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Mitigating Risks of Ochratoxin A Contamination in Oats

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MITIGATING RISKS OF OCHRATOXIN A CONTAMINATION IN OATS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Masters in Agronomy, Horticulture and Plant Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

ARS	Agriculture Research Station
a_w	Water activity
ºC	Centigrade
CFU	Colony Forming Unit
CO_2	Carbon dioxide
DG18	Dichloran 18% Glycerol
DYSG	Dichloran Yeast Extract Glycerol medium
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electron spray ionization
EU	European Union
G	Gram
HPLC	High-Performance Liquid Chromatography
IAC	Immuno affinity Column
KCl	Potassium Chloride
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
O_2	Oxygen

OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
Ppb	Parts per billion
Ppm	Parts per million
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
US	United states
USDA	United States Department of Agriculture
UV	Ultraviolet

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ABSTRACT

MITIGATING RISKS OF OCHRATOXIN A CONTAMINATION IN OAT

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Ochratoxin A (OTA) is a toxic metabolite produced by several species of fungal genera *Penicillium* and *Aspergillus*. The major OTA producer in temperate regions (i.e. U.S., Canada, Europe) is *Penicillium verrucosum*. Oat (*Avena sativa*), like any other cereal grain, can be contaminated with OTA when storage conditions are favorable for fungal growth. The presence of OTA exceeding the European Union maximum limit of 3 ppb in processed food has been reported in some samples of oat-based breakfast cereals from the US. The use of oat genotypes with limited OTA accumulation would be an effective way to reduce risks of OTA contamination in oat based products. The objectives of this study were to: 1) determine the frequently of *P. verrucosum* in oat grain produced in South Dakota, and 2) develop a methodology to screen oat genotypes for OTA accumulation.

Grain samples from twelve oat cultivars grown at five locations in South Dakota from 2014 to 2016 were analyzed for the presence of *P. verrucosum* by direct plating on Dichloran Yeast Extract Glycerol Agar (DYSG) medium. Twenty-three out of the 360 samples (6.3%) evaluated had a very low percentage (0-16%) of kernels with *P. verrucosum* contamination.

Three experiments with oat grain inoculated with *P. verrucosum* were carried out to study the effect of water activity, temperature, isolates of *P. verrucosum*, genotypes and time after incubation on OTA production. All these factors except genotype were found to

have significant effects on OTA production by *P. verrucosum* in oats. A water activity of 0.90, temperature of 22.5°C, and *P. verrucosum* isolate C1136-1 were found to be highly favorable for OTA production in grain and could be used to assess varietal differences in grain OTA accumulation.

Keywords: Oats, Ochratoxin A, genotype, environment, *Penicillium verrucosum,* DYSG

LITERATURE REVIEW

1. Oat

Oats (*Avena sativa*) is a cereal crop member of the Graminae family. It is believed that Western Asia and Eastern Europe are regions where oats originated (Hoffman, et al., 1987). Oldest oat grains from about 2000 B.C. were found in Egypt. Oats were first introduced to North America in 1602 B.C. along with other grains (Gibson, et al., 2002).

Oats are mainly used as forage, human food, and livestock feed. Oat grains consist of a hull (outer covering) and a groat (caryopsis). Hulls are separated from groats by dehulling process. Groats are used by the milling industry to develop oat-based food products, such as oat flakes (rolled oats), steel cut oats (cut groats), oat flour, oat bran, oat cereals, and cereal bars. Hulls are mostly used in ruminant feeds. Some insoluble cellulosic fibers from hulls have also been incorporated into food (Stevenson, et al., 2011).

Oats are a good source of protein, mineral and fiber (Suttie and Reynolds, 2004). Oats are rich in β-glucan, a soluble fiber. Beta glucan have been shown to decrease risks of heart disease, to lower cholesterol, and to prevent type-2 diabetes (Webster, 2011). According to the health claim approved by FDA in 1997, oat grain in diet can lower level of cholesterol, and reduce risks of health problem (LABELING, 1997). Whole oat grains also contain compounds like Vitamin E, phytic acids, phenolic compounds, flavonoids, sterols, and avenanthramides that have antioxidant properties (Peterson, 2001).

Awareness about these health-promoting constituents in oats has increased the consumption of oats in the United States (Marquart, et al., 2001).

Oats can be grown in a wide range of climatic and soil conditions. It can be grown in hot and dry conditions as well as cold climates such as encountered in Canada (Suttie, et al., 2004). According to the USDA-Foreign Agricultural Service, worldwide production of oats was 22,869 thousand metric tons in 2016/17. European Union, Russia, Canada, Australia, United States, and Brazil were major oat producers in 2016/17. A total of 940 thousand metric tons of oats were produced in the United States while 2,610 thousand metric tons were consumed in 2016/17 (USDA, FAS 2016/17). South Dakota, Wisconsin, Minnesota, and North Dakota are the main oat producing states in the United States. South Dakota was the leading oat grain producer in 2013, 2014, and 2016, and ranked second in 2015.

2. Ochratoxin A (OTA)

Ochratoxin A is a secondary metabolite of several fungi of *Aspergillus* and *Penicillium* genera. There are three families of Ochratoxins, A (OTA), B (OTB) and C (OTC). Among them OTA is the most abundant and harmful because it is chlorinated (Abouzied, et al., Steyn, 1995). Ochratoxin A is a colorless to white organic acid with crystalline structure. It gives a green-blue fluorescence when exposed to UV-light. The chemical name of Ochratoxin A is L-Phenylanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-0xo-1H-2-benzopyrane-7-yl) carbonyl]-isocumarin. The molar mass of OTA is 403.815 grams per mole (el Khoury, et al., 2010). The melting point of OTA is high (169°C) and it is not completely destroyed even at 250°C (Boudra, et al., 1995; Redgwell, et al., 2002; Scott, et al., 1984). Hence, OTA is quite heat stable and cannot be

destroyed by conventional cooking and food processing which often involve temperatures in the range 80 to 120°C (Lee, et al., 2015).

2.1. Ochratoxin A Toxicity

Ochratoxin A has been found to be hepatotoxic, carcinogenic, genotoxic, immunotoxic, teratogenic, and neurotoxic in animals (Lucchetta, et al., 2010; Amezqueta, et al., 2012; Hayes,et al., 1974). Studies on human blood from Balkan area suggest that the Balkan Endemic Nephropathy and Chronic Intestinal Nephropathy is associated with the consumption of OTA (Abouzied, et al., 2002; Lee, et al., 2015; Nguyen, et al., 2014; Petkova-Bocharova, et al., 1990; Pfohl-Leszkowicz, et al., 2007).

The Codex committee on food additives and contaminates of the Codex Alimentarius commission of World Health in December 1997 recommended the maximal residual level (MSL) of OTA to be 5 ppb in grains. Consumption of OTA in higher amount through feed and foodstuffs may impact animal and human health. The European Commission and most other countries have regulated the maximum limit for OTA at 5 ppb in unprocessed cereal grains, 3 ppb in processed cereal products (Juan, et al., 2008; Van Egmond, et al., 2004), and 0.5 ppb in infant cereal based food (Beretta, et al., 2002). There are currently no guidelines for OTA maximum limit in food at USA (Kuruc, et al., 2015).

2.2. Food and oat grain contamination with OTA

Contamination with OTA has been reported in several agricultural products such as rice, wheat, maize, oats, barley, grapes, nut, and coffee. Because of its thermal

stability, OTA can also be found in processed food, beer, wine, and infant foods (Czerwiecki, et al., 2002). OTA has also been detected in human urine, blood and breast milk samples from the United States (Nguyen, et al., 2014). Barley and wheat samples contaminated with OTA were detected in the Great Plains region of the United States (Kuruc, et al., 2014). Recent surveys of breakfast and infant cereals from the United States also reported the presence of samples containing OTA at a concentration exceeding the European Union maximum limit of 3ng/g and most of the contaminated products contained oats (Lee, et al., 2015; Nguyen, et al., 2014; Mitchell, et al., 2017). Infants and children are at higher exposure risks to OTA because they have low body weight, consume oat based food in lager fractions, have higher metabolic rate and less ability to detoxify toxins (Mitchelle, et al., 2017; Cappozzo, et al., 2017).

2.3. Ochratoxin A producing Fungi

There are about twenty species of OTA producing fungi (Cabañes, et al., 2010). *Penicillium verrucosum* (also known as *Penicillium viridicatum*), and *Aspergillus nordicum* are the major OTA producing fungi. *Petromyces alliaceus, Aspergillus niger, Aspergillus carrbonarius, Penicillium nordicum, Aspergillus lacticoffeatus, Aspergillus flocculosus, Aspergillus pseudoelegans, Aspergillus cretensis, Aspergillus roseoglobulosus, Aspergillus westerdijkiae, Aspergillus sulphorous, Aspergillus steynii, Neopetromyces muricatus* are other fungi able to produce OTA (Amezqueta, et al., 2012; el Khoury, et al., 2010).

In countries of South America, South Asia, and Africa where climate is hot and dry, *Aspergillus* species are the major OTA producers (Moss, 2002). In temperate countries

such as the United States, Canada, and Europe, where temperatures are moderate, the *Penicillium* genus is the major OTA producer (el Khoury, et al., 2010, Kuruc, et al., 2015).

Grain infestation with OTA producing fungi takes place mostly in the field (Pitt, et al., 2013). Soil and plant debris are major sources of these fungus (Kuruc, et al., 2015).

2.4. Factors affecting Ochratoxin A production

Growth of ochratoxigenic mold and OTA production take place on various feed and food commodities if stored under conditions favoring mold growth and toxin production (MacDonald, et al., 2016). Factors such as temperature, relative humidity, water activity, CO_2, storage history, storage time, type of transit and transit time, type of grains, presence of OTA producing strains, interaction with other fungal micro flora present in the grains influence the production of OTA (Chelack, et al., 1991).

2.4.1. Temperature

Temperature is an important factor affecting fungal growth and OTA production. *P. verrucosum* is more problematic in cool and damp climatic regions. *P. verrucosum* can grow at temperatures ranging from 0 to 35°C (Krogh, 1987). *P. verrucosum* can produce OTA at temperatures ranging from 4 to 31°C in favorable moisture conditions (Axberg, et al., 1997; Krogh, 1987).

Aspergillus ochraceous, the major OTA producer in the tropical areas, can produce OTA at temperatures ranging from 12 to 37°C with the highest production taking place between 24 and 37°C at a water activity of 0.80 (Moss, 2002).

2.4.2. Moisture content and water activity

Grain moisture content (MC) is one of the most important factor affecting fungal growth and mycotoxin production in grains both in the field and during storage. When large volumes of grains are stored in bins, low temperature in winter causes a difference in temperature. Grains near the wall of bins are cold, while grains at the center are still warm. This causes migration of moisture towards the top center of bins. The resulting increase in grain moisture can favor mold growth and toxin production. Alternate conditions occur due to transformation of moisture from warm grains near wall to cool grains inside bins during summer, which causes accumulation of moisture in bottom center of the bins (Figure 5) (Hellevang, 1990).

Penicillium verrucosum is found mostly in grains with MC higher than about 14.5% (MacDonald, et al., 2016). Czaban (2006) evaluated the growth of *P. verrucosum* and OTA production on wheat grains at moisture content ranging from 10 to 30%. Fungal growth and OTA production was detected only in samples with MC ranging from 18 to 22%. Cairns–Fuller (2005) also found no growth *of P. verrucosum* and OTA production below 17-18 % MC.

Water activity or equilibrium relative humidity in food provides a better measurement than MC to determine the critical conditions where spoilage by molds and fungus can occur (Troller, et al., 1978). Water activity is the freely available (unbound) water in food. Water activity can be defined as the ratio of partial pressure of water vapor in the product to that of pure water (Mathlouthi, 2001). Water activity gives a measure of water readily available to fungi. The response to water activity and temperature differs according to the fungal species.

For *P. verrucosum*, optimum water activity for fungal growth and OTA is above 0.80 (Cairns-Fuller, et al., 2005). The water activity range at which *P. verrucosum* can produce OTA is narrower than the range at which it can grow (Lindblad, et al., 2004). Magan (2003) and Cairns-Fuller (2005) reported that OTA contamination by *P. verrucosum* was highest at a water activity of 0.95 and a temperature of 25°C.

2.4.3. Gas composition

Cairns-Fuller (2005) evaluated the effect of gas composition on *P. verrucosum* growth and OTA production in inoculated wheat grains incubated for 28 days at 25°C. At an exposure of 25% CO_2, rate of mycelium growth was inhibited by about 40% at a water activity of 0.95. When exposed to 50% CO_2, mycelium growth rate was inhibited by 75% at 0.95 a_w and by 90% at 0.90 a_w. Hence increased level of CO_2 can inhibit OTA production in grains (Cairns-Fuller, et al., 2005).

2.4.4. Interaction with other contaminants

Grains in storage may contain a wide range of microorganisms. Grains can become contaminated either in the field, from handling devices or during storage. Other fungal species on grains may inhibit *P. verrucosum* growth and OTA production. The abundance of *P. verrucosum* (and OTA formation) on wheat grains is affected by the presence of some other fungal species (Czaban, et al., 2006). It was also found that there is an inverse relationship between the percentage of wheat kernels infested with fungus different than *P. verrucosum* and the abundance of *P. verrucosum* on wheat grains (Czaban, et al., 2006).

Lee (2000) also reported some interactions between *P. verrucosum* and other mycotoxigenic *Fusarium* species on fungal growth and OTA production in wheat grains

(Table 1). In the absence of competition with other fungus, OTA production by *P. verrucosum* was significantly higher in all storage conditions under study. There was an interaction between *Aspergillus ochraceous* and other spoilage fungi for OTA production at two water activity levels and two temperatures (Figure 6) (Lee, et al., 2000). *Aspergillus ochraceous* produced significantly more OTA at water activity 0.95 than 0.995 and temperature 30°C when there was no competition with other fungus (Pardo, et al., 2004).

2.5. Methods to detect the presence of Ochratoxin A producing fungus *Penicillium verrucosum* in grain samples

2.5.1 Plating in selective culture medium

To minimize the impacts of OTA, it is necessary to detect, identify and quantify the presence of OTA producing fungi. Plating in selective and indicative medium has been commonly used to detect the presence of *P. verrucosum*. Hocking (1980) developed Dichloran 18% Glycerol Agar (DG18) as a selective and diagnostic medium for *P. verrucosum*. Frisvad then developed Yeast Extract Sucrose (YES), Dichloran Rose Bengal Yeast Extract Sucrose Agar (DRYES) and Dichloran Yeast Extract Sucrose Glycerol Agar (DYSG) (Cabañas, et al.). Later, DYSG was determined as the best selective and indicative medium for recovery of fungus *P. verrucosum* in grains among YES, DYSG, DRYES and DG18 (Lund, et al., 2003).

Penicillium verrucosum growth on DYSG medium is characterized by terracotta pigmentation on the reverse side of the plate (Figure 7) (Frisvad, et al., 1992). This pigmentation is unique to *P. verrucosum, as o*ther fungal genera and other *Penicillum* species exhibit different growth patterns or colors. Hence this medium can be used for the diagnosis of ochratoxigenic mold *P. verrucosum* in substrates like soil and stored cereal (Frisvad, et al., 1992). The diameter of *P. verrucosum* colony growing around the wheat grain plated in DYSG medium is found to be correlated with count of colony forming unit (Czaban, et al., 2006). Direct plating of grain in DYSG medium is an easy and economical method to estimate infestation of *P. verrucosum* with good precision (Elmholt, et al., 1999).

2.5.2 Molecular techniques

Rapid, sensitive and specific DNA based methods have been developed for the identification of ochratoxigenic fungi. Desired fragment of DNA from the fungus can be isolated. It can be amplified by making several copies of it by Polymerase Chain Reaction (PCR). The amplified DNA fragment helps in detection and identification of the fungal species. Primers specific for the identification of the species are used for the PCR. Most of the primers used are targeting the sequence of DNA responsible for toxin production.

There are various genes encoding for enzymes in the OTA biosynthetic pathway. Gene (*otapks*PN) and a non-ribosomal peptide synthetase gene (*otanps*PN) are two most important genes involved in this process. Non-ribosomal peptide synthetase (*otanps*PN) gene is responsible for the linkage of the phenylalanine moiety to the polyketide, hence it is a key enzyme responsible for biosynthesis of OTA in ochratoxigenic molds (Bogs, et

al., 2006, Harris, et al., 2001). Various PCR methods targeting *otanps*PN genes in *Aspergill*us and *Penicillum* species have been developed to detect and quantify the ochratoxicgenic molds (Bogs, et al., 2006; Czerwiecki, et al., 2002; el Khoury, 2010; Kuruc, et al., 2015; Rodríguez et al., 2012).

Molecular techniques for the detection of toxigenic mold in grain are less time consuming and less laborious than the other methods of fungal identification such as fungal culture (el Khoury and Atoui, 2010, Kuruc, et al., 2015). However, the main drawback of this method is that it cannot discriminate between viable and non-viable fungi (Kuruc, 2014).

2.6. Prevention and control of Ochratoxin A

Because the presence of OTA in food and feed can cause health hazards to human being and animals, it is necessary to minimize the contamination of OTA in food and feed by designing strategies for the reduction, prevention, elimination, and control of OTA.

2.6.1 Harvesting

Grains can get contaminated with *P. verrucosum* during harvesting (Lund, et al., 2003). Harvesting equipment should be well adjusted to prevent breaking of grains, and soil and debris should be picked for reducing chances of contamination of cereal grains with molds (Chelkowski, et al., 1981). It is important to harvest crop when grain MC is optimum, this minimizes the chances of damage during harvesting (Commission, 2003).

2.6.2 Good storage practices

To minimize the risks of infection with OTA producing molds and chances of OTA contamination, grain should be dried rapidly after harvest (Amézqueta, et al., 2009; Magan, et al., 2005). Grain must be dried and maintained at a MC <14% (MC<13.5% in oats) to prevent OTA accumulation in food chain (Magan. et al., 2005, Canadian Grain commission, 2016). Grains can be stored a long time if dried and stored in well-controlled storage conditions; particularly low temperature and moisture to inhibit the establishment of toxigenic fungi and OTA production (Kouadio, et al., 2006). Storage system should have adequate drainage system (Amézqueta, et al., 2009).

Fissures and damages in the hull are easy entry point to grain for fungi. Some damages and fissures in the hulls can occur due to damage by insects, improper harvesting, milling, storage and handling techniques (Axberg, et al., 1998). Cracked, shriveled and broken grains are more susceptible to mold growth and insect infestation than whole grain (Scudamore, et al., 1999).

2.6.3 Decontamination of Ochratoxin A

Detoxification of grains can be an effective method to control intake of OTA (Miraglia, et al., 2002). There are different physical, biological and chemical methods for decontamination of mycotoxins.

About 25% of initial OTA in grains is reduced in removal of bran and hulls (Scudamore, et al., 2003). Some of the physical processes like continuous freezing and thawing, exposure to ultra violet beam or gamma irradiation (2-5 kilo Gay), and ozonization can destroy OTA (Deberghes, et al., 1995; Denvir, et al., 1999).

Some bacteria (*Phenylobacterium immobile, Acinetobacter calcoaceticus, Bacillus licheniformis*), protozoa, yeast (*Trichosporon*, *Rhodotorula* and *Cryptococcus)* and filamentous fungi (*Aspergillus fumigatus*, *A. japonicas, Rhizopus homothallicus*, *R. stolonifera*, *R. oryzae)* can be used to biodegrade, detoxify and absorb OTA (Abrunhosa, et al., 2010).

Chemical methods like treatment of grains with ethanol solution followed by intermittent ultrasonic treatment (Lindner, 1996), alkaline hydrogen peroxide, sodium hydroxide mono-methylamine or ammonium with calcium hydroxide can detoxify OTA (Scott, 1996).

The main disadvantage of these approaches is that the decontaminated products may not always be safe for consumption; some chemical residues and derivatives of chemical reactions may remain in the products. In addition, it is not certain whether there can be complete biodegradation and absorption of OTA in product by microorganisms in all cases. Micronutrient content and palatability of foodstuffs may also be reduced after treatment (Abrunhosa, et al., 2010).

2.6.4 Use of Resistant Varieties

Varietal difference in accumulation of aflatoxin has been reported in crops like rice, cowpea (El-Kady, et al., 1996), peanut (Wilson, et al., 1977), broad bean (El-Kady, et al., 1991), wheat and barley (Axberg, et al., 1998, Axberg, Jansson, et al., 1997).

Madhyastha (1993) studied the effect of wheat and rapeseeds varieties on OTA production by *A. alutaceus*. They found a significant effect of cultivar on OTA accumulation in grains. Time of incubation and glucosamine content affected the OTA

accumulation in seed. Varietal differences in rye, barley and wheat for resistance to *A. ochraceous* invasion and OTA production were also observed (Axberg, 1998).

Axberg (1998) also found that the OTA content in rice cultivars inoculated with *P. verrucosum* differed from less than 1 to 38 ppb. Axberg (1997) found a significant effect of wheat and barley cultivars on OTA concentration in grains that were inoculated with *P. verrucosum.* Ochratoxin A level ranged from 2 ppb to 3400 ppb after 4 weeks of inoculation in barley cultivars. Ochratoxin A accumulation in wheat cultivars ranged from less than 1 to 80 ppb two weeks after inoculation. Their results suggested that OTA accumulation might be function of amylose and protein content in barley and wheat cultivars respectively (Axberg, et al., 1997).

The endeavor of using plant breeding to develop and selecting cultivars with higher resistance to fungal infection and OTA contamination is a sustainable and effective way to prevent the contamination of grains with OTA (Varga. et al., 2010). Chelkowski (1981) also recommended that selection of cultivars resistant to fungal attack be an important method for prevention of OTA contamination. Resistance in initial stage of fungus establishment was found in some wheat varieties (Chelkowski, et al., 1981).

Although genotype was shown to affect the amount of OTA production in wheat, barley, and rice (Axberg, et al., 1997; Axberg, et al., 1998) it is not clear if different oat genotypes respond differently to fungal infection and if the use of resistant oat cultivars could be an effective way to limit the incidence of OTA contamination in oat products.

The overall goal of this project was to determine the effect of genotype on OTA accumulation in oat grain. The specific objectives of this research were to:

1. Determine the frequency of *P. verrucosum* present on oat grain produced in South Dakota,
2. Identify the optimal environmental conditions for OTA production by *P. verrucosum* in inoculated oat grain and develop a methodology to screen oat genotypes for differences in OTA accumulation.

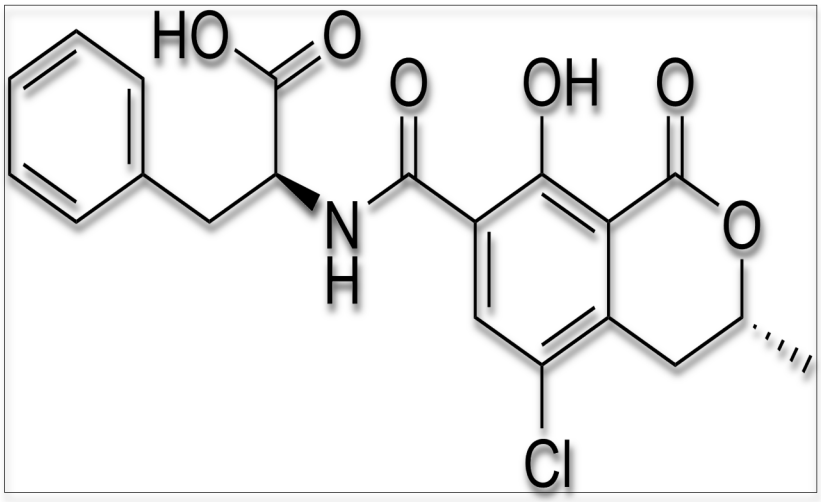

Figure 1. Chemical Structure of Ochratoxin A

Source: http://commons.wikimedium. org/wiki/File: Ochratoxin_A_structure.png

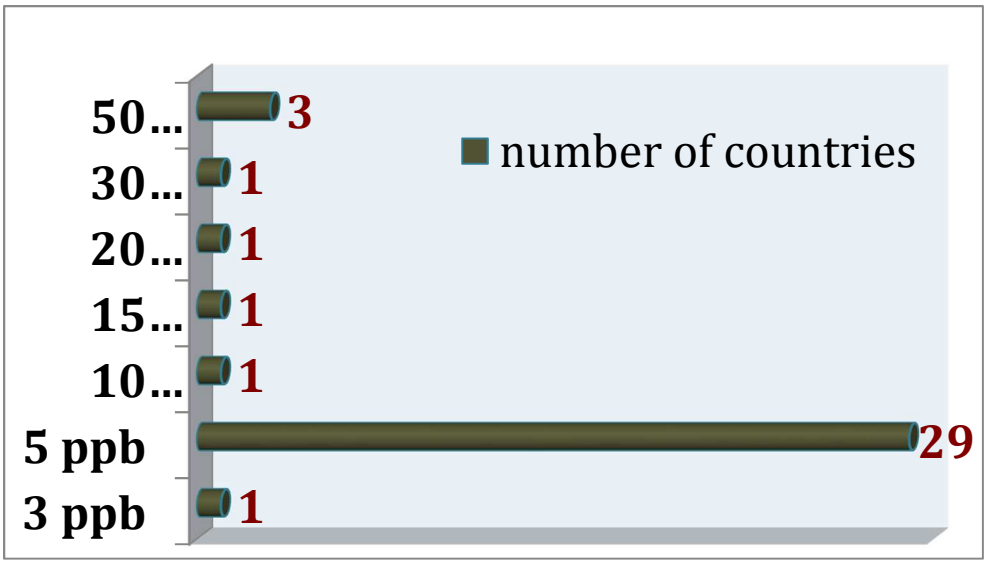

Figure 2. Guidelines for maximum OTA concentration in food around the world (Van Egmond et al. 2004)

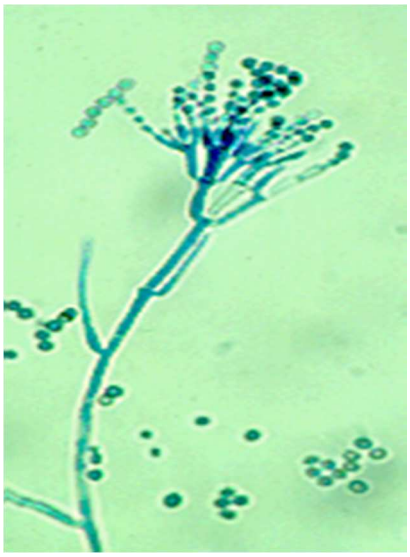

Figure 3. Conidiophores and conidia of P. verrucosum

Source: http://www.mycology.adelaide.edu.au

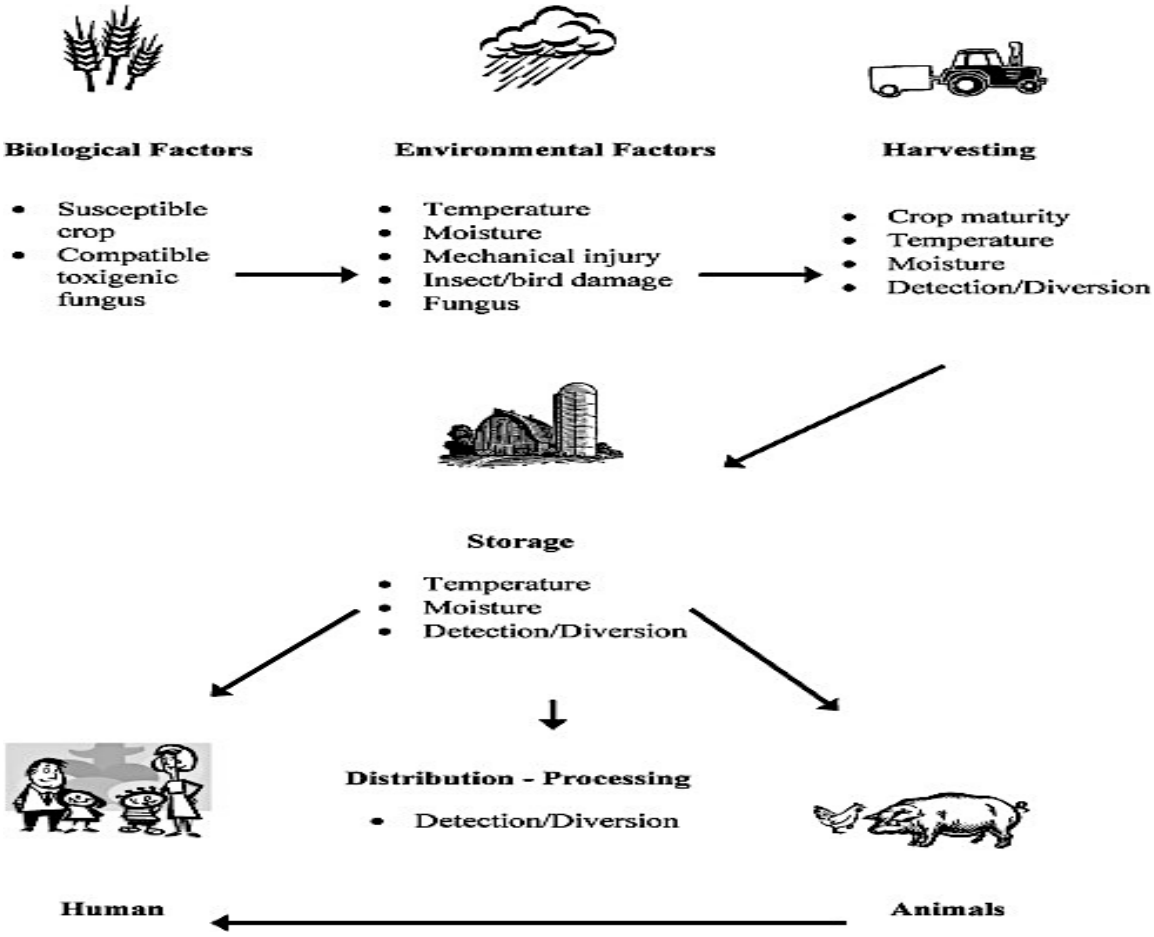

Figure 4. Factors affecting mycotoxin production (Bryden, 2012)

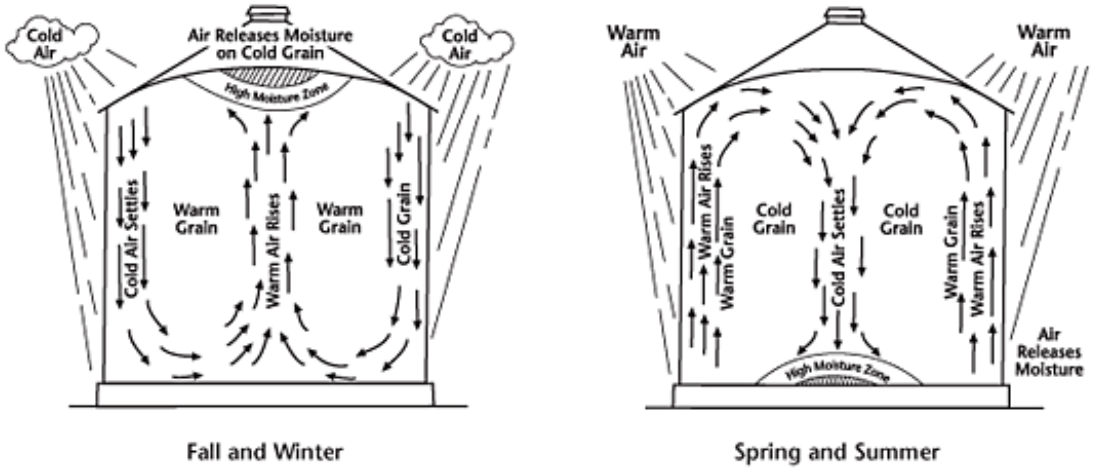

Figure 5. Air movement in stored grains inside bins and formation of high moisture zone

Source: http://www.canolacouncil.org/crop-production/canola-grower%27s-manual-contents/chapter-12-storage,-conditioning/chapter-12

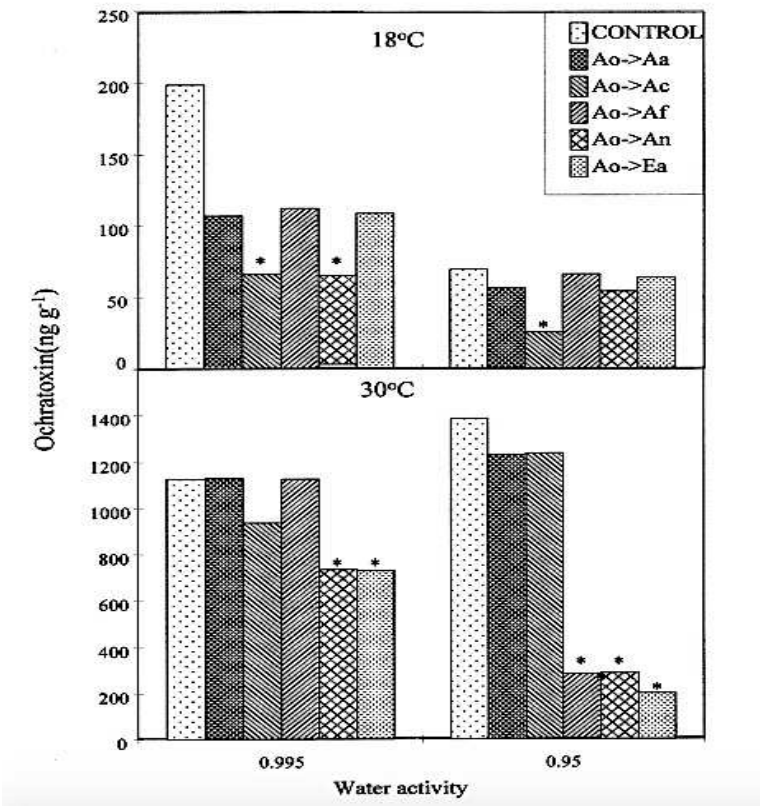

Figure 6. Effect of other fungal contaminants on OTA production on maize grain by Aspergillus ochraceus *at different storage conditions.*

** Indicate statistically significant differences (P=0.05) from*

Ao : Aspergillus ochraceous

Aa : Alternaria alternata;

Ac : Aspergillus candidus;

Af : Aspergillus flavus;

Ag: Aspergillus niger;

Ea : Eurotium amstelodami controls (Lee, et al., 2000).

Table 1. Interaction between *P. verrucosum* and other mycotoxigenic fungal contaminants on Ochratoxin A production on wheat-based medium (Magan, Hope, et al., 2003*)*.

Water activity	Temperature (°C)			
	15		25	
	0.99	0.95	0.99	0.95
P. verrucosum alone	3000 ± 327	1800 ± 645	150 ± 36	3600 ± 409
P. verrucosum + *F. culmorum*	0	10 ± 7	0	0
P. verrucosum + *F. poae*	60	200 ± 18	0	0

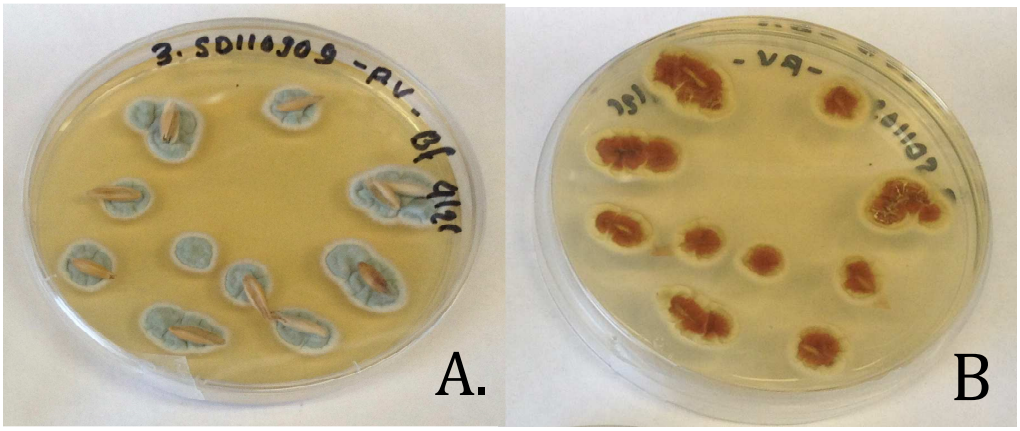

Figure 7. Penicillium verrucosum growing in DYSG media.

A. Penicillium verrucosum growing in oat kernels inoculated with P. verrucosum plated on DYSG medium (averse),

B. Penicillium verrucosum growing in inoculated oat kernels plated on DYSG medium (reverse).

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CHAPTER 2:

Abundance of *Penicillum verrucosum* in Grain Samples from Oat Varieties Commonly Grown in South Dakota

1. Abstract

Ochratoxin A (OTA) may cause toxicogenic effect in humans and animals when consumed in contaminated food products. Oat (*Avena sativa*), like any other cereal grain, can be contaminated with OTA when storage conditions are favorable for fungal growth and toxin production. The presence of OTA exceeding the European Union maximum limit of 3ng/g has been reported in samples of breakfast cereals, cereal based products and infant food collected in the United States. Some of these products are made with or contain oats. South Dakota is one of the main oat producing states in the United States, it is therefore important to determine how frequently *P. verrucosum* is present on oat grains produced in the state. This study was done to evaluate the abundance of *P. verrucosum* in oat grain samples collected in South Dakota. Kernels from twelve oat cultivars grown at five locations in South Dakota from 2014 to 2016 were plated on Dichloran Yeast Extract Sucrose Glycerol Agar (DYSG). *Penicillium verrucosum* was observed on 0.30%, 0.19% and 0.05% of the 2400 kernels tested in 2014, 2015 and 2016 respectively. Overall, twenty-two samples out of the 360 evaluated had a very low percentage of kernels contaminated with *P. verrucosum*. Most of the contaminated samples originated from one site. The samples from this site were likely contaminated prior to harvest.

Keywords: Oat, Ochratoxin A, Genotype, Environment, *Penicillium verrucosum*, DYSG medium.

2. Introduction

Ochratoxin A (OTA) is a naturally produced mycotoxin, occasionally found as a contaminant in a wide variety of agricultural commodities. It is a toxic compound with carcinogenic, nephrotoxic, neurotoxic, immunotoxic, and genotoxic effect in humans and animals (Cabañas, et al., 2008; O'Brien, et al., 2005). Ochratoxin A is also thought as one of the probable cause of nephropathies and urothelial tumors in humans (O'Brien, et al., 2005). International Agency for Research in Cancer (IRAC) classified OTA in Group 2B as a potential carcinogenic substance to humans (Organisation, et al., 1993).

Several species of *Penicillium* and *Aspergillus* genera produce OTA as a secondary metabolite when favorable conditions are encountered (Humans and Cancer, 1993, Samson, et al., 2004). *Penicillium verrucosum* is the most common and significant OTA producer in cold and temperate regions including Europe and North America (Ciegler, et al., 1973; Kuruc, et al., 2015; Sinha, et al., 1986). *Penicillium verrucosum* Diercks, is the only OTA producing species in the *Penicillium* genera (Czaban, et al., 2006, Frisvad, et al., 1991, Pitt, 1987). Between two distinct chemotypes of *P. verrucosum,* Chemotype II is the only one producing OTA in grain and grain-derived products (Frisvad, et al., 1989, Pitt, 1987). Dichloran Yeast Extract Sucrose Glycerol Agar medium (DYSG) was developed as a selective and diagnostic medium for examination of *P. verrucosum* (chemotype II) in soil and grain (Frisvad, et al., 1992, Lund, et al., 2003). Terracotta red coloration in reverse is characteristic of *P. verrucosum* growth on DYSG (Cabañas, et al., 2008; Elmholt, et al., 1999).

Under field conditions, cereal grains such as oats can be infected with *P. verrucosum* present in the soil and on plant residues (Miller, 1995). Other sources of

grains contamination are grain dryers, combines and silos (Hocking, et al., 2006). Proliferation of fungus and OTA contamination in grains can occur mostly during harvesting and post-harvesting (storage and processing) under suitable environmental storage conditions (Miller, 1995; Olsen, et al., 2006).

Optimum growth of *P. verrucosum* occurs at a water activity above 0.85 and a temperature of 20°C (Czaban, et al., 2006; Duarte, et al., 2010). However, *P. verrucosum* can grow slowly in a wide range of environmental conditions, it can thrive even at water activity conditions as low as 0.80 and temperature ranging from 0 to 30 °C (Pitt, et al., 1997; Cabañes, et al., 2010).

Penicillium verrucosum infestation and OTA contamination was detected in some barley and wheat grain samples from the Great Plains region of the United States (Kuruc, 2014). Oats as any other cereal grains can be contaminated with OTA when the storage conditions are favorable for the growth of the fungus. Humans can be exposed to OTA directly through consumption of contaminated cereal and cereal derived products and indirectly through consumption of animal products from animal fed with OTA contaminated feed. The presence of OTA exceeding the European Union maximum limit of 3ng/g has been reported in samples of breakfast cereals including some products made with oats from the United States (Lee, et al., 2015; Nguyen, et al., 2014). South Dakota is one of the leading states for oat production in the United States (USDA, NASS). It is therefore important to determine how frequently *P. verrucosum* is present on oat grain produced in South Dakota.

Several authors reported that the abundance of *P. verrucosum* in grain is a good indicator of possible OTA contamination (Lindblad, et al., 2004; Ramakrishna, et al.,

1991). The objective of this study was to determine the frequency of *P. verrucosum* in oat grain from commonly grown oat varieties in South Dakota.

3. Material and Methods

Oat Grain samples

Three hundred sixty grain samples from South Dakota Crop Performance Testing Oat Variety Trials were examined for the presence of *P. verrucosum*. Samples were collected from five locations in South Dakota (Brookings, South Shore, Beresford, Aberdeen, and Selby) in 2014 and 2015. In 2016, samples were collected from the same locations except that the Winner location was used instead of Beresford. Twelve oat cultivars most commonly grown in South Dakota (Colt, Deon, Goliath, Hayden, Horsepower, Jerry, Jury, Natty, Newburg, Shelby427, Rockford, and Stallion) were sampled. Ten grams of grain samples were collected after harvest from two field replications for each cultivar. The samples were stored at 4°C until they were plated.

Grains plating in DYSG medium

Dichloran Yeast Extract Sucrose Glycerol (DYSG) medium containing glycerol (anhydrous) 220 g/ 174.42 ml.L^{-1} yeast extract 20 g.L^{-1}; agar sucrose 20g.L^{-1}; 150 g.L^{-1}; MgSO4.7H_20 0.5 g.L^{-1}; $CuSO_4.H_2O$ 0.005 g.L^{-1}, ZnSO4.7H_2O 0.01 g.L^{-1}; Chloramphenicol 0.05 g.L^{-1}; Dichloran 0.002 g.L^{-1}, was used for grain plating. Grain infested with *P. verrucosum* produces red color colony and it serves as a marker to know if the grain is infested or not (Lund and Frisvad, 2003).

One hundred whole oat kernels were plated (10 kernels/plate; 10 replicates) for each sample in selective and indicative DYSG medium to determine the percentage of

kernels infested with *P. verrucosum* (Lund and Frisvad, 2003). The kernels were incubated for 7 days at 22.5°C in dark. A total of 36,000 grains were screened for *P. verrucosum* infestation (across all years, locations, and cultivars). The field experimental design was a randomized complete block design with four replications..

Reference fungal strain

United States Department of Agriculture, Agriculture Research Service (USDA, ARS), Peoria, IL kindly provided an isolate of *P. verrucosum* (NRRL 965) used as reference culture in the study. Fungal spores were increased in Potato Dextrose Agar (PDA) and stored with 40% glycerol at -80 °C.

Recording of grain infestation

Presence of *P. verrucosum* was identified by visual comparison between the fungal culture growing around the plated kernel in DYSG and the reference culture. Kernels with *P. verrucosum* had fungal colony with terracotta red coloration in reverse and bluish green colored mycelia in averse of petri dish. To confirm this identification, every developing colony around the kernel was isolated and re-plated in DYSG medium. The number of kernels contaminated with *P. verrucosum* was recorded. Infestation frequency (IF) was evaluated for total of 100 kernels per sample as follow:

IF (%) = (number of oat kernels contaminated with *P. verrucosum*/ total number of kernels evaluated) *100

4. Results

Grain contamination with *P. verrucosum* in oat samples collected from multiple locations in South Dakota was low. Overall 23 out of the 360 samples (6.3%) had at least one kernel infested with *P. verrucosum* and 65 out of 36,000 kernels

evaluated (0.18%) were contaminated with *P. verrucosum* (Table 2). Among the 23 contaminated samples, IF range was from 0 to 16 %.

In 2014, 0.30% of the 12,000 oat kernels screened were infested with *P. verrucosum*. None of the samples from Selby, Beresford or Aberdeen locations were infested with *P. verrucosum.* One sample from South Shore was infested with *P. verrucosum*. The Brookings location had the highest incidence of kernels contaminated with *P. verrucosum* in 2014. At this location, IF ranged from 0 to 5%. Seven out of twelve cultivars had few kernels infested with *P. verrucosum* in at least one of the two field replications. Oat cultivar Stallion grown at Brookings had the highest IF (4.5%) among the cultivars evaluated.

In 2015, 0.19% of the 12,000 oat kernels plated were infested with *P. verrucosum*. Again, no infested kernels were observed on samples from three of the five locations and only one kernel out of the 2,400 plated was infested with *P. verrucosum* in samples from Selby. In 2015, Brookings was again the location with the highest abundance of *P. verrucosum*, though even at that location, IF was less than 1% of the kernels evaluated. Samples from the cultivar Natty at the Brookings location had the highest level of IF (8%).

In 2016, *P. verrucosum* was observed on 0.05% of the 12,000 kernels plated. Samples from three locations did not have any kernels infested with *P. verrucosum*. Although samples from Brookings had the highest frequency of kernels infested in 2014 and 2015, no kernel infested with *P. verrucosum* was detected in 2016. Samples from South Shore and Selby had a few infested kernels (less than 0.2%). The highest level of

IF (0.015%) in 2016 was detected in the grain sample of cultivar Rockford grown at South Shore.

Over the three years of evaluation, cultivars Deon, Selby 427, and Rockford had the highest number of infested samples (four out of thirty evaluated). Cultivars Stallion, Colt, Jerry, Hayden, Natty, and Newburg also had few contaminated samples. The highest IF (16%) was observed in Natty at the Brookings location. None of the samples of cultivar Jury, Goliath, and Horsepower were infested with *P. verrucosum* in the three years of the experiment.

Overall, Brookings location had the highest number of kernels infested followed by South Shore and Selby. A total of 57 kernels among 7,200 kernels tested (less than 1%) were infested with *P. verrucosum* in Brookings location over the three years of evaluation. Five kernels (0.069%) and three kernels (0.042%) among 7,200 kernels tested in samples collected from South Shore and Selby respectively were infested with *P. verrucosum* over the three years of evaluation. None of the samples grown at the locations Beresford, Aberdeen, and Winner were infested with *P. verrucosum*.

5. Discussion

Previous research suggests that the level of infestation by *P. verrucosum* is related to the level of OTA contamination in foodstuff (Ramakrishna, et al., 1991). Therefore, knowledge of abundance of *P. verrucosum* in oat samples from commonly grown varieties in the Upper Midwest can be predictive of the risk of OTA contamination in oats, and oat derived products.

In this study, we found that oat samples collected in three growing seasons in SD (2014-2016) had low infestation of *P. verrucosum*. Frisvad (2003) stated that IF of more than 7% with *P. verrucosum* is a strong indicator of grain contamination with OTA. Only one sample out of the 360 samples tested had more than 7% of kernels infested with *P. verrucosum*. In that sample, 16% of the kernels were infested with of *P. verrucosum* suggesting that improper storage conditions would likely lead to grain OTA contamination for such a sample.

In comparison to other locations, Brookings had the highest incidence of kernels with *P. verrucosum.* This suggests that contamination occurred prior to harvest and that the growing environment may have an effect on oat grain contamination with *P. verrucosum*. The field from which grains are produced is the original source of storage fungi (Miller, 1995). If the grains are infected/infested with *P. verrucosum* and favorable conditions for the fungal proliferation present, OTA production can take place (Cabañas, et al., 2008). In our study, although *P. verrucosum* abundance was very low, improper storage conditions, especially for the samples with more than 7% IF could result in OTA contamination. It is therefore important to maintain proper grain storage conditions to avoid risks of OTA contamination.

The results warranted that oat grain samples should be periodically monitored for *P. verrucosum* and other OTA producing fungi in South Dakota. The *P. verrucosum* isolates recovered from oat grain should be screened for their ability to produce OTA.

Table 2. Abundance of *P. verrucosum* on oat grains at six South Dakota locations

	Kernels infested with *P. verrucosum* Number of kernels infested/total number of kernels plated (average infestation frequency)			
Years / Locations	2014	2015	2016	Total
Brookings	35/2400 (1.46)	22/2400 (0.92)	0/2400 (0)	57/7200 (0.79)
South shore	1/2400 (0.04)	0/2400 (0)	4/2400 (0.17)	5/7200 (0.069)
Selby	0/2400 (0)	1/2400 (0.04)	2/2400 (0.08)	3/7200 (0.041)
Beresford	0/2400 (0)	0/2400 (0)	-	0/4800 (0)
Aberdeen	0/2400 (0)	0/2400 (0)	0/2400 (0)	0/7200 (0)
Winner	-	-	0/2400 (0)	0/2400 (0)
Total	36/12000 (0.3)	23/12000 (0.19)	6/12000 (0.05)	65/36000 (0.18)

Table 3. Infestation frequency with *P. verrucosum* in oat grain samples from 6 South Dakota locations.

	Range in samples infestation frequency (%) (Number of samples infested/total number of samples)			
Years / Locations	2014	2015	2016	Total
Brookings	0-5 (9/24)	0-16 (6/24)	0 (0/24)	0-16 (15/72)
South shore	0-1 (1/24)	0 (0/24)	0-2 (3/24)	0-2 (4/72)
Selby	0 (0/24)	0-1 (1/24)	0-1 (2/24)	0-1 (3/72)
Beresford	0 (0/24)	0 (0/24)	-	0 (0/72)
Aberdeen	0 (0/24)	0 (0/24)	0 (0/24)	0 (0/72)
Winner	-	-	0 (0/24)	0 (0/72)
Total	0-5 (10/120)	0-16 (7/120)	0-2 (5/120)	0-16 (22/360)

Table 4. Number of samples infested with *P. verrucosum* and IF range in oat grain samples from 12 oat cultivars collected at 6 SD locations from 2014 to 2016.

Cultivar	Samples infested /Total number of samples	Average IF %	IF Range
Natty	2/30	0.83	0-16
Stallion	2/30	0.3	0-5
Selby 427	4/30	0.3	0-5
Deon	4/30	0.26	0-4
Jerry	2/30	0.16	0-4
Rockford	4/30	0.16	0-2
Colt	1/30	0.1	0-4
Hayden	2/30	0.03	0-1
Newburg	1/30	0.03	0-1
Jury	0/30	0	0
Horsepower	0/30	0	0
Goliath	0/30	0	0

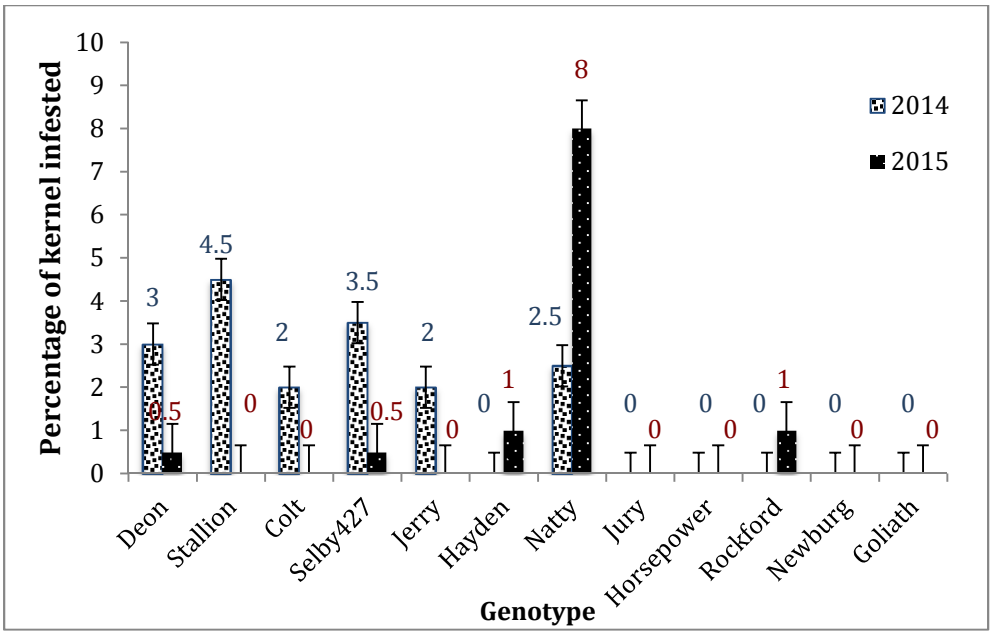

Figure 8. Infestation frequency with P. verrucosum in oat samples from Brookings in 2014 and 2015

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Chapter 3

Development of methodology to screen oat genotypes for Ochratoxin A accumulation

1. Abstract

Ochratoxin A production in oat grain infested with *P. verrucosum* depend on water activity, temperature and the ability of an isolate of *P. verrucosum* to produce OTA. Three experiments with oat grains inoculated with *P. verrucosum* were carried out. In Experiment I, OTA accumulation in *P. verrucosum* inoculated oat grains incubated at 22.5°C at two moisture conditions (0.85 water activity and 25% moisture conditions) were compared. Ochratoxin A accumulation was very low in both conditions, although higher at 0.85 water activity condition. In Experiment II, inoculated oat samples were incubated at three water activity levels (0.75, 0.85 and 0.95) and two temperatures (22.5°C and 27.5°C). Ochratoxin A accumulation was highest at a water activity of 0.95 and at a temperature of 22.5°C, 4 weeks after inoculation. In Experiment III, oat grains were inoculated with three different strains of *P. verrucosum* and incubated at two water activity levels (0.85 and 0.90) and two temperatures (and 22.5°C and 27.5°C) for 10 weeks. Significant differences among the isolates of *P. verrucosum* for OTA production were observed. Higher level of OTA production was observed when inoculated samples were incubated at 22.5°C and with a water activity of 0.90 for the cultivars studied.

2. Introduction

Occurrence of mycotoxins in cereal grains is a major concern for human and animal health worldwide (Liao, et al., 2011). Ochratoxin A is among one of the most prevalent mycotoxin in cereal grains including oats (Czerwiecki et al., 2002; Lund, et al., 2003; Lee, et al., 2015). This toxin is carcinogenic, nephrotoxic, immunotoxic, and embryotoxic to human and animals (Lucchetta, et al., 2010; Amezqueta, et al., 2012; Hayes,et al., 1974). It is produced by several species of genera *Aspergillus* and *Penicillium*. *Penicillium verrucosum* is the main OTA producer in all major cereal grains including corn, oats, wheat, barley and rice in temperate regions (Ciegler, et al., 1973; Kuruc, et al., 2015; Frisvad, et al 1993). Fifty to eighty percent of OTA intake by human is through contaminated cereal-based products (Miraglia, et al., 2002). Ochratoxin A contaminations were detected in corn, barley, oats, wheat, rice, beans, and nuts in United States (Trucksess, et al., 1988; Kuruc, et al., 2014).

Oat is used for feed and food. There is an increase in popularity of oats food consumption because of its health benefits (Mirmoghtadaie, et al., 2009). Oats are rich in nutrients like soluble fiber (beta-glucan), protein, fat and antioxidant (Lapvetelainen, et al., 1994). Even though it is considered a healthy food, it is not spared from the risk of mold infection and OTA contamination. The presence of OTA exceeding the European Union maximum limit of 3ng/g has been reported in some samples of breakfast cereals made with oats from the United States (Lee, et al., 2015; Nguyen, et al., 2014).

Abiotic factors like temperature, water activity and gas composition as well as biotic factors like presence of competitive microflora, aggressiveness of fungal isolates stimulate the growth of toxicogenic mold and OTA production in a substrate (Magan., et

al 2004, Cairns-fuller et al., 2005). Some properties of the cultivars of a crop species also may cause differences in susceptibility/resistance to fungal infection and mycotoxin accumulation (Axberg, et al., 1998). Varietal differences in mycotoxin accumulation are found in crops like rice, cowpea, peanut, broad bean, wheat, and barley (Axberg, et al., 1998; Axberg, et al., 1997; El-Kady, et al., 1996; Wilson, et al., 1977; El-Kady, et al., 1991). Significant effect of wheat and rapeseeds varieties on OTA production by *A. alutaceus* was also found by Madhyastha, 1993. Incubation time and glucosamine content was related to OTA accumulation (Madhyastha et al., 1993). Differences for OTA accumulation between varieties of wheat, rice and barley cultivars inoculated with *P. verrucosum* were observed (Axberg, et al., 1997; Axberg, et al., 1998; Chelkowski, et al., 1981). Their findings suggested that OTA accumulation was function of amylose and protein content present in barley and wheat cultivars respectively (Axberg, et al., 1997). It is also reported that kernels of some wheat, rye and barley varieties have differences in resistance to *P. verrucosum* infestation. Resistance to initial stage of fungus establishment was found in some varieties of wheat and barley (Chelkowski, et al., 1981).

Identification and use of low OTA genotypes, which are resistant to OTA production is an effective way to minimize the risks of OTA contamination in food and feed derived from oats (Miraglia, et al., 2002). Therefore, it is important to know if oat genotypes infested with ochratoxigenic mold respond differentially for OTA production.

In order to identify differences between oat cultivars for OTA accumulation, it is necessary to identify optimum conditions for OTA accumulation in oats infested with *P. verrucosum.* The objective of the study was to determine the optimum temperature and water activity for OTA production by high OTA producing strain of *P. verrucosum* in oats

3. Material and Methodology

Fungal strain

Fungal strains of *P. verrucosum* NRRL 965 was obtained from the United States Department of Agriculture (USDA), Agriculture Research Service (Peoria, IL). *Penicillum verrucosum* Strain C1136-1 and strain C84-2 were provided by Dr. Tom Graefenhan from the Canadian Grain Commission (Table 5). Fungal spores were increased in PDA and stored in 40% glycerol at -80 °C.

Development of moisture absorption curve:

A total of 10 petri dishes with 10-gram sub samples of oat grains in each were prepared. Known amount of water was added to each petri dish. They were kept at 4°C for 48 hours to equilibrate and were shaken periodically. The water activity of each sub sample was measured with an Aqualab Water Activity Meter (Model CX-2, Labcell, UK). Moisture content (MC) of oat grain sub-samples was determined by oven drying method for grains (22 hours at 130°C) (ASABE, 2012). The moisture adsorption curve was developed based on amount of water added and final water activity. This curve was used to determine the amount of water to add to reach the desired water activity level.

Grain infestation and incubation:

Three different experiments were carried out to identify the best conditions for OTA production in oats.

- Experiment I: Comparison of 25% moisture content and 0.85 water activity condition

Two different incubation conditions for OTA production in *P. verrucosum* inoculated oats were evaluated in this experiment.

Oat Grain samples

Grain samples from twelve oats varieties (Deon, Goliath, Horsepower, Jerry, Colt, Jury, Newburg, Rockford, Shelby 427, Stallion, Natty, and Hayden) grown at Miller, SD were used for this study. Samples were stored at room temperature following harvest and at 4°C following sub-sampling. In this design four varieties were from two field replications.

Sterilization of grains with gamma irradiation

Various other fungal species can be present in the non-sterilized grain; some of these fungi may influence the colonization of *P. verrucosum* and OTA production in grains. Literature shows that OTA accumulation when *P. verrucosum* is not the only fungal specie may be different than when it is the only fungal specie (Lee, et al., 2000). Hence it was necessary to sterilize oat samples before inoculation.

Surface sterilization with 2% $NaClO_3$ for 10 minutes could not remove all the microorganisms from the oat kernels. Therefore, samples were sterilized with gamma irradiation at a dose of 12 Kilo Gay at the 3M facility in Brookings, SD. Sterilized samples were stored at 4°C (Pardo, et al., 2004). This dose is supposed to be sufficient to kill all bacteria, fungi and yeasts present in the grain (Ramakrishna, et al., 1991). Gamma irradiation was chosen over autoclaving because it is expected to cause less physical and chemical changes in the grain (Lee, et al., 2000). Gamma irradiated oats retained germination capacity after gamma irradiation.

Inoculation

Penicillium verrucosum strain NRRL 965 obtained from USDA–ARS was used as the inoculum. Spore suspension was prepared from 8 to 10 days old culture of *P. verrucosum* grown in DYSG medium and prepared in 0.15% Tween 80. An haemocytometer was used to count the number of spores in solution. Gamma irradiated grains were inoculated with 4 mL of spore suspension. Concentration of spores was approximately 5000 spores per gram of oats (Axberg, et al., 1998).

Incubation

Condition 1:

Amount of water needed to reach a water activity of 0.85 a_w (according to moisture absorption curve for each sample) was added to sixty grams of inoculated grains. Sixteen jars of inoculated grains were stored inside a humidity chamber with constant temperature and water activity. Humidity chamber was prepared with saturated solution of potassium chloride placed in the bottom of a sealed box. This salt solution can maintain relative humidity of approximately 85% at 20°C (Winston, et al., 1960; Young, 1967). Jars with inoculated grain samples were placed on a perforated stand. Moisture loss was measured every week by weighting the samples (Axberg, et al., 1997). Sterile water was added to bring samples to initial water activity. The chamber/sealed box was placed inside an incubator set at a controlled temperature of 22.5°C. Inoculated grains were incubated for 4, 8 and 12 weeks.

Condition 2:

Initial moisture of the irradiated oat samples was measured using oven dry method for unground grains (ASABE, 2012). Total amount of water needed to obtain a

grain MC of 25% was calculated using the following formula (Tunde-Akintunde, et al., 2007):

Amount of water to be added = $[\frac{100-\%\text{ of intial moisture}}{100-\%\text{ of final moisture content}} - 1] \times \text{grams of seed}$

Moisture content in the sample was adjusted to 25% by adding sterile water and inoculum to jars containing 50 grams of irradiated oat samples. Each sample was inoculated with 4 mL of spore suspension containing spores to obtain 5000 spores per gram of oat grains. Inoculated samples in sixteen jars were incubated at 22.5°C with the lid tightly closed. Samples were weighted every week to monitor loss of moisture.

Sampling

About 12-gram sub-samples were collected in polythene bags at 6, 10 and 14 weeks after inoculation for OTA analysis. Two-gram sub-samples from each polythene bags were mixed in a separate bag representing a condition of incubation and sent for analysis.

- Experiment II: Comparison of six different incubation conditions

Oat Grain samples

Three oat cultivars were used in the study: Goliath, Horsepower, and Shelby 427. Grain samples were collected from two-field replications grown at Miller, SD in 2016. Samples were stored at room temperature following harvest. Each composite sample was sterilized with gamma irradiation at a dose of 12 Kilo Gay at 3M and stored at 4°C following irradiation.

Inoculation and incubation

Forty grams of gamma-irradiated grains were inoculated with 4 mL of spore suspension. Additional water (calculated from moisture adsorption curve) was added to bring inoculated oats to the desired water activity levels. Concentration of spores was 5000 spores per gram of oat grains (Axberg, et al., 1998). A total of thirty-six jars of inoculated grains were incubated at six water activity-temperature conditions. Temperature treatment were 22.5°C and 27.5°C, water activity levels were 0.75, 0.85 and 0.95.

Sampling

Sub-samples for OTA analysis were collected 4 and 8 weeks after inoculation. A 12 -gram subsample from each sample was collected in polythene bags. Two-gram sub-samples from each polythene bags were mixed in a separate bag representing a condition of incubation, which was sent for analysis at Romer labs.

- Experiment III: Comparison of three different isolates of *P. verrucosum* for OTA production at different incubation conditions.

Oat Grain samples

Three oats cultivars (Goliath, Horsepower, and Shelby 427) commonly grown in South Dakota were selected. Foundation seed was used in the study. Samples were stored at room temperature following harvest. Each sample was sterilized by autoclaving for 30 minutes at 121°C.

Inoculation and incubation

Autoclaved oat grain samples (40g) were inoculated with 4 mL of spore suspension from three different strains of *P. verrucosum* (strain NRRL 965, strain C1136-

1 and strain C84-2). Sterile water (amount calculated from moisture adsorption curve) was added to adjust inoculated grain samples to the desired water activity conditions. Final spore concentration was 5000 spores per gram of oat grains (Axberg, et al., 1998). A total of nine jars (three cultivars × three strains) of inoculated grains were enclosed in chambers with saturated salt solution. Incubation conditions were as follows:

- 22.5°C and 0.85 a_w
- 22.5°C and 0.95 a_w
- 27.5°C and 0.85 a_w
- 27.5°C and 0.95 a_w

Sampling

Sub-samples for OTA analysis were taken at 4, 7, and 10 weeks after inoculation. Approximately 12g from each sample was collected in polythene bags. Collected sub-samples were kept frozen at -20°C until analysis.

Quantification of Ochratoxin A with LC-MS/MS

Representative sub-samples were sent to Romer labs Inc., America at Union, Missouri for OTA quantification. Multitoxin mycotoxin detection method by LC-MSMS was used for quantification of OTA.

Extraction

Samples were grinded in a coffee grinder to make fine flour. Five grams of grinded sample was weighted and extracted with 20 mL of 84/16 Acetonitrile/ DI H_2O. The mixture was shaken in a gyratory shaker for 90 minutes; supernatant was filtered through four Whatman No.1 filter papers and 0.45 μm Micro cellulose filter paper using a suction pump.

QC samples (samples contaminated with known concentration of OTA) were used as check samples.

Clean up and purification

After filtration, 200 µL of filtrate was evaporated to dryness in a cuvette. It was reconstituted in 200 µL of Ammonium Formate solution containing internal standards solution. The mixture was mixed with a vortex and transferred to an auto sampler vial. Analysis on LC-MS/MS was done with an injection volume of 40 µL.

Calibration

Different concentrations of mixed standard stock solutions were prepared in a 5 mM Ammonium Formate with 0.1% Formic Acid solution in 50/50 Acetonitrile/DI H_2O. Standard solutions were dried down. They were reconstituted in Ammonium Formate solution containing the Internal Standards for positive mode analysis. Calibration curves were constructed for each analyte by plotting the analyte concentration versus the signal intensity. Plot with concentration of the internal standard versus the area of the analyte divided by the area of the internal standard was used for internal standards.

HPLC separation and MS/MS detection method

Shimadzu HPLC was used for the separation method. Total 40 mL of injected solution was passed through Phenomenox Gemini HPLC C18 column (4.6 x 150 mm, 5 µm) at flow rate of 1.0 mL/min. Column temperature was 40°C. Positive Electrospray ionization (ESI^+) 5nm Ammonium Formate with 0.1% Formic acid in DI H_20 was used as Mobile phase A and Acetonitrile was used as mobile phase B.

Mass-spectrometry was done using Applied Biosystems 3200 QTrap with ESI^+ sources for detection of OTA. Limit of detection (LOD) for Ochratoxin A test was 0.10 ppb.

Statistical analysis of data

Statistical analysis of data was done using R Software Version 1.0.136. Factorial ANOVA to determine significant differences among the factors and identify significant interaction could not be done because collected data for OTA concentration were not normally distributed. Several transformations method (log, ln(1+x), inverse, square and box-cox) could not transform the data to normal. Hence, the R-package "MINQUE" was used to estimate the variance components. Linear mixed models with resampling by jackknife were used to estimate the variance components and fixed effects. Graphs were developed using Microsoft-Excel 2011.

4. Results and Discussion

In this study, three different experiments were carried out in order to identify the best protocol to study varietal differences for OTA production in oat grains when inoculated with *P. verrucosum.*

Grain water activity and moisture content are the most important factors that affect the ability of *P. verrucosum* to grow and produce OTA. Some authors suggested that growth of *P. verrucosum* and OTA production takes place above 0.85 a_w (Carnis-Fuller, et al., 2005). Axberg (1997) detected significant amount of OTA in barley and wheat inoculated at 0.75 and 0.85 a_w. Moisture content of the barley and wheat samples used by Axberg (1997) ranged from 21.4 to 25% at 0.85 a_w. However, oat samples in our study

had MC around 16% at 0.85 a_w. Cairns-Fuller (2005) suggested that the best condition for OTA accumulation was at a grain MC of 25%. There are limited studies explaining the optimum water activity/moisture condition for oats. Hence, in Experiment-I, OTA accumulation in oat grain samples inoculated with *P. verrucosum* was compared between two incubation conditions: 0.85 a_w and 25% MC.

Accumulation of OTA at 25% moisture content at 6 and 14 weeks was lower than the limit of detection, and was 0.2 ppb at 10 weeks. When grain samples were at a water activity of 0.85, OTA accumulation increased with time of incubation. Level of OTA was 0.2, 0.3 and 0.5 ppb at 6, 10 and 14 weeks after inoculation.

Level of OTA detected was low in both conditions. The low OTA accumulation in grains at 25% MC may have been because of an inhibition of *P. verrucosum* growth inside the sealed jar. A decrease in oxygen, a rise in carbon dioxide level from respiring grains and mold, and the possible production of volatile fatty acid in anaerobic condition (sealed containers) could have inhibited mold growth and mycotoxin production (Navarro, et al., 1990, Navarro et al 1993, Navarro, et al., 1999, Navarro, et al., 2005, Moon, et al., 1983, Weinberg, et al., 1993). It is also possible that the environmental conditions i.e. a_w and temperature, evaluated may not have been optimal for OTA production by *P. verrucosum* in oats.

Experiment II was set up to identify the best incubation condition for OTA accumulation in *P. verrucosum* infested oats. Inoculated oat samples were incubated at 22.5 and 27.5°C. Three water activity levels (0.75, 0.85 and 0.95 a_w) were evaluated corresponding to low, medium and high-water activity.

Ochratoxin A accumulation was below the LOD at both temperatures when water activity was 0.75. Cairns-Fuller (2002) also experienced similar results on wheat grains incubated at 0.75 a_w (no growth of *P. verrucosum*). The highest level of OTA accumulation (0.9 ppb) was observed when inoculated grain was incubated for 4 weeks at a RH of 95% and a temperature of 22.5 °C. However, after 4 weeks of incubation, samples at 0.95 a_w started to decay and OTA level was much lower at 8 weeks (0.1 ppb at 22.5 °C and <0.1 ppb in 27.5°C). Because it was suspected that the samples at 0.95 were contaminated with microorganisms other than *P. verrucosum*, the experiment was terminated at 8 weeks of incubation. Some authors stated that some fungal isolates *Aspergillus, Mucor, Alterneria, Penicillium, Rhizopus, Claodosporium* (Abrunhosa, et al., 2002, Varga et al., 2000; Varga et al., 2005) and some bacteria (Skrinjar, et al 1996; Piotrowska et al., 2005) have the ability to decompose OTA. Hence, it is possible that contaminations with other microorganisms occurred and resulted in a reduction in OTA concentration at 0.95 a_w.

At 0.85 a_w condition, OTA (0.4 ppb) was detected at 27.5°C after 8 weeks of incubation. Ochratoxin A concentration was lower than the LOD in all other subsamples that were incubated at 0.85 a_w. The level of OTA detected in our experiment was low in comparison to levels reported in the literature at similar conditions. Axberg (1997) detected significant amount of OTA after four weeks of inoculation in barley (4-3400 ppb) and wheat (150-1200 ppb) grain samples inoculated with *P. verrucosum* and incubated at 20°C with a_w 0.85. Ochratoxin A concentration ranged from 4 to 12 ppb in barley samples and 11 to 590 ppb in wheat samples inoculated with *P. verrucosum* and incubated at 20°C with a_w 0.75 (Axberg, et al., 1998). Cairns-fuller (2005) reported OTA

concentration of 0.25 and 4.20 ppb in wheat grain samples inoculated with *P. verrucosum* and incubated for 28 days at 25°C with water activity level of 0.85 and 0.95 respectively. He also compared three isolates of *P. verrucosum* for OTA production in wheat agar, and found significant differences in their ability to produce OTA (Cairns-Fuller, et al., 2005). Pardo (2005) found significant differences for OTA production by different isolates of *Aspergillus ochraceous* and suggested that inter-specific differences can influence rate of OTA production in cereal grains and other medium.

Detailed information on the ability of the strain of *P. verrucosum* (NRRL-965) used in the study was not available. It is possible that NRRL-965 was not a high OTA producing strain. Experiment III, was developed to study the ability of different strains of *P. verrucosum* to produce OTA at two water activity levels (0.85 a_w and 0.90 a_w) and two temperatures (22.5°C and 27.5°C).

Experiment III showed that all the factors (water activity, temperature, *P. verrucosum* isolate, and inoculation duration) have a significant effect on OTA accumulation in artificially infested oats. Most of two-way, three-way, and four-way interactions of these factors were not significant at the 0.05 significance level. Average OTA production was higher at a water activity of 0.90 than at 0.85 (Figure 10). Grain moisture was approximately 15 and 18 % at 0.85 and 0.90 a_w, respectively. These results are consistent with results from Cairns-Fuller (2005) where levels of OTA production were higher at higher a_w and MC.

Higher OTA production was observed at 22.5°C than at 27.5°C (Figure 11). This result is consistent with results from Czaban (2006), where level of OTA was higher at 21°C compared to 28°C in grains at all moisture conditions (10, 20 and 30%) studied.

Average OTA accumulation was generally highest for cultivar Horsepower and lowest for cultivar Shelby 427 (Figure 12). However, genotype did not have a statistically significant effect on OTA accumulation when a reduced linear model was considered. Yet, the linear mixed model with fixed effect showed that Shelby 427 had a negative estimate value, which supports that it has negative effect on OTA production. The two other cultivars had a positive estimate value with Horsepower having the highest value. Varietal differences in moisture content were observed in initial moisture content in autoclaved grains and final moisture content after water activity adjustment. Cultivar Horsepower seemed to have higher moisture content followed by Goliath and Shelby427 at both 0.85a_w and 0.90 a_w. Grain OTA accumulation was linearly related (R square = 0.95%) to grain moisture content (Figure 13).

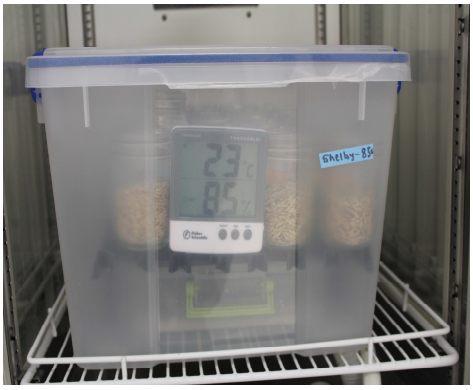
Statistical analysis showed that the strain of *P. verrucosum* can significantly affect the level of OTA accumulation in oat samples (Table 8). Strain NRRL-965 produced the least amount of OTA among the three strains evaluated (Figure 14). Strain C1136-1 produced the highest amount of OTA in all conditions. Greenish-blue fungal growth was visible on oats grain samples inoculated with Strain C1136-1 after 4 weeks of incubation. Differences in fungal mass on oat kernels between the different strains of *P. verrucousm* were visible (Figure 15). Statistical analysis with the linear mixed model indicated that *P. verrucousm* strain C1136-1 had a positive effect while NRRL-965 had a negative effect on OTA production.

Ochratoxin A production in all conditions was found to increase with time of incubation. Statistical analysis showed that duration of incubation was a significant factor

affecting OTA production. Extremely high levels of OTA were detected at 10 weeks of incubation in oats inoculated with strain C1136-1 and C-84 at 0.90 a_w.

Only three oat cultivars were used in this study. It is therefore difficult to conclude if there is a significant effect of the genotype. A larger study testing a large number of genotypes with replication needs to be conducted to conclude if there is a significant effect of the genotype on OTA accumulation when oat samples are inoculated with high OTA producing *P. verrucosum* strains and incubated at optimum condition.

Inoculated oats with 22.5% moisture content



Inoculated oats at 0.85 water activity conditions

Figure 9. Incubation of oat inoculated with P. verrucosum at two moisture conditions.

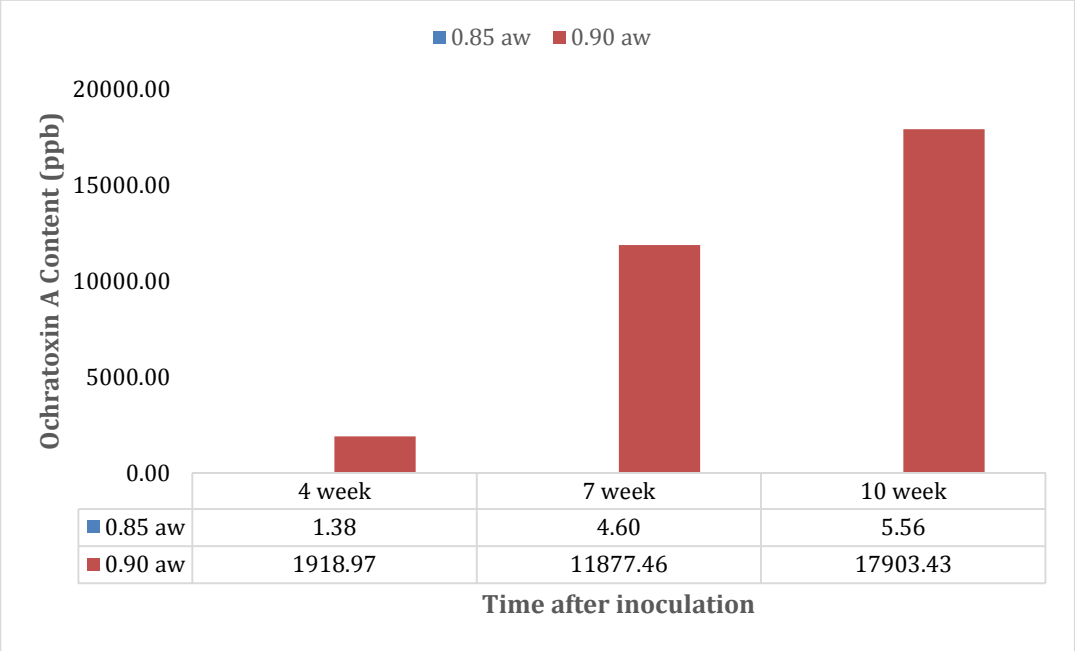


Figure 10. Ochratoxin A concentration in P. verrucosum inoculated oat grains following incubation at two different water activity levels following incubation for 4 ,7 and 10 weeks

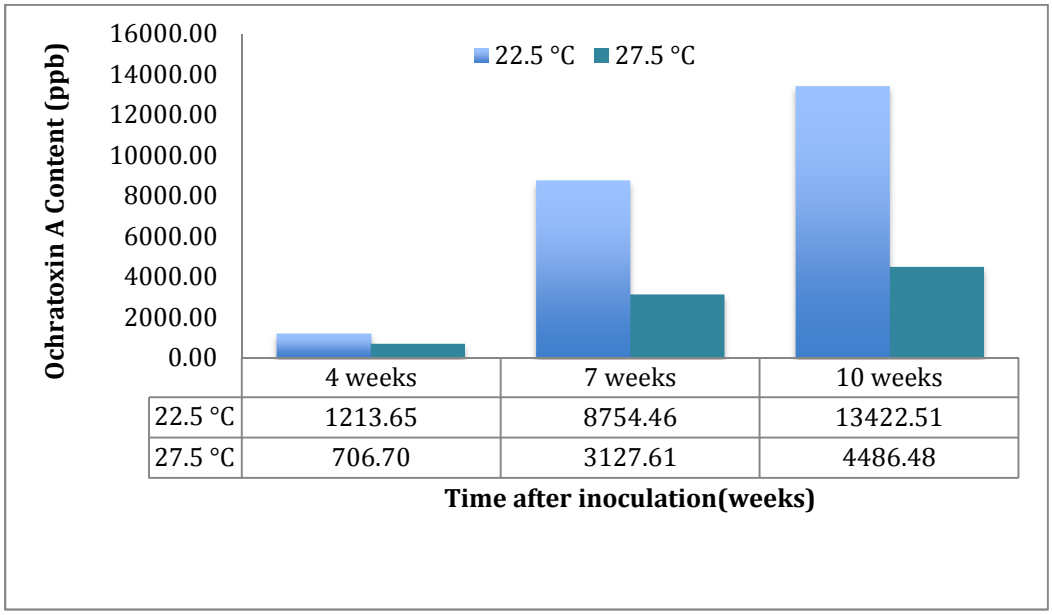


Figure 11. Ochratoxin A concentration in P. verrucosum inoculated oat grains following incubation at two different temperatures following incubation for 4 ,7 and 10 weeks.

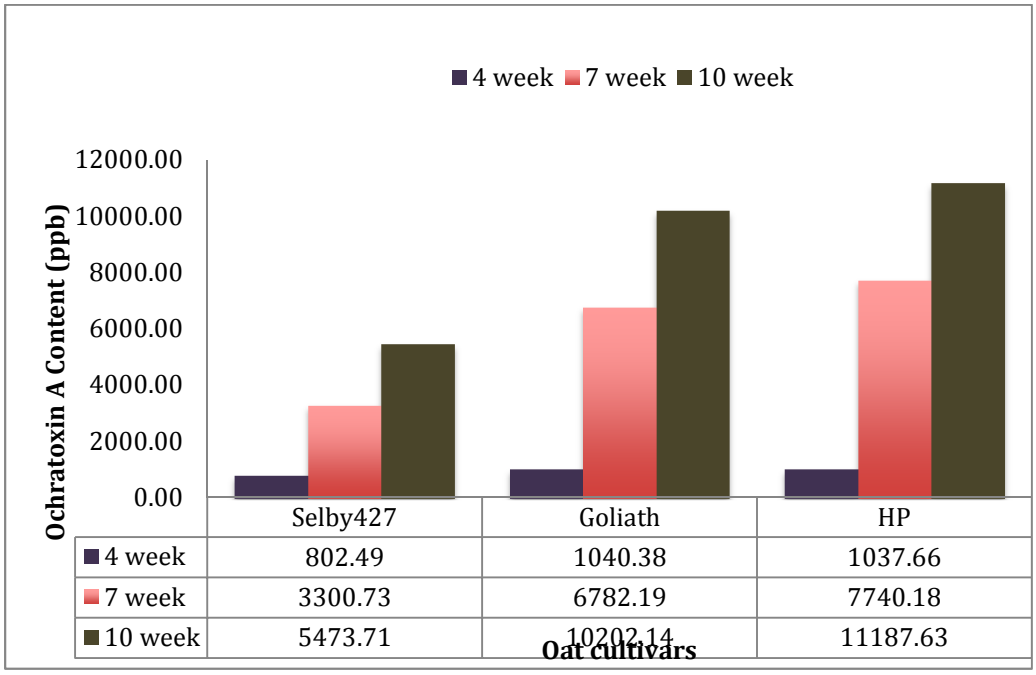


Figure 12. Ochratoxin A concentration in P. verrucosum inoculated oat grains from three cultivars incubated for 4 ,7 and 10 weeks.

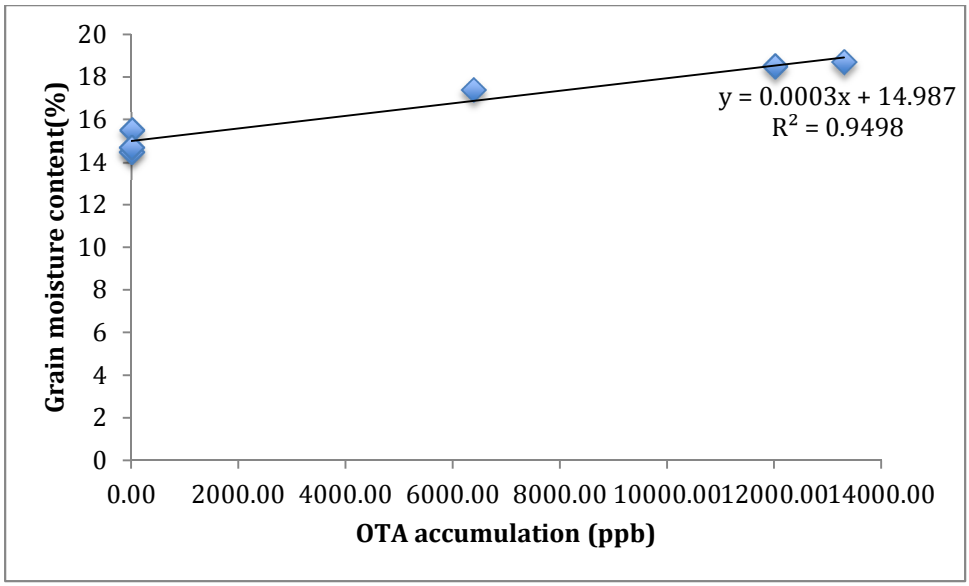
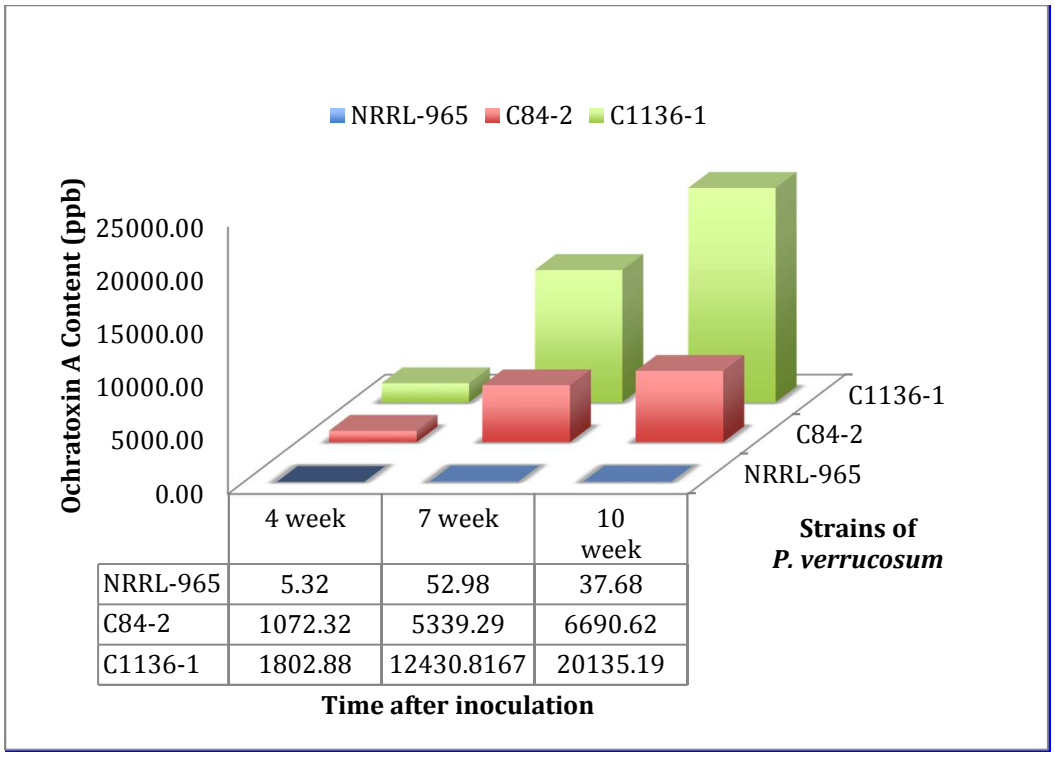


Figure 13. Relationship between grain OTA accumulation and grain moisture content in oat cultivars inoculated with P. verrucosum.



	4 week	7 week	10 week
NRRL-965	5.32	52.98	37.68
C84-2	1072.32	5339.29	6690.62
C1136-1	1802.88	12430.8167	20135.19

Figure 14. Ochratoxin A concentration in oat grains inoculated with three P. verrucosum isolates and incubated for 4, 7 and 10 weeks.

Table 5. Strains of *P. verrucosum* used in the study.

Species	Strain #	Host	Country of origin	Year of isolation	Provided by
Penicillium verrucosum	C1136-1	Barley	Canada	2010	Canadian Grain Commission
Penicillium verrucosum	C84-2	Oats	Canada	2010	Canadian Grain Commission
Penicillium verrucosum	NRRL-965	Biourge	Leuven, Belgium	N/A	USDA/ARS culture collection

Table 6. Ochratoxin A concentration in *P. verrucosum* inoculated after incubation at 22.5°C. for 6, 10 and 14 weeks at two different moisture conditions.

Incubation condition	Time after inoculation (weeks)		
	6	10	14
25% moisture content	<0.1 ppb	0.2 ppb	<0.1 ppb
0.85 water activity	0.2 ppb	0.3 ppb	0.5ppb

Table 7. Ochratoxin A concentration in oat grains inoculated with *P. verrucosum* at a three water activity levels and two temperature conditions.

Temperature	Water activity	Time after inoculation	
		4 weeks	8 weeks
22.5°C	0.95 a_w	0.9 ppb	0.1 ppb
27.5°C	0.95 a_w	0.3 ppb	<0.1 ppb
22.5°C	0.85 a_w	0.1 ppb	<0.1 ppb
27.5°C	0.85 a_w	<0.1 ppb	0.4 ppb
22.5°C	0.75 a_w	<0.1 ppb	<0.1 ppb
27.5°C	0.75 a_w	<0.1 ppb	<0.1 ppb

Table 8. Percentage of variance attributed to the main factors

	Estimate	P-Value	X2.5.LL	X97.5.UL
V(Time)	12975922	0.003469047	3588099.6	22363744
V(aw)	55303626	0.000744601	23113934.9	87493317
V(Temp)	11155936	0.032537435	-535998.8	22847870
V(Genotype)	548524	0.922569416	-2550887.8	3647936
V(Fungal isolate)	31077251	0.003494481	8569076.2	53585425
V(e)	111625359	0.000240202	55673825.6	167576893

Table 9. Linear mixed model estimates for fixed effects.

	Estimate	P-Value	2.5%LL	97.5%UL
Intercept	5241.5	2.53E-06	3733.8	6749.1
Time (4)	-4239.7	3.22E-05	-5892.0	-2587.3
Time (7)	533.5	7.20E-01	-1157.9	2224.8
Time (10)	3706.2	1.37E-04	1976.9	5435.5
Water activity (90)	5350	2.14E-07	4193.5	6506.5
Water activity (85)	-5350	2.14E-07	-6506.5	-4193.5
Temperature (22.5)	2589.8	7.54E-04	1079.8	4099.8
Temperature (27.5)	-2589.7	7.54E-04	-4099.8	-1079.8
Genotype (HP)	1447.8	1.03E-01	-488.7	3384.4
Genotype (Selby427)	-2037.5	1.01E-03	-3273.7	-801.4
Genotype (Goliath)	589.7	7.57E-01	-1408.8	2588.2
Strain (C84-2)	-863.5	5.22E-01	-2942.7	1215.6
Strain (C1136-1)	6231	4.04E-05	3734.7	8727.3
Strain (NRRL 965)	-5367.5	9.02E-07	-6736.7	-3998.2

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CONCLUSION

Ochratoxin A is a toxic fungal metabolite found in a variety of agriculture products including oats. Contaminations with ochratoxigenic molds often take place in the field. When conditions favorable for fungal growth and toxin production occur during storage, OTA can accumulate on grains. Little research has been done to determine how frequently the fungus responsible for OTA production is present on oat grains in the US. Estimation of abundance of ochratoxigenic mold can provide a rough estimation of risks of OTA contamination. South Dakota being one of the leading states in US for oat production, it provides a good area to study the abundance of the main OTA-producing fungal species *P. verrucosum* in oat samples from this region.

The results of the study show that there is very low abundance of *P. verrucosum* in oats produced in South Dakota. Most of the grain samples from the most common oat cultivars grown at multiple locations in South Dakota from 2014 to 2016 were free from *P. verrucosum* spores. Even though the frequency of infestation was small, there is still a risk that ochratoxigenic spores can infect the grains in the field or during storage and produce OTA if favorable environmental conditions occur during storage. Water activity/ moisture content and temperature are the most important factors affecting mold growth and toxin productions. Precautions during grain storage are very important to avoid OTA contamination.

Another promising method to prevent OTA contamination in oat would be to use cultivars resistant to infection with ochratoxigenic mold and OTA contamination. Different factors affecting OTA production by *P. verrucosum* were studied to develop a protocol for comparing oat cultivars for OTA accumulation in their grains. Among the

factors studied water activity was found to have the most influence on level of OTA production in oats. A water activity of 0.90 provided conditions ideal for OTA production. Level of OTA accumulation was found to increase with an increase in grain water activity/moisture and incubation time. Isolates of *P. verrucosum* were found to have differences in their ability to produce OTA in oats and this was the second most important factor. Isolate C1136-1 was the highest OTA producer among the isolates of *P. verrucosum* studied. Time after inoculation and temperature also had significant effect on OTA production. Significant effect of oat genotypes on OTA contamination was not found. However, oat cultivar Shelby427 had lower and Horsepower had higher OTA accumulation in their grains. A more thorough study including a larger number of oat cultivars is necessary to fully determine if cultivars differ in their ability to resist/support OTA production when infected by *P. verrucosum*.

Finally, the present study provides a guide to develop a protocol for oat grain inoculation with *P. verrucosum* and to determine optimal incubation conditions to assess for varietal differences in grain OTA accumulation. The identification of oat cultivars with limited OTA accumulation despite grain infestation with *P. verrucosum* could present a new tool in addition to optimal control of storage conditions to minimize oat grain contamination with OTA and reduce risks of consumers' exposure to OTA.