Diazinon Absorption, Translocation, and Metabolism in Bean Plants

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Diazinon (^{14}C-ring-labeled) was absorbed and initially accumulated in bean plant roots in higher quantities than in other regions of the plant. It also diffused out of roots when they were rinsed and placed in nutrient solution indicating movement along concentration gradients. Translocation to foliage occurred with small amounts of diazinon present in the primary leaves by two days but not thereafter. Although radioactivity steadily accumulated in the foliage, this was mainly the hydrolysis product (2-isopropyl-4-methylpyrimidin-6-ol) and unextractable metabolites, possibly conjugation products. Thus, the leaves appeared to be the main site for hydrolysis while diazinon persisted in the roots and nutrient solution for several days. Excised primary leaves also hydrolyzed diazinon. Metabolism of the pyrimidinol ring to [14]CO_2 was very minor, and no ^{14}C-diazoxon was detected at any interval.

D iazinon, O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate, is extensively applied to soil and crops to control phytophagous insects. Gasser (1953) first reported on its biological properties and effectiveness as a contact or vapor insecticide, although no systemic activity was demonstrated. Recent studies have shown that diazinon is absorbed and translocated in plants from leaf applications into the roots and root exudates (Gunner et al., 1966), from treated soil into the leaves in insecticidal quantities (Onsager and Rusk, 1967), and from planting water into seedlings (Miles et al., 1967). Metabolism of diazinon in plants has been shown to involve hydrolysis of the phosphorus pyrimidyl ester bond and subsequent metabolism of the 2-isopropyl-4-methylpyrimidin-6-ol to carbon dioxide. Small amounts of diazoxon, O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphate, also were detected in field-grown crops (Ralls et al., 1966).

In this study, ring-labeled ^{14}C-diazinon was used to investigate root absorption, translocation to the foliage, persistence, and metabolism of the insecticide in bean plants grown under controlled environmental conditions. Radioisotope-labeled compounds in various regions of the plants, in the nutrient solution, and [14]CO_2 in the atmosphere were determined at intervals to establish the significance of translocation and mechanisms of loss of diazinon in plants.

MATERIALS AND METHODS

Garden beans (Phaseolus vulgaris, Bountiful) were grown in nutrient solution in a laboratory growth chamber (E-57, Percival Refrigeration Co., Boone, Iowa) by the method of El-Refai and Hopkins (1966). The environmental conditions were controlled at 16 hours of light and 8 hours of dark with a light intensity of about 1100 foot candles. The corresponding temperatures during the light and dark cycles were 26° and 17° C. with a relative humidity of 40 ± 5%. The plants were treated when about 3 weeks old.

Excised Leaf Studies. The primary leaves of bean plants were excised by cutting the basal end of the petioles under water and inserting them into small tubes containing 1 ml. of an aqueous solution containing 80 p.p.m. of ^{14}C-diazinon and 0.1 ml. of acetone. After the solutions had been almost completely absorbed (3 to 5 hours), each stem was washed with water and transferred to a 20-ml. vial containing nutrient solution. A control tube of the solution showed that no diazinon degradation in water had occurred during the 5 hours. The treated leaves were then placed in the growth chamber under previously mentioned conditions, and three treated leaves were harvested for analysis immediately and at intervals through 15 days. In other experiments, treated leaves were placed in metabolism chambers to collect [14]CO_2. The nutrient solution also was analyzed for radioactive compounds in certain experiments. The leaves were macerated in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) with 50 ml. of benzene. The extracts were dried with sodium sulfate (anhydrous) and evaporated under nitrogen to a final volume of 0.5 ml. for chromatography.

Root Absorption Studies. The roots of intact bean plants were placed separately into 25-ml. vials, each containing 4 ml. of ^{14}C-diazinon aqueous solution. Plants roots were exposed to either 8 or 20 p.p.m. of diazinon dissolved in distilled water for the [14]CO_2 studies, and after 10 hours the closed metabolism chambers containing the treated plants were flushed with air for 2 hours to collect the accumulated CO_2, using the collection apparatus described later. The chambers were then opened, the plant roots rinsed thoroughly with water, and the rinses added to the ^{14}C-diazinon aqueous solution left in the vials. The pooled solution was made to known volume and assayed to determine the amount of diazinon absorbed by the roots. The roots were then placed in small jars containing nutrient solution, and these were again placed inside the metabolism chambers to collect [14]CO_2, as described later. Plants for translocation studies were treated with 20 p.p.m. in the same way, except they were not held in metabolism chambers. At intervals through eight days, plants were taken for analysis, the roots rinsed thoroughly with water, and the rinses combined with the nutrient solution remaining in the jar. The plants were then divided into roots, stem, primary leaves, and growing tip, and each part was homogenized with distilled water. Aliquots of the homogenates were combusted to determine total radioactivity and the remainder extracted with chloroform to recover soluble compounds. Total radioactivity in the chloroform

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was determined, and the extracts were then concentrated in a rotating vacuum evaporator for chromatographic analysis.

$^{14}CO_2$ **Production from Diazinon-2-^{14}C in Bean Plants.** Ten treated excised leaves in vials containing nutrient solution were placed in glass metabolism chambers inside a growth chamber and were exposed to 12-hour light and dark periods. Two intact bean plants per chamber were treated by root absorption of ^{14}C-diazinon in aqueous solution. The metabolism chamber was a rectangular chromatographic jar with a plate glass cover sealed with silicone grease. Inlet and outlet tubes, passed through rubber stoppers, were placed in two separate holes in the glass cover. Air was drawn into the chamber through the short inlet tube and out by a long outlet tube that extended nearly to the bottom of the jar. Water vapor was removed by passing the air through a column of anhydrous calcium sulfate, and CO_2 was trapped by bubbling the air through 5 ml. of the CO_2 absorption solution, described in a later section. A gas-washing bottle containing a saturated solution of barium hydroxide served as a backup trap to indicate any escape of CO_2 from the first trap. Air drawn through the system was regulated by a vacuum pressure pump joined to the outlet of the gas-washing bottle. The metabolism chamber containing the treated bean plants was flushed with air for 2 hours every 12 hours to collect CO_2 and to aerate the jar. A 2-ml. aliquot was taken from the carbon dioxide absorption solution and mixed with 10 ml. of toluene scintillation mixture and the radioactivity determined.

Radioisotope Techniques. Ring-labeled diazinon, O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl-2-^{14}C) phosphorothioate (Geigy Chemical Corp., Ardsley, N.Y.), had a specific activity of 4 μc. per mg. and was radiochemically pure as determined by thin-layer and paper chromatography. Aqueous solutions were prepared for root and petiole absorption as previously described.

Radioactive samples (including plant tissue homogenates and aqueous solutions) were combusted (Buyske *et al.*, 1962), and the resulting $^{14}CO_2$ was trapped in 5 ml. of an ethanolamine–ethylene glycol monomethyl ether mixture (1 to 2, v./v.) (Jeffay and Alvarez, 1961). A 2-ml. aliquot was mixed in 10 ml. of a scintillation mixture containing 0.4% PPO and 0.01% POPOP in a 2 to 1 mixture of toluene–ethylene glycol monomethyl ether. The samples were counted in a liquid scintillation spectrometer (Packard Instrument Corp., LaGrange, Ill.) to obtain a standard error of 5% or less.

Thin-Layer and Paper Chromatography. Separations of diazinon, diazoxon, and the pyrimidinol hydrolysis product were made on cellulose and silica gel thin layers and by paper chromatography (Table I). Apparatus, reagents, and washing of cellulose layers or paper was according to El-Refai and Hopkins (1965). Silica gel TLC was made with Eastman Type K301R plastic chromatographic sheets and apparatus (Distillation Products Industries, Rochester, N.Y.). Paper chromatography was done according to the techniques of Mitchell (1960). Several chromogenic sprays were used to detect and establish R_f values of diazinon and possible degradation products or metabolites.

Silver Nitrate–Bromophenol Blue (Getz, 1962). The

Table I. Thin-Layer and Paper Chromatographic Systems for Separating Diazinon, Diazoxon, and the Pyrimidinol Hydrolysis Product

		R_f Values		
No.	Chromatographic System	Di-azinon	Di-azoxon	2-Iso-propyl-4-methyl-pyrimidin-6-ol
1	25% Dimethyl formamide (stationary)[a] Isooctane (mobile)[a]	0.96	0.55	0.11
2	1.5% Vaseline in chloroform (stationary)[a] Water-ethanol (64.5 to 35.5) (mobile)[a]	0.32	0.93	0.92
3	Chloroform-benzene-ethyl acetate (40:40:20)[b]	0.95	0.70	0.28
4	Chloroform-benzene-ethyl acetate (48:48:4)[b]	0.93	0.48	0.33
5	30% Dimethyl formamide (stationary)[c] Isooctane (mobile)[c]	0.90	0.43	0.07
6	1.5% Vaseline in chloroform (stationary)[c] Water-ethanol (64.5 to 35.5) (mobile)[c]	0.40	0.94	0.90

[a] Cellulose layers on glass plates.
[b] Silica gel plastic plates (Eastman chromatogram sheets for thin-layer chromatography. Type K301R-silica gel. Distillation Products Industries, Rochester, N.Y. 14603, Division of Eastman Kodak Co.).
[c] Paper chromatography.

chromatograms were dried at 50° C., sprayed with reagent, and then reheated for 10 minutes.

Ammoniacal Silver Nitrate. The different sprays were prepared for TLC (El-Refai and Hopkins, 1965) and paper chromatography (Mitchell, 1960). To increase sensitivity of detection on cellulose layers, the chromatoplate was exposed to short-wave UV for 30 minutes and brominated for 30 seconds before the ammoniacal silver nitrate spray was applied. The plate was exposed again to short-wave UV for a few minutes, which gave white or yellow spots on a brown background. The spots turned purple when the plate was dipped into dilute hydrochloric acid. The color was permanent for diazoxon, while diazinon turned white again after drying. That modification resulted in detecting 1 μg. of either diazinon or diazoxon.

Bromine–Fluorescein–Silver Nitrate. The chromatograms were exposed to bromine vapor for 30 seconds, then sprayed with fluorescein solution (Walker and Beroza, 1963), followed by the silver nitrate prepared with 0.7 gram of silver nitrate in 5 ml. of distilled water and 20 ml. of 2-phenoxyethanol, and then diluted to 200 ml. with acetone. The thin-layer plates or paper chromatograms of extracts containing radiolabeled compounds were assayed with a windowless chromatogram scanner (Vanguard Instrument Co., LaGrange, Ill.). R_f values of the radioactive areas were compared with those of standards on the same chromatogram to establish their identity. Autoradiograms were also prepared by exposing x-ray film (Kodak Industrial Film, Type M) to the thin-layer plates for two weeks in a light-proof box.

RESULTS

Excised Leaf Experiments. Preliminary studies on diazinon metabolism were conducted with groups of excised primary leaves that had adsorbed variable amounts of an aqueous solution of ^{14}C-diazinon. After absorption, the leaf petioles were rinsed to remove any diazinon on the surface, transferred to nutrient solution, and then held in a growth chamber under the conditions previously mentioned. Analyses of control samples of the aqueous solution showed that no hydrolysis of diazinon occurred in the water during the absorption period. However, small amounts of the pyrimidinol hydrolysis product, 2-isopropyl-4-methylpyrimidin-6-ol-2-C^{14}, were detected in leaves analyzed immediately after absorption, demonstrating the beginning of metabolism. Metabolism increased to about 2% of the total radioactivity by 8 hours and to 12 to 14% in the next five days. The percentage of hydrolysis varied quite widely with extracts of some leaves containing up to 50% of the pyrimidinol compound. No other radioactive metabolites were detected in the extracts, and autoradiographs of thin-layer separations confirmed the absence of other trace metabolites. All radioactivity had disappeared from leaves held 22 days, indicating that the label had been lost as ^{14}CO$_2$, as indicated by Ralls et al. (1966), or that the radiolabeled compounds had been completely translocated into the nutrient solution. To determine these points, the leaves were held in metabolism chambers seven days to collect ^{14}CO$_2$, and then the nutrient solution and leaves were analyzed for labeled compounds. Extracts of both the leaves and the nutrient solution showed 13 to 15% of the radioactivity was the pyrimidinol compound while the remainder was diazinon, which closely compared with the first experiment. Metabolism apparently reached a plateau, and the insecticide and its hydrolysis product became evenly distributed between the leaves and nutrient solution by translocation. Some of the hydrolysis was probably due to enzyme activity in the leaves, since diazinon is very slowly hydrolyzed in water or dilute acids (Gasser, 1953). Metabolism of the pyrimidinol ring was very minor; only about 25-ng. equivalents of diazinon were released as ^{14}CO$_2$ or about 0.006% of the original diazinon initially absorbed by the leaves. The maximum production of ^{14}CO$_2$ occurred 24 hours after treatment. Radioactivity disappearing from the leaves in the first experiment, then, was due to translocation into the nutrient solution, with only trace amounts metabolized as ^{14}CO$_2$.

Root Absorption and Translocation in Bean Plants. Uptake of the ^{14}C-diazinon aqueous solution by roots of intact bean plants during the 5-hour treatment varied, ranging from about 1.5 to 5 ml. per plant. During that interval, most of the radioactivity was concentrated in the roots (80%), while the remainder was translocated to the stem and primary and terminal leaves in decreasing amounts (Figure 1).

When the roots were rinsed and transferred to nutrient solution, about 68% of the total radioactivity in the plants, mainly from the roots, diffused into the nutrient solution the next two days, which indicated that the diazinon passed freely into and from the roots with the movement of water. Radioactivity up to 13.5% reaccumulated in the roots by eight days. During the post-treatment interval, translocation of radiolabeled compounds continued into the foliage
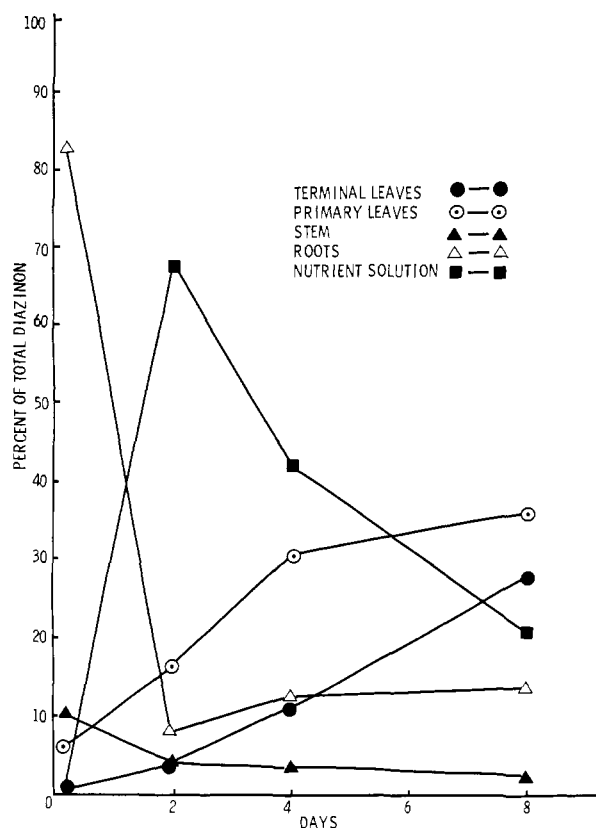

Figure 1. Translocation and distribution of radiolabeled compounds in bean plants and nutrient solution after root absorption of ^{14}C-diazinon in aqueous solution

with primary leaves accumulating 36% and terminal leaves 28% of the total radioactivity by eight days. The stem contained low levels, which diminished throughout the eight days.

Metabolism in Bean Plants. Degradation of diazinon was first investigated by extracting plant homogenates and nutrient solution with chloroform to recover unmetabolized diazinon and soluble metabolites (Table II). During the 5-hour absorption period, about 20% of the label was unrecoverable by solvent extraction, indicating early degradation to water-soluble or bound metabolites. About 60% of the recovered radioactivity was located in the roots and only minor quantities in the primary leaves and terminal foliage. When the roots were rinsed and transferred to nutrient solution, most of the radioactivity was released into the aqueous medium within two days. The foliage slowly increased in organosoluble compounds, but these amounts were low and did not exceed 4.5% in the primary leaves and 2.4% in the terminal leaves. Chloroform-soluble labeled compounds decreased from 77 to 16% in eight days.

Thin-layer chromatography of the extracts showed that the pyrimidinol hydrolysis product as well as diazinon was being recovered from both plant homogenates and the nutrient solution (Table III). Diazinon is quantitatively recovered by this procedure, but the pyrimidinol, because of its water solubility and polar characteristics, would not be expected to be thus recovered. The foliage appeared to be the most active site of diazinon metabolism in the plant.

Table II. Radiolabeled Compounds Extractable with Chloroform from ^{14}C-Diazinon-Treated Bean Plants

		Percentage of Total Diazinon Absorbed				
Interval	Nutrient Solution	Roots	Stem	Primary leaves	Terminal leaves	Total
5 hours	a	61.4	12.9	2.2	0.6	77.2
2 days	38.5	5.1	2.9	3.6	1.3	51.4
4 days	12.2	2.6	1.1	4.5	1.8	21.9
8 days	8.4	3.4	0.4	1.5	2.4	16.0

a Roots of the bean plants were placed in nutrient solution after absorption of diazinon aqueous solution.

Table IV. Distribution of Radiolabeled Compounds in Bean Plants, Nutrient Solution, and Carbon Dioxide 6 Days after Root Absorption of ^{14}C-Diazinon

Experiment No.	Total Diazinon Absorbed per Plant, μg.	Percentage of Total Diazinon Absorbed		
		Nutrient solution	Plant tissue	^{14}CO$_2$
1	29.7	21.7	78.2	0.1
2	52.1	39.2	60.7	0.1

Table III. Relative Percentage of Diazinon and the 2-Isopropyl-4-methypyrimidin-6-ol Hydrolysis Product in Chloroform Extracts of Bean Plants

	Nutrient Solution		Roots		Stem		Primary Leaves		Terminal Leaves	
Interval	Diaz.	Pyrim.	Diaz.	Pyrim.	Diaz.	Pyrim.	Diaz.	Pyrim.	Diaz.	Pyrim.
5 hours	...	...	99+	Trace	99+	Trace	a	a	a	a
2 days	86	14	99+	Trace	99+	Trace	20	80	0	100
4 days	66	34	84	16	a	a	Trace	99+	0	100
8 days	80	20	Trace	99+	a	a	Trace	99+	0	100

a Radioactivity insufficient for analysis.

Two days after treatment, roots still contained primarily diazinon while primary leaves showed 80% of the radioactivity to be the pyrimidinol, and no diazinon was detected in the terminal leaves. No diazinon was detected in foliage at four- and eight-day intervals or in roots at eight days. But a high percentage of diazinon remained in the nutrient solution through the experiment, which shows that diazinon is readily absorbed, but that only small amounts are translocated and persist in the foliage. Hydrolysis appears to proceed more rapidly in the foliage than in the roots. No radiolabeled diazoxon was detected in any extract.

To determine the amount of ^{14}CO$_2$ produced from the pyrimidinol ring-labeled diazinon, the plants were treated by root absorption and held in metabolism chambers six days. The distribution of total radioactivity in the plant tissue, nutrient solution, and as evolved ^{14}CO$_2$ is shown in Table IV. From 60 to nearly 80% of the radioactivity remained in the plant in two experiments in which the plants were treated with different quantities of diazinon. That compared well with data from plants used in the translocation studies. Only small amounts (about 0.1% of the total diazinon) were degraded and released as ^{14}CO$_2$. The nutrient solution correspondingly contained 20 to 40% of the radioactivity as root exudates.

The output of ^{14}CO$_2$ from treated excised leaves or intact plants reached a peak within 24 hours except with the whole plants, which had received a higher dosage of diazinon, in Experiment 2 (Figure 2); their peak occurred at three days. Metabolism thereafter dropped sharply and leveled off at a low rate. The production of ^{14}CO$_2$ during the 12-hour day period was found to be less than that produced during the 12 hours of darkness, so production probably correlates with photosynthetic cycles. During the daytime, part of the ^{14}CO$_2$ evolved was probably utilized in photosynthesis of plant carbohydrates.
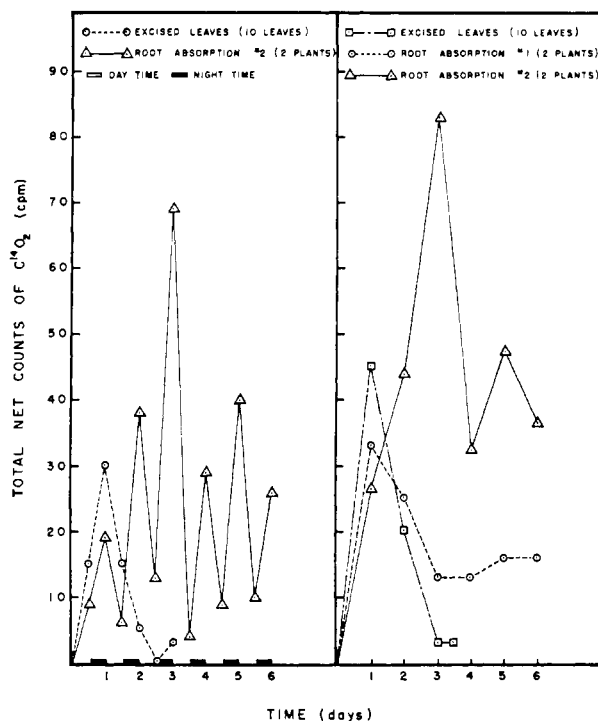

Figure 2. Rate of ^{14}CO$_2$ production from ^{14}C-diazinon-treated bean plants and excised leaves

Left, day and night ^{14}CO$_2$ production
Right, 24-hour interval ^{14}CO$_2$ production

DISCUSSION

Aqueous solutions of diazinon were readily absorbed by bean plant roots, and the insecticide accumulated there in much higher concentration than in the foliage. Root absorption and translocation of parathion, another insecticide

with little systemic activity, occurs in a very similar manner (El-Refai and Hopkins, 1966). Diazinon also rapidly diffused from the roots when they were placed in nutrient solution containing none of the insecticide, indicating movement into and from the roots to be along concentration gradients. Exudation of diazinon from bean plant roots after leaf applications also has been reported (Gunner et al., 1966).

Unmetabolized insecticide translocated to the foliage was found only in the primary leaves two days after treatment At longer intervals, the terminal leaves and foliage contained only the pyrimidinol hydrolysis product and metabolites that could not be extracted with chloroform. Translocation of systemic amounts of diazinon from treated soil into the leaves of sugar beet seedlings has been reported (Onsager and Rusk, 1967), indicating the insecticide may accumulate and persist in the foliage under continuous exposure of roots to diazinon. Treating roots of cabbage and tobacco seedlings with planting water preparations of diazinon also resulted in translocation and persistence in the foliage (Miles et al., 1967). Pea plants grown in diazinon-treated sand accumulated the insecticide in the foliage especially at a higher rate of treatment and a shorter harvest time (Lichtenstein et al., 1967). Diazinon persisting in soil for several weeks (Getzin, 1967) would account for translocation and possible accumulation of systemic residues over an extended time. However, the general lack of systemic properties of diazinon, as indicated in this study, is due to only minor quantities being translocated to the aerial parts of the plant and to diazinon being readily hydrolyzed in the foliage.

Metabolism of diazinon was predominantly due to hydrolysis of the phosphorus pyrimidinyl ester bond. That reaction seems to occur most rapidly in the foliage since the 2-^{14}C-2-isopropyl-4-methylpyrimidin-6-ol hydrolysis product was detected there first and continued to accumulate there in greater quantities. This was confirmed by metabolism with excised leaves in which absorbed diazinon was hydrolyzed in considerable quantities by the leaves. Diazinon persisted much longer in the roots and nutrient solution than in the foliage.

No ^{14}C-labeled diazoxon was detected in the plant extracts or nutrient solution, indicating that oxidation is very minor or that the oxon is hydrolyzed as rapidly as it is formed.

Metabolism of the pyrimidine ring is also exceedingly minor with only about 0.1% of the absorbed diazinon label being evolved as [14]CO_2 during several days. The peak of [14]CO_2 production was greater during dark than light periods; corresponding photosynthetic cycles and peak production occurred at 24 to 72 hours after treatment depending on the rate of treatment. Metabolism of the ring probably occurs following hydrolysis of diazinon.

Metabolites not extractable with chloroform steadily accumulated in the plants throughout the post-treatment period, suggesting conjugation of the pyrimidinol hydrolysis product.

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