GROWTH OF *FUSARIUM GRAMINEARUM* AND PRODUCTION OF TRICHOTHECENES DURING THE MALTING OF WINTER RYE AND TRITICALE

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

There is growing interest in malting and brewing with rye. However, previous research has shown a propensity for the development of deoxynivalenol (DON) in rye malts, even when levels on the grain is low. The main objective of this study was to assess the growth of *F. graminearum* and development of trichothecenes during malting of rye. Infected samples were obtained from 2016 variety trails in Minnesota. While DON levels were generally below 0.2 mg/kg, an average increase of 41% was seen after malting. The most significant increases in DON were at three days of germination. *Fusarium* Tri5 DNA levels were observed to increase at two days. When single kernels were tested, most were free from DON. Levels in the bulk grain sample were due to a small number of highly contaminated kernels. In the malted samples, a greater portion of kernels contained DON, and overall levels were much higher.

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1. INTRODUCTION

Rye (*Secale cereale* L.) is a cereal mainly grown in northern Europe and Asia between the Ural Mountains and the Nordic Sea, with minimal soil, fertilization and climate requirements (Anonymous, 2002). Rye's low demand of the environment and high source of fiber has made it a commonly used ingredient in bread for centuries. However, recently some craft breweries started using rye malt to add spicy, nutty, and robust flavor to beer. Also, adding rye malt can help to increase the range of beer color (Clemons et al, 2006). However, maltsters and brewers need to be aware that rye grains infected with Fusarium Head Blight (FHB) can pose food safety issue. Rye is a cereal that can be easily infected by *Fusarium* species. Some *Fusarium* species can produce the type B trichothecene mycotoxins such as deoxynivalenol (DON, also known as vomitoxin) and its acetylated forms, 3- and 15- acetyl-deoxynivalenol (3ADON and 15ADON). Deoxynivalenol-3-glucoside (D3G, also known as masked mycotoxin), the plant metabolite of DON, may also cause potential problems because it can be hydrolyzed by intestinal microbiota and release free mycotoxins (Gratz et al, 2013; Dall'Erta et al, 2013). In addition to health concerns, *Fusarium* species associate with FHB, a plant disease, can contribute to economic losses for producers of small grains such as wheat, barley, and rye.

Many studies have been published concerning the fate of mycotoxins, masked toxins, and *Fusarium* growth during the malting of barley (Schwarz et al, 1995; Lancova et al, 2008; Habler et al, 2016; Vegi et al, 2011). Also, previous research (Jin et al, 2018a) had demonstrated a marked propensity for the development of DON and several other tricothecenes during the malting of FHB infected rye. High levels were detected in malts from rye samples that had no detectable DON. This is not typically observed with barley and is problematic as maltsters

generally select grain based upon DON levels. This is of concern as there is growing interest in the production of rye for malt, beer, distilled beverages and artisan baking.

Therefore, the main objectives of this study were to assess the growth of *Fusarium graminearum* and the development of trichothecene toxins during the malting of rye. The development of D3G was monitored during malting to assess the relevance of DON with other conjugates. Also, the distribution of DON in single rye and triticale kernels was evaluated, before and after malting.

2. LITERATURE REVIEW

2.1. Fusarium Head Blight

2.1.1. Introduction of the Fusarium Head Blight

Chester and Arthur first described Fusarium Head Blight (FHB) and its associated term scab in the late 19th century (Shaner, 2003). In North America, FHB is a very common plant disease for small grains, such as barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.). Similarly, rye (*Secale cereale* L.) can be infected by FHB (Osborne and Stein, 2007; McMullen et al, 2011; Papouskova et al, 2015).

FHB is an important concern for cereal growers, due to crop yield reduction and lower economic returns. Significant yield losses and quality reductions can be caused by FHB due to shriveled, light test-weight kernels (McMullen et al, 2011). Yield losses can approach 100% when the infection occurs at the flowering or the early stage of kernel development due to sterile spikelets and extremely thin kernels (Steffenson, 2003).

Wilson et al (2017) summarized the economic value of crop losses suffered by US wheat and barley producers, and estimated the impact of risk mitigation tools (e.g. fungicide uses, resistant varieties development, and management practices) in reducing FHB. They indicated that the average production loss for hard wheat was around 113 million bushels with $427 million of average revenue loss during the base period of 1993 to 1996 (before widespread utilization of fungicides), but subsequently declined from 1997 until 2014 (fungicide started being applied to 70-80% of cereals). The average production loss in 2014 was around 18 million bushels or $120 million of average revenue loss. Similar results occurred for durum and soft wheats. The trends of average production losses and variability declined from the base period (1993-1996) for all years.

However, barley was an exception. The average production loss for barley declined from 11 million bushels during the base period (1993-1996) to 6 million bushels in 2012. However, in 2014, the average production loss was as high as 14 million bushels. Also, even during the years preceding the US Wheat and Barley Scab Initiative, there were still higher average revenue losses for some years compared with the base period (i.e. when the average revenue loss was $20 million). The average revenue loss was $78 million in 2014. The higher losses of production and revenue may be a result of the absence of resistant varieties (Wilson et al, 2017). Therefore, continued research on the development of resistant varieties development for barley is necessary. Furthermore, testing of DON can be an important strategy for producers to buy barley in epidemic years that is within DON advisory levels.

2.1.2. Symptoms of the Fusarium Head Blight

The symptoms of FHB are easily recognized in the field because no other plant disease produce the same symptoms (Shaner, 2003). The first and most typical symptoms on wheat are bleached spikelets. A brown discoloration of stem tissue, and infected grains on the spike and head are indicators of FHB infection (McMullen et al, 2011). Head-blight infected wheat is characterized by blight heads, brown peduncle tissues, and prematurely senesces (i.e. plant death), while infected barley kernels at harvest may show a pinkish discoloration. Kiecana and Mielniczuk (2012) reported field observations of infected rye kernels from heads that were diminished. Visible orange-rosy powder was present on the chaff, and consisted of the mycelium and spores of the *Fusarium* spp. Further, the infected heads contained smaller kernels with a white-greyish color.

2.1.3. Fusarium Head Blight Pathogens

The genus *Fusarium* was first named for fungus having fusiform spores by a German mycologist in 1809 (Stack et al, 2003). Many species from *Fusarium*, a large and common group of fungi, have been studied by mycologists. Over 1000 *Fusarium* species were discovered by the end of the 19th century, including the predominant species that causes FHB, *Fusarium graminearum*, which was first described by Schwabe in 1838 (Booth, 1971). There are approximately 20 *Fusarium* species that can cause FHB and damage crops. The most common species includes *Fusarium graminearum, F. culmorum, F. avenaceum, F. asiaticum,* and *F. sporotrichioides* (Osborne and Stein, 2007). *Fusarium graminearum* is the most common causal agent of FHB in North American.

Fusarium graminearum (teleomorph: *Giberella zeae*) was divided into two groups. The head blight pathogen, the most prevalent group, was designated as group 2, and includes members that readily form perithecia, which serve as inoculum and are transmitted by air. Group 1 members do not form perithecia in culture, and are associated with crown rot (Bushnell et al, 2003). Group 1 was later reclassified as *G. coronicola* based on molecular phylogenetic analysis.

2.1.4. Impact of the Fusarium Head Blight Disease on Grain Safety

FHB is a great concern to small grain producers and processors, not only because of the economic aspect, but also because of the possible presence of mycotoxins on grain that is utilized as animal feed or in food. There are many historical records of outbreaks of foodborne illness caused by the consumption of FHB infected grain. As of 1987, there have been 35 reported outbreaks in China due to the consumption of foods made with moldy corn and wheat, that affected 7818 people from 1961 to 1985 (Luo, 1988). Most people showed similar gastrointestinal illness symptoms include nausea, vomiting, and diarrhea. In 1987, 97 people

having similar symptoms in the Kashmir Valley, India, was reported (Bhat et al, 1989). After an investigation, Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium*, was detected in the range of 0.34 to 8.4 mg/kg in the wheat products they consumed (Pestka, 2010). The presence of DON and other mycotoxins in cereals suggests that additional research is needed to mitigate the problem of contaminated cereals from entering the food supply.

2.2. Deoxynivalenol and its Conjugates

2.2.1. Deoxynivalenol

2.2.1.1. Introduction

Over 200 toxins are in trichothecene family, which have been mainly classified into four groups, type A, B, C, and D (McCormick et al, 2011). The main mycotoxins produced by *Fusarium* including deoxynivalenol (DON), T-2 toxin, HT-2 toxin, and nivalenol (NIV), as well as zearalenone (ZEA) (Osborne and Stein, 2007; Schwarz et al, 1995). Type A trichothecene, such as T-2 toxin, HT-2 toxin, and neosolaniol (NEO), are mainly produced by *F. sporotrichioides, F. langsethiae, F. eqiuseti*, and *F. venenatum*. NIV, DON, and trichothecin belong to type B trichothecenes, and those are mainly produced by *F. graminearum, F. cerealis, F. culmorum*, and *F. poae* (McCormick et al, 2011; Leslie and Logrieco, 2014). DON (Figure 1) is the most commonly found trichothecene in cereals from Europe and North America when compared with other type A trichothecene toxins, such as T-2 and HT-2 (Bottalico and Perrone, 2002; Pestka, 2010). DON is an organic compound with a polar structure that contains three free hydroxyl groups (-OH). The hydroxyl groups are associated with the toxicity of DON.

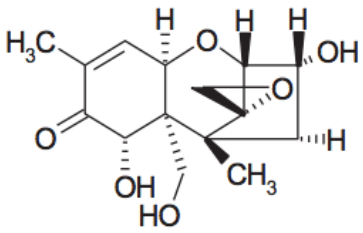


Figure 1. Structure of Deoxynivalenol (Berthiller et al, 2015).

2.2.1.2. Acute and Chronic Toxicity

Trichothecenes, are also known as 12, 13-epoxy trichothecenes due to the presence of an epoxide group at the C12-C13 position, which is essential to the biological activity and contributes to its toxic potential (Grove, 1988; Pestka, 2010). Trichothecenes can bind to eukaryotic ribosomes and inhibit protein synthesis (Ueno, 1983). Also, Pestka and Smolinski (2005) summarized some other toxic mechanisms of trichothecene from previous research, including impaired membrane function, altered intercellular communication, and deregulation of calcium homeostasis. DON is less toxic than other mycotoxins in the trichothecene group such as T-2 toxin, which is more common in Europe (Sobrova et al, 2010). However, it can still be poisonous in high doses, and shock-like death may occur when extremely high levels of DON are consumed. Pestka and Smolinski (2005) summarized the DON dose for acute effects, in which mice, ducks, hens, swine, cats and dogs were used. From acute toxicity studies, mortality or marked tissue injury was observed with extremely high DON (>27 mg/kg b.w.) levels, while emesis was observed with low doses (~50 µg/kg) of DON. In addition, Pestka and Smolinski (2005) summarized the sub-chronic and chronic toxicity of DON. Reduction of weight gain and anorexia were observed as common effects of prolonged dietary exposure. The no-observed-adverse-effect-levels (NOAELs) for rodents and swine are 0.1-0.15 mg/kg b.w./d and 0.03-0.12 mg/kg b.w./d, respectively. The results suggest that animals have different sensitivities to DON exposure because different species have different abilities to absorb and metabolize DON (Pestka

and Smolinski, 2005). For example, compared with rodents and dogs, swine are more sensitive to subchronic and chronic DON exposure. Overall, the sensitivity of animals to DON are as follows: swine > mice > rats > poultry ≈ ruminants. Prelusky and Trenholm (1991) reported the tissue distribution of DON in the swine dosed intravenously. DON level reached a maximum at 0.33-1 hour, and the highest levels were detected in plasma, kidney, and liver, followed by fat, lymph, lung, and adrenals. Pestka and Smolinski (2005) reported the distribution of DON in the organs of mice. The result showed that DON was rapidly distributed in all organs within 30 mins of exposure. Distribution of DON in the organs was ranked in the following order: kidney > heart > plasma > liver > thymus > spleen > brain.

2.2.2. Deoxynivalenol-3-glucoside (D3G)

2.2.2.1. Introduction

Deoxynivalennol-3-glucoside (D3G) was first reported in naturally contaminated maize and wheat in 2005 (Berthiller et al, 2005). D3G, a metabolite of DON (Figure 2), is formed by *Fusarium*-infected cereals as a response to the DON production by fungi (Berthiller et al, 2009). D3G is produced by glucosylation, a reaction occurring in phase II metabolism of plants, to reduce the toxicity of DON. Therefore, D3G represents a detoxification mechanism, and may be involved in the *Fusarium* resistance of cereal plant. Poppenberger et al (2003) reported that a UDP-glycosyltransferase from *Arabidopsis thaliana* can detoxify DON by catalyzing the transfer of glucose from UDP-glucose to the hydroxyl group at the carbon 3 of DON. D3G weakly binds to the ribosome, thus D3G has a reduced ability to inhibit the synthesis of protein. As such D3G less toxic when compared with other trichothecenes (Berthiller et al, 2013; Berthiller et al, 2015).

The production of D3G can occur in the field or during food-processing. Simsek et al (2012) reported the fate of D3G during milling and baking process. Results showed a significant

(P<0.05) reduction by 23.7% of D3G after milling of whole wheat, and a 51.6% reduction in D3G level in baked bread compared with mixed dough. D3G remained stable during fermentation and proofing, but was sensitive to heat.

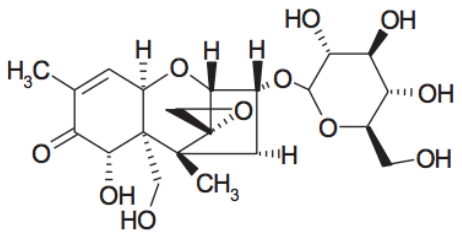


Figure 2. Structure of Deoxynivalenol-3-glucoside (Berthiller et al, 2015).

2.2.2.2. Toxicity

There has been limited number of research publications on the toxicity of D3G, although some in-*vitro* studies about the toxic effects have been published recently. The oral bioavailability and absorption of D3G have been investigated by Nagl et al (2012) and Broekaert et al (2017). Their results showed that the oral bioavailability of D3G was around 2-5 times lower than DON, and the absorption of D3G was significantly slower than DON. Several researchers reported that masked mycotoxin could be hydrolyzed by intestinal microbiota of humans and pigs to release free mycotoxins (Gratz et al, 2013; Dall'Erta et al, 2013). Additionally, Berthiller et al (2013) reported a positive correlation between the efficacy of conversion of D3G to DON and the composition and abundance of microbiota. In order to assess the hydrolytic ability of microbiota in different regions of the small and large intestines, Gratz et al (2018) collected samples from the jejunum, ileum, cecum, colon, and feces from pigs, and incubated the samples anaerobically with D3G for 72 hours. The results showed that most of the microbiota can hydrolyze D3G very rapidly except those in the jejunum.

2.2.3. Correlation Between DON and its Derivatives

Masked forms of DON including D3G and its acetylated forms, 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON), are the main derivatives that might contribute to DON exposure (JECFA, 2011). The presence of D3G, 3ADON, and 15ADON in food and feed has been reported previously. The conversion of D3G and acetylated forms to DON in cereals has been reported (Berthiller et al 2005; Berthiller et al, 2009). Berthiller et al (2009) reported that the average ratios of D3G/DON in wheat and maize samples were 17% and 14%, respectively. Schwarz et al (2014) reported D3G content in barley samples from the US was present at levels of <0.20-3.11 mg/kg, representing about 10-19% of the measured DON content, and the variation in the D3G/DON ratio was suggested to be due to environmental and cropping conditions. Formation of mycotoxin glucosides were observed not only in the raw grains, but the transformation of DON into its conjugated forms was also observed during germination (Maul, 2012). For instance, during germination, there is an increased amount of activated uridine diphosphate glucose that can be consecutively attached to the mycotoxin by uridine diphosphate glycosyltransferases, which are able to transform mycotoxin to mycotoxin glucosides. Therefore, germination may induce the glycosylation of DON in *Fusarium* infected grains. Maul et al (2012) reported the ability of various grains to transform DON into D3G, 3ADON, and 15ADON during germination. The results showed no formation of 3- and 15-ADON. However, D3G formation was observed for most of the grains, and there was a continuous increase of D3G content until the end of germination. The observed conversion was minimal at the beginning of germination, and generally increased from 17 h until 50 h, with an average formation rate of 4.8 nmol kg^{-1} $hour^{-1}$ (Maul et al, 2012). Habler et al (2016) reported a significant increase in D3G during the malting of barley.

2.3. Fusarium Head Blight Problems in Malting and Brewing

2.3.1. The Impact of Fusarium Head Blight in Grain Quality

FHB can affect grain quality, both directly and indirectly (Schwarz, 2003). An indirect impact includes the reduction of grain yield caused by the infection, especially when infection occurs at flowering or the early stage of kernel development. Yield loss result as *Fusarium* spores attach to kernels, mycelia spread on the surface, and even get into the endosperm, resulting in shriveled and light test-weight kernels (Schwarz, 2003; Steffenson, 2003). The direct impact includes the effects in grain composition or metabolism that are related to fungal growth or metabolites (Schwarz, 2003). Effects to grain composition include the reduction of kernel fill, resulting in thinner kernels and less endosperm in proportion to the husk. With light and thin kernels, less material can be extracted in brewing, and a higher proportion of protein and some enzymes, such as α-amylase, may result. Several researchers have documented the changes of the *Fusarium*-infected grain structure by using scanning electron microscopy (SEM) (Schwarz, 2003; Jackowiak et al, 2005; Packa et al, 2008), including infected barley, wheat, and triticale. Damage to the starch granules and destruction of the cellular structure were observed in infected grains.

2.3.2. The Impact of Fusarium Head Blight During Malting

Good malt quality is a precondition for making beer with a good quality. During the malting process, the environmental conditions, such as temperature and moisture, should be well-controlled (Mallet, 2014). Usually, a 36- to 52-hour steeping process at 12-20°C is required to bring grain moisture content up to 43-46% range prior to germination. Steeping can help rinse chaff, dirt, and germination inhibitors, and it helps to induce the release of hydrolytic enzymes required for proper malt modification during germination. Steeped barley with an ideal moisture

can be transferred to a germination unit. The germination process requires that temperature to be maintained at 16-21°C, and with humidified air recirculation to maintain the moisture at 42-48%. The green malt is then dried in the kilning process to reduce the moisture to 4% for storage and flavor development. The factors mentioned above can affect final beer quality. Only when grains are malted under regulated conditions can a proper modification be obtained, enzyme activity developed, and characteristic flavor, aroma, and color be formed. However, the environmental conditions of malting, with high grain moisture and moderate temperature, would be favorable for *Fusarium* to grow, and mycotoxin production may occur during the growth of *Fusarium* (Schwarz, 2017; Schwarz, 2003).

Schwarz et al (1995), focused on the fate of DON and other trichothecenes during malting and brewing, and reported the development of DON, 15ADON, and 3ADON during malting of barley, which were naturally infected *F. graminearum*. DON levels increased throughout the whole malting process except steeping, which lowers the DON level. The solubilization of mycotoxin into the steep water or loss of *Fusarium* biomass during washing were likely explanations for the decreased DON levels during steeping (Schwarz, 2017). After five days of germination, there was an 18 to 114% increase of DON concentrations compared with the original infected barley. Also, increases of ZEA and 15ADON were observed during germination. There was a slight increase of DON and other trichothecenes during kilning due to a reduction of the high moisture content of the green malt with the progressively increasing temperature (40-65°C) during the first stage of kilning. DON stability at higher temperature is supported by Wolf-Hall and Bullerman (1998) who found that DON was stable at neutral to acidic pH at temperature up to 170°C.

A similar fate of *Fusarium* mycotoxins was reported by Lancova et al (2008) and Habler et al (2016). Additionally, the formation of D3G was investigated during malting. According to Lancova et al (2008), D3G was 8.6 times higher in malt than in original barley, and Habler et al (2016) reported that the significant increase of D3G was from the steeping-out to kilning. As observed for DON, there was no change observed on D3G concentration after steeping. High deoxynivalenol-glycosyl-transferase activity was found from germination until at least the initial stage of kilning, and the D3G was observed to be 33-56 times higher in the barley malt than the original barley.

The growth of the host pathogen, *Fusarium* spp was examined by Vegi et al. (2011), who reported that the malting process had a significant effect on the growth of *F. graminearum* for both inoculated barley and naturally infected barley. The growth of *F. graminearum* was measured by Tri5 DNA concentration. The Tri5 gene encodes the enzyme, trichodiene synthase, which catalyzes the first step in the trichothecene biosynthetic pathway (Brown et al, 2001). They observed around an 8-fold increase in the DNA concentration level during malting of the *F. graminearum* infected samples.

2.3.3. The Impact of Fusarium Head Blight During Brewing

The brewing operation includes four major steps: brewhouse operations (mashing, lautering, and boiling), fermentation, cellar operations, and packaging (Schwarz, 2017). Mashing is the first step where the barley malt is ground and extracted with hot water. The proteolytic enzymes of barley are active in this process, and the starch from the malt and adjunct can be broken down by amylase produced during malting, and converted to fermentable sugars (Schwarz, 2017). However, DON and its derivatives can be extracted into wort. After filtration (i.e. lautering), wort can be separated from the insoluble material (spent grain), then boiled for

around an hour to kill spoilage organisms. Even though most of the spoilage organisms are killed during boiling, most mycotoxins with adequate heat stability remain stable. Wort is then fermented for about a week with yeast (*Saccharomyces cerevisiae* or *S. pastorianus*) (Schwarz, 2017). The fermented wort is aged, filtered, carbonated, and packaged.

FHB infected barley has been found to have many impacts when it is utilized for making beer, such as poor germination, grain damage, and effects on beer quality (Schwarz, 2017). The enzymes and the metabolites produced from *Fusarium*-infected barley may damage the grain components and result in the alteration of malt and beer quality. Moreover, the alteration of wort-soluble nitrogenous compounds by *Fusarium*-enzyme may affect color, flavor, and foam.

Raw material contaminated by toxigenic fungi are a major concern for brewers because they can greatly influence barley, malt, and beer quality and safety (Schwarz 2003; Hill, 2015).

However, the ultimate concern for brewers about mycotoxin contamination is food safety and the public's perception of safety. There have been numerous worldwide surveys of beer for DON contamination, and DON is frequently reported, albeit at low levels (Schwarz 2017). Varga et al (2013) analyzed 374 beer samples from 38 European countries for the presence of DON, 3ADON, and D3G. Beer in this study was categorized as pale, wheat, dark, bock, or nonalcoholic. The nonalcoholic beers showed the lowest contamination percentage compared with alcoholic beers. Similar results were found by Kostelanska et al (2009), who reported a positive correlation between alcohol content and mycotoxin concentration, which might be explained by the fact that more malt is required for higher alcohol beer.

In contrast, regardless of beer categories, 77% of beers were found to be contaminated with DON level while 93% of beers were contaminated with D3G (Varga et al, 2013). Average DON and D3G levels were 8.4 μg/L and 6.9 μg/L, respectively. Approximately 4% of beer

samples with DON levels above 30 μg/L, and one beer sample contained 90 μg/L DON. This data indicates that most barley is contaminated with *Fusarium* and thus preventing *Fusarium* contamination might be an approach to prevent DON contamination in beer.

2.3.4. Control of Fusarium Head Blight

As described above, a dramatic increase of *Fusarium* mycotoxin level might occur during malting if FHB-infected grain was used. Hence, the avoidance of FHB-infected grain in incoming grains can be an effective way to prevent production of DON in further malting (Schwarz, 2017). However, during a crop year when FHB is widespread, avoidance of FHB-infected grain in incoming grains may not be possible. Cleaning and sizing operations might be effective in reducing overall DON levels in grains for malting because FHB infected grains are sometimes thinner and smaller (Schwarz et al, 2006). According to Schwarz et al (2006), DON level in thin kernels was considerably higher than original unsized samples, but their removal often did not greatly reduce the DON levels of the bulk sample.

Moreover, reduction of *Fusarium* spp. with grain storage was reported by Beattie et al (1998). A significant reduction of DON production was observed during malting using infected barley stored for 3 months. DON levels developed in malting continued to drop even lower after 7 months of storage regardless of the storage conditions. DON developed in malting dropped faster when infected barley was stored at room temperature (≈24°C). Therefore, storage of FHB-infected barley prior to malting is desirable to lower the ability of *Fusarium* to produce DON during malting. In addition, some biological controls have been applied during malting to limit the growth of *Fusarium*. For example, *Wickerhamomyces anomalus*, a yeast commonly found in malting environment, was found to inhibit the growth of *Fusarium* spp. as well as beer gushing (Laitila et al 2011).

2.4. Rye Beer

Rye (*Secale cereale* L.) is a cereal mainly grown in northern Europe and Asia between the Ural Mountains and the Nordic Sea, and has minimal requirements regarding soil, fertilization and climate for growth (Anonymous, 2002). Since rye is agronomically undemanding, and has high yield, it became an important grain in addition to wheat and barley (Dornbusch, 2015). In Finland and Denmark, rye bread is consumed mainly as wholemeal bread and is a good source of dietary fiber, with approximately 40% of dietary fiber coming from rye foods (Bondia-Pons et al, 2009). In addition, other phenolic compounds in rye can provide health benefits.

Centuries ago, not only was rye used in bakeries, it was used for making beer. Rye was used in Roggenbier, an old European style of beer (Dredge, 2014). However, the Reinheitsgebot, the German Beer Purity Laws, specified the name of hops, for preservation, and required barley to be the only grain source for beer brewing. This was to avoid some unscrupulous brewers using unsafe ingredients as preservatives for beer products (Geitner, 2011). Therefore, rye was used primarily for bread-baking until recently, when many craft brewers started to use rye malt. Since then, rye beer has become popular due to the unique flavor that results from rye.

Rye is not an easy grain to malt and brew compared with barley. Maltsters need to take care at the steeping step of malting. Unlike barley kernels, rye is huskless, so it can more quickly absorb water relative to barley (Dornbusch, 2015). Moreover, its high arabinoxylan content causes slow lautering, and its high protein content causes haze. Additionally, rye requires longer modification time during germination compared with barley. Even though rye is a little bit hard to deal with, it is still used by many craft brewers to create distinctive characters for their beer. According to Clemons et al (2006), malts for making beer are usually from barley, but rye malt

is usually added as an adjunct to create a spicy, nutty, and robust flavor to beer. Also, rye malt can be used to add red-black color to a beer to create a wide color range, and usually dark version of beer has darker and richer malt flavor (Dredge, 2014; BJCP, 2015).

As mentioned in previous sections, rye is one of the small grains that can be infected by FHB, but there is limited research that has been reported about FHB and mycotoxins in rye malt. This is of concern as there is growing interest in the production of rye for malt, beer, and other distilled beverages. As a result, additional research is needed to understand the effect of FHB on the malting behavior of rye and rye malt quality.

3. OBJECTIVE

The objectives of this research were to assess the growth of *F. graminearum* and the development of DON during the malting process, as well as the distribution of DON in single rye and triticale kernels, before and after malting.

4. EXPERIMENT APPROACH

4.1. Materials

Rye samples were supplied by the University of Minnesota-Crookston, and were obtained from two different location in 2016 (Crookston and Kimball). Six winter rye varieties ACHazlet, KWS Bono, Rymin, SU Memphisto, and Wheeler were used in this study. Tulus, a triticale, was also included. All samples (n=32) selected were naturally infected in the field, and were screened for grain plumpness, moisture, germinative capacity, and other quality parameters (Table 1).

4.2. Methods

4.2.1. Grain Analysis

4.2.1.1. Grain Sample Separation

Grain plumpness was tested using the American Society of Brewing Chemists (ASBC) Method Barley- 2C (ASBC, 2009). Assortment of rye grains were determined by using sieves with slot widths of 7/64 in. (2.78 mm), 6/64 in. (2.38 mm), 5/64 in. (1.98 mm). Rye grain (100g) was placed on the top screen of the barley grader (Sortimat Pfeuffer, Germany) and shaken for 2 mins. Plumpness was determined by the percentage of grains remaining on the 7/64 in. (2.78 mm) and 6/64 in. (2.38 mm) sieves. The grains passed through 5/64 in. (1.98 mm) sieves were considered as thin. In this study, only the grains remaining above 5/64 in. (1.98 mm) was kept for future analysis and malting.

Table 1. Grain Quality of 2016 University of Minnesota Rye and Triticale Samples

Sample Code[1]	Location	Variety	% Plump	% Thin	Moisture	Protein (% dry basis)	Germinative Energy %
R156	Crookston	AC Hazlet	73.8	3.7	8.4	12.7	99
R161	Crookston	AC Hazlet	76.1	3.3	8.1	12.2	97
R136	Crookston	KWS Bono	42.6	5.4	8.3	11.2	99
R160	Crookston	KWS Bono	43.7	5.0	8.3	10.6	99
R179	Crookston	KWS Bono	52.1	4.7	8.9	10.3	97
R145	Crookston	Rymin	69.7	4.6	8.8	13.2	96
R176	Crookston	Rymin	73.7	2.9	8.0	12.6	99
R140	Crookston	SU Mephisto	65.2	5.6	8.7	10.7	100
R158	Crookston	SU Mephisto	59.0	6.2	8.3	11.1	98
R167	Crookston	SU Mephisto	71.0	4.8	8.9	10.7	99
T137	Crookston	Tulus	70.9	2.9	8.3	14.2	92
T148	Crookston	Tulus	69.2	4.1	9.0	14.4	91
T168	Crookston	Tulus	69.3	2.6	8.5	14.2	94
R144	Crookston	Wheeler	56.4	10.7	7.6	19.3	93
R180	Crookston	Wheeler	64.5	9.2	8.0	18.7	91
R260	Kimball	AC Hazlet	76.8	3.0	8.7	9.3	99
R270	Kimball	AC Hazlet	74.0	3.4	9.0	9.7	97
R295	Kimball	AC Hazlet	75.2	3.5	9.7	11.1	97
R261	Kimball	KWS Bono	54.3	3.4	8.2	8.7	100
R298	Kimball	KWS Bono	40.1	8.6	8.4	8.7	99
R255	Kimball	Rymin	75.8	3.6	8.6	9.7	100
R271	Kimball	Rymin	68.0	3.7	9.7	9.9	100
R288	Kimball	Rymin	73.0	3.2	9.3	10.7	98
R248	Kimball	SU Mephisto	67.6	4.2	8.7	9.6	98
R278	Kimball	SU Mephisto	58.8	6.6	9.2	10.3	98
R297	Kimball	SU Mephisto	60.1	6.8	9.2	9.5	99
T244	Kimball	Tulus	64.3	4.2	9.6	13.3	94
T272	Kimball	Tulus	58.7	5.3	9.1	13.3	95
T282	Kimball	Tulus	57.9	5.5	9.0	13.3	90
R254	Kimball	Wheeler	83.2	2.6	9.9	15.8	96
R267	Kimball	Wheeler	77.9	2.7	8.5	17.6	94
R296	Kimball	Wheeler	77.0	5.3	9.3	17.4	96

[1]R: rye and T: triticale. Number is code for the replication within the field trial.

4.2.1.2. Grain Moisture and Protein

The moisture and protein of grain samples were determined by Near Infrared Reflectance (Foss 1241 Grain Analyzer, Eden Prairie, MN), using the calibration supplied by the manufacturer.

4.2.1.3. Germinative Energy

Germinative energy of grain samples was determined by ASBC Barley Method-3C (2009). Samples were incubated in a cabinet at room temperature, and chitting kernels (i.e. the acrospires and rootlets starting initial growth after the rupture of the grain's seed coat) were removed after 24, 48, and 72 hours. All chitted grains were recorded to calculate the percentage of germinative energy.

4.2.2. Micro-malting

All the rye samples were micro-malted using the method described by Karababa et al (1993) and Wang et al (2017). Pilot steeping (10g dry basis) was used to determine the time for each sample to reach 45% moisture. Two malting replications were performed with the first in March 2017, and the second in April 2017. Thirty-two samples (80g dry basis d.b.) were steeped at 16°C. Steeping includes aeration for six minutes every four hours, and a one-hour air-rest for each 12 hours of steep time. After 37-54 hours of steeping, the samples then were taken out of steep tank, spread on paper towels to remove surface water. The weight (145.45g) of the sample was adjusted using distilled water to 45% moisture. After moisture adjustment, samples were placed in 400 mL beakers and put into a germination cabinet to germinate for five days at 16°C and 95% relative humidity. Sample moisture was adjusted daily with additional distilled water, and were hand-turned every day during germination to prevent matting of the rootlets. After germination, all samples were placed into kiln containers and dried by a laboratory-scale malt

kiln. The kiln temperature was increased in a stepwise manner from 49°C to 85°C for 24 hours. The malts were then de-rooted manually by hand rubbing. The clean samples were collected, weighed, and stored at room temperature prior to testing.

Based upon the DON levels detected in malts for the first experiment, six sub-samples were selected to more closely examine the development of DON during the malting process. Sample size for the second experiment was 10g (d.b.). These were malted as previously described for the first experiment. Sixteen subsets (10g d.b.) were separated for each of the six samples. These were for two repetitions of each sample, and 8 sampling times. These subsamples were removed at steep out, the first, second, third, fourth, and fifth day of germination, and after kilning (with and without de-rooting), respectively. All the subsamples, except for the kiln sample, were frozen at -80°C and then freeze-dried.

4.2.3. Cross-contamination in Malting

A third malting experiment was conducted to evaluate if the DON detected in some rye malts might be due to cross-contamination from more highly infected samples during steeping. The possibility was raised as all samples are steeped in the same tank, and the steep vessels are open. A DON-free rye sample (0 mg/kg) and a triticale sample with higher level of DON (1.60 mg/kg) were selected for the cross-contamination test. Each sample (10g d.b.) were placed into five 50 mL perforated round-bottom centrifuge tubes (3122-0050, Nalgenem Rochester, NY). The first five tubes set consisting of three clean rye samples and two high-DON samples were put into the same steep basket, another two tubes of clean rye samples and three of the high-DON samples were placed in another basket. Baskets were placed right beside each other in the steep tank. A third set consisting of three tubes of clean samples was placed in basket on the opposite corner of the steep tank. All the malting, germination, and kilning conditions were the

same as the previously described. After kilning, samples were de-rooted and stored at room temperature prior to testing.

4.2.4. Determination of Type B Trichothecenes

Type B Trichothecenes (DON, NIV, 3ADON, 15ADON) were detected by using gas chromatography with electron capture detection (GC-ECD) according to the procedures modified from Tacke and Casper (1996). Samples were ground on a Perten LM 3600 disc mill (Perten Instruments. Hägersten, Sweden). The ground sample (2.5g) and 84% acetonitrile water (20 mL) were place into a 50 mL conical bottom polypropylene centrifuge tube. The tubes were capped and shaken to suspend material. Tubes were put into a test tube rack and placed on a horizontal shaker for 1 hr at 150 cycle/min. Tubes were placed upright to allow the contents to settle. Solid phase extraction (SPE) column containing 1 g of 50/50% C18/alumina (Silicycle, Quebec City, Canada. SPM-R 1101030B) were placed into a 16 x100 mm glass culture tube. The sample extract (4 mL) was added to the SPE column and allowed to pass through the column. The eluant (2 mL) was transferred to a 13x100 mm screw-capped test tube, placed in a 55°C sand bath and dried with nitrogen gas. After the eluant was totally dried, samples were derivatized using trimethylsilayamidazole (TMSI): trimethylchlorosilane (TMCS): N, O, - Bis (trimethylsilyl) trifluoroacetamide (BSTFA): Pyridine (1:1:1:1 v/v/v/v). The tubes were capped, agitated on Vortex mixer for 10s, and allow to react 30 mins at room temperature. After derivatization, the samples were extracted into isooctane (1 mL). The 0.5-1.0 ml of upper layer was transferred into a 2.0 ml GC vial. The sample (1 µl) was injected onto the column in the Agilent 6890 GC-ECD (Santa Clara, CA) system for analysis. Electronic Pressure Control (EPC) cool on-column inlet was used with initial inlet temperature of 90°C. The samples were then separated on a 35% phenyl methyl siloxane column (30.0 m x 250 µm x 0.25 µm) (Agilent HP-35, 19091G-133).

Flow rate of Helium was 1.8 ml/min with pressure of 20 psi. The injector program was from 90°C, and heated at a 20°C /min ramp to a final temperature of 300°C. The oven program was from initial temperature of 70°C, and heated with a 25°C /min ramp to a temperature of 170°C, then heated at a rate of 5°C /min to a temperature of 300°C with a 10-min hold. The μ-EDU detector temperature was 300°C. Helium and $ArCH_4$ (argon/methane) gas were used as carrier gas and makeup gas, respectively. The limit of detection (LOD) for DON detection on the GC-ECD was 0.20 mg/kg, and the limit of quantification (LOQ) for DON detection on the GC-ECD was 0.50 mg/kg. The LOD and LOQ was determined as a signal to noise ratio as 3:1 and 10:1, respectively.

4.2.5. Determination of Deoxynivalenol-3-Glucoside

D3G was analyzed on an Agilent 1290 series liquid chromatography with a 6540 UHD Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS detector (Agilent Technologies, Santa Clara, CA) as described by Jin et al (2018b). The sample extract prepared for DON analysis were also used for D3G analysis. Approximately 1.0 mL of acetonitrile extract was filtered through a 0.20 μm nylon filter.

The mobile phase system consisted of 0.1% of formic/water (solvent A) and 0.1% of formic/acetonitrile (solvent B) with the flow rate of 0.30 ml/min. The injection volume was 10.0 μl. A ZORBAX SB-C18 Column (2.1 x 50 mm, 1.8 μm, Agilent, USA) was used for the separation at 30°C. The reference standards consisted of 2 ml of purine in 500 ml of 95% acetonitrile/water and 0.8ml of HP0921 ($C_{19}H_{19}O_8N_3P_3F_{24}$) in 500 mL 95% acetonitrile/water with reference masses of 121.05 and 922.01. The analytes were further analyzed by using Agilent jet stream electrospray ionization (AJS ESI) with the following settings: gas temperature was 300°C with a flow rate of 10 L/min; nebulizer pressure was 35 psig; sheath gas temperature

was 325°C with the flow rate of 10 L/min. D3G in samples was quantified with its sodium adduct of m/z 481.168. The D3G standards was supplied by Biopure (50.3 µg/µl, Biopure, MO, USA). The LOD for D3G detection on the LC/MS (Q-TOF) was 0.20 mg/kg, and the LOQ was 0.5 mg/kg.

4.2.6. Determination of Deoxynivalenol in Single Kernels

Levels of DON were determined in single kernels in order to determine the extent of infection in grain, and changes that occurred during malting. Six sub-samples were selected for the single kernel test. Most of the cleaned kernels were visibly unaffected (i.e. appearing normal with no visible signs of infection). For each sample, 80g (d.b.) of rye kernels were malted for 5 days, as previously described for the first micro-malting experiment. After kilning, 46 malted rye kernels, including visibly unaffected kernels and fusarium damaged pink color kernels were randomly picked from each malt sample. Original rye grains were also analyzed in the single kernel test. A note was made as to whether each kernel was visibly free of infection or pink colored (assumed to be *Fusarium* infection).

Single kernels were prepared and analyzed according to Mirocha et al (1998) with some modifications. Single kernels were weighed and then placed individually into 15 ml polypropylene centrifuge tubes along with 1.5 ml of acetonitrile: water (84:16, v/v). After capping the tubes, samples were soaked overnight. The kernels were then crushed using a glass rod and shaken on a horizontal shaker for 3 hours. The resultant extract (1 ml) was placed into test tubes, then evaporated and dried with nitrogen for 30-45 mins. The dried samples were derivatized with 50 µl of derivatization agent: trimethylsilayamidazole (TMSI): trimethylchlorosilane (TMCS): N, O, - Bis (trimethylsilyl) trifluoroacetamide (BSTFA): Pyridine (1:1:1:1 v/v/v/v). The tubes were capped, agitated on Vortex mixer for 10 s, and allowed to react

30 mins at room temperature. Isooctane (500 µl) was added, followed by 500 µl sodium bicarbonate to quench the reaction. The samples were vortexed for 10s until the milky isooctane become transparent. The upper layer (0.25-0.5 ml) was transferred to a 1.5 ml GC vial, and 1 µl was injected into the GC/ECD for analysis as previously described.

4.2.7. Determination of Tri5 DNA

Fusarium DNA levels in grain and malt samples were determined by real-time polymerase chain reaction (RT-PCR) as described by Presiser et al (2015) and Jin et al (2018a) with some modifications. DNA in grain and malt samples was extracted by using a DNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA, USA). Samples were ground using UDY mill (UDY Corp., CO, USA) equipped with a 0.5 mm screen, and stored at -80°C prior to DNA extraction. Sample (25mg) were extracted according to the procedures described in the DNeasy Plant Mini Kit Handbook.

The SYBR Green One-Step Kit (Bio-Rad, Hercules, CA, USA) was used for DNA quantification, and the primers Tri5 forward (5'-GATTGAGCAGTACAACTTTGG-3') and Tri5 reverse (5'-ACCATCCAGTTCTCCATCTG-3') (Sigma-Aldrich, MO, USA) were used for amplification (Preiser et al, 2015). A CFX96 Real-time System thermocycler (Bio-Rad, Hercules, CA, USA) was used to perform RT-PCR. All the PCR components were mixed in a 96-well PCR plate. The total reaction volume was 10.0 µl, including 0.5 µl of Forward primer (10 pmol) and 0.5 µl of Reverse primer (10 pmol), 3.0 µl of PCR grade water (Bio-Rad, Hercules, CA, USA), 5.0 µl of SYBR Green fluorescent dye, and 1.0 µl of DNA template.

For the Tri5 standard curve, the *Fusarium graminearum* genomic DNA was from a pure culture grown on Potato dextrose agar (PDA) media, and was extracted by using a DNeasy Plant Mini Kit. After the DNA was extracted, a polymerase chain reaction (PCR) was used for DNA

amplification. The gel piece with Tri5 DNA was cut off, then the Tri5 DNA was purified with E.Z.N.A. Gel Extraction Kit (OMEGA Bio-Tec Inc. GA, USA). DNA concentration was measured by using a Qubit dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific). Seven concentrations from serial dilutions of purified *Fusarium graminearum* Tri5 PCR amplicon (100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 ag) were prepared for the PCR reactions, which were performed simultaneously with other samples to quantify the initial amount of Tri5 DNA for each sample.

The qPCR reaction conditions include an initial denaturation at 95°C for 2 mins 45 s, followed by 40 cycles of 95°C, 15 s; 62°C, 30 s; and Plateread. The melt curve was at 60°C, 31 s; 43 cycles of 60°C, 5 s; +0.5°C /cycle; ramp 0.5°C; Plateread. The increase in fluorescence was measured at 60°C in each cycle. The cycle threshold (CT) only represents the positive reaction when the value was < 35. The results were analyzed by Bio Rad CFX Manager 3.1 software.

4.2.8. *Fusarium* Species Isolation

Four sub-samples were selected for *Fusarium* species isolation. Species were determined at the School of Integrative Plant Science, Plant Pathology and Plant-Microbe Biology Section (Cornell University, Ithaca, NY), using the following methods: 100 kernels of each sample were surface-sterilized in order of 95% ETOH for 1 min, 20% bleach for 1 min, and rinsed in sterile water. Kernels were then plated onto PDA++ agar (neomycin and kanamycin amended) with 4 replicates, and incubated at room temperature (around 24°C) under black light with 12 hours photoperiod for 5 to 7 days. All *Fusarium* colonies growing out of each kernel were transferred to individual PDA plates and incubated under the same conditions as previous described. Colonies were identified morphologically and representatives of each that were identified were confirmed via TEF1a sequencing.

5. RESULTS AND DISCUSSION

5.1. Samples and Grain Quality

A total of 26 winter rye and 6 winter triticale samples were selected from University of Minnesota variety trials conducted at Crookston and Kimball, MN in 2016 (Wiersma et al, 2017). Selections included both open-pollenated hybrid types, and represented seven varieties (Table 2).

Table 2. Origin and Characteristics of Rye and Triticale Samples Grown in University of Minnesota Trials in 2016

	Variety	Type[a]	Year of Release	Breeder[b]	Intended Use
Winter rye	AC Hazlet	OPV	2006	AAFC	Grain
	KWS Bono	Hybrid	2013	KWS	Grain
	Mephisto	Hybrid	2011	SU	Grain
	Rymin	OPV	1973	MNAES	Grain
	Wheeler	OPV	1971	MAES	Forage
Winter triticale	Tulus	PLV[c]	2008	SU	Grain

[a] OPV: open pollinated variety
[b] AAFC: Agriculture and Agri-Food Canada, KWS (Kleinwanzlebener Saatzucht, Germany), MAES: Michigan Agricultural Experiment Station, MNAES: Minnesota Agricultural Experiment Station, and SU; Saaten Union (Germany)
[c]PLV: pure line variety

Grain quality was tested prior to micro-malting to ensure the samples were suitable for malting. All samples were tested for kernel plumpness, moisture, protein, and germinative energy (Table 1). All samples exhibited acceptable germinative energy (90-100%, mean=96.5%), which is necessary for malting. Seventy-five percent of the samples displayed germinative energy of >95%, which is the normal standard for barley. Greater variability was seen in protein content (8.7-19.3%, mean=12.3%) and kernel plumpness (40.1-83.2%, mean 65.6%).

5.2. *Fusarium* Species and Toxins in Naturally Infected Winter Rye and Triticale

5.2.1. Determination of *Fusarium* Species

Fusarium species present on rye and triticale samples was determined as part of a previous study (Jin et al, 2018a). Species were determined at the School of Integrative Plant Science, Plant Pathology and Plant-Microbe Biology Section (Cornell University, Ithaca, NY) by morphological identification on PDA, and then by translation elongation factor 1-α (TEF1a) sequencing of isolates. One hundred kernels of each sample were plated in four replications. The predominant species isolated from the two triticale samples was *F. graminearum,* which represented 55-100% of all isolates (Table 3). *F. equiseti*, *F. poae* and *F. tricinctum* were found on the Crookston, MN triticale sample T137C. Surprisingly, the rye samples showed lesser percentages of *F. graminearum,* and a greater proportion of other species. This is of concern as, *F. sporotrichiodes* and *F. poae* are known to produce other mycotoxins such as NIV, 4,15-diacetoxyscirpenol (DAS), T2 toxin, and HT-2 toxin, several of which have greater toxicity than DON (Morcia et al, 2016). The type-A trichothecenes, DAS, T2 toxin and HT-2 toxin were not measured in the current study.

However, the results of this portion of the study must be viewed with some caution. Only 4 of 32 samples were tested, and the identification of species was performed 9 months after harvest. The viability of *Fusarium* is known to decline with storage (Beattie et al, 1998). Also, Jin et al (2018b) reported a 42% lower development of *Fusarium* Tri5 DNA during malting of hard spring wheat samples stored for 6 months compared with the samples stored for 2 months, which indicates storage impacted the viability of *Fusarium*.

Table 3. *Fusarium* Species Isolated from Rye and Triticale Samples

Sample[a]	Number of isolates per 100 kernels (mean of 4 plates)						
	Total *Fusarium*	*F. graminearum*	*F. sporotrichiodes*	*F. equiseti*	*F. poae*	*F. tricinctum*	*F. avenaceum*
T244K	8	8	nd	nd	nd	nd	nd
T137C	11	6	nd	2	1	2	nd
R261K	4	2	2	nd	nd	nd	nd
R180C	16	2	1	7	2	3	1

[a] T: triticale, R: rye, K: Kimball, C: Crookston.
nd: Not detected

5.2.2. Type B Trichothecene Toxins in Winter Rye and Triticale Grain Samples

DON levels of the harvested grain samples (unsized) ranged from below the LOD to 2.82 mg/kg (Table 4). Levels were highest in the triticale samples, with all 6 samples exhibiting levels >0.90 mg/kg, whereas the rye samples averaged 0.32 mg/kg (range 0-1.32 mg/kg) across the two locations. These results seem striking, but it must be remembered that only a single cultivar of triticale was included in the current study (3 replications/location), and the overall population size was very small. Nevertheless, this observation seems to support the previous findings of Góral et al (2016) who reported higher content of type B trichothecenes was observed in winter triticale compared with winter rye, even though there was lower percentage of FHB indices and *Fusarium* damaged kernels (FDK) in winter triticale.

Thin kernels were removed from all samples prior to malting, as thins take up water faster during steeping, and their inclusion can cause uneven malt modification in germination (Schwarz and Li, 2011). These sized samples were utilized for malting, and all subsequent testing of mycotoxins. Removal of the thin kernels almost always reduced DON levels (Table 4). This has been frequently observed in wheat, where FHB infection can cause reduction in kernel size, and the appearance of FDK, which is evidenced by shrunken and bleached kernels. Jin et al

(2014) reported a significant correlation between FDK and DON concentration in winter wheat samples.

When samples with DON levels of only ≥ 0.2 mg/kg are considered, removal of the thin kernels reduced DON by an average of approximately 50% (range: 0-86% reduction). However, where DON was ≥ 1.0 mg/kg, removal of thins reduced DON to below 0.5 mg/kg in only 2 of the 6 cases. This illustrates that cleaning (sizing) in itself is generally not capable of turning unacceptable samples into acceptable ones. Food and Drug Administration (FDA) advisory guidelines for DON are 1 mg/kg for finished grain products for human consumption (FDA, 2010). DON limits have often been quoted as 0.4-0.5 mg/kg for malting barley, but DON limits can vary on by-products from 0-10 mg/kg (Wilson et al, 2017).

After removal of thin kernels, DON levels in the sized rye samples decreased to an average of 0.13 mg/kg (range from LOD - 0.42 mg/kg) across the two locations. All of the sized rye samples were below the DON limit (<0.5 mg/kg) that is often selected by many maltsters. DON levels in the sized triticale samples ranged from 0.49 to 1.60 mg/kg, Three of the 6 triticale samples still had DON levels higher than the FDA advisory guideline for finished grain products (1 mg/kg).

Table 4. Deoxynivalenol Levels in Grain Before and After Removal of Thin Kernels[a]

Sample[c]	DON (mg/kg)[b]		Thin Grain (%)
	Unsized Grain	Sized Grain	
R156C	0.56	0.11	3.7
R161C	0.29	0.30	3.3
R136C	0.23	0.12	5.4
R160C	0.32	0.12	5.0
R179C	0.30	0.03	4.7
R145C	0.08	0.05	4.6
R176C	0.00	0.07	2.9
R140C	0.35	0.07	5.6
R158C	0.12	0.03	6.2
R167C	0.13	0.13	4.8
T137C	2.82	1.60	2.9
T148C	1.48	0.75	4.1
T168C	1.05	1.11	2.6
R144C	0.56	0.23	10.7
R180C	0.81	0.34	9.2
R260K	0.19	0.03	3.0
R270K	0.15	0.07	3.4
R295K	0.47	0.29	3.5
R261K	0.00	0.05	3.4
R298K	0.44	0.22	8.6
R255K	0.14	0.02	3.6
R271K	0.10	0.20	3.7
R288K	0.66	0.19	3.2
R248K	0.08	0.07	4.2
R278K	0.12	0.08	6.6
R297K	0.28	0.10	6.8
T244K	1.36	1.55	4.2
T272K	1.17	0.74	5.3
T282K	0.99	0.49	5.5
R254K	0.28	0.06	2.6
R267K	0.35	0.05	2.7
R296K	1.32	0.42	5.3

[a] Thin kernels: passing through a sieve with 2.00 x 19 mm rectangular openings

[b] Limit of quantification (LOQ) for DON by the gas chromatography combined with electron capture detector (GC-ECD): 0.20 mg/kg; Limit of detection (LOD): 0.20 mg/kg.

[c] R: rye; T: triticale; C: Crookston; K: Kimball; Number is code for the replication within the field trails

The levels of other type B trichothecenes and D3G in the sized grain are shown in Table 6. The levels of NIV, 3ADON, and 15ADON were all below the LOD. Similar results also were reported in a previous study. Jin et al (2018a) reported the levels of NIV, 3ADON, and 15ADON were all below the LOD (0.20 mg/kg) in all rye samples from ND, NY, and MN for the 2014 and 2015 crop years.

D3G is formed naturally by a plant detoxification mechanism, which functions to manage xenobiotics (including mycotoxins). This mechanism involves glycosylation reactions, in which, UDP-glucosyltransferases can detoxify DON by catalyzing the transfer of glucose from UDP-glucose to the hydroxyl group (Poppenberger et al, 2003). D3G is less toxic than DON, and has less ability to inhibit protein synthesis than DON (Berthiller et al, 2013).

In this study, D3G levels were below 0.20 mg/kg (LOD) for most of the samples, with highest level of 0.29 mg/kg being observed in sample T244K. Only 4 of 32 samples (2 rye and 2 triticale samples) had D3G levels higher than the LOD. It was not possible to determine the ratio of D3G/DON (mol%), since the D3G and DON of most samples were below the LOD. Even for the few samples where levels were above the LOD, the sample size was too small to give accurate results. In a previous study, Jin et al (2018a) reported an average ratio of D3G/DON in wheat samples was 23 mol% with a range of 7-57 mol%. The average ratio of D3G/DON in rye was 42 mol% (Jin et al, 2018a).

Results of general linear model analysis (Table 5) was used to determine the impact of rye variety and growing location on DON and D3G. Results for NIV, 3ADON, and 15ADON were not analyzed since all these mycotoxins were not detected in rye grains. This analysis included 5 varieties and 2 locations (2-3 replications of each variety) in 2016. Triticale samples were not included in this analysis. In this study, results showed no significant effect from variety

and growing location on DON, while variety had a significant effect ($P \leq 0.05$) on D3G. However, result of this portion of the study should be viewed with some caution, as there were only five rye varieties from two locations in one crop year. In addition, most of DON and D3G levels were below the LOD. Therefore, a larger samples size (more locations and varieties) is needed for statistical validity.

Table 5. General Linear Models (GLM) of the Effects of Rye Variety and Growing Location on Deoxynivalenol and Deoxynivalenol-3-glucoside

Variable	Source	df	Sum of Squares	Mean Square	F-value	*Pr>F*
DON						
	Location	1	1.75E-05	1.75E-05	–	–
	Rep(Location)	4	0.1071	0.026775	–	–
	Variety	4	0.0703	0.017575	1.81	0.2892
	Location*Variety	4	0.0388	0.0097	0.47	0.3328
D3G						
	Location	1	1.40E-07	1.40E-07	–	–
	Rep(Location)	4	0.0255	0.006375	–	–
	Variety	4	0.0384	0.0096	8.86	0.0287*
	Location*Variety	4	0.0434	0.01085	0.47	0.7569

Table 6. Type B Trichothecene Toxins and Deoxynivalenol-3-glucoside in Grain and Malt

Sample Code[a]	Trichothecenes in grain (mg/kg)					Malting Time[b]	Trichothecenes in malt (mg/kg)				
	DON	NIV	3ADON	15ADON	D3G		DON	NIV	3ADON	15ADON	D3G
R156C	0.11	0.00	0.00	0.00	0.10	1	7.99	0.08	0.73	0.72	1.80
						2	10.35	0.07	1.29	0.66	6.41
R161C	0.30	0.00	0.00	0.00	0.15	1	16.28	0.13	1.13	0.64	2.14
						2	14.78	0.12	2.11	0.70	2.73
R136C	0.12	0.00	0.00	0.00	0.04	1	3.59	0.01	0.38	0.21	1.19
						2	6.48	0.05	0.90	0.75	3.58
R160C	0.12	0.00	0.00	0.00	0.04	1	4.90	0.01	0.42	0.49	1.00
						2	7.11	0.05	0.09	0.75	3.29
R179C	0.03	0.00	0.00	0.00	0.02	1	4.81	0.03	0.51	0.41	1.19
						2	6.08	0.04	0.95	0.71	2.05
R145C	0.05	0.00	0.00	0.00	0.07	1	3.88	0.03	0.61	0.19	0.85
						2	3.1	0.10	0.50	0.29	3.86
R176C	0.07	0.00	0.00	0.00	0.07	1	3.38	0.03	0.55	0.15	0.85
						2	8.9	0.09	1.57	0.48	1.45
R140C	0.07	0.00	0.00	0.00	0.01	1	1.50	0.00	0.33	0.06	0.56
						2	3.76	0.03	0.75	0.28	1.37
R158C	0.03	0.00	0.00	0.00	0.01	1	1.67	0.00	0.09	0.19	0.75
						2	1.75	0.00	0.37	0.30	1.28
R167C	0.13	0.00	0.00	0.00	0.02	1	2.26	0.00	0.51	0.09	0.71
						2	0.86	0.06	0.19	0.06	0.36

[a] R: rye, T: triticale, C: Crookston; K: Kimball; Number is code for the replication within the field trial.
[b] 1: Malted around 9 months after harvest; 2: Malted around 10 month after harvest.

Table 6. Type B Trichothecene Toxins and Deoxynivalenol-3-glucoside in Grain and Malt (continued)

Sample Code[a]	Trichothecenes in grain (mg/kg)					Malting Time[b]	Trichothecenes in malt (mg/kg)				
	DON	NIV	3ADON	15ADON	D3G		DON	NIV	3ADON	15ADON	D3G
T137C	1.60	0.00	0.00	0.00	0.23	1	35.17	0.20	3.84	2.67	3.03
						2	39.14	0.35	5.39	3.82	11.66
T148C	0.75	0.00	0.00	0.00	0.17	1	20.13	0.11	1.92	1.81	2.38
						2	33.81	0.26	4.62	3.61	9.73
T168C	1.11	0.00	0.00	0.00	0.13	1	28.84	0.13	2.66	2.36	2.59
						2	40.37	0.35	6.02	3.36	12.70
R144C	0.23	0.00	0.00	0.00	0.16	1	12.84	0.09	1.89	0.64	1.41
						2	9.96	0.16	1.49	0.94	4.00
R180C	0.34	0.00	0.00	0.00	0.14	1	17.77	0.17	1.32	1.14	1.41
						2	17.04	0.23	2.23	1.67	6.60
R260K	0.03	0.00	0.00	0.00	0.04	1	3.22	0.01	0.05	0.42	1.24
						2	8.64	0.06	0.40	1.73	3.95
R270K	0.07	0.00	0.00	0.00	0.05	1	5.28	0.03	0.14	1.11	1.43
						2	6.68	0.05	0.15	0.90	3.82
R295K	0.29	0.00	0.00	0.00	0.19	1	5.43	0.02	0.18	1.13	1.50
						2	4.57	0.04	0.17	0.57	2.39
R261K	0.05	0.00	0.00	0.00	0.04	1	0.71	0.00	0.00	0.18	0.47
						2	0.8	0.00	0.04	0.14	0.94
R298K	0.22	0.00	0.00	0.00	0.03	1	3.15	0.00	0.07	0.80	1.18
						2	2.97	0.02	0.05	0.55	1.41
R255K	0.02	0.00	0.00	0.00	0.03	1	0.17	0.01	0.10	0.05	0.23
						2	0.82	0.00	0.03	0.07	0.75

[a] R: rye, T: triticale, C: Crookston; K: Kimball; Number is code for the replication within the field trial.
[b] 1: Malted around 9 months after harvest; 2: Malted around 10 month after harvest.

Table 6. Type B Trichothecene Toxins and Deoxynivalenol-3-glucoside in Grain and Malt (continued)

Sample Code[a]	Trichothecenes in grain (mg/kg)					Malting Time[b]	Trichothecenes in malt (mg/kg)				
	DON	NIV	3ADON	15ADON	D3G		DON	NIV	3ADON	15ADON	D3G
R271K	0.20	0.00	0.00	0.00	0.08	1	3.47	0.01	0.23	0.42	1.24
						2	2.31	0.00	0.03	0.04	1.89
R288K	0.19	0.00	0.00	0.00	0.04	1	1.98	0.00	0.04	0.31	0.63
						2	5.02	0.04	0.30	0.42	2.85
R248K	0.07	0.00	0.00	0.00	0.04	1	2.55	0.03	0.10	0.70	0.85
						2	0.85	0.00	0.06	0.10	1.32
R278K	0.08	0.00	0.00	0.00	0.03	1	1.44	0.01	0.08	0.39	0.54
						2	0.59	0.00	0.00	0.14	0.64
R297K	0.10	0.00	0.00	0.00	0.07	1	1.36	0.00	0.07	0.36	0.55
						2	1.57	0.01	0.02	0.43	2.18
T244K	1.55	0.00	0.00	0.00	0.29	1	31.56	0.13	1.50	4.34	2.34
						2	27.65	0.14	1.76	3.23	10.24
T272K	0.74	0.00	0.00	0.00	0.14	1	33.02	0.09	1.72	5.45	2.54
						2	23.22	0.09	1.38	4.02	8.41
T282K	0.49	0.00	0.00	0.00	0.25	1	21.61	0.05	0.92	3.98	2.15
						2	20.19	0.08	1.05	3.83	8.02
R254K	0.06	0.00	0.00	0.00	0.08	1	1.06	0.02	0.21	0.24	0.43
						2	1.4	0.03	0.02	0.19	1.34
R267K	0.05	0.00	0.00	0.00	0.03	1	3.27	0.03	0.37	0.44	0.51
						2	5.28	0.06	0.18	0.68	1.34
R296K	0.42	0.00	0.00	0.00	0.28	1	16.04	0.10	0.98	1.85	1.37
						2	10.3	0.06	0.03	1.55	4.65

[a] R: rye, T: triticale, C: Crookston; K: Kimball; Number is code for the replication within the field trial.
[b] 1: Malted around 9 months after harvest; 2: Malted around 10 month after harvest.

5.2.3. Type B Trichothecene Toxins in Winter Rye and Triticale Malt Samples

DON level in 100% of rye samples were below 0.5 mg/kg. However, there was a 40-fold increase of DON levels following malting, and DON levels in 85% of malted rye samples were in excess of 1.0 mg/kg (Table 7). DON levels in rye malt increased from 0.13 to 5.23 mg/kg on average, with the highest value of 17.77 mg/kg being measured (Table 7). These large increases in DON levels observed after malting of relative clean rye (<0.5 mg/kg) would be a concern as maltsters usually select grain by DON level. There were also large increases in DON levels in the triticale samples; 1.04 to 29.56 mg/kg. DON levels in all triticale malts were in excess of 20 mg/kg.

There were no obvious changes in NIV levels in rye malt. NIV levels in most samples were below the LOD (Table 6). Only two triticale samples had levels above 0.2 mg/kg, with the highest level at 0.35 mg/kg. Noticeable increases of 3ADON and 15ADON were observed after malting. The highest level of 3ADON was 6.02 mg/kg, and the highest 15ADON level was 5.45 mg/kg. Overall, the average levels of NIV, 3ADON and 15ADON in malts were <0.2, 0.90, 1.11 mg/kg, respectively.

Similar results also were seen in the previous work of Jin et al (2018a), who reported apparent increases of type B trichothecenes after the malting of rye. The increases of DON and acetylated forms of DON after malting are of concern because some toxicological research has shown that NIV and 15ADON have higher acute toxicity than DON, with LD_{50} values in mice of 39, 34, and 78 mg/kg (oral), respectively (IARC, 1993; Payros et al, 2016). In contrast, 3ADON is less toxic than DON.

After malting, the average D3G level in rye malt samples was 2.63 mg/kg, and ranged from 0.49-4.11 mg/kg (Table 8). This represents around a 20-fold increase in D3G levels after

malting. Even though there was a low level of D3G in rye samples, large increases of D3G was observed after malting. The average ratio of D3G/DON in the malts was 29 mol% after malting, and ranged between 5-100 mol%. The low D3G/DON ratios in some malt samples, especially triticale, may due to the higher DON levels that existed on the unmalted grain. Similar results were observed by Jin et al (2018a) who reported six- and 14-fold increases, respectively, in D3G levels after malting of NY and MN rye samples harvested in 2014 and 2015 crop years. The ratio of D3G/DON mol% increased from 42 to 60 mol% on average following malting, with a range between 26 and 160 mol%. Decreased D3G/DON ratio was observed in malt which were with higher DON levels on the rye. The results suggested that there might be a maximum capacity of DON transformation in the grain during the germination (Jin et al 2018a).

5.2.4. Relationship Between Deoxynivalenol and Deoxynivalenol-3-glucoside in Rye and Triticale

Thirty-two rye and triticale samples were used for this portion of study to access the correlation between DON and D3G in rye and triticale. The range of DON levels in original rye and triticale grains were less than the LOD to 1.6 mg/kg, and the D3G levels were ranged from the LOD to 0.29 mg/kg (Figure 3). The relationship was relatively weaker (r^2=0.55), and overall, DON is not a good predictor of D3G levels in grain. This observation seems to support the previous findings of Schwarz et al (2014) who reported weak relationship between DON and D3G levels on barley, and suggested DON on barley was found not to be a strong predictor of D3G levels.

Table 7. Development of Deoxynivalenol in Naturally Contaminated Rye After Malting

			DON in rye (mg/kg)						DON in rye malt (mg/kg)					
						% of Samples						% of Samples		
Location	Crop year	# of samples	Mean	MIN	MAX	<0.50	0.50-1.00	>1.00	Mean	MIN	MAX	<0.50	0.50-1.00	>1.00
Crookston	2016	12	<0.20[a]	<0.20[a]	0.34	100	0	0	7.13	0.86	17.77	0	4	96
Kimball	2016	14	<0.20[a]	<0.20[a]	0.42	100	0	0	3.6	0.17	16.04	0	21	79

Note: [a] LOD for DON detection on the GC-ECD in our lab is 0.20 mg/kg;

Table 8. Development of Deoxynivalenol-3-glucoside in Naturally Contaminated Rye After Malting

Location	Crop year	# of samples	D3G in Rye (mg/kg)			D3G in Malt (mg/kg)		
			Mean	MIN	MAX	Mean	MIN	MAX
Crookston	2016	12	<0.20[a]	<0.20[a]	0.16	2.12	0.36	6.60
Kimball	2016	14	<0.20[a]	<0.20[a]	0.28	1.49	0.23	4.65

Note: [a] LOD for D3G detection on the LC/MS(Q-TOF) in our lab is 0.20 mg/kg

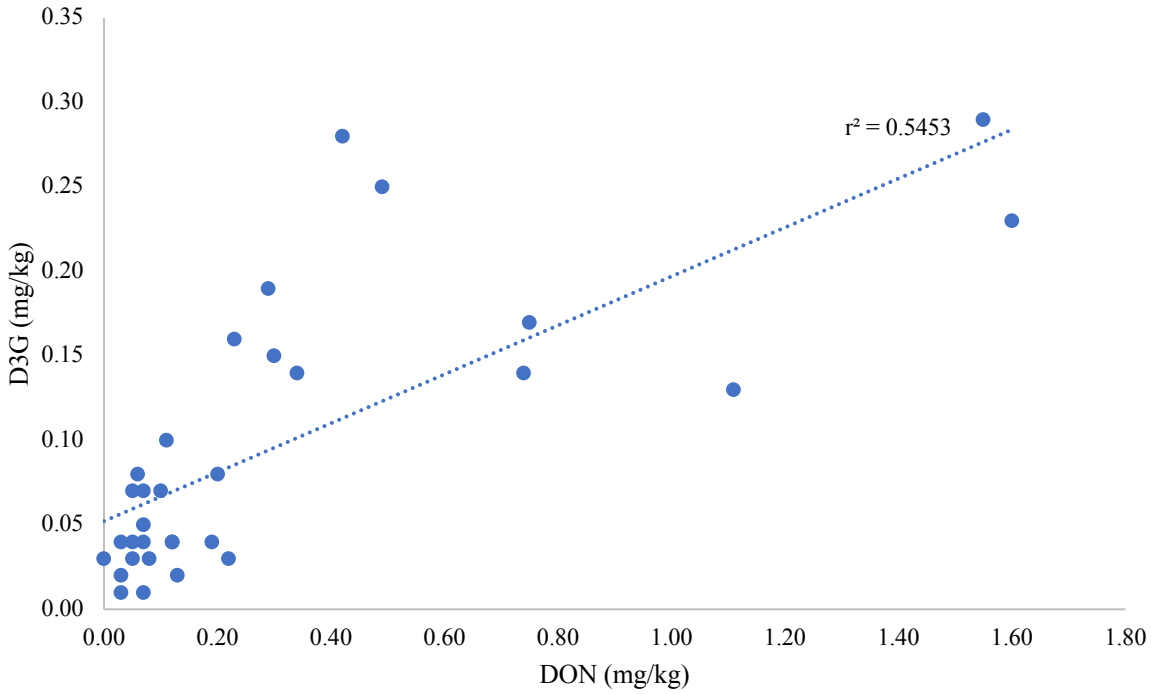


Figure 3. Correlation Between Deoxynivalenol (DON) and Deoxynivalenol-3-glucoside (D3G) in Rye (n=26) and Triticale (n=6).

5.2.5. Deoxynivalenol and Deoxynivalenol-3-glucoside on Rye and Triticale After Malting

The relationship between DON and D3G in malt was also examined on replicate malting of the same 32 samples (N=64) (Figure. 4). There was a one-month time gap between duplicate malting of the samples. DON levels of the malts prepared in March ranged from 0.17-35.17 mg/kg, and D3G levels ranged from 0.23-3.03 mg/kg. For the samples malted in April, DON and D3G ranged from 0.59-39.14 mg/kg and 0.36-11.66 mg/kg, respectively. Overall, D3G levels were higher in the second malting, while DON levels were slightly lower. Samples were stored at room temperature for the period between malting. The higher D3G levels with the second malting were surprising, although the lower DON levels were expected, as the viability of *Fusarium* generally decreases with time. Much of the D3G is formed during germination, where rye UDP-glucosyl transferases convert existing or newly formed DON to D3G. In support of this, strong correlations between malt DON and D3G levels were observed (Figure 4).

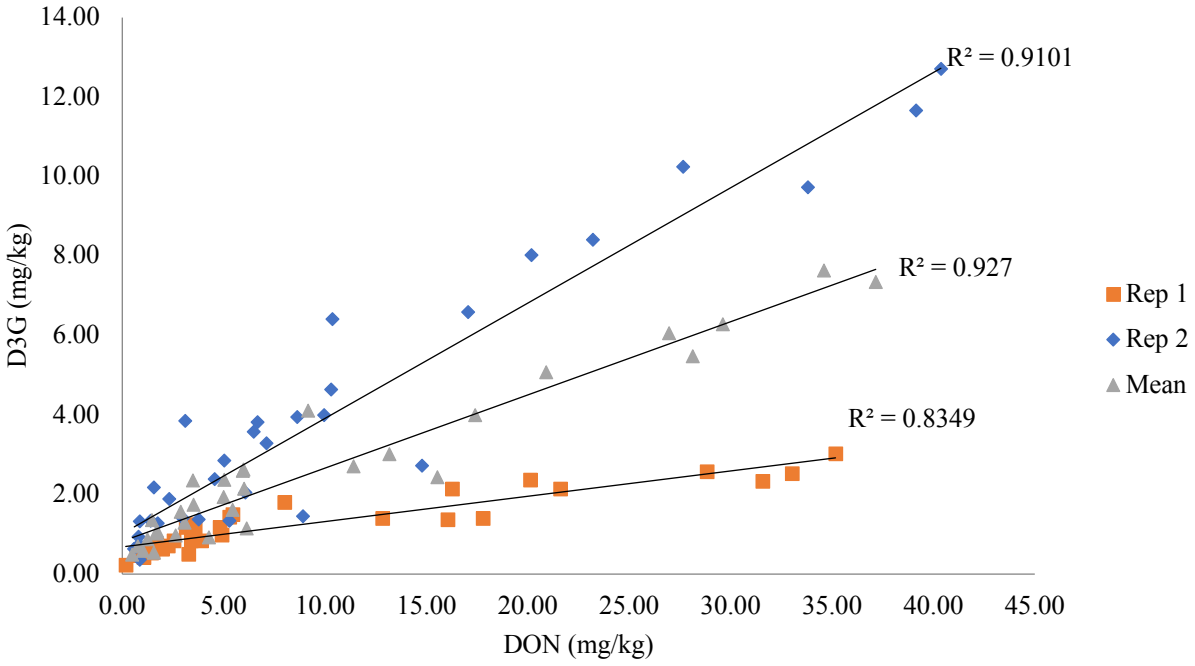


Figure 4. Correlation Between Deoxynivalenol (DON) and Deoxynivalenol-3-glucoside (D3G) in Rye Malt (n=26) and Triticale Malt (n=6). (Samples were malted at two different times points 1: 9 months after harvest; 2: 10 months after harvest).

5.3. Evaluation of Cross-contamination During Malting

This experiment was conducted to determine if cross-contamination of samples could occur during the malting process as conducted in our laboratory. Haikara et al. (1977) had previously shown that cross-contamination of uninfected kernels does occur during the germination of individual samples of FHB infected barley samples. In our laboratory, while samples are steeped in individual containers, they are placed in a common water bath. As the individual containers are perforated, cross contamination with spores or mycelium could be possible.

In order to test this hypothesis, individual samples of a highly infected rye sample (1.6 mg/kg DON) were steeped with individual samples rye, with DON below the LOD. Each individual sample (10g d.b.) was placed into a 50 mL perforated round-bottom centrifuge tube.

Five tubes (Table 9) were then placed inside a 100 x150 mm cylindrical copper mesh steep basket. The three baskets containing the samples were placed in the different positions in the steeping tank. Basket 1 and 3 were placed next to each other, while basket 2 was placed on the opposite side of the steep tank.

After steeping, all samples were geminated and kilned under the same conditions. DON levels were tested after de-rooting. The DON levels in the highly infected malts ranged from 8.42-24.88 mg/kg (Table 9), while DON levels in the malt from clean rye samples were all below the LOD. The results showed that the DON levels of clean samples remained below the LOD, even when placed directly adjacent to highly contaminated samples. This suggests that there is little possibility of cross-contamination of samples during steeping under our laboratory conditions. Therefore, the large increases of DON observed after malting (Table 6) were not due to the cross-contamination during steeping in prior malting tests. The large volume of steep water (130 Liter) relative to the small amount of grain (15 g) could be a factor. However, additional experiments, with either kernel plating or DNA extraction and real-time PCR, could be conducted after steeping to determine whether there was contamination with *Fusarium* existed in the “no DON” samples.

Table 9. Deoxynivalenol Levels in Samples Tested for Cross-contamination

Steep Container	Grain Sample [a, b]	Malt DON (mg/kg)
BASKET #1	Highly-infected [a]	16.15
	Clean [b]	<LOD
	Highly-infected [a]	8.42
	Clean [b]	<LOD
	Highly-infected [a]	24.88
BASKET #2		
	Clean [b]	<LOD
	Highly-infected [a]	16.71
	Clean [b]	<LOD
	Highly-infected [a]	24.11
	Clean [b]	<LOD
BASKET #3		
	Clean [b]	<LOD
	Clean [b]	<LOD
	Clean [b]	<LOD

[a]: T137C (triticale) from previous experiment was used as the highly infected sample.
[b]: Clean (rye) samples were have no detectable DON.

5.4. Development of *Fusarium* Toxins During Malting of Naturally Infected Winter Rye and Triticale

5.4.1. Development of *Fusarium* Toxins and Growth of *Fusarium* in Various Malting Stage

The objective of this portion of experiment was to assess the development of mycotoxins in winter rye and triticale samples over the entire malting process. Four rye samples (R261K, R297K, R161C, and R180C) and two triticale samples (T137C, T244K) with different DON levels (0.05 to 1.60 mg/kg) were selected for this portion of experiment. They will be subsequently referred to as samples A, B, C, D, E, and F, respectively. All samples were malted under the same malting conditions. Each day of the process, samples were removed for testing of mycotoxins (DON, D3G, 3ADON, 15ADON) and *Fusarium* DNA (Tri5 gene). Results of GLM

analysis (Table 10) shows that DON, D3G, 3ADON, 15ADON, and Tri5 DNA were all significantly ($P \leq 0.05$) affected by treatment and time, as well as the interaction between treatment and time. The significant interactions between treatment and time shows that samples behaved differently across time.

The general trends are shown in Figure 5. Overall, the development of DON increased over the time of malting, with a slight decrease during steeping as have been previously reported (Schwarz 2017). The overall trend for the development of D3G is shown in Figure 6. Levels were observed to increase during malting. However, there was a decrease after kilning, which could be due to cleavage of the bond between DON during heat-treatment as reported by Kostelanska et al (2011). Alternatively, it might be due to change in the sample matrix during kilning, such as binding of the D3G with compounds formed in the Maillard reaction.

The growth trend of *Fusarium* over malting time is shown in Figure 7. A decrease in Tri5 DNA was observed after steeping, which may due to loss on fungi from the surface of kernels. *Fusarium* continued to grow throughout germination until kilning.

Table 10. General Linear Models of the Effects of Deoxynivalenol Levels and Malting Time on Type B Trichothecene Toxins, Deoxynivalenol-3-glucoside, and *Fusarium* Tri5 DNA.

Variable	Source	df	Sum of Squares	Mean Square	F-value	*Pr>F*
DON						
	Rep	1	1.591	1.591	–	–
	Treatment	5	2782.438	556.4876	84.16	<0.0001
	Rep*Treatment	5	33.062	6.6124	–	–
	Time	7	4008.5045	572.6435	83.74	<0.0001
	Rep*Tine	7	47.87	6.838571429	–	–
	Treatment*Time	35	3937.19	112.4911429	17.57	<0.0001
D3G						
	Rep	1	10.166	10.166	–	–
	Treatment	5	2121.06	424.212	56.9	<0.0001
	Rep*Treatment	5	5.09	1.018	–	–
	Time	7	4731.76	675.9657143	416.4	<0.0001
	Rep*Tine	7	83.16	11.88	–	–
	Treatment*Time	35	2640.3	75.43714286	15.87	<0.0001
Tri5 DNA						
	Rep	1	2.181	2.181	–	–
	Treatment	5	8348.08	1669.616	82.09	<0.0001
	Rep*Treatment	5	101.70	20.34	–	–
	Time	7	8131.36	1161.622857	28.40	<0.0001
	Rep*Tine	7	286.32	40.90285714	–	–
	Treatment*Time	35	6252.11	178.6317143	9.65	<0.0001
NIV						
	Rep	1	0	0	–	–
	Treatment	5	0.02	0.004	409.67	<0.0001
	Rep*Treatment	5	5.00E-05	1.00E-05	–	–
	Time	7	0.0981	0.014014286	1.17E+31	<0.0001
	Rep*Tine	7	0	0	–	–
	Treatment*Time	35	0.1434	0.004097143	409.67	<0.0001
3ADON						
	Rep	1	0.524	0.524	–	–
	Treatment	5	11.156	2.2312	11.74	<0.0001
	Rep*Treatment	5	0.95	0.19	–	–
	Time	7	17.75	2.535714286	11.80	<0.0001
	Rep*Tine	7	1.5	0.214285714	–	–
	Treatment*Time	35	22.286	0.636742857	4.81	<0.0001
15ADON						
	Rep	1	0.333	0.333	–	–
	Treatment	5	52.731	10.5462	21.06	0.0023
	Rep*Treatment	5	2.55	0.51	–	–
	Time	7	73.32	10.47428571	14.81	0.001
	Rep*Tine	7	4.95	0.707142857	–	–
	Treatment*Time	35	120.01	3.428857143	5.15	<0.0001

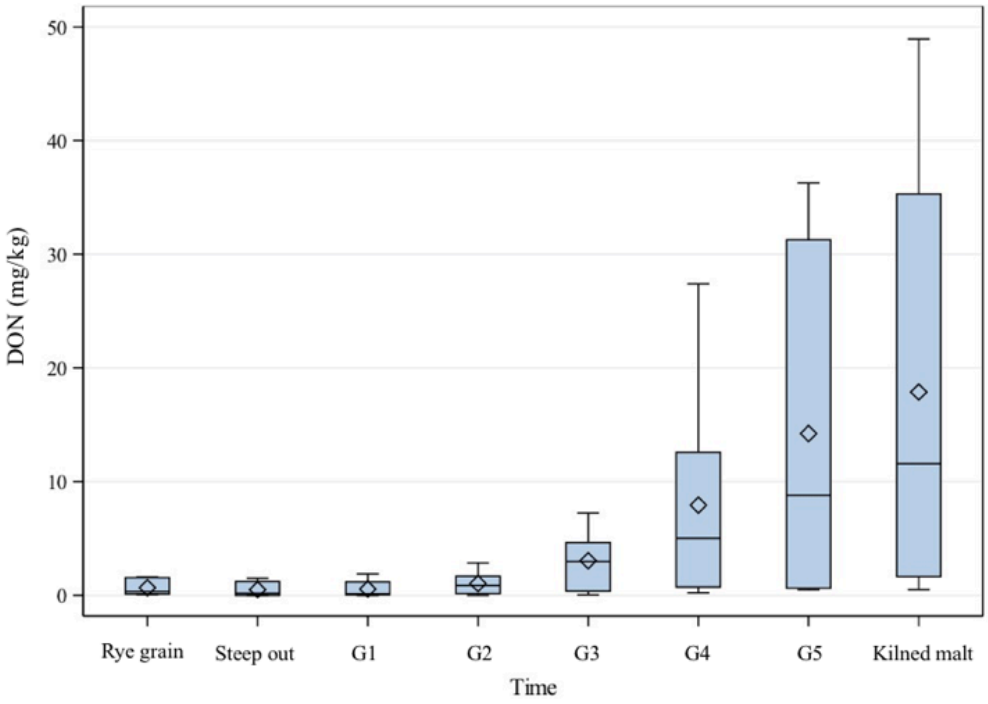


Figure 5. Trend of the Development of Deoxynivalenol During Malting.
(Each time point includes the mean of six treatments. Error bars represent standard error of the mean; Square points in the box represent mean, and line in the box represent median).

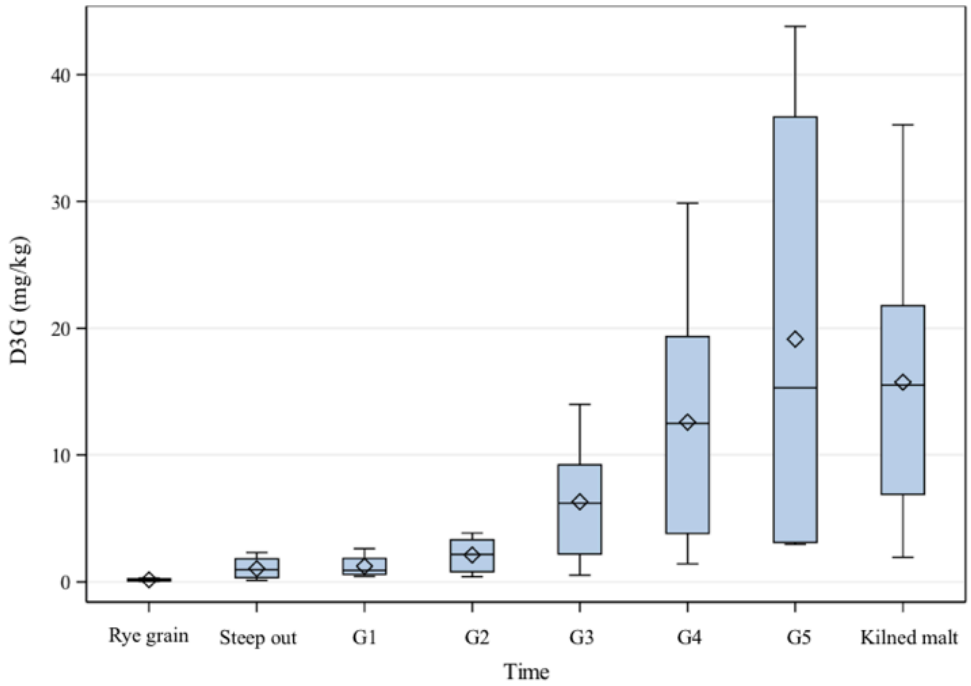


Figure 6. Trend of the Development of Deoxynivalenol-3-glucoside During Malting.
(Each time point includes the mean of six treatments. Error bars represent standard error of the mean; Square points in the box represent mean, and line in the box represent median).

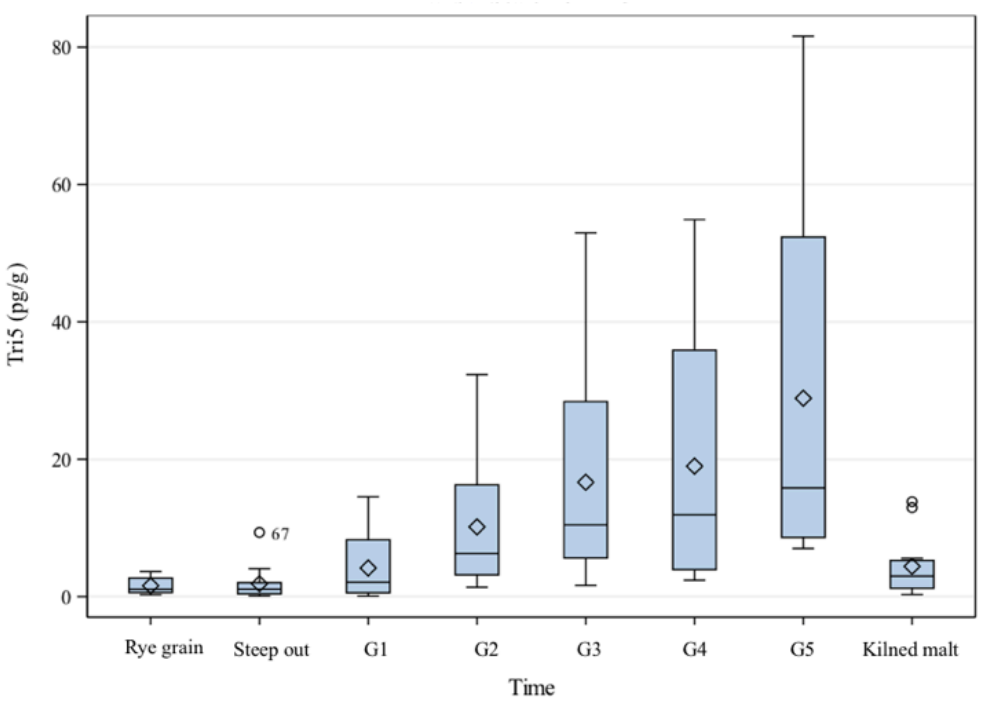


Figure 7. Trend of the Development of Tri5 Gene During Malting.
(Each time point includes the mean of six treatments. Error bars represent standard error of the mean; Square points in the box represent mean, and line in the box represent median).

5.4.2. Development of Deoxynivalenol, Deoxynivalenol-3-glucoside, and Tri5 DNA in Each Treatment

Based on the overall development of mycotoxins and metabolites, and the growth of *Fusarium*, further analyses were completed to compare the development among each treatment. Because there were low levels of NIV, 3ADON, and 15ADON detected in rye and malt, only DON, D3G, and Tri5 DNA were used for the analysis. Mean values were separated using least significant difference (LSD) analysis to determine the level of significance. Treatments A-D are rye grains from different varieties and DON levels, while treatments E and F are triticale.

5.4.3. Development of Deoxynivalenol During Malting

The DON levels detected in each subsamples collected each day during whole malting process from six treatments (i.e. A, B, C, D, E, and F) had original DON level of 0.05 mg/kg, 0.10 mg/kg, 0.30 mg/kg, 0.34 mg/kg, 1.60 mg/kg, and 1.55 mg/kg, respectively, in raw grain

(Table 11). There were decreases in DON level observed after steeping for all of treatments, which indicates the ability of steeping water to wash out DON on the kernel surface. The final malt had DON level 7-39 times higher than original grains, in which, the largest increase was found in treatment C and treatment D (39 and 34 times higher than original grains, respectively), and the DON levels in malt from triticale E and F increased 24 and 29 times, respectively.

DON concentration increased significantly for all treatments during malting. Significant increases in DON concentrations were observed at germination day 3 for most of treatments (A, C, and F), while DON increase at germination day 2 in treatment D, while B and E had significant increases in DON concentrations at germination day 4. Compared with rye, triticale had greater increases in DON levels after malting. Basically, significant increases appeared at germination day 3 for most of treatments, day 2 and day 4 were also observed for some treatments. Moreover, for treatment D and F, there were significant increases in DON concentrations observed every day after day 3.

Table 11. Deoxynivalenol Levels in Each Treatment over the Malting Process

Time	DON (mg/kg)					
	R261K (A)	R297K (B)	R161C (C)	R180C (D)	T137C (E)	T244K (F)
Grain	0.05 a	0.10 a	0.30 a	0.34 ab	1.60 a	1.55 a
Steep out	0.00 a	0.00 a	0.19 a	0.12 a	1.26 a	1.39 a
Germination Day 1	0.00 a	0.01 a	0.11 a	0.16 a	1.31 a	1.65 a
Germination Day 2	0.14 a	0.04 a	0.85 a	1.05 b	1.77 a	2.33 a
Germination Day 3	0.35 ab	0.05 a	4.50 b	2.38 c	5.54 a	5.39 b
Germination Day 4	0.55 ab	0.56 b	6.48 c	3.50 d	23.86 b	12.58 c
Germination Day 5	0.57 ab	0.57 b	10.65 d	6.80 e	31.28 b	35.47 d
Malt after kilning	1.53 b	0.65 b	11.63 d	11.58 f	37.64 b	44.25 e

5.4.4. Development of Deoxynivalenol-3-glucoside During Malting

Six treatments had detectable levels of D3G. All treatments had increased D3G after steeping, which is in contrast to that observed in the DON evaluation. The rising of D3G was

likely due to the grain synthesizing D3G by itself in the inner part of kernel for protection, so there was much less effect from steeping water. Significant increases in D3G were observed around germination day 3 (Table 12) (for B was in day 2, and for E was in day 4), which means there was significant conversion from DON to D3G. This finding may indicate that the maximum activity of UDP-glucosyl transferases in rye malt may not be reached until around the third day of germination. All of treatments had increased D3G from the beginning until the end of kilning. The D3G level in final kilned malt were 128, 78, 128, 111, 171, and 132 times higher than original grains A-F, respectively.

Table 12. Deoxynivalenol-3-glucoside Levels in Each Treatment over the Malting Process

Time	D3G (mg/kg)					
	R261K (A)	R297K (B)	R161C (C)	R180C (D)	T137C (E)	T244K (F)
Grain	0.04 a	0.07 a	0.15 a	0.14 a	0.23 a	0.29 a
Steep out	0.19 a	0.24 ab	0.78 a	0.64 a	1.55 a	1.81 ab
Germination Day 1	0.73 a	0.47 abc	1.69 a	1.53 a	1.65 a	2.20 ab
Germination Day 2	0.76 a	0.54 bc	2.35 a	2.10 a	3.35 a	3.70 ab
Germination Day 3	2.18 ab	0.81 c	9.82 b	5.56 b	9.06 a	10.42 bc
Germination Day 4	2.07 ab	3.07 d	16.86 cd	8.03 b	25.78 b	18.99 c
Germination Day 5	3.04 ab	3.81 e	19.26 d	15.60 d	39.49 c	38.42 d
Malt after kilning	5.12 b	5.48 f	15.20 c	11.38 c	23.14 b	30.07 d

5.4.5. Growth of *Fusarium* During Malting

The concentration of Tri5 DNA represents the *Fusarium* existed in malt samples collected at each time point. The original Tri5 DNA extracted from each treatment (A-F) were 0.89 pg/g, 0.51 pg/g, 1.03 pg/g, 0.92 pg/g, 2.72 pg/g, and 3.59 pg/g, respectively. Unlike neither DON levels, which decreased after steeping for all treatments, nor D3G levels, which increased after steeping for all treatments, Tri5 DNA decreased for some of the treatments (A, F), and increased for other treatments (B, C, D, and E). The variation may be due to different localization of *Fusarium* in the kernels. Some *Fusarium* spores may attach to the seed coat

before germination and hyphy development, so those spores are easily washed off by steeping water. However, seeds infected with *Fusarium* in the endosperm may result in growing of *Fusarium* during steeping due to ideal moisture concentrations.

After steep-out, all treatments had increased Tri5 DNA concentrations every day until the end of germination, and decreased during kilning (Table 13). The results indicate that the growth of *Fusarium* continued after steeping until the fifth day of germination due to ideal temperature and moisture. The highest Tri5 DNA content of each treatment were observed at the fifth day of germination, which were 8, 17, 16, 17, 20, and 20 times higher than the concentration of Tri5 DNA content in original grains for samples A, B, C, D, E, and F, respectively. The LSD results support the significant increase of Tri5 DNA concentration at germination day 2 for all treatments (Table 13).

However, during kilning, Tri5 DNA content was reduced due to the high temperature. The final Tri5 DNA extracted from kilned malt was low, ranging from 0.73 to 13.40 pg/g.

Table 13. Tri5 DNA in Each Treatment over the Malting Process

Sample	Tri5 DNA (pg/g)					
	R261K (A)	R297K (B)	R161C (C)	R180C (D)	T137C (E)	T244K (F)
Grain	0.89 a	0.51 a	1.03 a	0.92 a	2.72 a	3.59 ab
Steep out	0.12 a	0.58 a	1.11 a	1.15 a	6.72 ab	1.71 a
Germination Day 1	0.21 a	0.59 a	1.76 a	2.54 ab	8.96 ab	11.09 ab
Germination Day 2	3.71 b	1.68 b	8.28 b	4.47 b	16.28 bc	26.60 bc
Germination Day 3	3.76 b	1.69 b	12.88 c	8.00 c	28.40 cd	43.20 c
Germination Day 4	5.63 c	2.78 c	13.92 c	9.92 c	40.48 d	43.40 c
Germination Day 5	7.04 c	8.64 d	16.86 c	15.72 d	53.60 e	71.68 d
Malt after kilning	0.73 a	1.31 b	3.84 a	2.64 ab	4.64 ab	13.40 ab

5.5. Deoxynivalenol in Single Kernels

5.5.1. Deoxynivalenol in Single Rye Kernels of Rye and Triticale Grain

The objective of this portion of study were to assess the distribution of DON on individual kernels from the bulk grain sample. Forty-six kernels were randomly picked from

each of the six rye samples that were chosen for the previous malting experiment. The results (Table 14) indicate that for five of the samples (R297K, R161C, R180C, T137C, and T244K) more than 50% of kernels had no measurable DON (<LOQ). In rye samples R180C and R297K, 80 and 91%, respectively of the kernels were clean.

Table 14. Distribution of Deoxynivalenol in Single Kernels of Rye and Triticale

		DON in grain (mg/kg)			% of Samples			
Sample[a]	# of samples	Mean	MIN	MAX	<LOD	<0.50	0.50-1.00	>1.00
R261K	46	0.44	<0.20[b]	3.47	46	19	24	11
R297K	46	0.03	<0.20[b]	0.47	91	9	0	0
R161C	46	0.66	<0.20[b]	11.43	52	11	17	20
R180C	46	0.06	<0.20[b]	1.12	80	17	0	3
T137C	46	1.34	<0.20[b]	20.12	67	7	4	23
T244K	46	4.23	<0.20[b]	142.27	67	21	4	8

Note: [a] R: rye, T: triticale, C: Crookston; K: Kimball; Number is code for the replication within the field trial;
[b] LOD for DON detection on the GC-ECD in our lab is 0.20 mg/kg;

Overall, the result shows a low percentage of single kernels were highly contaminated with DON. The mean values for all bulk samples were low, and for some were close to zero (Figure 8). Only a few kernels in each treatment were detected with extremely high DON levels. The DON levels observed in a single kernel for each sample ranged from 0.47 to 142.27 mg/kg (Table 14). The majority of the kernels had no detectable DON, and a lesser portion of kernels had DON concentrations as high as 142 mg/kg, these results support the common supposition that DON in the bulk grain samples is due to a small number of kernels with higher DON.

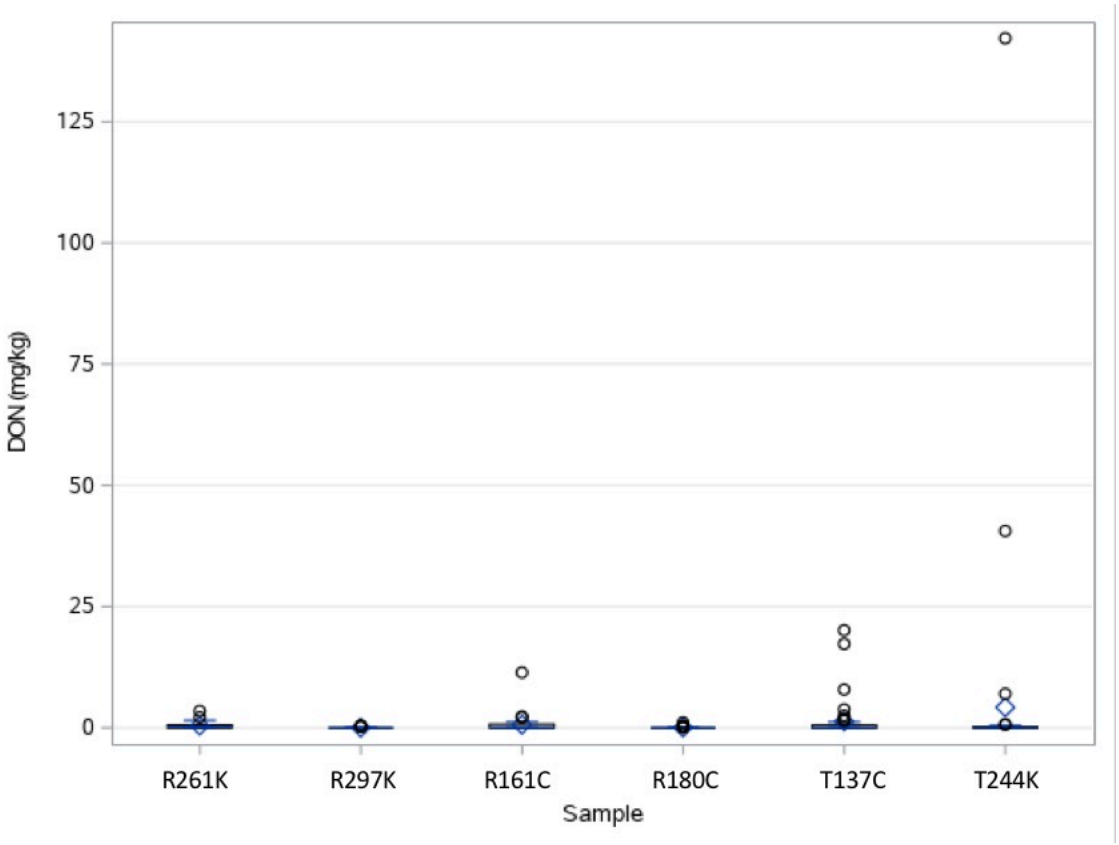


Figure 8. Deoxynivalenol in Single Kernels of Rye and Triticale.
(Error bars represent standard error of the mean; Square points in the box represent mean, and line in the box represent median; Circles outside the whiskers represents outliers, surprisingly high maximums or surprisingly low minimums).

5.5.2. Distribution of Deoxynivalenol in Single Rye and Triticale Malt

This portion of study was conducted to determined changes in single kernel DON distribution and levels following malting. Samples (80g, d.b.) were malted at two time points, as previous described, forty-six malt kernels from each malted sample were then randomly selected for DON measurement.

Contrary to that observed for unmalted grain, results on malt showed that in most cases more than 50% of kernels contained DON (Table 15). Contamination of 95-100% of kernels with DON was observed in some samples (R161C, R180C, T137C, and T244K). Also, in contrast to the results from unmalted grain, there was only a small percentage of malt kernels that had no detectable DON. This supports the results of earlier research that showed increase in *Fusarium* contamination following germination of FHB infected barley (Schwarz 2003).

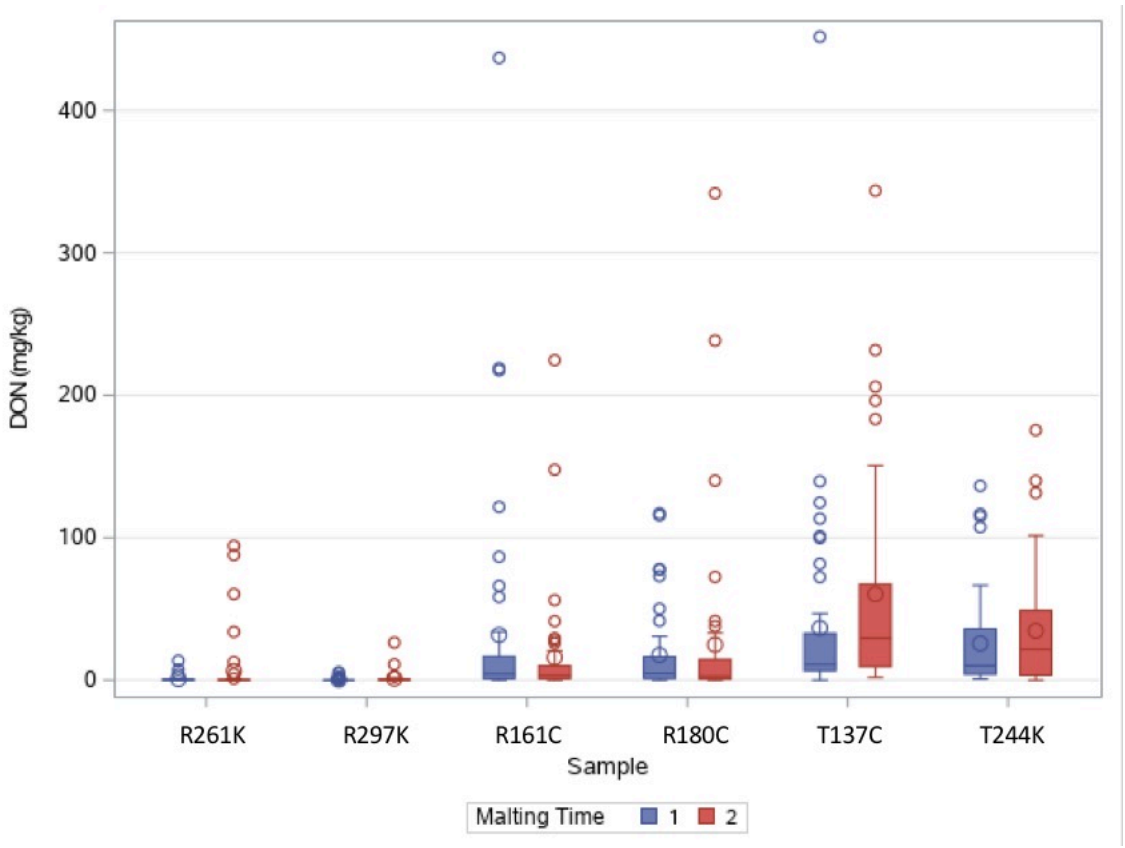
Overall, the mean of DON level in each treatment increased after malting compared with unmalted rye (Figure 9). Some variation between two replicates may be due to a single (or small amount) kernel with high DON level since only 46 kernels were used in the determination. Moreover, more highly infected kernels (outliers) were observed in rye malt (Figure 9), which indicates many kernels were infected and produced DON during malting.

The results from two replicates were variable due to different malting environments (i.e. storage time, tap water conditions) and variation of kernel infection. However, similar distribution trend proved that there was a higher percentage of malt that became infected during malting, which likely coincided with DON concentration being distributed in most of the malt kernels. Cross-contamination between green malt during germination is likely to be an explanation, and ideal temperature and moisture helped with DON development during germination.

Table 15. Distribution of Deoxynivalenol in Malted Rye and Triticale

				DON in rye (mg/kg)			% of Samples			
Sample[a]	Malting Time[b]	# of samples	# of pink kernels	Mean	MIN	MAX	<LOD	<0.50	0.50-1.00	>1.00
R261K	1	46	0	0.86	<0.20[c]	13.71	48	17	20	15
	2	46	0	6.52	<0.20[c]	94.35	67	11	4	18
R297K	1	46	0	0.39	<0.20[c]	5.67	80	7	2	11
	2	46	0	1.23	<0.20[c]	26.45	30	35	15	20
R161C	1	46	2	31.79	<0.20[c]	436.97	0	2	15	83
	2	46	1	16.01	<0.20[c]	224.69	4	2	13	81
R180C	1	46	1	17.71	<0.20[c]	117.18	4	0	13	83
	2	46	2	24.93	<0.20[c]	341.92	7	2	17	74
T137C	1	46	6	25.83	0.77	136.46	0	0	2	98
	2	46	8	42.75	<0.20[c]	175.6	9	4	4	83
T244K	1	46	3	36.69	<0.20[c]	451.77	2	0	2	96
	2	46	4	60.51	1.87	343.72	0	0	0	100

Note: [a] R: rye, T: triticale, C: Crookston; K: Kimball; Number is code for the replication within the field trial;
[b] Malting Time: 1: 9 months after harvest; 2: 10 months after harvest;
[c] LOD for DON detection on the GC-ECD in our lab is 0.20 mg/kg;



*Malting Time: 1: Malted around 9 month after harvest; 2: Malted around 10 month after harvest.

Figure 9. Deoxynivalenol in Single Kernels of Malted rye and Triticale.
(Lines in box and whiskers represent minimum, first quartile, median, third quartile, and maximum; Square points in the box represent mean; Circles outside the whiskers represents outliers, surprisingly high maximums or surprisingly low minimums).

6. CONCLUSION

In this study, rye grains were cleaned and sized before malting. The removal of thin kernels can help to avoid some shrunken FDK and reduce DON levels in some samples, which is what maltsters might do before process. After malting of sized rye grain, large increases in DON were observed in malts that were prepared from relatively clean grain (with average DON levels <0.2 mg/kg). This is of concern as, many maltsters generally select grain base upon DON levels, as DON limits often quoted as 0.4-0.5 mg/kg. In addition, these samples had been stored for several months prior to malting. In general, the increase in DON concentration began after the second day of germination, with significant increases observed at the third day of germination. The increases in D3G were generally observed at the second day to germination and a significant increase was observed at the third day of germination. DON is not a good predictor of D3G in grains, however, strong correlations were observed between DON and D3G levels after malting. The growth of *Fusarium*, as measured by Tri5 DNA, begins in the steep and with significant increases observed at the second day of germination. This makes sense as transcription and translation would proceed the actual synthesis of DON.

The distribution of DON on individual kernels from the bulk grain sample were tested. DON in the bulk grain samples is due to a small number of kernels with high DON. However, after malting, most kernels contain DON, which may be due to cross-contamination between green malt kernels, or growth of already existing *Fusarium*. Condition of germination provide favorable temperature and moisture conditions for fungal growth. Determining whether this is due to cross-contamination of grains, or the growth of *Fusarium* in “no DON” kernels will require additional experimentation such as kernel plating. Results support that *Fusarium* is not present on all grains.

The results of species isolation showed that *F. graminearum* was the predominated species detected from selected samples. However, results of this very small sample set do suggest other species may be associated with rye. In future study, larger sample size would be used, and the presence of type A trichothecene and other mycotoxins should be investigated. Reasons for the strong propensity for the development of DON on rye when compare to barley are still not clear. It might relate to the open pollinating nature of rye. Tissue localization should be investigated in future studies.

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