Soil Metabolism of [14C]Methiozolin under Aerobic and Anaerobic Flooded Conditions

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ABSTRACT: Methiozolin is a new turf herbicide controlling annual bluegrass in various cool- and warm-season turfgrasses. This study was conducted to investigate the fate of methiozolin in soil under aerobic and anaerobic flooded conditions using two radiolabeled tracers, [benzyl-14C]- and [isoxazole-14C]methiozolin. The mass balance of applied radioactivity ranged from 91.7 to 104.5% in both soil conditions. In the soil under the aerobic condition, [14C]methiozolin degraded with time to remain by 17.9 and 15.9% of the applied in soil at 120 days after treatment (DAT). [14C]Carbon dioxide and the nonextractable radioactivity increased as the soil aged to reach up to 41.5 and 35.7% for [benzyl-14C]methiozolin at 120 DAT, respectively, but 36.1 and 39.8% for [isoxazole-14C]methiozolin, respectively, during the same period. The nonextractable residue was associated more with humin and fulvic acid fractions under the aerobic condition. No significant volatile products or metabolites were detected during this study. The half-life of [14C]methiozolin was approximately 49 days in the soil under the aerobic condition; however, it could not be estimated in the soil under the anaerobic flooded condition because [14C]methiozolin degradation was limited. On the basis of these results, methiozolin is considered to undergo fast degradation by aerobic microbes, but not by anaerobic microbes in soil.

**KEYWORDS:** *methiozolin, soil metabolism, aerobic, anaerobic flooded*

■ INTRODUCTION

Methiozolin [5-(2,6-difluorobenzyl)oxymethyl-5-methyl-3-(3-methylthiophen-2-yl)-1,2-isoxazoline] is a novel turf herbicide developed by Moghu Research Center and registered in 2010 in Korea. This molecule was first invented as a rice herbicide candidate,[1] which controlled barnyardgrass (*Echinochloa* sp.) and several annual broad-leaved and sedge weeds from 125 g/ha in a paddy condition while showing excellent safety to transplanted rice up to 1.0 kg/ha.[2] Koo and Hwang[3] reported that the molecule effectively controlled annual bluegrass (*Poa annua*) and large crabgrass (*Digitaria sanguinalis*) at pre- and postemergence stages and was highly safe to various cool- and warm-season turfgrasses including creeping bentgrass, Kentucky bluegrass, perennial ryegrass, zoysiagrass, and bermudagrass. This herbicide greatly inhibited biosynthesis of both cellulose and hemicellulose fractions in corn roots;[4] however, the morphological symptoms did not resemble those of known cell wall synthesis inhibitors such as dichlobenil.[5] Up to now, the mechanism of action of the molecule is not thoroughly understood and appears to be a new action mechanism.

Koo et al.[6] reported that methiozolin had low mammalian and ecotoxicity. However, limited information on the environmental fate and metabolism was available until now. In this paper, we report on the soil metabolism of methiozolin under aerobic and anaerobic flooded conditions, describing the mass balance, the degradation pattern, and the formation of metabolites.

■ MATERIALS AND METHODS

Chemicals. The radiolabeled test compounds, [benzyl-14C]-methiozolin and [isoxazole-14C]methiozolin (Figure 1), were synthesized
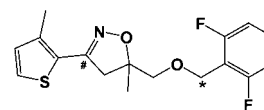
Figure 1. Chemical structure of methiozolin and 14C-labeled position: [benzyl-14C]Methiozolin (∗) and [isoxazole-14C]methiozolin (#).

at Korea Radiochemicals Center (Suwon, Korea). The purity of the both radiochemicals was >99%, and specific activities were 4.55 and 6.59 MBq/mg for [benzyl-14C] and [isoxazole-14C]methiozolin, respectively. They were used without further purification. HPLC grade acetone, acetonitrile, dichloromethane, and water were purchased from Duksan Co. (Ansan, Korea). All other reagents and common chemicals were of analytical grade and commercially available.

Test Soil. A sandy clay loam soil was sampled from the top 10 cm of a drained paddy field. The soil was sieved to remove plant debris and rocky particles and then air-dried at room temperature for 48 h for soil texture characterization (Table 1)[7−9] and then stored at 4 °C to maintain microbial activity prior to use.

Radioassay. Radioactivity of all liquid samples was measured by a liquid scintillation counter (LSC; Tri-Carb 2900TR, PerkinElmer,

USA). Radioactivity in a gross amount of less than twice the background was considered to be below the limit of determination accuracy. Ultima Gold AB scintillation cocktail (4 mL) was used for the acidic aqueous samples, and Insta-Gel Plus (4 mL) was used for the aquatic, organic

samples and CO_2 trapping agent. The nonextractable soil residue (0.2 g) was combusted by a sample oxidizer (model 307, PerkinElmer, USA) after mixing with Combustaid (0.2 mL). The [^{14}C]carbon dioxide produced was absorbed in Carbo-Sorb E (5 mL) and mixed with PermaFluor E$^+$ (10 mL) scintillator for LSC counting. All scintillation cocktails and Combustaid used in this study were purchased from PerkinElmer, USA. The efficiency of the oxidizer was determined using aliquots of the Spec-Chec-^{14}C check source for the automatic sample oxidizer and was >95%. Measurements of radioactivity were corrected by the oxidizer efficiency.

Chromatography. Identification of methiozolin and metabolites and determination of radioactivity were performed using a PerkinElmer series 200 HPLC system equipped with a UV−vis detector and flow scintillation analyzer (Radiomatic 610TR, PerkinElmer, USA) by cochromatography with an authentic compound. The detection wavelength was 254 nm. A reverse phase C_{18} column (Cosmosil, 5 μm, 250 × 4.6 mm i.d., Nacalai Tesque, Japan) was used. The elution solvents were acetonitrile and water (0.1% trifluoroacetic acid). A linear gradient (from 30% acetonitrile to 90% for 27 min) using a flow rate of

Table 1. Characterization of Test Soils

parameter	soil
particle size distribution (%)	
sand	53.75
silt	26.25
clay	20.00
texture class (USDA)	sandy clay loam
pH (1:5) in water	5.93
water-holding capacity	38.1
organic carbon (%)	1.82
organic matter (%)	3.14
cation exchange capacity (mequiv/100 g)	10.6

Table 2. Extractability of [^{14}C]Methiozolin with Different Solvent Systems

soil condition	compound	applied radioactivity (%)			
		acetone/water (7:3)	acetone/water (1:1)	acetone/water/36% HCl (35:35:2)	total
aerobic	[benzyl-^{14}C]	98.4 ± 0.32	4.2 ± 0.15	0.4 ± 0.02	103.0 ± 0.21
	[isoxazole-^{14}C]	95.7 ± 0.13	4.2 ± 0.09	0.8 ± 0.04	100.8 ± 0.16
anaerobic flooded	[benzyl-^{14}C]	88.7 ± 2.00	1.5 ± 0.05	2.2 ± 0.67	92.5 ± 1.35
	[isoxazole-^{14}C]	91.6 ± 2.69	1.1 ± 0.27	2.4 ± 0.09	95.1 ± 3.04
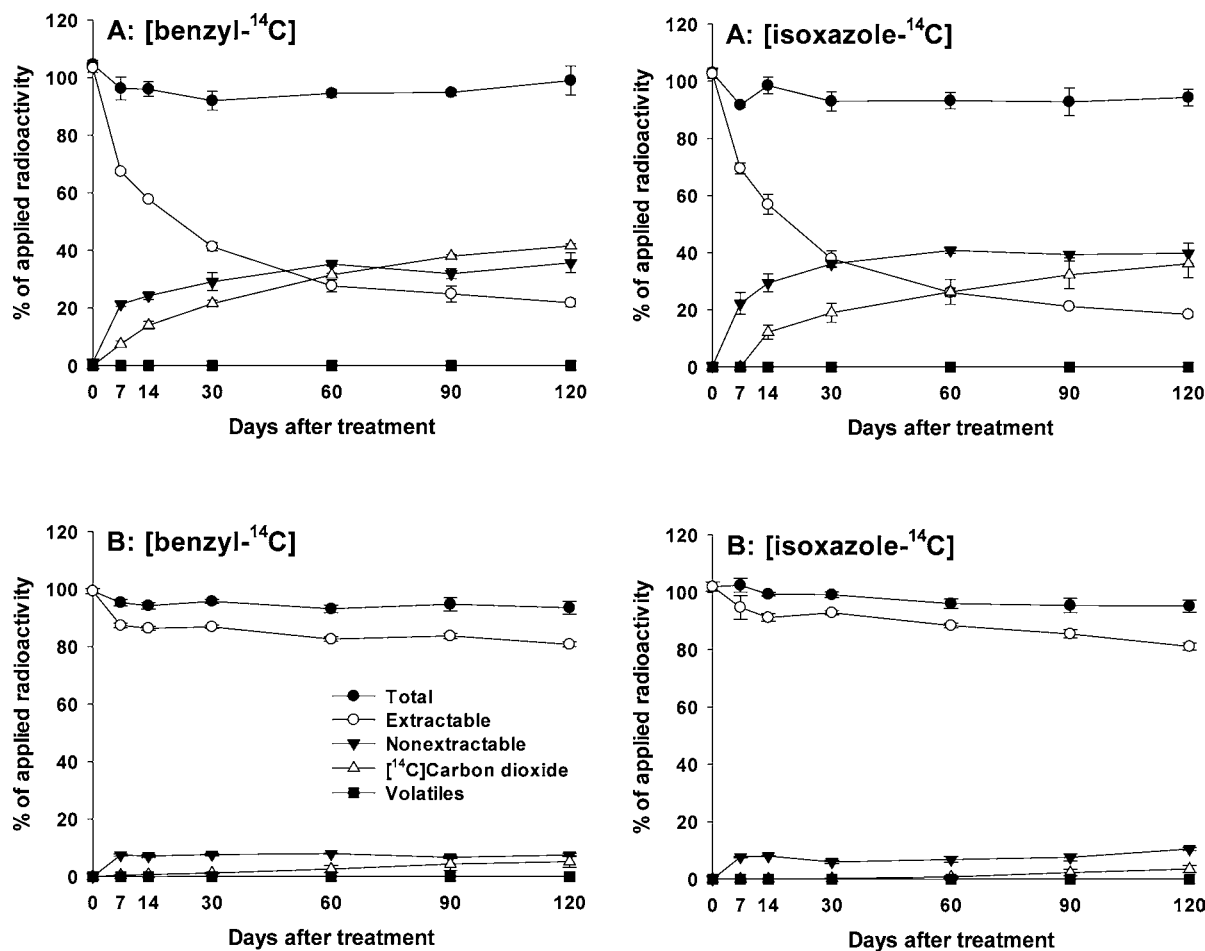
Figure 2. Distribution of radioactivity in the soil treated with [^{14}C]methiozolin under the aerobic (A) and anaerobic flooded (B) conditions. Each data point represents the mean ± SD (n = 3).

1.0 mL/min was adopted to separate the peaks of methiozolin and potential metabolites produced.

Extraction Efficiency. To the air-dried soil samples (60 g) was added 14 mL of distilled water to set the soil moisture at 60% field moisture capacity and to yield an aerobic condition. Alternatively, 44 mL of distilled water was added to flood the soil sample to yield an anaerobic flooded condition. Either [benzyl-^{14}C] or [isoxazole-^{14}C]methiozolin stock solution was prepared at 200 μg/mL in acetone, and then 150 μL of the stock solution containing 30 μg of [^{14}C]methiozolin was added to the soil sample. This methiozolin concentration in soil is equivalent to the field use rate (0.5 mg/kg soil). Following [^{14}C]methiozolin addition, the soil was thoroughly mixed. After 30 min, samples of the treated soil were extracted three times sequentially with acetone/water (7:3, v/v, 100 mL), acetone/water (1:1, v/v, 100 mL), and acetone/water/36% HCl (35:35:2, v/v/v, 100 mL). Triplicate aliquots (0.5 mL) of the soil extracts were analyzed by LSC to measure the radioactivity.

Soil Incubation. For the aerobic condition, the soil (60 g, air-dry weight) was weighed into a 100 mL incubation flask, and 14 mL of distilled water was added. For the anaerobic flooded condition, 60 g of the soil in the incubation flask was flooded with 44 mL of N$_2$ gas-purged and sterilized distilled water. The depth of water was maintained at approximately 2 cm.[10,11] All of the procedures were performed in a clean bench using a sterilized water to maintain sterility. All soil samples were kept in a flow-through metabolism chamber and preincubated at 25 ± 1 °C for 2 weeks in the dark prior to [^{14}C]methiozolin treatment. Air or N$_2$ gas for the aerobic or anaerobic condition, respectively, was passed through the system at a flow rate of 20 mL/min through a 0.5 N sodium hydroxide solution to remove carbon dioxide followed by distilled water to humidify. After preincubation, an aliquot of the [benzyl-^{14}C] or [isoxazole-^{14}C]methiozolin solutions was applied to the soil at a rate of 0.5 mg/kg as describe above. The soil samples treated were then incubated in a flow-through metabolism system at 25 ± 1 °C in the dark. The effluent gas from the chamber was passed through two XAD-2 resins (Supelco, USA) and two sodium hydroxide solution traps (0.5 N, 40 mL) in sequence to trap volatile compounds and [^{14}C]carbon dioxide, respectively. The treated soil was sampled at 0, 7, 14, 30, 60, 90, and 120 days after treatment (DAT). Readjustment of the soil moisture content and collection of [^{14}C]carbon dioxide and volatile products were carried out every 2 weeks. Volatile products were extracted with 5 mL of methanol from resins by sonication for 5 min at ambient temperature. Triplicate aliquots (1 to 4 mL) of the methanol extracts and sodium hydroxide solution from the traps were analyzed by LSC.

Extraction and Analysis of Soil. At each soil sampling date, three flasks per treatment were taken, and soil samples were extracted with acetone/water (7:3, v/v, 100 mL) by sonication (15 min) and shaking (30 min, 250 rpm). The extract was centrifuged at 5000 rpm for 15 min, and then the supernatant was taken and radioactivity was analyzed as described above. After acetone was removed under reduced pressure at 40 °C, 20 mL of the saturated sodium chloride solution and 20 mL of the 2 N hydrochloric acid solution were added to the aqueous solution, which was then extracted twice with 100 mL of ethyl acetate. The ethyl acetate solution was concentrated in vacuo at 40 °C and dissolved in 2 mL of acetonitrile. An aliquot of the solution was analyzed by radio-HPLC to determine the concentration of the parent and its metabolites.

After extraction with acetone/water (7:3, v/v), the remaining soil was then sequentially extracted with 100 mL of acetone/water (1:1, v/v) and 100 mL of acetone/water/36% HCl (35:35:2, v/v/v). In each procedure, the same extraction was repeated three times, and analysis was done by LSC. All of the postextracted soil samples were air-dried and then homogenized and weighed. Triplicate portions (0.2 g) were combusted with the oxidizer before LSC counting.

Distribution of Nonextractable Radioactivity in Soil. The nonextractable radioactivity residue by the extraction solvent system was fractionated with a strong base and acid into three fractions of humin, humic acid, and fulvic acid.[12,13] Ten grams (dry weight equivalent) of the nonextractable soil residue was extracted with 30 mL of 0.5 N sodium hydroxide solution by shaking for 24 h at room temperature.
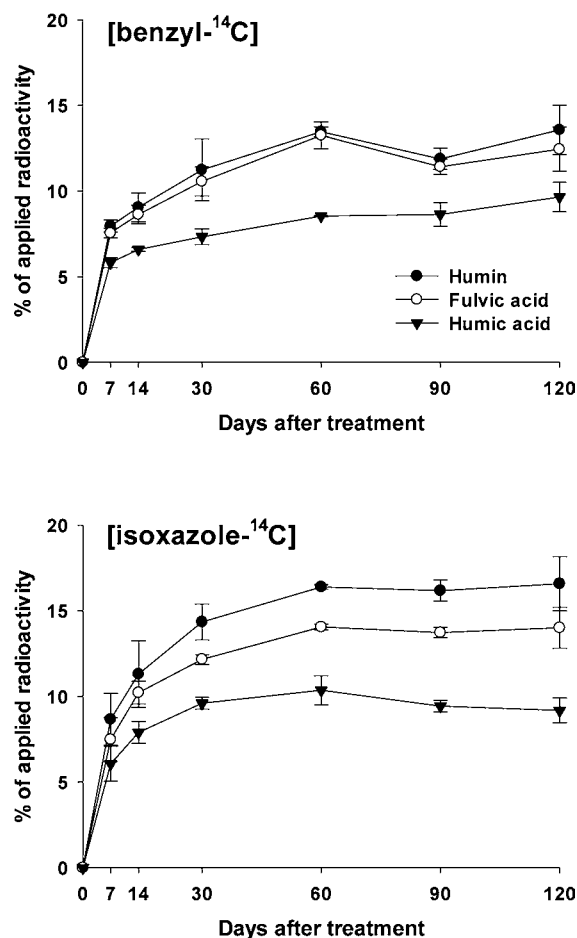

Figure 3. Distribution of nonextractable radioactivity in the soil treated with [benzyl-^{14}C]- and [isoxazole-^{14}C]methiozolin under the aerobic condition. Each data point represents the mean ± SD (n = 3).

The extract was centrifuged at 5000 rpm for 15 min and the supernatant (fulvic and humic acid fraction) was collected. This procedure was repeated until the radioactivity of supernatant reached the background level, and all of the supernatants were combined. Triplicate subsamples (0.2 g) of the precipitate (humin fraction) were combusted to determine the radioactivity content. Concentrated hydrochloric acid was added to the combined supernatant to adjust the pH to 1.0 and maintained at ambient temperature for 24 h. The mixture was then centrifuged at 5000 rpm for 15 min, and the supernatant (fulvic acid fraction) was collected. The resulting precipitate (humic acid fraction) was washed with 10 mL of 1 N hydrochloric acid solution and centrifuged. The supernatants were combined with the fulvic acid fraction. The volume of the combined solution was measured, and 1 mL of the solution was analyzed by LSC. The humic acid precipitate was redissolved in 20 mL of 0.5 N sodium hydroxide solution, the volume of the mixture was measured, and then triplicate aliquots (1 mL) were analyzed by LSC.

Calculation of Half-Life. A pseudo-first-order kinetics model was used to calculate the half-life.[14−17] The rates of degradation of [^{14}C]methiozolin were calculated by nonlinear regression analysis using SigmaPlot 10.0 software (SPSS Science, USA).

■ RESULTS AND DISCUSSION

Extraction Efficiency. [^{14}C]Methiozolin was recovered from the test soil with a high yield (88.7−98.4%) using the acetone/water (7:3, v/v) extraction system under both the aerobic and anaerobic flooded conditions (Table 2). A small amount (<4.2%) was detected in the acetone/water (1:1, v/v) extract. Although most of the radioactivity was recovered in the
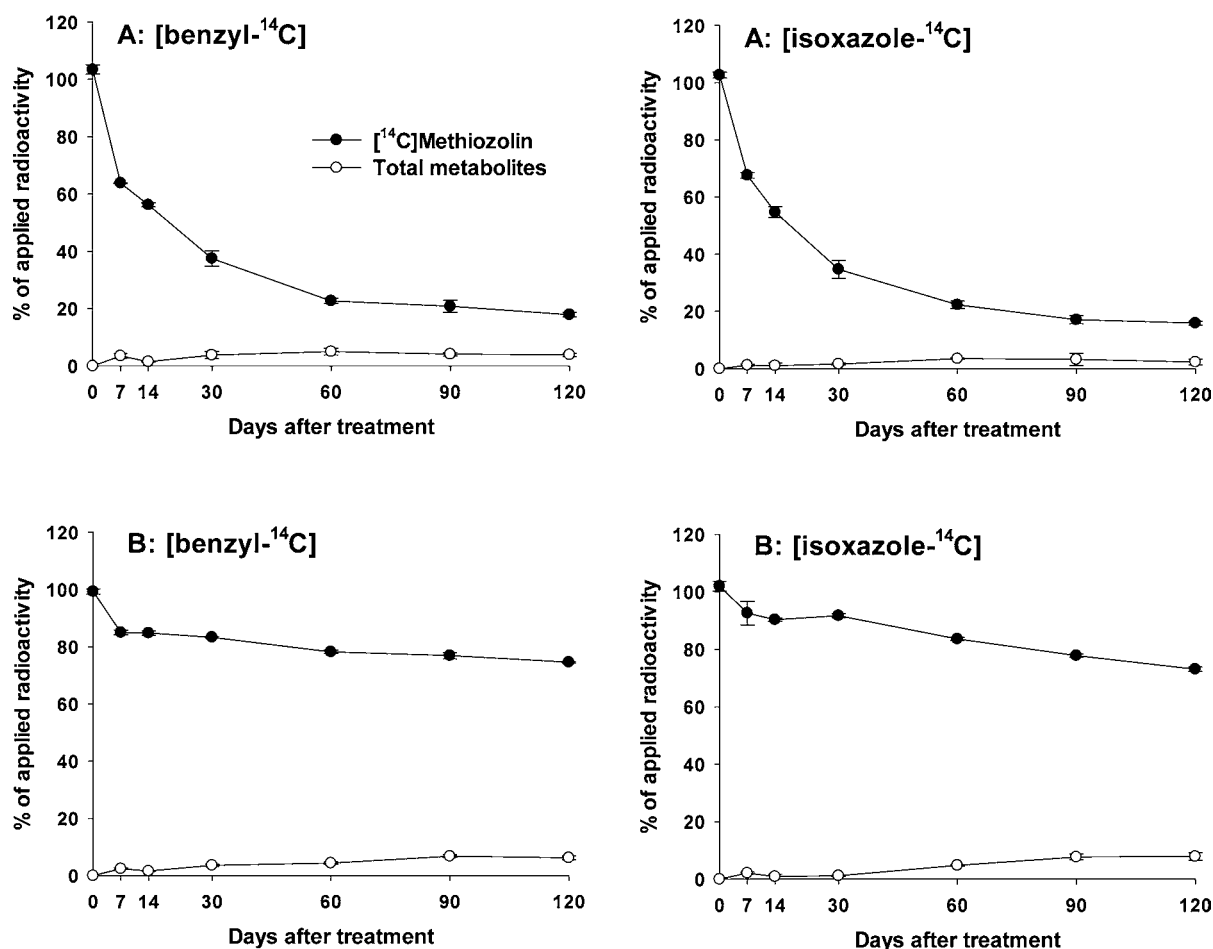
Figure 4. Degradation of methiozolin and formation of its metabolites in the soil under the aerobic (A) and anaerobic flooded (B) conditions. Each data point represents the mean ± SD (n = 3).

acetone/water fraction, the acetone/water/36% HCl (35:35:2, v/v/v) solution was used to extract metabolites, assuming they could be potentially more polar or acidic than the parent compound.[18]

Mass Balance. *Aerobic Condition.* Mass balance was calculated by determining the radioactivity recovered from the solvent extracts, [14C]carbon dioxide, volatile compounds, and solvent nonextractable residues.[19] The average mass balance throughout the study was 91.7−104.5% of applied radioactivity of [benzyl-14C] and [isoxazole-14C]methiozolin (Figure 2). The radioactivity of the solvent extract from the soil decreased with time and accounted for 21.8 and 18.4% of the applied radioactivity of [benzyl-14C] and [isoxazole-14C]methiozolin at 120 DAT, respectively. [14C]Carbon dioxide radioactivity levels from the soil steadily increased with time and accounted for 41.5 and 36.1% of the applied radioactivity of [benzyl-14C] and [isoxazole-14C]methiozolin at 120 DAT, respectively. The large production of [14C]carbon dioxide indicates that rapid and complete mineralization of methiozolin occurs by the soil microbes.[20−24] Volatile products from the soil were not detected throughout the study.

The nonextractable radioactivity levels steadily increased as the soil aged and accounted for 35.7% of [benzyl-14C] and 39.8% of [isoxazole-14C]methiozolin treated at 120 DAT, suggesting that binding of methiozolin or its degradation products to soil had occurred. Many pesticides are partially degraded, and the metabolites are involved in the formation of nonextractable residues. However, in most cases the metabolites are not known

and can be quantified by using [14C]-labeled parent compounds.[25] On the basis of these results, nonextractable formation occurred rapidly, especially within the first 7 days, but methiozolin or its metabolites were involved in nonextractable residue formation and which of those was more rapidly fast was unclear.[26] However, the similar rate of formation of the bound residue in soils treated with [benzyl-14C] and [isoxazole-14C]methiozolin suggests that the benzyl and isoxazole moiety might have similar binding affinity to the soil matrix, or the bound entity might have both the benzyl and isoxazole moieties.

Anaerobic Flooded Condition. In the soil under the anaerobic flooded condition, the average mass balances during the study were 93.2−102.5% of the applied radioactivity for both [benzyl-14C] and [isoxazole-14C]methiozolin (Figure 2). Solvent-extractable radioactivity levels decreased slightly with time and accounted for 80.8 and 81.1% of the applied radioactivity of [benzyl-14C] and [isoxazole-14C]methiozolin at 120 DAT, respectively. The nonextractable radioactivity levels were 6.7−8.0 and 6.0− 10.5% and [14C]carbon dioxide radioactivity levels were 0.4−5.2 and 0.02−3.5% of the applied radioactivity of [benzyl-14C] and [isoxazole-14C]methiozolin, respectively. No volatile products were detected. The results might be due to minimal activity of the soil microbes under the anaerobic flooded condition.[11]

Distribution of Nonextractable Radioactivity. Humic substance binds with many organic compounds including agrochemicals.[27] To find out the distribution of radioactivity of the applied [14C]methiozolin in the nonextractable soil residues,
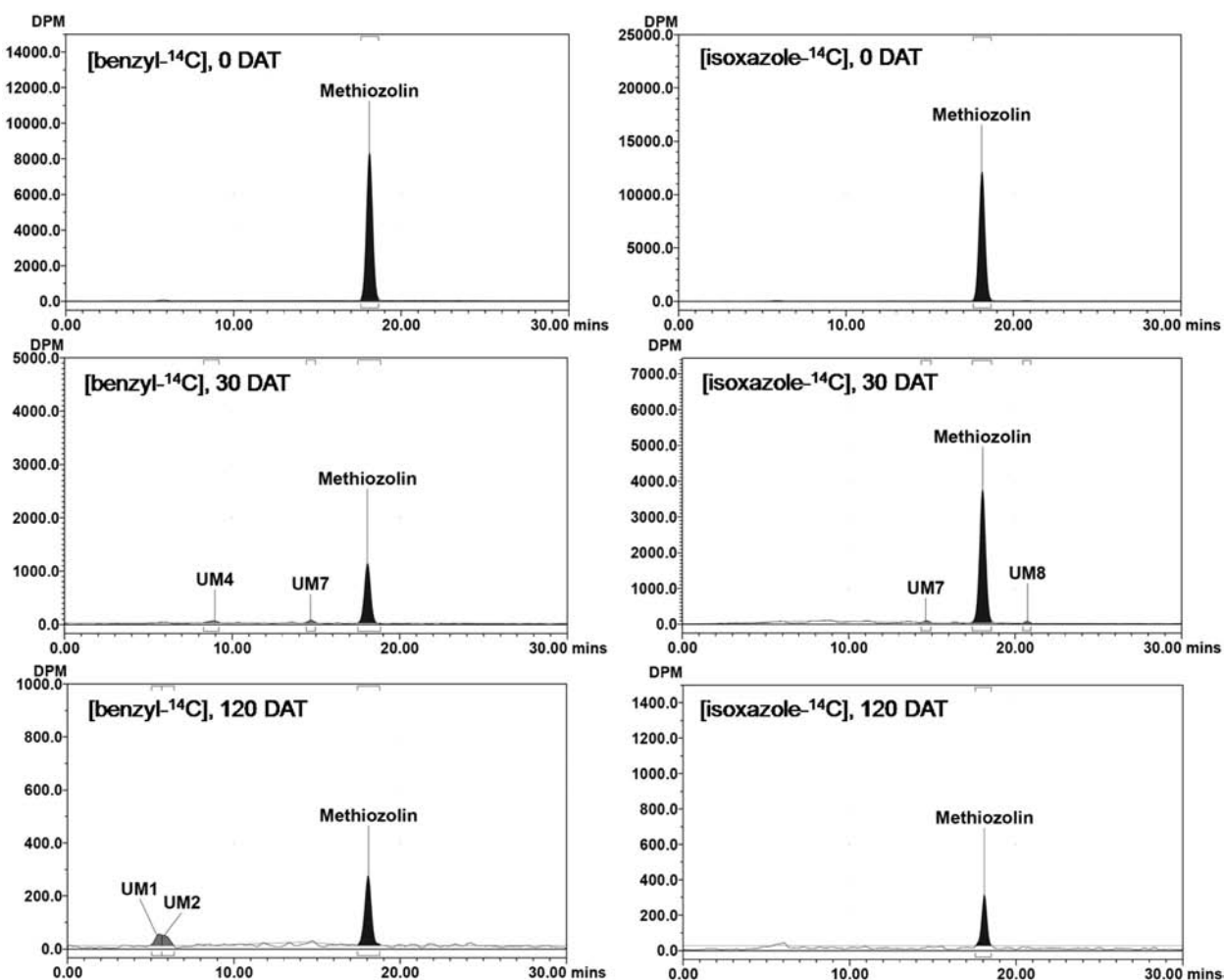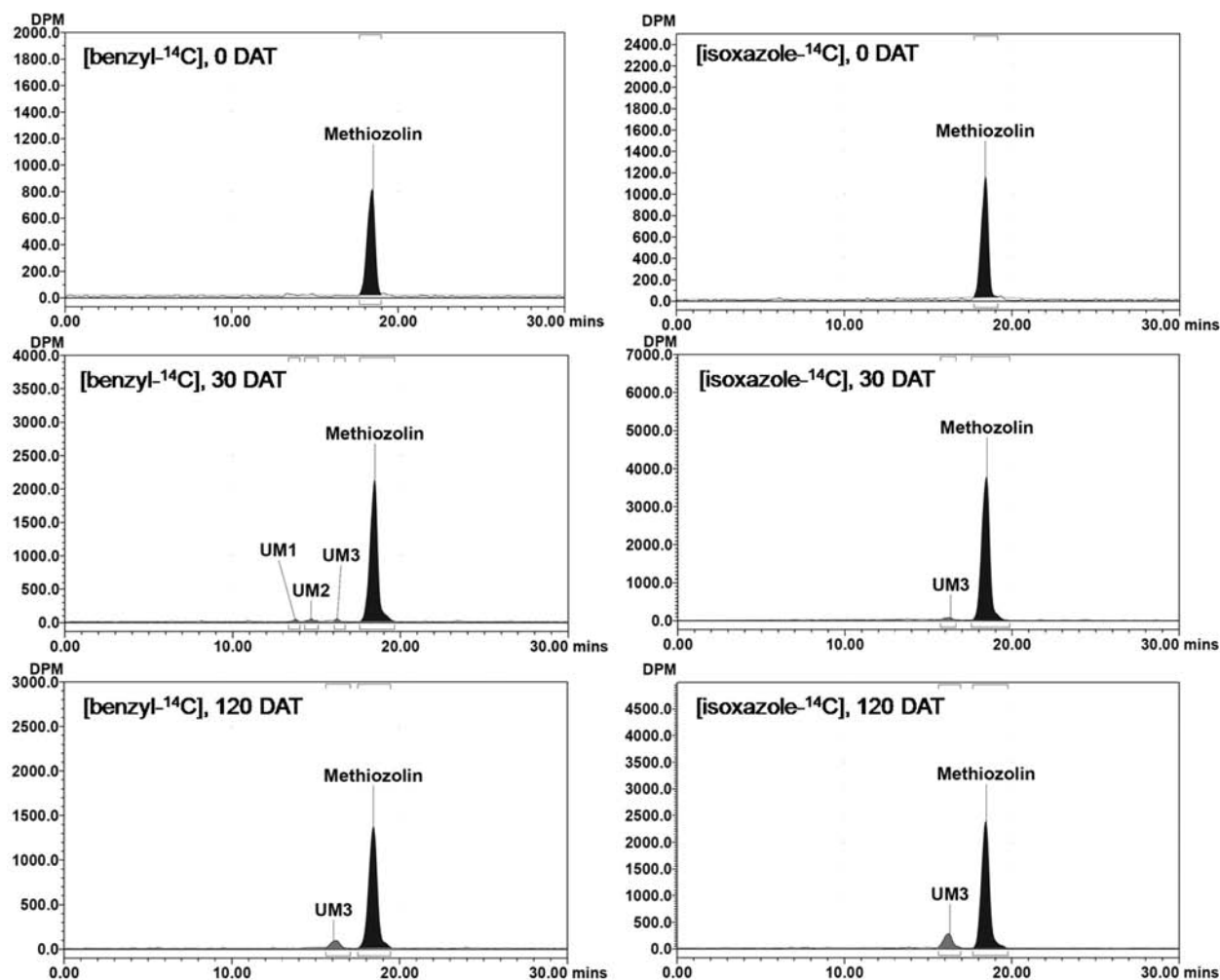
Figure 5. Representative radio-HPLC chromatograms of solvent extracts from the soil under the aerobic condition after application of [benzyl-^{14}C]- and [isoxazole-^{14}C]methiozolin. UM, unidentified metabolite.

further fractionation into fulvic acid, humic acid, and humin was performed. Of the radioactivity remaining in the soil under the aerobic condition treated with [benzyl-^{14}C]methiozolin at 120 DAT, 12.4, 9.7, and 13.6% of the applied radioactivity were found in fulvic acid, humic acid, and humin fractions, respectively (Figure 3). For [isoxazole-^{14}C]methiozolin, 14.0, 9.2, and 16.6% of the applied radioactivity were founded in fulvic acid, humic acid, and humin fractions, respectively. These results indicated that the bound residue was associated more with humin and fulvic acid fractions.[18] Fractionation of the bound residues under the anaerobic flooded condition was not conducted because only a small amount of radioactivity was detected.

Degradation of [^{14}C]Methiozolin and Formation of Metabolites. Degradation of [^{14}C]methiozolin and formation of metabolites in the soil under the aerobic and anaerobic flooded conditions is shown in Figure 4. In the soil under the aerobic condition, both [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin degraded rapidly until 30 DAT, and at that time, the amount of [^{14}C]methiozolin was 37.5 and 34.7% of the applied, respectively. After 30 DAT, [^{14}C]methiozolin degraded relatively more slowly than in the first 30 days, and 17.9 and 15.9% of the applied radioactivity were detected in the soil treated with [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin, respectively, at 120 DAT. The calculated half-lives in the soil were 51.7 and 46.8 days, respectively, for [benzyl-^{14}C] and [isoxazole-^{14}C]-

methiozolin. The similar degradation rated between [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin indicate that both the ^{14}C-labeled moieties have similar susceptibility to degradation by aerobic soil microbes. In the soil under the anaerobic flooded condition, <25% of applied [^{14}C]methiozolin was degraded until 120 DAT; therefore, the half-life could not be calculated. The results suggest that methiozolin degradation largely depends on the aerobic microbes, but not on anaerobic microbes in soil.

In the present study, in both soil conditions, although the amount of degradation products increased slightly with time, no major metabolite was detected (Figures 5, 6). In the soil under the aerobic condition, eight minor metabolites were found in the soil treated with [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin throughout the study (Figure 5). However, the amount of each metabolite was 0.5–2.2% of the applied. The combined percentage for all of the metabolites at each sampling date were 1.0–5.0% of the applied radioactivity, and the amount was maximized at 60 DAT in both soils. In the soil under the anaerobic flooded condition, three minor metabolites were detected, and the amount of total metabolites at each sampling date was 0.9–7.9% of the applied radioactivity (Figure 6). In this study, no significant metabolite was found in all of the soil conditions, suggesting that metabolites produced from methiozolin may rapidly bind to humic substances and/or further degrade rapidly into carbon dioxide.

Figure 6. Representative radio-HPLC chromatograms of solvent extracts from the soil under the anaerobic flooded condition after application of [benzyl-^{14}C] and [isoxazole-^{14}C]methiozolin. UM, unidentified metabolite.

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Notes

The authors declare no competing financial interest.

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