Effect of Pesticides on Zearalenone Production in Culture and in Corn Plants

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ABSTRACT

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The effect of pesticides on production of zearalenone by *Fusarium roseum* var. *graminearum* was studied. In potato-dextrose broth, pesticides at 100 ppm inhibited mycelial growth as follows: 56% by fonofos, 35% by EPN, 42% by toxaphene, 49% by heptachlor, 33% by fensulfothion, 69% by metalkamate, 71% by carbaryl, 78% by maneb, and 100% by naled. When pesticides were added to flasks containing autoclaved corn kernels and inoculated with *F. roseum*, zearalenone production was inhibited by 100 ppm of pesticide as follows: 90% by fonofos, 77% by metalkamate, 100% by maneb, 69% by fensulfothion, 93% by EPN, 94% by carbaryl, 97% by carbofuran, and 100% by naled. Corn inoculated with *F. roseum* while in the field and treated with fonofos, carbaryl or maneb after silking showed a significant reduction in zearalenone production.

Additional key words: chemical control, F-2 toxin, *Zea maydis*.

Zearalenone is an estrogenic compound which is produced as a secondary metabolite by various species of *Fusarium* including *F. roseum* var. *graminearum*. This compound has been implicated in causing hyperestrogenism, abnormal estrus, infertility, stillbirth, small litters, and fetal absorption when ingested by swine (3,13,16). Dairy cattle show decreased appetite, milk production, and fertility when fed zearalenone (10). However, when implanted into heifers, zearalenone increased feed utilization, stimulated growth, and improved carcass grade (15).

Stoloff et al (17) reported that 10% of the 1973 corn crop from the Corn Belt contained zearalenone and that positive samples averaged 117 μg/g. Zearalenone was found in 17% of 223 samples from terminal elevators and food processing establishments. Six processed corn samples, intended for human food use, were positive for zearalenone (8).

Berisford and Ayres (1) found that production of zearalenone was completely inhibited in culture by the insecticide naled. Results from another study (2) showed that a number of insecticides inhibited growth and zearalenone production by *F. roseum*. Since Christensen (4) reported that there is a close association between invasion of corn by the corn borer and secondary fungal rots by *Fusarium*, insecticides could provide a means of controlling corn borers and other destructive insects as well as controlling zearalenone-producing fungi.

Therefore, we undertook experiments to determine the inhibitory effect of selected insecticides and fungicides on growth of *Fusarium roseum* and zearalenone production in culture and in field corn.

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MATERIALS AND METHODS

Culture and inoculum. *F. roseum* var. *graminearum* NRRL 3376 (Northern Regional Research Laboratory, Peoria, IL), a zearalenone-producing organism (4), was used throughout this study. It was maintained on potato-dextrose agar (PDA) slants at 4 C and transferred monthly.

The inoculum for all experiments was prepared by growing *F. roseum* on PDA in 100 × 15-mm petri dishes for 3 wk at 21 C. Contents of two petri dishes, including agar and fungus, were homogenized in 40 ml of sterile water with a VirTis homogenizer for 1 min.

Pesticide preparation and source. Pesticides were dissolved in dimethylsulfoxide (DMSO) and added after autoclaving of broth or corn in amounts to make final pesticide concentrations (active

ingredients) of 0, 10, 50, or 100 ppm (w:w). All pesticides were of technical grade and were supplied directly by the manufacturer. Detailed information concerning the pesticides is provided in Table 1.

Growth inhibition study. For measuring effects of pesticides on growth, the fungus was grown in 500-ml Erlenmeyer flasks containing 100 ml of potato-dextrose broth (Difco). Cultures were inoculated with 1 ml of inoculum of *F. roseum* var. *graminearum* and incubated at 21 C for 3 wk. Mycelial mats were dried at 60 C for 12 hr for determination of dry weight. The inhibition in treated samples was calculated as percent of control.

Zearalenone inhibition study. To measure zearalenone production, the fungus was grown in 500-ml Erlenmeyer flasks containing 50 g of autoclaved dent corn kernels. Sterile distilled water (about 22 ml/50 g of corn) was added to corn to achieve a moisture content of 44%. Pesticide was added, and corn for the zearalenone inhibition study was inoculated with 1 ml of inoculum of *F. roseum* var. *graminearum* and incubated at room temperature for 1 wk prior to incubation for 3 wk at 15 C (4).

Extraction of and analysis for zearalenone. Corn was ground in a closed metal blending cup mounted on a Waring blender. To the entire contents of an Erlenmeyer flask of corn (50 g), 100 ml of chloroform was added. The samples were shaken for 15 min on a wrist action shaker. Fifty milliliters of the chloroform extract was added to a 250-ml separatory funnel and shaken with 50 ml of 4% sodium hydroxide solution. The bottom layer was discarded, and 50 ml of 0.7% citric acid solution and 50 ml of benzene was added to the funnel and the mixture was shaken for an additional minute. The bottom layer was discarded. The extract was filtered through anhydrous sodium sulfate and then evaporated to dryness with a flash evaporator at 63 C. Recoveries ranged from 64 to 81%. Values were corrected for loss based on a mean recovery of 74%.

Residues were redissolved in 2 ml of methanol. Samples were filtered through a 0.45 μm organic solvent filter (Millipore) prior to high-performance liquid chromatography (HPLC). Zearalenone standard was generously donated by C. J. Mirocha, University of Minnesota, St. Paul.

Zearalenone was analyzed by using an HPLC unit (Waters Associates Inc., Milford, MA) equipped with dual M6000 pumps, a programmer, a UK6 septumless injector, a model 440 ultraviolet detector (278 nm filter), and a model 420 fluorescence detector (excitation wavelength = 360 nm, and emission wavelength = 440 nm) linked in sequence. Zearalenone was eluted from a μ-Porasil column (Waters) at 3.5 min with a benzene:methanol:acetic acid (95:10:5, v/v) solvent system at a flow rate of 1 ml/min.

Thin-layer chromatography (TLC) was used to confirm the identity of compounds. Silica Gel G Prekote TLC plates (250 μm thick; Analtech, Pittsburgh, PA) were activated at 115 C for 30 min immediately before use. The developing solvent was chloroform:ethanol (97:3, v/v) (12). Presence of zearalenone was determined by comparing the R_f of a known zearalenone standard to the unknown samples under UV light and by spraying the plates with Fast Violet B salt solution (14).

Growth studies in the field. Field corn (*Zea maydis*) cultivar 3184 (Pioneer Seed Co.), commonly grown in the southeastern United States, was grown at the Plant and Soil Science Laboratory at The University of Tennessee. The experiment consisted of four randomized subplots per pesticide as follows: no pesticide and no fungal inoculation (15 plants), pesticide with no fungal inoculation (15 plants), no pesticide but with fungal inoculation (15 plants), and pesticide and fungal inoculation (15 plants). Two ears were collected from each plant. Two replications were performed.

All corn ears were cut from silk to middle through the husk in order to have consistency among the ears throughout the experiment. Ears were inoculated from silk to middle (approximately 5 cm) 20 days after silk development by injecting approximately 1 ml of a spore suspension (10^5 conidia per milliliter) of *F. roseum* var. *graminearum*. Ears not receiving fungal inoculation were injected with 1 ml of sterile distilled water. Corn ears scheduled for pesticide treatment were treated with fonofos, carbaryl, and maneb. Pesticide was applied by spraying the ear with 100-ppm pesticide solution (w:w, active ingredients) for 4 sec. Immediately after inoculation, each treated ear was completely enclosed in a waterproof paper bag which was removed only when pesticides were applied weekly. They were replaced on the ear after each pesticide application. Thirty ears from each subplot for each replicate were harvested 72 days after silking. The husk was removed, the ears were shelled, and the kernels were frozen until analysis. The kernels were ground, extracted, and analyzed as described earlier. Pesticide residues were analyzed by using standard FDA procedures (9). An analysis of variance was performed and significance of differences among means was determined according to Duncan's multiple range test.

RESULTS AND DISCUSSION

All pesticides, except carbofuran, caused a significant decrease

TABLE 1. Detailed information on pesticides tested for effects on the production of zearalenone by *Fusarium roseum* var. *graminearum* NRRL 3376 in laboratory culture and in corn plants (modified from Martin [11])

Chemical family and and individual chemical names	Common name	Trade name	Mol. wt.	LD_{50}[y] (mg/kg)	Registered by EPA[z]	Supplier
Carbamates						
m-(1-Methylbutyl) and *m*-(1-ethylpropyl) phenyl *N*-methylcarbamate	Metalkamate	Bux	221	87	NO	Chevron (Richmond, CA)
1-Napthyl carbaryl	Carbaryl	Sevin	201	307	YES	Union Carbide (Jacksonville, FL)
2,3-Dihydro-2,2-dimethyl-benzofuran-7-yl methyl carbamate	Carbofuran	Furadan	221	5	YES	FMC (Middleport, NY)
Polymeric manganese ethylene 1,2-bisdithiocarbamate	Maneb	Dithane M-22	265X	6,750	NO	DuPont (Wilmington, DE)
Organophosphates						
Diethyl 4-(methylsulphinyl) phenyl phosphorothionate	Fensulphothion	Dasanit	308	2	YES	Chemagro (Kansas City, MO)
O-Ethyl *S*-phenyl ethylphosphono-dithioate	Fonofos	Dyfonate	246	8	YES	Stauffer (Richmond, CA)
Ethyl 4-nitrophenyl phenyl-phosphonothioate	EPN	EPN	323	7	YES	DuPont (Wilmington, DE)
1,2-dibromo-2,2-dichloroethyl dimethyl phosphate	Naled	Dibrom	381	40	NO	Velsicol (Chicago, IL)
Chlorinated hydrocarbons						
Heptachlorodicyclopentadiene	Heptachlor	None	373	430	NO	Chevron (Richmond, CA)
Mixture of chlorinated camphenes (67–69% chlorine)	Camphechlor	Toxaphene	414	40	YES	Hercules (Wilmington, DE)

[y] Oral LD_{50} in the male rat.

[z] Registered for insect pests of corn (USDA, 1982). Toxaphene may not be used on potential silage corn intended for dairy animals or animals being finished for slaughter.

($P < 0.05$) in mycelial growth of *F. roseum* in the broth culture (Table 2). Three of the carbamate insecticides (metalkamate, maneb, and carbaryl) inhibited mycelium production by nearly the same degree whereas the other carbamate insecticide, carbofuran, did not result in significant inhibition. Likewise, two of the organophosphate (OP) insecticides, EPN and fensulfothion resulted in low levels of inhibition while fonofos inhibited growth by 56%. The antifungal action of the pesticides seemed to be dependent on the structure of the pesticide in question.

OP fungicides have specific structural features associated with antifungal activity. Unlike insects, fungi cannot oxidize the bond P=S to P=O, and therefore, pesticides having the P=S structural feature are generally inactive against fungi unless the phosphate group is bonded to nitrogen (HN-P=S) (5). It appears that this rule also applies to insecticides that have antifungal activity, since the least inhibitory OP insecticides were fensulfothion which has the P=S groups and EPN which has P-O-S group in their structure. The addition of side groups such as halogens (Cl,Br) or cyanide (CN) often increases antifungal activity of OP pesticides (18). Naled, which consistently has been shown to completely inhibit growth of *Aspergillus, Fusarium*, and *Penicillium* (1,6,7), has two bromine groups per molecule. Torgesson (18) reported that the presence of halogens in toxicants increases their rate of uptake by cells.

The chlorinated hydrocarbon insecticides heptachlor and toxaphene inhibited growth of *F. roseum* by <50%. The poor inhibition of growth of *F. roseum* by these insecticides suggests that they do not show promise for control of fungi (they are no longer permitted on corn for insect control).

Table 2 shows the results of the zearalenone inhibition studies in corn. All the pesticides at 100 ppm, except toxaphene and heptachlor, inhibited zearalenone production by at least 69%. Naled was the only pesticide which inhibited toxin production by 100% at each concentration. Overall, the results indicate that all carbamate and OP pesticides studied are strong inhibitors of zearalenone biosynthesis. Formula weights for pesticides are shown in Table 1. A weak negative correlation (−0.64) was calculated for antifungal activity as molecular weight increased. Therefore, compounds having lower formula weights did appear to contribute more active sites per gram of pesticide applied. The major exception to this trend was naled which is halogenated at two sites, and is probably taken up more quickly by cells. Toxin production is usually more sensitive than growth to inhibitors, since metabolites for synthesis of zearalenone are produced during fungal growth. Small decreases in primary biosynthesis (growth) generally resulted in a large decrease in secondary biosynthesis.

The selection of pesticides for the field study was based on growth inhibition, since zearalenone is primarily produced after harvest in corn stored in cribs (4). The initial growth of *F. roseum* must be prevented before harvest since pesticide residues at harvest must be within tolerance and would not be available for inhibition of zearalenone during storage. Therefore, fonofos, carbaryl, and maneb were selected for the field study. Results of this study are presented in Table 3. Corn inoculated with *F. roseum* but receiving no pesticide treatment had a mean zearalenone content of 168 ppb. Zearalenone levels were significantly reduced by fonofos, maneb, and carbaryl ($P < 0.05$).

Corn not inoculated with *F. roseum* was often moldy from natural contamination; however, the zearalenone content was only 30 ppb. Application of fonofos and carbaryl did not significantly reduce zearalenone production in uninoculated corn, possibly because levels were naturally low. However, zearalenone was not detected in corn treated with maneb.

Corn was analyzed for insecticide residues at harvest. Residues for fonofos, carbaryl, and maneb were 3, 2, and 0.01 ppm, respectively. Two samples of corn had carbaryl residues >10 ppm and four samples of corn had fonofos residues >10 ppm.

Under the conditions of this study, fonofos, carbaryl, and maneb inhibited zearalenone production in corn either extensively or moderately depending on the amount of zearalenone produced in controls. Future research should address the economic feasibility of using pesticides to control insects and fungi and the impact of specific insecticides on the fungal inhibition. The frequency of pesticide application and the appropriate use of a pesticide must be considered in determining the feasibility of controlling toxigenic fungi in preharvest corn.

TABLE 2. Effects of pesticides on growth and on zearalenone production of *Fusarium roseum* in potato-dextrose broth and in corn

Pesticide	Level (ppm)	Mycelial wt.[z] (mg/100 ml)	Inhibition (%)	Zearalenone[z] (μg/50 g corn)	Inhibition (%)
Naled (+ Control)	0	205.5 a	0	19.8 a	0
	10	0.0 b	100	0.0 b	100
	50	0.0 b	100	0.0 b	100
	100	0.0 b	100	0.0 b	100
Metalkamate	0	396.8 a	0	4.4 a	0
	10	336.1 b	15	2.6 b	41
	50	130.9 c	67	1.5 c	66
	100	122.0 c	69	1.0 d	77
Fensulfothion	0	279.3 a	0	1.6 a	0
	10	225.3 b	19	1.1 b	31
	50	198.6 b	29	1.0 b	38
	100	188.5 b	33	0.5 c	69
Fonofos	0	377.7 a	0	6.2 a	0
	10	328.2 b	13	1.1 b	82
	50	198.7 c	47	1.0 b	84
	100	166.8 c	56	0.6 b	90
Maneb	0	359.2 a	0	8.7 a	0
	10	39.6 b	89	5.6 b	36
	50	1.9 c	78	0 c	100
	100	1.9 c	78	0 c	100
Carbofuran	0	439.1 a	0	6.7 a	0
	10	430.3 a	2	1.6 b	76
	50	392.6 a	11	0.5 b	93
	100	372.5 a	15	0.2 b	97
Carbaryl	0	412.7 a	0	6.5 a	0
	10	337.6 b	18	2.0 b	69
	50	203.1 c	51	0.7 c	89
	100	146.1 d	71	0.4 c	94
Toxaphene	0	496.7 a	0	6.8 a	0
	10	325.3 b	35	6.7 a	1
	50	288.9 b	42	6.8 a	0
	100	287.1 b	42	6.5 a	4
Heptachlor	0	404.1 a	0	9.2 a	0
	10	238.3 b	41	9.3 a	0
	50	217.2 b	46	9.0 a	3
	100	207.1 b	49	9.0 a	3
EPN	0	458.7 a	0	36.3 a	0
	10	356.4 b	22	12.2 b	69
	50	343.5 b	25	7.3 c	87
	100	298.6 c	35	5.5 d	93

[z] Mean of two replications. Values within treatments in vertical columns followed by the same letter are significantly different at $P < 0.05$ according to Duncan's multiple range test.

TABLE 3. Effect of fonofos, carbaryl, and maneb on zearalenone production on Pioneer 3184 dent corn harvested 72 days after silking

Pesticide	Zearalenone production (mg/kg)	
	Inoculated[x]	Uninoculated
No pesticide (Control)	168 a[y]	30 b
Fonofos	22 b	22 b
Carbaryl	18 b	15 b
Maneb	0 c(ND)[z]	0 c(ND)

[x] Ears were inoculated 20 days after silk development with 1 ml of water containing 10^8 conidia of *F. roseum*.

[y] Values followed by different letters are significantly different ($P < 0.05$). All data are the mean of two replications of 30 samples per replicate.

[z] ND = none detected.

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