A Sensitive Bioassay for Quantification of Metalaxyl in Soils

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The authors are grateful to Homer M. LeBaron of Ciba-Geigy Corporation and to the California Avocado Commission for financial support, to D. P. Ryskiewich of Ciba-Geigy Corporation who supplied the ^{14}C-metalaxyl, and to Betty Ann Merrill for typing the manuscript.
Accepted for publication 3 February 1984.

ABSTRACT

Bailey, A. M., and Coffey, M. D. 1984. A sensitive bioassay for quantification of metalaxyl in soils. Phytopathology 74:667-669.

A sensitive bioassay was developed to detect low concentrations of metalaxyl in soils. Aqueous extracts of metalaxyl-treated soils were incorporated into cornmeal agar prior to autoclaving. The quantitative estimation of the amount of metalaxyl in soils was based on a highly significant positive relationship between the radial growth of an isolate of *Phytophthora boehmeriae* and the log concentration of the fungicide in the agar. The isolate of *P. boehmeriae* was chosen for its high sensitivity to metalaxyl as manifested in a linear growth response on cornmeal agar over the range of 2 to 30 ng/ml. The sensitivity and quantitative nature of the bioassay were confirmed by comparison with data obtained for scintillation counting using a common source of ^{14}C-metalaxyl.

Additional key words: fungicide biodegradation in soil.

Fungitoxic compounds can be assayed in soils either by the use of sensitive organisms (bioassay) or by more complex procedures (7). Bioassays that have been developed for fungicides involve either the diffusion of the chemical into agar bands (2) or bioautographic determination by using thin-layer chromatograms (3,5). More recently, specific bioassays have been developed for determining the levels of metalaxyl in plant tissues (4,8). A method was developed that involved applying methanolic extracts to filter paper, which was then overlaid with agar, and an isolate of *Pythium ultimum* Trow sensitive to metalaxyl was grown on the agar (8).

The trend towards increasing usage of metalaxyl for control of root diseases caused by *Pythium* and *Phytophthora* has focused attention on the need to develop a suitable soil bioassay for this fungicide. The purpose of this research was to develop a highly sensitive biological method for quantitative estimation of metalaxyl for use in studies of the mobility, adsorption and degradation of the fungicide in soils.

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MATERIALS AND METHODS

Selection and evaluation of test organism. Isolate P1257 of *Phytophthora boehmeriae* Sawada from the Phytophthora Collection at the University of California at Riverside was selected from a range of isolates for its extreme sensitivity to metalaxyl (1). The fungus was subcultured regularly on plates of cleared V-8 juice agar and incubated at 24 C in the dark.

The effect of autoclaving on the activity of metalaxyl was considered because filter-sterilized soil extracts caused some growth inhibition of *P. boehmeriae*. An experiment was performed by using 0.03, 0.06, 0.09, 0.12, 0.15, and 0.3 μg of metalaxyl (technical grade) per milliliter in cornmeal agar. In one half of the treatments, the medium with metalaxyl was autoclaved, while in the other half metalaxyl was added to the medium after autoclaving, when it had cooled to ~45 C.

A standard curve for *P. boehmeriae* was obtained starting with a stock solution of 1 μg of metalaxyl technical grade (94.3% a.i.) per milliliter of water. A range of concentrations from 0.001 to 0.09 μg/ml of the stock were applied to cornmeal agar to obtain a dosage-response curve. Each of the concentrations was tested three times, and after 4 days at 24 C, colony growth of three replicates was recorded in millimeters. The growth was plotted against the log concentration of metalaxyl.

Soil sampling and extractions. The influence of proportions of soil and water used to extract metalaxyl was investigated for three California soils (Table 1). Soils were adjusted to field capacity and 2,000 g (based on wet weight) were treated with 100 ml of a 4,000 μg/ml metalaxyl (E.C., 25.11% a.i.). The percent moisture content of each soil was determined before adding the metalaxyl solution. Samples of 10, 20, 40, and 100 g of soil, dried overnight at 70 C, were bioassayed.

The extraction procedure using 10 g of soil in 100 ml of distilled water was also performed with quantities of metalaxyl as low as 1 μg/ml applied to soil. A bioassay was carried out as described, and the percent recovery of metalaxyl was determined by using a standard curve.

Bioassay procedures. The bioassay for quantification of metalaxyl in soil was used with a selection of California soils (Table 1). Soils were adjusted to field capacity, and each sample, consisting of 200 g of soil based on dry weight, was treated with 40.0 mg of metalaxyl (technical grade, 94.3% a.i.) dissolved in 2 ml of methanol to give a final concentration of 200 μg/g of soil. The soil samples were incubated in the dark at 23 C in 500-ml Mason jars. A control sample, to which metalaxyl had not been added, was run for each soil. Samples consisting of about 20 g of soil were removed from each jar at 0, 14, 28, 42, 56, and 70 days. The samples were dried overnight at 75 C and the dried soil was pulverized into a fine powder using a mortar and pestle. Exactly 10 g of the dry soil was added to 100 ml of distilled water and the suspension agitated on a reciprocal shaker at ~160 strokes (2 cm) per minute for 1 hr. The suspension was passed through Whatman No. 1 filter paper and finally 1 ml of the diluted soil extract was added to 100 ml of cornmeal agar to give a concentration of 0.01 μg/ml. Cornmeal agar media containing soil extracts were autoclaved for 20 min at 120 C. Following inoculation with 5-mm-diameter disks of P1257, the plates were incubated at 24 C in the dark for 96 hr. At this stage the colony diameters were measured in millimeters. Actual concentrations of metalaxyl present in soils were calculated by reference to a standard response curve for P1257.

The bioassay was confirmed using ^{14}C-metalaxyl in California sandy loam soil B (Table 1). In each treatment, 10 g of soil based on dry weight was adjusted to field capacity and treated with ^{14}C-metalaxyl dissolved in water. The concentrations of ^{14}C-metalaxyl used were 2, 4, 8, and 12 μg/g soil. Each 10-g soil sample was treated as described above for the bioassay and the concentration of ^{14}C-metalaxyl was obtained by reference to a standard response curve for P1257.

Using the same ^{14}C-metalaxyl extracts, 1-ml duplicate samples at each concentration were mixed with 2 ml of liquid scintillation fluid (Beckman High Performance for aqueous samples) and counted in a Beckman (model 7500) liquid scintillation counter. The disintegrations per minute were recorded, converted to μg/ml of metalaxyl, and compared with the original amount of ^{14}C-metalaxyl added to the soil samples.

TABLE 1. Physical characteristics of seven soils used to determine soil and water volumes for maximum extraction, and biodegradation, of metalaxyl[a]

Soil and texture	Composition (%)			Organic matter (%)	Soil pH
	Sand	Silt	Clay		
Sandy loam A	76.4	13.6	10.0	3.6	7.1
Sandy loam B	77.0	14.6	8.4	3.8	7.2
Sandy loam C	59.3	30.7	10.0	2.1	6.8
Sandy loam D	71.2	16.4	12.4	0.7	7.7
Sandy loam E	77.4	10.3	12.3	1.8	6.9
Sandy loam F	63.2	20.0	16.8	3.3	6.9
Sandy clay loam G	55.8	21.2	23.0	3.3	7.4

[a] The percentage composition of the soils was determined by mechanical analysis using the hydrometric method. Percentage organic matter content was analyzed by the Walkley-Black method. Soil pH was measured using a saturated soil paste.

TABLE 2. Percentage of initial metalaxyl concentrations estimated with the bioassay procedure at six times after application[a]

Soil and texture	Days after application					
	0	14	28	42	56	70
Sandy loam B	88.8	89.8	89.8	86.5	88.8	90.8
Sandy loam D	94.02	87.5	87.5	90.8	87.5	90.8
Sandy loam E	88.8	87.5	89.8	81.0	86.5	92.1
Sandy loam F	90.8	89.8	89.8	86.5	86.5	90.0
Sandy clay loam G	94.0	87.4	80.9	75.0	35.2	2.5

[a] Each soil, consisting of 200 g dry wt, was treated with 40 mg of metalaxyl dissolved in 2 ml of methanol. Soils were incubated in the dark at 23 C. Samples, consisting of about 20 g of soil maintained at field capacity, were removed at 0, 14, 28, 42, 56, and 70 days. Exactly 10 g of the dry soil was extracted with 100 ml of distilled water and known volumes of the extract were incorporated into cornmeal agar. Metalaxyl concentrations were estimated by using the bioassay with *Phytophthora boehmeriae*.

RESULTS

Selection and evaluation of test organism. Autoclaving had no effect on the activity of metalaxyl against isolate P1257 of *P. boehmeriae*. The linear regression lines representing the radial growth of P1257 plotted against the log concentration of metalaxyl were identical, with correlation coefficients of 0.97 and 0.96 for the autoclaved and non-autoclaved samples, respectively.

Filter-sterilized soil extracts were frequently found to cause some inhibition of the growth of P1257. However, with a range of soil types, autoclaving always removed this inhibitory effect. Consequently soil extracts from metalaxyl-treated samples were autoclaved with the agar medium in the bioassay of soils.

A standard curve for *P. boehmeriae* was constructed by plotting the radial growth rate of the fungal colony against the log concentration of metalaxyl in the agar medium (Fig. 1). The linear regression obtained had a correlation coefficient of $r = -0.97$. The metalaxyl concentrations detectable by the bioassay with *P. boehmeriae* ranged from 2 to 30 ng/ml (Fig. 1).

Soil sampling and extractions. The influence of the size of the soil sample upon the extraction of metalaxyl was investigated with three sandy loams of differing physical composition (Table 1). The results indicated that the maximum extractability of the fungicide was assured with 10 g dry weight of soil in 100 ml of water, although with two of the three soils tested, more concentrated extracts gave equivalent results (Fig. 2).

Bioassay procedures. The use of ^{14}C-metalaxyl added to soil and then extracted immediately either for a bioassay determination or for scintillation counting, established the quantitative nature of the

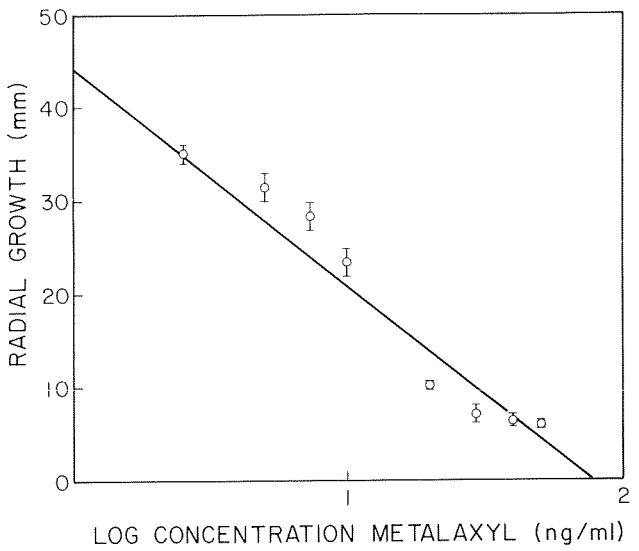

Fig. 1. Standard curve obtained for *Phytophthora boehmeriae*, isolate P1257, for radial growth on cornmeal agar of log concentrations of metalaxyl in nanograms per milliliter. Vertical bars represent the standard errors ($P = 0.05$).

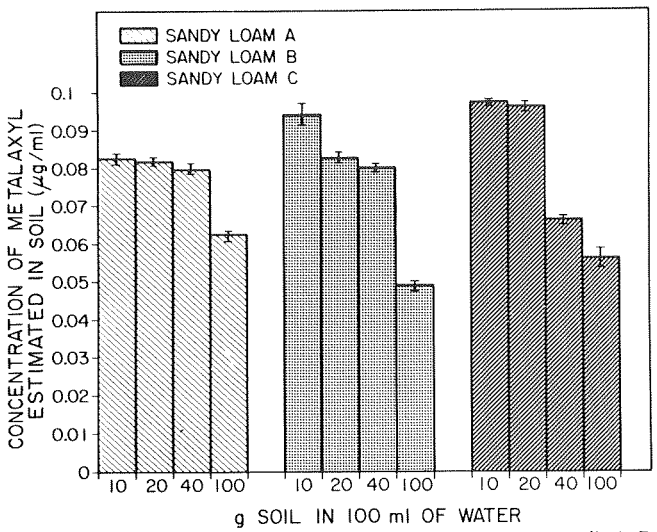

Fig. 2. Concentration of metalaxyl estimated in three sandy loam soils A, B, and C (see Table 1 for details of their physical composition) with different amounts of soil extracted in 100 ml of water. Bar lines represent the standard error of the mean based on three replicate values per treatment ($P = 0.05$).

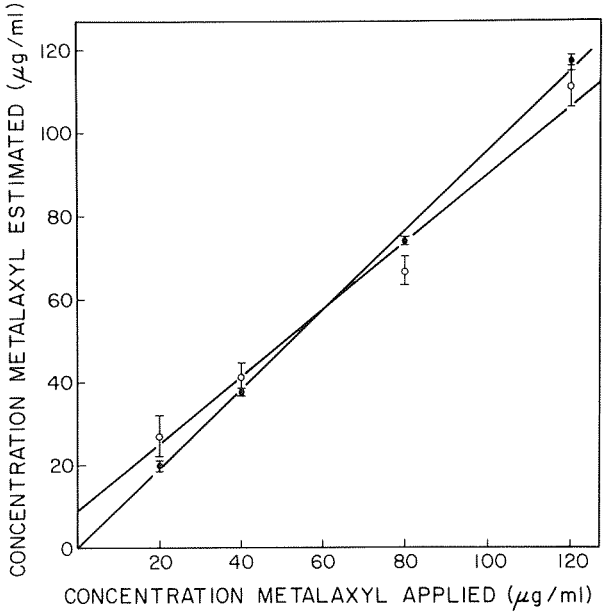

Fig. 3. Comparison of the linear regressions obtained for ^{14}C-metalaxyl extracted from soil using either the bioassay with *Phytophthora boehmeriae* (○) or scintillation counting (●). Vertical bars represent the standard errors for the two methods ($P = 0.05$).

procedure (Fig. 3). Linear regressions of the two sets of data showed identical correlation coefficients ($r = 0.99$). Standard errors ($P = 0.05$) obtained for the bioassay were very low, and demonstrated the high reproducibility of the method.

A series of soils, selected as characteristic of the types in which avocados (*Persea americana* Mill.) are cultivated in southern California, were tested, by using the bioassay procedure, for ability to degrade metalaxyl. Generally, the majority of the 200 μg/ml of metalaxyl added originally was recovered, even after 70 days of incubation (Table 2). In only one instance, with a sandy clay loam soil, was metalaxyl completely degraded by 70 days.

DISCUSSION

The bioassay developed by using the very sensitive isolate P1257 of *P. boehmeriae* was capable of detecting quantities of metalaxyl as low as 2 ng/ml. This sensitivity was over 10 times greater than that achieved with two other bioassays, developed for plant tissues. In one of them (8) *Pythium ultimum* Trow was used (8), and in the other *P. megasperma* Drechs. f. sp. *glycinea* (Hildeb.) Kuan and Erwin was used in a bioautographical assay which employed thin-layer chromatography (4).

Experiments with ^{14}C-metalaxyl established that the bioassay with *P. boehmeriae* was quantitative and highly reproducible. The standard errors obtained with this method were very low compared to the two previously described bioassays (4,8). The sensitivity was comparable to that achieved by gas liquid chromatography (6) or by use of ^{14}C-metalaxyl as in the present study. The simplicity of the method, adding the soil extract containing metalaxyl to the agar medium prior to autoclaving, was another useful feature. The extreme sensitivity of the bioassay would make it useful in investigations where there is a need for accurate determination of very low concentrations (<0.05 μg/ml) of metalaxyl in soils. Since the in vitro ED_{50} values for growth inhibition of many *Phytophthora* species is in the range of 0.05 to 0.5 μg/ml (1), assays at these levels are highly desirable.

In bioassays of field soils for metalaxyl, high sensitivity would be an important feature of any method used. However, the bioassay utilizing *P. boehmeriae* was only useful in the range of 2–30 ng of metalaxyl per milliliter, which represented the linear dosage response range for this isolate. For samples in which metalaxyl concentrations are not known it would be necessary to test several dilutions of the soil extract to avoid exceeding the range of sensitivity.

The biodegradability of metalaxyl in soils has been examined by Sharom and Edgington (6). In a loam soil containing 5.7% organic matter, the half-life of metalaxyl was found to be about 3 wk. In contrast, in a muck soil with 62.8% organic matter, the metalaxyl half-life was about 8 wk. This was attributed to the higher adsorptive capacity of the muck soil, due to its very high organic matter content, that may have made metalaxyl less available to the soil microorganisms (6). In our study, all the soils had low organic matter, and yet with the majority, which were sandy loams, there was no evidence of biodegradation even after 10 wk. With one soil, a sandy clay loam, there was extensive biodegradation of metalaxyl between 42 and 56 days. The initial levels of metalaxyl used in our study were high (200 μg/g soil) compared with the 4 μg/g soil used by Sharom and Edgington (6), and this may have influenced the results that were obtained.

In conclusion, the bioassay utilizing *P. boehmeriae* was developed primarily for use with metalaxyl-treated soils. However, it might also be useful for assaying plant extracts or aqueous media such as ground water. A limitation of the method would be its inability to differentiate between metalaxyl and any of its biodegradation products, or other toxicants, with fungistatic activity against *P. boehmeriae*.

LITERATURE CITED

1. Coffey, M. D., and Bower, L. A. 1984. In vitro variability among isolates of six *Phytophthora* species in response to metalaxyl. Phytopathology 74:(In press).
2. Edgington, L. V., Buchenauer, H., and Grossman, F. 1973. Bioassay and transcuticular movement of systemic fungicides. Pestic. Sci. 4:747-752.
3. Homans, A. L., and Fuchs, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. J. Chromatogr. 51:327-329.
4. Lazarovits, G., Brammall, R. A., and Ward, E. W. B. 1982. Bioassay of fungitoxic compounds on thin-layer chromatograms with *Pythium* and *Phytophthora* species. Phytopathology 72:61-63.
5. Peterson, C. A., and Edgington, L. V. 1969. Quantitative estimation of the fungicide benomyl using a bioautograph technique. J. Agric. Food Chem. 17:898-899.
6. Sharom, M. S., and Edgington, L. V. 1982. The adsorption, mobility, and persistence of metalaxyl in soil and aqueous systems. Can. J. Plant Pathol. 4:334-340.
7. Speck, M., and Dirr, E. 1980. Gas chromatographic determination of metalaxyl (Ridomil®) residues in tobacco. J. Chromatogr. 200:313-316.
8. Wynn, E. C., and Crute, I. R. 1983. Bioassay of metalaxyl in plant tissue. Ann. Appl. Biol. 102:117-121.