Translocation of Metalaxyl in Soybean Plants and Control of Stem Rot Caused by *Phytophthora megasperma* f. sp. *glycinea*

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The authors acknowledge the gift of Harosoy 63 soybean seeds from K. L. Athow, Department of Botany, Purdue University, Lafayette, IN 47907, and ^{14}C-metalaxyl and other formulations from CIBA-GEIGY Co., Greensboro, NC 27409.
Accepted for publication 11 March 1985.

ABSTRACT

Gupta, J. P., Erwin, D. C., Eckert, J. W., and Zaki, A. I. 1985. Translocation of metalaxyl in soybean plants and control of stem rot caused by *Phytophthora megasperma* f. sp. *glycinea*. Phytopathology 75:865-869.

Metalaxyl, applied to seeds, leaves, cotyledons, and soil, was translocated mainly in an upward direction in soybean plants. Treatment of seeds with metalaxyl resulted in partial control (40% survival) at 30 mg/100 g of seeds and complete control (100% survival) at 200 mg/100 g of seeds of stem rot when wounded hypocotyls of 7-day-old plants were inoculated with *Phytophthora megasperma* f. sp. *glycinea*. The percentage distribution of metalaxyl (measured by gas chromatography) in 7-day-old plants after seed dressing (200 mg/100 g of seeds) was 79.2% in the cotyledons, 20.3% in leaves and stems, and less than 0.12% in roots. When ^{14}C-metalaxyl (48.7×10^4 dpm per seed) was applied to seeds, 91.5% of the extractable radioactivity (metalaxyl and derivatives) in 7-day-old plants was detected in the cotyledons, 5.8% in leaves and stems, and 2.57% in roots. The extract of cotyledons from 7-day-old plants treated at 200 mg/100 g of seeds and assayed by thin-layer chromatography, contained 40% ^{14}C metalaxyl, while 60% of the radioactivity remained at the origin of the chromatogram. When ^{14}C-metalaxyl was applied to a leaf or to a cotyledon, 99% of the recovered radioactivity remained in the treated leaf or cotyledon. After ^{14}C-metalaxyl was applied to soil by drenching, 80.5% of the radioactivity taken up by the plant in 7 days accumulated in the cotyledons, 15.1% in the other aerial portions, and 4.4% in the roots.

Metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-DL-alanine, methyl ester] is a systemic fungicide which controls many diseases caused by fungi in the order Peronosporales (13,14). Staub et al (16) presented biological evidence that the predominant direction of movement of metalaxyl in plants was upward.

Control of many diseases caused by fungi classified in the Peronosporales has been obtained by seed dressing with metalaxyl (2,9,10,18). In most cases, the effect of seed treatment on disease development was limited in duration. Miller and de Whalley (10) showed that primary infection by *Peronospora viciae* on pea was more efficiently controlled by seed dressing than was secondary infection. Control of Phytophthora root and stem rot caused in soybean by *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin by seed dressing with metalaxyl has been reported (1,8,11,19). Radial growth of *P.m.* f. sp. *glycinea* was highly sensitive to metalaxyl with an ED_{50} value of 0.033 μg/ml (4). Ward et al (17) reported that control of infection of soybean hypocotyls by metalaxyl applied to soil caused a hypersensitive type of response accompanied by the production of a phytoalexin, glyceollin.

This host-pathogen system was selected for study of translocation of metalaxyl because the pathogen could be inoculated into hypocotyl tissue after treatment of seed with metalaxyl; this allowed correlation of disease control with the

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metalaxyl treatment and the movement of the fungicide in plant tissues. We report here the effect of seed treatment on the suppression of stem rot caused by *P.m.* f. sp. *glycinea* and quantitative data on the translocation of metalaxyl in soybean after application to seeds, soil, and to cotyledons and leaves. Part of this report has been published in an abstract (6).

MATERIALS AND METHODS

Plants and inoculation method. Soybean (*Glycine max* 'Harosoy 63') plants (7 days old) were inoculated with mycelium of a culture of *P.m.* f. sp. *glycinea*, race 7, grown on V8 juice agar at 21 C. The mycelium was applied under a shallow slash-wound flap (~3 mm long) in the cortical tissue of the stem immediately below the cotyledons (a modification of Keeling's [7] method). Plants were incubated in a mist chamber at 25 C for 36 hr and removed to a glasshouse at 20–25 C. The incidence (percent dead plants) of stem rot was recorded 3 days after inoculation. Treatments were replicated at least three times and experiments repeated twice.

Chemicals. Radioactive metalaxyl (>99% pure, U-ring-^{14}C, specific activity 24.6 μCi/mg), was mixed with either Ridomil® 2E (25.1% a.i.), or Ridomil 50 WP (50% a.i.) for use. Dosages are given as active ingredient.

Seed treatment. Seeds were treated with Ridomil 50 WP by shaking in a flask on a mechanical shaker for 20 min. ^{14}C-Metalaxyl (10 μCi) was dissolved in 4 ml of acetone and mixed thoroughly with 8 mg of unlabeled metalaxyl (Ridomil 50 WP). After the acetone evaporated, the metalaxyl–^{14}C-metalaxyl mixture, suspended in 0.5 ml of water, was applied to seeds (4 g), shaken in an Erlenmeyer flask for 20 min, and dried at room temperature for 24 hr. An average of 184 μg of metalaxyl (0.22 μCi = 48.7×10^4 dpm) was deposited on each seed. Treated and untreated seeds were planted singly in $10 \times 10 \times 10$-cm plastic pots. The soil used in all experiments was UC mix (sand:peat [1:1 v/v]).

Soil treatment. ^{14}C-Metalaxyl (0.5 μCi) was mixed with 2 mg of unlabeled metalaxyl (Ridomil 50 WP) in 10 ml of water and applied to the surface of the soil in each pot containing one 7-day-old soybean plant. The soil was irrigated to saturation 24 hr later.

Cotyledon and leaf treatment. ^{14}C-Metalaxyl was diluted with Ridomil 2E and water so that every 100 μl contained 100 μg of metalaxyl and 0.25 μCi ^{14}C-metalaxyl. A Pasteur pipette was used to spread 100 μl of the solution over the upper surface of a leaf or cotyledon of a 7-day-old plant. Aluminum foil covered with several paper towels was placed on the soil and wrapped around the stem of the plant at the soil level to prevent metalaxyl from inadvertently reaching the roots of the plant.

Autoradiography. Seven days after treatment with ^{14}C-metalaxyl, the intact plants were removed from soil, rinsed in water, dried between newspapers which were changed daily, and dried in an oven (70 C) for 12 hr. Plants were mounted on poster board. Control plants (no metalaxyl) were treated similarly. The plants were exposed to Kodak No-Screen X-ray film in darkness for 4 wk. Thin-layer chromatography plates were similarly exposed for 4 wk.

Measurement of ^{14}C-metalaxyl and radiolabeled derivatives in plants. ^{14}C-Metalaxyl was removed from intact treated seeds (about 0.11 g/seed) by extraction with five portions of acetone (5 ml per seed) (total 25 ml). A 1-ml portion of the combined extracts was placed in a liquid scintillation vial until the acetone evaporated. Ten milliliters of scintillation fluid (3 g 2,5-diphenyloxazole (PPO), 100 mg *p-bis*-[2-(5-phenyloxazole)]-benzene (POPOP), and toluene to make 1 L) was added to the vial, and radioactivity was measured in a Beckman LS7500 liquid scintillation spectrophotometer.

Soybean plants were harvested and the roots were washed in water 7 and 15 days after planting. Fresh weights of roots, hypocotyls, cotyledons, and aerial parts were recorded. The plant material was cut into small pieces, and the ^{14}C-labeled compounds were extracted by grinding it in a mortar and pestle with acetone (10 ml of acetone per gram of plant tissue) and repeated with two more volumes of acetone. The extracts were combined and filtered through a layer of cotton and the volume was reduced to 10 ml by evaporation under a stream of N_2. One milliliter of the concentrated extract was transferred to a scintillation vial, evaporated to dryness at room temperature, and the radioactivity was measured as described.

Extraction and cleanup of extracts. Plant tissue was homogenized in 95% ethanol (10 ml/g fresh weight). In some experiments, a known amount of metalaxyl was added to the tissue homogenate of untreated plants. The tissue was filtered and then extracted three more times with ethanol. A known amount of metalaxyl was added to the filtrate of untreated plants in some experiments. The combined extracts were concentrated to about 25 ml in vacuo, diluted with about 100 ml of distilled water, and partitioned with an equal volume of ethyl acetate. The aqueous phase was discarded, and the organic phase was partitioned with 1.0 N NaOH and then with water. The aqueous phases were discarded and the remaining water in the organic phase was removed with anhydrous $MgSO_4$. The organic solution was evaporated to dryness, and the residue was dissolved in about 20 ml of dichloromethane. This solution was loaded on a silica gel column (10 g of silica gel GF 254; E. Merck, Darmstadt, Germany) that was tightly packed dry inside a 50-ml fritted-disk Büchner funnel. The column was eluted with two 50-ml portions of dichloromethane and the eluate was discarded. Metalaxyl, which remained adsorbed on the silica gel, was then eluted with three 50-ml portions of ethyl acetate. Activated charcoal (about 5 g) was added to the eluate and stirred for 2 min. The charcoal was then removed by filtration and discarded. The filtered solution was evaporated to dryness. The residue was dissolved in an appropriate volume of acetone and analyzed by gas-liquid chromatography (GLC).

An Aerograph 600 gas chromatograph (Varian Associates, Palo Alto, CA) equipped with a hydrogen flame ionization detector (FID) was used. A GLC column (stainless steel tubing, 100 cm in length and 4 mm ID), was packed with 3.4% silicone (fluoro) QF-1 and 6.2% silicone DC 200 on Gas Chromosorb Q (80/100-mesh) (Varian Associates). Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. The flow rate of H_2 and that of air was 30 ml/min. Metalaxyl was eluted at a column temperature of 180 C, injector temperature 250 C, and detector temperature 250 C. The retention time under these conditions was 4.0 min. Peak area was determined by multiplying the peak height (mm) by the width of the peak (mm) at the midpoint. The concentration of metalaxyl was calculated by comparing the peak area of the unknown with the peak area of a standard solution of metalaxyl. The linear range of the detector was between 1 and 10 μg of metalaxyl. In some experiments, the internal standard *p,p'*-diethoxybiphenyl was used just before the GLC analysis. It eluted from the GLC column at a retention time of 6.0 min.

Acetone extracts of soybean tissue were spotted on thin-layer chromatograms (TLC) with a 2-mm-thick layer of silica gel (GF254; Brinkman Instruments, Westbury, NY). The prepared plates were developed by ascending chromatography in hexane:methanol:ethanol (40:2.5:60, v/v) and the ^{14}C-labeled compounds were localized by exposure of the TLC plates to X-ray film for 4 wk. The silica gel in the radioactive zones was scraped from the plate, the ^{14}C-labeled compounds were eluted with acetone or water, and the radioactivity was measured by scintillation spectrometry.

RESULTS AND DISCUSSION

Effect of seed treatment with metalaxyl on hypocotyl infection by *P.m.* f. sp. *glycinea*. All plants grown from untreated seed died following slash-wound-inoculation of the hypocotyls of 7-day-old seedlings with *P.m.* f. sp. *glycinea*. Treatment of seed with metalaxyl at several dosages increased survival of the seedlings in direct proportion to the dosage: 30 mg metalaxyl per 100 g of seeds, 40% survival; 60 mg, 60%; 100 mg, 80%; and 200 mg, 100% survival. Surviving plants were free of symptoms. Locke et al (9) reported control of Pythium blight of snap beans in greenhouse and field experiments by treatment of seed with metalaxyl (200 mg/kg of seeds).

TABLE 1. Distribution of metalaxyl in soybean plants grown from metalaxyl-treated seeds

Metalaxyl seed treatment			Metalaxyl extracted per plant					
			7-day-old			15-day-old		
Treatment rate[a] (mg a.i./100 g seed)	Analyzed[b] (μg/seed)	Plant part	Analyzed[c] (μg/plant part)	Applied (%)	Distribution (%)	Analyzed[c] (μg/plant part)	Applied (%)	Distribution (%)
30	44	Cotyledons	1.99	4.52	95.2	1.00	2.27	50.2
		Leaves[d]	<0.06	<0.14	<2.9	0.99	2.25	49.8
		Stems	<0.04	<0.09	<1.9	nd		
		Roots	1.09	4.75	99.0	nd		
		Total				1.99	4.52	100.0
60	67	Cotyledons	4.96	7.30	98.6	3.68	5.49	67.1
		Leaves[d]	<0.06	<0.09	1.2	1.65	2.46	30.1
		Stems	<0.07	<0.10	1.3	<0.09	<0.13	<1.5
		Roots	5.09	7.49	101.1	<0.07	<0.10	<1.2
		Total				5.49	8.18	99.9
100	124	Cotyledons	14.53	11.72	97.3	7.52	6.06	72.5
		Leaves[d]	0.21	0.17	1.4	2.69	2.17	25.8
		Stems	<0.06	<0.05	<0.4	0.10	0.08	0.95
		Roots	14.80	11.94	99.1	<0.09	<0.07	<0.8
		Total				10.40	8.38	100.05
200	288	Cotyledons	36.48	12.70	79.2	25.34	8.79	71.1
		Leaves[d]	9.62	3.30	20.3	10.03	3.48	28.1
		Stems	<0.56	<0.02	<0.12	<0.25	<0.09	<0.07
		Roots	46.66	16.02	99.62	<0.10	<0.003	<0.0
		Total				35.72	12.363	99.27

[a] As Ridomil® 50 WP.
[b] Deposit (five seeds pooled) determined by gas-liquid chromatography (GLC).
[c] Pooled parts from 30 plants; roots consisted of plant material below the soil surface; metalaxyl was extracted with 95% ethanol and analyzed by GLC. Limit of detection was 0.1 μg/g fresh weight. The "nd" indicates that metalaxyl was not detectable. Data were corrected for 88% recovery efficiency. See text for details.
[d] Data on leaves and stems (includes the hypocotyls) are combined on the 7-day sample but not on the 15-day sample.

TABLE 2. Distribution of radioactivity (metalaxyl plus derivatives) in soybean plants grown from seeds treated with ^{14}C-metalaxyl[a]

	Metalaxyl extracted per plant					
	7-day-old			15-day-old		
Plant part extracted[b]	^{14}C extracted from plant part (dpm × 10^{-4})	Percentage of ^{14}C applied	Distribution (%)	^{14}C extracted from plant part (dpm × 10^{-4})	Percentage of ^{14}C applied	Distribution (%)
Cotyledons	23.52	48.29	91.45	13.34	27.39	84.91
Aerial portion (including hypocotyls)	1.54	3.16	5.98	2.01	4.12	12.79
Roots[c]	0.66	1.36	2.57	0.36	0.73	2.29
Total	25.72	52.81	100.00	15.71	32.24	~100.00

[a] Approximately 184 μg metalaxyl (0.22 μCi = 48.7 × 10^4 dpm) were applied per seed.
[b] Acetone 10 ml/g fresh weight of plant tissue.
[c] Roots consisted of plant material below the soil surface.

TABLE 3. Distribution of ^{14}C-metalaxyl and derivatives in 14-day-old soybean plants 7 days after application of ^{14}C-metalaxyl to leaves or cotyledons

Plant part treated[a]	Plant part extracted[b]	^{14}C extracted from plant part (dpm × 10^{-4})	Percentage of ^{14}C applied	Distribution (%)
Cotyledon	Cotyledon where ^{14}C was applied	26.72	48.11	99.55
	Cotyledon opposite treated cotyledon	0.003	0.005	0.01
	Aerial portion (minus cotyledons)	0.100	0.18	0.37
	Hypocotyl	0.004	0.007	0.015
	Roots[c]	0.01	0.02	0.04
	Total	26.84	48.36	~100.00
Leaf	Leaf where ^{14}C was applied	21.29	38.36	99.30
	Leaf opposite treated leaf	0.05	0.09	0.23
	Aerial portion (minus treated leaf)	0.08	0.14	0.37
	Hypocotyl plus cotyledons	0.02	0.04	0.09
	Roots	0.003	0.005	0.01
	Total	21.44	38.63	~100.00

[a] Metalaxyl 110 μg (0.25 μCi = 55.5 × 10^4 dpm) applied per plant.
[b] Acetone 10 ml/g fresh weight of plant tissue.
[c] Roots consisted of plant tissue below the soil surface.

Translocation of metalaxyl from treated seeds. Fortification of plant tissue with metalaxyl prior to extraction showed that the GLC analytical method recovered 88% of the metalaxyl in the sample. All data were appropriately corrected (Table 1). Speck and Dirr (15) obtained 75–80% recovery from tobacco tissue by GLC. Analysis of treated soybean seed revealed the average deposit per seed to be approximately proportional to the dosage of metalaxyl applied (Table 1).

Most of the applied metalaxyl remained in the cotyledons with smaller percentages moving to leaves and stems. For example, after 7 days, 1.99 μg/plant (4.5%) was recovered in the cotyledons of plants grown from seeds treated with 30 mg/100 g and at 200 mg/100 g, 36.48 μg/plant (12.7%), was recovered from cotyledons, and 3.3% from leaves and stems. After 15 days at the 200 mg/100 g of seed dosage and the largest percentage of metalaxyl remained in the cotyledons (71.1% of that absorbed) and a lesser amount was distributed to leaves (28.1%). Although the amount of metalaxyl translocated from seeds was relatively small, 100% of the plants from seeds treated with metalaxyl at 200 gm/100 g of seed survived stem inoculation with *P.m.* f. sp. *glycinea*. Although hypocotyls were not separated from the aerial portions, 7 days after treatment the wet weight of aerial portions contained 10.67 μg, cotyledons 124.48 μg, and roots 7.18 μg of metalaxyl per gram fresh weight. Since Coffey and Bower (4) reported an ED_{50} of 0.033 μg/ml for metalaxyl on radial growth in agar, it would appear that the concentration of metalaxyl was high enough to prevent colonization by *P.m.* f. sp. *glycinea*.

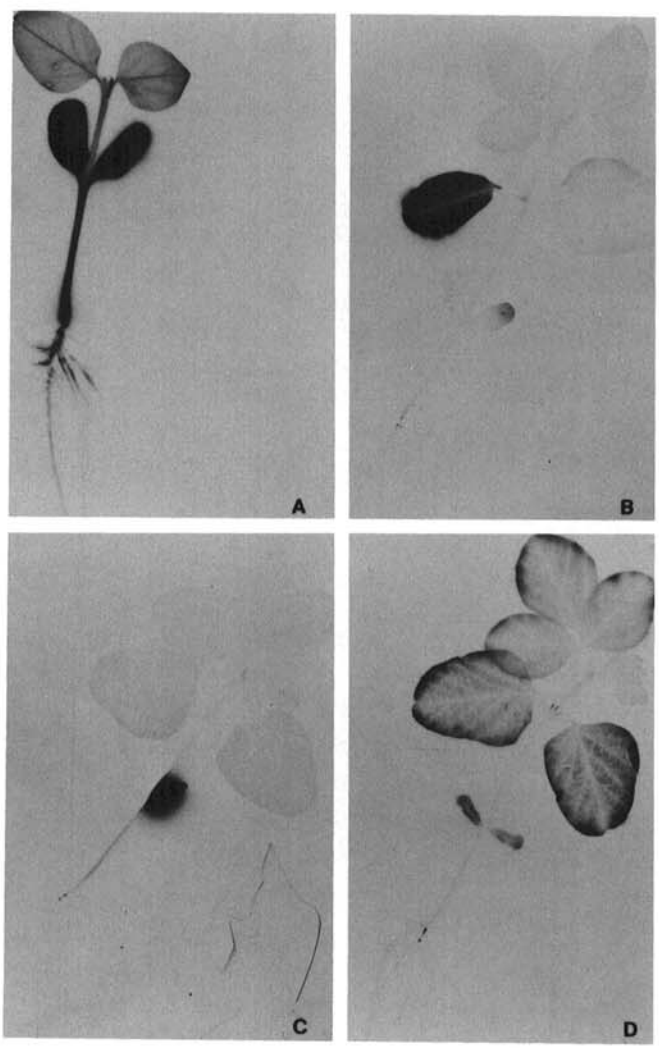

Fig. 1. Visualization of ^{14}C-metalaxyl and ^{14}C-derivatives in soybean tissue by autoradiography. Since only 40% of the radioactive material in acetone extracts of cotyledons from treated seeds was found to be authentic metalaxyl, autoradiographs should be interpreted accordingly. **A,** Plant was assayed 7 days after treatment of seed with ^{14}C-metalaxyl (0.22 μCi) and 184 μg metalaxyl per seed. **B,** Plant was assayed 7 days after spreading 100 μl of a ^{14}C-metalaxyl solution (100 μg metalaxyl, 0.25 μCi) ^{14}C-metalaxyl on a primary leaf. **C,** Plant was assayed 7 days after spreading 100 μl of a ^{14}C-metalaxyl solution (100 μg metalaxyl, 0.25 μCi) on a cotyledon as in **B**. **D,** Plant was assayed 7 days after 10 ml of a ^{14}C-metalaxyl solution (2.02 mg metalaxyl plus 0.5 μCi ^{14}C-metalaxyl) was drenched on soil in a 10 × 10 × 10-cm plastic pot.

Translocation of ^{14}C-metalaxyl from treated seeds. Assay of the acetone extract of the cotyledons of 7-day-old plants grown from seed treated with ^{14}C-metalaxyl (0.22 μCi per seed) by TLC revealed that only 40% of the radioactivity was in metalaxyl (R_f 0.25) and 60% consisted of more-polar ^{14}C-labeled derivatives that remained at the origin of the chromatogram. When seeds were treated and plants were assayed 7 and 15 days later, the total radioactivity (metalaxyl plus derivatives) recovered from 7-day-old plants was 52.81% and from 15-day-old plants was 32.24% of that applied to the seed. Most of the recovered ^{14}C was detected in the cotyledons of 7-day-old (91.45%) and 15-day-old (84.91%) plants. The aerial portions of the 7-day-old seedlings contained 5.98% and the roots 1.36% of the total radioactivity recovered from the plants. These values were slightly higher in the 15-day-old plants (Table 2). Distribution of ^{14}C-metalaxyl and derivatives is shown by autoradiography in Fig. 1A.

The data for distribution after seed treatment as measured by extraction of metalaxyl and assay by GLC (Table 1) were in general similar to the data obtained with ^{14}C-metalaxyl (Table 2) except that the metalaxyl was detected in roots only in much smaller quantities by GLC (Table 1). These data indicated that the amount of metalaxyl translocated was sufficient to control stem rot caused by artificial inoculation with *P.m.* f. sp. *glycinea*.

Translocation after foliar treatment with ^{14}C-metalaxyl. When ^{14}C-metalaxyl was applied to the upper surface of a leaf or a cotyledon of a 7-day-old plant and analyzed 7 days later, 99% of the radioactivity in the plant was recovered at the site of application in both leaves and cotyledons (Table 3). The distribution of radioactivity after application to a cotyledon was 0.01% in the opposite cotyledon, 0.37% in the aerial portion, 0.015% in the hypocotyl, and 0.04% in the roots.

After application to a leaf, only 0.23% of the activity was distributed to the opposite leaf, 0.37% to the aerial portion (minus the treated leaf), 0.09% to the hypocotyl (including cotyledons) and 0.01% to the roots (Table 3). Visualization by autoradiography was correlated with these data (Fig. 1B,C). Since only 40% of the radioactivity was authentic metalaxyl, the autoradiographs should be interpreted accordingly. Zaki et al (20) assumed that the radioactivity detected included authentic metalaxyl, but recognized the probability that only 30–60% of the total radioactivity might be authentic metalaxyl (CIBA-GEIGY Corp., *unpublished*).

Application to foliage may have limitations for control of a disease on untreated foliage. Cohen et al (5) reported rapid movement of metalaxyl from roots to leaves but not from one leaf to another.

Zaki et al (20) reported that more than 90% of the foliar-applied ^{14}C-metalaxyl (analyzed by combustion) remained in or on leaves of avocado up to 28 days after application. Only a small amount

TABLE 4. Distribution of ^{14}C-metalaxyl and derivatives in 14-day-old soybean plants 7 days after treatment with metalaxyl[a] by soil drench

Plant part extracted[b]	^{14}C in plant part (dpm × 10^{-4})	Percentage of ^{14}C applied	Distribution (%)
Cotyledons	2.19	1.97	80.5
Aerial portion (minus cotyledons)	0.41	0.37	15.1
Roots[c]	0.12	0.11	4.4
Total recovered	2.72	2.44	~100.0

[a] Metalaxyl 2.02 mg (0.5 μCi = 111 × 10^4 dpm) per 7-day-old plant.
[b] Acetone 10 ml/g fresh weight of plant tissue.
[c] Roots consisted of plant tissue below the soil surface.

(<1%) of the radioactivity applied to leaves was recovered in the roots. We are unable to account for >50% of the radioactivity applied to the foliage; it was not found in the soybean system 7 days later. However, it can be assumed that much of this could have been lost by vaporization based on other studies (CIBA-GEIGY Corp., *unpublished*). Caverly and Unwin (3) reported over 80% recovery of metalaxyl from various plant tissue and other media using acetone extraction, but these were to test analytical methods of extraction immediately following spiking of the samples.

Translocation of ^{14}C-metalaxyl in plants after soil treatment. When soil was drenched with ^{14}C-metalaxyl, only 2.44% of the radioactivity applied to soil was recovered in plants (Table 4) (Fig. 1D). The distribution of the total recovered radioactivity was greatest in the cotyledons (80.5%), much less in other aerial parts (15.1%), and least in the roots (4.4%) (Table 4).

These data substantiate other reports, in which bioassay methods were used, that movement of metalaxyl was largely acropetal and apoplastic (12,16).

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