A Visual Pattern of Mycotoxin Production in Maize Kernels by *Aspergillus* spp.

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ABSTRACT

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Different *Aspergillus* species (*flavus, parasiticus,* and *nidulans*), which produce different intermediates and end products of the aflatoxin pathway (norsolorinic acid, NOR; sterigmatocystin, ST; and aflatoxin, AF), are useful in studying the maize-*Aspergillus*-mycotoxin interaction. *Aspergillus* AF mutants, which produce NOR (a visible orange intermediate of both ST and AF), were used to visualize mycotoxin deposition in host and fungal tissues. NOR was seen in specific maize kernel tissues (embryo and aleurone) and specific fungal tissues (substrate mycelium but not sporulating mycelium) within 24 h after inoculation of kernels or growth media. ST and AF were found in the same maize tissues but only after organic extracts of these tissues were quantitated by time-consuming chromatography methodologies. Mycotoxin production and fungal ingress by all three *Aspergillus* spp. were subject to regulation by the developmental stage of the maize kernel: both fungal colonization and NOR deposition shifted from embryonic to endosperm tissues in germinating maize kernels. The appearance of NOR flagged the progress of fungal invasion through kernel tissues. We suggest that NOR mutants may be useful tools to identify likely infection sites in maize kernels and that the genetically characterized *A. nidulans* may be useful in helping identify global regulatory mechanisms in the maize-*Aspergillus*-mycotoxin interaction.

Additional keywords: polyketide, secondary metabolism, *Zea mays*.

Aspergillus spp. are seed-deteriorating fungi known for their ability to produce mycotoxins in crops such as maize, peanuts, tree nuts, and cottonseed (13–15,17). Aflatoxin (AF) and sterigmatocystin (ST), end products of the same biosynthetic pathway (Fig. 1), are two of the most common *Aspergillus* mycotoxins. AF is produced by *A. flavus* Link:Fr. and *A. parasiticus* Speare, and ST is produced by several *Aspergillus* spp. including *A. nidulans* (Eidam) G. Wint. and *A. versicolor* (Vuill.) Tiraboschi (1,13–15). These mycotoxins are derived from polyketides, which are complex molecules synthesized in a manner similar to the synthesis of fatty acids (16). Polyketides are among the most abundant secondary metabolites produced by fungi; other fungal polyketides include spore pigments (5), mycelial melanins (2), and phytotoxins (20,34). *Aspergillus* mycotoxin production is not thought to be necessary for pathogenesis and is induced only under certain nutritional and developmental environments (37). Additionally, there is a range in mycotoxin-producing ability among isolates of *Aspergillus* (7,10) and a consequent imperfect correlation between *Aspergillus* infection and AF formation in food products (32).

The maize crop is especially subject to infection by *A. flavus* and subsequent AF contamination. Other *Aspergillus* spp., especially *A. parasiticus,* have also been isolated from maize kernels (13,28). One difficulty in assessing AF contamination in maize kernels is the limited methodologies currently available for AF detection. Although the fungus is often seen growing on and around kernels, AF and ST are not visible. Instead, AF and ST are routinely extracted from pulverized host tissues by organic solvents. Such methodologies are uninformative in terms of identifying the spatial and temporal induction and deposition of mycotoxins in specific maize-kernel tissues.

To overcome these limitations, we chose to identify *Aspergillus* isolates that could improve our ability to investigate the biology of the maize-*Aspergillus*-AF interaction and allow us to see the pattern of AF deposition in the maize kernel. We first examined the AF biosynthetic pathway and the *Aspergillus* spp. that produce AF and/or ST (Fig. 1). Of significance was the fact that although all four AFs (B_1, B_2, G_1, and G_2) and ST are not visible in white light, all of the precursors in the pathway from norsolorinic acid (NOR) through versicolorin A are highly visible, pigmented compounds. Most *A. flavus* strains produce AFB_2 and AFB_1 as end point metabolites, and *A. parasiticus* isolates produce all four AFs as end point metabolites. However, there are several mutants of both species that are blocked in the pathway and accumulate various pigmented intermediates. We chose to work with one isolate of each species that accumulates NOR, a bright orange compound easily seen by the eye. These isolates gave us the ability to watch AF deposition in maize-kernel tissues. Also of interest was the fact that *A. nidulans,* a genetically defined fungus used as a model system for many biological studies (35), produces ST as an end point metabolite (19). We have recently characterized the *A. nidulans* gene, *verA,* necessary to convert versicolorin A to sterigmatocystin and have identified several *A. nidulans* metabolic mutants that could be useful in understanding the regulation of the AF-ST biosynthetic pathway (18). Because this *Aspergillus* sp. is so amenable to molecular studies, we wished to determine whether *A. nidulans* infected and produced mycotoxins in maize kernels in a manner similar to that of *A. flavus* and *A. parasiticus* so as to validate the use of *A. nidulans* in future studies of AF production in maize kernels.

MATERIALS AND METHODS

Fungal isolates. The following isolates were used: *A. flavus* NRRL 19772, *A. nidulans* FGSC 26 (Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City), *A. parasiticus* SK1 (courtesy of J. Bennett and S. Kale, to be deposited with ATCC), and *A. versicolor* SRRC 110. *A. flavus* NRRL 19772 and *A. parasiticus* SK1 are white-spored AF mutants that accumulate NOR. These NOR mutants are incompletely blocked at the step from NOR to averantin and produce some AF, which enables dual assessment of both NOR and AF production with these and related isolates (3,27; Fig. 1). *A. nidulans* FGSC 26 and *A. versicolor* SRRC 110 produce ST. All fungal isolates were maintained at −80 C as 33% glycerol stocks. Spore suspensions were prepared from 7-day-old cultures grown on potato-dextrose agar at 29 C in darkness.

Maize. Uninfected seeds of maize (*Zea mays* L.) hybrid TX804 × TX814 (courtesy of A. Bockholt), stored in an incubator at 4 C, were used for all maize-kernel experiments. Seeds were always surface sterilized in 0.26% (w/v) NaOCl for 3 min, rinsed in sterile distilled water, and air dried in a sterile hood before inoculation with 5 μl of a suspension (1×10^6 spores per milliliter) of the appropriate fungal isolate. All work was conducted in a biosafety hood, and gloves were worn throughout the entire procedure.

Production of mycotoxins in vitro. The two NOR-producing isolates (*A. flavus* NRRL 19772 and *A. parasiticus* SK1) were assessed for their ability to produce visible NOR on a known AF-inducing medium (2.0% yeast extract and 2.0% sucrose [YES]) and a non-AF-inducing medium (2.0% yeast extract and 2.0% peptone [YEP]) with and without 2.0% ethanol. *A. nidulans* FGSC 26 was similarly assessed for its ability to produce ST. Sterile 10-dram vials containing 3 ml of the appropriate agar medium were inoculated with 5 μl of a suspension (1×10^6 spores per milliliter); this procedure was replicated three times. A water control was included. NOR production was visually noted on a daily basis. After 1 wk of fungal growth on each medium, NOR, ST, and AF production was evaluated by standard chromatography methodologies. The metabolites were extracted by adding 1.5 ml of acetone to the vials for 1 h followed by the addition of 1.5 ml of chloroform. This extract was transferred to clean vials, dried down under a fume hood, and resuspended in chloroform. Two replicates were analyzed by spotting 20 μl of the solution on thin-layer chromatography plates. Plates were developed in ether-methanol-water (96:3:1, v/v/v) for AF and NOR and in benzene-glacial acetic acid (95:5, v/v) for ST. Fluorescent zones were compared to known standards of ST and AF (Sigma Chemical Company, St. Louis, MO) and a crude NOR extract previously prepared from *A. parasiticus* SRRC 162. One repetition was quantitated by high-performance liquid chromatography (HPLC) as follows.

For NOR and AF quantitation, the crude chloroform extract was dried under nitrogen and resuspended in 1 ml of chloroform. A 500-μl aliquot was used to quantitate all four AFs (AFB_1, AFB_2, AFG_1, and AFG_2), and 500 μl was dried under nitrogen and resuspended in methanol to quantitate NOR. NOR was detected by following the procedures of McCormick et al (24) with a Waters HPLC unit with dual pumps and WISP autosampler with a C18 (10-μm) Radial-Pak cartridge column. A gradient program with two solutions was employed. Solution A consisted of methanol and tetrahydrofuran (2:1), and solution B consisted of 0.1 M acetic acid. NOR was eluted on a gradient of 67–79% solution A at a flow rate of 2 ml/min. NOR was detected at 313 nm. AFs were eluted from a normal phase silica gel column by a single solvent system (750 ml of chloroform, 225 ml of cyclohexane, 25 ml of isopropanol, and 25 ml of acetonitrile) at a flow rate of 2 ml/min. AFs were detected at 365 nm.

For ST quantitation, the crude chloroform extract was dried under nitrogen and resuspended in 1 ml of chloroform. ST was eluted from a Waters C18 (5-mm) LiChrosorb Hibar column by a single solvent system (80% acetonitrile) at a flow rate of 1 ml/min. ST was detected at 245 nm.

Visual description of tissue specificity of fungal colonization and NOR production on seed halves. Maize kernels are composed largely of endosperm and embryo tissues (4). To better observe which of these tissues were preferentially colonized by different *Aspergillus* spp., maize seeds were cut in half and inoculated with *A. flavus* NRRL 19772, *A. nidulans* FGSC 26, *A. parasiticus* SK1, or *A. versicolor* SRRC 110. A water control was also included. For each *Aspergillus* sp., five seeds were longitudinally cut in half through the embryo and placed on moist filter paper in a single glass petri dish containing a vial cap filled with distilled water. This humidity chamber favored fungal growth but not kernel germination (seeds were damaged by this treatment, but some seed halves were still able to germinate after several days). Immediately upon being cut, seed halves were inoculated on the embryo-endosperm interface with 5 μl of the spore suspension. Seeds were incubated in the dark at 29 C and examined daily for fungal growth (i.e., abundant sporulation) on embryo and/or endosperm tissue. Hyphae and spores were washed off in a water-filled beaker to improve the visual assessment of NOR accumulation in randomly chosen seeds inoculated with *A. parasiticus* SK1 and *A. flavus* NRRL 19772. Positive responses were de-

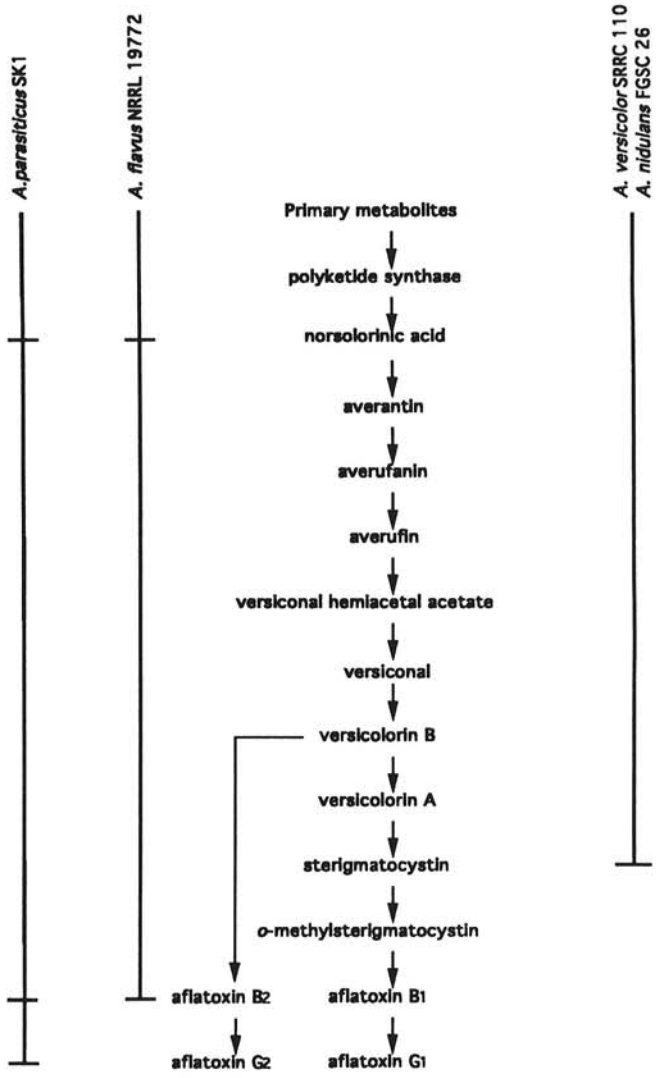

Fig. 1. Aflatoxin and sterigmatocystin are end products of the same biosynthetic pathway. The first stable intermediate in the aflatoxin-sterigmatocystin pathway is the red-pigmented intermediate norsolorinic acid (NOR), which is produced by a polyketide synthase. The pathway bifurcates at versicolorin B to produce the aflatoxins B_2, G_2 and B_1, G_1. *Aspergillus parasiticus* SK1 accumulates NOR as well as all four aflatoxins; *A. flavus* NRRL 19772 accumulates NOR and aflatoxins B_1 and B_2; and both *A. versicolor* SRRC 110 and *A. nidulans* FGSC 26 accumulate sterigmatocystin.

scriptive and consisted of observing 1) whether each *Aspergillus* sp. preferentially colonized embryo and/or endosperm tissues and 2) when and where NOR accumulated. Treatments were replicated five times (i.e., five plates per *Aspergillus* sp.). After 1 wk, seeds were autoclaved and discarded.

Production of mycotoxins in whole maize kernels. In excess of 300 seeds were wounded by piercing the pericarp covering the endosperm or embryo with a sterile needle. The seeds were then inoculated with 5 μl of the spore suspension of *A. parasiticus, A. flavus,* or *A. nidulans.* Unwounded seeds were also inoculated on either the embryo or endosperm side of each seed with *A. parasiticus* or *A. flavus* as controls to see where NOR first appeared in "natural" conditions. Water controls were also included. Five seeds were placed in one humidity chamber as described above. Treatments were replicated in excess of five times to allow investigation of mycotoxin production in ungerminated seeds. Germinated seeds were discarded. All seeds were incubated in the dark at 29 C and examined daily for the presence of mycelium and NOR accumulation.

After inoculation, seeds were chosen randomly at daily intervals and cut open to visually assess internal NOR accumulation. A pair of needle-nosed forceps was used to separately peel the pericarp and aleurone layers away from the endosperm of endosperm-inoculated seeds (the aleurone is a lipid-rich, one- to three-cell layer located between the endosperm and pericarp tissues). The embryo was dissected from the endosperm and discarded. The remaining separated tissues (pericarp, aleurone, and endosperm) were visually assessed for the presence of NOR.

In inoculated embryo-wounded kernels, NOR, AF, and ST production was quantitated over a 2-wk period. All seeds were inoculated on the same day, incubated in the dark at 29 C, and visually examined daily for the presence of mycelium and NOR accumulation in those inoculated with *A. flavus* and *A. parasiticus.* Ten embryo-wounded seeds (10 seeds were needed for ease-of-extraction methodology) were removed from the incubation chambers at intervals of 3, 6, 9, and 12 days for each treatment. Seeds were cleansed of mycelial growth and dissected. The pericarp was removed and discarded, and the embryo and endosperm tissues were separated from each other. The endosperm tissue included the aleurone layer. Embryo and endosperm fractions were then separately processed for NOR and AF content (*A. flavus* and *A. parasiticus*) or ST (*A. nidulans*). Maize tissue was ground into a fine powder under liquid nitrogen and extracted with acetone and water (70:30) followed by a hexane wash to remove lipids. The resulting solution was dried down under a fume hood and resuspended in chloroform. Mycotoxins were finally partitioned with chloroform and quantitated by HPLC as previously described.

Fig. 2. Visualization of norsolorinic acid (NOR) in substrate mycelium of *Aspergillus parasiticus* SK1, which readily produces NOR in YES (2.0% yeast extract and 2.0% sucrose) medium without (C) and with (E) 2.0% ethanol incorporated into the medium. However, *A. parasiticus* SK1 produces NOR in YEP (2.0% yeast extract and 2.0% peptone) medium only when ethanol is added to the medium. NOR production is restricted to the substrate mycelium and is not visually present in the aerial, sporulating mycelium. Similar results were observed with *A. flavus* NRRL 19772, except that this isolate does not grow as fast or produce as much NOR as does *A. parasiticus* SK1.

RESULTS

Production of mycotoxins in vitro. NOR, like AF, was produced consistently in YES medium and not in YEP medium by both *A. flavus* NRRL 19772 and *A. parasiticus* SK1 (Fig. 2). Thin-layer chromatography results showed that *A. nidulans* FGSC 26 produced slight amounts of ST on YEP medium and significant amounts on YES medium; this was confirmed by HPLC quantitation (Table 1). The addition of 2.0% ethanol to growth media stimulated mycotoxin production in YEP medium by all three species (Fig. 2, Table 1). NOR was produced in the substrate medium and was not visually present in the aerial, sporulating mycelium (Fig. 2). The same spatial separation of NOR-producing and non-NOR-producing fungal tissues was seen on infected kernels.

A. flavus, A. nidulans, and *A. parasiticus* preferentially colonize embryo tissues. Two patterns of maize-seed colonization were observed in half seeds. Visual observation showed that *A. flavus, A. nidulans,* and *A. parasiticus* preferentially grew and sporulated on the embryo tissue of nongerminating seed halves, whereas *A. versicolor* grew on the endosperm tissue (data not shown). This pattern of colonization was repeatable for each species. On seed halves that germinated, *A. flavus, A. nidulans,* and *A. parasiticus* grew poorly (if at all) on embryo tissues but invaded and sporulated heavily on endosperm tissues. This change in fungal colonization of specific kernel tissues was reflected in concurrent NOR production. Visual NOR accumulation was terminated in the embryo upon kernel germination and was initiated in endosperm tissue.

Mycotoxin accumulation in embryo and aleurone tissues. Inoculation of embryo-wounded whole seeds resulted in vigorous sporulation of *A. flavus, A. nidulans,* and *A. parasiticus* on the outside of the kernel. Sporulation was significantly decreased in both unwounded seed and endosperm-wounded seed for all three *Aspergillus* spp. Most striking was the pattern of NOR deposition in seed inoculated with *A. flavus* and *A. parasiticus* (Fig. 3). NOR deposition paralleled fungal growth in embryo and aleurone tissues. When embryo-inoculated kernels were cut open, NOR had accumulated throughout the entire embryo by day 4 in seed inoculated with *A. parasiticus.* Fungal mycelia and associated NOR pigment spread at a visibly slower rate in seed inoculated with *A. flavus.* When embryo-inoculated seeds were dissected and each tissue extracted to determine mycotoxin levels, it was observed that most of the mycotoxins remained in the embryo tissue,

Fig. 3. Visualization of norsolorinic acid (NOR) in the aleurone layer of maize kernels. Living maize kernels were dissected into endosperm, pericarp, and aleurone tissues 7 days after inoculation with *Aspergillus parasiticus* SK1 on wounded endosperm sites (wounds can be seen in some of the kernels in the endosperm dish). NOR was concentrated in the aleurone tissue.

although some was detected in the endosperm after a certain period of time (Table 2). In seeds infected with *A. parasiticus* and *A. flavus*, there was a tendency for a greater proportion of AF to be detected in endosperm tissues versus embryo tissues compared with NOR accumulation in these same tissues (Table 2). For undetermined reasons, the extracts from kernels inoculated with *A. nidulans* contained no ST after 9 days; this may have been due to a technical error.

In endosperm-inoculated seed, NOR deposition was seen in the tissues just below the pericarp. Upon dissection of the kernels, it was readily apparent that the NOR was contained within the aleurone tissue (Fig. 3). Microscopic inspection indicated that fungal mycelium was virtually limited to the aleurone tissue and that the NOR was contained within and immediately adjacent to the mycelium. Often, the embryo of endosperm-inoculated seed accumulated the NOR pigment after 11 days or more of incubation. By visually following NOR deposition in these kernels, we noted that the fungus appeared to reach the embryo primarily by growing along aleurone tissue and not by growing through the endosperm tissue. In those cases where the endosperm-inocu-

TABLE 1. Sterigmatocystin (ST), norsolorinic acid (NOR), and aflatoxin (AF) production by *Aspergillus nidulans, A. parasiticus*, and *A. flavus* in secondary metabolite-inhibiting and -inducing growth media

Isolate[a] / Mycotoxin	Media[b,c] YEP	YEP + ETOH	YES	YES + ETOH
A. nidulans FGSC 26				
ST	0.8 (2.6)	25.3 (87.9)	6.4 (22.2)	3.0 (10.5)
A. parasiticus SK1				
NOR	0	17.6 (61.2)	17.2 (59.8)	14.0 (48.5)
AFB_1	0	4.7 (16.2)	0.9 (3.1)	1.8 (6.2)
AFB_2	0	0	0	0
AFG_1	0	1.2 (4.3)	0.3 (0.9)	0.4 (1.4)
AFG_2	0	0	0	0
A. flavus NRRL 19772				
NOR	0	1.5 (5.2)	3.7 (12.7)	2.3 (7.9)
AFB_1	0	0	0.2 (0.8)	1.2 (4.1)
AFB_2	0	0	0	0
AFG_1	0	0	0	0
AFG_2	0	0	0	0
Control	0	0	0	0

[a] *A. nidulans* FGSC 26 can produce ST; *A. parasiticus* SK1 can produce NOR and AFB_1, AFB_2, AFG_1, and AFG_2; and *A. flavus* NRRL 19772 can produce NOR and AFB_1 and AFB_2.

[b] Three milliliters of different growth media (YEP = 2.0% yeast extract and 20% peptone; YEP + ETOH = addition of 2.0% ethanol to YEP; YES = 2.0% yeast extract and 2.0% sucrose; YES + ETOH = addition of 2.0% ethanol to YES) were inoculated with 5 μl of a spore suspension (1×10^6/ml) of the indicated fungal isolates. The control medium was inoculated with water only.

[c] Mycotoxins are reported in parts per million; total amounts of the mycotoxins (in micrograms) are shown in parentheses. For reference, food supplies are rejected at >20 parts per billion and feed supplies at 20–300 parts per billion, depending on the animal.

TABLE 2. Sterigmatocystin (ST), norsolorinic acid (NOR), and aflatoxin (AF) accumulation over time in wounded maize kernels inoculated with *Aspergillus nidulans, A. parasiticus*, and *A. flavus*[a]

Isolate[b] / Mycotoxin	3 days Em[c]	3 days En	6 days Em	6 days En	9 days Em	9 days En	12 days Em	12 days En
A. nidulans FGSC 26								
ST	308.2[d]	0	336.6	11.1	0[e]	0	360.8	0
A. parasiticus SK1	...	...	(360)[f]	(1)	(66)	(1)	2,368	(1)
NOR	510.1	0	2,307.2	6.4	417.0	6.3	710.6	0.3
AFB_1	14.6	0	75.7	0.3	88.4	3.2	49.1	0
AFB_2	7.6	0	22.3	0	27.7	0	0	0
AFG_1	18.3	0	387.6	2.5	336.2	19.7	310.2	1.1
AFG_2	3.1	0	51.0	0	45.4	2.1	43.3	0
Total AF	43.6	0	536.6	2.8	497.7	25.0	402.6	1.1
			(192)	(1)	(20)	(1)	(366)	(1)
A. flavus NRRL 19772	...	...	(345)	(1)	...	...	(290)	(1)
NOR	2.6	0	138.2	0.4	1.1	0	319.4	1.1
AFB_1	2.4	1.2	28.4	0.1	1.3	0.3	94.3	0.5
AFB_2	0	0	4.8	0	0	0	14.2	0
AFG_1	0	0	0	0	0	0	0	0
AFG_2	0	0	0	0	0	0	0	0
Total AF	2.4	1.2	33.2	0.1	1.3	0.3	108.5	0.5
	(2)	(1)	(232)	(1)	(4.3)	(1)	(217)	(1)
Control	0	0	0	0	0	0	0	0

[a] Maize kernels were wounded by piercing the pericarp covering the embryo with a sterile pin. Inoculum consisted of 5 μl of a spore suspension (1×10^6/ml) placed on the wound. Kernels (five per petri dish) were incubated at 29 C in the dark for 3, 6, 9, or 12 days. Control seeds were inoculated with water.

[b] *A. nidulans* FGSC 26 can produce ST; *A. parasiticus* SK1 can produce NOR and AFB_1, AFB_2, AFG_1, and AFG_2; and *A. flavus* NRRL 19772 can produce NOR and AFB_1 and AFB_2.

[c] Em = embryo; En = endosperm + aleurone. Ten kernels per treatment were separated into embryo and endosperm + aleurone tissues, and these tissues were extracted separately for mycotoxins.

[d] Mycotoxins are reported in parts per million.

[e] It is probable that technical errors resulted in no detection of ST in the maize embryos of the 9-day *A. nidulans* treatment.

[f] Numbers in parentheses show the proportion of NOR or total AF in embryo and endosperm tissues of a single treatment.

lated kernels germinated, NOR deposition increased in the aleurone tissues and also started to appear in endosperm tissues.

In unwounded seed, NOR appeared to accumulate in the embyro and/or aleurone tissues at different rates in individual seed. NOR sometimes accumulated in streaks in the aleurone tissue under natural tears in the pericarp (data not shown).

DISCUSSION

Mycotoxin contamination of seed crops by *Aspergillus* spp. has long been a serious problem in all agricultural communities. AF and ST contamination of cereal crops is of great concern because both of these compounds are known mammalian carcinogens (19,29,30). In general, AF has been the mycotoxin of concern in maize and ST on small grains, although both mycotoxins have been found in many agricultural commodities (1,14,15, 17,26). Preharvest AF contamination of maize is associated with environmental stress or insect damage during the growing season (13,28). The induction of NOR, ST, and AF by ethanol (Fig. 2, Table 1), a metabolite commonly used to induce stress responses in many organisms, may indicate that physiological stresses can affect AF biosynthesis, at least in vitro, but the effects of environmental stress on the maize-*Aspergillus* interaction is unclear.

Part of the uncertainty in determining what specific factors affect AF production in maize kernels lies in the current destructive methodologies used to assess AF contamination of maize kernels, which obscure observations of tissue-specific, fungus-mycotoxin locations in the various kernel compartments. Whereas it is easy to see *Aspergillus* growing on kernels, AF is not visible in white light. Also, the amount of *Aspergillus* present is not necessarily indicative of the level of AF contamination (21,32). AF detection involves grinding kernel tissue in organic solvents followed by specific methodologies such as enzyme-linked immunosorbent assay, HPLC, or thin-layer chromatography. These techniques can be time consuming, expensive, and/or labor intensive and can interfere in identifying the spatial and temporal induction and deposition of AF in maize kernels. One solution to this problem is an assay system that allows AF to be visible in and on the maize kernel. We have achieved this goal by using *A. flavus* and *A. parasiticus* mutants that produce visible quantities of the pigment NOR. NOR is the first stable intermediate in the ST-AF biosynthetic pathway and must be produced prior to ST-AF formation (Fig. 1). We have seen that NOR and AF are produced under the same conditions both in vitro and in vivo for the *Aspergillus* isolates used in the present study and consider NOR to be regulated in a fashion similar to that of AF in progenitor wild-type isolates.

Our results indicate that AF-NOR biosynthesis by *A. flavus* and *A. parasiticus* and ST biosynthesis by *A. nidulans* is initiated in and largely limited to embryonic or aleurone tissues invaded by the fungus. These results are in agreement with previous studies of AF contamination in maize kernels (28,33). All three species produced up to several hundred parts per million of NOR, AF, or ST in embryonic tissues of embryo-wounded kernels. There was also a significant amount of mycotoxin accumulation in the endosperm tissues. Observation of NOR deposition in these seeds suggested that endosperm invasion by the fungus usually originated from the aleurone layer or near the scutellum. Because in vitro (9) and in vivo (L. Lindner and N. P. Keller, *unpublished data*) studies indicate that AF is excreted by fungal mycelium but that NOR and several other ST-AF pathway intermediates are mostly contained within the mycelium, we thought there could be a difference in the relative proportion of NOR and AF accumulation in embryo versus endosperm tissues of the same seeds. In both *A. flavus*- and *A. parasiticus*-infected kernels, there was a tendency for a greater proportion of AF than of NOR to accumulate in the endosperm relative to the embryo (Table 2). For example, day 6 extracts from seeds infected with *A. parasiticus* showed that for every one part of NOR in the endosperm tissue, there were 360 parts of NOR in the embryo tissue. From this same extraction, there were only 192 parts of total AF in the embryo tissue for every one part in the endosperm tissue. We also noted a spatial separation of NOR within the fungal mycelium. Visually and microscopically, it appeared that NOR accumulated within colonizing mycelial tissues and not in conidiophores or conidia. However, other researchers have detected AF in conidia of several *Aspergillus* isolates (36). It may be that AF diffuses to the conidial apparatus after being produced in mycelium or that AF is produced in different tissues in different isolates of *Aspergillus*, as suggested by the previous study (36). We also note that the NOR-producing isolates we used are white spored because of a mutation in the polyketide synthase needed for conidial pigmentation, and we can not be sure that there is not a relationship between pigment and toxin polyketide production in *Aspergillus*. This could be an important issue in understanding the regulation of ST-AF biosynthesis. Other polyketides that are visually tissue specific include *Aspergillus* conidial (5) and ascospore pigments and *Streptomyces* antibiotics and mycelial pigments (12).

We saw that fungal colonization and NOR production were dependent on the developmental stage of the maize kernel. Upon kernel germination, visible NOR accumulation ceased in the embryo, even though some mycelium was still present, but started to appear in the endosperm tissue. The germinating embryo was resistant to *Aspergillus* infection and concomitant mycotoxin production. *Aspergillus*-inhibitory compounds have been reported in immature embryos of peanuts (23), and it is possible that an antifungal compound or compounds are generated in germinating maize embryos. Recent studies have identified maize lines with embryo-associated resistance to *Aspergillus* infection (8). With the particular hybrid used in this study, both unwounded and wounded living seed showed NOR contamination in the same tissues, but the unwounded seed were infected and therefore contaminated at slower rates. NOR accumulation was often obvious prior to significant fungal growth, and it is possible that the appearance of NOR could be used as a marker to identify the site of fungal ingress in unwounded seed.

In addition to using NOR producers (*A. flavus* NRRL 19772 and *A. parasiticus* SK1), we also used ST producers (*A. nidulans* FGSC 26 and *A. versicolor* SRRC 110) to study the maize-AF relationship. Similarities and differences in colonization of maize kernels by *Aspergillus* spp. can help elucidate general and specific factors necessary for compatible reactions in the maize-*Aspergillus* interaction. Similarities and differences in NOR, ST, and AF production may identify global regulatory mechanisms in polyketide production by these *Aspergillus* spp. Although our sample population was small (one isolate of each of four species), our results suggest that the differential ability of each *Aspergillus* sp. to colonize specific tissues on maize seed may reflect its host association in nature and consequent requirement for specific host degradative enzymes. *A. flavus, A. nidulans*, and *A. parasiticus* are frequently isolated from oil seed crops and, as we have shown here, preferentially colonize and produce mycotoxins in the embryo and aleurone of the maize kernel. In a typical maize kernel, the composition of the embryo is starch, 9%; lipid, 31%; and protein, 19%; whereas the composition of the endosperm is 88, <1, and 7%, respectively (4). The small percentage of lipid in the endosperm is due mostly to the presence of the aleurone layer (4). Smart et al (33) showed that lipid bodies, not starch granules, were destroyed in *A. flavus*-infected maize tissues, which demonstrates probable *Aspergillus* lipase activity during pathogenesis of oil seeds. We do not know whether the inability of *A. versicolor* SRRC 110 to colonize maize embryo tissue was typical of most *A. versicolor* isolates, although another study has shown that *A. versicolor* produces strong amylase and cellulase activities but weak lipase activity (25). *A. versicolor* is often isolated from small grains or roughage (22,26) where the bulk of the substrate biomass is starch or sugars; isolates from these environments would need enzyme activities different from those of the isolates from oil seed crops. Other studies have indicated a role for specific fungal degradative enzymes in *Aspergillus* pathogenesis (6,11,25).

A separate issue is the ability of the *Aspergillus* spp. to produce AF or ST in host tissues. AF and ST, which are end products of the same polyketide pathway, were produced under similar

conditions both in vitro and in vivo by the *A. flavus, A. nidulans*, and *A. parasiticus* isolates used in this study. We were most interested in observing the method of kernel colonization and ST production by *A. nidulans* because this fungus has a rich history as a model system for the study of fundamental biological problems (35). Recent results have shown that *A. nidulans* and *A. parasiticus* contain analogous ST-AF biosynthetic genes (18,31), and developmental mutants of *A. nidulans* have been shown to be incapable of producing ST or colonizing maize kernels in the same manner as does wild-type *A. nidulans* (N. P. Keller and T. H. Adams, *unpublished data*). It is reasonable that ST and AF would be induced in these *Aspergillus* spp. by similar environmental and physiological signals. Comparing and contrasting ST and AF production by these different species will be helpful in identifying global regulatory factors governing polyketide biosynthesis in the aspergilli.

Future studies are aimed at the exploration of which specific factors (host, fungal, and/or environmental) in the maize-*Aspergillus* interaction may act to regulate NOR-ST-AF production. We believe that using NOR- and ST-producers can help elucidate the biochemical, physiological, and molecular mechanisms governing polyketide mycotoxin production in *Aspergillus* spp. NOR mutants may prove to be useful tools to either select AF-tolerant hybrids or understand the nature of AF resistance, such as in the recently reported AF-resistant MAS maize lines (8). ST-producing isolates of *A. nidulans,* a fungus more genetically characterized than either *A. flavus* or *A. parasiticus,* could be helpful in determining whether there are global regulatory mechanisms in the maize-*Aspergillus*-mycotoxin interaction. The knowledge gained from these studies should lead to pertinent control strategies for *Aspergillus* mycotoxin contamination and provide insights into the mechanisms of elicitation of fungal secondary metabolites.

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