Delayed and Enhanced Degradation of Benomyl and Carbendazim in Disinfested and Fungicide-Treated Soils

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ABSTRACT

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Factors affecting the biodegradation of benomyl and of its fungitoxic hydrolysis product, carbendazim, in soil were studied. Soil disinfestation by fumigation or solarization prior to fungicide incorporation inhibited degradation and extended the persistence of carbendazim in soil. Carbendazim was present for a significantly longer time and at higher concentrations in leaves of pepper grown in a field soil disinfested and treated with benomyl than with plants grown in nondisinfested soil. The fungicides TMTD (thiram) and fentin acetate strongly inhibited the degradation of carbendazim, when added to the soil with carbendazim. A drastic reduction in general enzyme activity in soil amended with TMTD was observed. *Alternaria alternata* and *Bipolaris tetramera*, which are capable of degrading carbendazim, were isolated from the soil. The two fungi were sensitive to low concentrations of TMTD and fentin acetate. The combined application of TMTD with benomyl in the field, intended to prolong the biological activity of benomyl, further reduced the number of apothecia of *Sclerotinia sclerotiorum* compared with benomyl treatment alone. In soil with a history of benomyl treatment, carbendazim was degraded more rapidly than in a soil without such a history.

Additional key words: benzimidazole fungicides, pesticide persistence, pesticides, problem soils.

The increased use of pesticides in modern agriculture poses challenging problems for research workers. On the one hand, there is the need to extend the effectiveness of the pesticides in the field from both a pest control and economic point of view, but on the other hand there is the need to shorten their persistence due to environmental concerns and possible side effects on nontarget organisms. Microbial degradation usually affects the persistence of pesticides very significantly (12). Although many studies have dealt with biodegradation of pesticides in culture and field soil, only a few workers have studied the fate of pesticides in disinfested soils. Soil disinfestation by fumigation or solarization affects microbial activity in soil, and may affect the fate and behavior of pesticides (2,11,15,17,20). Rapid degradation of pesticides and a concommitant loss of pest control have been reported where pesticides have been applied repeatedly to soil. This might be due to a buildup of populations of microorganisms able to degrade the pesticides. These reports focus mainly on degradation of herbicides and insecticides (13,14). The possibility that the effectiveness of benomyl in mushroom casing is related to enhanced degradation of the fungicide has also been raised (6). The fate and microbial degradation of the fungicide benomyl and its fungitoxic hydrolysis product, carbendazim, have been studied under laboratory and field conditions. These compounds are exposed to physical and microbial transformations in soil. They are adsorbed to organic matter and clay minerals (1,10) and are degraded by soilborne microorganisms to nonfungitoxic compounds in periods ranging from weeks to months, depending on soil type, humidity, and temperature conditions (4,7–10).

In the present study, we investigated the persistence of benomyl and carbendazim in disinfested soils and their interaction in soil with other fungicides in an attempt to extend their effectiveness. Slowing down the degradation of benomyl and carbendazim may extend their persistence and consequently prolong their effectiveness in disease control. The possibility of enhanced degradation in soil with previous benomyl application was also studied.

MATERIALS AND METHODS

Fungicides. The following fungicides were used: carbendazim (technical grade; Agan Chemical Co., Israel), Benlate (benomyl 50 WP; E.I. du Pont de Nemours & Co.), thiram (tetramethylthiuram disulfide or TMTD) (Tirpa 80 WP; Machteshim Inc., Israel), and fentin acetate (Bdilan 60 WP; Machteshim Inc., Israel).

Medium. Potato-dextrose agar (PDA) was used for isolation and growth of fungi. Fungicide suspensions were prepared in acetone and appropriate dilutions were added to warm PDA (50 C) prior to pouring it into petri dishes (10 ml per dish). After they had been poured, the dishes were left open in a sterile hood to allow acetone residues to evaporate.

Cellophane disks. Clear cellophane disks were cut to fit a petri-dish diameter. After they had been rinsed (5 min in tap water followed by 5 min in distilled water), the disks were placed in boiling distilled water for 2 hr. After an additional rinse (5 min in distilled water), 10 disks, along with paper dividers between them, were placed in a petri dish and autoclaved for 20 min.

Laboratory studies of carbendazim degradation in soil. An acetone solution of carbendazim (100 μg/g) was added dropwise to 40 g of air-dried soil. After the solvent had evaporated, the soil was left uncovered in a ventilated hood for 24 hr to remove traces of the solvent and then mixed with 110 g of the same soil to provide a 150-g replicate. The soil samples were placed in biometer flasks (3) and moistened to the approximate equivalent of 80% field capacity by adding 12 ml of water to each 150-g replicate of soil. The flasks were sealed with thin polyethylene and incubated in the dark at 27 C. Twenty milliliters of water was put in the side arm of the flask to maintain a constant moisture level in the soil. Soil treatments were replicated three times in each experiment. At various time intervals,

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subsamples of 20 g each were taken from the flasks and stored at −18 C until analyzed.

Autoclaving (121 C at 1 atmosphere for 1 hr on 2 successive days) of wet soil (containing 75 ml water per kilogram of air-dried soil), when required, was carried out before any fungicides were added.

Extraction and analysis of carbendazim from soil. Extraction of carbendazim from soil was as follows: a 20-g soil sample was shaken in a 500-ml Erlenmeyer flask with 200 ml of 1 N NaOH for 10 min. The aqueous solution was separated from the soil and extracted three times with 150, 100, and 100 ml, respectively, of analytical-grade, glass-redistilled ethyl acetate. The ethyl acetate extracts were combined, concentrated in a Rotavapor to about 75 ml, and extracted with 75, 50, and 50 ml of 0.1 N HCl. Fifty milliliters of 3 N NaOH was added to the aqueous phase and extracted with 100, 80, and 80 ml of ethyl acetate. The ethyl acetate was dried over anhydrous sodium sulphate, evaporated to near dryness, and the residues were dissolved in methanol. Aliquots were injected into a Tracor 985 high-pressure liquid chromatography (HPLC) apparatus connected to a Perkin-Elmer model 204 fluorescence spectrophotometer set at 285 nm for excitation and 307 nm for carbendazim analysis. The HPLC system was equipped with a C-18 reversed-phase absorbent (10 μm) packed in a 20-cm-long, 4-mm (I.D.) column. The solvent used was methanol + H_2O (4:1, v/v) containing 10 drops of ammonium hydroxide per liter. Flow rate was 0.5 ml/min. Recovery of carbendazim from 20 g of soil at concentrations of 0, 1, 5, and 10 μg was 0, 88 ± 6, 89 ± 4, and 95 ± 4%, respectively.

Uptake of carbendazim by pepper. A randomized block design field trial was used to assess carbendazim uptake by pepper in untreated and disinfested soil. The trial was located on the experiment farm of the Faculty of Agriculture in Rehovot (red-brown sandy soil; 0.6% organic matter, 3.8% clay, pH 7.9, field capacity 9%). Disinfestation treatments consisted of fumigation with methyl bromide at 500 and 1,000 kg/ha. Soil disinfestation by soil solarization was accomplished by tarping the moist soil with transparent polyethylene for 1 mo prior to planting (11). Benlate was incorporated into the soil of the untreated and the disinfested plots at the rate of 10 kg/ha. One-month-old pepper (*Capsicum annuum* L. 'Maor') seedlings were planted after fungicide incorporation. The plants were uprooted at various time intervals for determination of carbendazim in the leaves.

Bioassay for carbendazim in leaves of pepper plants. The bioassay used was similar to that described by Solel and Edgington (19). Disks 13 mm in diameter were cut from the edge of the youngest leaf >40 mm in length from plants freshly uprooted from the field. Disks were submerged for 10 sec in liquid nitrogen to disrupt cell membranes. Immediately thereafter, the leaf disks were placed in the center of a petri dish containing 10 ml of PDA. The petri dishes were preinoculated with 0.1 ml of a conidial suspension in sterile water (5×10^6 conidia per milliliter of water) of an isolate of a carbendazim-sensitive isolate of *Penicillium digitatum* Sacc. that was obtained from the Department of Plant Pathology and Microbiology, Faculty of Agriculture, Rehovot. Inoculated dishes with the disks were stored in the dark for 24 hr at 4 C and then transferred to a dark incubator (27 C). After 48 hr, a clear zone of fungus growth inhibition was evident in cases where carbendazim had diffused from the leaf disks. Comparisons of fungus inhibition areas, caused by diffusion of carbendazim from the leaf disks of the various treatments, indicated the relative uptake of carbendazim by plants.

Isolation of carbendazim-degrading microorganisms. Soil dilutions on PDA containing 250 μg of chloramphenicol per milliliter were made to isolate a variety of soilborne fungi. Addition of carbendazim at 10 μg/ml to the medium served as a selective factor. The capacity of each tested fungus to degrade carbendazim was determined by the turnover method described here. Pure cultures of the tested fungi were placed in the center of petri dishes containing PDA amended with carbendazim at 1 μg/ml. After cultures had grown sufficiently to cover approximately one half of the medium surface, the agar (with the culture) was removed in one piece, overturned, and placed back in the same petri dish. A conidial suspension of *P. digitatum* (5×10^5 conidia per 0.1 ml of sterile water), sensitive to carbendazim, was spread over the newly exposed agar surface and incubated 48 hr at 27 C. If conidia on carbendazim-amended media germinated, indicating the possibility that carbendazim had disappeared, carbendazim degradation by the tested fungi was investigated further.

Actual carbendazim degradation by these select fungi was determined as follows: the fungi were reisolated from the preliminary test dishes and maintained on PDA (27 C) for 10 days. Disks (13 mm in diameter) of the newly grown cultures were placed in the center of a large (15-cm diameter) petri dish containing 50 ml of PDA amended with carbendazim at 10 μg/ml. This test was carried out in three replicates, and one disk was placed in the center of each large petri dish. After the fungus had grown to cover approximately half of the medium surface, round disks (13-mm diameter) were cut out along the diameter of the petri dish, and removed for bioassay of carbendazim. The bioassay consisted of placing the disks on PDA in petri dishes preinoculated with the sensitive strain of *P. digitatum*. The dishes (with the disks) were placed in the dark for 24 hr at 4 C and then transferred to a dark incubator (27 C). After 48 hr, a fungal inhibition zone was evident. The area of germination inhibition zone was presumed to be related to the amount of carbendazim in the tested disk; the higher the concentration of carbendazim in the tested disk, the larger the inhibition zone. This was verified by comparing the size of the inhibition zone to that obtained in tests with disks from medium containing various concentrations of carbendazim. A reduction in inhibition zone size in experiments with the tested fungi was considered as an indication of higher carbendazim-degradation capacity.

Another test was conducted to determine whether loss of carbendazim activity was due to degradation or rather to adsorption of the compound to the mycelium of the tested fungus, thus preventing it from affecting the bioassay organism. In similar dishes containing the same amended medium, a sterile cellophane paper disk was placed on the medium before the culture being tested was placed on it to serve as a physical barrier between the organism and the medium. After the growth period, the cellophane was removed, and along with the fungal mat attached, placed in acetone and shaken 15 min for carbendazim extraction. The extract, after concentration, was tested for carbendazim residues.

Determination of general enzyme activity in soil. The hydrolysis of fluorescein diacetate (FDA) (Sigma Chemical Co., St. Louis, MO) was used as an assay for the determination of general microbial activity in soil as indicated by enzyme activity levels. This test is an indicator of general microbial activity (18). Samples (5 g) of soil were preincubated with 50 ml of 0.06 M sodium phosphate buffer (pH 7.6) for 30 min at 24 C on a shaker. After preincubation, 250 μl of FDA solution (2 mg of acetone per milliliter) were added to the suspension. After 180 min of shaking at 24 C, 4 ml of the suspension was removed and transferred to a test tube with 4 ml of acetone to terminate the reaction. The suspension was centrifuged in a clinical centrifuge (HEC HN-SII) for 10 min at 1,500 rpm. Optical density (O.D.) of the supernatant was determined at 490 nm (Kontron Uvikon 810 spectrophotometer). All phases of the assay were carried out at minimal lighting conditions. The O.D. is proportional to enzyme activity.

Effect of benlate and TMTD on the number of apothecia of *Sclerotinia sclerotiorum* (de Bary). A field trial was carried out at Gilat experimental station (silty loam loessial sierozem type soil; 0.5% organic matter, 20% clay, pH 8.4, field capacity 20%) in a lettuce field naturally infested with sclerotia of *S. sclerotiorum*. A motorized sprayer (spraying boom of 1.2 m and 10 nozzles) was used to spray the soil surface with benlate (WP 50% a.i.) at the rate of 5 kg/ha. Other treatments included spraying with TMTD (WP 80% a.i.) at the rate of 5 kg/ha and a combination of both fungicides. The experiment consisted of four replicates in a randomized block design. All fungicide suspensions were sprayed at the equivalent of 2,000 L/ha. Fungicide application took place during December 1983, 36 days after planting. The number of apothecia was recorded at various time intervals following fungicide treatment during February and March 1984.

RESULTS

Effect of disinfestation and sterilization on persistence of carbendazim in soil and on its uptake by pepper plants. Carbendazim residues were determined after various incubation periods in biometer flasks containing autoclaved or disinfested and untreated soil. The most pronounced degradation occurred in the untreated soil; about 80% of the amount applied was degraded within 30 days (Fig. 1). Autoclaving resulted in a pronounced inhibition of degradation throughout the experiment. Both methods of soil disinfestation strongly retarded carbendazim degradation. Thus, the half life of carbendazim in the soil was extended from about 20 days in the untreated soil to 40–55 days in the disinfested soils.

Disinfestation of soil in the field, prior to incorporation of benlate, resulted in an increase of carbendazim uptake by pepper plants grown in this soil as compared to plants grown in the nondisinfested plots. The increase observed was both greater and longer at the higher dosage of methyl bromide (Table 1). Fourteen days after soil treatment, carbendazim could be detected only in plants grown in disinfested soil. Twenty-two days after soil treatment, only plants grown in a soil treated with 1,000 kg of methyl bromide per hectare contained carbendazim at significantly higher rates than plants in the untreated plots.

Isolation of carbendazim-degrading fungi from soil. While conducting the turnover assay for detection of carbendazim-degrading fungi, following the incubation period, germination of *P. digitatum* was very clear on carbendazim-free PDA dishes, and it was completely inhibited on carbendazim-amended dishes free of a tested culture. Germination inhibition occurred in most media in which a test culture had grown. However, in a few cases, germination of *P. digitatum* was evident over the medium above an overturned fungus colony grown on carbendazim-amended PDA. It was suspected that the medium below these germination spots did not contain carbendazim, possibly due to degradation of the fungicide by the fungus previously grown on the opposite side of the same medium. Of 20 fungi isolated from soil on carbendazim-amended dilution plates, two were determined to be capable of degrading this fungicide in culture. They were identified as *Alternaria alternata* (Fr.) Keissler and *Bipolaris tetramera* (McKinney) Shoemaker. Their degradation capacity was further verified by growing them on PDA supplemented with carbendazim at 10 μg/ml. After 2 wk of fungal growth on the plates, bioassays of agar disks taken from the center of the inoculated plates showed a 50% reduction in the amount of fungicide.

The apparent disappearance of carbendazim from the inoculated plates could be attributed either to degradation by the fungi or to inactivation by adsorption to mycelia. To examine these two possibilities, cellophane disks were used as mechanical barriers between the fungi and the medium. Chemical analyses showed that carbendazim was present only in the fungus-free medium, with no evidence of the compound in the fungal mat. Thus, fungicide disappearance could not be attributed to its adsorption to the mycelium. It was also found that both fungi are tolerant to carbendazim since the ED_{50} for growth on PDA amended with carbendazim was approximately 500 μg/ml.

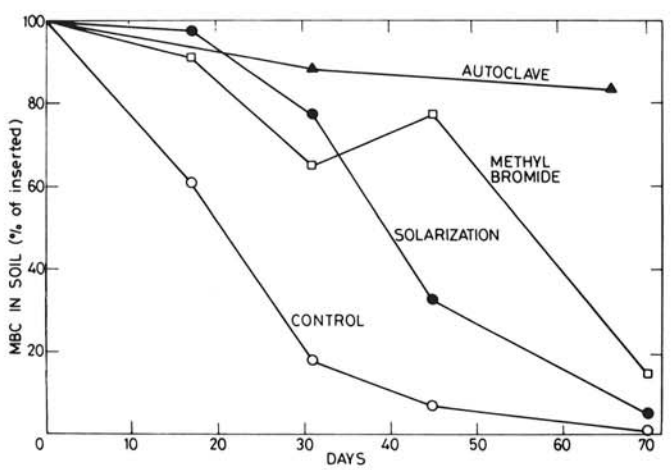

Fig. 1. Effect of soil sterilization by autoclaving, disinfestation with methyl bromide at 500 kg/ha, or solarization on persistence of carbendazim subsequently added to soils at 10 μg/g. Points represent the means of three replicates. Carbendazim was determined by fluorescence spectrophotometry following extraction and HPLC.

Inhibition of carbendazim degradation in soil by fungicides. The previous results indicated that treatments that potentially affect soil microbial activity inhibit carbendazim degradation. Thus, the effect of fungicides, which were found in preliminary experiments to be inhibitory towards the two carbendazim-degrading fungi, was examined. Two fungicides, TMTD and fentin acetate, which strongly reduced the growth of the two fungi at 20 μg/g in culture, were examimed for effects on carbendazim degradation in soil in biometer flasks. Both fungicides had a pronounced inhibitory effect on the degradation of carbendazim in soil, which was even more lasting than the effect of soil fumigation (Table 2). Soil microbial activity, as reflected by enzyme activity, was still low after 45 days of incubation in TMTD-treated soils in which enzyme activity was inhibited by about 50%. Enzymatic activity in the methyl-bromide-treated soil was similar to that of the control after 45 days of incubation, while soil sterilization almost completely inhibited it (Table 3).

Effect of soil treatment with benlate and TMTD on apothecia of *S. sclerotiorum* in a lettuce field. Soil treatment with benlate significantly reduced the number of apothecia in treated plots. An average of 11 apothecia per square meter was recorded throughout the growing season in untreated plots, whereas 1.6 were found on the surface of benlate-treated soil. The combined treatment of benlate and TMTD further reduced the number of apothecia to an average of 0.4 per square meter. Soil treatment with TMTD alone did not significantly affect the number of apothecia when compared to the untreated control (7.5 per square meter).

Enhanced degradation of carbendazim in field soil pretreated with benlate. Soil was taken from benlate-treated plots in the lettuce field described above, 3.5 mo after benlate incorporation. This soil proved to have the capability of more rapidly degrading carbendazim, as tested in biometer flasks, when compared to a soil from the same field that had no carbendazim history (Fig. 2). This enhanced biodegradation resulted in a substantial shortening of the half-life of the fungicide from 11 to about 4 days in this soil system. It is clearly evident that the major change in the degradation process "acquired" by the soil with carbendazim history is the drastic diminishing of the lag period before actual breakdown occurs.

DISCUSSION

The manipulation of microbial processes related to pesticide persistence in soil may prove to be of great value both when attempting to prolong or shorten the biological activity of these

TABLE 1. Effect of fumigation with methyl bromide and soil solarization on relative uptake of carbendazim from soil by pepper plants as determined by a bioassay with *Penicillium digitatum*

Soil treatment	Relative amount of carbendazim in pepper leaves at indicated days after application to soil[y]			
	6	14	22	29
No disinfestation	90 a[z]	2 a	0 a	0 a
Methyl bromide (500 kg/ha)	92 a	19 b	13 ab	0 a
Methyl bromide (1,000 kg/ha)	100 a	28 b	19 b	5 a
Soil solarization	96 a	32 b	3 a	1 a

[y] All bioassay results (inhibition zones) were compared with that of methyl bromide (1,000 kg/ha) after 6 days, which was assigned a value of 100.

[z] Within columns, numbers followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 2. Effect of fungicide treatments or soil fumigation with methyl bromide on persistence of carbendazim added to soil[y]

Soil treatment	Carbendazim remaining (%) 31 days	45 days
Untreated	0 b[z]	0 b
Carbendazim	7 b	2 b
Methyl bromide + carbendazim	83 a	9 b
Carbendazim + tetramethylthiuram disulfide (20 μg/g)	85 a	78 a
Carbendazim + tetramethylthiuram disulfide (100 μg/g)	93 a	94 a
Carbendazim + fentin acetate (20 μg/g)	94 a	79 a
Carbendazim + fentin acetate (100 μg/g)	95 a	98 a

[y] Methyl bromide was applied in the field at 500 kg/ha. Carbendazim was added to soil at 10 μg/g and analyzed by fluorescence spectrophotometry following extraction and HPLC.

[z] Within columns, numbers followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 3. Effect of soil treatments on general enzymatic activity and its relationship to carbendazim persistence in soil[x]

Soil treatment	Optical density (490 nm)	Carbendazim in soil (% of initial) after 45 days of incubation[y]
Untreated	0.35 a[z]	2 b
Methyl bromide	0.30 a	9 b
Tetramethylthiuram disulfide (20 μg/g)	0.18 b	78 a
Tetramethylthiuram disulfide (100 μg/g)	0.13 b	94 a
Autoclaved	0.01 c	95 a

[x] Enzymatic activity was determined by fluorescein diacetate hydrolysis as detected in soil, 45 days after adding carbendazim (10 μg/g). Optical density values represent relative enzymatic activity.

[y] Data for this column (with the exception of the autoclaved treatment) were taken from results described in Table 2 for comparison.

[z] Within columns, numbers followed by a common letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

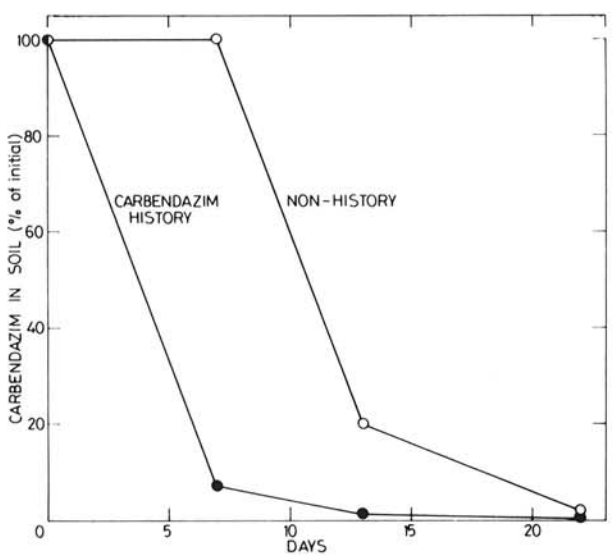

Fig. 2. Degradation of carbendazim in soils with or without a history of prior carbendazim treatment. Points represent the means of three replicates. Carbendazim was added to soils at 10 μg/g and determined by fluorescence spectrophotometry following extraction and HPLC. Initial treatment of soil with a carbendazim history was at 10 μg/g, 3 mo prior to the experiment.

compounds. This study has shown that inhibiting the breakdown of benomyl or carbendazim in soil by general biocidal (soil disinfestation) or relatively selective means (fungicides) extends its effectiveness under laboratory and field conditions. The implications of these observations could be used to reassess the use of carbendazim where the benefits of soil incorporation of this compound are considered marginal. It may well be that prolonging the persistence and consequently the effectiveness of benomyl or carbendazim as soil-applied fungicides may enable their use in a wider range of situations. Furthermore, other relatively short-lived compounds should be tested for improved performance by inhibiting the breakdown process. Degradation of parathion was inhibited by captafol (5) and of linuron by the fumigant vorlex (17). TMTD has special advantages in this respect since it also controls certain soilborne pathogens and even renders the soil suppressive to *Pythium* (16).

Degradation of benomyl and carbendazim is mainly microbial though a certain proportion might be immobilized by a nonbiological process (10). The determination of microorganisms involved in the process may prove to be an important step toward better control of the degradation process, especially when enhanced degradation occurs. The turnover method may be a useful tool in screening for such organisms. The excretion of antibiotic substances by the organism tested may inhibit the pesticide test organism. This would perhaps mask the ability to identify pesticide breakdown in cases where both degradation and antibiotic excretion occur simultaneously. We believe that such a situation would be rare, yet it must be taken into account while using the method. A similar problem could arise should the breakdown product be toxic to the test organism.

TMTD and methyl bromide suppress fungal populations in soil but not bacteria; they may even increase bacteria in certain cases (15,16). This indicates that fungi may play an important role in benomyl and carbendazim degradation, although this has yet to be established.

Attention should also be drawn to the expanding phenomenon of soils in which pesticides are rapidly degraded ("problem soils"). The enhanced degradation of pesticides can result from either frequent application of the same compound ("history soils") or from acquired degradation capability due to contact with other structurally related pesticides. This phenomenon may be analogous to cross-resistance as found in the case of pathogen resistance to structurally related pesticides. In the present study, enhanced degradation of benomyl in soil under field conditions was still evident 2 mo after crop harvest. Some reports on loss of effectiveness of benomyl may be related to such a phenomenon, as already has been shown with mushroom casing (6). The use of soil disinfestation or fungicidal amendments may serve not only to extend and increase effectiveness of soil fungicides, but also to prevent enhanced degradation or even to correct "problem soils" which already rapidly degrade the fungicide. It is not economically feasible to disinfest soils solely to inhibit pesticide degradation. However, this potential benefit should be taken into account when disinfestation is considered for pest control. Moreover, under the right circumstances, combining soil disinfestation with a fungicide may not only improve the performance of the fungicide but might also extend effectiveness of disinfestation by delaying reinfestation of the soil.

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