

Degradation of ^{14}C -Malathion in Stored Corn and Wheat Inoculated with *Aspergillus glaucus*¹

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ABSTRACT The degradation of ^{14}C -malathion ([1,2- ^{14}C] ethyl label) was investigated in stored corn and wheat inoculated with *Aspergillus glaucus*, a common grain storage fungus. Grains free of internal storage fungi were surface-sterilized before treatment with ^{14}C -malathion and fungal inoculation. After 6 months, inoculated corn contained $74.1 \pm 2.6\%$ of applied radiocarbon, compared with $91.9 \pm 5.5\%$ in sterilized controls. Similarly, inoculated wheat contained $86.0 \pm 2.1\%$ of applied radiocarbon, compared with $92.8 \pm 1.1\%$ in sterilized control wheat. The low quantities of radiocarbon recovered from the inoculated grain may have resulted from losses by volatilization or $^{14}\text{CO}_2$ evolution, neither of which was determined in this study. Although malathion was degraded in both sterilized and inoculated grain (4 to 18% of the applied radiocarbon was recovered as ^{14}C -malathion), sterilized grain contained significantly more ^{14}C -malathion than inoculated grain after 6 months.

Malathion applied to stored grain may be degraded by biotic agents such as the grain itself or fungi associated with the grain. In addition, abiotic factors such as the moisture content and the temperature of the grain may also affect the breakdown of the insecticide. Kadoum and LaHue (1972) found that, after 6 months, live sorghum grain contained 15.9% of the applied malathion as compared with 43.1% found in autoclaved sorghum grain. Thus, although abiotic factors contributed to the breakdown of malathion, significantly more malathion was degraded in viable sorghum than in autoclaved sorghum.

The effect of fungi on the breakdown of stored-grain protectants has not been investigated thoroughly. In his review of stored-grain protectant metabolism, Rowlands (1971) concluded that the extent of pesticide metabolism due to the parasitic microflora of cereal grains remained to be positively elucidated. Rowlands (1975) reported rapid degradation of lindane and two of its metabolites applied to moldy grain of 21% moisture content. However, his study did not distinguish between fungi-mediated insecticide degradation and that due to grain or abiotic factors. Fungi present in grain are generally grouped into two categories, field fungi and storage fungi (Christensen and Kaufmann 1969). Field fungi attack kernels before harvest and generally die during storage. The metabolism of protectant insecticides is more likely to be affected by storage fungi, which infect grain during storage and cause grain deterioration.

We conducted this study to determine the effect of *Aspergillus glaucus* group species, the most common storage fungus, on the degradation of ^{14}C -malathion in stored corn and wheat. Sterilized corn and wheat were used as controls to indicate the quantities of insecticide metabolized by the grain or abiotic factors. Unextract-

able (bound) residues as well as extractable residues were determined.

Materials and Methods

Chemicals

^{14}C -Malathion ([1,2- ^{14}C] ethyl label) was purchased from Amersham Corp. The insecticide was at least 99% pure as determined by thin-layer chromatography (TLC), autoradiography, and liquid scintillation counting (LSC). The ^{14}C -malathion was diluted with non-radioactive malathion to a specific activity of 480 $\mu\text{Ci}/\text{mmol}$ before use. Non-radioactive malathion, malaoxon, malathion monocarboxylic acid (MCA), malathion dicarboxylic acid, and *O*-demethyl malathion (potassium salt) were obtained from American Cyanamid Co. Diethyl 2-mercaptosuccinate acid was purchased from Aldrich Chemical Co., whereas thiomalic acid was purchased from Tridom Chemical Co. Acetone, benzene, and hexane were redistilled before use. Acetonitrile, chloroform, toluene, ethanol, and methyl cellosolve were analytical reagent grade.

Grain

1980 Crop hard red spring wheat, cv. 'Borah,' was stored at ca. 4°C, 50% relative humidity (RH), before use. Freshly harvested, 1981 crop yellow dent corn (hybrid 3183, Pioneer Brand) was purchased from a local farmer and air dried to 13.7% moisture content. The corn and wheat had not been pretreated with insecticides or fungicides and contained no internal storage fungi detectable by plating surface-sterilized kernels on agar medium as described below. Broken kernels and debris were removed from the grain by hand.

Kernels were surface sterilized by successive immersion in 100% ethanol for 30 sec, 2% aqueous sodium hypochlorite (Clorox brand) for 2 min, and sterile tap water for 30 sec. After this sterilization procedure, the wheat had a moisture content of 19.7% and the corn had a moisture content of 17.5%. The surface-sterilized corn and wheat were air dried to a moisture content of 15.5% on sterile absorbent paper in a sterile hood for 2 and 2.5 h, respectively. The grain was then incubated

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at 26°C, 60% RH, for 1 week. The moisture content of the grain was determined by drying 10 g of grain at 130°C for 18 h.

Grain Treatment

In a sterile hood, 132-g (dry weight) equivalents of corn or wheat were each treated with 8.6 ml of acetone containing 1.32 mg (2.11 μ Ci) of 14 C-malathion. The treated grain was mixed well and aerated in the hood for ca. 1 h until the acetone had evaporated completely. Preliminary experiments indicated that the moisture content of the grain was only slightly affected by this acetone treatment. Portions of the grain were then removed, extracted in duplicate, and analyzed to determine initial insecticide concentration.

An inoculum of *A. glaucus* was prepared by tempering corn to 25% moisture content and autoclaving it at 125°C for 20 min. The cooled, sterile corn was then inoculated with pure cultures of three members of the *A. glaucus* group species: *A. repens*, *A. amstelodami*, and *A. ruber*. The fungal inoculum was incubated for 3 weeks at room temperature before use.

Triplicate samples, a total of 53.25 g of insecticide-treated corn or wheat, were placed in each of four Erlenmeyer flasks. One day after insecticide treatment, 53.25 g of the corn or wheat, prepared as described above, was inoculated with four kernels of the fungal inoculum. A second 53.25-g portion of corn or wheat was maintained as a sterilized control. The grain was stored in darkness at 26°C, 60% RH, for 1 month and then at 26°C, 86% RH, for 5 months. The moisture contents of the sterilized and inoculated grains were not determined after the 6-month storage period. However, the moisture content of wheat which had been surface sterilized, inoculated with *A. glaucus*, and incubated at 26°C, 86% RH for 3 months was 16.2%.

To determine the presence of *A. glaucus* on the surface, kernels were plated directly, whereas internal fungal growth was determined by plating surface-sterilized grain. The whole-seed plating assay was performed by placing a total of 3 corn kernels or 13 wheat kernels on malt agar containing 4% NaCl. Fungi growing from the kernels were identified after incubation for 5 days at 25°C.

Extraction and Analysis

Malathion and its breakdown products were extracted from grain by using a modification of the procedure described by Cook and Moore (1976). Each 15-g (dry weight) equivalent of grain was extracted with 250 ml of acidified acetonitrile and 25 ml of distilled water at a high speed in a Waring blender. Extracts were vacuum filtered, concentrated on a rotary evaporator at 35°C, mixed with 50 ml of 2% (aq) Na_2SO_4 , and adjusted to pH 8.5 with 5% (aq) Na_2CO_3 . This extract was partitioned with two 35-ml portions of chloroform, resulting in a chloroform phase and an aqueous phase. The chloroform phase was dried over acidified Na_2SO_4 . The aqueous phase was mixed with 5% NaCl (wt/vol), adjusted to pH 2 with 6 N HCl, and partitioned with 50

ml of chloroform. The resulting chloroform phase was pooled with the previously described chloroform fraction, concentrated, and adjusted to a 10-ml vol.

Aliquots (0.5 ml) of the organic or water extraction phases were mixed with 10 ml of a scintillator composed of 12 g of Preblend 2a70 (Research Products Int. Corp.), 1,500 ml of toluene, and 1,500 ml of methyl cellosolve. Radiocarbon remaining in the grain after extraction was determined by combusting 0.08-g aliquots of previously extracted grain in a modified Schöniger apparatus as described by Buyske et al. (1963). Samples were analyzed in a Searle/Isocap/300 model 6872 liquid scintillation counter. Data were corrected for background, counter efficiency, and dilutions.

Malathion and its breakdown products in the chloroform extracts were characterized and quantitated by thin-layer chromatography, autoradiography, and liquid scintillation counting by using a modification of the procedure described by Lichtenstein et al. (1978). The TLC plates were first developed in benzene to separate interfering materials from the 14 C compounds, then redeveloped in the same direction in a mixture of hexane-benzene-acetic acid (3:1:1).

The identity of malathion monocarboxylic acid in the grain extracts was confirmed by high-performance liquid chromatography (HPLC). A mixture of HPLC-grade acetonitrile-0.1% acetic acid (1:1) was pumped at a flow rate of 1 ml/min through a Bio-Sil ODS-10 reverse-phase column (250 by 4 mm; Bio-Rad Laboratories). The effluent was monitored at 350 nm. Retention times of peaks produced by sample extracts were compared with those of authentic standards. The identity of malathion was confirmed by gas-liquid chromatography. A Tracor model 560 gas chromatograph equipped with a nitrogen phosphorus detector and a glass column (122 cm by 2 mm ID) packed with 3% OV-17 on 100/120-mesh Gas Chrom Q was used. The column was operated with a helium flow rate of 25 ml/min at 200°C, inlet at 215°C, and detector at 250°C.

Results and Discussion

Six weeks after fungal inoculation, results of the whole-seed plating assay indicated that no fungi were present either internally or externally in sterilized corn or wheat. Wheat inoculated with *A. glaucus* contained no internal fungi, although fungi were present on the surface of every kernel. Surface fungi were present on all inoculated corn kernels, and one-third of the kernels contained internal fungi. Six months after fungal inoculation, no internal fungi were detected from plating the uninoculated controls. Although *A. glaucus* was present on one wheat kernel surface (of 13 checked), there was no visible evidence of fungal growth. However, *A. glaucus* was detected both internally and externally in 100% of the inoculated corn and wheat. Visual inspection of the grain indicated that corn was more heavily infected with *A. glaucus* than wheat. The malt agar containing 4% NaCl was used because it enhances the growth of the storage fungi and suppresses the growth of other fungi and bacteria (Christensen 1946). Possibly, another kind of medium may have shown the presence of bacteria,

Table 1. Degradation of ¹⁴C-malathion in corn and wheat stored 6 months after inoculation with *A. glaucus*^a

Grain	Extraction phase	Radiocarbon recovered, % of applied ¹⁴ C-malathion	
		Sterilized grain (%T) ^b	Inoculated grain (%T)
Corn	Chloroform	20.9 ± 1.3 (22.7)	13.6 ± 1.0 ^d (18.4)
	Water	28.5 ± 1.7 (31.0)	25.2 ± 0.5 (34.0)
	Bound ^c	42.5 ± 5.6 (46.3)	35.3 ± 2.3 (47.6)
	Total	91.9 ± 5.5 (100.0)	74.1 ± 2.6 ^d (100.0)
Wheat	Chloroform	24.9 ± 1.1 (26.8)	19.2 ± 0.4 ^d (22.3)
	Water	22.4 ± 1.7 (24.2)	23.6 ± 0.4 (27.5)
	Bound	45.5 ± 3.3 (49.0)	43.2 ± 1.7 (50.2)
	Total	92.8 ± 1.1 (100.0)	86.0 ± 2.1 ^d (100.0)

^aValues represent means ± SD of triplicate tests. ¹⁴C-Malathion was applied at 10 ppm (0.016 μCi/g, dry weight) to grain at 15.5% moisture content 1 day before fungal inoculation. Treated grain was incubated at 26°C, 60% RH, for 1 month and at 26°C, 86% RH, for 5 months.

^b%T = ¹⁴C in percent total radiocarbon recovered.

^cUnextractable ¹⁴C residues determined by combustion to ¹⁴CO₂.

^dResults are significantly different from those for sterilized controls at the 1% (Students *t* test).

Table 2. ¹⁴C-Malathion and metabolites recovered from chloroform extracts of sterilized and inoculated grains^a

Grain	Compound ^b	Radiocarbon recovered, % of applied ¹⁴ C-malathion	
		Sterilized grain (%T) ^c	Inoculated grain (%T)
Corn	Malathion	8.3 ± 0.7 (39.8)	4.0 ± 0.4 ^d (29.4)
	MCA	4.3 ± 0.7 (20.4)	3.0 ± 0.8 (22.2)
	Other	8.3 ± 0.8 (39.8)	6.6 ± 1.7 (48.4)
	Total	20.9 ± 1.3 (100.0)	13.6 ± 1.0 ^d (100.0)
Wheat	Malathion	18.3 ± 1.3 (73.4)	12.9 ± 0.1 ^d (67.3)
	MCA	1.5 ± 0.5 (6.1)	2.7 ± 0.0 ^c (14.0)
	Other	5.1 ± 0.1 (20.5)	3.6 ± 0.5 ^d (18.7)
	Total	24.9 ± 1.1 (100.0)	19.2 ± 0.4 ^d (100.0)

^aSee footnote a to Table 1.

^bMalathion (*R_f* = 0.71); MCA = malathion monocarboxylic acid (*R_f* = 0.59); other = unknown compound I (*R_f* = 0.66) and unknown compound II (*R_f* = 0.16).

^cSee footnote b to Table 1.

^dResults are significantly different from those sterilized controls at the 1% or 2% level, respectively (Students *t* test).

although the moisture content of the grains were too low for active bacterial growth.

The degradation of ¹⁴C-malathion, as indicated by the production of chloroform-soluble, water-soluble, and unextractable residues, was investigated after a 6-month incubation period (Table 1). Malathion and its relatively apolar breakdown products such as malathion monocarboxylic acid are chloroform soluble. Walker and Stojanovic (1973) reported that malathion had 3.5 times the bovine acetylcholine-sterase inhibitory activity of malathion monocarboxylic acid. March et al. (1956) found that malathion monocarboxylic acid was over 10 times less toxic to house flies and 35 times less toxic to mosquito larvae than the parent compound. The relatively detoxified, polar residues partition into the water. Although the nature of unextractable (bound) residues is unknown, they are often formed under conditions favoring insecticide degradation (Lichtenstein 1980).

Corn and wheat inoculated with *A. glaucus* contained significantly less chloroform-soluble radiocarbon than their respective sterilized controls. Furthermore, the total quantities of radiocarbon recovered from inoculated corn and wheat were also significantly lower than those recovered from sterilized controls. The low total resi-

dues recovered in inoculated grain may have resulted from losses by volatilization or ¹⁴CO₂ evolution, neither of which were determined in this study. The quantities of water-soluble and bound radiocarbon recovered in grain inoculated with *A. glaucus* were not significantly different from those recovered in sterilized grain.

Since grain inoculated with *A. glaucus* contained significantly less chloroform-soluble radiocarbon than sterilized controls, ¹⁴C-malathion breakdown products in the chloroform extraction phase were analyzed both quantitatively and qualitatively (Table 2). Corn and wheat inoculated with *A. glaucus* contained significantly lower quantities of malathion than sterilized controls. However, the small quantities of ¹⁴C-malathion recovered from both sterilized and inoculated grain showed that ¹⁴C-malathion was degraded extensively in all treatments. Malathion monocarboxylic acid and two unidentified compounds were the principle degradation products recovered. Inoculated corn contained relatively larger quantities (in percentage of total recovered radiocarbon) of the two unknown degradation products than sterilized corn. However, similar amounts of MCA were recovered from both inoculated and control corn. Inoculated wheat contained more MCA and less of the two

unknown compounds than its sterilized control. Lewis et al. (1975) reported that malathion was rapidly degraded in liquid cultures of the fungus *A. oryzae*, resulting primarily in malathion monoacid. Similarly, Mostafa et al. (1972) showed that liquid cultures of *A. niger* metabolized 59% of applied malathion within 10 days through carboxylester hydrolysis and demethylation. In our study, the oxygen analog, malaaxon, was not recovered from either the corn or the wheat. Rowlands (1967) found significant quantities of malaaxon only in freshly harvested wheat of 18% moisture content which had been treated with malathion. Unknown compounds I and II did not correspond to malathion, malaaxon, malathion carboxylic acids, diethyl mercaptosuccinate acid, or the alkyl phosphate metabolites. Furthermore, unknown compound I did not correspond to malaaxon carboxylic acids, demethyl malathion, or thiomalic acid. Malathion dicarboxylic acid was not detectable in our system because it did not contain the radiocarbon label.

Not only did the freshly harvested corn support a heavier growth of *A. glaucus*, but also more of the insecticide was degraded in corn than in wheat. Both inoculated and sterilized corn contained less chloroform-soluble radiocarbon than respective wheat samples (Table 1). Furthermore, the chloroform fractions of both corn treatments also contained less malathion than corresponding wheat samples (Table 2). These results may have been related to the fact that the corn was freshly harvested, whereas the wheat had been stored almost a year before use. However, these results may reflect inherent differences in the ability of corn or wheat to metabolize malathion.

In summary, results presented in this paper suggest that storage fungi may be partially responsible for the accelerated degradation of malathion commonly observed in grain stored at high temperatures and moisture contents.

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