# Studies about *Fusarium* infection of emmer and naked barley during grain ripening and the post-harvest period

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# Preface

This study was conducted within the second phase of the subproject five of the joint project "Forschungsverbund Agrar- und Ernährungswissenschaften Niedersachsen" (FAEN) - Quality-related plant production- Mycotoxins in the context of production, quality and processing - finaced by the Ministry of Science and Culture of Lower Saxony, Germany.

In the first phase of the project Eggert (2010) studied the influence of *Fusarium* infection on mature, recently harvested emmer and naked barley grains.

In the focus of this study were the *Fusarium* infection of emmer and naked barley in the course of grain ripening and the influence of post-harvest conditions on selected grain quality parameters.

The determination of *Fusarium* mycotoxins as well as *Fusarium graminearum* DNA were performed by the cooperation partners in the laboratory of Molecular Phytopathology and Mycotoxin research, Department of Crop Sciences, University of Göttingen, Germany. Tryptic digestion of proteins and identification of proteins by mass spectrometry (MS) were performed at the Institute of Plant Proteomics, University of Hannover, Germany.

This PhD thesis contains five manuscripts prepared for publication. The analytical methods and the materials used for this research as well as the sample preparation are described in detail in the respective manuscripts.

**Reference**

Eggert, K. (2010). *Fusarium*-Befall bei Emmer und Nacktgerste. PhD thesis. Göttingen

# 1 Introduction

## 1.1 Fusarium head blight (FHB) of cereals

### 1.1.1 Infection

*Fusarium* head blight (FHB) is a disease of small-grain cereals (wheat, barley, oats, rye, and triticale) caused by several *Fusarium* species occurring worldwide were small-grain cereals are grown. *Fusarium* can survive saprophytically on residues on the field, such as crop stubble of small grains and maize and therfore provide an inoculum for *Fusarium* head blight (FHB) disease. Reduced tillage practices that leave crop stubble in the field may contribute to the survival of the spores and increase the amount of inoculums on the soil surface. The production of the principle inocula (macroconidia and ascospores) depends on the *Fusarium* species and the environmental conditions (Champeil et al., 2004). Ascospores can be spread widely through long distances by wind and macroconidia can be dispersed by rain splash. After contact with a host, spore germination occurs and the fungus begins the infection cycle (Gilbert and Fernando, 2004).

*F. graminearum, F avenaceum* and *F. culmorum* are the predominant *Fusarium* species associated with *Fusarium* head blight (FHB) in Europe (Bottalico and Perrone, 2002). *F. graminearum* is supposed to be the most virulent species worldwide (Bai and Shaner, 2004).

After establishing in crop residues the fungus is colonizing living host tissue such as wheat, barley, corn, and rice tissues at specific stages of the host life cycle. In wheat and barley, heads are most susceptible to *F. graminearum* compared to other parts of the plants. The infection of cereals can occur at any growth stage of kernel development, but the first half of the grain-filling period is the most vulnerable to infection (Bai and Shaner, 2004). The infection is promoted by long periods of high temperatures between 15 and 30 °C and a high relative humidity of more than 90% (McMullen et al., 2012).

At the beginning of the infection process the pathogen the hyphae develop on the surface of florets and glumes, allowing the fungus penetrate the tissues within the inflorescence, the anthers, the lemmae, the glumes or rachis depending on the infecting species and the time of infection. The hyphea penetrates into the ears passing around and through the plant cells, degrading them (Champeil et al., 2004, Goswami and

Kistler, 2004). Fungal spread in wheat from floret to floret or from spikelet to spikelet occurs through the vascular bundles in the rachis and rachilla (Goswami and Kistler, 2004).

### 1.1.2 Disease and symptoms

The first visible symptoms are brown spots on the glumes or on the rachis at the point of infection, frequently followed by spread in adjacent spikelets and discoloration of the ears (head blight). During later infection period, fungal growth appears along the edges of the glumes visible as characteristic pink spore masses (Parry et al., 1995). Figure 1.1 shows the brownish spikelets of wheat, emmer and naked barley at the milk ripe stage after *F. graminearum* spray inoculation during flowering.


**Figure 1.1:** *Fusarium* infected heads of A: wheat, B: emmer and C: naked barley at milk ripe stage after artificial *F. graminearum* inoculation. (Photo: Trümper 2011/2012)

An infection with *Fusarium spp.* results in reduced grain yield as well as highly shrivelled and damaged grains and mycotoxin contamination, which makes the cereal products harmful for human and animal consumtion (Kazan et al., 2012).

### 1.1.3 Fusarium mycotoxins

*Fusarium* mycotoxins are secondary metabolites of several *Fusarium* species that accumulate in cereal grains and are harmful for humans and animals health (Woloshuk and Shim, 2013). Natural occurring *Fusarium* mycotoxins usually belong to three main structural groups, that are trichothecenes (deoxynivalenol (DON), nivalenol (NIV), 3- and 15- acetyldeoxynivalenol (3, 15- AcDON), T-2-Toxin, HT-2-Toxin,

Diacetoxyscirpenol (DAS)), Zearalenone (ZEA) and moniliformin (MON). Furthermore, beauverecin (BEA) and fumonisin (FUM) have been found in *Fusarium* infected grains (Bottalico and Perrone, 2002). *Fusarium* species differ in their trichothecene profiles. DON and its acetylated derivates AcDON are the main trichothecene metabolites found in cereal grains in Europe and they are commonly produced by several *Fusarium* species, mainly by *F. graminearum* and *F. culmorum.* These *Fusarium* species are furthermore producers of NIV and ZEA (Bottalico and Perrone, 2002, Rocha et al., 2005). According to a large scale study in eleven European countries DON was detected in 57% of all analysed samples (Schothorst and van Egmond, 2004).

### 1.1.4 Toxicity of *Fusarium* mycotoxins

Trichothecenes are chemically stable and withstands high temperatures. Therefore, they are not degraded during food processing, which increases the risk for occurrence in foods and feeds (Maresca, 2013). For this reason highly *Fusarium* infected grains can lead to serious mycotoxin contaminated cereal products, harmful for human and animal health (Rocha et al., 2005). The prevalent trichothecene DON is not only the most detected trichothecene in cereal grains but is also proven to occur at the highest concentrations. Therefore, DON is of great importance due to its frequent occurrence in toxicological relevant concentrations in cereal products (Pestka, 2010). Human dietary exposure of DON through the consumption of contaminated food can result in acute intoxication inducing symptoms as vomiting, nausea and diarrhoea and gastroenteritis. Furthermore, DON can affect nutrient absorption (glucose, amino acids) by protein inhibition and induction of apoptosis in intestinal cells (Maresca et al., 2002) and alters functions of the immune system, the endocrine system and the brain (Maresca, 2013). Pigs have been shown to be the most sensitive animals to DON intake compared to ruminants and poultry concerning these symptoms, although all evaluated animal species are more or less susceptible to DON (EFSA, 2004, Pestka, 2007). The differences in metabolism and detoxification of DON among animal species might contribute to their different sensitivity (Pestka, 2007). The bioavailability of ingested DON and the respective metabolites (deepoxy-DON, DON- glucuronide) is strongly dependent on the metabolism, for example the localization of gut bacteria in the intestine. Several studies exhibited very low transfer of DON into animal tissues (Döll et al., 2008) or DON accumulation in the milk of cows (Keese et al., 2008) or in eggs of

laying hens (Valenta and Dänicke, 2005). According to this, no risk of high intake of DON is expected resulting from animal tissue consumption compared to direct consumption of cereal products through human dietary consumption. The mycotoxin ZEA is another frequently appearing contaminant of grains and cereal products. ZEA is known to be of a relatively low acute toxicity. Nevertheless, ZEA and its metabolites exhibit oestrogenic properties and are therefore associated with reproductive disorders of animals and occasionally with hypoesrogenic syndromes in humans (Zinedine et al., 2007). Several studies confirm the hypothesis that exposure to ZEA may contribute to the increasing occurrence of breast cancer by stimulating the growth of human breast cancer cells (Ahamed et al., 2001, Pazaiti et al., 2012). Furthermore, ZEA has been shown to be genotoxic and alters immunological parameters in vitro (Zinedine et al., 2007). Due to the toxic potential of mycotoxins, regulatory limits for cereals and cereal products for human dietary consumption have been introduced in Europe. The maximum levels for the *Fusarium* toxins DON, ZEA, Fumonisins, T-2 and HT-2 toxin are defined according to the commission regulation (EC) No 1881/2006. In unprocessed cereals the limit for DON is 1250 µg/kg and in breads and other cereal products the maximum value is 500 µg/kg, respectively.

## 1.2 *Fusarium* resistance and plant-pathogen interactions

It is essential for developing efficient strategies to control the FHB disease to understand the molecular crosstalk between *Fusarium spp.* and the cereal hosts. Therefore, various studies using genomic, transcriptomic and proteomic methods were conducted to investigate the complex mechanisms involved in *Fusarium* infection strategies, penetration and colonization of host tissues (Walter et al., 2010).

Different crops and cultivars exhibit a large variety concerning resistance to FHB. Two main types of resistance to FHB are widely recognized: type I to initial infection and type II resistance to fungal spread within adjacent spikelets. Additionally, three more types of resistance have been defined by (Mesterházy, 1995): resistance to kernel infection (type III resistance), tolerance to infection (type IV resistance) and resistance to DON accumulation (type V resistance). Morphological and physiological traits such as plant high, anther extrusion, flowering time and flowering duration, kernel density or awn presence are proposed to contribute to type 1 resistance (Lu et al., 2013, Yoshida et al., 2005a). Numerous physiological mechanisms are employed by the host plants to resist fungal establishment, penetration and mycotoxin-biosynthesis, such as the

formation or release of antifungal compounds and proteins (Walter et al., 2010). For example, plant secondary metabolites as phenolic acids have frequently been associated with FHB resistance. Ferulic acid and *p*-cumaric acid, the most prevalent phenolic acids in cereal grains have been shown to inhibit fungal growth in vitro and are supposed to be a predictor for susceptibility of host plants (McKeehen et al., 1999). Additionally, the induction of catechin synthesis in the grains of naked barley infected with *Fusarium spp.* was detected in a previous study (Eggert et al., 2010).

Particularly, host plants are able to employ a rage of proteins in response to pathogen attack that partly interact with fungal cell wall to nitice and inhibit fungal activities (Walter et al., 2010). Extensive proteomic studies are available concerning differently expressed proteins in *Fusarium* infected cereals, particularly during the first days after anthesis and inoculation, respectively (Yang et al., 2013). According to these studies many proteins involved in plant signalling pathways were up regulated. For example, Ding et al. (2011) and Zhou et al. (2006) found genes and proteins induced in wheat by *F. graminearum* infection related to antioxidant, jasmonic acid and ethylene plant signalling pathways and oxygen stress. Coincidently, Li and Yen (2008) suggested that FHB resistance in the wheat cultivar Sumai 3 is mediated by jasmonic acid and ethylene signaling pathways. High abundance of reactive oxygen species (ROS) in plant tissues are known to trigger hypersensitive response and accordingly plant cell death as defence response to pathogen attack (Torres, 2010). Plants use ROS as second messengers in signal transduction processes to transmit information concerning changes in the environment (Foyer and Noctor, 2005). Furthermore, proteins related to metabolism and photosynthesis has been differentially regulated due to *Fusarium* infection in wheat and barley (Yang et al., 2010, Shin et al., 2011, Zhou et al., 2006). According to various studies proteins related to defence response were induced after *Fusarium* infection, such as chitinases, glucanases, thaumatin like proteins (TLP), heat shock proteins, peroxidases as well as xylanase- and protease inhibitors of cereals spikes and grains (Wang et al., 2005, Geddes et al., 2008). For example chitin, the main component of fungal cell walls can be recognized by plant chitin binding proteins that are able to inhibit fungal growth. Chitinases and glucanases, further groups of antifungal proteins interact with chitin and glucan, respectively by catalysing its degradation causing weakened fungal cell walls and cell lysis (Theis and Stahl, 2004). Enzymes, such as protease inhibitors and xylanase inhibitors prevent fungal hydrolases from degrading

plant cell walls (Misas-Villamil and van der Hoorn, 2008). Thaumatin-like proteins have been demonstrated to have antifungal activity by acting on fungal membranes, showing glucanase activity or binding to actin (Wong et al., 2010). Furthermore, cystein rich peptides and proteins such as defensins and thionins and non specific lipid transfer proteins inhibit the growth of a broad range of pathogen fungi (Walter et al., 2010). Only a few studies were conducted concerning *Fusarium* infection during later ripening stages and later infection periods, respectively. Changes in proteins related to plant defence response and metabolism also occurred in mature grains of emmer and naked barley, showing that defence mechanism can be stimulated not only in the early *Fusarium* infection stage but also at maturity (Eggert and Pawelzik, 2011, Eggert et al., 2011b).

## 1.3 Principal factors contributing to FHB severity and mycotoxins-biosynthesis in cereals

The regulation of trichothecene biosynthesis has been shown to be controlled by various regulatory systems in response to environmental conditions (Merhej et al., 2011). The principal factor contributing to the development of FHB is known to be climatic conditions, such as temperature and rain fall. A warm and humid climate controls the mycelium growth as well as the mycotoxins production in the plant (Champeil et al., 2004, Parry et al., 1995). Especially temperatures around 20°C and humid climate during flowering can contribute to high *Fusarium* infection rates (Parry et al., 1995). Moreover, agricultural practices as crop rotation and soil tillage can influence the development of FHB. High amounts of low degradable crop debris on the soil surface lead to pathogen survival and growth on the Plants. Therefore, FHB disease has been shown to be more severe if the pre-crops are maize, rather than wheat, barley or rapeseed and sugar beet and if soil tillage is limited (Blandino et al., 2012). Higher FHB incidence can also be affected by improper fertiliser application (Pirgozliev et al., 2003). For example increased nitrogen application increases the occurrence of *Fusarium* infected grain in wheat and barley (Lemmens et al., 2004, Martin et al., 1991). However, some studies showed converse results finding no impact of nitrogen application (Yoshida et al., 2008). The effect of fungicides on the retardation of FHB disease has been well established and documented in several studies (Blandino et al., 2012, Mesterhazy et al., 2003). The efficacy depends on the type of fungicide used, the method of application and the time of application (Champeil et al., 2004). Fungicides

containing triazoles, which inhibit the biosynthesis of ergosterol as are supposed to be the most effective agents against FHB pathogens, especially when they are applied at anthesis (Beyer et al., 2006).

The most important factor for controlling FHB disease is the cultivation of resistant cultivars. So far, completely *Fusarium* resistant cereals do not exist. All varieties are more or less susceptible to FHB. Resistance to *Fusarium* development and trichothecene accumulation is controlled by different genes on 18 chromosomes. The most common source for resistance breeding in wheat is the chinese cultivar Sumai 3 (Champeil et al., 2004).

Several studies revealed different infection strategies of *Fusarium* in wheat and barley. Thus, *F. graminearum* genes expressed during the infection of wheat and barley has been shown to be different (Lysoe et al., 2011). Moreover, the trichothecene DON was proven to act as virulence factor in wheat but not in barley. In wheat DON inhibits cell wall thickening in the rachis node, preventing fungal growth to adjacent spikeltets but in barley fungal spread within spikelets did not occur with or without DON (Maier et al., 2006, Jansen et al., 2005). DON is an important virulence factor promoting *Fusarium* aggressiveness. Apart from triggering programmed cell death DON strongly induces defence gene expression in *Fusarium* infected plant tissues (Kazan et al., 2012). The treatment of wheat leaves with DON induced hydrogen peroxide production within six hours and induced the transcription of several defense genes (Desmond et al., 2008).

## 1.4 Impact of Fusarium infection on grain quality

### 1.4.1 Technological traits

*Fusarium* infection can cause negative effects on processing quality parameters of cereal products. *F. culmorum* infection of barley grains generates an increase of proteolytic activity compared to not infected grains, leading to altered protein composition and therefore effects malting quality (Oliveira et al., 2013). One established negative effect of *Fusarium spp.* infection on barley malt quality is an increased gushing potential of the malt (Sarlin et al., 2005). FHB in wheat affects baking quality, mainly by lowering dough stability and therefore negatively impact the loaf-shape of the breads (Gärtner et al., 2007, Capouchova et al., 2012). Eggert et al. (2011a) revealed a strong digestion of gluten protein-fractions by *Fusarium* proteases in vitro. Especially high molecular weight glutenins were digested, which are known to be

significant for dough properties (dough strength) and baking quality (loaf volume) of wheat (Wieser, 2007).

## 1.5 Post-harvest-management

### 1.5.1 Relevance

Cereal products are one of the most important staple foods in the world. About two billion tonnes of cereals are produced worldwide, requiring 65% of the world's agriculture crop land (Klingler, 2010). The rising world population requires increased food supply and therefore, a minimized post- harvest food loss is an urgent demand (Hodges et al., 2011). The most important risk to human health from stored products are fungal mycotoxins from various storage- and field fungi. Under suitable storage conditions, cereals can be stored for long periods without quality losses and without being harmful for human health. Depending on moisture contents and temperatures, complex biotic and abiotic interactions can occur during storage, such as pest immigration and emigration, as well as insect/fungal interactions. It is necessary to understand the relationship between fungal activity and quality losses during storage to minimize the risks of quality losses and prevent food waste (Magan et al., 2010).

### 1.5.2 Interaction between environment and fungi during storage

Grains are colonised by a range of microorganisms including yeasts and several filamentous fungi on the field, which can further develope during storage. The composition of the population depends on the climatic conditions on the field. Improper post harvest management can lead to contamination with a number of mycotoxins from field- and storage fungi and can cause rapid losses in grain quality, such as a reduced germination capacity, deterioration of milling and baking quality. Cereal grain is provided to be stored at a water activity ($a_w$) of $\leq 0.7$ corresponding to grain moisture of about 14%. Grains harvested at higher moisture contents require an effective ambient drying to prevent the development of spoilage fungi (Magan et al., 2003, Magan et al., 2010). Apart from inefficient drying systems mechanical damage of the grains also contributes to the entry of microorganisms and the degradation of grain starch and proteins by them. Furthermore, insect pests can contribute to fungal growth in stored grains by producing metabolic heat generating higher humidity through their respiratory activity. Warm and humid conditions can quickly result in fungal spoilage and

mycotoxin production (Magan et al., 2003). Moreover, growing of fungi can also result in a significant increase in their respiratory activity. This can result in an increase in temperature and sometimes spontaneous heating building a terrain for thermophilic fungi and actinomycetes (Magan et al., 2010). The most occurring fungi in stored cereal grains are *Aspergillus, Penicillium* and *Fusarium*. These pathogens mainly produce aflatoxins, ochratoxins and trichothecenes, respectively (Magan and Aldred, 2007). Several studies revealed that warm and humid storage of slight or moderate *Fusarium* infected wheat can lead to increased trichothecene contents (Homdork et al., 2000). Furthermore, the types of fungi and mycotoxins accumulating are dependent on the environment. An increase in storage temperature of wheat to 30ºC dramatically increased the DON contamination, while the presence of ZEA was reduced (Mylona et al., 2012). Consistently, Prange et al. (2005) found completely changed fungal flora after the storage of only four weeks at 20°C and grain moisture of 20%. Additionally strong competitive interactions between field fungi (*Fusarium*) and storage fungi (*Penicillium, Aspergillus*) were observed concerning mycotoxins production. Thus, trichothecene producers prevented the production of ochratoxins, whereas high ochratoxin contents seemed to prevent trichothecene formation of *Fusarium* species (Prange et al., 2005).

## 1.6 Emmer (Triticum turgidum L. ssp. dicoccum)

### 1.6.1 Domestication and characteristics

Emmer belongs to the earliest domesticated crops in the Fertile Crescent, a region at Mediterranean area in antiquity. The site of domestication of emmer is not certain, but it is postulated that the gene pool of domesticated emmer is enriched by genes from wild emmer of the southern Levant (Lebanon, Syria, Israel) (Luo et al., 2007). The domestication of hulled emmer was the first step towards the evolution of free-threshing tetraploid durum wheat and hexaploid bread wheat (Luo et al., 2007). A main beneficial trait is the property to give high yields on poor soils. The emmer ear is characterised by persistent enclosing hulls and strong glumes. The spikes are narrow and generally awned. The kernels are long and slender and red-brown or white in colour (Zaharieva et al., 2010). Figure 1.2 shows an emmer ear at hard dough ripening stage and emmer grains after harvest. In most areas in the world emmer has been replaced to a great extend by hull-les wheat species with several exceptions. It can still be considered as an

important crop in India, Ethiopia and Yemen, where it is used for traditional foods. At present, emmer cultivation makes up only one percent of the total world wheat area and the production is around 250,000 tons (Zaharieva et al., 2010). The yields reach from 19 to 35 dt $ha^{-1}$, which is about 40 % of the common wheat yields (Schwabe, 2012), (http://www.emmer-einkorn.ch/portrait/, date 12.11.2013).


**Figure 1.2:** Emmer ear at hard dough stage (A) and emmer grain after harvest (B). (Photo: Trümper 2011/2012).

### 1.6.2 Utilisation of emmer

Currently, emmer is predominantly used for human nutrition and partly for animal food. In Europe it is used as fodder for pigs and horses. In human nutrition it is mainly applied for bread making and beer production. In parts of Spain and Russia emmer is used for traditional breads and pancakes as well as for fermented products like beer and liquor. In India, emmer grains are polished and cooked or processed for fried wet gluten balls as traditional meals (Zaharieva et al., 2010). A renewed interest in emmer during the last years has been observed due to its ability to grow in soils with low fertility, in cold climate zones and its stress resistance as well as its nutritional valuable contents of fibre and antioxidant compounds. Emmer has high protein contents in the range of 18-23% and high mineral contents giving breads of good quality and valuable for human nutrition (Pagnotta et al., 2009). Emmer bread has better flavour, taste and crust colour compared to wheat bread (Zaharieva et al., 2010).

### 1.6.3 Susceptibility of emmer to Fusarium head blight (FHB)

Emmer cultivars are supposed to be resistant to fungal diseases such as stem rust, prevalent in wet areas. Furthermore some cultivars show tolerance to heat and drought stress. Thus, emmer represents a useful genetic resource for resistance breeding in wheat concerning biotic and abiotic stress (Zaharieva et al., 2010). Concerning FHB resistance a wide variation has been found from highly susceptible to highly resistant. Although most of the emmer cultivars are susceptible, the few resistant wild emmer cultivars represent potential new sources to enhance resistance to FHB in durum wheat (Buerstmayr et al., 2003, Oliver et al., 2007). The major component for FHB resistance in emmer is supposed to be the type II resistance to fungal spread. Apart from active plant defence mechanisms, plant morphology such as plant high and hulls may play a significant role in *Fusarium* and mycotoxins resistance. Especially the strong glumes surrounding the kernel may prevent fungal invasion to some extend (Buerstmayr et al., 2003).

## 1.7 Naked barley (Hordeum vulgare ssp. nudum)

### 1.7.1 Domestication and characteristics

Historically, naked barley has been an important crop in the ancient Egypt, Mesopotamia and Greece (Dickin et al., 2012). Archaeological remains of barley grains indicate that ancient barley was domesticated about 10000 years ago (Badr et al., 2000). Nevertheless, better food processing properties of wheat and rice considerably decreased the use of barley as food (Baik and Ullrich, 2008). The wild relative of cultivated barley *(Hordeum vulgare L.)* is known as *Hordeum spontaneum. H. vulgare* and *H. spontaneum* are morphological similar. The cultivated form has broader leaves, shorter stem and awns, shorter and thicker spikes and larger grains (Badr et al., 2000). Barley, which is genetically diverse, can be classified into spring or winter types, two-row or six-row barley, hulled or naked (hull-less) barley and additionally by the type of end-use quality (e.g. malting, feed) (Baik and Ullrich, 2008). Hulled barley and naked barley are close relatives that are distinguished by a single gene locus *(nud)*. The caryopsis of hulled barley is adhering to the grain, whereas naked barley has easily separable husk upon threshing (Taketa et al., 2004, Taketa et al., 2008). Figure 1.3 shows the ear and the grain of the naked barley cultivar Lawina.

Naked barley produces lower grain yields compared to the hulled genotypes. However, the weight of the hulls is included into the yield of hulled barley, which makes up about 15% (Dickin et al., 2012).


**Figure 1.3:** Naked barley ear at hard dough stage (A) and naked barley grain after harvest (B). (Photo: Trümper 2011/2012).

### 1.7.2 Utilisation of barley and naked barley

Barley is predominantly used for animal feed. Worldwide, about 30% of the produced amount is used for malting and brewing and only 2% is used for food directly (Baik and Ullrich, 2008). Due to the fact that naked barley does not require removing the hulls after harvest it is more suitable for food processing than hulled barley. Nevertheless, hulled barley is preferred for malting and brewing because the hulls contribute to beer flavour and are helpful for filtering procedures during brewing. In contrast to wheat, only little progress has been made in the past concerning improved food processing quality of barley, making selection of raw material suitable for special food more complicated. In human nutrition barley is used for many traditional dishes in Russia, Poland, Japan and India (Baik and Ullrich, 2008). In some countries pearled barley is used as rice substitute. In western countries flaked barley is used as breakfast cereal (Bhatty, 1986). Additionally, barley is mixed into wheat-based products, especially breads cakes and noodles (Baik and Ullrich, 2008). The replacement of wheat flour by 10% naked barley flour in bread had no negative effect on bread quality concerning loaf volume crumb firmness. However, the ß-glucan content is increased more than three-fold (Choi et al., 2011). Recently, the interest in using particularly naked barley for human nutrition is rising due to the high ß-glucan contents and the low glycemic load promising certain health benefits, such as reduced risk of coronary heart disease and a

cholesterol lowering effect (Dickin et al., 2012). Moreover, naked barley contains high levels of minerals such as calcium, magnesium and potassium (Dickin et al., 2012). It became apparent that naked barley is suitable for malt and whisky production. With modified malting conditions it was possible to achieve good alcohol yields and furthermore a shorter steeping circle and reduced germination time, saving water and energy during malting (Agu et al., 2009).

### 1.7.3 Susceptibility of naked barley to *Fusarium* head blight (FHB)

Barley genotypes vary in their susceptibility to *Fusarium* infection. Gene expression related to FHB resistance in barley is very complex. Some traits have been reported to be associated with FHB resistance, such as plant height and spike characters. In general, two-rowed barley is more resistant to FHB than six-rowed barley (Buerstmayr et al., 2004). Furthermore, the flowering type seems to play an important role. Cleistogamous (closed-flowering) cultivars are supposed to be more resistant to FHB than chasmogamous (open-flowering) cultivars (Yoshida et al., 2005b). Comparing hulled and naked barley, the hulled types appears to be more resistant (Warzecha et al., 2010). Contrary, Buerstmayr et al. (2004) partly detected very low toxin contents in naked barley cultivars, suspecting considerable proportions of toxins remaining in the hulls.

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# 2 Objectives

A key aspect of this work was to investigate the impact of *Fusarium* infection on the quality of emmer and naked barley grains during grain ripening and in the post-harvest period. One fundamental risk of *Fusarium* infection of cereals is the contamination with mycotoxins, making the products harmful for human and animal nutrition. Emmer and wheat are known to be more susceptible to FHB disease and mycotoxin accumulation compared to naked barley. It is crucial for controlling the final toxin concentrations to discover when fungal toxin production generally occurs during grain development and if there are differences between cereal genotypes. In this study, the mycotoxin accumulation during grain development of emmer, wheat and naked barley has been compared between natural infected grains and after *F. graminearum* inoculation at the time of anthesis.

Another major risk of *Fusarium* infected grains is the gegradation of valuable ingredients, such as proteins and polysaccharides by fungal enzymes. The protein composition of cereals is of considerable relevance for processing properties and the quality of the end products. The present study should provide an insight into the changes of proteins that occur during grain development and the impact of *F. graminearum* inoculation.

Furthermore, the impact of storage conditions on emmer and naked barley grains has been investigated as well as the impact of *Fusarium* infection during storage. Microbial interactions depend on the respective environmental conditions and are significant for the development of storage fungi or *Fusarium* species. Alterations in the mycotoxin profile and the changes of protein composition after six month of storage have been investigated in the present study.

Another focus was the investigation of possible resistance – related physiological mechanisms. For this reason it was explored, whether the formation of phenolic compounds during grain development in the grains and in the hull-tissues of emmer and naked barley grains are related to their FHB resistance.

Moreover, the regulation of specific proteins due to *Fusarium* inoculation depending on the ripening stage of emmer and naked barley grains and progressive *Fusarium* infection, respectively, was investigated. Earlier proteomic studies revealed different responses of emmer and naked barley grains after *Fusarium* infection at maturity. The

present study should give new information concerning possible resistance mechanisms depending on the crop genotype and the respective grain ripening stage.

# 3 Results and discussion

## 3.1 Changes of the protein composition in the grains of emmer, wheat and naked barley after artificial inoculation with *Fusarium graminearum* in the course of grain ripening

**Abstract**

Emmer, wheat and naked barley cultivars were investigated in terms of their susceptibility to *Fusarium graminearum* infection. In the focus of research was the impact of an artificial *Fusarium* inoculation compared to natural infection on the grains of field-grown emmer (*Triticum dicoccum*), wheat (*Triticum aestivum*) and naked barley (*Hordum vulgare nudum*) grains. This study permitted to compare the protein formation of three different types of grains in the course of grain development from the early milk ripe to mature grain. The influence on protein formation as well as the toxin accumulation after *F. graminearum* infection during a long infection period was investigated. Therefore, protein fractions separated according to Osborne were characterized and quantified by HPLC and SDS-Page methods and *Fusarium* toxins were quantified by mass spectrometry. An infection with *F. graminearum* affected the protein composition of all species from the early grain development on, whereupon emmer and wheat were more affected than naked barley. *Fusarium* Infection led to increased gliadin sub-fractions and decreased glutenin sub-fractions in emmer and wheat proteins. In addition to the lower susceptibility to *Fusarium* infection naked barley responded differently to fungal attack than emmer and wheat. Naked barley proteins also showed increased hordein and hordenin sub-fractions during early grain development. The hordenins were apparently degraded by fungal proteases in later grain development stages. Furthermore, emmer and wheat grains showed distinct higher toxin contents than naked barley. In naked barley grains the toxin contents increased with grain ripening, whereas they were rather constant in emmer and wheat.

**Introduction**

*Fusarium spp.* infection of cereals causes losses in yield, grain- and seed quality worldwide. In Europe the predominant *Fusarium* species occurring in cereals *F. culmorum* and *F. graminearum* (Kazan et al., 2012). These species produce numerous mycotoxins, such as the trichothecenes deoxynivalenol (DON), the acetylated derivates

(AcDON) and nivalenol (NIV) as well as zearalenone (ZEA) (Bottalico and Perrone, 2002). Emmer is closely related to the tetraploid durum wheat and exhibits lower values of high-molecular-weight-glutenins (HMW-GS) and a high ratio of gliadins/glutenins compared to other wheat species, which is negatively correlated with bread making quality (Wieser, 2000). Nevertheless, the interest in emmer products, such as bread, pasta and traditional foods lately increased worldwide due to its specific taste and flavour as well as nutritional qualities of emmer products compared to durum or bread wheat (Zaharieva et al., 2010). Furthermore, some emmer and wild emmer cultivars are resistant to pests, diseases and abiotic stresses, which increasingly serve as reservoir of genes in wheat breeding (Buerstmayr et al., 2003). Hull-less (naked) barley is mostly used for animal feed. Considering human nutrition, naked barley has the potential for good quality malt for whisky and beer production (Agu et al., 2009). Furthermore, naked barley flour is supposed to be a good supplement in wheat bread formulations, providing nutritional advantages in comparison to wheat, such as a high β-glucan and mineral contents (Choi et al., 2011).

An infection with *Fusarium spp*. is known to cause degradation of protein components in cereal grains. Recent studies provide information about adverse effects of Fusarium infection on the protein degradation and thus the processing quality of emmer-, wheat-, naked barley- and barley grains, showing that emmer and wheat were more affected than barley and naked barley (Eggert et al., 2010a, Eggert et al., 2010b, Oliveira et al., 2013).

Several studies confirm host-specific infection strategy and pathogenicity concerning the infection of wheat and barley with *F. graminearum*. For example, trichothecenes are a virulence factor in wheat, but obviously not in barley. The infection of adjacent spikelets is limited in barley, whereas this defence is inhibited by trichothecenes in wheat (Jansen et al., 2005, Maier et al., 2006). According to a comparative analysis of the transcriptome of wheat and barley during *F. graminearum* infection, the infection of wheat was more rapid than in barley. Furthermore, genes expressed during *Fusarium* infection of wheat compared to barley were remarkable different (Lysøe et al., 2011). Frequently, infection pattern, mycotoxin production, as well as defence response of wheat and barley in terms of *Fusarium spp.* infection is in the focus of research. Nevertheless, several studies were performed to investigate the impact of *Fusarium* infection on grain protein composition. Wang et al. (2005) and Boyacloglu and

Hettiarachchy (1995) analysed the impact of *Fusarium spp.* infection on protein quality parameters, showing the degradation of wheat protein and the effect on processing quality parameters. A current study describes the *in vitro* degradation of wheat gluten protein by *F. graminearum* proteases, finding out that the HMW- glutenin sub-fraction of wheat was most affected (Eggert et al., 2011). However, in these studies grains at the final stage of ripening were used and therefore the digestion of extracted protein from ripe grains was investigated. So far, the influence of a Fusarium infection during a long infection period on the storage protein composition in the ripening grain was not yet studied. Several studies showed that environmental factors, such as soil moisture and temperature regimes cause changes in gluten-protein composition already in the early grain development and therefore affect the processing quality of the final products (Panozzo et al., 2001, Daniel and Triboi, 2002, Naeem et al., 2012). It is likely that the synthesis of the different gluten-protein types is dependent on the timing and type of stress the plant is exposed to, and that the susceptibility to protein-degradation by fungal attack is dependent on the timing of protein biosynthesis and polymerisation. The aim of this work was to investigate the impact of *F. graminearum* infection on the storage protein composition of emmer, wheat and naked barley during grain development.

## Materials and methods

### Experimental design and sample preparation

The field trial was carried out at Marienstein (Nörten-Hardenberg), near Göttingen in 2011. Two emmer genotypes (Linie 9-102; Klein), three naked barley genotypes (Lawina; 00/900/5N; ZFS) and one summer wheat (Ameretto) was grown in a field trial, sowed in spring 20011. The plants were grown in eight replications. Each plot had a dimension of three to six meters. Four replications were artificially inoculated with an *F. graminearum* spore *suspension* ($20^5$ spores/mL; 50 mL/$m^2$ three times during flowering. Three DON producing strains of *F. graminearum* (FG 142, FG 143, FG 144) were cultured on an autoclaved wheat straw suspension, consisting of nine g straw (1.5 mm), 500 mL distilled water and 50 mg streptomycine sulfate for ten days at 20°C. The DON producing strains were reference stocks from the Division of Plant Pathology and Crop Protection at the Department of Crop Science of the Georg-August-University of Göttingen. After harvesting, the quantification of the conidiospores was conducted with a Fuchs-Rosenthal chamber (0.0625 $mm^2$; depth: 0.2 mm). Grain samples of the emmer cultivars from each plot (four inoculated, four naturally infected) were collected four times and naked barley grains as well as wheat grains were gathered five times during grain development. The ripening stages of the cereals were identified using the extended BBCH-scale. The BBCH-scale defines the phenological growth stages with a standardised decimal code. The abbreviation „BBCH" derives from Biologische Bundesanstalt, Bundessortenamt and chemical industry (Meier, 2001). The days after inoculation (dai), which are synonymous with the days after anthesis (daa) were documented (Table 3.1).

**Table 3.1:** Days after inoculation (dai) (anthesis: 0 dai) of emmer, wheat and naked barley plants and the corresponding phenological growth stages

| Phenological growth stage (BBHC code) | Days after inoculation (dai) | | |
|---|---|---|---|
| | Emmer | wheat | Naked barley |
| early milk (73) | -- | 19 | 7 |
| medium milk (75) | 19 | -- | -- |
| late milk (77) | | 26 | 14 |
| soft dough (85) | 25 | 36 | 21 |
| hard dough (87) | 32 | 47 | 36 |
| plant death (97) | 39 | 54 | 54 |

-- No sample was obtained

Fifty ears from each plot were harvested and freeze dried. Afterwards the grains were removed manually and milled with a ball mill (Mixer Mill MM 400, Retsch®, Han, Germany). Samples were stored at −80 °C prior to analysis.

**Protein extraction for RP-HPLC and SDS-Page**

The protein-fractions were extracted according to Wieser (1998). To extract the albumins and globulins together, 100 mg sample was extracted two times with 1 mL sodium phosphate-buffer (0.4 M NaCl; 0.067 M HKNaPO4; pH 7.6) under magnet-stirring at room temperature for 20 min. The samples were centrifuged for 15 min at 7500 rpm and 20°C and the supernatants were combined and filled up to 2 mL with the sodium phosphate-buffer. To receive the gliadins, the residual pellets were extracted with 500 µL 60% (v/v) ethanol under magnet-stirring for 15 min at room temperature. The samples were centrifuged (15 min; 7500 rpm; 20°C) and the supernatants were collected. This procedure was repeated three times, the supernatants were combined and the volumes are filled up with 60% (v/v) ethanol up to 2 mL. In a final step the glutenins were extracted two times with 1 mL extraction solution, containing 50% 1-propanol; 2 M urea; 0.05 M tris buffer (pH 7.5) and 1% DTT with shaking at 60°C for 30 min in a Thermomixer (Thermomixer comfort, Eppendorf, Hamburg). The samples were centrifuged (15 min; 7500 rpm; 20°C), the supernatants were combined and filled up to 2 mL with extraction solution. The extracts were filtered through 0.45 µm syringe filter and stored at -20°C prior to HPLC injection.

**Analysis of protein fractions with RP-HPLC**

For the separation of the gliadin and glutein subunits a PerfectSil 300 C8 (300 × 4.6 mm; 5 µm) analytical column (MZ Analysentechnik, Mainz, Germany) was used. The mobile phases were A: 0.1% TFA in $H_20$ and B: 0.1% trifluoroacetic acid (TFA) in acetonitrile. The flow rate was 1 mL/min and the column temperature was set to 50°C. The separation was performed with the following gradient: 0 min 100% A; 5 min 76% A; 50 min 50% A; 54 min 10% A. For the gliadins and hordeins 50 µL was injected and for the glutenins and hordenins 100 µL was injected for separation.

**Sample preparation for SDS-Page**

Aliquots of 500 µL of the albumin+globulin-; gliadin- and glutenin extracts were collected in a 2 mL tube and the containing protein was precipitated with 1.5 mL ice-cold acetone, containing 10% TCA. The suspension was stored at -20°C over night for

entire precipitation. Afterwards the samples were centrifuged (10 min; 4°C; 10000 rpm) and the residual pellets were washed three times with ice-cold acetone. The pellets were dried in a vacuum concentrator (RVC 2-25 CD, Christ, Germany) at 100 mbar for 10 min. The separation of the proteins was performed according to the method of Laemmli (1970). The pellets were dissolved in 500µL (albumin + globulin), and 250 µL (gliadin) sample buffer (2% SDS; 20% glycerol; 0.050 M Tris-HCl (pH8.8); 1% DTT; 0.01% bromophenol blue). For solving the protein pellets, the solutions were treated 5 min in an ultrasonic bath and are furthermore heated at 90°C for 3 min in a thermomixer (Thermomixer comfort, Eppendorf, Hamburg). The electrophoresis was conducted in a mini SDS-Page cell (Bio-Rad Laboratories GmbH, Munich, Germany) with 5% T stacking gels and 15% T separating gels. To each slot 10 µL sample solution was applied. Every gel was run with a Precision Plus Protein molecular weight standard (Bio-Rad Laboratories GmbH, Munich). Finally, the gels were stained with a modified colloidal coomassie G-250 staining (blue silver) (Candiano et al., 2004).

**Analysis of albumins and globulins**

The amount of albumin/globulin was determined from the albumin+globulin phosphate buffer- extract by using the BioRad Protein Assay Dye Reagent Concentrate (catalog number 500-0006), based on the Bradford method (Bradford, 1976).

**Quantitative nitrogen analysis**

For the nitrogen analysis each 100 mg sample was measured with the vario MAX C/N-analyser (elementar, Hanau, Germany).

**Quantitative LC-MS/MS of mycotoxins**

Trichothecene and ZEA determination were performed with small variations as described by (Adejumo et al. (2007a) and (Adejumo et al. (2007b), respectively. Both analyses were conducted in the laboratory of Molecular Phytopathology and Mycotoxin research, Department of Crop Sciences, University of Göttingen, Germany.

**Statistical analysis**

All analyses were run with four biological replications. Statistica 10 software (StatSoft Inc., Tulsa, USA) was used to calculate the mean values and standard deviations and to apply the normality test, equal variance test and ANCOVA analysis.

## Results and discussion

### The influence of *Fusarium graminearum* infection on the DON and ZEA concentrations in ripening grains of emmer, wheat and naked barley

An inoculation with *Fusarium graminearum* led to increased contents of DON and ZEA in all investigated cereal grains, whereas natural infected grains showed no detectable toxin concentrations. Table 3.2 shows the contents of DON and ZEA in emmer, wheat and naked barley after artificial inoculation in comparison to natural infection. Comparing the toxin contents of the inoculated emmer and wheat grains with naked barley grains, less than one-tenth was found in naked barley. These results demonstrate a successful inoculation of all cultivars as well as a higher infection pressure of *F. graminearum* in emmer and wheat than in naked barley. Considering the changes of toxin contents during grain ripening, clear differences can be seen between emmer, wheat and naked barley after artificial inoculation. Whereas the DON and ZEA contents during grain development of emmer and wheat were rather constant, the DON content in naked barley increased. Regarding wheat grains the DON content was slightly lower in the milk ripe stages and remained constant from the soft dough stage on. ZEA predominantly appeared in later development stages, except for the emmer grains, where it occured in all development stages. The higher toxin content in emmer and wheat compared to naked barley confirms the results of Eggert et al. (2010a; b,) finding higher resistance to *Fusarium* infection of barley and naked barley compared to wheat and emmer. Eggert et al. (2010b) suggested that the resistance to pathogen spread within spikelets and therefore the rather localized *Fusarium* infection, or the higher appearance of protease inhibitors and pathogenesis-related proteins were responsible for the higher resistance of barley and naked barley. On the other hand natural occurring mechanisms in cereals are known leading to a reduction of *Fusarium* trichothecene mycotoxins (Wegulo, 2012). Mechanisms causing low toxin accumulation are referred to as type V resistance to *Fusarium* infection (Champeil et al., 2004). There are two possibilities to reduce toxin contents in cereals. The trichothecenes can be detoxified by biochemical transformation of the chemical structure induced in the plants. The other possibility is the inhibition of fungal trichothecene biosynthesis as response to the pathogen attack by the plants (Boutigny et al., 2008). The regulation of trichothecene biosynthesis in *Fusarium spp.* has been demonstrated to be a response to plant defence mechanisms (Kazan et al., 2012). For example, DON is inhibiting the plant cell-wall

thickening in the rachis node, which is suspected to be a barrier for hyphal growth in wheat (Jansen et al., 2005). Several studies, comparing *F. graminearum* trichothecene knockout wheat mutants with the wild type, associated reduced DON levels with reduced virulence within adjacent spikelets. Barley showed natural type II resistance (spreading of the infection between adjacent spikelets), without altered pathogenesis according to DON-presence. (Jansen et al., 2005, Maier et al., 2006, Kazan et al., 2012) Furthermore, Ilgen et al. (2009) demonstrated that *TRI5* gene expression, a gene encoding trichothecene synthase, is induced in the plant tissue through which the mycelium is growing and therefore the developing wheat- kernel influences the trichothecene induction. The highest induction of *TRI5* was found in later infection stages in the rachis node and not in the anthers, suggesting that DON is not a virulence factor during initial infection (Ilgen et al., 2009). It is currently not completely understood how DON affects the virulence on plant tissues. Hardly information is available about the infection pattern and the function of mycotoxins in infected barley compared to wheat. It can be assumed, that trichothecene- production in emmer and wheat compared to barley have different relevance according to the pathogenicity of *F. graminearum* during grain development.

**Table 3.2:** Toxin concentrations (mg kg $^{-1}$) in artificially *Fusarium*- infected and in naturally infected emmer, wheat and naked barley grains during grain ripening

| | | **Natural infection** | | **Inoculation** | |
|---|---|---|---|---|---|
| | | **DON** | **ZEA** | **DON** | **ZEA** |
| Cereal | **BBCH** | **MV ± SD** | **MV ± SD** | **MV ± SD** | **MV ± SD** |
| Emmer | 75 | < LOD | < LOD | 13.1 ± 5.2 | 0.1 ± 0.2 |
| | 85 | < LOD | < LOD | 18.2± 6.3 | 0.2 ± 0.1 |
| | 87 | < LOD | < LOD | 16.2± 3.1 | 0.1 ± 0.1 |
| | 97 | < LOD | < LOD | 17.5± 4.4 | 0.2 ± 0.0 |
| Wheat | 73 | < LOD | < LOD | 6.1 ± 3.0 | < LOD |
| | 77 | < LOD | < LOD | 5.1 ± 1.9 | 0.0 ± 0.0 |
| | 85 | < LOD | < LOD | 18.0 ± 2.8 | 0.0 ± 0.0 |
| | 87 | < LOD | < LOD | 17.5 ± 10.7 | 0.1 ± 0.1 |
| Naked barley | 73 | < LOD | < LOD | < LOD | < LOD |
| | 77 | < LOD | < LOD | 0.4 ± 0.4 | < LOD |
| | 85 | < LOD | < LOD | 1.5 ± 0.7 | < LOD |
| | 87 | < LOD | < LOD | 2.6 ± 1.6 | 0.3 ± 0.6 |
| | 97 | < LOD | < LOD | 4.5 ± 7.8 | 0.2 ± 0.2 |

LOD= limit of detection; MV= mean value; SD= standard deviation

**Classification of cereal proteins**

The grain storage proteins are classified according to their solubility into albumins, globulins, prolamins and glutelins (Osborne, 1909). In most cereals, the prolamins are the major protein type. In wheat and emmer the prolamins are called gliadins and in barley hordeins, based on the Latin generic name. Therefore, the glutelins are called glutenins in wheat and hordenins in barley. According to Shewry and Halford (2002) the prolamins of triticeae (e.g. wheat, barley) can be classified into three groups on the basis of their amino acid composition: sulphur-rich (s-rich), sulphur poor (s-poor) and high molecular weight (HMW) prolamins. The α- and γ-gliadins and the γ-hordeins belong to the S-rich prolamins, whereas the ω-gliadins and the C-hordeins belong to the s-poor prolamins. The S-rich and S-poor prolamins also occur in polymeric forms with interchain disulfide bonds, which represent the predominant low-molecular-weight (LMW)-subunits of the glutelins with about 20% according to total gluten proteins (Wieser, 2007, Shewry and Tatham, 1990, Shewry and Halford, 2002). The prolamin and glutelin sub-fractions were separated with RP-HPLC and classified as described in Figure 3.1.

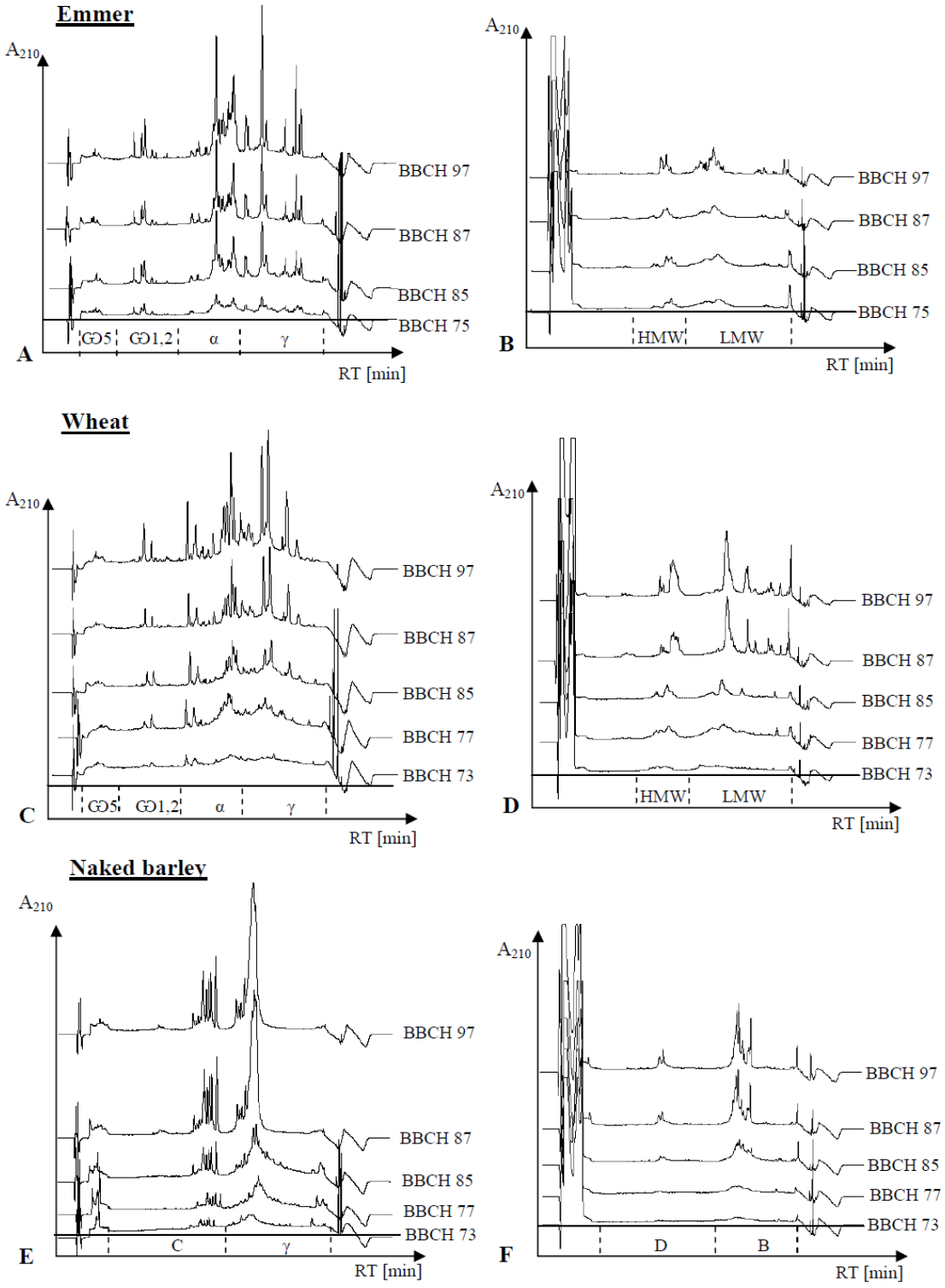



**Figure 3.1:** RP-HPLC-chromatograms of protein fractions in emmer (cv. Klein) **A** gliadins **B** glutenins; wheat (cv. Amaretto) **C** gliadins **D** glutenins and naked barley (cv. Lawina) **E** hordeins **F** hordenins. The phenological growth stages are related to the days after inoculation according to Table 3.1

**Accumulation of nitrogen and soluble proteins in ripening grains of emmer, wheat and naked barley and the effect of *F. graminearum* inoculation**

The biosynthesis of cereal storage protein, especially for wheat has been studied extensively e.g. (Kawakatsu and Takaiwa, 2010, Abonyi et al., 2007, Shewry and Halford, 2002). With the current study nitrogen and protein accumulation during grain filling and grain ripening of wheat, emmer and naked barley can be compared directly.

The ripening period of emmer was considerably shorter than that of wheat and naked barley. The period from flowering to harvest was 39 days for emmer and 54 days for wheat and naked barley. Milk ripe grains occurred at 19 days after anthesis (daa) for emmer and wheat. In contrast, naked barley cultivars showed milk ripe grains after less than 10 days. Figure 3.2 A, B and C shows changes of the nitrogen content and the albumin + globulin proteins during grain development of emmer, wheat and naked barley after natural infection and artificial inoculation, regarding the days after inoculation (dai). Overall, emmer grains reached about 10 percent higher nitrogen contents than naked barley and wheat. During grain development of natural infected wheat the nitrogen content remained nearly constant, whereas in emmer a significant increase from milk ripe stage to plant death stage was visible (Table 3.3). The nitrogenous compounds can be expressed as crude protein content by multiplying the corresponding total nitrogen content by a conventional factor according to ICC No. 105/2. This standard is only applied for mature grains for which reason the crude protein is not calculated for partly immature grains here.

After *F. graminearum* inoculation the nitrogen contents of emmer and wheat were significantly higher at all maturity stages in comparison to natural infection (Figure 3.2 and Table 3.3). The nitrogen content of developing naked barley was decreasing after the early milk stage and increasing again since hard dough stage. In emmer the difference between artificial inoculation and natural infection was increasing with grain ripening (Figure 2A). Naked barley showed no significant effect of *F. graminearum* infection on the nitrogen accumulation (Figure 3.2C and Table 3.4). Siuda et al. (2010) detected increased protein contents in *Fusarium culmorum* infected wheat grains and explained this as a possible result of the lower carbohydrate content caused by the developing fungus feeding from carbohydrate metabolites. In contrary, Wang et al. (2005) found no effect of *Fusarium* infection on the crude-protein content of wheat grains. Boyacloglu and Hettiarachchy (1995) detected increased protein contents in

moderately infected wheat and decreased protein contents in lightly infected wheat. In the current study naked barley had the lowest toxin contents and showed the lowest effect on the nitrogen contents.

The amounts of albumins + globulins in emmer were constant during grain development, whereas the concentrations of albumin + globulin were decreasing in wheat and naked barley (Figure 3.2). These differences can be possibly explained by the shorter grain ripening period of emmer compared to wheat and naked barley.

After *F. graminearum* inoculation the albumin + globulin fractions of emmer and naked barley were slightly decreasing since the early grain development. The decrease was probably caused by fungal degradation or by dectreased formation of these proteins in the grains. However this effect was not significant in wheat (Table 3.3 and 3.4).

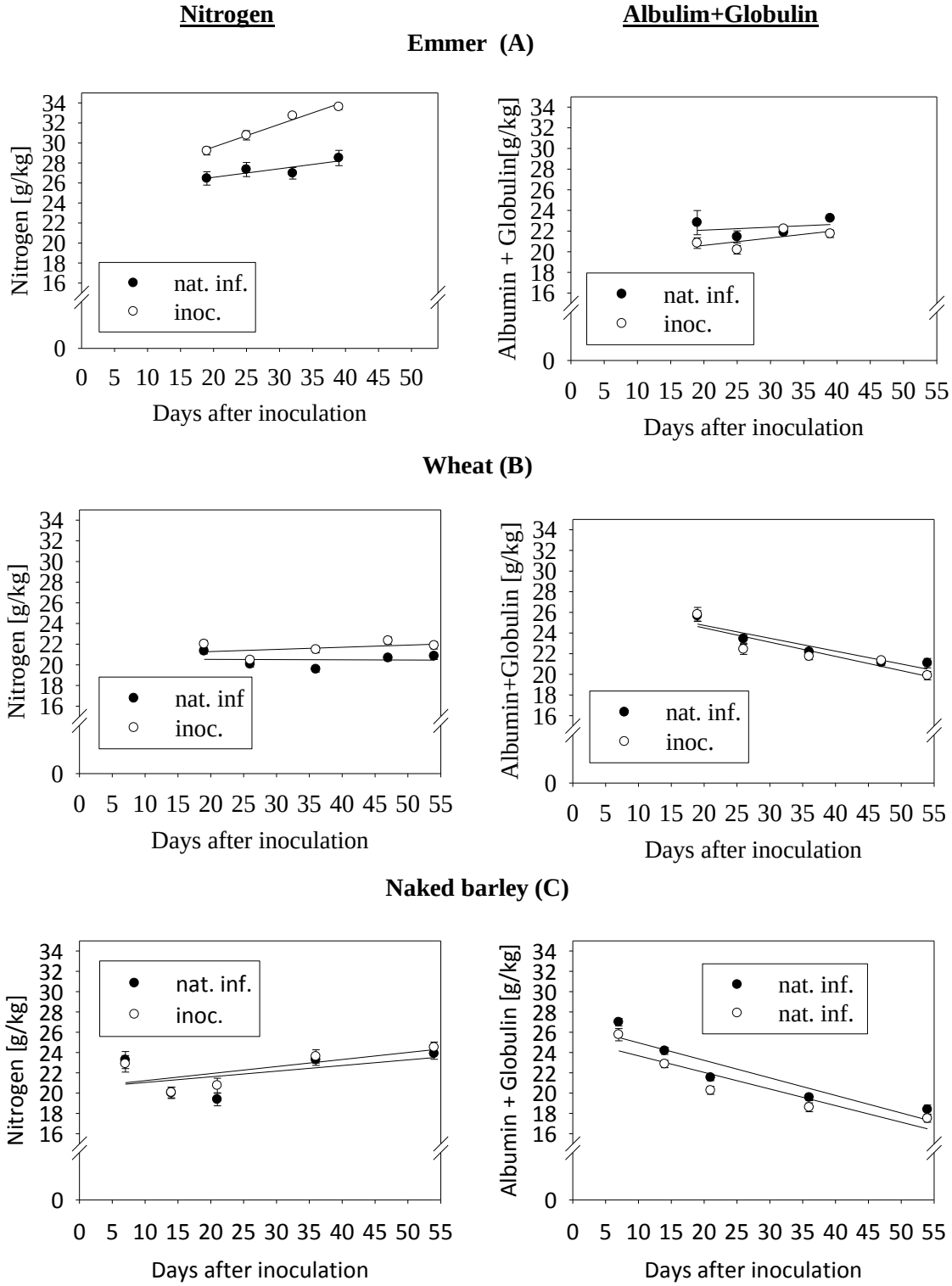



**Figure 3.2:** Nitrogen content and albumin+globulin content in naturally infected (nat. inf.) and inoculated (inoc.) samples of A emmer (n = 8), B wheat (n = 4) and C naked barley (n = 12) during grain ripening. The phenological growth stages are related to the days after inoculation according to Table 3.1

**Accumulation of gluten-forming proteins in ripening grains of emmer, wheat and naked barley and the effect of *F. graminearum* inoculation**

Among the gluten-forming proteins α- and γ- gliadins (S-rich) made up the major protein fraction of emmer and wheat. Both sub-fractions showed similar accumulation during grain ripening. However, the highest α- and γ- gliadins were detected in emmer (Figure 3.1 A-D and Figure 3.3 A, B). In emmer and wheat grains α- and γ- gliadins were doubled from the milk ripe to the plant death stage. The ω-gliadins (S-poor) of emmer and wheat were predominantly constant during the grain development with the exception of the emmer- ω1,2-gliadins, which were significantly increased during grain ripening (Table 3.3).

HMW-GS and LMW-GS subunits already occurred at the early grain development in both varieties. The glutenin subunits in emmer showed no significant increase during grain development, whereas the accumulation of HMW- and LMW subunits of wheat were distinctly increased in the course of grain ripening (Figure 3.5). These differences could be partly explained with the longer ripening period of wheat compared to emmer, accompanied by receiving earlier grain development stages for wheat in this experiment. However, emmer had lower proportions of LMW-GS and HMW-GS compared to wheat. Similar results are available from Wieser (2000) comparing the gluten proteins from different wheat species, including spring wheat and emmer.

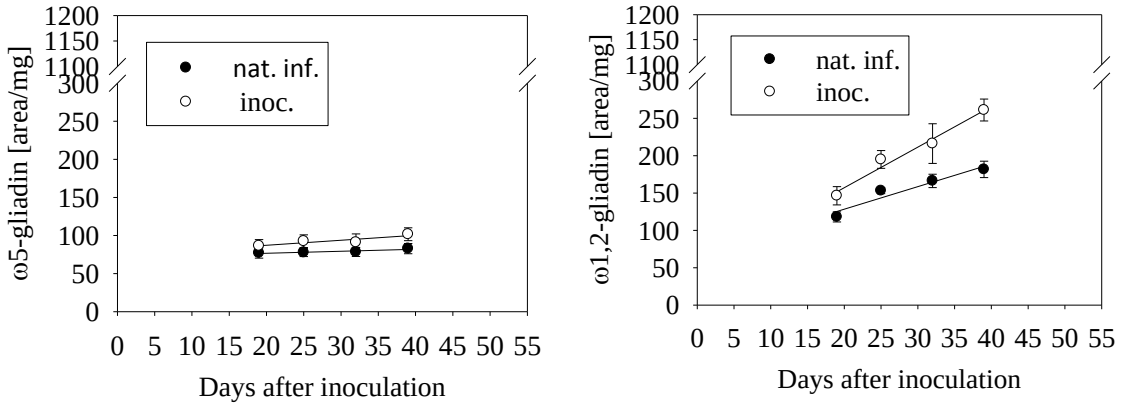
In naked barley grains the γ-hordeins made up the main fraction of prolamins, followed by C-hordeins (Figure 3.1 E, F). All protein sub fractions of naked barley were increasing significantly during grain ripening (Table 3.4). The formation of the prolamin-subunits occurs synchronously. In the early grain development a rapid increase of prolamine subunits can be observed, which is getting lower at later ripening stages, since hard dough stage (dai 36). The accumulation of polymeric hordenin sub-fractions is similar to the hordein sub-fractions (Figure 3.4 and 3.6).

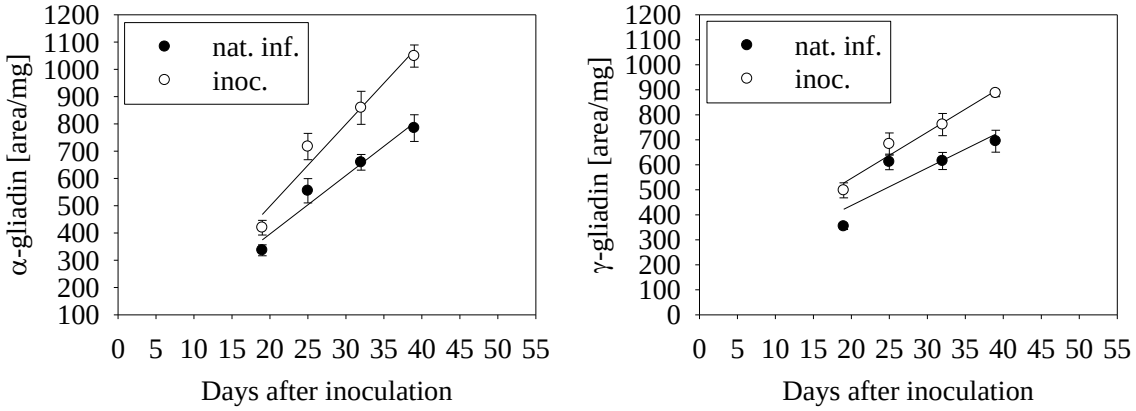
Regarding the effect of artificial *F. graminearum* infection on the gluten-forming proteins of emmer and wheat, all gliadin sub-fractions were increasing between 10- and and 40 percent, whereas the glutenin sub-fractions were decreasing due to Fusarium infection (Figure 3.3, 3.5). These results are consistent with other studies, focussing on the response of mature wheat grains to *Fusarium ssp.* infection and the impact on protein quality parameters. A distinct reduction of glutenins and a slight increase of gliadins were detected after *F. culmorum* infection in wheat grains in a study of Wang

et al. (2005). Eggert et al. (2010a) detected stronger effects of *Fusarium* infection on the gluten-protein of wheat compared to emmer, in spite of a lower infection degree of wheat. In the current study, from the early stage of grain development until maturity, the glutenin subunits of both emmer and wheat were significantly affected by *F. graminearum* infection (Table 3.3). The effect of *Fusarium* infection on gliadin and glutenin subunits due to *Fusarium* infection tended to be higher at later grain development stages.

Similar to emmer and wheat, *F. graminearum* infection of naked barley led to significantly increased ethanol-soluble hordein-fractions (Figure 3.4 and Table 3.4). However, this effect is lower in naked barley compared to emmer and wheat. In contrast, Eggert et al. (2010b) found a slight, but not significant reduction of hordeins and hordenins in mature naked barley grains after *F. graminearum* + *F.culmorum* infection. Regarding the effect of *F. graminearum* infection during grain ripening, the relative change of hordein subunits was slightly decreasing from the early milk stage the plant death stage (Figure 3.4). Contrary to emmer and wheat, the glutelin- (hordenin) subunits of naked barley grains were increased until soft dough stage (21 dai) after *Fusarium* infection, whereupon they were decreasing with beginning of the hard dough stage compared to natural infected grains (Figure 3.6). Apparently the hordenins in naked barley grains were first up-regulated due to *Fusarium* inoculation during the grain fill stage and were later digested by fungal proteases.

**Gliadins** **Emmer (A)**






**Wheat (B)**

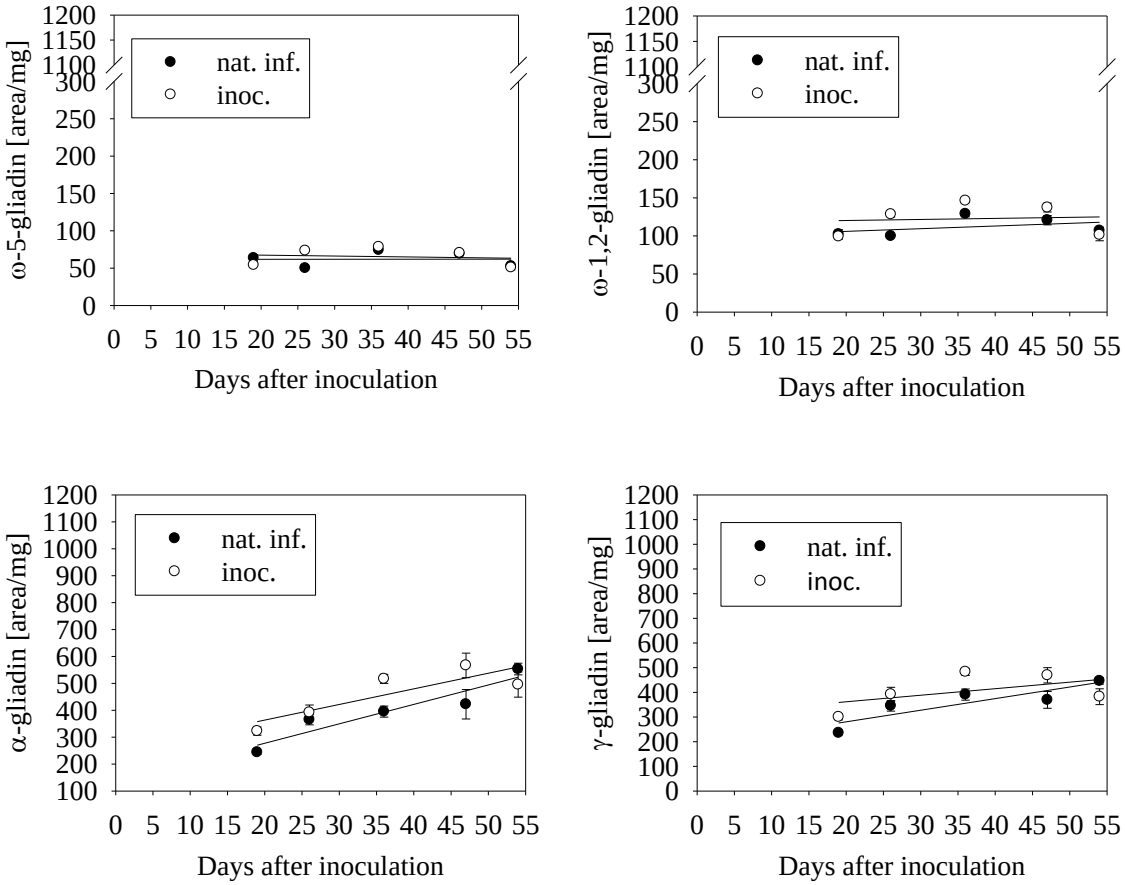



**Figure 3.3:** Accumulation of gliadin sub-fractions of naturally infected (nat. inf.) and inoculated (inoc.) A emmer (N=8) and B summer wheat (N=4) during grain ripening. The phenological growth stages are related to the days after inoculation according to Table 3.1

**Hordeins** **Naked barley**

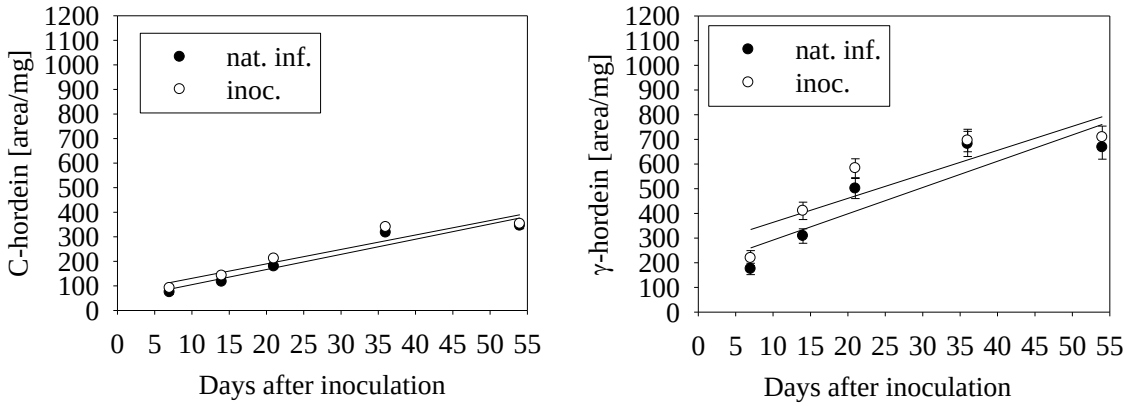
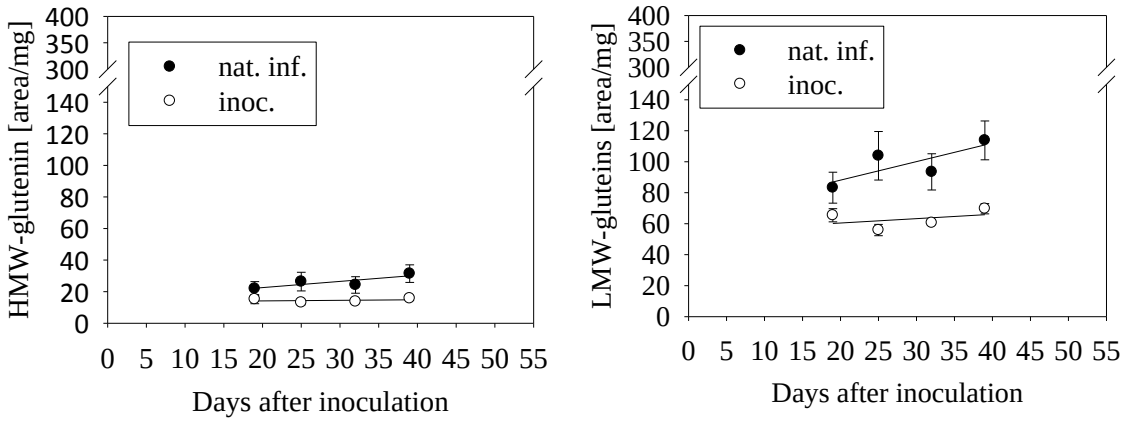



**Figure 3.4:** Accumulation of hordein sub-fractions of naturally infected (nat. inf.) and inoculated (inoc.) naked barley (n=12) during grain ripening. The phenological growth stages are related to the days after inoculation according to Table 3.1

**Glutenins** **Emmer (A)**




**Wheat (B)**

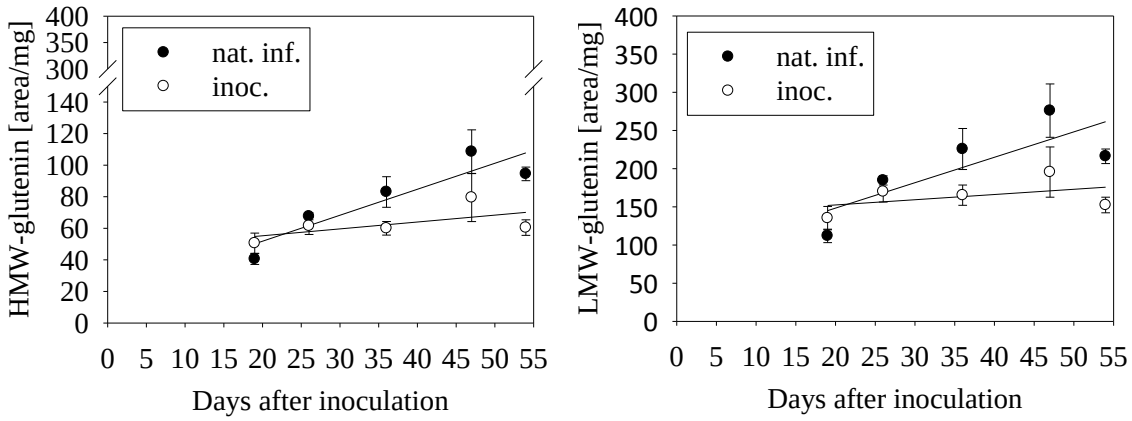



**Figure 3.5:** Accumulation of glutenin sub-fractions of naturally infected (nat. Inf.) and inoculated (inoc.) summer wheat (n=4) during grain ripening. The phenological growth stages are related to the days after inoculation according to Table 3.1

**Hordenins** **Naked barley**

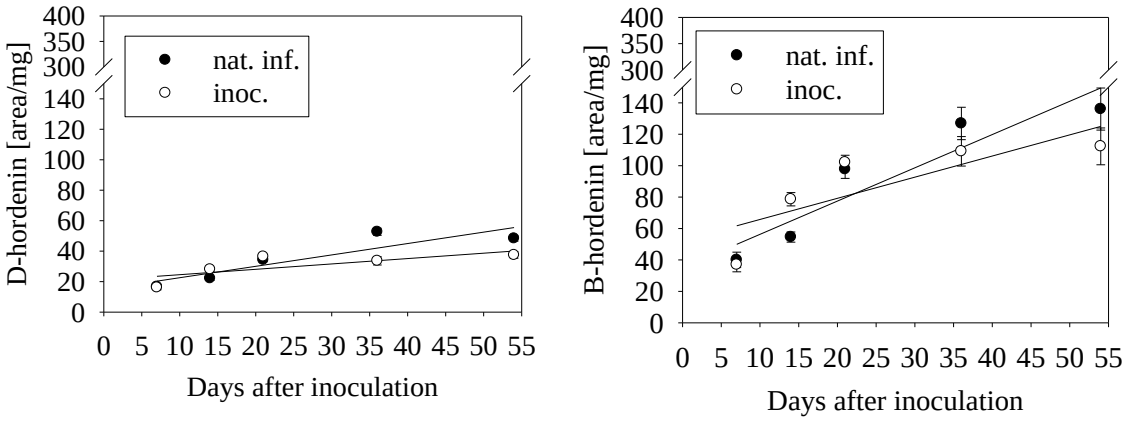



**Figure 3.6:** Accumulation of hordenin sub-fractions of naturally infected (nat. Inf.) and inoculated (inoc.) naked barley (n=12) during grain ripening. The days after inoculation (dai) are related to the phenological growth stages according to Table 3.1

**Table 3.3:** Significance (p-values) for changing of nitrogen and protein (sub)-fractions of emmer and wheat due to *F. graminearum* infection according to ANCOVA analysis (dai as co-factor)

| | | | | Gliadin fractions | | | | Glutenin fractions | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | | N | Alb+glob | GƆ5 | GƆ1,2 | α | γ | HMW | LMW |
| Emmer | Dai | 0.00*** | 0.06 ns | 0.19 ns | 0.00*** | 0.00*** | 0.00*** | 0.24 ns | 0.09 ns |
| | Inoc. | 0.00*** | 0.01** | 0.01** | 0.00*** | 0.00*** | 0.00*** | 0.00*** | 0.00*** |
| Wheat | Dai | 0.37 ns | 0.00*** | 0.68 ns | 0.27 ns | 0.00*** | 0.00*** | 0.00*** | 0.00*** |
| | Inoc. | 0.00 | 0.17 ns | 0.32 ns | 0.07 ns | 0.01** | 0.03* | 0.01** | 0.01** |

Dai= days after inoculation; Inoc. = inoculated; p significance: ***, **, * = p < 0.001, 0.01, 0.05 and ns= not significant

**Table 3.4:** Significance (p-values) for changing of nitrogen and protein (sub)-fractions of naked barley due to *F. graminearum* according to ANCOVA analysis (dai as co-factor)

| | Treatment | N | Alb+glob | Hordein fractions | | Hordenin fractions | |
|---|---|---|---|---|---|---|---|
| | | | | C | γ | D | B |
| Naked barley | Dai | 0.00*** | 0.00*** | 0.00*** | 0.00*** | 0.00*** | 0.00*** |
| | Inoc. | 0.40 ns | 0.00*** | 0.01** | 0.05* | 0.01** | 0.58 ns |

Dai= days after inoculation; Inoc. = inoculated; p significance: ***, **, * = p < 0.001, 0.01, 0.05 and ns= not significant

**SDS-Page analysis of *F. graminearum* induced degradation of emmer and naked barley storage proteins**

The inoculation of emmer grains with *F. graminearum* resulted in increased gliadin fragments especially in low molecular weight range since the early grain-development (Figure 3.7). Eggert et al. (2011) revealed an enzymatic digestion of wheat gliadin- and glutenin subunits by *Fusarium* proteases *in vitro*. The glutenin proteins were obviously degraded by *Fusarium* proteases into lower molecular weight proteins and peptides (Figure 3.8). According to a previous study of Eggert et al. (2011), degraded glutenin-fragments were partially co- extracted with the gliadins, pretending increased gliadin fragments. Therefore, the elevated gliadin fragments could partly derive from the degraded glutenin in the current study. The ethanol- soluble hordeins of the naked barley were degraded due to the *F. graminearum* infection similar to the emmer-gliadins (Figure 3.9). In contrast, hardly any changes are noticeable regarding the hordenins with SDS-Page analysis. A slightly higher fragmentation appeared in the inoculated samples (Figure 3.10).

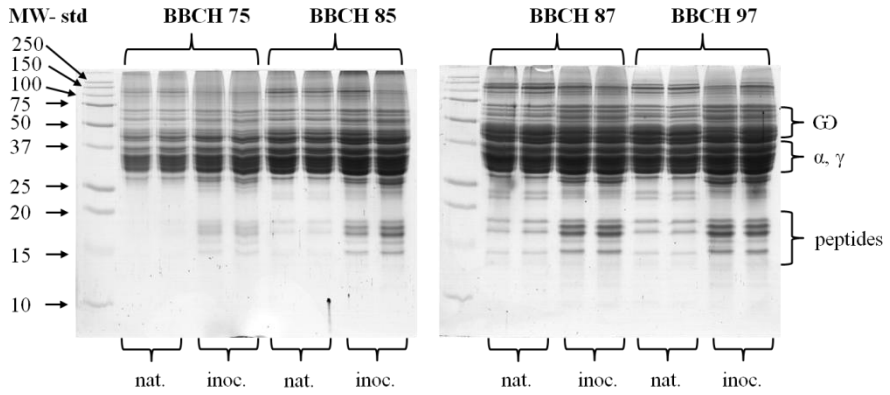



**Figure 3.7:** SDS-Page of emmer (cv. Klein) gliadins after artificial inoculation (inoc.) and natural infection (nat.) at four grain development stages. The phenological growth stages are related to the days after inoculation according to Table 3.1

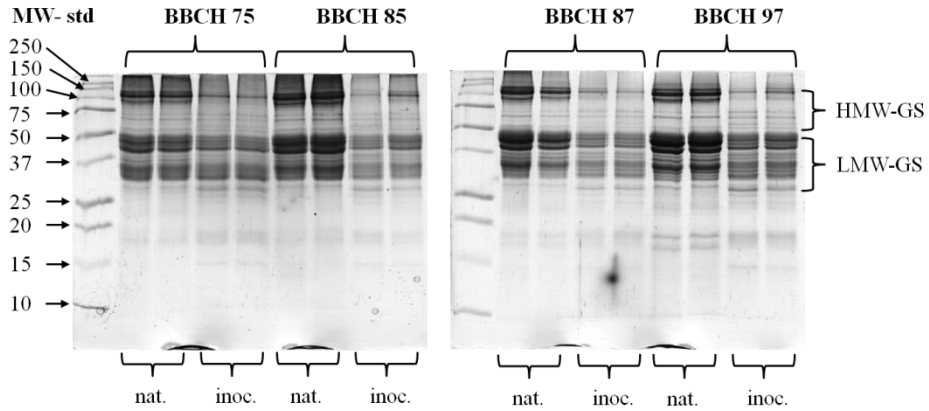



**Figure 3.8:** SDS-Page of emmer (cv. Klein) glutenins after artificial inoculation (inoc.) and natural infection (nat.) at four grain development stages. The phenological growth stages are related to the days after inoculation according to Table 3.1

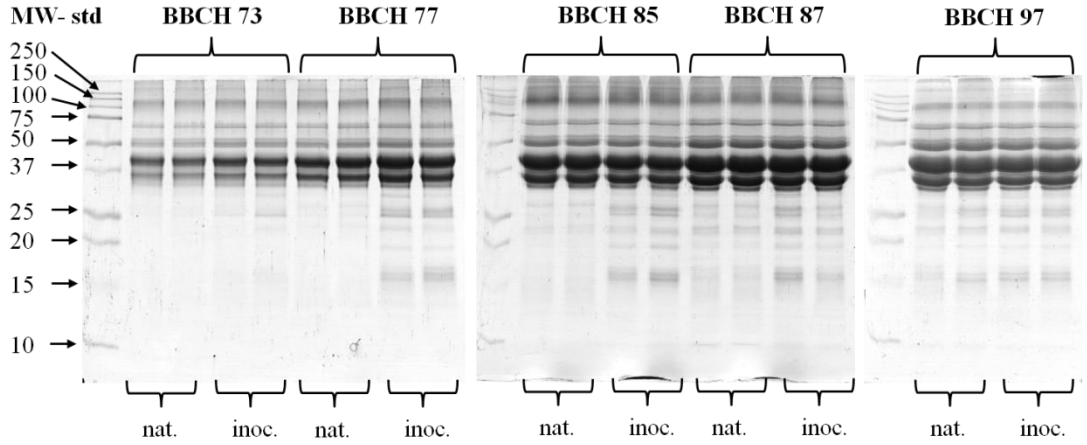



**Figure 3.9:** SDS-Page of naked barley (cv. Lawina) hordeins after artificial inoculation (inoc.) and natural infection (nat.) at five grain development stages. The phenological growth stages are related to the days after inoculation according to Table 3.1

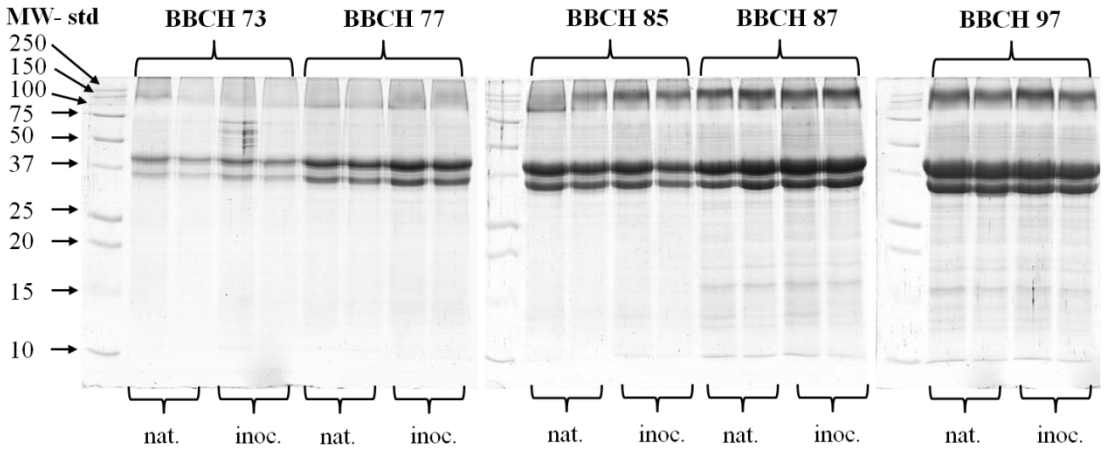



**Figure 3.10:** SDS-Page of naked barley (cv. Lawina) hordenins after artificial inoculation (inoc.) and natural infection (nat.) at five grain development stages. The phenological growth stages are related to the days after inoculation according to Table 3.1

It is known that *Fusarium* species produce several proteases, which are degrading cereal storage protein in infected cereals. These fungal enzymes are predominantly alkaline serine proteases, such as trypsin-like and subtilisin-like proteases (Pekkarinen et al., 2000, Pekkarinen et al., 2002).

The inhibition of fungal proteases seems to be an important resistance factor in cereals, as the pathogen needs the nutrients for expansion of the fungal mycelium in the plant. Protease inhibitors that are known to occur in cereals are the Bowman-Birk-, the chymotrypsin/subtilisin-, the bifunctional α-amylase/subtilisin (BASI) inhibitors and serpins (Pekkarinen and Jones, 2003, Gorjanović, 2009).

The weaker effect of *F. graminearum* infection on naked barley compared to emmer and wheat, especially in later development stages, could be explained by a more effective protease inhibition if the infected naked barley grains. Therefore, further studies should be established comparing the intensity of protease inhibition during grain development of wheat and barley after pathogen attack. Another possibility explaining the lower susceptibility of naked barley is an up-regulation of further pathogenesis related (PR) proteins, retarding fungal growth, such as chitinases, glucanases and thaumatin- like proteins as well as defensins and thionins (Gorjanović, 2009). Although the protein content and composition is in general genetically predetermined, several studies revealed the influence of abiotic environmental factors, such as high-temperature and water-deficit stress on the wheat grain development and protein composition (Daniel and Triboi, 2002, Dupont and Altenbach, 2003, Jia et al., 2012, Naeem et al., 2012). According to Naeem et al. (2012) and Jia et al. (2012) drought,

excess watering and heat stress influences glutenin biosynthesis and post-translational polymerisation of glutenin macropolymers (GMP). It is possible that pathogen attack induces similar molecular mechanisms as abiotic stress as response to the environment, leading to changes in the protein composition in mature grain. This could explain the decreased hordenin- fractions in the early naked barley-grain development, which cannot be explained by the inhibition of fungal growth and protease inhibition.

The impact of pathogen stress on emmer or wheat and (naked) barley storage protein composition during the entire grain development is poorly established. Predominantly the expression of specific proteins in wheat and barley spikes a few days after inoculation has been investigated using proteomic and transcriptome studies (Geddes et al., 2008, Yang et al., 2010, Lysøe et al., 2011). According to these studies, pathogenesis related proteins, such as chitinases and proteins involved in oxidative stress response were up regulated in barley spikelets at two days after inoculation with *F. graminearum*. Moreover, proteins were up regulated in wheat spikelets during initial infection suggesting an increased energy metabolism and protein synthesis during initial *F. graminearum* infection of barley. Further investigations concerning the molecular mechanisms taking place during the entire infection period are needed to understand the stress response and the fungal impact on cereal proteins.

With this study we can confirm, that *F. graminearum* infection causes changes in cereal grain protein composition since the early grain development. Wheat and emmer were more affected than naked barley considering the toxin contents and the protein composition, which confirms earlier studies, showing naked barley more resistant to Fusarium than wheat and emmer. These results indicate a different response of emmer, wheat and naked barley to *Fusarium* infection and a different impact on storage proteins, respectively.

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## 3.2 Effect of Fusarium infection in the field on mycotoxins production in emmer and naked barley grains and the impact on gluten proteins during storage

**Abstract**

In general, *Fusarium* infection in the field negatively influences the quality of cereal grains. Unfavorable post harvest management can strongly contribute to quality losses due to increased microbial activity during storage. Emmer and naked barley cultivars as well as one summer wheat cultivar were grown in a field trial at two locations during the years 2010-2012. In the years 2011 and 2012 the grains were additionally field-inoculated with *Fusarium graminearum*. The effect of storage time (three and six months, respectively) and two different storage conditions on the toxin contents in the grains of emmer and naked barley were investigated. Additionally, changes in protein composition during six months of storage of these grains were analyzed, depending on storage condition and *Fusarium* infection. Naked barley showed lower toxin contents after *F. graminearum* inoculation compared to emmer and wheat as well as different toxin spectra in both natural infected and artificially inoculated grains. Natural infected emmer and wheat showed deoxynivalenol (DON) and zearalenone (ZEA) accumulation, whereas nivalenol (NIV) only accumulated in naked barley. Due to inoculation with DON-producing *F. graminearum* strains, DON and ZEA accumulated strongly in emmer, wheat and naked barley. NIV was again only detected in naked barley. During storage the toxin composition in natural infected grains was hardly changed, whereas in inoculated grains the DON contents in emmer and naked barley cultivars were significantly reduced. The prolamins and glutelins of natural infected and artificial inoculated grains were significantly affected by storage of six months, particularly at rather warm and humid storage conditions. This effect was also observed in *F. graminearum* inoculated grains at colder and dryer storage conditions. However, both storage conditions had only little impact on the toxin contents of the grains, whereas the protein composition has been strongly affected by storage.

**Introduction**

*Fusarium* head blight (FHB) is a disease, caused by series of mycotoxins producing *Fusarium* species, such as *F. graminearum* and *F. culmorum* which are the most prevalent species in Europe (Foroud and Eudes, 2009). Trichothecenes from the B group, such as deoxynivalenol (DON), nivalenol (NIV) and the acetylated forms 3-acetyldeoxynivalenol (3-Ac-DON) and 15-acetyldeoxynivalenol (15-Ac-DON) as well

as zearalenone (ZEA) are the predominant occurring mycotoxins produced by these *Fusarium* species (Bottalico and Perrone, 2002). These mycotoxins accumulate in grains of *Fusarium* infected cereals, such as wheat, barley, oat, rye, maize and rice. DON represents the major *Fusarium* toxin which can be found in feed and food. Due to its high stability DON withstands processing and cooking and thus occurs frequently in processed products (Streit et al., 2012, Bonnet et al., 2012). Ingestion of DON-contaminated grain can seriously compromise humans and animals health (Foroud and Eudes, 2009). Several studies demonstrated a positive relationship between FHB intensity, *Fusarium* damaged kernels and pathogen growth with DON accumulation (Hernandez Nopsa et al., 2012, Sneller et al., 2012). However, it is denoted that FHB severity is not the only a crucial factor for toxin accumulation. *Fusarium* infections in late grain development can lead to DON accumulation in grains even without visible characteristics of the disease (Cowger and Arrellano, 2010, Yoshida and Nakajima, 2010, Del Ponte et al., 2007). Numerous environmental factors, for example climatic conditions (rain fall, temperature) and crop sequence as well as cultivar resistance and related plant defence mechanisms, such as cell wall thickening and production of reactive oxygen species, influence the DON accumulation in cereal grains in the field (Merhej et al., 2011, Popovski and Celar, 2013, Wegulo, 2012).

*Fusarium* infection and therefore mycotoxin production occurs in the field but can proceed during storage under appropriate conditions. Further development of *Fusarium* biomass or infection with typical storage fungi (*Aspergillus spp.*, *Penicillium spp.*) during storage can worsen toxin accumulation and quality losses of cereal grains. Therefore, a critical point for reduction of mycotoxins in the food chain is a proper post harvest management. Insufficient drying and high intergranular humidity enhance the risks for fungi development and subsequent mycotoxin contamination (Magan et al., 2010). According to a study of Prange et al. (2005) the storage of only four weeks at 20°C and 20% grain moisture content changed the fungal flora almost completely. In the same study the occurrence of *Fusarium spp.* decreased, whereas the amount of infected grains by storage fungi increased with storage time. Additionally, unsuitable storage of cereal grains can lead to losses in grain quality, as germination rates (Homdork et al., 2000) and losses of nutritional quality with regard to amino acid and carbohydrate composition (Rehman, 2006). A previous study of Eggert et al. (2011) revealed a strong digestion of high molecular mass glutenins (HMW-GS) and other storage protein fractions by *Fusarium* proteases. The gluten protein composition,

particularly the HMW-GS influence bread making properties like dough strength and therefore the quality of the resulting breads (loaf volume) (Halford et al., 1992). From the cited studies it can be assumed that proceeding activity and growth of *Fusarium* species and other microorganisms during storage can cause increased degradation of cereal storage proteins and thus decreased grain quality. In the current study emmer, wheat and naked barley were investigated concerning toxin formation under field conditions after natural *Fusarium* infection and artificial *F. graminearum* inoculation. The effect of two different storage conditions on the *Fusarium* toxin formation and the changes of gluten protein composition as important quality parameters were analysed.

## Materials and Methods

### Experimental design and storage conditions

Two emmer genotypes (Linie 9-102; Klein), three naked barley genotypes (Lawina; 00/900/5N; ZFS) and as a control one summer wheat (Ameretto) were grown at two locations near Göttingen (Torland and Gladebeck). The plants were grown in four replications (four plots). Each plot had a dimension of three to six meters. In 2010 and 2011 the field trial was carried out in Torland and Gladebeck and in 2012 it was conducted only in Torland. Additionally in 2011 and 2012, four replications of each genotype grown in Torland were artificially inoculated with an *F. graminearum* spore *suspension* ($20^5$ spores/mL; 50 mL/$m^2$) three times during flowering. For preparation of the spore suspension, three DON producing strains of *F. graminearum* (FG 142, FG 143, FG 144) were cultured on an autoclaved wheat straw suspension, consisting of nine g straw (particle size 1.5 mm), 500 mL distilled water and 50 mg streptomycine sulfate for ten days at 20°C. The DON producing strains were reference stocks from the Division of Plant Pathology and Crop Protection at the Department of Crop Science of the Georg-August-University of Göttingen. After harvesting, the quantification of the conidiospores was conducted with a Fuchs-Rosenthal chamber (0.0625 $mm^2$; depth: 0.2 mm). After harvesting the emmer grains were dehusked with a single-spike-thresher (Kurt Pelz, Bad-Godesberg, Germany). The grains of emmer, wheat and naked barley were milled (RetschZM 100, Retsch GmbH, Haan, Germany) to particle sizes of 0.5 mm.

For the storage trial both, naturally infected and artificially inoculated grains of one emmer genotype (Linie 9-102) and two naked barley genotypes (Lawina and 00/900/5N) grown in 2011 in Torland were stored in cotton bags each 2 kg for 6 months under two storage conditions; A: 60% air humidity, 14% grain moisture content, 15°C; B: 70% air humidity, 14% grain moisture content, 20 °C. These parameters were chosen to simulate comparable grain storage conditions to customary practice. The grains were wetted to a moisture content of 14% and samples were analysed before storage, after three and six months, respectively.

### Quantitative LC-MS/MS of mycotoxins

Trichothecene and ZEA determination were performed with small variations as described by (Adejumo et al. (2007a) and (Adejumo et al. (2007b), respectively. Both

analyses were conducted in the laboratory of Molecular Phytopathology and Mycotoxin research, Department of Crop Sciences, University of Göttingen, Germany

**Protein extraction for RP-HPLC**

The protein-fractions were extracted according to Wieser (1998). To extract the albumins and globulins together, 100 mg sample was extracted two times with 1 mL sodium phosphate-buffer (0.4M NaCl; 0.067M HKNaPO4; pH7.6) under magnet-stirring at room temperature for 20 min. The samples were centrifuged for 15 min at 7500 rpm and 20°C and the supernatants were removed. To receive the gliadins, the residual pellets were extracted with 500 µL 60% (v/v) ethanol under magnet-stirring for 15 min at room temperature. The samples were centrifuged (15 min; 7500 rpm; 20°C) and the supernatants were collected. This procedure was repeated three times, the supernatants were combined and the volumes were filled up with 60% (v/v) ethanol up to 2 ml. In a final step the glutenins were extracted two times with 1 mL extraction solution, containing 50% 1-propanol; 2 M urea; 0.05 M tris buffer (pH 7.5) and 1% DTT with shaking at 60°C for 30 min in a Thermomixer (Thermomixer comfort, Eppendorf, Hamburg). The samples were centrifuged (15 min; 7500 rpm; 20°C), the supernatants were combined and filled up to 2 mL with extraction solution. The extracts were filtered through 0.45 µm syringe filter and stored at -20°C prior to HPLC injection.

**Analysis of protein fractions with RP-HPLC**

For the separation of the gliadin and glutein subunits a PerfectSil 300 C8 (300 × 4.6 mm; 5 µm) analytical column (MZ Analysentechnik, Mainz, Germany) was used. The mobile phases were A: 0.1% TFA in $H_20$ and B: 0.1% trifluoroacetic acid (TFA) in acetonitrile. The flow rate was 1 mL/min and the column temperature was set to 50°C. The separation was performed with the following gradient: 0 min 100% A; 5 min 76% A; 50 min 50% A; 54 min 10% A. For the gliadins and hordeins 50 µL was injected and for the glutenins and hordenins 100 µL was injected for separation.

**Statistical analysis**

All analyses were run with four biological replications. Statistica 10 software (StatSoft Inc., Tulsa, USA) was used to calculate the mean values and standard deviations and to apply the normality test, Nested ANOVA analysis and Tukey HSD test.

## Results

### Effect of season, location and artificial *F. graminearum* inoculation on mycotoxins in emmer, wheat and naked barley grains

The mycotoxins deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) were detected in all analysed cereals, whereas the DON derivatives 3-acetyldeoxynivalenol (3-Ac-DON), 15-acetyldeoxynivalenol (15-Ac-DON) and deoxynivalenol-3-glucoside (D3G) were not detectable in the same grains. Interestingly, in naturally infected grains at both locations DON was only accumulated in emmer and wheat grains, whereas NIV occurred only in naked barley grains (Table 3.5). In samples from Torland the highest DON contents were found in emmer in 2012 and the highest NIV content was detected in naked barley in 2011. The toxin contents in emmer and naked barley grains at both locations were comparable. Some differences have been observed between the years. Regarding natural infected grains, the highest contents of DON and ZEA in emmer have been found 2012 and the highest NIV contents in naked barley have been found in 2011.

The toxin contents in the inoculated grains of emmer and naked barley also showed great variability during 2011 and 2012 (Table 3.5). An inoculation with *F. graminearum* led to incresed DON and ZEA contents in all cereals, compared to the natural infected grains. The DON contents were significantly higher in wheat and emmer compared to naked barley, whereas the ZEA contents of all genotypes varied strongly between the years 2011 and 2012. However, the highest ZEA contents were found in emmer with 956 µg/kg, followed by wheat with 640 µg/kg. The highest ZEA content in naked barley grains was found in the more susceptible naked barley genotype Lawina with 187 µg/kg.

**Table 3.5:** Mycotoxin contents in µg/kg of emmer, wheat and naked barley after natural *Fusarium* infection and after artificial *F. graminearum* inoculation, grown at two locations (Gladebeck and Torland) in 2010-2012

| | | Natural infected | | | | | | Artificially inoculated | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Gladebeck | | | Torland | | | Torland | | |
| **Cereal grain** | **Year** | **DON[a]** | **NIV[a]** | **ZEA[a]** | **DON[a]** | **NIV[a]** | **ZEA[a]** | **DON[a]** | **NIV[a]** | **ZEA[a]** |
| **Emmer** | | | | | | | | | | |
| | 2010 | 371.7±149.4 | < LOD | 22.9±20.6 | 280.0±141.4 | < LOD | < LOD | -- | -- | -- |
| Linie 9-102 | 2011 | < LOD | < LOD | < LOD | < LOD | < LOD | < LOD | 9606.3±1792.4 | < LOD | 50.1±13.9 |
| | 2012 | -- | -- | -- | 1744.6±2269.1 | < LOD | 165.8±271.7 | 14696.2±2861.9 | < LOD | 881.3±390.5 |
| | 2010 | 581.2±300.0 | < LOD | < LOD | 348.4±134.3 | < LOD | < LOD | -- | -- | -- |
| Klein | 2011 | < LOD | < LOD | < LOD | < LOD | < LOD | < LOD | 8484.9±2169.4 | < LOD | 34.6±12.5 |
| | 2012 | -- | -- | -- | 4271.4±169.2 | < LOD | 296.1±144.2 | 14219.0±1079.6 | 156.7±58.8 | 956.4±427.5 |
| **Wheat** | | | | | | | | | | |
| | 2010 | 276.7±84.5 | < LOD | 11.4±2.7 | 208.2±134.0 | < LOD | < LOD | -- | -- | -- |
| Amaretto | 2011 | <LOD | < LOD | < LOD | < LOD | < LOD | <LOD | 3833.5±509.9 | < LOD | 640.6±102.7 |
| | 2012 | -- | -- | -- | 255.8±86.2 | < LOD | 16.0±11.7 | 1857.9±358.2 | < LOD | 97.8±138.3 |
| **Naked Barley** | | | | | | | | | | |
| | 2010 | < LOD | 310.4±80.7 | < LOD | < LOD | 204.5±36.9 | < LOD | -- | -- | -- |
| Lawina | 2011 | < LOD | 185.3±88.2 | < LOD | < LOD | 659.8±122.1 | < LOD | 2395.8±748.1 | 848.3±362.1 | 187.8±238.3 |
| | 2012 | -- | -- | -- | < LOD | 89.0±33.8 | < LOD | 1144.1±365.2 | < LOD | 55.1±20.5 |
| | 2010 | < LOD | 178.3±68.9 | < LOD | < LOD | 219.6±95.7 | < LOD | -- | -- | -- |
| 00/900/5N | 2011 | < LOD | < LOD | < LOD | < LOD | 189.5±63.4 | < LOD | 1773.7±585.1 | 238.8±193.6 | 40.8±33.5 |
| | 2012 | -- | -- | -- | < LOD | < LOD | 21.4±27.5 | 1338.1±622.9 | < LOD | 88.6±50.7 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2010 | < LOD | 193.0±38.9 | < LOD | < LOD | <LOD | < LOD | -- | -- | -- |
| ZFS | 2011 | < LOD | 123.0±33.9 | < LOD | < LOD | 130.5±105.5 | < LOD | 833.8±293.8 | 302.9±170.2 | < LOD |
| | 2012 | -- | -- | -- | < LOD | < LOD | 11.5±1.0 | 534.9±81.5 | < LOD | 9.5±3.2 |

[a] Mean ± standard deviation; LOD = limit of detection; n = 4; DON = deoxynivalenol; NIV = nivalenol; ZEA = zearalenone; --no samples available

**Effect of storage on *Fusarium* toxin contents**

After storage of natural infected grains, DON was exclusively detected in emmer, whereas NIV was detected in naked barley (Table 3.6). The storage of emmer grains led to increased DON contents in the emmer genotype Linie 9-102 from below the detection limit before storage up to 146.0 µg/kg after six months. However, there were no significant differences between three and six months of storage. The DON content was significantly lower under storage condition B (70%; 20°C) than under storage condition A (60%; 15°C). In naked barley grains a slight increased NIV content was detectable under storage conditions B. Nevertheless, this effect was not significant. DON was not induced by the storage of naked barley grains with the exception of the less susceptible naked barley cultivar ZFS, where the toxin was detected after 3 months under storage condition B.

The storage of artificially inoculated and therefore highly infected grains of emmer surprisingly led to a significant decrease of DON and NIV contents in emmer and the rather susceptible naked barley genotype Lawina (Table 3.7). The resistant naked barley genotype ZFS showed no significant changes in DON contents after storage. Only under storage condition B a slight decrease of DON was detected regarding the genotype ZFS. The storage condition had no significant effect on the mycotoxin contents, but under storage condition B a higher degradation of DON in emmer and naked barley tended to occur. The degradation of DON in emmer and naked barley partly increased with storage time. NIV was decreased after three months of storage and increased again after six months of storage in both naked barley cultivars. The mycotoxin ZEA was only detected in emmer and in the naked barley cultivar Lawina. The storage had no influence on the ZEA contents in emmer, whereas in naked barley the toxin was only detected after storage of three month under condition A.

**Table 3.6:** Changes of *Fusarium* mycotoxins due to two different storage conditions and storage duration of natural infected grains

| Cereal | Storage conditions | | | *Fusarium* toxins [a] | | |
|---|---|---|---|---|---|---|
| | Air Humidity (%) | Temperature (°C) | Storage (months) | DON | NIV (µg/kg) | ZEA |
| **Emmer** | | | 0 | < LOD | < LOD | < LOD |
| | 60 (A) | 15 (A) | 3 | 120.4±31.5 | < LOD | < LOD |
| | | | 6 | 146.0±25.8 | < LOD | < LOD |
| **Linie 9-102** | | | 3 | 85.7±34.7 | < LOD | < LOD |
| | 70 (B) | 20 (B) | 6 | 67.2±20.5 | < LOD | < LOD |
| Effect of storage condition | | | | * | -- | -- |
| Effect of storage time | | | | n.s. | -- | -- |
| **Naked barley** | | | 0 | < LOD | 659.8±122.1 | < LOD |
| | 60 (A) | 15 (A) | 3 | < LOD | 673.0±229.2 | < LOD |
| | | | 6 | < LOD | 665.9±247.6 | < LOD |
| **Lawina** | | | 3 | < LOD | 782.5±335.0 | < LOD |
| | 70 (B) | 20 (B) | 6 | < LOD | 781.6±264.1 | < LOD |
| Effect of storage condition | | | | -- | n.s. | -- |
| Effect of storage time | | | | -- | n.s. | -- |
| | | | 0 | < LOD | 130.5±105.5 | < LOD |
| | 60 (A) | 15 (A) | 3 | < LOD | 320.2±46.7 | < LOD |
| | | | 6 | < LOD | 186.4±130.3 | < LOD |
| **ZFS** | | | 3 | 174.4±69.3 | 205.5±99.8 | < LOD |
| | 70 (B) | 20 (B) | 6 | < LOD | 198.0±105.9 | < LOD |

| | | | |
|---|---|---|---|
| Effect of storage condition | -- | n.s. | -- |
| Effect of storage time | -- | n.s. | -- |

[a] Mean ± standard deviation; n = 4; A = 60 % air humidity and 15 °C storage temperature; B = 70 % air humidity and 20 °C storage temperature; LOD = limit of detection; * = $p < 0.05$; n.s. = not significant according to Nested ANOVA with storage time as hierarchical factor; DON = deoxynivalenol; NIV = nivalenol; ZEA = zearalenone

**Table 3.7:** Changes of *Fusarium* mycotoxins due to two different storage conditions and storage duration of artificially inoculated grains

| Cereal | Storage conditions | | Storage (months) | Fusarium toxins [a] | | |
|---|---|---|---|---|---|---|
| | Air Humidity (%) | Temperature (°C) | | DON | NIV (µg/kg) | ZEA |
| **Emmer** | | | 0 | 9606.3±1792.4 | <LOD | 50.1±13.9 |
| | 60 (A) | 15 (A) | 3 | 6959.7±1220.6 | <LOD | 53.0±20.0 |
| | | | 6 | 6872.4±1178.4 | <LOD | 131.4±126.2 |
| **Linie** | | | 3 | 6539.9±963.9 | <LOD | 66.3±23.0 |
| | 70 (B) | 20 (B) | 6 | 6411.0±1509.6 | <LOD | 51.6±15.8 |
| Effect of storage condition | | | | n.s. | -- | n.s. |
| Effect of storage time | | | | * | -- | n.s. |
| **Naked barley** | | | 0 | 2395.8±748.1 | 848.3±362.1 | 187.8±238.3 |
| | 60 (A) | 15 (A) | 3 | 1689.5±330.3 | 362.5±60.2 | 12.0±2.4 |
| | | | 6 | 1186.6±873.1 | 528.4±51.1 | <LOD |
| **Lawina** | | | 3 | 1353.2±448.1 | 398.6±133.9 | <LOD |
| | 70 (B) | 20 (B) | 6 | 1169.7±796.1 | 461.5±207.7 | <LOD |
| Effect of storage condition | | | | n.s. | n.s. | -- |
| Effect of storage time | | | | * | * | -- |
| | | | 0 | 833.8±293.8 | 302.9±170.2 | <LOD |
| | 60 (A) | 15 (A) | 3 | 974.3±229.0 | <LOD | <LOD |
| **ZFS** | | | 6 | 830.4±194.4 | 216.6±37.6 | <LOD |
| | | | 3 | 673.9±104.1 | < LOD | <LOD |
| | 70 (B) | 20 (B) | 6 | 730.7±130.3 | 332.2±23.8 | <LOD |

| | | | |
|---|---|---|---|
| Effect of storage condition | n.s | n.s. | -- |
| Effect of storage time | n.s. | * | -- |

[a] Mean ± standard deviation; n = 4; A = 60 % air humidity and 15 °C storage temperature; B = 70 % air humidity and 20 °C storage temperature; LOD = limit of detection; * = $p < 0.05$n.s. = not significant according to Nested ANOVA with storage time as hierarchical factor; DON = deoxynivalenol; NIV = nivalenol; ZEA = zearalenone

**Effect of storage conditions on gluten-forming proteins**

In natural infected grains of emmer and naked barley the storage of six months at relatively low temperatures and air humidity under storage condition A (15 °C, 60%) had no significant influence on the prolamine and glutelin sub-fractions (Figures 3.11-3.12). However, the storage under condition B (20°C, 70% air humidity) led to significant decreased hordein sub-fractions in the susceptible naked barley genotype Lawina and to decreased gliadins in the emmer grains (Figures 3.11-3.12). Regarding the inoculated grains, the degradation of hordein and gliadin sub-fractions also occurred under storage condition A (Figures 3.11-3.12). Only the resistant naked barley genotype ZFS showed no significant degradation of hordeins due to storage.

The emmer glutenins were not significantly affected by the storage (Figure 3.13). The naked barley hordenins were affected to lower extend than the hordeins under the present storage conditions. A significant increase of hordenin sub-fractions in natural infected grains was only detected in the naked barley cultivar Lawina under storage condition B (Figure 3.14). Considering the inoculated grains, both naked barley cultivars showed increased hordenin sub-fractions under storage condition B.

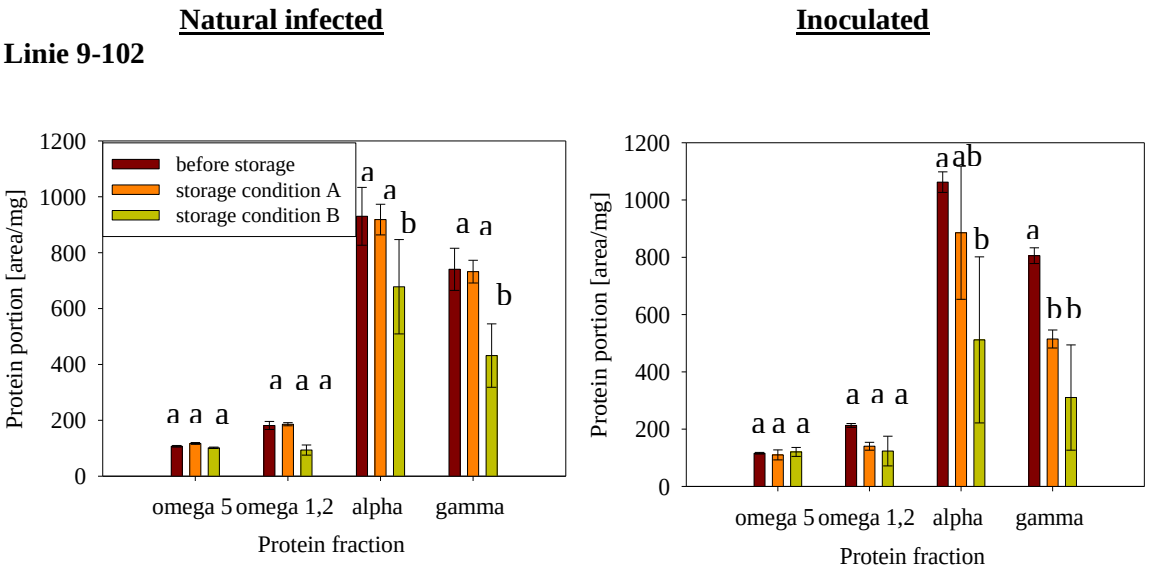



**Figure 3.11:** Effect of the storage condition on emmer (Linie) gliadins of natural infected and artificial *F. graminearum* inoculated grains. Storage condition A: 15°C/60% air humidity, Storage condition B: 20°C/70% air humidity. Mean values with the same letter (comparison within protein fraction) differ significantly according to Tukey HSD test ($p < 0.05$).

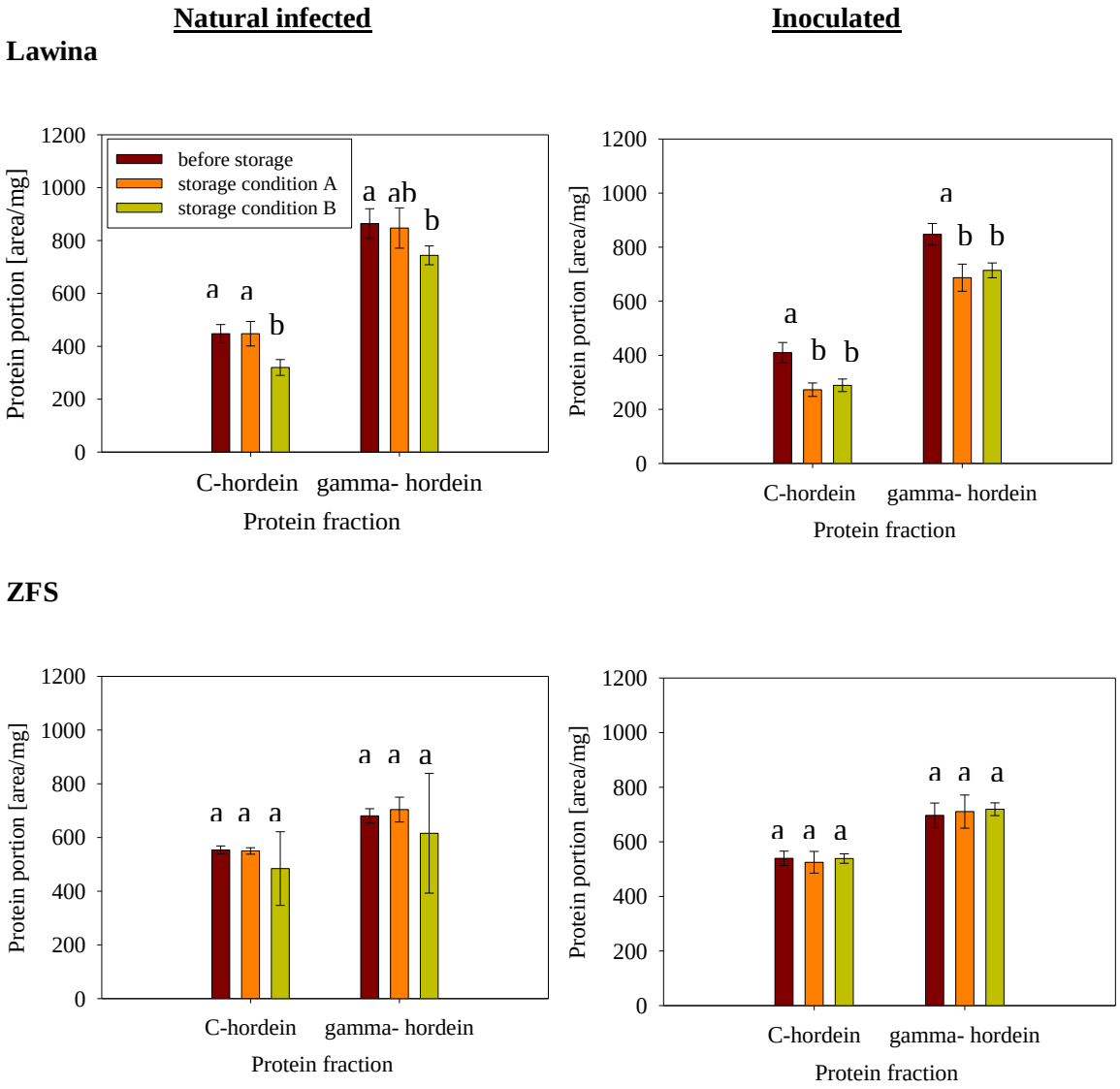



**Figure 3.12:** Effect of the storage condition on naked barley hordeins of natural infected and artificial *F. graminearum* inoculated grains. Storage condition A: 15°C/60% air humidity, Storage condition B: 20°C/70% air humidity. Mean values with the same letter (comparison within protein fraction) differ significantly according to Tukey HSD test ($p < 0.05$).

**Natural infected** **Inoculated**

**Linie 9-102**

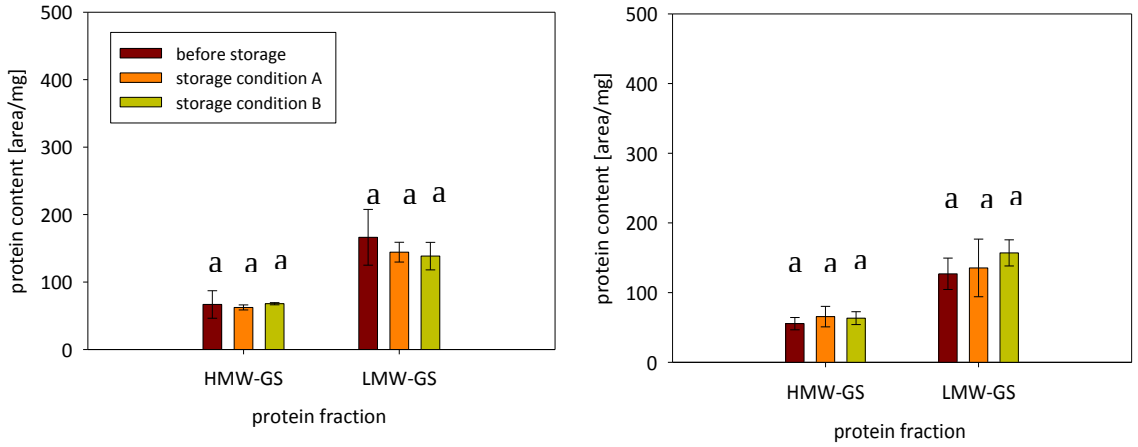
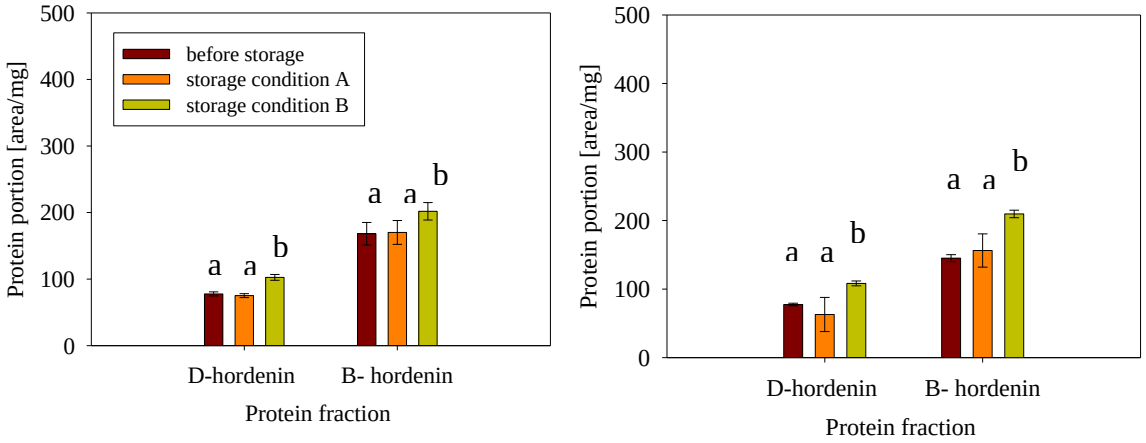



**Figure 3.13:** Effect of the storage condition on emmer (Linie) glutenins of natural infected and artificial *F. graminearum* inoculated grains. Storage condition A: 15°C/60% air humidity, Storage condition B: 20°C/70% air humidity. Mean values with the same letter (comparison within protein fraction) differ significantly according to Tukey HSD test ($p < 0.05$); HMW-GS= high molecular weight glutenins; LMW-GS= low molecular weight glutenins

**Natural infected** **Inoculated**

**Lawina**



**ZFS**

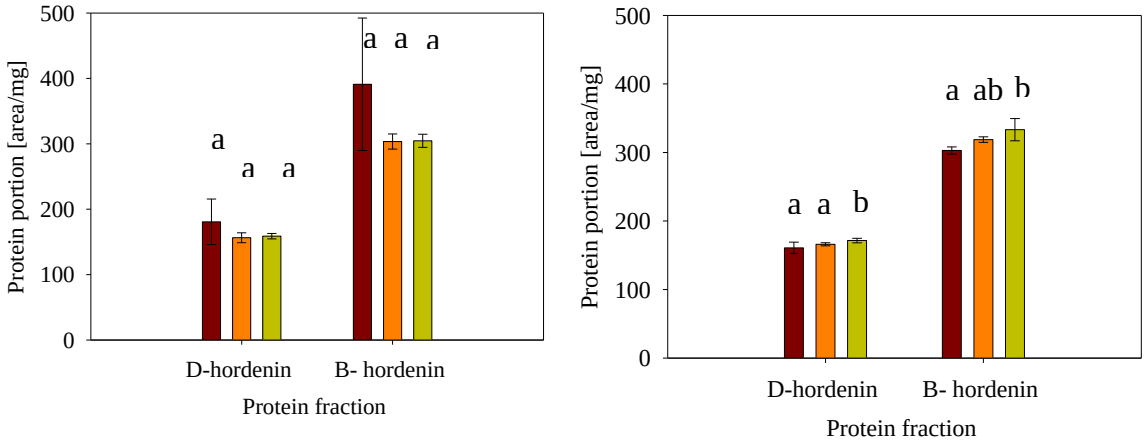



**Figure 3.14:** Effect of the storage condition on naked barley hordenins of natural infected and artificial *F. graminearum* inoculated grains. Storage condition A: 15°C/60% air humidity, Storage condition B: 20°C/70% air humidity. Mean values with the same letter (comparison within protein fraction) differ significantly according to Tukey HSD test ($p < 0.05$).

The ratio of prolamine/glutelin was significantly decreased under storage condition B in inoculated grains of emmer cultivar Linie 9-102 and in naked barley cultivar Lawina both, natural infected and inoculated grains (Table 3.8).

**Table 3.8:** Prolamime/glutelin proportion of natural infected and artificial inoculated grains of naked barley (Lawina, ZFS) and emmer (Linie 9-102) after storage for six months at two conditions; A (15 °C, 60% air humidity) and B (20 °C, 70% air humidity)

| | Natural infected | | | Inoculated | | |
|---|---|---|---|---|---|---|
| | Lawina | ZFS | Linie 9-102 | Lawina | ZFS | Linie 9-102 |
| Before strorage | 5.4±0.8$^{a}$ | 2.3±0.6$^{a}$ | 8.7±2.0$^{a}$ | 5.7±0.4$^{a}$ | 2.7±0.1$^{a}$ | 12.3±2.2$^{a}$ |
| Storage condition A | 5.3±0.9$^{a}$ | 2.7±0.1$^{a}$ | 9.5±0.5$^{a}$ | 4.6±1.4$^{ab}$ | 2.5±0.2$^{a}$ | 8.9±3.7$^{ab}$ |
| Storage condition B | 3.5±0.4$^{b}$ | 2.4±0.7$^{a}$ | 6.5±1.9$^{a}$ | 3.2±0.2$^{b}$ | 2.5±0.0$^{a}$ | 5.0±2.6$^{b}$ |

Small letters refer to comparisons within rows (one cultivar and treatment). Mean values with the same letter differ significantly according to Tukey HSD test ($p < 0.05$)

## Discussion

### Toxin accumulation depending on type of grain and *Fusarium* species

Natural infected grains of emmer exceeded in 2012 the maximum DON level of 1250 µg/kg according to the EU Regulation 1881/2006 with 1744.6 µg/kg and 4271.4 µg/kg, respectively (Table 3.5). Artificial inoculation with *F. graminearum* led to DON contents above this level in almost all samples. Only the resistant naked barley cultivar ZFS accumulated lower toxin contents from 500 to 800 µg/kg. Many studies showed that different genotypes of small grain cereals differ in their resistance to *Fusarium* infection and toxin accumulation (Sneller et al., 2012). Furthermore, the significance of trichothecenes in virulence is strongly dependent on the cereal genotype (Maier et al., 2006). According to previous studies of Eggert et al. (2010a), the analysed naked barley cultivars are more resistant to *Fusarium spp.* infection than emmer and wheat regarding the toxin accumulation at comparable environmental conditions. One reason for the higher resistance of naked barley compared to wheat and emmer could be the inherent type II resistance of naked barley, preventing fungal spread to adjacent spikelets (Foroud and Eudes, 2009). According to a study of Bai et al. (2001) DON production plays a significant role in the spread of FHB disease within wheat spikelets, but is not necessary for initial infection. Langevin et al. (2004) inoculated six different cereal species (soft and durum wheat, triticale, rye, barley and oat) with a trichothecene non-producing strain of *F. graminearum* (*Tri*-) and with the wild type strain (*Tri*+). Generally, the wild type strain was more aggressive than the non-producing strain with varying extend according to the crop species. According to this, reduced virulence of *Fusarium* in wheat and to some extend in barley may be achieved by inhibiting toxin formation by the pathogen or by the degradation of toxins by the host plants (Boutigny et al., 2008). In the present study no DON-3-glucoside was detectable in all three analysed types of grain, indicating no degradation (glycosylation) of DON.

The recently analysed wheat and emmer grains exhibited similar mycotoxins profiles in natural *Fusarium* infected and artificial *F. graminearum* inoculated grains (Table 3.5). Predominantly DON was accumulated in the grains, followed by ZEA. The significant higher DON contents after *F. graminearum* infection compared to natural infection can be explained by a higher disease severity as well due to the fact, that the selected *F. graminearum* strains were designated DON producers. NIV was accumulated predominantly in naked barley grains and hardly in natural infected and inoculated

grains of emmer and wheat. These results suggest different host-pathogen interactions between *F. graminearum* and wheat, emmer and naked barley, respectively. These findings are in accordance with a study of Suchowilska et al. (2009), who investigated mycotoxin profiles in the grains of einkorn, emmer and spelt. A discriminant analysis of a number of mycotoxins revealed that these species differ significantly in the mycotoxin profiles of their grains in the case of natural infection as well as after artificial *F. culmorum* inoculation. The authors suggested a possible synergistic effect between natural occurring *Fusarium* species and the applied *F. culmorum* strain, because of the appearance of mycotoxins that are not produced by *F. culmorum* (Suchowilska et al., 2009). Similar results were obtained by Wiwart et al. (2011), who observed significant differences in trichothecene profiles in the grains of wheat and spelt. These studies and the recent study confirm the co-occurrence of *Fusarium* species in cereal grains, depending on cereal genotypes and therefore depending on plant specific traits and defence strategies. Contributing to this, Ilgen et al. (2009) showed that the induction of *Tri5* gene is tissue specific during the hyphal invasion of wheat and Jansen et al. (2005) revealed different defence strategies of *F. graminearum* in wheat and barley. Finally, different relevance of trichothecenes production related to the virulence of *F. graminearum* in wheat and barley can be assumed.

**Effect of storage on *Fusarium* toxin accumulation**

Under the investigated storage conditions the natural infected grains of emmer and naked barley were only little effected concerning changes in the mycotoxin profiles and contents (Table 3.6). Only DON was significant increased in emmer grains at both storage conditions independent of storage time. A slight trend to higher NIV contents in natural infected naked barley grains was visible after storage of three and six months. In contrast, the storage of inoculated and therefore more heavily *Fusarium*-infected grains led to a significant decrease of DON contents in emmer and in the naked barley grains. Moreover, decreased NIV contents were detected after storage of naked barley grains (Table 3.7).

These results confirm several studies concerning the influence of storage time and storage conditions on *Fusarium* mycotoxin development. Dänicke et al. (2004) found no changes in DON and ZEA concentrations during the storage of heavily *Fusarium* infected wheat for one year under ambient conditions (7-22°C/44-55% relative air humidity). Similar results were obtained in stored heavily *F. culmorum* infected wheat

grains (Homdork et al., 2000). Nevertheless, these authors found an accumulation of ZEA under warm and humid storage conditions (25°C/ 90% relative air humidity) as well as an accumulation of DON in slight and moderate *Fusarium* infected wheat kernels and an overgrowing of *Fusarium* by competing storage fungi as *Aspergillus spp.* and *Penicillium spp.* (Homdork et al., 2000). In these studies the moisture content of the kernels was relatively low (11-14%). Coincidently, Prange et al. (2005) chose suboptimal storage conditions (20°C/ 90% relative air humidity, 20% grain moisture content) and found a decreased number of *Fusarium spp*. infected grains as well as an increased number of grains infected with other storage fungi. Interestingly, extremely high contents of DON and NIV were detected over the storage period of 12 weeks, whereas hardly ochratoxin (OTA) was detected. In contrast, high OTA values were found in the non *F. culmorum* inoculated water control originated from the same base material, assuming strong competitive interactions between field fungi and storage fungi during storage at suboptimal conditions (Prange et al., 2005).

In the present study both storage conditions were conducted at relatively low humidity (60% and 70%) as well as low grain moisture content of 14 % in combination with moderate temperatures of 15°C and 20°C, respectively. The results confirm that these storage conditions are not crucial concerning the mycotoxin accumulation on cereal grains. In addition, no visible moulds caused by storage fungi have been observed in the current study. Nevertheless, a reduction of mycotoxins in inoculated emmer and naked barley grains has been detected, particularly at storage conditions B (Table 3.7). Some reports concerning trichothecene transforming microorganisms maintain a possible biological detoxification of DON by environmental microorganisms (Zhou et al., 2008, Karlovsky, 2011. For example *Aspergillus tubingensis* and *Alternaria alternata* were reported to transform DON (He et al., 2008, Theisen and Berger, 2005). Furthermore, Sato et al. (2012) classified several DON-degrading bacteria from environmental samples, such as soil, wheat leafs and wheat spikelets. Since the samples were derived from field-grown samples, the DON-degradation by natural in the field occurring microorganisms is conceivable. However, the air humidity and grain moisture in the current study were relatively low, offering rather unsuitable conditions for microbial growth.

**Effect of storage on grain protein composition**

Regarding the current study, especially warm and humid conditions (storage condition B) affected the grain protein composition of both varieties (Figures 3.11-3.14). Naturally infected and inoculated grains of emmer and naked barley showed a decrease of prolamins (gliadins and hordeins). Emmer gliadins were most degraded during storage, particularly under storage condition B (Figure 3.11). According to Eggert et al. (2011) *Fusarium* proteases degraded wheat gliadin and glutenin sub-fractions in an *in vitro* study as well as *in vivo* after *Fusarium* inoculation of emmer (Eggert et al., 2010b) and naked barley (Eggert et al., 2010c). Fungal proteases may be active during storage and thus proceed in degrading cereal storage protein. Regarding *Fusarium* infected grains here, protein degradation were also observed at rather cold and dry storage conditions (condition A). It can be assumed that *Fusarium* infected grains exhibit higher respiratory activity, resulting in partly higher temperatures of the grain. This could possibly explain the changes of protein composition of *Fusarium* infected grains during storage at condition A. On the contrary, an increase of hordenins in naked barley grains was detected after storage, mainly under storage condition B (Figure 3.14). Chen and Schofield, (1996) and Mann et al. (2000) revealed decreased levels of free reduced glutathione and of free oxidised glutathione after short term storage as a result of disulfide bonds formation by oxidation processes. Disulfide bonds between the polypeptides of gluten proteins lead to higher polymerisation degrees of these proteins, which could explain an increase of hordenins in naked barley in the present study.

Despite no visible spoilage fungi were observed in the current investigation, growth of storage fungi is possible, which would contribute to storage protein degradation. Due to the fact that *Fusarium* toxins decreased, further development of *Fusarium spp.* during storage at the respective conditions cannot be verified, even though mycotoxin production alone is no sufficient evidence for *Fusarium* growth. However, the development of *Fusarium* species, other fungi and the changes of protein composition during storage and the contribution of microbial spoilage require further investigation.

The decrease of prolamins and the increase of glutelins, led to changed proportions of prolamin/glutelin, which may affect processing quality of certain cereal products (Table 3.8). Karaoğlu et al. (2010) reported that storage periods beyond three months worsened wheat quality with regard to gluten content, Zeleny sedimentation and amylase activity. Cereal grain proteins have an important impact on several functional properties for food

processing. It is generally known that the gliadin/glutenin ratio of wheat is an important factor for rheological behaviour of dough (Shewry et al., 2002, Janssen et al., 1996). High glutenin to gliadin ratio increases dough strength and therefore increases bread making quality (Sisons, 2008). According to this, storage of emmer grains can positively influence processing quality, due to the enhanced glutenin and degraded gliadin values. Nevertheless, gliadins can also strongly influence breadmaking properties of wheat (Van Lonkhuijsen et al., 1992). The addition of gliadin significantly improved loaf volumes of pan breads (Khatkar et al., 2002). The requirements for quality of cereals are complex and depend on respective processing parameters.

It can be concluded, that storage under warm and humid conditions influenced the protein composition and therefore changed several physiochemical properties. Especially *F. graminearum* infected grains showed changes in protein composition after storage, even at rather cold and dry conditions (Figures 3.11-3.14). However, the storage conditions were not suitable for further *Fusarium* development or toxin formation, respectively. Nevertheless, microbial activities are supposed to be responsible for storage protein degradation and thus for changes in end- of- use quality.

In conclusion, naked barley showed the lowest toxin concentrations compared to emmer and wheat grains. Moreover, naturally infected naked barley accumulated NIV, whereas emmer and wheat accumulated DON. After inoculation with declared DON producing *F. graminearum* strains, DON was also accumulated in naked barley grains. The storage of naturally infected grains led hardly to changed toxin contents in naked barley, but in emmer grains increased DON contents were observed after storage. In contrast, the toxin contamination of inoculated grains was significantly reduced after storage. It can be assumed, that storage of relatively dry grains (14% grain moisture) of emmer and naked barley under mild temperatures between 15-20°C and air humidity of 60-70% hardly worsen *Fusarium* infestation. Nevertheless, significant changes in protein composition were observed in emmer and the susceptible naked barley cultivar Lawina after storage of six months. The prolamins were degraded, whereas glutelins were accumulated particularly during warmer and more humid storage, supposing changed processing properties of the products. *Fusarium* inoculation increases the changes in protein composition even during storage at 15°C and 60% air humidity. Both storage conditions characterize customary storage conditions, where the development of spoilage fungi is not expected. Nevertheless, changes in gluten protein composition

occurred, which was aggravated due to *Fusarium* infection. Both storage conditions did not worsen the toxin contamination of the grains but the protein composition as an important quality parameter has been strongly affected.

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## 3.3 Studies on phenolic acid accumulation during grain development in emmer and naked barley and the impact of *Fusarium graminearum* infection


### Abstract

Three naked barley genotypes with different *Fusarium* resistance and two emmer genotypes being more susceptible to *Fusarium* infection compared to naked barley were investigated concerning their phenolic acid composition in the grains and in the hull-tissues (glumes, lemma, palea) at different stages of grain development after natural *Fusarium* infection and after *Fusarium graminearum* inoculation. It was distinguished between methanol soluble phenolic aids and bound to arabinoxylans and other cell wall components phenolic acids. In the grains and in the hulls of emmer and naked barley ferulic acid was the major phenolic acid, followed by *p*-coumaric and caffeic acid in soluble and bound forms. Regarding the soluble fraction, additionally catechin was detected in the grains and hulls of emmer and naked barley. Emmer and naked barley showed similar contents of ferulic acid and *p*-coumaric acids in the grains, whereas naked barley had distinct higher bound ferulic acid and *p*-coumaric acid in the hulls compared to emmer. Additionally, naked barley grains exhibited more catechin than emmer. However, the most susceptible naked barley genotype showed the highest catechin contents. An inoculation with *F. graminearum* led to reduced cinnamic acid derivatives, predominantly bound ferulic acid in emmer grains and hulls and in naked barley hulls. Naked barley grains were hardly affected by *Fusarium* infection concerning the analysed cinnamic derived phenolic acids. However, in one naked barley genotype catechin was strongly induced due to *Fusarium* inoculation.

The high contents of phenolic acids in naked barley may contribute to the higher resistance to *Fusarium* infection compared to emmer. The phenolic compounds in the hull-tissues possibly play an important role in inhibiting pathogen growth and toxin accumulation in the grains.


### Introduction

*Fusarium* head blight (FHB) occurs in most areas in the world where small grain cereals are grown. The disease causes great concern worldwide due to the ability to produce various mycotoxins appearing in processed food and feed. The major pathogens causing FHB of cereal grains in Europe are *Fusarium graminearum* and *Fusarium culmorum*. Both phytopathogens can produce B-type trichothecenes, such as deoxynivalenol

(DON), its 3- and 15 acetylated forms and other hazardous mycotoxins. (Parry et al., 1995, Bottalico and Perrone, 2002) Due to the fact that these molecules are highly stable, they are not degraded during processing mechanisms and thus occur in cereal based food products as well as in animal feed (Woloshuk and Shim, 2013). Developing strategies for reduced toxin formation in the field requires a better understanding towards the mechanisms leading to mycotoxin accumulation in cereal grains (Merhej et al., 2011). It is reasonable assumed that antioxidant compounds such as phenolic acids effect trichothecene biosynthesis and fungal growth (Ponts et al., 2011, Naoumkina et al., 2010, Dixon et al., 2002). Phenolic acids represent the most abundant form of phenolic compounds in cereals. They are concentrated in the outer layers of the grains (pericarp, aleurone layer). Phenolic acids are hydroxylated derivatives of benzoic or cinnamic acids (Mattila et al., 2005). Hydroxycinnamic acids, such as *p*-coumaric, caffeic, ferulic, and sinapic acids are more abundant in cereal grains than hydroxybenzoic acids (e.g. gallic acid, vanillic acid) (Wang et al., 2013). They are mainly bound to cell wall components through ester and ether bonds. Thus they have a significant importance in cross-linking of polysaccharides with cell wall components such as cellulose and lignin, making the plant cell wall more stable against enzymatic degradation (Parker et al., 2005). Phenolic acids also occur in free forms or conjugated, for example, to short carbohydrates. The free and soluble conjugated phenolic acids, which are esterified to sugars and other low molecular mass components, have been shown to be influenced by environmental conditions, whereas bound phenolic acids were more stable concerning environmental (temperature, precipitation) induced changes (Fernandez-Orozco et al., 2010).

The highest contnts of phenolic acids in wheat of more than 4000 mg $kg^{-1}$were found in the bran and in whole grain flour. *P*-coumaric acid was predominantly located in the hulls, whereas the aleurone layer contained high levels of ferulic acid. According to this, *p*-cumaric acid has been found to be lower in naked barley as compared to hulled genotypes (Quinde-Axtell and Baik, 2006). Additionally, in barley also polyphenols belonging to the family of flavanoids have been identified. A highly abundant flavan-3-ol monomer in is catechin. Naked barley grains showed higher catechin contents than hulled barley (Quinde-Axtell and Baik, 2006).

Phenolic compounds are supposed to play an important role in resistance to *Fusarium* infection. Siranidou et al. (2002) found significant higher amounts of free phenolic

compounds in the hulls of a resistant wheat cultivar after inoculation with *F. culmorum*. The susceptible genotypes showed no changes in free phenolic compounds concerning *Fusarium* infection. Additionally, previous studies revealed increased amounts of catechin in the grains of mature naked barley cultivars due to *Fusarium* infection (Eggert et al., 2010) and in the spikelets of resistant barley 72 hours after *Fusarium* inoculation (Kumaraswamy et al., 2012). Moreover, inoculation with *F. graminearum* induced *p*-coumaric acid in barley spikelets. Additionally, the induction of *p*-coumaric acid was stronger after inoculation with the trichothecene non producing *F. graminearum* strain than with the trichothecene producing strain. This may illustrate an impact of trichothecenes on the inhibition of plant derived resistance related metabolites (Kumaraswamy et al., 2012). Several *in vitro* studies confirm an inhibitory effect of phenolic acids on fungal growth (Ponts et al., 2011, McKeehen et al., 1999). Phenolic acid treatment of *F. culmorum in vitro* resulted in a reduced expression level of trichothecene biosynthesis (*Tri*) gens (Boutigny et al., 2010). Natural phenolic acid extracts, containing not only monomeric forms but also various oligomeric forms of mainly ferulic acid, have been shown to have a higher inhibitory effect than a mixture of commercial available monomeric forms (Boutigny et al., 2010). Ferulic acid is mainly esterified to arabinoxylan chains that are cross-linked to lignin subunits. *P*-coumaric acid is directly esterified to lignin subunits (Santiago et al., 2013). Lignification processes and cell-wall cross-linking associated with cell wall thickening may contribute to plant resistance against fungal invasion, providing mechanical barriers (Siranidou et al., 2002).

The objective of this study was to investigate the accumulation of phenolic compounds in the grains and in the hull tissues (glumes, lemmas, paleas) of emmer and naked barley during grain development. Furthermore, the effects of F. graminearum infection at anthesis on the formation of these compounds were studied considering a different susceptibility to *Fusarium* infection of these two genotypes. The compositions of phenolic compounds in emmer and naked barley tissues are suspected to be different contributing to a different *Fusarium* susceptibility of the grains depending on the respective grain ripening stage.

## Materials and Methods

### Experimental design and sample preparation

The field trial was carried out at Marienstein (Nörten-Hardenberg), near Göttingen in 2011. Two emmer genotypes (Linie 9-102; Klein) and three naked barley genotypes (Lawina; 00/900/5N; ZFS) were grown in a field trial, sowed in spring 2011. The two emmer cultivars are distinctly more susceptible than the naked barley cultivars. Within the naked barley cultivars, Lawina and 00/900/5N are more susceptible compared to the resistant cultivar ZFS. The plants were grown in eight replications. Each plot had a dimension of three to six meters. Four replications were artificially inoculated with an *F. graminearum* spore *suspension* ($20^5$ spores/mL; 50 mL/ $m^2$ three times during flowering. Three DON producing strains of *F. graminearum* (FG 142, FG 143, FG 144) were cultured on an autoclaved wheat straw suspension, consisting of nine g straw (1.5 mm), 500 mL distilled water and 50 mg streptomycine sulfate for ten days a 20 °C. The DON producing strains were reference stocks from the Division of Plant Pathology and Crop Protection at the Department of Crop Science of the Georg-August-University of Göttingen. After harvesting, the quantification of the conidiospores was conducted with a Fuchs-Rosenthal chamber (0.0625 $mm^2$; depth: 0.2 mm). The ears of the emmer cultivars from each plot (four inoculated, four naturally infected) were collected four times and naked barley ears were gathered five times during plant growth and grain development. The ripening stages of the cereals were identified using the extended BBCH-scale (Table 3.9). The BBCH-scale defines the phenological growth stages with a standardised decimal code. The abbreviation „BBCH" derives from Biologische Bundesanstalt, Bundessortenamt and chemical industry (Meier, 2001). The days after inoculation (dai), which are synonymous with the days after anthesis (daa) were documented.

**Table 3.9:** Days after inoculation (dai) (anthesis: 0 dai) of emmer and naked barley plants and the corresponding phenological growth stages

| Phenological growth stage (BBCH code) | Days after inoculation (dai) | |
|---|---|---|
| | Emmer | Naked barley |
| Early milk (73) | -- | 7 |
| Medium milk (75) | 19 | -- |
| Late milk (77) | -- | 14 |
| Soft dough (85) | 25 | 21 |
| Hard dough (87) | 32 | 36 |
| Plant death (97) | 39 | 54 |

-- No sample was obtained

Fifty ears from each plot were harvested and freeze dried. Afterwards the ears were divided into grains and the respective hull tissues (glumes, lemma, palea) and analysed individually. Therefore, the spikes were freeze dried and removed manually. Finally, the grains and the hulls were milled with a ball mill (Mixer Mill MM 400, Retsch®, Germany). Samples were stored at −80°C prior to analysis.

**Extraction of phenolic acids**

Two different groups of phenolic compounds were extracted. The first group was soluble in aqueous methanol (free phenolic compounds) and the second group were covalent-bound phenolic compounds. For receiving the aqueous methanol soluble phenolic acids, 500 mg of whole grain flour and husk, respectively was extracted three times with 5 mL (for 12, 12, and 12 h) of 80% aqueous methanol containing 1% acetic acid. The sample solutions were centrifuged for 15 min at 10000 rpm and the supernatants were pooled in a 15 mL falcon tube. Afterwards, the supernatants and the residual pellet were dried in a vacuum concentrator (RVC 2-25 CD, Christ, Germany) at room temperature and 5 mL of 0.1 M $H_2SO_4$ was added to the dried supernatants (soluble phenolic acids) as well as to the pellet (bound phenolic acids) followed by a hydrolysis for 1 h at 100°C. After the extracts were cooled to room temperature, 1.5 mL 1 M sodium acetate solution (pH 5.5) containing 125 units α-amylase (product code 10065-10G, 35.7 units/mg, Sigma Aldrich, Switzerland), was added to the samples and incubated for 30 °C for 2 h. In the next step, 1 mL 0.1 M sodium acetate solution (pH 5.5) containing 25 units cellulase (product code 22178-25G, 1.13 units/mg, Sigma Aldrich, Switzerland) was added to the mixture and incubated at 30°C for 18 h. Finally, 1 mL of 25% NaCl solution was added and then, the phenols were extracted three times, each with 4 mL of ethyl acetate. The three ethyl acetate fractions were pooled and dried in a vacuum concentrator at room temperature (RVC 2-25 CD, Christ, Germany). The dried extract was resuspended in 1 mL of 80% aqueous methanol containing 1% acetic acid and kept at -20 °C until analysis. Prior to HPLC analysis, the samples were centrifuged for 5 min at 10000 rpm and the supernatants were filtered through a 0.45 µm syringe filter transferred to a vial for HPLC-injection.

**RP-HPLC analysis**

Reference compounds used for phenol quantification in the experiment (+) catechin (sigma aldrich), caffeic-, ferulic- and *p*-cumaric acid (Merck). For HPLC a PerfectSil Target ODS-3 HD (200 × 4.0 mm, 5 µm) analytical column (MZ Analysentechnik, Mainz, Germany) was used. The mobile phases were A: 1% acetic acid in $H_20$ and B: 1% acetic acid in methanol. The flow rate was 1.2 mL/min and the column temperature was set to 40°C. The separation was performed with the following gradient: 2 min 90% A; 35 min 70% A; 50 min 10% A; 52 min 0% A; 56 min 100% A. The injection volume was 20 µL. The detection was performed with a diode array detector (Jasco MD-2015 Plus Multiwavelength Detector). The obtained chromatograms were analyzed with the Jasco ChromPass Chromatography Data Systems (Version 1.8.6.1).

**Quantitative LC-MS/MS of DON**

DON determination was performed with small variations as described by (Adejumo et al. (2007a) in the laboratory of Molecular Phytopathology and Mycotoxin research, Department of Crop Sciences, University of Göttingen, Germany

**Statistical analysis**

All analyses were run with four biological replications. Statistica 10 software (StatSoft Inc., Tulsa, USA) was used to calculate the mean values and standard deviations and to apply the normality test, the students *t*- test and the ANOVA analysis.

## Results

### DON accumulation in emmer and naked barley grains

The formation of DON in emmer and naked barley during grain ripening was determined to demonstrate the successful artificial infection with *Fusarium* compared to the natural infection. After *F. graminearum* inoculation, the DON contents were distinctly increased in all emmer and baked barley cultivars (Table 3.10). Natural infected grains showed no detectable DON (data not shown). Both emmer cultivars contained significant higher DON values than the naked barley cultivars, confirming the higher susceptibility of emmer compared to naked barley as reported by Eggert et al. (2010).

**Table 3.10:** DON concentrations in emmer and naked barley cultivars after artificial inoculation with *F. graminearum* in mg/ kg

| Crop | Cultivar | Dai | DON |
|---|---|---|---|
| | | | MV ± SD |
| Emmer | Linie 9-102 | 19 | 15.0 ± 7.3 |
| | | 25 | 18.6 ± 5.6 |
| | | 32 | 14.9 ± 3.2 |
| | | 39 | 15.9 ± 2.8 |
| | Klein | 19 | 11.2 ± 1.1 |
| | | 25 | 17.7 ± 7.8 |
| | | 32 | 17.4 ± 2.7 |
| | | 39 | 19.1 ± 5.4 |
| Naked barley | Lawina | 7 | < LOD |
| | | 14 | 0.2 ± 0.1 |
| | | 21 | 1 ± 0.3 |
| | | 36 | 1.6 ± 0.6 |
| | | 54 | 3.3 ± 1.7 |
| | 00/900/5N | 7 | < LOD |
| | | 14 | 0.9 ± 0.4 |
| | | 21 | 1.6 ± 0.9 |
| | | 36 | 4.0 ± 1.4 |
| | | 54 | 9.6 ± 12.8 |
| | ZFS | 7 | < LOD |
| | | 14 | 0.1 ± 0.0 |
| | | 21 | 1.9 ± 0.7 |
| | | 36 | 2.1 ± 1.7 |
| | | 54 | 0.7 ± 0.4 |

Dai= days after inoculation; LOD = limit of detection; MV = mean value; SD = standard deviation

Regarding the grain ripening, described as days after inoculation (dai), hardly changes concerning DON accumulation occurred in emmer grains, whereas naked barley grains showed slightly increased DON contents. However, the values refer to the dry matter content, so the per-kernel or per-ear values of fresh material, respectively were likely higher at later ripening stages. Both emmer cultivars showed similar DON concentrations, whereas all three naked barley cultivars contained different DON amounts. The DON accumulation in grains of the cultivar ZFS was slightly lower than in the other two cultivars, especially at late grain ripening stage.

**Formation of phenolic acids in emmer and naked barley during grain ripening and after *Fusarium graminearum* infection**

Phenolic compounds were determined in two different fractions: methanol soluble phenolic acids and bound (to cell wall and carbohydrates) phenolic acids. Distinct higher amounts of cinnamic-derived phenolic acids occurred in the bound form compared to the methanol soluble fraction (Tables 3.11- 3.14). Emmer and naked barley showed higher contents of cinnamic-derived phenolic acids in the hulls than in the grains. Especially bound *p*-coumaric acid occurred in distinct higher concentrations in the hulls. Furthermore, the concentration of soluble phenolic acids was higher in the hulls than in the grains.

The concentrations of cinnamic-derived phenolic acids found in emmer and naked barley grains were comparable, whereas the concentrations of the bound cinnamic-derived acids in the hulls of naked barley were distinctly higher than in the emmer hulls. Due to the fact that only minor differences between emmer and naked barley cultivars were detected concerning the cinnamates, these are not considered here.

In emmer grains and hulls the bound ferulic acid was clearly the most prevalent phenolic acid followed by *p*-coumaric acid. Most of the phenolic acids were decreasing during emmer grain ripening. Only the bound caffeic acid in the emmer hulls was slightly increasing. After *F. graminearum* infection of emmer grains the hydroxycinnamic acid derivatives, especially bound ferulic acid and *p*-coumaric acid in the grains and hulls were reduced. The most affected phenolic acid was ferulic acid in the hulls of emmer (Tables 3.11 and 3.12).

In naked barley, the highest phenolic acid concentrations were detected in the hulls at early ripening stage. The most abundant bound ferulic acid showed concentrations up to

1500 mg/kg in the hulls. Additionally, higher amounts of bound *p*-coumaric acid were observed in the hulls of naked barley compared to the grains. All determined soluble and most of the bound phenolic acids were decreasing during grain ripening, except the bound caffeic acid in naked barley grains and hulls, which were increasing during the ripening.

Regarding the effect of *Fusarium* inoculation on naked barley hardly any changes concerning phenolic acid contents were detected in the grains (Table 3.13), whereas some phenolic acids in the hulls were significantly reduced, especially at early grain development (Table 3.14).

**Formation of catechin in emmer and naked barley during grain ripening and after *Fusarium graminearum* infection**

The catechin contents of emmer grains were relatively low compared to naked barley grains, whereas the contents in emmer and naked barley hulls were similar. In contrast to the cinnamic acid derivatives, the catechin concentrations in the grains varied strongly within the genotypes.

Regarding the emmer cultivars, higher catechin concentrations have been found in the hulls compared to the grains (Table 3.15). The catechin contents in the grains of both emmer cultivars were similar and decreased from around 20 mg/kg at milk ripe stage to 7 mg/kg at plant death stage. The emmer cultivar Klein contained twofold more catechin in the hulls than the cultivar Linie 9-102. Minor effects were detected in emmer grains or hulls concerning changes in catechin contents due to *Fusarium* inoculation (Table 3.15).

Regarding the catechin contents in the naked barley grains, strong differences were detected between cultivars (Table 3.16). The highest catechin contents were observed in the grains of the susceptible cultivar Lawina (503.3 mg/kg) followed by 00/900/5N, whereas the resistant cultivar ZFS showed catechin contents similar them in the emmer grains, ranging from 25 mg/kg to 10 mg/kg. Interestingly, the catechin content in the hulls of the resistant cultivar ZFS is the highest at plant death stage. During grain ripening the cultivars Lawina and 00/900 reached a maximum catechin content at late milk stage (dai 14) and soft dough stage (dai 21), respectively before decreasing until plant death stage (dai 54). The resistant cultivar ZFS showed a slight decrease during

grain ripening. Regarding naked barley hulls, the catechin contents were decreasing at later development stages, since 36 dai.

Controversial findings were made concerning the effect of *F. graminearum* inoculation on the catechin contents in the grains. No effect was detected in naked barley hulls. Regarding grains of the resistant naked barley cultivar ZFS, no significant changes of the catechin content was observed. In contrast, the rather susceptible cultivar Lawina showed a high induction if catechin at the early milk ripe stage (dai 7) followed by a reduction until soft dough stage (dai 21) and later a slight induction that is not significant. The cultivar 00/900/5N, likewise susceptible, showed catechin induction at almost all development stages due to *F. graminearum* inoculation.

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**Table 3.11**: Soluble and bound phenolic acids in naturally infected (Nat. inf.) and *F. graminearum* inoculated (inoc.) emmer grains during grain ripening in mg/kg (corresponding phenological growth stage s. Table 3.9)

| | | 19 dai (BBCH 75) | | | 25 dai (BBCH 85) | | | 32 dai (BBCH 87) | | | 39 dai (BBCH 97) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | Phenolic acid | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p |
| Soluble | Caffeic acid | 11.8 | 9.2 | * | 7.6 | 7.1 | n.s. | 8.5 | 8.6 | n.s. | 8.6 | 8.6 | n.s. |
| | *P*-coumaric acid | 15.9 | 15.9 | n.s. | 13.4 | 14.2 | n.s. | 11.9 | 11.3 | n.s. | 5.5 | 5.1 | n.s. |
| | Ferulic acid | 75.7 | 59.8 | * | 38.3 | 25.3 | * | 16.3 | 17.4 | n.s. | 6.9 | 8.1 | n.s. |
| | Sum | 103.4 | 84.9 | * | 59.3 | 46.6 | n.s. | 36.7 | 37.3 | n.s. | 21.0 | 21.8 | n.s. |
| Bound | Caffeic acid | 8.6 | 10.2 | * | 11.2 | 8.2 | * | n.d. | n.d. | | n.d. | n.d. | |
| | *P*-coumaric acid | 16.9 | 13.5 | * | 12.8 | 11.9 | n.s. | 10.8 | 10.7 | n.s. | 9.3 | 9.8 | n.s. |
| | Ferulic acid | 638.4 | 577.3 | n.s. | 553.9 | 463.9 | * | 430.6 | 328.9 | * | 308.7 | 231.7 | * |
| | Sum | 663.9 | 601.0 | n.s. | 577.9 | 484.0 | * | 441.4 | 339.6 | * | 318.0 | 241.5 | * |

Dai= days after inoculation; P= significance * = significant ($p < 0.05$); n.s. = not significant according to paired Student *t*-test (n= 8); n.d. = not detected; Sum = total phenolic acids

**Table 3.12:** Soluble and bound phenolic acids in naturally infected (Nat. Inf.) and *F. graminearum* inoculated (inoc.) emmer hulls during grain development in mg/kg (corresponding phenological growth stage s. Table 3.9)

| | | 19 dai (BBCH 75) | | | 25 dai (BBCH 85) | | | 32 dai (BBCH 87) | | | 39 dai (BBCH 97) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | Phenolic acid | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p |
| Soluble | Caffeic acid | 23.5 | 25.3 | n.s. | 20.8 | 26.1 | n.s. | 39.0 | 27.0 | * | 18.4 | 14.8 | * |
| | *P*-coumaric acid | 116.6 | 108.9 | n.s. | 112.4 | 98.4 | n.s. | 101.2 | 79.7 | * | 67.2 | 51.7 | * |
| | Ferulic acid | 102.1 | 116.1 | n.s. | 108.6 | 111.7 | n.s. | 112.5 | 100.6 | n.s. | 60.8 | 63.3 | n.s. |
| | Sum | 242.2 | 250.3 | | 241.8 | 236.2 | | 252.7 | 207.3 | | 146.4 | 129.8 | |
| Bound | Caffeic acid | 8.6 | 9.2 | n.s. | 8.8 | 8.7 | n.s. | 11.0 | 8.4 | * | 10.3 | 10.2 | |
| | *P*-coumaric acid | 178.1 | 163.3 | n.s. | 140.3 | 109.7 | * | 121.3 | 92.2 | * | 129.9 | 110.3 | * |
| | Ferulic acid | 601.9 | 492.8 | * | 502.9 | 294.3 | * | 360.4 | 192.6 | * | 237.0 | 184.3 | * |
| | Sum | 788.6 | 665.3 | * | 652.0 | 412.7 | * | 492.7 | 293.2 | * | 377.2 | 304.8 | * |

Dai= days after inoculation; P= significance * = significant ($p < 0.05$); n.s. = not significant according to paired Student *t*-test (n= 8); Sum = total phenolic acids

**Table 3.13:** Soluble and bound phenolic acids in naturally infected (Nat. inf.) and *F. graminearum* inoculated (Inoc.) naked barley grains during grain development in mg/kg (corresponding phenological growth stage s. Table 3.9)

| | | 7 dai (BBCH 73) | | | 14 dai (BBCH 77) | | | 21 dai (BBCH 85) | | | 36 dai (BBCH 87) | | | 54 dai (BBCH 97) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | Phenolic acid | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p |
| Soluble | Caffeic acid | 10.5 | 10.7 | n.s. | 9.7 | 9.9 | n.s. | 9.0 | 8.6 | n.s. | 9.7 | 10.4 | n.s. | 8.2 | 8.1 | n.s. |
| | *P*-coumaric acid | 11.6 | 10.9 | n.s. | 12.4 | 11.4 | n.s. | 9.2 | 7.2 | * | 3.9 | 4.7 | n.s. | 2.9 | 3.1 | n.s. |
| | Ferulic acid | 27.5 | 28.2 | n.s. | 31.4 | 31.9 | n.s. | 25.6 | 23.0 | n.s. | 21.7 | 21.3 | n.s. | 11.7 | 11.1 | n.s. |
| | Sum | 49.6 | 49.8 | n.s. | 53.5 | 53.2 | n.s. | 43.8 | 38.8 | n.s. | 35.3 | 36.4 | n.s | 22.8 | 22.3 | n.s. |
| Bound | Caffeic acid | n.d. | n.d. | | n.d. | n.d. | | 5.3 | 4.0 | n.s. | 15.2 | 15.2 | n.s. | 21.2 | 19.6 | n.s. |
| | *P*-coumaric acid | 11.2 | 10.6 | n.s. | 10.0 | 9.1 | n.s. | 8.6 | 8.6 | n.s. | 7.3 | 7.3 | n.s. | 7.7 | 7.3 | n.s. |
| | Ferulic acid | 689.4 | 709.4 | * | 703.9 | 702.3 | n.s. | 594.3 | 585.2 | n.s. | 387.8 | 378.3 | n.s. | 334.8 | 343.3 | n.s. |
| | SumΣ | 700.6 | 720.0 | n.s. | 713.9 | 711.4 | n.s. | 608.2 | 597.8 | n.s. | 410.3 | 400.8 | n.s. | 363.7 | 370.2 | n.s. |

Dai = days after inoculation; P = significance * = significant ($p < 0.05$); n.s. = not significant according to paired Student *t*-test (n = 8); n.d. = not detected; Sum = total phenolic acids

**Table 3.14:** Soluble and bound phenolic acids in naturally infected (Nat. inf.) and *F. graminearum* inoculated (Inoc.) naked barley hulls during grain development in mg/kg (corresponding phenological growth stage s. Table 3.9)

| | | 7 dai (BBCH 73) | | | 14 dai (BBCH 77) | | | 21 dai (BBCH 85) | | | 36 dai (BBCH 87) | | | 54 dai (BBCH 97) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| fraction | Phenolic acid | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p |
| soluble | Caffeic acid | 56.5 | 47.9 | * | 54.8 | 47.7 | * | 56.6 | 56.2 | n.s. | 53.1 | 47.1 | n.s. | 7.6 | 9.6 | n.s. |
| | *P*-coumaric acid | 140.0 | 136.2 | n.s. | 142.3 | 126.1 | * | 118.5 | 106.3 | * | 41.5 | 35.9 | n.s. | 16.6 | 17.7 | n.s. |
| | Ferulic acid | 114.1 | 117.3 | n.s. | 99.5 | 120.0 | n.s. | 116.7 | 122.8 | * | 86.3 | 83.5 | n.s. | 37.8 | 42.3 | n.s. |
| | Sum | 310.6 | 301.4 | n.s. | 296.6 | 293.8 | | 291.8 | 285.3 | | 180.9 | 166.5 | n.s. | 62.0 | 69.6 | n.s. |
| bound | Caffeic acid | 16.4 | 15.0 | * | 17.6 | 14.3 | * | 20.6 | 20.8 | n.s. | 25.1 | 23.8 | n.s. | 21.3 | 20.8 | n.s. |
| | *P*-coumaric acid | 587.1 | 526.9 | n.s. | 419.0 | 362.5 | n.s. | 372.3 | 331.3 | * | 321.4 | 317.0 | n.s. | 370.0 | 430.6 | n.s. |
| | Ferulic acid | 1539.3 | 1257.9 | * | 1329.3 | 1037.4 | * | 1204.3 | 922.0 | * | 603.1 | 597.8 | n.s. | 572.5 | 573.9 | n.s. |
| | Sum | 2142.8 | 1799.8 | * | 1765.9 | 1414.2 | * | 1597.2 | 1274.1 | * | 949.6 | 938.6 | n.s. | 963.8 | 1025.3 | n.s. |

Dai= days after inoculation; P= significance * = significant ($p < 0.05$); n.s. = not significant according to paired Student *t*-test (n= 8) ; Sum = total phenolic acids

**Table 3.15:** Soluble catechin contents in naturally infected (Nat. inf.) and *F. graminearum* inoculated (Inoc.) emmer grains and hulls during grain development in mg/kg (corresponding phenological growth stage s. Table 3.9)

| emmer grain | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 19 dai (BBCH 75) | | | 25 dai (BBCH 85) | | | 32 dai (BBCH 87) | | | 39 dai (BBCH 97) | | |
| | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p |
| Klein | 18.3 a | 13 a | * | 11.8 a | 9.7 b | n.s. | 8.1 a | 8.1 a | n.s. | 7.2 a | 7.8 a | n.s. |
| Linie 9-102 | 21.1 a | 15.1 a | n.s. | 13.3 a | 12.5 a | n.s. | 8.4 a | 8.4 a | n.s. | 5.8 a | 6.7 a | n.s. |
| emmer hulls | | | | | | | | | | | | |
| Klein | 96.8 a | 102.7a | n.s. | 96 a | 93.9 a | n.s. | 94.3 a | 76.8 a | * | 71.3 a | 55.5 a | * |
| Linie 9-102 | 46.9 b | 45.7 b | n.s. | 49.4 b | 48.6 b | n.s. | 44.1 b | 40.4 b | n.s. | 34.6 b | 34 b | n.s. |

Dai = days after inoculation; P = significance * = significant ($p < 0.05$); n.s. = not significant according to paired Student *t*-test (n = 8); small letters refer to ANOVA analysis comparing the cultivars at each dai and treatment (same letters in a row are not related)

**Table 3.16:** Soluble catechin contents in naturally infected (Nat. inf.) and *F. graminearum* inoculated (Inoc.) naked barley grains and hulls during grain development in mg/kg (corresponding phenological growth stage s. Table 3.9)

| | naked barley grain | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 dai (BBCH 73) | | | 14 dai (BBCH 77) | | | 21 dai (BBCH 85) | | | 36 dai (BBCH 87) | | | 54 dai (BBCH 97) | | |
| | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p | Nat. inf. | Inoc. | p |
| Lawina | 124.6 a | 385.4 a | * | 503.3 a | 348.9 a | n.s | 167.6 a | 91.3 b | * | 188.9 a | 190.9 a | n.s. | 193.3 a | 204.2 a | n.s. |
| 00/900/5N | 27.7 b | 23.1 b | n.s. | 25.1 b | 67.9 b | * | 102.7 a | 139.2 a | n.s. | 64.9 b | 120.3 b | * | 70 b | 124.6 b | * |
| ZFS | 25.4 b | 27.8 b | n.s. | 15.6 b | 6.9 c | n.s. | 10.5 b | 9.6 c | n.s. | 17.4 c | 18.4 c | n.s. | 21.3 c | 11.4 c | n.s. |
| | naked barley hulls | | | | | | | | | | | | | | |
| Lawina | 76 a | 79.7 a | n.s. | 88.6 a | 80.7 a | n.s. | 80.4 a | 76.9 a | n.s. | 55.4 a | 53.2 a | n.s. | 13.7 c | 15.6 b | n.s. |
| 00/900/5N | 43.3 c | 41.1 c | n.s. | 40.9 c | 42.3 c | n.s. | 37.6 c | 42.3 c | n.s. | 37.5 b | 32.9 b | n.s. | 16.4 b | 16.8 b | n.s. |
| ZFS | 62.2 b | 61.1 b | n.s. | 55.1 b | 60.3 b | n.s. | 64.8 b | 62.9 b | n.s. | 49.3 ab | 37.7 b | n.s. | 22.6 a | 25.4 a | n.s. |

Dai = days after inoculation; P = significance * = significant ($p < 0.05$); n.s. = not significant according to paired Student *t*-test (n = 8); small letters refer to ANOVA analysis comparing the cultivars at each dai and treatment (same letters in a row are not related)

## Discussion

### DON accumulation in emmer and naked barley grains

The current DON values of emmer and naked barley confirm the higher susceptibility of emmer compared to naked barley (Table 3.10). The naked barley genotype ZFS has been proven to be more resistant to DON accumulation than Lawina and 00/900/5N. These findings are in accordance with previous experiments (Eggert et al., 2010). According to Boutigny et al. (2008) two different natural processes limit *Fusarium* trichothecene accumulation (type V resistance) in cereals. These processes include chemical transformation of the toxins by plant metabolic pathways, including glycosylation acetylation and the inhibition of mycotoxin biosynthesis by plant secondary metabolites, such as antioxidant compounds. Trichothecene biosynthesis has been enhanced under oxidative stress conditions and inhibited in the presence of hydrogen peroxide scavenging catalyse *in vitro* (Ponts et al., 2007). Trichothecenes are synthesised by a series of oxygen requiring mechanisms. Thus, antioxidant compounds, such as phenolic acids can influence the fungal redox status playing an important role during trichothecene biosynthesis (Boutigny et al., 2008, Ponts et al., 2007). Therefore, high amounts of antioxidant phenolic acids can possibly contribute to lower toxin accumulation in naked barley compared to emmer.

### Phenolic acids and *Fusarium* infection

The most abundant phenolic acid in emmer and naked barley has been found to be ferulic acid, followed by *p*-cumaric and caffeic acid (Tables 3.11-3.14). These three phenolic acids have been shown to inhibited mycelia growth of *Fusarium* species *in vitro* up to 50 % (Kumaraswamy et al., 2011, McKeehen et al., 1999). Furthermore, treatment of *F. culmorum* in liquid cultures with a mixture of phenolic acids and with a natural extract of phenolic acids from wheat bran resulted in a drastic reduction of trichothecene biosynthesis genes (Boutigny et al., 2010). According to Ponts et al. (2011) ferulic acid was considered to be the most toxic phenolic compound concerning fungal growth compared to other cinnamic-derived acids as caffeic-, *p*-coumaric-, syringic- and *p*-hydroxybenzoic acids. On the other hand, elevated toxin accumulation has been observed after addition of ferulic acid and *p*-coumaric acid in liquid cultures of *F. graminearum* strains at concentrations around 0.5 mmol/ liter in a study of Ponts et al. (2011). In accordance, ferulic acid treatment induced *TRI5* gene expression in an *F.*

*graminearum* strain (Ponts et al., 2011). Trichothecene biosynthesis has been strongly related to oxidative burst, thus *Fusarium* pathogens are suggested to adapt secondary metabolism to the oxidative state in favour of the infection process (Merhej et al., 2011). Nevertheless, most studies exhibited inhibition of fungal growth and trichothecene biosynthesis by phenolic acids. According to Ponts et al. (2011) these contradictory results can be explained by different culture media used in the respective *in vitro* experiments and by different experimental designs.

In the current study with emmer and naked barley higher phenolic acid concentrations were found in the hulls than in the grains (Tables 3.11-3.14). Cinnamic acid derivatives play a role in cross-linking the arabinoxylan chains and can provide higher rates of lignin linkages, reinforcing the cell walls in the hulls (Parker et al., 2005). This may inhibit pathogen growth through grain-hull tissues, saving the grains from fungal infestation and therefore improve resistance to *Fusarium* infection. Moreover, distinct higher amounts of phenolic acids in naked barley hulls compared to emmer hulls and grains, especially of bound *p*-coumaric and ferulic acid illustrates a major relevance of phenolic acids in naked barley hulls, which is possibly related to the higher *Fusarium* resistance of naked barley compared to emmer. Contributing to this, several studies found a relation between phenolic acid concentrations and *Fusarium* resistance. Thus, ferulic acid (soluble plus cell wall bound) occurrence in maize kernels has been proven to contribute to *Fusarium* resistance. Levels of ferulic acid were significantly higher in the more *Fusarium* ear rot resistant groups and significantly lower in the susceptible groups (Assabgui et al., 1993, Picot et al., 2013). Additionally, McKeehen et al. (1999) found higher ferulic acid accumulation rates over the first 25 days after anthesis (daa) in the grains of an FHB resistant wheat cultivar compared to a susceptible cultivar.

Moreover, several variations concerning phenolic acid concentrations have been noticed between emmer and naked barley genotypes which provides possible explanations for their different susceptibility to *Fusarium* infection. The bound caffeic acid concentrations in the grains of emmer were decreasing, whereas they were increasing in naked barley grains during grain development (Tables 3.1; 3.13). Thus, the bound caffeic acid concentration in naked barley was increased during grain development.

An inoculation with *F. graminearum* led to a slight reduction of the cinnamic derived acids in emmer grains and in the hulls of emmer and naked barley (Tables 3.11-3.14), whereupon ferulic acid was most affected. However, this effect was mostly not

significant, but the results are in accordance with previous studies finding a significant reduction of ferulic acid in mature grains of emmer and naked barley after *Fusarium* inoculation (Eggert et al., 2010). Eggert et al. (2010) proposed the inhibition of specific enzymes involved in phenylpropanoid pathways or the inhibition of DNA or RNA biosynthesis by trichothecenes to be responsible for phenolic acid reduction, since trichothecenes are potent inhibitors of protein biosynthesis in plants according to Rocha et al. (2005).

**Catechin and *Fusarium* infection**

Higher catechin contents in the grains of naked barley compared to emmer could contribute to the higher *Fusarium* resistance of naked barley compared to emmer. However, the most susceptible naked barley genotype Lawina showed by far the highest catechin concentrations in the grains (Table 3.15). The most resistant genotype ZFS contained relatively high catechin concentrations in the hulls, particularly at late grain ripening stage, supposing again a major relevance of phenolic acids in the hulls concerning *Fusarium* resistance. All naked barley cultivars were more resistant to toxin accumulation and showed higher catechin contents in the grains than the emmer cultivars.

An increase of catechin due to *Fusarium* inoculation was only detected in the grains of the naked barley cultivar 00/900/5N during the entire grain development. These results are partly in accordance with former studies, finding increased catechin contents in mature naked barley grains due to *Fusarium* spp. infection (Eggert et al., 2010). Contrary to the findings of the current study, Eggert et al. (2010) found no catechin in emmer grains. Though, the catechin concentrations found in emmer grains at plant death stage (maturity) were relatively low compared to the average abundance in mature naked barley grains (Tables 3.15-3.16). Catechin has been revealed to be part of a passive resistance mechanism by formation of a physical barrier in the testa layer of cereal grains. Furthermore, proanthocyanidins like catechin can inhibit fungal enzymes like pectinases and xylanases (Skadhauge et al., 1997, Treutter, 2006).

**Conclusion**

In conclusion, the highest concentrations of cinnamic acid derivatives have been found in the hulls of emmer and naked barley, whereupon the concentrations were distinctly higher in the hulls of naked barley compared to emmer. Contributing to these results,

naked barley showed the lowest toxin accumulation in the grains after *Fusarium* inoculation, assuming a protective effect against the pathogen of phenolic acids in the hulls of naked barley. This can be related particularly to the physiological functions of phenolic acids concerning cell-wall fortification and resistance to biodegradation by fungal enzymes.

However phenolic acids, especially ferulic acid decreased after *F. graminearum* inoculation, possibly by fungal inhibition of their biosynthesis. Mechanisms in plants depending on antioxidant capacity and the general redox status are complex. Thus, the influence of antioxidant secondary plant metabolites, like cinnamic derived acids and catechin on resistance to *Fusarium* infection and resistance to toxin accumulation in cereals may contribute to plant defence mechanisms.

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## 3.4 Identification of differently regulated proteins after Fusarium graminearum infection of emmer (Triticum dicoccum) at different grain ripening stages


### Abstract

This study was conducted to improve the knowledge of molecular mechanisms concerning the interaction between *Fusarium graminearum* and the cereal *Triticum dicoccum* in the course of grain ripening and progressive infection. Emmer plants were artificial inoculated with a *F. graminearum* spore suspension at anthesis. In the course of grain ripening from milk ripe to plant death stage four phenological growth stages were collected for analysis. The infection degree was evaluated based on the *F. graminearum* DNA conent on the kernel-surrounding tissues. For proteome analysis the albumin and globulin fraction of emmer grains, consisting of proteins with various functions related to development and stress response were analysed regarding the changes due to *Fusarium* infection by two-dimensional gel electrophoresis. Altogether 43 proteins could be identified by mass spectrometry. In the early grain development, proteins related to stress response, such as 2-cys peroxiredoxin, a chitinase, a xylanase inhibitor and a spermidine synthase were up regulated. During later grain development, stress related proteins, as chitinases, heat shock proteins and an α-amylase inhibitor-like protein were rather down regulated. During all ripening stages, especially during milk and soft dough stage, proteins related to carbon-metabolism, starch-and protein biosynthesis as well as photosynthesis increased in abundance due to *F. graminearum* infection. At plant death stage only two proteins related to metabolism were down regulated.


### Introduction

*Fusarium* head blight (FHB) is a cereal disease causing significant yield losses and in particular accumulation of several mycotoxins, such as trichothecenes like deoxynivalenol (DON) and the acetylated derivates or zearalenone (Bottalico and Perrone, 2002). The predominant species infesting cereals in Europe are *Fusarium graminearum* and *Fusarium culmorum* and the most critical period for infection and colonisation of cereal ears with *Fusarium spp.* has been defined as the anthesis and the first half of the grain filling stage (Kazan et al., 2012). Multiple mechanisms of defence or resistance (active or passive) are known to exist in plants. Currently five types of resistance are described: Type I resistance to initial infection by inhibiting fungal

penetration into the plant and resistance (type II) to fungal spread within adjacent tissues. Type III and IV resistance involve the ability to degrade DON and high tolerance to DON, respectively. Furthermore a resistance (type V) to grain infection is described, resulting in lower effects on the yield (Champeil et al., 2004). An effective method to control FHB is the cultivation of resistant cultivars (Ruckenbauer et al., 2001). Therefore more information about infection mechanisms of the pathogen and respective defence strategies of the plants is needed.

A few studies are available concerning the sources of FHB resistance in emmer (Buerstmayr et al., 2003, Oliver et al., 2007). Oliver et al. (2007) found a wide variation concerning the susceptibility to *F. graminearum*, ranging from highly resistant to highly susceptible and a study of Buerstmayr et al. (2003) analysing wild emmer cultivars from Israel detected that most of the plants were highly susceptible to *F. graminearum*. Nevertheless, occasional genotypes showing lower infection rates are supposed to be a possible source for resistance-breeding in emmer and durum wheat.

Several proteomic studies have been performed, analysing various proteins with a potential role in plant-fungus interaction. These studies provide an insight into pathogenicity and host resistance concerning *Fusarium spp.* infection of cereals. Most of them focussed on the initial infection of wheat spikes (Wang et al., 2005, Zhou et al., 2006, Shin et al., 2011) and barley spikes (Yang et al., 2010, Geddes et al., 2008) during the first days after inoculation at anthesis. According to these studies up to three days after inoculation the abundance of many proteins related to carbon metabolism, photosynthesis, oxidative stress and fungal cell-wall degradation were changed due to *F. graminearum* infection of the spikes. Regarding the proteins involved in carbon metabolism and photosynthesis controversial findings concerning up and down regulation were made. Wang et al. (2005) found out that most of these proteins decreased after infection in wheat spikes, whereas Yang et al. (2010), Shin et al. (2011) and Zhou et al. (2006) ascertained an increased energy metabolism in infected barley- and wheat heads. Furthermore, pathogenesis related proteins, such as chitinases and thaumatin-like proteins and proteins involved in oxidative stress response were predominantly accumulated during the early infection of wheat and barley according to these studies.

To our knowledge little research have been carried out considering the differential expression of proteins in response to *Fusarium spp.* infection during later stages of

infection in the course of grain ripening. Dornez et al. (2010) analyzed wheat kernels inoculated at anthesis with *F. graminearum* at 5, 15 and 25 days, respectively, post anthesis (water ripe, milky ripe and soft dough stage) focussing on different classes of xylanase inhibitor proteins (XIP). Additionally several PR proteins were identified, finding peroxidases, and chitinases predominantly down regulated and thaumatin-like proteins as well as a wheatwin-2 precurser up regulated since 5 days post anthesis (Dornez et al., 2010). Earlier studies of Eggert et al. (2011) and Eggert and Pawelzik, (2011) investigated the proteome of mature emmer- and naked barley grains after a long *Fusarium*-infection period. These studies also revealed an increase in stress related proteins, such as a serine protease inhibitor and a thaumatin-like protein and a decrease in proteins related to oxidative stress and a chitinase in emmer. In naked barley an up regulation of transcription regulation proteins and protease inhibitors were detected. Furthermore, proteins regulating starch synthesis were down regulated in both varieties (Eggert and Pawelzik, 2011, Eggert et al., 2011).

In the current study four different, well-defined grain-development stages from milk ripe to plant death of an emmer genotype (Linie 9-102) were investigated regarding the changes of specific proteins due to *F. graminearum* infection. The aim of this work was to investigate *F. graminearum* infection-induced changes in protein expression in emmer grain that are dependent on the respective ripening stage and the associated stage of infection. At different ripening stages of the kernel we expected different infection strategies of the pathogen and therefore different protein expression patterns of the emmer grain. Generally, albumins and globulins possess multiple functions during growth, development and stress response of cereals (Gao et al., 2009) and therefore, the investigation was focused on albumin and globulin protein fraction changes.

## Methods and materials

### Experimental design

The field trial was carried out at Marienstein (Nörten-Hardenberg), near Göttingen in 2011. The investigated emmer genotype was Linie 9-102. The plants were grown in eight plots, each with a dimension of three to six meters. Four plots were artificially inoculated with an *F. graminearum* spore suspension ($20^5$ spores/mL; 50 mL/$m^2$) three times during flowering. Three DON producing strains of *F. graminearum* (FG 142, FG 143, FG 144) were cultured on an autoclaved wheat straw suspension, consisting of nine g straw (1.5 mm), 500 mL distilled water and 50 mg streptomycine sulfate for ten days a 20°C. The DON producing strains were reference stocks from the Division of Plant Pathology and Crop Protection at the Department of Crop Science of the Georg-August-University of Göttingen. After harvesting, the quantification of the conidiospores was conducted with a Fuchs-Rosenthal chamber (0.0625 $mm^2$; depth: 0.2 mm).

### Sample preparation and protein extraction

For the analysis of proteomics, ears from each plot (four inoculated; four naturally infected) were collected four times during grain development. The ripening stages according to Table 3.17 of the emmer grains were identified using the extended BBCH scale. The BBCH-scale defines the phonological growth stages with a standardised decimal code. The abbreviation „BBCH" derives from Biologische Bundesanstalt, Bundessortenamt and chemical industry (Meier, 2001).

**Table 3.17:** Days after inoculation (dai) (anthesis: 0 dai) of emmer plants and the corresponding phenological growth stages

| Phenological growth stages (BBH code) | Days after inoculation (dai) |
|---|---|
| Medium milk (75) | 19 |
| Soft dough (85) | 25 |
| Hard dough (87) | 32 |
| Plant death and collapsing (97) | 39 |

Fifty ears were randomly collected and freeze dried. Afterwards the grains were manually removed from the ears and milled with a ball mill (Retsch®; Mixer Mill MM 400). Grain samples were stored at −80°C prior to analysis.

The albumins and globulins were extracted with an 50 mM sodium phosphate buffer (pH 7.8), containing 0.1 M NaCl and 0.2% protease inhibitor cocktail (Sigma-

Aldrich®). 100 mg flour was extracted with 1 mL of sodium phosphate buffer and stirred for 2 h at 4°C. After centrifugation (eppendorf; centrifuge 5804 R) at 8000g and 4°C for 10 min, 500 µL of the supernatant was transferred into a new tube. 1.5 mL of ice-cold TCA-acetone was added to the supernatants and stored at -20°C to precipitate the protein over night. The cold samples were centrifuged (8000 g; 4°C; 10 min) and the supernatants were removed. The pellets were rinsed three times with cold acetone under stirring for 10 min at 4°C and then centrifuged as before. The pellets were evaporated at 100 mbar for 10 min in a vacuum concentrator (Christ; RVC 2-25 CD) and resuspended in 500 µL lysis buffer (6 M urea, 2 M thiourea, 0.2% pharmalyte buffer (w/v pH 3-10), 2% CHAPS, 2%DTT , 0.2% protease inhibitor cocktail (Sigma-Aldrich®), 0.002% bromophenol blue). For solubilization of the protein, samples were shaken for 1h at 33°C in a Thermomixer (eppendorf). Finally, protein concentrations were determined with a 2-D Quant Kit (Amersham Bioscience) and adjusted to a concentration of 1µg/µL.

**Two-Dimensional Gel Electrophoresis (2D GE)**

For isoelectric focusing, commercially available immobilized pH gradient (IPG) stripes (pH 3-10, 17 cm, BioRad) were used. 300 µL of the protein sample, solved in the lysis buffer was loaded into a tray. The IPG stripes were rehydrated over night, at which the stripes were covert with 1 mL mineral oil to prevent evaporation. The IEF was performed in the Protean® IEF cell (BioRad) with the following conditions: 15 min 0-250 V; 3h 250 - 10000 V; 10000 V - 60000 V- hrs. The ramp was chosen rapid, the current was set to 50 µA per gel and the temperature was 20°C. Afterwards the IPG stripes were incubated one after another with two buffer agents for 15 min: the first containing 6 M urea, 2% SDS, 0.375 M Tris-HCL (pH 8.8), 20% glycerol, 2% DTT and the second containing 6 M urea, 2% SDS, 0.375 M Tris-HCL (pH 8.8), 20% glycerol, 2.5% iodoacetamide. Finally the strips were rinsed with SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). SDS-PAGE was performed in the Protean® II xi Cell (BioRad) by using 12% acryamide gels (20 cm, 20 cm, 1 mm) with a current of 30 mA per gel. The staining was performed with a modified colloidal Coomassie G-250 staining (blue silver) according to Candiano et al. (2004).

**Data analysis**

The gels were scanned with an image scanner (Epson Expression™ 10000 XL) using the LabScan 6.0 software. The gel images were saved as TIF files and analyzed using the PDQuest Basic 8.0.1 analysis software (BioRad). For statistical analysis four biological replications of each group were required, artificial inoculated and natural infected samples were used to create 'replicate groups' for each grain development stage. Spot detection and matching was carried out by using the automated 'spot Detection wizard'. Spots that were present in at least three of the group members were added to the analysis set. The spot quantities in four naturally infected samples were compared with the spot quantities in four inoculated samples. For statistical analysis sets, the student's t-test with significance level of 90 % was chosen. The standard deviation between four replicate groups was below 50 %. Protein spots were accepted to be regulated after *F. graminearum* infection if the difference between the mean value of natural infected and artificially inoculated samples were higher than the factor of two.

***Fusarium graminearum* DNA**

*F. graminearum* DNA was determined by real-time PCR as described by Brandfass and Karlovsky, (2008) and Brandfass and Karlovsky, (2006) in the laboratory of the group "Molecular Phytopathology and Mycotoxin Research", Department of Crop Sciences, University of Göttingen. DNA from *F. graminearum* was extracted from 100 mg of grain hull-tissues after removing the kernels.

**Protein identification by mass spectrometry**

Tryptic digestion of proteins and identification of proteins by mass spectrometry (MS) were performed as described by Klodmann et al. (2010) at the University of Hannover by the Institute of pant proteomics. Procedures were based on peptide separation using the EASY-nLC System (Proxeon; Thermo Scientific, Bremen, Germany) and coupled MS analyses using the MicrOTOF-Q II mass spectrometer (Bruker Bremen, Germany). MS data evaluation was carried out using ProteinScape2.1 software (Bruker, Bremen, Germany) and the Mascot search algorithm (Matrix Science, London, UK) against (1) the NCBI protein database (www.ncbi.nih.org), (2) the UniProt protein database (http://www.uniprot.org/) and (3) the Arabidopsis protein database (www.Arabidopsis.org; release TAIR 10). The following Mascot search parameters were used: enzyme, trypsin/P (up to one missed cleavage allowed); global modification,

carbamidomethylation (C), variable modifications, acetyl (N), oxidation (M); precursor ion mass tolerance, 15 ppm; fragment ion mass tolerance, 0.6 Da; peptide charge, 1+, 2+, and 3+; instrument type, electrospray ionization quadrupole time of flight. Minimum ion score was 30, minimum peptide length was 4 amino acids, significance threshold was set to 0.05 and protein and peptide assessments were carried out if the Mascot Score was greater than 30 for proteins and 20 for peptides.

## Results and discussion

### *Fusarium* DNA

*Fusarium* biomass was evaluated as *F. graminearum* DNA. Table 3.18 shows the *F. graminearum* DNA in the analysed ripening stages of the emmer genotype Linie 9-102. The DNA was determined on the spikelet tissues after removing the kernel. In the natural infected samples the *F. graminearum* DNA was below the limit of quantification. After artificial inoculation 4.1 mg/kg per dry matter (DM) *F. graminearum* DNA was detected already at milk ripe stage (BBCH 75) and increased up to 21.3 mg/kg at plant death stage (BBCH 97). These results demonstrate a significant higher infection rate of the inoculated plants compared to the natural infected plants and therefore a successful inoculation can be assumed.

**Table 3.18:** *Fusarium* DNA content of emmer grain hull-tissues (glumes + rachis) during grain ripening

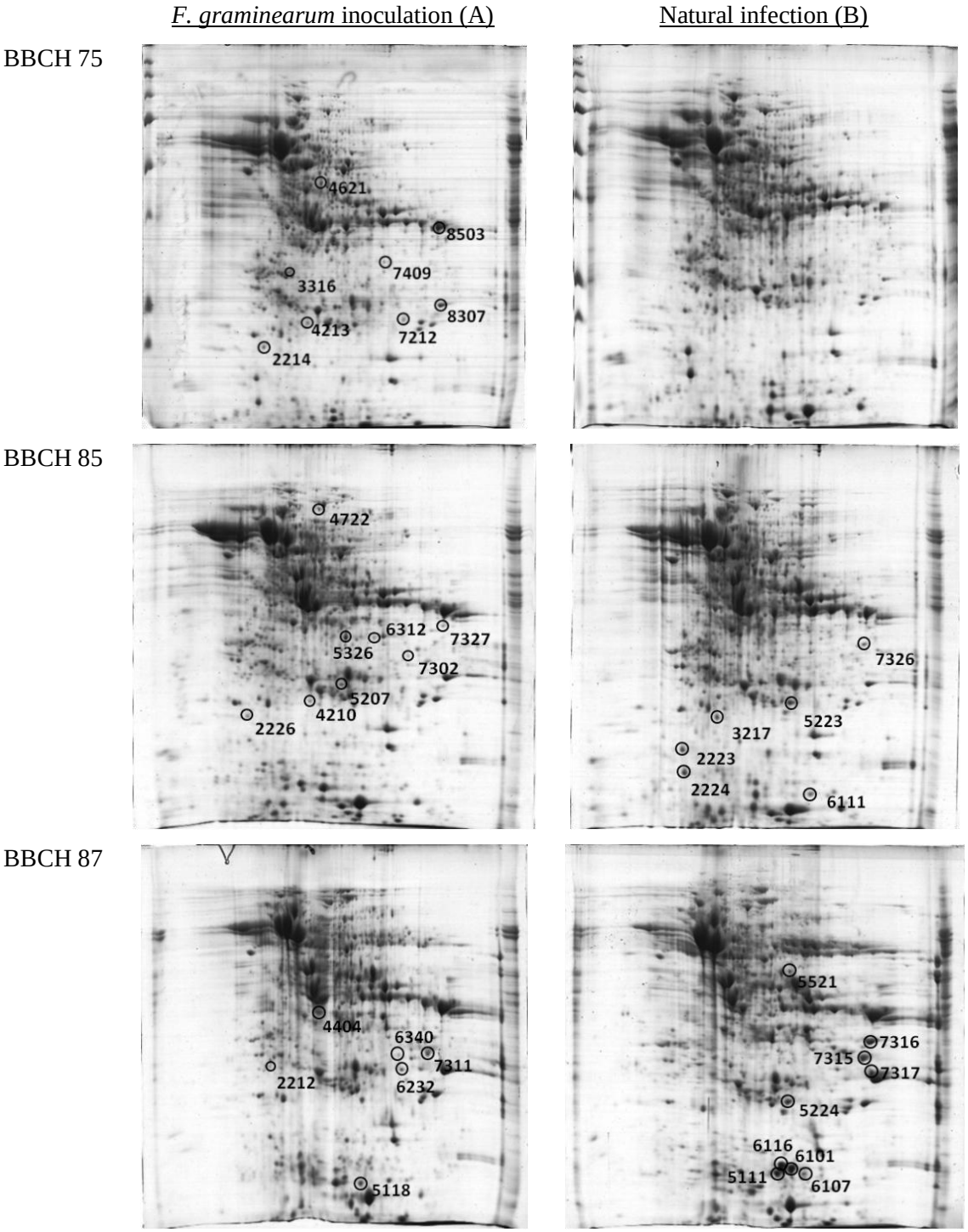
| BBCH | Natural infection | Inoculation |
|---|---|---|
| | ***Fusarium* DNA [mg kg$^{-1}$]** **MV ± SD** | ***Fusarium* DNA [mg kg$^{-1}$]** **MV ± SD** |
| *75* | <LOQ | 4.1 ± 1.6 |
| *85* | <LOQ | 7.4 ± 3.4 |
| *87* | <LOQ | 7.6 ± 3.8 |
| *97* | <LOQ | 21.3 ±9.7 |

LOQ= limit of quantification (0.02 mg/kg); MV= mean value; SD= standard deviation

### Proteome analysis

After artificial *F. graminearum* infection a total of 52 Proteins showed different expression patterns regarding all development stages (Figure 3.15). At BBCH 75 (milk ripe stage) and BBCH 85 (soft dough stage) eight proteins were up-regulated due to *Fusarium* infection, whereas at BBCH 87 (hard dough stage), six proteins and at BBCH 97 (plant death stage) only three proteins were up regulated (Figure 3.15A). On the contrary, no proteins were detected to be down regulated at BBCH 75, whereas six proteins showed reduced expression at BBCH 85. Nine proteins were down regulated at BBCH 87 as well as 12 proteins at BBCH 97 (Figure 3.15B). In summary, during earlier development stages protein expression rather increased due to artificial *F. graminearum* infection, whereas proteins were predominantly down regulated at later grain ripening stages. Forty two proteins were identified by LC-MS according to their peptide sequences after trypsin-digestion and database-search. At BBCH 85, BBCH 87

and BBCH 97 several predicted protein with currently unknown function were down regulated (Tables 3.22-3.25). Furthermore, some of the identified and not identified proteins were apparently proteolytic fragments.



BBCH 97

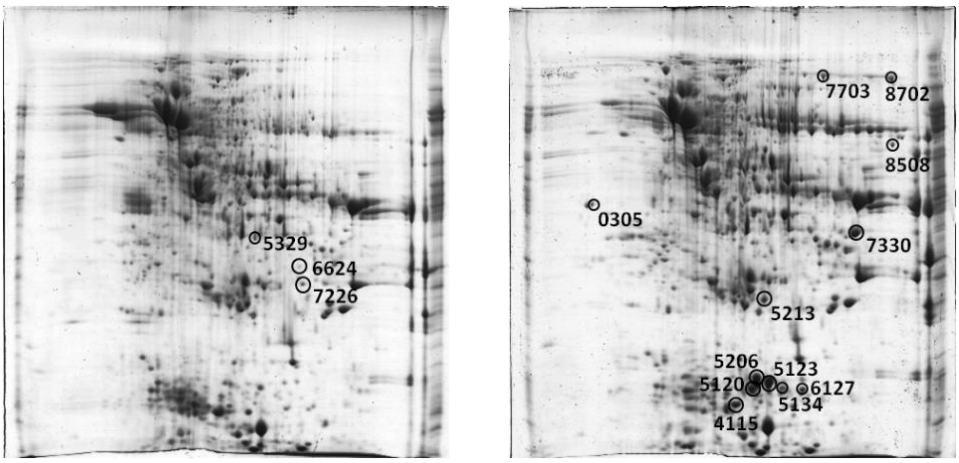



**Figure 3.15:** 2-D protein gels of emmer grains (12 % SDS-PAGE, IEP range: pH 3-10, 17 cm linear strip, coomassie G250–stained) at four development stages (BBCH 75, BBCH 85, BBCH 87, BBCH 97) after *F. graminearum* infection (A) and natural infection (B). The altered spots are each encircled and numbered with an ID according to Tables 3.22-3.25

**Oxidative burst related proteins**

In all development stages proteins related to oxidative stress were up- or down regulated due to *Fusarium* infection. Table 3.19 shows the changes of oxidative stress related enzymes regulated after *Fusarium* infection. Altogether, three antioxidative proteins were identified: a peroxidase, 2-cys peroxiredoxin and a manganese superoxide dismutase. In the early grain development at BBCH 75 the peroxidase 1 was up regulated by a factor of 2.9, and by a factor of 6.3 at BBCH 87 compared to the natural infected grains, whereupon the protein was exclusively expressed in the inoculated grains at BBCH 97. The 2-cys peroxiredoxin was up regulated in the early grain development at BBCH 75 and BBCH 85 and down regulated at BBCH 87 and BBCH 97. Furthermore, a manganese superoxide dismutase was found to be down regulated at BBCH 85.

**Table 3.19:** Changes of proteins related to oxidative burst due to *F. graminearum* infection compared to natural infection during grain ripening stages

| | **BBCH Stage** | | | |
|---|---|---|---|---|
| **Protein** | **75** | **85** | **87** | **97** |
| Peroxidase 1 | + 2.9 | | + 6.3 | + ∞ |
| 2-cys peroxiredoxin BAS1 | + ∞ | + 3.1 | -3.7 | -10.8 |
| Manganese superoxide dismutase | | - 4.2 | | |

(+) = up regulated; (-) = down regulated); +∞ = protein was only found at natural infected grains; - ∞ = protein was only found at artificial inoculated grains

The increased accumulation of antioxidant enzymes in the kernels suggests an oxidative burst after the infection with *F. graminearum*. Plants produce high levels of reactive oxygen species (ROS) by membrane bound NADPH oxidases, such as hydrogen

peroxide ($H_2O_2$) as response of biotic or abiotic stress (Torres, 2010). This oxidative burst contributes to the hypersensitive response and consequently to cell death at the site of the pathogen infection, limiting the pathogen spread to adjacent tissues. On the other hand, the plants produce antioxidants and ROS scavenging enzymes to detoxify these reactive molecules (Atkinson and Urwin, 2012). ROS are furthermore involved in cellular signalling pathways associated amongst others with the induction of defence mechanisms. Their regulation by antioxidative enzymes is proposed to play an important role in plant defence, since the ROS-levels during oxidative burst mediates complex cell signalling networks (Torres, 2010, O'Brien et al., 2012). Peroxidases are furthermore involved in several lignin-polysaccharides cross linking in plant cell walls, leading to higher resistance concerning enzymatic hydrolysis (Ralph et al., 2004). In addition ROS have been identified to induce DON biosynthesis in *F. graminearum*, whereas the presence of catalase reduces the DON- accumulation (Ponts et al., 2007).

**Pathogenesis related proteins**

An important plant strategy to inhibit fungal grows is the induction of pathogenesis related (PR) proteins. PR proteins are known to be induced in plants that are exposed to pathological or related situations and are assumed to protect plants against pathogenic microorganisms and various pests as well as abiotic stresses (van Loon and van Strien, 1999). In the present study different PR-proteins were identified being regulated due to *Fusarium* infection (Table 3.20).

**Table 3.20:** Changes of pathogenesis related proteins due to *F. graminearum* infection compared to natural infection during grain ripening stages of emmer

| | | BBCH Stage | | | |
|---|---|---|---|---|---|
| **Protein** | **Function** | **75** | **85** | **87** | **97** |
| Class II chitinase | chitin catabolic process | + 2.5 | | - 10.5 | |
| Basic endochitinase A (class I) | chitin catabolic process | | | - 12.5 | - 11.5 |
| Predicted (chitin binding) protein | chitin binding | | | + 3.6 | + ∞ |
| Xylanase inhibitor XIP-III | inhibition of fungal spread | | + 2.6 | | |
| Predicted (small heat shock) protein | protein folding | | | - ∞ | |
| HSP 70 (heat shock protein) | protein folding | | | - ∞ | |
| Hypothetical protein | defence response | | - 3.5 | | |
| Globulin | α - amylase inhibitor | | | - 8.1 | |

(+) = up regulated; (-) = down regulated); +∞ = protein was only found at natural infected grains; - ∞ = protein was only found at artificial inoculated grains

Several protein spots that were changed in abundance belong to the group of chitinases. Chitinases catalyse the hydrolysis of chitin, a linear polymer of β- 1,4 linked N-acetyl glucosamine. The amino acid sequence of the chitinase-subfamilies I and II are highly similar. The main difference is that class I chitinases possess beside the catalytic domain a cysteine-rich chitin binding domain, which has no catalytic function, but is suggested to promote the chitinase catabolic activity (Ubhayasekera, 2011). Chitinase production is beside pathogen attack dependent on various biotic an abiotic stress factors, such as wounding, heavy metals, drought- and cold stress. The enzymes are not only involved in plant defence by destroying fungal cell walls, the oligomers of digested chitin generate signal molecules that are supposed to play a role in plant defence (Ubhayasekera, 2011, Sarma et al., 2012). In the present study, chitinases were predominantly down regulated (Table 3.20). Nevertheless a class II endochitinase was up- regulated after *Fusarium* infection in the early grain development at BBCH 75 and later down-regulated at BBCH 87. Furthermore, a class I endochitinase was down-regulated at BBCH 87 and 97. Interestingly in the later stages of infection at BBCH 87 and BBCH 97 a "predicted protein" was up-regulated containing a chitin-binding domain. Coincident with a study of barley genotypes, analyzing the protein expression due to *F. graminearum* infection after three days post-inoculation, chitinase was up-regulated in spikelets during early infection (Geddes et al., 2008). Furthermore, a proteome analysis of mature emmer grains after *Fusarium* infection showed a reduction of a class II chitinase, discussing a fungal signal to be responsible (Eggert et al., 2011). Lutz et al. (2003) discovered that DON-producing *F. culmorum* and *F. graminearum* strains inhibit the expression of a chitinase gene from *Trichoderma atroviride*, demonstrating a possible fungal control of gene regulation concerning chitinase expression.

A xylanase inhibitor protein, playing a role in plant defence against fungi was up-regulated at BBCH 85 with a factor of 2.6 (Table 3.20). Xylanase inhibitor proteins (XIP) belong to the glycosyl hydrolase 18 family, similar to chitinases. They inhibit cell wall-degrading fungal xylanases and therefore inhibit pathogen spread. According to a study of Dornez et al. (2010) different xylanase inhibitor iso-forms were up regulated during grain ripening of wheat in response to *F. graminearum* infection. Two heat-shock proteins (HSP) were down regulated at BBCH 87. Heat shock proteins are known to assist in the correct folding of polypeptides as molecular chaperones and assist the

refolding of non-native proteins. They are known play a role in protecting plants from stress by securing the protein conformation under stress conditions (Wang et al., 2004). Furthermore, an α-amylase inhibitor was down regulated by a factor of 8.1 at BBCH 87. Alpha-amylase inhibitors protect the endosperm against fungal degradation of starch. Most of the pathogenesis related proteins were down regulated especially in the later grain development since BBCH 87. So far, it remains unclear if the pathogen inhibits the formation of PR-Proteins or if the infected plant focuses other defence mechanisms in the proceeded infection, such as cell-wall thickening or accumulating phytoalexins.

**Energy- carbohydrate metabolism and photosynthesis related proteins**

Various proteins being regulated after *F. graminearum* infection are involved in energy- and carbohydrate metabolisms, photosynthesis and starch- and protein synthesis. Especially in the earlier ripening stages proteins involved in glycolysis, citric acid cycle and electron transport chain are up regulated (Table 3.21). Zhou et al. (2006) suggested a possible "connection of glycolysis between *F. graminearum* and wheat" to benefit the carbon assimilation of the fungus. Two proteolytic digested proteins, both at BBCH 97 were down regulated: a glyceraldehyde-3-phosphate dehydrogenase, involved in glycolysis and a malate dehydrogenase involved in the citric acid cycle (Table 3.25).

Another protein, spermidine synthase was up regulated by a factor of 4.2 at BBCH 75 (Table 3.21). Spermidine synthase is involved in the biosynthesis of the polyamine spermidine. Polyamines occur in all living cells. They are involved in several cellular processes such as gene expression, translation, cell division and development as well as cell signalling (Kusano et al., 2008). Polyamines are also involved in stress response and resistance to pathogen infection (Walters, 2003). It has been determined, that some intermediates and products of the polyamine pathway, such as agmatine and putrescine are strong inducers of TRI5 gene expression in vitro and therefore inducers of DON production (Gardiner et al., 2009). DON is known to be a virulence factor promoting fungal spread within wheat spikelets (Jansen et al., 2005). Gardiner et al. (2010) observed a significant increase in putrescine and spermindine in *F. graminearum* infected wheat heads one to seven days after inoculation in comparison to a mock-inoculation. Furthermore, polyamines (putrescine, spermine and spermidine) are amongst other functions suggested to be involved in ROS-scavenging processes (Rhee et al., 2007). In a study of Jang et al. (2012) an increased polyamine biosynthesis prevented the accumulation of reactive oxygen species in rice. Additionally, an

enhanced expression of ROS detoxifying enzymes was found, associated with higher polyamine contents. At BBCH 85, BBCH 87 and BBH 97 several predicted proteins with currently unknown function were down regulated. Furthermore, some of the identified (Tables 3.22-3.25) and not identified proteins were apparently proteolytic fragments.

**Table 3.21:** Changes of proteins related to energy- and carbon metabolism and photosynthesis due to *F. graminearum* infection compared to natural infection during grain ripening stages of emmer

| | | BBCH Stage | | | |
|---|---|---|---|---|---|
| **Protein** | **Function** | **75** | **85** | **87** | **97** |
| Glucose and ribitol dehydrogenase homolog | carbohydrate metabolism | + 2.8 | + 9.9 | | + 5.9 |
| Glyceraldehyde-3-phosphate dehydrogenase | glycolysis | + 3.4 | | | |
| Succinate dehydrogenase | citric acid cycle, electron transport chain | | | + 10.1 | |
| Glucose-1-phosphate adenylyltransferase | glycan/starch biosynthesis, | + 3 | | | |
| Pyruvate, phosphate dikinase 1 (proteolytic fragment) | photosynthesis | + 5.4 | | | |
| Ferredoxin-NADP reductase | photosynthesis, energy metabolism | | + 6.6 | | |
| Eukaryotic translation initiation factor 6-2 | protein biosynthesis | | | + 2,5 | |
| 60S acidic ribosomal protein P0 | protein biosynthesis | | | + 4.9 | |
| Spermidine synthase | polyamine biosynthesis | + 4.2 | | | |

(+) = up regulated; (-) = down regulated); +∞ = protein was only found at natural infected grains; - ∞ = protein was only found at artificial inoculated grains

To our knowledge this was the first proteomic study analysing the effect of *Fusarium* infection on cereal grains during a long infection-period from different ripening stages beginning with early grain development until plant death. In conclusion, an inoculation of emmer grains with *F. graminearum* led to changes of protein expression in all analysed development stages. In the early ripening stages proteins, predominantly related to metabolism and photosynthesis as well as stress-related proteins, like PR-poteins and proteins related to oxidative stress were up regulated. Additionally a spermidine synthase was up regulated at BBCH 75. During later ripening stages (BBCH

87 and 97) predominantly stress-related proteins were down regulated. Nevertheless some stress-related proteins, such as peroxidase and chitin binding proteins were also increased in abundance after *F. graminearum* infection during later grain ripening stages, demonstrating some defence strategies were persistent during the whole infection period. It is imaginable that the pathogen profits from the enhanced metabolism, since the metabolites are beneficial for fungal growth. This study gives an overview of molecular mechanisms initiated by *F. graminearum* infection of emmer grains. Further studies are reasonable, for example comparing susceptible and resistant emmer- and wheat cultivars concerning their response to pathogen attack at different grain ripening stages to understand the response mechanisms of cereals during the entire infection period.

**Table 3.22:** Proteins changed in abundance after *F. graminearum* infection of emmer grains at milk ripe (BBCH 75). The ratio displays the difference in abundance of the respective proteins from artificially inoculated grain compared to natural infected grain, detected as protein spot (density) by two-dimensional gel electrophoresis.

| Spot ID[a] | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio[b] | Identification | Function | Protein homologue | pI[c] exp. | Mass[d] (kDa) exp. | pI [c] calc. | Mass[d] (kDa) calc. | Mascot score | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2214 | -- | 11.3 ± 1.9 | -- | 2-Cys peroxiredoxin BAS1 | response to oxidative stress | *Hordeum vulgare* | 5.1 | 19 | 5.4 | 23 | 224.19 | Q96468 |
| 3316 | 2.9 ± 1.6 | 12.1 ± 1.7 | + 4.2 | spermidine synthase | spermidine biosynthesis | *Oryza sativa* | 5.6 | 28 | 5.1 | 35 | 126.67 | Q9SMB1 |
| 4621 | 2.1 ± 0.6 | 6.5 ± 3.0 | + 3 | ADP-glucose pyrophosphorylase | glycan biosynthesis, starch biosynthesis | *Hordeum vulgare* | 6.3 | 42 | 6.2 | 57 | 988.43 | P30524 |
| 7212 | 3.0 ± 0.7 | 10.3 ± 6.0 | + 3.4 | glyceraldehyde-3-phosphate dehydrogenase C2 (GAPC2) | glycolysis | *Arabidopsis thaliana* | 8.1 | 23 | 6.8 | 36 | 62 | AT1G13440.1 |
| 7409 | 1.4 ± 0.5 | 3.9 ± 2.5 | + 2.8 | glucose and ribitol dehydrogenase homolog | Carbohydrate metabolism | *Hordeum vulgare* | 7.7 | 30 | 6.7 | 31 | 78.09 | T06212 |
| 8503 | 27.3 ± 10.2 | 78.8 ± 22.3 | + 2.9 | peroxisase 1 | response to oxidative stress | *Triticum aestivum* | 8.9 | 35 | 9.5 | 39 | 250.55 | Q8LK23 |
| 8307 | 9.1 ± 3.4 | 23 ± 7.5 | + 2.5 | class II chitinase | hydrolase, cell wall degradation | *Triticum aestivum* | 8.9 | 25 | 9.5 | 28 | 275.39 | Q4Z8L8 |
| 4213 | 0.9 ± 1.1 | 5.0 ± 2.3 | + 5.4 | pyruvate phosphate dikinase 1, Chloroplastic **(proteolytic fragment)** | photosynthesis | *Oryza sativa* | 6.1 | 22 | 5.9 | 102 | 103.03 | Q6AVA8 |

[a] related to Figure 1
[b] (+)= up regulated; (-) =down regulated
[c] (pI)= isoelectric point; experimental/calculated
[d] Mass = molecular Mass; experimental/calculated

**Table 3.23:** Proteins changed in abundance after *F. graminearum* infection of emmer grains at soft dough stage (BBCH 85). The ratio displays the difference in abundance of the respective proteins from artificially inoculated grain compared to natural infected grain, detected as protein spot (density) by two-dimensional gel electrophoresis.

| Spot ID [a] | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Function | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2226 | 3.3 ± 0.6 | 10.1 ± 2.7 | +3.1 | 2-Cys peroxiredoxin BAS1, chloroplastic | oxidoreductase | *Hordeum vulgare* | 4.3 | 22 | 5.3 | 23 | 767.51 | Q96468 |
| 4722 | 0.2±0.1 | 3.7±1.4 | +16.7 | hypothetical protein | transketolase | *Gibberella zeae PH-1* | 6.3 | 83 | 5.5 | 74 | 516.79 | FG09998.1 |
| 5326 | 7.1±3.1 | 31.3±1.5 | + 4.4 | glucose and ribitol dehydrogenase homolog | carbohydrate metabolism | *Hordeum vulgare* | 6.8 | 33 | 6.7 | 32 | 673.12 | T06212 |
| 6312 | 1.9±0.3 | 10.4 ± 2.3 | + 5.55 | glucose and ribitol dehydrogenase homolog | Carbohydrate metabolism | *Hordeum vulgare* | 7.3 | 33 | 6.7 | 32 | 173.95 | T06212 |
| 7302 | 1.5±0.6 | 3.8±0.5 | +2.6 | xylanase inhibitor XIP-III | inhibition of xylan degradation | *Triticum aestivum* | 9.9 | 32 | 7.8 | 33 | 386.93 | Q4W6G2 |
| 7327 | 0.9±0.7 | 6.2±0.9 | +6.6 | Ferredoxin--NADP Reductase, embryo isozyme, chloroplastic | oxidoreductase, energy metabolism, photosynthesis | *Oryza sativa* | 8.6 | 36 | 9.4 | 42 | 330.20 | O23877 |
| 2223 | 22.8 ± 6.5 | 6.5 ± 3.5 | - 3.5 | hypothetical protein | defence response | *Oryza sativa* | 4.9 | 21 | 4.6 | 17 | 1381.56 | A2XUF3 |
| 3217 | 15.8 ± 4.9 | 0.6 ± 0.7 | -25.1 | predicted protein | unknown | *Hordeum vulgare* | 5.6 | 24 | 5.4 | 26 | 123.75 | F2D074 |
| 5223 | 45.2±9.6 | 10.6±9.6 | - 4.2 | manganese superoxide dismutase | response to oxidative stress | *Triticum aestivum* | 7.1 | 25 | 8.8 | 25 | 105.34 | O82571 |
| 5207 | 0.9±0.3 | 13.8±4.9 | +15.4 | globulin-like protein **(proteolytic fragment)** | nutrition reservoir | *Oryza sativa* | 6.7 | 27 | 6.9 | 52 | 158.83 | Q8L8I0 |
| 6111 | 5.1±3.3 | 1.6±1.1 | - 3.2 | predicted protein **(proteolytic fragment)** | unknown | *Hordeum vulgare* | 7.3 | 17 | 9.4 | 34 | 212.57 | F2D224 |

[a] related to Figure 1
[b] (+)= up regulated; (-) =down regulated
[c] (pI)= isoelectric point; experimental/calculated
[d] Mass = molecular Mass; experimental/calculated

**Table 3.24:** Proteins changed in abundance after *F. graminearum* infection of emmer grains at hard dough stage (BBCH 87). The ratio displays the difference in abundance of the respective proteins from artificially inoculated grain compared to natural infected grain, detected as protein spot (density) by two-dimensional gel electrophoresis.

| Spot ID [a] | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Function | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2212 | 2.1±0.5 | 5.2±2.8 | + 2.5 | eukaryotic translation initiation factor 6-2 | protein biosynthesis | *Arabidopsis thaliana* | 5.2 | 28 | 4.5 | 26 | 284.28 | Q9M060 |
| 4404 | 5.2±0.8 | 25.6±9.5 | + 4.9 | 60S acidic ribosomal protein P0 | translational elongation | *Oryza sativa* | 6.2 | 36 | 5.2 | 34 | 373.84 | P41095 |
| 6232 | 1.8±1.1 | 11.4±1.0 | +6.3 | peroxidase 1 | response to oxidative stress | *Triticum aestivum* | 8.0 | 28 | 9.5 | 38 | 366.82 | Q8LK23 |
| 6340 | 1.3±0.8 | 4.9±0.7 | +3.6 | predicted protein | Chitin binding (type-1 domain) | *Hordeum vulgare* | 7.9 | 30 | 8.8 | 34 | 215.16 | F2CSS7 |
| 7311 | 1.6±0.9 | 16.3±7.8 | +10.1 | succinate dehydrogenase | Oxidoreductase activity | *Triticum aestivum* | 8.5 | 30 | 9.7 | 31 | 130.46 | Q84VR9 |
| 5111 | 43.9±14.1 | -- | -- | predicted: small heat shock protein (HSP20) | Protein folding | *Hordeum vulgare* | 7.0 | 14 | 5.4 | 17 | 91.81 | F2EH52 |
| 5224 | 29.3±3.8 | 7.8±2.2 | - 3.7 | 2-Cys peroxiredoxin BAS1. chloroplastic (Fragment) | antioxidant, oxidoreductase | *Hordeum vulgare* | 7.2 | 25 | 5.4 | 23 | 609.74 | Q96468 |
| 5521 | 6.2±1.7 | -- | -- | HSP70 (heat shock protein) | protein folding | *Hordeum vulgare* | 7.2 | 53 | 5.7 | 67 | 84.32 | Q40058 |
| 7315 | 44.3±4.1 | 5.5±3.9 | -8.1 | globulin | α- amylase inhibitor | *Triticum turgidum* | 8.6 | 31 | 9.7 | 25 | 891.55 | AAR95703 |
| 7316 | 39.7±7.2 | 3.2±1.3 | -12.5 | basic endochitinase A | chitin catabolic process | *Secale cereale* | 8.8 | 33 | 9.5 | 34 | 1713.89 | Q9FRV1 |
| 7317 | 49.6±34.2 | 4.7±3.7 | -10.5 | class II chitinase | glucosidase hydrolase | *Triticum aestivum* | 8.8 | 29 | 9.5 | 28 | 646.49 | Q4Z8L7 |
| 8501 | 2.2±0.5 | -- | -- | predicted protein | unknown | *Hordeum vulgare* | 9.5 | 58 | 11.2 | 48 | 204.37 | F2DS64 |

[a] related to Figure 1
[b] (+)= up regulated; (-) =down regulated
[c] (pI)= isoelectric point; experimental/calculated
[d] Mass = molecular Mass; experimental/calculated

**Table 3.25:** Proteins changed in abundance after *F. graminearum* infection of emmer grains at plant death (BBCH 97). The ratio displays the difference in abundance of the respective proteins from artificially inoculated grain compared to natural infected grain, detected as protein spot (density) by two-dimensional gel electrophoresis.

| Spot ID [a] | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Funktion | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5329 | 1.1±1.2 | 6.4±1.1 | + 5.9 | glucose and ribitol dehydrogenase homolog | oxidoreductase | *Hordeum vulgare* | 7.2 | 33 | 6.7 | 32 | 720.54 | T06212 |
| 6224 | -- | 3.2±0.4 | -- | predicted protein | chitin binding (type-1 domain) | *Hordeum vulgare* | 8.1 | 29 | 8.8 | 34 | 215.16 | F2CSS7 |
| 7226 | -- | 8.8±1.9 | -- | peroxidase 1 | response to oxidative stress | *Triticum aestivum* | 8.1 | 27 | 9.5 | 39 | 366.82 | Q8LK23 |
| 0305 | 9.4±2.3 | -- | -- | predicted protein | unknown | *Hordeum vulgare* | 4.4 | 28 | 4.1 | 28 | 695.56 | F2DNE8 |
| 7330 | 54.9±10.5 | 5.0±1.2 | - 11.0 | basic endochitinase A | defence against fungal pathogens | *Secale cereale* | 8.9 | 33 | 9.5 | 34 | 1875.66 | Q9FRV1 |
| 7703 | 11.4±2.6 | 0.3±0.3 | - 32.9 | predicted protein | unknown | *Hordeum vulgare* | 8.4 | 77 | 7.3 | 70 | 376.84 | F2EKY2 |
| 8508 | 7.2±0.5 | 1.3±0.6 | - 5.4 | predicted protein | unknown | *Hordeum vulgare* | 9.6 | 48 | 11.2 | 48 | 298.19 | F2DS64 |
| 8702 | 19.4±3.0 | -- | -- | predicted protein | unknown | *Hordeum vulgare* | 9.6 | 74 | 7.3 | 70 | 565.66 | F2DS64 |
| 5213 | 33.7±4.2 | 3.1±0.4 | - 10.8 | 2-Cys peroxiredoxin BAS1. chloroplastic (Fragment) | antioxidant, oxidoreductase | *Hordeum vulgare* | 7.4 | 25 | 5.4 | 23 | 609.74 | Q96468 |
| 4115 | 50.5±5.9 | 22.2±20.2 | - 2.3 | globulin 3 **(proteolytic Fragment)** | nutrition reservoir | *Triticum aestivum* | 6.9 | 16 | 8.8 | 66 | 356.10 | ACJ65514 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5134 | 20.4±9.6 | -- | -- | malate dehydrogenase **(proteolytic Fragment)** | tricarboxylic acid cycle | *Oryza sativa* | 7.7 | 18 | 5.7 | 35 | 2163.24 | F2D4W6 |
| 6127 | 22.5±4.0 | 2.1±0.7 | - 10.8 | Glyceraldehyde-3-phosphate Dehydrogenase **(proteolytic Fragment)** | glycolysis | *Hordeum vulgare* | 8.0 | 17 | 6.2 | 33 | 1386.85 | P08477 |

[a] related to Figure 1
[b] (+)= up regulated; (-) =down regulated
[c] (pI)= isoelectric point; experimental/calculated
[d] Mass = molecular Mass; experimental/calculated

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## 3.5 Identification of regulated proteins in naked barley *(Hordeum vulgare nudum)* after *Fusarium graminearum* infection at different grain ripening stages

**Abstract**

With this study we analysed the effect of *Fusarium graminearum* infection on field-grown naked barley. Naked barley ears were artificially inoculated with *F. graminearum* spores during anthesis. In the course of grain ripening five phenological growth stages of naked barley grains from milk ripe to plant death stage were analysed. The albumin and globulin proteins of artificially inoculated grains and natural infected grains were separated by two-dimensional gel electrophoresis. Forty-five proteins spots that were changed in abundance due to *F. graminearum* infection were identified by mass spectrometry. During early grain ripening stages more proteins changed in abundance than during later ripening stages. Various proteins showing altered expression pattern after *Fusarium* infection were related to stress response such as oxidative burst, antifungal proteins and proteins related to plant signal transduction pathway. Protease inhibition was exclusively relevant during milk ripe stage. Some antifungal proteins, such as thaumatin-like protein were still accumulated at plant death stage. Plant metabolism related proteins were predominantly changed in abundance during the first three ripening stages, which was not observed during late grain ripening stages. Proteins related to nitrogen metabolism and protein biosynthesis were predominantly down regulated, whereas proteins related to carbon metabolism were up regulated at infected grains.

### Introduction

Fusarium head blight (FHB) is a serious disease of small grain cereals, causing significant losses of crop yield due to the accumulation of mycotoxins, such as Deoxynivalenol (DON), Nivalenol (NIV) and Zearalenone (ZEA). The crucial agents for this disease in Europe are *Fusarium graminearum,* Fusarium *culmorum* and *Fusarium avenaceum* (Bottalico and Perrone, 2002). *F. graminearum* has been identified as the major pathogen, responsible for FHB during the last decades (Kazan et al., 2012).

There are differences concerning the pathogenicity of *Fusarium spp.* among different hosts (Maier et al., 2006). For example Jansen et al. (2005) demonstrated that DON is a

virulence factor during hyphal invasion of the rachis node in wheat spikes but not in barley. Buerstmayr et al. (2004) detected a broad variation for resistance to *F. graminearum* in 143 different spring barley lines. Moreover, in this study the two-row barley line has been found to be more resistant than the six-row barley lines. A comparison of *F. culmorum* susceptibility between hulled and hulless (naked) barley revealed hulless genotypes to be more susceptible than hulled genotypes, suggesting the adhering glumes to protect the seed from pathogen invasion (Warzecha et al., 2010). Nevertheless, studies of Eggert et al. (2010a) and Eggert et al. (2010b) showed higher resistance to *Fusarium spp.* infection of naked barley compared to emmer and wheat. According to these studies, beside higher toxin contents in emmer and wheat grains, their storage protein composition was found to be stronger affected by *Fusarium* infection than the proteins of barley and naked barley grains. *Fusarium* infection of barley grain affects the protein composition due to increased proteolytic activity and therefore influences the malting and brewing quality. Furthermore, fungal mycotoxins can be accumulated in products like beer (Oliveira et al., 2013). Certainly, the development of cereal genotypes with low FHB severity and therefore low mycotoxins contamination is an important issue in resistance breeding. Understanding plant defence mechanisms on molecular level is crucial for breeding programs. In recent time proteomic studies has become a useful tool for identifying proteins that are involved in plant defence response, host resistance and plant-pathogen interactions concerning *Fusarium spp.* infection of cereals, such as wheat and barley (Yang et al., 2013). Several proteomic studies focussed on the secreted proteins by the fungi, playing a role in pathogenicity. Phalip et al. (2005), Paper et al. (2007) and Yang et al. (2012) identified predominantly proteins involved in plant cell wall degradation like glycoside hydrolases and proteins involved in starch- and protein degradation. Extensive proteomic studies have been conducted to investigate the differentially expressed proteins of wheat (Zhou et al., 2006, Wang et al., 2005) and barley (Yang et al., 2010, Geddes et al., 2008) infected with *F. graminearum* within the first days during the initial infection. According to these studies, many proteins related to metabolism and photosynthesis, as well as fungal cell wall degradation were changed in abundance. Furthermore, pathogenesis related (PR) proteins such as chitinases, thaumatin-like proteins and proteins involved in oxidative stress response were predominantly accumulated during the early infection of wheat and barley. Studies on mature emmer and naked barley grains, which were infected by *Fusarium spp.* revealed also changes

of proteins serving as nutrient reservoirs and changes of proteins involved in oxidative stress, defence responses and transcription regulation, showing an initiation of defence strategies in harvested grains (Eggert and Pawelzik, 2011, Eggert et al., 2010a).

With the current study we investigated the impact of *F. graminearum* infection on the grains of a naked barley cultivar, regarding the whole course of grain ripening and the corresponding infection stage, respectively. Given that cereal albumins and globulins contain numerous proteins with relevant functions such as cell development, metabolism and stress response we expect novel information concerning pathogenicity and host response to *F. graminearum*.

## Methods and materials

### Experimental design

The field trial was carried out at Marienstein (Nörten-Hardenberg), near Göttingen in 2011. The investigated naked barley genotype was Lawina. The plants were grown in eight plots, each with a dimension of three to six meters. Four plots were artificially inoculated with an *F. graminearum* spore suspension ($20^5$ spores/mL; 50 mL/$m^2$) three times during flowering. Three DON producing strains of *F. graminearum* (FG 142, FG 143, FG 144) were cultured on an autoclaved wheat straw suspension, consisting of nine g straw (1.5 mm), 500 mL distilled water and 50 mg streptomycine sulfate for ten days a 20 °C. The DON producing strains were reference stocks from the Division of Plant Pathology and Crop Protection at the Department of Crop Science of the Georg-August-University of Göttingen. After harvesting, the quantification of the conidiospores was conducted with a Fuchs-Rosenthal chamber (0.0625 $mm^2$; depth: 0.2 mm).

### Sample preparation and protein extraction

For the analysis, ears from each plot (four inoculated; four naturally infected) were collected five times during grain development. The development stages were identified according to the BBCH-scale. The ripening stages according to Table 3.26 of the grains were identified using the extended BBCH-scale. The BBCH-scale defines the phonological growth stages with a standardised decimal code. The abbreviation „BBCH" derives from Biologische Bundesanstalt, Bundessortenamt and chemical industry (Meier, 2001).

**Table 3.26:** Days after inoculation (dai) (anthesis: 0 dai) of naked barley plants and the corresponding phenological growth stages

| Phenological growth stages (BBCH code) | Days after inoculation |
|---|---|
| Early milk (73) | 7 |
| Medium milk (77) | 14 |
| Soft dough (85) | 21 |
| Hard dough (87) | 36 |
| Plant death and collapsing (97) | 54 |

Fifty ears were randomly picked and freeze dried. Afterwards the grains were manually removed from the ears and milled with a ball mill (Retsch®; Mixer Mill MM 400). Grain samples were stored at −80°C prior to analysis. The albumins and globulins were extracted with an 50 mM sodium phosphate buffer (pH 7.8), containing 0.1 M NaCl and

0.2% protease inhibitor cocktail (Sigma-Aldrich®). 100 mg flour was extracted with 1 mL of sodium phosphate buffer and stirred for 2 h at 4°C. After centrifugation (Eppendorf; centrifuge 5804 R) at 8000g and 4°C for 10 min, 500 µL of the supernatant was transferred into a new tube. 1.5 mL of ice-cold TCA-acetone was added to the supernatants and stored at -20°C to precipitate the protein over night. The cold samples were centrifuged (8000 g; 4°C; 10 min) and the supernatants were removed. The pellets were rinsed three times with cold acetone under stirring for 10 min at 4°C and then centrifuged as before. The pellets were evaporated at 100 mbar for 10 min in a vacuum concentrator (Christ; RVC 2-25 CD) and resuspended in 500 µL lysis buffer (6 M urea, 2 M thiourea, 0.2 % pharmalyte buffer (w/v pH 3-10), 2% CHAPS, 2% DTT, 0.2% protease inhibitor cocktail (Sigma-Aldrich®), 0.002% bromophenol blue). For solubilisation of the protein, samples were shaken for 1h at 33°C in a Thermomixer (Eppendorf). Finally, protein concentrations were determined with a 2-D Quant Kit (Amersham Bioscience) and adjusted to a concentration of 1µg/µL.

**Two-Dimensional Gel Electrophoresis (2D GE)**

For isoelectric focusing, commercially available immobilized pH gradient (IPG) stripes (pH 3-10, 17 cm, BioRad) were used. 300 µL of the protein sample, solved in the lysis buffer was loaded into a tray. The IPG stripes were rehydrated over night, at which the stripes were covert with 1 mL mineral oil to prevent evaporation. The IEF was performed in the Protean® IEF cell (BioRad) with the following conditions: 15 min 0-250 V; 3h 250 - 10000 V; 10000 V - 60000 V- hrs. The ramp was chosen rapid, the current was set to 50 µA per gel and the Temperature was 20°C. Afterwards the IPG stripes were incubated one after another with two buffer agents for 15 min: the first containing 6 M urea, 2% SDS, 0.375 M Tris-HCL (pH 8.8), 20% glycerol, 2% DTT and the second containing 6 M urea, 2% SDS, 0.375 M Tris-HCL (pH 8.8), 20% glycerol, 2.5% iodoacetamide. Finally the strips were rinsed with SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS). SDS-PAGE was performed in the Protean® II xi Cell (BioRad) by using 12 % acryamide gels (20 cm, 20 cm, 1 mm) with a current of 30 mA per gel. The staining was performed with a modified colloidal Coomassie G-250 staining (blue silver) according to Candiano et al. (2004).

**Data analysis**

The Gels were scanned with an image scanner (Epson Expression™ 10000 XL) using the LabScan 6.0 software. The gel images were saved as TIF files and analyzed using the PDQuest Basic 8.0.1 analysis software (BioRad). Required for statistical analysis four biological replications of each group, artificial inoculation and natural infection were used to create 'replicate groups' for each grain development stage. Spot detection and matching was carried out by using the automated 'spot Detection wizard'. Spots that were present in at least three of the group members were added to the analysis set. The spot quantities in four naturally infected samples were compared with the spot quantities in four inoculated samples. For statistical analysis sets, the student's t-test with significance level of 90% was chosen. The standard deviation between four replicate groups was under 50%. Protein spots were accepted to change after *F. graminearum* infection if the difference between the mean value of natural infected and artificially inoculated samples were equal or higher than the factor of two.

**Protein identification by mass spectrometry**

Tryptic digestion of proteins and identification of proteins by mass spectrometry (MS) were performed as described by Klodmann et al. (2010) at the University of Hannover by The institute of Plant Proteomics. Procedures were based on peptide separation using the EASY-nLC System (Proxeon; Thermo Scientific, Bremen, Germany) and coupled MS analyses using the MicrOTOF-Q II mass spectrometer (Bruker Bremen, Germany). MS data evaluation was carried out using ProteinScape2.1 software (Bruker, Bremen, Germany) and the Mascot search algorithm (Matrix Science, London, UK) against (1) the NCBI protein database (www.ncbi.nih.org), (2) the UniProt protein database (http://www.uniprot.org/) and (3) the Arabidopsis protein database (www.Arabidopsis.org); release TAIR 10). The following Mascot search parameters were used: enzyme, trypsin/P (up to one missed cleavage allowed); global modification, carbamidomethylation (C), variable modifications, acetyl (N), oxidation (M); precursor ion mass tolerance, 15 ppm; fragment ion mass tolerance, 0.6 Da; peptide charge, 1+, 2+, and 3+; instrument type, electrospray ionization quadrupole time of flight. Minimum ion score was 30, minimum peptide length was 4 amino acids, significance threshold was set to 0.05 and protein and peptide assessments were carried out if the Mascot Score was greater than 30 for proteins and 20 for peptides.

**Quantitative LC-MS/MS of DON**

DON determination was performed with small variations as described by (Adejumo et al. (2007) in the laboratory of Molecular Phytopathology and Mycotoxin research, Department of Crop Sciences, University of Göttingen, Germany

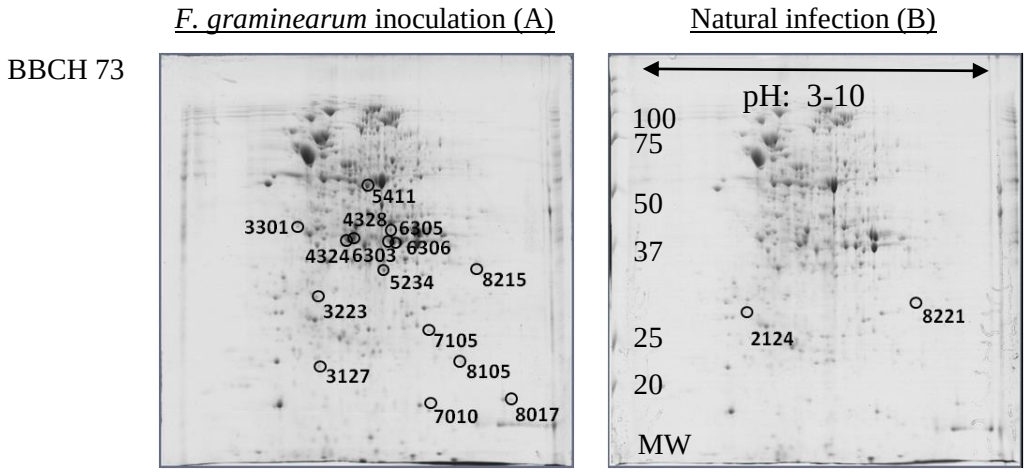
## Results and discussion

### DON accumulation in grains

The accumulation of DON in the grains of the naked barley cultivar Lawina is described in chapter 3.3. After *F. graminearum* inoculation, the DON contents were distinctly increased in all development stages (Table 3.10), whereas DON was not detectable in natural infected grains. These results demonstrate the successful *F. graminearum* inoculation of the ears.

### Proteome analyses

After artificial *F. graminearum* infection a total of 58 proteins showed different expression patterns regarding all five development stages (Tables 3.32-3.36 and Figure 3.16). A total of 45 proteins could be identified by mass spectrometry and database search. Thirteen proteins were not identified or are proteins with unknown function. During the early grain development more proteins were changed in abundance than during later grain development. At BBCH 73, BBCH 77 and BBCH 85 17, 15 and 13 proteins showed different abundance, whereas at BBCH 87 and BBCH 97 only 7 and 6 proteins, respectively, were found to be differently regulated (Figure 3.16). Moreover, from the BBCH 73 until BBCH 85 considerably more proteins were detected to be up-regulated due to *F. graminearum* infection (Figure 3.16A), whereas most of the differently expressed proteins during the later grain development (BBCH 87 and BBCH 97) were down regulated (Figure 3.16B).



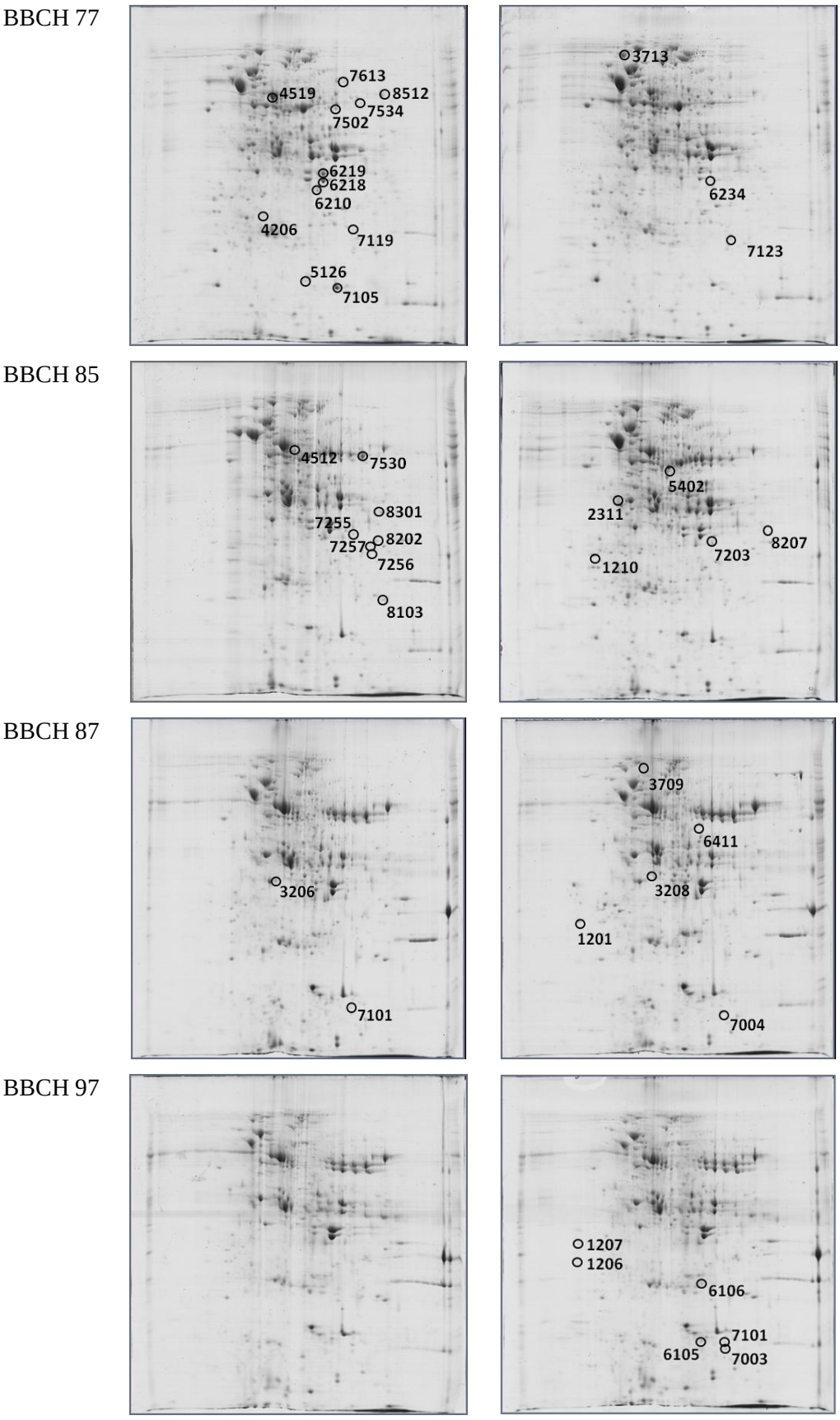



**Figure 3.16:** 2-D protein gels of naked barley grains (12 % SDS-PAGE, IEP range: pH 3-10, 17 cm linear strip, molecular weight (MW) in Kda, coomassie G250–stained) at five development stages (BBCH 73, BBCH 77, BBCH 85, BBCH 87, BBCH 97) after artificial *F. graminearum*

inoculation (A) and natural infection (B). Altered spots are each encircled and numbered with an ID according to Tables 3.32-3.36

**Pathogenesis related (PR) proteins**

Numerous proteins of the barley seed proteome are involved in stress response including PR proteins (Bak-Jensen et al., 2004, Østergaard et al., 2004). PR proteins are generally induced in plant tissues in response to pathogens or pests (van Loon et al., 2006). These proteins thus have various biological functions playing a role in resistance either direct by the inhibition of pathogen growth and spore germination or indirect, for example, by the prevention of pathogen penetration through plant cell walls or involvement in signal transduction concerning plant-pathogen interaction (Gorjanovic, 2009).

During all grain development stages PR proteins were regulated due to *F. graminearum* infection compared to natural infected grains (Tables 3.27 and 3.28). Exclusively during the early grain ripening stages (BBCH 73 and BBCH 77) proteins related to protease inhibition were found to be up regulated (Table 3.27). Two serine protease inhibitors (Z-type serpins) were accumulated by the factors 2.0 and 2.6 due to *Fusarium* infection at BBCH 73. At BBCH 73 and 77 a barley α-amylase/subtilisin inhibitor (BASI) was up-regulated by a factor of 1.6 and induced, respectively. Furthermore, a predicted protein containing an endo-peptidase inhibitor domain was up-regulated by a factor of 2.4 at BBCH 73 in *F. graminearum* infected barley grains. Protease inhibitors have the ability to form complexes with proteases to inhibit their proteolytic activity and therefore protect the plant proteins from being digested by foreign proteases (Ryan, 1990). The bi-functional barley α-amylase/subtilisin inhibitor additionally prevents the degradation of the endosperm starch due to fungal attack. Several PR proteins like α-amylase/subtilisin inhibitors and serpins have been observed to accumulate constitutively in barley seeds during grain filling and maturation (Finnie et al., 2002). During later ripening stages no protease inhibitors were found to be up- or down regulated after *F. graminearum* infection compared to natural infection in the current study. Nevertheless, serine protease inhibitors have been found to be up regulated after *Fusarium spp.* infection in mature naked barley grain (Eggert and Pawelzik, 2011). During the initial infection period of barley and wheat spikelets up to three days post inoculation with *F. graminearum* no protease inhibitors were detected by proteomic approach (Yang et al., 2010, Geddes et al., 2008, Bi et al., 2005), suggesting that protease inhibition is not significant for defence response during the initial infection.

Yang et al. (2010) observed *F. graminearum* induced degradation of β-amylase in the spikelets of a susceptible barley cultivar since the third day post inoculation, demonstrating the action of a specific endopeptidase and therefore determined the stage at which proteolytic degradation of *Fusarium* proteases occurred.

Furthermore, in the current study nucellin, an aspartic protease-like protein was down regulated at BBCH 73 by a factor of 3.0. Barley nucellin is reported to be expressed in the nucellus and is supposed to play a role in its programmed cell death. Furthermore, the nucellin gene is also strongly expressed in barley embryos (Bi et al., 2005, Chen and Fooland, 1997). The specific function of this aspartic protease-like protein in barley development or defence response is currently not explored. Additionally, a 20 S proteasome subunit was induced after *Fusarium* infection at BBCH 85. Proteasomes consist of multiple subunits and play a role in plant development and stress response. They are responsible for the degradation of proteins damaged or misfolded, for example, because of cellular stress (Kurepa et al., 2009). Furthermore, higher levels of the free 20S proteasome leaded to increased tolerance to oxidative stress (Kurepa et al., 2008).

**Table 3.27:** Changes of proteases and proteins related to protease inhibition due to *F. graminearum* infection compared to natural infection during grain ripening stages of naked barley

| | BBCH stage | | | | |
|---|---|---|---|---|---|
| Protein | 73 | 77 | 85 | 87 | 97 |
| Serpin z-type | + 2.0 | | | | |
| Serpin Z4 | + 2.6 | | | | |
| α-amylase/subtilisin inhibitor | + ∞ | + 1.6 | | | |
| Predicted: endopeptidase inhibitor | + 2.4 | | | | |
| Nucellin | - 3.0 | | | | |
| Proteasome subunit alpha type | | | + ∞ | | |

(+) = up regulated; (-) = down regulated); +∞ = protein was only found in natural infected grains

A group of antifungal proteins that were found to be up regulated due to *F. graminearum* infection in the current study were thaumatin-like proteins (TLP's). At BBCH 77, BBCH 85 and BBCH 97 thaumatin-like proteins were accumulated in the infected grains by the factors 2.1, 2.2 and 2.8, respectively (Table 3.28). Thaumatin-like proteins inhibit *in vitro* hyphal growth and mediate hyphal and spore lysis (De Lucca et al., 2005). Due to the fact that thaumatin-like proteins occurred since milk ripe grains to

plant death stage after *F. graminearum* infection it can be assumed that TLP's are fundamental for plant defence against the pathogen during the entire grain development.

In the analysed naked barley cultivar cell wall-degrading and related enzymes seem to play a role in plant defence against *F. graminearum*. A predicted chitin binding-domain protein belonging to the barley wound induced (Barwin) protein family (PR-4) was accumulated at BBCH 73 by the factor 2.3 (Table 3.28). These proteins show inhibitory activity on the growth of phytophatogenic fungi, as well as the potential to amplify the antifungal effect of TLP's and chitinases (Gorjanovic, 2009). Furthermore, a fungal cell wall-degrading enzyme, β-1,3-endoglucanase was up-regulated at BBCH 85 by a factor of 1.8 (Table 3.28). This finding is consistent with a study of Oliveira et al. (2013), who detected an up-regulation of β-1,3-glucanase in mature barley grain, infected with *F. culmorum*. During later grain development, at BBCH 87 a predicted glyoxalase 1 was induced after *F. graminearum* infection (Table 3.28). Glyoxalase detoxifies methylglyoxal, a cytotoxic metabolite of amino acid catabolic process and is known to be up regulated by some abiotic stress (Espartero et al., 1995). Lin et al. (2010) revealed an induction of a glyoxalase gen in wheat spikelets after an inoculation with *F. graminearum* 12 hours post inoculation, suggesting a possible role of glyaxalases in *Fusarium* resistance. The biochemical processes of glyoxalase related to FHB resistance is still unclear.

Moreover, several PR proteins were down regulated due to *F. graminearum* infection (Table 3.28). In accordance with a study of Yang et al. (2010) analysing barley spikelets three days after *F. graminearum* infection, a heat shock protein (Hsp) belonging to the Hsp90 family was down regulated at BBCH 77 by a factor of 3.0 in the current study. Additionally, two small Hsp's were down-regulated at BBCH 87 and BBCH 97. Hsp's are molecular chaperones, assisting the correct folding of polypeptides and the refolding of proteins. They play a role in saving plants under stress conditions by protecting their protein conformation (Wang et al., 2004). Moreover, a β-hordothionin was found to be down regulated at BBCH 97 by a factor of 2.3. Thionins belong to the PR-13 protein family, which have a putative role in plant defence (Gorjanovic, 2009). They have an inhibitory effect on fungi and bacteria. Thionins from barley, alternatively named hordothionins has been expressed in transgenic oat lines. The resulting transgenic hordothionin reduced the growth of *F. graminearum in vitro* (Carlson et al., 2006). In this study no accumulation of thionins were observed. However, a down regulation of β-

hordothionin occurred not until grain-maturity. Two more proteins, supposed to be involved in plant defence were down-regulated in the inoculated grains: a 14-3-3-like protein at BBCH 73 and a phospholipase D at BBCH 87 with a factor of 3.3. The 14-3-3 proteins act as regulators of a wide range of target proteins in eukaryotic cells involved in signal transduction. Therefore, these proteins play a crucial role in response to environmental stress as well as defence response to pathogen attack by activating for example the hypersensitive response (Roberts et al., 2002). In contrast a study of Manosalva et al. (2011) analysing the role of rice 14-3-3 protein in disease resistance revealed higher resistance to a necrotrophic fungal pathogen of gene-silenced rice-plants compared to plants capable of 14-3-3 protein expression. Additionally, a higher expression of a peroxidase gene and accumulation of reactive oxygen species have been observed in the plants lacking in 14-3-3 protein expression (Manosalva et al., 2011). Phospholipase D catalyses the hydrolysis of structural polypeptides to produce phosphatidic acid, a second messenger regulating various cellular functions, including signalling pathways related to plant defence (Canonne et al., 2011). Moreover phosphatidic acid has been shown to induce ROS production in Arabidopsis (Zhang et al., 2009). Interestingly, the suppression of a phospholipase D beta 1 gene in rice enhanced disease resistance in rice as well as the expression of defence related genes including those for glucanases, chitinases and thaumatin-like proteins (Yamaguchi et al., 2009). Furthermore, this transgenic rice showed enhanced accumulation of ROS and the induction of hypersensitive response-like reactions in the leaves, indicating a negative regulation of defence response of phospholipases in plants. Finally, a nascent polypeptide associated complex (αNAC) was down regulated due to *F. graminearum* infection at BBCH 97. The αNAC has transcriptional activating function and binds to DNA, rRNA and tRNA (Rospert et al., 2002). Hotokezaka et al. (2009) found that αNAC down regulation initiates apoptosis of human cells *in vitro* and plays a role in endoplasmatic reticulum stress.

**Table 3.28:** Changes of pathogenesis related proteins due to *F. graminearum* infection compared to natural infection during grain ripening stages of naked barley

| | BBCH stage | | | | |
|---|---|---|---|---|---|
| Protein | 73 | 77 | 85 | 87 | 97 |
| Thaumatin-like protein | | + 2.1 | + 2.2 | | + 2.8 |
| Predicted: chitin binding protein | + 2.3 | | | | |
| B-1,3-endoglucanase | | | + 1.8 | | |
| Glyoxalase 1 | | | | + ∞ | |
| Heat shock protein | | - 3.0 | | - 5.7 | - ∞ |
| B-hordothionin | | | | | - 2.3 |
| 14-3-3-like protein A | - 1.8 | | | | |
| Phospholipase D alpha 1 | | | | - 3.3 | |
| Nascent polypeptide-associated Complex (NAC), alpha subunit protein | | | | | - ∞ |

(+) = up regulated; (-) = down regulated); +∞ = protein was only found in natural infected grains; - ∞ = protein was only found in artificial inoculated grains

**Oxidative burst related proteins**

A total of three different proteins related to oxidative burst were regulated due to *F. graminearum* infection (Table 3.29). Peroxidase was accumulated in the infected barley grains at BBCH 73 by a factor of 1.9 and at BBCH 77 by the factors of 2.4 and 1.9 and furthermore induced at BBCH 85. Additionally, a catalyse isoenzyme was up-regulated at BBCH 77 with a factor of 2.3. The antioxidant protein 1-cys peroxiredoxin was found to be up-regulated at BBCH 77 by a factor of 2.6 and later down regulated at BBCH 97 by a factor of 6.0. Low levels of reactive oxygen species (ROS) are known to be constantly generated during metabolism. An increased occurrence of antioxidant proteins after *F. graminearum* infection demonstrates an oxidative burst, known as the rapid accumulation of ROS in the plants (Torres, 2010). ROS are associated with the hypersensitive response, leading to programmed cell death at the site of pathogen attack to prevent pathogen spread. Moreover these reactive molecules play a role in cellular signalling associated with the induction of defence responses as well as in cell-wall cross-linking to establish a physical barrier for the invading pathogen (O'Brien et al., 2012). Plants produce ROS-scavenging enzymes to reduce these reactive molecules, and therefore prevent cell damage and ensure redox homeostasis in the plant cells (Atkinson and Urwin, 2012). On the contrary Geddes et al. (2008) identified proteins associated with resistance to *Fusarium* head blight during the initial infection of spikelets from six barley genotypes with varying susceptibility. Interestingly Geddes et al. (2008) detected higher abundance of ROS-scavenging enzymes in the susceptible

barley genotypes compared to the more resistant ones, suspecting that an oxidative burst associated with hypersensitive response could contribute to the saprophytic *F. graminearum* invasion of the plant tissues. Anyway, in the analysed naked barley cultivar ROS-scavenging seemed to play a more crucial role in early grain ripening stages than during later ripening stages according to the present study. Additionally, two predicted cystathione β-synthase (CBS) domain containing proteins were found to be down regulated at BBCH 87 and BBCH 97 by a factor of 2.0 and 2.1, respectively (Table 3.29). The CBSX3 domain containing protein was shown to activate mitochondrial thioredoxin and therefore plays a role in redox regulation and cellular homeostasis and therefore regulates various physiological processes, such as redox signalling, radical scavenging, carbon assimilation, translation and detoxification (Yoo et al., 2011).

**Table 3.29:** Changes of proteins related to oxidative burst due to *F. graminearum* infection compared to natural infection during grain ripening stages of naked barley

| | BBCH stage | | | | |
|---|---|---|---|---|---|
| Protein | 73 | 77 | 85 | 87 | 97 |
| Peroxidase | + 1.9 | + 2.4 | + ∞ | | |
| | | + 1.9 | | | |
| 1-cys peroxiredoxin | | + 2.6 | | | - 6.0 |
| Catalase isoenzyme | | + 2.3 | | | |
| Predicted: CBS domain containing protein (CBSX3) | | | | - 2.0 | - 2.1 |

(+) = up regulated; (-) = down regulated); +∞ = protein was only found in natural infected grains

**Proteins associated with plant metabolism**

Twelve proteins related to metabolism showed altered expression patterns after *F. graminearum* infection (Tables 3.30 and 3.31). All of them were detected in the earlier grain ripening stages from BBCH 73 to BBCH 85 and no proteins related to metabolism were changed in abundance during the later grain ripening at BBCH 87 and BBCH 97. Remarkably, the majority of proteins related to nitrogen metabolism and amino acid synthesis were found to be down regulated due to Fusarium infection (Table 3.30), whereas all proteins related to carbohydrate metabolism were up regulated (Table 3.31). Only an alanine aminotransferase (AlaAT) was up regulated at BBCH 73 by a factor of 1.8 and later down regulated with a factor of 2.0 at BBCH 85. This enzyme plays a role in formation and degradation of the amino acid alanine. For several plant species a regulation of AlaAT has been observed in the context of environmental stress response

(Kendziorek et al., 2012). An analysis of barley roots showed an increase of AlaAT after 12-24 hours of hypoxic stress (Muench and Good, 1994). Kendziorek et al. (2012) found that AlaAT expression in wheat is regulated by light exposure, nitrogen availability and hypoxia, suspecting that these enzymes are crucial for the regulation of energy availability in plants exposed to environmental stress. Furthermore, the introduction of an AlaAT gene into rice plants resulted in significantly increased biomass, grain yield and a higher nitrogen content compared to the wild-type plants (Shrawat et al., 2008).

Two eukaryotic translation initiation factors were down regulated at BBCH 77 and BBCH 85. Furthermore, a predicted 40 S ribosomal protein and a 60S ribosomal protein and an elongation factor were down regulated at BBCH 85 and 87, indicating altered in protein biosynthesis due to *Fusarium* infection.

**Table 3.30:** Changes of proteins related to nitrogen metabolism due to *F. graminearum* infection compared to natural infection during grain ripening stages of naked barley

| | BBCH stage | | | | |
|---|---|---|---|---|---|
| Protein | 73 | 77 | 85 | 87 | 97 |
| L-alanine aminotransferase | + 1.8 | | - 2.0 | | |
| Predicted: eukaryotic translation initiation factor 3 subunit G | | - 2.2 | | | |
| Eukaryotic translation initiation factor | | | - ∞ | | |
| Predicted: 40 S ribosomal protein SA-like | | | - 2.4 | | |
| 60S acidic ribosomal protein | | | | - 3.2 | |
| Putative elongation factor 1 beta | | | - 2.1 | | |

(+) = up regulated; (-) = down regulated); - ∞ = protein was only found in artificial inoculated grains

Regarding the changes of carbohydrate metabolism (Table 3.31), only one protein, a 2-dehydro-3-deoxyphosphooctonate aldolase was down regulated due to *F. graminearum* infection in fact at BBCH 85. This enzyme belongs to the (DAHP) synthase family and therefore catalyzes the first committed step of the shikimate pathway (Maeda and Dudareva, 2012). All the other proteins related to carbohydtrat metabolism are accumulated after an infection with *F. graminearum*. β-amylase was up regulated by a factor of 2.8 at BBCH 77 and by factor of 2.9 at BBCH 85 showing increased polysaccharide degradation. At BBCH 73 two glyceraldehydes-3-phosphate dehydrogenases were up regulated with a factor of 2.9 and 2.0, respectively. Moreover, a glucose and ribitol dehydrogenase homolog and a predicted diphosphate-fructose-6-

phosphate 1- phosphotransferase were up regulated each with a factor of 2.8 at BBCH 77, showing changes in the pathway for carbohydrate metabolism at early grain development. Additionally, ferredoxin-NADP+ reductase, involved in energy metabolism have been induced at BBCH 73 in Fusarium infected plants. Geddes et al. (2008) suggested that a modification in expression pattern of proteins related to metabolism and regulation is a by-product of fungal invasion, as the fungus tries to acquire resources from the plant. Furthermore, β-glucosidase was up regulated by a factor of 2.3 at BBCH 77. β-glucosidases belong to the family-1- glycoside hydrolases, that catalyze the hydrolysis of β-glucosidic bonds. They are involved in many physiological processes in plants, such as cell wall degradation of pathogens, formation of intermediates in plant cell wall lignification and activation of non-active glycosylated defence compounds by hydrolysis of the glucosidic linkages (Morant et al., 2008).

**Table 3.31:** Changes of proteins related to carbon metabolism due to *F. graminearum* infection compared to natural infection during grain ripening stages of naked barley

| | BBCH stage | | | | |
|---|---|---|---|---|---|
| Protein | 73 | 77 | 85 | 87 | 97 |
| Predicted: 2-dehydro-3-deoxyphosphooctonate aldolase | | | - 2.7 | | |
| Glyceraldehydes-3-phosphate dehydrogenase | + 2.9 + 2.0 | | | | |
| B-amylase | | + 2.8 | + 2.9 | | |
| Glucose and ribitol dehydrogenase homolog | | + 2.8 | | | |
| Predicted: diphosphate-fructose-6-phosphate 1- phosphotransferase | | + 2.8 | | | |
| B-glucosidase | | + 2.3 | | | |
| Ferredoxin-NADP+ reductase | + ∞ | | | | |

(+) = up regulated; (-) = down regulated); + ∞ = protein was only found in natural infected grains

This work provides a first insight concerning the interaction between naked barley and *F. graminearum* during the entire period of grain ripening and therefore during a long period after Fusarium infection by proteomic approach. The investigation revealed different induced response patterns at different grain ripening stages. During the early grain development considerable more proteins are changed in abundance due to *F. graminearum* infection than in the later grain ripening sages. Whereas protease inhibition plays a major role at early milk ripe stage, several pathogenesis related

proteins and proteins associated with defence response, such as thaumatin-like proteins and ROS scavenging enzymes were regulated due to *F. graminearum* infection during the entire infection period. Interestingly, proteins related to nitrogen metabolism were predominantly down regulated, whereas proteins related to carbohydrate metabolism were predominantly up regulated in artificially inoculated grains compared to natural infected grains. These results demonstrate that complex mechanisms are initiated by *Fusarium* infection, which are supposed to be partly adapted to the respective infection strategy of the pathogen. For better understanding of the host-pathogen interactions during the entire infection period, more studies are necessary, including for example transcriptome analysis at accurately defined infection stages of the respective plant tissues.

**Table 3.32:** Proteins changed in abundance after *F. graminearum* infection of naked barley grains at early milk ripe stage (BBCH 73). The ratio displays the difference in abundance of the proteins from artificially inoculated grains compared to natural infected grains, detected as protein spot (density) by two-dimensional electrophoresis.

| Spot ID [a] | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Function | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2124 | 2.9 ± 0.4 | 1,6 ± 0,3 | - 1,8 | 14-3-3-like protein A | response to penetration of powdery mildew fungi. | Hordeum vulgare | 5.3 | 28 | 4.7 | 29 | 1380.04 | P29305 |
| 8221 | 2.9 ± 0.8 | 1,0 ± 0,4 | - 3,0 | nucellin | aspartyl protease | Hordeum vulgare | 8.5 | 28 | 7.0 | 45 | 402.26 | C3SBD3 |
| 3127 | 2.0 ± 0,8 | 5,2 ± 1,1 | + 2,5 | not identified | -- | -- | 5.7 | 22 | -- | -- | -- | -- |
| 3223 | 3.4 ± 1,1 | 5,7 ± 0,2 | + 1,7 | Not identified | -- | -- | 5.7 | 29 | -- | -- | -- | -- |
| 3301 | 1.7 ± ,09 | 3,3 ± 0,3 | + 2,0 | protein z-type serpin | serine protease inhibition | Hordeum vulgare | 5.4 | 40 | 5.5 | 43 | 171.65 | CAA66232 |
| 4324 | 28.1 ± 6,7 | 54,5 ± 9,8 | + 1,9 | not identified | -- | -- | 6.4 | 37 | -- | -- | -- | -- |
| 4328 | 25.0 ± 24,1 | 65,9 ± 12,0 | + 2,6 | Serpin-Z4 | aerine protease inhibition | Hordeum vulgare | 6.5 | 38 | 5.7 | 43 | 1124.99 | P06293 |
| 5234 | 7.7 ± 4,9 | 14,5 ± 2,7 | + 1,9 | peroxidase BP 1 | response to oxidative stress | Hordeum vulgare | 7.1 | 33 | 9.1 | 39 | 580.05 | AAA32973 |
| 5411 | 17.5 ± 4,1 | 32,3 ± 6,7 | + 1,8 | Alanine aminotransferase 2 | L-alanine catabolic process | Hordeum vulgare | 6.8 | 50 | 5.9 | 53 | 3284.06 | P52894 |
| 6303 | 8.6 ± 6,3 | 25,1 ± 4,8 | + 2,9 | Glyceraldehyde-3-phosphate dehydrogenase. cytosolic | glycolysis | Hordeum vulgare | 7.2 | 37 | 6.2 | 33 | 1143.78 | P08477 |
| 6305 | 7.9 ± 0,5 | 15,6 ± 4,9 | + 2,0 | Glyceraldehyde-3-phosphate dehydrogenase. cytosolic | glycolysis | Hordeum vulgare | 7.2 | 39 | 6.8 | 36 | 948.28 | P26517 |
| 6306 | 15.4 ± 3,8 | 59,2 ± 1,7 | + 3,8 | not identified | -- | -- | 7.2 | 37 | -- | -- | -- | -- |
| 7010 | -- | 1,3 ± 0,3 | + ∞ | Alpha-amylase/ subtilisin inhibitor | amylase/protease inhibition | Hordeum vulgare | 8.0 | 18 | 8.9 | 22 | 319.81 | P07596 |
| 7105 | 1.4 ± 0,2 | 3,3 ± 1,0 | + 2,3 | predicted protein | chitin binding | Hordeum vulgare | 8.0 | 26 | 8.7 | 34 | 508.90 | F2DYI6 |
| 8017 | 1.3 ± 0,4 | 3,2 ± 0,6 | + 2,4 | not identified | -- | -- | 9.6 | 18 | -- | -- | -- | -- |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8105 | 2.1 ± 0,8 | 5,0 ± 0,6 | + 2,4 | predicted protein | endopeptidase inhibitor activity | Hordeum vulgare | 8.7 | 22 | 7.8 | 26 | 409.56 | F2E994 |
| 8215 | -- | 2,5 ± 0,2 | + ∞ | predicted: ferredoxin-NADP+ reductase activity | photosynthesis, energy metabolism | Hordeum vulgare | 9.0 | 33 | 9.5 | 41 | 288.19 | BAJ93902 |

[a] related to Figure 1
[b] (+)= up regulated; (-) =down regulated
[c] (pI)= isoelectric point; experimental/calculated
[d] Mass = molecular Mass; experimental/calculated

**Table 3.33:** Proteins changed in abundance after *F. graminearum* infection of naked barley grains at early milk ripe stage (BBCH 77). The ratio displays the difference in abundance of the proteins from artificially inoculated grains compared to natural infected grains, detected as protein spot (density) by two-dimensional electrophoresis.

| Spot ID [a] | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Function | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3713 | 28,2 ± 2,5 | 9,6 ± 3,2 | - 3,0 | heat shock protein 90, cytosolic | Protein folding | Hordeum vulgare | 5.5 | 92 | 4.8 | 80 | 799.93 | Q7XJ80 |
| 6234 | 0,9 ± 0,1 | 0,4 ± 0,2 | - 2,2 | Predicted: Eukaryotic translation initiation factor 3 subunit G | Protein biosynthesis | Hordeum vulgare | 7.6 | 32 | 6.7 | 31 | 554.34 | BAJ99755 |
| 7123 | 1,5± 0,1 | 0,9 ± 0,1 | - 1,6 | Not identified | -- | -- | 8.1 | 23 | -- | -- | -- | -- |
| 4206 | 0,5 ± 0,1 | 1,3 ± 0,3 | + 2,6 | predicted protein | unknown | Hordeum vulgare | 5.9 | 27 | 5.5 | 26 | 341.15 | F2DJC5 |
| 4519 | 19,1 ± 5,8 | 53,8 ± 11,0 | + 2,8 | β-amylase | Polysaccharide degradation | Hordeum vulgare | 6.2 | 58 | 5.6 | 60 | 2433.38 | P82993 |
| 5126 | 1,8 ± 0,5 | 4,7 ± 2,4 | + 2,6 | 1-Cys peroxiredoxin | antioxidant | Hordeum vulgare | 6.9 | 18 | 6.3 | 24 | 212.17 | P52572 |
| 6210 | 1,9 ± 1,0 | 5,2 ± 1,7 | + 2,8 | glucose and ribitol dehydrogenase homolog | carbohydrate metabolism | Hordeum vulgare | 7.2 | 30 | 6.6 | 32 | 650.62 | T06212 |
| 6218 | 11,5± 1,6 | 22,4 ± 3,3 | + 1,9 | Chain A, Crystal Structure Of Barley Grain Peroxidase 1 | antioxidant | Hordeum vulgare | 7.4 | 32 | 6.5 | 34 | 935.10 | 1BGP_A |
| 6219 | 9,3 ± 2,4 | 22,2 ± 7,7 | + 2,4 | peroxidase BP 1 | antioxidant | Hordeum vulgare | 7.4 | 33 | 9.1 | 39 | 640.71 | Q40069 |
| 7105 | 22,3 ± 3,8 | 35,9 ± 2,9 | + 1,6 | Alpha-amylase/subtilisin inhibitor | Amylase/protease inhibition | Hordeum vulgare | 7.7 | 17 | 8.9 | 22 | 2069.30 | P07596 |
| 7119 | 0,7 ± 0,3 | 1,4 ± 0,1 | + 2,1 | Predicted: thaumatin-like protein | antifungal | Hordeum vulgare | 8.0 | 25 | 9.0 | 26 | 334.98 | BAK00635 |
| 7502 | 2,3 ± 0,4 | 5,4 ± 1,4 | + 2,3 | Catalase isozyme 1 | peroxidase | Hordeum vulgare | 7.7 | 51 | 6.7 | 56 | 218.15 | P55307 |
| 7534 | 0,1 ± 0,0 | 0,9 ± 0,5 | + 6,4 | Not identified | -- | -- | 8.1 | 54 | -- | -- | -- | -- |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7613 | 0,4 ± 0,1 | 1,1 ± 0,2 | + 2,8 | predicted: diphodphate-fructose-6-phosphate 1-phosphotransferase activity | glycolysis | Hordeum vulgare | 7.8 | 66 | 6.9 | 68 | 857.70 | BAJ94879 |
| 8512 | 3,1 ± 0,4 | 7,0 ± 1,9 | + 2,3 | beta-glucosidase | Carbohydrate metabolism | Hordeum vulgare | 8.7 | 59 | 7.7 | 57 | 1021.28 | AAA87339 |

[a] related to Figure 1
[b] (+)= up regulated; (-) =down regulated
[c] (pI)= isoelectric point; experimental/calculated
[d] Mass = molecular Mass; experimental/calculated

**Table 3.34:** Proteins changed in abundance after *F. graminearum* infection of naked barley grains at early milk ripe stage (BBCH 85). The ratio displays the difference in abundance of the proteins from artificially inoculated grains compared to natural infected grains, detected as protein spot (density) by two-dimensional electrophoresis.

| Spot ID [a] BBCH 85 | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Function | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1210 | 8,1 ± 2,4 | 4,0 ± 2,1 | - 2,1 | putative elongation factor 1 beta | Protein biosynthesis | Hordeum vulgare | 4.8 | 29 | 4.4 | 25 | 545.71 | CAB90214 |
| 2311 | 3,6 ± 0,8 | 1,5 ± 0,8 | - 2,4 | predicted: 40S ribosomal protein SA-like | translation | Brachypodium distachyon | 5.4 | 41 | 4.8 | 33 | 578.64 | XP_003558658 |
| 5402 | 28,2 ± 3,8 | 13,7 ± 4,3 | - 2,0 | Alanine aminotransferase 2 | L-alanine Catabolic process | Hordeum vulgare | 6.7 | 52 | 5.9 | 53 | 2507.89 | P52894 |
| 7203 | 1,3 ± 0,5 | -- | - ∞ | predicted: Eukaryotic translation initiation factor 3 subunit G | Protein biosynthesis | Hordeum vulgare | 7.7 | 32 | 6.7 | 31 | 967.94 | BAJ99755 |
| 8207 | 1,0 ± 0,2 | 0,4 ± 0,2 | - 2,7 | predicted: 2-dehydro-3-deoxy phosphooctonate aldolase | carbohydrate metabolism | Hordeum vulgare | 8.9 | 35 | 9.2 | 32 | 739.82 | BAJ85172 |
| 4512 | 2,2 ± 0,2 | 6,6 ± 1,3 | + 2,9 | β-amylase | Polysaccharide degradation | Hordeum vulgare | 6.5 | 57 | 5.5 | 59 | 2937.61 | P16098 |
| 7255 | -- | 1,9 ± 0,3 | + ∞ | peroxidase BP 1 | antioxidant | Hordeum vulgare | 8.0 | 32 | 9.1 | 39 | 100.44 | AAA32973 |
| 7256 | -- | 1,4 ± 0,5 | + ∞ | proteasome subunit alpha type-7-A | Cleavage of peptide bonds | Oryza sativa | 8.4 | 30 | 7.7 | 27 | 136.46 | A2YXU2 |
| 7257 | -- | 0,8 ± 0,1 | + ∞ | not identifid | -- | -- | 8.4 | 31 | -- | -- | -- | -- |
| 7530 | 9,8 ± 1,7 | 16,7± 0,9 | + 1,7 | hypothetical protein | unknown | Sorghum bicolor | 8.2 | 56 | 6.3 | 51 | 138.50 | XP_002466310 |
| 8103 | 2,4 ± 0,9 | 5,3 ± 0,3 | + 2,2 | predicted protein: thaumatin like | antifungal | Hordeum vulgare | 8.7 | 22 | 9.2 | 24 | 1494.88 | BAJ96714 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8202 | 2,0 ± 0,5 | 3,6 ± 0,6 | + 1,8 | β-1,3-endoglucanase GI | degrade fungal cell wall polysaccharides | Hordeum vulgare | 8.5 | 31 | 9.0 | 33 | 1340.92 | P34742 |
| 8301 | 2,0 ± 0,4 | 5,8 ± 1,3 | + 2,9 | not identified | -- | -- | 8.7 | 36 | -- | -- | -- | -- |

[a] related to Figure 1

[b] (+)= up regulated; (-) =down regulated

[c] (pI)= isoelectric point; experimental/calculated

[d] Mass = molecular Mass; experimental/calculated

**Table 3.35:** Proteins changed in abundance after *F. graminearum* infection of naked barley grains at early milk ripe stage (BBCH 87). The ratio displays the difference in abundance of the proteins from artificially inoculated grains compared to natural infected grains, detected as protein spot (density) by two-dimensional electrophoresis

| Spot ID [a] BBCH 87 | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Function | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1201 | 2,7 ± 1,3 | 0,5 ± 0,2 | - 5,7 | predicted: p23-hB-ind1-like (Small HSP- like) | protein folding | Hordeum vulgare | 4.0 | 27 | 4.1 | 24 | 586.83 | BAJ94677 |
| 3208 | 1,5 ± 0,3 | 0,5 ± 0,2 | - 3,2 | 60S acidic ribosomal protein P0 | translational elongation | Oryza sativa | 5.9 | 34 | 5.2 | 34 | 254.73 | P41095 |
| 3709 | 0,6 ± 0,3 | 0,2 ± 0,0 | - 3,3 | phospholipase D alpha 1 | lipid catabolic process, several cellular processes | Zea mays | 5.8 | 91 | 5.2 | 92 | 949.96 | Q43270 |
| 6411 | 0,7 ± 0,2 | -- | - ∞ | predicted protein | unknown | Hordeum vulgare | 7.0 | 46 | 7.3 | 70 | 1059.54 | BAK08004 |
| 7004 | 3,5± 0,5 | 1,7 ± 0,1 | - 2,0 | Predicted: CBS domain-containing protein CBSX3, mitochondrial-like | cell redox homeostasis | Brachypodium distachyon | 7.7 | 15 | 9.7 | 22 | 552.09 | XP_003560016 |
| 3206 | -- | 2,2 ± 1,5 | + ∞ | Predicted: Glyoxalase I | detoxification of methylglyoxal | Hordeum vulgare | 5.8 | 33 | 5.2 | 32 | 938.13 | BAJ85169 |
| 7101 | 0,6 ± 0,2 | 1,4 ± 0,3 | + 2,1 | Not identified | -- | -- | 7.7 | 16 | -- | -- | -- | -- |

[a] related to Figure 1

[b] (+)= up regulated; (-) =down regulated

[c] (pI)= isoelectric point; experimental/calculated

[d] Mass = molecular Mass; experimental/calculated

**Table 3.36:** Proteins changed in abundance after *F. graminearum* infection of naked barley grains at early milk ripe stage (BBCH 97). The ratio displays the difference in abundance of the proteins from artificially inoculated grains compared to natural infected grains, detected as protein spot (density) by two-dimensional electrophoresis

| Spot ID [a] BBCH 97 | Naturally infected [MW ± SD] | Artificially infected [MW ± SD] | Ratio [b] | Identification | Function | Protein homologue | pI [c] exp. | Mass [d] (kDa) exp. | pI [c] calc. | Mass [d] (kDa) calc. | Mascot score | Accession |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1206 | 1,3 ± 0,7 | -- | - ∞ | Predicted: p23-hB-ind1-like (Small HSP- like) | protein folding | Hordeum vulgare | 4.5 | 27 | 4.1 | 24 | 142.91 | BAJ94677 |
| 1207 | 1,2 ± 0,2 | -- | - ∞ | Nascent polypeptide-associated complex (NAC), alpha subunit family protein | response to salt stress | Arabidopsis thalina | 4.5 | 30 | 4.1 | 22 | 187.73 | AT3G12390.1 |
| 6105 | 4,6 ± 1,0 | 2,0 ± 0,3 | - 2,3 | Beta-hordothionin | defense response | Hordeum vulgare | 7.2 | 16 | 7.6 | 14 | 394.33 | P21742 |
| 6106 | 4,2 ± 2,0 | 0,7 ± 0,2 | - 6,0 | 1-Cys peroxiredoxin | antioxidant | Hordeum vulgare | 7.2 | 24 | 6.3 | 24 | 1407.64 | P52572 |
| 7003 | 4,0 ± 0,7 | 1,9 ± 0,3 | - 2,1 | Predicted: CBS domain-containing protein CBSX3, mitochondrial-like | cell redox homeostasis | Brachypodium distachyon | 7.8 | 15 | 9.7 | 22 | 283.52 | XP_003560016 |
| 7101 | 1,3 ± 0,5 | 3,6 ± 1,3 | + 2,8 | thaumatin-like protein TLP8 | anfifungal | Hordeum vulgare | 7.8 | 16 | 9.2 | 24 | 73.50 | AF355458_1149 |

[a] related to Figure 1

[b] (+)= up regulated; (-) =down regulated

[c] (pI)= isoelectric point; experimental/calculated

[d] Mass = molecular Mass; experimental/calculated

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# 4 Summary

The influence of *Fusarium graminearum* infection on mycotoxin formation and on the protein composition of emmer and naked barley grains was investigated during grain ripening from milk ripe grains until ripeness and during a storage period of six month.

*F. graminearum* inoculation affected the protein composition of emmer and naked barley grains from the early grain development on, whereupon emmer and wheat were more affected than naked barley. *F.graminearum* inoculation led to increased gliadin sub-fractions and decreased glutenin sub-fractions in emmer and wheat grains. However, naked barley proteins also showed increased hordein and hordenin sub-fractions during early grain development. The hordenins and the glutenins were apparently degraded by fungal proteases, particularly at later grain development stages. Furthermore, emmer and wheat grains showed distinct higher toxin contents than naked barley. In naked barley the toxin contents increased with grain ripening, whereas they were rather constant in emmer and wheat.

Furthermore, naked barley and emmer exhibited different toxin spectra in both natural infected and artificially inoculated grains. Natural infected emmer and wheat showed DON and ZEA accumulation, whereas naked barley accumulated only NIV, assuming different *Fusarium* species may establish depending on the grain variety. After inoculation with DON-producing *F. graminearum* strains, DON and ZEA accumulated strongly in emmer, wheat and naked barley, whereas NIV was again only detected in the naked barley cultivars.

During storage of mature grains the toxin composition in natural infected grains was hardly changed, whereas in inoculated grains the DON contents in emmer and naked barley cultivars were significantly reduced. Nevertheless, the prolamins and glutelins of natural infected and artificial inoculated grains were significantly affected during storage of six months, particularly at rather warm and humid storage conditions. This effect was also observed in *F. graminearum* inoculated grains at colder and dryer storage conditions.

Regarding the occurrence of phenolic compounds in emmer and naked barley grains and in the respective hulls, ferulic acid was the major phenolic acid, followed by *p*-coumaric and caffeic acid in soluble and bound forms. Furthermore, naked barley had higher amounts of cinnamic acid derived phenolics in the grains and particularly in the

hulls. However, phenolic acids, especially ferulic acid decreased after *F. graminearum* inoculation, possibly resulting from inhibition of their biosynthesis by the fungi. Mechanisms in plants depending on antioxidant capacity and the general redox status can interfere with the antioxidant secondary plant metabolites and thus may influence toxin accumulation and resistance to *Fusarium* infection in cereals. The phenolic compounds in the hull-tissues obviously play an important role in inhibiting pathogen growth and toxin accumulation in emmer and naked barley grains.

Proteomic studies were conducted to analyse the effect of *Fusarium* inoculation on emmer and naked barley grains during the entire grain ripening and infection-period beginning with early grain development (milk ripe) until plant death. The obtained results allow an overview of molecular mechanisms initiated by *F. graminearum* infection of emmer and naked barley grains and an insight into the interactions between two cereal grains with *F. graminearum* during the entire period of grain ripening. Different proteins have been induced depending on the grain ripening stages and the cereal genotype. An inoculation of emmer grains with *F. graminearum* led to changes of protein expression in all analysed development stages. In the early ripening stages proteins, predominantly related to metabolism and photosynthesis as well as stress-related proteins, like PR-poteins and proteins related to oxidative stress were up regulated. Additionally a spermidine synthase was up regulated at BBCH 75 (milk ripe). During later ripening stages at BBCH 87 and BBCH 97 predominantly stress-related proteins were down regulated. Nevertheless, some stress-related proteins such as peroxidase and chitin binding proteins were increased in abundance after *F. graminearum* infection, demonstrating some defence strategies were persistent during the whole infection period. The pathogen may profit from the enhanced metabolism, providing nutrients that are beneficial for fungal growth.

In naked barley grains, more proteins were changed in abundance during early grain development after *F. graminearum* inoculation than in the later grain ripening sages. Protease inhibition played a major role at early milk ripe stage, whereas several pathogenesis related proteins and proteins associated with defence response, such as thaumatin-like proteins and ROS scavenging enzymes were regulated due to *F. graminearum* infection during the entire infection period. Interestingly, proteins related to nitrogen metabolism were predominantly down regulated in naked barley, whereas proteins related to carbohydrate metabolism were predominantly up regulated in

artificially inoculated grains compared to natural infected grains. These results demonstrate that complex mechanisms are initiated by *Fusarium* infection, which are supposed to be partly adapted to the respective infection strategy of the pathogen.

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# 6 curriculum vitae

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