Transcuticular Movement of Fungicides

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ABSTRACT

Movement of fungicides through cuticle, chemically removed from apple leaves, was studied in a system in which a fungicide was applied to the external side of a cuticle, and the diffusion of the fungicide into potato-dextrose agar underneath the cuticle was bioassayed. In 24 hr, 0 to 87% of the dosage of fungicides applied to a cuticle disk had moved through it. Transcuticular movement of fungicides was more efficient with cuticles isolated from the abaxial than from the adaxial surface of leaves. Efficiency of transcuticular movement of benzimidazole fungicides decreased in the following order: thiophanate-methyl, thiophanate, benomyl, methyl 2-benzimidazolecarbamate (MBC), and thiabendazole (TBZ). Movement of triarimol, phenylmercurimonoethanol ammonium acetate, and carboxin was moderately rapid, although the last one was limited in movement through adaxial cuticle. Captafol, chlorothalonil, thiram, and captan exhibited relatively slow rates of transcuticular movement, particularly with abaxial cuticle. Dodine did not show transcuticular movement. Increasing the solubility of benomyl and TBZ in acidified water enhanced their transcuticular movement fourfold. Translaminar movement was evident with benomyl, MBC, thiophanate, and thiophanate-methyl, but not with captan, captafol, or chlorothalonil.

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The systemic fungicidal activity of chemicals applied to foliage depends upon their uptake and subsequent translocation within the plant. Uptake is determined by the ability of a chemical to move through the cuticle, a process which will be referred to as transcuticular movement. Cuticular structure and transcuticular movement were comprehensively reviewed by Martin & Juniper (10) and by Hull (7). Studies on transcuticular movement of fungicides are rare (9). Various authors (5, 9, 12, 16, 19) have used isolated cuticles in studying transcuticular movement of pesticides or nutrients. In all of these studies a two-compartment system was used, in which movement of a chemical from a solution in one compartment through the cuticle into water in another compartment was measured. Rates of transcuticular movement of chemicals reported for such systems in a 24-hr period often involved less than 2% of the chemical available for movement (5, 9, 12, 16, 19). These rates seem to be so low that they are in disagreement with the effective performance of those pesticides and nutrients in practice.

In the present study, we developed a system, which, although artificial, might more closely simulate leaf uptake from spray application. The transcuticular movement of systemic and nonsystemic fungicides was quantified, and the relation between values obtained and the fungicidal performance will be discussed.

MATERIALS AND METHODS.—*Fungicides.*—The chemical formulations tested were: benomyl (Benlate—WP 50%, E.I. du Pont de Nemours & Co., Wilmington, Delaware); captafol (Difolatan—WP 80%, Chevron Chemical, Cherry Hill, New Jersey); captan (Orthocide 50—WP 50%, Chevron Chemical Co.); carboxin (Vitavax—WP 75%, UniRoyal Chemical, Bethany, Connecticut); chlorothalonil (Bravo—WP 75%, Diamond Alkali Co., Cleveland, Ohio); dodine (Cyprex—WP 65%, American Cyanamid Co., Princeton, New Jersey); methyl 2-benzimidazolecarbamate (MBC) (technical, E. I. du Pont de Nemours & Co.); phenylmercurimonoethanol ammonium acetate (PMMAA) (Puratized Apple Spray—Soluble concentrate 11.5%, Gallowhur Chemicals Canada Ltd., Montreal, Canada); thiabendazole (TBZ) (Mertect 60—WP 60%, Merck & Co., Inc., Rahway, New Jersey); thiophanate, 1,2-bis-(3-ethoxy-carbonyl-2- thioureido)-benzene (Cercobin—WP 50%, Nippon Soda Co., Ltd., Ohtemachi, Tokyo, Japan); thiophanate-methyl, 1,2 -bis-(3-methoxy-carbonyl-2-thioureido) - benzene (NF-44-WP 70%, Nippon Soda Co., Ltd.); thiram (Arasan—WP 75%, E.I. du Pont de Nemours & Co.); triarimol (EL-273-WP 75%, Elanco Products Co., Indianapolis, Indiana).

Standard cuticle disks.—Throughout the study mature apple leaves (*Malus sylvestris* Mill.) were used unless otherwise stated. Cuticles were isolated from leaves by decomposing the pectic layer underneath the cuticle either enzymatically (15) or chemically (6). In preliminary trials with benzimidazole fungicides, permeability of cuticles was found to be independent of the method of isolation. As the chemical method was easier, it was adopted for further use. Disks 10 mm in diam were cut from leaves and soaked in a solution of 60% $ZnCl_2$ in 12N hydrochloric acid. The solution was infiltrated into the leaf disks by using intermittent vacuum. After 2 to 3 days, the disks were transferred into water and stirred gently, which separated the cuticles from

other tissues. The cuticle disks were then washed gently by several changes of the water in which they were floating. Subsequently, they were picked up and supported by rings of aluminum foil with an inner diam of 6.5 mm and an outer diam of 14 mm. Each disk was checked under a microscope for imperfections and to determine whether the cuticles were from the adaxial (astomatous) or the abaxial (stomatous) side of leaves. Cuticles always were mounted with the external side faced upward, using hairyness, vein elevation, and sometimes patches of unseparated cells of inner tissue as criteria. During all procedures the cuticle disks were manipulated rapidly to prevent dehydration.

Measuring transcuticular movement.—The mounted cuticle disks were placed on the surface of 10 ml of potato-dextrose agar (PDA) in petri plates. The PDA had a pH of 5.6 and was supplemented with aureomycin, 100 μg/ml, to prevent bacterial contamination. For testing transcuticular movement of thiophanates, the pH of the PDA medium was adjusted to 8.7 to induce conversion of the nonfungitoxic compounds to the toxic form (13, 18). The aluminum rings provided a uniform area of contact (33 mm^2) and prevented lateral diffusion of any applied fungicide into the medium. Spores of a fungus sensitive to the fungicide being evaluated were either sprayed on the medium with a fine atomizer 24 hr after application of the fungicide, or seeded in the medium just prior to pouring into plates. Fungicides were dissolved or suspended in water to the desired concentration and were applied to the cuticle in a 5-μliter droplet by a microsyringe. The plates were then maintained at a temperature optimal for development of each fungus, and when growth was apparent, the zone of inhibition (Fig. 1) was measured. The amount of chemical which moved through the cuticle during the experimental period was assessed from standard curves. The latter were obtained from a series of known amounts of the fungicide applied to filter paper disks of 6.5-mm diam. The diameter of the zone of inhibition was plotted against the log of the concentration of the fungicide and gave a straight line within the range of concentrations being evaluated. The rate of transcuticular movement expresses the amount of fungicide which moved through the cuticle within 24 hr as a percentage of the dosage applied to the cuticle. If not otherwise stated, 10 cuticles were used for each treatment. Test fungi used in the bioassay were as follows: *Penicillium cyclopium* for benomyl, captafol, captan, chlorothalonil, MBC, TBZ, thiophanate, and thiophanate-methyl; *Ustilago maydis* for carboxin and thiram; and *Venturia inaequalis* for dodine, PMMAA, and triarimol.

Extraction of TBZ and spectrophotometrical analysis.—Water agar, into which TBZ had been diffused, was blended in a Waring Blendor with ethyl acetate, and the supernatant liquid was filtered and evaporated under partial vacuum. The residue was redissolved in ethyl acetate and spread over the origin line of a silica gel chromatogram with a fluorescence indicator (No. 6060, Eastman Kodak Co., Rochester, New York), and the chromatogram was developed

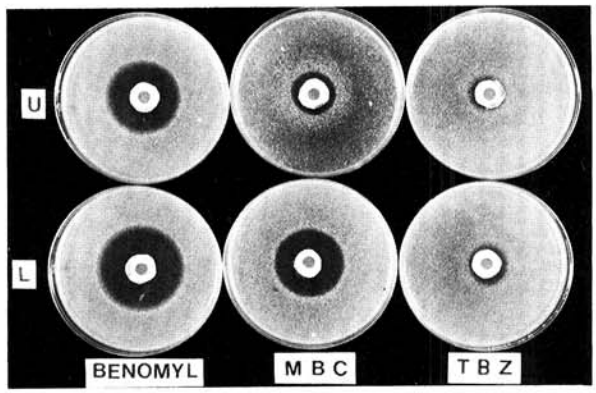

Fig. 1. Zone of inhibition of *Penicillium cyclopium* after 24-hr incubation. Adaxial (U) and abaxial (L) cuticle disks of apple leaves, supported by an aluminum ring, were placed on inoculated PDA plates, and 3.3 nmole of benomyl, methyl 2-benzimidazolecarbamate (MBC), and thiabendazole (TBZ) were applied on top of cuticles.

using ethyl acetate. The presence of a chemical with R_F value corresponding to TBZ was observed under ultraviolet light. A band of 20 mm centered at R_F 0.40 was eluted in 10 ml ethyl acetate. Likewise, a band from a chromatogram sheet of water agar control was eluted and served as a reference solution. The test solution was scanned with a spectrophotometer and gave an absorption curve similar to that of authentic TBZ. The concentration of TBZ was determined from absorption at 302 nm.

Fluorometric measurement.—The concentrations of TBZ in the range of 0.2-0.5 μg/ml in water were determined with a Turner fluorometer with exitation filter at 302 nm and emission filter of 360 nm.

RESULTS.—*Time course study of transcuticular movement.*—Aliquots of 1 μg of benomyl were applied to disks of adaxial and abaxial cuticles on PDA plates. After various time intervals, nine cuticle disks of each type were removed and the fungicide in the PDA medium was bioassayed with *Penicillium cyclopium*. The zones of inhibition were so large at 24 hr that the cuticle disks had to be transferred to other plates for the 24- to 48-hr period, and the amounts recovered were combined. The results (Fig. 2) show that movement of benomyl through the abaxial cuticle was more efficient than through the adaxial one. With the abaxial cuticle, movement was faster during the first 8 hr but gradually slowed down, whereas movement through the adaxial cuticle was constantly moderate.

Rates of transcuticular movement.—Fungicides were applied to disks of adaxial and abaxial cuticles in dosages adjusted to suit bioassay evaluation, considering both the amounts to be recovered and the sensitivity of the test fungi. Marked differences in efficiency of penetration through cuticle were observed, especially with the adaxial cuticle (Table 1). Among the benzimidazoles, movement through the adaxial cuticle within the 24-hr period ranked thiophanate-methyl > thiophanate > benomyl >

TABLE 1. Rate of transcuticular movement of fungicides applied to disks of cuticles isolated from adaxial or abaxial surfaces of apple leaves

Fungicide	Dosage applied (nmole)	% Transcuticular movement[a]	
		Adaxial cuticle	Abaxial cuticle
Thiophanate-methyl	3.3	56	87
Thiophanate	3.3	44	64
Benomyl	3.3	28	65
Methyl 2-benzimidazolecarbamate	3.3	8	17
Thiabendazole	5.0	6	42
Carboxin	10.6	18	51
Triarimol	3.8	48	49
Phenylmercurimonoethanol ammonium acetate	0.7	34	47
Dodine	17.4	0	traces
Captafol	5.7	6	24
Captan	16.6	4	13
Chlorothalonil	7.1	8	35
Thiram	10.4	13	34

[a] The amount of fungicide moved through standard cuticle disks within 24 hr is expressed as percentage of the dosage applied. Figures are an average of the results of two repeated tests, with 10 replicates in each test.

MBC > TBZ. All five benzimidazoles were quite efficient in movement through the abaxial cuticle, although thiophanate-methyl again excelled with 87% moving through the cuticle, and MBC was least efficient with only 17 percent transcuticular movement. Examples of benzimidazole bioassays are shown in Fig. 1. Carboxin, triarimol, and PMMAA moved through both adaxial and abaxial cuticles quite readily although carboxin was limited in movement through adaxial cuticle. Dodine did not diffuse through cuticles to any significant extent. Captafol, captan, chlorothalonil, and thiram demonstrated moderate rates of transcuticular movement, especially with abaxial cuticles.

The relation between the dosage applied and rate of transcuticular movement was also investigated. Benomyl was applied to adaxial cuticle disks in aliquots of 5 μliters containing 2.6, 5.2, and 10.4 nmole to each of seven disks. The amounts of fungicide bioassayed in the PDA medium after a 24-hr diffusion period averaged 1.1 (42.3% of dosage applied), 1.5 (28.8%), and 1.7 (16.3%) nmole, respectively. These figures show that multiplication of the dosages applied resulted in a diminishing increase in the amounts of fungicide that moved through the cuticle. The same trend was found with thiophanates. When thiophanate was applied at 3.3 and 7.5 nmole the amounts moved through cuticles were 2.1 (63.6%) and 2.6 (34.6%) nmole with abaxial cuticles and 1.4 (42.4%) and 1.8 (24.0%) nmole with adaxial cuticles, respectively. With the same dosages of thiophanate-methyl, the amounts were 2.9 (87.8%) and 4.5 (60.0%) nmole with abaxial cuticles and 1.9 (57.5%) and 2.0 (26.6%) nmole with adaxial cuticles, respectively.

Fungicides with relatively low efficiency of transcuticular movement, MBC and TBZ, responded to increased dosages by a proportional increase in movement. When MBC was applied at 3.3 and 7.5 nmole, the amounts moved through cuticles were, with abaxial cuticles 0.6 (18.1%) nmole, and 1.3 (17.3%) nmole, and with adaxial cuticles 0.3 (9.0%) and 0.6 (8.0%) nmole, respectively. With TBZ applied to adaxial cuticles in aliquots of 5 and 50 nmole, the average amounts bioassayed in the PDA medium were 0.3 (6.0%) and 2.75 (5.5%) nmole, respectively. The transcuticular movement of TBZ was verified by extracting the agar medium and assaying with a spectrophotometer. Results were virtually identical with the two techniques.

In another experiment, transcuticular movement of TBZ was assayed using an apparatus designed by Goodman & Addy (5). Diffusion rate of the fungicide from a 50 μg/ml aqueous solution in one compartment through cuticles into water in another compartment was measured. Fluorometric determinations with eight repeated diffusion tests for 24 hr revealed that less than 1% of the chemical in the donating compartment had moved through the cuticle.

The transcuticular movement of benomyl was also studied with cuticles isolated from leaves of lime [*Citrus aurantifolia* (Christm) Swingle] growing in a greenhouse. Aliquots of 3.3 nmole were applied to cuticle disks. Movement through adaxial (astomatous) and abaxial (stomatous) disks in 24 hr was 29 and 34%, respectively. These rates imply that the cuticle of the abaxial side of citrus leaf is less permeable than the corresponding apple cuticle.

Effect of solubilization of fungicides on their transcuticular movement.—TBZ and benomyl, only slightly soluble in neutral water, were solubilized to form concentrations of 200 μg/ml by acidifying the water with hydrochloric acid to pH 2.1 and 0.1, respectively. Aliquots of 5 μliters were applied to eight disks of adaxial cuticles. The transcuticular movement within a 24-hr period of TBZ and benomyl (average of two experiments) were 23 and 93%,

respectively. These values are nearly fourfold those of the respective wettable powder formulations in neutral water (Table 1). When similar aliquots were applied to filter paper disks of 6.5 mm and bioassayed, zones of inhibition were larger than those formed with the same dosages of the respective aqueous suspensions. The diameter of the zones of inhibition with suspended and solubilized formulations of TBZ were 24.2 and 34.9 mm, respectively, which when compared with standard curves on semilog paper, is equivalent to a 191% increase in amount of fungicide. Similarly, suspended and solubilized formulations of benomyl formed zones of 41.1 and 47.8 mm, respectively, equivalent to a 376% increase in fungicidal activity with solubilization. No inhibition was induced by the acidified water controls.

To elucidate the significance of the formulation on the performance, benomyl was dissolved in chloroform and compared with an aqueous suspension. The chloroform was evaporated from the filter paper disks, and then the disks were bioassayed. The solubilization with chloroform increased fungicidal activity by 425%. Upon evaporation of chloroform, a fine precipitate must have formed enhancing diffusion into agar.

Translaminar movement of fungicides.—Some fungicides, namely captan, captafol, and chlorothalonil, showed moderate transcuticular movement, yet they are not generally considered as systemic. Therefore, their movement within the leaf was assayed. Disks (18 mm), cut from leaves of bean (*Phaseolus vulgaris* L.), were mounted on rings of aluminum foil with inner and outer diameters of 13 and 24 mm, respectively, and were placed on PDA with the adaxial side upward. Five μg of MBC, 10 μg of benomyl and of each thiophanate fungicide, and 50 μg each of captan, captafol and chlorothalonil were applied in a 50 μliter aqueous suspension to each of 10 leaf disks. After 24 hr the leaves were removed and the plates were bioassayed. The experiment was replicated twice. Rates of translaminar movement of MBC and benomyl were 2 and 11% of the dosage applied, respectively. Thiophanate and thiophanate-methyl moved through the leaf to produce zones of inhibition. However, since standards of authentic thiophanates were not fungitoxic, their movement could not be determined. Captan, captafol, and chlorothalonil failed to produce zones of inhibition, indicating lack of translaminar movement. Leaves with the latter three fungicides were frozen, and compacted to form a moist pellet, which was bioassayed. Most of the amounts of fungicides applied to the leaf disks were recovered.

DISCUSSION.—Transcuticular movement is one of the major factors determining the systemic performance of fungicides. Therefore, a parameter expressing efficiency of transcuticular movement may contribute to the analysis of the results of in vivo studies. Also, comparison of results from field tests of systemic fungicides with the isolated in vitro cuticle system used in this study may throw light on the merit of the latter as a test. The dosages of fungicides evaluated resemble the order of magnitude of dosages used in agricultural practices. For example, we applied 1.0 μg (3.4 nmole) of benomyl in a 5-μliter droplet or 200 μg/ml, compared with 300 to 500 μg/ml of benomyl (active ingredient) used in practice. Several examples were selected from the available information and will be discussed.

Benomyl applied to foliage was superior to TBZ in the systemic control of powdery mildew of cucumber (L. V. Edgington & Janice Schooley, *unpublished data*) and Cercospora leaf spot of sugar beet (17). Furthermore, in both cases application of these fungicides to the abaxial leaf side gave a better control than application to the adaxial surface. These results parallel data on rates of transcuticular movement of these fungicides (Table 1). The superior disease control by benomyl could also be attributed in part to its greater inherent toxicity than TBZ. Thiophanate-methyl and, to a less extent, thiophanate, generally require higher dosages than benomyl to obtain an equivalent disease control (4, 17). Thiophanate, and particularly thiophanate-methyl, were superior to benomyl in transcuticular movement. However, cuticular penetration is not enough because the ultimate systemic fungicidal activity of thiophanate and thiophanate-methyl is dependent on conversion to ethyl 2-benzimidazolecarbamate (EBC) and MBC, respectively (18). At the pH of plant tissue, this is a bioconversion requiring time, and thiophanate-methyl is more rapidly changed to MBC than is thiophanate to EBC (13). Also, EBC is less fungitoxic than MBC (18). Conversely, benomyl in aqueous solution breaks down rapidly to MBC (3).

Acidified solutions of benomyl and TBZ were superior to the respective suspensions in the systemic control of Verticillium wilt of cotton, when applied to foliage (2). Our data demonstrate a prominent increase in transcuticular movement of the acidified formulations, which may well account for their superior field performance. The bioassay comparison revealed that zones of inhibition formed by solubilized benomyl and TBZ, applied to filter paper disks, were larger than those of the respective suspensions. This is due to faster diffusion apparently derived from a superior dispersion of the soluble form. Likewise, a superior dispersion of a soluble form on the cuticle is presumed, resulting in enhanced transcuticular movement.

Some fungicides not considered truly systemic have a therapeutic effect ("kickback") on scab development on apple leaves, when applied after inoculation. Efficiency of the therapeutic action is high with triarimol (1) and phenylmercurial fungicides (11), mediocre with dodine (11), and low with captafol (E. J. Klos, *personal communication*), thiram and captan (11). Since the causal organism of apple scab, *Venturia inaequalis*, develops subcuticularly only, penetration of the fungicide through the cuticle would be required to reach the pathogen. Rates of transcuticular movement of those fungicides (Table 1) may well account for the effective performance of triarimol and PMMAA and

for the moderate therapy obtained by captafol, captan, and thiram treatments. Dodine did not show any transcuticular movement and yet it is known to possess moderate "kickback" action. A previous abstract (8) has reported that dodine moves laterally on the cuticle rather than translaminarly. Preliminary tests verify excellent lateral movement on isolated cuticles. This could be due to surfactant properties of dodine. However, lateral movement would only improve distribution and protective action, but not therapeutic activity.

Systemicity of fungicides in leaves is dependent upon both transcuticular movement and subsequent translocation within the lamina. The translaminar movement of benomyl, MBC, and the thiophanate fungicides conforms with their systemic performance. Our test indicated no systemicity of captan, captafol, and chlorothalonil in bean leaf although the test of transcuticular movement and their therapeutic action on apple scab demonstrated their ability, though moderate, to move through cuticles. Since no inactivation of these fungicides by bean leaf was apparent in the in vitro test with mashed leaves, lack of systemicity might be due to retention of those fungicides by leaf tissue adjacent to the cuticle.

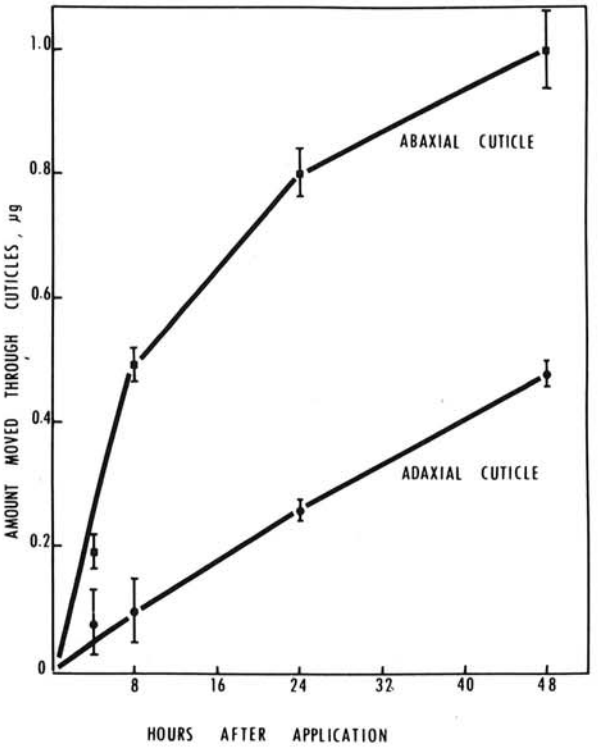

Fig. 2. Movement of benomyl through abaxial and adaxial cuticular disks of apple leaves. Dosage applied was 1 µg/33 mm² disk. Mean of nine replicate cuticle disks with standard error indicated by vertical bar.

It may be concluded that rates of transcuticular movement of fungicides as determined with the in vitro test correlate with many of the in vivo studies and therefore, may serve for interpretation of the performance of the fungicides as well as in screening for systemic fungicides. As an example, carboxin has only a moderate rate of transcuticular movement. Analogues of this fungicide might greatly enhance the movement and thus improve disease control.

The main component in this test, which may be questioned, is the isolated cuticle. With the light microscope we did not see any defect in the integrity of the cuticle. Neither did Norris & Bukovac (14) observe morphological differences between intact and isolated cuticles, with polarized light or electron microscopy. We would expect that the epiwax was partially removed in the course of processing the cuticle, as it is a very delicate surface structure.

If we compare rates of movement through the cuticle in our system with that of the liquid:cuticle:liquid system used by others (5, 9, 12, 16, 19), our rates of 50-80% of dosage applied moving through the cuticle appear much higher. However, if one considers the nmoles penetrating/cuticle area/unit time, the results are of a similar magnitude (ca. 0.3-3 nmole/33 mm²/24 hr). Our method seems closer to reality than the two-compartment system due to simulation of the components related to the dynamics of the agricultural practice. In both instances, a droplet of the fungicide suspension is applied to the cuticle surface and forms interfaces of air:spray droplet:cuticle. A relatively high concentration is in contact with the cuticle and is available for transcuticular movement by the process of diffusion. Our results show that the amounts of fungicides that diffuse through the cuticle during certain time intervals increases with increase in dosage applied, indicating that rate of transcuticular movement depends on the gradient in the concentration of the chemical on both sides of the cuticle. With fungicides that are more mobile with respect to transcuticular penetration the effect of concentration gradient is less pronounced. Thus, movement of benomyl was fast soon after application to abaxial cuticle (Fig. 2) when the concentration gradient was maximal, but slowed down later as a result of the rapid decrease in concentration gradient. With the adaxial cuticle, the drop in concentration gradient was slow and did not affect the speed of movement.

The concentration gradient is dependent on the concentration underneath the cuticle. Hence, removal of the chemical after passage through the cuticle seems to be an important component of the natural system, where diffusion in the apoplast and removal by the transpiration stream maintain the concentration gradient. In our system, diffusion in the agar medium simulates this removal process. Yet, the rates of transcuticular movement obtained in the in vitro study are not necessarily those existing in the in vivo system – the plant leaf, and should be considered as relative rates.

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