Effect of Bacterial Chitinases on the Growth and Aflatoxin Production of *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare

Stella Marie Mozar-Doyungan[1] and Ida F. Dalmacio[2]

Abstract

The ability of the crude bacterial chitinases produced from **Bacillus circulans** *B1037 (5Bc),* **Arthrobacter luteus** *B1077 (6A1),* **B. circulans** *B1045 (7Bc),* **Streptomyces griseus** *B1562 (9Sg),* **Serratia marcescens** *LPC19 B1748 (11Sm) and* **S. marcescens** *LPM42 B1749 (14Sm) to inhibit growth and aflatoxin production of* **Aspergillus flavus** *and* **A. parasiticus** *was studied.*

Results show that the crude chitinases can control the growth of the two aflatoxigenic molds, **A. flavus** *and* **A. parasiticus** *in corn kernels as indicated by the significant reduction in the number of infected kernels. In addition, aflatoxin B_1 was not detected in some-enzyme treated corn kernels previously infected with the test organisms.*

Keywords: aflatoxin, antifungal agents, chitinases, chitinolytic bacteria

Introduction

The growth of fungi in stored products should not be ignored. Their growth leads to the deterioration in appearance, in quality for processing, and often in the food value of the product and second, their production of mycotoxins (Pitt and Hocking, 1991). In many ways, these consequences cause economic losses to growers, feed and livestock manufacturers.

Two species of molds, *Aspergillus flavus* and *A. parasiticus* are widely known to produce aflatoxins. Aflatoxins are highly toxic metabolites. Acute disease in human and animals, as well as a chronic diseases such as primary liver cancer, have been linked to aflatoxin consumption (Bhat, 1991). Since aflatoxin is a secondary metabolite, one approach to control toxin contamination is to control the growth of the molds in the commodity.

Chitinases are enzymes which hydrolyze chitin, *B*-1,4 linked polymer of N-acetylglucosamine. As fungal cell walls contain chitin, their chitinolysis offers a method of their control. In addition, the use of chitinases as biocontrol agents holds prospect for an ecologically sound system for pest management.

Several studies reported the antifungal of bacterial chitinases. Chitinase from *Bacillus cereaus* along with laminarase cause lysis of *Fusarium oxysporum* (Mitchell and Alexander,

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[1] Department of Biological Sciences, College of Science and Mathematics, MSU-Iligan Institute of Technology, Iligan City

[2] Microbiology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños College, Laguna

1963), chitinase of *B. circulans* enhanced lysis of *Aspergillus oryzae* (Horikoshi and Iida, 1958; 1959), chitinolytic *Arthrobacter* inhibited the growth of *Fusarium moniliforme* var. *subglutinans* (Barrows-Broaddus and Kerr, 1981), chitinolytic bacteria: *B. cereus, B. chitinoporous* and *Serratia marcescens* were antagonistic against *Aspergillus flavus and A. parasiticus* and *Fusarium oxysporum* (Gay, 1992), and crude chitinase of *S. marcescens* cause lysis of fungal tips of *Sclerotium rolfsii* (Ordentlich et al, 1988).

This study determines the ability of the crude bacterial chitinases to control the growth and aflatoxin production of *Aspergillus flavus* and *A. parasiticus.*

Materials and Methods

The crude bacterial chitinases from the following bacteria namely; *Bacillus circulans* B1037 (5Bc), *Arthrobacter luteus* B1077 (6A1), *B. circulans* B1045 (7Bc), *Streptomyces griseus* B1562 (9Sg), *Serratia marcescens* LPC19 B1748 (11Sm) and *S. marcescens* LPM42 B1749 (14Sm) were produced and assayed by Doyungan, 1996.

Certified corn kernels IPB var. 1 and var. 7, purchased from the National Seed Foundation of the Institute of Plant Breeding (IPB), UPLB, were used. A total 28,800 healthy corn kernels were selected as substrates for the growth of *Aspergillus flavus* and *A. parasiticus.*

Corn kernels were disinfected with 10% solution of a commercially available sodium hypochlorite solution (5.25% by weight) for 1 min, followed by three rinses of sterile distilled water. The kernels were then dried in the oven at 75°C for about 6-8 hours to lower the moisture content in order to prevent germination of the corn seeds.

Spore suspensions of 10^5/ml of the test molds were prepared. Three experimental set-ups were used. Three trials were done for every set-up with two replications per trial.

First set-up. A total of 3,200 disinfected corn kernels were divided into eight lots, one lot each for the six crude chitinases and two controls (positive and negative). Each lot, consisting of 400 kernels, was divided further into two sublots, representing two replications. Each sublot was added with 1.0 ml spore suspension. Then, the kernels were shaken to disperse the spores. The inoculated kernels were placed in 10 Petri dishes lined with moist filter paper, 20 kernels per dish.

Seed treatment of the inoculated kernels was done by spraying with about 2ml of the crude enzyme per dish right after placing the kernels in the dishes. For controls, distilled water and 500 ppm Benlate were used. The percentage of infection was noted 7-10 days after treatment. To determine kernel infection, each kernel was examined visually for presence of hyphal growth.

Second set-up. Corn kernels were prepared as in the first set-up. Then, a volume of 20 ml crude enzyme was added to each sublot of spore-seeded corn kernels. For controls, distilled water and 500 ppm Benlate were used. The kernels were shaken again thoroughtly to mix the enzyme, afterwhich, they were decanted and placed in 10 Petri dishes lined with moist filter paper, 20 kernels per dish. The dishes were incubated for 7-10 days at room temperature and percent infection was determined.

Third set-up. The same number of corn seeds were divided into lots and sublots and inoculated with spore suspesion as in the previous set-ups. The inoculated kernels were placed in Petri dishes (20 kernels/dish) lined with moist filter paper and were incubated at room temperature for 48 hours. Then, each dish was sprayed with 2 ml crude enzyme or enough to drench all the seeds. For controls, distilled water and 500 ppm Benlate were used.

One hour, 3 hours, 7 hours and 24 hours after spraying, 50 kernels from each lot were placed in two Petri dishes line with moist filter paper (25 kernels per dish). Growth of the fungus was observed after 7-10 days incubation. After observation, the kernels soaked for 24 hours from this set-up were assayed for aflatoxin following the procedure of Pons and Goldblatt, 1965.

Results and Discussion

The antifungal activity of the crude bacterial chitinases were determined by assaying the percentage infection of spore-inoculated chitinase-treated corn kernels. Data on the antifungal activity of the crude chitinases on *Aspergillus flavus* and *A. parasiticus* based on the mean number of infected corn kernels in set-ups 1 and 2 are presented in Table 1. All the crude chitinases in set-up 1 produced significant reduction in the mean percentage of corn kernels infected with *A. flavus* compared to the negative control. Lowest percentage of infectivity was observed in lots treated with *Bacillus circulans* (7Bc) followed by *Serratia marcescens* (11Sm). Slightly different result was observed in set-up 2. Two crude chitinases from *B. circulans* (5Bc) and *Arthrobacter luteus* (6Al), did not show significant difference compared to the negative control and lowest percentage of infectivity was observed in lots treated with crude chitinases from *Streptomyces griseus* (9Sg) followed by *S. marcescens* (11Sm). In *A. parasiticus* spore-seeded lots, all but one *B. circulans* (5Bc) crude chitinase caused significant reduction in the mean percentage of infected corn kernels in set-up 1 compared to the negative control. Lowest percentage of infectivity was observed in lots treated with crude chitinases from 9Sg and 11Sm.

In set-up 3, antifungal activity of the crude chitinases was assayed on kernels seeded with spores of *A. flavus* and *A. parasiticus* and incubated for 48 hours. In *A flavus*, no significant differences were observed in the mean percentage of infected kernels of the lots treated with the crude enzymes assayed one hour after treatment compared to the negative control. A significant difference however was observed when compared with the positive control. Three hours after treatment, all crude enzymes except *B. circulans* (7Bc) and *S. marcescens* (14Sm) caused significant reduction in the mean percentage of infected kernels compared to the negative control. Seven hours hours after treatment, all the crude chitinases except 7Bc were found to cause a significant difference in the mean percentage of infected kernels. All of the crude enzymes caused significant reduction 24 hours after treatment (Table 2a).

Table 2b presents the chitinase activity of the crude enzymes against *A. parasiticus* as assayed at various times after treatment. One hour after treatment, only *B. circulans* (7Bc) and *Streptomyces griseus* (9Sg), caused significant reduction in the mean percentage of infected corn kernels compared to the negative control. After three hours, *Serratia marcescens* (14 Sm) in addition to 7Bc and 9Sg, showed significant effect on the percentage infected kernels. Seven hours after, only *B. circulans* (5Bc) did not show any significant difference in the percentage of infected kernels. All the crude chitinases, however, caused significant reduction in the percentage of infected kernels 24 hours after treatment compared to the negative control.

The above results further indicate that the chitinase can cause significant reduction in

infection when allowed to act for at least three hours.

It is apparent in the results of the three set-ups that the crude chitinases can control the growth of both *A. flavus* and *A. parasiticus* in inoculated corn kernels. The infection of some kernels in set-ups 1 and 2 can be attributed to the resistance of some spores to the enzyme. Probably, these spores might have germinated and some hyphae were acted upon by the enzyme. The enzyme, however, did not affect all the hyphae, thus causing the infection. The significant reduction in kernel infection caused by all crude enzymes 24 hours after treatment in set-up 3 can be due to the susceptibility of the newly-growing hyphae to the chitinases.

The differences in the results in set-ups 1 and 2 can be attributed to some factors like growth conditions and method of enzyme application. Each set-up was done at different times. In set-up 1, the enzyme was sprayed, and such, more enzyme was retained in the dish while in set-up 2, the enzyme was added to the kernels and then decanted after thorough shaking. It could be that little amount of the enzyme was left in the kernels. Furthermore, *B. circulans* (7Bc) might be more effective if applied through spraying than soaking. The reverse is observed with *Streptomyces griseus* (9Sg).

Results of the analysis for the presence of aflatoxin in the corn kernels obtained from set-up 3 are presented in Table 3. Aflatoxin B1 was present in all the corn kernels inoculated with *A. flavus*. Likewise, the same aflatoxin was present in kernels inoculated with *A. parasiticus* and were treated with *B. circulans* (7Bc), *Serratia marcescens* (11Sm) and *S. marcescens* (14Sm) in addition to the control. No aflatoxin was detected in kernels treated with B. *circulans* (5Bc), *Arthrobacter luteus* (6Al) and *Streptomyces griseus* (9Sg).

The presence of aflatoxin in the treated corn kernels can be accounted for by the fact that spores of *A. flavus* and *A. parasiticus* were inoculated into the corn and were allowed to grow on the corn before the crude enzyme extracts were added (set-up 3). With the 48-hour incubation, the molds had already grown. Dickens and Pattee (1966) reported that at least 48 hours from inoculation is required for aflatoxin to develop in peanut kernels at 90°F with moisture contents between 15 and 30%. This may also hold true for corn kernels.

Aflatoxin contamination of crop plants, grains and foodstuff is dependent on the successful colonization and growth of the aflatoxigenic molds. Since aflatoxins are secondary metabolites, their production is affected if growth is inhibited. Results have shown that crude chitinases can affect growth of *A. flavus* and *A. parasiticus*, and this might be the reason why no aflatoxin was detected in some enzyme-treated corn kernels. It is possible that the amount of toxin present was very low beyond the limit of sensitivity of thin layer chromatography (TLC) which is 1 ppb (Pons and Goldblatt, 1965).

Table 1. Growth of *Aspergillus flavus* and *A. parasiticus* on corn kernels treated with undiluted crude chitinases after 7-10 days of incubation (set-ups 1 and 2).

ENZYME	INFECTED CORN KERNELS (%)*							
	Aspergillus flavus				*A. parasiticus*			
	Set-up 1 (Mean+/- S.E.)		Set-up 2 (Mean+/- S.E.)		Set-up 1 (Mean+/- S.E.)		Set-up 2 (Mean+/- S.E.)	
Control (-)	77.75a	2.09	87.75a	5.22	70.38a	2.69	82.38a	4.86
Control (+)	0.50f	0.35	0.63f	0.38	0.63g	0.47	1.50e	0.65
5Bc	61.43b	4.28	72.38abc	6.21	62.25ab	4.91	59.25a	13.51
6Al	49.63c	4.56	81.52ab	4.94	45.88cd	5.78	58.00ab	12.62
7Bc	14.75e	5.13	64.88bc	3.24	9.13f	1.89	45.50bc	7.85
9Sg	53.80b	1.98	14.13e	4.68	52.25bc	2.77	10.13d	1.78
11Sm	32.37d	3.75	28.63d	4.74	30.63e	5.76	24.25d	7.36
14Sm	52.88bc	1.05	59.38c	13.94	38.78de	2.05	44.88bc	9.17

Mean values with dissimilar letters show significant differences based on DMRT.
*average of three trials with two replications per trial

Legend:

Control (-) - distilled water
Control (+) - Benlate
5Bc - *Bacillus circulans*
6Al - *Arthrobacter luteus*
7Bc - *B. circulans*
9Sg - *Streptomyces griseus*
11Sm - *Serratia marcescens*
14Sm - *S. marcescens*

Table 2a. Growth of *Aspergillus flavus* on corn kernels treated with undiluted crude chitinases after 7-10 days of incubation (set-up 3).

ENZYME	INFECTED CORN KERNELS (%)* Time of Assay							
	After 1 hour (Mean+/- S.E.)		After 3 hours (Mean+/- S.E.)		After 7 hours (Mean+/- S.E.)		After 24 hours (Mean+/- S.E.)	
Control (-)	40.00a	6.45	58.00a	11.44	46.67a	8.37	72.00a	12.00
Control (+)	0.67b	0.67	0b	0	0c	0	1.33b	1.30
5Bc	26.67a	7.42	26.00a	7.21	20.67b	8.09	17.33b	6.98
6Al	21.33a	5.13	24.00b	5.16	9.33bc	1.98	10.67b	3.68
7Bc	35.33a	3.33	47.33a	3.49	28.67ab	6.57	22.67b	5.81
9Sg	21.33a	8.31	19.33b	5.51	6.00c	2.88	3.33b	1.23
11Sm	26.67a	15.00	12.00b	4.84	7.33c	2.40	27.33b	16.41
14Sm	28.00a	7.84	31.33a	17.20	14.67bc	5.81	22.67b	10.36

Mean values with dissimilar letters show significant differences based on DMRT.
*average of three trials with two replications per trial

Legend:

Control (-) - distilled water
Control (+) - Benlate
5Bc - *Bacillus circulans*
6Al - *Arthrobacter luteus*
7Bc - *B. circulans*
9Sg - *Streptomyces griseus*
11Sm - *Serratia marcescens*
14Sm - *S. marcescens*

Table 2b. Growth of *Aspergillus* parasiticus on corn kernels treated with undiluted crude chitinases after 7-10 days of incubation (set-up 3).

ENZYME	INFECTED CORN KERNELS (%)*							
	Time of Assay							
	After 1 hour (Mean+/- S.E.)		After 3 hours (Mean+/- S.E.)		After 7 hours (Mean+/- S.E.)		After 24 hours (Mean+/- S.E.)	
Control (-)	60.67a	9.77	51.33a	5.79	57.33a	16.77	52.00a	10.12
Control (+)	1.33b	1.33	0c	0	0.67b	0.67	1.33b	1.33
5Bc	33.33ab	5.79	24.00ab	4.95	24.67ab	12.83	13.33b	3.21
6Al	36.67ab	3.33	25.33ab	8.56	10.67b	3.33	14.00b	4.82
7Bc	24.67b	5.97	21.33bc	3.68	14.66b	4.09	22.00b	9.73
9Sg	17.33b	5.13	12.00bc	5.27	16.67b	8.67	10.00b	4.10
11Sm	34.67ab	8.68	25.00ab	13.60	12.00b	5.84	14.00b	6.18
14Sm	35.33ab	15.23	8.67bc	2.81	20.67b	16.08	19.33b	16.34

Mean values with dissimilar letters show significant differences based on DMRT.
*average of three trials with two replications per trial

Legend:

Control (-)	- distilled water
Control (+)	- Benlate
5Bc	- *Bacillus circulans*
6Al	- *Arthrobacter luteus*
7Bc	- *B. circulans*
9Sg	- *Streptomyces griseus*
11Sm	- *Serratia marcescens*
14Sm	- *S. marcescens*

Table 3. Aflatoxin production by *Aspergillus flavus* and *A. parasiticus* in corn kernels treated with the crude chitinases incubated for 7-10 days.

ENZYME	AFLATOXIN CONTENT (in ppb B1)		(%) REDUCTION	
	A. flavus	*A. parasiticus*	*A. flavus*	*A. parasiticus*
Control (+)	3333.33	3333.33	0	0
Control (-)	below 1 ppb*	below 1 ppb*	-	-
5Bc	trace of B1	below 1 ppb*	-	-
6Al	833.33	below 1 ppb*	75	-
7Bc	500.00	333.33	85	90
9Sg	833.33	below 1 ppb*	75	-
11Sm	500.00	1333.33	85	60
14Sm	500.00	333.33	85	90

*Limit of detection is 1ppb (Pons and Goldblatt, 1965)

Legend:

Control (+) - distilled water
Control (-) - Benlate
5Bc - *B. circulans*
6Al - *Arthrobacter luteus*
7Bc - *B. circulans*
9Sg - *Streptomyces griseus*
11Sm - *Serratia marcescens*
14Sm - *S. marcescens*

References Cited

Barrows-Broaddus, J., and T.K. Kerr. 1981. Inhibition of *Fusarium moniliforme* var. *subglutinans*, the causal agent of pine pitch canker, by the soil bacterium *Arthrobacter* sp. Can. J. Microbiol. 27:20-27.

Bhat, R.V. 1991. Aflatoxins: Successes and failures of three decades of research, In B.R. Champ, E. Highly, A.D. Hocking and J.L. Pitt (eds), Fungi and mycotoxins in stored products. Proceedings of an international conference held at Bangkok, Thailand, 23-26 April 1991.

Dickens, J.W. and H.E. Pattee. 1966. The effects of time, temperature and moisture on aflatoxin production in peanuts inoculated with a toxic of *Aspergillus flavus*. Trop. Sci. 8:11-22.

Doyungan, S.M. 1996. Control of *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare growth using bacterial chitinases. Unpublished Ph.D. Thesis, UP at Los Banos. 109p.

Gay, P.A., K.V. Saikumar, T.E. Cleveland and S. Tuzon. 1992. Antagonistic effect of chitinolytic bacteria against toxin-producing fungi. Phytopathology. 82:1074 (Abstr.)

Horikoshi, K. and Iida. 1958. Lysis of fungal mycelia by bacterial enzyme. Nature 181:917-918.

Horikoshi, K. and S. Iid. 1959. Effect of lytic enzyme from *Baccilus circulans* and chitinase from *Streptomyces sp.* on *Aspergillus oryzae*. Nature 183:186-187.

Mitchell, R. and M. Alexander. 1963. Lysis of soil fungi by bacteria. Can J. Microbiol. 9:169-177.

Ordentlich, A., Y. Elad and I.Chet. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78:84-88.

Pitt, J.I. and A. Hocking. 1991 Significance of fungi in stored products. IN: Fungi and Mycotoxins in Stored Products. B.R. Champ, R.E. highly, A.D. Hocking and J.L. Pitt (eds). Proceeding of an international conference at Bangkok, Thailand, 23-26 April.

Pons, W.A. JR. and L.A. Goldblatt. 1965. The determination of aflatoxins in cottonseed products. J. Amer. Oil Soc. 42:461-475.