

EVALUATION OF FUNGICIDES FOR CONTROL OF TREE DISEASES

- I. Preliminary Screening Against the
Dutch Elm Disease *Ceratocystis ulmi*
(Buism) Moreau Under Laboratory Conditions.

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INTRODUCTION

At a recent conference on Human Environment in Stockholm (1972), the United Nations recommended, under its Man and Biosphere program, that an accelerated effort in the beautification of the metropolitan and municipal surroundings be made by increased plantings of green trees in the form of ornamental, shade and amenity plants. The reason for this was that by the end of this century, over 90% of the population would reside in the urban environments and hence it is important to create attractive landscape, greenbelts and parks around the cities (Jorgensen 1973). Therefore it is logical to be concerned about the preservation and protection of urban and amenity trees and the aim of the present investigation is to find ecologically acceptable chemicals for the control of tree diseases.

Elm (Ulmus americana L.) is one of the popular shade trees in the urban and suburban localities and its protection against the Dutch elm disease (DED) caused by Ceratocystis ulmi is urgently needed. Even though considerable effort has been made during the past 40 years to save the elm from DED, no practical and effective cure is available to date. However, with the discovery of a new fungicide (benomyl[®]) by the Dupont Co., U.S.A., (Delp and Klopping 1968) it seems that DED can now be contained and combatted (Smalley 1972, Kondo 1972, Prasad 1972). Although benomyl shows great promise as a chemotherapeutant against the DED (Stipes 1969, Biehn and Diamond 1971) its lack of solubility in aqueous formulations has been one of the serious drawbacks. Also if strains of DED become resistant to benomyl or if benomyl inhibits turnover of DNA (Clemons & Sisler 1971) then there is a need to screen

other compounds which possess greater solubility, compatibility with spray formulations and potency against the tree diseases. With this objective in mind, a screening program was undertaken and the present report summarizes the relative fungicidal activities of ten compounds.

MATERIALS AND METHODS

(i) Chemical Compounds

Samples of ten commercially available compounds were obtained from various chemical companies and such details are given in the Appendix I. Since many of these compounds were not absolutely pure in their composition, a 100% activity factor was calculated on the basis of the active ingredient and then the adjusted amounts were employed to prepare stock solutions or suspensions. At this point the hydrogen ion concentration of each solution was checked with a combination electrode pH meter. Further dilutions were made to obtain a range of concentration for each candidate compound under study. In order to prevent undue decomposition or contamination, all solutions were stored in a cool and dark place. Five to seven concentrations of each compound were prepared and used against the test organism.

(ii) Culture of Fungi

The screening trials were carried out with two fungi, the DED organism (Ceratocystis ulmi (Buism.) C. Moreau and the citrus mold (Penicillium expansum L.). Pure cultures of both were obtained from Shade and Ornamental Tree Laboratory, Delaware, Ohio, and Great Lakes Forest Research Centre, Sault Ste. Marie. The fungi were first grown on potato dextrose agar (PDA) and then subcultured in the same medium at

weekly intervals for maintenance of their vigor. For large scale screening program, standard agar plates containing 15 ml. sterile agar (Difco PDA) were employed. These plates were seeded with P. expansum or C. ulmi spores according to a procedure described by Hock (1970). Briefly, spores were harvested from fully grown cultures, suspended in sterile water and filtered through a cheesecloth. Optical density of this suspension was then measured at 450 nm in a colorimeter. Only such a suspension exhibiting $OD = 0.1$ (10^6 spores/ml) was mixed at the rate of 15 ml/L with the agar media because it produced excellent growth of the organisms at room temperature. Hundreds of agar plates seeded with the fungi were thus prepared and stored in a refrigerator for 24-48 hours.

(iii) Fungicide Treatment

The method of application of each candidate chemical consisted of cutting out a uniform 10 mm well in the centre of the agar medium with a sterilized # 7 cork borer and pipetting of 0.1 ml of each of the compound into it. All petri dishes were then held in an incubator at $25 \pm 1^\circ\text{C}$ for 48 hours. To minimize variability, all treatments were replicated three fold. In some cases where persistence of inhibition was assayed for longer periods, petri dishes were allowed to stay for 144 hours in the incubator. The zones of inhibition produced by each compound were then measured and the concentrations reducing the growth by 25 or 50% obtained by plotting these data on a graph paper.

RESULTS

Since P. expansum has been used as a bioassay organism for benomyl and benomyl like compounds and is far more sensitive than the

DED organism, preliminary screening trials were carried out with this fungus first. Concentrations ranging from 0, 1, 10, 100, 1000 and 5000 ppm were tried. Sterile distilled water was used for appropriate controls. Dose-response curves for each compound are presented in Fig. 1, 2 & 3. As can be seen, some candidate chemicals are more potent than others. Morestan, HOE EC, and Vancide TH were the least effective while the other 7 produced varying degrees of inhibition. It seems that time of exposure to some compound is an important factor in toxicity since treatment for 2, 4 and 6 days, produced different degree of responses. When the dosage was plotted on a logarithmic scale against the percentage inhibition, none of the compounds yielded a typical sigmoid curve and this complicated application of probit technique for determination of ED_{50} . Therefore ED_{25} and ED_{50} for each compound were estimated by approximation and the results are presented in Tables I & II. Clearly out of all compounds tested MBC-P is the most potent in reducing the growth by 25% and 50%. The order of relative inhibition was as follows: ED_{25} : MBC-P > Benomyl-C > Mertect Fl > Vancide Maneb Fl > Benomyl-P > Thiophanate methyl > Vancide 40 > Vancide TH = HOE EC = Morestan. ED_{50} : MBC-P > Vancide Maneb Fl > Mertect Fl > Benomyl-C > Vancide 40 > Benomyl-P > Thiophanate methyl > Morestan = HOE EC = Vancide TH.

When these groups of compounds were evaluated against the DED pathogen the response was somewhat different (See Tables III and IV). Even though much higher dosages up to 20,000 ppm were employed against the DED organism, the degree of inhibition was poor and small. Still ED_{50} & ED_{25} were estimated and judging the performance of all candidates, the most potent compounds were Vancides. The order of inhibition was in the

following manner: ED_{50} : Vancide 40 > Vancide Maneb > Vancide TH > MBC-P > Benomyl-P = Benomyl-C = Thiophanate methyl = Morestan = Mertect Fl = HOE EC. ED_{25} : Vancide Maneb Fl > Vancide 40 > Vancide TH > MBC-P > Benomyl-C > HOE EC Benomyl-P = Thiophanate methyl = Morestan = Mertect Fl. The comparative effects of all compounds on both organisms are summarized and illustrated in Figs. 4, 5 and Table V.

PENICILLIUM EXPANSUM

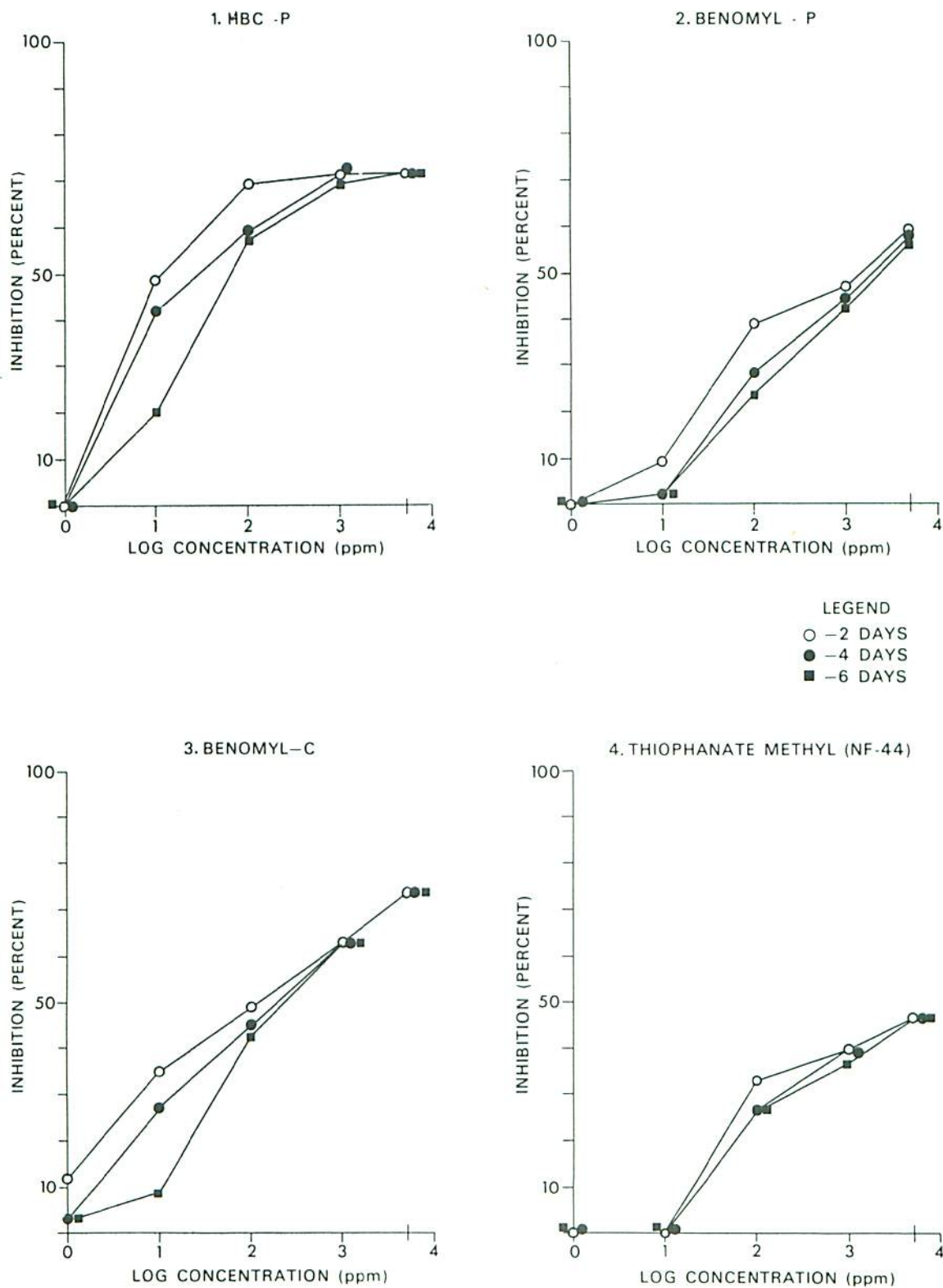


Fig. 1 Dose-response curves for *Penicillium expansum* following treatment with MBC-P, Benomyl-P, Benomyl-C and Thiophanate methyl (NF44) after 2, 4 and 6 days.

PENICILLIUM EXPANSUM

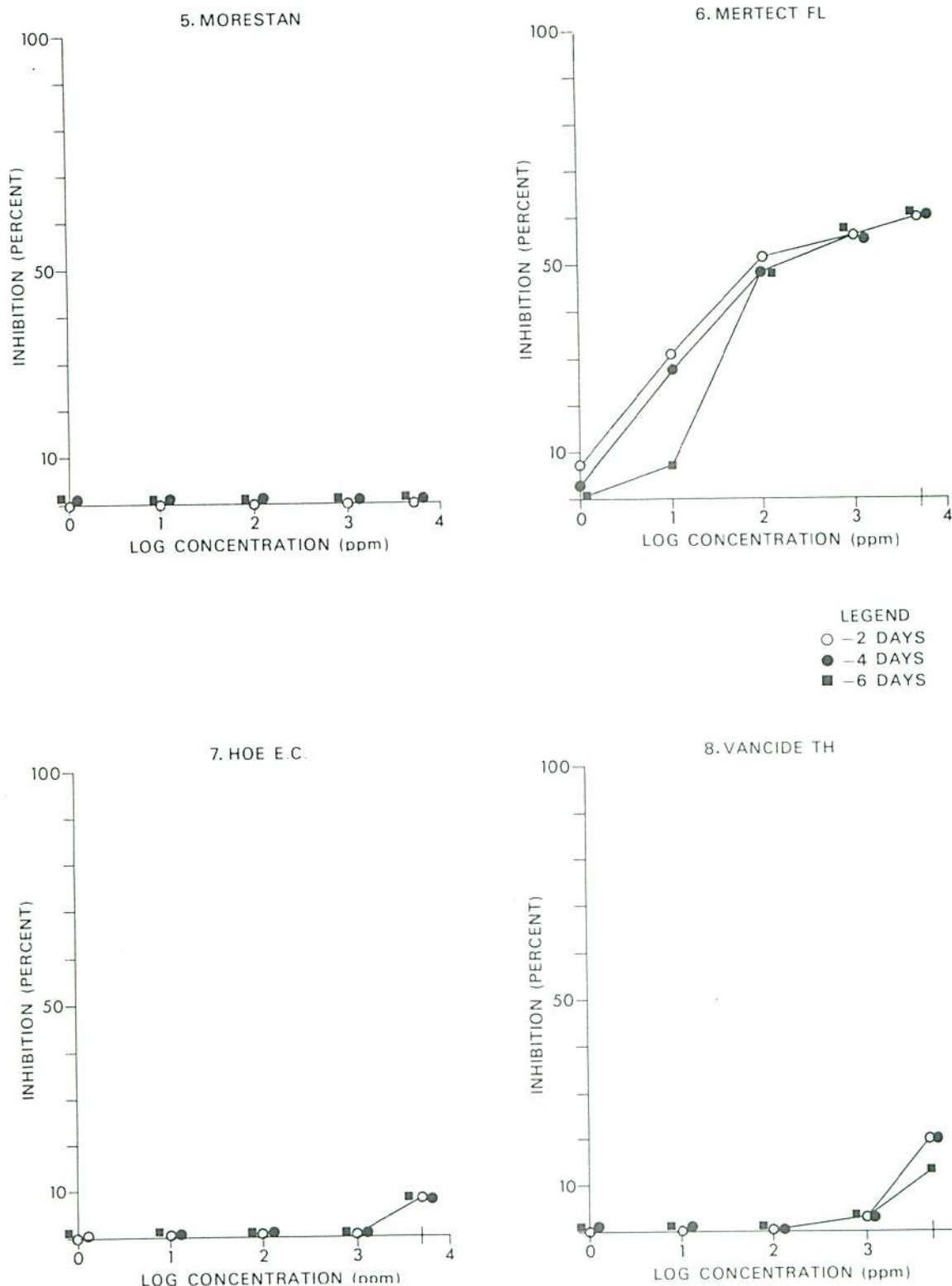


Fig. 2. Dose-response curves for *Penicillium expansum* following treatment with Morestan, Mertect FL, HOE EC and Vancide TH after 2, 4 and 6 days.

PENICILLIUM EXPANSUM

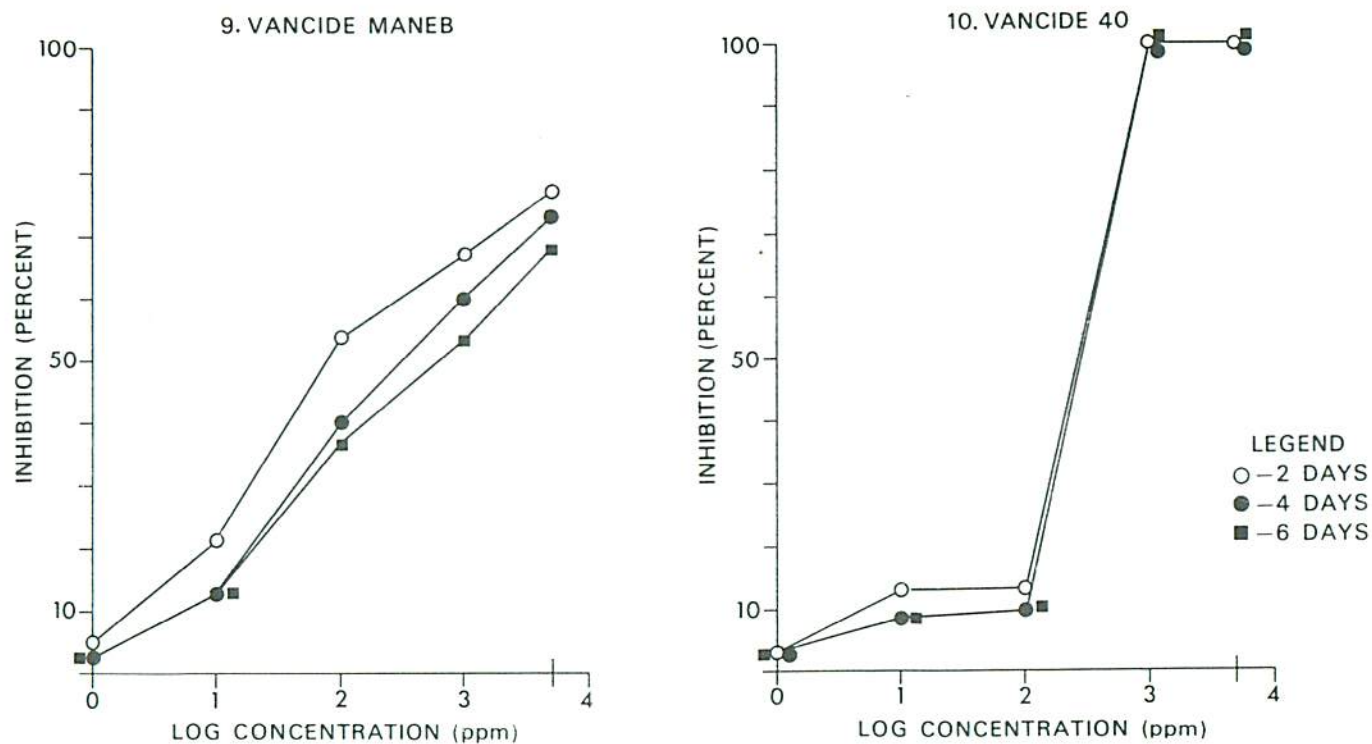


Fig. 3. Dose-response curves for Penicillium expansum following treatment with Vancide Maneb and Vancide 40 after 2, 4 and 6 days.

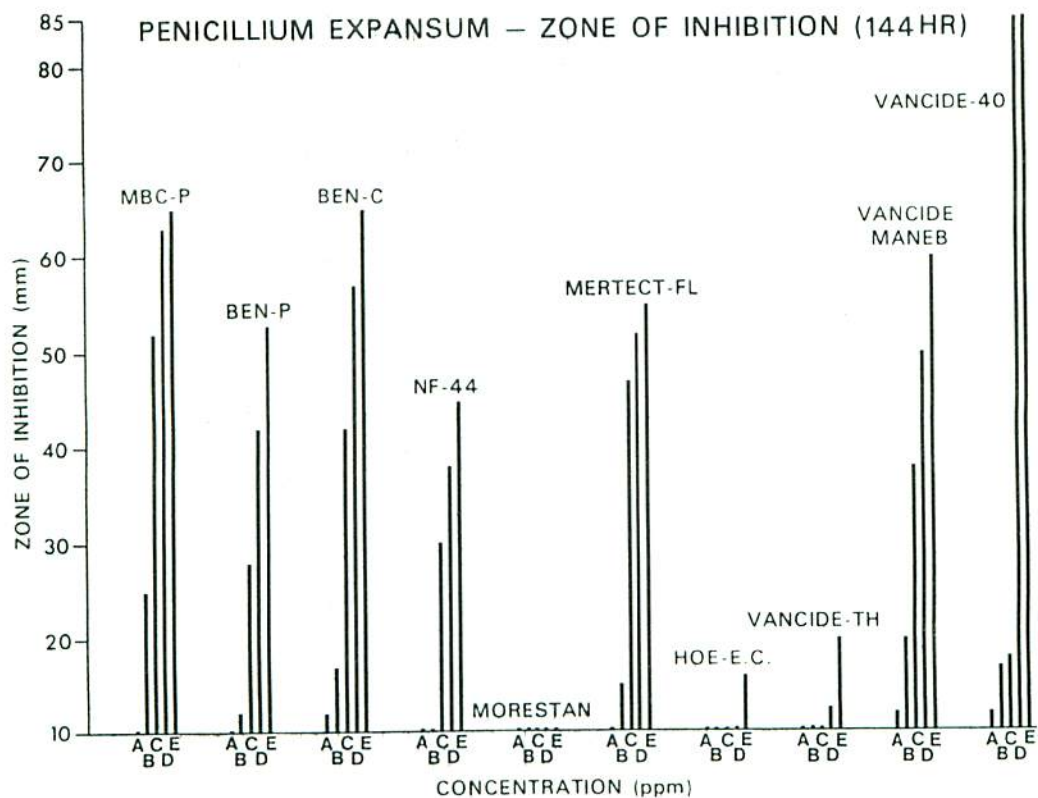
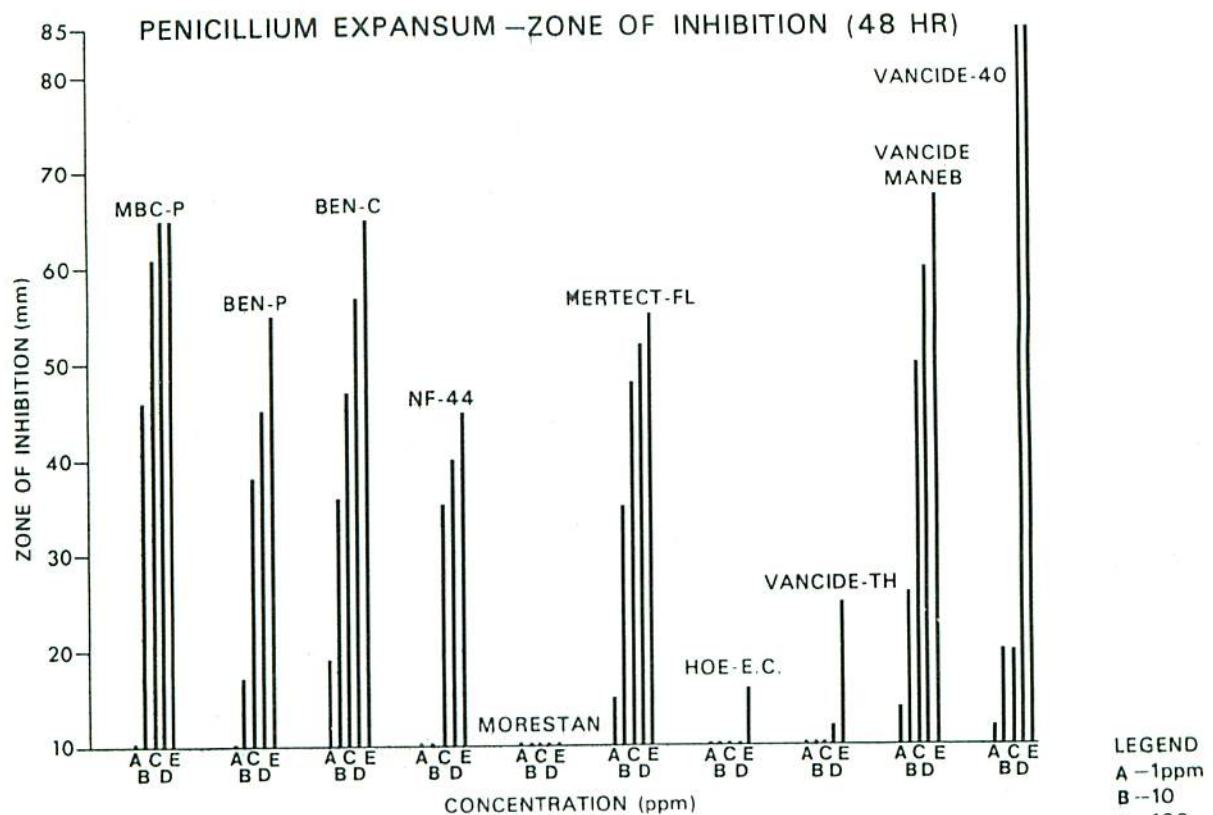


Fig. 4. A summarized account of effectiveness of ten compounds on colony formation of *P. expansum* after 2 and 6 days.

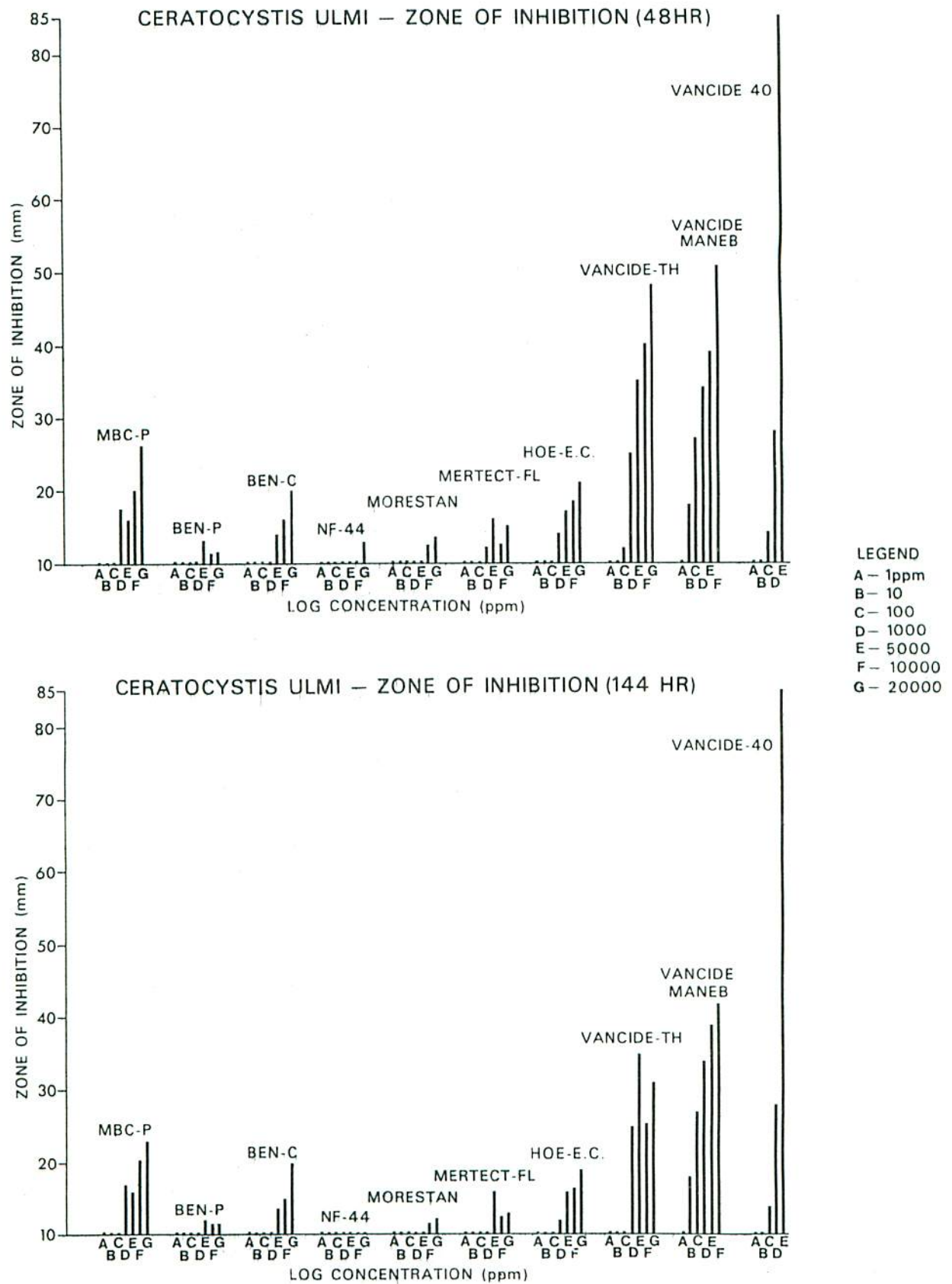


Fig 5. A summarized account of effectiveness of ten compounds on growth and colony formation of C. ulmi after 2 and 6 days.

TABLE I

ED₅₀ and Relative Inhibition of Penicillium expansum after 2 days

<u>Compound</u>	<u>ED₅₀ (ppm)</u>	<u>Relative Inhibition</u>	<u>Inhibition Index</u>
MBC-P	12.6	1	100
Benomyl-P	2000	0.006	0.6
Benomyl-C	158	0.08	8
Thiophanate Methyl	7080	0.002	0.2
Morestan	-	-	-
Mertect FL.	81.3	0.16	16
Hoe E.C.	-	-	-
Vancide TH	-	-	-
Vancide Maneb FL.	79.5	0.16	16
Vancide 40	282	0.045	4.5

TABLE II

ED₂₅ and Relative Inhibition of Penicillium expansum after 2 days

<u>Compound</u>	<u>ED₂₅ (ppm)</u>	<u>Relative Inhibition</u>	<u>Inhibition Index</u>
MBC-P	3.17	1	100
Benomyl-P	32.4	0.098	9.8
Benomyl-C	3.24	0.98	98
Thiophanate Methyl	56.3	0.056	5.6
Morestan	-	-	-
Mertect FL.	5.63	0.56	56
Hoe E.C.	-	-	-
Vancide TH	7080	-	-
Vancide Maneb FL.	13.2	0.24	24
Vancide 40	142	0.022	2.2

TABLE III

ED₅₀ and Relative Inhibition of Ceratocystis ulmi after 2 days.

<u>Compound</u>	<u>ED₅₀ (ppm)</u>	<u>Relative Inhibition</u>	<u>Inhibition Index</u>
MBC-P	*563000	1	100
Benomyl-P	-	-	-
Benomyl-C	-	-	-
Thiophanate methyl	-	-	-
Morestan	-	-	-
Mertect FL	-	-	-
HOE E.C.	-	-	-
Vancide TH	20000	28	2800
Vancide Maneb FL.	4470	126	12600
Vancide 40	1780	316	31600

TABLE IV

ED₂₅ and Relative Inhibition of Ceratocystis ulmi after 2 days.

<u>Compound</u>	<u>ED₂₅ (ppm)</u>	<u>Relative Inhibition</u>	<u>Inhibition Index</u>
MBC-P	* 31700	1	100
Benomyl-P	-	-	-
Benomyl-C	* 56300	0.56	56
Thiophanate Methyl	-	-	-
Morestan	-	-	-
Mertect FL	-	-	-
HOE E.C.	* 89100	0.36	36
Vancide TH	1780	17.8	1780
Vancide Maneb FL.	159	199	19900
Vancide 40	1000	31.7	3170

* Estimated values.

TABLE V

A Comparison of Performance of Penicillium expansum and Ceratocystis ulmi in terms of ED₂₅ after being exposed to various compounds for 2 to 6 days.

Compound	<u>Penicillium expansum</u>		<u>Ceratocystis ulmi</u>	
	2 days	6 days	2 days	6 days
MBC-P	3.17ppm	14.1 ppm	*31700 ppm	63100 ppm
Benomyl-P	32.4	126	-	-
Benomyl-C	3.24	28.2	*56300	*56200
Thiophanate Methyl	56.3	79.4	-	-
Morestan	-	-	-	-
Mertect Fl.	5.63	28.2	-	-
HOE E.C.	-	-	*89100	*89100
Vancide TH	*7080	*50100	1780	1780
Vancide Maneb Fl.	13.2	31.6	159	159
Vancide 40	142	158	1000	1000

* Estimated values

TABLE VI

Physical and Chemical Characteristics of
Various Chemical Compounds used in the Screening Trials

<u>Compound</u>	<u>Physical State</u>	<u>pH of Stock Solution</u>	<u>Solubility in Water</u>
1. MBC-P	Solid	6.21	Nil - Precipitate
2. Benomyl-P	Solid	6.32	Nil - Precipitate
3. Benomyl-C	Solid	6.51	Poor - Suspension
4. Thiophanate Methyl	Solid	5.99	Poor - Suspension
5. Morestan	Solid	7.51	Poor - Suspension
6. Mertect Flowable	Liquid	6.87	Poor - Suspension
7. HOE E.C.	Liquid	3.70	Poor - Suspension
8. Vancide TH	Liquid	3.70	Poor - Suspension
9. Vancide Maneb Fl.	Liquid	6.91	Good-Solution-Suspension
10. Vancide 40	Liquid	7.70	Good-Solution-Suspension
11. Distilled water	Liquid	6.98	Excellent-Solution

DISCUSSION

From the foregoing results it is evident that fungicidal compounds of different chemical structures show differential toxicity to P. expansum and C. ulmi (Table I & II). Because P. expansum is more sensitive to these groups of chemicals, it can be employed as a bioassay organism for the screening of these compounds. What is more interesting is the differential behaviour of each compound on the germination of DED spores. While some compounds are not effective at all, some are slightly so while others are highly inhibitory. This suggested that penetrability, solubility and toxic action at cellular level might be different for different compounds. Since the commercial formulations of each candidate compound contained impurities (Appendix I) and possessed a wide range of solubility in aqueous solutions (Table VI), it was thought pertinent to investigate the pH of each solution. Table VI also shows the variability in pH and it is possible that this factor contributed to differential toxicity (Simon & Beevers 1952, Prasad 1972). Similarly some chemicals were soluble in water, some formed suspension and others precipitated out of solution (Table VI). Again, since this screening technique involves the diffusion of each compound into the agar seeded with spores of P. expansum or C. ulmi, physical factors (pH, solubility, etc.) are bound to influence diffusion and hence effectiveness of each compound (Horsfall 1956). Perhaps this may be one of the reasons why there was no sigmoid response to applied dosages. Even though this screening trial was preliminary, it does indicate that Vancide fungicides are superior to benomyls in fungitoxicity against the DED.

However, further experiments should be carried out with higher dosages to permit at least 50% reduction in colony formation of C. ulmi. This may be achieved by solubilization of each compound into aqueous medium, since the determination of ED_{50} hinges on the diffusion of each compound into the agar plate. Formulations containing organic solvents are, by and large, inhibitory to fungal growth and their use in screening must be treated with caution. Alternatively, a different procedure of evaluation of insoluble fungicides should be restored to. Perhaps the suspension should be thoroughly mixed with the agar first so as to ensure uniform distribution and then the seeding of each petri dish with C. ulmi spores should be carried out. Following this, some of the promising compounds should be tested for systemicity under greenhouse and field conditions.

SUMMARY

Ten chemical compounds of different chemical structures were tested for fungitoxicity in agar plates against Penicillium expansum and Ceratocystis ulmi. The order of inhibition as judged by the concentration required to reduce colony formation by 25% (ED_{25}) were different for different species. For P. expansum the order was: MBC-P > Benomyl-C > Mertect Fl > Vancide Maneb > Benomyl-P > Thiophanate methyl > Vancide TH = HOE EC = Morestan. For C. ulmi the order was: Vancide Maneb > Vancide 40 > Vancide TH > MBC-P > Benomyl-C > HOE EC = Morestan = Mertect Fl = Thiophanate methyl = Benomyl-P.

P. expansum was more sensitive than C. ulmi and is suggested for use in bioassay of these compounds. The relative toxicities of some

compounds began to decline 48 hours after treatment and this suggested as if the fungi metabolized the compounds. The superior action of Vancide Maneb over benomyl appeared to be partly related to water solubility.

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APPENDIX-I

CHEMICAL NOMENCLATURE OF COMPOUNDS & THEIR SOURCES

Compound	Type	Chemical Name	Source
MBC - P	Benzimidazole	1-(Butylcarbamoyl)-2-Benzimidazole Carbamic Acid, Methyl Ester	Dupont Co. U.S.A.
Benomyl - P	Benzimidazole	1-(Butylcarbamoyl)-2-Benzimidazole Carbamic Acid, Methyl Ester	Dupont Co. U.S.A.
Benomyl - C	Benzimidazole	1-(Butylcarbamoyl)-2-Benzimidazole Carbamic Acid, Methyl Ester, 50% W.P.	Dupont Co. U.S.A.
Thiophanate Methyl (NF-44)	Thiourea	1,2-BIS(3-Methoxycarbonic-2-Thioureido) Benzene	Nippon Soda Co. Japan
Morestan	Carbamate	6-Methyl,-2,3-Quinoxalinedithiol Cyclic S,S-Dithiocarbonate 25% W.P.	Chemagro, U.S.A.
Mertect Fl.	Benzimidazole	2-(4 Thiazolyl) Benzimidazole Flowable	Merck & Co. U.S.A.
HOE EC 2873	Phosphorus Ester	2-10,0-Diethyl-Thionophosphoryl)-5- Methyl-6-Carbethoxy-Pyrazolo-(1,5,a) Pyrimidine	Canadian Hoechst Co.
Vancide TH	Triazine	Hexahydro-1,3,5-Triethyl-S-Triazine	R.T. Vanderbilt Co. U.S.A.
Vancide Maneb FL (+ Zn)	Carbamate	Manganese Ethylenebisdithiocarbamate with added zinc	R.T. Vanderbilt Co. U.S.A.
Vancide 40	Morpholine	(N(2-Nitrobutyl) Morpholine	R.T. Vanderbilt Co. U.S.A.