2015

Seedborne black Aspergillus species as maize seedling pathogens: role of fumonisin production and interaction with soilborne Pythium species

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Seedborne black *Aspergillus* species as maize seedling pathogens:

Role of fumonisin production and interaction with soilborne *Pythium* species

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Plant Pathology

Program of Study Committee:
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Iowa State University

Ames, Iowa

2015

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ACKNOWLEDGEMENTS

I would like to thank my major professors, Gary Munkvold and Alison Robertson, for their guidance and help throughout the course of this research, and especially for their attention to detail in the writing of the final report. Thank you also to Tom Kaspar for his help in ensuring clarity throughout. Both of my labs have also been immensely helpful and supportive, especially my lab managers, who patiently answered dozens of logistical questions, and Sally Mallowa, who welcomed and encouraged me from day one.

I would like to acknowledge Dr. Robert Proctor (USDA-ARS, Peoria, IL) and Dr. Antonio Moretti (ISPA, Bari, Italy) for providing the Aspergillus isolates from Illinois and Italy, and the Leath Assistantship, which funded a portion of my studies.

I would also like to thank my family and friends for all their support and encouragement along the way, and for reminding me that while academics isn't all there is to life, God is honored by hard work and perseverance in every area of our lives. Finally, thank you Jesus for giving me life and everything else.

ABSTRACT

Twenty-six strains of black *Aspergillus* (*Aspergillus* section Nigri) were studied as seedling pathogens of maize. There were two major research components. The first component was an evaluation of the pathogenicity of isolates from several black *Aspergillus* species and a comparison of the pathogenicity of isolates with and without fumonisin production. This was accomplished by testing inoculated seeds in warm germination and cold tests, and by evaluating growth of inoculated seeds in rolled paper towel assays. In the second component, four of these species were selected for evaluation of interactions between *Aspergillus* and *Pythium* as a seedling disease complex in maize. Seeds inoculated with *Aspergillus* were planted in soil infested with *Pythium sylvaticum*, *Pythium torulosum*, or a control in a cup assay, and scored on several criteria for seedling growth.

Species of *Aspergillus* in section Nigri are commonly associated with maize kernels but there is little information about their effects on maize seed germination and seedling health. It has recently been discovered that some strains in this group have the capacity for producing the fumonisin mycotoxins, but it is not known what effect, if any, this has on pathogenicity in seedling disease. We compared 9 strains from Iowa, 4 from Illinois, and 13 from Italy in two seed-inoculation assays assessing their ability to affect germination and seedling growth. Some were fumonisin-producing strains and others were non-producing. Representatives of *A. awamori*, *A. niger*, *A. phoenicis*, *A. tubingensis*, and a single strain of *A. carbonarius* were included. Maize seeds of two different hybrids were inoculated with spores of each of these strains. They were evaluated for germination and seedling growth using a warm germination test, a cold test, and a rolled paper towel assay. Strains of each species reduced germination or

seedling growth of one or both hybrids, but there was high variability among strains within species. There were no consistent differences between fumonisin-producing and non-producing strains.

While many pathogens of maize seedlings have been studied extensively in isolation, little is known about their interactions with each other. In the second portion of this study we investigated the relationship between *Aspergillus* section Nigri, seedborne fungi that cause ear rot and seedling disease in maize, and *Pythium* spp., which are soilborne and cause seedling disease. Maize seeds inoculated with one of four strains of *Aspergillus* or not inoculated were planted in cups filled with non-infested sterile field soil, or soil infested with *P. sylvaticum* or *P. torulosum*. The cups were placed in a growth chamber at 25°C in a randomized complete block design (RCBD) and assessments done at 7 and 14 days after planting (DAP). An interaction was detected between *Pythium* and *Aspergillus* on seedling height at 7 DAP and percent emergence. Percentage healthy mesocotyl, height at 14 DAP, and shoot weight were reduced by *Pythium* only. Root weight was affected by both *Pythium* and *Aspergillus*, but with no interaction. For the variables with an interaction, *P. torulosum* caused more severe symptoms when associated with seed-borne *Aspergillus*, while *P. sylvaticum* caused severe symptoms regardless of the presence of *Aspergillus* spp. The results suggest that seedborne *Aspergillus* can exacerbate seedling disease caused by *Pythium* spp. under some conditions. This highlights the need for further study of seedling pathogens with reference to the entire soil ecosystem, rather than simply in isolation.

CHAPTER I

INTRODUCTION

Goals and Rationale

Aspergillus section Nigri is prevalent in soils worldwide and is a pathogen of several crops, including maize. It is of particular importance because it produces mycotoxins hazardous to both human and animal health, including ochratoxin and fumonisin. It infects the maize kernels in the ear, and can remain as a seedborne pathogen, infecting the seedlings as they germinate (3).

In light of the recent discovery of fumonisin production in *Aspergillus* section Nigri species (1), there has been substantial study of the genetics of fumonisin production and its implications for food safety. However, there has not been any attempt to date to determine what role, if any, fumonisins play in *Aspergillus* seedling disease of maize. We designed our study to compare a wide selection of strains from *Aspergillus* section Nigri, including both fumonisin-producing and non-producing strains. We hypothesized that fumonisin production aids the fungus in infecting the maize seedling by interfering with its metabolism, so we expected that there would be detectable differences in pathogenicity between fumonisin-producing and non-producing strains.

In addition, we wanted to investigate whether *Aspergillus* section Nigri, which is a common seedborne pathogen (4), has an interaction with soilborne *Pythium*. Since *Pythium* is one of the most important soilborne seedling pathogens of maize, it is important to understand how it interacts with other pathogens in the soil and on the seed surface. Although the

comparison between the *Aspergillus* strains used in the first part of this study did not show any clear effect of fumonisin production on pathogenicity, strains with and without fumonisin production capability were included to see if fumonisin had an important effect on the interaction. Since seedling pathogens have a range of possible interactions with each other, from no increase in symptoms from an additional pathogen, to disease at least equal to the individual symptoms added together (2), we could not anticipate beforehand what sort of effect we would discover in exposing maize seedlings to both pathogens simultaneously.

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CHAPTER 2

REVIEW OF LITERATURE

Introduction

Maize has long been important as a food crop and as animal feed. More recently, it has also become a major component of energy production as a biofuel crop for fermentation and ethanol distillation. Like all crops, it has many economically important diseases, which significantly reduce production capacity. Pathogens can attack maize plants at any stage in their lifecycle, causing seed rots and seedling diseases, stem and foliar diseases, ear rots in the field, and storage molds of harvested kernels. In the United States, it is estimated that maize diseases of all kinds cause anywhere from a 2% to a 15% reduction in yield each year (43).

Seedling Diseases of Maize

Prevalence

Collectively, seedling diseases have a major impact on maize yield. In 2013, maize seedling diseases in the United States and Ontario collectively caused more yield loss in maize than any one pathogen (21). Total losses from seedling diseases in the top 21 maize-producing states and Ontario were estimated at 149.8 million bushels for 2013 (21), which equates to 1.1% of the total maize crop produced in the United States that year (4). In Illinois, Iowa, Minnesota, and Nebraska alone, maize seedling diseases caused an estimated loss of 126.5 million bushels in 2013, nearly double the losses from Goss's wilt, the next most damaging pathogen (21). Since

the United States produces 30% of the world's maize (3), even a small percentage loss translates into a major impact on global food production.

Seedling diseases include both pre-emergence death of the seed or seedling, and post-emergence death, or damping off. In maize, seedling disease is often caused by fungi and oomycetes, which can be either seedborne or soilborne. Symptoms in maize are often similar, regardless of causal organisms, and include reduced emergence and stunting, discoloration, and wilting of the plants that do emerge (24).

If a large portion of the field has poor emergence or damping off, it may be necessary to replant to achieve the maximum profit from that field (10). However, as the season goes on, it becomes less and less advantageous to go to the expense of replanting, since yield potential of the replanted crop will be low due to the reduced growing season (10).

Moreover, although a maize plant may survive the initial pathogen attack, damage to roots or other organs often results in stunting even in the mature plant (6). This can also cause yield loss, especially when plants in the field are unevenly affected. If some plants are delayed in their development compared to others in the same row, this may result in as much as a 5-10% yield loss, as the smaller plants draw nutrients away from the larger ones, but are unable to produce a large yield themselves (10). In extreme cases, highly uneven growth may also warrant replanting a field (10).

Fungicide seed treatments are highly effective at reducing seedling diseases in maize, but cannot completely eliminate them (22). Disease is generally most severe when seedlings are already under stress from other factors, such as drought, chilling injury, misuse of chemicals, or seed damage prior to planting (43). In Iowa, seedling disease has been particularly problematic in south and southeast Iowa, especially in years with cool, wet conditions near planting (33). The

risk of seedling disease is increased if temperatures are below 13°C at or soon after planting (22). Standing water in the field both stresses the seedling and provides favorable conditions for zoospore dispersal in pathogens such as *Pythium* (24). In addition, there is a greater risk for seedling disease when there are high levels of inoculum in the soil from the previous year, such as when maize is planted in consecutive years without rotation to non-host crops (34).

Causal organisms

Seedling diseases are caused by numerous fungi and oomycetes, both seedborne and soilborne. Many common maize ear rot fungi, such as *Fusarium* spp., *Aspergillus* spp., *Rhizoctonia* spp., *Nigrospora* spp., *Trichoderma* spp., and *Stenocarpella maydis*, are also seed-transmitted and may cause disease on seedlings which germinate from infected seed (43). In addition to pathogens present on the seed itself, many soilborne fungi and oomycetes can also cause disease in maize seedlings, including *Pythium* spp., *Fusarium* spp., and *Penicillium oxalicum* (43).

In Iowa, the most common seedling disease pathogens of maize are in the genera *Pythium* and *Fusarium* (24). *Pythium* species primarily infect maize seeds and seedlings in cool, wet soils, and cause dark, water-soaked lesions on the mesocotyl and root, while *Fusuarium* species result in lighter lesions and shriveled roots and mesocotyl (32). Both are more prevalent when seedling emergence is delayed, as often happens with early planting (32). They can be managed by use of seed treatment fungicides, and by waiting to plant until the soil temperature is above 10°C and there is minimum excess soil moisture (32).

Black *Aspergillus*

Aspergillus niger and related species

Several species of *Aspergillus* are common among seedborne fungi in maize. The genus *Aspergillus* contains over 200 species, primarily distinguished by their distinctive spore-bearing structures, consisting of a long conidiophore with a round cluster of conidia chains at the end (30). Many of these species are important as plant, animal, and human pathogens, and several produce mycotoxins hazardous to human and animal health (30). *A. flavus* is well-known for its aflatoxin production (43), and several species of *Aspergillus* section Nigri produce ochratoxin A, fumonisins, or both (12).

Aspergillus species are placed into major groups based on their spore color, including the *A. niger* group (*Aspergillus* section Nigri), which comprises the species which produce black conidia (30). Classification within this subdivision is difficult since the species are morphologically quite similar, and thus genetic analysis is relied on heavily (1). Many species of the *A. niger* aggregate, which include *A. niger*, *A. tubingensis*, *A. awamori*, and *A. phoenicis*, among others, are particularly difficult to differentiate, while *A. carbonarius* is more readily identified because of its larger spore size (1).

Aspergillus species are prevalent saprophytes in soils worldwide, and are especially common in subtropical regions, between 25-35 degrees latitude (15). *Aspergillus niger* has optimum growth between 30-37°C, but can grow well at temperatures up to 42°C (19). It does not tolerate low temperatures, and shows little to no growth at 15°C (19). *Aspergillus niger* can achieve stable growth at a pH as high as 8, but when grown in an unbuffered medium, it will produce various forms of acid, rapidly reducing the pH to below 2 (2). This characteristic has been harnessed for the commercial production of citric acid (2). It is thought that aggressive

acidification gives *A. niger* a competitive advantage against organisms that are unable to grow well at such a low pH (2). *A. niger* also has a very high salt tolerance and has even been identified as a common species in the hypersaline waters of salt evaporation ponds (7).

Black *Aspergillus* species are primarily associated with diseases in maize, peanut, grape, and onion (28). Many species of *Aspergillus*, including those in *Aspergillus* section Nigri, cause ear rot on maize and can also become a problem as storage molds if the grain moisture content is too high (16). In the United States, *Aspergillus* species are not typically a major concern as a seedling pathogen, although seedborne infection of up to 62% has been reported (18). A survey of untreated commercial seed lots in the United States found an overall contamination of 2.8% (36).

Fumonisin production in *Aspergillus* section Nigri

Fumonisins are a family of mycotoxins primarily produced by fungi in the genus *Fusarium*. They are toxic to humans and to many domestic animals (43). They strongly affect sphingolipid metabolism, which is thought to interfere with cellular regulation, causing effects which vary according to the species of animal affected (29). These include neurotoxicity in horses and pulmonary edema in swine (29). In humans, fumonisins are associated with esophageal cancer, gastrointestinal disease, liver cancer, and neural tube defects (42). There are more than 20 different forms of fumonisins, but the most important are fumonisin B_1, B_2, and B_3.

It was recently discovered that some *Aspergillus* species contain a cluster of genes similar to those controlling fumonisin production in *Fusarium*. However, *Aspergillus* species do not possess orthologues to all genes in the *Fusarium* fumonisin cluster; in particular, the lack of an orthologue to the *Fusarium fum2* gene results in a lack of fumonisin B_1 production (39). Instead, the primary fumonisin produced in *Aspergillus* is fumonisin B_2 (37), biosynthesis of which

requires the *fum8* gene, found in some strains of *Aspergillus*. In-vitro production of fumonisin B_2 in *Aspergillus niger* was first confirmed in 2007 (12). Some strains of Aspergillus isolated from raisins were later reported to produce fumonisin B_1 and fumonisin B_3 as well (40), though this finding has been disputed (25, 26, 41). The fumonisin B_2 produced by *A. niger* was compared to that produced by *Fusarium* spp., and no differences could be detected in the chemical structures, even though the genes for the synthases differ somewhat (17). Two additional fumonisins are also produced by *A. niger*: fumonisin B_4, which is also present in small amounts in *Fusarium* species (27), and fumonisin B_6, which has the same elemental composition as fumonisin B_1, but differs in structure (17).

Fumonisin production in *Aspergillus* species is affected by the environment in which they are grown. In a comparison between media types, *A. niger* produced fumonisins primarily in media with low water potential, consistent with its growth as a storage mold, in contrast with *Fusarium verticillioides*, which produced fumonisins predominantly on media containing plant extracts (12). *A. niger* has optimal growth around 37°C, but comparatively low fumonisin production at that temperature, with the greatest production of fumonisin B_2 occurring around 25-30°C, a somewhat higher temperature than the maximum production observed in *Fusarium* spp. (19). Most *Aspergillus* strains showed an increase in fumonisin B_2 production with increasing NaCl and sucrose, but a decrease in production with increasing glycerol, though the results were rather variable with respect to the response to water activity (19). In addition, lactate has been shown to increase fumonisin B_2 production in the presence of starch (38).

Fumonisin and plant disease

It is unclear how fumonisin production benefits fumonisin-producing fungi. Preventing fumonisin B_2 production by disruption of the *fum8* gene does not affect vegetative growth, sensitivity to temperature, or sensitivity to UV light, suggesting it does not play a role in normal growth of the fungus (37).

In *Fusarium*, the role of fumonisins in pathogenicity is controversial. It is not necessary for disease, since it has been shown that some strains of *F. verticillioides* can infect maize without it (8). However, another study by Desjardins et al. found that nearly all highly virulent strains of *F. moniliforme* also produced high levels of fumonisin B_1 , while many less virulent strains did not (9). Additionally, disease symptoms and stunting can be induced in maize seedlings by fumonisins alone, though the seedlings eventually outgrew the effects at the lower concentrations tested (5). Maize seedlings have some ability to detoxify low levels of fumonisin, but higher levels have detrimental effects, possibly by inducing premature senescence through their interference with sphingolipid metabolism (5).

It is possible that fumonisins contribute to virulence or pathogenicity in *Aspergillus* species, but this has not yet been experimentally tested. This study is intended to begin an examination of this question by comparing the virulence of a range of fumonisin-producing and non-producing strains of *Aspergillus* section Nigri.

Pythium and Seedling Disease

Classification and general information

Many species in the genus *Pythium* cause disease, most commonly root rot, on a wide variety of plant hosts. Historically, their abundance in the environment has been underestimated

because they compete poorly with saprophytes in culture, but they are now frequently isolated from diverse environments through the use of selective media. (13). Classification of *Pythium* species is based primarily on characteristics of the sporangia and oogonia, which is somewhat problematic because these morphological characteristics can be highly dependent on cultural conditions (13). Additionally, many currently described "species" may merely represent the extreme ends of species complexes, with no clear dividing line separating them (13). Most *Pythium* species are homothallic, but a few, including *P. sylvaticum*, are heterothallic, and distinct mating populations have been identified (13).

Pythium species primarily infect young roots and often cause pre- and post-emergence damping off in the seedlings of their various hosts, which can cause localized heavy losses (13). Later in the seedling development, *Pythium* is generally restricted to infecting secondary roots, but can still cause root rot, resulting in stunting and chlorosis which may permanently damage the young plant (13). Even in adult trees and other perennials, *Pythium* species can continue to attack new root growth, causing decline and eventual death of the host plant (13). Though *Pythium* is primarily a root pathogen, under favorable conditions it can infect succulent stems, leaves, and fruits as well (13). In maize, *Pythium* can continue to infect root tips in the adult plants throughout the growing season, especially in poorly drained soils (43).

There is wide variation among *Pythium* species with regard to their optimum temperatures and moisture conditions (13). Some species only cause disease under high-moisture conditions, as much as 90% moisture holding capacity, while others are relatively insensitive to moisture, and some were inhibited by 90% soil moisture, but were effective pathogens at lower soil moisture contents (13). For some species, temperature is a more important factor in pathogenicity than soil moisture, but the optimum temperature for growth is not always the same

as the optimum temperature for disease development (13). In maize seedlings, *Pythium* infection is most commonly associated with the cool wet conditions early in the season (6, 31).

Pythium primarily infects maize at the seedling stage, resulting in seed rot and damping off (6). Typical symptoms include dark lesions on the roots and mesocotyl and poor root development (24). In 2012, damping off from *Pythium* caused an estimated loss of 63.3 million bushels of maize in Illinois, Iowa, Minnesota, and Nebraska alone (20).

Pythium species can survive as saprophytes, but are not strong competitors with other saprophytic organisms (13). They can, however, survive for years in the soil as a saprophyte or as resting structures, such as long-lasting oospores (13). In Iowa, maize-soybean rotation is of limited use in controlling *Pythium* infection, since many of the same strains that infect maize are also highly virulent on soybean (35, 44).

Many species of *Pythium* can infect maize, with the most common species varying by region. An Ohio study found *P. dissotocum* and *P. sylvaticum* to be the most common species recovered from diseased maize seedlings, with 7 other species identified (6), while a recent survey of diseased maize seedlings in Iowa found predominantly *P. torulosum*, in addition to 8 other *Pythium* species (33). There are also large differences in virulence on maize between species of *Pythium* and even between strains within a species (6, 44).

Interactions with other organisms

Seedling diseases in nature do not occur in a vacuum. In fact, it is often difficult to determine which pathogen is actually responsible for seed or seedling death, and which are merely acting as opportunistic saprophytes (23). Since there are many other pathogens present in the soil and sometimes in the seeds as well, it is likely that some of them interact with *Pythium* species. Some may compete for resources as saprophytes, or as seedling pathogens. Others may

even facilitate infection by *Pythium*, or may themselves cause increased disease in the presence of *Pythium*. Understanding these interactions could allow for more effective and comprehensive management strategies, but little work has been done on this topic to date.

Some work has been done on the interaction of *Pythium* with soil fungal pathogens. In perhaps the first attempt to explore this subject, Ho (14) inoculated maize seedlings with several pairs of seedling pathogens, including *Pythium* and *A. niger*, and found varying responses, ranging from a roughly additive effect in disease severity to no effect at all from the addition of the second pathogen. However, this study only tested a single isolate from each pathogen, and used a rather imprecise rating system for evaluating disease severity. In addition, there was no statistical analysis, making it impossible to determine whether the results observed were significant. There was no consistent pattern of interaction discernible between either species of *Pythium* and *Aspergillus* in this study (14).

A study by Foley found that infection of maize seed with *Diplodia zeae*, *Gibberella zeae*, or *Nigrospora oryzae*, though symptomless, dramatically reduced germination when placed onto cultures of *P. debaryannum*, as opposed to germination in the presence of *P. debaryannum* alone (11). As the author noted, it was not possible to control for all pathogens, particularly since *Fusarium moniliforme* was endemic in the seeds, but it was assumed that this background level of disease was constant, regardless of whether there was an additional infection of one of the three species studied (11). Though this study did not have a formal statistical analysis of the interaction, the magnitude of the differences observed is too great to plausibly attribute them to chance (11).

There is evidence for an interaction between *Pythium* and *Fusarium* species in peanuts and peas (13), however, to our knowledge, no study has rigorously evaluated the interaction of

Pythium with any species of *Aspergillus*. There appears to have been little work of any kind on interactions between maize seedling diseases in recent decades.

In this study, we compared two of the most common *Pythium* strains that cause seedling disease in maize, *P. sylvaticum* and *P. torulosum*, and evaluated them for evidence of an interaction with *Aspergillus* in a cup assay. Since it is possible that fumonisin production could play a role in the interaction, either by weakening the plant or by making the *Aspergillus* strains more competitive against *Pythium*, we selected two fumonisin-producing *Aspergillus* strains and two non-producing strains for inclusion in the study.

Summary and Conclusion

Seedling diseases are a major issue in maize production, perhaps more so than any other single disease. Although seed treatments have come a long way in mitigating their effects, seedling disease still causes heavy losses each year. If disease is severe enough, it may be more economical to replant the whole field and start over than to take the losses from the reduced stand count and stunted, unproductive plants. However, the time lost from crop growth can still result in significant yield reductions. It is important to understand seedling diseases and their interactions with each other so that they can be effectively managed and losses reduced.

Aspergillus section Nigri generally causes more damage as an ear rot than as a seedling pathogen in maize. However, the recent discovery of fumonisin production in certain strains has made it important to understand whether the presence of mycotoxin affects any stage of the disease cycle. If fumonisin production gives *Aspergillus* species an advantage in pathogenesis, or in competing against other microorganisms, the fumonisin-producing strains may have a larger economic impact. This study addresses this question by comparing a large range of fumonisin-

producing and non-producing strains in three different kinds of germination and seedling growth assays.

Since *Aspergillus* section Nigri is a common seedborne pathogen (18, 36), it is important to understand how it may interact with other seedling disease fungi and oomycetes already present in the soil. One such pathogen is *Pythium*, a common soilborne oomycete which is also one of the most important pathogens in Iowa maize production. *Aspergillus* alone does not often cause major seedling disease, but if it exacerbates disease caused by *Pythium* and other organisms, control of *Aspergillus* contamination in maize seed could be a viable strategy for reducing overall seedling disease. In this study, maize seed inoculated with one of four Iowa strains of *Aspergillus* section Nigri was planted into cups containing soil containing *P. sylvaticum* or *P. torulosum*, two of the most common species that cause disease in maize, and the resulting seedlings assessed for evidence of an interaction.

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CHAPTER 3

PATHOGENICITY OF *ASPERGILLUS* SPECIES IN SECTION NIGRI TO MAIZE SEEDLINGS

Abstract

Species of *Aspergillus* in section Nigri are commonly associated with maize kernels but there is little information about their effects on maize seed germination and seedling health. It has recently been discovered that some strains in this group have the capacity for producing the fumonisin mycotoxins, but it is not known what effect, if any, this has on pathogenicity in seedling disease. We compared 9 strains from Iowa, 4 from Illinois, and 13 from Italy in two seed-inoculation assays assessing their ability to affect germination and seedling growth. Some were fumonisin-producing strains and others were non-producing. Representatives of *A. awamori*, *A. niger*, *A. phoenicis*, *A. tubingensis*, and a single strain of *A. carbonarius* were included. Maize seeds of two different hybrids were inoculated with spores of each of these strains. They were evaluated for germination and seedling growth using a warm germination test, a cold test, and a rolled paper towel assay. Strains of each species reduced germination or seedling growth of one or both hybrids, but there was high variability among strains within species. There were no consistent differences between fumonisin-producing and non-producing strains.

Introduction

Several species of *Aspergillus* are common among seedborne fungi in maize. The genus *Aspergillus* contains over 200 species, including the *A. niger* group (*Aspergillus* section Nigri),

comprising the species which produce black conidia (15). Black *Aspergillus* species are primarily associated with diseases in maize, peanut, grape, and onion (14). Many species of *Aspergillus*, including *Aspergillus* section Nigri, cause ear rot on maize and can also cause damage in storage if grain moisture content is too high (8). In the United States, they are not typically a major concern as seedling pathogens, although seedborne infection of up to 62% has been reported on maize, and occasional serious outbreaks of *Aspergillus* ear rot have occurred (10). A survey of untreated commercial seed lots in the United States found an overall contamination of 2.8% (16).

It was recently discovered that some *Aspergillus* species contain a cluster of genes similar to those controlling fumonisin production in *Fusarium*. However, *Aspergillus* species do not possess orthologues to all genes in the *Fusarium* fumonisin cluster; in particular, the lack of an orthologue to the *Fusarium fum2* gene results in a lack of fumonisin B_1 production (22). Instead, the primary fumonisin produced in *Aspergillus* is fumonisin B_2 (19), biosynthesis of which requires the *fum8* gene, found in some strains of *Aspergillus*. In-vitro production of fumonisin B_2 in *Aspergillus niger* was first confirmed in 2007 (6). Unlike *Fusarium verticillioides*, which produces fumonisin B_1, fumonisin B_2, and fumonisin B_3, the *A. niger* strains were initially only observed to produce fumonisin B_2 (6). Some strains of Aspergillus isolated from raisins were later reported to produce fumonisin B_1 and fumonisin B_3 as well (23), though this finding has been disputed (11, 12, 24). The fumonisin B_2 produced by *A. niger* was compared to that produced by *Fusarium* spp., and no differences could be detected in the chemical structures, even though the genes for the synthases differ somewhat (9). Two additional fumonisins are also produced by *A. niger*: fumonisin B_4, which is also present in small amounts in *Fusarium* species

(13), and fumonisin B_6, which has the same elemental composition as fumonisin B_1, but differs in structure (9).

It is unclear how fumonisin production benefits fumonisin-producing fungi. Preventing fumonisin B_2 production by disruption of the *fum8* gene in *A. niger* does not affect vegetative growth, sensitivity to temperature, or sensitivity to UV light, suggesting it does not play a role in normal growth of the fungus (19).

In *Fusarium*, the role of fumonisins in pathogenicity is controversial. It is not necessary for pathogenicity, since it has been shown that some strains can infect maize without it (4). However, a study by Desjardins et al. (5) found that nearly all highly virulent strains also produced high levels of fumonisin B_1 , while many less virulent strains did not. Additionally, disease symptoms and stunting can be induced in maize seedlings by fumonisins alone, though the seedlings eventually outgrew the effects at the lower concentrations tested (2). Maize seedlings have some ability to detoxify low levels of fumonisin, but higher levels have detrimental effects, possibly by inducing premature senescence through their interference with sphingolipid metabolism (2).

It is possible that fumonisins contribute to virulence or pathogenicity in *Aspergillus* species, but this has not yet been experimentally tested. This study is intended to begin an examination of this question by comparing the virulence of a range of fumonisin-producing and non-producing strains of *Aspergillus* section Nigri.

We designed our study to compare a wide selection of strains from *Aspergillus* section Nigri, including both fumonisin-producing and non-producing strains. Warm germination and cold tests were used to evaluate seed germination under ideal and cold stress conditions, and rolled paper towel assays were used to measure seedling growth after germination, as well as

examine the seedlings for lesions. We hypothesized that fumonisin production aids the fungus in infecting the maize seedling by interfering with its metabolism, so we expected that there would be detectable differences in pathogenicity between fumonisin-producing and non-producing strains.

Materials and Methods

Fungal Strains

Twenty-six strains of black *Aspergillus* were obtained from maize kernel samples from Iowa, Illinois, and Italy, and were selected to provide a broad representation of black *Aspergillus* species occurring on maize, including strains with and without fumonisin B_2 production, as determined in a previous study (21) (Table 1). The strains had initially been placed in *Aspergillus* section *Nigri* based on morphological characteristics, and were further identified to species based on β-tubulin and calmodulin gene sequences (21).

Preparation of spore suspensions for laboratory experiments

Each *Aspergillus* strain was grown on potato dextrose agar under fluorescent light at 20-25°C for 7 days. The spores were rinsed off the plates, gently dislodging them from the colonies, and suspended in sterile distilled water. The spore concentrations of the suspensions were determined using a hemaocytometer, and they were diluted to a concentration of 10^6 spores/ml. For all spore suspensions, Tween 80 was added as a surfactant at a rate of approximately 0.125ml per liter of water for the first run of the warm germination and cold tests, and 0.5ml per liter for the second run and rolled paper towel assay. In the second run of the laboratory experiments, one strain (ITEM 15167) produced insufficient spores to reach the necessary volume of 10^6 spores/ml, so 5×10^5 was used instead.

Warm germination and cold tests

Two maize hybrids were used in the two repetitions of the warm germination, cold, and rolled paper towel tests. Hybrid A was an experimental hybrid obtained from Syngenta, and hybrid B was Syngenta hybrid 85v88-300GT (Syngenta Seeds, Northfield, MN). The maize kernels were first surface sterilized by submerging for 5 minutes in 0.6% sodium hypochlorite, followed by 3 minutes in 75% ethanol, and finally 2 minutes in sterile water. The kernels were then added to flasks containing the spore suspensions (or the control of Tween water), and placed on a shaker for 12 hours at 130 rpm for the first run, and 80rpm for the second, both at room temperature (20-25°C). For the first run of the warm germination and cold tests, 820 seeds were inoculated for each treatment, divided between two 250ml flasks with 100ml of suspension in each. For the second run, 850 seeds were placed in a single 500ml flask, with 300ml suspension. After the 12 hours, the suspension was drained off, and the kernels spread on paper towels to dry in a biosafety hood.

After the kernels had dried, they were submitted to the Iowa State University Seed Testing Laboratory for a standard warm germination test and a cold test (1). In the warm germination test, the seeds were grown on moist blotter paper at 25°C, and evaluated after one week for the percentage germination. The cold test approximates stresses of early spring planting by covering seeds with a layer of moist sand-soil mixture, chilling for one week at 10°C, and then warming up to 25°C for an additional week before evaluation of the percentage germination.

For both tests, four replicates of 100 seeds each were used, and each test was run twice, first with hybrid A, and then with hybrid B.

Rolled paper towel assay

Maize seeds from the same two hybrids were used for the rolled paper towel assay. Each replicate consisted of 15 seeds, placed two thirds of the way up on two layers of damp paper towel, and covered with a third towel after inoculation. There were 3 replicates for each treatment. In the first run (hybrid A) the U. S. strains (Iowa and Illinois) were run as one experiment, and the Italian strains were run as a second, due to logistical considerations. In the second run (hybrid B), all strains were run in the same experiment.

To inoculate, 0.1mL of spore suspension (or the control of Tween water) was pipetted over the top of each seed. They were covered with a third damp towel, loosely rolled vertically, and placed in individual unsealed plastic bags. These bags were stood upright in 5-gallon buckets and covered by placing a clear plastic bag over the top of each bucket to prevent contamination or drying. After 7 days of growth in ambient conditions (florescent lighting, 20-25°C), the seedlings were evaluated for growth.

Data collection and analysis

In the warm germination test, seedlings are scored as normal, abnormal, or dead, and the official germination percentage is equal to the percentage of normal seedlings. In the cold test, germination is evaluated on the basis of percentage emergence.

In the rolled paper towel assay, the length of the longest root and shoot was measured on each seedling, at the point of the furthest distance from the seed. The roots and shoots were separated and weighed immediately after unwrapping each replicate, to avoid drying. The seed itself was not weighed, so non-germinated seeds were recorded as 0 for both weights. For all measurements, the values for the fifteen seedlings on each towel were averaged and analyzed as a single observation.

All data were analyzed using ANOVA (SAS version 6.1). Fisher's protected least significant difference (LSD) was used to compare treatments, with the level of significance $P < 0.05$.

Results

Warm germination and cold tests

Aspergillus strains varied substantially in their effects on warm germination and cold test results for both hybrids. Several strains differed significantly from the non-inoculated control in each test. Some strains ranked more consistently near the high or low end in all four tests, while the ranking of other strains varied widely among experiments.

For hybrid A, the warm germination percentage varied considerably among the treatments, from approximately 75% to nearly 100%, with the control near the highest end of the range at 96.5% (Fig 1). Twelve of the 24 strains reduced warm germination significantly compared to the control. Among the 13 fumonisin-producing strains, seven reduced warm germination significantly. In the cold test, the differences were even more pronounced, with cold test germination ranging from less than 40% to over 80% (Fig 2). In this case, the control was near the middle of the range at 62.5%, with some strains of *Aspergillus* resulting in significantly higher cold test germination than the control. Eleven of the 26 strains significantly reduced cold test germination, 11 strains did not differ from the control, and four strains had cold test germination values significantly higher than the control. Among the 14 fumonisin-producing strains, seven reduced cold test germination, five did not differ from the control, and two were significantly higher than the control.

In hybrid B, in the warm germination test results were much lower overall, but still showed a wide variation among treatments, from a minimum of approximately 45%, to the control at 71.0% (Fig. 3). Nineteen of the 25 strains significantly reduced warm germination compared to the control. Eleven of the 13 fumonisin-producing strains significantly warm reduced germination compared to the control. Hybrid B performed even worse than hybrid A in the cold test, with no treatment resulting in cold test germination greater than 60%, and some near 40% (Fig. 4). The control was at the lower end of the range at 49.8%. Among the 25 strains, only two strains (both fumonisin-producing) had cold test germination values significantly lower than the control, and four strains had results significantly higher. Among the 13 fumonisin-producing strains, two reduced cold test germination, 10 did not differ from the control, and one was significantly higher than the control.

For both maize hybrids, there were no significant differences in overall means for warm germination or cold test results between the fumonisin-producing *Aspergillus* strains and the non-producing strains (hybrid A: P = 0.3893 and P = 0.8225; hybrid B: P = 0.1723 and P = 0.2058).

There were significant differences among species for effects on warm germination, but not cold test germination, for hybrid A, (Table 2). Warm germination was significantly lower for *A. tubingensis* and *A. niger* compared to *A. phoenicis*, and *A. awamori*, with no significant differences within either of these two groups. For hybrid B, there were differences by species in both warm germination and cold test germination (Table 2). Inoculation with *A. awamori, A. niger, A. phoenicis*, or *A. tubingensis* reduced warm germination relative to *A. carbonarius*. In the cold test, inoculation with *A. niger* reduced germination relative to *A. tubingensis, A. phoenicis*, and *A. awamori*.

Rolled paper towel assays

With maize hybrid A, there were significant differences among the U.S. strains for effects on root weight, shoot weight, and shoot length, but not for root length (Table 3). Three of the 10 strains reduced root weight), including two of the six fumonisin-producing strains. Eight strains reduced shoot weight, including five fumonisin-producing strains. Nine of the strains reduced shoot length, including five fumonisin-producing strains.

In the trial with the Italian strains, there were significant differences among the strains in all four variables for maize hybrid A (Table 4). In root weight, all 11 of the strains differed significantly from the control, with one of the six fumonisin-producing strains increasing the root weight, and all other strains decreasing it. Ten strains reduced shoot weight, including five fumonisin-producing strains. Four strains increased root length, including two fumonisin-producing strains. Ten strains reduced shoot length, including five fumonisin-producing strains.

For hybrid B, there also were significant effects on all four variables (Table 5). Twenty-four of the 25 strains reduced root weight, including all 13 fumonisin-producing strains; 22 strains reduced shoot weight, including 12 fumonisin-producing strains; 13 strains reduced root length, including eight fumonisin-producing strains, and 21 strains reduced shoot length, including 11 fumonisin-producing strains.

Differences in overall means of the various measures of seedling growth between fumonisin-producing and non-producing strains were not consistent. For hybrid A inoculated with U.S. strains, there was no detectible difference between fumonisin-producing and non-producing strains with regard to seedling root weight, shoot weight, root length, or shoot length (Table 6). However, among the Italian strains, inoculation with fumonisin-producing strains

resulted in significantly higher seedling shoot weight and length compared to the non-producing strains, with no difference in root weight or length (Tables 4 and 6).

With maize hybrid B, seedlings inoculated with fumonisin-producing strains resulted in seedlings with decreased root and shoot weight compared to the strains without fumonisin production (Tables 5 and 6). The root and shoot lengths were not significantly different.

In the rolled paper towel assay for hybrid A inoculated with the U. S. strains of *Aspergillus*, inoculation with *A. phoenicis* resulted in significantly lower root weight, shoot weight, and shoot length relative to *A. awamori*, and root weight relative to *A. niger* (Table 7). Among the Italian strains, there were no significant differences among species (Table 8).

On hybrid B, inoculation with *A. awamori*, *A. niger*, *A. phoenicis*, or *A. tubingensis* reduced root weight, shoot weight, and shoot length relative to *A. carbonarius*. Inoculation with *A. niger* reduced root weight relative to *A. awamori*, and reduced shoot weight and length relative to both *A. tubingensis* and *A. awamori*. Inoculation with *A. phoenicis* reduced shoot weight relative to *A. tubingensis* as well (Table 9).

Discussion

The results of this study indicate that fungal strains in *Aspergillus* section Nigri can be pathogenic to germinating seeds and to seedlings. Although storage molds in general are associated with reduced emergence (18) and seedling disease(20), there are no previously published studies reporting the pathogenicity of *Aspergillus* section Nigri on maize seedlings. Windham and Williams (25) reported on kernel infection following the inoculation of maize ears with *A. niger*, but they did not report effects on germination or seedling disease.

In both the warm germination and cold tests, there was a wide range in pathogenicity among the *Aspergillus* strains compared. Results were variable between the warm germination and cold tests, and between the two hybrids, suggesting that the pathogenicity of these *Aspergillus* strains depends strongly on experimental conditions and host genotype. Hybrid B performed particularly poorly, even in the absence of added pathogens, suggesting poor quality seed. Results confirm that some *Aspergillus* section *Nigri* strains can reduce germination and emergence in maize, but other strains have little capacity to do so.

Among the species compared, *A. niger* inoculation often resulted in reduced germination, but results varied among experiments. The single strain of *A carbonarius* did not show a high level of pathogenicity, often not differing from the control. However, little can be concluded about this species based on only one strain.

In the rolled paper towel assays, nearly all the strains of *Aspergillus* reduced shoot length and weight. Among the species, strains of *A. phoenicis* tended to result in shoot and root lengths and weights lower than the other species, but these differences were not always significant. Root length was less strongly affected by *Aspergillus*, and in some cases, root length actually increased, even though root weight was reduced. It is possible that growth of feeder roots was reduced by these strains, causing more nutrients to be directed to lengthening the radicle and other main roots, but no data were collected that could indicate whether this was the case.

All strains of *Aspergillus* tested showed some level of capacity to act as seedling pathogens in maize, but some were only weakly pathogenic. In addition, there was no clear relationship between fumonisin production and aggressiveness as a seedling pathogen. One of the most aggressive strains overall was ITEM 15114, not a fumonisin-producer, while one of the fumonisin-producing strains, strain 7, produced consistently low levels of symptoms.

The lack of association between fumonisin B_2 production and effects on germination for either hybrid indicates that fumonisin production is not necessary for, and probably has little or no role in the aggressiveness of black *Aspergillus* strains as seedborne pathogens. The fact that inoculation with some strains resulted in higher emergence than the control in both cold tests may be due to competition with other pathogens in the media, as the sand used in the standard cold test is not sterile. It is also possible that other seedborne pathogens played a role, if there were some that needed the cold conditions to show strong effects on germination. It is possible that a weakly pathogenic strain of *Aspergillus* may outcompete another, more strongly pathogenic organism which is already present, resulting in a net increase in germination.

In the rolled-towel assays, there was some evidence for greater aggressiveness of fumonisin-producing strains toward hybrid B, but this was not consistent for hybrid A. For hybrid A, shoot length and weight were reduced to a greater extent by fumonisin non-producing strains from Italy compared to the fumonisin-producing strains. This suggests that the differences observed were due to characteristics of the strains themselves, rather than their ability to produce fumonisin. It is also possible that hybrid B was more susceptible to fumonisin than hybrid A, though neither hybrid showed an effect of fumonisin in the germination tests.

Taken together, the results of this study suggest that fumonisin-producing and non-producing strains of *Aspergillus* spp. have similar levels of aggressiveness as pathogens of maize seedlings. Pathogenicity tests comparing wild-type *Aspergillus* strains against strains mutated by disruption of the *fum8* gene would provide a more definitive test of the role of fumonisin B_2 production in the pathogenicity of fungi in *Aspergillus* section Nigri. Most studies with fumonisin-producing *Fusarium* species have concluded that there is little or no role of fumonisins in disease development (3); however, fumonisins are phytotoxic (2) and some

researchers have concluded that fumonisins play a role in pathogenicity or virulence (7, 17). Fumonisin production by *Aspergillus* spp. does not include fumonisin B_1, and overall levels of production are much lower than in *Fusarium* spp. (21). If fumonisins can influence seedling disease, the effects may not be evident with the low levels of fumonisin B_2 production that occur with *Aspergillus* spp.

Differences in aggressiveness among *Aspergillus* species were not consistently evident; a larger number of strains of each species may be required in order to detect significant species differences, if they exist.

Table 1. Fumonisin production of *Aspergillus* strains used. Fumonisin production as determined by R. Proctor (pers. comm.) (Illinois strains only) or Susca et al. (2014) (all other strains).

Origin	**Strain name**	**Species**	**FB_2**
Iowa	Strain 2	*A. tubingensis*	-
Iowa	Strain 7	*A. awamori*	+
Iowa	Strain 16	*A. tubingensis*	-
Iowa	Strain 28	*A. tubingensis*	-
Iowa	Strain 33	*A. awamori*	+
Iowa	Strain 35	*A. niger*	+
Iowa	Strain 47	*A. tubingensis*	-
Iowa	Strain 51	*A. niger*	+
Iowa	Strain 74	*A. niger*	-
Illinois	NRRL 62518	*A. niger*	+
Illinois	NRRL 62522	*A. phoenicis*	+
Illinois	NRRL 62526	*A. phoenicis*	+
Illinois	ENDO 3233	*A. niger*	+
Italy	ITEM 15065	*A. carbonarius*	-
Italy	ITEM 15078	*A. niger*	+
Italy	ITEM 15096	*A. niger*	-
Italy	ITEM 15099	*A. niger*	+
Italy	ITEM 15114	*A. niger*	-
Italy	ITEM 15129	*A. awamori*	-
Italy	ITEM 15132	*A. awamori*	+
Italy	ITEM 15165	*A. niger*	+
Italy	ITEM 15167	*A. niger*	+
Italy	ITEM 15178	*A. niger*	-
Italy	ITEM 15187	*A. awamori*	-
Italy	ITEM 15206	*A. niger*	+
Italy	ITEM 15225	*A. niger*	-

Table 2. Warm germination and cold test percentages, by *Aspergillus* species.

Species	Warm Germ %, hybrid A	Cold Germ %, hybrid A	Warm Germ %, hybrid B	Cold Germ %, hybrid B
A. awamori	94.6 a	50.9 a	57.7 b	52.7 a
A. carbonarius	--	56.3 a	69.8 a	52.0 ab
A. niger	86.5 b	57.9 a	55.1 b	49.5 b
A. phoenicis	95.3 a	54.6 a	55.6 b	53.6 a
A. tubingensis	87.3 b	61.9 a	53.7 b	55.6 a

Table 3. Effect of *Aspergillus* strain inoculation on seedling growth in rolled paper towel assay, U. S. strains, hybrid A. Strain names in italics indicate fumonisin production. Asterisks indicate treatments significantly different from the non-inoculated control ($\alpha = 0.05$).

Strain	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
Control	1.85	5.24	13.22	9.69
ENDO 3233	1.33	3.06*	16.42	5.80*
NRRL 62522	1.12*	2.52*	13.33	5.00*
NRRL 62526	1.00*	2.28*	12.20	4.44*
Strain 2	0.87*	1.89*	9.95	3.38*
Strain 7	2.05	5.00	14.47	9.26
Strain 16	1.43	3.93	15.73	6.85*
Strain 33	1.36	2.99*	15.91	5.21*
Strain 35	1.48	2.92*	13.21	5.43*
Strain 47	1.42	3.56*	15.76	5.87*
Strain 74	1.72	3.51*	15.47	6.40*

Table 4. Effect of *Aspergillus* strain inoculation on seedling growth in rolled paper towel assay, Italian strains, hybrid A. Strain names in italics indicate fumonisin production. Asterisks indicate treatments significantly different from the non-inoculated control ($\alpha = 0.05$).

Strain	Root weight (g) LSD = 0.40	Shoot weight (g) LSD = 0.87	Root length (cm) LSD = 2.39	Shoot length (cm) LSD = 1.55
Control	2.61	6.33	15.88	10.94
ITEM 15065	1.75*	3.54*	16.84	5.65*
ITEM 15078	2.15*	3.79*	15.92	6.50*
ITEM 15096	1.73*	3.38*	18.05	6.06*
ITEM 15099	1.64*	3.60*	17.55	6.12*
ITEM 15114	1.64*	2.72*	18.27*	4.83*
ITEM 15129	1.79*	4.25*	19.38*	6.48*
ITEM 15132	1.77*	3.91*	18.74*	6.53*
ITEM 15165	3.24*	6.29	20.28*	10.70
ITEM 15167	1.66*	4.08*	18.24	7.11*
ITEM 15206	1.43*	3.28*	15.98	5.21*
ITEM 15225	2.16*	3.46*	17.87	5.82*

Table 5. Effect of *Aspergillus* strain inoculation on seedling growth in rolled paper towel assay, U. S. and Italian strains, hybrid B. Strain names in italics indicate fumonisin production. Asterisks indicate treatments significantly different from the non-inoculated control ($\alpha = 0.05$).

Strain	**Root weight (g)** LSD = 0.66	**Shoot weight (g)** LSD = 1.08	**Root length (cm)** LSD = 3.48	**Shoot length (cm)** LSD = 1.68
Control	4.45	4.89	18.45	8.26
ENDO 3233	1.81*	2.10*	12.33*	3.92*
NRRL 62518	1.55*	1.98*	12.13*	4.09*
NRRL 62522	2.10*	2.48*	15.32	5.00*
NRRL 62526	2.04*	2.02*	15.12	4.21*
Strain 2	2.81*	3.13*	16.29	5.46*
Strain 7	3.46*	3.80*	16.43	7.44
Strain 16	2.78*	3.54*	18.02	6.39*
Strain 28	2.04*	2.50*	12.54*	4.61*
Strain 33	2.35*	2.51*	16.00	4.86*
Strain 35	1.79*	1.99*	13.88*	4.16*
Strain 47	2.51*	3.36*	15.61	5.81*
Strain 51	2.36*	2.22*	14.88*	4.87*
Strain 74	2.51*	2.27*	14.26*	4.54*
ITEM 15065	4.06	4.25	18.77	8.04
ITEM 15078	2.22*	1.69*	14.18*	3.26*
ITEM 15096	1.91*	1.82*	13.37*	3.86*
ITEM 15114	1.89*	1.78*	13.29*	3.73*
ITEM 15129	1.93*	1.96*	11.11*	3.87*
ITEM 15132	2.13*	2.49*	14.34*	5.04*
ITEM 15165	2.08*	2.41*	13.67*	4.68*
ITEM 15167	2.82*	3.85	18.01	7.30
ITEM 15178	2.18*	2.39*	15.58	4.80*
ITEM 15187	3.16*	3.62*	19.34	6.50*
ITEM 15206	2.21*	2.32*	14.65*	4.37*
ITEM 15225	3.53*	3.87	19.26	6.90

Table 6. P-values from ANOVA tables for effects of fumonisin production, by run.

	Root weight	Shoot weight	Root length	Shoot length
Hybrid A, U. S. strains	0.7303	0.4497	0.7212	0.8359
Hybrid A, Italian strains	0.3665	0.0436	0.6318	0.0313
Hybrid B, all strains	0.0313	0.0363	0.1565	0.1498

Table 7. Rolled paper towel assay results by *Aspergillus* species for hybrid A, U. S. strains. Means with the same letter within each column are not significantly different ($\alpha = 0.05$)

Species	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
A. awamori	1.70 a	3.99 a	15.19 a	7.24 a
A. niger	1.51 a	3.16 ab	15.03 a	5.88 ab
A. phoenicis	1.06 b	2.40 b	12.77 a	4.72 b
A. tubingensis	1.28 ab	3.28 ab	14.30 a	5.61 ab

Table 8. Rolled paper towel assay results by *Aspergillus* species for hybrid A, Italian strains. Means with the same letter within each column are not significantly different ($\alpha = 0.05$)

Species	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
A. awamori	1.78 a	4.08 a	19.06 a	6.50 a
A. carbonarius	1.75 a	3.54 a	16.84 a	5.65 a
A. niger	1.96 a	3.83 a	17.77 a	6.54 a

Table 9. Rolled paper towel assay results by *Aspergillus* species for hybrid B, all strains. Means with the same letter within each column are not significantly different ($\alpha = 0.05$)

Species	Root weight (g)	Shoot weight (g)	Root length (cm)	Shoot length (cm)
A. awamori	2.60 b	2.88 bc	15.44 a	5.54 b
A. carbonarius	4.06 a	4.25 a	18.77 a	8.04 a
A. niger	2.22 c	2.36 d	14.58 a	4.65 c
A. phoenicis	2.07 bc	2.25 cd	15.22 a	4.60 bc
A. tubingensis	2.53 bc	3.13 b	15.62 a	5.57 b

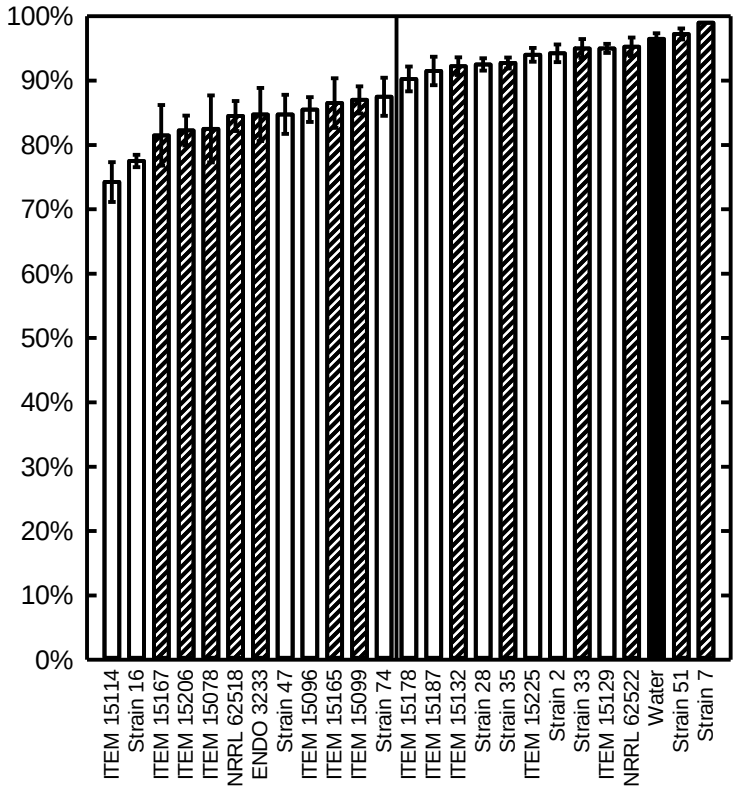

Fig. 1. Germination percentages in warm germination test for maize kernels (hybrid A) inoculated with strains of *Aspergillus* with and without production of fumonisin B2. Hatch marks indicate strains with fumonisin production. Strains to the right of the vertical line were not significantly different from the control (LSD = 6.9%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

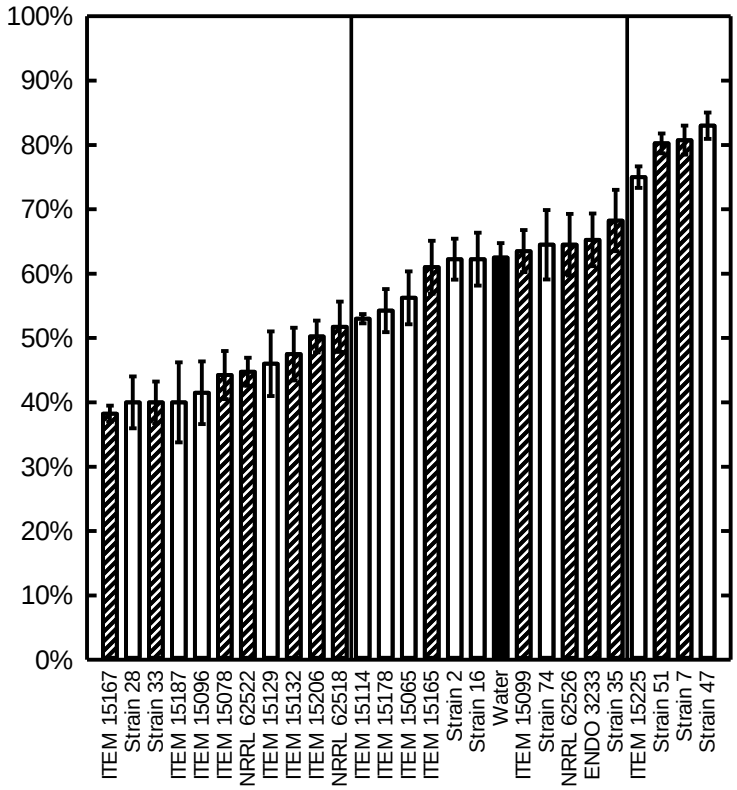

Fig. 2. Germination percentages in cold test for maize kernels (hybrid A) inoculated with strains of *Aspergillus* with and without production of fumonisin B2. Hatch marks indicate strains with fumonisin production. Strains between the two vertical lines were not significantly different from the control (LSD = 10.4%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

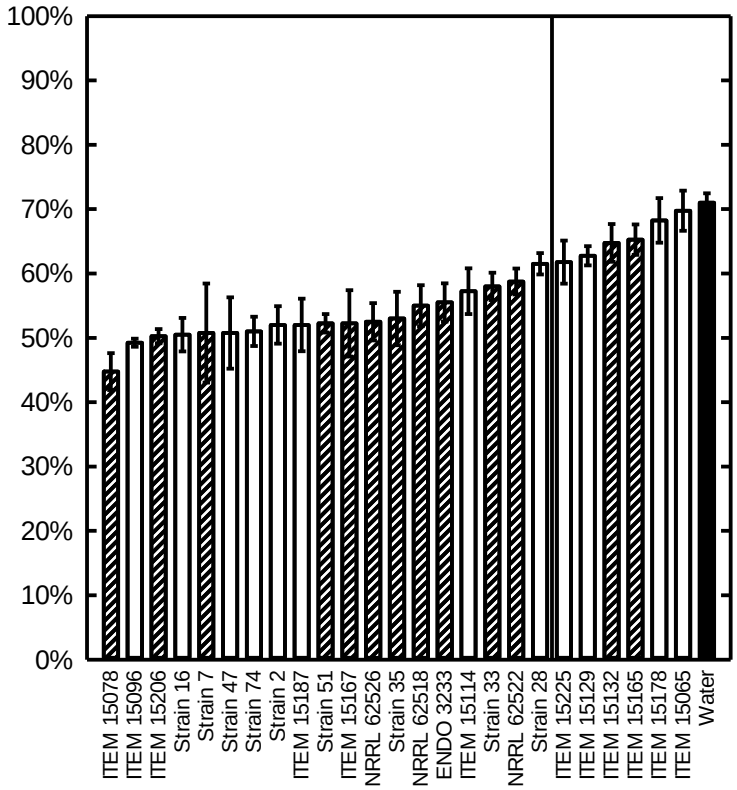

Fig. 3. Germination percentages in warm germination test for maize kernels (hybrid B) inoculated with strains of *Aspergillus* with and without production of fumonisin B2. Hatch marks indicate strains with fumonisin production. Strains to the right of the vertical line were not significantly different from the control (LSD = 9.3%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

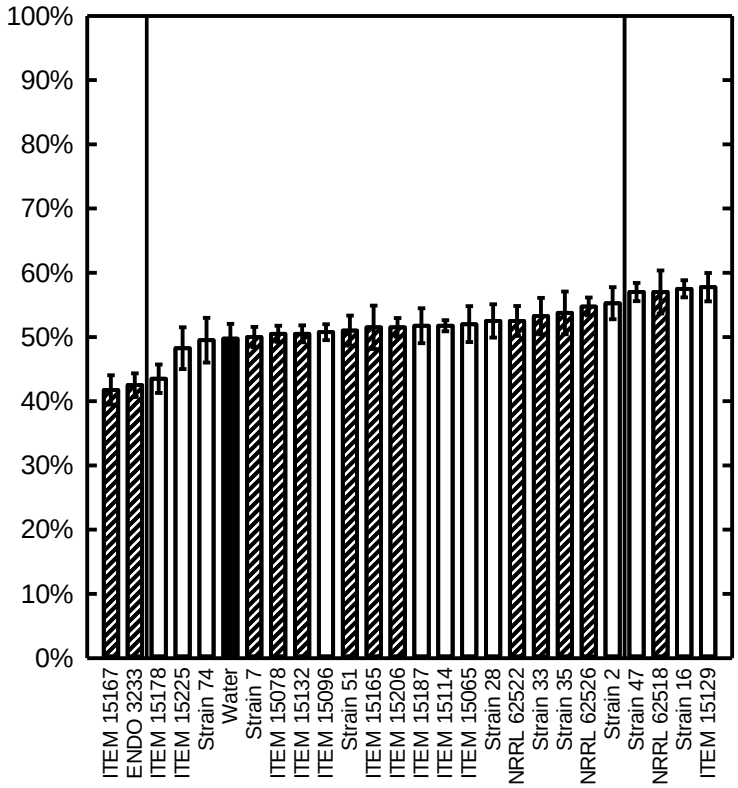

Fig. 4. Germination percentages in cold test for maize kernels (hybrid B) inoculated with strains of *Aspergillus* with and without production of fumonisin B2. Hatch marks indicate strains with fumonisin production. Strains between the two vertical lines were not significantly different from the control (LSD = 6.6%, $\alpha = 0.05$). Error bars represent the standard error of the mean.

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CHAPTER 4

INTERACTION BETWEEN SEEDBORNE ASPERGILLUS SPECIES AND SOILBORNE FUSARIUM SPECIES

Abstract

While many pathogens of maize seedlings have been studied extensively in isolation, little is known about their interactions with each other. In the second portion of this study we investigated the relationship between *Aspergillus* section Nigri, seedborne fungi that cause ear rot and seedling disease in maize, and *Pythium* spp., which are soilborne and cause seedling disease. Maize seeds inoculated with one of four strains of *Aspergillus* or not inoculated were planted in cups filled with non-infested sterile field soil, or soil infested with *P. sylvaticum* or *P. torulosum*. The cups were placed in a growth chamber at 25°C in a randomized complete block design (RCBD) and assessments done at 7 and 14 days after planting (DAP). An interaction was detected between *Pythium* and *Aspergillus* on seedling height at 7 DAP and percent emergence. Percentage healthy mesocotyl, height at 14 DAP, and shoot weight were reduced by *Pythium* only. Root weight was affected by both *Pythium* and *Aspergillus*, but with no interaction. For the variables with an interaction, *P. torulosum* caused more severe symptoms when associated with seed-borne *Aspergillus*, while *P. sylvaticum* caused severe symptoms regardless of the presence of *Aspergillus* spp. The results suggest that seedborne *Aspergillus* can exacerbate seedling disease caused by *Pythium* spp. under some conditions. This highlights the need for further study of seedling pathogens with reference to the entire soil ecosystem, rather than simply in isolation.

Introduction

Collectively, seedling diseases have a major impact on maize yield. In 2013, maize seedling diseases in the United States and Ontario collectively caused more yield loss in maize than any single pathogen (11). In Illinois, Iowa, Minnesota, and Nebraska, corn seedling diseases caused an estimated loss of 126.5 million bushels in 2013, nearly double the losses from Goss's wilt, the next most damaging disease (11).

Seedling disease pathogens may be either seedborne or soilborne, with *Pythium* species among the most common (13). This genus primarily infects maize seeds and seedlings in cool wet soils, causing poor root development and dark water-soaked lesions on the mesocotyl and root (13, 14). Infected seedlings often wilt and die, or are stunted. If disease incidence is high enough, a farmer may have to replant the diseased areas, or in some cases the entire field.

Many species of *Pythium* can infect maize, with the most common species varying by region. In Ohio, *P. dissotocum* and *P. sylvaticum* were the most common species recovered from diseased maize seedlings, with 7 other species identified (1), while a recent survey of diseased corn seedlings in Iowa found predominantly *P. torulosum*, in addition to 8 other *Pythium* species (15). Moreover, large differences in virulence on maize may exist between species of *Pythium* and even between strains within a species (1, 19).

Seedling diseases in nature do not occur in a vacuum. In fact, it is often difficult to determine which pathogen is actually responsible for seed or seedling death, and which are merely acting as opportunistic saprophytes (1, 12). Since there are so many other pathogens present in the soil, as well as on the seed, it is likely that some of them interact with *Pythium* species. Some may compete for resources as saprophytes, or as seedling pathogens. Others may facilitate infection by *Pythium*, or may themselves cause increased disease in the presence of

Pythium. Understanding these interactions could allow for more effective and comprehensive management strategies, but little work has been done on this topic to date.

Aspergillus section Nigri is a common seedborne pathogen (10, 16), that may interact with soilborne *Pythium*. *Aspergillus* alone does not often cause major seedling disease (10), but if it exacerbates disease caused by *Pythium* and other organisms, control of *Aspergillus* contamination of maize seed could be a viable strategy for reducing overall seedling disease. In addition, it is possible that fumonisin produced by *Aspergillus* (6) could either weaken the *Pythium* or facilitate its entry into the seedling roots, adding another facet to the interaction.

Little work has been done on the interaction of *Pythium* with other pathogens. Ho (8) inoculated maize seedlings with several pairs of seedling pathogens, including *Pythium* and *A. niger*, and observed no consistent pattern of interaction between either species of *Pythium* and *Aspergillus*. In this study however, disease severity was not critically assessed and no statistical analysis was done, thus is was difficult to draw conclusions regarding the various interactions between pathogens in the assessment. In another study, germination of seed infected with *Diplodia zeae*, *Gibberella zeae*, or *Nigrospora oryzae* was considerably reduced when it was placed onto cultures of *P. debaryannum*, as compared to non-infected seed germinating on *P. debaryannum* alone (5). Evidence for an interaction between *Pythium* and *Fusarium* species in peanuts and peas has also been reported (7). However, to our knowledge, no study has rigorously evaluated the interaction of *Pythium* with any species of *Aspergillus*. There appears to have been little work of any kind on interactions between maize seedling diseases in recent decades.

In this study, we compared two of the most common *Pythium* strains in Iowa maize, *P. sylvaticum* and *P. torulosum*, and evaluated them for evidence of an interaction with *Aspergillus* in a cup assay. Since it is possible that fumonisin production could play a role in the interaction,

either by weakening the plant or by making the *Aspergillus* strains more competitive against *Pythium*, we selected two fumonisin-producing *Aspergillus* strains and two non-producing strains for inclusion in the study. Thus, the objectives of this study were to determine whether such an interaction existed, and if so, to determine whether it was competitive or synergistic interaction.

Materials and Methods

Strains used in this study

Four strains of black *Aspergillus* were obtained from maize kernel samples from Iowa: two strains with fumonisin production, 7 and 35, and two without, 28 and 47, as determined in a previous study (18). The two strains in each category were chosen to include one each near the high and low end of virulence on maize seedlings, as determined by our comparison of strains discussed in the previous chapter (Table 1). The strains were identified as belonging to *Aspergillus* section *Nigri* based on morphological characteristics, and were identified to species based on β-tubulin and calmodulin gene sequences (18).

Aspergillus inoculation

Each *Aspergillus* strain was grown on potato dextrose agar under fluorescent light at 20-25°C for 7 days. The spores were rinsed off the plates, gently dislodging them from the colonies, and suspended in sterile distilled water. The spore concentrations of the suspensions were determined using a hemaocytometer, and they were diluted to a concentration of 10^6 spores/ml. Tween 80 was added as a surfactant at a rate of approximately 0.5ml per liter of water.

The prepared suspensions were used to inoculate maize kernels of hybrid B from the first part of this study, Syngenta hybrid 85v88-300GT (Syngenta Seeds, Northfield, MN). Prior to inoculation, maize kernels were surface sterilized by soaking for 5 minutes in 0.6% sodium hypochlorite, followed by 3 minutes in 75% ethanol, and finally 2 minutes in sterile water. After sterilization, 800 seeds per treatment were placed in 500mL-flasks, each containing 300mL of an *Aspergillus* spore suspension (or the control of Tween water), and placed on a shaker for 12 hours at room temperature. The suspension was drained off, and the kernels spread on paper towels to dry at room temperature. After the kernels were thoroughly air-dried (4 days), they were stored in sealed plastic bags at approximately 2°C until use.

***Pythium* inoculum**

Two species of *Pythium* were used in this experiment: *P. sylvaticum*, which grows well and is pathogenic on maize near room temperature, and *P. torulosum*, which is pathogenic on maize at 13°C, and is not as pathogenic at 18°C or 23°C (9). The *P. sylvaticum* strain used, Gr8, was isolated from symptomatic soybean seedlings in 2011 Poweshiek county, Iowa. The *P. torulosum* strain, I-8, was isolated from symptomatic maize seedlings in 2012 in Mahaska county, Iowa.

Pythium inoculum to infest the soil was prepared using the method from Stewart et al. (17) with some modifications. Each species of Pythium was grown on half-strength V8 juice agar (17) until the plates were well colonized with mycelia. Parboiled rice (440 g) and 323 mL of distilled water were placed in clear autoclave bags (12x24 – VWR international, West Chester, PA, USA) that were sealed with tape and autoclaved for 60 minutes at 121°C. The following day, one plate of *Pythium*-colonized agar per bag was cut up into approximately 0.5cm cubes and added to a bag of autoclaved rice under sterile conditions. The bags were resealed, the rice and

agar thoroughly mixed by carefully rotating the bags by hand, and placed in the dark at room temperature. The rice in the bags was mixed every 1-3 days, until mycelial growth was visible on most of the rice (5-6 days). Bags with visible contamination (dark colored mycelium or clumping from bacteria) were discarded. The *Pythium*-infested rice was spread out to dry on newspaper-lined trays, and when thoroughly dry, the inoculum was stored in sealed plastic bags at room temperature and used within 60 days.

Planting and setup

Field soil was sieved through a 60 mm mesh, double-bagged, and autoclaved twice on two consecutive days at 121 °C for 90 minutes each. Infested soil was prepared for each species of *Pythium* by mixing 15 mL of inoculum per 237 mL of soil. Three drainage holes were punched in the base of 237 mL Styrofoam cups that were then filled to approximately 2.5 cm below the rim with *Pythium*-infested soil or the non-infested control soil. Three maize seeds were placed in each cup, spaced evenly such that they did not touch the sides of the cup or each other. In the first run, the remainder of the cup was filled with infested soil, but this resulted in a substantial amount of mycelial growth on the surface of the cups, so in the second and third runs, the top 1.25 cm of the cups was filled with non-infested soil, regardless of the treatment.

The cups were set in trays and placed in growth chambers at 25°C under constant light for 2 weeks after planting, and were watered as needed, which was whenever the surface of the soil began to dry out. After watering, trays were allowed to drip for a minimum of five minutes before they were placed back over any other tray, to minimize cross-contamination.

Data collection and analysis

Seedling height was measured at 7 and 14 days after planting (DAP) as the height from the soil surface to the tip of the highest leaf, and the heights of the three seedlings in each cup

were averaged, including zeros. The percentage of healthy mesocotyl was estimated visually as the percent of mesocotyl tissue that was not discolored nor water soaked. Seeds with no mesocotyl were rated as 0% healthy. Emergence was recorded daily, until no more seedlings emerged and percent emergence calculated. Average wet shoot weight and wet root weight per seedling were recorded at 14 DAP, immediately after the seedlings were removed from the soil and rinsed in running water to remove the soil. The shoot and root were separated at the point where they attached to the seed and weighed separately. The seed itself was not weighed, so non-germinated seeds were recorded as 0 for both weights. For all measurements, the values for the three seedlings in each cup were averaged and analyzed as a single observation.

Each run of the experiment was laid out in randomized complete block design in a single growth chamber. All cups in each block were placed in random order in a single tray, with additional non-inoculated cups placed to fill in the empty spaces. The first run of the experiment had two blocks, and the second and third runs had four blocks each. For the second and third runs, two trays (blocks) were placed on each shelf of the growth chamber, and the trays rotated between the two shelves at each watering.

All data were analyzed using ANOVA (SAS version 6.1). Fisher's protected least significant difference (LSD) was used to compare treatments, with the level of significance $P < 0.10$.

Results

No significant treatment by run interaction was detected for height at 7 DAP ($P = 0.7346$) or 14 DAP ($P = 0.6246$), percentage of healthy mesocotyl ($P = 0.7910$), emergence ($P = 0.9751$), or shoot weight ($P = 0.4836$), so the three runs were combined for analysis. Root weight data for

the first run were not recorded quickly enough to avoid drying, so they were not included in the analysis. There was no significant interaction between run and treatment for the remaining two experimental runs ($P = 0.2081$), so root weight data was combined as well.

Percentage healthy mesocotyl was significantly affected by *Pythium*, but not by *Aspergillus* or the interaction (Table 2). Both species of *Pythium* reduced healthy mesocotyl compared to the control (soil with no *Pythium*) ($P < 0.1$; Fig 1) but were not significantly different from each other.

An interaction effect at was detected between *Pythium* and *Aspergillus* treatments on seedling height at 7 DAP (Table 2). When analyzed by *Pythium* treatment, no treatment effect on seedling height was detected for seedlings grown from *Aspergillus*-inoculated seed in non-inoculated soil ($P = 0.2527$) or in *P. sylvaticum*-infested soil ($P = 0.8498$). There was, however, an effect of *Aspergillus* with *P. torulosum* ($P = 0.0280$). Seedlings inoculated with *Aspergillus* strains 28, 35, and 47 were shorter than the control seedlings with no *Aspergillus*, and Strain 35 was shorter than Strain 7 as well when grown in *P. torulosum*-infested soil ($P < 0.1$; Fig 2).

At 14 DAP, a significant effect of *Pythium* ($P < 0.0001$), but not of *Aspergillus* was detected on seedling height (Table 2). Seedlings grown in *Pythium*-infested soil were shorter than those grown in non-infested soil for both species evaluated ($P < 0.1$; Fig 3).

Percent emergence showed a significant interaction effect between *Pythium* and *Aspergillus* (Table 2). When analyzed by *Pythium* treatment, in the non-infested soil, a greater number of seedlings emerged from seeds inoculated with *Aspergillus* strain 35 than the non-inoculated seed or seed treated with *Aspergillus* strains 28 or 47 ($P < 0.1$). None of the *Aspergillus* treatments differed from each other in the soil infested with *P. sylvaticum*, while in

the soil infested with *P. torulosum*, all four strains of *Aspergillus* resulted in reduced emergence compared to the non-inoculated seed ($P < 0.1$; Fig 4).

Both *Pythium* species reduced the average seedling shoot weight at 14 DAP ($P < 0.1$), but *Aspergillus* had no effect on shoot weight, and the interaction was not significant (Table 2; Fig. 5).

At 14 DAP, an effect of both *Aspergillus* and *Pythium* on average seedling root weight was detected (Table 2). Treatments where seedlings were grown in soil infested with either species of *Pythium* had lower root weights than any treatment with seedlings grown in non-infested soil. Additionally, among the treatments with non-infested soil, seeds inoculated with *Aspergillus* strains 28, 35, and 47 had significantly lower root weights compared to the non-inoculated control and *Aspergillus* strain 7 ($P < 0.1$; Fig. 6).

Discussion

In this study, we examined the interaction of seedborne *Aspergillus* with soilborne *Pythium* species on maize seedling development. Under the conditions used in this study, there was clearly a larger effect of the *Pythium* species than *Aspergillus* section Nigri on overall seedling health. Presence in the soil of either *P. sylvaticum* or *P. torulosum* dramatically reduced the percentage of healthy mesocotyl, height at 14 DAP, shoot weight, and root weight. The presence of various black *Aspergillus* species had comparatively little effect on any of these. In order to have a favorable temperature for *Aspergillus* growth and pathogenicity, the temperature used in our study, 25°C, was warmer than normal planting conditions in Iowa, but even with a favorable temperature, *Aspergillus* did not have nearly as large an effect as the *Pythium* species. It is possible that this might be due to the relative proportions of inoculum of each pathogen

present, however it is more likely that *Aspergillus* section Nigri was a weaker pathogen under these conditions.

There were some differences observed between *Aspergillus* strains, but these differences were not consistent between assays, nor did they correspond to previously identified virulence. This seems to indicate that virulence may vary depending on the assessment conditions, so different strains could be more or less virulent depending on environmental conditions.

In addition, there was no apparent relationship between virulence and fumonisin production, although it is impossible to draw any firm conclusions with only two strains from each category. In *Fusarium*, the role of fumonisins in pathogenicity is controversial. Though it is not necessary for pathogenicity (2), a study by Desjardins et al. (3) found that nearly all highly virulent strains also produced high levels of fumonisin B_1, while many less virulent strains did not. In *Aspergillus* section Nigri, the amounts of fumonisin produced are less than in *Fusarium* species (18), so the quantities produced may be too small to produce any measurable effects.

In contrast to previous studies (1, 4), which reported *P. sylvaticum* to be only moderately virulent on maize, and *P. torulosum* weakly virulent, our isolates of both species caused severe symptoms. Both dramatically affected root and shoot weight, plant height, and mesocotyl rot, regardless of whether *Aspergillus* was present or not. This may be due to a difference in the strains themselves, or it be related to the experimental conditions, since the other studies used a petri dish assay. The temperatures used for evaluation were similar in their and our studies, so this was likely not a factor.

In this study, the two *Pythium* species responded differently to the presence of seedborne *Aspergillus*. Unlike *P. sylvaticum* or the non-inoculated control, infection with *P. torulosum* showed more severe symptoms (reduction of height at 7 DAP and reduced emergence) when any

of the 4 species of *Aspergillus* section Nigri was present (with the exception of strain 7, which did not show a measurable effect on height at 7 DAP). This suggests a synergistic interaction between *P. torulosum* and *Aspergillus* section Nigri, though the mechanism of this interaction is not known. This may be because *P. torulosum* is less virulent than *P. sylvaticum* near 25°C, as reported by Broders et al. and Matthiesen and Robertson (1, 9), and benefited from the already weakened plants due to *Aspergillus* infection early in germination and growth. Foley (5) reported an interaction between *P. debaryanum* and other minor seedling pathogens, even though under the conditions he used, *P. debaryanum* alone was highly virulent (5). This suggests that the interactions may be specific to the individual pathogen species, rather than being directly related to the virulence of the pathogens involved.

To our knowledge, this is the first study to demonstrate an interaction between seedling pathogens in soil conditions. Although the growth chamber is not completely analogous to field conditions, use of field soil allowed for a closer approximation of the soil environment in the field than can be achieved by plate assays. However, we acknowledge that it is likely that there are far more interactions at play under field conditions, given the sheer number of pathogens and other microorganisms present in the soil and on the seeds themselves. Further study of these interactions could be very important in understanding the dynamics involved in maize seedling disease.

Since this study showed a synergistic effect between a weak pathogen, namely *Aspergillus* section Nigri, and *P. torulosum*, the most prevalent Pythium species associated with diseased maize seedlings in Iowa, this suggests that it could be important to control seedborne *Aspergillus* if it enables other pathogens to be more virulent. Further research is needed to

explore interactions between seedling disease pathogens of maize to improve our understanding of seedling diseases and maize and thereby develop effective management strategies.

Table 1. Black *Aspergillus* strains isolated from Iowa seedlings, with and without fumonisin production. Pathogenicity was assessed in an earlier study comparing *Aspergillus* strains on maize seedlings.

Strain Name	Fumonisin	Pathogenicity	Species	Strain Name
Strain 7	FB+	Low	*A. awamori*	Strain 7
Strain 28	FB-	High	*A. tubingensis*	Strain 28
Strain 35	FB+	High	*A. niger*	Strain 35
Strain 47	FB-	Low	*A. tubingensis*	Strain 47

Table 2. P-values from ANOVA tables for effects of two *Pythium* species, four strains of *Aspergillus* and their interaction on maize germination and seedling growth.

Source	% Healthy Mesocotyl	Height, 7 DAP	Height, 14 DAP	Percent Emergence	Shoot Weight	Root Weight
Pythium	0.0000	0.0000	0.0000	0.0016	0.0000	0.0000
Aspergillus	0.6653	0.0078	0.3962	0.4586	0.5866	0.0835
Interaction	0.2602	0.0676	0.3913	0.0614	0.9130	0.1392

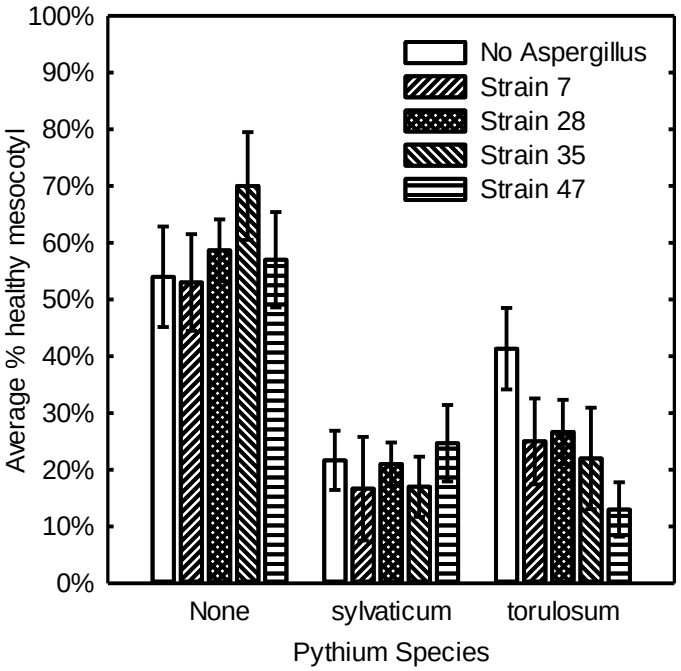

Fig 1. Average percentage healthy mesocotyl tissue of seedlings at 14 days after planting for maize seed inoculated with *Aspergillus* species and grown in soil infested with *Pythium sylvaticum* or *P. torulosum*. Error bars represent the standard error of the mean.

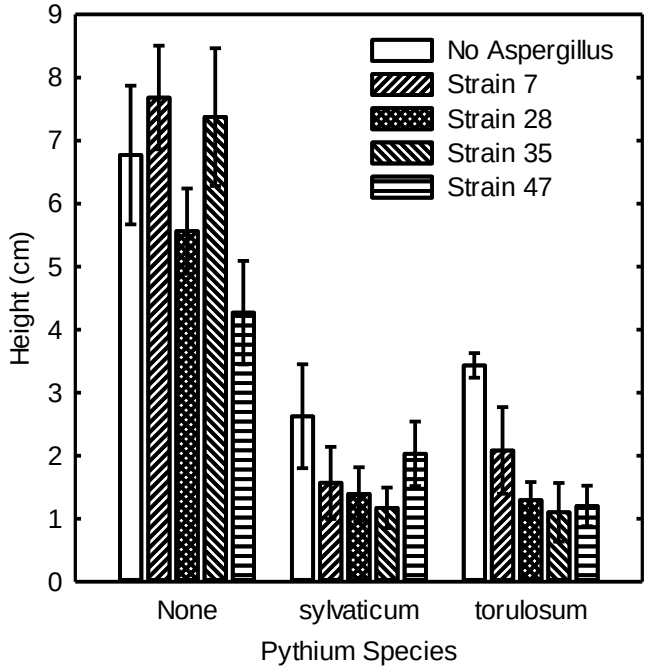

Fig 2. Average height at 7 days after planting of maize seedlings grown from seed inoculated with *Aspergillus* species in soil infested with *Pythium sylvaticum* or *P. torulosum*. Height was measured from the soil surface to tip of highest extended leaf. Error bars represent the standard error of the mean.

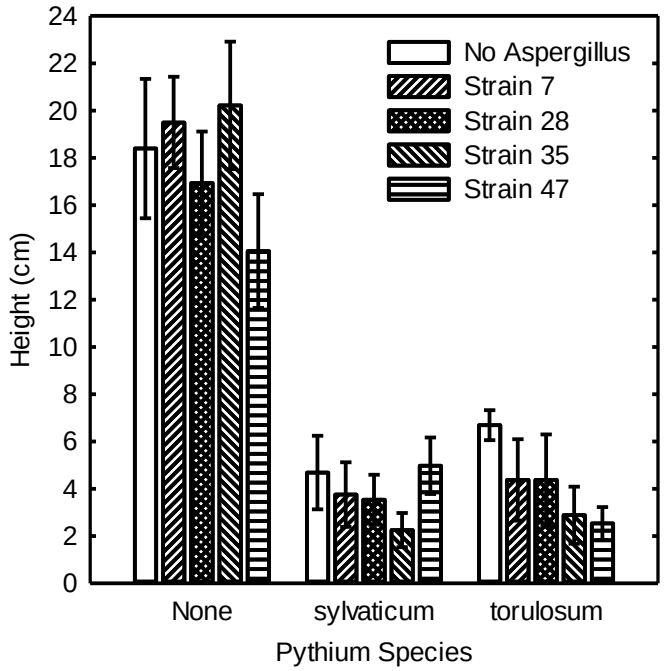

Fig 3. Average height at 14 days after planting of maize seedlings grown from seed inoculated with *Aspergillus* species in soil infested with *Pythium sylvaticum* or *P. torulosum*. Height was measured from the soil surface to tip of highest extended leaf. Error bars represent the standard error of the mean.

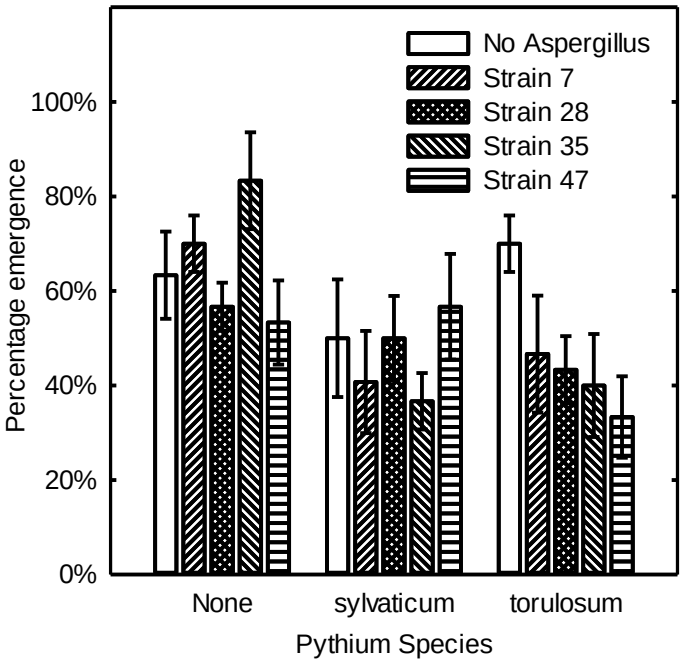

Fig 4. Percentage emergence for maize seed inoculated with *Aspergillus* species and grown in soil infested with *Pythium sylvaticum* or *P. torulosum*. Error bars represent the standard error of the mean.

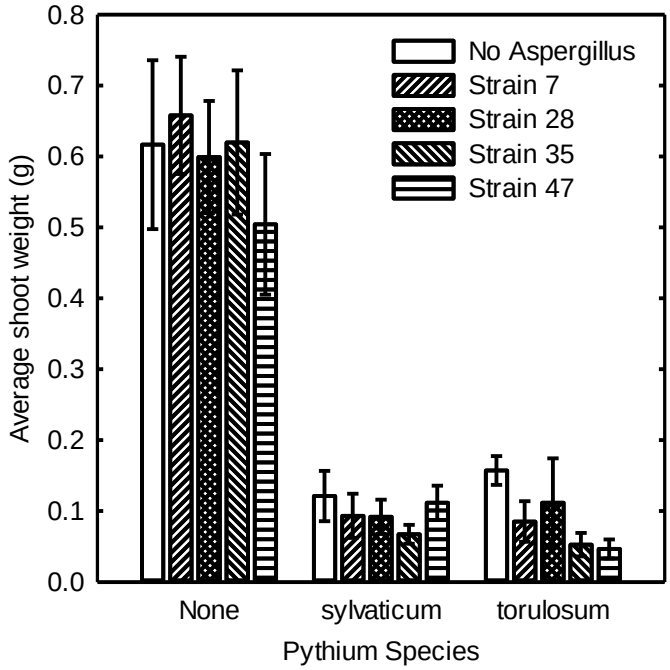

Fig 5. Average seedling shoot weight at 14 days after planting for maize seed inoculated with *Aspergillus* species and grown in soil infested with *Pythium sylvaticum* or *P. torulosum*. Error bars represent the standard error of the mean.

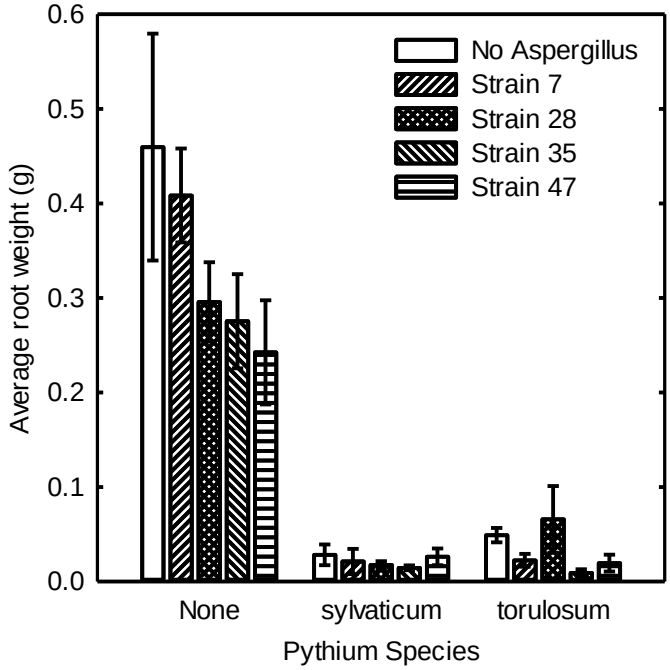

Fig 6. Average seedling root weight at 14 days after planting for maize seed inoculated with *Aspergillus* species and grown in soil infested with *Pythium sylvaticum* or *P. torulosum* (runs 2 and 3). Error bars represent the standard error of the mean.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Summary of Results

In the comparison of *Aspergillus* section Nigri strains, there was a wide range of virulence observed between the strains in both germination tests and the rolled paper towel assay. Results were variable among the three tests, and between the two maize hybrids, suggesting that the pathogenicity of these *Aspergillus* strains depends strongly on experimental conditions and host genotype. There were some differences observed among species, but these were not consistent, and there appears to be more variation within species than among them. In the rolled paper towel assay, nearly all strains reduced shoot length and weight, though roots were less strongly affected.

All strains of *Aspergillus* tested showed some level of capacity to act as seedling pathogens in maize, but some were only weakly pathogenic. In addition, there was no clear relationship between fumonisin production and aggressiveness as a seedling pathogen. Though there were some differences between the overall means for the fumonisin-producing and non-producing strains in the rolled paper towel assay, these were not all in the same direction, suggesting that the differences observed were due to the characteristics of the strains themselves, rather than their ability to produce fumonisin.

In the interaction study with *Pythium*, there was clearly a larger effect of either *Pythium* species than of *Aspergillus* section Nigri on overall seedling health under the conditions used. This is despite the fact that the temperature used in the experiments was closer to the optimum

for *Aspergillus* than for *P. torulosum*. Presence in the soil of either *P. sylvaticum* or *P. torulosum* dramatically reduced the percentage of healthy mesocotyl, height at 14 DAP, shoot weight, and root weight. The presence of various black *Aspergillus* species had comparatively little effect on any of these. It is possible that this is due to the relative proportions of inoculum of each pathogen present, however it is more likely that *Aspergillus* section Nigri was a weaker pathogen under these conditions.

There were some differences observed between *Aspergillus* strains in their interaction with *Pythium* and individually, but these differences were not consistent between assays, nor did they correspond to previously identified virulence or fumonisin production. This indicates that virulence may vary depending on the assessment conditions, so different strains could be more or less virulent depending on environmental conditions.

In contrast to previous studies (1, 2), which reported *P. sylvaticum* to be only moderately virulent on maize, and *P. torulosum* weakly virulent, our isolates of both species caused severe symptoms. Both dramatically affected root and shoot weight, plant height, and mesocotyl rot, regardless of whether *Aspergillus* was present or not. This may be due to a difference in the strains themselves, or it be related to the experimental conditions.

Though both caused strong disease symptoms, the two *Pythium* species responded differently to the presence of seedborne *Aspergillus*. Unlike *P. sylvaticum* or the non-inoculated control, infection with *P. torulosum* showed more severe symptoms when *Aspergillus* section Nigri was present. This suggests a synergistic interaction between *P. torulosum* and *Aspergillus* section Nigri, possibly because *P. torulosum* is a weaker pathogen than *P. sylvaticum* near 25°C, as reported by Broders et al. and Matthiesen and Robertson (1, 3), and benefited from the already weakened plants due to *Aspergillus* infection early in germination and growth.

Conclusions

The lack of a clear association between fumonisin B_2 production in *Aspergillus* section Nigri and maize seedling disease suggests that fumonisin-producing and non-producing strains of *Aspergillus* spp. have similar levels of aggressiveness as pathogens of maize seedlings. Pathogenicity tests comparing wild-type *Aspergillus* strains against strains mutated by disruption of the *fum8* gene would provide a more definitive test of the role of fumonisin B_2 production in the pathogenicity of fungi in *Aspergillus* section Nigri, since there would not be the confounding factors of differing genetic backgrounds in the various strains. Similarly, differences in aggressiveness among *Aspergillus* species were not consistently evident; a larger number of strains of each species may be required in order to detect significant species differences.

To our knowledge, this is the first study to demonstrate an interaction between seedling pathogens in soil conditions. Since this study showed a synergistic effect between a weak pathogen namely *Aspergillus* section Nigri, and *P. torulosum*, the most prevalent Pythium species associated with diseased maize seedlings in Iowa, this suggests that it could be important to control seedborne *Aspergillus* if it enables other pathogens be more virulent. However, the temperature of 25° was chosen to promote good growth of both *Aspergillus* and *Pythium* species, and is warmer than temperatures common when maize is planted in Iowa. Further study at cooler temperatures would indicate whether this interaction is likely to affect maize planted in Iowa fields. In addition, interactions with seed treatments should also be investigated, since maize seed planted in Iowa is usually coated with some form of fungicide, and this could completely alter the dynamics of the interaction.

Finally, it is likely that there are far more interactions at play under field conditions, given the sheer number of pathogens and other microorganisms present in the soil and on the

seeds themselves. Further research is needed to explore interactions between seedling disease pathogens of maize to improve our understanding of seedling diseases and maize and thereby develop effective management strategies.

References

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