

Modelling the colonisation of maize by toxigenic and non-toxigenic *Aspergillus flavus* strains: implications for biological control

H.K. Abbas¹, R.M. Zablotowicz² and H.A. Bruns¹

¹USDA-Agricultural Research Service, Crop Genetics & Production Research Unit, Post Office Box 345, Stoneville, MS 38776, USA; ²USDA-Agricultural Research Service, Southern Weed Science Research Unit, Post Office Box 350, Stoneville, MS 38776, USA; Hamed.Abbas@ars.usda.gov

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Abstract

To successfully exploit biological control it is desirable to understand how the introduced agent colonises the host and interferes with establishment of the pest. This study assessed field colonisation of maize by *Aspergillus flavus* strains as biological control agents to reduce aflatoxin contamination. Maize (corn, *Zea mays* L.) ears were inoculated with *A. flavus* using a pin-bar technique in 2004 and 2005. Non-aflatoxigenic strains K49 (NRRL 30797) & CT3 (NRRL 30798) and toxigenic F3W4 (NRRL 30798) were compared against a carrier control (0.2% aqueous Tween 20). Ten ears were sampled over 12 to 20 days, visually assessed, and curves fit to a three compartment Gompertz equation or other best appropriate regressions. Aflatoxin was determined by HPLC and cyclopiazonic acid (CPA) by LC/MS. The Gompertz model describes growth parameters, e.g. growth constant, lag phase and maximum colonisation characterised patterns of maize colonisation for most inoculated treatments. Aflatoxin accumulation in maize inoculated with F3W4 was about 35,000 ng/g in 2004 and 2005, with kinetics of aflatoxin accumulation in 2005 well described by the Gompertz equation. Less than 200 ng/g was observed in maize inoculated with strains CT3 & K49 and accumulation was described by a linear or logistic model. Maize inoculated with strains CT3 and F3W4 accumulated a maximum of 220 and 169 µg/kg CPA, respectively, compared to 22 and 0.2 µg/kg in the control and K49 inoculated, respectively. This technique can be used to elucidate colonisation potential of non-toxigenic *A. flavus* in maize in relation to biological control of aflatoxin. The greatest reduction of aflatoxin and CPA in maize inoculated with strain K49 and Gompertz parameters on colonisation indicates its superiority to CT3 as a biological control agent. The dynamics of maize colonisation by *A. flavus* strains and subsequent mycotoxin accumulation generated by using the pin-bar technique has implications for characterising the competence of biocontrol strains for reducing aflatoxin contamination.

Keywords: aflatoxin, *Aspergillus flavus*, biological control, cyclopiazonic acid, Gompertz growth model, maize

1. Introduction

Aflatoxin contamination is a major problem in maize (corn, *Zea mays* L.), particularly in the South-Eastern United States (Abbas *et al.*, 2002, 2006a; Diener *et al.*, 1987; Payne, 1992). This group of toxins is produced by *Aspergillus flavus* Link, *A. parasiticus* Speare, and *A. nomius* Kurtzman, Horn & Hesseline (Abbas *et al.*, 2004; Abbas, 2005; CAST, 2003). The increase in maize production in the Mid-Southern

United States can be attributed to increased demand for maize to support the aquaculture and poultry industry and food exports. Recently, there is a trend to use maize for ethanol production to stabilise domestic fuel source availability (Mojovic *et al.*, 2006; Latif and Rajoka, 2001).

Mississippi's humid sub-tropical climate and usual precipitation deficit in the summer is conducive to aflatoxin production by *A. flavus*. The potential for

aflatoxin contamination varies greatly from year to year (Bruns, 2003). Aflatoxin is a serious food safety concern due to its toxic effects in humans and animals (Robens and Cardwell, 2003). These include poultry, fish, livestock, and pets (Stenske *et al.*, 2006; CAST, 2003) and recently human mortality in Kenya (Shepherd, 2005; Peraica *et al.*, 1999; Lewis *et al.*, 2005). Because of these concerns, the US Food and Drug Administration has limited the aflatoxin content in maize to 20 µg/kg for human use (Van Egmond and Jonker, 2005). Cyclopiazonic acid (CPA) is another toxin produced by many *Aspergillus* species and other fungi (Trucksess *et al.*, 1987). This toxin is found in contaminated maize, beans and nuts, and can have deleterious effects on livestock (Bryden, 1991).

Strategies to reduce aflatoxin in crops have received much attention (Robens and Brown, 2004; Robens and Riley, 2002). One approach is using *A. flavus* strains that do not produce aflatoxin, also known as non-aflatoxigenic strains (Abbas *et al.*, 2006b). These strains have been shown to reduce aflatoxin contamination in peanuts (*Arachis hypogaea* L.) (Dorner, 2005), cotton (Cotty, 1994) and maize (Abbas *et al.*, 2006b) through competition with strains that produce aflatoxin.

Many microorganisms, including fungi, typically undergo a three or four phase growth curve (Righelato, 1975). Following inoculation, most fungi undergo a lag phase, which is a period of adaption to a new environment and growth substrates. Following the lag phase is the period of exponential growth until there is depletion of available substrates or space that limit further growth. Once available substrates and/or space is utilised, the organism undergoes a stationary phase that can be followed by either asexual reproduction into a quiescent form (e.g. conidia), or undergo decline. The development of a model to describe colonisation of artificial media by *A. flavus* and aflatoxin formation in pure culture was originally described by Pitt (1993). Recently, a more in-depth relationship describing growth on ground maize grain and interactions between temperature and water activity was modelled by Samapundo *et al.* (2007) for *A. flavus* and *A. parasiticus*. In complex natural environments, spatio-temporal availability of nutrients and competition with other microorganisms affect patterns of fungal colonisation (Boswell *et al.*, 2007), and rather sophisticated models have been developed to characterise mycelial colonisation on natural substrates.

This study assessed the use of the pin-bar technique, an artificial inoculation method using a pin-bar device, to study the potential for colonisation of maize by various toxigenic and non-toxigenic *A. flavus* strains. The pin-bar technique has proven very useful in characterising maize lines showing potential resistance to aflatoxin contamination (Moore *et al.*, 2004; Windham *et al.*, 2003). The current study uses a growth model developed by

Gompertz (Gompertz, 1825) to characterise patterns of colonisation of maize ears following pin-bar inoculation in two years of field trials. Aflatoxin and CPA accumulation following pin-bar inoculation were monitored in the second year of field studies.

2. Materials and methods

Aspergillus strains

Non-toxigenic *A. flavus* strains K49 (NRRL 30797 isolated from maize) and CT3 (NRRL 30798 isolated from soil) and toxigenic F3W4 (NRRL 30796 isolated from soil) (Abbas *et al.*, 2006b) were used in this study. Strains CT3 and F3W4 also produce cyclopiazonic acid (CPA). *Aspergillus* cultures were grown on potato dextrose agar (PDA) for 8 d, at 28 °C in the dark. All strains were verified for phenotypic characteristics based on toxin production and cultural characteristics (Abbas *et al.*, 2006b). Conidia were removed by scraping, suspended in aqueous Tween 20 (0.2%), and adjusted to a concentration of about 5×10^7 conidia/ml based on microscopic counts using a haemocytometer.

To correlate *in vitro* characteristics with field experimentation, the growth and aflatoxin accumulation by toxigenic strain F3W4 was assessed on PDA (9 cm) plates. Plates were inoculated with 200 µl of a spore suspension of $\sim 10^6$ conidia/ml, spread evenly using a glass rod and turntable, and incubated at 28 °C in the dark. After 1, 2, 4, 6 and 8 days incubation, triplicate plates were sampled by scraping mycelia into tared scintillation vials covered with cheese cloth, oven-dried for 24 h at 50 °C, and dry weight recorded. The mycelia was extracted with 70% methanol (5 ml/g of fungal material) and analysed by HPLC for aflatoxin (Sobolev and Dorner, 2002).

Field trial experimental design

This experiment was conducted at the Mississippi State University Delta Branch Experiment Station in Stoneville, MS in 2004 and 2005. The maize hybrid Pioneer 32R25 (Johnston, Iowa) was planted in 27 m rows 1.1 m wide in 2004 and 40 m long in 2005. A non-inoculated buffer row of corn surrounded each side of the inoculated treatment maize. The centre 20 m and 30 m of plants in the treated row was inoculated in 2004 and 2005, respectively.

Pin-bar inoculation

Maize ears were inoculated at 20 d after anthesis using a pin-bar inoculator (King and Scott, 1982). The inoculating device consisted of three 100 mm-long rows of 12 sewing needles mounted on a wood bar, with 6 mm of the points exposed. Pin-bars were surface sterilised with NaHOCl (0.5%), rinsed several times with sterile water and again with isopropanol (70%), and air dried. Pins were dipped in

conidial suspensions, and the excess solution was removed by gently shaking against the wall of the container. Finally, the bars were pressed through the husk into the centre of the ear, penetrating the kernels to a depth of 5 to 10 mm as illustrated in (Figure 1A to D).

Experimental parameters

In 2004, maize was assayed five times at 0, 2, 5, 7, 9 and 12 d after inoculation. A more rigorous sampling was conducted in 2005 with nine samples up to 21 d. Ten inoculated ears were harvested per treatment at random, and the number of colonised kernels and total number of kernels was visually determined. In 2004, aflatoxin was determined in the colonised kernels at 12 d after inoculation by 70% methanol extraction and ELISA (Abbas *et al.*, 2002).

In 2005, aflatoxin was determined from a composite of all ten ears in each sample, and analysed by HPLC and CPA by LC/MS. After a three-minute extraction period on a reciprocal shaker, samples were filtered and the supernatant was further clarified. Sample clean-up was performed according to Sobolev and Dorner (2002). The re-constituted sample (800 μ l) was eluted through a 1.5 ml extract-clean reservoir minicolumn packed with aluminium oxide (Alltech Co., Deerfield, IL), and 20 μ l

was injected on a Waters HPLC system equipped with a Nova-Pak C18 column (150 x 3.9 mm, 4 μ m) and a 474 model fluorescence detector (Waters Co., Milford, MA). Separation was carried out at 30 °C, with a mobile phase consisting of water-methanol-1-butanol (700:360:12.5) and a flow rate of 0.9 ml/min. The sample was subjected to post-column derivatisation using a PHRED, photochemical reactor (Aura Industries Inc., New York, NY). Total aflatoxin concentrations were based on the summation of aflatoxin B1 and aflatoxin B2 measured at 365 nm (excitation) and 440 nm (emission) compared to aflatoxin standards (Sigma Aldrich, St. Louis, MO). LC/ESI/MS/MS analyses for CPA were carried using a Thermo Finnegan LCQ Advantage, coupled to a Thermo Finnigan Surveyor MS, and a Thermo Finnigan Surveyor MS Pump (Thermo Electron Corporation, West Palm Beach, FL). Samples were run using 10 μ l partial loop injections analysed in full-scan mode producing a molecular ion of 337.1 (M+H). For MS-MS fragmentation, a collision energy of 36V with an isolation width of two was applied to the 337.1 molecular ion producing two significant ions 196.1 and 182.1, where these two ions of interest were used for quantitation purposes in MS-MS selective reaction monitoring mode (MS-MS SRM). The limit of detection for CPA by LC/ESI/MS/MS was 12 ng/g.

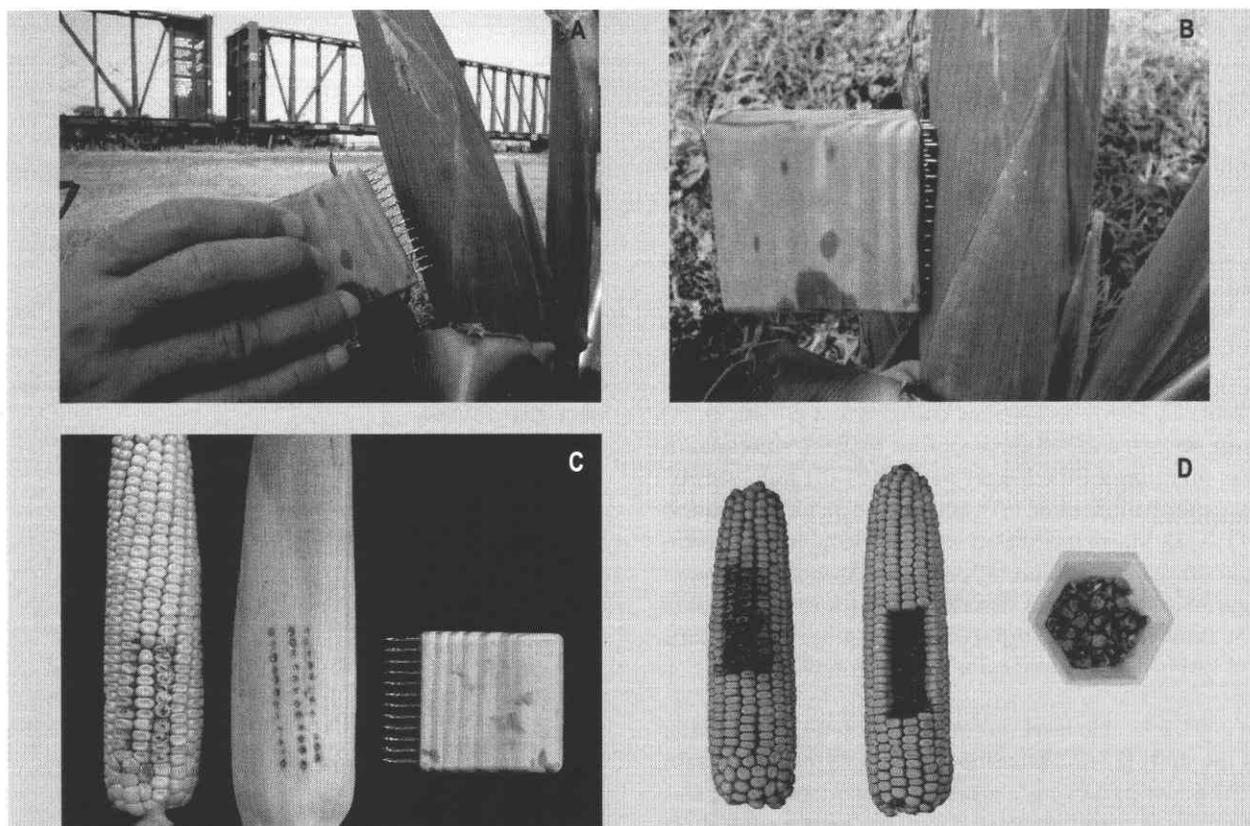


Figure 1. Pin-bar inoculation device and inoculation technique. (A) Insertion of device into maize ear. (B) Complete penetration of pin-bar into maize ear. (C) Wounds on husk following inoculation and subsequent colonisation by *Aspergillus flavus*. (D) Removal of inoculated kernels from maize ear for toxin analysis.

Gompertz growth model

Growth and aflatoxin accumulation were modelled using Sigma plot version 10.0 (Jandell Scientific Software, San Rafael, CA). Ideally the curves were initially fit to a three compartment Gompertz equation or the best fit model. The Gompertz equation used in these studies is:

$$y = ae - e^{-k(t - ti)} + ct \tag{1}$$

where:

a = maximum colonisation, toxin concentration, or fungal mass;

k = growth constant;

ti = lag;

t = time;

y = percentage colonised kernels, aflatoxin concentration, or fungal mass.

This growth curve has been widely used to describe biological populations and processes as diverse as the degradation of herbicides in soil (Zablotowicz *et al.*, 2006) and characterising the growth and mortality of medflies and nematodes (Easton, 1997).

3. Results and discussion

As previously described by Pitt (1993), aflatoxin accumulation by *A. flavus* generally follows biomass accumulation on solid media (Figure 2) with maximum aflatoxin accumulation observed after 4 days following conidia formation within 2 days. Both parameters were described well with the Gompertz model, yielding R^2 values of 0.997 for fungal biomass and 0.951 for aflatoxin accumulation.

The colonisation of maize kernels after pin-bar inoculation with three *A. flavus* strains is presented in Figures 3 and 4. In 2004, K49 and F3W4 exhibited greater maximum colonisation than CT3; this is evident comparing the a values generated from the Gompertz growth model (Table 1). However, similar colonisation constants (k) were observed for all three strains. In 2005, colonisation was monitored for a longer period (21 d), and a similar maximum colonisation (a) was observed for all three strains (Table 2). Maize inoculated with K49 exhibited a shorter lag before kernel colonisation, and subsequently a more aggressive infection as described by a lower colonisation constant for K49 compared to the other two strains indicating a more rapid colonisation by K49.

In 2004, aflatoxin level was only measured at termination of the study (12 d). Maize inoculated with toxigenic strain F3W4 had 34,184 ng/g aflatoxin while 7,000 ng/g was observed in maize inoculated with the carrier control. By contrast, maize inoculated with K49 and CT3 had only 4 and 190 ng/g aflatoxin, respectively. In 2005, similar levels of aflatoxin for these treatments were observed at 12 d

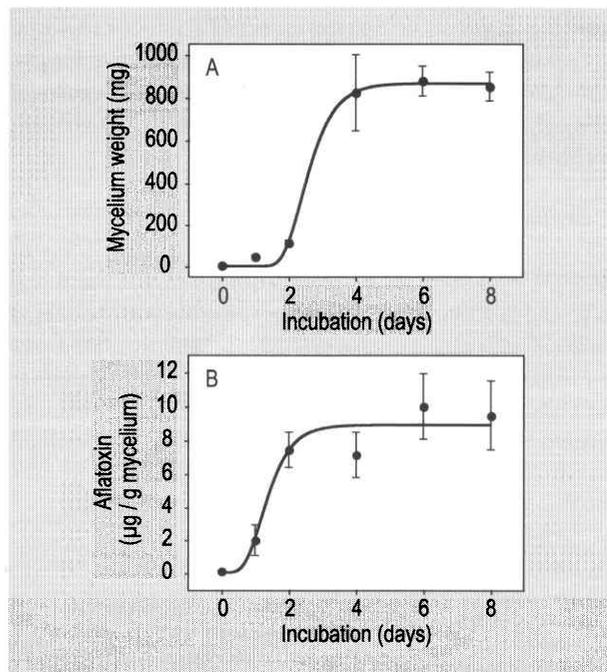


Figure 2. (A) Mycelial dry weight accumulation and (B) aflatoxin production by *A. flavus* strain F3W4 on potato dextrose agar. Mean and standard deviation of three replicates and curve modelled using the Gompertz equation.

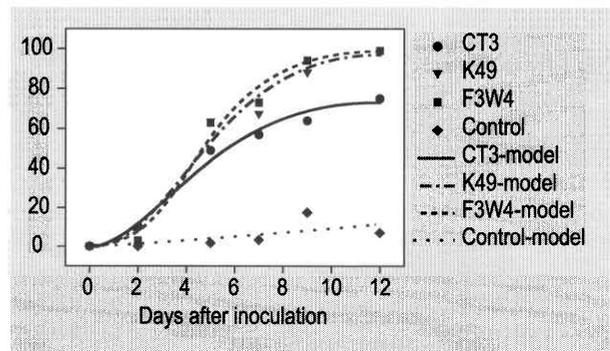


Figure 3. Observed and modelled patterns of colonisation of maize kernels by *Aspergillus flavus* strains following pin-bar inoculation in 2004.

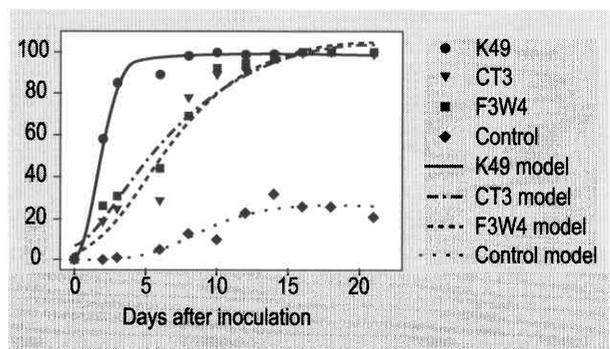


Figure 4. Observed and modelled patterns of colonisation of maize kernels by *Aspergillus flavus* strains following pin-bar inoculation in 2005.

Table 1. Gompertz growth parameters describing colonisation of maize by various *Aspergillus flavus* strains when introduced by pin-bar inoculation technique (2004).

Inoculum	r ²	a	ti	k
Non-inoculated	0.740	12.3	7.0	nd
K49	0.986	98.1	3.8	1.9
CT3	0.989	72.9	3.4	2.2
F3W4	0.971	94.7	3.8	1.8

a = maximum colonisation (% of total kernels colonised); ti = duration of lag phase (days); k = growth constant; nd = not determined.

Table 2. Gompertz growth parameters describing colonisation of maize by various *Aspergillus flavus* strains when introduced by pin-bar inoculation technique (2005).

Inoculum	r ²	a	ti	k
Non-inoculated	0.890	26.4	3.3	7.7
K49	0.990	98.1	0.8	1.5
CT3	0.989	104.5	3.6	4.2
F3W4	0.971	104.8	4.2	4.2

a = maximum colonisation (% of total kernels colonised); ti = duration of lag phase (days); k = growth constant.

after inoculation (Figures 5 A-D). Patterns of aflatoxin accumulation in maize inoculated with F3W4 also fit the Gompertz growth equation corresponding with the level of colonisation. A maximum accumulation of 36,720 ng/g was observed with F3W4 inoculated maize, with a lag of 5.1 d before aflatoxin accumulation (Table 3, Figure 5A). The maximum aflatoxin accumulation in maize inoculated with CT3 and the carrier control were 119 and 3,800 ng/g, respectively (Table 3, Figures 5B and D). By contrast, aflatoxin accumulation in K49 inoculated maize was best described as a linear pattern with only 57 ng/g accumulating at 21 d (Figure 5C). A sigmoidal curve describing aflatoxin accumulation was only observed in maize inoculated with F3W4 (Figure 5A). A logistic model lacking a lag phase was apparent for maize inoculated with CT3 (Figure 5D). The non-inoculated control could be fit to a Gompertz model, however the large variation in maximum accumulation suggests a random nature of aflatoxin accumulation in this treatment (Figure 5B). A maximum accumulation of about 60 ng/g aflatoxin was observed in maize inoculated with K49 (Figure 5C) compared to 116 ng/g in maize inoculated with the other non-toxicogenic strain CT3 (Figure 5D). These results indicate a competitive displacement of indigenous *A. flavus*. Use of either non-aflatoxigenic strain virtually eliminated aflatoxin accumulation. These results also indicate a greater suppression of aflatoxin production by K49.

Cyclopiazonic acid accumulation data in maize inoculated with various strains are presented in (Figure 6A-D). In contrast to aflatoxin, CPA accumulation was not described

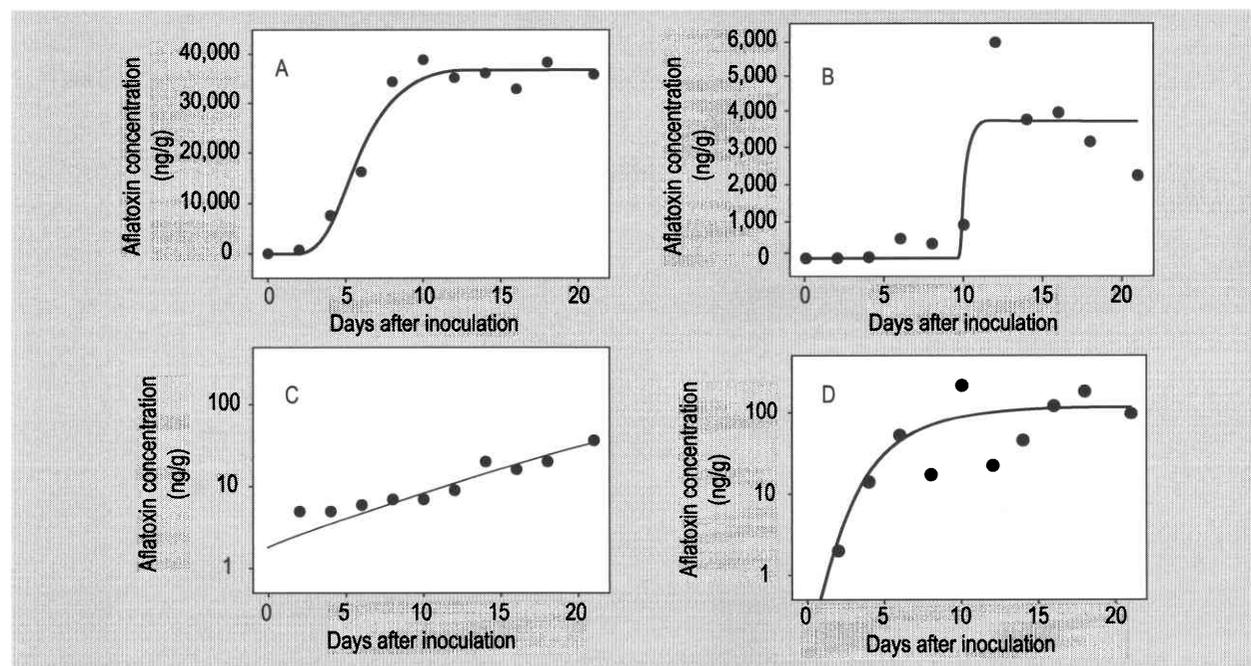

Figure 5. Observed and modelled patterns of aflatoxin accumulation in inoculated area of maize kernels by *Aspergillus flavus* strains following pin-bar inoculation in 2005, inoculated with (A) strain F3W4; (B) 0.2% Tween 20 control; (C) strain K49; and (D) strain CT3.

Table 3. Gompertz growth parameters describing aflatoxin accumulation in maize by various *Aspergillus flavus* strains when introduced by pin-bar inoculation technique (2005).

Inoculum	r ²	a	ti	k
Non-inoculated	0.806	3,745	9.93	0.19
K49	0.900	nd ¹	nd ¹	nd ¹
CT3	0.989	118	0.25	0.14
F3W4	0.991	36,720	5.3	1.59

a = Maximum aflatoxin accumulation (ng/g); ti = duration of lag phase (days); k = growth constant.
¹ Linear relationship not described by a multivariate equation, nd = not determined.

as well by the Gompertz model. This suggests that factors other than growth and fungal colonisation may affect CPA accumulation. The r² observed were 0.8501 for K49, and 0.619 and 0.639 for CT3 and F3W4, respectively. Maximum accumulation of CPA was 0.22 µg/kg for maize inoculated with K49 and 22 µg/kg for the formulation control (Figure 6A-D). Maize inoculated with CPA-producing strains CT3 and F3W4 had maximum CPA accumulation (a) of 169 and 77 µg/kg, respectively. These results demonstrate that inoculation with K49 has the potential to reduce both aflatoxin and CPA accumulation. However, inoculation with non-aflatoxigenic strain CT3 accumulated twice as much

CPA as F3W4. Cyclopiazonic acid has been inferred as a toxin in livestock, perturbing cellular calcium metabolism (Burdock and Flamm, 2000). Potential hazards of CPA accumulation need to be addressed when developing biocontrol strategies to reduce aflatoxin contamination.

In pure culture studies, Pitt (1993) described the production of aflatoxin by *A. flavus* at various temperatures relative to fungal growth. This work demonstrates aflatoxin accumulation parallels growth of *A. flavus* when the temperature regimen is appropriate for aflatoxin production (20 to 27 °C). A similar relationship was observed between colonisation of maize by F3W4 and toxin accumulation. The Gompertz model for colonisation by F3W4 predicted a lag (ti) of 4.3 d, while a ti value of 5.32 was observed for aflatoxin accumulation, while both parameters approached maximum colonisation and concentration 10 days after inoculation, respectively. Modelling of environmental restraints of colonisation by *A. flavus* and *A. parasiticus* on maize was attempted by Samapundo *et al.* (2007). However, these researchers used homogenous ground maize for a substrate rather than actual ears in the field. Empirical parameters developed under controlled laboratory studies can provide interesting insights into the possible interactions of a fungus and environmental conditions. However, extrapolation to field conditions is not always possible as interactions in the laboratory do not necessarily reflect what occurs between the living plant host and invasive fungal pathogen under natural conditions. Pin-bar inoculation techniques have been used with a moderate

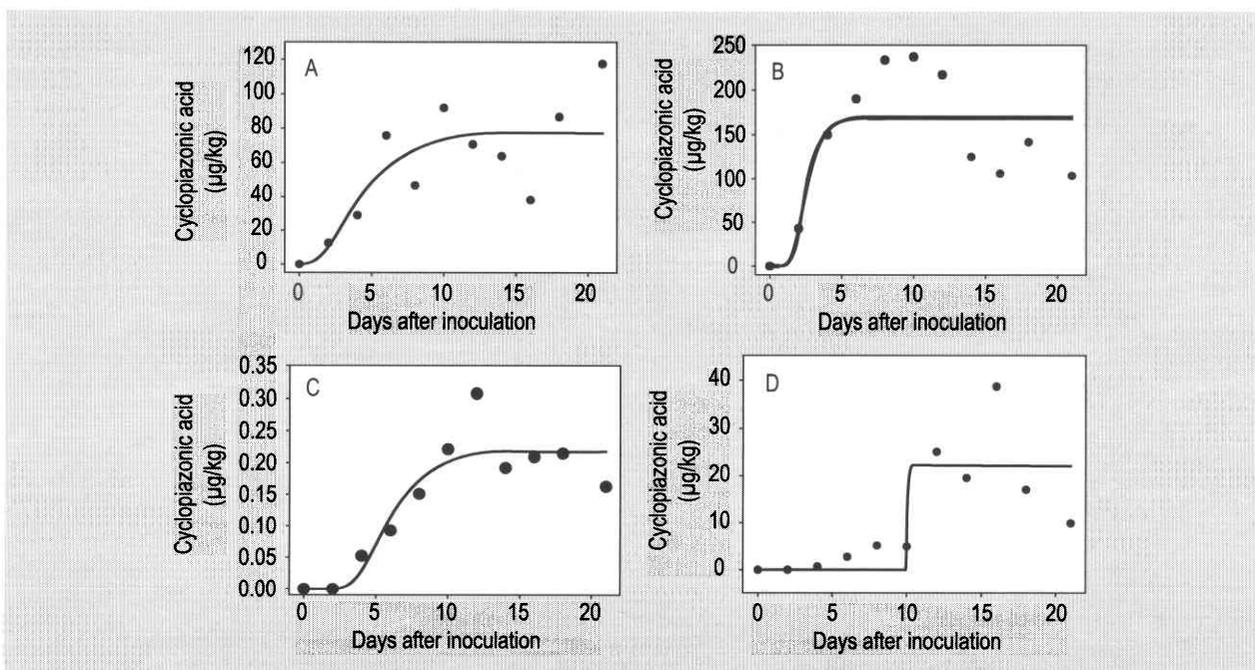


Figure 6. Observed and modeled patterns of cyclopiazonic acid (CPA) accumulation in inoculated area of maize kernels by *Aspergillus flavus* strains following pin-bar inoculation in 2005 inoculated with (A) strain F3W4; (B) strain CT3; (C) strain K49; and (D) 0.2% Tween 20 control.

degree of success in screening for maize lines with resistance to aflatoxin in breeding programs (Campbell and White, 1994, 1995; Windham *et al.*, 2003).

The current studies illustrate how the pin-bar technique can be used to quantify colonisation potential and reduction of aflatoxin and/or CPA contamination by non-toxicogenic *A. flavus* strains. These results from pin-bar inoculation studies indicate the non-toxicogenic *A. flavus* strain K49 (isolated from maize), is superior in colonising maize compared to non-aflatoxigenic strain CT3 (isolated from soil). The use of the Gompertz growth model and other regressions is rather simple and does not require sophisticated expertise in differential calculus. Thus, these models should be widely available to most researchers to quantify colonisation of corn or other crops by *Aspergillus* or perhaps other fungi. Studies using *Aspergillus* strains containing reporter genes such as GUS (β -glucuronidase) (Brown *et al.*, 1995) or GFP (green fluorescent protein) (Du *et al.*, 1999) would improve the ability to monitor colonisation by introduced *A. flavus* if permissible to conduct these studies under field conditions. Research is ongoing using the pin-bar technique to characterise competition among toxigenic and non-toxicogenic isolates for colonisation of maize.

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