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***Fusarium* species on barley:**
Steps towards an integrated management

Katharina Marlene Hofer

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RYCHLIK M, HOFER K, GASTL M, HABLER K, GEIßINGER C, HESS, M (2015) *From the barley to the bottle – How can we safeguard the quality of our beer?*. Lab and More int. 2, 10-15.

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HOHENEDER F, GRUHN S, HESS M, HOFER K (2016). *Gerste gesund düngen*. dlz agrarmagazin 4, 48-51.

HOFER K, HESS M (2016). *Fusarium in Gerste*. Landwirt 11, 48-49.

CONTENTS

ABBREVIATIONS

ABC	ATP-binding cassette
ATP	adenosine triphosphate
BC	backcross
BEA	beauvericin
Bgh	*Blumeria graminis* f. sp. *hordei*
Bst	*Bacillus stearothermophilus*
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
D3G	deoxynivalenol-3-glycosid
dai	day after inoculation
DAMP	damage-associated molecular pattern
DAS	diacetoxyscirpenol
DON	deoxynivalenol
DPRE	number of days with precipitation > 1mm
EC	European Commission
ENN	enniatin
ET	ethylene
F.	*Fusarium*
FAO	Food and Agriculture Organization of the U.N.
FHB	Fusarium head blight
HD	heading date
IPM	Integrated Pest Management
JA	jasmonic acid
LAMP	loop-mediated isothermal amplification
LC-MS/MS	liquid chromatography tandem mass spectrometry
MAPK	mitogen-activated kinase
MDR	multidrug-resistant protein
MLO	Mildew locus O
NIV	nivalenol
nsLTP	non-specific lipid transfer protein
PAMP	pathogen-associated molecular pattern
PR	pathogenesis-related
PRE	sum of precipitation
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait locus
R.	*Ramularia*
RH	relative humidity
SA	salicylic acid
spp.	species
T	temperature
UDP	uridine diphosphate
USDA	U.S. Department of Agriculture
USFDA	U.S. Food and Drug Administration
WT	wild type
ZEA	zearalenon

SUMMARY

Fusarium species on barley cause Fusarium head blight (FHB) a devastating fungal disease on small grain cereals occurring worldwide. Similar to what is reported for wheat, FHB on barley results in yield reductions and mycotoxin contaminations. In the case of spring barley, which serves mainly as raw material for malt and subsequent beer production, the thread of FHB comprises also quality degradations and interferences during processing of malt.

Integrated management approaches for disease control usually focus on crop cultivation by combining different agronomical measures. However, integrated management has to go beyond barley production and needs extension to harvest, processing, and warehousing in the case of barley-FHB and its wide-ranging impact on various steps within the barley-to-beer value-added chain, in which several stakeholders are involved.

The present study aimed to contribute further steps towards an integrated management of barley-FHB by filling up relevant gaps of knowledge regarding pathogen occurrence, diagnosis, epidemiology, impact, and control. Monitoring studies characterized the *Fusarium* complex on barley and malt and detected *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae*, and *F. sporotrichioides* as predominant species. Correlation analysis of climate data and *Fusarium* DNA in grain material revealed species-specific differences in weather-modulated epidemiology. The impact of as well as the influence of agronomic measures on barley-FHB was tested in inoculation trials. Low effects on yield are suggested, but *Fusarium* infection was found to endanger product safety and in particular product quality. Both, the usage of powdery-mildew-resistant *mlo*-genotypes as well as the enhanced application of nitrogen were suspected as supportive for FHB. Present data did not support these assumptions and found contrarily increased *Fusarium* infection upon nitrogen starvation. In downstream processes maltsters and brewers apply malt symptom-based methods to identify *Fusarium* contaminations and to appraise associated risks. Data of the present study suggest that the visual assessment is insufficient to assess the risk of malt contaminations with *Fusarium* DNA and mycotoxins.

Taken together, the present study added more detail on the picture of barley-FHB. This information is intended to support future integrated management of *Fusarium* control from the field to the finally processed consumer product.

ZUSAMMENFASSUNG

Der Befall mit *Fusarium*-Arten an Gerste führt zur Ährenfusariose, einer weltweit auftretenden Pilzkrankheit an kleinkörnigen Getreiden. Vergleichbar mit dem was für Weizen bekannt ist, resultieren Ährenfusariosen an Gerste in Ertragsverlusten und Kontaminationen mit Mykotoxinen. Im Fall von Sommergerste, die vor allem als Rohmaterial in der Malz- und Bierherstellung Verwendung findet, rufen Ährenfusariosen auch Qualitätsreduktionen und Prozessstörungen hervor.

Integrierte Ansätze zur Kontrolle von Pflanzenkrankheiten fokussieren sich für gewöhnlich auf den Anbau und implizieren die Kombination aus verschiedenen agronomischen Maßnahmen. Ährenfusariosen an Gerste verursachen weitrechende Probleme an verschiedenen Stellen der Wertschöpfungskette, an der mehrere Interessensvertreter beteiligt sind. Deshalb sollte das integrierte Management über den Anbau hinausgehen und auch die Ernte, die Verarbeitung und die Lagerung mit einschließen.

Das Ziel der vorliegende Arbeit war es, relevante Wissenslücken hinsichtlich des Auftretens, der Diagnose, der Epidemiologie, des Schadpotentials und der Kontrolle von Ährenfusariosen an Gerste zu schließen, um somit weitere Schritte hin zu einem integrierten Management beizutragen. Monitoringuntersuchungen charakterisierten den *Fusarium*-Komplex an Gerste sowie Malz und detektierten *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae* und *F. sporotrichioides* als dominierende Arten. Korrelationsanalysen mit Klimadaten und *Fusarium*-DNA-Gehalten im Kornmaterial zeigten artspezifische Unterschiede in der wettermodulierten Epidemiologie. Das Schadpotential von sowie der Einfluss agronomischer Maßnahmen auf Ährenfusariosen in Gerste, wurde in Inokulationsexperimenten untersucht. Während ein geringer Einfluss auf Kornerträge festgestellt wurde, gefährden *Fusarium*-Infektion an Gerste die Produktsicherheit und vor allem die Produktqualität. Die Verwendung von mehltauresistenten *mlo*-Sorten sowie der erhöhte Einsatz von Stickstoff wurden als förderlich für das Auftreten von Ährenfusariosen angenommen. Die vorliegenden Daten konnten diese Vermutungen nicht stützen und fanden dementgegen erhöhten *Fusarium*-Befall bei Stickstoffmangel vor. Um in der nachgelagerten Verarbeitung *Fusarium*-Kontaminationen und damit verbundene Risiken zu identifizieren, verwenden Mälzer

und Brauer symptombasierte Methoden. Die Ergebnisse der vorliegenden Arbeit sprechen dafür, dass die visuelle Bonitur von Malz die *Fusarium*-Kontamination in Form von DNA und Mykotoxinen nur unzureichend anzeigt.

Zusammenfassend konnten die Ergebnisse der vorliegenden Arbeit ein klareres Bild von Ährenfusariosen an Gerste zeichnen. Die gewonnene Information ist dazu vorgesehen ein zukünftiges integriertes Management von Ährenfusariosen an Gerste zu unterstützen, vom Feld bis hin zum Endprodukt.

1 INTRODUCTION

1.1 The Fusarium head blight complex on small grain cereals

Fusarium head blight (FHB) is a fungal disease attacking a broad range of *Gramineae* hosts, including wheat and barley (PARRY *et al*., 1995; OSBORNE AND STEIN, 2007). The disease is caused by a complex of *Fusarium* species (PARRY *et al*., 1995; LIDDELL *et al*., 2003). Several species were isolated from small-grain cereals, comprising *F. acuminatum*, *F. arthrosporioides*, *F. avenaceum, F. cortaderiae*, *F. culmorum*, *F. crookwellense*, *F. equiseti*, *F. flocciferum*, *F. graminearum* sensu lato, *F. langsethiae*, *F. lumulosporum*, *F. moniliforme*, *F. oxysporum*, *F. poae*, *F. pseudograminearum*, *F. proliferatum*, *F. sambucinum*, *F. semitectum*, *F. sporotrichioides*, *F. solani*, *F. subglutinans*, *F. torulosum*, *F. tricinctum*, *F. venenatum*, *F. ventricosum*, and *F. verticillioides* (YLIMÄKI *et al*., 1979; PARRY *et al*., 1995; AOKI AND O'DONNELL, 1999; GALE *et al*., 2002; KOSIAK *et al*., 2003; LOIVEKE *et al*., 2003; YLI-MATTILA *et al*., 2004; MONDS *et al*., 2005; YLI-MATTILA, 2010; NIELSEN *et al*., 2011). Phylogenetic studies suggest further subdivision of the *F. graminearum* clade into *F. graminearum sensu strictu*, *F. austroamericanum*, *F. meridionale*, *F. boothii*, *F. mesoamericanum*, *F. acacia-mearnsii*, *F. asiaticum*, *F. cortaderiae*, *F. brasilicum*, *F. vorosii*, and *F. gerlachii* (WARD *et al*., 2002; O'DONNELL *et al*., 2004; STARKEY *et al*., 2007). *Microdochium nivale* and *M. majus*, formerly known as '*F. nivale'*, are often added to the complex as both species are able to cause head blight (BOTTALICO AND PERRONE, 2002; XU AND NICHOLSON, 2009).

F. graminearum, *F. culmorum*, and *F. avenaceum* are internationally perceived as predominant, albeit complex compositions differ between hosts, regions, and years (PARRY *et al*., *1995*; BOTTALICO AND PERRONE, 2002; LOIVEKE *et al*., 2003; NIELSEN *et al*., 2011): *F. graminearum* is noticed as the major causal agent of wheat-FHB (PARRY *et al*., 1995; GILBERT AND FERNANDO, 2004; OSBORNE AND STEIN, 2007). Additionally, *F. culmorum* and *F. avenaceum* were detected as important pathogens on rye and triticale, whereas *F. langsethiae* appears to be a major problem in oat cultivation (IMATHIU, 2008; NIELSEN *et al*. 2011; HOFGAARD *et al*., 2016). On barley, *F. culmorum*, *F. avenaceum*, and *F. langsethiae* are among the most relevant species (NIELSEN *et al*., 2011). Beside hosts, complex compositions differ between continents and regions. In spite of an international predominance of *F. graminearum*

other species are important on a regional scale (XU AND NICHOLSON, 2009). *F. culmorum*, *F. avenaceum*, and *F. poae* are other relevant species in Europe and *F. asiaticum* has high importance in China and Japan (BOTTALICO AND PERRONE, 2002; QU *et al*., 2008; SUGA *et al*., 2008). Alongside *F. graminearum*, *F. pseudograminearum* and *F. cortaderiae* predominate in Australia and New Zealand, respectively (AKINSANMI *et al*., 2004; MONDS *et al*., 2005). Under German cultivation conditions, *F. avenaceum*, *F. culmorum*, and *F. poae* were, beside *F. graminearum*, frequently isolated from wheat grain (ELLNER, 2000; BIRZELE *et al*., 2002; LIENEMANN *et al*., 2001).

Diverse compositions of *Fusarium* species complexes are perceived as unstable. On the one hand, long-term shifts can be observed: *F. culmorum*, *F. avenaceum*, and *F. poae* increase in central and southeastern Europe over time (BOTTALICO AND PERRONE, 2002) comparable to *F. poae*, *F. tricinctum* as well as *Microdochium* species becoming more relevant in northwest Europe (XU *et al*., 2005, 2008; NIELSEN *et al*., 2011). On the other hand, annual predominance structures were recorded: For instance *F. culmorum* was shown to be more important in Canada in years with lower temperatures (MILLER, 2002). Environmental conditions as well as agronomic factors are recognized as driving forces for predominance structures within the *Fusarium* complex (PARRY *et al*., 1995; JENNINGS *et al*., 2000; ROSSI *et al*., 2001).

1.2 The epidemiology of Fusarium head blight pathogens

FHB epidemiology is summarized in several reviews (PARRY *et al*., 1995; CHAMPEIL *et al*., 2004; GILBERT AND FERNANDO, 2004; XU AND BERRIE, 2005; OSBORNE AND STEIN, 2007; TRAIL, 2009; XU AND NICHOLSON, 2009). Major aspects regarding inoculum sources and distribution pathways are presented in figure 1. Among all *Fusarium* species within the FHB complex, the epidemiology of *F. graminearum* is most completely documented. The basic principles appear to be similar for all *Fusarium* species, but also evident differences are observable.

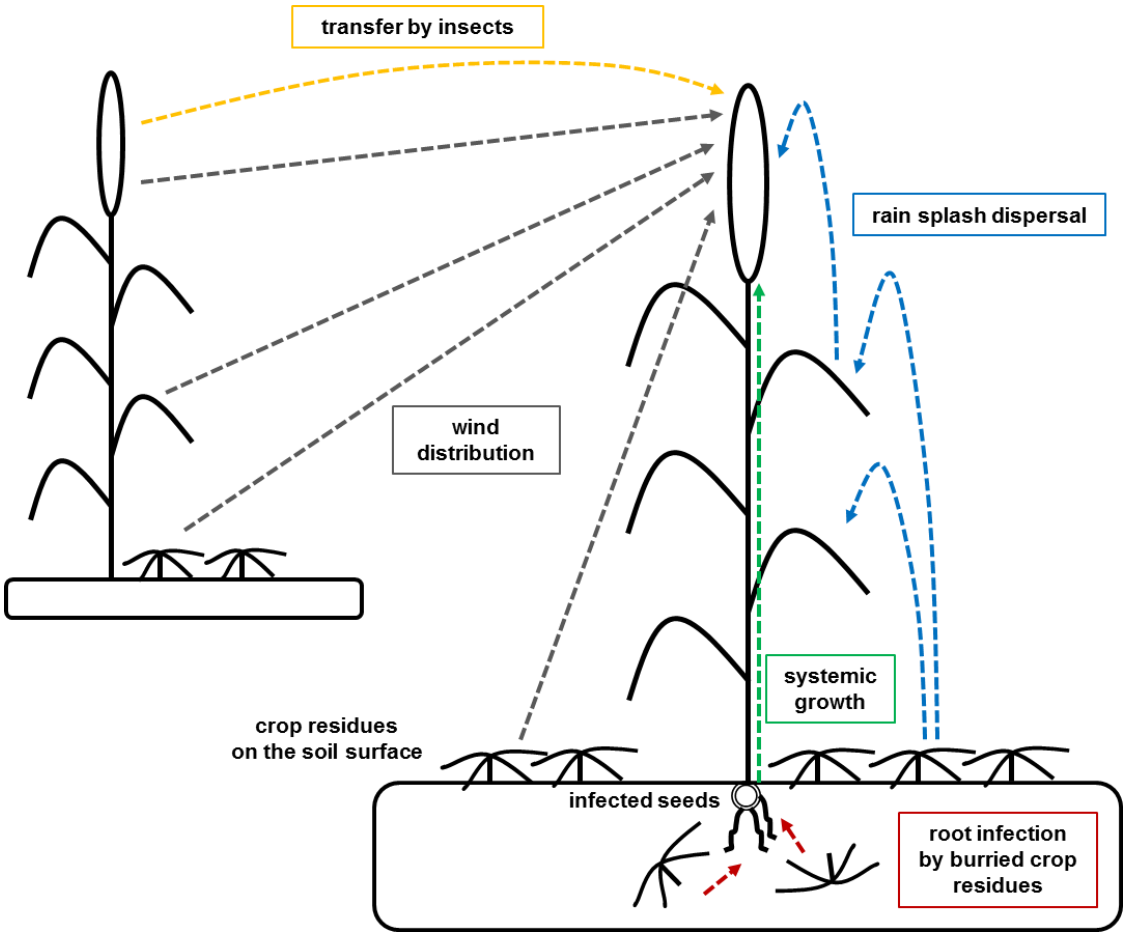

Figure 1: Different sources of *Fusarium* infection under field conditions

1.2.1 Inoculum sources and production

FHB pathogens overwinter as saprophytic mycelium or as clamydospores on maize stalks or cereal root, stem, and grain tissue. Thus, crop residues on the soil surface are the primary source of inoculum for head infections (ATANASOFF, 1920; SUTTON, 1982; KHONGA AND SUTTON, 1988; PARRY *et al*., 1994; XU, 2003; GILBERT AND FERNANDO, 2004; GUENTHER AND TRAIL, 2005). Infections of plant roots and seedlings occur from contaminated buried material in the soil (CHONGO *et al*., 2001). Infected seed material is discussed as a further initial source of inoculum. GILBERT *et al*. (2003) and XI *et al*. (2008) found stem blight as a resulting symptom of seed infection, but failed to show subsequent FHB occurrence. A relationship between blighted stems as another source of inoculum and resulting FHB on other plants has so far not been described. Additional inoculum input arises from other gramineous hosts such as wild grasses or other field crops such as beans or canola (JENKINSON AND PARRY, 1994; INCH AND GILBERT, 2003; PEREYRA AND DILL-MACKY, 2008).

Individual *Fusarium* species form different types of infectious propagules: Hyphal fragments, conidia, and ascospores serve as inoculum for FHB development (SUTTON, 1982; BAI AND SHANER, 1994). Most of the FHB pathogens produce asexual spores (conidia), whereas *F. graminearum* additionally generates fruit bodies (perithecia) for the formation of sexual spores, called ascospores (KHONGA AND SUTTON, 1988; XU AND NICHOLSON, 2009). These spores are noticed as primary inoculum of *F. graminearum* emerging from crop residues (SUTTON, 1982; BAI AND SHANER, 1994; PARRY *et al*., 1995; DESJARDIN, 2006).

Mycelial growth as well as conidia and ascospore production is crucially influenced by climatic factors (CHAMPEIL *et al*., 2004; OSBORNE AND STEIN, 2007). BRENNAN *et al*. (2003) investigated *in vitro* the mycelial growth rates of various FHB pathogens under different temperature regimes. The temperature optimum for *F. graminearum* was at 25°C, whereas *F. culmorum* and *F. poae* showed maximum growth rates at 20-25°C. Growth of *F. avenaceum* and *M. nivale* reached highest values at 20°C. Conidia sporulation is also influenced by temperature. *In vitro* tests resulted in species-specific temperature optima for *F. graminearum* (32°C), *F. culmorum* (32°C), *F. avenaceum* (28°C), and *M. nivale* (26°C) (XU, 2003). Studies agree on a climatic influence on ascospore appearance, but discussed precise light, temperature, and moisture conditions for perithecia formation and maturation (TSCHANZ *et al*., 1976; SUNG AND COOK, 1981; PAULITZ, 1996; ANDRIES *et al*., 2000; INCH *et al*., 2000; DUFAULT *et al*., 2002a, 2002b, 2006; TRAIL *et al*., 2002). Most recent studies of MANSTRETTA AND ROSSI (2015) assessed perithecia development and maturation under laboratory and field conditions. In controlled environments, perithecia formation was optimal at 21.7°C and ≥75% relative humidity, but was restricted by temperatures lower than 5°C and higher than 30°C. Maturation occurred between 20°C and 25°C, but only with ≥85% relative humidity. The prevalence of sufficient moisture was also found to be decisive for perithecia development and maturation in outdoor trials. The production of ascospores occurs in the range of 13°C and 33°C, but is ideally between 25°C and 28°C. Asospores are actively released from perithecia at temperatures of about 15-25°C and relative humidities higher than 76% (SUTTON, 1982; MANSTRETTA AND ROSSI, 2015). Light regimes were recognized as another decisive factor. Under lab conditions higher amounts of spores were ejected in light than in darkness (TRAIL *et al*., 2002). Under natural

conditions, contrasting results with diurnal variation were obtained. PAULITZ (1996), INCH *et al*. (2000), and SCHMALE III *et al*. (2002) trapped more ascospores in dark compared to daylight periods.

1.2.2 Inoculum dispersal and infection routes

Inoculum of *Fusarium* pathogens reaches cereal heads via various routes of dispersion (PARRY *et al*., 1995). Insects were considered as distributors of fungal inoculum. Several head blight pathogens, including *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* were isolated from mites, midges, flies, or beetles (CHEREWICK AND ROBINSON, 1958; GORDON, 1959; WINDELS *et al*., 1976, MONGRAIN *et al*. 2000). Systemic growth resulting from infected seeds or contaminated soil was studied as another infection pathway of *Fusarium* pathogens causing FHB, but is controversially discussed (WAGACHA AND MUTHOMI, 2007). Some researchers were able to re-isolate pathogens from internodes after soil inoculation, but failed to show FHB (SNIJDERS, 1990; CLEMENT AND PARRY, 1998). Others could recover previously applied fungal material to seedlings in subsequently appearing heads, indicating systemic growth (JORDAN AND FIELDING, 1988; HUTCHEON AND JORDAN, 1992). More recent studies used microscopic approaches to elucidate systemic distribution. Inoculating stem bases of plant seedlings, MUDGE *et al*. (2006) detected subsequent wheat head colonization by *F. graminearum* and *F. pseudograminearum*, whereas COVARELLI *et al*. (2012) did not retrieve *F. culmorum* in heads of soft wheat.

Wind and rain-splash are recognized as the major transfer means of FHB inoculum (MADDEN, 1992; PAUL *et al*., 2004). Air-borne prevalence was documented for the ascospore-producing species *F. graminearum*, but as well for conidiogenous fungi such as *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. crookwellense* *F. moniliforme*, *F. poae*, *F. sporotrichioides*, *F. subglutinans*, *F. tricinctum*, *M. nivale* (MARTIN, 1988; OBST *et al*., 1995; PAULITZ, 1996; FERNANDO *et al*., 2000; ROSSI *et al*., 2002; MALDONADO-RAMIREZ *et al*., 2005; OSBORNE AND STEIN, 2006; KELLER *et al*., 2014). Air dispersal of ascospores without wind occurs via forcible ejection out of a perithecium. However, travel distances might not exceed 12 mm (TRAIL *et al*., 2005; DAVID *et al*., 2016). In the presence of wind, ascospores are suggested to be distributed several meters following density gradients; the number of spores decreases in proportion to the distance from the inoculum source (FERNANDO *et al*.,

1996; PAULITZ *et al*., 1999; DE LUNA *et al*., 2002; PRUSSIN *et al*., 2014). SCHMALE III *et al*. (2012) trapped ascospores at a height of 324 m indicating long-distance transport, which was also reported in other studies (FRANCL *et al*., 1999; MALDONADO-RAMIREZ *et al*., 2005; PRUSSIN *et al*., 2014). Comparing air-dispersed spore types of *F. graminearum*, ascospores were more highly abundant than conidia (FERNANDO *et al*., 2000; MAKELL AND FRANCL, 2003).

Rain-splash dispersal is perceived as major transportation mode of conidial spores (SUTTON, 1982; PARRY *et al*., 1995), but also for ascospores (GILBERT AND TEKAUZ, 2000; PAUL *et al*., 2004). Fungal propagules are splashed by drops hitting spore-bearing surfaces. Resulting spore-incorporating droplets overcome distances and transfer inoculum to higher situated plant organs (FITT AND LYSANDROU, 1984). The extent and height of splash dispersion is favored by several physical and biological factors, comprising high amount and intensity of precipitation, large drop sizes, supporting features of spore-containing tissues, and pathogen-specific characteristics contributive for dissemination (FITT AND LYSANDROU, 1984; BRENNAN *et al*., 1985; WALKLATE, 1989; WALKLATE *et al*., 1989; MADDEN, 1997; HUBER *et al*., 1998). Similar to wind dispersion, density of splashed spores decreases with increasing distance from the inoculum source (JENKINSON AND PARRY, 1994; HÖRBERG, 2002; PAUL *et al*., 2004; MANSTRETTA *et al*., 2015). Rain-splashed spores of *F. graminearum* were collected in a maximum height of 100 cm (PAUL *et al*., 2004). Studies of JENKINSON AND PARRY (1994) and HÖRBERG (2002) measured lower vertical distances for the transport of *F. poae* (70 cm); *F. culmorum* (60 cm), and *F. avenaceum* (45 cm). An effect of spore size on distribution distance was excluded by HÖRBERG (2002). As only a small amount of fungal propagules was observed to overcome a distance comparable to the spacing between soil-borne inoculum and cereal heads, leaves were considered to work as temporary storage (PARRY *et al*., 1995). The possibility of saprophytic survival of *Fusarium* fungi and deposition of viable spores on leaves was obtained by ALI AND FRANCL (2001). Consequently, dispersed ascospores and conidia can either been directly blown or splashed from crop residues located on the soil surface or further disseminated from higher plant organs such as leaves and small grain heads (OSBORNE *et al*., 2002).

1.2.3 Infection and colonization process

The infection and colonization biology of *Fusarium* species on wheat spikes is well documented (STRANGE AND SMITH, 1971; KANG AND BUCHENAUER, 1999, 2000a, 2000b, 2002; PRITSCH *et al*., 2000; WANJIRU *et al*., 2002; GUENTHER AND TRAIL, 2005; KANG *et al*., 2005; JANSEN *et al*., 2005; BROWN *et al*., 2010; BOENISCH AND SCHÄFER, 2011). Only a few studies obtained information about infection in barley or oat (LEWANDOWSKI AND BUSHNELL, 2001; JANSEN *et al*., 2005; LEWANDOWSKI *et al*., 2006; YOSHIDA *et al*., 2007; TEKLE *et al*., 2012, LINKMEYER *et al*., 2013). The major components of a cereal spike and occurring colonization pathways are illustrated in figure 2.

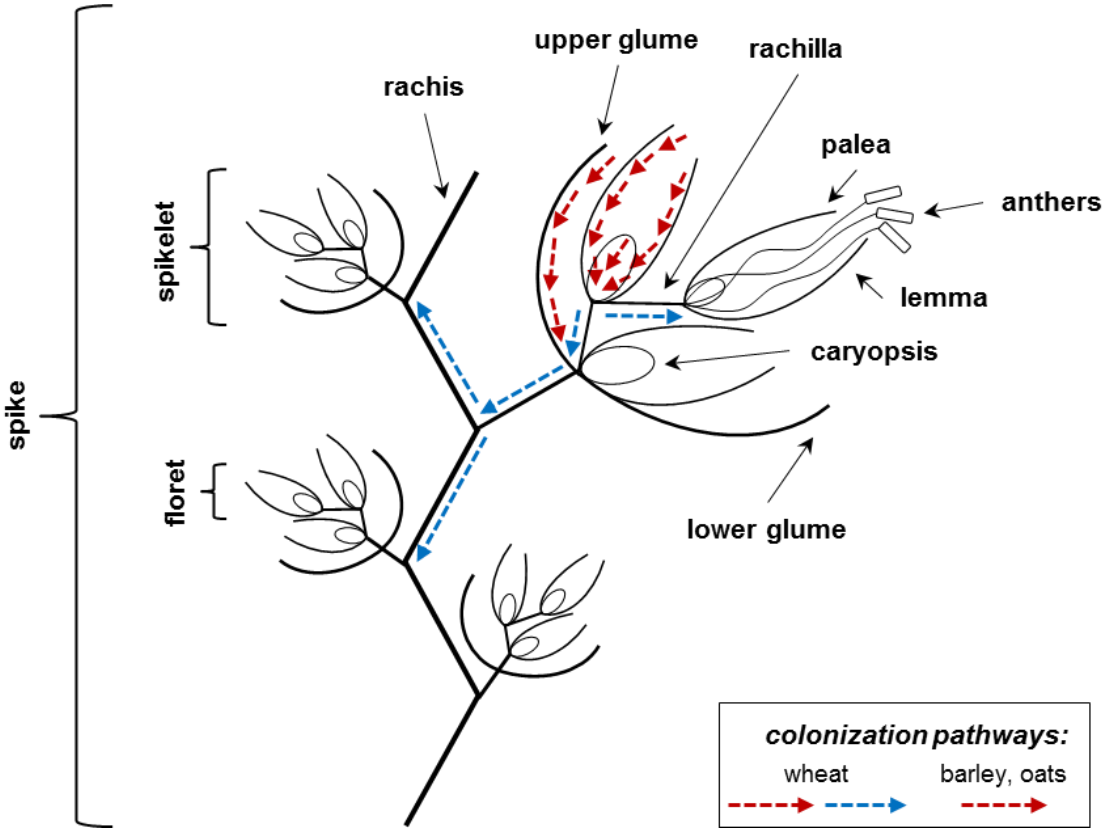

Figure 2: Cereal spike structure and *Fusarium* colonization pathways

Fungal material enters single florets through gaps between palea and lemma, the apical floret mouth via extruded antheres, or glume stomata. Spores germinate on all internal and external surfaces of host tissues, but hyphal networks establish mainly on internal surfaces of husks and on the caryopsis. Subsequently developed infection hyphae penetrate the cuticle of host tissues (husks and caryopsis) using

penetration pegs. Pathogens further develop subcuticular before forming additional penetration pegs for further hyphal spread into the epidermal cell wall. In the process, parenchyma cells are destroyed and the cytoplasm, including cell organelles, disintegrates. This stage can be recognized by the appearance of visual symptoms (brown and water-soaked). *Fusarium* species colonize vertically by inter- and intracellular growth towards rachilla and rachis. Further spread within the wheat spike occurs via colonization of cortical tissue and vasculature of the rachis. This is in contrast to barley spikes. Colonization of barley and oat florets is limited to the rachilla and the rachis node. Thus, floret to floret or spikelet to spikelet infection, respectively, is inhibited (JANSEN *et al*., 2005; TEKLE *et al*., 2012).

As the colonization process is divisible in a biotrophic phase characterized by symptomless epiphytic and intercellular development, and a necrotrophic phase apparent by damage of host-tissue, several *Fusarium* species are suggested to be hemibiotrophs (BUSHNELL *et al*., 2003; MAKANDER *et al*., 2010; LINKMEYER, 2012; AUDENAERT *et al*., 2013). The basic mechanisms of infection and colonization are fundamentally comparable between several species (XU AND NICHOLSON, 2009), but infection rate is species-specific dependent on temperature and humidity (ROSSI *et al*., 2001). High temperatures (29°C, 28°C, and 26.5°C) were optimal for the infection by *F. graminearum*, *F. avenaceum*, and *F. culmorum*, respectively. By contrast, *Microdochium* spp. benefited from cooler conditions (18°C). Increasing relative humidity during infection promoted infection by *F. graminearum*, *F. avenaceum*, and *Microdochium* spp., whereas infection by *F. culmorum* was hampered.

1.2.4 Pathogenicity of *Fusarium* species and cereal defense mechanisms

Fusarium pathogens use general pathogenicity factors such as production of cell wall degrading enzymes and toxins as well as host-specific factors such as effectors. The host counteracts with general defense mechanisms such as generation of antifungal proteins or with pathogen-specific mechanisms such as detoxification of fungal toxins. This molecular crosstalk during host-pathogen-interaction is thoroughly reviewed in WALTER *et al*. (2010), KAZAN *et al*. (2012), and MA *et al*. (2013). Most available information is based on studies in wheat and the wheat-FHB relevant species *F. graminearum* and *F. culmorum*. Figure 3 outlines the basic mechanisms of *Fusarium*-cereal-interaction.

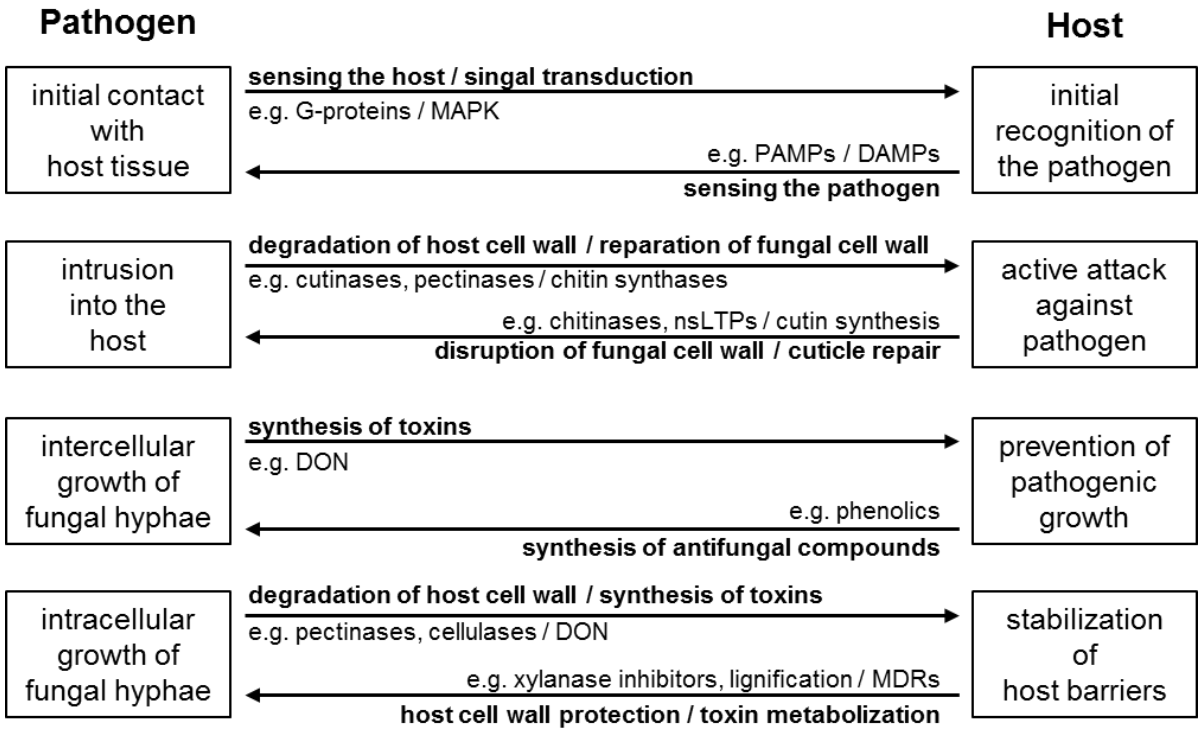

Figure 3. Mechanisms in *Fusarium*-host-interaction

Abbreviations: MAPK=mitogen-activated protein kinase, PAMP=pathogen-associated molecular pattern, DAMP=damage-associated molecular patterns, nsLTP=non-specific lipid transfer protein, DON=deoxynivalenol, MDR=multidrug-resistant protein.

Following the initial contact, pathogen and host are sense each other. Specific receptor proteins such as G-protein-coupled receptors and the transducin beta-subunit enable host recognition by *Fusarium* (CUOMO *et al*., 2007; DING *et al*., 2009) and involve further transmission of signals by for instance mitogen-activated protein kinase (MAPK) and cyclic adenosine monophosphate (cAMP) pathways (DI PIETRO *et al*., 2001; HOU *et al*., 2002; URBAN *et al*., 2003; GARCÍA-MARTÍNEZ *et al*., 2012). As suggested for other pathosystems, cereal hosts might recognize *Fusarium* pathogens by sensing pathogen-associated molecular patterns (PAMPs) such as chitin or glucan and/or damage-associated molecular patterns (DAMPs) such as cutin monomers. Transcriptional studies indicated enhanced formation of chitinases and glucanases in wheat after inoculation with *F. graminearum* and *F. culmorum* (PRITSCH *et al*., 2000; LI *et al*., 2001). BLEIN *et al*. (2002) detected plant defense responses mediated by complexes consisting of cutin monomers, liberated by fungal cutinases, and constitutively formed plant non-specific lipid transfer proteins (nsLTPs). Fungal intrusion into the host might be facilitated by degradation of plant cell wall

components (KIKOT *et al*. 2009). Cutin and cell wall degrading enzymes such as cutinases, cellulases, xylanases, and pectinases were found to be produced by *F. culmorum* and *F. graminearum* during infection and colonization of wheat spikes (KANG AND BUCHENAUER, 2000c; WANJIRU *et al*., 2002; JENCZMIONKA AND SCHÄFER, 2005; CUOMO *et al*., 2007). Further inter- and intracellular fungal growth might be promoted by the formation of mycotoxins. Several studies strongly suggested deoxynivalenol (DON) being a virulence factor in colonization of wheat (PROCTOR *et al*., 1995; DESJARDINS *et al*., 1996; BAI *et al*., 2002; EUDES *et al*., 2001; SCHERM *et al*., 2011). DON supports cellular damage and subsequent cell death by destruction of plasma membranes and further damage of chloroplasts and ribosomes as well as by provoking the production of hydrogen peroxide in host cells (MILLER AND EWEN, 1997; DESMOND *et al*., 2008). As DON is manipulating and suppressing host defense responses (AUDENAERT *et al*., 2013), it was speculated that DON functions as a virulence effector. Even if toxins may not be essential for early steps of host infection by *Fusarium* (JANSEN *et al*., 2005; MAIER *et al*., 2006; BOENISCH AND SCHÄFER, 2011), DON was shown to play a key role in the fungal spread within the wheat spike (PROCTOR *et al*. 1995; HARRIS *et al*. 1999; DESJARDINS *et al*. 2000; BAI *et al*. 2002; LANGEVIN *et al*., 2004; JANSEN *et al*., 2005; ILGEN *et al*., 2008). Biosynthesis of DON was shown to be induced by specific amine sources and acidity states of growth media as well as by present levels of reactive oxygen and phenolic acids (KAZAN *et al*., 2012).

Responses of cereal hosts to *Fusarium* infection imply the formation of defense-related hormones, the production of pathogenesis-related (PR) proteins, oxidative burst-associated enzymes, and components involved in cell wall fortification and cellular detoxification (KANG AND BUCHENAUER, 2000a; BODDU et al., 2006; ZHOU *et a*l., 2006; GEDDES *et al*., 2008; KAZAN *et al*., 2012; KOSAKA *et al*., 2015). Phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are suggested to function as mediators of the FHB defense responses (LI AND YEN, 2008; DING *et al*., 2011; MAKANDAR *et al*., 2012; QI *et al*. 2012). Fungal cell walls are disrupted by chitinases, glucanases, thionins, nsLTPs, and puroindolines (PRITSCH *et al*., 2000; LI *et al*., 2001; LAY AND ANDERSON, 2005; VAN LOON *et al*., 2006). Further growth of *Fusarium* is prevented by the synthesis of phytoalexins such as cyclic hydroxamic acids or phenolics and by antifungal peptides such as defensins and

hydroxyproline-rich proteins (FRIEBE *et al*., 1998; MCKEEHEN *et al*., 1999; SØLTOFT *et al*., 2008). Host cell wall degradation through *Fusarium* attack can be impeded by the synthesis of polygalacturonase inhibitor proteins and xylanase inhibitors (IGAWA *et al*. 2005; FEDERICI *et al*. 2006). Host cell wall fortification against *Fusarium* might occur via increased cell wall thickening, enhanced formation of cell wall apposition, and lignification (KANG AND BUCHENAUER, 2000c; RIBICHICH *et al*., 2000; MOHAMMADI AND KAZEMI, 2002). Beside systemic and local defense responses, detoxification processes were shown to be induced in cereal hosts upon *Fusarium* infection. Treatment with *F. graminearum* or DON triggers expression of cereal genes involved in dilution or detoxification mechanisms for example ATP (adenosine triphosphate)-binding cassette (ABC)-transporters, multidrug-resistant proteins (MDR), uridine diphosphate (UDP)-glucosyltransferases, cytochrome P450s, and gluthathione-S-transferases (BODDU *et al*., 2007; WALTER *et al*., 2008; GARDINER *et al*., 2010; SCHWEIGER *et al*., 2010; LI *et al*., 2010; KOSAKA *et al*., 2015).

1.3 The impact of Fusarium head blight infections

FHB is a devastating fungal disease recognized in all cereal growing areas of the world (SNIJDERS 1990; HUTCHEN AND JORDAN, 1992; SALAS AND STEFFENSON, 1999; BAI AND SHANER, 2004; GOSWAMI AND KISTLER, 2004; XU AND NICHOLSON, 2009; MCMULLEN *et al*., 1997, 2012). FHB results in direct economic damages due to yield reductions as well as in indirect detriments due to reductions of product safety and quality (MCMULLEN *et al*., 1997). Depending on the regions included and the observed time period, estimates for economic losses ascribed to *Fusarium* infections amount to several million U.S.$ per year (WINDELS, 2000; NGANJE *et al*., 2004a, 2004b; MCMULLEN *et al*., 1997, 2012; MURRAY AND BRENNAN, 2009; OBANOR AND CHAKRABORTY, 2014).

1.3.1 Yield quantity

Severe outbreaks of FHB in Europe, Asia, Australia, and North and South America can be accompanied by high yield losses of up to 70% (TUSA *et al*., 1981; MARTIN AND JOHNSTON, 1982; MIHUTA-GRIMM AND FORSTER, 1989; ZHUPING, 1994; PERKOWSKI AND KIECANA, 1997; MOSCHINI *et al*., 2001; PEREYRA *et al*., 2006; MURRAY AND BRENNAN, 2009). FHB infections diminish yield by reducing the kernel number

per head and the thousand kernel weight (SNIJDERS AND PERKOWSKI, 1990; ARSENIUK *et al*., 1993; SURMA *et al*., 2000; NIELSEN *et al*., 2014).

1.3.2 Product safety

Fusarium pathogens endanger cereal product safety due to their capability to form secondary metabolites. Relevant species and associated substances are listed in table 1. *F. graminearum* and *F. culmorum* synthesize type B trichothecenes such as nivalenol (NIV) or DON as well as zearalenon (ZEA). *F. langsethiae* and *F. sporotrichioides* produce type A trichothecenes such as T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (DAS). *F. poae* is known to biosynthesize both type B and type A trichothecenes. *F. avenaceum* and *F. tricinctum* are non-trichothecenes producers, but form beauvericin (BEA) and/or enniatins (ENNs), respectively (DESJARDIN, 2006). Some of these mycotoxins, in particular type B trichothecenes, are phytotoxic and therefore perceived as virulence factors (see 1.2.4). Furthermore, the biosynthesis of these compounds is considered to play a primary role in competition with other microorganisms (XU AND NICHOLSON, 2009). Dependent on the environmental conditions and competitors, co-inoculation experiments showed altered contents of secondary metabolites compared to inoculation with single isolates (XU *et al*., 2007). Fungal interaction is thereby not restricted to interspecies competition. MIEDANER *et al*. (2004a) compared toxin contents in winter rye after inoculation with individual strains or mixtures of *F. culmorum* isolates differing in chemotype. Most comparisons indicated suppressed trichothecene formation in grain treated with the mixed inoculum, indicating competition within the species.

Table 1. Selected *Fusarium* species and most relevant associated mycotoxins

***Fusarium* species**	**mycotoxins**
F. graminearum *F. culmorum*	DON, NIV (type B trichothecenes), ZEA
F. langsethiae *F. sporotrichioides*	T-2 toxin, HT-2 toxin, DAS (type A trichothecenes)
F. poae	NIV, DAS
F. avenaceum	ENNs, BEA
F. tricinctum	ENNs

Abbreviations: DON=deoxynivalenol, NIV=nivalenol, ZEA=zearalenon, DAS=diacetoxyscirpenol, ENN=enniatin, BEA=beauvericin.

Several *Fusarium* toxins are harmful to mammalian and avian individuals (DESJARDIN, 2006; ANTONISSEN *et al*. 2014; PIERRON *et al*., 2016). Trichothecenes are cytotoxic, impede protein synthesis, and interfere with immune functions (SUGITA-KONISHI AND PESTKA, 2001; DA ROCHA *et al*., 2005, 2014; PESTKA, 2010). Depending on dose, duration of exposure, type of secondary metabolite and animal species as well as physical constitution of the animal, mycotoxins of *Fusarium* can lead to acute and chronic mycotoxicoses (D'MELLO *et al*., 1999). These appear as gastroenteritis, immunological dysfunction, vomiting, and anorexia (PESTKA, 2007; TEP *et al*., 2007). For other compounds, such as ZEA, BEA, or ENN, no hazardous effects in terms of mycotoxicoses have been detected. Nevertheless, ZEA was reported to cause estrogenic syndrome in swine (DESJARDIN, 2006).

To ensure public health, maximum limits for contaminants in foodstuff were set by the European Commission (EC) in 2006. The legal amount of DON in unprocessed durum wheat or oat is limited to 1750 µg/kg. In other unprocessed cereals the maximum limit of DON is 1250 µg/kg. The content of ZEA in unprocessed cereals must not exceed 100 µg/kg. DON and ZEA limits for cereals intended for direct human consumption or cereal foodstuff range lower (EC, 2006a). In 2013 the European Commission passed a recommendation for the presence of T-2 and HT-2 toxin in cereal products. Indicative levels of T-2 and HT-2 toxin in unprocessed oats, barley, and wheat are 1000 µg/kg, 200 µg/kg, and 100 µg/kg, respectively. Cereal grain intended for direct human consumption should not contain more than 200 µg/kg (oat) or 50 µg/kg (other cereals) of T-2 and HT-2 toxin (EC, 2013). No legislation or recommendation exists for NIV or DAS (EC, 2006a, 2013). According to the regulatory guidance of the U.S. Food and Drug Administration (USFDA) the advisory level for DON in finished wheat products intended for the U.S. human consumption is 1000 µg/kg (USFDA, 2010). Similar to Europe, there are currently no legislations concerning T-2 or HT-2 toxin in U.S. products.

1.3.3 Product quality

Beside product safety, FHB also downgrades cereal product quality. *Fusarium* pathogens are able to modify the composition of grain components such as starch, celluloses and proteins (BOYACIOĞLU AND HETTIARACHCHY, 1995; DEXTER *et al*., 1996, 1997; WANG *et al*. 2005a; SIUDA *et al*., 2010; SCHMIDT *et al*., 2016) and therefore

reduce baking quality (DEXTER *et al*., 1996; WANG *et al*. 2005b; LANCOVA *et al*., 2008a).

Cereal grain, in particular barley, is furthermore used for malting purposes (FAO, 2009). ULLRICH (2011) collected information about the international malt output: The worldwide malt production capacity is about 20 million tons per year, with Europe producing a major part (42%). Most of the malt is produced in Germany, the United Kingdom, France, Belgium, Spain, and the Czech Republic. The U.S. and Canadian malt production capacity is around 1.8 and 1.7 million tons per year, respectively. Australia and China produce 0.7 and 4.0 million tons per year, respectively. The major part of malt is utilized for beer production (94%), whereas only small amounts are distilled (4%) or used as food.

FHB infections in barley grain were shown to downgrade raw material quality and to interfere with the malting and brewing process. *Fusarium* pathogens reduce kernel weight as well as germinating capacity or energy and enhance water sensitivity (OLIVIERA *et al*., 2012; NIELSEN *et al*., 2014). By changing the ultrastructure and the enzymatic status of barley grain, *Fusarium* infections alter solubility characteristics during malting, negatively affecting subsequent brewing quality attributes (SARLIN *et al*., 2005a; SCHWARZ *et al*., 2006; OLIVIERA *et al*., 2012, 2013).

1.3.4 Process reliability

Fusarium infections are connected to the ‘gushing’ problem in beer production, the phenomenon of the spontaneously over foaming of beer immediately upon opening the bottle (SCHWARZ *et al*., 1996; SARLIN *et al*., 2005b; SHOKRIBOUSJEIN *et al*., 2011). Gushing factors such as hydrophobins or nsLTPs result from host parasite interactions (WALTER *et al*., 2010). Hydrophobins are small, surface-active fungal proteins (WESSELS, 1996; LINDNER *et al*., 2005) and were considered to support spore adhesion and growth of aerial mycelium on host surfaces by reducing water surface tension at the medium-air interface. Exposed to the interface of water and air or any hydrophobic surface such as the cuticle, hydrophobin monomers spontaneously self-assemble to a stable amphipathic membrane accompanied by lowering water surface tension (TUCKER AND TALBOT, 2001). Several *Fusarium* species were shown to form hydrophobins (SARLIN *et al*., 2007, 2012). The defined role of these proteins in beer gushing is still discussed. Hydrophobin compounds might function as nucleation sites

for CO_2 bubble formation (LINDNER, 2009). In carbonated liquids hydrophobins enclose gaseous CO_2-molecules by self-assembling. These bubbles remain stable in a closed container, but suddenly expand when pressure drops due to bottle opening (SHOKRIBOUSJEIN *et al*., 2011). Beside fungal components, surface-active molecules deriving from plant defense responses, for instance nsLTPs or associated degradation products, were discussed to induce gushing (HIPPELI AND ELSTNER, 2002; HIPPELI AND HECHT, 2008) as they are highly relevant for beer foam quality (SØRENSEN *et al*., 1993).

1.4 The control of Fusarium head blight

Several agronomical control measures encompassing cultural, genetic, biological, and chemical tools were shown to reduce FHB in cereals (reviewed in PIRGOZLIEV *et al*., 2003; EDWARDS, 2004; GILBERT AND TEKAUZ, 2011; WEGULO *et al*., 2015). Combining multiple measures can further reduce infection compared to single measures (KOCH *et al*., 2006; MCMULLEN *et al*., 2008; WEGULO *et al*., 2011; BLANDINO *et al*., 2012) and is necessary to comply with the intention of 'Good Agricultural Practice'.

1.4.1 Crop rotation and tillage

Crop debris on the soil surface harboring pathogens is the primary source of inoculum for FHB infections (see section 1.2.1). Both cultivating non-hosts within the crop rotation as well as burying host crop residues by appropriate tillage was found to reduce *Fusarium* infections and associated mycotoxin contamination (WEGULO *et al*., 2015). Cereal host in general and maize in particular were shown to support FHB in subsequent crops compared to non-hosts (DILL-MACKY AND JONES, 2000; CHAMPEIL *et al*., 2004; BATEMAN *et al*. 2007; EIBLMEIER AND VON GLEISSENTHALL, 2007; MAIORANO *et al*., 2008; BLANDINO *et al*., 2010). WEBER *et al*. (2016) found further a negative correlation between height of preceding crop stubble and infection by *F. culmorum*. Compared to minimum or zero tillage, ploughing was most effective in reducing FHB inoculum or FHB occurrence on wheat (DILL-MACKY AND JONES, 2000; YI *et al*., 2001; BAI AND SHANER, 2004; EDWARDS AND RAY, 2005; BATEMAN *et al*. 2007; EIBLMEIER AND VON GLEISSENTHALL, 2007; BLANDINO *et al*., 2010, GUO *et al*., 2010; WEBER *et al*., 2016; WEST *et al*., 2012).

1.4.2 Host resistance

Different components of resistance towards FHB have been characterized (SCHROEDER AND CHRISTENSEN, 1963; MESTERHÁZY, 1995; MILLER *et al*., 1985; BOUTIGNY *et al*., 2008): Type I (resistance to initial infection), type II (resistance to spread), type III (resistance to grain infection), type IV (tolerance against FHB and trichothecenes), and type V (resistance to trichothecene accumulation). The last type of resistance can be subdivided into class 1 and 2 describing resistance by chemical modification of trichothecenes or inhibition of their synthesis, respectively. Because FHB resistance is quantitatively inherited and expression of resistance strongly underlies environmental factors, mapping of quantitative trait loci (QTL) was established as the primary method in breeding approaches (reviewed in BUERSTMAYR *et al*., 2009). Several QTLs for FHB resistance are characterized in wheat, most of them associated to type II resistance, for instance *Fhb1* (SCHWEIGER *et al*., 2016). Barley naturally exhibits type II resistance, wherefore type I and III are in the focus of breeders (BAI AND SHANER, 2004; LINKMEYER, 2012). Unfortunately, much fewer FHB resistance sources are available for barley (RUDD *et al*., 2001; BAI AND SHANER, 2004), as most of the resistant cultivars exhibit negative characteristics concerning agronomic features and malting quality (ZHU *et al*., 1999).

1.4.3 Fertilization

The influence of nitrogen fertilization on wheat-FHB was extensively studied in field experiments, but contradicting data outputs were generated: LEMMENS *et al*. (2004) and MA *et al*. (2004) noted increasing FHB upon increased nitrogen input, whereas other research approaches have not found nitrogen influence on FHB in wheat (AUFHAMMER *et al*., 2000; FAUZI AND PAULITZ, 1994; TEICH AND HAMILTON, 1985) or detected inconsistency (HEIER *et al*., 2005; SUBEDI *et al*., 2007). Few studies have investigated the influence of nitrogen input on barley-FHB. MUHAMMED *et al*. (2010) and YANG *et al*. (2010) conducted greenhouse trials and detected increasing and decreasing effects, respectively. Comprehensive field studies investigating the effect of nitrogen fertilization on barley-FHB as well as the effect of other macronutrients or micronutrients on FHB in general are lacking so far.

1.4.4 Biological and chemical plant protection

The effect of chemical compounds on wheat-FHB was tested in several studies, differing in treatment factors like product type, dose, timing, and method as well as in environmental factors such as pathogen pressure, cultivar susceptibility, and climate (PAUL *et al*., 2006). Most of the approaches resulted in FHB reduction upon fungicide application (BOYACIOĞLU *et al*., 1992; HOMDORK *et al*., 2000; PIRGOZLIEV *et al*., 2002; HAIDUKOWSKI *et al*., 2005). Azoles in general and triazoles such as tebuconazole, metconazole, and prothioconazole in particular, were most efficient (BOYACIOĞLU et al. 1992; HOMDORK *et al*., 2000; EDWARDS *et al*. 2001; SIMPSON *et al*., 2001; IOOS *et al*. 2005; BEYER *et al*., 2006; PAUL *et al*., 2008). On the contrary, a supportive effect on FHB was shown for strobilurines (SIMPSON *et al*., 2001; CROMEY *et al*. 2002; ELLNER *et al*., 2006). Elimination of competitors and delayed senescence due to greening of hosts were suggested as possible reasons for enhanced *Fusarium* infection and contamination. Beside active ingredients, treatment timing is decisive for the success of FHB control. Treatment at early stages of plant development was shown to reduce FHB (EDWARDS AND GODLEY, 2010), but applications appeared to be most efficient during anthesis or grain development (YOSHIDA *et al*., 2008, 2012; D'ANGELO *et al*., 2014).

Some studies detected potential biocontrol agents for FHB reduction. Biocontrol of soil-borne pathogens is based on antagonism, which encompasses antibiosis, competition, and exploitation (BAKER, 1968). Several strains of bacterial (*Bacillus*, *Pseudomonas*, *Streptomyces*), yeast (*Rhodotorula*, *Sporobolomyces*, *Cryptococcus*) and fungal (*Trichoderma*) genera were found to reduce FHB or associated mycotoxin production (FERNANDEZ, 1992; SCHISLER *et al*., 2002, 2006; KHAN *et al*., 2001, 2004; PALAZZINI *et al*., 2007; MATARESE *et al*., 2012). To date, the potential of chemical or biological fungicides to reduce barley-FHB has hardly been investigated.

1.5 The concept of Integrated Pest Management (IPM)

The concept of Integrated Pest Management (IPM) is perceived as the guiding principle of the 'Good Agricultural Practice' and is defined accordingly by the European Union in directive 91/414/EEC as follows: "The rational application of a combination of biological, biotechnical, chemical, cultural or plant-breeding measures, whereby the use of plant protection products is limited to the strict minimum necessary to maintain the pest population at levels below those causing economically unacceptable damage or loss" (EC, 1991). Similar definitions are made by other institutions such as the Food and Agriculture Organization of the U.N. (FAO) or the U.S. Department of Agriculture (USDA). According to that definition, integrated control of FHB is often perceived as combination of available agronomical control measures for reducing infection (LEMMENS *et al*., 2004; MCMULLEN *et al*., 2008; LORI *et al*., 2009; WILLYERD *et al*., 2012). However, additional components are required to establish successful IPM (figure 4).

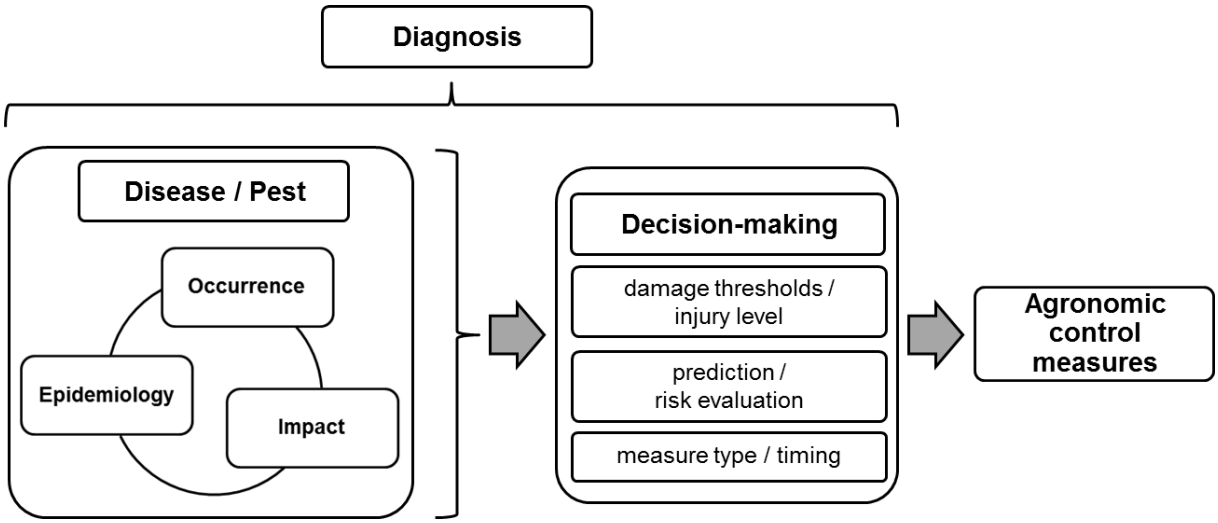

Figure 4. Components of Integrated Pest Management (IPM)

See the next paragraph for a detailed explanation.

Knowledge of pathogen occurrence, epidemiology, and impact is the basis for decision-making regarding the agronomic control. This is supported by definitions for 'economic injury levels' and for 'economic damage thresholds' as well as by corresponding instructions for types and timings of control measures (FLINT, 2012; PHILIPS *et al*., 2014). In integrated control concepts, the 'economic injury level'

indicates “the lowest population density that will cause economic damage” and the ‘economic damage threshold’ marks “the density at which control measures should be determined to prevent an increasing pest population from reaching the economic injury level” (STERN *et al*., 1959). Prediction models, which forecast infections and/or contaminations, became valuable tools in IPM as they provide information on the need and timing of control measures, usually pesticide treatments (WAY AND EMDEN, 2000; PRANDINI *et al*., 2009). Precise diagnostics of a pathogen is another essential element of IPM. Accurate pathogen identification is needed for determining pathogen occurrence in extent and intensity and for elucidating pathogen-specific epidemiology and impact. Furthermore, pathogen diagnostics serves as supervisory element for the compliance with damage thresholds and injury levels (VERREET *et al*., 2000; EHLER, 2006; FLINT, 2012).

German examples for practically applied IPM-concepts are the ‘PhytophthoraModell Weihenstephan’ (HAUSLADEN AND HABERMEYER, 2001), ‘The IPM Wheat model’ (VERREET *et al*., 2000), the ‘Gerstenmodell Bayern’ (LFL, 2016), or ‘The IPM Sugar Beet Model’ (WOLF AND VERREET, 2002). These models support decision-making of farmers in terms of fungicide application.

1.6 Objectives and methodology

Fusarium species infect small grain cereals such as wheat and barley and cause FHB, a devastating fungal disease affecting yield quantity as well as product quality and safety. The majority of scientific studies focused on wheat-FHB and its most relevant causal agent *F. graminearum*. In that pathosystem, research has generated extensive knowledge of epidemiology as well as impact and could develop effective control strategies. Much less information about FHB on barley is available. This impedes the implementation of an effective integrated management strategy. The present work aimed to contribute further steps towards integrated management of FHB on barley through strategic research into the most relevant knowledge gaps. The concrete research objectives were:

- To characterize the *Fusarium* complex on German barley and malt (Papers I and V).
- To explore the influence of climatic factors on epidemiological aspects of *Fusarium* species relevant for barley (Papers I and III).
- To determine species-specific damage potential in barley with regard to symptomatology and yield quantity as well as to product quality and safety (Papers I and IV).
- To test the influence of the Mildew locus O (MLO) gene in spring barley cultivars on FHB (Paper II).
- To analyze the influence of nitrogen fertilization on barley-FHB (Paper III).
- To evaluate the visual assessment of malt in regard to its validity for predicting *Fusarium* contaminations (Paper V, in preparation).

Several diagnostic tools were used to detect and quantify *Fusarium* infection. Visual investigations rated incidence on immature barley spike (Paper I) as well as on malted grain (Paper V) material. Mycological analyses revealed fungal infection on dry grain (Paper I) and malt (Paper V). Quantitative polymerase chain reaction (qPCR) and liquid chromatography tandem mass spectrometry (LC-MS/MS, in cooperation with the Chair of Analytical Food Chemistry, Technische Universität München) determined *Fusarium* DNA (Papers I-V) and secondary metabolites, respectively (Paper I, III, and V), in immature spike material, dry grain, and/or malt.

Monitoring studies were conducted to characterize the German barley-FHB complex. Therefore, naturally infested barley grain (Paper I) and malt (Paper V)

samples, originating from different years and locations, were mycological assessed for the occurence of *Fusarium* species. Further analysis by qPCR and LC-MS/MS gave more detailed information about complex composition and dominance patterns.

Correlation analyses of *Fusarium* DNA contents in harvested grain and either weather (Paper I) or microclimate (Paper III) data, recorded at relevant phases during plant development, revealed further insights into barley-FHB epidemiology.

Inoculation trials under field (Papers I-III) and greenhouse (Papers III and IV) conditions were carried out to artificially increase pathogen pressure. Therefore, barley plants were spray-infected with species-specific spore suspensions at the time period of anthesis (Papers I-IV) or soil surface-inoculated with species-specific infected grain material at growth stages around stem elongation (Paper III). In each experiment a corresponding number of controls were included. On the one hand, inoculation experiments served for the assessment of species-specific damage potentials (Papers I and IV). Symptom formation, yield reduction, DNA and mycotoxin contamination as well as gene regulation was evaluated by comparing data of inoculated and non-inoculated samples. On the other hand, inoculation trials were used to investigate the influence of Mlo-resistance (Paper II) and nitrogen fertilization (Paper III) on barley-FHB. For that purpose, *Fusarium* DNA was quantified in harvested grain of near-isogenic lines differing in powdery mildew resistance (Paper II) or of plants differing in nitrogen supply (Paper III). The comparison of variants was undertaken in backgrounds with natural and artificially enhanced pathogen pressure.

Naturally infested and artificially generated malt samples were used to evaluate the validity of the practically applied visual assessment to predict malt quality and safety (Paper V). Correlation analyses of symptomatology data, malt quality parameters as well as DNA and mycotoxin contents in naturally infested samples gave information about the connectivity of these factors under practical conditions. Comparisons of specifically colored or artificially contaminated samples with regard to DNA and mycotoxins shed light on the question of whether reliability and sensitivity of this commonly applied method is sufficient.

Finally, the possible implementation of own and literature results into a strategy for the integrated management of spring barley-FHB is discussed.

2 RESULTS

2.1 The influence of inoculum and climatic factors on the severity of Fusarium head blight in German spring and winter barley

2.1.1 Summary of the publication LINKMEYER *et al*., 2016 (Paper I)

Fusarium head blight is known as a destructive disease on small grain cereals such as wheat and barley. Up to now, *Fusarium* research has focused mainly on wheat and *F. graminearum* as the major causal agent of the disease. Little information about barley-FHB and associated *Fusarium* species is available. This study aimed to address the lack of detail about the relevant barley-FHB complex and species-specific epidemiology as well as to evaluate the damage potential of individual *Fusarium* species.

In a five-year monitoring approach grain samples of naturally infected German spring and winter barley were screened for the presence of individual *Fusarium* species. The complex composition was dominated by *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae*, *F. sporotrichioides* and *F. poae*, but differed between years and cultivars. In parallel, DNA and mycotoxin content of trichothecene-producing *Fusarium* species in grain were determined by qPCR and LC-MS/MS, respectively. Data suggested the importance of *F. langsethiae* for toxin contamination in spring barley and of *F. graminearum* in winter barley.

In the process, correlation analysis of DNA contents and meteorological data in connection with plant development was carried out to identify the influence of weather parameters and barley flowering time on *Fusarium* infection. Results indicate species-specific associations with mean temperature (T), relative humidity (RH), sum of precipitation (PRE), and number of days with precipitation of more than 1 mm (DPRE) around anthesis as well as with heading date (HD) of plants. The occurrence of *F. graminaerum* was positively associated with RH, PRE and DPRE, but negatively with T. By contrast, correlations were positive for the appearance of *F. culmorum* and *F. langsethiae* and T, but negative for DPRE. No significant associations of weather parameters and *F. sporotrichioides* were detected. HD was found be positively connected to infection with *F. culmorum* and *F. langsethiae*, but not to infections with *F. graminearum* and *F. sporotrichioides*.

Field inoculation experiments were conducted in spring barley to study species-specific damage potential in terms of symptom formation, yield reduction and mycotoxin contamination. Among the tested species, symptom formation as well as yield reduction was strongest after artificial infection with *F. culmorum* and *F. avenaceum*. Effects were lower or absent after inoculation with *F. tricinctum*, *F. langsethiae*, and *F. sporotrichioides*. Regarding natural or artificial infection with the type B trichothecene producer *F. culmorum*, a significant association between grain infection and mycotoxin contamination was observed. This was contrary for the type A trichothecene-producing species *F. langsethiae* and *F. sporotrichioides*. Contamination with T-2 and HT-2 also occurred in the absence of significant visible damage to grain and hence did not necessarily promote fungal colonization.

Taken together, the barley-FHB complex was shown to include several species differing in dominance, most likely driven by species-specific environmental requirements and damage potentials.

2.1.2 Own contributions to the publication LINKMEYER *et al*., 2016 (Paper I)

Experimentation: Quantification of *Fusarium* DNA in samples of 2010 for monitoring studies.

Data analysis: Critical processing and analysis of data, including statistical analysis; Contributory interpretation of data.

Writing: Substantial rewriting of the original manuscript for resubmission.

2.2 MILDEW LOCUS O mutation does not affect resistance to grain infections with *Fusarium* spp. and *Ramularia collo-cygni*

2.2.1 Summary of the publication HOFER *et al*., 2015 (Paper II)

The *Mildew Locus O* (*MLO*) defines a major susceptibility gene for powdery mildew in spring barley caused by the biotrophic pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*). Recessive *mlo* resistance alleles mediate monogenic, race-nonspecific, and durable resistance against *Bgh* and are widely used for breeding spring barley. The pleiotropic effect of spontaneous formation of leaf lesions and associated yield reductions could be removed in modern *mlo* varieties by rearranging genes in other parts of the genome. However, *mlo* resistance was considered to be also costly in terms of increased susceptibility to cell-death inducing pathogens such as *Magnaporte oryzae*, *Bipolaris sorokiniana*, *Ramularia-collo cygni*, and *Fusarium graminearum*. The present study investigates whether *mlo* powdery mildew resistance increases infection of barley grain with relevant Fusarium head blight pathogens and the partially seed-transmitted leaf pathogen *R. collo-cygni* when produced under field conditions.

In a four-year microplot experiment plants of two spring barley varieties (Ingrid and Pallas) and their near-isogenic, powdery-mildew-resistant backcross (BC) lines (BC Ingrid-*mlo5* and BC Pallas-*mlo5*) were cultivated under field conditions. At the time period of anthesis plants were spray-inoculated with *Fusarium* species-specific spore solutions. A corresponding number of plants remained non-inoculated and were only exposed to natural infection. Matured grain was harvested and assessed for fungal DNA of relevant FHB pathogens (*F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae*, *F. sporotrichioides*) and *R. collo-cygni*. In four consecutive years DNA of *Fusarium* species and *R. collo-cygni* were not present higher in grain from *Bgh*-resistant *mlo5* genotypes than in near-isogenic wild-type (WT) lines. On the contrary, similar or in several cases lower infection was detected in grain of *mlo5* genotypes compared to corresponding WT-lines.

These data suggest no enhanced field susceptibility of powdery-mildew-resistant *mlo5* spring barley lines toward grain infection by hemibiotrophic ascomycete fungi of the *Fusarium* genus or *R. collo-cygni*. Data might be considered relevant for decision making in barley breeding for pathogen resistance.

2.2.2 Own contributions to the publication HOFER *et al*., 2015 (Paper II)

Experimentation: Design and performance of the field trial in 2013; Quantification of *Fusarium* DNA in samples of 2013 and of *R. collo-cygni* DNA in samples of 2010-2013.

Data analysis: Data processing and analysis, including statistical analysis; Contributory interpretation of data.

Writing: Preparation of tables; Writing of the manuscript draft and manuscript editing for 1st and final submission.

2.3 Effect of nitrogen fertilization on Fusarium head blight in spring barley

2.3.1 Summary of the publication HOFER *et al*., 2016 (Paper III)

Nitrogen fertilization has been shown to affect the incidence of Fusarium head blight in wheat. Only little information about effects on barley-FHB is available. Spring barley is primarily used for malting purposes and subsequent beer production. Maltsters and brewers set distinct specifications for raw barley, because its condition is decisive for malt and beer quality. These imply limits for protein as well as for microorganisms and toxins. Consequently, nitrogen input may play a key role in adjusting barley quality to the requirements of the malting industry as it determines the protein content and likely the occurrence and impact of FHB. The present study investigated the influence of nitrogen fertilization on the incidence of relevant *Fusarium* species in spring barley under field and greenhouse conditions.

In a two-year field trial plants of the spring barley cultivar Grace were grown under conditions differing in nitrogen input. Grain material from differently fertilized field plots was analyzed for *Fusarium* DNA and associated mycotoxins by qPCR and LC-MS/MS, respectively. Under natural pathogen pressure no effect of nitrogen fertilization on *Fusarium* infection was observed. Under conditions with high pathogen pressure (*F. culmorum* and *F. avenaceum*), artificially increased via species-specific soil-surface inoculation at the stage of stem elongation, nitrogen application reduced contents of *Fusarium* DNA and associated mycotoxins in barley grain. Additionally, nitrogen-dependent canopy parameters were recorded over the season and correlated with DNA and mycotoxin data. Sparser canopy and reduced plant height permitted more *Fusarium* infections.

A greenhouse trial was carried out to exclude potential canopy factors. Plants of the cultivar Grace were differentially fertilized with nitrogen and spray-inoculated with spores of *F. culmorum* at the time period of anthesis. Immature spike material was sampled at specific time points after infection and tested for *Fusarium* DNA content. Plants with high nitrogen-fertilization allowed less fungal development, indicating nitrogen effects on plant defense.

These data suggest restricting effects of nitrogen fertilization on *Fusarium* grain infection in barley by influencing canopy characteristics and possibly plant physiology.

2.3.2 Own contributions to the publication HOFER *et al*., 2016 (Paper III)

Experimentation: Design and performance of field trials; Design and performance of the greenhouse trial; Determination and collection of yield and microclimate data; Quantification of *Fusarium* DNA.

Data analysis: Data processing and analysis, including statistical analysis; Interpretation of data.

Writing: Design and preparation of figures and tables; Writing of the manuscript.

2.4 Influence of *Fusarium* isolates on the expression of barley genes related to plant defense and malting quality

2.4.1 Summary of the publication HOFER AND GEIßINGER *et al*., 2016 (Paper IV)

Fusarium head blight in barley leads to yield reduction, to mycotoxin contamination as well as to interferences with malt and subsequent beer production. Solubility characteristics of barley grain material are decisive for the production of high quality malt, but were shown to be influenced by *Fusarium* infection. Underlying enzymatic processes are not well understood. This pilot study aimed to increase the understanding of transcriptional alterations in barley grain in response to species-specific *Fusarium* infection during early and late plant development as well as during subsequent steps of malt preparation.

Plants of two spring barley varieties (Grace and Scarlett) were cultivated under greenhouse conditions and spray-inoculated with spores of *F. culmorum*, *F. avenaceum*, *F. langsethiae*, and *F. sporotrichioides* at the time period of anthesis. A corresponding number of control plants was treated with an equivalent mock-solution instead and served as reference. Spike material of developing plants was sampled at specific days after inoculation (dai): 2dai, 4dai, 7dai, and 21dai. Matured grain was harvested and malted according to a standard protocol. Additional samples were obtained at specific time points in the malting process: dry grain, malt after steeping, after germination, and after kilning. From the samples genomic *Fusarium* DNA was isolated and quantified to determine the success of inoculation and severity of infection. This revealed much stronger infection by *F. culmorum* and *F. avenaceum* compared to *F. langsethiae* and *F. sporotrichioides*. RNA was isolated from the same samples, reverse transcribed to cDNA (complementary DNA), and further used in gene expression experiments. A subset of candidate genes, either relating to plant defense (*thaumatin-like protein*, *b-1,3-glucanase*) or to malting quality (*a-amylase/trypsin inhibitor*, *a-amylase*, *b-amylase 1*, *b-amylase 2*, *a-glucosidase*, *limit dextrinase*, and *b-ketoacyl synthase*), was selected.

Gene expression data disclosed alteration of defense and malting-quality related genes as a response to *Fusarium* infection. This was observed shortly after inoculation in developing spikes as well as later during the malting process. The

temporal expression profiles of the target genes were comparable in both varieties and alterations to these expression profiles were largely similar after inoculation.

Defense gene regulation was used as one marker for fungal contamination level. Both *thaumatin-like protein* and *b-1,3-glucanase* were further upregulated after infection with *F. culmorum* and *F. avenaceum*. This was less apparent in *F. langsethiae*- and *F. sporotrichioides*-infected samples. On the contrary, fungal contamination level has not always represented the alteration strength in target genes associated with malting quality. Especially at some time points before harvest *a-amylase/trypsin inhibitor*, *a-amylase*, and *b-amylase 1* were consistently upregulated after infection by all *Fusarium* species.

In summary, present data revealed a potential of *Fusarium* infection to influence gene expression associated with defense and malting quality during plant development and the subsequent malting process following seed dormancy. This pilot study shows the possibility for studying the impact of spike infections with different *Fusarium* species on the expression of genes which determine malting quality, from pre-harvest infected tissues to the malting process.

2.4.2 Own contributions to the publication HOFER AND GEIßINGER *et al*., 2016 (Paper IV)

Experimentation: Design and performance of greenhouse trials; Quantification of *Fusarium* DNA in spike, grain, and malt samples.

Data analysis: Processing and analysis of DNA and gene expression data; Contributory interpretation of data.

Writing: Design and preparation of figures; Critical reading and editing of the manuscript.

2.5 *Fusarium* species on barley malt – Visual assessment as an appropriate tool for detection?

2.5.1 Summary of the manuscript GEIßINGER AND HOFER *et al*. (Paper V)

Fusarium infections in malting barley cause mycotoxin contaminations, quality degradations and interfere with processing. The visual assessment of barley malt is a commonly applied, but critically viewed practice in the malting and brewing industry to screen cereal commodities for fungal infection and to appraise associated risks for product safety and processing. The method assumes a direct connection between occurring symptomatology and actual fungal contamination. The exceedance of a defined limit of red colored kernels (usually five to seven) in a 200 g subsample of malt is associated with an unjustifiable risk in terms of DON contamination or gushing and can lead to reductions in price or the rejection of the entire batch. The present study evaluated the visual assessment in terms of its suitability to ensure product quality and safety. It was further intended to resolve the presumed linkage between kernel discoloration and *Fusarium* infection.

A total number of 243 malt samples, produced from commercially cultivated barley, were visually assessed and examined for quality features. A subset was further mycologically and molecularly (qPCR and LC-MS/MS) analyzed for the degree of *Fusarium* infection. Correlation analysis revealed a generally low predictability of symptomatology for *Fusarium* contamination. However, significant correlations became apparent between the number of discolored kernels and fungal DNA contents under conditions of higher levels of infection, although this was not the case for mycotoxin levels. Specific colored malt samples were generated and analyzed for *Fusarium* DNA as well as for associated toxins and compared to asymptomatic malt samples. Samples with discolored kernels were enriched with fungal DNA and mycotoxins, in particular with DNA of *F. avenaceum*, *F. tricinctum*, and with ENNs. Serial dilution experiments were conducted to evaluate the validity of the visual assessment. Therefore, defined numbers of *Fusarium*-infected red kernels were added to potentially pathogen-free malt. DNA and toxin levels in the produced malt series were quantified. Although *Fusarium* contamination increased with the rising amount of red kernels, the collected data suggest that only high numbers of discolored kernels could reliably indicate *Fusarium* contamination.

Taken together, the visual assessment of red kernel discoloration was found to be overvalued in predicting DON. In general, symptomatology is likely overestimated with regard to its reliability as an indicator for *Fusarium* contaminations. Nevertheless, it might still assist in the assessment of risk of fungal contaminants, especially as long as no better tool is available in practice.

2.5.2 Own contributions to the manuscript GEIßINGER AND HOFER *et al*. (Paper V)

Experimentation: Mycological assessment of barley malt; Quantification of *Fusarium* DNA; Design and performance of the experiment with specific colored malt samples; Performance of the serial dilution experiment.

Data analysis: Data processing and analysis, including statistical analysis.

Writing: Contributory writing of the manuscript.

3 DISCUSSION

Fusarium head blight is a devastating fungal disease on small grain cereals. Numerous studies have contributed to IPM by evaluating FHB-restricting agronomic measures, by improving accuracy and efficiency of diagnostic methods, and by assessing the occurrence, the epidemiology, and the impact of individual *Fusarium* species. For wheat production, FHB is recognized as a limiting factor (DUBIN, 1997). Therefore, research approaches focused on wheat cultivation and wheat-relevant *Fusarium* species such as *F. graminearum* as well as on associated mycotoxins, for instance DON. However, findings which contribute to the management of an individual pathogen or the protection of a specific crop cannot easily be transferred to related pathosystems (EDWARDS, 2004).

For barley cultivation, *Fusarium* infection was so far not perceived as problematical as for wheat. Several facts might be relevant in that regard: Firstly, barley production is lower in both amount and area than that of wheat (FAO, 2016). Secondly, the yield reduction potential of barley-FHB appears moderate compared to wheat-FHB (LINKMEYER, 2012; LINKMEYER *et al*., 2013). Thirdly, barley is mostly used for animal nutrition and beer production and not ususally intended for direct human consumption (ULLRICH, 2011). This might diminish the risk perception of producers, processors, consumers, and authorities for mycotoxin contaminations. In particular spring barley serves as raw material for malting and subsequently beer production (ULLRICH, 2011). The malting and brewing industry pre-define specific product safety and quality requirements to guarantee functional manufacturing and to comply with end-product standards (FAO, 2009; JACOB, 2011). In this context, FHB pathogens negatively affecting malt and beer safety and quality, are unwanted invaders. At the same time, trends towards biofuels and increased meat consumption lead to decreasing acreage for malting barley as it competes with other crops for arable land (FAO, 2009). Hence, the establishment of an integrated management strategy for barley-FHB is essential to provide the required quantities of safe and high-quality barley. The barley-to-beer value-added chain comprises several production steps and the concerns of various stakeholders (figure 5). Therefore, the integrated management of FHB in malting barley has to go beyond the pure cultivation-oriented IPM as described in chapter 1.5.

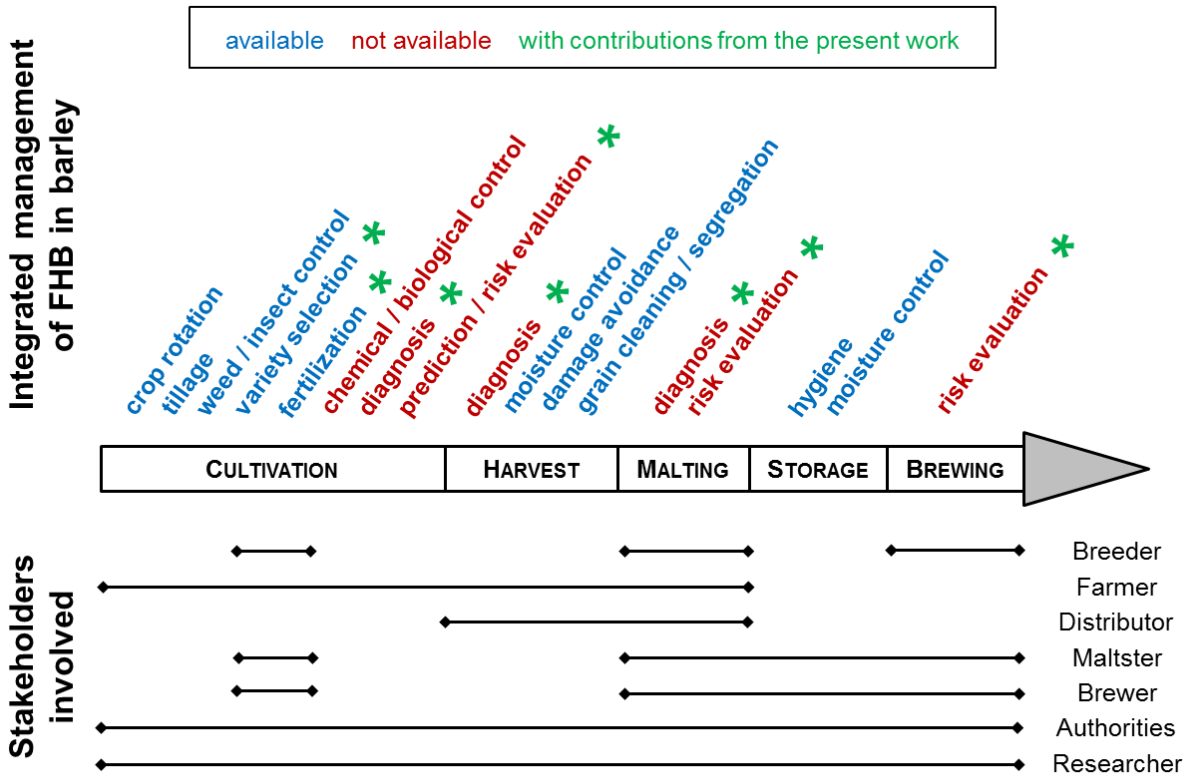

Figure 5. Modell for the integrated management of FHB in barley

A detailed explanation of this figure is given in the course of the discussion.

First and foremost, the present work provides further information on FHB in barley. This concerns agronomical control measures as well as diagnosis, prediction, and risk evaluation during production and processing (figure 5). Regarding control measures, nitrogen fertilization and the use of powdery-mildew-resistant genotypes were assessed for their FHB-affecting potential. *Fusarium* diagnosis was carried out optical, molecular, and bioanalytical techniques. Monitoring studies revealed the occurrence of the most relevant species within the German *Fusarium* complex on barley. Correlation analysis of *Fusarium* DNA contents in grain and weather as well as microclimate variables identified species-specific characteristics of epidemiology. The quantity-, safety-, and quality-related impacts of individual species were assessed in inoculation experiments. Possibilities for the implementation of own and literature results into an integrated management strategy for FHB in malting barley as well as associated obstacles are discussed. Over and above, findings are supposed to contribute steps towards an integrated management of FHB on barley (figure 5).

3.1 Agronomic control needs to be adjusted to barley-FHB

A broad range of agronomic tools is available to control FHB (see 1.4). Availability and effectiveness depend on several factors and need to be evaluated for individual crops. Measures for the control of wheat-FHB were extensively studied, but knowledge regarding the effectiveness of preventive and responsive practices towards barley-FHB appeared much lower. Under German law, chemical agents for the control of barley-FHB are not registered and biological control is not commercialized so far. Consequently, preventative methods were in the focus of attention (Papers II and III).

Reduction of inoculum sources

Managing debris of previous crops is one of the most effective control practices to reduce wheat-FHB (YI *et al*., 2001; PIRGOZLIEV *et al*., 2003). Soil-surface inoculation of barley with *Fusarium*-infected bruised grain material at the stage of stem elongation was shown to increase *Fusarium* DNA contents in harvested grain (Paper III). This shows that soil-borne inoculum can contribute to barley-FHB and suggests that prevention of pathogen-harboring crop residues is likely essential. Increasing proportions of non-hosts in crop rotations or at least omitting maize as pre crop together with conventional soil tillage by ploughing was shown to effectively limit FHB in wheat (see section 1.4.1). Adequate weed control further supports the reduction of inoculum sources (PARRY *et al*., 1995). The same measures are proposed to work for the control of barley-FHB.

Selection of varieties with low FHB susceptibility

Cultivation of moderately resistant wheat genotypes was beneficial for reducing FHB and associated contamination with DON (MESTERHÁZY *et al*., 2005; TÓTH *et al*., 2008). FHB resistance sources in barley are rare (RUDD *et al*., 2001; BAI AND SHANER, 2004) and at present no barley cultivars exhibiting specific host resistance are commercially available. For integrated management approaches, indirect resistance due to plant architecture, morphological or physiological traits might consequently be even more important to avoid FHB. Plant height was negatively correlated with grain infection by *F. culmorum* and *F. avenaceum* (Paper III) and heading date was shown to markedly influence infection with *F. culmorum* and *F. langsethiae* (Paper I). These factors were already suggested to

play a role in FHB-resistance as well as factors relating to spike characteristics (MESFIN *et al*., 2003; BAI AND SHANER, 2004; BUERSTMAYER *et al*., 2004, 2009; YOSHIDA *et al*., 2005; HORSLEY *et al*., 2006).

Powdery-mildew-resistant spring barley lines containing the *mlo5* resistance allele were suggested to exhibit enhanced susceptibility to cell-death inducing pathogens such as *F. graminearum* (JANSEN *et al*. 2005). Powdery mildew resistance conferred by *mlo* genes is widely distributed in commonly used spring barley varieties (BUNDESSORTENAMT, 2015). No evidence for enhanced susceptibility of powdery-mildew-resistant varieties to *F. graminearum* or other barley-FHB relevant species was found when comparing two spring barley varieties (Ingrid and Pallas) with their near-isogenic *mlo5* backcross lines under field conditions, varying in climate and pathogen pressure (Paper II). Consequently, present data gave no reason for excluding powdery-mildew-resistant spring barley cultivars from barley-FHB specific integrated management approaches.

Adaption of fertilizer regimes

The influence of nitrogen fertilization on wheat-FHB was extensively studied and due to conflicting results controversially discussed (see section 1.4.3). Enhanced nitrogen input was shown to extend anthesis of plants, to delay maturation (WEINERT AND WOLF, 1995), and to increase biomass (MUHAMMED *et al*., 2010). Associated prolonged opening of infection and colonization windows as well as creation of favorable microclimate were suggested to promote FHB in wheat (LEMMENS *et al*., 2004). The present study investigated the influence of nitrogen fertilization on barley grain infection with relevant FHB-pathogens, in particular *F. culmorum* and *F. avenaceum* (Paper III). An association of enhanced nitrogen application to FHB-supportive microclimate could be established. However, factors related to canopy structure (plant height, soil coverage) and plant physiology (nutrition status, defense potential) were considered to have a greater effect than microclimate (biomass). No significant nitrogen effect on barley-FHB was observed under conditions with natural infection, but enhancement of soil-borne pathogen pressure resulted in highest *Fusarium* contaminations in non- or low fertilized variants. Present results do not match with observations for wheat-FHB which are consequently not unreservedly transferable to barley cultivation. Nitrogen fertilization could be even taken into account for reducing barley-FHB.

Opportunities and obstacles for the implementation of FHB-reductive agronomic measures in an integrated management approach

The present study suggested several measures to be contributive or at least compatible for the management of barley-FHB in the field. Prior to implementation into an integrated management strategy, several unresolved issues need to be addressed. The effect of single methods might vary dependent on the conditions prevalent and therefore awaits further verification. The same is true for the effect of measure combinations. Their application is likely more efficient than usage of single methods, as shown for wheat-FHB (WEGULO *et al*., 2015), and necessary to comply with the concept of IPM (see chapter 1.5). Moreover, compatibility with current regional farming systems, control strategies for other diseases, and specific requirements to product quality need to be clarified. The following examples demonstrate possible obstacles due to interdependences within cultivation and due to conflicts of interest between stakeholders involved (see figure 5):

(i) Trends in the direction of intensified maize cultivation (DESTATIS, 2016; EUROSTAT, 2016) and conserved tillage systems (MAIORANO *et al*., 2008) are observed and thus conflict with the concept of reducing soil-borne FHB inoculum by ploughing.

(ii) The adaption of nitrogen fertilization could be beneficial for limiting FHB in barley, but might simultaneously promote other relevant diseases as shown for powdery mildew (LAST, 1962; JENSEN AND MUNK, 1997; *own unpublished data*).

(iii) Quality assurance of raw barley is of main interest in the production of malt, because it has decisive influence on the end product quality of beer. Therefore, several specifications are set for malting barley, encompassing purity and low protein content (FAO, 2009; JACOB, 2011). Accordingly, production of barley commodities is strongly influenced by product requirements, which are mostly contractually fixed between producers and processors (*personal communication* W. KÖNIG, managing director Braugersten-Gemeinschaft e.V.). Non-compliance of specification often results in commodity rejections or at least in price discounts for farmers or distributors. German breeders, maltsters, and brewers run great efforts to generate high quality malting barley. Selection factors are quality of barley, malt, wort, and beer. Agronomic properties are also in the focus and imply phenotypic parameters as well as yield factors and disease resistance, for instance against powdery mildew

(BRAUGERSTEN-GEMEINSCHAFT, 2008; BUNDESSORTENAMT, 2015). FHB resistance in barley cultivars is often linked to poor performance concerning agronomical features and malting quality (ZHU *et al*., 1999), which might lead to early rejection of promising lines in current breeding strategies. Prioritizing selection for FHB resistance in the early phases of breeding programs, as suggested by STEFFENSON AND SMITH (2006), is considered to be conducive for implementing host-resistance in the long run. In the short run, supply of information concerning susceptibility to FHB of currently available cultivars, could facilitate responsible action of producers. Farmers, if not anyway fixed to contractual provisions with distributors or processors, have so far no indications for FHB-limiting selection of barley varieties. Explicit investigation and subsequent transparent publication by breeders and authorities in variety lists or in seed guides, like it is conducted in Canada (LEGGE *et al*., 2004), is required to support integrated management of barley-FHB. Beside variety selection, nitrogen fertilization warrants critical reflection. Nitrogen fertilization in malting barley cultivation is restricted to comparably low levels as protein contents of grain must not exceed 11.5% (FAO, 2009; JACOB, 2011). But at the same time nitrogen restriction limits yield and might promote *Fusarium* infection, especially under conditions with high pathogen pressure (Paper III).

Looking at FHB and the barley-to-beer value-added chain, the aforementioned examples illustrated high complexity of implementing agronomical control measures in an integrated management strategy. Among all stakeholders involved (figure 5), farmers most obviously should prevent FHB, because negative effects of *Fusarium* infections can be hardly corrected post-harvest (POSTULKOVA *et al*., 2016). At the same time, farmers bear the greatest entrepreneurial risk, because achieving profitable yields appears as balancing act, complying with other crop management systems as well as with strict contract specifications, and simultaneously controlling barley-FHB. This issue would be elegantly mastered by breeding for FHB-resistant high-quality barley varieties.

3.2 Diagnosis of *Fusarium* in barley is complicated

Several direct and indirect techniques are available to identify diseases, including visual, serological, molecular, and biomarker-based methods (reviewed in SCHAAD *et al*., 2003; SANKARAN *et al*., 2010). Precise diagnosis of a pathogen is an essential element of IPM (see chapter 1.5). Accurate disease identification is indispensable for assessing pathogen occurrence, epidemiology, and impact. Furthermore, pathogen diagnosis is needed to verify compliance with defined damage thresholds and injury limits (VERREET *et al*., 2000; FLINT, 2012). The present work explored *Fusarium* appearance on barley pre-harvest by symptom detection (Paper I) as well as post-malting by symptom detection and mycological assessments (Paper V). *Fusarium* DNA was determined in immature spike material, dry grain and/or malt by qPCR (Papers I-V). Secondary metabolites were measured via LC-MS/MS (Paper I, III, and V).

Visual diagnosis

For the visual detection of FHB, symptom scoring is mandatory. Pre-screening of FHB-incidence in wheat on the field was shown to be a useful indicator for estimating impacts on yield parameters and for appraising risks of mycotoxin contamination (SNIJDERS AND PERKOWSKI, 1990; MIEDANER *et al*., 2004b; PAUL *et al*., 2005). Scoring of symptoms was also used to evaluate the *Fusarium*-reductive potential of agronomic measures (DILL-MACKY AND JONES, 2000; LEMMENS *et al*., 2004; LORI *et al*., 2009; CHRPOVÁ *et al*., 2011; BÉRUBÉ *et al*., 2012; YOSHIDA *et al*., 2012; D'ANGELO *et al*., 2014; WACHOWSKA AND GŁOWACKA, 2014). As manual determination of FHB in the field is time consuming, mobile remote sensing-based detection techniques have come to the fore in recent studies (DAMMER *et al*., 2011; BAURIEGEL AND HERPPICH, 2014). Initial FHB symptoms on wheat spikes appear as necrotic lesions on glumes of infected spikelets soon after infection (BUSHNELL *et al*., 2003). At advanced stages, characteristic 'head blight' symptoms can be observed in wheat. The fungus spreads in the rachis and clogs vascular bundles. Deficiency of water and nutrients results in premature ripening of the head, resulting in insuffiecient or incomplete filling of kernels (BUSHNELL *et al*., 2003; BAI AND SHANER, 2004). This distinct symptomatology permits pre-harvest detection of wheat-FHB.

Symptoms in barley appear as brown discolorations on individual spikelets and are perceived as unspecific and similar to those caused by other field pathogens

infecting the ear (GOSWAMI AND KISTLER, 2004). Inoculation experiments in the field were conducted to assess the symptom formation potential of individual *Fusarium* species (Paper I). In all three years of observation, symptoms were detectable even without spore application. Head necrotization was significantly increased after inoculation with spores of *F. culmorum* (3 of 3 years), *F. avenaceum* (2 of 3 years), *F. langsethiae* (1 of 2 years), and *F. sporotrichioides* (1 of 3 years). No markedly enhanced symptom formation followed application of *F. tricinctum* spores. CHRPOVÁ *et al*. (2011) mentioned difficulties in assessing barley according to symptoms, because scoring data for head necrotizations do not reliably correlate with mycotoxin contamination. Data of the present inoculation experiments could associate symptomatology and mycotoxin contents for the DON-producer *F. culmorum*, but not for the type A trichothecene producing species *F. sporotrichioides* (Paper I). Because four of five tested *Fusarium* species were shown to cause head necrotization and the low informative value regarding mycotoxin contamination, the visual pre-harvest detection has to be considered lacking specificity and sensitivity. Performance may be even lower when applied in conditions with natural infection.

Taking into account that FHB is caused by a complex of at least seven relevant *Fusarium* pathogens (Paper I), differing in epidemiology and damage potential (Papers I and IV), identification on the species level is relevant for integrated management purposes. The mycological assessment of fungal colonies grown on agar is a commonly used laboratory technique to visually detect seed-borne microorganisms on cereal grain (FLANNIGAN, 1970), however it is time-consuming as cultivation of fungal colonies takes up to 14 days. Using this method, the genus *Fusarium* was found to be relevant in contaminating barley and malt, with *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae*, *F. sporotrichioides*, and *F. poae* playing the most dominant roles (Papers I and V). Differentiation of fungal genera was often hampered by overgrowth of single colonies. This might be caused by differing requirements of single genera and species to prevalent environment conditions affecting fungal growth (BRENNAN *et al*., 2003). Fungi show mutual antagonistic effects concerning colony development (WHIPPS, 1987), resulting in suppression of single species. Consequently, there is a risk of over- or underestimation of the present infection with single fungal species (NICHOLSON *et al*., 2003). Determination of fungi at species level is moreover

constricted by similarities in macroscopic and microscopic characteristics (GERLACH AND NIRENBERG, 1983). Accurate classification requires therefore expert knowledge (DEMECKE *et al*., 2005).

Visual methods are also applied in post-malting *Fusarium* identification. The visual assessment of malt is a conventional procedure in the malting and brewing industry to appraise quality reductions, safety risks, and interferences with processing caused by *Fusarium* infection. For this purpose, the amount of discolored kernels in a batch of 200 g serves as indicator for *Fusarium* contamination (JACOB, 2011). Paper V evaluated this method and found a positive connection of color symptoms and *Fusarium* incidence, suggesting a certain informative value for this method. *Fusarium* contamination in the form of DNA and mycotoxins was, however, also detected in asymptomatic material. This indicates incomplete representation of *Fusarium* emergence by this method.

DNA-based diagnosis

In contrast to visual methods, molecular techniques show higher specificity and sensitivity, and enable assessment of fungal biomass (DEMECKE *et al*., 2005; NICOLAISEN *et al*. 2009). Specific primers and reaction conditions have been designed and evaluated to quantify DNA of individual *Fusarium* species (NIESSEN, 2007). In the present work (Papers I-V), quantitative PCR was carried out on the basis of NICOLAISEN *et al*. (2009). As reproducibility, specificity, and sensitivity of the method were confirmed by LINKMEYER (2012) and LINKMEYER *et al*. (2013) for the current lab, data outputs were considered as reliable. This technique requires high resources of time and money, because accurate sample preparation is essential for the quality of data outputs and consumables for PCR reactions are expensive, especially when used in high-throughput screenings.

Chemical diagnosis

Chemical methods determine *Fusarium* contamination in terms of secondary metabolites by using for instance LC-MS/MS (ASAM AND RHYCHLIK, 2006; HU *et al*., 2014; HABLER AND RYCHLIK, 2016). Latest research generated a highly specific and sensitive multi-mycotoxin stable isotope dilution LC-MS/MS method for the quantification of *Fusarium* toxins. Up to 14 secondary metabolites of *Fusarium* species, including associated derivatives such as 3-acetyldeoxynivalenol or modified

mycotoxins such as deoxynivalenol-3-glycosid (D3G), can be measured in parallel (HABLER AND RYCHLIK, 2016). In the present work, LC-MS/MS methods were used (Papers I and V; HABLER *et al*., 2016) and considered as optimal tools for appraising the risk potential of barley-FHB concerning food safety. These methods can also be used as diagnostic tool for assessing epidemiological aspects when applied to experiments with species-specific inoculation, in which fungal invaders are known (Papers I and III). However, the determination of secondary metabolites is not sufficient for concluding on a pathogen-specific invasion when used for practical purposes, because several *Fusarium* species share similar toxin profiles (see chapter 1.3, table 1) and toxin production is influenced by environmental factors. Although high mycotoxin specificity is given, quantification of secondary metabolites by LC-MS/MS lacks species specificity and thus has to be complemented by other methods as applied during this thesis (Papers I, III, V).

Opportunities and obstacles for the implementation of sufficient Fusarium diagnosis in an integrated management approach

The present study applied several methods to diagnose *Fusarium* infection on barley. For scientific purposes the combination of diagnostic methods is necessary and was successfully used in the present work to obtain more comprehensive and detailed knowledge of pathogen occurrence, epidemiology, and impact. The application of just a single method might either result in a lack of specificity and sensitivity or of feasibility with regard to time and costs. Both lead to absent practicality for IPM purposes (table 2). Consequently, new approaches are required to reliably identify *Fusarium* species on barley and malt in a fast and affordable way.

Table 2. Evaluation of applied methods for integrated management purposes

method	specificity	sensitivity	effort (time)	effort (costs)	practicality for IM
visual (field symptoms)	o	+	+	+	o
visual (malt symptoms)	o	+	+	+	o
classical mycology	++	++	+++	+	o
qPCR	+++	+++	++	+++	o
LC-MS/MS	++	+++	++	+++	o

Strength of method: o=absent, +=low, ++=intermediate, +++=high; Abbreviation: IM=integrated management.

Recent studies developed loop-mediated isothermal amplification (LAMP) assays to detect and quantify DNA of various *Fusarium* species on cereals (DENSCHLAG *et al*., 2012, 2013, 2014; NIESSEN *et al*., 2012). By LAMP the amplification of target sequences occurs at constant temperatures (isothermal) and therefore negates the use of a costly PCR-thermocycler. The use of a target-specific as well as loop primers and *Bacillus stearothermophilus* (*Bst*) DNA polymerase, provides fast and specific amplification of high concentrations of the desired target product (NOTOMI *et al*., 2000; NAGAMINE, 2002). Calcein flourescence enables indirect in-tube detection under daylight conditions with the naked eye (TOMITA *et al*., 2008). The technique is suggested to be simple, time-saving, robust, specific, sensitive as well as high throughput appropriate and therefore suitable for field and malt house application (NIESSEN AND VOGEL, 2010; DENSCHLAG *et al*., 2014). Hence, LAMP-detection might be considered as promising tool for the implementation in integrated management strategies. Connecting LAMP data outputs to actual *Fusarium* impacts warrants further research. If properly introduced, LAMP can be used as decision guidance for the compliance with damage thresholds and injury levels or as valuable support for the selection of further and more targeted diagnostics.

In this context *Fusarium* diagnosis is conceivable at different time points within the barley-to-beer chain (figure 5). The relevance of on-field diagnosis appears low, because subsequent options for action such as fungicide treatments are not available in German barley cultivation. Post-harvest detection of *Fusarium* could support decision-making whether barley commodities are sufficient for malt production or preferably intended for alternative usage. Post-malting diagnosis is considered as highly relevant, because it is the last option to prevent the brewing process from negative effects. The replacement of the currently applied visual assessment by molecular methods such as LAMP or more targeted tools such as mycotoxin measurements is suggested as expedient for the integrated management of barley-FHB.

3.3 Prediction requires knowledge of *Fusarium* occurrence and epidemiology

Numerous prediction models were developed to support the control of wheat-FHB as decision-making tools for fungicide applications or as forecasting for contaminations. These models differ in complexity regarding FHB-determinative factors taken into account, but inclusion of meteorological data is a common feature (PRANDINI *et al*., 2009). First studies to develop models for barley-FHB were carried out recently. BONDALAPATI *et al*. (2012) aimed to predict *F. graminearum* appearance and associated DON contamination in barley with a weather-based approach. Prediction systems for other barley-relevant *Fusarium* species are not available yet, most likely due to the perceived lower relevance of barley compared to wheat, but also because of the incomplete information about the prevalent *Fusarium* complex and the epidemiology of *Fusarium* species other than *F. graminearum*. The present work determined the occurrence of *Fusarium* species on German barley (Paper I) and malt (Paper V) and explored the influence of climatic factors on epidemiological aspects and grain infection (Papers I and III).

FHB on barley is caused by a broad range of Fusarium species

A monitoring approach of Paper I characterized the *Fusarium* complex of German winter and spring barley and found *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae*, *F. sporotrichioides*, and *F. poae* to be relevant on naturally infested grain. Comparable results were generated by NIELSEN *et al*. (2011), describing the Danish FHB complex on barley. These authors identified the same species and *Microdochium* spp. as important threads for barley cultivation. Both studies detected annual variation in complex composition, but ascertained *F. langsethiae* a dominant role. *F. graminearum* was also detected as dominating pathogen in German barley, in particular in winter cultivars. This species played a minor role in Denmark. Paper V assessed primarily German barley malt and found a comparable species complex, also differing between years. On malt, *F. avenaceum*, *F. graminearum*, and *F. tricinctum* were most present, indicating a shift in dominance structures from raw grain to malt. This was already suggested by PETTERS *et al*. (1988). Studies of HABLER *et al*. (2016), analyzing the fate of *Fusarium* DNA and toxins throughout malting of artificially infected barley, confirmed species-specific changes. DNA amounts of *F. culmorum* were enhanced in malt compared to

corresponding barley grain, whereas DNA of *F. sporotrichioides* and *F. avenaceum* was reduced or unaltered, respectively. Both Papers (I and V) found a considerable heterogeneous *Fusarium* complex on barley and malt. It differed in composition and dominance structure between years and material, likely caused by climatic conditions prevalent during barley cultivation and malt production.

Fusarium species differ in epidemiology

Climate factors were already shown to affect aspects of *Fusarium* epidemiology such as inoculum production and dispersal as well as spike infection and colonization (ROSSI *et al*., 2001; DOOHAN *et al*., 2003; OSBORNE AND STEIN, 2007; XU AND NICHOLSON, 2009). Previous research focused basically on the main causal agents of wheat-FHB such as *F. graminearum*. A more differentiating look at individual species and hosts however, revealed variances. In the present work, barley grain infection by individual *Fusarium* pathogens was associated with meteorological parameters and plant development. After spray-inoculation at anthesis under field conditions, grain of fertilized plants had higher content of *Fusarium* DNA than grain of unfertilized plants (Paper III). Lower temperatures and higher humidity in denser (fertilized) canopies were connected to increased *Fusarium* DNA contents. Soil-surface inoculation at the time period of stem elongation had the opposite effect. *Fusarium* infection was higher in grain of unfertilized plots (Paper III). Reduced soil surface coverage and plant height was suggested to promote rain-splash dispersion of soil-borne inoculum. In another experiment, a correlation analysis between weather data and natural *Fusarium* occurrence on spring and winter barley grain revealed markedly differences between individual *Fusarium* species (Paper I). The infection of spring and winter barley grain with *F. graminearum* was negatively associated with temperature, but positively with relative humidity, sum of precipitation, and the number of days with precipitation. By contrast, correlations with temperature were positive for the infection with *F. culmorum* and *F. langsethiae*, but negative for the number of days with precipitation. No significant associations of weather parameters and infection with *F. sporotrichioides* were detected. Plant development in terms of heading date was found be positively connected to infection with *F. culmorum* and *F. langsethiae*, but not to infections with *F. graminearum* and *F. sporotrichioides*.

Beside requirements to environmental conditions, pathogenicity and virulence of individual species is relevant for infection success and extent (XU AND NICHOLSON, 2009). In the present study application of *F. culmorum* and *F. avenaceum* inoculum on spring barley under field or greenhouse conditions resulted in high DNA contents, indicating strong grain colonization by those species. *F. tricinctum* and *F. sporotrichioides* DNA contents after artificial infection with similar amounts of inoculum ranged lower. Almost no or very low grain colonization was observed after inoculation with *F. langsethiae* (Papers I-IV). Previous studies already suggested species-specific aggressiveness. *F. graminearum* and *F. culmorum* are perceived as highly virulent, whereas *F. poae* and *F. langsethiae* are characterized as weak pathogens (BRENNAN *et al*., 2003; XU *et al*., 2007; DIVON *et al*., 2012). Surprisingly, *F. langsethiae* was shown to play the dominant role on naturally infested German spring barley (Paper I). This raises the question, whether the epidemiological features of this relatively recently discovered species differ fundamentally from those of other *Fusarium* pathogens. Important aspects of *F. langsethiae* epidemiology such as production and dispersal of infectious units, growth behavior, plant infection, and colonization have been addressed, but focused basically on oat as the most relevant host (EDWARDS, 2004; TORP AND ADLER, 2004; IMATHIU, 2008; IMATHIU *et al*., 2009, 2010; MEDINA AND MAGAN, 2010; ORLANDO *et al*., 2010; DIVON *et al*., 2012). A more barley specific picture awaits further study.

Opportunities and obstacles for the implementation of Fusarium prediction in an integrated management approach

Data of the present work could link *Fusarium* species occurrence to prevalence of weather conditions at the time period after heading (Paper I). These findings might support prediction of barley-FHB. Prediction models are key instruments in IPM (see chapter 1.5) and aid the control of wheat-FHB by deducing relevance and timing of fungicide treatments and by forecasting contamination levels (PRANDINI *et al*., 2009). Chemical control of FHB in barley is unavailable under German cultivation conditions, but prediction might be useful for the integrated management of barley-FHB by prognosticating post-harvest grain contamination. This could facilitate decision-making of farmers and distributers, whether grain is appropriate to go into the barley-to-beer chain and would allow pre-harvest estimation by maltsters and brewers on availability of processable barley

commodities. Pre-harvest prediction of *Fusarium* contaminations could moreover support the organization of appropriate harvest and storage conditions (figure 5). This encompasses for instance control of moisture, avoidance of mechanical grain damage, cleaning and segregation of grain as well as maintenance of general hygiene (EC, 2006b). Against the background that barley-FHB diagnostics is complicated (see chapter 3.2) and that the FHB complex of barley is relatively heterogenic (Papers I and V), prediction could enable indication for diagnostics on which species or secondary metabolite should be thoroughly tested post-harvest, pre-malting, and pre-brewing.

Prediction models might be highly beneficial for the integrated management of barley-FHB, but implementation involves considerable research effort. The present findings concerning climatic factors and pathogenicity need to be verified and connected to other FHB relevant risk factors such as inoculum density and host susceptibility. Furthermore, epidemiological aspects of relevant, but less studied, species such as *F. langsethiae* have to be fully elucidated prior to modelling.

Prediction models for wheat-FHB focus on the DON-producing species *F. graminearum* and *F. culmorum* (HOOKER *et al*., 2002; DE WOLF *et al*. 2003; ROSSI *et al*., 2003; DEL PONTE *et al*., 2005; KLEM *et al*., 2007; VÁŇOVÁ *et al*., 2009; VAN DER FELS-KLERX *et al*., 2010). The broad range of occurring *Fusarium* species and associated mycotoxin contaminations on barley require comprehensive prediction approaches, which respect the whole FHB complex. Constant monitoring is essential to designate relevant species for distinct regions, but also to recognize potential long-term shifts in complex compositions. In that manner, climate change could restrict heterogeneity together with biased cultivation methods. XU *et al*. (2008) detected a lower range of species on sites with higher temperatures. Climate change-dependent rise of temperature might therefore lead to dominance of individual species suppressing or replacing others. *F. graminearum* is already dominant in most regions of the world, most likely due to its broad adaption to climatic variability (OSBORNE AND STEIN, 2007). Recent studies of HOFGAARD *et al*. (2010) reported increasing prevalence of this species in Nordic regions of Europe. *F. graminearum* is characterized by high virulence compared to other species and was shown to adapt quickly to climate change (XU AND NICHOLSON, 2009). Further extended and intensified maize cultivation is additionally perceived as a driving force of FHB

caused by *F. graminearum* (PARIKKA *et al*., 2012). It is likely that *F. graminearum* will stay in the focus of attention in the following decades. Thus, the risk of underestimating the presence and hazard of other species in barley rises (SALAS *et al.*, 1999). Control of one distinct pathogen might additionally promote the occurrence of other species by creating ecological niches for less dominant species, which might become highly relevant in the future, at least in distinct years. Under German conditions, enhanced maize cultivation (DESTATIS, 2016) and climate change associated more frequent and intense rainfall events might promote infections by *F. graminearum*. Nevertheless, infection by *F. culmorum* and *F. langsethiae* was associated with higher temperatures (Paper I). These species might consequently also benefit from climate change and therefore require particular attention.

In order to manage FHB contamination in downstream sectors, producers, processors, researchers, and authorities are well-advised to develop and integrate adapted prediction models. This involves also subsequent risk evaluation.

3.4 Risk evaluation requires knowledge of *Fusarium* impact

The ubiquitous nature of *Fusarium* pathogens makes total control of FHB impossible (PIRGOZLIEV *et al*., 2003). Risk evaluation at different stages of the barley-to-beer chain is consequently mandatory for the integrated management of barley-FHB (figure 5) and requires knowledge of *Fusarium* impact. The thread of FHB was shown to be multifactorial (see chapter 1.3). With regard to wheat cultivation, yield reductions and contaminations with DON were in the majority of cases subjects of risk evaluation. Concerning spring barley, downgrading of malt and beer quality as well as degradation of process reliability have to be included. The present study investigated the impact of barley-FHB on yield quantity (Paper I) as well as on product safety (Paper I) and quality (Paper IV).

Impact on yield quantity

Reduction of yield quantity caused by FHB might be lower in barley than in wheat, most likely due to the different degree of systemic fungal growth in the ear. In barley, prevalent type II resistance inhibits *Fusarium* spread within the spike (BAI AND SHANER, 2004), whereas in wheat the infection of a single spikelet can induce mortification of the whole or at least big parts of the head (see section 1.2.3).

However, barley-FHB might cause economically detrimental effects in years or at locations with environmental conditions beneficial for *Fusarium* infections. Inoculation trials were considered as an appropriate approach to simulate high pathogen pressure. Paper I registered significant yield losses after spray-inoculating spring barley with spores of *F. culmorum* and *F. avenaceum*. In similar trials of SARLIN *et al*. (2005a) yield was significantly decreased by inoculation with *F. graminearum*. No such effects were observed for other species, including the spring barley relevant pathogen *F. langsethiae* (Paper I; SARLIN *et al*., 2005a). The applied spore solutions as well as the application techniques used are considered as hardly representative for commercial crop production. Firstly, barley grain might be infested with several (*Fusarium*) pathogens at the same time causing cumulative damage (Nielsen *et al*., 2014). Secondly, the all at once top-down application of high doses of spores is suggested to not sufficiently reflect the infection pathway of a soil-borne disease. Indeed, more natural inoculation methods such as soil surface application of infected grain for enhancing soil-borne pathogen pressure resulted in significantly enhanced *Fusarium* infection (DNA and mycotoxins), but did not markedly reduce yield (Paper III). Under commercial production conditions, the present study indicates a low impact of barley-FHB on yield quantity. To finally state more precise definitions, the cumulative effect of species-specific yield damage potential is required, but up to date not yet assessed (NIELSEN *et al*., 2014). Moreover, agronomical and environmental factors need to be incorporated into the valuation, because marked yield losses in distinct years or areas cannot be excluded.

Impact on product safety

Fusarium infection endangers product safety by contaminating cereals with mycotoxins. These secondary metabolites such as type A and B trichothecenes function in host-pathogen interaction (see section 1.2.4) as well as in pathogen-pathogen competition (MIEDANER *et al*., 2004a; XU AND NICHOLSON, 2009) and affect animal and human health (DESJARDIN, 2006; ANTONISSEN *et al*. 2014; PIERRON *et al*., 2016). As a result, maximum levels for DON and ZEA as well as recommended maximum levels for T-2/HT-2 in “unprocessed” cereals and products “intended for direct human consumption” were introduced by the European Commission (EC, 2006a, 2013). The limits were exceeded in samples of experiments with artificial infection (Paper I). Amounts of DON and T-2/HT-2 in naturally infested barley varied

between years, sites, and cultivars, but mostly ranged on a moderate level. Exceedance of maximum DON-levels occurred occasionally in winter barley (Paper I). Several studies indicated transmission of fungal DNA or mycotoxins from barley throughout the malting and brewing process into beer (SCHWARZ *et al*., 2006; HABLER *et al*., 2016). So far there are no mycotoxin limits for beer set by the EC, but the mathematically determined mycotoxin content according to EC-limits for 'unprocessed cereals' was not exceeded in commercially available beers (*unpublished results* K. HABLER, Chair of Analytical Food Chemistry, Technische Universität München). Therefore, no serious safety risks for human consumers could be concluded. On the basis of the current legislation and recommendation of the European Commission, results of the present study suggest a moderate impact of barley-FHB on consumer product safety.

Impact on product quality

Fusarium infection affects product quality in various ways. Germination capacity of grain is reduced (SCHWARZ *et al*., 2001; SARLIN *et al*., 2005a) and beer is contaminated with hydrophobic compounds (SARLIN *et al*., 2005b). Furthermore, *Fusarium* has the capability to change solution behavior of sugars and proteins in malt and consequently affect the quality of beer (SARLIN *et al*., 2005a; OLIVIERA *et al*., 2012, 2013). The underlying processes of altered enzyme activity are so far not well understood. A gene expression approach gave further insights into *Fusarium*-dependent regulation of genes related to malting quality (Paper IV). Regulation of genes was observed during plant development and throughout the malting process, in some cases independently from the fungal contamination level. This indicated that even low amounts of *Fusarium* infection have a high potential to affect expression of genes relevant for carbohydrate solubility characteristics of malt and hence to influence malt quality. Effective links between determined *Fusarium*-dependent gene expression and actual alteration of solubility characteristics of grain compounds during malting and brewing need to be clarified, but the present results indicate a potential high impact of barley-FHB on product quality.

Opportunities and obstacles for the implementation of risk evaluation in an integrated management approach

The results of the present work indicate a wide-ranging impact of barley-FHB. Yield quantity was marginally affected, but a moderate and potentially high impact on product safety and quality was determined. These insights might contribute to the development of injury levels and damage thresholds useful for the integrated management of barley-FHB. Both dimensions are known from cultivation-oriented IPM (see chapter 1.5) for suggestion of direct fungicide strategies for preventing yield losses. Yield reductions due to barley-FHB were found to be low (Paper I) and chemical control is not released for barley in Germany. However, injury levels in terms of product safety and quality could be applied in post-harvest risk evaluation and might facilitate subsequent decision-making of barley processors.

The maximal levels for *Fusarium* toxins set or recommended by the European Commission (EC, 2006a, 2013) might be perceived as injury levels for product safety, but require modification for the purpose of integrated barley-FHB management. Agricultural commodities and associated products can be contaminated by a number of secondary metabolites, because they are often infested by several fungi, most of them able to simultaneously form a range of mycotoxins (STREIT *et al*., 2012). The present study revealed a broad spectrum of relevant *Fusarium* species and other toxin producing fungal genera such as *Alternaria* spp., *Aspergillus* spp., and *Penicillium* spp. on naturally infested malt (Paper V). Examining different products and intermediates of the barley-to-beer value-added chain, several secondary metabolites of *Fusarium* species were detected (Paper V, LANCOVA *et al*., 2008b; HABLER *et al*., 2016). Detected compounds were DON-derivates and the modified DON-metabolite D3G. Metabolization during digestion to the original mycotoxin DON might endanger humans and animals (BERTHILLER *et al.*, 2011; NAGL *et al*., 2012). However, DON-derivates are so far not regulated by law and are generally not tested by default in practice or in monitoring programs. Existing injury levels as already defined by the EC may prevent from the immediate danger of the most relevant mycotoxins, but appear incomplete on closer inspection. The integration of maximum levels for cumulative toxin contents (e.g. *Fusarium* toxins, Alternariol, Aflatoxins, Ochratoxin) and the extension of already existing levels in terms of derivates and modified metabolites are considered as important for managing barley-FHB. Because

malt is neither “unprocessed”, nor “intended for direct human consumption” and the injury levels for beer are just mathematically determinable, EC-limits for trichothecenes appear difficult to apply in barley processing. Existing legislation has therefore to be completed with precise definitions. Relevant amounts of mycotoxins could be also found in spent grains (*unpublished results* K. HABLER, Chair of Analytical Food Chemistry, Technische Universität München), which accrue as by-product of beer production and are used for animal nutrition. Extension and more detailed defining might therefore be also necessary in legislation for animal fodder.

The brewing industry defined also injury levels to prevent from mycotoxin contaminations, in particular DON, and from problems in manufacturing (JACOB, 2011). Malt is visually assessed for the amount of red kernels prior to further processing and is based on the assumption that occurring symptomatology is linked to *Fusarium* infection. Exceedance of specified limits (usually 5-7 red kernels in 200 g of malt) is associated to an unjustifiable risk and often leads to commodity rejections or price reductions for farmers, distributers, or maltsters. The present study evaluated the value of the visual assessment (Paper V). Data from commercial samples suggested a generally low predictability of *Fusarium* contamination. However, under conditions with higher infection levels, significant correlations became apparent between the number of red kernels and fungal DNA. No such correlations were detected for mycotoxins. Specific malt samples with higher numbers of red kernels were particularly enriched with *F. avenaceum* and associated ENNs, but not with trichothecenes. Due to the present data, the visual assessment is considered to be overestimated regarding its reliability as an indicator for *Fusarium* contaminations. Against the background of lacking alternative methods the visual assessment of barley might still be helpful to assess the risk of fungal contamination, but reconsideration of defined injury levels is suggested to prevent barley and malt producers from unjustified penalties. At the same time the brewing industry need to establish more specific and reliable methods for safety and quality assurance in barley products.

3.5 Conclusion and outlook

The present work achieved several findings, which will contribute steps towards an integrated management strategy against FHB in barley. At the same time important gaps of required knowledge were indicated and challenges for implementation into management practice were pointed out.

The integrated management of barley-FHB is necessary to supply the need of high-quality malting barley. It is a way from raw barley grain to finished beer and a lot of stakeholders are involved. Consequently, the management of FHB in barley has to go beyond the cultivation-oriented IPM and needs to be extended to harvest, warehousing, and processing (figure 5).

Limitation of initial *Fusarium* infection in the field could be facilitated by reduction of inoculum sources and adaption of fertilizer regimes. On the long run, it might be most efficiently achieved by breeding for FHB-resistant or little susceptible high-quality malting barley varieties. So far methods for the management of *Fusarium* contaminations in barley processing sectors are lacking. The development of precise and easy-to-apply diagnostic tools as well as of reliable prediction models and references for risk evaluation is considered as most expedient for processors.

The integrated management of FHB in barley implies also compatibility with other cropping systems and distribution of risks among stakeholders in cases of failing control as well as distribution of responsibilities for the implementation and the further development of control strategies.

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5 APPENDIX

5.1 GEIßINGER AND HOFER *et al.* (Paper V)

Fusarium Species on Barley Malt – Visual Assessment as an Appropriate Tool for Detection?

Cajetan Geißinger[1]*, Katharina Hofer[2]*, Katharina Habler[3], Michael Heß[2], Ralph Hückelhoven[2], Michael Rychlik[3], Thomas Becker[1], Martina Gastl[1#]

[1] Chair of Brewing and Beverage Technology, Technical University of Munich (TUM), Weihenstephaner Steig 20, 85354 Freising, Germany

[2] Chair of Phytopathology, Technical University of Munich (TUM)
Emil-Ramann-Str. 2, 85354 Freising, Germany

[3] Chair of Analytical Food Chemistry, Technical University of Munich (TUM)
Alte Akademie 10, 85354 Freising, Germany

* These authors contributed equally to this work

[#] Corresponding author: Chair of Brewing and Beverage Technology, Technical University of Munich (TUM), Weihenstephaner Steig 20, 85354 Freising, Germany
Martina.Gastl@tum.de, Tel.: +49 8161 713266, Fax: +49 8161 713263

Keywords: visible assessment, *Fusarium* species, malt quality, food safety

19 **Abstract**

20 *Fusarium* infections in malting barley cause mycotoxin contamination, quality
21 degradation, and interfere with processing The visual assessment of barley malt
22 is a commonly applied, but critically viewed practice in the malting and brewing
23 industry to screen cereal batches for fungal infection, as it assumes a direct
24 connection between occurring symptomatology and actual fungal
25 contamination. The exceedance of a defined limit of red kernels (usually five to
26 seven) in a 200 g subsample of malt is associated with an unjustifiable risk for
27 further processing, and can lead to reductions in price or the rejection of the
28 entire batch. The present study evaluated the suitability of this method to
29 ensure product quality and safety. It was further intended to resolve the
30 presumed linkage between kernel discoloration and *Fusarium* infection. In
31 general, symptomatology showed low predictability for *Fusarium* contamination.
32 However, significant correlations became apparent between the number of
33 discolored kernels and fungal DNA contents under conditions of higher levels of
34 infection, although this was not the case for mycotoxin levels. Although
35 symptomatology is likely overestimated with regard to its reliability as an
36 indicator for *Fusarium* contaminations, it might still assist in the assessment of
37 risk of fungal contaminants.

38

Introduction

The range of food control and safety laws and regulations has been steadily increasing over recent decades. Strict regulatory enforcement has impacted all countries and food chain operators, including the beverage and brewing industry. Delivering a high quality product is of great importance to the beverage and brewing industry. Of the components required for brewing, the primary ingredient apart from water is malt. Barley (*Hordeum vulgare* L.) is the most widely used cereal for malting and brewing (Gupta et al. 2010; Ullrich 2011). In cereal processing, quality characteristics can be classified into three groups: food safety, processing quality, and product quality. For barley crop and malt, food safety primarily refers to the level of mycotoxins. The processing quality of a top malt is regarded as good amylolytic as well as balanced cytolytic and proteolytic properties. Product quality is further assessed via visible fungal contaminations or kernel deformities (symptoms).

A common cause of poor quality crop/malt is microbial contamination, in particular by *Fusarium* (*F.*) species (spp.). Barley is naturally infected by microorganisms, including several genera of filamentous field and storage fungi (Flannigan 1969; 1970). The infection of barley with *Fusarium* spp. can result in Fusarium head blight (FHB), which is regarded as a severe plant disease and presents a challenge for cultivating small grain cereals globally (Bai and Shaner 2004; Jansen et al. 2005; McMullen et al. 1997; Parry et al. 1995; Trail 2009; Wang et al. 2006; Yang et al. 2011). FHB is a soil-borne disease persisting on harvest residues over winter. Among the field fungi, infection by *Fusarium* spp. is perceived as a severe threat to subsequent production processes of cereals. FHB is caused by a pathogen complex comprising up to 17 species, with *F. graminearum*, *F. culmorum*, and *F. avenaceum* being predominant. Fungal spores are mainly distributed by wind or rain-splash during the vegetation period and infect cereal spikes during the flowering stage (Parry et al. 1995). In wheat production, FHB can be considered as possible yield limiting factor (Dubin et al. 1997; McMullen et al. 1997). Yield losses due to FHB have also been reported in barley, but are not considered as significant as the resulting quality deterioration (Linkmeyer et al. 2016).

Fusarium spp. are able to form a variety of secondary metabolites (Desjardins

2006). *F. graminearum* and *F. culmorum* are known to produce deoxynivalenol (DON) and nivalenol (NIV), both type B trichothecenes, as well as zearalenone (ZEA), whereas *F. sporotrichioides* and *F. langsethiae* form type A trichothecenes (T2- and HT2-toxin). *F. poae* is known to produce type A trichothecenes (diacetoxyscirpenol (DAS)) and type B trichothecenes (NIV). The non-trichothecene producing species *F. tricinctum* and *F. avenaceum* metabolize moniliformine (MON) and enniatins (ENNs). Moreover, *F. avenaceum* forms beauvericin (BEA), a toxic cyclopeptide. Due to the negative health effects, the European Commission has set maximum DON and ZEA levels for unprocessed cereals of 1,250 and 100 µg/kg, respectively (European Commission 2006). To date, there are no established maximum levels for the other mycotoxins found in barley and malt (European Commission 2006). The content of mycotoxins can increase during malting (Habler et al. 2016; Hu et al. 2014; Lancova 2008; Schwarz 1995; Vaclavikova et al. 2013), thereby presenting a challenge to the brewing industry, not only to remain in compliance with food safety regulations but also malt quality specifications. Some mycotoxins persist in the malting and brewing process or are modified during the procedure, and are found in finished beer (Habler et al. 2016; Habler and Rychlik 2016). However, the amount of soluble mycotoxins typically found in beer is considerably below regulations (Varga et al. 2013).

Oliveira et al. (2012) and Sarlin et al. (2005) concluded that depending on the barley variety, the crop year, and the *Fusarium* species, the germinative capacity and energy of infected barley samples were significantly lowered by *Fusarium* infection. The distribution of the pathogen complex changes throughout the malting process; however, the type of fungal contamination in malt is similar to that in barley (Petters et al. 1988). The negative effects of *Fusarium* infections on malt and beer quality have been intensively studied (Oliveira et al. 2012; Sarlin et al. 2005). The proteolytic properties of malt are particularly enhanced in *Fusarium* infected barley (Oliveira et al. 2013). The expression of amylolytic and cytolytic genes can also increase when malting infected barley (Hofer et al. 2016). Malt infected with *Fusarium* tends to deliver a higher free amino nitrogen (FAN) and soluble nitrogen content as well as a darker wort color (Sarlin et al. 2005). *Fusarium* also causes the β-glucan and viscosity levels to decrease and hinders activities of α-amylase, β-amylase, and

106 β-glucanase (Oliveira et al. 2013; Sarlin et al. 2005). Enhanced amylolysis,
107 cytolysis, and especially proteolysis can lead to processing difficulties while
108 brewing.

109 The risks and quality problems associated with *Fusarium* infections present a
110 continual challenge to the malting and brewing industry. Consequently,
111 appropriate tools are required to detect *Fusarium* contamination in barley malt.
112 To this end, several analytical methods are available. Currently, the most
113 commonly used method is visual assessment. Malt symptoms such as reddish
114 and black discolorations are directly linked to *Fusarium* infections. However, the
115 validity and reliability of the visual assessment method for determining *Fusarium*
116 contamination in malt is not scientifically proven. The results are mostly
117 associated with the occurrence of mycotoxins or gushing quality aspects;
118 however, processability tends to be neglected. Malt batches not meeting visual
119 quality standards are rejected or lowered in price.

120 Visual assessment is a rapid and low-cost method used in practice to screen
121 cereal batches for fungal infection. The method consists of counting the
122 discolored kernels in 200 g of a malt subsample. The collected data provide an
123 indication of fungal stocking in brewing malt and help appraise the associated
124 risk potential. Initially, this method was used to evaluate wheat malt batches.
125 Niessen (1991) established that five to seven red kernels in a 200 g subsample
126 enhance the risk of gushing in beers brewed with infected wheat malt. The
127 method was later used to evaluate barley malt without adapting it to barley.
128 Moreover, no thorough evaluation of the validity and reliability of the method to
129 evaluate barley commodities has been performed. In most malt trading
130 contracts, the number of red kernels in a batch is a critical aspect; however,
131 black kernel discoloration is often ignored. The MEBAK (Mitteleuropäische
132 Brautechnische Analysenkommission) recommends that malt batches with a
133 high amount of discolored kernels should be further analyzed for *Fusarium*
134 infection and mycotoxins (Anger 2006).

135 Reddish, black, or black pointed discoloration is regarded as a symptom of
136 fungal infection (primarily *Alternaria* and *Fusarium* spp.). However, not all fungal
137 species are likely to discolor the kernels in the same way (Hudec 2007; Tangni
138 and Larondelle 2002). In the visual assessment guidelines, only red kernels are

considered to have quality lowering effects (e.g., enhanced color, free amino nitrogen (FAN), soluble nitrogen). Rath (2009) found that red kernels can be highly infected with *Fusarium* species; in this study, the modified Carlsberg test was used to conduct gushing experiments. The data showed that adding of five red kernels to gushing-negative reference malt leads to positive gushing test results. However, gushing was also induced by symptomless malt batches. Based on these results, data collected by visual assessment appears to be insufficient for preventing undesirable effects caused by *Fusarium* spp. occurring in beer. Beside mycotoxins, *Fusarium* spp. are known to lead to the accumulation of other undesirable substances such as hydrophobins and saturated fatty acids. These substances also have the potential to induce gushing in bottled beer (Christian et al. 2011; Postulkova et al. 2016; Shokribousjein et al. 2011).

The present study aims to determine if information gathered through visual assessment is sufficient to ensure product quality and safety. Furthermore, it is intended to determine if kernel discoloration is directly associated with *Fusarium* infections. In the present study, 243 samples, produced from commercially cultivated barley, were visually assessed and examined for quality. A subset was further mycologically (agar plate method) and molecularly (quantitative polymerase chain reaction (qPCR) and liquid chromatography tandem mass spectrometry (LC-MS/MS)) analyzed for the degree of *Fusarium* infection. Subsequently, specific colored (discolored, red, black) malt samples were created and analyzed for *Fusarium* DNA as well as associated toxins and compared to asymptomatic malt samples. Additionally, serial dilution experiments were conducted to evaluate the validity of the visual assessment method. A defined number of *Fusarium*-infected kernels were added to potentially pathogen free malt. DNA and toxin levels in the produced malt series were quantified by qPCR and LC-MS/MS, respectively. Different analytical methods were used in the present study to collect accurate information, explore possibilities, and conclusively determine the validity and reliability of visual malt assessment for the malting and brewing industry. Finally, the applicability of the methodology to accurately evaluate the quality of barley and barley malt samples is discussed.

Materials and methods

Grain material

A total of 243 commonly grown (i.e., grown under practical cultivation conditions) barley samples were collected from harvests during 2012 (*n* = 82), 2013 (*n* = 77), and 2014 (*n* = 84). The samples derived from 91 different varieties (winter barley (*n* = 27); spring barley (*n* = 216)) and six European countries ((Austria (*n* = 1), Poland (*n* = 2), Sweden (*n* = 2), Denmark (*n* = 17), Switzerland (*n* = 19), and Germany (*n* = 202)).

Additional grain material was produced for serial dilution experiments. Potentially pathogen-free barley grain material as well as artificially species-specific infected material was generated under greenhouse conditions.

Quality assessment of unmalted barley

To assess the unmalted barley samples, standard analyses were performed as described in the approved standard MEBAK methods (Anger 2006). All analyses were conducted in triplicate. Moisture content of the barley samples was determined following MEBAK method 1.5.1.1(Anger 2006). Raw protein was quantified using a Foss Ticator™ digestion system and a Kjeltec™ 8460 System (Foss GmbH, Hillerød, Denmark) as described in MEBAK method 1.5.2.1 (Anger 2006). The germinative energy of the samples was assessed based on the Schönfeld test as described in MEBAK method 1.4.2.2 (Anger 2006). According to MEBAK standards, the germinative energy should not be below 95% (Anger 2006). The water sensitivity of the barley samples was also tested (MEBAK method 1.4.3 (Anger 2006)). The sprouting of barley was optically inspected following MEBAK method 1.4.5.1 (Anger 2006); the barley kernels were first treated with a 20% $CuSO_4$ solution before counting the kernels which showed acrospire growth. A Sortimat (Pfeuffer, Kitzingen, Germany) with three sieve inserts (2.2, 2.5, and 2.8 mm) was used to determine the kernel size distribution of the samples, as described in MEBAK method 1.3.1 (Anger 2006).

Malting procedure

All malting trials were performed at the pilot scale plant located at the Institute of Brewing and Beverage Technology, Technische Universität München,

Germany. A standard malting regime, as described in MEBAK method 1.5.3 (Anger 2006), was conducted to malt all barley samples (*n* = 243). The samples were processed in thermostatically controlled chambers at a relative humidity of 95-98%. The degree of steeping was set to 45% during the first three days of the malting process (i.e., the steeping phase) and was maintained at 45% throughout germination. The barley samples were left to germinate for an additional three days at 14.5 ± 0.5°C. All samples were turned twice daily during the remaining germination period. The initial withering ('*Schwelke*') temperature was set to 50°C for 16 h followed by 1 h at 60°C and 1 h at 70°C. The kilning temperature was adjusted to 80°C and maintained for 5 h. After kilning, rootlets were removed and malt samples were stored in a dry and dark location until further processing.

Malt standard analyses

To characterize the produced malts, standard malt analyses were performed according to the approved standard MEBAK methods (Anger 2006). All analyses were conducted in triplicate. Moisture content in malt was determined as described above. To assess the amylolytic properties of the samples, malt extract, α-amylase activity, gelatinization temperature, and final attenuation were measured. The malt extract was measured using an Anton Paar Alcolyzer DMA 4500 (Anton Paar, Graz, Austria) following MEBAK method 3.1.4.2.2 (Anger 2006). A Ceralpha α-amylase assay kit (Megazyme, Wicklow, Ireland) was used to measure the α-amylase activity (Anger 2006; McCleary 2002). The gelatinization temperature was measured with a rapid visco analyzer (RVA) Super 4 (Newport Scientific Pty. Ltd., Warriewood, Australia) as described in MEBAK method 2.7 (Anger 2006). The final attenuation (i.e., fermentation potential indicator) was determined according to MEBAK method 3.1.4.10.1.3 (Anger 2006).

The cytolytic properties—viscosity, friability, steelinees, and β-glucan—were also determined. Viscosity (congress mashing) was measured using a falling ball viscometer, AMVn-Automated Micro Viscometer (Anton Paar, Graz, Austria) as described in MEBAK method 3.1.4.4.2 (Anger 2006). The friability and steeliness of the malt kernels were measured using a friabilimeter (Pfeuffer Gmbh, Kitzingen, Germany) following MEBAK method 3.1.3.6 (Anger 2006).

MEBAK method 3.1.4.9.2 (Anger 2006) was modified to a high-throughput approach to quantify the β-glucan content in the malt samples. In this assay, a BioTek Synergy H4 Hybrid microplate reader (BioTek, Winoosky, USA) was used to record the fluorescence.

Proteolytic activity of the malted samples was also assessed. The Kolbach index (MEBAK method 3.1.4.5.3 (Anger 2006)) was calculated from the measured soluble nitrogen and raw protein values obtained using a Foss Ticator™ digestion system and a Kjeltec™ 8460 System (Foss GmbH, Hillerød, Denmark) following MEBAK methods 3.1.4.5.2 (Anger 2006) and 1.5.2.1 (Anger 2006), respectively. The FAN content was quantified using a BioTek Synergy H4 Hybrid microplate reader (BioTek, Winoosky, USA) as described in MEBAK method 3.1.4.5.5.1 (Anger 2006). In addition, dimethyl sulfide precursor (DMSP) of all samples was measured according to MEBAK method 3.1.4.17 (Anger 2006) using gas chromatography (GC) coupled with a flame photometric detector (FPD) (Hewlett Packard, Wilmington, USA). To obtain reproducible results, all samples from the same harvest year were analyzed on the same day.

The symptomatic status of all barley malt samples (n = 243) was visually evaluated. To ensure the representativeness of the samples and the reproducibility of the analysis, a Retsch PT100 sample divider (Retsch GmbH, Haan, Germany) was used to evenly divide the malt samples into 200 g batches. Each aliquot was then spread out and the discolored kernels (number of red (NR), Fig. 1A; number of black (NB), Fig. 1B) were removed and counted. The visual assessment of each sample was performed in duplicate.

Determination of *Fusarium* contamination

Of the total sample pool (n = 243), representative samples (2012 (n = 20), 2013 (n = 19), and 2014 (n = 20)) were chosen. The selection criterion was kernel symptomatology. The inclusion of the maximum representativeness and heterogeneity (from low to high numbers of symptomatic kernels) for each crop year was attempted. In the selected malt samples (n = 59), the mycological status was recorded by the agar plate method. *Fusarium* species-specific DNA content was quantified via qPCR. Of the 59 samples, ten per harvest year (n = 30) were further analyzed for specific mycotoxin contents using LC-MS/MS.

To clarify the relationship between kernel symptomatology and DNA content, discolored kernels and asymptomatic kernels (i.e., control) were hand-selected from the commercially produced samples of 2014. The DNA contents of the six most common *Fusarium* spp. in Europe were measured in red, black, and asymptomatic samples as well as the content of associated mycotoxins. Discolored samples were compared to asymptomatic samples derived from the same batch of malt. From the collected data, it was possible to calculate a factor for each *Fusarium* species and mycotoxin, respectively. The factors were determined as the ratio of the amount of DNA or mycotoxin in red or black samples and the corresponding amounts in asymptomatic samples (i.e., red/asymptomatic or black/asymptomatic). The larger the factor, the higher the enrichment with the particular *Fusarium* spp. DNA or mycotoxin in association with kernel discoloration.

Serial dilution experiments were conducted in triplicate. Greenhouse cultivated and therefore potentially pathogen-free barley was malted. In the dilution series, zero, five, ten, twenty, or forty artificially *Fusarium*-infected malt kernels were mixed with the pathogen-free malt to a final weight of 200 g. For each dilution step, the amount of genomic DNA and associated secondary metabolites were quantified.

Mycological assessment

Under sterile conditions, a representative number of kernels (approximately 5 g) was surface sterilized with 1% NaOCl solution rinsed with autoclaved deionized water, and subsequently dried on filter sheets. Of each sample, 80 kernels were placed on synthetic nutrient-poor agar (SNA). The agar plates were incubated for 14 days at 16°C with a 12h/12h darkness/ultraviolet (UV)-light rhythm. Upon incubation, fungal species were identified based on macro- and microscopic properties (e.g., color of colony, amount of mycelium, or shape of the spores) (Nelson et al. 1983).

Quantification of *Fusarium* DNA via qPCR

Isolation of genomic DNA from malt was performed according to a protocol published in Linkmeyer et al. (2013). In brief, 2 g milled barley malt was lysed and DNA was precipitated. Chloroform/isoamylalcohol was used to remove proteins and polysaccharides. DNA pellets were washed with ethanol and

dissolved in double-distilled water to a final concentration of 20 ng/µL. Quantification of species-specific *Fusarium* DNA in malt was based on the protocol of Nicolaisen et al. (2009) reproduced by Linkmeyer et al. (2013). PCR amplification was conducted in an MX3000P Cycler (Stratagene, Santa Clara, CA). Specific primers for individual *Fusarium* spp. (FgramB379fwd/FgramB411rev, FculC561fwd/FculC614rev, FspoA18fwd/FspoA85rev, FlangA29fwd/FlangA95rev, Fave574fwd/Fave627rev, and Ftri573fwd/Ftri630rev) were used. An assay for measuring barley DNA (Hor1f/Hor2r) was included for normalization. To perform absolute quantification of barley and *Fusarium* DNA, an external standard calibration was conducted. Therefore, dilution series (100, 10, 1, 0.1, and 0.01 ng of DNA) of pure fungal and barley DNA were included in the assays. *Fusarium* DNA amounts in samples were determined in duplicate and normalized to barley DNA.

Mycotoxin analysis via LC-MS/MS

Several *Fusarium* mycotoxins (deoxynivalenol; deoxynivalenol-3-glucoside (DON-3-G); 3-acetyldeoxynivalenol (3-AC-DON); 15-acetyldeoxynivalenol (15-AC-DON); ZEA, nivalenol; T2-toxin; HT2-toxin; ENNs B, B1, A, and A1; BEA) were quantified in barley malt. Sample preparation was performed as recently published by Habler and Rychlik (2016). A volume of 10 mL acetonitrile/water (84/16, v/v) was added to 1 g milled barley malt. The mixture was shaken and the internal standards (80 µL of $[^{15}N]_3$-ENN B and 80 µL of $[^{15}N]_3$-ENN B1, (0.1 µg/mL)) were added to 4 mL of supernatant. After vortexing, the mixture was completely applied to a Bond Elut Mycotoxin cartridge (Agilent Technologies, Santa Clara, CA, USA), and the liquid was passed through the cartridge by vacuum suction and evaporated until dry. The sample was resuspended with 200 µL acetonitrile/water (1/1, v/v) and membrane filtered (0.45 µm), after which LC-MS/MS analysis was performed (Habler and Rychlik 2016). LC-MS/MS was performed on a Shimadzu LC-20A Prominence system (Shimadzu, Kyoto, Japan) using a Hydrosphere RP-C_{18} column (150 × 3.0 mm^2, S-3 µm, 12 nm, YMC Europe GmbH, Dinslaken, Germany) with a C_{18}-guard column (Phenomenex, Aschaffenburg, Germany) as a stationary phase maintained at 40°C. The injection volume of each sample was 10 µL. The binary gradient system consisted of (A) 0.1% formic acid and (B) methanol with 0.1% formic acid at a flow rate of 0.2 mL/min. The gradient was initiated and

maintained at 10% B for 2 min, raised linearly from 10 to 87% B during the next 6 min, held at 87% for 7 min, raised to 100% B during the next 5 min, and then maintained at 100% B for 3.5 min. Next, the mobile phase returned to 10% B within 2 min and the system was equilibrated for 9.5 min before the next run. The LC was coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems Inc., Foster City, CA, USA). The ion source parameters were set as follows: curtain gas 20 psi, CAD gas pressure high, ion spray voltage 4,500 eV, spray gas 80 psi, dry gas 75 psi, and temperature 450°C. All samples were analyzed in quadruplicate.

Data analysis

Statistical analysis was conducted using SPSS Statistics 22 (IBM Corporation, New York, USA). Correlation analyses were performed by computing Pearson correlation coefficients. Statistically significant correlations are indicated by *($P \leq 0.05$) and **($P \leq 0.01$). Comparison of mean values was conducted using analysis of variance (ANOVA) including a Tukey-B test ($P \leq 0.05$).

Results

Barley and malt standard analyses

A total of 243 commonly grown barley samples were collected from the harvests of 2012, 2013, and 2014. To characterize the samples, standard analyses of barley and barley malt were performed. The detailed quantitative data for the average barley and the corresponding barley malts of each year of harvest are summarized in Supplementary Table 1. The collected data confirmed that the sample pool is highly heterogeneous and revealed year-dependent distribution patterns.

Visual assessment of barley malt

The symptomatic status of all barley malt samples was visually inspected. The distribution of the identified NR and NB for individual samples from all three years is shown in Figure 2. The maximum numbers of red kernels in samples of 2012, 2013, and 2014 were 85, 18, and 22, respectively. The mean numbers of the discolored kernels present in each crop were calculated (Supplementary

Table 1). The highest mean number of red kernels was found in the 2012 samples (NR = 10). On average, three and four red kernels were counted in the crops of 2013 and 2014, respectively. For 2012, the average NB was higher than the NR. A maximum of 53 and 54 black kernels were counted in the samples from 2012 and 2013, respectively. A maximum of 19 black kernels was counted in the crop of 2014. On average, six black kernels were found in samples from 2012 and 2013, respectively, and only one in the samples from 2014.

Relationship between kernel discoloration and malt quality attributes

In the present study, numerous quality parameters of barley and barley malt samples were screened. To identify and measure the associations between symptomatology (i.e., NR or NB) and quality attributes, correlation analysis was used. The statistically relevant correlation coefficients are shown in Table 1 and Table 2. From the computed data, it may be concluded that not all quality parameters were influenced to the same degree, and year-dependent effects were observed. The strongest relationship was evident between NR and NB. The correlation coefficients ranged between $r = 0.23^*$ and $r = 0.70^{**}$. The degree of association between NR and other quality attributes was weak, with scattered coefficients ranging between $r = 0.14^*$ and $r = 0.47^{**}$. More associations between NR and malt quality parameters were identified than for NB. The relationships between NB and the malt quality attributes were generally weaker than those of NR.

Characterization of the *Fusarium* complex in malt

For further characterization of the malt samples, a highly diverse sample set was created based on the collected NR and NB data. Of the total sample pool ($n = 243$), 59 representative malt samples from the 2012 ($n = 20$), 2013 ($n = 19$), and 2014 ($n = 20$) harvests were mycologically assessed (agar plate method) for fungal contamination. The mycological analysis of the naturally infected, surface sterilized barley malt samples revealed fungal species of several genera (Figure 3). Filamentous fungi grew out of surface-sterilized malt kernels in almost all cases. Fungal spp. or genera were recorded and their relative frequency of occurrence calculated. Species belonging to genera *Alternaria*, *Aspergillus*, *Fusarium*, *Epicoccum*, *Drechslera*, *Mucor*, and

401 *Microdochium* were predominantly detected. Other seed-borne pathogens were
402 also identified, including *Acremonium* spp., *Penicillium* spp., *Ascochyta* spp.,
403 *Botrytis* spp., *Ulocladium* spp., and *Rhynchosporium* spp. Following *Alternaria*
404 (35%) and Aspergillus spp. (15%), *Fusarium* represented the third-most
405 frequent genus in barley malt (13%), suggesting an important role of
406 *Fusarium* spp. as barley contaminants.

407 The *Fusarium* complex comprises a large percentage of the seed-borne
408 mycobiota in barley. The incidence of *F. crookwellense*, *F. equiseti*, *F. poae*,
409 and *F. acuminatum* on barley malt was low. Conversely, *F. culmorum*,
410 *F. graminearum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae*, and
411 *F. sporotrichioides* were the most predominant *Fusarium* spp. in the examined
412 sample set. Species-specific qPCR of the most commonly found *Fusarium*
413 species was conducted to quantify the amount of genomic DNA present. The
414 obtained results revealed that the cumulative total DNA content of
415 *Fusarium* spp. was highest in the samples from 2012 (30.05 pg/ng barley DNA).
416 The DNA concentrations were lower in 2013 and 2014 (1.56 and 5.81 pg/ng
417 barley DNA, respectively). The distribution of the *Fusarium* DNA complex is
418 depicted in Figure 4. Independent of the degree of infestation, *F. avenaceum*,
419 *F. graminearum*, and *F. tricinctum* were the dominant *Fusarium* species in every
420 year of observation.

421 **Relationship between kernel discoloration and *Fusarium* DNA**
422 **concentration**

423 Statistical analyses were performed to elucidate potential relationships between
424 kernel symptoms and the molecular data of barley malt. For each harvest, the
425 visual assessment data (NR and NB) of the barley malt samples (n = 59) were
426 correlated to the corresponding measured contents of *Fusarium* DNA from the
427 six predominant species (Table 3). The computed correlation coefficients were
428 low in most of the tested relationships. The Pearson correlation analysis of the
429 red kernels and the six *Fusarium* spp. revealed only positive associations when
430 significant. The strongest positive correlation was between NR in the malt of
431 2012 samples and DNA of *F. avenaceum* (r = 0.87**). Moderate correlations
432 were computed for DNA of *F. culmorum* and *F. sporotrichioides* (r = 0.56*) for
433 2012. In 2013, correlations between NR and fungal DNA contents were not

significant. In 2014, three moderate associations were calculated for the DNA concentration of *F. culmorum*, *F. avenaceum*; and *F. tricinctum* and the NR, with Pearson correlation coefficients of r = 0.49*, r = 0.56*, and r = 0.53, respectively. The calculated correlation coefficients between the NB and the *Fusarium* DNA showed no clear trend. In 2012, NB correlated significantly with DNA content of *F. sporotrichioides* (r = 0.85**), *F. culmorum* (r = 0.79*), and *F. avenaceum* (r = 0.60**). Only a moderate degree of association was identified in the 2013 harvest between NB and *F. tricinctum* (r = 0.48*). No relevant correlations were calculated in the 2014 samples. In most cases the correlations of kernel discoloration with DNA content of *Fusarium* spp. showed little reproducibility over individual years of sampling.

In Figure 5, the associations of NR in samples from 2012, 2013, and 2014 with the DNA concentrations of *F. avenaceum* (Figure 5A) and *F. culmorum* (Figure 5B) are shown. The associations of NR and DNA contents of other relevant *Fusarium* species are presented in Supplementary Figure 1. Scatter plots reveal high heterogeneity in the distribution of the correlation of NR and *Fusarium* DNA. Within each *Fusarium* species, high variability between the three years was observed. A particularly high coefficient of determination ($R^2 = 0.76$) was observed only for samples from 2012 for the correlation of NR and DNA of *F. avenaceum*.

Enriched *Fusarium* spp. in discolored kernels

Color-specific samples were selected from the analyzed malt samples from 2014 ($n = 84$). These were molecularly analyzed and compared to the symptomless control samples. From the collected data, it was intended to disclose the role of the relevant *Fusarium* pathogens in kernel discoloration. Moreover, this should possibly validate the potential associations between symptomatology (NR or NB) and *Fusarium* DNA concentration. The computed factors for the samples from 2014 are shown in Table 4. The factors were calculated as the ratio of the amount of DNA in red or black kernels and that in asymptomatic kernels. The highest factors in the red samples were identified for *F. tricinctum* (×268) and *F. avenaceum* (×143). The calculated factors for the four other *Fusarium* species ranged lower. The lowest factor was computed for *F. langsethiae* (×5) followed by *F. graminearum* (×15) and *F. culmorum* (×17). A

467 moderate factor was determined for *F. sporotrichioides* (×69). The calculated
468 factors in the black kernel samples were generally lower. The *Fusarium* species
469 can be classified into three groups: 1) *F. graminearum* and *F. langsethiae* had
470 high factors of ×37 and ×31, respectively; 2) moderate factors were calculated
471 for *F. tricinctum* (×18) and *F. avenaceum* (×16); 3) a low factor of ×6 was
472 computed for both, *F. culmorum* and *F. sporotrichioides*.

473 The comparison of discolored and control samples revealed high DNA
474 concentrations of all tested *Fusarium* species in the discolored samples. In the
475 red kernel samples, *F. avenaceum* and *F. tricinctum* were found in very high
476 concentrations. However, *Fusarium* DNA was also detected in non-discolored
477 control samples. The data show possible *Fusarium* infection in asymptomatic
478 samples and support high DNA content of *Fusarium* spp. in discolored kernels.

479 **Validity of the visual assessment method**

480 *F. avenaceum* and *F. tricinctum* were identified as predominant species within
481 the barley malt *Fusarium* complex (Figure 4) and the most abundant species in
482 association with kernel discoloration (Table 4). Therefore, the validity and
483 reliability of the visual assessment method to detect contaminations caused by
484 *F. avenaceum* and *F. tricinctum* were assessed. For that purpose, barley was
485 grown in the greenhouse and artificially infected with the individual *Fusarium*
486 species. A serial dilution experiment was conducted by adding red discolored
487 malt kernels from barley artificially infected with either *F. avenaceum* or
488 *F. tricinctum* to potentially pathogen-free barley malt. In this dilution series, zero,
489 five, ten, twenty, or forty infected kernels were combined with symptomless malt
490 from the greenhouse cultivated non-infected control barley to a final weight of
491 200 g. For each dilution step, qPCR analysis was conducted to quantify the
492 amount of *Fusarium* DNA in the sample (Figure 6).

493 For both examined *Fusarium* species, *F. avenaceum* and *F. tricinctum*, the DNA
494 concentrations increased with higher numbers of infected kernels. In the
495 *F. avenaceum* data (Figure 6A), no statistical differences between the samples
496 containing zero, five, and ten symptomatic kernels were established, whereas
497 samples with twenty and forty red kernels were contaminated to a significantly
498 higher degree. As evident in Figure 6B, a clear distinction based on the
499 *F. tricinctum* DNA concentration can only be made in the sample containing

forty symptomatic kernels. Although coefficients of determination were high, the collected data suggest that only high numbers of reddish discolored kernels could indicate *Fusarium* contamination above the control level.

Relationship between symptomatology and mycotoxin content

Fusarium contamination might cause discoloration and higher *Fusarium* DNA concentrations in kernels. The secondary metabolite (i.e., mycotoxin) levels can also be enhanced in *Fusarium* infected samples. An additional sample subset was created (n = 10 per year) to reveal the degree of association between kernel discoloration (NR and NB) and secondary metabolites (DON, DON-3-G, 3-AC-DON, 15-AC-DON, ZEA, NIV, T2, HT2, ENN B, ENN B1, ENN A, ENN A1, and BEA). The secondary metabolites were quantified using LC-MS/MS. The Pearson correlation analysis of the data revealed no distinct relationships (Supplementary Table 2). The results were inconsistent with regard to individual secondary metabolites, specific kernel color, and harvest year.

Therefore, the toxins were quantified in color-specific and asymptomatic samples from the year 2014 (Supplementary Table 3). Several mycotoxins (3-AC-DON, 15-AC-DON, NIV, ZEA, T2, HT2, ENN A, and BEA) were not found in asymptomatic kernels. However, DON (19 µg/kg) and DON-3G (544 µg/kg) as well as ENN B (136 µg/kg), ENNB1 (43 µg/kg), and ENN A1 (11 µg/kg) were detected in asymptomatic control samples. These results indicate latent toxin contamination in the malt samples.

To further evaluate the validity and reliability of the visual assessment method to detect specific mycotoxin contamination in malt samples, the serial dilution experiment (see Figure 6) was supplemented with LC-MS/MS data. Figure 7 illustrates the measured ENN B and ENN B1 concentrations in the artificially created malt samples. Potentially pathogen-free barley malt was therefore spiked with a known number of *F. avenaceum*- or *F. tricinctum*-infected red kernels. The ENN B and ENN B1 concentrations increased with higher numbers of infected kernels. From the ENN B (*F. avenaceum*) data (Figure 7A), no statistical differences between the samples containing zero, five, and ten symptomatic kernels were revealed. As seen in Figure 7C, a conclusive distinction based on the ENN B level in *F. tricinctum*-infected material can only be made in the sample containing forty symptomatic kernels. From the ENN B1

data, significant discrimination can be identified in the *F. avenaceum* (see Figure 7B) and *F. tricinctum* (see Figure 7D) samples containing ten and forty symptomatic kernels, respectively. Similar to that observed for fungal DNA, predictions of mycotoxin contents are difficult when based on the number of discolored kernels. Significantly enhanced levels of mycotoxins in malt samples were only obtained when high numbers of discolored kernels were added.

Summary – Validation of the visible assessment

For further evaluation of validity of the visual assessment, the present study focused on a subset of samples from the three years of the trial. Of the total sample pool, ten representative samples were chosen from each of the harvests (n = 30) for specific mycotoxin analysis. From the 30 malt samples, it was evident that at low NR (≤20), the correlation between visible assessment, mycological assessment, genomic DNA concentrations, and mycotoxin analysis was generally not significant (data not shown). Consequently, the correlation analyses between kernel symptomatology and the other examined analytical methods were tested with samples containing more than twenty red kernels (n = 16). Table 5 shows coefficients of determination (R^2) of *Fusarium* detection methods and malt quality attributes of samples with NR >20. Data support the possibility of *F. avenaceum* being responsible for discoloration of malt samples with a high number of red kernels (NR >20) and corresponding contamination with ENN B. However, DON content and other quality parameters did not correlate with NR.

Discussion

Fusarium infections in barley malt compromise product quality and safety. Maltsters and brewers perform a visual examination of the samples to evaluate for infection and risk level in malt samples. However, in current practice, the validity and reliability of visual assessment tends to be overrated. The purpose of the present study was therefore to explore the possibilities and evaluate the validity of the visual assessment method to conclusively determine malt batch product quality and safety. In an effort to further elucidate the extent to which a *Fusarium* infestation in malt could potentially affect product quality and safety, the collected experimental data were statistically analyzed.

566 **Symptomatology and malt quality attributes**

567 Year-dependent malt quality variations can pose a challenge to brewers and
568 maltsters. In addition to the yearly fluctuating protein contents and gelatinization
569 temperatures, the degree of *Fusarium* infection in barley and barley malt
570 changes with each harvest. Fluctuating crop quality is mainly attributed to
571 weather conditions during plant development. In the present study, in which
572 three harvests (2012-2014) were thoroughly evaluated, it was observed that
573 proteolysis was particularly enhanced during 2012. The proteolytic parameters
574 of the 2012 samples correlated with high numbers of red and black kernels
575 (Tables 2 and 3). These slight effects could possibly be ascribed to *Fusarium*
576 infestations. Data suggest that in individual years, the number of discolored
577 kernels can be associated with enhanced proteolytic activities in malt.

578 Several studies have evaluated the influence of *Fusarium* on malt quality
579 attributes (Oliveira et al. 2012; Sarlin et al. 2005; Schwarz et al. 2002). These
580 studies worked with material artificially inoculated in a greenhouse, the field, or
581 subsequently in climate chambers. In the present study, the impact of
582 *Fusarium* spp. in naturally infected samples was investigated. This could
583 explain the discrepancies in the collected data. The influence of *Fusarium* spp.
584 on malt quality parameters (Supplementary Table 1) was not as high as
585 previously reported. Previous studies focused on *F. graminearum*, *F. poae*, and
586 *F. culmorum*. The data generated in the present study revealed that in
587 commercial samples *F. avenaceum, F. culmorum*, and *F. tricinctum* are the
588 predominant *Fusarium* species and were associated with kernel discoloration in
589 the 2012-2014 harvests. This is supported by statistically significant correlations
590 (Table 4). However, *Fusarium* content could still represent a secondary effect
591 without causing the color itself. When considering the correlations of NR or NB
592 with either malt quality parameters or fungal DNA content, one has to conclude
593 that the number of discolored kernels is indicative of malt quality attributes only
594 in single years, with relatively low correlation coefficients found. Moreover,
595 kernel discoloration does not reliably indicate the presence of single
596 *Fusarium* spp. when considering an individual batch of malt (Table 4, Figure 5,
597 and Supplementary Figure 1).

598 **Characterization of the *Fusarium* complex in malt**

599 The mycological analysis of the naturally infected, surface sterilized barley malt
600 samples revealed fungal species of several genera. Amongst others, species
601 belonging to genera *Alternaria*, *Aspergillus*, *Fusarium*, *Epicoccum*, *Drechslera*,
602 *Mucor*, and *Microdochium* were predominantly detected. The vast range of
603 microorganisms found in barley malt might greatly influence malt quality and
604 value. However, few general practical tools exist to assess the risk of
605 microorganisms for barley malt quality.

606 **Relationship of symptomatology and content of *Fusarium* DNA**

607 The six tested *Fusarium* species (*F. culmorum*, *F. graminearum*, *F. avenaceum*,
608 *F. tricinctum*, *F. langsethiae*, and *F. sporotrichioides*) were reported in previous
609 studies to play an important role as barley pathogens (Linkmeyer et al. 2016;
610 Nielsen 2011). Infection by these species is mainly disadvantageous to product
611 and processing quality as well as to food safety issues. Among other negative
612 effects, infection can reduce crop yield, enhance mycotoxin contamination
613 (Linkmeyer et al. 2016; Nielsen et al. 2014), and interfere with the malting
614 process (Hofer et al. 2016; Oliveira et al. 2012; Sarlin et al. 2005). Parry et al.
615 (1995) listed 17 species associated with the *Fusarium* head blight complex on
616 small grain cereals. Furthermore, *Fusarium* symptomatology in barley is
617 considered to be unspecific, and the symptoms are similar to those caused by
618 other microorganisms (Goswami and Kistler 2004). Therefore, the possibility
619 should not be disregarded that barley malt discoloration could be the result of
620 other, low abundant Fusarium species or other contaminants not yet thoroughly
621 characterized. In the present study, the genomic DNA concentrations of six
622 *Fusarium* spp. (*F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. tricinctum*,
623 *F. sporotrichioides*, and *F. langsethiae*) were quantified using qPCR.
624 Subsequently, the generated molecular data were correlated to NR and NB in
625 the samples. The strongest correlation between visual assessment (NR) and
626 DNA concentration was found for *F. avenaceum* and *F. culmorum*.

627 **Possible agents of symptomatology**

628 Under practical/commercial conditions, it is assumed that up to five to seven red
629 kernels per 200 g are acceptable in malt batches for brewing purposes (Anger
630 2006; Niessen 1991). If the number of red kernels exceeds this limit, the risk of

631 gushing or mycotoxin contaminations is considered too high (Anger 2006). More
632 than seven red kernels in 200 g were found in 22% of the assessed sample
633 pool in the present study (*n* = 243), thus predicting a high risk of *Fusarium*
634 contamination for further brewing processes. To clarify the potential causal
635 agents of kernel discoloration, *Fusarium* DNA in selected symptomatic and
636 asymptomatic kernels was quantified.

637 Experiments comparing specific-colored samples to symptomless malt revealed
638 several *Fusarium* species that strongly associate with kernel discoloration. A
639 relationship between *Fusarium* species and red discoloration was
640 predominantly identified for *F. avenaceum* and *F. tricinctum*. The potentially
641 gushing-inducing species, *F. culmorum* and *F. graminearum* (Niessen et al.
642 1991), were only moderately associated with reddish kernel discoloration.
643 Hudec (2007) determined the isolation frequency of several fungal species on
644 specific colored barley grain. In agreement with our data on fungal DNA content
645 from the present study, *F. avenaceum* was clearly the most abundant species
646 associated with red/pink kernels. In accordance to Mathre (1997), the gathered
647 data identified *F. graminearum* as a *Fusarium* species associated with black
648 discolorations in barley malt. The comparison of symptomatic and control
649 samples revealed enhanced DNA levels in discolored samples for all tested
650 *Fusarium* species, in particular *F. avenaceum* and *F. tricinctum*. *Fusarium* DNA
651 was also detected in non-discolored control samples, thus indicating
652 asymptomatic infection in barley malt by *Fusarium* species.

653 **Validity of visual assessment – Product quality and food safety**

654 From the serial dilution experiment in which a greenhouse cultivated potentially
655 pathogen-free sample was spiked with an increasing number of discolored
656 *F. avenaceum*- or *F. tricinctum*-infected kernels, it was concluded that there is
657 no statistical difference between the samples containing zero, five, or ten
658 symptomatic kernels. A possible explanation could be the unique microflora
659 composition (i.e., DNA or toxin distribution) on each discolored kernel. The
660 heterogeneity of the toxin composition in kernels was confirmed in single kernel
661 analysis (*unpublished data*; e.g., the ENN B levels ranged from 8.93 to 18.38 µg
662 in single red kernels of similar weight). However, when considering the

663 coefficient of determination, *Fusarium* DNA and ENN contents were strictly
664 associated with the number of artificially added red kernels (Figure 7).

665 It is possible to identify *Fusarium* contaminations caused primarily by
666 *F. avenaceum* and *F. tricinctum*, in barley malt by visual evaluation. However,
667 statistical analysis revealed that high accuracy was achieved when greater than
668 twenty red kernels were found in or added to 200 g of malt (Figure 6 and
669 Table 5). This suggests that the set limit of five to seven red kernels should be
670 critically reviewed. Kernel discoloration provides only an indication for the
671 presence of *F. avenaceum* and *F. tricinctum*, but cannot conclusively determine
672 it. One might conclude that the number of discolored kernels is insufficient to
673 reliably predict high contamination of *Fusarium* spp. However, for assessment
674 of the risk of fungal contamination, there is currently no better and more easily
675 applicable tool available. Several methods are available for the assessment of
676 *Fusarium* infections in malt samples. Quantitative PCR is a powerful tool to
677 detect and determine the degree of the *Fusarium* contamination. However, the
678 need for a multiplexed method which can simultaneously detect all
679 *Fusarium* spp. remains. An alternative would be to quantify the mycotoxins in
680 barley or barley malt.

681 Hitherto published studies attempted to correlate *Fusarium* infection rates with
682 DON levels. In the total commercial malt samples of the present study, no
683 significant correlation was found between mycotoxins and number of colored
684 kernels. However, when considering only samples with a high NR (>20), ENN
685 was enriched (Table 5). When considering selected red or black kernels from
686 malt samples, red kernels were enriched in ENN and black kernels were
687 enriched in DON and ZEA. Generated DNA data revealed that the highest DNA
688 concentrations in the *Fusarium* complex corresponded to *F. graminearum*,
689 *F. avenaceum*, and *F. tricinctum*. Both, *F. avenaceum* and *F. tricinctum*, are
690 ENN producing species, and cannot produce DON. Hence, low numbers of
691 discolored kernels are insufficient to assess the risk of mycotoxin contamination
692 in single samples of malt. In particular, red kernels appear to offer little
693 information in regard to the contents of DON. However, similar to what was
694 discussed for *Fusarium* DNA, while no more accurate tool has been
695 implemented in practice, the number of discolored kernels cannot be neglected
696 for the management of mycotoxin risk. If it is intended to examine the mycotoxin

contamination, a robust method which can detect all mycotoxins and their modified metabolites should be used. The published methods (Habler and Rychlik 2016) tend to be time consuming; therefore, they are not suitable for quality control during the production process.

Conclusion

Visual inspection of barley remains an important step in selection procedures for commercial malt samples by brewers. For practical malting and brewing, five to seven red, potentially *Fusarium*-infected kernels in 200 g of a malt sample is the limit recommended by MEBAK (Anger 2006). The significance of this limit is based on modified quality characteristics of malt and the high gushing potential in bottled beer. The relationship between discolored kernels and metabolites, which arises during *Fusarium* infection, has often been discussed between corresponding stakeholders. Problems associated with *Fusarium* contamination affect processing and final product quality, as well as the safety of the product. More specific effects include enhanced malt modification, proteolysis, extract losses during malting (processing quality), the risk of gushing in bottled beer (product quality), and the transfer of mycotoxins to spent grains used for animal feed and to beer (food safety). For acquiring information on the modification or germination energy, it is also useful to identify the degree of *Fusarium* infection, because *Fusarium* is known to enhance proteolysis which could lead to technological problems during processing.

A visible assessment of discoloration of malt kernels can be an indicator of enhanced *Fusarium* infections. Assessed kernels do not reflect the total extent of infection. An infection with *Fusarium* is multifaceted and results among others in kernel discoloration, enrichment of fungal DNA and mycotoxins, and possibly other substances, which could contribute to the gushing phenomenon. To complete risk estimation, available detection methods should be combined. However, in brewing practice, this is likely difficult to implement. Hence, avoidance of a *Fusarium* infection during the development of barley crop in the field is the most desirable route to minimize the infection of kernels. However, in barley production practice, only limited tools are available to control Fusarium head blight (Wegulo et al. 2015). In addition, some options to reduce the

730 infection level after malting using sorter systems are available. It should be
731 noted however, that removal of symptomatic kernels might not sufficiently
732 reduce the total amount of fungal DNA and mycotoxins in individual malt
733 samples.

734 In the present study, different methods for detection of fungal contamination
735 were used to evaluate the value of the visual assessment of malt kernels. In
736 general, data from commercial samples showed low predictability of fungal
737 contamination in samples with discolored kernels. However, in single years with
738 high levels of infection, correlations between NR or NB and fungal DNA were
739 significant. Importantly, this was not the case when considering mycotoxin
740 levels. However, when considering selected discolored kernels or specific malt
741 samples with high NR, an enrichment of fungal DNA and mycotoxins was
742 evident. This was particularly the case for *F. avenaceum* and ENN. Hence,
743 visual assessment of red kernel discoloration is likely overrated in terms of its
744 value in predicting the content of DON. Possibly, one has to consider NB rather
745 than NR to assess the risk of DON contamination. However, although NR is
746 likely overestimated in regard to its reliability as an indicator for *Fusarium*
747 contamination, it can still assist in assessing the risk of fungal contaminants.
748 NR >20 was associated with the risk of ENN and *Fusarium* contamination.
749 Hence, the recommendation of MEBAK for recruiting additional methodology of
750 risk assessment if at least five to seven red kernels are detected in 200 g of
751 malt appears reasonable (even when neglecting the risk of gushing), provided
752 no better tool than visual assessment is available. Maltsters may have a great
753 interest in future development of more reliable and easy-to-apply tools that
754 predict the risk of *Fusarium* contamination in malt samples with a lower rate of
755 false positive outcomes.

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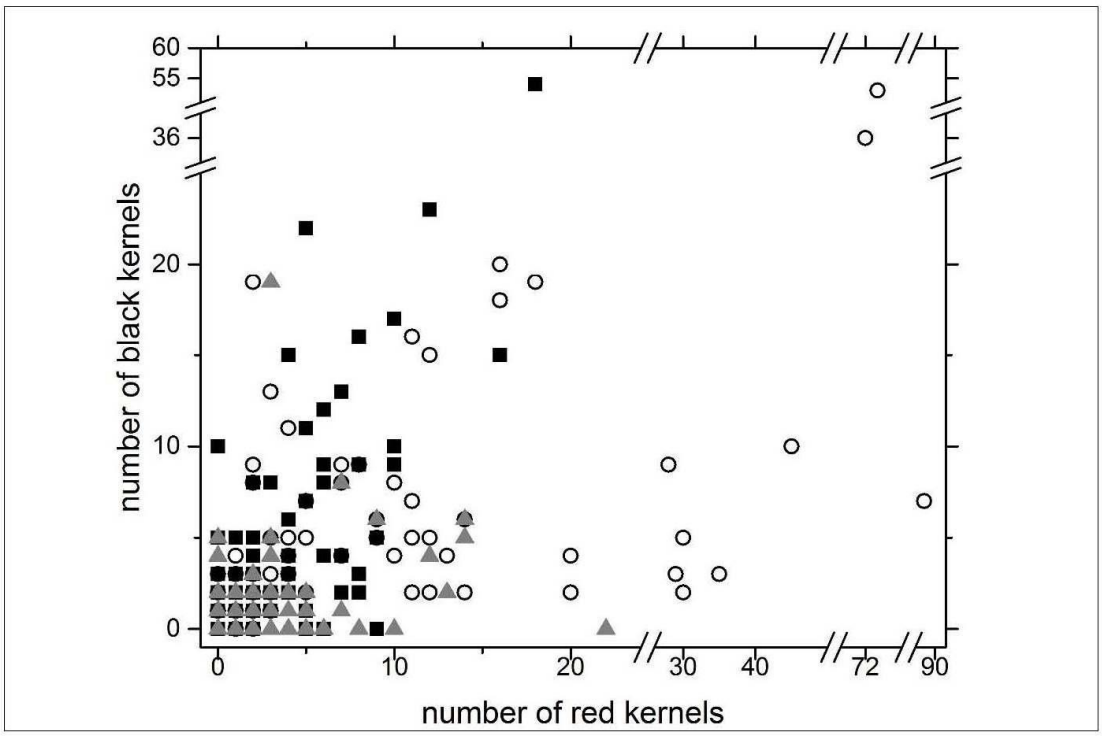
882

883 *Figure 1: Examples for discolored kernels in barley malt. Red symptomatology (A), black symptomatology (B).*

884 *Supplementary Table 1: Malting quality attributes of commercially produced samples. Commercially produced grain samples, derived from different years and*
885 *locations, were processed and analyzed for malt quality attributes according to MEBAK (Mitteleuropäische Brautechnische Analysenkommission) (Anger 2006).*
886 *Data represent mean values for individual years of observation (2012: n = 82; 2013: n = 79; 2014: n = 84) and for the total number of samples (n = 243).*
887 *Abbreviations: d.m. = dry matter, DU = dextrinizing units, DMSP = dimethyl sulfide precursor.*

quality attribute	unit	year				MEBAK method	analysis referring to
		2012	2013	2014	total		
moisture content barley	%	12.53	12.83	11.38	12.25	1.5.1.1	barley
raw protein barley	% d.m.	10.12	9.82	9.75	9.90	1.5.2.1	
germinative energy 3rd day	%	91.80	94.49	95.54	93.94	1.4.2.2	
germinative energy 5th day	%	94.67	96.47	96.99	96.04	1.4.2.2	
water sensitivity	%	36.20	24.58	35.64	32.14	1.4.3	
grading >2.8 mm barley	%	83.88	74.99	74.88	77.91		
grading 2.5-2.8 mm barley	%	13.66	20.78	19.53	17.99		
grading 2.2-2.5 mm barley	%	1.85	3.49	4.19	3.18	1.3.1	
screenings barley	%	0.60	0.74	1.40	0.91		
grade 1 barley	%	97.55	95.77	94.41	95.91		
percentage >2.8 mm grade 1	%	85.96	78.11	78.87	80.98		
sprouting	%	0.00	0.00	1.69	0.56	1.4.5	
malting loss	%	7.96	8.59	8.97	8.51	1.5.3	
respiration loss	%	3.90	4.33	3.92	4.05	1.5.3	
red kernels (NR)	n/200g	10.29	4.00	2.81	5.70	3.1.4.16	visual assessment
black kernels (NB)	n/200g	5.89	5.81	1.43	4.37		
extract malt d.m.	% d.m.	82.85	83.15	82.36	82.78	3.1.4.2.2	amylolytic properties
α-amylase	DU d.m.	62.54	59.84	61.85	61.41	3.1.4.7	
gelatinization temperature	°C	62.90	63.07	62.34	62.77	2.7	
final attenuation	% d.m.	83.48	83.96	86.74	84.72	3.1.4.10.1.3	
viscosity (8.6% m/m)	mPa s	1.47	1.45	1.46	1.46	3.1.4.4.2	cytolytic properties
friability	%	87.55	92.91	90.72	90.39	3.1.3.6	
steeliness	%	1.24	0.34	0.32	0.64	3.1.3.6	
β-glucan 65°C	mg/l	272.94	214.47	137.77	208.39	3.1.4.9	
raw protein malt	% d.m.	9.72	9.20	9.43	9.45	1.5.2.1	proteolytic properties
soluble nitrogen malt d.m.	mg/100g d.m.	704.29	656.21	670.52	677.01	3.1.4.5.2	
Kolbach index	%	45.51	44.80	44.66	44.99	3.1.4.5.3	
free amino nitrogen	mg/100g d.m.	182.16	161.70	154.31	166.06	3.4.5.5.1	
DMSP	ppm	3.88	6.58	8.37	6.28	3.1.4.17	other properties
moisture content malt	%	4.94	4.67	4.83	4.81	1.5.1.1	



889

890 *Figure 2: Number of discolored kernels in commercially produced malt samples. Commercially*
891 *produced grain samples derived from different years and locations were malted and visually assessed*
892 *for the number of red and black kernels according to MEBAK (Mitteleuropäische Brautechnische*
893 *Analysenkommission)* (Anger 2006)*. The scatter plot illustrates year-dependent (2012* (o)*: n = 82;*
894 *2013* (■)*: n = 79; 2014* (▲)*: n = 84) distribution patterns of kernel symptomatology.*

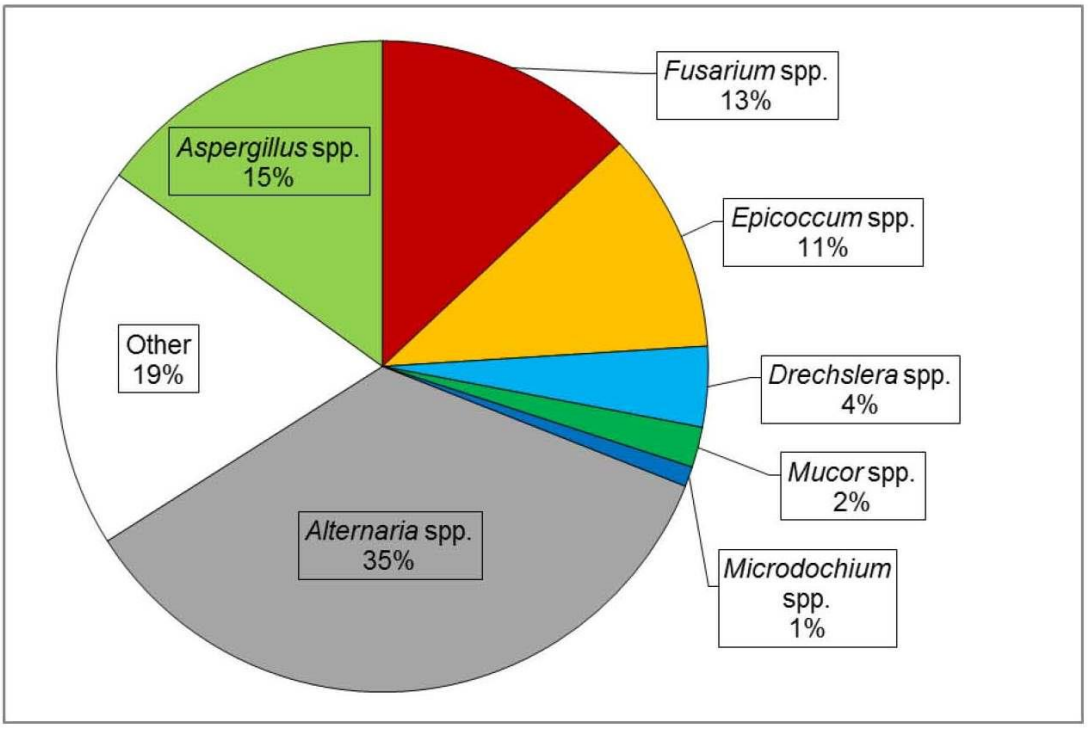
895

*Table 1: Correlation of quality attributes with red discoloration. Commercially produced grain samples (n = 243), derived from different years (2012: n = 82; 2013: n = 79; 2014: n = 84) and locations, were malted and analyzed for malt quality attributes according to MEBAK (Mitteleuropäische Brautechnische Analysenkommission) (Anger 2006). Data represent significant correlation coefficients (*p ≤0.05 and ** p ≤0.01) resulting from Pearson correlation analysis of the number of red kernels with data of all other measured quality attributes (Supplementary Table 1). Positive correlation coefficients suggest positive relationships between kernel discoloration and the tested quality attribute. Negative coefficients indicate negative connections. The size of correlation coefficients indicates strength of connectivity. Abbreviations: d.m. = dry matter.*

		Pearson correlation coefficients			
quality attributes	unit	2012	2013	2014	mean
moisture content barley	%				0.14 *
water sensitivity	%			0.31 **	0.19 **
grading >2.8 mm barley	%				0.17 **
grading 2.5-2.8 mm barley	%				-0.18 **
malting loss	%	0.26 *			
respiration loss	%	0.32 **		0.23 *	0.15 *
black kernels	n/200g	0.60 **	0.70 **	0.23 *	0.53 **
gelatinization temperature	°C		0.47 **	0.34 **	0.23 **
final attenuation	% d.m.			0.24 *	
viscosity (8.6% m/m)	mPa s			-0.25 *	
friability	%		0.27 *		
soluble nitrogen	mg/100g d.m	0.36 **			0.32 **
Kolbach index	%	0.37 **		0.23 *	0.27 **
free amino nitrogen	mg/100g d.m	0.30 **			0.30 **
moisture content malt	%		-0.30 *		

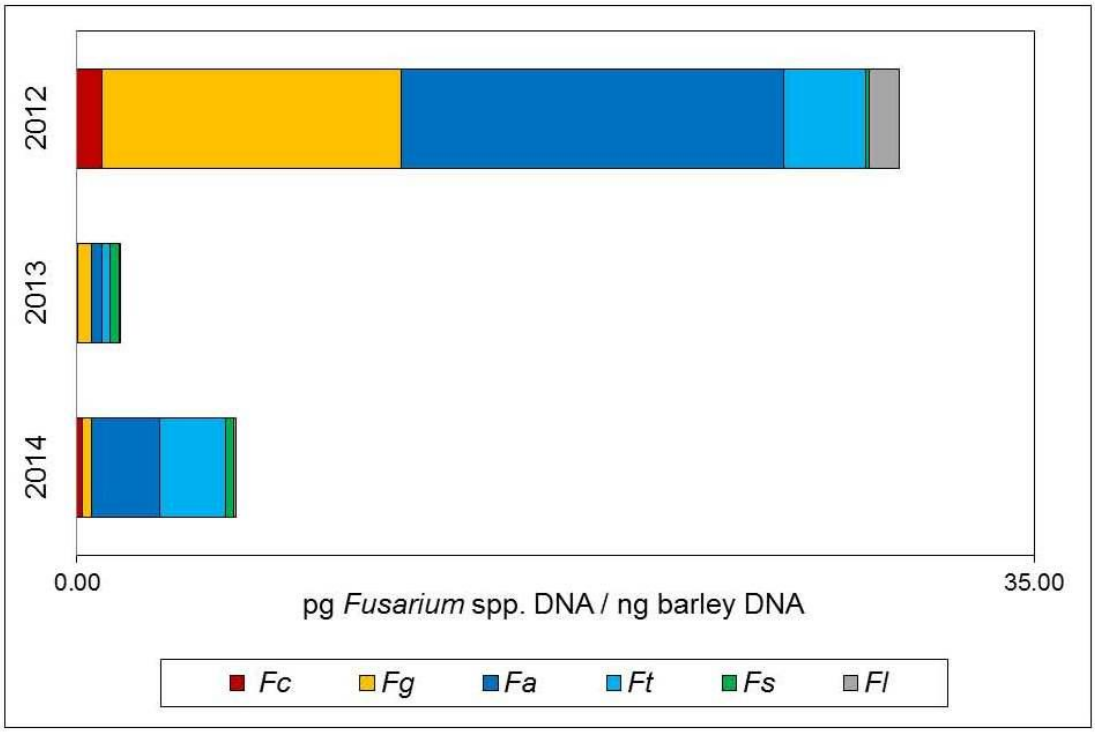
*Table 2: Correlation of quality attributes with black discoloration. Commercially produced grain samples (n = 243), derived from different years (2012: n = 82; 2013: n = 79; 2014: n = 84) and locations were malted and analyzed for malt quality attributes according to MEBAK (Mitteleuropäische Brautechnische Analysenkommission) (Anger 2006). Data represent significant correlation coefficients (*p ≤0.05 and ** p ≤0.01) resulting from Pearson correlation analysis of the number of red kernels with data of all other measured quality attributes (Supplementary Table 1). Positive correlation coefficients suggest positive relationships between kernel discoloration and the tested quality attribute. Negative coefficients indicate negative connections. The size of correlation coefficients indicates strength of connectivity. Abbreviations: d.m. = dry matter.*

		Pearson correlation coefficients			
quality attributes	unit	2012	2013	2014	mean
moisture content barley	%			-0.34 **	0.17 **
germinative energy 3rd day	%			-0.26 *	
sprouting	%			0.30 **	
red kernels	n/200g	0.60 **	0.70 **	0.23 *	0.53 **
extract malt d.m.	% w.w.			-0.28 *	
gelatinization temperature	°C		0.24 *		0.19 **
final attenuation	% d.m.				-0.16 *
friability	%		0.25 *	-0.22 *	
soluble nitrogen	mg/100g d.m	0.26 *			0.18 **
free amino nitrogen	mg/100g d.m	0.30 **			0.22 **
moisture content malt	%		-0.26 *		



916

917 *Figure 3: Mycological status of commercially produced barley malt. Malt was produced from*
918 *commercially cultivated barley samples according to MEBAK (Mitteleuropäische Brautechnische*
919 *Analysenkommission) (Anger 2006) and analyzed for mycological status using an agar plate test. Data*
920 *represent percentage of genus-specific fungal infestation resulting from mean values (n = 59) of three*
921 *years of observation (2012: n = 20; 2013: n = 19; 2014: n = 20).*



922

923 *Figure 4: Fusarium DNA in commercially produced barley malt. Malt was produced from commercially*
924 *cultivated barley samples according to MEBAK (Mitteleuropäische Brautechnische*
925 *Analysenkommission) (Anger 2006) and tested for the Fusarium DNA content using quantitative*
926 *polymerase chain reaction (qPCR). Data represent mean values for individual years of observation*
927 *(2012: n = 20; 2013: n = 19; 2014: n = 20). Abbreviations: Fc = F. culmorum, Fg = F. graminearum,*
928 *Fa = F. avenaceum, Ft = F. tricinctum, Fs = F. sporotrichioides, Fl = F. langsethiae.*

929

930 *Table 3: Correlation of Fusarium DNA content and kernel symptomatology. Pearson correlation*
931 *analysis of species-specific Fusarium DNA contents determined by quantitative polymerase chain*
932 *reaction (qPCR) and numbers of red (NR) and black (NB) kernels identified by visual assessment in*
933 *malt produced according to MEBAK (Mitteleuropäische Brautechnische Analysenkommission) (Anger*
934 *2006) from commercially cultivated grain samples (2012: n = 20; 2013: n = 19; 2014: n = 20). Data*
935 *represent Pearson correlation coefficients. Positive and negative values indicate positive and negative*
936 *relationships, respectively. Size of correlation coefficients suggests strength of connectivity.*
937 *Statistically significant correlations are indicated by *(p ≤0.05) and **(p ≤0.01). Abbreviations: Fc =*
938 *F. culmorum, Fg = F. graminearum, Fa = F. avenaceum, Ft = F. tricinctum, Fs = F. sporotrichioides,*
939 *Fl = F. langsethiae.*

		Pearson correlation coefficients					
		Fc-DNA	*Fg*-DNA	*Fa*-DNA	*Ft*-DNA	*Fs*-DNA	*Fl*-DNA
NR	2012	0.56 *	0.38	0.87 **	0.16	0.56 *	0.05
	2013	0.14	0.29	0.22	0.44	-0.03	0.17
	2014	0.49 *	0.30	0.56 *	0.53 *	0.27	0.03
NB	2012	0.79 **	0.13	0.60 **	-0.18	0.85 **	-0.17
	2013	0.14	0.12	0.26	0.48*	0.16	0.33
	2014	-0.02	-0.08	0.30	-0.22	0.28	-0.09

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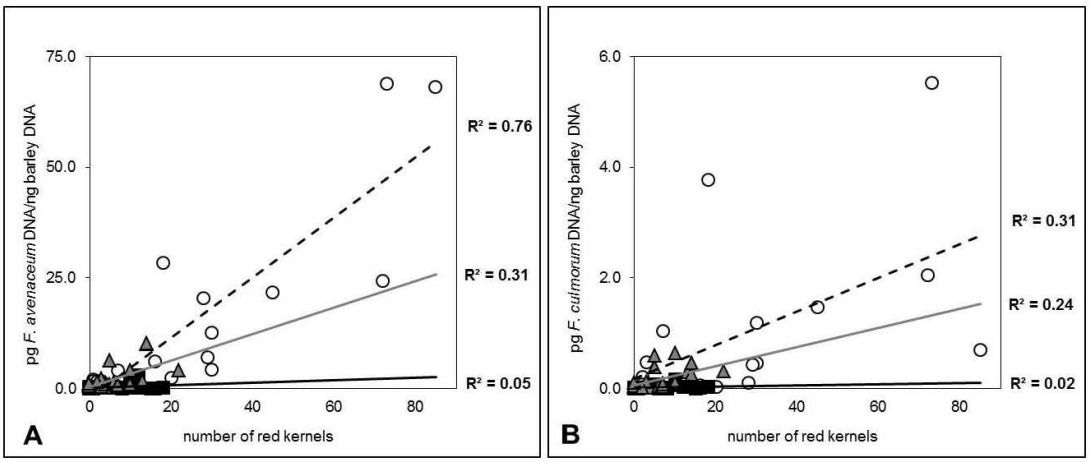
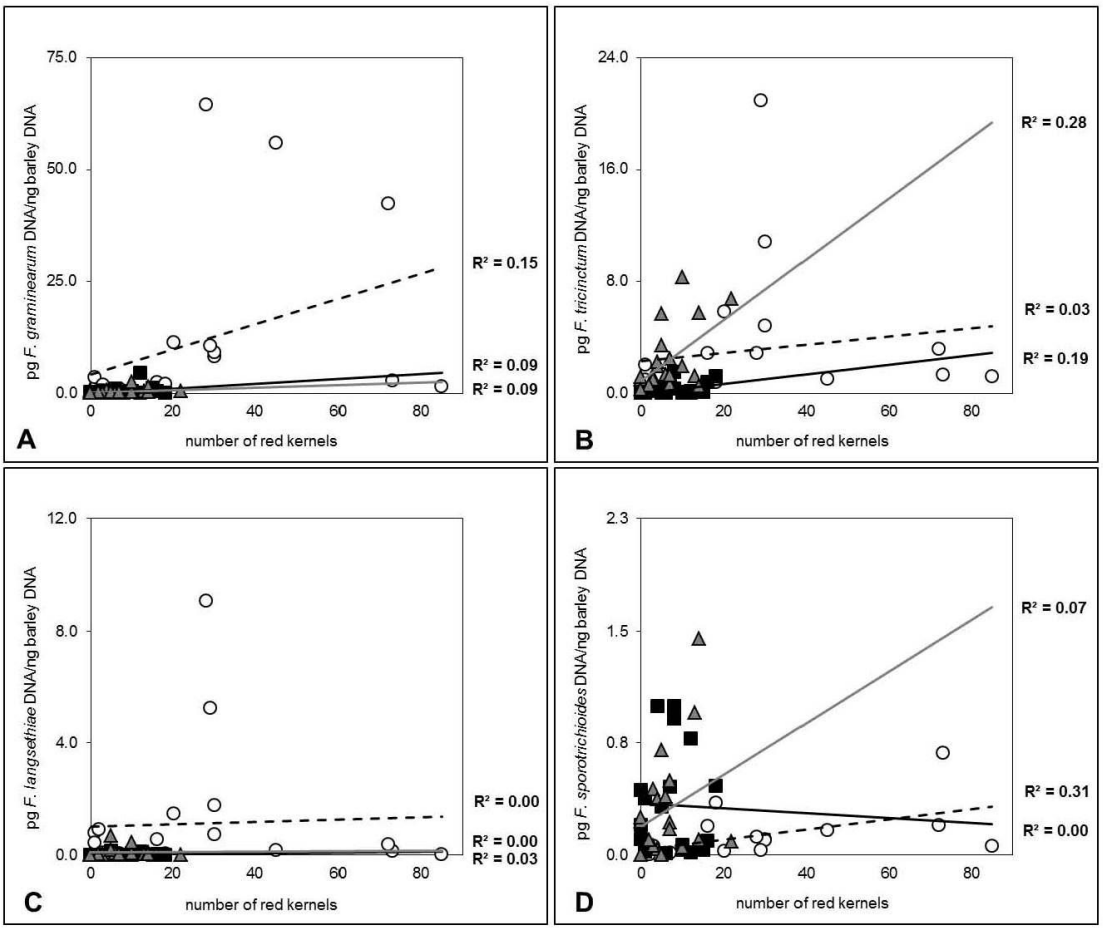


Figure 5: Correlation of Fusarium DNA contents and red discoloration. Scatter plots illustrate correlation of species-specific Fusarium DNA contents (F. avenaceum (A) and F. culmorum (B)) determined by quantitative polymerase chain reaction (qPCR) and the number of red kernels identified by visual assessment in individual malt samples produced according to MEBAK (Mitteleuropäische Brautechnische Analysenkommission) (Anger 2006) from commercially cultivated grain in three years of observation (2012 (o): n = 20; 2013 (■): n = 19; 2014 (▲): n = 20). Trend lines are shown as 2012 (---), 2013 (—), and 2014 (—). R^2 indicates strength of connectivity in individual years.



Supplementary Figure 1: Correlation of Fusarium DNA contents and red symptomatology. Scatterplots illustrate correlation of species-specific Fusarium DNA contents (F. graminearum (A), F. tricinctum (B), F. langsethiae (C), and F. sporotrichioides (D)) determined by quantitative polymerase chain reaction (qPCR) and number of red kernels identified by visual assessment in individual malt samples produced according to MEBAK (Mitteleuropäische Brautechnische Analysenkommission) (Anger 2006) from commercially cultivated grain in three years of observation (2012 (o): n = 20; 2013 (■): n = 19; 2014 (▲): n = 20). Trend lines are shown as 2012 (---), 2013 (—), and 2014 (—). R^2 suggests strength of connectivity in individual years.

962 *Table 4: Fusarium DNA content in selected symptomatic and asymptomatic malt kernels. Fusarium*
963 *DNA content determined by quantitative polymerase chain reaction (qPCR) analysis in selected*
964 *symptomatic (red or black) and asymptomatic (control) malt kernels selected from commercially*
965 *produced malt of 2014. Factors were calculated by the ratio: pg Fusarium spp. DNA/ng barley DNA of*
966 *the discolored sample divided by pg Fusarium spp. DNA/ng barley DNA of the asymptotic sample.*
967 *Abbreviations: Fc = F. culmorum, Fg = F. graminearum, Fa = F. avenaceum, Ft = F. tricinctum, Fs =*
968 *F. sporotrichioides, Fl = F. langsethiae.*

	Fusarium species DNA (pg/ng barley DNA)					
	Fc	*Fg*	*Fa*	*Ft*	*Fl*	*Fs*
red	1.71	4.66	157.71	165.02	0.98	0.86
asymptomatic (control red)	0.10	0.32	1.10	0.62	0.18	0.01
factor (= red/asymptomatic)	**17**	**15**	**143**	**268**	**5**	**69**
black	0.49	3.96	12.46	11.02	6.25	0.10
asymptomatic (control black)	0.08	0.11	0.76	0.60	0.20	0.02
factor (= black/asymptomatic)	**6**	**37**	**16**	**18**	**31**	**6**

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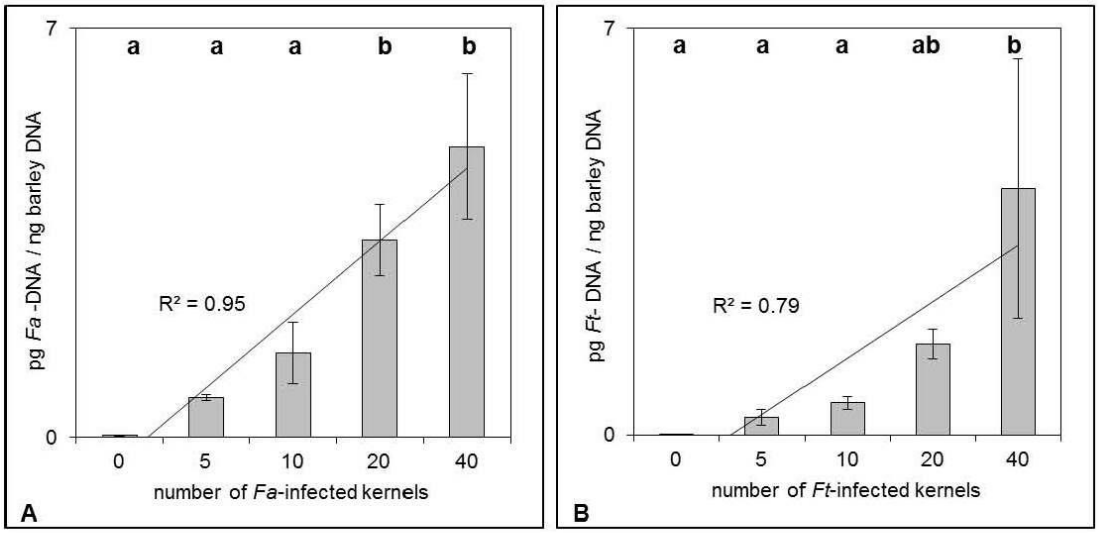

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971 *Figure 6: Fusarium DNA content in artificially contaminated malt samples. Mean values (n = 3) of*
972 *Fusarium DNA determined by quantitative polymerase chain reaction (qPCR) analysis in malt samples*
973 *generated from pathogen-free tested malt, enriched with a distinct number of F. avenaceum (A) or*
974 *F. tricinctum-infected (B), symptomatic malt kernels. Characters above error bars (standard deviation)*
975 *indicate statistically significant differences in DNA content by Tukey B test (P ≤0.05). Abbreviations:*
976 *Fa = F. avenaceum, Ft = F. tricinctum.*

*Supplementary Table 2: Correlation of mycotoxin content and kernel symptomatology. Pearson correlation analysis of Fusarium toxin content determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis and numbers of red (NR) and black (NB) kernels identified by visual assessment in malt produced according to MEBAK (Mitteleuropäische Brautechnische Analysenkommission) (Anger 2006) from commercially cultivated grain samples (2012: n = 10; 2013: n = 10; 2014: n = 10). Data represent Pearson correlation coefficients. Positive and negative values indicate positive and negative relationships, respectively. Height of correlation coefficients suggests strength of connectivity. Statistically significant correlations are indicated by *(p ≤0.05) and **(p ≤0.01). Abbreviations: DON: deoxynivalenol; 3AcDON: 3-acetyl-deoxynivalonol; 15AcDON: 15-acetyl-deoxynivalonol; D3G: deoxynivalenol-3-glucoside; NIV: nivalenol; ZEA: zearalenone; ENN B: enniatin B; ENN B1: enniatin B1; ENN A1: enniatin A1; ENN A: enniatin A; BEA: beauvericin; HT2: HT2-toxin; T2: T2-toxin.*

		Pearson correlation coefficients											
		DON	3AcDON	15AcDON	D3G	ZEA	NIV	HT2	T2	ENN B	ENN B1	ENN A1	ENN A	BEA
	2012	0.29	0.29	0.30	0.39	0.38	0.50			0.71 *	0.52	0.38	0.28	0.50
NR	2013	0.28	0.37	0.41	0.26	0.43	-0.35	0.11		-0.14	-0.16	-0.07	-0.04	-0.35
	2014	0.12		0.00	-0.26		-0.06	0.02	0.62	0.54	0.51	0.56	-0.36	-0.45
	2012	0.20	0.21	0.21	0.26	0.35	0.95 **			-0.01	-0.14	-0.18	-0.20	0.95 **
NB	2014	-0.33	-0.26	-0.21	-0.35	-0.14	-0.28	0.44		-0.15	-0.20	-0.22	-0.28	-0.28
	2013	-0.22		-0.36	-0.32		-0.14	-0.04	0.09	0.00	-0.13	-0.01	-0.11	-0.06

985 *Supplementary Table 3: Fusarium toxin content in selected symptomatic and asymptomatic malt kernels. Fusarium toxin content determined by liquid*
986 *chromatography tandem mass spectrometry (LC-MS/MS) analysis in distinct symptomatic (red or black) and asymptomatic (control) malt kernels selected from*
987 *commercially produced malt of 2014. Factors were calculated by the ratio: µg/kg mycotoxin of the discolored sample divided by µg/kg mycotoxin of the*
988 *asymptotic sample. Abbreviations: DON: deoxynivalenol; 3AcDON: 3-acetyl-deoxynivalonol; 15AcDON: 15-acetyl-deoxynivalonol; D3G: deoxynivalenol-3-*
989 *glucoside; ZEA: zearalenone; ENN B: enniatin B; ENN B1: enniatin B1; ENN A1: enniatin A1; ENN A: enniatin A; BEA: beauvericin; HT2: HT2-toxin; T2: T2-toxin.*

	mycotoxin (µg/kg)											
	DON	3AcDON	15AcDON	D3G	ZEA	HT2	T2	ENN B	ENNB1	ENN A1	ENN A	BEA
red	790.0	38.0	68.8	2171.8	45.6	65.9	15.4	38250.0	20125.0	8700.0	1625.0	12.7
asymptomatic (control red)	19.4	0	0	544.4	0	0	0	92.6	30.5	7.8	0	0
factor (= red/asymptomatic)	**41**	**38**	**69**	**4**	**46**	**66**	**15**	**413**	**660**	**1121**	**1625**	**13**
black	127.8	0	0	1060.9	30.8	301.9	472.5	3425.0	953.8	351.9	55.1	36.8
asymptomatic (control black)	1	0	0	27	0.1	0	0	135.6	43.2	10,7	1.3	0
factor (= black/asymptomatic)	**123**	**0**	**0**	**39**	**212**	**302**	**473**	**25**	**22**	**33**	**44**	**37**

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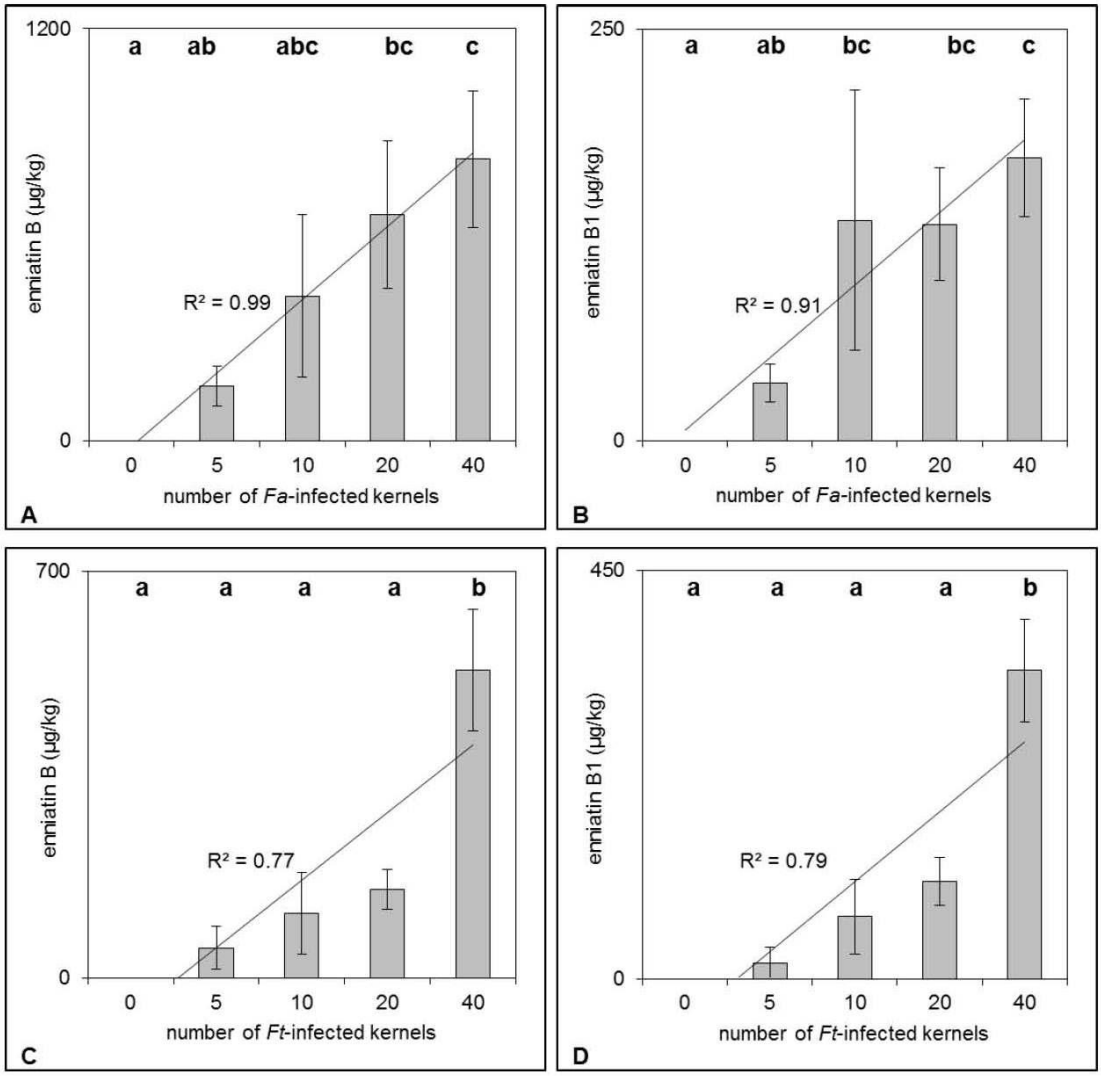

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992 *Figure 7: Fusarium toxin content in artificially contaminated malt samples. Mean values (n = 3) of*
993 *Fusarium toxins enniatin B (A + C) and B1 (B + D) determined by liquid chromatography tandem mass*
994 *spectrometry (LC-MS/MS) analysis in malt samples generated from pathogen-free tested malt,*
995 *enriched with a distinct number of F. avenaceum- (A + B) or F. tricinctum-infected (C + D),*
996 *symptomatic malt kernels. Characters above error bars (standard deviation) indicate statistically*
997 *significant differences in toxin content by Tukey B test (P ≤0.05). Abbreviations: Fa = F. avenaceum,*
998 *Ft = F. tricinctum.*

Table 5: Coefficients of determination (R^2) of Fusarium detection methods and malt quality attributes of samples with NR of >20 (n = 16); FAN: free amino nitrogen; total Fusarium DNA: sum of all Fusarium DNA; F. avenaceum DNA; total mycological assessment: sum of all mycological assessed pathogens; total mycotoxins: sum of all measured toxins. Abbreviations: FAN = free amino nitrogen, DON = deoxynivalenol, ENN B = enniatin B.

	attribute	coefficients of determination (R^2)
Food quality	FAN	0.17
	soluble nitrogen	0.12
	F. avenaceum DNA	0.80 **
	total *Fusarium* DNA	0.90 **
	total mycological assessment	0.05
Food safety	DON	0.11
	ENN B	0.54 **
	total mycotoxins	0.67 **

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Die Zeit am Lehrstuhl für Phytopathologie habe ich sehr genossen, vor allem weil viele liebe Menschen meinen Weg zur Doktorarbeit begleitet haben. Für ihr Vertrauen, ihre Unterstützung und Motivation bin ich sehr dankbar.

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Curriculum vitae

Persönliche Daten

Name	:	Katharina Marlene Hofer
Geburtsdatum	:	15. September 1987
Geburtsort	:	Landshut

Studium und Beruf

01/2013 – heute	:	Wissenschaftliche Mitarbeiterin am Lehrstuhl für Phytopathologie der Technischen Universität München
10/2010 – 12/2012	:	Masterstudiengang Agrarwissenschaften, Fachrichtung Agrarökosysteme, an der Technischen Universität München
		Abschluss: Master of Science
10/2007 – 09/2010	:	Bachelorstudiengang Agrarwissenschaften an der Technischen Universität München
		Abschluss: Bachelor of Science

Schulbildung

1998 – 2007	:	Gymnasium der Schulstiftung Seligenthal, Landshut
		Abschluss: Allgemeine Hochschulreife
1994 – 1998	:	Volksschule Kronwinkl, Kronwinkl-Eching