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FUNGI AND MYCOTOXINS IN FRESH AND ENSILED MAIZE AND THE AFFECTS OF AGRONOMIC PRACTICES, WEATHER CONDITIONS AND SILAGE CHARACTERISTICS ON TOXIN CONTAMINATION

A Thesis in Plant Pathology

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DISSERTATION ABSTRACT

Maize silage, which constitutes a significant portion of the cattle diet, can become contaminated by mycotoxins produced by *Aspergillus, Alternaria, Fusarium* and *Penicillium* and the presence of these toxins has been associated with serious herd health problems (226). The objectives of this work were to study the mycoflora and mycotoxins in maize silage and investigate how agronomic practices, weather conditions and the process of ensiling affect the frequency and concentrations of several classes of mycotoxins. Silage was collected in Pennsylvania from 30-40 dairies during harvest and six months after storage in 2001 and 2002. *Fusarium* and *Penicillium* were the most commonly occurring toxigenic fungi although *Aspergillus fumigatus*, two *Alternaria* species and a novel species, *Penicillium farinosum*, were also present. The most commonly detected mycotoxins were those produced by *Fusarium* species deoxynivalenol (DON) and fumonisins. AAL-TA and AAL-TB, produced by *Alternaria* species, were also present and this is the first report of AAL-TB in maize silage. All four *Penicillium* toxins, cyclopiazonic acid, mycophenolic acid, patulin and roquefortine C were present in both fresh and ensiled maize, although it was previously believed that contamination by these toxins occurs almost exclusively during storage. The majority of silage samples were contaminated by multiple mycotoxins simultaneously and only 4 of 120 samples appeared to be free of detectable levels of contamination. The implications of theses findings are that multi-toxin contamination is likely to be wide-spread in silage and therefore, management strategies to prevent contamination must have a multi-facted approach rather than focusing on a single toxin.

TABLE OF CONTENTS

NTRODUCTION: The economic impact of mycotoxins in maize silage

Maize silage is a whole plant fermented feed that can make up to 60% of the diet of dairy and beef cattle. Silage can become contaminated with a group of fungal-produced secondary metabolites known as mycotoxins, which are harmful to humans and animals at low concentrations. Mycotoxin contamination of foods and feeds has gained increased awareness since the initial discovery of aflatoxin, a hepatotoxin with carcinogenic activity that caused a fatal disease referred to as "Turkey-X" disease in the 1960's (182). Since then, several other classes of mycotoxins have been discovered as contaminants of foods and feeds and many are now federally regulated in terms of levels considered safe for humans and animals (254).

Although it is difficult to assess the economic impact of mycotoxins, it is generally agreed that the cost is substantial (42). In 2003, the Council of Agricultural Sciences and Technology (135) reported that the estimated average annual loss in the United States due to contamination by major classes of mycotoxins (aflatoxin, deoxynivalenol and fumonisins) was $932 million; $6 million of which were attributed to losses in the livetock industry. The costs associated with mycotoxins may result from preventative strategies or costs incurred if contamination does occur. Unsurprisingly, preventative measures against mycotoxins involve monetary expenditures that producers are often willing to make because these options may greatly reduce the overall financial burden compared to losses from contamination. Prevention may include practices such as utilizing maize hybrids with drought, disease, or pest resistance, amending soil to promote plant health and applying pest control to prevent crop damage. In general, practices that promote or ensure plant health are inhibitory towards mycotoxin formation, as many mycotoxigenic fungi are pathogenic, particularly if the plant is stressed or damaged. Before the feed can be safely stored, silo repairs may be necessary to prevent air entry and subsequent mycofloral growth. Bacterial inoculants or additives may be added to ensure that fermentation occurs quickly and uniformly throughout the feed so that fungal growth and toxin production are rapidly inhibited (123, 168).

When mycotoxin contamination does occur, the resulting expenses are dependent on the severity of the problem, which in turn depends on the type of toxin and the level of contamination. In many cases, mycotoxins may cause subtle symptoms that are easily overlooked or difficult to diagnose, resulting in reduced animal health, decreased productivity and financial losses. In cases where symptoms of mycotoxicoses are more obvious, a veterinarian or other animal health specialist is often needed to assess the situation and provide supportive therapy. Serious cases may result in fatalities whereby affected animals need to be replaced. Testing feed for toxins may also incur a significant cost and if contamination is believed to be extensive, the feed may need to be discarded and replaced. A specific concern for dairy

producers is that some mycotoxins, such as aflatoxin and cyclopiazonic acid, are excreted in milk (67, 149). Aflatoxin content in milk is federally regulated (254) and if a situation arises where the contamination is suspected, the milk may require testing and disposal if toxin concentration is above allowable levels.

To date, the majority of practices utilized to prevent or reduce mycotoxins in maize silage are often ineffective or inefficient. As such, animal producers, consumers, federal regulation agencies and other institutions are continually faced with the issues and costs that arise from mycotoxin contamination. A better understanding of what fungi and mycotoxins are problematic in feeds and how environmental conditions impact mycotoxin load is an important first step in developing targeted, effective strategies towards prevention or management of toxins in maize silage.

Chapter 1. Review of mycotoxigenic fungi and mycotoxins.

ABSTRACT

This chapter will briefly review the biology and ecology of mycotoxigenic fungi, genetic potential and environmental stimuli that impact mycotoxin production, the effects of toxin contamination on cattle and current strategies to control mycotoxins. The four mycotoxigenic genera associated with maize are *Alternaria, Aspergillus, Fusarium* and *Penicillium*. To date, biosynthetic genes for several major mycotoxins have been characterized and found to be clustered. Although mycotoxin biosynthesis is dependent on genetic potential, toxin production is also affected by environmental conditions, substrate, interactions with other organisms and other unknown factors. It is often unclear why fungi produce mycotoxins, although some mycotoxins appear to facilitate the pathogenicity or virulence of fungi on maize and other plant hosts. Several reports have found that maize silage is frequently contaminated with multiple mycotoxins but to date, the effects of many of these toxins on cattle are unknown. Current management to prevent mycotoxins in maize relies primarily on planting disease resistant maize hybrids and cultural practices, but in most cases these approaches have had limited success, suggesting that alternative strategies need to be developed or the contemporary methods improved.

1-1 The four major mycotoxigenic genera associated with maize and their reported mycotoxins

Mycotoxigenic fungi associated with maize have often been loosely categorized as either field or storage concerns, although there is considerable ecological overlap, which is dependent on environmental conditions favorable for fungal growth and toxin production. The four major toxin-producing genera are *Alternaria, Aspergillus, Fusarium* and *Penicillium*. *Fusarium* species are infamous pathogens of maize and other cereals and produce some of the most commonly encountered mycotoxins in maize; the trichothecenes and fumonisins (53). Some species also produce the estrogenic compound zearalenone. *Alternaria* species are a cosmopolitan group of organisms, many of which cause major plant diseases. However, they are not usually pathogens of maize, but rather exist as epiphytic saprophytes (30). Both *Alternaria* and *Fusarium* spp. require relatively high water activities and therefore, are unlikely to occur in the low moisture and low oxygen conditions post-ensiling (29, 87). However, members of both genera have been isolated from stored silage that had been exposed to oxygen or was ensiled incorrectly (128, 191). Conversely, several *Aspergillus* and *Penicillium* species will survive in environments with low moisture, oxygen and pH, which is probably why they are often the most frequently encountered mycotoxigenic fungi in silage (31, 49, 154). In the field, members of both genera are opportunistic

pathogens of maize (190), although some *Aspergilli* are overtly pathogenic if environmental conditions are favorable (148). In general however, most species are ubiquitous soil or plant saprophytes that can be isolated regularly from environmental samples (197).

Several species of *Alternaria* have been reported on maize grain and silage, with the most frequently reported species being *A. alternata* and *A. tenuissima*. Isolates of *A. alternata* f. sp. *lycopersici* (renamed *A. arborescens*) (234) are reported to produce the AAL-toxins, which are structurally related to the fumonisins produced by *Fusarium* species (4). High frequencies of *Alternaria* have been reported as epiphytes on cereals in the field, particularly after periods of heavy rainfall (88). *Alternaria* species are also reported as opportunistic pathogens of maize and have been found colonizing bird and insect damaged ears (190).

Fusarium species cause some of the most devastating diseases of maize worldwide (53). Economically, some of the the most important *Fusarium* species on maize include *F. graminearum, F. culmorum, F. sporotrichioides*, *F. verticillioides* and *F. proliferatum*. *F. graminearum* and *F. culmorum* produce deoxynivalenol and other acetylated forms of this type-B trichothecene as well as zearalenone, a compound with estrogenic activity (13, 39). *F. sporotrichioides* produces type-A trichothecenes including T-2 toxin and diacetoxyscirpenol (107), while *F. verticillioides* and *F. proliferatum* produce the type-A, B, C and P series of fumonisins (211). Of the fumonisin analogs, the B series, including fumonisin B_1, B_2 and B_3, are the most important in an agricultural setting. In general, major *Fusarium* diseases of concern are ear and stalk and rots, particularly Gibberella ear and stalk rot (caused primarily by *F. graminearum* teleomorph *Gibberella zeae*) and Fusarium ear rot (primarily *F. verticillioides* teleomorph *Gibberella fujikori* mating population D). Other *Fusarium* species will also cause ear and stalk rot of maize, but in North America, *F. graminearum* and *F. verticillioides* are the most common causal agents (158). Although *F. verticillioides* is a pathogen of maize, it also occurs quite frequently as an endophyte (122). It is often the most common *Fusarium* species encountered in maize and is frequently isolated from asymptomatic tissue (18).

Although they are not usually considered serious pathogens in cooler climates, *Aspergillus flavus* and *A. parasiticus* can cause considerable economic losses in maize in the southern part of the United States and in the north during hot, dry seasons (218, 270). Much of the economic value of the crop is lost to contamination by aflatoxins, a group of acutely toxic and highly carcinogenic compounds produced by both species. In addition to producing aflatoxin, *A. flavus* isolates also produce cyclopiazonic acid, an ability this species shares with several members of the genus *Penicillium* (80, 82). *A. fumigatus* also has

mycotoxin-producing capability but is more often encountered in ensiled maize than in the field. Isolates of *A. fumigatus* from maize silage have been reported to produce fumitremorgens B and C, fumigaclavines B and C, and gliotoxin (49, 154). *A. fumigatus*, which is thermotolerant, is often reported as the most frequently encountered fungus in maize silage, particularly if the feed becomes overheated or is not ensiled correctly (226).

Several species of *Penicillium*, including *P. crustosum, P. commune, P. roqueforti, P. paneum* and *P. expansum* have been isolated from the root rhizosphere of maize and maize silage (8, 120, 127, 191, 197). *P. crustosum* and *P. commune* are closely related and produce cyclopiazonic acid and roquefortine C (80, 236). *P. roqueforti*, like *A. fumigatus* discussed above, is also reported as the most commonly isolated fungus in maize silage (31, 226). Recently, *P. roqueforti* has been divided into three species, now referred to as *P. roqueforti, P. paneum* and *P. carneum*, based on analysis of the internal transcribed spacer regions 236 1 and 2 and the 5.8 S ribosomal gene as well as mycotoxin profiles (31). Of the three species, only *P. roqueforti* and *P. paneum* have been reported in silage (31, 246). Both *P. roqueforti* and *P. paneum* will grow in substrates with low water activity, low pH (to pH 3.0), and low oxygen (4.2% versus atmospheric oxygen content of 21%) content. Both species produce roquefortine C, although *P. roqueforti* also produces PR-toxin and mycophenolic acid, while *P. paneum* produces patulin. *P. expansum*, which has also recently been reported in silage, will also tolerate low pH and low oxygen conditions and like *P. paneum*, produces roquefortine C and patulin (31).

1-2 Mycotoxin production

1-2-1 Genetic control of mycotoxin production

To date, many of the genes responsible for the biosynthesis of major mycotoxins (trichothecenes, fumonisins, aflatoxins) have been identified and studied. Interestingly, thus far the majority of mycotoxin biosynthesis genes identified are clustered within the genome of producing species (33, 203, 279). It is unknown why fungi (and other organisms) cluster genes involved in secondary metabolite production, although it has been suggested that clustering is a mechanism to ensure that these genes are more likely to be passed together by vertical or horizontal transfer (85). In addition, as clustered genes are often highly co-regulated by only a few transcriptional factors, clustering may allow for more finely tuned control of biosynthesis (261).

Currently, 25 genes and 23 enzymatic steps have been identified for the aflatoxin biosynthetic pathways of *Aspergillus flavus*, which produces aflatoxin (AF) B_1 and B_2 and *A. parasiticus*, which produces

AFB_1, AFB_2, AFG_1 and AFG_2 (279). Of the 16 different aflatoxin molecules, only AFB_1, AFB_2, AFG_1 and AFG_2 are present in foods and feeds (27).

The biosynthesis of the type A and type B trichothecenes is also fairly well understood based on work with *F. sporotrichioides*, which produces type A trichothecenes including T-2 toxin (T2) and diacetoxyscirpenol (38) and *F. graminearum* which produces deoxynivalenol (DON), nivalenol (NIV) and other acylated forms of these molecules (34). Type A and B trichothecens differ at C-8 of the 12,13-epoxy-trichothec-9-ene skeleton, where type B molecules have a keto group at this position and type A compounds often have a hydroxyl or acetyl group substitution (28). Approximately ten to twelve genes make up the core trichothecene cluster in *F. graminearum* and *F. sporotrichoides* respectively but an additional three genes located elsewhere in the genome are also involved in the 15 enzymatic steps of biosynthesis (33).

The fumonisin cluster appears to consist of at least 15 concurrently expressed genes within a 75-kb region (203). Work is ongoing to determine the function of these genes and to identify additional factors involved in regulating the expression of genes within the cluster (77).

The biosynthesis of the AAL-toxins appears to be remarkably similar to that of the fumonisins (38) suggesting that the genes responsible for AAL production may be clustered and similar to those present in *F. verticillioides* and *F. proliferatum*. Currently, little is known about the genes responsible for AAL production (147), although *A. alternata* mutants, deficient in the ability to produce the toxin, have been generated by restriction enzyme mediated integration (REMI) (7). In a similar respect, few of the genes responsible for production of *Penicillium* mycotoxins, including cyclopiazonic acid, patulin, mycophenolic acid and PR-toxin have been elucidated, although the biosynthesis of some of these toxins has been studied (104, 175, 242, 272). Proctor *et al* (202), in a study on sesquiterpenoid biosynthesis of *P. roqueforti*, isolated *Ari1*, a gene thought to be responsible for production of aristolochene, a precursor to PR-toxin. To date, many of the genes responsible for patulin are also unknown, although a gene encoding 6-methylsalicylic acid synthase, a polyketide synthase involved in patulin biosynthesis, has been isolated from *P. patulum* and *P. urticae* (24, 264). However, there have been few recent studies on characterizing additional genes responsible for the biosynthesis of *Penicillium* toxins.

1-2-2 Mycotoxin production in maize grain and silage

Fungal occurrence and mycotoxin production in maize are affected by many external factors, including weather conditions, cultural practices and interactions with other microorganisms. Unsurprisingly weather conditions are often the delineating factor that results in toxin contamination of maize, although

other factors, such as maize hybrid, plant maturity, insect damage, preceding crop, tillage system, soil fertility and other variables may also significantly affect toxin production.

Aflatoxin contamination of maize is often common during sustained periods of high temperature and humidity (148) during the growing season. *A. flavus* will infect the silk in these conditions and then spread down the silk channel and into the ear and kernels. Colonization is also often common in kernels damaged by birds or insects (108). *A. flavus* will also form sclerotia in damaged kernels that can serve as a source of inoculum the following season (148), although it is generally present saprophytically in soil as well (100). Other factors known to affect aflatoxin production in maize include: maize hybrid cultivar (274), insect damage (275) and the presence of other fungi (144).

Like *Aspergillus* species, *F. graminearum* can also infect maize via the silks although under different conditions. The majority of successful silk infections by *F. graminearum* take place 6-8 days after silk emergence (206) and are favored by warm (25-28º C) humid (above 80% relative humidity) weather (158, 173, 247, 260). In contrast to silk infections, fungal growth in the stalk of maize appears to be favored by warm, dry conditions that are thought to stress the plant and facilitate fungal colonization (66, 273). DON production, like fungal growth, is favored by similar conditions as fungal growth (158, 173, 247, 260) although other factors such as maize genotype (13), fungal strain (257) and microbial competition also affect toxin production (208). Interestingly, several studies have found that DON production also varies within the maize plant and often the vegetative portion of the plant have much higher DON concentrations than the kernels (62, 125, 130).

Favorable conditions for zearalenone (ZEA) production *in vitro* are similar to those reported for DON, however, there are several variations reported for optimal production temperature. While some authors have reported that a constant temperature of 20-25 º C promoted the highest yields of ZEA on maize kernels (89, 133, 156, 165), others (106, 219) found that incubation at 25 º C followed by incubation at a cooler temperature (12-15 º C) gave the highest concentration of ZEA. Conversely, both Milano and Lopez (156) and Montani *et al* (165) found that changing the incubation temperature from 25 º C to lower temperatures inhibited or prevented ZEA formation. Moisture availability also influences optimal ZEA production where cultures with water activities between 0.95 to 0.97 a_w had the highest production and those at 0.90 and below had little or no production (133, 165). Other factors reported to affect ZEA production include culture substrate and pH (89). Like observations made on DON concentrations in various portions of the maize plant, ZEA also appears to be produced in much higher concentrations in the

vegetative portions of maize rather than the kernels, which often have little or no ZEA present (62, 125, 130).

Unlike the conditions favorable for *F. graminearum* and DON production, infection and ear rot by *F. verticilliodes* and *F. proliferatum* and subsequent fumonisin contamination are more common in warm, dry weather (159, 160, 173). Insect damage also plays a large role in fumonisin contamination of maize as several species of coleopteran and lepidoptoran larva have been found to "vector" fungal spores (68, 173). Fumonisin production is also dependent on the isolate (61), maize hybrid (47, 189), plant maturity (37, 266), interactions with other fungi (146, 255) and insect wounding (68, 240).

Although *Alternaria* and *Penicillium* spp. are usually not pathogenic on maize, they can cause rot on ears damaged by insects and birds (190, 46). High population of *Alternaria* have also been reported on cereals during sustained periods of warm, wet weather (30). Little is known about conditions favorable for AAL-toxin production on maize, although one study has looked at *A. alternata* growth on maize grain and found that 30º C and 0.98 a_w were optimal for kernel colonization *in vitro* (249). During storage, any of the aforementioned fungi will grow in silage if the feed is exposed to oxygen and there is sufficient moisture, although some species, such as *P. roqueforti* and *A. fumigatus*, are particularly well adapted to colonize silage.

1-3 The role of mycotoxins in fungal biology and ecology

Mycotoxins are structurally diverse compounds that appear to have different functions regarding the biology and ecology of their fungal producers. Some toxins appear to play a role in virulence or pathogenicity, others are thought to act as chemical warfare agents against other organisms and some may have signaling functions. However, for the majority of mycotoxins it is unclear why their fungal producers devote resources to synthesize these secondary metabolites. This is particularly true for the *Penicillium* toxins, however, it has been suggested that many of these toxins play a role in helping their fungal producers secure and defend ecological niches (197).

Trichothecenes have been observed to have phytotoxic properties (58). Additionally, mutant strains of *F. graminearum*, containing a non-functional trichodiene synthase gene and thus not able to produce DON, had reduced ability to cause ear rot in maize and scab on wheat compared to the wild type strain. Interestingly, DON production is not an absolute requirement for the fungus to successfully cause disease in wheat; rather it appears to facilitate disease progression, categorizing it as a virulence factor (124). Conversely, *Alternaria* isolates pathogenic towards tomato must produce AAL-toxin to cause disease on the plant host, earning this particular toxin the label of a host-specific pathogenicity factor (115).

This name is somewhat misleading however, as AAL-toxin has been detected in agricultural samples other than tomatoes or tomato products (280). As *Alternaria* species do not appear to be overtly pathogenic towards maize, it is unclear why the fungus produces this toxin in a setting other than a disease situation. Interestingly, the fumonisins, which are structurally similar to AAL-toxins and are phytotoxic on plant cell lines (3) do not play a direct role in the initiation or development of maize ear rot, suggesting that they are not pathogenicity or virulence factors (59). Mutants of *F. verticillioides,* with a disrupted polyketide synthase gene and unable to produce fumonisin, caused ear rot in maize equivalent to disease caused the wild type strain. Interestingly, although fumonisin does not appear to play a major role in the development of ear rot in mature plants, fumonisin-producing strains are more virulent towards maize seedlings, suggesting that this toxin may be involved in seedling blight caused by *F. verticillioides* (56, 281).

1-4 Mycotoxins in maize silage and co-occurrence of mycotoxins

Currently, mycotoxins from each of the four major mycotoxin-producing genera have been detected in maize silage. The following tables (1.1 and 1.2) indicate which mycotoxins are reported in silage and other maize-based feeds and which toxins have been reported to co-occur. Most species of mycotoxigenic fungi are capable of producing more than one toxin simultaneously and many inhabit the same or similar niches on maize. The co-occurrence of mycotoxins in feeds is of concern and several authors have noted that multi-toxin contamination may lead to additive or synergistic affects on animals that consume them (213, 226).

Table 1.1. Mycotoxins reported in silage and other maize based feeds

FUNGAL GENUS	TOXIN	REFERENCE
Fusarium	[1]T-2 toxin	101
	Deoxynivalenol	280
	Fumonisin B_1, B_2, B_3	114
	Zearalenone	128
Alternaria	[1]AAL-toxin	280
Aspergillus	Aflatoxin B_1	268
Aspergillus/Penicillium	Cyclopiazonic acid	280
Penicillium	Patulin	169
	Roquefortine C	14
	Mycophenolic acid	224

[1] Toxin was identified in a maize-based feed other than silage

Table 1.2. Mycotoxins reported to co-occur in maize-based feeds.

TOXINS[1]	REFERENCE[2]
T-2, DON, ZEA	188[a]
FA, DON, ZEA, AFB1, FB1	239[a]
AFB1, DON, FB1	262[a]
CPA, AFB1	253[a]
CPA, DON, FB1, ZEA, PR, AAL	[3]280[b]
PAT, PR, MPA	169[b]

[1] Abbreviations: T2-toxin (T2), deoxynivalenol (DON), zearalenone (ZEA), aflatoxin B_1 (AFB1), fumonisin B_1 (FB1), cyclopiazonic acid (CPA), AAL-toxin (AAL) patulin (PAT), mycophenolic acid (MPA)
[2] a = grain corn or b = corn silage
[3] Authors did not specify the number of toxins present in each individual sample

1-5 Cellular and pathological effects of mycotoxin exposure on cattle and other animal species

For many of the mycotoxins discussed here, there are little data available on how these toxins affect cattle health and productivity, although in many cases there is anecdotal evidence suggesting that these compounds cause serious health problems. Investigations on toxin exposure in cattle have focused primarily on acute toxicoses during short term feeding studies, due to the difficulty and expense of conducting research on long-term chronic exposure. Occasionally information is available with other animals, such as rats, chickens and swine, but it is difficult to relate these studies to cattle because of differences in size and physiology. Another major concern is that although maize silage can be contaminated with multiple toxins simultaneously, the affects of multi-toxin exposure are virtually unknown.

1-5-1. *Aspergillus*: aflatoxins and cyclopiazonic acid

Mycotoxicosis, or more specifically aflatoxicosis, in cattle that have ingested aflatoxins has been well documented. Symptoms of aflatoxicosis include feed refusal, decreased milk production, gastrointestinal hemorrhaging, and jaundice, cirrhosis, and hemorrhaging of the liver (50). Experimental evidence suggests that 10 mg/kg of aflatoxin B_1 (AFB1) is sufficient to cause acute symptoms and death in adult cattle (48). Physiologically, when cattle or other animals (including humans) ingest aflatoxin-contaminated products, it is converted by hepatic P450 enzymes into several different compounds (131). This reaction produces the highly reactive aflatoxin B_1 8,9-expoxide, which is believed to be responsible for the majority of cellular damage resulting from AFB1 exposure. On a molecular level, AFB1 8,9-epoxide will form adducts with proteins and nucleic acids causing extensive damage, particularly in tissues with high metabolic activity such as the liver. Long term exposure may eventually result in cancer development, which is why this toxin is regulated in foods and feeds (2, 254). Dairy producers have a dual concern in

that not only can animals suffer acute affects of aflatoxicosis, but the toxin can be passed in milk. One of the other side products formed when cattle metabolize AFB1 is aflatoxin M_1 (AFM1) (149). Although less toxic than AFB1, AFM1 has similar carcinogenic activity, is stable following pasteurization and can remain so for several years (245).

Although cyclopiazonic acid (CPA) has not been studied with dairy cattle or other ruminants, it appears to have tremorgenic activity and causes lesions on the liver, kidney and the gastrointestinal tract in rats (121) and similar affects in other species (35). CPA will chelate metal ions (82), including calcium, and in rats inhibits the P-type ATPase enzyme, which is responsible for regulating intracellular calcium concentrations. This inhibition is believed to affect *in vivo* calcium levels, which in turn interferes with downstream cellular signaling. CPA is also problematic because it, like aflatoxin, is excreted in milk (67). Once CPA is present in milk and other dairy products, it is stable following pasteurization and appears to remain indefinitely (200).

1-5-2. *Fusarium*: trichothecenes, zearalenone and fumonisins

The acute effects on cattle from exposure to trichothecenes are well documented. Symptoms of T-2 mycotoxicosis in cattle include gastrointestinal hemorrhaging, inactivity and weakness, decreased body temperature, disease resistance and milk production (101). Acute symptoms have been reported from feeding concentrations as low as 0.5 mg/kg of T-2 toxin (55). There is also evidence that trichothecene ingestion, including T-2 toxin, weakens the immune system, causing a higher incidence of pathogenic illnesses in herds exposed to these compounds (36). In comparison to T-2 toxin, dairy cattle show considerable tolerance to DON (103), presumably because rumen microorganisms are able to degrade this compound into less toxic derivatives (116). Rumen microbes will also metabolize T-2 toxin (113), although the derivatives are only marginally less toxic than the original compound.

On a molecular level, trichothecene mycotoxins inhibit protein translation (75) by binding to the small ribosomal subunit, leading to obstruction of DNA and RNA synthesis. Another affect of trichothecenes includes interaction with cellular membranes, which causes lipid peroxidation and membrane instability (252). At the cellular level, trichothecenes inhibit the function of macrophages, T cells, and B cells of the immune system, directly suppressing immune function in several animals including cattle (36,193). Trichothecenes also affect erythrocyte production by causing damage directly to the cells and to bone marrow, which may explain why hemorrhagic symptoms are associated with trichothecene mycotoxicoses (187).

Unlike the trichothecenes, which may be acutely toxic, zearalenone (ZEA) affects the productivity of the cattle, rather than their general health. ZEA is an estrogen analog which mimics the activity estrogen, disrupting the reproductive cycles of many animals, including cattle (91). When animals consume ZEA contaminated feed, they metabolize the compound to either α or β-zearalenol, which bind to estrogen receptors in the liver, kidneys, and uterus, and cause reduced conception rates and missed breeding cycles (250).

Similar to DON, the physiological impact of B-series fumonisins (FB) on cattle appears to be marginal, even when animals are exposed to fairly high levels of the compound (186). One explanation for this tolerance is that FB are not readily absorbed into tissues and are rapidly excreted by ruminants (201). More recent data suggest that on a cellular level, damage does occur to the liver and immune system upon exposure to FB (150). Fumonisins have a similar structure to sphinganine, a precursor to sphingolipid production and the substrate of the ceramide synthase enzyme (5). Because of this similarity in structure FB will bind to ceramide synthase and prevent the formation of sphingolipids, which are molecules important in cell signalling. Disruption of intercellular communication then leads to cell dysfunction and apoptosis (3).

1-5-3. *Alternaria*: AAL-toxins

To date, AAL-toxins have only been studied with cultured cell lines rather than animal subjects. As mentioned previously, the AAL-toxins and fumonisins have similar structures in that they both resemble the sphinganine molecule (38). Plant and animal cells exposed to AAL-TA had an increased rate of apoptosis (4), however, the effect was less dramatic than when the same cell lines were exposed to fumonisin (263). As both AAL-toxins and fumonisins have a similar mode of action at the molecular level, refer to the information above on how fumonisin disrupts sphingolipid biosynthesis and causes cellular damage (5).

1-5-4. *Penicillium*: patulin, mycophenolic acid and roquefortine C

Although anecdotal evidence and case studies suggest that *Penicillium* mycotoxins have caused severe toxicoses in cattle, clinical studies have yet to be conducted. Patulin was recently implicated in a case of cattle toxicoses where several beef herds experienced tremors, paralysis and death and autopsy revealed serious damage to the nervous system (220). Although the causal agent in this case was *Aspergillus clavatus* rather than a *Penicillium* species, patulin was detected in fodder samples and was produced by isolates in culture. A second incident occurred when cattle consumed silage contaminated with *P. roqueforti* and experienced a loss of appetite, gastrointestinal hemorrhaging, and abortion (256). The suspected agents were PR-toxin and patulin, although no tests were performed to verify this

hypothesis. Studies on the toxicity of patulin (74) on rats have found that this compound is a potent hemorrhagic agent, causing hemorrhaging in the lungs, kidneys, liver and brain of exposed animals. Patulin has also been observed to inhibit DNA, RNA and protein synthesis and has been implicated as an immunotoxic agent (51).

Unlike patulin, mycophenolic acid (MPA) (40) does not appear to be acutely toxic; however it is considered a potent immunosuppressive agent (241). In the medical field, it is used in anti-rejection therapy for organ transplant patients. MPA acts as an inhibitor of several enzymes responsible for purine synthesis (76). This disruption of purine synthesis inhibits subsequent formation of T and B lymphocyte cells, which are important in initiating the immune response of mammals. As a result, proliferation of other immune-related cells and downstream antibody formation is suppressed. Recently, MPA was reported in 38 of 135 maize silage samples and in some silage, the amount of MPA was equivalent to 10% of the dose given to patients undergoing immunosuppressive therapy. To date however, the effects of MPA on cattle are unknown so the implications of these findings are still inconclusive although it is thought that the presence of MPA in feeds may make animals more susceptible to diseases and more sensitive to other mycotoxins that may be present (213, 226).

Roquefortine C has been implicated as the causal agent of paralysis, abortion and placental retention in cattle in two reports (90, 244). However, as the isolates of *P. roqueforti* studied also produced other mycotoxins, including patulin and PR-toxin, it is difficult to determine if roquefortine C was the only toxin involved. The role of roquefortine C as a neurotoxin has also been demonstrated with dogs. In two separate incidences animals suffered paralysis, tremors and convulsions when they consumed food contaminated with *P. roqueforti* (32, 277). Roquefortine C was also found in the stomach contents of one of the dogs in these cases (32).

1-6 Management strategies to prevent and control mycotoxins in silage

The majority of practices to prevent or control mycotoxin contamination of silage and other feeds rely on a combination of pre-harvest cultural practices, including hybrid selection, crop rotation, residue management (tillage), controlling insect pests. For many of the major mycotoxins, especially those produced by *Fusarium* species, prevention and control has not been reliable with these measures (172). Much of the inability to successfully control mycotoxins is a lack of understanding the factors that contribute to mycotoxin contamination. For some toxins the environmental conditions favorable for toxin production are well understood, but the affects of agronomic practices are sometimes not clear. Many of the studies on fungi and mycotoxin contamination of silage have been conducted in Europe and Asia, making it difficult

to determine if the situation in the United States is similar and if control strategies being used are comparably effective. Additionally, for many of mycotoxins, particularly *Penicillium* toxins, although there is anecdotal evidence suggesting that they are problematic in silage, little is known about how frequently they occur and at what levels. As such it is difficult to determine what mycotoxins are problematic and which should receive the greatest attention in terms of prevention and control.

Tillage is one cultural strategy that is thought to assist in preventing inoculum build up and subsequent infection and mycotoxin contamination in maize. This approach has been suggested for aflatoxin where sclerotia are buried with deep tillage, exposing them to soil microorganisms that will parasitize them and prevent re-infection of the following season's crop (71). However, as *Aspergilli* are often well adapted soil saprophytes, this strategy may have marginal benefit (153). Deep tillage may also help limit infection and DON contamination by *F. graminearum* as this fungus relies on maize stalk residue to produce inoculum the following season (247). This has been demonstrated for other cereals impacted by *F. graminearum*, such as wheat (64).

Crop rotation with a broadleaf crop is another strategy thought to reduce infection by *F. graminearum,* as this fungus will infect multiple cereal species (44), causing head scab on wheat, rye, barley and other gramineous crops. When grass species are grown in successive seasons the fungus can build up inoculum to infect the next season's cereal crop. Therefore, incorporating non-cereal into the crop rotation scheme may be an effective measure against re-occurring infection, over-season survival and mycotoxin contamination. To date however, there is no evidence suggesting that this strategy reduces *F. graminearum* infection or DON contamination (78).

Insect control is another important management practice as many mycotoxigenic fungi are associated with insect-damaged crops (68). This is certainly the case for aflatoxin, where maize crops protected from insect damage had lower concentrations of this toxin (54). Disease caused by *F. verticillioides* and fumonisin contamination are also related to insect damage (157) and several studies have demonstrated that transgenic maize lines, containing a gene encoding BT toxin from the bacterium *Bacillus thuringensis* that is toxic to lepidopteran larva, have considerably lower fumonisin levels than corresponding isolines (92, 174). Pre-harvest contamination of *Alternaria* and *Pencillium* mycotoxins may also be prevented if insect control is practiced as most infections caused by these species are facilitated by insect damage (190).

As many pre-harvest strategies have provided insufficient or inconsistent control of mycotoxins, research has been done on degrading or binding toxins in storage with chemical agents or microorganisms.

Some microorganisms are reported to bind DON to their cell surfaces possibly reducing the biological availability of the toxin in feeds (73), while others appeared to degrade DON (97). Zearalenone degradation by microorganisms in rumen fluid was reported, but in this same study the authors could find no evidence of aflatoxin or DON metabolism (113). A reduction in zearalenone concentration was also reported when grain was treated with an organic acid mixture (110). Fumonisins also appear to be degraded by microorganisms (26, 40, 41) although these microbes, like the ones mentioned above, have yet to be formulated for commercial application. Binding agents, such as clay compounds and activated charcoal, may also be effective against limiting the bioavailability of toxins. Several agents appear to bind aflatoxin in such a manner (63) and at least one product may be successful at controlling fumonisin contamination as well (16). Interestingly, there are reports that ensiling maize and other fermentative processes reduce the concentration of the *Penicillium* toxins patulin and mycophenolic acid (111, 170).

Chapter 2. Project justification, objectives and major goals: Fungi and mycotoxins in fresh and ensiled maize and the affects of agronomic practices, weather conditions and silage characteristics on toxin contamination.

The future of successful mycotoxin prevention and control in silage will most likely rely on a combination of pre and post-harvest control strategies. Improving current strategies and developing novel and efficient controls against toxin contamination requires a better understanding of the fungal species present in North American silages, what mycotoxins are common in silage and at what levels and how environmental factors such as weather, agronomic practices and ensiling conditions impact the fungi and the production of mycotoxins in silage. As such, the objectives of this research were to (1) characterize mycoflora in silage using traditional microbiological approaches and more recent molecular techniques (2) detect and quantify several classes of mycotoxins, including trichothecenes (T-2 toxin, deoxynivalenol and 15-acetyl-deoxynivalenol), zearalenone, fumonisins (B_1 and B_2), AAL-toxins (TA and TB), patulin, mycophenolic acid, and roquefortine C and (3) determine how weather conditions during the growing season, producers' agronomic practices and the process of ensiling affect the occurrence and levels of these toxins in fresh and ensiled maize. Understanding more about the dynamics of fungal contamination and mycotoxin contamination will provide insight regarding mycotoxins of concern in North America, where and possibly how management strategies might be applied in the silage-producing process to prevent or control toxin accumulation. Additionally, this work aims to understand what variables are important in influencing toxin formation so that in the future, predictive models can be developed to indicate when conditions are favorable for the formation of a particular mycotoxin. Once a through investigation has been conducted on what mycotoxins are present in maize silage, relevant studies regarding animal effects can also be carried out. The following chapters 3-7 provide detailed information on how the objectives of this work were met, the results of these studies and the implications for cattle health and mycotoxin control.

Chapter 3: Microbiological and molecular determination of fungal flora in fresh and ensiled maize silage

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ABSTRACT

The fungal flora of fresh and ensiled maize was studied using culturing techniques and a DNA sequence-based approach. Silage was collected for two years from twelve farms in Pennsylvania at harvest and after ensiling. Samples were plated on selective media and isolates identified by morphology and sequences of the internal transcribed spacer regions of rDNA (all isolates), 800-900 bp of the 5' end of the translation elongation factor 1-alpha gene (*Fusarium* species only) and a portion of the *rodA* gene (*Aspergillus fumigatus* only). ITS regions were amplified from total silage DNA, cloned, sequenced and compared to fungal ITS sequences in Genbank using the BLAST-N algorithm. For samples analyzed by both methods, the molecular technique detected a greater number of species than selective plating. Plating recovered several *Penicillium* and *Fusarium* species and *Aspergillus fumigatus,* while molecular analysis detected *Alternaria*, *Penicillium* and *Fusarium* species. Data from both methods found that *Fusarium* and *Penicillium* were the dominant mycotoxigenic fungi in silage, while yeast made up the majority all fungi recovered or detected. Known mycotoxigenic species often accounted for 50% or more of the total number of species isolated or detected at each site. Viable Fusaria were not isolated from or detected in ensiled maize, suggesting that *Fusarium* species do not survive the ensiling process. Results from this study suggest that while *Fusarium* toxins are likely to occur in the final product due to pre-harvest contaminants, those produced by *Penicillium* spp. may be produced in the field or during storage.

INTRODUCTION

Fungal spoilage of maize silage reduces the nutritional value and palatability of the feed, increases its allergenic potential and may result in mycotoxin contamination (226). Mycotoxigenic fungi in silage are associated with animal health problems such as acute toxicoses, decreased productivity, fertility, and disease susceptibility (6, 53, 226, 238, 244). Human health may also be affected as dust from molded silages has been implicated as a causal agent in organic dust toxic syndrome, a neurological and respiratory illness (192).

There are reports on the co-occurrence of mycotoxigenic fungi (8, 17, 112, 127, 171, 222) and mycotoxins (169, 280) in silage and many species are known to produce several toxins simultaneously (31, 49, 128, 154). Multi-toxin contamination is of particular concern because of potential additive or synergistic effects on animals and humans exposed to molded silages (213). Another issue is that some fungal species present in silage have been linked to mycotic infections in cattle, particularly *Aspergillus fumigatus* (105). To date, known toxigenic genera reported in silage include *Aspergillus, Alternaria, Fusarium,* and *Penicillium.* Other commonly isolated fungi include *Mucor, Epicoccum, Cladosporium*, and a variety of yeasts (8, 127, 155, 167, 228).

Mycotoxigenic fungi are often associated with maize either as field or storage organisms. For example, many *Fusarium* spp. are maize pathogens and/or endophytes, while *Alternaria* spp. are epiphytic saprophytes or weak pathogens (29). As such, toxins produced by these genera are usually considered to be field-borne problems. Conversely, because *Aspergillus* and *Penicillium* are often isolated as post-harvest spoilage organisms, their toxins are regarded as a storage concern (196, 226). However, under favorable conditions, including hot dry weather and insect damage, members of both genera and their toxins may be found on maize in the field (46, 108, 148). In ensiled maize, the majority of fungal growth is limited by low oxygen content and the production of organic acids by lactic acid bacteria (152). However, some species, such as *Penicillium roqueforti* and *Aspergillus fumigatus*, can survive the microaerophilic low pH environment of silage likely explaining the high reported frequency of toxigenic fungi isolated from ensiled maize (8, 31, 49, 127, 154, 191, 256). *Fusarium* and *Alternaria* species may also grow and contaminate silage if the low oxygen tension in the feed is not maintained (8, 17, 191).

The development of strategies to reduce toxin contamination of silage will be aided greatly by improved surveys of fungi present. Methods to determine the fungi present in silage usually rely on selective media (212, 235). However, this approach only isolates viable fungi and may have difficulty recovering those with fastidious growth requirements (237). Another concern is that selective media may

mask the presence of slower-growing fungi. Likewise, antagonistic interactions between fungi in culture make it difficult to recover sensitive species. An alternative approach is to identify fungi present in silage by characterizing their ribosomal DNA sequences. This approach has successfully detected fungi in environmental samples including soil from the wheat rhizosphere (237), grasslands (102) and arctic tundra soils (222). Another benefit of this approach, as illustrated by the work of Schadt *et* (222), is that it can detect previously unidentified species.

Many studies have been conducted to characterize fungi in maize silage (8, 17, 112, 127, 151, 171, 228) but the majority have been conducted in Europe and Asia, making it difficult to directly relate them to the what may be true in North America. Additionally, most of these studies relied on culturing techniques, with the exception of May *et al* (151). These researchers used denaturing gradient gel electrophoresis on fungal SSU rDNA amplified from total silage to identified fungi in maize silage. However, this approach had some difficulty resolving closely related fungal sequences. To provide a more comprehensive assessment of mycoflora in North American silage, we analyzed fresh and ensiled whole plant maize using two methods: (1) a microbiological evaluation with selective media and morphological identification, and (2) a molecular assessment using DNA sequences. This two-method design allowed us to compare our results to earlier work, assess the efficacy of the DNA sequence-based technique and potentially reveal species not previously reported in silage. By examining fresh and ensiled maize, we can investigate what fungi are more prevalent in the field versus those predominant in storage and better understand how the process of ensiling affects the fungal community in maize silage.

MATERIALS & METHODS

Sample collection

Maize silage was collected from four geographic regions (Figure 1) in Pennsylvania in 2001 and 2002, from farms with a history of known or suspected mycotoxin contamination. Samples were collected at harvest and then again at the same site approximately 3-6 months after ensiling. Harvest samples consisted of freshly chopped maize that was collected from a mechanical harvester as it made several passes within a field intended for ensiling. Ensiled maize was either removed from several locations at the face where bunker or trench silos were used, or as the silage was being emptied from the silo during “feed out” where upright silos were used. All samples were mixed by hand and a 1-3 kg was removed for further study. Samples for molecular analysis were stored at -80 °C while those for microbiological assessment were stored at 4 ºC.

Fungal isolation

Microbiological assessment was conducted on harvest and ensiled maize collected during 2001 and 2002 from three farms in each of the four regions. Fungi were isolated using a protocol developed by Skaar and Steinwig (235) with some modifications. Fifty grams of maize silage was ground with an M-2 model Stein mill (Fred Stein Lab, Inc., Atchinson, KS) and a 10 g portion of the sample soaked in 50 mL of sterile 0.9% aqueous NaCl solution (wt/vol) for 30 minutes at room temperature. After incubation, the mixture was manually shaken for 1 minute and filtered through four layers of cheese cloth. The resulting suspension was diluted to 10^{-3} with sterile 0.9% NaCl solution and 100 µL of the diluted solution was spread onto a plate of Malt-Yeast Sucrose Agar (MYSA). The media consisted of 15 g malt extract, 2 g tryptone, 2 g oxgall, 30 g of sucrose, 5 g of yeast extract, 0.5 g of sodium nitrate and 20 g of agar (all materials purchased from Difco, Sparks, MD) to which 1 L of water was added. A total of 15 MYSA plates were inoculated for each silage sample. MYSA medium was prepared according to the original protocol (235). Plates were incubated for 7 days at room temperature. During this period plates were examined and fungal colonies were transferred at first observation to Potato Dextrose Agar (PDA) (Becton-Dickinson Microbiology Systems, Sparks, MD). Plating was done in duplicate for each silage sample, producing a total of 30 MYSA plates.

Because the MYSA method yielded low numbers of *Fusarium* species, a separate protocol was developed for isolation of these fungi. After grinding (described above) 10 g of silage, the sample was soaked in 50 mL of 50% aqueous Clorox solution for 5 minutes. The sample was rinsed three times with 100 mL of sterile distilled water for 2 minutes per wash. Approximately 0.5 g of surface sterilized silage was placed directly onto a plate containing Nash medium (177). Nash medium was prepared with materials purchased from Difco (Sparks, MD) and consisted of 15 g peptone, 1 g KH_2PO_4, 0.5 $MgSO_4*7H_2O$, 20 g agar, 1 g of pentachloronitrobenezene (Uniroyal Chemical Company, INC, Middlebury, CT) to which 1 L of water was added. A total of 5 plates were used for each sample. This procedure was performed twice for a total of 10 plates and a maximum of 20 *Fusarium* isolates per silage sample. For each sample, using the MYSA and NASH isolation protocols, a total of 100 isolates were collected.

Ensiled samples (*N*=12) from 2002 only were analyzed with a method to detect *Aspergillus fumigatus* that was modified from Melo dos Santos *et al* (154). Silage was ground (described above) and a 10 g portion was removed for analysis. One-hundred mL of sterile distilled water with 0.01% Tween 20® (Merck, Darmstadt, Germany) was added to the sample and shaken by hand for 1 minute. The extract was diluted to 10^{-1} and 200 µL spread onto plates of Dichloran Rose Bengal Medium (DRBM) (100), and grown for 5 days at 45 ºC. All materials for the DRBM medium were purchased from Difco (Sparks, MD) and the medium consisted of 10 g glucose, 2.5 g peptone, 0.5 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4*7H_2O$, 20 g agar, 25

mg rose bengal to which 1 L of water was added and the pH adjusted to 5.0. Five plates of DRBM were used per silage sample. After incubation, 5-20 isolates per sample were collected.

Isolate identification

Isolates from the MYSA, NASH, and DRBM plates were inoculated onto PDA, grown for 7 days and transferred to appropriate media for identification. *Aspergillus* and *Penicillium* species were transferred to Czapek Yeast Autolysate Agar and Malt Extract Agar (195), while *Fusarium* species were placed on fresh PDA and Carnation Leaf Agar (181). All other fungi were transferred to fresh PDA prior to identification. Isolates were identified morphologically using the reference cultures listed in Table 1 and identification guides (23, 117, 181, 195).

To validate the morphological identification of each isolate, the internal transcribed spacer regions of the nuclear ribosomal RNA gene repeat were sequenced (267). For *Fusarium*, the ITS and the 5' coding region and introns of the translation elongation factor 1-alpha (TEF) gene were sequenced (84, 183) were confirmed by sequencing the ITS region and a region flanking the introns of the *rodA* gene, which encodes a hydrophobin (83). All sequence data were aligned using Clustal X (72) and manually edited using ABI Prism Sequencing Analysis software version 3.4.1 (Applied Biosystems, Foster City, CA). ITS and *A. fumigatus rodA* sequence data were compared to the Genbank database (National Center for Biotechnology Information, Bethesda, MD) using the BLAST-N algorithm (10) and the closest match was recorded (≥98% sequence identity in all cases). Similarly, TEF sequence data were compared to sequences in the online FUSARIUM-ID Database (84) with BLAST-N.

Fungal isolates were grown in 10 mL of Potato Dextrose Broth (Becton Dickinson Microbiology Systems, Sparks, MD) , shaking at 150 rpm at room temperature for 7 days. Mycelia were harvested by filtering the culture and scraping the material into a 1.7 mL centrifuge tube. Yeast cells were collected by centrifugation (Sorvall RT7 centrifuge with RTH-750 rotor; Thermo Electron Corp., Boston, MA) of the cultures at 6000 x *g* for 5 minutes, decanting the media and re-suspending the cells in 1 mL of sterile distilled water. This suspension was transferred to a 1.7 mL centrifuge tube, spun at 13000 x *g* for 1 min (Heraeus Biofuge pico centrifuge, Thermo Electron Corp, Boston, MA) and the water decanted. After harvest, fungal isolates were lyophilized in an ADVantage Freeze Dryer (VirTis, Gardiner, NY). Cultures of known mycotoxigenic species are available on request.

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Isolates for DNA sequence analysis were then subjected to the polymerase chain reaction (PCR) in 50 µl reactions using 1 µL of sample DNA, and a final concentration of the following: 0.2 µM of each primer, 0.2 mM dNTP mixture, 0.1X

PCR buffer (0.5 M KCl, 0.1M TrisHCl pH 8.3, and 0.025M $MgCl_2$), and 0.01 U/µL of Taq polymerase (Promega, Madison, WI). All reactions were carried out in a PTC-200 thermocycler (MJ Research, Waltham, MA). The PCR and sequencing primers used were ITS5 and ITS4 (267) for the ITS region, EF1 and EF2 for the translation elongation factor gene (183) and rodA1 and rodA2 for the *RodA* gene (83). The PCR amplification conditions were as follows: for ITS, (1) 96 ºC for 1 min, (2) 55 ºC for 30 s, (3) 72 ºC for 1 min, 30 cycles of (4) 94 ºC for 30 s, (5) 55 ºC for 20 s, (6) 72 ºC for 1 min, and a final polymerization step of (8) 72 ºC for 2 min. The amplification conditions for TEF analysis were as follows: (1) 95 ºC for 1 min, followed by 35 cycles of (2) 94 ºC for 1 min, (3) 53 ºC for 1 min, (4) 72 ºC for 1 min, followed by a final polymerization step of (6) 72 ºC for 5 min. The amplification conditions for the *RodA* gene were as follows: (1) 94 ºC for 2 min, followed by 35 cycles of (2) 94 ºC for 1 min, (3) 56 ºC for 1 min, (4) 72 ºC for 1 min, followed by a final polymerization step of (5) 72 ºC for 2 min.

PCR products were cleaned up for DNA sequencing using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA). Sequencing reactions were performed using the BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA) kit according to the manufacturer's instructions, except that reaction volumes were scaled down to 8 µl. Sequence reactions were purified with Performa® DTR 384-Well Plates (Edge BioSystems, Gaithersburg, MD). Sequence analysis was performed on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Genomic DNA extraction and purification from maize silage

For molecular analysis, one sampling site was selected from each of the four regions from which one harvest and one ensiled sample from 2001 and 2002 was analyzed, for a total of 16 samples. Fifty grams of maize silage was ground as described above and 10 g of sample removed for DNA extraction. Total genomic DNA was extracted using the UltraClean™ Mega Prep Soil DNA Kit (MoBio Laboratories, Inc., Solona Beach, CA). DNA was further purified using Genomic-tip 20/G DNA clean up columns (Qiagen, Valencia, CA).

PCR amplification and purification

PCR was performed using purified total genomic DNA from silage, with primers ITS5 and ITS4 (267) and the high-fidelity PfuTurbo® DNA Polymerase (Stratagene, La Jolla, CA). The remaining reaction reagents and conditions are described above. A total of 10 amplification reactions were performed for each sample. Following amplification, the reactions were pooled and subjected to electrophoresis in a 1.5% agarose gel in 1X TAE buffer (15). The gel was visualized with ethidium bromide and the ITS

fragments (a smear between 400 to 900 bp in size) were manually excised from the gel using a scalpel and purified using QIAquick® Gel Extraction Kits (Qiagen, Valencia, CA).

Generation of ITS clone library

Purified ITS fragments were ligated into the pGEM®-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions and recombinant plasmids were transformed into competent JM106 *Escherichia coli* cells (Promega, Madison, WI). One hundred µL of undiluted transformed cells were plated onto Luria-Bertani (LB) medium containing 100 µg/mL ampicillin (Shelton Scientific, Shelton, CT), 40 µg/mL X-gal (Inalco, Milano, Italy) and 0.5 mM IPTG (Inalco, Milano, Italy) for blue-white screening. Clones included in the libraries were selected by picking white colonies with a sterile toothpick and inoculating wells in a 96-well microtiter dish (BD Biosciences, Bedford, MA) containing 200 µL of LB broth amended with 100 µg/mL amplicillin. Cultures were incubated overnight at 37ºC with shaking at 100 rpm. Libraries were preserved for long-term storage by adding 60 µL of 50% glycerol (Shelton Scientific, Shelton, CT) prior to storage at -80 ºC.

Clone library analysis

Preliminary sequencing analysis revealed that the majority of the population of each library consisted of several yeast species. To avoid repetitively sequencing clones of a few species, a hybridization method was used to identify clones of interest. To determine predominant yeast species in each library, plasmid DNA was extracted from 24 randomly selected clones using the QIAprep® Miniprep kit (Qiagen, Valencia, CA) and sequenced using the SP6 or T7 primer according to the manufacturer's instructions. Based on the resulting data, yeast sequences, found to make up 25% or more of the library, were chosen for selective screening to avoid repetitious sequencing. Template DNA for the hybridization probes was generated by growing clones of the selected yeast species and extracting their plasmid DNA as noted above. *EcoR1* restricted plasmid DNA was run on a 1.0% agarose gel and the 400-900 bp ITS insert band manually excised and purified as described above. After template DNA purification, the NEBlot® Phototype Kit (New England Biolabs, Beverly, MA) was used to generate biotinylated ITS probes for colony hybridization using the manufacturer's protocol.

Southern blots were prepared by stamping each library with a 96-well replicating tool onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ) set on a plate of ampicillin-amended LB media (100 µg/µL). Membranes were incubated overnight at 37 ºC. Following incubation, membranes were soaked for 1 minute in 10% aqueous sodium dodecyl sulfate solution (SDS) and washed.

Membranes were then prepared according to the NEBlot® Phototope ® Kit instructions for colony hybridizations.

Membranes were prehybridized at 68 ºC for 1 hour in a prehybridization solution and hybridization was performed immediately afterward under the same conditions with approximately 20 ng/mL of probe added to the prehybridization solution according to the NEBlot® Phototope ® Kit instructions. The membrane was hybridized overnight, washed and incubated in CDP-Star reagent (New England Biolabs, Beverly, MA) per the Phototope®-Star Detection Kit manual. The treated membranes were exposed for 5 minutes to BioMax Light Audioradiography film (Eastman Kodak, Rochester, NY) and the film developed according to company instructions. Membranes were stripped and stored according to the Phototope®-Star Detection Kit instructions.

After screening the libraries, 96 clones that did not hybridize with any of the targeted yeast species were selected for sequencing analysis. Selected clones were grown overnight and subjected to plasmid extraction, sequencing and data analysis as described above.

RESULTS

Microbiological assessment.

Using a combination of three different plating methods, we isolated six different species of *Penicillium* (*P. roqueforti, P. paneum, P. expansum, P. crustosum, P. commune* and *P. citrium*) seven species of *Fusarium* (*F. avenaceum, F. culmorum, F. graminearum, F. pseudograminearum, F. proliferatum, F. sporotrichioides* and *F. verticillioides*; Figure 2) and one species of *Aspergillus, A. fumigatus.* In terms of species abundance, *P. roqueforti* and *F. graminearum* were the most commonly isolated species known to be mycotoxigenic. *P. roqueforti* was isolated from 50% of the harvest (*N*=24) and 75% of the ensiled (*N*=24) samples, while *F. graminearum* was found in 58% of the harvest samples and in none of the ensiled samples. *P. paneum,* which is closely related to *P. roqueforti* and only recently defined as a separate species (31), was isolated from two harvest (*N*=24) and four ensiled samples (*N*=24). The third member of the *P. roqueforti* group, *P. carneum* was not isolated from any of the samples.

Although the MYSA medium was formulated to recover fungi from big bale grass silage, it was not successful in isolating *Fusarium* species from maize silage. Samples plated on MYSA quickly became overgrown by yeast and zygomycetes before colonies of *Fusarium* could be successfully isolated. Therefore, a method with the *Fusarium*-specific NASH media was incorporated to address this issue. The MYSA method also failed to recover *A. fumigatus* from the 2001 ensiled and harvest samples or the 2002 harvest samples. However, addition of the DRBM plating method yielded several isolates of *A. fumigatus*

from 5 of the 12 ensiled samples from 2002. None of the silage contaminated with *A. fumigatus* was included in the sample set for molecular analysis with the DNA sequence based technique so we were not able to address if this species could be detected in silage by molecular analysis.

In addition to the mycotoxigenic species isolated from silage, several other filamentous and yeast species were recovered as well. Filamentous fungal genera isolated included *Acremonium, Cladosporium, Cordyceps, Epicoccum, Mortierella* and *Mucor* (Table 2). In general, yeast made up the majority of the fungi isolated from silage. *Geotrichum candidum* was the most frequently encountered species identified by sequence, and was isolated in 75% of the harvest samples (*N*=24) and 21% of the ensiled samples (*N*=24). Other yeasts that occurred at relatively high frequency included *Candida intermedia, Candida sake, Debaryomyces hansenii, Issatchenkia orientalis, Pichia anomala, Pichia fermentans* and *Pichia membranifaciens*.

Molecular analysis

Molecular analysis was able to detect the same mycotoxigenic species isolated by plating, as well as exact matches with several additional species in the GenBank database, including *Alternaria alternata, A. tenuissima, Fusarium chlamydosporum, F. oxysporum, F. subglutinans, Penicillium aurantiogriseum* and *P. farinosum. P. farinosum* was detected from the NE site in a harvest and ensiled sample from 2001. As far as we are aware, this is the first report of *Penicillium farinosum* in maize silage. Other fungi detected only by molecular assessment included *Candida fragi, Cladosporium cladosporioides, Cryptococcus flavescens, Cryptococcus laurentii* and *Orbilia luteorubella*. The yeast species listed above that were found to occur at a high frequency in plated silage samples also made up a large percentage of the fungal ITS clones. In general, three or fewer species accounted for 80% or more of the clones in each library.

For silage samples analyzed by plating and DNA sequences, molecular analysis detected at least twice the number of species identified by plating (Figure 3). Data from plating and molecular analysis revealed that mycotoxigenic fungi often made up 50% or more of the species isolated or molecularly detected from harvest and ensiled samples at each site (Figure 4a and b). However, we found that in general, species diversity decreased as a result of ensiling (Figure 5a and b). Ten out of twelve sites examined by plating had fewer fungal species in their ensiled samples compared to those from harvest (Figure 5a), whereas all four sites analyzed with the molecular technique had fewer species in their ensiled samples (Figure 5b). Interestingly, we observed that while *Fusarium* and *Alternaria* species could be isolated or detected from harvest samples, none were found in ensiled material (Figure 2). In contrast, several *Penicillium* species,

including *P. roqueforti, P. paneum, P. crustosum* and *P. commune* were found in both ensiled and harvest samples.

DISCUSSION

To our knowledge, this is the first study to successfully use ITS sequence-based identification to study the fungal population in maize silage. We found that in general, the molecular method detected a greater abundance of species per sample than plating alone. However, this method utilized a selective screening process, which may have limited our ability to detect species closely related to the hybridization targets. As such, it is possible we underestimated the true number of fungal species present in silage. We did find it interesting that this method detected *Penicillium farinosum*, which has not reported in silage prior to this study. *P. farinosum* is closely related if not synonymous with *P. crustosum* (194), and is reported to produce the diketopiperazine alkaloid roquefortine C (119) on both artificial media and wheat kernels (210).

Because the majority of the *Fusarium* species encountered in this study are known pathogens or endophytic inhabitants of maize (190), their presence in the harvest samples was not unexpected. In particular, *F. graminearum*, the most frequently isolated *Fusarium* species from harvest samples, is one of the most commonly encountered maize pathogens in North America (190) and it is reported to be a late-season colonizer of maize stalk tissue (273), which may explain its high frequency in the harvest samples. We did find it surprising that *F. verticillioides* was not isolated with greater frequency (isolated from only 4 of 24 samples), although it was detected in 6 of the 8 harvest samples analyzed with the molecular technique. *F. verticillioides*, like *F. graminearum*, is a pathogen of maize (190) but can also be isolated with relatively high frequency from symptomless kernels, stalks and leaves (18).

Consistent with previous studies (8, 14, 127, 191, 256), we found *P. roqueforti* to be the most commonly isolated mycotoxigenic fungus in ensiled samples and the second most frequently isolated from harvest samples. Although *A. fumigatus* was also recovered we did not isolate it as frequently as *P. roqueforti.* This may be due in part to unfavorable culturing conditions prior to the addition of the DRBM method, although other methods have successfully isolated *A. fumigatus* onto non-selective media at 25ºC (49). Therefore, it may be that *A. fumigatus* was simply not present as abundantly as *P. roqueforti* in silage from this study. Similar to observations of Boysen *et al* (31), we did recover *P. paneum* from maize silage but at a lower frequency than *P. roqueforti*. Likewise, we did not isolate or detect *P. carneum*, the third member of the *P. roqueforti* complex, in any of the silage samples. The isolation of *P. roqueforti* and *P. paneum* is expected as their ability to survive ensiling is well documented (14,31). *Penicillium expansum*

was also isolated in this study, and like *P. roqueforti* and *P. paneum,* it is reported to tolerate a low oxygen concentration and the presence of organic acids (198), which likely explains its presence in silage (31).

Interestingly, no viable *Fusarium* species were isolated from ensiled samples. The inability of *Fusarium* to persist in ensiled maize was observed by Golosov *et al* (87) and suggests that they are not capable of surviving the low oxygen low pH environment of silage. As such, the majority of *Fusarium* mycotoxins in silage are probably produced in the field and strategies to reduce *Fusarium* mycotoxins in silage should focus on the crop prior to ensiling. This is particularly important, because although ensiling may successfully eliminate fusaria from maize, many of their toxins, such as the trichothecenes and zearalenone, are stable in storage (128, 129). Therefore, once formation has occurred, toxin levels in the silo may not drop appreciably before consumption. However, based on our observations the concentration of one trichothecene, deoxynivalenol, does decrease from harvest to approximately 6 months after ensiling (139). One possible explanation for this phenomenon is that microbial agents within silage are capable of degrading or binding this toxin (73, 97). Although *Fusarium* spp. are not tolerant of conditions during ensiling, most *Penicillium* isolated in this study were found both in fresh and ensiled maize, so it is possible that toxin formation by these fungi may occur in the field and during storage. Toxin production by *Penicillium* in the field is perhaps less common than it is for *Fusarium,* as most *Penicillium* spp.are cosmopolitan saprophytes rather then pathogens. However, toxins may be produced in the field if damaged kernels are colonized (46) in a disease known as “blue-eye” on maize (190). Most *Penicillium* species grow well in low water activity, which is why they are usually considered post-harvest spoilage organisms and their toxins are generally produced in maize after ensiling (196, 226). Consequently, since *Penicillium* spp. require oxygen, prevention of toxin production during storage should focus on maintaining the anaerobic conditions of the feed.

Although mycotoxigenic fungi accounted for 50% or more of the fungal species isolated from each of the study sites, the majority of the fungal population consisted of yeast. This was also true for the clone libraries, which in many cases were made up almost entirely by three or more species of yeast. Middlehoven (155) isolated several predominant species in ensiled maize including *Issatchenkia orientalis, Geotrichum candidum, Pichia anaomala,* and *Pichia fermentans*. He found that while some species, such as the basidiomycete yeast *Cryptococcus laurentii* (detected in this study by molecular analysis in harvest samples only) did not persist after ensiling, the species listed above were present for the duration of the 122 day study. May *et al* (151) also noted that yeasts, including *P. anomala*, were the dominant fungi present 2-3 months after ensiling. Interestingly, Middlehoven also noted that when the silage was exposed

to oxygen, *P. fermentans, P. anomala* and *G. candidium* were all able to assimilate lactic and acetic acids from the feed (155). By degrading organic acids, yeast have been suggested to pre-condition silage for colonization by other fungi when anaerobic conditions are not maintained (154). Contamination of silage with high populations of yeast may also be problematic as species such as *G. candidum* can produce unpleasant odors that cause the cattle to reject the feed (226).

The results from this work confirm that multiple mycotoxigenic species can occur in a single whole-plant maize sample both at harvest and after ensiling, and suggest that a mixture of toxins can be present simultaneously (17, 127, 169, 228). Table 3 presents fungal species isolated or detected in this study and lists the toxins they are reported to produce. Many of these toxins, including several trichothecenes, zearalenone, AAL-toxin, TA, and those produced by *Penicillium roqueforti* and *P. paneum*, have been detected in maize silage (31, 141, 169, 280). It is likely that the other toxins listed in this table are also present but they have yet to be investigated. For example, although several studies have cultured *A. fumigatus* from silage and tested the ability of isolates to produce mycotoxins (49, 154, 238), as far as we are aware no studies have been conducted to directly detect these toxins in silage. Data from this study provide additional guidance as to what mycotoxins may be expected in North American silages and invites further research toward their detection. In addition, by expanding our understanding of what fungi occur in North American silages, more targeted management strategies can be developed to prevent fungal and mycotoxin contamination in the field and during storage.

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Table 3-1. Reference Cultures

Fungal species	Reference number
Aspergillus parasiticus	NRRL 465[a]
Aspergillus flavus	NRRL 500[a]
Aspergillus fumigatus	NRRL 164 [a]
Alternaria alternata	NRRL 5255 [a]
Penicillium crustosum	NRRL 968 [a]
Penicillium chrysogenum	NRRL 807 [a]
Penicillium citrinum	NRRL 1847 [a]
Penicillium expansum	NRRL 976 [a]
Penicillium commune	NRRL 890 [a]
Penicillium griseofulvum	NRRL 989 [a]
Penicillium roqueforti var *roqueforti*	IBT 6754[b]
Penicillium roqueforti var *paneum*	IBT 12392 [b]
Penicillium roqueforti var *carneum*	IBT 6884 [b]
Fusarium sporotrichioides	T918[c]
Fusarium equiseti	R5619[c]
Fusarium poae	T919 [c]
Fusarium cerealis	R3090 [c]
Fusarium proliferatum	M1597 [c]
Fusarium graminearum	R9527 [c]
Fusarium pseudograminearum	R2268 [c]
Fusarium culmorum	R6563 [c]
Fusarium verticillioides	M8632 [c]

Culture sources:
a - Agricultural Research Service Culture Collection National Center for Agricultural Utilization Research Peoria, Illinois USA.
b - IBT, Culture Collection of Fungi, Technical University of Denmark, Department of Biotechnology, Lyngby, Denmark.
c - Fusarium Research Center, the Pennsylvania State University, University Park, PA

Table 3-2. Filamentous fungi and yeast isolated from silage by plating.

Fungal species	Harvest	Ensiled
*Acremonium strictum**	✓	
Brettanomyces bruxellensis	✓	✓
Candida cleridarum	✓	
Candida intermedia	✓	✓
Candida quinlingensis	✓	
Candida sake	✓	✓
*Cladosporium tenuissimum**	✓	✓
Clavispora lusitaniae	✓	✓
*Cordyceps sinesis**	✓	
Debaryomyces hansenii	✓	✓
*Epicoccum nigrum**	✓	✓
Geotrichum candidum	✓	✓
Hanseniaspora uvarum	✓	
Issatchenkia orientalis	✓	✓
Kluyveromyces marxianus		✓
Metschiakowia pulcherrima	✓	✓
*Mortierella hyaline**	✓	
*Mucor circinellioides**	✓	✓
*Mucor racemosus**	✓	✓
*Mucor rouxii**	✓	✓
Pichia anomala	✓	✓
Pichia fermentans	✓	✓
Pichia membranifaciens	✓	✓
Pichia segobiensis	✓	
Saccharomyces castelli	✓	
Saccharomyces paradoxus		✓

* Indicates filamentous species.

Table 3-3. Mycotoxigenic fungi isolated or detected in maize silage and their reported toxin production profiles.

Fungal species	Reported toxins	References
Alternaria alternata	Altenuene, alternariol, alternariol monomethyl ether, altertoxins, tenuazonic acid, AAL-toxins	11, 86
A. tenuissima	Altenuene, alternariol, alternariol monomethyl ether, altertoxins, tenuazonic acid	12
Aspergillus fumigatus	fumitremorgins, gliotoxin, verrucologen	49, 154
Fusarium avenaceum	moniliformin, fusarins, beauvericins	57, 135
F. chlamydosporum	chlamydosporol, moniliformin	176, 221
F. culmorum	deoxynivalenol, 15-acetyl-deoxynivalenol nivalenol, zearalenone, fusaranone X	22, 164
F. equiseti	nivalenol, diacetoxyscirpenol, neosolaniol zearalenone, fusarochromanone	167
F. graminearum	deoxynivalenol, nivalenol, fusarenon-x zearalenone	164
F. oxysporum	moniliformin, fusaric acid, wortmannin	19
F. pseudograminearum	deoxynivalenol, nivalenol	57
F. proliferatum	fumonisins, moniliformin, beauveracin, fusaric acid	19, 180
F. subglutinans	fumonisins, moniliformin, beauveracin, fusaric acid, fusaproliferin	19, 60, 230
F. verticillioides	fumonisins, fusaric acid, fusarins	60
Penicillium aurantiogriseum	penicillic acid, roquefortines, verrocosidin	46, 80
P. citrinum	citrinin	80
P. commune	cyclopiazonic acid	80
P. crustosum	penitrem A, roquefortines	80
P. expansum	patulin, roquefortine, chaetoglobosin	80
P. farinosum	roquefortines	210
P. paneum	mycophenolic acid, roquefortines	31
P. roqueforti	mycophenolic acid, roquefortines, PR-toxin	31

Figure 3-1. Location of study daries. Figure 1. Locations of twelve Pennsylvania dairy farms where silage was collected in this study. Individual dairies are represented by one of four geographic regions in the state, designated as ▲ (Northwest), ★ (Northeast), ● (South-central) and ■ (Southeast).

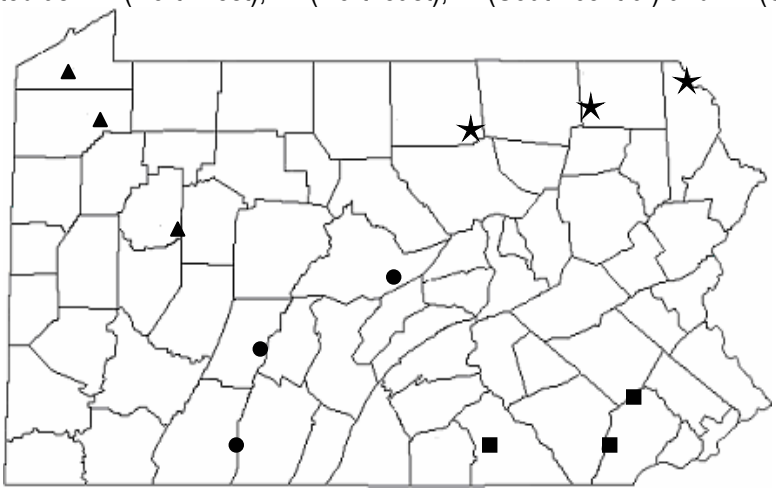


Figure 3-2. Mycotoxigenic species isolated by plating from maize at harvest and after ensiling. Isolations were made from fresh and ensiled silage plated on to MYSA and Nash media.

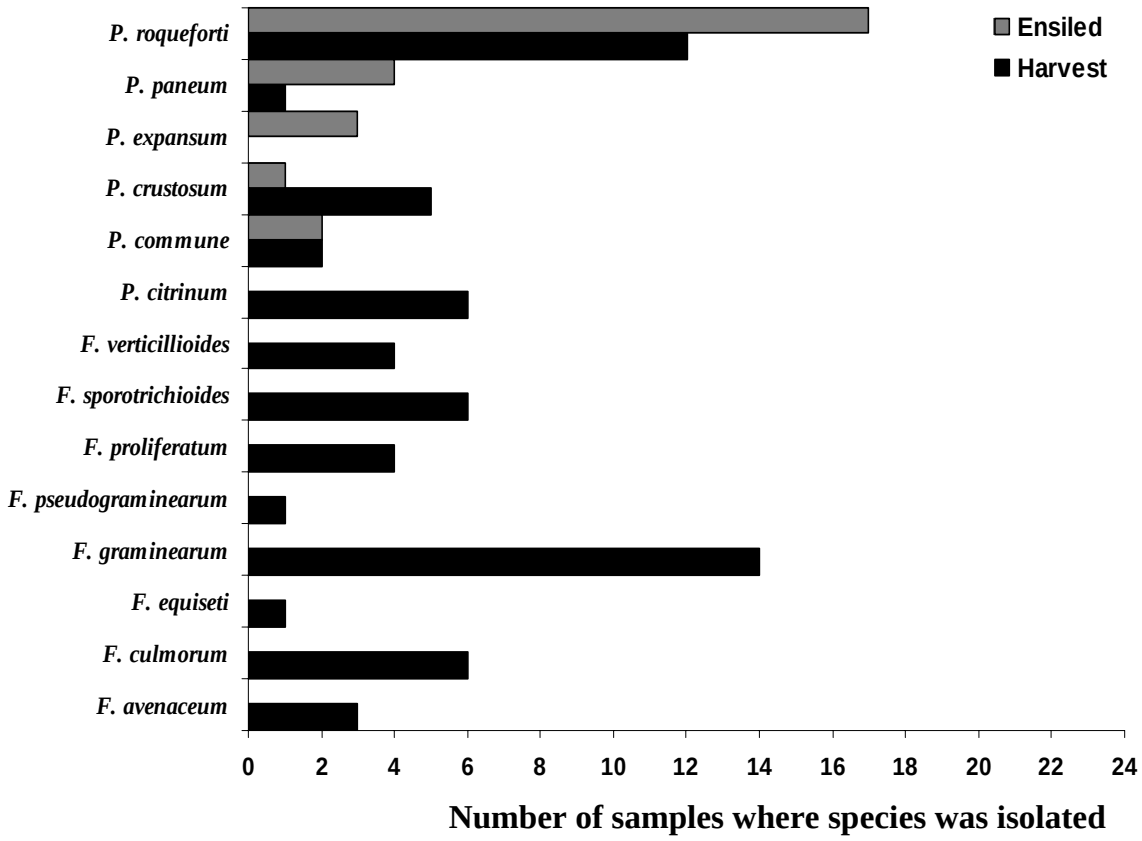


Figure 3-3. Number of toxigenic and non-toxigenic species isolated from each site. Comparison of the number of toxigenic and non-toxigenic fungal species isolated by A. plating and B. molecular detection from maize at harvest and after ensiling.

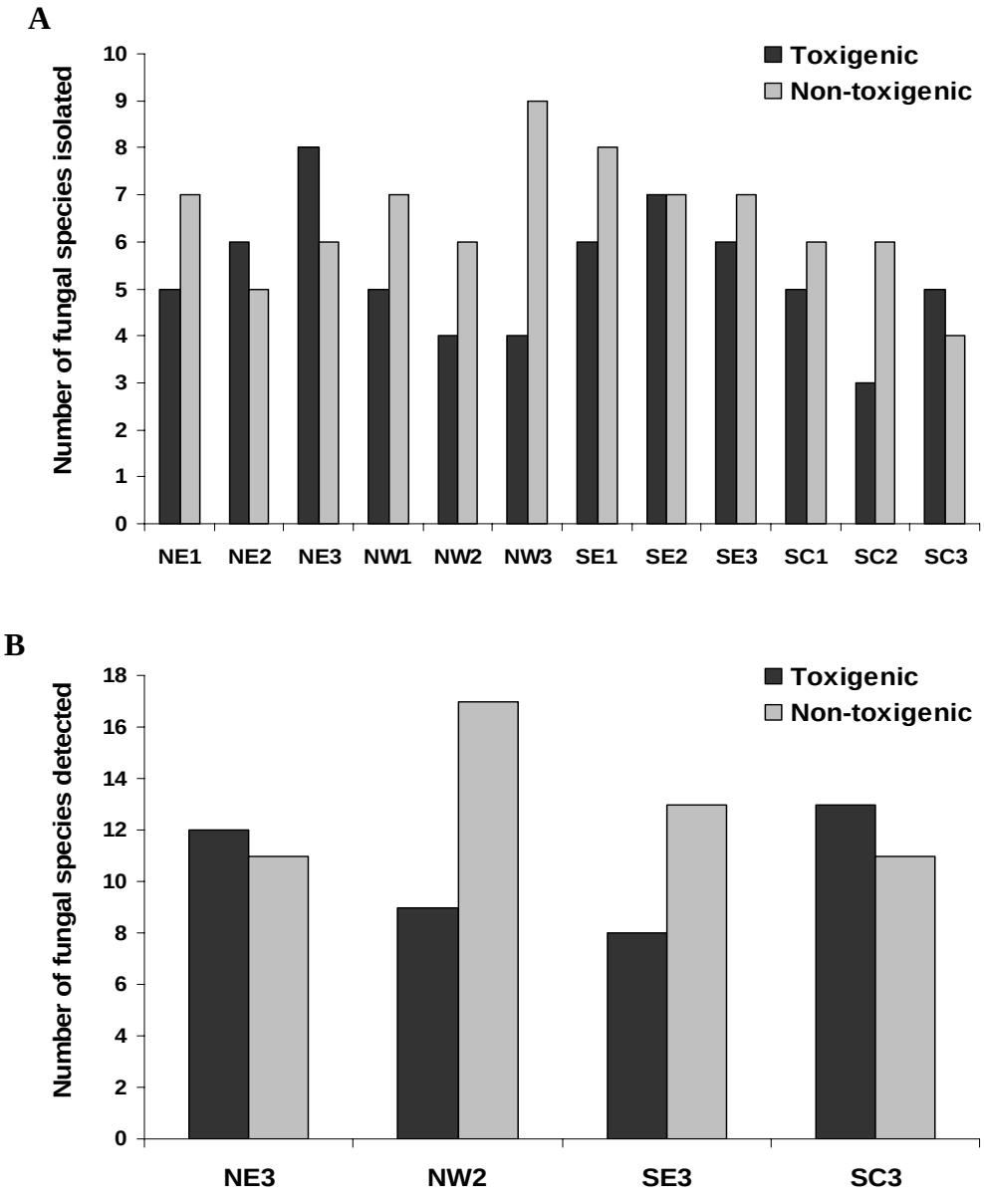
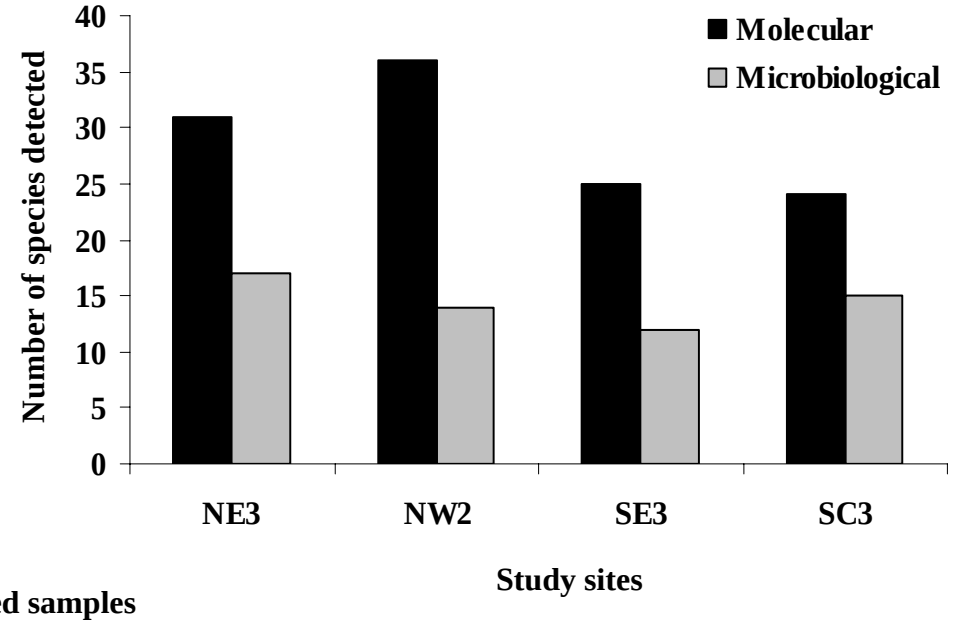


Figure 3-4. Number of species detected in maize silage by microbiological and molecular analysis. Comparison between the number of species detected by each of the study methods. Data are from A. freshly harvested whole plant maize samples and B. ensiled maize.

A – harvest samples



B – ensiled samples

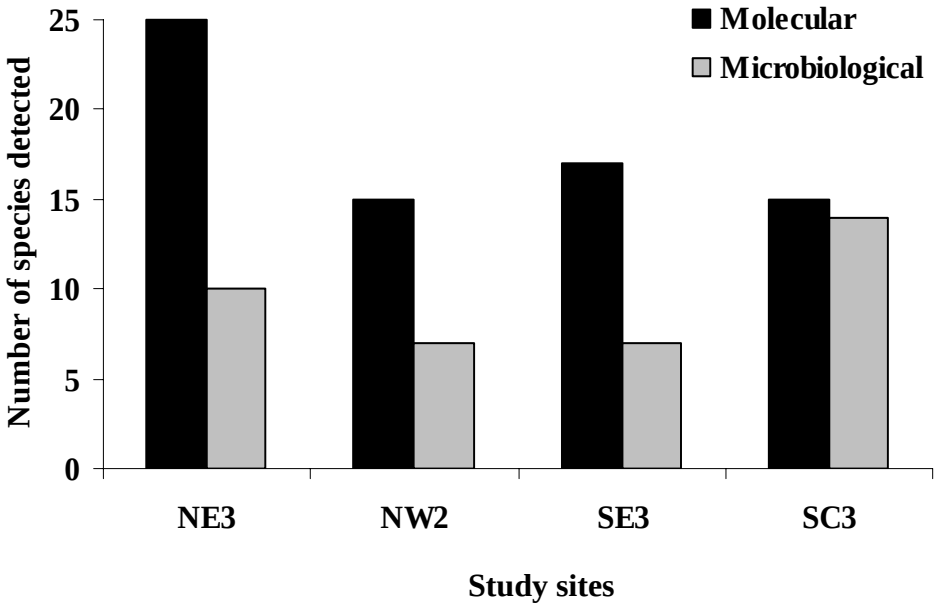
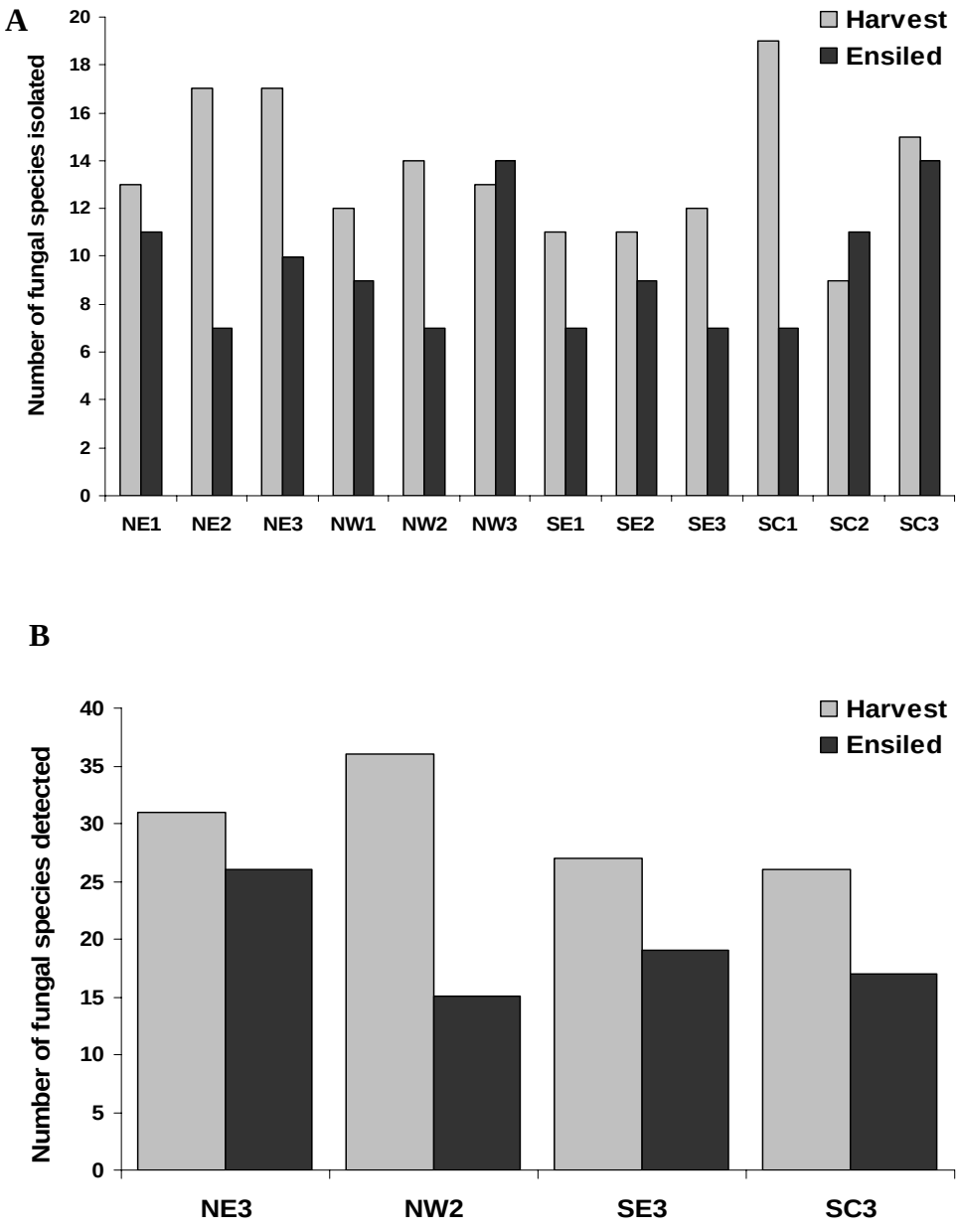


Figure 3-5. Number of fungal species identified from study sites. A. Number of fungal species isolated by plating and B. detected by the molecular method from fresh and ensiled maize.



Chapter 4: Relationships between weather conditions, agronomic practices and fermentation characteristics with deoxynivalenol content in fresh and ensiled maize.

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ABSTRACT

The deoxynivalenol (DON) content of maize silage was determined in samples collected at harvest and after ensiling in 2001 and 2002 from 30-40 Pennsylvania dairies. Information on cultural practices, hybrid maturity, planting and harvest date was collected from each site. Site-specific weather data and a corn development model were used to estimate hybrid development at each site. Correlation analysis was used to assess the relationship between weather data, hybrid development, and cultural practices and pre-harvest DON. Fermentation characteristics (moisture, pH, etc.) of ensiled samples were measured to study their relationship to post-harvest DON contamination. No significant difference ($P \leq 0.05$) was noted between the numbers of samples containing DON in 2001 versus 2002, although concentration was higher in 2002 samples. A positive correlation was observed between DON concentration of harvest samples and daily average temperature, minimum temperature and growing degree day during tasselling, silking, and milk stages. A negative correlation was observed between daily average precipitation at blister stage and DON concentration in harvest samples. Samples from no-till or minimum-till locations had higher DON concentration than moldboard or mixed till locations. Harvest samples had higher DON concentration than ensiled samples, suggesting that physical, chemical or microbiological changes resulting from ensiling may reduce DON in storage.

INTRODUCTION

The mycotoxin deoxynivalenol (DON) is one of the most widely reported natural contaminants of maize and other cereals (188, 225). Several species of *Fusarium* produce DON and its acetylated derivatives, but *F. graminearum* Schwabe is the most commonly isolated DON producer in North America (184). The presence of DON in maize-based feeds, including silage, is associated with animal health problems and poor performance (53, 226, 269, 271), although cattle are less acutely sensitive than other animals (43). However, *F. graminearum* is capable of producing mycotoxins other than DON (89) and is often isolated simultaneously with other mycotoxigenic fungi (17), increasing the risk of synergistic or additive toxic effects on animal consumers (213). To date no studies have investigated the chronic effects of sub-acute levels of DON on dairy cattle.

F. graminearum is a serious pathogen of maize causing Gibberella ear and stalk rot in reference to the telomorph *Gibberella zeae* Petch (190). Much of the research on the relationship between *F. graminearum* and maize has focused on factors that influence infection and colonization in the ear and kernels. However, because maize silage consists of the whole plant, understanding the infection process in vegetative portions of the plant such as the leaves and stalks is also critical. Interestingly, there are reports that the ear and kernels often have significantly lower concentrations of DON compared to the leaves and stalk (62, 125, 130).

There are several routes by which the fungus gains entry into the plant, including wounds by insects and birds (173), seed transmission (109) and potentially root colonization (166). The majority of infections occur approximately 6-8 days after silk emergence when ascospores are moved by wind or conidia by splash dispersal (173, 206, 223, 247). Ear infection may subsequently lead to colonization in areas adjacent to the initial infection including the stalk, leaves and roots (161, 276). Stalk colonization has been reported to increase late in the season (273) and is thought to result from an increase in tissue susceptibility as carbohydrates and other nutrients are redirected to developing kernels (66). Premature ripening caused by early season plant stress may also play a role in stalk infection (273). Stalk infection plays an important role in the epidemiology of Gibberella ear and stalk rot as this tissue is the primary substrate for over wintering and inoculum production the following season (118).

Ideal weather conditions for *F. graminearum* silk infection and spread into the ear are high humidity (≥80%) and sustained temperatures between 24-28ºC (158, 173, 247, 259). Conversely, there is an association between drought-stressed plants and increased stalk colonization (66, 273). Epidemiologically, temperature and moisture appear to be the most influential factors affecting the development of ear and stalk rot (158, 173, 247). This also appears to be the case for DON biosynthesis both *in vitro* and *in planta* (89, 134, 205, 259), which is consistent with reports that DON is a virulence factor in Gibberella rot of maize (93, 204). Optimal conditions for production *in vitro* on maize kernels are temperatures between 28-30 ºC and a moisture content of approximately 30-40% (89, 134). Vigier *et al.* (259), with data from field inoculated ears, used regression analysis to study DON in maize and found that that higher toxin concentrations were associated with a relative humidity ≥ 80% during July to September in eastern Canada . Some variation in DON production is also associated with factors such as fungal strain (257), microbial competition (208) and culture substrate (89). A differential response in DON accumulation has also been observed in relation to maize hybrid types (13).

Prevention of Gibberella ear and stalk rot and DON contamination traditionally focuses on agronomic practices that temporally or spatially separate the crop from fungal inoculum (172). The most important source of inoculum for Gibberella ear and stalk rot is crop debris, particularly maize stalk residue, left on the field after harvest (118). The fungus is also pathogenic on cereals such as wheat, barley, and rye (44) and residue from these crops are also inoculum sources (158, 173, 247). Rotation with a non-host broadleaf crop temporally separates susceptible plants from inoculum and several studies have demonstrated that such a rotation sequence decreased incidence and severity of disease and final DON concentration (132, 229). Tillage systems that are aggressive in burying crop residue physically separate the crop from inoculum and may serve to reduce disease and DON in wheat (64), but this has been an inconsistent trend (173). There has also been considerable focus towards improving host resistance through breeding and genetic engineering, but to date, these strategies have limited success or are still in developmental stages (172). Therefore, there is incentive to develop post-harvest strategies to control the toxin in storage. When performed correctly, ensiling eliminates *F. graminearum* populations, implying that additional DON production is unlikely to occur as long as anaerobic conditions are maintained (87). However, once DON is formed in the field, neither storage nor ensiling have been found to affect toxin concentration (129). Several bacterial strains show promise in their ability to reduce or eliminate DON (73, 97, 233), but no attempts have been made to apply them for post-harvest DON control. Organic acid application is also reported to reduce mycotoxin levels in silage, but the effect on DON is not known (110).

Currently, there are no management practices that provide consistent control of DON in maize silage. This results in part from an incomplete understanding of the factors that influence DON production in parts of the plant other than the ear. To compound this problem, many studies utilize artificial inoculation rather than natural infection, which makes them difficult to relate to field conditions. The effect of agronomic practices on DON in maize is poorly understood and few studies have addressed this issue. In response to these concerns, our objective was to investigate how natural DON contamination in maize silage is affected by different agronomic practices, weather conditions and ensiling. The agronomic practices used to manage the silage in this study were based solely on producers' management decisions and weather conditions and were not under artificial control. It is our hope that this work will improve the current understanding of factors that influence DON in maize silage so that more effective control strategies can be developed to control this toxin.

MATERIALS AND METHODS

Maize silage samples were collected from four regions in Pennsylvania (Fig. 1) in 2001 and 2002 from farms with a history of known or suspected mycotoxin contamination. Thirty-two farms participated in 2001 and thirty-nine in 2002. Samples were collected at harvest and approximately three to six months after ensiling. Harvest samples consisted of freshly chopped maize that was collected from a mechanical harvester as it made several passes within a field intended for ensiling. Samples of ensiled maize were either removed from several locations at the face of a structure (bunker or trench silo), or as the silage was being emptied from a silo during feed-out (upright silo). Sample collection was based on the amount of silage fed per day, with sites feeding 1-2 tons collecting samples every other day for 14 days and sites feeding 3-4 (or more) tons per day collecting for 7 days consecutively. Both the harvest and ensiled samples were mixed by hand and a 1-3 kg portion removed and stored at -80 °C until further analysis.

Analysis of the deoxynivalenol (DON) content of harvest and ensiled samples from 2001 and 2002 was performed by Cumberland Valley Analytical Services Inc. (CVAS) (Haggerstown, MD) using a modified thin layer chromatography method (251) verified by high-performance liquid chromatography (72). The limit of quantification (the amount of toxin that can be reliably quantified by the method 95% of the time) for DON in silage was 0.5 µg of DON per g of silage. Fermentation characteristics of ensiled samples, including percent dry matter, pH, ammonia content, and the concentrations of lactic, acetic, proprionic, butyric, and isobutyric acids were also analyzed by CVAS (2001 and 2002).

All statistical tests used in the data analysis were performed using a 95% confidence level. The Pearson Correlation Coefficient was used to examine the relationships between silage fermentation characteristics and DON concentration in the ensiled samples from 2001 and 2002. The Kendall Correlation Coefficient was used to evaluate the relationship between fermentation characteristics and the frequency of DON contamination in ensiled samples (incidence). This nonparametric test was deemed necessary because of the binary nature of the DON incidence data (1 = DON presence and 0 = DON absence). These data violated the requirements of the parametric correlation procedure (Pearson's), which assumes normally distributed data and continuous variables. The nonparametric procedure also allowed for the detection of non-linear relationships. Both correlation coefficients were calculated using the PROC CORR procedure in SAS statistical software (SAS Institute Inc., Cary, NC.).

Using the non-parametric Mann-Whitney test, DON incidence was compared between the 2001 and 2002 growing seasons and between harvest and ensiled samples (Minitab Inc., State College, PA). The non-parametric Kruskal-Wallis test was used to compare DON incidence (2001 and 2002 harvest and ensiled samples) between the four maize silage production regions in Pennsylvania. Non-parametric tests

were applied to DON incidence data to avoid violating the assumptions of equivalent parametric tests. DON concentration was compared between samples from the 2001 and 2002 growing seasons and between the harvest and storage samples using the paired t-test. DON levels (2001 and 2002 harvest and ensiled samples) between the four regions of Pennsylvania were compared using a general linear model.

ZedX Inc. (Bellfonte, PA) provided estimated daily weather data for each site using algorithmns (136) and the coordinates and elevation of each location. Weather variables included the minimum, maximum, and average temperature, total precipitation, average relative humidity, total wetness hours, growing degree day (base 10 ºC), accumulated growing degree day (with day 1 beginning at planting date), precipitation minus evaporation, and the accumulated precipitation minus evaporation (with day 1 beginning at the planting date). Using planting date information and hybrid type planted at each farm, ZedX, Inc. provided a daily prediction of hybrid growth during the growing season using a method developed from the work of Allen (9). Prior to data analysis, the daily weather variables for each site were averaged for each growth stage. For example, if silking occurred over a five day period at a site, five days of data for each variable were averaged and the single value used to represent conditions during silking. Growth stages of particular interest included tasselling (VT) through physiological maturity (R6) (214). Pearson's correlation was used to determine the relationship between weather variables during each growth stage and the DON levels in the 2001 and 2002 harvest samples. The relationship between DON incidence in 2001 and 2002 harvest samples and weather variables was assessed using Kendall's correlation.

During silage harvest, information on agronomic practices used to manage the crop was collected. Practices under consideration included whether rotation with a broadleaf crop occurred, the type of tillage system used, if an inoculant of lactic acid bacteria or an organic acid was added prior to ensiling, and the type of silo used to store the feed. Crop rotation was designated by whether the producer did [1] or did not [0] rotate corn with a broadleaf crop. Tillage systems utilized were classified as no till [1], minimum till [2], moldboard or deep till [3] and mixed tillage [4], where a variety of systems were used to manage crop residue. No till would be considered the least aggressive system in terms of burying crop residue, followed by minimum tillage, mixed tillage, and finally moldboard tillage, which would bury the most debris. Inoculant use was indicated by use [1] or omission [0] prior to ensiling. Silo type was categorized as upright [1], trench or bunker [2], or a bag silo [3]. Kendall's correlation was used to determine agronomic practices related to DON incidence and levels in harvest and ensiled samples. Practices found to have a relationship to DON incidence or levels were further evaluated with the Tukey-Kramer test to determine differences between categorical levels.

RESULTS

The incidence of DON contamination in silage samples (harvest and ensiled) was not significantly different between the two growing seasons (P=0.2644). In 2001, 84% of the total number of samples (n=62) contained DON, while 66% were contaminated in 2002. Although incidence was not different between seasons, there were significantly higher levels of DON present in 2002 (harvest and ensiled) compared to 2001 ($P \leq 0.0001$) (Fig. 2). The average DON concentration of 2001 (harvest and ensiled) samples was 0.8 µg/g (range: 0-3.7 µg/g), while the average for 2002 was 1.1 µg/g (range: 0-5.1 µg/g). Of the total number of 2002 samples (n=62), 68% had higher concentrations of DON than 2001 samples with 42% having ≥1 µg/g DON than their 2001 counterparts. When DON incidence was compared between harvest and ensiled samples from 2001 and 2002, harvest samples were more frequently contaminated than ensiled samples ($P \leq 0.0001$). For example, while 75% of the total number of harvest samples (n=71) contained DON, only 42% of the ensiled samples were positive. DON concentration was also different between harvest to ensiled ($P \leq 0.0001$) (Fig. 3). The average DON concentration of harvest samples (2001 and 2002) was 1.4 µg/g (range: 0-5.1 µg/g), while the average of ensiled samples (2001 and 2002) was 0.6 µg/g (range: 0-3.7 µg/g). In 2001, 68% of the harvest samples had ≥1 µg/g DON than their ensiled counterparts, whereas in 2002 39% of the harvest samples had ≥1 µg/g DON. Neither DON incidence nor concentration was significantly different between the regions for either year (P=0.4628).

Weather conditions and seasonal plant development. Statewide, both the 2001 and 2002 growing seasons (April to October) had higher than average temperatures. Out of 108 recorded growing seasons, 2001 ranked 53rd and 2002 6th as the warmest seasons on record (National Climatic Data Center, Ashville, NC). Both seasons also received less than average precipitation, with 2001 ranking 28th and 2002 24th in terms of being the driest years.

Comparison between the seasons found that the 2002 crop took fewer days to reach several developmental stages compared to 2001 despite the fact that the planting dates were comparable (2001 – day 124/365 and 2002 – day 129/365) (Fig. 4). A paired t-test indicated these differences to be significant for tassel (VT) (P=0.013), silking (R1) (P=0.015), blister (R2) (P=0.024) and milk (R3) (P=0.010) stages with the average harvest maturity in 2001 at dough (R4) stage and in 2002 at dent stage (R5). The recommended stage for silage harvest is physiological maturity (R6) (216).

Temperature and moisture variables during VT, R1, R2, and R3 growth stages had the most significant relationship to DON concentration in 2001 and 2002 harvest samples (Table 1). A positive relationship was observed between DON levels and average daily temperatures during VT, R1, and R3 and

a negative relationship between DON and average daily precipitation during R2. Average daily temperatures and minimum daily temperatures during VT, R2, and R3 were higher in 2002 versus 2001, whereas the average daily precipitation, relative humidity, and wetness hours (only R2) during R2 and R3 were lower in 2002 than in 2001 (Table 1 and Fig. 5). No significant relationship was observed between DON levels and weather conditions from R4 to R6 in 2001 and 2002.

Agronomic practices. Correlation analysis between agronomic practices and DON incidence and levels in harvest and ensiled samples from 2001 and 2002 revealed that only tillage type had a significant affect (*P*=0.0108) and only on DON levels. Neither crop rotation (harvest samples), additive use before ensiling (ensiled samples), nor silo type (ensiled samples) had an effect on DON incidence or levels. Although tillage type did not significantly affect DON incidence (*P*=0.960), it did impact DON concentration (*P*=0.031) (Fig. 6). The average DON concentration in samples (harvest and ensiled from 2001 and 2002) managed by different tillage systems is as follows: no till 2.4 µg/g (range: 0-5.1 µg/g), minimum till 1.7 µg/g (range: 0-5.1), mixed till 1.1 µg/g (range: 0-4.3 µg/g) and moldboard till 0.6 µg/g (range: 0-1.8 µg/g). Comparison of DON levels between samples under different tillage systems revealed that samples from no till sites had higher DON concentrations than moldboard (deep tillage) or mixed tillage samples (Fig. 6). The average DON content from no till sites was approximately four times higher than samples from moldboard sites and twice as high as samples from mixed till sites. Although the average DON concentration in minimum till samples was lower than no till by an average of 0.7 µg/g, the difference was not significant.

Forage quality. Fermentation analysis on percent dry matter, pH, ammonia content, lactic acid, acetic, proprionic, butyric, and isobutyric acid content found that the majority of ensiled samples (2001 and 2002) were in the range of what is considered normal for maize silage (216). No relationship was observed between percent dry matter, pH, ammonia content, lactic acid, acetic, proprionic, butyric, and isobutyric acid content and DON incidence or DON levels in the samples.

DISCUSSION

The levels and frequency of DON contamination in maize silage from this study are comparable to those reported for maize grain and silage samples from other North American studies (188, 225, 280). In general, the concentrations of DON encountered in this study were not high enough to warrant excessive concern for dairy cattle health. We did find it surprising that DON levels in 2002 were significantly higher than in 2001, as this season was a drought year. Numerous studies have demonstrated that warm temperatures in combination with frequent precipitation are favorable for *F. graminearum* infection,

colonization and DON production in maize (158, 207, 243, 247, 259). However, the observation that DON concentration is higher in warm, wet years versus hot, dry seasons is based on studies of the ear and grain, not the whole plant (126, 205, 207, 208, 243, 259). Although temperatures in 2002 were closer to the optimal DON production temperature of 28ºC (89, 134), the moisture availability in this season was less than 2001, particularly during the R2 and R3 stages of development. Dodd (66) noted that there was an association between drought stress and increased stalk rot. Therefore, drought stress in the 2002 crop may have resulted in increased fungal colonization in the stalk and subsequently, higher levels of DON compared to 2001. We also observed that the 2002 crop matured more quickly but remained in the field an equal amount of time as 2001 silage. Several studies have found that premature maturation and a delay in harvest are associated with greater disease severity and higher final DON concentration (66, 126, 273). However, because plants were not evaluated for fungal colonization, it is difficult to say the conditions we observed are the only elements contributing to higher DON in 2002.

Our observation that temperature and moisture were important factors in influencing DON concentration in maize agrees with findings reported by other researchers (205, 207, 208, 243, 259). While temperature (within the temperature range considered in this study) had a positive relationship to DON, indicating that as temperature increased DON concentration did as well, moisture had a negative affect in that as moisture decreased, DON increased. These relationships again reflect that warm temperatures and decreased moisture availability may have resulted in plant stress, increased fungal colonization and greater DON accumulation, rather than suggesting that warm temperatures and low moisture availability are directly responsible for increased DON production *in planta*.

The timing of potential drought stress may also play an important role in final DON concentration, as weather conditions during tassel (R1) to milk (R3) had the strongest correlation to DON levels. During these stages, the majority of plant nutrients are diverted to embryo and kernel development, somewhat to the detriment of vegetative tissue like the stalk. Therefore, stressful conditions during these developmental periods may have an additive effect on stalk susceptibility to *F. graminearum* infection.

Although weather conditions likely have the greatest influence on DON in maize silage according to our findings and those of previous research (205, 207-209, 243) some affect may also be attributed to agronomic practices, including crop rotation and tillage type. Studies with wheat found that less DON was present in the grain when it was grown after a broadleaf crop then when it was grown after wheat or maize (229). Ear rot severity in maize decreased when rotation with a broadleaf crop was incorporated in corn production (132) but the implications for DON accumulation under non-inoculated and non-irrigated

conditions are not clear (172, 209). The results of this investigation indicate that crop rotation with a broad leaf crop did not significantly impact DON contamination of maize silage. However, few sites included in this study practiced crop rotation, limiting our ability to detect differences. Further research is needed to clarify the influence of crop rotation on DON contamination.

In this study we observed that while tillage type had no effect on DON incidence, it did influence DON concentration. This is somewhat surprising because one might expect that burying crop debris would reduce disease and DON incidence rather than DON concentration. Flett *et al.* (78) found no relationship between tillage practices and severity of Gibberella ear rot; however the impact on DON accumulation was not considered. Synthesis of previous reports with the current study suggests that deep tillage alone is unlikely to adequately reduce DON incidence and concentration. Although we observed that silage from moldboard tilled sites had the lowest DON concentration, deep tillage is known to reduce soil moisture retention (21). As such, deep tillage may exacerbate the mycotoxin situation by intensifying drought stress and predisposing the crop to stalk rot. Until the impact of tillage on DON in maize is more fully understood, an approach to mycotoxin management that integrates disease control strategies, including tillage, crop rotation and host resistance, along with other critical factors such as soil conservation and financial considerations of the farm, is likely to be the most successful in the future.

In contrast to previous reports that DON concentration does not change after ensiling (129), we found that DON concentration was lower in ensiled samples compared to those collected at harvest. When we evaluated the relationship between physical and chemical characteristics of ensiled samples, we found that none of the factors considered had a significant affect on DON concentration. Therefore, we think that toxin reduction may be due to the activity of silage microflora. Several bacteria have been found to degrade or bind DON to their cell membranes (73, 97, 233) when cultured *in vitro*. Interestingly, microbial degradation also appears to take place in the rumen of cattle and other animals (113, 116) and it is possible that animals acquire these organisms by consuming silage. Our findings lend credence to the idea that DON may be managed in a post-harvest situation. Future work should potentially focus on isolating organisms in silage that can degrade DON and determining how their activity can be enhanced in contaminated silages.

To our knowledge, this is the first study to investigate the combination of weather conditions, agronomic practices and ensiling on natural DON contamination at a large number of locations. Although managing pre-harvest DON contamination may be difficult due to the large and relatively uncontrollable affect of weather, utilizing a combination of pre- and post-harvest control strategies may prove to be fruitful.

Likewise, future studies should focus on improving our understanding of DON accumulation in the whole plant and determine if post-harvest toxin degradation can be enhanced in maize silage.

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Table 4-1. Relationship between DON content in maize at harvest and weather conditions during select maize developmental stages in 2001 and 2002.

Growth stage[a]	Weather variables[b,c]	2001[d] Average 221	2002[d] Average 221	Correlation coeff.[e]	p-value
VT	Min. temp. (ºC)	17.4 (14.4-20.1)	18.3 (11.8 - 21.8)	0.395	0.001
	Ave. temp. (ºC)	22.8 (20.1-25.2)	23.6 (18.7-26.6)	0.324	0.006
	AGDD[c]	3820.1 (3858.6-4294.5)	4085.7 (3605.8-4767.0)	0.332	0.004
R1	AGDD[c]	3996.0 (3758.3-4487.8)	4267.3 (3751.5-4966.0)	0.324	0.005
R2	Precip. 92	0.26 (0.14-0.53)	0.12 (0.05-0.23)	-0.402	>0.0001
	RH (%)	73.7 (69.3-77.8)	68.6 (60.2-76.9)	-0.378	0.001
	WH (hr.)	12.4 (9.7-16.2)	7.1 (3.2-14.3)	-0.446	>0.0001
R3	Min. temp. (ºC)	13.1 (8.2-20.1)	16.3 (11.5-21.1)	0.257	0.030
	Ave. temp. (ºC)	18.6 (13.7-24.5)	22.0 (17.7-27.1)	0.235	0.047
	AGDD[c]	5015.6 (4653.3-5488.0)	5222.9 (4550.3-6036.0)	0.235	0.047

[a] Growth stages: VT=tassel, R1=silking, R2=blister, R3-milk (ref).
[b] Weather variables: Min. temp.= minimum temperature, Ave. temp. = average temperature, AGDD= accumulated growing degree day, Precip.= precipitation, RH= relative humidity, WH=wetness hours.
[c] Accumulated growing degree day shown has a base of 50 ºF.
[d] Data shown are the averaged values for each growing stage.
[e] Pearson's correlation coefficients were calculated for the combined data set from 2001 and 2002 (n=124 samples).

Figure 4-1. Relative location of sample dairies in the four major production areas of Pennsylvania. Dairies are represented by region as triangles ▲ in the Northwest, ★ in the Northeast, ● in the South central, and ■ in the Southeast.

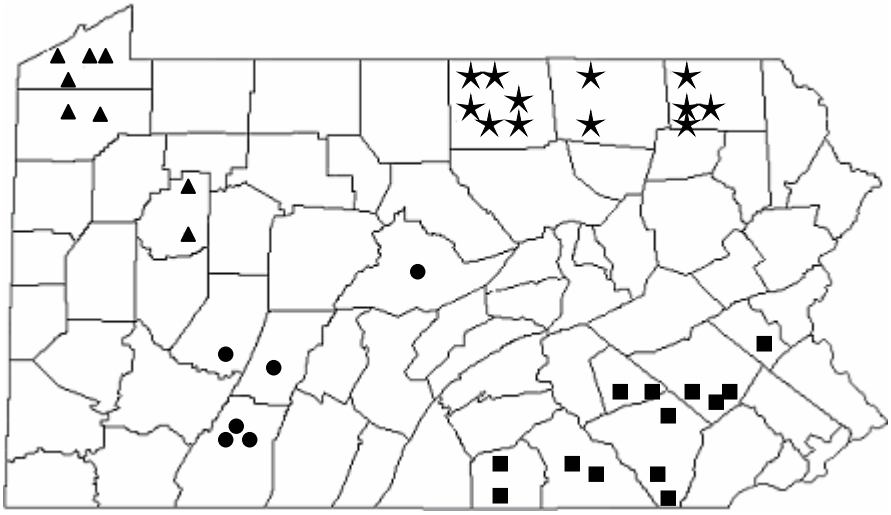


Figure 4-2. Average DON concentration in maize at harvest and after ensiling from 2001 and 2002. Asterisks over 2002 sample bars had significantly higher concentrations of DON than their 2001 sample counterparts.

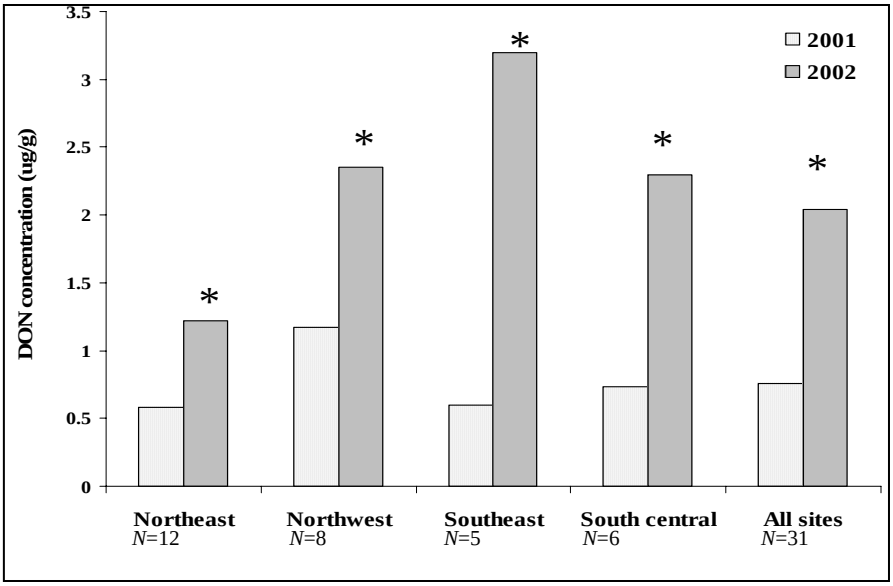


Figure 4-3. Average DON concentration in maize at harvest and after ensiling in 2001 and 2002. Asterisks over harvest sample bars indicate that these samples had significantly higher concentrations of DON than their ensiled counterparts.

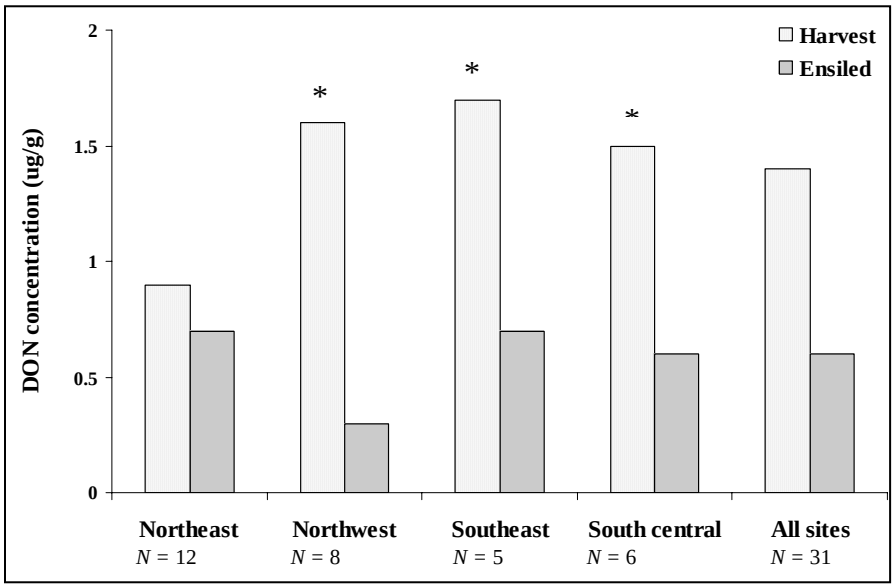


Figure 4-4. Number of days in 2001 and 2002 from the planting date to each developmental stage considered in the study. Asterisks over 2001 bars indicate that it took significantly more days to reach a growth stage compared to the 2002 crop. Developmental stages are as follows: VT=tasselling, R1=silking, R2=blister, R3=milk, R4=dough, R5=dent and R6=physiological maturity (45).

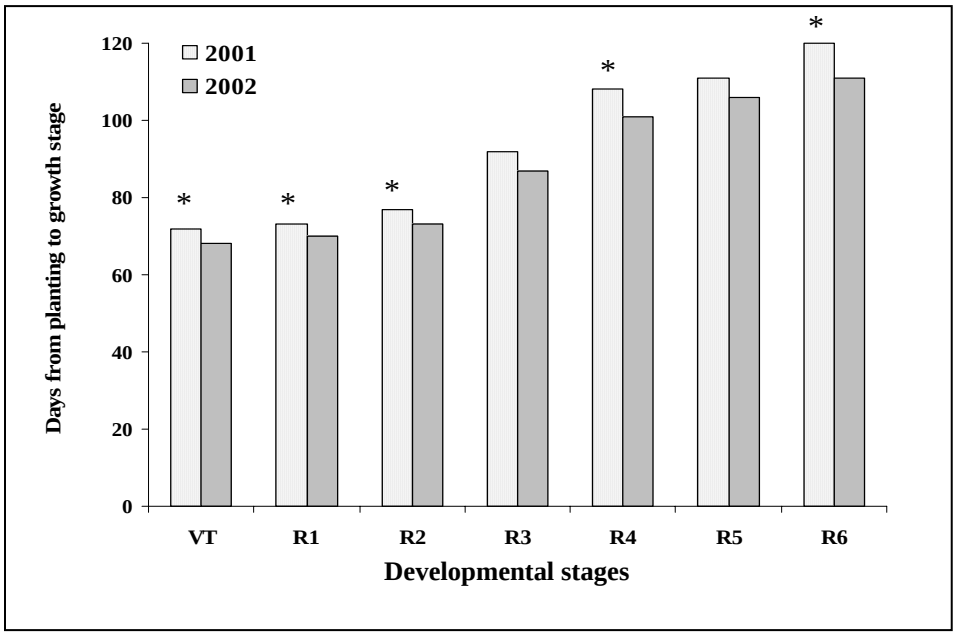


Figure 4-5. A. Comparison of DON concentrations in 2001 and 2002. DON concentration is expressed in parts per million (ppm) which is equivalent to µg of DON per g of silage. B. Comparison of averages and ranges of weather variables in 2001 and 2002 during B. VT, C. R2 and D. R3 stages.

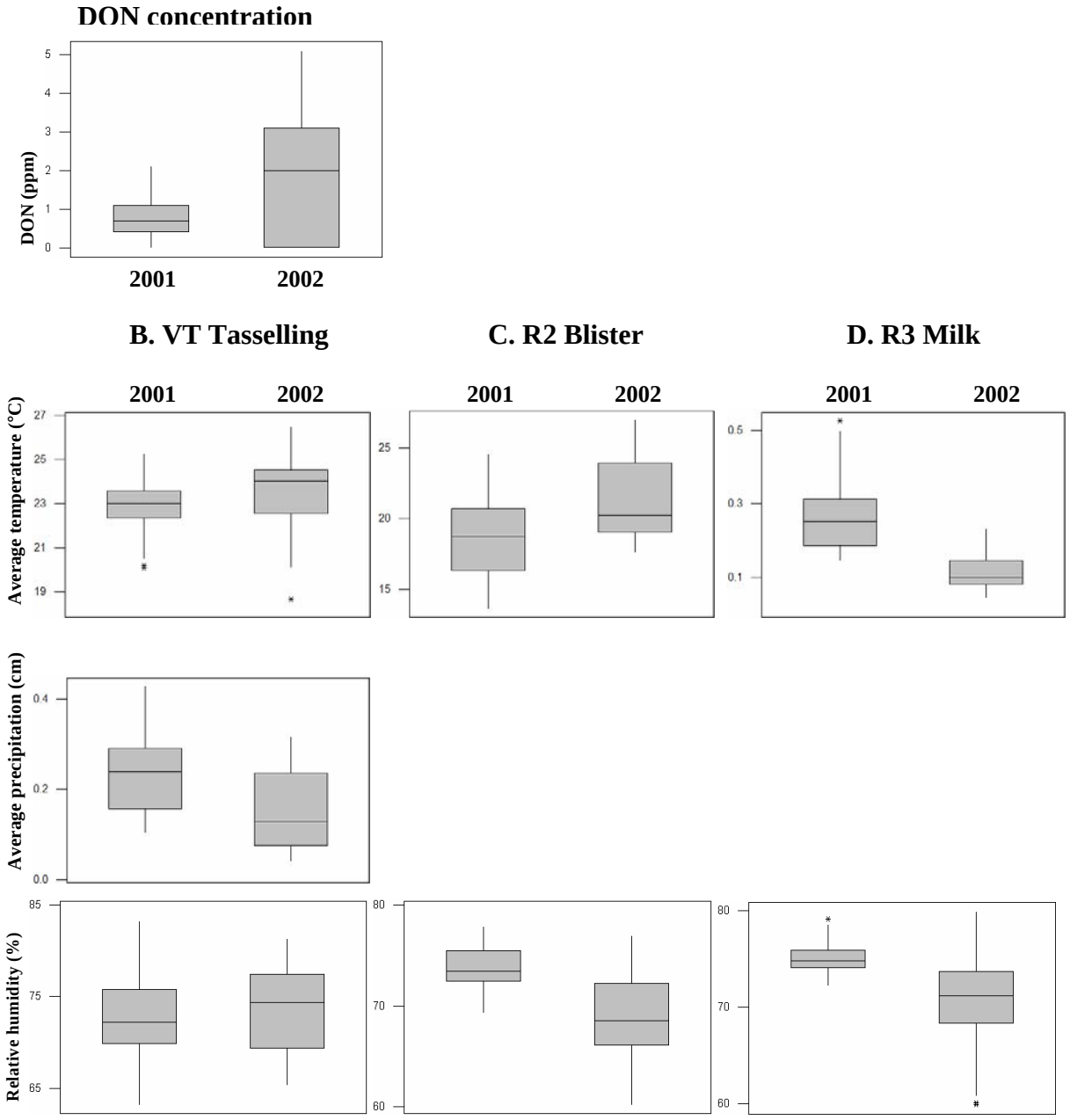
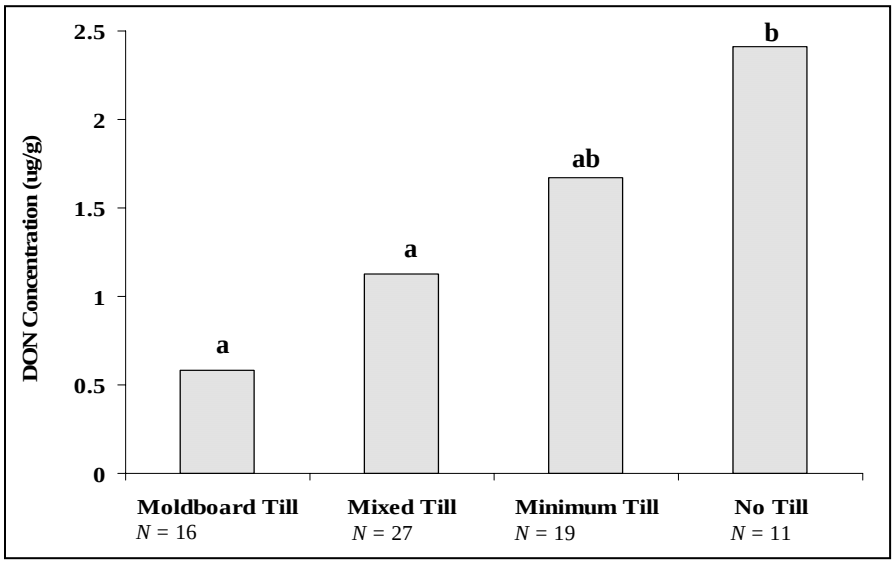


Figure 4-6. Average DON concentration in 2001 and 2002 silage samples managed under different tillage systems. Mixed tillage refers to systems where more than one tillage type was used. Letters over bars indicate significant differences in DON concentration between tillage systems detected by the Tukey-Kramer test. Bars marked by the same letter are not significantly different.



Chapter 5: Analysis of sphinganine analog mycotoxins in fresh and ensiled maize (*Zea mays*) to examine the impact of weather conditions, agronomic practices, and silage characteristics on mycotoxin frequency and concentration.

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ABSTRACT

Spinganine analog mycotoxins are fungal contaminants reported in maize and maize based feeds. Our objectives were to detect and quantify fumonisin B_1, B_2, AAL-TA and TB to determine how agronomic practices, weather conditions and ensiling affected the occurrence and levels in maize silage. Silage was collected at harvest and after ensiling in 2001 and 2002 from 30-40 Pennsylvania dairies. SAMs were quantified using HPLC and HPLC-MS. The average concentration and range of SAMs in silage are as follows: fumonisin B_1: 2.02 µg/g (0.20-10.1), fumonisin B_2: 0.98 µg/g (0.20 – 20.3), AAL-TA: 0.17 µg/g (0.20 – 2.0) and TB: 0.05 µg/g (0.03 – 0.90). Fumonisn B_1 was the most frequently detected toxin (92%), followed by fumonisin B_2 (55%), AAL-TA (23%) and TB (13%). Temperature during maize development was positively correlated with fumonisin occurrence and levels and negatively with AAL-TA, while moisture variables were negatively correlated with fumonisins and positively with AAL-TA. Fumonisin levels were higher in silage harvested at later developmental stages (R4 dough through R6 physiological maturity). Ensiling did not affect toxin concentration nor did the agronomic practices (rotation with a broadleaf crop, tillage system, inoculant use, silo type) or silage characteristics considered (dry matter, pH, organic acid concentrations) in this study. This is the first report of AAL-TB in silage as well as the first to report on factors that affect SAM frequency and levels in whole plant maize silage.

INTRODUCTION

Sphinganine analog mycotoxins (SAMs) are structurally similar compounds (Fig. 1) produced by members of the fungal genera *Fusarium* and *Alternaria* (163, 179, 211). Two classes of SAMs, the fumonisins and AAL toxins (AAL-TA), have been reported in maize-based feeds including silage (114, 142, 199, 280). Although both classes of SAMs have similar cytotoxic activity (231), the fumonisins are considerably more toxic (258), are associated with several human and animal diseases (45, 142, 143, 179, 215), and are federally regulated in foods and feeds (162). To date, the cellular affects of AAL-toxin exposure have been studied and found to be similar to those caused by fumonisins (3, 4); however, the toxicological impact on humans and animals is unclear. Feeding studies with fumonisin B_1 have revealed that although cattle are not acutely sensitive to this toxin (186), ingestion resulted in abnormal hepatic and

immune system pathology (150). To our knowledge, no studies have investigated chronic effects on dairy cattle exposed to sub-acute levels of fumonisins or AAL-toxins. An additional concern is that SAMs do co-occur with other mycotoxins (280), increasing the risk of additive or synergistic toxicological affects (213).

SAM-producing fungi are diverse in terms of their behaviors toward maize and the niches they inhabit. The most commonly encountered fumonisin-producing *Fusarium* spp. are *F. verticillioides* (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis*) and *F. proliferatum* (Matushima, T) Nirenberg (teleomorph *Gibberella fujikori* mating population D). Although both species cause an ear rot referred to as Fusarium ear rot (190), *F. verticillioides* is also an endophyte of maize (79, 122, 178). This endophytic association is concerning as fumonisins may still be present in infected plant parts even in the absence of symptoms (61). In contrast to *Fusarium* species, *Alternaria* spp., such as *Alternaria alternata* and *A. tenuissima* (138, 171) may be found as epiphytic saprophytes(30) although they may act as opportunistic pathogens if the plant is wounded (190) and as the plant naturally senesces during the growing season. Both species have been reported on maize and in silage (8, 120, 138, 171).

Thus far, studies on fumonisin have been limited to understanding factors that affect toxin contamination of the ear and grain rather than the whole plant, which is significant for understanding toxin accumulation in silage. High levels of fumonisin in maize grain are often associated with warm weather (260) as 30 ºC is optimal for toxin production *in vitro* (145). Fumonisin contamination is also dependent on other factors, including fungal strain (61), maize genotype (47, 189), plant maturity (37, 266), interactions with other fungi (146, 255) and insect wounding (68, 240). Much less is known about factors that affect *Alternaria* growth and there have been no published reports on conditions for toxin production on maize. Torres et al (249) found that when *A. alternata* was grown on maize kernels at temperatures of 20º and 30º C and water activities of 0.92, 0.95 and 0.98, the fungus was able to grow at all conditions studied, although optimal growth occurred at 30º C with 0.98 a_w. Under field conditions, high frequencies of *Alternaria* have been found on cereals during sustained periods of warm, wet weather (88).

Current management of fumonisin contamination in maize relies on the use of resistant hybrids and cultural practices (47, 70, 172, 189), although other strategies, such as the use of transgenic maize lines, microbial biocontrol and *in planta* detoxification by genetically modified plants are under investigation (20, 25, 26, 41, 70, 174). Natural resistance to Fusarium ear rot pathogens and fumonisin contamination appears to be contingent on a number of physical and genetic characteristics, which do not always provide consistent control (47, 98, 265). Transgenic hybrids, particularly Bt lines, have been recommended as their toxicity towards lepidopteran larvae results in less feeding damage and generally lower concentrations of

fumonisin than non-transgenic isolines (92, 174). However, the cost-effectiveness of these hybrids over traditional lines may vary by region (65, 69). The impact of cultural management practices has been the focus of few investigations, however, the information provided by Flett *et al* (78) found that different tillage systems had no affect on Fusarium ear rot. Post-harvest approaches, such as identifying microorganisms that degrade fumonisin (26, 40, 41), are also under investigation, although most studies are still in preliminary stages. Commercial binding agents have also received some attention and some show promise for reducing the biological availability of fumonisin (16), although this has yet to be tested with ruminants. As the overall risk of AAL-toxin contamination of maize is not well understood, to date, no effort has been made to address control measures.

The current inability to prevent or control SAM contamination of maize –based feeds, including silage, is likely due to a lack of understanding factors that are conducive or inhibitory to toxin formation. Although there is a significant amount of information on fumonisin production in maize grain, it is not known whether conditions affecting toxin occurrence in grain are the same for a whole plant feed such as silage. Additionally, the prevalence and levels of AAL-toxins in maize silage has not been studied rigorously and little is known about stimuli that impact toxin occurrence. As such, our objectives were to (1) determine the levels and frequencies of fumonisins B_1, B_2, AAL-toxins TA and TB and (2) to investigate how weather conditions, agronomic practices and the process of ensiling affect the concentration and frequency of SAMs in maize silage.

MATERIALS & METHODS

Silage collection

Maize silage was collected from four regions in Pennsylvania (Fig. 2) at dairies with a history of known or suspected mycotoxin contamination. In 2001, thirty-two farms participated in the study and in 2002, thirty-nine. Samples were collected at harvest and three to six months after ensiling to compare the affect ensiling had on the content of SAMs in silage. Harvest samples consisted of 1-3 kg freshly chopped maize, while samples of ensiled maize were either removed from several locations at the face of a structure (bunker or trench silo), or as the silage was being emptied from a silo during feed out (upright silo). Silage collection was based on the amount of silage fed per day, with sites feeding 1-2 tons collecting samples every other day for 14 days and sites feeding 3-4 (or more) tons per day collecting for 7 days consecutively. After collection, samples were stored at -80 °C until analysis.

Extraction and purification of SAMs

Prior to toxin extraction, silage samples were dried at 55 ºC for 5-7 days, milled to a flour-like consistency, mixed sub-sampled in 500 g quantities for analysis. Using a method modified from Syndenham et al (248), 25 g of ground silage was extracted with 100 mL of HPLC-grade methanol-water (3/1 v/v) and placed on a rotary shaker at 310 rpm for 1 hour. The samples were then centrifuged at 6000 x *g* for 10 min. Purification was performed using 8 mL polypropylene columns (Alltech, State College, PA) packed with 500 mg of strong anion exchange media (SAX) (Bondesil, Varian, Palo Alto, CA) and polypropylene frits with a 20 µm pore size (Alltech). Columns were primed by passing 5 mL of methanol through followed by 5 mL of methanol/water (3/1 v/v). The pH of each sample was checked with an pH probe, adjusted to 5.8-6.5 with 2-3 drops of 1 M NaOH and 10 mL passed through the SAX column. The column was then washed with 5 mL of methanol/water (3/1 v/v) and 3 mL of methanol. SAMs were eluted by adding 10 mL of methanol amended with 1% glacial acetic acid. Sample eluate was concentrated to dryness under a stream of nitrogen, resuspended in 200 µL methanol and stored at -20 ºC prior to analysis. Toxin was extraction was done twice from two sub samples of each silage sample. The results of each extraction were then compared and if the difference was geater than 1.0 µg/g for the fumonisins and 0.5 µg/g for AALTA and TB, the extraction procedure was repeated with a third sub sample.

SAM standards

A mixed standard of fumonisin B_1 (100 µg/mL) and B_2 (75 µg/mL) (Romer Labs Inc., Union, MO) was used to determine toxin frequency and quantity by HPLC analysis. AAL toxin TA and TB were generated from culture ATCC 28329 of *A. arborescens* from the American Type Culture Collection (Manassas, VA) to generate a standard to assess method recovery and quantitative analysis. The fungus was grown on Potato Dextrose agar (Difco, Sparks, MD) for 7 days at room temperature and 2 mL of sterile distilled water were added to the plate to create a spore suspension. Rice cultures were prepared by adding 2 g of rice to a 15 mL polypropylene tube, adding 2 mL of water, incubating the cultures at room temperature for 4 h and autoclaving at 121 ºC for 30 min. After autoclaving, 500 µL of spore suspension was added to the cultures, which were then incubated in the light at room temperature for 14 days. Cultures were extracted as described above although they were proportionately scaled down to extract 2 g of material.

SAM derivatization

For fluorescence HPLC detection, samples were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) (Sigma-Aldrich, St. Louis, MO) (273) using a method modified from Manica *et al*

(137). For each silage extract, 12.5 µL was transferred to a new autosampler vial and desolvated under nitrogen. The sample was resuspended in 179 µL of borate buffer (10 mM, pH 9.2) to which 8 µL of potassium cyanide solution (80 mM in borate buffer) was added followed by 16 µL of NDA solution (55 mM in acetonitrile). Samples were then incubated for 15 minutes at room temperature and filtered with a 0.2 µm PTFE filter (Pall Life Sciences, East Hills, NY).

Detection of fumonisins B_1 and B_2 by HPLC

Fumonisins were detected and quantified using an Agilent 1100 HPLC with fluorescence detection (Agilent, Palo Alto, CA). Chromatographic separation was achieved using a reverse phase Thermo Betasil C-18 column (150 x 2.1 mm; 5 µM particle size, pore size 60 Å) (Thermo, Bellefonte, PA). Solvents consisted of: A – water 1% acetic acid, B – acetonitrile 1% acetic acid and C – methanol. The program followed a linear gradient beginning with A at 100% to 70% and B from 0% to 30% over 5 minutes, followed by A at 70% to 24% and B from 30% to 76% from minutes 5 to 38, holding at C for 38 to 42 minutes and re-equilibrating at A from 42 to 46 minutes before the next injection. Total run time was 46 min. Fluorescence detection was set at 420 nm excitation with a 480 nm emission for NDA-derivatized SAM. Data were analyzed using ChemStation for LC Rev. A.08.01 analysis software (Agilent, Palo Alto, PA).

HPLC-MS Analysis of AAL toxins TA and TB

Preliminary analysis indicated that although the fumonisins were well resolved from the silage matrix and from one another by the chromatography method described above, the AAL toxins were not easily separated from components of the silage matrix. Therefore, we chose to analyze these toxins by LC-MS. Twenty-five µL of silage extract were transferred to an amber autosampler vial, desolvated under nitrogen to dryness and resuspended in 496 µL of acetonitrile. Four µL of retinoic acid (in a stock acetonitrile at a concentration of 1 µg/µL) at a concentration of 1 µg/µL (Sigma, St. Louis, MO) was then added to each sample as an internal standard. The solvent delivery system consisted of a LC10ADvp ternary solvent delivery system (Shimadzu, Columbia, MD) to which a Quattro II mass spectrometer (Micromass, Beverly, MA) was attached for detection. Solvents for HPLC-MS analysis were the same as A and B described above. The method began with A at 100% following a linear gradient to 30% and B at 0% going to 30% from time 0 to 2 min, followed by A from 70% to 24% and B from 30% to 76% from 2 to 12 min, a wash with 100% B from 12 to 14 min followed by a re-equilibration step at 100% A from 14 to 15 min. Negative electrospray ionization (ESI-) was used to detect AAL-TA and TB by MS analysis and the instrument was set up for selected ion monitoring for retinoic acid (m/*z* 299), AAL-TA (m/*z* 520) and TB (m/*z* 504). Data analysis was performed using Mass Lynx v.3.5 mass spectrometry software (Milford, MA).

Evaluation of agronomic practices, weather data and silage characteristics

Information was collected from each of the study dairies regarding the agronomic practices they used to manage the crop. These practices included whether rotation with a broadleaf crop occurred, the type of tillage system used, if an inoculant of lactic acid bacteria or an organic acid was added prior to ensiling and the type of silo used to store the feed. Crop rotation was designated by whether the producer did [1] or did not [0] rotate corn with a broadleaf crop. Tillage systems utilized were classified as no till [1], minimum till [2], moldboard or deep till [3] and mixed tillage [4], where a variety of systems were used to manage crop residue. Silo type was categorized as upright [1], trench or bunker [2], or a bag silo [3].

ZedX Inc. (Bellfonte, PA) provided site-specific estimated daily weather data for each dairy using algorithms (136) and the coordinates and elevation of each location. Weather variables included the minimum, maximum, and average temperature, total precipitation, average relative humidity, total wetness hours, growing degree day (base 10 ºC), accumulated growing degree day (with day 1 beginning at planting date), precipitation minus evaporation, and the accumulated precipitation minus evaporation (with day 1 beginning at the planting date). Using planting date information and hybrid type planted at each farm, ZedX, Inc. provided a daily prediction of hybrid growth during the growing season using a method developed from the work of Allen (9). Growth stages included in analysis were tasselling (VT) through physiological maturity (R6) (214).

Biochemical evaluations of silage quality for 2001 and 2002 ensiled samples, including percent dry matter, pH, ammonia content, and the concentrations of lactic, acetic, propionic, butyric, and isobutyric acids were performed by Cumberland Valley Analytical Services Inc. (Haggerstown, MD) (1).

Statistical analysis

All statistical tests for data analysis were performed at a 95% confidence level. Using the non-parametric Mann-Whitney test, SAM frequency (incidence) was compared between the 2001 and 2002 growing seasons and between harvest and ensiled samples (Minitab Inc., State College, PA). The non-parametric Kruskal-Wallis test was used to compare SAM incidence (2001 and 2002 harvest and ensiled samples) between the four maize silage production regions in Pennsylvania. SAM concentration was compared between samples from the 2001 and 2002 growing seasons and the harvest and storage samples using the paired t-test. SAM levels (2001 and 2002 harvest and ensiled samples) between the four regions of Pennsylvania were compared using a general linear model.

Kendall's correlation was used to determine agronomic practices related to SAM incidence and levels in harvest and ensiled samples with the PROC CORR procedure in SAS statistical software (SAS

Institute Inc., Cary, NC). Practices found to have a relationship to SAM incidence or levels were further evaluated with the Tukey-Kramer test to determine differences between categorical levels.

Daily weather variables for each site were averaged for each growth stage prior to statistical analysis. For example, if silking occurred over a five day period at a site, five days of data for each variable were averaged and the single value used to represent conditions during silking. Pearson's correlation was used to determine the relationship between weather variables during each growth stage and the SAM levels in the 2001 and 2002 harvest samples. The relationship between SAM incidence in 2001 and 2002 harvest samples and weather variables was assessed using Kendall's correlation.

The Pearson Correlation Coefficient (SAS Institute Inc., Cary, NC) was used to examine the relationships between silage fermentation characteristics and SAM concentration in the ensiled samples from 2001 and 2002. The Kendall Correlation Coefficient was used to evaluate the relationship between fermentation characteristics and the frequency of SAM contamination in ensiled samples (incidence).

RESULTS/DISCUSSION

Evaluation of extraction and detection method for SAMs

As silage is a particularly difficult matrix to work with, several recent publications have focused on using antibody-based clean up methods for SAMs in maize grain and silage (114, 278, 280). We determined that this approach, although quite selective, was not necessarily cost-effective for processing a large number of samples. Therefore, for our analysis we chose to exploit the structural similarity of the fumonisins and AAL-toxins (Fig. 1) to develop a more general, but less expensive method to study the four toxins in question. Our extraction method was modified from Syndenham et al (248), which was originally intended to extract fumonisins B_1, B_2 and B_3 from maize grain. This was successful as both classes of SAMs willl bind to strong anion exchange media within the pH range of 5.8-6.5, making this a simple and efficient method to extract and select for both groups of toxins simultaneously from silage. Using the HPLC and HPLC-MS methods described above, we were able to separate the fumonisins and AAL-toxins from one another as well as from the silage matrix (Fig. 3 and 4) and detect the toxins at levels well below those of concern for cattle. When silage samples (*N*=12) were spiked with each of the four toxins at a range of 0.5 – 5 µg of toxin to g of silage, the percent recoveries were as follows: FB_1 = 79-85%, FB_2 = 74-88%, AALTA = 71-84% and AALTB = 77-82%. The limit of detection for the fumonisin HPLC method was 30 ng of toxin per g of silage and 20 ng/g of silage for the AAL-toxins. We found that the sensitivity and recovery ability of this method was comparable to the immunoaffinity-HPLC methods of Yu et al (280) and Kim et al (114).

Levels and occurrence of SAMs

There was no statistically significant difference between silage at harvest and after ensiling in terms of the levels and frequency of SAMs detected. The average concentration and range in parentheses of SAMs in fresh and ensiled maize from 2001 and 2002 (*N*=120) are as follows for FB_1: 2.02 µg/g (0.20-10.1), FB_2: 0.98 µg/g (0.20 – 20.3), AAL-TA: 0.17 µg/g (0.20 – 2.0) and TB: 0.05 µg/g (0.03 – 0.90). FB_1 was the most frequently detected toxin (92%), followed by FB_2 (55%), AAL-TA (23%) and TB (13%). The frequency at which we detected FB_1 and FB_2 in silage is comparable to what was reported by Kim *et al* (114), although the average concentration and range for both toxins in our study was higher. This may be due to the fact that relatively severe droughts occurred during both seasons of the study, a condition that is reported to favor fumonisin contamination (158). In comparison to the findings of Yu *et al* (280), we detected FB_1 more frequently at higher levels but AAL-TA less frequently at a slightly lower average concentration. To our knowledge, this is the first report of AAL-TB in silage.

Relationship of weather conditions during plant development to SAM frequency and concentrations

The frequency and concentration of the fumonisins in 2002 was significantly higher than 2001 for most of the study regions (Fig. 5 and 6). Conversely, the frequency of the AAL toxins was slightly higher in 2001, although the average concentration was low in both seasons. The differences in frequency and concentration for the four toxins in the two seasons are likely a result of the weather conditions in each year. Although both the 2001 and 2002 growing seasons (April to October) had higher than average temperatures, 2001 ranked 53rd and 2002 6th out of 108 recorded seasons as the warmest on record (National Climatic Data Center, Asheville, NC). The 2002 season was also drier than 2001, ranking 24th in terms of driest years compared to 2001, which was 28th. Previous studies have observed that fumonisin concentrations are higher during drought years (157, 159) and have suggested that this may be due to greater insect activity, decreased plant resistance from water stress and other conditions favorable for fungal proliferation and toxin production. Although little is known about conditions favorable for AAL toxin production, the warm, dry weather prevalent during this study may have been deleterious to *Alternaria* growth and toxin production, which may explain the low frequencies and concentrations we observed both years. Interestingly, Torres *et al* (249) found that *A. alternata* was able to grow at a wider range of temperatures and water activities than *F. verticillioides*. However, although *A. alternata* may be able to grow *in vitro* at a wide range of temperatures, based on our observations, favorable temperatures and moisture requirements for toxin production in the field may be more restrictive.

Correlation analysis on the relationship between weather conditions during maize developmental stages and SAM occurrence and concentration indicated that in general, the relationships of temperature and moisture to toxin contamination in silage are similar to those with grain. For FB_1 there was a consistent pattern throughout most maize developmental stages (within the stages considered in this study) where temperature was positively correlated to FB_1, while moisture was negatively correlated (Table 1). There was some deviation of this trend at R1 (silking) when FB_1 occurrence was positively correlated with average relative humidity and wetness hours. It appears that from R2 to R6 (blister to maturity) FB_1 contamination was more likely to be present if temperatures were higher and moisture availability to the plant lower, as indicated by the negative relationship with the average precipitation minus evaporation during R3 (milk) and R6 (maturity) stages. We observed a similar relationship pattern between FB_1 concentration and temperature and moisture variables from VT through R6, where temperature was positively correlated to FB_1 and negatively correlated with moisture. Therefore, this relationship suggests that warmer temperatures and decreased moisture availability lead to higher final concentrations of FB_1 in silage compared to the average. Although the relationship pattern was similar for toxin occurrence and concentration, the relationship between concentration, temperature and moisture was stronger than between these variables and occurrence.

The relationship between FB_2 occurrence and weather conditions appeared to be similar to that of FB_1, although environmental conditions during silking (VT) seemed to have a stronger impact on occurrence of this toxin than they did on FB_1 occurrece (Table 1). During silking, there was a negative relationship between several moisture variables, including average precipitation, relative humidity, wetness hours and precipitation minus evaporation, while temperature had a positive affect. Interestingly, while FB_1 occurrence was affected during most of the developmental stages of maize, no relationship was observed between FB_2 occurrence and weather conditions until the mid to later stages of kernel maturation, (R3 to R5), although this observation may be an artifact of detection. Again, temperature was found to be positively correlated, while moisture variables had a negative relationship to FB_2. The same pattern was observed between toxin concentration, moisture and temperature, although the relationship was stronger between environment and concentration than for occurrence (Table 2).

In contrast to the fumonisins, AAL-TA occurrence was affected by weather conditions during R2 and R6 where temperature during these stages was negatively correlated and moisture variables positively correlated. The same pattern was observed for AAL-TA toxin levels where temperature was negatively

correlated during R2, R3 and R6, and positively correlated with precipitation. For AAL-TB, too few samples contained this toxin to achieve reliable correlation results.

Based on these correlation analyses, moisture and temperature variables appeared to have the strongest relationship on the occurrence and finial concentration of SAMs. For the fumonisins, the relationship we observed is similar to previous reports on maize grain, where warm temperatures and low moisture availability positively affected final fumonisin concentrations (157, 158, 160, 173, 260). Because this investigation is unique in investigating how environment and plant maturity impact AAL toxin levels and occurrence, it is difficult to compare our results to previous studies.

Relationship between plant maturity at harvest and FB_1 concentrations

As indicated by correlation analyses, conditions during the later stages of plant maturation appeared to have the strongest relationship with final FB_1 concentration. This is reflected by our observation that maize silage harvested at later maturation stages, R5 and R6 had slightly higher frequencies of contamination and significantly higher final FB_1 concentrations than silage harvested earlier (Fig. 7). This observation may indicate that the majority of fumonisin production may occur in the grain as higher fumonisin concentration occurred in crops harvested at later kernel maturation stages. A similar trend has been observed in grain, where maize harvested later in the season had higher average fumonisin concentration (37). Differential fumonisin production has also been observed *in vitro* on kernels harvested at different maturation stages (266) and it was found that kernels at R5 or dent stage supported the greatest production of fumonisin. Shim *et al* (232) found that the degermed kernel tissue, containing the endosperm and pericarp, supported almost 5 times higher levels of fumonisin production than did the germ portion. Therefore, as older more mature kernels contain more endosperm and thus more starch than kernels at earlier stages of development (R1-R3), this may explain why we observed higher fumonsisn concentrations in silage harvested at later maturities. Similarly, Bush *et al* (37) also observed that more mature kernels (R5 and R6) supported much greater fumonisin production compared to kernels at earlier stages of development.

Regional variation in SAM frequency and concentration

The four silage production areas in Pennsylvania were significantly different from one another in terms of SAM occurrence and toxin levels (Fig. 8). For FB_1, there was a north to south variation in that the SE and SC were more often contaminated and at higher average concentrations (t-test $p \leq 0.05$). This pattern was not observed with FB_2 however, as the NW and SE both had lower average concentrations of this toxin compared to the NE and SC. Again, this may be an artifact of detection rather than an actual

observation. Although the AAL toxins were more often present in samples from the NE and NW (t-test $p \leq 0.05$) than from the southern part of the state, the levels were not significantly different between the regions. These differences in toxin frequency and concentration between the regions may be due in part to geographical differences in temperature. During the stages of maize development (VT-R6) the only consistent significant difference between the northern sites and those in the south was that the south had higher average temperatures from VT to R6. This suggests that temperature may be the overriding weather factor that determines the occurrence and levels of SAMs.

Influence of agronomic practices and silage characteristics on SAMs in silage

Correlation analysis between agronomic practices and SAM occurrence and levels revealed that none of the agronomic practices in question, crop rotation, additive use before ensiling, tillage system or silo type, affected toxin incidence or concentration. Likewise, no relationship was observed between percent dry matter, pH, ammonia content, lactic acid, acetic, propionic, butyric, and isobutyric acid content and SAM incidence or levels in silage. As such, it does not appear that pre or post-harvest strategies, such as tillage, crop rotation, pre-inoculant or other additives, confer any benefit in terms of SAM reduction in silage.

Conclusions and the implications for control of SAMs in silage

As fumonisins were the most commonly encountered toxins and occurred at the highest concentrations, it is likely that these toxins are more of a concern to cattle health and productivity than the AAL-toxins. However, for most samples, the levels of fumonisins were still lower than those considered to be problematic for cattle. Although fumonisins were more frequently encountered at higher levels than AAL-toxins, during cooler and wetter seasons the reverse may be true. In addition, as there are no published studies on the affects of AAL-toxins on cattle, their presence is a concern.

Based on our observations, weather conditions during plant development and maturation appear to be the most influential factors affecting SAM occurrence and concentrations in silage within those considered in this study. Therefore, management of SAMs should focus on silage while the crop is in the field, although pre-harvest control of SAM contamination may be difficult to achieve due to the large and relatively uncontrollable influence of weather. However, the observations stated here provide more detail to develop accurate predictive models for SAM contamination, which may lead to control strategies similar to the the warning systems being developed for mycotoxins in other crops (99). As crop rotation and tillage also did not appear to have an affect on toxin contamination, strategies such as using resistant hybrids or chemical controls should continue to be employed. In terms of post-harvest management, we did not

observe a reduction in SAMs from harvest to ensiling, indicating that microorganisms is silage may not be capable of degrading these toxins. However, as some microorganisms are known to degrade fumonisins, these organisms should continue to be studied so that their abilities can be exploited for post-harvest detoxification.

ACKNOWLEDGEMENTS

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Table 5-1. Significant relationships ($p \leq 0.05$) between weather variables and SAM occurrence in maize silage.

Toxin	Stage[1]	Variable[2]	Average	Range	Corr. coefficient[3]
FB_1	R1	RHUM	69.7	58.0 – 81.8	0.243
		WHR	8.4	0 – 17.5	0.241
	R2	TMAX	27.6	23.1 – 31.8	0.396
		ATEMP	22.1	17.4 – 26.0	0.321
	R3	TMAX	25.7	19.0 – 32.9	0.387
		TMIN	14.5	8.2 – 20.9	0.379
		ATEMP	20.0	13.7 – 27.0	0.381
		PVAP	-63.0	-82.0 – -46.2	-0.280
	R4	TMAX	23.7	15.3 – 34.7	0.396
		TMIN	12.6	2.8 – 21.0	0.395
		ATEMP	18.1	8.6 – 27.5	0.419
	R5	TMAX	23.6	15.6 – 29.0	0.324
	R6	PREC	0.2	0.0 – 0.71	-0.453
		PVAP	-0.23	-0.84 – 0.43	-0.429
FB_2	VT	TMAX	28.8	24.8– 32.8	0.396
		PREC	0.18	0.0 – 0.76	-0.266
		RHUM	73.1	60.2 – 83.2	-0.344
		WHR	10.5	0.0 – 19.3	-0.337
		PVAP	-0.51	-0.91– 0.76	-0.367
	R3	RHUM	73.0	59.8 – 79.9	-0.259
	R4	TMAX	74.8	59.5 – 94.5	0.301
		RHUM	72.3	60.5 – 82.3	-0.392
		PVAP	-0.48	-0.71 – 0.43	-0.287
	R5	TMIN	12.8	8.8 – 18.8	0.313
		PREC	0.23	0.0 – 1.02	-0.389
AALTA	R2	TMAX	27.7	23.1 – 31.8	-0.287
	R6	PVAP	-0.23	-0.84 – 0.43	0.378

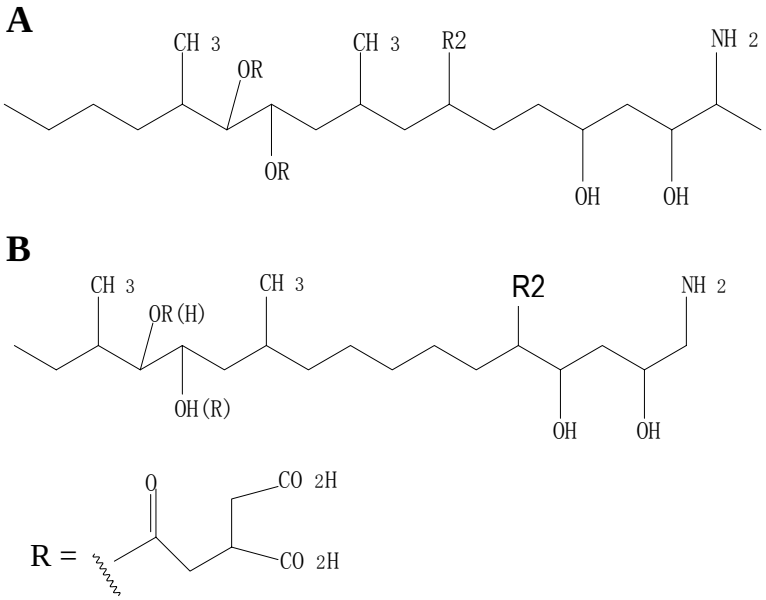
[1] Growth stages are as follows: VT – tassel, R1 – silking, R2 – blister, R3 – milk, R4 – dough, R5 – dent, R6 – maturity (214)
[2] Weather variable abbreviations:RHUM – relative humidity, WHR – wetness hours, TMAX – maximum temperature, TMIN – minimum temperature, ATEMP – average temperature, PVAP – precipitation minus evaporation, PREC - precipitation
[3] Kendall's Correlation coefficient

Table 5-2. Significant relationships ($p \leq 0.05$) between weather variables and SAM concentration in silage.

Toxin	Stage	Variable	Average	Range	Corr. Coefficient[1]
FB_1	VT	TMIN	17.8	11.8 – 21.8	0.357
		ATEMP	23.1	18.7 – 26.4	0.345
	R2	TMAX	27.7	23.1 – 31.8	0.417
		TMIN	16.6	11.2– 21.4	0.403
		RHUM	71.2	60.2 – 77.1	-0.491
		WHR	9.8	3.2 – 16.2	-0.439
		PVAP	-0.46	-0.15 – -0.76	-0.426
	R3	TMAX	25.7	19.0 – 32.9	0.602
		ATEMP	20.0	13.7 – 27.0	0.578
		RHUM	73.0	59.8 – 79.9	-0.433
		PVAP	-0.36	-0.76 – 0.08	-0.429
	R4	TMAX	23.8	15.3 – 34.7	0.415
		TMIN	12.6	2.9 – 21.0	0.434
		ATEMP	18.1	8.6– 27.5	0.441
	R5	PVAP	-0.25	-0.76 – 0.74	-0.691
	R6	TMAX	23.2	14.2 – 33.8	0.506
		ATEMP	17.6	10.7– 26.2	0.512
		PREC	0.20	0.0 – 0.71	-0.546
		PVAP	-0.23	-0.84 – 0.43	-0.728
FB_2	VT	TMAX	28.8	24.8 – 32.8	0.296
	R3	TMAX	25.7	19.0 – 32.0	0.294
		TMIN	14.5	8.2 – 20.9	0.341
		ATEMP	20.0	13.7 – 27.0	0.335
		PVAP	-0.36	-0.76 – 0.08	-0.258
	R4	TMAX	23.8	15.3 – 34.7	0.333
		TMIN	12.6	2.9 – 21.0	0.495
		ATEMP	18.1	8.6 – 27.5	0.449
		RHUM	72.3	60.5 – 82.3	-0.301
		WHR	8.5	2.2 – 17.8	-0.291
	R6	PREC	0.28	0.05 – 0.74	-0.564
AALTA	R2	TMAX	81.8	73.6 – 89.2	-0.362
		RHUM	71.2	60.2 – 77.1	0.325
	R3	TMAX	25.7	19.0– 32.9	-0.260
		PREC	0.20	0.05 – 0.43	0.264
		PVAP	-0.36	-0.76 – 0.08	0.274
	R6	TMAX	23.2	14.2 – 33.8	-0.392
		PREC	0.20	0.0 – 0.71	0.491
		PVAP	-0.23	-0.84 – 0.43	0.441

[1] Growth stages are as follows: VT – tassel, R1 – silking, R2 – blister, R3 – milk, R4 – dough, R5 – dent, R6 – maturity (214).
[2] Weather variable abbreviations:RHUM – relative humidity, WHR – wetness hours, TMAX – maximum temperature, TMIN – minimum temperature, ATEMP – average temperature, PVAP – precipitation minus evaporation, PREC - precipitation
[3] Pearson's Correlation coefficient

Figure 5-1. Chemical structure of A. fumonisins and B. AAL toxins.



R2: FB_1/AALTA = OH
R2: FB_2/AAL-TB = H

Figure 5-2. Map of the relative locations of sample dairies. Symbols represent the four major dairy production regions Northeast - ★ , Northwest - ▲, Southeast - ■ and South central – ●.

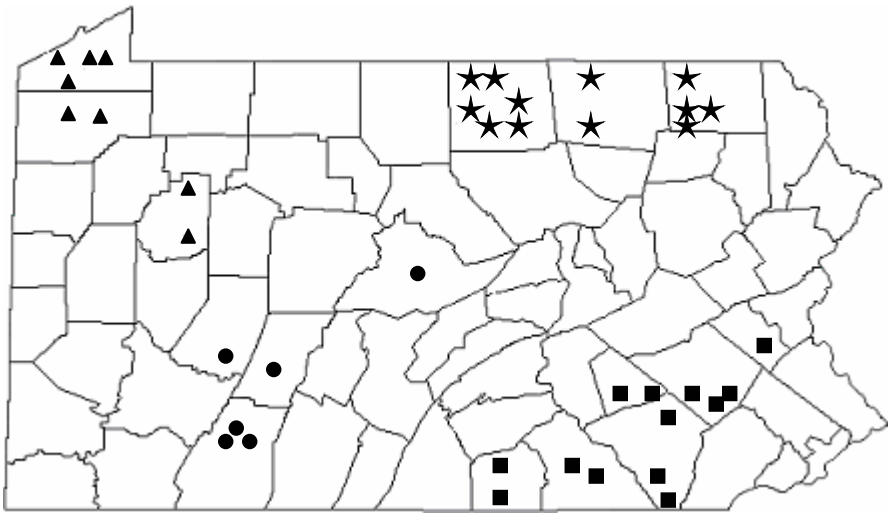


Figure 5-3. HPLC chromatogram of A. silage sample naturally contaminated with fumonisin B_1 and fumonisin B_2 and B. a standard of the two toxins. The standard peaks represent 61.6 ng and 46.2 ng respectively for fumonisin B_1 and fumonisin B_2. Asterisks in panel A are over peaks identified as FB_1 and FB_2.

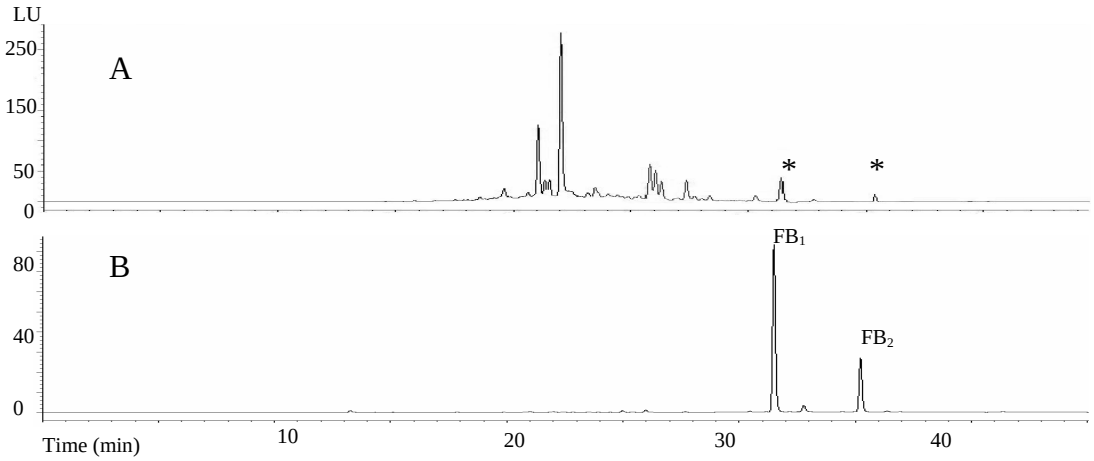


Figure 5-4. LC-MS chromatograms of A. AAL-TA standard, B. sample with AAL-TA, C. AAL-TB standard, D. sample with AAL-TB and E. retinoic acid (4 µg) in a spiked sample. AAL-TA and TB occur as double peaks due to the presence of two isomers. Mass is at the upper right and corresponds to the loss of H^+ from the parent compound. The number at the lower right is ion intensity.

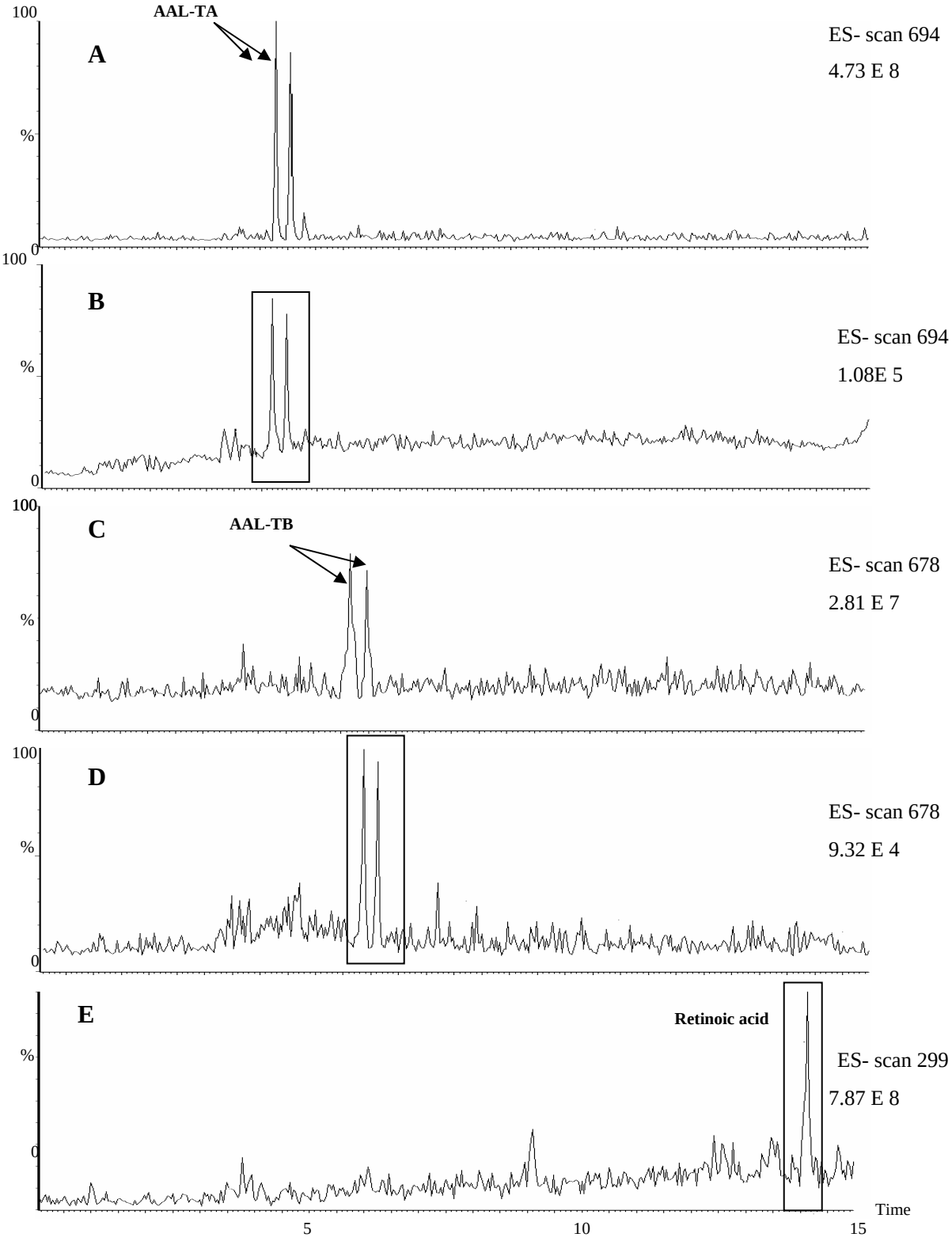


Figure 5-5. Frequency of SAMs in fresh and ensiled maize from 2001 and 2002. A – Fumonisin B_1, B - Fumonisin B_2, C – AAL-TA and D – AAL-TB . The number of samples included in analysis for each region NE – 24, NW – 16, SE – 10 and SC – 10.

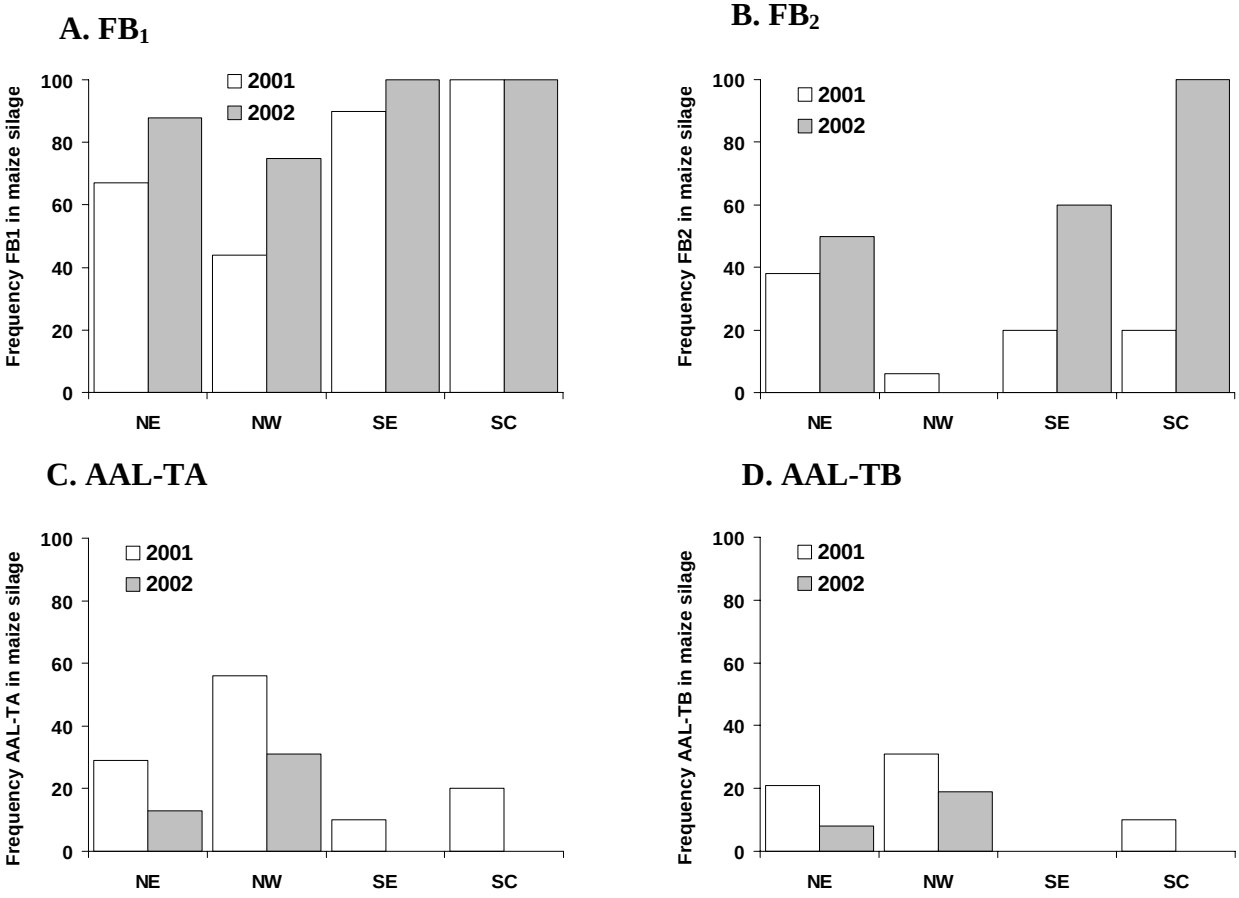


Figure 5-6. Average concentration of SAMs in maize silage: A – fumonisin B_1, B - fumonisin B_2, C – AAL-TA and D – AAL-TB. The number of samples for each region NE – 24, NW – 16, SE – 10 and SC – 10. Asterisks over bars indicate a season (2001 or 2002) with significantly (≤0.05) higher toxin concentration.

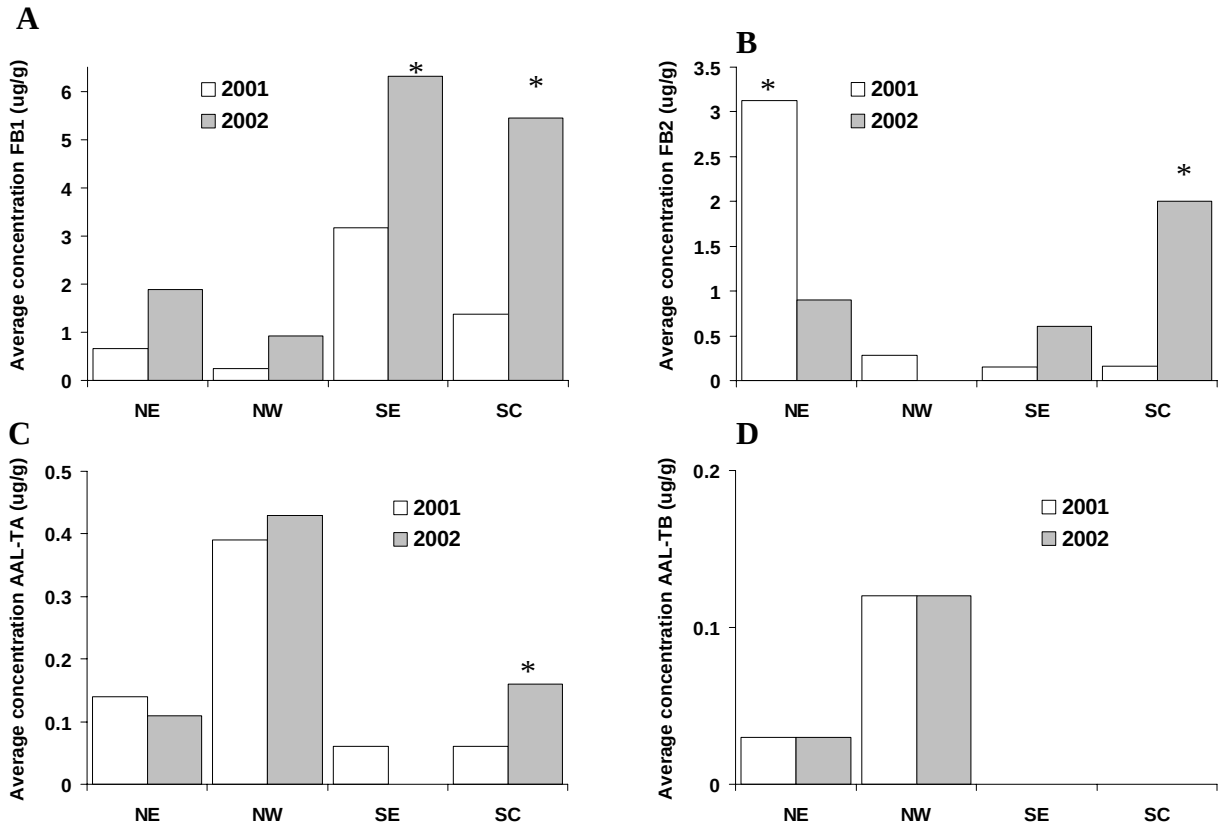


Figure 5-7. A. Frequency and B. average concentration of SAMs in samples harvested at different maturities. Bars of the same toxin followed by a different letter are significantly different at $p \leq 0.05$.

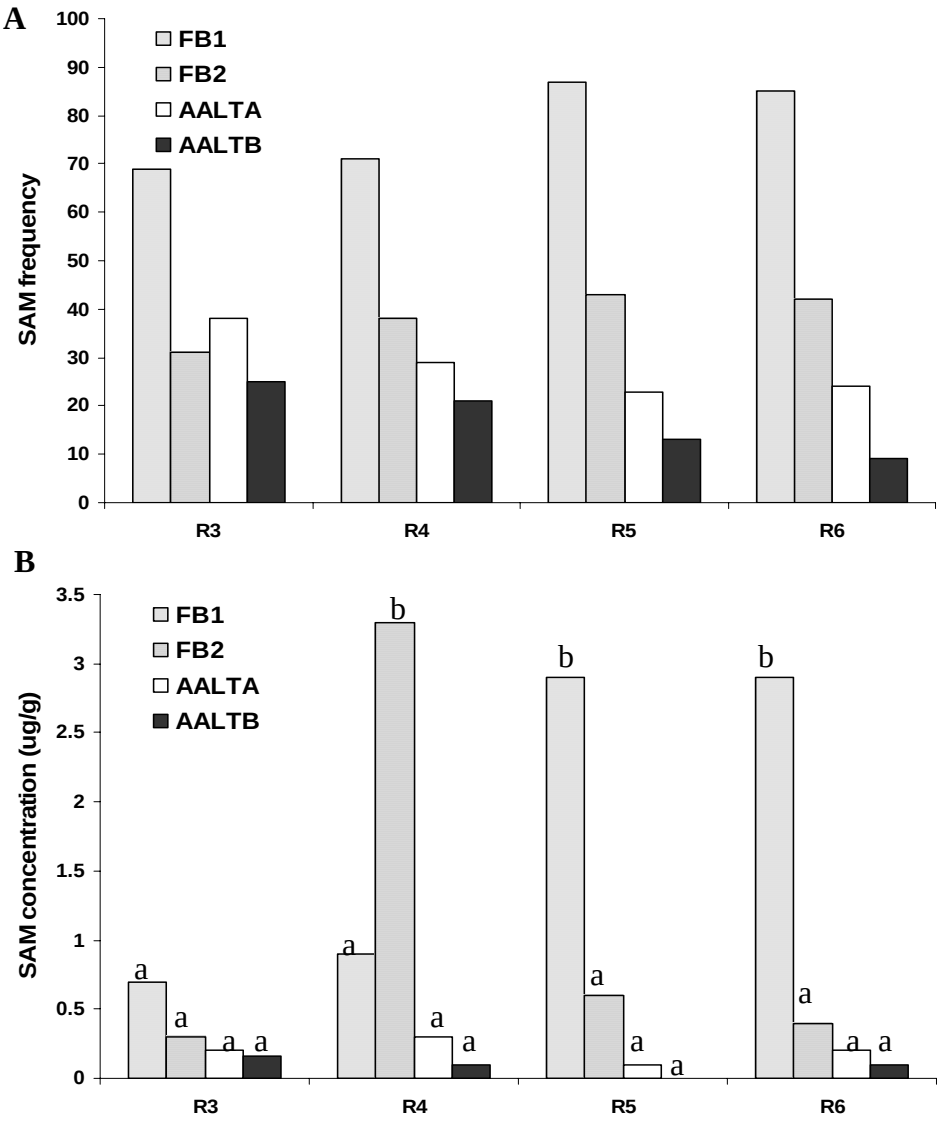
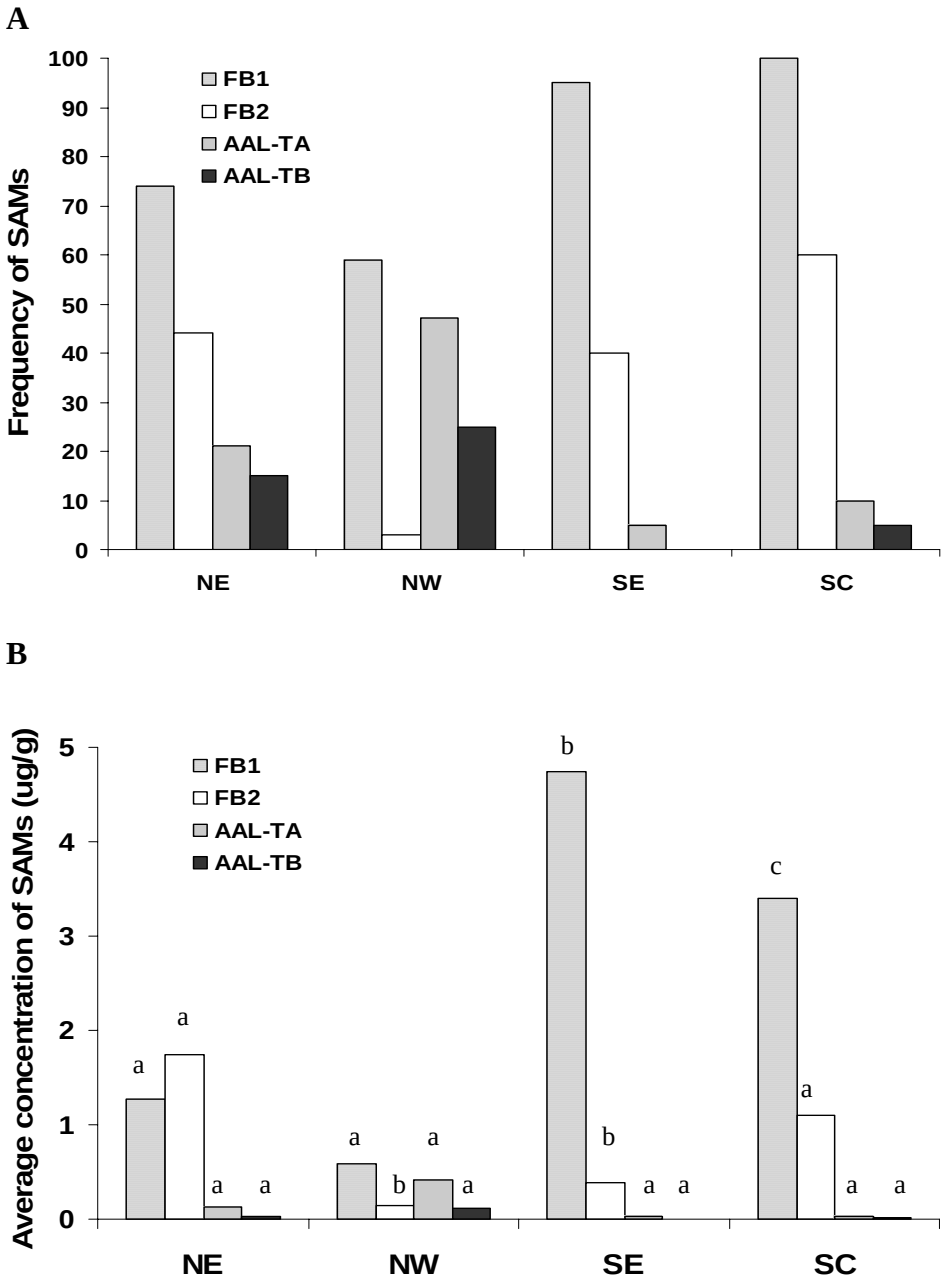


Figure 5-8. A. Frequency and B. average concentration of SAMs in each the four silage production regions. The number of samples included in analysis for each region NE – 24, NW – 16, SE – 10 and SC – 10. Bars that represent the same toxin with different letters over them are significantly different at $p \leq 0.05$ between regions.



Chapter 6. Contamination of fresh and ensiled maize with four mycotoxins produced by *Penicillium* species.

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In preparation for:

ABSTRACT

Mycotoxins produced by *Penicillium* are reported in maize silage and are associated with health problems in cattle. Our objective was to develop a method to detect four toxins patulin (PAT), mycophenolic acid 40, cyclopiazonic acid (CPA) and roquefortine C (ROC) in fresh and ensiled maize. Silage was collected at harvest and after ensiling in 2001 and 2002 from 30-40 Pennsylvania dairies. HPLC-MS was used to detect the four toxins in silage. The average concentration of the toxins in positive samples with the range in parentheses was: PAT 0.48 µg/g (0.03-0.91), MPA 0.46 µg/g (0.08 – 0.71), CPA 0.18 µg/g (0.03 – 0.86) and ROC 0.79 µg/g (0.03 – 3.7). ROC was the most frequently detected toxin (42%), followed by MPA (34%), CPA (28%) and PAT (16%). All four toxins were found in fresh and ensiled maize samples and up to 40% were contaminated with two toxins and 12% with three toxins. Overall ROC was the most commonly encountered toxin, occurred at the highest levels and was the most common co-contaminant with the other mycotoxins. Based on our observations, *Penicillium* mycotoxins can be produced while the crop is in the field and after ensiling, suggesting that preventative measures should begin before the crop is ensiled.

INTRODUCTION

Mycotoxins produced by *Penicillium* are known to occur in maize based feeds such as silage and many are related to serious health problems in cattle (226). Some of the toxins reported in silage include cyclopiazonic acid (CPA) (280), patulin (PAT) (169), mycophenolic acid 40 (224) and roquefortine C (ROC) (90). The majority of contamination is thought to take place during storage, as many of these mycotoxigenic species will grow on substrates with low water activities, and some can also withstand low pH and oxygen concentration (31, 197). Additionally, some species appear to be ubiquitous in silage and they, as well as their toxins, can be isolated from material that is not visibly molded (31).

Many toxigenic species of *Penicillium* are ubiquitous saprophytes on plant material and in the soil (197) and some have been found to cause ear rot on maize damaged by birds and insects (190). In ensiled maize they are often the most commonly isolated filamentous fungi. Several species of *Penicillium* have been reported in maize silage including the closely related *P. crustosum* and *P. commune*, which produce the compounds cyclopiazonic acid and roquefortine C (80, 236, 246). The most frequently isolated

Penicillium in silage is *P. roqueforti* (31, 226), which has recently been divided into three species, *P. roqueforti, P. paneum* and *P. carneum,* based on analysis of rDNA genes and chemotaxonomic profiles (31). Of the *P. roqueforti* group, only *P. roqueforti* and *P. paneum* have been reported in silage (31, 138, 246). Both species will grow in substrates with low water activity, low pH (to pH 3.0), and low oxygen content and produce roquefortine C (31). Additionally, *P. roqueforti* also produces PR-toxin and mycophenolic acid, while *P. paneum* produces patulin. *P. expansum*, which has also recently been reported in silage, will also tolerate low pH and low oxygen conditions and like *P. paneum*, produces roquefortine C and patulin (31).

Both PAT and ROC have been implicated in cattle toxicoses, although clinical data on the effects of toxin exposure in cattle are not available for either these toxins or for MPA or CPA. ROC was suspected as the causal agent in several cases of paralysis, abortion and placental retention in cattle in two separate reports (90, 244). However, as the strains of *P. roqueforti* produced other mycotoxins, including PAT and PR-toxin, it is difficult to determine if ROC was the only toxin involved. The neurotoxic effects of ROC have also been demonstrated with dogs, where animals suffered paralysis, tremors and convulsions when they consumed food contaminated with *P. roqueforti* (32, 277). Recently PAT was implicated in a case of cattle toxicoses where several beef herds experienced tremors, paralysis and death and autopsy revealed serious damage to the nervous system (220). PAT was detected both in culture and in the feed. In an earlier incident, cattle that consumed *P. roqueforti*-contaminated silage experienced a loss of appetite, gastrointestinal hemorrhaging, and abortion (256). The suspected agents were PR-toxin and PAT, although no tests were performed to verify this hypothesis. Unlike PAT, MPA does not appear to be acutely toxic; however it is a potent immunosuppressive agent (224, 241) and in the medical field it is used in anti-rejection therapy for organ transplant patients. In one study MPA was reported in 38 of 135 maize silage samples and in some silage, the concentration was equivalent to 10% of the dose given to patients undergoing immunosuppressive therapy. MPA in feeds is believed to make animals more susceptible to infectious diseases and more sensitive to other mycotoxins that may be present (213, 226). Although CPA has not been studied with dairy cattle or other ruminants, it causes tremorgenic activity and lesions on the liver, kidney and gastrointestinal tract in rats (121) and similar affects in other species (35). An additional concern with CPA is that it is excreted in milk (67), remains stable following pasteurization and for long periods of time during storage (200).

To date there are no effective strategies that offer complete protection against *Penicillium*-toxin contamination, although some toxins (PAT and MPA) are reported to be degraded during storage of ensiled

maize (111). Despite the belief that the majority of toxin formation occurs during storage, this has not been demonstrated conclusively and it may be that prevention of contamination should actually begin in the field. Routine testing of these toxins is also not performed, although they are often associated with serious health problems in cattle. The lack of information on the frequency and levels of these toxins also makes it difficult to determine which are the most important in terms of prevention and under what conditions are they generally present. Part of the lack of understanding on how frequently these toxins occur in silage and the impacts they have on cattle health is probably due to difficulties related to performing multi-toxin analysis, which is generally time consuming, laborious and expensive. Also, the majority of studies on *Penicillium* and *Aspergillus* mycotoxins have been conducted in Europe and Asia, making it difficult to relate these findings to the situation in North America. With these concerns in mind, our objective was to develop an analytical method to detect and quantify several toxins simultaneously including cyclopiazonic acid, mycophenolic acid, patulin and roquefortine C from fresh and ensiled maize from Pennsylvania. The overall goal of this work is to determine the frequency and levels of these toxins so that we can better understand which mycotoxins are of concern in fresh and ensiled maize and how the process of ensiling impacts toxin concentrations.

MATERIALS & METHODS

Sample collection and preparation

Maize silage was collected from four regions in Pennsylvania (Fig. 1) at dairies with a history of known or suspected mycotoxin contamination. To date, preliminary analysis has been performed on samples from the Northeastern (NE) region, from twelve farms. Samples were collected at harvest and three to six months after ensiling to compare the affect ensiling had on the toxin concentration of silage. Harvest samples consisted of 1-3 kg freshly chopped maize, while samples of ensiled maize were either removed from several locations at the face of a structure (bunker or trench silo), or as the silage was being emptied from a silo during feed out (upright silo). Silage collection was based on the amount of silage fed per day, with sites feeding 1-2 tons collecting samples every other day for 14 days and sites feeding 3-4 (or more) tons per day collecting for 7 days consecutively. After collection, samples stored at -80 °C until analysis. Prior to toxin extraction, silage samples were dried at 55 ºC for 5-7 days, milled to a flour-like consistency, mixed and 500 g sub-sampled for analysis.

Toxin extraction and cleanup

The method used to extract cyclopiazonic acid, mycophenolic acid, patulin and roquefortine C was modified from that of Rundberget and Wilkins (217). All solvents used for extraction were of HPLC grade.

For each sample, 10 g of silage was weighed and extracted for 1 hour with 60 mL acetonitrile-water (9:1 v/v) with 0.1% formic acid on an automatic shaker at 300 rpm. Samples were centrifuged at 6000 x *g* for 10 min and 10 mL was decanted for cleanup. Samples were defatted two times with 10 mL of hexane and the lower aqueous phase was removed for analysis. Defatted samples were transferred to 11 mL vial and desolvated to dryness under a stream of nitrogen at 65 ºC. The residue was then redissolved in 1 mL acetonitrile, filtered with a pre-wet nylon filter (company and pore size), desolvated a second time and resuspended in 500 µL of acetonitrile. Samples were stored in 2 mL amber vials at 4ºC until HPLC-MS analysis.

Standards

HPLC-MS Analysis

The solvent delivery system consisted of a LC10ADvp ternary solvent delivery system (Shimadzu, Columbia, MD) to which a Quattro II mass spectrometer (Micromass, Beverly, MA) was attached for detection. Chromatographic separation was achieved using a reverse phase Thermo Betasil C-18 column (150 x 2.1 mm; 5 µM particle size, pore size 60 Å) (Thermo, Bellefonte, PA). Solvents consisted of: A – water amended with 0.05 M ammonium acetate B – methanol with 0.05 M ammonium acetate and C – methanol. The method began with A at 100% with a linear gradient program moving to 100% B from time 0 to 15 min, holding at B from 15 to 25 min and then moving to an isocratic wash with C from 25 to 30 min and equilibrating with A for 1 min prior to the next injection. The flow rate was constant at 0.3 mL solvent per minute. The toxins were detected using Atmospheric Pressure Chemical Ionization in the positive mode (APCI+). Standards of cyclopiazonic acid, mycophenolic acid, patulin and roquefortine C were all purchased from Sigma-Aldrich (St. Louis, MO). 10 µL of a mixed standard (50 ng/µL in acetonitrile) or each redissolved samle extract were injected for analysis. The instrument was set to detect masses between 100 to 600 and the detected masses of the toxins were those of the of the parent compound plus H^+: PAT – m/*z* 155, MPA – m/*z* 321, CPA – m/*z* 337, ROC – m/*z* 390. Data analyses were performed using Mass Lynx v. 3.5 mass spectrometry software (Milford, MA).

RESULTS & DISCUSSION

Evaluation of extraction and detection method

The method of Rundberget and Wilkins (217) was originally developed using a complex matrix of several different food stuffs. We successfully modified this HPLC-mass spectrometry method for use with maize silage and found that when silage samples (*N*=12) were spiked with each of the four toxins at a range of 0.5 – 5 µg of toxin per g of silage, the percent recoveries were as follows: patulin = 79-85%,

mycophenolic acid = 74-88%, cyclopiazonic acid = 71-84% and roquefortine C = 77-82%. With this method the toxins could be separated from one another and from interfering compounds in the silage matrix (Fig. 2). The limit of detection for the toxins was 30 ng/g.

Levels and occurrence of patulin, mycophenolic acid, cyclopiazonic acid and roquefortine C

There was no significant difference between silage at harvest and after ensiling in terms of the levels and frequency of the four toxins (paired t-test and Mann-Whitney respectively $p \geq 0.05$). We did find it interesting that freshly harvested samples contained comparable (though slightly lower) concentrations of toxins compared to ensiled maize and were contaminated slightly less often (Fig 3). The average concentration and range in parentheses of the four toxins in fresh and ensiled maize from 2001 and 2002 (N=48) are as follows: PAT 0.48 µg/g (0.03-0.91), MPA 0.46 µg/g (0.08 – 0.71), CPA 0.18 µg/g (0.03 – 0.86) and ROC 0.79 µg/g (0.03 – 3.7). ROC was the most frequently detected toxin (42%), followed by MPA (34%), CPA (28%) and PAT (16%).

The incidence and levels of some of the toxins observed in this study are similar to what has been observed in previous investigations. Schneweis *et al* (224) found that 32% of the silage samples analyzed were contaminated with MPA at a range of 0.2 to 350 µg/g with an average of 14 µg/g. The higher concentrations observed by these authors may be due to the fact that they purposely selected samples that were visibly molded. Auerbach *et al* (14) also found higher frequency and levels of ROC than we observed, with 54% of the total number of samples containing the toxin, 8.3% of the non-molded (visual assessment) and 100% of the molded samples. In terms of toxin concentration, they observed an average of 8.5 µg/g of toxin at a range of 0.2 to 36.0 µg/g. For CPA, Yu *et al* (280) found that 87% of the samples were contaminated with an average range of 0.34 µg/g and a range of 0.12-1.82 µg/g. Although these levels are similar to what we observed, the frequency of contamination was higher. In a laboratory silo Muller *et al* (169) found that PAT was produced in silage at concentrations up to 15 µg/g in material that was inoculated with *P. roqueforti*. As far as we are aware, this study is the first to report naturally occurring patulin contamination of maize silage so it is difficult to compare our observations to others' work.

Multi-toxin contamination

ROC was the most common co-contaminant with the other toxins considered in this work (Fig 4). Of the total number of samples analyzed, 34% were contaminated with a single toxin, 40% with two toxins and 12% with three toxins. Overall, these observations are not surprising as three of these toxins, PAT, MPA and ROC, are produced by members of the *P. roqueforti* group. In earlier work, we isolated *P. roqueforti, P. paneum, P. commune, P. crustosum* and *P. expansum* from silage samples, although *P. roqueforti* was

by far the most commonly encountered species (138). This likely explains why ROC was the most frequent contaminant of silage, although the low frequency of MPA is somewhat surprising. It is possible that conditions for producing this toxin were not as favorable as they were for ROC. We did find it interesting that none of the samples were contaminated with all four toxins simultaneously.

Conclusions thus far and future work

Given that there has been little work addressing the toxicity of PAT, MPA, CPA and ROQ to cattle, it is difficult to say if the levels we observed in fresh and ensiled maize are dangerous. Certainly the presence of multiple toxins in samples is of some concern as they may have additive or synergistic affects on animals exposed to them (213). Although it has traditionally been thought that toxin formation occurs after ensiling and during storage, based on our observations a limited amount of formation begins in the field. We did observe a slight increase in the numbers of ensiled samples contaminated with the toxins and an equally slight increase in toxin concentration. However, based on analysis thus far, we did not observe a decrease in toxin concentration compared to the reports by Karlovsky (111) for PAT and MPA.

Given that maize silage appears to be contaminated in the field as well as in storage, management practices aimed at toxin control may need to focus on the crop both before it is ensiled and during storage. To date there are no effective control strategies to prevent or control these toxins in silage and much of that lack is probably due to not fully understanding what conditions affect toxin contamination. Future work on this project will focus on completing the analysis of maize silage from the other three regions and determining how agronomic practices, weather conditions and silage quality affect the levels and frequencies of the four toxins. With these data we hope to define some of the factors that affect toxin contamination in silage with the goal providing accurate information to develop effective prevention or control strategies against mycotoxin in silage. In addition, a more throurough understanding of *Penicillium* toxin contamination of silage may lead to clinical studies on cattle exposure to these toxins.

ACKNOWLEDGEMENTS

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Figure 6-1. Map of the relative locations of sample dairies in the Northeast - ★.

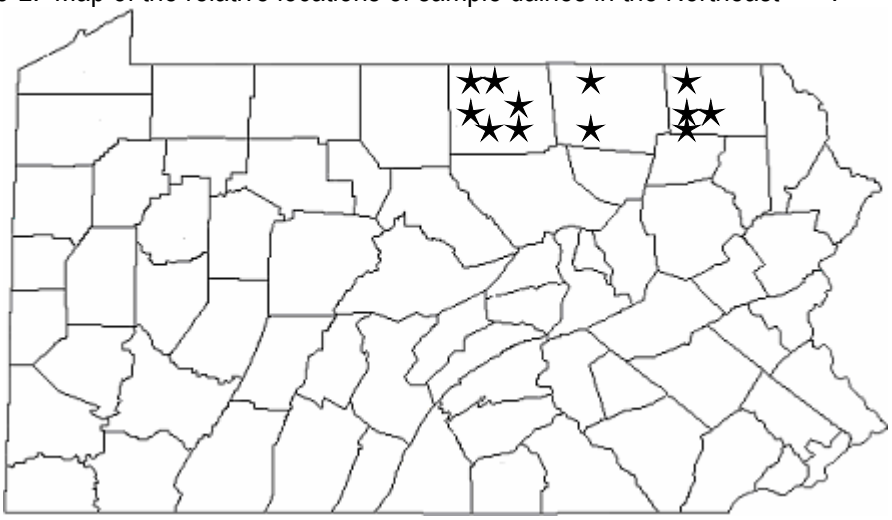


Figure 6-2. LC/MS chromatograms showing (A) total ion chromatogram for a mixed standard of four mycotoxins and reconstructed ion chromatograms showing silage samples contaminated with (B) patulin (PAT) m/z 155, (C) mycophenolic acid (MPA) m/z 321, (D) cyclopiazonic acid (CPA) m/z 337 and (E) roquefortine C (ROC) m/z 390. Mass is at the upper right of each chromatogram and corresponds to the gain of H^+ compared to the parent compound, the total ion count (TIC) is given for the standard. The nuber at the lower right is absolute signal intensity corresponding to he 100% level indicated on the axis.

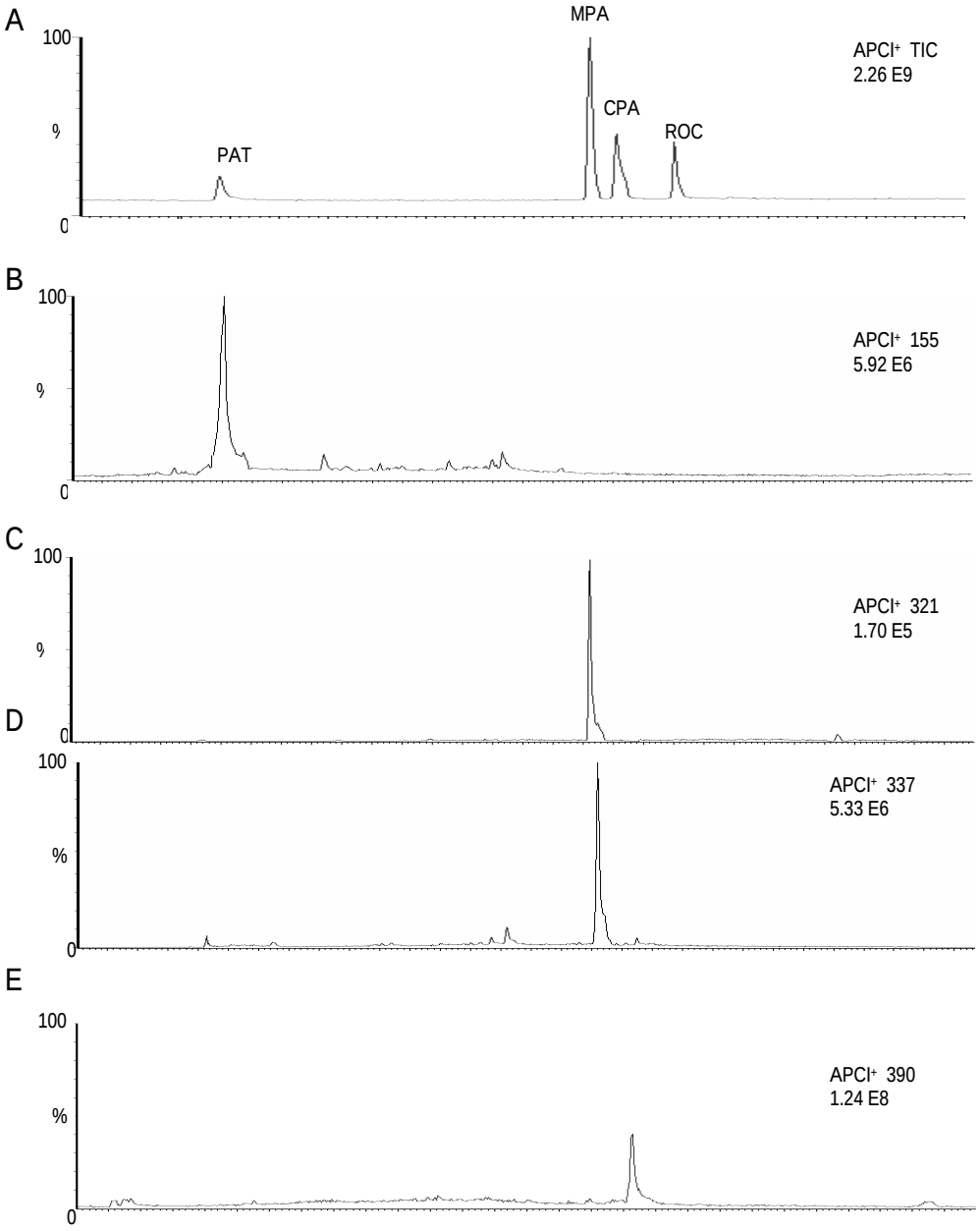


Figure 6-3. The frequency A. and levels B. at which PAT, MPA, CPA and ROC occurred in fresh and ensiled maize in 2001 and 2002.

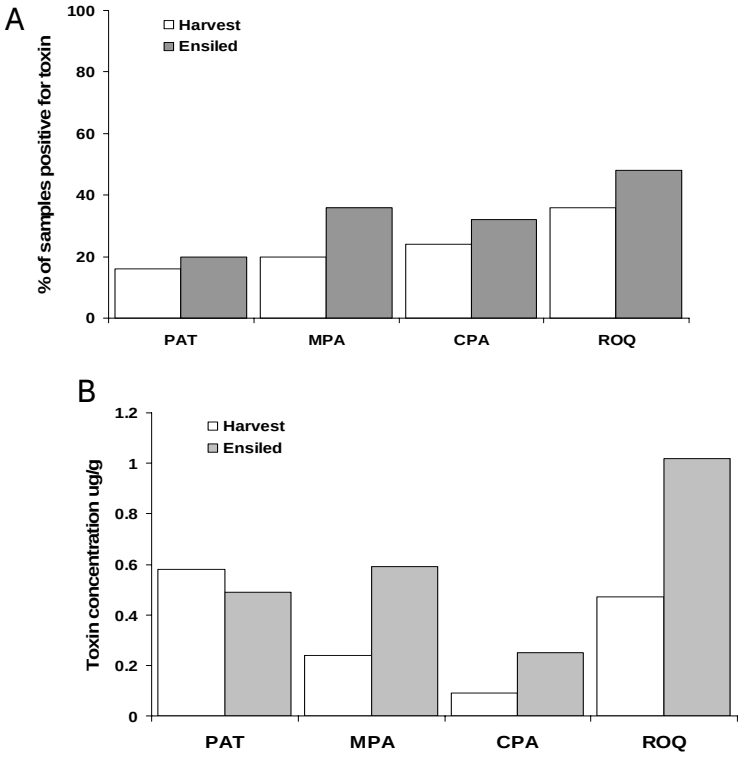
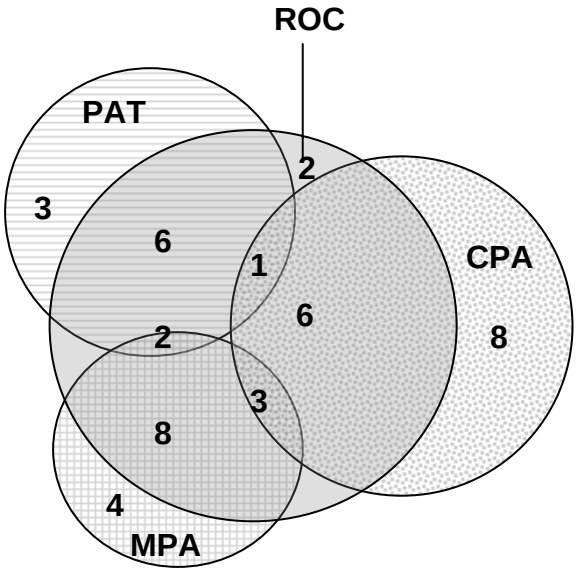


Figure 6-4. Venn diagram illustrating the number of samples co-contaminated with PAT, MPA, CPA and ROC.



Chapter 7: Multi-toxin contamination of fresh and ensiled maize

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ABSTRACT

Silage and other maize based feeds can become contaminated with toxins produced by the fungal genera *Aspergillus, Alternaria, Fusarium* and *Penicillium*. Most mycotoxigenic species can produce more than one toxin simultaneously and co-occurrence of toxigenic species on maize has been reported. We investigated sixteen different mycotoxins in maize silage including aflatoxins B_1, B_2, G_1, and G_2, AAL-toxin TA and TB, cyclopiazonic acid, deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, fumonisin B_1 and B_2, patulin, mycophenolic acid, roquefortine C, T-2 toxin and zearalenone. Silage samples were collected from four regions in Pennsylvania from 30-40 dairies in 2001 and 2002. Most samples contanined at least one mycotoxin and many contained three or more toxins simultaneously. These findings may have serious implications for cattle health and welfare and suggest that in the future more focus should be placed on strategies that prevent the formation of multiple mycotoxins rather than focusing on a single toxin.

INTRODUCTION

Multi-toxin contamination of silage and other maize based feeds has been reported in maize production areas around the world (62, 188, 225, 227, 253, 268). Many fungi are capable of producing different toxins simultaneously (17, 31, 112, 128) and several species can co-occur in maize and silage (8, 120, 127, 138, 228). To date, the most comprehensive study on multi-toxin contamination was conducted by Yu *et al* (280) where six different toxins, AAL-TA, cyclopiazonic acid (CPA), deoxynivalenol (DON), fumonisin B_1 (FB1), PR-toxin (PR) and zearalenone (ZEA) were detected and quantified using an immunoassay method. These toxins were selected to represent the presence of the four major myotoxin producing genera known to occur in silage *Aspergillus* and *Penicillium* producing CPA (both genera), *Penicillium* PR-toxin, *Alternaria* AAL-TA and *Fusarium* producing DON, ZEA and FB1. Although most of toxins occurred fairly frequently, they were generally present at low levels. However, this work did not report on the number of samples co-contaminated with toxins or how many toxins occurred in each sample. In addition, the authors indicated that in the future their observations should be verified by other analytical methods such as HPLC or MS.

Several reviews have focused on the the implication of of multiple toxin occurrence in feeds including silage (226, 52, 213). Many mycotoxins are either known or suspected to cause health problems when cattle consume them (6, 36, 48, 52, 90, 101, 150, 220, 226) and it is believed that the presence of several

toxins may exacerbate these health problems (53, 213). Several studies on swine and poultry exposure to *Fusarium* trichothecenes and fumonisins in combination with *Aspergillus* mycotoxins (aflatoxin and cyclopiazonic acid), revealed that in many cases toxin co-occurrence resulted in additive or synergistic toxicological affects (94-96, 121).

Expanding on the work of Yu *et al* (280), we conducted a multi-toxin survey of fresh and ensiled maize collected in Pennsylvania in 2001 and 2002. Our goal was to detect and quantify multiple mycotoxins in fresh and ensiled maize to more thouroughly understand of what toxin combinations are present in silage that may pose a threat to cattle. Toxins included in this study were aflatoxins (AF) B_1, B_2, G_1, and G_2, AAL-toxin TA and TB, cyclopiazonic acid (CPA), deoxynivalenol (DON), 15-acetyldeoxynivalenol (15ADON), 3-acetyldeoxynivalenol (3DON), T-2 toxin (T2), zearalenone (ZEA), fumonisin (FB) B_1 and B_2, patulin (PAT), mycophenolic acid and roquefortine C (ROC). AF1, AF2, AG1, AG2, DON, 15DON, 3DON, T2 and ZEA were analyzed by a commercial lab and the remaining toxins were analyzed with methods developed in our lab.

MATERIALS AND METHODS

Silage collection and preparation

Maize silage was collected from four regions in Pennsylvania (Fig. 1) at dairies with a history of known or suspected mycotoxin contamination. In 2001, thirty-two farms participated in the study and in 2002, thirty-nine. Samples were collected at harvest and then three to six months after ensiling to compare the affect ensiling had on the content of SAMs in silage. Harvest samples consisted of 1-3 kg freshly chopped maize, while samples of ensiled maize were either removed from several locations at the face of a structure (bunker or trench silo), or as the silage was being emptied from a silo during feed out (upright silo). The frequency of silage collection was based on the amount of silage fed per day, with sites feeding 1-2 tons collecting samples every other day for 14 days and sites feeding 3-4 (or more) tons per day collecting for 7 days consecutively. After collection, samples stored at -80 °C until analysis. Prior to in-house toxin extraction, silage samples were dried at 55 ºC for 5-7 days, milled to a flour-like consistency, mixed and 500 g sub-sampled for analysis. Samples analyzed by the commercial lab, Cumberland Valley Analytical Services (CVAS) (Haggerstown, MD) were shipped overnight and stored at -20 ºC until analysis.

Toxin analysis by commercial lab

Samples screened by CVAS for AF1, AF2, AF1, AF2, DON, 15DON, 3DON, T2 and ZEA were analyzed by TLC and positive samples verified by HPLC according published methods for maize (1). The

limit of quantification (the amount of toxin that can be reliably quantified by the method 95% of the time) for all toxins in silage was 0.5 µg of toxin per g of silage with the exception of the aflatoxins which was 20 ng/g.

In-house HPLC/HPLC-MS toxin analysis

Analysis of FB1, FB2, AALTA, AALTB, CPA, MPA, PAT and ROC were performed with methods developed for multi-toxin extraction (140, 141). FB1, FB2, AALTA and AALTB were extracted with a modified method from Syndenham *et al* (248) using solid phase extraction. A mixed FB1/FB2 standard from Romer Labs (Union, MO) was used f or analysis. Samples for fumonisin analysis were derivatized with naphthalene-2,3-dicarboxaldehyde (Sigma-Aldrich, St. Louis, MO) and detected by HPLC with fluorescence detection as described in Mansfield *et al* (141). Data were analyzed using ChemStation for LC Rev. A.08.01 analysis software (Agilent, Palo Alto, PA). AAL-toxins were detected by HPLC-MS using negative electrospray ionization and retinoic acid as an internal standard. A standard was generated from culture ATCC 28329 of *A. arborescens* as described in Mansfield *et al* (140). The limit of detection for the fumonisin HPLC method was 30 ng/g and 20 ng/g of with the HPLC-MS method for AAL-toxins. CPA, MPA, PAT and ROC were extracted using a method modified from Rundberget and Wilkins (217). Standards of each toxin were purchased from Sigma-Aldrich (St. Louis, MO). Toxins were detected using HPLC-MS with positive atmospheric pressure chemical ionization as described by Mansfield *et al* (140). The limit of detection for all four *Penicillium* toxins was 30 ng/g. MS data were analyzed using Mass Lynx v. 3.5 mass spectrometry software (Milford, MA). Chromatographic separation of all toxins was achieved using a reverse phase Thermo Betasil C-18 column (150 x 2.1 mm; 5 µM particle size, pore size 60 Å) (Thermo, Bellefonte, PA).

RESULTS/DISCUSSION

To date, analysis for CPA, MPA, PAT and ROC has been conducted on samples from the NE region (*N*=12); analysis for the remaning toxins has been completed for all samples. Table 1 provides the average, range and frequency of the different mycotoxins detected in silage from the NE. Neither the aflatoxins nor 3ADON were detected in these samples. Toxins produced by *Alternaria* (AAL-TA and AAL-TB), *Fusarium* (DON, 15ADON, FB1, FB2 and ZEA), *Penicillium* (CPA, MPA, PAT and ROC) and *Aspergillus* (CPA) were detected in these samples, representing each of the four mycotoxigenic genera reported in silage. The fumonisins were the most frequently encountered toxins and this may be explained by the drought conditions during 2001 and 2002 (141), which are thought to favor fumonisin contamination (158). In addition, *F. verticillioides* is one of the most commonly isolated fungi from maize. Toxins produced by *Penicillium roqueforti* (MPA, PAT and ROC), the most commonly isolated filamentous fungus

in maize silage (31), and its close relative *P. paneum* were also found fairly frequently in these samples. Interestingly, in a related study on the mycotoxigenic fungi occurring in these samples we did not isolate any *Alternaria* species (138). However, we did identify *A. alternata* by molecular methods and detected toxins produced by this species in these samples (141).

In the total sample set (NE, NW, SE and SC) the most frequently occurring mycotoxins were DON and the FB1, although it is difficult to say if this will be true once the data analysis is complete on the *Penicillium* toxins. The average, range and frequencies (*N* = 120) of these toxins are provided in table 2 along with FB2, AAL-TA and AAL-TB. In addition to these toxins, six samples contained 15ADON (ave 1.16 µg/g; range 0.6 – 2.6 µg/g), five zearalenone (ave 1.1 µg/g; range 1.0 – 1.3), and one sample contained AF1 and AF2 with a concentration of AF1 at 23.5 ng/g and AF2 at 13.5 ng/g. The total amount of aflatoxin in these samples (AF1 + AF2), may be high enough for cattle to excrete aflatoxin M_1 in milk (81) at a concentration above the Food and Drug Administration action level of 0.5 ng/g (ppb) (185).

Multi-toxin contamination was evident in the NE samples where 70% of the samples were contaminated by three or more different toxins (Fig. 2). Of greater concern is that over a quarter (28%) of these samples were contaminated with five or more mycotoxins. In the total data set (*N* = 120 samples), four samples either had no toxin present (of the toxins tested in this study) or toxin concentrations lower than the detection limit, 33 contained one toxin, 40 two toxins, 35 three toxins and 8 had four toxins. As these samples have not yet been analyzed for the *Penicillium* mycotoxins, these numbers will likely change.

The data we have provided here indicate that multi-toxin contamination occurs frequently in silages produced in the the northeastern United States. Samples from the NE were found to contain toxins produced by all four mycotoxigenic genera reported in silage. Thus far in the total data set, FB1 and DON were the most common contaminants. Almost none of the samples were completely free of toxin, suggesting that multi-toxin contamination may be common in other silage producing areas in North America. Although most of the toxins occurred at levels below those of concern for cattle, the implications of multi-toxin contamination in silage are not well understood. Additionally, although the toxicity of several toxins investigated here, particularly the *Penicillium* mycotoxins, have not been studied with dairy cattle, case studies suggest that they are quite toxic to cattle (90, 220). Cattle do appear to be less sensitive to DON and the fumonisins compared to species such as horses or swine, (103, 186) but the affects of these toxins in combination with other myoctoxins have not been investigated.

The majority of studies on mycotoxin control or prevention focus on a single toxin. Given the evidence provided here, it is important to consider that silage may become contaminated with multiple toxins simultaneously. In the future, studies should take this factor into account when developing strategies to combat mycotoxin contamination in silage. This likely means that a combination of pre- and post-harvest cultural practices and possibly chemical controls will be necessary to reduce the overall mycotoxin load present in silage.

Table 7.1. The average, range and frequency of mycotoxins in maize silage from the NE in 2001 and 2002.

Toxin[1]	Average (µg/g)	Range (µg/g)	Frequency % (*N*=50)
AF1	0	0	0
AF2	0	0	0
AG1	0	0	0
AG2	0	0	0
AALTA	0.13	0.20 – 1.42	20
AALTB	0.03	0.03 – 0.07	14
CPA	0.05	0.02 – 0.86	26
DON	0.79	0.40 – 5.12	25
3ADON	0	0	0
15ADON	0.09	0.60 – 2.61	6
FB1	1.24	0.32 – 5.23	76
FB2	1.72	0.02 – 20.33	42
MPA	0.17	0.02 – 1.31	30
PAT	0.08	0.02 – 0.91	16
ROC	0.35	0.08 – 3.74	38
ZEA	0.06	1.02 – 1.10	4

[1] Toxin abbreviations: aflatoxin B1 and 2 – AF1, AF2, aflatoxin G1 and 2 – AG1, AG2, cyclopiazonic acid – CPA, deoxynivalenol – DON, 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), fumonisin B1 and B2 – FB1, FB2, mycophenolic acid – MPA, patulin – PAT, roquefortine C – ROC and zearalenone – ZEA.

Table 7.2. The average, range and frequency of mycotoxins in maize silage from all regions.

Toxin[1]	Average (µg/g)	Range (µg/g)	Frequency % (*N*=120)
AALTA	0.17	0.20 – 2.00	23
AALTB	0.05	0.03 – 0.90	13
AF1*	23.5 ng/g		0.08
AF2*	13.5 ng/g		0.08
AG1	0		0
AG2	0		0
DON	0.99	0.40 – 5.12	58
3ADON	0		0
15ADON	1.16	0.60 – 2.61	5
FB1	2.01	0.20 – 10.10	78
FB2	0.98	0.02 – 20.33	35
ZEA	1.10	1.00 – 1.30	4

* only one sample was positive for this toxin

[1] Toxin abbreviations: aflatoxin B1 and 2 – AF1, AF2, aflatoxin G1 and 2 – AG1, AG2, cyclopiazonic acid – CPA, deoxynivalenol – DON, 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), fumonisin B1 and B2 – FB1, FB2, mycophenolic acid – MPA, patulin – PAT, roquefortine C – ROC and zearalenone – ZEA.

Figure 7-1. Map of the relative locations of sample dairies. Symbols represent the four major dairy production regions Northeast - ★ , Northwest - ▲, Southeast - ■ and South central – ●.

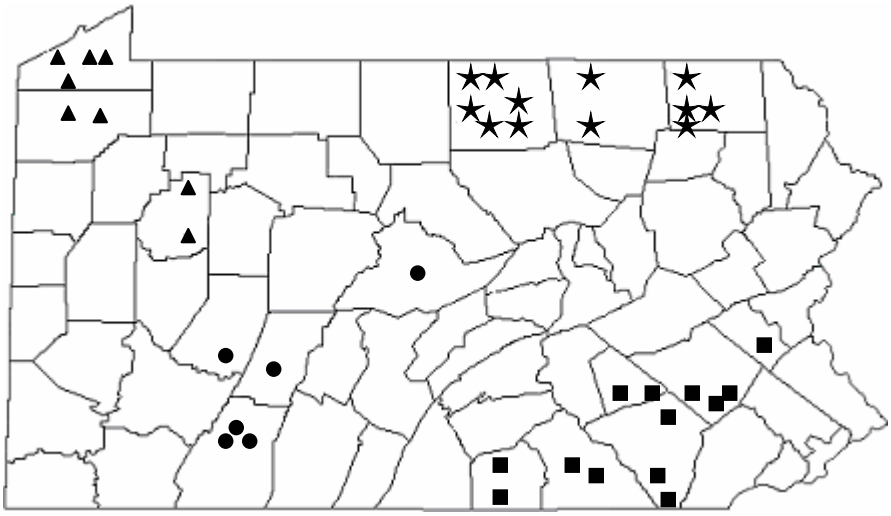
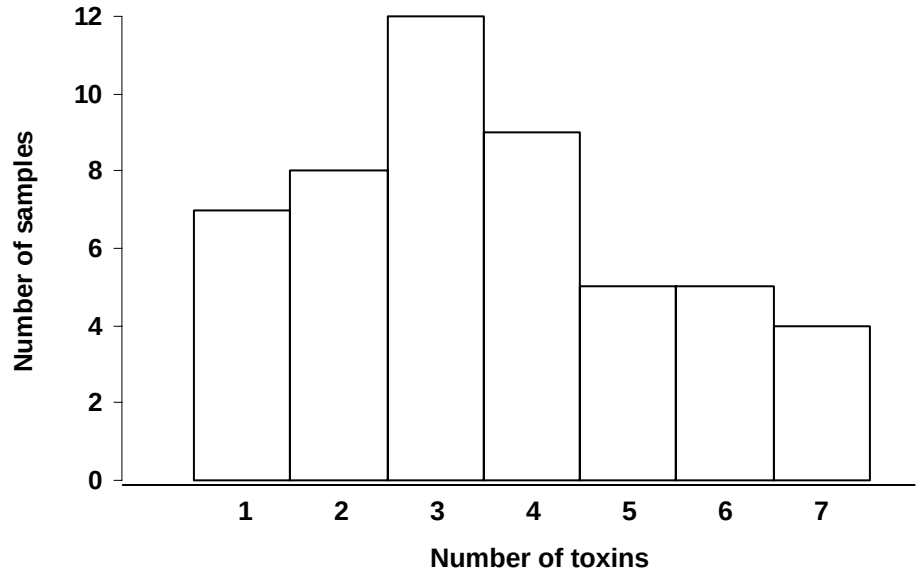


Figure 7.2. The number of samples (NE – *N* = 50) contaminated by one or more mycotoxins.



Chapter 8. Project summary, conclusions and the future of mycotoxin research in maize silage

Although mycotoxin contamination in maize silage has serious and detrimental impacts on the economic well-being of the livestock and dairy industry, few studies have addressed mycoflora and mycotoxins in North American maize silage. European and Asian studies on this topic usually focus on a single fungal species or toxin, although several toxigenic fungi occur in silage and there is considerable evidence for multi-toxin contamination. Also, little is known about what factors affect mycotoxin occurrence in maize silage as the majority of studies on toxins and maize have concentrated on maize grain rather than toxin distribution in the whole plant. Many of the strategies to prevent or manage mycotoxins maize silage provide limited or inconsistent control and this is partly due to our lack of understanding what fungi and mycotoxins are present and what factors impact toxin production in silage. In response to these concerns, the objectives of this research were to (1) study mycoflora in silage using traditional microbiological approaches and molecular techniques, (2) detect and quantify several classes of mycotoxins, including trichothecenes (T-2 toxin, deoxynivalenol and 15-acetyl-deoxynivalenol), zearalenone, fumonisins (B_1 and B_2), AAL-toxins (TA and TB), patulin, mycophenolic acid, and roquefortine C and (3) determine how weather conditions during the growing season, producers agronomic practices and the process of ensiling affect the occurrence and levels of these toxins in fresh and ensiled maize.

8-1: Summary of major findings

8:1-1 Maize silage mycoflora

There have been several published reports on the mycoflora of maize silage (8, 17, 112, 127, 151, 171, 228) and the majority of these studies have used culturing and morphological identification to detect and identify fungi from silage. To provide a more comprehensive assessment of mycoflora in North American silage, we analyzed fresh and ensiled whole plant maize using two methods: (1) a microbiological evaluation with selective media and morphological identification, and (2) a molecular assessment using DNA sequences. Although both methods used increased our understanding of fungi in silage, we found that the molecular technique detected a greater number of species than selective plating. Plating recovered several *Penicillium* and *Fusarium* species and *Aspergillus fumigatus,* while molecular analysis detected *Alternaria*, *Penicillium* and *Fusarium* species. *Alternaria alternata* and *A. tenuissima* were detected only using the molecular method. In addition, the molecular technique identified a novel species in silage, *Penicillium farinosum*. Data from both methods found that *Fusarium* and *Penicillium* were the dominant mycotoxigenic genera in silage, while yeast made up the majority all fungi recovered or detected. Fungi

known or suspected to produce mycotoxins often accounted for 50% or more of the total number of species isolated or detected from each study site, although ensiling reduced the total number of species recovered. Results from this study suggest that ensiling is an effective strategy to reduce the total number of toxigenic fungal species in maize, particularly *Fusarium* species. In addition, while *Fusarium* toxins are likely to be produced on maize while it is still in the field, *Penicillium* mycotoxins have the potential to be present in fresh and ensiled maize. The presence of multiple toxigenic species in the same silage sample suggested that multi-toxin contamination is a likely occurrence in North American maize silage and in later investigations this was found to be true.

8:1-2 Toxins in maize silage and factors that influence contamination

Although deoxynivalenol (DON) is one of the most well-studied and frequently encountered mycotoxins in maize, there are currently no management practices that provide consistent control of DON in maize silage. Much of what is known about DON contamination of maize is based on studies of the ear and kernels and most of these investigations have looked at disease development and toxin formation after artificial inoculation rather than natural infection. In response to these concerns, our objective was to investigate how natural DON contamination in maize silage is affected by different agronomic practices, weather conditions and ensiling. Overall, temperature and moisture during the early reproductive and maturation stages of plant development had the strongest affect on final DON concentration in silage. We did find it interesting that the 2002 growing season, which was warmer and drier than 2001, had higher average DON concentration. Our hypothesis for what we observed is that drought conditions favored premature plant development and were more conducive to Gibberella stalk rot, which has been described by Dodd (66, 273). These findings have significant implications in that DON contamination of silage may occur even under conditions not considered to be particularly favorable for toxin accumulation in grain and therefore, current models to predict the likelihood of toxin in grain are probably not valuable for silage.

Although agronomic practices such as deep tillage and crop rotation have long been suggested to as methods to control DON in maize, until now evidence to support these claims has been lacking. Based on our observations, samples from no till or minimum till locations had higher DON concentration than moldboard or mixed till locations although the incidence of toxin contamination was not different for any of the tillage systems studied. In terms of management, it is unlikely that deep tillage alone will prevent DON accumulation in silage given the strong influence of moisture and temperature. In both years of this study we consistently observed that ensiled samples had lower DON concentration that freshly harvested

samples. As we could find no evidence that the physical and chemical characteristics of these samples affected toxin concentration, we believe that toxin reduction may be a result of silage microflora.

Fumonisins make up another group of mycotoxins that like DON, frequently occur in maize silage, are not easily or consistently controlled in maize and are generally studied in maize grain rather than in the whole plant. AAL-toxin TA, which is structurally related to the fumonisins, has been reported in silage although almost nothing is known about toxin formation in maize. Due to the structural similarity of the fumonisins and AAL-toxins, we developed a method to simultaneously extract these two classes of toxins to (1) determine the levels and frequencies of fumonisins B_1, B_2, AAL-toxins TA and TB and (2) investigate how weather conditions, agronomic practices and the process of ensiling affect the concentration and frequency of SAMs in maize silage. Unlike DON, where final toxin concentration was affected during the early stages of grain development, there was a relationship with temperature and moisture throughout plant maturation for the fumonisins and at the later stages for AAL-toxin TA. Greater fumonisin concentrations were also found in silage that was harvested at later maturity stages, which may reflect the association observed by Dodd (66) that increased susceptibility to fungal colonization comes as the plant is relocates photosynthates from the stalk for grain fill.

Unlike the *Fusarium* toxins considered in this study, mycotoxins produced by *Penicillium* species have not been thoroughly investigated in maize or silage. Toxin production is thought to occur primarily during storage as many *Penicillium* spp. grow well at low water activities, but this has not been demonstrated rigorously. Our objective was to develop a multi-toxin method to detect and quantify cyclopiazonic acid, mycophenolic acid, patulin and roquefortine C in fresh and ensiled maize and determine the frequency and levels of these toxins to better understand which are of concern and how the process of ensiling impacts toxin concentrations. Results of this work indicate that all four of the *Penicillium* mycotoxins were present fairly frequently (30-40%) but at relatively low concentrations, although for all of these toxins it is unknown what levels are of concern for cattle. Contrary to the long-held thought that toxin formation occurs primarily during storage, our data suggest that as many of the freshly harvested samples also contained these toxins, toxin formation may begin in the field and continue during ensiling. Given that maize silage appears to be contaminated in the field as well as in storage, management practices aimed at toxin control may need to focus on the crop both before it is ensiled and during storage.

Preliminary data from our toxin analyses suggest that multi-toxin contamination of silage occurs regularly, which is of concern as the majority of studies on mycotoxin control or prevention focus on a single toxin. Although some toxins occurred at levels below those of concern for cattle (DON and the

fumonisins), the implications of multi-toxin contamination in silage are not well understood and in some cases the affects of even a single toxin, particularly those produced by *Penicillium* species are unknown. Given the results of this investigation, future work on stragies to combat mycotoxin contamination in silage will rquire a broader wider perspective and focus on mycotoxins occurring in combination rather than in isolation. As it is unlikely that any single activity will reduce toxin contamination, control may require the incorporation of multiple pre and post-harvest practices to reduce the overall mycotoxin load in silage.

8:3 Future investigations on mycotoxigenic fungi and mycotoxins

8:2-1 Issues not addressed by this work

As so few of the samples in this study contained aflatoxins (B_1, B_2, G_1 and G_2), the acetylated forms of deoxynivalenol 3 and 15 acetyldeoxynivalenol), T-2 toxin and zearalenone, we were not able to accurately address the affects of weather conditions, agronomic practices and the process of ensiling on the concentrations of these toxins in maize silage. It may be that the environmental conditions were not favorable for the accumulation of these toxins, indicating that study over several more seasons should be conducted. Another possibility is that fungi producing these toxins are not commonly present within the study area, suggesting that study encompassing a wider geographical area would be required.

Because many of the study sites did not incorporate rotation with a broad leaf crop as part of their routine agronomic practice, it was difficult to determine what, if any, affect this had on the frequency and concentration of the *Fusarium* toxins. As so few studies have addressed the efficacy of crop rotation to control mycotoxins in maize, future studies should focus on how rotation affects the levels and concentrations of these toxins. Such an investigation could either recruit interested participants that incorporate different rotation strategies or could utilize field trials to assess the impact on mycotoxins.

Although this study was able to consider a large and varied number of mycotoxins in maize silage, there are several toxins that may be present, based on the fungal species isolated from silage, that were not investigated. Some of these toxins include: tenuazonic acid (*Alternaria*), fusaric acid (*Fusarium*), moniliformin (*Fusarium*) and toxins produced by *Aspergillus fumigatus*, including fumitremorgens B and C, fumigaclavines B and C, and gliotoxin. To date, none of these toxins have been reported in silage.

A final consideration is that both seasons considered in this study were unusually hot and dry. As such, it may be that the observations noted here on fungi, toxins and the affects of environmental conditions are considerably different that what one might see during a season with average temperatures and precipitation. To address these concerns, it would be beneficial to conduct a longer study (3-5 years) where one would be more likely to be able to oberve conditions over a range of seasonal variables.

8:2-2 Other future directions for investigations on mycotoxigenic fungi and mycotoxins

As noted in the introduction, there are many unknowns in terms of our understanding of mycotoxigenic fungi and mycotoxins. Although great progress is being made in understanding the genetics behind toxin production, there is still relatively little known about *Alternaria* and *Penicillium* toxins which is of concern as these fungi are frequent contaminants of foods and feeds. Another subject that has not been adequately addressed for many mycotoxigenic fungi is why they invest energy in producing mycotoxins. This is particularly true again for the *Aspergillus* and *Penicillium* toxins, although this is still unclear for some *Fusarium* toxins such as the fumonisins and zearalenone.

8:3 Implications of this work for the future of mycotoxin control in maize silage

Within the framework of this investigation we have successfully (1) developed sensitive assays to detect both mycoflora and mycotoxins in fresh and ensiled maize, (2) conducted a through survey of fungi and mycotoxins in maize silage and (3) identified factors that impact the occurrence and levels of major mycotoxins. As many of the toxin analysis methods we have developed will detect several mycotoxins simultaneously, this may spur further research into toxin prevalence in maize throughout North America. In addition, as we identified many factors that influence toxin contamination of maize silage the data provided by our work may lay the foundation for developing predictive models to warn producers of the likelihood of toxin contamination in silage and other maize-based feeds. As some of the agronomic practices considered in this study were not widely performed, continuing work should focus on how practices such as tillage and crop rotation and how they affect mycotoxin levels. In conclusion, this study has provided valuable information for future researchers as well as producers in understanding mycotoxins found in maize silage and factors that may be exploited to reduce toxin occurrence in silage.

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EDUCATION

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The Pennsylvania State University, University Park, PA 16802. Began Ph.D. dissertation work in Plant Pathology in August of 2001. Completed the requirements of the Doctor of Philosophy in Plant Pathology in December 2005.

PROFESSIONAL SOCIETIES

The American Phytopathological Society. 2002 to the present.
The Mycological Society of America. 2002 to the present.
Sigma Gamma Delta. 2003 to the present.

PUBLICATIONS/ PRESENTATIONS & ABSTRACTS AT NATIONAL MEETINGS

Nagy, M.A. and Kuldau, G.A. 2003. Mycotoxigenic fungi and mycotoxins in Pennsylvanian corn silage. Abstract and presentation prepared for the 4th annual Melhus Symposium sponsored by the American Phytopathological Society.

Mansfield, M.A. and Kuldau, G. A. 2004. Survey of fungi in maize silage using microbiological and molecular techniques. Abstract and poster prepared for The Mycological Society of America 2004 Annual Meeting in Asheville, N.C.

Kuldau, G.A., Mansfield, M.A., Archibald, D.D., Jones, A.D. 2004. Development of a liquid chromatographic method for determination of two classes of mycotoxins in maize silage. Abstract and poster prepared for the American Phytopathological Society 2004 Annual Meeting in Anaheim, CA.

Mansfield, M. A., Jones, A. D., and Kuldau, G. A. 2005. Levels and frequencies of *Penicillium* mycotoxins in fresh and ensiled maize silage from Pennsylvania. Abstract and presentation prepared for the American Phytopathological Society 2005 Annual Meeting in Austin, TX.

PROFESSIONAL ACHIEVEMENTS

Recipient of the I.E. Melhus award to travel to the annual meeting for The American Phytopathological Society in Charlotte, N.C. and present at the fourth annual Melhus Symposium in August 2003.

Recipient of the Popp Award presented by the Penn State Department of Plant Pathology in August 2004.