Sci. Rep. Fac. Agr. OKAYAMA Univ, (54), 1–8 (1979)

Effect of Fungicides, Benomyl and Thiram on Soil Microflora and Some Soil Inhabitant Fungi[a)]

Hachiro Oku, Kazuo Oki[b)], Tomonori Shiraishi, Kenji Sato, and Seiji Ouchi
(*Laboratory of Plant Pathology*,)

Received June 30, 1979

Summary

Effects of Benomyl and Thiram on the soil microflora and some soil inhabitant fungi were studied using the soil (sandy loam) of the experimental field, Okayama University. Under field conditions, heavy application of Benomyl did not affect significantly the soil microflora. Thiram, however, reduced the fungal population in soil to 1/6 at the next day of treatment, but recovered to the normal level after 6 days. Under laboratory conditions, both fungicides did not affect soil microflora. Population of Benomyl-to lerant fungi was about 1/10 of the total fungi and increased slightly in the field soil by treatment with Benomyl at the later stage of experiment during June to October. Neither Thiram-tolerant fungus nor bacterium was found in both Thiram-treated and non-treated soils. A fungus highly tolerant to Benomyl was isolated and identified as *Aspergillus versicolor*, and found to not have the metabolic activity to degrade BCM. The absorption of BCM by the mycelia of this tolerant fungus, *A. versicolor*, was less than half of the BCM-sensitive one, such as *Cladosporium harbarum*.

Introduction

One of the most serious problems in modern agriculture is believed to be harmful side effects of pesticides on living organisms and their environments. Some pesticides are known to reside on food materials and in soil. The other alters the microflora of soil. For example, repeated application of an organochlorine fungicide, Daconil, increased the population of fungi tolerant to the fungicide[6)].

Benomyl, the most widely used fungicide, was reported by Raynal and Ferrari[8)], Hofer et al.[2)] that it did not disturb the microflora in soil under the normal condition encountered in agriculture. However, Gowda and Patil[1)] found that Benomyl application increased the bacterial population in Bangalore sandy soil during the last two weeks of a 4-week-incubation period. According to Mazur[4)], Benomyl, Dyrene and Maneb reduced nitrification rates when incorporatad in soil. Thus, the data hitherto obtained seems to be somewhat diverse, and this difference may be resulted from the difference of experimental conditions.

In this experiments, the effect of Benomyl on soil microflora was examined under natural and constant laboratory conditions. In addition, the uptake and the degradation of Benomyl by Benomyl-sensitive and tolerant fungi were compared to know the mechanism of Benomyl-tolerance.

The effect of Thiram on microflora in soil was also studied.

---

a) This work was supported by a grant from the Ministry of Education, Science and Culture of Japan (Grant No. 248049)

b) Okayama Agricultural Experiment Station

Materials and Methods

Soil

Soil (sandy loam) of the experimental field of Okayama University was used throughout this study after sifted out a 50-mesh sieve.

Fungicides

Benomyl : (1) Authentic sample of Benomyl supplied by Okayama Agricultural Experiment Station was used for the study on the metabolic degradation by tolerant fungus. Pure BCM was supplied by prof. T. Kondo of Kyushu University.

(2) Benlate wettable powder (50%) was used for the studies of the effect on soil microflora. According to our examination, almost all the active ingredient of this wettable powder was benzimidazole-2-carbamic acid methyl ester (BCM).

Thiram : Sankyo Thiuram 80 was used throughout this study.

Treatment of soil with Benomyl and Thiram under field conditions

Three holes of $1 \times 1 \times 0.2$ m were made in the experimental field of Okayama University, covered with plastic sheets, and soils were put in them. One of which was sprayed 3 times with the water suspension of Benlate wettable powder (20 g BCM in 2,000 ml of water). The other one was sprayed 3 times with the water suspension of Sankyo Thiuram 80 (20 g pure Thiram in 2,000 ml of water). The remaining one was sprayed with water as a control. The sprayings were performed every 7 day. These soils were left under field conditions during June to October.

Treatment of soil with fungicides under the constant laboratory conditions

Thirty grams of soil were put into 500 ml-shake flasks and 9 ml of water suspension of Benlate wettable powder (3 mg pure BCM) were poured in it. The other soil samples were poured with the same concentration of Thiram. The control soil was poured with 9 ml of distilled water. These samples were kept in the incubator at 22°C.

Cultivation, and Calculation of microorganisms in soil samples

Thirty grams of soil samples were agitated with 270 ml of sterilized water at 22°C for 10 min. on a rotary shaker, 1 ml of the supernatant was diluted with 1,000 ml of sterilized water, and subjected for the cultivation of fungi. That is, 0.2 ml of the 1,000 times diluent of soil extract was mixed with 15 ml of Martin's medium (1% glucose, 0.5% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 2% agar) containing 30 ppm of streptomycin and rose bengal at 45°C in a petri-dish, and incubated at 22°C for 7 days.

For cultivation of bacteria, 0.2 ml of 1,000,000 times diluent of soil extract was mixed with 15 ml of beaf extract agar medium at 45°C and incubated at 22°C for 3 days.

The number of fungal or bacterial colonies developed were counted and their populations were expresed as the number per g of soil (dry weight).

The number of BCM-tolerant or Thiram-tolerant fungi was assessed by counting colonies which developed on the Martin's medium containing 100 ppm of BCM or Thiram. The tolerant dacteria to these fungicides were estimated as the same manner as above.

Quantitative analysis of residual BCM in soil

Fifty grams of soil were put into many 300 ml-Erlenmeyer flasks. The half of which was sterilized by autoclaving and the remaining half was left unsterilized. Then, Benlate wettable powder was added into each flask to give a final concentration of 100 ppm and incubated at 22°C. These soil samples were extracted 0, 1, 3, 5, 7, and 9 weeks after incubation with a solvent mixture of methanol : ethylacetate=1 : 1 (v/v) and the extracts were concentrated under reduced pressure to dryness. The residues were dissolved into 5 ml of ethanol, 50 μl of each were spotted quantitatively on TLC plate of Silica Gel-GF_{254} (Merck) and developed with a solvent system of chloroform : ethanol=19 : 1 (v/v). After drying, the plate was searched for BCM under UV

light. The silica gel which correspond to the *Rf* value of BCM was scratched and BCM was eluted with 5 ml of ethanol. The concentration of BCM was determined spectrophotometrically by measuring absorbance at 280 nm, pure BCM as a standard.

Isolation and identification of Benomyl (BCM)-tolerant fungus

A BCM-tolerant fungus was isolated from a colony which developed on the Martin's medium containing 100 ppm of BCM. This fungus was cultured on Czapek agar medium and identified according to the method of Thom and Raper[9].

Absorption of BCM by sensitive and tolerant fungi

Aspergillus versicolor, the BCM-tolerant fungus, and *Cladosporium harbarum*, sensitive fungus, were cultured in modified Czapek liquid medium[5] at 22°C for 5 days on a rotary shaker. The mycelia were collected on a Büchner funnel by filtration, and 25g (wet weight) of each fungal mat were added into shake flasks containing 100 ml of 4 ppm solution of BCM. Shake flasks were shaked on a rotary shaker at 22°C. Five ml of filtrate were collected at 30, 60, and 120 min. after treatment, the remaining BCM was extracted with 5 ml of ethyl acetate and analyzed quantitatively by the method as descrived above.

The amount of BCM absorbed by fungal mycelia was calculated and expressed as μg/g dry weight of mycelia.

Svrvay of Benomyl-metabolite in mycelia and culture filtrate of tolerant fungus

Aspergillus versicolor, the BCM-tolerant fungus was cultured in the modified Czapek liquid medium at 22°C for 5 days, then 100 μg of pure Benomyl was added into each flasks, and continued to culture. The fungal mycelia were separated from the filtrate by filtration at 1, 3, 5, 7, and 9 days after treatment.

The mycelia were homogenized with 20 ml of water, 20 ml of ethyl acetate and glass-beads by Vertis homogenizer for 10 min. The homogenate was filtered through Büchner funnel with Hyflo-super-cel and the ethyl acetate phase of the filtrate was concentrated to dryness. The residue was dissolved in 2 ml of ethanol, and chromatographed by TLC as described above. After drying, the plate was searched for metabolite of Benomyl under UV light.

The amount of BCM was also determined by the method described above.

Results

Effect of fungicides on soil microflora

1). *Effect under field conditions*

As shown in Fig. 1, the population of soil inhabitant fungi was not affected seriously by heavy treatment with BCM under field conditions. Total number of soil fungi reduced to about 1/6 at next day after the last treatment with Thiram. The population, however, recovered to the normal (untreated) level at 7 th day.

The population of BCM-tolerant fungi seemed to increased slightly at later stage of experiment by treatment with BCM. No Thiram-tolerant fungi could be found in both Thiram-treated and non-treated soil during 121 days of experiment.

The bacterial population in soil was not affected significantly by treatment with BCM and also with Thiram (Fig. 2).

2). *Effect under laboratory conditions*

Incubation of soil with fungicides, BCM or Thiram in laboratory condition did not affect the total number of soil inhabitant fungi (Fig. 3). The number of BCM-tolerant fungi was less than 1/10 of the total number of fungi, and the treatment with BCM did not affect significantly the number of tolerant fungi in the soil (Fig. 3). No Thiram-tolerant fungus was found in all the soil samples tested as well as the experiment under the field conditions.

The population of bacteria in soil was not affected significantly by the treatment with

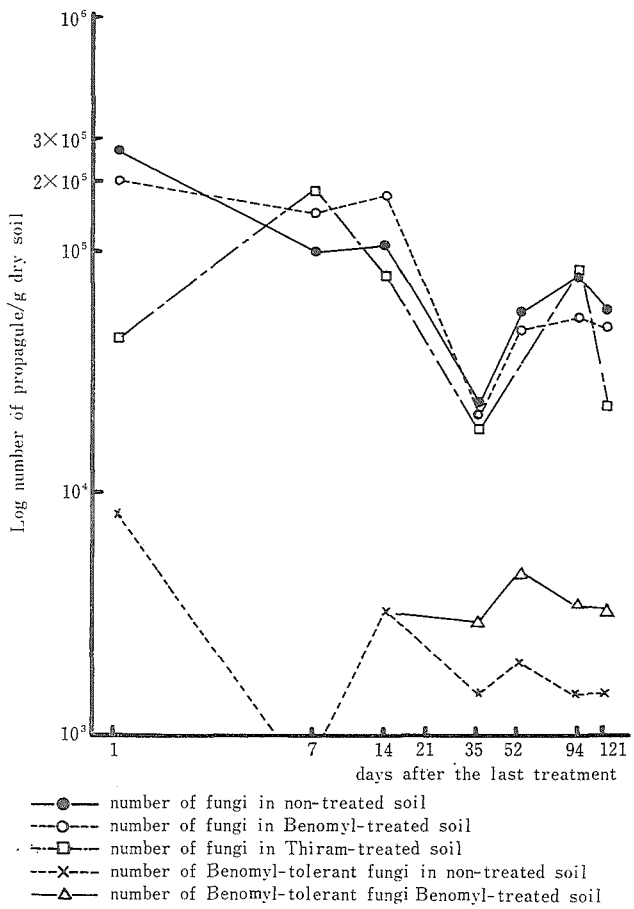

Fig. 1. Effect of fungicides on the population of soil fungi under field conditions

both fungicides. Number of BCM-tolerant bacteria developed on the BCM-containing medium (100 ppm) was almost the same as the number that developed on BCM-free medium, suggesting that BCM has no antibacterial activity. Thiram-tolerant bacteria, however, did not found in both Thiram-treated and non-treated soil.

Recovery of BCM from sterilized and unsterilized soil

Results of quantitative analysis of residual BCM in sterilized and unsterilized soil were shown in Fig. 4. As seen in the Figure, added BCM did not decrease in sterilized soil, but decreased rapidly in unsterilized soil. This indicates that the decrease of BCM in unsterilized soil is dependent on the metabolic activity of soil microorganisms.

Mechanism on BCM-tolerance of Aspergillus versicolor

1). *Isolation and identification of BCM-tolerant fungus*

A fungus which grew on the medium containing 100 ppm of BCM was isolated as the BCM-tolerant fungus. This fungus was quite tolerant showing good growth on the media containing 1,000 ppm of BCM.

The natures of the tolerant fungus on Czapek agar medium was as follows :

Vegetative mycelium……septate, colorless
Conidiophore ……enlarging upward and bearing sterigmata, smooth wall, not colored
Sterigmata……two sereis

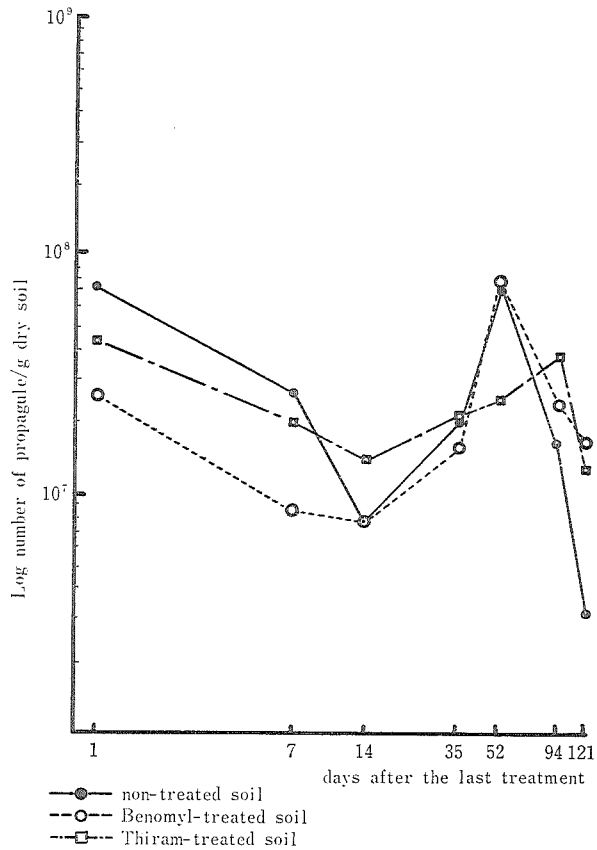

Fig. 2. Effect of fungicides on the population of soil bacteria under the field conditions

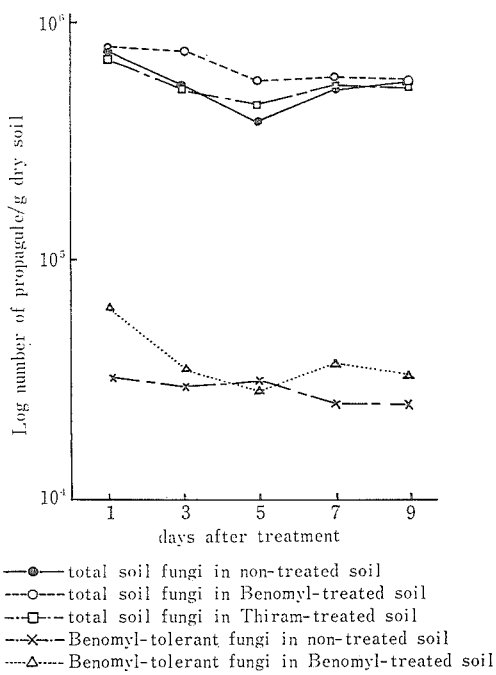

Fig. 3. Effect of fungicides on the population of soil fungi under laboratory conditions

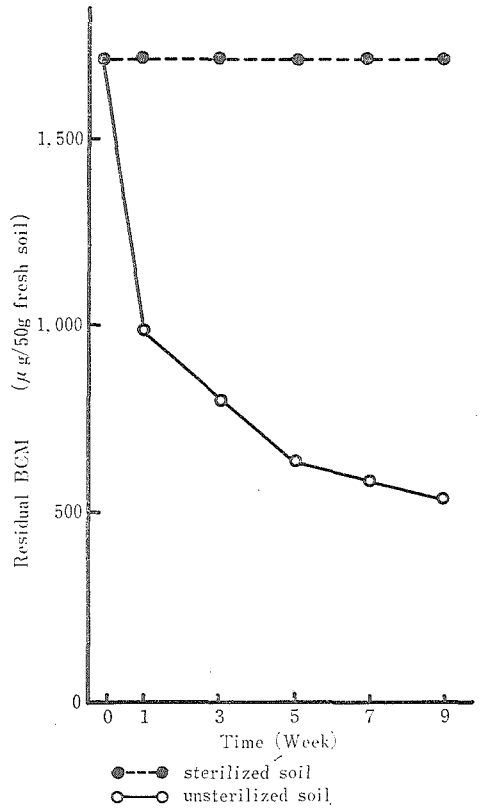

Fig. 4. Time course of residual BCM in sterilized and unsterilized soil

Conidial head ····· globose, not cylindrical-clavate
Perithecia ····· not produced
Color of colony ····· white, but gave greenish color at some stage of development
Conidial area of colony···dull green or tan

According to the systematic classification method of Thom and Raper[9], the above properties coincided with those of *Aspergillus versicolor* group.

2). *Survay of degradation product of Benomyl in culture of tolerant fungus*

One of the most important causes of tolerance of some microorganisms to some toxicants is the detoxication mechanism due to the metabolic activity of the microorganisms.

To know whether the BCM-tolerant fungus, *A. versicolor*, has the detoxication mechanism or not, we searched the degradation products of added authentic Benomyl in the culture of this fungus. However, no compound other than Benomyl and BCM was found by thin layer chromatography of the extracts from both fungal mycelia and culture filtrate. The sum total of the amount of Benomyl and BCM in culture filtrate and in fungal mycelia did not change during 7 days of culture.

These results indicated that the tolerance of this fungus to Benomyl does not depend on the metabolic degradation of this fungicide.

3). *Absorption of BCM by sensitive and tolant fungi*

The difference in absorption of BCM by sensitive and tolerant fungi were examined.

As shown in Fig. 5, the sensitive fungus, *Cladosporium harbarum* took up BCM twice as much compared to the tolerant fungus, *A. versicolor*. The maximum absorption

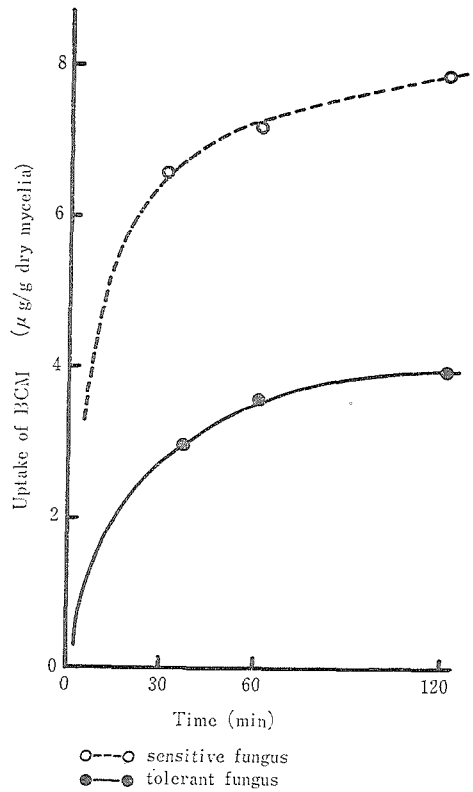

Fig. 5. Time course of BCM-Uptake by tolerant aud sensitive fungi

occred within the first 30 min. after addition of BCM, and then, reached plateau in both sensitive and tolerant fungi.

Discussion

The effect of pesticides on the metabolic activities of soil, such as respiration, nitrification and so on, has been studied by many workers to know indirectly the effect on soil microorganisms. These reports indicete that some pesticides, especially organochlorine compounds inhibit these metabolic activity regardless of the kind of pesticides. The inhibition, however, appears at very much higher concentrations than used in the field[10].

Recently, many reports regarding the direct effect of pesticides on soil microflora have deen appeared, and indicated that some pesticides affect significantly to the microflora. For example, the fungal populations were 40-75 % less in Captan-treated soil than those in non-treated soil at 44 th day, but the bacterial populations increased[3]. The effects, however, were known to be variable depending on the environmental conditions, such as kinds of soil, temperature, pH, humidity and the kind of soil microorganisms.

Our experimental results of the present reports indicated that Benomyl and Thiram, the most widely used agricultural fungicides, did not affect seriously the soil microflora of the experimental field of Okayama University (sandy loam), as have been reported by several workers[2,7,8]. Thiram reduced the fugal population in the soil to about 1/6 at the next day of the last treatment, but recovered to the level in non-treated soil after 6 days.

The largest reason why these active fungicides showed little or no effect on soil microflora might be due to the buffer action of soil particles, since 100 ppm of Thiram inhibit completely the growth of soil microorganisms in vitro but not in soil.

Soil microorganisms were known to play a key role in the breakdown of residual pesticides. Our results also indicated that BCM was degraded by microorganisms in soil because autoclaving of the soil inhibited completely the degradation of this fungicide. Therefore, we searched the tolerant microorganism to BCM and studied to know whether the tolerant one has the metabolic activity to degrade BCM. A highly tolerant fungus to BCM, *A. versicolor*, however, did not show any activity to decompose BCM. One of the tolerant mechanisms of this fungus seemed to be due to the low absorption ability to this fungicide.

Acknowledgement

The authors wish to thank Prof. T. Kondo of Kyushu University for supplying the authentic sample of BCM. Financial support from Sankyo Co., Ltd, is also acknowledged.

References

1) GOWDA, T. K. S. and R. B. PATIL : Biochem. J. **128**, 56–57 (1972).
2) HOFER, I., T. BECK and P. WALLNOEFER : Z. Pflanzenkrankh. Pflanzenschutz **78**, 398–405 (1971)
3) HOUSEWORTH, L. D. and B. G TWEEDY : Plant Soil **38**, 493–500 (1973).
4) MAZUR, A. R. : Disertation Abstr. Intern. B. **39**, 4162 (1974).
5) OKU, H. : Ann. Phytopath. Soc. Japan **23**, 169–175 (1968).
6) OKU, H., T. SHIRAISHI, S. OUCHI and S. HORIUCHI : Sci. Rep. Fac. Agr. Okayama Univ. **47**, 7–13 (1976).
7) PEEPLES, J. L. : Phytopatholgy **64**, 857–860 (1972).
8) RAYNAL, G and F. FERRARI : Phytiat. Phytopharm. **22**, 259–272 (1973).
9) THOM, C. and K. B. RAPAER : A Manual of the Aspergilli, Williams & Wilkins Co. Bartimore (1945).
10) YAMAMOTO, I. and J. FUKAMI ed. Pesticide Design -Strategy and Tactics-p.1099, Soft Science Inc. Tokyo (1979).

殺菌剤，ベノミル，チウラムの土壌微生物相と２～３の土壌生息性糸状菌に及ぼす影響

奥　八郎，沖　和生[a)]，白石友紀，佐藤健二，大内成志
岡山大学農学部（植物病学研究室）

ベノミルとチウラムの土壌微生物相と２～３の土壌生息性糸状菌に及ぼす影響を，岡山大学農学部圃場の土壌を用いてしらべた．

自然条件下においては，ベノミルの多用は土壌微生物相にたいした影響を与えない．チウラムで処理すると，翌日には土壌生息性糸状菌の密度が約1/6に減少したが，６日前にはもとの密度に回復した．室内実験では，両殺菌剤とも土壌微生物相に影響を与えなかった．ベノミル耐性糸状菌数は，総糸状菌数の約1/10，この数はベノミル処理土壌において，120日間に及ぶ実験期間の後期にはやゝ増加した．チウラム処理土壌，無処理土壌中にはともに，チウラム耐性糸状菌，細菌は存在しなかった．ベノミルに高度耐性を示す糸状菌を分離し，*Aspergillus versicolor* と固定した．この菌にはベノミルを代謝分解する能力はなく，ベノミル吸収力が感受性の *Cladosporium harbarum* に比して約半分であった．

a) 岡山県農業試験場