AFLATOXIN CONTROL IN POSTHARVEST CORN KERNELS: EFFECTS OF CHITOSAN AND BACILLUS SUBTILIS

CUERO, R.G., G. OSUJI AND E. DUFFUS

PRAIRIE VIEW A & M UNIVERSITY, TEXAS A & M UNIVERSITY SYSTEM
CARC - P.O. BOX U, PRAIRIE VIEW, TEXAS 77446, USA

ABSTRACT

Aspergillus flavus growth and aflatoxin production in postharvest corn kernels was determined after treatment with chitosan and Bacillus subtilis. Treatment effect was determined in maize extract agar amended with chitosan and in cracked corn kernels at various water activities (0.80, 0.85, 0.90 and 0.95) at 25 C. Single or combined treatments were applied simultaneously. Chitosan reduced (50%) colony diameter and sporulation of A. flavus in maize extract agar. B. subtilis inhibited A. flavus at distance. Both chitosan and B. subtilis reduced A. flavus population on corn kernels at all Aw(s). Aflatoxin production was significantly ($p<0.05$) reduced by single chitosan treatment and by combined chitosan-B. subtilis treatment at 0.90 Aw as compared to control and other treatments. There was no aflatoxin production at lower Aw(s) (0.80 and 0.85). There was an overall correlation ($p=0.0001$) of the relationship between Aw level and A. flavus population ($r=0.50$) and aflatoxin production (0.80).

INTRODUCTION

Postharvest cereal grains develop a succession of microorganisms including toxigenic Aspergillus flavus Link ex Fries and strains of bacteria such as Bacillus spp. Microbial profile in stored commodities are determined by environmental conditions, especially water activity (Aw), temperature, and composition of the intergranular air (Magan and Lacey, 1984). Components of the microflora interact and produce transient or permanent changes within the population (Cuero et al., 1987; 1987a; 1988). Variation in microorganism populations seem to affect aflatoxin formation in cereal grains (Cuero et al., 1987; 1987a; 1988). Most studies of aflatoxin formation have used liquid media or autoclaved (nonviable) grain in axenic cultures and only rarely have viable grains and open systems been used.

Numerous attempts have been made to control aflatoxin production by limiting growth of the toxigenic fungus through the application of chemical or microbial agents with limited success.

Bacillus subtilis strains isolated from soils inhibited growth and aflatoxin production by A. parasiticus and A. flavus in corn and peanut under laboratory conditions (Kimura and Hirano, 1988). Cuero et al., (1987) reported that B. amyloliquefasciens stimulated the aflatoxin production by A. flavus in irradiated maize and rice grains. Cuero et al., (1989

unpublished) found that B. subtilis GUS 2000 caused formation of abnormal mycelium of A. flavus in liquid culture.

Chitosan is a cationic carbohydrate polymer of B-1,4 glucosamine residues that is chemically derived by deacetylation of natural-occurring chitin; the parent material is obtained primarily from crustacean shells (Muzzarelli, 1988) (Fig 1).

Cuero et al., (1988b, 1989), found that chitosan exhibits extensive inhibitory effects on A. flavus growth and/or aflatoxin production in liquid culture, and also in preharvest crops. Cuero et al., 1988b) demonstrated by microscopy that chitosan affects spore germination and production by A. flavus. Other reports have elucidated the antifungal effect of chitosan on a broad range of plant pathogenic fungi (Hadwiger et al., (1984).

The main objective of the current study was to acquire information for development of practical methods that utilize chitin/chitosan polymers alone or in combination with microbial agents to inhibit growth of aflatoxin-producing fungal species and control aflatoxin production in stored corn.

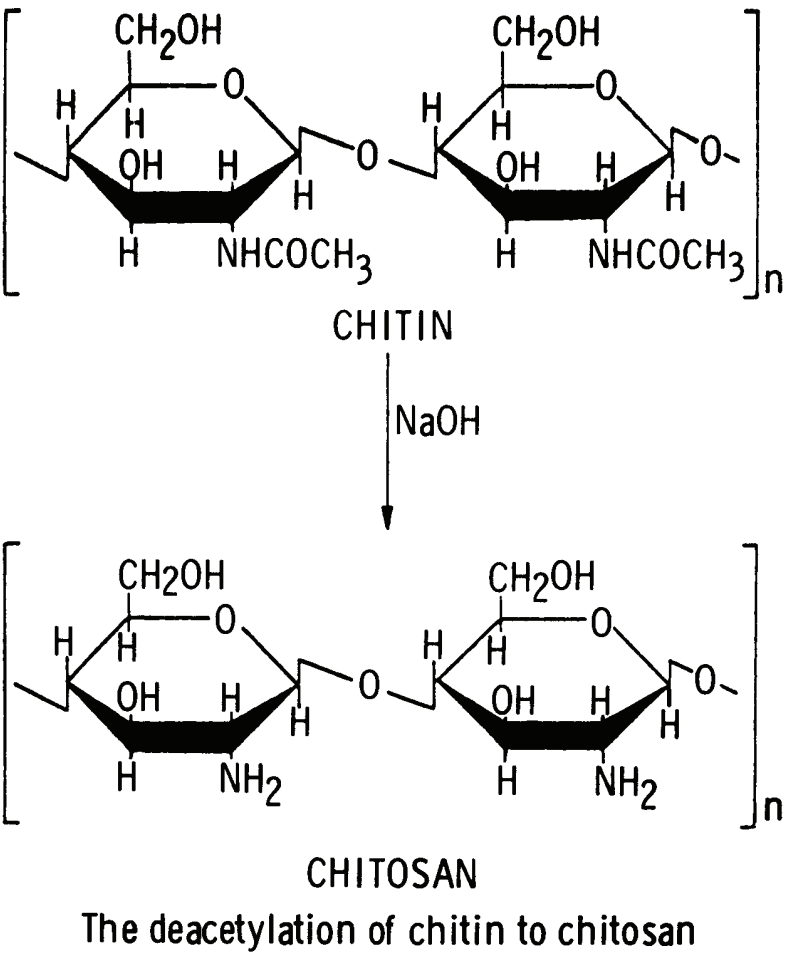
MATERIALS AND METHODS

Preparation of A. flavus and B. subtilis. An isolate of A. flavus (NRRL 3357) was used throughout the study; this isolate was maintained on potato dextrose agar (PDA). Fresh fungal inoculum (10^6 spore/ml) was prepared from 3-4 day-old cultures by recovering spores in sterile water. B. subtilis (isolate GUS 2000, Gustafson, Inc., Dallas, Texas) was grown on nutrient agar slopes at 25 C. The cells were harvested in sterile distilled water and shaken to obtain a homogeneous suspension. Fungal and bacterial cell concentrations were determined by dilution plating on nutrient agar plates with subsequent incubation for 72 h at 25 C. A suspension of 10^5 bacterial cells per ml was sed as the inoculum.

Microbial Development on Maize Extract Agar Alone or Amended with Chitosan. Maize extract agar was prepared by adding 3% aqueous maize extract to potato dextrose agar (PDA). Maize extract was obtained by boiling 3 g of ground maize in 100 ml of water for 3 m. Treatment agar plates were prepared by amendment with chitosan (0.5% v/v).

Preparation of Chitosan. Chitosan was prepared as 1% solution in water:acetic acid mixture (1:1 v/v) according to directions by the supplier, Protan Lab. Inc., Seattle, WA.)

Treatment effect was determined in a microbial dominance test; the procedure was developed by Magan and Lacey (1984). For single cultures of A. flavus (10^6 spore/ml) or B. subtilis (10^5 cells/ml) each plate was point inoculated at the center(Pitt, 1979). In combined microbial treatments, B. subtilis was inoculated 24 h after A. flavus. After inoculation three replicate plates were stacked, sealed in microporous polypropylene bags and incubated in a controlled environmental growth chamber at 95% relative humidity and 25 C. Plates were examined regularly for up to 8 days; colony diameters and the distance separating colonies were measured. After incubation microbial interactions was determined by visual assessment of



The deacetylation of chitin to chitosan

Figure 1. The structure of chitin and chitosan

colony development and scored on the following scale (Johnson and Curl, 1978; Magan and Lacey, 1984):

Classification type	Numeral value
Mutual intermingling	1
Mutual inhibition on contact or with space between the two colonies small	2
Mutual inhibition at a distance	3
Inhibition of one organism on contact, the antagonist continues to grow unchanged or at a reduced rate through the colony of the inhibited organism	4

Inoculation and Treatment Application. Cracked corn kernels (cultivar B73xMO17) susceptible to A. flavus infection and aflatoxin production was used. Corn kernels were treated with hot water (60 C for 30 minutes) to achieve surface sterilization and to maintain kernel viability (95%). Surface sterilized kernels were soaked in sterile water to achieve inbibition and adjusted to 0.80, 0.85, 0.90 and 0.95 Aw (Cuero et al., 1987). Subsequent treatments included soaking in aqueous A. flavus spore suspension, B. subtilis cells and with or without chitosan.Treatments include: 1) A. flavus, 2) B. subtilis, 3) chitosan and 4) chitosan + B. subtilis. Single and combined treatments included final levels of chitosan (1%) and B. subtilis (10^5 cells/ml) with suspensions of toxigenic A. flavus (10^6 spore/ml). Inoculated Inoculated kernels (50 grams/replicate) were placed in separate microporous film bags (Cuero et al., (1985)) (Fig 2). Bags were incubated at 25 C for 8 days. The bags were removed daily and carefully shaken to prevent mycelial compaction. Fungal growth was visually assessed at the end of the incubation period using a scale of: 0, no growth; 1, little growth; 2, 50% of the kernel substrate covered; 3, 75% of kernel substrate covered; and 4, 100% kernels substrate covered. A. flavus colony forming units (CFU) were quantitated by dilution plate count method using potato dextrose agar (PDA).

Mycotoxin Extraction and Quantification. Ground blended corn samples were assayed for aflatoxin B1 by the Official First Action Method (Association of Official Analytical Chemists, (1980).

Statistical Analysis. Data were analysed by using the statistical analysis system (SAS) computer program.

RESULTS

Growth and Int eractions Between A. flavus and Either chitosan or B. subtilis on Maize-extract agar. Chitosan reduced (50%) colony diameter and sporulation of A. flavus in maize extract agar (Fig 3). Chitosan did not affect B. subtilis growth. B. subtilis inhibited A. flavus at distance with an index of dominance (ID) of 4 (Magan and Lacey, 1984; Cuero et al., 1987)and also the bacterium continued to grow unchanged through the colony of the inhibited fungus.(Fig 4)

Fungal Culture on Corn kernels. The effect of chitosan or B. subtilis as single treatments on reducing the number of A.

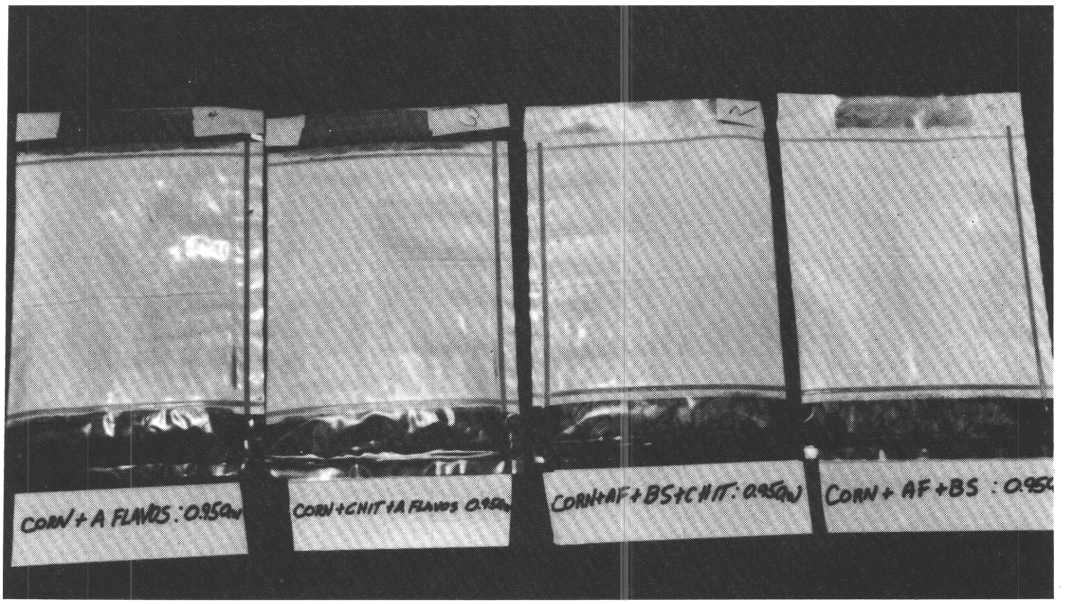


Figure 2. Growth of Aspergillus flavus alone or in mixture with chitosan or Bacillus subtilis in cracked corn kernels at 0.95 Aw at 25 C after 8 days incubation

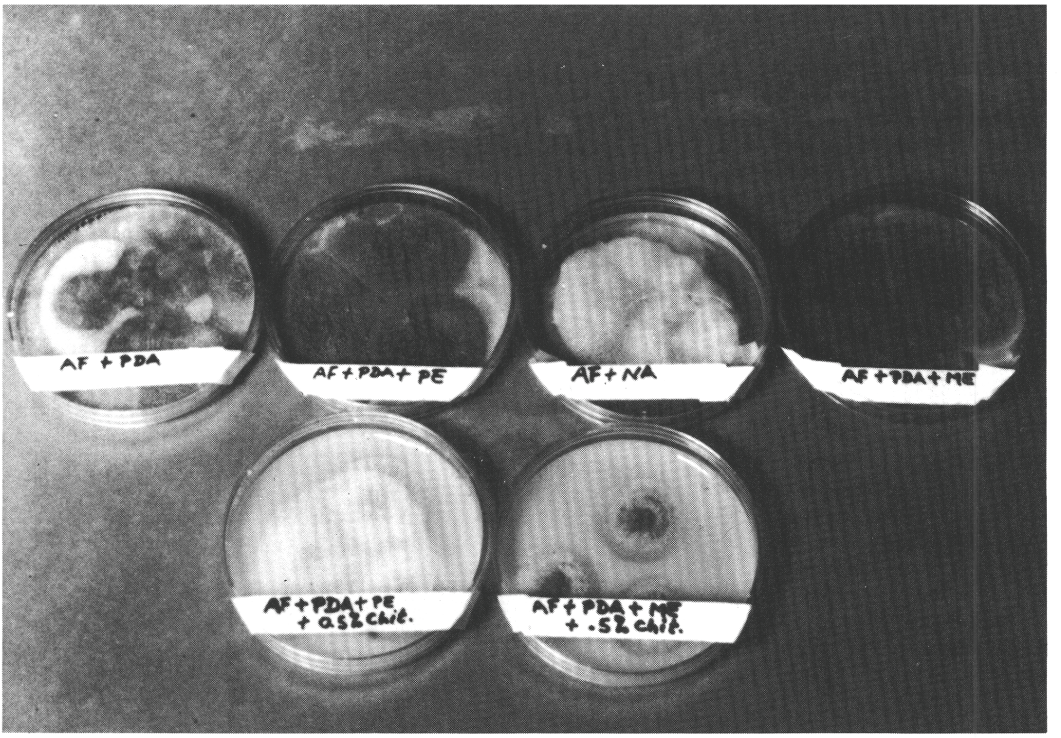


Figure 3. Inhibition on growth of aflatoxigenic Aspergillus flavus on chitosan-amended agar with maize extract

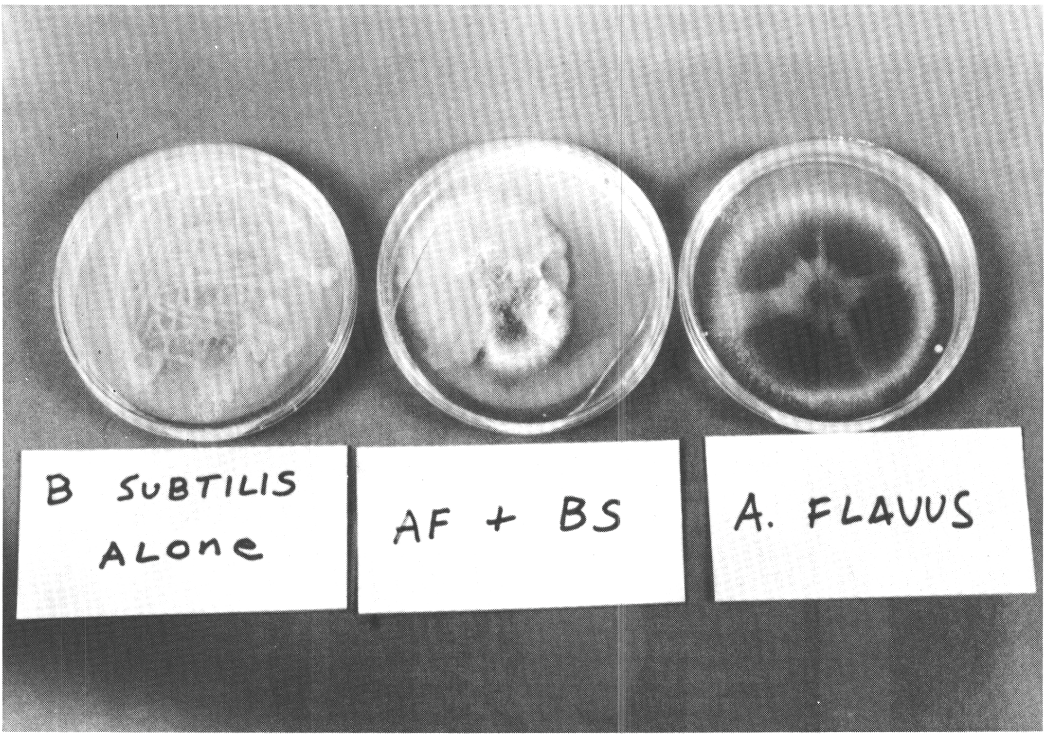


Figure 4. Inhibition on growth of aflatoxigenic Aspergillus flavus by strain of Bacillus subtilis (Gus 2000)

flavus CFU (population) in corn kernels was observed at all Aw(s), but the visible growth was evident only at higher Aw(s) (0.90 and 0.95) (Table 1). Mycelia formation was visibly observed at highest Aw (0.95) only.

Aflatoxin Production in cracked corn. Toxin production by A. flavus in cracked corn is shown in Figure 5. Aflatoxin production was significantly ($p<0.05$) reduced by single chitosan and B. subtilis treatment at 0.90 Aw as compared to control and other treatments. At 0.90 Aw, single chitosan and B. subtilis showed the most effective treatment to control aflatoxin production by A. flavus. B. subtilis and chitosan single treatments significantly reduced aflatoxin production as compared to other treatments at 0.90 Aw. At the highest Aw (0.95) production of aflatoxin was decreased by only 20% and 11% by single chitosan and B. subtilis treatments respectively. Combined chitosan-B. subtilis treatment did not affect aflatoxin production at the highest Aw (0.95). In general, the highest Aw (0.95), environment did not affect the ability treatment reduction of aflatoxin production. There was no aflatoxin production at lower Aw (s) (0.80 and 0.85).

There was an overall correlation ($p=0.0001$) of the relationship between Aw level and A. flavus CFU ($r=0.47$), VFG ($r=0.82$) and aflatoxin production (0.80).

DISCUSSION

Growth of A. flavus in cracked corn substrate in the microporous bag system (Figure 2) and on agar (Figure 3) point inoculation method, showed that the interacting fungus was usually inhibited by single chitosan or B. subtilis treatment as well as by combined chitosan-B. subtilis treatment. Reduction of fungal colony diameters and sporulation on agar plates, and inhibition of mycelia formation in cracked corn kernels at Aw(s) lower than 0.95, might suggest that, on the surface of both agar plates and corn, the multispore A. flavus inoculum has been reduced to small but discrete colonies of the fungus although the extent of aerial development and sporulation may be less easily distinguished than in point inoculation experiments. Cuero et al., (1988b) demonstrated that chitosan affected sporulation of A. flavus species and inhibited germination especially during the first 48 h of fungal growth in liquid culture. Cuero et al., (1990 unpublished) have found increased elicitation of phenolic compounds in peanut seeds treated with chitosan, at higher Aw(s). The inhibitory effect by B. subtilis of A. flavus growth might be a result of antibiosis action by the bacteria or a consequence of competition for nutrient availability (Cuero, 1989).

Correlations between Aw level, fungal growth and toxin production indicates that chitosan and B. subtilis treatments are most effective at Aw lower than 0.95.

CONCLUSION

1. Undoubtedly chitosan and B. subtilis either as single or combined treatments are very effective biocontrol agents of both

TABLE I. ASPERGILLUS FLAVUS GROWTH IN SINGLE AND COMBINED TREATMENTS AT VARIOUS WATER ACTIVITIES AT 25 C, AFTER 8 DAYS INCUBATION.

A_w [1]	0.80		0.85		0.90		0.95	
TREATMENT [2]	CFU [3]	VFG [4]	CFU	VFG	CFU	VFG	CFU	VFG
AFL ALONE	2×10^4	0	6×10^4	0	2×10^5	1	2.5×10^6	3
AFL + CHIT	1×10^4	0	4×10^3	0	1×10^4	0	1×10^6	3
AFL + BSUB	1×10^4	0	9×10^2	0	1.5×10^5	0.5	7×10^5	2
AFL + CHIT + BSUB	9×10^3	0	2×10^3	0	3×10^4	0	2.5×10^6	3
LSD	1×10^4	0	4×10^4	0	1×10^4	0	3×10^6	0.5

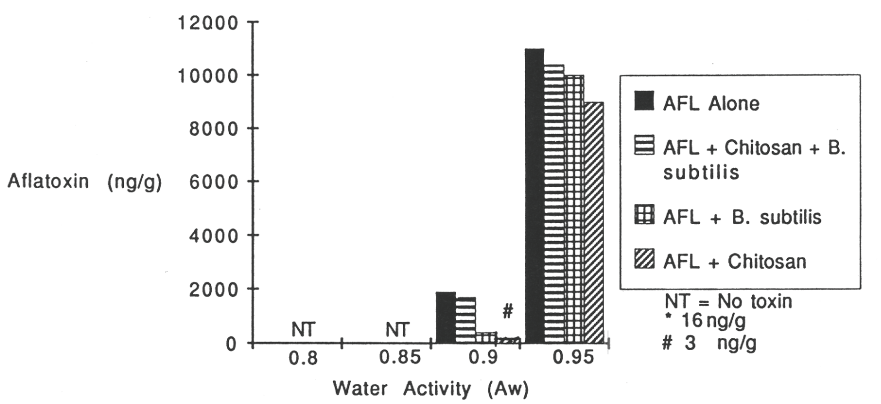
1 A_w = water activity.

2 AFL = Aspergillus Flavus, CHIT = Chitosan, BSUB = Bacillus Subtilis.

3 CFU = Colony forming unit.

4 VFG = Visible fungal growth; scale, 0 to 5: 0 = No visible growth; 1 = very little growth; 2 = 25% of the grain covered; 3 = 50% of the grain covered; 4 = 75% of the grain covered; 5 = 100% of the grain covered.

Figure 5. Aflatoxin production by A. flavus alone or in combined treatment with chitosan or B. subtilis in corn kernels, after 8 days of incubation at 25°c and various water activities



A. flavus and concomitant aflatoxin production in corn.

2. Clearly, effectiveness of chitosan and B. subtilis on controlling both growth and aflatoxin production by A. flavus depends upon water content of the substrate.

3. The use of chitosan and B. subtilis as biocontrol agents of toxigenic A. flavus and aflatoxin in corn seem considerable, practical and economic.

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ELIMINATION DES AFLATOXINES DU MAIS ET DES ARACHIDES A DIFFERENTES TEMPERATURES ET A DIFFERENTES ACTIVITES DE L'EAU : EFFETS DE *BACILLUS SUBTILIS* ET DU CHITOSAN

Raul G. CUERO, E. DUFFUS et G. OSUJI

Prairie View A&M University, Texas A&M University System
Cooperative Agricultural Research Center
P.O. Box Drawer U, Prairie View, Texas 77446

RESUME

On a déterminé la croissance d'*Aspergillus flavus* et la production d'aflatoxines B chez le maïs et les arachides à différentes activités de l'eau (0,80 ; 0,85 ; 0,90 et 0,95) et à deux températures différentes (25 et 30 C), après traitement au chitosan ou avec *Bacillus subtilis* (GUS 2000). Des traitements de désinfection, uniques ou combinés, ont été appliqués simultanément avec l'inoculum d'*Aspergillus flavus*. Les effets du chitosan sur la réduction de la population de *A. flavus* (CFU) ont été observés à 0,85 et à 0,90 A_W à toutes températures mais il n'y a eu aucun effet à 0,95 A_W, ni dans les grains de maïs, ni sur les arachides Cependant, l'effet du chitosan sur la croissance de *A. flavus* était plus marqué chez les grains de maïs. La production d'aflatoxines a été réduite de 65 % dans les arachides traitées à 0,90 A_W par rapport à celles qui n'avaient pas été soumises au traitement. Aucune aflatoxine n'est apparue à 0,80 et à 0,85 A_W, ceci pour les deux espèces, aussi bien dans les grains traités que dans les grains non traités. *B. subtilis* seul a réduit à la fois la croissance de *A. flavus* (86 %) et de l'aflatoxine à une A_W inférieure à 0,90 % chez le maïs et l'arachide mais le traitement combiné, *B. subtilis* + chitosan, a complètement éliminé la production d'aflatoxine. A des A_W plus élevées (0,90 et 0,95), *B. subtilis* seul s'est montré plus efficace pour éliminer le production d'aflatoxines (55 %) à toutes les températures, chez le maïs et l'arachide, bien que la combinaison de *B. subtilis* et du chitosan se soit avérée donner le meilleur résultat dans la réduction du taux d'aflatoxines (80 %). Les effets obtenus par les traitements sur les populations de *A. flavus* et sur la production d'aflatoxines se sont avérés globalement plus efficaces sur le grain de maïs que sur l'arachide.