A Metalaxyl Bioassay for Large Numbers of Small Foliar Samples

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ABSTRACT

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A simple bioassay was developed for quantifying the fungicide metalaxyl in small samples of potato leaf tissue. Metalaxyl was extracted from leaf disks (1–4 cm^2) by soaking in 100% methanol (2 ml) in 12 × 75 mm glass culture tubes. Disks were removed after 24 hr and methanol was evaporated by placing tubes in a water bath (75 C) for approximately 2 hr, which left crude leaf extracts in the tube. Extraction efficiency in this method was 86–91% as efficient as maceration and centrifugation. One milliliter of molten cornmeal agar was pipetted into each tube, autoclaved, and poured into 35 × 10 mm plastic petri dishes. Radial growth of *Phytophthora boehmeriae* after 96 hr accurately quantified metalaxyl in the 5–50 ng/ml range. In samples determined to be >50 ng/ml, the *P. boehmeriae* colony was removed and *P. citrophthora* was used in a similar way on the remaining medium to quantify metalaxyl in the 50–500 ng/ml range. The main advantage of this method is that many samples can be measured accurately in a short time.

Additional key words: fungicide bioassay.

Quantitative studies on uptake, transport, or degradation of metalaxyl in plants depend on reliable detection techniques. Among the techniques used to quantify metalaxyl have been gas chromatography (8,10), thin-layer chromatography (9), ^{14}C labeling (13), ELISA (8), and bioassays (2,14). Each method has advantages and disadvantages, and the method of choice depends on the needs in a particular study.

For a study of the dynamics of metalaxyl in field-grown potato plants we needed a method for assaying a large number of small foliar samples. Because fungicide residues are often highly variable within a crop canopy (4,5), we wanted to be able to describe the variability by fitting a probability distribution to the data. Therefore, we needed a large number of samples for each plot each time samples were taken. Furthermore, we wanted small tissue samples (up to 4 cm^2 leaf area) rather than whole leaflets in order to describe the variability in fungicide residue on a size scale closer to that encountered by a single pathogen lesion. None of the previously reported methods satisfied these needs for a field study. Gas chromatography and thin-layer chromatography were too time-consuming. ELISA may be applicable but still needs to be adapted to handle a large number of samples. Radioactively labeled (^{14}C) metalaxyl was inappropriate because of environmental constraints.

Bioassay techniques are more amenable to dealing with large numbers of samples relatively easily. The bioassay method for metalaxyl proposed by Wynn and Crute (14), however, is not sensitive enough to detect small quantities of metalaxyl biologically active in potato leaves. Bailey and Coffey (2) reported a very sensitive bioassay method for detecting metalaxyl in soil, but it needs to be adapted for plant tissues. A disadvantage of their method is that the range of detection is very narrow (2–30 ng/ml), and a dilution series is necessary to be assured of getting a sample in the appropriate range of concentrations.

The bioassay for metalaxyl reported in this paper differs from other bioassay methods (2,14) in being appropriate for a large number of samples, very sensitive so that small tissue samples can be used, and useful over a broad range of detection without needing dilution series. Preliminary reports of this method have appeared previously (7,11).

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MATERIALS AND METHODS

Metalaxyl extraction. Metalaxyl was extracted from leaves of potato cultivars Norchip and Hudson. Disks of leaf tissue were cut with a cork borer to provide uniform samples 14 mm in diameter. Metalaxyl was extracted by soaking single leaf disks for 24 hr at room temperature in 2 ml of 100% methanol in 12 × 75 mm glass tubes. Leaf tissue was removed after 24 hr, and the extract was dried by evaporation in a 75 C water bath for approximately 2 hr.

The efficiency of extraction by soaking in methanol was compared with that of grinding and centrifugation, which has been shown to extract >99% of metalaxyl from lettuce leaves (14). Extraction efficiency of the two methods was compared on leaves treated with metalaxyl (Apron 25WP) in two ways: 1) Leaflets were dipped in metalaxyl solutions (100 μg a.i./ml) for 5 sec, then shaken gently and allowed to dry adaxial side up on paper towels, and 2) single compound leaves attached to 3-cm stem segments took up metalaxyl by transpiration when the stem segment was submerged in a metalaxyl solution (100 μg a.i./ml) for 6 hr. Terminal leaflets were removed after 6 hr for metalaxyl analysis. Leaflets from both application methods were treated the same way. Two disks (14 mm diameter) were cut from each leaflet and placed in 2 ml of methanol. One disk from each leaflet was randomly chosen and ground by hand with a glass rod until all the tissue was completely macerated (approximately 2 min per sample). Ground samples were then centrifuged at 1,000 *g* for 5 min. The supernatant was decanted to new glass tubes. The other leaf disk in each pair was soaked in methanol for 24 hr. All extracts were then dried and assayed.

Molten cornmeal agar (1.0 ml, CMA, Difco, 17 g/L) was pipetted into each tube containing dried extracts. The tubes were then covered with aluminum foil, placed in a pan of water (3–4 cm deep), and autoclaved for 15 min at 121 C. Metalaxyl, which is soluble in water and stable to autoclaving (2), becomes incorporated into the medium along with some of the leaf extract during autoclaving. We used water in the pan to keep the agar molten after autoclaving; otherwise, the small volume of medium in each tube would cool too quickly. After autoclaving, the medium from each tube was poured into 35 × 10 mm plastic culture dishes; 1 ml of medium covered the bottom of the dish with a thin layer.

Bioassay organisms. Two different bioassay organisms were used, depending on the range of metalaxyl concentrations encountered. *Phytophthora boehmeriae* Sawada, isolate P1257, was obtained from M.D. Coffey at the University of California,

Riverside. This isolate had been used in a consistently sensitive bioassay for metalaxyl in soils (2). Because of the narrow range of detection reported for *P. boehmeriae* (2), a second bioassay organism was used for higher concentrations. *P. citrophthora* (Sm. & Sm.) Leonian, isolate P1210 (originally from the Riverside collection), was obtained from W. Wilcox at the New York State Agricultural Experiment Station, Cornell University, Geneva.

Standard curves. Leaf disks were cut from untreated leaflets and placed in methanol. Known amounts of metalaxyl were pipetted into each tube in 50-μl aliquots from stock solutions (made from analytical-grade metalaxyl and stored at 4 C) just after the disks were put into the methanol. The final concentrations of metalaxyl in the medium were: 0, 5, 10, 15, 30, and 50 ng/ml for the *P. boehmeriae* standard curve and 0, 30, 50, 75, 100, 200, 350, 500, 650, and 800 ng/ml for the *P. citrophthora* standard curve. Three replicates per concentration were used.

Incubation and growth measurements. *P. boehmeriae* and *P. citrophthora* were both grown at 24 C on 20% V-8 medium (200 ml of unclarified V-8 juice, 800 ml of distilled H_2O, 3 g of $CaCO_3$, 15 g of agar). *P. boehmeriae* was grown for 5–7 days before use and *P. citrophthora*, for 4–5 days. Mycelial plugs (4 mm diameter) were cut with a cork borer from just behind the margin of a *P. boehmeriae* colony. A plug was placed mycelial side down on the edge of each 35 × 10 mm culture dish containing CMA. The volume of V-8 medium (18 ml) in the culture plates (100 × 15 mm) from which plugs were cut was kept uniform to obtain uniform plug thickness.

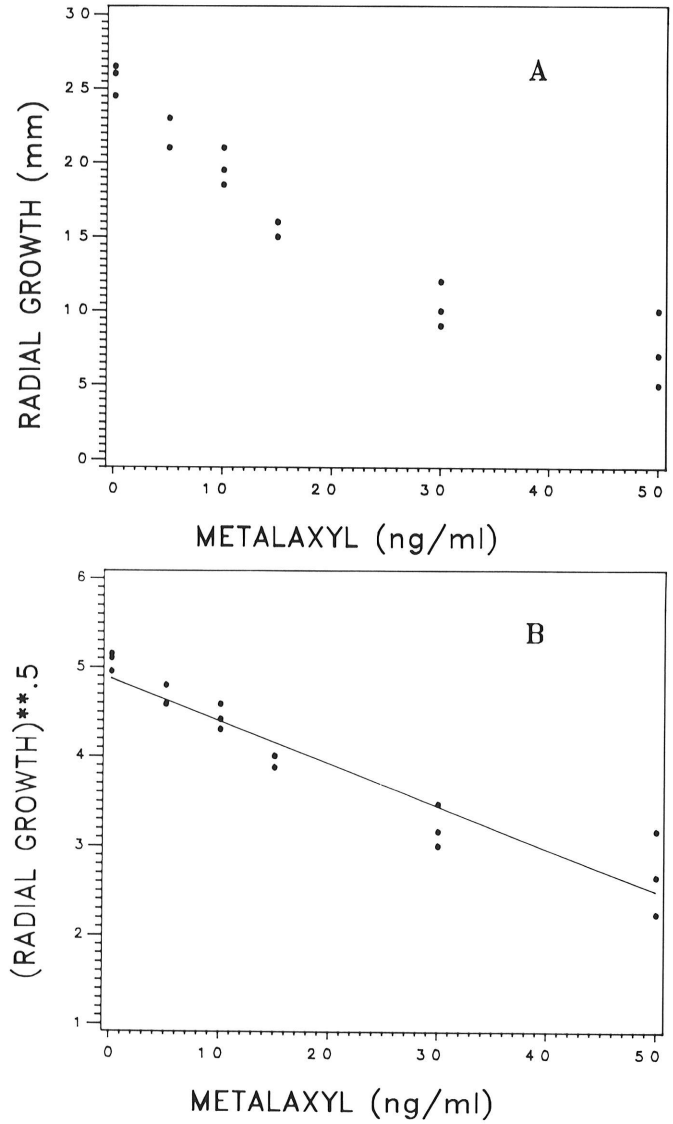

Fig. 1. A, Response of *Phytophthora boehmeriae* to known concentrations of metalaxyl added to extracts of 14-mm-diameter leaf disks from the potato cultivar Hudson. Radial growth (mm) was measured after 96 hr of incubation at 24 C. There were three replications per metalaxyl concentration. **B,** Transformed data for *P. boehmeriae* plotted against metalaxyl concentration. The transformation used was the square root of radial growth. R^2 for the plotted regression line is 0.91.

Trays of culture dishes containing extract-amended medium and seeded with *P. boehmeriae* or *P. citrophthora* were sealed in plastic bags with some wet paper towels inside to prevent drying. The sealed bags were then placed in a dark, 24 C incubator for 85–96 hr. After incubation, the radial growth of *P. boehmeriae* or *P. citrophthora* colonies was measured. Measurements were taken to the nearest 0.5 mm with a clear plastic ruler from the edge of the mycelial plug to the margin of the colony. When mycelial growth was irregular, an average of three colony radii was used. When many samples were done at the same time, colony margins were marked with a scalpel on the bottom of the culture dish for later measurement.

If radial growth of *P. boehmeriae* in an unknown sample was less than the average growth on the 50 ng/ml standard curve samples, the *P. boehmeriae* colony was removed aseptically with a scalpel or spatula. A plug from *P. citrophthora* was then placed mycelial side down 90° from where *P. boehmeriae* had been. *P. citrophthora* was used to quantify metalaxyl in the same manner as described for *P. boehmeriae* except that incubation was for only 85–90 hr.

Effects of leaf extracts on the sensitivity of the assay. To assess whether extracts of various sized leaf disks affect the sensitivity of the bioassay in detecting small quantities of metalaxyl, extracts from 6-, 10-, 14-, and 22.5-mm-diameter disks were incorporated into the medium as previously described. Medium was also prepared without addition of leaf extracts. Metalaxyl was added to achieve final concentrations of 0, 3, 5, 7.5, and 10 ng/ml in all extracts except for the 22.5-mm disk, which had 0, 5, 7.5, 10, 12.5, and 15 ng/ml. The influence of extracts and metalaxyl on radial growth of *P. boehmeriae* was then measured.

Effects of mancozeb on the bioassay. In the United States, metalaxyl is registered for use on potatoes as a mixture with mancozeb (Ridomil MZ 58 contains 10% a.i. metalaxyl and 48% a.i. mancozeb) (1). Therefore, to ensure that mancozeb does not confound the estimation of metalaxyl from field-sprayed plants, we needed to show that *P. boehmeriae* and *P. citrophthora* were not affected by this fungicide under conditions used in this assay. Potato leaflets (cv. Norchip) were dipped into a mancozeb suspension (1.0 mg a.i./ml, Manzate 200) for 5 sec, then shaken gently and allowed to dry. This concentration of mancozeb is equivalent to that achieved when Ridomil MZ 58 is applied at 1.97 kg/ha (1.75 lb/acre) in 935 L/ha (100 gal/acre). Control leaflets were dipped in distilled water. Disks from each leaflet were then assayed as described with both *P. boehmeriae* and *P. citrophthora* to detect any differences in radial growth.

RESULTS

Extraction efficiency. The passive extraction method of soaking leaf disks in methanol for 24 hr is almost as efficient as maceration and centrifugation. Estimates for metalaxyl concentration tended to be slightly greater when tissue was macerated. The mean amount of metalaxyl detected from leaf disks in the passive extraction method was 91.2% as much as the mean for the grinding extraction when metalaxyl was applied by dipping the leaves ($\bar{x}$ = 317.0 ng/cm, SD = 123.0 for grinding extraction; $\bar{x}$ = 278.5 ng/cm, SD = 99.0 for passive extraction; n = 18 for both). Similarly, 86.5% as much metalaxyl was detected in the passive extraction method as in the grinding extraction when metalaxyl was applied via the transpiration stream ($\bar{x}$ = 62.2 ng/cm, SD = 57.4 for grinding extraction; $\bar{x}$ = 53.8 ng/cm, SD = 32.5 for passive extraction; n = 16 for both). However, lower one-sided 95% confidence limits for the differences in extraction methods (grinding minus passive) were both less than 0.0 (−1.2 and −13.9 ng/cm for the dip and transpiration treatments, respectively), indicating that maceration and centrifugation do not extract significantly more metalaxyl than passive extraction at the 5% level of significance (P = 0.06 and P = 0.26, respectively).

Responses of *P. boehmeriae* and *P. citrophthora* to metalaxyl. Radial growth of either *P. boehmeriae* or *P. citrophthora* decreases curvilinearly as metalaxyl concentration increases (Figs. 1A and 2A). The coefficients of variation calculated on radial growth at each metalaxyl concentration for the three replications were 6.4% or less for 0, 5, 10, and 15 ng/ml in the *P. boehmeriae* standard curve and 5.9% or less for all concentrations in the *P. citrophthora* standard curve except 200, 650, and 800 ng/ml. The 200 ng/ml level had a coefficient of variation of 9.8%. The higher concentrations of metalaxyl in both curves had greater coefficients of variation, indicating that estimation at these levels is less precise.

Transformation of the response variable, radial growth (y), was done to linearize the standard curves. The transformation used was $f(y) = y^k$, where k was determined according to the method described by Box and Cox (3). If $k = 0$, then $f(y) = \ln y$ was used. The transformations used for these curves were $f(y) = y^{1/2}$ for *P. boehmeriae* (Fig. 1B) and $f(y) = \ln y$ for *P. citrophthora* (Fig. 2B). Regressing the transformed variable on metalaxyl provided a better fit to these data than the more conventional approaches of regressing untransformed radial growth or probits of percent inhibition on the logarithm of metalaxyl. Linear regressions of $f(y)$ on metalaxyl concentrations have coefficients of determination (R^2) 0.91 for *P. boehmeriae* and 0.96 for *P. citrophthora*. Metalaxyl concentrations were estimated using the regression of $f(y)$ on metalaxyl in the standard curve as a reference. *P. boehmeriae* was used for estimates of metalaxyl in the 5–50 ng/ml range and *P. citrophthora* for estimates in the 50–500 ng/ml range.

Effects of leaf extracts on assay sensitivity. Extracts from the 14- and 22.5-mm-diameter leaf disks reduced the sensitivity of *P. boehmeriae* for detecting small quantities of metalaxyl. Radial growth for each metalaxyl concentration within a leaf disk size was compared to the control (no metalaxyl). For each test observation on metalaxyl, the number of control observations with greater radial growth was recorded. The limit of detection was defined to be the lowest concentration at which 5% or fewer of the comparisons showed test observations greater than the control. The detection limits by this criterion were 5 ng/ml of metalaxyl without leaf extracts and 3, 5, 7.5, and 10 ng/ml of metalaxyl on leaf disks 6, 10, 14, and 22.5 mm in diameter, respectively (Table 1).

When detection limits are expressed per unit area of leaf, the 22.5-mm disks have the lowest limit. Detection limits are: 10.6, 3.8, 4.9, and 2.5 ng/cm^2 for the 6-, 10-, 14-, and 22.5-mm disks, respectively. Although the 22.5-mm disk has the lowest detection when based on leaf area, the variability is greatest for the larger (14- and 22.5-mm) disks, as can be seen by the greater coefficients of variation (Table 1).

Effects of mancozeb on the bioassay. There were no measurable effects of mancozeb on radial growth of either *P. boehmeriae* or *P. citrophthora* under conditions used in this assay. Mean radial growth of *P. boehmeriae* on extracts from mancozeb-treated leaflets was 25.2 mm (SD = 1.14, n = 10), compared with the control with a mean of 25.3 mm (SD = 1.65, n = 10). Mean radial growth of *P. citrophthora* was 27.7 mm for both control and mancozeb treatments (SD = 0.47 for mancozeb and 0.71 for control, n = 10).

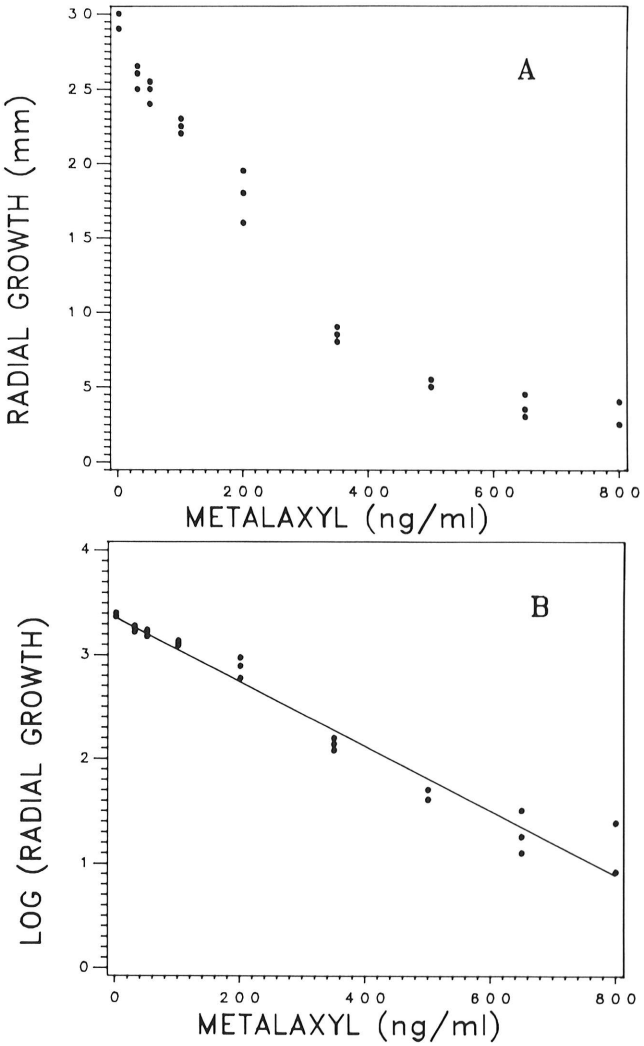

Fig. 2. A, Response of *Phytophthora citrophthora* to known concentrations of metalaxyl added to extracts of 14-mm-diameter leaf disks from the potato cultivar Hudson. Radial growth (mm) was measured after 90 hr of incubation at 24 C. There were three replications per metalaxyl concentration. **B,** Transformed data for *P. citrophthora* plotted against metalaxyl concentration. The transformation used was the natural logarithm of radial growth. R^2 for the plotted regression line is 0.96.

TABLE 1. Effects of extracts from different sized leaf disks on radial growth (mm) of *Phytophthora boehmeriae* on medium amended with small amounts of metalaxyl

	Leaf disk diameter (mm)														
	No leaf extracts			6			10			14			22.5		
Metalaxyl (ng/ml)	Radial growth[a]	C.V.[b]	p[c]	Radial growth	C.V.	p	Radial growth	C.V.	p	Radial growth	C.V.	p	Radial growth	C.V.	p
0	24.3	3.7	na[d]	26.4	2.2	na	26.6	1.7	na	25.8	3.6	na	26.2	3.9	na
3	22.9	4.7	20	24.5	3.8	4	25.1	3.3	6	24.5	5.2	22	...[e]	...	...
5	19.2	3.9	0	22.4	1.9	0	23.7	4.9	2	24.4	7.7	26	24.0	5.9	8
7.5	16.9	4.4	0	21.2	3.9	0	21.8	4.8	0	22.3	7.0	4	23.7	9.5	22
10	15.0	5.8	0	20.2	3.6	0	19.6	4.2	0	22.0	11.7	4	21.8	6.6	0
12.5	...	...	...	...	...	...	...	...	...	...	...	...	20.5	5.2	0
15	...	...	...	...	...	...	...	...	...	...	...	...	17.7	11.9	0

[a] Mean radial growth of *P. boehmeriae*; n = 10 for samples without and 5 for samples with metalaxyl.
[b] Coefficient of variation (%).
[c] Percent of comparisons with radial growth of test observations on metalaxyl greater than control (see text).
[d] Not applicable.
[e] Not done.

DISCUSSION

The described bioassay method for metalaxyl compares favorably with other bioassay methods. The limits of detection are 3–10 ng/ml, depending on the size of the leaf disks. Bailey and Coffey (2) reported a sensitivity of 2 ng/ml when soil is assayed for metalaxyl using the same isolate of *P. boehmeriae*. The addition of potato leaf extracts from 14- and 22.5-mm diameter disks reduced the sensitivity of *P. boehmeriae* to metalaxyl to 7.5 and 10 ng/ml, respectively. Plant extracts also reduced the sensitivity of *Pythium ultimum* Trow to low concentrations of metalaxyl (compare standard curves in Figs. 1 and 3 of Wynn and Crute [14]). Detection limits of 3–10 ng/ml in our method are much more sensitive than Wynn and Crute's detection limit of 25 ng per sample. The greater sensitivity in our method is due not only to using a different bioassay organism but also to incorporating the extracts into only 1 ml of medium, rather than the 4 ml used by Wynn and Crute (14). Edgington et al (6) doubled the sensitivity of a bioassay for benomyl by reducing the volume of medium into which the fungicide diffused. The sensitivity of our method could be increased even more by using 0.75 ml of CMA, but ensuring even coverage of the culture dish with such a small volume of medium is difficult.

Coefficients of variation calculated on radial growth at each metalaxyl concentration in the standard curves are a measure of the precision of this method. At low concentrations in both curves, the coefficients of variation were approximately 6% or less. Coefficients of variation reported for gas chromatography and ELISA were 5.9 and 4.2%, respectively (8). Variability in Wynn and Crute's (14) method is roughly equivalent. Bailey and Coffey (2) claimed to have much less variability than Wynn and Crute using *P. boehmeriae* in their soil assay. The greater variability in our method is due to the effects of leaf extracts from 14-mm-diameter disks. Variability was not as great for small leaf disks.

Mancozeb applied at normal field rates apparently does not interfere with the assay for metalaxyl, thus allowing the assay to be useful when metalaxyl and mancozeb are applied as a mixture. The lack of inhibition of mancozeb on the bioassay organisms under assay conditions is not fully understood. Probably, mancozeb is not fully extracted from the leaf disk in methanol or is not resuspended in the agar, or both. Mancozeb is known to be practically insoluble in most organic solvents and water (12). Furthermore, mancozeb that does resuspend in the agar may be destroyed during autoclaving. Mancozeb is decomposed at high temperatures by moisture (12). Regardless of the mechanism, the important result is that the assay for metalaxyl is not affected.

The major advantage of this method is the number of samples that can be analyzed in a short time. The number of samples per day that can be adequately analyzed by gas chromatography and ELISA has been estimated at only 8–36 (8) (although the estimate of 36 samples per day for ELISA is based on four subsamples). Wynn and Crute (14) estimate that with their method, 300 samples could be analyzed in 3 days (2 days for incubation). With the bioassay method presented here, over 1,000 samples have been analyzed in approximately 10 days (*unpublished*). Furthermore, this included nearly 8 days of incubation that required no active work and during which many more samples were begun. The time saving is attributable to three simplifying procedures. First, tissue samples do not need to be weighed; all metalaxyl determinations are expressed relative to leaf area instead of leaf weight. Second, the extraction process requires only soaking the tissue in methanol; no grinding, centrifugation, or cleanup steps are necessary. In addition, this passive extraction is nearly as efficient as grinding, which was reported to extract 99% of metalaxyl from lettuce leaves (14). Finally, because two bioassay organisms that detect metalaxyl in different ranges are used, no dilution series of samples is necessary. This method is also applicable to other plant parts.

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