Systemic Movement in Herbaceous Plants of Benomyl and Its Methyl Isocyanate Homologue

Lesley Crawford and Alan W. Bown

Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada

Mikio Chiba*

Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, Vineland Station, Ontario L0R 2E0, Canada

^{14}C-labeled Azindoyle (MBC-MIC), the methyl isocyanate homologue of benomyl, was found to exhibit more systemic movement than benomyl in herbaceous plants. Of the total radioactivity applied to the leaf surface, the movement was mainly toward the growing tips, with some sign of downward movement. On broad beans the percentage of translocated activities were 31.5 and 12.0% of that applied for Azindoyle and benomyl, respectively. Both Azindoyle and benomyl were relatively stable at the site of application, and after 48 h, the percentages of intact parent compound were 47.5 and 52.2% for Azindoyle and benomyl, respectively. However, after 48 h, the percentage of parent compound at the translocated site was lower than that at the site of application, at 6.8 and 3.8% for Azindoyle and benomyl, respectively.

Keywords: *Azindoyle; benomyl; benomyl homologues; systemic movement; radioautographs*

INTRODUCTION

Benomyl, methyl [1-(butylcarbamoyl)-1*H*-benzimidazol-2-yl]carbamate (MBC-BIC), is a widely used fungicide effective against many diseases of commercially important crops. However, its repeated use has resulted in the selection of fungal strains resistant to benomyl (Dekker, 1976; Delp, 1980; Ishii et al., 1984, 1985; Northover, 1986; Northover and Matteoni, 1986), and an effective substitute is required. Chiba and Northover (1988) and Northover and Chiba (1989) demonstrated that methyl, ethyl, and propyl isocynate homologues of benomyl (MBC-MIC, MBC-EIC, and MBC-PIC, respectively) were effective against benomyl-resistant *Botrytis cinerea* Perf.: Fr., one of the most important pathogens of grapes (Northover and Matteoni, 1986). The three homologues were effective against both benomyl-sensitive (S) and benomyl-resistant (R) isolates of *B. cinerea*, and the use of these homologues to overcome benomyl resistance was patented in the United States (Chiba, 1989). Azindoyle is a trade name registered by Sanex Inc. for these homologues. When germ tube elongation was measured, they were as effective as benomyl against the S isolate but more effective against the R isolate than benomyl. The sensitivity of spore germination of the S and R isolates to MBC-EIC and MBC-PIC was the reverse of their sensitivity to benomyl, which constitutes an example of negative cross-resistance. These findings clearly indicate that the mode of action of these homologues against R isolates is not the same as that of benomyl, despite the fact that these homologues, like benomyl, produce carbendazim (MBC), methyl 2-benzimidazolecarbamate, as a common major degradation compound after their application. The primary mode of action of MBC is generally considered to be its binding to tubulin (Ishii and Davidse, 1986), and the resistance of *B. cinerea* against benomyl is due to the reduced binding of MBC to tubulin (Davidse, 1986).

Systemic movement of benomyl and MBC has been reported for several herbaceous plant species (Upham and Delp, 1973). They used ^{14}C-labeled benomyl and MBC and found that benomyl was substantially more mobile than MBC when applied to the leaf surface. Systemic movement is a very important characteristic for any fungicide. Here we report studies conducted to investigate the relative systemic movement of Azindoyle, benomyl, and MBC on kidney bean, broad bean, and apple seedlings.

MATERIALS AND METHODS

Application of Radiolabeled Azindoyle, Benomyl, and MBC to Plant Material. Radiolabeled Azindoyle, benomyl, and MBC, each with the benzene ring uniformly labeled with ^{14}C, specific activity of 1.15 mCi/mmol, and radiochemical purity >95%, were provided by Dalton Chemical Laboratories, Inc., North York, ON. Aqueous suspensions were prepared by first vigorously grinding the chemical with an equal quantity of blank formulation (containing the same ingredients as Azindoyle WP except the active ingredient), such that the particle size of the labeled active ingredient was <3 μm in length. The blank formulation was provided by CBR Research International Corp., Sidney, BC. All plants were grown in Agriculture and Agri-Food Canada greenhouses, but all experiments were carried out at Brock University. The surfactant Agral 90 (supplied by I.C.I. Chipman, Stoney Creek, ON), at 0.02 μL/mL, was added to the suspension when applications were to be made to plant materials. Translocation was studied in mature broad beans [*Vicia faba* cv. Toto (left leaf of the fourth doublet)], kidney bean [*Phaseolus vulgaris* cv. California Red (middle leaf of the mature first trifoliate)], and McIntosh apple seedlings [*Malus* × *domestica* Borkh. (sixth leaf)]. Application of the aqueous suspension, 250 μg (active ingredient)/mL (ppm), was to the abaxial surface, adjacent to the midrib, at the midpoint of the leaf. The veins of the leaf were

10.1021/jf980357a CCC: $15.00
Published on Web 11/19/1998

used as a pattern to define the actual application area, and a micropipet was used to apply 10 μL of the suspension to an area approximating 1 cm^2. A second 10 μL aliquot was transferred to a scintillation vial to measure the total radioactivity applied to the leaf. The leaves were held horizontally for 15 min for drying. Plants were kept in a growth chamber for 48 h at 23 °C, 50−60% relative humidity, with a 16 h light and 8 h dark cycle.

Extraction of Radiolabeled Products of Azindoyle and Benomyl from Plant Materials. After incubation, treated leaves were sectioned with a razor. Sections analyzed included the base (closest to the petiole), the actual application area, and the tip (furthest from the petiole). The different sections were immediately placed in 0.01 N NaOH solution (pH 12) and ground in a mortar for 10 min. This procedure was a modification of the orginial method reported by Chiba and Singh (1986). After 30 min (for Azindoyle) and 60 min (for benomyl), these parent compounds were quantitatively converted to either 3-methyl-2,4-dioxo[1,2-*a*]-*s*-triazinobenzimidazole (STM) or 3-butyl-2,4-dioxo[1,2-*a*]triazinobenzimidazole (STB), respectively. No side products were detected while MBC already present was unaffected (Lundrigan, 1997). The slurry was washed with ethyl acetate (3:1 v/v ethyl acetate/0.01 N NaOH) and centrifuged for 10 min at 2350*g*. This procedure was repeated three times to collect the labeled breakdown products in the ethyl acetate fraction. The fractions were combined, and ethyl acetate was removed under vacuum with a rotary evaporator. The residue was redissolved in either 1 mL of MeOH (Azindoyle) or acetonitrile (benomyl) and used for scintillation counting and thin-layer chromatographic (TLC) analysis. An aliquot of the NaOH fraction was also counted for radioactivity to confirm transfer of all the labeled products to the ethyl acetate fraction. Samples were counted in a Beckman 1800 LS scintillation counter and corrected for quenching using an internal standard. Counts were converted to a percent recovery of the total radioactivity applied to the leaf and normalized.

Separation and Identification of Breakdown Products of Azindoyle and Benomyl. Redissolved samples were sonicated in a water bath for 10 min before being spotted on plastic-backed silica gel TLC plates containing a fluorescent indicator (silica gel 60 F254). A total of four unlabeled reference compounds [MBC, 2-aminobenzimidazole (2-AB), STM, or STB and 1-(2-benzimidazolyl)-3-*n*-methylurea, (BMU) or 1-(2-benzimidazolyl)-3-*n*-butylurea (BBU)] were spotted at the origin of each sample. The plates containing samples from Azindoyle-treated leaves were developed to 7.5 cm in ethyl acetate, methanol, and ammonium hydroxide (100:25:1 v/v/v; Baude et al., 1973). For benomyl, however, the plates were developed to 15 cm in the above system to separate MBC, BBU, and 2-AB. STB and STM remained at the origin in the above TLC systems. Consequently, once the plates had dried, they were developed 6 cm in a second solvent system, consisting of ethyl acetate, dioxane, methanol, and ammonium hydroxide (32:4:1:1 v/v/v/v; Baude et al., 1973), to move STB and STM from the origin. Compounds were visualized and their areas noted using a UV illuminator. The visualized areas were scraped from the plastic-backed plates and dissolved in MeOH in a scintillation vial, and a scintillant (ACS, Amersham) was added prior to radioactive counting. Recovery of ^{14}C activity by this method was >95%. The percentage of the total radioactivity recovered as STM or STB was used to represent the respective parent compound.

Autoradiography of Intact Plant Tissue after Application of Azindoyle and Benomyl. Plants were treated and incubated as outlined above. At the end of 48 h, the treated leaves were placed between sheets of glass and pressed at −20 °C. The tissue was then covered with a single layer of plastic wrap and placed on an autoradiographic film (Biomax MR film, Amersham). The exposed film was kept at −70 °C and developed at the end of 35 days of exposure.

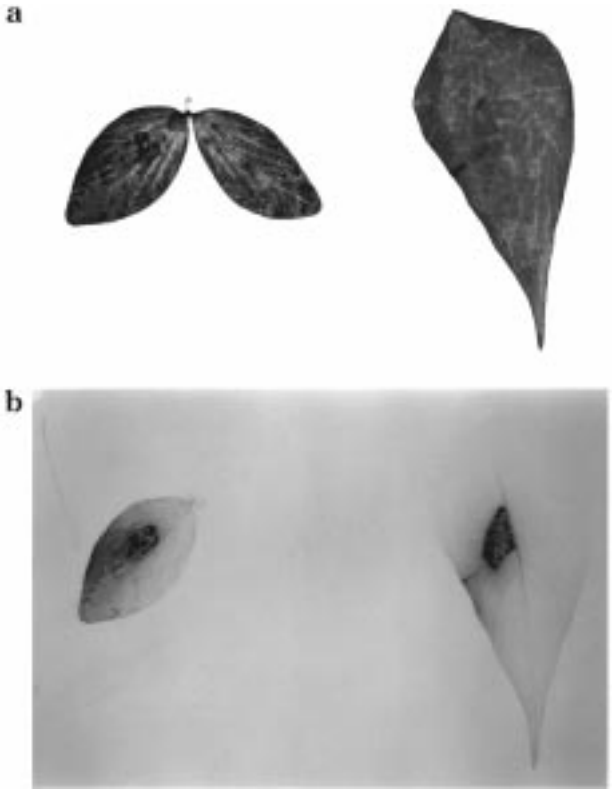

Figure 1. Broad bean (left) and kidney bean (right) leaves treated with ^{14}C-MBC-MIC (a) and the corresponding autoradiograph (b). Treated sections are the darkened areas along the midrib at the center of each leaf (b).

RESULTS AND DISCUSSION

Translocation of Radioactive Compounds. The autoradiographs clearly indicate the translocation of ^{14}C-labeled compounds by darkening of the film at areas not corresponding to the leaf application area. The application area is the darkest spot along the midrib of the leaf. Movement of ^{14}C-containing compounds, from Azindoyle, in both broad and kidney bean leaves is generally outward along the veins and toward the tip of the leaf, furthest from the petiole, (Figure 1). Although the trend was similar with ^{14}C-benomyl, the movement of ^{14}C-MBC toward the tip was not observed (data not shown). These data support similar results obtained after application of ^{14}C-benomyl and ^{14}C-MBC to leaf surfaces of herbaceous plants (Baude et al., 1973; Upham and Delp, 1973).

Quantification of the differences in the amount of translocated product for each treatment was determined by sectioning the treated leaves and extracting ^{14}C-containing compounds from the tissue with NaOH (pH 12) and ethyl acetate. The results of scintillation counting indicate that in all of the plants tested, radioactivity from Azindoyle was translocated more than that from benomyl (Table 1). In all cases the major destination after translocation was the tip of the leaf (Table 1). These data are in agreement with previous studies using benomyl (Upham and Delp, 1973). In Azindoyle-treated broad bean leaves 31.5% of the recovered ^{14}C had been translocated to the tip, base side leaf, and petiole. In contrast, in benomyl-treated broad bean leaves only 12.0% of the recovered ^{14}C had been translocated from the application area. In Azindoyle-treated kidney bean leaves 12.1% of the recovered ^{14}C had been translocated, whereas only 0.6% ^{14}C had been

Table 1. Percent Distribution of Radioactive Carbon from ^{14}C-Azindoyle and ^{14}C-Benomyl within the Leaf

leaf type	leaf location	% of recovered activity (SE) Azindoyle ($n = 5$)	benomyl ($n = 6$)
broad bean	application area	68.5 (3.0)	88.0 (1.8)
	tip	16.6 (3.4)	8.0 (0.8)
	base	5.6 (0.9)	0.8 (0.5)
	side leaf	7.1 (2.2)	1.4 (0.9)
	petiole	2.2 (1.8)	1.8 (1.5)
kidney bean	application area	87.9 (3.3)	99.4 (0.4)
	tip	11.1 (2.3)	0.6 (0.4)
	base	1.0 (1.0)	0
apple	application area	93.4 (1.5)[a]	93.5 (3.0)
	tip	6.2 (1.6)[a]	3.1 (1.7)
	base	0.1 (0.1)[a]	1.4 (0.9)
	fifth leaf	0.1 (0.1)[b]	2.1[c]
	seventh leaf	0.3 (0.2)[b]	0[c]

[a] $n = 7$. [b] $n = 3$. [c] $n = 2$.

Table 2. Percent Distribution of Radioactivity Identified as Parent Compound and MBC in Different Parts of Leaves after Application of ^{14}C-Azindoyle and ^{14}C-Benomyl to Bean Leaves

leaf type	leaf location	Azindoyle ($n = 5$) MBC	parent	benomyl ($n = 5$) MBC	parent
broad bean	application	52.5 (2.8)	47.5 (2.1)	47.8 (6.2)	52.2 (6.2)
	tip	93.2 (5.8)	6.8 (5.8)	96.2 (1.1)	3.8 (2.5)
kidney bean	application	44.1 (3.1)	55.9 (3.8)	53.3 (9.5)[a]	46.7 (9.5)[a]
	tip	97.0 (1.8)	3.0 (1.8)	100[b]	0[b]

[a] $n = 6$. [b] $n = 3$.

translocated in benomyl-treated leaves. There was clear evidence for translocation of both Azindoyle and benomyl on apple seedlings, but the difference between the two was substantially less than that found on the bean plants (Table 1).

Preliminary data also indicated translocation of ^{14}C from Azindoyle in grape leaves (cv. Chardonnay). In addition, the amount of translocated ^{14}C from Azindoyle in apple leaves increased to 21.0% at the tip when given a 96 h incubation period. This is a substantial increase from 6.2% after a 48 h incubation. In this last experiment ^{14}C was also detected in the base section of the apple leaf. These results indicate that the movement of Azindoyle was slower in apple seedlings than in broad beans and kidney beans and also that more radioactivity could be translocated in both broad beans and kidney beans if given a longer period of time.

Identification of Compounds at the Application Site and after Translocation. Forty-eight hours after application, Azindoyle, benomyl, and their breakdown products were identified by TLC of the extracted samples. At the point of application of Azindoyle and benomyl to bean leaves, ~50% of the recovered ^{14}C was found to be MBC and 50% was in the intact parent compound (Table 2). These results are similar to previous studies showing 25% breakdown of benomyl 72 h after application to the fully expanded third trifoliate of pinto beans (Baude et al., 1973). In contrast, the percentage of parent compound at the tip of broad bean leaves after translocation was substantially less, being 6.8 and 3.8% for Azindoyle and benomyl, respectively. Corresponding data for kidney beans were 3.0 and 0%. The balance was MBC (Table 2). It is clear that the percent of the parent compound at the translocation site is substantially lower than that at the application site. Because the movement of MBC is limited (Baude et al., 1973; Upham and Delp, 1973; this study), these data indicate that translocation of the parent compound to the tip occurred prior to degradation to MBC.

In conclusion, the data obtained in this study consistently indicated that a larger percentage of radioactivity was translocated after application of Azindoyle compared to benomyl. Also, the data consistently indicated that compared to benomyl a larger percentage of intact parent compound was found at a translocation site after application of Azindoyle to herbaceous plant leaves. These findings clearly indicate that Azindoyle exhibits a very favorable characteristic as a fungicide by demonstrating systemic movement superior to that of benomyl.

Factors Affecting the Penetration and Translocation of Fungicides. The performance of pesticidal chemicals when applied to leaf surfaces is markedly influenced by their penetration and translocation in plant tissues. The physicochemical properties of these chemicals must be considered when penetration and translocation are discussed (Yanase and Andoh, 1992; Baker et al., 1992). In a study of the translocation of 21 photosynthesis-inhibiting herbicides, the strongest positive correlation was found between translocation and water solubility. There was little correlation between translocation and photosynthesis-inhibiting activity (Yanase and Andoh, 1992). These findings support the results presented here. As indicated in Table 1, translocation was more obvious with Azindoyle than with benomyl in all three plant species tested. This is explained by the greater water solubility of Azindoyle than of benomyl. Azindoyle is 10 times more soluble than benomyl (10 and 1 μg/mL, respectively) in the pH range of 3−10 (Lundrigan, 1997). The study found that the rates of decomposition of both fungicides were little different between pH 3 and 6, although both decomposed more rapidly at pH >10. Also, both compounds decomposed at ~100 °C without showing any sign of melting (Lundrigan, 1997). These results indicate that, apart from water solubility, it is difficult to demonstrate the significance of a specific physicochemical property of these two fungicides which correlates with the extent of translocation.

Another study demonstrated that it is difficult to prove a clear correlation between rates of penetration and physicochemical properties of active ingredients and formulants. This study used data from 26 chemicals (herbicides, fungicides, growth regulators, insecticides, and model compounds) and regression analysis to demonstrate poor correlations between uptake (foliar penetration) and individual variables (partition coefficient, water solubility, molecular weight, and melting point). Penetration rates, however, were greatest with low-melting, lipophilic compounds. It was also found that chemicals penetrated more rapidly into waxy leaves of rape and strawberry than into less waxy sugar beet leaves (Baker et al., 1992). This aspect was also observed in the present study, where the percentage uptake was substantially higher with broad beans than with kidney beans (Table 1; Figure 1). The former is substantially more waxy than the latter.

Although the presence of the surfactant Ethylan TU enhanced uptake in the first 24 h after droplet application, many chemicals entered leaves in significant amount in the absence of the surfactant (Baker et al., 1992). However, in studies with Azindoyle and benomyl, the use of an appropriate surfactant was essential

to suspend these fungicides in water (Chiba and Northover, 1988). In the present study, a blank WP formulation (which contained a surfactant) was used to suspend the labeled compounds in water, and prepared aqueous suspensions were applied to leaf surfaces. In addition, chemicals were ground to <3 μm particle length before making an aqueous suspension, because preliminary findings indicated that the fungicidal efficacy was substantially better when the particle size was smaller. Similarly, other authors observed that uptake was increased as the size of the crystals decreased (Baker et al., 1992).

In summary, Azindoyle exhibits greater foliar translocation than benomyl, and this characteristic correlates with Azindoyle's greater solubility in water. Field experiments will be required to substantiate the results presented, which were obtained under controlled laboratory conditions.

ABBREVIATIONS USED

Azindoyle, MBC-MIC, methyl [1-(methylcarbamoyl)-1*H*-benzimidazol-2-yl] carbamate; benomyl, MBC-BIC, methyl [1-(butylcarbamoyl)-1*H*-benzimidazol-2-yl] carbamate; carbendazim, MBC, methyl 2-benzimidazolecarbamate; STM, 3-methyl-2,4-dioxo[1,2-*a*]-*s*-triazinobenzimidazole; STB, 3-butyl-2,4-dioxo[1,2-*a*]-*s*-triazinobenzimidazole; BMU, 1-(2-benzimidazolyl)-3 -*n*-methylurea; BBU, 1-(2-benzimidazolyl)-3-*n*-butylurea; 2-AB, 2-aminobenzimidazole.

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Received for review April 6, 1998. Revised manuscript received September 1, 1998. Accepted September 25, 1998.

JF980357A