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Influence of *Fusarium avenaceum* infections on barley malt: Monitoring changes in the albumin fraction of barley during the malting process

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ABSTRACT

Fusarium spp. are ubiquitous field pathogens, which are known to affect quality characteristics of cereals. Infections with *Fusarium* pathogens in brewing cereals are problematic and augur for a poor malt quality. The negative effects of *Fusarium* infections are various. Besides causing agronomic losses, kernel discoloration, and the production of several mycotoxins, *Fusarium* spp. are known to enhance the proteolytic activity of infected barley malt. Enhanced proteolysis can be observed in higher values in free amino nitrogen (FAN) as well as in soluble nitrogen. Previous studies showed that, in recent crop years, the predominant *Fusarium* spp. detected in European malt was *F. avenaceum*. This study focuses on the changes in the albumin fraction during the malting process of barley samples artificially infected with *F. avenaceum* in comparison to uninfected control samples. The barley samples were grown under controlled conditions in a greenhouse, and two different sample types were studied: One was not infected with *Fusarium* spores whereas the other sample was inoculated during anthesis with a spore suspension of *F. avenaceum*. Malting trials were carried out in a micromalting facility under defined conditions. To monitor the changes in protein profiles due to *Fusarium* infection, samples were analyzed at different stages of the malting process: barley (raw grain), poststeeping, postgermination, and postkilning. To evaluate the changes in the protein composition, different fractionation techniques (Osborne fractionation, isoelectric focusing, and capillary gel electrophoresis) were utilized. The albumins of the Osborne fractionation were further analyzed as they represent the main metabolically active proteins. Protein maps of the different malting steps were plotted with a custom-programmed visualization tool for comparing the infected and uninfected samples at each malting step. The results elucidate the changes in the albumin fraction during the malting process and reveal the influence of *Fusarium* infection on the albumin composition of barley, barley malt, and the intermediate products of malting.

1. Introduction

Fusarium head blight (FHB) is a serious problem in barley cultivation and a devastating plant disease with respect to barley yield and quality (McMullen et al., 1997; Steffenson, 2003; Windels, 2000). Farmers are facing rejection of their harvest due to possible mycotoxin contamination in years of high FHB occurrence (Bai and Shaner, 2004) and consequently the risk of economic losses. Infection of plants with *Fusarium* spp. and the growth of these species are promoted by high humidity (e.g. rainy weather conditions) and warm temperatures in barley fields during anthesis (Bushnell et al., 2003; Osborne and Stein, 2007; Wilcoxson et al., 1992). As a result, maltsters are confronted with the problem of hard-to-handle barley in the malt house since the affected samples have a tendency toward higher malt modification, which is beyond of control (Oliveira et al., 2013; Sarlin et al., 2005; Schwarz et al., 2006). Besides cytolysis and enzyme generation, proteolysis is a main reason for malting. Brewers need high-quality malt with special quality attributes to brew high-quality beers. Hence, brewers are challenged with these altered malts, which might cause problems in flavor stability, foam stability, and turbidity of their beer (Hippeli and Elstner, 2002; Sørensen et al., 1993; Steiner et al., 2011). This problem is exacerbated because the altered malt quality characteristics are sometimes not evident in analytical data.

Previous studies showed that, in recent crop years, the predominant

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Fusarium spp. in Europe was *F. avenaceum* (Geißinger et al., 2017; Linkmeyer et al., 2016; Nielsen et al., 2011). *Fusarium* infection is generally not obvious in barley except in highly infected lots, where the presence of dark dots on the barley husks can be a hint of *Fusarium* infection (Murray et al., 2008). During malting procedure the conditions for *Fusarium* growth are favorable, because of humidity and temperature during germination. Furthermore, *Fusarium* growth continues during withering as long as the lot is infected by *Fusarium* species; as a result, symptoms (reddish or black discoloration of the kernel surfaces) can become visible (Geißinger et al., 2017). These visible symptoms, especially red kernels, have mainly been caused by *F. avenaceum* in recent crop years (Geißinger et al., 2017).

Additionally, malt infected with *Fusarium* spp. is consequently contaminated with a spectrum of *Fusarium* mycotoxins (Habler et al., 2016). These pose a risk in terms of food safety and hence human or animal health in the final product (Desjardins and Proctor, 2007). Furthermore, mycotoxins and other fungal metabolites can disturb biosynthesis in plants as well as kernel development (Hofer et al., 2016; Rocha et al., 2005) and should be monitored in suspicious cases. As a consequence, *Fusarium* infection can influence the quality characteristics of malt.

During the malting process, the conditions for fungal growth are favorable because humidity is high (a_w value > 0.95) and temperature (between 14.5 and 20 °C during germination, over 50 °C during withering, and up to 85 °C during kilning) is conducive to fungal growth (Vegi et al., 2011). The presence of *Fusarium* fungi can result in altered expression rates of barley malt enzymes, which are needed during the malting and brewing processes (Hofer et al., 2016). But also the enzymes of *Fusarium* ssp. can influence malt quality attributes by these altered enzyme levels (Laitila et al., 2007).

Besides the production of metabolites such as mycotoxins or surface-active substances (e.g. hydrophobins), changes in amylase activity have also been reported (Oliveira et al., 2012; Oliveira et al., 2013; Sarlin et al., 2005; Sarlin et al., 2007). An enhanced expression of amylolytic enzymes (α- and β-amylase as well as limit dextrinase) and cytolytic enzymes in infected samples has also been reported previously (Hofer et al., 2016). Moreover, cytolytic malt specifications such as beta-glucan and sample viscosity are decreased by *Fusarium* infection.

Furthermore, proteolysis is also altered by *Fusarium* infection. It has been reported that values for soluble nitrogen, Kolbach index (KI), and free amino nitrogen (FAN) are increased significantly when kernels are infected with *Fusarium* spp. (Oliveira et al., 2012; Oliveira et al., 2013). Proteins play a key role in barley growth and later in the malting process (Gupta et al., 2010) as well as in beer production. They are also involved in host-pathogen interactions when *Fusarium* spp. attack plants (Boddu et al., 2006; Yang et al., 2012). Some barley proteins are partially degraded during malting (Weiss et al., 1992), but others (e.g. α-amylases) are also synthesized as de novo enzymes during malting (Georg-Kraemer et al., 2001; Hofer et al., 2016). Fungal enzymes that degrade barley cell walls during barley growth are versatile (Federici et al., 2006; Igawa et al., 2005). But also starch and proteins are degraded in an enhanced manner after fungal infection with *Fusarium* spp. (Oliveira et al., 2012).

Since enzymes in the albumin (water-soluble) fraction are associated with metabolism and the protection from pathogens, albumins play a key role in host-pathogen interactions as well as in the degradation processes in barley and barley malt when kernels are attacked by *Fusarium* fungi (Goesaert et al., 2005). Globulins are related to kernel texture, whereas prolamins and glutelins are the main storage proteins in barley (Tatham and Shewry, 2012).

The present study aimed to investigate changes in protein profiles during the malting process with respect to albumins. The focus was on checking what influences *F. avenaceum* infection has on the albumin profile of barley malt. Therefore, greenhouse-grown infected samples (INOC) and uninfected samples (MOCK) were fractionated before, during, and after malting and were analyzed using Osborne fractionation, isoelectric focusing, and capillary gel electrophoresis. The data were compared using a newly designed graphing software. The results elucidated the changes in the albumin profile during the malting process and revealed the influence of *Fusarium* infection on the protein composition of malt.

2. Materials and methods

2.1. Materials

2.1.1. Barley grain material

Barley plants were cultivated according to the procedure published by Hofer et al. (2016). In short, the double row spring barley (variety Grace) was used for the trial. For each test series (INOC and MOCK) 20 pots with 8 plants each were cultivated in a greenhouse chamber under controlled conditions (16 h/8 h day/night rhythm, 15/12 °C, 60% rel. humidity). At growth stage (GS) 65–69, during anthesis, each spike (INOC) was inoculated with a *F. avenaceum* spore suspension (50,000 spores/mL in 0.02% Tween 80). Spikes of control plants (MOCK) were not inoculated with *Fusarium* spores but sprayed with a 0.02% Tween 80 solution. After spray treatment, spikes were covered with polythene bags for 48 h to maintain high humidity for facilitating fungal infection (control plants were also covered with bags). Additional air humidifiers were positioned to increase the relative humidity up to > 90% for seven days. Inoculated (INOC) and MOCK-treated barley spikes were harvested from ripened barley plants at GS 93 and threshed using a single spike thresher. The threshed grains of each variant were then pooled to obtain a sample quantity of approximately 250 g. The samples for analysis and malting were taken from the sample pool to obtain maximal homogeneity.

2.1.2. Malting

INOC and MOCK barley grains were malted using a pattern for pale malt according to MEBAK guidelines for micromalting (R-110.00.008 [2016-03]; (Jacob, 2016)). Malting procedure was done once using 150 g grain material of the pooled sample of each treatment. INOC and MOCK-treated samples were processed separately to prevent from cross contamination. The steeping procedure was performed at defined conditions for three days and was split into wet steeping and aeration. Wet steeping was performed in a stainless steel steeping tank (5 h), followed by air rest (19 h) in a climatic chamber (14 ± 0.1 °C, 95–98% rel. humidity). A steeping degree of 44.5% was achieved on the second day (wet steeping for 4 h and air rest for 20 h).

The steeping degree was adjusted by spraying on the third day when deviations occurred. Germination was performed in a climate chamber (14 ± 0.1 °C, 95–98% rel. humidity) for 96 h by turning the green malt twice a day. After germination was complete, the green malt was dried starting the kilning process with withering (16 h, 50 °C), before temperatures were gradually increased (1 h, 60 °C; 1 h, 70 °C; 5 h, 80 °C).

2.2. Methods

2.2.1. MEBAK methods

Standard malt analyses were performed in technical triplicates according to the approved standard MEBAK methods (Jacob, 2016). The moisture content of the barley and malt samples was determined following MEBAK method R-200.18.020 [2016-03] (Jacob, 2016). To assess the amylolytic properties of the samples, extract levels and α-amylase activity were measured. The malt extract was measured using an Anton Paar DMA 4500 (Anton Paar, Graz, Austria) following MEBAK method R-205.01.080 [2016-03] (Jacob, 2016). A Ceralpha α-amylase assay kit (Megazyme, Wicklow, Ireland) was used to measure the α-amylase activity (Jacob, 2016; McCleary et al., 2002).

The proteolytic properties of the malted samples were also assessed. The KI (Jacob, 2016) was calculated from the measured soluble nitrogen and raw protein values obtained using a FOSS Tecator™

digestion system and a Kjeltec™ 8460 system (FOSS GmbH, Hillerød, Denmark) following MEBAK methods R-205.11.030 [2016-03] and R-200.20.030 [2016-03] (Jacob, 2016), respectively. The FAN content was quantified using a BioTek Synergy H4 Hybrid microplate reader (BioTek, Winooski, VT, USA) as described in MEBAK method R-205.14.111 [2016-03] (Jacob, 2016). All samples were analyzed in technical triplicates.

2.2.2. Determination of mycotoxins

Fusarium enniatins [ENNs *B*, *B1*, *A*, and *A1* and *beauvericin* (*BEA*)] were quantified in barley and barley malt. Sample preparation was performed the same way as published before, with the same liquid chromatography-tandem mass spectrometry (LC-MS/MS) settings (Habler and Rychlik, 2016). Briefly, 1 g of sample was mixed with a volume of 10 mL of acetonitrile/water mixture. The mixture was shaken and internal standards were added to 4 mL of the supernatant. Internal standard were *BEA* (purchased from supplier AnaSpec, San Jose, USA), *ENN B* (purchased from supplier Bioaustralis, New South Wales, Australia), and further enniatins *B1*, *A1*, and *A* (purchased from supplier Enzo life Sciences, Lörrach, Germany). After homogenization, the entire mixture was applied to a Bond Elut Mycotoxin cartridge (Agilent Technologies, Santa Clara, CA, USA), drawn through the cartridge by vacuum suction, and evaporated to dryness. The sample was re-suspended with acetonitrile/water and membrane-filtered. LC-MS/MS analysis was then performed at 40 °C using a Shimadzu LC-20A Prominence system (Shimadzu, Kyoto, Japan) fitted with a Hydrosphere RP-C18 column (YMC Europe GmbH, Dinslaken, Germany) and a C_{18}-guard column as the stationary phase (Phenomenex, Aschaffenburg, Germany). The injection volume of each sample was 10 μL. 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, 4500 eV; spray gas, 80 psi; dry gas, 75 psi; and temperature, 450 °C. All samples were extracted and analyzed in technical duplicates.

2.2.3. Fungal biomass quantification

Isolation of genomic DNA from grain and malt was performed as described previously by Linkmeyer et al. (2013). Briefly, 2 g of barley or malt flour was lysed and the DNA was precipitated using different types of salt buffer. Chloroform/isoamyl alcohol and RNAse were used to remove proteins, polysaccharides, and RNA, respectively. Produced DNA pellets were washed with ethanol followed by resolving in water to a final concentration of 20 ng/μL. Quantification of species-specific *Fusarium* DNA in grain and malt of INOC and MOCK samples was performed using the published qPCR protocol of Nicolaisen et al. (2009), adapted by Linkmeyer et al. (2013). Genomic DNA of *F. avenaceum* and barley plant DNA was amplified in an MX3005P Cycler (Stratagene, Santa Clara, CA, USA) using 2X SYBR Green qPCR Master Mix (Thermo Scientific, Schwerte, Germany) and specific primer pairs. The absolute quantification of *Fusarium* and barley DNA was calibrated using standard curves made from a dilution series of pure fungal DNA and referenced to the barley DNA content. All samples were analyzed in technical duplicates. The amount of fungal DNA was normalized to the content of plant DNA in a sample and is given as 'pg/ng plant DNA'.

2.2.4. Osborne fractionation

Osborne fractionation was performed with a modification of a previously published procedure (Faltermaier et al., 2013). A 0.2 g milled sample of every specimen was extracted with 0.5 mL of distilled MilliQ water by vortexing and then shaking for 30 min on a vertical shaker. Samples were then centrifuged for 10 min at 4 °C and the supernatants were collected. Extraction was then repeated on the pellets with a further 0.5 mL of water to produce a second supernatant. Both supernatant extracts from each sample were pooled and stored at −20 °C until needed.

2.2.5. Isoelectric focusing

Isoelectric point- (pI-) based OFFGEL fractionation was performed using a 3100 OFFGEL electrophoresis system (Agilent Technologies, Santa Clara, CA, USA) combined with an OFFGEL Kit, pH 3–10, with a 24-well setup following the protocol of the supplier and the procedure published by Munoz-Insa et al. (2016). First, the gel strips (linear pH gradient ranging from 3 to 10) were rehydrated 10 min before samples were loaded. The extracted samples were then dispensed above all wells in the OFFGEL strip and positioned in the OFFGEL rig. When applying a voltage, individual proteins stop migrating when the pH becomes equal to the protein pI. The fractionated samples were recovered using a pipette and processed for the following analysis using capillary gel electrophoresis.

2.2.6. Capillary gel electrophoresis

Molecular weight-based separation procedures were performed on an Agilent 2100 Bioanalyzer in combination with the Protein 80 Kit and Agilent 2100 Bioanalyzer Expert Software. All samples were prepared according to the procedure of Munoz-Insa et al. (2016) and according to the protocol provided by the supplier (Agilent Technologies, Santa Clara, CA, USA). The samples were extracted and analyzed in technical duplicates.

2.2.7. Visualization tool

Visualization to facilitate comparison between samples was done using software developed in-house using Matlab (version R2016a). This tool compares peak levels, as percentages of total, between two samples at the various pIs of the proteins. The software tool calculates whether peaks present in the reference sample increase or decrease and whether peaks had appeared or disappeared. The software was designed with a 1% tolerance for "peak drift," which allows the kDa values of peaks to shift up to 1% relative to the reference sample and still be recognized as the reference peak in the comparator sample. The software also allows for 5% sensitivity in peak size changes and only recognizes changes greater than this threshold. The tolerance and sensitivity values were based on previous experience with the expected variance of sample analyses in the Agilent Bioanalyzer, allowing for an α of 0.05.

The software visualizes these changes by plotting a graph with pI as the horizontal axis and kDa as the vertical axis. The peaks that grow in the comparator sample are represented by circles, whereas those that shrink are represented by triangles. New peaks are represented by squares, and disappearing peaks are represented by crosses. In the case of shrinkage or growth, the size of the points on the graph is directly proportional to the magnitude of the change in peak size; for new or lost peaks, the point sizes are directly proportional to the size of the peak in question. The particular spots in the protein maps are assigned to regions.

2.2.8. Data analysis

The statistical analysis was performed using SPSS statistics 22 software (IBM Corporation, New York, NY,USA). Correlation analyses of malt quality characteristics were conducted by computing Pearson correlation coefficients, were the statistically significant correlations are indicated by **($P \leq 0.01$).

3. Results and discussion

In this study, *F. avenaceum* was chosen for grain infection experiments since an earlier publication assessing European barley grain material has revealed a predominant role of this species when it comes to kernel discoloration (Geißinger et al., 2017). *F. avenaceum* is not only known to be involved in kernel discoloration, but also to have an impact on kernel processing qualities such as enhanced amylolysis and especially, proteolysis. In addition to greenhouse-grown kernel material, which was purposefully infected with *Fusarium* spores (INOC), uninfected (MOCK) barley grain was also produced and processed

Table 1

Intensity of fungal infection indicated by the amount of *F. avenaceum* DNA as well as the level of contamination indicated by the amount of mycotoxins in uninoculated (MOCK) and in inoculated (INOC) barley and barley malt samples. Analysis was performed in duplicates. *Abbreviations*. ENN B: enniatin B; ENN B1: enniatin B1; ENN A1: enniatin A1; ENN A: enniatin A; LOD: limit of detection;

		F. avenaceum DNA	Mycotoxins							
Material	Treatment	pg/ng barley DNA	ENN B (μg/kg)	SD	ENN B1 (μg/kg)	SD	ENN A1 (μg/kg)	SD	ENN A (μg/kg)	SD
Barley	MOCK	0.03	2.8	0.4	< LOD	–	< LOD	–	< LOD	–
Malt	MOCK	4.25	12.3	0.1	< LOD	–	< LOD	–	< LOD	–
Barley	INOC	100.40	17,840	420	600	11	596	47	49	4
Malt	INOC	93.68	10,948	630	620	21	476	29	36	1

identically.

3.1. Fungal growth during the malting procedure

To evaluate fungal growth during malting, changes in fungal biomass were quantified using qPCR (Table 1). In barley and malt MOCK samples, the amount of *Fusarium* DNA was 0.03 and 4.25 pg/ng plant DNA, respectively. This low DNA amounts were not expected in MOCK samples, but can be explained by slight cross contamination while cultivation; MOCK and INOC samples were cultivated in one greenhouse chamber to ensure comparability. In contrast, the *F. avenaceum* DNA levels in INOC samples was around 3000-fold higher, with values of 100.4 and 93.7 pg/ng in raw barley grain and malt, respectively. This indicates that very little *Fusarium* biomass is sufficient in our trials to produce substantial *Fusarium* growth during the malting process. In the case of the INOC sample, *Fusarium* DNA decreased by 6.7% after malting. DNA levels were also measured after steeping and germination and increased continuously until kilning (data not shown). After malt cleaning, *Fusarium* DNA levels decreased slightly because of the removal of rootlets and dust as it was shown earlier by Habler et al. (2016).

3.2. Fusarium mycotoxins

Since *F. avenaceum* produces mycotoxins, in particular enniatins (ENNs) and beauvericin (BEA), the mycotoxin contents of barley and the corresponding malts were determined (Table 1), because mycotoxins have implications for food safety aspects and can affect protein biosynthesis. BEA was not found in any samples, whereas ENNs were found in high amounts in INOC samples. The barley MOCK samples contained very low mycotoxin concentrations that were mostly below the limit of detection (LOD); ENNs were found in only one case, where ENN B was present at very low concentrations by possible cross contaminations in cultivation. ENNs B1, A1, and A were below the limit of detection. In the malt MOCK sample, the ENN B content was four-fold higher. Other mycotoxins were below the LOD. The barley INOC samples contained high mycotoxin concentrations due to the metabolites of fungal growth. Interestingly the ENN B content decreased over the malting procedure by a factor of 1.6. The decrease of mycotoxins can be the result of malt cleaning, when rootlets and dust are removed by a malt cleaner as it was shown earlier by Habler et al. (2016).

3.3. Malt quality attributes

Fusarium spp. are known to alter malt quality characteristics (Geißinger et al., 2017; Oliveira et al., 2012; Sarlin et al., 2005). The malt quality attributes analyzed in this study are shown in Table 2 for both, the MOCK and INOC samples. The extract in the INOC sample was 1.18% higher than in the MOCK sample, as were the amylolytic activities of α- and β-amylase 14.29% and 13.37% respectively. Raw protein was a little lower in infected samples, but soluble nitrogen increased (not significant). This resulted in KIs of 40.8% in the infected sample and 38.6% in the uninfected one. Since *Fusarium* is known to enhance the proteolytic activity (Oliveira et al., 2013; Sarlin et al., 2007), this behavior is consistent, as it also is with regard to FAN values.

3.4. Protein plots

Since albumins play an important role in pathogen-host interactions as well as in enzyme activity in barley and corresponding malts (Sarlin et al., 2005), albumins were analyzed after extraction from *Fusarium*-infected (INOC) and uninfected (MOCK) samples. To evaluate the changes in protein profiles during the malting process, samples were taken and immediately frozen in liquid nitrogen after four malting steps (barley, poststeeping, postgermination, and postkilning). These samples were later analyzed by Osborne fractionation, isoelectric focusing, and ultimately microfluidic gel electrophoresis.

Resulting spots in the protein maps that changed by more than ± 5% when comparing MOCK and INOC samples were selected and the degree of change was calculated and plotted by our newly in-house developed visualization tool (Figs. 1–4). Protein maps with all spots including changes lower 5% are pictured in Supplementary Figs. 1–4. The resulting spots were later identified using the protein databases uniprot.org and expasy.org and are later discussed according to the literature (Table 3). The relevant spots are classified into different regions and are referred to from the left to the right direction.

Albumins in Barley: Changes in the Albumin Fraction of Barley caused by F. avenaceum. When barley is infected with *Fusarium* spp., the protein profile, especially that of albumins (leucosins), changes markedly. Due to water solubility, albumins are important for brewers and maltsters, such as *nonspecific lipid transfer proteins* (*ns-LTPs*), *protein Z-type serine protease inhibitors* (*serpins*), which are involved in host–pathogen interactions, as well as amylases and defensive proteins, such as *defensin/thionin*, *barwin*, *trypsin/α-amylase inhibitors*, *thaumatin-like proteins* (*TLPs*), *barley monomeric α-amylase inhibitor* (*BMAI*), and *barley dimeric α-amylase inhibitor* (*BDAI*).

The virtual 2D-PAGE maps generated by our in-house developed visualization tool (Figs. 1–4) show the differences (changes) between the MOCK and INOC barley samples. These plots show spots that increased or decreased, as well as those that appeared or disappeared, due to fungal infection. In regions 1 and 2 (Figs. 1–4), the *LTP* protein class was located where changes in subregions were determined. *LTPs* are proteins with a molecular weight of 6–10 kDa (Finkina et al., 2016), which exhibit a wide pI range from 6 to 9 (Gorjanović, 2009). Other authors have reported even higher pI values (pI ~ 9–10) in the *LTP* group (Finkina et al., 2016). Many authors have suggested that *LTP* possesses antifungal properties when it interacts with fungal membranes (Carvalho and Gomes, 2007; Finkina et al., 2016; Gorjanović et al., 2005; Iimure and Sato, 2013; Perrocheau et al., 2006; Salminen et al., 2016; van Loon et al., 2006). Region 1 contained spots that have been previously reported as *LTP1* (Alexander and Morris, 2006; Finkina et al., 2016; Gorjanović et al., 2005; Hippeli and Elstner, 2002). *LTP1* spots in region 1 (Fig. 1) increased in barley samples after *Fusarium* infection by 38.5%, 7.2%, and 42.7%. After the steeping step (Fig. 2), the differences between the MOCK and INOC samples were inconsistent and the changes varied between −9.8% (decrease) and +15.5%

Table 2

Malt quality attributes of infected (INOC) and uninfected (MOCK) barley malt samples. Values represent means of triplicate extraction and measurement of each sample. Abbreviations: d. m.: dry matter; FAN: free amino nitrogen; SD: standard deviation; DU: dextrin building unit; DP: diastatic power; Except soluble nitrogen, the data represent significant correlation coefficients (** indicates $P \leq 0.01$) resulting from Pearson correlation analysis of treatment with data of quality attributes.

Analysis	Method (MEBAK; (Jacob, 2016))	Unit	INOC	SD	MOCK	SD	Difference due to fungal infection (%)
Moisture malt	R-200.18.020 [2016-03]	%	8.0	0.0	8.1	0.0	−1.23**
Extract malt	R-205.01.080 [2016-03]	%, as is	78.3	0.03	77.3	0.01	+1.29**
Extract malt d.m.	R-205.01.080 [2016-03]	%, d.m.	85.1	0.03	84.1	0.01	+1.18**
Viscosity (8.6%)	R-205.10.282 [2016-03]	mPa × s	1.954	0.007	1.890	0.018	+3.28**
pH value	R-205.06.040 [2016-03]		5.79	0.01	5.85	0.00	−1.04**
Raw protein	R-200.20.030 [2016-03]	%, d.m.	11.3	0.02	11.7	0.13	−3.54**
Soluble nitrogen d.m.	R-205.11.030 [2016-03]	mg/100 g malt d.m.	737	13.4	723	2.4	+1.90
Kolbach index	R-205.12.999 [2016-03]	%	40.8	0.75	38.6	0.20	+5.39**
FAN d.m.	R-205.14.111 [2016-03]	mg/100 g malt d.m.	140	3.7	128	2.5	+8.57**
α-Amylase	R-200.24.731 [2016-03]	DU	91	1.4	78	0.7	+14.29**
β-Amylase	R-200.22.111 [2016-03]	DP	636	0.0	551	4.2	+13.37**

(increase), whereas after germination (Fig. 3), the changes were between +6.9% and +37.4%. Finally, after malting was complete (Fig. 4), the *LTP1* spot changes varied between +6.3% and +24.4%. Gorjanović et al. (2005) posited that *LTP* served a protective function in host-pathogen interactions since it is classified as a pathogen-related protein (PR-14). In a previous study *LTP* was reported as a probable *α-amylase/protease inhibitor* (*PAPI*) (Jegou et al., 2001), but the discussed inhibitory effects were controversial (Perrocheau et al., 2005). The expression of *LTP1* might have increased, because of the high infective load in our trials but, despite this, the amount of *F. avenaceum* DNA did not drop. That indicates that *Fusarium* growth was not inhibited by the high *LTP1* levels (see also Table 1). *LTP2*, which has been described by Finkina et al. (2016) and Douliez et al. (2001), was possibly localized to region 2 (Fig. 1). In the INOC sample, the presumptive *LTP2* spots in region 2 disappeared, except in the stage after steeping. The putative *LTP2* could only be detected in the infected green malt sample after steeping, but it was reduced by −7.7% (Fig. 2). At the time points 'barley', 'post-germination', and 'post-kilning', this 6–7 kDa peptide was no longer detectable in the inoculated samples. All forms of *LTP* are known to bind lipids, with different binding potentials for saturated *cis*- and *trans*-fatty acids and lipids (Salminen et al., 2016). It is also known that *Fusarium* infection enhances the amount of fatty acids in cereal grains (Stuper-Szablewska et al., 2014). The lipid transfer activity of cereal *LTP2* has been reported to be fivefold higher than that of *LTP1* (Douliez et al., 2001). Thus, the reduction of the putative *LTP2* may be linked to its possible binding to fatty acids (Jones and Marinac, 1995), since after such binding this peptide may not be detectable with the methods used.

The *protein Z-type serpins* were located in region 3 (Figs. 1–4). These *protein Z-type serpins* are the most abundant proteins in barley and barley malt. This protein family is also found in finished beer, because it can be converted in a heat- and also protease-stable form (Gorjanović, 2009) analogous to *LTP* (Gorjanović et al., 2005). The theoretical molecular mass of barley *serpins* is around 43 kDa (Evans and Hejgaard, 1999; Gorjanović, 2009; Hejgaard, 1982; Perrocheau et al., 2005; Steiner et al., 2011), and the isoelectric points are between 5.5 and 6.8 (Rasmussen et al., 1996; Roberts et al., 2003). Except for two spots, the *serpins* increased in *Fusarium*-infected barley, with the values being −19.0%, +17.1%, +24.4%, −25.9%, and +12.1% relative to the uninfected control (Fig. 1). Eggert et al. also reported an increase of *protein Z* in emmer grains when kernels were infected with *Fusarium* (Eggert et al., 2011). But the samples in this study were infected with *F.*

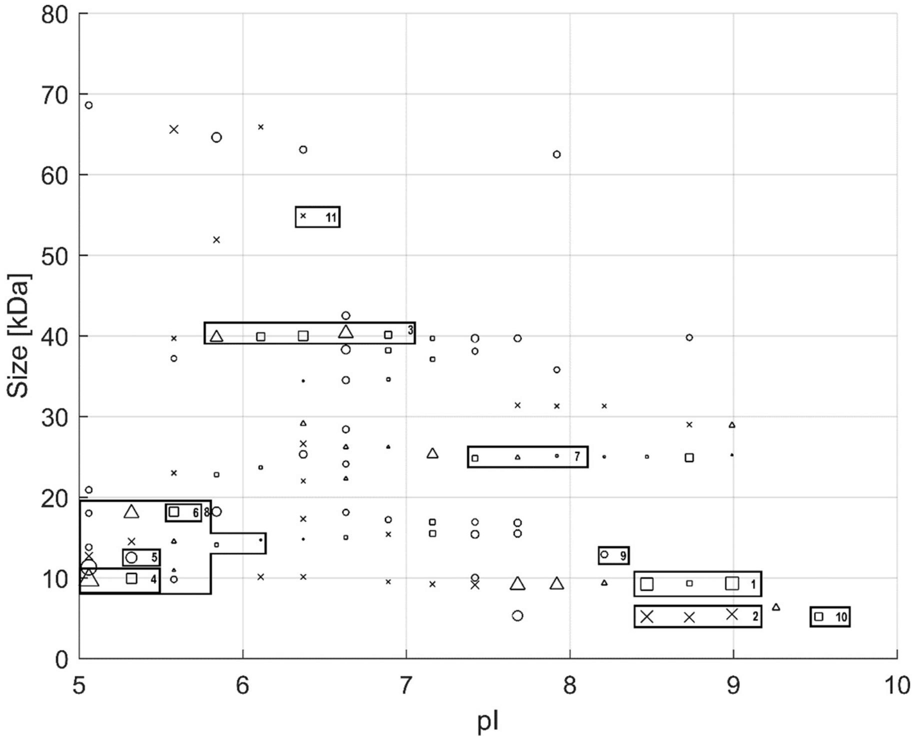

Fig. 1. Barley protein map. The calculated differences of spots (barley MOCK-barley INOC); ○: new spot (appeared due to Fusarium infection); ×: extinct spot (disappeared due to Fusarium infection); □: increased spot (increased due to Fusarium infection); Δ: decreased spot (decreased due to Fusarium infection). Figure indicates spots of proteins, which contribute more than 5% at each pI. Sizes of spots reflect the magnitude of the changes from MOCK to INOC sample. The relevant spots are disposed in different regions and are referred to always from the left to the right direction. Abbreviations: kDa: kilodalton; pI: isoelectric point.

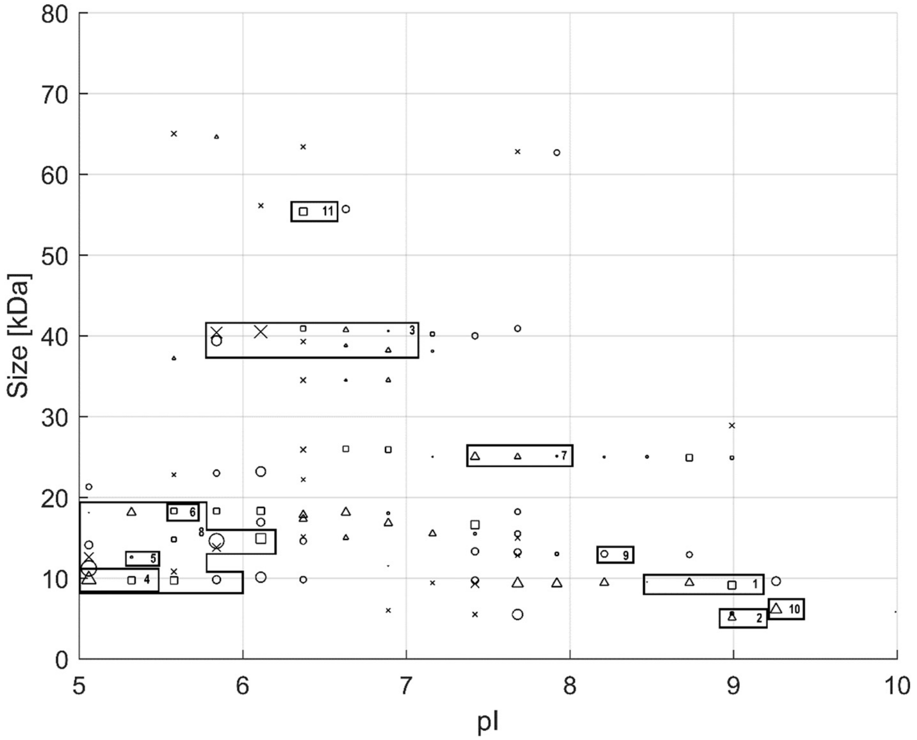

Fig. 2. Steeping protein map. The calculated differences of spots (steeping MOCK–steeping SPRAY); ○: new spot (appeared by Fusarium infection); ×: extinct spot (disappeared by Fusarium infection); □: increased spot (increased by Fusarium infection); Δ: decreased spot (decreased by Fusarium infection). Sizes of spots indicate the magnitude of the changes. The relevant spots are disposed in different regions and are referred always from the left to the right direction. Abbreviations: kDa: kilodalton; pI: isoelectric point.

culmorum and *F. graminearum*, but not with *F. avenaceum*. After steeping, these values ranged between −4.1% and +7.0% (Fig. 2), and after germination, except for one spot, all the *serpins* were reduced by *Fusarium* infection; only the spot with a pI between 6.76 and 7.03 increased (by 10.8%) when compared to the uninfected sample. After kilning, the differences between the MOCK and INOC samples were −6.0%, −3.3%, +0.3%, +10.5%, and +15.2%.

Subtilisin-chymotrypsin inhibitors were located in region 4 (Fig. 1). Two spots were identified, which probably correspond to the *subtilisin-chymotrypsin inhibitors CI-1a* and *CI-1b* (Greagg et al., 1994; Perrocheau et al., 2005). Several isoinhibitors with the same function are found both in plants and in animals (Chang and Tsen, 1981). These types of inhibitors act on serine proteases and belong to the potato chymotrypsin inhibitor family I (Perrocheau et al., 2005). Putative *CI-1a* levels decreased after *Fusarium* infection by −48.8% in barley, −25.4% post-steeping, −50.8% post-germination, and −65.9% in the malt sample. In contrast, the putative *CI-1b* increased by +26.3%, +12.8%, +2.0%, and +8.9% from barley to malt (Figs. 1–4). This suggests that the

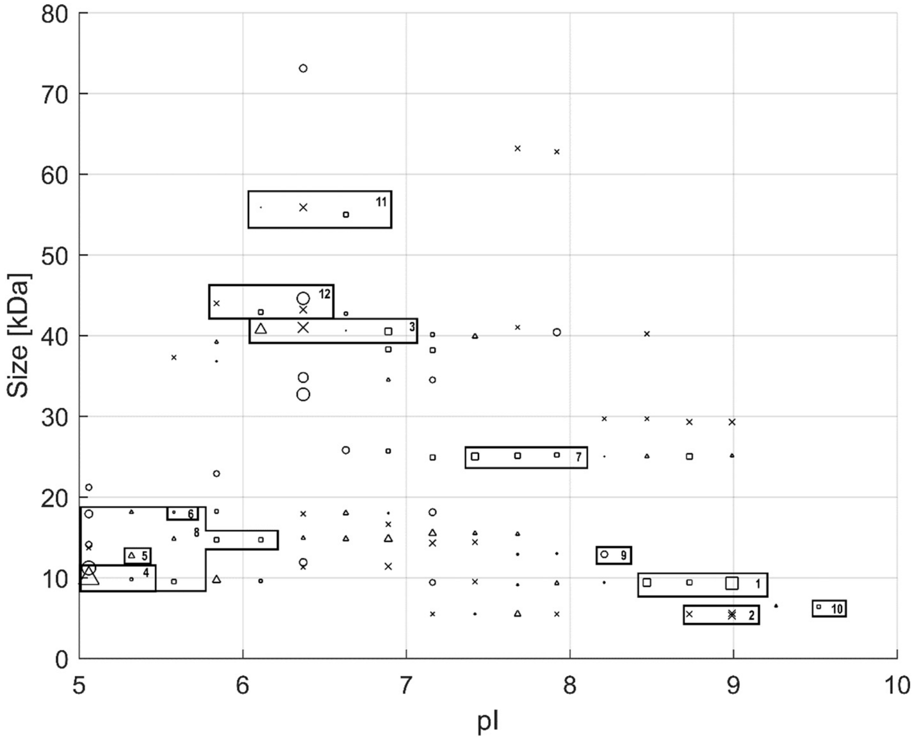

Fig. 3. Germination protein map. The calculated differences of spots (germination MOCK–germination SPRAY); ○: new spot (appeared by Fusarium infection); ×: extinct spot (disappeared by Fusarium infection); □: increased spot (increased by Fusarium infection); Δ: decreased spot (decreased by Fusarium infection). Sizes of spots indicate the magnitude of the changes. The relevant spots are disposed in different regions and are referred always from the left to the right direction. Abbreviations: kDa: kilodalton; pI: isoelectric point.

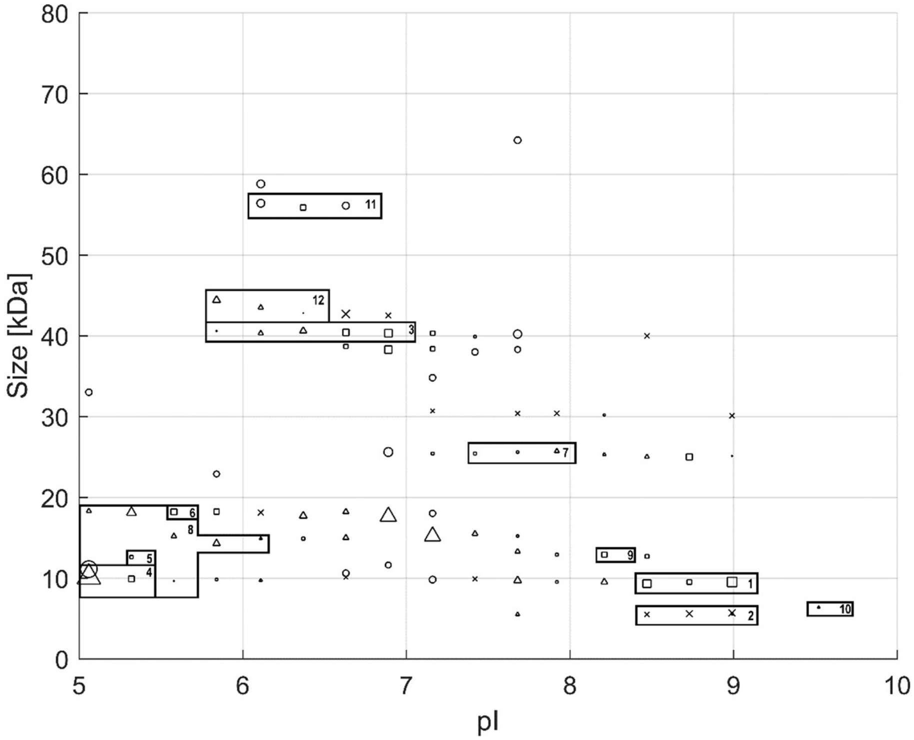

Fig. 4. Kilning protein map. The calculated differences of spots (kilning MOCK–kilning SPRAY); ○: new spot (appeared by Fusarium infection); ×: extinct spot (disappeared by Fusarium infection); □: increased spot (increased by Fusarium infection); Δ: decreased spot (decreased by Fusarium infection). Sizes of spots indicate the magnitude of the changes. The relevant spots are disposed in different regions and are referred always from the left to the right direction. Abbreviations: kDa: kilodalton; pI: isoelectric point.

pathogen-barley interaction leads to a striking decrease in putative *CI-1a* over the malting process, whereas *CI-1b* conversely increases over the same period as a response of the kernels to the *Fusarium* infection.

The spot in region 5 (Fig. 1) may be a *cysteine proteinase inhibitor* (Santamaria et al., 2015). This protein was first found in rice (Kondo et al., 1990), but has also been reported in barley (Santamaria et al., 2015). It has been reported to be involved in the control of germination by inhibition of endogenous cysteine proteases, but it may also play a role in host-pathogen interactions by inhibiting exogenous proteases. Region 5 was only found in *Fusarium*-infected samples in both barley and poststeeping processes (Figs. 1 and 2), which could indicate the involvement of this protein in the defense response. After germination, this spot was also found in the MOCK sample and was calculated to be reduced by −5.1% in response to *Fusarium* infection (Fig. 3). In the finished malt sample, this protein increased by +3.0% in the infected samples.

Putative *thaumatin-like proteins* (*TLPs*) were located in regions 6 and 7 (Figs. 1–4). The spot in region 6 could be *TLP4*, with a molecular mass of 17.8 kDa and a pI of 5.69 (Gorjanović, 2009). *TLP4* is categorized as a weak-acidic-pI *TLP* (Singh et al., 2017) and its levels were +22.0% higher in *Fusarium*-infected barley compared to the corresponding MOCK sample. After steeping, the difference was −10.3%, whereas after germination and in malt, the differences were +1.2% and +8.8%, respectively. Putative *TLP5*/*TLP6*, *TLP7*, and *TLP8* could be found in region 7 (Figs. 1–4), with molecular masses of around 25 kDa and pI between 7.5 and 8.0 (basic-pI *TLPs*; (Singh et al., 2017)). *TLP5*/*TLP6* increased by 8.6%, *TLP7* decreased by 2.9%, and *TLP8* was newly synthesized in *Fusarium*-infected barley. After steeping, *TLP5*/*TLP6* and *TLP7* decreased by 10.2% and 5.3%, respectively, whereas *TLP8* was newly synthesized in the INOC sample relative to the MOCK sample. After germination, all spots increased by 12.3%, 6.9%, and 4.9%, but after kilning, the magnitude of the differences decreased and was +2.4% (*TLP5*/*TLP6*), +1.7% (*TLP6*), and −3.1% (*TLP8*). *TLPs* are products of a diverse gene family and are involved in host-pathogen interactions for defensive (antifungal) purposes, as well as in plant development (Liu et al., 2010). They have been reported to inhibit hyphal growth and sporulation in a multiplicity of fungi (Kuwabara et al., 2002), and the antifungal effect is mediated by the electrostatically polarized surface of the protein (Gorjanović, 2009). Some *TLP* spots decreased in infected samples, whereas others appeared in the infected barley and were absent in the uninfected control. The *TLPs* of ascomycetes are similar to those of barley (Liu et al., 2010) and are found to be close to each other in 2D-PAGEs. The new and elevated spots in this region could, therefore, be either of fungal origin or the result of the barley-pathogen interaction.

Furthermore, the putative proteins *trypsin/α-amylase inhibitors* and *α-amylase inhibitors* (Gorjanović, 2009; Oliveira et al., 2013; Perrocheau et al., 2005; Sultan et al., 2017), also known as *BMAI* and *BDAI*, were located nearby in region 8 (Figs. 1–4). In this region, we found up to 11 spots, besides regions 4, 5, and 6, which either increased or decreased with *Fusarium* infection (Figs. 1–4). These spots could be, among other possibilities, the *trypsin/α-amylase inhibitors CMa*, *CMb*, and *CMd* (Gorjanović, 2009; Sultan et al., 2017). Other authors have described 14 spots on gels in this area (Perrocheau et al., 2005). These inhibitors are varied, and ranges between 12 and 16 kDa in molecular weight and 4 and 7 in pI have been reported (Perrocheau et al., 2005). Other authors have described these inhibitors as having a pI range of 5–6 with a molecular weight range of 10–15 kDa (Sultan et al., 2017). *The trypsin/α-amylase inhibitor CMa* varied by +3.4%, +34.5%, +4.8%, and −6.5% in the four steps from barley to malt, whereas changes in *CMb* varied by −3.0%, +5.2%, −2.3%, and −3.5%, respectively. *CMd* disappeared in *Fusarium*-infected barley and was not found in any of the subsequent malting steps. *BMAI* increased by 24.9% after steeping, but changes were smaller (< 4.2%) at the other processing steps. In case of *BDAI*, spots decreased in all four malting steps from barley to malt by −26.8%, −10.3%, −2.1%, and −11.8%, indicating that *Fusarium* infection lowers the relative amount of *BDAI*, but increases the amount of *BMAI* during steeping and germination.

The spot in region 9 (Fig. 1) is putatively *barwin*, a small protein (13.7 kDa) with a basic pI (Finnie and Svensson, 2014; Svensson et al., 1992). As most of the proteins identified in this work, *barwin* also possesses an antifungal potential and has been reported to intensify the antifungal effects of *TLP* as well as *chitinase* (Yang et al., 2010). *Barwin* was only found in the infected barley sample, which implies that it is

Table 3

Spot numbers of putative targets (Fig. 1–4). The table shows the change in putative proteins at four malting steps: barley, poststeeping, postgermination, and kilned malt. Changes in protein levels (% of target protein of each pI) are expressed as a percentage after calculating the amount of protein found in the MOCK sample minus the amount found in the INOC sample (M-S) in an each well after isoelectric focusing. Theoretical molecular weights and pIs were calculated using the ExPASy calculation tool at https://web.expasy.org/compute_pi/ using target entries from www.uniprot.org. *Abbreviations*. MW: molecular weight; pI: isoelectric point; theor.: theoretical; obs.: observed.

Spot #	Putative protein	Change barley M-S (%)	Change steeping M-S (%)	Change germination M-S (%)	Change malt M-S (%)	MW theor./obs.	pI theor./obs.	Theor. reference	UniProt entry
1	*LTP1*	+38.5	+0.1	+ 14.6	+16.7	9.33/9.20–9.40	8.66/8.34–8.60	(Finkina et al., 2016)	P07597
1	*LTP1*	+7.2	−9.8	+6.9	+6.3	9.35/9.30–9.40	8.78/8.60–8.86	(Finkina et al., 2016)	P07597
1	*LTP1*	+42.7	+15.5	+37.4	+24.4	9.47/9.3–9.5	9.26/8.86–9.13	(Finkina et al., 2016)	P07597
2	*LTP2*	−44.9	± 0	± 0	−7.8	6.96/5.1–5.2	8.22/8.34–8.60	(Finkina et al., 2016)	O81135
2	*LTP2*	−31.7	± 0	−10.7	−13.6	6.96/5.5	8.22/8.60–8.86	(Finkina et al., 2016)	O81135
2	*LTP2*	−37.5	−7.7	−31.4	−14.4	6.96/5.3–5.7	8.22/8.86–9.13	(Finkina et al., 2016)	O81135
3	*Protein Z*	−19.0	± 0	−1.5	+0.3	42.69/39.2–40.6	5.45/5.71–5.98	(Gorjanović, 2009; Perrocheau et al., 2005)	Q43492
3	*Protein Z*	+17.1	± 0	−15.8	−3.3	43.28/39.9–40.7	5.72/5.98–6.24	(Gorjanović, 2009; Perrocheau et al., 2005)	P06293
3	*Protein Z*	+24.4	+7.0	± 0	−6.0	−/40.0–40.6	−/6.24–6.50	(Gorjanović, 2009; Perrocheau et al., 2005)	
3	*Protein Z*	−25.9	−4.1	−0.1	+10.5	42.95/40.3–40.7	6.77/6.50–6.76	(Gorjanović, 2009; Perrocheau et al., 2005)	Q40066
3	*Protein Z*	+12.1	−0.4	+10.8	+15.2	42.95/40.1–40.6	6.77/6.76–7.03	(Gorjanović, 2009; Perrocheau et al., 2005)	Q40066
4	*Subtilisin-chymotrypsin inhibitor*	−48.8	−25.4	−50.8	−65.90	8.88/9.70–10.10	5.24/4.93–5.19	(Greagg et al., 1994)	P16062
4	*Subtilisin-chymotrypsin inhibitor*	+26.3	+12.8	+2.0	+8.9	8.96/9.70–9.90	5.33/5.19–5.45	(Greagg et al., 1994)	P16063
5	*Cysteine proteinase inhibitor*	+18.4	+1.2	−5.1	+3.0	12.78/12.5–12.7	5.22/5.19–5.45	(Kondo et al., 1990)	Q1ENF6
6	*TLP4*	+22.0	−10.3	+1.2	+8.8	17.8/18.1–18.2	5.69/5.45–71	(Gorjanović, 2009)	Q946Z1
7	*TLP5/TLP6*	+8.6	−10.2	+12.3	+2.4	21.35/24.8–25.4	7.53/7.29–7.55	(Singh et al., 2017)	Q946Z0
7	*TLP7*	−2.9	−5.3	+6.9	+1.7	21.38/24.9–25.6	7.91/7.55–7.81	(Singh et al., 2017)	Q946Y9
7	*TLP8*	+1.5	+0.7	+4.9	−3.1	21.85/25.1–25.6	8.15/7.81–8.08	(Singh et al., 2017)	Q946Y8
8	*Trypsin/α-amylase inhibitor CMa*	+3.4	+34.5	+4.8	−6.5	16.1/14.1–14.7	5.87/5.45–7.71	(Gorjanović, 2009)	P28041
8	*Trypsin/α-amylase inhibitor CMb*	−3.0	+5.2	−2.3	−3.5	17.2/14.5–15.2	5.77/5.71–5.98	(Gorjanović, 2009; Sultan et al., 2017)	P32936
8	*Trypsin/α-amylase inhibitor CMd*	−15.2 d	–	–	–	17.9/14.5	5.24/5.19–5.45	(Gorjanović, 2009)	P11643
8	*BMAI*	+1.0	+24.9	+4.2	−1.1	14.44/14.7–14.9	6.16/5.98–6.24	(Perrocheau et al., 2005)	P16968
8	*BDAI*	−26.8	−10.3	−2.1	−11.8	17.05/18.0–18.1	5.36/5.19–5.45	(Sultan et al., 2017)	P13691
9	*Barwin*	+7.0	+7.0	+7.0	+7.2	13.74/12.9–13.0	7.76/8.08–8.34	(Gorjanović, 2009; Svensson et al., 1992)	P28814
10	*Defensin*	+15.0	−15.7	+3.5	−0.9	5.25/5.2–6.4	9.77/9.39–6.65	(Portieles et al., 2006)	P20230
11	*β-Amylase*	−6.0	+14.4	−16.4	+7.4	55.19/54.9–55.9	5.99/6.24–6.50	(Finnie et al., 2011)	P82993
12	*α-Amylase*	–	–	−8.1	−7.8	45.42/44.0–44.4	5.42/5.71–5.98	(Rogers and Milliman, 1983)	P00693
12	*α-Amylase*	–	–	+4.8	−3.3	44.93/42.9–43.5	5.66/5.98–6.24	(Rogers, 1985; Vallée et al., 1998)	P04063
12	*α-Amylase*	–	–	+23.0	−0.1	45.46/42.8–44.6	5.88/6.24–6.50	(Alexander and Morris, 2006)	P04750

involved in the plants defense response to fungal attacks. Its levels were elevated by +7.0%/+7.2% at all malting stages in *Fusarium*-infected samples. This implies that the putative *barwin* is produced by *Fusarium* during barley growth, is still present after harvest, and does not vary. Besides *LTP1*, *barwin* was the only protein that increased over all the malting steps due to the *Fusarium* infection.

The spot in region 10 (Fig. 1) could be a *thionin* or *defensin* barley grain peptide (PRP-13) (Gorjanović, 2009) and it increased after infection. *Defensins* are small plant polypeptides (~5 kDa) with basic pIs and antimicrobial properties (Lay and Anderson, 2005). *Thionins* as well as *defensins* and *ns-LTPs* have been found in several cereal seeds (Egorov et al., 2005) and are classified as antimicrobial peptides with protective functions against plant pathogens (Gorjanović, 2009). This spot increased by 15.0% in infected barley but decreased by 15.7% after steeping and then changed by +3.5% and −0.9% in the final two steps of the malting process.

The spot in region 11 (Fig. 1) is a putative *β-amylase* in barley, which has a molecular weight of 55 kDa and a pI of ~6 (Eggert et al., 2011; Finnie et al., 2011). We did expect an increase in β-amylase in this sample because previous work based on gene expression studies has shown that β-amylase in barley was upregulated in infected samples at the transcriptome level (Hofer et al., 2016). Here, we found that the putative *β-amylase* level decreased in the proteome in a *F. avenaceum* infected barley sample, whereas other authors have described an increase of *β-amylase* in *F. culmorum*-infected and *F. graminearum*-infected emmer grains (Eggert et al., 2011). The use of different *Fusarium* spp. could explain the discrepancies in the studies. A further possible reason for these discrepancies could be the enzymatic degradation of *β-amylase*, since Yang et al. (2010) have described the degradation processes of *β-amylase* in a *Fusarium*-infected sample. In a proteome-based study, they found fragments of *β-amylase* in a *Fusarium*-infected sample, which were products of a specific endoprotease, whereas the *β-amylase* of uninfected samples was not degraded (Yang et al., 2010). This might explain why *β-amylase* did not appear in the infected barley sample of this study. At the steeping stage, we found a slight increase of 14.4% in *β-amylase* levels (Fig. 2). However, after germination, *β-amylase* decreased by 8.1% compared to the MOCK sample (Fig. 3). In malt, we found an increase of 7.4% as well as two new spots in this region, which could be attributed to isoforms of *β-amylase* (Fig. 4) and which is in accordance with the malt quality attributes were we determined 13.37% more *β-amylase* activity in the INOC sample. In the gene expression study mentioned earlier, there was downregulation in *β-amylase 2* at germination and slight upregulation after kilning in the *F. avenaceum* infected sample (Hofer et al., 2016), which is in accordance with the results in this study.

Besides *β-amylase*, *α-amylase* is the main starch debranching enzyme in the brewing process. While isoforms of *β-amylase* are present in barley kernels, *α-amylase* is synthesized de novo during germination (Georg-Kraemer et al., 2001). Therefore, *α-amylase* was not found in either barley or samples after steeping. During germination, *α-amylase* was produced and we found three spots in region 12 at this stage that could be assigned to *α-amylase* isoforms (Figs. 3 and 4). The molecular weight of *α-amylase* was ~45 kDa, and its pI was between 5.7 and 6.5. The relative values of the three *α-amylase* spots in MOCK barley changed by −8.1%, +4.8%, and +23.0% due to *Fusarium* infection and were −7.8%, −3.3%, and −0.1% after kilning.

4. Conclusion

Fusarium infection in small grain cereals has an enormous impact on quality. It can result in yield losses, malt quality reductions (from proteolysis) and is concerning in terms of food safety (mycotoxins). This study illustrated the impact of *F. avenaceum* infection (the primary representative of *Fusarium* species found on European malt in recent years) on the quality characteristics of barley and barley malt. By using artificial infection measures under greenhouse conditions, two different barley sample types varying in infestation severity (*Fusarium* DNA content) and metabolite contamination (such as by *ENN*s) were produced and subsequently processed in malting experiments. In the study we used the *F. avenaceum* isolate to inoculate the barley plants in greenhouse conditions. It is still unclear how the knowledge from the study is transferable to samples grown under field conditions were infections of a multitude of pathogens are common. This research is the first systematic analysis of the impact of *F. avenaceum* infection on the albumin fractions in barley and barley malt as well as on albumin fractions in the intermediate stages during the malting process. Besides the major barley proteins, *LTP*s and *serpins*, various proteins underwent changes in their abundance in kernels infected by *F. avenaceum* when compared to the uninfected control. These changes were mostly not evident when looking at malt quality characteristics, but had an impact on specifications and barley protein profiles. The visualization software for virtual 2D-PAGE, which was newly developed in this study, is a powerful tool for carving out protein profile changes over the malting process or between different types of samples.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.12.026.

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