ENDOPHYTIC ASSOCIATIONS OF SPECIES IN THE *ASPERGILLUS* SECTION *NIGRI* WITH MAIZE (*ZEA MAYS*) AND PEANUT (*ARACHIS HYPOGEA*) HOSTS, AND THEIR MYCOTOXINS

by

EDWIN RENE PALENCIA

(Under the Direction of Charles W. Bacon)

ABSTRACT

Filamentous fungi in the *Aspergillus* section *Nigri,* the black aspergilli, are associated with several crops including maize, peanut, and grape where their infections can cause maize kernel rot, peanut blight, and grape rot, respectively. New evidence suggests black aspergilli can colonize plant tissue as endophytes. We developed a system to identify black aspergilli from peanut and maize in the southeastern United States. A survey indicated that 86.7 % (*n*= 150) of the isolates characterized by a rep-PCR system were *A. niger,* suggesting that this species complex is predominant in maize and peanut fields. To gain a better understanding of the plant-fungi interactions, we developed a genetic transformation system to generate fluorescent black *Aspergillus* strains. Microscopy showed that both *A. niger* and *A. carbonarius* fluorescent transformants colonized root tissue as intercellular hyphae, which is characteristic of endophytes. Plant experiments were carried out to determine the potential benefits of crop plants harboring endophytic fungi. Eleven black *Aspergillus* species were used to inoculate maize seeds, but only *A. carbonarius* and *A. niger* were able to systemically colonize seedlings; none of the isolates significantly promoted plant growth on two maize cultivars. In greenhouse studies in peanut, *A.*

niger and its fluorescent transformants systemically colonized above and below-ground peanut tissue, but infections with both species had neutral effects on plant growth in the Florida 07 cultivar. We also tested the capacity of 150 isolates to produce fumonisins, carcinogenic secondary metabolites. We found that 27.8% (*n*=54) of the isolates produced FB_1, 59.2% FB_2, and 44.5% FB_3. In conclusion, we report here that *A. carbonarius* and *A. niger* strains form endophytic associations with maize and peanut hosts. We also documented that some black *Aspergillus* isolates are capable of producing fumonisins.

INDEX WORDS: *Aspergillus*, endophyte, mycotoxin, maize, corn, peanut

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2012

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DEDICATION

To my parents Eduarda del Rosario and Jose Victor Palencia for making me dream the fascinating game of life. To my beautiful wife, Kimberley, for filling my life with joy and happiness. The world is out there waiting for us.

ACKNOWLEDGEMENTS

First and foremost, I am grateful to my major advisor and mentor Dr. Charles Bacon for his patience, positive influence, guidance and continuous support through the years in the PhD program. He has shown me, by example, how to pursue excellence not only in science but in life in general. For the years to come, I will make sure to apply everything I have learned from him in any event of my life. Thanks Dr. Bacon, I deeply appreciate everything what you have done for me. I owe you much.

I would also like to thank my advisory committee: Drs. Scott Gold, Anthony Glenn, and Harald Scherm for their invaluable encouragement, support, and for providing scientific guidance during the years of graduate school. I am thankful to Dr. Ronald Riley, for his encouragement, science, and mainly for his kind support throughout the years.

My sincere thanks go to Dorothy M. Hinton for being instrumental in many aspects of this doctoral research, for her kindness, but especially for her friendship. Most of the experiments described in this doctoral dissertation would not have been possible without her assistance. Thanks Dot.

I would also like to thank Dr. Maurice Snook for his help in the chemical analysis. I am thankful to Trevor Mitchell for devoting part of his time to the chemical analysis and for his friendship. I want to thank Johnna Garrish for being a great help in several experiments of this research, but especially for always helping me out with a great smile. Thanks to Britton Ormiston for her support, especially in my early years of graduate school.

I also want thank my friend Ada Bacetty for the great moments we have spent throughout all these years. It has been great to see how far you have gone, and you keep it coming. I am proud to be considered your friend.

I thank my ARS-USDA-TMRU family who have helped me to discover this wonderful world of science. To all of you, past and present members of my USDA family, I will take you in my heart wherever I go. Last but not least, I want to thank Latoya Wiggins, Susan Brooks, Takiyah Ball, Rhonda Gantt, Calvin Williams, Nathaniel Upshaw (R.I.P), Susan Mize, and Joyce, for their invaluable friendship. You provided me with family love, especially in those days when a hug and hand shake were essential to pass through difficult times.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

BACKGROUND

Maize (corn, *Zea mays*) and peanut (*Arachis hypogaea*) are two major agronomic crops in the state of Georgia. In the last decade, the production of maize in Georgia has increased, with 720,000 tons produced in 2010 (20). In the same year, Georgia was the top peanut producer in the United States market, with almost 48% of the national production, contributing approximately $2 billion to the economy. Fungal pathogens pose a potential threat that can result in yield reduction and poor kernel quality for both crops annually.

Kernel and seedling rot in maize and Aspergillus crown rot in peanut are important plant diseases that, depending on environmental factors and the cultivar, can drastically reduce quality and yield. In both cases, members of the *Aspergillus* section *Nigri* (black aspergilli), especially *A. niger*, are consistently associated with such diseases. This fungal group consists of 18 cryptic species, which are characterized by dark-pigmented conidia with uniseriate and biseriate conidiophores (2).

Generally, black aspergilli are pre and post-harvest opportunistic plant pathogens which are able to infect different plant hosts, including onion, garlic, maize, peanut, coffee and grape. The evidence indicates that among black aspergilli, *A. carbonarius* is mainly responsible for producing rots of grapes; whereas *A. niger* causes kernel rot in maize and peanut. However, details of the host-parasite relationship and the role of toxins (pathotoxins and mycotoxins) in this interaction have not been elucidated for the black aspergilli. Furthermore, due to the cryptic

nature of species within this larger subspecies complex, knowledge of contributing species to specific disease and associated toxins is limited.

The basic biology of black aspergilli in maize and peanut are equally unknown. Past studies suggest that the black aspergilli are associated with plants as latent infections. Presently, information exists to suggest that most infections that are endophytic are in fact very active metabolically. Such endophytes are also interactive with their host, where some are able to produce a disease. The concept of endophytism has been known for decades, but it has acquired a greater significance since Bacon et al (7) showed in a classical paper that the endophytic fungus, *Neotyphodium coenophialum* in tall fescue grasses (*Festuca arundinaceae*) was involved in toxicity syndrome suffered by cattle fed with infected fescue. This clavicipitalean fungus, along with diverse fungi such as *Fusarium verticillioides*, is endophytic and capable of producing a diversity of mammalian toxins as well as plant diseases. The question posed in this dissertation is whether any of the black-spored aspergilli described earlier as latent are in fact endophytic, and as such, capable of producing mycotoxins that are characteristic of the species within the complex.

Fungal endophytes produce a wide range of mycotoxins. Mycotoxins have gained considerable world-wide attention due to their negative implications in both human health and agricultural production. In humans, mycotoxicoses, diseases produced by mycotoxins, are an important human health issue, especially in humid and hot climates, where conditions favor their biosynthesis (16). In agricultural fields, mycotoxin contamination reduces crop quality and yield, and according to the Food and Agriculture Organization of the United Nations, it is estimated that economic losses due to mycotoxin contamination accounts for $923 million per year in the United States (12). The consumption of mycotoxin-contaminated staple foods and feeds also

constitutes a human and animal health issue because their ingestion produces a myriad of deleterious effects ranging from chronic illness to death. Among the most important mycotoxins, based on their prevalence and toxic effects in humans, Ochratoxin A (OTA) is the third most studied mycotoxin, just after aflatoxins and Fumonisin B_1. Recent studies indicate that the biseriate black aspergilli *A. niger* and *A. carbonarius* are major producers of OTA, and it has been estimated that between 0.6-50% of the field isolates of *A. niger* are capable of producing OTA, and 25-100% of the *A. carbonarius* isolates produce this mycotoxin (1, 11, 18). OTA is nephrotoxic, immunosuppressive, teratogenic and classified as a potential carcinogenic mycotoxin in animals by the International Agency for Research on Cancer (14). In humans, OTA has been associated with Balkan Endemic Nephropathy (BEN), an irreversible chronic nephropathy that leads to renal failure that is geographically confined to rural areas in Eastern Europe (8). Patients suffering from BEN have high levels of OTA in urine and blood serum. Recently, OTA has been strongly associated with the Tunisian Chronic Interstitial Nephropathy (CIN) (23). High levels of OTA in cereals and cereal-based foods consumed by inhabitants of rural areas of Tunisia are strongly correlated with the incidence of CIN (13). OTA is produced by several species in the genera *Aspergillus* and *Penicillium*, which are responsible for contaminating different commodities. Originally, OTA was discovered as a mycotoxin produced by *A. ochraceus*; however, new studies have confirmed that species within the *Aspergillus* section *Nigri* are able to produce this secondary metabolite (reviewed in 15).

The fumonisins FB_1, FB_2, and FB_3 have been considered among the most potent carcinogenic mycotoxins produced exclusively by the genera *Fusarium*. However, new evidence has shown that strains of *A. niger* can produce fumonisins (22). From an agronomical perspective, these findings have severe implications due the number of food and feed

commodities that *A. niger* can contaminate, especially peanut and maize, where *A. niger* is among the most isolated fungal species. It also poses a serious problem to the biotechnological industry, where *A. niger* is extensively used for several industrial applications and has been granted the Generally Recognized As Safe (GRAS) status by the Food and Drug Administration (FDA).

Although fungal endophytes and their associations may become pathogenic and harmful to humans and domestic animals, it is the benefits of mutualistic interaction provided by fungal endophytes to the plant host that has attracted the attention of microbiologists and plant pathologists. Over the last two decades, an increased public concern about the non-target effects of chemical treatments, utilized to ensure high agronomic yield, has led to an explosion of a new and rapidly growing area of research to investigate ecologically compatible alternatives to agrichemicals. Fungal endophytes are postulated as such an alternative to chemical pesticides and fertilizers. Systemic colonization of plants by non-pathogenic fungi promotes plant growth, provides protection against plant pathogens and herbivores, and improves tolerance to salt, drought and hot weather (3, 4, 17, 21).

Field surveys showed that the black aspergillus species *A. niger* is the second most frequent isolated fungus, after *F. verticillioides*, and depending upon environmental factors such as water activity and temperature, *A. niger* can outcompete *F. verticillioides* for natural substrates in agronomical fields (5, 6, 9). Additional reports indicate that OTA and the fumonisins can co-occur in maize, perhaps indicating that both *A. niger* and *F. verticillioides* might interact as endophytes during the pre-harvest stage (10, 19).

PROJECT OBJECTIVES

The main focus of this research was to gain a better understanding of the fungal biology of the *A.* section *Nigri* species that are able to colonize peanut and maize hosts, their endophytic nature, and their capacity to produce mycotoxins. The basic hypothesis of this research project is that there are species of the *Aspergillus* section *Nigri* present as endophytes in kernels and plants of two major agronomic crops, maize and peanut. In order to test this hypothesis, the following three specific objectives were developed:

Objective 1: To determine the predominant species in the *A.* section *Nigri* that are able to infect maize and peanut in the southeastern United States.

a) To develop *in vitro* procedures to isolate these fungi from seeds and plant tissue.

b) To develop morphological and molecular protocols to characterize the black aspergilli isolated from plant environments.

Objective 2: To study the plant-endophyte interactions between maize/ peanut hosts and species of the uniseriate and biseriate clade of the *Aspergillus* section *Nigri* group.

a) To design fluorescent transformants of black aspergilli to assess the endophytic development and colonization patterns.

b) To assess the effects of endophytic colonizations by black aspergilli on plant growth and biomass in maize and peanut.

Objective 3: To study the production of ochratoxins and fumonisins by black aspergilli strains isolated from peanut and maize fields.

a) To determine the capacity of black aspergilli to produce ochratoxins and fumonisins in different crop seeds.

b) To develop chromatographic procedures to detect the production of mycotoxins by fungal species of the *Aspergillus* section *Nigri*.

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CHAPTER 2

THE BLACK *ASPERGILLUS* SPECIES OF MAIZE AND PEANUT AND THEIR POTENTIAL FOR MYCOTOXIN PRODUCTION[1]

[1]Palencia, E.R., Hinton, D. M., and Bacon, C. W. 2010. Toxins. 2:399-416.

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ABSTRACT

The black-spored fungi of the subgenera *Circumdata*, the section *Nigri (=Aspergillus niger* group), is reviewed relative to their production of mycotoxins and their effects on plants as pathogens. Molecular methods have revealed more than 18 cryptic species, of which several have been characterized as potential mycotoxin producers. Others are defined as benign relative to their ability to produce mycotoxins. However, these characterizations are based on *in vitro* culture and toxin production. Several can produce the ochratoxins that are toxic to livestock, poultry, and humans. The black aspergilli produce rots of grape, maize, and numerous other fruits and grain, and they are generally viewed as post-harvest pathogens. Data are reviewed to suggest that black aspergilli, as so many others, are symptomless endophytes. These fungi and their mycotoxins contaminate several major grains, foodstuffs, and products made from them such as wine and coffee. Evidence is presented that the black aspergilli are producers of other classes of mycotoxins such as the fumonisins, which are known carcinogens and known from prior investigations as being produced by *Fusarium* species. Three species are identified in U.S. maize and peanut as symptomless endophytes, which suggests the potential for concern as pathogens and as a food safety hazard.

1. Introduction

Fungi of the genus *Aspergillus* have a long history of associations with humankind. The genus was first described by Micheli in 1729 *[1]* to include those fungi with long stalks and spore heads that radiated in long chains from a central structure resembling an aspergillum, the brush-like structure used in religious ceremonies for sprinkling holy water. Species of this genus are common, originally applied to the first species of this genus, *A. glaucus* by Link *[2]*. They are extremely diverse in their habitats, and extraordinarily versatile in terms of their ability to

produce secondary metabolites. Several are important human and plant pathogens, while others are useful in the production of several fermented food products highly regarded by humankind. Due to their metabolic versatility, species of this genus have great biotechnological potential and several are used for the production of numerous food and nonfood by-products. The plant pathogens are of concern not only for their ability to destroy several agronomically important food crops, but also due to their ability to produce several mycotoxins. These mycotoxins are associated with specific species or subgenera of *Aspergillus* and are in general toxic to livestock, poultry, fish, and humans.

Early attention was devoted to species of *Aspergillus* as causes of animal toxicities, mainly poultry *[3]*. Immediately after this report, it was established that this toxicity, described as the Turkey-X disease of peanut, was caused by *A. flavus* and *A. parasiticus [4]*. Subsequent studies resulted in identifying the aflatoxins as the toxicological agent *[5]*, which initiated mycotoxicology as a serious and complex problem of food safety. Continued studies resulted in distinguishing another class of mycotoxins produced by species of aspergilli, the ochratoxins, which along with the aflatoxins, were established as important carcinogenic mycotoxins. Ochratoxin A was discovered in maize by Van der Merwe *et al. [6]* as the mycotoxin produced by strains of *Aspergillus ochraceus*, and *Penicillium verrucosum*. One of the first reports for the natural occurrences of ochratoxicosis was in poultry, which consisted of five independent episodes including about 970,000 turkeys, two episodes of about 70,000 laying hens, and two episodes in about 12,000,000 broilers *[7]*. These observations served to establish ochratoxins as important toxins in agriculture, and in food safety. Recently the ochratoxins have been reported from several other species of *Aspergillus* sections *Circumdati* (*A. ochraceus* group), and by *Eurotium herbariorum*, a member of the *Aspergillus* section (*A. glaucus* group). Recently, the

black species of *Aspergillus* have been shown to be able to produce ochratoxins *[8]*, which extends the geographic area of concern from the temperate zone to the tropical and sub-temperate zone due to the distribution of these black species.

The black aspergilli are commonly found as soil organisms on decomposing dead plant residues *[9]*, and they are pathogenic on several crops. As discussed below, the majority of the black *Aspergillus* species are associated with grape, onion, maize, and peanut, where they are cited as pathogens causing such diseases as peanut and maize seedling blight, and maize kernel rot. However, there are numerous examples of fungi associated with plants as symptomless endophytes, and there is evidence that this life habit may be practiced by the *Aspergillus* species as well. In association with several hosts, these symptomless endophytes have the capacity to either develop as pathogens or as saprophytes, and in either state become producers of mycotoxins. Symptomless expressions of several black aspergilli are indicated in the literature, but nothing is indicated about their ability to produce mycotoxins and any associated pathology. Further, the black species of *Aspergillus* associated with any plant pathological problems were indicated in early publications as *A. niger*, the black species. In this review, we refer to *A. niger sensu strictu*, i.e., *A. niger* var. *niger*, to designate or distinguish the present day description from the older published accounts, which in most instances were applied *sensu lato* and will be referred to here as simply *A. niger*. While several cryptic species within this subgenus have been delineated, this recent taxonomic revision creates a large gap of knowledge of black *Aspergillus* species that are presently defined, and which is important to food safety and plant pathology. This review focuses on identifying the species of the subgenera *Circumdati*, the section *Nigri* (= *A. niger* group) (Table 2.1) encountered in cereals and other plants, with an emphasis on maize

and peanut in particular. The nature of the association with their hosts and their potential to produce ochratoxins and other toxins relative to specific species are also reviewed.

2. The Black *Aspergillus* Species.

There are well over 190 *Aspergillus* species, and these can be conveniently separated into several distinct morphospecies, and several of these are based on colors according to the earlier classification of Raper and Fennell *[10]*. However, phylogenetic analyses of sequence data resulted in separating the *Aspergillus* genus into eight subgenera *[11]*. Following these analyses, the economically important species that produce ochratoxins were divided to include those species of the subgenera *Circumdati*, the sections *Circumdati* (= *Aspergillus ochraceus* group) and *Nigri* (*A. niger* group). There are no known teleomorphic species of group *Nigri*. In recent years, members of the *Aspergillus* section *Nigri* have undergone an extensive taxonomic revision resulting in several new taxa, such as *A. niger* var. *niger*, *A. melleus*, *A. sulphureus*, *A. brasilensis*, *A. ostianus*, *A. petrakii*, *A. scletotium*, *A. carbonarius*, *A. aculeatus*, *A. japonicus*, *A. tubingensis*, *A. ibericus* and *Eurotium herbariorum [12-14]* (Table 2.1).

However, these new taxa have not been identified as to the responsible species in diseases of food crops, such as maize seedling blight, maize ear rot and seedling blight of peanuts. Further, any role they may play in the pathogenic expression of maize kernels and plants prior infected with *Fusarium verticillioides*, a common maize pathogen usually co-associated with black aspergilli as a symptomless infection, is unknown.

Klich's *[9]* classic paper on the biography of the *Aspergillus* genus indicated that most of the species occurred in the tropical latitudes below 25 degree north and south, with greater than expected frequencies in the subtropical to warm temperate zones at latitudes between 26 and 35 degrees. This study suggested that species abundance peaked in the subtropics. This distribution

is attributed to several biotic and abiotic interacting factors with the major factor temperature *[9]*. In general, the black species of aspergilli were found to occur more frequently in forest and cultivated soils and less frequency in desert soils. However, *A. niger* var. *niger* was uniformly distributed throughout the entire sampling areas including forest, grassland, wetlands, deserts, and cultivated soils *[9]*. Thus, this documents this species as the most common species in both subgenera of *Circumdati.*

In a recent survey of maize and peanut using rep-PCR to distinguish morphotypic and molecularly derived species (Figure 2.1), several basic black *Aspergilli* were distinguished in peanut and maize *[15]*. The survey was designed to analyze for endophytic species of these two plants using surface disinfection of kernels and plant parts. This survey indicated that several species were present in these two plants as seedborne systemic and endophytic infections (Table 2.2). This survey indicates that the *A. niger* var. *niger* was the major species isolated from these two plants, with *A. foetidus* and *A. japonicus* occurring as minor species. Peanut accounted for three species, while only two were isolated from maize. The work is similar to that of Magnoli *et al. [16]* who found that of the black aspergilli, *A. niger* var. *niger* along with *A. japonicus* var. *japonicus* was isolated from surface-disinfested maize kernels from Argentina.

3. Mycotoxins Produced by Black *Aspergillus* sp.

The earliest report of toxicity from the black *Aspergillus* species was by Frischbier and Richtesteiger *[17]* who reported on the experimental poisoning of pigs fed bread that was inoculated with *A. niger* and that the toxic component was oxalic acid. Later, Wilson and Wilson *[18]* indirectly indicated *A. niger* as the toxic organisms since oxalic acid was isolated from moldy feedstuffs that was toxic to livestock. Presently, the type of the section, *A. niger,* has important industrial application and most strains of this specific species hold the Generally

Recognized as Safe status issued by FDA. What is not clear, however, is what species is intended when the term "A. niger" is used for all black-spored aspergilli. Thus, correct taxonomic descriptions of species within this group are extremely important as this can serve to distinguish those that are phytopathogens and mycotoxic from those that have technological applications.

3.1 Ochratoxins

Ochratoxin A is the more toxic of two dihydroisocoumarins initially isolated from broth cultures of *A. ochraceus*. The other is ochratoxin B, which is the dechlorinated analog of ochratoxin A. Both A and B occur in smaller amounts as the methyl and ethyl esters. Ochratoxin A is one of the world's most important mycotoxin, rated third of the top six, primarily due to the documented deaths of humans, primarily in Europe. The International Agency for Research on Cancer classifies this mycotoxin in the 2B group, possible carcinogen to humans *[19]*, but it is not regulated in the United States, although it is in Europe (FAO Food and Nutrition Paper No. 81, 2004). Ochratoxin A is nephrotoxic, teratogenic, carcinogenic, and immunosuppressive in animals, and it is cytotoxic in hepatic cell lines. In humans, ochratoxin A has been associated with Balkan Endemic Nephropathy, a tubule- interstitial nephropathy leading to a chronic renal failure, that is characterized by high concentration of ochratoxin A in blood serum and urine of patients suffering from this disease *[20]*.

In addition to the above *in vivo* description of toxicity from ochratoxin A, there is a recent finding of its mode of action at the cellular level. This toxin is reported to interact with tight junction pores, which regulate paracellular transport across cell and tissue membranes, by altering the four isoforms of cell-to-cell specific cell membrane adhesion proteins called claudins *[21]*. Thus, this toxin affects cell membrane integrity, producing non-regulated transports in and

out of cells. This can have a high economic impact at the agricultural level affecting food products ranging from eggshell damage to increased bacterial infections.

3.2 Ochratoxin contaminated products and producing species

Ochratoxin A was isolated as a natural contaminant from maize *[22]*, and since this report it has been isolated from many agronomically important crops, and processed food and feed. Commodities contaminated with this toxin predominantly include cereal and derived products including maize, wheat, rice, sorghum, mixed livestock, and poultry feed. Ochratoxin has also been found in green coffee beans, peanuts, olives, beer, grapes, raisins, peas, beans, barley, oats, rice, and wheat. It has been detected in processed food such as cheese, wine, grape juice, powdered milk, fruits, and black pepper *[8, 23-25]*. Ochratoxin A is isolated from meats of poultry and swine consuming contaminated feed *[26]*, and has been detected in human blood and milk *[27-29]*. This indicates the widespread occurrence, and the saprophytic and parasitic nature of the black *Aspergillus* species.

Aspergillus niger var. *niger* and *A. carbonarius* are two major producers of ochratoxin A. For example, 25 to 100% of the isolates of *A. carbonarius* are ochratoxigenic, and 0.6 to 50% of the isolates of *A. niger* var. *niger* are ochratoxigenic *[13]*. Recent surveys indicate that these two black-spored aspergilli are the main source of ochratoxin A in major food products, including maize and wheat, in both tropical and subtropical zones of the world *[30, 31]*. Curiously, the *A. niger* strains that do produce ochratoxin have been placed in the type N RFLP pattern, while ochratoxin production is not associated with strains from the type T RFLP stains *[32]*. However, this difference might reflect nutritional requirement in culture more than those required on natural substrates *[33]*. Both *A. niger,* var. *niger* and *A. carbonarius* are common in the United States *[9, 15]*, along with other ochratoxigenic species, *A. foetidus,* and *A. tubingensis,* but their

ability to produce the ochratoxins is unknown. However, the species as currently defined that occur on maize and peanut or grape have not been identified, nor has ochratoxin production been established on maize or peanut by these species.

In the past, the occurrence of ochratoxin A in maize has always indicated that the maize was contaminated during storage and that the maize was grown in a temperate climate because the fungi that produce it, mainly *A. ochraceus, Penicillium verrucosum,* and *P. nordicum, [34, 35],* grow well under cool to cold conditions. Currently, additional temperate species are indicated as being able to produce ochratoxin A and B. These include *A. alliaceus, A. sclerotiorum, A. sulphureus, A. albertensis, A. auricomus, A. melleus, A. glaucus, A. wentii, Neopetromyces muricatus,* and *A. westerdijkiae [34-36]*.

Members of a different *Aspergillus* section, the *Aspergillus* section *Nigri* (formerly known as *A. niger* aggregate) have now been isolated from maize and peanut (Table 2.2). The *Aspergillus* section *Nigri* group occurs in the warmer temperate and tropical zones *[9]*. The highest percentages of ochratoxigenic strains were found within the *Aspergillus* section *Nigri*, the taxa responsible for the main source of ochratoxin A in animal feeds, especially in locations with less than desirable storage and humidity-temperature conditions *[20, 37, 38]*. Surveys of poultry feed and the poultry environment indicated that the most frequently isolated fungus, second to *F. verticillioides*, was a black-spored species of the *A. niger* aggregate *[39, 40]*. Maize and mixed diets prepared with maize, especially for poultry, contain a high number of *A. niger* CFU *[39-41]*. In Georgia, a black spored species, i.e. *A. niger* var. *niger,* is a major pathogen of peanut, causing seedling blight of all the major cultivars. However, there are additional species that are found in lesser frequencies (Table 2.3) *[15]*. The problem is compounded because maize is a rotational crop for peanut in several areas of the state, which results in infected maize.

Neither the precise identify of the pathogen nor the biological nature of this association have been defined. Thus, ochratoxin A potentially can occur throughout the temperate, tropical, and subtropical climates of the world.

3.3 Fumonisins

Additional toxic substances are presented in Table 2.1. It was recently discovered that four strains of *A. niger* were able to produce another type of mycotoxins, the fumonisins, which are commonly associated with strains of the maize pathogen *Fusarium verticillioides (Gibberella moniliformis]* and other *Fusarium* species *[42]*. *Fusarium verticillioides*-contaminated maize is correlated with human esophageal cancer, and the fumonisins are highly toxic to horses, pigs, and poultry *[43]*. The fumonisin mycotoxins are carcinogenic, although B_2 is more cytotoxic than B_1. The most commonly isolated fumonisin is the B_1 homolog, while B_2 is isolated less frequently. Only strains of *A. niger* var. *niger* have currently been shown to produce the fumonisins [34, 44] and the B_4 homologues, the biosynthetic precursor that lacks the hydroxyl group. So far only the strains of *A. niger* var. *niger* from grape have been reported as producers of the fumonisins.

A survey of black aspergilli isolated from raisins indicated that 77% of *A. niger* from grapes produced fumonisin B_2 and B_4, and interestingly none of these strains produced the ochratoxins *[34, 44]*, suggesting that the ability to produce each mycotoxin depends on the genetics of a strain. The black aspergilli are co-isolated with *F. verticillioides* from maize and peanut suggesting that the source of fumonisin accumulation on these substrates might be derived from either fungus. However, the production by strains of *A. niger* var. *niger* from maize has not been demonstrated. Since the black aspergilli, especially *A. niger* var. *niger*, are

used in so many biotechnological processes for food use, the production of the fumonisins by the commonly occurring species increases the concern for food safety.

3.4 Other mycotoxins

The black aspergilli have produced a variety of biologically active compounds *[36]*, some of which are phytotoxic. One not indicated as being produced by the black aspergilli in the extensive review is penicillic acid, which we consider important not only for it biological activity as a mycotoxin but also due to its numerous other properties. These properties are reviewed with the hope that attention is drawn to it as a metabolite of the black aspergilli. Penicillic acid (γ - keto- β-methoxy- δ-methylene-A^{α} –henenoic acid) is particularly produced by most of the golden colored species of section *Circumdati* (= *A. ochraceus* group), and is oftentimes co-produced with ochratoxin A by strains of *A. ochraceus [40, 45]*. Besides the *A. ochraceus* group, penicillic acid is also produced by numerous other species of *Aspergillus* and *Penicillium [45-47]*, which suggest that penicillic acid is ubiquitous in strains and species of other subsections of these two genera. Penicillic acid is mycotoxic and synergistic with ochratoxin A in several animal studies *[48-50]*, and phytotoxic to seedlings *[51, 52]*. While the production of penicillic acid has not been reported in the most recent review of secondary metabolites reported in the black aspergilli *[36]*, its production might explain a role for this species in this subsection as pathogens in several seedling diseases.

Penicillic acid is also inhibitory to microorganisms, particularly Gram-negative bacteria *[53]*, and may be intricately involved with competition and ecological success of producing species. Recently, penicillic acid was established as an effective quorum sensing inhibitor *[54, 55]*, therefore interfering with cellular communication, and producing disruptive effects on virulence expression by pathogenic species, especially Gram-negative species. Its mode of

action with bacteria might reflect it activity as an inhibitor of quorum sensing. Quorum sensing affects cellular activities of bacteria by interfering with several aspects of bacterial metabolism ranging from those necessary for growth to those responsible for cellular motility *[54, 56]* to biofilm formation *[57]*. However, it is argued that quorum sensing is highly specific and it probably cannot be broadly applied to control all bacteria where quorum sensing is expressed *[58]*. This range of biological activity for penicillic acid indicates several desirable pharmacological benefits such as quorum sensing to undesirable one such as an inhibitor to bacteria used as biocontrol agents against penicillic acid positive pathogenic fungi. Penicillic acid therefore should be viewed also as an antibiotic where it mode of microbial antagonism is one of interfering with important signaling mechanisms used by colonizing bacteria, conceivably reducing their competition with the producing *Aspergillus* species. Since the biological activities described for penicillic acid above are important from several control measures, its production by the black aspergilli should be investigated.

4. Host Associations and Plant Pathology

Black aspergilli are reported as pre- and post-harvest pathogens in maize, other cereal grain, bunch grape, onion, garlic, soybean, apple, mango, and peanut *[16, 41, 59-61]*, although the extent of damage on each host depends on unknown predisposing environmental factors. The inoculum source of most species is the soil and litter, particular the vineyard soils *[9, 62-64]*. Other sources include the seed of most crops *[15, 62]* or fruit *[63]*. Most black aspergilli are indicated as opportunistic pathogens of fruits such as grapes and some spices *[62, 63]*. In a small survey *[15]*, several species of black aspergilli were isolated from surface disinfected maize and peanut, which indicated that these species were endophytes. Endophytic associations have also been reported in onion and garlic (*62]*, although these were characterized as latent

infections. The better term for these infections is symptomless, as endophytic infections have proved in all instances as not latent or dormant as implied, but metabolically active, colonizing the host, producing several classes of secondary metabolites, some of which are toxic. The major difference is the absence of disease symptoms produced during these infections. These endophytic infections can become, however, pathogenic under some biotic and abiotic conditions either pre- or post-harvest. Symptomless infections pose grave problems from a food safety concern, as commodities contaminated by such infections are not obvious, appear normal, but can contain toxic metabolites.

An investigation of the endophytic nature of maize seedling was conducted. *Aspergillus carbonarius SRRC 2131 and A. niger* var. *niger* SRRC 13 were transformed with a yellow fluorescent protein vector that was used to measure colonization of maize seedlings *[15, 65]*. The transformed black aspergilli did not affect the ability of these strains to colonize maize seedlings (Figure 2.3). These transformed species were isolated from surface disinfected seedling roots and leaves of plants grown under ideal growth room conditions, and there were no significant differences in seedling height and stem thickness *[15]*. The infection remained symptomless and attempts at inducing seedling blight by inducing drought did not produce the disease. The endophytic nature of the black aspergilli was indicated from the recovery of *A. niger* from surface-disinfested plant materials of onion by Hayden and Maude *[62]*. The infection in onion was also symptomless and infection was proved to develop from contaminated onion seed. Seedling onions were, as in the maize seedlings, similar to non-infected except in shoot length *[62]*.

In grape, another situation is described. *Aspergillus* rot in grape is caused by the black species, *A. niger*, var. *niger, A. carbonarius* and *A. aculeatus [63]*. These black aspergilli are

reported as opportunistic pathogens of damaged berries of grape, since in the absence of damage, spores remain on the surface of grapes without causing visible pathology. Further, *A. niger* is known to cause kernel rot of maize (Figure 2.4), which is similar to ear/kernel rot produced by *Fusarium verticillioides,* and *F. graminearum*. However, black aspergilli are isolated from surface-disinfested kernels of maize (Figure 2.2). Symptomless infections in onion by *A. niger* var. *niger* can develop into a postharvest disease, but perhaps due to injury to the bulbs or unsuitable storage conditions *[62, 66]*. In the case of maize, infection and the resulting kernel rot may occur from wounds produced by earworms and other damaging insects, similar to that which occurs from infections by *A. flavus.* Nevertheless, endophytic infections do occur as demonstrated from isolations from surface-disinfested kernels, and these endophytic infections can colonize plant tissue. Therefore, some black aspergilli are capable of a biotrophic endophytic existence with maize and onion. Since these two plants are widely separated taxonomically, it is possible that endophytic infections by the black aspergilli exist in numerous plant taxa.

Since ochratoxin A is present in maize, and other cereal grain under field conditions *[16]*, there is a suggestion that the black aspergilli species may form some relationship with maize during its growth under field conditions or that it is interactive with other field fungi. In a laboratory study, it was demonstrated that the fumonisin-producing fungus, *F. verticillioides,* can exclude *Aspergillus* species, such as *A. niger var. niger,* from colonizing maize, but this depended on optimum temperature and specific water activity levels *[67, 68]*. The interaction of antagonistic substances was not examined in that study, but conceivable competition may also relate to inhibitory substances as well as the abiotic factors measured.

SUMMARY

In the past most descriptions and concerns relative to the black aspergilli have been referred to as *A. niger*, resulting in a great deal of confusions concerning the actual species observed. Recent molecular analysis of the black aspergilli indicated several cryptic species, which now should be aligned with recent phytopathological and toxicological concerns. To prevent this confusion, the older *A. niger* has been replaced by *A. niger, var. niger* in order to exclude the prior confusion in discussions of both the earlier and recent descriptions. The subgenus *Circumdati* section *Nigri* consists of at least 19 species of black spore species of which *A. niger* var. *niger* is probably the most dominant species in the US where it is found in most soil types and on dozens of fruits and cereal grains. Of major concern is the relationship of the black aspergilli with maize and peanut, two plants of major economic concern in the US. Strains of *A. niger* var. *niger*, along with others, can produce the ochratoxins and the fumonisin B_1 mycotoxins. In addition to the effect of the mycotoxins on animals and humans, these toxins are interactive with other secondary metabolites to produce synergistic effects. Others are also phytotoxic *[69]*, suggesting a role for this mycotoxin in the pathology of seedling blight of peanut and maize. The production of both the ochratoxins and the fumonisins by the black aspergilli extends the concern, since food and feeds not inductive to one mycotoxin can be inductive to the other. Similarly, attempts at control of one might not control the other. Current disease descriptions are based on the casual identification of the black aspergilli as belonging to the older descriptions of *A. niger*. Certain species have been characterized as producing specific toxins and associated pathology. This indicates the need for reexamination of taxa associated with specific food crops. Additionally, detailed studies of their host relationship are also indicated, as some species are associated with crops as symptomless endophytes. There are also

reports on the co-occurrence of both ochratoxin A with the fumonisins in maize *[70, 71]*, which suggests interactions between producing endophytic fungi on maize, such as the maize endophyte *F. verticillioides*. Further, evidence of co-infection of *A. niger* with *A. ochraceus* in maize observed is also a concern as this presents difficulty in identifying the correct offending mycotoxic species. The black aspergilli are known pathogens in peanut culture where major problems are indicated from the seedling stage or during peanut set late in matured plants. The result is poor quality seeds that are rated too low for human consumption. Thus, pathogenic expressions in maize, cotton, grape, peanut and other plants, the production of the ochratoxins and fumonisins on these products, and the unknown species associated with specific pathological expressions on these important crop plants underlines the need for further studies of the black aspergilli. Finally, the prediction of rising global temperatures should influence the population patterns and shifts of species within the *A. niger* group to more northern latitudes that should increase the number of additional crops, adding to the mycotoxigenic potential globally for all species of the subgenera *Circumdata.*

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Table 2.1. Toxins isolated from black *Aspergillus* species.

Species[a]	Host[b]	Ochratoxin	Biologically active metabolites
A. niger var niger[c]	Maize, peanuts, grapes, and grape products, coffee, tea, beans, spices[d]	+[i]	Fumonisin B2 Fumonisin B4 Nigragillin[e], Malformin[f]
A. carbonarius	Grapes, java coffee bean	+	Carbonarones[g]
A. tubingensis	Arabica coffee bean	+	Malformin, nigranillin
A. brasiliensis	Grapes	-	Malformin
A. acidus	Raisins	-	uk
A. ibericus	Grapes	-	uk
A homomorphus:	Soil, nh	-	Secalonic acid[h]
A. ellipticus	Soil, nh	-	Terphenyllin[f]
A. aculeatinus	Arabica coffee bean	-	uk
A. aculeatus	Green coffee bean	-	Secalonic acid, Aspergillusol A[g]
A. japonicus	Grapes, maize, peanut	+	Cycloclavin
A. uvarum	Healthy grapes	-	Secalonic acid
A. piperis	Black pepper	-	Aflavinines[e]
A. sclerotiicarbonarius	Robusta coffee bean	-	uk
A. sclerotioniger	Coffee bean		Aflavinines
A. heteromorphus	Soil, nh	-	uk

[a] *A. niger* var. *niger* consists of several synonyms including *A. awamori, A. phoenicis, A. kawachii, A. saitoi, A. usamii, A. foetidus, A. citricus,* and *A. ficuum,* and oftentimes these synonyms are listed by several authors as varieties of *A. niger, i.e., A. niger* var. *awamori,* and as such they are ochratoxin *A* producers *[36]*. [b]Indicates the principal plant host associated with the species as a parasitic relationship if known, does not preclude nor exclude soil and other saprophytic habitats, which is indicated as nh, no host known. [c]Data indicated for this species may or may not reflect information for *A. niger sensu stricto*, rather than generalized descriptive placement of black aspergilli in this species complex. [d]

The species include isolations from various plant parts of black cumin, fennel, lime tree, absinthium, ginger, cinnamon, peppermint, carob tree, chamomile, saffron, curcuma, wormwood, rose, and lesser galangel [72]. [e]Insecticidal. [f] Phytotoxic. [g]Antibiotic. [h]Weak mycotoxic activity. [i]Symbols, +, -, present or absent; uk, unknown.

Table 2.2. Field collection of *Aspergillus* isolates with predicted species results based on rep-PCR [15].

Strain Number[a]	Location	Species
RRC 453	Peanut, South Georgia	*A. niger*
RRC 454	Peanut, South Georgia	*A. foetidus*
RRC 455	Peanut, South Georgia	*A. niger*
RRC 456	Peanut, South Georgia	*A. niger*
RRC 457	Peanut, South Georgia	*A. niger*
RRC 458	Peanut, South Georgia	*A. foetidus*
RRC 459	Peanut, South Georgia	*A. niger*
RRC 460	Peanut, South Georgia	*A. japonicus*
RRC 475	Peanut slurries, Dawson, Georgia	*A. niger*
RRC 476	Peanut slurries, Dawson, Georgia	*A. niger*
RRC 477	Peanut slurries, Dawson, Georgia	*A. foetidus*
RRC 478	Peanut slurries, Dawson, Georgia	*A. niger*
RRC 479	Peanut slurries, Dawson, Georgia	*A. japonicus*
RRC 480	Peanut slurries, Dawson, Georgia	*A. niger*
RRC 481	Peanut slurries, Dawson, Georgia	*A. niger*
RRC 482	Maize kernels, Midwestern USA	*A. niger*
RRC 483	Maize kernels, Midwestern USA	*A. niger*
RRC 484	Maize kernels, Midwestern USA	*A. niger*
RRC 485	Maize kernels, Midwestern USA	*A. niger*
RRC 486	Maize, kernels, Midwestern USA	*A. niger*
RRC 487	Maize kernels, Midwestern USA	*A. niger*
RRC 488	Maize kernels, Midwestern USA	*A. niger*
RRC 489	Maize kernels, Midwestern USA	*A. niger*
RRC 490	Maize kernels, Midwestern USA	*A. niger*
RRC 493	Maize kernels, Midwestern USA	*A. niger*

RRC 494	Maize kernels, Midwestern USA	*A. niger*
RRC 495	Maize kernels, Midwestern USA	*A. niger*
RRC 497	Maize kernels, Midwestern USA	*A. niger*
RRC 500	Maize kernels, Midwestern USA	*A. niger*
RRC 501	Maize kernels, Midwestern USA	*A. niger*
RRC 503	Maize kernels, Midwestern USA	*A. niger*
RRC 504	Maize kernels, Midwestern USA	*A. niger*
RRC 507	Maize kernels, Midwestern USA	*A. foetidus*
RRC 510	Maize kernels, Midwestern USA	*A. niger*

[a]RRC, Russell Research Center culture collection, Athen, GA.

Table 2.3. Species of black-spored aspergilli isolated from surface-disinfested maize kernels and peanut samples obtained from the Midwest and south Georgia, USA.

	% Isolation Frequency[a]	
Aspergillus Species	Maize	Peanuts
A. niger	95	67
A. foetidus	-	20
A. japonicus	5	13

[a]Data modified from Palencia *et al.* [15].

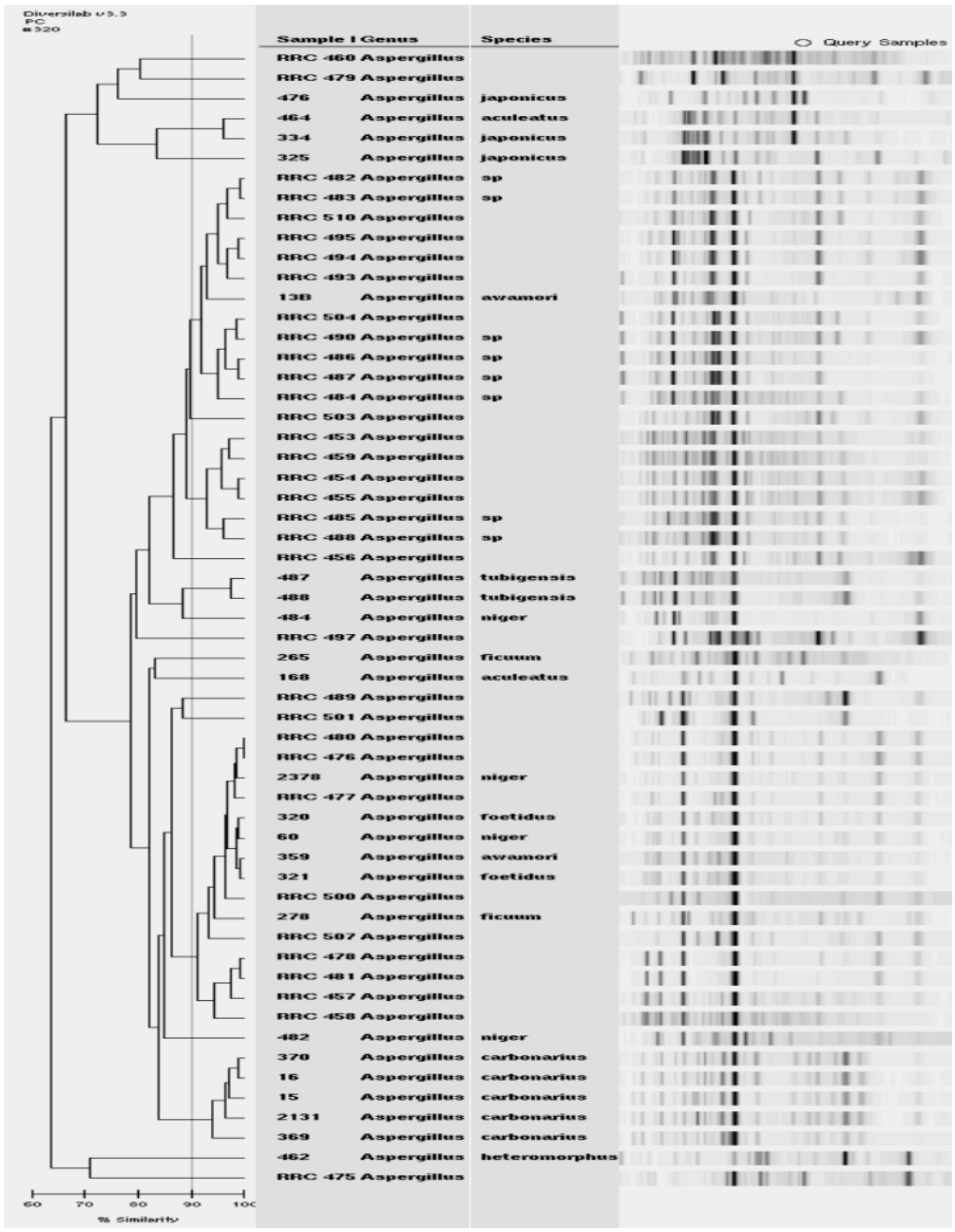

Figure 2.1. Identity of 54 *Aspergillus* section *Nigri* isolates, designated RRC from corn and peanut samples, analyzed along with reference species of black aspergilli by a rep-PCR barcoding procedure [15].

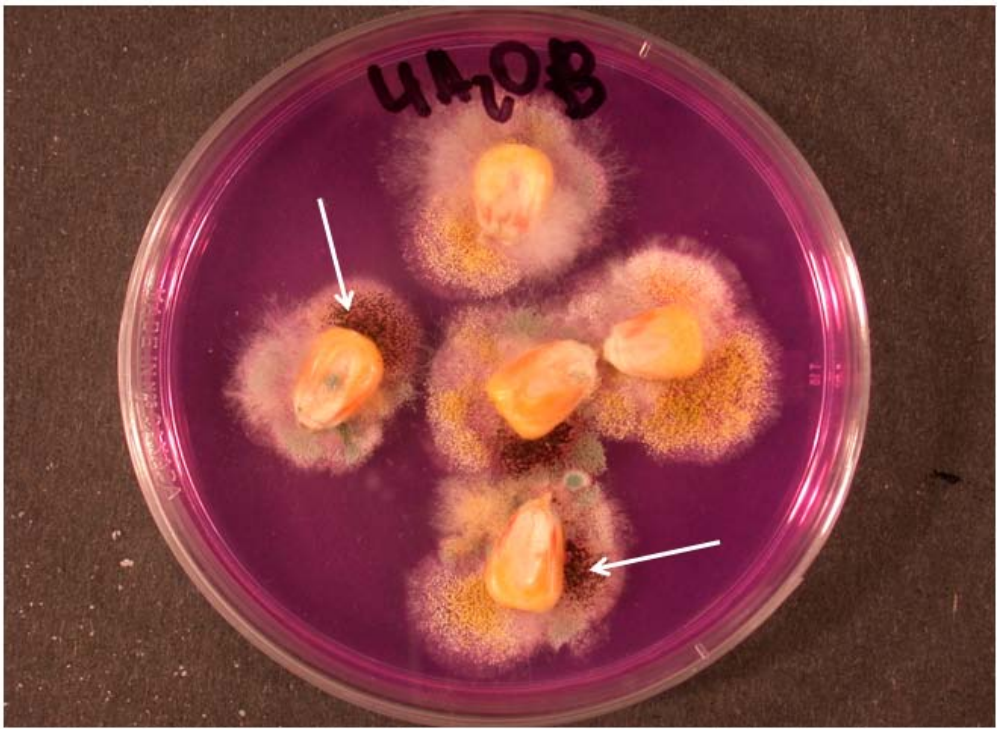

Figure 2.2. Isolation of black aspergilli from surface-disinfested maize kernels, showing a black *Aspergillus* species (arrows) along with *A. ochraceus* on an isolation medium.

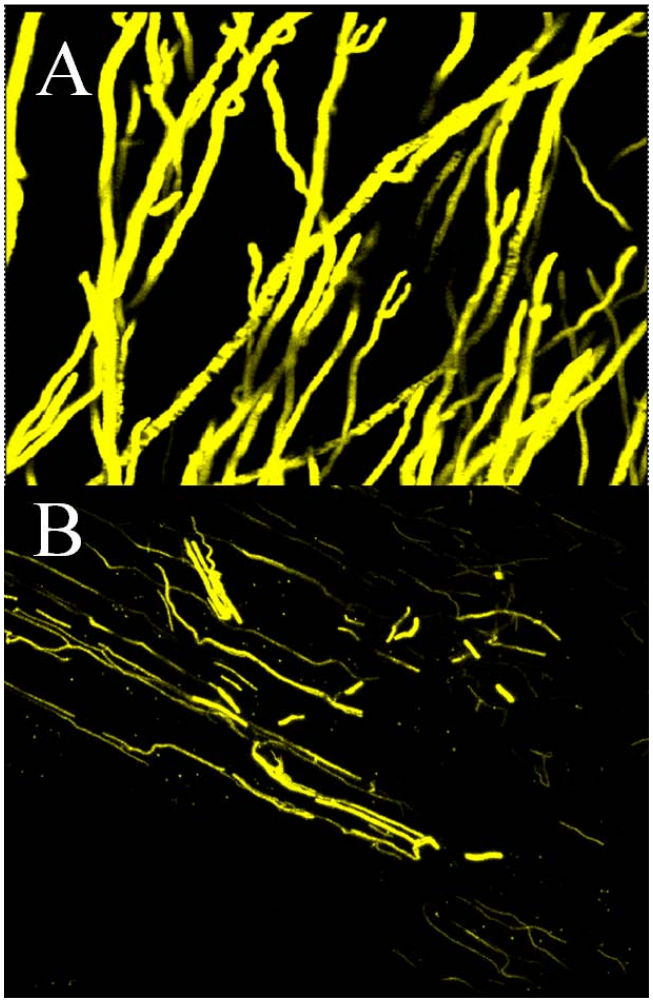

Figure 2.3. *Aspergillus carbonarius* SRRC 2131 transformed with yellow fluorescent protein growing on potato dextrose agar medium (A) and in the roots of maize seedlings, illustrating the symptomless endophytic colonization of maize following soil inoculation with the fungus (B), which persists even under drought conditions *[65]*.

Figure 2.4. Maize kernel rot produced by *Aspergillus niger*

CHAPTER 3

USE OF A REP-PCR SYSTEM TO PREDICT SPECIES IN THE *ASPERGILLUS* SECTION *NIGRI*[1]

[1]Palencia, E.R., Klich, M.A., Glenn, A. E., Bacon, C.W. 2009. Journal of Microbiological Methods. 79:1-7. Reprinted here with permission of the publisher.

ABSTRACT

The *Aspergillus niger* aggregate within the *Aspergillus* section *Nigri* is a group of black-spored aspergilli of great agronomic importance whose well defined taxonomy has been elusive. Rep-PCR has become a rapid and cost-effective method for genotyping fungi and bacteria. In the present study, we evaluated the discriminatory power of a semi-automated rep-PCR barcoding system to distinguish morphotypic species and compare the results with the data obtained from ITS and partial calmodulin regions. For this purpose, 20 morphotyped black-spored *Aspergillus* species were used to create the *Aspergillus* section *Nigri* library in this barcoding system that served to identify 34 field isolates. A pair-wise similarity matrix was calculated using the cone-based Pearson correlation method and the dendrogram was generated by the unweighted pair group method with arithmetic mean (UPGMA), illustrating four different clustered groups: the uniseriate cluster (I), the *Aspergillus carbonarius* cluster (II), and. the two *A. niger* aggregate clusters (named III.A and III.B). Rep-PCR showed higher resolution than the ITS and the partial calmodulin gene analytical procedures. The data of the 34 unknown field isolates, collected from different locations in the United States, indicated that only 12% of the field isolates were >95% similar to one of the genotypes included in the *Aspergillus* section *Nigri* library. However, 64% of the field isolates matched genotypes with the reference library (similarity values >90%). Based on these results, this barcoding procedure has the potential for use as a reproducible tool for identifying the black-spored aspergilli.

INTRODUCTION

Members of the *Aspergillus* section *Nigri* (also known as black-spored aspergilli) are an important group of fungi because of their dual impact on food safety, medical mycology, and in the biotechnology industry. On one hand they have been isolated mainly from soil, but they also have been found in several other substrates (19, 20, 29, 39, 44, 45), where they are known to cause detrimental effects, especially as food spoilage and opportunistic animal and human pathogens. On the other hand, some black-spored aspergilli are beneficial organisms, such as *Aspergillus niger* in the biotechnology industry, where this species has been granted the GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (34). Compounding the economical importance of black-spored aspergilli, are the recent studies that some members of the section *Nigri* are able to produce two mycotoxins, ochratoxin A (2, 3, 4, 6, 11) and the B2 fumonisin (8).

Ochratoxin A is teratogenic, immunosuppressive, and is a potential carcinogenic agent in animals and humans (1). Ochratoxin A has been detected in several agricultural products and their derivatives including wine, coffee drinks, and beer (7, 22). The fumonisins are common mycotoxins of the genus *Fusarium*, but have recently been demonstrated as being produced by black-spored aspergilli. The fumonisins are very important carcinogenic mycotoxins that are produced by *Fusarium* species on maize and maize by-products worldwide, and are toxic to all livestock species and poultry. They are also associated with human esophageal cancer and recently associated with human neural tube defects (21, 40, 41). The frequency of fumonisin production by species of the *Aspergillus* section *Nigri* has not been established.

Black-spored aspergilli are difficult to classify and the taxonomy of this section is still unclear (9). Traditionally, the classification of this section was based on morphological

characteristics. However, recognition of phenotypic characters (34) within this group by an inexperienced diagnostician is a tremendous challenge. A diagnostic phenotypic procedure based on biochemical traits on agar media along with some molecular approaches has been recently reviewed (33). Complicating identification of species within this section is the revelation by molecular examination that there exist several cryptic species within the major recognized morphospecies, creating at least 15 provisional species with in this black-spored group (32). Even under molecular scrutiny recent examination has reduced several species to either synonyms or as a variety of *A. niger* (9, 27, 30), although the more recently described species have not been so scrutinized.

Molecular tools such as RAPD's, RFLPs, ribosomal RNA and protein coding gene sequences have been used to differentiate between taxa within the section *Nigri*, and some of them showed relative high biodiversity in the *A. niger* aggregate (24, 25, 30, 35). The use of molecular tools such as the internal transcribed spacer (ITS) rDNA region and partial calmodulin gene sequencing analysis has been documented as a good approach. However, the main limitations of these techniques are their low discriminatory power to differentiate black-spored aspergilli, low reproducibility, high price and the requirement of highly specialized technicians to perform such analyses. A molecular-based approach known as rep-PCR is based on the amplification of intervening sequences located within short repetitive DNA sequences that are dispersed throughout the genome of prokaryotes and eukaryotes (38). Rep-PCR uses primers that target repetitive extragenic palindromic (rep) regions of bacterial and fungal genomes. Most of these approaches are ideal for phylogenetic analyses (27). Recently, a semi-automated rep-PCR barcoding system was developed that might overcome the low reproducibility and limitations of relative low discriminatory resolution at species level of taxonomic molecular tools (14).

Molecular techniques useful for fungal identification center on barcoding because it shows enormous promise for the very rapid identification of organisms at the species level, although there is considerable debate for this approach for phylogenetics due to uncertainty over the length of barcoding sequences that should be used. However, current data now indicate that standard short barcoding sequences are sufficient for species identification (12, 23, 37). Rep-PCR was originally developed for bacterial typing, but it has also been recently used in fungi (13, 15). While the work of Hansen et al. (13) specifically addressed several *Aspergillus* species, it included only two black-spored species, and no type species of these. They concluded that there were multiple clusters of their *A. niger* isolates and suggested that these represented subspecies.

In this study, we assessed the discriminatory power of the semi-automated DiversiLab rep-PCR system for typing the *Aspergillus* section *Nigri* species compared to conventional DNA sequencing approaches. The species selected were the generally accepted species that usually included the type of these. As a second objective, we created a black-spored aspergilli library for subsequent use with this system as a tool to identify field isolates of this section.

MATERIALS AND METHODS

Fungi

Fifty-four black-spored aspegilli isolates were used in this study. Of these, 20 strains representing 7 species (Table 3.1) were previously identified to the species level by morphological characteristics (17). These strains were used to construct a validated *Aspergillus* section *Nigri* library. Thirty-four black-spored aspergilli strains were isolated from surface sterilized (5) field maize (*Zea maize*) and peanuts (*Arachis hypogaea*) kernels. Field isolates were obtained from maize and peanut meats on Czapek Yeast Agar (CYA) and single-spore

cultures were made of each isolate (Table 3.2). All strains were stored as frozen stocks at −80 °C in 0.01% Tween 15% glycerol solution or on working slants of CYA maintained for up to 6 months at room temperature. Cultural morphology of these isolates was examined on CYA following the procedure of Klich (17). The black-spored aspergilli isolated from seed were reported as relative isolation frequency and expressed as a percent.

DNA extraction

All fungal isolates were cultured in 125-ml baffle-bottom Erlenmeyer flasks containing 20-ml Yeast Extract Sucrose (YES) broth (Difco Laboratories, Detroit, Michigan), and closed with Styrofoam plugs. The inoculum used was prepared from 6-day-old fungi grown on CYA. Inoculated flasks were incubated at 25 °C for 72 h as stationary cultures. Genomic DNA was extracted from a 10-µl loop of individual black-spored aspergilli hyphae using the UltraClean™ Microbial DNA isolation Kit (Mo Bio Laboratories, Solana Beach, California) following the manufacturer's instructions. To increase yields, hyphal material was heated (65 °C for 10 min) and the bead mechanical lysis stepwas extended to 30 min. DNA concentration was determined using a NanoDropTM 1000 spectrophotometer (Nano-Drop Technologies, Wilmington, Delaware) at 260 nm and adjusted to approximately 25 ng/µl.

Rep-PCR DNA barcodes

DNA samples were amplified using the DiversiLab Aspergillus Kit for DNA fingerprinting (bioMerieux, Inc, Durham, North Carolina), following the manufacturer's instructions. Briefly, approximately 2 µl (or 50 ng) of genomic DNA was added to 0.5 µl (2.5 U) of AmpliTaq polymerase (Applied Biosystems, Foster City, California), 2-µl kit supplied primer mix, 2.5-µl GeneAmp 10× PCR Buffer (Applied Biosystems), and 18-µl kit-supplied rep-PCR mix (MM1). The PCR step was performed using a PTC 200 Peltier thermal cycler. (Bio-Rad, Hercules,

California) under the following thermocycler conditions: initial denaturation of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 70 °C for 90 s, and a final extension of 70 °C for 3 min. The rep-PCR amplicons were separated using the chip-based technology LabChip® (Caliper Technologies Corp., Mountain View, California), and analyzed using an Agilent 2100 bioanalyzer version B.02.06 51418 (Agilent Techonologies, Palo Alto, California). Finally, the resulting DNA barcode patterns were reported as electropherograms and the genetic relationships among the isolates were analyzed using DiversiLab software version 3.3 and Pearson's correlation coefficient to generate the distance matrices and the unweighted pair group method with arithmetic average (UPGMA) dendrogram. All the analyses were automatically uploaded to the DiversiLab Microbial Typing system (www.diversilab.com).

DNA sequencing analysis

A two-phase DNA sequencing approach was utilized to confirm the identities of the black-spored isolates. First, a 688 bp DNA fragment of the partial calmodulin gene was amplified and sequenced using CL1 and CL2A primers, as described by O'Donnell et al. (24). Second, a 710 bp DNA fragment of the internal transcribed spacer ribosomal region (ITS) was amplified using ITS-4 and ITS-5 primers (42). In both cases, the amplicons were purified using QIAquick® PCR purification Kit (Qiagen Sciences, Germantown, Maryland). Bidirectional sequencing for each amplicon was performed at the USDA-ARS Eastern Regional Research Center Facility (Wyndmoor, PA) using the same primer sets. DNA contiguous sequences were constructed and edited using a Sequencer v. 4.7 software (Gene Codes Corporation, Ann Arbor, Michigan). DNA sequence alignments were generated using ClustalW2 software, and

phylogenetic analyses for both the partial calmodulin gene and ITS region were conducted using MEGA version 4 software (36). Phylogenetic trees were generated using the neighbor-joining algorithm, complete deletion, Nucleotide: Maximum Composite Likelihood method with 1000 bootstrap replications.

RESULTS AND DISCUSSION

The two main aims of this study were: i) to compare the taxonomic resolution of the semi-automated DiversiLab rep-PCR barcoding system with ITS and partial calmodulin gene DNA sequences analysis, and ii) to determine if this barcoding system can provide an additional taxonomic tool to differentiate between members of the *Aspergillus* section *Nigri*, especially members of the *A. niger* aggregate. One feature of this barcoding system is the generation of gel-like images or virtual gel images of the DNA amplicons (Fig. 3.1). An *Aspergillus* section *Nigri* library was created using 20 black-spored aspergilli that were previously identified at species level by morphological characters (Table 3.1).

From the dendrogram analysis, four major clusters (shown as roman numbers) were identified: the uniseriate cluster (the *Aspergillus aculeatus*/*Aspergillus japonicus*, Fig. 3.1 cluster I), the *Aspergillus carbonarius* cluster (Fig. 3.1, cluster II), and two clusters from the *A. niger* aggregate (Fig. 3.1, clusters III.A and III.B). The rep-PCR system clearly differentiated between two morphological groups in *Aspergillus* section *Nigri*: the uniseriate (*A. japonicus*/*aculeatus*) and the biseriate group, which includes species within the *A. niger* aggregate and the *A. carbonarius* cluster. The uniseriate species formed a cluster (Fig. 3.1, cluster I) consisting of *A. japonicus* and *A. aculeatus*, that showed low similarity (>85%). A second *A. aculeatus* isolate, SRRC 168, formed another branch, however this branch is strongly associated with the biseriate section *Nigri* species (Fig. 3.1). The SRRC 168 isolate was analyzed in triplicate and using

different frozen stock material; however, after performing the dendrogram analysis, this sample was always placed outside of the uniseriate cluster (I). One possible explanation might be that our stock material might be contaminated with material from other fungal/bacterial organism, which lead to a different barcoding pattern.

The five *A. carbonarius* isolates formed a lineage with concordance and statistical support (90% cutoff) (Fig. 3.1, cluster II) and their genetic relatedness can be visualized using the gel-like images. The *A. niger* aggregate (clusters III.A and III.B in Fig. 3.1) showed high biodiversity among the analyzed isolates. The cluster III.A showed high similarity, with most of the isolates showing >90% similarity. Magnani et al. (18) demonstrated that *A. niger* and *A. tubingensis* can be differentiated by RFLPs, and in our analysis using the DiversiLab dendrogram, '*A. niger*' and '*A. tubingensis*' clusters are clearly separate from each other. The '*A. tubingensis*' cluster (III.B) formed a distinct group which, based on our analysis, is a separate group from the '*A. niger*' cluster (III.A). The difference between clusters III.A and III.B is clearly evident when comparing the barcoding pattern of each cluster.

Thirty-four field black-spored isolates obtained from different substrates in the United States (Table 3.2) were scrutinized at the species level using the DiversiLab system using the Top-match feature. This feature compares the queried sample (field isolate) fingerprints with the other ones included in the *Aspergillus* section *Nigri* library. As a result, the percentage of similarity between the queried samples and the five most similar matches, based on the fingerprinting patterns, are reported (Fig. 3.2, Table 3.2). The data reflect the large diversity within the section, possibly reflecting subspecies and strains with large and varied relationships. For the interpretation, 95% similarities between the queried and library isolates were considered a positive designation. In our analysis, 4 (12%) of the field isolates fingerprints had >95%

similarity (Table 3.2), with all the isolates classified in the *A. niger* aggregate. Twenty (59%) isolates were <90% similar to fingerprints in the database. From these data, only 3 (9%) of the field isolates were <80% similar to any of the black-spored aspergilli included in the *Aspergilus* section *Nigri* database.

The data generated do show high promise as an aid in providing the quick and reproducible identification of this group. However, it is evident that an extended *Aspergillus* section *Nigri* database will be required to improve performance and potentially to replace the highly subjective morphological identification systems within this very difficult section. The second objective of our study was to compare the resolution power of the rep-PCR approach with the conventional DNA sequencing approaches for species identification. A comparative analysis between the DiversiLab dendrogram results (Fig. 3.1) with both ITS and partial calmodulin gene sequence analysis (Fig. 3.3) showed high discriminatory power of the DiversiLab analysis relative to the ITS rDNA and the partial calmodulin gene sequencing analysis (Fig. 3.3). One of the limitations of the DNA sequencing analyses is their low discriminatory power for species belonging to the *A. niger* aggregate. In our analysis, it is clear that the *A. niger* aggregate showed high biodiversity using the DiversiLab system compared to the sequencing analyses. A small cluster within the *A. niger* aggregate, consisting of six isolates (SRRC 320, SRRC 60, SRRC 2378, SSRC 278, SSRC 321, and SSRC 359) showed consistent similarities using both ITS and partial calmodulin gene sequencing trees (Fig. 3.3). However, in the system described here, these isolates showed high variability. The discriminatory power of the rep-PCR approach technique is higher than with the ITS rDNA and calmodulin sequencing approaches, which is more evident in the cryptic species within the *A. niger* aggregate.

Another advantage of this barcoding system is the use of a semi-automated system for black-spored classification. This system allows, once a reference library has been created (i.e. the *Aspergillus* section *Nigri* library), the comparison of the DNA barcode pattern of the queried (field) isolate with the barcode pattern of the isolates included in the reference library. The predicted species data (Table 3.2) offer some important suggestions for consideration. These data indicate that *A. niger* has the highest relative frequency of occurrence (82%) of all the predicted black-spored species, followed by *Aspergillus foetidus* (12.1%). This is in concordance with prior data that suggested *A. niger* is the species that is usually reported as being occurring in soils (16) and the cause of seedling and kernel diseases of maize and peanuts. *A. carbonarius* is another species reported as being an ochratoxin A producer and somewhat cosmopolitan (16, 43) but was absent in our rather limited survey of maize and peanuts. We are not aware of a comprehensive survey of maize and peanuts for black-spored aspergilli. These data suggest that peanuts are infected by several black-spored species, primarily by *A. niger*, followed by *A. foetidus*.

Maize is infected primarily by *A. niger* as the dominant species or 94% of the maize samples were infected by this species. Since the kernels were surfaced sterilized, the results indicate that in addition to being seed-borne, some species have the potential of being endophytic in their association with maize and peanut. A more comprehensive study of the black-spored species, both endophytic and non-endophytic, is warranted. Of the accepted black-spored *Aspergillus* species (33), several were isolated from maize and peanuts in this study, and of these two, *A. niger* and *A. foetidus*, are reported as producers of ochratoxin A (25, 31). However, based on beta-tubulin analysis, these two species were considered synonyms (27), i.e., *A. foetidus* was made a synonym of *A. niger*, which is supported by the data from our work. *A. foetidus* was

erected in 1945 to include the black-spored fungi with persistently dark grayish brown to olive-brown heads, to distinguish it from the carbon black heads of *A. niger*. Similarly, both *A. ficuum* and *A. awamori* were made synonyms of *A. niger*. This indicates the highly superficial nature of morphology and reflects the complexity and problematic nature of the taxonomy of this section. However, this barcode procedure could distinguish *A. japonicus*, *A. aculeatus*, *A. niger*, *A. tubingensis*, *A. carbonarius*, *A. foetidus*, and *Aspergillus heteromorphus*. Based on preliminary data, we believe that others within this section can also be differentiated using this procedure. Due to the great diversity observed in isolates of *A. niger*, and in other related black-spored species, our data suggest that studies must be conducted on the biology, potential for ochratoxin A and fumonisin production, and plant pathology of the black-spored species now identified on maize and peanuts, two commodities of great agronomic importance in the USA.

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Table 3.1. *Aspergillus* section *Nigri* isolates used in this study.

Isolate[a]	Species	Source[b]
SRRC 13B	*Aspergillus niger*	NRRL 2042
SRR 16	*Aspergillus carbonarius*	NRRL 2007
SRRC 60	*Aspergillus niger*	NRRL 3 (ATCC 9029)
SRRC 278	*Aspergillus niger*	NRRL 364, ATCC 16882
SRRC 320	*Aspergillus foetidus*	NRRL 337, ATCC 10254
SRRC 321	*Aspergillus foetidus*	NRRL 341 [T]
SRRC 325	*Aspergillus japonicus*	Soil, Panama, NRRL 1782(ATCC 16873)
SRRC 334	*Aspergillus japonicus*	NRRL 5118
SRRC 359	*Aspergillus niger*	Bran, NRRL 3112
SRRC 369	*Aspergillus carbonarius*	Honduras, NRRL 346
SRRC 370	*Aspergillus carbonarius*	NRRL 368, ATCC 6276
SRRC 462	*Aspergillus heteromorphus*	Culture contaminant, Brazil, NRRL 4747 [T]
SRRC 464	*Aspergillus aculeatus*	NRRL 5119
SRRC 476	*Aspergillus japonicus*	NRRL 359
SRRC 484	*Aspergillus tubingensis*	NRRL 4875, CBS 128.48
SRRC 487	*Aspergillus tubingensis*	NRRL 4700
SRRC 488	*Aspergillus tubingensis*	NRRL 4866
SRRC 2131	*Aspergillus carbonarius*	FRR 369T
SRRC 2378	*Aspergillus niger*	NRRL 3536 [T]
SRRC 168	*Aspergillus aculeatus*	NRRL 5094

[a]SRRC, obtained from culture collection of the USDA, ARS, Southern Regional Research Center, New Orleans, LA.

[b]Type isolates are designated by a superscript T.

Table 3.2. Field collection of *Aspergillus* isolates with predicted species results based on rep-PCR.

Strain number [a]	Location	Similarity % of top match	Predicted species
RRC 453	Peanut, South Georgia	90.0	*A. niger*
RRC 454	Peanut, South Georgia	90.6	*A. foetidus*
RRC 455	Peanut, South Georgia	89.6	*A. niger*
RRC 456	Peanut, South Georgia	85.5	*A. niger*
RRC 457	Peanut, South Georgia	93.8	*A. niger*
RRC 458	Peanut, South Georgia	91.3	*A. foetidus*
RRC 459	Peanut, South Georgia	87.5	*A. niger*
RRC 460	Peanut, South Georgia	78.7	*A. japonicus*
RRC 475	Peanut slurries, Dawson, Georgia	79.3	*A. niger*
RRC 476	Peanut slurries, Dawson, Georgia	98.3	*A. niger*
RRC 477	Peanut slurries, Dawson, Georgia	98.4	*A. foetidus*
RRC 478	Peanut slurries, Dawson, Georgia	93.3	*A. niger*
RRC 479	Peanut slurries, Dawson, Georgia	75.1	*A. japonicus*
RRC 480	Peanut slurries, Dawson, Georgia	98.5	*A. niger*
RRC 481	Peanut slurries, Dawson, Georgia	93.1	*A. niger*
RRC 482	Maize kernels, Midwestern USA	92.7	*A. niger*
RRC 483	Maize kernels, Midwestern USA	93.3	*A. niger*
RRC 484	Maize kernels, Midwestern USA	93.4	*A. niger*
RRC 485	Maize kernels, Midwestern USA	93.4	*A. niger*
RRC 486	Maize, kernels, Midwestern USA	90.7	*A. niger*
RRC 487	Maize kernels, Midwestern USA	90.7	*A. niger*
RRC 488	Maize kernels, Midwestern USA	89.9	*A. niger*
RRC 489	Maize kernels, Midwestern USA	88.4	*A. niger*
RRC 490	Maize kernels, Midwestern USA	90.1	*A. niger*
RRC 493	Maize kernels, Midwestern USA	92.8	*A. niger*
RRC 494	Maize kernels, Midwestern USA	92.9	*A. niger*
RRC 495	Maize kernels, Midwestern USA	93.4	*A. niger*
RRC 497	Maize kernels, Midwestern USA	82.2	*A. niger*
RRC 500	Maize kernels, Midwestern USA	97.0	*A. niger*
RRC 501	Maize kernels, Midwestern USA	88.6	*A. niger*
RRC 503	Maize kernels, Midwestern USA	85.2	*A. niger*
RRC 504	Maize kernels, Midwestern USA	89.4	*A. niger*
RRC 507	Maize kernels, Midwestern USA	94.4	*A. foetidus*
RRC 510	Maize kernels, Midwestern USA	92.1	*A. niger*

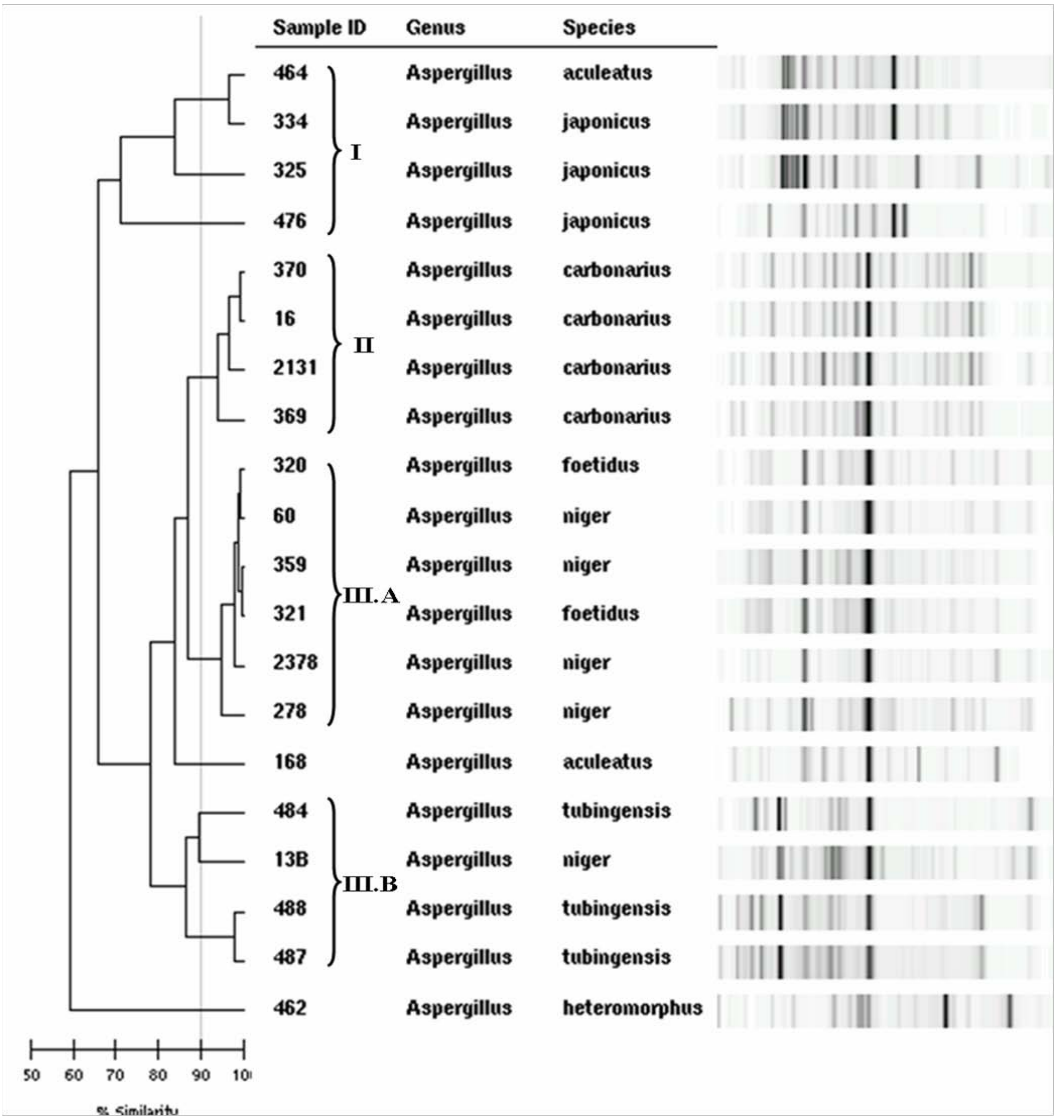

Figure 3.1. Dendogram and gel-like images resulting from rep-PCR amplicons of black-spored aspergilli used to create the *Aspergillus* section *Nigri* library in the DiversiLab system. A pair-wise percent similarity matrix was generated using the Pearson's correlation coefficient, which was used to generate the UPGMA dendrogram. All isolates were identified previously by morphology. Roman numbers indicate the different clusters in the *Aspergillus* section *Nigri* analysis using rep-PCR. The vertical line on the dendrogram indicates 90% similarity cutoff.

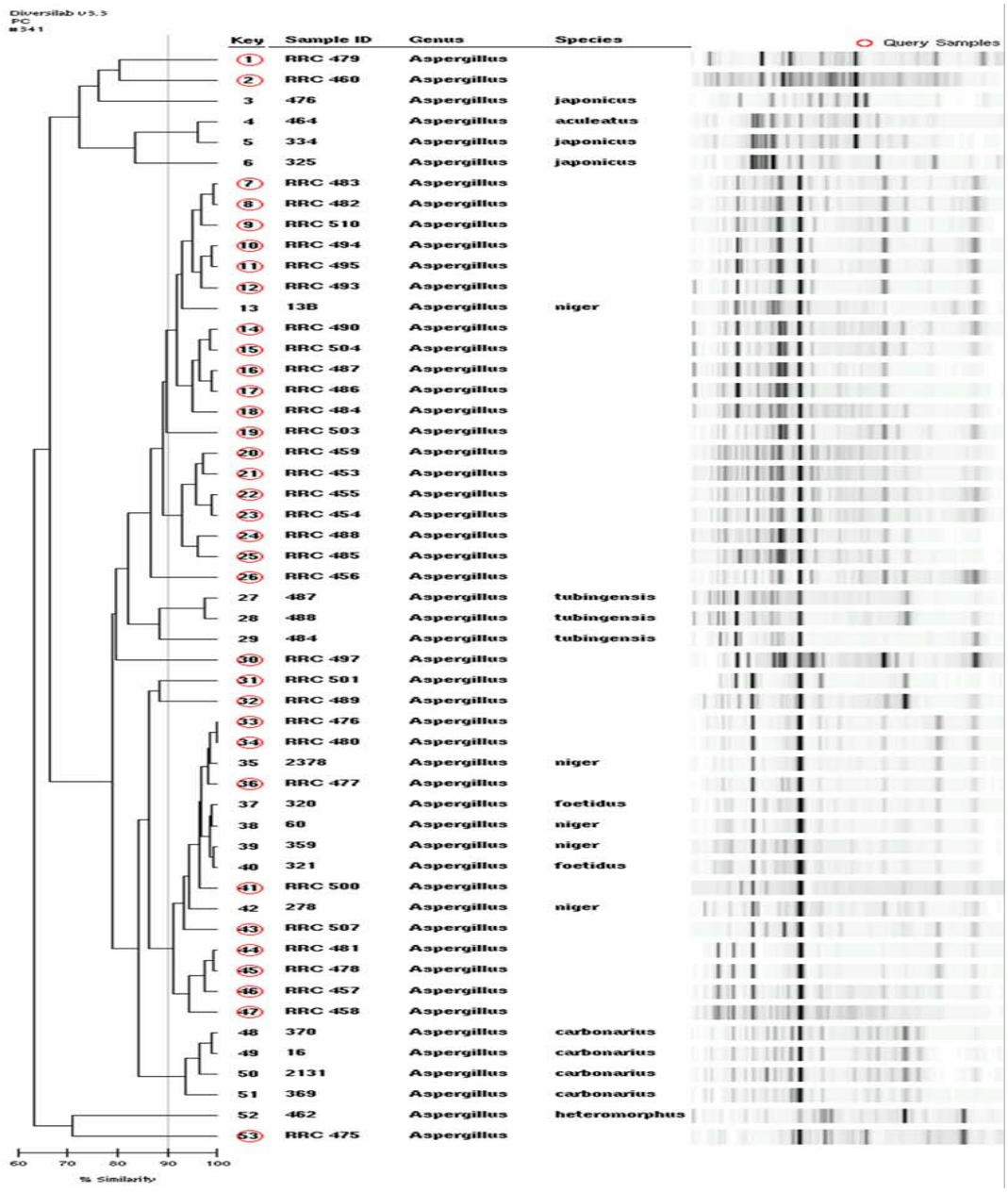

Figure 3.2. Dendogram and gel-like images illustrating the rep-PCR barcodes of all 54 *Aspergillus* section *Nigri* isolates. A pair-wise percent similarity matrix was generated using the Pearson's correlation coefficient, which was used to generate the UPGMA dendrogram. Queried sample numbers are indicated by circles and established identities of these are indicated in Table 3.2.

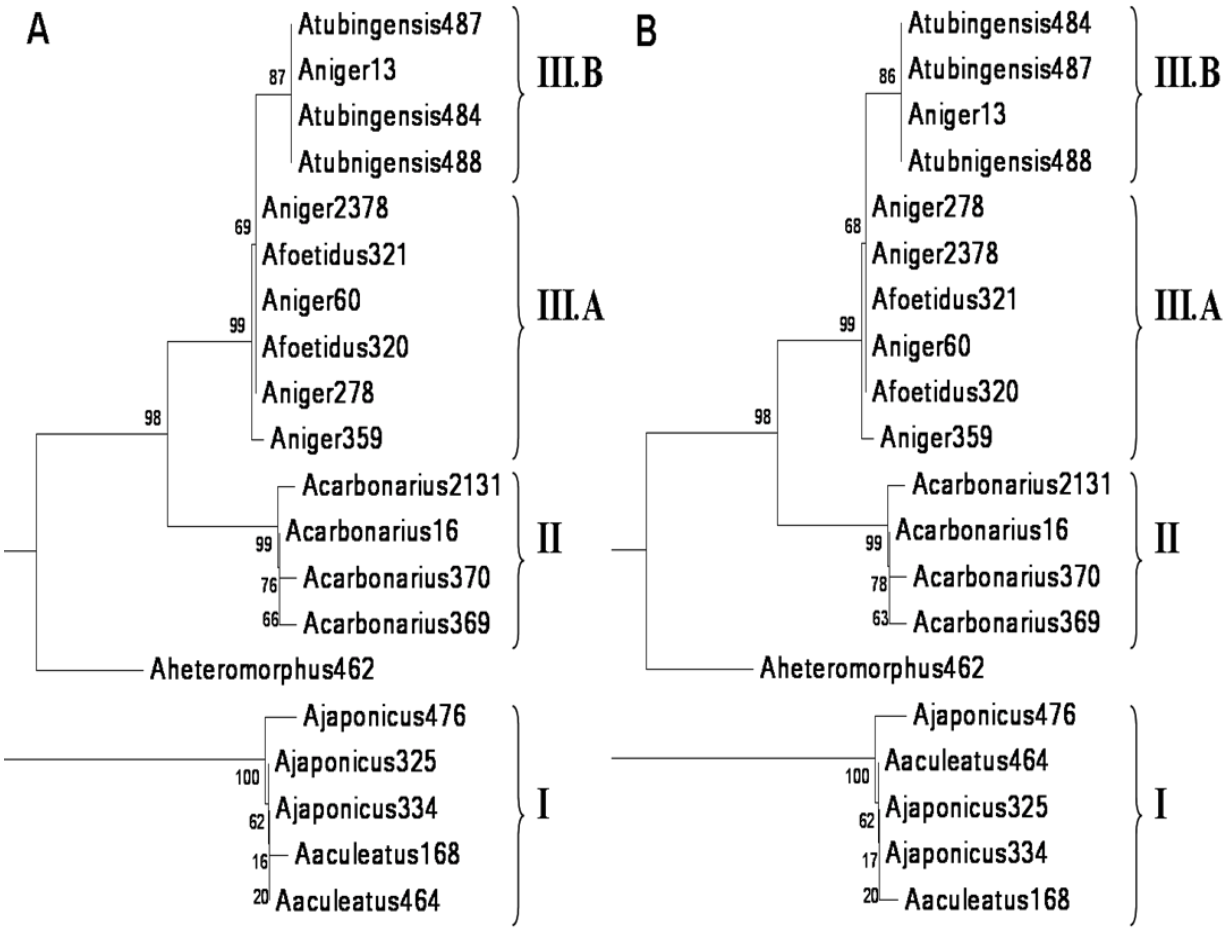

Figure 3.3. Collapsed trees generated by the neighbor-joining algorithm of the ITS-4 rDNA ITS-5 (panel A) and partial calmodulin gene (panel B) DNA sequences. Bootstrap percentages were calculated from 1000 re-sampling replications and are indicated at nodes. In both panels A and B, I, II, IIB indicate the different phylogenetic groups.

CHAPTER 4

GENETIC TRANSFORMATION OF THE FUNGI *ASPERGILLUS NIGER* VAR *NIGER* AND *ASPERGILLUS CARBONARIUS* USING FLUORESCENT MARKERS[1]

[1]Palencia, E.R., Glenn, A.E., Hinton, D.M., and Bacon, C.W. To be submitted to *Phytopathology*.

ABSTRACT

Aspergillus niger and *A. carbonarius* are two species in the *Aspergillus* section *Nigri* (black-spored aspergilli) frequently associated with peanut (*Arachis hypogea*), maize (*Zea mays*), and onion (*Allium cepa*), where they infect plant tissues as symptomless endophytes. These endophytic associations are a major concern since black aspergilli are known to produce mycotoxins, most significantly ochratoxins and fumonisins. To facilitate the study of the black aspergilli-maize interactions during the early stages of symptomless infections, we used the enhanced yellow fluorescent protein (EYFP) and the monomeric red fluorescent protein ($mRFP_1$) to transform *A. niger* and *A. carbonarius*, respectively. The constitutive expression of the fluorescent markers was stable in the cytoplasm of the transgenic strains in both hyphae and conidia. Differences in fungal distribution and seedling development between *A. niger* and *A. carbonarius* wild type and their fluorescent expressing strains were studied using 21 day old maize seedlings. The *in planta* studies showed that both wild type and fluorescent black aspergilli were re-isolated from leaf, stem and root tissues of infected maize seedlings, without any visible disease symptoms. When compared with maize seedlings grown from uninoculated seeds, seedlings inoculated with the yellow fluorescent *A. niger* transgenic strain showed shoot and biomass suppression of the above ground tissue. Conversely, height, shoot diameter, and biomass of seedlings inoculated with the red fluorescent *A. carbonarius* strain were similar to the control. Fluorescent microscopy analysis revealed similar colonization patterns of both black aspergilli fluorescent transformants. Yellow and red fluorescent strains were capable of invading epidermal cells of maize roots intercellularly within the first 3 days after inoculation, but intracellular hyphal growth was more evident after 7 days of inoculation. We also tested the capacity of fluorescent transformants to produce ochratoxin A. Our results with *A. carbonarius*

showed that transgenic strains had similar production of this secondary metabolite. This is the first report on the use of fluorescent markers to study the colonization patterns of two endophytic fungi in the *Aspergillus* section *Nigri*.

INTRODUCTION

The *Aspergillus* subgenus *Circumdati* section *Nigri* (black aspergilli) is an important group of fungal species because of their worldwide distribution and their positive impact in the biotechnological industry. *A. niger* is widely used in industrial processes and has been granted the Generally Recognized As Safe (GRAS) status by the U.S. Food and Drug Administration (44). Conversely, some species within this section are frequently linked to negative impacts in agriculture where they are known as pre and post-harvest plant pathogens, causing a wide range of plant diseases in different hosts, including: grape, onion, garlic, peanut, maize, coffee, fruits and vegetables (24, 27, 39, 49). Recent evidence showed that some black aspergilli are able to colonize plant hosts as symptomless endophytes (31, 50). Symptomless endophytic states occur in plants characterized as balanced symbionts in that the fungus and plant host are highly compatible. The ingestion of contaminated crop products is a potential threat to humans and animal health since some black aspergilli, especially *A. niger* and *A. carbonarius*, are known to produce toxic secondary metabolites.

The species of the *Aspergillus* section *Nigri* isolated from natural substrates including peanut, maize, and grape are able to produce and accumulate mycotoxins such as ochratoxins and fumonisins (3, 4, 16, 28). Ochratoxin A (OTA) is nephrotoxic, teratogenic, immunosuppressive, and classified as a potential carcinogen in humans by the International Agency on Research on Cancer (21). Ingestion of staple foods contaminated with elevated levels of OTA by humans is associated with Balkanic Endemic Nephropathy, a chronic condition that

leads to kidney failure (48). OTA was originally described as a mycotoxin produced by *A. ochraceus*; however since *A. niger* was reported as OTA producer (1), other black aspergilli are reported to produce this secondary metabolite. Recent studies also reported that *A. niger* strains are able to produce fumonisins, another group of mycotoxins described as secondary metabolite produced by filamentous fungi in the genus *Fusarium* (23, 47). In humans, the ingestion of contaminated crop grains with fumonisins has been strongly associated with esophageal cancer and neural tube defects in areas where maize is a dietary staple (18, 45). The production of ochratoxins and fumonisins by black aspergilli presents a potential food safety problem for human and animal health because the fungal species within this group can infect important crops such as maize, grape, and peanut.

The visualization of plant-pathogen interactions has been facilitated by using molecular markers such as the green fluorescent protein (GFP) isolated from *Aequorea victoria* (36), especially for dissecting the endophytic-plant host associations for bacteria and fungi. Recent studies showed the efficient integration of the GFP gene into fungal endophytes, including the slow-growing endophytic fungus *Undifilum oxytropis* (29), the natural root endophyte *Fusarium equiseti* (26), and the wide host range fungus *Muscodor albus* (12). The success of GFP as live imaging marker for fungal cells has increased the interest of many researchers to develop and optimize vectors to express fluorescent proteins with different excitation and emission wavelengths, including the enhanced yellow fluorescent protein (EFYP), and the monomeric red fluorescent protein ($mRFP_1$) (2). The main advantage of using fluorescent markers to monitor microbe-plant associations over traditional techniques relies on the fact that fluorescent proteins do not require preparatory steps, which might affect the structure of living cells.

A major aim of this study was to develop an efficient protoplast-based genetic system to transform the black aspergilli *A. niger* and *A. carbonarius* and determine plant fungus interactions in maize. Another aim of this study was to determine the production of ochratoxin A and fumonisins by black-spored *Aspergillus*.

MATERIALS AND METHODS

Black aspergilli strains and plasmids. The two black-spored species used for genetic transformation were provided by Maren Klich, USDA-ARS, Southern Regional Research Center (SRRC), New Orleans, LA. *A. niger* var *niger* SRRC 13 (=NRRL 2042, National Research Laboratory, USDA-ARS, Peoria, IL) and *A. carbonarius* SRRC 2131 (=FRR 639, Food Research in North Ryde, New South Wales, Australia) as described earlier (31). The plasmids used for genetic transformation of *A. niger* SRRC 13 and *A. carbonarius* SRRC 2131 included pCA45, expressing enhanced yellow fluorescent protein (EYFP), and pCA51, expressing the monomeric red fluorescent protein ($mRFP_1$) (2). These plasmids were provided by Lynda Ciuffetti, Department of Botany and Plant Pathology, Oregon State University. Plasmids, pCA45 and pCA51, contained the hygromycin resistance gene (*hph)* as selective marker and the *Tox A* promoter from *Pyrenospora tritici-repentis* to drive the constitutive expression of EYFP and $mRFP_1$ (8).

Culture and protoplasting conditions. The fungi were stored at -80 °C in a 0.01 % Tween 80, 15 % glycerol solution. Potato dextrose agar slants were inoculated with a 10-µl loop sample of *A. niger* SRRC 13 and *A. carbonarius* SRRC 2131 stock solutions and incubated at 25°C for 10 days. Spore suspensions were prepared by adding 10 ml of sterile 0.01% Tween 80 solution to the PDA slants and filtering the resulting suspension through sterile cheesecloth. Fungal suspensions were concentrated by centrifugation at 3000 rpm for 10 min. Protoplasts

were generated using a modified method described in Szewczyk et al (46). Briefly, a suspension of 1 x 10^8 spores in 20 ml of complete medium (1 g $NaNO_3$, 1.25 g N-Z case, 0.5 g yeast extract, 15 g sucrose, 0.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.25 g, 0.1 ml trace elements, 5 ml vitamin solution, and 500 ml autoclaved distilled water) was incubated at 30°C and 200 rpm for 16 hours in a incubator shaker (Innova 4300, New Brunswick Scientific, Edison, NJ). After incubation, the hyphal mat was collected using sterile cheesecloth and aseptically transferred to 8 ml of the protoplasting-enzyme mix and incubated in a rotary incubator at 80 rpm for 4 hours at 30° C. The protoplasting solution was prepared by mixing 10 ml of a citric acid buffer (1.1 M KCl, 0.1M citric acid pH 8) with 1.34 g of Vinoflow enzyme (Gusmer Enterprises Inc., Napa, CA), followed by a filtration step using an Acrodisc 0.2 µm HT Tuffryn membrane syringe filter (Pall Corporation, Ann Arbor, MI). Protoplasts were harvested by slow addition of 20 ml of a cold 1.2 M sucrose solution and centrifuged at 3400 rpm for 20 min. After forming an interface, protoplasts were transferred to a sterile 50 ml conical tube and suspended in 40 ml pre-chilled sorbitol-tris-calcium (STC) buffer: 1.2 M sorbitol, 50 mM $CaCl_2.2H_20$, and 10 mM Tris-HCl pH 8.0. The suspension was centrifuged at 2500 rpm for 15 min at room temperature. The concentration of the protoplast suspension was determined by using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY).

Transformation of black-spored *Aspergillus* species. Genetic transformation of *A. niger* SRRC 13 and *A. carbonarius* SRRC 2131 was performed using a modified polyethylene glycol (PEG) mediated transformation method previously described (19). Briefly, 10 µg of DNA plasmid vectors were separately mixed with 100 µl of STC buffer, 100 µl protoplast solution (1 x 10^7 protoplasts), and 50 µl of 30% PEG (Sigma Aldrich, Milwaukee, WI). The mixture was incubated for 20 min at room temperature, followed by the addition of 2 ml of 30% PEG.

Finally, the resulting solution was mixed with a 2 ml-aliquot of STC buffer and 36 ml of molten overlay medium (0.22 g yeast extract, 0.22 g casein enzymatic hydrolysate, 2.2 g agarose in 110 ml of distilled deionized water). A 5-ml aliquot of the mixture was poured over 20 ml of solidified regeneration medium (2.0 g yeast extract, 2.0 g casein enzymatic hydrolysate, 32 g agar, 1.6 M sucrose) in 100 mm diameter petri dishes. Dishes were incubated at 30°C for 48 hours, during which time single colonies were visible on the regeneration medium. Because both plasmid vectors carry the *hph* gene, putative fluorescent transformants were selected after overlaying cultures with10 ml of 1% water agar amended with hygromycin B (Roche Diagnostics, Indianapolis, IN). Two concentrations of hygromycin B were used: 300 µg/ml for *A. niger* yellow fluorescent transformants; and 150 µg/ml for *A. carbonarius* red fluorescent transformants. Mitotic stability of fluorescent transformants was monitored by transferring transformants to non-selective medium. The yellow and red fluorescent transformants were subcultured on PDA slants without the selective pressure of hygromycin B for five generations.

DNA manipulation and molecular biology analysis. Plasmids, pCA45 and pCA51, were shipped and recovered as described earlier (40). The recovered plasmids were transformed into TOP10 electrocompetent *E. coli* strain (Invitrogen, Carlsbad, CA) by electroporation, following the manufacturer's intructions. Individual putative transformed *E. coli* colonies were picked and propagated in Luria-Bertani medium amended with ampicillin (50 µg/ml) as selective agent. Transformed *E. coli* cells resistant to ampicillin were stored at -80°C in a 15% glycerol solution. For high DNA yields, the DNA extraction for fluorescent expression plasmid vectors was performed using a QIAGEN large-construct kit (Qiagen, Valencia, CA). Putative *A. niger* and *A. carbonarius* transformants were preliminarily screened by PCR using a rapid method previously described (51). Briefly, putative transformants resistant to hygromycin were

individually inoculated onto PDA dishes and incubated at room temperature in darkness. After 3 days, sterile toothpicks were used to aseptically transfer hyphal tissue into 100 µl of sterile water in a 1.5-ml centrifuge tube. The fungal suspension was vortexed and centrifuged at 10,000x*g* for 1 min to remove PCR inhibitors. Water was discarded and the pellet was resuspended in 100 µl of autoclaved lysis buffer (50 mM sodium phosphate at pH 7.4, 1mM EDTA and 15% glycerol) and incubated at 80° C for 30 min. DNA from the putative transformants was screened by individual PCR reactions containing: 2.5 µl of 10X PCR buffer, 0.5 µl 25 mM MgCl2, 1.5 µl of 10mM dNTP mix, 1.0 µl of each primer, 0.25 µl Amplitaq DNA polymerase (5 units/µl), and 18.25 µl sterile water. PCR reactions consisted of a denaturating step at 94° C for 5 min followed by 35 cycles of 94° C for 1 min, annealing step (ITS region= 53° C for 1 min; hygromycin = 54° C for 1 min; ToxA gene= 61° C for 30 s), and a final extension step of 72° C for 7 min.

Plant material and growth conditions. For the endophytic colonization of maize (*Zea mays*) seedlings by black aspergilli and the expression of their fluorescent proteins *in planta*, maize cultivar Pioneer 33K81 (Pioneer Hi-Bred Inc, Johnston, IA) was used. Seeds were subjected to external and internal sterilization using the heat shock procedure described earlier (7). Briefly, kernels were placed in sterile plastic cups and rinsed with sterile water for 3 min in an orbital shaker. The water was discarded and kernels were surface disinfested using commercial bleach (6.15% sodium hypochlorite) for 10 min in an orbital shaker, followed by a heat-shock treatment at 60° C for 5 min. Seeds were rinsed three times with sterile water and dried aseptically on autoclaved filter paper placed in 100 x 15 mm Petri dishes for 3 h. Maize seed viability was tested by pre-germinating them on a germination medium per liter of sterile distilled water: 12 g agar, 10 g glucose, 0.1 g peptone, and 0.1 g yeast extract) for 2 days at 25° C as described earlier (25). Germinated seeds were separately inoculated using fungal spore

suspensions of wild type and fluorescent transformants ($1x10^5$ cfu/ml) in 0.01% Tween 80 for 16 hours at room temperature. Maize inoculation was performed by incubating sterile kernels in 10 ml of a spore suspension (1×10^5cfu/ml) overnight in a petri dish. After incubation, the maize seeds were dried on a sterile Whatman paper #4 for 3 hours at room temperature under ventilated aseptic conditions.

After inoculation, both inoculated and un-inoculated seeds were used to evaluate differences in plant responses from maize seedlings to the black aspergilli wild types and fluorescent transformants. This involved the use of four replicates of 10 inoculated and germinated seeds planted in sterile potting mix (Fafard, Agaman, MA) containing sphagnum peat moss (40-50%), horticultural vermiculite, horticultural perlite, limestone, and processed pine bark. The control group consisted of germinated seeds treated with sterilized 0.01% Tween 80 without fungal spores. All plants were grown under aseptic conditions in a plant growth room for 21 days under 14 hours of light (cool-white, high-output fluorescent tube at an average 254 μmol m^{-2} s^{-1}) at 26°C and 10 hours of darkessn at 22°C. After 21 days, maize seedlings were removed from pots and roots were freed of planting mix soil by shaking plants in autoclave bags. After separating root and aerial tissue, plant tissue were cut into 3-5 cm sections and placed in sterile screw-cap disposable specimen containers. Sectioned plant tissue was rinsed in sterile water for 3 min in an orbital shaker, followed by a surface disinfestation step in 1 % chloramine-T hydrate solution (Sigma-Aldrich, Saint Louis, MO) for 30 min on a rotary shaker. The solution was removed and the plant material was rinsed 3 times in sterile water. Plant segments were trimmed (1.0 cm from each end) using sterile razor blades and the resulting segments were placed on dichloran rose bengal chloramphenicol medium (DRBC, Difco laboratories, Detroit, MI) amended with 18% glycerol for fungal isolation. Five segments from each plant tissue (blades,

stems, and root tissue) were aseptically placed on DRBC dishes and incubated at 25° C for 7 days. Plant material was separated into root and aerial tissues and oven-dried at 60° C to a constant weight to determine plant biomass.

Isolation and fingerprinting. To confirm the identity of the black aspergilli isolated from surface-disinfested plant tissue, a rep-PCR DNA fingerprinting analysis was performed. After 7 days, hyphae growing out from the plant tissue were aseptically transferred with sterile toothpicks into 10-ml of yeast extract sucrose (YES) medium and incubated at 25° C for 48 hours. Young hyphae on YES medium were transferred into 1.5-ml centrifuge tubes and centrifuged at 3000 rpm for 5 min. DNA was extracted from the pellet and subjected to the DNA fingerprinting analysis described earlier (31). Dendogram and the gel-like images were generated using the Agilent 2100 bioanalyzer version B.02.06 51418 (Agilent Technologies, Palo Alto, CA) and the DiversiLab software version 3.3 (bioMerieux Inc, Durham, NC)

Microscopic analysis. Fresh hyphae and spores from fluorescent transformants were visualized using a Leica DM6000 B fluorescent microscope (Leica, Wetzlar, Germany) equipped with an external light source Leica EL6000 with a mercury metal halide bulb. Two filter cubes were used for this study: for the enhanced yellow fluorescent protein (EYFP), the excitation/emission wavelengths were 514/527 nm, and for the monomeric red fluorescent protein (mRFP1), the excitation/emission wavelengths were 558/582 nm. Micrographs of fungal material sections were recorded as several Z-stack of TIFF-images, and compatible Z-stack images were created by using the LAS Stackon v 1.0.B13 software (Leica). The resulting images were loaded into the Montage module of Leica Application Suite (LAS) V 3.7.0 to visualize extended focus images. Fluorescent proteins from inoculated tissue were visualized using a microslide mount in a 5 µl solution of Mowiol® (Calbiochem, La Jolla, CA) anti-fade medium

for direct observation under the microscope. The Z-stack images and montages were generated by using the LAS Stackon and the LAS application suite softwares as described earlier.

Mycotoxin production and analysis. *Aspergillus niger* and *A. carbonarius* wild types and fluorescent transformants were tested for production of ochratoxins and fumonisins using maize kernels as natural substrate. Maize kernels were pre-treated before the fermentation process as follows: 10 g maize kernels were placed in 40 ml of sterile deionized distilled water and incubated at room temperature for 12 hours in 125 ml Styrofoam stopped flasks. The residual liquid was discarded and the kernels were autoclaved at 121°C for 15 min. Each flask was inoculated with three agar plugs (5 mm) of *A. niger* or *A. carbonarius* SRRC 2131 wild type and their fluorescent transformants, and incubated at 30°C under dark conditions. The production of mycotoxins in maize kernels was analyzed after 7 days incubation. Harvested kernel samples were lyophilized in a Benchtop K freeze dryer (Virtis, Gardiner, NY) at -43°C, 60 mTorr for 4 days, and ground to a fine powder using mortar and pestle.

The extraction method for OTA consisted of adding approximately 2.5 g of the ground lyophilized samples to 10 ml of a methanol: chloroform 1:1 (vol/vol) mixture. The suspension was placed in a rotary shaker for 3 hours at room temperature and sonicated for 30 min. Extracts were filtered using Whatman #4 filter paper. The methanolic solution was extracted three times with 8 ml of 0.5 N $NaHCO_3$ and the pooled aqueous solution was collected in a 25 ml beaker. The alkaline-aqueous solution was acidified to pH 2-3 with 0.2 M HCl and mixed 3 times with 20 ml $CHCl_3$. The organic layer was collected and transferred to a 250 ml boiling flask. The solvent was removed by using a rotary evaporator (Buchi, Flawil, Switzerland) and the extracts were redissolved in 0.5 ml methanol. A preliminary thin-layer chromatography procedure was used to assess the production of OTA by black aspergilli and their transgenic fluorescent strains.

Methanolic extracts (30 µl) were spotted onto a silica gel coating TLC sheet (aluminum backing, 250-µm layer, 20 x 20 cm. Whatman, Maidstone, England), and dried at room temperature for 5 min. The TLC sheet was developed with a mobile phase containing toluene-ethylene acetate-formic acid 50:40:10 (vol:vol:vol) in a saturated thick glass tank for 40 min, followed by a examination under UV light (254 nm). OTA spots were detected and photographed using an Alpha Innotech imaging station (Alpha Innotech, San Leandro, CA). Quantification of OTA was performed by high performance liquid chromatography (HPLC). The Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) used for mycotoxin analysis was equipped with a vacuum degasser G1322A, a quaternary pump G1311A, a fluorescent detector FLD G1321A, and a Diode-array detector DAD G 1315D. For OTA quantification analysis, the fluorescence detector was set at 334 nm (λ_{ex}) and 460 nm (λ_{em}). The chromatographic separation was carried out using an analytical C18 column (Zorbax Eclipse XDB; 4.6 x150 mm; particle size 5 µm) with a mobile phase of acetonitrile/water/acetic acid (52:46:2) at a flow rate of 1.0 ml/min. The photomultiplier gain was set at 10.00 for the mycotoxin analysis.

Statistical analysis. Sigma Plot v. 9.0 and Sigma Stat v 3.1 (Systat Software, Point Richmond, CA) were used to perform analysis of variance (ANOVA) to compare the effect of plant seedling inoculation and the controls on plant growth.

RESULTS

Selection of transformants and frequency of transformation. Both transformation vectors pCA45 and pCA51 contained the hygromycin phosphotransferase B encoding gene as selectable marker. The sensitivity to different hygromycin B concentrations by *A. niger* var *niger* SRRC 13 and *A. carbonarius* SRRC 2131 was determined using a PDAplate assay (data not shown). The *A. niger* strain showed complete growth inhibition at 250 µg/ml; whereas the data

from *A. carbonarius* indicated complete growth inhibition at 75 µg/ml. Transformants were selected by a top agar overlay method using two different hygromycin B final concentrations. The *A. ni*ger yellow fluorescent transformants were selected by overlaying 1% agar at 300 µg/ml hygromycin B; whereas *A. carbonarius* red fluorescent transformants were selected at 150 µg/ml. Transformation frequencies varied from 34-50 transformants for *A. niger* and 38-45 transformants *A. carbonarius* per 10^7 conidia.

Mitotic stability and analysis of transformants. The mitotic stability of the hygromycin-resistant transformants was tested by transferring ten transformants onto PDA medium without the selective pressure of hygromycin. After five generations, transformants remained mitotically stable and were single-spored on PDA and transferred to PDA slants amended with hygromycin B (150 µg/ml). For the analysis of putative transformants, 24 single-spored isolates for each species were subjected to genomic DNA extraction using the method described earlier (51). The insertion of pCA45 and pCA51 vectors into *A. niger* and *A. carbonarius* transformants, respectively, was determined by PCR amplification of the *hph* and *Tox A* regions of the black aspergilli transformants genomic DNA samples. The *hph* gene and the *Tox A* regions were amplified using two set of primers. Our results indicated that 22 (91%) transformants of *A. niger,* and 20 (83%) of *A. carbonarius* were positive for the integration of the *hph* gene and the *Tox A* promoter. Ten transgenic strains from each species were subjected to microscopic analysis under fluorescent light to determine the stable expression of the EYFP and $mRFP_1$ fluorescent markers *in vitro*. All transformants showed a wide range of fluorescence intensities, with 8 (80%) of the transformants subjected to fluorescence assay for *A. niger* expressing the EYFP marker, and 7 (70%) of the *A. carbonarius* expressing the mRFP1 marker

(Fig. 4.1). In both cases the transformants with the brightest intensities were used to study the endophytic interactions with maize seedlings.

Microscopic and fluorescent analysis. *In vitro* expression of EYFP and $mRFP_1$ markers by the fluorescent transformants was observed using a fluorescent microscope system equipped with filters for detecting each fluorochrome (Fig. 4.1 a-k). The constitutive expression of EYFP and mRFP1 markers was bright and stable in both fungal strains. For better contrast and visualization of viable hyphae in Petri dishes, fungal inoculum was placed on malt extract agar, which did not show autofluorescence at the wavelengths used to detect EYFP (λ_{exc} 514/ λ_{emm} 527 nm) and $mRFP_1$ (λ_{exc} 558/ λ_{emm} 582 nm). Conidia were harvested 5 days after inoculation since older spores were difficult to observe under fluorescent light due to their dark pigmentation. The $mRFP_1$ fluorescent *A. carbonarius* strain was especially affected by this phenomenon, where mature spores producing characteristic melanized pigmentation were bearly visible under fluorescent light (Fig 4.1, k), even when the gain and intensity parameters were increased. The production of the secondary metabolite melanin is a hallmark of this fungal group (43). Although melanization also occurred in the *A. niger* yellow fluorescent strain (Fig 4.1, i), the expression of this fluorescent marker was stronger than the red fluorescent marker. Thus, production of dark-pigmented spores in *A. niger* yellow fluorescent transformants did not affect the visualization of EYFP.

In planta expression of the fluorescent markers allowed the study of colonization pattern of *A. niger* and *A. carbonarius* in maize seedlings. By day 3, moderate apical growth of hyphae was observed for *A. niger* at the elongation zone (Fig. 4.2 a). This growth was characterized by intercellular growth of hyphae (Fig. 4.2 b). However, by day 14, both inter- and intra-cellular hyphal growth was detected (Fig. 4.2 c), not only in the elongation zone but also near the root

cap. The intracellular growth was characterized by thick bright hyphae, perhaps indicating a more complex interaction between hyphae and plant tissue after 14 days of the inoculation. Cross-sections of plant root tissue analyzed after 14 days of inoculation indicated the epidermal and subepidermal growth of hyphae (Fig 4.2 d and e). The subepidermal growth of hyphae was more evident in elongation zones where hyphae were clearly colonizing root tissue. For *A. carbonarius*, the colonization of elongation zones in maize seedlings (Fig. 4.3 a) before 7 days of the inoculation exhibited a similar pattern as shown in the *A. niger* fluorescent mutant. At this stage, the colonization of the elongation zone was more evident than in *A. niger*, and it was characterized by intercellular growth. Fungal growth was evident in the elongation zone (Fig 4.3 b) by day 14, with inter- and intracellular hyphae. The attachment of fluorescent spores in the elongation zone by *A. carbonarius* was evident. After 14 days both elongation zone and root cap tissue were heavily colonized with intra- and inter-cellular hyphae (Fig 4.3 d). Cross-section of the root cap section, by 14 days after inoculation, showed development of conidiophores (Fig. 4.3 e), with massive growth of hyphae at epidermal and subepidermal tissues (Fig. 4.3 f).

Seedling growth and re-isolation of black aspergilli strains from plant tissue. To study the systemic infection and the plant-pathogen interactions by endophytic black aspergilli in maize seedlings, transformed and untransformed black spored aspergilli were used to infect surface disinfested kernels. Maize seedlings grown from treated kernels were compared with a control (water) group. Our studies revealed that both fluorescent and wild type strains of *A. niger* and *A. carbonarius* were able to infect 21-day-old seedlings. We compared the DNA fingerprint profiles of the re-isolated fluorescent transgenic black aspergilli using a rep-PCR approach described earlier (31). The results indicated that the *A. niger* yellow fluorescent strains isolated from leaves, stems, hypocotyl, and roots (Fig 4.4) were >93% similar to the genotype of their

wild type. For the red fluorescent *A. carbonarius* transformants, the re-isolated strain was >95% similar to its wild type. In both cases, our findings showed that transformation of transgenic *A. niger* and *A. carbonarius* did not affect the capacity of the fluorescent transgenic strains to endophytically colonize maize seedlings.

We determined the effects of black aspergilli colonization on 3-week-old maize seedlings using *A. niger*, *A. carbonarius* and their respective fluorescent strains (Fig. 4.5). The height and stem thickness (Fig. 4.5 a,b) of the seedlings were measured from maize plants treated with black aspergilli strains, and compared with the control. In terms of plant height, the maize seedlings grown from inoculated seeds with fluorescent black aspergilli did not show significant differences when compared with the control group ($P > 0.05$, Fig. 4.5 a). However, when stem thickness of the *A. niger* strains was compared with the control (Fig 4.5 b), the treated maize seedlings showed a reduction in the stem diameter ($P<0.05$). We also measured the plant biomass of the above- and below-ground tissue (Fig. 4.5 c), of the treated and untreated maize seedlings. For the above-ground tissue, plant biomass of seedlings treated with *A. niger* and its transformants were significantly reduced ($P<0.05$). For the below-ground tissue, the maize seedlings treated with the red fluorescent *A. carbonarius* had significantly increased root tissue ($P<0.05$).

Mycotoxin production. To address the question whether the genetic transformation of black aspergilli wild types affected OTA production in the fluorescent transformants, a TLC assay (Fig 4.6 A) was performed. The TLC analysis revealed that both *A. niger* EYFP and *A. carbonarius* $mRFP_1$ transformants were able to maintain their ability to produce OTA. The quantification of OTA by HPLC showed that the *A. carbonarius* red fluorescent transformant produced the highest amount of OTA (27.2 µg/ml ± SD 1.51) which was not significantly

different compared with its wild type *A. carbonarius* SRRC 2131 (25.9 µg/ml ± 1.0435) (Fig 4.6 B). For the *A. niger* strains, the yellow fluorescent transformant produced a similar amount of OTA (0.068 µg/ml ± 0.017) when compared with the *A. niger* wild type (0.06 µg/ml ± 0.016). Generally, *A. carbonarius* is able to produce higher amounts of OTA than *A. niger*, and this trend was observed in our studies. Both *A. carbonarius* strains were able to produce more than 500 times the amount of OTA than *A. niger* strains.

DISCUSSION

The use of fluorescent markers as a molecular tool to study the fate of microorganisms in complex plant-microbial associations for endophytic microorganisms has increased (26; 32, 33, 37, 41). In this study, we report the development of a genetic transformation method to create fluorescent transgenic strains of two black aspergilli, *A. niger* and *A. carbonarius*. These strains allowed the study of colonization patterns of black aspergilli in 3-weeks-old maize seedlings grown from inoculated seeds. The fluorescent transformants constitutively expressed the EYFP (*A. niger*) and $mRFP_1$ (*A. carbonarius*) reporters (2) in both *in vitro* and *in planta* conditions.

The genetic transformation of fungal species has been hampered by the lack of efficient transformation procedures that meet the specific requirements for each species. The protoplast-mediated transformation system is based on the treatment of protoplasts with polyethylene glycol (PEG). This system has been successfully used in fungal species (42). The lack of efficient cell wall degrading enzyme cocktails to prepare protoplasts has proven to be the main limiting factor for developing protoplast-based approaches for transformation. We applied a protoplast-generating system which involved treating young hyphae with the inexpensive winemaking enzyme Vinoflow® FCE, a blend of pectinase and beta 1,3-1,6 glucanase activity that has been used with other fungal species (11, 13, 17). This enzyme preparation successfully degraded

fungal cell walls, resulting in high protoplast yield for *A. niger* and *A. carbonarius.* Further, Szewczyk et al (46), reported similar results in *A. nidulans*. High yields were reported by de Bekker et al (11) who used a mixture of lysing enzymes from *Trichoderma harzianum*, a chitinase from *Streptomyces gris*eus, and a glucuronidase from *Helix pomatia* to develop protoplasts in *A. niger*. Unlike de Bekker et al (11), where the cell wall degrading enzymatic cocktail was not suitable to generate protoplasts in another black aspergillus species, *A. awamori*, our procedure was suitable for other black aspergilli (data not shown), including the ochratoxigenic species *A. carbonarius*. Although we obtained high protoplasts yields with black aspergilli, this enzyme has been unsuccessfully used for degrading cell walls of *A. fumigatus* (15), suggesting a variable cell wall composition among *Aspergillus* species.

In our studies, we have shown that by using a protoplast mediated transformation method we successfully inserted the DNA from pCA45 and pCA51 vectors into the genome of the black aspergilli *A. niger* and *A. carbonarius*. Both species constitutively expressed the yellow and red fluorescent markers that were used to monitor fungal interactions in maize root tissue. Transformation frequencies varied from 34-50 transformants for *A. niger* and 38-45 transformants *A. carbonarius* per 10^7 conidia. Compared with other transformation methods used for black aspergilli transformation, such as that using *Agrobacterium tumefaciens*, our results showed lower transformation frequencies (10, 20). Although *A. tumefaciens*-mediated transformation resulted in higher transformation frequencies, the resulting transformants were characterized by silencing of the fluorescent phenotypes after long storage (14) and a low number of DNA insertions (11). Our fluorescent transformants have kept their fluorescent phenotype after long periods of storage, so this protocol is not only suitable for protoplast development, but also more effective for black aspergilli genetic transformation.

An inherent problem observed in fluorescent strains of black aspergilli is that the time of maturation decreased the fluorescence intensity of spores of the transformed black aspergilli. This phenomenon has also been documented in other living systems, where the production of certain pigments such as melanin has became a major challenge for cell imaging (9). It is believed that melanin and other compounds absorb the light intensity when irradiated with fluorescent light at the wavelength of several fluorescent markers. However, a characteristic feature of black aspergilli is the production of melanin, which is the taxonomical feature of this group. We did not encounter any difficulty with our microscopic system.

In this study, the production of ochratoxins by the wild types and fluorescent transformants was evaluated. Although our studies did not address the role of ochratoxins in plant-fungal associations, we showed that even after insertion of foreign DNA into *A. niger* and *A. carbonarius*, the production of these secondary metabolites remained the same compared to their respective wild types.

Fungal root colonization in maize seedlings was monitored using the fluorescent strains. Intercellular and intracellular hyphae were visible in symptomless root tissue 24 hours after inoculation. Fluorescent strains were separately used to inoculate maize seeds, and our observations showed that *A. niger* is the more aggressive colonizer of the surface of the root tissue compared with *A. carbonarius*. Surface colonization was also observed in *Fusarium verticillioides* studies (5), where it was concluded that this symptomless endophyte might be a cortical root fungus. We also observed that both *A. niger* and *A. carbonarius* strains did not show specificity for infections sites in root tissue and both fluorescent strains were not visible at the root cap. Similar results were shown by Olivain et al (30), who used fluorescent markers in *F. oxysporum* strains to study of colonization on tomato root tissue by pathogenic and

nonpathogenic strains. They concluded that there was no specificity for infection sites in plant tissue, and both strains were visible on the surface of the root tissue but not in the apical zone.

In natural environments, where only few fungi are able to overcome the host's defenses and cause disease, endophytic fungi are usually described as weak pathogens (35) because they lack the molecular arsenal to actively penetrate, invade and colonize the plant tissue to cause disease. However, under special conditions such as drought, infections by endophytic fungi can become pathogenic and cause significant damage in the plant tissue. We demonstrated that maize seedlings grown from seed inoculated with *A. niger* strains showed a reduced biomass of the upper and root tissue, but developed more lateral roots.

The importance of this study relies on the potential of endophytes to influence the plant hosts responses to cope with biotic and abiotic challenges, due to their capacity to live within the plant host without developing disease symptoms and their ubiquitous symbiotic associations with almost all plants (6; 38). The biosynthesis of mycotoxins by filamentous fungi is hypothesized to play a role in the plant-endophytic associations. OTA research has primarily been conducted on animal toxicity; however its role in plant-fungal interactions still remains unclear. Peng et al (34) observed that *Arabidopsis thaliana* seedlings grown on culture media ammended with OTA inhibited plant growth and induced the production of reactive oxygen species, hydrogen peroxide and superoxide radicals, leading to a hypersensitive response in detached leaves.

The study of fumonisins and their role in plant-endophyte interactions has been documented. Glenn et al (19) showed that non-fumonisin producing *Fusarium verticillioides* strains were unable to cause foliar symptoms, whereas maize seedlings inoculated with fumonisin-producing strains developed symptoms of disease, suggesting that fumonisins might play a role in the incidence of disease in maize seedlings. A better understanding of the

mechanisms leading to beneficial effects to the plant partner in symbiotic interactions with endophytes may provide alternative approaches to improve the agronomic performance of important field crops. The use and improvement of microscopy tools, such as the use of fluorescent markers, to study plant-microbe associations will reveal the molecular basis of the mechanisms used by fungal endophytes to protect their plant hosts. To the best of our knowledge, this is the first report on the use of molecular markers to elucidate the root colonization patterns by fluorescent strains of species within the *Aspergillus* section *Nigri*.

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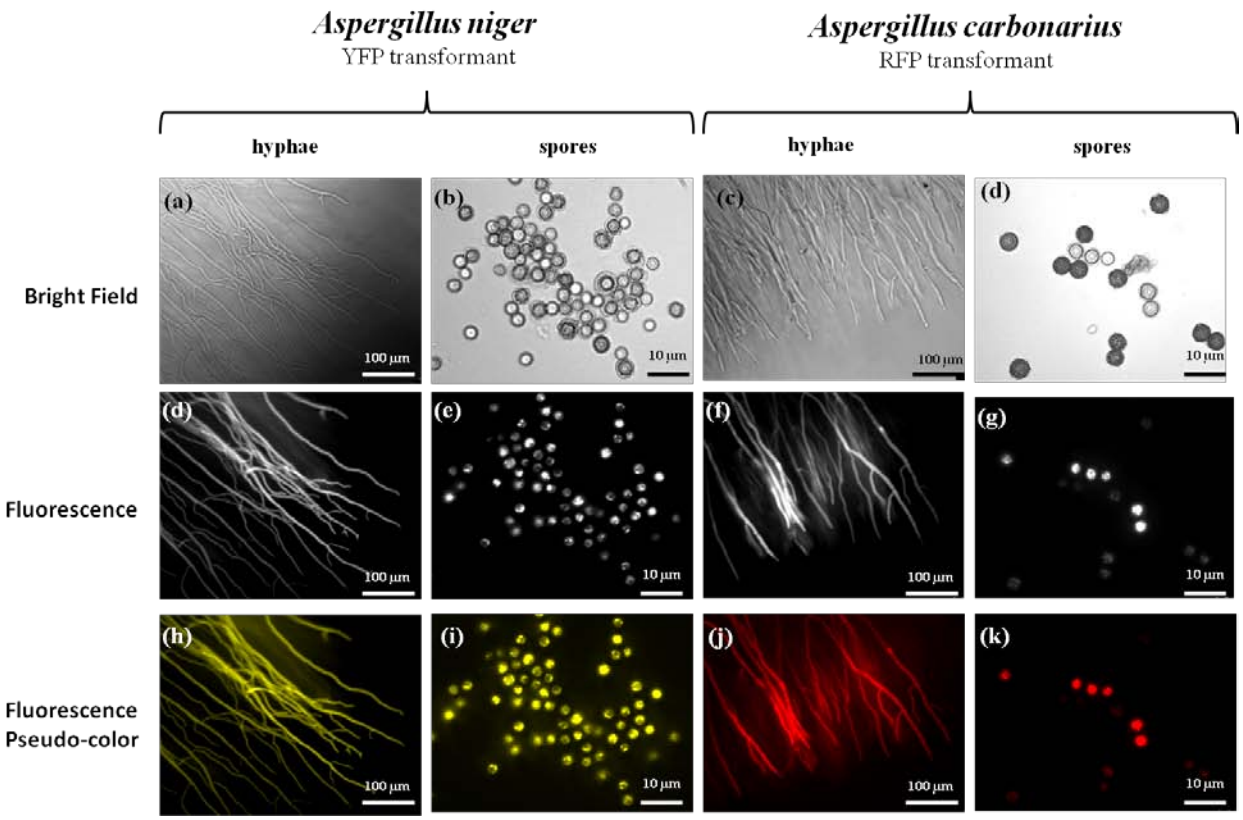

Figure 4.1. Black aspergilli transformants expressing the EYFP (yellow) and $mRFP_1$ (red) fluorescent proteins under the *ToxA* promoter from *Pyrenophora tritici-repentis*. Bright-field (a-d), fluorescent no pseudo-color (d-g), and fluorescent with pseudo-color (h-k) micrographs for *A. niger* var *niger* SRRC 13 and *A. carbonarius* SRRC 2131 fluorescent transformants. Hyphal mats and spore suspension of fungal species were acquired using a Leica DM 6000B fluorescence microscope with a 40x objective for hyphal mats and 100x immersion oil objective for spore suspensions mounted in a coverslip using mounting solution Mowiol. Hyphae and spores fluorescent micrographs for the *A. niger* YFP transformant were acquired using emission 527nm/excitation 514 nm; whereas the *A. carbonarius* mRFP1 transformant images used emission 582nm/excitation 558 nm.

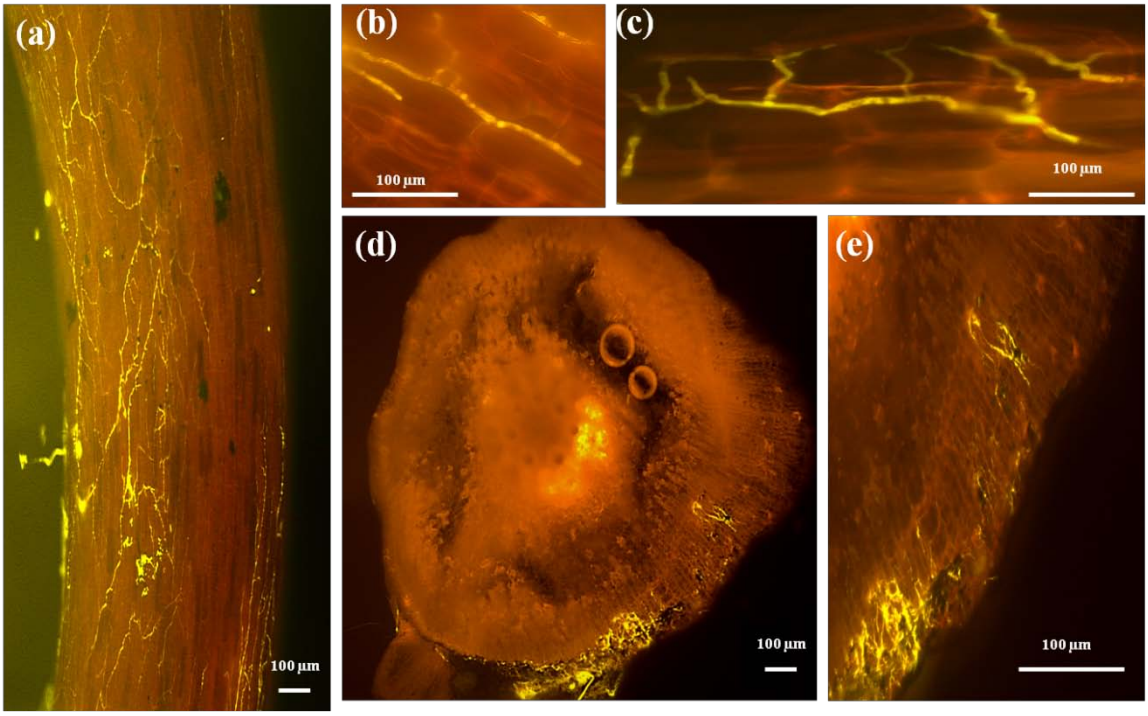

Figure 4.2. Colonization pattern of maize seedlings by the yellow fluorescent *A. niger* strain. (a) External colonization of the root elongation zone by day 3. The zone close to the inoculated seed showed moderate colonization. (b) Longitudinal section showing intercellular hyphae in 3-day-old roots. (c) At 14 days after inoculation, the colonization pattern of *A. niger* showed intra and inter-cellular hyphae. (d) Cross-section of root showing colonization of subepidermal tissue. (e) Higher magnification of root tissue in (d).

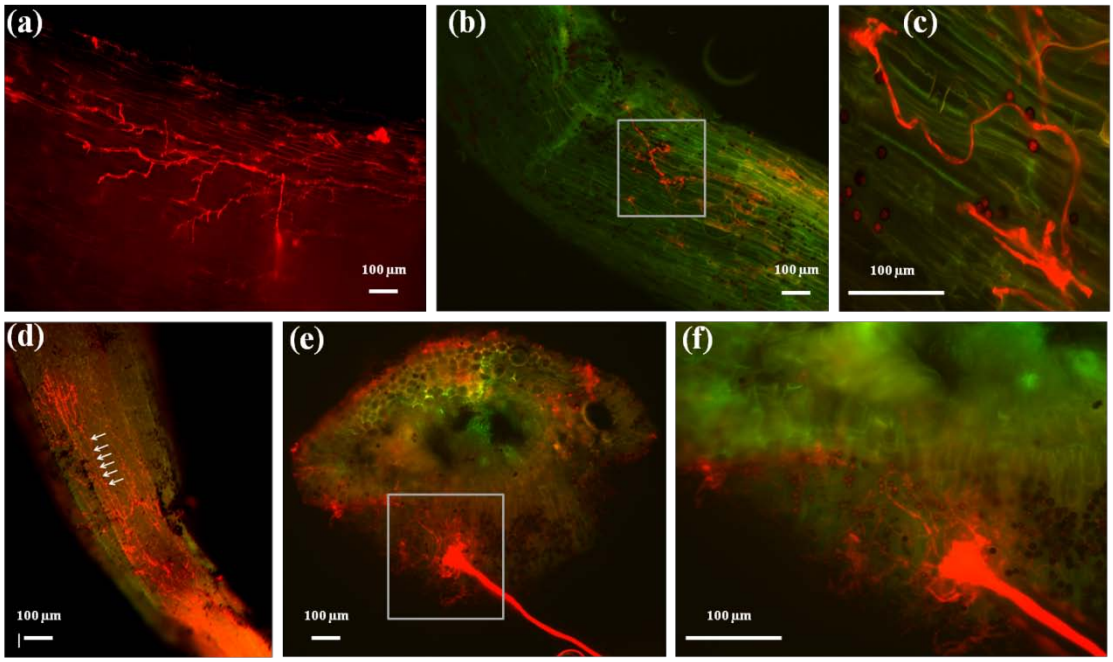

Figure 4.3. *A. carbonarius* fluorescent strain expressing the $mRFP_1$ marker in root tissue. (a) Fungal hyphae (red) growing intercellularly on the epidermal side of maize root by 3 days after inoculation (dai). (b) Elongation zone colonization by hyphae at 7 dai. Hyphae are characterized by both intra- and intercellular apical growth. (c) Higher magnification of (b) where characteristic apical growth is shown. (d) Heavy hyphal growth on maize seedling roots by 14 dai. The fungal growth is massive at both elongation zone and root cap. Arrows indicate hyphal growth on maize root tissue. (e) Cross-section of infected root tissue with development of conidiophores at the epidermal surface. (f) Higher magnificationof (e) showing hyphal mass.

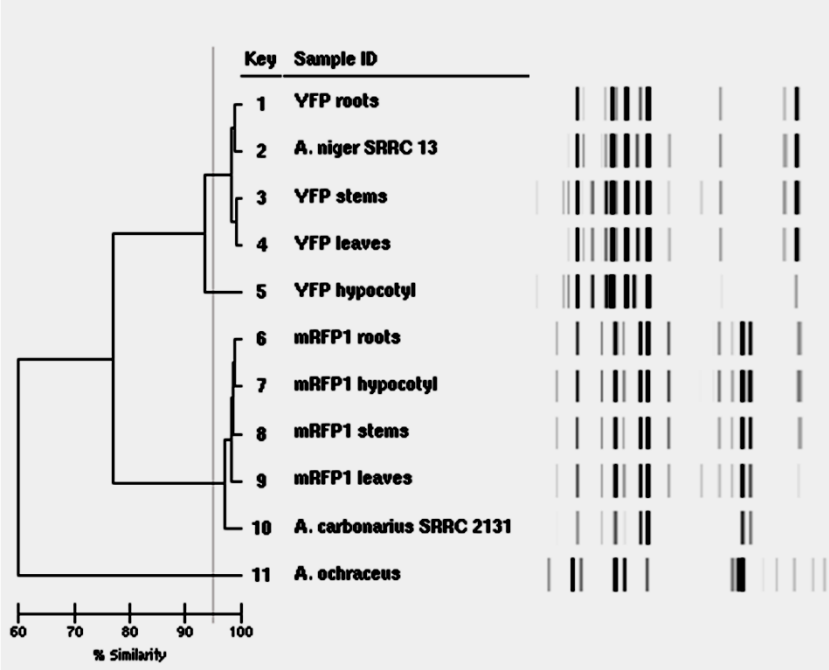

Figure. 4.4. Dendogram and virtual gel images generated by the DiversiLab genotyping system. A rep-PCR analysis was carried out using *A. niger* var *nige*r SRRC 13, *A. carbonarius* SRRC 2131 as morphotypes, and their fluorescent transformants (EYFP and $mRFP_1$) to determine percent similarity between the fungal morphotypes and the re-isolated fungal transformants from maize seedlings. *A. ochraceus* was used as an outgroup for the genotypic analysis. The dendrogram was generated by the Pearson's correlation coefficient algorithm, with a 95% cutoff to indicate high similarity among fungal strains.

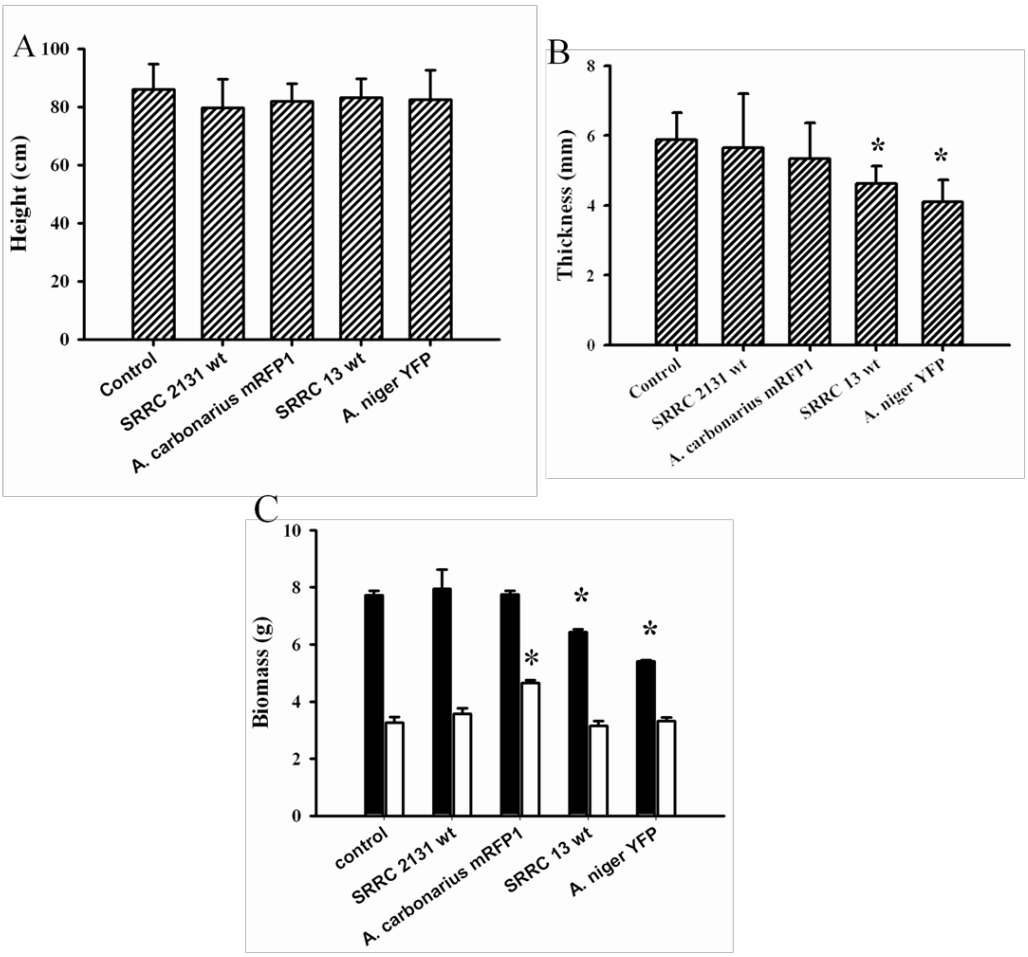

Figure 4.5. Effects of black aspergilli strains and their transformants on colonization of 3-week-old maize seedlings. A) Plant height. B) Stem thickness measured at the first internode. C) Comparison of biomass for 3-week-old maize seedlings grown from uninoculated (control) and inoculated seeds. Solid (black) bars represent biomass for leaf and stem tissue, and open (white) bars represent biomass for root tissue. For all data, an asterisk indicates a significant difference ($P < 0.05$) between the uninfected seedlings and the inoculated seedlings treated with black aspergilli strains. Values are means ± SD with *n*=3 replicates with 10 maize seedlings per treatment.

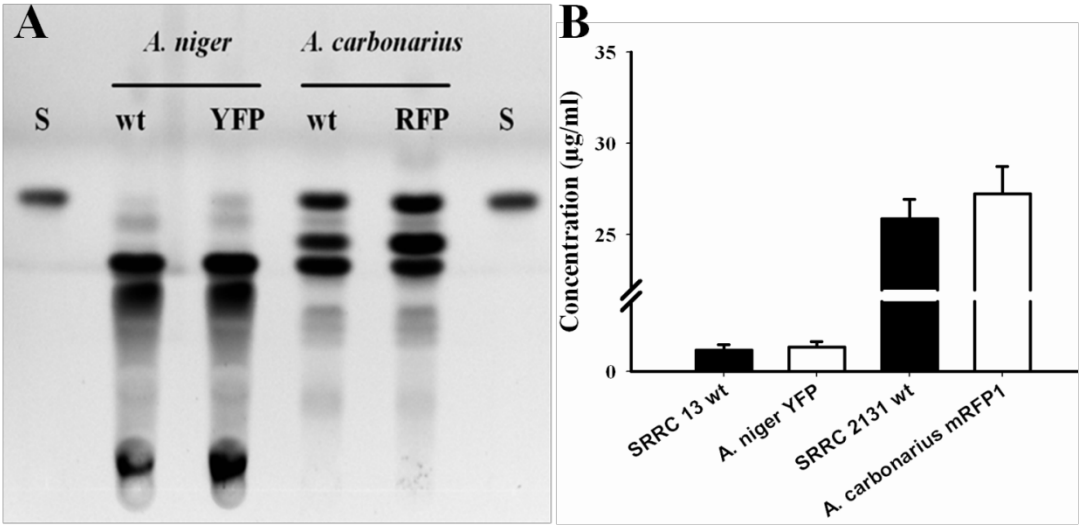

Figure 4.6. Ochratoxin A production on maize seeds by black aspergilli. (A) TLC plate showing results for the wild types (wt) *A. niger* SRRC 13 and *A. carbonarius* SRRC 2131and their fluorescent transformants (yellow, YFP; red, RFP) on corn. All samples were extracted after incubation for 7 days at 28°C using an alkaline process (sodium bicarbonate). Two aliquots (550 ng each) of the ochratoxin A standard (S) were loaded onto silica TLC sheets and developed with a mobile phase of toluene-ethyl acetate-formic acid (50:40:10). (B) OTA production determined by HPLC from wild types and transgenic strains. Values are means ± SD with *n*=3 replicates per strain. The two-tailed t-test indicated that both fluorescent strains did not significantly differ in OTA production from their wild types.

CHAPTER 5

ENDOPHYTIC ASSOCIATIONS BETWEEN BLACK ASPERGILLI AND MAIZE/PEANUT AS PLANT HOSTS[1]

[1]Palencia, E.R., Hinton, D.M., and Bacon, C.W. To be submitted to *Phytopathology*

ABSTRACT

Although fungal endophytes are ubiquitous in natural and cultivated environments, information on their interactions with agronomic crops at the microscopic level and the effects on plant development are scarce. Here, we report on the effects of plant-fungal colonization by 11 isolates of the *Aspergillus* section *Nigri* group, also known as black aspergilli, in two maize (*Zea mays*) cultivars. Our results indicated that fungal isolates belonging to the biseriate clade of the *Aspergillus* section *Nigri* were able to endophytically colonize 3-week old plant seedlings grown from inoculated seeds; whereas species of the uniseriate clade were limited to below-ground plant colonization. In terms of plant growth, the effects of inoculation of maize seedlings with 11 black aspergilli showed a neutral effect on height and stem thickness after 3 weeks. We also report the study of fungal colonization by *A. niger* and *A. carbonarius* in 15-week-old peanut (*Arachis hypogaea*) plants, showing that both *A. niger* wild type and its yellow fluorescent transformant were able to systemically colonize Tifguard and Florida 07 peanut cultivars. However, *A. carbonarius* and its red fluorescent transformant were limited to underground plant tissue colonization in both cultivars. Peanut plants inoculated with *A. niger* and *A. carbonarius* showed a decreased plant biomass in the above-ground tissue, but the root-limited colonization of *A. carbonarius* showed an increased development of root tissue in Tifguard. Our results confirmed the endophytic habit of some black species of the *Aspergillus* genus in two agronomic crops.

INTRODUCTION

Endophytic organisms, including prokaryotes and eukaryotes, colonize internal plant tissues, developing transient symptomless infections without any immediate damage to the plant host (35). Virtually all plant species shelter at least one endophytic organism, with a wide range

of plant-endophyte associations from antagonists to mutualists (33). Unlike their bacterial counterparts that colonize vascular tissue, most fungal endophytes colonize the root host cortex with either inter- or intra-cellular growth patterns (3, 11). Evidence suggests that the extensive colonization by endophytic fungi is a passive process where hyphae grow into the inner surface of the xylem elements thus reaching all plant organs (13).

Fungal species in the *Aspergillus* section *Nigri* group, also known as black aspergilli due to their characteristic melanized conidia, have been associated with opportunistic infections of several cereal grains and other plant hosts (9, 24, 31). Additionally, recent reports indicate that several black aspergilli are isolated from different natural environments as fungal endophytes (29; 42). Black aspergilli endophytic associations have been described as latent infections, because it was believed that the fungus remained dormant as subcuticular hyphae until external conditions were suitable for disease development. However, many of these infections are metabolically active and perennially associated with plants (16).

Definitions for fungal endophytes vary, but all studies, used to demonstrate an endophytic organism, are based on its recovery from surface disinfested plants and seeds. Histological approaches, light or electron microscopy and confocal fluorescent techniques, have been used to demonstrate endophytic life strategies of fungal species. The microscopic analysis of root tissue shows variable colonization patterns by endophytic fungi since both inter- and intracellular hyphae can be visualized (34). Nonetheless, the growth of hyphae in parallel within intercellular spaces of root tissue is considered a confirmatory test of the endophytic habit of fungi, as demonstrated with histological studies on the endophytic fungus *Fusarium verticillioides in maize* (4). Recently the use of advanced microscopy tools, including live-cell imaging, has improved the study of fungal endophytes (21, 22). One of the advantages of live-cell imaging

over *in vitro* approaches is that the former provides more detail about the dynamics of plant-fungal interactions without any apparent damage to the plant tissue. Also, the combination of autofluorescent proteins and live-cell imaging systems allows a clear visualization of plant-fungal associations based on the optical sectioning of plant tissue by the laser component (19). Furthermore, the results from live-cell imaging can be used as confirmatory tests for other microscopy techniques (8).

What is the advantage for plant hosts to harbor a number of endophytic fungi? Although there can be several negative effects of plant-fungal interactions, the positive effects provided by these systemic and often asymptomatic interactions include protection against plant pathogens and suppression of herbivores in cultivated grass, the activation of the stress response system, promotion of plant growth and yield, drought tolerance, and heavy metal tolerance (10, 15, 32, 37, 39). The common use of chemical pesticides in agricultural fields to control plant diseases, has led to an increased public concern because of its potential environmental consequences and the development of resistance to agrichemicals by plant pathogen populations. As a result, scientists have recognized the use of endophytic fungi as biocontrol agents based on the properties of these fungi to confer benefits to the plant, such as protection against pathogens. Biocontrol, as defined by Pal and McSpadden (28), is an introduced or native living organism that is deliberately used to suppress plant pathogens activities. Recently, *Aureobasidium pullulans,* an endophytic fungus isolated in the Colombian Highlands, has shown a great potential to control *Ralstonia solanacearum*, the causal agent of bacterial wilt in tomato (25). In greenhouse studies, the endophytic fungus in tall fescue *Neotyphodium coenophialum* was effectively used to control the parasitic nematode *Pratylenchus scribneri* by a mechanism that involves the production of nematitoxic compounds such as alkaloids and polyphenolics (2).

The main objective of this study was to provide evidence, based on anatomical and histological studies, for endophytic relationships among species of the black aspergillus group, with maize and peanut cultivars.

MATERIALS AND METHODS

Fungi and plant material. Uniseriate and biseriate fungal species used in this study are listed in Table 5.1. Black aspergilli were provided by Maren Klich (Southern Regional Research Center, ARS-USDA, New Orleans, LA), and stored as frozen stock conidial suspensions in sterile 0.01% Tween 80, 15% glycerol solution at -80 °C. Two maize cultivars Pioneer 3245 and 3140 (Pioneer HiBred, Johnston, IA) were utilized due to their different levels of resistance to *F. verticillioides* colonization. The two peanut cultivars used in this study were provided by Corley Holbrook, USDA-ARS, Tifton, GA: 'Tifguard', a runner-type peanut (*Arachis hypogaea* L. subsp. *hypogaea* var. *hypogaea*), which is resistant to root-knot nematode (*Meloidogyne arenaeria* race 1), and *Tomato spotted wilt virus*, and 'Florida 07', a medium to late runner market type peanut, which is susceptible to root-knot nematode, moderate resistance to white mold caused by *Sclerotium rolfsii*, and tolerance to early and late leaf spot caused by *Cercospora arachidicola* and *Cercosporidium personatum*, respectively.

Fluorescent transformants of black aspergilli. *Aspergillus niger* and *A. carbonarius* were transformed using a protoplast-mediated transformation approach described in Chapter 4. The plasmids pCA45, containing the enhanced yellow fluorescent marker (EYFP), and pCA51, carrying the monomeric red fluorescent marker ($mRPF_1$), were under the control of the *ToxA* promoter from *Pyrenosphora tritici-repentis* (1). Plasmids pCA45 and pCA51 contained the hygromycin B phosphotransferase marker, *hph*, as the selective gene. Putative transformants were selected using two final hygromycin B (Roche Diagnostics, Indianapolis, IN)

concentrations: 300 µg/ml for *A. niger* yellow fluorescent transformants, and 150 µg/ml for *A. carbonarius.* Mitotic stability was tested on potato dextrose agar (PDA) dishes without hygromycin B for nine generations. Expression of fluorescent markers, was visualized utilizing a Leica DM6000 B fluorescent microscope (Leica, Wetzlar, Germany) as described in Chapter 4.

Colonization of maize and peanut cultivars by black-spored aspergilli. Fresh conidia were obtained by inoculating 10 ml PDA slants with a 10-µl loop from frozen stock conidial suspensions. PDA slants were incubated at 25°C for 7 days in the dark. The inoculum (mycelium and conidia) was harvested by flooding the PDA slants with 10 ml sterile 0.01% Tween 80 and filtered through cheesecloth to remove fungal mycelium. For all fungal species, the concentrated conidial suspensions were diluted to 1 x 10^5 cfu/ml with a 0.01% Tween solution and used to inoculate maize kernels. Controls consisted of maize kernels treated with 0.01% Tween solution without inoculum. Maize seeds were surface disinfested using 40 ml commercial bleach (6.15% sodium hypochlorite) for 10 min. in a sterile propylene specimen container (Fisher Scientific, Pittsburg, PA), rinsed three times with sterile water, and imbibed for 4 hours at room temperature in sterile water. A heat shock treatment was performed on maize kernels to remove internal contamination of seed-borne microorganisms. The kernels were subjected to a heat shock treatment by placing them in a 60°C water bath for 5 min. (5). After sterilization, viability was tested by placing kernels on growing medium (per liter :12 g agar, 10 g glucose, 0.1 g peptone, and 0.1 g yeast extract) for 2 days at 25° C. The kernels were inoculated by placing them in a 100 mm petri dish containing 20 ml of spore suspension (1 x 10^5 spores/ml) and incubated at room temperature for 16 hours. The negative control consisted of kernels treated with sterile water and no other treatment instead of the spore suspension treatments. After inoculation, kernels were placed on sterile filter paper and dried for 30 min under aseptic conditions. Three

replicates of 10 kernels per pot were grown in 12.5-cm pots containing commercial potting mix (40-50% Canadian sphagnum peat moss, processed pine bark, horticultural vermiculite, horticultural perlite, limestone; Farfard, Agawam, MA) in a plant growth room at 26 °C under 16 hours of light (cool-white, high output fluorescent tubes, average 250 µmol m^{-2} s^{-1}): 8 hours of darkness at 32 °C:29 °C (light:dark).

Black aspergilli symptomless colonization in maize seedlings was monitored by the method described earlier (3). In brief, plant seedlings were harvested after 21 days of growth. Plant tissues from leaves, stems and primary roots were sectioned into 3-5 cm pieces and placed in sterile propylene specimen container (Fisher Scientific). Plant tissues were rinsed with sterile water in a rotary shaker for 3 min, and water was aseptically drained in a hood. Surface disinfestation of plant tissue was performed by using a 1% Chloramine T salt hydrate solution (Sigma-Aldrich, St. Louis, MO) in a rotary shaker for 30 min. The ssolution was removed and plant material was rinsed twice in sterile water for 3 min each time. Sterile tissues were aseptically placed in a sterile paper towel for 10 min. The edges (0.5-1 cm) of root, stem, and leaf tissues were removed and discarded using sterile razor blades. Finally, plant tissue (five sections) were placed on a semi-selective dichloran rose bengal chloramphenicol (DRBC) culture medium, amended with 15% glycerol. Plant sections on DRBC plates were incubated in the dark at 25°C for 4 days.

For peanut, two successive greenhouse experiments were conducted to determine the endophytic infections and disease potential by black aspergilli on peanuts. All peanut seeds were surface disinfested for 3 min using commercial bleach (sodium hypochlorite 6.19%, Clorox®) and rinsed three times with sterile distilled water. Seeds were dried aseptically at room temperature and grouped into five treatments (5 replicates per treatment). The individual

treatments were as follows: T1, *A. carbonarius* SRRC 2131 wild type; T2, *A. carbonarius* red fluorescent transformant; T3, *A. niger* var *niger* SRRC 13; T4, *A. niger* yellow fluorescent transformant, and T5 the uninoculated soil as control. For inoculum preparation, each fungal strain was grown on PDA slants for 7 days at 25°C and harvested with 8 ml of a 0.01% Tween solution. Plants were grown in a sterile soil mix in 20-cm pots containing a 50:50 mix of professional potting mix (Fafard) and sandy soil. The top 5 cm of soil was inoculated and mixed with 20 ml of the spore suspension (final concentration 1 x 10^5 spores/ml) for each treatment. Three days after inoculation, the surface-disinfested peanut seeds from each cultivar were planted in the experimental soil and grown at 30-35° C for 15 weeks under green house conditions. Pots were watered as needed and 2 weeks after planting, peanut seedlings were fertilized with 250 ml of Miracle-Gro® (Scotts, Maryville, OH) and water-soluble tomato plant food (18% nitrogen, 18% phosphate, and 21% soluble potassium) After 15 weeks, plants were harvested and subjected to a fungal colonization assay as described above with modifications. Briefly, plants were separated into roots, stems, petioles and leaflets, which were sectioned into 2-3-cm segments. Plant segments were surface disinfested using Chloramine-T (Sigma-Aldrich) for 30 min and dried aseptically for 10 min in a paper towel. Sections were trimmed down at the edges with a sterile blade and placed on the semi-selective DRBC medium as indicated above. DRBC dishes were incubated at 25 °C for 7 days.

Microscopy. Fluorescent analysis of black aspergilli colonization in plant tissue was performed by utilizing a Leica DM6000 B fluorescent system (Leica Microsystems,). For fluorescence imaging, an external Leica EL6000 light source with a mercury metal halide bulb was attached to the microscope system. The expression EYFP and $mRFP_1$ was visualized using the following conditions: for EYFP λ_{exc} 514nm/λ_{emm} 527nm , and for mRFP1 λ_{exc} 558nm/ λ_{emm}

582nm. Fluorescent images were acquired with a high resolution digital camera, Hamamatsu orca-ER (Hamamatsu Photonics K.K., Hamamatsu City, Japan), whereas bright field images from plant sections were acquired using a digital microscope camera Leica DFC 450 (Leica) attached to the microscope system. Micrographs were recorded as Z-stack images (a multifocus image) using the LAS Stackon v 1.0.B13 software (Leica), and the Leica Application Suite v 3.7.0.

Plant sections were observed under fluorescent microscopy for 2 weeks to monitor fungal colonization patterns by the transformed strains of *A. niger* and *A. carbonarius*. For each time point, 5 inoculated and 5 uninoculated roots were examined under the microscope in two consecutive experiments. Longitudinal and cross sections were obtained by methods described earlier (13; 21). For fluorescent images, plant sections were flooded with 25 µl of the anti-fade medium Mowiol® (Calbiochem, La Jolla, CA) and observed under the microscope using the conditions described above.

Statistical analyses. GraphPad Instat software version 3.05 (GraphPad, San Diego, CA) was used for the statistical analyses. Plant seedling growth variables were analyzed by one-way ANOVA followed byTukey's test (P= 0.05). Graphs were generated using SigmaPlot software version 9.0 (Systat Software, Inc., Chicago, IL).

RESULTS

Endophytic colonization of maize and peanut. Inoculated maize seedlings were harvested 3 weeks after planting to study the ability of black aspergilli to endophytically colonize two maize cultivars. Endophytic colonization of black aspergilli in plant tissue was monitored using fluorescent genetic transformants (Figure 5.1). The characteristic intercellular hyphal growth was visualized 3 days after inoculation in both differential and elongation zones of roots.

Maize seeds were inoculated with black *Aspergillus* spore suspensions at 1x 10^5 cfu/ml, from eleven black aspergilli isolates (Table 5.1). The black aspergilli used in this experiment included two species of the uniseriate clade, *A. japonicus* SRRC 325, and *A. aculeatus* SRRC 168. Also four *A. carbonarius* strains, five strains within the *A. niger* group. Fungal isolation frequency was calculated as the number of plant tissue segments with fungal growth over the total of segments (*n*=5) in the DRBC medium. The frequencies of isolation of three independent experiments showed that the uniseriate black aspergilli were poor colonizers of maize cultivars (Table 5.2). Pioneer 33K81 showed resistance to endophytic colonization by the uniseriate black aspergilli. These strains were not re-isolated from surface-disinfested leaf, stems, and root tissues (Table 5.2); whereas in Pioneer 3140, both uniseriate species showed weak systemic endophytic associations. The isolation frequency ranged from 6 to 33% in root tissue, and 6 to 13.3% in stems. Neither Pioneer 33K81 nor Pioneer 3140 were subjected to endophytic colonization by the uniseriate black aspergilli as shown by the low percentage frequency of colonization of the fungal strains (0% for all leaf tissue).

For the biseriate species, the frequencies of isolation varied within strains and for each maize cultivar. Three *A. carbonarius* strains were strong systemic colonizers of maize tissue, including SRRC 2131, SRRC 370, and SRRC 15 (Table 5.2). These three *A. carbonarius* strains, showed high isolation frequencies line Pioneer 33K81 (100% for all plant organs). Although the same *A. carbonarius* SRRC 2131, SRRC 370, and SRRC 16 strains were also able to systemically colonize Pioneer 3140, they showed slightly lower frequency of isolation than from Pioneer 33k81 (Table 5.2). Interestingly, the *A. carbonarius* SRRC 369 strain showed a different colonization pattern than the other *A. carbonarius* strains used in this experiment. *A. carbonarius* SRRC 369 was not isolated from leaf tissue in Pioneer 33K81 (Table 5.2); whereas this same

strain poorly colonized Pioneer 3140 tissue, showing lower frequencies of isolation (20% for leaf, 13.3% for stem, and 13.3% for root tissue) in this maize cultivar.

Three *A. niger* strains, SRRC 359, SRRC 13, and SRRC 60, were able to systemically colonize Pioneer 33K81, with high percentage frequencies of isolation (100% for leaf, stem, and root tissue). Only two of these *A. niger* strains, SRRC 13 and SRRC 60, remained as systemic colonizers of the Pioneer 3140. Although *A. niger* SRRC 60 showed lower frequencies of colonization (80% for leaf and stem tissue, and 93% for root tissue (Table 5.2), it was demonstrated that this strain was also a systemic endophyte of Pioneer 3140. *A. niger* SRRC 359 was a poor systemic endophyte for Pioneer 3140 (Table 5.2), showing low isolation frequencies.

Two strains (*A. niger* SRRC 1158 and the closely related *A. foetidus* SRRC 321; Table 5.1), showed low isolation frequencies in both maize cultivars (Table 5.2). Slight differences were observed for these two species. In Pioneer 33K81, these black aspergilli were limited to vigorous colonization of the root tissue (isolation frequency 100%), but they were poorly recovered from upper parts of this maize cultivar. However, the same two species were recovered from leaf tissue in Pioneer 3140, reaching the leaf and stem tissues. Their low isolation frequencies ranged from 6.6 to 26.6% for leaf tissue, and 13.3 to 40% for stem tissue, perhaps indicating that these two species slowly develop a systemic endophytic association with this plant cultivar.

In peanut plants two successive greenhouse experiments were performed to determine the distribution of *A. niger* SRRC 2131and *A. carbonarius* SRRC 2131 in 15-week old plants. Also, the isolation frequency of the two fluorescent transformants of *A. niger* SRRC 13 (yellow) and *A. carbonarius* SRRC 2131 (red) was determined. The greenhouse experiments showed that both *A. niger* and its fluorescent transformant were strong endophytic colonizers of Florida 07 and

Tifguard cultivars (Table 5.3; Figures 5.2 and 5.3). The isolation frequencies for *A. niger* and its transformants were high (100% for all plant organs) in both peanut cultivars. The strains grew vigorously from the plant segments onto the selective DRBC medium (Figure 5.2 and 5.3) after 5 days of incubation.

In contrast, *A. carbonarius* and its fluorescent transformant were limited to root colonization, with low isolation frequencies (Table 5.3). The endophytic association of *A. carbonarius* and its transformant with root tissue in both peanut cultivars is clear (isolation frequency 100% for Florida 07, and 90% in Tifguard; Table 5.3). *A. carbonarius* SRRC 2131 was a weak endophytic colonizer of above-ground tissue, only reaching stem tissue (isolation frequency 90%) in Florida 07r.

Impacts of black aspergilli endophytic colonization on plant growth. Maize seedlings from three successive growth room experiments were also used to determine the impact of black aspergilli colonization on plant growth (Table 5.4). Two *A. carbonarius* strains SRRC 15 and SRRC 369 significantly increased the height in Pioneer 3140 ($P< 0.05$) and Pioneer 33K81($P<0.01$), indicating that maize seedlings grown from inoculated seeds with these two strains showed a positive impact on height. However, only *A. carbonarius* SRRC 15 significantly increased the stem diameter in Pioneer 3140. The impact of *A. niger* group strains on maize seedling development ranged from neutral (SRRC 359, SRRC 60, SRRC 1158, and SRRC 321; $P > 0.05$) to negative impact (SRRC 13; $P<0.01$) (Figure 5.4).

Two black aspergilli (*A. niger* SRRC 13 and *A. carbonarius* SRRC 2131) and their fluorescent transformants were used to colonize two peanut cultivars. After 15 weeks post-inoculation, the impact of fungal endophytic colonization on plant growth was measured (Table 5.5 and Figure 5.5). The two black *Aspergillus* species and their fluorescent transformants had a

neutral effect on above ground plant biomass for both peanut cultivars ($P > 0.05$). For the root tissue, the effect on plant growth was also neutral ($P>0.05$).

DISCUSSION

Black aspergilli are a closely related group of filamentous fungi that infect many plants including onion, garlic, maize, peanut, and grape (6, 23, 29, 38). Initially black aspergilli infections were known as latent or dormant. Species in the genus *Aspergilus* are known for causing deleterious effects in animals and some important crops, but little is known about the endophytic habits of species in this genus. Evidence has demonstrated that some species in the *Aspergillus* section *Nigri* can colonize plant tissue as metabolically active endophytes (30, 40, 42). Although some research has indicated that species within the *Aspergillus* section *Nigri* are fungal endophytes (42), to our knowledge this is the first time that morphologically and genetically identified black aspergilli (30) have been used to demonstrate their ability to colonize maize and peanut plant tissue. Compounding the phytopathology of these fungi, they are known to produce secondary metabolites that have been associated with protection against other plant pathogens and herbivores (2, 27).

In the present study we performed histological studies to visually demonstrate the endophytic habit of black aspergilli as inconspicuous intercellular infections. Isolation of black aspergilli from surface disinfested plant tissue and demonstration of this habit with microscopic examination as symptomless infections within intercellular spaces are two confirmatory tests to determine the endophytic fungi (4, 41). Intercellular spaces are free spaces between plant cells located in the cortex of the root tissue that form the apoplasm (18). This tissue provides the fungus with an environment from which it can obtain nutrients and in which there is less competition than in other sites on or inplant tissue. Fungal endophytes do not form specialized

structures to obtain nutrients, but it has been hypothesized that hydrolytic enzymes and specialized hyphae of some endophytes might be involved in acquiring nutrients from the host (17, 18).

When fungal distribution of black aspergilli on 3-week old maize seedlings was assessed, three *A. carbonarius* strains were able to systemically colonize the plant hosts; whereas one *A. carbonarius* strain was limited to poorly colonize root tissue. This difference in colonization among the strains of the same species has also been documented in the endophytic fungus *Fusarium oxysporum* in barley, where avirulent strains were located on the surface of root tissue, but virulent strains rapidly colonized the plant host (7). The variability of the plant-endophytic fungi association is dependent on the physiology and genotype of the plant host, and also the genotype and virulence of the fungal component.

Inoculated maize seedlings were harvested 3 weeks after planting to determine the impact of black aspergilli endophytic association with maize seedlings. It was observed that two *A. carbonarius* strains, SRRC 15 and SRRC 369, improved plant height in two maize cultivars; whereas two other *A. carbonarius* strains, SRRC 2131 and SRRC 370, showed no impact on maize plant height. Neutral and negative impacts of endophytic fungi are not rare in nature. We only measured one component of plant development. Definite conclusions can only be reached when additional measurements are made including yield, photosynthesis, rate of plant development and disease expression. Further, Schulz and Boyle (36) hypothesized that instead of a lack of an interaction, neutral endophytic associations are balanced antagonisms between the fungus and the plant host. Such balanced associations permits fungal growth in the plant host, but in an unbalanced state there is a ‘tug of war’.

Although the mechanisms leading to promotion of plant growth are still unclear, evidence suggests that directly or indirectly, endophytic fungi use varous approaches to promote plant growth, including, increased absorption of phosphorus and nitrogen by the plant roots (39), the production of phytohormones (26), and production of plant growth promoting secondary metabolites (14)

In conclusion, we demonstrated that species of the *Aspergillus* section *Nigri* are able to develop endophytic associations with maize and peanut hosts. Although systemic colonization of plant tissue was variable and depended on the plant species and cultivar, some *A. carbonarius* strains were strong endophytic colonizers of maize seedlings, which were found in all organs of the plant host. In contrast, *A. niger* was a strong colonizer of peanut hosts with high isolation frequency from all plant organs.

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Table 5.1. Black aspergilli strains used in this study.

Number [a]	Species	Other accession numbers
SRRC 325	*A. japonicus*	NRRL 1782 [b]
SRRC 168	*A. aculeatus*	NRRL 5094
SRRC 2131	*A. carbonarius*	FRR 369 [c]
SRRC 370	*A. carbonarius*	NRRL 368
SRRC 15	*A. carbonarius*	---
SRRC 369	*A. carbonarius*	NRRL 346
SRRC 359	*A. niger*	NRRL 3112
SRRC 60	*A. niger*	NRRL 3
SRRC 1158	*A. niger*	---
SRRC 321	*A. foetidus*	NRRL 341

[a] SRRC, obtained from culture collection of the USDA-ARS, Southern Regional Research Center, New Orleans, LA

[b] NRRL= Northern Regional Research Laboratory collection

[c] FRR= Food Research in North Ryde, New South Wales, Australia

Table 5.2. Recovery of black aspergilli from two maize cultivars after infiltration of seeds with fungal conidia[a].

Cultivar	Treatment	Fungal Isolation(%)		
		Leaf	Stem	Root
Pioneer 3140	*A. japonicus* SRRC 325	0	13.3	33.3
	A. aculeatus SRRC 168	0	6.66	6.66
	A. carbonarius SRRC 2131	93.3	93.3	100
	A. carbonarius SRRC 370	93.3	93.3	93.3
	A. carbonarius SRRC 15	86.6	93.3	100
	A. carbonarius SRRC 369	20	13.3	13.3
	A. niger SRRC 359	33.3	33.3	60
	A. niger SRRC 13	100	100	100
	A. niger SRRC 60	80	80	93
	A. niger SRRC 1158	26.6	40	100
	A. foetidus SRRC 321	6.66	13.3	66.6
Pioneer 33K81	*A. japonicus* SRRC 325	0	0	0
	A. aculeatus SRRC 168	0	0	0
	A. carbonarius SRRC 2131	100	100	100
	A. carbonarius SRRC 370	100	100	100
	A. carbonarius SRRC 15	100	100	100
	A. carbonarius SRRC 369	0	13.3	13.3
	A. niger SRRC 359	100	100	100
	A. niger SRRC 13	100	100	100
	A. niger SRRC 60	100	100	100
	A. niger SRRC 1158	0	13.3	100
	A. foetidus SRRC 321	0	0	100

[a]Values are based on five plant tissue segements plated in three replicons in three growth room experiments.

Table 5.3. Recovery of black aspergilli from two peanut cultivars after infiltration of seeds with fungal conidia[a].

Cultivar	Treatment	Fungal isolation (%)			
		leaflet	petiole	stem	root
Tifguard	Control	0	0	0	10
	A. niger wt [a]	100	100	100	100
	A. niger YFP transformant	100	100	100	100
	A. carbonarius wt [a]	0	0	20	90
	A. carbonarius mRFP1 transformant	0	0	0	90
Florida 07	Control	0	0	0	10
	A. niger wt [a]	100	100	100	100
	A. niger YFP transformant	100	100	100	100
	A. carbonarius wt [a]	0	0	90	100
	A. carbonarius mRFP1 transformant	0	0	0	100

[a]Values based on five plant tissue segments plated in three replications in two greenhouse experiments.

Table 5.4. Effects of black aspergilli on Pioneer 3140 maize growth.

Treatment [a]	Experiment A		Experiment B		ExperimentC	
	Height (cm)	ST[b] (mm)	Height (cm)	ST (mm)	Height (cm)	ST (mm)
Control	71.162 ±5.316	4.087 ± 0.551	72.489 ±5.065	3.842 ±0.430	71.063 ± 6.738	4.108 ±0.426
A. japonicus SRRC 325	67.900 ±4.068	4.548 ±0.228	75.371 ± 3.621	4.533 ± 0.856	69.500 ±4.093	4.453 ±0.154
A. aculeatus SRRC 168	69.45 ±13.618	4.285 ±0.714	68.987 ±5.206	5.286 ±0.286	71.300 ±6.258	4.277 ±0.373
A. carbonarius SRRC 2131	76.000 ±4.663	4.009 ±0.762	78.800 ±1.395	3.736 ±0.705	75.825 ±8.928	4.282 0.378
A. carbonarius SRRC 370	66.383 ±12.477	4.608 ±0.525	68.550 ±2.177	4.160 ±0.134	76.425 ±8.681	3.551 ±0.996
A. carbonarius SRRC 15	79.425 ±11.475	5.462 ±0.524	78.786 ±8.143	6.063 ±0.428	85.720 ±7.053	5.410 3.188
A. carbonarius SRRC 369	70.067 ±5.532	3.133 ±0.656	67.544 ±7.908	3.086 ±0.373	69.400 ±6.394	3.749 ±0.272
A. niger SRRC 359	66.425 ±5.894	4.364 ±0.965	60.038 ±5.170	4.619 ±0.703	67.875 ±8.811	4.784 ±1.823
A. niger SRRC 13	57.475 ±13.657	4.016 ±0.938	67.057 ±12.556	4.720 ±0.527	60.722 ±23.957	4.266 ±0.299
A. niger SRRC 60	71.050 ±12.737	4.275 ±1.011	81.140 ±4.451	4.410 ±0.153	78.000 ±10.280	4.950 ±0.617
A. niger SRRC 1158	69.325 ±5.526	3.780 ±0.667	69.088 ±8.458	4.583 ±1.218	67.188 ±11.932	4.127 ±0.576
A. foetidus SRRC 321	77.460 ±4.352	4.064 ±0.734	78.100 ±11.831	3.943 ±1.558	80.017 13.378	3.510 1.613

[a] Inoculated and uninoculated plants from three successive growth room experiments were harvested after 21. Measurements indicate the means ± standard deviations. Three replicates with 10 plants per pot.

[b] ST = stem thickness.

Table 5.5. Effects of black aspergilli on Pioneer 33K81 maize growth.

Treatment [a]	Experiment A		Experiment B		Experiment C	
	Height (cm)	ST [b] (mm)	Height (cm)	ST (mm)	Height (cm)	ST (mm)
Control	70.544 ±7.350	4.010 ±1.130	75.289 ±8.102	4.284 ±0.950	65.922 ±7.180	4.182 ±0.592
A. japonicus SRRC 325	73.425 ±10.092	4.809 ±0.529	75.063 ±8.352	4.442 ±0.281	67.688 ±22.340	4.566 ±0.269
A. aculeatus SRRC 168	66.080 ±8.768	3.724 ±0.709	67.950 ±50.608	4.080 ±0.218	71.533 ±12.711	8.163 ±1.009
A. carbonarius SRRC 2131	67.533 ±10.812	3.802 ±1.927	72.511 ±3.803	4.990 ±0.566	63.486 ±8.146	3.854 ±0.76
A. carbonarius SRRC 370	66.388 ±5.163	4.501 ±0.704	69.211 ±8.872	4.310 ±1.560	79.771 ±9.417	3.486 ±0.940
A. carbonarius SRRC 15	73.060 ±6.985	4.358 ±0.654	75.970 ±10.187	3.943 ±1.067	62.920 ±4.230	3.775 ±0.934
A. carbonarius SRRC 369	75.520 ±5.051	3.896 ±0.800	83.578 ±4.542	3.182 ±0.825	79.900 ±9.539	4.142 ±0.494
A. niger SRRC 359	69.978 ±6.002	4.712 ±2.825	64.989 ±7.139	3.511 ±0.889	70.880 ±5.885	3.839 ±0.823
A. niger SRRC 13	69.844 ±7.079	9.327 ±12.407	68.667 ±4.026	4.297 ±1.020	71.656 ±5.936	5.444 ±0.875
A. niger SRRC 60	64.460 ±8.714	4.212 ±4.212	67.230 ±9.869	4.191 ±1.018	62.956 ±5.699	4.243 ±1.105
A. niger SRRC 1158	63.310 ±14.457	3.603 ±0.779	68.750 ±13.795	4.304 ±0.270	72.189 ±10.294	3.200 ±0.687
A. foetidus SRRC 321	72.567 ±15.013	4.253 ±1.973	78.878 ±5.266	3.834 ±0.730	70.367 ±8.966	3.660 ±0.461

[a] Inoculated and uninoculated plants from three successive growth room experiments were harvested after 21 days. Measurements indicate means ± standard deviations. Three replicates with 10 plants per pot

[b] ST = stem thickness

Table 5.6. Effects of black aspergilli on peanut plant growth (dry weight).

Cultivar	Treatment [a]	Experiment A		Experiment B	
		Aerial (g)	Roots (g)	Aerial (g)	Roots (g)
Tifguard	Control	2.672 ±0.371	1.128 ±0.341	2.572 ±0.973	3.386 ±1.126
	A. carbonarius SRRC 2131	2.128 ±0.631	1.106 ±0.479	1.802 ±1.257	4.566 ±1.445
	A. carbonarius mRFP1	2.416 ±0.802	1.902 ±0.448	2.360 ±0.819	2.132 ±0.437
	A. niger SRRC 13	2.162 ±0.342	0.936 ±0.194	1.588 ±0.866	3.870 ±0.669
	A. niger YFP	1.802 ±0.651	0.908 ±0.333	2.008 ±1.580	1.580 ±0.638
Florida 07	Control	2.148 ±0.571	2.096 ±0.654	2.462 ±0.978	3.684 ±0.814
	A. carbonarius SRRC 2131	1.948 ±0.532	1.442 ±0.332	2.534 ±1.923	3.162 ±1.087
	A. carbonarius mRFP1	2.288 ±0.445	2.046 ±0.760	2.538 ±0.641	4.766 ±0.833
	A. niger SRRC 13	2.366 ±0.323	1.728 ±0.366	2.312 ±0.723	4.088 ±0.367
	A. niger YFP	2.230 ±0.615	2.136 ±0.343	2.162 ±0.802	2.114 ±0.608

[a] Inoculated and uninoculated plants from two successive greenhouse experiments were harvested after 15 weeks. Measurement indicate the means ± standard deviations Six replicates.

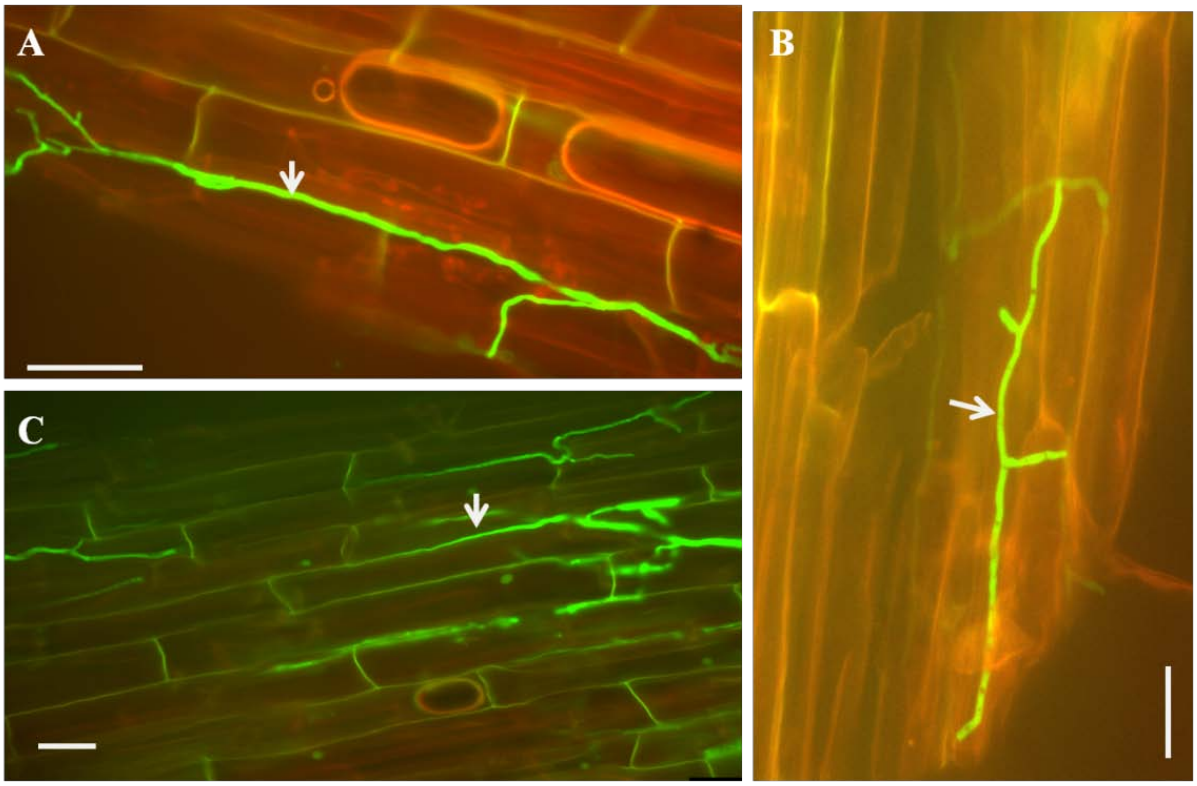

Figure 5.1. Endophytic colonization by *A. niger* yellow fluorescent transformant (green) in maize root tissue. The characteristic hyphal growth in the intercellular spaces of *A. niger* in root tissue after 3 days A) in the differentiation zone and in the B) in the elongation zone. C) Inter- and intracellular growth of *A. niger* fluorescent transformant in the elongation zone 7 days after inoculation. Bars indicate 50 µm . Arrows indicate hyphae growing intercellularly in root tissue.

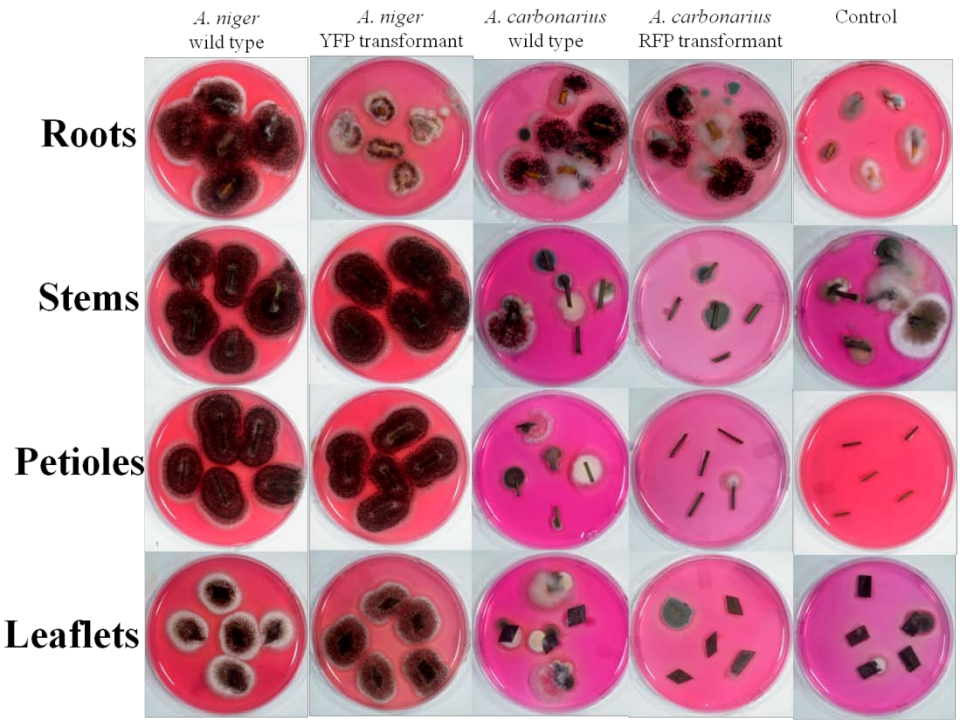

Figure 5.2. Black aspergilli isolated from 15-week-old above and below-ground tissues from peanut cultivar Tifguard.

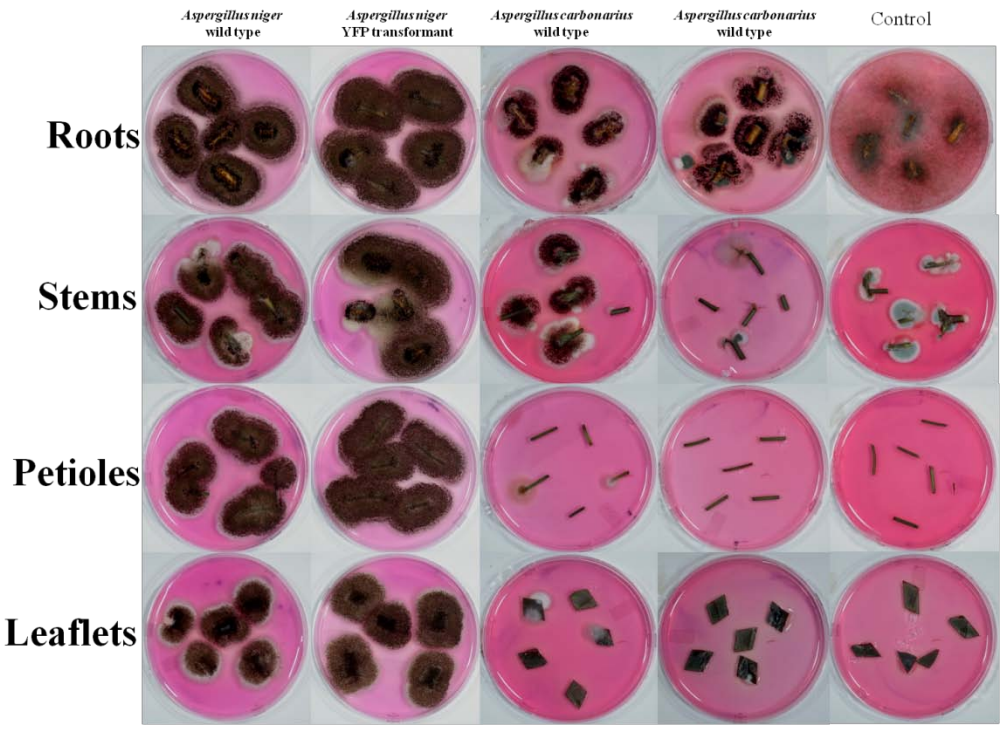

Figure 5.3. Black aspergilli isolated from 15-week-old above and below-ground tissues from peanut cultivar Florida 07.

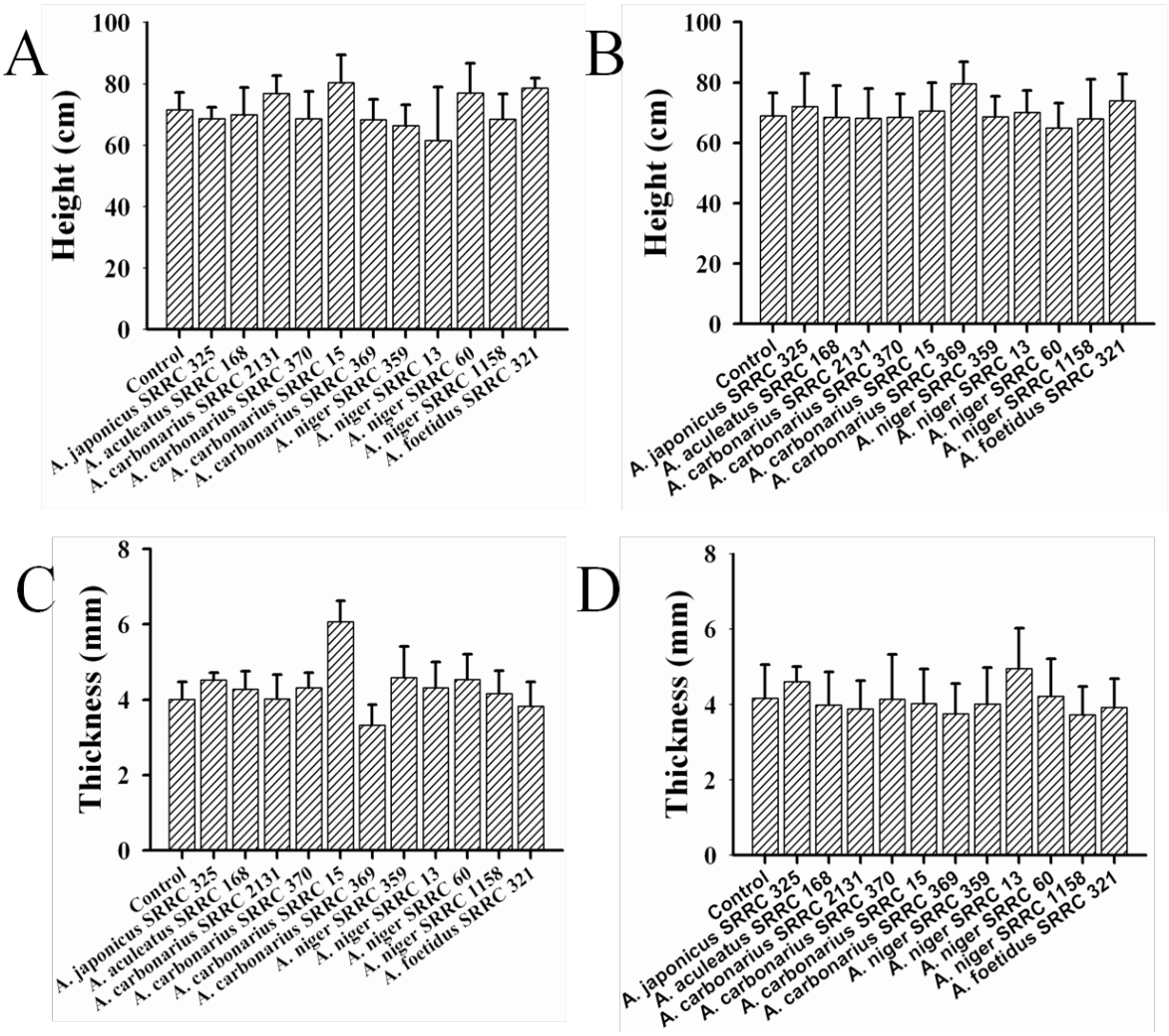

Figure 5.4. Black aspergilli effects on 3-week-old maize stems. A and C, Pioneer 3140; B, and D Pioneer 33K81. Three consecutive experiments. Values are means ± SD with *n* =3 replicates with 10 plants per pot.

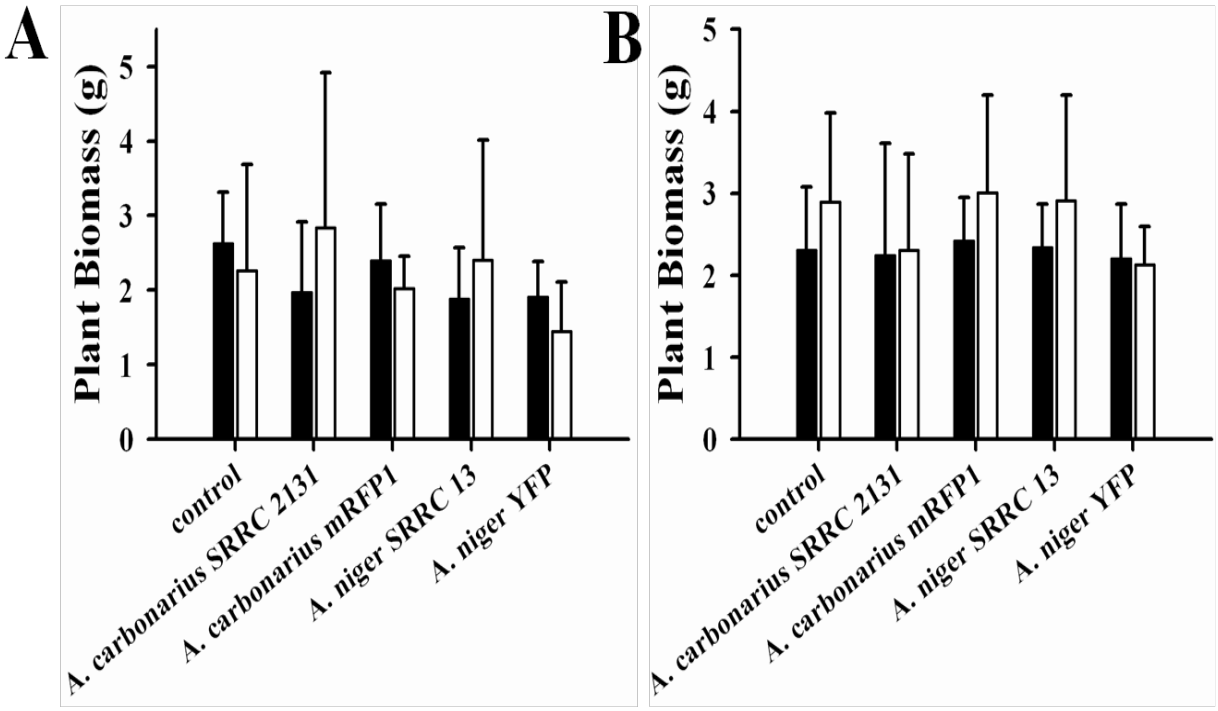

Figure 5.5. Effects of black aspergilli inoculation on peanut seedling growth (biomass dry weight). A) Tifguard, and B) Florida 07. Black bars represent plant biomass in above-ground tissue and white bars represent below-ground plant biomass. Results from two consecutive greenhouse experiments. Values are means ± SD with 6 replicates per treatment.

CHAPTER 6

PRODUCTION OF OCHRATOXINS AND FUMONISINS BY MEMBERS OF THE *ASPERGILLUS* SECTION *NIGRI* ISOLATED FROM PEANUT AND MAIZE FIELDS[1]

[1]Palencia, E.R., Mitchel, T., Riley, R., Snook, M., Bacon, C. W. To be submitted to *Phytopathology*.

ABSTRACT

The genus *Aspergillus* produces a wide variety of bioactive low-molecular weight metabolites, known as mycotoxins, which are not considered essential for growth and energy maintenance. The *Aspergillus* section *Nigri* group, the black aspergilli, represents a genetically closely related fungal species complex that produce mycotoxins, including the secondary metabolites ochratoxins and the fumonisins. Ochratoxin A is a teratogenic, nephrotoxic, and carcinogenic metabolite that has been linked to Balkan Endemic Nephropathy syndrome, a kidney health problem in Central Europe. Fumonisin FB_1 is the most commonly isolated fumonisin in maize and is linked to livestock toxicities, neural tube defects, and esophageal cancer in humans. The production of mycotoxins by black-spored *Aspergillus* poses a threat to human and livestock food safety, since these fungal species are usually associated as infections in dried fruits, grape, maize, peanut and other important crop commodities. Recent data indicate black aspergilli can develop asymptomatic endophytic interactions with maize and peanut. An endophytic infection is a potential problem in agriculture because, under favorable environmental conditions, these infections can lead to the accumulation of ochratoxins and fumonisins in cereal grains and other crops. In the present report, we studied the capacity of black aspergilli isolated as endophytes from peanut and maize in the southeastern United States to produce ochratoxins A and B, and the fumonisins B_1, B_2, and B_3 on autoclaved corn using HPLC and mass spectral analysis. Our results indicated that 54 isolates produced at least one of the three fumonisins included for this analysis. Specifically, 27.8 , 59.2, and 44.4 % of the 54 isolates produced FB_1, FB_2, and FB_3, respectively. None of the field isolates produced detectable amounts of ochratoxins.

INTRODUCTION

Mycotoxins are toxic metabolites characterized by small molecular weight, diverse toxicological activity, and unrelated chemical structure. Fungal species in the genera *Penicillium*, *Fusarium*, and *Aspergillus* are known to produce a plethora of toxic compounds that contaminate several agricultural commodities. In the genus *Aspergillus*, the species within *Aspergillus* section *Nigri*, the black aspergilli, are frequently isolated from agricultural environments, including maize, peanut, grape, coffee, or onion (19, 21, 31, 33, 34). Black aspergilli have been isolated as opportunistic pathogens responsible for the pre- and post-harvest spoilage of grapes and berries. However, recent evidence suggests that some species form endophytic associations with plant hosts (13, 30, 31, 46, 48). Plants infected by mycotoxigenic black aspergilli may, under certain environmental conditions, accumulate mycotoxins in edible parts of the plant, especially in the grain of agronomical crops. This presents a potential threat for human and animal health since the occurrence of elevated levels of mycotoxins in cereal crops is strongly associated with chronic diseases, including cancer.

The black aspergilli *A. carbonarius* and *A. niger* are soil inhabitants and are usually associated with several plant hosts, such as berries and coffee, where they are responsible for post-harvest spoilage (17, 28). *A. niger* is also widely used in industrial processes to produce extracellular enzymes and citric acid. In the biotechnology industry, *A. niger* var *niger* has been granted the GRAS (Generally Recognized As Safe) status by the United States Food and Drug Administration (40). In recent years, the black aspergilli have attracted the attention of many researchers because they have been reported to produce the secondary metabolites ochratoxin A and the fumonisins (1, 7).

Ochratoxins are a group of chemically related metabolites produced during secondary metabolism in some fungi (Figure 6.1). Ochratoxin A is widely identified as a contaminant of cereal crops, which are considered the main source of contamination for human and domesticated animals (reviewed in 35). Ochratoxin A is the most important natural occurring mycotoxin of this group that is comprised of a dihydrocoumarin moiety linked to a molecule of β-phenylalanine. Although ochratoxin A was originally identified in *A. ochraceus* isolated from maize; it is now known to be produced by several species in *Aspergillus* and *Penicillium*, and it is isolated from different natural environments, reflective of this species diversity (39, 42). Other ochratoxin A-related metabolites have been detected, including its chlorine-lacking analogue ochratoxin B, the ethyl ester ochratoxin C analogue, and the dihydroisocoumarin moiety ochratoxin α. The occurrence of either ochratoxin B or ochratoxin C in crop commodities in the United States is rare; nonetheless, sporadic reports have indicated high concentrations of ochratoxin B and ochratoxin C in different substrates (12, 44).

Ochratoxin A is a nephrotoxic, teratogenic, immunosuppressive and carcinogenic metabolite in domestic animals and laboratory rats (3, 11, 16). In humans, ochratoxin A has been consistently associated with a fatal disease known as Balkan Endemic Nephropathy (BEN) that affects inhabitants of countries in southeastern Europe (45). BEN is a chronic tubulo-interstitial nephropathy characterized by a heavy damage to the tubular epithelium. Recent evidence shows that ochratoxin A plays an important role in the incidence of a group of nephropathies known as Chronic Interstitial Nephropathies (CIN) that affect countries in northern Africa (47). The ingestion of cereals contaminated with elevated concentrations of ochratoxin A is directly associated with high incidence of CIN.

The fumonisins form a group of mycotoxins (Figure 6.2) that are produced by several *Fusarium* species, particularly the endophytic maize fungus *Fusarium verticillioides* (4). Recent studies now indicate that the European and South American black *Aspergillus* species are also able to produce fumonisin B_2, fumonisin B_4, and fumonisin B_6 (FB_2, FB_4, and FB_6) (18, 23, 26). Fumonisins resemble the chemical structure of sphingolipids, and strong evidence suggests that the most abundant and toxic metabolite of this group, fumonisin B_1, is involved in the disruption of lipid metabolism leading to chronic animal diseases and cancer (37). The consumption of maize contaminated with fumonisins is correlated with porcine pulmonary edema and equine leucoencephalomalacia in farm animals (38, 49). Esophageal cancer, neural tube defects, and kidney failure in humans are diseases usually associated with the consumption of food contaminated with fumonisins (9, 24, 41).

The prevalence and host association of the black aspergilli with United States peanuts and corn is unknown. Further specific mycotoxins produced by peanut and maize-associated black aspergilli are also unknown. The major objective of this research is to determine the capacity of black aspergilli isolated as endophytes from maize and peanut to produce ochratoxins and fumonisins.

MATERIALS AND METHODS

Fungal and plant material. *Aspergillus niger* SRRC 13 (=NRRL 2042) and *A. carbonarius* SRRC 2131 (=FRR 369) were used to determine conditions for both ochratoxins and the fumonisins production on the cereal grain. In addition 150 isolates of black aspergilli were obtained from peanut and maize. The identities of these were based on morphological characteristics (15) and by using a rep-PCR method developed as molecular tool for species identification (31). Fungal material was isolated, single-spored and stored at -80 °C in a 0.01%

Tween, 15% glycerol solution. Potato dextrose agar (PDA) dishes were inoculated with a 10-µl loop of a frozen stock and incubated for 7 days at 25° C. Three-5-mm agar plugs were aseptically transferred to 10 g of kernels in a 125-ml baffled flask, incubated at 30° C for 14 days. After the incubation period, inoculated kernels were lyophilized.

The seed from five cereals were used as natural substrates to determine ochratoxin and fumonisin production by members of the *Aspergillus* section *Nigri* isolated from maize and peanut and these included: rye (*Secale cereale*), oats (*Avena sativa*), barley (*Hordum vulgare*), and wheat (*Triticum aestivum*). They were purchased from Johnny' Selected Seeds (Winslow, ME). Maize (*Zea mays*) seeds were provided by Pioneer Hi bred (Johnston, IA). A seed sample (approx. 10 g) of each crop was allowed to imbibe in 40-ml of sterile water for 16 h, and then the water excess was removed. The moist kernels were autoclaved at 121° C, 10kPa for 15 minutes. The inocula for the cereal were prepared from PDA dishes were inoculated with a 10-µl loop of a frozen stock of black spored aspergilli isolates and incubated for 7 days at 25° C in darkness.

Mycotoxin production. To determine the maximum time period for ochratoxin production, sterile maize kernels were inoculated with *A. niger* var *niger* SRRC 13 and *A. carbonarius* SRRC 2131 and incubated at 25°C and 30°C in the dark. They were analyzed for mycotoxin production after 3, 6, 9, 20, and 30 days of inoculation. After the incubation period, colonized kernels were stored at -25° C for 2 h, and then lyophilized in a Benchtop K freeze dryer (Virtis, Gardiner, NY) at -43° C, 80 Pa for 48 h. Lyophilized samples were ground to a powder with liquid nitrogen and stored at -25° C until the ochratoxin and fumonisin analyses.

Analytical solvents and standards. All solvents used for this experiment were of analytical reagent-grade. Methanol and chloroform (Fisher Scientific, Fair Lawn, NJ), acetonitrile (MeCN, Burdick & Jackson, Muskegon, MI), water (J.T. Baker Phillipsburg, NJ),

and formic acid (Sigma-Aldrich, St. Louis, MO) were HPLC-grade. Stock standard solutions of ochratoxin A (Sigma-Aldrich) and ochratoxin B (Santa Cruz Biotechnology, Santa Cruz, CA) were prepared by dissolving an aliquot of the standards in methanol to a 10 µg/ml final concentration followed by a sonication step for 10 min at room temperature, and stored at -25 °C until required. Working standard solutions were prepared from stock solutions and used for calculating the standard curve. The ochratoxin $^{13}C_{20}$ OEKANAL® solution (10 µg/ml in acetonitrile; Sigma Aldrich) was used as an internal standard to quantify the production of ochratoxin A and ochratoxin B by black aspergilli and stored at -25 °C. FB_1, FB_2, and FB_3 were kindly provided by Ronald Plattner, National Center for Agricultural Utilization Research, NCAUR, USDA-ARS, Peoria, IL. Standard solutions of fumonisins were prepared by adding 970 µl of acetonitrile: water 30:70 (v/v) + 1% formic acid to a final concentration of 10 µg/ml. The quantification of fumonisins was performed using the internal standard [^{13}C] FB_1 (10 µg/ml in methanol; Sigma-Aldrich).

Mycotoxin extraction. For the extraction of ochratoxins, an alkaline-acid based method was performed by adding approximately 2.5 g ground samples to 10 ml of a methanol:chloroform 1:1: mixture in a 10-ml glass tube. The samples were extracted for 16 h by using a rotary shaker at room temperature, and subsequently sonicated for 1 h in a 5510 Branson ultrasonic cleaner (Branson Ultrasonics, Danbury, CT). The extracts were filtered using Whatman #1 filter paper on a glass funnel, and the supernatant was collected into a 60-ml separation funnel. The methanolic supernatant was mixed three times with 20 ml of 0.5 N $NaHCO_3$, and the aqueous phase was collected in a 125-ml Erlenmeyer flask. The pH of the aqueous layer was adjusted to pH 2-3 with 2.5 ml of 0.2 M HCl, and after appropriate mixing, ochratoxin A and ochratoxin B were extracted three times from the acidified aqueous phase with

20-ml portions of chloroform. The combined chloroform fractions were collected and transferred to a 100-ml boiling flask and the chloroform was evaporated on a rotary evaporator (Buchi, Flawil, Switzerland) at 42°C. The residue was dissolved in 1-ml of chloroform, and transferred to crimp top vials. The extracts were blow-dried with nitrogen at 37 °C, 0.5 ml of methanol were added and the solids were dissolved by sonication. Individual extracts were filtered using 0.45 µm Costar® nylon centrifuge tube filters (Corning, NY) at 4000 rpm for 3 min and transferred to 2-ml screw cap glass PTFE/silicone vials (National Scientific, Rockwood, TN) for LC-MS/MS analysis.

The fumonisin extraction was carried out using a modified method described in (36). Briefly, 12.5 ml of acetonitrile:water 1:1: (v/v) was added to approximately 2.5 g of the lyophilized sample, and pH was adjusted to 4.5 with 200 µl 1 N HCl. The extraction mixture was shaken at full speed in a reciprocal shaker for 16 h, and subsequently sonicated in a Branson ultrasonic cleaner for 30 min. The extracts were centrifuged at 1000 x g for 3 min and the supernatant was transferred into glass tubes using Pasteur pipettes. The extract, 2 ml, was combined and mixed with 6 ml of HPLC water. The diluted extracts were slowly filtered by loading them onto a sep-pak C_{18} cartridges (Waters Corporation, Milford, MA). Loaded cartridges were sequentially rinsed with 2 ml of water, and 2 ml of acetonitrile:water 70:30 (v/v) was used to elute the samples from the C_{18} sorbent package. The filtered extracts (0.5 ml) were mixed with 50 µl of the ^{13}C fumonisin internal standard for LC-MS analysis.

Thin Layer Chromatography and Liquid Chromatography/Mass Spectrometry. Thin layer chromatographic (TLC) analysis was carried out to assess the production of ochratoxin A by black aspergilli on crop seeds. Briefly, methanolic extracts (30 µl) were spotted onto silica gel coating TLC sheet (Whatman, Maidstone, England), and dried for 5 min. The

TLC sheet was developed by using toluene/ethylene acetate/formic acid 50:40:10 (vol:vol:vol) in a saturated thick glass chromatographic tank for 40 min. Ochratoxin A production was detected using UV light (λ_{nm} 254) and recorded in a photographic system (Alpha Innotech, San Leandro, CA). The LC-MS detection and quantification was performed with a Finnigan LCQ Duo ion-trap mass spectrometer (ThermoQuest, San Jose, CA) with an electrospray ion source (ESI), and equipped with a SpectraSystem autoinjector and Finnigan surveyor MS pump plus (Thermo Electronic, San Jose, CA). The Finnigan LCQ system was controlled by the Thermoquest Xcalibur software version 2.0.7. Chromatographic separations were carried out by passing samples through an Ultrasphere Beckham Coulter ODS C_{18} column (5 μm particle size, Fullerton, CA), 250 x 4.6 mm, attached to a C_{18} guard column. Samples were eluted with a mobile phase system: solvent A (99% acetonitrile and 1% formic acid) and solvent B (water and 1% formic acid). An elution gradient started with 10 min solvent A, increasing solvent B to 100% over 40 min. For ochratoxin A and ochratoxin B detection, water with 1% formic acid (solvent A) and acetonitrile (solvent B) were delivered using the following program: 0% solvent A and 100% solvent B for 20 min, 50% solvent A and 50% solvent B for the next 20 min.

RESULTS AND DISCUSSION

The black species of *Aspergillus* are known to produce a wide variety of secondary metabolites (reviewed in 8, 27). However, the demonstration of mycotoxin on liquid media varies with the strain, and most media are developed for a particular strain isolated from a specific cereal. What is desired is a procedure that would ensure that a strain or species indicated as negative is not due to the specific media being tested being unsuitable. In the present study we developed a procedure that will conveniently demonstrate the ability of a variety of species

and strains of black *Aspergillus*, under laboratory conditions, to produce both ochratoxins and fumonisins on autoclaved cereals.

To assist in screening the black aspergilli for their ability to produce ochratoxins and fumonisisn, a rapid TLC approach was used to determine whether *A. niger* SRRC 13 and *A. carbonarius* 2131 isolates were able to produce ochratoxin A on autoclaved corn, rye, oat, wheat and barley kernels. After three consecutive experiments, the results indicated that *A. niger* SRRC 13 produced ochratoxin A exclusively on corn seeds, but not detectable amounts of this mycotoxins were observed on rye, barley, wheat, and oat (Figure 6.3). However, *A. carbonarius* SRRC 2131, produced ochratoxin A on corn, rye, and barley (Figure 6.3). The production of these mycotoxins differed in maximum amounts and appeared to be temperature dependent. When corn was used as the substrate, both *A. niger* SRRC 13 and *A. carbonarius* SRRC 2131 (Figure 6.4) were able to produce ochratoxin A. After monitoring the production of ochratoxins in these two black aspergilli for 30 days at 25 °C and 30 °C, *A. carbonarius* SRRC 2131 achieved maximum synthesis after 20 days of incubation at 25° C (Figure 6.4 B). The maximum synthesis by *A. niger* SRRC 13 occurred after 12 days of incubation at 25° C (Figure 6.4 A).

Since both black aspergilli expressed maximum syntheses of ochratoxin A after incubation at 25° C as opposed to 30° C, temperature apparently is an important environmental factor for these two black aspergilli. Temperature optima for the production of this mycotoxin by these two species are expected to reflect their maximum growth at these temperatures. In this regard, the temperature optimum for growth of *A. carbonarius* on laboratory media occurs at 30° C, while the optimum for *A. niger* occurs at temperatures above 30° C (29). Our data are indicative of these temperature and growth requirements. On liquid media, Esteban et al (6) presented evidence that optimum temperature for ochratoxin A production by *A. niger* was

between 20-25 °C, and differed from the conditions required by *A. carbonarius* which occurred between 15-20 °C (5). However, moisture or water activity is also important and is interactive with the production of this mycotoxin (2, 25). Nevertheless the data indicate that on autoclaved corn, ochratoxin A is produced by these species. This therefore, substantiates the earlier reports (7,27, 28, 42) that American isolates from peanut and maize have the potential for producing ochratoxin A.

Ochratoxins and fumonisins belong to the group of the polyketide biopolymers, which are natural products formed after multiple condensation steps of acetate (14). The polyketide biosynthesis depends on the bioavailability of acetyl-CoA, the fundamental unit in polyketide biosynthesis. Line of evidence has suggested that seed composition, especially seed fatty acids, is a major contributor for polyketide biosynthesis (20).We believe our work demonstrating the ability of isolates to produce ochratoxin A on seeds is a valid assessment of the potential of an isolate as opposed to media-based *in vitro* assays.

Recent surveys have suggested that cryptic species within the *A. niger* group may be responsible for ochratoxin A contamination in corn and corn-based products (22; 31). Ochratoxin A is usually found as a contaminant of cereal grains such as rye, wheat, oats, and barley; however, the source of such of contamination is not clear since several species from *Aspergillus* and *Penicillium*, known to produce ochratoxin A, are also isolated from these cereal crops. More evidence is needed to determine which species are responsible for ochratoxin contamination in important agronomical crops. Based on our results, corn and other cereals should be used to screen for ochratoxin A production by black *Aspergillus* species isolated from peanut and maize fields. A similar conclusion was reached by Belli et al. (2) who used grape medium to determine ochratoxin A synthesis by grape isolates.

The second mycotoxin test for the corn and peanut isolates was for the ability to produce fumonisins. The results of screening for fumonisin B_1, B_2, and B_3 showed that 54 of the black isolates produced at least one of the analyzed fumonisins (Table 6.2). The fumonisin producing isolates consisted of 49 (90.7%) *A. niger*, 4 (7.4%) *A. foetidus*, and 1 (1.8%) *A. tubingensis* isolated as endophytes from maize and peanut tissue. A total of 15 (27.8%,) field isolates produced detectable amounts of FB_1, with 10 (66.6 %,) field strains isolated from peanut fields, and 5 (33.3%,) strains isolated from maize. A total of 32 isolates produced FB_2, which consisted of 13 (40.6%,) isolates from maize, and 19 (59.3%) from peanut. The remaining 24 isolates produced FB_3 that consisted of 12 (50.0%) black aspergilli isolates obtained from peanut fields, and 12 (50.0%) isolated from maize fields. Only one field strain (1.8%), *A. niger* RRC 579, produced detectable amounts of all isomers of fumonisin analyzed in this study (Fig.6.5).

The fumonisins were first isolated from *Fusarium verticillioides* (10) and their natural occurrence was first associated with maize. The fumonisins consist of a mixture of isomers, FB_1, FB_2, and FB_3. The synthesis is from FB_3 to FB_2 and finally the end product FB_1. One, two, or all isomers have been isolated from natural substrates or in liquid media. In contrast with other reports that determined the production of mycotoxins on liquid synthetic or semi-synthetic media, we screened the black aspergilli for the production of both ochratoxins and fumonisins using cereal grain. The production of FB_1, by 16 black aspergilli ranged between 0.236-4.053 ng/ml with the highest concentration produced by the RRC 537strain that was isolated from a maize field (Table 6.2). This is not the first report of the production of FB_1 by a black *Aspergillus* species. Varga et al. (43) reported the production of FB_1 and FB_3 by *A. niger* strains, which were isolated from California raisins. However, there is controversy with the production of FB_1 and FB_3 by black aspergilli. Our study adds to the controversy since the isolates in this

study produced FB_1. Several early studies suggest that the black aspergilli isolated from raisins do not produce FB_1, but rather FB_2 (23, 26, 32). Regardless of the genetic mechanism involved in this controversy, we believe the inability of the strains to produce FB_1 may well lie with the difference in the medium and the origin of the strain. So the question is whether these differences are due to culture conditions *in vitro* or to genetic differences in strains from the two crops. An answer to the culture conditions might be obtained from culturing grape isolates on corn and the reverse, i.e., culture of maize isolates on the medium used to determine fumonisin production by the grape isolates. The unavailability of suitable substrates for completing the synthesis of FB_1 might be a problem. However, studies demonstrating that only FB_2 was produced by the grape isolates used either a complex medium of natural components (23, 26) or a very simple synthetic agar medium (32). Perhaps the substrate necessary for the complete synthesis of FB_1 was not available in this synthetic medium resulting in the reaction stopping at FB_2.

An explanation has been put forward to indicate that genetic differences in grape isolates may be responsible for the absence of FB_1 production. This suggestion is based on the revelation that high percentages of black aspergilli from grape contain the fumonisin gene clusters but do not produce any fumonisins, indicating that those that do are defective or different from genes from the *Fusarium* isolates. Further, Palumbo et al. (32) suggest that the presence of the gene cluster for fumonisin production in *A. niger* cannot be used to predict or detect FB_2 production. In general these data indicate that there are functional differences among populations within grape, and we might extend this to suggest that there are differences among populations on other crops such as maize resulting in populations that are capable of producing FB_1. To our knowledge, however, these studies have only been determined in vitro culture of these organisms.

It is important to remember that the production of FB_2 in culture does not predict it production *in planta*. In our work we confirm the earlier report that on another natural substrate, maize, the black aspergilli can produce the carcinogenic and cytotoxic isomers of fumonisins. FB_1, FB_2, and FB_3.(10). Structure-activity-relationships of fumonisin in short-term carcinogenesis and cytotoxicity assays, and the production of these isomers from black *Aspergillus* species from maize and peanut extend the concerns for human and animal health.

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Table 6.1. Molecular characterization of black aspergilli isolates from peanut and maize fields in the southeastern United States using rep-PCR.

No.	Strain number [a]	Location	Top match (% similarity)	Predicted species
1	RRC 453	Peanut, South Georgia	90.0	*A. niger*
2	RRC 454	Peanut, South Georgia	90.6	*A. foetidus*
3	RRC 455	Peanut, South Georgia	89.6	*A. niger*
4	RRC 456	Peanut, South Georgia	85.5	*A. niger*
5	RRC 457	Peanut, South Georgia	93.8	*A. niger*
6	RRC 458	Peanut, South Georgia	91.3	*A. foetidus*
7	RRC 459	Peanut, South Georgia	87.5	*A. niger*
8	RRC 460	Peanut, South Georgia	78.7	*A. japonicus*
9	RRC 462	Peanut, South Georgia	90.2	*A. niger*
10	RRC 465	Peanut slurries, Dawson, Georgia	94.5	*A. niger*
11	RRC 469	Peanut slurries, Dawson, Georgia	95.5	*A. niger*
12	RRC 470	Peanut slurries, Dawson, Georgia	90.5	*A. niger*
13	RRC 475	Peanut slurries, Dawson, Georgia	79.3	*A. niger*
14	RRC 476	Peanut slurries, Dawson, Georgia	98.3	*A. niger*
15	RRC 477	Peanut slurries, Dawson, Georgia	98.4	*A. foetidus*
16	RRC 478	Peanut slurries, Dawson, Georgia	93.3	*A. niger*
17	RRC 479	Peanut slurries, Dawson, Georgia	75.1	*A. japonicus*
18	RRC 480	Peanut slurries, Dawson, Georgia	98.5	*A. niger*
19	RRC 481	Peanut slurries, Dawson, Georgia	93.1	*A. niger*
20	RRC 482	Maize kernels, Midwestern USA	92.7	*A. niger*
21	RRC 483	Maize kernels, Midwestern USA	93.3	*A. niger*
22	RRC 484	Maize kernels, Midwestern USA	93.4	*A. niger*
23	RRC 485	Maize kernels, Midwestern USA	93.4	*A. niger*
24	RRC 486	Maize, kernels, Midwestern USA	90.7	*A. niger*
25	RRC 487	Maize kernels, Midwestern USA	90.7	*A. niger*

26	RRC 488	Maize kernels, Midwestern USA	89.9	*A. niger*
27	RRC 489	Maize kernels, Midwestern USA	88.4	*A. niger*
28	RRC 490	Maize kernels, Midwestern USA	90.1	*A. niger*
29	RRC 491	Maize kernels, Midwestern USA	95.1	*A. niger*
30	RRC 493	Maize kernels, Midwestern USA	92.8	*A. niger*
31	RRC 494	Maize kernels, Midwestern USA	92.9	*A. niger*
32	RRC 495	Maize kernels, Midwestern USA	93.4	*A. niger*
33	RRC 497	Maize kernels, Midwestern USA	82.2	*A. niger*
34	RRC 500	Maize kernels, Midwestern USA	97.0	*A. niger*
35	RRC 501	Maize kernels, Midwestern USA	88.6	*A. niger*
36	RRC 503	Maize kernels, Midwestern USA	85.2	*A. niger*
37	RRC 504	Maize kernels, Midwestern USA	89.4	*A. niger*
38	RRC 505	Maize kernels, Midwestern USA	94.1	*A. niger*
39	RRC 507	Maize kernels, Midwestern USA	94.4	*A. foetidus*
40	RRC 509	Maize kernels, Midwestern USA	95.1	*A. niger*
41	RRC 510	Maize kernels, Midwestern USA	92.1	*A. niger*
42	RRC 511	Maize kernels, Midwestern USA	89.6	*A. niger*
43	RRC 512	Maize kernels, Midwestern USA	89.6	*A. tubingensis*
44	RRC 513	Maize kernels, Midwestern USA	89.6	*A. niger*
45	RRC 514	Maize kernels, Midwestern USA	89.5	*A. niger*
46	RRC 515	Maize kernels, Midwestern USA	90.3	*A. niger*
47	RRC 516	Maize kernels, Midwestern USA	89.4	*A. niger*
48	RRC 517	Maize kernels, Midwestern USA	89.7	*A. niger*
49	RRC 518	Maize kernels, Midwestern USA	90.1	*A. niger*
50	RRC 519	Maize kernels, Midwestern USA	94.5	*A. niger*
51	RRC 520	Maize kernels, Midwestern USA	89.4	*A. niger*
52	RRC 521	Maize kernels, Midwestern USA	85.9	*A. niger*
53	RRC 522	Maize kernels, Midwestern USA	86.3	*A. niger*
54	RRC 523	Maize kernels, Midwestern USA	88.6	*A. foetidus*

55	RRC 524	Maize kernels, Midwestern USA	86.5	*A. niger*
56	RRC 525	Maize kernels, Midwestern USA	92.7	*A. niger*
57	RRC 526	Maize kernels, Midwestern USA	91.4	*A. niger*
58	RRC 527	Maize kernels, Midwestern USA	95.5	*A. niger*
59	RRC 528	Maize kernels, Midwestern USA	93.3	*A. niger*
60	RRC 529	Maize kernels, Midwestern USA	91.6	*A. niger*
61	RRC 530	Maize kernels, Midwestern USA	91.0	*A. niger*
62	RRC 531	Maize kernels, Midwestern USA	89.2	*A. niger*
63	RRC 532	Maize kernels, Midwestern USA	92.0	*A. niger*
64	RRC 533	Maize kernels, Midwestern USA	91.0	*A. niger*
65	RRC 534	Maize kernels, Midwestern USA	85.4	*A. niger*
66	RRC 535	Maize kernels, Midwestern USA	84.0	*A. niger*
67	RRC 536	Maize kernels, Midwestern USA	84.0	*A. niger*
68	RRC 537	Maize kernels, Midwestern USA	94.1	*A. niger*
69	RRC 540	Peanut kernels, Pearsall, Texas	95.7	*A. niger*
70	RRC 541	Peanut kernels, Pearsall, Texas	95.2	*A. niger*
71	RRC 542	Peanut kernels, Pearsall, Texas	95.4	*A. niger*
72	RRC 543	Peanut kernels Rochester, Texas	94.6	*A. niger*
73	RRC 544	Peanut kernels Rochester, Texas	94.2	*A. niger*
74	RRC 545	Peanut kernels Rochester, Texas	94.4	*A. niger*
75	RRC 546	Peanut kernels Rochester, Texas	94.4	*A. niger*
76	RRC 547	Peanut kernels Rochester, Texas	95.4	*A. niger*
77	RRC 548	Peanut kernels Rochester, Texas	94.4	*A. niger*
78	RRC 550	Peanut kernels, Pearsall, Texas	78.1	*A. aculeatus*
79	RRC 551	Peanut kernels, Pearsall, Texas	78.3	*A. aculeatus*
80	RRC 552	Peanut kernels, Pearsall, Texas	92.4	*A. niger*
81	RRC 553	Peanut kernels, Headland Alabama	90.9	*A. niger*
82	RRC 554	Peanut kernels, Pearsall, Texas	94.2	*A. niger*
83	RRC 555	Peanut kernels, Pearsall, Texas	98.4	*A. niger*

84	RRC 556	Peanut kernels, Headland Alabama	91.7	*A. niger*
85	RRC 557	Peanut kernels, Rochester, Texas	93.3	*A. niger*
86	RRC 558	Peanut kernels, Headland Alabama	92.3	*A. niger*
87	RRC 559	Peanut kernels, Rochester, Texas	91.6	*A. niger*
88	RRC 560	Peanut kernels, Rochester, Texas	85.3	*A. niger*
89	RRC 561	Peanut kernels, Rochester, Texas	96.3	*A. niger*
90	RRC 562	Peanut kernels, Rochester, Texas	87.4	*A. niger*
91	RRC 563	Peanut kernels, Rochester, Texas	92.1	*A. niger*
92	RRC 564	Peanut kernels, North Carolina	98.5	*A. niger*
93	RRC 565	Peanut kernels, North Carolina	95.5	*A. niger*
94	RRC 566	Peanut kernels, North Carolina	92.7	*A. niger*
95	RRC 567	Peanut kernels, North Carolina	98.5	*A. foetidus*
96	RRC 568	Peanut kernels, Ashford, Alabama	93.8	*A. niger*
97	RRC 569	Peanut kernels, Ashford, Alabama	93.5	*A. niger*
98	RRC 570	Peanut kernels, Ashford, Alabama	93.8	*A. niger*
99	RRC 571	Peanut kernels, Ashford, Alabama	89.5	*A. niger*
100	RRC 572	Peanut kernels, Ashford, Alabama	90.1	*A. niger*
101	RRC 573	Peanut kernels, Ashford, Alabama	94.1	*A. niger*
102	RRC 574	Peanut kernels, Seminole, Texas	90.4	*A. foetidus*
103	RRC 575	Peanut kernels, Seminole, Texas	98	*A. niger*
104	RRC 576	Peanut kernels, Seminole, Texas	92.2	*A. niger*
105	RRC 577	Peanut kernels, Seminole, Texas	98.9	*A. niger*
106	RRC 578	Peanut kernels, Seminole, Texas	95	*A. niger*
107	RRC 579	Peanut kernels, Seminole, Texas	98.4	*A. niger*
107	RRC 580	Peanut kernels, Seminole, Texas	98.2	*A. niger*
109	RRC 581	Peanut kernels, Seminole, Texas	90.4	*A. niger*
110	RRC 582	Peanut kernels, Levelland, Texas	88.2	*A. foetidus*
111	RRC 583	Peanut kernels, Levelland, Texas	91.4	*A. foetidus*
112	RRC 584	Peanut kernels, Levelland, Texas	98.5	*A. niger*

113	RRC 585	Peanut kernels, Levelland, Texas	94.5	*A. niger*
114	RRC 586	Peanut kernels, Levelland, Texas	97.8	*A. foetidus*
115	RRC 587	Peanut kernels, Levelland, Texas	98.3	*A. niger*
116	RRC 588	Peanut kernels, Levelland, Texas	93.5	*A. niger*
117	RRC 589	Peanut kernels, Levelland, Texas	90.1	*A. niger*
118	RRC 590	Peanut kernels, Levelland, Texas	91.9	*A. foetidus*
119	RRC 591	Peanut kernels, Levelland, Texas	95	*A. niger*
120	RRC 592	Peanut kernels, Levelland, Texas	95.4	*A. foetidus*
121	RRC 593	Peanut kernels, Wakefield, Virginia	97.2	*A. niger*
122	RRC 594	Peanut kernels, Lubbock, Texas	91.9	*A. niger*
123	RRC 595	Peanut kernels, Lubbock, Texas	97.5	*A. niger*
124	RRC 596	Peanut kernels, Lubbock, Texas	95.8	*A. niger*
125	RRC 597	Peanut kernels, Wakefield, Virginia	90.5	*A. niger*
126	RRC 598	Peanut kernels, Wakefield, Virginia	91	*A. niger*
127	RRC 599	Peanut kernels, Wakefield, Virginia	94	*A. niger*
128	RRC 600	Peanut kernels, Wakefield, Virginia	91.2	*A. niger*
129	RRC 601	Peanut kernels, Wakefield, Virginia	93	*A. niger*
130	RRC 602	Peanut kernels, Wakefield, Virginia	90.4	*A. niger*
131	RRC 603	Peanut kernels, Wakefield, Virginia	90.5	*A. niger*
132	RRC 604	Peanut kernels, Wakefield, Virginia	88.4	*A. niger*
133	RRC 605	Peanut kernels, Wakefield, Virginia	91.1	*A. niger*
134	RRC 606	Peanut kernels, Pearsall, Texas	94.8	*A. niger*
135	RRC 607	Peanut kernels, Seminole, Texas	97.8	*A. niger*
136	RRC 608	Peanut kernels, Seminole, Texas	85.5	*A. niger*
137	RRC 609	Peanut kernels, Pearsall, Texas	93.1	*A. carbonarius*
138	RRC 610	Peanut kernels, Pearsall, Texas	94.3	*A. niger*
139	RRC 611	Peanut kernels, Pearsall, Texas	93.2	*A. niger*
140	RRC 612	Peanut kernels, Headland Alabama	96.1	*A. niger*
141	RRC 613	Peanut kernels, Headland Alabama	93.3	*A. niger*

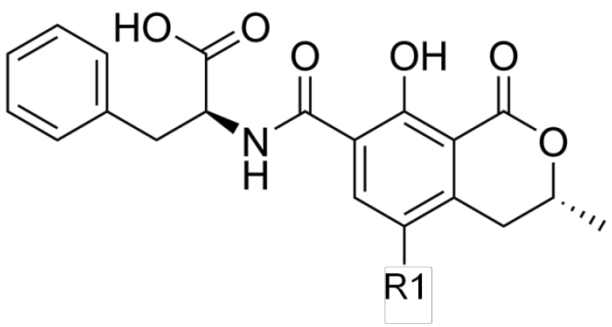
142	RRC 614	Peanut kernels, Headland Alabama	91.3	*A. niger*
143	RRC 615	Peanut kernels, Rochester, Texas	91.6	*A. niger*
144	RRC 616	Peanut kernels, Rochester, Texas	93	*A. niger*
145	RRC 617	Peanut kernels, North Carolina	97.6	*A. niger*
146	RRC 618	Peanut kernels, Ashford, Alabama	80.1	*A. carbonarius*
147	RRC 619	Peanut kernels, Wakefield, Virginia	90.6	*A. niger*
148	RRC 620	Peanut kernels, Levelland, Texas	92.3	*A. niger*
149	RRC 621	Peanut kernels, Levelland, Texas	93.4	*A. niger*
150	RRC 622	Peanut kernels, Wakefield, Virginia	90.5	*A. niger*

Table 6.2. Fumonisin production by black aspergilli isolates.

Isolate	Species	Substrate	Fumonisins[a] (ng/ml)		
			FB_1	FB_2	FB_3
RRC 458	*A. foetidus*	Peanut	0.349	0.354	nd
RRC 462	*A. niger*	Peanut	nd	0.468	nd
RRC 465	*A. niger*	Peanut	0.532	nd	nd
RRC 469	*A. niger*	Peanut	nd	nd	4.283
RRC 470	*A. niger*	Peanut	1.29	nd	0.151
RRC 478	*A. niger*	Peanut	1.564	nd	nd
RRC 480	*A. niger*	Peanut	0.534	nd	nd
RRC 482	*A. niger*	Maize	nd	0.506	nd
RRC 488	*A. niger*	Maize	nd	nd	0.421
RRC 491	*A. niger*	Maize	nd	0.485	nd
RRC 495	*A. niger*	Maize	nd	0.477	nd
RRC 500	*A. niger*	Maize	nd	nd	9.7
RRC 501	*A. niger*	Maize	nd	0.601	0.159
RRC 503	*A.niger*	Maize	nd	0.700	0.110
RRC 505	*A. niger*	Maize	nd	nd	16.123
RRC 507	*A. foetidus*	Maize	nd	nd	7.206
RRC 509	*A. niger*	Maize	0.375	nd	nd
RRC 511	*A. niger*	Maize	nd	0.581	nd
RRC 512	*A. tubingensis*	Maize	nd	nd	0.303
RRC 513	*A. niger*	Maize	nd	0.568	nd
RRC 515	*A. niger*	Maize	0.406	nd	0.522
RRC 516	*A. niger*	Maize	nd	0.548	nd
RRC 517	*A. niger*	Maize	nd	0.612	nd
RRC 519	*A. niger*	Maize	nd	1.133	0.884
RRC 522	*A. niger*	Maize	nd	0.321	0.279
RRC 524	*A. niger*	Maize	0.462	nd	nd
RRC 526	*A. niger*	Maize	nd	0.668	nd
RRC 527	*A. niger*	Maize	2.374	nd	nd
RRC 530	*A. niger*	Maize	nd	0.737	0.289
RRC 537	*A. niger*	Maize	4.053	nd	0565

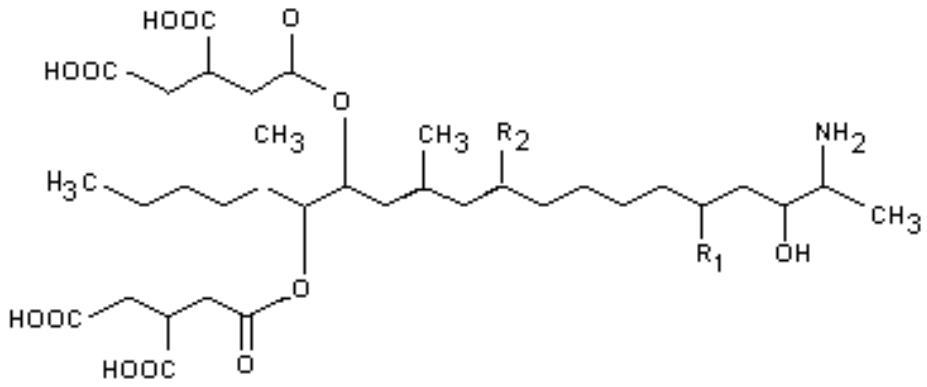
RRC 543	*A. niger*	Peanut	nd	0.510	0.524
RRC 546	*A. niger*	Peanut	nd	0.534	nd
RRC 547	*A. niger*	Peanut	nd	1.557	nd
RRC 548	*A. niger*	Peanut	0.730	nd	nd
RRC 552	*A. niger*	Peanut	nd	0.838	0.343
RRC 556	*A. niger*	Peanut	0.783	nd	nd
RRC 564	*A. niger*	Peanut	0.346	0.761	nd
RRC 566	*A. niger*	Peanut	nd	0.646	nd
RRC 568	*A. niger*	Peanut	nd	0.878	nd
RRC 569	*A. niger*	Peanut	nd	0.466	nd
RRC 571	*A. niger*	Peanut	nd	1.052	nd
RRC 573	*A. niger*	Peanut	0.324	nd	nd
RRC 576	*A. niger*	Peanut	nd	0.831	0.355
RRC 579	*A. niger*	Peanut	0.545	0.731	0.412
RRC 583	*A. foetidus*	Peanut	nd	0.645	nd
RRC 592	*A. foetidus*	Peanut	nd	0.869	0.208
RRC 596	*A. niger*	Peanut	nd	0.934	nd
RRC 599	*A. niger*	Peanut	nd	0.737	nd
RRC 603	*A. niger*	Peanut	nd	nd	0.453
RRC 606	*A. niger*	Peanut	nd	nd	0.355
RRC 610	*A. niger*	Peanut	nd	0.814	0.242
RRC 615	*A. niger*	Peanut	nd	0.875	0.438
RRC 620	*A. niger*	Peanut	nd	nd	1.186
RRC 622	*A. niger*	Peanut	0.236	0.429	nd

[a] nd =not detected



Mycotoxin	R1	Formula	Molecular mass
Ochratoxin A	Cl	$C_{20}H_{18}ClNO_6$	403.8
Ochratoxin B	H	$C_{20}H_{19}NO_6$	369.4

Figure 6.1. Chemical structure of ochratoxins.



Mycotoxin	R_1	R_2	Formula	Molecular Mass
Fumonisin B1	OH	OH	$C_{34}H_{59}NO_{15}$	721.84
Fumonisin B2	OH	H	$C_{34}H_{59}NO_{14}$	705.84
Fumonisin B3	H	OH	$C_{34}H_{59}NO_{13}$	689.84

Figure 6.2. Chemical structure of fumonisins.

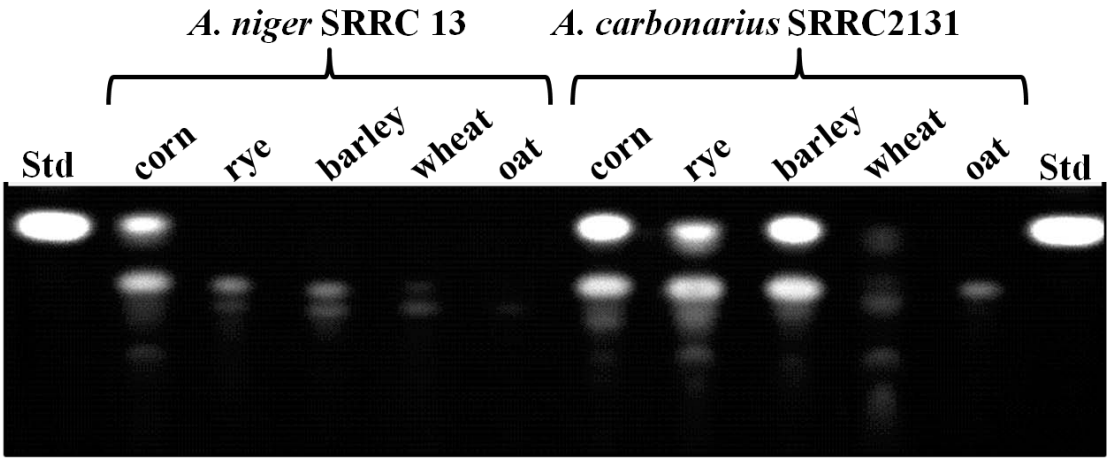


Figure 6.3. Ochratoxin A production by black *Aspergillus* species on crop seeds. *A. niger* SRRC 13 and A. *carbonarius* SRRC 2131 methanolic extracts on silica gel TLC sheets. ST denotes ochratoxin A standard spots (1 µg).

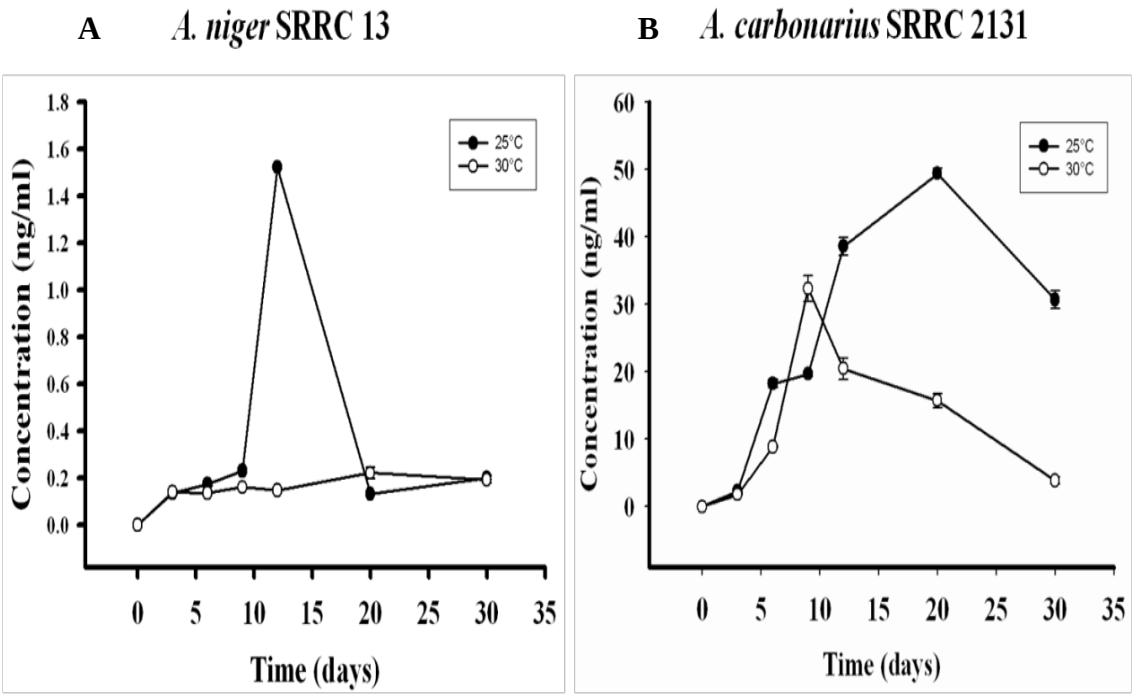


Figure 6.4. Monitoring ochratoxin A production by black aspergilli during 30 days in darkness. (A) *A. niger* SRRC 13 and (B) *A. carbonarius* SRRC 2131. Black circles represent ochratoxin A production at 25 °C and white circles represent ochratoxin A production at 30 °C

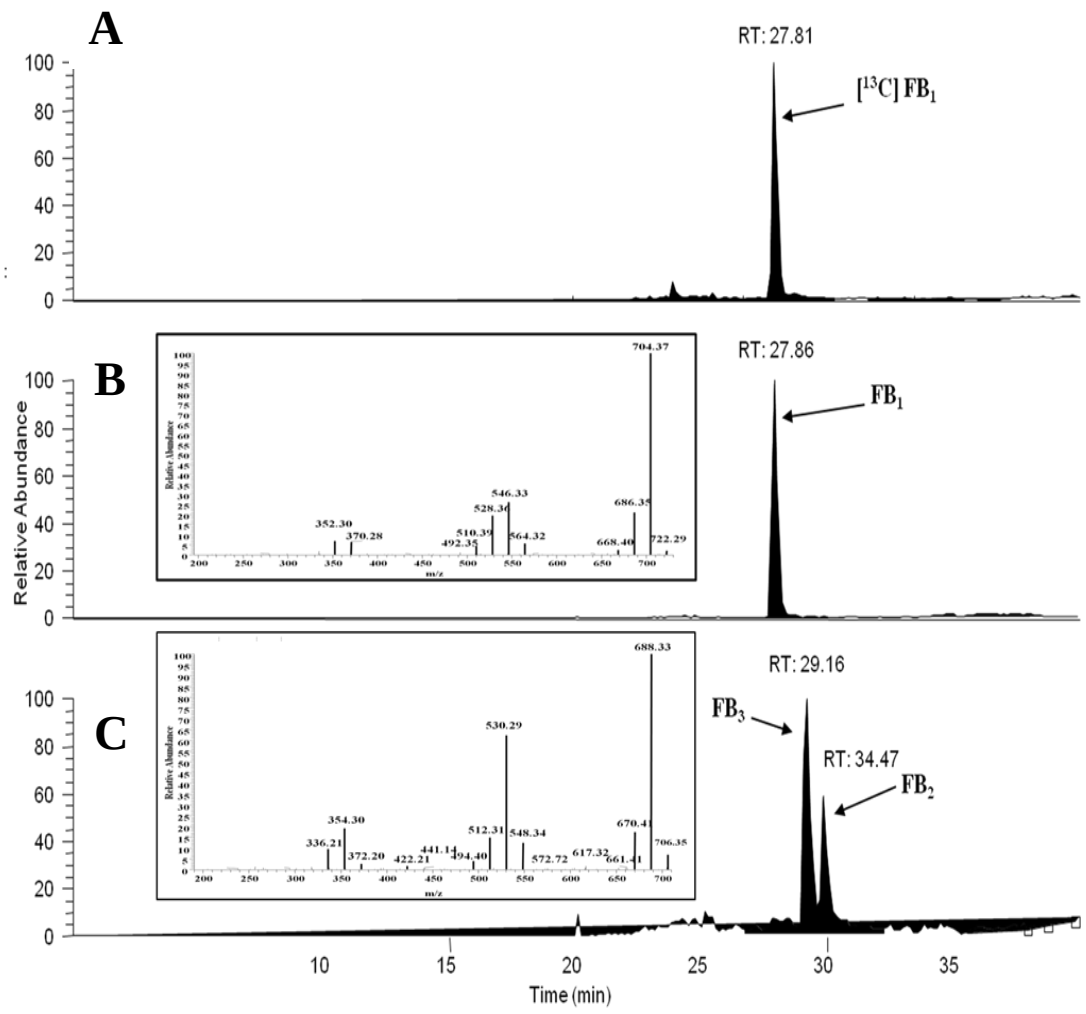


Figure 6.5. HPLC-MS chromatograms for isolate RRC 579 *A. niger*. (A) ^{13}C FB_1 internal standard, (B) FB_1, (C) FB_2 and FB_3 detected from the RRC 579 extract. Inserts in (B) and (C) show the fragmentation patterns for FB_1, and FB_2/FB_3 isomers.

CHAPTER 7

SUMMARY

Filamentous fungi in the *Aspergillus* section *Nigri* (black aspergilli) are important species in mycology, biotechnology and plant pathology. In this group, several species are considered important tools in the biotechnological industry, especially *A. niger*, which is widely used in industrial processes to produce citric acid and organic acid. This group is also known to produce a wide range of secondary metabolites, including ochratoxin A and recently the fumonisins. Ochratoxin A is nephrotoxic, teratogenic, immunosuppressive and potentially carcinogenic in humans and animals. The incidence of elevated concentrations of ochratoxin A in urine and blood samples from human patients is strongly associated with two nephropathies, the Balkan Endemic Neprhopathy (BEN) and the Tunisian Chronic Interstitial Nephropathy (CIN). In both BEN and CIN the ingestion of cereals and cereal-based products is linked to the incidence of these nephropathies, which usually lead to renal failure.

Black aspergilli are usually isolated from soil in cultivated fields. Some species are considered weak or latent pathogens. However, evidence is presented here that endophytic colonization by black species of *Aspergillus* may be common and problematic.

The primary objective of this research project was to determine the incidence of black aspergilli in peanut and maize, which are two important crops in the southeastern United States. The growth of black aspergilli from surface-disinfested seeds and plant tissue on the semi-selective, dichloran rose-bengal chloramphenicol (DRBC) medium, was the first confirmatory step in the identification of the endophytic nature of black aspergilli.

To gain a better understanding of endophytic interactions of black aspergilli with maize and peanut hosts, we studied the colonization patterns of black aspergilli with a live-cell imaging system. For this purpose, we developed a cost-effective genetic transformation system to generate fungal fluorescent transformants. The *A. niger* yellow fluorescent transformants and the *A. carbonarius* red fluorescent transformants were effectively used to monitor fungal colonization in maize and peanut cultivars. When compared with their wild types, the mitotically stable fluorescent transformants did not show difference in terms of phenotype, plant colonization, or mycotoxin production. To confirm intercellular growth of black aspergilli in maize and peanut seedlings, we examined this interaction at the microscopic and ultrastructural level. Both *A. niger* and *A. carbonarius* wild types colonized the intracellular spaces of both hosts. The transformed species can be used in future experiments involving genetic studies to understand the fungal biology of black aspergilli.

To determine the effects of endophytic colonization in maize and peanut, we used 11 black aspergilli strains, encompassing species from the uniseriate and biseriate clade of the *A.* section *Nigri* group. The fungal distribution studies in maize revealed that *A. japonicus* SRRC 325 and *A. aculeatus* SRRC 168 were not able to systemically colonize maize plant tissue, whereas strains of *A. carbonarius* and *A. niger* were able to systemically colonize the plant tissue. Interestingly, even within strains of the same species, there were differences in endophytic colonization of maize cultivars. For instance, *A. carbonarius* SRRC 370 was able to colonize the Pioneer 3140 cultivar, but in another maize cultivar, endophytic infection was limited to the root tissue. Maize plant growth was not affected by endophytic colonization by the black aspergilli. Greenhouse studies of the peanut plant revealed that *A. niger* and its yellow fluorescent transformants were able to endophytically colonize, root, stem, petiole and leaflet

tissue in both Tifguard and Florida 07 cultivars. As for A. *carbonarius* and its red fluorescent transformant, their growth was limited to the root tissue. The effects of fungal colonization on peanut plant biomass indicated that the Tifgard peanut cultivar infected by *A. carbonarius* SRRC 2131 and A. *niger* var. *niger* had a decrease in above-ground biomass, whereas below-ground tissue plant biomass did not change significantly. In the Florida 07 cultivar, *A. carbonarius* decreased root tissue biomass, but *A. niger* did not affect the plant biomass.

Besides the phytopathological importance of black aspergilli as potential biocontrol agents, this fungal group is known for the production of ochratoxin A and the fumonisins. In order to explore the capacity of black *Aspergillus* isolates from peanut and maize fields to produce mycotoxins, we used autoclaved cereal seeds of rye, oat, corn, wheat, and barley to monitor the mycotoxin production by *A. carbonarius* SRRC 2131 and *A. niger* SRRC 13. Our results indicated that *A. niger* produced ochratoxin A exclusively in corn; whereas *A. carbonarius* was able to produce ochratoxin A in corn, rye, and barley seeds. As a result, corn was used as natural substrate to study the production of ochratoxin A by our field isolates. None of the isolates (>90% classified as *A. niger* var *niger*) were able to produce detectable amounts of ochratoxin A. However, when performing a fumonisins analysis on the field isolates, we found that 37% (n = 55) of the black aspergilli isolated from peanut and maize fields produced at least one of the studied isomers of fumonisins, FB_1, FB_2, or FB_3 . From these results 27% (n = 15) were able to produce FB_1, 58% (n=32) produced FB_2, and 44% (n=24) produced FB_3. This is the first report of fumonisins, especially FB1, from the black aspergilli of corn and peanut.

The potential for black aspergilli to become a problem in these two crops is still to be determined. Nevertheless, the results detailed in this dissertation demonstrate that specific species of black aspergilli are present in corn and peanut produced in the United States, and our

study of their ability to endophytically colonize these crops suggests the nature of potential mycotoxin contamination. Thus, this research project may be considered a milestone. Future investigations will need to focus on determining the endophytic associations of black aspergilli with other important crops such as cotton and tomato. More research is needed to study additional effects (beneficial or harmful) of black aspergilli colonization, including plant responses.