RECENT RESEARCH FOR THE CONTROL OF MYCOTOXINS IN CEREAL

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ABSTRACT

This paper briefly reviews several areas of research at the Northern Regional Research Laboratory. The first study involves the effects of insecticide treatment of sterile and unsterile wheat on the formation of aflatoxin and ochratoxin. In at least one treatment, increased yields were observed (carbon tetrachloride–carbon disulphide). The second study concerns our recent surveys on incidence of ochratoxin, aflatoxin and zearalenone in white and yellow corn (maize). An aflatoxin problem of some magnitude existed in 1971 in the southern part of the United States, even though only 4 per cent of the yearly corn production comes from this region. The third aspect covers the numbers of species of *Aspergillus* and *Penicillium* that produce penicillic acid and ochratoxin. Ochratoxin is now known to be produced by the following species of *Aspergillus: sulphureus*, *sclerotiorum*, *alliaceus*, *melleus*, *ostianus*, *petrakii* and *ochraceus*; also by the following species of *Penicillium*: *viridicatum*, *commune*, *cyclopium*, *purpurresces* and *variable*. The fourth study deals with the effect of ammonia treatment on a laboratory scale on the internal mould flora of corn.

Since this Conference is concerned with the control of mycotoxins, I would like to briefly review our recent research at the Northern Regional Research Laboratory regarding control of mycotoxins in cereals.

As I see it, preventing mycotoxins from getting into food and feeds involves several distinct lines of defence.

(*i*) Prevention of mould growth. This approach has three parts: (*a*) prevention of fungal growth on the crop in the field; (*b*) prevention of grain damage in the field during harvest; and (*c*) prevention of product moulding during storage, processing and further storage.

(*ii*) Detection methods. These are discussed in my other paper on aflatoxin‡.

(*iii*) Removal of the toxin if present (*a*) chemically, (*b*) physically and (*c*) enzymatically.

(*iv*) Identification of new possible mycotoxin threats.

At the Northern Regional Research Laboratory, we are not involved in studies on the prevention of mould growth except after the crop has been harvested. At this stage, we wondered about two things. First, what levels of

† Agricultural Research Service, US Department of Agriculture. Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

‡ This volume p. 51.

toxins were found in grain which was inoculated while retaining its normal microbial flora? Secondly, what effect did insecticides have on grain which again became moist enough for moulds to grow? Did they protect the grain against mould growth, and if not, was more or less toxin likely to be found?

The effect of insecticides on soil fungi has been studied by a number of investigators. However, the effects of gaseous insecticides on fungi in stored grain and the metabolites produced by these fungi have not been so thoroughly investigated. During storage of agricultural commodities, conditions can become favourable for both growth and production of mycotoxins by fungi even after the crop is dried below the moisture level at which fungi grow. Corn is often blended in such a way as to mix excessively wet corn with old dried corn to reach a suitable moisture level. Sometimes moisture levels in pockets left by uneven mixing become high enough for growth of *Aspergillus flavus*. Should corn treated with insecticides be used in blending, and if hot spots develop will aflatoxin be formed? Likewise, treated corn might be accidently moistened by condensation or leaking roofs and again have high enough moisture to allow the corn to support mould growth.

The action of several chemicals, including fungicides and insecticides on moulds from the series *A. flavus* have been reported from other laboratories. The literature indicates that aflatoxin synthesized by *A. flavus* in a defined medium was inhibited by dimethyl sulphoxide, although aflatoxin was not degraded. Chemicals used in foods or feeds as preservatives or stabilizers were screened for prevention or reduction of *A. flavus*, *A. parasiticus*, and/or aflatoxin accumulation in peanut pods. Results in the literature varied from complete inhibition of the fungi with no aflatoxin formation to aflatoxin accumulation in amounts of up to twice that of the control.

A paper, accepted for publication, by Vandegraft *et al.*[1] (in the press) reports our study on the effects of two insecticides used to protect stored grain; namely, phosphine and a carbon tetrachloride–carbon disulphide mixture (80:20, w/w). Specifically, we investigated their effects on aflatoxin production by strains of *A. flavus* and *A. parasiticus*, and ochratoxin production by *A. ochraceus* and *Penicillium viridicatum*. For production purposes, pearled wheat was sterilized to achieve maximum yields of ochratoxin and aflatoxin. Under normal conditions, stored grain would never be sterilized. Hence, we studied the effects of insecticides on mycotoxin production with slightly cracked wheat, both sterilized and non-sterilized, instead of corn because corn had not been previously tested, and we had no way of knowing what to expect as far as yields were concerned.

Wheat was treated with phosphine for 48 h at 21–27°C, and then aerated. Treatment with the second insecticide was for 3 days at the rate of 3.9 ml per 25 lb of wheat. Each lot was stored in containers at 0°C. For the first series the fermentation was conducted in Fernbach flasks with 150 g of grain wet with 45 ml of tap water, left unsterilized, and inoculated with 8 ml of the spore suspension. The Aspergilli were incubated for 6 days at 28°C on a Gump shaker while *P. viridicatum* were incubated for 12 days at 20°C. The second series of flasks was treated as above but autoclaved and cooled before inoculation. Each experiment was done in duplicate. Ochratoxin and aflatoxin were assayed in the usual manner (*Table 1*). One notes that the unsterilized grain produced less toxin whether treated with phosphine or not.

Table 1. Effect of phosphine on mycotoxin production (μg g^{-1}) on wheat

	Aspergillus flavus NRRL 3251	*Aspergillus parasiticus* NRRL 3145	*Aspergillus ochraceus* NRRL 3174	
	Aflatoxin B_1	Aflatoxin B_1	Ochratoxin A	Ochratoxin B
Sterilized				
untreated	2850	150	2890	80
treated	3380	120	3380	120
Unsterilized				
untreated	620	30	1720	30
treated	860	20	2340	60

However, with the insecticide treated, the levels of toxin produced by *A. flavus* and *A. ochraceus* were higher than the untreated, whereas they were lower with *A. parasiticus*. *Table 2* contains results of wheat treated with the CCl_4–CS_2 mixture and inoculated with *A. parasiticus*, *P. viridicatum* and *A. ochraceus*. On the unsterilized wheat, yields of ochratoxin and aflatoxin were less than on the sterilized wheat. Treated wheat gave higher yields than the untreated, except in the case of the yield of ochratoxin B by *A. ochraceus* NRRL 3174.

Table 2. Effect of a CCl_4–CS_2 mixture on mycotoxin production (μg g^{-1}) on wheat

	Aspergillus parasiticus			*Penicillium viridicatum*	*Aspergillus ochraceus*
	NRRL 2999		NRRL 3145	NRRL 3712	NRRL 3174
	Aflatoxin B_1	Aflatoxin G_1	Aflatoxin G_1	Ochratoxin A	Ochratoxin B
Sterilized					
untreated	510	260	80	510	250
treated	680	310	160	1820	140
Unsterilized					
untreated	100	40	50	310	140
treated	160	70	60	690	100

The conclusions to this study are: (*a*) treatment with phosphine caused both increases and decreases in ochratoxin and aflatoxin depending on the fungi; (*b*) increased yields of mycotoxin were observed on the sterilized wheat treated with the carbon tetrachloride–carbon disulphide mixture in all fermentations but one (NRRL 3174); (*c*) sterilization of wheat by autoclaving before inoculation usually increased mycotoxin production (the two exceptions were only slightly larger); and (*d*) the effect of insecticide treatment on mycotoxin production sometimes depended upon whether the wheat was sterilized or not.

Our current renewed interest in aflatoxin in corn is the result of difficulties with this mycotoxin in white corn from the southern part of the United States in 1971. As you know, we have conducted a number of surveys in the US Corn Belt for the presence of aflatoxin, ochratoxin and zearalenone. Surveys of corn (maize) were made in 1965, 1967 and 1968–69; and our findings indicated that aflatoxin was restricted to poorer grades with aflatoxin levels

Table 3. Summary of surveys for aflatoxin conducted at the Northern Regional Research Laboratory

Corn grade	Crop years 1964 and 1965 Assayed No.	%	Positive No.	%	Crop year 1967 Assayed No.	%	Positive No.	%	Export cargo Assayed No.	%	Positive No.	%
No. grade given	0				0				3	1	0	
1	40	3	0		3	1	0		0		0	
2	253	19	0		27	9	0		67	23	1	1
3	210	16	0		51	18	1	2	126	43	3	2
4	174	13	0		52	18	0		65	23	3	4
5	281	21	5	2	46	16	0		7	2	0	
Sample grade	353	27	25	7	104	37	5	5	25	8	1	4
Total	1311		30	2.3	283		6	2.1	293		8	2.7

less than 50 ppb (*Table 3*). Surveys of wheat, sorghum, soybeans and oats showed none or a very low incidence of aflatoxin with the levels in the few positive samples being extremely low. White corn is produced mainly in the warmer regions of the United States, but these regions produce only a small percentage of our total corn crop. However, white corn is extremely important because it is used widely in human food.

Table 4 shows that corn surveyed in 1969 from three south-eastern states was all negative, but that in 1970, considerable contamination was encountered even in the higher grades.

Table 4. South-eastern corn (Virginia, South Carolina, North Carolina)

Grade US No.	Crop year 1969 (No positive samples) No. assayed	Crop year 1970 (corn blight) No. assayed	No. positive	Levels Aflatoxin B_1 (ppb)
2	3	4	2	62, 62
3	3	6	4	83, 25, 12, 6
4	2	5	3	12, 12, 6
5	2	4	3	124, 42, 8
Sample grade	2	1	0	
Total	12	20	12	

Aspergillus flavus was easily isolated from all positive samples

Table 5 contains some preliminary data on white corn in the 1969–70 crops. It should be pointed out that corn was infected with southern corn leaf blight in 1970.

Table 6 shows the situation in yellow corn based upon 47 corn samples. Details on the southern corn survey were presented by Shotwell, Hesseltine and Goulden[2] at the meeting of the American Association of Cereal Chemists in the autumn of 1972. You will agree that these data indicate a problem. Whether it will be a further problem will depend on more surveys of this year's crop (1972) as well as future ones.

Table 5. Southern white corn; 1969–70 crops

Grade US No.	Samples assayed	Positive samples	Levels aflatoxin B_1 (ppb)
2	2	—	—
3	2	—	—
4	3	2	12, 6
5	3	2	141, 42
Sample grade	1	—	
	11	4	

Official first action assay method used

Table 6. Southern yellow corn; 1969–70 crops

Grade US No.	Samples assayed	Positive samples	Levels aflatoxin B_1 (ppb)
2	8	2	62, 62
3	16	5	83, 25, 13, 12, 6
4	10	3	31, 12, 6
5	7	3	308, 124, 8
Sample grade	6	2	98, 4
	47	15	

Official first action assay method used

In connection with our surveys of mycotoxins in grain, we have taken part of the ground-corn sample not used for assay, plated it onto appropriate media with a bacterial inhibitor, and determined the presence or absence of *A. flavus* series. Besides determining whether the *A. flavus* series was present or not, we looked for other Aspergilli that can be identified without isolation, as well as *Fusarium*, *Penicillium* and Mucorales, and in addition other fungi that can be identified without making individual pure cultures. With but one exception, all aflatoxin-positive samples of grain have shown the presence of members of the *A. flavus* series.

We are now concerned with just how the aflatoxin came about in the white corn grown in the areas described. Consequently, we have a large research programme underway this year to determine how *A. flavus* gets into the corn. Does this occur in the field before harvest, as has been suggested by some, or only after storage? Are spores of the *A. flavus* series insect borne? If infection occurs in the field, at what time and how do the kernels get infected? On the other hand, does infection occur immediately after picking because moisture is not rapidly reduced? We hope to answer these and many other questions in the field study now being conducted by Dr Lillehoj.

Another investigation, which we have conducted, is an investigation of the moulds that produce ochratoxin. The Northern Laboratory was the first to report its natural occurrence in any product. We detected it in corn. Originally, this mycotoxin was reported to be synthesized by *A. ochraceus*. Since aflatoxin is produced by at least two species of the *A. flavus* series, we

were curious as to how many species of the *A. ochraceus* series produced ochratoxin. In a paper recently published[3], we took 10 strains of each species in the *A. ochraceus* series available (in some uncommon species in the series only one or two strains were available to us) and tested them for their ability to produce ochratoxin. Each strain (44 were tested) was grown on sterilized cracked corn or pearled wheat at 28°C for 5 days and continuously agitated on a shaker. The results of the ochratoxin assays indicated that some strains of all species in the series produce ochratoxin except *A. elegans* (only one strain tested) and *A. auricomus* (five strains). Some of the positive species are of no economic significance (*A. sulphureus*, *A. melleus*, *A. ostianus* and *A. petrakii*) since they occur very rarely or not at all in foods and feeds. However, three species are significant: *A. sclerotiorum* grows on apples and pears; *A. alliaceus* is a world-wide parasite on onions, and *A. ochraceus* has been reported on chilli, black and red pepper, corn, rice, wheat and oats.

In a second study from our laboratory, six species of the same series, *A. ochraceus*, were grown on a liquid medium rather than on a solid substrate as used above. In addition to producing ochratoxin, they also produced penicillic acid. At low temperatures of 10 to 20°C, penicillic acid synthesis is favoured, whereas at 28°C ochratoxin A is produced. Generally, penicillic acid was produced in yields of about one to three magnitudes greater than ochratoxin A. Penicillic acid is less toxic on a weight basis; but unlike ochratoxin, it is a carcinogen. Under the conditions we used, some strains produced only one of these toxins and not the other, or vice versa. Van Walbeek *et al.*[4] recently reported the production of ochratoxin by *P. viridicatum*, and in another study made by Ciegler[5] it is now known that ochratoxin is produced not only by *P. viridicatum*, but also by *P. variable*, *P. commune*, *P. purpurescens* and *P. cyclopium*.

As mentioned above, the last line of defence against aflatoxin is a means for detoxification of contaminated material. Whatever method is followed, it must have the following attributes. (*a*) It must be economically feasible. To detoxify aflatoxin in an inferior mouldy corn, which must sell at a low price, requires that the process be cheap. (*b*) The aflatoxin must be destroyed and the resulting destruction products must be non-toxic. (*c*) The process must not reduce the nutritional value of the product. (*d*) The process must be simple and fast so that it can be used in different places with unskilled labour. (*e*) The process must not contaminate the surrounding environment whether air, soil, or water.

Work at the Northern Laboratory has involved laboratory studies with ammonia to detoxify aflatoxin in corn. The use of ammonia on contaminated cottonseed has already been discussed in this Symposium. The results of our work are too premature to detail, but I can describe some findings of the effect of 0.5 and 2.0 per cent ammonia as ammonia hydroxide on the microbiological flora of corn. White corn was tempered to 26 per cent moisture for 24 h and then 2.0 per cent NH_3 (w/w) was added. A microbiological examination was done on samples from the original corn and from corn after 24 h of tempering, 1 h after ammoniation, and 14 days after ammoniation. Details of the experiments and the results are described in a paper by Bothast *et al.*[6] The various microbiological examinations showed that bacteria increased rapidly during the 24-hour tempering while moulds increased slightly.

However, 1 h after the 2 per cent ammonia was added, corn became sterile as far as fungi were concerned, including the internal mould flora. Ammonia has an amazing ability to penetrate corn and destroy the vegetative fungus mycelium. Although the bacterial count was reduced it persisted even to the end of the experiment at 14 days. Corn was sterile so far as moulds are concerned, but it still retained a selected bacterial population. The data are shown in *Figure 1*. With 0.5 per cent ammonia, similar results were obtained except

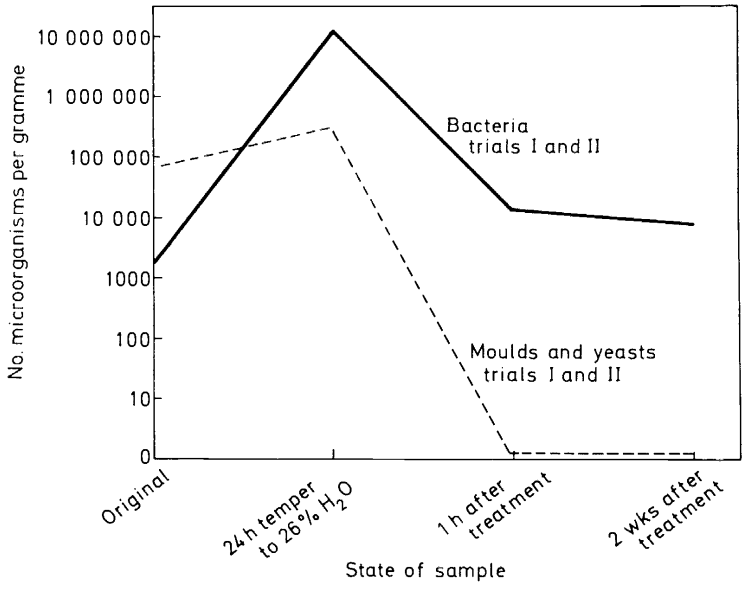

Figure 1. Response of the microbial flora of corn to treatment with 0.5 per cent ammonia

some moulds were still present at 1 h after treatment although they were all destroyed at 14 days.

When corn is treated with ammonia, it becomes darker in colour—the more ammonia, the darker the colour. However, in mice-feeding preference tests in which mice were allowed to choose between ammoniated white corn and untreated corn, no preference occurred between the untreated versus the treated.

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