Quantitative Estimation of the Fungicide Benomyl Using a Bioautograph Technique

When the fungicide benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] was dissolved in water or chloroform, two fungitoxic chemicals were detected. One was the original benomyl and the other, a breakdown product (methyl 2-benzimidazolecarbamate). The breakdown product was relatively stable in aqueous solution and within plants, but the benomyl disappeared within a week. Fungitoxic chemicals were determined both quantitatively and qualitatively using a bioautograph technique in which a thin-layer plate was sprayed with a mixture of agar and *Penicillium* spores; the diameter of the zone of inhibited growth above the fungitoxic spot was related to the amount of fungitoxic chemical in the spot.

Erwin *et al.* (1968) have described a bioassay method for the quantitative determination of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate], but their procedure did not allow for a separation of possible fungitoxic breakdown products before assay.

In the present study, a bioautograph technique similar to those of Ferenczy *et al.* (1968) and Dekhuijzen (1964) was used for identification and quantitative determination of fungitoxic materials. This communication describes preliminary results on the breakdown of benomyl in aqueous solution and in plants.

EXPERIMENTAL

A standard solution of benomyl at 5 p.p.m. (technical grade, duPont, Wilmington, Del.) was prepared in chloroform. Small volumes were spotted on a heat-activated Eastman Chromagram sheet 6061 (silica gel without fluorescent indicator) so as to give a series of standard amounts of benomyl ranging from 0.03 to 0.4 μg. Spot sizes were kept as uniform and small as possible. The chromatogram was run using acetone as the solvent system. Following drying, the plate was sprayed with a suspension of agar and spores of *Penicillium sp.* of the cyclopium series (Raper and Thom, 1949) prepared as follows: A spore suspension in 1% glucose and 0.2% KNO_3 with 0.15 absorbance at 450 mμ was mixed with a 1.5% solution of warm malt agar (spore suspension–agar, 7.5 to 10) and a thin layer of this mixture was sprayed onto the chromatogram. The plate was incubated in a humid chamber for 20 hours at room temperature. Fungitoxic spots then appeared as clear areas against the opaque growth of the fungus. As seen in Figure 1, two fungitoxic spots, A and B, were present in each case. A represents the original benomyl and B, a fungitoxic breakdown product. This breakdown product was identified as methyl 2-benzimidazolecarbamate [as suggested by Ryker (1968)] by observing that it chromatographed with the same R_f as a standard in two solvent systems. The diameters of the spots increased linearly with an increase in the log of the concentration of benomyl applied (Figure 2). As expected, best linearity was obtained when one solution and one pipette were used to spot the samples, different amounts being obtained by applying different volumes. Bioautography of a series of amounts of B (technical grade >95% supplied by duPont) showed that the equilibrium concentration of benomyl as applied to the plates from chloroform solutions was about 46% B by weight.

This technique was used to study the breakdown of benomyl in leaves of *Phaseolus vulgaris* L. cultivar, Tender Green. The seeds were germinated in moist vermiculite for 7 days and then transferred to one-half strength Knop's solution until the first trifoliate leaves were mature. The plants were then placed in a saturated solution of benomyl (approximately 40 p.p.m.) for 4 days. Following treatment, the roots were rinsed thoroughly and the plants returned to nutrient solution. At this time, only B remained in the treatment solution.

To determine the initial variation in the amounts of B present in the three leaflets of the first trifoliate leaf, all three leaflets were harvested at once from four plants. Fresh weights of leaflets were determined and each was extracted by grinding in a Ten Broeck homogenizer in 10 ml. of chloroform. Any fibrous plant material remaining was removed and the

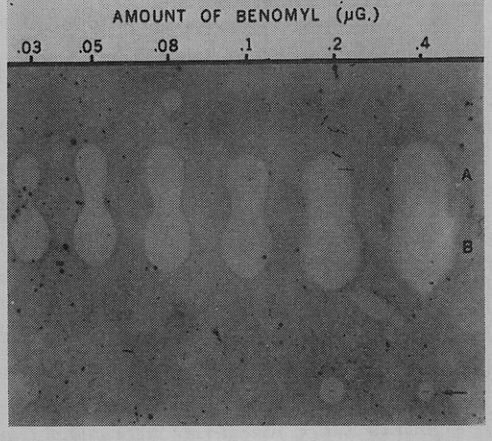

Figure 1. Bioautograph of a series of amounts of benomyl (solvent system, acetone)
Arrow indicates line of origins

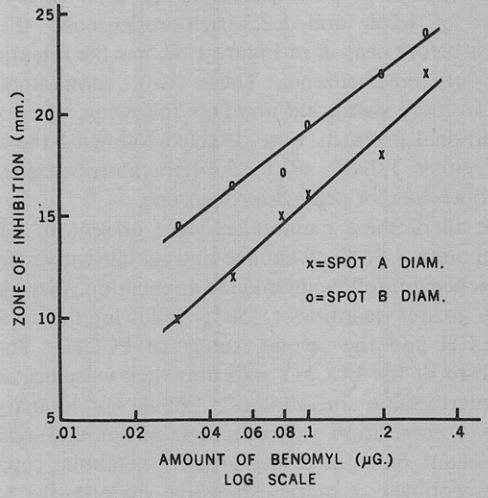

Figure 2. Graph showing semilogarithmic relationship between diameters of spots (Figure 1) and amounts of benomyl applied to the chromatogram

chloroform extract dried under vacuum at room temperature. The residue was redissolved in 5 ml. of chloroform and a 5-μl. sample was used for chromatography. Table I gives the amounts of B extracted from each of the leaflets. No significant differences (at the 5% level of statistical significance) in the amounts of B (on a fresh weight basis) were found among the three leaflets of each leaf, indicating that the amount of benomyl present in one of the leaflets gives a good estimation of the amount in the other two. Consequently, the stability of B was studied by analyzing leaflets over a time period. In four replicate plants, one leaflet was removed from each trifoliate leaf 1 day after treatment, the second was removed 3 days after treatment, and the third was removed 7 days after treatment. The results of this study are presented in Table II. A statistical analysis showed that B (expressed on the basis of fresh weight) was not significantly altered at the 5% level of significance.

Table I. Amounts of B(μG./G. Fresh Wt.) Extracted from Leaflets of the First Trifoliate Leaves, of *P. vulgaris*[a]

	Plant			
Leaflet	1	2	3	5
1	341	251	44	14
2	116	195	42	15
3	164	254	52	19

[a] Leaflets were harvested simultaneously.

Table II. Amounts of B(μG./G. Fresh Wt.) Extracted from Leaflets of the First Trifoliate Leaves of *P. vulgaris*[a]

		Plant			
Leaflet	Day	1	2	3	4
1	1	209	274	212	44
2	3	214	210	304	39
3	7	207	220	310	38

[a] One leaflet from each plant was harvested 1, 3, and 7 days following the 4-day period of uptake of the fungicide.

DISCUSSION

The bioautograph method is extremely sensitive, capable of detecting less than 0.05 μg. of benomyl, while the agar plate bioassay as used by Erwin *et al.* (1968) will detect about 0.7 μg. in our experience. The bioautograph is also about 10-fold more sensitive than ultraviolet light for detection of benomyl. The diameters of the zones of inhibition in our bioassay are linearly related to the log of the concentration of the fungicide.

Since benomyl breaks down to compound B in aqueous solution within 4 days, one would expect to obtain only compound B in plants after applying the fungicide in an aqueous solution to the roots for 4 days. Compound B appears to be stable in plants, at least for 6 days, but this does not indicate it is unaltered in its distribution pattern within leaves, or leaflets, during this period. Although benomyl breaks down completely to compound B in aqueous solution, the proportions of benomyl to B do not change in a month's time when the benomyl is dissolved in chloroform or acetone. This difference in stability of benomyl in an aqueous *vs.* a nonaqueous medium is to be expected since the breakdown reaction is hydrolytic. Although the method reported here has been used only for benomyl and its breakdown product, it could readily be adapted for quantitative measurements of many other chemicals since the diameter of the spot is dependent on diffusion. Theoretically, any organism whose growth is significantly depressed or enhanced by a chemical could be used in a similar bioautograph for that chemical.

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Carol A. Peterson
L. V. Edgington

Department of Botany
University of Guelph
Guelph, Ontario, Canada

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