Absorption and Metabolism of 3-Hydroxy-5-methylisoxazole in Plants and the Biological Activites of its Metabolites

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ABSTRACT

The absorption and metabolism of the soil fungicide, 3-hydroxy-5-methylisoxazole (Tachigaren, Code No. F-319), by cucumber, tomato, and rice plants was investigated. Absorption of (3-^{14}C)-F-319 into roots of these seedlings from nutrient solutions was rapid; translocation into leaves occurred 24 h after treatment, movement was readily traced from treated to untreated rootlets, which also exuded small amounts of the chemical.

F-319 was converted into two major metabolites [MU: 3-(β-D-glucopyranosyloxy)-5-methylisoxazole; and ML: 2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one] in roots and shoots of the treated plants. MU and ML were isolated and purified as crystals by column chromatography, and their structure was determined by spectroscopic analyses of mass, nuclear magnetic resonance, infrared and ultraviolet spectra, optical rotatory dispersion curves, and acid hydrolysis. Synthesized samples were identical with the products of F-319 metabolism in plants.

No phytotoxic effect of ML on cucumber and rice seedling growth was observed. MU retarded the growth of germinated rice seed when applied at the high concn of 1,500 μg/ml, as did F-319 at the same concn. However, MU exerted less of a suppressive effect on water uptake by rice seedlings than did F-319. Both F-319 and MU displayed toxic activity against *Pythium debaryanum,* but ML was inactive in this respect.

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Additional key words: hymexazol, distribution, glycosidation.

The soil-fungicide, 3-hydroxy-5-methylisoxazole (Tachigaren, Code No. F-319) has been used for the control of soil-borne diseases, such as damping-off and Fusarium wilt of rice seedlings (*Pythium* spp., *Rhizoctonia* spp., and *Fusarium* spp.), cucumber (*Pythium aphanidermatum* Edson and *Fusarium oxysporum* Schlecht. f. *cucumerinum* Oven), tomato [*Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans.] and beets (*Pythium debaryanum* Hesse), and has shown promise for use on other crops.

F-319 was originally selected as one of several highly effective chemicals in a greenhouse evaluation for control of fusarial disease in cucumber plants. Diseases incited by *Fusarium* spp. are among the most troublesome in plant production. Chemicals having isoxazole-ring appear to have considerable potential against *Fusarium* spp., and our studies led to the finding of 3-hydroxy-5-methylisoxazole (19). Selective toxicity of the chemical for certain species of soil-borne fungi may be due not only to its antimicrobial activity, but also to its metabolic alteration in plants and by microbial life in soils.

The study reported here was undertaken to determine the absorption and translocation pattern and the metabolic fate of F-319 in plants. We also sought to obtain fundamental information for residue analysis and to determine the biological activities of the metabolites. Portions of these studies were reported previously (7, 8).

MATERIALS AND METHODS.—Fungicide, F-319, labeled with ^{14}C in position-3 of the isoxazole ring with a specific activity of 1.381 mCi/mM was synthesized by H. Fujita and R. Endo of the Central Research Laboratories of our company (Sankyo Co., Ltd., Shinagawa-ku, Tokyo, Japan). Radiochemical purity of ^{14}C-F-319 was determined by thin-layer chromatography after purification by silica gel column chromatography and repeated recrystallization in *n*-hexane. In some experiments, the radioactive chemical was further diluted with an unlabeled one. Aqueous solutions of the chemical were prepared for root and foliar treatment. Soil treatment was carried out with a 500 μg/ml (1.9 mM) aqueous solution at a rate of 3 liter/m^2 applied as a drench after sowing.

Plant culture.—Seeds of cucumber (*Cucumis sativus* L. 'Midorifushinari'), tomato (*Lycopersicum esculentum* Mill.'Shinfukuju') and two varieties of rice (*Oryza sativa* 'Kinmaze' and 'Shigaasahi No. 27') were germinated in either sandy soil or vermiculite. After the seedlings attained a ht of approximately 10 cm, they were transferred to a hydroponic solution unless otherwise stated. The nutrient solution made of Daika No. 2 Hydroponics (Dainihon Ink Co., Ltd., Tokyo, Japan) at a dilution of 1:1,000 supported good growth. One week was allowed for their adjustment to the new growing conditions. The plants were used at about the three-leaf stage of growth. The plants were maintained in a greenhouse programmed for 28 C, 16-h days and 8-h, 20 C nights, and 60% relative humidity (RH). The plants were set in the center of the benches in order to avoid the higher wind velocities at edges, which could increase transpiration and consequently uptake of the chemical (17).

Absorption and translocation.—Seedlings of cucumber, tomato and rice plants having two leaves were placed with their roots in 100 ml of nutrient solution with concn of 0.73 μCi of ^{14}C-F-319. The individual plants were sampled at 0.5, 1, 3, 6, 24, and 48 h after the initiation

of treatment. After thoroughly washing the roots of all the plants in running tap water to remove radioactivity not absorbed, three of the whole plants, in duplicate, were surface-dried, weighed, measured, and autoradiographed. Another set of three plants per treatment was divided into shoots and roots, and freeze-dried. These organs were homogenized in 90% ethanol in a blender, then filtered through a glass filter. The filtrate was evaporated in vacuo almost to dryness at 50 C. The residue was dissolved in 90% ethanol and made up to volume for radioactivity measurements. Sediments on the filter were dried and combusted to CO_2 in a Schoeniger flask and the resulting ^{14}C-CO_2 evolved was absorbed in 5 ml of hyamine solution and the radioactivity determined. The amount of radioactivity absorbed by plants was expressed as the sum of radioactivities in the liquid and solid phases.

Radioactivity in the ethanol extracts was measured using a Model 3002 Packard Tri-Carb liquid scintillation spectrometer with a counting efficiency of 85%. The scintillation dioxane:toluene solution 4:1, v/v) contained 8 g of diphenyloxazol (PPO) and 0.2 g of 1,4-bis-2-(4-methyl-5-phenyloxazole)-benzene (dimethyl-POPOP)/liter. Counting efficiency for each sample was determined by the adding of a ^{14}C-labeled benzoic acid standard.

To follow the movement of the label after foliar application of ^{14}C-F-319, 50 μl of an aqueous solution of ^{14}C-F-319 (0.1 μCi) in 0.03% Tween 20 (polyoxyethylene sorbitan monolaurate) was applied to half of the upper surface of fully expanded primary leaves of cucumber and rice plants grown in individual pots containing greenhouse potting soil. The solution dried in 30 min. Immediately prior to harvest, the treated area was covered with masking tape to prevent contamination of other plant parts. Intact plants in duplicate were harvested 1, 3, and 7 days after the treatment and autoradiographed.

Cucumber seedlings at two-leaf stage were removed from the nutrient solution and suspended in air, supported by wool thread and tape. The rootlets were separated individually and each of rootlet tips was immersed in vials containing 2 ml of nutrient solution. The remaining exposed parts of the roots were carefully covered with absorbent cotton which had been moistened with water. This experiment was performed in moist chamber. ^{14}C-F-319 (0.69 μCi) was applied to either 1, 3, or 5 rootlet tips in vials, respectively. The rootlet was removed from the solution 24 h after treatment. After the treated root was marked, the whole plant was carefully spread on a filter paper, surface-dried, and autoradiographed. The movement of ^{14}C from the treated rootlets to the untreated ones in the root system was measured by counting radioactivity in the solution in the vials in which the untreated rootlets had been immersed.

Autoradiography was performed according to the procedures of Nelson and Hamilton (14) and Stiasni et al. (18) using Sakura X-ray film (Industrial Type N., Konishiroku Photo Ind. Co., Ltd., Tokyo, Japan).

Metabolism in plants.—The roots and shoots of cucumber, tomato, and rice plants, treated with ^{14}C-F-319 (1.4 μCi) for 5-20 days in nutrient solutions or soil, were analyzed for F-319 and its metabolites. They were homogenized for 5 min in a blender in at least three times their volume of 90% ethanol, the homogenate was filtered through Whatman No. 2 filter paper, and the extract was evaporated to dryness in vacuo at 50 C. The residue was redissolved in methanol. Thin-layer chromatography (TLC) of the extract was performed by using 20 × 20-cm glass plates coated with silica gel G (Merck, 0.25 mm) previously washed with acetone and methanol. The chromatograms were developed ascendingly for 15 cm in a filterlined glass jar using the solvents indicated in the legend of Fig. 4. The air-dried plates were autoradiographed.

Parallel experiments were done under aseptic conditions to determine whether the metabolites of F-319 were enzymatically produced exclusively by plants and/or could also be produced by microbial transformation. As an additional control, cucumber and rice seedlings were treated with ^{14}C-F-319 (0.73 μCi) just prior to homogenization.

Cucumber and rice plants, which were germinated in aseptic conditions after seed-treatment with a 2% sodium hypochlorite solution for 15 min, were grown in the autoclaved nutrient solution for 3 wk, and fed ^{14}C-F-319 (0.73 μCi) aseptically via the roots. Isolation and identification of the metabolites in the sterile plant environment was performed by chromatography.

The distribution of radioactivity in roots and shoots of plants that had received 4.2 μCi of ^{14}C-F-319 via their roots in liquid culture was studied over a time course of 6-144 h after treatment. Root and shoot extracts were prepared as described above. Radioactivity was detected by TLC and measured in a liquid scintillation counter.

Isolation of metabolites and determination of their chemical structure.—Isolation and identification of the metabolites were conducted mainly using cucumber roots, which were fed ^{14}C-F-319 via the roots in a liquid culture, since they absorbed and metabolized the chemical most abundantly. Cucumber seedlings at the four-leaf stage of growth were transferred for 4 days to nutrient solutions which contained either ^{14}C-F-319 (1.4 μCi) or 100 μg/ml of the unlabeled chemical. Then the plants were harvested, the roots and shoots were excised and extraction of ^{14}C-materials was performed on the roots only, since it was found that about 70% of the radioactivity accumulated there. The roots were homogenized, and extracted with 90% ethanol at pH 6.0. The organic solvent portion of the extract was evaporated, leaving an aqueous phase which was washed three times with *n*-hexane chloroform and then dialized. The dialyzates were concd in vacuo, dissolved in ethanol, applied to Sephadex LH 20 columns, and eluted with ethanol. The eluate was evaporated to dryness in vacuo. The extracts of both the labeled and unlabeled F-319 treatments were combined, applied to a silica gel column, and eluted step-wise with chloroform-methanol solutions. The radioactivity of the effluent was monitored and three fractions were collected. This chromatographic separation was repeated three times for each of the three fractionated samples corresponding to F-319, MU, and ML.

In order to determine the chemical composition of the carbohydrate moiety of the ^{14}C-labeled metabolites, the metabolites from the three fractions were hydrolyzed in

0.1-6.0 N hydrochloric acid for 0.5-24 h, concd in vacuo, and diluted with methanol. This process was repeated several times, until there was no detectable acidity in the solution. The alcohol extract was then evaporated to a convenient volume and spotted on Whatman No. 1 paper along with various hexoses as standards. Chromatograms were developed descendingly in ethyl acetate:pyridine:water (10:4:3, v/v). The air-dried papers were sprayed with silver nitrate solution by the method of Trevelyan et al. (21).

Hydrolyzates of MU and ML, glucose, and galactose were trimethylsilylated by the method of Yamakawa and Ueta (22). The trimethylsilyl derivatives were subjected to gas-liquid chromatography under the conditions described below. To determine the bond position of the carbohydrate to F-319, the metabolites and several polysaccharides were also subjected to gas chromatographic analysis. The two metabolites, laminarine, trehalose, cellobiose, and gentibiose were methylated by the method of Hakomori (5) and then trimethylsilylated. Pure methly α-D-glucoside and a mixture of methyl D-glucosides were used as standards. The columns and conditions for gas-liquid chromatography were 3 mm, I.D., stainless, 180-cm-long column coated with 15% butane-1,4-diol succinate on 80- to 100-mesh Celite 545 and also 15% polyethylene glycol succinate on 80- to 100-mesh Chromsorb AW operated isothermally at 150 C (GC-1C GLC, Shimazu Co., Kyoto, Japan) with a hydrogen flame detector. In addition to the acidic digestion, an enzymatic hydrolysis with β-glucosidase (Sigma Chemical Co.), in pH 4.5 citric acid-phosphate buffer, was carried out at 37 C for 24 h to ascertain whether it would liberate glucose from the metabolites.

The authentic samples were synthesized as follows: 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (10), an excess amount of F-319 potassium carbonate, anhydrous calcium sulfate, and anhydrous acetonitrile were placed in a flask, and the contents were stirred during refluxing. The mixture of tetra-*O*-acetyl-α-D-glucopyranosides thus obtained was resolved into a main product and a minor one by means of column chromatography on silica gel using ethyl acetate-*n*-hexane (2:3 and 7:3, v/v) as eluent. After de-*O*-acetylation with sodium methoxide (20), the former gave MU and the latter ML, which were recrystallized from *n*-propanol.

The spectrum analysis of the metabolites was done as follows: optical rotatory dispersion curves were determined on a JASCO ORD UV-5 in methanol. Nuclear magnetic resonance spectra were determined on a Varian HA-100 in CD_3OD with tetramethylsilane as an internal standard. The mass spectra of the trimethylsilated metabolites were produced using the direct insertion probe of a JMS-OISG mass spectrometer. Infrared spectra of the metabolites were recorded on a Perkin-Elmer 221 grating infrared spectrophotometer in Nujol.

Biological activities of the metabolites.—Ten seeds of rice and cucumber plants were placed on filter paper in 9-cm diam petri dishes, to which 5 ml of a sample solution at a desired concn was added. These were incubated for 3 days at 28 C in the dark, and the lengths of subsequent shoot and root growth were measured. The test was conducted with both of the isolated metabolites, MU and ML, and the synthesized samples. The concn of the metabolites used in this and the following experiments was three times as high as that of F-319 in order to obtain comparable concns in all samples.

The effect of MU on physiological activities of the plants was estimated by comparing its influence on water uptake activity with plants treated with F-319. Two pairs of three cucumber seedlings were cultured in 25 ml of nutrient solution in graduated tubes, and the experiment was repeated. Due to the paucity of MU isolated from plant tissue, synthesized MU was used in this experiment. The MU and F-319 were added to the solution at the concns of 3, 30, 90, 300, 900, and 1,500 μg/ml and 1, 10, 30, 100, 300, and 500 μg/ml, respectively. The amount of water absorbed in each sample solution was recorded at 24-h intervals and all of the solution was renewed every day during 7-day incubation period.

F-319 has a relatively weak toxic activity in vitro against many kinds of soil-borne fungi, with the exception of *Pythium debaryanum* (19). Hence, it was postulated that F-319 might be transformed in plants to products more toxic against soil microorganisms. One-ml aqueous solutions of the plant-produced and synthesized samples of MU and ML at the desired concns were incorporated at 45 C in 9 ml of potato-dextrose agar and poured into petri dishes. Agar disks (5 mm diam) containing mycelium of *Pythium debaryanum,* were placed at the center of the dishes, and the diam of mycelial growth was measured after incubation for 3 days at 27 C. The experiments were triplicated.

RESULTS.—*Absorption and translocation.*—Uptake of ^{14}C-F-319 via the roots of the intact cucumber seedlings appeared to be very rapid as indicated by the autoradiograms of the whole cucumber plants (Fig. 1). Rice roots also rapidly absorbed the chemical, while tomato roots did so somewhat more slowly. Large amounts of radioactive materials had entered the cucumber roots within 1 h, translocation from the roots to stems occurred in 3 h, but translocation throughout the leaves was not detected until 24 h after treatment.

The examination of radioactivity in plants at varying periods following application of ^{14}C-F-319 to a full-grown primary leaf, showed that the applied chemical moved mainly in the direction of the transpiration stream. The autoradiogram on day 7 shows that some of the chemical moved into the upper leaf from the treated leaf and accumulated in the shoot-apex (Fig. 2). A lesser amount of ^{14}C was translocated basipetally. In tomato plants, the same translocation pattern was obtained, and in rice plants, the basipetal movement was slightly greater than that observed in cucumber plants.

Treatment of cucumber rootlet-tips with ^{14}C-F-319 resulted in movement to all the untreated rootlets, even by the single rootlet tip treatment (Fig. 3). The quantity of label in the solution in which the untreated rootlets had been incubated was 0.08, 0.09, and 0.28% of the amounts applied, when ^{14}C-F-319 was applied to 1, 3, and 5 rootlets, respectively.

Metabolism in plants.—Ethanolic extracts of the roots and shoots of cucumber, tomato, and rice plants treated with ^{14}C-F-319 via the roots or leaves in nutrient solution,

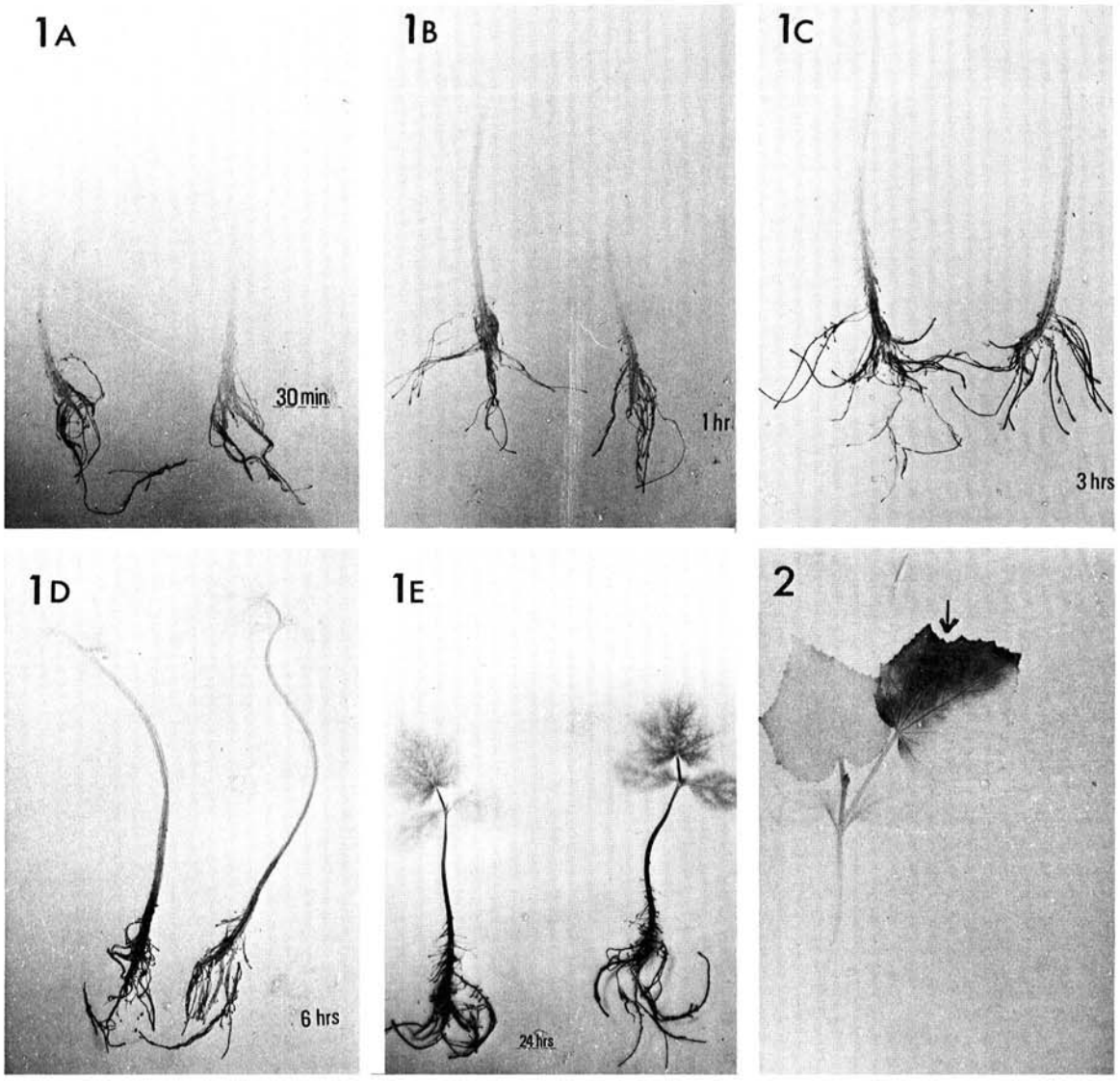

Fig. 1-(A to E), 2. Autoradiography of living cucumber seedlings. **1-(A to E)**: Autoradiographys of the time-rate of distribution of radioactivity in cucumber seedlings (two-leaf stage) treated with 0.73 μCi of ^{14}C-F-319 (3-hydroxy-5-methylisoxazole) via the roots in liquid culture. Time period after treatment: **A)** 0.5 h; **B)** 1.0 h; **C)** 3.0 h; **D)** 6.0 h; and **E)** 24 h. The tests were duplicated with three replicate plants for each treatment. **2)** Autoradiograph of translocation of radioactivity in a cucumber seedling treated with 0.1 μCi of ^{14}C-F-319 in 50 ml aqueous solution on half of the upper-surface of a primary leaf. Three replicate plants were used. Arrow marks point of application. The plant was autoradiographed 7 days after treatment.

or in soil, yielded the same metabolites, although the amounts varied.

More than 90% of the applied radioactivity was extractable. TLC analyses revealed that more than about 60% of the applied chemical was converted to the metabolites, and that the extracts consisted of the unaltered ^{14}C-F-319, two major metabolites (MU and ML), and at least two minor metabolites (Ma and Mb), as shown in Fig. 4. The minor metabolites were present only in trace amounts, and no further attempt was made to determine their structures.

The control plants treated with 1.01 μM ^{14}C-F-319 aqueous solution just prior to homogenization revealed no metabolites. The aseptically grown rice and cucumber plants grown produced the same metabolites of ^{14}C-F-319 as those grown in the nonsterile condition. Although ^{14}C-F-319 is metabolized by a large number of microorganisms in soils and nutrient solutions (Kamimura et al., *unpublished*), 90% ethanol extracts of fungal mycelia, culture filtrates, or soil did not yield metabolites which corresponded to those extracted from F-319-treated plants.

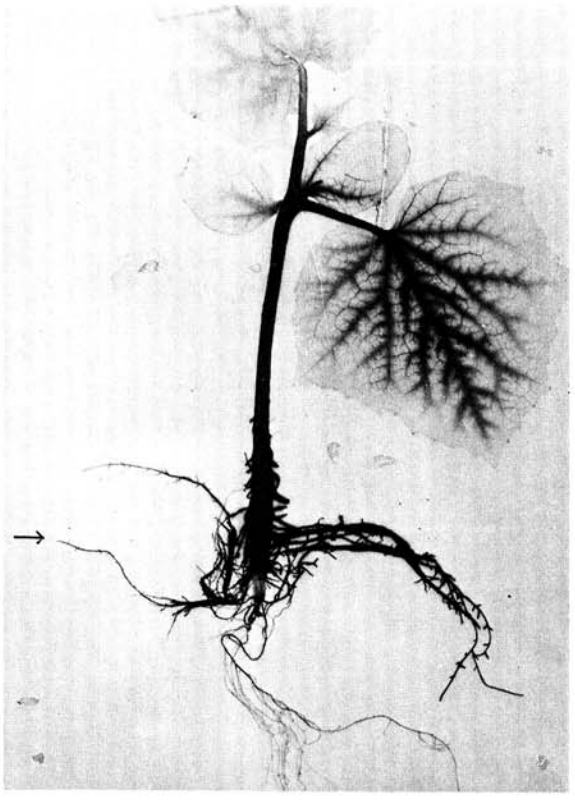

Fig. 3. Autoradiograph of movement of radioactivity in cucumber roots treated with 0.69 μCi of ^{14}C-F-319 (3-hydroxy-5-methylisoxazole) via a single rootlet tip. Three replicate plants at the two-leaf stage grown in nutrient solution were used. Each of the rootlet tips of a plant was incubated in 2 ml of nutrient solution in a vial. The chemical was applied to one vial containing a single rootlet tip for 24 h. Arrow marks the point of application.

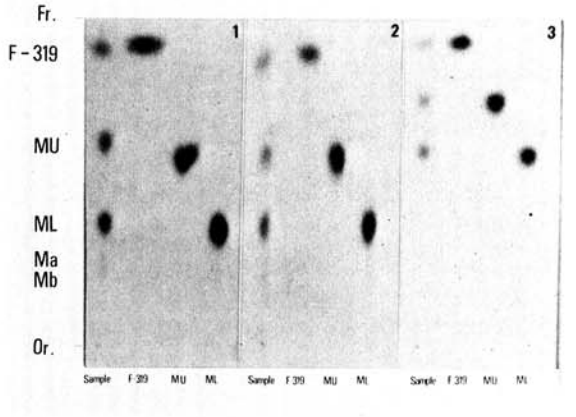

Fig. 4. Autoradiograph of thin-layer chromatogram of an ethanol extract of cucumber seedling roots exposed to ^{14}C-F-319 (3-hydroxy-5-methylisoxazole). Three chromatogram solvent systems were used: 1) *n*-butanol:acetic acid:water (8:2:3, v/v), 2) ethyl acetate:methylethyl ketone:formic acid:water (5:3:1:1, v/v), 3) ethyl acetate:pyridine:water (10:4:3, v/v). Adsorbent;silica gel G. Fr;front. Or;origin. Metabolites were named MU and ML [major metabolites; MU:3-(β-D-glucopyranosyloxy)-5-methylisoxazole and ML:2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one], Ma and Mb (minor metabolites) on the ordinate. On the abscissa, Sample; the extracts of roots treated with the ^{14}C-F-319 (1.4 μCi) via the roots for 4 days in liquid culture. MU and ML spotted on the abscissa were purified following extraction from previously treated plants.

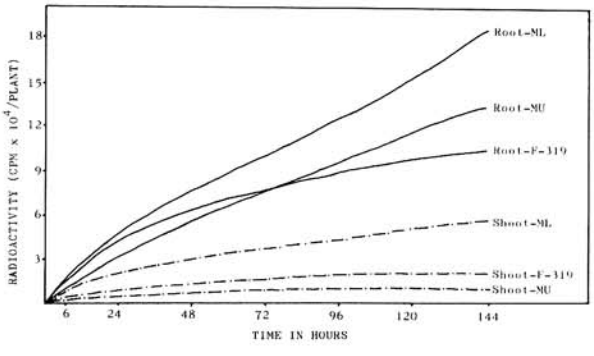

Fig. 5. Radioactivity of the metabolites, MU [3-(β-D-glucopyranosyloxy)-5-methylisoxazole] and ML [2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one] of F-319[3-hydroxy-5-methylisoxazole] detected in the roots and shoots of rice seedlings. Distribution of ^{14}C in ethanol extracts of the plants treated for 6 days via the roots with ^{14}C-F-319 (4.2 μCi). Values are the mean of three replicate plants and are given as radioactivity per plant.

The rate of formation of the metabolites by rice seedlings after continuous exposure of roots to ^{14}C-F-319 in nutrient solution is shown in Fig. 5. More radioactivity was detected in the roots than in the shoots of rice seedlings. Distribution of ^{14}C in the ethanol extracts showed that more ML was formed than MU in the roots and shoots, and that the level of both metabolites, MU and ML in the roots and ML in the shoots, was increased with time. MU concn in the shoots leveled off after 48 h.

Isolation of metabolites and determination of their chemical structure.—Plant-produced MU and ML were purified in *n*-propanol to crystal form and their melting points were 152-155 C for MU and 213-215 C for ML, respectively. Both metabolites were sufficiently pure to permit application of physical methods for identification. The metabolites were negative to Ehrlich's reagent and ninhydrin, but positive to 0.2% anthrone-sulfuric acid and α-naphthol reagent.

Both MU and ML were shown to have the molecular formula of $C_{10}H_{15}N$ by the elemental analyses and the mass spectra of the trimethylsilyl derivatives, which showed peaks m/e 549 (M^+) and m/e 98 (F-319-H). In addition, NMR spectra determinations for both compounds indicated that the signals due to a methyl proton at δ 2.33, an olefinic proton at δ 5.90, an anomeric proton at δ 5.1, and carbinyl protons at δ 3.3 - 3.7 which are attached to the carbons bearing hydroxy groups.

Furthermore, a color test of these metabolites using 0.2% anthrone-sulfuric acid and α-naphthol also presented evidence that they might be glycosides.

Hydrolysis of MU was effected with 0.2 N hydrochloric acid at 100 C for 0.5 h; whereas for ML a treatment with 6 N hydrochloric acid for 6 h was required. From the hydrolyzates of both metabolites, F-319 was detected by thin-layer chromatography, and glucose was identified by paper and gas chromatography of the trimethylsilyl derivative.

To determine the linkage of glucose with F-319, both metabolites, MU and ML, were permethylated and methanolyzed. Resulting methyl sugar was identified with methyl penta-*O*-methyl-β-glucopyranoside and its α-anomer by gas-chromatographic analysis. In addition to the above results, negative color reaction of these metabolites with aniline hydrogen phthalate provided the evidence that F-319 was bonded to position-1 of glucose in these molecules.

The sugar linkage in MU and ML was considered to be of the β-configuration, when their optical rotatory dispersion was compared with methyl α- and phenyl β-D-glucopyranoside. It was assumed that the naturally occurring glucose in plants was D-glucose. In addition, when the metabolites were treated with β-glucosidase, MU readily liberated glucose, and ML yielded only a little.

Thus, MU and ML are recognized to be isomeric. Since it was already known that N-alkyl-4-isoxazolin-3-one derivatives exhibited an absorption maximum at about 230 nm in the ultraviolet spectrum (13), the absorption at 230 nm of ML indicated that it corresponded to *N*-β-glucoside whereas MU corresponded to *O*-β-glucoside. Furthermore, the infrared spectrum (IR) of MU showed the characteristic band of 5-alkyl isoxazole ring at about 1,630 and 1,510 cm^{-1}, while the IR of ML had a strong band at 1,690 cm^{-1} due to the carbonyl group of 4-isoxazolin-3-one. Finally, the structure of the metabolites, MU and ML, was confirmed by comparison with the synthesized authentic samples. Chromatograms as well as spectrum analyses revealed MU to be 3-(β-D-glucopyranosyloxy)-5-methylisoxazole and ML to be 2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one (Fig. 6).

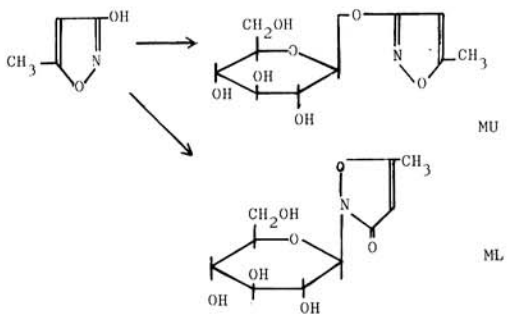

Fig. 6. Metabolic pathway of the soil fungicide 3-hydroxy-5-methylisoxazole (F-319) in plants. **MU**= 3-(β-D-glucopyranosyloxy)-5-methylisoxazole and **ML** = 2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one.

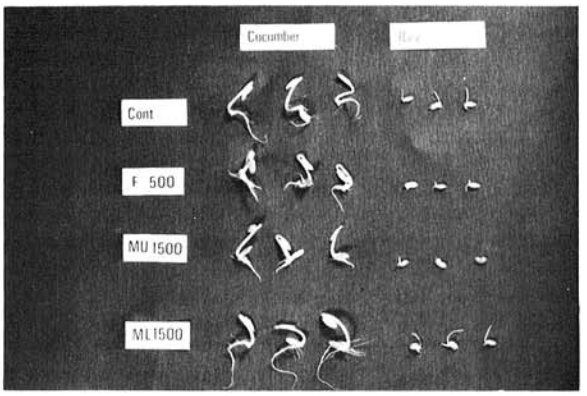

Fig. 7. Phytotoxic effect of 3-hydroxy-5-methylisoxazole (F-319) and its metabolites, 3-(β-D-glucopyranosyloxy)-5-methylisoxazole (MU) and 2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one (ML), on the germination and subsequent growth of cucumber and rice plants. Ten seeds were placed in petri-dishes, in which 5 ml of a sample solution at the indicated concn was added. They were incubated for 3 days at 28C. The test was triplicated and the representative seedlings were photographed. The test was also repeated using synthesized MU and ML, and the same results were obtained. Cont=control, F 500 = F-319 at 500 μg/ml, MU and ML each were at 1,500 μg/ml.

Biological activities of the metabolites.—Data on the metabolism of ^{14}C-F-319 in plants indicated that more than 60% of it was converted to MU and ML. F-319 does not have unfavorable effects on plants at concns at which it would normally be used. Nevertheless the phytotoxic properties of the metabolites were investigated as was their antimicrobial activity.

F-319 and MU inhibited the germination and subsequent growth of rice seedlings, especially in the early stages of growth and at the high concn such as 500 and 1,500 μg/ml. This was not as apparent in the case of cucumber seeldings. ML, on the other hand, did not affect their growth or germination (Fig. 7). At the low concns of 0.01 - 30 μg/ml, the metabolites had no detectable effects on seedling growth.

MU had an inhibitory effect on the seedling growth of rice plants nearly equal to F-319 (Fig. 7). Since MU and F-319 affected the test plants differentially, their influence on water uptake of cucumber and rice plants were investigated. Measurements were made every day for 7 days after treatment. The water uptake of rice plants was decreased to some extent by application of 100-1,000 μg F-319/ml, but not appreciably by MU over the concn range studied. Thus, this response was less intense in plants treated with MU which tended to recover their water absorption equilibrium after a time (Fig. 8). Cucumber plants were little affected. In general, it appeared that the inhibitory effect of MU on water uptake was less than the original chemical, F-319.

Mycelial growth of *P. debaryanum* was inhibited by F-319 at a concn of 1.0 μg/ml, and by MU at 3.0 μg/ml, but not by ML even at 300 μg/ml (Table 1).

The synthesized metabolites were equally as effective as

those isolated from plants vis-a-vis phytotoxicity and fungitoxicity (Table 1).

DISCUSSION.—*Absorption and translocation.*—Aqueous solutions of ^{14}C-F-319 were rapidly absorbed by roots of cucumber, tomato, and rice plants. The chemical accumulated there in much higher concn than in the foliage during the 24-h treatment. This was apparent from autoradiograms and from the time-course study on absorption and translocation (Fig. 1). These results also indicated that the concn of ^{14}C-F-319 and its ^{14}C-metabolites had to reach a certain level in the roots before translocation to the foliar portion of the plants would occur. Root absorption and translocation of the other pesticides, eg. Bioxone and Diazinon (6,9) which have systemic activity, occur in a similar manner.

Autoradiograms of the cucumber plants which received foliar applications of ^{14}C-F-319 showed that labeled material moved from the treated leaf toward the other portions with the transpiration stream (Fig. 2). Lesser amounts of label were translocated basipetally. This distribution pattern may indicate that movement in the treated leaf was mainly in the apoplast and minimally in the symplast (16).

TABLE 1. Antifungal activity of 3-hydroxy-5-methylisoxazole (F-319) and its metabolites, MU and ML to the mycelial growth of *Pythium debaryanum* on potato-dextrose agar medium

Concn (μg/ml)	Diam of colony at 3rd day after inoculation (cm)			
	F-319	Concn (μg/ml)	MU[a]	ML[a]
0.0	9.0	0.0	9.0	9.0
0.1	9.0	0.3	9.0	9.0
0.3	6.5	0.9	6.7	9.0
1.0	0.0	3.0	0.0	9.0
3.0	0.0	9.0	0.0	9.0
10.0	0.0	30.0	0.0	9.0
100.0	0.0	300.0	0.0	9.0

[a]Mean of triplicate test are given. a; The tests were also repeated using the synthesized metabolites, MU and ML, and the same results were obtained. MU = 3-(β-D-glucopyranosyloxy)-5-methylisoxazole and ML = 2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one.

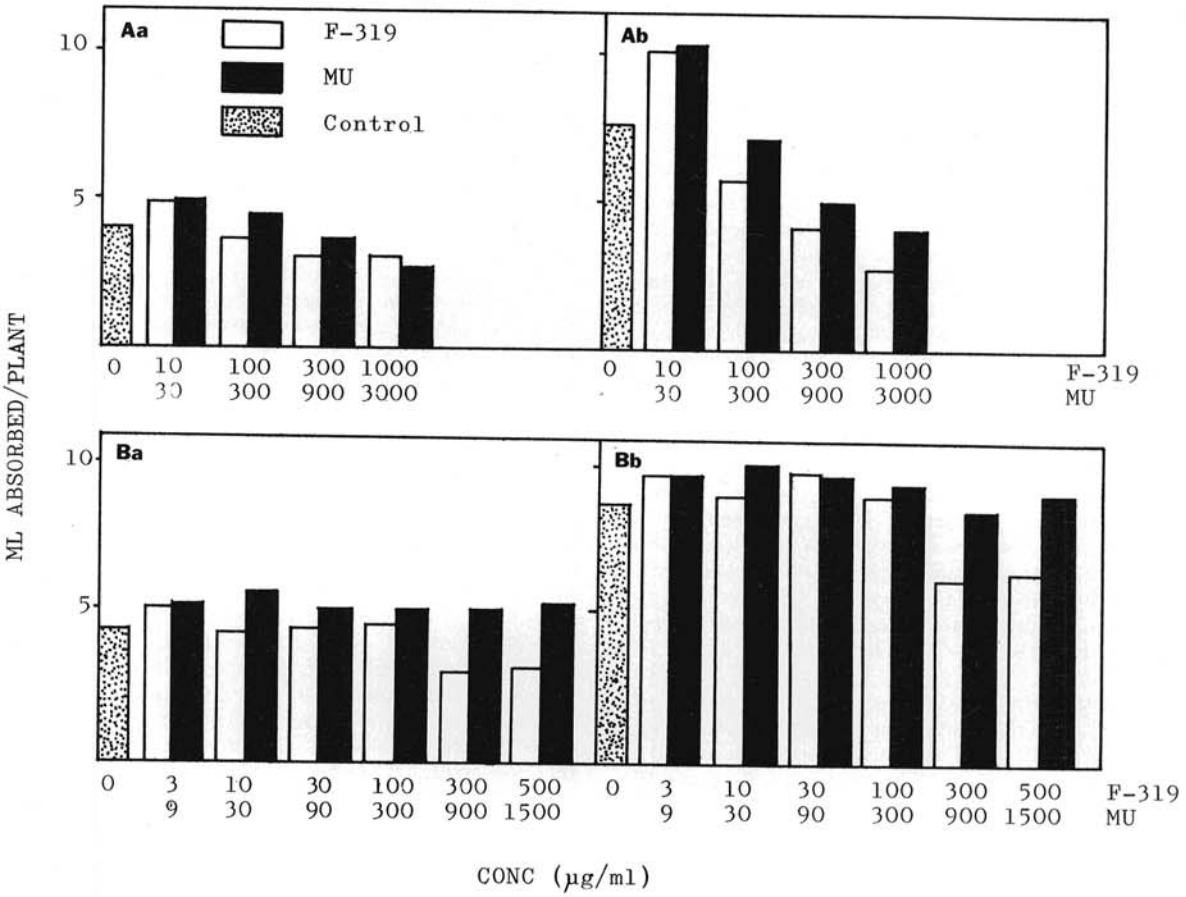

Fig. 8-[A-(a,b) and B-(a,b)]. Effects of 3-hydroxy-5-methylisoxazole (F-319) and 3-(β-D-glucopyranosyloxy)-5-methylisoxazole (MU, synthesized compound) on water uptake by **A)** rice and **B)** cucumber seedlings during a 4-day **a)** and a 7-day **b)** absorption period. The amount absorbed per day was recorded and the solution was renewed every day. Mean of three replicate plants are given. Plants used were at the third- to fourth-leaf stage of growth.

In contrast, the downward movement of foliarly-applied ^{14}C-F-319 was more evident in rice plants than in cucumber. This might be due to morphological differences between the two species. One of the merits of this soil fungicide is its utility as either a post- or pre-planting treatment. Since F-319 does not move basipetally extensively, it would appear to be more effective in postplanting treatments applied to the soil near the roots of plants than as a foliar spray.

F-319 taken up by the rootlet tip seemed to move readily and became distributed throughout the entire root system (Fig. 3). ^{14}C-materials diffused out the untreated rootlets following their translocation from the one-to-five treated rootlet tips. Diffusion of some ^{14}C into surrounding absorbent cotton precluded a quantitative assessment of the translocation of F-319. However, the data reveal lateral movement of F-319, and its uniform distribution throughout the root system.

In a separate study on the movement of F-319 in the soil, we found that the chemical applied as soil drench did not move readily, but rather stayed at the soil surface (Kamimura et al., *unpublished*). This evidence led us to suspect that F-319 applied to the soil moved from plant to plant via root systems, concentrating primarily in the rhizosphere, and thus bringing about effective control of root-invading pathogens.

Metabolism in plants and the structure of the metabolites.—The metabolites were exclusively produced by the plants per se and the plants did not absorb metabolites resulting from microbial decomposition of F-319 in soil.

In other research, F-319 was found to be metabolized by a large number of microorganisms in soil and in artificial media. However, no metabolites corresponding to those elaborated by the plants were detected (Kamimura et al., *unpublished*). Thus, it is contended that root absorption of metabolites produced in soil by microbial degradation of ^{14}C-F-319 during the course of our experiments influenced neither the quantity nor the quality of the metabolites detected in the plants.

The pattern of metabolism and quality of the metabolites detected in cucumber, tomato and rice plants were essentially identical. The metabolites were also the same as those produced by callus tissues of tobacco plants. In these experiments, ^{14}C-CO_2 evolved from ^{14}C-F-319 by callus tissue was traced in a completely closed system using an aseptic liquid tissue culture technique (8). Metabolism due to the cleavage of the isoxazole ring seemed to be minor, because only about 0.1-1.0% of the absorbed F-319 label was evolved as ^{14}C-CO_2 during a 10-day period.

The metabolism of F-319 was predominantly due to the glycosidation in the position-2 and 3 of the isoxazole ring. The structure of both metabolites was determined by spectroscopic analysis, and MU was found to be 3-(β-D-glucopyranosyloxy)-5-methylisoxazole and ML: 2-(β-D-glucopyranosyl)-5-methyl-4-isoxazolin-3-one (Fig. 8). The glycosidation of pesticides by plants is a phenomenon that has been reported previously (2, 3, 12).

The proportions of MU and ML formed to unaltered F-319 appeared to be extremely variable and dependent on environmental and morphological factors. To evaluate the basis for the wide quantitative variation in metabolite formation, such factors as age, plant organ involved, cultural conditions, time following treatment, light intensity, temp, etc. were examined. It was found that the metabolism of ^{14}C-F-319 was much enhanced and more MU was formed than ML in the shoots of cucumber kept in the light. This effect could be reversed in the dark (Kamimura et al., *unpublished*).

Attention is called to the rapid rate of glycoside formation of ^{14}C-F-319 in plants and the gradual increase in production of ML with time (8).

Biological nature of metabolites.—The phytotoxicity, fungitoxicity, hormonal activity, and mammalian toxicity of the metabolites indicated the following: ML did not limit the growth of germinated rice or cucumber seeds; MU, like F-319, caused retardation of growth of germinated rice when applied at the high concn of 1,500 μg/ml. On the other hand, the effect of MU on water uptake by rice seedlings was less suppressive than F-319. Thus, phytotoxic effects of the metabolites appeared less intense than the original chemical. It seems likely that the metabolite formation is a mechanism for the detoxification of F-319 in the plants. This is more pronounced with the passage of time. Only ML was detected in the plants at the latter stage of growth (8).

In a separate experiment, the fate of MU and ML in plants was investigated. Labeled MU or ML previously isolated from plant tissue was applied to the cucumber seedlings via the roots in liquid culture. Five days after treatment, the metabolites were re-extracted from the plants and TLC showed that when MU was applied, a large amount of ML, a smaller amount of F-319, and a trace of MU were detectable. When ML was applied, ML predominated, F-319 was found in trace amounts, and MU was not detected. These findings suggest that F-319 undergoes a detoxification process in plants.

Though fungitoxicity of F-319 in vitro was relatively low against certain pathogens of soil-borne diseases, the fungicide was fairly effective in vivo. Accordingly, we at first postulated that the applied chemical might be biotransformed in plants to more toxic products. The data in Table 1 showed that this was probably not the case; nevertheless, the chemotherapeutic activity of the metabolites has been studied (11).

Interesting evidence for the possible conversion of F-319 to compounds with hormonal qualities was reported by Ogawa et al. (15).

Finally, a problem that could not be overlooked was the possible conversion of the applied fungicide to compounds that are potentially hazardous to the health of consumers of F-319-treated foods. As reported previously (1, 4), there is a possibility that the transformation products are potentially more toxic than the parent compound. However, it would appear that this is not the case with F-319. Toxicological evidence reveals that the acute oral LD_{50} of F-319 for rats and mice is 1,968-2,148 and 3,090-4,678 mg/kg, respectively, whereas both MU and ML were nonlethal to either mice or rats at >6,000 mg/kg.

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