Inhibition of *Aspergillus flavus* Growth by Silk Extracts of Resistant and Susceptible Corn

Keywords: *Aflatoxins; antifungal; enzyme inhibitors; hydrolases*

INTRODUCTION

The problems of recognition and reaction in defense mechanisms are shared by virtually all plants, and different strategies have evolved for combating pathological challenges (Albersheim and Anderson-Prouty, 1975). New concepts of protein chemistry and molecular biology now allow a better understanding of the types and functions of molecules involved in plant defense mechanisms (Boller, 1985).

A major problem in corn and other cereal crops concerns contamination with aflatoxins that are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. These compounds constitute a number of structurally related secondary metabolites, which differ considerably in their biological effects (Bennett and Papa, 1988). One of the goals of corn research is to determine differences in susceptibility to *A. flavus* infection among varieties attributable to intrinsic agents such as volatile organic compounds, polysaccharides, and polypeptides. A recent study in our laboratory using agar assays showed that saline-soluble proteins extracted from kernels of corn inhibited growth of *A. flavus* (Neucere and Zeringue, 1987).

In this paper, we describe the response of mycelial growth and sporulation of *A. flavus* in vitro to water extracts of corn silks or styles. We chose silks from open-pollinated varieties with extremes in aflatoxin contamination due to *A. flavus* infection, namely Yellow Creole (highly resistant) and Huffman (highly susceptible). These two varieties are among several that were field tested in recent years (Zuber et al., 1983). Correlation of fungal growth inhibition and characterization of protein and hydrolases in silk extracts from the two varieties were assessed. Glucanases and chitinases have been reported to be involved in resistance of plants against diverse fungi (Boiler et al., 1983; Neucere et al., 1995).

EXPERIMENTAL PROCEDURES

Sample Preparation and Protein Extraction. Sixty milligram samples of mature dried silks from the two varieties were extracted in 1.0 mL of water using a mortar and pestle at 25 °C. The homogenates were clarified by centrifugation at 5000*g* for 30 min at 25 °C. Protein contents of each solution were determined according to the standard BCA procedure (Pierce Chemical Co.).

Analytical Procedures. Separation of native cationic proteins by cathodic polyacrylamide gel electrophoresis, PAGE (Bio-Rad), was conducted on vertical slabs at pH 8.5 according to the procedure of Mikola (1965) using a borate−potassium hydroxide buffer system. The stacking gels contained 4% (w/v) acrylamide, and the running gels contained 7.5% (w/v) acrylamide. Electrophoresis was conducted at a constant current of 50 mA/slab for approximately 2 h at 10 °C. Standard anionic PAGE with detergent was conducted according to the procedure of Pan et al. (1989). The active β-1,3-glucanase enzyme bands in the two procedures were visualized on the gels as described by Pan et al. (1989). Each gel was incubated at 40 °C for 30 min in a mixture containing 75 mL 0.05 M potassium acetate (pH 5.0) and 1 g of laminarin dissolved in 75 mL of water by heating in a boiling water bath. The gel was washed three times with distilled water after incubation. The PAGE gels were then put into a glass tray containing 0.3 g of 2,3,5-triphenyltetrazolium chloride in 200 mL of 1.0 M NaOH. The tray was kept in a boiling water bath until red bands appeared (about 10 min). The β-1,3-glucanase activity of the extracts was detected by the laminarin−dinitrosalicylate assay of Abeles and Forrence (1970). Chitinase assays were performed according to the procedure of Hackman and Goldberg (1964). Bioassays of fungal growth inhibition were carried out in tissue cluster plates using potato dextrose agar as described in an earlier study (Neucere and Zeringue, 1987). The test samples were dialyzed against distilled water before application to the agar wells.

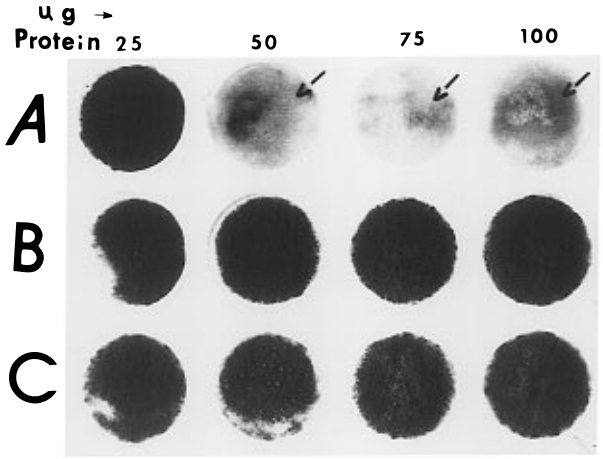

Figure 1. Evaluation of antifungal activity in agar imparted by water-soluble extracts of corn silks from Yellow Creole (A) and Huffman (B). The wells contained 25, 50, 75, and 100 μg of protein from each variety. The wells in row C contained 50 μL aliquots of fungal spores as controls. The inoculum consisted of 50 μL of *A. flavus* spores for each sample well application.

RESULTS AND DISCUSSION

Representative data of fungal growth inhibition by the water extracts in solid media are shown in Figure 1. No inhibition was detected in wells containing up to 100 μg of protein in the Huffman fraction (B). The Yellow Creole sample (A) showed inhibition for levels greater than 25 μg of protein. These experiments were designed to show only the differences in inhibition of spore germination by the two extracts. Further studies will be required to determine whether structural proteins or enzymes in silks do indeed play a significant role in defense against fungi. Row C corresponds to spores only.

Glucanases in extracts from the two silk fractions were detected following electrophoresis on native PAGE gels (Figure 2) with a cathodic buffer system (gel 1) to separate basic proteins and with the standard anodic system with detergent (gel 2) to separate acidic enzymes. The glucanase activity appeared as bright red bands on the gels. The β-1,3-glucanase isozyme bands were different in both the native and the detergent gels. The results describe direct detection of glucanase patterns for comparing resistant and susceptible varieties of corn.

Comparative protein content and relative content of total chitinase and glucanase activities in the two silk

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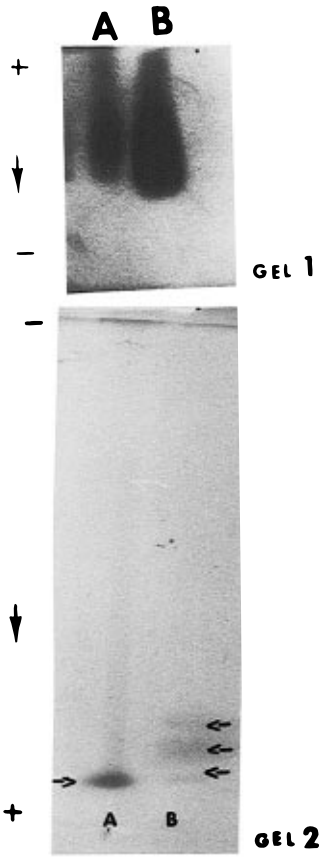

Figure 2. Cathodic native PAGE (gel 1) and LDS−PAGE (gel 2) for detecting β-1,3-glucanase activity in water-soluble extracts of corn silks from Yellow Creole (A) and Huffman (B). These semiquantitative colorimetric assays show relative intensity of enzyme activity in the two extracts. The arrows (gel 2) indicate bands in (B) not present in (A).

Table 1. Measurement of Protein, β-1,3-Glucanases, and Chitinases in Silk Extracts from Varieties That Are Resistant (r) and Susceptible (s) to *A. flavus* Infection

genotype	protein[a] (mg/mL)	β-1,3-glucanase[b] (sp act.)	chitinase[c] (sp act.)
Yellow Creole (r)	0.55	3.2	4.7
Huffman (s)	0.16	41.2	16.0

[a] Data obtained from water extracts by the standard BCA procedure. [b] One unit of β-1,3-glucanase activity per milligram of protein liberates 1 mg of glucose from laminarin after 2 h, pH 5.5, 50 °C. [c] One unit of chitinase activity per milligram of protein liberates 1 mg of *N*-acetyl-D-glucosamine from chitin in 48 h, pH 5.1, 37 °C. Colorimetric procedure using Chitin Azure as substrate.

extracts are presented as the average of duplicate analyses in Table 1. The protein content, based on dry weight, in Yellow Creole silk is 4-fold that of the Huffman silk. Both chitinase and glucanase assays, however, showed that the Huffman variety had much higher activities than those observed in Yellow Creole.

In summary, these preliminary results show the presence of water-soluble factor(s) in the silks of a variety of *A. flavus* resistant corn that was (were) not detected in a susceptible variety. Electrophoretic analysis of the extracts showed moderate heterogeneity in glucanase banding patterns. Per unit of dry weight, a larger quantity of protein was detected in the resistant variety. The isozyme profile of glucanase activity in the susceptible variety was more complex than in the resistant variety. Chitinases, like glucanases, are present in the water-soluble extracts of both varieties and are more abundant in the susceptible variety. Work continues in an attempt to identify the factors in corn silks and other tissues that seem to be involved in the inhibition of *A. flavus* growth and aflatoxin production in the field.

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