Phytotoxicity of the Fungicide Metalaxyl and Its Optical Isomers

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ABSTRACT

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Metalaxyl is a systemic fungicide commonly used in citrus production to control *Phytophthora* root rot and foot rot. When applied as a drench, injury was observed on newly planted, young citrus trees. The visual injury symptoms ranged from light- to bright-yellow leaves. It was learned that the commercial formulation of metalaxyl contained various isomers and that these isomers may vary in phytotoxic effects on citrus leaves. The objective of this study was to determine the difference in herbicidal activity between the two optical isomers of metalaxyl on pepper plants and citrus leaves. The phytotoxicity of the fungicide metalaxyl and its optical isomers (CGA76538, S⁺; CGA76539, R⁻) was determined using a pepper seedling growth bioassay and by measuring protein synthesis as estimated by the incorporation of ¹⁴C-leucine by citrus mesophyll cells. The two isomers and metalaxyl differed in their herbicidal activity to pepper plants and citrus cells. Pepper seedlings treated with R⁻ had significantly higher mean fresh weight than plants treated with metalaxyl or S⁺ at 0.1, 1.0, 10.0, and 100 ppm. Protein synthesis, as measured by the inhibition of ¹⁴C-leucine incorporation by citrus mesophyll cells, also was inhibited more by metalaxyl and the S⁺ isomer than by the R⁻ isomer. After 30 min of incubation at 100 μ M, the R⁻ isomer inhibited ¹⁴C-leucine incorporation by 29%, whereas incorporation of ¹⁴C-leucine in the metalaxyl and the S⁺ isomer treatments was higher (46 and 81%, respectively). Similarly, the highest ¹⁴C-leucine uptake at 60 min was obtained by R⁻ at all concentrations. The assays showed that the R⁻ and S⁺ isomers differ in their biological activity as expressed by weight loss of pepper plants and inhibition of protein synthesis and that the S⁺ isomer is responsible for the phytotoxicity of metalaxyl. The findings in this study show that the phytotoxicity of metalaxyl was due to the presence of the S⁺ optical isomer. Removal of this isomer from metalaxyl has enabled the continued use of this fungicide for control of foot rot and root rot in citrus.

Additional keywords: *Capsicum frutescens* L., *Citrus sinensis* L. Osbeck, Ridge pineapple, 'Yolo Wonder' pepper

The systemic fungicide metalaxyl is widely used in citrus production to control root rot and foot rot diseases caused by *Phytophthora* spp. when applied as a soil drench, a stem paint, or foliar spray (3,6,7,13,15). In young citrus trees, the fungicide usually is applied as a drench or ring treatment and, in Florida, it often is applied as a tank mix with various herbicides. In young field-grown citrus trees, bright yellow foliage with some leaf necrosis was observed following metalaxyl treatments. The yellowing was observed on both young and old leaves. Initially, herbicide injury was suspected; however, the symptoms were not typical of the damage

caused by the commonly used herbicides. In preliminary greenhouse studies (M. Singh, unpublished) with sweet orange (*Citrus sinensis* L. Osbeck 'Ridge pineapple') and grapefruit (*C. paradisi* Macfad.), plants treated with metalaxyl and metalaxyl mixed with herbicides (simazine and bromacil) produced symptoms similar to those observed on field plants. Confirmation of the phytotoxicity of metalaxyl was shown by Singh and Mersie (14). Metalaxyl-treated plants developed mild yellowing foliar symptoms similar to those observed on field plants.

Metalaxyl is an acylalanine fungicide that is closely related chemically to an important class of herbicides, the chloracetamides, which include metolachlor and alachlor. Members of the acylalanine family have an asymmetrical carbon atom that gives rise to a pair of optical isomers. This chirality in metalaxyl is caused by the asymmetric carbon in the alkyl side chain (Fig. 1, symbol #). These optical isomers, or enantiomers, are designated as CGA 76538 (S⁺) and CGA 76539 (R⁻) and differ markedly in biological activity. The R⁻ isomer is more active than the S⁺ against

fungi (11). In these in vitro tests, selected fungi (of the order Peronosporales) were inhibited in the inhibitory concentration 50% (IC₅₀) value range of 0.1 to 0.3 ppm for R⁻ and 100 ppm for the S⁺ isomer.

Metalaxyl is similar in chemical structure to the herbicide metolachlor, which also is a mixture of R⁻ and S⁺ isomers. The S⁺ isomer is more active as a herbicide than the R⁻ and has been selected for commercial production due to its activity on susceptible plants and its efficacy at low rates (16). Metalaxyl was a mixture of both isomers and caused visible herbicide type symptoms in greenhouse tests; therefore, studies were initiated to determine the herbicidal activity of both isomers on citrus leaves and pepper (*Capsicum frutescens* L.).

The objective of this study was to determine the difference in herbicidal activity between the two optical isomers of metalaxyl on pepper plants and citrus leaves.

MATERIALS AND METHODS

Pepper growth assay. Pepper plants ('Yolo Wonder') previously were found in preliminary experiments to be highly sensitive to metalaxyl (W. Mersie and M. Singh, unpublished). Pepper seeds were sown (1 cm deep) in pots filled with vermiculite and germinated in a greenhouse. At the three-leaf stage, the seedlings were removed from the vermiculite, their roots were washed, and fresh weights were measured. The seedlings then were trans-

CGA 76538 S (+)
CGA 76539 R (-)

Fig. 1. Metalaxyl and its two isomers (CGA 76538 S⁺ and CGA 76539 R⁻). The asymmetric (#) carbon in the alkyl side chain.

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ferred to 50-ml Erlenmeyer flasks containing 50 ml of half strength Hoagland and Arnon solution (10) and were grown in a laboratory under $250 \mu\text{E m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density provided by fluorescence lamps with a 14-h photoperiod at a room temperature of $24 \pm 1^\circ\text{C}$.

After 48 h, the solution was changed with a Hoagland and Arnon solution, and metalaxyl or CGA 76538 (S^+ isomer) or CGA 76539 (R^- isomer) at 0 (control), 0.1, 1.0, 10.0, 100.0, and 250 ppm (wt/vol) were directly added to the solution. Metalaxyl at 250 ppm is equivalent to the recommended field application rate of metalaxyl on citrus. After 7 days, the seedlings were harvested and their fresh weights were determined. Experiments were repeated three times with three replications and the combined data were subjected to analysis of covariance to remove variation in final weight that is associated with initial weight (8). The adjusted means of fresh weight of seedlings were compared with a paired t test.

Citrus cellular assay. Eight-month-old sweet orange seedlings (Ridge Pineapple) were grown from seed in planter flats in a greenhouse with a 14-h photoperiod. The growing medium used was Promix-BX³ (peat:perlite:vermiculite, 3:1:1, vol/vol/vol). The plants were watered daily and fertilized once per week with 1% Nutrilife (N-P-K, 20:20:20) solution. The average photosynthetic photon flux density was $250 \mu\text{E m}^{-2} \text{s}^{-1}$ at plant level. Day and night temperatures were 25 and $26 \pm 2^\circ\text{C}$, respectively.

Mature citrus leaves (third or fourth youngest) were detached from the experimental trees, rinsed with distilled water, blotted, deveined, and cut into 1-mm-by-1-cm strips with a razor blade. About 5 g of leaf tissue then were vacuum infiltrated with 30 ml of infiltration medium (20 mM MES [2-(*N*-morpholino)ethanesulfonic acid], pH 5.8, with 0.1% macerascase, 2% pectinase, and 0.3% potassium dextran sulfate; 9). The leaf tissue was filtered through a 200- μm nylon net and the tissue was transferred to a beaker with 30 ml of maceration medium (20 mM MES, pH 5.8, containing 0.2% pectinase, 2% cellulose, 0.3% potassium dextran sulfate, and 0.6 M mannitol; 9). The tissue was stirred slowly on a magnetic stirrer for 10 min, the suspension was filtered through the 200- μm nylon net, and the filtrate was discarded. The leaf tissue was transferred to another 30 ml of fresh maceration medium and was stirred for 2 h. The cells released during this period were filtered again through the 200- μm nylon net and washed three times with 10-ml aliquots of incubation medium and centrifuged at $60 \times g$ for 3 min. The pelleted cells were resuspended in incubation medium (0.5 mM mannitol, 5 mM KNO_3 , 2 mM $\text{Mg}(\text{NO}_3)_2$, 1 mM CaCl_2 , 50 mM HEPES-KOH buffer, pH 7.8). For chlorophyll determination, three 1-ml sam-

ples of the final cell suspension were placed in three 15-ml centrifuge tubes. To each tube, 4 ml of 80% acetone was added and the tubes were vortexed. The supernatant fluid then was assayed spectrophotometrically for its chlorophyll content according to the methods of Arnon (1). The chlorophyll content of cell preparations used in this study was 15 to 25 μg chlorophyll/ml of incubation medium.

^{14}C -leucine incorporation into protein of citrus mesophyll was measured after 30 and 60 min of incubation as described by Hatzios (9). Briefly, 2 ml of the cell preparation and 0.1 ml of L-[U- ^{14}C] leucine (specific activity 290 M Ci/m mol) containing 0.1 μCi of radioactivity were placed in 50-ml Erlenmeyer flasks. Analytical grades of metalaxyl, CGA 76538 (S^+ isomer), and CGA 76539 (R^- isomer) were dissolved in methanol and made up to volume with distilled water so that the final methanol concentration was less than 1%. Concentrations of the metalaxyl and its isomers were 0.1, 1.0, 10.0, and 100 ppm. Flasks with the assay mixtures (cell preparation, ^{14}C -leucine and metalaxyl) as prepared above were placed on a shaker at 100 oscillation/min under a fluorescent lamp providing $250 \mu\text{E m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density at the top of the flasks. A control blank with only distilled water was used for comparison. At the end of 30 and 60 min of incubation time, a 500- μl sample was collected from each flask with a micropipetter and placed in a 20-ml vial. To each tube, 2 ml of ice-cold trichloroacetic acid (TCA) (10 wt/vol) was added and the vials were left overnight at 4°C in a refrigerator. The TCA-insoluble protein precipitates then were collected by filtering

each sample through 2.1-cm glass fiber filter disks. The filter disks were washed successively with 6 ml (3 by 2 ml) of ice-cold TCA, 6 ml (3 by 2 ml) of 80% ethanol, 2 ml of acetone, and 4 ml (2 by 2 ml) of diethylether. The disks were dried, put into vials with 20 ml of scintillation fluid, and radioassayed.

Counts per minute (CPM) of ^{14}C were measured and were converted to disintegration per minute (DPM) based on the counting efficiency of the liquid scintillation counter ($>97\%$). The results were converted to DPM/mg/chlorophyll and data was subjected to analysis of variance. There were three replications in an experiment and all experiments were conducted twice. The combined data was subject to analysis of variance and the means were compared using Duncan's multiple range test.

RESULTS AND DISCUSSION

Pepper growth assay. Metalaxyl and its S^+ isomer reduced the fresh weight of pepper compared with the isomer R^- (Fig. 2). The difference was evident even at 0.1 ppm. At this lowest concentration, the average fresh weight of pepper seedlings treated with the control, S^+ , and R^- was 600, 425, and 707 mg, respectively. Pepper plants treated with metalaxyl or the S^+ isomer had significantly lower fresh weights than control plants at all concentrations, whereas the R^- caused lower weights than the controls only at ≥ 100 ppm.

The similarity in pepper weight reductions caused by metalaxyl and the S^+ at all concentrations indicates that S^+ isomer is the herbicidal component of the mixture. Another acylalanine fungicide, CGA 29212

Fig. 2. Fresh weights of pepper plants treated with metalaxyl and its two optical isomers. Means followed by the same letter are not significantly different according to the t test at 0.05 probability level.

(methyl N-(2,6-dimethylphenyl)-N-chloroacetylalaninate), also is reported to be composed of two isomers that differ in their biological activity (11). In this study, the dextrorotatory S^- enantiomer had no fungicidal activity but was highly herbicidal, whereas the R^- enantiomer has the opposite properties, with no herbicidal activity but highly active as a fungicide (11).

Citrus cellular assay. Preliminary experiments on the uptake of ^{14}C -metalaxyl were conducted for up to 60 min. The results showed that absorption increased steadily up to 30 min and there was no increase thereafter (*data not presented*). Two incubation periods (30 and 60 min) were selected for this study because maximum uptake of metalaxyl by the cells oc-

curred over 30 min. At 60 min of incubation, the untreated cells incorporated almost twice the ^{14}C -leucine compared with the 30-min period (Figs. 3 and 4). There also was an increase in the absorption of ^{14}C -leucine in all the treatments in the 60-min incubation.

After 30 min, 0.1 ppm of metalaxyl and its isomers had no effect on incorporation of ^{14}C -leucine by cells compared with the control (Fig. 3). At 1.0 ppm, metalaxyl and the S^+ isomer significantly reduced the incorporation of ^{14}C -leucine by cells compared with the control. The R^- isomer, however, had no ^{14}C -leucine incorporation at 1.0 ppm. At 10 ppm, all three treatments reduced ^{14}C -leucine incorporation relative to the control, but metalaxyl and the R^- isomer reduced incorporation more than the S^+ isomer. At 30 min of incubation, the maximum reduction of ^{14}C -leucine incorporation by the R^- isomer (at 100 ppm) was only 29% relative to the untreated control; whereas, for metalaxyl and the S^+ isomer, ^{14}C -leucine incorporation was 46 and 81% lower than the control, respectively.

Inhibition of ^{14}C -leucine uptake in cells at 60 min was greater than the control when treated with metalaxyl and the S^+ isomer at 1, 10, and 100 ppm. At the highest concentration tested (100 ppm), the R^- isomer inhibited ^{14}C -leucine incorporation by 65%; whereas, for metalaxyl and the S^- isomer, inhibition was greater. The inhibition of ^{14}C -leucine incorporation by metalaxyl and the S^+ isomer appeared to be time dependent. At 30 min, the inhibition at 1.0 ppm by metalaxyl was 27% and, by the S^+ isomer, 36%; whereas, at 60 min, it increased to 47 and 61%, respectively. For the R^- isomer, inhibition percentages in the 30- and 60-min experiments were similar (21 and 18%). Similar effects of incubation time on inhibition of incorporation of radioactive precursors into protein by cultured cells were observed for 2-chloro-N,N-di-2-propenylacetamide (CDAA; 2) and S-ethyl dipropylcarbamothioate (EPTC; 5). This could be because of a delay in absorption or subcellular transport, a delay in the induction of the inhibition, or because the chemicals were inhibiting a metabolic site that supplied an essential factor for protein synthesis rather than affecting it directly. Based on our data, it is not possible to determine which of these factors may be responsible for the delayed inhibition of ^{14}C -leucine incorporation.

The incorporation of ^{14}C -leucine by cells appears to be a sensitive assay to identify the herbicidal effect of metalaxyl and its isomers. Photosynthesis also was examined by determining the fixation of $^{14}CO_2$ from $NaH^{14}CO_3$ by citrus cells (*data not presented*). However, photosynthesis was not significantly inhibited by treatment concentrations up to 100 ppm after 60 min of incubation. This suggests that metalaxyl,

Fig. 3. Effect of metalaxyl and its optical isomers on ^{14}C -leucine incorporation by mesophyll cells of citrus (expressed as disintegrations per minute [DPM] μg chlorophyll $^{-1}$) incubated for 30 min. Means followed by the same letter are not significantly different according to the least significant difference test at $P = 0.05$ probability level.

Fig. 4. Effect of metalaxyl and its optical isomers on ^{14}C -leucine incorporation by mesophyll cells of citrus (expressed as disintegrations per minute [DPM] μg chlorophyll $^{-1}$) incubated for 60 min. Means followed by the same letter are not significantly different according to the least significant difference test at $P = 0.05$ probability level.

like the other acylalanines, does not have a direct effect on CO₂ fixation. On the other hand, the inhibition of ¹⁴C-leucine incorporation by metalaxyl is similar to the effect produced by other acylalanines. For example, chloroacetamide herbicides, including alachlor and CDAA, have been reported to strongly inhibit protein synthesis in various systems (2,12).

The results presented in this report indicate that the herbicidal effect of the fungicide metalaxyl is caused by the S⁺ isomer. This clearly is shown by data from the pepper growth assay and ¹⁴C-leucine incorporation. Our results also further confirm previous findings (4) that, for nonphotosynthetic inhibitors, the ¹⁴C-leucine incorporation assay can be used to identify toxicity of chemicals at the cellular level.

The S⁺ isomer was removed from the formulation of metalaxyl and has enabled the continued use of this important fungicide for the control of citrus root and foot rot.

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