FATE OF DEOXYNIVALENOL AND DEOXYNIVALENOL-3-GLUCOSIDE DURING THE MALTING PROCESS

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Fate of Deoxynivalenol and Deoxynivalenol-3-Glucoside during the Malting Process

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ABSTRACT

Deoxynivalenol (DON) is commonly found on small grains and causes food safety issues. Deoxynivalenol-3-Glucoside (DON-3-G) is a conjugate, formed as a defense response by the host plant. Past studies have shown both to be present in *Fusarium* infected small grains, and processed products like beer, but there is limited information on DON-3-G in malt. Objectives were to determine the levels of DON-3-G in barley and wheat, and to study its fate during malting of inoculated and commercial samples. Commercial barley and wheat samples were used to determine levels in naturally infected grain. During malting, barley DON declined 48% on average, but DON-3-G increased by 115%. Both compounds increased in malted wheat. The genotype x crop year interactions were significant for both toxins, indicating that the genotypes did not respond similarly in the two years. The potential for large amounts of DON-3-G to be formed during malting has not been reported.

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LIST OF ABBREVIATIONS

ASBC ....American Society of Brewing Chemists

DON...Deoxynivalenol

D-3-G Deoxynivalenol-3-Glucoside

EFSA..European Food Safety Authority

FDA..Food and Drug Administration

FHB..Fusarium Head Blight

GC-EDC..Gas Chromatography – Electron Capture Detector

HRS..Hard Red Spring

LC-QTOF-MS...................................Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry

LOD ....Limits of Detection

LOQ ....Limits of Quantitation

NDSU..North Dakota State University

SAS ....Statistical Analysis System

SCF ....Scientific Committee on Food

RCBD..Randomized Complete Block Design

TDI..Tolerable Daily Intake

UPLC-MS/MS ....Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

USDA..United States Department of Agriculture

1. LITERATURE REVIEW

1.1. Introduction

1.1.1. Fusarium Head Blight (FHB)

Fusarium Head Blight (FHB) is a fungal disease that commonly infects spring and winter wheat (*Triticumaestivum*L.), durum wheat (*Triticumturgidum*L.), oat *(Avena*sativaL.) and barley (*Hordeumvulgare*L.) (Schmale and Bergstrom, 2010). The symptoms of FHB on wheat and barley exhibit some differences. For example, the first symptoms of FHB appear shortly after flowering in wheat. The infected spikelets of wheat appear partially or totally bleached. The pathogen can eventually begin growing and diffusing inside of wheat grain and the entire spike can be infected. As the disease progress, the fungus colonizes the developing grain causing it to shrink and wrinkle inside the spike. Often, the infected kernels have a rough, shriveled appearance, ranging in color from pink, soft-gray, to light-brown. Compared to the healthy grain, the infected grains appear shriveled, discolored, and are lightweight (Wise and Woloshuk, 2010). In barley, the tendency for infection to spread throughout the spikeis less pronounced, and only individual spikelets tend to be infected. Infected spikelets may show a bleached appearance or a brown or water-soaked appearance. Severely infected barley kernels at harvest may show a pinkish discoloration (McMullen et al., 2008). Infection of barley, unlike wheat, occurs after pollination because pollination occurs before the spike emerges from the plant. In the late stages of FHB infection, bluish-black spherical bodies (perithicia) may appear on the grain. In both wheat and barley, the grain quality is impacted by the presence of mycotoxins, or by actual damage to the grain caused by infection. An example of this would be damage to proteins by Fusarium proteases.

The main pathogen causing FHB in United States is *Fusarium graminearum* Schwabe, which can produce trichothecene mycotoxins (Bennett and Kilich, 2003). Other pathogens, such as *F. poae* and *F. avenaceum*, may also cause FHB on barley (McMullen et al., 2008). The spores of *Fusarium* species can persist and overwinter on crop debris above or on the soil surface. After the new crop is sown, the spores of the fungi can be windblown or rain-splashed on to the spikesof the new plants. Severity of infection depends on several factors including environment, species of *Fusarium*, and crop genotype. FHB can cause significant yield losses and quality reductions. At the same time, FHB also has a great effect on the economics of wheat and barley. The United States Department of Agriculture (USDA) ranks FHB as the worst plant disease in the US since the rust epidemics of the 1950s (Schmale and Bergstrom. 2010).

Barley is the most important ingredient in beer. It is fourth most widely produced cereal grain in the USA, after maize (*Zea mays* L.), wheat, and sorghum (*Sorghum bicolor* L.). The importance of maintaining the safety of this supply cannot be ignored. The mycotoxins produced by the FHB pathogen, if ingested by livestock, can cause feed refusal and reduced weight gain. However, a greater worry is if DON enters the human food chain through infected grain, as it can cause gastroenteritis and decreased immune function in humans (Pestka and Smoliske, 2005). The production of mycotoxins cannot be effectively eliminated, even though FHB infection can be reduced with fungicides (Malachova et al., 2010). Plant breeding had resulted in wheat and barley cultivars with some FHB resistance, but total immunity is likely not possible. As such, FHB infected grain will need to be monitored for mycotoxins and there is also a need for additional research.

1.1.2. Deoxynivalenol

Bennett and Klich (2003) defined mycotoxins as "secondary metabolites produced by fungi that are capable of causing disease and death in humans and other animals." The mycotoxin deoxynivalenol (DON) is mostly produced by *Fusarium* species, but can also be produced by *Stachybotryschartarum* (Sobrova et al., 2010). DON belongs to a class of mycotoxins referred to as tricothecenes (Desjardins, 2006), which is a large class of structurally related compounds. The chemical structure of deoxynivalenol is shown in Figure 1.

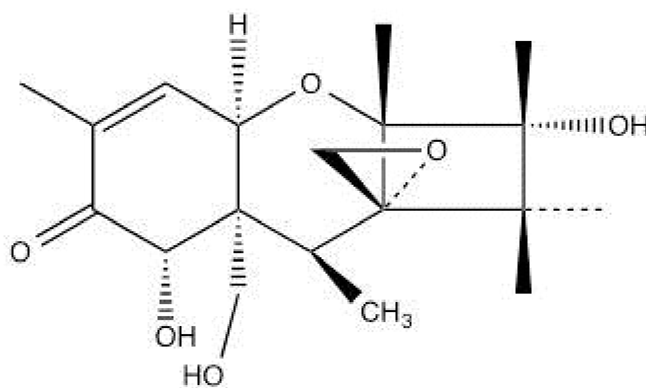

Figure 1. Chemical structure of deoxynivalenol (DON) (Schwarz et al, 2014)

Tricothecenes are tricyclic sesquiterpenes that contain a double bond between C-9 and C-10, and a 12, 13-epoxide ring. They are divided into two classes based upon the functional group at C-8. Type A tricothecenesare represented by T-2 toxin and diacetoxyscirpenol. Type B tricothecenes are represented by nivalenol and DON. The *Fusarium* species and specific chemotypes, within species, determine which toxins are produced. *F.graminearum* produces type B tricothecenes.

DON is frequently determined in the laboratory using gas chromatography–electron capture detection (GC–ECD) (Schwarz et al, 1995), although other chromatographic techniques are also used, including ultra-performance liquid chromatography-tandem mass spectrometry

(UPLC-MS/MS) (Malachova et al, 2011) and high-performance liquid chromatography (HPLC) (Zachariasova et al, 2012). In the grain industry, enzyme-linked immune sorbent assay (ELISA) test kits are commonly used to determine DON. This is due to the fact that the ELISA tests kit cost less money, require less training, but still yield results comparable to chromatographic methods.

1.1.3. Deoxynivalenol-3-Glucoside

Deoxynivalenol-3-glucoside (DON-3-G) is a product of a detoxification mechanism present in many plants, whereby DON is conjugated to glucose by UDP-glucosyltransferases*in planta* (Berthiller et al, 2013). DON-3-G has been detected in FHB infected wheat, corn, oat, and barley. It is often referred to as a masked (or bound) mycotoxin, which are defined as those which escaped detection, or are not extractable, during routine analytical procedures. There is concern over masked toxins, as it is possible that they might be released to free toxin under food processing conditions, or during digestion. The chemical structure of deoxynivalenol-3-glucoside (DON-3-G) is shown in Figure 2.

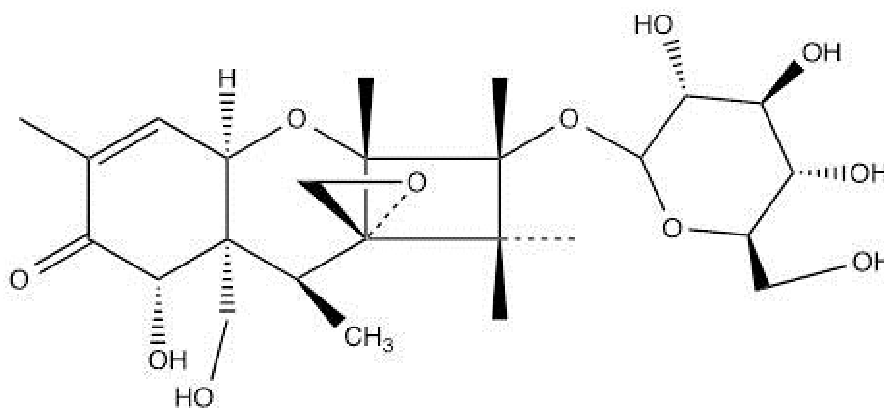

Figure 2. Chemical structure of deoxynivalenol-3-glucoside (DON-3-G) (Schwarz et al, 2014)

DON-3-Glucoside is generally determined in the laboratory by some form of liquid chromatography-mass spectrometry (e.g. UPLC-MS/MS) (Malachova et al, 2011; Schwarz et al,

2014). Simsek et al. (2013) stated DON-3-G cannot be measured by gas chromatography. It is referred to as a masked mycotoxin, again, because it is not typically measured by methods commonly used for measuring DON. DON-3-G can show cross-reactivity with the DON antibody used in some DON test kits (Lancova et al, 2008). In fact, the American Society of Brewing Chemists (ASBC) recommends against the use of test kits for measuring DON in malt (Bartlett et al, 2010).

1.1.4. Toxicity of Deoxynivalenol (DON)

Toxicology studies have shown DON has acute and chronic toxicity (Bennett and Klich, 2003). Acute toxicity means that when a higher dose of toxin is ingested in a single exposure, an undesired effect of the toxin will have a rapid onset. Chronic toxicity occurs when a lower dose of the toxin is consumed over a long time period, and appearance of the effects is delayed. The toxicity of DON was first seen mainly with farm animals when contaminated feed grains were fed. The symptoms seen in agricultural animals that consumed a high dose of DON were vomiting, nausea, and diarrhea. When the daily intake of DON was low, the animals exhibited weight loss and food refusal. Because of these symptoms, the DON toxin has also been called vomitoxin.

The principle of DON effects on human or animals is that it disrupts normal cell function by inhibiting protein synthesis via binding to the ribosome and by activating cellular kinases. These kinases are involved in signal transduction and impact cellular proliferation, differentiation, and apoptosis" (Pestka and Smolinske, 2005). As with other trichothecenes, leukocytes are also targets of the DON toxin. Leukocytes, also known as white blood cells, function to defend the body from disease and foreign invaders. The effect of DON on the leukocytes can be both immunostimulatory and immunosuppressive. For acute DON exposure,

Pestka (2008) stated that “DON promotes leukocyte apoptosis with associated immune suppression”. The toxin can damage the transducer of downstream signaling; thus, causing immune stimulation and apoptosis. Chronic exposure to DON results in up-regulated expression of cytokines, chemokines and inflammatory genes with concurrent immune stimulation.”

It has been hypothesized that gastroenteritis was related to DON ingestion in humans because of an outbreak of gastroenteritis following possible DON exposure in India in the 1990s (Pestka and Smolinske, 2005). The outbreak occurred after thousands of people ate wheat that was damaged by rain and mold. This outbreak was very similar to two cases reported earlier in China. From 1961 to 1981, a total of 32 outbreaks of diarrhea, headache, and fever occurred in people who ingested scabby or moldy wheat, barley, or corn. Onset of these symptoms was within 30 minutes. More than half of the people who ate the moldy food got sick, which was a very high incidence that brought these cases to public attention. A similar occurrence of people getting sick after eating scabby or moldy grains was reported in China in 1991. There were two similarities among these outbreaks. The first one was the symptoms, which included diarrhea, headache, and fever. The second similarity was the grains ingested were all moldy. With technological advances in mycotoxin analysis not being available in the 1960’s and 70’s, researchers were not able to identify DON.

Luo and coworkers (1991) have proposed that DON may have a relationship with esophageal cancer. Samples of corn and wheat were collected from different families at locations in a Chinese province. Each family had an esophageal cancer patient. Also, these families lived in two different locations but in the same province in China. One location had higher esophageal cancer risk and the other location had much lower risk. The results showed that the samples from the high risk location had 2.4-3.3 times higher DON content than the lower risk location (Luo et

al, 1990). While the results suggest DON as a factor of causing cancer, other factors such as pollution, genetics, environment, water conditions, soil conditions, cannot be ignored.

There is limited information about the toxicity of DON-3-G. Nagl et al (2012) indicated that DON-3-G is considered less toxic compared to DON, at least in rats, and might be hydrolyzed in the digestive tract of mammals. Because of the lack of the in vivo tests data, DON-3-G has not been considered in food law or regulations.

1.2. Regulation of Deoxynivalenol (DON)

As DON is harmful to human or animal health, the daily intake levels need to be controlled in an appropriate manor. Tolerable daily intake (TDI) is defined as an estimate of the amount of a substance in food or drinking water that can be taken in daily over a lifetime without appreciable health risk (Gunnar F, 1979, *p. 283*). TDIs are calculated on the basis of laboratory toxicity data to which uncertainty factors are applied. Uncertainty factors or safety factors mean the differences converting the no-observed-adverse-effect-level (NOAEL) in a group of animals into a level of human intake. The calculation used for TDI is NOAEL x uncertainty factor.

In United States, there is no TDI level for DON. Only an advisory guideline for DON has been published by the U.S. Food and Drug Administration (FDA) (2010). In processed products, the advisory level is 1.0 ppm (mg/kg) of DON. In 2001, the Scientific Committee on Food (SCF) in Europe stated the TDI of DON as 1µg/kg body weight (EFSA, 2013). Other data provided from European Food Safety Authority (EFSA) shows maximum TDI levels from 0.2 – 1.25 mg/kg in processed and unprocessed grain products.

1.3. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Malting and Brewing

1.3.1. The Malting and Brewing Processes

Malting and brewing are the two major steps for making beer. There are several steps in malting processing. Cleaning is performed to remove all foreign materials. In steeping, barley grains are soaked in water for approximately 48 h to raise the moisture content from 12 to around 45%. Steeping promotes germination and diffusion of enzymes into the endosperm. After steeping, barley is germinated for 4-5 day low temperature (e.g. 16 ^{o}C) and high relative humidity (e.g. >90%). Enzymes (amylase and proteases) that are important in brewing are synthesized. The last step of malting is kilning, where the grain is dried from 45% moisture to 4% moisture over 24 h. Temperatures are raised from 50 to 80 ^{o}C in step-wise manner to help preserve the enzymes and to develop color and flavor. Following kilning the rootlets are removed, and the final product is malt.

Mashing, lautering, wort boiling, fermentation and aging are the major steps of the brewing process. For mashing, the malt is ground and extracted with water in a mash tun. Starch and protein are converted to sugars and amino acids by amylase and protease enzymes. After mashing, the malt extract (wort) is separated from the insoluble portion (spent grains) in a process called lautering. The wort is then boiled and hops are added to impart bitterness and aroma. In the fermentation step, yeast and oxygen are added to the wort. Alcoholic fermentation requires about one week at about 12 ^{o}C. Aging is conducted to settle yeast, make the beer clearer, and to mature flavors.

1.3.2. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Brewing Grains

DON has been reported to occur on all small grains. Barley is the grain most commonly used in malting and brewing, but wheat and rye are occasionally used in specialty products,

especially by craft brewers. Maize is frequently used as an adjunct grain in brewing. Many surveys have been conducted on commercial barley for the presence of DON (Trucksess et al, 1995; Schwarz et al, 2006).

Deooxynivalenol-3-Glucoside was first reported in naturally contaminated wheat and maize by Berthiller et al. in 2005. DON-3-G and DON levels ranged from 0.05 to 0.20 mg/kg and 0.50-1.50 mg/kg, respectively. Later, Wang et al. (2012) surveyed 969 maize and maize-based samples from 24 provinces of China over three years. In maize kernels, the DON and DON-3-G level across years were 0.30-44 and 3.00-500.00 μg/kg. The DON-3-G to the DON ranged from 1.0 to 440.0 mol%, and the average was 27.0mol%. In some samples, the amount of DON-3-G was found to be more than the amount of DON. Based upon their results, the authors concluded that DON-3-G should be included in the risk assessment and TDI estimates for DON.

However, the information on DON-3-G in barley and malt is limited. Malachova et al. (2010) reported the analysis of 148 barley samples from experimental nurseries in the Czech Republic (2005-2008). Deoxynivalenol was found in83% of samples (average level of 28 μg/kg), while DON-3-G was found in only 7.2% of samples (average level of 3.6 μg/kg). The average of DON-3-G/DON ratio was 3.9 mol %. Schwarz et al. (2014) studied commercial barley samples from the upper Midwest USA that were purposely selected based on FHB infection. Deoxynivalenol was reported in 59% of samples. Deoxynivalenol-3-glucoside was detected in 72% of these DON positive samples. The average levels of DON and DON-3-G were 2.99 and 1.03 mg/kg, respectively. The average DON-3-G/DON ratio was 19.0 mol%, with values ranging from 8.0 to 45.0 mol%.

Simsek et al. (2013) surveyed DON and DON-3-G in commercial USA Midwest and Western samples of hard red spring (HRS) wheat. The samples were obtained from HRS wheat

growing region in U.S.; North Dakota, South Dakota, Montana and Minnesota. The results indicated that samples from different growing regions had different levels of DON and DON-3-G. Levels of DON-3-G have shown a positive relationship with DON content. When the amount of DON increased, it appeared that the level of DON-3-G resulting from the plant detoxification mechanism also increased. In addition, DON-3-G was significantly correlated with the percent damaged kernels. However, to date there is no information about DON and DON-3-G on malted wheat. This is important as malted wheat has become a very important ingredient for some specialty beers, and may actually account for 5-10% of all malt use in the USA (Bond et al, 2015).

Rye is currently a trendy grain with USA craft brewers and distillers (Julia S, 2012). Again there is very little information available on DON and DON-3-G in rye or rye malt. Rasmussen et al. (2003) surveyed the levels of DON in rye samples from Danish market. Samples (N=25) were collected from 1998 to 2000 from both mills and retail markets in Denmark. Deoxynivalenol was found on 50% of samples (average level of 49 µg/kg), and the range was from 20-257 µg/kg. In 2012, Rasmussen et al. conducted another experiment to test for DON and DON-3-G in rye samples. They believed that there was a correlation between DON and DON-3-G in rye grain. However, the results showed that the DON level of 12 rye samples from 2007 and 2008 were lower than 50 µg/kg, and DON-3-G was not detectable. Their limit of detection (LOD) for DON-3-G was 35 µg/kg. There are no data to date on levels of DON and DON-3-G on malted rye.

1.3.3. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Malting

The changes in DON during the malting of naturally infected barley was first reported by Schwarz et al. in 1995. In barley samples that are contaminated with *Fusarium*, there is the

possibility that the *Fusarium* will continue to grow during the germination phase of malting and produce additional DON (Schwarz PB, 2003, *p. 395-414*). However, *Fusarium* is not a storage mold, and its viability and ability to produce DON generally declines with storage time (Beattie et al, 1998).

For almost all samples, a decrease in DON is seen during steeping and the DON remains low in the finished malt. Maltsters and brewers generally have a limit on DON in barley and malt. These limits can vary between companies and with crop years. Barley with over 1 to 2 mg/kg is generally not accepted, and many brewers specify that levels on malt must be below the LOQ.

Lancova and coworkers (2007) showed that the amount of DON-3-G can increase during the germination stage of malting, with the content of DON decreasing (Lancova et al., 2007). They speculated that the increase in DON-3-G might be due to the occurrence of enzymatic activities during germination. Following the steeping process, they found that DON was either eliminated or reduced to below 10% of the original concentration on barley. Deoxynivalenol-3-glucoside started to appear during the germination stage. Investigations at NDSU later showed that levels of DON-3-G could increase dramatically during malting, and might actually be several-fold higher in concentration than DON (P. Schwarz personal communication).

Formation of DON-3-G during germination is likely due to the presence of the enzyme UDP-gluocsyltransferase in the grain. This had been postulated to be a plant defense mechanism to detoxify DON (Gardiner et al, 2010). Increased solubility of the conjugated toxin might facilitate its transport from the cytoplasm to a vacuole or intercellular space. This mechanism had been proposed to be associated with FHB resistance in cereals, and the presence of UDP-Gluocsyl-transferase capable of detoxifying DON in barley had been reported (Shin et al, 2012).

Information on DON-3-G in malt is limited, but the fact that it has been detected in commercial beers (Kostelanska et al., 2009; Yelko et al., 2015; Zachariasova et al., 2012), suggests that it is present in malt. Maul et al. (2011) showed that germinated grains of barley, millet, oat, ray, spelt, and wheat have the ability to convert applied DON to DON-3-G. This is presumably through the action of plant UDP-glucosyl-transferases.

Maul and coworkers investigated the conversion of DON to DON-3-G by barley, millet (*Pennisetumglaucum* L.), oat, rye, spelt (*Triticumspelta* L.), and wheat grains. The grain samples were comprised of healthy looking grains, but low amounts of background contamination could not be excluded. Grains were steeped overnight in 2 mL of either distilled water (control) or an aqueous DON solution. Both DON and DON-3-G concentrations were measured over the next 5 dof germination. Barley grain was found to convert 50% of the applied DON, and DON-3-G was the only major conversion product for barley. Also in the same experiment, the time of DON-3-G formatted in wheat samples was been observed. Furthermore, Maul et al. found that DON-3-G formation was most relevant between 17 and 46 h during germination. At hour 50, it appeared that DON and DON-3-G reached concentration equilibrium, and no more DON-3-G was formed after that time. They speculated that during the first day of germination, there was a large quality of glucosidase enzymes and free glucose present in the kernel. Glucose might be activated as uridine diphosphate (UDP) glucose, and these could be conjugated to DON by the glycosyl-transferases. Figure 3 shows diagram of DON and DON-3-G overtime changing during germination time (hr.).

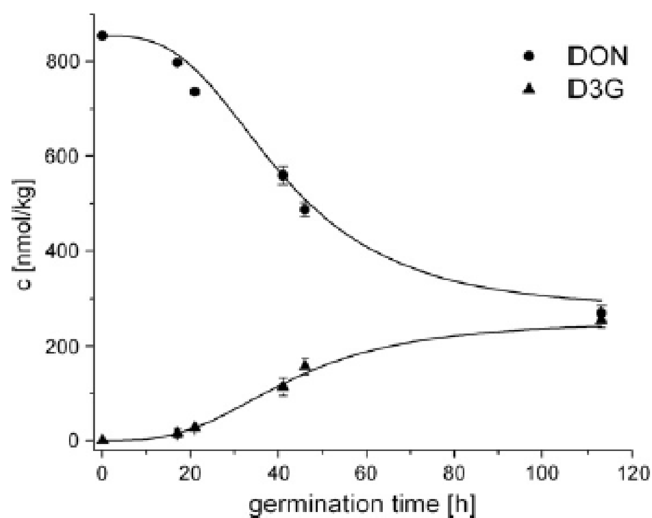

Figure 3. The changes of DON and DON-3-G during germination time (h) of barley (Maul et al., 2012).

In summary, during the malting process it appears that DON generally decreases throughout the process, while levels of DON-3-G started appearing during germination and continued to increase. The final step of malting is removal of the rootlets. Lancova et al. (2007) suggested that the rootlets contained the highest mycotoxin levels, and concern is appropriate when these are used for food or animal feed.

1.4. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Beer

There have been several recent reports on the presence of DON and DON-3-G in beer. Kostelanska et al. (2009) tested 176 commercial beers from both the European and North American markets. DON was detected in 74% of samples and levels ranged from 1.0 to 35.9 µg/L. Deoxynivalenol-3-glucoside was detected in 64% of samples and levels ranged from 1.2-37.0 µg/L. In several cases the levels of DON-3-G were actually higher than DON. A similar study was conducted by Yelko et al. (2015) where 159 commercial beers from the Spanish market in 2013 where surveyed. Deoxynivalenol was found in 60% of the beer samples at an average level 17.2 µg/L. Another 15 beer samples were tested by Zachariasova et al. (2012) in

2012 from the Czech Republic, where DON and DON-3-G were detected in all samples. DON level range from 5.6 to 45.5 μg/L, and DON-3-G ranged from 6.0 to 82.1 μg/L. In 2009, Kostelanska et al. (2009) indicated that DON and DON-3-G were found in both light and dark beers, with the content being greatest in higher alcohol beers.

2. OBJECTIVES

1. Determine levels of the DON and DON-3-G in barley samples from inoculated FHB nurseries
    a. Determine impact of genotype on the levels of DON and DON-3-G in barley.
    b. Investigate the changes that occur in DON and DON-3-G with the malting of inoculated barley.
2. Determine levels of the DON and DON-3-G in commercial barley and HRS wheat samples.
    a. Determine the changes in DON and DON-3-G content that occurs during malting of barley and wheat.

3. MATERIALS

3.1. Barley

Naturally infected and inoculated barley samples were used for experiments involving the determination of DON and DON-3-G levels in barley and malt. Naturally infected commercial samples (N=29, 2013) were obtained from a commercial barley buyer in ND. These samples were collected at the time of delivery from the farm to the buyer.

Inoculated samples were obtained from the NDSU Langdon Research Extension Center. These trials included three replicated of each entry. Details on the inoculation procedure have been described by both Urrea et al. (2002) and Schwarz et al (2014). One hundred and eight and 111samples were obtained in 2013 and 2014, respectively. A list of genotypes obtained from both the 2013 and 2014 nurseries is shown in Table 1.

3.2. Wheat

Naturally infected hard red spring wheat samples (N=19) were obtained from the NDSU wheat quality program and were part of the 2014 Hard Red Spring Wheat Crop Survey (U.S. Hard Red Spring Wheat Regional Quality Report, 2014). A single sample of *Fusarium* infected rye was obtained from a commercial grower in New York (2014 crop year).

Table 1. The pedigree information of 2013 and 2014 inoculated nurseries.

Genotype	Row type	Pedigree
2B05-0811	2	2B99-2763 / 2B00-0719
2ND25276	2	ND20802/3/ND19922//ND19929/ND20177
2ND27705	2	2ND24393/TR05285
2ND28065	2	2ND21867/2ND24383
AC METCALFE	2	AC OXBOW/MANLEY
CDC COPELAND	2	WM861-5/TR118
CELEBRATION	6	LEGACY/6B94-7862
CONLON	2	Bowman*2/DWS1008//ND10232
CONRAD	2	B1215//B1202/TR488
INNOVATION	6	6B98-9438 / 6B97-2311
LACEY	6	M78/M79
ND22421	6	ND18546/ND19656
ND26891	6	ND21376/ND20299
ND27177	6	Stellar-ND/ND23530
ND28554	6	ND23497/ND22421
ND28555	6	ND23497/ND22421
ND29196	6	ND20299/ND21786
ND29380	6	ND25025/ND21843
PINNACLE	2	ND18172/ND19130
QUEST	6	FEG18-20 / M110
RAWSON	2	ND15403-3/ND15368//ND16453
ROBUST	6	MOREX/MANKER
STELLAR-ND	6	FOSTER/ND12200/6B88-3213
TRADITION	6	6B89-2126/ND10981

4. METHODS

4.1. Measurement of Protein and Moisture

Protein and barley moisture content were determined by Near Infrared Reflectance using a Foss 1241 Grain Analyzer (Eden Prairie, MN).

4.2. Micro-Malting

Commercial barley samples and select samples (N=70) from the Langdon nursery were micro-malted using our standard laboratory methods (Karababa et al., 1993). The prepared 50 g sample (dry basis) was malted. The time required for each sample to reach 45% steep-out moisture was first determined by pilot-steeping a 10g sample. Steeping of all samples was performed at 16°C with 2 h of air rest included with each 12 h of steeping. Germination was performed for 96 hat 16°C and ~95% relative humidity. Samples were turned daily by hand to prevent matting, and sample weight was adjusted to 45% moisture with distilled water. Kilning was conducted in a forced air laboratory kiln. Total kiln time was 24 h, during which temperatures was ramped from 49 to 85°C. Rootlets were removed from kilned malt prior to analysis. The same procedure was used for the malting of wheat and rye samples.

4.3. Determination of DON

A modification of the method of Tacke and Casper (1996) was used to determine DON. Grain was ground using a Perten laboratory model 3610 mill (Perten Instruments, Hagersten, Sweden). The ground samples (2.5 g) were weighed into 50mL conical bottom polypropylene centrifuge tubes, and 20 mL of 84% acetonitrile/water was added. Samples were extracted by shaking on a horizontal shaker for 1 h. The speed of the shaking was 100/sec. After shaking, sample was allowed to settle before transferring a 4 mL aliquot of the supernatant to a solid phase extraction (SPE) column containing 1 g of 50/50% C18/alumina. The eluant (2 mL) was

transferred to 15x150 mm tubes and dried under a stream of nitrogen gas. Next, TMSI (trimethlysilayamidazole): TMCS (trimethylchorosilane) (10:1) was used for derivatization. After derivatization, the sample was extracted into 1mL of isooctane, and analyzed by gas chromatography with electron capture detection (GC ECD) analysis on an Agilent 6890 GC ECD (Santa Clara, CA).

Sample separation was achieved on a 5% phenyl methyl siloxane column (30 m×0.25 mm, 0.25 µm film thickness) (Agilent HP-5). Flow was 40 cm/second with a pressure of 20 psi. An intermediate polarity deactivated column (1-2 m×0.53 mm) (Restek. Bellefonte, PA) was used as a guard column. Injection volume was 1 µL of sample, with cool-on column injection. The initial inlet temperature was 90 °C. The inlet was heated at a rate of 20 °C/min to a final temperature of 300 °C. The initial oven temperature was 70 °C, and ramping at 25 °C/min, and then increased oven temperature to 170 °C. A subsequent ramp at a rate of 5 °C/min was used to increase oven temperature to 300 °C. The u-ECD detector was 300°C. Makeup gas was $ArCH_4$ (argon/methane) at flow rate of 60 mL/min. Mirex was used as the internal standard (Mirex, ULTRA Scientific, Kingstown, RI) at 0.5 mg/mL.

The DON standard was supplied by Biopure (Romer Labs Inc., Union, MO). A standard curve from 0.1 to 40 ng/µL was used which equates to 0.40 to 120 mg/kg in grain. The standard was spiked into an extract of DON-free grain sample. The LOQ and limit of detection (LOD) were 0.4 and 0.1 mg/kg, respectively.

4.4. Determination of DON-3-G

The same extract prepared for DON analysis was used for the determination of DON-3-G. Approximately 1 mL of supernatant from the 84% acetonitrile extracts (2.5 g/20 mL) was

filtered through a 25 mm nylon syringe filter (0.2 μm). Samples were analyzed on an Agilent Technologies 6540 UHD Accurate-Mass LC-QTOF-MS (Santa Clara, CA).

Samples were placed on auto sampler that was maintained at a temperature of 4 °C. An Agilent 1290 pump was used for the reference standards. These reference standards were 1.0 mL purine in 500 mL 95 ACN: 5 H_2O, and 0.8 mL HP-0921 in 500 mL 95ACN:5H_2O. The masses of these references were 118.086 and 922.010. The flow rate of the reference standards was 0.004 mL/min. The solvent system consisted of 0.01% formic/water (solvent A) and 0.01% formic/acetonitrile (solvent B). The ISO. Infusion Pump (Model: F1310B) was used, and the flow rate was 0.004 mL/min. Five μL of sample was injected and separation was achieved using an Eclipse Plus C18 column (2.1x50 mm, 1.8 μm film thickness). For data collection, the gas temperature and gas flow were 300 °C and 7 liter/min, respectively. The nebulizer pressure was 20.8 kPa. Sheath Gas temperature and flow were 325 °C and 12 L/min.

The standard was spiked into an extract of DON-free grain and malted grain samples. The LOQ and LOD were 0.1 and 0.05 mg/kg, respectively.

4.5. Statistical Analysis

The data for *Fusarium* inoculated barley and the corresponding malted barley samples were analyzed in a randomized complete block design (RCBD) using PROC GLM procedures of SAS System (version 9.1, SAS Institute, Cary, NC). *F*-tests were considered significant at $P \leq 0.05$. Means were separated using least significant differences (LSD) to determine the level of significance at $P = 0.05$.

5. RESULTS AND DISCUSSION

5.1. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Barley from Fusarium Head Blight Inoculated Nurseries

The objectives of this portion of the experiment were to determine the relationship between DON and DON-3-G in samples that were inoculated with *Fusarium,* and if genotype had a significant impact on the levels. Thirty-six genotypes (n=3 replicates) were analyzed from the 2013 Langdon nursery and 37 from the 2014 nursery. However, not all genotypes were present in both years. For purposes of the statistical analyzes only data from the 24 genotypes present in both years was analyzed.

Results of the ANOVA procedure for DON, DON-3-G and DON-3-G/DON as mol% are given in Table 2. Genotype and the interaction of year*genotype had a significant ($P \leq 0.05$) effect on both DON and DON-3-G. Only genotype was observed to significantly impact mol%. Examination of the data presented in Figures 4 and 5 shows that there were large differences in the magnitude of DON and DON-3-G levels between the 2013 and 2014 nurseries.

Table 2. Combined analysis across years for DON, DON-3-G, and mol% of barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014.

Variable	Source	df	Sum of Squares	Mean Square	*F*-value	*Pr>F*
DON						
	Year	1	2547.43	2547.43	--	--
	Rep(Year)	4	448.26	112.07	--	--
	Genotype	23	840.03	35.52	1.86	0.0199
	Year*Genotype	23	744.14	32.35	1.65	0.0493
DON-3-G						
	Year	1	453.89	453.89	--	--
	Rep(Year)	4	67.43	16.86	--	--
	Genotype	23	105.09	4.57	2.59	0.0007
	Year*Genotype	23	76.98	3.35	1.9	0.017
Mol %						
	Year	1	596.58	596.58	--	--
	Rep(Year)	4	4137.61	1034.40	--	--
	Genotype	23	4458.72	193.86	2.5	0.0011
	Year*Genotype	23	1310.73	56.97	0.74	0.7968

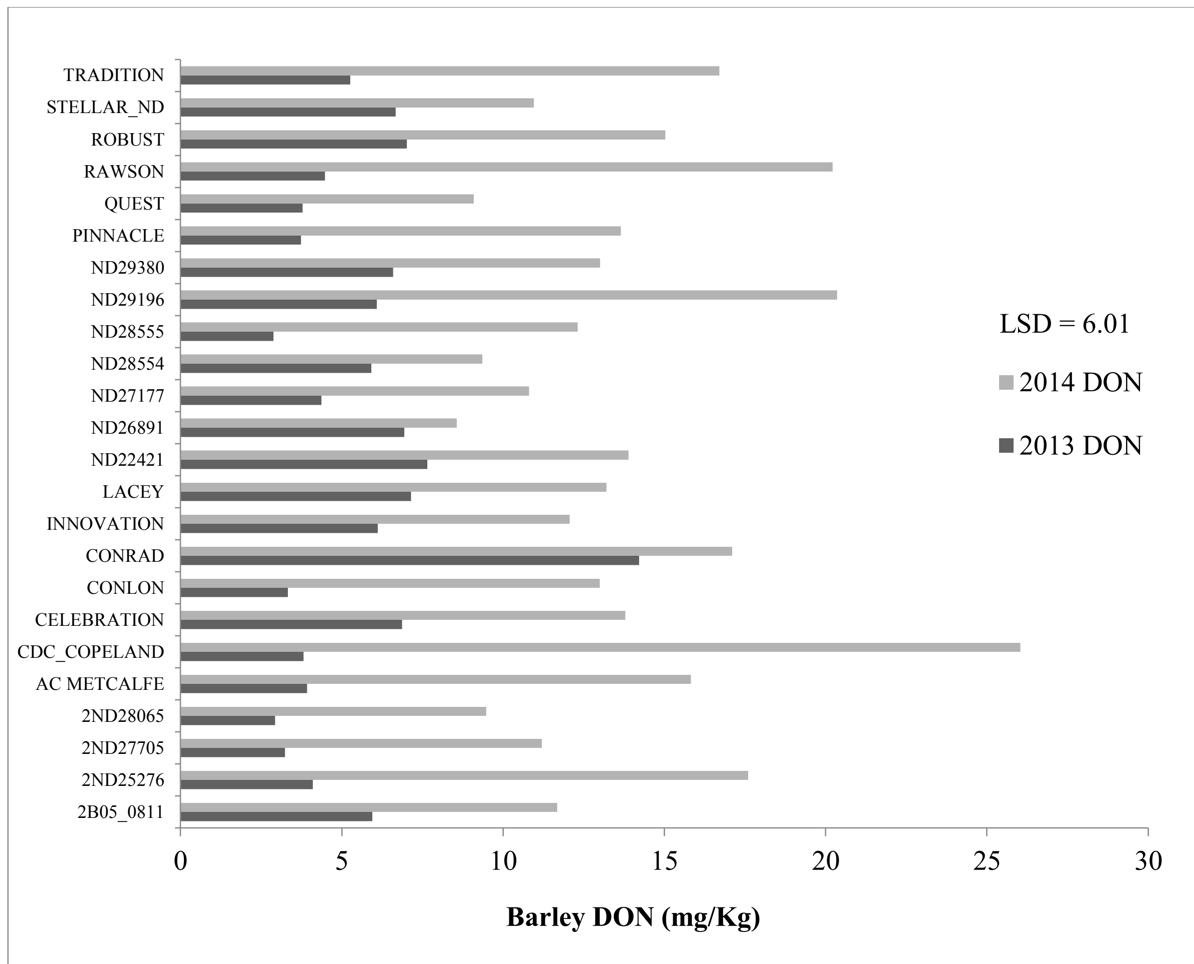

Figure 4. Mean deoxynivalenol of barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in the 2013 and 2014.

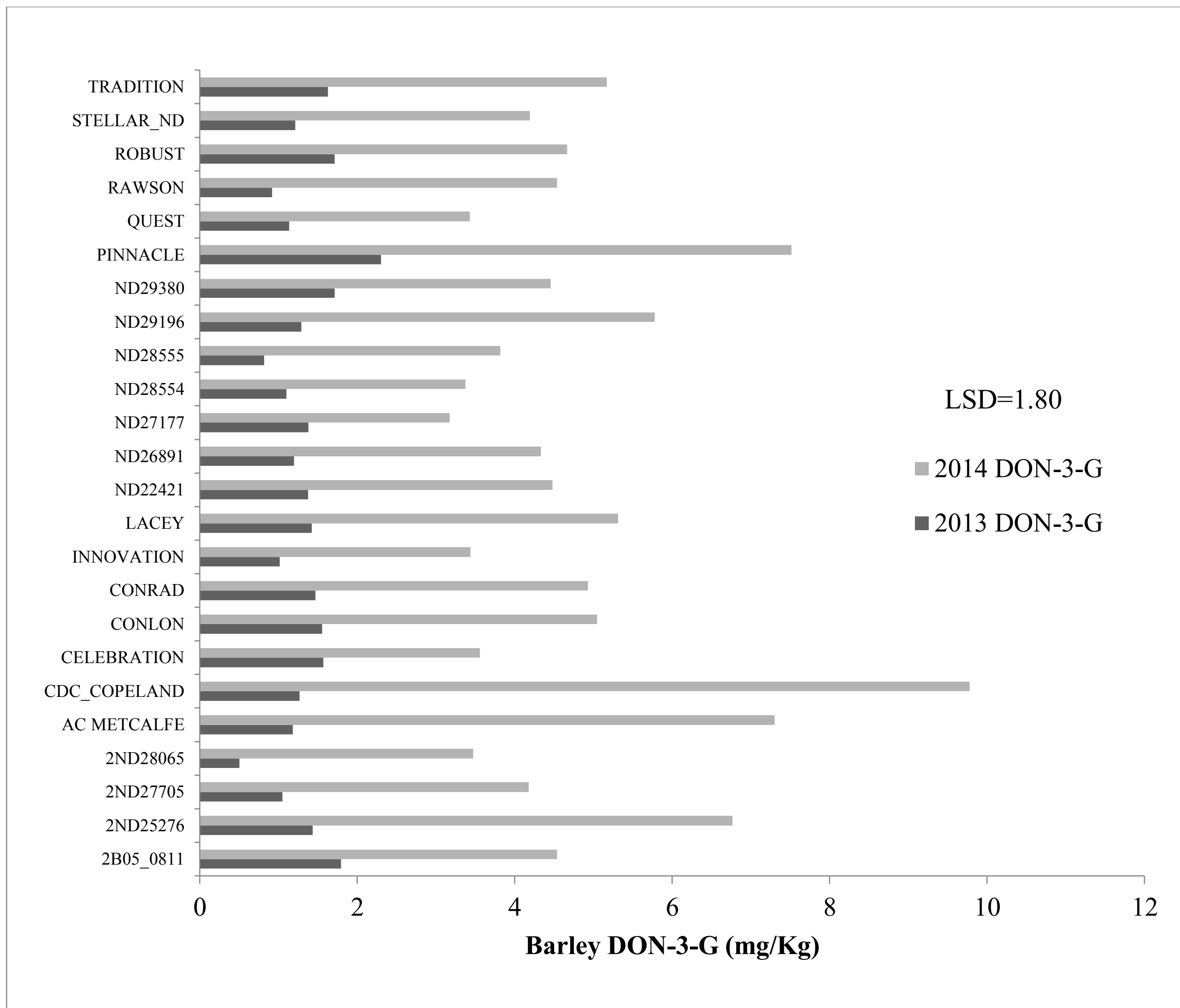

Figure 5. Mean deoxynivalenol-3-glucoside grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center Mean deoxynivalenol of barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in the 2013 and 2014 years

Both DON and DON-3-G were detected in all inoculated barley samples, but levels were much higher in 2014. This suggests a greater level of infection. However, as can be seen from Figure 4 the rank of samples changed between years. CDC Copeland had the highest level of DON in 2014, but ranked 18th in 2013. ND26891 was lowest in 2014, but ranked 5th highest in 2013. Similar observations can be made when comparing years for DON-3-G. CDC Copeland was highest in DON-3-G in 2014 but ranked 14th in 2013.

As such, the genotype*year interactions for DON and DON-3-G were true interactions, and simply not due to differences in magnitude. In 2013, the average level of DON was 5.54 mg/kg and ranged from 2.88 to 14.22 mg/kg (Table 3). Average DON of the 2014 samples was 13.95 mg/kg, and ranged from 8.56 to 26.03. The LSD value for DON was 6.01 for both years. When this LSD is used to examine the data in Figure 4, it can be seen that there were not significant differences in DON between many of the samples.

Table 3. Mean deoxynivalenol and deoxynivalenol-3-glucoside averaged across genotypes grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014.

	Deoxynivalenol				**Deoxynivalenol-3-Glucoside**				**Deoxynivalenol-3-Glucoside/ Deoxynivalenol* mol%**			
		mg/kg				mg/kg				mg/kg		
Year	Samples ≥ LOQ[a]	Average	Minimum	Maximum	Samples ≥ LOQ[b]	Average	Minimum	Maximum	Samples ≥ LOQ	Average	Minimum	Maximum
2013	72	5.54	2.88	14.22	72	1.33	0.50	2.3	72	19.46	7.47	40.83
2014	72	13.95	8.56	26.03	72	4.88	3.17	9.78	72	23.53	14.8	37.5

[a] Limit of Quantitation for deoxynivalenol was 0.4 mg/kg

[b] Limit of Quantitation for deoxynivalenol-3-glucoside was 0.1 mg/kg

* Deoxynivalenol-3-glucoside/ deoxynivalenol should be only for samples with DON ≥ LOQ and DON-3-G ≥ LOQ.

DON-3-G was detected in all of the samples from the inoculated barley nurseries in both years. For the 2013 crop year, the meanDON-3-G level across genotypes was 1.33 mg/kg, and ranged from 0.5 to 2.3 mg/kg. In 2014, the meanDON-3-G level averaged across genotypes was 4.88 mg/kg. The minimum and maximum levels were 3.17 and 9.78 mg/kg. As previously discussed, there were big differences in DON-3-G between years. Figure 5 shows the DON-3-G differences for each genotype between crop years.

The LSD (least significant difference) value for DON-3-G was 1.80 mg/kg, and was calculated as described for DON. This suggests no significant differences in DON-3-G between cultivars in 2013, while some differences can be detected in 2014 (Figure 5)

The relationship between DON and DON-3-G for the two crop years can be seen in Figures 6 and 7. The relationship was quite weak in 2013 (r=0.25). Most the 2013 samples fell in a range between 2 and 8 mg/kg DON and 0.5 to <2.5 mg/kg DON-3-G. The ranges were larger in 2014, and barley DON levels were found to be a better predictor of DON-3-G.Similar observations on crop year difference have been made by Schwarz et al (2014).

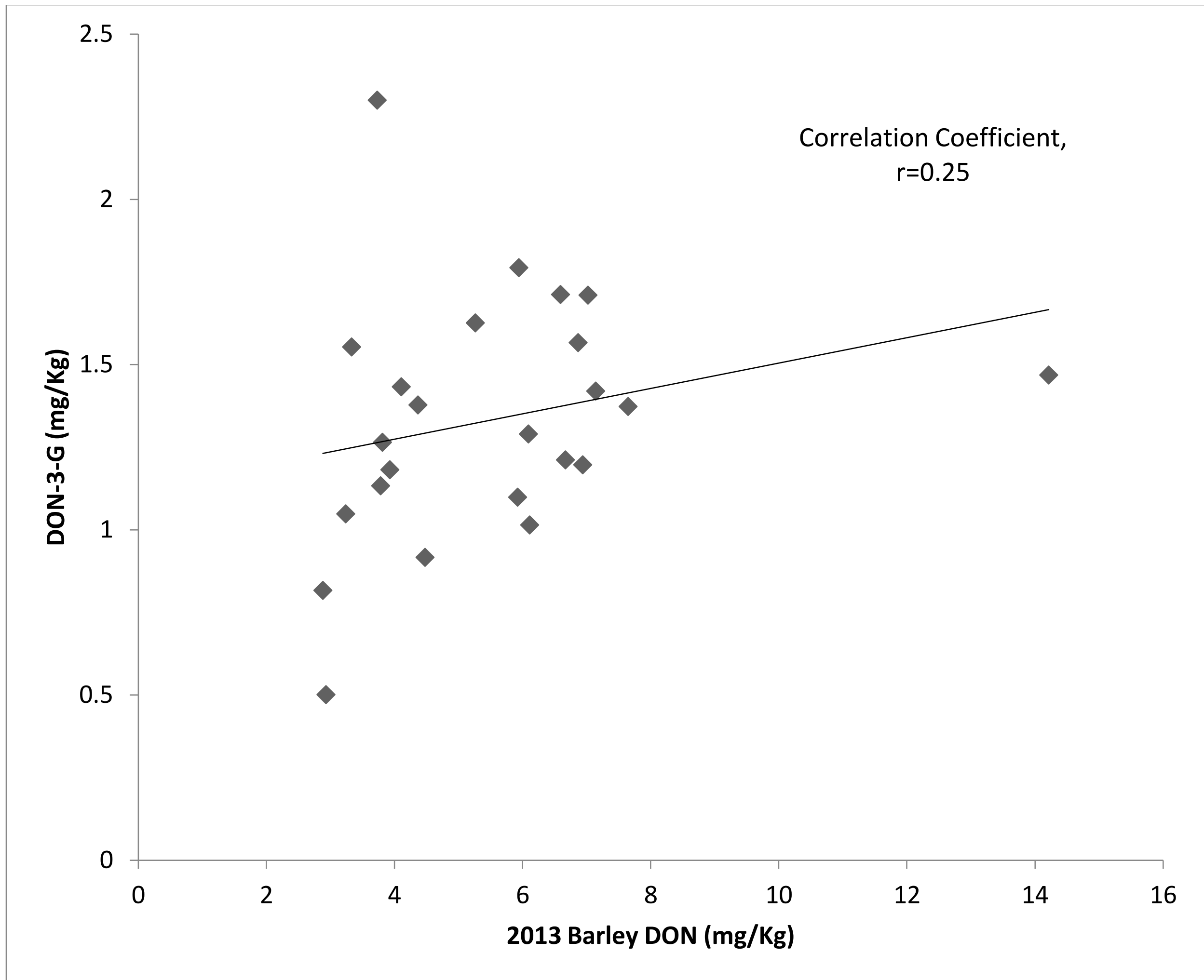

Figure 6. Comparison of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) of genotypes grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 (N=24) in North Dakota.

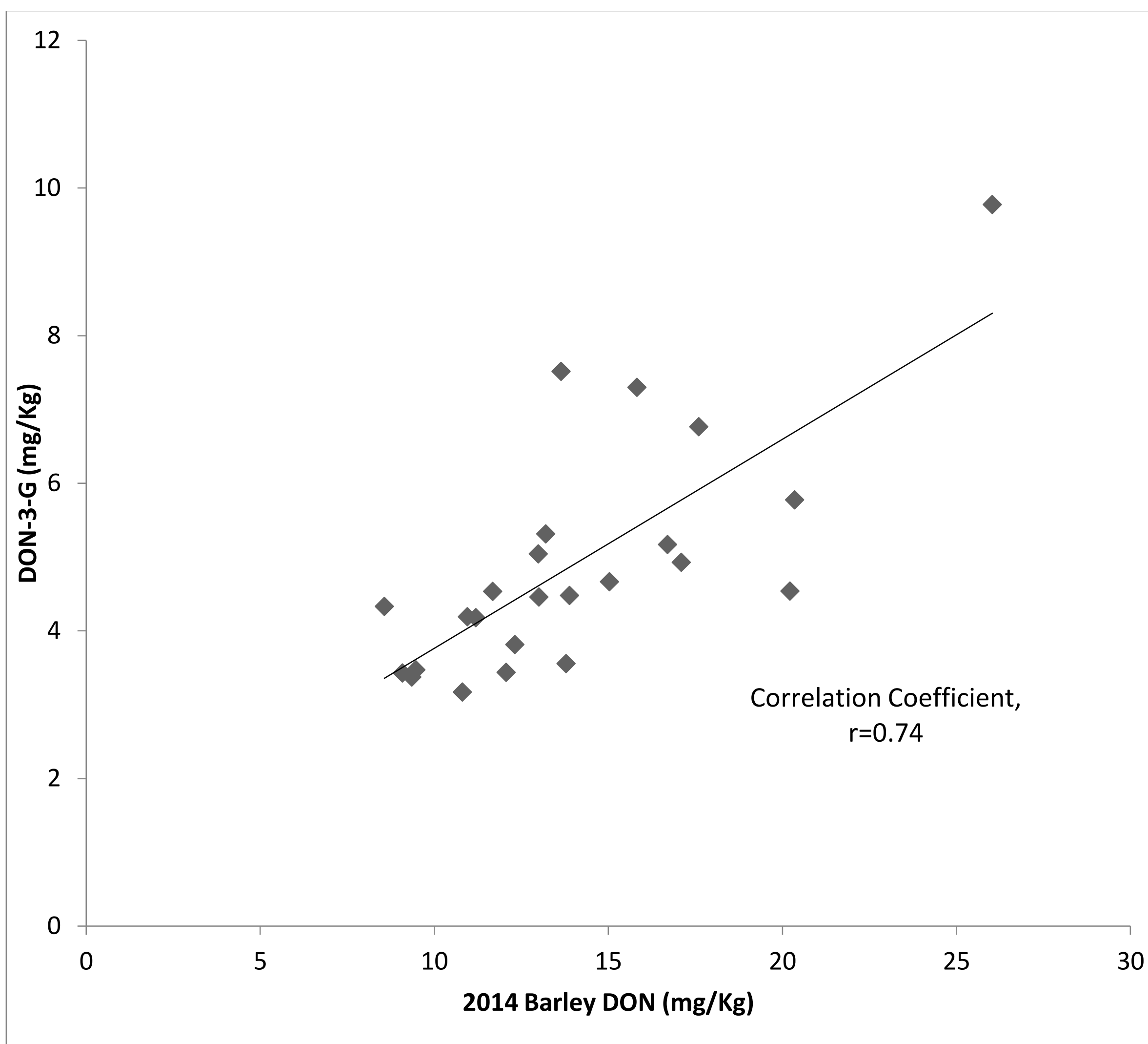

Figure 7. Comparison of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) levels of genotypes grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2014 (N=24) in North Dakota.

The ratio of DON-3-G/DON in mol% is an indication of the portion of DON that has been converted to DON-3-G by the plant. It is of interest, as some regulators have suggested that DON-3-G and DON congeners (3- and 15-ADON) should be included in TDI estimates for DON. Mol% was significantly impacted by genotype; the year*genotype interaction was not significant (Table 2). The average DON-3-G/DON ratio was 19.5 mol% for samples containing both compounds at levels above the respective LOQs (0.40 mg/kg DON, 0.10 mg/kg DON-3-G) from 2013 inoculated nurseries. The range in DON-3-G/DON for these 2013 samples was 7.5 to

40.8 mol%. For the 2014 year crop samples, the average mol% was 23.5, and values ranged from 14.8 to 37.5 mol%. Since the year*genotype interaction was non-significant for mol%, Figure 8 shows the average mol% for each genotype across crop years. The LSD values for mol% was 10.09.

The results of study showed similar DON levels when compared to the results Schwarz et al (2014) who also studied inoculated barley. However, the levels of DON-3-G detected in the current study were higher. They reported only 0.20 to 1.04 mg/kg DON-3-G, current results showed DON-3-G levels ranging from 0.50 to 9.78 mg/kg. The results of Schwarz et al (2014) indicated an average of 0.7 mol% in barley with very little range. Malachova et al (2010) observed an average of 3.9 mol% in infected barley. The average reported here was higher, but, not outside what has been reported in other cereals. Wang et al (2012) measured DON and DON-3-G in naturally infected samples of maize collected in China. They reported DON and DON-3-G levels ranged from 0.30-4374 and 3.00-500 µg/kg, across the years studied. The DON-3-G/DON ranged (mol, %) was from 1.00 to 440%.

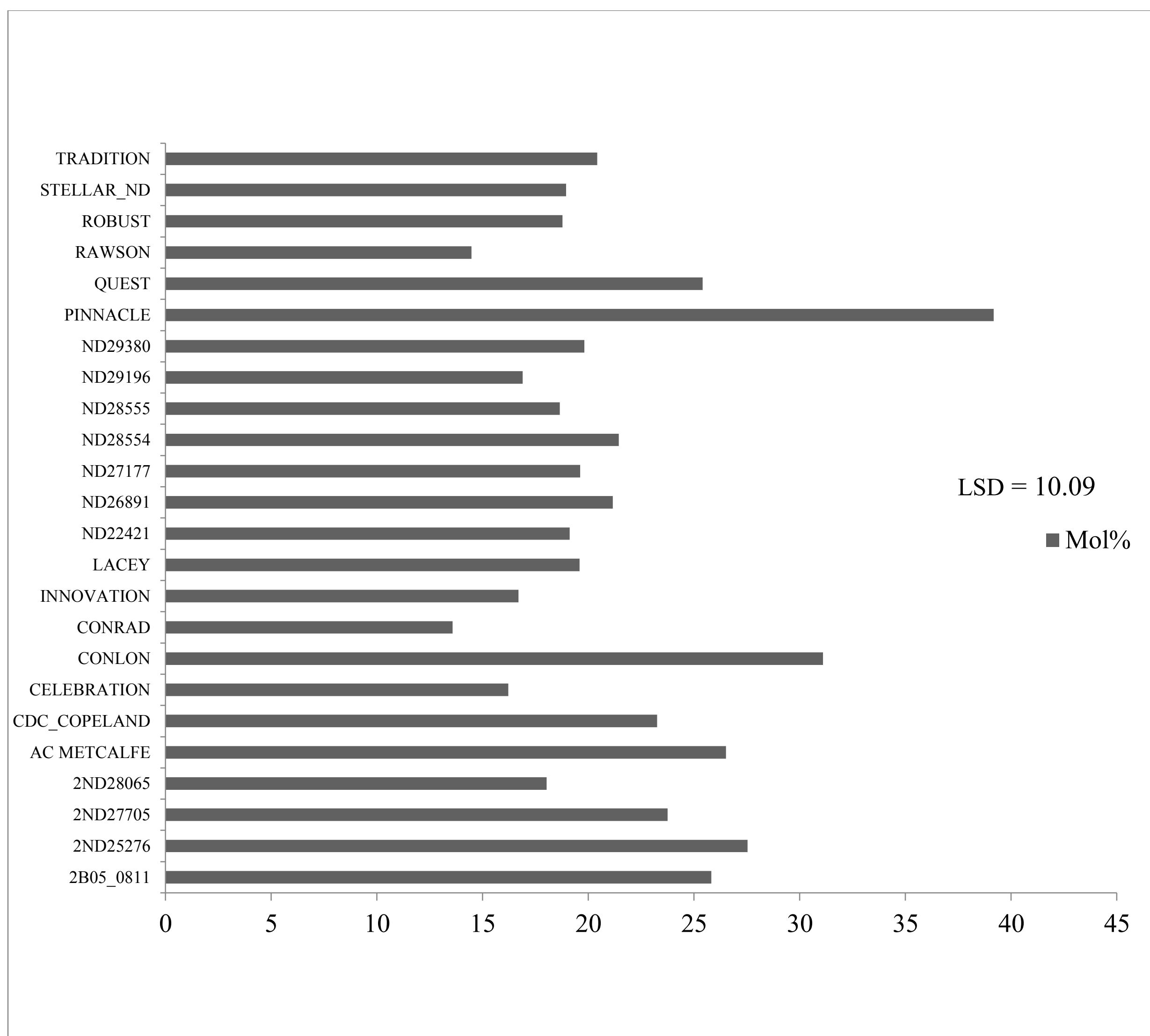

Figure 8. The mean ratio of DON-3-G/DON in mol% across years (2013 and 2014) for inoculated barley in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center.

5.2. Association of Deoxynivalenol-3-Glucoside to Fusarium Head Blight Resistance Levels in Barley

Schwarz et al. (2014) attempted to relate FHB resistance to levels of DON and DON-3-G in inoculated barley. Barley genotypes were grouped into classes of moderately resistant (MR), moderately susceptible (MS), and susceptible (S) based upon breeder experience with the

materials. Significant differences were detected in DON levels between the MR and MS classes, and between the MR and S classes. No differences were seen between levels of DON-3-G.

In the current study, genotypes were class based on DON data relative to the MR checks Quest (six-rowed) and Conlon (two-rowed) checks. Genotypes were considered MR when their values of DON were <115% of the appropriate six- or two-row check. Susceptible (S) samples had DON levels of >165% of the check. Any sample in between 115% and 165% considered as moderately susceptible (MS). These data are shown in Table 4.

Table 4. Estimated resistance levels in *Fusarium* Inoculated Barley for Langdon Hill Plots across years of 2013 and 2014.

Genotype	**Row type**	**DON**	**DON-3-G**	**mol%**	**% to QUEST**	**% to CONLON**	**Disease Classification**
2B05-0811	2	8.81	3.17	25.82	/	108%	MR
2ND27705	2	7.22	2.62	23.75	/	88%	MR
2ND28065	2	6.2	1.99	18.03	/	76%	MR
CONLON	2	8.16	3.3	31.1	/	100%	MR
PINNACLE	2	8.69	4.91	39.17	/	106%	MR
QUEST	6	6.44	2.28	25.4	100%	/	MR
2ND25276	2	10.86	4.1	27.54	/	133%	MS
AC METCALFE	2	9.88	4.24	26.52	/	121%	MS
RAWSON	2	12.35	2.73	14.47	/	151%	MS
ND26891	6	7.75	2.77	21.15	120%	/	MS
ND27177	6	7.59	2.28	19.62	118%	/	MS
ND28554	6	7.64	2.24	21.43	119%	/	MS
ND28555	6	7.6	2.32	18.65	118%	/	MS
CELEBRATION	6	10.33	2.57	16.22	160%	/	MS
INNOVATION	6	9.09	2.23	16.7	141%	/	MS
LACEY	6	10.18	3.37	19.58	158%	/	MS
ND29380	6	9.8	3.09	19.82	152%	/	MS
STELLAR-ND	6	8.82	2.7	18.95	137%	/	MS
CDC COPELAND	2	14.92	5.53	23.25	/	183%	S
CONRAD	2	15.66	3.2	13.59	/	192%	S
ND22421	6	10.77	2.93	19.12	167%	/	S
ND29196	6	13.22	3.54	16.9	205%	/	S
ROBUST	6	11.03	3.19	18.78	171%	/	S
TRADITION	6	10.99	3.4	20.42	171%	/	S

5.3. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Malted Barley from Fusarium Head Blight Inoculated Nurseries

The objectives of this portion of the thesis were to determine the relationship between levels of DON and DON-3-G on barley samples that were inoculated with *Fusarium*, and levels on the corresponding malted barley samples. Thirty-six genotypes (n=3 replicates) were analyzed from the 2013 Langdon nursery and 37 form the 2014 nursery. However, not all genotypes were present in both years. For purposes of the statistical analyses, only data from the 16 genotypes present in both years was used.

Results of the ANOVA procedure for DON, DON-3-G and DON-3-G/DON mol% in barley and malted barley samples are given in Table 5. Genotype and the year*genotype interaction were significant ($P \leq 0.05$) for DON and DON-3-G in barley and malted barley. Only genotype was significant impact on mol%.

Table 5. ANOVA results for DON, DON-3-G, and mol% of barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014.

Variable	Source	df	Sum of Squares	Mean Square	*F*-value	*Pr>F*
Barley DON						
	Year	1	1047.129150	1047.129150	--	--
	Rep(year)	2	1.659623	0.829811	--	--
	Genotype	15	401.959000	26.797267	2.04	0.0466
	Year*Genotype	15	444.253118	29.616875	2.26	0.0280
Barley DON-3-G						
	Year	1	188.7547508	188.7547508	--	--
	Rep(year)	2	0.3388311	0.1694155	--	--
	Genotype	15	90.1737870	6.0115858	3.16	0.0036
	Year*Genotype	15	57.8673725	3.8578248	2.02	0.0488
Barley Mol %						
	Year	1	3.443891	3.443891	--	--
	Rep(year)	2	355.600237	177.800118	--	--
	Genotype	15	6696.646559	446.443104	3.13	0.0038
	Year*Genotype	15	1733.319385	115.554626	0.81	0.6589

Deoxynivalenol and deoxynivalenol-3-glucoside were detected in all samples from 2013 (N=32) and 2014 (N=32) but levels were much higher in 2014. This suggests a greater level of infection. In 2013, mean DON across genotypes was 4.33 mg/kg and ranged from 0.92 to 12.73 mg/kg (Table 6). In 2014, mean DON averaged across genotypes was 12.43 mg/kg, and ranged from 4.85 to 30.00. The LSD values for DON was 7.39 for both years. When this LSD is used to examine the data in Figure 9, it can be seen that there were some significant differences in DON between many of the samples and same sample between different years. For example, CDC Copeland had a DON level in 2014 at 26.65 mg/kg, and it is significant different with ND28554 in 2014, which had a DON level at 5.28 mg/kg. However, within the same cultivar of CDC Copeland, DON levels in 2013 and 2014 were at 3.44and 26.65 mg/kg, respectively.

Table 6. Mean deoxynivalenol and deoxynivalenol-3-glucoside averaged across genotypes grown in a mist-irrigated nursery inoculated with *Fusariumgraminearum*located at the Langdon Research Extension Center in 2013 and 2014.

	Deoxynivalenol				**Deoxynivalenol-3-Glucoside**				**Deoxynivalenol-3-Glucoside/ Deoxynivalenol* mol%**			
		mg/kg				mg/kg				mg/kg		
Year	Samples ≥ LOQ[a]	Average	Minimum	Maximum	Samples ≥ LOQ[b]	Average	Minimum	Maximum	Samples ≥ LOQ	Average	Minimum	Maximum
2013	32	4.33	0.92	12.73	32	1.44	0.47	5.13	32	26.02	6.73	91.34
2014	32	12.43	4.85	30.00	32	4.88	1.47	10.32	32	26.49	11.54	57.36

[a] Limit of Quantitation for deoxynivalenol was 0.4 mg/kg
[b] Limit of Quantitation for deoxynivalenol-3-glucoside was 0.1 mg/kg
* Deoxynivalenol-3-glucoside/ deoxynivalenol should be only for samples with DON ≥ LOQ and DON-3-G ≥ LOQ

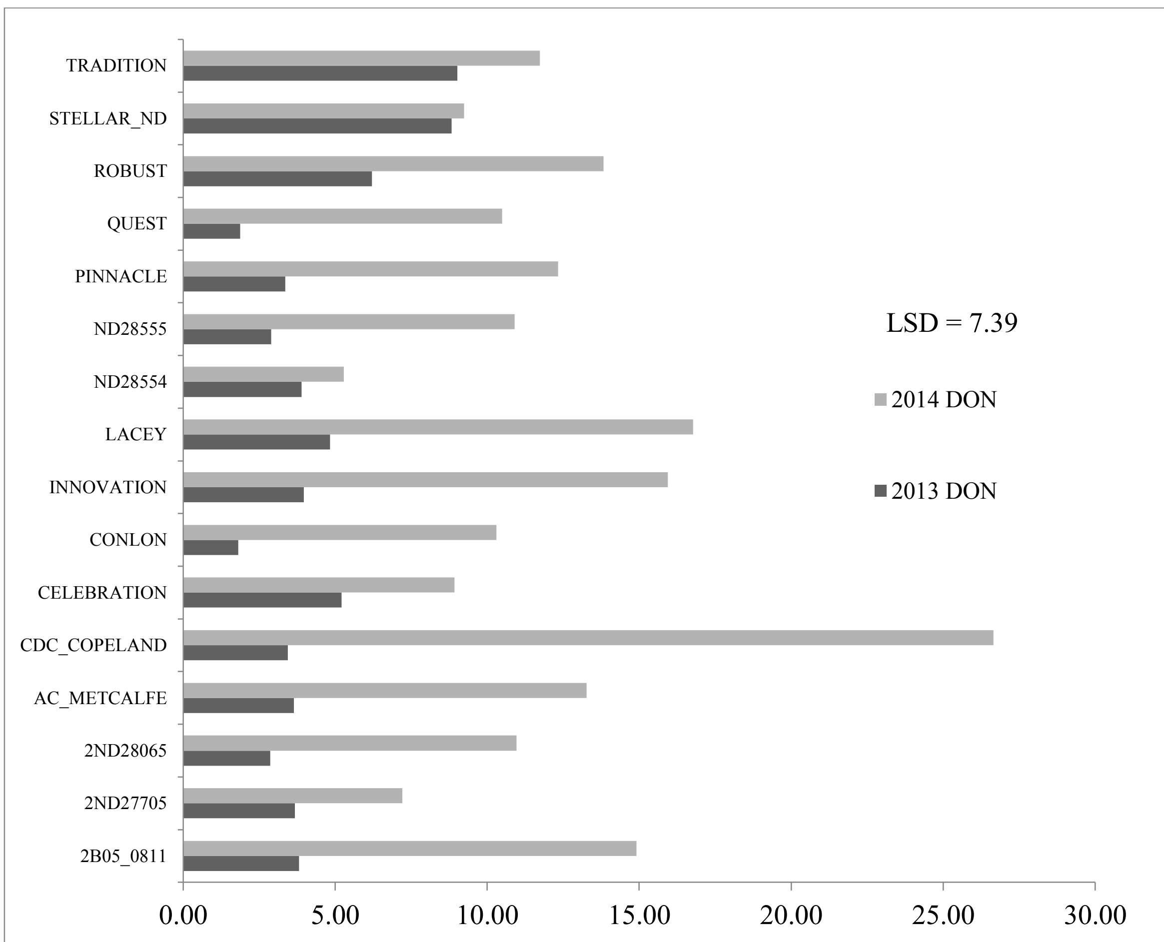

Figure 9. Mean deoxynivalenolof barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014.

Deoxynivalenol-3-glucoside was detected in all of the *Fusarium* inoculated barley nurseries from both years. For the 2013 crop year, the meanDON-3-G level across genotypes was 1.4 mg/kg, and ranged from 0.5 to 5.1 mg/kg. In 2014, the meanDON-3-G level across genotypes was at 4.88 mg/kg. The minimum and maximum levels were 1.47 and 10.32 mg/kg, respectively. The LSD value for DON-3-G was 2.82 for both years. When this LSD is used to examine the data in Figure 10, it can be seen that there were nosignificant differences in DON between many of the samples in 2013; however, there were some significant differences between

years. For example, Pinnacle had a DON-3-G level at 8.48 mg/kg in 2014, and it is significantly different from the value obtained for the same genotype in 2013, which was 3.00 mg/kg.

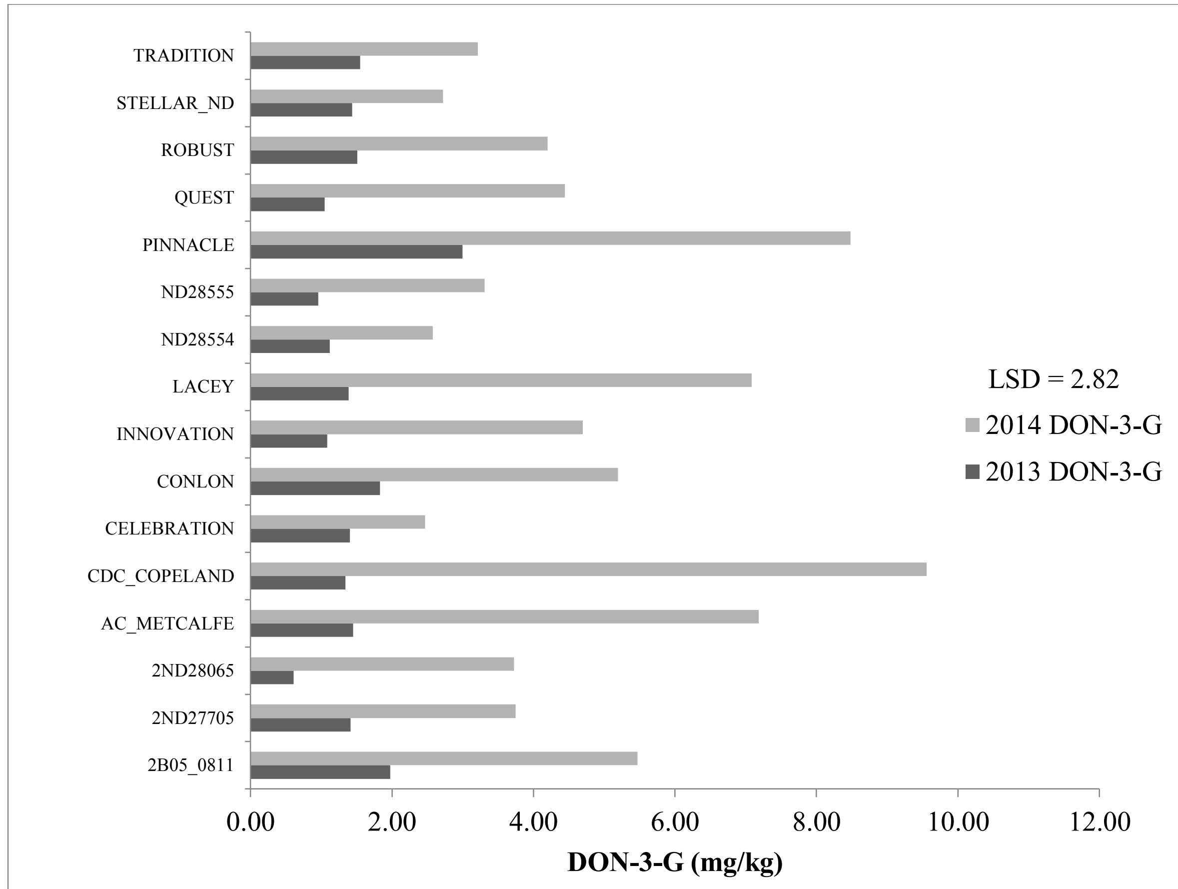

Figure 10. Mean deoxynivalenol-3-glucoside of barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014.

Barley genotype was found to have a significant impact on DON-3-G/DON mol%. Mean DON-3-G/DON ratio across genotypes was 26.0 mol% from 2013 inoculated nurseries. The range in DON-3-G/DON for these 2013 samples was 6.7 to 91.3 mol%. In 2014, mean mol% across genotypes was 26.5, and values ranged from 11.5 to 57.4 mol%.

The genotype*year interaction was not significant for % mol, but there were significant differences between genotypes. Figure11 shows the mean mol% for each genotype across years. The LSD values for mol% was 17.24 (error mean square =142.64). The varieties that had highest

and lowest DON-3-G/DON mol% were Pinnacle and Stellar ND, and average levels of these two varieties across years are 49.9 and 15.2 mol%.

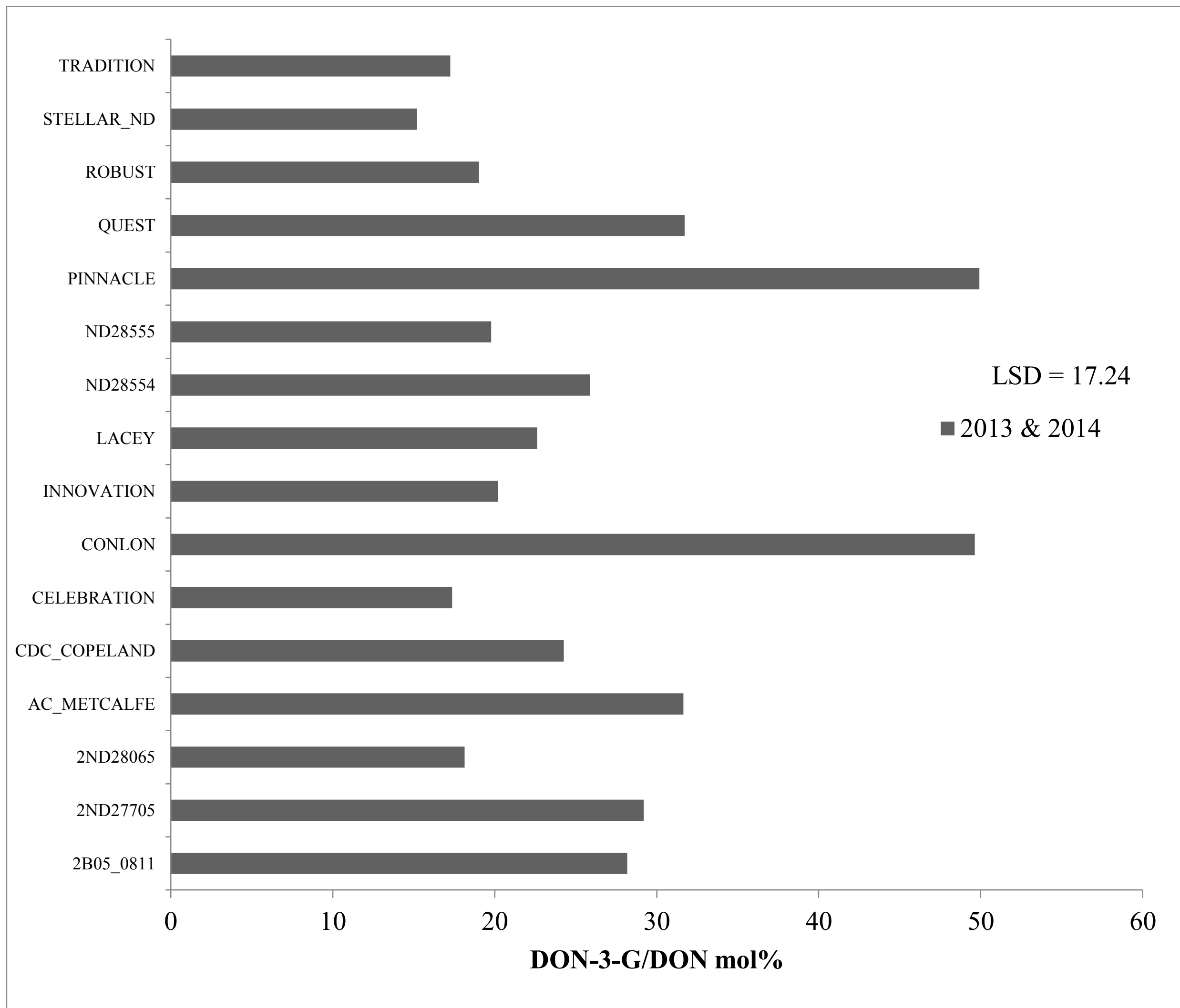

Figure 11. The ratio of DON-3-G/DON in mol% across years (2013 and 2014) of barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014for inoculated barley from the 2013 and 2014 crop years.

The 2013 and 2014 inoculated barley samples were malted, and results of the ANOVA are shown in Table 7. Genotype and genotype*year were significant for DON, DON-3-G, and mol%. DON and DON-3-G were found in 97% and 100% of 2013 malt samples, and in 100% of 2014 malt samples (Table 8). DON and DON-3-G levels were generally declined following

malting. In 2103 the average level of malt DON was 1.84 mg/kg and values ranged from 0.44 to 6.60 mg/kg. The average of the 2014 samples was 4.31 mg/kg, and values ranged from 0.43 to 17.74 mg/kg. The average of DON-3-G for the 2013 malts was 1.53 mg/kg, and values ranged from 0.15 to 4.19 mg/kg. For same genotypes in 2014, the average DON-3-G level was at 2.61 mg/kg. The minimum and maximum levels were 0.84 to 6.38 mg/kg. The average DON-3-G/DON ratio was 88.9 mol% from 2013 inoculated nurseries. The range was 2.7 to 424.5 mol%. For the 2014 year crop samples, the average mol% was 58.5, and values ranged from 20.0 to 159.3 mol%.

The sample ND28555 from 2014 had the highest DON-3-G/DON mol% ratio at 424.5%. The high mol% usually happens with a sample which has low DON level near the LOQ (0.40 mg/kg). In this case, the levels of DON and DON-3-G of this sample were 0.44 and 2.89 mg/kg. The Equation of DON-3-G/DON mol% is showing in the following below:

$$\text{Deoxynivalenol-3-glucoside relative to the deoxynivalenol (mol, \%)} = [(\text{Deoxynivalenol-3-glucoside}_{conc} * M_{Deoxynivalenol}) / (M_{Dexynivalenol\text{-}3\text{-}glucoside} * \text{Dexynivalenol}_{conc})] * 100 \quad (1)$$

Table 7. ANOVA results for DON, DON-3-G, and mol% for corresponding malted barley of barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014.

Variable	Source	df	Sum of Squares	Mean Square	*F*-value	*Pr>F*
Malt DON						
	Year	1	102.4397016	102.4397016	--	--
	Rep(year)	2	4.1121406	2.0560703	--	--
	Genotype	15	318.0317984	21.2021199	11.20	<.0001
	Year*Genotype	15	214.0774234	14.2718282	7.54	<.0001
Malt DON-3-G						
	Year	1	18.74890000	18.74890000	--	--
	Rep(year)	2	11.79235625	5.89617813	--	--
	Genotype	15	39.52229375	2.63481958	5.66	<.0001
	Year*Genotype	15	15.52320000	1.03488000	2.22	0.0305
Malt Mol %						
	Year	1	12218.28375	12218.28375	--	--
	Rep(year)	2	61393.45651	30696.72825	--	--
	Genotype	15	88153.86381	5876.92425	3.97	0.0006
	Year*Genotype	15	85195.82232	5679.72149	3.83	0.0008

Figure 12 shows the relationship between DON and DON-3-G in the 2014 inoculated barley and malted barley samples. Both DON and DON-3-G declined following malting. For example, CDC Copland had the highest DON and DON-3-G levels at 26.65 and 9.56 mg/kg among the *Fusarium* inoculated barley sample from 2014. Following malting, both DON and DON-3-G levels were reduced to 16.90 and 6.18 mg/kg. This result can be seen from Figure 13.

Table 8. Mean deoxynivalenol and deoxynivalenol-3-glucoside averaged across genotypes of malt made from barley grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2013 and 2014.

	Deoxynivalenol				**Deoxynivalenol-3-Glucoside**				**Deoxynivalenol-3-Glucoside/ Deoxynivalenol* mol%**			
	mg/kg				mg/kg				mg/kg			
Year	Samples ≥ LOQ[a]	Average	Minimum	Maximum	Samples ≥ LOQ[b]	Average	Minimum	Maximum	Samples ≥ LOQ	Average	Minimum	Maximum
2013	31	1.84	0.44	6.60	32	1.53	0.15	4.19	31	88.86	2.74	424.53
2014	32	4.31	0.43	17.74	32	2.61	0.84	6.38	32	58.45	20.01	159.33

[a] Limit of Quantitation for deoxynivalenol was 0.4 mg/kg
[b] Limit of Quantitation for deoxynivalenol-3-glucoside was 0.1 mg/kg
* Deoxynivalenol-3-glucoside/ deoxynivalenol should be only for samples with DON ≥ LOQ and DON-3-G ≥ LOQ.

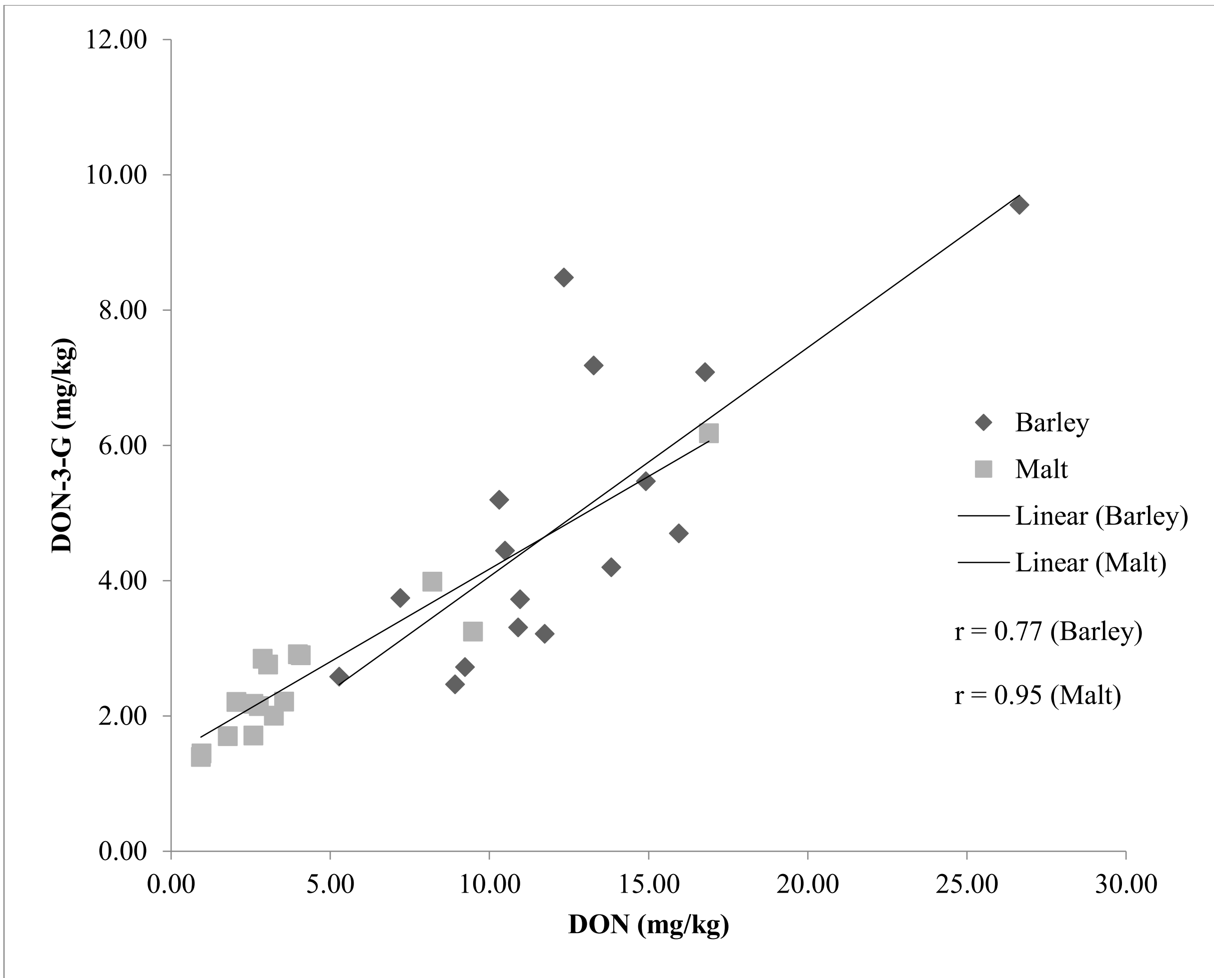

Figure 12. Comparison of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) of genotypes grown in a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2014 (N=16) in North Dakota.

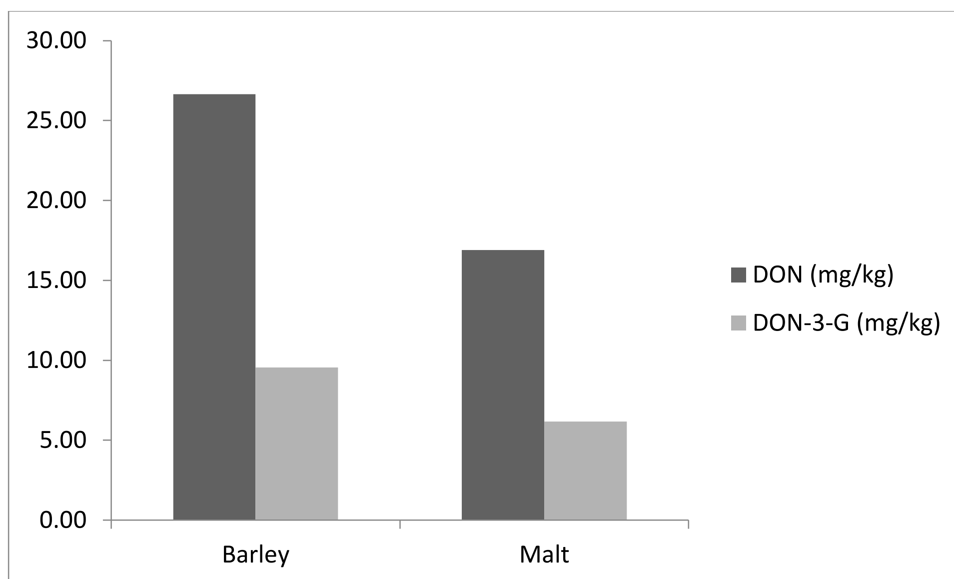

Figure 13. Mean deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) levels in barley and malt of CDC Copland. Barley was obtained from a mist-irrigated nursery inoculated with *Fusarium graminearum* located at the Langdon Research Extension Center in 2014.

5.4. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Commercial Barley, Wheat, and Corresponding Malts

Naturally infected commercial barley samples (N=29, 2013) were obtained from commercial barley buyer in ND. Eighty-three percent of the samples contained the mycotoxin DON that was higher than the limit of quantitation (LOQ= 0.4 mg/kg). Table 9 shows DON and DON-3-G levels on commercial barley samples from year 2013, and commercial wheat samples from 2014. The average DON level of 2013 commercial barley DON was 1.46 mg/kg, and it ranged from 0.45 to 4.82 mg/kg. The masked mycotoxin DON-3-G was detected in 59% of samples at an average level 0.48 mg/kg. The minimum value of DON-3-G detected was 0.1 mg/kg, and the maximum value was 1.25 mg/kg. The ratio of DON-3-G/DON was 19.4 mol%. These results were very similar to the results of Schwarz et al (2014) for commercial barley samples collected from the same region in 2001-2012.

Table 9. Mean deoxynivalenol and deoxynivalenol-3-Glucoside Levels of commercial barley (N=29) and wheat (N=19) samples collected in North Dakota in 2013 and 2014.

	Deoxynivalenol				**Deoxynivalenol-3-Glucoside**				**Deoxynivalenol-3-Glucoside/ Deoxynivalenol* mol%**			
		mg/kg				mg/kg				mg/kg		
	Samples ≥ LOQ[a]	Average	Minimum	Maximum	Samples ≥ LOQ[b]	Average	Minimum	Maximum	Samples ≥ LOQ	Average	Minimum	Maximum
Commercial Barley 2013	24	1.46	0.45	4.82	17	0.48	0.10	1.25	16	19.43	4.87	99.81
Commercial Wheat 2014	16	1.73	0.41	4.80	18	0.25	0.12	0.53	16	12.78	5.28	27.37

[a] Limit of Quantitation for deoxynivalenol was 0.4 mg/kg

[b] Limit of Quantitation for deoxynivalenol-3-glucoside was 0.1 mg/kg

* Deoxynivalenol-3-glucoside/deoxynivalenol should be only for samples with DON ≥ LOQ and DON-3-G ≥ LOQ.

The 2013 barley samples were malted, and the data are shown in Table 10. During malting, DON levels declined while amount of DON-3-G increased. DON and DON-3-G were found in 79% and 100% of malt samples, respectively. The average change in DON was a decrease of 41%, when compared to the original barley. The mean level in malt across all samples was 0.76 mg/kg, but values ranged from 0.40 to 1.78 mg/kg. The average increase in DON-3-G following malting was 314%. The mean value of DON-3-G in malt averaged across samples was 1.03 mg/kg, and values ranged to 0.22 to 2.55 mg/kg. The ratio of DON-3-G/DON was 110.8 mol%. This represents a significant increase from values in barley, and the plant is clearly transforming DON to DON-3-G during malting. Figures14 and 15 show the comparison of DON and DON-3-G levels on commercial barley samples before and after malting. The relationship between DON and DON-3-G and the commercial barley and malted barley samples is shown in Figure 16.

Table 10. Mean deoxynivalenol and deoxynivalenol-3-glucoside levels averaged across samples following malting of commercial barley (N=29) and wheat (N=19) samples obtained in North Dakota in 2013 and 2014.

	Deoxynivalenol				Deoxynivalenol-3-Glucoside				Deoxynivalenol-3-Glucoside/ Deoxynivalenol* mol%			
		mg/kg				mg/kg				mg/kg		
	Samples ≥ LOQ[a]	Average	Minimum	Maximum	Samples ≥ LOQ[b]	Average	Minimum	Maximum	Samples ≥ LOQ	Average	Minimum	Maximum
Commercial Barley 2013	23	0.76	0.40	1.78	29	1.03	0.22	2.55	23	110.79	43.79	206.57
Commercial Wheat 2014	19	8.61	4.44	13.19	19	2.73	2.11	3.88	19	23.85	12.98	40.66

[a] Limit of Quantitation for deoxynivalenol was 0.4 mg/kg

[b] Limit of Quantitation for deoxynivalenol-3-glucoside was 0.1 mg/kg

* Deoxynivalenol-3-glucoside/ deoxynivalenol should be only for samples with DON ≥ LOQ and DON-3-G ≥ LOQ.

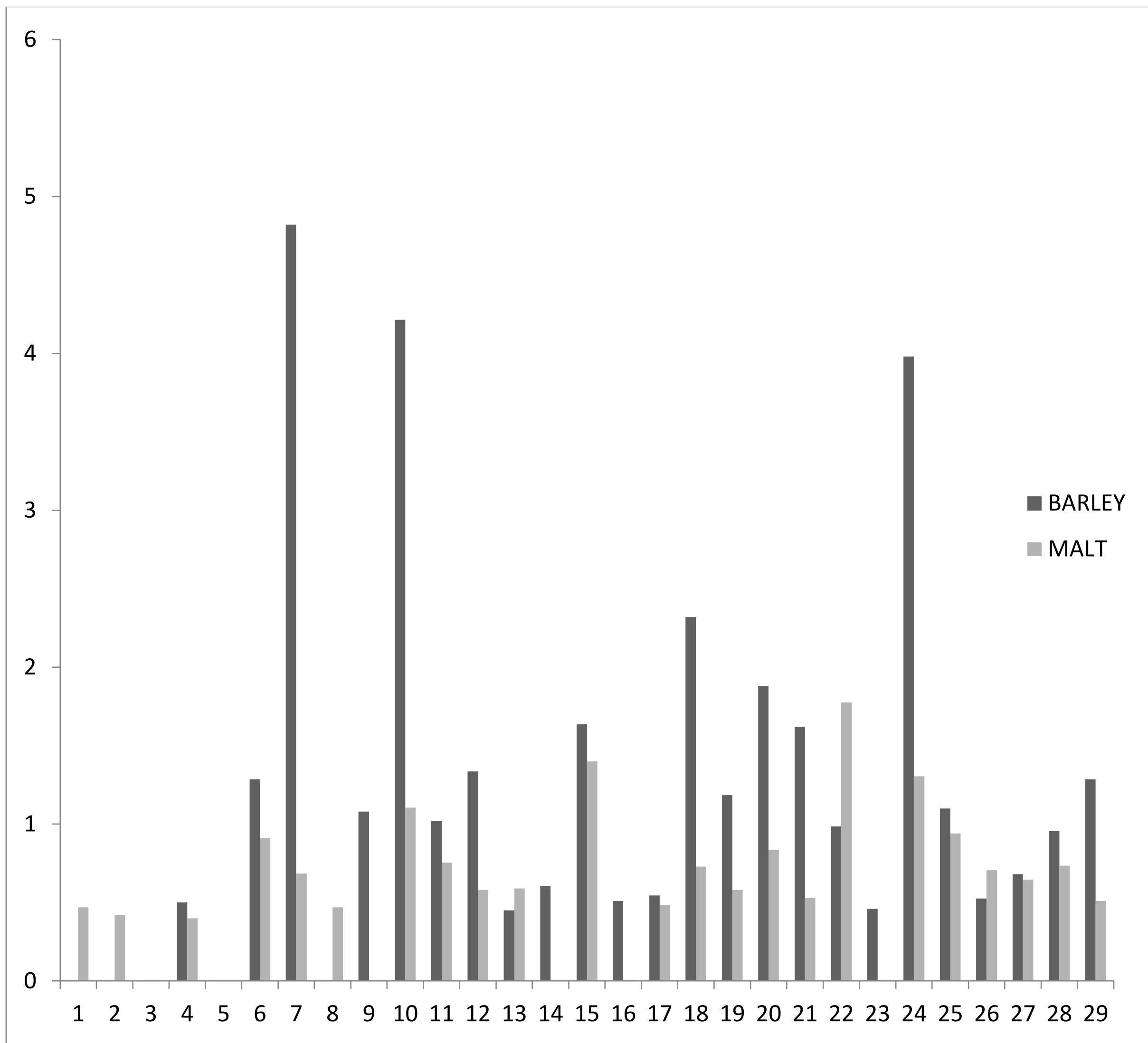

Figure 14. Comparison of deoxynivalenol (DON)levels commercial barley and its corresponding malt samples obtained in North Dakota in2013.

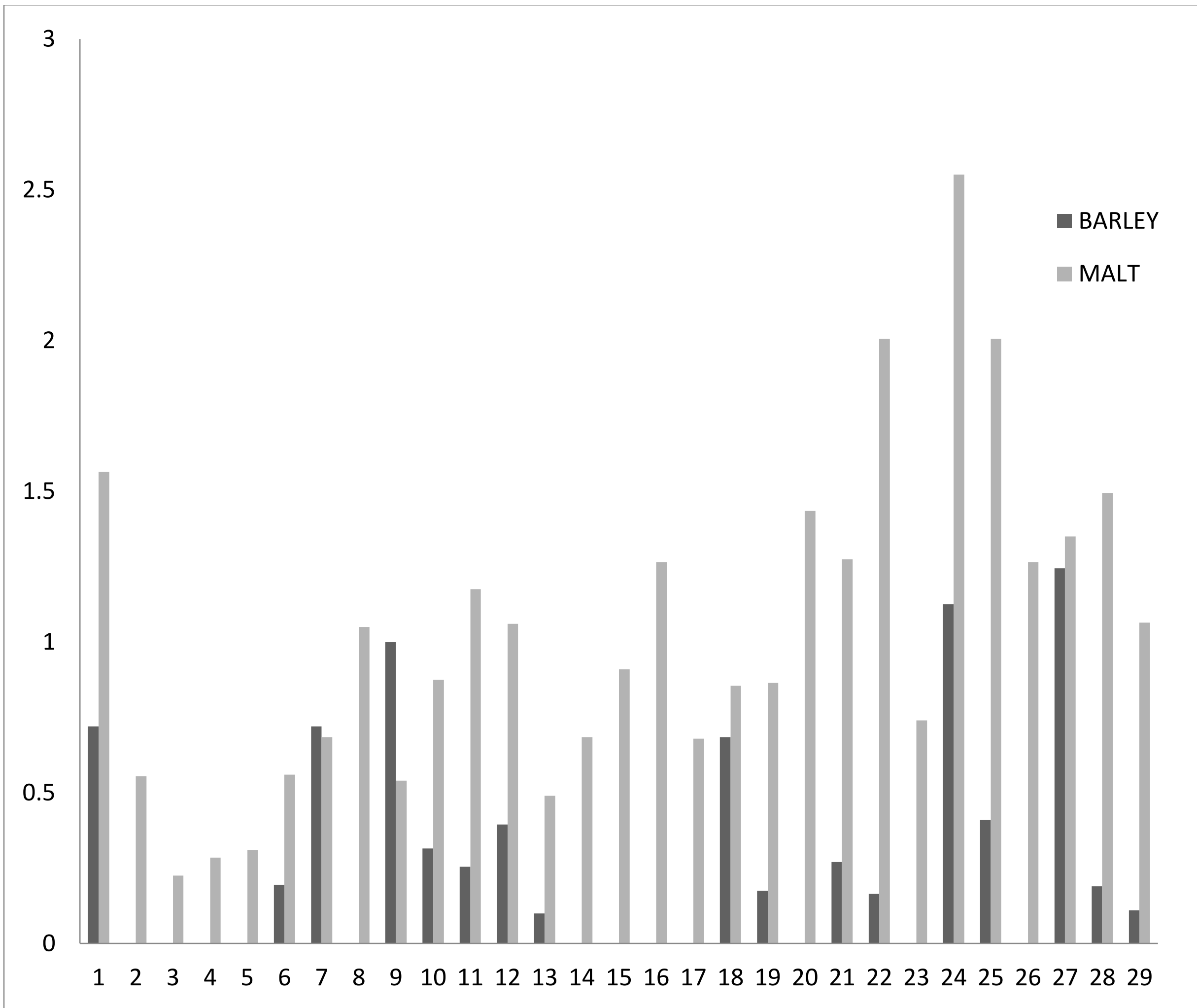

Figure 15. Comparison of deoxynivalenol-3-glucoside (DON-3-G)levels commercial barley and its corresponding malt samples obtained in North Dakota in 2013.

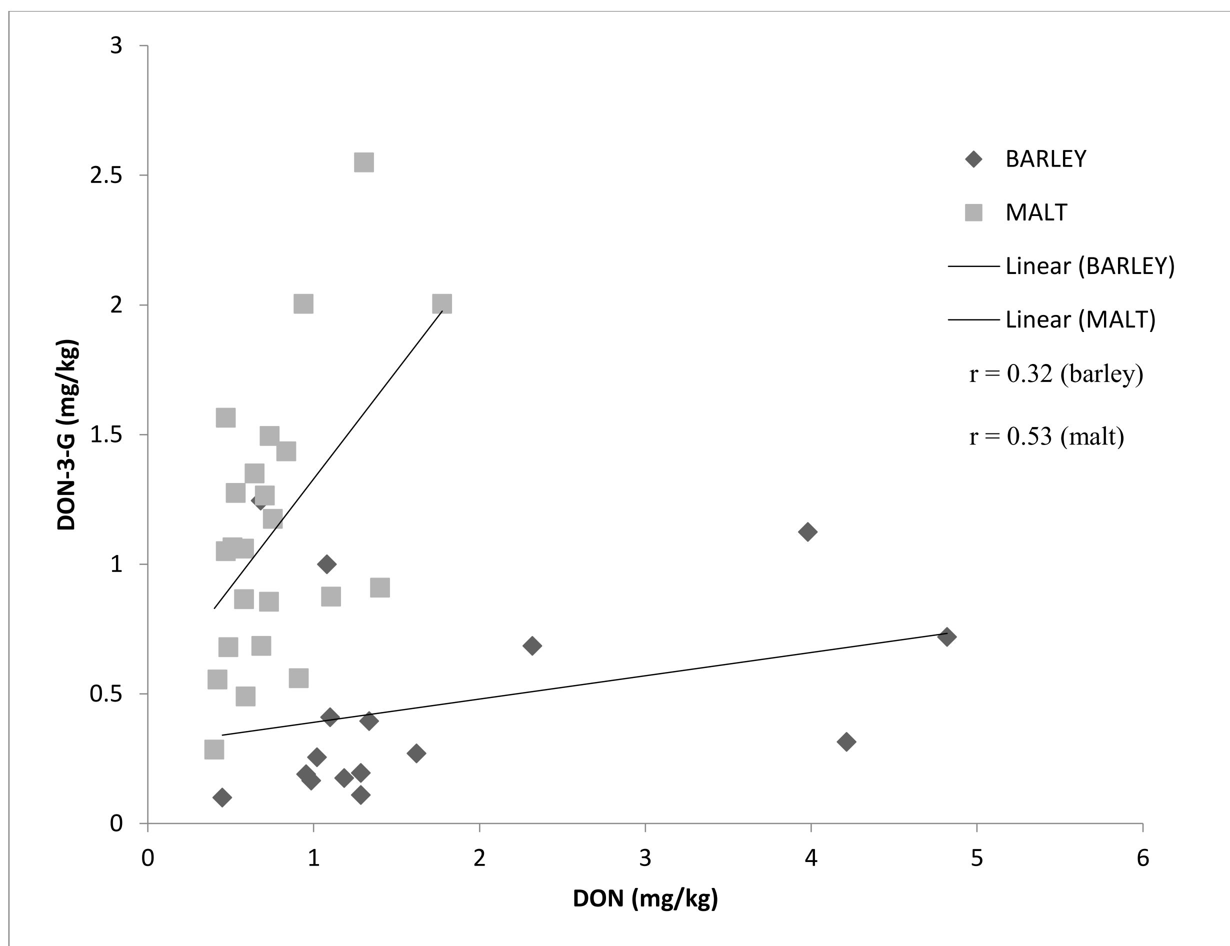

Figure 16. Comparison of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) of 2013commercial barley samples (N=29) obtained from North Dakota before and after malting.

Naturally infected hard red spring wheat samples (N=19) were obtained as part of the 2014 Hard Red Spring Crop Survey (2015). Results of this experiment (Table 9) showed both DON and DON-3-G were found 84% and 95% of obtained wheat samples, respectively. Averaged across samples, levels of DON and DON-3-G were 1.73 mg/kg and 0.25 mg/kg, respectively. DON values ranged from 0.41 to 4.80 mg/kg, and DON-3-G ranged from 0.12 to 0.53 mg/kg. The mean ratio of DON-3-G/DON across samples was 13.2 mol%. This ratio is similar to that previously observed in wheat (Simsek et al., 2013).

Following malting, DON and DON-3-G were found in all wheat samples. Unlike as observed for barley, both the levels of DON and DON-3-G in the wheat malts were increased. This can be seen by comparing Figures 17 and 18. During malting, mean DON across samples increased from 1.73 mg/kg to 8.61 mg/kg. The lowest DON level on malted wheat was 4.44 mg/kg, and the highest level was 13.19 mg/kg. The mean value of DON-3-G across samples in malted wheat increased from 0.25 mg/kg to 2.73 mg/kg, and ranged from 2.11 mg/kg to 3.88 mg/kg. The meanDON-3-G/DON ratio of malted wheat was 23.9 mol%, compared 12.8 mol% for the unmalted grain. The relationship between DON and DON-3-G for the wheat and malted wheat samples is shown in Figure 19.

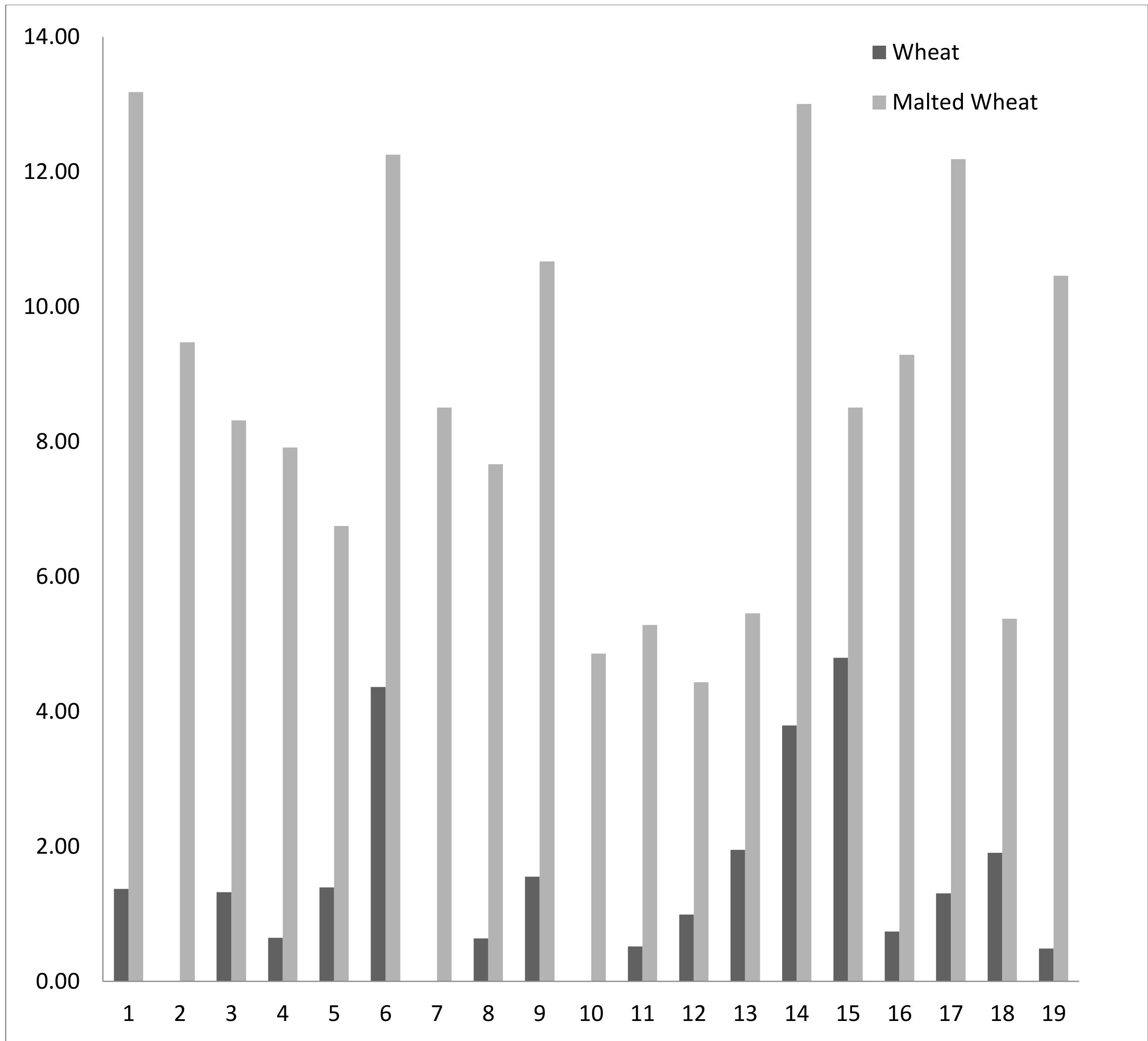

Figure 17. Comparison of deoxynivalenol (DON)levels commercial wheat and its corresponding malt samples obtained in North Dakota in2014.

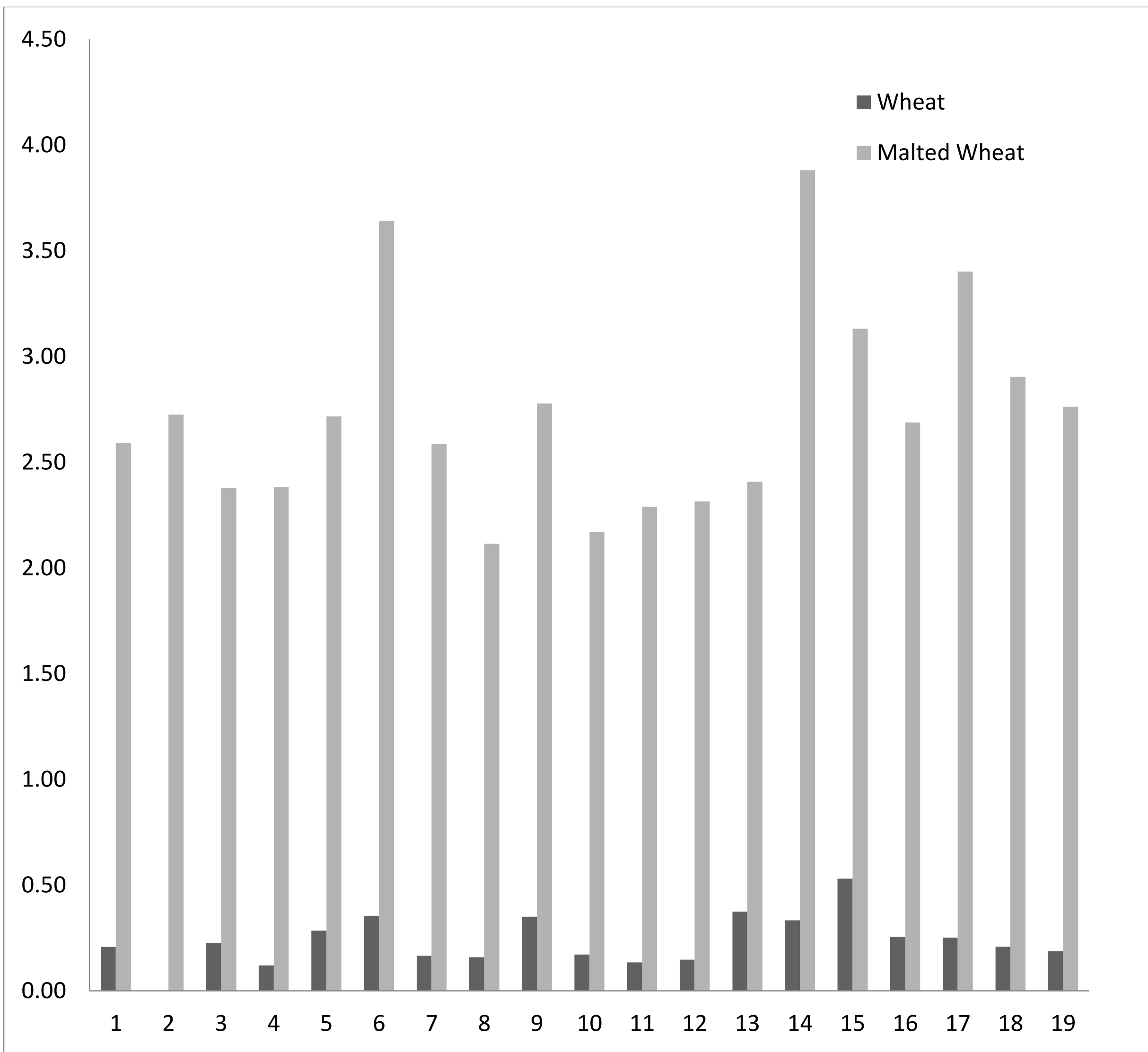

Figure 18. Comparison of deoxynivalenol-3-glucoside (DON-3-G)levels commercial wheat and its corresponding malt samples obtained in North Dakota in2014.

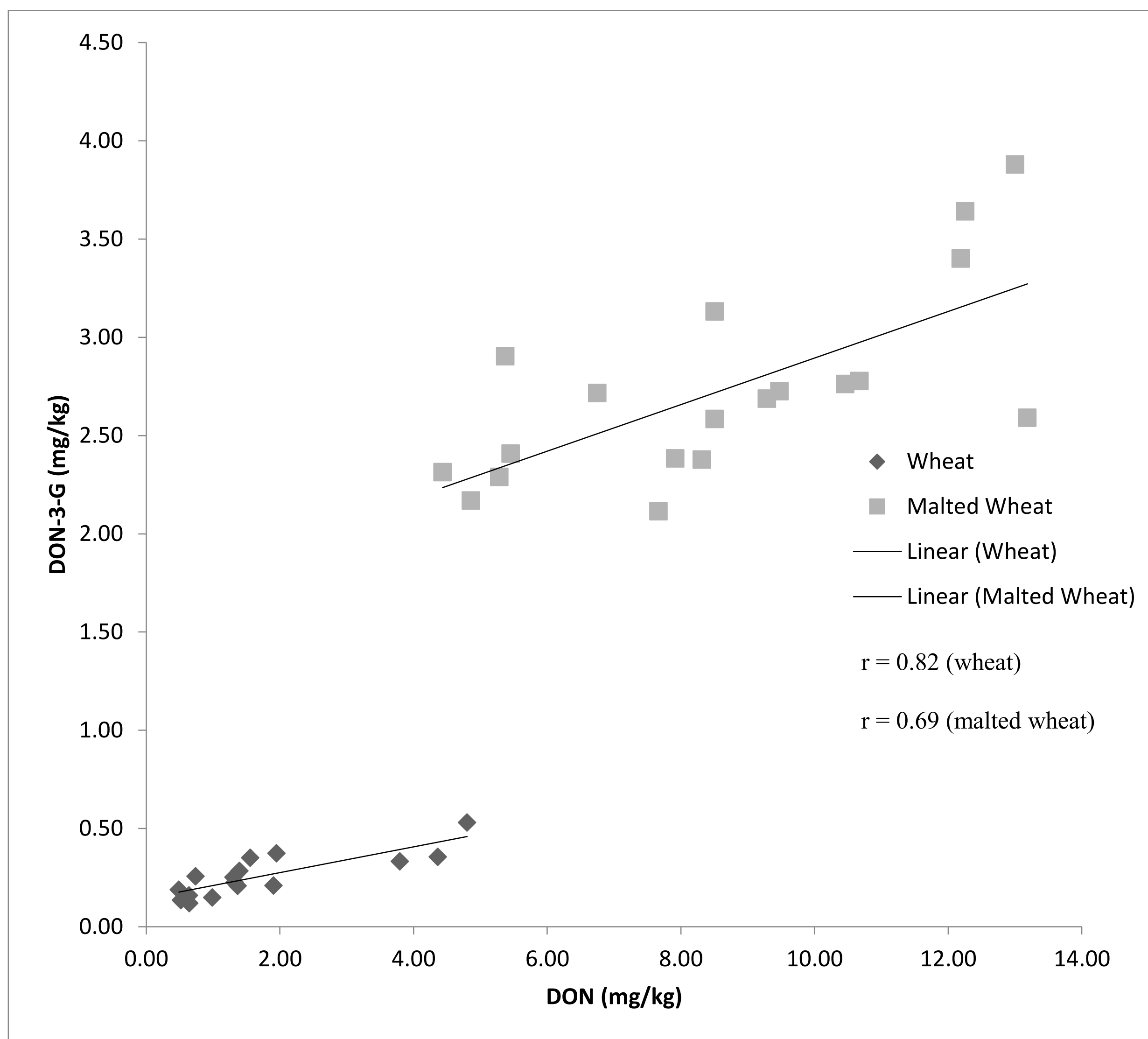

Figure 19. Comparison of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) of 2014 commercial wheat samples (N=14) obtained from North Dakota before and after malting.

At the same time the wheat samples were being malted, a single sample of FHB infected rye sample was tested. Three replicates of this sample were malted. The DON and DON-3-G in the sample before malting were 1.46 mg/kg and 1.73 mg/kg. The DON-3-G/DON ratio in the unmalted grain was 32.5mol%. Following malting, the average DON and DON-3-G values of three replicates increased to 12.24 mg/kg and 2.36 mg/kg, respectively. The DON value was

increased over eight times during malting in this case. DON-3-G also increased, but not as much as DON increased. The average ratio of DON-3-G/DON was 12.8 mol %.

5.5. Deoxynivalenol and Deoxynivalenol-3-Glucoside in Malt Rootlets

In this experiment, the rootlets of barley and wheat malt samples were tested for DON and DON-3-G following kilning. According to Lancova et al. (2007), rootlets contained the highest mycotoxin level of all brewing intermediates. In the current study, rootlets were collected from all commercial barley (N=29) and all commercial wheat samples (N=14). In addition, combined rootlets from 2013 and 2014 malts from the inoculated Langdon nursery(N=108, 111) were also analyzed. A total of four composite rootlet samples were tested. Table 11 shows the test results for these composites. Table 12 compares the rootlet data, to mean values for the respective grain samples.

All four rootlet samples contained DON and DON-3-G. The wheat rootlet sample had the highest contamination with DON (152.70 mg/kg). The rootlet samples from commercial barley (2013) and inoculated barley (2013, 2014) also contained DON at levels of 8.98 mg/kg, 14.73 mg/kg, and 28.42 mg/kg. The average ratio of DON-3-G to DON among these rootlet samples was 6.1 mol%, and ranged from 0.9 to 14.2 mol%.

In the Table 11, the average DON levels of malt ranked low to high from 0.76 mg/kg, 1.78 mg/kg, 4.30 mg/kg, and 8.61 mg/kg were from naturally infected commercial barley (2013), inoculated barley (2013), inoculated barley (2014), and naturally infected commercial wheat (2014), respectively. The same low to high rank was observed for the rootlet samples, 8.96 mg/kg (2013 commercial barley), 14.73 mg/kg (2013 inoculated barley), 28.42 mg/kg (2014 inoculated barley), and 152.70 mg/kg (2014 commercial wheat). Respective to the ranking order,

the DON change of the rootlet samples compared to their malt samples was10.79, 7.28, 5.61, and 16.74. The average of the change was 10.11.

Table 11. Mean deoxynivalenol (DON), deoxynivalenol-3-Glucoside (DON-3-G), and ration of DON-3-G relative to the DON (mol, %) of rootlets obtained from malted barley and wheat.

	DON	**DON-3-G**	**DON-3-G/DON**
	mg/kg		**mol %**
Commercial Barley	8.96	1.97	14.21
Commercial Wheat	152.70	2.10	0.89
Inoculated Barley 2013	14.73	0.79	3.47
Inoculated Barley 2014	28.42	2.61	5.94

Table 12. Comparison of Deoxynivalenol (DON) and Deoxynivalenol-3-Glucoside (DON-3-G) Levels on Malt and its Rootlets Samples.

	Malt		**Rootlet**	
	DON	DON-3-G	DON	DON-3-G
	mg/kg			
Commercial Barley	0.76	1.03	8.96	1.97
Commercial Wheat	8.61	2.73	152.70	2.10
Inoculated Barley 2013	1.78	1.44	14.73	0.79
Inoculated Barley 2014	4.30	2.76	28.42	2.61

6. CONCLUSION

This research provided additional information on the level of DON and DON-3-G in barley and malt. A key component was information on the formation of DON-3-G during malting. DON-3-G increased significantly during the malting process in barley, wheat, and rye. In fact, this was the first study to show the large increase in DON and DON-3-G during the malting of wheat, and also the first study of malting of rye. The increase in DON-3-G is of interest because DON-3-G is not measured by standard methods, the toxicity of DON-3-G in mammals is currently unknown, and there is no TDI limit for DON-3-G. DON-3-G is also known to cross-react with anti-bodies in some DON test kits, thus giving inflated results for DON in malt.

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