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The maize rachis affects *Aspergillus flavus* spread during ear development

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Abstract

Aspergillus flavus that transgenically expressed the green fluorescent protein was used to follow infection in ears of maize hybrids resistant and susceptible to the fungus. Developing ears were needle-inoculated with GFP-transformed *A. flavus* 20 days after silk emergence, and GFP fluorescence in the pith was evaluated at 1, 2, 3, 4, 5, 10, and 20 days after inoculation. Fluorescence levels in the pith of susceptible lines were significantly higher ($P < 0.0001$) than in resistant lines at all time points. Pith sections apical to the inoculation point displayed higher fluorescence levels compared to other sections of the ear, suggesting fungal spread via the water/nutrient transport system. Fluorescence levels in resistant lines did not change significantly over time, implying spread of the fungus but not growth. Fluorescence in susceptible ears was highest at early time points, suggesting that conditions were more conducive to spread than at the later time points. These results suggest that the rachis could retard the spread and/or growth of the fungus inside the developing maize ear. Although fluorescence was observed in kernels from resistant ears, it occurred at a much higher frequency in those from susceptible hybrids. Together, these results suggest that the rachis is used by maize as a defense structure similar to other preformed types of resistance.

Keywords: *Zea mays*, rachis, *Aspergillus flavus*, fluorescence microscopy

Introduction

Invasion of *Zea mays* L ears by *Aspergillus flavus* frequently produces aflatoxins, one of a family of toxic, carcinogenic, mutagenic, and teratogenic mycotoxins that causes acute poisoning, liver damage, and chronic toxicity in humans and animals. Aspergillus flavus infection and aflatoxin contamination of maize can occur either pre- or post-harvest. In the field, drought stress aggravates invasion of husks by insects, such as corn earworm and corn borer, which facilitate *A. flavus* transmission (McMillan 1983, Rodriguez et al 1983). This physical injury provides a site for entry of plant tissues by fungal conidia. For example, *A. flavus* did not infect non-wounded bean leaves or corn kernels at 22°C, but caused moderate to severe symptoms when these tissues were injured during inoculation (St. Leger et al 2000). Injured plants could also be infected by wind-borne conidia. Sclerotia that germinate in the soil and disperse airborne conidia are believed to be the primary inoculum in maize fields (Wicklow 1983). Once introduced into the ears, the conidia germinate resulting in fungal growth and establishment in the tissues. Aflatoxin is a metabolite that can be produced from this growth. *A. flavus* conidia can also germinate on the surface of silks, progress to the glumes and ultimately colonize the kernel (Marsh and Payne, 1984).

Although research and breeding efforts to identify quantitative trait loci (QTL) for maize resistance to *A. flavus* infection and/or aflatoxin accumulation (Busboom and White, 2004; Brooks et al, 2005; Kelley et al, 2010; Warburton et al, 2010) are active, there still is much to learn about fungal growth within the maize tissues once inoculation has occurred. Microscopic examination of ears of a nonresistant maize line that were wound-inoculated with a toothpick through the husk to the edge of the rachis (Smart et al, 1990) indicated that *A. flavus* spread from the wound 14 days post-inoculation (dpi) and could be found throughout all rachis tissues except the pith and lignified fibers at 28 dpi. The fungus entered the rachillae of adjacent spikelets from the rachis and the insertion points of the bracts. Infection of kernels was always through the rachilla, and hyphae did not enter the endosperm through the exterior of the pericarp. Clearly, spread of the fungus through the rachis was an important infection mechanism in wound-inoculated maize ears. Aflatoxin was not detected in non-infected kernels

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indicating that it was not translocated through the rachis (Smart et al, 1990). *Aspergillus flavus* infection through the silk, on the other hand, typically occurred after pollination, which initiates silk senescence (Marsh and Payne, 1984). After progressing through the silk, hyphae penetrated the kernels through the pedicel, which appeared to provide the primary route for fungal invasion (Lillehoj, 1983).

While a number of maize lines with enhanced resistance to *A. flavus* have been developed (Windham and Williams, 2002), limited work has been done to elucidate the resistance mechanism. One possible resistance factor is the ability of specific tissues to impede fungal invasion and spread. Consistent with this premise is a study by Pechanova et al (2011), which showed that the developing rachis of resistant genotypes constitutively expressed stress-related proteins and enzymes catalyzing reactions in the phenylpropanoid pathway whereas susceptible genotypes did not express these proteins until they were infected with *A. flavus*. Since wound inoculation by insect vectors is a major route of pre-harvest *A. flavus* infection, and fungal spread within the ear and subsequent infection of the kernels could occur through the rachis and rachillae (Smart et al, 1990), resistance of the rachis to fungal spread could be an important component of overall resistance.

In this study, we used an *A. flavus* strain transformed with the gene for green fluorescent protein to test the hypothesis that fungal growth is retarded in the rachis of maize lines resistant to *A. flavus* infection. The progress of fungal infection within developing ears was followed from the point of inoculation in the center towards each end of the of the ear. We compared the path of infection in resistant and susceptible ears and quantified fluorescence levels in rachis cross-sections. The spread of the fungus within the rachis and kernels was examined in an attempt to visualize its spread through the ear and into the kernels.

Materials and Methods

Aspergillus flavus propagation

Aspergillus flavus strain GAP 2-8 (Magbanua et al, 2007), transformed with and expressing the Aequorea victoria Green Fluorescent Protein (GFP), was used to monitor growth of the fungus within a developing maize ear. The plasmid used for transformation incorporated the CMV promoter fused to the GFP open reading frame (personal communication, Dr. Gary Payne). The transformed fungus was maintained in Czapek agar solution supplemented with NaCl (7.5% NaCl, 4.9% agar) at 28°C and transferred once a month. Inoculum was prepared by transferring the fungus into sterile 500-ml flasks, each containing 50 g corn grits and 100 ml sterile distilled water, and incubating at 28°C for 3 weeks. Conidia were washed from the grits with 500 ml autoclaved distilled water containing 20 drops l^{-1} of Tween 20 and filtered through four layers of sterile cheesecloth. The concentration of the conidia in the filtrate was measured using a hemocytometer and adjusted to 9 x 10^7 conidia ml^{-1} using sterile distilled water. The filtered inoculum was stored at 4°C until use.

Plant material and inoculation with A. flavus

Hybrids produced from the resistant inbreds Mp313E, Mp420 and Mp494 and the susceptible inbred lines Mp339, SC212m and GA209 were used in this study (Windham and Williams 2002). The resistant hybrids Mp313E x Mp420 and Mp313E x Mp494 and susceptible hybrids GA209 x SC212m and Mp339 x SC212m were planted on April 23, 2001 and silk emergence was tagged for each ear. Plants were allowed to undergo open-pollination. Approximately 20 days after silk emergence (DAS), the midpoint of the ear was inoculated through the husk using a modified pin bar technique (Reid et al, 1996). Approximately 100 µl of inoculum was delivered to the ear. The inoculated area was marked with a waterproof pen. Ears were inoculated at the midpoint so the fungus could move in the apical and/or the basal direction. The mode of entry of the fungus was through mechanical wounding of 1 to 4 kernels located in the middle of the ear. The fungus was delivered into the wounded kernels and approximately ¼ inch into the rachis where the kernels were attached. Negative controls were inoculated with either sterile water or non-GFP transformed A. flavus.

Ear collection and preparation

Inoculated ears were collected at 1, 2, 3, 4, 5, 10, and 20 days after inoculation (DAI). There were three replicates for each time point. With the inoculation point marked, the collected ears were husked and cut into 1-cm cross-sections from tip to base. The sections were stored in the correct spatial order in brown paper bags (Lawson Showerproof No. 504), dried at 50°C for 3 days, and stored at room temperature until analyzed. Earlier results had indicated that drying was required to reduce autofluorescence in the rachis tissues (data not shown).

Pith cultures

Pith sections excised from resistant and susceptible ears at 1, 3, and 5 DAI were cultured on Czapek agar solution. Three cross-sections were selected from each ear, representing the inoculation point and locations apical (towards the tip of the ear) and basal (towards the base of the ear) relative to the inoculation point. Pith sections from each location were aseptically removed, surface-sterilized with 70% ethanol, and quickly flamed to vaporize the ethanol. The sections were cut into smaller pieces and placed on labeled Petri dishes containing Czapek growth medium. The plates were incubated at 28°C for 24 hours and the fluorescence in each section assessed.

Microscopy

An Olympus SZX12 dissecting microscope was used to visualize and analyze the sections and for

Table 1 - Fluorescence levels in sampled ear sections from the hybrids by different categories.

Genotype	Mp313xMp420 (resistant)	Mp313xMp494 (resistant)	GA209xSC212m (susceptible)	Mp339xSC212m (susceptible)
	Mean Fluorescence Level mm^{-2}			
Category				
Overall	398.52A	378.65A	988.57B	754.24C
By location in ears				
Basal	368.85a	343.00a	918.24a	667.57a
Inoculation point	405.08ab	371.72a	941.10a	725.19a
Apical	421.63b	421.24b	1106.36b	869.94b
By time after inoculation				
1 DAI	351.85ac		1168.9a	767.40abc
2 DAI		401.07b	1446.4b	753.25ac
3 DAI	408.81ab	398.73b	1177.9a	933.32b
4 DAI	299.70c	357.36b	818.1c	689.02acd
5 DAI	440.25b	377.83b	970.0ac	820.32ab
10 DAI	367.18abc	368.30b	971.6ac	621.55cd
20 DAI	414.11ab	365.92b	556.0d	570.09d

Data within a single column bearing the same upper case superscript do not differ significantly ($\alpha = 0.05$). Data within a single row bearing the same lowercase superscript do not differ significantly ($\alpha = 0.05$). Missing time points correspond to samples or replicates damaged in the field. Category: "Overall" - For each genotype, sampled ear sections from the different positions and time after inoculation were pooled and fluorescence was analyzed; "By location in ear" - For each genotype, sampled sections were pooled and grouped based on their position in the ear (basal, inoculation point or apical) and fluorescence was analyzed; "By time after inoculation" - For each genotype, sampled sections were pooled and grouped based on time after inoculation and fluorescence was analyzed.

digital image capture. For the different hybrids, the fluorescence level in inoculated ears was assessed at approximately the midpoint, where the ear was needle-inoculated, and in sections apical and basal relative to the midpoint. Optimum fluorescence was obtained (Du et al, 1999) using excitation and emission wavelengths of 395 nm (filter BP460-490) and 509 nm (filter BA510IF), respectively. The baseline exposure time for each genotype was the longest exposure time at which no fluorescence was detected in the water- or wild type *A. flavus*-inoculated negative control. Quantity One (BioRad, Hercules, CA) image analysis software was utilized to quantify the fluorescence from each section. The fluorescence levels were measured in the pith area to obtain standardized measurements for all samples. Fluorescence was normalized with respect to the area of the pith section and expressed as intensity mm^{-2} to offset size differences between individual ears and the variation in pith diameter between the different sections of an ear. Fluorescence data were analyzed using Excel (Microsoft, Redmond, WA) and the GLM procedure in SAS (The SAS Institute Inc, Cary, NC).

Results and Discussion

To evaluate whether the rachis contributes to maize resistance to *A. flavus* infection, we used a strain of the fungus expressing GFP to follow its path of accumulation through the maize ear. The fungus was inoculated in the middle of the ear through the kernels and into the rachis via a modified pinbar (Reid et al, 1996). GFP fluorescence was evaluated and compared among all samples as an indirect measure of *A. flavus* invasion. Studies of *A. flavus* gene expression and colonization in corn kernels using the same GFP-transformed strain indicated that GFP expression could be used for screening genotypes that are resistant to the fungus (Du et al, 1999). We believe that the use of a reporter such as GFP increased the sensitivity of this assay, permitting visualization of fungal spread at the time points selected. Extreme care was undertaken in the analyses to ensure accurate results, through the use of controls, proper fluorescence detection protocols to avoid autofluorescence interference and validation of the presence of viable fungi using pith cultures (subsection Recovery of GFP-tagged *A. flavus* strain from cultured pith sections).

The pith, at the center of the maize ear, provides support and conducts nutrients to the kernels, and is comprised of ground cells, which are highly vacuolated, unlignified, parenchyma cells. The pith is circumscribed by the main vascular bundles that serve as the terminus for each rachilla and from which radiate a thick layer of increasingly lignified parenchyma tissues (Smart et al, 1990). Lignin is a potential source of autofluorescence (Billinton and Knight, 2001), and its interference was eliminated by viewing the samples at the optimum wavelengths of GFP excitation and emission, 395 nm and 509 nm, respectively (Du et al, 1999). Autofluorescence effects were further minimized by drying the ear sections. In addition, cross-sections of ears that were mock inoculated with water or with wild-type *A. flavus* controls were examined. The background fluorescence levels for all hybrids were very low for both types of controls (data

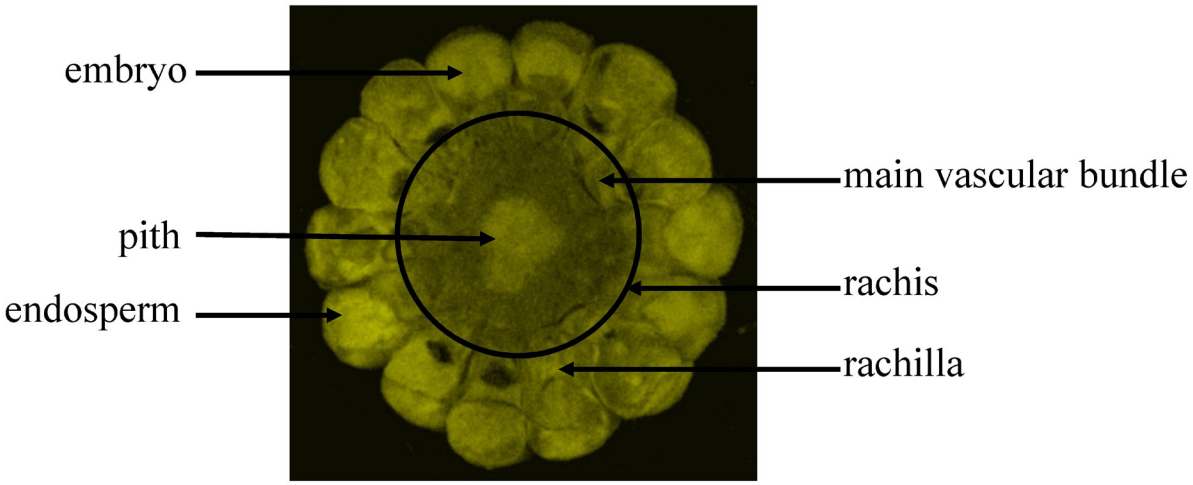

Figure 1 - Photograph of a cross-section of a maize ear in white light. The drying process caused a slight distortion, but the different parts are still distinguishable and are labeled. Encircled section represents the rachis where the fluorescence photomicrographs were taken.

not shown) and the settings used to view them were used as the exposure time for digital image capture of the experimental samples.

Fluorescence levels and patterns in wound-inoculated ears of susceptible and resistant hybrids are different via microscopy

To aid in the visualization of the images, Figure 1 is presented. It is a white light picture of the cross section of a maize ear with the pith area of interest indicated by a circle. GFP fluorescence was examined in pith sections of the ears taken from base (I-B), middle (I) and tip (I-T) from the ears of the susceptible hybrids GA209xSC212m and Mp339xSC212m. Strong fluorescence in these genotypes was apparent at 1 and 3 DAI, respectively. The pattern of fungal spread was similar for both susceptible hybrids and that of GA209xSC212m is shown in Figure 2. Ears collected from GA209xSC212m exhibited fluorescence in the pith area, lignified parenchyma cells around the pith, rachilla and pericarp (Figure 2). At these time points, some ear cross-sections already exhibited extensive penetration of the pith and main vascular bundles. The fluorescence radiated into individual rachillae from the circumference of the pith and then into the black abscission layer and pericarp. This pattern of fluorescence was observed in all of the replicates examined.

In contrast, fluorescence in the ear sections of the resistant hybrids Mp313ExMp420 and Mp313E x Mp494 at 1 DAI (data not shown) was barely visible and only slightly higher than background. In most sections examined, fluorescence was detected only at the periphery and not in the center of the pith. Very little fluorescence was detected beyond the rachis. Fungal spread through the ear at 3 and 5 DAI was not as pronounced in the resistant hybrids as in the susceptible hybrids. It was clearly visible that the fluorescence levels and patterns were different in resistant and susceptible genotypes.

Longitudinal distribution of fluorescence in wound-inoculated resistant and susceptible developing ears

To determine if there were differences in fungal abundance in the base, center and tip of the developing ear, fluorescence was quantified using Quantity One and compared from representative piths taken from each of these cross-sections, which was an indirect way of comparing the fluorescence along the longitudinal axis. These results are summarized in Table 1. Between the two susceptible hybrids, the total fluorescence measured in the sampled sections was higher ($p < 0.001$) in GA209xSC212m than in Mp339xSC212m. The peak fluorescence level was observed at 2 DAI in GA209xSC212m and at 3 DAI in Mp339xSC212m. At 20 DAI, however, fluorescence levels did not differ significantly between the two hybrids. The spatial distribution of fluorescence was similar in the ears of both lines. In most ears examined, all sections apical of the inoculation site (and thus downstream of nutrient flow) displayed higher fluorescence levels compared to the inoculation site and all sections basal (upstream) of it ($p = 0.0008$). The higher level of fluorescence in the apical sections was manifested as an increased intensity or a larger area of fluorescence in the pith, or both. The center and basal sections exhibited similar levels of fluorescence (Table 1).

In comparison, fluorescence in the resistant hybrids was 2.5- to 5-fold lower ($p < 0.0001$) than in the susceptible hybrids, indicating a much lower rate of fungal spread. In fact, the fluorescence in the ear sections was barely visible and only slightly higher than background. In most sections examined, fluorescence was detected only at the periphery and not in the center of the pith (data no shown). Comparison of the two resistant hybrids indicated no significant

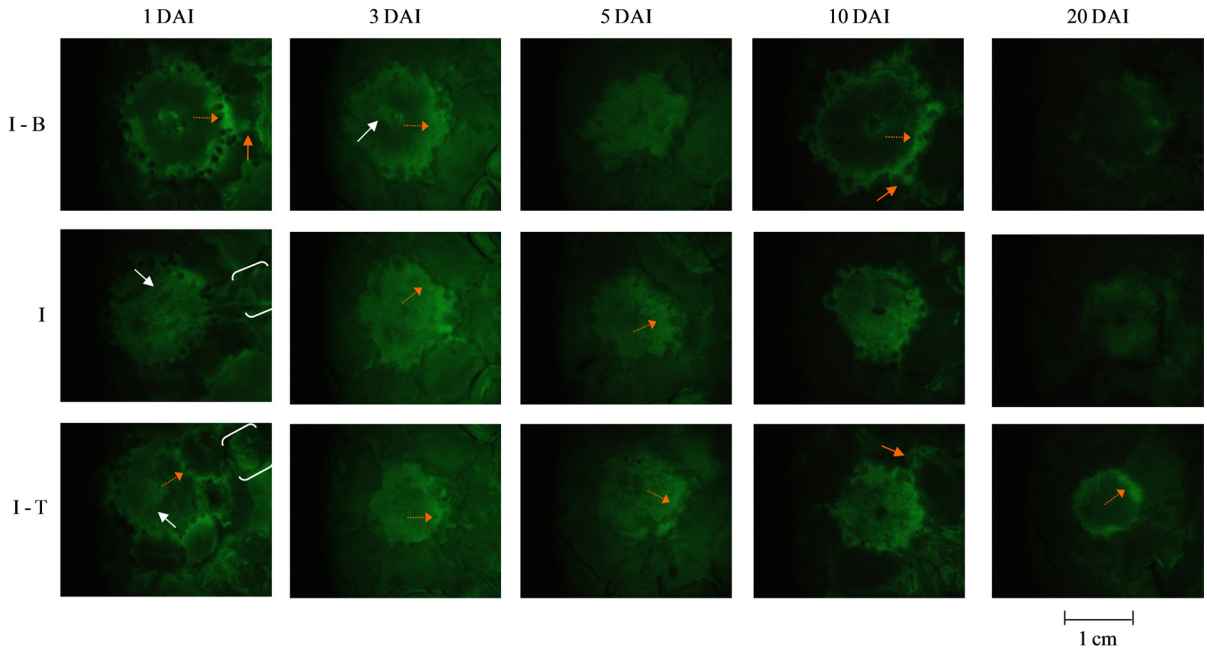

Figure 2 - Fluorescence photomicrographs of cross-sections of the pith area in ears of the susceptible hybrid GA209xSC212m inoculated with GFP-tagged *A. flavus* and collected at 1, 3, 5, 10, and 20 DAI. White and orange arrows indicate apparent movement of the fungus into the pith and kernel, respectively, and the dashed orange arrow points to the fluorescing main vascular bundle. Brackets enclose kernels exhibiting fluorescence, while yellow arrowheads indicate fluorescing endosperm. The kernels were situated on different planes. I - B, a section between the base and the point of inoculation; I, inoculated section; I - T, a section between the inoculated section and the apical end.

difference in overall fluorescence (Table 1). However, the spatial pattern of fluorescence, with higher levels in the apical sections than in the inoculated and basal sections, was similar in all four hybrids studied. Fluorescence occurred in the pith area, through which nutrients are conveyed to the kernels in an apical direction. Taken together, these results suggest that *A. flavus* could exploit the water/nutrient transport system for spread and possibly growth within susceptible ears. The center of the pith is composed of potentially unlignified, highly vacuolated parenchyma cells that are unlikely to deter the spread of *A. flavus*. In comparison, significantly less fungal spread was observed in resistant ears.

Inoculation was performed at 20 DAS when the kernels were in the milk stage, which is characterized by rapid embryo growth and starch accumulation in the endosperm. This condition is favorable for transport of photosynthate to the kernels and could explain why the highest fluorescence levels were observed at 1 or 3 DAI in susceptible hybrid lines. At 20 DAI (40 DAS), on the other hand, the kernels were in the dent stage, with a moisture content of 55%. This could account for the reduced fluorescence in the susceptible lines at 10 and 20 DAI, as fungal growth could be reduced by moisture limitations, particularly in the rachis. An alternative explanation is suggested by the findings of Pechanova et al (2011), who reported that stress-associated proteins in the rachis of susceptible genotypes are induced only upon exposure to the fungus. Hence it is possible that, at the earlier time points, the susceptible rachis contained insufficient resistance factors to hinder fungal spread, but within 20 d accumulated sufficient levels of these proteins to overcome the fungus. This would suggest that, given sufficient time after inoculation, fungal growth and colonization would be deterred to a certain degree even in susceptible rachis. The resistant rachis, on the other hand, contained high levels of the resistance factors even before infection, and immediately impeded fungal spread or growth. GFP fluorescence levels in resistant hybrids were significantly lower than in the susceptible hybrids and were almost uniform over time, but slightly higher in the apical sections. This observation suggests that the fungus was sustained and able to survive in these lines, but its spread and/or growth was arrested by resistance factors in the ear, and particularly in the rachis. The resistant rachis has been shown to express stress-related proteins constitutively (Pechanova et al, 2011), hence high levels of the proteins were maintained even prior to infection, and fungal spread was immediately impeded after inoculation. The fluorescence observed at rachis sections away from the inoculation point may have been due to translocation of the fungus via the water/nutrient transport system, but the amount of fluorescence in this region of the resistant ear was far less than in the susceptible ear. Additional studies have shown that the rachis of the resistant inbred Mp313e has the potential to form a

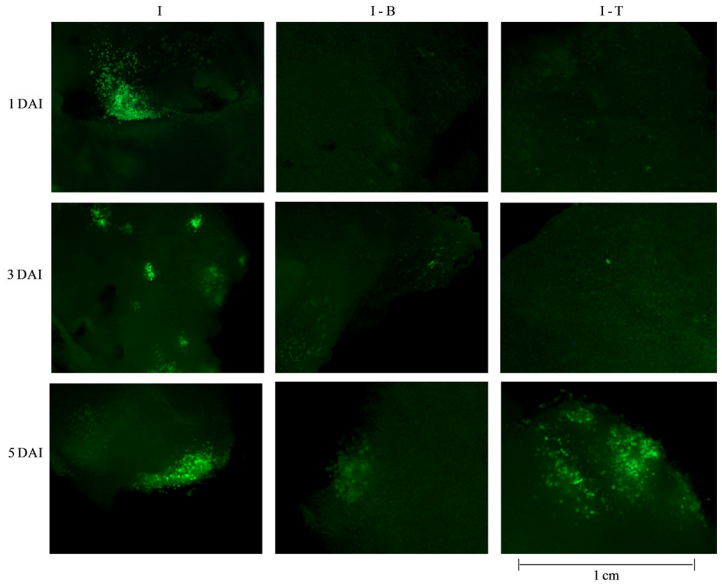

Figure 3 - Fluorescence photomicrographs of cultured pith sections from ears of the susceptible hybrid GA209xSC212m. Ears were inoculated with GFP-tagged *A. flavus*, collected at 1, 3 and 5 DAI and oven dried to minimize background fluorescence. Pith sections were excised, surfaced-sterilized, cultured on Czapek growth medium for 24 hours and viewed under a microscope.

more highly cross-linked lignin structure than the susceptible inbred SC212m, which could further impede fungal spread through the ear (unpublished data, DS Luthe).

Recovery of GFP-tagged A. flavus strain from cultured pith sections

Rachis sections excised from ears inoculated with the *A. flavus* strain expressing GFP were also aseptically grown on agar plates to determine if fungal growth as determined by GFP fluorescence could be obtained from sections distal from the inoculation site. We proposed that this would determine if viable GFP-labeled fungi could be cultured from the rachis and verify its spread in the ear. Although direct microscopic examination of ear sections from resistant hybrids revealed fluorescence in all sections at 3 and 5 DAI, and inoculated and apical sections at 1 DAI, growth of GFP-labeled fungus was only recovered from pith cultures collected from the inoculated sections of the 3 and 5 DAI samples. When pith samples from inoculated and apical sections at the 1, 3 and 5 DAI time points, and basal sections of the 3 and 5 DAI time points from the susceptible hybrids were cultured, GFP-labeled labeled fungus grew in all samples (Figure 3). The recovery of GFP-labeled fungi in the apical section at 1 DAI shows rapid spread of the fungus through the rachis in the initial stages of infection. The spread appears to continue until 5 DAI when GFP-tagged fungi were recovered at the apical and basal sections. These results are consistent with the hypothesis that the susceptible rachis "allowed" spread of the fungus. Currently, we do not know the mechanism of fungal spread within the developing ear. The fungus could be dispersed through the ear via vascular system or apoplastically early in the infection process. Also, while the highest fluorescence levels were visualized at 1 DAI in the developing ears, these samples did not yield the highest fluorescence levels in culture. Nevertheless, the pith cultures demonstrated that actively growing fungi could be recovered from sections that did not come into direct contact with it during inoculation. The differences in the fungal spread pattern indicated by fluorescence microscopy *vis-à-vis* the pith cultures could have been due to the differences between growth conditions in the culture medium and the ear.

Conclusions

Although it is well known that the rachis provides mechanical support for the grain and transports water and essential nutrients to the developing kernels, its potential role in protecting the developing ear from *A. flavus* infection and aflatoxin accumulation has been overlooked. The results of this study are significant because they demonstrate that the developing rachis from genotypes that are resistant and susceptible to *A. flavus* infection both appear to utilize the same mechanism to defend against fungal attack. However, the resistant rachis retards the abundance of the fungus and limits invasion of the developing kernels more effectively.

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