FURTHER STUDIES ON THE FUNGICIDAL PROPERTIES OF MAKROLIN

S. Shahid Husain and Mohammad Yousuf

Phytopathology Section, Central Laboratories, Pakistan Council of Scientific and Industrial Research, Karachi

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Makrolin, a chlorinated hydrocarbon, was further tested for its fungicidal properties and compared with colloidal copper and zerlate. *Alternaria tenuis*, *Helminthosporium anomalum* and *Fusarium dimerum* were used as test fungi. Poisoned food and slide-spore germination techniques were employed and the results of the two methods compared. In poisoned food technique, Makrolin was found to be as effective as zerlate in controlling the growth of *F. dimerum* and *H. anomalum*. 50 ppm. of both compounds gave 100% inhibition of the two fungi. Makrolin was less effective on *A. tenuis*. The third compound, colloidal copper, was not employed in this technique due to its unstable suspendability in the liquid media.

In the slide spore-germination technique, Makrolin was found to be as effective as colloidal copper in checking the spore-germination of *F. dimerum* and *H. anomalum* but less effective as compared to zerlate.

Similarly zerlate and colloidal copper were more effective in controlling the spore-germination of *A. tenuis* than Makrolin.

On the basis of above results Makrolin can easily be recommended for further exploitation.

Introduction

Since the beginning of planned research for new or improved fungicides, in the Laboratories of this Council, studies have been made in the hope that from the hundreds of indigenous compounds which can be tested by simple laboratory methods, a few might be selected for costly field trials and ultimate commercialization in Pakistan.

Quraishi and Husain[1] and later Ahmed and Quraishi[2] made preliminary studies on the fungicidal properties of two indigenous products, Makerol and Sharigol, obtained during the desulphurisation of coal.

Recently fungicidal properties of a new indigenous product, "gasoline," which is a waste product of Daud-Khel fertilizer factory, and is converted into a highly chlorinated liquid and termed as "Makrolin"[3] have been tested. Ashrafi and Ghayasuddin[4] and later Ashrafi *et al.*[5] carried out some preliminary tests on its fungicidal properties and have reported that Makrolin is quite effective in checking the growth of *Fusarium*, *Alternaria* spp., and *Aspergillus niger*, as compared to colloidal copper and copper naphthenate.

Many characters of fungi such as growth, morphology, sporulation and spore-germination, are altered by treatment with chemicals, and it is the first manifestation (i.e. growth) of chemical effect on fungi that has been taken into consideration for the quantitative determination of the fungicidal effects. The use of poisoned food technique was made for the first time by Falck,[6] primarily for the purpose of testing various types of wood preservatives.

The fact that contact between a fungicide and a fungus is more true in liquid medium than in solid medium was emphasized by various workers.[7–10] The liquid culture technique was thus adopted for this very reason. The most widely used and standardized method of evaluating fungicides has been the spore-germination technique.[11–12]

The results of the present investigations are, therefore, based on these two quantitative techniques, namely, the poisoned food and the spore-germination.

Experimental

Many characters of fungi are altered through chemical treatments.[6] Two of the character, growth and spore-germination have been taken for measuring the effect of different chemicals used in present studies.

Makrolin,[3] along with Zerlate[13] (zinc dimethyl di-thiocarbamate) and Colloidal copper,[14] was tested for its fungicidal properties. *Alternaria tenuis* Nees (Cotton leaf), *Fusarium dimerum* Penzig (A.R.I. Tandojam), and *Helminthosporium anomalum* Gilman and Abott (Soil) three common plant pathogenic fungi causing various diseases of economic importance, were used as test organisms.

Liquid Culture Technique

Czapeck's Dox synthetic medium was used for the growth of the various fungi. This liquid culture poisoned food technique had also been employed by many previous investigators[7-10,15-16] for the quantitative determination of fungicidal effects of different substances.

The medium was prepared according to Riker's[17] formula. All the constituents of the medium were dissolved in distilled water, including potassium dihydrogen phosphate,[18] and distributed in 300 ml. capacity flasks as required.

0.5% solution of Makrolin was prepared in acetone and 0.125, 0.25, 0.5, 1.0, and 2.0 ml. of this solution was added to 99.875, 99.75, 99.5, 99.0 and 98.0 ml. of sterilized Czapek's medium in flasks to obtain final concentrations of 6.2, 12.5, 25, 50 and 100 ppm., respectively. These concentrations have been recommended [11,12] and used by various other workers as well. Similarly 0.5% solution of Zerlate was prepared in 2% sodium hydroxide solution (since this compound is soluble in water upto 65 ppm. only, but more soluble in alkali [20]) and pipetted into flasks containing Czapek's medium. The final concentrations of Zerlate being the same as for Makrolin.

All the flasks including controls were inoculated with 4 mm. discs of fungal mycelium, cut from the advancing edges of a four day old culture grown in potato dextrose agar in Petridishes.[19] All these procedures were carried out most asceptically and the flasks were incubated at room temperature (28 ± 2°C.) for three weeks.

The mycelium from all the flasks was collected on filter paper 12 cm. in diameter. These filter papers did not show any appreciable change in their weights on repeated washing and drying. The papers were weighed before the mycelium was collected. The mycelium was filtered, washed thrice with distilled water and dried in an hot air oven at 55°C. for 24 hours. The papers were then re-weighed. The difference between the original and final weights of filter paper gave the dry weight of the mycelium.

By substracting the weights of mycelium in different concentrations of fungicides from that of controls, the percentage of inhibition was calculated and the Dosage-Response curves one plotted.[6,19,21]

SLIDE SPORE-GERMINATION TECHNIQUE

Makrolin alongwith Zerlate and Colloidal copper against *A. tenuis*, *H. anomalum*, and *F.* was used *dimerum*, in this technique. The technique as recommended by the "Committee on Standardization of Slide Spore-germination Technique [11,12] set up by the American Phytopathological Society, along other modified and improved techniques of various workers[7,14,19,21-28] was employed for determining the effect of Makrolin on fungus spores. It is because of the tremendous importance and precision of this method that most of the fungicides screened and developed during the last 25 years were through this technique alone.[29]

Making of Dilution.—The different concentrations of the fingicides as recommended and used [19] were prepared in 25 ml. capacity tubes as follows.

Makrolin: 50% emulsifiable concentrate was prepared with the help of a locally prepared emulsifier (containing mineral turpentine and Turkey red oil). From this emulsion the required concentrations of 0.32, 0.16, 0.08, 0.04, and 0.02% were made with distilled water.

Zerlate: One percent solution of Zerlate in 2% sodium hydroxide was prepared and from this, concentrations of 0.02, 0.01, 0.005, 0.0025, and 0.00175% were prepared.

Colloidal Copper: The suspension was prepared directly in distilled water and the desired concentrations of 0.02, 0.01, 0.005, 0.0025 and 0.00175% were made. The concentrations were altered as desired and seperate pipettes were employed.

Ordinary glass slides of $3'' \times 1''$ were washed thoroughly by boiling in dilute chromic acid solution for 10 to 12 minutes and then in tap and distilled water.[11,17] The slides were dried and immersed in 10% Turkey red oil solution for one hour. This oil works as a spreading agent and fulfils all the conditions of a good surfactant.[23]

Three circles of 15 mm. diameter were made on each slide by slightly touching the open end of a tube which was previously heated and rotated on the solidified wax surface for a few seconds. This method of confining the standardized drop of fungicide and fungus spore was found to be quite satisfactory and advantageous in contrast to other methods like scratching, engraving, cavity slide or the wax pencil marking techniques.[14,17,23,30]

Obtaining of Spores.—five ml. of distilled water was added to the seven days' old culture (except in the case of *H. anomalum*, where a ten-day old culture was used) grown in P.D.A. medium and the tube was shaken gently so as to avoid the breaking of agar surface. Since it was difficult to dislodge spores merely by shaking, the mycelium was rubbed gently with a camel hair brush.[11,12] The suspension was then filtered through four layers of fine muslin cloth. Filtered spore suspension was then centrifuged for one minute[24] at 1200 round per minute, though additional centrifugation with greater force and for a longer time would not alter germination.[23]

The supernatant liquid containing pieces of agar or mycelium was decanted leaving the spores at the bottom of tube. 10 ml. of freshly prepared and sterilized medium[22,23] (0.0001% potassium citrate and 2% sucrose) was added in the tube containing spores. The spore suspension was standardized in such a way as to contain about 50,000 spore/ml, [11,12,24] or 30 ± 5 spore in low magnification of microscope.

Unlike Horsfall's[31] horizontal spraying or McCallen's and Wilcoxon's [26] settling tower technique, simple pipetting method as used by Montgomery and Moore[14] and other workers[23,28,30] was employed for transferring the measured amount of the fungicide material into the circles. With the help of a sterilized 1 ml. pipette. 0.1 ml. of the fungicide solution or suspension was pipetted in each of the three circles on a slide. Pipetting was started from the lower towards the higher dilution and separate pipettes were employed for each fingicide. The slides were then exposed for drying for about 2 to $2\frac{1}{2}$ hours. In this manner three sets of five slides each for the three fungicides and one slide for control were prepared to observe the inhibition of spore germination.

In all dried circles 0.1 ml. of standardized spore suspension was pipetted to obtain the original concentrations of fungicides. Slight mixing of the spore suspension with the dried deposit was done by gently shaking the slides. Immediately the slides were transferred to moist chambers. The moist chambers were made by putting wet filter papers on the bottom of petri plates and then two small glass rods were placed on those papers for resting the slides.[22] Such chambers provided sufficient humidity for spore-germination without being water sealed.[11]

Slides of different concentrations were kept in different chambers to avoid the effect of other concentrations of fungicides on spore-germination. The slides were incubated at room temperature $28 \pm 2°C$ and observations taken after 17 and 41 hours.[12,24] The final observation for *A. tenuis* were taken after 41 hours, while for *F. dimerum* and *H. anomalum*, after 17 hours, as their spores germinated very rapidly.

The observation were taken by counting the number of germinated and non-germinated spores in each circle under low power of microscope.[12] The spores were regarded as germinated if the length of the germ tube was $\frac{1}{4}$th the size of spore.

Results

POISONED FOOD TECHNIQUE

The results of the poisoned food technique are the mean of three experiments and each experiment was triplicated. The degree of inhibition equals to the mean difference, in dry weights, between the treatment and controls.[10] the Dosage-Response curves (DR) have been drawn on log-probit scales[6,19] (Figs. 1 to 6).

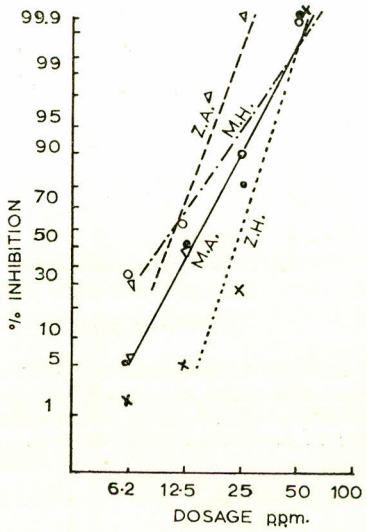

Fig. 1.—Showing the effect of Makrolin and Zerlate on the growth of *A. tenuis* and *H. anomalum*. (Z. H., Zerlate with *H. onomalum;* Z. A., Zerlate with *A. tenuis;* M. A., Makrolin with *A. tenuis;* M. H., Makrolin with *H. anomalum*).

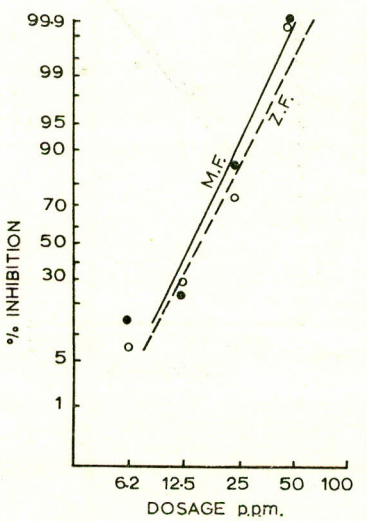

Fig. 2.—Showing the effect of Makrolin and Zerlate on *F. dimerum;* growth. (M. F., Makrolin with *F. dimerum;* Z. F., Zerlate with *F. dimerum*).

Makrolin checked the growth of *A. tenuis* at 50 ppm., while Zerlate gave the same results at

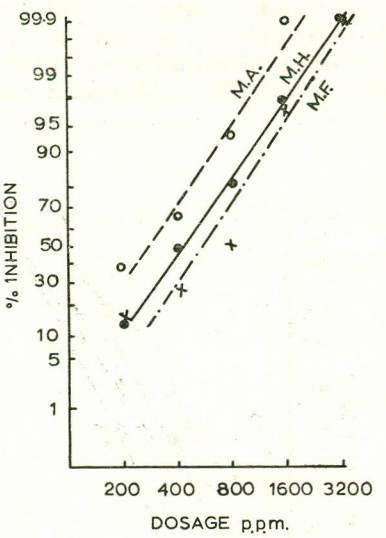

Fig. 3.—Showing dosage-response curves of Makrolin with *A. tenuis; H. anomalum* and *F. dimerum*—% inhibtion of spores-(M. A., Makrolin with *A. tenuis;* M. H., Makrolin with *H. anomalum;* M. F. Makrolin with *F. dimerum*).

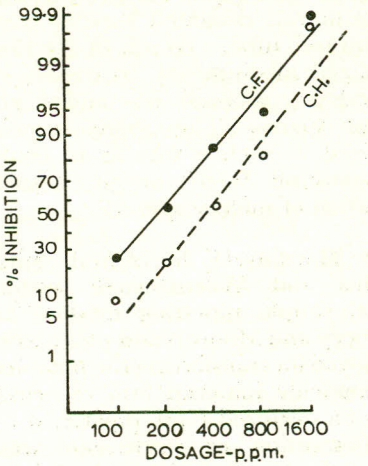

Fig. 5.—Showing dosege-response curves of colloidal copper with *F. dimerum* and *H. anomalum*—% inhibition spores-(C. F., colloidal copper with *F. dimerum;* C. H., colloidal copper with *H. anomalum*).

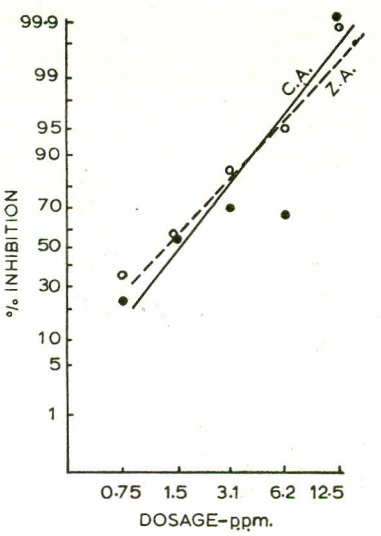

Fig. 4.—Showing dosage-response of colloidal Copper and Zerlate with *A. tenuis*—% inhibition of spores-(C.A., colloidal copper with *A. tenuis;* Z. A., Zerlate with *A. tenuis*).

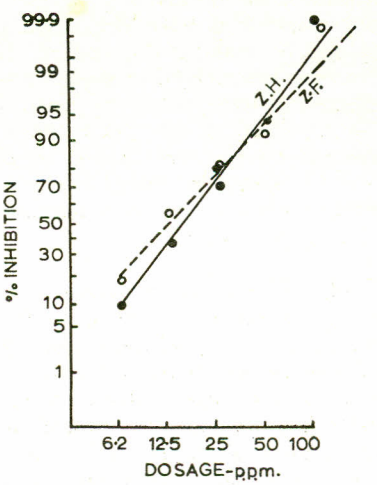

Fig. 6.—Showing dosage-responsc curves of Zerlate with *H. anomalum* and *F. dimerum* (Z. H., Zerlate with *H. anomalum;* Z. F., Zerlate with *F. dimerum*).

25 ppm. (Table 1 and Fig. 1). The growth of *F. dimerum* and *H. anomalum* was completely controlled at 50 ppm. by both Makrolin and Zerlate (Table 1 and Figs. 1,2). The 20,000 ppm. (2%) of acetone control run with all the experiments showed little growth inhibition of the three fungi. (Acetone controls were run due to its being used as a solvent).

STIDE SPORE-GERMINATION TECHNIQUE

Each final observation in this method is the mean of three triplicated experiments. The spore inhibition percentage was calculated by counting the germinated and non-germinated spores.

A. tenuis spores were inhibited completely at 1600 ppm. of Makrolin and 12.5 ppm. of both Zerlate and Colloidal copper (Tables 2,3 and 5 and Figs. 3,4). The spores of *F. dimerum* and *H. anomalum* were inhibited at 1600 ppm. by both Makrolin and colloidal copper but at 100 ppm. of Zerlate (Tables 2,4,6 and Figs 3,5,6.). The 10,000 ppm. emulsifier control showed no inhibition in any of the experiments.

The mode of action of the fungicides can be campared and ascertained from the DR curves, as suggested by Horsfall.[6] From these graphs the ED 95 values can also be calculated. The curves for identical concentrations have been drawn together to economise space.

TABLE 3.—PERCENT INHIBITION OF *A. tenuis* SPORES DUE TO COLLOIDAL COPPER AFTER 41 HOURS AT ROOM TEMPERATURE. THE RESULTS ARE MEANS OF 3 EXPERIMENTS.

Concentrations (ppm)	% Inhibition with average standard deviations
0.75	23.8±2.38
1.5	52.0±1.22
3.1	69.9±3.46
6.2	63.2±2.00
12.5	100.0
Control	0.0

Discussion

The results obtained demonstrate that *A, tenuis*, *H. anomalum* and *F. dimerum* differ in their response to colloidal copper, Makrolin and Zerlate in each of the technique employed during the present studies. In the case of *A. tenuis* 100% growth in-

TABLE 1.—EFFECT OF DIFFERENT CONCENTRATIONS OF MAKROLIN AND ZERLATE ON THE GROWTH OF *A. tenuis*, *F. dimerum* AND *H. anomalum* AFTER 21 DAYS AT ROOM TEMPERATURE 28±2°C.

Fungicides	Fungi	Mean weight (mg). of mycelium of three triplicated experiments with average standard deviations. Concentrations (ppm.) 6.2	12.5	25	50	100	Control
Makrolin.	*A. tenuis*	1318±11.67	601.3±52.60	288.6±32.00	—	—	1390±6.48
% Inhibition		5.2%	49.5%	79.2%	100%	100%	0%
	F. dimerum	809±20.34	689±56.43	138±26.05	—	—	954±26.00
% Inhibition		15.2%	27.7%	85.5%	100%	100%	0%
	H. anomalum	753.3±45.81	417.3±54.02	155.3±4.27	—	—	1111±44.90
% Inhibition		32.2%	62.4%	86.0%	100%	100%	0%
Zerlate.	*A. tenuis*	517±11.79	397±17.64	—	—	—	746±12.56
% Inhibition		30.7%	46.7%	100%	100%	100%	0%
	F. dimerum.	905±9.27	702±8.85	254±15.34	—	—	975±6.38
% Inhibition		7.1%	28%	74%	100%	100%	0%
	H. anomalum	1119.4±2.21	1192±43.87	841±25.08	—	—	1141±62.00
% Inhibition		1.9%	4.3%	26.3%	100%	100%	0%

TABLE 2.—PERCENTAGE INHIBITION OF SPORES OF *A. Tenuis. F. Dimerum and H. Anomalum*, DUE TO MAKROLIN, AFTER 41, 17 AND 17 HOURS AT ROOM TEMPERATURE, RESPECTIVELY. THE RESULTS ARE MEANS OF 3 EXPERIMENTS.

Fungi	Concentrations (ppm) 200	400	800	1600	3200	Control
A. tenuis ..	38.00±2.19	65.4±4.91	93.0±3.14	100	100	1.5±0.35
F. dimerum ..	12.9±1.85	28.0±2.19	50.6±2.68	97.6**±0.41	100	0.0
H. anomalum ..	12.2±0.54	48.0±2.12	80.0±2.83	97.0±1.41	100	0.0

* This experiment was performed at a later date than the one with Makrolin, hence the general decrease in growth may be due to prolong sub-culturing which may have decreased the vigor of the fungus.

** 2-3% of spore-germination is negligible.

TABLE 4.—PERCENT INHIBITION OF *F. dimerum* AND *H. anomalum* SPORES DUE TO COLLOIDAL COPPER AFTER 17 HOURS AT ROOM TEMPERATURE. THE RESULTS ARE MEANS OF 3 EXPERIMENTS.

Fungi	Concentrations (ppm.)					
	100	200	400	800	1600	Control
F. dimerum	25.3 ±1.14	55.6 ±1.78	84.0 ±0.70	94.0 ±2.45	100 ..	0.0 ..
H. anomalum	9.6 ±1.08	21.3 ±7.09	55.6 ±2.86	80.3 ±5.11	100 ..	0.0 ..

TABLE 5.—PERCENTAGE OF SPORE-INHIBITION OF *A. tenuis* DUE TO ZERLATE AFTER 41 HOURS AT ROOM TEMPERATURE. (MEAN OF THREE TRIPLICATED EXPERIMENTS WITH STANDARD DEVIATIONS).

Concentrations ppm.	% inhibition with average standard deviations
0.75	33.0±1.01
1.5	54.8±13.68
3.1	82.6±5.41
6.2	95.6±1.78
12.5	100.0 —
Control	0.0

TABLE 6.—PERCENT INHIBITION OF *F. dimerum* AND *H. anomalum* SPORES DUE TO ZERLATE AFTER 17 HOURS AT ROOM TEMPERATURE. (MEAN OF THREE TRIPLICATED EXPERIMENTS WITH STANDARD DEVIATIONS).

Fungi	Concentration ppm.					
	6.2	12.5	25	50	100	Control
F. dimerum	19.6 ±1.05	53.3 ±4.26	78.0 ±6.28	92.0 ±2.12	100 —	0.0 —
H. anomalum	10.0 ±2.55	37.0 ±6.04	70.0 ±3.08	93.3 ±2.37	100 —	0.0 —

hibition was obtained at 25 ppm. of Zerlate and on the contrary to obtain the same inhibition of *A. tenuis* 50 ppm. of Makrolin were required. *F. dimerum*, and *H. anomalum* have been checked at 50 ppm. by both Makrolin and Zerlate.

The germination of *A. tenuis* spores was inhibited at much lower concentrations by Zerlate and Colloidal copper, but higher concentrations of Makrolin were needed for their complete check. Spores of *F. dimerum* and *H. anomalum* were inhibited at 1600 ppm. of Makrolin and colloidal copper but 100 ppm. of Zerlate were required to obtain the same inhibition.

Difference in the efficacy of a compound against a particular fungus have also been reported by earlier workers. Gottleib[32] reported trials of 80 compounds on *Stemphylium sarciniforme:* 50% of compounds gave same ED 50 values by both the techniques, 35% compounds were more toxic to growth than to spore germination and 15% more toxic to spore germination than to growth.

Rich and Horsfall [21] tested 1000 compounds by both the methods on *Monilinia fructicola:* of these 53% were inactive by either test, 24% were active by either test, 18% were active on spore germination but not on growth and 5% were active on growth but not on germination of spores.

Manten *et al.*[33] compared these two methods with 14 fungi and one fungicide and reported differences in ED95 values for the two techniques.

One of the main reasons, as also reported by Horsfall and Rich[21] is that the physiology of growing hyphae is different from that of germinating spores and hence the two methods rate the compounds in different order. Specificity and availability of a compound to a particular fungus may also be the other factor.

An other example of such differences can be that of the dithiocarbamates. Zerlate would not have been proved to be a potent fungicide if it were not tested against *Alternaria solani*, since this compound proved a total failure against *Phytophthora infestens* in its initial stages of development.[34]

The two additives, acetone and emulsifier, being ineffective in checking either the spore-germination or the growth, do not add to the efficacy of Makrolin.

The differences in the slope or the steepnesses of the DR curves in three compounds in respect to the three fungi depicts a different mode of action.

From the present studies a conclusion that Makrolin can prove to be a good future fungicide, can easily be drawn. Field trials in the different regions of West Pakistan are in progress, with this compound.

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