Effect of Steryl Cinnamic Acid Derivatives from Corn Bran on *Aspergillus flavus*, Corn Earworm Larvae, and Driedfruit Beetle Larvae and Adults

Robert A. Norton* and Patrick F. Dowd

National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Mycotoxin Research, Peoria, Illinois 61604

Steryl esters of ferulic and *p*-coumaric acids were tested for their effect on corn fungal pathogens and insects. The steryl ester fraction from corn bran had no effect on corn earworm larvae or driedfruit beetle adults, but driedfruit beetle larvae showed a significant increase in weight and the triglyceride fraction was active. Bioassays of pure ferulate esters and the ester fraction from both corn and rice showed no appreciable effect at 100 ppm on spore germination or germ tube growth of *Aspergillus flavus*, *Fusarium moniliforme*, or *Diplodia maydis*. *Sclerotinia sclerotiorum* was inhibited 75−90% by the total steryl ester fraction from corn and approximately 50% by the ester fraction from rice bran oil. Stigmastanyl and campestanyl ferulate showed little or no inhibition. The steryl ester fraction increased aflatoxin B_1 level in *A. flavus* NRRL 6536 significantly at 0.33 and 1.0 mg/mL. However, the esters had no effect on *A. flavus* NRRL 3357. The steryl ferulate and *p*-coumarate ester fraction from corn was not inhibitory to the corn pathogens tested, but it may increase the level of aflatoxin formation in corn infected by some strains of *A. flavus*.

Keywords: *Aspergillus flavus; aflatoxin; steryl ferulates; driedfruit beetle; corn earworm; corn bran; triglycerides*

INTRODUCTION

Preharvest corn is susceptible to a number of pathogens which affect its value and may cause formation of toxic metabolites in the developing kernels. Among these is *Aspergillus flavus*, many strains of which produce aflatoxin B_1 (Klich and Pitt, 1988), one of the most potent natural carcinogens known (Squire, 1981). Currently the primary method of controlling the level of aflatoxins in corn is to test the grain after harvest and segregate that which exceeds regulatory levels (Nichols, 1983). Consequently there is growing interest in developing corn lines resistant to infection by *A. flavus* (Davis et al., 1985). Research was initiated on compounds in the pericarp and aleurone layers as a part of a project to identify chemical factors that may affect susceptibility of maturing corn kernels to invasion by *A. flavus* and other preharvest pathogens or the amount of the aflatoxin produced in infected kernels. These layers could play a crucial part in the ability of the fungus to grow within the kernel after gaining entry though the silk or through breaks in the pericarp, especially from the stress resulting from drought conditions which are associated with outbreaks of *A. flavus* in the Midwest (Diener et al., 1987). Insect damage is positively correlated with aflatoxin levels (Lillehoj et al., 1980); therefore factors affecting insect damage should have an effect on aflatoxin level.

The bran and germ fractions of rice (Schroeder et al., 1968; Takahashi et al., 1989), wheat (Schnurer, 1991), and corn (Keller et al., 1994) have been found to contain a disproportionate amount of the aflatoxin or fungal biomass from *A. flavus* in infected grains. All of these cereals also contain significant amounts of sterols esterified to ferulic acid and, to a minor extent, *p*-coumaric acid in the bran and germ (Endo et al., 1968; Seitz, 1989; Norton, 1994). Seitz (1989) has shown that these compounds are highly localized within the seeds of corn, rye, wheat, and triticale; in corn and wheat the esters are found mostly in the inner pericarp region and the aleurone layer but they also apparently occur in the germ of corn (Norton, 1995a). Smart et al. (1990) have shown that the scutellum in corn is colonized heavily in infected kernels. There is evidence that ferulic acid, the major phenolic acid esterified, inhibits aflatoxin synthesis and growth at levels of 100−500 ppm (Chipley and Uraih, 1980; Sinha and Singh, 1981). If *A. flavus* were to hydrolyze the esters during infection and growth, it would release both the ferulic and *p*-coumaric acids and the set of sterols esterified to them and the level of free ferulic acid would be higher than 300 ppm if completely released. Alternatively the intact esters could be inhibitory. These two lines of evidence suggested that steryl ferulate esters could be involved in inhibition of growth and/or aflatoxin synthesis in *A. flavus*. The work reported here was undertaken to determine what effect these compounds had on *A. flavus* and on some insect pests of preharvest corn.

MATERIALS AND METHODS

Compounds/Fractions Tested. Hexane extracts of corn bran (ConAgra Grain, Omaha, NE) were prepared and worked up as described previously (Norton, 1994); briefly, bran was extracted overnight in hexane, rinsed twice, evaporated and then partitioned between hexane and basic aqueous methanol followed by partitioning between acidified aqueous methanol and hexane. The hexane extract from the acidic methanol was fractionated by use of rotation planar chromatography (Chromatotron, Harrison Research, Palo Alto, CA). Extract (0.6−0.8 g) was dissolved in hexane and applied to a 2 mm thick Chromatotron plate which had previously been equilibrated by passing ca. 200 mL of hexane through it. The sample was

* Address correspondence to this author at USDA, ARS, National Center for Agricultural Utilization Research, 1815 N. University, Peoria, IL 61604 [telephone (309)681-6251; fax (309)681-6686; e-mail nortonra@ncaur1.ncaur.gov].

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Table 1. Results for Assay of Crude Hexane Fractions from Corn Bran for Activity against Corn Earworm and Driedfruit Beetle

	corn earworm larvae		driedfruit beetle		
			% reduction in feeding[b]		
fraction[a]	mean weight (mg)	% control	adults	larvae	weight (mg)
control (solvent)	23.1				4.5
ferulic acid (250 ppm)	30.4		56.2[d]	12.5	3.4
complete extract	nd[c]		all dead	37.5[d]	nd
fraction 1 steryl esters	24.4		6.25	0	nd
fraction 2 triglycerides	4.3	18.5[d]	75.0[d]	68.8[d]	1.3[d]
fraction 3 unknowns	33.7		12.5	6.25	nd
fraction 4 steryl CAD esters	21.8	94.3	12.5	18.75	6.1[d]
fraction 6 sterols	28.6		6.25	0	nd
fraction 7 unknowns	29.7		6.25	6.25	

[a] Concentration of test compounds in medium at 3 times the level in bran. [b] Maximum value is 75%. [c] Not determined. [d] Treated diets are significantly different from controls ($p < 0.05$) by analysis of variance.

eluted with the following solvents and appropriate fractions were collected as judged by the elution of bands from the plate when viewed with a long-wave UV lamp: hexane:ethyl ether 95:5 (v/v), 60 mL; 85:15, 40 mL; 65:35, 50 mL; 50:50, 60 mL; 100% ether, 80 mL; ether:acetone 80:20, 40 mL; 100% acetone, 60 mL. Fractions were evaporated and portions spotted on a 0.25 mm thin layer chromatographic plate developed in $CHCl_3$:acetone (98:2 v/v) and visualized by spraying with 50% H_2SO_4 in methanol and heating at 100 °C for a few minutes. Similar fractions were then combined.

Bioassays. *Insects*. The corn earworm *Helicoverpa zea* (Boddie) colony was originally obtained from the Department of Entomology of the University of Illinois, Urbana, IL. The driedfruit beetle *Carpophilus hemipterus* (L.) colony was originally obtained from the Department of Biological Sciences, University of Illinois—Chicago. Both insects were reared on pinto bean-based diet at 27 ± 1 °C, 40 ± 10% relative humidity, 14:10 light:dark photoperiod (Dowd, 1988). Newly hatched, first instar, 1 day old corn earworm larvae were used in assays. Second instar, 4 day old driedfruit beetle larvae were used in assays. Assays were run for 7 days under the same conditions used for rearing.

Silica gel column fractions of corn hexane extract were tested for activity against corn earworm larvae and driedfruit beetle larvae (Dowd, 1988) and adults by dissolving the test material in acetone and adding to the synthetic diet. Activity was measured by either effect on growth (weight gain) for 20 individual corn earworm larvae per treatment or reduction in feeding, which was rated on a visually estimated scale of 1−4, 4 being the control level of normal feeding for each of four blocks caged with five adult driedfruit beetles per block per treatment (Dowd, 1990). Extracts were added at a level 3 times that found in the bran.

Fungi. *Aspergillus flavus*, *Fusarium moniliforme*, *Diplodia maydis*, corn pathogens, and *Sclerotinia sclerotiorum*, a dicot pathogen, were evaluated for effect on spore germination and germ tube growth against selected esters and fractions at the laboratories of Pioneer Hi-Bred, Inc., Johnston, IA. Compounds were incorporated at 100 ug/mL in microtiter wells and reactions scored on a scale of 0−4 (0 = no observable fungal inhibition relative to water control; 1 = slight inhibition, approximately 50%; 2 = substantial inhibition, 75−90%; 3 = almost complete inhibition; 4 = complete inhibition). Details of the assay have been reported previously (Duvick et al., 1992).

Incubations with *Aspergillus flavus* NRRL 6536 and NRRL 3357 were carried out using a suspended disk culture method as previously described (Norton, 1995b). Briefly, a scintillation vial with 1 mL of water in the bottom and closed by an open type cap with a thick septum was used. The septum was pierced by a pin and a sterile 1 cm glass fiber disk affixed (extra thick glass fiber filter, Gelman Sciences, Ann Arbor, MI). Filters were washed in acetone, benzene, chloroform, and methanol before use. To facilitate incorporation of the esters within the filters and allow the filters to take up the medium/spore suspension, esters were dissolved in a solution of benzene with 1% crude L-α-phosphatidylcholine (PC; Sigma, type II-S, catalog no. P-5638, approximately 17% PC) and pipetted onto the bottom of disks, and the solvent was evaporated under reduced pressure in a sterile desiccation chamber. The disk was then inoculated with 90 μL of medium containing the spores at 5000/mL. The medium was SL medium salts containing (per liter) 10 g of asparagine, 3.5 g of $(NH_4)_2SO_4$, 750 mg of KH_2PO_4, 350 mg of $MgSO_4 \cdot 7H_2O$, 75 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 5 mg of $MnCl_2 \cdot 4H_2O$, 2 mg of $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$, 2 mg of $Na_2B_4O_7$, 2 mg of $FeSO_4 \cdot 7H_2O$ and 50 g of glucose made up to 1 L with deionized water (Reddy et al., 1971). Disks were inoculated by pipetting small volumes at five points on the top and bottom of the disks and incubated at 25 °C in darkness for 5 days.

Aflatoxin Analysis. Aflatoxins were extracted overnight from disks two times with 2 mL of $CHCl_3$. Extracts were combined, evaporated with nitrogen, and made up to volume with acetonitrile:water (9:1 v/v). An aliquot was derivatized and analyzed by high-performance liquid chromatography as previously described (Norton, 1995b). NRRL 6536 and NRRL 3357 produce both aflatoxin B_1 and B_2; however the level of the latter was usually between 0.5 and 1.0% of the former.

RESULTS

Insect Assays. All fractions of the bran hexane extract, including the steryl cinnamic acid derivative (CAD) fraction, were tested for activity against corn earworm larvae and driedfruit beetle larvae and adults as shown in Table 1. To the authors' knowledge the hexane extract of corn bran has not previously been tested on insects. It was possible that ferulic acid would be released in the insect by hydrolysis of the steryl ferulate esters and that this would affect the insect feeding or weight gain, and therefore a ferulic acid supplemented diet was included in the assay. At the level tested (250 ppm) there was no effect from ferulic acid on corn earworm larvae, but feeding by driedfruit beetle adults was significantly inhibited. No effect on the weight of the corn earworm larvae or on feeding in driedfruit beetles was found when the steryl CAD fraction was tested at 3 times the level the esters occurred in the bran. A significant increase in the weight of the driedfruit beetle larvae from the steryl CAD fractions was seen, however. Although the steryl esters appear not to be inhibitory, and promoted the growth of the driedfruit beetle larvae, the triglyceride fraction (fraction 2) from the bran did have a marked effect on weight of corn earworm larvae, producing an 81.5% decrease in weight compared to the control and significantly decreased feeding in both driedfruit beetle adults and larvae (Table 1). None of the other fractions showed significant inhibition of any insects.

Microtiter Plate Test. The two principal components of the steryl ferulates in corn, sitostanyl and

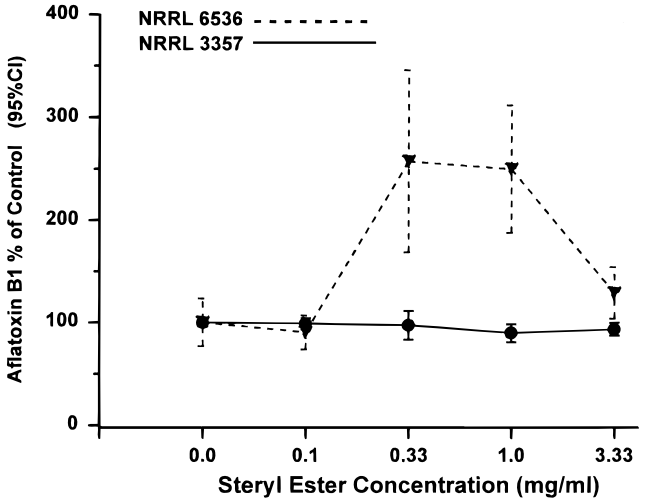

Figure 1. Effect of steryl ester fraction from corn bran on aflatoxin B_1 production of two strains of *Aspergillus flavus*, NRRL 6536 and NRRL 3357. Aflatoxin production is expressed as percent of control with 95% confidence interval.

campestanyl ferulate, along with a mixture of all the esters for both corn and rice were tested for activity against the following crop plant pathogens: *Sclerotinia sclerotiorum*, *Aspergillus flavus*, *Fusarium moniliforme*, and *Diplodia maydis*. All compounds and fractions were tested at 100 ppm against all four fungi; however, only *S. sclerotiorum* was inhibited by any of the materials tested. Following are the materials tested and the score shown by *S. sclerotiorum*: total steryl CAD fraction from corn, score 2 (75−90% inhibition); stigmastanyl ferulate, score 0 (no observable inhibition relative to water control); campestanyl ferulate, score 0.5 (<50% inhibition); and total steryl CAD fraction from rice bran oil, score 1 (ca. 50% inhibition). The two major esters present in the corn mixture, stigmastanyl and campestanyl ferulate, showed little or no activity although the complete ester fraction showed 75−95% inhibition for corn and approximately 50% for the ester fraction from rice bran oil. Stigmastanyl and campestanyl ferulate account for approximately 80% of the total esters in corn (Norton, 1994), which suggests that either there is a synergistic effect with the major components or that one or more of the minor components (no one of which exceeds 5% of the esters) is proportionately more active against *S. sclerotiorum*. The same compound may be present in rice bran oil at a reduced level. Seitz (1989) tested the steryl ferulate fraction from wheat for activity against spores of *Aspergillus amstelodami*, which is a stored grain pathogen, and found that the esters alone did not support growth or germination and did not inhibit germination or growth when included in nutrient medium at 1 mg/mL.

Suspended Disk Culture. The effect of the complete steryl CAD fraction from corn on aflatoxin B_1 production was assessed by using the suspended disk culture system and these data are shown in Figure 1. The data shown are the average for three experiments of five replicates each for NRRL 6536 and one experiment of five replicates for NRRL 3357. NRRL 6536 consistently has higher variation in aflatoxin than NRRL 3357 (Norton, 1995b). As shown in Figure 1, the total ester fraction had no effect on aflatoxin B_1 by NRRL 6536 at the lowest level tested (0.1 mg/mL), but at 0.33 and 1.0 mg/mL medium a sharp increase in aflatoxin B_1 occurred which subsequently decreased as the level of ester was increased. However, even at the highest concentration aflatoxin B_1 was still slightly above the control. NRRL 3357 showed no effect on toxin production at any level of the esters. Incubation of NRRL 3357 with an equimolar series of γ-oryzanol, the related esters from rice bran, also produced no change in aflatoxin from control levels (data not shown). Incubation of NRRL 3357 with 0.88 and 3.0 mg/mL ferulic acid resulted in inhibition of aflatoxin B_1 by 18% and 72.2%, respectively (data not shown). Quantitative growth data were not obtained from this experiment, but visual observation showed that the disks with the highest level of ester fraction had more visible growth at 60 h than the PC control; the other ester levels graded down to the PC control. Results were similar for the third day, and by the end of the fourth day the PC control and the disks with ester were all uniformly covered with a layer of yellow-green conidial heads. There was little visual difference between any of the disks except the PC control which showed a rather irregular pattern of conidial heads (data not shown).

DISCUSSION

Plant-derived lipid fractions have generally received limited testing against insects compared to other allelochemicals such as phenolics or flavonoids. Most of the related work has been with long-chain fatty acids. Structure−activity work has indicated optimum chain lengths, saturation, and salts for fatty acid activity against insects (e.g., Kabara 1987); commercial materials are on the market. Otherwise, work has been more limited. The amount of extractable leaf cuticle components was inversely proportional to resistance of pear leaves to the pear psylla (*Cacopsylla pyricola* Forester) (Ge'rard et al., 1993).

Differences in extractable leaf waxes of cabbage has been associated with resistance to diamondback moth larvae (Eigenbrode et al., 1991). Differences in cuticular lipid content are thought to be associated with resistance of peanuts to fall armyworm and thrips (Yang et al., 1993a). Cuticular lipid extracts from bermuda grass leaves can inhibit the growth of the fall armyworm [(*Spodoptera frugiperda* (J. E. Smith)] (Quisenberry et al., 1988). When cuticular lipids have been extracted from corn silks, the resulting residue is less toxic than the original material (Yang et al., 1991, 1992). Cuticular lipid extracts of corn leaves inhibited growth of *S. frugiperda* when incorporated into defined diets (Yang et al., 1991). However, some individual leaf lipid components stimulated growth of *S. frugiperda* (Yang et al., 1993b). We also noted that the steryl CAD ester fraction stimulated growth of driedfruit beetle larvae.

It was hypothesized that hydrolysis of the esters by *A. flavus* or insects could release ferulic acid and inhibit feeding or aflatoxin production. Ferulic acid at 0.5 mg/mL was found to inhibit aflatoxin B_1 production by *A. flavus* NRRL 3145 by 50% at 0.5 mg/mL and *A. parasiticus* NRRL 3240 by 76.6% (Chipley and Uraih (1980); NRRL 3145 is now identified as *A. parasiticus*). If nearly all of the esters are localized in the inner pericarp and aleurone layer, as reported by Seitz (1989), and if this region comprises about half the aleurone/pericarp tissues, as indicated by micrographs of the corn pericarp (Randolph, 1936), and using a figure of 5% for the bran fraction (Earle, 1946), then the *in situ* concentration of esters is estimated at 1 mg/g dry weight of tissue, based on a value of 400 μg/g bran for the esters (Norton, 1995a). This would yield 0.33 mg/g ferulic acid with complete hydrolysis which should show significant

inhibition of aflatoxin. At the highest level of esters tested, 3.33 mg/mL, the amount of ferulic acid which would be released if complete hydrolysis occurred is calculated to be 1.09 mg/mL. As mentioned above, incubation of NRRL 3357 with ferulic acid resulted in inhibition of aflatoxin B_1 by 18% and 72.2% for concentrations of 0.88 and 3.0 mg/mL, respectively, with a calculated inhibition for 1.09 mg/mL of ca. 28%, assuming inhibition to be a linear function of the log of concentration. Since no inhibition occurred with either NRRL 3357 or NRRL 6536, either the amount of the ester hydrolyzed was substantially less or inhibition was offset by stimulation from the sitostanol released. Since the results for both corn esters and rice bran esters with NRRL 3357 were not statistically different from the controls; the type of sterol involved, sitostanol and campestanol vs cycloartenol and 24-methylenecycloartenol, does not appear affect the response of the fungus. The strains used for the present work appear to be substantially less sensitive to ferulic acid than those used by previous workers.

The lack of inhibition of aflatoxin formation suggests that possibly the effect was offset by stimulation by the sterol portion of the ester. A few studies have reported on the effect of sterols on *A. flavus* and *A. parasiticus*: Fanelli et al. (1981) found that a mixture of sterols, fatty acids, and phosphatidylcholine inhibited both growth and aflatoxins. When *A. flavus* was incubated with the sterol fraction and the saponified (fatty acid) fractions from wheat germ, aflatoxin levels were much higher with the saponified fraction (Jemmali et al., 1974). The unsaponified (sterol) fraction of sunflower meal had no effect on aflatoxin level when added to defatted meal inoculated with *A. parasiticus* (Chulze et al., 1990). The little evidence available, therefore, suggests that plant membrane sterols do not promote or inhibit aflatoxin formation to any appreciable extent.

Keller et al. (1994) have shown that *A. flavus* grows most vigorously and produces the highest levels of aflatoxin when it infects the embryo. However, if infection occurs through a break in the pericarp distal to the embryo, the mycelium grows almost exclusively within the aleurone layer, and not the endosperm tissue, until it reaches the embryo as visually shown by Keller et al. (1994) with a mutant which produced norsolorinic acid, a red precursor of aflatoxins. Similar results were reported by Smart et al. (1990), who suggested that growth occurred in the layer of thin-walled tissue (endocarp, inner pericarp) between the outer pericarp and the aleurone/testa layer and possibly entered the aleurone through breaks in the testa. The composition of this layer of the kernel appears to be critical, then, for the course of infection when it occurs at a point on the pericarp not over the germ. The layers containing the steryl esters probably only become a factor when drought or stress conditions result in cracks in the pericarp that allow access to the inner pericarp or internal cracks in the seed coat (testa), as discussed by Smart et al. (1990), which would also allow access to the aleurone layer and germ. Then the esters in the inner pericarp layer and in the aleurone could provide either a metabolizable substrate for the fungus or a signal that the fungus has reached a particular region in the kernel and stimulate sporulation.

Lillehoj (1983), in reviewing factors affecting aflatoxin formation in developing corn, has suggested that emphasis on determining the "... nutrient properties of silks and developing corn kernels at critical stages in terms of substrates for *A. flavus* and *A. parasiticus* mycelial growth and aflatoxin biosynthesis..." is a critical element in the microenvironmental interaction between corn and *A. flavus*. The results presented above show that the fraction containing the steryl esters of ferulic and *p*-coumaric acids is one component of the pericarp and germ which can influence the level of aflatoxin produced by some *A. flavus* strains in infected kernels.

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