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ESTIMATING BENZIMIDAZOLE RESIDUES IN THATCH AND TURFGRASS BY BIOASSAY

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Abstract: Bioassays using pellets of agar, thatch-agar and turfgrass-agar were developed using benzimidazole-sensitive *Penicillium expansum*, to detect the fungicide methyl 2-benzimidazole carbamate (MBC) which is the major fungitoxic degradation product of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] in thatch and turfgrass clippings. These bioassays were used to estimate the amount of fungicide that was biologically available and hence by subtraction from that applied, the amount that remained bound and biologically unavailable. The limit of quantitation was 0.5 mg kg^{-1}. From 19.9% to 93.2% of the applied fungicide was bound by thatch and 46.2% to 56.9% was bound to turfgrass clippings depending on the concentrations used. *In vitro* degradation studies showed that MBC had a half life of approximately 2.5 weeks at 23C in non-sterilized thatch.

1. INTRODUCTION

Bioassay techniques have been developed previously to detect residues of pesticides in plant and soil and to study the dissipation and movement of pesticides in soil.[1-5] Many bioassays for fungicides are based on the diffusion of the fungicide from a paper disc or a sample-agar pellet to media inoculated with spores of the test fungus. The fungicide can then be determined quantitatively from the resulting growth inhibition of the fungus. The inhibition zone from a known quantity of fungicide permits calculation of a standard dosage-response curve from which unknown concentrations of the fungicide in samples can be estimated.[2,4,6]

Chemical analysis of pesticide residues in soil and plant tissue involves many extraction steps, which may be laborious and costly, and only a few samples can be processed in a short period.[7] The measurement of benomyl through chemical analysis is difficult because benomyl decomposes rapidly in solution, plants and soil, and during analysis, to methyl 2-benzimidazole carbamate (MBC).[8-10] The fungicidal activity of benomyl is primarily due to MBC.[8-9]

For turfgrass disease control, Tersan 1991® (50% a.i. benomyl) is usually applied as a wettable powder in water suspension. After application, the recommendation is to water the fungicide into the turf.[11] However, one obstacle preventing the fungicide from reaching the rooting zone in the soil is the presence of thatch.[12-13]

Thatch is a tightly intermingled layer of dead, poorly functioning, and live roots, rhizomes, stolons and organic debris between the zone of exposed leaf blades (turfgrass) and the soil surface.[14] The turfgrass/thatch/soil system can adsorb significant amounts of applied pesticides, reduce the amount reaching the roots in thatch and soil, and reduce plant uptake of the systemic pesticides.[13,15]

In a previous study, we developed bioassays using paper discs and soil-agar pellets and applied them to study the dissipation and movement of MBC in soil using a benzimidazole-sensitive isolate of *Penicillium expansum* Link as a test fungus.[5] The objective of the present work was to further develop bioassays for the detection of total, bound, and biologically available MBC in thatch and turfgrass.

2. MATERIALS AND METHODS

2.1 Thatch-agar and turfgrass-agar pellet bioassays

Thatch collected from creeping bentgrass (*Agrostis palustris* Huds.) at the Cambridge Research Station, University of Guelph, Guelph, Ontario was air-dried and cut into small pieces (< 3 mm). Using a small pressure spray pack (100 ml), standard concentrations of MBC (analytic grade, supplied by Dupont

Canada Ltd.) in thatch were prepared by mixing thatch (100 g) with MBC-methanol solution (50 ml) as uniformly as possible to obtain a concentration range of 0.5 to 50 mg kg^{-1}. The thatch was left in a fume hood overnight to allow methanol to evaporate and then kept in a freezer at -20C.

Samples of freshly mown creeping bentgrass clippings (100 g) were sprayed uniformly with MBC-methanol solutions (10 ml) amended with 0.01% surfactant Aqua-Gro (Aquatrols Corp. of America, Pennsauken, NJ) which increases the uptake of the chemical. The concentrations of MBC ranged from 0.25 to 10 mg kg^{-1}. The clippings were well-mixed and left in a fume hood overnight to allow methanol to evaporate, then stored in a freezer at -20C. The integrity of cells was destroyed by freezing, and this may have permitted MBC which was taken up systemically by the grass to be released into the agar.

Eight samples of thatch or clippings (0.2 g) with different MBC concentrations were placed in separate wells (12.7 mm diam) of a 24-well culture plate (Corning Glass Works, Corning, NY) followed by mixing with 0.8 ml of 45C water agar (1.5%) using a steel needle. After the agar solidified, pellets were transferred onto the middle of a Petri dish (10 cm diam) containing 10 ml of potato dextrose agar (PDA) mixed with 0.1 ml conidial suspension (2×10^6 conidia ml^{-1}) of benzimidazole sensitive *P. expansum* and 0.1 ml streptomycin sulphate (3000 mg $litre^{-1}$). Water-agar pellets without thatch or clippings were amended with MBC dissolved in methanol to give MBC concentrations equivalent to those in thatch- or turfgrass-agar pellets. The bioassay dishes were incubated in the dark at 4C for 24 h and then at 23C for 24 h.

The mean width of the zone of the inhibition (diameter of fungal growth inhibition less the diameter of pellet) was plotted against the log concentration of MBC. The amount of MBC bound to thatch or turfgrass was calculated using the regression equations for the agar pellet bioassay and the thatch- or turfgrass-agar pellet bioassay.

Samples of turfgrass clippings treated with different concentrations of MBC-methanol solution amended with 0.01% Aqua-Gro were tested by both turfgrass-agar pellet bioassay and UV-spectrophotometry after extraction with methanol according to Fernandes and Cole.[16] The results from the two methods were compared. The experiment was repeated.

2.2 Bioassay for degradation of MBC in thatch

Two kilograms of air-dried sterilized or non-sterilized thatch were adjusted to pH 7.0 with NaOH or HCl solution. This was determined by further dilution and testing in water of a subsample. The thatch was then mixed with a MBC-methanol solution to obtain a concentration of 10.0 mg kg^{-1}. The thatch samples were placed in a fume hood overnight to allow methanol evaporation, and 200 g were placed in a biometer flask.[17] The thatch was adjusted to a moisture content of approximately 80% field capacity with sterilized water. The top opening of the flask was sealed with parafilm. The flasks were incubated in the dark at 23C. There were four replicates.

At weekly intervals for 5 weeks, thatch equivalent to 40 g dry weight was removed from each flask and kept at -20C until testing. The thatch was then air-dried and four subsamples (0.2 g each) tested for MBC concentration with the thatch-agar pellet bioassay. Three subsamples (12 g each) were extracted, cleaned-up and measured at 282 nm with a spectrophotometer following the procedure of Austin and Briggs.[18] The experiment was repeated.

2.3 Statistical analysis

Regression was performed between width of the zone of inhibition and log concentration of MBC. Student's *t*-tests ($P = 0.05$) were performed to compare the results from turfgrass-agar and thatch-agar pellet bioassays against UV-spectrophotometry.

3 RESULTS AND DISCUSSION

3.1 Thatch-agar and turfgrass-agar pellet bioassays

Standard curves for agar pellet, thatch-agar pellet, and turfgrass-agar pellet bioassays were established by plotting the log concentration of MBC against the width of the zone of inhibition (Figs. 1 and 2). There was a linear relationship between width of the zone of inhibition and log concentration of MBC for all

bioassays, and the relationships were very strong ($r^2 > 0.99$). The regression lines for agar pellet bioassay reflected the full effects of a concentration of MBC without pesticide binding. By comparing this regression against the regression for treated thatch samples or treated grass samples, we could estimate the amount bound to thatch or grass. Portions of a pesticide tightly bound to thatch or turfgrass usually cannot be extracted with water or even methanol,[7,19] and are generally considered biologically unavailable.[19,20]

The slopes of the regression lines for the thatch bioassay (Fig. 1) had a different pattern than the slopes of the grass bioassay regression lines (Fig. 2). In the grass bioassay (Fig. 2), the slopes of the agar pellet and the turfgrass-agar pellet regression lines were parallel, with the gap between the lines reflecting a consistent proportion of residues bound in the grass. The thatch bioassay had regression lines for thatch-agar pellet and agar pellet bioassays that differed from each other (Fig. 1). This indicated that the proportion of the fungicide bound by thatch was not constant, but was proportional to the MBC residue levels. It also showed that thatch could retain a larger, presumably bound, portion of the MBC than turfgrass. Similar results have been reported by Niemczyk and Filary[12] who found that despite 1.25 cm of irrigation water immediately after treatment, 96-99% of the residues of nine applied pesticides remained adsorbed by the thatch during the seven days following the application.

The amount of MBC bound to thatch could be calculated using the regression equations for agar pellet and thatch-agar pellet bioassays. For example, from the dosage-response curve (Fig. 1) for the thatch containing 10.0 mg kg^{-1} of MBC, the amount of MBC adsorbed by the thatch was calculated using the following formulae:

$Y_1 = 14.5 + 29.9 \text{ Log } X_1$, ($r^2 = 0.998$). (Equation 1).

$Y_2 = 6.9 + 13.8 \text{ Log } X_2$, ($r^2 = 0.995$). (Equation 2).

Equation 1 is the regression for agar pellet bioassay, where X_1 is the concentration of MBC in thatch in a corresponding thatch-agar pellet. Equation 2 is the regression equation for thatch-agar pellet bioassay, where X_2 is the concentration of MBC in thatch.

The amount of MBC bound to thatch can be calculated as follows:

Let $Y_1 = Y_2$; then, if $X_2 = 10.0$ mg kg^{-1}, X_1 can be calculated by equating the two equations:

$14.5 + 29.9 \text{ Log } X_1 = 6.9 + 13.8 \text{ Log } (10.0)$, therefore $X_1 = 1.61$.

MBC bound to thatch: $[(X_2 - X_1) / X_2] = [(10.0 - 1.61)/10.0] = 83.9\%$ at 10.0 mg kg^{-1}.

The amount of MBC bound to the turfgrass clippings could be calculated in the same way as that for thatch but using regressions equations derived for the grass bioassay (Fig. 1). For turfgrass clippings, 46.2% to 56.9% of the MBC originally applied was bound (Table 1). This small variation is consistent with parallel regression lines found in the grass bioassay (Fig. 2). For thatch, 19.9% to 93.2% of the MBC originally applied was bound (Table 1). As previously noted, the regression lines of the thatch bioassay (Fig. 1) differed significantly which allowed for greater proportions of residues to be bound with increased fungicide concentration. The reason for this differential binding of fungicide residues in thatch at increased fungicide concentrations is uncertain. Perhaps at low concentrations, the fungicide becomes attached to superficial binding sites, whereas with high concentrations, the fungicide binds to sites more strongly and permanently.

3.2 Comparison between thatch- or turfgrass-pellet bioassays and spectrophotometry

The degradation of MBC in non-sterile thatch as tested by spectrophotometry and thatch-agar pellet bioassay was not significantly different ($P = 0.05$) at weekly intervals during the 5-week study (Fig. 3). Results using both methods showed a half life for MBC of 2.5 weeks in thatch *in vitro*. There was no significant degradation of MBC in autoclaved thatch. Only thatch which was not sterilized showed significant degradation of MBC (Fig. 3). Microorganisms were presumed to be responsible for the degradation.

Previous work has found the half-life of total benzimidazole residues to be 3-6 months in turf and 6-12 months in bare soil.[21] Degradation studies of benomyl in thatch have not been previously reported, although an accelerated rate of pesticide degradation is found in the thatch layer.[22,23]

For turfgrass clippings that were spiked with a known amount of MBC, the extracts did not differ significantly ($P = 0.05$) in MBC concentration when tested either by the turfgrass-agar pellet bioassay or the

UV-spectrophotometry method (Table 2). More detailed comparisons between soil-agar pellet bioassay and UV-spectrophotometry were made in our previous studies which showed no significant difference.[5]

The limit of quantitation of this bioassay technique was found to be 0.5 mg kg^{-1}.[5] Although chemical analyses such as spectrophotometry or chromatography may be able to quantify much lower concentrations, residue levels that are required to control turfgrass diseases are well above this amount.[24]

4 CONCLUSIONS

The bioassays developed were fast, less costly and could be used to directly detect the biologically-available MBC in thatch and turfgrass. Using agar pellets amended with the same concentration of fungicide as in thatch-agar or turfgrass-agar pellets, the fungicide bound to thatch or turfgrass was estimated. This method to detect fungicide adsorption or binding did not require extraction processes necessary in most chemical analyses. Thatch-agar pellet bioassay was also used to study the degradation of the fungicide in thatch. Turfgrass, unlike other crops is frequently mowed, and the fungicide levels in clippings can vary greatly with time after fungicide treatment depending on frequency of mowing. The turfgrass-agar pellet bioassay could be used as a tool to monitor the fungicide levels in turfgrass clippings, which may then be used to determine the critical time for fungicide treatment. One drawback of these bioassays in field application is lack of specificity, since other fungitoxic substances may affect the bioassay fungus. This may be a major limitation if such bioassays were to be used in regulatory processes; however, the bioassays can be used to further studies of MBC dissipation and factors which may limit or increase uptake of MBC in turfgrass.

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TABLE 1

MBC Concentration and Proportion Bound by Thatch and Turfgrass Clippings Estimated by Agar Pellet and Thatch- or Turfgrass-Agar Pellet Bioassay.

MBC concentration ($mg\ kg^{-1}$)	MBC bound (%)	
	Thatch	Turfgrass
0.5	19.9[a]	46.2
1.0	44.3	48.0
2.0	61.7	49.7
4.0	73.6	51.3
6.0	78.8	52.3
8.0	81.8	52.9
10.0	83.9	53.5
20.0	88.9	55.0
40.0	92.4	56.5
50.0	93.2	56.9

[a] Each number is the mean of data from two experiments with four replicates in each experiment.

TABLE 2

MBC in Turfgrass Clippings Estimated by Turfgrass-agar Pellet Bioassay and UV-spectrophotometry.

Method of analysis	Concentration of MBC ($mg\ kg^{-1}$) Sample Number 1	2	3	4
Turfgrass-agar pellet	0.8a[a]	2.3a	4.8a	8.2a
UV-spectrophotometry	0.9a	2.1a	4.5a	7.9a

[a] Means with the same letter in a column are not significantly different from each other at $P = 0.05$ (*t*-test). Each number is the mean of combined data from two experiments with eight replicates for turfgrass-agar pellet bioassay and four replicates for UV-spectrophotometry in each experiment.

Fig. 1. Relationship between width of the zone of inhibition and concentration of MBC in thatch tested by agar pellet bioassay (○ $Y_1 = 14.5 + 29.9\text{Log}X_1$, $r^2 = 0.998$) and thatch-agar pellet bioassay (□ $Y_2 = 6.9 + 13.8\text{Log}X_2$, $r^2 = 0.995$). Each data point is the mean of combined data from two experiments with eight replicates in each experiment (standard error bars shown).

Fig. 2. Relationship between width of the zone of inhibition and concentration of MBC in grass tested by agar pellet bioassay (○ $Y_1 = 18.4 + 26.8\text{Log}X_1$, $r^2 = 0.994$) and grass-agar pellet bioassay (□ $Y_2 = 10.8 + 25.5\text{Log}X_2$, $r^2 = 0.994$). Each data point is the mean of combined data from two experiments with eight replicates in each experiment (standard error bars shown).

Fig. 3. *In vitro* degradation of MBC in thatch over five weeks, determined by thatch-agar pellet bioassay (□) and spectrophotometry (○). Results from the two methods were not significantly different ($P = 0.05$) from each other at each time interval (*t*-test). Each data point is the mean of combined data from two experiments with four replicates for thatch-agar pellet bioassay and three replicates for spectrophotometry in each experiment.