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Effect of Microorganisms on Gluten Quality in Wheat

Effekt av mikroorganismer på glutenkvalitet i hvete

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Master of Science in Plant Science

Abstract

Food security is a current issue of both domestic and international concern. Faced with a growing population and the increased food demands it entails, the Norwegian government has recently renewed its commitment to increase food production nationally. In accordance, efforts are being made in Norwegian wheat production, but there are challenges to overcome. Fluctuation in gluten content and functionality in Norwegian-grown wheat in recent years has created economic and practical difficulties for the bread baking industry. Studies of samples of wheat with poor baking quality have found possible links to microorganisms, particularly *Fusarium* ssp., and environmental conditions conducive to their growth. As part of the "Quality Wheat" project started in 2014, this study aims to elucidate possible connections between microorganisms and reduced baking quality in wheat. A greenhouse inoculation experiment with wheat and microorganisms and a media experiment testing microorganisms' ability to use gluten as a nitrogen source facilitated exploration of this topic.

In the greenhouse inoculation experiment, spring wheat was inoculated with *Fusarium avenaceum, Fusarium graminearum, Microdochium majus,* and *Luteibacter rhizovicinus*. Harvested grain from wheat inoculated with *F. graminearum* and *F. avenaceum* exhibited symptoms of infection and was found to contain fungal DNA. Analysis of flour made from the same material also revealed protease activity on gluten and gelatin substrates in samples of wheat inoculated with *F. graminearum* and *F. avenaceum*. When baking quality of the flour from this experiment was analyzed, it was found that flour from wheat severely infected with *F. graminearum* contained a reduced proportion of unextractable polymeric proteins (%UPP) known to confer dough with its elastic strength. This was accompanied by a reduction in measured maximum resistance to stretching (R_{MAX}) when using the same flour for analysis. In flour made from wheat inoculated with *F. graminearum* in this study, *F. graminearum* DNA content was found to be significantly negatively correlated to both %UPP and R_{MAX} values.

An experiment was designed to test fungal growth on cultural media containing different nitrogen sources. The experiment allowed for testing of whether *F. avenaceum, F. graminearum, M. majus* and *Microdochium nivale* could grow on a medium with gluten as the sole nitrogen source and detection of protease activity in the form of gluten degradation. All four species grew on the gluten-containing medium and gluten degradation was detected in the medium. *F. avenaceum, F. graminearum, M. majus and M. nivale* all exhibited the ability to degrade gluten.

Results from this study support the hypothesis that *F. graminearum* can infect wheat and produce proteases that degrade gluten proteins to the extent that it negatively affects baking quality. *F. avenaceum* also exhibited the ability to affect baking quality negatively. *F. graminearum, F. avenaceum, M. majus* and *M. nivale* all showed the ability to degrade gluten proteins and are therefore potentially capable of negatively affecting baking quality. *Microdochium* ssp.' connection to gluten degradation and reduced baking quality in wheat remains uncertain but merits further study, especially in the case of *M. majus*.

Abstrakt

Mattrygghet er et viktig tema både nasjonalt og internasjonalt. Den forventede befolkningsøkningen krever tilsvarende økning i matproduksjon. I tråd med dette har den norske regjering nylig forpliktet seg til å øke matproduksjon i Norge, noe som også inkluderer produksjon av hvete. Imidlertid har variasjon i gluten-innhold og funksjonalitet i norske hvete i de siste årene skapt praktiske og økonomiske vanskeligheter for mølle- og bakeindustrien. Studier har vist at dårlig bakekvalitet i hvete kan være forårsaket av mikroorganismer, slik som *Fusarium* ssp., i kombinasjon med værforhold som er gunstige for soppvekst. Prosjektet HveteKvalitet (Norsk mathvete med riktig proteininnhold og god baketeknisk kvalitet) som startet i 2014, har som ett av målene å undersøke sammenhenger mellom infeksjon med mikroorganismer og redusert bakekvalitet. Temaet har blitt utforsket i dette mastergradsarbeidet ved å gjennomføre et smitteforsøk i veksthus med ulike mikroorganismer i hvete og et medieforsøk for å vurdere mikroorganismers evne til å utnytte glutenproteiner som nitrogenkilde.

Følgende mikroorganismer ble brukt i smitteforsøket: *Fusarium avenaceum, Fusarium graminearum, Microdochium majus,* og *Luteibacter rhizovicinus*. Korn høstet fra vårhvete som var inokulert med *F. graminearum* og *F. avenaceum* hadde klare sjukdomssymptomer og inneholdt DNA av de respektive soppartene. Det ble påvist proteaseaktivitet i mel fra det samme materialet. I tillegg ble det påvist en reduksjon i andelen av ikke-ekstraherbare polymeriske proteiner (unextractable polymeric proteins, %UPP) i korn med høy grad av *F. graminearum*-infeksjon. Det ble også registrert en reduksjon i R_{MAX} (maksimum motstand mot strekking) i gluten fra disse prøvene. Innhold av *F. graminearum*-DNA i korn fra hvete inokulert med *F. graminearum* var signifikant, negativt korrelert med både %UPP og R_{MAX}. Basert på resultatene fra smitteforsøket ble det i tillegg satt opp et forsøk for å avdekke om *F. avenaceum, F. graminearum, M. majus* og *Microdochium nivale* har evne til å utnytte glutenproteiner som nitrogenkilde. Farging av agaren disse soppene ble dyrket på etter endt forsøk viste at *F. avenaceum, F. graminearum, M. majus* og *M. nivale* alle har evne til å bryte ned glutenproteiner.

Resultatene fra dette studiet støtter hypotesen om at *F. graminearum* kan produsere proteaser som bryter ned glutenproteiner, noe som kan medføre at bakekvaliteten kan bli redusert i mel fra *F. graminearum* infisert korn. *F. avenaceum* viste også evne til å forårsake en reduksjon i bakekvalitet. *F. graminearum, F. avenaceum, M. majus* and *M. nivale* viste alle evne til å bryte ned gluten og er derfor i stand til å ha en negativ effekt på bakekvalitet. Sammenhengen mellom forekomst av *Microdochium* ssp. og bakekvalitet i hvete er fremdeles uavklart, og videre forskning bør gjennomføres for å undersøke dette, og da særlig med tanke på *M. majus*.

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Introduction

Food security is a current issue of both domestic and international concern. Citing population growth, climate change, finite natural resource availability, and ever-rising commodity prices, the Norwegian government presented in 2011 a report to Parliament outlining goals to increase food production in Norway by 20% by 2030 (Landbruks- og matdepartementet). The most recent report from the Ministry of Agriculture and Food (2016) has confirmed the government's continued commitment to the goal of increased Norwegian food production. In accordance with these goals, efforts are being made in Norwegian wheat production.

To meet the goals of increased production of food-grade wheat, Norwegian farmers have challenges to overcome. Fluctuation in gluten content and functionality in Norwegian-grown wheat in recent years has created economic and practical difficulties for the bread baking industry. Challenges are compounded by a system in which assessment of commercial quality is based on cultivar class and protein content. These have been shown to be poor predictors of gluten quality (Moldestad et al. 2011). While Moldestad (2011) observed that temperature during the growing season may be related to gluten quality, it is likely not the only environmental parameter of relevance and exogenous proteases, especially, deserve further attention (Koga et al. 2016). A possible source of exogenous proteases is microorganisms. Other studies have already linked *Fusarium* ssp. to gluten protein degradation or reduced baking quality (Boyacloglu & Hettiarachchy 1995; Capouchová et al. 2012; Dexter et al. 1996; Eggert et al. 2011; Pekkarinen et al. 2000; Wang et al. 2005).

As part of the "Quality Wheat" project started in 2014, this study aims to elucidate connections between microorganisms and reduced baking quality in wheat. A greenhouse inoculation experiment with wheat and microorganisms and a media experiment testing microorganisms' ability to use gluten as a nitrogen source facilitated exploration of this topic.

Thesis Statement

Fusarium ssp., and potentially other microorganisms, have the ability to infect wheat and produce enzymes capable of degrading gluten proteins, thus rendering the resulting flour unsuitable for baking bread.

Gluten

Protein is generally known to be the component in flour which has the most influence on baking quality (Weegels et al. 1996), and composition and structure of the gluten proteins specifically have the most significant impact (Tronsmo et al. 2002). Protein makes up approximately 10-12% of wheat flour, and gluten proteins comprise approximately 80-85% of total protein (Goesaert et al. 2005). Gluten proteins are found in the starchy endosperm of wheat kernels as a matrix around starch granules and function chiefly as storage molecules for the seed's use in germination. It is these storage proteins in the flour which, when mixed with water to form a dough, form a continuous network and confer dough with viscoelastic properties necessary for baking bread (Shewry et al. 1995).

Examination of gluten proteins often involves classification according to solubility and function. Early solubility classification was described by Osborne (1924), who divided wheat protein fractions based on solute. He found that albumins and globulins could be removed from wheat flour protein by water and a salt solution, respectively. Osborne then described the remaining gluten proteins as comprising, for the most part, the remaining classes labeled as gliadin, glutenin, and residue. He separated the monomeric gliadin fraction with aqueous alcohol, and he found some of the glutenin fraction to be soluble in dilute acetic acid. The remaining

insoluble residue was then made up of the rest of the HMW gluten polymers and other non-gluten polymers. The largest polymers are most insoluble (Goesaert et al. 2005).

Classification of gluten proteins by function also involves division into two main groups: gliadins and glutenins. Gliadins and glutenins are generally present in similar quantities. Gliadins include monomers in the molecular weight range of 30000 to 80000, are soluble in alcohol, and affect viscosity of dough. Glutenins are poorly soluble polymers with molecular weights larger than or equal to 80000, and they give dough its elasticity, or strength (Shewry et al. 1995; Goesaert et al. 2005). Glutenin polymer structure is rod-like and made up of β-spirals, and the secondary polymeric structure is characterized by tangled, branched network polymers connected by intermolecular disulphide bonds and hydrogen bonds (Dobraszczyk 2004).

The largest, least soluble glutenin polymers have received considerable attention in studies and are considered to be strongly related to bread making quality (Belitz et al. 1986; Weegels et al. 1996; Macritchie 1987; Dobraszczyk 2004; Shewry et al. 1995). The larger gluten polymers give wheat dough strength for expansion of air bubbles during fermentation, allowing loaf volume to increase while maintaining desired shape (Dobraszczyk 2004).

To use information about the largest protein polymers in flour to make predictions about baking quality, protein characterization is required. One method, presented by Singh et al. (1990) involves dissolving flour proteins in sodium dodecyl sulfate (SDS) with and without sonication and then using size-exclusion high-performance liquid chromatography (SE-HPLC) to separate the dissolved protein molecules by size. Resulting chromatogram curves can then be integrated to obtain quantities of molecules present in different size categories. Using SDS as a solvent is advantageous because it has been found to cause minimal reduction of disulphide bonds. This means that after the first extraction with SDS, the largest protein polymers, including the largest glutenin polymers, are left intact and undissolved. Sonication is then used to disrupt the structure of the unextractable polymers, making them smaller and therefore soluble in SDS. SE-HPLC analysis is then used again on these dissolved proteins, called the SDS-unextractable fraction, to determine the quantity of the largest polymers in the flour sample. Using SE-HPLC is applicable for small samples sizes (Singh et al. 1990) such as those from the inoculation experiment of this study.

Integrating the area under the various peaks of the chromatogram curves from SE-HPLC provides data that can be used to predict baking quality. Gupta et al. (1993) demonstrated that by using the areas under the curves of the first peaks of both the SDS-extractable fraction (F1) and the SDS-unextractable fraction (F1*), percent unextractable polymeric protein of total polymeric proteins (%UPP) could be calculated. Flour samples with higher %UPP values have, therefore, higher proportions of the larger polymeric proteins which equates with a stronger, more elastic dough. Polymeric protein size distribution has also been found to be highly correlated with R_{MAX}, maximum resistance to stretching (Gupta et al. 1993). Finding %UPP of samples of flour is a commonly used method to characterize gluten protein.

There are different methods available for measuring viscoelastic properties of gluten. The Kieffer Extensibility test is based on principles similar to those applied in the well-known and traditional Brabender Extensograph where a kneaded dough's maximum resistance against stretching is measured. Advantages of using the improved Kieffer Extensibility test include the option to use either dough or gluten to be stretched, a much smaller required quantity of sample material, improved instrumentation, and parameters recorded in SI units. R_{MAX} and extensibility of gluten can be measured using the method described by Kieffer et al. (1998). In this protocol, gluten is washed out of a small dough made with only 10 g of flour. Following centrifugation and incubation, gluten is stretched and resistance and extensibility measured. R_{MAX} values were found to be

correlated with loaf volume and are therefore a useful indication of baking quality. Higher R_{MAX} values indicate stronger gluten that will have better baking quality. Extensibility is also relevant for baking quality. Monomeric gliadins are known to confer doughs with their viscous qualities (Shewry et al. 1995), and viscosity of the dough is measured as extensibility using the Kieffer extensograph. A satisfactory baking result requires the correct balance of elasticity and extensibility in the dough. Kieffer extensograph is suitable for analyzing small samples sizes (Kieffer et al. 1998), so it will also be useful in this study.

Gluten quality in wheat produced in Norway has varied in recent years. Results from field trials have shown that quality varies both by location and season (Moldestad et al. 2011; Uhlen et al. 2015). Both these studies also found low temperatures and precipitation at grain filling to be associated with samples of poor quality. In addition, it has been suggested that proteases produced by microorganisms, specifically *Fusarium* ssp., may be a cause of the observed decreased gluten quality (Uhlen et al. 2015; Koga et al. 2016).

Microorganisms

Several studies have linked *Fusarium graminearum* to reduced baking quality or decreased gluten content in wheat (Bechtel et al. 1985; Boyacloglu & Hettiarachchy 1995; Dexter et al. 1996; Capouchová et al. 2012; Nightingale et al. 1999). Proteases produced by *F. graminearum* have been shown to have the ability to degrade gluten in medium (Pekkarinen et al. 2000) and *in vitro* (Eggert et al. 2011). A pathogen in the fusarium head blight (FHB) complex, *Fusarium avenaceum,* has also been implicated in grain damage (Clear & Patrick 2000; Wang et al. 2005), and has been one of the prevalent FHB pathogens in Norway (Brodal et al. 2016). *Microdochium nivale* has also been observed in connection wheat samples that exhibited poor baking quality, but the pathogen's potential role is not known (Koga et al. 2016). Research conducted as part of the "Quality Wheat" project found *Microdochium majus* DNA in samples of wheat with poor baking quality (not published). *F. graminearum, F. avenaceum,* and *Microdochium* ssp. have all been shown to be important plant pathogens in Norway (Brodal et al. 2016; Bernhoft et al. 2013; Hofgaard et al. 2016)

Microorganisms included in this study were chosen based on various analyses completed in the first years of the "Quality Wheat" project (results not shown). These microorganisms included *F. avenaceum, F. graminearum, M. majus, M. nivale,* and *Luteibacter rhizovicinus*.

Fusarium ssp. and *Microdochium* ssp. are known plant pathogens and are included in the group of fungi which cause FHB, also known as scab or fusarium ear blight. *F. avenaceum* and *F. graminearum* are among the most prevalent *Fusarium* species found in Norway (Hofgaard et al. 2016; Sundheim et al. 2013). They are saprophytic fungi which survive on infected plants and debris from grain and other grasses. Spread occurs via ascospores, mainly by wind dispersal, and conidia, mainly by splash dispersal. Infection of grain generally takes place in warm wet/humid conditions during anthesis (Chakraborty & Newton 2011; Doohan et al. 2003; Brodal 2012).

While part of the FHB complex of microorganisms, *Microdochium* ssp. are also known to cause snow mold, leaf blotch in oats, stem rot, and fusarium patch in turf grasses (Tronsmo 2013). *M. majus* and *M. nivale* were classified as varieties of the same species, *M. nivale*, until the work of Glynn et al. elevated them separately to species status (2005). These two species are common in cold and cool areas and are favored by wet weather. Optimum growth temperature is 18 to 21°C. Like *Fusarium* ssp., *M. nivale* and *M. majus* survive on debris and infected plant residues, and spread occurs via mycelia, conidia, and ascospores. The development of snow mold on grasses and grains requires approximately two months of snow cover after which melting reveals typical pink-white patches of mycelium on infected plants which then die and develop a paper-like appearance (Tronsmo et al. 2001). There is some indication of host specialization within *Microdochium* ssp. as *M. majus*

has been observed to be less pathogenic on turf grasses and more pathogenic than *M. nivale* on wheat (Hofgaard et al. 2006; Glynn et al. 2005). Of the diseases caused by *Microdochium* ssp., FHB is of most relevance for grain quality.

FHB is a disease of global importance, and it is on the rise. Increasingly warmer and wetter weather during grain anthesis has provided optimum conditions for infection by *Fusarium* ssp. Simultaneously, agricultural practices such as reduced tillage and unfavorable crop rotation have led to increased inoculum production (Aamot et al. 2015; Chakraborty & Newton 2011; Dill-Macky & Jones 2000). This combination has fueled the increase of the disease seen around the world. Projections relating to climate change indicate possible further increases in FHB (Parikka et al. 2012). Changing flowering times could result in more wet weather during anthesis, which is conducive to infection. In addition, increased atmospheric CO_2 concentrations are considered to have a positive effect on fungal growth and plant biomass, meaning there will be more plant material available to sustain more inoculum (Madgwick et al. 2011; Chakraborty & Newton 2011).

Reduced grain yield and quality may be the consequences of *Fusarium* infection in wheat, and, in a recent two-year period, FHB resulted in $2.7 billion in losses in central USA and the great plains area alone (Chakraborty & Newton 2011). Another reason FHB receives considerable global attention concerns mycotoxin production. Several of the *Fusarium* species that make up the FHB complex produce toxins which contaminate grain and pose a serious health threat to humans and animals. *F. graminearum* is a known producer of the mycotoxin deoxynivalenol (DON) (Sundheim et al. 2013). Growing concern regarding this and other mycotoxins in the food chain led to the commissioning of the Norwegian Scientific Committee of Food Safety to complete an official risk assessment (Bernhoft et al. 2013). The committee's findings did reveal that children up to the age of two in Norway consume over the calculated tolerable daily intake of DON even in low DON years, so concern is justified. *F. avenaceum* produces a range of mycotoxins, including enniatins, which are commonly detected in Norwegian grains (Hofgaard et al. 2016). *M. nivale* and *M. majus* are not known to produce mycotoxins (Tronsmo et al. 2001).

The only non-fungal microorganism used in this study was the bacteria *L. rhizovicinus*, identified by Johansen et al. (2005) and recently sequenced by Aamot et al. (2017). It is a gammaproteobacterium isolated from the rhizosphere in a field of organically grown barley in Denmark. It was found to be able to grow in a temperature range of 5 to 30°C and is capable of hydrolyzing aesculin, starch, and gelatin (Johansen et al. 2005). A study involving "plant growth promoting rhizobactia" identified a possible candidate for bio-fertilizer as *L. rhizovicinus* (Guglielmetti et al. 2013). A study of fungus-feeding rhizopheric bacteria also identified *L. rhizovicinus* among isolated bacteria (Ballhausen et al. 2015). *L. rhizovicinus* is not a known plant pathogen.

For this study's inoculation experiment, a method was required to examine potential protease activity in flour. Lantz and Ciborowski (1994) describe the use of zymography to successfully detect and characterize bacterial proteases. They show how protein substrates, such as gluten, can be copolymerized into polyacrylamide gels which can then be used for running electrophoresis. Successful polymerization of gels most often involves using ammonium persulfate (APS) to generate free radicals for polymerization and N,N,N',N'-tetramethylethylenediamene (TEMED) to speed up APS degradation and subsequent polymerization. Using a discontinuous buffer system and a tracking dye allows the progress of the buffer front through the gel to be monitored during electrophoresis. This allows electrophoresis to be stopped at the appropriate time even when aspects of how the samples interact with buffers may be unknown and therefore make necessary running time unknown (Garfin 2003). SDS is often used to denature proteins, rendering them inactive during electrophoresis. Renaturing of proteins with Triton x-100, incubation, and staining then allow for detection of proteolytic activity. Coomassie Brilliant Blue R-250, for example, stains the portions of the zymogram where

the protein substrate has not been degraded by protease activity. Bands in the gel that are devoid of staining are, therefore, interpreted as bands of protease activity. This sensitive method for detecting protease activity (Lantz & Ciborowski 1994) should be a useful method for analyzing samples of protein extracted from flour. A disadvantage to consider when using zymography is that proteases could fail to renature after using SDS (Lantz & Ciborowski 1994). Streaking might also be observed in the gels if proteases are active during electrophoresis (Lantz & Ciborowski 1994) or as a result of stacking, causing a high concentration of proteins to enter the gel slowly (Garfin 2003).

To interpret the effect microorganisms may have on baking quality in wheat, it is necessary to detect their presence and relative quantities in the inoculated samples. A method is required that is specific enough to detect inoculation species' DNA even in the presence of plant DNA and the potential presence of DNA from other microorganisms. For those samples where DNA from the inoculation microorganism is detected, it would be beneficial to compare quantities of microorganism DNA to values obtained from baking quality analysis. Real time quantitative PCR (qPCR) is a useful method. In their article entitled *Real Time Quantitative PCR*, Heid at al. (1995) describe the successful use of a TaqMan probe that emits a peak of fluorescence during the extension phase of PCR due to its degradation by Taq polymerase. For this process the probe is labeled with both a reporter (in this case 6-carboxyfluorescein, or FAM) and a quencher (6-carboxy-tetramtheyl-rhodamine, or TAMRA). When the probe is cut during PCR, the quencher no longer performs its function and the increased fluorescent emission from the reporter is detected and can be used to quantify the DNA in the sample for which the probe and primers were designed. Several studies have employed qPCR as a means to identify and quantify FHB pathogens (Divon et al. 2012; Halstensen et al. 2006; Reischer et al. 2004; Waalwijk et al. 2004). Advantages of using this method to detect and quantify DNA include that it is specific, reduces contamination risks of handling PCR products, is reproducible, and is high throughput. Factors that can affect the accuracy of results include PCR target composition and size, primer sequences, reaction conditions such as temperature and time, and sample purity (Heid et al. 1995).

Results from qPCR analysis of material in this study influenced the decision to conduct the media experiment. Not all the microorganisms used for inoculation in the greenhouse produced infection. In addition, there was interest in examining the ability of *Fusarium* ssp. and *Microdochium* ssp. to utilize different sources of nitrogen, and, more specifically, degrade gluten.

Nitrogen is one of the macronutrients fungi require. Ammonium is considered to be the most usable form, but *Fusarium* ssp., with the exception of mutants, are generally able to reduce nitrate and utilize it as a source of nitrogen as well (Leslie & Summerall 2006). Fungi obtain necessary nutrients through excretion of exocellular enzymes, or exozymes, which digest nutrient sources. Exozymes may also play a role in pathogenesis (Griffin 1994). Several studies have shown that manipulating necessary nutrients, such as nitrogen and carbon, in media will induce changes in *F. graminearum* gene expression, expressed sequence tags (ESTs), and protein production (Güldener et al. 2006; Paper et al. 2007; Trail et al. 2003). Correll et al. (1987) used a minimal medium (MM) in studies of nitrate nonutilizing mutants (*nit*) of *Fusarium oxysporum*. Trail et al. (2003) found that using carbon and nitrogen starvation mediums facilitated detection of genes known to be connected with pathogenicity. Media can also be chosen to favor mycelial growth or spore production depending research objectives (Leslie & Summerall 2006).

The complete medium (CM) is a nutrient sufficient medium which contains various nitrogen sources and facilitates growth in nearly all *Fusarium* ssp. (Leslie & Summerall 2006). Recording growth rates of *Fusarium* ssp. and *Microdochium* ssp. on complete medium (CM) compared to various minimal media with different nitrogen sources, including gluten, will facilitate studying which nitrogen sources these pathogens can utilize.

In several of the aforementioned studies, *Fusarium* ssp. have shown poor growth when placed in conditions of nitrogen starvation or given a nitrogen source they cannot utilize (Correll et al. 1987; Güldener et al. 2006; Paper et al. 2007; Trail et al. 2003). It will therefore be useful to examine MM containing gluten after the fungal species have been allowed to grow on it to see whether there is evidence of gluten degradation. The staining method used for zymogram gels, in which Coomassie blue R-250 is used to stain protein, may also be useful in revealing areas in the medium where proteases have degraded the gluten. If a species grows well on the MM with a gluten nitrogen source and the media shows signs of protease activity in the form of gluten degradation when examined, it will be an indication that the particular species in question may be able to produce the protease(s) necessary to utilize gluten as a source of nitrogen. This would be useful information in dealing with FHB and its consequences for baking quality in wheat.

Materials and Methods

Greenhouse Experiment to Explore Microorganisms' Effect on Baking Quality in Wheat

Experimental Design: Greenhouse I and II

The purpose of the inoculation experiment in the greenhouse was first to inoculate wheat with different microorganisms combined with various incubation treatments and assess the harvested grains for fungal infection. Then, flour made from the inoculated wheat was assessed for protease activity and changes in baking quality. The inoculation experiment was carried out twice and will be referred to as Greenhouse I and Greenhouse II. Greenhouse I was sown February 1, 2016 and inoculated April 6, 2016. Greenhouse II was sown March 4, 2016 and inoculated May 10, 2016. Plants were grown in the greenhouses on the Ås campus of the Norwegian University of Life Sciences.

Within both Greenhouse I and II, there were two replicates of four pots, eight pots total, of wheat that received each inoculation/incubation treatment combination. Inoculation treatments included a control without microorganisms and inoculation with the following microorganisms: three isolates of *F. avenaceum,* three isolates of *F. graminearum*, three isolates of *M. majus,* and one isolate of *L. rhizovicinus*. Incubation treatments included 2-day and 4-day incubation performed by covering inoculated ears with clear plastic bags. At harvest, inoculated ears from each four-pot replication were combined to comprise one sample.

Plant Material and Growing Conditions

Seed grain of spring wheat, *Triticum aestivium*, cv. Zebra was provided by Kimen Seed Laboratory (Kimen Såvarelaboratoriet AS). The sample (201411463) was selected based on the criteria of high germination percent (97% without fungicide seed treatment) and low infection (1% *Fusarium*, 0% *Stagonospora nodorum,* and 0.5% *M. nivale*). Ten seeds were planted in each of 200 black plastic, 3.5 liter pots (Aeroplas) filled to the brim with 86% peat potting soil (see Appendix Table 6) that had been crumbled by hand. Pots were distributed amongst three greenhouse tables, watered as needed, and rearranged periodically prior to inoculation to maintain even growth and development. The plants were supported with bamboo rods tied together with twine.

Light fixtures above each table were fitted with a total of three white (Powerstar, HQI-BT 400 WD Daylight E40, Osram, Germany) and two yellow bulbs (Lucolux PSL Photosynthesis Light, Son-T LU400W PSL T E40, General Electric, Hungary). Supplemental lighting was set to 200 µmol m^{-2} s^{-1} for a 16 hour photoperiod. Photosynthetic active radiation was measured in the greenhouse with lights on after dark February 24, 2016 with a LI-185B Quantum/Radiometer/Photometer (LI-COR, Inc. Nebraska, USA), and average photosynthetic

active radiation was measured to be 139.17 µmol m^{-2} s^{-1} two pot heights above the table with foliage extending approximately 15 cm above the level of the flat sensor. Supplemental lighting was turned off after inoculation during incubation to avoid contributing to increasing temperatures. After incubation, supplementary lighting was turned back on and photoperiod adjusted to 18 hours.

Due to difficulties controlling temperature in the greenhouse and availability of alternative locations, there were some adjustments made during the inoculation experiment which mostly affected temperature. Facilities were not available to do the same with Greenhouse I and II, so temperature regimes are described separately.

For Greenhouse I, temperatures before inoculation were set to 15°C day/10°C night with a 16-hour day and relative humidity (RH) 65%. After 2-day incubation, plants were moved to different rooms with 18°C day/ 12°C night and a 12-hour day and RH 70% (average). The temperature for the 4-day incubation plants was increased to 18°C day/ 12°C night for the last two days of incubation before they too were moved to the other rooms with 18°C day/ 12°C night and a 12-hour day. Plants remained in these conditions for approximately one month after inoculation and then had to be moved back to the original greenhouse room where temperatures were often over 28°C before harvesting.

For Greenhouse II, temperatures before inoculation were set to 15°C day/10°C night with a 16-hour day. After inoculation, daytime temperatures were increased to 20°C day/ 15°C night and RH to 75%.

Plants were fertilized 10 times prior to inoculation with 0.5 L liquid fertilizer composed of 50 % Solution A and 50 % Solution B. Solution A was made of 25 parts calcium nitrate, one part ammonium nitrate, and 25 parts Kristalon (YARA, Inc) mixed with water to obtain ionic strength 1.5 mS cm^{-1}. Solution B was 25 parts Rød Superba, also mixed with water to 1.5 mS cm^{-1}.

Microorganisms

Inoculum for fungal isolates was prepared shortly before inoculation and stored at -20°C. Three isolates each of *F. avenaceum*, *F. graminearum*, and *M. majus* were grown on mung bean agar (MBA) (Leonard & Bushnell 2003) made with organically grown mung beans. Brand of organic mungs beans was chosen based on a sporulation experiment (data not shown). Mycelium plugs were first taken up from -80°C, transferred to potato dextrose agar (PDA) and grown in the dark at 9°C for 10-14 days. Thereafter new plugs were stamped out from the edge of mycelium growth, transferred to MBA petri dishes, and grown in a cabinet at 20-22°C with 12 hours of white and UV-light. Spores were collected by washing petri dishes with 5 ml distilled water and collecting the water and spores with a pipette. Spores were counted using a microscope and Bürk counting chamber slide. Spore suspensions of varying concentration (500 µl 1.13-6.53·10^5 spores ml^{-1} for Greenhouse I and 500-700 µl 10^5 spores ml^{-1} for Greenhouse II) were transferred to new MBA petri dishes and the process repeated until an adequate volume of 10^5 spores ml^{-1} spore suspension was obtained. Inoculum was frozen in 45 ml plastic tubes at -20°C until the day of inoculation.

For Greenhouse II, *M. majus* spores were grown of PDA with cellophane but otherwise produced using the same method with the growth cabinet temperature set to 20°C. Difficulties in spore harvesting were encountered with some isolates for Greenhouse I, so the following had lower spore concentrations than 10^5 spores ml^{-1} for inoculation: *F. avenaceum* isolate 2 (0.65·10^5 spores ml^{-1}), *M. majus* isolate 1 (0.15·10^5 spores ml^{-1}), and *M. majus* isolate 2 (0.69·10^5 spores ml^{-1}).

L. rhizovicinus inoculum was produced by transferring the bacteria to nutrient glycerol agar (NGA) slant tubes. Bacteria grew at room temperature and was collected and added to sodium phosphate buffered saline (SPBS)

in order to attain a suspension with measured optical density (OD) of 0.2 absorbance at 600nm on a UV-spectrophotometer. This absorbance value is normal for a bacterial cell concentration of approximately 10^8 cells ml^{-1}. Most bacterial cells in the inoculum had been grown the day before inoculation, but supplementation with some older cells was necessary to reach desired concentration. Inoculum concentration was tested on NGA petri dishes and calculated to be $6.4 \cdot 10^7$cells ml^{-1} for Greenhouse I and estimated (due to some lab difficulties) to be $2.383 \cdot 10^8$ cells ml^{-1} for Greenhouse II.

Inoculation

Prior to inoculation, ears at growth stages Zadoks 61-65 for Greenhouse I and Zadoks 65-69 for Greenhouse II were selected and marked with plastic-coated wire twist ties on stems. 320 ml of prepared inoculum was applied to each group of 16 pots designated to be inoculated with the same microorganism. Tween®20 (Sigma-Aldrich) was added to each volume of inoculum (1µl ml^{-1}) into order to help with spore dispersal. Inoculum was applied with a compressed air spray gun with pressure set to approximately 1 bar. The nozzle and container used for inoculum were disinfected between species of inoculum and rinsed between isolates of the same species of inoculum. Replicates were kept together but placed randomly in relation to replicates inoculated with other microorganisms on greenhouse tables.

Marked ears of wheat were counted and harvested at maturity. Threshing was completed with a threshing machine (Seedboy, F. Walter-H. Wintersteiger, Austria) or by hand in the case of some of the very infected samples which would have been lost in the threshing machine. Samples were weighed and thousand-grain weight was calculated with the help of a seed counter. Kernels from the four pots which made up one replicate were combined and given a sample number. Samples were milled on a Retsch ZM 200 mill with a 1mm ring sieve (Retsch GmbH, Germany) to whole grain flour, and care was taken to disassemble and vacuum equipment between samples. Flour samples designated for qPCR analysis were stored at -80°C. The rest was stored at 4°C.

Table 1 Microorganisms used in the inoculation experiment (Greenhouse I and II) and the media experiment of this study. Species include Fusarium avenaceum, Fusarium graminearum, Microdochium majus, Microdochium nivale, and a bacteria, Luteibacter rhizovicinus.

Species	Isolate	Year	Host	Origin	Additional information
F. avenaceum 1	201030	2004	Wheat, grain	Ås, Norway	
F. avenaceum 2	201063	2005	Wheat, leaf	unknown	
F. avenaceum 3	202021	2012	Wheat, straw	Solør, Norway	
F. graminearum 1	200630	2006	Wheat	Østlandet, Norway	aggressive
F. graminearum 2	201196	2012	Wheat, straw	Solør, Norway	3-ADON genotype
F. graminearum 3	202058	2013	Wheat, grain	Rakkestad, Norway	15-ADON genotype
M. majus 1	200345	2003	Wheat, grain	Rakkestad, Norway	
M. majus 2	200417	2003	Wheat, grain	Kapp, Norway	
M. majus 3	200430	2004	Wheat, leaf	Ås, Norway	fast-growing
M. nivale 1	200231	1996	Ryegrass	Løken, Norway	re-isolated in 1998, ryegrass, Ås
M. nivale 2	200272	1999	Meadow fescue	Ås, Norway	
M. nivale 3	201050	2010	Festulolium	Bjørke, Norway	
L. rhizovicinus	LJ96^T	2005	-	Taastrup, Denmark	from barley rhizosphere

Fungal DNA Analysis

To assess whether inoculated wheat had been infected, the content of fungal DNA in harvested kernels was measured with Quantitative Polymerase Chain Reaction (qPCR). DNA was extracted from 150 mg flour samples

using a FastDNA SPIN Kit for Soil (MP Biomedicals, Ohio, USA). This yielded 100 µl eluate per sample. A 10x dilution plate was prepared by transferring 10 µl of each DNA eluate to a separate well of a 96-well plate and adding 90 µl Milli-Q water to each. Extracted DNA was stored at -20°C until used. Quantitative PCR analysis was run for wheat, *F. avenaceum*, *F. graminearum*, and *M. majus* DNA in all flour samples. Table 2 shows assays used for probes as well as forward and reverse primers.

Table 2 Assays used for qPCR analysis of Fusarium avenaceum, Fusarium graminearum, Microdochium majus, and cereal DNA in samples of flour made with inoculated wheat.

Species	Primers, Probes	Sequences (including fluorophores and quenchers in present study)	Publication
F. avenaceum	TMAV-f	AGA TCG GAC AAT GGT GCA TTA TAA	Halstensen et al. 2006
	TMAV-r	GGC CCT ACT ATT TAC TCT TGC TTT TG	
	TMAV-p	Cy5-CTC CTG AGA GGT CCC AGA GAT GAA CAT AAC TTC-BHQ3	
F. graminearum	gramMGB-f	GGC GCT TCT CGT GAA CAC A	Waalwijk et al. 2004
	gramMGB-r	TGG CTA AAC AGC ACG AAT GC	
	gramMGB-p	6-FAM-AGA TAT GTC TCT TCA AGT CT-MGB	
M. majus	majus 2-F	CGC CAA GGA CTC CTC CAG TAG	Waalwijk et al. 2004
	majus 2-R	GCC GAC GAA TGG ATA TTA AGA ACT	
	majus probe	6-FAM-TCC CGC CTT CAC GGT GGA AAG C-TAMRA	
Cereal DNA	Cox554-f	GGT TGT TGC CAC CAA GTC TCT T	Divon et al. 2012
	Cox554-r	TGC CGC TGC CAA CTT C	
	Cox554-p	VIC-CTC CTA TTA AGC TCA GCC TT-MGB	

Stock solutions of forward and reverse primers were diluted to 7.5 pmol/µl. Stock solutions for probes were diluted to 2.5 pmol/µl. Template DNA of each of the fungal species was used in standard dilutions series which were used to generate standard curves to determine DNA quantities in the flour samples.

Plates for qPCR were prepared by adding 4 µl of 10x diluted DNA extract (or standard DNA with concentration range of 1-4000 pg) in a total volume of 25 µl per reaction for the specific species. Each reaction consisted of SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories, USA), 300 nM forward primer (Invitrogen, Thermo Fisher Scientific, USA), 300 nM reverse primer (Invitrogen, Thermo Fisher Scientific, USA), and 100 nM probe (Life Technologies, Thermo Fisher Scientific, USA for *F. graminearum, M. majus,* and cereal DNA; Sigma-Aldrich, USA for *F. avenaceum*). Plates were centrifuged and qPCR run with a C1000 Touch Thermal Cycler, CFX Real-Time System (Bio-Rad Laboratories, Singapore). PCR reactions were run with the following program: 3 minutes at 95°C followed by 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Each sample and standard was run in duplicate and repeated in cases where the absolute value of the difference of the replicate Cq values was greater than 1 and Cq values themselves were less than or equal to 30.

Protease Activity Analysis

Zymography was used to detect protease activity in protein extracted from flour made from inoculated wheat. To extract salt-soluble protein from flour samples 1000 µl (800 µl for some small samples) 0.5M NaCl extraction buffer was added to 200 mg (100 mg for some small samples) of flour. Samples were homogenized in the buffer with a Precellys Evolution machine (Bertin Technologies, Monigny le Bretonneux, France). Samples were homogenized at 6600 rpm for two periods of 15 seconds each with a 30-second pause in between. Afterwards, samples were centrifuged for at 4°C for 15 minutes at 13000 rpm and aliquoted into

new Eppendorf tubes. Sample material designated for subsequent protein concentration measurement was stored at -20°C and the rest at -80°C for future use.

Protein concentrations of the extracts were measured using a *DC*™ Protein Assay Kit (Bio-Rad, USA). Polyacrylamide separating gels (12.5% (w/v) Acryl/Bis-Acrylamide (37.5:1), 275 mM Tris-HCl pH 8.8, 0.05% (w/v) Ammonium persulfate and 0.05% (v/v) Tetramethylethylenediamine) were cast with a gluten (final concentration, 2 mg/ml, 0.5% (w/v) SDS and 2.5 mM Tris(2-carboxyethyl)phosphine hydrochloride) or gelatin (final concentration, 1 mg/ml) substrate. The gluten solution (8 mg/ml) was made by mixing 240 mg gluten powder with 30 ml protein extraction buffer (375 mM Tris/HCl pH 8.8, 2% SDS, 10 mM TCEP) for 5-6 hours at 60°C. The mixture was then centrifuged and the supernatant obtained. The gelatin solution (10 mg/ml) was made by dissolving gelatin in distilled water at 37°C. Stacking gels (4% (w/v) Acryl/Bis-Acrylamide (37.5:1), 126 mM Tris-HCl pH 6.8, 0.1% SDS, 0.05% (w/v) APS and 0.1 % (v/v) Tetramethylethylenediamine) were cast atop separating gels. Loading buffer (250 mM Tris-HCl pH 6.8, 10 % Glycerol, 62% SDS, 0.005 % Bromphenol blue) was added each protein sample that had been diluted with distilled water to a concentration of 1 µg/µl. 10 µg protein was pipetted into the gel standing in the gel apparatus. Running buffer (25 mM Tris, 0.192 M glycine, 0.1 % SDS) was added to the apparatus to cover the gels, and electrophoresis was run at 100V for approximately four hours at 4°C to separate extracted protein samples. Gels were then removed, renatured twice with 2.5 % w/v Triton X-100, and washed once with 50 mM Tris-HCl (pH 7.6). Afterwards, gels were incubated at 37°C overnight in incubation buffer (50 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2{\cdot}2H_20$, 0.2 M NaCl). The following day gels were stained with staining solution (0.1 % (w/v) Coomassie blue R-250, 50% (v/v) methanol, 7% (v/v) acetic acid) for one hour and destained with destaining solution (20% (v/v) methanol, 7% (v/v) acetic acid) for two hours to reveal protease activity. Gels were then scanned.

Protein Characterization

Protein characterization with Size Exclusion Fast Performance Liquid Chromatography (SE-FPLC) facilitated calculation of %UPP values used in assessing baking quality of flour made from inoculated wheat. SDS-extractable proteins (SUP fraction) were extracted from 240 mg flour samples by first mixing with 30 ml extraction buffer (0.1 M Sodium Phosphate buffer pH 6.9 with 1 % SDS) in a 40 ml centrifuge tube at 60 °C for 80 minutes. Samples were then centrifuged for 30 minutes at 19500 rpm (Beckman Coulter Avanti J-26 XP, USA) and resulting supernatant poured off. To obtain the SDS-unextractable proteins (SON fraction) from the remaining flour sample, another 30 ml extraction buffer was added and stirred to loosen the flour pellet. Samples were then sonicated at 70 % automatic amplitude compensation (Sonics VC130, Sonics and Materials, Newton, CT 06470-1614, USA) for 3 minutes, centrifuged again (see above), and supernatant obtained. SUP and SON fractions were filtered through a 45 µm membrane before analysis.

SUP and SON fractions were obtained for all samples from Greenhouse I and Greenhouse II that received 4-day incubation following inoculation. 100 µl samples from both fractions were separated at 0.4 ml $minute^{-1}$ on a Superose® 12HR 10/300 GL column connected to an ÄKTA SE-FPLC (GE Healthcare Life Science, UK). A 0.05 M sodium phosphate buffer with 0.1 % SDS (pH 6.9) and 0.8 M NaCl were used for running samples. Proteins within the fractions were then eluted by size and results presented as curves, chromatograms, for SUP and SON fractions (Figure 4). The SUP chromatogram was divided into five peaks. The first two SUP chromatogram peaks represent extractable polymeric protein. The last three SUP chromatogram peaks represent extractable monomeric protein, gliadin (F3 and F4), and albumin and globulin (F5). The SON chromatogram was divided into two peaks representing unextractable polymeric proteins, the main F1* and the much smaller F2*. Percent unextractable polymeric proteins of total polymeric proteins (%UPP) was calculated by dividing the area under the F1* peak from the SON fraction by the sum of the areas of both the F1 and F1* peaks and multiplying by 100.

Measuring Viscoelastic Properties of Gluten

By stretching gluten from flour made with inoculated wheat, maximum resistance to stretching (R_{MAX}) and extensibility could be measured with the Kieffer extensograph and results used to indicate baking quality. Flour samples were mixed with commercial whole wheat flour (Regal) in order to ensure adequate quantities of gluten for analysis. 30% sample material was used for all analyses except those with the inoculation/incubation combination of *F. graminearum* with 4-day-incubation, in which case the sample material amount was further reduced to 10-20% to obtain readings. Selected 2-day and 4-day incubated samples from Greenhouse II were analyzed. The most severely infected flour samples, based on fungal DNA content and symptoms of infection, were quite small and replicates of both the *F. graminearum* 1 and *F. graminearum* 2 4-day incubated samples from Greenhouse II had to be combined in order to have enough for analysis.

10 g of sample flour blended with commercial flour were weighed out and 5 ml of 2% NaCl solution was added. The the Glutomatic 2100 (Perten Instruments AB, Sweden) was used to knead a dough for one minute, and then rinse away starch and other particles from the gluten with the same 2 % NaCl solution for 10 minutes. Gluten was transferred to molds for centrifugation at 20°C for 10 minutes at 4100 rpm (rotor 5-5.1, ACC/DEC 9, TJ-25 Centrifuge, Beckman Coulter, Germany). Excess water was poured out of the mold and the gluten was transferred to another mold press that was placed in a plastic bag and allowed to rest for 45 minutes at 30°C. Gluten was stretched in the SMS/Kieffer Dough & Gluten Extensibility Rig connected to a TA.XT Plus Texture Analyser (Stable Micro Systems, UK) that recorded resistance to stretching and extensibility in addition to generating curves, extensograms, for analysis. The recorded maximum resistance to stretching, R_{MAX}, is associated with gluten elasticity while extensibility is associated with viscosity of gluten.

Commercial whole wheat flour used in this study contained ascorbic acid. Kieffer et al. (1998) found that the addition of ascorbic acid to flour increased R_{MAX} values for dough but not for gluten. This would suggest that ascorbic acid was washed away with the starch and other particles when the gluten was rinsed and should not have affect R_{MAX} values.

Media Experiment to Explore Gluten Degradation Capabilities of Fungi

Experimental Design

The purpose of the media experiment was to establish whether the fungal species in this study could grow on a medium with gluten as the sole nitrogen source and to detect protease activity, gluten degradation, in the medium. Four types of media were included in this study, each containing different sources of nitrogen. The complete medium (CM) contained $NaNO_3$, casein (N-Z Amine), and yeast extract and was meant to facilitate growth in all fungal isolates being used. The remaining three types of media included minimal media (MM) with only one of the following as a source of nitrogen: $NaNO_3$, gluten, or casein (Table 3). Recipes for basal medium and vitamin stock solution were based on a modified version of Czapek-Dox found in *The Fusarium Laboratory Manual* (Leslie & Summerall 2006) (Appendix Tables 7-9). Three isolates each of *F. avenaceum, F. graminearum, M. majus,* and *M. nivale* (Table 1) were grown on each of the four media types.

Mycelium plugs were taken out of storage at -80°C and transferred to potato dextrose agar (PDA) medium in petri dishes, placed in the dark at 9°C for six days, and then allowed to stand on the lab bench at room temperature for four days. The amount of PDA medium in the petri dishes was reduced to roughly half the usual amount (25ml) to minimize transfer of PDA medium when new mycelium plugs were collected for use on media to be tested in the experiment. Mycelium plugs approximately 5 mm in diameter were punched out

from peripheral mycelium and transferred to petri dishes containing 25 ml of the media to be tested. Each isolate-medium combination had a total of three replicates in the experiment. Two extra plates containing minimal medium with gluten were prepared per isolate for protein staining.

Table 3 Media used to test the ability of Fusarium graminearum, Fusarium avenaceum, Microdochium majus, and Microdochium nivale to utilize different sources of nitrogen. Amounts of protein/amino acids used in the media correspond to 0.33 g L^{-1} nitrogen. CM refers to control medium and MM refers to minimal medium.

Medium	Basal Medium	Sucrose	$NaN0_3$	Protein/Amino Acid	Vitamin Stock Solution
CM	1L	30 g	2 g	N-Z Amine 2.5 g, Yeast extract 1 g	10 ml
MM	1L	30 g	2 g	-	-
MM	1L	30 g	-	Gluten 2.4 g	-
MM	1L	30 g	-	N-Z Amine 3 g	-

Fungal Growth Rates on Media with Various Nitrogen Sources

The fungi grew in the dark at 15°C for eight days on each of the four media types. Petri dishes were stacked upside down to reduce condensation and isolates within each replicate were kept in separate plastic bags. Mycelial growth was registered daily starting on day three and ending on day eight by marking the extent of observable growth on each of four perpendicular axes drawn on the underside of the petri dishes with marker. Average mycelial growth rates were then determined by measuring the distances between these marks.

Detecting Gluten Degradation in Medium

For each of the fungal isolates, two petri dishes of gluten minimal medium were designated for protein staining. One dish was kept in the dark for three days at 15°C and four days at 6°C whereas the other dish remained at 15°C for the entire growing period. Mycelium was scraped off the agar and media stained and destained according to the same protocol previously described for zymography. Stained media was then photographed.

Statistics

Statistical analyses were performed in R Commander (NMBU settings). Thousand-grain weight, %UPP, and growth rates for the media experiment were analysed using multi-way ANOVA and Tukey HSD on a 95% confidence interval. In some of the above-mentioned analyses, one-way ANOVA was also run with data within Greenhouse I or II to compare with multi-way results.

Fitted regression plots were generated for the natural log of the quantity of *F. graminearum* DNA plotted against both %UPP and R_{MAX} (Figure 8). DNA quantities were calculated separately for each sample based on the amount of flour used for the specific analysis and the specific amount of DNA (pg mg^{-1} flour) for the same sample material as measured with qPCR analysis. The transformed amount of DNA was then used in linear regression analysis with both % UPP and R_{MAX} values.

Results

Greenhouse Experiment to Explore Microorganisms' Effect on Baking Quality in Wheat

Symptoms of Infection

Figure 1 Grains of spring wheat, cv. Zebra, harvested from the second greenhouse experiment. Grains shown are from the second greenhouse experiment and were incubated for four days following inoculation. Shown are a non-inoculated control sample (A) and samples of wheat inoculated with Microdochium majus (B), Fusarium avenaceum (C), and Fusarium graminearum (D).

Grains of wheat inoculated with *F. avenaceum* and *F. graminearum* exhibited symptoms of infection including shrunken appearance and discoloration (Figure 1, C & D). No symptoms were detected in grains of wheat inoculated with *M. majus* (Figure 1, B). Thousand-grain weight values were reduced for *F. graminearum*-inoculated kernels. Multi-way ANOVA and Tukey's HSD on a 95% confidence interval were run separately for Greenhouse I and Greenhouse II material and show that mean values were significantly reduced compared to control for all *F. graminearum*-inoculated material except Greenhouse I with 2-day incubation (Table 4).

Table 4 Mean values for thousand-grain weight (g) for kernels of spring wheat, cv. Zebra, from the inoculation experiment. Different superscript letters indicated statistical difference in Tukey's HSD on a 95% confidence interval within the same greenhouse experiment. 2-day and 4-day refer to periods of incubation following inoculation with Fusarium avenaceum, Fusarium graminearum, Microdochium majus, Luteibacter rhizovicinus, or control.

	Greenhouse I		Greenhouse II	
	2-day	4-day	2-day	4-day
Control	55.92^{a}	56.18^{a}	50.29^{a}	49.53^{a}
F. avenaceum	54.90^{ab}	55.19^{ab}	48.42^{a}	43.99^{a}
F. graminearum	52.74^{ab}	46.33^{b}	25.72^{b}	6.12^{c}
M. majus	55.26^{ab}	55.82^{a}	47.05^{a}	48.83^{a}
L. rhizovicinus	57.51^{a}	56.44^{a}	48.53^{a}	48.51^{a}

Fungal DNA Analysis

Analysis of flour samples with qPCR showed that Greenhouse II samples of wheat inoculated with *F. graminearum* and *F. avenaceum* generally contained more fungal DNA per mg flour compared to corresponding sample material from Greenhouse I (Figure 2). Longer incubation time also resulted in higher fungal DNA content in flour from *F. graminearum*- and *F. avenaceum*-inoculated wheat samples in both Greenhouse I and II. The quantities of *M. majus* DNA measured for inoculated samples were negligible and nearly the same for both Greenhouse I and II. Data from qPCR analysis also revealed some cross-contamination, particularly from *F. graminearum* and *F. avenaceum*.

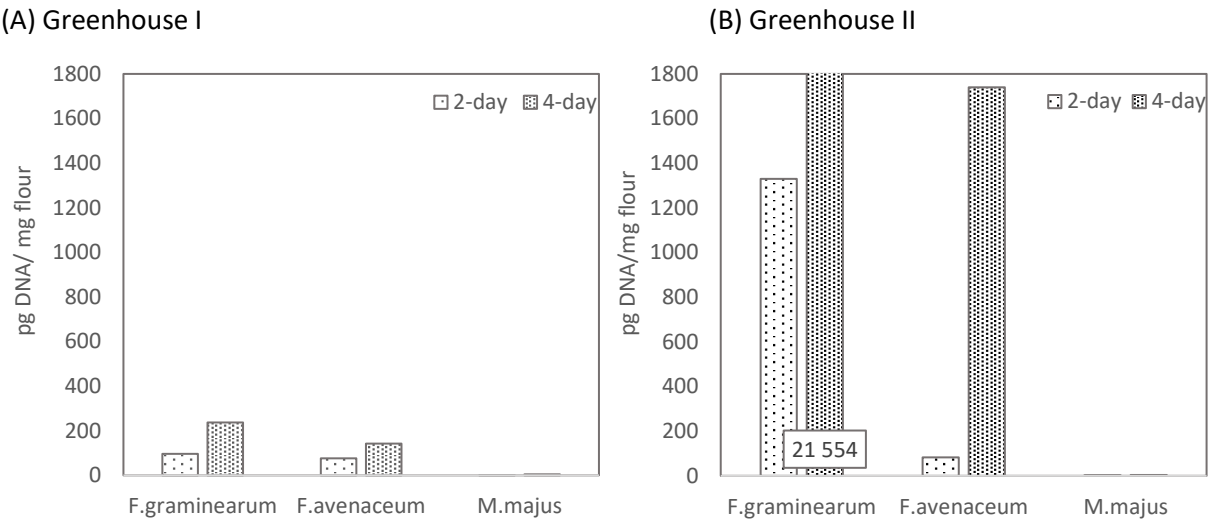


Figure 2 Average DNA content in harvested kernels of wheat from the greenhouse inoculation experiment, Greenhouse I (A) and Greenhouse II (B). Ears were inoculated with one of the following fungal species: Fusarium graminearum, Fusarium avenaceum, or Microdochium majus. Plants were incubated for either two or four days following inoculation. Note: In figure B, the DNA content of F. graminearum in kernels that received 4-day incubation is shown at the base of the column and is in pg DNA mg^{-1} flour.

Protease Activity Analysis

Zymography analysis showed that flour made from wheat inoculated with both *F. graminearum* and *F. avenaceum* in Greenhouse I and II contained proteases capable of degrading the gluten substrate in the gel (Table 5).

Table 5 Protease activity on zymograms with a gluten substrate for Greenhouse I and II of the incubation experiment. Sample material is protein extracted from flour made from wheat inoculated with Fusarium avenaceum and Fusarium graminearum isolates (isolate number given in parentheses) and incubated for two or four days.

	Greenhouse I		Greenhouse II	
	2-day	4-day	2-day	4-day
F. avenaceum 1	+	+	+	+
F. avenaceum 2	-	-	-	+
F. avenaceum 3	-	+	-	+
F. graminearum 1	-	+	+	+
F. graminearum 2	+	+	+	+
F. graminearum 3	-	+	+	+

Staining of zymogram gels allows protease activity to be identified as Coomassie blue R-250 only stains protein. Light bands in the gel are indicative of protease activity. Images of zymogram gels (Figure 3) show similar patterns of protease activity on gels with gluten and gelatin substrates for samples from Greenhouse II with 4-day incubation. Relative band thickness indicates levels of protease activity with thicker bands indicating higher levels. Wheat inoculated with *F. graminearum* showed higher levels of protease activity compared to *F. avenaceum.* Unlike the zymogram gels with a gluten substrate, the zymogram gels with a gelatin substrate had a distinct band of halfway down the gel in addition to the thick bands extending down from the loading wells. In zymogram gels with a gluten substrate, both higher protease activity and activity in more samples were observed in Greenhouse II compared to Greenhouse I. There was a general trend of higher activity levels in 4-day versus 2-day incubation samples. Flour from wheat inoculated with *M. majus* isolates and *L. rhizovicinus* showed no protease activity on any of the zymogram gels.

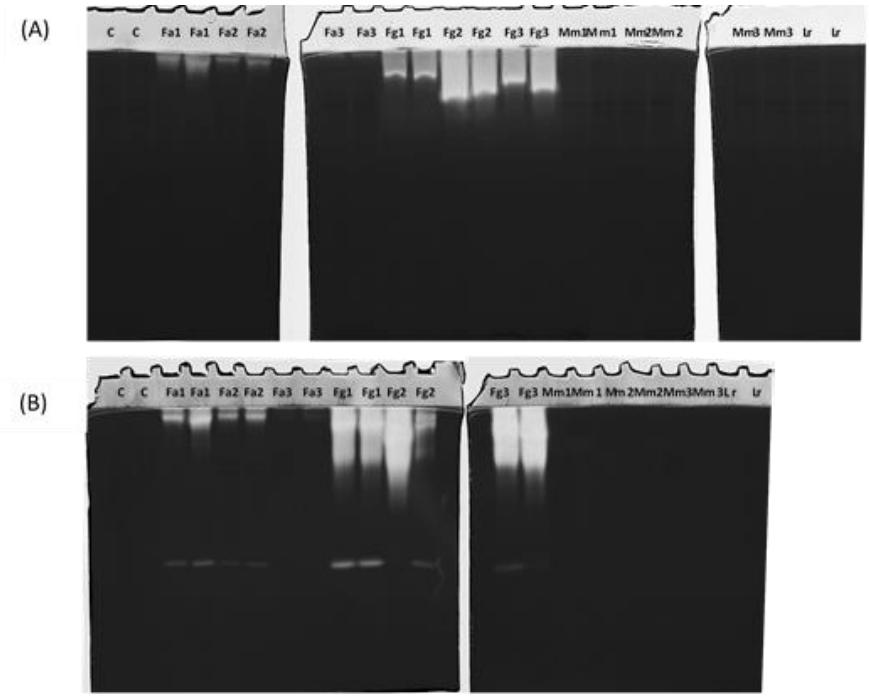


Figure 3 Zymogram gels used to identify protease activity in flour from wheat inoculated with Fusarium avenaceum (Fa), Fusarium graminearum (Fg), Microdochium majus (Mm), and Luteibacter rhizovicinus (Lr). Non-inoculated control samples (C) are also included. Numbers following abbreviations correspond to unique isolates. Sample material is from Greenhouse II of the inoculation experiment and received 4-day incubation following inoculation. Gels contain gluten (A) and gelatin (B) substrates.

Protein Characterization

Generally, samples analyzed with SE-FPLC exhibited curves comparable to that of the control sample in Figure 4. The curves labelled "Fg3" were generated from one of two samples that produced such atypical chromatograms. Both of these samples came from wheat in Greenhouse II that received 4-day incubation, and was inoculated with *F. graminearum*. These two samples showed changes over the whole chromatogram, not merely in the F1 and F1* peaks which were used to calculate %UPP. An increase in the F4 peak can indicate a

shift in gluten molecule size from large to small. The increase in peaks after peak 4 may be explained by the presence of fungal proteins in the sample.

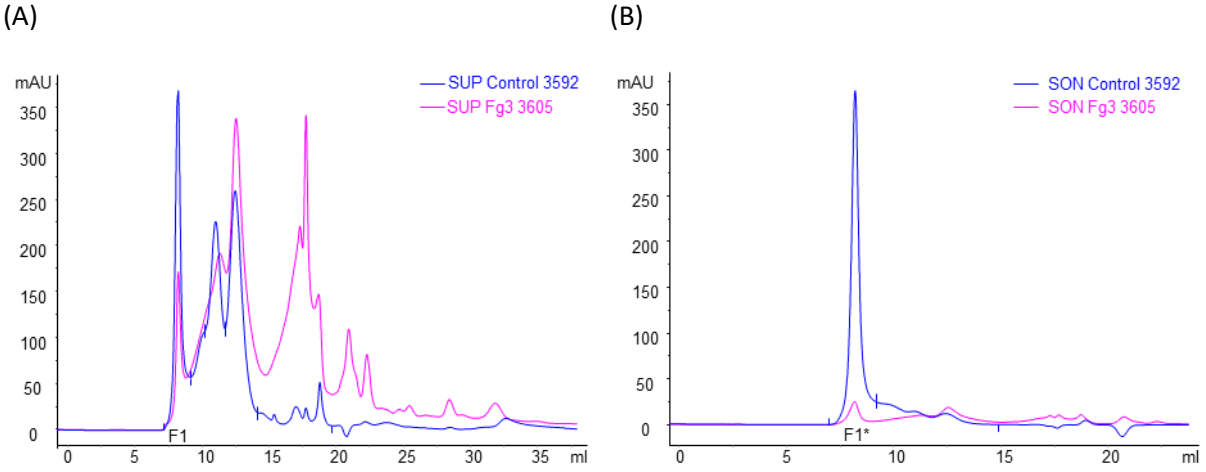


Figure 4 Chromatograms from SE-FPLC analysis for both SDS-extractable (A) and SDS-unextractable (B) fractions of protein from a control sample (blue) and a sample from wheat inoculated with Fusarium graminearum isolate 3 (pink). Samples are from Greenhouse II of the inoculation experiment and received 4-day incubation.

No statistically significant differences in the proportion of unextractable polymeric proteins were detected in flour from inoculated versus non-inoculated plants in Greenhouse I (Figure 5). However, significantly lower %UPP values were detected in flour samples from wheat inoculated with *F. avenaceum* 1 and all three isolates of *F. graminearum* compared to non-inoculated samples in Greenhouse II (Figure 5). When the statistical analysis was performed separately for samples from Greenhouse I and II, only the *F. graminearum*-inoculated wheat samples were significantly different from the control samples in Greenhouse II.

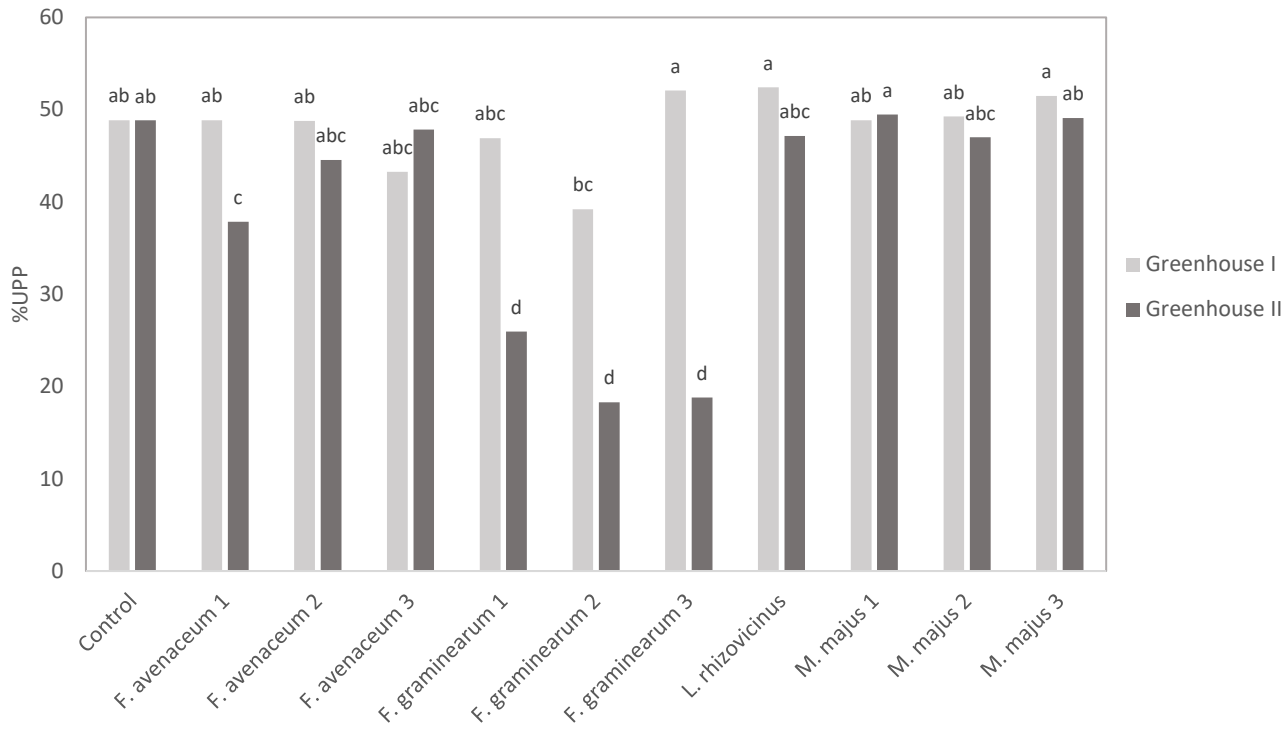
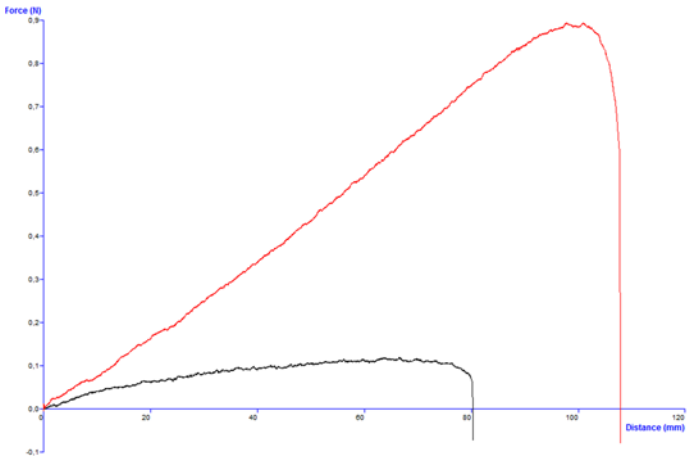


Figure 5 Means of proportion of unextractable polymeric protein (%UPP) in flour samples. In addition to a non-inoculated control, samples include wheat inoculated with the following microorganisms: Fusarium avenaceum, Fusarium graminearum, Microdochium majus, and Luteibacter rhizovicinus. Samples are from Greenhouse I and II of the inoculation experiment and received 4-day incubation. Numbers following species indicate unique isolates. Letters above columns correspond to Tukey's HSD analysis on a 95% confidence interval. Columns that do not share a letter have statistically different values.

Measuring Viscoelastic Properties of Gluten

Flour from *F. graminearum*-inoculated wheat in Greenhouse II was selected for this study since both protease activity and affected %UPP values were detected in these samples. Of these, a notable effect on R_{MAX} was detected in samples with 4-day incubation (Figure 7). The effect observed for *F. graminearum*-inoculated samples was so substantial that the proportion of sample flour mixed into commercial flour had to be reduced to 20% attain readings. In one instance, sample flour had to be reduced further to 10% to obtain a reading, and it was this sample, inoculated with *F. graminearum* 1, that produced one of the extensograms in Figure 6. It stands in stark contrast to the control sample extensogram. The most severely infected flour samples, based on fungal DNA content and symptoms of infection, were quite small and replicates of both the *F. graminearum* 1 and *F. graminearum* 2 4-day incubated samples from Greenhouse II had to be combined in order to have enough for analysis. Limited sample material also precluded producing adequate data for statistical analysis of R_{MAX} values. Nevertheless, Figure 7 makes the dramatic effect of *F. graminearum*-inoculation with 4-day incubation apparent. Tendencies to increased extensibility values were observed for some samples inoculated with *Fusarium* spp., but differences were not significant.

(A)



(B)

Inoculation (% sample material)	R_{MAX} (N)	Extensibility (mm)
Control (30%)	0.895	107.991
F. graminearum (10%)	0.121	80.318

Figure 6 Representative extensograms (A) and R_{MAX} and extensibility values (B) for two flour samples: control (red) and a sample of flour made from wheat inoculated with Fusarium graminearum isolate 1 (black). Both received 4-day incubation in Greenhouse II. Percentages listed after inoculation treatment in the table (B) indicate proportion of sample material of total flour used for analysis. Commercial whole wheat flour comprised the rest.

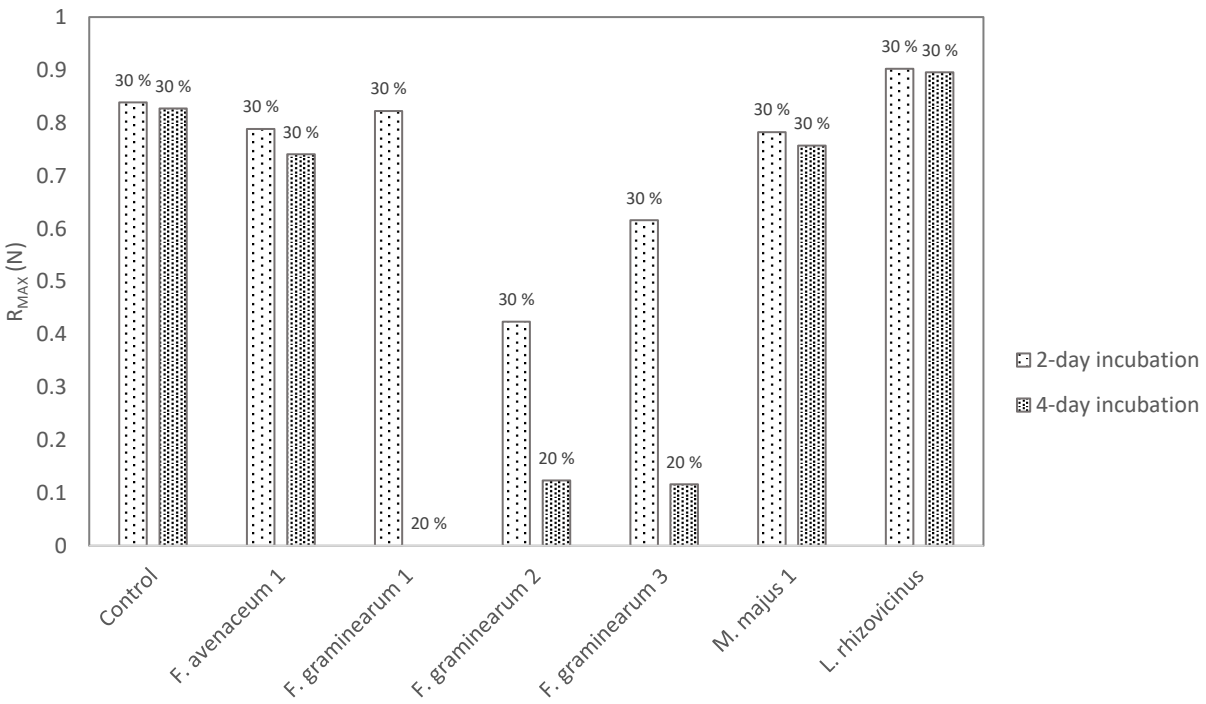


Figure 7 Mean maximum resistance to extension (R_{MAX}) values generated by stretching gluten obtained from flour made from inoculated wheat and commercial grade flour. In addition to a control, sample flour was made from wheat inoculated with one of the following species: Fusarium avenaceum, Fusarium graminearum, Microdochium majus, and Luteibacter rhizovicinus. The numbers appearing after the species in the figure indicate isolates. Samples are from Greenhouse II of the inoculation experiment and received 2-day or 4-day incubation following inoculation. Percentages above columns indicate proportion of sample material used for analysis. Commercial whole wheat flour comprised the remaining amount.

Correlations between *F. graminearum* DNA Content and %UPP and R_{MAX}

Since it was observed that flour made from *F. graminearum*-inoculated wheat samples had reduced %UPP and R_{MAX} values, qPCR data was used to explore possible correlations with quantity of fungal DNA. The amount of sample flour used for each analysis, SE-FPLC and Kieffer extensograph, was multiplied by pg mg^{-1} *F. graminearum* DNA obtained for each specific sample with qPCR analysis. The natural log of total *F. graminearum* DNA content in the sample tested was then plotted against both %UPP and R_{MAX} values (Figure 8). Linear regression analysis showed the transformed quantity of *F. graminearum* DNA to be significantly negatively correlated to both R_{MAX} and %UPP values ($p<0.001$ for both).

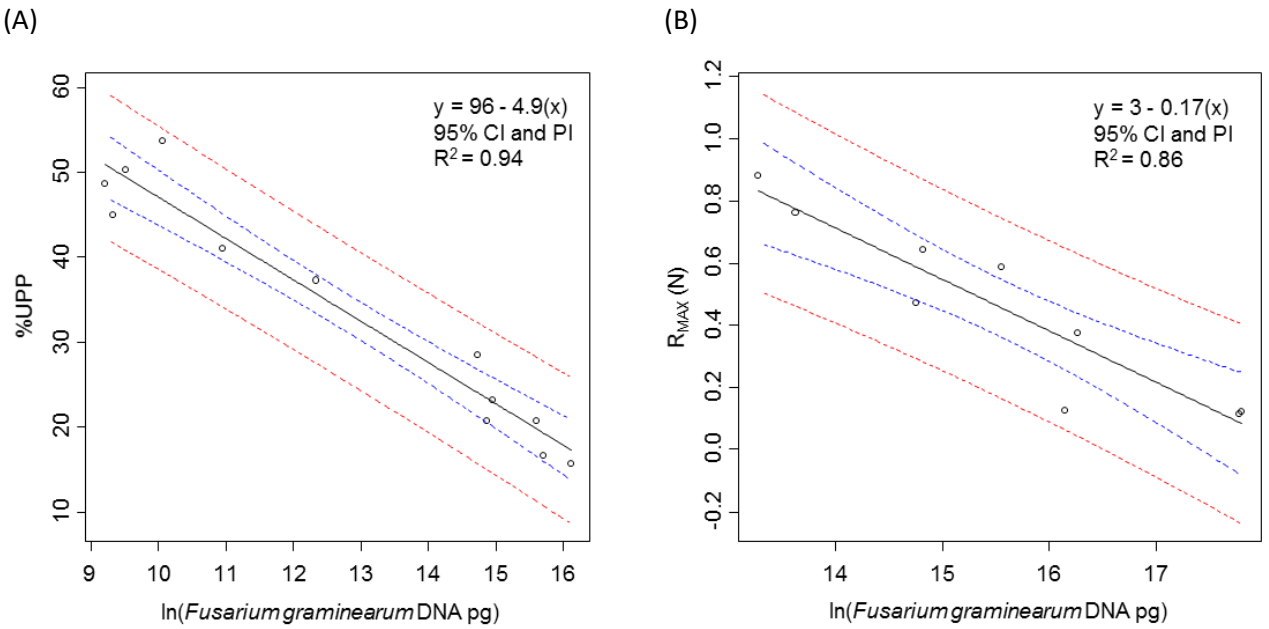


Figure 8 Fitted regression plots showing association between the transformed content of Fusarium graminearum DNA in whole wheat flour and the proportion of unextractable polymeric protein (%UPP, A) and the resistance to stretching (R_{MAX}, B). Sample flour used to obtain %UPP values was from wheat from Greenhouse I and II of the inoculation experiment and received 4-day incubation following inoculation. Sample flour used to obtain R_{MAX} values was from wheat from Greenhouse II of the inoculation experiment and received 2-day or 4-day incubation following inoculation. The flour samples are from wheat inoculated with Fusarium graminearum. The blue dashed lines mark the 95% confidence intervals and the red dashed lines mark the 95% prediction intervals.

Media Experiment to Explore Gluten Degradation Capabilities of Fungi

Fungal Growth Rates on Media with Various Nitrogen Sources

In the media experiment, all four fungal species grew on all four types of media at 15°C (Figure 9). *M. majus* and *M. nivale* generally grew faster than *F. avenaceum* and *F. graminearum* on all media with the exception of the one with $NaNO_3$ as a nitrogen source. Generally, *M. majus* and *M. nivale* grew faster than *F. avenaceum and F. graminearum* on CM. All species except *F. avenaceum* had slightly faster growth rates (not statistically significant) on the medium with gluten as the only nitrogen source than on the complete medium. *M. majus* had a significantly higher growth rate than both *F. avenaceum* and *F. graminearum* on the gluten-containing medium. *M. nivale* had a significantly higher growth rate than *F. avenaceum* on gluten-containing medium.

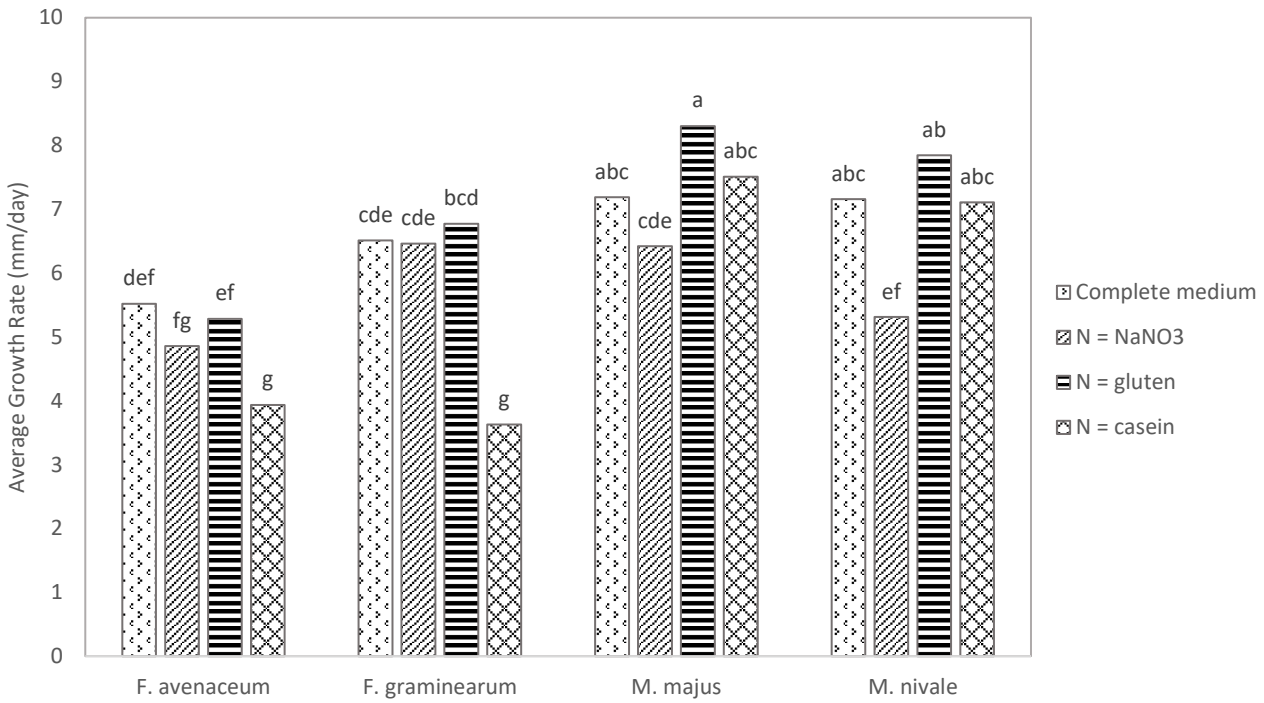


Figure 9 Average growth rates for Fusarium avenaceum, Fusarium graminearum, Microdochium majus, and Microdochium nivale on media with different nitrogen sources (complete medium with yeast extract, $NaNO_3$, and casein; and three minimal media each with one of $NaNO_3$, gluten, or casein). Mycelium plugs grew on petri dishes in the dark at 15°C. Letters above the columns correspond to groups from Tukey's HSD, 95% confidence interval. Columns which do not share letters are statistically different.

Detecting Gluten Degradation in Medium

The extra replicates of MM petri dishes with gluten as a nitrogen source exhibited areas of gluten degredation in the medium. This was easiest to observe in the replicate set for which the temperature had been reduced to 6°C approximately halfway through the growing period. This slowed growth enough to prevent mycelium from reaching the edges of the petri dish. When the medium was dyed there was a visible edge showing where degradation of gluten in the medium corresponded to the extent of the fungal growth on the medium (Figure 10).

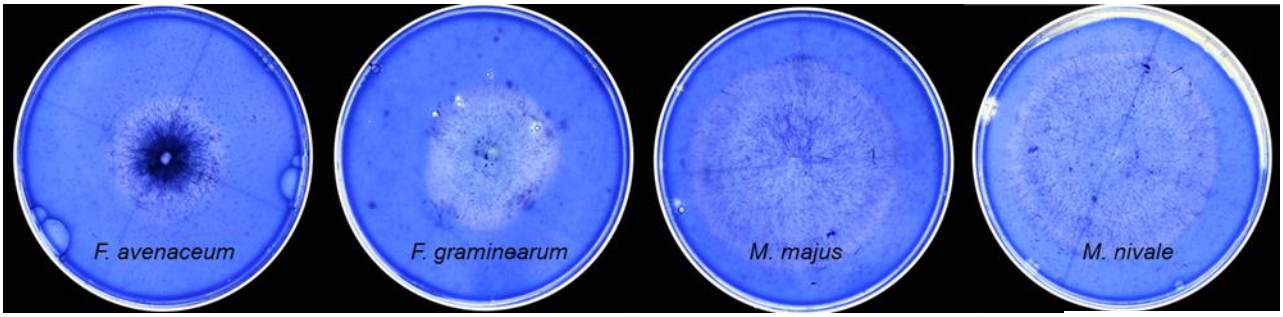


Photo: U. Böcker

Figure 10 Medium containing gluten as a sole nitrogen source stained after Fusarium avenaceum, Fusarium graminearum, Microdochium majus and Microdochium nivale were grown. The petri dishes were kept in the dark, first for three days at 15°C, and then four additional days at 6°C. Fungal mycelium growth was scraped off the medium and Coomassie blue R-250 used to stain remaining gluten in the medium.

Discussion

The objective of this study was to explore whether microorganisms, particularly *Fusarium* ssp., have the ability to produce gluten-degrading enzymes and have a negative impact on the baking quality of wheat flour. Analysis of protease activity in flour from inoculated wheat and in gluten-containing medium inoculated with a microorganism provided an indication of microorganisms' ability to degrade gluten proteins. Finally, analysis of baking quality exhibited whether infection and protease activity had negative consequences for flour when used for baking.

Disease Assessment of Kernels from Wheat Inoculated with Selected Microorganisms

The observation of symptoms in harvested wheat kernels and DNA quantification of the different microorganisms provided a means by which to determine whether inoculation with microorganisms had been successful.

Inoculation with *F. graminearum* was successful in both Greenhouse I and II of the inoculation experiment. Harvested kernels exhibited typical symptoms of infection including a shriveled appearance and discoloration. In addition, *F. graminearum*-inoculated grain in both Greenhouse I and II with 4-day incubation and Greenhouse II with 2-day incubation had significantly lower thousand-grain weights as a result of the characteristic shriveling. This is consistent with *Fusarium* damage described by Dexter et al. (1996). It should also be noted that in the most infected samples from Greenhouse II with 4-day incubation, threshing had to be done by hand in order to avoid losing the entire sample to the threshing machine. It can be assumed that kernels with this degree of infection would not likely enter the food chain. They would presumably be lost in threshing, or likely have DON-levels far above the established safe limits and be discarded at screening. Analysis of flour samples with qPCR confirmed high levels of infection observed visually. *F. graminearum* DNA quantities in Greenhouse II material that had received 4-day incubation, especially, were extremely high. In both Greenhouse I and II, *F. graminearum* infection increased with a longer incubation time following inoculation. Considerably higher DNA quantities detected by qPCR for Greenhouse II may be attributed to developmental stage of ears at time of inoculation and temperatures during incubation. At inoculation, Greenhouse I ears were at early-flowering, whereas Greenhouse II ears were closer to late-flowering. This

means that there would have been more material at a more susceptible stage for infection when ears were sprayed with *F. graminearum* spores for Greenhouse II (Del Ponte et al. 2007). In addition, temperatures of over 30°C were measured in the incubation bags for Greenhouse II. *F. graminearum* has been shown to flourish at warmer temperatures (Brennan et al. 2003; Ramirez et al. 2006). This combined with the humid conditions inside the plastic bags would have provided ideal infection conditions. Following incubation, temperatures rose to above 20°C in Greenhouse II nearly every day until harvest, providing conducive conditions for *F. graminearum* to continue to grow at temperatures near its optimum growth temperature of 25°(Brennan et al. 2003).

Inoculation with *F. avenaceum* also resulted in infection of wheat ears in both Greenhouse I and II of the inoculation experiment, but to a lesser extent than was the case for *F. graminearum*. Some discoloration and shriveling was observed in inoculated kernels. Shriveling, however, did not result in significantly decreased thousand-grain weight. Detection of *F. avenaceum* DNA with qPCR supported the conclusion of successful infection. In both Greenhouse I and II, DNA levels were increased with longer incubation times. This effect was stronger in Greenhouse II, yet less extreme than the increase seen for *F. graminearum*. Longer periods of high humidity providing by incubation may have contributed to higher DNA levels associated with longer incubation times for both Greenhouse I and II. High temperatures and ears closer to late-flowering at time of inoculation, as with *F. graminearum* inoculation, may have contributed to the highest levels of *F. avenaceum* observed in Greenhouse II with 4-day incubation. *F. avenaceum* DNA levels may have been lower than *F. graminearum* levels due to *F. avenaceum* having a lower optimum growth temperature (Brennan et al. 2003). In addition, *F. avenaceum* has been shown to be less aggressive in wheat compared to *F. graminearum* (Mesterházy et al. 2005).

M. majus inoculation in Greenhouse I and II was not deemed to be successful. Harvested, threshed kernels showed no symptoms of infection, and qPCR analysis confirmed the presence of only very low levels of *M. majus* DNA in inoculated kernels. Failure is assumed to be directly linked to very warm conditions that developed in the greenhouse. Temperatures of over 30°C were either in the greenhouse or in the incubation bags for both Greenhouse I and II of the inoculation experiment. *M. nivale* can grow at temperatures up to 28°C (Årsvoll 1975; Hofgaard et al. 2006), but the optimum temperature for growth is closer to 20°C (Brennan et al. 2003). The temperature constraints for *M. majus* growth would presumably be similar, and a study completed by Xu et al. (2008) even indicates that *M. majus* may have a lower colonization temperature than *M. nivale*. Tests indicated that spores used for inoculation were viable for both Greenhouse I and II, so inoculation failure is assumed to be due to temperature in the greenhouse exceeding the optimal range for this pathogen. *M. majus* isolates 1 and 2 also had lower spore concentrations in the suspensions used for inoculation in Greenhouse I, but DNA levels attained from qPCR did not reveal differences that could be attributed to this.

Inoculation with *L. rhizovicinus* produced no symptoms, and *L. rhizovicinus*-inoculated wheat samples were not analyzed with qPCR due to the fact that the species' DNA had not yet been sequenced, so the necessary materials were not available. The lack of visible effect of inoculation was not completely unexpected. The microorganism's role in relation to a wheat plant is unknown. If it is in fact a "plant growth promoting rhizobacteria" as one study suggests it could be (Guglielmetti et al. 2013), then the inoculation procedure was actually a poorly aimed bio-fertilization attempt. Another study (Ballhausen et al. 2015) isolated a bacteria in the *Luteibacter* genera that was found to have some antifungal activity, but association with the fungi in this preliminary study could also be attributed living off hyphal exudates, using the fungal hyphae as a sort of vector to infect plants, or merely using fungi as an alternate host. *L. rhizovicinus* can hydrolyze starch, which

could be a reason it would be associated with samples of poor baking quality. Regardless of whether *L. rhizovicinus* has any effect on wheat, its growth may have been hindered by high temperatures in the greenhouse as seemed to be the case for *M. majus.* In the original article describing the species, Johansen et al. (2005) found that *L. rhizovicinus* can grow at temperatures from 5 to 30°C, but absolutely not at 37°C.

There was some cross-contamination detected in the inoculation experiment. Results from qPCR revealed, however, that DNA quantities detected from species other that used for inoculation were low in nearly all cases. Only the *L. rhizovicinus*-inoculated wheat samples from Greenhouse II with 2-day incubation contained more than 1 pg mg^{-1} flour of DNA not belonging to *L. rhizovicinus*. This does not seem to have had a noticeable effect on results of the analyses conducted, so keeping pots inoculated with the same microorganism together but randomized in relation to others was sufficient to keep cross-contamination at a minimum for this study. Despite the observed disadvantage of low levels of cross-contamination, conducting inoculation experiments in the greenhouse facilitated limiting variation in factors such as cultivar, temperature, relative humidity, nutrient availability, light, and water availability. All plants within each greenhouse experiment received the same treatment except for inoculation microorganism, and this helps make it possible to assess the effects of microorganisms in relation to protease activity and baking quality.

Ability of Microorganisms to Degrade Gluten

Protease activity in flour samples from the inoculation experiment was analyzed with zymography, and microorganisms' ability to degrade gluten *in vitro* was assessed in a media experiment.

Protease activity was detected in flour samples from *F. graminearum*-inoculated wheat from both Greenhouse I and II of the inoculation experiment. Zymograms showed protease activity on both gluten and gelatin substrates in the gel. The streaking seen extending down from the loading well can be an indication that proteases were not denatured by SDS. If this was the case then these proteases were active during electrophoresis in these samples, meaning that electrophoresis was run in the "binding mode" and the length of the streak is then interpreted as an indication of relative protease quantity in the sample due to substrate hydrolysis (Lantz & Ciborowski 1994). Another interpretation of the streaking could be that high concentrations of protein in a sample moved into the gel slowly during the run (Garfin 2003). Either way, the wider bands lacking staining indicate higher relative levels of protease activity. In the zymogram with a gelatin substrate, the thick, streaked bands directly under the loading wells were, for most of the flour samples made from *F. graminearum*-inoculated wheat, accompanied by a distinct band approximately halfway down the zymogram. Perhaps this was an enzyme that was not active during electrophoresis that then degraded the gelatin substrate during incubation after proteases had been renatured. Protease activity observed on the zymogram containing gelatin shows that there were also proteases in flour made from wheat inoculated with *F. graminearum* that were able to degrade gelatin. That *F. graminearum* would produce different types of enzymes when infecting wheat is consistent with the results from the study conducted by Paper et al. (Paper et al. 2007) in which they identified several enzymes produced by *F. graminearum in planta*. In the media experiment, *F. graminearum* grew on a medium with gluten as the only nitrogen source at a rate not significantly different from the growth rate on complete medium. Staining of the gluten-containing medium also revealed gluten degradation. These results are consistent with other studies which also found that *F. graminearum* had the ability to degrade gluten proteins (Pekkarinen et al. 2000; Eggert et al. 2011).

F. avenaceum-inoculated wheat samples also showed protease activity. The zymogram with a gluten substrate showed activity for all isolates from Greenhouse II with 4-day incubation, two isolates for Greenhouse I with

4-day incubation, and one isolate for both Greenhouse I and II with 2-day incubation. Bands showing protease activity in the zymogram were thinner and less prone to streaking compared to *F. graminearum*. If this is interpreted to mean that protease amounts were lower in flour made from *F. avenaceum*-inoculated wheat, then it is consistent with qPCR findings of less DNA. Less severe infection would be expected to be reflected by less protease activity. The zymogram with a gelatin substrate shows the same distinct band for flour samples made from *F. avenaceum*-inoculated wheat as for those inoculated with *F. graminearum*. Zymography with other *Fusarium* ssp. could show whether the enzyme this band represents is common for the genera. All three *F. avenaceum* isolates grew on medium with gluten as the only nitrogen source at a combined average rate not significantly different their combined average rate on complete medium. Staining of the gluten minimal medium revealed degradation of gluten protein where *F. avenaceum* grew. Results from these experiments show that *F. avenaceum* can degrade gluten, which is consistent with findings of Nightingale et al. (1999). Results from this study showed that *F. avenaceum* degraded gluten to a lesser extent than *F. graminearum.*

M. majus protease activity was not observed with zymography. This may be a result of the very low quantity of this fungus present in grains from *M. majus*-inoculated plants, as described previously. Results from the media experiment revealed no significant differences in growth rates of *M. majus* on minimal medium containing gluten as the only nitrogen source compared to complete medium containing various nitrogen sources. Staining of the gluten-containing medium revealed gluten protein degradation by this fungus, and indicates *M. majus* can produce proteases that degrade gluten *in vitro*. The possibility that *M. majus* could produce gluten-degrading proteases when infecting wheat seems plausible but needs further testing.

M. nivale was not included in the inoculation experiment in the greenhouse, but as a FHB pathogen, it was interesting to include in the media experiment. *M. nivale* displayed the ability to utilize gluten as a sole nitrogen source as there was no significant difference between growth rate on complete medium containing several nitrogen sources and minimal medium containing only gluten as a nitrogen source. Staining of gluten-containing medium revealed gluten-degrading protease activity for *M. nivale* as well. Results from the media experiment could indicate that *Microdochium* ssp. have an advantage over *F. avenaceum* and *F. graminearum* in utilizing gluten as a sole source of nitrogen at 15°C. Perhaps a cooler growing season would favor *Microdochium* ssp. in the FHB complex of microorganisms.

This study offers no grounds upon which to draw conclusions regarding the ability of *L. rhizovicinus* to produce proteolytic enzymes that degrade gluten. Only fungal species were included in the media experiment, and the inoculation experiment was inconclusive, as discussed previously.

In the discussion thus far, the assumption has been made that protease activity observed in samples from the inoculation experiment came from the microorganisms. These are flour samples and could conceivably contain enzymes of plant origin. Koga et al. (2016) addressed this topic of endogenous proteases in their study. Indicating pre-harvest sprouting as a development associated with high protease activity within the wheat kernels, they used a protease inhibitor when assessing gluten protein degradation with incubation and SDS-PAGE. Despite inhibiting the main protease associated with pre-harvest sprouting, cysteine protease, levels of protease activity in the samples remained unaffected. These findings contributed to the conclusion that exogenous proteases were the cause of protease activity they observed. In dealing with plant pathogens, however, it is feasible that a plant's response to infection involves activation of different pathways and production of different products than those associated with pre-harvest sprouting. For example, *Fusarium* ssp. infection in plants is met with partial (also known as polygenic, quantitative, or horizontal) resistance, meaning that the plant relies on several tactics to deal with the intruding pathogen. This can include production of

pathogenesis proteins (Agrios 2005). Attributing the cause of this study's results to enzymes of plant origin, however, does not seem to be the best explanation. Results from qPCR clearly show a fungal presence in samples of poor quality. In addition, the media experiment showed that all of the isolates of *Fusarium* ssp. used in this study are perfectly capable of producing enzymes that degrade gluten. It seems logical to assume the same as Koga et al. (2016), and state that exogenous proteases can be considered the main source of the protease activity observed on the zymograms and in the media experiment in this study.

Influence of Microorganisms on Baking Quality in Wheat

SE-FPLC was used to analyze how microorganisms influence glutenin polymer size in flour made from inoculated wheat and the Kieffer extensograph test was used to test viscoelastic properties of gluten from the samples. These analyses helped to determine whether *F. graminearum*- and *F. avenaceum*-infected flour that had been shown to have protease activity also exhibited reduced baking quality.

Microorganisms Observed in Connection with Reduced Baking Quality

SE-FPLC analysis was completed and %UPP values generated for all 4-day incubation samples from both Greenhouse I and II of the inoculation experiment. Of these, flour samples from *F. graminearum*-inoculated wheat from Greenhouse II contained very high levels of fungal DNA, and a representative chromatogram for these samples was noticeably different from the chromatograms generated by the rest of the sample material. Only a negligible amount of unextractable polymeric protein (F1* peak) was detected in these Greenhouse II, 4-day incubation samples *F. graminearum*-inoculated wheat. The F1 peak was also only half the area of the corresponding peak for the control sample, so proportion of total polymers, not just unextractable polymers, in these samples was also low. Glutenin polymers were not only reduced in size, proportion of glutenin polymers was also drastically reduced in flour from wheat inoculated with *F. graminearum*. Multi-way ANOVA analysis showed that flour from *F. graminearum*-inoculated wheat from Greenhouse II with 4-day incubation had significantly decreased %UPP values. Flour samples with such low %UPP values would accordingly be expected to have poor baking quality based on the work of Gupta et al. (1993) who found %UPP to be positively correlated with dough strength. In addition to having low peaks corresponding to polymeric protein, these heavily infected *F. graminearum* samples from Greenhouse II had a greater quantity of smaller molecules. One possible explanation is that the analysis detected protein molecules from fungi which would dominate in such heavily colonized wheat kernels. Although flour from *F. graminearum*-inoculated wheat from Greenhouse I with 4-day incubation was also shown to be infected and have protease activity, it did not have significantly reduced %UPP values. Thus, protein characterization only indicated reduced baking quality for the *F. graminearum*-infected flour samples from the inoculation experiment which showed the worst symptoms of infection and had the highest fungal DNA content.

Flour from *F. graminearum*-inoculated wheat from Greenhouse II with 4-day incubation also had dramatically reduced R_{MAX} values for gluten, despite using only 10-20% sample material for the analysis. Since SE-FPLC analysis of these *F. graminearum*-inoculated wheat samples from Greenhouse II with 4-day incubation showed that the flour contained only negligible amounts of unextractable polymeric protein, the question is whether that would that have had enough effect on the gluten protein distribution to cause such a dramatic reduction in R_{MAX} when 80-90% of the flour was of strong commercial grade. Perhaps gluten preparation including the 45 minutes the gluten rested at 30°C in high humidity conditions allowed proteases from the infected sample to degrade functional gluten from the commercial flour. If this is the case, it could have ramifications for the bread baking industry. It means that proteases present in infected flour could degrade gluten protein in the

flour it is mixed with during the baking process and cause quality reduction. This was also suggested by Dexter et al. (1996) after observing reduced baking quality in a sample only after a procedure involving a significant fermentation period. Study of the fungal protease produced by *F. culmorum* was found to have an active temperature range from 10 to 100°C with a 50°C maximum (Wang et al. 2005). This protease was also associated with reduced baking quality in wheat, and it is possible that *F. graminearum*, being in the same genera, could produce proteases that are active in an equally broad temperature range. Whether it is possible that grain infected with *F. graminearum* can make it past DON screening and into the food chain with a high enough protease content to cause problems needs to be investigated.

Linear regression analysis results showed both %UPP and R_{MAX} values to be significantly negatively correlated with the natural log of *F. graminearum* DNA content (pg) of the flour used for analysis ($P<0.001$ for both). For this dataset from the inoculation experiment in the greenhouse, transformed *F. graminearum* DNA content accounted for 94% of variation observed in %UPP values and 86% of variation observed in R_{MAX} values for flour from *F. graminearum*-inoculated wheat. It is, however, important to consider this result in the context of the inoculation experiment where other environmental factors were approximately the same for all plants within each greenhouse experiment. The effect on R_{MAX} values may actually be a combination of having a lower proportion of unextractable polymeric protein to start with and protease activity during dough preparation prior to gluten stretching.

The gluten obtained from flour inoculated with *F. graminearum* (mixed with commercial flour) which failed to generate R_{MAX} values was sticky and difficult to handle. Gluten from one of the flour samples from *F. graminearum*-inoculated wheat from Greenhouse II with 2-day incubation was also somewhat sticky. Other studies have reported gluten stickiness in connection with *Fusarium* infection (Dexter et al. 1996; Capouchová et al. 2012). Capouchová et al. (2012) suggested the observed stickiness might be related to the starch in the samples also being affected by *Fusarium*. In an examination of *F. culmorum* damaged wheat kernels, Jackowiak et al. (2005) found that not only did *F. culmorum* produce enzymes capable of degrading starch, but that these enzymes could diffuse through the endosperm such that direct contact with fungal hyphae was not necessary for degradation.

F. avenaceum isolates also caused infection and protease activity was detected in resulting flour. Analysis of baking quality, however, was not as conclusive as for flour samples from *F. graminearum*-inoculated wheat. Flour from *F. avenaceum*-inoculated wheat generated chromatograms comparable to controls and only in the case of *F. avenaceum* 1 from Greenhouse II with 4-day incubation, were %UPP values significantly reduced. Given these results, flour samples from wheat inoculated with *F. avenaceum* were not prioritized in Kieffer extensograph analysis, and only flour from *F. avenaceum* 1-inoculated wheat from Greenhouse II with 2-day and 4-day incubation was analyzed. Results showed some reduction in R_{MAX} in 4-day incubated compared to 2-day incubated sample material when it made up 30% of total flour used. The %UPP results show that *F. avenaceum* infection can result in reduced baking quality and R_{MAX} values were also reduced, but this observation was limited to only one of three isolates used for this study.

Based on the results of this study, *F. graminearum* infection can have a negative impact on gluten protein and baking quality. This was also demonstrated for *F. avenaceum*, but to a lesser extent.

Microorganisms with Potential to Reduce Baking Quality

As foreseen based on inoculation failure and lack of protease activity on the zymograms, baking quality of flour made from wheat inoculated with *M. majus* was not different from control samples. The results of the media experiment, however, clearly demonstrated that both *M. majus* and *M. nivale* are capable of producing

proteases necessary to degrade gluten and use it as a nitrogen source *in vitro*. According to Brodal et al. (2016), *Microdochium* ssp. are among the most common FHB pathogens found in Norwegian seed grain. Compared to other FHB pathogens, *Microdochium* ssp. are more common in cool to moderate conditions with frequent precipitation events (Xu et al. 2008). Interestingly, several Norwegian wheat samples which have exhibited poor baking quality have been from cooler, wetter seasons (Koga et al. 2016). *Microdochium* ssp. produce no known mycotoxins, so there is no test, such as the test for DON content at grain delivery, that would prevent grain infected with *Microdochium* ssp. from entering the food chain. Despite other FHB pathogens, such as *F. graminearum*, being more common in warmer conditions (Xu et al. 2008), *Microdochium* ssp. may still be able to overcome predicted temperature increases due to climate change. *Microdochium* ssp. has shown adaptability, showing up in places such as Hawaii and California, and the anticipated lengthening of the growing season in Norway could allow for increased inoculum production (Tronsmo 2013). *Microdochium* ssp. are prevalent in Norway and may continue to be, even in the face of climate change. The media experiment in this study also demonstrated that both *M. majus* and *M. nivale* can degrade gluten and their connection to reduced baking quality in wheat deserves further attention.

Conclusion

Results from this study support the hypothesis that *F. graminearum* can infect wheat and produce proteases that degrade gluten proteins to the extent that it negatively affects baking quality. Negative effects on baking quality were also indicated for *F. avenaceum*. When grown on medium containing gluten as a sole nitrogen source, all fungal species analyzed in this study exhibited the ability to degrade gluten. *F. graminearum, F. avenaceum, M. majus* and *M. nivale* are therefore potentially capable of negatively affecting baking quality. *Microdochium* ssp.' connection to gluten degradation and reduced baking quality in wheat remains uncertain but merits further study, especially in the case of *M. majus*.

References

Agrios, G.N., 2005. How Plants Defend Themselves Against Pathogens. In *Plant Pathology*. Amsterdam: Elsevier Academic Press.

Ballhausen, M.-B. et al., 2015. Methods for Baiting and Enriching Fungus-Feeding (Mycophagous) Rhizosphere Bacteria. *Frontiers in Microbiology*, 6(1416).

Bechtel, D.B. et al., 1985. The Effects of Fusarium graminearum Infection on Wheat Kernels. *Cereal Chemistry*, 62(3), pp.191–197.

Belitz, H.-D. et al., 1986. Structure and Function of Gluten Proteins. *Cereal Chemistry*, 63(4), pp.336–341.

Bernhoft, A. et al., 2013. Risk assessment of mycotoxins in cereal grain in Norway.

Boyacloglu, D. & Hettiarachchy, N.S., 1995. Changes in some Biochemical Components of Wheat Grain that was Infected with Fusarium graminearum. *Journal of Cereal Science*, 21, pp.57–62.

Brennan, J.M. et al., 2003. Studies on in vitro growth and pathogenicity of European Fusarium fungi. *European Journal of Plant Pathology*, 109, pp.577–587.

Brodal, G., 2012. Fusarioser i korn (Fusarium spp). *Plantevernleksikonet*. Available at: http://leksikon.nibio.no/vieworganisms_pdf.php?mode=currentOrganism [Accessed April 24, 2017].

Brodal, G., Tangerås, H. & Henriksen, B., 2016. Smitte av fusariose på såkorn i Norge gjennom 45 år. In E. Strand, ed. *Jord- og Plantekultur 2016: Forsøk i korn, olje- og proteinvekster, engfrøavl og potet 2015*. Norwegian Institute of Bioeconomy Research (NIBIO).

Capouchová, I. et al., 2012. Effect of Different Intensities of Fusarium Infestation on Grain Yield, Deoxynivalenol Content and Baking Quality of Winter Wheat. *Romanian Agricultural Research*, 29.

Chakraborty, S. & Newton, A.C., 2011. Climate change, plant diseases and food security: An overview. *Plant Pathology*, 60(1), pp.2–14.

Clear, R.M. & Patrick, S.K., 2000. Fusarium head blight pathogens isolated from fusarium-damaged kernels of wheat in western Canada, 1993 to 1998. *Canadian Journal of Plant Pathology*, 22(1), p.51–6o.

Correll, J.C., Klittich, C.J.R. & Leslie, J.F., 1987. Nitrate Nonutilizing Mutants of Fusarium oxysporum and Their Use in Vegetative Compatability Tests. *Phytopathology*, 77(12), pp.1640–1646.

Dexter, J.E., Clear, R.M. & Preston, K.R., 1996. Fusarium Head Blight: Effect on the Milling and Baking of Some Canadian Wheats. *Cereal Chemistry*, 73(6), pp.695–701.

Dill-Macky, R. & Jones, R.K., 2000. The Effect of Previous Crop Residues and Tillage on Fusarium Head Blight of Wheat. *Plant Disease*, 84(1), pp.71–76.

Divon, H.H. et al., 2012. Fusarium langsethiae (Torp and Nirenberg), investigation of alternative infection routes in oats. *European Journal of Plant Pathology*, 132(1), pp.147–161.

Dobraszczyk, B.J., 2004. The physics of baking: rheological and polymer molecular structure–function relationships in breadmaking. *J. Non-Newtonian Fluid Mech*, 124, pp.61–69.

Doohan, F.M., Brennan, J. & Cooke, B.M., 2003. Influence of climatic factors on Fusarium species pathogenic to cereals. *European Journal of Plant Pathology*, 109, pp.755–768.

Eggert, K., Rawel, H.M. & Pawelzik, E., 2011. In vitro degradation of wheat gluten fractions by Fusarium graminearum proteases. *European Food Research and Technology*, 233, pp.697–705.

Garfin, D.E., 2003. Gel Electrophoresis of Proteins. In J. Davey & M. Lord, eds. *Essential Cell Biology*. Oxford: Oxford University Press, pp. 197–268.

Glynn, N.C. et al., 2005. Phylogenetic analysis of EF-1 alpha gene sequences from isolates of Microdochium nivale leads to elevation of varieties majus and nivale to species status. *Mycological Research*, 109(8), pp.872–880.

Goesaert, H. et al., 2005. Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in Food Science & Technology*, 16, pp.12–30.

Griffin, D.H., 1994. Fungal Physiology. In New York: Wiley-Liss.

Guglielmetti, S. et al., 2013. Luteibacter rhizovicinus MIMR1 promotes root development in barley (Hordeum vulgare L.) under laboratory conditions. *World Journal of Microbiology and Biotechnology*, 29(11), pp.2025–2032.

Gupta, R.B., Khan, K. & MacRitchie, F., 1993. Biochemical Basis of Flour Properties in Bread Wheats. I. Effects of Variation in the Quantity and Size Distribution of Polymeric Protein. *Journal of Cereal Science*, 18, pp.23–41.

Güldener, U. et al., 2006. Development of a Fusarium graminearum Affymetrix GeneChip for profiling fungal gene expression in vitro and in planta. *Fungal Genetics and Biology*, 43(5), pp.316–325.

Halstensen, A.S. et al., 2006. Real-time PCR detection of toxigenic Fusarium in airborne and settled grain dust and associations with trichothecene mycotoxins. *Journal of Environmental Monitoring*, 8, pp.1235–1241.

Heid, C.A. et al., 1995. Real Time Quantitative PCR. *Genome Research*, 6, pp.986–994.

Hofgaard, I.S. et al., 2016. Associations between Fusarium species and mycotoxins in oats and spring wheat from farmers' fields in Norway over a six-year period. *World Mycotoxin Journal*, 9(3), pp.365–378.

Hofgaard, I.S. et al., 2006. Isolates of Microdochium nivale and M-majus differentiated by pathogenicity on perennial ryegrass (Lolium perenne L.) and in vitro growth at low temperature. *Journal of Phytopathology*, 154, pp.267–274.

Jackowiak, H. et al., 2005. Scanning electron microscopy of Fusarium damaged kernels of spring wheat. *International Journal of Food Microbiology*, 98(2), pp.113–123.

Johansen, J.E. et al., 2005. Luteibacter rhizovicinus gen. nov., sp. nov., a yellow-pigmented gammaproteobacterium isolated from the rhizosphere of barley (Hordeum vulgare L.). *International Journal of Systematic and Evolutionary Microbiology*, 55(6), pp.2285–2291.

Kieffer, R. et al., 1998. Correlations of the Breadmaking Performance of Wheat Flour with Rheological Measurements on a Micro-scale. *Journal of Cereal Science*, 27, pp.53–60.

Koga, S. et al., 2016. Investigating environmental factors that cause extreme gluten quality deficiency in winter wheat (Triticum aestivum L.) Investigating environmental factors that cause extreme gluten qual. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science*, 66(3), pp.237–256.

Landbruks- og matdepartementet, 2016. *Melding til Stortinget 11 (2016–2017) Endring og utvikling: En fremtidsrettet jordbruksproduksjon*,

Landbruks- og matdepartementet, 2011. *Melding til Stortinget 9 (2011-2012) Landbruks- og matpolitikken*, Available at: https://www.regjeringen.no/contentassets/adb6bd7b2dd84c299aa9bd540569e836/no/pdfs/stm201120120009000dddpdfs.pdf [Accessed April 22, 2017].

Lantz, M.S. & Ciborowski, P., 1994. [45] Zymographic techniques for detection and characterization of microbial proteases. *Methods in Enzymology*, 235, pp.563–594.

Leonard, K.J. & Bushnell, W.R., 2003. *Fusarium head blight of wheat and barley*, St. Paul: APS Press.

Leslie, J.F. & Summerall, B.A., 2006. *The Fusarium Laboratory Manual*, Oxford: Blackwell Publishing Ltd.

Macritchie, F., 1987. Evaluation of Contributions from Wheat Protein Fractions to Dough Mixing and Breadmaking. *Journal of Cereal Science*, 6, pp.259–268.

Madgwick, J.W. et al., 2011. Impacts of climate change on wheat anthesis and fusarium ear blight in the UK. *European Journal of Plant Pathology*, 130(1), pp.117–131.

Mesterházy, Á. et al., 2005. Common resistance to different Fusarium spp. causing Fusarium head blight in wheat. *European Journal of Plant Pathology*, 112(3), pp.267–281.

Moldestad, A. et al., 2011. Effect of temperature variation during grain filling on wheat gluten resistance. *Journal of Cereal Science*, 53, pp.347–354.

Nightingale, M.J. et al., 1999. Fusarium head blight: effect of fungal proteases on what storage proteins. *Cereal Chemistry*, 76(1), pp.150–158.

Osborne, T.B., 1924. *The Vegetable Proteins* 2nd ed., London: Longmans,Green And Co.

Paper, J.M. et al., 2007. Comparative proteomics of extracellular proteins in vitro and in planta from the pathogenic fungus Fusarium graminearum. *Proteomics*, 7(17), pp.3171–3183.

Parikka, P., Hakala, K. & Tiilikkala, K., 2012. Expected shifts in Fusarium species' composition on cereal grain in Northern Europe due to climatic change. *Food Additives & Contaminants: Part A*, 29(10), pp.1543–1555.

Pekkarinen, A. et al., 2000. Production of Proteases by Fusarium Species Grown on Barley Grains and in Media Containing Cereal Proteins. *Journal of Cereal Science*, 31, pp.253–261.

Del Ponte, E.M., Fernandes, J.M.C. & Bergstrom, G.C., 2007. Influence of growth stage on fusarium head blight and deoxynivalenol production in wheat. *Journal of Phytopathology*, 155(10), pp.577–581.

Ramirez, M.L., Chulze, S. & Magan, N., 2006. Temperature and water activity effects on growth and temporal deoxynivalenol production by two Argentinean strains of Fusarium graminearum on irradiated wheat grain. *International Journal of Food Microbiology*, 106(3), pp.291–296.

Reischer, G.H. et al., 2004. Quantification of Fusarium graminearum in infected wheat by species specific real-time PCR applying a TaqMan Probe. *Journal of Microbiological Methods*, 59, pp.141–146.

Shewry, P.R. et al., 1995. Biotechnology of Breadmaking: Unraveling and Manipulating the Multi-Protein Gluten Complex. *Biotechnology*, 13, pp.1185–1190.

Singh, N.K. et al., 1990. Use of Sonication and Size-Exclusion High-Performance Liquid Chromatography in the Study of Wheat Flour Proteins. I. Dissolution of Total Proteins in the Absence of Reducing Agents. *Cereal Chemistry*, 67(2), pp.150–161.

Sundheim, L. et al., 2013. Temporal Variation of Mycotoxin Producing Fungi in Norwegian Cereals. *Microorganisms*, 1(1), pp.188–198.

Trail, F. et al., 2003. Analysis of expressed sequence tags from Gibberella zeae (anamorph Fusarium graminearum). *Fungal Genetics and Biology*, 38(2), pp.187–197.

Tronsmo, A.M. et al., 2001. Low temperature diseases caused by Microdochium nivale. In N. Iriki et al., eds. *Low Temperature Plant Microbe Interactions Under Snow*. Hokkaido National Agricultural Experiment Station, pp. 75–86.

Tronsmo, A.M., 2013. Snow Moulds in a Changing Environment—A Scandinavian Perspective. In R. Imai, M. Yoshida, & N. Matsumoto, eds. *Plant and Microbe Adaptations to Cold in a Changing World*. New York, NY: Springer New York, pp. 305–317.

Tronsmo, K.M. et al., 2002. A Study of how Size Distribution of Gluten Proteins, Surface Properties of Gluten and Dough Mixing Properties Relate to Baking Properties of Wheat Flours. *Journal of Cereal Science*, 35, pp.201–214.

Uhlen, A.K. et al., 2015. Variation in gluten quality parameters of spring wheat varieties of different origin grown in contrasting environments. *Journal of Cereal Science*, 62, pp.110–116.

Wang, J. et al., 2005. Impact of the fungal protease produced by Fusarium culmorum on the protein quality and breadmaking properties of winter wheat. *Eur Food Res Technol*, 220, pp.552–559.

Weegels, P.L., Hamer, R.J. & Schofield, J.D., 1996. Critical Review: Functional Properties of Wheat Glutenin. *Journal of Cereal Science*, 23, pp.1–18.

Waalwijk, C. et al., 2004. Quantitative detection of Fusarium species in wheat using TaqMan. *European Journal of Plant Pathology*, 110, pp.481–494.

Xu, X.-M. et al., 2008. Relationship Between the Fungal Complex Causing Fusarium Head Blight of Wheat and Environmental Conditions. *Phytopathology*, 98(1).

Aamot, H.U. et al., 2015. Genetic and phenotypic diversity within the Fusarium graminearum species complex in Norway. *European Journal of Plant Pathology*, 142(3), pp.501–519.

Aamot, H.U., Hofgaard, I.S. & Lysøe, E., 2017. Complete genome sequence of Luteibacter rhizovicinus strain LJ96T, isolated from the rhizosphere of barley (Hordeum vulgare L.) in Denmark. *Genomics Data*, 11, pp.104–105.

Årsvoll, K., 1975. Fungi causing winter damage on cultivated grasses in Norway. *Scientific Reports of the Agricultural University of Norway*, 54(9), pp.1–49.

Appendix

Table 6 Information on potting soil used for inoculation experiment in the greenhouse (Tjerbo Torvfabrikk, Rakkestad, Norway). Information is translated from the label (original text in Norwegian).

Contents	86 vol. % sphagnum peat, H2-H5, 10 vol. % sand, 4 vol. % granulated clay
Added pr. m^3	4 kg pulverized limestone, 1 kg pulverized dolomite, 1 kg Multimix with micronutrients
Dry matter	160 g $liter^{-1}$
Organic content	45 % of dry matter
Acidity	pH 5.5-6.5
Conductivity	25 mS m^{-1}
Phosphorus (P-CAT)	35 mg L^{-1}
Potassium (K-CAT)	190 mg L^{-1}
Total N	900 mg L^{-1}
Volume	50 liter
Density	340 kg m^{-3}

Table 7 Basal medium recipe (1 L) used for media experiment.

Ingredient	Amount
KH_2PO_4	1 g
$MgSO_4$ $7H_20$	0.5 g
KCl	0.5 g
Agar, Bacto	20 g
Trace element solution	0.2 ml
Distilled H_2O	to 1 L

Table 8 Trace element solution recipe (100 ml) used in basal medium recipe for media experiment.

Ingredient	Amount
Citric acid $1H_2O$	5,46 g
$ZnSO_4$ $7H_2O$	5,33 g
$Fe(NH_4)_2(SO_4)_2$ $6H_2O$	1 g
$CuSO_4$ $5H_2O$	250 mg
$MnSO_4$ $4H_2O$	73.83 mg
H_3BO_4 (Boric acid)	50 mg
Na_2MoO_4 $2H_2O$	50 mg
Distilled H_2O	95 ml

Table 9 Vitamin stock solution recipe (1 L) used in complete medium (CM) for media experiment.

Ingredient	**Mengde**
myo-inositol	4 g
hemicalcium salt	200 mg
Choline Cl	200 mg
Thiamine HCl	100 mg
Pyridoxine	75 mg
Nicotinamide	75 mg
L-Ascorbic acid	50 mg
4-aminobenzoic acid	5 mg
Folic acid	5 mg
Biotin	5 mg
50:50 ethanol:H_2O	to 1 L

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