

Wheat Seed Colonized with Atoxigenic *Aspergillus flavus*: Characterization and Production of a Biopesticide for Aflatoxin Control

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*Biocontrol of aflatoxin contamination using atoxigenic *Aspergillus flavus* to competitively exclude aflatoxin-producing strains has previously been reported, and is currently in the third year of commercial-scale tests (treating 50–200 ha per annum). Wheat seed colonized with atoxigenic *A. flavus* has been used in the commercial trials. Requirements for production of this colonized wheat seed are described and the spore yield of wheat is compared to other substrates. The study suggests that the most cost-effective inoculum production would require colonization of wheat (10^6 conidia kg^{-1} of wheat seed) at 25% (w/w) moisture for 18 h at 31 °C. To prevent fungal growth and associated wheat aggregation in storage, seed had to be dried below 15% (w/w) moisture, although a moisture content of 35% (w/w) did not reduce viability in sealed containers stored at 18–25 °C over an 8-month period. The dry biopesticide had multi-year stability without refrigeration and withstood temperatures of 70 °C for 20 min. Sporulation of the product occurred within 3 days at 31 °C and 100% relative humidity with yields averaging 4.9×10^9 conidia g^{-1} by day 7.*

Keywords: *Aspergillus flavus*, aflatoxin, biopesticide, formulation

INTRODUCTION

Aflatoxins are extremely toxic mycotoxins produced by some members of *Aspergillus* section *flavi*. These fungi are ubiquitous and infect many crops and crop products including peanuts, corn, cottonseed and a variety of nuts (Diener *et al.*, 1987; Cotty *et al.*, 1994). In order to keep the aflatoxin content of foods and feeds below potentially dangerous levels, maximum permissible aflatoxin contents are mandated in most countries. Produce with aflatoxin exceeding these limits has reduced value. Both health risks and the reduced profitability of contaminated crops create a need to prevent the formation of aflatoxins (Park *et al.*, 1988).

Affected material may be decontaminated using ammonia (Park *et al.*, 1988). The process

is costly, not universally available, and ammoniated crops generally have reduced value. Methods to reduce aflatoxin formation include manipulation of harvest date (Bock & Cotty, 1999), irrigation practice (Russell *et al.*, 1976), harvest method (Russell *et al.*, 1981) and storage practice (Batson *et al.*, 1997).

Biocontrol of aflatoxin-producing strains with atoxigenic strains of *A. flavus* is being developed on corn (Brown *et al.*, 1991), cottonseed (Cotty, 1994) and peanuts (Dorner *et al.*, 1992). Atoxigenic strains are used to competitively exclude aflatoxin producing strains during crop colonization (Cotty & Bayman, 1993; Cotty *et al.*, 1994). Atoxigenic strains have been applied with rice kernels and wheat seed, in alginate pellets and in conidial suspensions (Dorner *et al.*, 1992; Daigle & Cotty, 1995; Cotty, 1994). When applied as a solid formulation, the biocontrol agent is activated by moisture (from irrigation, rainfall or dew) and produces conidia which are dispersed to the crop.

For commercial use, formulations of biocontrol agents must allow cost effective production and adequate stability to allow the product to be transported, stored, and applied in a commercially compatible manner without significant loss in viability. Cotty (1994) initially used colonized wheat seed as a source of atoxigenic *A. flavus* in field plot experiments. Daigle and Cotty (1995) experimented with various forms of alginate pellet and found that those containing wheat gluten and mycelia of atoxigenic *A. flavus* produced more conidia than wheat seed ($4.0 \times 10^9 \text{ g}^{-1}$ vs $1.0 \times 10^9 \text{ g}^{-1}$ after 7 days incubation). However, alginate pellet production is a multi-stage process requiring constituents that are expensive compared to non-processed wheat (Daigle & Cotty, 1997). The cost of the bulk product for alginate pellets was estimated to be US\$2.53–5.76 kg^{-1} while the cost of wheat is US\$0.18–0.26 kg^{-1} . Inherent difficulties and expense of producing sufficient alginate pellets for large-scale field trials hampered further testing. New methods for the production of alginate pellets may help reduce the cost of encapsulating biocontrol agents (Daigle *et al.*, 1997; Daigle *et al.*, 1998). However, the simplicity and field success of colonized wheat has led us to develop that alternative.

Since 1989, the wheat seed formulation has been empirically improved, and large scale field trials (200 ha in 1997 and 1998) directed at preventing aflatoxin contamination of cottonseed have been undertaken, using this product as the sole inoculum source (Cotty, 1997). These trials have been strongly supported by the agricultural community in Arizona (Anon, 1996a, b; Rayner, 1998). The process for colonizing wheat with *A. flavus* has been incrementally modified and the current process, used to produce 2200 kg of colonized wheat in a laboratory in two months, has only been reported in materials submitted to the United States Environmental Protection Agency (EPA).

Any method used to control an agricultural problem must fit the economics of the production system. The following paper outlines the production of an inexpensive and stable biopesticide intended for use in preventing aflatoxin contamination. Experiments were undertaken to characterize better the colonized wheat used in commercial field tests currently underway, while optimizing and refining the process used to produce this product.

MATERIALS AND METHODS

General Methods and Protocol

Inoculation, incubation and drying of material. A standard process for manufacturing *A. flavus* colonized wheat was allowed by the EPA under Experimental Use Permit 69224-EUP-R. In that procedure, 1 kg of hard red winter wheat seed (Organic Grade, Arrowhead Mills, Hereford, TX, USA) was added to 2 l Nalgene plastic canisters (Nalgene Labware, Rochester, NY, USA) with 70 ml of water. The wheat seed-containing bottles were rolled for 20 min, to allow even distribution of the moisture and autoclaved for 60 min. After setting overnight at room temperature, the wheat seed was autoclaved for a second 60 min. After cooling, the wheat seed was ready for inoculation. *A. flavus* strain AF 36 (Cotty, 1994)

was cultured on V8 juice agar (5% V8 juice, 2% agar, pH 5.2) in Petri dishes. Conidia were harvested from 7-day-old cultures with sterile swabs and suspended in sterile distilled water. Conidial concentrations were measured using a turbidity meter (Orbeco-Hellige Digital Direct-Reading Turbidimeter, Orbeco Analysis Systems Inc., New York, USA). A linear nephelometric turbidity unit (NTU) vs colony forming unit (CFU) standard curve was developed to relate turbidity to conidial concentration. Spore suspension (150 ml , 1.0×10^6 conidia ml^{-1}) was added to each canister and the canisters were rolled horizontally for 3 h at 3 rpm on a tissue culture roller (Modular Cell Production Roller Apparatus, Wheaton, Millville, NJ, USA) to allow even absorption of the liquid and even dispersal of the inoculum. After rolling, canisters were incubated static at 31°C for 24 h. At initiation of incubation, the wheat moisture ranged from 28–32% (w/w) depending on the moisture content of the starting grain. Moisture was determined using an Ohaus Moisture Determination Balance (Model MB200, Ohaus Corporation, Florham Park, NJ, USA). After incubation, the seed was placed in sterile cotton pillowcases ($72.5 \text{ cm} \times 47.5 \text{ cm}$, seven canisters per bag) and placed in a forced-air tray oven (Proctor & Schwartz, Inc., Philadelphia, PA, USA) at 58°C to dry for 48 h. Incoming air was ducted through a HEPA-filter. Seed was then transferred to 19 l polyethylene food containers (Letica Corp, Rochester, MI, USA) for storage and transport.

A series of tests were performed to optimize the above procedure and to characterize the resulting product. Unless otherwise stated, treatments were replicated three times, and the experiments were repeated twice. All experiments were fully randomized.

Spore yield. The spore yield of the colonized wheat was quantified by placing one seed in each cell of a sterile 12-cell multi-well plate (Corning Glass Works, Corning, NY, USA). Replicates consisted of one multi-well plate (12 cells). Each treatment was replicated three times. Water (10–12 ml) was added only in the inter-cell spaces; plates were covered, placed in sealed plastic containers to prevent evaporation and transferred to a water-jacketed incubator at 31°C . Spore yield was measured on four seed randomly selected from each plate. The seed were placed in a 100-ml bottle containing 50 ml of 70% aqueous ethanol. Conidia were dislodged by agitation (10 s) and the turbidity of the solution measured by turbimetry as described previously. The spore concentration was extrapolated from the NTU / CFU standard curve. Tests showed that wheat seed alone did not contribute significantly to the NTU of the spore suspensions measured. After considering the kinetics of sporulation in this system, 7 days was chosen as the standard incubation period for spore yield assessment. The percentage of seeds with sporulation was also recorded for each assessment.

Visual assessment of colonization. For some experiments, the seed surface colonization was assessed visually. Ten seeds were fixed in an acetic acid : ethanol (50 : 50 v/v) fixative and stained with 0.5% methylene blue in lactophenol. Seed were examined with a dissecting microscope (X63) with oblique illumination. Fungal colonization of the dorsal (upper) and ventral or crease (lower) surfaces was scored separately using a 1–5 scale: 1 = no colonization visible; 2 = 1–5 mycelial strands visible; 3 = 6–20 mycelial strands visible; 4 = 21–50 mycelial strands visible and 5 = >50 mycelial strands colonizing the surface.

Effect of Concentration of Conidia on Colonization of Wheat Seed

Influence of initial spore concentration on colonization of wheat seed was assessed by altering the concentration of conidia used in the standard procedure. Evaluated concentrations included 0, 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 or 10^8 conidia kg^{-1} of wheat seed.

Incubation Period

In order to optimize the required incubation period, subsequent to rolling for 3 h, the inoculated wheat seed was placed at 31°C for 0, 4, 8, 18, 24 and 48 h. After incubation, seed was dried according to the standard procedure and spore yield was quantified. Colonization

was visually assessed. To assess further the degree of colonization, seed from the 0, 8, 24 and 48 h incubation periods were surface disinfected with 70% ethanol for 0, 15, 30, 60, 120 and 180 min. After ethanol treatment, seed were washed twice in sterile distilled water and fungal growth was visually assessed and spore yield quantified after 7 days (100% relative humidity (RH), 31°C) as described previously.

Effect of Moisture Content on Colonization of Wheat Seed

The moisture content required for successful product manufacture was determined by adjusting wheat moisture during incubation to either 10, 12, 15, 20, 25, 30 or 35% (w/w). The desired moisture content was produced by adding different quantities of water to wheat seed that had been pre-sterilized and dried. After the standard sterilization process, the seed were dried to 3% (w/w) moisture in the forced air oven at 80°C. A moisture content of 10% (w/w) was obtained by adding 70 ml of spore suspension (comprising 50 ml spore suspension, 1.0×10^8 conidia ml⁻¹ plus 20 ml water) to 1 kg of dried wheat. The volume of water was adjusted to achieve target moistures. After 24 h incubation (31°C), seed was dried according to the standard procedure and colonization was visually assessed. Colonization was also assessed by surface disinfection of seed samples from each treatment with 70% ethanol for 20 min (shown to be an effective time period in the previous experiment). The seed was washed twice in sterile distilled water and fungal growth was visually assessed after 7 days as previously described.

Effect of Seed Substrate on Spore Yield

Wheat seed, red sorghum, black-eyed peas, black beans, soy beans, barley, rye, oats, corn, Pima cottonseed, finger millet seed and rice grains were compared for ability to support conidial production by *A. flavus*. The standard procedure was followed for inoculation, incubation, drying and quantification of spore yield. For each substrate, 100-grain weights were measured and used to calculate spore yield g⁻¹.

Stability and Viability of the Wheat Seed/*A. flavus* formulation

Effect of time. Colonized wheat seed manufactured by the standard process was stored in plastic bags (Quart Size, Ziploc, Dow Brands L.P., Indianapolis, IN, USA) at room temperature from 12 May 1995–15 October 1997. Viability and spore production were assessed at approximately monthly intervals over this 29-month period. Seed were randomly selected from an individual bag on each occasion. Spore yield was quantified using the procedure already described for incubating seed in multi-well plates. However, on each occasion five NTU measurements were made on individual seeds.

Effect of moisture content (and drying period). To assess the impact of drying period on product performance, colonized wheat seed produced by the standard process was dried in a forced air oven for 0–138.5 h. At each sampling time, moisture content, viability and spore yield were determined. In order to assess the impact of moisture content on stability and viability, at each sampling time a 50 g subsample was sealed in an air-tight polypropylene container. Samples were stored at room temperature (18–25°C) for 8 months and subsequently assessed for viability and spore yield.

Effect of heat. Heat tolerance was assessed by heating colonized wheat seed to either 60, 70, 80, 90, 100 or 110°C for 20 min. Heating was performed in 5 ml sterile glass vials (with loose caps) containing 20 seed each. In a second experiment, seed was heated for 20 min at either 70, 74, 76, 78, 80, 82, 84, 86 or 90°C. In a third experiment, colonized wheat was heated to 80°C for either 0, 5, 10, 20, 40 or 80 min. In all experiments viability of the biopesticide was tested after treatment by incubating seed in multi-well plates as previously described. Each experiment was replicated three times and was performed twice. In order to

determine the actual heat exposure of the product during on farm storage in western Arizona, temperature dataloggers (HOBO-Temp, Onset Computer Corporation, Pocasset, MA, USA) were placed in wheat containers shipped to Arizona for use on the 1997 and 1998 crops. The biopesticide, *A. flavus* AF36 (EPA Registration No. 69224-E UP-1), is labelled for packaging in 19 l polyethylene food containers. Dataloggers were placed in plastic bags within the packaged product and thus were exposed to the same conditions as the product to the point of application in farmer's fields.

Data Analysis

SAS (SAS Institute Inc., SAS Campus Drive, Cary, NC, USA) was used to analyze the data. Analysis of variance (ANOVA) was applied to all mean comparisons and mean separations were performed using Tukey's HSD test ($P = 0.05$). Regression analysis was used to investigate and describe the relationships between different variables (effects of drying period on moisture content, temperature on viability, storage period on viability, and incubation period on sporulation). Prior to analysis, spore yield data from the substrate-type tests were square root transformed to reduce heterogeneity of variance between treatments.

RESULTS

Spore Production on Wheat Seed Colonized by *A. flavus*

At 31°C, significant numbers of spores (5.4×10^8 conidia g^{-1}) were produced within 3 days (Figure 1) and the quantity produced rapidly increased from 4–7 days. Thereafter, the rate of spore production slowed until the test was terminated at 14 days (6.5×10^9 conidia g^{-1}).

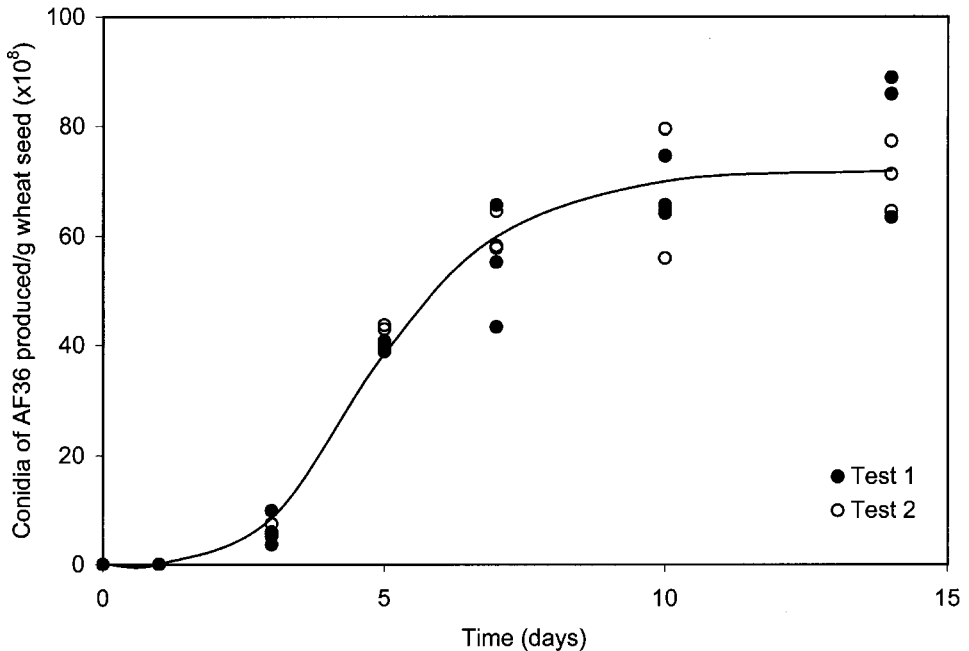


FIGURE 1. Sporulation of *A. flavus* strain AF36 on colonized wheat seed after different periods of incubation. The regression solution is a Gompertz sigmoidal model, $Y = e^{(-4.28) - 13.55(0.54^Y)}$, $R^2 = 0.96$.

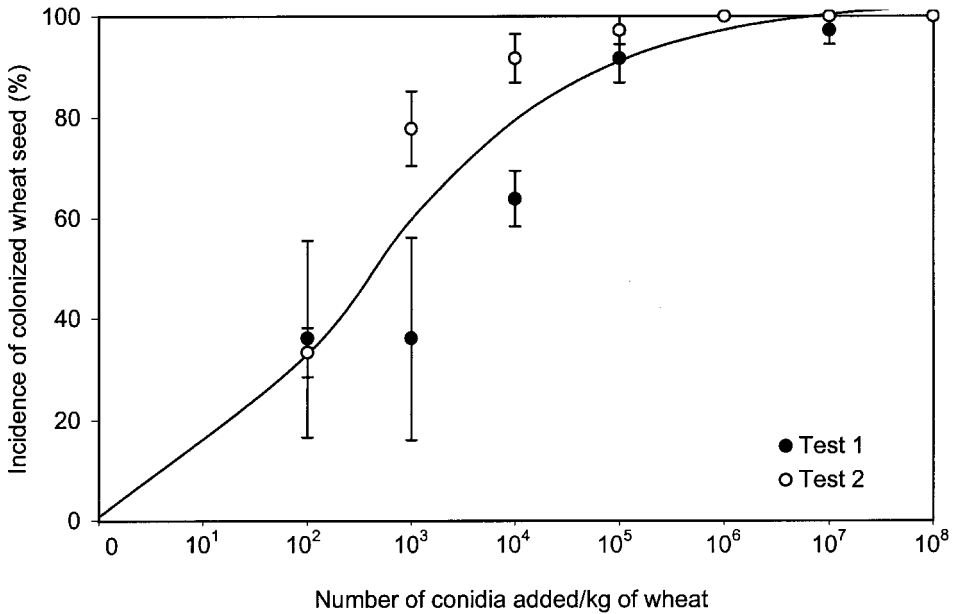


FIGURE 2 Effect of spore concentration of *A. flavus* strain AF36 on wheat seed colonization. Regression solution is a Gompertz-sigmoidal model, $y = e^{0.933 - 4.96(0.48)^x}$, $R^2 = 0.85$.

Effect of Concentration of Conidia on Colonization of Wheat Seed

Increased conidial concentration resulted in higher incidence of colonized wheat seed (Figure 2). Colonization increased significantly with greater conidial concentration up to 10^5 conidia kg^{-1} . Increases in colonization above 97% were not significant (Tukey's HSD, $P = 0.05$).

Effect of Incubation Period on Colonization of Wheat Seed

Initial evaluations suggested incubation period does not influence the extent of wheat colonization because 100% of the evaluated seed from all incubation periods produced statistically similar ($P < 0.05$) spore yields ($1.5\text{--}12.5 \times 10^8$ conidia g^{-1} wheat seed). However, microscopic examination of the surface indicated an influence of incubation period on seed surface colonization (Table 1). Wheat incubated for 18–48 h had greater ($P = 0.05$) colonization on the dorsal surface than wheat incubated for 0–8 h, and colonization of the ventral surface was greater than on the dorsal surface at 4 and 8 h. At time 0, colonization did not exceed uninoculated controls and no fungal mycelia were observed on dorsal surfaces. At 18-h incubation or more, the two surfaces had similar levels of colonization. Surface disinfection of the wheat seed also indicated that seed incubated for 24 h or more was better colonized by *A. flavus* than seed incubated for 8 h or less (Figure 3). A low percentage (5–10%) of seed colonized for 24 or 48 h retained viable *A. flavus* after washing in 70% ethanol for 3 h.

Effect of Moisture Content on Colonization of Wheat Seed

Microscopic examination revealed significant ($P < 0.05$) differences in the amount of fungal growth on seed incubated with different moisture contents (Table 2). Below 25% moisture, fungal growth was sparse on both seed surfaces. At 25% moisture and above, mycelial growth was dense. Similarly, the surface disinfection with 70% ethanol showed greatest reduction in survival when seed was colonized at 20% moisture or less (Figure 4).

TABLE 1. Effect of incubation period on wheat seed colonization by *A. flavus* strain AF36

Incubation period (h)	Colonization (visual assessment scale 1–5) ^a	
	Upper surface	Lower surface
Control ^b	1.0 a	1.8 a
0	1.0 ab	1.9 a
4	1.3 b	2.6 a
8	1.9 b	4.3 b
18	4.9 c	4.6 b
24	4.7 c	4.6 b
48	5.0 c	4.8 b

^aNumbers in a column followed by a common letter are not significantly different using Tukey's HSD test. Fungal colonizations of the dorsal (upper) and ventral or crease (lower) surfaces were scored separately using a 1–5 scale where 1 = no colonization visible; 2 = 1–5 mycelial strands visible; 3 = 6–20 mycelial strands visible; 4 = 21–50 mycelial strands visible and 5 = >50 mycelial strands visible.

^bControl not inoculated.

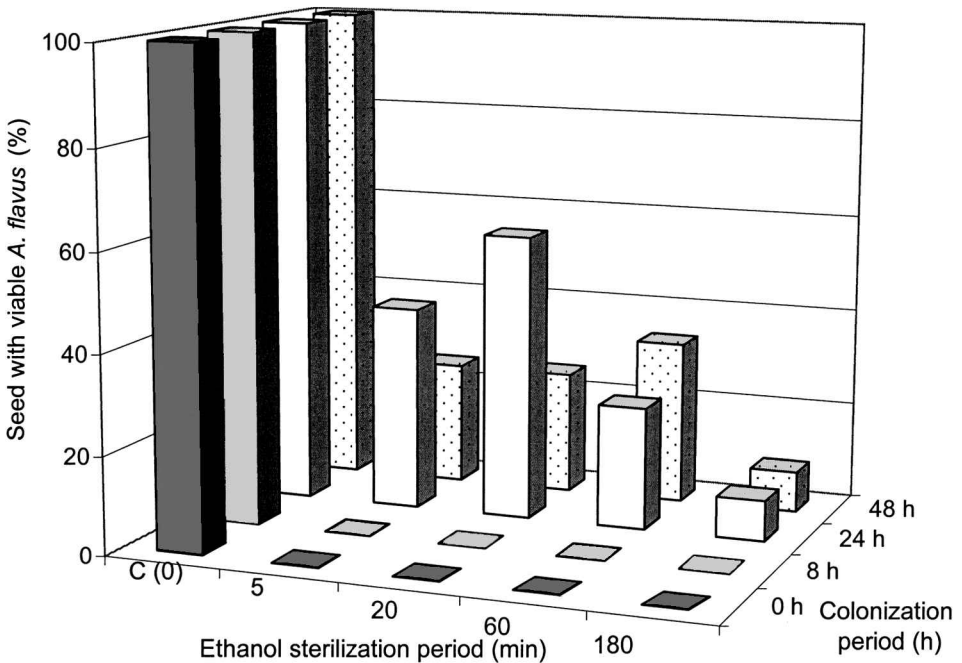


FIGURE 3. Effect of disinfection for various periods with 70% ethanol on survival of *A. flavus* strain AF36 in sterile wheat colonized for different periods at 31°C.

Effect of Seed Substrate on Spore Yield of *A. flavus*

Seed differed significantly ($P < 0.001$) in ability to support spore production (Table 3). Both wheat (11.4 and 7.2×10^9 conidia g^{-1} in tests 1 and 2, respectively) and oats (7.6 and 9.2×10^9 conidia g^{-1} in tests 1 and 2, respectively) gave consistently high spore counts in the two tests. Pima cottonseed (0.8 and 1.7×10^9 conidia g^{-1} in tests 1 and 2, respectively) and corn (0.6×10^9 conidia g^{-1}) appeared to be the least effective substrates for sporulation.

TABLE 2. Effect of moisture content on wheat seed colonization by *A. flavus* strain AF36 incubated for 24 h

Moisture content (%)	Colonization (visual assessment scale 1-5) ^a	
	Upper surface	Lower surface
Control ^b	1.0 a	1.8 a
10	1.0 a	1.8 a
12	1.0 a	2.1 a
15	1.0 a	2.0 a
20	1.6 a	2.9 a
25	4.6 b	4.8 b
30	4.3 b	4.5 b
35	4.6 b	4.6 b

^aNumbers in a column followed by a common letter are not significantly different using Tukey's HSD test. Fungal colonizations of the dorsal (upper) and ventral or crease (lower) surfaces were scored separately using a 1-5 scale where 1 = no colonization visible; 2 = 1-5 mycelial strands visible; 3 = 6-20 mycelial strands visible; 4 = 21-50 mycelial strands visible and 5 = >50 mycelial strands visible.

^bControl not inoculated.

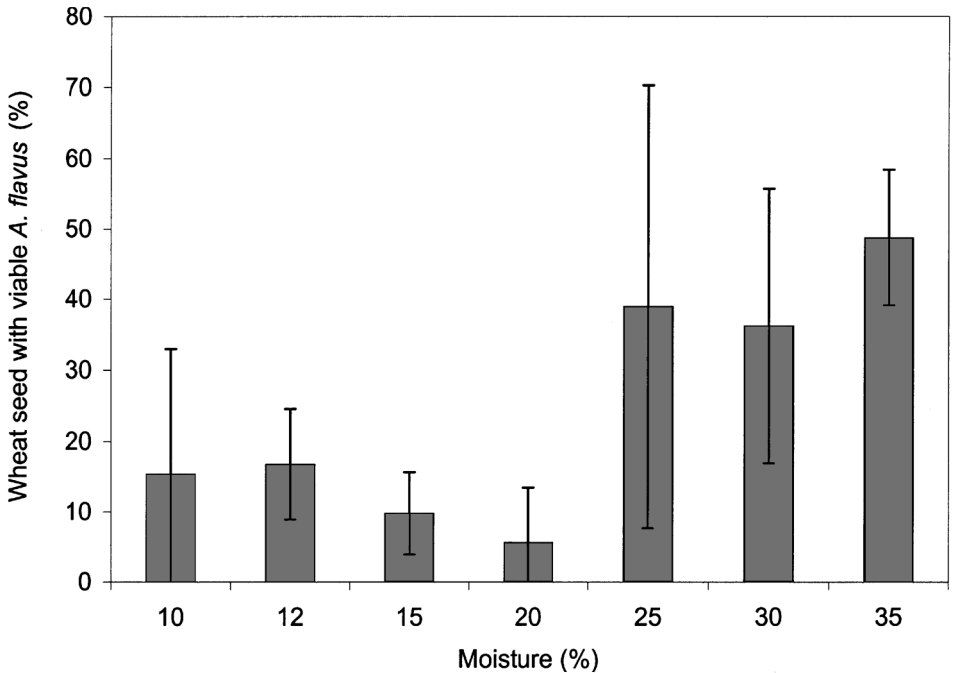


FIGURE 4. Effect of disinfection with 70% ethanol for 20 min on survival of *A. flavus* strain AF36 in wheat incubated at different moisture contents. Standard deviations (SD) of the means are indicated.

TABLE 3. Effect of substrate on the quantity of conidia produced by *A. flavus* strain AF36 during 7 days incubation

Test 1		Test 2		Substrate	Hundred grain wt g (SD)	Grains/g
Substrate	Conidia ^a	Substrate	Conidia			
Wheat	11.4 a	Oats	9.2 a	Oats	2.8 (0.12)	36
Rye	10.6 ab	Wheat	7.2 ab	Wheat	2.8 (0.07)	36
Rice	9.3 ab	Rice	6.3 abc	Rice	1.5 (0.01)	67
Oats	7.6 ab	Finger millet	6.0 abcd	Finger millet	0.6 (0.02)	176
Finger millet	6.5 bc	Sorghum	4.6 bcd	Sorghum	2.6 (0.13)	38
Soybeans	3.8 cd	Soybeans	3.8 bcde	Soybeans	20.1 (1.25)	5
Black-eyed peas	3.3 d	Rye	3.5 cde	Rye	3.5 (0.10)	28
Black beans	2.7 d	Black-eyed peas	3.3 cde	Black-eyed peas	22.6 (0.13)	4
Pima cottonseed	0.8 e ^b	Black beans	3.2 cde	Black beans	19.3 (0.76)	5
		Barley	3.2 de	Barley	4.7 (0.04)	21
		Pima cottonseed	1.7 ef	Pima cottonseed	12.4 (0.68)	8
		Corn	0.6 f	Corn	36.0 (0.34)	3

^aConidia g⁻¹ × 10⁹.

^bMeans separation based on square root transformed data using Tukey's HSD test at $P = 0.05$. Numbers followed by the same letter are not significantly different.

Not all substrates were consistent between the two test runs. For example, rye produced 10.6 and 3.5×10^9 conidia g⁻¹ in tests 1 and 2, respectively. In addition, seed size varied greatly (100 grain weights ranged from 0.6 g for finger millet to 36 g for corn). There was a consistent negative correlation between sporulation capacity of the inoculated substrate and seed weight (in test 1, -0.7360 , $P = 0.001$, and in test 2, -0.6590 , $P = 0.001$). Smaller seeds tended to produce greater numbers of spores/g of substrate.

Stability and Viability of the Wheat Seed/AF36 Formulation

Effect of time. Regression analysis ($y = 0.005x - 10.08$, $R^2 = 0.001$) showed there was no effect of storage period at room temperature (18–25°C) on spore yield of the biopesticide over a 29-month period from 12 May 1995–15 October 1997 (Figure 5).

Effect of moisture content (and drying period). In both tests, moisture content as high as 37% (w/w) in sealed containers did not influence viability over an 8-month period at room temperature (18–25°C). The colonized seed retained full capacity to produce conidia at all moisture contents (3–37% (w/w)). However, the fungus grew with atypical, fluffy white growth from a few of the seed stored at 26–30% (w/w) moisture. The fungus grew during storage at moistures above 15% (w/w). This growth resulted in undesirable clumping of the product. The moisture content of the colonized wheat rapidly fell in the drying oven from ca 35% (w/w) to ca 8% (w/w) over 48 h (Figure 6). Wheat moisture was reduced to below 15% (w/w) within 24 h.

Effect of heat. Extreme heat killed *A. flavus* (Table 4). When seed was heated for 20 min (Figure 7), viability decreased between 70 and 90°C ($r = -0.7508$, $P = 0.01$, for tests 1 and 2 combined). At 80°C, viability of AF36 on wheat declined rapidly ($r = -0.6377$, $P = 0.05$, for tests 1 and 2 combined), with a 50% loss of viability within 4 min and 90% loss after 40 min. Typical temperatures of exposure that the biocontrol agent was subjected to during shipping and on farm storage in western Arizona are shown in Figure 8. Maxima did not exceed 58.6°C.

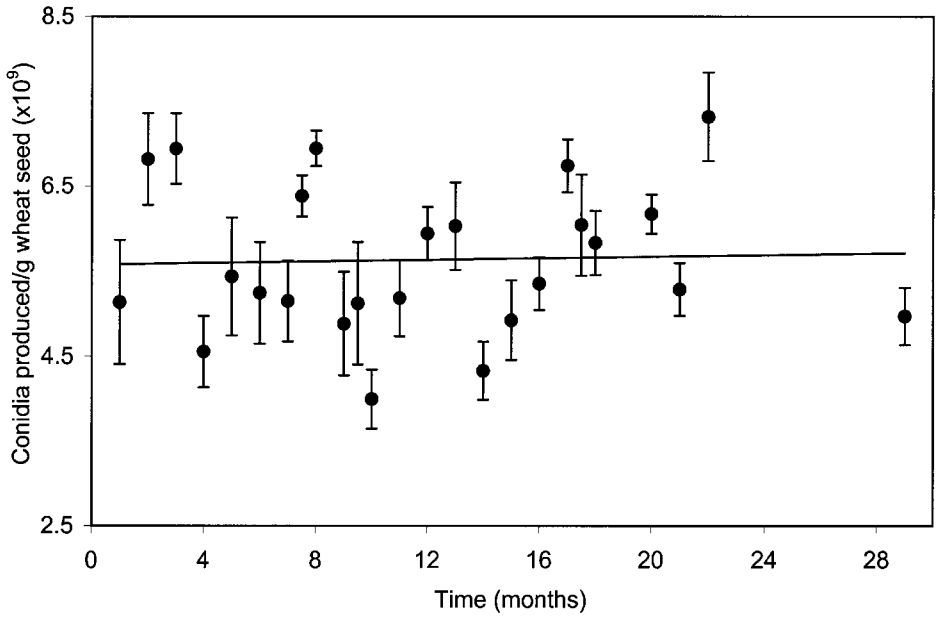


FIGURE 5. Viability of wheat seed inoculated with *Aspergillus flavus* strain AF36 at room temperature (18–25°C) during a 29 months period. The regression solution is linear, $y = 0.005x - 10.08$, $R^2 = 0.001$.

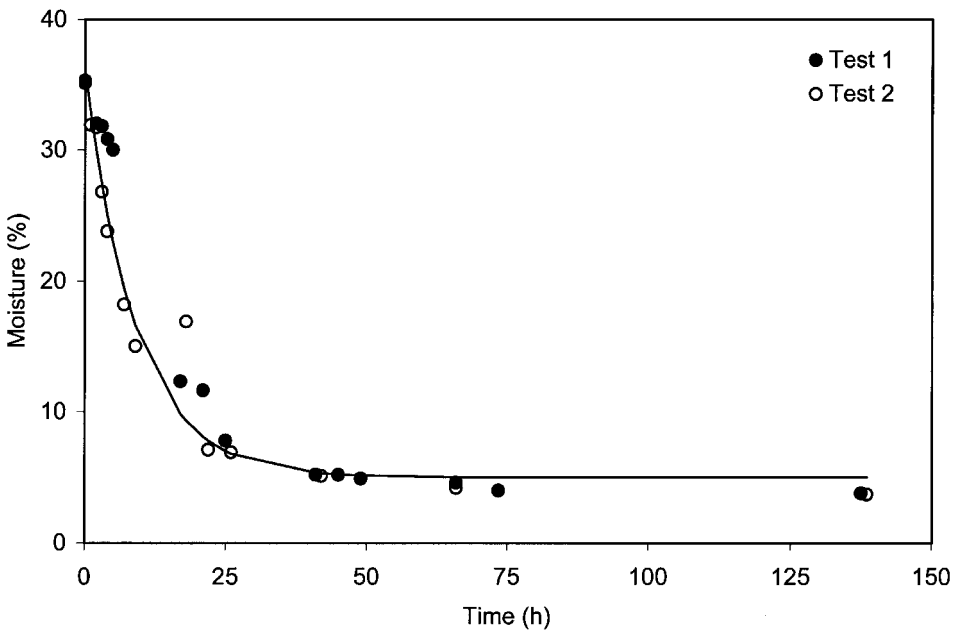


FIGURE 6. Influence of drying at 58°C on moisture content of wheat seed colonized by *A. flavus* strain AF36. The regression solution is an asymptotic model, $y = 0.05 - (-0.312e^{-0.11x})$, $R^2 = 0.95$.

TABLE 4. Effect of temperature on survival of *A. flavus* strain AF36 on colonized wheat seed

Temperature (°C) ^a	Survival (%) ^b	
	Test 1	Test 2
Control	100 a	100 a
60	100 a	100 a
70	100 a	95.8 a
80	33.3 b	19.4 b
90	0 c	0 c
100	0 c	0 c
110	0 c	0 c

^aTemperature maintained for 20 min.

^bNumbers are means of three replicates. Values followed by a common letter are not significantly different using Tukey's HSD test.

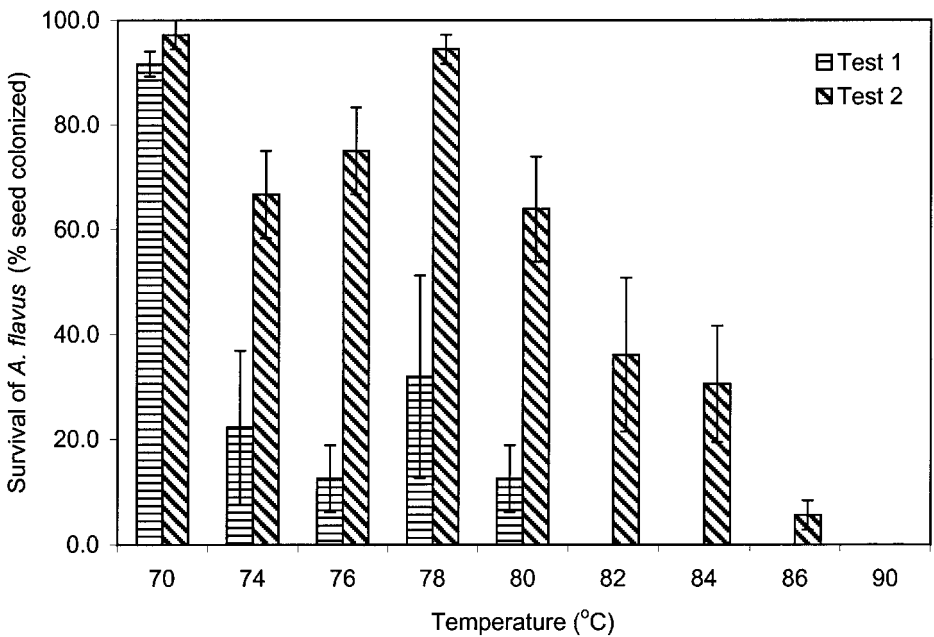


FIGURE 7. Effect of temperature on viability of *A. flavus* strain AF36 on wheat seed. Standard errors of the means are indicated.

DISCUSSION

In order for a biopesticide to be useful it must meet specific criteria; it must have sufficient growth both during culture and in the release environment, have adequate efficacy, minimal production costs, and good shelf life, preferably at least 18 months (Couch & Ignoffo, 1981; Feng *et al.*, 1994). Efficacy of wheat seed colonized by *A. flavus* in displacing aflatoxin-producing strains and reducing aflatoxin contamination of cottonseed has been demonstrated in field plot experiments (Cotty, 1994), and pilot studies in commercial settings (Rayner, 1998). The current study highlights some of the advantages of the wheat seed formulation compared to potential alternatives already considered (Daigle & Cotty, 1995, 1997). The wheat seed/*A. flavus* product is particularly straightforward to produce and eliminates

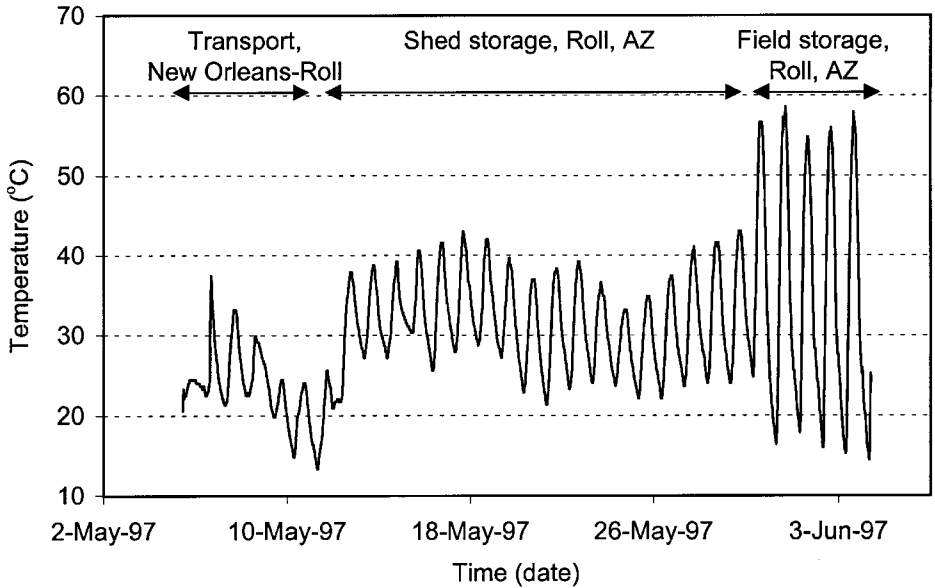


FIGURE 8. Temperatures experienced by wheat seed colonized by *A. flavus* strain AF36 during commercial use in western Arizona. The material was manufactured in New Orleans, Louisiana, and shipped to Roll, Arizona, where it was stored on farm until use.

cumbersome manufacturing needs required in alginate pellet formulation (Daigle & Cotty, 1997).

Using a natural substrate like wheat seed is practical. Wheat seed is relatively inexpensive (*ca* US\$0.18–0.26 kg⁻¹), readily available and already has characteristics that are ideal for application (granular and flowable). Wheat seed also has tough outer coats that prevent damage to the dried product. Colonization of the wheat prior to application reduces the likelihood that the wheat will be exploited by other, unintended microorganisms. The product has been successfully applied in commercial operations at 10 kg ha⁻¹ using a granular applicator (for example, a Gandy Box), a muck spreader, and an aerial applicator. The latter will be particularly important if large areas must be treated over relatively short periods.

A. flavus grows extremely well on wheat, and produces excellent spore yields (5.7×10^9 – 7.9×10^9) after 7 days incubation. Previous studies with artificial media (Daigle & Cotty, 1995, 1997) indicated that alginate formulations produce more conidia than wheat seed (4.0×10^9 g⁻¹ vs 1.0×10^9 g⁻¹, respectively after 7 days incubation). However, the advantage in conidial production may not warrant the additional cost of alginate production (total bulk costs up to US\$5.76 kg⁻¹; Daigle & Cotty, 1997), although as simplified mass production procedures are developed, the cost for alginate may fall considerably (Daigle *et al.*, 1997; Daigle *et al.*, 1998). A further potential advantage of wheat over alginate that has not been tested may be the duration of its integrity under field conditions, thereby prolonging spore production. In the current study, wheat was consistently an excellent substrate for spore production. Substrates with larger particle sizes produced fewer spores. This may be explained by the surface area to volume ratios of the different seeds. Large seeds have a proportionately smaller surface area from which to produce conidia. In the current test, seed size variation may have interfered with detection of influences from nutritional differences among substrates. To evaluate these influences, the various substrates should probably have been ground, and uniformly pelletized.

Sterile wheat colonized by *A. flavus* shows remarkable long term stability and tolerance of heat and moisture. In these studies the fungus survived for 29 months with no loss in viability when stored at room temperature (and *ca* 6% moisture). Even at 35% (w/w) moisture, survival extended throughout the 8 month study.

At 6% (w/w) moisture, the end use product survived 70°C for at least 20 min. Indeed, the product is routinely dried during manufacture at 58°C for 48 h and retains 100% viability. Previous studies (Daigle & Cotty, 1995) using alginate formulations indicated temperature in excess of 32°C and RH in excess of 50% resulted in reduced viability within a few months. The extensive product stability observed in the current study makes production, transport, and use more commercially compatible. This high stability should also allow survival during unfavorable periods in the field after application. Air temperatures in the field in Arizona frequently exceed 40°C (AZMET data, Roll, Arizona, July–September on the world wide web at <http://www.ag.arizona.edu/azmet>; Bock, unpublished data), but maxima exceed 50°C only occasionally. However, after application wheat may be exposed in full sun on the soil surface to temperatures over 70°C (Bock, unpublished data). If applications are made immediately prior to irrigation, prolonged exposure to temperatures in excess of 60°C will be avoided. It might be prudent to delay applications until canopy shade can ameliorate the temperature to which the product is exposed.

Cost saving may be made at several stages in the manufacturing process. Colonization can occur with minimal water (25% moisture) and reduced incubation (18 h). In the current study drying to 15% was sufficient to prevent fungal growth in storage. This is considerably less drying than previously employed. If fungal growth occurs before application flowability and dispersability will be reduced. Furthermore, premature fungal growth wastes resources needed after application.

The amount of colonized wheat seed added in the field is relatively low (10 kg ha⁻¹). This application rate effectively reduces aflatoxin without increasing the overall quantity of *A. flavus* on the crop (Cotty, 1994). The production of colonized wheat, as outlined here, requires relatively small quantities of conidia. To put the required quantity in perspective, consider growing *A. flavus* on wheat seed and using the conidia from that seed for the bulk inoculation of further seed. Assuming 1 g (about eight grains) of wheat seed produces 7.0×10^9 conidia, and each kg of seed requires 10^6 conidia for adequate colonization, it should be possible to produce 1000 kg of final product (a quantity sufficient to treat 100 ha!), with the conidia produced from just 1 g of colonized sterile wheat. In addition, the *A. flavus*/wheat seed formulation is axenic, while the alginate system suffers microbial contamination during the production cycle (Daigle *et al.*, 1998).

Wheat seed colonized with a toxigenic *A. flavus* may have application on many crops where aflatoxin contamination is a recurrent problem (Brown *et al.*, 1991; Dorner *et al.*, 1992). Similar formulations may also be relevant to aflatoxin control in less developed countries where aflatoxin can be a serious problem on staple food like corn (Sematou *et al.*, 1997) and groundnuts (McDonald & Mehan, 1989). It seems likely that specific strains well adapted to, and native in target regions and crops will be needed. However, the manufacturing procedures outlined here should be widely adaptable even in relatively low technology areas.

Finally, grain and other natural products are routinely used as media for culture of diverse fungi (CABI, 1983). This may indicate that similar, inexpensive production methods may be applicable to formulation of other biopesticides. For example, *Trichoderma* sp. is effectively applied in a pregelatinized starch/flour granular formulation (Lewis *et al.*, 1995), but work in India (Sarwant *et al.*, 1995) indicates *Trichoderma* sp. grown on agricultural waste also controls fungal pathogens.

Ultimate commercialization of the wheat seed *A. flavus* formulation and manufacturing process will depend on economics of production versus the benefits of aflatoxin control. It seems likely that barring capital investment, the costs of producing sterile wheat colonized by an atoxigenic strain of *A. flavus* will be low. The ultimate success of such a venture may

lie with the perception of the economic benefits and risks both within agricultural communities and within society as a whole.

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