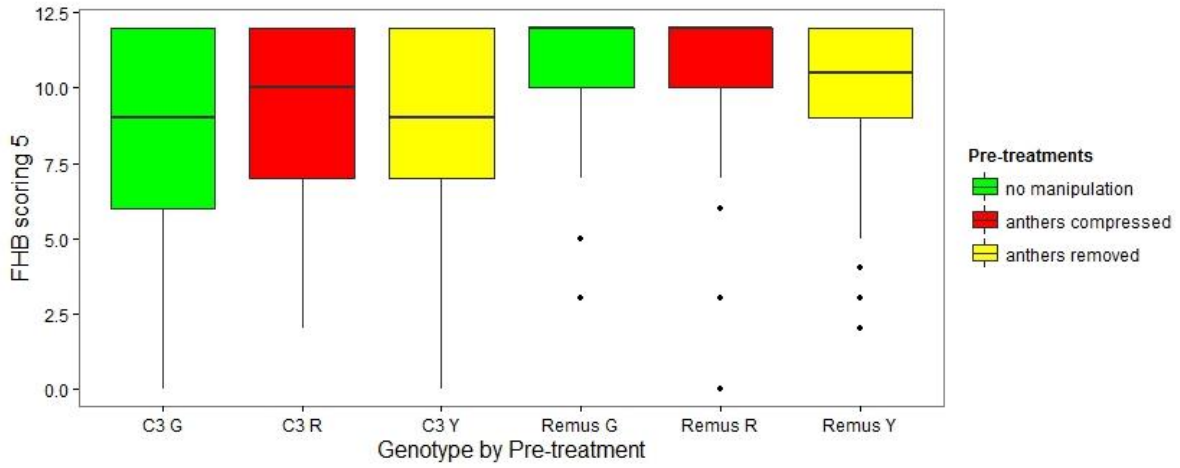

Figure 16: FHB severity in experiment 2, 22 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.

The figures indicate a declining difference between the genotypes and pre-treatments at later scoring dates. For S5 (22 dai), the observed difference in FHB severity is negligible. For the factor genotype, statistical analysis did not reveal any significant difference between the pre-treatments for S5 ($P < 0.001$).

When I looked at the genotype by pre-treatment interaction for S5, there was only a significant difference between 'C3 G' and 'Remus G'. For pre-treatments 'R' and 'Y', no significant difference was detected among genotypes. As stated in the beginning of this section, this result was expected, bearing in mind that anther retention affects type 1 resistance, but cannot impede the spread of the fungus. To analyse the role of anthers in FHB infection, one should look at the beginning of infection. I considered S2 an appropriate scoring date to show the influence of the anthers on the resistance to initial infection.

Genotype by pre-treatment means for S2 in experiment 2 and their statistical significance are illustrated in table 7. For S2, a significant difference was found between 'C3 G' and 'C3 R'. Pre-Treatment 'R', with the anthers placed inside, caused a higher disease severity in genotype 'C3'. In 'Remus', however, the opposite was the case. Pre-treatment 'G' resulted in a higher disease severity than pre-treatment 'R'.

Removing the anthers in pre-treatment 'Y' provoked a significantly lower disease severity in both genotypes. 'C3 Y' resulted in a 58.6% reduction of diseased spikelets/spike when compared to 'C3 G'. 'Remus Y' resulted in a 73.5% reduction of diseased spikelets/spike when compared to 'Remus G'. This shows that the effect of removing the anthers was considerably larger in 'Remus'.

Table 11: Genotype by pre-treatment means in experiment 2 for the disease scoring S2, 10 days after inoculation.

	Pre-treatment		
Genotype	G	R	Y
C3	$2.9^{A\,a}$	$4.3^{B\,b}$	$1.2^{C\,c}$
Remus	$6.9^{A\,d}$	$5.7^{B\,e}$	$1.8^{C\,c}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

When it comes to the genotype by pre-treatment interaction, the differences/similarities between 'C3 Y' and 'Remus Y', and between 'C3 R' and 'Remus R' are highlighted in table 11.

It is evident that there has to be a significant difference between 'C3 G' and 'Remus G'. Without manipulation, 'Remus' is more susceptible than 'C3'. However, when both genotypes have their anthers removed, no significant difference can be detected between 'Remus' and 'C3' for S2. This result clearly shows that infection does occur without the presence of anthers, but there are considerably less infection sites.

When both genotypes have their anthers placed inside in pre-treatment 'R', 'Remus' is still slightly more susceptible, with on average 5.7 diseased spikelets/spike, than 'C3' with 4.3 diseased spikelets/spike. This indicates that resistance factors other than anthers may play a role in the infection process.

In addition to the genotype by pre-treatment means for the disease scoring S2, 10 days after inoculation depicted in table 7 above, genotype by pre-treatment means for all other disease scorings are presented in the following tables (Table 12 to Table 15).

Table 12: Genotype by pre-treatment means in experiment 2 for the disease scoring S1, 6 dai.

	Pre-treatment		
Genotype	G	R	Y
C3	$1.5^{A\,a}$	$1.9^{A\,b}$	$0.2^{B\,c}$
Remus	$4.0^{A\,d}$	$3.2^{A\,e}$	$0.4^{B\,c}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

Table 13: Genotype by pre-treatment means in experiment 2 for the disease scoring S3, 14 dai.

	Pre-treatment		
Genotype	G	R	Y
C3	$5.2^{A\,a}$	$6.4^{A\,b}$	$3.9^{B\,c}$
Remus	$9.1^{A\,d}$	$7.9^{A\,e}$	$5.6^{B\,ab}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

Table 14: Genotype by pre-treatment means in experiment 2 for the disease scoring S4, 18 dai.

	Pre-treatment		
Genotype	G	R	Y
C3	$7.4^{AB\,a}$	$8.3^{A\,b}$	$6.6^{B\,c}$
Remus	$10.0^{A\,d}$	$9.5^{A\,b}$	$8.1^{B\,ab}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

Table 15: Genotype by pre-treatment means in experiment 2 for the disease scoring S5, 22 dai.

	Pre-treatment		
Genotype	G	R	Y
C3	$8.4^{A\,a}$	$9.4^{A\,b}$	$8.5^{A\,c}$
Remus	$10.9^{A\,d}$	$10.5^{A\,b}$	$9.7^{A\,bc}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

5 Discussion

5.1 FHB resistance and anther retention

The role of anthers in FHB infection has already been mentioned in the beginning of the last century by Pugh et al. (1933). Retained anthers were found to promote kernel infection and histological studies of inoculated spikelets identified retained anthers as the first points of infection. More recent studies confirmed that the presence of anthers can increase disease severity (Kang and Buchenauer 2000; Miller et al. 2004). The enhanced hyphal growth on anthers might be attributed to growth promoting components in anthers, which have been identified as choline and betaine (Strange et al. 1974). Lately, there has been a growing interest in the influence of anthers on FHB infection. Several authors assessed anther retention in field trials and evaluated its correlation with FHB severity (Buerstmayr and Buerstmayr 2015; He et al. 2014; Lu et al. 2013; Skinnes et al. 2010).

In this study, a total of 403 wheat breeding lines were assessed for anther retention (AR) in a field trial in 2015 by inspecting 20 florets per genotype. The wheat lines were also evaluated for FHB severity on four scoring dates, 14, 18, 22 and 26 days after spray inoculation. A significant variation for AR as well as for FHB severity was observed between the lines. The within-year-heritability of anther retention was quite high ($H^2 = 0.85$). A significant positive correlation was found between AR and FHB severity ($r = 0.50$, $P < 0.0001$), which means that lines with few retained anthers are less diseased. This finding is in good agreement with the results of other studies.

Skinnes et al. (2008) found a correlation of $r = 0.40$ ($P < 0.0001$) between AE and FHB resistance for the evaluated winter wheat varieties. He et al. (2014) characterized Chinese bread wheat lines for FHB resistance and identified a correlation of $r = -0.43$ ($P < 0.001$) between AE and FHB severity. Similar correlation coefficients ($r = -0.45$ to -0.64, $P < 0.0001$) were reported by Lu et al. (2013), who evaluated a RIL population from the cross of Shanghai-3/Catbird and the German spring wheat cultivar Naxos. A considerable weaker correlation ($r = 26$, $P < 0.05$) was found by Graham and Browne (2009), who scored FHB by natural infection in their experiment.

The fan-shaped distribution pattern between AE and FHB that has been reported by some authors (Skinnes et al. 2008; He et al. 2014) also holds true for this study. Lines with few retained anthers show less variation in FHB severity than lines with many retained anthers. Lines with high anther retention may have a low or a high degree of resistance, whereas lines with low anther retention consistently showed relatively low FHB severity. Thus, when selecting against lines with high anther retention some lines with good resistance level might be lost.

However, selecting for lines with high anther extrusion seems to select lines with a good level of resistance.

When looking separately at the different sets, lines from the 'PrecW' set showed significantly less anther retention than lines from 'YoungEast' ($P < 0.0001$) and lines from 'YoungWest' ($P < 0.05$). In addition, the FHB severity of 'PrecW' was significantly lower ($P < 0.0001$) than the FHB severity of 'YoungWest' lines, but it did not differ significantly from 'YoungOst'. The lower anther retention of the F_7 lines (PrecW) may be attributed to the selection against FHB in the breeding program. Selection for low FHB severity appears to select lines with low AR.

It is to note, however, that the correlation between AR and FHB for 'PrecW' set is not significant. It appears that the pre-commercial breeding lines possess FHB resistance QTL that are not associated with AR, such as type 2 resistance QTL. Type 2 resistance QTL lower the spread of the fungus, but do not influence resistance to initial infection.

In addition to the 403 genotypes evaluated in the field, two lines with similar genetic background, 'Remus' and 'C3' were assessed for AR, FHB severity and FHB incidence. 'C3' exhibits a 98.5% 'Remus' background and differs in the possession of the FHB resistance QTL *Qfhs.ifa-5A.* 'C3' showed a much higher degree of AE in the field evaluation. It was less diseased than the susceptible cultivar 'Remus' without the resistance QTL. This observation suggests that *Qfhs.ifa-5A* might act as a passive resistance mechanism controlling anther extrusion. To gain further evidence for this assumption, a glasshouse experiment, which will be discussed in the following subsection, was conducted.

5.2 Relationship of anther retention and *Qfhs.ifa-5A*

In the glasshouse experiment, the role of anthers in FHB resistance was studied by manipulating the florets of two wheat lines differing in the possession of the FHB resistance QTL *Qfhs.ifa-5A*. The wheat plants in the experiment belonged to two genotypes, the susceptible 'Remus' and the near isogenic line 'Remus_NIL3' (C3), which carries the *Qfhs.ifa.-5A* QTL, conferring type 1 resistance.

Two experiments were conducted. In experiment 1 the manipulation included four pre-treatments. Wheat heads were emasculated in pre-treatment 'Y' by removing the anthers with a forceps, anthers were compressed between palea and lemma in pre-treatment 'R', only the middle florets were removed in pre-treatment 'B', and no manipulation was done in pre-treatment 'G'. In experiment 2 only three pre-treatments were applied, namely 'R', 'Y' and 'G', and middle florets were not removed in neither of them.

Anther retention is a morphological trait that influences the rate of initial infection, called type 1 resistance and thus, earlier disease scorings are more appropriate to investigate the

role of AR in FHB resistance. Disease symptoms at later scoring dates are, to a great extent, provoked by the spread of the pathogen, reflecting type 2 resistance instead of initial infection. For this reason, I considered the disease scoring S2, i.e. 10 dai, as the most appropriate parameter to evaluate the influence of AR on FHB type 1 resistance. The disease scoring S1, i.e. 6 dai, would be too early, since in some spikes the first disease symptoms were not observed until S2.

Contrary to expectations, pre-treatment ‘G’ and ‘B’ differed significantly from each other for both genotypes. Surprisingly, pre-treatment ‘B’ resulted in a higher disease incidence. The reason for this could be that more inoculum adhered to the spikes of pre-treatment ‘B’, owing to the cavity that had been produced by removing the middle florets. This means, that by removing the smaller middle florets for simplifying the manipulation of the anthers, plants were made more susceptible to FHB. To avoid the undesirable bias produced by removing the middle florets, experiment 2, with only three pre-treatments, was included in this study.

In experiment 1 the effect of the presence or absence of anthers was already visible. Plants with removed anthers tended to be less diseased than plants with the anthers compressed inside the florets. In the following, the results of experiment 2 will be discussed.

As expected from the evaluation in the field, ‘Remus G’, the control without manipulation, was significantly more diseased than ‘C3 G’. It showed 6.8 symptomatic spikelets/spike compared to 2.9. Since this result confirmed the assumption from the field trial, the single head inoculation in the glasshouse was continued.

Pre-treatment ‘Y’ resulted in a significant reduction of FHB severity in both genotypes, when evaluated 10 days after inoculation. Wheat plants that had their anthers removed consistently showed lower FHB severity. ‘Remus Y’ showed a mean FHB score of 1.8 compared to 6.8 in the control treatment ‘G’ without manipulation. The mean value for ‘C3 Y’ was 1.2 compared to 2.9 in the control treatment. The effect of removing the anthers was considerably higher in the susceptible ‘Remus’.

FHB severity of ‘Remus Y’ and ‘C3 Y’ did not differ significantly 10 days after inoculation. When anthers were removed, both genotypes showed a similar resistance response. This means that by removing the anthers, the susceptible ‘Remus’ showed the same level of resistance to initial infection as the resistant ‘C3’. Since removing the anthers mimics the natural situation of complete AE, this result provides further evidence that AE plays a vital role in type 1 resistance. By removing the anthers it was possible to gain the same level of resistance for both genotypes, the susceptible ‘Remus’ and the resistant ‘C3’.

At later disease scorings, however, ‘Remus Y’ was more susceptible than ‘C3 Y’, but still significantly more resistant than ‘Remus G’ and ‘Remus R’. Later disease scoring do not only

measure disease incidence, but also spread of the symptoms. The resistance allele of 'C3', *Qfhs.ifa-5A*, confers type 1 and to a lesser extent type 2 resistance (Buerstmayr et al. 2003), resulting in a reduced spread of the fungus.

FHB severity of 'C3' in pre-treatment 'R' was significantly higher than in pre-treatment 'G', when evaluated 10 days after inoculation. When anthers were removed and subsequently compressed inside the floret, so that they could not be extruded, the FHB score for 'C3' was 4.3, compared to 2.9 for the plants without manipulation. This might indicate that the *Qfhs.ifa-5A* resistance allele of 'C3' confers higher AE. If this was definitely not the case, the resistance QTL should also lower initial infection in pre-treatment 'R'. When 'C3' was assessed for AR in the field, on average only 4.25 out of 20 florets showed retained or trapped anthers. This is a very small number compared to 'Remus', which showed 11.54 florets with retained anthers. However, no QTL study so far could associate the resistance QTL *Qfhs.ifa-5A* with AE.

At present, no QTL for AE have been identified on chromosome 5A that overlapped with the resistance QTL. The only QTL analysis that detected a QTL for AE on 5AS was done by Buerstmayr and Buerstmayr (2015). However, this QTL did not coincide with a QTL for FHB severity. The identification of several different major and minor QTL associated with AE in various mapping studies (Buerstmayr and Buerstmayr 2015; Skinnes et al. 2010; Lu et al. 2013), as well as the continuous distribution of the trait, confirm that several genes are involved in the inheritance of AE. Further research is needed to identify QTL for AE that are associated with FHB resistance and to determine why the QTL for AE co-localize with FHB resistance QTL. It would also be interesting to investigate the factors that influence the extent of AE, such as filament length, swelling of lodicules and together with this, opening width and duration.

'Remus' did not show the same response to pre-treatment 'R' as 'C3'. FHB severity differed significantly between pre-treatment 'R' and 'G', and surprisingly, the compression of the anthers in the florets resulted in lower FHB severity than the un-manipulated pre-treatment 'G'. A possible explanation for this can be found when looking at the anther retention characteristics of 'Remus'. When AR of 'Remus' was assessed under natural field conditions, its mean anther retention score was 11.54, meaning 11.5 florets with retained anthers out of 20 inspected florets.

Kubo et al. (2013) showed in pot and field experiments that plants with no AE (closed flowering) exhibited a significantly lower FHB score than plants with partially extruded anthers. This could be the reason why 'Remus' plants of pre-treatment 'R' with no AE had a slightly, but significantly lower FHB score than 'Remus' plants of pre-treatment 'G'. 'Remus R' showed 5.7 diseased spikelets/spike compared to 6.8 of 'Remus G'. In pre-treatment 'G', without

manipulation, ‘Remus’ manifested its natural anther retention characteristics, with a relatively high amount of partially extruded anthers. As demonstrated by Kubo et al. (2013), plants with partially extruded anthers are more susceptible to initial infection than plants with no anther extrusion.

A significant genotype by treatment interaction was found in pre-treatment ‘R’. When anthers were compressed inside, ‘Remus’ was more susceptible than ‘C3’, with 5.7 and 4.3 diseased spikelets/spike respectively. This indicates that *Qfhs.ifa-5A* is possibly not only one gene controlling AE and FHB resistance. When there was no AE in pre-treatment ‘R’, ‘C3’ was still more resistant than ‘Remus’. This suggests an additional resistance gene in the QTL interval, which is not associated with AR.

It should be remarked that AR only has an influence on initial FHB infection. For S3 (14 dai), no significant differences were observed between pre-treatment ‘R’ and ‘G’, as well as ‘Y’ and ‘G’, for both genotypes, except for ‘Remus Y’. ‘Remus Y’ was significantly less diseased than ‘Remus G’, until S5, 22 days after inoculation.

Finally, a number of limitations of this study need to be considered. First, in the field trial only one year of field evaluation was done. Nevertheless, two replications were included. Second, DON accumulation was not analysed in the field trial. The correlation analysis between AR and FHB only included FHB severity. However, FHB severity was evaluated on four scoring dates, which is quite a lot when compared to other studies that only performed one or two FHB scorings.

6 Conclusions and Outlook

In conclusion, this work supports the view of numerous studies that AE lowers initial FHB infection. This suggests that selection for high AE in breeding programmes can enhance type 1 resistance. Anther extrusion is a highly heritable morphological trait and therefore, including it in breeding programmes is a promising approach to develop resistant cultivars. However, to obtain a satisfactory level of resistance under high disease pressure, type 1 resistance should be combined with type 2 resistance. High resistance against initial infection without resistance against spread of the fungus cannot prevent FHB.

Furthermore, the results suggest that the major resistance QTL *Qfhs.ifa-5A* is a passive resistance mechanism controlling AE and FHB resistance. Also, additional resistance genes seem to be present in the QTL interval.

For future research, an interesting issue would be to identify more QTL for AE associated with FHB resistance. Also, the encouraging results from the glasshouse experiment need to be validated in a broader range of genotypes and in an additional experiment.

The present work investigated the role of AE in FHB resistance in hexaploid wheat. Anther retention is correlated with FHB severity, thus wheat lines with few retained anthers tend to show less FHB severity by enhancing the resistance to initial infection. By emasculating the wheat plants in the glasshouse, it was shown that FHB infection is possible without the presence of anthers, notwithstanding it is significantly lower.

The results from this study are encouraging and I am confident that including the evaluation of anther retention in breeding programs will help to select lines with high FHB resistance.

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8 Appendix

8.1 ANOVA outputs for experiment 1 and experiment 2

ANOVA output for disease scoring S1, in experiment 1

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	404.98	126.75	126.75	63.31	< 0.001
treatment	406.60	240.07	80.02	39.97	< 0.001
genotype:treatment	406.89	65.34	21.78	10.88	< 0.001

ANOVA output for disease scoring S2, in experiment 1

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	408.27	434.99	434.99	96.52	< 0.001
treatment	409.81	1358.72	452.91	100.50	< 0.001
genotype:treatment	410.10	115.60	38.53	8.55	< 0.001

ANOVA output for disease scoring S3, in experiment 1

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	409.21	797.06	797.06	127.43	< 0.001
treatment	410.68	1335.71	445.24	71.18	< 0.001
genotype:treatment	411.53	86.07	28.69	4.59	< 0.01

ANOVA output for disease scoring S4, in experiment 1

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	409.21	775.91	775.91	106.94	< 0.001
treatment	410.69	1143.39	381.13	52.53	< 0.001
genotype:treatment	411.54	63.70	21.23	2.93	< 0.05

ANOVA output for disease scoring S5, in experiment 1

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	405.03	643.55	643.55	83.82	< 0.001
treatment	406.54	1121.22	373.74	48.68	< 0.001
genotype:treatment	407.41	62.18	20.73	2.70	< 0.05

ANOVA output for disease scoring S1, in experiment 2

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	349.19	189.72	189.72	57.50	< 0.001
treatment	342.22	502.09	251.04	76.09	< 0.001
genotype:treatment	341.66	101.78	50.89	15.42	< 0.001

ANOVA output for disease scoring S2, in experiment 2

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	347.15	401.13	401.13	78.72	< 0.001
treatment	340.02	1106.83	553.41	108.61	< 0.001
genotype:treatment	339.44	201.92	100.96	19.81	< 0.001

ANOVA output for disease scoring S3, in experiment 2

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	405	589.28	589.28	76.75	< 0.001
treatment	405	526.87	263.43	34.31	< 0.001
genotype:treatment	405	122.07	61.04	7.95	< 0.001

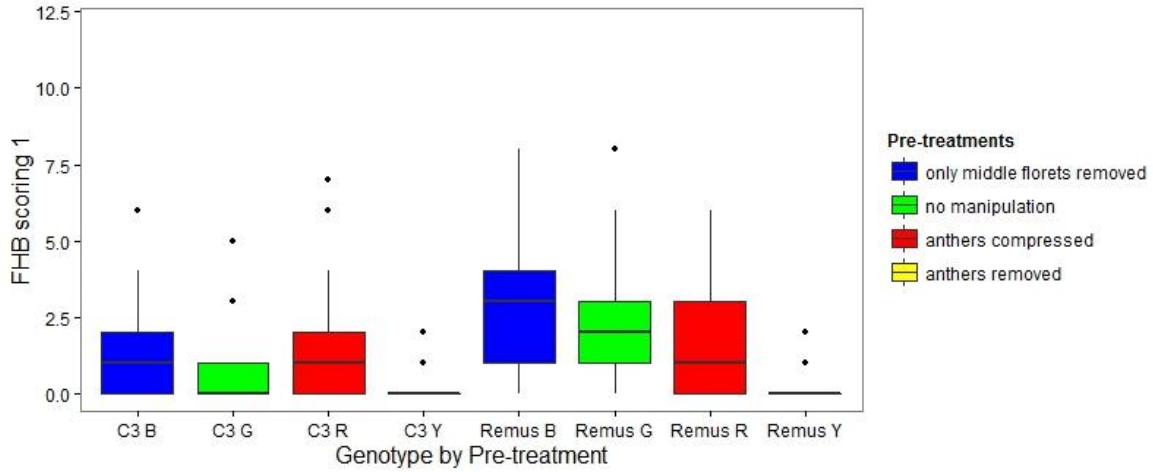
ANOVA output for disease scoring S4, in experiment 2

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	405	323.52	323.52	36.21	< 0.001
treatment	405	205.87	102.93	11.52	< 0.001
genotype:treatment	405	37.67	18.83	2.11	> 0.1

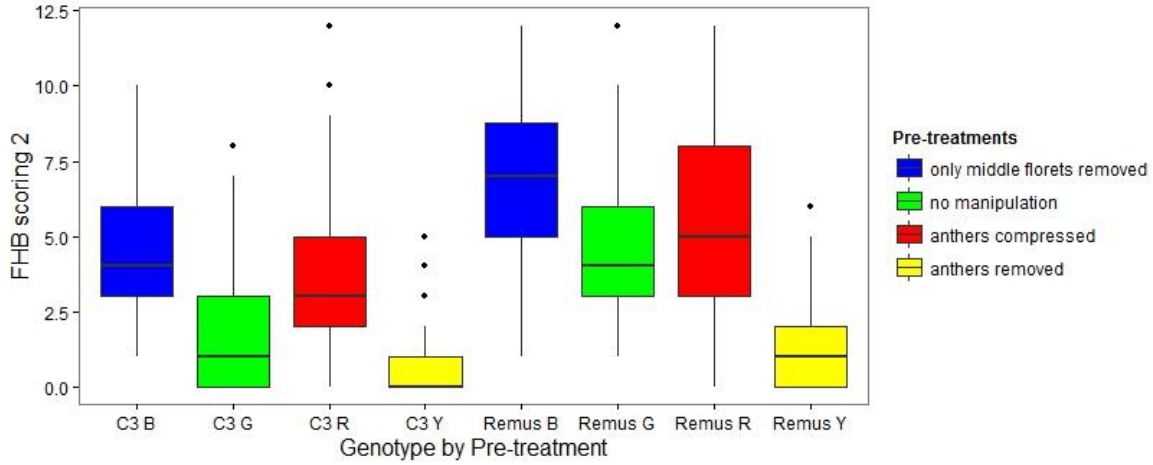
ANOVA output for disease scoring S5, in experiment 2

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value	p value
genotype	403	255.34	255.34	30.28	< 0.001
treatment	403	49.67	24.84	2.95	> 0.1
genotype:treatment	403	42.43	21.21	2.52	> 0.1

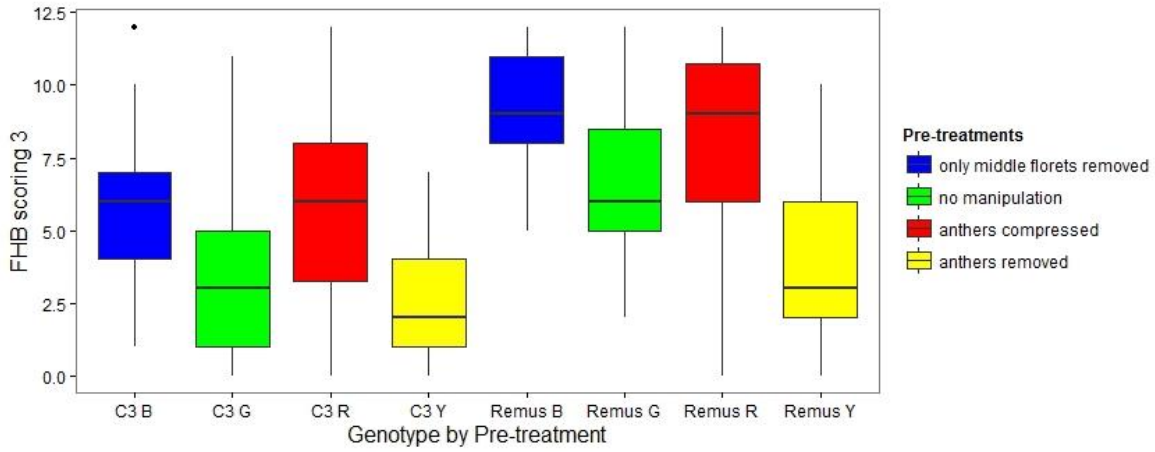
8.2 Boxplots for experiment 1



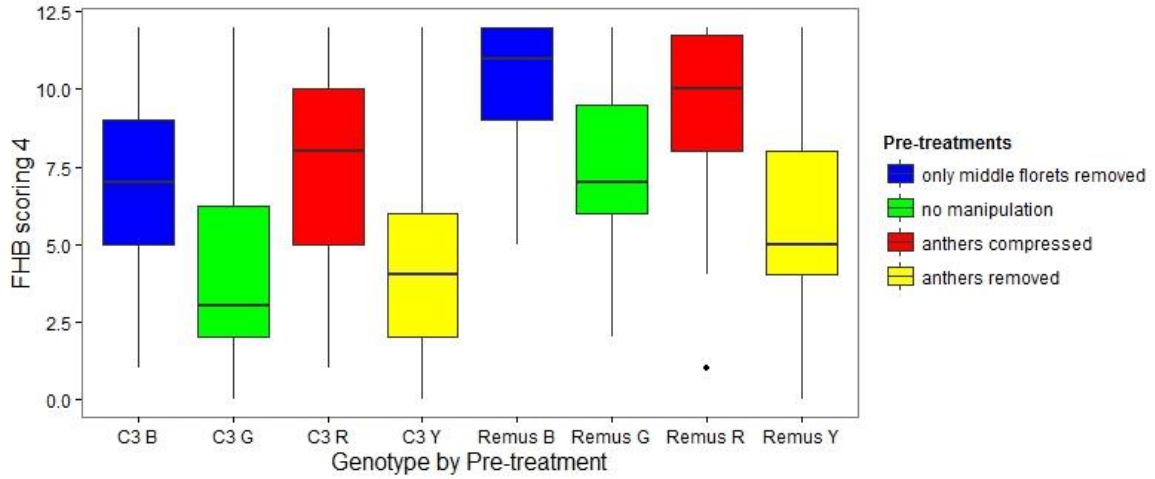
FHB severity in experiment 1, 6 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.



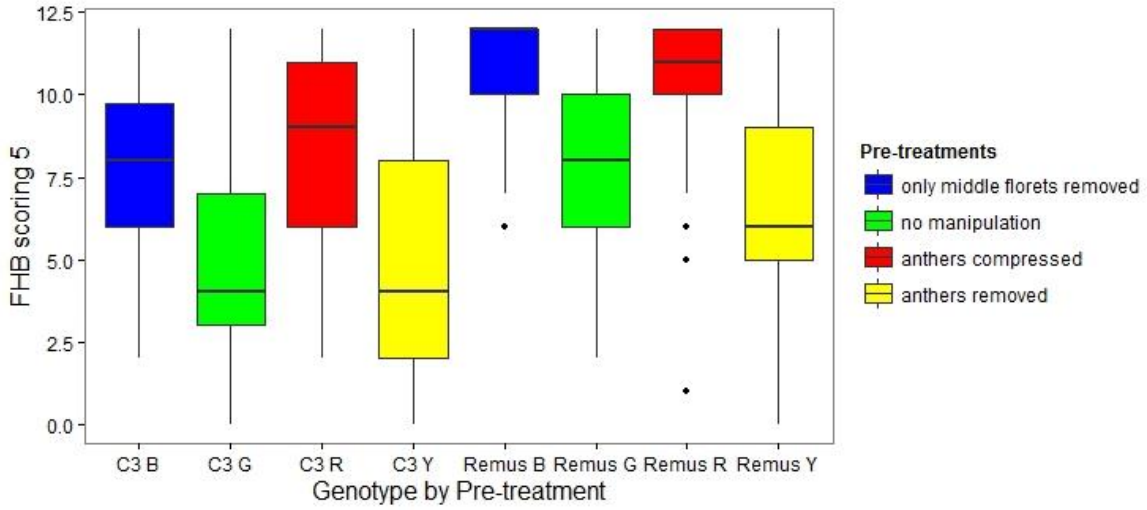
FHB severity in experiment 1, 10 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.



FHB severity in experiment 1, 14 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.



FHB severity in experiment 1, 18 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.



FHB severity in experiment 1, 22 dai for the two genotypes and the different pre-treatments, FHB severity is expressed in number of symptomatic spikelets/spike.

8.3 Genotype by pre-treatment means and significant differences for experiment 1

Genotype by pre-treatment means for the number of diseased spikelets in experiment 1, for the disease scoring S1, 6 dai.

	Pre-treatment			
Genotype	B	G	R	Y
C3	$1.3^{A\,a}$	$0.4^{B\,b}$	$1.2^{A\,c}$	$0.1^{B\,d}$
Remus	$3.0^{A\,e}$	$2.3^{AB\,f}$	$1.7^{B\,ac}$	$0.2^{C\,bd}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

Genotype by pre-treatment means for the number of diseased spikelets in experiment 1, for the disease scoring S3, 14 dai.

	Pre-treatment			
Genotype	B	G	R	Y
C3	$5.7^{A\,a}$	$3.3^{B\,b}$	$6.0^{A\,c}$	$2.6^{B\,d}$
Remus	$9.1^{A\,e}$	$6.6^{B\,ac}$	$8.2^{A\,f}$	$3.9^{C\,b}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

Genotype by pre-treatment means for the number of diseased spikelets in experiment 1, for the disease scoring S4, 18 dai.

	Pre-treatment			
Genotype	B	G	R	Y
C3	$6.8^{A\,a}$	$4.4^{B\,b}$	$7.4^{A\,c}$	$4.1^{B\,d}$
Remus	$10.0^{A\,e}$	$7.6^{B\,ac}$	$9.5^{A\,f}$	$5.6^{C\,ab}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

Genotype by pre-treatment means for the number of diseased spikelets in experiment 1, for the disease scoring S5, 22 dai.

	Pre-treatment			
Genotype	B	G	R	Y
C3	$7.7^{A\,a}$	$5.1^{B\,b}$	$8.4^{A\,c}$	$5.1^{B\,d}$
Remus	$10.7^{A\,e}$	$8.2^{B\,ac}$	$10.2^{A\,f}$	$6.5^{C\,a}$

Capital letters indicate means in a row that are not significantly different from each other, small letters indicate means in a column that are not significantly different from each other ($\propto$=1%).

8.4 R script for the evaluation of breeding lines for FHB resistance and anther retention in the field

```
#Traits: AR (Anther retention),HD (Anthesis date),WUH (Plant
height),B1-B4 (Fusarium)

setwd("C:/Users/andrea/Desktop/Sebastian/GENIE Fusarium Tulln 2015")
library(lme4)
library(lmerTest)
#library(lsmeans)

#>>>import the data as from the csv file
DATA <- read.table("Genie.csv",header=T,sep=";",dec=",")
DATA$REP <- as.factor(DATA$REP)
DATA$SET <- as.factor(DATA$SET)
DATA$GEN <- as.factor(DATA$GEN)
str(DATA)
#####################################################################
#>>>fit a linear model for anther retention

#Treatment: GEN
#Design:    REP/SET/Plot = REP + REP:SET +  error

#--->fit an effect SET to disciminate between the PRECW, YOUNG West
and East lines

model <- lmer(AR~1+ (1|REP) + (1|REP:SET) + (1|GEN),DATA)

#>>>QQ-Plot and a residual plot to check the model assumptions of
#   normality and homogeneity of variance of the residuals
qqnorm(resid(model))
plot(model,form=resid(.)~fitted(.),xlab="Fitted
value",ylab="Studendized residuals")

#-->looks quite good

#>>>print and extract the variance components
print(VarCorr(model),comp=c("Variance","Std.Dev."),digits=6)
VC.COMPS <- as.data.frame(VarCorr(model))
VC.COMPS

#>>>compute the heritability based on the classical formula
#h² = vg / (vg + ve/r)
h2 <- VC.COMPS[1,4]/(VC.COMPS[1,4] + VC.COMPS[4,4]/2)
```

```
h2

#>>>compute the LS-MEANS or BLUES
modelF <- lm(AR~GEN + REP + REP:SET,DATA)
anova(modelF)
lsmeans.list <- lsmeans(modelF,test.effs="GEN")
LSMEANS <- lsmeans.list$lsmeans.table

#write.table(LSMEANS,"LSMEANS.AR.txt",quote=TRUE,col.names=TRUE,row.
names=FALSE)
LSMEANS.AR <- read.table("LSMEANS.AR.txt",header=T,sep="",dec=".")

#>>>plot the data in a histogram
hist(LSMEANS.AR$Estimate,xlab="Number of retained
anthers",col="orange1",ylab="Number of lines",ylim=c(0,100),
     main="Anther retention")
text(x=0,y=100,pos=4,expression("h² = 0.77"))

LSD <- sqrt(2*7.824639/2)*1.96
LSD

arrows(14.5,60,20,60,length=0.08,code=3,angle=90) #x,y Beginn  ;x,y
Ende
text(14.5,60,pos=2,bquote("LSD "*alpha*"=5%"))



##########################################
##########################################
#>>>now have a look at the fusarium data

#-->compute the heritability for the individual scorings and the
average
str(DATA)

DATA$B.MEAN <- (DATA$B1 + DATA$B2+DATA$B3+DATA$B4)/4
h2 <- numeric(5)
count <- 1

for(i in 11:15){
  DATA$FUS <- DATA[,i]

  model <- lmer(FUS~1+ (1|REP) + (1|REP:SET) + (1|GEN),DATA)
  VC.COMPS <- as.data.frame(VarCorr(model))

  h2[count] <- VC.COMPS[1,4]/(VC.COMPS[1,4] + VC.COMPS[4,4]/2)
```

```
  count <- count + 1
}

H2.FUS <- data.frame(Scoring=c(1:4,"Mean"),heritability=h2)
H2.FUS
#-->h² = 0.85 for the average of the 4 scorings (looks good!)
#-->perform a full mixed model analysis and get the lsmeans

model <- lmer(B.MEAN~1+ (1|REP) + (1|REP:SET) + (1|GEN),DATA)

#>>>QQ-Plot and a residual plot to check the model assumptions of
#   normality and homogeneity of variance of the residuals
qqnorm(resid(model))
plot(model,form=resid(.)~fitted(.),xlab="Fitted
value",ylab="Studendized residuals")
#-->looks good with some slight outliers

#>>>print and extract the variance components
print(VarCorr(model),comp=c("Variance","Std.Dev."),digits=6)
VC.COMPS <- as.data.frame(VarCorr(model))
VC.COMPS

#>>>compute the LS-MEANS or BLUES
modelF <- lm(B.MEAN~GEN + REP + REP:SET,DATA)
anova(modelF
lsmeans.list <- lsmeans(modelF,test.effs="GEN")
LSMEANS <- lsmeans.list$lsmeans.table

#write.table(LSMEANS,"LSMEANS.FUS.txt",quote=TRUE,col.names=TRUE,row
.names=FALSE)
LSMEANS.FUS <- read.table("LSMEANS.FUS.txt",header=T,sep="",dec=".")


#>>>plot the data in a histogram
hist(LSMEANS.FUS$Estimate,xlab="FHB severity
(%)",col="firebrick2",ylab="Number of lines",ylim=c(0,150),
     main="Fusarium head blight")
text(x=0,y=150,pos=4,expression("h² = 0.85"))

LSD <- sqrt(2*20.34570/2)*1.96
LSD

arrows(40,60,49,60,length=0.08,code=3,angle=90) #x,y Beginn  ;x,y
Ende
```

```
text(40,60,pos=2,bquote("LSD "*alpha*"=5%"))

##########################################################
#>>>also test the phenotypic correlation betweem AR and FUS
BLUES.FUS <- LSMEANS.FUS[,c(1:2)]
names(BLUES.FUS)[2] <- "FUS"
BLUES.AR <- LSMEANS.AR[,c(1:2)]
names(BLUES.AR)[2] <- "AR"

BLUES.COMPLETE <-
merge(BLUES.FUS,BLUES.AR,by.x="GEN",by.y="GEN",all.x=T,all.y=T)
cor.test(BLUES.COMPLETE$FUS,BLUES.COMPLETE$AR)

#>>>check the correlation by set
SETS <- DATA[,c(4,7)]
SETS <- SETS[!duplicated(SETS$GEN),]

BLUES.COMPLETE <-
merge(BLUES.COMPLETE,SETS,by.x="GEN",by.y="GEN",all.x=T,all.y=T)

CORRELATION <- numeric(3)
P.VALUE <- numeric(3)

for(i in 1:3){
  BLUES.CORTEST <- droplevels(BLUES.COMPLETE[BLUES.COMPLETE$SET %in%
levels(BLUES.COMPLETE$SET)[i],])
  RESULT.COR <- cor.test(BLUES.CORTEST$FUS,BLUES.CORTEST$AR)
  CORRELATION[i] <- RESULT.COR$estimate
  P.VALUE[i] <- RESULT.COR$p.value
}

RESULT.SET <-
data.frame(SET=levels(BLUES.COMPLETE$SET),CORRELATION=CORRELATION,P.
VALUE=P.VALUE)
RESULT.SET

# show significant differences for AR between sets
model<-lmer(AR~SET+(1|REP)+(1|SET:GEN),DATA)
anova(model)
lsmeans.list<-lsmeans(model)
LSMEANS<-lsmeans.list$lsmeans.table
write.table(LSMEANS,"LSMEANS.AR.SET.txt",quote =
TRUE,col.names=TRUE,row.names=FALSE)
differences.list<-difflsmeans(model)
LSMEANS.DIFF<-differences.list$diffs.lsmeans.table
```

```
LSMEANS.DIFF$COMPARISON<-row.names(LSMEANS.DIFF)
write.table(LSMEANS.DIFF,"LSMEANS.DIFF.SET.txt", quote=TRUE,
col.names = TRUE, row.names = FALSE)

#show significant differences for B.MEAN between sets
model<-lmer(B.MEAN~SET+(1|REP)+(1|SET:GEN),DATA)
anova(model)
lsmeans.list<-lsmeans(model)
LSMEANS<-lsmeans.list$lsmeans.table
write.table(LSMEANS,"LSMEANS.B.MEAN.SET.txt",quote =
TRUE,col.names=TRUE,row.names=FALSE)
differences.list<-difflsmeans(model)
LSMEANS.DIFF<-differences.list$diffs.lsmeans.table
LSMEANS.DIFF$COMPARISON<-row.names(LSMEANS.DIFF)
write.table(LSMEANS.DIFF,"LSMEANS.DIFF.SET.FUS.txt", quote=TRUE,
col.names = TRUE, row.names = FALSE)
```

8.5 R script for the assessment of anther retention and FHB severity of 'Remus' and 'C3' in the field

```
setwd("C:/Users/andrea/Desktop/EigeneVersuche")
DATA<-read.table("Antherenbonitur2.csv",header=T,sep=";",dec=".")
Genotype<-as.factor
Antheren<-as.numeric

# boxplot for anthers
boxplot(Antheren~Genotype,
data=DATA,col=c("slateblue","orangered1"),names=c("C3","Remus"))
title(xlab="Genotype",font.lab=2)
title(ylab="Number of florets with retained anthers", cex.lab=0.8,
font.lab=2)
AU5<-as.numeric

# boxplot for AU5
boxplot(AU5~Genotype,
data=DATA,col=c("slateblue","orangered1"),names=c("C3","Remus"))
title(xlab="Genotype",ylab="AUDPC", font.lab=2)

# boxplot for incidence
boxplot(Incidence~Genotype,
data=DATA,col=c("slateblue","orangered1"),names=c("C3","Remus"),ylim
=c(10,110))
title(xlab="Genotype",ylab="Incidence (%)", font.lab=2)
```

```
# ANOVA to see significant differences between the genotypes for
anthers, incidence and AU5
model <- lm( Antheren ~ Genotype, data = DATA)
anova(model)
model <- lm( Incidence ~ Genotype, data = DATA)
anova(model)
model <- lm( AU5 ~ Genotype, data = DATA)
anova(model)
```

8.6 R script for the analysis of the relationship between *Qfhs.if-5A* and anther retention

```
setwd("C:/Users/andrea/Desktop/EigeneVersuche")
library(lme4)
library(lmerTest)
library(ggplot2)

#>>>import the data as from the csv file
DATA <- read.table("Andrea.csv",header=TRUE,sep=";",dec=".")
str(DATA)
DATA$pot <- as.factor(DATA$pot)
DATA$head_2 <- as.factor(DATA$head_2)
DATA$genotyp <- as.factor(DATA$genotyp)
DATA$experiment <- as.factor(DATA$experiment)

#set up the model
#Treatment: genotyp x treatment = genotyp + treatment +
genotyp:treatment
#Design: pot/genotyp:treatment = pot + error

#>>>fit a mixed model for experiment 1, for disease scoring 1
model <- lmer(S1 ~ genotyp*treatment + (1|head_2),EXPERIMENT.1)
anova(model)
#summary(model)
#>>>QQ-Plot and a residual plot to check the model assumptions of
#   normality and homogeneity of variance of the residuals

qqnorm(resid(model))
plot(model,form=resid(.)~fitted(.),xlab="Fitted
value",ylab="Studendized residuals")
#>>>print and extract the variance components
print(VarCorr(model),comp=c("Variance","Std.Dev."),digits=6)
VC.COMPS <- as.data.frame(VarCorr(model))
VC.COMPS
```

```
#>>>compute the LS-MEANS or BLUES
lsmeans.list <- lsmeans(model)
LSMEANS <- lsmeans.list$lsmeans.table

write.table(LSMEANS,"LSMEANS.S1.EXP1.txt",quote=TRUE,col.names=TRUE,
row.names=FALSE)

#>>>compute all pairwise differences between the BLUES
differences.list <- difflsmeans(model)
LSMEANS.DIFF <- differences.list$diffs.lsmeans.table
LSMEANS.DIFF$COMPARISON <- row.names(LSMEANS.DIFF)

write.table(LSMEANS.DIFF,"LSMEANS.DIFF.S1.txt",quote=TRUE,col.names=
TRUE,row.names=FALSE)

#a boxplot
COLORS <-
c("blue","green","red","yellow","blue","green","red","yellow")
LABELS <- c("C3 B","C3 G","C3 R","C3 Y",
            "Remus B","Remus G","Remus R","Remus Y")

ggplot(data=EXPERIMENT.1,aes(x=factor(EXPERIMENT.1$geno_treat),y=EXP
ERIMENT.1$mS)) +
  geom_boxplot(aes(fill = factor(EXPERIMENT.1$treat))) +
  theme_bw() +
  ggtitle("") +

#scale_fill_brewer(palette="Paired",labels=c("CM","Remus"),name="Gen
otype") +

scale_fill_manual(values=COLORS,labels=c("B","G","R","Y"),name="Grou
p") +
  scale_y_continuous(limits=c(0,12),name="mS") +
  scale_x_discrete(name="Treatment",labels=LABELS) +
  theme(title = element_text(size=18,color="black"),
        axis.title.x = element_text(size=18,color="black"),
        axis.title.y = element_text(size=18,color="black"),
        axis.text = element_text(size=16,color="black"),
        panel.grid.major = element_line(color = "white"),
        panel.grid.minor = element_line(color = "white"),
        #legend.title = element_blank(),
        #legend.justification=c(1,0),
        #legend.position=c(1,0),
        legend.title = element_text(size=16),
        legend.text = element_text(size=16),
        legend.key = element_rect(fill="white",linetype=0))
```

```
COLORS <-
c("blue","green","red","yellow","blue","green","red","yellow")
LABELS <- c("C3 B","C3 G","C3 R","C3 Y",
            "Remus B","Remus G","Remus R","Remus Y")

ggplot(data=EXPERIMENT.1,aes(x=factor(EXPERIMENT.1$geno_treat),y=EXP
ERIMENT.1$S1)) +
  geom_boxplot(aes(fill = factor(EXPERIMENT.1$treat))) +
  theme_bw() +
  ggtitle("") +

#scale_fill_brewer(palette="Paired",labels=c("CM","Remus"),name="Gen
otype") +
  scale_fill_manual(values=COLORS,labels=c("only middle florets
removed", "no manipulation","anthers compressed","anthers
removed"),name="Pre-treatments") +
  scale_y_continuous(limits=c(0,12),name="FHB scoring 1") +
  scale_x_discrete(name="Genotype by Pre-treatment",labels=LABELS) +
  theme(title = element_text(size=18,color="black"),
        axis.title.x = element_text(size=16,color="black"),
        axis.title.y = element_text(size=16,color="black"),
        axis.text = element_text(size=12,color="black"),
        panel.grid.major = element_line(color = "white"),
        panel.grid.minor = element_line(color = "white"),
        #legend.title = element_blank(),
        #legend.justification=c(1,0),
        #legend.position=c(1,0),
        legend.title = element_text(size=12),
        legend.text = element_text(size=12),
        legend.key = element_rect(fill="white",linetype=0))
```

8.7 Production of Inoculum

BOKU	Page: 1 von 7 Valid per: 20.05.2007	Standard operating procedure SOP 3-04
	Revision: 0	**Production of Inoculum of *F. culmorum***

Contents:

Page

1. Purpose / Principle

This SOP regulates the production of inoculum for plant inoculation experiments with *F. culmorum*. This SOP also regulates the handling of the inoculum and determination plus calculation of conidial concentrations, the area of application and the responsibility.

2. Area of application

Laboratory Fusarium Resistance Breeding, growth medium kitchen
IFA Tulln, Institut for Plant Production
responsible person for execution and calculation: Ing. Andrea Koutnik

3. Measurement principle/Basics

Seed a wheat-oat-mixture, swollen in babyfood-jars, with *F. culmorum*, grown on agar before, and leave it to grow for app. 2 weeks in diffuse daylight at room temperature. For better aeration and complete colonisation of the medium, vigorously shake the jars daily. Thereafter, store the jars in a refrigerator at 4 - 8°C. If needed, wash the kernels, count and calculate the conidia in the suspension.

4. Procedure data / Validating

Not available

5. Equipment, Equipment settings and Material

5.1. Equipment

Autoclave Varioklav
LaminAir Heraeus
Microscope Nikon Labophot 2

5.2. Equipment settings

Autoclave Varioklav: 121°C, 20 min, „forciert“
LaminAir: switch on 20 min before use, disinfect with alcohol directly before use
Microscope: Objective : 10 / 0,25 (yellow) and 40 / 0,55 (light blue)

5.3. Material

Wheat- oat- mixture (1 part oat and 2 parts wheat, volume/volume)
Baby food jars
plastic lid Magenta B-Cup, Sigma, part number: B86486.
Tea-filter
Collecting container (glass-flask, beaker)
Buerker Tuerk counting chamber

6. Chemicals

Not available

7. Analysis Procedure

7.1. Sample collection

Take the required Fusarium culture from the existing master culture collection at the IFA Tulln (laboratory/resistance breeding room: BP/E/24 B) and scatter it on SNA (special Nirenberg agar). After app. 72 h the grown Fusarium shall be used for the inoculation of the wheat - oat - mixture.

7.2. Sample Preparation

Put app. 50 g wheat - oat - mixture (1 part oat, 2 parts wheat) into baby food-jars, fill up with osmose water and leave it over night.. After app. 24 hours decant the surplus water and lock the glasses with plastic lids. Then autoclave at 121°C for 20 min, after cooling inoculate in the LaminAir with *F. culmorum* (one small piece of Fusarium-overgrown agar/jar).

Keep the inoculated, locked glasses for two weeks in diffuse daylight at room temperature, until the grains are well through-grown (brown-orange discoloration of the kernels). For better aeration and colonisation shake the jars (app. once per day). Subsequently the jars are stored in the refrigerator at 4-8°C.

For the production of inoculum of *F. culmorum* the glasses are filled with osmose water, shaken well and poured through a filter into a collecting container. Repeat the procedure again if necessary.

The conidia in this solution are counted and processed further accordingly.

7.3. Calibration

Not available

7.4. Measurement

Counting of the conidia takes place in a Buerker - Tuerk - chamber.

App. 10 small chambers are counted out and the average value/chamber is calculated. A small chamber corresponds to $25x10^{-5}$ mm³ = $25x10^{-8}$ ml

x conidia are in $25x10^{-8}$ ml
in 1 ml → y

Example: on average 6 conidia are contained in 25 x 10^{-8} ml

6 / 25 x 10^{-8} ml = 0,24 x 10^{8} conidia / ml =

C = 24 Mio. conidia/ml

7.5. Evaluation

The dilution factor is calculated with the following formula:

$$C1 \times V1 = C2 \times V2$$

C1 = known concentration of conidia-suspension
V1 = unknown volume of conidia-suspension
C2 = known concentration of inoculum
V2 = known volume of inoculum

Example:
From the example mentioned in Pt. 7.4 10 litres of inoculum in a concentration of 50.000 conidia/ml should be prepared.

$$V1 = \frac{50.000 \text{ con/ml} \times 10000 \text{ ml}}{24 \times 1.000.000 \text{ con/ml}} = 20{,}8 \text{ ml}$$

Production of the desired inoculum:
20,8 ml from the counted conidia-suspension are filled up to 10 L.

7.6. Handling

The conidia-suspension is labelled as follows in the desired concentrations in appropriate PP-tubes and frozen (in our example 20,8 ml of conidia-suspension is frozen):

Fusarium xy / IFA-Nr Dissolved in xx L Is equivalent to yy.yyy K/ml

Example:

F.culmorum / IFA104 Dissolved in 10 L Is equivalent to 50.000 K/ml

The necessary quantity of frozen conidia-suspension tubes are thawed and vibrated in handwarm water and diluted accordingly to the data on the label.

7.7. Units of the results

The unit of concentration is K/ml (conidiens/ml).

The exact dilution data are, as described in Pt. 7.6., on the label.

8. Applicable Documents

Production of growth media (Test- SOP 3 - 03)
Manufacturing of master cultures
Laboratory order from 06 December 2005

9. Literature

Not available

10. Attachments

SOP-Validity proof
SOP-mailing list