# BEHAVIOR OF AN ATOXIGENIC STRAIN OF *ASPERGILLUS FLAVUS* ON STERILE WHEAT: INSIGHTS RELATED TO FIELD USE

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## Abstract

Strains of *A. flavus* that do not produce aflatoxins (atoxigenic strains) are being applied to agricultural fields to reduce aflatoxin contamination in crops through competitive exclusion of aflatoxin producers. The atoxigenic strains are applied on sterile wheat seed that serves as a nutrient for the fungus. In Arizona, crops are grown under flood irrigation that may cause prolonged soil saturation and burial of the atoxigenic product. Influences of flooding and temperature on spore yield were assessed. Colonized seed was buried in non-sterile soil and flooded for 24 h at temperatures between 10° C and 42° C. Survival of the atoxigenic strain was highest below 25° C and decreased at 31° C and above. Increasing the duration of flooding decreased survival of the atoxigenic strain with a concomitant increase in colonization by other fungi. Incubation period during product manufacture influenced product performance when it was subjected to flooding. Colonization for 20-24 hours was optimal while shorter colonization decreased sporulation and longer incubations, up to 72 hours, conferred no advantage. The initial concentration of spores, ($1 \times 10^7$ spores/kg to $1 \times 10^9$ spores/kg) applied to the product during manufacture did not influence product performance.

## Introduction

Aflatoxins are a group of toxic, carcinogenic fungal metabolites that affect a number of agricultural commodities including cottonseed, corn, and peanuts. In cottonseed, which is susceptible to infection both during development and after maturation, *Aspergillus flavus* is the chief causal agent of aflatoxin contamination. Aflatoxins have habitually been a concern for the cottonseed industry because regulatory limitations restrict quantities of aflatoxins allowable in food and feed and consequently limit the use and value of contaminated products (Cotty, 1997).

Biocontrol of aflatoxin-producing strains of *A. flavus* with atoxigenic strains (those which do not produce aflatoxin) has been developed as a method to reduce aflatoxin contamination (Cotty, 1990; Dorner et al., 1992). In western Arizona, where aflatoxin contamination of cottonseed occurs perennially, the use of an atoxigenic strain of *A. flavus* (strain AF36) is being implemented in an area-wide biological control program for the management of aflatoxins in cottonseed. Existing practices apply sterile wheat seed colonized with the atoxigenic strain to soil surfaces preceding irrigation. Irrigation provides the humidity needed to promote sporulation of *A. flavus* on the colonized grain. Applications are made to cotton fields at lay-by, or immediately prior to first bloom. This gives the atoxigenic strain preferential exposure to the developing crop and an advantage in the competition for resources during crop infection (Cotty et al., 1994). This approach effectively alters communities of *A. flavus* in the soil and on the crop as well as reduces aflatoxin quantities in cottonseed (Cotty, 1994). In both 1999 and 2000 more than 10,000 acres in Arizona were treated using this procedure.

Influences of environmental variables on performance of colonized wheat seed after application to the field are uncertain. Undoubtedly, environmental dynamics such as temperature, moisture, and humidity can affect *A. flavus* growth and thus product performance (Karunaratne and Bullerman, 1990; Lacey, 1994). In Arizona, cotton production is completely dependent on irrigation. Flood irrigation, the predominant form, can result in standing water and can saturate the soil for several days. During irrigation the colonized wheat seed can become immersed and/or buried with unknown repercussions. Furthermore the effects of temperature on product performance during critical periods after application are undefined. These aspects must be described in order to develop recommendations for effective use of the colonized wheat seed product in Arizona.

The current study assessed sporulation of AF36 on wheat seed under constant and diurnal temperatures with the goal of defining optimal ranges for product application. An in vitro system was developed to examine influences of; temperature during flooding, flood duration, and seed placement within the soil during flooding. In addition, a standard flooding assay was developed to further optimize manufacture of the atoxigenic strain formulation. Collectively, this information provides a basis for improved recommendations for manufacture and use of atoxigenic strain products.

## Material and Methods

### Effects of Temperature on Spore Yield

To determine influences of temperature on sporulation, seeds colonized by AF36 were placed in multi-well plates (1 seed per well, 3 replicates of 12 seeds per temperature treatment). Spaces between wells were filled with water to provide humidity and plates were covered and incubated in sealed plastic containers (to prevent moisture loss) throughout incubation. To examine effects of constant temperature colonized seeds were incubated continually at 13° C, 19° C, 25° C, 31° C, and 37° C. Effects of a diurnal temperature cycle were assessed by incubating colonized seeds at 31° C for 12 hours per day and at 3° C, 8° C, 13° C, 16° C, or 19° C for the remaining 12 hours. Spore yield was measured at 2-3 day intervals over a two week period. Four samples of two randomly selected seeds from each replicate were washed with 95% ethanol. The washings from the two seeds were combined, brought to a final volume of 23 ml, and added to an equal volume of deionized water. The spore suspension was mixed and measured with a turbidimeter (Orbeco-Hellige Digital Direct-Reading Turdibimeter, Orbeco Analysis Systems Inc., New York) to obtain a nephelometric turbidity unit (NTU) value. Spore yield was extrapolated from an NTU versus colony forming unit (CFU) standard curve.

### Influences of Flooding on Product Performance

Soils collected from Arizona cotton fields targeted for inclusion in the area-wide aflatoxin management program were air-dried, hammered to pass a No. 12 screen, and homogenized in a V-mixer. In a sterile test tube 10 g of soil, 10 ml sterile deionized water, and 20 seeds colonized by the atoxigenic strain were combined and mixed to distribute the seeds throughout the soil. Following flooding, test tubes were shaken to mix the compacted soil and seeds were recovered by passing the contents through a sterile No. 12 screen. Remaining soil was rinsed from the seeds with sterile water and 12 seeds were transferred to 24-well plates that were arranged as previously described. In all flooding experiments the multi-well plates were incubated at 31° C for 5 days after which the seed was assessed under a dissecting microscope (20x-50x) for incidence of *A. flavus* and other fungi. Sporulation of *A. flavus* was visually rated from 0 to 5. In addition, controls of colonized wheat seed in sterile water and sterile wheat seed in non-sterile field soil were evaluated. Ten isolates of *A. flavus* per temperature treatment were isolated and tested to confirm AF36 identity using VCG analysis.

Using the flooding procedure product behavior was evaluated under various conditions. To determine the influences of temperature on product performance colonized wheat seed was subjected to flooding in non-sterile soil. Atoxigenic product was flooded for 24 hours at 10° C, 15° C, 20° C, 25° C, 31° C, 37°, and 42° C. Similarly, the impacts of flood duration were

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assessed by flooding for 1, 2, 4, 8, 16, 24, 48, 72, or 96 hour periods at 25° C. To assess influences of positioning in flooded soil on product performance, colonized seed was either placed on top of or buried within the soil. Buried seed treatments were set up using standard flooding procedures. Unburied treatments were prepared by positioning seeds on top of the soil after it had settled. Buried and unburied seed treatments were exposed to flooding at 25° C, 31° C, and 37° C for 48 hours. To assess potential variability among soils influence on colonized wheat seed during flooding, 7 soils from various locations in Arizona were assayed using a 48 hour flood duration at 31° C.

**Optimizing Manufacturing of Atoxigenic Product**

Product was manufactured using established procedures (Bock and Cotty, 1999). Seventy ml of water was added to 1 kg hard red winter wheat seed in a 2 L bottle and was rolled for 20 minutes before being autoclaved twice (60 minutes each, 121°C). A 200 ml conidial suspension containing 2 x $10^8$ conidia was added to the sterile wheat seed and subsequently rolled for 3 hours at room temperature. After rolling, the bottles were incubated at 31° C for 24 hours. Following incubation, the colonized seed was transferred into sterile cotton pillowcases and placed in a forced-air drying oven for 48 hours at 58° C. Quality control was performed on each product batch. This consisted of examining the product for bacterial and fungal contaminants and assessing product spore yield in a standardized assay (Bock and Cotty, 1999).

The potential use of a flooding assay in optimization of atoxigenic strain product manufacture was assessed. The manufacture process was altered by varying the incubation period after rolling. Product was made with incubation lengths ranging from 5 to 72 hours. The concentration of the spore suspension used to inoculate the sterile wheat was also altered and product was manufactured using quantities from 1 x $10^7$ to 1 x $10^9$ spores per kg. The resulting material was subjected to the flooding for 48 hours at 25° C, 31° C, and 37° C. Products resulting from varied manufacturing were subject to the standard flooding for 48 hours at 25° C, 31° C, and 37° C.

## Results

**Effects of Temperature on Spore Yield**

Temperature influenced production of spores by the colonized wheat seed product (Figure 1). Final spore yield was similar at temperatures exceeding 25° C. However, initial sporulation was slower at 25° C than at temperatures (31° C and 37° C) within the optimal growth range of *A. flavus*. Temperatures below 25° C delayed or prevented sporulation. Constant temperature below 19° C prevented sporulation of *A. flavus* on the wheat seed. Twelve hour diurnal cycles of 13°/31° C reduced sporulation on the colonized seed 88 to 91% after two weeks in comparison with the control (31° C continuous) but did not prevent sporulation as did cycles of 8°/31° C and 3°/31° C. Diurnal cycles of 16°/31° C and 19°/31° C delayed sporulation in relation to the control, but did not preclude atoxigenic strain growth (Figure 2). Results from repeated experiments were similar.

**Influences of Flooding on Product Performance**

Temperature affected product performance during flooding in non-sterile soil. Colonized seed in sterile water controls survived and sporulated equally once incubated in multi-well plates, regardless of temperature during flooding. In non-sterile soil, however, temperature had a strong influence on product survival during immersion. High temperatures (37° and 42° C) resulted in low *A. flavus* survival rates, accompanied by high incidences of other fungi colonizing the seed (Figure 3). Cool temperatures favored *A. flavus* survival on the colonized wheat seed and inhibited utilization of the seed by fungal competitors. At 25° C there was little influence from other fungi and 80 to 100% of *A. flavus* survived the 24 hour flood period. At 31° C *A. flavus* survived on 50 to 100% of colonized wheat seed and at 37° C on 0 to 50%. No *A. flavus* survived flooding for 24 hours at 40° C. As incidence of competitive fungi on the wheat increased, sporulation of *A. flavus* decreased. Endemic fungi from the soil readily colonized the sterile seed controls. Length of immersion in non-sterile soil also influenced the atoxigenic strain. Incubation of *A. flavus* colonized seed at 25° C in flooded soil resulted in a gradual decline in the percent of seeds retaining viable *A. flavus* with less than 50% of the seeds having *A. flavus* after 96 hours. As the period of flooding increased the rates of *A. flavus* sporulation decreased (Figure 4). Decline in the quantity of spores produced coincided with colonization of the product with fungal competitors. Fungi other than *A. flavus* began colonizing both the sterile wheat seed controls and the atoxigenic strain product after as few as 8 hours.

Buried product was effected similarly to product that was immersed but on top of the soil layer at all three temperatures assayed (Figure 5). Soils from different locations in Arizona were similar in their effect on product survival during flooding (Figure 6).

**Optimizing Manufacture of Atoxigenic Product**

Manufacturing techniques influenced the ability of atoxigenic strain products to survive flood conditions. Varying incubation period during manufacture altered the ability of *A. flavus* to survive flooding at all temperatures assayed. Optimal survival was achieved with an incubation period of at least than 20 hours (Figure 7). When exposed to flooding at 37° C survival and sporulation rates were higher in treatments incubated for 20 and 24 hours during manufacture than for treatments incubated 5, 12, or 16 hours (Figure 8). At 25° C and 31° C survival rates were reduced only for seeds from the 5 hour incubation period. Sporulation after exposure to flooded soil at 31° C increased as incubation period during manufacture was increased. The quantity of spores used during manufacture did not influence survival of *A. flavus* on the colonized wheat in flooded soil within the range of conidial concentrations evaluated (1 x $10^7$ to 1 x$10^9$).

## Discussion

Regions of Arizona that experience perennial aflatoxin contamination of cottonseed are participating in an aflatoxin management program that utilizes an atoxigenic strain of *A. flavus* to competitively exclude aflatoxin producers. The intent of the program is to diminish the aflatoxin producing potential of the fungi within treatment areas and consequently, reduce the vulnerability of crops to contamination. The atoxigenic strain is applied on colonized wheat seed which serves both as the delivery mechanism and as the primary source of nutrition on which the fungus reproduces and spreads in the field. In order for the biocontrol application to be effective, the atoxigenic strain must grow and sporulate on the seed before the product is either removed or degraded by animal grazing, microbial competition, or agronomic practice. In general, early application is preferred because competitive exclusion relies on application of the atoxigenic strain prior to the annual increase of aflatoxin producing *A. flavus*. However, temperature and humidity requirements for growth and development of *A. flavus* on wheat limit the timing of applications. Results of the current study suggest temperature maxima should exceed 31° C and temperature minima should exceed 19° C for acceptable product performance. As temperature minima decrease, the time required for sporulation increases and spore yield declines. Delayed sporulation is particularly critical in Arizona's arid environment where product performance relies on irrigation to supply moisture to activate fungal growth. If the fungus grows too slowly, the humidity provided by irrigation may not be sufficient for initiation of spore production. In this situation, drying out of the delivered product is a potentially serious problem. Early in the season, a combination of low temperature, lack of a canopy, and limited irrigation all reduce the likelihood of optimal product performance. As the season progresses, fuller canopies, more frequent irrigation, and higher temperatures approach the optimal conditions described here. However, as the season proceeds, aflatoxin producing strains of *A. flavus* also increase in number and become

established on the developing crop. Therefore, it is best to treat as early as possible once minima temperatures exceed 19° C and the grower can terminate cultivation.

Producers utilizing atoxigenic strain material should consider soil type, slope of the land and crop size when modifying irrigation practices to assist atoxigenic strain applications. Crops in Arizona are completely dependent on irrigation which is predominantly delivered via flooding either in furrows or on the flat. The result is immersion and in some cases burial of the seed in a non-sterile soil suspension. The current study indicates that the colonized seed, when exposed to flooding in non-sterile soil, is highly susceptible to either co-infection or take over by other soil microorganisms. This detrimental influence increases with temperature. Immersion (flooding) in sterile water does not influence AF36 survival on the wheat seed product at any temperature (10° – 42° C for 24 hr or at 25° C for up to 96 hr). However, flooding in non-sterile soil results in a decline in viability of the atoxigenic strain. *Aspergillus flavus* retained colonization of the seed at temperatures below 31° C and subsequently yielded significant quantities of spores. At higher temperatures survival began to decline and the trend continued until there was no atoxigenic strain survival at 42° C. Reduced *A. flavus* survival on the colonized wheat was accompanied by an increase in seed colonization by competing microorganisms and as a consequence a reduction in the spore yield of *A. flavus*. Damage to product performance increased with flood duration. Soil type may also partially dictate product performance and survival. Heavy soils stay saturated longer and potentially expose the product to longer periods at high soil moisture. Potential problems might be mitigated by either altering irrigation practice or by applying the product after irrigation but while sufficient moisture for product activation remains.

In flooded environments, buried product was equally, but not more, susceptible to degradation than product resting on top of the soil. Microbial competition and environmental elements may be comparable in both situations. Buried product, however, must be capable of growing to and sporulating on the soil surface in order to be effective in crop colonization. Further information detailing the potential for *A. flavus* spread through the soil strata are needed to adequately assess the actual impact of burial on the atoxigenic strain's ability to colonize the crop and alter the soil microbiota.

The atoxigenic strain product is applied by air and ground. Both types of application can distribute the product on top of the crop and into the furrows. Material landing in furrows has increased exposure to flooding, burial, and subsequent degradation. However, some producers band the product directly on top of the seed bed beneath the canopy. Considering the results of the current study, this type of directed application must be preferred.

Survival of colonized wheat seed during field flooding and subsequent burial may be necessary for optimal performance of the product under field conditions. Therefore, an assay was developed to evaluate performance of varying batches of product under flooding in non-sterile soil. This was used to optimize the manufacturing process in order to produce atoxigenic strain material that would perform best under flood conditions. The results indicate that the manufacturing incubation period should be no less than 20 hours for the atoxigenic strain to colonize the wheat seed sufficiently to survive high temperature (37° C) flooding. Incubation periods longer than 48 hours resulted in a decline in survival rate, possibly due to degradation of the seed by the atoxigenic strain. All conidial concentrations tested (1 x $10^7$ to 1 x $10^9$) yielded product with equal potential to survive flooding. The primary determining factor in product quality appears to be incubation period. The standard assay has also been effectively used as a quality control assay to measure the quality of product batches produced during development of a scaled-up facility for manufacturing atoxigenic strain product. Research and development on this commercial scale process is being pursued through a partnership between the Arizona Cotton Research and Protection Council and the Agricultural Research Service.

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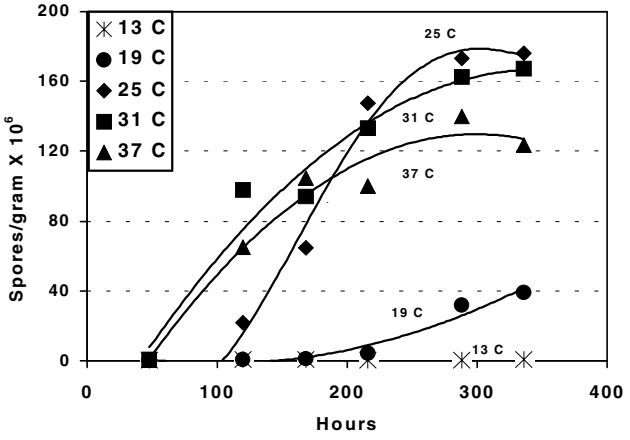


Figure 1. Influence of temperature on spore yield by atoxigenic *A. flavus* strain AF36 on sterile wheat seed.

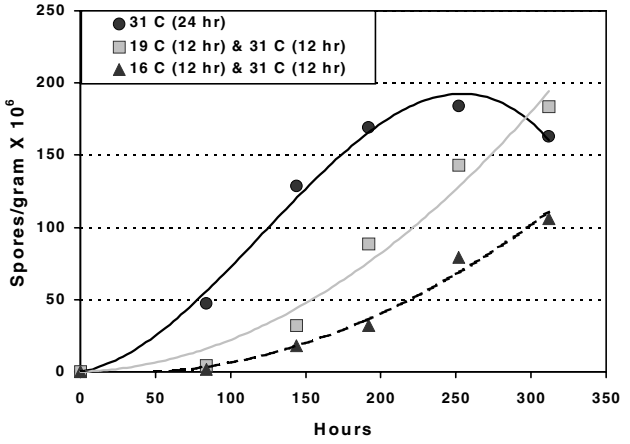


Figure 2. Effects of diurnal cycles on spore yield by atoxigenic *A. flavus* strain AF36 on sterile wheat seed.

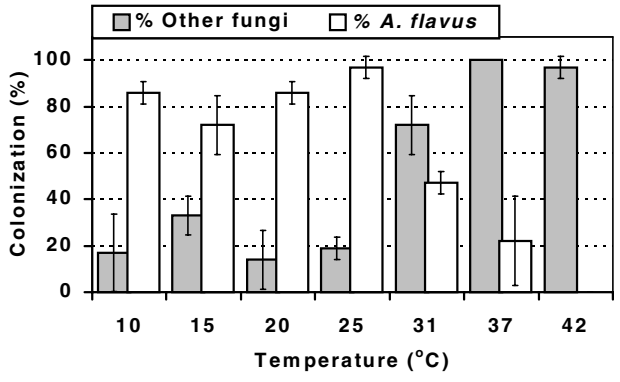


Figure 3. Wheat seed colonization by *A. flavus* and other fungi after incubation of *A. flavus* colonized wheat seed in flooded non-sterile soil for 24 hours at various temperatures.

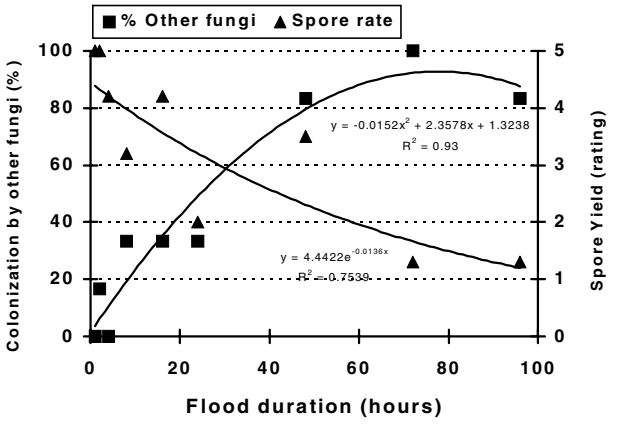


Figure 4. Spore yield of atoxigenic strain AF36 and colonization by other fungi after incubation of the atoxigenic strain colonized wheat seed product in flooded soil for various periods.

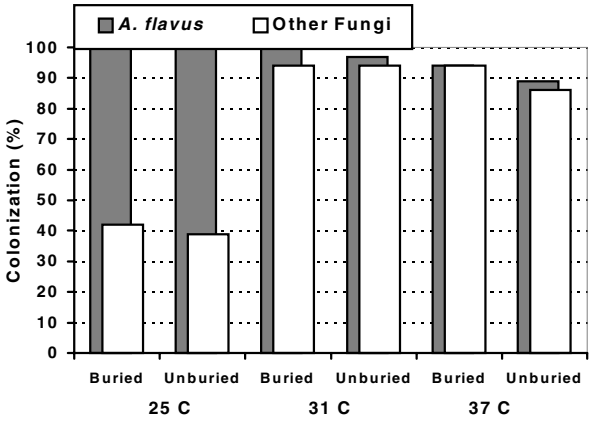


Figure 5. Percent of wheat seeds retaining *A. flavus* colonization after 48 hours flooding. Treatments included burial in flooded soil and immersion in flood water on top of the soil.

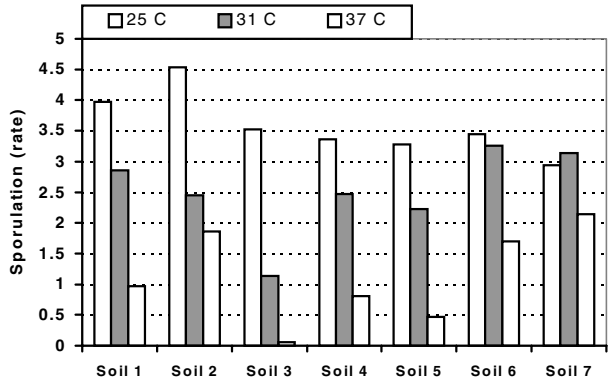


Figure 6. Impacts of flooding for 48 hours in different soils on atoxigenic *A. flavus* sporulation on colonized wheat seed.

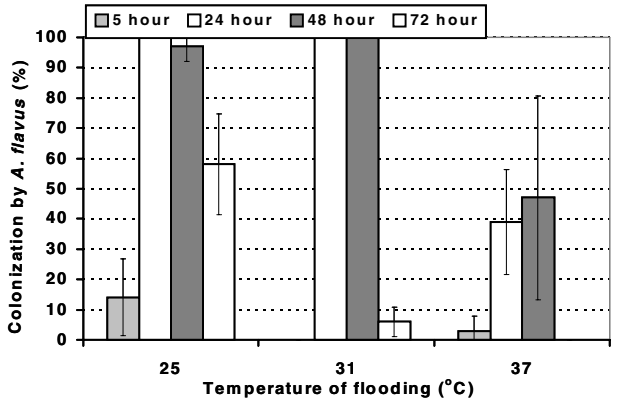


Figure 7. Influence of incubation period during manufacture on *A. flavus* survival on the colonized wheat seed in flooded soil for 48 hours at 25° C, 31° C, and 37° C.

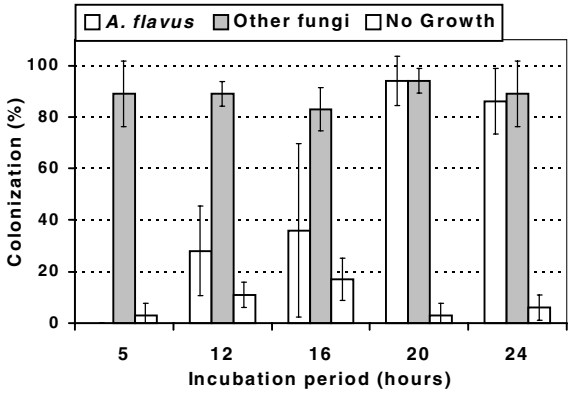


Figure 8. Influence of incubation period during manufacture on *A. flavus* survival on the colonized wheat seed in flooded soil for 48 hours at 37° C. Colonization of the seed by fungi other than *A. flavus* during the 48 hour incubation is also indicated.